

Effects of Temperature and Moisture on the Ecotoxicity of Metal-Based Fungicides Towards Earthworms

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“In the name of Allah, The Most Gracious and The Most Merciful “

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PREFACE

This study was done for the degree Master of Science in Environmental Sciences and was carried out in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, South Africa. The laboratory work was conducted following standard test procedures and used a common earthworm species (*Eisenia andrei*) as test organisms. This study was conducted full-time during the period of January 2020 to January 2022, under the supervision of Prof. Mark Maboeta and Dr. Prosper Opute. The thesis was written following the format provided by the NWU.

SUMMARY

Climate change is a major threat to biotic and abiotic ecosystems worldwide and might potentially affect the mobility and bioavailability of metals in soil. Anthropogenic activities such as mining, agriculture and the use of pesticides and fungicides all contribute to soil pollution. These activities coupled with climate change could have a greater effect on soil organisms. The aim of this study was to determine if varying soil moisture contents and different temperatures will influence the toxicity of single and binary mixtures of the fungicides, copper oxychloride and mancozeb, (in different concentrations) towards earthworms (*Eisenia andrei*) as bioindicators of soil quality.

Tests were done using artificial soil in accordance with the OECD guidelines under two different temperatures viz. 20°C and 25°C and two different moisture contents viz. 30% and 50%. The concentrations for copper oxychloride (CuOx) were 200, 500 and 1000 mg/kg and mancozeb (MnZn) 44, 850 and 1250 mg/kg in single and binary mixtures. Endpoints measured included earthworm growth, reproduction, avoidance-behaviour, body metal analyses, comet assays (DNA analysis) and soil enzymatic analyses.

The most significant results in mortality manifested in the 25°C 30% exposure group which had a 73% and 70% mortality rate in the CuOx1000 and CuOx1000 + MnZn1250 mg/kg treatments respectively. In terms of growth and reproduction a decrease in both biomass and reproductive viability was observed with increasing concentrations. In the copper oxychloride treatment, there was a significant difference ($p < 0.05$) between 20°C 30% and 20°C 50%, and between 25°C 30% and 25°C 50%, which implies that soil moisture had a significant effect. Mancozeb treatments had significant differences between the 25°C 30% exposure and the 20°C 30%, 20°C 50%, 25°C 50% exposures. Binary treatments showed a significant difference only at the CuOx1000 + MnZn1250 mg/kg (20°C 50%) exposure when compared to the other temperatures and moistures 20°C 30%; 25°C 30%; 25°C 50%. The avoidance test indicated that earthworms avoided soils spiked with (CuOx500, CuOx1000, MnZn850, MnZn1250, CuOx500 + MnZn850, CuOx1000 + MnZn1250 mg/kg) under all the different temperatures and soil moistures (20°C 30; 20°C 50%; 25°C 30%; 25°C 50%). DNA damage increased as the concentrations of the treatments increased. Significant differences ($p < 0.05$) were found between the different temperatures and soil moistures although the different experiments produced different results. Body metal analysis test corresponded with the comet assay test viz. an increase in body metal concentration as the concentrations of the treatments increased. Significant differences ($p < 0.05$) were found in the copper and manganese concentrations although no significant differences were found in the zinc concentrations. Soil enzymatic analysis indicated significant differences in the alkaline phosphatase and glucosidase tests for the single and binary (copper oxychloride and mancozeb) treatments and for the 25°C 50% exposure it was the most significant. It is evident that fluctuating temperatures and soil moistures had varying effects on the single and binary mixtures of copper oxychloride and mancozeb.

It was concluded that varying temperatures and soil moistures would have a significantly different result on the effects of metal-based fungicides towards earthworms based on the title of this study. Increasing the temperature and lowering the soil moisture had the most detrimental ecotoxicity effects on earthworms exposed to metal-based fungicides.

Keywords: Alkaline phosphatase; Bioindicators; Climate change; Copper oxychloride; *Eisenia andrei*; Glucosidase; Mancozeb; Moisture; Temperature

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CHAPTER 1: INTRODUCTION

1.1 General introduction

Anthropogenic activities resulting in pollution has increased rapidly over the past few decades due to mining (Jubileus *et al.* 2013; McGuirk *et al.* 2020), agricultural practices (Maboeta *et al.* 2002), industrial practices (Maboeta and Fouche, 2014; Maboeta *et al.* 2003) and the use of fertilizers (Eijsackers *et al.* 2005). Metal-based fungicide use increases the concentration of metals available to soil organisms. All these activities put a strain on the environment and the organisms that live in it, which might be exacerbated by climate change (Gelybó *et al.* 2018; Siebert *et al.* 2019). The effects of climate change on biodiversity are likely to include changes in ecological interactions, habitat preference, species abundance and distribution, phenology and increases in invasive species (Eggleton *et al.* 2009; Singh *et al.* 2019). Climate change affects both above-ground and below ground ecosystems and might possibly influence the ecotoxicity of pollutants in these environments. It is expected that climate change will alter the behaviour and distribution of metals in the soil and alter the ecological function of soil organisms in contaminated environments (González-Alcaraz and van Gestel, 2015). Higher soil temperatures because of climate change will decrease the soil water content (González-Alcaraz and Van Gestel, 2016; Siebert *et al.* 2019). This temperature and moisture change can have effects on the bioavailability of heavy metals in the soil. Many soil organisms have integuments which are permeable to water and thus rely on the moist soil environment for survival (Siebert *et al.* 2019). Changes in climate conditions will influence contaminants in the soil as well as any organisms residing in the soil ecosystem with possible negative impacts on soil processes making the soil less productive.

1.2 Climate change

It is widely recognized that climate change will be one of the most substantial threats to biodiversity over the next century, with significant consequences for many ecosystems in terms of their functioning and service provisioning (Sala *et al.* 2000; Singh *et al.* 2019). Climate change might affect ecosystems due to impacts on species composition and biodiversity (Bates *et al.* 2008); these changes could include changes in ecological interactions, habitat preferences, species abundance and distribution, as well as an increase in invasive species (Eggleton *et al.* 2009). Temperature and soil moisture fluctuations will affect soil organisms, processes, and pollutants. Pesticide toxicity is enhanced or decreased by changes in temperature and soil moisture (Hackenberger *et al.* 2018) and could have both direct and indirect effects on pesticides. Indirect effects could include changes in the duration of exposure to pesticides due to shifts in cultivation (Hackenberger *et al.* 2018). As a result, the potential enhancement of volatility and degradation of pesticides could affect their efficiency against pests and, therefore, pesticide application rates could increase. Both the amount and the extent of pesticide application could increase as a result of this increase (Kookana *et al.* 2010). Direct effects of climate change include

effects on pesticide decomposition and toxicity due to the expected temperature and precipitation fluctuations (Hackenberger *et al.* 2018). These fluctuations are of paramount importance, since they can have an impact on the performance of soil organisms as well as the speciation and consequently the bioavailability of metals (González-Alcaraz *et al.* 2018). It has been shown that pesticide degradation is dependent on soil moisture and temperature.

1.3 Soil

The fundamental regulatory compartment for all benthic and terrestrial ecosystems is soil. Soil filters polluted water, acts as a buffer and provides a habitat for organisms. The breakdown of primary minerals and the incorporation of organic matter over a long period of time results in the formation of soil (Blouin *et al.* 2013). Soil's vital role in the ecosystem should be protected from threats due to the long process of soil formation.

There is a worldwide decline in soil quality due to environmental impacts e.g. mining, industry and agriculture (Fouché *et al.* 2017). Soils are a major sink for metals released into the environment by these anthropogenic activities, and unlike organic contaminants which are oxidized to carbon (IV) oxide by microbial action, most metals do not undergo microbial or chemical degradation, and their total concentration in soils persists long after their introduction (Wuana and Okieimen, 2011). Metal pollution is persistent and permanent, and its accumulation in food chains, poses a major threat to the health of organisms and humans (Li *et al.* 2019). Therefore, the utilisation of agrochemicals should be weighed up against food security because agricultural practices impact soil quality (Fouché *et al.* 2017).

Chemical and physical characteristics of soil plays an important role in the ecotoxicity of metals and thus is an important factor to consider when doing research and applying it to field conditions (Maboeta and Fouche, 2014). According to Eijsackers *et al.* (2017) soil ecotoxicological tests are done at 22°C and 60% soil moisture content which is reasonable from a European Union (EU) regulator's point of view. Although temperatures on the African Continent are higher than that of Europe, the parameters of these experiments are based on international standards (OECD). In temperature extremes, earthworm mortality is higher because of desiccation (Eijsackers *et al.* 2017). Higher temperatures and altered soil moisture can have a major impact on the toxicity of metals and organic pollutants with regards to soil organisms.

1.4 Copper, manganese and zinc fungicides used in the agricultural sector

The agricultural sector has been using synthetic pesticides for many years to combat weeds, insects, and pathogens to minimize economic loss (Gowri and Thangaraj, 2020; Jovana *et al.* 2014). Pesticides are one of many cost-effective methods used for controlling pests and other threats to crops, such as genetically modified (GM) crops and biological methods, but they can also be detrimental to non-target and beneficial organisms such as earthworms (Gowri and Thangaraj, 2020; Wang *et al.* 2016). Metal-based fungicides can accumulate in soil and have detrimental effects on soil health and non-target species, which needs more investigation.

One of the sources of copper, manganese and zinc contamination usually occurs through agricultural practices which use copper oxychloride and mancozeb (both fungicides) on crops to protect them from fungal diseases. Copper oxychloride is used mainly on tomato and grape crops while mancozeb is used on potato and citrus crops (Dabrowski *et al.* 2014). The migration of pesticides to non-target environments is caused by leaching, runoff, and spray drift (Dabrowski *et al.* 2014). Climate change could possibly result in increased temperatures and lowered soil moisture contents which might influence the ecotoxicity of any metals already in the soil. According to previous studies (e.g. Černohlávková *et al.* 2009; Eijsackers *et al.* 2005; Oladipo *et al.* 2019; Voua Otomo *et al.* 2013) the use of mancozeb and copper oxychloride could have detrimental effects on the soil mesofauna due to the resulting increase of manganese, zinc and copper in the soil. There is a possibility that human health might be at risk for the reason being that the water and soil quality can degrade due to soil metal pollution (Oladipo *et al.* 2019).

Copper oxychloride ($\text{H}_3\text{Cl}_2\text{Cu}_2\text{O}_3$) is frequently used in the global agricultural sector, in vineyards mainly, as a fungicide (Reinecke *et al.* 2002; Maboeta *et al.* 2004). Copper is a vital trace element to all living organisms as it contributes to various metabolic processes and this micronutrient is controlled by the body via complicated homeostatic processes, but excess of copper has harmful effects to the organism as it stimulates free radical production in the living cell, and it disrupts the whole-body antioxidant capacity (Mahmoud *et al.* 2019). In natural and uncontaminated soils, copper concentrations are relatively low unless the parent rock from which the soil was derived is high in copper. Elevated concentrations of copper in soils are toxic and may result in a range of effects including reduced biological activity and subsequent loss of fertility (Dumestre *et al.* 1999).

Mancozeb ($\text{C}_8\text{H}_{12}\text{MnN}_4\text{S}_8\text{Zn}$), which contains manganese and zinc, is a synthetic Ethelene-bis-dithiocarbamate which is also used to treat fungal diseases in plants. Černohlávková *et al.* (2009) reported that mancozeb is used globally in the agricultural sector mainly on vineyards, wine production, and many other fruits and vegetable crops. Manganese (Mn) is a micronutrient essential for plant growth and is relatively abundant in the upper crust of the earth. Despite it being water-soluble, it has low mobility and soil pH plays an important role in the bioavailability of manganese. Zinc is an essential

trace element that is readily available in soils (Sankhla *et al.* 2019) and is required for the healthy functioning of biological systems but at elevated levels it becomes toxic (Sankhla *et al.* 2019).

1.5 Earthworms

Earthworms contribute significantly to good soil quality due to their effect on the development of soil aggregates, their continuous species-specific burrowing behaviour, which regularly mixes the upper soil layer, and which results in litter being introduced into the soil thus improving soil drainage (Eijsackers *et al.* 2005; Jouquet *et al.* 2014; Singh *et al.* 2019).

The burrowing and casting of earthworms allow stabilized aggregates to form, allowing easier penetration of water and air, thus, improving the physical properties of soils (Sankhla *et al.* 2019). These activities also improve nutrient uptake by plants and prevent or reduce anoxic conditions, allowing aerobic microorganisms to thrive (Sankhla *et al.* 2019). A crucial role is played by earthworms in the food chain by channelling waste energy from dead organic matter to higher trophic levels (Sankhla *et al.* 2019).

Earthworms are good examples of biological indicator species, due to their involvement in soil functioning and they are vastly distributed. They are relatively easy to sample, and have measurable responses such as growth, reproduction, and body tissue contaminant concentration. As such, earthworms have been used extensively in soil ecotoxicological research (Bandeira *et al.* 2020; Eijsackers *et al.* 2005), due to their contact with soil (Andriuzzi *et al.* 2015; Velki and Ečimović, 2015), their impact on nutrient cycling and because they have very few external barriers to the soil (Fouché *et al.* 2017; González-Alcaraz and Van Gestel, 2016). They are used widely as bioindicators of environmental pollution, and they are keystone species within the ecosystem. The above-mentioned characteristics make the earthworm species ideal for evaluating soil toxicity (Oladipo *et al.* 2019).

1.6 Problem statement

Climate change is a global phenomenon affecting the world. Changes in temperature and soil moisture might have an influence on contaminants in the soil, making it more bioavailable to organisms. Soils become contaminated by the accumulation of metals and metalloids through emissions from the rapidly increasing industrial activities, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Eijsackers *et al.* 2017, 2005; Wuana and Okieimen, 2011). Copper, manganese and zinc contamination usually occurs due to agricultural practices which use copper oxychloride and mancozeb fungicides on their crops to protect them from fungal diseases. These fungicides eventually enter the soil and contaminate it. Factors such as temperature and soil moisture contents vary depending on the area, time of the year and the effect of

climate change and these factors will influence the results obtained in the laboratory. Usually when experiments are done in the laboratory, these factors are kept constant and thus the impact it can have, is usually not considered. It is uncertain how rainfall will change over sub-Saharan Africa in the mid- and late 21st century (James and Washington, 2013). Severe drought conditions are predicted for South Africa, Asia, and the Mediterranean region. Climate change is predicted to increase the frequency and severity of heat waves, precipitation, and storms (Niang *et al*, 2014). Changes in precipitation regimes and temperature may increase or decrease chemical toxicity in the environment (Noyes *et al*, 2009). Therefore for this research to be more relevant to field conditions, differences in moisture and temperature need to be taken into account.

1.7 Research aim and objectives

The aim of this study was to determine if varying soil moisture contents and different temperatures influence the ecotoxicity of single and binary mixtures of copper oxychloride and mancozeb concentrations towards earthworms (*Eisenia andrei*) as bioindicators of soil quality. Specific objectives to achieve this were to:

- Determine the effects of copper oxychloride and mancozeb on the survival, growth, reproduction, and avoidance behaviour of *E. andrei* using standardised toxicity tests (OECD, ISO) under different temperatures and soil moisture conditions.
- Determine the effects of temperature and soil moisture content on the bioaccumulation of metals in earthworm body tissues.
- Assess DNA damage of earthworm cells as biomarker using the comet assay.
- Evaluate soil enzymatic activities (β -Glucosidase, Phosphatase, Urease) in contaminated soils under different temperature and moisture contents.

1.8 Outline of chapters

Chapter 1: Introduction, problem statement, aims and objectives.

Chapter 2: Literature review which is a published article (H. Kaka, P. A. Opute, M. S. Maboeta, "Potential Impacts of Climate Change on the Toxicity of Pesticides towards Earthworms", Journal of Toxicology, vol. 2021, Article ID 8527991, 14 pages, 2021. <https://doi.org/10.1155/2021/8527991>). Details are given on the effect of climate change on the soil environment as well as its effect on soil temperature and moisture content. The role of earthworms as bioindicators and the fungicides used will also be explained. Previous studies on these topics were also considered.

Chapter 3: Materials and Methods gives detailed procedures on artificial soil, test substances used, concentrations of chemicals used and how the different temperatures and moisture contents are controlled and details on the toxicity tests, namely the avoidance,

growth and reproduction test, body metal analysis tests, DNA analysis tests using comet assay and the soil enzymatic tests.

Chapter 4: The results on the effects of temperature and moisture on the ecotoxicity of copper oxychloride towards earthworms.

Chapter 5: The discussion explains the effects of temperature and moisture on the ecotoxicity of mancozeb towards earthworms.

Chapter 6: Conclusions and recommendations. This will include all the crucial outcomes of this study as well as indicate if the objectives were accomplished. Recommendations will also be done on future experiments similar to this study. Lastly, a combined list of references for all the chapters will be provided.

CHAPTER 2: LITERATURE REVIEW

Potential Impacts of Climate Change on the Toxicity of Pesticides towards Earthworms

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ABSTRACT

This review examined one of the effects of climate change that has only recently received attention i.e. climate change impacts on the distribution and toxicity of chemical contaminants in the environment. As ecosystem engineers, earthworms are (potentially) threatened by the increasing use of pesticides. Increases in temperature, precipitation regime changes, and related extreme climate events can potentially affect pesticide toxicity. This review of original research articles, reviews, governmental, and intergovernmental reports focused on the interactions between toxicants and environmental parameters. The latter included temperature, moisture, acidification, hypoxia, soil carbon cycle and soil dynamics, as altered by climate change. Dynamic interactions between climate change and contaminants can be particularly problematic for organisms since organisms have an upper and lower physiological range, resulting in impacts on their acclimatization capacity. Climate change variables such as temperature and soil moisture also have an impact on acidification. An increase in temperature will impact precipitation which might impact soil pH. Also, an increase in precipitation can result in flooding which can reduce the population of earthworms by not giving juvenile earthworms enough time to develop into reproductive adults. As an independent stressor, hypoxia can affect soil organisms, alter bioavailability, and increase the toxicity of chemicals in some cases. Climate change variables, especially temperature and soil moisture, significantly affect the bioavailability of pesticides in the soil and the growth and reproduction of earthworm species.

Key words: Climate change, toxicity, pesticides, earthworm, temperature, moisture

2.1 Introduction

Climate change is defined as the alteration in the climate state, evidenced by changes in the variability of its properties such as temperature, precipitation, and wind (IPCC 2013). These variables persist for an extended period (typically decades or longer) which coincides with an increased likelihood in the intensity of extreme climate events, such as drought and flooding (Akinsanya *et al.* 2016; Singh *et al.* 2019). Soil biodiversity includes many different types of organisms ranging from microscopic organisms to large animals and plants. The soil supports a wide range of ecosystem functions, processes and services, making it essential to human life (Bardgett and Van Der Putten, 2014; Blouin *et al.* 2013; Jouquet *et al.* 2014; Wall *et al.* 2015). At some point, environmental processes in the air, water, and soil interact with each other, and this interaction is essential for these processes to function at an optimal level. Anthropogenic activities including agricultural and mining activities are primarily responsible for soil pollution (Jubileus *et al.* 2013; Maboeta *et al.* 2018; McGuirk *et al.* 2020) which impacts the biodiversity of soil organisms which are therefore reduced or lost (Gelybó *et al.* 2018).

Earthworms makeup 40-90 % of the soil macro-faunal biomass in many terrestrial ecosystems and thus are vital soil organisms (Singh *et al.* 2019). There are 23 families, and over 700 genera with more than 7,000 species of earthworms already described worldwide, although the number of species is much more (Csuzdi, 2012; Singh *et al.* 2019). They play an important role as ecosystem engineers, thus making them keystone species (Singh *et al.* 2019). The environment is modified physically and chemically by earthworms; they create, transform and maintain the habitat for soil organisms and plant communities (Blouin *et al.* 2013). These modifications are done by processes such as litter fragmentation, burrowing, and casting activities. These activities drive soil processes such as nutrient cycling, soil aggregate stability, water infiltration, plant growth, and soil carbon storage (Coleman. *et al.* 2018). They have many roles in the ecosystem, making them very useful as bio-indicators in soil eco-toxicological studies (Fründ *et al.* 2011; Maboeta and Fouche, 2014; Oladipo *et al.* 2019). The diversity of earthworm species is studied worldwide, and Phillips *et al.* (2019) highlighted climate change drivers such as increasing air temperature and changing mean annual rainfall as key factors affecting its global distribution. Climate change alters soil properties such as moisture, temperature, pH, and texture, affecting not only earthworm communities but also all life interacting with the soil environment (Singh *et al.* 2019).

2.2 Predicted global change scenarios

Climate change is a global problem. Generally, when models are used to predict future changes in climate, it usually suggests an increase in more extreme and severe events in the coming decades (IPCC, 2013). The main drivers of climate change affecting the soil ecosystem are greenhouse gases, air and soil temperature increases, extreme precipitation, and erosion (Biswas *et al.* 2018; Patil and Lamnganbi, 2012). According to the Intergovernmental Panel on Climate Change (IPCC, 2018), global warming for 2006-2015 was 0.87°C, which is higher than the average between 1850-1900. The estimated global warming caused by anthropogenic activity is currently increasing at 0.2°C per decade due to past and ongoing impact of pollutants (IPCC, 2018). Activities by humans have caused global warming to increase by 1°C above pre-industrial levels, and this is likely to reach 1.5°C between 2030 and 2052 if it continues to increase at this current rate (IPCC, 2018). When comparing regional changes of the pre-industrial levels to the 1.5°C increase in global warming, it estimates extreme increased temperatures, increased heavy precipitation and an increase in drought in many regions, for example, the projected increase in precipitation in West Africa, the increase in temperature in the Indian Ocean resulting in increased rain in East Africa and the increased global surface temperature (IPCC, 2018). These predicted climate change events may have significant effects on the bioavailability of pollutants in the soil.

The ability of greenhouse gases such as carbon dioxide, methane, nitrous oxide and fluorinated gases to capture heat from the sun's energy causes the greenhouse effect (Kweku *et al.* 2018). The primary sources of air pollution are the combustion of fossil fuels, as well as industrial and agricultural activities. As a result of these events, more of the sun's radiation is trapped in the atmosphere, causing global warming (Figure 1). According to Olivier and Peters (2020), since the year 2012, the global annual increase in CO₂ emissions has slowed down to about 1.5%, and in 2015-2016 it has remained flat. In 2017, 2018 and 2019, the global CO₂ emissions increased by 1.4, 2.4 and 0.9% respectively. The increase was a result of increased consumption in coal. The annual global carbon sequestration potential is about 0.4-0.7 Pg C y⁻¹, which means that if these rates remain constant until the year 2100, the soil carbon sequestration would only contribute to a maximum of 1-3% towards reducing the carbon emissions (Smith, 2008). This scenario suggests that carbon sequestration into the soil will play a minimal role in reducing carbon dioxide emissions. The study by Marhan *et al.* (2015) indicated a higher loss of nitrogen in the form of N₂O from the soil with high earthworm population and a warmer climate.

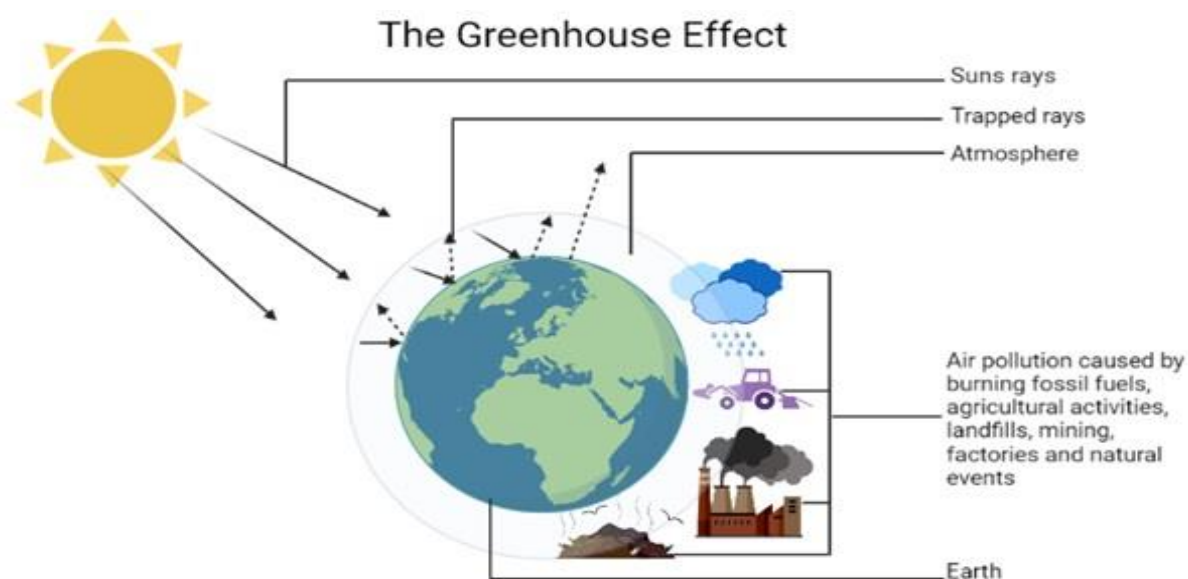


Figure 1: Simple representation of the greenhouse effect.

The main drivers of air pollution: burning of fossil fuels and industrial and agricultural activities. These events trap more of the sun's radiation in the atmosphere resulting in global warming.

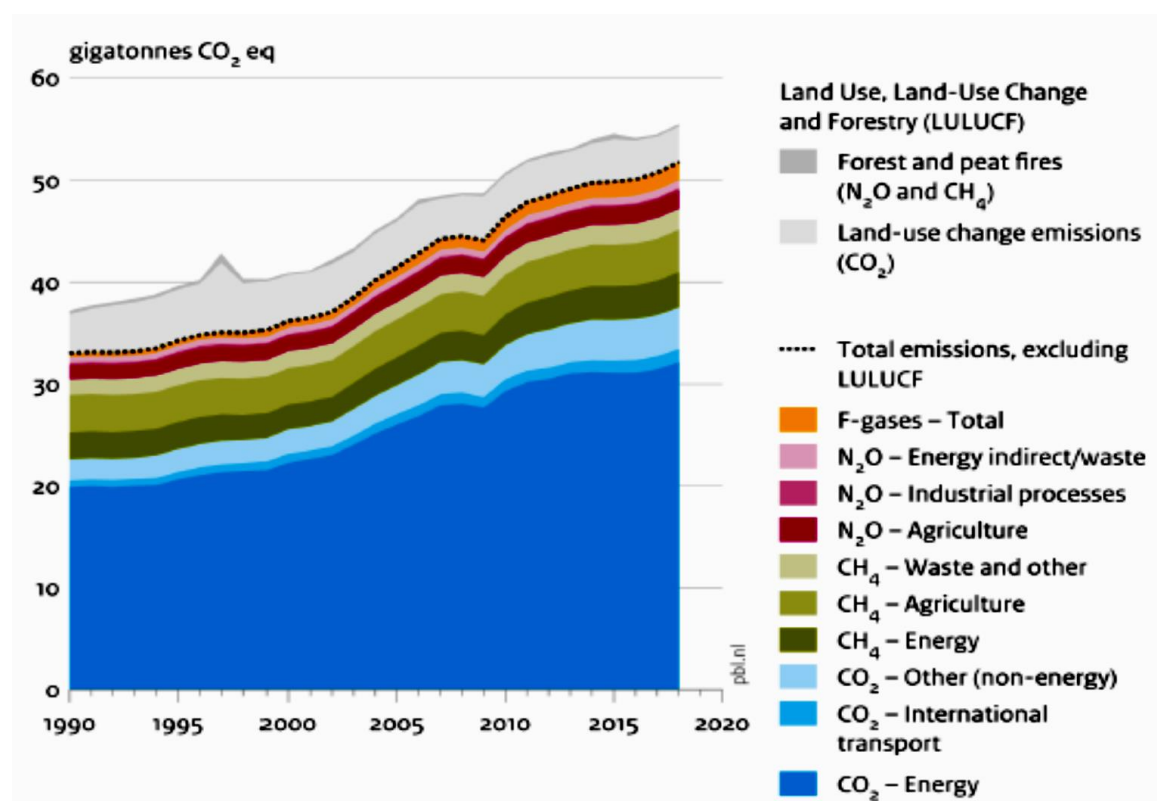


Figure 2: Global greenhouse gas emissions, per type of gas and source, including LULUCF (Source with permission: Olivier J.G.J. and Peters J.A.H.W. (2020). Trends in global CO₂ and total greenhouse gas emissions: 2019 report. PBL Netherlands Environmental Assessment Agency, The Hague).

As shown in Figure 2, greenhouse gases are increasing at an alarming rate, and the increase has been exponential over the last ± 60 years. If left unchecked, this extreme increase will keep on having a massive effect on environments worldwide. Human activity is responsible for the rapid rise in greenhouse gases such as carbon dioxide, methane and nitrous oxide (Smith *et al.* 2003). Most of the gas emitted is carbon dioxide, although gases such as methane and nitrous oxide are much better at absorbing infrared radiation and contributing to global warming (Smith *et al.* 2003). Over 100 years, 1 kg of methane has a warming potential 23 times greater than 1kg of carbon dioxide and 1 kg of nitrous oxide has a warming potential of nearly 300 times that of carbon dioxide (Smith *et al.* 2003). Approximately two-thirds of the nitrous oxide emissions and one-third of the atmosphere's methane emissions come from soils (Smith *et al.* 2003). If global warming continues unabated, it can have many devastating effects such as rising sea levels, ocean acidification, and severe weather events. However, there have been international initiatives such as the Kyoto Protocol, the signing of the Paris Agreement on Climate Change, and other initiatives set up to combat this effect globally (Kweku *et al.* 2018). According to Olivier *et al.* (2017), the record for the warmest year in 140 years was 2016 with an increase of $+0.99^{\circ}\text{C}$ and in 2019 it was the second warmest year with the global land and ocean surface temperatures increasing by $+0.95^{\circ}\text{C}$ above the average. They further stated that since 1880, 9 out of the 10 warmest years have occurred since 2005.

Anthropogenic activity has had an influence on the global water cycle and in turn affected the global precipitation regime by globally increasing the amount of atmospheric moisture and the precipitation patterns over the land (IPCC 2013). Singh *et al.* (2019) stated that precipitation regime changes might show significant heterogeneity and thus be difficult to predict. However, climate models project an increase in the frequency of extreme precipitation events. Also, an increase in rainfall due to climate change can result in soil loss and soil erosion by increasing sedimentation in streams and reservoirs (Patil and Lamnganbi, 2012). This soil loss will have detrimental effects on soil organisms as well as soil functions in an ecosystem. Once these functions are disrupted, changes in the environment can occur. Most of the water which supplies soil moisture comes in the form of precipitation and is evaporated from the ocean and transported to land by the atmosphere, and this movement of water in a climate system is an essential part of life on land.

Ultraviolet radiation is one of the components of solar radiation and it is separated into UV-A, UV-B, and UV-C (Chuang *et al.* 2006). The stratospheric ozone usually reflects UV-C and UV-B, thus only UV-A and very little UV-B reach the earth, although due to the depletion of atmospheric ozone there is an increase in UV-B reaching the earth (Chuang *et al.* 2006; Formánek *et al.* 2014). Higher UV-B radiation modifies soil microbial communities and decreases populations of soil meso- and macrofauna (Formánek *et al.* 2014). According to Formánek *et al.* (2014), UV-B is known to increase degradation of some pollutants such as phenylurea herbicides, p,p'-DDT, 2,4-dichlorophenoxyacetic acid, biphenol

and PAHs, although it can also inhibit rhizoremediation of organic pollutants. In arid and semi-arid regions, ultraviolet radiation is one of the main drivers of increasing litter decay through photodegradation (Wang *et al.* 2017). Abiotic photodegradation can mineralize the decomposition of litter into gases such as CO₂, CO and CH₄, and at the same time, alter the chemistry of organic materials (Wang *et al.* 2017). Complex carbon structures can be broken down by ultraviolet radiation into smaller molecular compounds, increasing dissolved organic carbon concentration in the litter (Wang *et al.* 2017).

2.3. Consequences of climate change for the structure and function of soil ecosystems

According to Bellard *et al.* (2012), in fifty years from now, we are likely to see an increase in the rate of species loss due to climate change, although the evidence that this will happen is still inconclusive. The aboveground and belowground ecosystems have many interactions and they rely on one another for specific functions (Wardle *et al.* 2004). However, the aboveground biodiversity is usually observed and acts as a basis for predicting environmental changes (Eisenhauer *et al.* 2019). The need to study the biodiversity of aboveground and belowground ecosystems simultaneously is essential because we might not find the differences found in one ecosystem in the other (Cameron *et al.* 2019). Anthropogenic activities contribute to the loss of biodiversity and ecosystem services in soil. Mining and agriculture, for example, exploit the soil ecosystem in an unsustainable manner, potentially resulting in a 60% depletion of ecosystem services (Veresoglou *et al.* 2015; Wall *et al.* 2015). Soils and soil processes will primarily be affected by climate change through changes in rainfall patterns and temperature. Earthworm populations will be affected by climate change variables such as drought, irregular precipitation and increasing temperatures (Wever *et al.* 2001; Perreault and Whalen, 2006; Staley *et al.* 2008). Additional research is required on climate change effects on soil organisms to develop and improve models (Moreau-Valancogne *et al.* 2013). Irrespective of the fact that earthworms and other soil organisms play a vital role in soil ecosystems and functions and the noticeable danger of climate change, there is still no conclusive overview of climate change effects on soil organisms, particularly earthworms given the increasing pesticide pollution in the environment.

2.3.1 Rise in soil temperature

Temperature is one of the most important climate change drivers and it is influential in determining soil biological activity and the decomposition process (Singh *et al.* 2019). The increase in temperature has shown to be the cause of various alterations to many plant and animal species, with it being massively influenced by biotic interactions (Chen *et al.* 2011; Gilman *et al.* 2010). Earthworms are a poikilothermic species, which means that their body temperatures fluctuate with their respective environments, therefore, the activity, density, growth, metabolism, respiration, and reproduction of

earthworms are all affected by temperature variations. They tend to gather in areas where conditions are ideal for their metabolism, suggesting that both high and low temperatures produce a direct response; these effects include: reduced growth rate, feeding activity, cocoon production and juvenile development both at the individual and community level (Singh *et al.* 2019). Although more is known about the higher lethal temperatures than the lower lethal temperatures, the response of earthworms to temperature fluctuations is also dependent on the species involved because every species has its specific tolerance range for different variables. The increase in temperature can also affect the bioavailability of the soil's metal pollutants. Besides the fact that temperature will influence soil organisms in the natural world, many other variables, such as the soil moisture content will also affect the ecosystem at the same time.

A study by Eggleton *et al.* (2009) showed earthworm diversity over six years, took into account temperature and soil moisture as climate change variables. They reported a decrease in earthworm numbers in the dry winter months, and during the moist summer months, there was an increase in earthworm numbers; dry summer months negatively affected epigeic species such as *Dendrobaena octaedra*, *Dendrobaena attemsi* and *Satchellius mammalis*, and in wetter months the earthworm numbers increased. Species such as *Aporrectodea caliginosa* and *Lumbricus rubellus* had a high tolerance to temperature changes. Also the study by Berman and Meshcheryakova, (2013) indicated a similar result: in higher temperatures with higher soil moisture, earthworm numbers increased rapidly. The study by Hughes *et al.* (2019) showed that higher air temperatures with lower soil moisture could reduce earthworm population. The abundance of earthworms usually increases when climatic conditions are favourable, such as when the temperature is moderate, and the soil moisture content is higher (Phillips *et al.* 2019). This abundance tends to decrease in colder climates, where the soil moisture content is lower (Walsh and Johnson-Maynard, 2016).

2.3.2 Change in soil moisture content

The soil environment is affected directly and indirectly by drought (Coyle *et al.* 2017). The deficiency of precipitation or drought does not immediately affect the deeper soil layers but will reduce the water content of the upper soil layer relatively quickly (Nepstad, 2002). The movement of soil organisms is inhibited by reduced soil water content, which hardens the soil (Anh *et al.* 2014), and diminishes the extent of water film (Coleman, *et al.* 2018). The reduction of vegetation cover by drought can cause increased temperatures, an altered microclimate on the soil surface, and reduced resource availability (Franklin *et al.* 2016). The biological activity and earthworm diversity of soils is reduced through increased soil temperature both directly and indirectly. The frequency and significance of droughts are increasing around the world (Dai, 2013) and there are only a few studies on the effects of drought on earthworms (Plum and Filser, 2005; Petersen *et al.* 2008; Owojori and Reinecke, 2010; Morón-Ríos *et*

al. 2010; Holmstrup *et al.* 2016). An increase in temperature is associated with the volatilization and degradation of organic and inorganic pollutants in the soil (Biswas *et al.* 2018). Thus, increase in soil temperature increases contaminants' transportation in the ground (Biswas *et al.* 2018).

Earthworms are only active if free water is available in the soil. They are very limited in morphologically or physiologically maintaining moisture in the body using the cuticle (Singh *et al.* 2019). When soils become too dry, earthworms usually lose weight, decrease their burrowing activity, and may also enter diapause or become dormant (Owojori and Reinecke, 2010). The cocoons produced by epigeic species are drought resistant, although it represent an essential strategy for the survival of the species in drought-stricken areas (Petersen *et al.* 2008) and may rapidly recover after the drought conditions end (Eggerton *et al.* 2009). Anecic earthworms can form permanent vertical burrows in the soil, during the dry periods, and can enter a diapause, and stay dormant for several months (Singh *et al.* 2019). Some endogeic species such as (*Aporrectodea caliginosa*) can form non-permanent horizontal burrows in the topsoil and form aestivation chambers covered with mucus and gut content and protect themselves against water loss (Singh *et al.* 2019). The composition of earthworm community's response to droughts can likely change due to the different ecological strategies these species use. Plum and Filser (2005) suggested that soil's organic matter and water holding capacity were critical factors in modifying earthworms' responses to drought conditions.

Floods on the other hand can cause massive changes to the soil (Zhang *et al.* 2015) by oversaturation and this can result in a lack of oxygen in the soil resulting in hypoxia, although the floodplains contain sediment which is very nutrient-rich making it some of the most productive ecosystems around the world (Tockner and Stanford, 2002). The diffusion and oxygen availability of the soil is reduced during flooding, resulting in reduced soil nutrient availability since decomposition processes are halted (Singh *et al.* 2019). Anaerobic conditions occur in flooded soils, which will significantly affect the composition of soil food webs, microbial biomass, and soil microbial community structure (Plum, 2005; Unger *et al.* 2009). Plum, (2005) stated that flooding reduced the abundance, biomass and diversity of all groups of soil organisms in grasslands and increase in temperature and flood duration increased its effects. The ability of soil invertebrates to survive depends on their behavioural, morphological, and physiological traits (Plum, 2005). Singh *et al.* (2019), reported that the abundance of earthworms was reduced in floodplain areas, and that periodical flooding had species-specific effects on earthworm populations. Another study by Bullinger-Weber *et al.* (2012) also indicated that an absence of anecic earthworms would suggest that more erosion and sedimentation processes have taken place, and an increase in epigeic earthworms may have positive effects on the texture of the topsoil and the organic matter quality. Many earthworms can survive long periods submerged in water (Singh *et al.* 2019), and some earthworm species can survive in flooded soils (Zorn *et al.* 2008). A critical factor to consider is the period between two flooding events. The study by Klok *et al.* (2006) suggested that earthworms from a

frequently flooded area mature much quicker than earthworms from a non-flooding site. Floods can also reduce earthworm populations by not giving juvenile earthworms enough time to develop into reproductive adults. Short flooding periods followed by long recovery periods can be beneficial to certain earthworm species e.g. *Lumbricus rubellus* and *Allolobophora chlorotica*. Schütz *et al.* (2008) and Bullinger-Weber *et al.* (2012) suggested that it could favour epigeic earthworm species. A study by Plum and Filser (2005) indicated that controlled flooding should be kept short in winters and natural summer flooding should follow and that a recovery period of six months should be sufficient for the re-establishment of earthworm populations.

2.4 Soil acidification

The soil acidification process is a gradual and continuous natural process that occurs during pedogenesis and is aided by water leaching basic cations to lower subsoil (Rengel, 2011). The acidification is accelerated by increase in nitrogen and sulphur deposition which is associated with human activity (Meng *et al.* 2019). The soil pH has a fundamental effect on the solubility and availability of potentially toxic ions, and while a low pH (acidic) will benefit free metal cations and protonated anions, a higher pH (alkaline) will favour carbonates and hydroxyl complexes (Rengel, 2011). Acidification can be enhanced by high precipitation which will allow leaching of soil cations. However, the soil itself is a buffer for external H ions (Meng *et al.* 2019) and only once it exceeds its maximum capacity will the soil become acidic.

One of the main drivers of soil acidification in terrestrial environments is the accumulated nitrogen deposition, and according to Tian and Niu (2015), during 2000-2010, more than 50kg ha⁻¹ of nitrogen was accumulated in terrestrial environments. This nitrogen-induced acidification has posed a significant threat to terrestrial ecosystem functioning and species diversity. Using nitrogen fertilizers can have a variety of effects on soil acidification. The NH₄⁺ ions can displace base cations such as Ca₂⁺, Mg₂⁺ and Na⁺ by binding them and making them more easily leached out of the soils, thus reducing their buffering against acidification and therefore when plant roots absorb the NH₄⁺ ions, the H⁺ ion is released into the soil solution, and this can cause soil acidification (Tian and Niu, 2015). Climate change variables such as temperature and soil moisture affect the acidification process. Higher soil moisture will allow leaching to occur more easily promoting soil acidification. Reducing the nitrogen cycles by lowered temperatures will reduce the ecosystems' ability to sequester nitrogen resulting in more acidification (Rengel, 2011). When precipitation is higher, it accelerates the leaching of cations, further aggravating acidification (Meng *et al.* 2019).

2.5 Soil Organic Carbon (SOC) cycling and SOC dynamics

The carbon cycle involves the soil, plants and all animal life, including humans, thus, the disruption of the carbon cycle would mean disaster for all living organisms. Soil organic matter is expressed as soil organic carbon, which plays a vital role in the sorption of soil pollutants (Biswas *et al.* 2018). The basic processes involved in the global carbon cycle start when plants take up carbon dioxide from the atmosphere. Through photosynthesis, sunlight energy is trapped in the carbon-to-carbon bonds of organic molecules. Some of these organic molecules are used as energy sources, while carbon is returned to the atmosphere as carbon dioxide. The remaining organic materials are temporarily stored as constituents of the standing vegetation, which eventually adds to the soil as plant litter or root deposition.

The productivity of the vegetation growing on the soil is related to the soil's carbon input rate, measured by the net primary production (NPP) (Smith, 2008). The NPP varies with climate, land cover, species composition and soil type, and also shows seasonal variations due to its dependence on light and temperature. Smith, (2008) explained that over long periods, an amount of NPP enters the soil as organic matter (decomposition of plant matter) and converted to carbon dioxide and methane via soil heterotrophic respiration processes. The remaining carbon is referred to as net ecosystem production (NEP). Although harvesting, fires, and insect damage can also remove carbon, when combined with heterotrophic processes, they can counterbalance the terrestrial carbon dioxide input from global primary production, and the residual carbon is known as net biome production (NBP), which can be positive or negative depending on whether the ecosystem is a carbon source or sink (Smith, 2008).

Human impact disrupts the carbon cycle balance because carbon enters the atmosphere from burning of fossil, cement production, the ocean, land use, animals, plants, and the geological reservoirs through mining. The emission rate of carbon in the atmosphere remains unbalanced and the speed at which carbon is used makes it non-renewable, which disrupts the global carbon cycle. Climate, vegetation, parent material, topography, and time are all important factors influencing soil carbon. Soil carbon reservoir has been suggested as both a sink and a source of atmospheric carbon dioxide. It is considered a source when net decomposition exceeds carbon inputs into the soil, which could be due to human activities or global warming, and it is considered a sink when the difference between net ecosystem carbon uptake and tree growth rates exceeds the net ecosystem carbon uptake (Trumbore, 1997). The degree and timing of response are determined by the amount of carbon available to respond rapidly to climate and vegetation changes, as well as the time lag between carbon fixation in plants and subsequent release to the atmosphere during decomposition.

2.6 Toxicity of pesticides towards earthworms under climate change scenarios

2.6.1 The fate of pesticides

Climate change has left indelible imprints on the ecosystem, including extreme temperature and rainfall events, as well as an increase in atmospheric carbon dioxide (CO₂) concentrations, which may significantly affect the usage, distribution, and degradation patterns of pollutants such as pesticides (Saha *et al.* 2019). Weather variables such as rainfall, temperature, and wind extremes influence pesticide fate and behaviour in the environment. It is essential to understand the environmental fate of pesticides because they are biologically active and designed to interfere with metabolic processes. The potential health risks that pesticides can have on human and environmental health need to be evaluated, after which one must take precautions to mitigate their effects. Applying pesticides to targeted areas predictably allows these chemicals to affect organisms and the environment. It is in the understanding of pesticides' physical, chemical and biological processes that one can realize their impact on target and non-target species. This understanding is crucial for improving pest management strategies that must have minimal adverse effects on human and environmental health. One of the most critical factors in the transport of pesticides in the soil is the sorption-desorption process because it controls the volume of pesticides available for distribution (Rice *et al.* 2007).

In terms of pesticide efficacy, it was widely assumed that some pesticides are more hazardous to insect pests at higher temperatures (Noyes and Lema 2015). However, recent reports such as de Beeck *et al.* (2017), discovered that chlorpyrifos decomposed more at higher temperatures, resulting in decreased mortality and oxidative damage to insect pests. This is attributable to the fact that organophosphate insecticides hydrolyze at a faster rate at higher temperatures (Hooper *et al.* 2013). Pesticides that undergo aqueous phase hydrolysis, such as organophosphates, carbamates, synthetic pyrethroids, and sulfonylureas, are generally temperature-sensitive (Choudhury and Saha, 2020). When exposed to intense sunlight, some insecticides undergo substantial photodegradation. The loss of pesticides due to volatilization is also favoured by rising temperatures (Otieno *et al.* 2013). Microbial activity in soil will also increase when temperature and moisture levels rise. As a result, the rate of pesticide degradation induced by soil microbes will increase. Thus, with the higher dissipation of pesticides and the climate adaptation of pests as a result of global warming, pesticides will most likely be used more frequently and at a higher application rate. (Choudhury and Saha, 2020). The uncertainties related to climate predictions make it challenging to predict the effect of climate change on pesticides. However, according to Bloomfield *et al.* (2006), changes in temperature and seasonality and intensity of rainfall are the main drivers in pesticide fate and transport. Also, changes in land use accompanied by climate change events will substantially affect the fate of pesticides in the soil environment.

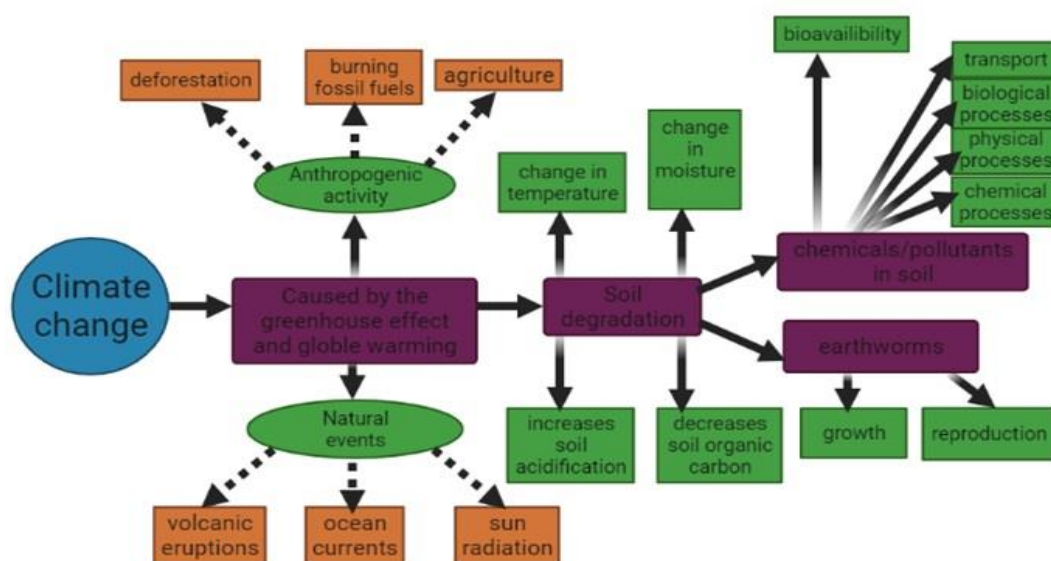


Figure 3: Fate and behaviour of pesticides towards earthworms under the influence of climate change.

Figure 3 illustrates a simple representation of how climate change affects soil pesticides and inevitably soil organisms, specifically earthworms. Climate change is mainly caused by the greenhouse effect which is a natural effect that is accelerated by anthropogenic activities such as deforestation, burning of fossil fuels and agricultural practices. Deforestation and agriculture can go hand in hand, because according to Bennett (2017), 25% of the world's greenhouse gas emissions comes from deforestation through practices such as logging and burning of biomass. Thus, making agriculture one of the most important causes of deforestation. As stated above, climate change does have a great effect on the soil ecosystem and as a result it will influence soil organisms as well as chemicals in the ground. Several studies have found that climate change affects soil dynamics, which in turn affects pesticide toxicity to earthworms (Table 1).

2.6.2 Effect of climate change drivers on pesticide toxicity to earthworms

Chemical pollutants including pesticides can be altered by climate change when the climate change drivers alter the physical, chemical and biological properties of ecosystems (González-Alcaraz and van Gestel, 2015). The impacts of climate change on pesticides may be both indirect and direct (Kattwinkel *et al.* 2011). The indirect effects include changes in pesticide exposure due to the shifts in cultivation towards higher latitudes and extension of cultivation periods (Tubiello *et al.* 2002; Bloomfield *et al.* 2006). Potential enhancement of pesticide volatility and degradation could affect the effectiveness of pesticides against pests and thus could increase application levels for pesticides (Noyes *et al.* 2009). This rise in the use of pesticides may be in both quantities but also the scope of use (Koleva and Schneider, 2009). In terms of direct effects, climate change can affect the decomposition and toxicity

of the pesticides (Kattwinkel *et al.* 2011), especially in the expected temperature and precipitation alternations (Noyes *et al.* 2009). Degradation of the pesticide is dependent on soil moisture and temperature (Kookana *et al.* 2010). Soil temperature and soil moisture are key climate change drivers influencing earthworm growth, survival, fecundity, and behaviour (Edwards and Bohlen, 1996), and indirectly influencing the environment of the earthworm and food availability (Curry, 2004). Also, soil temperature and moisture influence most characteristics of the life cycle, such as weight, cocoon incubation time, sexual maturity initiation, reproduction and life span. Temperature rise can speed earthworm growth and reproduction rate (Uvarov *et al.* 2011). It has also been shown that, in *Aporrectodea caliginosa* earthworms, soil moisture and temperature affect biomarkers (Booth *et al.* 2000). Synergistic responses have been reported in many studies dealing with the impact of soil moisture on effects of various chemicals (Bindesbøl *et al.* 2005; Friis *et al.* 2004; Long *et al.* 2009; Lima *et al.* 2011).

A study done by González-Alcaraz *et al.* (2018) assessed how different combinations of air temperatures and soil moisture contents (20°C and 25°C; 30% and 50%) affected the bioaccumulation kinetics of zinc and cadmium in the earthworm species *Eisenia andrei*. The results of this study indicated that the earthworms accumulated zinc rapidly in contaminated soils. Still, when put in uncontaminated soil, they quickly eliminated the zinc. The air temperatures of 20 and 25°C and the soil moisture contents of 30% and 50% had no significant impacts on zinc bioaccumulation in the earthworms. However, higher temperatures and moisture allowed cadmium to be taken up and eliminated at faster rates. The kinetics of cadmium reduced when the temperatures were higher, coupled with lower moisture contents. Analysis already performed in other studies indicates the value of ecotoxicological studies and the fact that climate change variables influence the bioavailability of pollutants in variables of soil climate change must be considered while performing these experiments. The study on *Eisenia fetida* species by Pelosi *et al.* (2014) also reported how climate change can affect soil organisms as well as contaminants in the soil. According to their findings, temperature and soil moisture content influenced soil enzyme behaviour, as earthworms responded to pesticides differently. The toxicity of earthworms varied when exposed to the same pesticide concentration at different temperatures, decreasing at lower temperatures and increasing at higher temperatures. This report shows that temperature variability can modify the effects of pesticides on earthworm populations. However, more research is required to better understand the complex relationship between environmental factors and the toxicity of chemicals especially the dynamic interaction between climate change drivers and pesticide toxicity to earthworms. Toxicokinetics, bioaccumulation, and detailed life cycle traits in earthworm toxicology research can help explain the exposure pathways taken by soil pollutants in tested species under current projected climate change scenarios which will help in better understanding the effects these pollutants in soil and soil vertebrates with the aim of taking comprehensive mitigation strategies as well as sustainable use of agricultural pesticides.

Table 1: Effects of climate change parameters on the toxicity of pesticides towards earthworms.

Study	Climate change parameters					Earthworm species	Pesticide used	Effects on the life cycle traits of earthworms and biomarkers
	Temperature	Precipitation/Soil moisture	pH	Ultraviolet radiation	Acidification			
Friis <i>et al.</i> 2004	N/A	Soil water potentials from pF 1.5 (wet) to pF 5 (very dry) were obtained	N/A	N/A	N/A	<i>Apporectodea caliginosa</i>	Copper	With increasing drought level, the whole-body burden of copper increased from about 40 microg Cu g(-1) dry weight to about 90 microg Cu g(-1). When the worms were exposed to drought, the osmolality in their body fluids increased.
Booth <i>et al.</i> 2008	5-20 °C	15-30% WHC	N/A	N/A	N/A	<i>Apporectodea caliginosa</i>	N/A	ChE and GST activity increased with temperature
Uvarov <i>et al.</i> 2011	Temperature fluctuations (2 °C ± 5 °C)	N/A	N/A	N/A	N/A	<i>Lumbricus rubellus</i> and <i>Dendrobaena octaedra</i>	N/A	Increase in temperature increased earthworm growth and reproduction rate
Velki and Ečimović, 2015	15, 20 and 25 °C	NA	NA	NA	NA	<i>Eisenia fetida</i>	Imidacloprid, Alpha-cypermethrin, Indoxacarb, combined (Chlorpyrifos and cypermethrin), Lambda-cyhalothrin, combined (Difenoconazole and propiconazole), combined (Azoxystrobin and cyproconazole), Tembotrione, Imazamox, Diquat, Fluazifop-p-butyl, Glyphosate	Increase in temperature mostly resulted in increased pesticide toxicity; whereas toxicity decreased at lower temperature; Investigation of mechanisms by which temperature affects the toxicity is required.

Lima <i>et al.</i> 2015	8, 20 and 28 °C	55% WHC	pH 5.8	NA	NA	<i>Eisenia andrei</i>	Carbaryl	Synergistic ratios showed a tendency to synergism at high temperatures; <i>temperature</i> increased the deleterious effects of carbaryl to <i>Eisenia andrei</i>
De Silva <i>et al.</i> 2009	20 ± 2 °C, 26 ± 2 °C	50% WHC	pH of 5.9–6.1	NA	NA	<i>Eisenia Andrei</i>	Carbendazim, and carbofuran, chlorpyrifos	Survival was more sensitive at the higher temperature; effects on reproduction and growth varied inconsistently with temperature and soil types; Pesticide toxicity decreased in the order carbendazim > carbofuran > chlorpyrifos
Bandeira <i>et al.</i> 2020	20, 25 and 28 °C	60% WHC	pH of 6.0	NA	NA	<i>Eisenia andrei</i>	Imidacloprid	Toxicity of insecticide showed a tendency to increase with increase in temperature and this was evident both in the number of juveniles and the percentage of initial weight
Zou <i>et al.</i> 2018	20 °C	35% WHC	Varied	NA	Acidified soils (pH = 5.5, 4.3 and 3.1)	<i>Eisenia fetida</i>	Chlorpyrifos, triazophos and dimethoate	The toxicity of OPs was slightly increased with the decrease of soil pH; Bioavailability and toxicodynamics are key factors for toxicity variation.
Hackenberger <i>et al.</i> 2018	20 °C and 25 °C	30% and 50% WHC	pH of 6.0 ± 0.5	NA	NA	<i>Eisenia fetida</i>	Propiconazole and chlorantraniliprole	Applied temperature and soil moisture combinations affected the selected biomarkers; the most important interaction was between concentration and temperature.

2.7 Discussion and conclusions

The soil ecosystem is a critical environment that provides many services. It stores carbon, provides a medium for plant growth, and serves as a natural environment for organisms. The soil environment interacts with the atmosphere and hydrosphere and provides many human civilisation materials and food to consume. Thus, the soil environment's health is crucial for human, plant and animal life, and protecting this natural resource is essential for survival. Activities such as mining and agriculture provide a source of pollution to the soil environment. Mining disrupts the earth's crust and displaces large volumes of soil, ruining the environment. Refining mined materials pollute the soil and water systems. Also, large scale agricultural practices alter the natural ecosystem and the use of pesticides can pollute the soil and water systems through runoff and leaching. Earthworms are keystone species and are generally used in ecotoxicological studies since they interact with the soil directly and perform many functions in soil processes (Maboeta *et al.* 2002; Reinecke *et al.* 2002; Reinecke and Reinecke, 2004; Maboeta and Fouche, 2014). The interaction between earthworms and soil makes them a relevant species to be used in laboratory tests. There are many different earthworm species used depending on the region and tests done. Most soil ecotoxicological studies use earthworms in their tests (Maboeta *et al.* 2002; Reinecke *et al.* 2002; Jubileus *et al.* 2013; Maboeta and Fouche, 2014; Lima *et al.* 2015; González-Alcaraz *et al.* 2018; Oladipo *et al.* 2019) to name a few, and this gives further evidence that earthworms are a suitable species to use in these studies. Table 1 above shows the results of studies on the effect of pesticides towards earthworms under climate change scenarios. Different concentrations of pesticides had different effects on the various life cycle traits of earthworms and biomarkers. When climate change variables are introduced, these same concentrations had different effects on the on the same tested parameters, and this will again supply more evidence for researchers to take these variables into account.

Global climate change has an impact on ecosystems worldwide. The focus of this review is on the potential effects of climate change on pesticides and their implications for earthworms in the soil ecosystem. Climate change has been shown to have a significant impact on the toxicity of pollutants in soil by altering weather patterns and natural global cycles. Previous research has found that varying temperature and moisture affect the bioavailability of pollutants in the soil ecosystem. According to many analyses from Intergovernmental Panel on Climate Change (IPCC) report, the global temperature and other climate change drivers are becoming more severe and erratic; therefore, current research must consider environmental concerns related to climate change. The year 2019 was the second warmest year in 140 with an increase of 0.95°C (Olivier and Peters, 2020), and the reality is that global warming is affecting the human and natural world. This review highlights some of the impact of climate change on the soil and the pollutants in the soil. Most toxicological analysis using earthworms does not take these climate change variables into account; thus, they use standard temperatures and soil moisture contents while focusing mainly on single pollutants and their effects. However, these variables undoubtedly affect the bioavailability of these pollutants in the soil. To accurately determine the effects of these pollutants in natural ecosystems, one must consider these variables. Studies such as (Chen *et al.* 2011; Gilman *et al.* 2010; González-Alcaraz and van Gestel, 2015; González-Alcaraz and Van Gestel,

2016a; Olivier and Peters, 2020), to name a few all indicate that climate change can directly affect the soil temperature and moisture. When air temperatures increase, the temperature of the soil surface increases as well and an increase in temperature will also reduce the soil moisture. An increase in the temperature and a decrease in the soil moisture will affect the characteristics as well as the bioavailability of pollutants in the soil. Climate change also makes rainfall patterns more unpredictable, and this could lead to flooding and droughts. Both events can cause the soil moisture to become excessive (flooding) or minimal (droughts). Excess water can cause leaching in the soil which will promote acidification and runoff from agricultural areas and mines which can pollute nearby areas. Droughts can reduce the vegetation cover resulting in increased soil erosion. These conditions will also affect earthworm populations as well as the bioavailability of contaminants in the soil. Future studies must consider collecting relevant data as climate change becomes a more urgent issue. Several studies such as (Giska *et al.* 2014; González-Alcaraz *et al.* 2018b; González-Alcaraz and van Gestel, 2015; González-Alcaraz and Van Gestel, 2016; Lee and Kim, 2013; Rice *et al.* 2007), all show that other factors such as temperature and soil moisture affect the bioavailability of pesticides in the soil, thus making it essential to be taken into account and not only using the concentrations of pollutants as an endpoint.

The soil itself has capabilities to act as a buffer against pollutants as well as variables such as temperature, moisture and acidification. The properties and make-up of the soil give it these characteristics to act as a buffer. The organic matter (carbon) in the soil can help reduce the effects of heavy metals for instance in the soil. The variety of microbial communities in the soil also helps to break down organic pollutants. The soil surface itself acts as a buffer to temperature changes for the lower soil levels. Certain soil types like clay can retain water for longer periods helping with soil moisture. With regards to the acidification process, it is a slow and natural process although it is hastened by pollution. Soil is the buffer against acidification because soil buffers external H^+ ions, and only when the H^+ input exceeds the maximum of soil buffer capacity, it will cause soil acidification (Meng *et al.* 2019). Soil health can maintain the health of animals, plants, humans and the environment (Patil and Lamnganbi, 2012), making it a vital system that needs to be monitored and protected. Soil pollutants such as heavy metals, organic contaminants and persistent organic pollutants have been found in soil ecosystems and pose a threat to animal, human and plant health (Biswas *et al.* 2018). The possibility of these pollutants affecting human and animal life is increasing with increasing pollution. Climate change effects are becoming more and more evident. The impact they have on soil pollutants is seen in ecosystems worldwide, making it a critical variable when doing ecotoxicological research. More research is needed to obtain adequate data on the interaction of environmental factors such as the temperature and moisture and other extreme climate change drivers. This has become more relevant with the acceleration of global climate and to enable relevant key climate regulatory authorities to implement laws to mitigate and manage the effects of soil pollution on ecosystem health.

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Declaration of interests

There is no conflict of interest.

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CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental design

The laboratory experiment was done at the North-West University, South Africa. [Figure 4](#) is a graphical representation of the experimental design.

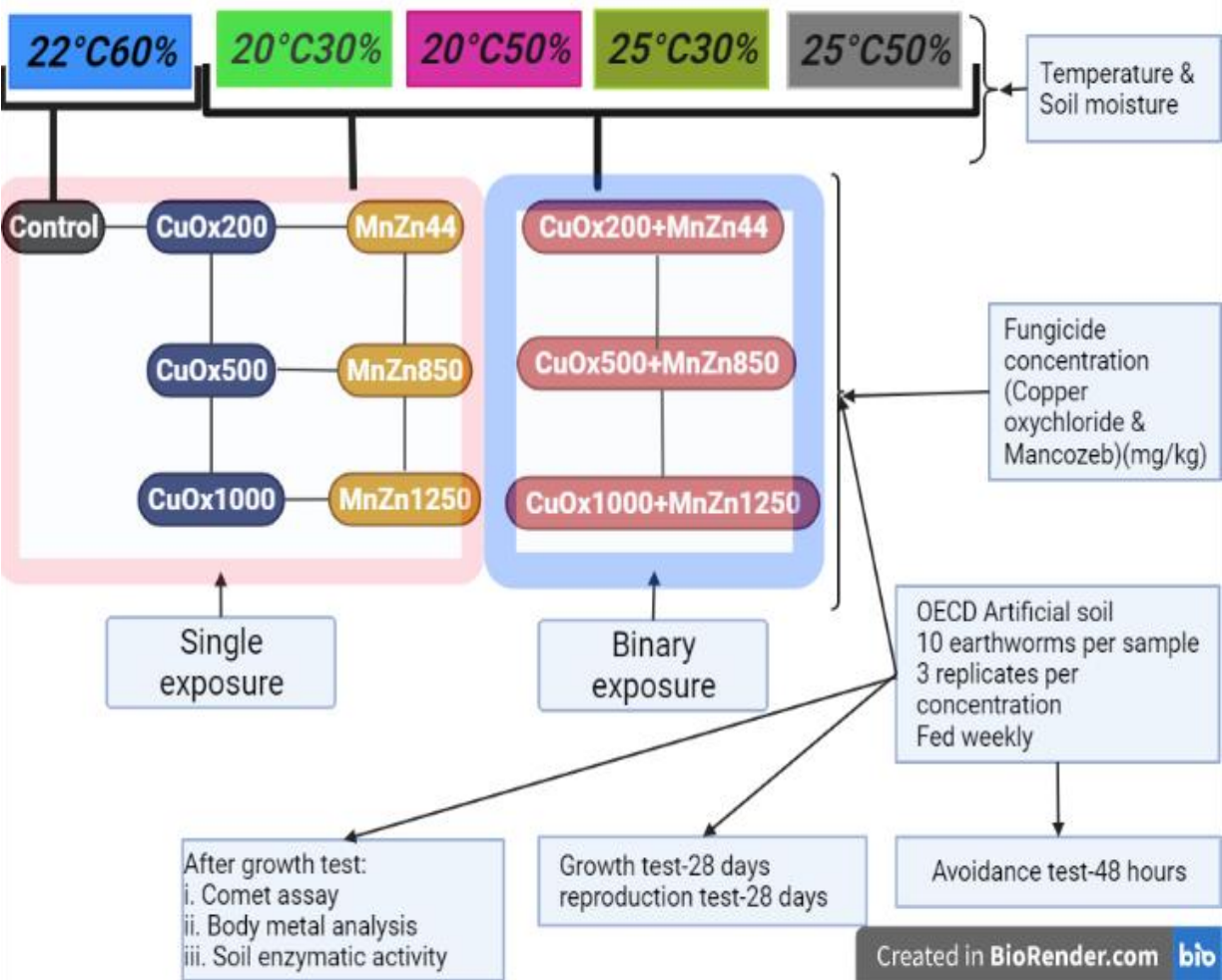


Figure 4: Summary of the experimental design to test the effect of climate change on fungicides in the soil using earthworms as bioindicators.

As shown above in [Figure 4](#), these experiments were done under two different temperatures namely 20°C and 25°C and two different moisture contents viz. 30% and 50%. A control was done only at 22°C60% because that is the temperature and soil moisture used in standardised tests according to the protocol.

3.2 Soil preparation

All exposures in this study were done using artificial soil prepared according to the standard guideline (OECD 222) set out by the Organisation for Economic Co-operation and Development (OECD, 2015). The artificial soil was prepared seven days prior to the start of the experiment and consisted of three substances (based on the dry weight): 10% sphagnum-peat, 70% silica sand and 20% kaolin clay. These three substances are thoroughly mixed to ensure a uniform substrate.

The pH of the soil is then measured using the Hanna instruments edge pH meter. It is measured in a mixed sample of 1:5 ratio using 1 M solution potassium chloride (KCl) or 0.1 M calcium chloride (CaCl₂) (OECD, 2015). The pH of the soil should be 6-6.5, if not it is adjusted using 0.3-1% pulverised calcium carbonate (CaCO₃) (OECD, 2015).

The water holding capacity was done also using the (OECD, 2015) guidelines. To determine the water holding capacity (WHC) a Sartorius moisture analyser was used (Sartorius MA35M-000230V2). Triplicate samples of 5 g each were used. Filter paper was used to seal the bottom of each tube, and they were placed into a glass beaker with water and left for three hours. Afterwards, these tubes were then placed into a beaker with fine silica sand and left for a further two hours in order for the soil in the tubes to reach a 100% WHC. After the two hours, each tube was analysed using the Sartorius moisture analyser. The readings obtained was used to calculate the 30%, 50%, 60% and 100% WHC.

Equation 1: $WHC \text{ (in of dry mass)} = \frac{(S-D)}{D} \times 100$

Where S is water saturated soil and D is dry soil (OECD, 2015). The mathematical mean of the samples was calculated and used to obtain the desired moisture content.

3.3 Spiking of soil samples

Two fungicides were used in this experiment viz. copper oxychloride and mancozeb. These metal-based fungicides were used in single and binary mixtures. The concentrations of copper oxychloride and mancozeb used in this study was as follows 200 mg/kg, 500 mg/kg and 1000 mg/kg and 44 mg/kg, 850 mg/kg and 1250 mg/kg respectively. These concentrations were based on previous studies (Eijsackers *et al.* 2005; Helling *et al.* 2000; Maboeta *et al.* 2003; Oladipo *et al.* 2019; Vermeulen *et al.* 2001). The concentrations were mixed in single and binary mixtures as shown in [Figure 4: Summary of the experimental design to test the effect of climate change on fungicides in the soil using earthworms as bioindicators.](#) It was dissolved in distilled water before adding it and mixing it into the soil in the precise amount of H₂O needed to achieve moisture contents of 30% and 50% respectively.

3.4 Earthworm bioassay

The earthworm growth and reproduction test was done using OECD guideline 222 (OECD, 2015), and the avoidance test was done in accordance to the ISO guidelines (ISO 527-2, 2003) and the artificial soil used in these experiments was made using the OECD 222 guidelines (OECD, 2015) as stated above. Adult earthworms (*Eisenia andrei*), with a visible clitellum, were used in these experiments. The earthworms were purchased from Ecolife Vermikompos Pty Lmt.

Three replicates per concentration was tested. Ten earthworms were placed in each container and pre-weighed individually to ensure the individual weights were between 0,250-0,600 g (OECD, 2015). The weighing is done by rinsing the earthworms in deionised water, then drying them on paper towels and weighing them individually.

The tests were conducted at 20°C and 25°C in climate-controlled rooms. The soil moisture was kept at either 30% or 50% for the entire experiment. Soil moisture was maintained by regularly weighing the containers and comparing it to the weight at the start of the experiment. When there was a difference in weight deionized water was added using a spray bottle at the surface of the soil. According to the OECD (2015) guidelines the water content should not vary by more than 10%.

After the 28-day period the adult earthworms were removed, and 5 g of manure was placed in each container and left for a further 28 days. This 5 g is placed there as feed for any juveniles. There is no other feeding taking place for the remainder of the reproduction test.

3.4.1 Growth, reproduction and mortality

Containers (500 ml) were filled with 500 g of artificial soil and ten adult earthworms were added to each container. The earthworms were fed with horse manure collected from a farm outside Potchefstroom. The manure was air dried prior to being used. Each container received 5 g of finely crushed and moistened horse manure every seven days for the entire 28-day growth test. The manure was moistened with distilled water. Containers were observed every week and any uneaten food was removed from the container. The containers with uneaten feed had the ratio of feed reduced to avoid fungal growth. The relative growth rate (RGR) of the earthworms was calculated as follows:

$$\text{Equation 2: } RGR = \frac{(W_t - W_o)}{W_o} \times 100\%$$

RGR = Relative growth rate

W_o = Initial average weight of earthworms (day 1)

W_t = the average weight on day 7, 14, 21 and 28

Every seven days, the earthworms were removed from their containers, cleaned with distilled water, and dried on paper towels. They were then weighed before being returned to their respective containers; however, any unresponsive earthworms after poking were recorded as dead.

After the 28-day period, the mature earthworms were removed from the container and only the juveniles and cocoons were left behind. The mature earthworms were used in body metal analysis as well as comet assay. The cocoons were left for a further 28 days before the juveniles were counted and recorded for reproductive success. Thereafter, soil samples were removed and used in the soil enzymatic tests.

3.4.2 Earthworm avoidance test

The concentrations used in avoidance-behaviour trials were the same as with the growth and reproduction experiment. As shown in [Figure 4](#), each container was divided equally in the middle. One side had the control soil, and the other side had the test soil. To place the soil appropriately without mixing a divider was placed in the middle when adding the soil, once done the divider is removed and ten adult earthworms are placed in the middle and left for 48 hours in environmental chambers under the desired temperature. After the 48-hour period, the two soils are divided again and the earthworms on each half are counted and recorded. Any earthworms found in the middle of the two soils were counted as half for each side. Missing earthworms were recorded as escaped. The avoidance response was calculated as follows (ISO 527-2, 2003):

$$\text{Equation 3: } AR = \frac{N_c - N_t}{N} \times 100$$

AR = Avoidance Response

N_c = Number of earthworms in control soil

N_t = Number of earthworms in test soil

N = Total number of earthworms (control soil + test soil)

3.5 Comet assay

The comet assay experiment was carried out using the protocol described by Dhawan *et al.* (2003), and Reinecke and Reinecke (2004). Three earthworms per replicate was used for the comet assay and were taken from the growth experiment exposure after the 28-day period. Earthworms were placed in petri dishes with moistened paper towels 24 hrs before the test was done for them to empty their gut content so that no soil contamination occurred while preparing the suspension cell samples. All the comet assay solutions (lysis solution, buffer solution, alkaline solution, extrusion solution and electrophoresis solution) were prepared prior to the start of the test and left in a refrigerator to chill to 4 °C. The earthworms were individually placed in 1 ml extrusion fluid (95 % PBS, 5 % absolute ethanol, 10 mg/ml guaiacol glycerol ether and 2.5 mg/ml EDTA) in Eppendorf tubes for a period of three minutes.

The extrusion solution was then centrifuged at 3000 rpm for five minutes at 4°C, the process was repeated three times with an ice-cold PBS to wash the cell samples. Once completed it is re-suspended in 0.5 ml PBS (Voua Otomo and Reinecke, 2010). An aliquot of 10 µl of suspension was mixed with 100 µl of low melting agarose which was pre-heated to 40°C. The mixture was then transferred onto microscope slides pre-coated with agarose. The slides were orientated horizontally and were kept at 4°C in the dark for 15 minutes.

Thereafter, the slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO per 1000 mL) for one hour at 4°C in the dark. Thereafter, the lysis solution was aspirated and the slides placed in chilled electrophoresis solution (10 N NaOH (200 g/500 mL dH₂O) and 200 mM EDTA (14.89 g/200 mL dH₂O, pH 10)) and left for a further thirty minutes in the dark thereafter. The slides then went through electrophoresis for thirty minutes under 20 V (1 V/cm) and 300 mA in electrophoresis solution to allow for the denaturing and movement of the broken ends of the nucleus (Reinecke and Reinecke, 2004; Voua Otomo and Reinecke, 2010). Once electrophoresis was completed, the slides were then placed in a buffer solution (30 mL NaOH, 5.0 mL EDTA and 0.4 M Tris, pH 7.5) for five minutes, then rinsed twice in cold water for two minutes. The slides were then dehydrated in absolute ethanol for twenty minutes, allowed to air dry and stored in darkness until staining and analysis could be done.

Before analysis, the slides were rehydrated in pre-chilled distilled water for thirty minutes followed by staining with a fluorescent dye (ethidium bromide) for 15 minutes. Once the staining was completed, the slides were kept on moistened paper towels and viewed on an inverted research microscope (Nikon's Eclipse TE2000). The images of the comets were then viewed and captured. The comet images were scored with Image-J software, version 1.53e, which quantifies the extent of the DNA damage.

3.6 Body metal analysis

This method was done according to USEPA (1996). After 28 days of exposure, earthworms were removed from the substrate and kept on moist paper towels in petri dishes for 24 hours in order for them to extrude their gut content. Earthworms were then euthanized by placing them individually in 70% ethanol. The earthworms were then placed individually in Eppendorf tubes, frozen at -86°C for 24 hrs and then freeze-dried using the Labnet Prism R freeze drier for 72 hrs.

The freeze-dried samples were then digested in 2 ml nitric acid (HNO₃) and placed in a heat-block which is then covered by a watch glass. Samples are then heated to 95°C ± 5°C without boiling it, and refluxed for 10 to 15. Once the samples are cooled, 5 ml of concentrated HNO₃ is added and this solution is refluxed for 30 minutes. The formation of brown fumes is an indication of oxidation of the sample by HNO₃, and once this occurs the process is repeated until no fumes occur. This solution is then evaporated to 5 ml without boiling (USEPA, 1996). The solution is then diluted with distilled water to obtain a ten times dilution (1: 9, v/v). Once the dilution is completed, the solution is filtered through a 0.45 µm Whatman no. 41 filter paper, and analysed using inductively coupled plasma mass spectrometry (ICP-MS) for heavy metals. Copper (Cu), zinc (Zn), and manganese (Mn) were the heavy metals tested for.

3.7 Soil enzymatic activity

The soil samples (50 g) were taken from all the exposure concentration groups after the 28-days growth test and then frozen at -86°C in sealed bags. Prior to the soil enzymatic activity test, β -glucosidase, urease, and alkaline phosphatase, the sample soils were air dried (Alef *et al.* 1996; Claassens *et al.* 2006). The tests were done at the Agricultural Research Council (ARC) in Potchefstroom, South Africa.

3.7.1 β -glucosidase activity

To determine the β -glucosidase activity Dick *et al.* (1996) was used. The substrate used in the soil was p-nitrophenyl- β -D-glucosidase and the liberated p-nitrophenol by enzymatic hydrolysis was determined by THAM–NaOH (0.1M, pH 12). Thereafter the optical density was measured and compared to the optical density of the p-nitrophenol standard solution (Salazar *et al.* 2011). The β -glucosidase enzymatic activity is thus expressed in μg p-nitrophenol g^{-1} dwt h^{-1} . The chemicals used during this assay were toluene, modified universal buffer (MUB), p-nitrophenyl- β -D-glucosidase (PNG), calcium chloride (CaCl_2), and sodium hydroxide (NaOH).

One gram of soil was weighed and added into a 50 ml glass bottle. A solution made up of 0.25 ml of toluene, 4 ml of MUB, and 1 ml of PNG was then mixed with the soil. The caps of the bottles were closed and the bottle was swirled in order to mix the contents and thereafter incubated in a water bath at 37°C for 1 h. Thereafter, 1 ml of calcium chloride (CaCl_2) and 4 ml of hydroxy methyl-aminomethane (THAM) buffer (pH 12) were added. The contents were swirled again in order for it to be mixed and immediately after mixing the solution was filtered through a funnel with Whatman No. 1 filter paper. The control was made by adding the PNG substrate after the incubation but no CaCl_2 and THAM buffer. A calibration curve was prepared using the standard p-nitrophenol solution in the concentrations of (0, 10, 20, 30, 40, 50 and 100 μg).

Thereafter, 300 μL of the supernatant from each sample was pipetted into a 96-well transparent microplate. The colour change was measured spectrophotometrically at a wavelength of 410 nm using a spectrophotometer (Biotek ELx800). The β -glucosidase activity was then determined for each sample as μg g^{-1} dry weight of soil h^{-1} using the linear standard curve. The β -glucosidase activity was calculated using the following calculation:

$$\text{Equation 4: } P\text{-Nitrophenol } (\mu\text{g g}^{-1} \text{ dwt h}^{-1}) = \frac{C \times V}{\text{dwt} \times \text{SW} \times T}$$

C = measured concentration of p-nitrophenol ($\mu\text{g ml}^{-1}$ filtrate)

Dwt = dry weight of 1 g moist sample

V = total volume of the material suspension in ml

SW = weight of samples used (1 g)

T = incubation time in hours

3.7.2 Phosphatase alkaline activity

The alkaline phosphatase was done according to Alef and Nannipieri (1995). Soil alkaline phosphatase activities were determined by determining the release of p-nitrophenol in the soil and this is done by incubating the soil with p-nitrophenyl. The enzymatic activity is thus expressed in $\mu\text{g p-nitrophenol g}^{-1} \text{ dwt h}^{-1}$. The optical density was measured and compared with the optical density of the p-nitrophenol standard solution (Salazar *et al.* 2011).

A solution made up of 0.2 ml toluene, 4 ml MUB, and 1 ml of p-nitrophenyl phosphate solution (PNP) was added to one gram of soil in a 50 ml glass bottle. The bottle was swirled to mix the contents and incubated in a water bath for 1 h at 37°C. After 1 h, 1 ml of CaCl_2 and 4 ml of NaOH were added, swirled to mix the contents, and filtered with Whatman No.1 filter paper. The control was prepared the same way but without any substrates. A calibration curve was prepared using the standard (p-nitrophenol) solution in the concentration range of 0, 10, 20, 30, 40, 50, and 100 μg . An aliquot of 300 μL of the filtrate was pipetted into a 96-well microplate and a spectrophotometer (Biotek ELx800) was used to read the intensity of the colour development at 410 nm. The soil alkaline phosphatase activities were determined for each sample as $\mu\text{g g}^{-1}$ dry weight of soil h^{-1} interpolated from the linear standard curve. The phosphatase alkaline activity was calculated using the following calculation:

$$\text{Equation 5: P-Nitrophenol } (\mu\text{g g}^{-1} \text{ dwt h}^{-1}) = \frac{C \times v}{\text{dwt} \times \text{SW} \times t}$$

C = measured concentration of p-nitrophenol ($\mu\text{g ml}^{-1}$ filtrate)

Dwt = dry weight of 1 g moist sample

V = total volume of the material suspension in ml

SW = weight of samples used (1 g)

T = incubation time in hours

3.7.3 Urease activity

The urease activity was done as described by Kandeler and Gerber (1988). This assay determined the amount of released ammonia from the soil samples. The chemical solutions used during this assay were urea solution, potassium chloride solution (KCl), sodium hydroxide (NaOH), sodium salicylate solution, sodium dichloroisocyanide, borate buffer, ammonium standard solution I and ammonium standard solution II.

A 2.5 ml solution of urea was mixed with five grams of soil in a 100 ml glass bottle. Caps were used to close the glass bottles and it was then incubated for 2 h in a water bath at 37°C. Thereafter, 50 ml of KCl solution was added to extract the liberated ammonium and the bottle was placed on a mechanical shaker for 30 min. The suspension was filtered through Whatman No.1 filter paper. The control was prepared by adding 2.5 ml of distilled water in place of the urea solution before adding KCl (Kandeler and Gerber, 1988). To determine the ammonium concentration, 1 ml of clear filtrate was pipetted into a 50 ml glass bottle. Then 9 ml of distilled

water, 5 ml of the sodium salicylate and sodium hydroxide solutions, and 2 ml of the dichloroisocyanide solution were added. The solution was left at room temperature for 30 min, thereafter the optical density (ammonium content) was measured at 600 nm using a spectrophotometer (Biotek ELx800). The method is based on the reaction of sodium salicylate with NH₃ in the presence of sodium dichloroisocyanurate, which forms a colour complex (green) under an alkaline pH condition (Attademo *et al.* 2021). The calibration curve was constructed using ammonium standard solution II in the concentration range (0, 10, 20, 30, 40, 50, and 100 µg) and the urease activities for each sample were expressed as µg NH₄ N g⁻¹ dry weight of soil 2 h⁻¹ interpolated from the linear standard curve. The results were calculated using the following formula:

$$\text{Equation 6: } (\mu\text{g NH}_4 - \text{N g}^{-1} \text{ dwt } 2 \text{ h}^{-1}) = \frac{\mu\text{g NH}_4 - \text{N ml}^{-1} \times V \times 10}{\text{dwt} \times 5}$$

Dwt = dry weight of 1 g moist material

V = total volume of the extract (52.5 ml)

10 = the dilution factor

5 = weight of soil used in the assay

3.8 Statistical analysis

Statistical analyses for the earthworm assays were done using the SigmaStat® program. The difference between sample groups were determined with the one-way analysis of variance (ANOVA). The relative growth rate (RGR) was used for the growth tests. For the earthworm body metal analysis Fisher's LSD method (pairwise comparison) was used.

CHAPTER 4: RESULTS

4.1 Bioassays

The influence of temperature and soil moisture on the toxicity of mancozeb and copper oxychloride to earthworms was evaluated. Biological endpoints included earthworm mortality, growth, reproduction, avoidance behaviour, comet assays and soil enzymatic activities. Physiochemical endpoints included body metal analysis.

4.1.1 Mortality

The percentage mortality of earthworms exposed to copper oxychloride and mancozeb in single and binary mixtures over a 28-day period at various temperatures and soil moistures is reported in [Table 2](#)~~Table-2~~. According to the OECD 222 guidelines for the testing of chemicals under the validity of the test (OECD, 2015), the mortality should not exceed 10% in the control and therefore, this value as an endpoint was considered as a significant response. For the duration of the experiment, mortality in the control, CuOx200, CuOx500, MnZn44, MnZn850, MnZn1250, CuOx200 + MnZn44 and CuOx500 + MnZn850 mg/kg groups were all lower than 10% under the different temperature and moisture combinations. Although, the CuOx500 + MnZn850 mg/kg group under 20°C30% was 13%. However, the mortality in the CuOx1000 and CuOx1000 + MnZn1250 mg/kg groups exceeded 10% in all the temperature-moisture combinations and in the CuOx500 + MnZn850 mg/kg group a significant mortality was only observed at the 20°C30% exposure (13 %). For the CuOx1000 mg/kg group, the mortality was 53% (20°C30%), 13% (20°C50%), 73% (25°C30%) and 23% (25°C50%) respectively. For the CuOx1000 + MnZn1250 mg/kg group, the mortality was 47% (20°C30%), 37% (20°C50%), 70% (25°C30%), and 30% (25°C50%) respectively.

Table 2 The percentage mortality for the copper oxychloride (CuOx) and mancozeb (MnZn) single and binary mixtures under different temperatures (T) and soil moisture (SM) conditions over a 28-day period (n=30).

Percentage mortality (different T and SM conditions)				
Treatments (mg/kg)	20°C30%	20°C50%	25°C30%	25°C50%
Control	0% ^a	0% ^a	0% ^a	0% ^a
CuOx200	0% ^a	0% ^a	0% ^a	0% ^a
CuOx500	0% ^a	0% ^a	3% ±5.77 ^a	0% ^a
CuOx1000	53%±11.54 ^b	13%±15.27 ^b	73%±5.77 ^b	23%±11.55 ^b
MnZn44	0% ^a	3%±5.77 ^a	3%±5.77 ^a	3%±5.77 ^a
MnZn850	0% ^a	0% ^a	3%±5.77 ^a	0% ^a
MnZn1250	0% ^a	0% ^a	3%±5.77 ^a	3%±5.77 ^a
CuOx200+ MnZn44	3%±5.77 ^a	0% ^a	7%±10 ^a	0% ^a
CuOx500+ MnZn850	13%±5.77 ^b	0% ^a	7%±10 ^a	3%±5.77 ^a
CuOx1000+ MnZn1250	47%±15.28 ^b	37%±23.09 ^b	70%±10 ^b	30%±10 ^b

Different superscript letters^{a-b} (rows) indicate significantly different values ($p<0.05$). All the values with the “a” superscript are significantly different from the values with the “b” superscript.

4.1.2 Earthworm growth

The relative growth rates of the earthworms in the different exposures/treatments (data not shown) was done to determine if there was a difference between the prescribed OECD control conditions (22°C60%) and the controls used in the present study (20°C30%; 20°C50%; 25°C30%; 25°C50%). The preceding sentence is incomplete. There was no significant difference ($p>0.05$) between the OECD control conditions (22°C60%) and the controls used in this study. Therefore, the control treatments in this experiment were considered valid controls.

Table 3 summarises the relative growth rate of CuOx treatments under different temperature and soil moisture conditions. The CuOx200, CuOx500 and CuOx1000 mg/kg under 20°C30% were significantly different ($p<0.05$) from each other as well as the control after the 28-day period. Under 20°C50% only the CuOx1000 mg/kg treatment was significantly different ($p<0.05$) from the control group. The CuOx500 and CuOx1000 mg/kg treatments under 25°C30% were significantly different from each other as well as the control treatment. The data from the 25°C50% exposure indicated that there was a significant difference between the CuOx200 and CuOx1000 mg/kg and the control ($P<0.05$). With regards to all three treatments (CuOx200;

CuOx500; CuOx1000 mg/kg) there was a significant difference between 20°C 30% and 20°C 50%, and between 25°C 30% and 25°C 50%.

When comparing the same treatments with each other (indicated by symbols in superscript on day 28 in Table 3) there was a significant difference ($p < 0.05$) between the same treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) under (20°C30%) and (20°C50%), and between (25°C30%) and (25°C50%).

Table 3: The relative growth rate (RGR) (%) of earthworms in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) over a 28-day period under different temperature and soil moisture conditions. (n= 30).

Treatment (mg/kg)	RGR (%)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control 20°C30%	0,46	-3,78	-8,27	-19,41	-28,93 ^{a■}
CuOx200 20°C30%	0,43	-5,88	-11,89	-20,59	-28,55 ^{b•}
CuOx500 20°C30%	0,44	-19,34	-26,74	-32,06	-40,75 ^{c▲}
CuOx1000 20°C30%	0,40	-31,06	-46,35	-52,93	-59,07 ^{d♦}
Control 20°C50%	0,38	5,77	0,53	-10,70	-20,06 ^{a■}
CuOx200 20°C50%	0,40	-4,03	-9,67	-15,74	-25,09 ^{a■}
CuOx500 20°C50%	0,41	-10,46	-17,01	-24,37	-31,73 ^{a■}
CuOx1000 20°C50%	0,38	-22,31	-28,12	-34,58	-41,50 ^{b•}
Control 25°C30%	0,30	23,06	16,55	10,62	1,74 ^{a■}
CuOx200 25°C30%	0,32	5,99	-0,36	-7,09	-14,32 ^{a■}
CuOx500 25°C30%	0,31	1,90	-3,80	-14,30	-24,37 ^{b•}
CuOx1000 25°C30%	0,31	-20,60	-29,57	-30,48	-34,29 ^{c▲}
Control 25°C50%	0,27	23,49	12,93	4,51	15,80 ^{a■}
CuOx200 25°C50%	0,27	16,84	12,04	4,49	-1,56 ^{b•}
CuOx500 25°C50%	0,46	-16,84	-23,90	-25,78	-35,91 ^{a■}
CuOx1000 25°C50%	0,47	-22,19	-31,49	-33,74	-40,00 ^{b•}

Different superscript letters^{a-d} (rows) and symbols^{■•▲♦} (columns) (Day 1 – Day 28) indicate significantly different values ($p < 0.05$). The superscript letters indicate whether there was a significant difference in the concentrations between day 1 – day 28. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 4 summarizes the relative growth rate of the MnZn treatment under different temperatures and soil moistures. The MnZn1250 mg/kg under 20°C30% and 20°C50% was the only treatment that showed a significant difference ($p<0.05$) in these exposures after the 28-day period. Under 25°C30%, all three treatments (MnZn44 mg/kg, MnZn850 mg/kg and MnZn1250 mg/kg) showed significant differences between them and the control. There was no significant difference between treatments and the control under 25°C50%.

MnZn44 mg/kg, MnZn850 mg/kg and MnZn1250 mg/kg (25°C30%) was significantly different ($p<0.05$) from the other MnZn44 mg/kg, MnZn850 mg/kg and MnZn1250 mg/kg treatments under different temperatures and moistures (20°C30%; 20°C50%; 25°C50%).

Table 4: The relative growth rate (RGR) (%) of earthworms in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) over a 28-day period under different temperatures and soil moistures. (n= 30).

Treatment (mg/kg)	RGR (%)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control 20°C30%	0,46	-3,78	-8,27	-19,41	-28,93 ^{a■}
MnZn44 20°C30%	0,40	4,33	-1,17	-9,61	-24,53 ^{a■}
MnZn850 20°C30%	0,46	-16,32	-19,66	-25,46	-30,11 ^{a■}
MnZn1250 20°C30%	0,39	-15,09	-21,20	-28,39	-35,58 ^{b•}
Control 20°C50%	0,38	5,77	0,53	-10,70	-20,06 ^{a■}
MnZn44 20°C50%	0,38	10,58	1,68	-12,91	-21,94 ^{a■}
MnZn850 20°C50%	0,39	-9,42	-14,52	-21,63	-27,86 ^{a■}
MnZn1250 20°C50%	0,38	-10,43	-17,36	-24,34	-32,57 ^{b•}
Control 25°C30%	0,30	23,06	16,55	10,62	1,74 ^{a■}
MnZn44 25°C30%	0,30	12,36	1,87	-2,09	-8,83 ^{b•}
MnZn850 25°C30%	0,31	-4,02	-10,30	-15,72	-20,49 ^{b•}
MnZn1250 25°C30%	0,29	-5,46	-18,76	-25,31	-32,11 ^{c▲}
Control 25°C50%	0,27	23,49	12,93	4,51	15,80 ^{a■}
MnZn44 25°C50%	0,44	-10,39	-14,13	-21,79	-30,37 ^{a■}
MnZn850 25°C50%	0,44	-8,01	-12,82	-19,70	-25,47 ^{a■}
MnZn1250 25°C50%	0,40	-6,13	-11,48	-21,02	-27,82 ^{a■}

Different superscript letters^{a-d} (rows) and symbols^{■•▲} (columns) (Day 1 – Day 28) indicate significantly different values ($p<0.05$). The superscript letters indicate whether there was a significant difference in the

concentrations between day 1 – day 28. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

The binary treatments of (CuOx + MnZn) under different temperatures and soil moistures is summarised in

Table 5

Table 5. Under all the different temperatures and soil moistures (20°C 30%; 20°C 50%; 25°C 30%; 25°C 50%) the CuOx500 + MnZn850 mg/kg and CuOx 1000 + MnZn 1250 mg/kg were significantly different ($p < 0.05$) from the control treatment. Only under 20°C50% were both these treatments (CuOx500 + MnZn850 mg/kg and CuOx 1000 + MnZn 1250 mg/kg) significantly different from each other.

When comparing the same treatments with each other (indicated by symbols in superscript) under different temperatures and moistures (20°C 30%; 20°C 50%; 25°C 30%; 25°C 50%), there was no significant differences ($p > 0.05$) between the CuOx200 + MnZn44 mg/kg and CuOx500 + MnZn850 mg/kg treatments. There was only a significant difference between the CuOx1000 + MnZn1250 mg/kg (20°C50%) and the other temperatures and moistures (20°C 30%; 25°C 30%; 25°C 50%).

Table 5: The relative growth rate (RGR) (%) of earthworms in the binary treatments of copper oxychloride + mancozeb (CuOx + MnZn) (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) over a 28-day period under different temperature and soil moistures. (n= 30).

Treatment (mg/kg)	RGR (%)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control 20°C30%	0,46	-3,78	-8,27	-19,41	-28,93 ^{a■}
CuOx200+MnZn44 20°C30%	0,46	-11,16	-16,98	-25,91	-31,01 ^{a■}
CuOx500+MnZn850 20°C30%	0,40	-22,15	-30,05	-35,47	-41,19 ^{b•}
CuOx1000+MnZn1250 20°C30%	0,39	-20,44	-36,14	-41,59	-50,42 ^{b•}
Control 20°C50%	0,38	5,77	0,53	-10,70	-20,06 ^{a■}
CuOx200+MnZn44 20°C50%	0,41	-1,86	-10,56	-15,97	-24,64 ^{a■}
CuOx500+MnZn850 20°C50%	0,36	-7,74	-11,31	-21,87	-27,21 ^{b•}
CuOx1000+MnZn1250 20°C50%	0,36	-18,88	-36,03	-40,02	-48,80 ^{c▲}
Control 25°C30%	0,30	23,06	16,55	10,62	1,74 ^{a■}
CuOx200+MnZn44 25°C30%	0,31	11,17	1,98	-1,58	-8,31 ^{a■}
CuOx500+MnZn850 25°C30%	0,29	3,90	-5,93	-13,13	-18,60 ^{b•}
CuOx1000+MnZn1250 25°C30%	0,30	-10,84	-18,09	-30,82	-32,06 ^{b•}

Control 25°C50%	0,27	23,49	12,93	4,51	15,80 ^{ab}
CuOx200+MnZn44 25°C50%	0,42	-13,65	-16,72	-17,94	-26,85 ^{ab}
CuOx500+MnZn850 25°C50%	0,37	-15,93	-17,98	-28,68	-37,58 ^{b•}
CuOx1000+MnZn1250 25°C50%	0,40	-8,99	-21,23	-24,82	-38,12 ^{b•}

Different superscript letters^{a-d} (rows) and symbols^{•▲■} (columns) (Day 1 – Day 28) indicate significantly different values ($p < 0.05$). The superscript letters indicate whether there was a significant difference in the concentrations between day 1 – day 28. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

4.1.3 Earthworm reproductive success

The reproductive success was determined by counting the number of cocoons and the number of juveniles hatched over the 56-day period. The average number of cocoons produced, average number of hatchlings and number of hatchlings per cocoon are all shown in Tables 6-8. The highest ($P < 0.05$) average number of cocoons and hatchlings produced in the control groups were the 25°C50%; the average number of cocoons in this treatment was $20,67 \pm 2,52$ and the number of hatchlings was $18,33 \pm 1,15$.

The average number of cocoons and number of hatchlings in the CuOx treatments decreased significantly ($p < 0.05$) as the exposure concentration increased for all the temperature-moisture combinations (Table 6). There was a significant difference in the number of cocoons ($14,67 \pm 2,08$) as well as the number of hatchlings ($11,67 \pm 3,51$) between the control group (20°C30%) and the treatments of CuOx200 mg/kg (20°C30%) ($7,33 \pm 0,58$; 5 ± 1), CuOx500 mg/kg (20°C30%) ($4,33 \pm 0,58$; $3,33 \pm 0,58$), CuOx1000 mg/kg (20°C30%) ($1,33 \pm 1,15$; $1,33 \pm 1,52$), respectively. There was a significant difference in the number of cocoons ($19,67 \pm 1,53$) as well as the number of hatchlings ($17,33 \pm 2,51$) between the control group (20°C50%) and the treatments of CuOx200 mg/kg (20°C50%) ($6,67 \pm 2,08$; $5,33 \pm 1,53$), CuOx500 mg/kg (20°C50%) ($3,67 \pm 0,58$; $2,67 \pm 0,58$), and CuOx1000 mg/kg (20°C50%) ($1,33 \pm 1,15$; $1,33 \pm 1,52$), respectively. Similar to the 20°C30% and 20°C50% combinations, the 25°C30% ones had a significant difference between the control group ($18,67 \pm 3,51$; $3,05 \pm 1,67$) and the treatments of CuOx200 mg/kg ($9,33 \pm 2,08$; $7 \pm 1,52$), CuOx500 mg/kg ($5,67 \pm 1,53$; $4,33 \pm 1,16$), and CuOx1000 mg/kg (0; 0) respectively, under 25°C30%. There was a significant difference in the number of cocoons ($20,67 \pm 2,52$) as well as the number of hatchlings ($18,33 \pm 1,15$) between the control group (25°C50%) and the treatments of CuOx200 mg/kg (25°C50%) ($8 \pm 1,21$; $7 \pm 1,14$), CuOx500 mg/kg (25°C50%) ($6,33 \pm 1,53$; $5 \pm 1,03$), and CuOx1000 mg/kg (25°C50%) ($1,67 \pm 1,53$; $1,33 \pm 1,15$), respectively. No reproduction was observed in the CuOx1000mg/kg (25°C30%) for the duration of the experiment.

Table 6: The average number of cocoons, hatchlings and number of hatchlings per cocoon (reproductive output) of the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) under different temperatures and moistures. (n= 30).

Treatments(mg/kg)	No. of cocoons	No. of hatchlings	No. of hatchlings per cocoon
Control (20°C30%)	14,67±2,08 ^{a■}	11,67±3,51 ^{a■}	0,79±1,01
CuOx200 (20°C30%)	7,33±0,58 ^{b•}	5±1 ^{b•}	0,68±0,29
CuOx500 (20°C30%)	4,33±0,58 ^{c▲}	3,33±0,58 ^{b•}	0,77±0
CuOx1000 (20°C30%)	0,33±0,58 ^{d♦}	0,33±0,58 ^{c▲}	1±0.08
Control (20°C50%)	19,67±1,53 ^{a■}	17,33±2,51 ^{a■}	0,88±0,69
CuOx200 (20°C50%)	6,67±2,08 ^{b•}	5,33±1,53 ^{b•}	0,8±0,39
CuOx500 (20°C50%)	3,67±0,58 ^{c▲}	2,67±0,58 ^{bc•▲}	0,73±00.23
CuOx1000 (20°C50%)	1,33±1,15 ^{c▲}	1,33±1,52 ^{c▲}	1±0,26
Control (25°C30%)	18,67±3,51 ^{a■}	3,05±1,67 ^{a■}	0,89±0,32
CuOx200 (25°C30%)	9,33±2,08 ^{b•}	7±1,52 ^{a■}	0,75±0,06
CuOx500 (25°C30%)	5,67±1,53 ^{b•}	4,33±1,16 ^{a■}	0,76±0,26
CuOx1000 (25°C30%)	0 ^{c▲}	0 ^{b•}	-
Control (25°C50%)	20,67±2,52 ^{a■}	18,33±1,15 ^{a■}	0,89±0,96
CuOx200 (25°C50%)	8±1.21 ^{b•}	7±1.14 ^{a■}	0,875±0.13
CuOx500 (25°C50%)	6,33±1,53 ^{b•}	5±1.03 ^{b•}	0,79±0,37
CuOx1000 (25°C50%)	1,67±1,53 ^{c▲}	1,33±1,15 ^{c▲}	0,8±0,26

Different superscript letters^{a-d} (rows) and symbols^{■•▲♦} (columns) indicate significantly different values (p<0.05). The superscript letters indicate whether there was a significant difference in the number of cocoons as well as the number of hatchlings between the different groups. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 7 summarises the reproductive output for the MnZn treatments under different temperatures and moistures. Similar to the CuOx treatments, as the exposure concentration increased the reproductive output decreased. There was a significant difference in the number of cocoons as well as the number of hatchlings between the control group (20°C30%) (14,67±2,08; 11,67±3,51) and the treatments of MnZn44 mg/kg (20°C30%) (6,66±1,53; 5±1,73), MnZn850 mg/kg (20°C30%) (5,33±0,58; 3,66±0,58), and MnZn1250 mg/kg (20°C30%) (2,67±0,58; 1,33±0,58). The (20°C50%) exposure had similar outcomes to the (20°C30%) exposure, with there being a significant difference in the number of cocoons as well as the number of hatchlings

between the control group (20°C50%) (19,67±1,53; 17,33±2,51) and the treatments of MnZn44 mg/kg (20°C50%) (8,66±0,58; 6,67±0,58), MnZn850 mg/kg (20°C50%) (6±1; 4,66±1,15), and MnZn1250 mg/kg (20°C50%) (4,33±1,15; 3±1). With regards to the number of cocoons in the (25°C30%) exposure, there was a significant difference between the control group (18,67±3,51) and the MnZn1250 (25°C30%) (4,33±1,15). There was a significant difference in the number of hatchlings in the control group (25°C30%) (3,05±1,67) and all the treatments (MnZn44 (25°C30%) (6,67±0,58); MnZn850 (25°C30%) (4,66±1,15); MnZn1250 (25°C30%) (3±1). There was a significant difference in the number of cocoons as well as the number of hatchlings between the control group (25°C50%) (20,67±2,52; 18,33±1,15) and the treatments of MnZn44 mg/kg (25°C50%) (13,33±2,52; 10,33±3,05), MnZn850 mg/kg (25°C50%) (8,33±0,58; 6±1), and MnZn1250 mg/kg (25°C50%) (6,67±0,58; 4,67±0,58).

Table 7: The average number of cocoons, hatchlings and number of hatchlings per cocoon (reproductive output) of the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) under different temperatures and moistures. (n= 30).

Treatments (mg/kg)	No. of Cocoons	No. of Hatchlings	No. of Hatchlings per Cocoon
Control (20°C30%)	14,67±2,08 ^{a■}	11,67±3,51 ^{a■}	0,79±1,01
MnZn44 (20°C30%)	6,66±1,53 ^{b•}	5±1,73 ^{b•}	0,75±0,14
MnZn850 (20°C30%)	5,33±0,58 ^{b•}	3,66±0,58 ^{b•}	0,69±0
MnZn1250 (20°C30%)	2,67±0,58 ^{c▲}	1,33±0,58 ^{c▲}	0,5±0
Control (20°C50%)	19,67±1,53 ^{a■}	17,33±2,51 ^{a■}	0,88±0,69
MnZn44 (20°C50%)	9±1 ^{b•}	6,33±0,58 ^{b•}	0,70±0,29
MnZn850 (20°C50%)	4,66±1,15 ^{c▲}	3,33±0,58 ^{c▲}	0,71±0,41
MnZn1250 (20°C50%)	2,67±1,53 ^{c▲}	1,67±1,53 ^{c▲}	0,625±0
Control (25°C30%)	18,67±3,51 ^{a■}	3,05±1,67 ^{a■}	0,89±0,32
MnZn44 (25°C30%)	8,66±0,58 ^{a■}	6,67±0,58 ^{b•}	0,77±0
MnZn850 (25°C30%)	6±1 ^{a■}	4,66±1,15 ^{c▲}	0,78±0,11
MnZn1250 (25°C30%)	4,33±1,15 ^{b•}	3±1 ^{c▲}	0,69±0,11
Control (25°C50%)	20,67±2,52 ^{a■}	18,33±1,15 ^{a■}	0,89±0,96
MnZn44 (25°C50%)	13,33±2,52 ^{b•}	10,33±3,05 ^{b•}	0,775±0,38
MnZn850 (25°C50%)	8,33±0,58 ^{c▲}	6±1 ^{b•}	0,72±0,29
MnZn1250 (25°C50%)	6,67±0,58 ^{c▲}	4,67±0,58 ^{c▲}	0,70±0

Different superscript letters^{a-d} (rows) and symbols^{■●▲♦} (columns) indicate significantly different values ($p < 0.05$). The superscript letters indicate whether there was a significant difference in the number of cocoons as well as the number of hatchlings between the different groups. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

The reproductive output for the binary mixtures of fungicides (CuOx + MnZn) is shown in Table 8. The binary mixtures had similar results to both single fungicide treatments. There was a significant difference in the number of cocoons as well as the number of hatchlings between the control group (20°C30%) (14,67±2,08; 11,67±3,51) and the treatments of CuOx200 + MnZn44 mg/kg (20°C30%) (6,00±1,32; 3,66±1,53), CuOx500 + MnZn850 mg/kg (20°C30%) (1,67±1,53; 1±0,1), and CuOx1000 + MnZn1250 mg/kg (20°C30%) (0,67±1,15; 0,33±0,58). The (20°C50%) exposure had similar outcomes to the (20°C30%) exposure, with there being a significant difference between the number of cocoons as well as the number of hatchlings in the control group (20°C50%) (19,67±1,53; 17,33±2,51) and the treatments of CuOx200 + MnZn44 mg/kg (20°C50%) (7,67±1,15; 4,66±0,58), CuOx500 + MnZn850 mg/kg (20°C50%) (3,67±0,58; 2,33±0,58), and CuOx1000 + MnZn1250 mg/kg (20°C50%) (0,33±0,58; 0,33±0,58). There was a significant difference in the number of cocoons produced between the control (25°C30%) (18,67±3,51) and the CuOx1000 + MnZn1250 mg/kg (25°C30%) (0) treatment. The number of hatchlings in this exposure (25°C30%) showed a significant difference between the control (25°C30%) (3,05±1,67) and all the treatments (CuOx200 + MnZn44 mg/kg (25°C30%) (6,66±1,94), CuOx500 + MnZn850 mg/kg (25°C30%) (2,33±1,15), and CuOx1000 + MnZn1250 mg/kg (25°C30%) (0)). There was a significant difference in the number of cocoons as well as the number of hatchlings between the control group (25°C50%) (20,67±2,52; 18,33±1,15) and all the treatments (CuOx200 + MnZn44 mg/kg (25°C50%) (10,33±1,53; 7±1), CuOx500 + MnZn850 mg/kg (25°C50%) (6±1; 4,67±1,15), and CuOx1000 + MnZn1250 mg/kg (25°C50%) (1,33±1,15; 1±0,14)). The CuOx1000+MnZn1250 and the CuOx1000 mg/kg under (25°C30%) showed no reproduction.

Table 8: The average number of cocoons, hatchlings and number of hatchlings per cocoon (reproductive output) of the binary mixtures consisting of copper oxychloride + mancozeb (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) under different temperatures and moistures. (n= 30).

Treatments (mg/kg)	No. of Cocoons	No. of Hatchlings	No. of Hatchlings per Cocoon
Control (20°C30%)	14,67±2,08 ^{a■}	11,67±3,51 ^{a■}	0,79±1,01
CuOx200+ MnZn 44 (20°C30%)	6,00±1.32 ^{b•}	3,66±1,53 ^{b•}	0,61±0,37
CuOx 500+ MnZn 850 (20°C30%)	1,67±1,53 ^{c▲}	1±0.1 ^{b•}	0,6±0,37
CuOx 1000+ MnZn 1250 (20°C30%)	0,67±1,15 ^{c▲}	0,33±0,58 ^{b•}	0,5±0,41
Control (20°C50%)	19,67±1,53 ^{a■}	17,33±2,51 ^{a■}	0,88±0,69
CuOx 200+ MnZn 44 (20°C50%)	7,67±1,15 ^{b•}	4,66±0,58 ^{b•}	0,61±0,41
CuOx 500+ MnZn 850 (20°C50%)	3,67±0,58 ^{d♦}	2,33±0,58 ^{bc•▲}	0,64±0.07
CuOx 1000+ MnZn 1250 (20°C50%)	0,33±0,58 ^{c▲}	0,33±0,58 ^{c▲}	1,00±0.23
Control (25°C30%)	18,67±3,51 ^{a■}	3,05±1,67 ^{a■}	0,89±0,32
CuOx 200+ MnZn 44 (25°C30%)	8,67±0,58 ^{a■}	6,66±1.94 ^{b•}	0,77±0,29
CuOx 500+ MnZn 850 (25°C30%)	3±1 ^{a■}	2,33±1,15 ^{c•}	0,78±0,11
CuOx 1000+ MnZn 1250 (25°C30%)	0 ^{b•}	0 ^{d♦}	-
Control (25°C50%)	20,67±2,52 ^{a■}	18,33±1,15 ^{a■}	0,89±0,96
CuOx 200+ MnZn 44 (25°C50%)	10,33±1,53 ^{b•}	7±1 ^{b•}	0,678±0,37
CuOx 500+ MnZn 850 (25°C50%)	6±1 ^{c▲}	4,67±1,15 ^{b•}	0,78±0,11
CuOx 1000+ MnZn 1250 (25°C50%)	1,33±1,15 ^{d♦}	1±0.14 ^{d♦}	0,75±0,11

Different superscript letters^{a-d} (rows) and symbols^{■•▲♦} (columns) (Day 1 – Day 28) indicate significantly different values (p<0.05). The superscript letters indicate whether there was a significant difference in the number of cocoons as well as the number of hatchlings between the different groups. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

4.1.4 Avoidance response behaviour

Figure 5 illustrates the avoidance response behaviour for the 20°C30% exposure. According to the ISO guidelines (3.3: limited habitat function), when 80% of the exposed population is found in the control it is an indication of the impact on behaviour (ISO 527-2, 2003). All the treatments showed an avoidance response (above 80%) throughout the 48 h exposure except for the MnZn44 mg/kg which showed no response (13.33 %).

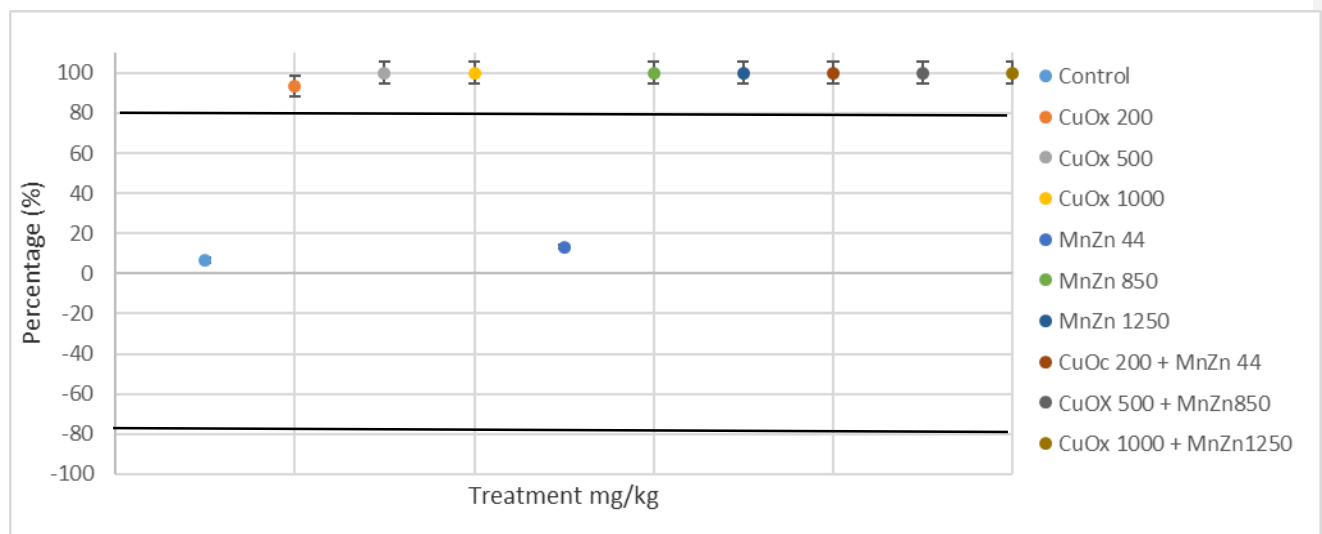


Figure 5: Avoidance response behaviour (%) of *E. andrei* under 20°C30% (temperature and soil moisture) after exposure to copper oxychloride (CuOx) and mancozeb (MnZn) in single and binary mixtures (n= 30)

Figure 6 illustrates avoidance response behaviour for the 20°C50% exposure. The CuOx200 mg/kg, MnZn44 mg/kg and the binary mixture of CuOx200+MnZn44 mg/kg had no response, with (73.33 %), (26,67 %) and (40 %) respectively while the rest of the treatments had an avoidance response (above 80 %).

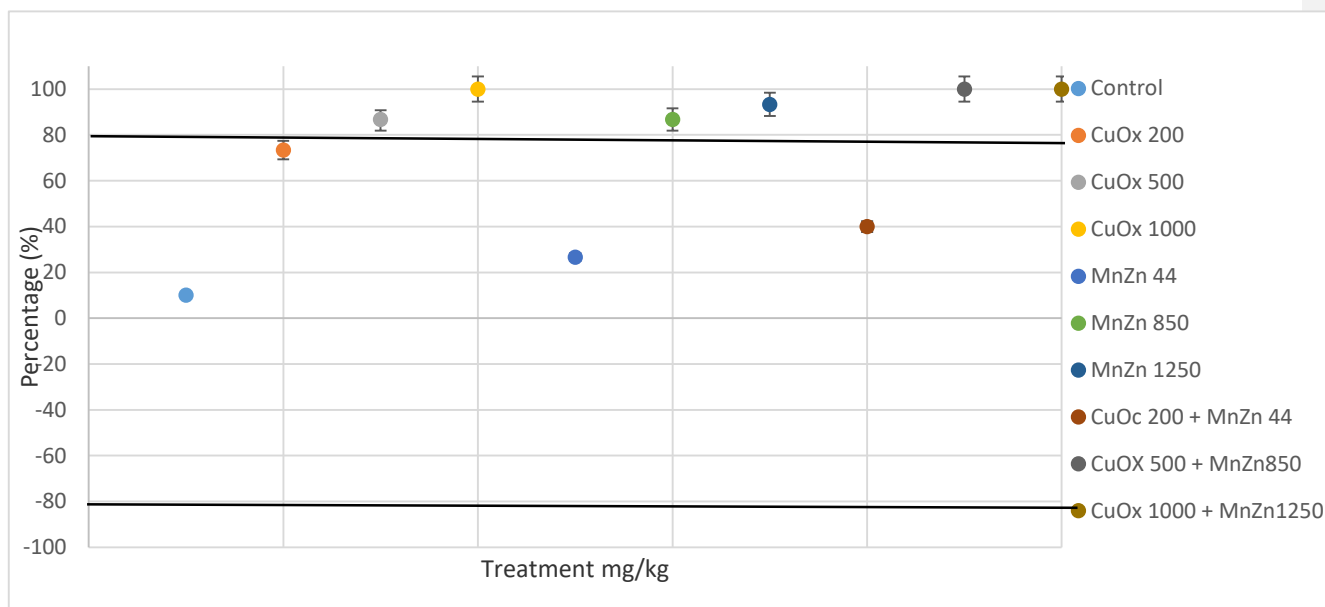


Figure 6: Avoidance response behaviour (%) of *E. andrei* under 20°C 50% (temperature and soil moisture) after exposure to copper oxychloride (CuOx) and mancozeb (MnZn) in single and binary mixtures (n= 30).

Figure 7 illustrates avoidance response behaviour for the 25°C 30% experiment. All the treatments showed an avoidance response behaviour (80 %) except the CuOx 200 mg/kg (73.33 %) and the MnZn 44 mg/kg (33.33 %) which had no response.

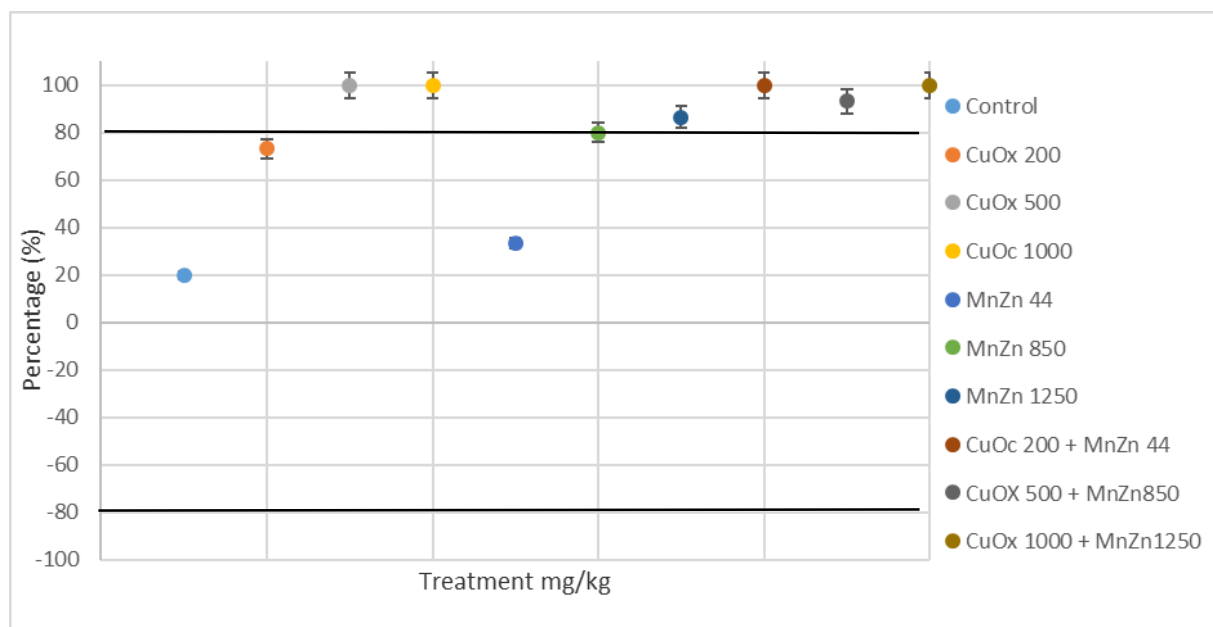


Figure 7: Avoidance response behaviour (%) of *E. andrei* under 25°C 30% (temperature and soil moisture) after exposure to copper oxychloride (CuOx) and mancozeb (MnZn) in single and binary mixtures (n= 30).

Figure 8 illustrates avoidance response behaviour for the 25°C50% experiment. Similarly, to the other avoidance response behaviour exposures (20°C30%); (20°C50%) and (25°C30%), all the treatments showed an avoidance response behaviour (above 80 %) except the MnZn44mg/kg, which was 33.33 % (no response).

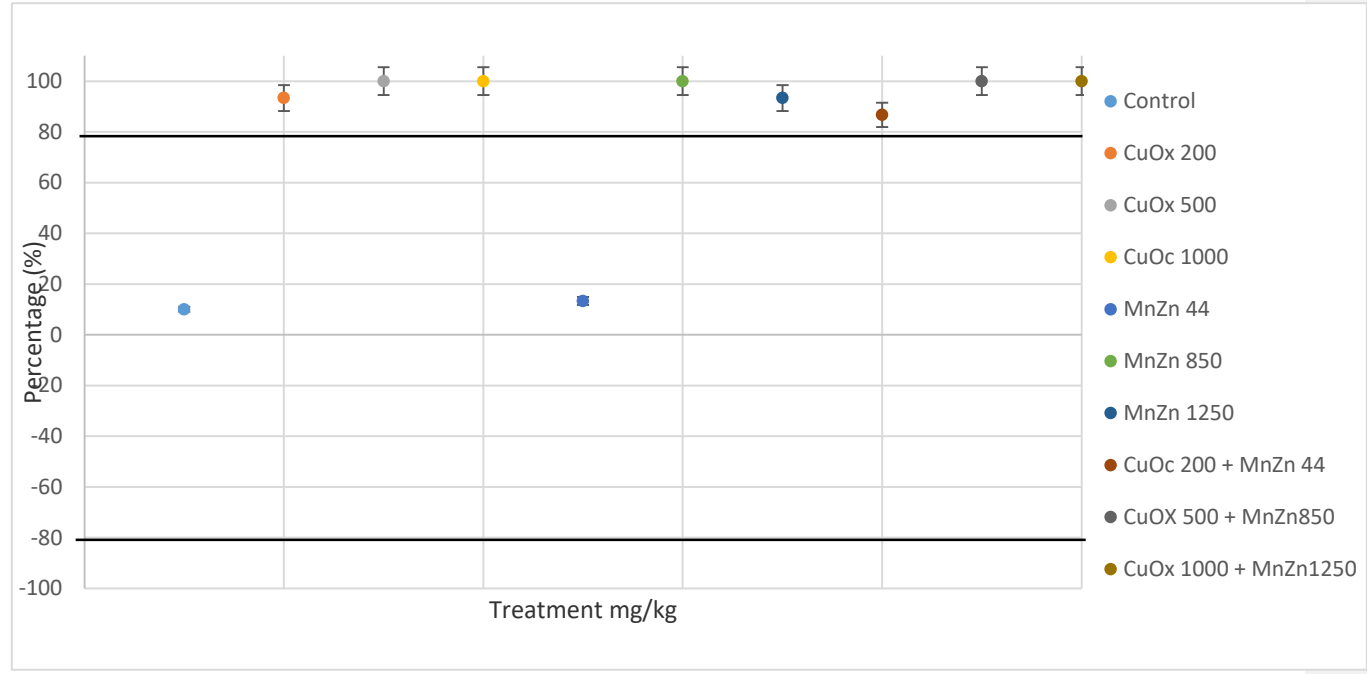


Figure 8: Avoidance response behaviour (%) of *E. andrei* under 25°C50% (temperature and soil moisture) after exposure to copper oxychloride (CuOx) and mancozeb (MnZn) in single and binary mixtures (n= 30).

4.2 Comet assay

Figure 9 and Figure 10 below illustrate the comets under the Nikon eclipse TE2000 inverted microscope. Figure 9 illustrates comets which have not migrated and kept their shape, while Figure 10 illustrates comets which have migrated.

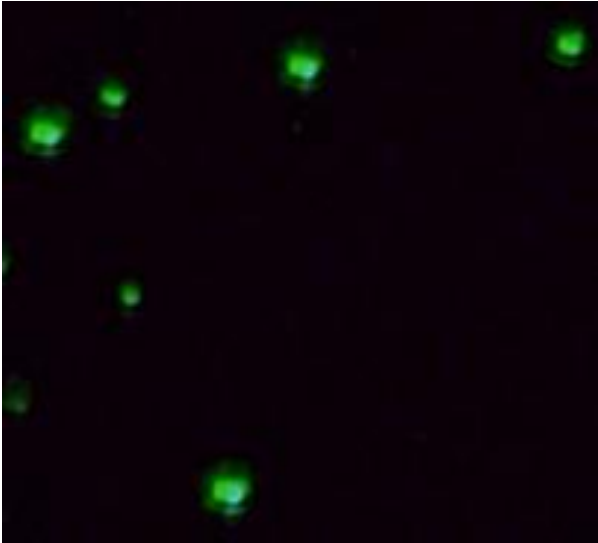


Figure 9: Comets under the Nikon eclipse TE2000 inverted microscope that have not migrated.

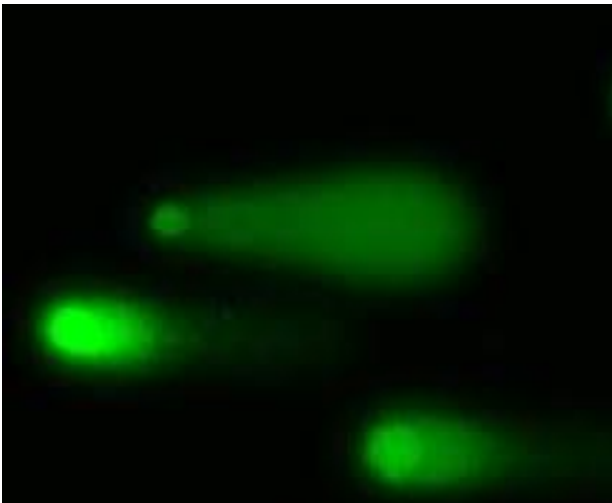


Figure 10: Comets under the Nikon eclipse TE2000 inverted microscope showing migration.

Figure 11 illustrates the mean tail length (μm) of earthworms in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg). There was a significant difference ($p < 0.05$) between the CuOx500 mg/kg (20°C50%) and the control (20°C50%) as well as between the CuOx500 mg/kg (20°C50%) and the other CuOx500 mg/kg (20°C30%), (25°C30%), (25°C50%). There was a significant difference between the CuOx1000 mg/kg (20°C30%), (20°C50%), (25°C30%), (25°C50%) and their respective controls (20°C30%), (20°C50%), (25°C30%), (25°C50%). There was also a significant difference between the CuOx1000 mg/kg (25°C30%) and the CuOx1000 mg/kg (20°C30%), (20°C50%) and (25°C50%).

Form

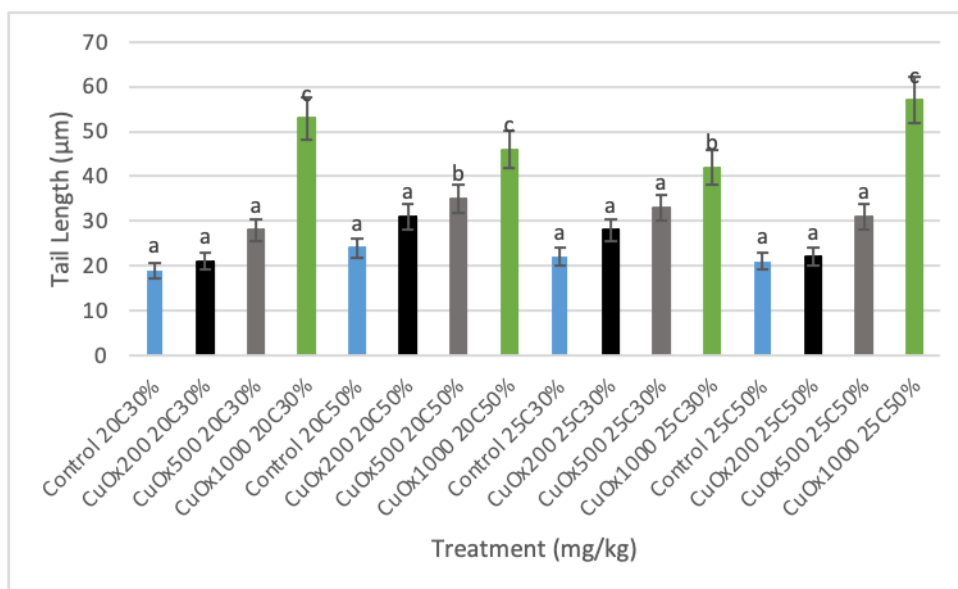


Figure 11: The mean tail length (μm) of earthworms in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) after a 28-day period under different temperature and soil moisture conditions. ($n = 30$). Different superscript letters^{a-c} indicate significantly different values ($p < 0.05$).

Figure 12 illustrates the mean tail length (μm) of earthworms in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg). There was a significant difference ($p < 0.05$) between the MnZn850 mg/kg (25°C50%) and the control (20°C50%) as well as between the MnZn850 mg/kg (25°C50%) and the other MnZn850 mg/kg (20°C30%), (20°C50%), (25°C30%). There was a significant difference between the MnZn1250 mg/kg (20°C30%), (20°C50%), (25°C30%), (25°C50%) and their respective controls (20°C30%), (20°C50%), (25°C30%), (25°C50%). There was also a significant difference between the MnZn1250 mg/kg (20°C30%) and (20°C50%) and the MnZn1250 mg/kg (25°C30%) and (25°C50%).

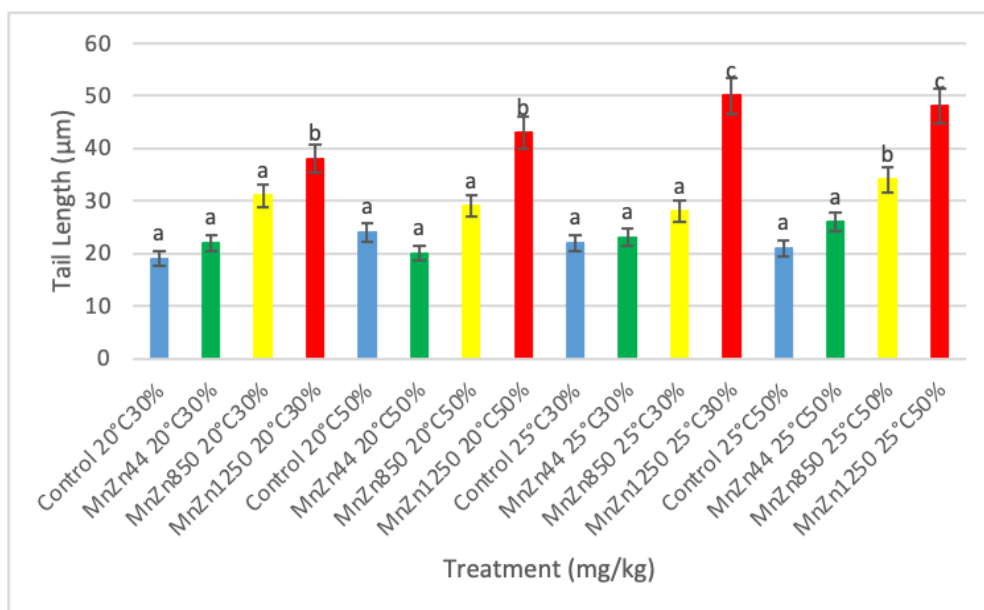


Figure 12: The mean tail length (µm) of earthworms in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) after a 28-day period under different temperatures and soil moistures. (n= 30). Different superscript letters a-c indicates significantly different values (p<0.05).

Figure 13 illustrates the mean tail length (µm) of earthworms in the binary treatments of copper oxychloride + mancozeb (CuOx + MnZn) (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg). There was a significant difference (p<0.05) between the CuOx500 + MnZn850 mg/kg (25°C50%) and the control (25°C50%) as well as between the CuOx500 + MnZn850 mg/kg (20°C50%) and the other CuOx500 + MnZn850 mg/kg (20°C30%), (20°C50%), (25°C30%). There was a significant difference between the CuOx1000 + MnZn1250 mg/kg (20°C30%), (20°C50%), (25°C30%), (25°C50%) and their respective controls (20°C30%), (20°C50%), (25°C30%), (25°C50%). There was also a significant difference between the CuOx1000 + MnZn1250 mg/kg (20°C50%) and the CuOx1000 + MnZn1250 mg/kg (20°C30%), (25°C30%) and (25°C50%).

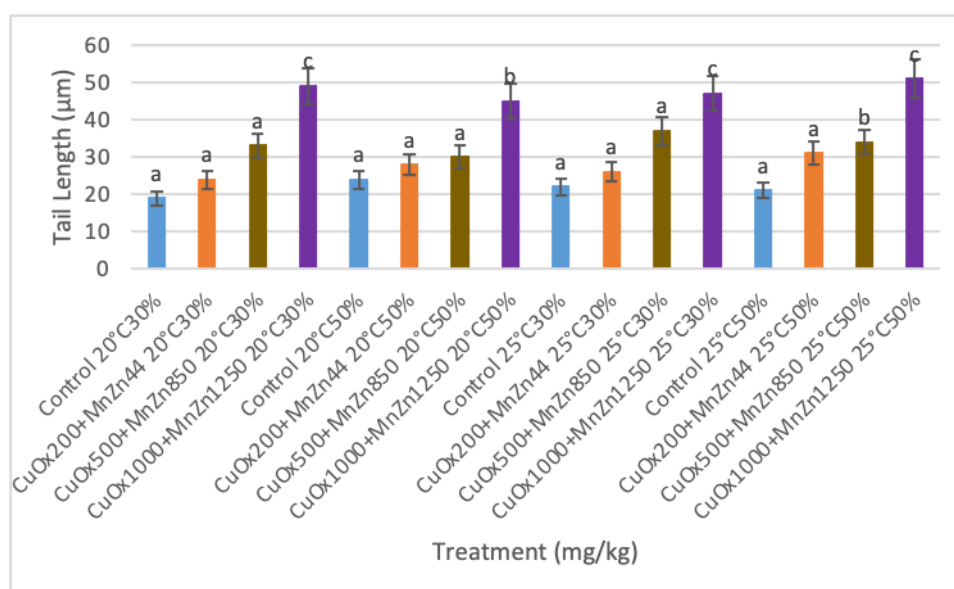


Figure 13: The mean tail length (μm) of earthworms in the binary treatment of copper oxychloride + mancozeb (CuOx + MnZn) treatment (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) after a 28-day period under different temperatures and soil moistures. (n= 30). Different superscript letters^{a-c} indicate significantly different values ($p<0.05$).

4.3 Body metal analysis

Table 9 summarises the mean body metal concentration of earthworms in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg). There was no significant difference ($p>0.05$) in the copper (Cu) concentrations under (20°C 30%), (20°C 50%) and (25°C 30%). There was a significant difference ($p<0.05$) in the (25°C50%) between the treatments (CuOx200; CuOx500 and CuOx1000 mg/kg) and the control.

When comparing the same treatments with each other (indicated by symbols in superscript) there was a significant difference ($p<0.05$) between the Cu200 mg/kg (20°C30%) and the CuOx200 mg/kg (25°C50%). There was a significant difference between the Cu500 mg/kg (25°C50%) and the CuOx500 mg/kg (20°C30%). There was a significant difference between the CuOx1000 mg/kg (25°C50%) and the CuOx1000 mg/kg (20°C30%).

Table 9: The mean body metal concentration of earthworms in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) after a 28-day period under different temperatures and soil moistures. N= 30

Treatments (mg/kg)	Average concentration mg/kg		
	Copper (Cu)	Manganese (Mn)	Zinc (Zn)
Control (20°C30%)	18,69±1,21 ^{a■}	15,77±2,33 ^{a■}	48,2±0,16 ^{a■}
CuOx200 (20°C30%)	32,92±3,02 ^{b•}	38±0,4 ^{b•}	38±3,36 ^{a■}
CuOx500 (20°C30%)	52,2±2,3 ^{b•}	40,5±2,1 ^{a b•}	40,5±6,8 ^{a■}

CuOx1000 (20°C30%)	69,5±3,9 ^{c▲}	55,8±7,4 ^{c▲}	55,8±3,5 ^{a■}
Control (20°C50%)	6,22±3,68 ^{a■}	13,96±3,15 ^{a■}	31,5±1,98 ^{a■}
CuOx200 (20°C50%)	37,4±7,5 ^{b•}	39,5±1,1 ^{b•}	39,5±0,79 ^{a■}
CuOx500 (20°C50%)	46,9±1,7 ^{c▲}	41,2±2,8 ^{b•}	41,20,3 ^{a■}
CuOx1000 (20°C50%)	51,5±1,6 ^{c▲}	44,9±6,5 ^{b•}	44,9±1,5 ^{a■}
Control (25°C30%)	7,1±7,2 ^{a■}	16,1±3,01 ^{a■}	41,4±0,16 ^{a■}
CuOx200 (25°C30%)	43,3±3,4 ^{b•}	42,4±4 ^{b•}	42,4±4,53 ^{a■}
CuOx500 (25°C30%)	50,4±0,54 ^{b•}	45,7±7,3 ^{b•}	45,7±7,8 ^{a■}
CuOx1000 (25°C30%)	65,5±5,6 ^{c▲}	57,2±8,8 ^{c▲}	57,2±1,6 ^{a■}
Control (25°C50%)	7,38±2,52 ^{a■}	11,73±3,7 ^{a■}	32,9±4,21 ^{a■}
CuOx200 (25°C50%)	41,3±1,4 ^{b•}	34,1±4,3 ^{b•}	34,1±3,77 ^{a■}
CuOx500 (25°C50%)	44,3±4,6 ^{b•}	39,8±1,4 ^{b•}	39,8±0,6 ^{a■}
CuOx1000 (25°C50%)	44,5±4,4 ^{b•}	43,8±5,4 ^{b•}	43,8±0,72 ^{a■}

Different superscript letters^{a-c} (rows) and symbols^{■•▲} (columns) indicate significantly different values ($p < 0.05$). The superscript letters indicate whether there was a significant difference in the copper, manganese and zinc concentrations. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 10 summarises the mean body metal concentration of earthworms in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg). There were no significant differences ($p > 0.05$) in the zinc (Zn) concentrations in any of the treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) under the different temperatures and moistures (20°C30%), (20°C50%), (25°C30%), (25°C50%).

There were no significant differences in the manganese (Mn) concentration in any of the treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) under (20°C30%) and (20°C50%). There was a significant difference ($p < 0.05$) between the manganese concentrations in the (MnZn850 and MnZn1250 mg/kg) treatments when compared to the control under (25°C30%). There was a significant difference between the manganese concentrations in the (MnZn44, MnZn850 and MnZn1250 mg/kg) treatments when compared to the control under (25°C50%).

When comparing the same treatments with each other (indicated by symbols in superscript) there was no significant difference ($p > 0.05$) in the zinc concentrations. There were significant differences ($p < 0.05$) in the manganese concentrations. There was a significant difference between the MnZn44 mg/kg (25°C50%) and the MnZn44 mg/kg (20°C30%) and (20°C50%). There was a significant difference between the MnZn850 mg/kg (25°C30%) and the MnZn850 mg/kg (20°C30%). There was also a significant difference between the MnZn850 mg/kg (25°C50%) and the MnZn850 mg/kg (20°C30%). There was a significant difference between the MnZn1250 mg/kg (25°C30%) and the MnZn1250 mg/kg (20°C30%). There was also a significant difference between the MnZn1250 mg/kg (25°C50%) and the MnZn1250 mg/kg (20°C30%).

Table 10: The mean body metal concentration of earthworms in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) after a 28-day period under different temperatures and soil moistures. (n= 30).

Treatments (mg/kg)	Average concentration mg/kg		
	Copper (Cu)	Manganese (Mn)	Zinc (Zn)
Control (20°C30%)	18,69±1,21 ^{a■}	15,77±2,33 ^{a■}	48,2±7,7 ^{a■}
MnZn44 (20°C30%)	12,78±0,45 ^{a■}	19,4±3,71 ^{a■}	45,3±4,8 ^{a■}
MnZn850 (20°C30%)	23,8±0,4 ^{a■}	54,18±1,08 ^{c▲}	59,3±8,8 ^{a■}
MnZn1250 (20°C30%)	48,7±4,5 ^{c▲}	57,8±2,7 ^{c▲}	60,6±4,1 ^{a■}
Control (20°C50%)	6,22±3,68 ^{a■}	13,96±3,15 ^{a■}	31,5±0,87 ^{a■}
MnZn44 (20°C50%)	7,4±0,83 ^{a■}	20,14±2,96 ^{ab■●}	41,7±1,3 ^{a■}
MnZn850 (20°C50%)	7,83±0,54 ^{a■}	40,09±4,8 ^{c▲}	41,9±1,5 ^{a■}
MnZn1250 (20°C50%)	11,51±2,1 ^{b●}	47,55±7,8 ^{c▲}	42,8±2,4 ^{a■}
Control (25°C30%)	7,1±7,2 ^{a■}	16,1±3,01 ^{a■}	41,4±0,9 ^a
MnZn44 (25°C30%)	7,84±0,25 ^{a■}	26,8±3,7 ^{b●}	36,8±3,7 ^{a■}
MnZn850 (25°C30%)	9,025±0,97 ^{a■}	51,5±2,9 ^{c▲}	39,7±0,8 ^{a■}
MnZn1250 (25°C30%)	16,45±3,45 ^{b●}	51,81±2,7 ^{c▲}	43±2,5 ^{a■}
Control (25°C50%)	7,38±2,52 ^{a■}	11,73±3,7 ^{a■}	32,9±1,7 ^a
MnZn44 (25°C50%)	6,75±0,64 ^{a■}	29±5,9 ^{b●}	36,5±4,0 ^{a■}
MnZn850 (25°C50%)	10,62±2,78 ^{a■}	55,8±2,7 ^{c▲}	39,3±1,2 ^{a■}
MnZn1250 (25°C50%)	11,89±3,14 ^{b●}	56,3±2,8 ^{c▲}	40,9±0,5 ^{a■}

Different superscript letters^{a-c} (rows) and symbols^{●▲} (columns) indicate significantly different values (p<0.05). The superscript letters indicate whether there was a significant difference in the copper, manganese and zinc concentrations. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 11

Table 11 summarises the mean body metal concentration of earthworms in the copper oxychloride (CuOx) + mancozeb (MnZn) treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg). There were no significant differences (p>0.05) in the copper, concentrations in any of the treatments under (20°C30%), (20°C50%) and (25°C30%). There was a significant difference (p<0.05) in the copper concentration under all the treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) when compared to the control under (25°C50%).

There was no significant difference (p>0.05) in the manganese concentration in any of the treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) under 20°C30%). There were significant differences (p<0.05) in the manganese concentration in the treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) when compared to the control under (20°C50), (25°C30%) and (25°C50%). There were no significant differences in the zinc concentrations under the different temperatures and soil moistures.

When comparing the same treatments with each other (indicated by symbols in superscript) there was a ($p < 0.05$) significant difference between the CuOx1000 + MnZn1250 mg/kg (25°C50%) and CuOx1000 + MnZn1250 mg/kg (20°C30%). There were no other significant differences.

Table 11: The mean body metal concentration of earthworms in the binary treatments of copper oxychloride + mancozeb (CuOx + MnZn) (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) after a 28-day period under different temperatures and soil moistures. $n = 30$

Treatments (mg/kg)	Average concentration mg/kg		
	Copper (Cu)	Manganese (Mn)	Zinc (Zn)
Control (20°C30%)	18,69±1,21 ^{a■}	15,77±2,33 ^{a■}	48,2±8,5 ^{a■}
CuOx200+ MnZn 44 (20°C30%)	38,79±1,75 ^{b•}	23,75±6,5 ^{b•}	47±7,4 ^{a■}
CuOx 500+ MnZn 850 (20°C30%)	47,18±1,14 ^{b•}	28,36±2,6 ^{b•}	51,3±7,4 ^{a■}
CuOx 1000+ MnZn 1250 (20°C30%)	64,5±7,5 ^{c▲}	29,88±1,78 ^{b•}	51,6±2,9 ^{a■}
Control (20°C50%)	6,22±3,68 ^{a■}	13,96±3,15 ^{a■}	31,5±2,9 ^{a■}
CuOx 200+ MnZn 44 (20°C50%)	41,18±4,14 ^{b•}	28,37±5,27 ^{b•}	39,8±0,1 ^{a■}
CuOx 500+ MnZn 850 (20°C50%)	58,6±1,6 ^{c▲}	39,09±7,7 ^{b•}	42,8±3,1 ^{a■}
CuOx 1000+ MnZn 1250 (20°C50%)	71,2±4,2 ^{d•}	28,37±5,27 ^{b•}	45,8±6,1 ^{a■}
Control (25°C30%)	7,1±7,2 ^{a■}	16,1±3,01 ^{a■}	41,4±1,7 ^{a■}
CuOx 200+ MnZn 44 (25°C30%)	42,7±4,7 ^{b•}	31,2±5,1 ^{b•}	38,9±0,8 ^{a■}
CuOx 500+ MnZn 850 (25°C30%)	48,6±1,5 ^{b•}	35±0,79 ^{b•}	42,4±2,7 ^{a■}
CuOx 1000+ MnZn 1250 (25°C30%)	58,5±2,5 ^{c▲}	38,2±1,05 ^{b•}	43±3,3 ^{a■}
Control (25°C50%)	7,38±2,52 ^{a■}	11,73±3,7 ^{a■}	32,9±6,8 ^a
CuOx 200+ MnZn 44 (25°C50%)	43±1,2 ^{b•}	31,6±0,54 ^{b•}	36,3±3,4 ^{a■}
CuOx 500+ MnZn 850 (25°C50%)	49±2,45 ^{b•}	36,2±2,87 ^{b•}	37,5±2,2 ^{a■}
CuOx 1000+ MnZn 1250 (25°C50%)	66±3,9 ^{d•}	40,8±2,7 ^{c▲}	44,6±5,0 ^{a■}

Different superscript letters^{a-d} (rows) and symbols^{•▲■} (columns) indicate significantly different values ($p < 0.05$). The superscript letters indicate whether there was a significant difference in the copper, manganese and zinc concentrations. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

4.4 Soil enzymatic activity

Table 12 summarises the mean concentration of alkaline phosphatase, glucosidase and urease activity in the copper oxychloride (CuOx) treatments under different temperature and soil moisture conditions. There were no significant differences ($p > 0.05$) in the alkaline phosphatase activity for the CuOx200, CuOx500 and CuOx1000 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%). There were significant differences ($p < 0.05$) in the glucosidase test for the (20°C50%) exposure between the CuOx200 and CuOx500 mg/kg treatment and their respective controls. There were also

significant differences in the (25°C30%;) exposure between CuOx500 mg/kg and its control. There were no significant differences in the urease activity for the CuOx200, CuOx500 and CuOx1000 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

When comparing the same treatments with each other (indicated by symbols in superscript) there was a significant difference ($p < 0.05$) in the glucosidase test between the same treatments CuOx200 mg/kg under (20°C30) and (20°C50), and between (25°C30) and (25°C50). For the CuOx500 mg/kg there was a significant difference between the (20°C30) exposure and the other exposures (20°C50; 25°C30 and 25°C50). For the CuOx1000 mg/kg treatment there was a significant difference between the (25°C50) exposure and the other exposures (20°C30; 20°C50 and 25°C30). There were no significant differences in the urease activity for the CuOx200, CuOx500 and CuOx1000 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

Table 12: The mean concentration of alkaline phosphatase, glucosidase and urease activity in the copper oxychloride (CuOx) treatments (CuOx200 mg/kg; CuOx500 mg/kg; CuOx1000 mg/kg) after a 56-day period under different temperatures and soil moistures. N= 3

Treatment (mg/kg)	Average Concentration		
	Alkaline phosphatase Activity (P-nitrophenol mg/kg/h)	Glucosidase Activity (P-nitrophenol mg/kg/h)	Urease Activity (NH ₄ -N mg/kg/h)
Control (20C30%)	7365±1191 ^{a■}	3108±584 ^{e∅}	4,75±1 ^{a■}
CuOx200 (20C30%)	1883±983 ^{b•}	2889±209 ^{e∅}	4,36±1 ^{a■}
CuOx500 (20C30%)	1557±266 ^{b•}	2808±254 ^{e∅}	21,43±11 ^{a■}
CuOx1000 (20C30%)	1847 ±712 ^{b•}	2889±527 ^{e∅}	12,24±7 ^{a■}
Control (20C50%)	2311±362 ^{a■}	3163±1070 ^{e∅}	18,71±11 ^{a■}

CuOx200 (20C50%)	2899±1869 ^{a■}	2774±470 ^{c◊}	12,15±5 ^{a■}
CuOx500 (20C50%)	3578±1209 ^{a■}	14848±3967 ^{c▲}	11,62±3 ^{a■}
CuOx1000 (20C50%)	16811±3159 ^{c▲}	11545±2928 ^{d♦}	19,76±12 ^{a■}
Control (25C30%)	19735±10097 ^{c▲}	9284±2791 ^{b●}	-2,4±2 ^{a■}
CuOx200 (25C30%)	6816±1871 ^{a■}	13136±1351 ^{c▲}	9,24±2 ^{a■}
CuOx500 (25C30%)	9884±6362 ^{a■}	18664±2354 ^{d♦}	10,387±1 ^{a■}
CuOx1000 (25C30%)	4089±3733 ^{a■}	8496±818 ^{b●}	8,71±3 ^{a■}
Control (25C50%)	33138±6139 ^{d♦}	15090±2803 ^{a■}	29,66±13 ^{a■}
CuOx200 (25C50%)	12715±1016 ^{a■}	18175±976 ^{a■}	22,09±3 ^{a■}
CuOx500 (25C50%)	9986±6692 ^{a■}	21193±5604 ^{a■}	29,31±7 ^{a■}
CuOx1000 (25C50%)	11661±3612 ^{a■}	16103±4259 ^{a■}	10,044±1 ^{a■}

Different superscript letters^{a-e} (rows) and symbols^{●,▲,♦,◊} (columns) indicate significantly different values ($p<0.05$). The superscript letters indicate whether there was a significant difference in the alkaline phosphatase, glucosidase and urease activity concentrations for copper oxychloride. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 13 summarises the mean concentration of alkaline phosphatase, glucosidase and urease activity in the mancozeb (MnZn) treatments under different temperature and soil moisture conditions. There were significant differences ($p<0.05$) in the alkaline phosphatase activity for the MnZn44 and MnZn850 mg/kg treatments under the (25°C50%) exposure. There were significant differences in the glucosidase test for the (20°C50% and 25°C50%) exposure in the MnZn1250 mg/kg treatment. There were no significant differences in the urease activity for the MnZn44, MnZn850 and MnZn1250 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

When comparing the same treatments with each other (indicated by symbols in superscript) there was a significant difference ($p<0.05$) in the alkaline phosphatase test between the treatments of MnZn44 mg/kg under (25°C50) and the other exposures (20°C30; 20°C50; 25°C30). There was a significant difference in the alkaline phosphatase test between the treatments of MnZn850 mg/kg under (25°C50) and the other exposures (20°C30; 20°C50; 25°C30). There was a significant difference in the alkaline phosphatase test between the treatments of MnZn1250 mg/kg under (25°C50) and the other exposures (20°C30 and 20°C50). There was a significant difference in the glucosidase test between the same treatments MnZn44 mg/kg under (25°C50) and (20°C50), and between (25°C30) and (25°C50). For the MnZn850 mg/kg there was a significant difference between the (20°C50) exposure and the (25°C50) exposure and between the 20°C30 and 25°C30 exposures. For the MnZn1250 mg/kg treatment there was a significant difference between the (20°C30) exposure and the other exposures (20°C50; 25°C30 and 25°C50). There were no significant differences in the urease activity for the MnZn44, MnZn850 and MnZn1250 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

Table 13: The mean concentration of alkaline phosphatase, glucosidase and urease activity in the mancozeb (MnZn) treatments (MnZn44 mg/kg; MnZn850 mg/kg; MnZn1250 mg/kg) after a 56-day period under different temperatures and soil moistures. N= 3

Treatment (mg/kg)	Average Concentration		
	Alkaline phosphatase Activity (P-nitrophenol mg/kg/h)	Glucosidase Activity (P-nitrophenol mg/kg/h)	Urease Activity (NH ₄ -N mg/kg/h)
Control (20C30%)	7365±1191 ^{a■}	3108±584 ^{e◇}	4,75±1 ^{a■}
MnZn44 (20C30%)	2147±1697 ^{e◇}	3403±727 ^{e◇}	4,18±2 ^{a■}
MnZn850 (20C30%)	4061±823 ^{d♦}	2611±1031 ^{e◇}	-4,8±3 ^{a■}
MnZn1250 (20C30%)	3444±1395 ^{d♦}	1778531 ^{e◇}	44,1±32 ^{c▲}
Control (20C50%)	2311±362 ^{b•}	3163±1070 ^{e◇}	18,71±11 ^{a■}
MnZn44 (20C50%)	16641±2158 ^{a■}	12629±4744 ^{c▲}	-10,6±±4 ^{b•}
MnZn850 (20C50%)	2147±753 ^{a■}	11795±1530 ^{c▲}	13,8±9 ^{a■}
MnZn1250 (20C50%)	12018±2375 ^{a■}	14848±2047 ^{c▲}	9,8±7 ^{a■}
Control (25C30%)	19735±10097 ^{a■}	9284±2791 ^{b•}	-2,4±2 ^{a■}
MnZn44 (25C30%)	12774±3041 ^{a■}	15265±5858 ^{b•}	16±2 ^{b•}
MnZn850 (25C30%)	34226±8201 ^{b•}	14391±2021 ^{c▲}	15±13 ^{b▲}
MnZn1250 (25C30%)	42895±10084 ^{b•}	127542605 ^{b•}	15,98±7 ^{b•}
Control (25C50%)	33138±6139 ^{a■}	15090±2803 ^{b•}	29,66±13 ^{c▲}
MnZn44 (25C50%)	82348±23849 ^{c▲}	25972±5134 ^{c▲}	38,1±21 ^{c▲}
MnZn850 (25C50%)	81829±18898 ^{c▲}	22516±3513 ^{c▲}	27,9±18 ^{c▲}
MnZn1250 (25C50%)	53417±12021 ^{b•}	20565±1093 ^{c▲}	19,32±5 ^{a■}

Different superscript letters^{a-e} (rows) and symbols^{■•▲◇♦} (columns) indicate significantly different values (p<0.05). The superscript letters indicate whether there was a significant difference in the alkaline phosphatase, glucosidase and urease activity concentrations for mancozeb. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

Table 14 summarises the mean concentration of alkaline phosphatase, glucosidase and urease activity in the binary treatment of copper oxychloride (CuOx) and mancozeb (MnZn) under different temperature and soil moisture conditions. There were no significant differences (p>0.05) in the alkaline phosphatase activity for the CuOx200 + MnZn44, CuOx500 + MnZn850 and CuOx1000 + MnZn1250 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%). There were significant differences (p>0.05) in the glucosidase activity for the CuOx200 + MnZn44, CuOx500 + MnZn850 and CuOx1000 + MnZn1000 mg/kg treatments under the (25°C50%) exposure. There were no significant differences in the urease activity for the CuOx200 + MnZn44, CuOx500 + MnZn850 and CuOx1000 +

MnZn1250 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

When comparing the same treatments with each other (indicated by symbols in superscript) there was a significant difference ($p < 0.05$) in the alkaline phosphatase test between the treatments of CuOx200 + MnZn44 mg/kg under (25°C50) and the other exposures (20°C30; 20°C50; 25°C30). There was a significant difference in the glucosidase test between the same treatments CuOx200 + MnZn44 mg/kg under (20°C30) and (20°C50; 25°C30; 25°C50), and between (25°C50) and (20°C30; 20°C50; 25°C30). For the CuOx500 + MnZn850 mg/kg there was a significant difference between the (20°C30) exposure and the (25°C50) exposure and between the 20°C30 and 25°C30 exposures. For the CuOx1000 + MnZn1250 mg/kg treatment there was a significant difference between the (20°C30) exposure and the (25°C30) exposure. There were no significant differences in the urease activity for the CuOx200 + MnZn44, CuOx500 + MnZn850 and CuOx1000 + MnZn1250 mg/kg treatments under the different temperatures and soil moistures (20°C30%; 20°C50%; 25°C30%; 25°C50%).

Table 14: The mean concentration of alkaline phosphatase, glucosidase and urease activity in the binary copper oxychloride (CuOx) and mancozeb (MnZn) treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) after a 56-day period under different temperatures and soil moistures. N= 3

Treatment (mg/kg)	Concentration		
	Alkaline phosphatase Activity (P-nitrophenol mg/kg/h)	Glucosidase Activity (P-nitrophenol mg/kg/h)	Urease Activity (NH ₄ -N mg/kg/h)
Control (20C30%)	7365±1191 ^{a■}	3108±584 ^{e◇}	4,75±1 ^{a■}
CuOx200 + MnZn44 (20C30%)	2211±1177 ^{e◇}	3011±118 ^{e◇}	13,2±6 ^{a■}
CuOx500 + MnZn850 (20C30%)	991±822 ^{e◇}	2974±731 ^{e◇}	16,37±12 ^{a■}

CuOx1000 + MnZn1250 (20C30%)	10774±2001 ^{b•}	2835±194 ^{e◇}	7,04±6 ^{a■}
Control (20C50%)	2311±362 ^{e◇}	3163±1070 ^{e◇}	18,71±11 ^{a■}
CuOx200 + MnZn44 (20C50%)	10207±3245 ^{b•}	13228±5622 ^{b•}	13,03±9 ^{a■}
CuOx500 + MnZn850 (20C50%)	8270 ±2277 ^{a■}	11320±1331 ^{b•}	-5,5±1 ^{a■}
CuOx1000 + MnZn1250 (20C50%)	1997±757 ^{e◇}	9890±2577 ^{b•}	-6,5 ±7 ^{a■}
Control (25C30%)	19735 ±10097 ^{c▲}	9284±2791 ^{b•}	-2,4±2 ^{a■}
CuOx200 + MnZn44 (25C30%)	26330±8803 ^{d♦}	14716±1949 ^{c▲}	14,52±4 ^{a■}
CuOx500 + MnZn850 (25C30%)	13633±5520 ^{b•}	14758±3174 ^{c▲}	8,32±6 ^{a■}
CuOx1000 + MnZn1250 (25C30%)	8873±7263 ^{a■}	13881±4243 ^{c▲}	-19,1±19 ^{c▲}
Control (25C50%)	33138±6139 ^{d♦}	15090 ±2803 ^{c▲}	29,66 ±13 ^{a■}
CuOx200 + MnZn44 (25C50%)	39070±5029 ^{d♦}	25201±7938 ^{d♦}	29±26 ^{a■}
CuOx500 + MnZn850 (25C50%)	193954783 ^{c▲}	13560±4178 ^{b•}	3,96±6 ^{a■}
CuOx1000 + MnZn1250 (25C50%)	19080±2442 ^{c▲}	12594±2984 ^{b•}	11,44±5 ^{a■}

Different superscript letters^{a-e} (rows) and symbols^{■•▲◇} (columns) indicate significantly different values (p<0.05). The superscript letters indicate whether there was a significant difference in the alkaline phosphatase, glucosidase and urease activity concentrations for the binary mixture of copper oxychloride and mancozeb. Similarly, the superscript symbols indicate significant differences between the same concentrations under the different temperature and moisture groups.

CHAPTER 5: DISCUSSION

5.1 Mortality

Mortality is the end point of the earthworm acute toxicity test, but it is unlikely to be either the most sensitive or ecologically relevant parameter when predicting the effect on field populations (Spurgeon *et al.* 1994). The mortality did not exceed 10% (validity for the control groups) for all the treatments except for the CuOx1000, CuOx500 + MnZn850 and CuOx1000 + MnZn1250 mg/kg (OECD, 2015). Kilpi-Koski *et al.* (2020) tested the effect of binary mixtures of copper, chromium and arsenic to the earthworm *E. andrei* and it was reported that 557 mg/kg of Cu did not affect the survival of *E. andrei*. Vermeulen *et al.* (2001) tested the sublethal and acute toxicity of mancozeb to *E. fetida* and also reported no mortality with mancozeb concentrations below 400 mg/kg. This was also the case in the experiment with single and binary exposures of copper oxychloride and mancozeb. The mortality in the CuOx1000 and CuOx1000 + MnZn1250 mg/kg groups exceeded 10% in all the temperature-moisture combinations but in the CuOx500 + MnZn850 mg/kg treatment, significant mortality was only observed at the 20°C30% exposure. For the CuOx1000 and CuOx1000 + MnZn1250 mg/kg groups the (25°C30%) exposure recorded the highest mortality rate. A study by Maboeta and Fouche (2014) on bioassays of soil from a copper manufacturing site found that copper concentrations of 6925 $\mu\text{g g}^{-1}$ resulted in a mortality rate of 37%. The results from the current study and the Maboeta and Fouche (2014) study indicate that there can be a difference in results when field samples are used. Nonetheless this experiment indicated that different temperatures and soil moistures can have an effect on the mortality of earthworms exposed to single and binary mixtures of copper oxychloride and mancozeb because there were significant differences in the mortality rate of treatments under different temperatures and moistures. However, these significant differences were only observed at higher concentrations which makes mortality a less sensitive endpoint than growth and reproduction. An increase in mortality will negatively impact earthworms on an individual and population level. The increased mortality will reduce the reproductive output of the population as well as the ecological impact that earthworms have on their environment.

5.2 Earthworm growth

As a result of climate change, soil temperature and soil moisture are major factors that affect earthworm growth, survival, fecundity, and behaviour, and indirectly affect the soil environment and food availability (Kaka *et al.* 2021). In addition to soil properties such as pH, organic matter content, salinity, and particle size distribution, changes in climatic conditions (e.g. air temperature and soil moisture content) can affect the behaviour and speciation of metals and metalloids through changes in specific soil properties (González-Alcaraz and Van Gestel, 2016). The relative growth rate of the earthworms under different temperatures and soil moisture exposures varied between the different treatments. In this study, although there were significant differences between exposure concentrations, the different temperature and moisture exposures had varying effects on the single and binary treatments. In the copper oxychloride and mancozeb treatments the relative growth rates decreased over time. There were significant differences between the different treatments although,

the most significant decrease in growth rate was seen in the highest concentrations (CuOx1000, MnZn1250 and CuOx1000 + MnZn1250 mg/kg) and at 25°C30%. Oladipo *et al.* (2019) found that copper oxychloride at higher concentrations (675 and 1000 mg/kg) had a significant negative impact on earthworm biomass. In addition, studies done by Helling *et al.* (2000); Maboeta *et al.* (2004); and Eijsackers *et al.* (2005) found that copper oxychloride had adverse effects on earthworm growth and survival at concentrations ranging from 519-883 $\mu\text{g g}^{-1}$. Although these studies did not take varying temperatures and soil moistures into account, the detrimental effect of copper oxychloride is clear and from this study it can be noted that varying temperatures and soil moistures had an effect on the relative growth rate of the earthworms.

Eggleton *et al.* (2009) reported varying temperature and soil moisture without the addition of a pollutant will also affect the life cycle of earthworm species. They worked on the following species of earthworms, *D. octaedra*, *D. attemsi*, *S. mammalis*, *A. caliginosa*, and *L. rubellus*. In their study, they observed a decrease in earthworm numbers during the dry winter months, and an increase during the moist summer months (Eggleton *et al.*, 2009). Berman and Meshcheryakova (2013) also indicated a similar pattern in their study. From these studies and the current experiment, it can be noted that temperature and soil moisture will have an effect on earthworm populations.

Lima *et al.* (2015) tested carbaryl in LUFA 2.2 standard soil with concentrations between 20-100 mg/kg at temperatures varying between 28, 20 and 8 °C. They found that higher temperatures had more adverse effects on *E. andrei*. González-Alcaraz and Van Gestel (2016) exposed earthworms to arsenic, cadmium and zinc under different temperatures and soil moisture conditions. They observed a loss in biomass that was more pronounced in the soils with the highest concentrations and at 25°C30%. This was also found in the current experiment, although different chemicals were used (copper oxychloride and mancozeb), which indicates that higher temperatures and lower soil moistures can have more adverse effects when compared to normal test conditions. The varying results between exposures suggest that the temperature and soil moisture can affect the bioavailability of heavy metals. This is highlighted by Friis *et al.* (2004); De Silva *et al.* (2009); and Bandeira *et al.* (2020). Friis *et al.* (2004) found that the copper burden in *A. caliginosa* species increased with increasing drought, suggesting that soil moisture might play a significant role in copper toxicity. Carbendazim, carbofuran, and chlorpyrifos were tested by De Silva *et al.* (2009) on *E. andrei* at 20 and 26°C, with the results indicating that the survival was more sensitive at higher temperatures. According to a study by Bandeira *et al.* (2020) on *E. andrei*, imidacloprid toxicity increases when the temperature increases. Although the studies by Friis *et al.* (2004); De Silva *et al.* (2009) and Bandeira *et al.* (2020) did not use metal-based fungicides the effects of varying temperatures and moisture on pesticides is clearly evident and needs to be taken into account.

5.3 Reproductive success

There was a significant difference ($p < 0.05$) in the number of cocoons, as well as the number of hatchlings between the control groups (20°C30%), (20°C50%), (25°C30%), (25°C50%) and the treatments of CuOx200 mg/kg, CuOx500 mg/kg and CuOx1000 mg/kg, respectively. The average number of cocoons and number of hatchlings in the copper oxychloride (CuOx) treatment decreased significantly ($p < 0.05$) as the concentration

increased for all the temperature-moisture combinations. Increasing the temperature and lowering moisture caused even more unfavourable conditions for earthworm reproduction. It is clear from this that reproduction of earthworms is concentration dependent, but also that varying temperatures and soil moisture factors can negatively impact reproduction. There were no juveniles or cocoons in the CuOx1000 mg/kg treatment at (25°C30%). Oladipo *et al.* (2019) had reported a similar trend where no juveniles or cocoons was observed at 675 and 1000 mg/kg of copper oxychloride. Maboeta and Fouche (2014) found that cocoon production was severely lowered in field soils with copper concentrations up to 1400 mg/kg. The Oladipo *et al.* (2019) study used the standard temperature and soil moisture (20±2°C and 60%) and the Maboeta and Fouche (2014) study used temperature and soil moisture at 25°C and 60%. The effects of these chemicals are clearly observed. This experiment clearly indicates that varying temperature and soil moisture does affect the bioavailability of copper oxychloride and mancozeb towards earthworms as well as their reproductive output. When comparing the same treatments with each other under different temperatures and soil moistures this study showed that there was a significant difference in the number of hatchlings in the CuOx200 mg/kg treatment between 20°C and 25°C. Similarly, in the CuOx500 mg/kg group, there was a significant difference between 20°C and 25°C in terms of the number of cocoons. Although this trend was not observed in the CuOx1000 mg/kg treatment, it does indicate that there is a significant response at different temperatures. The 20°C50% exposure had the lowest average number of hatchlings and cocoons for the CuOx200 and CuOx500 mg/kg treatment. The 25°C30% exposure had the lowest average number of hatchlings and cocoons for the CuOx1000 mg/kg treatment. Under the highest concentration, the high temperature and low soil moisture had the most detrimental effect on earthworm reproduction.

There was a significant difference ($p < 0.05$) in the number of cocoons, as well as the number of hatchlings between the control groups (20°C30%), (20°C50%), (25°C50%) and in the treatments MnZn44 mg/kg, MnZn850 mg/kg and MnZn1250 mg/kg. There was only a significant difference in the number of cocoons in the highest concentration (MnZn1250 mg/kg) at (25°C30%). Although this study observed a low mean number of cocoons (61) and hatchlings (4,661, 15; 31) under MnZn850 and MnZn1250 mg/kg, the study by Oladipo *et al.* (2019) (22 °C and 60% moisture) reported no cocoons or juveniles with MnZn800 and MnZn1250 mg/kg. The disparity in the number of cocoons may be due to the different moisture and temperature conditions in the present study. The differences in results obtained from the varying exposures further indicates the effects that varying temperatures and moistures can have on earthworm populations. Dry soils can result in cocoon dehydration, which may prevent embryonic development (Lowe and Butt, 2005). The implications of climate change (increasing temperature and decreasing soil moisture) will have a negative impact on the fecundity of earthworm populations. Vermeulen *et al.* (2001) reported no statistically significant differences between the control and the MnZn8 and MnZn44 mg/kg treatments (25 °C and 75% moisture). This report is similar to the results obtained in this study under the 25°C30% condition, although the soil moisture in the Vermeulen *et al.* (2001) study was 75% and the test species used was *E. fetida*. However, comparing the same treatments at different temperatures and soil moisture levels revealed a significant difference in the number of cocoons and hatchlings in the MnZn850 mg/kg treatment at 20°C and 25°C. This finding implies that soil moisture and temperature played a significant role in the reproductive success of the MnZn850 mg/kg treatment.

There was a significant difference ($p < 0.05$) in the number of cocoons, as well as the number of hatchlings between the control groups (20°C30%, 20°C50%, 25°C50%) and the binary treatments (CuOx200 + MnZn44 mg/kg, CuOx500 + MnZn850 mg/kg and CuOx1000 + MnZn1250 mg/kg).

In the 25°C30% exposure, there was only a significant difference in the number of cocoons under the highest concentration (CuOx1000 + MnZn1250 mg/kg). The result above is consistent with the findings of the mancozeb experiment. Furthermore, at 25°C30%, the CuOx1000 + MnZn1250 mg/kg treatment produced no juveniles or cocoons, and this was also observed in the CuOx1000 mg/kg treatment under the same temperature-moisture conditions. It suggests that the single CuOx1000 mg/kg exposure had the same outcome as the binary CuOx1000 + MnZn1250 mg/kg treatment. Thus, the presence of mancozeb had no antagonistic effect on the copper oxychloride. According to a study by Gowri and Thangaraj (2020) on the monocrotophos pesticide, the amount of cocoon production decreased for *P. barotensis* and *E. eugeniae* when concentrations were increased. This study also indicated that although temperature and soil moisture affected the bioavailability of copper oxychloride and mancozeb, as the concentration increased, the detrimental effects increased. The lower moisture and temperature exposure had the most detrimental effects on reproduction. It is thought that earthworms are susceptible to copper because most tissues are unable to synthesize copper-binding ligands as a response to the metal, and this is managed by regulation rather than accumulative detoxification (Helling *et al.* 2000).

5.4 Avoidance behaviour

For some organisms, avoiding contaminants functions as a survival mechanism to reduce exposure to harmful substances and its ecological consequence can impact populations in the same way as lethal effects, possibly leading to population extinction (Gainer *et al.* 2022). In the (20°C30%) and (25°C50%) exposures, all the treatments showed an avoidance response behaviour (above 80%) throughout the 48-h exposure except in the MnZn44 mg/kg treatment, which showed no response. The (20°C50%) and (25°C30%) exposures presented an avoidance response behaviour for all the treatments except the CuOx200 mg/kg, MnZn44 mg/kg and the binary mixture of CuOx200+MnZn44 mg/kg only in the (20°C50%) exposure. The MnZn44 mg/kg treatment showed no avoidance under any of the exposures. This indicates that the earthworms did not actively avoid substrates with low concentrations of mancozeb and copper oxychloride under the different soil moistures and temperatures. The different temperatures and soil moistures did not affect the avoidance response behaviour of the higher concentrations in any major way. The low concentration of copper oxychloride (CuOX200 mg/kg) showed no avoidance in the (20°C50%) and (25°C30%) exposures while in the (20°C30%) and (25°C50%) exposures it indicated an avoidance response. This result indicates that varying temperatures and soil moistures can impact the avoidance behaviour of earthworms to copper oxychloride. Although this test is rapid, under prolonged exposure there might be different results. Vermeulen *et al.* (2001) tested the effects of mancozeb on the earthworm species *E. fetida* in a cow manure substrate. Their results indicated that *E. fetida* was highly sensitive to mancozeb, as evidenced by its avoidance response to low concentrations. The results from this study differ from those results; *E. andrei* species showed no avoidance under low concentrations

(MnZn44 mg/kg), under the varying temperature and soil moisture exposures. However, the difference in earthworm species and the difference in substrate (OECD soil) used might be a possible the reason for this. Jordaan *et al.* (2012) tested the pesticide azinphos-methyl (20-100 mg/kg) on the *E. andrei* species at 20 °C and 35-40% soil moisture and found that it did not cause any avoidance in the *E. andrei* species. Although this study did not have varying temperatures and soil moistures it did indicate the relevance of avoidance behaviour tests on pesticides. Oladipo *et al.* (2019) tested the effects of *Bacillus cereus* on the ecotoxicity of metal-based fungicide spiked soils towards the earthworm species *E. andrei* at 22°C and 60% soil moisture. They found that at lower concentrations of mancozeb (8 and 44 mg/kg), earthworms significantly preferred inoculated substrates, while at high concentrations (800 and 1250 mg/kg), earthworms completely avoided both inoculated and non-inoculated substrates. Earthworms were observed to prefer inoculated copper oxychloride substrates at 200 mg/kg while they avoided non-inoculated substrates at the same concentration. Copper oxychloride treatments at higher concentrations (450, 675 and 1000 mg/kg) displayed 100% net avoidance response. Although this study did not take different temperatures or soil moistures into account the results were similar especially at higher concentrations. The fact that *Bacillus cereus* had an effect on the avoidance response behaviour and the varying results of the CuOx200 mg/kg treatment from this study supports the idea that other factors such as changes in temperature and soil moisture can affect the avoidance response behaviour of earthworms. Earthworms avoided the higher concentrations (CuOx500, CuOx1000, MnZn850, MnZn1250, CuOx500 + MnZn850, CuOx1000 + MnZn1250 mg/kg) at different temperatures and soil moistures; an indication that this has adverse effects on earthworm populations. This is further noted in the growth and reproduction results which highlight the detrimental effect these chemicals can have.

5.5 Comet assay

This study used the average tail length (µm) of the comets to assess the DNA damage of the coelomic cells. There were no significant differences ($p>0.05$) in average tail length under the lowest treatments (CuOx200, MnZn44 and CuOx200 + MnZn44 mg/kg). There was however a significant difference ($p<0.05$) in all the other treatments. The CuOx500, MnZn850, MnZn1250, CuOx500 + MnZn850 and CuOx1000 + MnZn1250 mg/kg treatments all had significant differences from their respective treatments under the (20°C50%) exposure. The only treatment which differed was the CuOx1000 mg/kg treatment which had significant differences under the (25°C30%) exposure. There was a relationship in the exposure of (20°C50%) which had similar significant differences in the CuOx500, MnZn850, MnZn1250, CuOx500 + MnZn850 and CuOx1000 + MnZn1250 mg/kg treatments. All these treatments were significantly different from their respective treatments under the other exposures (20°C30%), (25°C30%), (25°C50%). These results reiterate the notion that differences in temperature and soil moisture will have an influence on the toxicity of copper oxychloride and mancozeb towards earthworms.

Comet assays can provide an advantage over other strand break assays because measurements are made on individual cells (Reinecke and Reinecke, 2004). Previous studies have indicated the potential benefit of using comet assay in genotoxic studies (Dhawan *et al.* 2003; Fourie *et al.* 2007; Reinecke and Reinecke, 2004; Voua

Otomo and Reinecke, 2010; Wang *et al.* 2016). Mincarelli *et al.* (2019) examined the effects of copper sulphate (30, 60 and 120 mg/kg) on *E. andrei* under 22 °C and 40% soil moisture. After 3 and 6 days there was a significant increase in copper in exposed organisms, although after 9 days despite the fact that there was higher Cu absorption, there was a slightly lower response in DNA damage. Reinecke and Reinecke (2004) tested nickel spiked substrates under 20 °C and 70% relative humidity using the species *E. fetida* and they observed a significant difference in nickel spiked substrates when compared to their negative control. Fourie *et al.* (2007) compared DNA damage of cadmium (01-50 mg/l) with 5 different earthworm species namely, *D. rubidus*, *A. caliginosa*, *E. fetida*, *A. diffringens* and *M. benhami* in a soil water medium for a exposure period of 48 h. They found that the cadmium had significant levels of DNA damage in the following species *D. rubidus*, *A. caliginosa* and *E. fetida*, although no DNA damage was found in *A. diffringens* and *M. benhami* species. It was suggested that higher concentrations of cadmium could induce DNA damage in the 2 species that did not show DNA damage, but this will be above the lethal concentration of *E. fetida* species. Although these studies did not use varying temperatures and soil moistures, they indicate the relevance of using genotoxic studies in ecotoxicology and the need to test climate change conditions in these tests.

5.6 Body metal analysis

There were significant differences in the single and binary exposures under the different temperatures and soil moistures. These significant differences were found in the copper and manganese concentrations but not in the zinc concentration for the single and binary exposures. There were significant differences between the CuOx200, CuOx500 and the CuOx1000 mg/kg treatments of the (20°C30%) exposure and their respective treatments under the (25°C50%) exposure. This indicates that temperature and soil moisture played a vital role in the copper uptake of the earthworm species. The mancozeb results indicate that temperature played an important role in the body metal concentration of manganese in earthworms. With significant differences between 20 and 25°C there is a potential that higher temperatures can have more adverse effects with regards to manganese uptake in earthworms. Similarly, to the single copper oxychloride treatments, the binary treatment showed a significant difference in the copper concentrations in the (25°C50%) exposure under all the treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) when compared to the control. This indicates that the addition of mancozeb to the copper oxychloride did not affect the bioavailability of copper to the earthworms. There were significant differences in the manganese concentration of the (20°C50), (25°C30%) and (25°C50%) exposures in the treatments (CuOx200 + MnZn44 mg/kg; CuOx500 + MnZn850 mg/kg; CuOx1000 + MnZn1250 mg/kg) when compared to the control. There were more significant differences in manganese concentrations in the binary exposure when compared to the single mancozeb exposure. This indicates that the addition of copper oxychloride influenced the manganese concentration. When comparing the same treatments with each other there was significant differences between the (20°C30%) and (25°C50%) exposures in the CuOx1000 + MnZn1250 mg/kg treatment with regards to the copper concentration. This was similarly found in the single copper oxychloride treatment which would indicate again that the addition of mancozeb did not affect the bioavailability of copper to the earthworms.

Although the body metal analysis did not indicate many differences when comparing the single and binary exposures at varying temperatures and soil moistures when looking at the mortality there was a higher mortality rate in the binary treatments at 25 °C 30% when compared to the single treatments, thus making it vital for further testing to take place to establish the relationship that temperature and moisture can have on single and binary treatments.

Earthworms have specific mechanisms for transporting and storing heavy metals, and a redistribution system for maintaining body levels of heavy metals, specifically trace metals, which may assist in maintaining a balance between uptake and excretion, but they must be regulated or differentially expressed to function properly (Burgos *et al.* 2005; Parihar *et al.* 2019). Smith *et al.* (2012) found that essential metals such as copper and zinc are regulated in the earthworm's body by aid of metallothioneins (Smith *et al.* 2012). According to Morgan and Morgan (1990) it was found that lead, zinc, and cadmium concentrations increased with soil metal concentrations in selected tissue fractions; each tissue fraction accumulated these metals, although the posterior alimentary canal (PAC) fraction contained the highest concentrations of these three metals, as well as the highest proportion of whole-body metal burdens. There is much more evidence that earthworms living in heavily Cu-contaminated soils have a much more effective physiology for controlling Cu concentrations than Pb or Cd concentrations (Morgan and Morgan, 1990). Oladipo *et al.* (2019) indicated no significant differences in body metal concentration of manganese and zinc in any mancozeb treatments although, at 800 and 1250 mg/kg there was significantly lower zinc tissue levels. González-Alcaraz and Van Gestel (2016) found that zinc concentration in earthworms reached a steady state after 1 day of exposure in both polluted study soils. Therefore, in both polluted study soils, increasing temperature and reducing soil moisture content had no significant impact on zinc bioaccumulation (González-Alcaraz and Van Gestel, 2016). Reinecke and Reinecke, (2004) found that essential micronutrients such as copper and iron serve multiple physiological functions, which are handled by endogenous transport and control pathways. This indicates that temperature and soil moisture played a vital role in the copper uptake of the earthworm species. Smith *et al.* (2012) indicated that copper concentrations between 50 and 60 mg/kg are indicative of earthworm mortality. This is also viewed in this experiment with high mortality rates at CuOx1000 and CuOx1000 + MnZn1250 mg/kg and the average copper concentration in this treatment was between 44 and 64 mg/kg.

5.7 Soil enzymatic analysis

Soil enzyme activities indicate the decomposition of soil organic matter (SOM), making them excellent sensors of soil microbial health and soil physicochemical conditions, and they have currently attracted great attention as biological indicators of soil quality due to their relative simplicity, environmental sensitivity, and rapid response to land management changes (Kotroczo *et al.* 2014). Enzyme activity triggers chemical reactions occurring in nature and is among the most important key components of the environment (Devi *et al.* 2018). A number of other factors can directly influence extracellular enzyme activities, including temperature, moisture, pH, nutrients and chemical properties (Kotroczo *et al.* 2014). Enzymes are associated with microbial populations in soil, so pesticide application may negatively impact enzymatic activity and biological processes.

The magnitude to which heavy metals (or metal-containing pesticides) affect enzyme activity is dependent on the availability of heavy metals, which is influenced by base saturation and soil acidity, the volume and properties of SOM and clay content as well as the interaction with other inorganic constituents (Sethi and Gupta, 2015). Phosphatases are a broad class of enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid and are considered to be crucial to the phosphorus cycle in soil ecosystems (Riah *et al.* 2014). Both b-glucosidase and cellulase are very important enzymes that are involved in the transformation and decomposition of organic matter in soil, generating glucose that is used by soil microorganisms for energy (Riah *et al.* 2014). Urease hydrolyzes urea into carbon dioxide and ammonia in soil; urease plays an important role in the nitrogen cycle (Madejón *et al.* 2001).

The copper oxychloride treatment indicated significant differences in the glucosidase test. CuOx200 and CuOx500 mg/kg under (20°C50%) was significantly different from their control and when comparing the same treatment with each other, there were significant differences in the CuOx200 mg/kg between 20 °C and 25 °C. the CuOx1000 mg/kg indicated significant differences between the (25°C50%) exposure and the other exposures (20°C30%; 20°C50% and 25°C30%). The mancozeb treatment indicated significant differences in the alkaline phosphatase and glucosidase tests under the (20°C50% and 25°C50%) exposures. When comparing the same treatments with each other the mancozeb treatments under (25°C50%) were significantly different from the other exposures which indicated that higher temperatures and soil moistures had a significant impact on the mancozeb treatments. The binary treatment indicated a significant difference in the glucosidase test in all the treatments (CuOx200 + MnZn44, CuOx500 + MnZn850 and CuOx1000 + MnZn1000 mg/kg) at (25°C50%) exposure. In the alkaline phosphatase test only the CuOx200 + MnZn44 mg/kg treatment indicated a significant difference at (25°C50%) when compared to the other exposures (20°C30%; 20°C50%; 25°C30%). The urease test showed no significant differences in any of the treatments (single and binary) and under any exposures. The mancozeb treatment as well as the binary treatment indicated that higher temperature and soil moisture had a greater influence on the soil enzymatic activity. Since water dissolves pesticides and aids in their movement, diffusion, and microbial degradation, dry soils have a slow rate of pesticide degradation (Devi *et al.* 2018). As water content increases, pesticide transformation also increases. Devi *et al.* (2018) found that temperature significantly impacted both the adsorption and hydrolysis of pesticides in soil, since it determines the solubility of the substance and encourages microbial activity.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study was conducted to determine the effects of temperature and soil moisture changes on the ecotoxicity of copper oxychloride and mancozeb spiked soils in single and binary mixtures towards earthworms. Copper oxychloride and mancozeb are known to have a detrimental effect on the survival growth and reproduction of earthworms. Copper oxychloride is more harmful to earthworms than mancozeb and this was evident in the binary mixture experiments. These chemicals can cause adverse ecological effects on earthworms' ecosystem functions. Changes in temperature and soil moisture can have an effect on the bioavailability of heavy metals in the soil. The relationship between temperature change, varying soil moisture content and heavy metals in the soil is extremely important in understanding this combined effect on the environment. This is evident in this study with results varying under different temperatures and soil moistures.

The growth and reproduction test indicated severe mortality at high concentrations of copper oxychloride (1000 mg/kg) and the binary mixture (CuOx1000 + MnZn1250 mg/kg) although, the highest mortality was recorded at (25°C30%) which suggested that higher temperature and lower soil moisture could have more adverse effects. The growth and reproduction both decreased as the concentrations of the treatments increased and at lower soil moisture and higher temperature (25°C30%) the effects of these treatments were more severe. There were no real notable differences between the different temperatures and soil moistures in the avoidance behaviour experiment. All the treatments showed an avoidance response behaviour except for the CuOx200 mg/kg (20°C50), (25°C30), MnZn44 mg/kg the (20°C30) (20°C50), (25°C30), (25°C50) and the binary mixture of CuOx200+MnZn44 mg/kg (20°C50), which showed no response. No significant difference was found between the different temperatures and soil moistures in the avoidance response experiment. The comet assay experiment was done to evaluate the genotoxic effects of single and binary mixtures of copper oxychloride and mancozeb. There were no significant differences under lower concentration treatments (CuOx200, MnZn44 and CuOx200 + MnZn44 mg/kg). There were significant differences in the other treatments and as expected higher soil concentrations resulted in more severe DNA damage. Similarly, to the growth and reproduction experiment higher temperatures and lower soil moistures had a more severe effect on the earthworms. This was expressed by having longer tail lengths at these conditions. The body metal analysis experiment showed significant differences between treatments under different exposures. All three copper oxychloride treatments indicated significant differences between the different exposures. The single mancozeb treatment and binary treatment only showed significant differences at higher treatments (MnZn850, MnZn1250 and CuOx1000 + MnZn1250 mg/kg) but only regarding the manganese concentration (binary treatment both manganese and copper). There were no significant differences in the zinc concentrations throughout the experiment and this is because zinc is an essential element in the earthworm's body, and they regulate their zinc body concentrations although very high concentrations can be lethal. The soil enzymatic analysis provided further evidence of the influence of varying temperature and soil moisture on single and binary mixtures of copper oxychloride and mancozeb. The (25°C50) exposure showed the most significant difference in the alkaline phosphatase and glucosidase tests.

As seen with the avoidance, growth, and reproduction tests copper oxychloride and mancozeb in single and binary mixtures had negative impacts on the growth and survival of earthworms. Lower moisture contents and higher temperatures had a more severe impact on these organisms, although this was not clearly seen in the comet assay experiment. Therefore, to obtain a clearer picture on the effects that climate change can have on single and binary mixtures of fungicides further studies need to be done.

It can be concluded that different temperature and soil moisture contents did have varying effects on the single and binary mixtures of copper oxychloride and mancozeb. Mortality increased in lower soil moisture. Growth and reproduction decreased under lower soil moisture as well the DNA damage and body metal analysis varied between exposures. The soil enzymatic analysis also indicated significant differences under different temperatures and soil moistures. Understanding the influence of temperature and soil moisture on the bioavailability of heavy metals in the soil is crucial to understanding the effects that these pollutants have in the natural world and help to remediate these effects.

6.2 Recommendations

This experiment compared how different temperatures and soil moistures would affect the toxicity of copper oxychloride and mancozeb towards earthworms. Further research can also include the soil pH as a variable and adjusting the pH can affect the toxicity of these chemicals.

The growth and reproduction tests can be examined through the second generation of earthworms exposed to copper oxychloride and mancozeb under these different temperatures and soil moistures and this can further indicate the relationship between toxicity and temperature and soil moisture.

Further studies should focus on the viability of cocoon production. The viability of the cocoons as well as the production of cocoons is affected by contaminated soils as well as varying temperature and soil moisture.

Long term studies can be done to investigate the temporal nature of DNA strand breaks and if DNA repair will occur after long term exposure. Including this can indicate if temperature change and soil moisture had an effect on DNA damage and repair. Generational studies will also indicate the effects that these pollutants and temperature and soil moisture changes will have on the genotoxicity of individual earthworms, and this should be linked to population growth rates.

Long term studies can be done to investigate the effects of temperature change and soil moisture on soil enzymatic analysis. The soil pH can also be adjusted and considered for such tests.

7. References

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