# Ecotoxicological assessment of aflatoxin in soil under different temperature and moisture conditions utilising earthworms

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“Nature uses only the longest threads to weave her patterns, so that each small piece of her fabric reveals the organization of the entire tapestry.”

Richard P. Feynman

# ABSTRACT

Aflatoxins are secondary metabolites produced by specific strains of fungi. These natural toxins are mainly found in soil, decaying vegetation, and food storage systems and are particularly abundant during drought stress. Aflatoxin contamination is one of the most important threats to food safety and human health, however, limited information about its toxicological consequences in the soil ecosystem is available. Recent studies suggest that aflatoxin contamination might become a bigger problem if the climate continues to change. This research aimed to define the risk of aflatoxin contamination in the soil ecosystem by investigating the toxicological consequences of aflatoxins to earthworms (*Eisenia andrei*) under different temperature and moisture conditions. Further, the biological control of aflatoxins by earthworms were investigated. The review concluded that many aspects of aflatoxin occurrence, degradation, and the effects of its transformation products in the soil environment are still unknown and remain an important area of research for soil health and productivity. Standard toxicity tests with different combinations of air temperature (21 and 26 °C) and soil moisture (30 and 50% of the soil water holding capacity-WHC) were used to investigate aflatoxins' toxicological consequences to earthworms in a soil medium. Sublethal endpoints, including growth, reproductive success and genotoxicity, were monitored. Additionally, aflatoxin concentrations in the soil were monitored over four weeks in the presence and absence of earthworms to establish earthworms' role in aflatoxin degradation. Negligible effects on earthworm survival, growth, and reproduction were observed at aflatoxin concentrations between 10 – 100 µg/kg, but a concentration-dependent increase in DNA damage at standard testing conditions was observed. The influence of temperature and moisture changed the exposure effect outcomes of aflatoxin in soil. Drought conditions (30% WHC) resulted in significantly more negative effects on earthworm reproductive and genetic status under increasing aflatoxin concentrations. The research highlighted the possible risk of environmentally relevant aflatoxin levels to the functional ability of important soil organisms for providing essential ecosystem services under changing climate conditions. However, it also highlighted the potential of earthworms to contribute to the biological control of aflatoxins under favourable environmental conditions.



**Keywords:** aflatoxins; earthworms; soil ecotoxicology; soil moisture; temperature, climate change

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To my mother – I regret not having finished my thesis while you were still with us, but I appreciate that you always believed in my ability to finish. Rest in eternal peace (3 September 2021).

# PREFACE

The work presented in this thesis is for the degree Doctor of Philosophy in Environmental Sciences. The experimental work was done at the Unit for Environmental Sciences and Management, North-West University, Potchefstroom Campus, Potchefstroom, South Africa. The study was conducted on a part-time basis from January 2018 to November 2021, under the supervision of Prof. Mark Maboeta and Prof Sarina Claassens

The research and results presented in this thesis signify original work undertaken by the author and has not been submitted for degree purposes to any other university. The research was supported by the University of South Africa's master's and doctoral support programme (MDSP). Appropriate acknowledgements have been made in the text where the work conducted by other researchers was included.

- All samples were kept at the NWU-Potchefstroom laboratories. The soil preparation (toxin spiking), husbandry, standard toxicity testing and genetic studies of the earthworms were conducted at the NWU laboratories in Potchefstroom.
- The analysis of the soil samples using the ELISA was conducted at the laboratories of the University of South Africa, Florida campus.

The referencing style used in this thesis follows the requirements of the journal *Mycotoxin Research.*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AF | Aflatoxins |
| $AFB_1$ | Aflatoxin $B_1$ |
| $AFB_2$ | Aflatoxin $B_2$ |
| AFG | Aflatoxin $G_1$ |
| ANOVA | Analysis of variance |
| DON | Deoxynivalinol - mycotoxin |
| ELISA | Enzyme-linked immunosorbent assay |
| EW | Earthworms |
| FAO | Food and Agricultural Organisation |
| HPLC | High performance liquid chromatography |
| IOBC | International Organization for Biological and Integrated Control |
| IPCC | Intergovernmental Panel on Climate Change |
| LC-MS | Liquid chromatography coupled with mass spectrophotometry |
| NWU | North-West University |
| OECD | Organisation for Economic Co-operation and Development |
| TLC | Thin layer chromotography |
| µg/kg | Micro gram per one kilogram |
| UNISA | University of South Africa |
| WHC | Water holding capacity of soil |
| WHO | World Health Organisation |

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# CHAPTER 1 – INTRODUCTION

## 1.1. Background

Certain fungi produce harmful toxins, called mycotoxins, as secondary metabolites during normal metabolic processes (Bennet and Klich, 2003). These fungi, also referred to as mycotoxigenic fungi, most commonly occur on crops growing in the field (pre-harvest) or during storage of the agricultural products post-harvest. The mycotoxins from infected plant material can leach into the soil and has the potential to contaminate the groundwater table (Kolpin et al. 2014) and negatively affect soil quality (Elmholt, 2008). When contaminated agricultural products are consumed, it can cause various toxic effects in different organisms, including animals and humans. The five food-borne classes of mycotoxins that are of most concern to food safety are deoxynivalenol, ochratoxin, fumonisins, zearalenone and aflatoxins, of which aflatoxins are considered the most toxic to humans. A large percentage of the world's food crops are destroyed each year due to mycotoxin contamination, which results in substantial annual income losses for producers and traders of edible crops (Guchi, 2015). A recent global review by Eskola et al. (2020), using extensive datasets, suggests that the World Health Organizations' (WHO, 2018) estimation that 25% of the world's food crops are destroyed annually is underestimated. The authors concluded that much higher detected values (up to 80% of crop contamination) have been reported in some areas of the world between 2010 – 2015.

Aflatoxins are produced by specific strains of fungi, namely *Aspergillus flavus* and *Aspergillus parasiticus* and are mainly found in soil, decaying vegetation, compost material, and food storage systems. Close to 20 different types of aflatoxins have been identified. Of these, aflatoxins B1 ($AFB_1$), B2 ($AFB_2$), G1 ($AFG_1$), G2 ($AFG_2$), and a derivative of B1, namely M1 ($AFM_1$), are of most concern due to the severe public health problems it may cause for humans, animals and food safety (Bradford et al*.* 2018). Aflatoxins in food and feed commodities are regulated through monitoring programmes in most countries in the world. Food crops contaminated with aflatoxins exceeding the recommended safe limits must be used for other purposes, for example, animal feed. In severe cases, regulations recommend disposal of the crops either through incineration or incorporation into the soil (EAC Policy Brief, 2018). When contaminated crops are worked into the soil for natural degradation, it increases natural concentrations, altering the ecological balance.

The impact of aflatoxins in humans, other mammals, and food commodities have been studied extensively since the 1960s when aflatoxin first became a concern. However, aflatoxins' occurrence and toxicological consequences in the soil ecosystem have received considerably less attention, and very little is known about the possible consequences of aflatoxin contamination for soil organisms. Decomposing plant residues contaminated with aflatoxigenic fungi introduce aflatoxins into the surface soil (Mishra and Das, 2003; Accinelli et al*.* 2008). Studies from the early 1980s found that

aflatoxin $B_1$ is rapidly transformed into other forms that decompose for much longer in the soil, up to 120 days in some cases (Angle and Wagner 1980). The longer persistence suggests that exposure of soil-dwelling organisms to the other forms of aflatoxin is very likely. Later, it was found that a large percentage of the aflatoxin $B_1$ is retained in the upper soil layer due to adsorption to the soil binding sites (Goldberg and Angle 1985), which can prolong the period of aflatoxin contamination (Accinelli et al. 2008). Many soil organisms that live in and ingest soil could be at risk of aflatoxin exposure. Aflatoxin interacts with DNA and proteins and may alter the normal biochemical functions of these molecules and lead to harmful effects. Azab et al. (2001) found that ingestion of food-borne mycotoxins, including aflatoxins, adversely affect the developmental rate, fertility and fitness of Lepidoptera larvae that feed off contaminated plant material and is thought to be linked to the inhibition of protein translation by the toxins in the insect cells. Mishra and Das (2003) also describe the biological effects of aflatoxin directly linked to DNA damage and inhibition of protein synthesis.

Earthworms play a key role in various soil functions and are widely acknowledged as good bioindicators of soil health. They are frequently used during risk assessments and ecotoxicological studies because they are sensitive to chemical and physical changes in soil and can detect negative effects early in the soil ecosystem (van Gestel 2012). Earthworms further influence the activity of many other important soil organisms and can regulate plant pathogens. Fungi are common food sources for earthworms (Zirbes et al. 2011) which means they can exhibit antifungal activity. A study by Wolfarth et al. (2016) showed that earthworms (*Lumbricus terrestris*) contribute to the sustainable control of the mycotoxin deoxynivalenol (DON) in wheat straw by feeding on *Fusarium spp*, the fungi responsible for the production of these mycotoxins, thus reducing the risk of environmental pollution as an ecosystem service. In a study by Schrader et al. (2013) it was demonstrated that earthworms could take up the mycotoxin DON after feeding on the fungi infested crop residues and incorporate it into their gut and body wall tissue where it might have unfavourable effects on their physiological and reproductive health. Although earthworms are fungivorous organisms that can regulate fungal populations, it is not currently known to what extent earthworms regulate the aflatoxin production by fungi in the soil. Szabó-Fodor et al. (2017) exposed earthworms to $AFB_1$ via the contact paper test and detected harmful effects on the physiology and reproduction of *E. fetida*. Further studies investigating the effects of aflatoxin on earthworms in a soil medium and whether the same negative effects might occur could not be found. Any long-term interference with their biochemical processes could negatively affect their functional response and alter soil fertility (OECD, 2016).

## 1.2. Problem statement

Aflatoxin contamination of food crops and stored food commodities remains one of the most important threats to food safety and human health. However, the fate and consequences of aflatoxin contamination in soil (Accinelli et al. 2008) and on soil organisms providing essential ecological services (Szabó-Fodor et al. 2017) remain unclear and could potentially pose a risk to soil health, agricultural productivity and food safety. Aflatoxin is considered low risk in the soil environment, and food contamination after research in the 1980s determined its adsorption potential to soil binding sites and natural degradation by soil microbes. However, once adsorbed to the soil binding sites, it becomes unavailable for microbial degradation and can remain in the soil for much longer periods (Angle 1986), where the exposure of soil organisms are prolonged. Since then, very little research has been done on this topic, and the effects of aflatoxin-contamination on organisms that live in and ingest soil were not considered.

The effects of the breakdown products of aflatoxin are seldom considered. Due to technological advances, the presence of different and previously unknown breakdown products have been revealed after microbial degradation (Starr et al. 2017) that might still have cytotoxic and genotoxic properties (Krifaton et al. 2011). More attention needs to be focused on the ecological risks of genotoxic and carcinogenic toxins for their chronic and long-term effect on the soil environment. In addition, pre-saturated soil reduces the adsorption of $AFB_1$ to soil particles (Starr and Selim, 2008), making the toxins and the transformation products more prone to leaching and increasing the bioavailable portion of $AFB_1$ in the soil solution before degradation. Recent studies also suggest that aflatoxins levels are probably much higher than was previously thought (Eskola et al. 2020). Finally, when contaminated crops are worked into agricultural soil for natural degradation as recommended by current regulations, it will increase natural concentrations and potentially change the soil's physicochemical characteristics and biotic parameters, thus affecting the ecological balance.

Aflatoxin production by *A. flavus* generally increases during drought stress (Fountain et al. 2014). Aflatoxin production is a common problem in Africa because soil moisture stress is one of the overriding constraints in most parts of Africa (Eswaran et al. 1996). According to the Intergovernmental Panel on Climate Change report (IPCC 2019) (Jia et al. 2019), the variability in natural climates will continue to impact terrestrial ecosystems in the future. Several studies have indicated the increased risk of post-harvest aflatoxigenic fungi becoming more frequent at pre-harvest stages under changing climates (Medina et al. 2017). It has further been suggested that climate change, especially drought stress, may increase the geographical distribution of mycotoxigenic fungi and their mycotoxins (Moretti et al. 2018). If climate conditions continue to change and drought stress increase in many parts of the world, it could potentially make aflatoxin-contamination a bigger risk to ecosystems, organisms, food crop quality, and human health (Medina et al. 2017). Therefore, a climatic approach is important for future risk assessments of aflatoxins in natural environments (González-Alcaraz et al. 2018).

The mitigation of aflatoxin contamination and the physical, chemical and biological methods to detoxify contaminted food products, has been extensively studied but remains an ongoing challenge for the food industry (Pankaj et al. 2018). Although decontamination by physical and chemical means are effective, it has proved uneconomical. Biological methods are becoming a promising alternative (Hackbart et al. 2014). More research is being focused on the biological control methods because it holds promise as an effective management strategy (Weaver et al. 2015; Raksha Rao et al. 2017) with fewer negative effects for the food industry than chemical methods. If earthworms act as a suitable remedial agent for pre-harvest aflatoxin, it might have economic benefits by reducing the need for harmful antifungal agents in crop systems infected by aflatoxins.

### 1.3. Justification and rationale of the study

The soil's physical, chemical, and biological conditions must be optimised to ensure sustainable crop production and food security. However, any effect of climate change on the ability of soil ecosystems to function normally and that might impact food supply must be considered and integrated into research. Determining the effect of aflatoxin contamination in the soil is an important area of research for protecting soil biodiversity, sustainable food crop production and the toxin's risk to human health. This study will focus on understanding how environmental variables altered by changing temperatures and soil moisture conditions might influence aflatoxins' occurrence and toxicology in the soil ecosystem. A comprehensive review of the current knowledge of aflatoxin contamination in soil ecosystems will be done to evaluate if aflatoxin contamination should still be considered a low-risk problem in soil.

A research-based approach will be followed to investigate how aflatoxin contamination might affect important soil organisms, specifically earthworms' functional ability and physiological health. Knowledge gained from this data intends to increase our understanding of the ecotoxicological effect of aflatoxin in soil under different climatic conditions as well as how predicted future climatic conditions (temperature and moisture content) will influence the occurrence and the toxicity of these harmful toxins in our soil ecosystems. Potential negative effects on beneficial soil organisms that play a key role in soil ecosystems will negatively affect the overall soil quality and, in turn, food security. A better understanding of the risks will allow for improved soil management strategies to work towards a more sustainable soil environment during changing climate conditions. Further, it will be of interest to the public health agencies responsible for monitoring the toxin to address or prepare for potential hazards.

This research aligns with the aims of the National Development Plan (NDP) of South Africa to ensure a "decent standard of living" for all in line with the NDP vision statement chapter five, "ensuring environmental sustainability" and chapter ten, "promoting health" by focusing on soil health and healthy food production. It further aligns with the aims and targets set by the United Nations Sustainable Development goals 12, 13 and 15 by contributing knowledge towards "sustainable

management and efficient use of our natural resources", understanding better the "adaptive capacity to climate-related hazards, impact reduction and early warning" and " protect, restore and promote sustainable use of our of terrestrial ecosystems" particularly the conservation of our soil resources and soil biodiversity that provide so many essential functions towards a healthy society.

### 1.4. Aim and Objectives

This study aimed to define the risk of aflatoxin contamination in the soil ecosystem by investigating the toxicological consequences of aflatoxins to earthworms (*Eisenia andrei*) under different temperature and moisture conditions and whether earthworms contribute to the biological control of aflatoxins. The specific objectives were:

1. Review the current knowledge on the occurrence, fate, and effects of aflatoxins in the soil ecosystem (Chapter 3).
2. Investigate the effect of aflatoxins on the survival, growth and reproduction of earthworms (*Eisenia andrei*), using standard (OECD) toxicity testing (Chapter 4).
3. Assess the level of DNA integrity in earthworm cells after exposure to $AFB_1$ utilising the comet assay (Chapter 4).
4. Evaluate if different combinations of air temperature and soil moisture affect the toxicity of $AFB_1$ to earthworms (Chapter 4).
5. Investigate if earthworms (*Eisenia andrei*) play a role in and affect the degradation of aflatoxins in the soil (Chapter 5).
6. Investigate if different temperatures and moisture conditions affect the aflatoxin degradation potential of earthworms (Chapter 5).

Here the null hypothesis was tested that :

- Aflatoxin does not cause a difference in the life-cycle processes of earthworms.
- No significant genotoxic effects are caused in earthworms after $AFB_1$ exposure at different concentrations.
- Soil climatic conditions do not significantly contribute to the toxicology of AFB1 to earthworms.
- The presence of earthworms does not significantly decrease the aflatoxin concentrations in soil.
- Soil climatic conditions do not significantly affect the ability of earthworms to decrease aflatoxin concentrations.

### 1.5. Ethical considerations

Ethical approval was obtained from the NWU - Faculty of Natural and Agricultural Sciences Ethics Committee (FNAS-Rec) and the Unisa - CAES Health research ethics committee . After review, the study was categorised as minimal risk.

- FNAS-Rec approval number: NWU- 01532-20-A9 (Annexure A)
- Unisa – Rec approval reference number: 2019/CAES_HREC/141 (Annexure B)

The risks identified before the start of the study:

- Aflatoxin is a carcinogen in humans and highly toxic, and all dermal and oral contact with the toxin was prevented at all times by wearing the appropriate protective gear.
- During the study, general laboratory safety precautions concerning the handling, storage, and disposal of toxic and carcinogenic substances were read and adhered to (Mycotoxin operating and safety handling, ORA lab manual, vol 4 section 7).

### 1.6. Outline of the thesis

Chapter 1 – The *Introduction* will provide a brief background to the study, including a problem statement highlighting the gap in the knowledge, the aim, and the study's objectives.

Chapter 2 – In the *Literature Review,* relevant literature not discussed in further chapters are discussed, including the importance and management of soil as a natural resource; the effects of climate change on soil health and productivity; natural toxins and their impact on food security with specific emphasis on aflatoxin, the essential role and ecosystem services of soil organisms, for example, earthworms and microbial communities.

Chapter 3 – *Aflatoxins in the soil ecosystem: an overview of its occurrence, fate and effects*. This chapter will provide an overview of the current knowledge on the occurrence, fate, and effects of aflatoxins in the soil ecosystem to clarify the focus that future ecological studies should have to define the risks associated with aflatoxin contamination in soil ecosystems.

Chapter 4 – *Ecotoxicological effect of aflatoxins on earthworms (Eisenia andrei).* This chapter will investigate the ecotoxicological effects of aflatoxins on earthworms (survival, growth, reproduction and genotoxicity) under different temperature and moisture conditions.

Chapter 5 – *The role of earthworms in aflatoxin degradation in soil under different temperature and moisture conditions.* Chapter 5 investigates whether the presence of earthworms affects the natural degradation of aflatoxin $B_1$ in soil and whether climatic conditions (soil temperatures and moisture) influence their ability to degrade the toxins.

Chapter 6 – The *Conclusions and Recommendations* is a summative chapter that provides the overall conclusions of the study based on the original objectives. It also provides general recommendations for future studies based on the findings of the current investigation.

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# CHAPTER 2 - LITERATURE REVIEW

## 2.1. Soil health and productivity

The soil is a dynamic environment and central to all earth systems. It is the fundamental regulatory compartment for all terrestrial life and provides essential ecosystem services. Ecosystem services refer to the subset of processes provided by an environmental compartment (Daily, 1997). In soil, these supporting services include clean water and air due to the filtering functions in soil, habitat, food production and poverty alleviation (Pereira et al. 2018). Further, soils play a fundamental role in regulating climate, including thermal and moisture balance, greenhouse gases and particulates in the atmosphere (FAO, 2015). Unfortunately, most of the world's soil resources are in poor condition. Soil degradation due to human activities are already a global problem but compounded by climate change, can become an even larger societal problem, compromising food security and ecosystem stability. The decline in soil condition is mostly attributed to soil erosion, organic carbon loss, soil contamination, soil acidification and salinisation, biodiversity loss, compaction and surface effects and soil moisture conditions (FAO, 2015). It affects various soil functions and the provision of important ecosystem services. The Soil Framework Directive of the European Union recognises seven soil functions that are most at risk (FAO, 2015):

a. biomass production
b. storing, filtering and transforming nutrients
c. biodiversity pool, such as habitats, species and genes
d. physical and cultural environment for humans and human activities
e. source of raw materials
f. acting as a carbon pool
g. archive of geological and archaeological heritage.

Soil quality is one of the three components of environmental quality, besides air and water quality. Soil quality, however, is more complex than water and air quality because it not only focuses on the degree of soil pollution but also on its capacity to function in a specific ecosystem (i) to sustain productivity, (ii) promote plant and animal health within its specific ecosystem and (iii) maintain overall environmental quality (Bünemann et al. 2018). The terms soil health and soil quality are directly linked and often used synonymously (Brevic, 2009). However, soil health is more related to the biological integrity of the soil, whereas soil quality is more related to its ability to function and relates to a combination of its physical and biological integrity. Healthy soils improve the soil quality but do not provide for soil quality on its own. Soil quality depends on the structure as well as the healthy functioning of the ecosystem. The physical characteristics of the environment, such as the annual cycles of temperature and rainfall, shape the soil structure and the characteristics of the biological communities in the ecosystem, whereas the function is determined by the kinds and

combinations of species that make up the system. Negative effects on soil health and quality translate into negative effects on soil productivity (Drobnik et al. 2018). Reduced soil productivity affects agricultural and livestock production, food security, and environmental sustainability, indirectly affecting economic growth and well-being, especially in developing countries where people are most vulnerable (Nielson, 2020).

In recent years, the contribution of soil biodiversity in providing, regulating, and maintaining a diverse range of soil ecosystem services has become more apparent (Perreira et al. 2018) and received attention outside the scientific community. The United Nations Decade on Ecosystem Restoration (2021 – 2030) recognises that to prevent, halt and reverse the degradation of soil ecosystems greatly depends on the protection of soil biodiversity (FAO, 2020). The protection of soil biodiversity has environmental and economic value because it benefits the environment and provides for the sustainable production of goods and services to the benefit of humanity (Plaas et al. 2019; FAO, 2020).

## 2.2. Soil biodiversity and their ecosystem services

The soil includes an immense complexity of biological life, from genes to species to communities (FAO, 2020). Soil biodiversity includes micro-, meso, and macro-organisms with the microbial communities forming the largest part of the soil biodiversity. This grouping according to size, however, does not explain their ecological function in the soil. Although many of these organisms are functionally redundant, many have unique roles to play.

Bacterial and fungal communities can be found in almost any environment due to their ability to live in and adapt to a wide range of pH and temperatures (Dubey et al. 2019). Their contribution to soil function is most critical due to their abundance and play an important role in the productivity and management of agricultural systems, especially the decomposition and turnover of soil organic matter (SOM), nitrogen fixation, disease suppression and producing plant growth stimulators (Elmer, 2009; Magdoff and van Es, 2021). They form mutualistic relationships with plants and root systems for nitrogen fixation, hormone production and pathogens control. Microbial communities are especially important in controlling plant-parasitic nematodes and fungi without harming the plant (Piskiewicz et al. 2009; Magdoff and van Es, 2021). In addition, bacterial and fungal communities have great potential for the bioremediation of contaminated sites. They affect the concentration and movement of pollutants and can detoxify harmful toxins (Madden & Stahr, 1993). Many species of fungi possess the ability to bioaccumulate toxic metals, e.g. copper, cadmium, mercury and zinc) in their fruiting bodies (Frąc, 2018). Soil microbial communities are considered the most effective management tool for environmental pollution control (Verma et al. 2017). Although fungal and plant interactions provide significant benefits for crop production, fungal diseases and toxins also represent some of the greatest threats to crop yields and food security (Willis, 2018).

Apart from soil microorganisms, several invertebrate species, e.g. earthworms, also provide many important ecosystem services in the soil environment (van Gestel, 2012). Earthworms and nematodes represent a large proportion of the soil biomass and contribute significantly to the soil's physical, chemical, and biological processes. Like microorganisms, nematodes and earthworms also play an important role in the soil organic matter dynamics and nutrient cycling, especially due to their interaction with the soil microbial communities (Costa et al. 2012). Some nematode groups are biocontrol agents of soil-borne plant fungal pathogens and snails (Askary and Abd-Elgawad, 2017). However, like fungi, not all nematode species are beneficial and many live as parasites on plant roots.

Earthworms are considered ecosystem service mediators (Plaas et al. 2019) due to their contribution of key aspects such as decomposition of organic matter, bioturbation (mixing of soil layers and organic matter) activities for improved soil structure and aeration. They are categorised into three ecological groups based on their feeding and burrowing behaviour: *epigeic*, *anecic* and *endogeic* (Bouché 1977). Epigeic earthworm species live in and feed on accumulated organic matter in the upper soil layers, while anecic species are detritivorous and forage on organic residues at the soil surface, which they pull into their burrows extending deep into the soil. The epigeic and anecic species are considered the primary decomposers. Endogeic species are usually geophagous and feed on a combination of organic residue and soil, and form shallow and semi-permanent burrows.

The earthworm tube-like digestive system host an abundance of microbes (bacteria, fungi and mesozoans) that play an important regulatory role in the host nutrient metabolism, immune system and other physiological functions (Liu et al. 2018). Several studies indicate the benefits of earthworms for cropping systems and their ability to advance agricultural sustainability (Bertrand et al. 2015) directly through their burrowing and feeding activity and indirectly by regulating the activity of other essential soil macro- and micro-fauna (Hoeffner et al. 2018, Plaas et al. 2019) and flora (Thouvenot et al. 2021). As soil and decomposing organic material move through the earthworm digestive tract, it creates castings full of organic matter and unique groups of microbial taxa that may enrich the soil. The earthworm gut fluid excreted in the castings has also been shown to regulate certain microbial communities by limiting the growth of some species while enhancing the proliferation of others (Byzov et al. 2007) and can even facilitate disease suppression (Elmer 2009). They are also essential biological regulators of plant pathogens such as fungi (Schrader et al. 2013). Earthworms detoxify the soil by aiding in the degradation of toxins, soil remediation, and land restoration (Edwards 1998; Zeb et al.2020), although it has been suggested that their remediation potential is often mediated by their intestinal bacteria (Sun et al. 2020). The specific mechanism of pollutant transformation is not yet known.

The activity, ecology, and dynamics of soil organisms are affected by anthropogenic activities, including but not limited to mining, agricultural management practices and the use of agricultural chemicals and veterinary medicines (Jensen et al. 2007; Hackenberger et al. 2018). In addition, it is

affected by several natural environmental factors such as the pH, temperature, the availability of nutrients and water, and the relationships and interactions with other organisms (FAO 2015).

### 2.3. Effects of climate on soil and soil communities

Air temperature and soil moisture affect the physical, chemical and biological processes in soil (Eswaran et al. 1997). Climatic changes can have different implications for different geographical areas (Jia et al. 2019). Although some areas could benefit from increased temperatures and altered rainfall, most areas may be negatively affected due to more frequent and extreme conditions, e.g. irregular precipitation patterns, increased temperatures and severe drought or waterlogging. Climatic changes may also result in several direct and indirect effects for soil quality and soil health. Several studies have reported possible changes in soil processes and species functional ability in ecosystems due to climate change, including soil organic carbon and nutrient dynamics (Doran et al. 1996; Acharya and Mishra, 2019). Classen et al. 2015 reviewed the direct and indirect effects of climatic changes on soil microbes-plant interactions, and it was suggested that the indirect effects on microbes, mediated by plant and soil litter inputs, might be stronger than the direct effects of climate on soil microbial communities. Soil respiration (the release of carbon dioxide) during the decomposition of organic matter by soil organisms generally increases in warmer soil temperatures and increased soil moisture (Doran et al. 1996). According to the IPCC (2019) report, there is a high probability that increasing soil temperatures and the resulting increased litter inputs will accelerate microbial respiration and increase carbon losses from the soil (Jia et al. 2019). Soil communities' nutrient cycling and decomposition activity are also affected during droughts when primary production becomes limited due to the changes in the soil-water-gas equilibrium. The altered vegetation type and density reduce organic matter accumulation rates (Perreira et al. 2018; FAO, 2015).

Direct effects of climate on soil food webs are equally a serious problem and might alter the behaviour and demographics of terrestrial species (Sing et al. 2019). Direct negative effects of climatic changes, especially temperature and soil moisture, on the biochemical processes of soil organisms have been observed in several soil species, including fungi (Bidartondo et al. 2018) and earthworms (Acharya and Mishra, 2019; González-Alcaraz et al. 2018). Consequently, these impacts on individual species might transfer into changes in species composition and ecosystem services. Some data suggest that warmer soil conditions combined with atmospheric $CO_2$ and altered soil moisture might modify host-resistance and host-pathogen interactions. It may shift towards fungal dominance in some areas instead of bacterial dominance, which increases the risk of fungal diseases and mycotoxin production (Pritchard 2011; Moretti et al. 2019). Some studies have also reported on altered toxicokinetics of toxic substances due to changing climate conditions (Hooper et al. 2013). González-Alcaraz et al. 2018 observed increased bioavailability and toxicity of cadmium in earthworms under warmer and drier conditions and an increased risk of transfer to higher species. Similar findings have been reported in studies investigating different temperature regimes

on the toxicity of agricultural pesticides (Bandow et al. 2014; Velki and Ečimović, 2016; Jegede et al. 2017).

Managing and protecting soil biodiversity can mitigate some of the effects of climate change in the soil environment (Pritchard 2011). Microbial communities can increase plants' resistance to various abiotic and biotic stresses (Dubey et al. 2018), while shifts in fungal diversity are directly linked with tree tolerance to climate change (Gehring et al. 2017). A study by Siebert et al. (2019) suggests that earthworms can reduce some of the negative effects of climate change, specifically increased temperatures, on the taxon richness of meso and macro-organisms.

## 2.4. Biological toxins

Important biological toxins are derived from bacteria (e.g. botulinum neurotoxins, staphylococcal enterotoxins), plants (e.g., ricin and abrin), marine animals (e.g. saxitoxins, tetrodotoxins, brevetoxins, ciguatera, domoic acid, okadaic acid, azaspiracids, and palytoxins) and fungi (e.g. mycotoxins) (Henkel 2010; Gerssen, 2010, WHO, 2018).

Mycotoxins (secondary metabolites produced by fungi) are small, low molecular weight, non-proteinaceous molecules, or peptides and proteins (WHO, 2018). The type and the amount of toxin produced by the fungi vary considerably and depend on the environmental conditions (pH, moisture, oxygen concentration and temperature) or the inter- and intraspecies competition with other organisms (Bräse et al. 2009). Fungal biomass usually increases during favourable (warm and humid) conditions, whereas toxin production mostly occurs during unfavourable conditions such as drought stress (Medina et al. 2017).

Close to 400 different types of mycotoxins have been identified, and they are mostly grouped according to their toxicity to vertebrates as mutagenic, carcinogenic or teratogenic (physical abnormalities during fetal development) (Cimbalo et al. 2020). Five food-borne mycotoxins usually cause the most economic and health concerns and are known as deoxynivalenol, ochratoxins, fumonisins, zearalenone and aflatoxins. Of these, aflatoxins are considered to be the most toxic. It is estimated that approximately 25% of the world's crops are contaminated by mycotoxins (FAO, 2015), although studies have shown that this is much higher in specific areas of the world (Eskola et al. 2020).

### 2.4.1. Aflatoxin

Aflatoxins are produced by specific strains of fungi, especially *Aspergillus flavus* and *Aspergillus parasiticus*. They are some of the best known and most studied mycotoxins (Cimbalo et al. 2020). Close to twenty different types of aflatoxins have been discovered, but aflatoxins B1 ($AFB_1$), B2 (AFB2), G1 (AFG1), G2 ($AFG_2$) and M1 ($AFM_1$) are of most concern due to their impact on human and animal health (Zain, 2011), and food safety (Bradford et al. 2018). The fungi produce $AFB_1$ or

$AFG_1$ as a result of unfavourable environmental conditions during their metabolic processes. These are then transformed into $AFB_2$ and $AFG_2$, respectively. Aflatoxin contamination most commonly occurs on crops and food products, including cereals, nuts, spices and dried fruits (WHO, 2018). Aflatoxin $M_1$ occurs in milk and dairy products. It is a metabolite of $AFB_1$ produced in dairy cows when they feed on contaminated food.

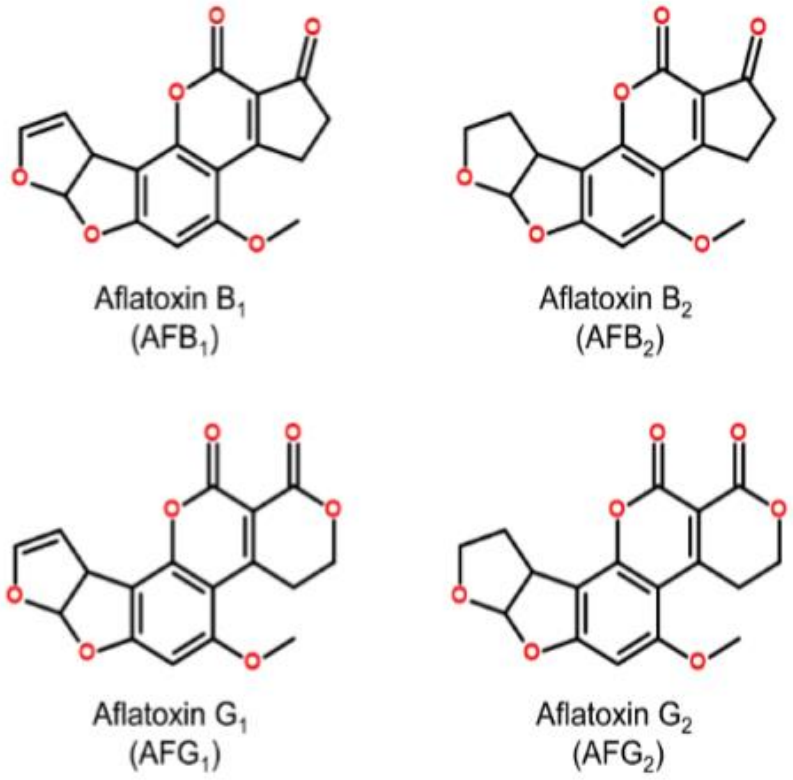


**Figure 2.1:**The chemical structure of the four major aflatoxins, $B_1$ and $G_1$, and their derivatives $B_2$ and $G_2$.

These toxins have similar structures and form a unique group of highly oxygenated, heterocyclic compounds. The B in AFB refers to the blue colour emitted by the toxins when they are viewed under fluorescent light, whereas the G in AFG refers to the green colour (Fig 2.2) emitted when it absorbs ultraviolet light and are viewed under fluorescent light (Eaton and Groopman, 1993).

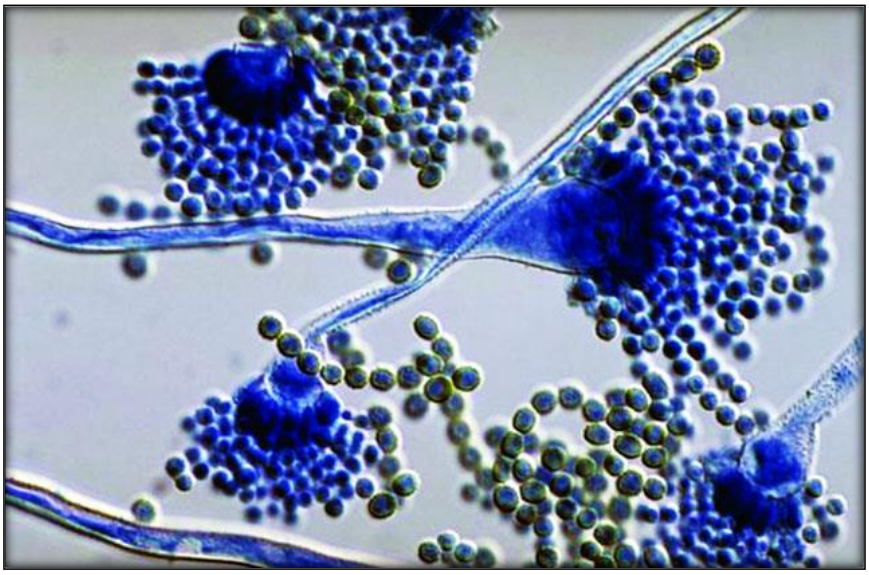

**Figure 2.2 :**Aflatoxin B (AFB) reflects blue and aflatoxin G (AFG) green after absorbing UV light
Source: Innotech.com

Aflatoxin may be cytotoxic, genotoxic, mutagenic and immunosuppressive, and classified as a group 1 carcinogen (Ostry et al. 2017). $AFB_1$ is one of the major causes of liver cancer (hepatocellular carcinoma) in Asia (Liu & Wu 2010) and Africa (Wagacha & Muthomi, 2008). In addition to the public health concerns posed by aflatoxins, their presence can also have serious economic implications for food and feed crop farmers (Bradford et al. 2018).

Aflatoxins have thus far been considered as low environmental risk (Angle and Wagner, 1981). However, the risk is based on its leaching potential into the soil water and is considered low risk because the adsorption onto soil prevents $AFB_1$ from leaching into groundwater. Angle and Wagner (1981) found that $AFB_1$ forms a conjugate with the clay or organic component in the soil. Goldberg and Angle (1985) found that soil with a high absorption coefficient, especially silty clay loam, retained between 80 and 92% of the aflatoxins posing little risk for leaching into the ground-water but increasing its persistence in the soil. The risk to organisms living in and ingesting the soil was not considered, questioning its low-risk status in the natural environment. Subsequently, low concentrations of $AFB_1$ (0.5 µg/ml) have been reported to cause phytotoxic effects in young plants due to alterations in their chloroplast morphology (McLean et al. 1995). Adverse negative effects have also been reported in living organisms, including reptiles, insects, birds and fish. Elshafie et al. (2007) observed high mortality of green sea turtles (*Chelonia mydas*) embryos on the beaches of Oman. It was suggested that aflatoxigenic fungi and their toxins in the soil and on the eggshells were the cause of the egg hatching failure and mortality of the developing embryos. Studies also show that $AFB_1$ causes negative effects on the reproduction, larval developmental or pupae stages of insects (Azab, 2001; Zhao et al. 2018) that feed on contaminated plant residue. In addition, negative effects on birds and fish have been reported and are usually due to anthropogenic interventions. Lawson et al. (2006) documented the first findings of positive hepatic aflatoxin residues in garden bird species in Britain after feeding on contaminated birdseed. In fish, chronic and acute aflatoxicosis

(ingestion of one or more types of aflatoxins) has been reported, resulting in severe toxic effects and even fish mortalities (Santacroce et al. 2008; Anater et al. 2016). According to Anater et al. (2016), fish can bioaccumulate dietary $AFB_1$ in their muscle and organ tissue, posing a health risk for consumers that feed on contaminated fish.

Research into the effects of aflatoxin on microbes normally focuses on the microbes' ability to detoxify or degrade aflatoxin with little consideration for the effects on community diversity. Angle and Wagner (1981) assessed the effects of $AFB_1$ on soil microbial communities using plate counts and a soil respiration study. Soil microbial communities were unaffected at low concentrations (1 and 100 µg/kg soil), whereas higher concentrations (10 000 µg/kg) had statistically significant effects on soil respiration. Very little research has been done to determine the effects on the structure and function of the microbial communities, but Wang et al. (2016) found that $AFB_1$ decreases the diversity of gut microbial communities in male rats while increasing community composition evenness, highlighting the possibility of structural changes in soil microbial communities when exposed to aflatoxins.

Post-harvest management strategies to control aflatoxin contamination focuses mainly on the correct storage temperatures and humidity. Drying foods prevents the accumulation of aflatoxins during storage (Bradford et al. 2018). Contaminated foods may be decontaminated or detoxified by physical (Grant and Phillips 1998; Jaynes et al. 2007) and chemical (Luo et al. 2014). methods to allow them to still be used. Physical detoxification in animal feed through the addition of clay additives are frequently used to decrease the bioavailability of the aflatoxins in the digestion system and have shown promise in preventing aflatoxicosis in animals (Jaynes et al.2007). Biological control of aflatoxin by fungi, bacteria, and their enzymes has been widely reported and is frequently used in the food industry to minimise $AFB_1$ contamination (Krifaton et al. 2011; Verheecke et al. 2016).

Several techniques have been developed for the detection and determination of aflatoxins in food and feedstuff. As aflatoxins are strictly regulated in most parts of the world, advanced and accurate analytical methods have been developed to achieve results with very high precision (Yao et al. 2015). Common techniques used in aflatoxin analysis include but are not limited to thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrophotometry (LC-MS) and enzyme-linked immunosorbent assays (ELISA). The HPLC and LC-MS are the most sensitive analytical techniques and can detect several types of mycotoxins simultaneously. However, it requires expensive equipment, specialist setup and validation of the specific commodity being monitored. Because aflatoxins were previously considered innocuous in soil, specific HPLC and LC-MS methods to detect and quantify aflatoxins in the soil have only recently been validated (Albert et al. 2021).

ELISA techniques are rapid and use inexpensive equipment, but it is not as sensitive as the chromatographic techniques. Matrix interference is possible, especially in plasma samples, and confirmatory LC analyses are often required (Yao et al. 2015). However, ELISA methods are used

for pre-screening purposes because it is a simple and rapid technique and the most frequently used analytical tool for detecting and quantifying aflatoxins in agricultural products (Anfossi et al. 2016). With the competitive ELISA method, a microtiter plate is pre-coated with aflatoxin. The sample is added along with a primary antibody specific for aflatoxin. After the addition of the sample, the aflatoxin in the sample competes with and prevents the antibody from binding to the pre-coated toxin attached to the well. During analysis, the resulting colour intensity has an inverse relationship with the sample's target concentration, meaning that there is a negative correlation between the optical density value of samples and the concentration of aflatoxins. The optical density of the known aflatoxins standards is then used to generate a standard curve from which the aflatoxin concentrations in the samples can be calculated (Nix and Wild, 2001).

### 2.5. Bioindicators and biomarkers in soil ecotoxicology

An ecological risk assessment requires information that reflects the potential exposure in the field and estimates the effects on terrestrial organisms and their functions. Various soil quality assessment indicators (chemical, physical and biological) exist and are used for monitoring approaches. Indicators are chosen based on their relevance to a specific soil threat, function or ecosystem service (Bünemann et al. 2018). The ecological risk of toxins cannot be evaluated only by measuring the total concentration of the chemicals/toxins in the soil. Therefore, a combination of biological (bioassays), chemical and physical indicators are often used as tools to monitor the effects of the toxins in the ecosystem and greatly improve quality assessment (Bünemann et al. 2018). Applying bioassays during risk assessments and ecotoxicological studies have great value in the estimation of the biochemical and cellular responses (i.e. biomarkers) of biological organisms (bioindicators) after exposure to a contaminant or toxin and are more closely related to soil functions (Bouma et al. 2014).

Biomarkers can include biochemical, physiological, histological and morphological measurements within an organism to assess its health (van Gestel and van Brummelen, 1995). The effects of contaminants at the cellular, biochemical and physiological level indicate the effects at the individual level and often occur more rapidly than at the population level. Biomarkers are therefore considered an early warning of toxicological effects within an ecosystem (Lionetto et al. 2012). Biomarkers that evaluate the bioindicators' functions are more useful in investigating the fate and effects of hazardous substances at the inspection site. Soil ecotoxicological tests considering contaminant or chemical mixtures (van Gestel 2012) and the interaction of contaminants with environmental stress factors like temperature and soil moisture (Velki and Ečimović, 2015; Jegede et al. 2017; Hackenberger et al. 2018) have developed over the past ten years and become a focal point in climate change-related studies.

Soil microbes, nematodes, and other invertebrate classes are often used as bioindicator organisms in ecotoxicological studies due to their close contact with the soil and their ecological significance in ecosystems. (van Gestel, 2012). Bioindicator species used in laboratory-based toxicity assays are

often chosen based on their short life span and how easy it is to culture (Yeates et al. 2011). A review by Bünemann et al. (2018) reported the frequency of different indicators used in soil quality assessment approaches and found that nitrogen mineralisation and soil respiration by microbes, microbial biomass, and earthworm biomarkers were some of the most frequently used indicators.

Soil microbes as bioindicators in soil are mostly based on their functional and structural traits. Many microbial-based metrics are used, including the more traditional methods based on biomass, microbial activity parameters, and potential enzyme activities. Other traditional methods include correlating soil nutrients with the quantity or the composition of bacteria, fungi and actinomycetes in soils (Zhou and Ding, 2007). DNA-based indicators assess the diversity patterns of the respective microbial groups, e.g. ribosomal genes like the 16S rRNA gene for bacteria and archaea or the ITS2 region for fungi assess the abundance of selected microbial gene sequences by quantitative PCR. Recent studies used soil microbial DNA concentrations as an indicator to determine the biomass of soil carbon and nitrogen functional groups (Gong et al. 2021). Soil microbial DNA based methods are continuously being improved, allowing for more genes to be identified that encode specific enzymes responsible for specific soil functions (Scholter et al. 2017).

Earthworms (Oligochaeta) are classified as good bioindicators of soil quality because they are widespread, robust and resistant, but at the same time sensitive to the slightest changes in their environment (Fründ et al. 2011). These organisms live in and ingest large amounts of soil and are continuously exposed to contaminants adsorbed to solid particles via their digestive tract or skin contact. They are frequently used in ecological risk assessment studies due to their ability to reflect trends in other species (Spurgeon et al. 2003; van Gestel 2012). The Organization for Economic Co-operation and Development (OECD) and the International Organization for Biological and Integrated Control (IOBC) developed a series of tests that may be used to determine the risk factors and potential adverse effects of harmful contaminants to non-target soil species. Physiological approaches in ecotoxicological studies include behavioural ecotoxicology, body burden measurement to quantify exposure, and biomarker methods (Spurgeon, 2003).

Some of the more frequently used cellular biomarkers in earthworms include:

- Enzyme biomarkers, e.g. acetylcholinesterase, metallothionein and antioxidant enzymes (Yang et al. 2012; Hackenbeger et al. 2018)
- cellular function and integrity biomarkers, e.g. lysosomal membrane integrity and coelomocyte function (Lionetto et al. 2012)
- genotoxicity biomarkers (Reinecke and Reinecke, 2004; Fouche et al. 2016)
- haemoglobin oxidation biomarkers (Calisi et al. 2012)

The coelomic fluid of earthworms plays an important role in their internal defensive against unknown toxicants or pollutants. Any impairment of coelomocyte functioning can negatively affect the health of the organism (Lionetto et al. 2012). Several cellular biomarkers have been standardised on

earthworm coelomocytes, including genotoxic biomarkers. Genotoxic biomarkers are often more sensitive when evaluating contaminants in the soil than whole organism responses such as survival, growth and reproduction (Smit et al. 2009). Genotoxic biomarkers, however, only relay information about the individual, while others (survival, behaviour, growth and reproduction biomarkers) may better predict changes at the population level. (Vasseur and Bonnard, 2014). A combination of biomarkers is often used to investigate earthworm population dynamics in response to fluctuating environmental stressors (Bertrand 2015). This study includes complementary investigations using earthworm survival, growth, reproduction, and genotoxicity biomarkers.

Although survival is a frequently used parameter during toxicological tests, it only provides information about acute toxicity and is often included to validate other tests. In the OECD test for testing chemicals (OECD, 2016), a test is considered valid only if 90% or more of the test organisms survives. Sublethal endpoints, e.g. reproduction, are more sensitive measures in ecotoxicology (Spurgeon et al. 2003). Growth and reproduction are considered an important measure of an individuals' fitness after exposure to a chemical or toxin (van Gestel and van Brummelen, 1996) and are accepted regulatory test methods in ecotoxicological and risk assessments.

Genotoxicity biomarkers assess cellular deoxyribonucleic acid (DNA) damage caused by environmental pollutants (Reinecke and Reinecke, 2004). Chemical or physical injuries to the DNA structure refers to genetic lesions such as DNA strand breaks that can promote changes or result in more permanent genetic alterations (Cestari, 2013) that may result in mutations during cell division and carried forward to future populations. A variety of techniques exist to test for possible damage to genetic material by environmental pollutants, e.g. the formation of adducts, chromosomal aberrations, breakage in the individual strands of DNA (comet assay) and the frequency of sister chromatid exchange (SCE) (Cestari, 2013).

The single-cell gel electrophoresis test (SCGE) or comet assay is a rapid and inexpensive technique that identify and quantify genotoxicity in any eukaryotic cell. Ostling and Johanson originally introduced it in 1984 to measure DNA strand breaks in individual mammalian cells (Cotelle and Ferard 1999). A modified comet assay protocol was developed by Singh et al. (1988) (in Cestari, 2013) in which alkaline conditions are used to detect single-strand breaks of DNA, making it especially significant for toxicological research and has become a valuable tool in environmental risk assessments. Under fluorescence microscopy, the migration of the damaged DNA material can be seen as the characteristic comet-like pattern (Reinecke and Reinecke, 2004). The comet assay as a genotoxicity biomarker has been successfully applied in ecological studies to detect DNA damage in freshwater organisms (Meland et al. 2019; Pellegri et al. 2020) and other terrestrial invertebrates (Reinecke and Reinecke, 2004; Fouché et al. 2016, Cavaliere et al. 2019) after exposure to different organic and inorganic substances. DNA damage in these organisms are often more sensitive than other physiological or behavioural biomarkers. Smit et al. (2009) found DNA damage to be 35 – 50 times more sensitive for evaluating oil toxicity in marine species than whole organism responses.

Several gaps in the knowledge about aflatoxin contamination in soil were identified from the literature discussed above, highlighting many questions about the potential risk under changing climate conditions.

i. Should aflatoxin contamination still be considered a low-risk in soil as was classified in the 1980s?

ii. Will the exposure of free and adsorbed $AFB_1$ in a soil medium cause any toxicological or genotoxic effect to non-target soil organisms that provide ecological functions, e.g. earthworms?

iii. Do earthworms play any role in the biological control and degradation of aflatoxins in the soil ecosystem?

iv. Do the variability in temperature and soil moisture change aflatoxins' toxicological effect on earthworms or their ability to control aflatoxins in soil?

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# CHAPTER 3 – AFLATOXINS IN THE SOIL ECOSYSTEM: AN OVERVIEW OF ITS OCCURRENCE, FATE AND EFFECTS

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## 3.1. Introduction

Mycotoxins are natural toxins produced by fungi as secondary metabolites during their metabolic processes (Bennet and Klich, 2003). Aflatoxins are a class of mycotoxins produced by specific strains of fungi, especially *Aspergillus flavus* and *Aspergillus parasiticus*. Of the more than 20 different types identified, aflatoxins B1 ($AFB_1$), B2 ($AFB_2$), G1 ($AFG_1$), and G2 ($AFG_2$) are of most concern due to its impact on human and animal health (Zain, 2011) and food safety (Bradford et al. 2018). Some of the most severe public health problems caused by aflatoxins include cytotoxic effects on blood cells (Iheanacho, 2015), autoimmune disorders and cancer (Ray et al. 1991; Theumer et al. 2018) and have been studied extensively since the 1960s when it first became a concern (Escrivá et al. 2017). In addition to the public health concerns posed by aflatoxins, its presence can also have serious economic implications for food and feed crop farmers, especially when producing maise and peanuts and the related processing industries (Krifaton et al. 2011). According to the Food Safety Digest report by the World Health Organization (WHO, 2018), an estimated 25% of the world's food crops are destroyed annually due to aflatoxin contamination, which results in annual income losses of millions of dollars for producers and traders of edible crops (Guchi, 2015).

Aflatoxins most commonly occur in soil, decaying vegetation such as compost material and food storage systems. Aflatoxins are introduced into the soil environment when contaminated plant residues are left to decompose (Accinelli et al. 2008) or when contaminated food from storage systems are worked back into the soil for natural degradation. These food-borne mycotoxins have the potential to contaminate groundwater (Kolpin et al. 2014) and negatively affect soil quality (Elmholt, 2008). Soil quality is of particular importance as it directly affects sustainable food production. Post-harvest contamination of food and feedstuffs and the reduction of aflatoxin contamination at pre-harvest stages have received considerable attention since the late 1970s (Spencer Smith et al. 2019). Several studies have shown the potential risk of aflatoxin exposure to living organisms in various ecosystems. Plants and crops grown in contaminated soil can absorb aflatoxins into their leaf, stem and root tissues (Mertz et al. 1981; Hariprasad et al. 2015). The developmental rate, fertility, and fitness of birds (Lawson et al. 2007) and insects (Azab et al. 2001; Zhao et al. 2018) that feed on these crops might be affected. However, the fate and consequences of aflatoxin contamination in soil (Accinelli et al. 2008) and important soil organisms (Szabó-Fodor et al. 2017) providing essential ecological services remain unclear and could potentially pose a risk to soil health.

Current regulations provide minimal options for the disposal of aflatoxin-contaminated crops. The most common options are incineration or incorporation into the soil for microbial degradation (EAC Policy Brief, 2018). This form of aflatoxin loading into the soil increases natural contamination and has the potential to alter the ecological balance. Further, changes in climatic patterns, particularly moisture and temperature conditions, have a major impact on toxigenic fungi's development and geographical distribution (Battilani et al. 2016). This could potentially make aflatoxin contamination a bigger risk to ecosystems, food crop quality, and human health in the future (Medina et al. 2017).

This review provides an overview of the current state of knowledge about the occurrence, fate and effects of aflatoxins in the soil ecosystem. The aim is to achieve more clarity regarding the environmental and toxicological consequences of soil contamination with aflatoxins that might increase risks on soil productivity, food and feed quality and human health.

### 3.2. Behaviour of aflatoxins in soil

Soil is the natural habitat for Aspergillus *flavus*, which is the most abundant aflatoxigenic species (Accinelli et al. 2008). Plant residues contaminated with aflatoxigenic *Aspergillus spp*. and left to decompose, introduce aflatoxins in the surface soil (Mishra and Das, 2003; Accinelli et al. 2008). Studies by Angle (1986) reported that natural concentrations of $AFB_1$ and its transformation products are expected to be low in soil due to the rapid degradation by soil microbes or the adsorption of the toxins to soil binding sites. However, when contaminated crops are left on the land or worked back into the soil for natural degradation, it can significantly increase the concentrations of aflatoxins in the soil and prolong the period of aflatoxin contamination (Accinelli et al. 2008). These toxins are particularly abundant in agricultural soil when crops are grown in a monoculture over several seasons, which increases the risk of successive aflatoxin accumulation in the soil. In a laboratory experiment under controlled temperature and moisture conditions, it was demonstrated that $AFB_1$ rapidly degrades once it is in the soil, but in a corn field study, $AFB_1$ was still detected five months after harvest most probably due to its gradual release from the contaminated plant debris (Accinelli et al. 2008). Aflatoxigenic fungi and their toxins are not just relevant in agricultural ecosystems but can also have consequences in other soil environments. The presence of aflatoxigenic fungi and its toxins in the soil and eggshells on the beaches of Oman were some of the most important factors implicated for egg hatching failure and high mortality during the embryonic development of green sea turtles (*Chelonia mydas*) (Elshafie et al. 2007). Of the six species of *Aspergillus* found, *A. flavus* was the dominant species and 75% of the *A. flavus* strains were aflatoxigenic, producing concentrations ranging from 0.3 to 28 µg/kg. These low concentrations of aflatoxins were enough to cause embryo mortality.

Leaching and adsorption studies carried out in the 1980s suggest that different types of soils retain aflatoxins differently. Angle and Wagner (1981) determined the persistence of aflatoxins in different

soil types over a 120-day incubation period and found that silty clay loam soil retained the highest percentage of the aflatoxins because $AFB_1$ formed a conjugate with the clay or organic component in the soil. Studies by Goldberg and Angle (1985) found that soil with a high adsorption coefficient, especially silty clay loam, retained between 80 and 92% of $AFB_1$ and that the bound aflatoxins would degrade at a much slower rate or only upon desorption from the soil binding sites. Due to this adsorption ability, it was proposed that aflatoxins pose relatively little harmful effects in the environment. Further studies showed that $AFB_1$ rapidly transform (half-life of ≤ 5 days) to $AFB_2$ and $AFG_2$ (Angle, 1986; Accinelli et al. 2008). These transformation products decompose at a much slower rate and could be detected in the soil for up to 77 days (Angle, 1986). The adsorption ability of aflatoxins to soil binding sites and its leaching potential were studied by Madden and Stahr (1993) using soil-water column systems. Eluates from contaminated corn and pure aflatoxin water mixtures were tested for both $AFB_1$ and its transformation product, $AFB_2$. Results obtained confirmed earlier findings by Goldberg and Angle (1985) that 50% of silty clay loam will prevent aflatoxins and its derivatives from leaching into groundwater due to the adsorption to the soil material. However, in 20% silty clay loam soil with a slightly lower adsorption ability, the leaching of aflatoxin was reduced but not eliminated. Other studies also suggest that light, sandy type soils could influence the occurrence and proliferation of *A. flavus* and might result in higher aflatoxin contamination (Achaglinkame et al. 2017). In all these studies, aflatoxins were introduced into the soil using appropriate solvents, however, in nature, these toxins are mostly found in organic mixtures with plant or animal-based material that might alter its adsorption characteristics (Elmholt, 2008).

Recent studies have identified $AFB_{2a}$ as the major transformation product of $AFB_1$ instead of $AFB_2$ and $AFG_2$, as previously reported (Starr et al. 2017). Aflatoxin $B_{2a}$ is the monohydroxylated derivative obtained from the addition of water to the double bond of the terminal furan of $AFB_1$ (Lillehoj and Ciegler, 1969). Although $AFB_{2a}$ is much less toxic than $AFB_1$, it still has DNA-binding capacity and is more polar than the other transformation products suggesting a higher possibility of reaction with other molecules or leaching in natural settings with water-saturated soils (Starr et al. 2017). The study by Starr et al. (2017) did not use reactive organic solvents such as methanol, but rather opted to replicate natural aqueous soil environments. Their findings suggest that leaching of both $AFB_1$ and its derivatives should still be of concern in areas where soils are sandier, shallow, or where there might be episodes of water saturation after flooding events.

### 3.3. Soil Ecotoxicology

So far little work has been done to determine the fate of aflatoxins once they are already present in the soil environment (Zhang et al. 2016; Szabó-Fodor et al. 2017). The high complexity and heterogeneity of the soil environment make it very difficult to study the ecological functions of secondary metabolites such as aflatoxins in the soil (Karlovsky, 2008). The exposure of soil organisms to soil trace elements is influenced by various mechanisms such as adsorption and

release from the soil binding sites, interactions with the soil microbial community and the metabolic transformations of the toxin in the soil solution (Kumpiene et al. 2017).

Soil organisms such as microbes, earthworms, and nematodes provide many important ecosystem services in the soil and are considered important biological indicators, allowing ecological risk assessments posed by chemicals (van Gestel, 2012; Fouche et al. 2017) or organic toxins in soils (Parelho et al. 2018). Microbial ecotoxicology has become a recognised field to assess the impacts of toxins on the structure and functional diversity of microorganisms (Ghiglione et al. 2016). Research into aflatoxins and soil microbial communities thus far, have focused on the aflatoxin degradation and detoxification ability of soil microbes rather than the effects that $AFB_1$ might have on the compositional and functional changes of soil microorganism, which support soil functions and ensure their stability and recovery. Angle and Wagner (1981) assessed the effects of $AFB_1$ on the population and activity of soil microorganisms exposed to rising concentrations of the toxin in agar media and soil respiration tests. Soil microbial communities were unaffected at low concentrations (1 and 100 µg/kg soil), whereas higher concentrations had statistically significant effects on soil respiration.

Studies by Feng et al. (2017) investigated the effect of $AFB_1$ on the growth, reproduction and DNA damage in the soil nematode, *Caenorhabditis elegans*, via a filter paper contact test. Results indicated toxin-induced DNA damage, germline cell death and significant inhibition of growth and reproduction. Preliminary studies by Szabó-Fodor et al. (2017), exposed earthworms to $AFB_1$, via the filter paper contact test. Harmful effects on *Eisenia fetida* were observed related to their physical fitness and behaviour, for example, coiling, excessive mucus secretion, sluggish movement and swelling of the clitellum. There is no information available on whether earthworms will be able to bioaccumulate these toxins and their effects on their survival, growth, and reproduction. In both studies(earthworms and nematodes), filter paper contact tests were performed. Further research is needed to establish if aflatoxins will have similar harmful effects to these species when exposed via contaminated soil or food. Avoidance-behaviour tests that determine if these organisms can detect and avoid aflatoxins would also have an ecological predictive value.

Furthermore, it has not been established whether earthworms and nematodes have the potential to reduce and degrade aflatoxins. *Aspergillus spp*, are common food sources for earthworms (Zirbes et al. 2011). As fungivorous soil organisms, earthworms have the potential to exhibit antifungal activity against various fungal species. Pižl and Nováková (2003) assessed the possible effects of several fungal species on the growth rates of earthworms, *E. andrei*, and found that their growth rate was the greatest in feeding trials with *A. flavus*. In addition to their ability to influence fungal succession, earthworms have been shown to sustainably regulate other types of toxic mycotoxins such as deoxynivalenol (Wolfarth et al. 2016). However, there is no literature available on whether

earthworms also have the potential to regulate aflatoxins. In our quest to find practical ways of controlling aflatoxin contamination in agricultural soils and crops, the regulative potential of natural soil organisms should be explored further.

### 3.4. Detoxification and degradation of aflatoxins

Methods to detoxify aflatoxins in food and feed by means of chemical, physical and biological technologies are widely studied (Luo et al. 2014; Ehrlich, 2014 Pankaj et al. 2018). Due to the adsorptive abilities of soil colloids, particularly clay, soil components can reduce detrimental bioactivities of aflatoxins by binding reactions. Physical detoxification of contaminated feed is often achieved by the addition of clay additives (Desheng et al. 2005; Jaynes et al. 2007). Grant and Phillips (1998) found that $AFB_1$ chemisorbs to the soil binding sites on a phyllosilicate clay, namely hydrated sodium calcium aluminosilicate (HSCAS), which significantly decreases the bioavailability of the toxin. The Food and Agriculture Organisation (FAO) seldom approve these methods because they do not always conform to the requirements in terms of nutritional values but also due to residual toxicity even remaining after respective treatments (Verheecke et al. 2016).

Aflatoxin degradation in the soil is mainly achieved by biological means (bacteria and fungi) (Cotty, 2006). Soil microbial communities play an important part in the soil ecology to enhance soil quality and productivity, especially with respect to the transformative role in soil nutrient availability, health, and fertility. Soil microorganisms affect the concentration and movement of aflatoxin (Madden and Stahr, 1993), and play an important part in the biodegradation process. The persistence of $AFB_1$ in autoclaved soil highlights the important role of microbial processes to drive aflatoxin degradation (Accinelli et al. 2008). Actinobacteria like *Nocardia corynebacteroides*, *Mycobacterium fluoranthenivorans*, *Corynebacterium rubrum*, and *Rhodococcus erythropolis* were reported as microbes able to effectively degrade aflatoxins (Krifaton et al. 2011; Cserháti et al. 2013). The aflatoxin degradation potential of soil microbial species is a complex process and varies considerably. In some cases microbial degradation processes are very effective, for example, *Bacillus spp.* are reported to effectively (85 – 100%) degrade $AFB_1$ (Raksha Rao et al. 2017). Isolates of the same species are, however, not always equally effective in degrading $AFB_1$. The intra-species degradation ability of *Rhodococcus erythropolis* varied between 20% in some strains and close to 100% in other strains of the same species (Cserháti et al. 2013).

Most research on the biological effects of aflatoxins focuses on the effect of $AFB_1$ but does not consider the effects of the transformation or by-products that are produced during degradation. Hackbart et al. (2014) found that fungal strains of *R. oryzae* did not have the same efficiency to decrease derivatives of $AFB_1$ such as aflatoxin M1 than it has been demonstrated for $AFB_1$. Soil microbes with the potential to significantly (> 80%) degrade aflatoxins do not necessarily eliminate the most detrimental effects of aflatoxins. Krifaton et al. (2011) found that microbial degradation of

$AFB_1$ may result in harmful by-products that cause cytotoxicity and/or genotoxicity. *R. erythropolis* strains retained genotoxicity after effectively degrading $AFB_1$ suggesting high degradation potential but low detoxifying potential (Cserháti et al. 2013). It is important to identify the transformation products of $AFB_1$ as well as the processes by which environmental degradation occurs (Starr et al. 2017) to get a better understanding of the long-term effects.

### 3.5. Prevention and control of aflatoxin contamination

Research on pre-harvest contamination mainly focuses on controlling *Aspergillus spp* (Rajesh et al. 2014) or on genetically based host-plant resistance (Spencer Smith et al. 2019). Soil and crop management practices are considered the primary means of preventing pre-harvest aflatoxigenic fungal infestation and subsequent aflatoxin contamination (Verheecke et al. 2016). This includes high-quality seed, proper irrigation, tillage practices, crop rotation and harvest at optimal maturity stage. Conservation tillage practices, that leave more than 30% of the previous crop in the field to conserve organic matter and reduce carbon losses in soil, are becoming more common. However, this could increase the risk of contaminated plant residues to re-contaminate the soil and result in increased pre-harvest aflatoxin contamination of subsequent crops (Accinelli et al. 2008). Biocontrol by fungi is emerging as a promising strategy for aflatoxin management. Naturally occurring, non-toxigenic *A. flavus* isolates have the potential to act as a biocontrol in soil and pre-harvest contamination of crops. This displacement strategy suggests that these non-toxigenic isolates of *A.flavus* have the potential to significantly (> 80%) reduce the toxigenic population (Cotty, 2006). Guidelines on how to dispose of aflatoxin-contaminated feed and crops are limited and currently, the only options for disposal are incineration or to work the contaminated crops or feed, back into the soil (EAC Policy Brief, 2018). The Pennsylvania Department of Agriculture provided a Mycotoxin Management guidance document in February 2012. Although it mainly focuses on the mycotoxin deoxynivalenol in corn, their document states that there are no on-farm disposal options for any mycotoxin-contaminated grain and feedstuff including aflatoxins and suggest that land application such as incorporating it into the soil, is not a recommended agronomic practice.

The potential hazard of aflatoxins to human health has been a serious concern on an international level for many years and has led to worldwide monitoring programs and regulatory actions by nearly all countries (WHO, 2018). However, monitoring of aflatoxins in the soil is not considered a necessity. To monitor and detect aflatoxins in soil requires sophisticated extraction and identification methods to establish its actual concentrations but are often complicated by the adsorption ability of aflatoxins to soil binding sites. Starr and Selim (2008), found supercritical fluid extraction (SFE) to be a highly sensitive and selective method for the extraction of $AFB_1$ from the soil. Using optimised SFE conditions, 72% of the total aflatoxins were extracted from dry soil, compared to an 18% recovery rate in studies that used liquid extraction methods. In addition, it was found that higher temperatures

(40 - 70 °C) increased that recovery rate. Although aflatoxins are quite stable to heat (Doyle et al. 1982), the presence of moisture directly influences the rate at which heat can degrade aflatoxins. In addition, pre-saturated soil reduces the adsorption of $AFB_1$ to soil particles (Starr and Selim, 2008) which means that the toxins or its transformation products are more prone to leaching into groundwater or being bioavailable in soil solution prior to degradation.

### 3.6. Climate change and aflatoxins

Soil temperature and moisture strongly affect soil microbial activity including the growth and distribution of the mycotoxigenic fungi, but it can also modify the host-resistance and host-pathogen interactions (Moretti et al. 2018). Several studies have indicated that the impact of climate change might influence toxin production by aflatoxigenic fungi (Battilani et al. 2016; Medina et al. 2017). The quantity of aflatoxigenic fungi associated with soils and crops varies with climate (Cotty and Jaime-Garcia, 2007). *Aspergillus* species generally grow and proliferate in temperatures above 20 °C whereas toxin production by these fungi is usually optimal at temperatures ranging between 25 – 37 °C (Cotty and Jaime-Garcia, 2007). Environmental factors such as changes in temperature, pH, drought conditions or periods of waterlogging may cause post-harvest aflatoxigenic fungi to become more frequent at pre-harvest stages (Medina et al. 2015). Sanders et al. (1984) found relationships between soil moisture and temperature, the percentage of plants colonised by *Aspergillus* sp. and the total aflatoxin concentrations in the plants. In irrigated soil, the incidence of fungal infestation in peanut plants was moderate but there was no evidence of aflatoxin production. However, drought-affected soil at cooler temperatures (≤ 21.5 °C), had a high incidence of *Aspergillus flavus* leading to aflatoxin contamination up to 19 µg/kg in edible and 2553 µg/kg in the oil plants, respectively. In warmer (≥ 25°C), drought-affected soil, the total aflatoxin concentration ranged at a higher level between 417 and 10 516 µg/kg in plants. In later studies, Jaime-Garcia and Cotty (2010) found that soil temperature and crop rotation influence the *A.flavus* community structure. In the summer months, the aflatoxigenic strains of *A.flavus* also referred to as the “S” strain, were dominant over the less aflatoxigenic strains or “L” strains. Fountain et al. (2014) observed similar increases in aflatoxin production by *A. flavus* during drought stress. Medina et al. (2017) studied the impact of various climatic scenarios including the increase in temperatures, drought stress and changing carbon dioxide concentrations on the growth and aflatoxin production by *A. flavus*. Results indicated that although the fungal growth remained constant in these scenarios, the aflatoxin production by the fungi increased significantly during drought stress.

Apart from the increased risk of aflatoxin production in areas where the fungi already occur, there is also a risk in terms of the geographic distribution of aflatoxigenic fungi due to climate change (IPCC, 2019). Prior to 2000, aflatoxins were of minor concern to maise and wheat crops in more temperate regions of Europe and more frequently and more severely affected crops in tropical and sub-tropical areas, especially Africa, Asia and the USA (Battilani et al. 2016; Medina et al. 2017). Battilani et al. (2016) used a modelling approach to investigate the risk of $AFB_1$ contamination in maise and wheat

in Europe under a +2 °C and +5 °C climate change scenario. Their findings predict that $AFB_1$ will become a bigger food safety issue for maise in Europe, especially in the +2 °C scenario, which, is also the most probable warming scenario expected in the coming years. This highlights the importance of further research on the relationship between changes in soil moisture and temperatures, aflatoxin production and occurrence as well as the ecotoxicity of these harmful toxins.

## 3.7. Conclusion

Aflatoxin contamination remains one of the most important threats to food safety and human health. It is currently unclear to what extent soil contamination with aflatoxins might endanger soil health and possibly lead to higher risks with respect to agricultural productivity and product quality. Due to the rate of degradation and the adsorption of aflatoxin to clays and organic material, it has so far been regarded as not posing any long-term environmental risk. There is only limited information available about the effects of aflatoxins on the structure and functions of the soil communities and the important ecosystem services they provide. The ability of soil organisms to regulate aflatoxins in the soil environment is either not studied so far. In soils containing a higher clay or organic content e.g. topsoil layers, the rate of aflatoxin degradation could significantly be prolonged due to the soil adsorptive properties, posing a long-term risk to the present soil organisms. From recent studies, we do not yet have a comprehensive understanding of $AFB_1$ transformation in natural environments and therefore this area of research should be explored in more detail. The genotoxic and cytotoxic potential of the transformation products may still pose a threat to soil ecosystems even after microbial degradation. More attention needs to be placed on the ecological risks of natural toxins that have genotoxic potential in the long-term perspective.

Air temperature and soil moisture affect the physical, chemical and biological processes in soil. Climate change will have a significant impact on the quality and productivity of soil for food crop production by affecting phytopathogens and pests as well as host-pathogen interactions that might enhance the frequency and severity of plant diseases. As drought events increase in many parts of the world, the occurrence of aflatoxins might increase, posing significant health risks to the soil ecosystem, food crop production, and human health. A climatic approach, in terms of changes in soil moisture and air temperature conditions, is regarded to be of major importance to enable reliable risk assessments relating to aflatoxin contamination. As progressive global warming is expected to endanger soil health and productivity substantially in the future, it is recommended to intensify research in this area to fill the gaps still existing about the consequences of aflatoxin contamination in the soil environment.

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# CHAPTER 4 - ECOTOXICOLOGICAL EFFECTS OF AFLATOXINS ON EARTHWORMS UNDER DIFFERENT TEMPERATURE AND MOISTURE CONDITIONS

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## 4.1. Introduction

Fungal toxins (mycotoxins) are often toxic to plants, animals and humans and are a common threat to food safety. Of the more than 400 types of mycotoxins, aflatoxins are considered to be the most toxic and carcinogenic. Exposure to aflatoxin B1 ($AFB_1$) poses a significant health risk for humans (Ostry et al. 2017; Theumer et al. 2018) and other living organisms, including plants (Mertz et al. 1981, Hariprasad et al. 2017), mammals (Corcuera et al. 2015), birds (Lawson et al. 2007), insects (Azab et al. 2001; Feng et al. 2016; Zhao et al. 2018) and fish (Anater et al. 2016). Most countries in the world regulate aflatoxin concentrations in food and feed products. When aflatoxins reach concentrations exceeding the accepted levels, regulations suggest that contaminated food products are discarded by burning or working the material back into the soil for natural degradation (EAC, 2018). When contaminated crops are worked into the soil, it increases natural concentrations and prolongs the duration of contamination due to the gradual release of the toxin (Accinelli et al. 2008). Moreover, increased concentrations can alter the ecological balance, potentially posing a risk to soil health.

So far, only a few studies have investigated the consequences of aflatoxins for soil organisms (Accinelli et al. 2008). The exposure of soil organisms to toxins in the soil is influenced by various mechanisms such as adsorption and release from the soil binding sites, interactions with the soil microbial community and the metabolic transformations of the toxin in the soil solution (Kumpiene et al. 2017), as well as the environmental conditions under which it occurs. Studies from the early 1980s found that $AFB_1$ does not persist in the soil for long as soil microbes degrade it into less toxic metabolites ($AFB_2$ and $AFG_1$ and $AFG_2$) in a relatively short period. Later studies found that $AFB_1$ may persist in soil for up to 120 days when adsorbed to the soil binding sites. Once bound to the soil binding sites, $AFB_1$ is mostly resistant to microbial degradation (Angle 1986). The effect of $AFB_1$ on the growth, reproduction and DNA damage in the soil nematode (*Caenorhabditis elegans*) were investigated via a filter paper contact test (Feng et al. 2016). Results indicated toxin-induced DNA damage, germline cell death and significant inhibition of growth and reproduction at concentrations between 30 and 100 µg/L (Leung et al. 2010; Feng et al. 2016). Harmful effects on earthworms (*Eisenia fetida*) exposed to $AFB_1$ via the filter paper contact test were observed related to their physical fitness and behaviour, including excessive mucus secretion, sluggish movement, coiling and swelling of the reproductive organs (Szabó-Fodor et al. 2017). These studies used filter paper tests and did not include exposure in soil media. Schrader et al. (2009) demonstrated that the mycotoxin, deoxynivalenol (DON), is incorporated into earthworm gut and body wall tissue after

feeding on the fungi-infested crop residues in a soil medium. Although the DON concentrations in the earthworm gut declined over time, the possible toxic effects on the earthworm demographic processes (growth and reproduction) were not established. Similarly, the consequences of $AFB_1$ and its degradation products for soil organisms (Accinelli et al. 2008) and soil biodiversity (Szabó-Fodor et al. 2017) remain unclear (Fouché et al. 2020).

The loss of soil biodiversity has become a serious issue for global soil quality, especially in arable soils under intensive agriculture. Soil environments are complex systems considered the main reservoir of global biodiversity (FAO 2020) and include diverse soil communities consisting of micro- and macro-organisms, e.g. bacteria, fungi, nematodes, mites, enchytraeids, springtails, ants, beetles and earthworms. The World Soil Charter recognises the critical importance of soil biodiversity for supporting soil functions and, therefore, providing, regulating and maintaining a diverse range of ecosystem services (FAO, 2015). Ecosystem services refer to the subset of processes provided by an environmental compartment. The value of protecting soil biodiversity and ecosystem services to meet various sustainable development goals (as proposed by the United Nations) is widely acknowledged (Pereira et al. 2018; FAO 2020). Economic growth and human well-being, therefore, depend on healthy soil. Unfortunately, the Status of the World's Soils Resources report (FAO, 2015) concluded that most of the world's soil resources are in poor or very poor condition, and urgent action is required, especially in developing countries where people are more vulnerable. The protection of soil biodiversity has also become essential for the success of the declared United Nations Decade on Ecosystem Restoration (2021–2030) (FAO 2020).

Earthworms play an ecologically significant role in the soil ecosystem. They are considered ecosystem service mediators (Plaas et al. 2019) due to their significant contribution to soil's physical, chemical and biological processes. The activity of earthworms affects many essential soil processes, including the soil organic matter and nutrient dynamics and the activity of many other essential soil macro- and micro-fauna (Hoeffner et al. 2018, Plaas et al. 2019) and flora (Thouvenot et al. 2021). They are also essential biological regulators of plant pathogens such as fungi (Schrader et al. 2013). By regulating the fungal population, earthworms may regulate some of the harmful toxins associated with fungal populations, thus reducing the risk of environmental pollution as an ecosystem service (Wolfarth et al. 2016). They are resilient, widespread and have relatively uniform characteristics that classify them as good bioindicators of soil health (Fründ et al. 2011). They have been used extensively in ecotoxicological studies due to their ability to reflect trends in other species and their sensitivity to even the slightest changes in their environment (van Gestel 2012, Hackenberger et al. 2018).

According to the Intergovernmental Panel on Climate Change report (IPCC) (Jia et al. 2019), it is very likely that the variability in natural climates will continue to impact terrestrial ecosystems in the future. Extreme climatic events over the past decade, such as floods and extreme drought conditions, have resulted in more sudden and severe changes in soil temperature and moisture

conditions rather than gradual shifts (FAO, 2020). Several studies have indicated that climate change might influence mycotoxin production (Battilani et al. 2016; Medina et al. 2017), making it a more considerable risk in the future. It is suggested that climate change significantly impacts the stages and rates of toxigenic fungi development and toxin production, which might modify host-resistance and host-pathogen interactions (Moretti et al. (2019). A study by Sanders et al. (1984) found a relationship between soil moisture, temperatures, the percentage of peanut plants colonised by *Aspergillus* spp. and the total aflatoxin concentrations on the plant material. In irrigated soil, aflatoxin could not be detected; however, in cooler, drought soil, total aflatoxin concentrations ranged between 0 and 19 µg/kg for edible plant crops and between 66 and 2553 µg/kg in oil crops. In drought-heated soil, the total aflatoxin concentrations range of contaminated plants increased to 417–10, 516 µg/kg. Similar findings report increased aflatoxin contamination in groundnut (Sibakwe et al. 2017) and corn (Damianidis et al. 2018) after prolonged drought conditions. The increased concentrations during drought conditions highlight the necessity to better understand how climate change may influence the risk of pre-and postharvest aflatoxins in the soil environment.

Risk assessments of toxic substances historically relied only on whole-organism endpoints directly related to demographic processes such as survival, growth and reproduction (Hooper et al. 2013). While organisms can show some tolerance towards toxicants, especially at lower concentrations, it does not imply that there are no effects, as there could be some physiological changes, even in the absence of mortality (Lin et al. 2020). Complementary investigations that incorporate both demographic and mechanistic aspects of the biological effects of toxic substances, sometimes overlooked in ecological risk assessments, have developed over the past 20 years (van Gestel 2012). Mechanistic aspects include the biochemical and molecular basis by which the toxin exerts an effect; for example, the consequences of exposure on metabolism can be evaluated by its genotoxicity. The comet assay or single-cell gel electrophoresis assay is a sensitive biomarker to identify and quantify genotoxicity. It can examine the double-strand breaks of DNA in any individual eukaryotic cell and has been successfully applied as a monitoring tool to detect DNA damage in humans (Collins 2004), other mammals (Olive & Banath, 2006), plants, freshwater organisms (Pellegri et al. 2020) and invertebrates (Augustyniak, 2016; Fouché et al. 2016).

Risk assessments applied in laboratory tests are generally based on standard temperature and moisture conditions for reproducibility and comparison between different studies (Giska et al. 2014). The Organisation for Economic Co-operation and Development (OECD) provides standard temperatures (20 ± 2 °C) and often moisture conditions when testing chemicals in soil organisms (OECD, 2016). However, existing monitoring and assessment methods may no longer be robust enough to detect adverse changes in organisms after exposure (Balbus et al. 2013). Changing climate conditions can alter the toxicokinetics of toxic substances (Hooper et al. 2013, González-Alcaraz et al. 2018). Several studies report the impact of different temperature regimes on the toxicity of agricultural pesticides (Garcia 2004, Bandow et al. 2014; Lima et al. 2015, Velki and Ečimović,

2016; Jegede et al. 2017) and metals (Gonzàlez-Alcaraz and van Gestel 2016) to soil organisms. These impacts vary as soil organisms respond differently to different toxins and chemicals under different temperature conditions. Garcia (2004) assessed the impact of temperature on the toxicity of two fungicides and an insecticide on two different invertebrate species (earthworms and isopods). Lower toxicity was indicated for the fungicides at higher temperatures (28 °C) but higher toxicity for the insecticides. Bandow et al. (2014) reported increased susceptibility of Collembola to the fungicide pyrimethanil at 26 °C compared to 20 °C. Increased toxicity of several common pesticides has been reported under increased temperatures (25 °C) (Velki and Ečimović, 2016). Similarly, increased toxicity of three common agricultural pesticides (chlorpyrifos, dimethoate and deltamethrin) were observed under tropical temperatures (26–28 °C), even if the concentrations were not considered a risk in their study (Jegede et al. 2017). Further, temperature-induced variations in earthworm enzymatic activities and proteins that may contribute to compensatory changes at the cellular metabolic level have also been reported (Tripathi et al. 2011).

Temperature is generally not the only environmental factor that plays a role. In most cases, increased temperature is also associated with other soil factors such as moisture conditions. Most studies primarily focus on different temperatures and do not always consider a combination of temperature and moisture conditions. Gonzàlez-Alcaraz and van Gestel (2016) and Gonzàlez-Alcaraz et al. (2018) studied the bioaccumulation and toxicity of metals in earthworms and enchytraeids under different climate change scenarios. Findings suggest that different air temperature and soil moisture combinations affect metal bioaccumulation kinetics in these organisms. Hackenberger et al. (2018) found that different temperature and moisture combinations affected earthworm enzyme activity and the organism's behavioural response after exposure to agricultural pesticides. Low moisture and high temperature in soil have been reported to increase earthworms' physiological stress, resulting in decreased protein synthesis and tissue protein levels (Acharya and Mishra, 2020). The temperature and moisture-induced decrease in protein synthesis might affect DNA repair activities in organisms (Qiao et al. 2007). It has, therefore, become necessary to monitor and assess the effects of different toxic substances under a broader range of environmental conditions. If climate conditions continue to change, it could potentially increase the risks of aflatoxin contamination in soil ecosystems in the future (Moretti et al. 2019).

To address some of these knowledge gaps, the current study aimed to investigate the toxicological consequences of aflatoxins to earthworms (*Eisenia andrei*) under different temperature and moisture conditions. The specific objectives of the study were:

1. Assess whether aflatoxin affects earthworms' life-cycle processes (survival, growth and reproduction) using a standard OECD test.
2. Assess the genotoxicity of aflatoxin to earthworms using the comet assay.
3. Assess whether different temperatures (21 °C and 26 °C) and soil moisture conditions (30% and 50% of soil water holding capacity) affect the toxicity of aflatoxins to earthworms.

## 4.2. Materials and Methods

### 4.2.1. Experimental design

A laboratory experiment was conducted at the North-West University, South Africa, to assess the effect of $AFB_1$ on the survival, growth, reproductive output and DNA integrity of earthworms. The dynamic nature of the soil environment makes it very difficult to interpret and measure the ecological functions of secondary metabolites in the soil (Karlovsky, 2008). Therefore, toxicological studies often use artificial soil prepared according to standard guidelines to overcome some of the complexity and heterogeneity of the soil environment. Although some variables of natural ecosystems are excluded, which can affect bioavailability, it has practical advantages. Using artificial soil manipulates essential parameters such as soil organic matter (SOM) and the variability in adsorptive properties of different soil types. Artificial soil was prepared ten days before starting the experiment according to the standard guidelines set out by the Organisation for Economic Co-operation and Development (OECD, 2016) and used in all the treatment exposures. The artificial OECD soil consisted of (based on dry weight):

- 10% sphagnum-peat (Mystics).
- 69% quartz sand with a grain size of between 50 - 200 µm.
- 20% kaolinite clay (obtained from Atlas Clay Group in Potchefstroom, South Africa.
- Chemically pure calcium carbonate (< 1%) to obtain a pH of 6.5 - 7.

The water holding capacity (WHC) of the artificial soil was determined using a Sartorius moisture analyser. Four 10 ml tubes were each filled with dry artificial soil. Filter paper was used to seal the bottom of each tube, placed into a glass beaker with water and left for three hours. After this period, the tubes were removed and placed in a beaker with moistened silicate sand for a further two hours for the soil in the tubes to reach 100% WHC. Two replicate samples of each tube were analysed using the Sartorius moisture analyser. Readings of the 100% WHC were obtained and used to calculate the desired 30% and 50% WHC (Annexure C ).

For this study, modifications were made in terms of the standard OECD guidelines for air temperature and soil moisture to represent a range of temperature and moisture conditions to assess if changing climate conditions might affect the toxicity of aflatoxins to earthworms. Four combinations of air temperature and soil moisture (as soil water holding capacity) were used and based on previous studies by other researchers (González-Alcaraz et al. 2018; Hackenberger et al. 2018).

- 21 ± 1 °C + 50% (WHC) – standard temperature and moisture conditions prescribed by the OECD
- 21 ± 1 °C + 30% (WHC) – standard temperature with drier soil conditions
- 26 ± 1 °C + 50% (WHC) – increased temperatures with standard moisture conditions
- 26 ± 1 °C + 30% (WHC) - increased temperatures with decreases soil moisture conditions

### 4.2.2. Introduction of aflatoxin into the soil.

Two different concentration treatments (10 and 100 µg/kg) were used for the study. The concentrations selected are comparable to actual concentrations found in environmental soil samples (Table 4.1).

Before introducing the toxin, the soil was moistened with $dH_2O$ to the desired water holding capacity (30% and 50%). Methanol was used as a solvent to prepare the liquid aliquots of the powdered $AFB_1$ (Enzo Life Science, through Biocom Africa). Aflatoxins are typically introduced into the surface soil by infected plant material left to decompose (Mertz et al. 1981; Accinelli et al. 2008). The powdered aflatoxin was dissolved with methanol to obtain 10 µg/ml for the higher concentration treatment. One ml of the 10 µg/ml aflatoxin was further diluted with methanol to obtain 1 µg/ml for the lower concentration treatment. For each vessel containing 600 g of soil, 6 ml of the aflatoxin solvent was mixed with 5 g dried horse manure containing sufficient quantities of wheat straw and placed on top of the moistened soil to introduce the desired aflatoxin concentration into the soil. The soil samples were weighed individually and placed under an extractor fume hood overnight in the dark at room temperature to allow the solvent to evaporate. After 12 hours, the soil was weighed again, and any loss of weight was compensated for by replacing lost moisture with $dH_2O$. The soils were left for another 12 hours to stabilise before introducing the earthworms. Control soil samples (three replicates for each WHC) with 5 g of uncontaminated horse manure were moistened with $dH_2O$ to achieve the desired WHC of 30% and 50%, respectively and left to stabilise for 12 hours.

**Table 4.1** Aflatoxin concentrations detected in environmental soil samples

| Author | Aflatoxin Concentration | Environmental sample analysed | Detection method |
|---|---|---|---|
| Mertz et al. (1981) | 0.1 – 10 µg/kg | Agricultural soil | TLC |
| Elshafe (2007) | 3.6 – 8.4 µg/kg | Eggshells and soil | HPLC |
| Accinelli et al. (2008) | 0.6 - 5.5 µg/kg | Soil | HPLC |
| | 145 – 275 µg/kg | Decomposing corn residues | |
| Rajkumar et al. (2013) | 10 – 100 µg/kg | Soil samples | LC-MS |
| | 50 – 1700 µg/kg | Decomposing maise residues | |
| Hariprasad et al. (2015) | 0.5 – 22 µg/kg | Soil samples | Indirect competitive (ic)ELISA |

### 4.2.3. Aflatoxin Concentrations

Soil samples were analysed 72 hours (week 1) and 32 days (week 4) after spiking with $AFB_1$ to monitor and quantify bioavailable $AFB_1$ concentrations during the experimental period. Aflatoxin ($B_1$) concentrations were analysed using an indirect, competitive enzyme-linked immunosorbent assay (ELISA, Elabscience total aflatoxin ELISA kit, E-TO-E006) according to the suppliers' protocol.

Briefly, 20 g of soil samples were taken randomly from the container to get a representative sample. Special care was taken to ensure that no earthworms or cocoons were included in the sample. The soil samples were air-dried overnight, crushed, and 2 g of homogenate was mixed with 10 mL of 70% methanol in a 50mL centrifuge tube. The content in the tube was mixed in a shaking incubator for 30 - 40 minutes and centrifuged at 4 x 1000 rpm for 10 minutes to obtain a 10 times dilution. A further 10 times dilution was done for the week one samples due to the high aflatoxin concentrations in the soil samples. The 96 well ELISA microtiter plate was prepared using 50 µL of the extracted solution. The optical density (OD) for each well was determined using a microplate reader. A standard curve was generated from the standard solutions' OD values and the samples' concentrations calculated from the OD values using a Four Parameter logistics regression model.

### 4.2.4. Earthworm survival, weight and reproduction test

Lab cultured adult earthworms (*Eisenia andrei)* were used for this study. *Eisenia* are amongst the most popular earthworm species found in compost and organic layers. Contaminated plant material introduces aflatoxin into the organic and compost layers and the soil (Accinelli et al. 2008), where exposure to these organisms occur.

Adult earthworms of reproductive age (visible clitellum) were acclimatised in clean experimental soil for 72 hours. The earthworms were briefly rinsed, placed on absorbent paper and then weighed individually. The mean starting weight of the worms was noted. Ten earthworms were introduced into each replicate vessel containing the soil. The weight of each vessel containing the soil, moisture at the desired WHC, 5 g of horse manure as food substrate (contaminated in aflatoxin treatments) and 10 earthworms was noted and incubated in an environmental climate chamber at 21 ± 1 °C for 30 days. The experimental layout was duplicated in a second trial, using new soil and different earthworms, but the soil samples were incubated at 26 ± 1 °C.

The soil moisture balance was maintained throughout the period by weighing the vessels every seven days and adding $dH_2O$ to maintain the original water holding capacity percentage. In addition, 5 g of re-wetted horse manure were added weekly as a food source for the earthworms as per the guidelines set by the OECD (2016) to ensure a normal cocoon production rate (Reinecke & Reinecke, 2004). After 30 days, the adult earthworms were removed from the vessels, counted (survival) and individually weighed after briefly rinsing and removing excess water. The average change in body weight as a percentage for the ten earthworms over the 30-day incubation period was determined for each vessel. Immediately after weighing the adult worms, three individuals per

vessel (n = 9 per treatment) were placed on moist paper to extrude their gut content overnight for use in the comet assay (4.2.5).

The vessels containing the cocoons and juvenile earthworms were incubated for an additional 30 - 40 days under the same conditions. Food (5 g of horse manure) was provided only once at the start of the second incubation period, and the moisture balance was monitored weekly. After the second incubation period, the number of juveniles was collected and counted using a hand sorting technique (OECD, 2016). Each vessel was checked in triplicate (over three days) to remove all the juveniles and cocoons.

### 4.2.5. Comet assay

The single-cell gel electrophoresis assay (comet assay) was conducted 30 days after earthworm exposure according to the protocol used by Reinecke and Reinecke (2004) to evaluate the extent of cellular DNA damage in earthworm coelomic cells. The OxiSelect™ Comet Assay kit (Cell Biolabs, Inc) was used. All the comet assay solutions (lysis solution – pH 10, Mg and Ca free phosphate-buffered saline (PBS), alkaline solution and electrophoresis running solution- pH 13) were prepared according to the Cell Biolabs protocol a day before the start of the assay and refrigerated at 4 °C. Twelve hours after removing the adult worms from the soil, coelomic cells were harvested using a non-invasive technique originally described by Eyambe et al. (1991). Each worm was first rinsed in° clean phosphate-buffered solution and then placed into an Eppendorf tube containing 1 ml ice-cold extrusion fluid (95% PBS, 10 mg/ml guaiacol glycerol ether, 2.5 mg/ml EDTA and 5% absolute ethanol). After three minutes, the earthworms were removed, rinsed and returned to clean soil. The tubes containing the cell suspension were centrifuged at 700 x g for 10 minutes at 4 °C. The supernatant was carefully removed, resuspended in 0.5 ml PBS and centrifuged for 3 minutes to wash the cells. The process was repeated twice to remove all excess coelomic mucous (Voua Otoma and Reinecke, 2010).

Cell samples were mixed with 37 °C comet agarose at a 1:10 ratio (v/v), after which 75 µl of the cell agarose mixture was pipetted onto a precoated microscope slide (1% normal melting point agarose) and placed on ice for 15 minutes. The slides were transferred to the lysis solution (4 °C) and kept at 4 °C in the dark for 1 hour to allow cell lysis. After aspirating the lysis solution, the slides were transferred into the alkaline solution (pH 13) for 30 minutes to allow denaturation. Finally, the slides were transferred into a horizontal electrophoresis chamber and covered with fresh alkaline solution. Voltage was applied for 30 minutes at 1 volt/cm, and the volume of the electrophoresis solution was adjusted to produce a current of 300 mA. After electrophoresis, the slides were rinsed twice in cold distilled water and once in 90% ethanol for five minutes each. Slides were air-dried and stored in the dark until analysis.

For analysis, the slides were rehydrated in de-ionised water for ten minutes, stained with 100 µl Vista Green DNA dye and incubated at room temperature for 15 minutes. The stained microscope slides

were viewed under a Leitz Diaplan fluorescence microscope (with the required FITC filters), and a minimum of fifty randomly selected cells were analysed per sample. The Comet IV software package was used to analyse the DNA damage in the coelomic cells quantitatively. The percentage of DNA that migrated away from the nucleus, represented by the tail intensity parameter (% tail DNA), was selected. The percentage of tail DNA was measured by the intensity of the pixels located in the comet tail. Previous studies have shown the tail intensity parameter to be the most meaningful of the various comet parameters to assess genotoxicity (Collins 2004) because the amount of DNA in the comet tail has a positive correlation with the level of DNA damage.

### 4.2.6. Statistical analysis

Statistical analysis and graphical representations were performed using R version 4.1.0 (R Core Team, 2021) and R Studio packages *vegan* and *ggplot2*. Assumptions of normality (Shapiro Wilk test) and homoscedasticity (Levene's test) for one-way analysis of variance (ANOVA) were met using log-transformed data for the reproduction and comet assay results. The analysis of controls between temperature and moisture treatments were done using two-way ANOVA. Further, three-way ANOVA was performed to test the possible interaction effect of concentration x moisture (WHC) x temperature. *Post hoc* analysis (Tukey's HSD) was performed following ANOVA. The MyCurveFit Add-in for Microsoft Excel was used for the aflatoxin concentration data to create a standard curve and perform a four-parameter logistics regression model (4PL) for concentration calculations from the optical density values.

## 4.3. Results

### 4.3.1. Aflatoxin concentrations in the soil

A total aflatoxin ELISA kit was used to detect and quantify the $AFB_1$ and its breakdown products ($AFB_2$ and $AFG_1$ and $AFG_2$). Low available concentrations were detected in the OECD soil (Figure 4.1) but confirmed that aflatoxin was present in the soil for the study duration. Higher concentrations were generally detected at 21 °C than 26 °C for both concentration treatments (10 µg/kg and 100 µg/kg) in week 4 (Figure 4.1). The percentage (%) decrease in detected concentration levels over four weeks was lower in the drier (30% WHC) soil, suggesting that the aflatoxin concentration degraded slower in dry soil. The degradation potential (% decrease) was significantly ($p < 0.05$) more at increased temperatures and moisture conditions.

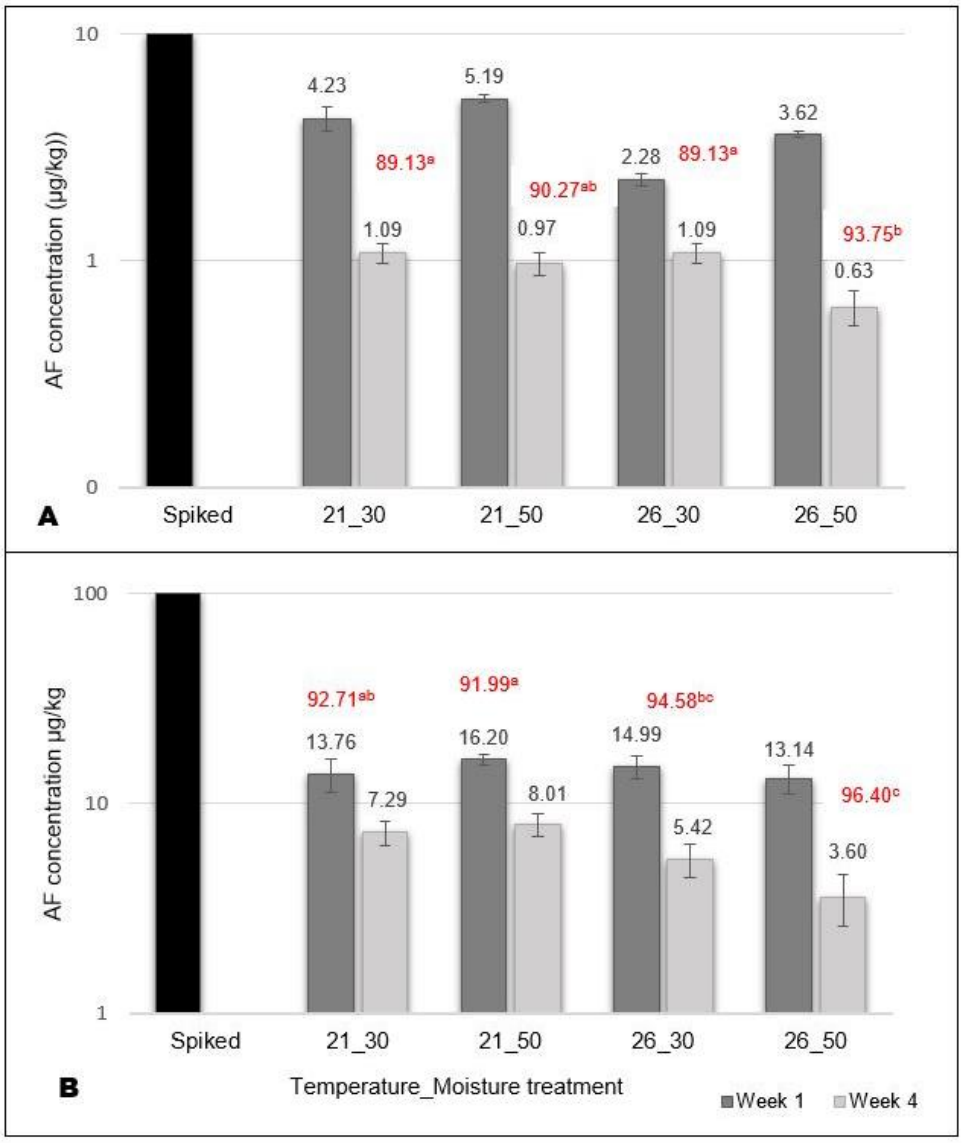


**Figure 4.1** Mean aflatoxin concentrations (AF) detected in soil 72 hours (Week 1) and 32 days (Week 4) after spiking the soil. **A**: Soil spiked with 10 µg/kg, and **B:** Soil spiked with 100 µg/kg. Percentage (%) decrease from the initial spiked amount is indicated in red. Different alphabetical letter (a,b and c) indicate a significantly ($p < 0.05$) different decrease (%) in detected concentrations *1*.

### 4.3.2. Earthworm survival, weight change and reproduction

The aflatoxin concentrations used in this experiment were at sublethal doses for earthworms. Szabó-Fodor et al. (2017) conducted a contact paper test that determined the $LD_{50}$ of $AFB_1$ to earthworms as 168.5 µg/mL. All control treatments had a survival rate above 90%, which met the validity criteria of the OECD 222 (OECD, 2016).

There was no significant ($p > 0.05$) difference in the survival rate between treatments at the same temperature, but the survival was lower at 26 °C than at 21 °C. The percentage change in the earthworms' mean body weight was noted as a measure of their growth. Results for the average weight change indicated a decrease in the mean body weight of less than 10% in all the groups. The earthworms were cultured in a different substrate with a higher percentage of available food. The weight loss percentage of earthworms after 4 weeks in the aflatoxin-treated soil was generally less than the control groups in each environmental group (same temperature and moisture conditions); however, the analysis of variance (ANOVA) found no statistical difference ($p = 0.06$) between the control and aflatoxin treatments.

Figure 4.2 shows the difference in the reproduction of earthworms at different aflatoxin concentrations and different environmental conditions. The number of juveniles at low aflatoxin concentrations (10 µg/kg) at 21 °C with 30% WHC was significantly ($p = 0.03$) less than its control sample (indicated by *). There was no difference in the number of juveniles at any other aflatoxin treatment compared to its control sample at the same environmental conditions. However, there were significant differences in the number of juveniles hatched at the same concentration under different environmental conditions.

Soil temperature and moisture are key factors that influence earthworm growth, survival, reproduction (Edwards and Bohlen, 1996) and other life cycle traits such as weight and cocoon incubation time. Drier soil conditions (30% WHC) decreased the number of juveniles hatched, especially at increased temperatures (26 °C with 30% WHC). Conversely, increased soil moisture (50% WCH) increased the number of juveniles hatched. In the aflatoxin treatment groups, the biggest difference was observed at the increased temperatures (26 °C) because the least number of juveniles hatched in the treatments with 30% WHC. In contrast, the highest number of juveniles were produced in the 50% WHC group, but the adults also had the highest weight loss percentage in this treatment. In the control group (0 µg/kg), the highest number of juveniles were produced at standard temperatures (21 °C) with 50% WHC.

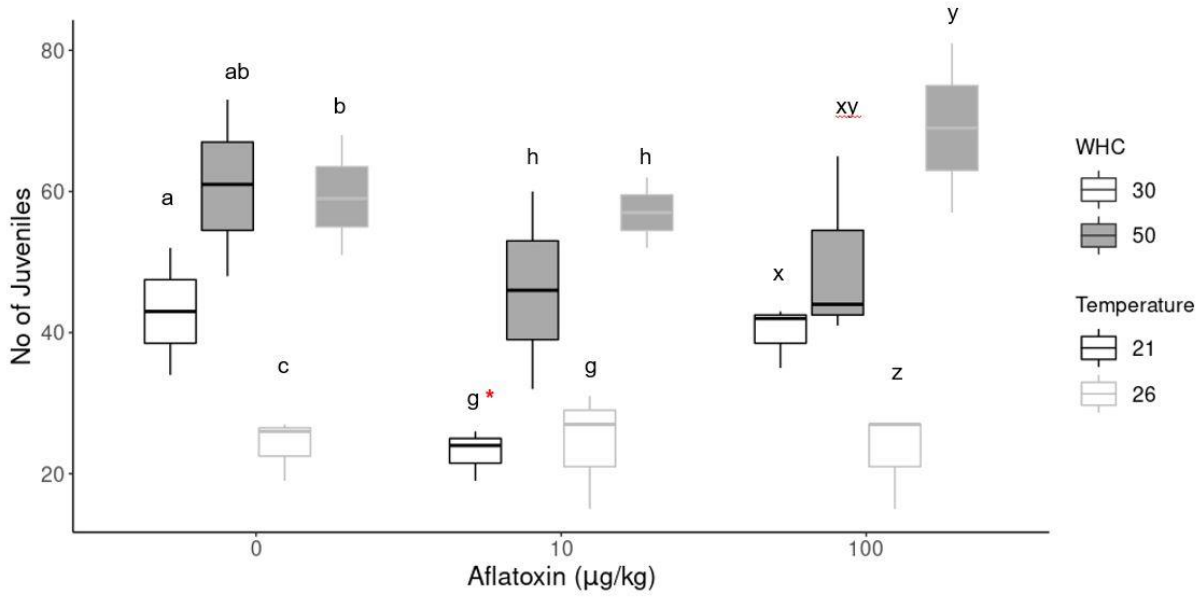


**Figure 4.2:**Reproductive output of earthworms. The * indicates a significant difference ($p < 0.05$) between concentration treatments at the same environmental conditions. Different alphabetical letters (a, b, c in 0 µg/kg; g, h in 10 µg/kg; x, y, z in 100µg/kg) indicate significant differences ($p < 0.05$) within the same concentration group.

One noticeable observation worth mentioning, although it was not measured, was a considerable size difference of the juveniles in the 30% WHC treatments compared to the 50% WHC treatments. In all cases (control and aflatoxin treatments and at both temperatures), the 50% WHC juveniles were considerably smaller at 10 weeks than the juveniles of the 30% WHC treatments.

### 4.3.3. Genotoxicity - Comet Assay results

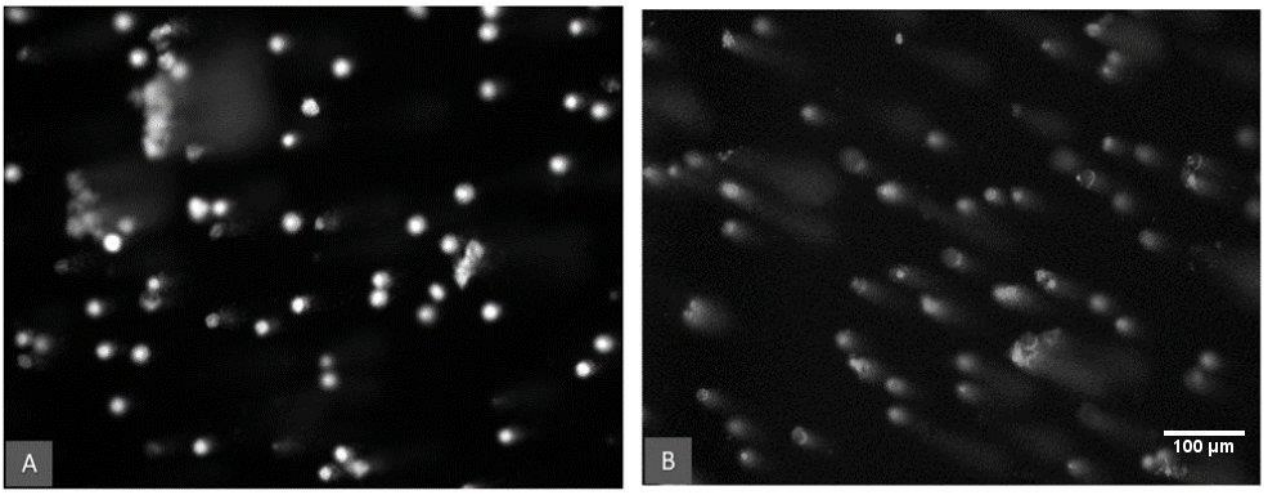


**Figure 4.3:**DNA damage of earthworm coelomic cells as viewed under a fluorescence microscope. Comparison of the aflatoxin 100 µg/kg treatment at 21 °C. **A** shows cells at 50% water holding capacity treatment. **B** shows cells in the 30% water holding capacity treatment and a significantly ($p < 0.001$) higher percentage of cells with DNA damage represented by the characteristic comet tails

Figure 4.3 shows an example of the observed DNA damage in earthworm coelomic cells measured as the tail intensity of the DNA strand breaks (% tail DNA) for the aflatoxin 100 µg/kg treatment group. The comet assay data were compared based on the aflatoxin concentration, the temperature and the moisture treatments. DNA damage in the control samples (0 µg/kg) is assumed to be the background values derived from endogenous and natural exogenous sources (Qiao et al. 2007). These background levels differed significantly ($p < 0.05$) between the two temperature treatments (Figure 4.4). Control earthworms had significantly lower DNA damage at 26 °C compared to 21 °C.

In the aflatoxin treatments, analysis of variance (ANOVA) indicated significant differences between the concentration groups at the same environmental conditions. Tukey's HSD indicated a significant difference ($p < 0.05$) in the DNA damage of the highest concentration group (100 µg/kg) at 30% WHC compared to the control (0 µg/kg) and 10 µg/kg groups (indicated with * in Figure 4.4). The aflatoxin treatment group at 21 °C was significantly higher ($p < 0.001$) than all other treatments. The DNA damage of 100 µg/kg group with 50% WHC was slightly increased at both temperatures but not statistically significant compared to its control group. These results indicate genotoxicity at the increased concentration group under drought conditions. There was also no statistical difference between the control and 10 µg/kg groups.

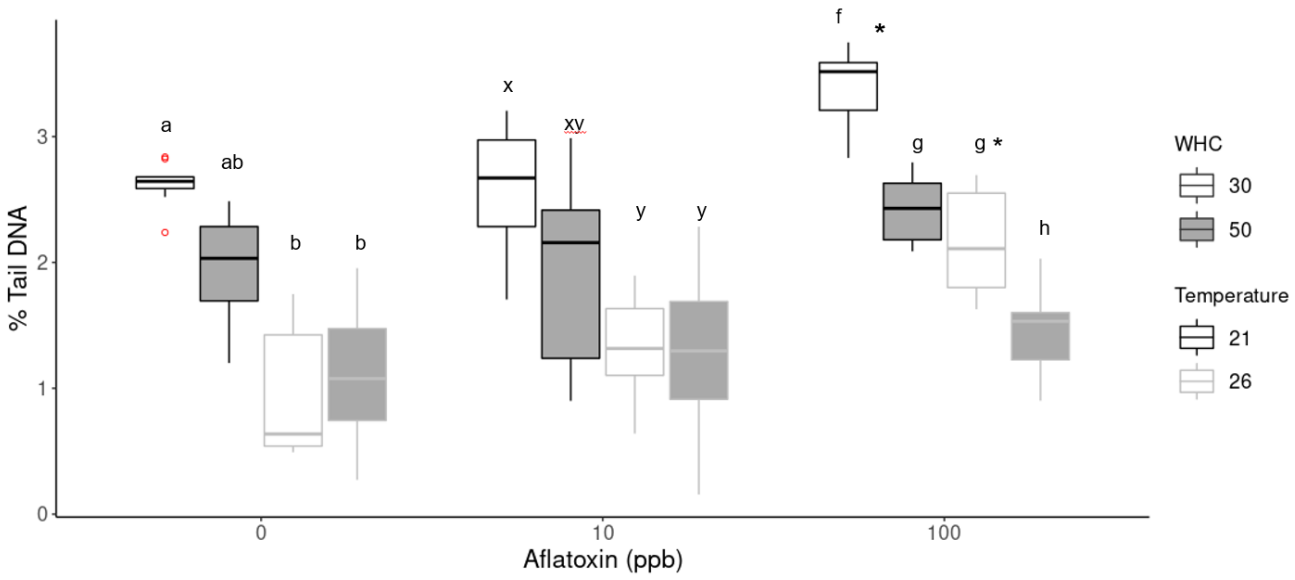


**Figure 4.4:** DNA damage in earthworm cells as % tail DNA at different aflatoxin concentrations (0, 10 and 100 µg/kg), temperature and moisture treatments. An * indicates a significant difference ($p < 0.05$) between the aflatoxin concentration and its control treatment at the same moisture and temperature conditions. Significant differences ($p < 0.05$) within the same concentration groups are indicated by different alphabetical letters (a, b in 0 µg/kg; x, y in 10 µg/kg; f, g, h in 100µg/kg).

The comet assay results were further compared at the same concentration with different temperatures and soil moistures (Figure 4.4). The same trend was observed in the 10 µg/kg and 100 µg/kg groups. Increased soil temperatures (26 °C) resulted in less DNA damage of earthworm coelomic cells than standard temperature (21 °C). The lower DNA damage at 26 °C correlates with the lower detected aflatoxin concentrations at 26 °C compared to 21 °C (Figure 4.1). The lowest DNA damage was observed in earthworms kept at 26 °C and 50% WHC. In contrast, the highest level of DNA damage was observed in earthworms at standard temperatures (21 °C) with drier soil conditions (30% WHC), irrespective of the aflatoxin concentration.

### 4.3.4. Interaction effect of climate conditions on the toxicity of aflatoxin

Three-way ANOVA assessed the interaction effect of the aflatoxin concentration, soil moisture (WHC) and temperature on reproduction and DNA damage (Table 4.2). The study demonstrated that different environmental conditions might affect the toxicity of aflatoxin in soil. Results indicated a statistically significant ($p < 0.001$) interaction effect between moisture vs temperature on reproduction and DNA damage. There was also a significant interaction effect of the aflatoxin concentration vs moisture in the genotoxic study.

**Table 4.2:** Summary of the three-way ANOVA on the effect of aflatoxin concentration (C), moisture (WHC) and temperature on DNA damage and reproduction after 30 days of exposure, *df* - degrees of freedom. Statistically, significant differences are indicated with asterisks

| | DNA damage | | | Reproduction | | |
|---|---|---|---|---|---|---|
| | **df** | **F-value** | | **df** | **F-value** | |
| Concentration (C) | 2 | 17.616 | *** | 2 | 3.509 | * |
| Moisture (WHC) | 1 | 36.458 | *** | 1 | 80.222 | *** |
| Tempertature (T) | 1 | 131.683 | *** | 1 | 0.107 | |
| C x WHC | 2 | 2.895 | * | 2 | 0.024 | |
| C x T | 2 | 0.852 | | 2 | 2.474 | |
| T x WHC | 1 | 8.817 | ** | 1 | 11.867 | ** |
| C x T x WHC | 2 | 0.698 | | 2 | 1.618 | |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Post-hoc analysis shows the specific combinations of moisture and temperature that resulted in an interaction effect (Table 4.3). There was no statistical interaction effect of soil moisture and temperature on weight loss. The same moisture levels at different temperature combinations did not prove to be significant for the reproduction test. However, there was a significant ($p < 0.05$) interaction effect between moisture and temperature in the genotoxicity study. The only combination that did not have a significant interaction effect was different moisture levels at the increased temperature

**Table 4.3:** Pairwise comparisons with Tukey's HSD to determine the specific interaction effect of moisture (M) and temperature (T) during the comet assay (DNA damage) and reproduction test. Statistically, significant ($p < 0.05$) interaction effects are indicated by asterisks.

Moisture (M) x Temperature (T)

| | DNA damage | Reproduction |
|---|---|---|
| 21_50 x 21_30 | *** | ** |
| 26_30 x 21_30 | *** | |
| 26_50 x 21_30 | *** | *** |
| 26_30 x 21_50 | *** | *** |
| 26_50 x 21_50 | *** | |
| 26_50 x 26_30 | | *** |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

## 4.4. Discussion

### 4.4.1. Aflatoxin concentration

Aflatoxin $AFB_1$ has a relatively short half-life (≤ 5 days at 28 °C) in soil (Accinelli et al. 2008) and degrades quickly into other metabolites ($AFB_2$ and $AFG_1$ and $AFG_2$). However, soil with higher organic matter and clay content form an aflatoxin conjugate with the soil-binding sites resistant to microbial degradation and may result in the $AFB_1$ persisting in the soil for much longer (Angle, 1986). ELISA is a quick and reliable technique to detect and quantify aflatoxins. These assays are mostly produced for detection in food and feed products. The recovery rates for soil matrices using an ELISA is very low because organic solvents, such as chloroform or methanol used in this study, seldom extract the toxin bound to the soil binding sites effectively (Mertz 1981). However, for this study, the ELISA results sufficiently quantified available aflatoxin concentrations and indicated low aflatoxin levels still present in the treated soil four weeks after spiking. These low concentrations mostly represent the free aflatoxin in the soil. *Eisenia andrei* are primarily detritivorous organisms that prefer to feed on organic matter such as decaying plant material. However, they also exhibit geophagous feeding traits (Jager et al. 2003), suggesting that the earthworms could have been exposed to the soil's free aflatoxin concentrations and the bound aflatoxin.

The ELISA results showed that different temperature and moisture combinations affected the concentration decrease over four weeks. The slower concentration decrease % in the drier soil suggested that the toxin persisted longer in the drier soil compared to the wetter soil. The natural degradation of aflatoxin under different temperature and pH values have been investigated (Doyle et al. 1982), but little information is available about the effects of moisture conditions on the natural degradation of aflatoxin. It is, however, known that the sorption of $AFB_1$ onto the soil particles is reduced in pre-saturated soil (Starr & Selim, 2008), suggesting that more $AFB_1$ will be available for microbial degradation under moist conditions, whereas in dry conditions, the $AFB_1$ will become less available for microbial degradation.

### 4.4.2. Earthworm survival, growth and reproduction

Growth is considered an important measure of an individuals' fitness after exposure to a chemical or toxin (van Gestel and van Brummelen, 1996). The environmentally relevant aflatoxin concentrations used in this study did not significantly affect earthworm survival, growth, and reproduction. Higher concentrations may, however, still result in harmful effects on earthworms. A concentration-dependent decrease in reproduction and a 40–60% size reduction were reported in nematodes (C. elegans) after aflatoxin exposure of as low as 3 µM (Leung et al. 2010). Degenerative changes have been reported in the reproductive areas of earthworms exposed to levels between 150 and 400 µg/L (Szabó-Fodor et al. 2017). According to Sing et al. (2020), increased temperatures typically increase earthworm abundance and may accelerate earthworm growth, whereas extreme climates such as drought and flooding might have more deleterious effects. The environmental

conditions had a more pronounced effect on the earthworm survival, growth and reproduction than the aflatoxin. Increased temperatures generally resulted in a higher weight loss percentage, decreased survival and increased reproduction under standard moisture conditions, although it was not statistically significant compared to 21 °C. Soil moisture did not affect weight loss but significantly affected reproduction. Even in the absence of the toxin, the significantly reduced reproduction rates at higher temperatures and decreased moisture indicate the physiological stress (Acharya and Mishra, 2020) for the earthworms at these conditions. Similar body weight changes in E. andrei under various climate scenarios were reported (Lima et al. 2011; González-Alcaraz and van Gestel, 2016). Lima et al. (2011) found a synergistic effect between carbaryl toxicity and soil moisture on survival and weight loss in earthworms, whereas González-Alcaraz and van Gestel (2016) reported increased weight loss of control earthworms kept at 25 °C compared to 20 °C with no effect by soil moisture. In contrast, Diehl and Williams in Sing et al. (2020) found decreasing body weight due to lowered soil moisture in E. fetida. There was evidence of a trade-off between earthworm reproduction and growth that may affect their response to toxicants (van Gestel 2012). In the aflatoxin treatments, the groups with the highest weight loss (26 °C and 50% WHC) also had the highest number of hatched juveniles, suggesting a different pattern of individual resource expenditure. The group with the lowest weight loss (10 µg/kg at 21 °C with 30% WHC) resulted in a significant decrease in the number of juveniles. The slower concentration decrease in the drier soil (Figure 4.1) could also have contributed to this.

This size difference observed in the juveniles from the different moisture treatments suggests increased moisture delayed cocoon hatching. Asynchronous and delayed hatching and the ability of the cocoons to remain viable for extended periods under favourable environmental conditions allow them to maximise their reproductive output (Lowe and Butt, 2014). In this study, increased moisture prolonged the cocoon incubation period. In contrast, drier soil conditions decreased the cocoon incubation period, suggesting that the juveniles hatched earlier, which is why they were bigger at 10 weeks. Optimal temperatures for most earthworm cocoons have been reported as 15 °C and 24 h darkness, and increased temperatures may increase the cocoon incubation period (Lowe and Butt, 2014). This study found that soil moisture had a more considerable effect on the cocoon incubation period than the temperature or the aflatoxin treatments.

### 4.4.3. Genotoxicity

Once $AFB_1$ is metabolised, it forms a genotoxic metabolic intermediate, AFBO, that can bind to DNA to form an aflatoxin-DNA adduct or induce DNA damage (Feng et al. 2016). Increased levels of DNA damage were observed in earthworm coelomocytes of the 100 µg/kg group, indicating the possible genotoxicity of aflatoxin in the soil at these concentrations. The groups with the highest DNA damage correlated with the higher detected aflatoxin concentrations in the soil. The genotoxicity of aflatoxin to soil nematodes (*C. elegans*) was indicated after exposure to concentrations between 30 and 100

µM (9–32 ppm) at optimal temperatures (15–20 °C) (Leung et al. 2010), although the study was not conducted in a soil medium, and soil moisture was not a determining factor.

As poikilothermic organisms, earthworms generally have increased metabolic activity at increased temperatures up to a threshold level (González-Alcarez et al. 2018). The results indicated lower DNA damage at increased temperatures in both the control and toxin groups. Although the higher temperature is considered sub-optimal for *Eisenisa* species (van Gestel et al. 1992), they can respond to temperature changes up to 28 °C by adjusting their enzymes capacities (Tripathi et al. 2011). Tripathi et al. (2011) investigated the temperature-dependent changes in metabolic enzymes and proteins in earthworms ranging from 12–44 °C and found that increased temperatures up to 28 °C decreased the activity of enzymes involved in energy production but contributed to compensatory changes in enzymes involved in the cellular metabolism, such as increased protein synthesis and possibly gene expression. DNA repair mechanisms in earthworms are facilitated by enzymes, although the exact mechanisms are unknown (Qiao et al. 2007). Studies have shown that the DNA repair can be very rapid, with strand breaks being repaired with a half time of less than thirty minutes and as short as three minutes (Olive and Banáth, 2006). The lower DNA damage at increased temperatures is possibly due to the increased metabolic activity and compensatory enzymatic changes in the earthworm metabolism. These results are consistent with the findings by Garcia (2004) that found a temperature-dependent decrease in the toxicity of fungicides under tropical conditions (28 °C).

In contrast, elevated DNA damage was observed at the increased temperature in combination with low moisture and high aflatoxin concentrations (100 µg/kg with 30% WHC). Although soil moisture is a known stressor to earthworms (Sing et al. 2020), the effect of one environmental factor cannot be interpreted on its own because the interaction of more than one environmental factor with the chemical stressor might increase the toxicity of these chemicals for organisms (Hooper et al. 2012; Booth et al. 2000). Further, external factors such as temperature, moisture and the accumulation of reactive oxygen species (ROS) due to a chemical stressor may also impact the enzymes involved in metabolic detoxification (Vasseur and Bonnard, 2014). The significantly ($p < 0.05$) higher levels of DNA damage of the 100 µg/kg group at increased temperatures and low moisture indicated the interaction of the toxin with the environmental conditions. Similarly, other studies reported enhanced metal detoxification under increased temperature (25 °C) with 50% moisture but significantly lower metal detoxification at the same temperature with low (30%) moisture (González-Alcaraz and van Gestel, 2016; González-Alcarez et al. 2018) and suggested that the combination of the warmer and drier environment could have hindered the earthworm metabolic performance.

### 4.4.4. Temperature and Moisture interaction with aflatoxin toxicity

Several studies report on the interaction of environmental conditions with chemical stressors and how it might increase the toxicity of these chemicals for organisms (Hooper et al. 2013). An important two-way interaction occurred between the concentration and moisture. DNA damage of the 100 µg/kg group in the low moisture soil (30% WHC) was statistically different to its control groups suggesting that the DNA damage was most likely impacted by the interaction of the toxin with the moisture conditions in the soil. Further, the interaction of soil moisture and temperature was indicated in both the reproduction and genotoxicity test, highlighting the importance of climate factors in the performance of soil organisms.

In many studies, the interaction of only one environmental factor with a chemical stressor is considered; however, the interaction of more than one environmental factor with the chemical stressor is more realistic as temperature and moisture are inherently linked in the soil (Hooper et al. 2012). A significant three-way interaction effect of soil type vs moisture content vs temperature was observed on the earthworm growth (Booth et al. 2000). Similarly, Hackenberger et al. (2018) found a statistically significant three-way interaction effect of pesticide concentration, temperature and moisture that affected earthworm enzyme activity and their response to pesticides. There was no evidence of a three-way interaction effect between concentration levels, temperature and moisture in this study. However, the possibility at higher concentrations should not be excluded because the moisture and temperature interaction was indicated in both the reproduction and genotoxicity tests. High levels of DNA damage can lead to genome disturbances that may impair growth, reproduction and population dynamics in the long term (Vasseur and Bonnard, 2014). The fact that the DNA damage was still elevated after 30 days of exposure suggests the possibility that the toxin may cause more permanent damage during drought conditions.

The results demonstrate the sensitivity of the comet assay to determine the effect of different environmental conditions (temperature and soil moisture) on the genomic functioning of the earthworm. The increased levels of DNA damage detected in uncontaminated soil in low soil moisture may have consequences for cell functioning and how they deal with other stressors (toxins) in their environment (Vasseur and Bonnard, 2014). The genotoxic biomarker proved to be more sensitive to evaluating aflatoxin's toxicity in the soil than whole organism responses (survival, growth and reproduction). Smit et al. (2009) found DNA damage to be 35 – 50 times more sensitive for evaluating oil toxicity in marine species than whole organism responses. However, DNA damage alone can only relay information about the individual. DNA damage must be complemented with growth and reproduction responses to predict possible effects at the population level (Vasseur and Bonnard, 2014). Complementary investigations on the reproductive output and the genotoxicity in earthworms in this study suggest that aflatoxin might be harmful at the population level during climate change.

## 4.5. Conclusions

Results indicated an insignificant effect of aflatoxin concentrations between 10 – 100 µg/kg on the earthworms' (*E. andrei*) survival, growth, and reproduction in an OECD soil medium. The presence of the toxin reduced the number of juveniles but also prevented the same level of weight loss compared to the control groups, although it was not statistically significant. Comet assay results indicated a concentration-dependant increase in DNA damage after 30 days of exposure to aflatoxin, suggesting that increasing aflatoxin concentrations might influence the health of soil organisms.

Different combinations of temperature and soil moisture conditions resulted in different effects. Increased temperatures generally resulted in lower survival rates and increased weight loss but increased reproductive output and showed less DNA damage, indicating their ability to adapt a different pattern of individual resource use. Moisture had a more pronounced effect on the population performance in terms of reproduction and DNA damage. Significantly reduced reproduction rates at higher temperatures and decreased moisture, even in the absence of the toxin, indicate the physiological stress for the earthworms at these conditions.

Although limited effects of the toxin were observed at standard testing conditions, the exposure-effect outcomes of aflatoxin might be influenced by climate change due to the interaction of the toxin with environmental conditions. In particular, decreased moisture treatments resulted in a significantly decreased reproductive output at low aflatoxin concentrations and significantly more DNA damage with increasing aflatoxin concentrations. Complementary investigations on the reproductive output and the genotoxicity in earthworms suggest that aflatoxin might be harmful at the population level during climate change. Future studies using contaminated agricultural soil will be valuable in predicting aflatoxins' effect in the natural environment. When using OECD soil, some variables in natural ecosystems that can affect the toxins' bioavailability might be excluded and can alter the results.

## 4.6. References


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# CHAPTER 5 – BIOLOGICAL CONTROL OF AFLATOXINS BY EARTHWORMS

## 5.1. Introduction

Aflatoxin (AF) contamination of food and feed products is one of the most important threats to food safety under changing climate conditions (Medina et al. 2017). Aflatoxins are chemically and thermally (< 160 °C) stable and not easily destroyed (Raters and Matissek, 2008). Extensive research has been done since the 1960s to find suitable control strategies for AF contamination at the pre-and post-harvest stages (Payne et al. 1986). Unfortunately, AF contamination in the production, storage and final food products remains a challenge and detected levels frequently exceed the regulatory limits for human consumption of between 4 – 20 µg/kg set by the USA and European Union (Jallow et al. 2021). In addition, the geographical distribution of AF continues to expand mainly due to climatic factors (Moretti et al. 2019). Changes in temperature, pH, periods of waterlogging and drought may cause post-harvest aflatoxigenic fungi to become more prevalent at pre-harvest stages (Medina et al. 2017). The persistence of AF contamination highlights the importance to continue our efforts to find effective control strategies to prevent contamination.

Management strategies include chemical, physical (Pakaj et al. 2018) and biological processes (Wu et al. 2009) to control mycotoxigenic fungi and manage AF contamination in food products. Chemical and physical processes are efficient but generally expensive, time-consuming and not always ideal for the safety and quality of treated products (Shcherbakova et al. 2015). Biological processes include using microorganisms and their enzymes to reduce or detoxify aflatoxins and are economically more viable, environmentally friendly, and a promising alternative management strategy for AF contamination (Wu et al. 2009; Verheecke et al. 2016). Most of these methods are designed to manage AFs in post-harvest agricultural products without considering the pre-harvest plant and soil ecosystems. The prevention and biological control of AF contamination at pre-harvest stages provide the greatest opportunity for immediate mitigation and limit post-harvest synthetic strategies that might affect the quality of products (Peles et al. 2021). Pre-harvest strategies are mostly centred around good agricultural practices, including using healthy seed, correct irrigation and tillage practices (Payne et al. 1986), crop rotation and harvesting times (Verheecke et al. 2016). Some research has also been done on genetically engineered peanut, corn and cotton varieties that contain resistant factors to inhibit toxin production by the fungi (Cary et al. 2000). Other strategies include antifungal agents, although research has shown that not all fungicides are effective against aflatoxigenic fungi (Weaver et al. 2015).

Once AF levels in food and feed products exceed safe usage limits, regulations suggest that the contaminated products be incinerated or worked into the soil for microbial degradation (EAC, 2018). When it enters the soil, factors such as the physiochemical properties (soil organic matter and clay

content), soil moisture (Star and Selim, 2008), and soil microorganisms (Accinelli et al. 2008) affect the fate of the $AFB_1$. A laboratory experiment under controlled temperature and moisture conditions demonstrated that $AFB_1$ is transformed into its degradation products ($AFB_2$, $AFG_1$ and $AFG_2$) in a relatively short time with a reported half-life of ≤ 5 days at 28 °C (Accinelli et al. 2008). However, in soil with a high adsorption coefficient, in other words, soil with high clay and organic matter content, $AFB_1$ might be adsorbed to the soil binding sites. When adsorbed to the soil binding sites, $AFB_1$ becomes unavailable for microbial degradation and persist in the soil for much longer (Angle, 1986). Goldberg and Angle (1985) reported adsorption coefficients of 238, 76, 46, and 17 mg/kg for silty clay loam, silt loam, clay loam, and sandy loam, respectively. The adsorption ability of aflatoxins to soil binding sites and its leaching potential were studied by Madden and Stahr (1993) using soil-water column systems. Results obtained confirmed findings by Goldberg and Angle (1985) that 50% of silty clay loam results in high adsorption of $AFB_1$ to the soil material. However, in soil with a slightly lower adsorption ability (20% silty clay loam), the leaching of AFs was possible, suggesting the presence of higher levels of the degradation products and possible exposure to soil organisms. The degradation products can remain in the soil for up to 77 days (Angle 1986). In a field study by Accinelli et al. (2008), $AFB_1$ was detected in the soil five months after harvest, and it was suggested that $AFB_1$ exposure might be prolonged due to its gradual release from contaminated plant debris during decomposition. Conservation and no-tillage practices are becoming more common in agriculture because of its benefits in preventing the loss of soil organic matter and reducing erosion. The disadvantage of these methods is the increased risk of contaminated crops re-contaminating the soil and subsequent crops with aflatoxigenic fungi. Appaw et al. (2020) reported a 188- to 226-fold increase in $AFB_1$ concentrations when maize kernels were left to dry on the soil, indicating the possibility of prolonged and continued exposure of soil organisms.

Biodegradation of AF in the soil is mainly achieved by microbial populations (Wu et al. 2009). The role of soil microbes and fungi in the degradation of AF has been widely reported (Wu et al. 2009; Raksha Rao et al. 2017; Peles et al. 2021). Soil microbes can effectively degrade AFs into other, less toxic forms, e.g. $AFB_2$, $AFB_{2a}$, $AFG_1$ and $AFB_2$. Actinobacteria species such as *Nocardia corynebacteroides*, *Mycobacterium fluoranthenivorans* (Hormisch et al. 2004), *Streptomyces lividans* and *Rhodococcus erythropolis* (Cserháti et al. 2013) have all been reported to significantly reduce (70 – 80% of initial concentrations) $AFB_1$ in a very short time under optimal conditions (Wu et al. 2009). In many of these cases, the efficiency or increase in efficiency was due to increased temperatures. Raksha Rao et al. (2017) reported optimal (> 90%) degradation and detoxification of $AFB_1$ by *Bacillus licheniformis* at 37 °C. The detoxification potential is also not the same for all microbial species, and some species may result in different by-products with harmful effects such as cytotoxicity or genotoxicity (Krifaton et al. 2011). Genotoxicity was retained in *R. erythropolis* strains after effectively degrading $AFB_1$, suggesting high degradation potential but low detoxifying potential (Cserháti et al. 2013). Specific enzymes purified from microbial systems have also been reported to degrade AFs (Liu et al. 2001) but are mostly used to detoxify food products. Biocontrol by fungi

occurs mainly by a displacement strategy in that naturally occurring and non-toxigenic *A. flavus* isolates reduce the toxigenic population and is considered one of the most effective pre-harvest methods currently (Xu et al. 2021). In other cases, it was reported that these non-toxigenic isolates and other fungal strains might also degrade $AFB_1$ (Wu et al. 2009), thereby acting as a biocontrol in soil and pre-harvest contamination of economically important crops (Weaver et al. 2015).

The AF degradation potential of other soil organisms involved during the decomposition processes is not frequently reported. The activities of detritivorous (feeding on dead organic material and plant detritus) and fungivorous (feeding of fungi) soil organisms such as earthworms affects many essential soil processes. Through the physical and chemical modification of their environment, for example, by modifying soil structure, earthworms create and maintain favourable habitats for microbial activity (Bertrand et al. 2015). They are also essential biological regulators of plant pathogens such as fungi and have been shown to regulate harmful toxins associated with some fungal populations (Schrader et al. 2013). *Aspergillus spp* such as *A. niger, A. flavus* and *A. fumigatu*s are common food sources for earthworms (Zirbes et al*.* 2011), and by feeding on fungi, they have the potential to exhibit antifungal activity against harmful species. Oldenburg et al. (2008), Wolfarth et al*.* (2016) and van Capelle et al. (2021) all reported that earthworms (*L. terrestris*) contribute to the sustainable control of the mycotoxin deoxynivalenol in wheat straw by feeding on *Fusarium,* thus reducing the risk of toxin contamination as an ecosystem service. Earthworm activities have great potential for the remediation of soil contamination, for example, heavy metals and other organic contaminants (Zeb et al. 2020). Burrowing, casting and mixing soil and litter (bioturbation) can displace strongly adsorbed contaminants (Bacarro et al. 2019). Further, previous studies indicate that earthworm intestinal bacteria play an important role in the remediation potential of earthworms (Sun et al. 2020), although the specific mechanism of pollutant transformation is not yet known. It is not currently known whether earthworm activity also affects $AFB_1$ in soil.

Detection of AF in soil is complicated by its adsorption potential to the soil binding sites. In soil with a high adsorption coefficient, the detection of AF is limited by the desorption from soil binding sites (Madden and Stahr 1993). Previous studies investigating $AFB_1$ in soil (Angle and Wagner, 1981, Madden and Stahr 1993, Starr and Selim, 2008) reported recovery rates of between 1 – 70% from soil. The highest recovery rate (70%) was obtained in pre-saturated soil before AF contamination. The sorption of $AFB_1$ onto the soil particles is reduced in pre-saturated soil (Starr and Selim, 2008), increasing the recovery of AF during extraction. Analytical methods to detect and monitor AF concentrations must be sensitive, accurate, reproducible, and easy to use (Jallow et al. 2021). Immunochemical-based methods like enzyme-linked immunosorbent assay (ELISA) and chromatography analysis have been widely applied for the visual detection and semi-quantification of various mycotoxins. The chromatographic methods are accepted and approved reference methods for quantitatively analysing mycotoxins (Jallow et al. 2021). These techniques provide sensitive and simultaneous analysis of fungal metabolites, including AF (Yao et al. 2015) but are

costly and time-consuming. Effective pre-screening methods like ELISA are often used to select relevant samples for further specific analysis to reduce costs. The ELISA is a simple and rapid technique and the most-used analytical tool for detecting and quantifying AFs in agricultural products (Anfossi et al. 2016). Commercially available AF ELISA kits have been a powerful tool to provide a high-throughput rate for AF detection and monitoring in food safety (Pereira et al. 2014). Although the ELISA method was designed specifically to detect mycotoxins in food commodities such as grains and peanuts, it has been successfully used in previous studies to quantitatively detect mycotoxins in soil samples (Wolfarth et al. 2016).

Under changing climate conditions, the need to identify other natural methods or organisms to degrade aflatoxins is important to ensure sustainable soil ecosystems and food safety. Although the role of earthworms in providing many important ecosystem services, including the protection against harmful toxins for the sustainability of agrosystems, is established (Plaas et al. 2020, van Capelle et al. 2021), some questions remain in terms of their role in aflatoxin degradation. In light of the information discussed, this study aimed to investigate the role of earthworms in the degradation of aflatoxins under different soil conditions.

The specific objectives were to:

- Investigate if earthworms (*Eisenia andrei*) improve the natural degradation of aflatoxins in soil.
- Determine if soil climatic conditions (temperature and soil moisture) affect the aflatoxin degradation potential of earthworms.

## 5.2. Materials and Methods

### 5.2.1. Experimental design

A laboratory experiment was conducted at the North-West University, South Africa and science labs of the University of South Africa to assess the degradation of AFs in the presence and absence of earthworms at two different temperatures (21 and 26 °C) and soil moisture (30% and 50% water holding capacity (WHC) conditions over four weeks. Two different AF concentrations (10 µg/kg and 100 µg/kg) were used for spiking the soil based on concentrations measured in environmental samples from previous studies (Table 2, Chapter 4).

A standardised soil medium was used in the experiment based on the Organisation for Economic Co-operation and Development (OECD, 2016) guidelines to overcome some of the complexity and heterogeneity of the soil environment. The water holding capacity (WHC) of the OECD soil was determined before the start of the experiment using a Sartorius moisture analyser (Annexure C). For each concentration group, four treatments were prepared based on a combination of air temperature and soil moisture (as soil water holding capacity) (González-Alcarez et al. 2018). In each treatment, three replicates contained earthworms, and three replicates were prepared with no earthworms (Table 5.1).

**Table 5.1**:The laboratory experiment was designed to include a combination of two temperature and two moisture treatments at two aflatoxin concentrations. Each treatment was replicated six times, three with earthworms and three without earthworms. WHC: water holding capacity

| | | Aflatoxin 10 µg/kg | | Aflatoxin 100 µg/kg | |
|---|---|---|---|---|---|
| **Treatment** | | **No earthworm** | **Earthworm** | **No earthworm** | **Earthworm** |
| 1. | 21 °C + 50% WHC | 3 replicates | 3 replicates | 3 replicates | 3 replicates |
| 2. | 21 °C + 30% WHC | 3 replicates | 3 replicates | 3 replicates | 3 replicates |
| 3. | 26 °C + 50% WHC | 3 replicates | 3 replicates | 3 replicates | 3 replicates |
| 4. | 26 °C + 30% WHC | 3 replicates | 3 replicates | 3 replicates | 3 replicates |

### 5.2.2. Soil spiking with aflatoxin

Before introducing the AF, the soil was pre-moistened with $dH_2O$ to the desired water holding capacity (30% and 50%). Methanol was used as a solvent to prepare the liquid aliquots of the powdered $AFB_1$ (Enzo Life Science, through Biocom Africa). Aflatoxins are typically introduced into the surface soil by infected plant material left to decompose (Accinelli et al. 2008). For this experiment, dried horse manure containing sufficient quantities of wheat straw was used to introduce the toxin into the soil. Horse manure is also the recommended food source for earthworms in toxicology studies. The powdered AF was dissolved with methanol to obtain 10 µg/ml for the higher concentration group. One ml of the 10 µg/ml AF was further diluted with methanol to obtain 1 µg/ml for the lower concentration group. For each vessel containing 600 g of soil, 6 ml of the AF solvent was mixed with 5 g dried horse manure to obtain the final concentrations of 10 and 100 µg/kg. The contaminated horse manure was placed on top of the moistened soil. The soil samples were weighed individually and placed under an extractor fume hood overnight at room temperature to allow the solvent to evaporate. After 12 hours, the soil was weighed again, and any loss of weight due to moisture loss was compensated for by adding $dH_2O$. The soils were left for another 12 hours to stabilise, after which ten adult earthworms were introduced into each of the replicate vessels for the earthworm treatments.

### 5.2.3. Enzyme-linked immunosorbent assay (ELISA)

An indirect, competitive ELISA kit (ELABScience total aflatoxin ELISA kit, E-TO-E006) was used to analyse total AF concentrations in the soil. The cross-reactivity of the kit was given as $AFB_1$ - 100%, $AFB_2$ - 80%, $AFG_1$ - 75%, $AFG_2$ - 45%. The ELABScience protocol provides several sample pre-treatment methods (grain, formula feed, high-fat products and liquids, e.g. sauce and carbonated liquids). These methods were tested, and the pre-treatment method for formula feed was most effective for aflatoxin extraction from soil.

Soil samples were taken 72 hours and 32 days after soil spiking for ELISA analysis. 20 g of soil were taken randomly from the 600 g vessel to get a representative sample. Care was taken not to include

earthworms or juveniles in the samples. The soil samples were crushed, and 2 g of homogenate was mixed with 10 mL of 70% methanol in a 50 ml centrifuge tube. The tube was mixed in a shaking incubator for 30 minutes and centrifuged at 4 x 1000 rpm for 10 minutes. To obtain a ten times dilution, 0.5 mL of the supernatant was mixed with 0.5 mL of de-ionised water. When optical density values were outside the detection limits of the kit, usually due to high AF concentrations in the sample, a further ten times dilution was done by mixing the supernatant mixture with 35% methanol to obtain a 100 times dilution (10 times from first step x 10 times from the second step). This step was only required during the first 72-hour sampling period.

For detection analysis, 50 µL of the extracted supernatant was used to inoculate a 96 well microtiter plate. Each sample (n=3) was inoculated in duplicate to ensure a consistent and accurate result. Equal quantities of the horseradish peroxidase conjugate and antibody working solution were added to each well and incubated for 30 minutes at 25 °C. After incubation, the liquid in the wells was carefully discarded, and the plate was washed five times with a phosphate buffer and water wash solution (1:19). A tetramethylbenzidine (TMB) reagent was added, and the plates were incubated for 15 minutes to allow for colour development. Each well's optical density was determined with a microplate reader at a wavelength of 450 nm.

### 5.2.4. Concentration calculation (4PL)

The optical density readings at 450 nm from a range of six standard solutions (0 – 0.32 µg/ml) provided with the kit were used to generate a non-linear standard curve. A four-parameter logistics (4PL) regression model was used to calculate the concentrations in the samples based on the standard curve. The equation used for the four-parameter logistics (4PL) model is:

$$y = d + \frac{a - d}{1 + \left(\frac{x}{c}\right)^b}$$

Where y (the concentration) is calculated from the four unknown parameters:
a = the minimum value that can be obtained
d = the maximum value that can be obtained
c = the point of inflection
b = Hill's slope of the curve

The absorbance value of the samples was first corrected by subtracting the blank reading. The corrected absorbance values of the samples were fit onto the standard curve to calculate the AF concentrations in the samples, and the dilution factors were applied. The correlation coefficient ($R^2$) was used to indicate the accuracy of fit. An $R^2$ value of ≥ 0.98 is usually recommended for non-linear regression models like the 4PL (Nix and Wild, 2001).

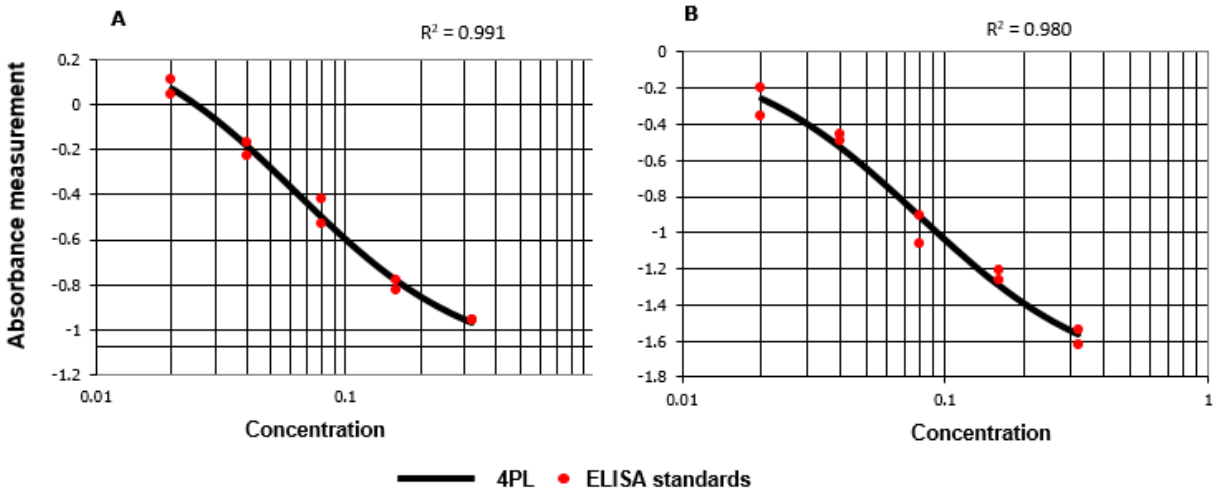


**Figure 5.1**:Standard curves based on the four-parameter logistics (4PL) model generated from the absorbance values of standards. A – standard curve generated at 72 hours. B – standard curve generated at 32 days. $R^2$ values indicate the "goodness of fit".

### 5.2.5. Statistical analysis

The Four Parameter Logistic Curve (4PL) online data analysis tool (MyAssays Ltd.) was used to construct standard curves and calculate sample concentrations (Figure 5.1). Statistical analysis and graphical representations were performed using R version 4.1.0 (R Core Team, 2021) and R Studio packages *vegan* and *ggplot2*. Shapiro Wilk was performed to test for normality of data. All datasets were normally distributed. Homogeneity of variance was tested with the Levene Test. When homogeneity of variance was violated, the Welch test was performed. Analysis of variance (ANOVA) was performed to test for differences in the calculated concentrations within the same faunal groups at different environmental conditions. *Post-hoc* Tukey's HSD was performed for multiple comparisons between environmental conditions and t-tests for differences between earthworm and non-earthworm treatments. Three-way ANOVA (a 2 x 2 x 2 design) was performed to test for possible interaction effects between faunal treatments (earthworm or no-earthworm), temperature (T) and moisture (WHC).

## 5.3. Results

The total AF concentrations ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$) detected at 72 hours and 32 days (4 weeks) after soil spiking for each concentration group are presented in Figure 5.2. The recovery rate from soil was generally low (mean of between 12 – 39%). Nevertheless, the ELISA method detected AF in all the samples at both time intervals and was sufficient to confirm differences in the total AFs between treatments.

Within 72 hours after spiking the soil with 10 µg/kg, lower soil AF concentrations were detected in the non-earthworm treatments at 21 °C, and it was statistically significant ($p < 0.001$) in the increased moisture (50% WHC) group. In contrast, at 26 °C, significantly ($p < 0.05$) lower soil AF concentrations were detected in the earthworm treatments. After 32 days, the soil from the earthworm test group at 26 °C and 30% WHC was still significantly ($p < 0.05$) lower than the non-earthworm group at the same conditions.

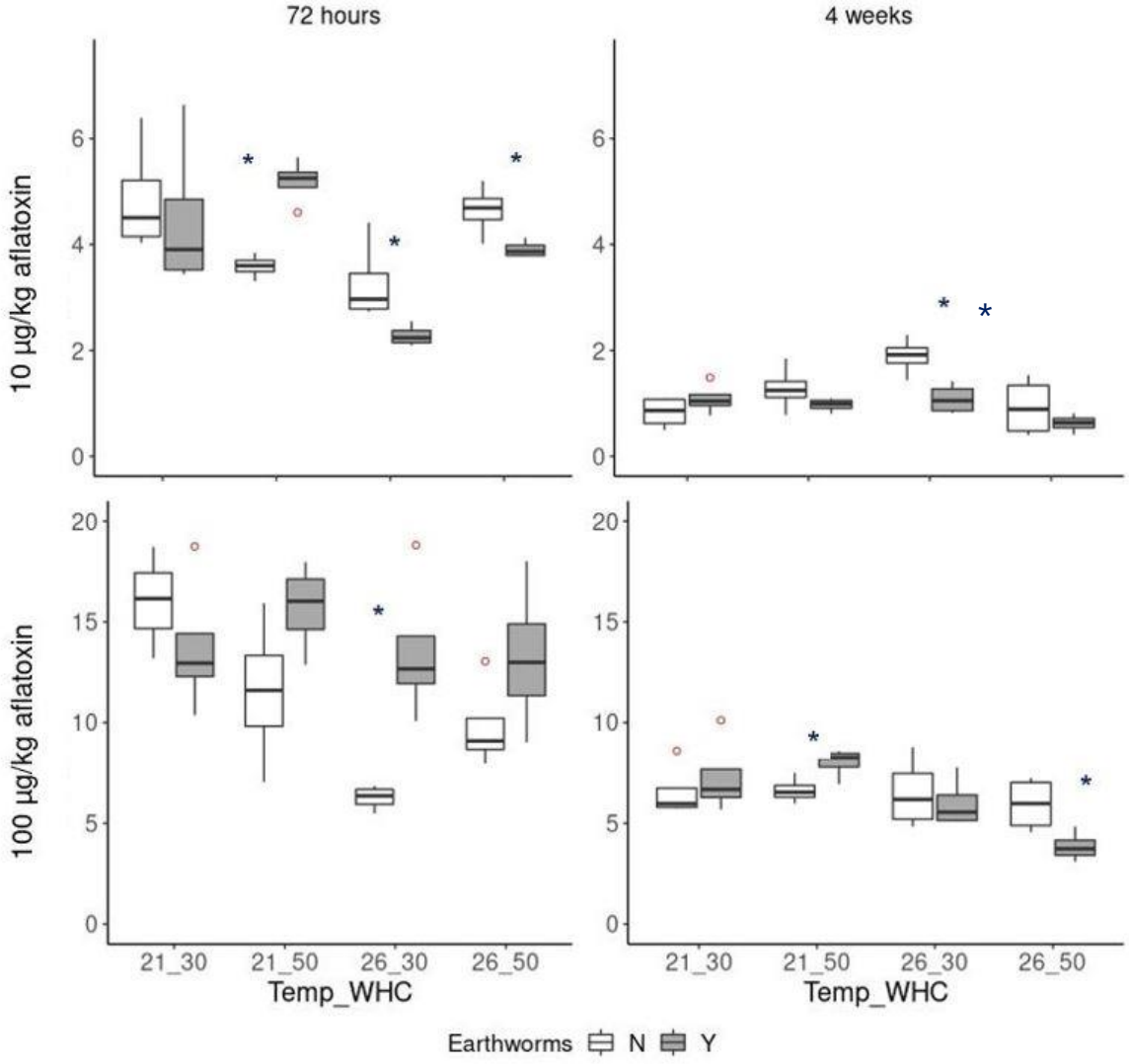


**Figure 5.2** Total aflatoxin concentrations detected in soil at 72 hours and 32 days (4 weeks) after spiking the soil for each concentration group (10 and 100 µg/kg), and at different temperature (Temp) and soil moisture as water holding capacity (WHC) combinations. Significant differences between earthworm and non-earthworm treatments are indicated with asterisks (*). Outliers in the data set are indicated by open circles.

In the 100 µg/kg spiked concentration, higher AF concentrations were initially detected in all the earthworm treated soil than the non-earthworm soil. The higher concentrations in the samples were possibly due to the bioturbation activity of the earthworms in the early stages before AF degradation. However, after 32 days, the mean AF concentration in the earthworm groups at 26 °C was lower than the non-earthworm groups, indicating improved degradation. The earthworm group at 26 °C

and 50% WHC was statistically ($p < 0.05$) lower than the non-earthworm group at the same conditions. In contrast, the mean AF concentrations in the earthworm treatments at 21 °C remained higher than the non-earthworm treatments, and t-tests revealed statistically ($p < 0.05$) higher concentrations at standard conditions (21 °C and 50% WHC).

**Table 5.2:** Percentage (%) decrease in soil aflatoxin (AF) concentrations after four weeks. Different alphabetical letters indicate significant ($p < 0.05$) differences within the same faunal group at the same spiked concentration (AF10 = 10 µg/kg and AF100 = 100 µg/kg). Significant differences between earthworm (EW) and non-earthworm groups at the same environmental conditions are indicated in bold.The asterisk (*) indicate the level of significance * $p < 0.05$; ** $p < 0.01$. Temperature (Temp), moisture as water holding capacity (WHC)

**Percentage (%) decrease**

| **Temp_WHC** | **AF10** | | | | **AF100** | | | |
|---|---|---|---|---|---|---|---|---|
| | Faunal groups | | | | | | | |
| | *Non-EW* | | *EW* | | *Non-EW* | | *EW* | |
| *21_30* | 89.68 | x | 89.13 | a | 93.43 | Z | 92.71 | ab |
| *21_50* | 87.18 | xy | 90.27 | ab | 93.36 | Z | 91.99 | a * |
| *26_30* | 81.08 | y | 89.13 | a ** | 93.50 | Z | 94.58 | bc |
| *26_50* | 90.69 | x | 93.75 | b | 94.06 | Z | 96.40 | c ** |

The detected soil concentrations varied considerably from 72 hours to 32 days within the same treatment. Therefore, further analysis was done to compare the percentage (%) decrease from the initial spiked concentration over four weeks (Table 5.2). On average, the total soil AFs decreased by 80 – 95% in the lower (10 µg/kg) spiked concentrations and decreased by 91 – 96.5% in the higher (100 µg/kg) spiked concentrations in 32 days. The percentage decrease was used as an indication of the AF degradation potential in each of the treatments.

At 10 µg/kg, the earthworm treated soil generally had a higher percentage (%) decrease or degradation in soil AF concentrations than the non-earthworm treatments, but ANOVA results indicated that only the group at 26 °C with 30% WHC was statistically significant ($p = 0.012$). When comparing between temperature and moisture (WHC) conditions within the same faunal group, the earthworm group had a significantly ($p < 0.05$) higher level of AF degradation at increased moisture (50% WHC) conditions and lower AF degradation in the drier soil (30% WHC) conditions. The lowest degradation potential was observed at 26 °C with 30% WHC and was statistically ($p = 0.03$) different from the soil with the highest AF degradation at 26 °C with 50% WHC in the earthworm and non-earthworm treatments.

At increased spiked concentrations (100 µg/kg), the earthworm treatments at 21 °C had a lower AF degradation potential than the non-earthworm treatments. At standard testing conditions (21 °C with 50% WHC), the AF degradation in earthworm treated soil was statistically lower than the non-earthworm treated soil. In contrast, at 26 °C, earthworm treatments generally showed a higher

percentage decrease in soil AF concentration than non-earthworm soil. The earthworm group at 26 °C and 50% WHC had a significantly ($p = 0.001$) higher % decrease after four weeks, indicating improved degradation compared to the non-earthworm group at the same conditions. Within the earthworm treatments across the different environmental conditions, there was also statistically ($p < 0.02$) higher degradation at increased temperatures (26 °C). There was no difference between the % decrease of the non-earthworm treatments across the different temperature and moisture conditions. A three-way analysis of variance (ANOVA) was performed to determine if there were any interacting effects between the three variables namely earthworm presence vs tempertuare vs moisture that affected the soil AF degradation, in addition to the specific main effects that were established.

**Table 5.3**: Main and interaction effects of temperature (T) and moisture (WHC) with the presesnce or absence of earthworms (EW) during the degradation of aflatoxins (AF10 = 10 µg/kg and AF100 = 100 µg/kg).

| | | **AF10** | **AF100** |
|---|---|---|---|
| *Main effects* | *df* | *F-value* | *F-value* |
| Moisture (WHC) | 1 | 5.071 * | 0.844 |
| Temperature (T) | 1 | 0.560 | 16.675 *** |
| *Interaction effects* | | | |
| EW x WHC | 1 | 0.18 | 0.126 |
| EW x T | 1 | 4.817 * | 10.181 ** |
| WHC x T | 1 | 13.308 ** | 3.343 |
| EW x T x WHC | 1 | 4.865 * | 1.232 |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Results confirmed the main effects observed in the one-way analysis and indicated significant interaction effects between the different environmental conditions (Table 5.3). The main effect of moisture (WHC) was significant ($p < 0.05$) in the lower spiked (10 µg/kg) concentration. The observation occurred at the increased temperatures (26 °C) only, but for both the earthworm and non-earthworm groups and not at the higher spiked (100 µg/kg) concentration group. The temperature main effect played a bigger role at increased concentrations (100 µg/kg). The temperature main effect was dependent on the presence of earthworms. A significant ($p < 0.05$) two-way interaction was observed between temperature and EW treatments at both concentration groups. Further analysis indicated that the interaction effect observed, occurred at increased temperatures indicating the specific interaction of increased temperatures and the presence of EW for improved soil AF degradation. A significant ($p <0.01$) interaction effect was reported between the moisture (WHC) and temperature (T) at the lower spiked concentrations (AF10), but it was dependent on a third variable because it was only observed in the non-earthworm group. No interaction effect was observed between moisture and temperature at increased concentrations.

However, when excluding the non-earthworm results, and focusing on the earthworm treated soil only, the combination of increased moisture and temperature resulted in a a significant ($p = 0.031$, F-value = 5.10) interaction effect in the earthworm treatments at 100 µg/kg, which explains the greatest degradation rate for the earthworm treatments at 26°C at 50% WHC.

### 5.4. Discussion

Aflatoxin degradation occurred at a faster rate at the higher concentrations (100 µg/kg). The increased degradation rate was possibly due to the soil sorption reaching a threshold level causing any additional free $AFB_1$ to be exposed to microbial degradation. In natural soil with low soil organic matter, AF degradation might occur at a faster rate. However, the presence of moisture also influences the $AFB_1$ sorption to soil particles. Although the soil in this study was not saturated to 100% WHC, the results verified temperature and moisture influence. The soil with increased temperature and moisture (50% WHC) had higher AF degradation (Table 5.2). The moisture might have reduced the sorption of $AFB_1$ to soil binding sites and accelerated the degradation process (Starr and Selim, 2008). This highlights the possible risk in areas with low annual rainfall or more frequent drought conditions as degradation is slower in dry soil. These findings are consistent with field studies conducted by Sanders et al. (1984) and Sibakwe et al. (2017) that reported increased aflatoxin contamination in groundnut at pre-harvest stages after prolonged drought conditions.

The AF degradation processes observed in the soil with no earthworms indicate degradation exclusively by soil microbial activity, while earthworm treated soil indicate single and combined effects of the earthworms and soil microorganisms. Results did not show earthworm treated soil to contribute significantly more to the AF degradation at 21 °C. However, at increased temperatures (26 °C), the presence of earthworms significantly ($p < 0.05$) contributed to the degradation of aflatoxins. Wolfarth et al. (2016) and van Capelle et al. (2021) found that earthworms (*L. terrestris)* significantly contribute to the degradation of the mycotoxin deoxynivalenol in a field study that was conducted at much lower soil temperatures (10 - 12.5 °C). Similarly, Zeb et al. (2020) reported that earthworms (*Eisenia fetida*) improve hydrocarbon degradation by stimulating microbial growth and activity. The increased microbial activity was reported to be due to degradable carbon being excreted by earthworms and their ability to aerate the soil. Their role in heavy metal detoxification and organic pollutants such as polycyclic aromatic hydrocarbons have also been linked to their intestinal bacteria (Sun et al. 2020). Although microbial activity was not measured, it is proposed that the increased degradation by the earthworms observed in this study was attributed to the earthworm activity and excretions that stimulated higher microbial activity.

Correlations between the toxicological effects described in Chapter 4 and the degradation potential investigated in this chapter, show a high negative correlation ($r = 0.93$) between DNA damage and the % decrease in the 100 µg/kg treatments and a positive correlation ($r = 0.91$) between reproduction and % decrease at 10 µg/kg. The lowest DNA damage in earthworm coelomic cells and

highest reproductive output was observed in the soil with the highest % decrease of AF concentrations.

Overall the results indicated that temperature had the biggest effect on the biological degradation of AF by earthworms. At increased temperatures, the earthworm treatments had a higher % AF decrease, indicating the interaction of temperature and the presence of earthworms for improved soil AF degradation. However, temperature and moisture are inherently linked in the soil, and the interaction of more than one environmental factor is important to understand biological results fully (González-Alcarez et al. 2018). Earthworms are poikilothermic organisms meaning that their metabolic, burrowing, and casting activity typically increases with increasing soil temperature if soil moisture is sufficiently high (Singh et al. 2019). The increased AF degradation at 26 °C would suggest that the earthworm behavioural alterations at these temperatures might have contributed to the higher decrease in AF concentrations. Burrowing, casting and mixing soil and litter (bioturbation) can displace strongly adsorbed contaminants (Bacarro et al. 2019). The greatest degradation potential was observed in the increased moisture treatments (50% WCH), possibly due to increased burrowing activity (Wen et al. 2020).

### 5.5. Conclusions

The aflatoxin regulatory potential is not the same for all soil organisms and is affected by environmental conditions. After four weeks of exposure, earthworm treatments did not affect the aflatoxin degradation at standard testing conditions (with 50% WHC). In contrast, improved AF degradation was observed in the earthworm treatments at 26 °C. This highlights the importance of reviewing standard toxicity protocols for laboratory studies to include wider environmental conditions during ecotoxicology studies. The results demonstrate the complex mechanisms underlying the interaction between toxicity level, temperature and moisture. The % decrease in AF concentrations (e.g. due to adsorption or degradation) was significantly influenced by temperature or soil moisture or a combination of the two factors. The interaction of temperature and soil moisture in the earthworm treatments at the increased concentrations further highlight the importance of climate factors in the performance of soil organisms.

Despite the low sensitivity of the ELISA method to detect total AFs in soil, it adequately identified significant differences in soil concentration between treatments. It is, however, recommended that detected values be confirmed with more sensitive and reliable test methods specific for detecting AFs in soil (Albert et al. 2021). It is also recommended that field-based studies be conducted to understand the effects in natural settings better.

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# CHAPTER 6 – GENERAL CONCLUSIONS AND RECOMMENDATIONS

Aflatoxins are well known fungal toxins produced by specific strains of soil fungi (*Aspergillus* sp). It commonly occurs in soil, compost and food storage systems. Due to its toxic and carcinogenic nature, extensive research has been done on the impact of aflatoxin contamination for the food and feed industries. Research has mainly focused on post-harvest contamination with little consideration at pre-harvest stages. Aflatoxin $B_1$ has so far been regarded not to pose any long-term environmental risk because it has a short half-life in soil due to microbial degradation and adsorption to soil organic matter. Limited research has been done on this topic since the 1980s.

The aim of this study was to clarify the risk of aflatoxins in the soil ecosystem under different soil climate (temperature and soil moisture) conditions. In the first phase of the study, existing literature about the occurrence, fate and effects of aflatoxins in soil, was reviewed. Results from the review led to focused experimental work to test several hypotheses about the toxicological effects of aflatoxin exposure to soil organisms that contribute to essential ecosystem services in the soil. Earthworms (*Eisenia andrei*) were used as bioindicator species, and established biomarkers were selected to conduct the toxicology tests in the laboratory.

## 6.1. Aflatoxins in the soil ecosystem: an overview of its occurrence, fate, effects and future perspectives

A detailed review of the literature was done to *determine the current knowledge on aflatoxins' occurrence, fate and effects specific to the soil environment*. The research from this phase of the study was published.

Fouché TC; Claassens S; Maboeta MS (2020) Aflatoxins in the soil ecosystem: an overview of its occurrence, fate, effects and future perspectives. *Mycotoxin Research*. 36 (3):303-309 https://doi.org/10.1007/s12550-020-00393-w

From the review, it was concluded that:

i. Aflatoxin contamination is still a serious worldwide problem that results in significant income losses for producers of edible crops. More focused research is required into the prevention at pre-harvest stages for improved management at post-harvest stages.
ii. Low aflatoxin concentrations are generally detected in soil and considered low risk for environmental contamination. However, soil concentrations may significantly increase during drought stress and when contaminated crops are left to decompose or worked into the soil as suggested by regulations, it increases the natural concentrations and prolongs exposure due to the gradual release of the toxins, thus altering the natural ecological balance over time. Insufficient information is available on the effects of aflatoxin exposure on soil organisms and it is currently unclear to what extent increased aflatoxin concentrations might endanger soil health. The functional response of soil organisms after aflatoxin exposure and the consequences on the

soil communities' structure and functions are unknown. It is an area of research that should be explored in more detail.

iii. Climate change has resulted in aflatoxin becoming a bigger risk in areas where it already occurs. Environmental factors such as changes in temperature, pH, drought conditions or periods of waterlogging may cause post-harvest aflatoxigenic fungi to become more prevalent at pre-harvest stages (Medina et al. 2017). Drought stress, in particular, causes increased aflatoxin production by fungi. In addition, studies show that changes in temperature and soil moisture might increase the risk of aflatoxin contamination in areas where it did not previously occur (Battilani et al. 2016). Therefore, it is recommended that future risk assessments of aflatoxin contamination take on a climatic approach and consider a wider geographic distribution.

iv. We do not yet have a comprehensive understanding of $AFB_1$ transformation in natural environments. Original studies from more than ten years ago found that when $AFB_1$ enters the soil, it is transformed by soil microbes into less toxic transformation products, namely $AFB_2$, $AFG_1$ and $AFG_2$, in a relatively short time (Accinelli, 2008). However, subsequent studies found $AFB_{2a}$ as the major transformation product (Starr et al. 2017) in addition to the other less toxic forms. $AFB_{2a}$ has DNA-binding capacity and is more polar than the other transformation products, suggesting a higher possibility of reaction with other molecules and an increased leaching potential in natural settings. The genotoxic and cytotoxic potential of the transformation products may still threaten the functional ability of soil organisms even after microbial degradation (Krifaton et al. 2011). There is insufficient information about the genotoxic effects of aflatoxins on soil organisms.

v. Several soil microbes (bacteria, fungi and yeasts) have been identified to effectively degrade aflatoxins in the soil environment, yet the potential of other soil organisms to regulate aflatoxins remains unclear and warrant further research.

### 6.2. Ecotoxicological effects of aflatoxins on earthworms under different temperature and moisture conditions

Earthworms play an important role in supporting soil functions, soil biodiversity and providing, regulating, and maintaining various ecosystem services. Potential negative effects for earthworms will have negative effects on soil health. This phase of the study investigated the potential effects of aflatoxins on earthworms in a soil medium. Complementary investigations that included life-cycle and mechanistic type biomarkers across a range of soil climatic conditions were conducted to make better-informed conclusions about the possible risks of aflatoxin exposure to soil organisms. Work from this phase of the study was submitted to the journal "Toxins" for review in November 2021 accepted for publication in December 2021 and published online in January 2022.

Fouché TC, Claassen S, Maboeta MS (2022) *Toxins 2022, 14(2), 75* *https://doi.org/10.3390/toxins14020075*

The first objective was to *assess if aflatoxin affects earthworms' survival, growth, and reproduction using a standard OECD test.* The null hypothesis that aflatoxin does not cause a difference in the earthworm life-cycle processes could not be rejected based on the observation that earthworms exposed to aflatoxins at environmentally relevant concentrations (10 – 100 µg/kg) did not demonstrate any negative effects on survival, growth or reproduction during standard testing conditions (Chapter 4). However, previous studies observed significant negative effects on earthworm physiology during a contact paper test with higher concentrations. It was concluded that higher concentrations in a soil medium might still negatively affect earthworm demographic processes.

The second objective *assessed the genotoxicity of aflatoxins to earthworms using the comet assay*. The null hypothesis that aflatoxin exposure at environmentally relevant concentrations does not cause significant DNA damage in earthworms was rejected. Significant DNA damage was indicated at higher exposure concentrations but not at low concentrations.

The third objective of the study *evaluated if different temperatures (21 °C and 26 °C) and soil moisture conditions (30% and 60% of soil water holding capacity) affect the toxicity of aflatoxins to earthworms*. The null hypothesis that soil climatic conditions do not significantly contribute to the toxicology of $AFB_1$ to earthworms was rejected. Changes in soil climate conditions, especially a decrease in moisture, significantly affected the population performance in terms of reproduction and genotoxicity. Increased temperatures alone generally had a positive effect on the population performance. However, the interaction of moisture with temperature significantly affected the exposure effect outcomes because statistically significant ($p < 0.001$) negative effects were indicated in the earthworm reproduction and DNA damage. There was also a significant increase in DNA damage and decreased reproduction due to the interactive effect of decreased moisture with increased aflatoxin concentration.

## 6.3. Biological control of aflatoxins in soil by earthworms

Numerous researches have investigated the biological control of aflatoxins by soil microorganisms, and several bacterial and fungal species have been identified that effectively degrade aflatoxins in soil. However, the aflatoxin degradation potential of other soil organisms involved during decomposition is not frequently reported. The activity of earthworms affects many essential soil processes and act as essential biological regulators of plant pathogens. Previous research reports earthworms' role in the degradation of another type of mycotoxin, deoxynivalenol (Wolfarth et al. 2016), but no literature is available on the role of earthworms in the degradation of aflatoxins in soil.

The first objective *investigated if the presence of earthworms improves the aflatoxin degradation potential in soil*. The percentage decrease in aflatoxin concentrations from the initial spiked concentrations were monitored over four weeks. The null hypothesis that the presence of

earthworms does not significantly decrease the aflatoxin concentrations in soil was accepted because observations did not find significant differences in the percentage decreased between earthworm and non-earthworm treated soil at standard testing conditions. At more favourable conditions (increased temperatures and sufficient moisture), the presence of earthworms significantly improved aflatoxin degradation, possibly due to increased burrowing activity and the stimulation of specific soil bacteria.

The second objective investigated if *different temperature and moisture conditions affect the earthworms' ability to degrade aflatoxins in soil.* The null hypothesis that soil climatic conditions do not significantly affect their degradation ability was rejected because improved aflatoxin degradation was observed in the earthworm treated soil at increased temperatures. There was also significantly improved aflatoxin degradation in the earthworm treated soil at increased moisture at the low concentrations. Interacting effects between temperature and moisture were not observed in the earthworm treated soil, but an interaction of temperature with increased concentrations was indicated.

Aflatoxin concentrations were monitored in the earthworm and non-earthworm treated soil, and increased degradation was observed at the higher concentrations, but the rate of degradation was slower in the soil with low moisture. During drought conditions, the soil environment does not only be have an increased risk of toxin production by fungi (Medina et al. 2017), but also has an increased risk of prolonged exposure due to the slower aflatoxin degradation rate.

### 6.4. Research highlights and key contributions of the study

This research highlighted several gaps in the knowledge about the consequences of aflatoxin contamination in the soil environment. Under changing climate conditions, aflatoxin contamination cannot be considered a low risk in soil ecosystems. Results indicated the influence of changes in temperature and moisture on the exposure effect outcomes of aflatoxin in soil and the importance of reviewing standard testing conditions to include broader environmental conditions. It highlighted the possible risk of environmentally relevant aflatoxin levels to the functional ability of important soil organisms, specifically earthworms. Finally, it highlighted the potential of earthworms to contribute to the biological control of aflatoxins under favourable environmental conditions.

### 6.5. Recommendations for further research

Although ELISA is a reliable and recognised method for aflatoxin detection, more sensitive and reliable test methods, specific for detecting aflatoxins in soil (Albert et al. 2021), are recommended to confirm concentrations.

This study took a single-species-based ecotoxicological approach to assess the ecological risk of a natural toxin and interactions of two environmental conditions. Future studies using real contaminated agricultural soil will be valuable for predicting aflatoxins' effect in the natural environment. Investigations that allow for the assessment of community-level effects due to species interactions are recommended and will add value to this body of knowledge. Further, it will be valuable to characterise the soil microbiome associated with aflatoxin degradation under these same climatic variables and in the presence and absence of earthworms.

The scope of this study did not allow the mechanisms of toxicity to be investigated, and the application of profiling based techniques (metabolomics) would complement and validate the biomarkers that were used. Therefore, further investigations are recommended to assess aflatoxin toxicity on a suite of earthworm metabolites involved in cellular processes such as growth, cellular proliferation, DNA stability, cell death, and the production of amino acids and proteins.

## 6.6. References


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# ANNEXURE A – NWU ETHICS APPROVAL

NORTH-WEST UNIVERSITY
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## ETHICS APPROVAL LETTER OF STUDY

Based on approval by the **Faculty of Natural and Agricultural Sciences Ethics Committee (FNAS-REC),** the Faculty of Natural and Agricultural Sciences Ethics Committee hereby **approves** your study as indicated below. This implies that the North-West University Senate Committee for Research Ethics (NWU-SCRE) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

**Study title: Ecotoxicological assessment of aflatoxin in soil under different temperature and moisture conditions utilising earthworms**
**Study Leader/Supervisor: Prof M Maboeta**
**Student: TC Fouche**

**Ethics number:** N W U - 0 1 5 3 2 - 2 0 - A 9
Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

**Application type: Single** **Risk Category:** Minimal

**Commencement date: 01/02/2020**
**Expiry date: 31/12/2022**

**Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt and review of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.**

Special in process conditions of the research for approval (if applicable):

- The following documentation are archived by FNASREC and should be complete and kept up to date:
  - Research proposal
  - Signed approval from the scientific committee indicating the proposed risk category
- All researchers involved in the study should submit signed NWU code of conduct statements annually.
- All researchers of low risk studies should submit proof of relevant ethics training every two years.
- All researchers that take part in activities that pose a safety and security threat to the researchers or the environment should submit a risk assessment form annually.
- All research involving human interaction should follow best ethical practise and keep documents as proof. This includes informed consent, questionnaires, incorporation of risk-benefit, and responsible data management.
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the FNASREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

# ANNEXURE B- UNISA ETHICS APPROVAL

UNISA university of south africa

**UNISA-CAES HEALTH RESEARCH ETHICS COMMITTEE**

Date: 27/08/2020

NHREC Registration # : REC-170616-051
REC Reference # : 2019/CAES_HREC/141
Name : Ms T Fouche
Staff #: 90207939

Dear Ms Fouche

**Decision: Ethics Approval Renewal after First Review from 01/08/2020 to completion**

---

**Researcher(s):** Ms T Fouche
fouchtc@unisa.ac.za

**Working title of research:**

Ecotoxicological effect of aflatoxin on earthworms (*Eisenie andrei*) under different temperature and moisture conditions

**Qualification:** Staff application

---

Thank you for the submission of your progress report to the Unisa-CAES Health Research Ethics Committee for the above mentioned research. Ethics approval is renewed until the completion of the project, **subject to submission of yearly progress reports**. **Failure to submit the progress report will lead to withdrawal of the ethics clearance until the report has been submitted.**

**Due date for progress report: 31 July 2021**

*The **minimal risk application** was **reviewed** by the UNISA-CAES Health Research Ethics Committee on 01 August 2019 in compliance with the Unisa Policy on Research Ethics and the Standard Operating Procedure on Research Ethics Risk Assessment.*

The proposed research may now commence with the provisions that:

1. The researcher will ensure that the research project adheres to the relevant guidelines set out in the Unisa Covid-19 position statement on research ethics attached.

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# ANNEXURE C – WATER HOLDING CAPACITY

Water holding capacity (WHC) calculation of OECD soil.

WHC (% of dry mass) =

$$\frac{S - D}{D} \times 100$$

Where S = weight (g) of saturated soil and D = weight (g) of dry soil.

1. Sample 1: S = 3.371 g ; D = 2.256 g

$$\therefore \frac{3.371 - 2.256}{2.256} \times 100 = 49.42\ \%$$

2. Sample 2: S = 2.243 g ; D = 1.476 g

$$\therefore \frac{2.243\ - 1.476}{1.476} \times 100 = 51.96\ \%$$

3. Sample 3: S = 3.800 g ; D = 2.523 g

$$\therefore \frac{3.8 - 2.523}{2.523} \times 100 = 50.61\ \%$$

4. Sample 4: S = 3.731 g; D = 2.508 g

$$\therefore \frac{3.731 - 2.508}{2.508} \times 100 = 48.76\ \%$$

- the mean WHC (%) per dry mass is =50.19 %
- In 600 g soil sample: 301 ml = 100% WHC

i. 30% WHC – *representative of dry soil*

301 x 0.30 = 90 ml / 600 g soil.

ii. 50% WHC – *representative of standard soil moisture*

301 x 0.50 = 150 ml / 600 g soil.

# ANNEXURE D- FRONTPAGE OF PUBLICATION IN MYCOTOXIN RESEARCH

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REVIEW

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# Aflatoxins in the soil ecosystem: an overview of its occurrence, fate, effects and future perspectives

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**Abstract**
Aflatoxins are secondary metabolites produced by specific strains of fungi, especially *Aspergillus* spp. These natural toxins are mainly found in soil, decaying vegetation and food storage systems and are particularly abundant during drought stress. Aflatoxin contamination is one of the most important threats to food safety and human health due to its toxic, mutagenic and carcinogenic properties. Therefore, most research focuses on post-harvest contamination of aflatoxins in feed and food commodities but very limited information is available about aflatoxin contamination and its toxicological consequences in the soil ecosystem. Current regulations provide minimal options for the disposal of aflatoxin-contaminated crops, amongst which is the incorporation of residues into the soil for natural degradation. This form of mycotoxin loading into the soil could potentially change its physico-chemical characteristics and biotic parameters. Recent studies suggest that as climate conditions change, the occurrence and geographical distribution of aflatoxins might increase, posing significant health risks to the soil ecosystem, food crop production and human health. This review will focus on studies that look at the environmental and toxicological consequences of aflatoxin contamination with the aim of clarifying the risk that aflatoxin contamination poses to soil ecosystems. Many aspects of aflatoxin occurrence, degradation and the effects of its transformation products in the soil environment are still unknown and remain an important area of research for soil health and productivity. A climatic approach, in terms of changes in soil moisture and air temperature, is important for future risk assessments of aflatoxin contamination.

**Keywords** Aflatoxins · Ecotoxicology · Soil · Climate change · Food safety

## Introduction

Mycotoxins are natural toxins produced by fungi as secondary metabolites during their metabolic processes (Bennet and Klich 2003). Aflatoxins are a class of mycotoxins produced by specific strains of fungi, especially *Aspergillus flavus* and *Aspergillus parasiticus*. Of the more than 20 different types identified, aflatoxins B1 ($AFB_1$), B2 ($AFB_2$), G1 ($AFG_1$) and G2 ($AFG_2$) are of most concern due to its impact on human and animal health (Zain 2011), and food safety (Bradford et al. 2018). Some of the most severe public health problems caused by aflatoxins include cytotoxic effects on blood cells (Iheanacho 2015), autoimmune disorders and cancer (Ray et al. 1991; Theumer et al. 2018) and have been studied extensively since the 1960s when it first became a concern (Escrivá et al. 2017). In addition to the public health concerns posed by aflatoxins, its presence can also have serious economic implications for food and feed crop farmers, especially when producing maize and peanuts, and the related processing industries (Krifaton et al. 2011). According to the Food Safety Digest report by the World Health Organization (WHO 2018), an estimated 25% of the world's food crops are destroyed annually due to aflatoxin contamination, which results in annual income losses of millions of dollars for producers and traders of edible crops (Guchi 2015).

Aflatoxins most commonly occur in soil, decaying vegetation such as compost material and food storage systems. Aflatoxins are introduced into the soil environment when contaminated plant residues are left to decompose (Accinelli et al. 2008) or when contaminated food from storage systems is worked back into the soil for natural degradation. These foodborne mycotoxins have the potential to contaminate

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*toxins*

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# Ecotoxicological Effects of Aflatoxins on Earthworms under Different Temperature and Moisture Conditions

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**Abstract:** Aflatoxin contamination remains one of the most important threats to food safety and human health. Aflatoxins are mainly found in soil, decaying plant material and food storage systems and are particularly abundant during drought stress. Regulations suggest the disposal of aflatoxin-contaminated crops by incorporation into the soil for natural degradation. However, the fate and consequences of aflatoxin in soil and on soil organisms providing essential ecological services remain unclear and could potentially pose a risk to soil health and productivity. The protection of soil biodiversity and ecosystem services are essential for the success of the declared United Nations Decade on Ecosystem Restoration. The focus of this study was to investigate the toxicological consequences of aflatoxins to earthworms' survival, growth, reproduction and genotoxicity under different temperature and moisture conditions. Results indicated an insignificant effect of aflatoxin concentrations between 10 and 100 µg/kg on the survival, growth and reproduction but indicated a concentration-dependent increase in DNA damage at standard testing conditions. However, the interaction of the toxin with different environmental conditions, particularly low moisture, resulted in significantly reduced reproduction rates and increased DNA damage in earthworms.

**Keywords:** aflatoxins; earthworms; soil ecotoxicology; soil moisture; temperature; climate change

**Key Contribution:** Results indicate the influence of temperature and moisture changes on the exposure effect outcomes of aflatoxin in soil. It highlights the possible risk of environmentally relevant aflatoxin levels to the functional ability of important soil organisms for providing essential ecosystem services.

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## 1. Introduction

Fungal toxins (mycotoxins) are often toxic to plants, animals and humans and are a common threat to food safety. Of the more than 400 types of mycotoxins, aflatoxins are considered to be the most toxic and carcinogenic. Exposure to aflatoxin $B_1$ ($AFB_1$) poses a significant health risk for humans [1,2] and other living organisms, including plants [3,4], mammals [5], birds [6], insects [7–9] and fish [10]. Most countries in the world regulate aflatoxin concentrations in food and feed products. When aflatoxins reach concentrations exceeding the accepted levels, regulations suggest that contaminated food products are discarded by burning or working the material back into the soil for natural degradation [11]. When contaminated crops are worked into the soil, it increases natural concentrations and prolongs the duration of contamination due to the gradual release of the toxin [12]. Moreover, increased concentrations can alter the ecological balance, potentially posing a risk to soil health.

So far, only a few studies have investigated the consequences of aflatoxins for soil organisms [12]. The exposure of soil organisms to toxins in the soil is influenced by various mechanisms such as adsorption and release from the soil binding sites, interactions with