

Assessing the effects of biochar as an ameliorant for biopesticide polluted soils utilising earthworms

HH Smith



orcid.org/0000-0002-1952-917X

Dissertation accepted in fulfilment of the requirements for the degree *Master of Science in Zoology* at the North-West University

Supervisor: Prof MS Maboeta

Co-Supervisor: Dr OA Idris

Graduation May 2023

24966622

Preface

Attached in Annexure A is the full article (with reference list), that is integrated into the thesis, as published in the Journal of Toxicology.

Acknowledgements

First and most importantly, all the glory to God for His grace, without Him nothing is possible.

Second, I dedicate my Master's degree to Mrs. Elsabé Thiart, forever in our hearts.

I would like to thank my supervisor Prof Mark Maboeta for his patience, guidance, support and words of encouragement, I am eternally grateful for everything.

My co-supervisor Dr Idris Oladayo for going above and beyond to help me whenever I needed it, I truly appreciate your time and effort.

Mr. Owen Rhode and Ms. Connie Abrams from the Agricultural Research Council, for all their help with the soil enzymatic activity.

A special thanks to my girlfriend Teneal Nel for all your support and words of encouragement, I appreciate every cup of coffee and visit to lighten my spirit.

To my friends Elsje Kemp and Christo Botes, thank you for always being an outlet and listening to my silly problems, it is much appreciated.

My co-masters student and friend Hussain Kaka, thanks for all your help with the laboratory work, our SS lunch breaks in your bakkie and endless conversations and jokes.

My parents, Hein and Ita Smith, and my brothers thank you for all the support, encouragement and patience throughout my studies.

Abstract

Two plant biopesticides, *Bidens pilosa* L. and *Tagetes minuta* L., were investigated for possible effects on earthworms and soil enzymatic activity, compared to the conventional pesticide, Fenamiphos. The efficacy of biochar as an ameliorant was also investigated to determine if the possible effects of plant biopesticides could be mitigated.

The growth and reproduction of earthworms were monitored at sublethal endpoints and the genotoxicity of the plant biopesticide extracts was evaluated using the comet assay. The influence of the test substance in soil on earthworm behaviour was evaluated with an avoidance behaviour assay. The relative growth rate (%RGR) was determined by the change in biomass of the earthworms at 28 days of the experiment. Fecundity success was measured by counting the number of cocoons and the number of juveniles on day 56. The DNA damage in earthworm coelomocytes was evaluated using single-cell gel electrophoresis, by scoring the tail length of the comet formed because of the deoxyribonucleic acid (DNA) strand breaks in the cell. The soil enzyme activities were evaluated by utilising the alkaline phosphatase, β -glucosidase and urease assay, and analysed using the spectrophotometer method. Artificial soil used for all treatments was prepared according to the OECD standard guidelines.

The soil treated with Fenamiphos had a greater negative effect on earthworm relative growth rate (%RGR) and reproduction than both plant biopesticides (*B. pilosa* and *T. minuta*). Biochar amended treatments, had the greatest effect on earthworm %RGR. However, there was no significant difference in earthworm reproduction in the biochar supplemented treatments compared to treatments without biochar.

Mixed results were obtained in the DNA damage of the earthworms caused by the plant biopesticides (*B. pilosa* and *T. minuta*). Overall, *B. pilosa* exposures induced more DNA strand breaks than *T. minuta* exposures, except for the 1000 mg/kg *T. minuta* concentration, which surprisingly has very high DNA strand breaks, with an average tail length of 49 μ m. Fenamiphos induced more DNA strand breaks at much lower concentrations (12.5 – 100 mg/kg) than the plant biopesticides. All the treatments supplemented with biochar showed no DNA damage and had no significant difference ($p < 0.05$) from the control, suggesting biochar prevented DNA damage.

There were no statistical differences among all treatments (*B. pilosa*, *T. minuta*, and Fenamiphos), including the control of the alkaline phosphatase and urease activities in the soil. There is an increase in p-nitrophenol released in Fenamiphos-treated soil (25 and 100 mg/kg) and, surprisingly, *T. minuta* (250 mg/kg) indicating an increase in β -glucosidase activity. All treatments (*B. pilosa*, *T. minuta*, and Fenamiphos) supplemented with biochar have no significantly different soil enzymatic activity (alkaline phosphatase, β -glucosidase, and urease activity) compared to the control. In conclusion, Fenamiphos reported more distinct negative effects on soil organisms than the plant biopesticides used in this study, and biochar does not act as an efficient ameliorant for the plant biopesticides (*B. pilosa* and *T. minuta*), and the chemical pesticide (Fenamiphos).

Keywords: Plant biopesticides, Fenamiphos, biochar, earthworm biomarkers, comet assay, soil enzymatic activity

Table of contents

Preface.....	i
Acknowledgements	ii
Abstract	iii
Table of contents.....	v
List of figures.....	viii
List of tables	ix
Chapter 1: Introduction and background to the study.....	1
1.1 Background of study	1
1.2 Introduction	2
1.2.1 Plant biopesticides	2
1.2.2 Biochar	4
1.3 Problem statement	5
1.4 Study justification	5
1.5 Aim and objectives	6
1.6 Chapter layout:	7
Chapter 2: Literature review	8
2.1 Introduction to biopesticides.....	8
2.2 Global trends of plant biopesticides	9
2.3 Advantages of biopesticides.....	11
2.4 Constraints of biopesticides	12
2.5 <i>Bidens pilosa</i>	13
2.5.1 Phytochemical properties of <i>B. pilosa</i>	13
2.5.2 Efficacy of <i>B. pilosa</i> for pest management	15
2.6 <i>Tagetes minuta</i>	15
2.6.1 Phytochemical properties of <i>T. minuta</i>	15
2.6.2 Efficacy of <i>T. minuta</i> for pest management.....	16

2.7 Mode of action of plant biopesticides	17
2.8 Fenamiphos	17
2.9 Biochar	18
2.10 Conclusion	18
Chapter 3: Materials and methods	20
3.1 Global trends of plant biopesticides: Bibliometric analysis	20
3.2 Experimental design.....	20
3.3 Plant material, collection and authentication	22
3.3.1 Plant extract	23
3.4 Artificial soil (test substrate)	23
3.5 Test organisms.....	24
3.6 Synthetic pesticide	24
3.7 Ecotoxicological studies	25
3.7.1 Acute toxicity using the contact filter paper test	25
3.7.2 Acute toxicity using the artificial soil test	25
3.7.3 Earthworm avoidance test.....	26
3.7.4 Earthworm growth and reproduction test	27
3.8 Comet assay	27
3.9 Soil enzyme analysis.....	29
3.9.1 β -glucosidase	29
3.9.2 Alkaline phosphatase	30
3.9.3 Urease	31
3.10 Statistical analysis	32
Chapter 4: Results.....	33
4.1.1 Green pesticide research production	33
4.1.2 Keywords on green pesticide research	35
4.1.3 Productivity on green pesticide research per country.....	37

4.1.4 Most cited documents on green pesticides	39
4.2 Ecotoxicological assays	42
4.2.1 Contact filter paper test	43
4.2.2 Artificial soil test	44
4.2.3 Avoidance behaviour assay	45
4.2.4 Earthworm growth	47
4.2.5 Reproduction.....	49
4.2.6 Comet assay	51
4.2.7 Soil enzymatic activity	53
Chapter 5: Discussion	58
5.1 Green pesticide research	58
5.2. Contact filter paper test	59
5.3 Artificial soil test	60
5.4 Avoidance behaviour assay	60
5.5 Earthworm growth	61
5.6 Reproduction.....	63
5.7 Comet assay	64
5.8 Soil enzymatic activity	65
Chapter 6: Conclusions and recommendations.....	68
6.1 Conclusions.....	68
6.2 Recommendations	70
References.....	71
Annexure A	81
Annexure B	92
Annexure C	93

List of figures

Figure 1: A) Aerial segment, B) flowers and seeds of <i>Bidens pilosa</i>	3
Figure 2: A) Aerial segment, B) leaves and C) flowers of <i>Tagetes minuta</i>	4
Figure 3: Summary of the experimental design to determine the effect of pesticides on earthworms and the efficacy of biochar as an ameliorant.	21
Figure 4: The location of the sampling site of plant species <i>B. pilosa</i> (24° 19' 17" S, 29° 11' 1" E) and <i>T. minuta</i> (24° 19' 18" S, 29° 11' 3" E) in Limpopo, South Africa..	22
Figure 5: Annual scientific publications of documents on biopesticides from 1994 to 2019.	44
Figure 6: Keyword co-occurrences network of the top 30 keywords on green pesticide research. The circle size of the keywords represents the frequency of occurrence in articles. The thickness of the line between any two keywords displays the degree of cooperation. Co-occurrence was used to measure cooperation.	45
Figure 7: Most productive countries and collaboration in the research of green pesticides from 1994 to 2019.	46
Figure 8: The percentage mortality of <i>E. andrei</i> in the contact filter paper test after 48 h exposure with different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos. Different letters (a-c) represent significant difference ($p < 0.05$).	44
Figure 9: Avoidance response (%) of <i>E. andrei</i> after exposure to different concentrations of <i>B. pilosa</i> over a 48 h period. (n=10).	45
Figure 10: Avoidance response (%) of <i>E. andrei</i> after exposure to different concentrations of <i>T. minuta</i> over a 48 h period. (n=10).	46
Figure 11: Avoidance response (%) of <i>E. andrei</i> after exposure to different concentrations of Fenamiphos over a 48 h period. (n=10).	47
Figure 12: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of <i>B. pilosa</i> treatments at the end of the 56-day time period. Error bars indicate the standard deviation of the mean. (n=3). No significant difference ($p < 0.05$) occurred.	50
Figure 13: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of <i>T. minuta</i> treatments at the end of the 56-day time period. Different letters (a-b) indicate significant difference ($p < 0.05$). Error bars indicate the standard deviation of the mean. (n=3).	50

Figure 14: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of Fenamiphos treatments at the end of the 56-day time period. Different letters (a-c) indicate significant difference ($p<0.05$). Error bars indicate the standard deviation of the mean. (n=3).....	51
Figure 15: The mean comet tail length of coelomic cells of <i>E. andrei</i> after exposure to different concentrations of <i>B. pilosa</i> (A), <i>T. minuta</i> (B) and Fenamiphos (C) with and without biochar for 28 days. Different letters (a-d) represent the significant difference ($p<0.05$) between treatments with and without biochar. Error bars indicate the standard deviation of the mean. (n=9).....	52
Figure 16: Fluorescence microscope images of earthworm coelomic cells observed during the comet assay. Image A shows the control group with intact DNA and image B shows the damaged DNA (taken from the 100 mg/kg Fenamiphos treatment). Image A taken at 10x magnification and Image B at 100x magnification.	53
Figure 17: Enzymatic analyses of the alkaline phosphatase activity after exposure to different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos with and without biochar. Treatments: Bp (<i>B. pilosa</i>), Tm (<i>T. minuta</i>) and F (Fenamiphos). Different letters represent the significant difference ($p<0.05$). Error bars indicate the standard deviation of the mean. (n=3).....	54
Figure 18: Enzymatic analyses of the β -glucosidase activity after exposure to different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos with and without biochar. Treatments: Bp (<i>B. pilosa</i>), Tm (<i>T. minuta</i>) and F (Fenamiphos). Different letters represent the significant difference ($p<0.05$). Error bars indicate standard deviation of the mean. (n=3).....	55
Figure 19: Urease activity after exposure to different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos with and without biochar. Treatments: Bp (<i>B. pilosa</i>), Tm (<i>T. minuta</i>) and F (Fenamiphos). Different letters represent the significant difference ($p<0.05$). Error bars indicate the standard deviation of the mean. (n=3).	56

List of tables

Table 1: Chemical structures and formulas of <i>B. pilosa</i> chemical compounds.....	14
Table 2: Chemical structures and formulas of <i>T. minuta</i> chemical compounds.....	16
Table 3: Taxonomy of <i>B. pilosa</i> and <i>T. minuta</i>	23
Table 4: Summary of data retrieved from the Scopus database	48

Table 5: Top 30 relevant keywords on green pesticide research from 1994 to 2019.	49
Table 6: Total citations per country in terms of green pesticide research from 1994 to 2019	39
Table 7: The 30 most cited documents on green pesticide research from 1994 to 2019 from Scopus.	36
Table 8: Top 30 productive journals, the total number of publications, total citations, h-index and publication start year, from 1994 to 2019.....	39
Table 9: The relative growth rate (RGR) (%) of <i>E. andrei</i> over 28 days of exposure to different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos.....	48
Table 10: The relative growth rate (RGR) (%) of <i>E. andrei</i> over 28 days of exposure to different concentrations of <i>B. pilosa</i> , <i>T. minuta</i> and Fenamiphos, with biochar (ameliorant) in all treatments.	49

Chapter 1: Introduction and background to the study

Biological indicators are often used during ecotoxicological studies to determine or predict changes in soil health (Bartlett *et al.* 2010). A common biological indicator is earthworms. Earthworms play an important role in terrestrial ecosystems by breaking down organic matter and aiding in soil formation (Lionetto *et al.* 2012), because of earthworms' interaction with soil they are greatly affected by pollutants present in the soil. Contaminants present in the soil can be absorbed by the earthworm's permeable skin, and also ingested due to their feeding habits (Lionetto *et al.* 2012). Due to the close interactions between earthworms and the soil they are exposed to, they are regarded as important biological indicators for changes in soil health.

1.1 Background of study

The global population is increasing at a rapid pace and with it the demand for food production. The agricultural sector faces a number of difficulties such as pests, weeds and pathogens that causes yield losses (Popp *et al.* 2013). The most common technique used for crop protection is the use of pesticides (Popp *et al.* 2013). Pesticides provide a way to ensure the quantity and quality of crops as well as the management of pests such as rodents and insects (Quinn *et al.* 2011). According to Aktar *et al.* (2009) the term pesticides include a number of compounds: herbicides, insecticides, fungicides, molluscicides, rodenticides and nematicides. Using pesticides on a regular basis has detrimental effects on beneficial soil organisms and the environment (Quinn *et al.* 2011) and have a direct impact on the human population as well. During the production of pesticides workers in these facilities are exposed to toxic chemicals, while the people applying the pesticides on farms are also exposed to the toxic chemicals (Aktar *et al.* 2009).

Exposure to pesticide residues poses further risk to human health, and can occur through various pathways (Boobis *et al.* 2008) at different stages of crop production. Pesticide residues can be found in food and drinking water. With South Africa being the largest consumer of pesticides in sub-Saharan Africa, the occurrence of pesticide residues in water resources poses a big problem (Dabrowski *et al.* 2014). The indiscriminate use of pesticides affects soil fertility as it negatively affects the beneficial soil organisms that many plants depend on to transform atmospheric nitrogen into

nitrites (Aktar *et al.* 2009). Although the use of pesticides is an effective method for pest management, it contributes to air, soil and water pollution, which has a negative impact on non-target organisms. The cost of reversing the impacts of chemical pesticides is significant, and one of the various approaches is the usage of biochar (Khorram *et al.* 2016). The multiple detrimental effects of pesticides on the environment and human health have escalated the demand for a more environmental friendly substitute (Walia, 2017). Biopesticides are a promising alternative to chemical pesticides, with plant biopesticides being one of the most preferred, and several countries are working to enhance biopesticide production and use (Miresmailli and Isman, 2014).

1.2 Introduction

Biopesticides are a possible natural substitute for synthetic pesticides. According to Dimetry (2014) the term biopesticide includes a wide array of pest control methods such as microbial organisms, plant-derived pesticides, secondary metabolites, insect pheromones applied for mating disruption, genes used to make crops resistant to insect, fungi and viral diseases. Studies done by Pavela and Benelli (2016) has shown promising results when using essential oils against pest arthropods. According to Walia (2017), biopesticides derived from plants are potentially environmental friendly; less toxic, less persistent and not harmful to non-target organisms, which makes it a promising substitute for synthetic pesticides. The plant-derived biopesticide (extracts) from *Bidens pilosa* and *Tagetes minuta*, proved to be an effective insecticide against Mexican bean weevils and the cowpea beetle (Goudoum *et al.* 2016). *B. pilosa* and *T. minuta* are the chosen plant species for this study. Biopesticides are generally known to be biodegradable, pose minimum threat to human health and targeted pests do not develop resistance due to several bioactive substances in biopesticides (Dimetry, 2014).

1.2.1 Plant biopesticides

Plant biopesticides are increasingly gaining ground in food production. However, the use of plant products for pest management is not a new concept as there is a long

history of herbs that were used against pests in China (Liu *et al.* 2007). Other countries such as Egypt, Greece, India and the United States have also been using botanical insecticides before synthetic pesticides were discovered (Pavela, 2014). Given all the advantages of plant biopesticides it is worth further research as a probable substitute for conventional synthetic pesticides.

B. pilosa is a broadly distributed erect, perennial herb with serrated green opposite leaves, white flowers and black long narrow ridged seeds (Kuo *et al.* 2021) (Figure 1). It is found across most temperate and tropical regions and is believed to have originated in South America (Yang, 2014).



Figure 1: A) Aerial segment, B) flowers and seeds of *Bidens pilosa*.

T. minuta is an annual perennial herb, belonging to the Asteraceae family and is native to South America (Garcia *et al.* 2012). At first the stems are green but turns brownish or yellowish after flowering and the leaves are pinnately dissected into four or six pairs of pinnae. This aromatic plant produces clusters of dull yellow flowers and has a wild-citrus-like smell (Batish *et al.* 2007) (Figure 2).

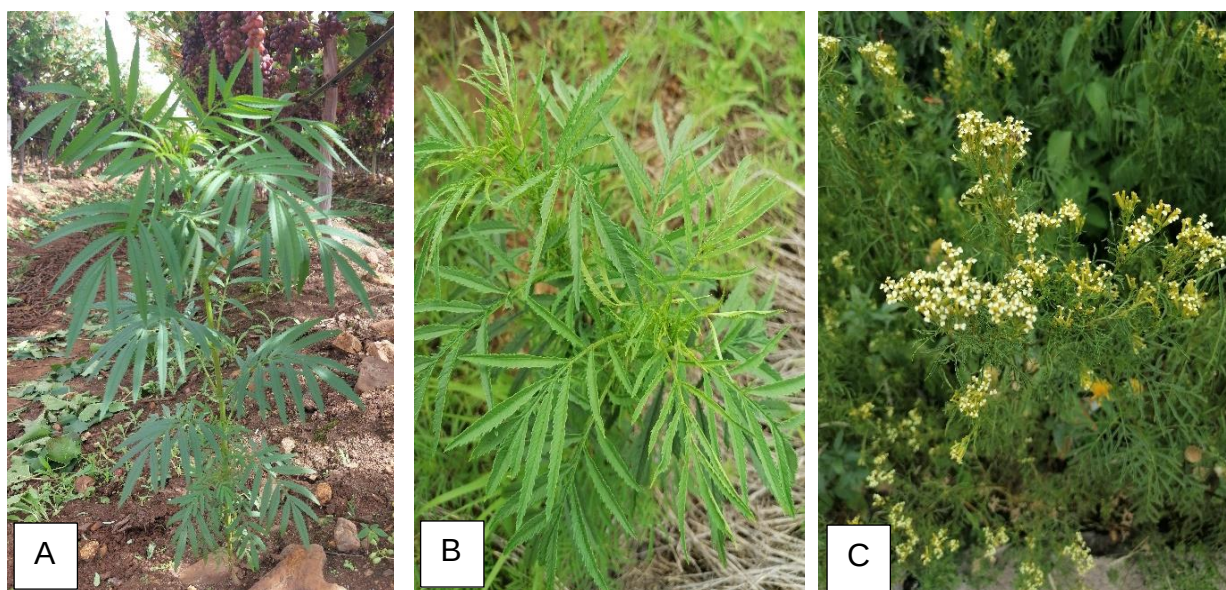


Figure 2: A) Aerial segment, B) leaves and C) flowers of *Tagetes minuta*.

1.2.2 Biochar

The use of biochar is a highly recommended technique for polluted soil restoration (Beesley *et al.* 2011). According to Zhu *et al.* (2017), biochar is “a carbon-rich solid that is produced by the pyrolysis of biomass in oxygen-limited conditions.” According to Gomez-Eyles *et al.* (2011), the use of biochar for soil amendment has a number of benefits such as carbon sequestration and the potential to reduce the bioavailability of organic and inorganic contaminants in soil. Biochar and other carbonaceous sorbents have the ability to reduce the bioavailability of organic contaminants in soil and can also decrease dissolved organic carbon as well as increase soil pH and cation exchange capacity.

According to Khorram *et al.* (2016), one of the most attracting qualities of biochar is its ability to reduce pesticide bioavailability. Biochar has the ability to adsorb several pesticides in soil which in turn can affect other processes such as bioavailability, leaching and ecotoxicological effects on non-target organisms (Varjani *et al.* 2019). The addition of biochar to soil enhances the adsorption and decreases the desorption of pesticides, which alters their mobility (Khorram *et al.* 2016). This means that biochar decreases leaching of pesticides in soil by entrapping the pesticide residues in the biochar particles. With biochar’s ability to increase adsorption and decrease pesticide

dissipation it typically lowers environmental pollution and risk of human exposure (Varjani *et al.* 2019).

1.3 Problem statement

Synthetic pesticides can accumulate in soil and have detrimental effects on soil health (Quinn *et al.* 2011). The natural substitute for synthetic pesticides, plant biopesticides, are more environmental friendly (Dimetry, 2014) but might still have effects on the environment. The effects plant biopesticides might have on the environment can be determined, as proposed by this study, by using earthworms as bioindicators for soil treated with the biopesticides. The bioremediating ability of biochar has been investigated by different researchers and was proven to be effective in decreasing metal mobility (Gomez-Eyles *et al.* 2011), but this has not been tested on plant biopesticide spiked soil. The efficacy of biochar as an ameliorant needs to be investigated by determining how effective it can rehabilitate the soil spiked with plant biopesticides.

1.4 Study justification

The detrimental effects of synthetic pesticides on the environment and beneficial soil organisms are well established. The use of plant biopesticides have been tested and confirmed to be efficient against insects, insect larvae, plant pathogens and soil nematodes. Although plant biopesticides are a promising alternative for pest management in agriculture, the threat posed by the indiscriminate use thereof in the environment cannot be ignored and needs to be investigated.

This study will provide valuable information regarding the effect of biopesticides, extracted from *B. pilosa* and *T. minuta*, on soil organisms (model organism, the earthworm *Eisenia andrei*) as well as the efficacy of biochar as an ameliorant for spiked soils.

1.5 Aim and objectives

Aim

This study aims to determine the effects of biopesticide spiked soils on *E. andrei* compared to the synthetic pesticide Fenamiphos, as well as the efficiency of biochar as a mean of remediation for the pesticide spiked soils.

Objectives

- i. Analyse the global research activities on botanical pesticides from 1994 to 2019 in the Scopus database by studying the research progression using bibliometric parameters.
- ii. Determine the toxicity of *B. pilosa* and *T. minuta* extracts on earthworms (*E. andrei*) using the filter paper and artificial soil assay according to the 207 OECD protocol (OECD, 1984).
- iii. Determine the effects of *B. pilosa* and *T. minuta* extracts on spiked soil and earthworms (*E. andrei*) and compare the effects to the synthetic pesticide Fenamiphos, using the standardized OECD 222 protocol (OECD, 2004).
- iv. Determine the DNA damage of *E. andrei* coelomic cells by using the comet assay as a biomarker for molecular damage caused by exposure to pesticides (*B. pilosa*, *T. minuta* and Fenamiphos).
- v. Evaluate the efficacy of biochar as an ameliorant for pesticide (*B. pilosa*, *T. minuta* and Fenamiphos) polluted soil using the standardized OECD 222 protocol (OECD, 2004).
- vi. Determine the soil enzymatic activities in soils spiked with *B. pilosa*, *T. minuta* and Fenamiphos, and the soil remediated with biochar, using Phosphatase, β -Glucosidase and Urease tests.

1.6 Chapter layout

Chapter 1: General introduction and background to the study – An overview of biopesticides and the test species for this study (*B. pilosa* and *T. minuta*) is given as well as the detrimental effects of pesticide use and possible remediating abilities of biochar on polluted soil.

Chapter 2: Literature review – An in-depth review of existing literature entailing the environmental impact of pesticides and biopesticides on the soil environment as well as on soil organisms (earthworms). The role of biochar in the rehabilitation of pesticide-polluted soil was also reviewed. Part of the literature review has been published in the Journal of Toxicology (Smith *et al.* 2021), which is included in this chapter.

Chapter 3: Materials and methods – The chapter describes the methodology used during this study. This includes the basis for the selection of the plant species (*B. pilosa* and *T. minuta*) used as biopesticides, outlines the experimental design and protocols used for this study.

Chapter 4: Results – The results obtained from the study are tabulated, graphically presented, and comprehensively discussed.

Chapter 5: Discussion – The results are comprehensively discussed using relevant studies from existing literature to support the findings.

Chapter 6: Conclusions and recommendations – Conclusions for the aims of this study are provided along with recommendations for future studies based on the findings of the current research.

References: A combined list of references for all chapters.

Chapter 2: Literature review

2.1 Introduction to biopesticides

Biopesticides are defined by Sporleder and Lacey (2013) as the managing of agricultural pests by compounds with specific biological effects and are derived from natural materials. Biopesticides have recently received more attention as a more environmental friendly technique of pest management (Karunamoorthi, 2012). The term biopesticide refers to a wide range of pest management strategies, including the use of microbial organisms, plant-derived pesticides, disrupting insect mating using pheromones, crop genome editing for insect, fungal, and viral resistance, as well as herbicide tolerance (Dimetry, 2014). Furthermore the potential products obtained from biopesticides can be divided into the following categories: Microbial pesticides, plant-incorporated protectants (PIPs) and biochemical pesticides (Dimetry, 2014). Microbial pesticides are produced to target specific pests and contain microorganisms such as viruses, bacteria, or fungi. Plants that produce pesticidal substances due to genetic modification are known as plant-incorporated protectants (Liu *et al.* 2021). Biochemical pesticides are based on substances that occur naturally and use non-toxic mechanisms for pest control (Dimetry, 2014), while chemical pesticides use synthetic molecules to eliminate pests directly (Sporleder and Lacey, 2013). Biochemical pesticides can further be classified into different biologically functional classes: Pheromones, plant extracts and natural growth regulators (Sporleder and Lacey, 2013).

Plant biopesticides are considered a valuable source of crop protectants as it contains a mixture of active biological substances and are less harmful to the environment and humans (Koul *et al.* 2008). The mixture of active biological substances greatly reduces the probability for pests and pathogens to develop resistance to botanical pesticides. The use of plant biopesticides for pest management is not a new concept. According to Dimetry (2014), there are written references from Rome dating back as far as 400 B.C. where plant extracts were used for pest management. Before the discovery of synthetic pesticides in the late 1930's China, Greece, India, Egypt, the United States and some European countries used plant biopesticides (Isman, 2006) (Dimetry, 2014). Although the history of plant biopesticides is not well documented, it has been established that European countries used plants for protection against insects over 3000 years ago (Pavela, 2016).

Plants containing phytochemicals, traditionally known as secondary metabolites, have the ability of biological activity against insects (Castillo-Sánchez *et al.* 2010). A number of popular plant species which has been researched for pest control and other biological properties include lemon grass (*Cymbopogon winteriana*), basil (*Ocimum basilicum*), eucalyptus (*Eucalyptus globulus*), rosemary (*Rosmarinus officinalis*), marigold (*Tagetes erecta*), thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum zeylanicum*), lavender (*Lavandula angustifolia*) and many more (Walia, 2017). According to Karunamoorthi (2012), more than 2000 plant species, from different families, which contain potential toxic abilities to pests have been identified. Plant biopesticides cover a wide range of spectrum in pest management because they contain a mixture of phytochemicals such as flavonoids, glycosides, terpenoids, alkaloids, tannins and phenols. However, phytochemicals are primarily vital for the plant's defence and protection, which can have physiological and behavioural effects on insects (Karunamoorthi, 2012). Solvent extractions is one of the most common methods used to obtain phytochemicals, in the form of crude extracts and essential oils (Castillo-Sánchez *et al.* 2010). Due to different levels of plant extract solubility different solvents are used, including water, ethanol, acetone, chloroform, petroleum ether and hexane. Numerous plants have been researched for pest management, however, only a minimal amount have been commercialized (Mathew 2016). According to Pavela and Benelli (2016), strict legislation on the use of endangered plant species, short persistence in the environment, and a lack of quality and sufficient quantities of plant material at affordable prices are the main reasons for the low commercial manufacturing of plant biopesticides.

2.2 Global trends of plant biopesticides

The detrimental effects of synthetic pesticides on the environment are well established as it affects animal and plant biodiversity as well as terrestrial and aquatic ecosystems (Mahmood *et al.* 2016). Other effects include toxicity towards non-target organisms, soil and groundwater pollution and harmful residues that contaminate crops and food (Walia *et al.* 2017). The excessive use of synthetic pesticides also leads to increased resistance in pests. The negative effects of synthetic pesticides have created the need for a safer and more environmentally friendly substitute. Biopesticides are believed to

be less toxic, environmentally friendly and not harmful to humans and non-target organisms (Walia *et al.* 2017). The use of botanical insecticides is not a new practice. As early as the 17th century, botanical insecticides have been used in agricultural practices by ancient China, Egypt, Greece and India (Isman, 2006). Over the past decade, the interest in biopesticides has grown immensely, with the intention to substitute with synthetic pesticides. According to a study done by Isman and Grieneisen (2014), the annual papers published on botanical insecticides has grown from 61 papers in 1980 to 1207 papers in 2012 (Isman and Grieneisen, 2014). This signifies an increase in the awareness and uses of plant biopesticides. In spite of the growing need for natural pest management, plant biopesticides contribute less than 1% of the total use of pesticides (Walia *et al.* 2017). The annual amount of synthetic pesticides used for crop protection is estimated at 2.5 million tons causing damages worth about \$100 billion, due to residues in crops, soil and water, nonbiodegradable properties and high toxicity towards non-target organisms (Koul *et al.* 2008). Synthetic pesticides are no doubt a threat to public health and demands an urgent alternative with better crop protection and less harmful properties.

The term 'green pesticides' entails all naturally acquired types of pest management from plant extracts, plant-derived pesticides, plant secondary metabolites and plant-based pesticides (Koul *et al.* 2008). The green pesticides are also referred to as botanical pesticides and are used as repellents, nematocides, insecticides, fungicides and bactericides, obtained in the form of isolated substances or complex mixtures (Campos *et al.* 2018). Although there has been significant growth in botanical pesticide research, the commercial availability of these pesticides remain limited (Grumezescu, 2007). According to Isman and Grieneisen (2014), there are a few limitations of the exploitation of botanical pesticides such as lack of availability of good quality plant pesticides at affordable prices, strict regulation particularly on endangered plant species, short persistence of the phytochemical in the environment and the extraction of the phytochemical from plants grown under different climatic conditions resulting in different compositions in terms of active agents, which leads to variability in effectiveness (Isman and Grieneisen, 2014). One of the main factors preventing the advancement of commercializing botanical pesticides is sustainability (Campos *et al.* 2018) of the product. In order to produce botanical pesticides on a commercial scale, it is necessary to obtain large amounts of the source plant, which requires large scale

production (Campos *et al.* 2018). However, most biopesticide plants are still not grown commercially, making it difficult to source for the plants. Although research on botanical pesticides has increased significantly over the past two decades (Isman, 2019), the above-mentioned limitations still contribute to the lack of commercialization of botanical pesticides.

With the continuously growing interest in botanical pesticides, it is necessary to carry out a bibliometric compilation to better understand the research trend. The fast growing number of academic publications makes it extremely difficult to stay up to date with everything being published (Aria and Cuccurullo, 2017). To help organise and better understand previous research, different quantitative and qualitative literature reviewing approaches are used. Bibliometrics is one such approach and according to Aria and Cuccurullo (2017) it has the potential to perform a “systematic, transparent, and reproducible review process based on the statistical measurement of science, scientists, or scientific activity.” Various research fields make use of bibliometrics to measure the impact or influence of certain research articles. For example, in the field of Engineering (Bao *et al.* 2019) used bibliometric analysis to evaluate the publications in the field of robotic exoskeletons and (He *et al.* 2020) used bibliometrics to analyse articles related to ‘metastatic castration-resistant prostate cancer’ in the field health and medicine.

2.3 Advantages of biopesticides

Given all the negative effects of synthetic pesticides research regarding natural products and plant extracts for pest management have increased. Studies done by Pavela and Benelli (2016) has shown promising results using essential oils against arthropods. According to Walia (2017), biopesticides derived from plants are environmental friendly, less toxic, less persistent and not harmful to non-target organisms. Most secondary metabolites or phytochemicals of plants used for pest control have an insectistatic effect on insects (Castillo-Sánchez *et al.* 2010). This means it interferes with the development and behaviour of the insects. Repellent activity is also found in plants containing compounds with a foul odour or irritating effect to repel insects. Some plant compounds are capable of causing anti-feeding activity in insects after ingestion and ultimately starving the insect to death (Isman,

2006). Growth regulating compounds are also found in plants and are capable of altering the growth regulating hormones in insects which leads to sterility, malformations or death (Castillo-Sánchez *et al.* 2010). All these are advantages of plant biopesticides over synthetic pesticides, as they occur naturally, are less persistent in the environment, are suitable for organic agriculture, are less toxic to non-target organisms and mammals, are safer for farm workers during the application, and targeted organisms are less likely to develop resistance due to the complexity of the phytochemicals (Seiber *et al.* 2014).

2.4 Constraints of biopesticides

One of the biggest constraints facing plant biopesticides is efficacy when compared to synthetic pesticides. Plant biopesticides are often less effective but safer (Koul *et al.* 2008). Additionally, challenges in the commercial application of plant biopesticides include the availability of adequate plant material, standardization of plant-based biopesticide products, and regulatory approval.

While many studies focus on the efficacy of biopesticides on target organisms, only a few focus on what effects it has on non-target organisms. The study of biopesticides on non-target organisms is necessary for the development and authorization of plant-based pesticides for commercial use and production. As with any new pesticide, the procedure for authorization is complex, costly, and requires in-depth toxicological research to acquire the necessary safety documentation (Pavela and Benelli, 2016). Contact effects of plant biopesticides are considered good but biodegradation of the active ingredients occurs progressively over time which leads to low persistence of the effect (Pavela, 2016). A number of plant species show promising pesticidal properties but it is expensive and often disadvantageous to cultivate these species (Essiedu *et al.* 2020). Other factors such as soil acidity and climate also affect the secondary metabolism of plants directly which makes it challenging to acquire a standardized product.

2.5 *Bidens pilosa*

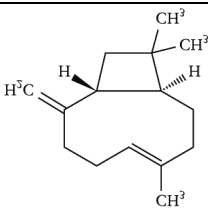
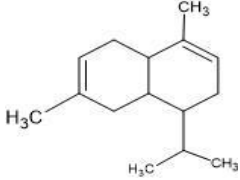
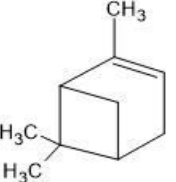
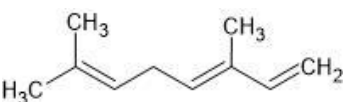
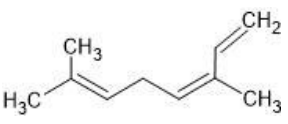
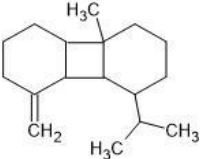
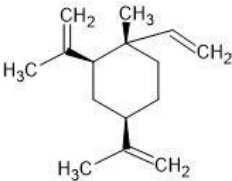
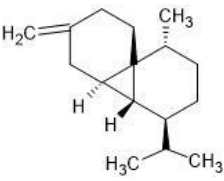
More than 230 *Bidens* species are known and are globally distributed (Bartolome *et al.* 2013). The plant has traditionally been used for food and medicine. In 1975, the Food and Agriculture Organization of the United Nations regarded this species as an edible plant (Kuo *et al.* 2021).

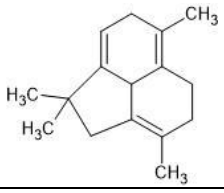
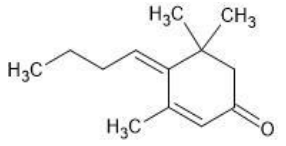
B. pilosa is traditionally used as an ingredient in teas and herbal medicines (Bartolome *et al.* 2013). The plant as a whole is often used to treat a variety of ailments, including digestive disorders, inflammation, infectious diseases, immunological disorders, metabolic syndrome and wounds. It is safe to ingest the plant but is generally used externally.

2.5.1 Phytochemical properties of *B. pilosa*

According to Xuan and Khanh (2016), the species *B. pilosa* comprises about 301 phytochemical compounds. The compounds are classified in the subsequent major chemical classes: polyacetylenes, flavonoids, phenolic acids, terpenes, pheophytins, fatty acids and phytosterols (Xuan and Khanh, 2016). According to the study done by Souza and Silva, (2013) flavonoids, saponins and tannins are the chemical compounds that have biocidal activity in plants. The chemical composition of the essential oil of *B. pilosa* revealed it has terpenes such as B-caryophyllene and τ -cadinene (Deba *et al.* 2008). Other compounds that were reported from the plant extracts include α -pinene, β -trans-ocimene, β -cis-ocimene, β -bourbonene, β -elemene, β -cubebene, α -caryophyllene and megastigmatrienone (Deba *et al.* 2008). In Table 1 the structures and formulas of the phytochemicals found in *B. pilosa* are presented.

Table 1: Chemical structures and formulas of *B. pilosa* chemical compounds

Chemical compound	Formula	Structure	Reference
β -caryophyllene	$C_{15}H_{24}$		(Xuan and Khanh, 2016)
τ -cadinene	$C_{15}H_{24}$		(Deba et al. 2008)
α -pinene	$C_{10}H_{16}$		(Deba et al. 2008)
β -trans-ocimene	$C_{10}H_{16}$		(Xuan and Khanh, 2016)
β -cis-ocimene	$C_{10}H_{16}$		(Deba et al. 2008)
β -bourbonene	$C_{15}H_{24}$		(Xuan and Khanh, 2016)
β -elemene	$C_{15}H_{24}$		(Deba et al. 2008)
β -cubebene	$C_{15}H_{24}$		(Deba et al. 2008)

α -caryophyllene	$C_{15}H_{24}$		(Deba <i>et al.</i> 2008)
megastigmatrienone	$C_{13}H_{18}O$		(Deba <i>et al.</i> 2008)

2.5.2 Efficacy of *B. pilosa* for pest management

Saponins are responsible for a number of biological properties due to its ability to form complexes with proteins, membrane phospholipids and steroids (Souza and Silva 2013). Saponins are well known for their actions on cell membranes, which can cause damage. These features could explain the haemolytic effect in snails reported in the study of Souza and Silva (2013). A study by Taba *et al.* (2020) found the aqueous extract of *B. pilosa* to have nematocidal and insecticidal activities on pine-wood nematode and Japanese pine sawyer. Methanol and acetone extracts from the leaves, flowers, seeds and stems of *B. pilosa* were found to have effective insecticidal properties against two species of bean weevils, where acetone extracts resulted in 100% mortality regardless of the concentration (Thakur and Sharma 2014).

2.6 *Tagetes minuta*

Tagetes minuta is an annual aromatic herb from the family Asteraceae. *Tagetes* is widely distributed and comprises over 23 000 species of which *T. minuta* is one (Gakuubi *et al.* 2016). In South Africa it is commonly known as khaki bush or “kakie bos”. This plant species is native to the southern part of South America and has spread to countless other countries (Gakuubi *et al.* 2016).

2.6.1 Phytochemical properties of *T. minuta*

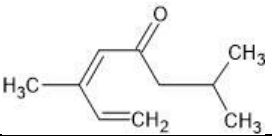
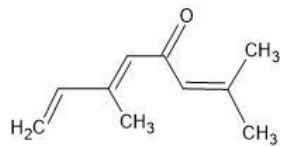
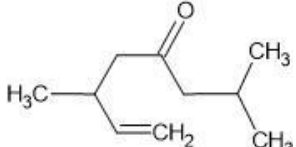
A study carried out by Garcia *et al.* (2012), determined that the main chemical compounds in the essential oil of *T. minuta* were B-ocimene, limonene, tagetone and dihydrotagetone. Other compounds found by Tankeu *et al.* (2013) and Chamorro *et*

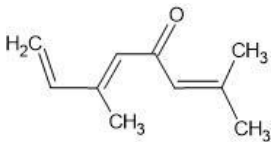
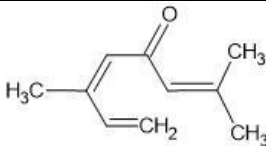
al. (2008), were (*E*)-ocimenone, (*Z*)-ocimenone, and tagetenone. Although there are many more chemical compounds, (*E*)-ocimenone, (*Z*)-ocimenone, and tagetenone are regarded as the main compounds responsible for the species bioactivity against pests (Tankeu *et al.* 2013). The phytochemical compounds of *T. minuta* are tabulated in Table 2.

2.6.2 Efficacy of *T. minuta* for pest management

Many studies utilized the essential oil of *T. minuta* against a number of pests including, mosquitoes, lice, moths and beetles. The essential oil of *T. minuta* has been reported to be effective against *Pediculus humanus capitis*, a common head louse known to cause pediculosis in humans, which is a problem worldwide (Cestari *et al.* 2004). Another study by Chantraine *et al.*, (1998) also reported positive insecticidal activity of *T. minuta* essential oil on the larvae of the mosquito species, *Aedes aegypti*. *T. minuta* essential oil also showed toxicity against three beetle species (*Callosobruchus maculatus*, *Sitophilus oryzae* and *Tribolium castaneum*) which are commonly found in stored grain (Krishna *et al.* 2005). Essential oils of *T. minuta* also had repellent and antifeedant activity against the diamondback moth (Reddy *et al.* 2016), which is a common pest found in vegetable crops.

Table 2: Chemical structures and formulas of *T. minuta* chemical compounds

Chemical compound	Formula	Structure	Reference
tagetone	C ₁₀ H ₁₆ O		(Chamorro <i>et al.</i> 2008)
tagetenone	C ₁₀ H ₁₄ O		(Chamorro <i>et al.</i> 2008)
dihydrotagetone	C ₁₀ H ₁₈ O		(Chamorro <i>et al.</i> 2008)

(E)-ocimenone	C ₁₀ H ₁₄ O		(Tankeu <i>et al.</i> 2013)
(Z)-ocimenone	C ₁₀ H ₁₄ O		(Tankeu <i>et al.</i> 2013)

2.7 Mode of action of plant biopesticides

The secondary metabolites of plant biopesticides, particularly saponins, are known to be toxic to cell walls, cell membranes and fungal cell organelles (Essiedu *et al.* 2020). The nematocidal properties of plant biopesticides are able to subdue nematode populations by inhibiting the hatching of eggs, as well as disorientate insects which affect mating, egg-laying and feeding (Essiedu *et al.* 2020).

B. pilosa and *T. minuta* consist of chemical compounds that have the ability to repel, deter and affect different insect life cycles. This makes the two plant species viable options for effective pest management given their wide distribution and pesticide abilities.

2.8 Fenamiphos

The organophosphate Fenamiphos is a widely used pesticide to control nematodes and insects (Cáceres *et al.* 2011). Fenamiphos is applied via spray equipment to a variety of crops including bananas, citrus, cotton seed, ginger, grapes, groundnuts, guavas, litchis, onions, papayas, peaches, peas, nuts, pineapples, potatoes and tomatoes (Quinn *et al.* 2011). According to the Pesticide Properties DataBase (PPDB) developed by the Agriculture & Environment Research Unit (AERU) at the University of Hertfordshire, Fenamiphos is moderately soluble in water, has low volatility and is highly toxic to mammals (Lewis *et al.* 2016). A study done by Cáceres *et al.* (2011), revealed the LC₅₀ of fenamiphos to be 228 mg/kg (after 28 days) to the earthworm species *Eisenia fetida*. The mode of action of Fenamiphos includes systemic, contact action and can be absorbed through plant roots (Lewis *et al.* 2016). Nematodes are immobilized by Fenamiphos by blocking neuro-enzymes, resulting in irregular movement, paralysis and death.

2.9 Biochar

Biochar is an organic carbon compound which is created by heating biomass in temperatures ranging from 300 to 1000°C, preferably in the absence oxygen (Jeffery *et al.* 2011). This process is commonly known as pyrolysis. The most common benefit of adding biochar to soil is increased crop yields, but varies due to soil properties and conditions. Biochar has the ability to supply and retain nutrients as well as improve soil physical and biological properties, which increases soil fertility and plant growth (Houben *et al.* 2013). Another main attraction of biochar application is its capability to remediate soil contaminated with heavy metals. Although studies have established that biochar has sorbent capabilities, there are a number of variables which influences these capabilities (Ahmad *et al.* 2014). According to Tang *et al.* (2013), the two main factors contributing to the properties of biochar are preparation temperature and the parent material. Biochar and other carbonaceous sorbents have the ability to reduce the bioavailability of organic contaminants in soil and can also decrease dissolved organic carbon, as well as increase soil pH and cation exchange capacity. Considering these factors biochar is a promising ameliorant for polluted soil.

2.10 Conclusion

Research has confirmed the efficacy of plant biopesticides against a variety of pests, although it is not as efficient as synthetic pesticides (Koul *et al.* 2008). Plant biopesticides require much greater application rates and more frequent reapplication in order to fulfil the same effects as synthetic pesticides. Insufficient quantities of available plant material contribute to the lack of commercial application, and the short persistence time due to rapid biodegradation which hinders the efficacy of the phytochemicals (Pavela, 2014).

A variety of factors have an impact on the chemical profile of plant species, this leads to natural variation and requires additional steps during production to ensure the products perform consistently (Koul *et al.* 2008). The factors that affect the chemical profile of plant species are climate, genetic variations, and geographic distribution. Although plant biopesticides only represent approximately 1% of the international market, the positive aspects cannot be denied (Pavela, 2014). Plant biopesticides

should be viewed as an important alternative for pest management, especially due to the considerably lower risks for both human and environmental health.

Chapter 3: Materials and methods

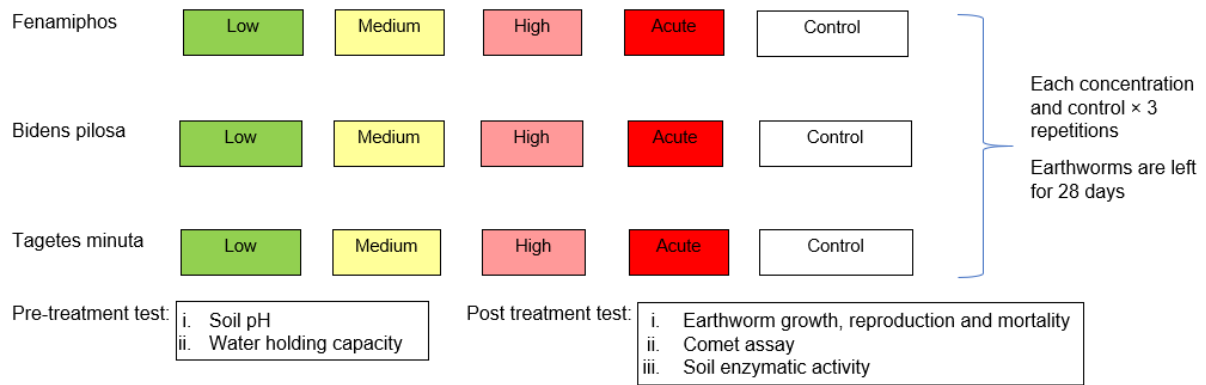
3.1 Global trends of plant biopesticides: Bibliometric analysis

The published research output on botanical pesticides was retrieved from the Scopus database on 7 August 2020. The Scopus database provides access to 27 million abstracts with citations dating back to 1966 (Burnham, 2006). The key terms used to query Scopus database in this study were “plant pesticides”, “plant biopesticides”, “plant-derived pesticides”, “green pesticides”, “botanical pesticides” and “phyto-pesticides”, using the Boolean operator “OR”. These key terms were used to retrieve title-specific research from 1994 – 2019 which returned 586 documents. The documents were cleaned and downloaded in BibTex file format (Olisah, Okoh and Okoh, 2018) and then analysed for a bibliometric statistics with R-project version 4.0.2. The codes used to extract the bibliometric indices were in accordance with Aria and Cuccurullo (2017) in the Rstudio interface. The bibliometric indices: annual scientific production, average article citation per year, average total citations per year, bibliographic coupling, most productive authors, most productive countries, most relevant keywords, and most productive journals/source were used in this study to evaluate the current and possible future trends of the research in plant biopesticides.

3.2 Experimental design

The effects of biopesticides (*B. pilosa* and *T. minuta*) and synthetic pesticide (Fenamiphos) spiked soils on earthworms (*E. andrei*) as well as the efficacy of biochar as a mean of remediation for polluted soils were determined by utilising the guidelines set out by the Organisation for Economic Co-operation and Development (OECD, 2004). The laboratory experiment was conducted in triplicate at the North-West University, South Africa and distilled water is used as the control. Figure 3 summarizes the experimental design in a schematic diagram.

Experiment 1 setup: Effect of pesticides on soil and soil organisms



Experiment 2 setup: Biochar as an ameliorant

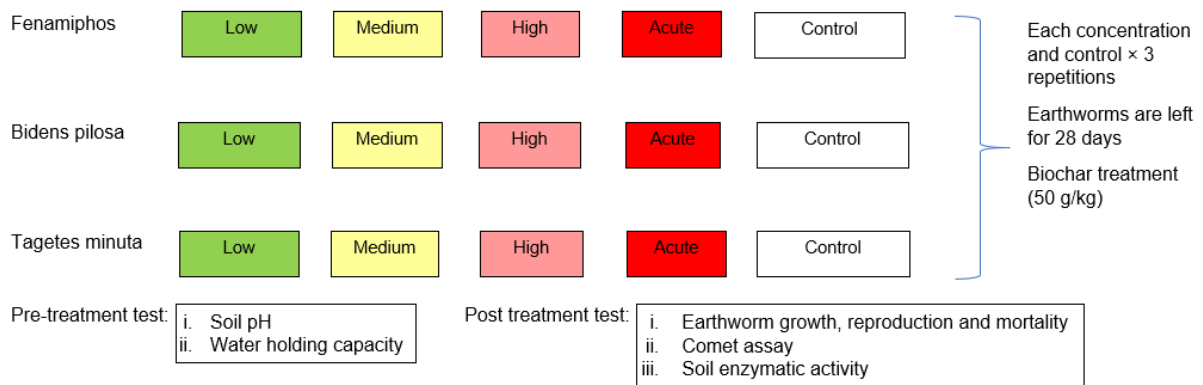


Figure 3: Summary of the experimental design to determine the effect of pesticides on earthworms and the efficacy of biochar as an ameliorant.

3.3 Plant material, collection and authentication

B. pilosa and *T. minuta* (taxonomic descriptions Table 3) were the chosen biopesticides. The aerial parts of the plant of both species were collected in May 2020 on a farm in Limpopo, South Africa (Figure 4). After collection, the plant materials were spread out on a large plastic canvas to dry in a green house, and pulverized, after drying to a constant weight, using a mill. Three replicates of each plant were authenticated and preserved at the A.P. Goossens herbarium of the North-West University. *B. pilosa* and *T. minuta* was authenticated and given barcode numbers PUC0015452 and PUC0015453 respectively and then added to the BRAHMS database.

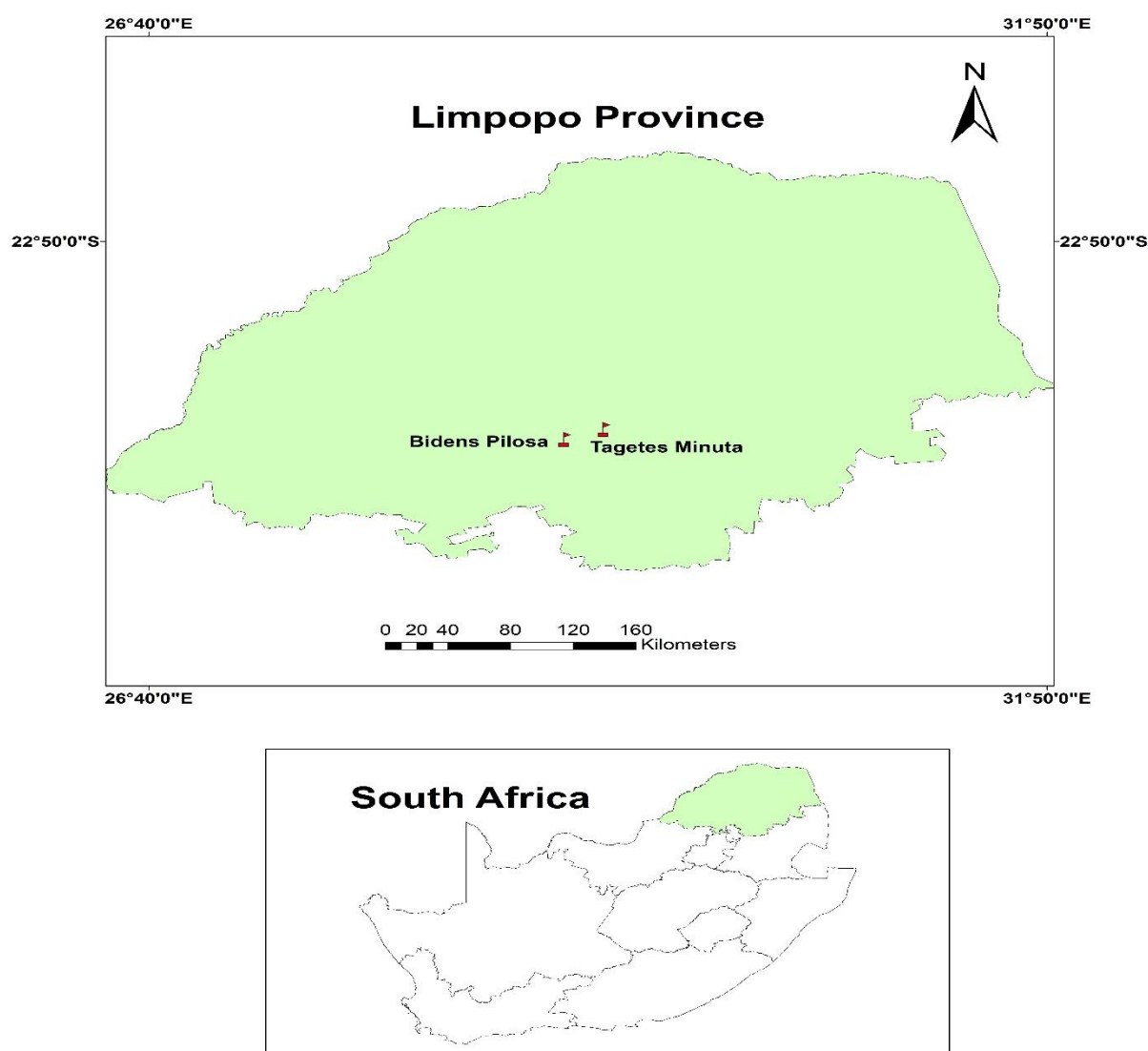


Figure 4: The location of the sampling site of plant species *B. pilosa* (24° 19' 17" S, 29° 11' 1" E) and *T. minuta* (24° 19' 18" S, 29° 11' 3" E) in Limpopo, South Africa.

Table 3: Taxonomy of *B. pilosa* and *T. minuta*

	<i>B. pilosa</i>	<i>T. minuta</i>
Domain	Eukaryota	Eukaryota
Kingdom	Plantae	Plantae
Phylum	Spermatophyta	Spermatophyta
Subphylum	Angiospermae	Angiospermae
Class	Dicotyledonae	Dicotyledonae
Order	Asterales	Asterales
Family	Asteraceae	Asteraceae
Genus	<i>Bidens</i>	<i>Tagetes</i>
Species	<i>Bidens pilosa</i> L.	<i>Tagetes minuta</i> L.

3.3.1 Plant extract

There are a number of extraction methods including, maceration, percolation, hydro distillation and steam distillation (to extract volatile oil) etc. the water extraction method was used during this study to replicate the method used by sustenance farmers (Bartolome *et al.* 2013). The plant extract procedure was done as described by (Idris *et al.* 2017). In the laboratory, 80 g pulverized plant material was mixed with 800 ml distilled water in a clean 1000 ml glass bottle. The glass bottles were left on a mechanical shaker for 24 h after which the contents were filtrated using a Buchner funnel, Whatman no. 1 filter paper, suction flask and vacuum pump. After filtration it was stored in 50 ml tubes and left in a freezer (−80°C) for 24 h. The filtrate was freeze-dried at −60°C until all moisture was extracted. The dried extracts were stored at 4°C until use.

The extract was reconstituted with 5 ml distilled water in selected concentrations which formed part of the total water necessary to obtain the final WHC of 60% (OECD 2004).

3.4 Artificial soil (test substrate)

Artificial soil was prepared in the laboratory according to the OECD 222 protocol (OECD, 2004). The soil consisted of 10% sphagnum peat, 20% kaolin clay, calcium carbonate to obtain an initial pH of 6 and 70% air-dried quartz sand. The pH of the soil was determined by mixing 5 g soil in 5 times its volume of 1 mol.L^{−1} solution of

potassium chloride (OECD, 2004). The mixture was shaken for 5 minutes and left to settle for 2 h, after which the pH was determined using a calibrated pH-meter.

The moisture content of the soil was adjusted to 60% of its maximum water holding capacity (WHC). Two gram of the artificial soil was placed in an auger tube. The bottom of the tube was covered with filter paper and placed in a water bath. The water level was adjusted to cover the soil sample and left for 3 h. Thereafter the auger tube was placed in a sealed container filled with wet finely ground quartz sand for 2 h to allow the soil sample to drain. The soil sample was weighed and dried to a constant mass at 105°C using a Sartorius moisture analyser and the readings were used to calculate the WHC with the following formula:

$$100\% \text{ WHC} = \frac{(\text{gram of soil} \times \% \text{ moisture})}{100} \quad (1)$$

3.5 Test organisms

E. andrei was the selected earthworm specie to conduct the experiments with. The earthworms were provided by Ecolife Vermikompos (Pty Ltd). The earthworms were acclimatised in a climate-controlled room at 20±2°C for at least 48 h. Adult worms with a visible clitellum and weights between 250 mg and 600 mg were selected (OECD, 2004). Each adult earthworm was washed in distilled water, momentarily placed on a paper towel, and weighed before being assigned to the test containers. The selected worms were acclimated for 24 h in the artificial soil prior to the start of the experiment.

3.6 Synthetic pesticide

Fenamiphos (Sigma-Aldrich), a synthetic pesticide, was used as a reference compound in this study. This organophosphate is a well-known systematic and contact insecticide and nematicide (Cáceres *et al.*, 2008). Each selected concentration of Fenamiphos was mixed with 5 ml distilled water, which was accounted for to obtain the final WHC of 60% (OECD, 2004), and shaken until dissolved.

3.7 Ecotoxicological studies

3.7.1 Acute toxicity using the contact filter paper test

The contact toxicity test was carried out as an initial screening test to identify whether the plant biopesticides (*B. pilosa* and *T. minuta*) and synthetic pesticide (Fenamiphos) are potentially toxic to earthworms. The test was conducted according to the OECD 207 guideline (OECD, 1984). Five concentrations in geometric series were used for all three substances. Each treatment consisted of 10 replicates with one earthworm per Petri dish. Prior to the test, the worms were left for 3 h on moist filter paper to allow them to empty their gut. The worms were then washed and dried before adding them to the respective Petri dishes.

Petri dishes were lined with filter paper which was cut to a suitable size (9 cm diameter) to avoid any overlap (OECD, 1984). Test substances were prepared in a series of concentrations by dissolving it in distilled water. A pipette was used to add 2 ml of each concentration onto the filter paper. The filter paper was then left to dry, after which 1 ml of distilled water was added to remoisten the filter paper. The five concentrations used for both *B. pilosa* and *T. minuta* were: 0.125 mg/cm², 0.25 mg/cm², 0.5 mg/cm², 1 mg/cm² and 2 mg/cm². The concentrations for Fenamiphos were: 0.00625 mg/cm², 0.0125 mg/cm², 0.025 mg/cm², 0.05 mg/cm² and 0.1 mg/cm². The Petri dishes containing the earthworms were placed in the dark for 48 h in a climate-controlled room at a temperature of 20±2°C. After 48 h the mortality was determined. Worms were considered dead when not responding to a gentle mechanical stimulus at the anterior end (OECD, 1984).

The concentrations derived from this experiment did not inform the concentrations used during the artificial soil tests (Chapter 3.6.2) as it was only used as a preliminary screening test to determine whether these pesticides are toxic to earthworms.

3.7.2 Acute toxicity using the artificial soil test

The acute toxicity artificial soil test was carried out as a preliminary range-finding test to determine the concentrations for the earthworm avoidance, growth and reproduction tests. Different concentrations in series were tested to determine at which concentration the respective pesticides were low, medium, high and acutely toxic to

earthworms. The test was conducted according to the OECD 207 guidelines (OECD, 1984). One-litre plastic containers were filled with 500 g of artificial soil. Soils were spiked with *B. pilosa*, *T. minuta* and Fenamiphos, with three replicates each. The concentrations for *B. pilosa* and *T. minuta* extracts were: 1, 10, 100, 1000 and 2000 mg/kg. The Fenamiphos concentrations were: 12.5, 25, 50, 100 and 250 mg/kg. The test containers were kept in a climate-controlled room at 20±2°C in continuous light to ensure the earthworms remain in the artificial soil. Mortality was assessed at day 7 and day 14 by examining if the earthworms respond to a gentle mechanical stimulus at the anterior end (OECD, 1984).

3.7.3 Earthworm avoidance test

This test was carried out in compliance with International Organization for Standardization guideline 17512 (ISO, 2008) to assess the impact of pollutants in soil on earthworm avoidance behaviour. The concentrations used during this test was determined using the acute toxicity test in artificial soil (Chapter 3.6.2). The spiked concentrations for both *B. pilosa* and *T. minuta* were: 250 mg/kg, 500 mg/kg, 1000 mg/kg and 2000 mg/kg. The spiked concentration for Fenamiphos were: 12.5 mg/kg, 25 mg/kg, 50 mg/kg, 100 mg/kg and 200 mg/kg. Adult earthworms were exposed to both control and spiked soil simultaneously by filling a container with 250 g control soil (distilled water) and 250 g spiked soil (test substance), with a divider in the middle to ensure the soil does not mix. After removing the divider, ten earthworms were placed on the dividing line and placed in a climate-controlled room for 48 h at 20±2°C. After 48 h the number of earthworms present in the control and spiked soil was counted respectively. Five replicates per concentration were used (ISO, 2008). Worms found on the dividing line were counted as 0.5 for both the control and spiked soil. The net response of the earthworms was calculated using the following formula:

$$NR = \left(\frac{C-T}{N} \right) \times 100 \quad (2)$$

Where NR = net response, C = worms observed in control soil, T = worms observed in spiked soil and N = total number of worms per replicate.

3.7.4 Earthworm growth and reproduction test

This test was performed according to the OECD 222 guidelines (OECD, 2004). One-litre plastic containers were filled with 500 g of artificial soil. Soils were spiked with *B. pilosa*, *T. minuta* and Fenamiphos at the same concentrations as the avoidance behaviour test, with three replicates each. The initial biomass of the worms (ten per replicates) was determined by weighing them individually before adding them to the test containers. The test containers were kept in the dark in a climate-controlled room at $20\pm 2^{\circ}\text{C}$. The earthworms were fed with 5 g of horse manure on day 1 and the same amount was added again every 7 days (OECD, 2004). At 7-day intervals, the earthworms were counted, washed and weighed and to observe if any mortalities occurred. The dead earthworms were removed. At the end of the 28-day growth experiment, the remaining earthworms were removed, weighed and counted. Afterwards the number of cocoons were recorded and left for a further 28 days under the same test conditions. Horse manure was added only at the beginning of the second 28-day period. After the 28 days (day 56) the number of juveniles were counted using the hand sorting technique (OECD, 2004).

Earthworm growth and reproduction test with biochar:

The test to assess the effect of biochar on ecotoxicity was identical to the methodology described above, with the same concentrations of treatments in the first test, the only difference being the addition of biochar (Organics Matter SA (Pty) Ltd) as an ameliorant. Biochar was mixed with the artificial soil according to the methods described by Yang *et al.* (2016) at 5% of the artificial soil.

3.8 Comet assay

After the earthworm growth test was completed (day 28), the surviving adult earthworms were used to perform the comet assay. Comet assay is a method to assess DNA damage in earthworm coelomic cells. The assay was carried out using a modified protocol by Reinecke and Reinecke (2004), originally prepared by Singh *et al.* (1988).

Three earthworms from each replicate were collected to assess cellular DNA damage. A day prior to the comet assay the earthworms were removed from the soil and placed on a moist paper towel so they could empty their guts and avoid soil contamination

while preparing the cell suspension samples. The solutions (PBS solution, lysing solution, electrophoresis buffer and neutralization buffer) used during the comet assay were prepared the day before the start of the experiment and left in a refrigerator at 4°C. The extrusion fluid, consisting of 90% PBS, 10% ethanol, 2.5 mg/ml EDTA and 10 mg/ml guaiacol glycerol ether, was prepared on the day of the test.

An aliquot of 1 ml of the extrusion fluid was pipetted into the Eppendorf tubes and live earthworms were introduced for 1 minute to extract the coelomic cells. After the extrusion of coelomic cells, they are removed from the extrusion fluid, rinsed in distilled water and returned to substrates. The Eppendorf tubes, with the extracted coelomic cells, were then centrifuged at 3000 rpm for 5 minutes, supernatant carefully removed and 0.5 ml of PBS added to wash the coelomic cells. The centrifuge process was then repeated twice. Low melting agarose and the cell suspension were mixed at a ratio of 1:10 and then spread onto a 3-well microscope slide. The microscope slides were left for 15 minutes in a 4°C refrigerator for the agarose gel to set. The slides were placed in a container, the lysing solution was added and left for 1h in the dark at 4°C. The slides were then removed and placed in a cold electrophoresis buffer for 30 minutes. The slides were transferred to an electrophoresis chamber and filled with electrophoresis buffer until the slides were covered. Electrophoresis was conducted for 30 minutes at 20 V and 300 mA, which allowed the migration of broken DNA strands from the nucleus (Reinecke and Reinecke, 2004). Once electrophoresis was done the slides were rinsed three times in cold distilled water for 2 minutes. The slides were then placed in 70% ethanol for 20 minutes, removed and allowed to air dry before storing in the dark at 4°C until staining.

On the day of analysis, the slides were rehydrated in cold distilled water for 30 minutes and stained with ethidium bromide for 15 minutes. After staining, the slides were kept on moist paper towels inside a container. The comet images were viewed under a fluorescence microscope (Nikon Eclipse TE2000 Inverted Research Microscope). After capturing the comet images, ImageJ (version 1.53e) software was used to quantitatively assess the extent of DNA damage in the cells by measuring the length of DNA migration (comet tail). The broken strands in the nuclear DNA increases the mobility of DNA fragments during electrophoresis, thus creating longer comet tails indicating greater DNA damage. ImageJ identifies the comet tail and measures the

distance from the comet head to the end of the tail to quantify the amount of DNA migration and determine the DNA damage.

3.9 Soil enzyme analysis

Soil samples (50 g) were collected from each replicate on termination of the earthworm growth test (day 28), placed in sealable plastic bags and frozen at -80°C . The analyses were done at the Agricultural Research Council (ARC), Potchefstroom, South Africa. The enzymatic activities in the soil were examined through; β -glucosidase (carbon), phosphatase (phosphorous) and urease (nitrogen) assays.

3.9.1 β -glucosidase

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

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

Afterwards, 300 μL of supernatant from each sample was pipetted into the corresponding 96-well transparent microplate. The colour change was measured

spectrophotometrically at a wavelength of 410 nm using a spectrophotometer (Biotek ELx800). The β -glucosidase activity was then determined for each sample as $\mu\text{g g}^{-1}$ dry weight of soil h^{-1} using the linear standard curve.

The β -glucosidase activity was calculated using the following formula:

$$p - \text{nitrophenol } (\mu\text{g g}^{-1} \text{ dwt h}^{-1}) = \frac{C \times v}{\text{dwt} \times \text{SW} \times t} \quad (3)$$

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

dwt = dry weight of 1 g moist soil

v = total volume of the soil suspension in ml

SW = weight of soil sample used (1 g)

t = incubation time in hours

3.9.2 Alkaline phosphatase

Alkaline phosphatase was done according to Alef and Nannipieri (1995). Soil alkaline phosphatase activities were determined by incubating soil with *p*-nitrophenol substrate and determining the release of *p*-nitrophenol in the soil. The optical density was measured and compared with the optical density of the *p*-nitrophenol standard solution (Salazar *et al.* 2011). The enzymatic activity is thus expressed in $\mu\text{g p-nitrophenol g}^{-1}$ dwt h^{-1} . Although the procedure is similar to the β -glucosidase activity, different chemicals are used to determine the alkaline phosphatase activity but are expressed the same ($\mu\text{g p-nitrophenol g}^{-1}$ dry soil weight).

One gram of soil was added to a 50 ml glass bottle with 0.2 ml toluene, 4 ml MUB, and 1 ml of *p*-nitrophenyl phosphate solution (PNP). The bottle was swirled to mix the contents and incubated in a water bath for 1 h at 37°C. After 1 h, 1 ml of CaCl_2 and 4 ml of NaOH were added, swirled to mix the contents, and filtered with Whatman No.1 filter paper. The control was prepared the same way but without any substrates. A calibration curve was prepared using the standard (*p*-nitrophenol) solution in the concentration range of 0, 10, 20, 30, 40, 50, and 100 μg . An aliquot of 300 μL of the filtrate was pipetted into a 96-well microplate and a spectrophotometer (Biotek ELx800) was used to read the intensity of the colour development at 410 nm. The soil

alkaline phosphatase activities were determined for each sample as $\mu\text{g g}^{-1}$ dry weight of soil h^{-1} interpolated from the linear standard curve.

The Alkaline phosphatase activity was calculated using the following formula:

$$p - \text{nitrophenol } (\mu\text{g g}^{-1} \text{ dwt h}^{-1}) = \frac{C \times v}{\text{dwt} \times \text{SW} \times t} \quad (4)$$

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

dwt = dry weight of 1 g moist soil

v = total volume of the soil suspension in ml

SW = weight of soil sample used (1 g)

t = incubation time in hours

3.9.3 Urease

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

Five grams of soil were mixed with 2.5 ml of urea solution in a 100 ml glass bottle. The glass bottle was closed with a cap and incubated for 2 h in a water bath at 37°C. Afterwards, 50 ml of KCl solution was added to extract the liberated ammonium and the bottle was placed on a mechanical shaker for 30 min. The suspension was filtered through Whatman No.1 filter paper. The control was prepared by adding 2.5 ml of distilled water in place of the urea solution before adding KCl (Kandeler and Gerber 1988). To determine the ammonium concentration, 1 ml of clear filtrate was pipetted into a 50 ml glass bottle. Then 9 ml of distilled water, 5 ml of the sodium salicylate and sodium hydroxide solutions, and 2 ml of the dichloroisocyanide solution were added. The solution was left at room temperature for 30 min, whereafter the optical density (ammonium content) was measured at 600 nm using a spectrophotometer (Biotek ELx800). The method is based on the reaction of sodium salicylate with NH_3 in the

presence of sodium dichloroisocyanurate, which forms a colour complex (green) under an alkaline pH condition (Attademo *et al.* 2021).

The calibration curve was constructed using ammonium standard solution II in the concentration range (0, 10, 20, 30, 40, 50, and 100 µg) and the urease activities for each sample were expressed as µg NH₄ N g⁻¹ dry weight of soil 2 h⁻¹ interpolated from the linear standard curve.

The Urease activity was calculated using the following formula:

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

dwt = dry weight of 1 g moist soil

V = total volume of the extract (52,5 ml)

10 = dilution factor

5 = weight of soil used in the assay

3.10 Statistical analysis

All experiments were performed in triplicates and mean values were obtained. The data was presented as the mean ± standard deviation (S.D.). Differences in the growth rate, reproduction, and soil enzyme between treatment groups were analysed with Minitab® 17.1.0, using one-way analysis of variance (ANOVA), and Tukey's multiple pairwise comparison at 95% confidence intervals (CI). The LC₅₀ values of the acute toxicity test were obtained by Probit analysis using IBM® SPSS Statistic version 23. Comets were scored using an image analysis software (ImageJ, version 1.53e), specifically for images of fluorescent stained nuclei, followed by ANOVA. The level of significance was p<0.05. Bibliometric data was analysed in BibTeX file format in RStudio version 3.6.3 using the installed bibliometric package and dependencies.

Chapter 4: Results

4.1.1 Green pesticide research production

To determine the progression of research on green pesticides in this study, a wide spectrum of biopesticide keywords was used to query the database. A total of 540 published documents, from 332 sources were recorded on green pesticides. These documents include articles, books, book chapters, conference papers, conference reviews, editorials, erratum, notes, reviews and short surveys. An average citation of 12.63 per document makes them relevant in the scientific community (Table 4). The annual trend of scientific production from 1994 to 2008 showed a minimal increase, with the highest increases in 2000, 2004 and 2007 (Figure 5). Production increased significantly from 2009, with the most documents published in 2019 (74 documents). A decline in publications, however, occurred during 2015 and 2016, with 32 and 38 publications respectively. The reason for such a decline is not known. The annual percentage growth rate from 1994 to 2019 was 13.68%. It is also worth noting that although there is a steady increase in the annual scientific production, fluctuations do occur between some years. Possible explanations could include improved laboratories, evolvement of new researchers, development of new research methodology and funding support (Olisah *et al.* 2019).

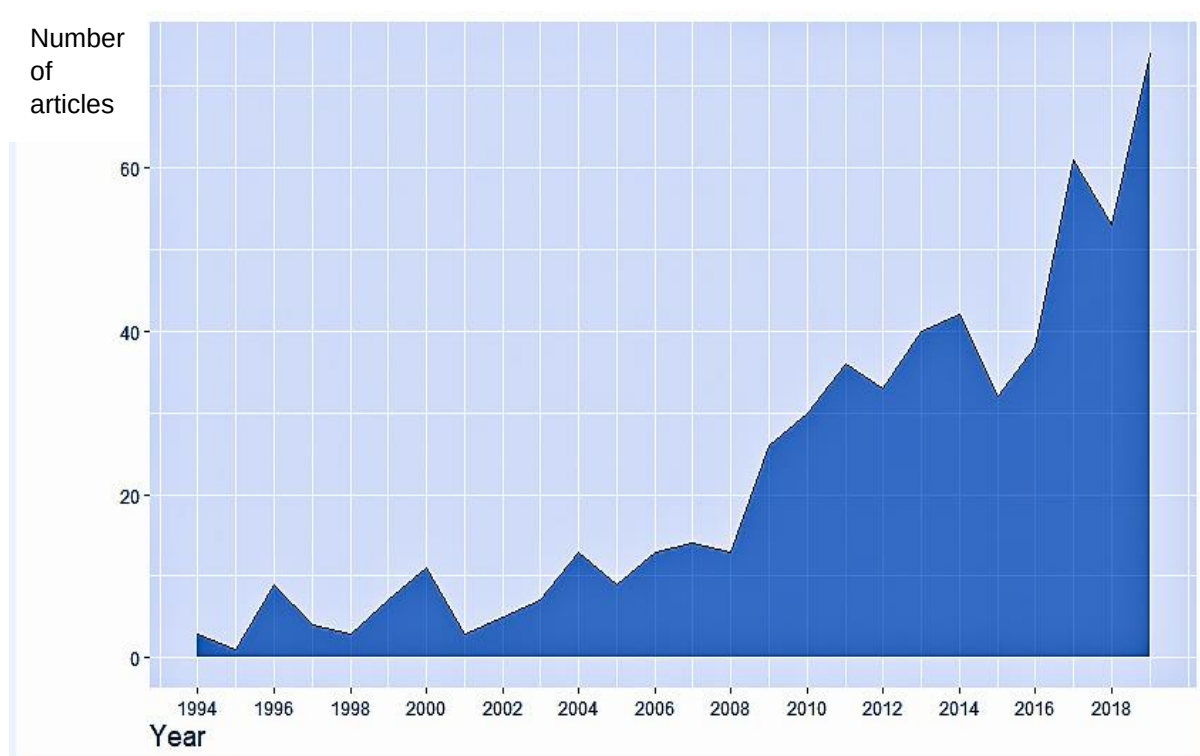


Figure 5: Annual scientific publications of documents on biopesticides from 1994 to 2019.

Table 4: Summary of data retrieved from the Scopus database

Description	Count
Documents	540
Source (Journals, books, etc.)	332
Keywords plus (ID)	4064
Author's keywords (DE)	1732
Period	1994 - 2019
Average citations per documents	12.63
Authors	1867
Author appearances	2408
Authors of single-authored documents	68
Authors of multi-authored documents	1799
Single-authored documents	79
Documents per author	0.311
Authors per document	3.22
Co-authors per documents	4.15
Collaboration index	3.59

4.1.2 Keywords on green pesticide research

Most published documents contain a number of keywords to assist with online searches and identify certain editors to a document (Olisah *et al.* 2019). To study the research trends of green pesticides both the singular and plural form of the author keywords were used. This aids in understanding the research evolution of the study field (Canas-Guerrero *et al.* 2013). Both the author keywords and keyword plus were included in the study. The author keywords are a list of terms provided by the author/s that best represents the content of the document, while keyword plus are terms or phrases that occur in the titles of a document's references but do not appear in the document's title itself (Zhang *et al.* 2015). Between 1994 and 2019 a total of 1732 author keywords and 4064 keyword-plus were retrieved from the documents on green pesticide research (Table 5). The most used author keyword was botanical pesticide/s occurring in 159 articles, followed by essential oil/s (41 articles) and pesticide/s (25 articles) respectively. Other author keywords occurring in the top 30 include green pesticides (22 articles), biopesticides (17 articles), pest management (14 articles), toxicity (12 articles) and plant extracts (13 articles) etc, all these are relevant to pest control using plant biopesticides. The most used keyword-plus was animal/s, occurring in 220 articles, followed by pesticide/s (217 articles) and insecticide/s (175 articles) respectively as shown in Table 5. It is not surprising to get a broad range of terms in keyword-plus because it is derived from the titles of documents cited by the author. Other keyword-plus terms in the top 30 are plant extract (102 articles), pest control (82 articles), essential oil (62 articles) and toxicity (38 articles).

In Figure 6, the co-occurrence network and interrelationship of the top 30 terms on green pesticide research are represented with a pictographic network. Each coloured circle represents a cluster of terms and the connecting lines represent the cooperation degree. Pesticide related terms are in blue clusters and the response related terms in red clusters (Figure 6).

Table 5: Top 30 relevant keywords on green pesticide research from 1994 to 2019

Rank	Author keywords (DE)	Articles	Keyword-plus (ID)	Articles
1	Botanical pesticide/s	159	Animal/s	220
2	Essential oil/s	41	Pesticide/s	217
3	Pesticide/s	25	Insecticide/s	175
4	Azadirachtin	24	Plant extract/s	151
5	Green pesticides	22	Article	117
6	Biopesticides	17	Chemistry	90
7	Neem	15	Pest control	82
8	Pest management	14	Hexapoda	79
9	Botanical insecticide/s	14	Nonhuman	78
10	Azadirachta indica	13	Drug effect	69
11	Botanicals	13	Azadirachta indica	65
12	Plant extracts	13	Essential oil	62
13	Spodoptera litura	12	Botanical pesticides	60
14	Toxicity	12	Controlled study	57
15	Mortality	11	Larva	50
16	Integrated pest management	10	Biopesticide	49
17	Antifeedant	9	Unclassified drug	43
18	Biological control	9	Female	42
19	Insecticidal activity	9	Lepidoptera	39
20	Repellency	9	Metabolism	38
21	Bioassay	8	Toxicity	38
22	Contact toxicity	8	Human	37
23	Insecticide	8	Bioassay	35
24	Pest control	8	Agriculture	33
25	Aphids	6	Biological control	33
26	Heteropneustes fossils	6	Insect	32
27	Phytochemicals	6	Mortality	32
28	Plutella xylostella	6	Fungi	31
29	Tetranychus urticae	6	Moth	31
30	Acute toxicity	5	Physiology	27

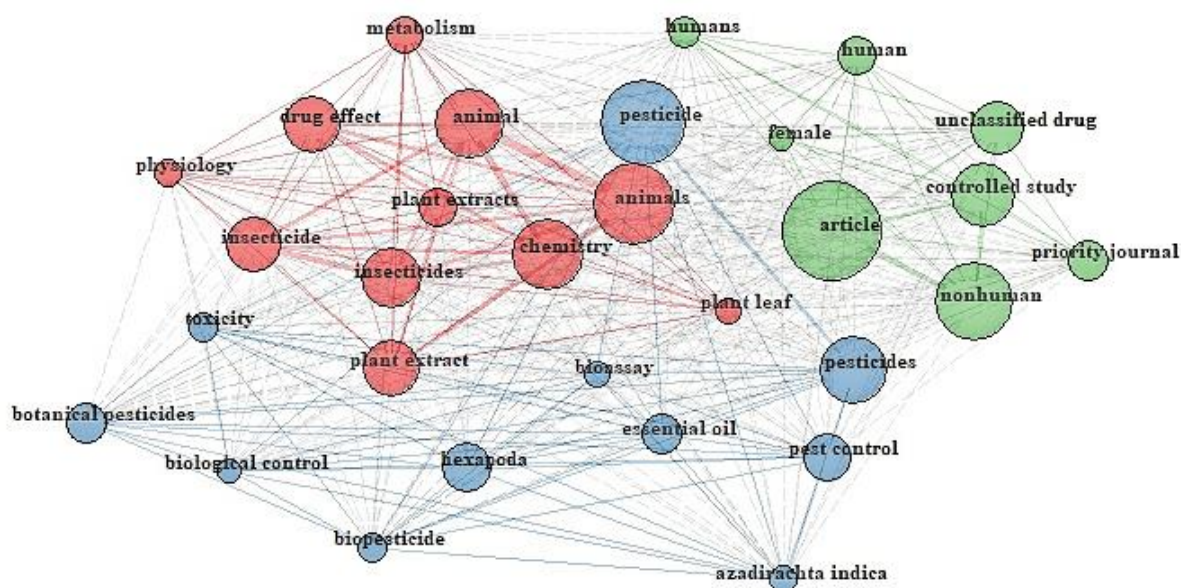


Figure 6: Keyword co-occurrences network of the top 30 keywords on green pesticide research. The circle size of the keywords represents the frequency of occurrence in articles. The thickness of the line between any two keywords displays the degree of cooperation. Co-occurrence was used to measure cooperation.

4.1.3 Productivity on green pesticide research per country

India is ranked first in the most productive countries with a total of 98 publications, among which 4.08% are published out of collaboration. China and USA are ranked second and third with 68 and 32 publications respectively. China has 2.94% and USA has 12.5% multiple country publications (MCP) which indicate the USA collaborate more while China relies more on local research. It is quite interesting to know that Italy and South Africa have fewer single country publications (SCP) compared to their MCP, hence they have a higher MCP: SCP ratio. The collaboration on publications of Italy, Netherlands and South Africa are 60.86%, 75.0% and 80.0% respectively, making these the countries with the most collaboration network (Figure 7).

Table 6 shows that India is ranked first with a total of 1080 citations; h-index 18, followed by USA (979 citations; h-index 17) and Italy (868 citations; h-index 16) second and third respectively. African countries in the top 30 list are Egypt in 8th (184 citations), South Africa 12th (92 citations), Zimbabwe 13th (90 citations), Nigeria 15th (69 citations), Kenya 20th (54 citations) and Tanzania 22nd (52 citations). It is debated that citations reflect the scientific impact and relevance of an article, but with limitation to

the measures of the research quality (Nieminen *et al.* 2006) (Aksnes *et al.* 2019). Hence, the citation ranking the USA first and other countries follow, could be the measure of their study relevancy. It is important to note that Europe is the largest consumer of pesticides with Asia the second largest and the leading countries in pesticide production are China, USA, France, Brazil and Japan (Zhang *et al.* 2011). In Figure 7 and Table 6 the above-mentioned countries are ranked in the top 30 of the categories of the most productive and most cited country in plant biopesticide study, but with the exception of Japan, indicating the countries are focusing on plant biopesticides as an alternative to synthetic pesticides and these studies are relevant in the scientific community.

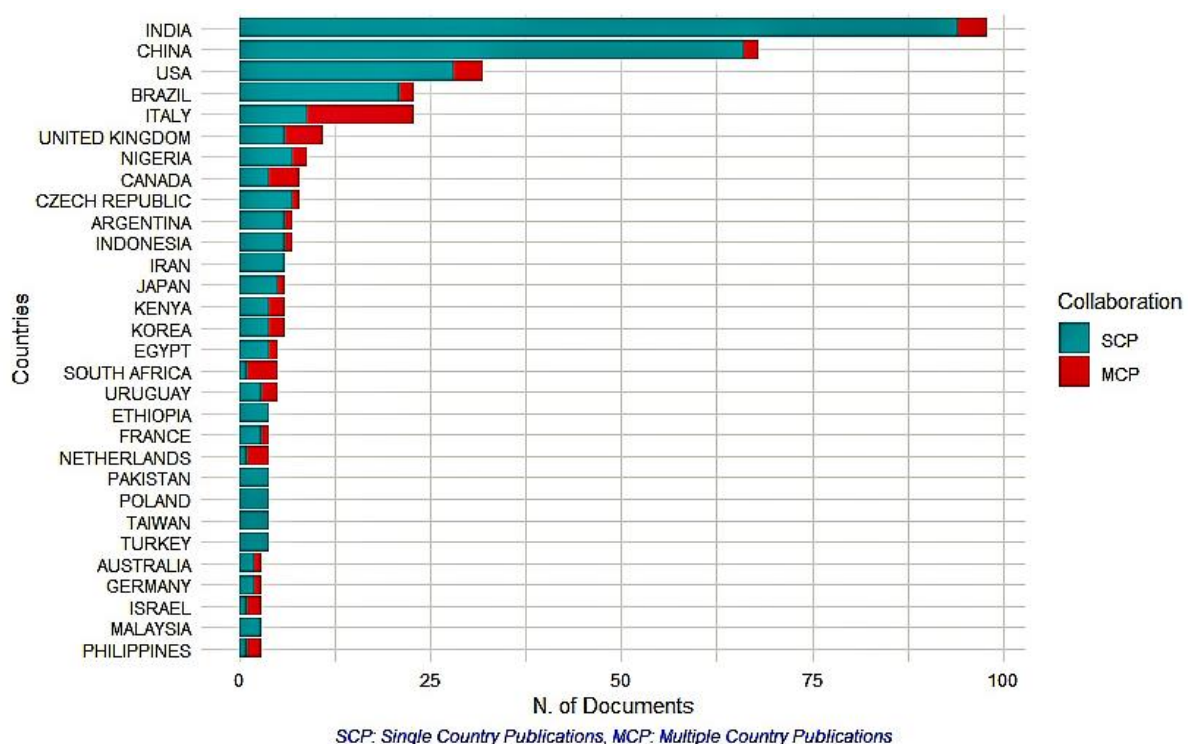


Figure 7: Most productive countries and collaboration in the research of green pesticides from 1994 to 2019.

Table 6: Total citations per country in terms of green pesticide research from 1994 to 2019

Rank	Country/Region	Total citations	Average article citations (%)	MCP_ratio	h_index
1	India	1080	11.020	0.041	18
2	USA	979	30.594	0.125	17
3	Italy	868	37.739	0.609	16
4	China	682	10.029	0.029	14
5	United Kingdom	441	40.091	0.455	11
6	Brazil	389	16.913	0.087	10
7	Canada	251	31.375	0.500	9
8	Egypt	184	36.800	0.200	7
9	Argentina	162	23.143	0.143	7
10	Belgium	105	52.500	1.000	6
11	Czech Republic	94	11.750	0.125	5
12	South Africa	92	18.400	0.800	5
13	Zimbabwe	90	45.000	1.000	5
14	Iran	86	14.333	0	5
15	Nigeria	69	7.667	0.222	4
16	Korea	68	11.333	0.333	4
17	Uruguay	64	12.800	0.400	4
18	Turkey	59	14.750	0	4
19	Israel	55	18.333	0.667	4
20	Kenya	54	9.000	0.333	4
21	Netherlands	52	13.000	0.750	4
22	Tanzania	52	17.333	0.667	4
23	Poland	44	11.000	0	3
24	Romania	44	44.000	0	3
25	Australia	42	14.000	0.333	3
26	Taiwan	39	9.750	0	3
27	France	38	9.500	0.250	3
28	Greece	38	38.000	0	3
29	Germany	27	9.000	0.333	2
30	Lebanon	27	27.000	0	2

MCP: Multiple Country Publications

4.1.4 Most cited documents on green pesticides

Table 7 presents the most cited papers on plant biopesticides. The top-ranked article published by Pavela and Benelli, (2016) had a total of 288 citations at an average of 57.6 citations per year. In the article, the strengths, challenges and constraints of essential oil-based biopesticides were analysed (Pavela and Benelli, 2016), considering the effectiveness, toxicity and the mechanisms of action. The high citation accredited by the article may be due to the wide spectrum of the biological activity such as; nematicidal, ovicidal, fungicidal, insecticidal and bactericidal effect of

essential oil-based biopesticides making the article relevant in a wide field of research. On the other hand, surprisingly, only 3 articles from the 1990s are listed in the top 30 most cited documents. This may indicate that more recent researchers are becoming more interested in plant biopesticides, as search for alternative synthetic pesticides grows.

In Table 8 the top 30 productive journals in terms of the number of publications, total citations and h-index are reported. The top 30 journals have published 219 articles which represent 37.76% of the documents in this study. The Pestology journal is ranked first (NP: 24; h-index; 4), Industrial Crops and Products second (NP: 21; h-index: 12) and Pest Management Science third (NP: 14; h-index: 10).

Table 7: The 30 most cited documents on green pesticide research from 1994 to 2019 from Scopus.

Rank	Reference	Journal	TC	TC/Year
1	Pavela R, 2016	<i>Trends Plant Sci</i>	288	57,6
2	Mulla Ms, 1999	<i>J Am Mosq Control Assoc</i>	242	11
3	Morgan Ed, 2009	<i>Bioorg Med Chem</i>	161	13,42
4	Son Tg, 2010	<i>J Neurochem</i>	135	12,27
5	Miresmailli S, 2014	<i>Trends Plant Sci</i>	134	19,14
6	Dodge Ja, 1996	<i>J Steroid Biochem Mol Biol</i>	130	5,2
7	Benelli G, 2017	<i>J Cluster Sci</i>	101	25,25
8	Attia S, 2013	<i>J Pest Sci</i>	99	12,37
9	El-Wakeil Ne, 2013	<i>Gesunde Pflanz</i>	95	11,87
10	Qian X, 2010	<i>J Agric Food Chem</i>	89	8,09
11	Cresswell Je, 2012	<i>Pest Manage Sci</i>	71	7,89
12	Sibanda T, 2000	<i>Crop Prot</i>	71	3,38
13	Mossa Ath, 2016	<i>J Environ Sci Technol</i>	68	13,6
14	Koul O, 2009	<i>Cab Rev Perspect Agric Vet Sci Nutr Nat Resour</i>	68	5,67
15	Kotkar Hm, 2002	<i>Pest Manage Sci</i>	68	3,58
16	Charleston Ds, 2005	<i>Biol Control</i>	64	4
17	Martin Km, 2000	<i>J Am Vet Med Assoc</i>	64	3,05
18	Benelli G, 2017	<i>Parasitol Int</i>	62	15,5
19	Hunt Pr, 2011	<i>Plos One</i>	62	6,2
20	Dubey Nk, 2010	<i>Curr Sci</i>	61	5,55
21	Zheng K, 2012	<i>Acta Chim Sin</i>	60	6,67
22	Mann Rs, 2012	<i>Mini-Rev Org Chem</i>	59	6,56
23	Rao Kj, 2013	<i>Rsc Adv</i>	56	7
24	Vidal Estrela Jl, 2006	<i>Pesqui Agropecu Bras</i>	54	3,6
25	Benelli G, 2018	<i>Ind Crops Prod-A</i>	53	17,67
26	Caboni P, 2012	<i>J Agric Food Chem</i>	51	5,67
27	Zibae A, 2010	<i>Bull Entomol Res</i>	51	4,64
28	Moreira Md, 2007	<i>Pest Manage Sci</i>	51	3,64
29	Mansour F, 2004	<i>Phytoparasitica</i>	50	2,94
30	Valladares G, 1997	<i>J Econ Entomol</i>	49	2,04

Table 8: Top 30 productive journals, the total number of publications, total citations, h-index and publication start year, from 1994 to 2019.

Source	NP	TC	h-index	PY-Start
<i>Pestology</i>	24	48	4	1996
<i>Industrial Crops And Products</i>	21	334	12	2010
<i>Pest Management Science</i>	14	378	10	2000
<i>Environmental Science And Pollution Research</i>	13	155	6	2010
<i>Advances In Plant Biopesticides</i>	13	91	7	2014
<i>Journal Of Agricultural And Food Chemistry</i>	12	342	8	2003
<i>Crop Protection</i>	10	258	9	1997
<i>Acta Horticulturae</i>	10	20	2	1997
<i>Scientific Reports</i>	9	75	5	2016
<i>Journal Of Biopesticides</i>	8	28	4	2010
<i>Plos One</i>	7	150	5	2006
<i>Journal Of Pest Science</i>	6	172	6	2009
<i>Molecules</i>	6	39	4	2010
<i>Green Pesticides Handbook: Essential Oils For Pest Control</i>	6	5	2	2017
<i>Acs Symposium Series</i>	5	50	2	1996
<i>Journal Of Pesticide Science</i>	5	23	3	2009
<i>Biopesticides International</i>	5	5	2	2011
<i>Phytoparasitica</i>	4	85	3	2004
<i>Pesticide Biochemistry And Physiology</i>	4	54	3	2011
<i>Journal Of Insect Science</i>	4	28	2	2014
<i>Archives Of Phytopathology And Plant Protection</i>	4	16	2	2011
<i>Zhongguo Zhongyao Zazhi</i>	4	7	2	2004
<i>Iop Conference Series: Earth And Environmental Science</i>	4	2	1	2018
<i>Trends In Plant Science</i>	3	426	3	2014
<i>Journal Of Economic Entomology</i>	3	92	3	1997
<i>Medical And Veterinary Entomology</i>	3	76	3	2008
<i>Phytochemistry Reviews</i>	3	61	3	2011
<i>International Journal Of Tropical Insect Science</i>	3	49	2	2006
<i>Ecotoxicology And Environmental Safety</i>	3	31	2	2010
<i>Acs Sustainable Chemistry And Engineering</i>	3	26	2	2016

4.2 Ecotoxicological assays

The ecotoxicity of plant biopesticides (*B. pilosa* and *T. minuta*) were evaluated with the contact filter paper and artificial soil tests utilizing earthworms (*E. andrei*) and the

treatments (plant biopesticides and synthetic pesticide Fenamiphos) were tested in soils with and without biochar and compared. In this study, the ecotoxicity of the plant biopesticides and synthetic pesticide was tested using acute toxicity tests, sublethal tests such as earthworm avoidance behaviour, chronic toxicity (growth and reproduction), and biomarkers (comet assay).

4.2.1 Contact filter paper test

The filter paper toxicity test of *B. pilosa*, *T. minuta* and Fenamiphos on *E. andrei* showed a significant response ($p < 0.05$) proportional with an increase in concentration (Figure 8). No mortality was observed in the control treatment during the 48 h of the exposure period. All three treatments (both plant biopesticides and Fenamiphos) had 100% mortality at 2 mg/cm². The mortality (%) of *E. andrei* exposed to *B. pilosa* were as follow: 20% mortality at 0.125 mg/cm², 40% mortality at 0.25 mg/cm², 50% mortality at 0.5 mg/cm², 70% mortality at 1 mg/cm² and 100% mortality at 2 mg/cm². The percentage mortality of the *T. minuta* exposures was significantly different ($p < 0.05$) from *B. pilosa*. A 30% mortality occurred at 0.125 mg/cm², 50% at 0.25 mg/cm², 70% at 0.5 mg/cm² and 80% at 1 mg/cm².

The mortality of *E. andrei* exposed to Fenamiphos was significantly ($p < 0.05$) higher than *B. pilosa* and *T. minuta* at all concentrations except at 2 mg/cm². Fenamiphos concentrations 0.006 mg/cm², 0.0125 mg/cm² and 0.025 mg/cm², resulted in 60%, 80% and 100% mortality respectively.

The median lethal concentration (LC₅₀) for *B. pilosa*, *T. minuta* and Fenamiphos for the contact filter paper test was 0.387 mg/cm² (upper bound 0.643 mg/cm², lower bound 0.212 mg/cm²), 0.255 mg/cm² (upper bound 0.417 mg/cm², lower bound 0.106 mg/cm²) and 0.006 mg/cm² (upper bound 0.009 mg/cm², lower bound 0.0002 mg/cm²) respectively.

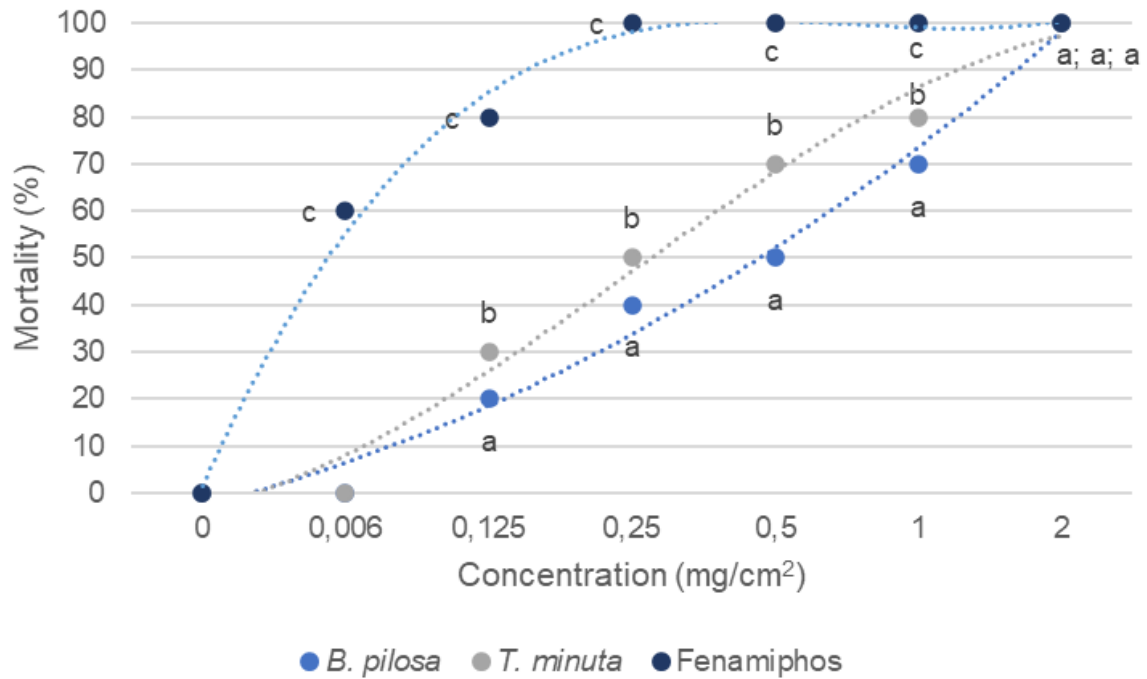


Figure 8: The percentage mortality of *E. andrei* in the contact filter paper test after 48 h exposure with different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos. Different letters (a-c) represent significant difference ($p < 0.05$).

4.2.2 Artificial soil test

No mortalities occurred during the 14-day test period in the artificial soil spiked with varying concentrations of *B. pilosa* and *T. minuta* extract, with concentrations as high as 2000 mg/kg. Mortalities did occur with the synthetic pesticide (Fenamiphos) treatments, and the LC_{50} was evaluated to be 163.853 mg/kg (upper bound 207.972 mg/kg, lower bound 197.914 mg/kg).

The difference in concentrations of the plant biopesticides (*B. pilosa* and *T. minuta*) and synthetic pesticide (Fenamiphos) before mortalities occurred can be attributed to the more toxic effects of Fenamiphos towards earthworms. However, this does not mean the different pesticides are not comparable, as the test was designed to include low, medium, high and acute concentrations. Due to the different compounds present in plant biopesticides and synthetic pesticides the lethal concentrations will differ.

4.2.3 Avoidance behaviour assay

The results of the 48 h avoidance test on *E. andrei*, when exposed to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos respectively, are presented in Figure 9, Figure 10 and Figure 11. All concentrations of *B. pilosa* and *T. minuta* showed no significant ($\geq 80\%$) effect in the avoidance or attraction of *E. andrei*.

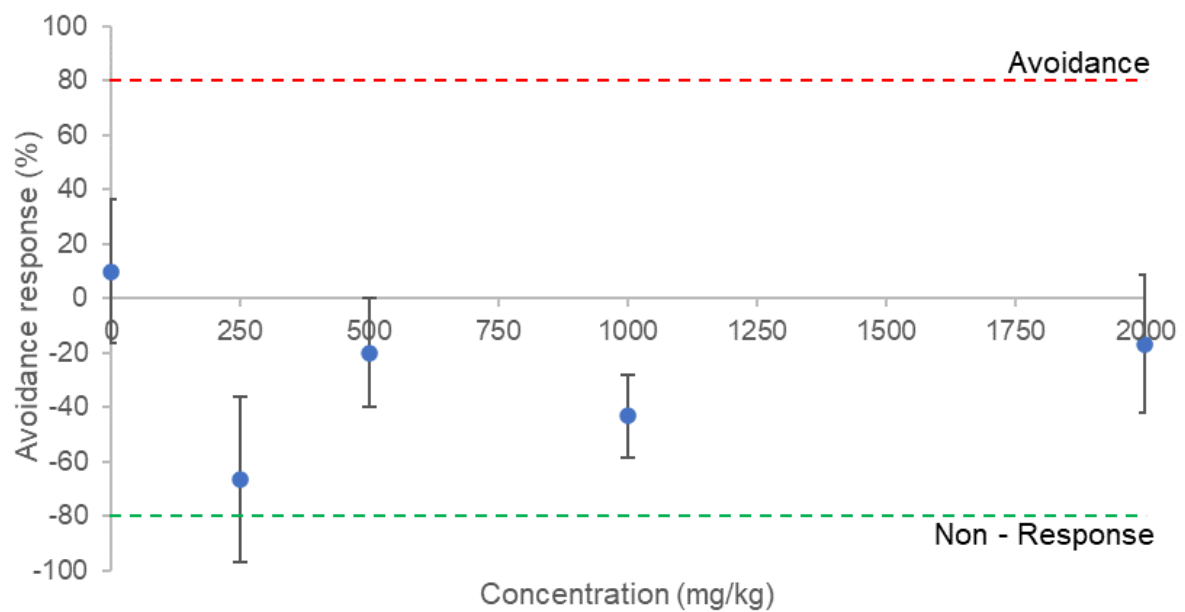


Figure 9: Avoidance response (%) of *E. andrei* after exposure to different concentrations of *B. pilosa* over a 48 h period. (n=10).

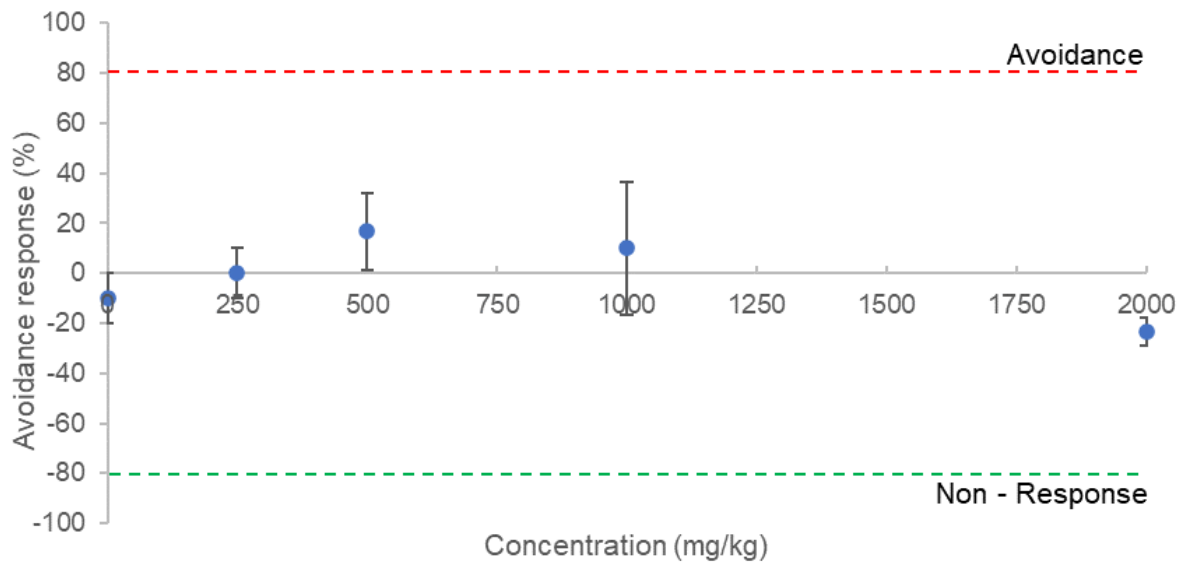


Figure 10: Avoidance response (%) of *E. andrei* after exposure to different concentrations of *T. minuta* over a 48 h period. (n=10).

The avoidance response (%) of the Fenamiphos exposures was higher than the plant biopesticides (*B. pilosa* and *T. minuta*), however, not above the threshold considered significant ($\geq 80\%$), according to the ISO guidelines (ISO, 2008). The highest concentration (200 mg/kg) had the highest avoidance response at 70% (Figure 11).

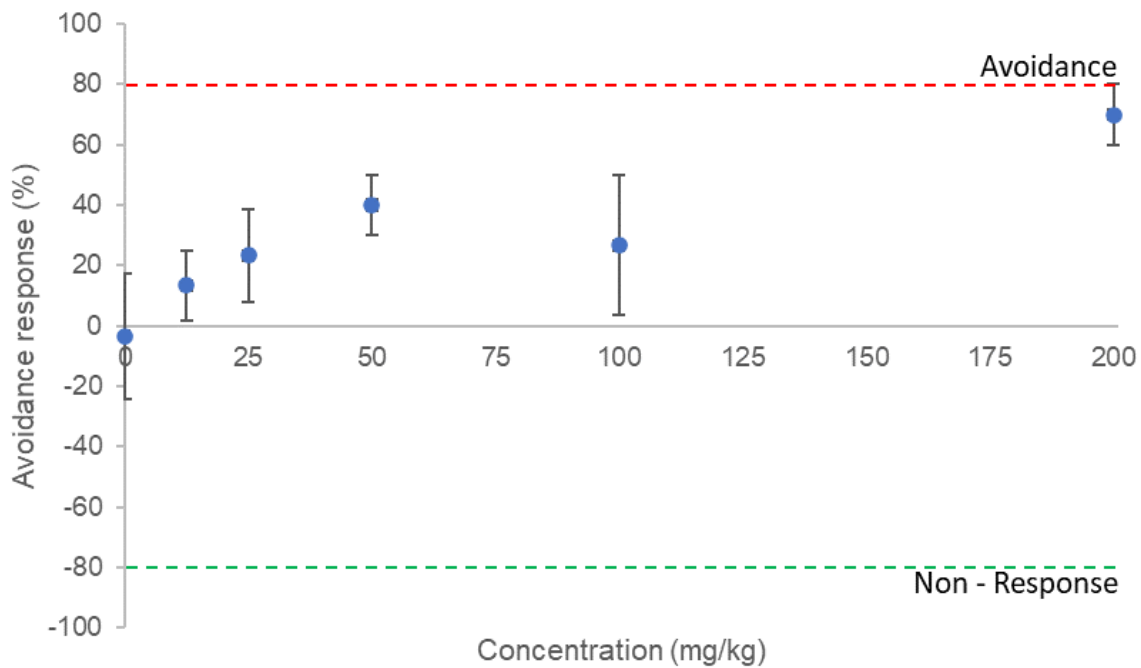


Figure 11: Avoidance response (%) of *E. andrei* after exposure to different concentrations of Fenamiphos over a 48 h period. (n=10).

4.2.4 Earthworm growth

Tables 9 and 10 show the relative growth rate (RGR%) of earthworms in soil spiked with *B. pilosa*, *T. minuta* and Fenamiphos, as well as the remediation of their potential effects by biochar. The control group showed a significant increase ($p < 0.05$) in biomass from day 1 to day 28 in all treatments. Table 9 shows that there was an increase in earthworm biomass in all concentrations of *B. pilosa* and *T. minuta* from day 1 to day 7 but declined gradually from day 14 to day 28. The biomass of the earthworms exposed to the Fenamiphos concentrations also increased during the first 7 days but declined rapidly from day 14 to day 28 in all concentrations (Table 9).

Table 9: The relative growth rate (RGR) (%) of *E. andrei* over 28 days of exposure to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos.

Treatment (mg/kg)	RGR (%)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.35 ^b	5.64 ^b	10.25 ^{ab}	20.96 ^a	20.59 ^a
Bp 250	0.32 ^b	26.85 ^a	20.17 ^a	15.90 ^a	14.19 ^{ab}
Bp 500	0.33 ^b	32.31 ^a	26.23 ^a	20.48 ^a	20.23 ^a
Bp 1000	0.33 ^b	31.69 ^a	27.31 ^a	21.02 ^a	22.60 ^a
Bp 2000	0.32 ^b	38.13 ^a	29.30 ^a	24.90 ^a	21.68 ^a
Tm 250	0.30 ^b	31.62 ^a	28.40 ^a	27.32 ^a	25.17 ^a
Tm 500	0.31 ^b	34.90 ^a	27.82 ^a	26.23 ^a	23.63 ^a
Tm 1000	0.30 ^b	34.67 ^a	31.43 ^a	29.57 ^a	27.18 ^a
Tm 2000	0.31 ^b	33.11 ^a	30.53 ^a	25.35 ^a	22.39 ^a
F 12.5	0.31 ^b	30.41 ^a	14.45 ^b	11.67 ^b	11.89 ^b
F 25	0.29 ^b	23.20 ^a	4.18 ^b	3.36 ^b	2.13 ^b
F 50	0.29 ^b	17.52 ^a	0.85 ^b	-2.93 ^b	-5.36 ^b
F 100	0.29 ^b	18.35 ^a	-0.14 ^b	-4.86 ^b	-8.46 ^b

Treatments: Bp (*B. pilosa*), Tm (*T. minuta*), F (Fenamiphos). Mean values with the same superscript letters across the row (Day 1 – Day 28) are not significantly different ($p < 0.05$). (n=30)

Growth assay with biochar

The growth assay with biochar as an ameliorant showed a reduction in relative growth rate of the earthworms for the plant biopesticide treatments (Table 10), compared to the growth in Table 9. The biomass of the earthworms treated with *B. pilosa* and *T. minuta* increased from day 1 to day 14, thereafter it decreases, but not statistically significant ($p > 0.05$), from day 14 to day 28. There is no significant difference ($p < 0.05$) in the growth of *E. andrei* between the two plant biopesticides. However, there was a significant ($p < 0.05$) decrease in biomass of the earthworms exposed to Fenamiphos. Importantly all concentrations from all treatments (*B. pilosa*, *T. minuta* and Fenamiphos) with biochar significantly differed ($p < 0.05$) in %RGR from all treatments without biochar from day 7 to day 28.

Table 10: The relative growth rate (RGR) (%) of *E. andrei* over 28 days of exposure to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos, with biochar (ameliorant) in all treatments.

Treatment (mg/kg)	RGR (%)				
	Day 1	Day 7	Day 14	Day 21	Day 28
Control	0.43 ^c	5.30 ^{bc}	16.06 ^{ab}	22.85 ^a	20.91 ^a
Bp 250	0.45 ^a	5.17 ^a	12.21 ^a	3.71 ^a	-0.43 ^a
Bp 500	0.41 ^a	6.51 ^a	12.00 ^a	7.51 ^a	0.92 ^a
Bp 1000	0.38 ^b	8.60 ^{ab}	19.36 ^a	8.90 ^{ab}	1.23 ^{ab}
Bp 2000	0.41 ^b	8.02 ^{ab}	15.88 ^a	7.34 ^{ab}	-0.60 ^b
Tm 250	0.42 ^a	6.06 ^a	12.31 ^a	5.54 ^a	0.94 ^a
Tm 500	0.43 ^b	6.99 ^{ab}	17.54 ^a	10.27 ^{ab}	0.06 ^b
Tm 1000	0.44 ^a	2.77 ^a	12.33 ^a	3.34 ^a	0.70 ^a
Tm 2000	0.43 ^b	8.03 ^{ab}	16.87 ^a	9.43 ^{ab}	1.16 ^b
F 12.5	0.43 ^a	5.64 ^a	9.23 ^a	2.79 ^a	-0.87 ^a
F 25	0.44 ^a	2.57 ^a	3.35 ^a	-5.87 ^a	-7.78 ^a
F 50	0.48 ^a	-1.19 ^a	-2.91 ^a	-6.06 ^a	-10.23 ^a
F 100	0.47 ^a	2.03 ^a	-2.37 ^{ab}	-8.57 ^{ab}	-12.18 ^b

Treatments: Bp (*B. pilosa*), Tm (*T. minuta*), F (Fenamiphos). Mean values with the same superscript letters across the row (Day 1 – Day 28) are not significantly different ($p < 0.05$). (n=30)

4.2.5 Reproduction

Reproductive success was measured by counting the number of cocoons and the number of juveniles that hatched during the 56-day exposure time. There was no significant difference ($p < 0.05$) in the mean number of cocoons in the *B. pilosa* and *T. minuta* treatments, as well as the lowest Fenamiphos concentration (12.5 mg/kg) compared to the control (Figure 12, 13 and 14). The concentrations (25 mg/kg, 50 mg/kg and 100 mg/kg) of Fenamiphos shows a significantly lower mean number of cocoons compared to the control and plant biopesticides (Figure 14 A).

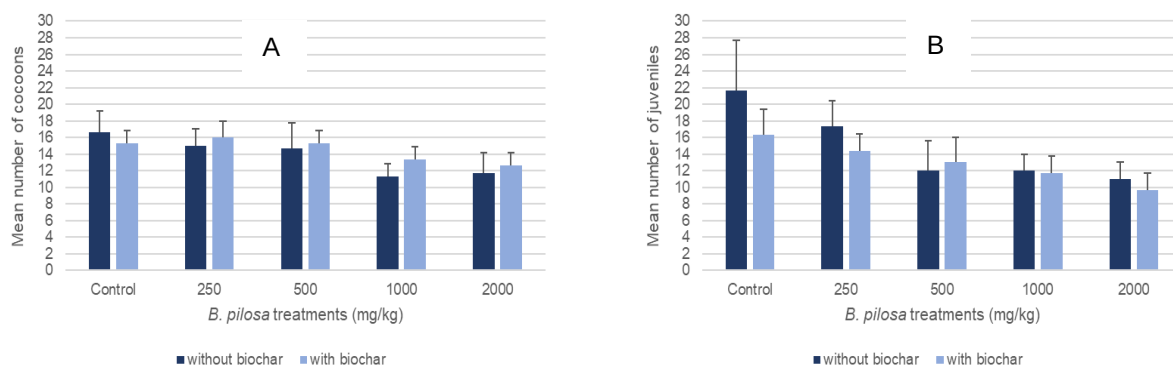


Figure 12: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of *B. pilosa* treatments at the end of the 56-day time period. Error bars indicate the standard deviation of the mean. (n=3). No significant difference ($p<0.05$) occurred.

None of the concentrations of the *B. pilosa* treatments and 250 mg/kg concentration of *T. minuta* showed significant difference ($p<0.05$) in the mean number of juveniles compared to the control. The 500 mg/kg and 2000 mg/kg concentrations of *T. minuta* differed significantly ($p<0.05$) from the control as well as all concentrations (12.5 mg/kg, 25 mg/kg, 50 mg/kg and 100 mg/kg) of the Fenamiphos treatments. The highest concentration (100 mg/kg) of Fenamiphos recorded the lowest mean number of juveniles and cocoons in all the treatments.

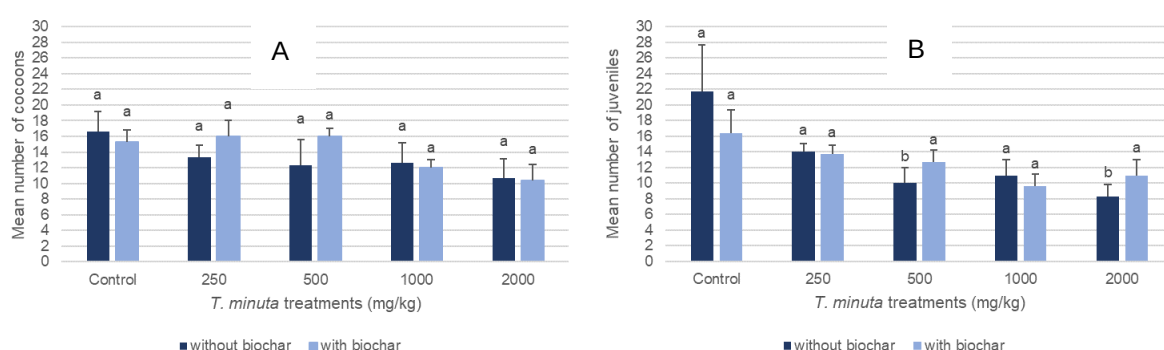


Figure 13: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of *T. minuta* treatments at the end of the 56-day time period. Different letters (a-b) indicate significant difference ($p<0.05$). Error bars indicate the standard deviation of the mean. (n=3).

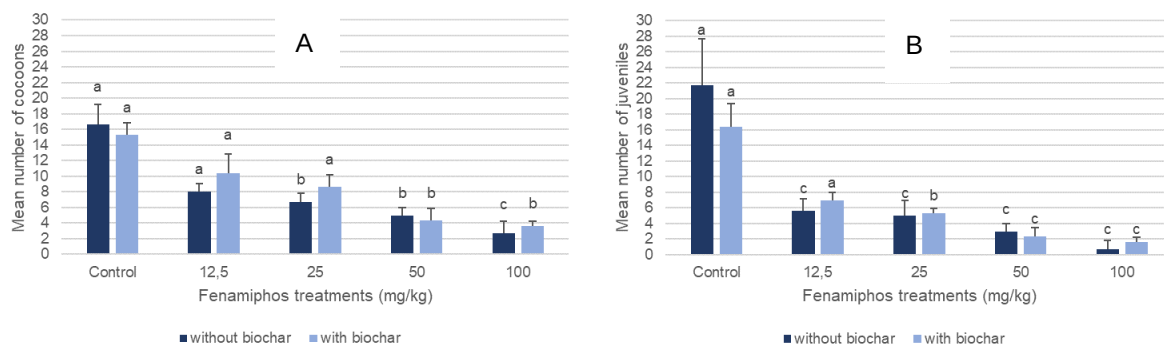


Figure 14: The mean number of cocoons (A) and juveniles (B) recorded in all concentrations of Fenamiphos treatments at the end of the 56-day time period. Different letters (a-c) indicate significant difference ($p < 0.05$). Error bars indicate the standard deviation of the mean. ($n=3$).

Biochar treatments for reproduction

None of the concentrations of *B. pilosa* and *T. minuta* treatments with biochar showed significant difference ($p < 0.05$) in the mean number of cocoons and juveniles compared to the control without biochar (Figure 12 and 13).

Likewise, the lowest Fenamiphos concentration (12.5 mg/kg) showed that there was no significant difference ($p < 0.05$) in both the mean number of cocoons and juveniles when compared to the controls as well as the treatments without biochar (Figure 14). The 25 mg/kg concentration of Fenamiphos showed no significant difference in the mean number of cocoons but the mean number of juveniles was significantly less ($p < 0.05$) than the control. The mean number of cocoons and juveniles in the two highest Fenamiphos concentrations (50 mg/kg and 100 mg/kg) was significantly less ($p < 0.05$) than the control and plant biopesticides (*B. pilosa* and *T. minuta*), with the highest Fenamiphos concentration (100 mg/kg) recording the lowest mean number of cocoons and juveniles of all treatments with biochar. When treatments with biochar were compared to treatments without biochar, no significant difference ($p < 0.05$) were observed (Figure 14).

4.2.6 Comet assay

The comet tail length was significantly longer ($p < 0.05$) in the 250 mg/kg, 500 mg/kg and 1000 mg/kg treatments of *B. pilosa* and 1000 mg/kg concentration of *T. minuta*

compared to the control. Interestingly the highest concentration (2000 mg/kg) of *B. pilosa* and *T. minuta* did not result in a significant change in comet tail length. All concentrations (12.5 mg/kg, 25 mg/kg, 50 mg/kg and 100 mg/kg) of Fenamiphos treatments without biochar had a significantly longer tail length ($p<0.05$) than the control and Fenamiphos treatments with biochar, especially the highest concentration of 100 mg/kg which was significantly longer than both the control and plant biopesticides (*B. pilosa* and *T. minuta*). Interestingly, both *B. pilosa* and *T. minuta* highest concentrations (2000 mg/kg) did not differ significantly ($p<0.05$) from the control (Figure 15 A and B).

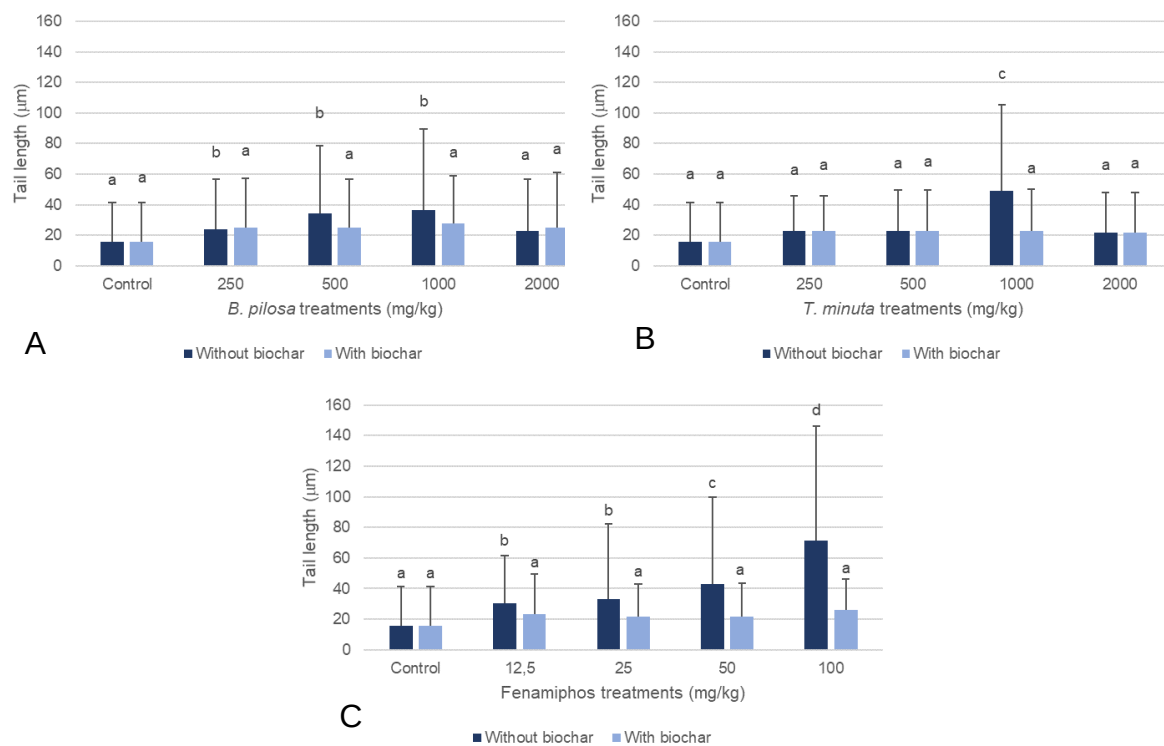


Figure 15: The mean comet tail length of coelomic cells of *E. andrei* after exposure to different concentrations of *B. pilosa* (A), *T. minuta* (B) and Fenamiphos (C) with and without biochar for 28 days. Different letters (a-d) represent the significant difference ($p<0.05$) between treatments with and without biochar. Error bars indicate the standard deviation of the mean. (n=9).

Comet assay with biochar

The result of the biochar comet assay shows no statistical difference ($p < 0.05$) in any of the treatments (*B. pilosa*, *T. minuta* and Fenamiphos) compared to the control (Figure 15 A-C). Compared to the treatments without biochar, the 250 and 2000 mg/kg concentrations of *B. pilosa* and 250, 500 and 2000 mg/kg concentrations of *T. minuta* resulted in no statistically significant difference ($p < 0.05$) compared to the treatments with biochar. This suggests that the DNA damage could have been ameliorated by the biochar, especially for the Fenamiphos treatments as overall it resulted in higher DNA damage.

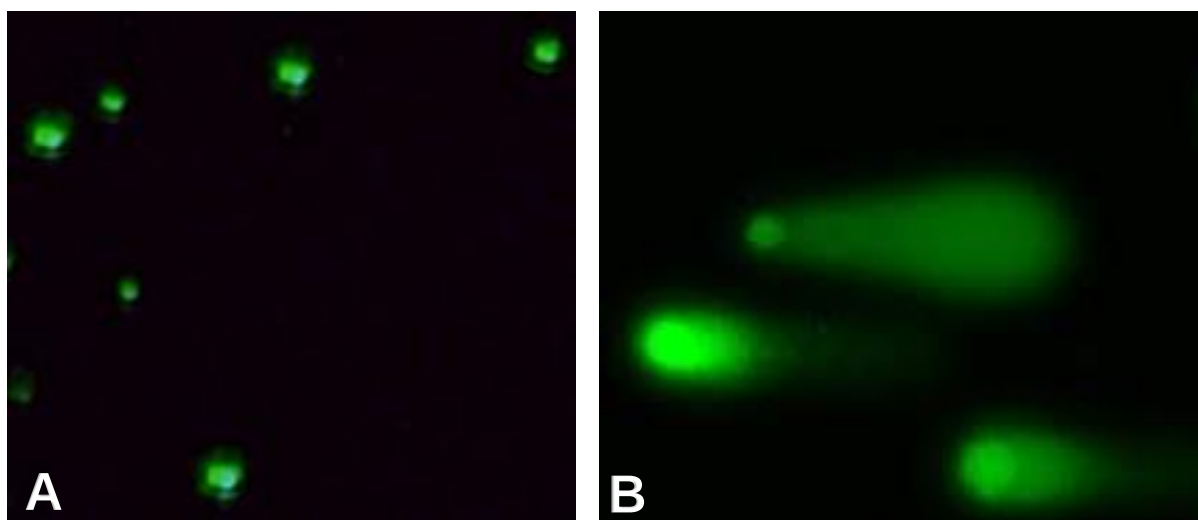


Figure 16: Fluorescence microscope images of earthworm coelomic cells observed during the comet assay. Image A shows the control group with intact DNA and image B shows the damaged DNA (taken from the 100 mg/kg Fenamiphos treatment). Image A taken at 10x magnification and Image B at 100x magnification.

4.2.7 Soil enzymatic activity

Tukey's honest significant difference test revealed that all concentrations of all treatments (*B. pilosa*, *T. minuta*, and Fenamiphos) shared at least one letter with the control treatment, indicating no significant difference ($p < 0.05$) in the alkaline phosphatase activity of the soils (Figure 14). Interestingly the 500 mg/kg *B. pilosa* concentration had the least alkaline phosphatase activity and the 25 mg/kg

Fenamiphos concentration had the most. This study reveals that the alkaline phosphatase activity of the soil is not concentration dependent.

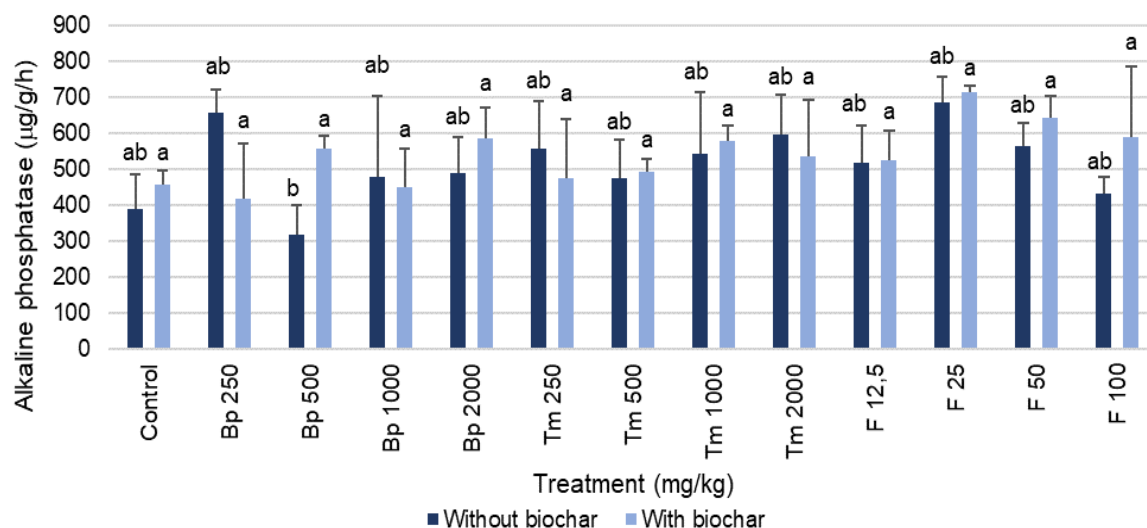


Figure 17: Enzymatic analyses of the alkaline phosphatase activity after exposure to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos with and without biochar. Treatments: Bp (*B. pilosa*), Tm (*T. minuta*) and F (Fenamiphos). Different letters represent the significant difference ($p < 0.05$). Error bars indicate the standard deviation of the mean. ($n=3$).

The β -glucosidase activity of the soil, all treatments except the 250 mg/kg of *T. minuta* treatment, 25 and 100 mg/kg Fenamiphos treatments did not statistically differ ($p < 0.05$) from the control (Figure 18). Overall, the 25 mg/kg Fenamiphos treatment soil had the highest β -glucosidase activity among all the treatments (Figure 18).

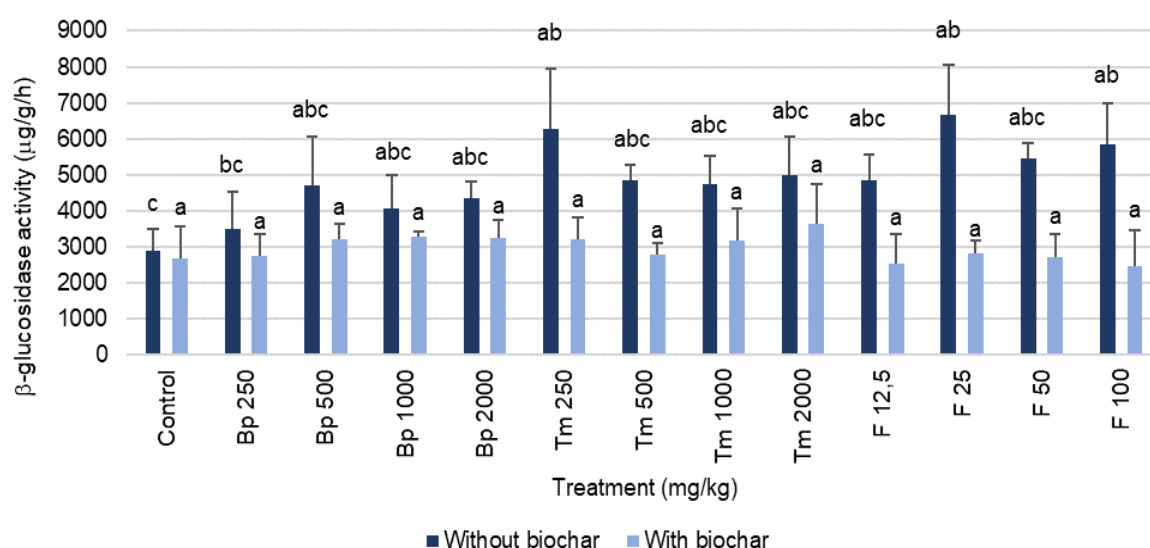


Figure 18: Enzymatic analyses of the β -glucosidase activity after exposure to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos with and without biochar. Treatments: Bp (*B. pilosa*), Tm (*T. minuta*) and F (Fenamiphos). Different letters represent the significant difference ($p < 0.05$). Error bars indicate standard deviation of the mean. ($n=3$).

The Urease activity of the soils shows no significant difference ($p < 0.05$) between all treatments (*B. pilosa*, *T. minuta* and Fenamiphos) and the control (Figure 19). Once again, the 25 mg/kg Fenamiphos treatment had the highest activity and was the only treatment that significantly differed ($p < 0.05$) from the plant biopesticides (*B. pilosa* and *T. minuta*).

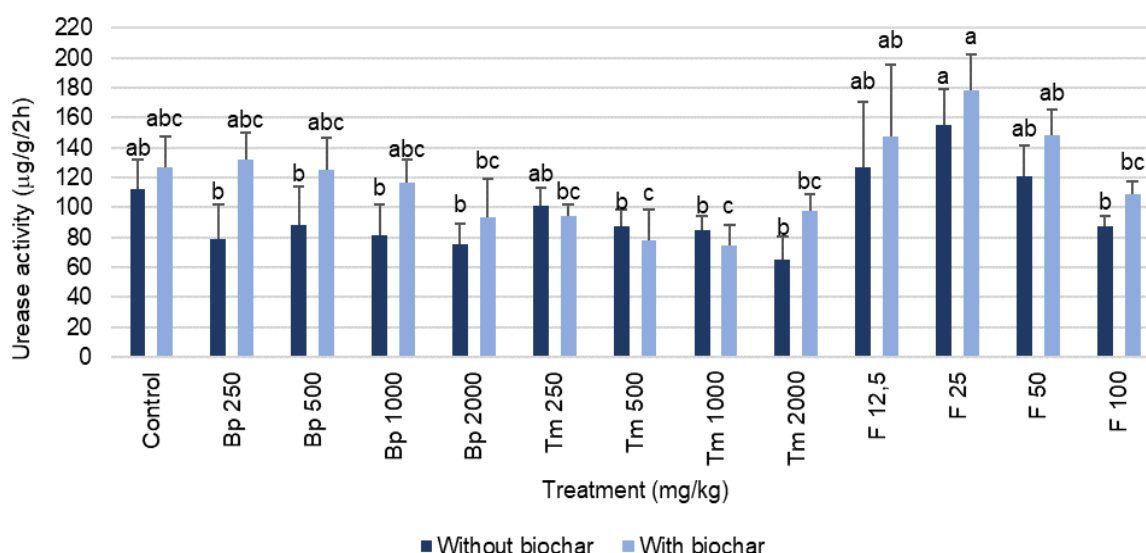


Figure 15: Urease activity after exposure to different concentrations of *B. pilosa*, *T. minuta* and Fenamiphos with and without biochar. Treatments: Bp (*B. pilosa*), Tm (*T. minuta*) and F (Fenamiphos). Different letters represent the significant difference ($p < 0.05$). Error bars indicate the standard deviation of the mean. ($n=3$).

Enzymatic activity of biochar treated soil:

The alkaline phosphatase assay with biochar resulted in no significant difference ($p < 0.05$) between all treatments (*B. pilosa*, *T. minuta* and Fenamiphos) and the control (Figure 17). However, the 25 mg/kg Fenamiphos treatment with biochar had the highest alkaline phosphatase activity.

Interestingly, only the 500 mg/kg concentration of the *B. pilosa* treatments with biochar had significantly more ($p < 0.05$) alkaline phosphatase activity compared to the 500 mg/kg concentration of *B. pilosa* without biochar.

The β -glucosidase assay with biochar likewise shows no significant difference ($p < 0.05$) between all the treatments (*B. pilosa*, *T. minuta* and Fenamiphos) and the control (Figure 18).

Overall, all treatments with biochar showed lower β -glucosidase activity than the treatments without biochar. None of the *B. pilosa* treatments with biochar showed significant difference ($p < 0.05$) compared to the *B. pilosa* treatments without biochar. The 250 and 500 mg/kg concentrations of the *T. minuta* treatments had significantly

less ($p < 0.05$) β -glucosidase activity than the same concentrations of *T. minuta* treatments without biochar. All concentrations of the Fenamiphos treatments with biochar had significantly less ($p < 0.05$) β -glucosidase activity than the Fenamiphos treatments without biochar.

All treatments (*B. pilosa*, *T. minuta* and Fenamiphos) from the urease assay with biochar shared at least one letter with the control, indicating no significant difference ($p < 0.05$). However, the 12.5 and 50 mg/kg Fenamiphos treatments with biochar did significantly differ ($p < 0.05$) from the 500 and 1000 mg/kg *T. minuta* treatments with biochar. The 25 mg/kg Fenamiphos treatment with biochar had the highest urease activity and significantly differed ($p < 0.05$) from all concentrations of the *T. minuta* treatments with biochar, the 2000 mg/kg *B. pilosa* treatment, and 100 mg/kg Fenamiphos treatment (Figure 19).

In general, the Fenamiphos treatments with biochar had higher urease activity than the *B. pilosa* and *T. minuta* treatments with biochar. Only the 250 mg/kg *B. pilosa*, 2000 mg/kg *T. minuta* and 100 mg/kg Fenamiphos treatments with biochar had significantly more ($p < 0.05$) urease activity than the same concentrations of *B. pilosa*, *T. minuta* and Fenamiphos treatments without biochar (Figure 19).

Chapter 5: Discussion

Plant biopesticides contain phytochemicals such as alkaloids, terpenoids, phenolics, and other secondary metabolites that allow them to be used for pest management (Tijjani *et al.* 2017). The mixture of biologically active substances found in plant biopesticides prevents pests from developing resistance (Dimetry, 2014). The secondary metabolites found in plant extracts, in their natural or isolated form, are a promising alternative to synthetic pesticides as they are more environmentally friendly and leave no residue on crops or the environment.

5.1 Green pesticide research

The increase in publications from 2009 could be due to the increasing realization of the detrimental effects of synthetic pesticides on the environment (Mahmood *et al.* 2016), the growing need for a sustainable alternative, government policies on environmental protection and pressure groups (Rosell *et al.* 2008).

To determine the productivity of a research field based on a bibliometric study, the number of scientific publications are investigated (Sun *et al.* 2018). A change in the number of publications could indicate a possible change in the research field. Considering the data collected in this study as shown in Table 6, a total of 580 documents from 332 sources were retrieved from the Scopus database. These documents were authored by 1867 researchers, on green pesticides from 1994 to 2019 with an estimate of 0.311 documents per author and 3.22 authors per document. Out of the 1867 authors, 1799 were authors of multi-authored documents with the remaining 68 being authors of single-authored documents. The average number of authors per document is referred to as collaboration index (Siamaki *et al.* 2014). Collaboration has a great impact on the citation of a document, particularly internationally co-authored papers received twice the average citation as local co-authored papers (Nguyen *et al.* 2017). The collaboration index for this study was 3.59, which is considered as high participation of co-authorship (Siamaki *et al.* 2014). A study by (Liu *et al.* 2011) on global biodiversity research, reported the worldwide research collaboration index to be 4.45, which is higher than this study but it is important to note that biodiversity research dates further back than green pesticide research, however, research on green pesticide is increasing rapidly.

In Table 7, the keyword-plus; 'animal/s' is ranked first. This could be due to the increasing use of plant phytochemicals for ectoparasites on livestock (Nyahangare *et al.* 2015). The use of plant biopesticides is common especially among communities in rural areas that are dependent on livestock for survival. Aside from the cultural practice, these communities often do not have the financial capability to buy pesticides and instead make use of biopesticides for pest management (Nyahangare *et al.* 2015).

In Figure 17, India and China are ranked first and second respectively in green pesticide research, this may be attributed to their long history of the use of botanical insecticides (Isman, 2006). The African continent has 5 countries in the top 30 most productive countries: Nigeria (7th), Kenya (14th), Egypt (16th), South Africa (17th) and Ethiopia (19th) with 9, 6, 5, 5 and 4 publications respectively. Although Africa consumes less than 5% of the global pesticides, most countries in Africa still use the most toxic pesticides in food production (Williamson, 2011). According to Dinham (2003), small-scale farmers apply these toxic pesticides weekly, sometimes more often, during the growing season. This raises several concerns to both environmental and human health experts in Africa. The above-mentioned factors indicate that African countries are not doing enough in pesticide regulations and research towards an alternative.

5.2. Contact filter paper test

The filter paper contact toxicity test was used as a pre-screening method to determine whether the plant biopesticides and a synthetic pesticide (Fenamiphos) were potentially toxic to earthworms. This was carried out according to the OECD 207 guideline (OECD, 1984). The percentage mortality increased with an increase in the concentration of *B. pilosa* and *T. minuta* extracts, as well as Fenamiphos. The study revealed there was overall higher earthworm mortality recorded for *T. minuta* extract compared to *B. pilosa* extract, suggesting that the former is more toxic. Each concentration of *T. minuta* resulted in a higher percentage mortality than *B. pilosa*.

The synthetic pesticide, Fenamiphos, had significantly higher percentage mortality at lower concentrations ($LC_{50} = 0,006 \text{ mg/cm}^2$) than both plant biopesticides (*B. pilosa* and *T. minuta*). The earthworms showed signs of agitation (wriggling) when exposed to the filter paper treated with Fenamiphos and climbed to the lids of the petri dishes

to avoid contact, indicating more agitation compared to the plant biopesticides (*B. pilosa* and *T. minuta*). This indicates that soil contaminated with Fenamiphos might have a detrimental impact on earthworm communities and could have a cascading effect on the soil ecosystem.

5.3 Artificial soil test

The toxicity test in artificial soil resulted in no earthworm mortalities for both *B. pilosa* and *T. minuta*, despite the concentration being increased up to 2000 mg/kg. The artificial soil spiked with Fenamiphos on the other hand, did result in mortalities and the median lethal concentration (LC₅₀) was evaluated to be 163,85 mg/kg. However, the LC₅₀ value for Fenamiphos varies in different studies. According to EFSA (2006), the LC₅₀ for Fenamiphos towards earthworms in soil with 10% organic matter is 888 mg/kg after 28 days of exposure. However, the study done by Càceres *et al.* (2011) determined the LC₅₀ to be 228 mg/kg for earthworms after 28 days of exposure, which is relatively close to the LC₅₀ determined during this study. The variation in the LC₅₀ value among studies could be ascribed to different soil types, the selection of earthworm species that could be more or less sensitive to pesticides, and the behaviour of pesticides in soil (van Gestel, 1992).

5.4 Avoidance behaviour assay

The avoidance response of the earthworms for all three test substances (*B. pilosa*, *T. minuta* and Fenamiphos) was not statistically significant. The highest concentration of Fenamiphos resulted in a 70% avoidance response, thus it is not considered significant ($\geq 80\%$) (Amorim *et al.* 2005). However, these results contradict the findings of Càceres *et al.* (2011) where they tested the toxicity of Fenamiphos towards *Eisenia fetida* in alkaline, neutral and acidic soil. In their study the earthworms avoided contact with the soil at concentrations of 100, 175 and 200 mg/kg by climbing to the lids of the containers, but after 24h burrowed into the soil at the 100 and 175 mg/kg concentrations. The differences in the outcome of the avoidance response could be due to the different species of earthworm used during the respective studies, as some species respond differently when exposed to pesticides (van Gestel, 1992). Volatility,

most likely, did not play a role in the avoidance behaviour of the earthworms as Fenamiphos has low volatility (Lewis *et al.* 2016).

5.5 Earthworm growth

Growth and reproduction are important measures to evaluate an individual's fitness after being exposed to a possible contaminant and are regarded as valuable ecotoxicology parameters (van Gestel and van Brummelen, 1996). The results indicated that the plant biopesticide (*B. pilosa* and *T. minuta*) treatments decreased earthworm relative growth rate (%RGR) from day 7 to day 28. Although there was no significant difference ($p < 0.05$) between the *B. pilosa* and *T. minuta* treatments, however, the relative growth rate of both plant biopesticides were significantly different ($p < 0.05$) from the controls (positive and negative). Studies regarding the effect of plant biopesticides on earthworms are scarce, however, the study by Ponsankar *et al.* (2016) used crude extracts derived from the flowers of *Couroupita guianensis* to determine the toxicity towards earthworms (*Eisenia fetida*) and compare it to the synthetic pesticides monoprothos and cypermethrin. The results obtained from this study correlate with the study of Ponsankar *et al.* (2016), where the synthetic pesticide is significantly more toxic to earthworms at much lower concentrations than the plant biopesticides.

The Fenamiphos treatments significantly ($p < 0.05$) decreased the earthworm relative growth rate (%). An increase in Fenamiphos concentration resulted in a decrease of relative growth rate (%), with the highest concentration (100 mg/kg) decreasing the %RGR the most, indicating that the response is concentration dependent. A similar trend was reported in the studies done by Caceres *et al.* (2011) and Yasmin and D'Souza (2010), where the earthworm biomass decreased with an increase in the concentration of Fenamiphos. Although the %RGR of earthworms exposed to Fenamiphos decreases significantly with an increase in concentration, there were no mortalities recorded at all concentrations (12.5, 25, 50 and 100 mg/kg) during the 28-day experiment. This is contrary to the study done by Caceres *et al.* (2011), where the 25, 50 and 100 mg/kg Fenamiphos concentrations resulted in 10%, 20% and 30% earthworm mortality respectively. However, our study revealed that Fenamiphos treatments, even at lower concentrations, indicate higher toxicity towards earthworms

than the plant biopesticides (*B. pilosa* and *T. minuta*) treatments and control. A possible explanation for the lower toxicity of the plant biopesticides could be that it does not affect non-target organisms and has a short half-life whereas synthetic pesticides are harmful to non-target organisms and remain in the environment for a long period (Walia, 2017). It is also important to note that only water soluble compounds were extracted with the method used during this study, which excludes certain compounds (which can be extracted using alternative methods) that could possibly affect non-target organisms.

Biochar treatments

A few studies revealed that growth assays with biochar as an ameliorant to soil contaminated with heavy metals and organic waste, improved the percentage relative growth rate (%RGR) of the earthworms compared to the growth assay without biochar. This can be attributed to the adsorptive capacity of biochar for contaminants (Nartey & Zhao, 2014). In this study, contrary to the result of the earthworm growth (without biochar), both plant biopesticide (*B. pilosa* and *T. minuta*) extracts gave a positive %RGR from day 1 to day 14, where after the %RGR declined until the end of the test period (day 28) in soil treated with biochar, resulting in a negative %RGR which was significantly lower ($p<0.05$) than the %RGR on day 28 of the test without biochar. To the best of our knowledge, the effect of biochar as an ameliorant for biopesticide spiked soil has not been researched. Therefore, these results are a possible indication that biochar does not improve earthworm growth in plant biopesticide spiked soil.

All the Fenamiphos treatments showed a negative relative growth rate (%) at the end of the 28-day test period, with the highest concentration (100 mg/kg) having the biggest negative impact on earthworm growth. Interestingly, the 50 mg/kg concentration showed a negative relative growth rate from day 7 to day 28 while the 100 mg/kg concentration showed a positive relative growth rate on day 7 before continuously declining until the end of the test period (day 28). By comparison, the relative growth rate of earthworms in soil with Fenamiphos treatments with biochar was significantly lower ($p<0.05$) than the control, both plant biopesticide treatments (*B. pilosa* and *T. minuta*), and the Fenamiphos treatments without biochar. The %RGR remained negative with wider margins at the end of the test period (day 28). All

treatments (*B. pilosa*, *T. minuta* and Fenamiphos) amended with biochar had significantly lower ($p<0.05$) %RGR compared to the treatments without biochar, indicating that biochar does not ameliorate the detrimental effects of plant biopesticides and synthetic pesticides. Ghani *et al.* (2018) conducted a study to determine the effect of biochar on the sorption, dissipation and uptake of Fenamiphos which concluded that biochar did not affect the efficacy of Fenamiphos. The study also indicated that biochar could increase the half-life of Fenamiphos from 2.7 days to 18.3 days (Ghani *et al.* 2018). This supports the results of this study where the Fenamiphos and plant biopesticides (*B. pilosa* and *T. minuta*) spiked soils, treated with biochar, had a greater negative effect on the earthworm %RGR than the treatments without biochar.

5.6 Reproduction

The reproduction was evaluated by counting the number of cocoons on day 28 and the number of juveniles on day 56. It was recorded that the extracts of the plant biopesticides (*B. pilosa* and *T. minuta*) at varying concentrations had no significant difference ($p<0.05$) compared to the control, indicating the extracts does not affect the cocoon production of *E. andrei*.

However, the mean number of juveniles from the 500 and 2000 mg/kg concentrations of *T. minuta* treatments was significantly less ($p<0.05$) compared to the control. As there is little to no research on the effect of biopesticides on the reproduction of earthworms, it is difficult to explain why *T. minuta* at these concentrations had a detrimental effect on the mean number of juveniles. A possible explanation could be ascribed to the chemical compounds (limonene, tagetone, tagetenone etc.) reported being present in the *T. minuta* extracts (Chamorro *et al.* 2008) which could negatively affect the number of juveniles that hatch.

All the treatments of Fenamiphos resulted in a significant ($p<0.05$) reduction in cocoon production and juveniles compared to the control and plant biopesticide (*B. pilosa* and *T. minuta*) treatments, except the lowest Fenamiphos concentration (12.5 mg/kg) treatment that did not significantly differ from the control. The higher the concentration of Fenamiphos the more detrimental the effect on the number of cocoons and juveniles. A study done by Farrukh and Ali (2011), found that cocoon production was

inhibited by the organophosphate Dichlorovos and was also concentration-dependent. They reported that at 38 and 76 mg/kg, the number of cocoons was significantly different from the control whereas the 19 mg/kg concentration was not significantly different from the control. This aligns with the results of this study where the number of cocoons was dependent on the concentration of Fenamiphos applied to the soil.

Biochar treatments

Interestingly, the biochar treated samples do not have a statistically different mean number of cocoons and juveniles for both plant biopesticide (*B. pilosa* and *T. minuta*) treatments compared to the control. This suggests that biochar is able to ameliorate the possible impact of biopesticides on earthworm reproduction (Han *et al.* 2021). The 50 and 100 mg/kg Fenamiphos treatments with biochar had the most severe effect on the mean number of cocoons and juveniles. These results indicate that biochar did not act as an effective ameliorant for Fenamiphos spiked soils. This is supported by the study done by Ghani *et al.* (2018), where biochar produced from date palm and *eucalyptus* was used to amend soils treated with Fenamiphos and Cadusafos. The results showed that the half-life of Fenamiphos was increased from 2.7 days to 18.3 days due to adsorption.

5.7 Comet assay

The mean comet tail length of the coelomic cells increased with an increase in concentration for the *B. pilosa* treatments, except for the highest concentration which surprisingly did not have a significant difference ($p < 0.05$) compared to the control. On the other hand, only the 1000 mg/kg concentration of the *T. minuta* treatments showed significant ($p < 0.05$) DNA damage compared to the control. It is thus difficult to compare the result with literature as the information on the genotoxicity of biopesticides towards earthworms is limited. However, a study by Muangphra and Gooneratne (2011), tested the toxicity of Neem extract on earthworms, and no significant difference occurred in the comet tail length compared to the control, which contradicts the results of this study. It is noteworthy that Neem extract consists of different chemical compounds and also a different species of earthworm was used in their study.

The Fenamiphos treatments showed an increase in mean comet tail length with an increase in concentration. The genotoxicity of Fenamiphos was the highest at the 100 mg/kg concentration. These results correspond with the outcome of the study conducted by Zhu *et al.* (2020), who evaluated the genotoxicity of chlorpyrifos (an organophosphate insecticide) on earthworms.

Biochar treatments

All treatments (*B. pilosa*, *T. minuta* and Fenamiphos) ameliorated with biochar resulted in no significant difference ($p < 0.05$) in the mean comet tail length compared to the control. This suggests that biochar prevent damage at the DNA level. The study by Zhang *et al.* (2019), also found that biochar could reduce the level of DNA damage in earthworms caused by a herbicide (mesotrione). However, other studies have found that the application rate of different types of biochar itself could lead to DNA damage in earthworms (Kim *et al.* 2014; Han *et al.* 2021). It is thus important to determine the application rate for different types of biochar which could influence the contaminant bioavailability and soil organism health (Zhang *et al.* 2019).

5.8 Soil enzymatic activity

No significant difference was recorded between the control, Fenamiphos and plant biopesticides (*B. pilosa* and *T. minuta*) treatments during the alkaline phosphatase assay. These results correlate with the study of Mishra *et al.* (2019), where they compared the effect of three plant extracts (*Cleistanthus collinus*, *Lantana camara* and *Strychnos nux-vomica*) and the synthetic pesticide, Chlorpyrifos, on soil enzyme activities. After 30 days of exposure to the respective plant extracts and Chlorpyrifos, no significant difference was reported between the treatments and the control.

Treatments with Fenamiphos produce more β -glucosidase activity in the soil than the biopesticides (*B. pilosa* and *T. minuta*) and the control. This increases with an increase in the concentrations of Fenamiphos in the soil. This contradicts the results of the study done by Mishra *et al.* (2019), where the pesticide Chlorpyrifos had the lowest β -glucosidase activity compared to the plant extracts of *C. collinus*, *L. camara* and *S. nux-vomica*.

The results of the plant biopesticides (*B. pilosa* and *T. minuta*) correlate with the results of the study done by Campolo *et al.* (2020), where the effect of citrus essential oil on soil enzymes was tested, and no significant difference was recorded compared to the control. However, Campolo *et al.* (2020), did record that the pesticide Chlorpyrifos had significantly lower urease activity than the citrus essential oil, which contradicts the results of this study.

Overall, neither the plant biopesticides (*B. pilosa* and *T. minuta*) nor synthetic pesticide (Fenamiphos) negatively impacted soil enzymatic activity which is a positive outcome as lower soil enzyme activity can affect nutrient availability to plants.

Biochar treatments

The environment plays a role in determining the interaction between biochar and soil enzymes. The environment can either increase or decrease enzyme activity (Foster, *et al.* 2018). A number of factors can also attribute to field results, such as the rate of biochar application, type of soil enzymes and soil type (Oleszczuk *et al.* 2014).

All concentrations of the treatments with biochar resulted in no significant difference ($p < 0.05$) from the control and treatments without biochar in the alkaline phosphatase assay. The same result was recorded in the study by Manori *et al.* (2021), where pine needle biochar was added to soil spiked with different cadmium concentrations.

The β -glucosidase activity also resulted in no significant difference ($p < 0.05$) between all treatments of *B. pilosa* and *T. minuta* at different concentrations, with biochar as an ameliorant compared to treatments without biochar. Overall the spiked soil amended with biochar resulted in less β -glucosidase activity compared to the spiked soil without biochar. The β -glucosidase was significantly lower ($p < 0.05$) in all concentrations of Fenamiphos treatments with biochar compared to the treatments without biochar. The results correlate with the study done by Foster *et al.* (2018) where they tested the effect of pine biochar, used as an ameliorant for agricultural soil, on β -glucosidase and acid phosphatase activity. They also found that pine biochar decreased the β -glucosidase activity in the soil, and a possible explanation could be the sorption characteristic of biochar that influences enzyme activity (Foster *et al.* 2018).

Overall, the urease activity is high in all treatments and higher in the Fenamiphos treatments at the 25 mg/kg concentration in both biochar and non-biochar treatments. The results from this study contradict the findings of Liu *et al.* (2018), where biochar made from corn cobs at different temperatures, inhibited the urease activity in soil. This could be because of different types of biochar being used, as the biochar for this study was made from pinewood. It is also noteworthy that the temperature for the pyrolysis of biochar production can alter the effect it has on urease activity (Liu *et al.* 2018).

Chapter 6: Conclusions and recommendations

6.1 Conclusions

The bibliometric study discussed the global trend of green pesticide research from 1994 to 2019 based on documents retrieved from Scopus. Despite the increasing research on green pesticides, commercial applications remain limited. Trends in the annual scientific production suggest that research on green pesticides will continue to increase. The detrimental effects of synthetic pesticides on the environment and human health could be the possible drivers for research productivity in the alternative field of plant biopesticides. This study also found that India, China and USA are the leading countries in the production of green biopesticide research articles and amass the highest total citations per country. Countries in Europe and Africa, although high consumers of pesticides have significantly less published research compared to the later mentioned countries. These countries need to intensify their research on alternative pesticides either through government or privately funded programs to promote environmentally friendly pesticides, especially in Africa where pesticides with high toxicity are still being used.

The effects of biopesticides were assessed on the avoidance behaviour, survival, growth, and reproduction of earthworms, and whether biochar acted as efficient ameliorant for spiked soil. Overall, the synthetic pesticide Fenamiphos, had more detrimental effects on earthworms than the plant biopesticides (*B. pilosa* and *T. minuta*). The plant biopesticides (*B. pilosa* and *T. minuta*) had no significant effect on the %RGR or reproduction of the earthworms compared to the control. Fenamiphos negatively affected the earthworm's %RGR, cocoon production and number of juveniles compared to the control and plant biopesticides (*B. pilosa* and *T. minuta*).

With biochar added to the spiked soils as ameliorant, it negatively impacted the earthworm %RGR exposed to Fenamiphos and the plant biopesticides (*B. pilosa* and *T. minuta*) when compared to the treatments without biochar. Indicating biochar possibly contributes to the detrimental effects of Fenamiphos and extracts of *B. pilosa* and *T. minuta* towards earthworms. Biochar did not affect the reproduction of the earthworms in all treatments.

The genotoxicity of Fenamiphos and the plant biopesticides (*B. pilosa* and *T. minuta*) were assessed utilising the comet assay. Mixed results were obtained from the genotoxicity of the plant biopesticides. The highest concentration (2000 mg/kg) for both plant extracts (*B. pilosa* and *T. minuta*) resulted in no significant earthworm DNA damage. However, the lower concentrations of *B. pilosa* had a genotoxic effect on earthworms, as well as the 1000 mg/kg *T. minuta* concentration. The genotoxic effect of Fenamiphos was more severe than the plant biopesticides (*B. pilosa* and *T. minuta*) at much lower concentrations. The high level of strand breaks in the earthworm DNA indicates the cellular stress when earthworms are exposed to Fenamiphos. Treatments with biochar shows decreased genotoxicity compared to treatments without biochar, indicating biochar prevents the cellular stress of earthworms when exposed to Fenamiphos, and plant biopesticides *B. pilosa* and *T. minuta*.

Analysis of the soil enzymes revealed there is no significant difference for all treatments (Fenamiphos, *B. pilosa* and *T. minuta*) in the alkaline phosphatase assay, indicating the treatments used in this study do not affect the alkaline phosphatase activity of the soil. The β -glucosidase activity of the soil is significantly high only in *T. minuta* and Fenamiphos concentrations. The soil's urease activity was overall higher for the Fenamiphos treatments.

The soils amended with biochar had no effect on the alkaline phosphatase and β -glucosidase activities of the soil. The urease activity was overall decreased in the biochar amended treatments.

In conclusion, biochar may have been an effective ameliorant for soil contaminated with heavy metals and organic waste. This study found that biochar does not improve earthworms' percentage relative growth rate (%RGR). Soil enzymatic activities such as alkaline phosphatase and β -glucosidase activities of the soil were unaffected by biochar. On the other hand, biochar was able to improve the fecundity of *E. andrei* by increasing the number of cocoons and juveniles. In addition, biochar protected *E. andrei* DNA from genotoxicity.

6.2 Recommendations

New questions arose after conducting this study and below are recommendations for future studies.

Further research is required on the effects of plant biopesticides on earthworms under realistic agricultural conditions to form a better understanding between pest management and soil organisms. It is also recommended to use a registered biopesticide, as self-produced plant biopesticides could be unregulated and extracts could possibly have negative effects on soil organisms and soil health.

Different application methods of the plant biopesticides could be studied and incorporated into future studies.

Different types of biochar are recommended for future studies, as it can influence soil enzyme activity, earthworm growth and prevent DNA damage. Biochar efficacy are also application rate dependent, and it is recommended to test different application rates to optimize the efficacy.

Other variables that could affect the toxicity of plant biopesticides are also recommended for future studies. Variables such as temperature and soil moisture are important parameters to consider.

Histology should be incorporated in future studies to determine if plant biopesticides could cause tissue damage in soil organisms.

References

- Ahmad, M., Rajapaksha, A.U., Lim, J.E., Zhang, M., Bolan, N., Mohan, D., Vithanage, M., Lee, S.S., and Ok, Y.S. 2014. Biochar as a sorbent for contaminant management in soil and water: A review. *Chemosphere* 99: 19–33. Elsevier Ltd. doi:10.1016/j.chemosphere.2013.10.071.
- Aktar, W., Sengupta, D., and Chowdhury, A. 2009. Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary Toxicology* 2(1): 1–12. doi:10.2478/v10102-009-0001-7.
- Alef, K., and Nannipieri, P. 1995. *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press.
- Amorim, M.J.B., Rombke, J., and Soares, A.M.V.M. 2005. Avoidance behaviour of *Enchytraeus albidus*: Effects of Benomyl, Carbendazim, phenmedipham and different soil types. *Chemosphere* 59: 501–510. doi:10.1016/j.chemosphere.2005.01.057.
- Attademo, A.M., Sanchez-Hernandez, J.C., Lajmanovich, R.C., Repetti, M.R., and Peltzer, P.M. 2021. Enzyme Activities as Indicators of Soil Quality: Response to Intensive Soybean and Rice Crops. *Water, Air, and Soil Pollution* 232(7): 1–12. Springer Science and Business Media Deutschland GmbH. doi:10.1007/S11270-021-05211-2/TABLES/2.
- Bartlett, M.D., Briones, M.J.I., Neilson, R., Schmidt, O., Spurgeon, D., and Creamer, R.E. 2010. A critical review of current methods in earthworm ecology: From individuals to populations. *European Journal of Soil Biology* 46: 67–73. doi:10.1016/j.ejsobi.2009.11.006.
- Bartolome, A.P., Villaseñor, I.M., and Yang, W. 2013. *Bidens pilosa* L. (Asteraceae): Botanical Properties , Traditional Uses , Phytochemistry , and Pharmacology. *Evidence-based Complementary and Alternative Medicine* 2: 1–51.
- Batish, D.R., Arora, K., Singh, H.P., and Kohli, R.K. 2007. Potential utilization of dried powder of *Tagetes minuta* as a natural herbicide for managing rice weeds. *Crop Protection* 26: 566–571. doi:10.1016/j.cropro.2006.05.008.
- Beesley, L., Moreno-Jiménez, E., Gomez-Eyles, J.L., Harris, E., Robinson, B., and Sizmur, T. 2011. A review of biochars' potential role in the remediation, revegetation

and restoration of contaminated soils. *Environmental Pollution* 159(12): 3269–3282. Elsevier Ltd. doi:10.1016/j.envpol.2011.07.023.

Boobis, A.R., Ossendorp, B.C., Banasiak, U., Hamey, P.Y., Sebestyen, I., and Moretto, A. 2008. Cumulative risk assessment of pesticide residues in food. *Toxicology Letters* 180(2): 137–150. doi:10.1016/j.toxlet.2008.06.004.

Cáceres, T., Ying, G.G., and Kookana, R. 2002. Sorption of pesticides used in banana production on soils of Ecuador. *Australian Journal of Soil Research* 40(7): 1085–1094. doi:10.1071/SR02015.

Cáceres, T.P., Megharaj, M., and Naidu, R. 2011. Toxicity and transformation of insecticide fenamiphos to the earthworm *Eisenia fetida*. *Ecotoxicology* 20: 20–28. doi:10.1007/s10646-010-0552-6.

Cáceres, T.P., Megharaj, M., and Naidu, R. 2008. Biodegradation of the pesticide fenamiphos by ten different species of green algae and cyanobacteria. *Current Microbiology* 57(6): 643–646. doi:10.1007/s00284-008-9293-7.

Campolo, O., Puglisi, I., Barbagallo, R.N., Cherif, A., Ricupero, M., Biondi, A., Palmeri, V., Baglieri, A., and Zappalà, L. 2020. Side effects of two citrus essential oil formulations on a generalist insect predator, plant and soil enzymatic activities. *Chemosphere* 257: 1–12. doi:10.1016/j.chemosphere.2020.127252.

Castillo-Sánchez, L.E., Jiménez-Osornio, J.J., and Delgado-Herrera, M.A. 2010. Tropical and Subtropical Agroecosystems. *Tropical and Subtropical Agroecosystems* 12: 445–462.

Cestari, I.M., Sarti, S.J., Waib, C.M., and Branco, A.C. 2004. Evaluation of the Potential Insecticide Activity of *Tagetes minuta* (Asteraceae) Essential Oil Against the Head Lice *Pediculus humanus capitis* (Phthiraptera : Pediculidae). *Neotropical Entomology* 33(6): 805–807.

Chamorro, E.R., Ballerini, G., Sequeira, A.F., Velasco, G.A., and Zalazar, M.F. 2008. Chemical composition of essential oil from *Tagetes minuta* L. *Journal of the Argentine Chemical Society* 96(1): 80–86.

Chantraine, J., Laurent, D., Ballivian, C., Saavedra, G., Ibanez, R., and Vilaseca, L.A. 1998. Insecticidal Activity of Essential Oils on *Aedes aegypti* Larvae. *Phytotherapy*

Research 12: 350–354.

Dabrowski, J.M., Shadung, J.M., and Wepener, V. 2014. Prioritizing agricultural pesticides used in South Africa based on their environmental mobility and potential human health effects. *Environment International* 62: 31–40. Elsevier Ltd. doi:10.1016/j.envint.2013.10.001.

Deba, F., Xuan, T.D., Yasuda, M., and Tawata, S. 2008. Chemical composition and antioxidant, antibacterial and antifungal activities of the essential oils from *Bidens pilosa* Linn. var. *Radiata*. *Food Control* 19(4): 346–352. doi:10.1016/j.foodcont.2007.04.011.

Dick, R.P., Breakwell, D.P., and Turco, R.F. 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In *Methods for assessing soil quality*, 49th edition. Edited by J.W. Doran and A.J. Jones. Madison WI, USA. pp. 247–272.

Dimetry, N.. 2014. Different Plant Families as Bioresource for Pesticides. In *Advances in Plant Biopesticides*. Edited by D. Singh. Springer India, New Delhi. pp. 1–20.

EFSA. 2006. Conclusion regarding the peer review of the pesticide risk assessment of the active substance fenamiphos.

Essiedu, J.A., Adepoju, F.O., and Ivantsova, M.N. 2020. Benefits and limitations in using biopesticides: A review. In *AIP Conference Proceedings* 2313. pp. 1–6. doi:10.1063/5.0032223.

Farrukh, S., and Ali, A.S. 2011. Effects of Dichlorovos Organophosphate on Growth, Reproduction, and Avoidance Behavior of Earthworm *Eisenia foetida*. *Iranian Journal of Toxicology* 5(14): 495–501.

Foster, E.J., Fogle, E.J., and Cotrufo, M.F. 2018. Sorption to Biochar Impacts β -Glucosidase and Phosphatase Enzyme Activities. *Agriculture* 8(158): 1–12. doi:10.3390/agriculture8100158.

Gakuubi, M.M., Wanzala, W., Wagacha, J.M., and Dossaji, S.F. 2016. Bioactive properties of *Tagetes minuta* L. (Asteraceae) essential oils: A review. *American Journal of Essential Oils and Natural Products* 4(2): 27–36.

Garcia, M. V., Matias, J., Barros, J.C., Lima, D.P., Lopes, R.S., and Andreotti, R. 2012.

Chemical identification of *Tagetes minuta* Linnaeus (Asteraceae) essential oil and its acaricidal effect on ticks. *Brazilian Journal of Veterinary Parasitology* 21(4): 405–411.

Ghani, S.B.A., Al-Rehiayani, S., Agamy, M.E., and Lucini, L. 2018. Effects of biochar amendment on sorption, dissipation, and uptake of fenamiphos and cadusafos nematicides in sandy soil. *Pest Management Science* 74(11): 2652–2659. doi:10.1002/ps.5075.

Gomez-Eyles, J.L., Sizmur, T., Collins, C.D., and Hodson, M.E. 2011. Effects of biochar and the earthworm *Eisenia fetida* on the bioavailability of polycyclic aromatic hydrocarbons and potentially toxic elements. *Environmental Pollution* 159(2): 616–622. Elsevier Ltd. doi:10.1016/j.envpol.2010.09.037.

Goudoum, A., Tinkeu, L.S.N., Ngassoum, M.B., and Mbofung, C.M. 2016. Insecticidal and Antifungal Properties of Essential Oil of *Bidens pilosa* Linn . Var . Radita (Asteraceae) Towards Stored Bambara Groundnut Insect and Fungi Pests. *Asian Journal of Agriculture and Food Sciences* 04(02): 66–72.

Han, J., Huang, Y., Meng, J., Fan, C., Yang, F., Tan, H., and Zhang, J. 2021. Exposure of earthworm (*Eisenia fetida*) to rice straw biochar : Ecotoxicity assessments for soil-amended programmes. *Science of the Total Environment* 794: 148802. Elsevier B.V. doi:10.1016/j.scitotenv.2021.148802.

Houben, D., Evrard, L., and Sonnet, P. 2013. Beneficial effects of biochar application to contaminated soils on the bioavailability of Cd, Pb and Zn and the biomass production of rapeseed (*Brassica napus* L.). *Biomass and Bioenergy* 57: 196–204. Elsevier Ltd. doi:10.1016/j.biombioe.2013.07.019.

Idris, O.A., Wintola, O.A., and Afolayan, A.J. 2017. Phytochemical and antioxidant activities of *Rumex crispus* L. in treatment of gastrointestinal helminths in Eastern Cape Province, South Africa. *Asian Pacific Journal of Tropical Biomedicine* 7(12): 1071–1078. Elsevier B.V. doi:10.1016/j.apjtb.2017.10.008.

Isman, M.B. 2006. Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. *Annual Review of Entomology* 51: 45–66.

ISO. 2008. Soil quality - Avoidance test for determining the quality of soils and effects

of chemicals on behaviour. Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*). No. 17512-.

Jeffery, S., Verheijen, F.G.A., van der Velde, M., and Bastos, A.C. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. *Agriculture, Ecosystems and Environment* 144(1): 175–187. Elsevier B.V. doi:10.1016/j.agee.2011.08.015.

Kandeler, E., and Gerber, H. 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biology and Fertility of Soils* 6: 68–72. doi:10.1007/BF00257924.

Karunamoorthi, K. 2012. Medicinal and Aromatic Plants: A Major Source of Green Pesticides/Risk-Reduced Pesticides. *Medicinal & Aromatic Plants* 01(08). doi:10.4172/2167-0412.1000e137.

Khorram, M.S., Zhang, Q., Lin, D., Zheng, Y., Fang, H., and Yu, Y. 2016. Biochar : A review of its impact on pesticide behavior in soil environments and its potential applications. *Journal of Environmental Sciences* 44: 269–279. Elsevier B.V. doi:10.1016/j.jes.2015.12.027.

Kim, W. Il, Kunhikrishnan, A., Go, W.R., Jeong, S.H., Kim, G.J., Lee, S., Yoo, J.H., Cho, N., and Lee, J.H. 2014. Influence of Various Biochars on the Survival , Growth , and Oxidative DNA Damage in the Earthworm *Eisenia Fetida*. *Korean Journal of Environmental Agriculture* 33(4): 231–238.

Koul, O., Walia, S., and Dhaliwal, G.S. 2008. Essential Oils as Green Pesticides : Potential and Constraints. *Biopesticides International* 4(1): 63–84.

Krishna, A., Prajapati, V., Bhasney, S., Tripathi, A.K., and Kumar, S. 2005. Potential toxicity of new genotypes of *Tagetes* (Asteraceae) species against stored grain insect pests. *International Journal of Tropical Insect Science* 25(2): 122–128. doi:10.1079/IJT200560.

Kuo, T., Yang, G., Chen, T., Wu, Y., Tran Nguyen Minh, H., Chen, L., Chen, W., Huang, M., Liang, Y., and Yang, W. 2021. *Bidens pilosa* : Nutritional value and benefits for metabolic syndrome . *Food Frontiers* 2(1): 32–45. doi:10.1002/fft2.63.

Lewis, K.A., Tzilivakis, J., Warner, D.J., and Green, A. 2016. An international database

for pesticide risk assessments and management. *Human and Ecological Risk Assessment* 22(4): 1050–1064. Taylor & Francis. doi:10.1080/10807039.2015.1133242.

Lionetto, M.G., Calisi, A., and Schettino, T. 2012. Earthworm biomarkers as tools for soil pollution assessment. *Soil health and land use management* 16: 305–331.

Liu, X., Cao, A., Yan, D., Ouyang, C., Wang, Q., and Li, Y. 2021. Overview of mechanisms and uses of biopesticides. *International Journal of Pest Management* 67(1): 65–72. Taylor & Francis. doi:10.1080/09670874.2019.1664789.

Liu, Y., Dai, Q., Jin, X., Dong, X., Peng, J., Wu, M., Liang, N., Pan, B., and Zing, B. 2018. Negative Impacts of Biochars on Urease Activity: High pH, Heavy Metals, Polycyclic Aromatic Hydrocarbons, or Free Radicals? *Environmental Science and Technology* 52: 12740–12747. doi:10.1021/acs.est.8b00672.

Liu, Z.L., Goh, S.H., and Ho, S.H. 2007. Screening of Chinese medicinal herbs for bioactivity against *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst). 43: 290–296. doi:10.1016/j.jspr.2006.06.010.

Manori, S., Shah, V., Soni, V., Dutta, K., and Daverey, A. 2021. Phytoremediation of cadmium-contaminated soil by *Bidens pilosa* L.: impact of pine needle biochar amendment. *Environmental Science and Pollution Research* 28: 58872–58884. Environmental Science and Pollution Research.

Mathew, L.K. 2016. Botanicals as Biopesticides: A Review. *International Journal of Advanced Research* 4(3): 1734–1739.

Miresmailli, S., and Isman, M.B. 2014. Botanical insecticides inspired by plant-herbivore chemical interactions. *Trends in Plant Science* 19(1): 29–35. Elsevier Ltd. doi:10.1016/j.tplants.2013.10.002.

Mishra, T., Govindharaj, G., Gadratagi, B.G., Patil, N.B., Yadav, M.K., Munda, S., Mukherjee, A.K., Jena, M., and Adak, T. 2019. Deciphering the associated risk on soil microbes upon use of biopesticides in rice ecosystem. *Environmental Monitoring and Assessment* 191: 654–663. Environmental Monitoring and Assessment.

Muangphra, P., and Gooneratne, R. 2011. Toxicity of Commercial Neem Extract to Earthworms (*Pheretima peguana*). *Applied and Environmental Soil Science*: 1–8.

doi:10.1155/2011/925950.

Nartey, O.D., and Zhao, B. 2014. Biochar preparation, characterization, and adsorptive capacity and its effect on bioavailability of contaminants: An overview. *Advances in Materials Science and Engineering*: 1–12. doi:10.1155/2014/715398.

OECD. 1984. Earthworm, Acute Toxicity Tests. 1-9.

OECD. 2004. Earthworm reproduction test (*Eisenia fetida*/*Eisenia andrei*). 1–18.

Oleszczuk, P., Jo, I., Futa, B., Pasieczna-patkowska, S., Pa, E., and Kraska, P. 2014. Effect of pesticides on microorganisms, enzymatic activity and plant in biochar-amended soil. *Geoderma* 214: 10–18. doi:10.1016/j.geoderma.2013.10.010.

Pavela, R. 2014. Limitation of Plant Biopesticides. *In* *Advances in Plant Biopesticides*. Edited by D. Singh. Springer India, New Delhi. pp. 21–34.

Pavela, R. 2016. History, presence and perspective of using plant extracts as commercial botanical insecticides and farm products for protection against insects - A review. *Plant Protection Science* 52(4): 229–241. doi:10.17221/31/2016-PPS.

Pavela, R., and Benelli, G. 2016. Essential Oils as Ecofriendly Biopesticides? Challenges and Constraints. *Trends in Plant Science* 21(12): 1000–1007. Elsevier Ltd. doi:10.1016/j.tplants.2016.10.005.

Ponsankar, A., Vasantha-Srinivasan, P., Senthil-Nathan, S., Thanigaivel, A., Edwin, E.S., Selin-Rani, S., Kalaivani, K., Hunter, W.B., Alessandro, R.T., Abdel-Megeed, A., Paik, C.H., Duraipandiyan, V., and Al-Dhabi, N.A. 2016. Target and non-target toxicity of botanical insecticide derived from *Couroupita guianensis* L. flower against generalist herbivore, *Spodoptera litura* Fab. and an earthworm, *Eisenia foetida* Savigny. *Ecotoxicology and Environmental Safety* 133: 260–270. Elsevier. doi:10.1016/j.ecoenv.2016.06.043.

Popp, J., Pető, K., and Nagy, J. 2013. Pesticide productivity and food security. A review. *Agronomy for Sustainable Development* 33(1): 243–255. doi:10.1007/s13593-012-0105-x.

Quinn, L.P., B, de, J., Fernandes-Whaley, M., Roos, C., Bouwman, H., Kylin, H., Pieters, R., and den Berg, J. van. 2011. Pesticide Use in South Africa: One of the Largest Importers of Pesticides in Africa. *Pesticides in the Modern World - Pesticides*

Use and Management, (4):49-96. doi:10.5772/16995.

Reddy, S.G.E., Dolma, S.K., Koundal, R., and Singh, B. 2016. Chemical composition and insecticidal activities of essential oils against diamondback moth , *Plutella xylostella* (L .) (Lepidoptera : Yponomeutidae). *Natural Product Research* 30(16): 1834–1838. Taylor & Francis. doi:10.1080/14786419.2015.1068772.

Reinecke, S.A., and Reinecke, A.J. 2004. The Comet Assay as Biomarker of Heavy Metal Genotoxicity in Earthworms. *Archives of Environmental Contamination and Toxicology* 46: 208–215. doi:10.1007/s00244-003-2253-0.

Salazar, S., Sánchez, L.E., Alvarez, J., Valverde, A., Galindo, P., Igual, J.M., Peix, A., and Santa-Regina, I. 2011. Correlation among soil enzyme activities under different forest system management practices. *Ecological Engineering* 37(8): 1123–1131. Elsevier. doi:10.1016/J.ECOLENG.2011.02.007.

Seiber, J.N., Coats, J., Duke, S.O., and Gross, A.D. 2014. Biopesticides: State of the Art and Future Opportunities. *Journal of Agricultural and Food Chemistry* 62(48): 11613–11619. doi:10.1021/jf504252n.

Singh, N.P., McCoy, M.T., Tice, R.R., and Schneider, E.L. 1988. A simple technique for quantitation of low level of DNA damage in individual cells. *Experimental Cell Research* 175(1): 184–191.

Souza, B.A., and Silva, L.C.D.A. 2013. Preliminary phytochemical screening and molluscicidal activity of the aqueous extract of *Bidens pilosa* Linné (Asteraceae) in *Subulina octona* (Mollusca , Subulinidade). *Anais da Academia Brasileira de Ciências* 85(4): 1557–1566.

Sporleder, M., and Lacey, L.A. 2013. Biopesticides. In *Insect Pests of Potato*, 1st edition. Edited by P. Giordanengo, C. Vincent, and A. Alyokhin. Elsevier / Academic Press, London. pp. 463–497.

Taba, S., Ashikaga, K., Oohama, T., Ajitomi, A., Kiyuna, C., Kinjo, M., and Sekine, K.T. 2020. *Bidens pilosa* extract effects on pine wilt: Causal agents and their natural enemies. *Forest Science* 66(3): 284–290. doi:10.1093/forsci/fxz076.

Tang, J., Zhu, W., Kookana, R., and Katayama, A. 2013. Characteristics of biochar and its application in remediation of contaminated soil. *Journal of Bioscience and*

Bioengineering 116(6): 653–659. Elsevier Ltd. doi:10.1016/j.jbiosc.2013.05.035.

Tankeu, S.Y., Vermaak, I., Viljoen, A.M., Sandasi, M., and Kamatou, G.P.P. 2013. Essential oil variation of *Tagetes minuta* in South Africa - A chemometric approach. *Biochemical Systematics and Ecology* 51: 320–327. Elsevier Ltd. doi:10.1016/j.bse.2013.10.003.

Thakur, D.R., and Sharma, R.K. 2014. Bioefficacy of *Bidens pilosa* L . against *Acanthoscelides obtectus* (Say) and *Zabrotes subfasciatus* (Boheman), stored pests of kidney beans , world wide. *International Journal of Agriculture and Crop Sciences* 7(15): 1470–1477.

Tijjani, A., Bashir, K.A., Mohammed, I., Muhammad, A., Gambo, A., and Musa, H. 2017. Biopesticides for pests control: a Review. *Journal of Biopesticides and Agriculture*, 3(1): 6–13.

van Gestel, C.A.. 1992. Validation of earthworm toxicity tests by comparison with field studies: a review of benomyl, carbendazim, carbofuran, and carbaryl. *Ecotoxicology and Environmental Safety* 23(2): 221–236.

van Gestel, C.A., and van Brummelen, T.. 1996. Incorporation of the biomarker concept in ecotoxicology calls for a redefinition of terms. *Ecotoxicology* 54: 217–225.

Varjani, S., Kumar, G., and Rene, E.R. 2019. Developments in biochar application for pesticide remediation : Current knowledge and future research directions. *Journal of Environmental Management* 232: 505–513. Elsevier. doi:10.1016/j.jenvman.2018.11.043.

Walia, S. 2017. Phytochemical biopesticides: some recent developments. *Phytochemistry Reviews* 16(5): 989–1007. Springer Netherlands. doi:10.1007/s11101-017-9512-6.

Xuan, T.D., and Khanh, T.D. 2016. Chemistry and pharmacology of *Bidens pilosa*: an overview. *Journal of Pharmaceutical Investigation* 46(2): 91–132. Springer Netherlands. doi:10.1007/s40005-016-0231-6.

Yang, W. 2014. Botanical, Pharmacological, Phytochemical, and Toxicological Aspects of the Antidiabetic Plant *Bidens pilosa* L. *Evidence-Based Complementary and Alternative Medicine*: 1–14.

- Yang, X., Liu, J., Mcgrouter, K., Huang, H., Lu, K., Guo, X., He, L., Lin, X., Che, L., Ye, Z., and Wang, H. 2016. Effect of biochar on the extractability of heavy metals (Cd , Cu , Pb , and Zn) and enzyme activity in soil. *Environmental Science and Pollution Research* 23: 974–984. doi:10.1007/s11356-015-4233-0.
- Yasmin, S., and D'Souza, D. 2010. Effects of Pesticides on the Growth and Reproduction of Earthworm: A Review. *Applied and Environmental Soil Science*: 1–9. doi:10.1155/2010/678360.
- Zhang, Q., Saleem, M., and Wang, C. 2019. Effects of biochar on the earthworm (*Eisenia foetida*) in soil contaminated with and / or without pesticide mesotrione. *Science of the Total Environment* 671: 52–58. Elsevier B.V. doi:10.1016/j.scitotenv.2019.03.364.
- Zhu, L., Li, B., Wu, R., Li, W., Wang, J., Wang, J., Du, Z., Juhasz, A., and Zhu, L. 2020. Acute toxicity, oxidative stress and DNA damage of chlorpyrifos to earthworms (*Eisenia fetida*): The difference between artificial and natural soils. *Chemosphere* 255: 126982. Elsevier Ltd. doi:10.1016/j.chemosphere.2020.126982.
- Zhu, X., Chen, B., Zhu, L., and Xing, B. 2017. Effects and mechanisms of biochar-microbe interactions in soil improvement and pollution remediation: A review. *Environmental Pollution* 227: 98–115. Elsevier Ltd. doi:10.1016/j.envpol.2017.04.032.

Research Article

Global Trends of Green Pesticide Research from 1994 to 2019: A Bibliometric Analysis

Hein Hendrik Smith, Oladayo Amed Idris , and Mark Steve Maboeta 

Unit for Environmental Sciences and Management (UESM), Faculty of Natural and Agricultural Sciences,
North-West University, Potchefstroom, North-West, South Africa

Correspondence should be addressed to Mark Steve Maboeta; mark.maboeta@nwu.ac.za

Received 6 December 2020; Revised 9 February 2021; Accepted 2 March 2021; Published 20 March 2021

Academic Editor: Zongming Ren

Copyright © 2021 Hein Hendrik Smith et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The fast-growing world population places food production under enormous pressure to ensure food security. One of the most common methods to increase food production is the use of pesticides, but the continuous use thereof has numerous detrimental effects on the environment. The interest in biopesticides for a possible substitute has grown over the past two decades. To determine the research evolution of biopesticides (green pesticides), a bibliometric analysis from 1994 to 2019 was carried out. A total of 580 documents were found eligible in the Scopus database for this analysis. Parameters such as the number of articles, article citations, keywords, source impact, and countries of publication were used to analyse the documents and rank countries based on authors, productivity, article citations, and co-authorship. The analysis reveals production increased significantly from 2009 and has the most published documents in 2019 with a total of 74 articles. Asia's most populous countries, India and China, were ranked first and second, respectively, and the USA third in terms of the most productive countries in the field of plant biopesticides. Countries in Europe and Africa however have fewer publications than expected in this field, given the fact that they are high consumers of pesticides. India, China, and the USA have 4.08%, 2.94%, and 12.5% multiple country publications (MCPs), respectively, with the USA having a stronger collaboration. Finally, there is a clear indication in this study that India and China are taking the lead in substituting synthetic pesticides with the alternative natural plant biopesticide.

1. Introduction

The detrimental effects of synthetic pesticides on the environment are well established as it affects animal and plant biodiversity as well as terrestrial and aquatic ecosystems [1]. Other effects include toxicity, soil and groundwater pollution, and harmful residues that contaminate crops and food. It also affects non-target organisms and the excessive use of synthetic pesticides leads to increased resistance in pests [2]. The negative effects of synthetic pesticides have created the need for a safer and more environmentally friendly substitute. Biopesticides are believed to be less toxic, environmentally friendly, and not harmful to humans and non-target organisms [2]. The use of botanical insecticides is not a new practice. As early as the 17th century, botanical insecticides have been used in agricultural practices by ancient

China, Egypt, Greece, and India [3]. Over the past decade, the interest in biopesticides has grown immensely, with the intention to substitute with synthetic pesticides. According to a study done by Isman and Grieneisen [4], the number of annual papers published on botanical insecticides has grown from 61 papers in 1980 to 1207 papers in 2012 [4]. This signifies an increase in the awareness and uses of plant biopesticides. In spite of the growing need for natural pest management, plant biopesticides contribute less than 1% of the total use of pesticides [2]. The annual amount of synthetic pesticides used for crop protection is estimated at 2.5 million tons causing damages worth about \$100 billion, due to residues in crops, soil and water, nonbiodegradable properties, and high toxicity [5]. Synthetic pesticides are no doubt a threat to public health and demand an urgent alternative with better crop protection and less harmful

properties. The term “green pesticides” entails all naturally acquired types of pest management from plant extracts, plant-derived pesticides, plant secondary metabolites, and plant-based pesticides [5]. The green pesticides are also referred to as botanical pesticides and are used as repellents, nematocides, insecticides, fungicides, and bactericides, obtained in the form of isolated substances or complex mixtures [6]. Although there has been significant growth in botanical pesticide research, the commercial availability of these pesticides remains limited [7]. According to Isman and Grieneisen [4], there are a few limitations of the exploitation of botanical pesticides such as lack of availability of good quality plant pesticides at affordable prices, strict regulation particularly on endangered plant species, short persistence of the phytochemical in the environment, and the extraction of the phytochemical from plants grown under different climatic conditions resulting in different compositions in terms of active agents, which leads to variability in effectiveness [4]. One of the main factors preventing the advancement of commercializing botanical pesticides is sustainability [6] of the product. In order to produce botanical pesticides on a commercial scale, it is necessary to obtain large amounts of the source plant, which requires large scale production [6]. However, most biopesticide plants are still not grown commercially, making it difficult to source for the plants. Although research on botanical pesticides has increased significantly over the past two decades [8], the above-mentioned limitations still contribute to the lack of commercialization of botanical pesticides.

With the continuously growing interest in botanical pesticides, it is necessary to carry out a bibliometric compilation to better understand the research trend. The fast-growing number of academic publications makes it extremely difficult to stay up to date with everything being published [9]. To help organise and better understand previous research, different quantitative and qualitative literature reviewing approaches are used. Bibliometrics is one such approach and according to Aria and Cuccurullo [9] it has the potential to perform a “systematic, transparent, and reproducible review process based on the statistical measurement of science, scientists, or scientific activity.” Various research fields make use of bibliometrics to measure the impact or influence of certain research articles. For example, in the field of engineering, the authors Bao et al. [10] used bibliometric analysis to evaluate the publications in the field of robotic exoskeletons and the authors He et al. [11] used bibliometrics to analyse articles related to “metastatic castration-resistant prostate cancer” in the field of health and medicine. Muhuri et al. [12] projected the growth structure of the ongoing industrial revolution “Industry 4.0” using bibliometric analysis in the field of intelligent engineering [12]. All these examples mentioned but few indicate bibliometric study is relevant in many fields.

During this study, the objective was to analyse the global research activities on botanical pesticides from 1994 to 2019, in the Scopus database. The data obtained was then used to study the research progression using bibliometric parameters and indicators such as most productive authors, most productive countries, annual scientific production, country

collaboration, average article citations per year, average total citations per year, most relevant keywords, bibliographic coupling, and documents production source impact. The Scopus database was chosen as it includes a wider spectrum of journals in citation analysis and keyword searching than PubMed and Web of Science [13]. It also offers an easy trajectory through the extrapolation of all the bibliometric indicators proposed in this study. The period 1994–2019 was chosen based on the fact that previous studies indicated that research on green pesticides has only become of increasing interest over the past two decades, due to increase in pesticides and agrochemicals residues which are causing significant contamination in the environment [14].

Due to the detrimental effects of synthetic pesticides on both the environment and human population, this study aims to carry out a descriptive bibliometric analysis on green pesticide research from 1994 to 2019, in order to present a general overview of global research trends towards finding an alternative for synthetic pesticides.

2. Materials and Methods

The published research outputs on botanical pesticides were retrieved from the Scopus database on 7 August 2020. The Scopus database provides access to 27 million abstracts with citations dating back to 1966 [15]. The key terms used to query Scopus database in this study were “plant pesticides”, “plant biopesticides”, “plant-derived pesticides”, “green pesticides”, “botanical pesticides” and “phyto-pesticides”, using the Boolean operator “OR”. These key terms were used to retrieve title-specific research from 1994 to 2019 which returned 586 documents. The documents were cleaned and downloaded in BibTex file format [16] and then analysed for a bibliometric statistics with R-project version 4.0.2. The codes used to extract the bibliometric indices were in accordance with Aria and Cuccurullo [9] in Rstudio interface. The bibliometric indices, annual scientific production, article citation per year, bibliographic coupling, most productive authors, most productive countries, most relevant keywords, and most productive journals/source, were used in this study to evaluate the current and possible future trends of the research in plant biopesticides. In this study, the annual scientific indicator is tallied up by institute, organisation, author, and countries. The citation analysis measures the relative importance or impact of an author or publication has been cited by others. However, the citation counts were used to measure the impact per country on plant biopesticides. The publication-citation matrix was used to analyse most productive authors/countries, co-citation, and bibliographic coupling as described [9, 17]. The keywords extracted directly from the documents with the R-project interface are used to study the conceptual structure of the green pesticide research field.

3. Results

3.1. Green Pesticide Research Production. To determine the progression of research on green pesticides in this study, a wide spectrum of biopesticide keywords were used to query

the database. A total of 540 published documents, from 332 sources, were recorded on green pesticides. These documents include articles, books, book chapters, conference papers, conference reviews, editorials, erratum, notes, reviews, and short surveys. An average citation of 12.63 per document makes them relevant in the scientific community (Table 1). The annual trend of scientific production from 1994 to 2008 showed a minimal increase, with the highest increases in 2000, 2004, and 2007 (Figure 1). Production increased significantly from 2009, with the most documents published in 2019 (74 documents). A decline in publications, however, occurred during 2015 and 2016, with 32 and 38 publications, respectively. The reason for such a decline is not known. The annual percentage growth rate from 1994 to 2019 was 13.68%. It is also worth noting that although there is a steady increase in the annual scientific production, fluctuations do occur between some years. Possible explanations could include improved laboratories, the evolution of new researchers, the development of new research methodology, and funding support [18].

3.2. Keywords on Green Pesticide Research. Most published documents contain a number of keywords to assist with online searches and identify certain editors to a document [18]. To study the research trends of green pesticides, both the singular and plural form of the author keywords were used. This aids in understanding the research evolution of the study field [19]. Both the author keywords and keyword plus were included in the study. The author keywords are a list of terms provided by the author/s that best represents the content of the document, while keyword-plus are terms or phrases that occur in the titles of a document's references but do not appear in the document's title itself [20]. Between 1994 and 2019, a total of 1732 author keywords and 4064 keyword-plus terms were retrieved from the documents on green pesticide research (Table 2). The most used author keyword was botanical pesticide/s occurring in 159 articles, followed by essential oil/s (41 articles) and pesticide/s (25 articles), second and third, respectively. Other author keywords occurring in the top 30 include green pesticides (22 articles), biopesticides (17 articles), pest management (14 articles), toxicity (12 articles), and plant extracts (13 articles) etc.; all these are relevant to pest control using plant biopesticides. The most used keyword-plus was animal/s, occurring in 220 articles, followed by pesticide/s (217 articles) and insecticide/s (175 articles), second and third, respectively, as shown in Table 2. It is not surprising to get a broad range of terms in keyword-plus because it is derived from the titles of documents cited by the author. Other keyword-plus terms in the top 30 are plant extract (102 articles), pest control (82 articles), essential oil (62 articles), and toxicity (38 articles). Although it is presumed that green pesticides are harmless to the environment, it is important to note that the keyword toxicity is listed in the top 30 of both the author keywords and keyword-plus terms, which could be referring to the potency of biopesticide plants. Most biopesticide plants are toxic but have a very short half-life [21]. This could have contributed to the general belief that plant biopesticides

TABLE 1: Summary of data retrieved from Scopus database.

Description	Count
Documents	540
Source (journals, books, etc.)	332
Keywords plus (ID)	4064
Author's keywords (DE)	1732
Period	1994–2019
Average citations per document	12.63
Authors	1867
Author appearances	2408
Authors of single-authored documents	68
Authors of multiauthored documents	1799
Single-authored documents	79
Documents per author	0.311
Authors per document	3.22
Co-authors per document	4.15
Collaboration index	3.59

are natural and therefore harmless, which is not always the case. In Figure 2, the co-occurrence network and interrelationship of the top 30 terms on green pesticide research are represented with a pictographic network. Each coloured circle represents a cluster of terms and the connecting lines represent the cooperation degree. Pesticide related terms are in blue clusters and the response related terms in red clusters (Figure 2).

3.3. Productivity on Green Pesticide Research per Country. India is ranked first in the most productive countries with a total of 98 publications, among which 4.08% are published out of collaboration. China and USA are ranked second and third with 68 and 32 publications, respectively. China has 2.94% and USA has 12.5% multiple country publications (MCP) which indicate the USA collaborates more while China relies more on local research. It is quite interesting to know that Italy and South Africa have fewer single country publications (SCP) compared to their MCP; hence they have a higher MCP: SCP ratio. The collaboration on publications of Italy, Netherlands, and South Africa is 60.86%, 75.0%, and 80.0%, respectively, making these the countries with the most collaboration network (Figure 3).

Table 3 shows that India is ranked first (1080 citations; h-index 18), followed by USA (979 citations; h-index 17) and Italy (868 citations; h-index 16), second and third, respectively. African countries in the top 30 list are Egypt in 8th (184 citations), South Africa 12th (92 citations), Zimbabwe 13th (90 citations), Nigeria 15th (69 citations), Kenya 20th (54 citations), and Tanzania 22nd (52 citations). It is debated that citations reflect the scientific impact and relevance of an article, but with limitation to the measures of the research quality [22, 23]. Hence, the citation ranking the USA first and other countries following could be the measure of their study relevancy. It is important to note that Europe is the largest consumer of pesticides with Asia the second largest and the leading countries in pesticide production are China, USA, France, Brazil, and Japan [24]. In Figure 3 and Table 3, the above-mentioned countries are ranked in the top 30 of the categories of the most productive and most cited country

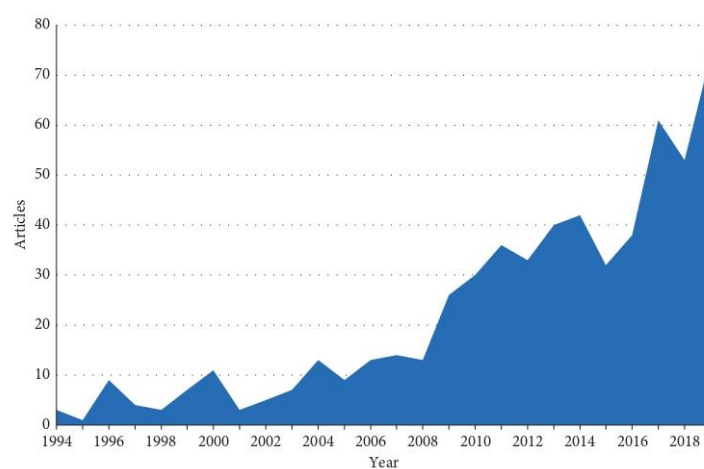


FIGURE 1: Annual scientific publications of documents on plant biopesticides from 1994 to 2019 in the Scopus database.

TABLE 2: Top 30 most relevant keywords on green pesticide research from 1994 to 2019.

Rank	Author keywords (DE)	Articles	Keyword plus (ID)	Articles
1	Botanical pesticide/s	159	Animal/s	220
2	Essential oil/s	41	Pesticide/s	217
3	Pesticide/s	25	Insecticide/s	175
4	Azadirachtin	24	Plant extract/s	151
5	Green pesticides	22	Article	117
6	Biopesticides	17	Chemistry	90
7	Neem	15	Pest control	82
8	Pest management	14	Hexapoda	79
9	Botanical insecticide/s	14	Nonhuman	78
10	<i>Azadirachta indica</i>	13	Drug effect	69
11	Botanicals	13	<i>Azadirachta indica</i>	65
12	Plant extracts	13	Essential oil	62
13	<i>Spodoptera litura</i>	12	Botanical pesticides	60
14	Toxicity	12	Controlled study	57
15	Mortality	11	Larva	50
16	Integrated pest management	10	Biopesticide	49
17	Antifeedant	9	Unclassified drug	43
18	Biological control	9	Female	42
19	Insecticidal activity	9	Lepidoptera	39
20	Repellency	9	Metabolism	38
21	Bioassay	8	Toxicity	38
22	Contact toxicity	8	Human	37
23	Insecticide	8	Bioassay	35
24	Pest control	8	Agriculture	33
25	Aphids	6	Biological control	33
26	<i>Heteropneustes fossilis</i>	6	Insect	32
27	Phytochemicals	6	Mortality	32
28	<i>Plutella xylostella</i>	6	Fungi	31
29	<i>Tetranychus urticae</i>	6	Moth	31
30	Acute toxicity	5	Physiology	27

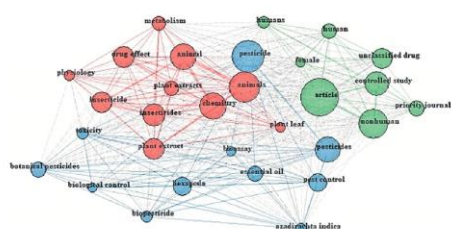


FIGURE 2: Keyword co-occurrences network of the top 30 keywords on green pesticide research. The circle size of the keywords represents the frequency of occurrence in articles. The thickness of the line between any two keywords displays the degree of cooperation.

in plant biopesticide study, but with the exception of Japan, indicating the countries are focusing on plant biopesticides as an alternative to synthetic pesticides, and these studies are relevant in the scientific community [25].

According to Lee and Bozeman [26], scientific collaboration in research has become the standard. Modern science is becoming ever more interdisciplinary and complex, which encourages research collaboration. Many funding agencies encourage collaborative research by incorporating this as one of their funding conditions [26]. The top 30 country collaboration network on green pesticides is shown in Figure 4. The coloured circles represent the countries and the circle size represents the number of collaborations with other countries. Thicker lines between countries illustrate collaborative strength between the countries. As revealed by the bibliometric analysis, Italy, United Kingdom, USA, Canada, South Africa, India, and Netherlands have the most collaborations with other countries. Figure 4 shows the frequency and network of collaboration between the countries. India, United Kingdom, Netherlands, Brazil, Canada, and the USA collaborate often. Italy, Czech Republic, Saudi Arabia, and India are regular collaborators. And Netherlands, South Africa, and the USA also frequently collaborate, all these forming a network. The collaborative strength of some of these countries could be ascribed to the respective countries history of pesticide use and government funding encouraging collaboration [18].

3.4. Most Cited Documents on Green Pesticides and Source Impact. Table 4 presents the most cited papers on plant biopesticides. The top-ranked article published by Pavla and Benelli [27] had a total of 288 citations at an average of 57.6 citations per year. In the article, the strengths, challenges, and constraints of essential oil-based biopesticides were analysed [27], considering the effectiveness, toxicity, and the mechanisms of action. The high citation accredited by the article may be due to the wide spectrum of the biological activity such as nematocidal, ovicidal, fungicidal, insecticidal, and bactericidal effect of essential oil-based biopesticides making the article relevant in a wide field of research. Surprisingly, only 3 articles from the 1990s are listed in the top 30 most cited documents. This may indicate that more recent researchers are becoming more interested

in plant biopesticides, as search for alternative synthetic pesticides grows.

In Table 5, the top 30 productive journals in terms of the number of publications, total citations, and h-index are reported. The top 30 journals have published 219 articles which represent 37.76% of the documents in this study. The Pestology journal is ranked first (NP: 24; h-index: 4), Industrial Crops and Products second (NP: 21; h-index: 12), and Pest Management Science third (NP: 14; h-index: 10). The source growth of the top 10 productive journals is shown in Figure 5. The journals "Environmental Science and Pollution Research," "Industrial Crops and Products," and "Scientific Reports" have grown exponentially over the last few years. On the contrary, the impact of the journals "Pestology," "Acta Horticulture," and "Advances in Plant Biopesticides" has drastically reduced in the field of plant biopesticides over time. It is also noteworthy that 25 years ago very few journals published articles on green pesticides. Recently, there are more publications on green pesticides indicating that the research area is growing.

4. Discussion

The increase in publications from 2009 could be due to the increasing realization of the detrimental effects of synthetic pesticides on the environment [1], the growing need for a sustainable alternative, government policies on environmental protection, and pressure groups [56].

To determine the productivity of a research field based on a bibliometric study, the number of scientific publications is investigated [57]. Globally, demand for biopesticides has increased due to rising interest in organic products as a potential alternative to synthetic insecticides [58]. The demand could have driven the raising research and publication in the field of plant biopesticides as shown in Figure 1. Andreo-Martinez et al. [59] in their study reported an increase of publication related to pesticides bioavailability in food, vegetables, and wine from 1 in 1976 to 154 in 2018 [59]. This is in accordance with our study as there is a steady and sharp increase in the publication of biopesticides related work from 2009. A change in the number of publications in a research field could indicate a possible change in demand and technology. In Table 2, the keyword-plus "animal/s" is ranked first. This could be due to the increasing demand and use of plant phytochemicals for ectoparasites on livestock [60]. The use of plant biopesticides is common especially among rural areas communities that are dependent on livestock for survival. Aside from the cultural practices, these communities often do not have the financial capability to buy pesticides and instead make use of biopesticides for pest management [60].

Considering the data collected in this study as shown in Table 1, a total of 580 documents from 332 sources were retrieved from the Scopus database. These documents were authored by 1867 researchers, on green pesticides from 1994 to 2019 with an estimate of 0.311 documents per author and 3.22 authors per document. Out of the 1867 authors, 1799 were authors of multi-authored documents with the remaining 68 being authors of single-authored documents.

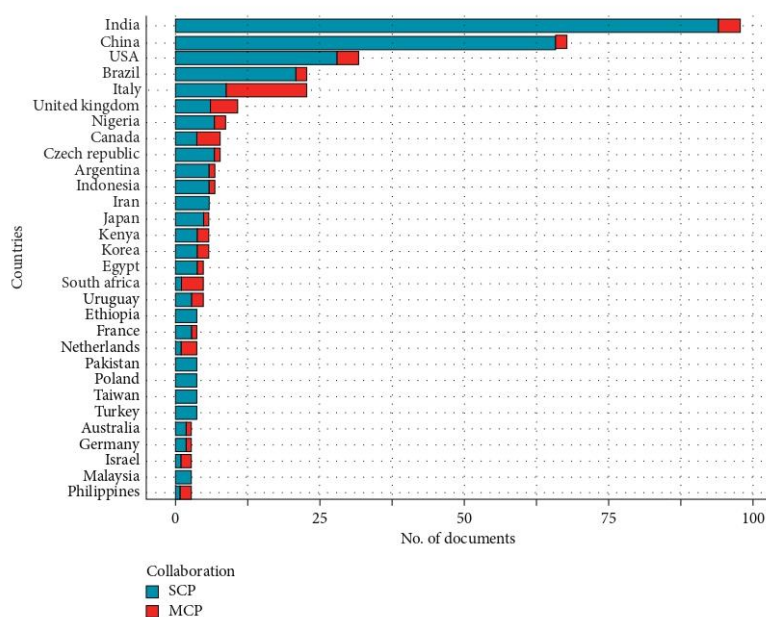


FIGURE 3: Most productive countries and collaboration in the research of green pesticides from 1994 to 2019. SCP: Single Country Publications, MCP: Multiple Country Publications.

TABLE 3: Citations per country in terms of green pesticide research from 1994 to 2019.

Rank	Country/region	Total citations	Average article citations (%)	MCP ratio	h-index
1	India	1080	11.020	0.041	18
2	USA	979	30.594	0.125	17
3	Italy	868	37.739	0.609	16
4	China	682	10.029	0.029	14
5	United Kingdom	441	40.091	0.455	11
6	Brazil	389	16.913	0.087	10
7	Canada	251	31.375	0.500	9
8	Egypt	184	36.800	0.200	7
9	Argentina	162	23.143	0.143	7
10	Belgium	105	52.500	1.000	6
11	Czech Republic	94	11.750	0.125	5
12	South Africa	92	18.400	0.800	5
13	Zimbabwe	90	45.000	1.000	5
14	Iran	86	14.333	0	5
15	Nigeria	69	7.667	0.222	4
16	Korea	68	11.333	0.333	4
17	Uruguay	64	12.800	0.400	4
18	Turkey	59	14.750	0	4
19	Israel	55	18.333	0.667	4
20	Kenya	54	9.000	0.333	4
21	Netherlands	52	13.000	0.750	4
22	Tanzania	52	17.333	0.667	4
23	Poland	44	11.000	0	3
24	Romania	44	44.000	0	3
25	Australia	42	14.000	0.333	3
26	Taiwan	39	9.750	0	3
27	France	38	9.500	0.250	3
28	Greece	38	38.000	0	3
29	Germany	27	9.000	0.333	2
30	Lebanon	27	27.000	0	2

MCPs: multiple country publications.

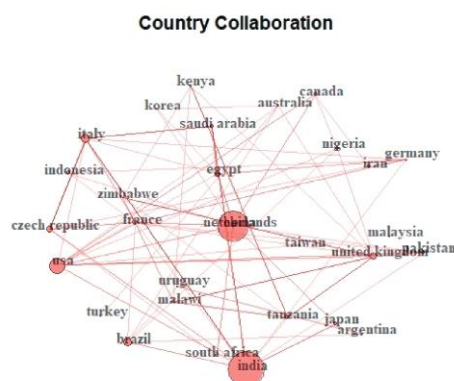


FIGURE 4: Country collaboration network of the top 30 most productive countries on green pesticide research. Line thickness represents the collaboration strength between countries.

TABLE 4: The top 30 most cited documents on green pesticide research from 1994 to 2019 from Scopus.

Rank	First authors	Journal	TC	TC/year
1	Pavela Roman, 2016	Trends Plant Sci	288	57.6
2	Mulla S. Mir, 1999	J Am Mosq Control Assoc	242	11.0
3	Morgan E. David, 2009	Bioorg Med Chem	161	13.42
4	Son T. Gen, 2010	J Neurochem	135	12.27
5	Miresmailli Saber, 2014	Trends Plant Sci	134	19.14
6	Dodge Jeffrey A, 1996	J Steroid Biochem Mol Biol	130	5.2
7	Benelli Giovanni, 2017	J Cluster Sci	101	25.25
8	Attia Sabrine, 2013	J Pest Sci	99	12.37
9	El-Wakeil Nabil E, 2013	Gesunde Pflanz	95	11.87
10	Qian Xuhong, 2010	J Agric Food Chem	89	8.09
11	Cresswell James E, 2012	Pest Manage Sci	71	7.89
12	Sibanda T, 2000	Crop Prot	71	3.38
13	Mossa Abdel Tawab H, 2016	J Environ Sci Technol	68	13.6
14	Koul Opende, 2009	Cab Rev Perspect Agric Vet Sci Nutr Nat Resour	68	5.67
15	Kotkar Hemlata M, 2002	Pest Manage Sci	68	3.58
16	Charleston Deidre S, 2005	Biol Control	64	4.0
17	Martin Krista M, 2000	J Am Vet Med Assoc	64	3.05
18	Benelli Giovanni, 2017	Parasitol Int	62	15.5
19	Hunt Piper R, 2011	Plos One	62	6.2
20	Dubey NK, 2010	Curr Sci	61	5.55
21	Zheng Ke, 2012	Acta Chim Sin	60	6.67
22	Mann Rs, 2012	Mini-Rev Org Chem	59	6.56
23	Rao K Jagajjanani, 2013	Rsc Adv	56	7.0
24	Vidal Estrela Joelma Lima, 2006	Pesqui Agropecu Bras	54	3.6
25	Benelli Giovanni, 2018	Ind Crops Prod-A	53	17.67
26	Caboni Pierluigi, 2012	J Agric Food Chem	51	5.67
27	Zibae A, 2010	Bull Entomol Res	51	4.64
28	Moreira Márcio D, 2007	Pest Manage Sci	51	3.64
29	Mansour F, 2004	Phytoparasitica	50	2.94
30	Valladares G, 1997	J Econ Entomol	49	2.04

TC= total citations.

The average number of authors per document is referred to as collaboration index [61]. Research collaboration and country collaboration network as shown in Figures 3 and 4 of this study are the bibliometric indicators that reflect the social structure among countries, institutions, and researchers. Many previous bibliometric studies in different

fields ranked the United States as being dominant in international collaboration [62, 63]; on the contrary, our analysis reveals Italy dominates international collaboration on green biopesticide followed by the United Kingdom. International research collaboration allows a synergy of knowledge, idea, and research fund that enhances the quality

TABLE 5: Top 30 productive journals, the total number of publications, total citations, h-index, and publication start year, from 1994 to 2019.

Source	NP	TC	h-index	PY-Start
Pestology	24	48	4	1996
Industrial Crops and Products	21	334	12	2010
Pest Management Science	14	378	10	2000
Environmental Science and Pollution Research	13	155	6	2010
Advances in Plant Biopesticides	13	91	7	2014
Journal of Agricultural and Food Chemistry	12	342	8	2003
Crop Protection	10	258	9	1997
Acta Horticulturae	10	20	2	1997
Scientific Reports	9	75	5	2016
Journal of Biopesticides	8	28	4	2010
Plos One	7	150	5	2006
Journal of Pest Science	6	172	6	2009
Molecules	6	39	4	2010
Green Pesticides Handbook: Essential Oils for Pest Control	6	5	2	2017
ACS Symposium Series	5	50	2	1996
Journal of Pesticide Science	5	23	3	2009
Biopesticides International	5	5	2	2011
Phytoparasitica	4	85	3	2004
Pesticide Biochemistry and Physiology	4	54	3	2011
Journal of Insect Science	4	28	2	2014
Archives of Phytopathology and Plant Protection	4	16	2	2011
Zhongguo Zhongyao Zazhi	4	7	2	2004
IOP Conference Series: Earth and Environmental Science	4	2	1	2018
Trends in Plant Science	3	426	3	2014
Journal of Economic Entomology	3	92	3	1997
Medical and Veterinary Entomology	3	76	3	2008
Phytochemistry Reviews	3	61	3	2011
International Journal of Tropical Insect Science	3	49	2	2006
Ecotoxicology and Environmental Safety	3	31	2	2010
ACS Sustainable Chemistry and Engineering	3	26	2	2016

NP: number of publications; TC: total citations; PY-Start: publication year start.

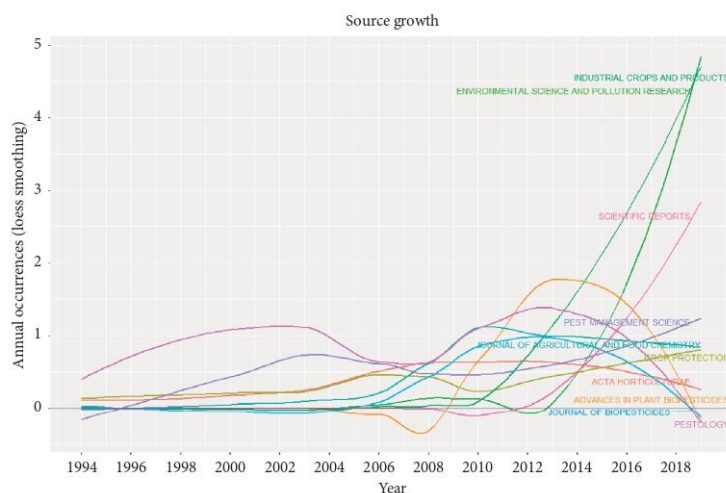


FIGURE 5: Source growth of the top 10 most productive journals from 1994 to 2019.

of research. Collaboration has a great impact on the citation of a document; particularly internationally co-authored papers were reported to receive twice the average citation as local co-authored papers [64]. India, however, tops the most cited countries on green biopesticide papers and most of these countries in Table 3 are also reported in the previous bibliometric study of pesticides [59]. The collaboration index (CI) for green biopesticide as established in this study is 3.59, which is considered as high participation of co-authorship [61]. A study by Liu et al. [24] on global biodiversity research reported the worldwide research collaboration index to be 4.45 [24], which is higher than the CI reported in this study. The CI of global biodiversity research as reported could be the ripple effect of climate change which is a major driver of biodiversity [65].

In Figure 3, India and China are ranked first and second, respectively, in green pesticide research; this may be attributed to their long history of the use of botanical insecticides [3]. The African continent has 5 countries in the top 30 most productive countries: Nigeria (7th), Kenya (14th), Egypt (16th), South Africa (17th), and Ethiopia (19th) with 9, 6, 5, 5, and 4 publications, respectively. Although Africa consumes less than 5% of global pesticides, most countries in Africa still use the most toxic pesticides in food production [66]. According to Dinham [67], small-scale farmers apply these toxic pesticides weekly, sometimes more often, during the growing season [67]. This raises a number of concerns to both environmental and human health experts in Africa. Finally, the above-mentioned factors indicate that African countries are not doing enough in pesticide regulations and the research towards an alternative which means the continent still needs to do more in pest management while jacking up crop production.

The limitations that arose during this study were language, the fact that the quality of published documents could not be ascertained, and the research not available on Scopus being missed out. The documents retrieved from the Scopus database were limited to "English written documents." Documents published in other languages also contribute to research productivity but were unfortunately eliminated in this study. The quality of published documents cannot be measured by total citations alone while the annual scientific production of an author or a country is also not a measure of research quality [18]. It is worth noting that bibliometric analysis does not criticize any part of a research document; hence quality control could be problematic. Regardless of the limitations associated with this study, it provided a global overview of green pesticide research productivity from 1994 to 2019.

5. Conclusion

This bibliometric study discussed the global trend of green pesticide research from 1994 to 2019 based on documents retrieved from Scopus. Despite the increasing research on green pesticides, commercial applications remain limited. Trends in the annual scientific production suggest that research on green pesticides will continue to increase. The detrimental effects of synthetic pesticides on the

environment and human health could be the possible drivers for research productivity in the alternative field of plant biopesticides. This study also found that India, China, and USA are the leading countries in the production of green biopesticide research documents articles and amass the highest total citations per country. Countries in Europe and Africa, although high consumers of pesticides, have significantly less published research documents compared to the earlier mentioned countries. Europe and Africa countries need to intensify their research on alternative pesticides through either government or privately funded programs to promote environmentally friendly pesticides, especially in Africa where pesticides with high toxicity are still being used.

Data Availability

Data are available on request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

- [1] I. Mahmood, S. R. Imadi, K. Shazadi, A. Gul, and K. R. Hakeem, "Effects of pesticides on environment," in *Plant, Soil and Microbes: Volume 1: Implications in Crop Science*, pp. 253–269, Springer, Berlin, Germany, 2016.
- [2] S. Lima, S. Saha, V. Tripathi, and K. K. Sharma, "Phytochemical biopesticides: some recent developments," *Phytochemistry Reviews*, vol. 16, 2017.
- [3] M. B. Isman, "Botanical insecticides, deterrents, and repellents IN modern agriculture and an increasingly regulated world," *Annual Review of Entomology*, vol. 51, no. 1, pp. 45–66, 2006.
- [4] M. B. Isman and M. L. Grieneisen, "Botanical insecticide research: many publications, limited useful data," *Trends in plant science*, vol. 19, 2014.
- [5] O. Koul, S. Walia, and G. S. Dhaliwal, "Essential oils as green pesticides: potential and constraints," *Biopesticides International*, vol. 4, pp. 63–84, 2008.
- [6] E. V. R. Campos, P. L. F. Proença, J. L. Oliveira, M. Bakshi, P. C. Abhilash, and L. F. Fraceto, "Use of botanical insecticides for sustainable agriculture: future perspectives," *Ecological Indicators*, vol. 105, pp. 483–495, 2019.
- [7] A. M. Grumezescu, *New Pesticides and Soil Sensors*, Academic Press, Cambridge, MA, USA, 2017.
- [8] M. B. Isman, "Commercial development of plant essential oils and their constituents as active ingredients in bioinsecticides," *Phytochemistry Reviews*, vol. 19, no. 2, pp. 235–241, 2020.
- [9] M. Aria and C. Cuccurullo, "Bibliometric: an R-tool for comprehensive science mapping analysis," *Journal of Informetrics*, vol. 11, no. 4, pp. 959–975, 2017.
- [10] G. Bao, L. Pan, H. Fang et al., "Academic review and perspectives on robotic exoskeletons," *IEEE Transactions on Neural Systems and Rehabilitation Engineering*, vol. 27, no. 11, pp. 2294–2304, 2019.
- [11] L. He, H. Fang, C. Chen et al., "Metastatic castration-resistant prostate cancer: academic insights and perspectives through bibliometric analysis," *Medicine (Baltimore)*, vol. 99, 2020.
- [12] P. K. Muhuri, A. K. Shukla, and A. Abraham, "Industry 4.0: a bibliometric analysis and detailed overview," *Engineering*

- Applications of Artificial Intelligence*, vol. 78, pp. 218–235, 2019.
- [13] M. E. Falagas, E. I. Pitsouni, G. A. Malietzis, and G. Pappas, "Comparison of PubMed, Scopus, Web of science, and google scholar: strengths and weaknesses," *The FASEB Journal*, vol. 22, no. 2, pp. 338–342, 2008.
 - [14] F. P. Carvalho, "Pesticides, environment, and food safety," *Food and Energy Security*, vol. 6, no. 2, pp. 48–60, 2017.
 - [15] J. F. Burnham, "Scopus database: a review," *Biomedical Digital Libraries*, vol. 3, 2006.
 - [16] R. Larayetan, C. Olisah, and O. A. Idris, "Research progression on studies related to green synthesis nanoparticles: a bibliometric review," in *Green Synthesis of Nanoparticles: Applications and Prospects*, pp. 1–22, Springer, Berlin, Germany, 2020.
 - [17] A. Perianes-Rodriguez, L. Waltman, and N. J. van Eck, "Constructing bibliometric networks: a comparison between full and fractional counting," *Journal of Informetrics*, vol. 10, no. 4, pp. 1178–1195, 2016.
 - [18] C. Olisah, O. O. Okoh, and A. I. Okoh, "Global evolution of organochlorine pesticides research in biological and environmental matrices from 1992 to 2018: a bibliometric approach," *Emerging Contaminants*, vol. 5, 2019.
 - [19] I. Cañas-Guerrero, F. R. Mazarrón, A. Pou-Merina, C. Calleja-Perucho, and G. Díaz-Rubio, "Bibliometric analysis of research activity in the 'Agronomy' category from the Web of Science, 1997–2011," *European Journal of Agronomy*, vol. 50, pp. 19–28, 2013.
 - [20] J. Zhang, Q. Yu, F. Zheng, C. Long, Z. Lu, and Z. Duan, "Comparing keywords plus of WOS and author keywords: a case study of patient adherence research," *Journal of the Association for Information Science and Technology*, vol. 67, no. 4, pp. 967–972, 2016.
 - [21] H. Y. Kang, Z. S. Othman, and S. I. Zubairi, "Accumulation of biopesticide-based rotenone from an optimized [BMIM][OTf] green binary solvent mixture in different parts of terong plant (*solanum melongena*)," in *Procedia Engineering*, pp. 702–709, Elsevier, Amsterdam, Netherlands, 2016.
 - [22] D. W. Aksnes, L. Langfeldt, and P. Wouters, "Citations, citation indicators, and research quality: an overview of basic concepts and theories," *SAGE Open*, vol. 9, no. 1, 2019.
 - [23] P. Nieminen, J. Carpenter, G. Rucker, and M. Schumacher, "The relationship between quality of research and citation frequency," *BMC Medical Research Methodology*, vol. 6, 2006.
 - [24] X. Liu, L. Zhang, and S. Hong, "Global biodiversity research during 1900–2009: a bibliometric analysis," *Biodiversity and Conservation*, vol. 20, no. 4, pp. 807–826, 2011.
 - [25] T. Riss, A. Niles, R. Moravec, N. Karassina, and J. Vidugiriene, "Cytotoxicity assays: in vitro methods to measure dead cells," in *Assay Guidance Manual*, 2019.
 - [26] S. Lee and B. Bozeman, "The impact of research collaboration on scientific productivity," *Social Studies of Science*, vol. 35, no. 5, pp. 673–702, 2005.
 - [27] R. Pavela and G. Benelli, "Essential oils as ecofriendly bio-pesticides? Challenges and constraints," *Trends in Plant Science*, vol. 12, 2016.
 - [28] M. S. Mulla and T. Su, "Activity and biological effects of neem products against arthropods of medical and veterinary importance," *Journal of the American Mosquito Control Association*, vol. 15, no. 2, pp. 133–152, 1999.
 - [29] E. D. Morgan, "Azadirachtin, a scientific gold mine," *Bio-organic & Medicinal Chemistry*, vol. 17, no. 12, pp. 4096–4105, 2009.
 - [30] T. G. Son, S. Camandola, T. V. Arumugam et al., "Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia," *Journal of Neurochemistry*, vol. 112, no. 5, pp. 1316–1326, 2010.
 - [31] S. Miresmailli and M. B. Isman, "Botanical insecticides inspired by plant-herbivore chemical interactions," *Trends in Plant Science*, vol. 19, no. 1, pp. 29–35, 2014.
 - [32] J. A. Dodge, A. L. Glasebrook, D. E. Magee et al., "Environmental estrogens: effects on cholesterol lowering and bone in the ovariectomized rat," *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 59, no. 2, pp. 155–161, 1996.
 - [33] G. Benelli, R. Pavela, F. Maggi, R. Petrelli, and M. Nicoletti, "Commentary: making green pesticides greener? The potential of plant products for nanosynthesis and pest control," *Journal of Cluster Science*, vol. 28, no. 1, pp. 3–10, 2017.
 - [34] S. Attia, K. L. Grissa, G. Lognay, E. Bitume, T. Hance, and A. C. Maillieux, "A review of the major biological approaches to control the worldwide pest *Tetranychus urticae* (Acari: tetranychidae) with special reference to natural pesticides," *Journal of Pest Science*, vol. 86, no. 3, pp. 361–386, 2013.
 - [35] N. E. El-Wakeil, "Retracted article: botanical pesticides and their mode of action," *Gesunde Pflanzen*, vol. 65, no. 4, pp. 125–149, 2013.
 - [36] X. Qian, P. W. Lee, and S. Cao, "China: forward to the green pesticides via a basic research program," *Journal of Agricultural and Food Chemistry*, vol. 58, no. 5, pp. 2613–2623, 2010.
 - [37] J. E. Cresswell, N. Desneux, and D. vanEngelsdorp, "Dietary traces of neonicotinoid pesticides as a cause of population declines in honey bees: an evaluation by Hill's epidemiological criteria," *Pest Management Science*, vol. 68, no. 6, pp. 819–827, 2012.
 - [38] T. Sibanda, H. M. Dobson, J. F. Cooper, W. Manyangarirwa, and W. Chiimba, "Pest management challenges for smallholder vegetable farmers in Zimbabwe," *Crop Protection*, vol. 19, no. 8–10, pp. 807–815, 2000.
 - [39] A.-T. H. Mossa, "Green Pesticides: essential oils as bio-pesticides in insect-pest management," *Journal of Environmental Science and Technology*, vol. 9, no. 5, pp. 354–378, 2016.
 - [40] O. Koul and S. Walia, "Comparing impacts of plant extracts and pure allelochemicals and implications for pest control," *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, vol. 4, pp. 1–30, 2009.
 - [41] H. M. Kotkar, P. S. Mendki, S. V. G. S. Sadan, S. R. Jha, S. M. Upasani, and V. L. Maheshwari, "Antimicrobial and pesticidal activity of partially purified flavonoids of *Annona squamosa*," *Pest Management Science*, vol. 58, no. 1, pp. 33–37, 2002.
 - [42] D. S. Charleston, R. Kfir, M. Dicke, and L. E. M. Vet, "Impact of botanical pesticides derived from *Melia azedarach* and *Azadirachta indica* on the biology of two parasitoid species of the diamondback moth," *Biological Control*, vol. 33, no. 2, pp. 131–142, 2005.
 - [43] K. M. Martin, M. A. Rossing, L. M. Ryland, R. F. DiGiacomo, and W. A. Freitag, "Evaluation of dietary and environmental risk factors for hyperthyroidism in cats," *Journal of the American Veterinary Medical Association*, vol. 217, no. 6, pp. 853–856, 2000.
 - [44] P. R. Hunt, T. G. Son, M. A. Wilson et al., "Extension of lifespan in *C. elegans* by naphthoquinones that act through stress hormesis mechanisms," *PLoS One*, vol. 6, Article ID e21922, 2011.

- [45] N. K. Dubey, R. Shukla, A. Kumar, P. Singh, and B. Prakash, "Prospects of botanical pesticides in sustainable agriculture," *Current Science*, vol. 98, pp. 479–480, 2010.
- [46] K. Zheng, L. Lin, and X. Feng, "Chiral N,N'-Dioxide-Ni(II) complex catalyzed asymmetric carbonyl-ene reaction of ethyl trifluoropyruvate," *Acta Chimica Sinica*, vol. 70, no. 17, pp. 1785–1790, 2012.
- [47] R. S. Mann and P. E. Kaufman, "Natural product pesticides: their development, delivery and use against insect vectors," *Mini-Reviews in Organic Chemistry*, vol. 9, pp. 185–202, 2012.
- [48] K. J. Rao and S. Paria, "Use of sulfur nanoparticles as a green pesticide on *Fusarium solani* and *Venturia inaequalis* phytopathogens," *RSC Advances*, vol. 3, no. 26, pp. 10471–10478, 2013.
- [49] J. L. V. Estrela, M. Fazolin, V. Catani, and M. R. Alc cio, "Toxicidade de  leos essenciais de *Piper aduncum* e *Piper hispidinervum* em *Sitophilus zeamais*," *Pesquisa Agropecu ria Brasileira*, vol. 41, no. 2, pp. 217–222, 2006.
- [50] G. Benelli, R. Pavela, R. Petrelli et al., "The essential oil from industrial hemp (*Cannabis sativa* L.) by-products as an effective tool for insect pest management in organic crops," *Industrial Crops and Products*, vol. 122, pp. 308–315, 2018.
- [51] P. Caboni, N. G. Ntalli, N. Aissani, I. Cavoski, and A. Angioni, "Nematicidal activity of (E,E)-2,4-Decadienal and (E)-2-Decenal from *Ailanthus altissima* against *Meloidogyne javanica*," *Journal of Agricultural and Food Chemistry*, vol. 60, no. 4, pp. 1146–1151, 2012.
- [52] A. Zibae and A. R. Bandani, "Effects of *Artemisia annua* L. (Asteraceae) on the digestive enzymatic profiles and the cellular immune reactions of the Sunn pest, *Eurygaster integriceps* (Heteroptera: scutellaridae), against *Beauveria bassiana*," *Bulletin of Entomological Research*, vol. 100, no. 2, pp. 185–196, 2010.
- [53] M. D. Moreira, M. C. Pican o, L. C. A. Barbosa, R. N. C. Guedes, E. C. Barros, and M. R. Campos, "Compounds from *Ageratum conyzoides*: isolation, structural elucidation and insecticidal activity," *Pest Management Science*, vol. 63, no. 6, pp. 615–621, 2007.
- [54] F. Mansour, H. Azaizah, B. Saad, Y. Tadmor, F. Abo-Moch, and O. Said, "The potential of middle eastern flora as a source of new safe bio-acaricides to control *Tetranychus cinnabarinus*, the carmine spider mite," *Phytoparasitica*, vol. 32, no. 1, pp. 66–72, 2004.
- [55] G. Valladares, M. T. Defago, S. Palacios, and M. C. Carpinella, "Laboratory evaluation of *Melia azedarach* (meliaceae) extracts against the elm leaf beetle (Coleoptera: chrysomelidae)," *Journal of Economic Entomology*, vol. 90, no. 3, pp. 747–750, 1997.
- [56] G. Rosell, C. Quero, J. Coll, and A. Guerrero, "Biorational insecticides in pest management," *Journal of Pesticide Science*, vol. 33, no. 2, pp. 103–121, 2008.
- [57] J. Sun, Y. Guo, M. M. Scarlat, G. Lv, X. G. Yang, and Y. C. Hu, "Bibliometric study of the orthopaedic publications from China," *International Orthopaedics*, vol. 42, 2018.
- [58] E. Hassan and A. G k e, "Production and consumption of biopesticides," in *Advances in Plant Biopesticides*, pp. 361–379, Springer, Berlin, Germany, 2014.
- [59] P. Andre -Mart nez, V. M. Ortiz-Mart nez, N. Garc a-Mart nez et al., "A descriptive bibliometric study on bio-availability of pesticides in vegetables, food or wine research (1976–2018)," *Environmental Toxicology and Pharmacology*, vol. 77, 2020.
- [60] E. T. Nyahangare, B. M. Mvumi, and T. Mutibvu, "Ethno-veterinary plants and practices used for ecto-parasite control in semi-arid smallholder farming areas of Zimbabwe," *Journal of Ethnobiology and Ethnomedicine*, vol. 11, pp. 1–16, 2015.
- [61] S. Siamaki, E. Geraei, and F. Zare-Farashbandi, "A study on scientific collaboration and co-authorship patterns in library and information science studies in Iran between 2005 and 2009," *Journal of Education and Health Promotion*, vol. 3, p. 99, 2014.
- [62] S. e. H. Zyoud, "Investigating global trends in paraquat intoxication research from 1962 to 2015 using bibliometric analysis," *American Journal of Industrial Medicine*, vol. 61, no. 6, pp. 462–470, 2018.
- [63] S. Zyoud, W. Waring, S. Al-Jabi, and W. Sweileh, "Global research production in glyphosate intoxication from 1978 to 2015: a bibliometric analysis," *Human & Experimental Toxicology*, vol. 36, no. 10, pp. 997–1006, 2017.
- [64] T. V. Nguyen, T. P. Ho-Le, and U. V. Le, "International collaboration in scientific research in Vietnam: an analysis of patterns and impact," *Scientometrics*, vol. 110, no. 2, pp. 1035–1051, 2017.
- [65] O. E. Sala, F. S. Chapin, J. J. Armesto et al., "Global biodiversity scenarios for the year 2100," *Science*, vol. 287, 2000.
- [66] S. Williamson, "Understanding the full costs of pesticides: experience from the field, with a focus on Africa," in *Pesticides-The Impacts of Pesticides Exposure* InTech Open, London, UK, 2011.
- [67] B. Dinham, "Growing vegetables in developing countries for local urban populations and export markets: problems confronting small-scale producers," *Pest Management Science*, vol. 59, no. 5, pp. 575–582, 2003.

Annexure B

Calculation of water holding capacity (WHC) of artificial soil:

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

Where S = water saturated soil and D = dry soil

$$\text{Sample 1: } S = 2,384 \text{ g and } D = 1,804 \text{ g} \rightarrow \frac{2,384 - 1,804}{1,804} \times 100 = 32,15\%$$

$$\text{Sample 2: } S = 1,251 \text{ g and } D = 0,981 \text{ g} \rightarrow \frac{1,251 - 0,981}{0,981} \times 100 = 27,52\%$$

$$\text{Sample 3: } S = 1,635 \text{ g and } D = 1,249 \text{ g} \rightarrow \frac{1,635 - 1,249}{1,249} \times 100 = 30,90\%$$

$$\rightarrow \text{mean WHC is } (32,15\% + 27,52\% + 30,09\%) \div 3 = 29,92\%$$

Annexure C

Biochar analysis

Sample	C (%)	N (%)	Ca (%)	Mg (%)	K (%)	P (%)	Na (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Fe (mg/kg)	Al (mg/kg)
1	49,33	0,71	0,44	0,14	0,32	0.05	1558,1	4	8	884	118	1103
2	42,90	0,56	0,46	0,14	0,37	0.05	1462,1	4	8,8	936	121	1045
3	46,05	0,61	0,46	0,14	,035	0.05	1341,5	4	8,8	947	122	1065