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Synergistic effects of a domestic (Thiacloprid based) neonicotinoid pesticide and Nitrogen, Phosphorus and Potassium fertiliser on common earthworms (Lumbricus terrestris).

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Synergistic effects of a domestic (Thiacloprid based) neonicotinoid pesticide and Nitrogen, Phosphorus and Potassium fertiliser on common earthworms (*Lumbricus terrestris*).

By

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Synergistic effects of a domestic (Thiacloprid based) neonicotinoid pesticide and Nitrogen, Phosphorus and Potassium fertiliser on common earthworms (*Lumbricus terrestris*).

Abstract

Neonicotinoids are designed to target insect pests, but their extensive use and long persistence in soils mean that non-target soil organisms such as earthworms are likely to be chronically exposed to them. The common use of neonicotinoids in agricultural systems makes them highly likely to come into contact with other agro-chemicals that may give rise to synergistic effects.

Chronic exposure of common earthworm (*Lumbricus terrestris*) to neonicotinoids in single or combined use with inorganic NPK fertiliser may pose problems that are not accounted for in most biological risk assessments under laboratory conditions.

To assess the impacts of such chronic exposure on earthworms, different concentrations of a neonicotinoid, in single or combined use with granulated NPK fertiliser were applied under controlled field conditions in three separate experiments, to mesocosms each containing 10 worms. Response variables measured were nocturnal activity and copulations, survival, change in mass and cocoon production. The neonicotinoid used was Thiacloprid, in the domestic formula most readily available to the public from 2017 to 2019 ('Provada Ultimate Bug Killer Concentrate 2' manufactured by Bayer (BPUBKC2)). Soils used included either standardised test soil 'LUFA2.2' or 'Levington's Organic Blend Topsoil' (OBT).

Two experiments used LUFA2.2 and one used OBT. The former was used to investigate whether LUFA2.2 is a suitable test soil for adaptation of the OECD earthworm reproduction test (ERT) for controlled field conditions. Each experiment applied different concentrations of Thiacloprid in single or combined use with NPK to assess effects of the chemicals in isolation and if mixtures caused synergistic effects.

Applications of Thiacloprid in single and combined use with NPK can cause high levels of mortality to *L. terrestris* at a range of concentrations. All Thiacloprid treatments caused 100 % mortality to *L. terrestris* when accommodated in LUFA2.2. As such, it was not conclusive that NPK gave rise to synergistic effects on mortality. Mortality in OBT was not significantly affected by exposure to combined treatments. However, NPK alone can have great effect on mortality of *L. terrestris*. Survival was significantly reduced by the presence of NPK in LUFA2.2 but not in OBT.

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Table of abbreviations

Abbreviation	Explanation
AC_{50}	Avoidance Response (50% spatial avoidance response in the population)
As	Arsenic
AST	OECD, Artificial Soil test
BPUBKC2	Provado Ultimate Bug Killer Concentrate 2
CCD	Colony Collapse Disorder (Honey bees (*Apis mellifera*))
CFS	Center for Food Safety
EU	European Union
DEFRA	Department for Environment, Food and Rural Affairs
DNA	deoxyribonucleic acid
EC_{50}	Effective Concentration (Half maximal effective concentration of toxin which induces a response halfway between the baseline and maximum after a specified exposure time)
EFSA	European Food Safety Authority
ERT	OECD, Earthworm Reproduction Test
FERA	Food and Environment Research Agency
FPCT	OECD, Filter paper contact test
FYM	Farm yard manure
LC_{50}	Lethal Concentration (Concentration of test chemical it takes to kill 50% of a given population)
LUFA2.2	LUFA2.2, standardised natural occurring soil type
MIN	Mineral Fertilisers
nAChRs	Nicotinic acetylcholine receptors
NGO	Non-Government Organisations
NPK	Nitrogen, Phosphorus, Potassium
OCC	Organic Carbon Content
OBT	Organic Blend Topsoil
OECD	Organisation for Economic Co-operation and Development
Pb	Lead
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (EU regulation)
RSPB	Royal Society for the Protection of Birds
SA	Soil Association
T	Thiacloprid suspo-emulsion formulation
TPM	Tooth Pick Method
WHC	Water Holding Capacity
XRT	X-Ray Tomography

Glossary

Word	Description
AC_{50} value	Avoidance Response (50% spatial avoidance response in the population).
Agricultural Intensification	The process of maximising outputs such as crops and livestock fodder yield per hectare by increasing inputs including labour, water, agro-chemicals and fertilisers, often to the detriment of the environment.
Agri-systems	Practices used to farm livestock, fodder, crops and energy for human consumption and economics.
Agro-chemicals	Chemicals used in farming for the control of plant or animal pests and disease or the fertilisation of soil.
Anecic	Earthworm species that produce and live in permanent, vertical burrows (middens).
Anthropogenic	Human generated change to environments or impacts on them (e.g., human induced pollution or climate change).
Artificial soil test	Experiments using simulated soil types to determine effects (biological risk assessment) of chemicals and/or pollutants on flora, fauna and other living ecosystem counterparts.
Bio-availability	The quantity of a given substance (e.g., thiacloprid and NPK) that has an active effect on the body after entering a living organism.
Bio-accumulation	The gradual build-up of a given substance such as a pesticide within the body of a living organism.
Biocides	A given substance or micro-organism that is used for the control or any living/destructive organism by causing harm or elimination.
Biodiversity	The variety and variability of life on planet earth.
Bio-indicator	Living organisms useful for the assessment of ecosystem and the environmental health.
Bio-magnification	The concentration of a given substance such as a pesticide, present in the tissues of living organisms which become successively greater at higher trophic levels of food chains.
Bio-marker (earthworms)	A behaviour or response (e.g., weight change) used as a measurable indicator from a state a good health to assess the effects of a given substance on the body.
Carcinogenic	A substance that has the potential to cause cancer in living organisms.
Cellular compartmentation	The organisation of organelles of eukaryotic cells into sperate compartments with different microclimatic conditions that allow for each to perform its specific function with greater efficiency.

Chemical applicants	Any chemical substances added to farmland to maximise the production of crops such as pesticides, herbicides and fertilisers.
Chitinous organelles	Small, specialized, structures made of chitin, held in cells which function like organs by carrying out specific bodily tasks. Chitin (a nitrogen containing polysaccharide associated chemically to cellulose which forms hard, protective exoskeletons or outer coverings).
Chlorpyrifos	A pesticide in the organophosphate class used to control insects and worm infestation of crops, livestock and buildings.
Chronically exposed	The continuous or recurring contact with a given toxic substance over long periods of time.
Clitellum	The glandular, saddle like thickening near the head of sexually mature earthworms. It allows the exchange of sperm by secreting mucus which helps keep the bodies of partnered worms engaged. Following reproduction, the skin of the clitellum sloughs off over the head, containing fertilised eggs and forming a protective cocoon.
Colony collapse disorder	The sudden vanishing of the majority of worker bees in honey bee (*Apis mellifera*) colonies, often abandoning queen, abundant food availability, nursing bees and larvae.
EC_{50} value	Effective Concentration (Half maximal effective concentration of toxin which induces a response halfway between the baseline and maximum after a specified exposure time).
Ecological functioning	Collective behavioural activities (e.g., foraging, locomotion and excretion) of living organisms and the effects they have on the physical and chemical health of the environment and/or combined effects of every natural process that maintains ecosystems, i.e., the combined effects of individual functions, with the overall rate of functioning being governed by the interrelationships of abiotic (non-living) and/or biotic (living) factors.
Ecosystem cascade	Secondary species extinctions instigated by the primary extinction of key species within a given ecosystem.
Ecosystem engineers	An organism significantly capable of creating, modifying, maintaining or destroying habitats, through its environmental activities. Their activities can significantly affect species richness and landscape heterogeneity (compositional heterogeneity (diversity of habitat types) or configurational heterogeneity (number, size and arrangement of habitat patches)) where they occur.

Ecosystem services	Recourses or physiological benefits that humans receive from the natural functioning of ecosystems. Services are grouped into 4 key types including provisioning (e.g., raw materials, foods and water), regulating (e.g., water filtration, nutrient cycling and carbon sequester), supporting (help maintain other ecosystem services e.g., the hydrogen cycle) and cultural (recreational activities such as fishing, wildlife watching and activities that aid physical and mental health and wellbeing).
Endocrine	Secretion of hormones released into the circulatory system by glands of the endocrine system, to regulate the functioning of the bodies organs.
Epidemiology	The study of study and analysis of the distribution, patterns and determinants of health and disease conditions in populations.
Epidermis	The first outer layer of a given organisms skin (epithelium).
Fertilisers (Inorganic, Organic)	Organic fertilisers are naturally produced, high in carbon and added to soils to add nutrients to aid crop growth. Inorganic fertilisers are synthetically produced and consist of mined minerals and synthetic chemicals to aid and intensify crop growth.
Filter paper contact test	A method used to measure the toxic effects of test substances by exposing earthworms to such chemicals on moist filter paper.
Food security	Abundant access to a sufficient quantity of affordable, nutritious food.
Fungicides	Chemicals used to kill unwanted fungus.
Genotoxicity	The properties of substances that causes damage to the genetic information within cells, instigating mutations that can activate cancer.
Half life	The time it takes for a specified quantity of a chemical in the environment to be reduced by 50%.
Herbicides (Glyphosate, Atrazine)	Chemicals used to kill unwanted plants.
Insecticides (Lambda-cyhalothrin)	Chemicals used to kill unwanted insects.
Key indicators	Species used as indicators of ecosystem health and other species existing in that ecosystem.
LC_{50} value	Lethal Concentration (Concentration of test chemical it takes to kill 50% of a given population)
Mesocosm	An outdoor experimental system that analyses the natural environment under controlled conditions.
Middens	Piles of organic matter left behind by earthworms.
Morphology	The study of a particular form, shape or structure.
Neonicotinoids (Thiacloprid, Imidacloprid,	A class of neuro-toxic insecticides chemically similar to nicotine, designed to bind to the nicotinic

Clothianidin, Thiamethoxam	acetylcholine receptors of the central nervous system and kill insects.
Nicotinic acetylcholine receptors	Central nervous system receptor polypeptides that respond to the neurotransmitter acetylcholine.
Non target organism	Species that are directly or indirectly effected by the application of substances which are not the intended targets of such a chemical.
NPK	Nitrogen, phosphorus, and potassium fertiliser manufactured from natural or synthetic materials, applied to plants or soil to aid nutrition and growth.
Organic carbon content	Portion of soil measured as carbon in organic form, excluding living soil fauna and plant matter.
Peripheral blood lymphocytes	Peripheral blood lymphocytes are mature lymphocytes (white blood cells) that circulate in the blood, instead of confined to organs.
Pesticide	Umbrella term for chemicals used to kill pests.
Pollinators	Any animal that transports pollen from the male anther to the female stigma of flowers to fertilise the ovules by the male gametes from pollen grains.
Precautionary principle	Allows decision makers to implement precautionary measures when scientific evidence on an environmental or human health hazard is tentative and presents possible high-risk factors.
Reproduction test	A method used to measure the toxic effects of test substances on reproduction by exposing earthworms to such chemicals in test soils for a specified time frame.
Setae (setal)	Chitinous, stiff bristles along the body of the earthworm that helps to connect with the soil surface and prevent backsliding during peristaltic movement.
Soil characteristics	The chemical, physical and biological properties that make up a given soil type.
Soil fauna	Numerous taxonomic groups of terrestrial and aquatic animals that regulate soil nutrient cycling by directly consuming organic matter, increasing its decomposition rates and distributing its nutrients.
Stressors	A physical factor (e.g., a toxin) has an adverse effect on an ecosystem or its biotic counterparts, which may result fatality or hinder growth or reproduction.
Sub-lethal	An effect that is less severe than lethal (e.g., weight loss, decreased fertility and reduced reproduction).
Suspo-emulsion	A formulation used for combining two active ingredients with different physical properties into one substance.
Synergistic	An interaction of two or more substances that produce a combined effect greater than their individual impact.

Systemic	Water solubility is adequate enough to allow a given substance to be absorbed by a plant and flow around its tissues.
Trophic level	The position a given to organisms with similar feeding traits that reside in a food web or ecological pyramid.
Water holding capacity	The ability of a soil type to physically retain water against gravitational pull.
X-RAY Tomography	A radiologic method of achieving clear X-ray images of internal structures.

Introduction

Intensive, agro-chemical farming practices using synthetic chemical applications to maximise crop productivity is held partially responsible for adverse impacts on ecosystems and ecosystem services (Goulson, 2013; Kurwadkar and Evans, 2016). Neonicotinoids are commonly applied to intensively managed, arable land to protect crops from pests and disease, thus increasing crop yields (Jeschke *et al*., 2011: Goulson, 2013). However, it is difficult to assess if widespread application of neonicotinoids has contributed to yield increases in agriculture and whether they offer greater economic benefits compared to alternative pest control practices (Goulson, 2013; Kurwadkar and Evans, 2016). Therefore, it is essential to research and assess the economic costs of loss of ecosystem services to ensure they are accounted for by policy makers and protected for food security of future generations. Modern inorganic fertilisers such as nitrogen (N), phosphorus (P) and potassium (K) (NPK) are widely used to improve soil quality by replenishing nutrients lost through intensive farming methods (Motesharezadeh *et al*., 2017; Techen and Helming, 2017). It is therefore likely that NPK and neonicotinoids are commonly used in combination in agricultural environments and may initiate synergistic effects on beneficial soil fauna such as earthworms (Holmes, 2017). Earthworms such as the common earthworm (*Lumbricus terrestris*) are key eco-system engineers and healthy populations are essential for long-term sustainability of food production by creating highly nutritious soil through the breakdown of organic matter (Mediene *et al*., 2011; Wang *et al*., 2012; Chagnon *et al*., 2014; Velki and Ečimović, 2015). Therefore, the loss of earthworm populations and ecosystem services they provide through widespread chemical application would be a catastrophic loss to European agriculture and economy, thus one incentive for research.

1. Agro-chemicals

1.1 Neonicotinoids

Neonicotinoids are synthetic, water-soluble chemicals easily absorbed by ground roots of plants where they are ingested by invertebrates that consume saturated tissue (Pelosi *et al*., 2013; van der Sluijs *et al*., 2014). They are designed to block neural transmission by binding to the nicotinic acetylcholine receptors (nAChRs) of the insects' central nervous system, causing loss of coordination and ability to feed (Jeschke *et al*., 2011; Gill *et al*., 2012; Eng *et al*., 2017), thereby protecting treated crops from pest damage. In spite of the apparent ability of neonicotinoids to target specific insects, there is tremendous concern among researchers regarding negative impacts on non-target organisms such as insect pollinators and ecosystem services they provide and vertebrates including people (Goulson, 2013; Kishandar, 2012).

Neonicotinoids are considered to have minimal impacts on non-target organisms that do not directly feed from treated crop tissue (Jeschke *et al*., 2011) but there appears to be a lack of literature supporting this claim. However, there have been recent surges in literature investigating toxic effects of neonicotinoid application on non-target organisms (Holmes, 2017). Re-analysis of the UK Food and Environment Research Agency (FERA) 2012 experiment investigating impacts of neonicotinoid seed dressings on bumblebee colonies (Goulson, 2015), found that when exposed to realistic levels of the neonicotinoid Imidacloprid, there was an 85 % reduction in the ability of bumblebee colonies to produce new queens. The implications of this include reduction in new colony establishment, decreasing pollination rates and having negative impacts on crop fertility (Goulson, 2015) which could have the adverse effect of incurring economic costs in future years.

Due to their systemic nature, neonicotinoids are marketed as low risk for non-target organisms whilst having maximum impact on crop specific pests (Jeschke *et al*., 2011; Soil Association, 2011; Goulson, 2014). Seed coating represents 60 % of neonicotinoid application with a combination of foliar sprays and soil drenching accounting for the rest (Jeschke *et al*., 2011; Soil Association, 2011). It is suggested that a short half-life in soil combined with seasonality and method of application can increase the volume of active ingredients available systemically to plants whilst preventing harm to non-target organisms (Jeschke *et al*., 2011). However, up to 94% of active ingredients from seed coating can remain in soil for up to 1000 days, thereby interacting with other agro-chemicals (Soil Association, 2011; Goulson, 2014; Basley and Gouson, 2017).

Considering this, is it possible that different methods of application where neonicotinoids are dissolved in water lend themselves to greater percentages of active ingredients entering soil and water networks compared to seed coating (Holmes, 2017) and could they be adversely affecting non-target soil fauna such as *L. terrestris*? Only 6 % of active ingredients of neonicotinoids applied as seed coating are claimed to be absorbed by crops and of that only 1 % given off in pollen, nectar or dust directly affects insect pollinators (Soil Association, 2011; Goulson, 2014). More research to quantify amounts, distributions, fates and consequences of application of pesticides and of neonicotinoids in particular in single or combined use with other chemical applicants such as NPK is surely needed.

1.2. Inorganic fertilisers

Common use of modern inorganic fertilisers such as NPK caused a global division of arable and pastural farming. Over time, this division reduced the use of traditional

livestock manures as fertilisers, known to increase earthworm biomass by 15 % compared to mineralised nitrogen fertiliser (Blanchet *et al*., 2016). The total global volume of NPK used in 2015 was 285.15 million tonnes, of which 245.77 was supplied for farming (FAO, 2017; Motesharezadeh *et al*., 2017; Holmes, 2017). There is limited research on the effect of inorganic fertilisers of this scale on earthworms but there are some long-term field trials, spanning the 1800s to date. Clements *et al*., (1991) demonstrated that surface leaf litter mass increases when exposed to concentrations of N, ranging from (612 g (188 kg N/ha)) at the lowest dose to (3730 g (752 kg N/ha)) at the highest. When earthworms are absent there is zero transfer of leaf litter from soil surface to lower depths, reducing nutrient availability and crop yield (Clements *et al*., 1991; Holmes, 2017) which would surely incur economic costs.

Pfiffner and Mädder (1997) assessed the differences in earthworm abundance between N fertiliser, Farm Yard Manure (FYM) and control treatments. Average abundance of earthworms was 144.5 m^{-2} with application of FYM, 67.9 m^{-2} in control treatments, and 60.9 m^{-2} with N, indicating that application of manure may benefit earthworms, but that inorganic fertiliser does not. Nitrogen fertilisers as single treatments have shown to enhance earthworm populations up to certain concentrations if soil pH is not altered (Blanchet *et al*., 2016). Earthworms prefer soils with pH values between 6.5 to 7.5 (Edward *et al*. 1995, Edwards and Bohlen 1996). Lordache and Borza, 2010, found the largest number of earthworms in treatments with the highest concentration of nitrogen fertiliser (by 85.85 % higher compared to the control treatment). Despite this, the greatest negative factor on earthworm abundance was pH, 26.67 /m^2 at pH 6.12, 14.67 /m^2 at pH 5.95 and 9.33 /m^2 at pH 6.67 (Lordache and Borza, 2010), indicating a narrow tolerance of earthworms to pH above and below the range of 6. Taking this into account,

the cumulative effects of NPK and synergisms with neonicotinoids and other chemical applicants could further alter soil pH beyond the tolerance of earthworm species and emphasises the need to research this knowledge gap with urgency (Wang *et al*., 2016; Mao *et al*., 2017; Van Hoesel *et al*., 2017).

A variety of fertilisers are used in combination with many agro-chemicals including fungicides, herbicides and insecticides, to maximise yield by protecting crops from infestation and disease (Pelosi *et al*., 2013; van der Sluijs *et al*., 2014; Wang *et al*., 2016). The neonicotinoid (Imidacloprid) accounts for 41 % value of the entire agrochemical market alone and is registered for use on 140 crop types in 120 countries (Jeschke *et al*., 2011; Goulson, 2014; Wang *et al*., 2015). Its widespread use indicates that neonicotinoids frequently come into contact with inorganic fertilisers that could give rise to synergistic effects on beneficial non-target soil fauna (Holmes, 2017). There is no evidence of literature on combined effect of NPK and neonicotinoids on non-target organisms including earthworms and given the probability that they are chronically exposed to cocktails of synthetic chemicals, deserves further investigation (Basley and Goulson, 2017; Holmes, 2017).

1.3. Neonicotinoids and Pollinators

In 2013, a devastating article was released by German researchers reporting enormous reduction in biomass of insects caught in Malaise traps positioned in 63 nature reserves in Germany beginning 1989 (Sorg et al., 2013: Leather, 2018). Hallmann *et al*., (2017) released a re-analysis of the data in the article by Sorg *et al*., (2013) demonstrating declines in average airborne insect biomass of 76 % (up to 82 % in midsummer) in just 27 years. This decline is apparent regardless of habitat-type and other factors including

changes in weather, land-use, and habitat characteristics cannot explain this overall decline (Hallmann *et al*., 2017; Leather, 2018). Although the driving force of this reduction in flying insect biomass remains unknown, it is likely that agricultural intensification through use of chemical applicants is partially to blame (Hallmann *et al*., 2017; Leather, 2018). A large amount of this biomass is likely to include insect pollinators responsible for the pollination of 70 % of global crop species and yield, of which 35 % is essential for human nutrition (Birkin and Goulson, 2015; Cole et al., 2015; Wood *et al*., 2015). The annual global economic cost associated with the loss of agricultural pollination services through population declines is valued in the region of € 153 billion (Birkin and Goulson, 2015).

Pollination is essential to crop fertility, health and yields but agro-chemical applications are held partially responsible for insect pollinator declines (EFSA, 2013; Thompson *et al*., 2013; Birkin and Goulson, 2015). The neonicotinoid thiacloprid is believed by some to be a 'bee-safe' neonicotinoid (Jeschke, 2011), yet bees are insects with similar nicotinic acetylcholine receptors to target, pest insects which would suggest otherwise. In fact, the phenomenon of colony collapse disorder in honey bees (*Apis mellifera*) has been partially attributed to widespread application of neonicotinoids along with other associated stressors (van der Sluijs *et al*., 2013).

It is important, however, to understand that neonicotinoids are unlikely to be the only contributor to adverse effects on pollinators with other agrochemicals possibly producing similar effects in single and combined use, as such a key driver of this research. The loss of soil fauna including earthworms responsible for nutrient cycling will have a negative effect on plant abundance, driving downwards ecosystem cascades that will undoubtably contribute further to pollinator declines and associated economic

costs. It is crucial to understand the economic cost of ecosystem service losses, including those provided by soil organisms, through intensive chemical farming practices (Carvell *et al*., 2015; Goulson, 2015; Wood *et al*., 2015), address government accountability and global food security (Holmes, 2017).

1.4. Regulation/legislations

Neonicotinoids are considered highly toxic to insects whilst appearing safe to vertebrates, increase agricultural yields, reduce poverty and control the spread of crop disease by targeting vectors (Jeschke *et al*., 2011; Goulson, 2013). These are arguments often used to convince governments to support their application for agricultural intensification to nourish the growing human population (Jeschke *et al*., 2011; Goulson, 2013; Wang *et al*., 2015). In spite of this, the UK recently backed an EU total ban on pesticides that can harm bees (Carrington, 2017; Collier, 2017). The European Commission consequently adopted regulations to completely ban the outdoor use of imidacloprid, clothianidin and thiamethoxam on 29 May 2018 (European Commission, 2018). However, Britain's exit from the EU creates uncertainty about whether this ban will extend to the UK and if it does, whilst good for non-target organisms, may have other implications. Such a shift in legislation may favour greater use of other neonicotinoids and pest control chemicals considered by some to be safe for bees such as thiacloprid (Jeschke *et al*., 2011). Thiacloprid is a neonicotinoid that is already being considered by the European Commission as a substitute due to its endocrine disrupting properties (European Commission, 2018). If greater use of these chemicals causes their concentrations in the soil to increase, then there may be detrimental implications for soil fauna and associated ecosystem services. A procedure to renew the approval of

thiacloprid (under Regulation (EU) No 844/2012) is ongoing with its current approval expiring on 30 April 2020.

Here in the UK, Brexit could provide opportunities to protect and enhance soil health through 'The Agricultural Bill', setting out how farmers and land managers will in future be paid for "public goods", such as better air and water quality, improved soil health, higher animal welfare standards, public access to the countryside and measures to reduce flooding (DEFRA and Gove, 2018). Under the new agricultural bill, farmers and land-managers who provide the greatest environmental benefits will secure the largest subsidies, laying the foundations for an apparent greener, cleaner and healthier countryside for future generations (DEFRA and Gove, 2018). If the Government implements this green agricultural bill, we should expect to see a reduction in the use of synthetic chemicals in favour of more environmentally friendly farming methods that protect soil fauna, ecosystem services and build soil. However, we cannot rely on Government to better regulate agro-chemical use without evidence of negative effects on the environment, ecosystem services and economy. Furthermore, we cannot rely on them to better regulate agro-chemical use of a matter of precaution, given track records and the current state of the environment. Taking this into account, the unreliability of Governments to implement chemical regulation using the 'Precautionary Principle' is a key driver of this research. The UK government appears to take a slower "sound science" approach to regulating environmental risk posed by agro-chemicals (Patterson and McLean, 2019) but the damage could be done before researchers can demonstrate adverse outcome. This highlights a need to research the effect of neonicotinoids in single and combined use on ecosystem counterparts including earthworms and services provided. Further research could ensure that beneficial non-target organisms are

accounted for by decision makers when improving legislation aimed at protecting biodiversity and food security by reforming the way agro-chemicals are regulated (Goulson, 2013; Goulson, 2014; Birkin and Goulson, 2015; Kumar, 2016).

1.5. Neonicotinoids: impacts on ecosystem services and economy

Biological interactions are crucial to maintaining healthy ecosystems including the soil food web, which is key to fertility. Earthworms may influence multiple functions of ecosystems through nutrient cycling and water infiltration but agro-chemicals in single or combined use may pose a risk to such interactions and put crop yields in jeopardy (Shennan, 2008). It is crucial to understand the economic cost of ecosystem service losses, including those provided by soil organisms, through intensive chemical farming practices (Carvell *et al*., 2015; Goulson, 2015; Wood *et al*., 2015), address government accountability and global food security (Carvell *et al*., 2015; Goulson, 2015; Wood *et al*., 2015).

During the 1970s French beekeepers began to debate possible causes for the new phenomenon of Colony Collapse Disorder (CCD) on honey bees (*Apis mellifera*) including natural pest infestation by the varroa mite (*Varroa destructor*) and pesticide applications (Goulson, 2014). Apiculturists quickly began to associate the beginning of CCD with the first agricultural use of a neonicotinoid launched under the trade name Calypso, against sucking and chewing pests (Jeschke *et al*., 2011). Over 30 years later, The United States Centre for Food Safety (CFS) (2014) determined on assessment of 19 scientific, peer review articles that there is minimal research evidencing that neonicotinoids maximise agricultural yields but highlight significant relationships between application, pollinator decline and reduced crop productivity. Assessment included the so called ‘Bee Safe’

pesticide thiacloprid, widely used across the European Union (EU) to combat infestation of pollen beetles (*Meligethes* spp.) on mass flowering crops such as oilseed rape (*Brassica napus*), despite agricultural pollination services provided (Goulson, 2013; Wood *et al*., 2015; Dewar; 2017). Since the systemic qualities of thiacloprid allow it to target *Meligethes* spp. through ingestion of pollen and nectar, the term 'Bee Safe' seems contradictory when non-target organisms are also exposed in this way (Goulson, 2013; Wood *et al*., 2015; Dewar; 2017).

The EU and all member states subsequently banned the use of three key bee-killing neonicotinoids (clothianidin, imidacloprid and thiamethoxam) in 2013 pending further investigation (Dewer, 2017; Zhang *et al*., 2017). However, thiacloprid remained in use across the EU and Elizabeth Truss, Environment Minister of the UK from 2014 to 2016 supported the use of neonicotinoids, subsequently securing a vote for lifting the ban in 2013 for use on mass flowering crops (Dewer, 2017; Zhang *et al*., 2017). This despite research by Goulson (2015), demonstrating that low level exposure to neonicotinoids reduced the ability of queen bumblebees (*Bombus* spp.) to reproduce and caused navigation loss of workers that leads to the death of entire colonies (Goulson, 2015; Jensen, 2015). It is therefore imperative that research continues to investigate the negative impacts of neonicotinoid application on population declines of pollinators, (Gouldson, 2013; Goulson, 2014; Birkin and Goulson, 2015; Stout and Finn, 2015) and other beneficial non-target organisms such as *L. terrestris*, so that the economic costs associated with losses of ecosystem services can be stressed. This will ultimately ensure that beneficial non-target organisms are accounted for by decision makers when improving legislation aimed at protecting biodiversity and food security by reforming

the way neonicotinoids are regulated (Goulson, 2013; Goulson, 2014; Birkin and Goulson, 2015; Kumar, 2016).

For example, economic costs may arise from loss of natural pest control through neonicotinoid related declines in populations of predators (Goulson, 2014; Hallamann *et al*., 2014) and reduced soil health via toxic effects on soil fauna such as earthworms (van der Sluijs *et al*., 2014; Wang *et al*., 2015; Velki and Ečimović, 2015).

Recently, there has been an influx of neonicotinoid-based products available in both agriculture and for domestic use (Jeschke *et al*., 2011; Goulson, 2014). Imidacloprid accounts for 41.5 % of the entire agro-chemical market, however competition through introduction of new substances threatens to erode the prices of generic major brand applicants (Jeschke *et al*., 2011; Goulson, 2014). This has led to opportunities for product creation within lower priced markets and increase affordability for agricultural and domestic use (Jeschke *et al*., 2011). Such opportunities will increase the amount of people able to acquire neonicotinoid-based products and the area of land exposed to treatment, having further implications for non-target organisms and possible adverse health impacts on people (Chen *et al*., 2014; Goulson, 2014; Hallamann *et al*., 2014; Cimino *et al*., 2016).

1.6. Neonicotinoid based domestic products

Price erosion has led to the development of many neonicotinoid-based domestic products in the public sector. Products range from parasite treatments for pets such as 'Advocate; Bayer Animal Health', as spot-on formulations or flea collars using imidacloprid as the key ingredient, to a number of products authorised for use on domestic crops, lawns and gardens against pests (Jeschke *et al*., 2011; Goulson, 2014).

The domestic, thiacloprid-based product 'Provado Ultimate Bug Killer Concentrate 2' made by Bayer (BPUBKC2) is one example of a neonicotinoid available at low cost at most supermarkets and garden centres. It is increasingly concerning that such products are widely available as foliar sprays and soil drenches for public use, especially since many researchers and Non-Government Organisations (NGOs) such as the Royal Society for the Protection of Birds (RSPB) are attempting to persuade the UK public that gardens are important refuges for wildlife from anthropogenic practices such as intensive agriculture (Rupprecht *et al*., 2015, Holmes, 2017). This suggests that an unknown and unprecedented amount of urban wildlife and soil organisms may be chronically exposed to fluctuating concentrations of neonicotinoids in gardens and public spaces (Holmes, 2017). Almost all neonicotinoid research has focussed on agriculture and there appears to be no evidence of research on the effects of domestic insecticide formulas and inorganic fertilisers on non-target organisms following authorised use in public, presenting a knowledge gap (Goulson, 2014). Furthermore, there is no evidence of chemical cocktails and synergistic effects on non-target organisms in the public realm, again few studies concentrate on agriculture in lab-based biological risk assessment studies, a further catalyst for this research in assessing lethal and sub-lethal effects of BPUBKC2 in single and combined use with NPK granules on *L. terrestris*.

1.7. Neonicotinoids: vertebrates

More recently there has been concern for the effects of neonicotinoids on non-target vertebrates such as birds and thought to be attributed to food contamination (Hallmann *et al*., 2013; Eng *et al*., 2017). There is currently concern among some researchers that neonicotinoids may have detrimental impacts on the health of people (Calderón-Segura

et al., 2012; Hallmann *et al*., 2017). This is despite statements by agrochemical producers that neonicotinoids are not harmful to mammals or birds, yet are selective in their literature by drawing attention away from toxic effects on fish (Calderón-Segura *et al*., 2012; Hallmann *et al*., 2017). There are few studies on the effects of neonicotinoid application on vertebrates, however research by Hallmann *et al* (2017) was able to demonstrate that significant reductions of 15 insectivorous bird species populations in the Netherland's were associated with high neonicotinoid concentrations, following introduction of imidacloprid in the 1990s. It is likely that the decline of all 15 species resulted from a combination of stressors associated with neonicotinoid treatment including loss of invertebrates exclusively used for chick rearing or direct consumption and poisoning from neonicotinoid coated seeds or contaminated live foods (Pelosi *et al*., 2013; Goulson, 2014; Hallamann *et al*., 2014).

The direct loss of species populations via loss of invertebrates or poisoning from trophic bio-accumulation and magnification by contaminated food should be a concern to us all (Calderón-Segura *et al*., 2012; Goulson, 2014; Hallamann *et al*., 2014). Due to the systemic nature of neonicotinoids, human ingestion through treated crops is unavoidable (Calderón-Segura *et al*., 2012; Kimura-Kuroda *et al*. 2012). Neonicotinoids have been found to bind with the mammalian nAChRs, similarly to nicotine, altering the density of neuro-receptors critical for human brain function and development including memory, cognition and behaviour (Calderón-Segura *et al*., 2012; Kimura-Kuroda *et al*. 2012; Chen *et al*., 2014). Changes in the density of neuro-receptors are associated with various nervous system disorders in people including Alzheimer's disease, Parkinson's disease, schizophrenia, and depression (Calderón-Segura *et al*., 2012; Kimura-Kuroda *et al*. 2012; Chen *et al*., 2014).

Various laboratory studies have also demonstrated adverse impacts on mammalian subjects including reduced sperm production and function, reduced pregnancy rates, increased embryo death, still and premature birth and slow development (Kumar, 2012; Chen *et al*., 2014; Cimino *et al*., 2016). Thiacloprid can be carcinogenic to mammals, having induced ovarian tumours in mice and uterine tumours in rats (Kumar, 2012; Chen *et al*., 2014; Cimino *et al*., 2016). Epidemiological studies have demonstrated that damage to human deoxyribonucleic acid (DNA) strands alter functions of the nervous, respiratory, reproductive and immune system and are all associated with increased risk of cancer (Kumar, 2012; Chen *et al*., 2014; Cimino *et al*., 2016). This is stressed by Cimino *et al* (2016), demonstrating exposure of human peripheral blood lymphocytes to neonicotinoid products caused DNA damage resulting in genotoxicity that instigates carcinogenic activity.

Taking the above information into consideration, there is reason to question whether vertebrate populations will be adversely impacted by earthworm exposure to thiacloprid including loss of food source and bio-accumulation and magnification causing bodily system failures and diseases associated with DNA damage (Calderón-Segura *et al*., 2012; Kimura-Kuroda *et al*. 2012; Chen *et al*., 2014; Cimino *et al*., 2016). These concerns are further catalysts for this research into lethal and sub-lethal effects of the domestic, thiacloprid based neonicotinoid BPUBKC2 in single and combined use with NPK granules on *L. terrestris* and to help develop foundations for future studies into species population declines (Pelosi *et al*., 2013; Goulson, 2014; Hallamann *et al*., 2014).

1.8. Neonicotinoids: Soil fauna

Tremendous international concern now exists among researchers regarding land contaminated with neonicotinoids and impacts on soil fauna (Wang *et al*., 2012; Wang *et al*., 2016). Internationally, seed coating represents the most frequent method of neonicotinoid application in agriculture at 60 %, with a combination of foliar sprays and soil drenching accounting for the rest (Jeschke *et al*., 2011; Soil Association, 2011). Jeschke *et al* (2011) suggests that the timing and method of application such as soil drenching increases the volume of neonicotinoid compounds available systemically to plants, thus preventing harm to non-target organisms. However, the Soil Association (SA) recently suggested that around 6 % of active ingredients from seed coating is up taken systemically by crops and that 80 to 90 % remains in soil for around 1000 days (Soil Association, 2011; Goulson, 2014; van der Sluijs *et al*., 2014). Taking this into consideration, it is conceivable that application methods such as foliar sprays and soil drenches where neonicotinoids are dissolved in water could lend themselves to increased percentages of active ingredients entering soil or water networks compared to that of seed coating (Soil Association, 2011; Goulson, 2014). This is likely to adversely affect a variety of soil fauna and is therefore a catalyst for investigating lethal and sub-lethal effects of domestic product BPUBKC2, a liquid-based formula containing thiacloprid as the active ingredient (Bayer, 2014) in single and combined use with NPK granules on *L. terrestris*, using the soil drench method of application.

There is extensive research on the impacts of neonicotinoid application on bees, but of the active ingredient applied, only 1 % is present in pollen or nectar used by pollinators (Soil Association, 2011; Goulson, 2013; Jeschke *et al*., 2013). Despite larger percentage of active ingredients remaining in soil, studies on the effects of neonicotinoid

accumulation in soil and consequences for soil fauna are few and remains poorly understood (Pelosi *et al*., 2013; Goulson, 2014). This disparity in effort between taxa and amounts of potential contaminant in soil provides further incentive for this research.

1.9. Earthworms

Earthworms are ecosystem engineers and key indicators of good soil conditions (Chen *et al*., 2014; Wang *et al*., 2016; Van Hoesel *et al*., 2017). There are three key ecological groups of earthworms (epegeic, anecic and epi-endogenic) representing three niches in soil habitats (Figure 1) (Kollath, 2021). Healthy populations are crucial for normal ecological functioning in terrestrial habitats including nutrient and energy cycling, decomposition of organic matter, soil formation and aeration and water infiltration (Wang *et al*., 2012; Chagnon *et al*., 2014; Velki and Ečimović, 2015). Earthworm behaviour is vital for soil organic matter dynamics and microbial activity (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016) as burrowing allows transportation of organic matter from soil surface to lower depths where further microbial decomposition occurs (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016). Earthworm burrows are also important for increasing soils water holding capacity (Capowiez *et al*., 2006; Murchie and Gordon, 2013). Earthworm eradication in terrestrial environments may increase soil compaction, impede water infiltration, increase runoff, erosion and drought, and flooding (Capowiez *et al*., 2006; Murchie and Gordon, 2013). Furthermore, earthworms are the foundations of many food chains and a reduction in populations will be detrimental at all trophic levels (Wang *et al*., 2012, Wang *et al*., 2016; Zaller *et al*., 2016). Therefore, it is important that biological risk assessment continues to investigate toxic effects of neonicotinoids on earthworms (Mao *et al*., 2017; Van Hoesel *et al*.,

2017), so that adverse effects on ecosystems and economics can be stressed and ensure accountability of legislators (Goulson, 2013; Goulson, 2014; Birkin and Goulson, 2015).

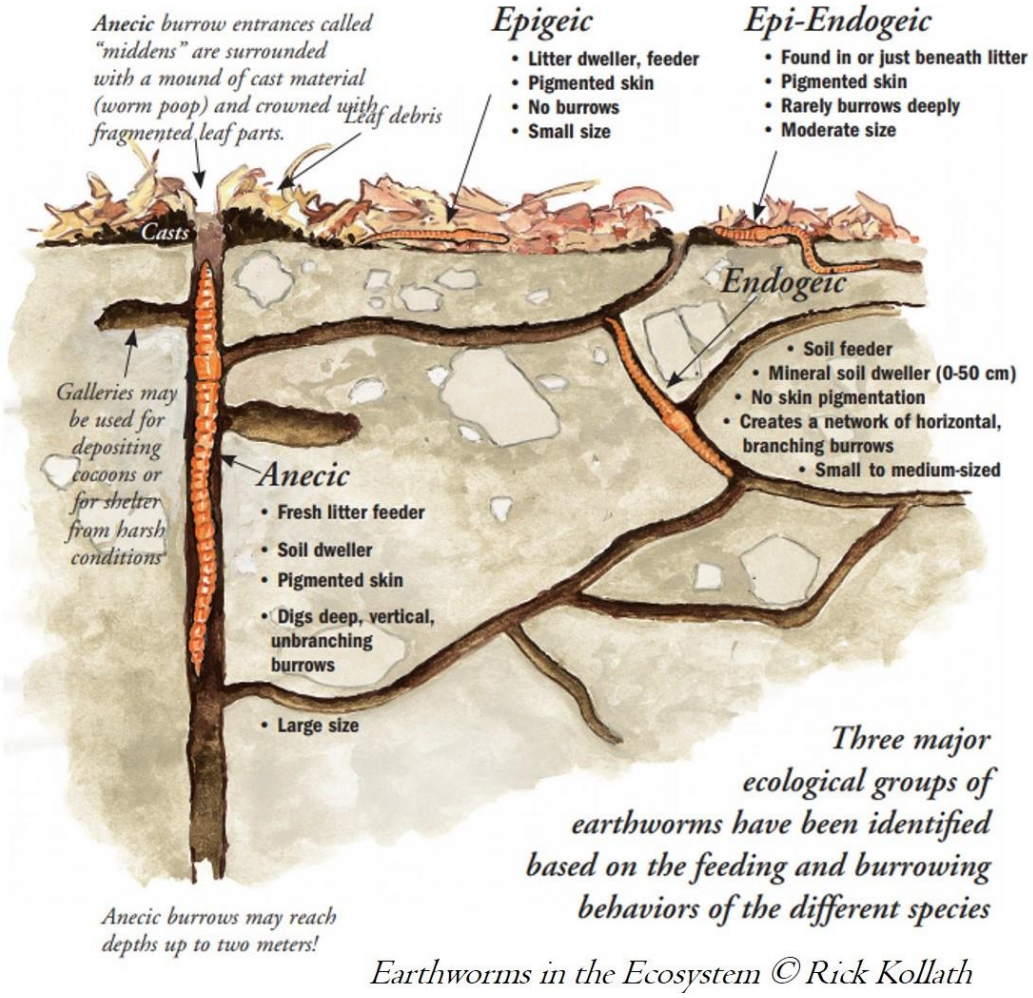

Figure 1: Earthworms in the ecosystem (Kollath, 2021)

Given the ecological importance of earthworms, research on the toxic effects of single agro-chemical applications has escalated, with the notable exception of neonicotinoids (Wang *et al*., 2016; Van Hoesel *et al*., 2017) leaving a gap in knowledge about their effects on a key component of the agricultural environment. Chemicals are also seldom applied in isolation and combined effects have received very little attention and this is even more marked for soil organisms (Wang *et al*., 2016; Mao *et al*., 2017; Van Hoesel

et al., 2017) despite evident concerns of multiple authors. As such, effects of agro-chemical mixtures on soil organisms deserves further investigation to ensure that services provided are safe-guarded (Wang *et al*., 2016; Mao *et al*., 2017; Van Hoesel *et al*., 2017).

Earthworm are key indicators of good soil conditions and healthy populations are vital for ecological functioning including nutrient cycling, decomposition of organic matter, soil formation and aeration and water infiltration (Luo *et al*., 1999; Wang *et al*., 2012; Chagnon *et al*., 2014; Velki and Ečimović, 2015). Earthworm behaviour is particularly crucial for soil functions, soil organic matter dynamics and microbial activity (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016). Behaviours such as burrowing, feeding and casting influence soil structure by creating passages that aid transportation of water and nutrients and aggregates that improve soil water retention (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016). One of the most common consequences of eradication of earthworms in forest and farmland ecosystems from various anthropogenic activities is soil compaction, which impedes water infiltration, increases agricultural runoff and erosion and drought, resulting in flooding (Capowiez *et al*., 2006; Murchie and Gordon, 2012; Widyatmani and Masateru, 2015).

Flooding events caused by ecological damage have detrimental impacts on people, including fatality and economic losses, affecting communities, businesses and government poverty reduction efforts (Surminski, 2018). The frequency with which random flooding events occur and associated effects are expected to be exacerbated by climate change (Surminski, 2018). The current costs of random flooding events and annual damage caused by them is estimated to cost the UK economy £ 1.3 billion (Energy and Climate Intelligence, 2021). Taking this into account, adverse impacts such as

flooding due to impaired ecosystem functions caused by eradication of earthworms is of huge concern, since neonicotinoids have proved highly toxic to the red worm (*Eisenia fetida*) and significantly reduce cocoon production and hatchability (Wang *et al*., 2015, Holmes, 2017). Therefore, the application of neonicotinoids in the field are likely to adversely affect or eradicate earthworm populations and reduce ecosystem functions they provide (Capowiez *et al*., 2006; Murchie and Gordon, 2012; Widyatmani and Masateru, 2015; Bawa *et al*., 2016).

Taking the above information into account and assumptions by some agro-chemical researchers that neonicotinoids are harmless to non-target organisms including earthworms provides further incentive for this research.

1.10. Agro-chemical mixtures on worms

Studies on effects of agro-chemicals on earthworms are scarce and there are virtually none that consider combined or synergistic effects. Studies on bees are more readily available but with large variation in results and conclusions. Nevertheless, they may be used to inform research about earthworms.

Most use traditional international standardised methodologies published by the Organisation for Economic Co-operation and Development (OECD) for testing chemicals on earthworms (OECD, 1984, OECD, 2015). The guidelines include three key experiments used within EU regulation, Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) for biological risk assessment and are all conducted under laboratory conditions (Alves *et al*., 2012; Pelosi *et al*., 2014; Wang *et al*., 2015). During such tests, biomarkers are used to distinguish diminishing fitness from a state of 'good health' when exposed to contaminants at variable concentrations and may include weight loss,

avoidance and reduced reproduction (Capowiez *et al*., 2003; Gomez-Eyles *et al*., 2007; Pelosi *et al*., 2014).

The Filter Paper Contact Test (FPCT) was used in conjunction with the Artificial Soil Test (AST) Wang *et al*., (2012) to compare methods of determining Lethal Concentration (LC) values of varying concentrations of 45 pesticides to red worms *E. fetida* in isolation and found that neonicotinoid, clothianidin had the highest toxicity, rating 'super toxic' with 0.28 (0.24 – 0.35) µg cm^{-2} LC capacity to kill 50 % (LC_{50}) of exposed worms (Wang *et al*., 2012). The results of the AST were consistent, with LC_{50} concentrations for clothianidin at 7.44 (6.65 – 9.06) mg kg^{-1} at 7 days and 6.06 (5.60 – 6.77) at 14 days exposure, remaining the most toxic of pesticides tested (Wang *et al*., 2012). Substantially lower concentrations may cause adverse sub-lethal effects on earthworm populations, especially when two or more chemicals act synergistically (Eng *et al*., 2017; Mao *et al*., 2017 Van Hoesel *et al*., 2017). In reality, agro-chemicals are likely to be applied as formulations and in combination and since these studies only measure single effects of active ingredients are deficient at accurately predicting biological responses of earthworms in the environment (Feng *et al*., 2016; Wang *et al*., 2016).

Recent research, using the toothpick method (TPM) found that common earthworm *L. terrestris* activity is significantly reduced by 9.2 % when exposed to imidacloprid seed dressing and further by 19.3 % when used combined with the herbicide glyphosate (Van Hoesel *et al*., 2017) indicating greater effects when in chemical synergism. Despite this, the TPM is fraught with potential criticisms of unreliability given that human error introduces a high-risk factor when disturbing toothpicks during husbandry. The TPM involves randomly inserting 12 toothpicks, vertically with the tip just penetrating the soils surface. Above ground earthworm activity alters the angle of the toothpicks and

the number of toothpicks differing from their original position (0.1 slight disturbance, 0.5 toothpicks tilted more than 45 º and 1 horizontal). The number of toothpicks in each category is then multiplied by the category value and summed as an index of above ground activity levels (Van Hosel *et al*., 2017). Surely the toothpicks are vulnerable to being disturbed by human activity and one earthworm just as likely to disturb 12 toothpicks as a small group.

More accurate for determining differences in worm activity is X-Ray Tomography (XRT), which Capowiez (2006) used to assess effects of imidacloprid on burrowing behaviour of *Allolobophora icterica*. Imidacloprid (0.1 or 0. 5 mg kg^{-1}) caused *A. icterica* to produce fewer, shorter and less continuous burrows, than those in control treatments. XRT could be a good method to assess synergistic effects of chemical cocktails on burrowing behaviour of earthworms and give a more realistic interpretations of possible effects in the field. This is because the length, depth and direction of developing soil macropores can be measured and compared with rising concentration of test chemicals in single and combined treatments against controls.

Reduced activity of worms via lethal or sub-lethal concentrations of chemical combinations could have greater adverse impacts on ecosystem services than use of single additives (EFSA, 2013; Goulson, 2013; Thompson *et al*., 2013). The OECD Earthworm Reproduction Test (ERT) may be a suitable method for testing sub-lethal effects of NPK and neonicotinoid synergisms on *L. terrestris*. Fecundity (cocoons per worm) in *E. fetida* was significantly less with application of thiacloprid at 1.50 mg/kg-1 soil (2.45 ± 0.35) than in controls (4.05 ± 0.05) (Wang *et al*., 2015). Thiacloprid also had significant, negative effects on cocoon hatchability, being 18.33 % less at 1.50 mg/kg-1 (3.97 ± 0.22) than controls (1.25 ± 0.25). Laboratory tests generally do not take into

account chemical cocktails and there is a distinct lack of literature on synergistic effects on any taxonomic groups (Wang *et al*., 2016; Mao *et al*., 2017 Van Hoesel *et al*., 2017). If different taxonomic groups differ in tolerance to agro-chemical contamination (Eng *et al*., 2017; Mao *et al*., 2017 Van Hoesel *et al*., 2017), investigation is warranted into impacts on reproduction to other British species of agricultural importance such as *L. terrestris*.

The current OECD methods are not representative of true field conditions due to short exposure time to treatments, active chemical ingredients are used rather than commercial formulae and confined to mesocosms causing pooling of chemicals at the bottom and model species do not commonly occur in agricultural habitats. Consequently, the OECD methods require reform, however, remain useful for isolating chemicals and cocktails for further field-testing (Pelosi *et al*., 2014; Wang *et al*., 2016; Mao *et al*., 2017; Van Hoesel *et al*., 2017). The ERT has qualities that could be transferable to controlled field conditions which current literature implies has not yet been applied to test single or combined effects of agro-chemicals on earthworms (Wang *et al*., 2016; Mao *et al*., 2017 Van Hoesel *et al*., 2017). As such, it may be a more realistic choice for research on agro-chemical synergies on earthworms, under controlled field conditions.

1.11. Aims and Objectives

Examination of '*Bayer Gardens: safety data sheet*' according to Regulation (EC) No. 1907/2006, shows there has been no biological risk assessment relating to effects on soil fauna (Bayer, 2014). Using the ERT, it is predicted that combinations of NPK and thiacloprid will have greater impacts on *L. terrestris* behaviours and reproduction than

single treatment and rising concentrations will increase lethality whilst reducing cocoon production and hatchability. Therefore, the biological responses of earthworms under controlled field conditions to single and combined use of NPK and Thaicloprid will be compared to predict synergistic effects in agricultural environments. The first rationale is to identify if the ERT is a suitable candidate for assessing toxicity of agro-chemicals to earthworms and evaluate suitability for identifying synergistic effects if two or more chemicals are used in combination. The second, to evaluate if the ERT may be suitably transferable to controlled field conditions or successfully adapted to improve experimental design for the intended study, including soil type. Below is listed a set of aims and objectives, this study hopes to achieve.

- Identify suitable biomarkers for testing toxicity of agro-chemical combinations on *L. terrestris* under controlled field conditions.

- Identify lethal and sub-lethal concentrations of test chemicals as single and combined application to *L. terrestris*.

- Identify and understand biological responses of *L. terrestris* to test chemicals in single and combined use that may have significant impacts on ecosystem structure, functioning and health to the detriment of ecosystem services, environment, food security and economy.

- Evaluate whether current international guidelines for testing chemicals on earthworms, under laboratory conditions are suitably transferable to controlled field conditions.

- Identify how methodology used in this research could be used to influence UK government and decision makers to develop policy and legislation aimed at protecting the environment, ecosystem services and food security outside of the EU.

The identification of adverse biological responses of *L. terrestris* under controlled field conditions to combined use of BPUBKC2 and NPK granules could help predict population declines in real agricultural environments (Holmes, 2017). For example, significantly reduced cocoon production and hatchability could reduce earthworm populations, resulting in losses of ecosystem services such as nutrient cycling, lowering crop yield and inflicting economic costs (Holmes, 2017). Significant effect on earthworm abundance in response to BPUBKC2 and NPK granules could be used as foundations for research into impacts of other agro-chemical mixtures on crop nutrition, growth and yield as a consequence of reduced nutrient cycling. Reduced abundance could highlight lower density of earthworm burrows and associated consequences including soil compaction which impedes water infiltration (Holmes, 2017). This will increase flooding and decrease space availability for arable agriculture, spoil crops and incur economic costs (Holmes, 2017). This study could be used to influence research seeking sustainable alternatives to intensive chemical farming practices to protect species that provide ecosystem services that benefit agriculture and have high economic value (Holmes, 2017). The study could be used to inform Government and Non-Government Organisations of adverse impacts of chemical combinations on earthworms and ecosystem services to ensure they are accounted for in policy or legislation development aimed at protecting food security (Holmes, 2017). The research could also help inform the public of environmental dangers of thiacloprid as a neonicotinoid licenced for domestic use in Europe and consequences for garden wildlife and crop production. It

may help change public perception on small scale, organic, bio-intensively produced crops as alternatives to chemical intensive agriculture, using natural predators as pest control, thus protects soil fauna and retains ecosystem services.

1.12. Hypothesis

The results will be gathered to reflect on whether there are any negative correlations between different concentrations of a neonicotinoid, thiacloprid-based domestic product in single and combined use with NPK fertiliser granules on *L. terrestris* under controlled field conditions. Experiment one will test if standardised soil LUFA 2.2, regularly used under laboratory conditions for biological risk assessment of agro-chemicals (Handy, 2018) with experiment two providing replication to give confidence about whether observations from experiment one was anomalous. The third experiment, was undertaken using Levington's organic blend topsoil (OBT) to create conditions that reflect natural conditions more closely and increase understanding and uncertainty of the outcome of experiments using LUFA 2.2.

1.12.1. Experiments 1 and 2:

H1: There are significant differences among treatments on (a) head counts, (b) copulation (c) survivors and (d) mass change in LUFA 2.2 soil, predicting negative behavioural responses to increasing concentrations of thiacloprid in single or combined use of NPK.

1. There are significant differences between the Controls and all other treatments on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single or combined use with NPK.

2. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single use.
3. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid when NPK is present.
4. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) and different concentrations of thiacloprid when NPK is present (NPK + T25 %, NPK + T50 %, NPK + T75 % and NPK + T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single and furthermore when combined use with NPK.

1.12.2. Experiment 3: Organic Blend Topsoil

H1: There are significant differences among treatments on (a) head counts, (b) copulation (c) survivors and (d) mass change in OBT, predicting negative behavioural responses to increasing concentrations of thiacloprid in single or combined use of NPK.

1. There are significant differences between the Controls and all other treatments on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single or combined use with NPK.

2. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single use.
3. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid when NPK is present.
4. There are significant differences between the different concentrations of thiacloprid (T25 %, T50 %, T75 % and T100 %) and different concentrations of thiacloprid when NPK is present (NPK + T25 %, NPK + T50 %, NPK + T75 % and NPK + T100 %) on (a) head counts (b) copulation (c) survivors and (d) mass change, predicting negative response to increasing concentrations of thiacloprid in single and furthermore when combined use with NPK.

2. Methodology

2.1. Acclimatisation

Fourteen days prior to each experiment, farmed *L. terrestris* purchased from '*The happy worm's company*' were acclimatised outside in 35 L of Levington Organic Blend Topsoil (OBT) and accommodated in a 45 L plastic bin with a ventilated lid (5 mm holes). During this time, the accommodation was located in an area where environmental conditions were similar to that of the research site. Whilst undergoing acclimatisation, the worms' diet consisted of mixed, shredded dried, deciduous tree leaves including sycamore maple (*Acer pseudoplatanus*), pedunculate oak (*Quercus robur*), wild cherry (*Prunus avium*) and white willow (*Salix alba*) to prepare them for a more natural diet compared to that of modern worm farming methods (Van Hoesel *et al*., 2017). The leaves were collected from the grounds around the experiment enclosure and freeze dried and thawed before use. The shredded leaves were applied evenly to the soil surface at 20 g per mesocosm. Mesocosms were topped up with an additional 20 g of when all leaves had disappeared.

2.2. Test soils

2.2.1. LUFA 2.2

LUFA 2.2 is a standard soil used especially for licenced studies investigating leaching, degradation and metabolism, influence on soil microflora and fauna, adsorption/desorption characteristics of pesticides in soils as well as for pot experiments and investigations in the laboratory and the field (LUFA Speyer, 2021). LUFA 2.2 is a natural occurring soil type from selected areas in Germany. The soil is used for agriculture without application of pesticides, biocidal fertilizers or organic manure for at

least 5 years before processing into a laboratory product. Mineral fertilizers are used 3 months before sampling. The soils are normally sampled from 0 - 20 cm depth, prepared and sieved with a 2 mm screen (LUFA Speyer, 2021).

Table 1: Physico-chemical characteristics of LUFA 2.2 soil including organic carbon content, pH and nitrogen content (average ± SD) and the soil type (LUFA Speyer, 2020).

Soil parameter	**LUFA 2.2**
Organic Carbon (%)	1.77 +- 0.56
pH (0.01 M CaCl2)	5.6 +- 0.3
Nitrogen (%)	0.20 +- 0.06
Cation exchange capacity (meq/100g)	8.5 +- 2.0
Soil type	Loamy sand (LS)

2.2.2. Levington's organic blend topsoil

OBT was chosen as a test soil, for its high organic matter content. The supplier was contacted to seek measurements of soil composition and parameters, with no success. There is no evidence that this topsoil has been certified as an organic product by the Soil Association (SA), with no official logo visible on the bag. The product is described as having a high content of organic matter derived from adding green garden waste as a soil amendment. It is not clear whether the garden waste used to create the soil amendment had previously been grown organically, thus cannot be considered an organic product.

2.3. Experimental design

The experiment was designed and adapted from the OECD guideline for the testing of chemicals on earthworm reproduction test (ERT) (*Eisenia fetida* / *Eisenia andrei*) for optimal breeding conditions and appropriate housing and husbandry of *L. terrestris*.

Exposure time for the ERT was increased from 28 days recommended by the OECD for *E. fetida* and *E. andrei* to give *L. terrestris* (as a slow reproducing species) sufficient time for cocoon production. The parameters measured and compared on exposure to Thiacloprid in single or combined use with NPK included number of worms active at the surface, number of copulations, number of survivors, change in mass and cocoon production. The experiments commenced outside under controlled field conditions from April to September to optimise favourable environmental conditions for breeding for *L. terrestris* and incubation of cocoons (Curry and Bolger, 1984).

The experiment was conducted within the boundaries of a secure enclosure (Appendix 1) to safeguard people and wildlife from direct contact with test chemicals. For health and safety reasons, preparation of test chemicals was carried out in the laboratory and sealed in plastic pots before transfer to the experimental area. The thiacloprid formula was diluted with rain water in a plastic measuring jug to 4 different concentrations. The greatest concentration was the manufacturer's recommended strength (20 ml/L H_20), referred to here as 100 %. Three weaker concentrations were 75, 50 and 25 % of the recommended strength. The concentrations of thiacloprid formula were used as a single application. Four other treatments used the same concentrations, but were also mixed with NPK. The two remaining treatments were NPK in single application and a procedural control of water only (Table 2). Thus, each experiment included 10 different treatments. Each treatment was replicated with n = 6 mesocosms per treatment for experiments 1 & 2, LUFA2.2 and n = 4 for experiment 3, OBT (Figure 2).

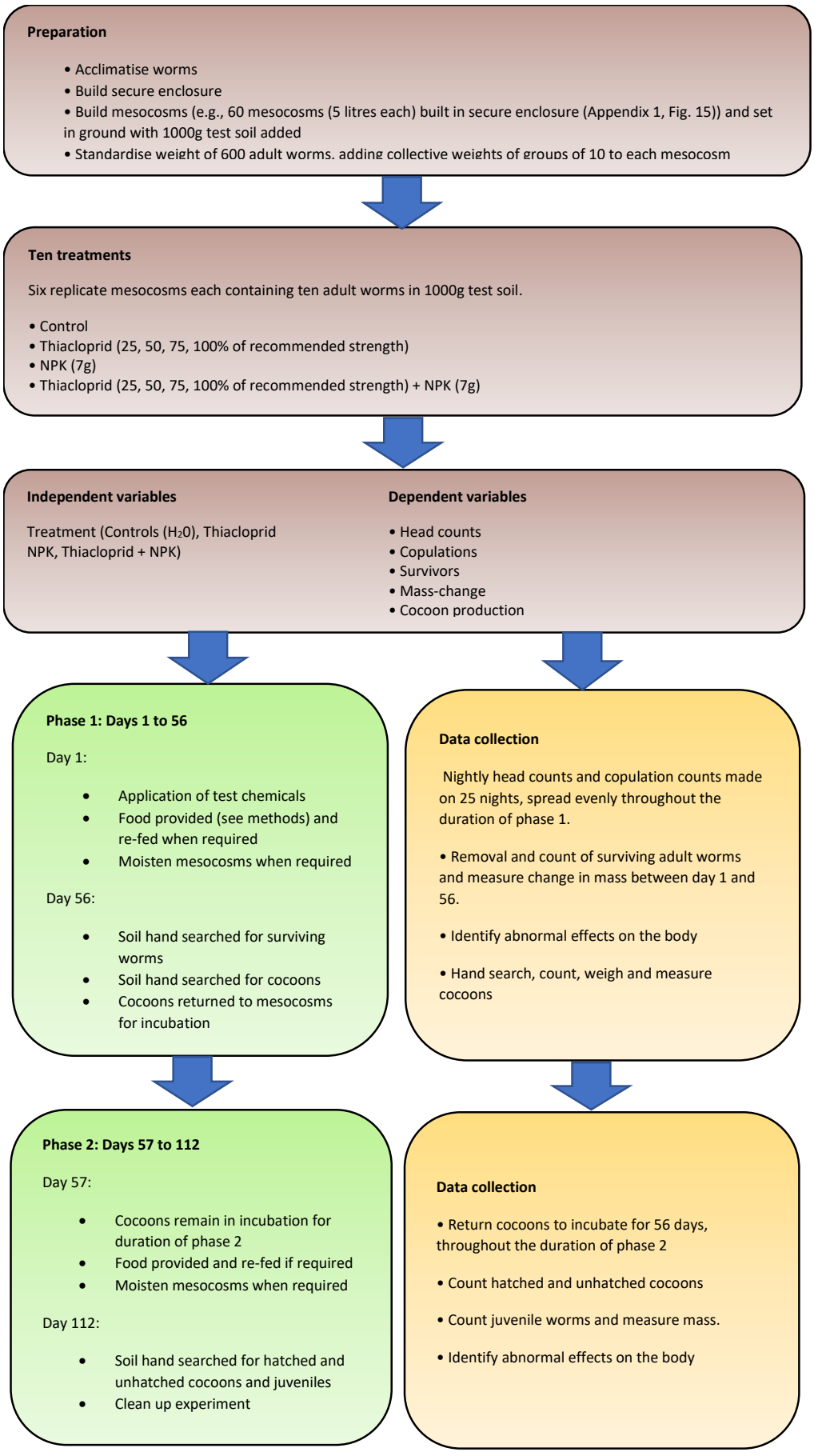

Figure 2: Schematical diagram of experimental design.

Table 2: Single and combined agro-chemical concentrations (Thiacloprid 9 g/L as a suspo-emulsion formulation, also containing 5-chloro-2-methyl-isothiazol-3-one/2 methyl-isothiazol-3-one,1,2-Benzisothiazolin-3-one)

Chemical concentration designed for this study. The solutions were mixed according to this table but applied as 250 ml per mesocosm containing 1000 g soil (6 replicates for each of experiment 1 and 2 (LUFA 2.2) and 4 for experiment 3 (OBT) per treatment). Percentages of Thiacloprid are fractions of the manufacturers recommended strength (20 ml/L H_20), falling in increments of 25 % (e.g., T75 = 15 ml or NPK+T75 = 15 ml +NPK).

Treatment	H_20(ml)	NPK(g/10cm^{2}soil)	THIA Suspo(ml)	THIA(mg)	THIA(ppb)
Control	1000	0	0	0	0
T25	1000	0	5	0.045	45
T50	1000	0	10	0.09	90
T75	1000	0	15	0.13	130
T100	1000	0	20	0.18	180
NPK	1000	7	0	0	0
NPK+T25	1000	7	5	0.045	45
NPK+T50	1000	7	10	0.09	90
NPK+T75	1000	7	15	0.13	130
NPK+T100	1000	7	20	0.18	180

2.3.1. Phase 1

Mesocosms forming the worms' accommodation were constructed from 5 L (diameter top 22 cm, bottom 17 cm and depth 17 cm) plastic plant pots (Figure 3) set 30 cm apart and buried into the ground so that the experimental soil was level with that of the natural ground level. The mesocosms were filled with 1000 g of LUFA2.2 or OBT and 10 worms (OECD, 2015; Wang *et al*., 2015). The worms were selected based on the presence of a well-developed clitellum (Figure 4), indicating mature reproductive status and had similar weights once washed with rain water (OECD, 2015; Wang *et al*., 2015).

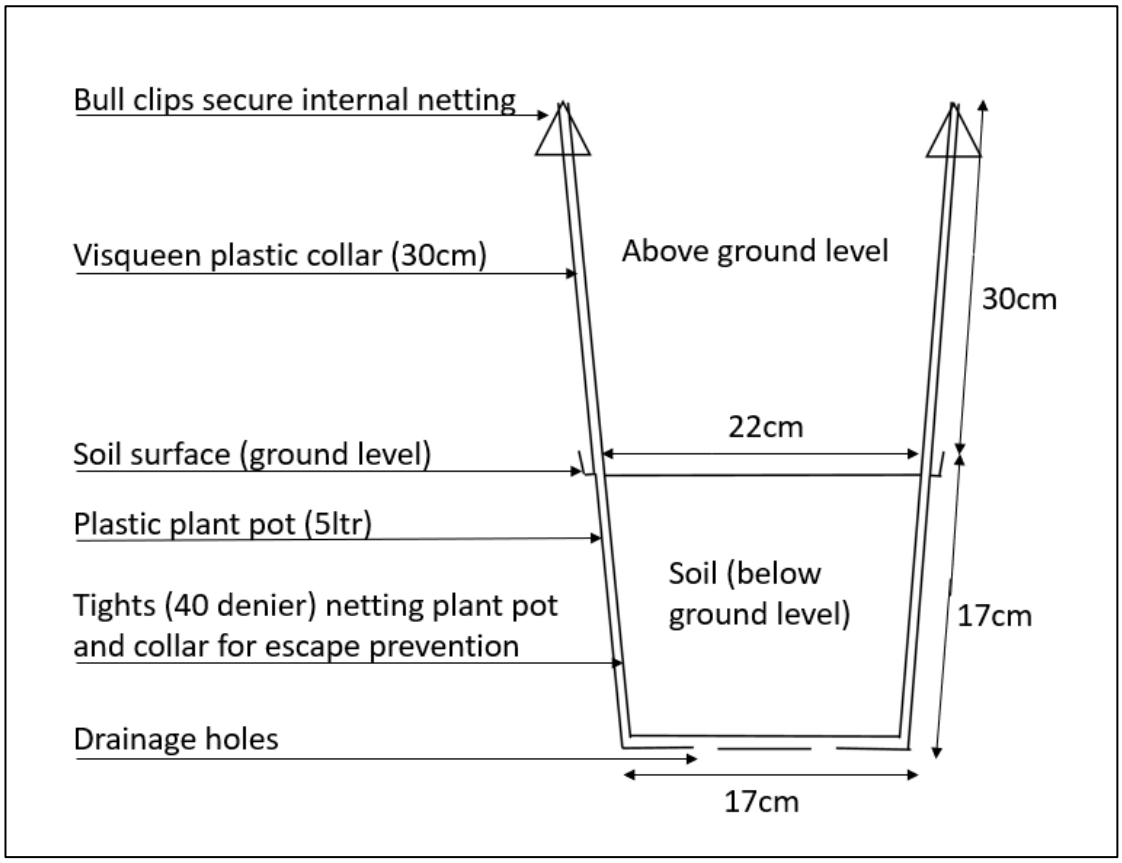

Figure 3: Mesocosm design used during this experiment (Holmes, 2017).

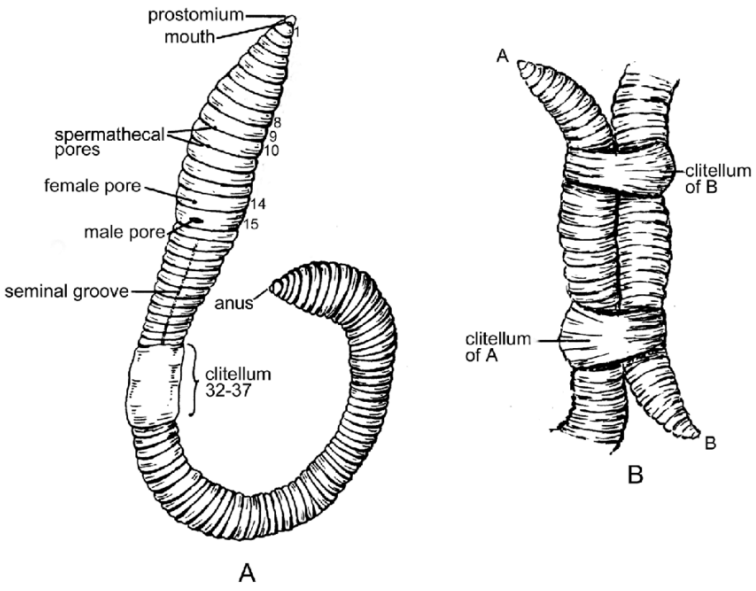

Figure 4: A. External features of *Lumbricus terrestris* turned slightly to one side to show genital pores, seminal groove and clitellum. B. Two worms in coition. The slime tube encloses the clitellum and apposed spermathecal pores. Relabelled after Jepson, M. 1951. Biological Drawings. Part II. John Murray, London, p. 31. (Jamieson and Ferraguti, 2006).

In total, there were 60 samples (LUFA 2.2, experiment 1 and 2) and 40 (OBT, experiment 3). Ten worms were randomly allocated accommodation using individual mesocosm numbers and a random number generator. The worms were placed on the topsoil surface and allowed to burrow. Any individual remaining at the surface beyond 15 minutes was assumed unhealthy and replaced. After 1 day of further acclimatisation, the designed treatments (Table 1) were applied to the soil surface as per manufacturer's mixing specifications. Thiacloprid solutions and water (250 ml/1000 g) was applied evenly to the soil surface as a drench using a 1 L watering can with rose attachment and NPK granules were applied as a surface scatter to mimic manufacturer's guidance for domestic use. Throughout the experiment, temperature, humidity and weather conditions were monitored as possible confounding factors.

Throughout the first phase of the experiments, direct observation was used to count the abundance of worms foraging or number of copulations taking place on the soil surface. Observations took place nightly between 10:00 p.m. and 12:00 a.m., each mesocosm was observed for two minutes and the starting point was alternated between the first and last sample each evening. Worms were counted using a red beam head torch before switching to a low-level white beam to observe surface behaviours such as reproduction (Figure 5) and responses to light. Any adverse or unusual behaviour and evident death was also recorded at each visit. After 56 days, surviving adult worms were counted, abnormal behaviours, changes in weight and morphology recorded and cocoons (Figure 6) counted using the hand search method (OECD, 2015; Wang *et al*., 2015). The adult worms were then removed permanently from the experiment and relocated to fresh accommodation.

Figure 5: Earthworm's copulating to show a well-developed clitellum and engagement of the seminal groove (See Figure 4) (Peacock, 2015).

Figure 6: Earthworm cocoons of Lumbricus terrestris (Edwards, 2021).

2.3.2. Phase 2

Mesocosms containing cocoons were returned to the enclosure to continue incubating for 60 days and provided with food for the remainder of the experiment (OECD, 2015; Wang *et al*., 2015). Mesocosms were provided with food as above (Acclimatisation). At the end of this period, hatched and unhatched cocoons and juvenile worms were counted by hand search and any abnormal behaviour or morphology documented.

2.3.3. Repeated experiment

To establish whether patterns observed were not just a one-off event, the application of the same treatments was repeated, again using LUFA 2.2. soil. If patterns were similar between the two experiments, this would give much greater credence to them being caused by the experimental treatments rather than chance events. If patterns differed between two near-identical experiments, then external influences may have had a greater effect on the outcomes than the treatments themselves.

2.4. Data analysis

The response variables measured against the chemical solutions designed for this study included nightly head counts, nightly copulations, number of survivors, change in mass and number of cocoons produced. In experiment 1, LUFA2.2, numbers of worms on the surface and the number of copulating pairs were counted for 20 consecutive nights and in experiment 3, OBT counts were done for 25 consecutive nights, beginning 36 hours after the chemical solutions were applied. For these two variables, the value used for each replicate mesocosm was the mean of the nightly observations. The number of survivors was the number of worms left alive in each mesocosm after 56 days. Change

in mass was standardised to starting weight (%) of the number of worms left alive after 56 days. The number of cocoons was the number of cocoons found in each mesocosm by hand searches of the soil after 56 days.

Differences in responses by worms among treatments were compared using one-factor ANOVA (for parametric data) or Kruskall-Wallis test (for non-parametric data). Normality of distribution of data was assessed using the Shapiro test. The single, fixed factor was chemical treatment, with 10 levels. Where significant differences occurred ($p < 0.05$) *post-hoc* pairwise tests Tukey (parametric) or Dunns test (non-parametric) were used to find which treatments differed. Where only two treatment types containing surviving worms remained, Wilcoxon rank sum test with continuity correction was used to identify if significant differences occurred ($p < 0.05$) between them.

All analyses were completed in the R software environment (Version 1.3.1073).

3. Results

3.1. Thiacloprid and NPK in LUFA 2.2: Experiment 1

3.1.1 Nocturnal activity

Nightly counts of worms at the surface were greatest in control treatments, averaging nearly one individual per replicate pot per night compared to only very seldom observations in all other treatments (Figure 7). There were significant differences in the numbers of worms active among the treatments (Figure 7; Kruskal-Wallis chi-squared = 38.003, df = 9, $p < 0.001$). Post–hoc pairwise Dunn's tests revealed significant differences between the Control and all other treatments ($p<0.05$) apart from Control vs T25 (Table 3, H1 light grey). For the treatments containing only Thiacloprid, there was no consistent pattern with increasing concentrations of Thiacloprid, but the four treatments fell into two clear groups. There were significantly more worms visible in T25 than in T50, T75 or T100 (Figure 7), which were all similar (Table 3, H2 mid grey cells). Of the five treatments that included NPK, there were no clear groups nor any consistent pattern with increasing concentrations of Thiacloprid. There were more worms visible in the NPK treatment than in any of the treatments including Thiacloprid and NPK (Figure 7), but differences were not significant (Table 1, H3 dark grey cells). For each of the four concentrations of Thiacloprid, the number of worms appeared similar (Figure 7) when the neonicotinoid was combined with NPK, and differences were not significant (Table 3, H4 darker grey).

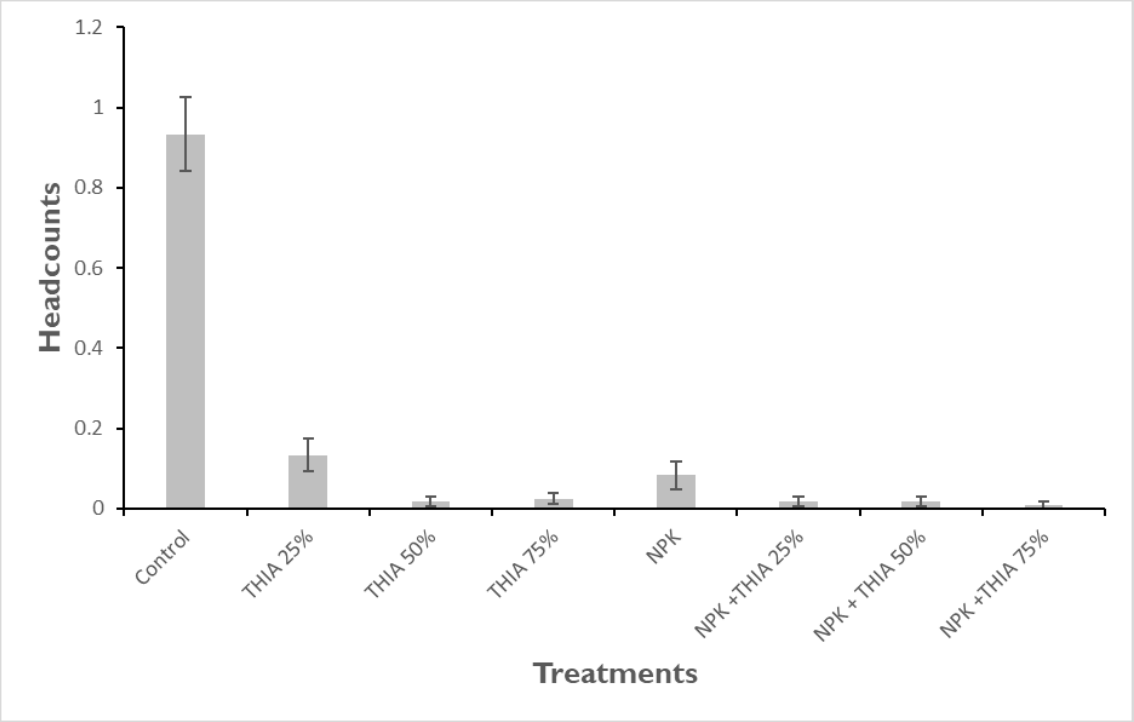

Figure 7: Mean (± s.e., n = 6 mesocosms) number of *Lumbricus terrestris* visible at night at the surface of LUFA2.2 artificial soil in mesocosms exposed to single or combined treatments of Thiacloprid and/or granulated NPK fertiliser (7 g/10 cm²). The value for each mesocosm was the average of 20 nightly observations. Concentrations of Thiacloprid were percentages of the manufacturer's maximum recommended strength (20 ml/L H20 = T100; 15 ml/L H20 = T75, etc.).

Table 3: Probabilities from post-hoc pairwise Dunn's test for nightly headcounts in experiment 1, LUFA2.2. Key: H1 light grey, H2 mid grey, H3 dark grey and H4 darker grey.

NPK+ T100	<0.001				0.500					
NPK+ T75	<0.001			0.486						0.412
NPK+ T50	<0.001		0.444						0.494	0.415
NPK+ T25	0.001	0.025						0.432	0.436	0.327
NPK	0.013						0.338	0.121	0.252	0.121
T100	<0.001									
T75	<0.001				0.433					
T50	0.001			0.442	0.369					
T25	0.289		0.027	0.012	0.003					
Control										
	Control	T25	T50	T75	T100	NPK	NPK+ T25	NPK+ T50	NPK+ T75	NPK+ T100

3.1.2. Copulation

Copulations were observed only in control mesocosms (0.175 ± 0.034), hence no analysis was possible.

3.1.3. Survivors

After 56 days, only the Control and NPK treatments contained survivors (Figure 8). A small number of survivors were present in only two mesocosms for the NPK treatments, whereas almost all worms survived in each of the six Control mesocosms (Figure 8). Treatments with no survivors were excluded from further analysis. There were significantly more survivors in the Control than in the NPK treatment (Figure 8: Wilcoxon rank sum test with continuity correction, ($p < 0.05$).

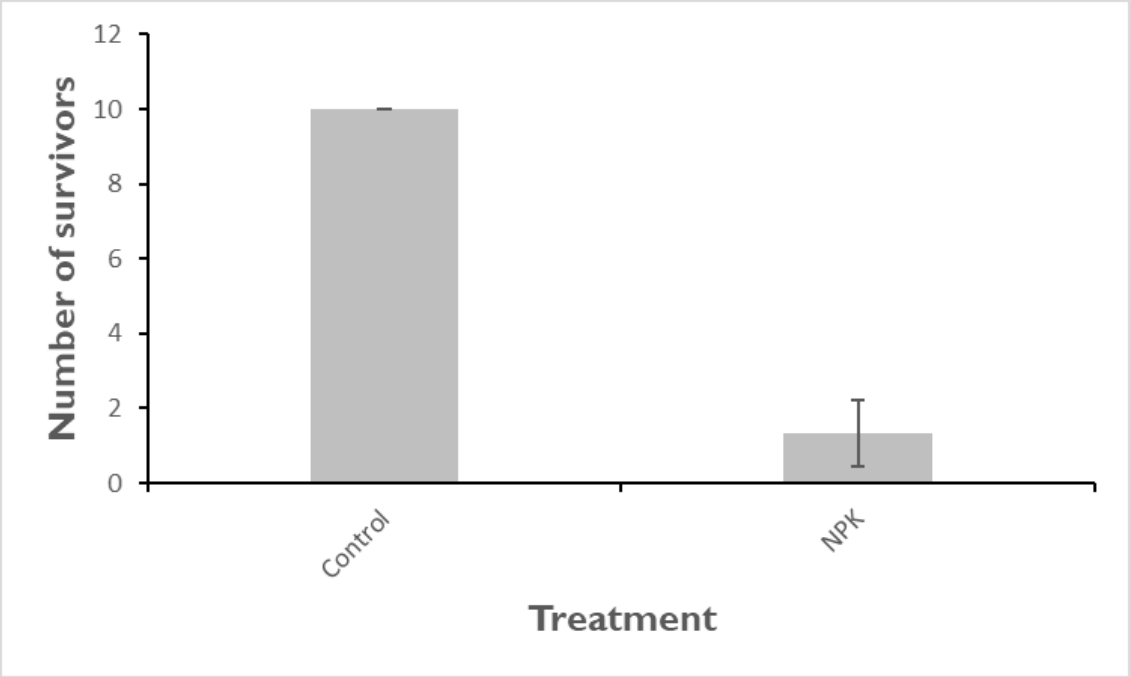

Figure 8: Mean (± s.e.) number of survivors of *Lumbricus terrestris* in LUFA2.2 artificial soil with Control (water only) or granulated NPK treatment (7 g/10 cm²) after 56 days.

3.1.4. Weight-change

The starting weight of each individual *L. terrestris* for LUFA2.2 was 4.94 ± 0.04 g. After 56 days, worms survived only in the Control and NPK treatments, so it was possible to compare weight-change in worms only for these treatments. Worms in the NPK treatment lost significantly more mass than did those in the Control (Figure 9; Wilcoxon rank sum exact test revealed significant differences between the Control and NPK treatments ($p<0.05$). Only two of the mesocosms with NPK treatment contained survivors, whereas all six of the Control mesocosms had survivors, so the sample size for each treatment differed (n= 2 and n = 6, respectively).

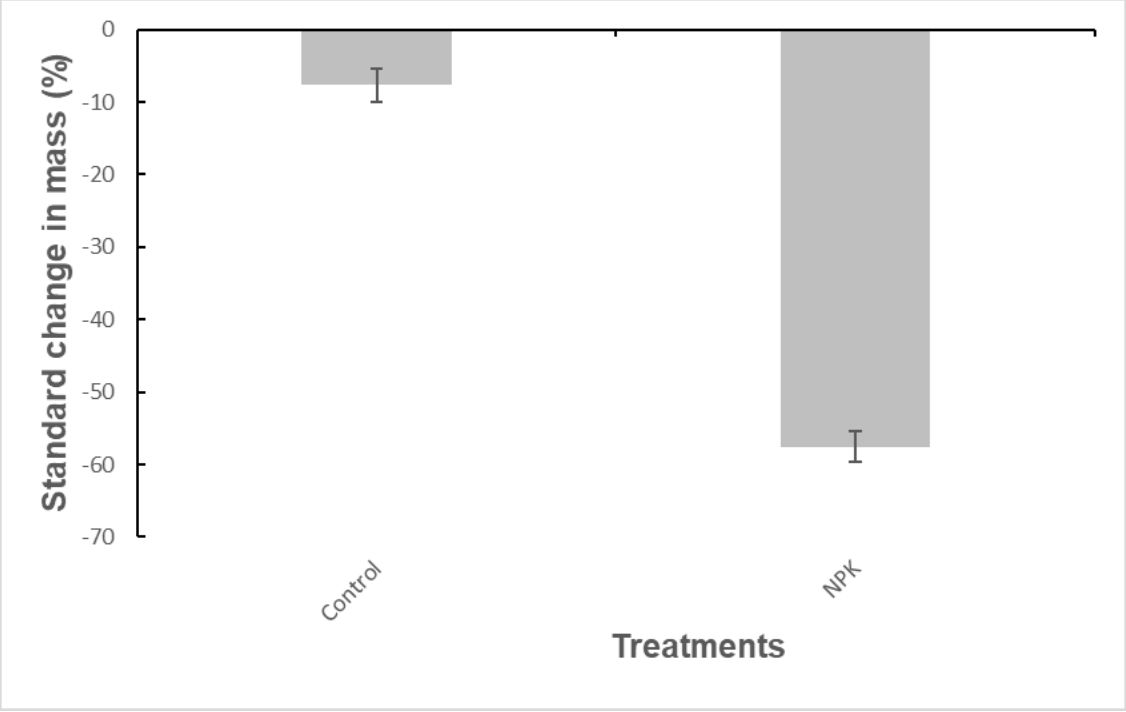

Figure 9: Mean (± s.e.) standard change in mass from starting weight of *Lumbricus terrestris* in LUFA2.2 artificial soil with Control (water only) or granulated NPK treatment (7 g/10 cm²) after 56 days.

3.1.5. Number of cocoons produced

Thorough hand searching of all mesocosms revealed no cocoons and so no analysis was possible.

3.2. Thiacloprid and NPK in LUFA 2.2: Experiment 2

Due to the large mortality in many of the treatments in Experiment 1, LUFA2.2, the experiment was repeated exactly, but this time survival was assessed after 7 days, rather than allowing 56 days to elapse. All worms in all treatments except the Controls died within the first 7 days.

Thus, analyses to compare surface activity, copulations, weight-change, survival and cocoon production were not possible. Despite the absence of analyses, the overall pattern (large survival in controls, massive mortality in other treatments) was consistent with that from Experiment 1, LUFA2.2 but the outcome was even more extreme.

3.3. Thiacloprid and NPK in organic blend topsoil: Experiment 3

In contrast with the experiments using LUFA2.2 soil (Figure 8), survival by worms across all treatments was much greater in OBT (Figure 12).

3.3.1. Nocturnal activity

There were significant differences in the numbers of worms counted among the ten treatments (Figure 10; Kruskal-Wallis chi-squared = 21.516, df = 9, $p < 0.05$). Post–hoc pairwise Dunn's tests revealed significant values between Control vs T50, T75 and T100 ($p<0.05$) but not for other treatments (Table 4, H1 light grey). Of the four treatments

containing only Thiacloprid there were no clear groups nor any consistent pattern with increasing concentrations of Thiacloprid (P>0.05) (Table 4, H2 mid grey). Of the five treatments that included NPK, there were no clear groups nor any consistent pattern with increasing concentrations of Thiacloprid (P>0.05) (Table 4, H3 dark grey). For each of the four concentrations of Thiacloprid, there were fewer worms visible than when the neonicotinoid was combined with NPK (Figure 10) but differences were not significant (Table 4, H4 darker grey).

Table 4: Probabilities from post-hoc pairwise Dunn's test for nightly headcounts in experiment 3, OBT. Key: H1 light grey, H2 mid grey, H3 dark grey and H4 darker grey.

NPK+ T100	0.073				0.369					
NPK+ T75	0.071			0.182						0.472
NPK+ T50%	0.167		0.156						0.307	0.339
NPK+ T25	0.267	0.276						0.411	0.261	0.266
NPK	0.143						0.378	0.420	0.374	0.374
T100	0.040									
T75	0.002				0.288					
T50	0.012			0.390	0.381					
T25	0.077		0.268	0.174	0.372					
Control										
	Control	T25	T50	T75	T100	NPK	NPK+ T25	NPK+ T50	NPK+ T75	NPK+ T100

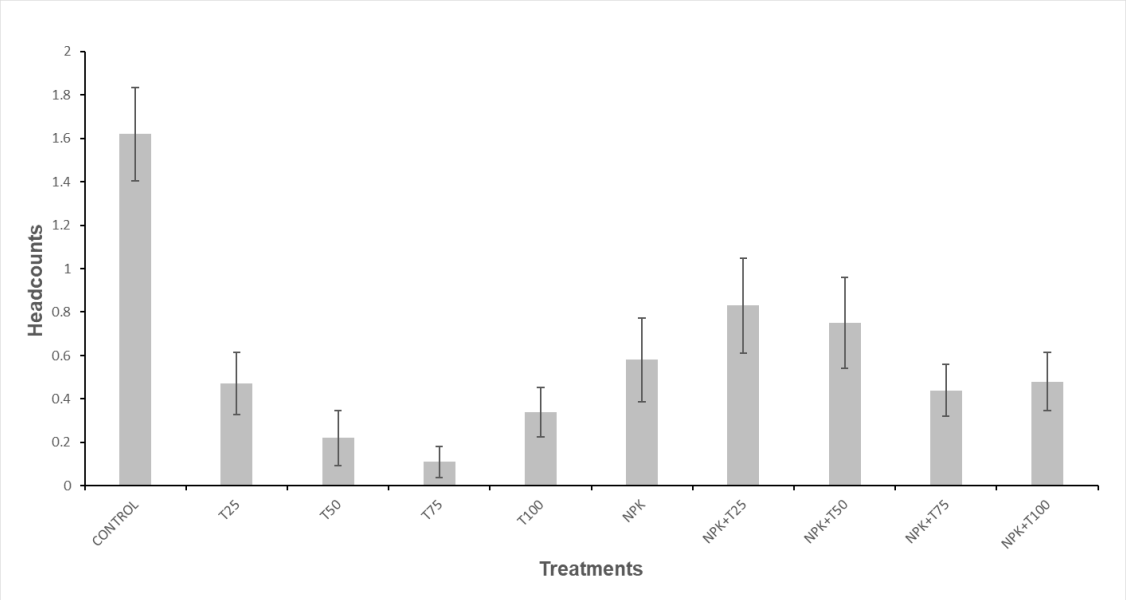

Figure 10: Mean (± s.e., n = 4 mesocosms) number of *Lumbricus terrestris* visible at night at the surface of OBT in mesocosms exposed to single or combined treatments of Thiacloprid and/or granulated NPK fertiliser (7 g/10 cm²). The value for each mesocosm was the average of 25 nightly observations. Concentrations of Thiacloprid were percentages of the manufacturer's maximum recommended strength (20 ml/L H20 = T100; 15 ml/L H20 = T75, etc.).

3.3.2. Copulations

With exception of NPK+T25, no copulations were observed in treatments containing thiacloprid; copulations were also observed in the Control and NPK treatments (Figure 11). There were significant differences in the numbers of copulations among the ten treatments (Figure 11; Kruskal-Wallis chi-squared = 24.422, df = 9, $p < 0.05$). Post–hoc pairwise Dunn's tests was not powerful enough to find a significant difference between Control vs NPK+T25% ($p=0.059$) (Table 5, H1 light grey) or NPK vs NPK+T25 ($p<0.073$) (Table 5, dark grey). The three remaining treatments fell into two groups; Control and NPK which were similar and each greater than the number of copulations observed in T25 (Figure 11).

Table 5: Probability values from post-hoc pairwise Dunn's test for nightly copulations in experiment 3, OBT. Key: H1 light grey, H2 mid grey, H3 dark grey and H4 darker grey.

NPK+ T100	0.013				0.535					
NPK+ T75	0.019			0.803						0.511
NPK+ T50	0.016		0.661						0.523	0.500
NPK+ T25	0.059	0.502						0.433	0.454	0.414
NPK	0.852						0.073	0.010	0.011	0.009
T100	0.024									
T75	0.096				0.833					
T50	0.048			0.900	0.7031					
T25	0.032		0.725	0.865	0.625					
Control										
	Control	T25	T50	T75	T100	NPK	NPK+ T25	NPK+ T50	NPK+ T75	NPK+ T100

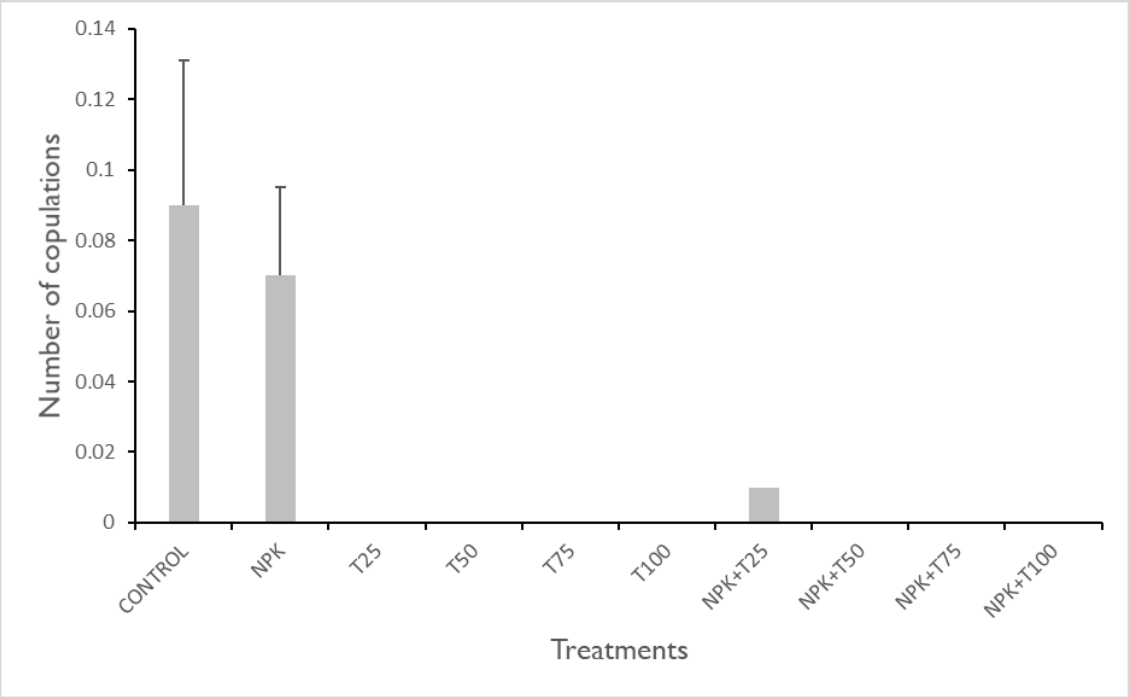

Figure 11: Mean (± s.e., n = 4 mesocosms) copulations of *Lumbricus terrestris* visible at night at the surface of OBT in mesocosms exposed to single or combined treatments of Thiacloprid and/or granulated NPK fertiliser (7 g/10 cm²). The value for each mesocosm was the average of 25 nightly observations. Concentrations of Thiacloprid were percentages of the manufacturer's maximum recommended strength (20 ml/L H20 = T100; 15 ml/L H20 = T75, etc.).

3.3.3. Survivors

There were no significant differences in the numbers of survivors counted among the ten treatments (Figure 12; Kruskal-Wallis chi-squared = 4.5884, df = 9, $p > 0.05$).

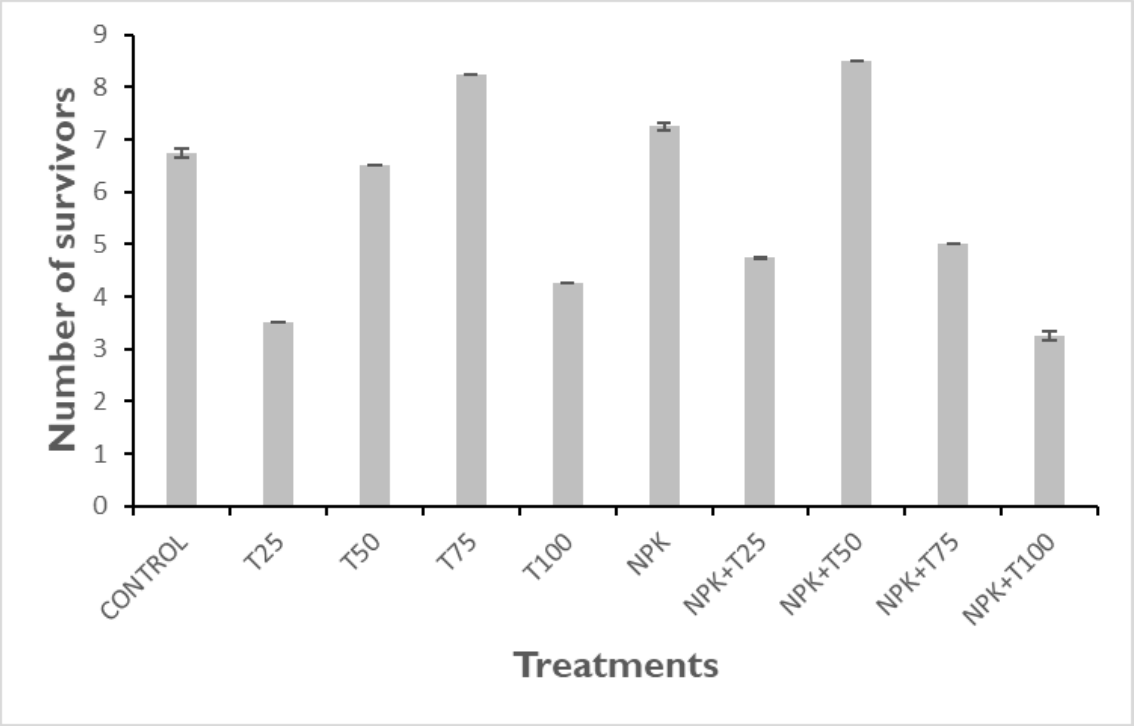

Figure 12: Mean (± s.e.) number of survivors of *Lumbricus terrestris* in OBT exposed to single or combined treatments of Thiacloprid and/or granulated NPK fertiliser (7 g/ 10cm²) after 56days.

3.3.4. Weight Change

The starting weight of each individual *L. terrestris* for OBT was 3.87 ± 0.03g There were significant differences in the numbers of worms counted among the ten treatments (Figure 13; One way ANOVA = 7.637, df = 9, $p < 0.001$). Post–hoc Tukeys test was not powerful enough to identify where those differences are. However, it is noteworthy that the Controls and NPK were the only treatments to gain weight, in comparison to all other treatments containing Thiacloprid, apart from NPK+Thia25. Of the eight treatments containing thiacloprid, only one showed an increase in mass. The probability of worms in seven of eight treatments with Thiacloprid losing mass, is small and significantly

different to expectation, if increases or decreases in mass were equally likely (Binomial test, p = 0.035). Thus, it is likely that the loss of mass observed is not due to chance, but to the application of Thiacloprid.

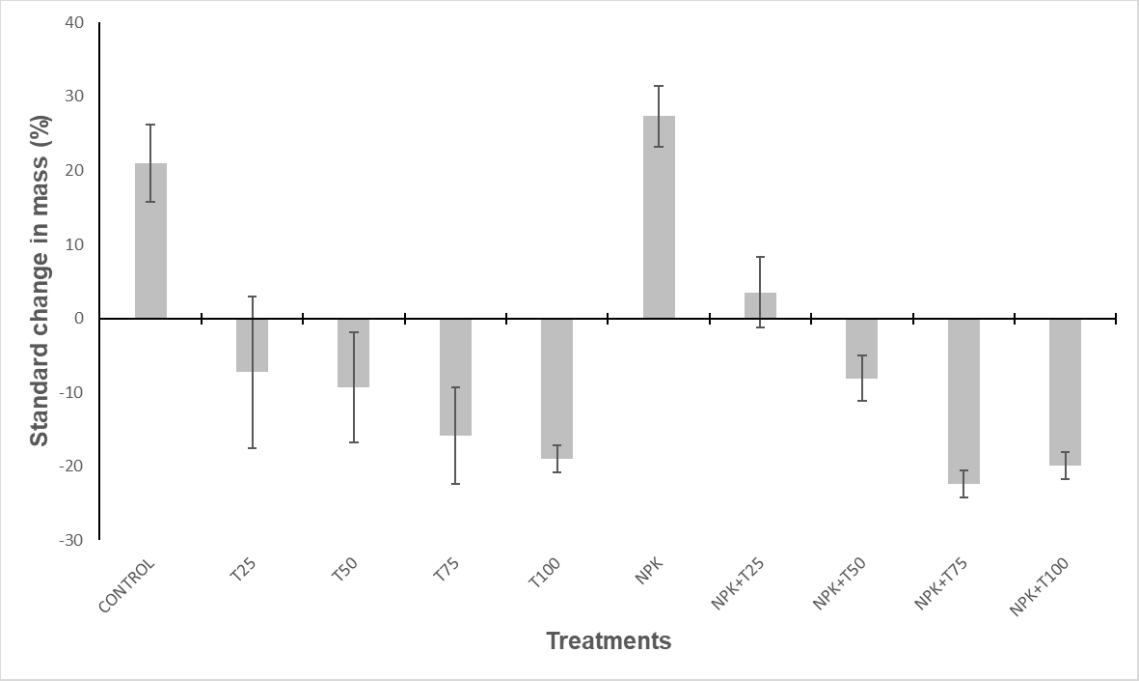

Figure 13: Mean (± s.e.) standard difference in mass from starting weight of *Lumbricus terrestris* in OBT exposed to single or combined treatments of Thiacloprid and/or granulated NPK fertiliser (7 g/10 cm²) after 56days.

3.3.5. Number of cocoons produced

Thorough hand searching of all mesocosms revealed no cocoons and so no analysis was possible.

4. Discussion

These experiments demonstrated great effects of single or combined agro-chemical mixtures on survival, mass and behavioural responses of earthworms. Criteria and conditions used for ERT in the laboratory may not be appropriate for transfer to field-experiments using real-life conditions. The results were gathered to reflect on whether there are any negative trends between different concentrations of the neonicotinoid, Thiacloprid-based domestic product, BPUBKC2 in single and combined use with NPK fertiliser granules on *L. terrestris*. The parameters measured and compared on exposure to Thiacloprid in single or combined use with NPK included number of worms active at the surface, number of copulations, number of survivors, change in mass and cocoon production. For this study, the ERT, usually carried out under laboratory conditions, was adapted to take place under controlled field conditions to better replicate real agricultural environments. The test substrate used in experiment 1 was standardised soil LUFA 2.2, regularly used under laboratory conditions for biological risk assessment of agro-chemicals (R. Handy pers. comm.). Given the extreme and unanticipated results in experiment 1, LUFA2.2, experiment 2, LUFA2.2, was an identical repeat of experiment 1, LUFA2.2 with the intention of providing confidence about whether observations from experiment 1, LUFA2.2 were anomalous or not. The final experiment, used OBT to provide conditions closer to those in the agricultural environment and to establish whether effects of application of treatments were consistent across different types of soil. It was predicted that Thiacloprid with added NPK would have greater impacts on *L. terrestris* behaviours and reproduction than single treatments and increasing concentrations would reduce survival and cocoon production, whilst increasing behavioural responses and changes in mass.

4.1. Nocturnal activity

4.1.1 Experiments 1 & 2: LUFA2.2

Experiment 1, LUFA2.2 indicated that both Thiacloprid, in single and combined use with in LUFA 2.2 soil had significant, negative impacts on numbers of worms active on the surface at night. For the Control treatments, there was almost 1 worm visible for each nightly observation, compared to 0 in all other treatments (Figure 7). This was in support of H1 (There are significant differences between the Controls and all other treatments on head counts) (Table 3, H1 light grey). A rational explanation for there being few worms on the surface, may be because they were dead or dying below ground. There is strong indication for death in the repeat Experiment 2, LUFA2.2. However, it is not known for sure, when the worms in Experiment 1, LUFA2.2 died in relation to nocturnal observations, which were carried out throughout the 56 days exposure to treatments.

For Experiment 1, LUFA2.2 treatments containing Thiacloprid only, fell into 2 groups, where the effect of concentrations ≥ 50 % of the manufacturers recommended strength had a significantly greater effect on head counts than treatments with 25 %. There was no evidence for a linear effect of concentration on activity at the surface, but the pattern observed was consistent with the prediction that stronger concentrations would have a greater effect (Table 3, H2 mid grey cells). Whilst this indicates that just T50, half the manufacturers recommended strength can have a negative impact on night time headcounts of *L. terrestris*, care should be taken to recognise that T25 also had significant negative effect on headcounts against the Controls H1 (There are significant differences between the Controls and all other treatments on head counts) (Table 3, H1 light grey).

This result is comparable to Capoweiz (2006) finding that the neonicotinoid Imidacloprid (0.1 or 0. 5 mg kg^{-1}) in single use caused *A. icterica* to produce fewer, shorter and less continuous burrows, than those in control treatments. The findings are also consistent with Van Hosel *et al* (2017) finding that *L. terrestris* activity is significantly reduced by 9.2 % when exposed to Imidacloprid seed dressing. If normal behaviours such as burrowing and activity levels are significantly reduced by the application of neonicotinoids such as Imidacloprid applied as seed coatings, it is likely to have an impact on the number of worms visible at the surface as observed in the present study.

When NPK was present with Thiacloprid, the effect of greater concentrations of Thiacloprid on head counts at the surface was not apparent. The number of worms was nearing zero, regardless of strength of Thiacloprid (Figure 7). For each of the four concentrations of Thiacloprid, the number of worms appeared to follow a similar pattern when NPK was present (Figure 7). The differences between single and combined treatments were not significant (Table 3, H4 darker grey). Despite indications that presence of NPK did not give rise to synergistic effects on head counts, it is difficult to ascertain the truth when all concentrations of Thiacloprid and all mixtures are zero. However, it is evident that all treatments containing Thiacloprid and the single use of NPK have a negative impact on headcounts (Figure 6). Ge *et al* (2018) demonstrated that Avoidance Response (AC_{50}) value of *E. fetida* to Thiacloprid was significant, at a dose of 7.87 mg/kg. Unfortunately, it is difficult to make a comparison between the pure active ingredient used by Ge *et al* (2018) and the domestic suspo-emulsion formulation used in this research.

4.1.2. Experiment 3: OBT

When OBT was used, more worms survived in all the treatments. There were significantly more worms visible in the Control and T25 mesocosms than in those for the three stronger concentrations of Thiacloprid. When the different levels of Thiacloprid in single use were compared (Table 4, H2 mid grey), there were, however, no significant differences in headcounts among treatments. This makes it difficult to say unequivocally, that application of Thiacloprid decreased significantly the number of worms at the surface. The overall pattern was similar to that from Experiment 1, LUFA2.2, providing some evidence that Thiacloprid has a negative effect on headcounts in OBT and cause to accept H1 (There are significant differences between the Controls and all other treatments on head counts). There was a weak indication that the presence of NPK has an antagonistic effect rather than being additive or multiplicative because the number of worms in combined NPK treatments were greater than Thiacloprid only (Figure 10), however the potential reason for this was not identified or understood.

Combinations of pesticides and heavy metals generally give rise to synergistic interactions, however various mixtures can present a dual behaviour where both synergism and antagonism are found in the same mixture depending on the dose-effect level (Uwizeyimana *et al*., 2017). When cadmium (Cd) is mixed with the neonicotinoid, Imidacloprid and λ-cyhalothrin there is an antagonistic response on *E. fetida* (Uwizeyimana *et al*., 2017). Chemical interactions that take place externally of an exposed organism can influence the availability of others (Uwizeyimana *et al*., 2017). The herbicide Atrazine has been reported to potentially interact with Cd ions (Cd2+) generating anhydrous and hydrated complexes, which affect the absorption process of these two compounds (Uwizeyimana *et al*., 2017). The interactions between Atrazine

and Cd outside of the target organism might reduce the absorption process resulting in antagonistic effects (Wang *et al*., 2012a, Uwizeyimana *et al*., 2017). Taking this into account, it is possible that interactions between N, P, K and Thiacloprid could have reduced the absorption rate of *L. terrestris*, accounting for the antagonistic result seen in Figure 10 on headcounts.

When comparing Experiment 1, LUFA2.2 with experiment 3, OBT and the different results between the two, LUFA2.2 had significant differences between the Controls and all other treatments ($p<0.05$) whereas OBT did not ($p>0.05$). This raises the question whether responses of earthworms to application of chemical can be affected by soil characteristics? It is possible that different characteristics, such as Organic Carbon Content (OCC) and Water Holding Capacity (WHC) can affect how much of the test chemicals the worms are directly exposed to. LUFA2.2 is a natural occurring soil type from selected areas in Germany. The soil is used for agriculture without application of pesticides, biocidal fertilizers or organic manure for at least 5 years before processing into a laboratory product (LUFA Speyer, 2021). Although the OCC of the LUFA2.2 was not provided, typical agricultural soil has a maximum OCC of 5% (Renaud *et al*., 2018) compared to OBT, marketed for its high OCC (Levington's, 2021). Taking this into account, variations in OCC, could account for differences in the significance values for headcounts in the two different soils. Organic carbon content can have important influences on the way in which organisms experience their environment and potentially harmful chemicals in it. For example, the bioavailability and therefore the toxicity of heavy metals (e.g., Arsenic (As)), to organisms in terrestrial ecosystems are largely influenced by soil properties such as OCC and WHC (Romero-Freire *et al*., 2015). Soils with the greatest OCC had smaller water-soluble soil concentrations of As than did soils

with less OCC. Soils richer in OCC also absorb water-soluble test chemicals more strongly than those with less OCC (Romero-Freire *et al*., 2015). Thus, it is possible that in experiments using OBT (with high OCC), *L. terrestris* may be less exposed to test chemicals than in experiments using LUFA2.2 (with small OCC). Further research manipulating OCC within the same soil type would be needed to find definitive answers to whether biological responses of earthworms are affected by agro-chemical application.

Despite the above explanation, the method used to measure nocturnal head counts does not give a complete picture of potential abnormal behaviours caused by agricultural chemicals. The presence of earthworms at the surface explains little about why earthworms are there or if other aspects of behaviour have been affected by the test chemical without distinguishing different behaviours at the time of head counts. During observations, it was noted that earthworms at the surface that were exposed to treatments containing Thiacloprid, often reacted unusually to white light, when changed from the red beam. Worms in the Control treatments would immediately retreat into their burrows, whilst those exposed to Thiacloprid were much slower to retreat or actively approached the light. Worms also responded differently to touch when gently prodded with the blunt end of a pencil; those in Control treatments retracted immediately into their burrows, while those exposed to Thiacloprid reacted very slowly or not at all. Neonicotinoids are designed to block neural transmission by binding to the nicotinic acetylcholine receptors (nAChRs) of the insects' central nervous system, causing loss of coordination and ability to feed (Jeschke *et al*., 2011; Gill *et al*., 2012; Eng *et al*., 2017). Very low levels of neonicotinoids can significantly affect the neural functioning of bees (Basely and Goulson, 2017). Jin *et al* (2015) found that ingestion of

0.76 ng Clothianidin per *Osmia* bees (within the range of estimated ingestion rate from treated oilseed crops between 4.27 and 13.65 ng/bee per day) was enough to prevent memory retrieval necessary for navigating towards learned locations. These doses are extremely small in comparison to the lowest dose in this study. However, if such small doses are found to be similarly ingested by earthworms in treated soil and the effects of neonicotinoids on neural functioning on bees is found to be similar in earthworms (Basely and Goulson, 2017), it could be that impaired neural functioning is a feasible explanation for abnormal behaviours displayed by *L. terrestris* exposed to Thiacloprid during this study. Abnormal behaviours, such as slower reaction time in the field, could leave *L. terrestris* more vulnerable to predation and cause predators of earthworms to be at more frequent risk of direct contact with neonicotinoids.

4.2. Copulations

4.2.1. Experiment 1&2: LUFA2.2

The lack of any observed copulations in all but Control treatments in Experiment 1, LUFA2.2 can be explained by the same mechanisms as the lack of worms at the surface. In Experiment 2, LUFA2.2 all worms were dead within the first 7 days except in Control mesocosms, so the lack of copulations in these treatments is perhaps not surprising.

4.2.2. Experiment 3: OBT

In the experiment with OBT, copulations were only present in the Controls, NPK and NPK+T25 in marked contrast with the LUFA 2.2. experiments. The patterns were not distinct, but most treatments containing Thiacloprid had significantly fewer copulations

than did the Control. (Table 5, HI light grey). Single application of NPK did not significantly affect number of copulations against the Controls. Nitrogen fertilisers as single treatments up to certain concentrations can enhance earthworm populations, if soil pH is not altered (Blanchet *et al*., 2016). Earthworms prefer soils with pH 6.5 to 7.5 (Edward *et al*. 1995, Edwards and Bohlen 1996). If the pH value for NPK in single use was not altered beyond the tolerance level of *L. terrestris*, normal reproductive patterns and behaviours may not be affected and could explain why copulations observed were not significantly different for NPK vs Controls. Although, there is no literature on the visual observations of sexual activity on *L. terrestris* under neonicotinoid exposure, number of copulations is likely to be affected in similar ways to nightly headcounts because copulation takes place at the surface after a premating courtship sequence involving burrow visits (Nuutinen *et al*., 2014). If more worms are visible at the surface, there is a greater chance of observing copulations. There is no significant difference between the single use of Thiacloprid at any concentration and Thiacloprid combined with NPK on copulations (Table 4, H4 darker grey), indicating that the presence of NPK does not give rise to synergistic effects. This all suggests that application of Thiacloprid at the concentrations considered here, is likely to have detrimental effects on rates of copulation, whether or not NPK is present, which is partially consistent with H1 (There are significant differences between the Controls and all other treatments on copulations).

4.3. Survival

4.3.1. Experiment 1&2: LUFA2.2

In experiment 1, LUFA2.2 at the end of 56 days, there were no survivors for any treatments where Thiacloprid was present. This contrasted with all 10 worms surviving in each of 6 Control mesocosms (Figure 8), giving strong support for H1 (There are significant differences between the Controls and all other treatments on survivors). Escape from the mesocosms was not possible, so worms in Thiacloprid treatments are assumed to have died. On average, 1 worm per mesocosm survived in the NPK treatment., which was significantly fewer than in the Control. Figure 14, below demonstrates observable differences in body condition of *L. terrestris* between the control and NPK treatments when accommodated in LUFA2.2, Whilst Figure 15, demonstrates difference in the ability of LUFA2.2 to hold its structure where *L. terrestris* were and were no longer present after 56 days.

Figure 14: *L. terrestris* ending phase 1 of the experiment 1 LUFA2.2. Earthworms accommodated in control treatments (Left) show no observable effects of LUFA2.2 and rainwater on the epidermis. In contrast, earthworms exposed to NPK, under the same soil conditions show noticeable wrinkling of the epidermis and sores.

Figure 15: LUFA2.2 ability to hold its structure when removed from the mesocosms at the end of 56days was noticeably different between treatments containing worms and those that did not. LUFA2.2 in control samples (Left) where all worms survived, was moist to the touch and held its structure well before intentionally breaking apart to count worms. LUFA2.2 in NPK treatments (Middle) had significantly fewer worms present was similar to the controls but was easier to break apart in the hand. LUFA2.2 in all other treatments (Right) where all worms died, did not hold its structure and fell apart easily in the hand.

There are few studies on the effects of inorganic fertilisers on earthworms and these present variable results. Stroud *et al*., 2016 counted the number of *L. terrestris* middens per m^2 and calculated that the abundance of *L. terrestris* was significantly less in plots treated with inorganic N at 0.08/m^2, being 3 to 8-fold lower than in plots with straw amendments added. This is evidence that NPK can have a negative impact on *L. terrestris* abundance and is consistent with Blanchet *et al*., 2016, who found that, on average the number of individual worms in plots treated with NPK was 184/m^2 whereas plots amended with manure was 273/m^2. This is also comparable to Pfiffner and Mädder (1997) assessing the differences in earthworm abundance between N fertiliser, FYM and control treatments, where average abundance of earthworms was 144.5/m^2 with application of FYM, 67.9/m^2 in control treatments, and 60.9/m^2 with N, indicating that application of manure may benefit earthworms, but inorganic fertiliser does not.

Although research presented here is consistent with the literature, there were significantly fewer survivors in NPK compared to Control treatments. Nitrogen fertilisers as single treatments can enhance earthworm populations up to certain concentrations, if soil pH is not altered (Blanchet *et al*., 2016). Earthworms prefer soils with pH values between 6.5 to 7.5 (Edward *et al*. 1995, Edwards and Bohlen 1996). Lordache and Borza (2010), found the largest number of earthworms in treatments with the highest concentration of N fertiliser ($N_{200}P_0$ 26.67 worms/m^2), which was 85.85 % higher than control treatment (N_0P_0 9.33 worms/m^2). Despite this, the greatest negative factor on earthworm abundance was pH, 26.67/m^2 at pH 6.12, 14.67/m^2 at pH 5.95 and 9.33/m^2 at pH 6.67 (Lordache and Borza, 2010), indicating a narrow tolerance of earthworms to a pH around the range of 6. Taking this into account, NPK could further alter soil pH beyond the tolerance of *L. terrestris* (Lordache and Borza, 2010) and be a potential cause

of the patterns observed, although pH was not measured. LUFA2.2 has a pH of 5.4 ± 0.2, indicating that the acidity of the soil alone may not be a favourable environment for *L. terrestris* to thrive. This pH, without added stressors associated with chemical application, whilst not harsh enough to preclude survival (e.g., Figure 8 Control), sub-lethal effects could account for the limited surface activity and lack of copulations and cocoon production if soil conditions were not favourable.

There was no support for hypotheses that survival would: decrease with increasing concentration of Thiacloprid applied; would differ between the different concentrations of Thiacloprid when NPK is present; and for each concentration of Thiacloprid applied, would differ when NPK was present or not. No worms survived in these treatments 56 days after exposure. This lack of survival meant it is difficult to assess if any concentrations of Thiacloprid in single use or when combined with NPK gave rise to synergistic effects. A few studies report on adverse effects of Thiacloprid on mortality of worms in the lab but not as extreme as in the present study.

4.3.2. Experiment 3: OBT

The results indicated that there were no significant differences in the numbers of survivors counted among the ten treatments ($p > 0.05$) (Figure 12). Therefore, it cannot be concluded that either H1, H2, H3 or H4 (See Hypothesis) is true. This result could be said to be comparable to Basley and Goulson (2017), where mortality levels of *L. terrestris* in food and soil treated with Clothianidin at concentrations ≤100 ppb were not significant and concluded to have at worst, a weak effect. However, during this study there were observable abnormal behaviours and effects on the body (discussed in nocturnal activity above) of *L. terrestris* that could result in reduction of populations

and/or earthworm activity and could adversely impact on normal soil functioning and health (Pelosi *et al*., 2013). Even though there was no significant effect on survival, the stress caused by Thiacloprid in single or combined use with NPK at various concentrations on *L. terrestris* may cause them to divert energy from normal behaviours such as growth and reproduction to maximise the chances of survival (Choo and Baker, 1998; Yasmin and D'Souza, 2010; Pelosi *et al*., 2013). This could be a legitimate explanation for relatively low mortality rate seen in this study.

However, during the first week of this study, it was noted that dead worms collected at the surface, exposed to Thiacloprid in single and combined use with NPK and NPK alone developed wrinkling of the skin, with lesions (Figure 14), irrelevant of concentration. This abnormality is consistent with Samal *et al* (2019) when exposing *L. terrestris* to high concentrations of urea. Samal *et al* (2019) found extensive setal damage consistent with wrinkling of the epidermis in worms exposed to a high concentration of urea compared to Control treatments where no morphological abnormalities were visible in the cuticle or epidermis. If setal damage and associated wrinkling of skin was attributable to test chemicals used by Samal *et al* (2019) then it is conceivable that Thiacloprid and NPK as single or combinations of differing concentrations may also cause such damage. Epidermal damage is also consistent with Wang *et al* (2015) AST, finding that 14 days exposure of *E. fetida* to Thiacloprid caused deterioration of the epidermal cells with increasing concentrations of Thiacloprid, inducing irregular cellular compartmentation and an irregular surface of epidermis.

The setae of earthworms are chitinous organelles (Figure 16) acting as anchors to aid burrowing through the earth (Vijaya *et al*. 2012). The structural integrity of the setae is therefore important if the earthworm is to accomplish vital ecological function in the

soil (Samal *et al*., 2019), thus wrinkling of the skin and associated damage to setae could also be a valid predictor of reduced earthworm activity at the soil surface and significantly lower headcounts in all treatments compared to the controls, even if the ability to survive is not hindered. Together, the result of this study and supporting literature indicates that Thiacloprid reduces the number of worms visible at the surface and therefore likely to have negative impacts on normal behaviours including burrowing, and nutrient cycling (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016; Samal *et al*., 2019). The consequences including hindrance of normal ecological functioning and health of soil, incurring a reduction in soil fertility associated with crop abundance, health and growth and long-term food security (Holmes, 2017).

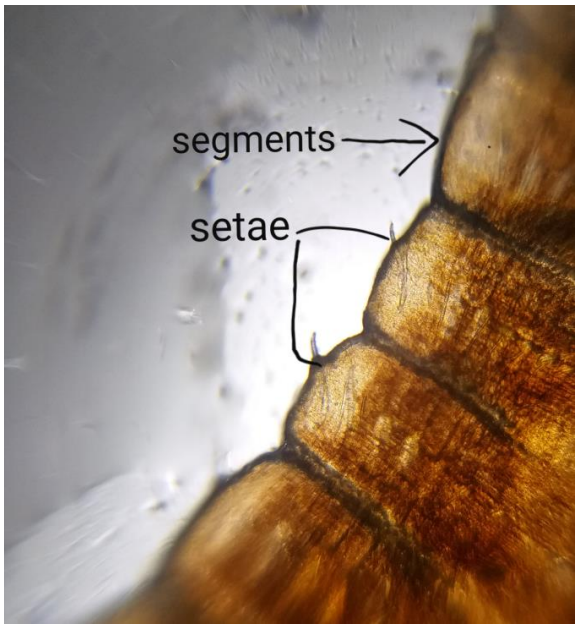

Figure 16: The chitinous organelles known as setae, attached to each body segment act as anchors to aid burrowing through the earth (Veena30, 2018).

de Lima E Silva *et al* (2017) tested Lethal Concentration values (LC_{50}) of Thiacloprid in its pure form on *E. andrei* using LUFA2.2 under laboratory conditions. It was found that the LC_{50} values for Thiacloprid was 2.7 to 3.9 mg/kg dry weight LUFA2.2. The finding is

consistent with LC_{50} values between 2.68 (Wang *et al*., 2015) and 10.96 mg/kg (Wang *et al*., 2012) for *E. fetida* after 14 days exposure to Thiacloprid in OECD artificial soil. The effects of Thiacloprid on LC_{50} of *E. fetida* was found to be significantly increased. Thiacloprid was the second most potent neonicotinoid next to Clothianidin at LC_{50} 0.9 mg/kg compared to LC_{50} 3.05 mg/kg of Imidacloprid, the most commonly used neonicotinoid globally (Jeschke *et al*., 2011; Goulson, 2014; Wang *et al*., 2015). The results of the present study are consistent with previous research, finding that Thiacloprid is toxic to earthworms, however it is important to recognise that there may be differences in the effects of the chemical between its pure form and domestic formulation used in this study (Renaud *et al*., 2018). BPUBKC2 contains components that could change the amount of active ingredient and its bioavailability to *L. terrestris*, thus it is questionable if the commercial formulas can have a greater impact on mortality, than the pure active ingredient alone, often used under laboratory conditions for biological risk assessment. This represents a knowledge gap in research that surely needs to be met to understand the true biological risks of neonicotinoids in commercial forms used in the field. Different species of worm have different tolerances to soil pH (Eng *et al*., 2017; Mao *et al*., 2017 Van Hoesel *et al*., 2017) meaning that biological response to test chemicals may vary between species. The species used in laboratory studies (e.g., *E. fetida* and *E. andrei*) may be better suited to the pH values of LUFA2.2 than are *L. terrestris*. Future studies could better understand biological risk assessments of test chemicals by experimenting on various species and soils that are more realistic of true agricultural environments.

4.4. Mass change

4.4.1. Experiments 1&2: LUFA2.2

For Experiment 1, LUFA2.2 56 days after exposure to single and combined use of Thiacloprid with NPK, only the Controls and NPK treatments contained survivors for measurable changes in mass (Figure 9). Surprisingly, both the Control and NPK treatments lost mass, however there were significant difference in mass change among the two remaining treatments, in support of H1 (There are significant differences between the Controls and all other treatments on mass change). Worms in the NPK treatments lost significantly more mass than the controls. Change in mass was an average of 57 % weight loss for NPK, compared to 7 % for the Controls. Edwards and Lofty (1982) reports under the long-term Broadbalk experiment, beginning 1843, that *L. terrestris* biomass was 51.4 g/m^2 in plots with FYM applied, compared to just 2.5 g/m^2 in those treated with inorganic N and 2.2 g/m^2 in untreated plots. This is consistent with Blanchet *et al* (2016), reporting that the highest earthworm biomass was recorded in soils with FYM added and mineral fertilisers (MIN) has the lowest. On average, the biomass for anecic species such as *L. terrestris* was around 40 g/m^2 for MIN and 60 g/m^2 for FYM (Blanchet *et al*., 2016). Both articles indicate that application of MIN can have a small, positive effect on the biomass of *L. terrestris* but was greatly increased by the addition of FYM. If inorganic N can have a positive impact on the biomass of *L. terrestris*, it raises the possibility that some of the negative results seen in this research are related to unknown soil mechanisms or other environmental factors.

There are no results to assess change in mass for LUFA 2.2 H2, H3 H4. It was not possible to identify if different concentrations of Thiacloprid in single use have greater effects on

mass change or whether the presents of NPK gives rise to synergistic effects as no worms survived in any treatments containing Thiacloprid.

When researching individual and combined toxic effects of the herbicide atrazine with 3 pesticides (Imidacloprid, Chlopyrifos and Lambda-cyhalothrin) on *E. fetida*, Wang *et al*., (2016) demonstrated, using the OECD, Artificial Soil Test (AST) that LC_{50} values in all treatments containing Imidacloprid in single, binary, ternary and quaternary mixtures were greater at 7 days, compared to 14 days exposure. This indicates time-dependent effects, as less of the active ingredients are needed to cause 50 % of the worms to die at 14 days compared to 7 days. However, the results for time-dependent synergism were not significant for all treatments that contained Imidacloprid (Wang *et al*., 2016) supporting the results of this study. Wang *et al* (2016) found that LC_{50} values of *E. fetida* for Imidacloprid in single use was 3.15 at 7 days and 2.82 at 14 days compared to 7.85 at 7 days and 4.28 at 14 days when mixed with Atrazine, indicating time dependent synergism, however the result was not significant. Furthermore, there was no significant difference of LC_{50} values of *E. fetida* for ternary mixtures with the addition of Chlorpyrifos at 4.39 for 7 days exposure and 2.56 at 14 days and once more for quaternary mixture with the addition of Lambda-cyhalothrin at 4.61 at 7 days compared to 2.41 at 14 days (Wang *et al*., 2016). If combinations of test chemicals and LC_{50} values are not significantly more for single use of Imidacloprid than mixtures, it indicates that some neonicotinoids are highly toxic to earthworms regardless of the addition of other chemicals and may not always initiate synergistic effects. This is despite all mixtures displaying a similar pattern with increased LC_{50} values and therefore lethality when exposed to test chemicals for increased periods of time, which is likely to be directly related to change in biomass.

For Experiment 2, LUFA2.2, the outcome for change in mass was the same, only the results were more extreme with all worms except in Control mesocosms died within the first 7 days, thus no comparisons could be made. There are a few studies reporting negative impacts of single application of Thiacloprid on worms under laboratory conditions. However, of the five articles identified where Thiacloprid was one of the target chemicals researched (Gomez-Eyles *et al*., 2007; Wang *et al*., 2012; Wang *et al*., 2015; de Lima E Silva *et al*., 2017; Renaud *et al*., 2018), none reported on changes in biomass of worms, focusing attention on cocoon production and LC_{50} values.

4.4.2. Experiment 3: OBT

For Experiment 3, OBT after 56 days exposure to single and combined use of Thiacloprid with NPK, all treatments contained survivors that were measured for changes in mass (Figure 13). There was a significant difference in mass change among treatments containing worms ($p < 0.001$), in support of H1 (There are significant differences between the Controls and all other treatments on mass change). Post–hoc Tukeys test was not powerful enough to identify where those differences are, therefore, it cannot be concluded that either H2, H3 or H4 (See Hypotheses) is true. Despite this, it is of note that the Control, NPK and NPK+Thia25% were the only treatments to gain weight compared to all other treatments containing Thiacloprid. Of the eight treatments in this experiment containing Thiacloprid, only 1 showed an increase in mass. This study found that the probability of 7 treatments decreasing and 1 increasing from the original weights was significant. The average weight gain per mesocosm for *L. terrestris* was around 30 % when exposed to NPK only, compared to 20 % for Control treatments (Figure 13). This result indicates that the application of NPK has no greater significance

on weight gain than the Controls. N fertilisers as single treatments have shown to enhance earthworm populations up to certain concentrations, if soil pH is not altered (Blanchet *et al*., 2016). If true, it suggests that NPK could enhance reproduction and therefore populations of *L. terrestris* in the field, of which the internal development of cocoons by individual worms could induce weight gain. Despite this, no cocoons were found in either treatment where copulations were witnessed in Controls and NPK and could account for the non-significant result for standardised change in mass. *L. terrestris* is a slow reproducing species (Butt *et al*., 1994; Lowe and Butt, 2014) and it is possible that the 28-day time extension used in this adaptation of the ERT, was not sufficient enough for the development and laying of cocoons. It is also possible that external influences such as timing, temperature and humidity did not provide favourable conditions for reproduction. For example, *L. terrestris* has shown to have only a single emergence period for copulations in the UK during spring to early summer (Lowe and Butt, 2014). During 2019 (the year of study), the UK's hottest daytime temperature on record was documented (Statistica, 2021), reaching 25.8 °C in Newquay, Cornwall (Newquay Weather Station, 2021), where the experiments commenced. However, average night-time temperatures around 14.4 °C were close to optimum incubation temperatures for cocoon incubation of *L. terrestris*, of which is 15 °C (Butt et al., 1994; Lowe and Butt, 2014), indicating that temperature may not be the single contributory factor.

There was a small weight increase for NPK+Thia25% at around 4 %, although the Post–hoc Tukeys test was not powerful enough to identify if differences between the Control and NPK treatment was significant. It is clear from the binomial test that most treatments containing Thiacloprid, whether in single or combined use with NPK has a

negative impact on weight change with 7 out of 8 treatments showing an average reduction of weight per mesocosm between -7 % and -20 %. This is inconsistent with Basley and Goulson (2017) finding that Clothianidin had no significant impact on body mass at 16 weeks exposure, double that of the time *L. terrestris* was exposed to Thiacloprid in this experiment., *Lumbircus terrestris* may, however, have different time-dependent tolerances to different chemicals of the neonicotinoid class (Wang *et al*., 2016; Mao *et al*., 2017 Van Hoesel *et al*., 2017). Different patterns observed could also be down to food contained in the gut. As in Basley and Goulson (2017), the guts of each worm in this study were not voided before weighing, thus it is possible that changes in mass could be exaggerated by differences in initial gut content. This was the reason for standardising the starting weights of worms in this study. However, differences in gut content at the end of experimentation could add weight to adverse behavioural effects of Thiacloprid. If binding to the nAChRs of *L. terrestris* central nervous system caused neurological functioning to hinder coordination, locomotion and ability to consume (Jeschke *et al*., 2011; Gill *et al*., 2012; Eng *et al*., 2017), food may be present in the guts of worms contained in Control and NPK treatments but not Thiacloprid treatments, which could exaggerate the results seen in this study. That being said, the use of mass change as a bioindicator is considered to be ecologically relevant, with large losses in weight leading to negative effects on survival and reproduction (Dittbrenner *et al*., 2010; Basley and Goulson, 2017). The use of change in mass may be a better bioindicator than survival at relatively short exposure times to test chemicals because survival could be mistaken as a positive biomarker, if abnormal behaviours consistent with the application of neonicotinoids are not recognised and energy reserves from normal activities are being redirected to survival (Choo and Baker, 1998; Yasmin and D'Souza, 2010; Pelosi *et al*., 2016). As such, more research is required to recognise different observable,

behavioural responses of earthworms to exposure of different neonicotinoids and chemical cocktails, if science is to use and completely understand the use of survivability as an accurate bioindicator in conjunction with change in mass.

4.5. Cocoons

No cocoons were found for any treatment in any experiment. Given that this is a standard variable measured in the ERT, it is important to ask why this was. It is possible that neither test soil created conditions favourable for breeding by *L. terrestris*, although copulations were observed in Control mesocosms of both soils and also in NPK and NPK+T25 treatments for OBT (Figure 10). This indicates that the presence of Thiacloprid has a significant impact on copulation and therefore on cocoon production. The duration of experiments in the present study was double the 28 days recommended by the OECD because *L. terrestris* have a slower reproductive cycle (3 cocoons/ worm/ month, (Butt *et al*., 1992)) under intensive breeding laboratory conditions throughout the year at 20 °C, than do *E. andrei* (5.5 cocoons/ worm/ week, (Latif *et al*., 2018)) under natural field conditions. Despite this, it remains possible that there had been insufficient time for the earthworms to produce cocoons as *L. terrestris* do not reproduce throughout the year in real agricultural environments (Butt *et al*., 1992). Ge *et al* (2018) found that the number of cocoons produced by *E. fetida* exposed to 6.0mg/kg of Thiacloprid was significantly less at 0.33 ± 0.11 compared to the Controls where 4.03 ± 0.32 were observed per worm. Wang *et al*., (2015) also found that number of cocoons produced by *E. fetida* was significantly less when exposed to just 1.50 mg/kg^{-1} Thiacloprid at 2.4 ± 0.35 cocoons per worm compared to 4.05 ± 0.05 in Control treatments. Together, these results are a good indication that Thiacloprid has a negative impact on the number of

cocoons produced by *L. terrestris* and would therefore reduce densities and population sizes, in turn leading to a loss of ecosystem services, reduced crop health and yield, food security and incurred economic costs. This highlights the need for further research into the impacts of neonicotinoids on earthworm reproduction to improve its influence on policy change aimed at protecting ecosystems, ecosystem counterparts, the services they provide for free to ensure food security is maintained for future generations.

4.6. Soil type

There is an indication that there is a relationship between biological responses of *L. terrestris* to exposure of test chemicals and soil type they are accommodated in. This is best demonstrated by mortality where all worms died in LUFA2.2 (Figure 8) when exposed to any treatment containing Thiacloprid, in marked contrast to all treatments containing survivors in OBT treatments (Figure 12). Effects on body mass between the two different soil types for the Controls and NPK treatments also showed a different pattern, with loss of mass in LUFA2.2 compared to increased mass in OBT. Headcounts were also less frequent in LUFA2.2 compared to OBT. For LUFA2.2 copulations only occurred in Control treatments, where in contrast sexual activity was present in NPK and NPK+T25. These stark differences indicate that different soil types may affect biological responses of earthworms in different ways and cause for further investigation to completely understand the consequences of chemical exposure to soil fauna including earthworms, key ecosystem engineers in real agricultural environments (Chen *et al*., 2014; Wang *et al*., 2016; Van Hoesel *et al*., 2017). Lanno *et al* (2019) found that lead (Pb) had a large range of half maximal effective concentration values (EC_{50}) on *E. fetida* reproduction. EC_{50} ranged over approximately 46-fold, from soil in Borris, Denmark at

110 mg Pb/kg compared to 5080 mg Pb/kg soil from De Meern, The Netherlands. The soil from Borris is characterised by 15 g/kg Organic carbon, 80 % sand, 17 % silt and 3 % clay in comparison to De Meern, at 50 g/kg Organic carbon, 12 % sand, 28 % silt and 60 % clay. If soils from different geographic areas with varying properties and characteristics is found to affect level of exposure of earthworms to heavy metals, it is possible that earthworm exposure to agro-chemicals could be affected in a similar way (Lanno *et al*., 2019). There is minimal research into soil properties and characteristics effecting chemical exposure to earthworms and represents a knowledge gap which should be addressed in order to acknowledge the true effects of agro-chemical exposure to key ecosystem engineers in the field and consequences for ecosystems and incurred impacts on people.

Conclusion

Applications of Thiacloprid in single and combined use with NPK can cause high levels of mortality to *L. terrestris* at a range of concentrations. All Thiacloprid treatments caused 100 % mortality to *L. terrestris* when accommodated in LUFA2.2. As such, it was not conclusive that NPK gave rise to synergistic effects on mortality. Mortality in OBT was not significantly affected by exposure to combined treatments. However, NPK alone can have great effect on mortality of *L. terrestris*. Survival was significantly reduced by the presence of NPK in LUFA2.2 but not in OBT.

Both nightly headcounts, copulations and body mass were significantly reduced by single use of Thiacloprid and when mixed with NPK at all concentrations for each soil type. NPK alone also caused adverse impacts on earthworm activity, copulations, survivors and mass. The single use of NPK alone may not hinder the ability of

earthworms to survive in all soil types, but can decrease headcounts. NPK can cause *L. terrestris* to gain mass and may be linked to greater sexual behaviour observed in OBT compared to LUFA2.2 treatments.

Ability of earthworms to survive and biological responses to Thiacloprid exposure in single or combined use with NPK could be dictated by soil type. It is vital to understand and take into account soil type when applying agro-chemicals to accurately predict biological responses of key ecosystem engineers, so that they and ecosystem services provided can be accounted for when adapting policy for protecting agricultural soils. The ability to survive exposure alongside adverse sublethal effects leaves *L. terrestris* and non-target organisms such as predators vulnerable to exposure via food contamination (Hallmann *et al*., 2017; Eng *et al*., 2017). If populations of predators are in jeopardy because of chemical cocktails that include neonicotinoids, it could be disastrous for pest control services provided by nature. Decreasing predator populations could lead to lack of crop protection from pests, poor plant health and yield and further associated problems such as food security and economic loss (Holmes, 2017).

The ERT could be useful for assessing toxicity of agro-chemicals to earthworms under controlled field conditions if species-specific soil conditions are met for appropriate test earthworms. This could increase our understanding of biological responses of *L. terrestris* to test chemicals and predict impacts on ecosystem structure, functioning and health to the detriment of ecosystem services, environment, food security and economy. Survival is not a suitable biomarker for biological risk assessments with toxins having greater sublethal effects than mortality in some test soils. ERT, under controlled field conditions could influence UK government and decision makers to develop policy

and legislation aimed at protecting environment, ecosystem services and food security outside of the EU.

Future aspects

This study showed that criteria and conditions used for ERT in the laboratory may not be appropriate for transfer to field-experiments using real-life conditions. However, reasoning for this may be the major limiting factor of sample size for this study. The OECD ERT guidelines recommend a minimum of two replicate samples (OECD, 2015) of which this research tried to mitigate for by increasing to four or six samples per treatment type. Although this study doubles sample size for OBT and triples for LUFA2.2, still relatively small sample size may significantly reduce the power of this research at finding synergistic effects. Despite this find, increasing sample size again per treatment, coupled with the versatility of the ERT for use with many different soil types and taxonomic groups could make it a powerful method for identifying non-interaction and/or chemical interactions between chemicals used in combination in the field. After identifying such interactions, research could focus analysis on whether such interactions give rise to synergistic or antagonistic effects and if those interactions influence behavioural response of soil fauna (Wang, 2012a; Uwizeyimana *et al*., 2017). It is vital that future work continues to investigate the implications of exposure of soil fauna to agro-chemical applicants in single and combined use and how it may be affected by different soil types.

Future studies should also seek to assess both acute effects that usually occur rapidly, as a result of short-term exposure to chemical cocktails and chronic effects where symptoms develop over long time periods. One limitation of this study is that the

relatively short-term experiment may not have sufficient time to show long-term effects of test chemicals on reproduction, thus increasing the length of the experiment may increase its effectiveness at identifying chronic effects on *L. terrestris* and of other beneficial soil fauna, allowing for more accurate biological risk assessment. It may be beneficial during the experiment to hand search for earthworms more frequently throughout phase 1. This would allow the researcher to identify effects of short time exposure including when worms died during the experiments. Identifying early death of slow reproducing species such as *L. terrestris* as a response to chemical exposure is extremely important if we are to protect such valuable species whose populations may take a decade or more to recover in real environments (Ashworth *et al*., 2017; Stroud, 2019). Future studies should seek to prioritise research to quantify amounts, distributions, fates and consequences of pesticides application and of neonicotinoids in particular in single or combined use with other chemicals. Future studies should aim to better allow Governments to understand biological risk assessments of test chemicals by experimenting on various beneficial earthworm species and soils that are more realistic of true agricultural environments. The versatility of the methods used in this study makes it easily adaptable for study of synergistic and antagonistic effects of chemical mixtures on a variety of taxonomic groups that exist in soil. Better understanding the effects of agro-chemicals on multiple beneficial groups of organisms and further consequences for ecosystem services they provide, should be helpful at allowing decision makers to implement protective policy and legislation aimed at improving future food security. Healthy earthworm populations are key to long-term sustainability of arable food production by building soil through the breakdown of organic matter and cycling of nutrients from soil surface to lower depths where they provide nourishment for crops. Due to the importance of earthworms in ecosystem

functioning and services provided, Governments should develop legislation aimed at protecting soil and soil fauna from pesticides (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016). Governments should recognise the value of earthworms to agriculture and their potential for mitigating impacts of climate change and rehabilitation of degraded and polluted land (Capowiez *et al*., 2006; Bawa *et al*., 2016; Pauli *et al*., 2016).

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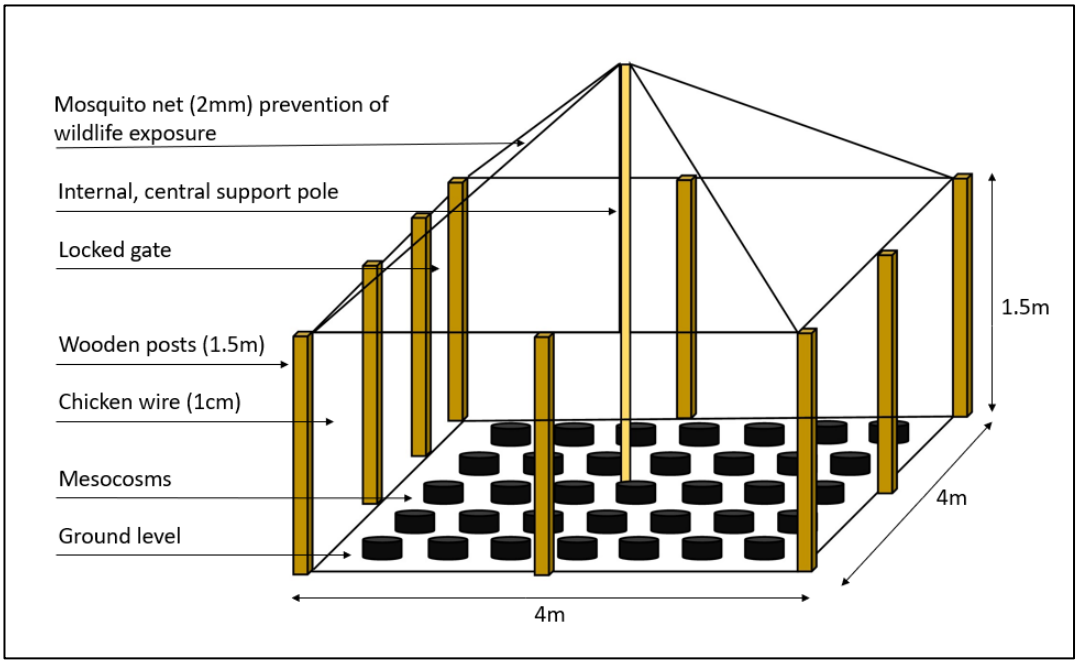
Appendices

Appendix 1



Secure enclosure used to protect people and wildlife from exposure to thiacloprid.