Biological indicators to assess the impacts of silver nanoparticles in different forms on agroecosystems

by

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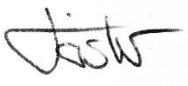
Signature of Candidate _____  _____

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Abstract

Silver nanoparticles (AgNPs) have been used increasingly in consumer products from medical supplies, clothing, cleaning equipment, plant protection products, to household appliances. This increased use leads to an increased presence in the environment, especially in agroecosystems by direct utilisation of plant protection products in addition to the application of biosolids to soils as a fertiliser. Silver in biosolids has increased in parallel with more consumer usage, as an increasingly larger amount of AgNPs is washed into the waste-water system. However, during wastewater treatment, AgNPs react with sulphur and are transformed into silver sulphide (Ag_2S), and research concerning this substance is less prevalent than it could be. In this thesis, a variety of aspects concerning potential effects of silver in the agricultural environment have been investigated, mainly through the use of *Aporrectodea caliginosa* (an endogeic earthworm species) as a representative of the soil faunal community.

Laboratory-based experiments investigated the toxicity of AgNPs in soil on *A. caliginosa* and AgNPs were found to be fatal at high doses (20% at 500; 60% at 750; and 80% at 1000 mg kg^{-1}), but only if the particles were pristine, as aged AgNPs had little to no effect. Earthworms also lost mass with increased levels of AgNPs. Pristine AgNPs caused avoidance behaviour of the earthworms, however in a reproductive study it was found that over the course of several weeks, *A. caliginosa* acclimatised to the adverse conditions, and initial negative effects diminished.

These experiments were repeated with Ag_2S, the form of silver more likely to occur after biosolid application, so that findings between the effects of AgNPs and Ag_2S, could be directly compared. Ag_2S caused no mortality and had limited negative effects on mass and reproduction of *A. caliginosa*. However, they still avoided Ag_2S-containing soil in an avoidance experiment, suggesting a low level of toxicity.

A range of plant-based experiments were undertaken to assess the impact of AgNP-containing plant protection products in addition to soil containing Ag_2S. In a germination experiment, silver products caused a significant decrease in germination and plumule emergence of lettuce seeds. A spray application of silver-containing products onto lettuce caused a decrease in mass at high silver concentration, but Ag_2S-containing soil caused an increased mass in radish plants in line with the increased concentration. Results from these experiments overall posed more questions worthy of investigation.

From the given experimental work, the potential hazards of increased spread of AgNPs onto agricultural fields via the application of biosolids have been shown to be low due to their transformation to Ag_2S. However, more research should be undertaken which acknowledges the prevalence of Ag_2S in agroecosystems.

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1 Introduction

1.1 General introduction

For centuries, humans have used this planet with abandon, not considering the consequences left behind. Whether it is the air, the water, or the soil, pollution is everywhere. From microplastics in our oceans, increasing levels of Carbon dioxide (CO_2) from burning of fossil fuels, to toxic waste leaching from landfills into the soil, there are many known negative effects day-to-day life has on the planet. And there are also some substances whose effect on the environment is still unknown, or currently in the process of being discovered. One of these is silver.

Antimicrobial effects of silver have been known for centuries; even before bacteria were discovered, silver containers were known to help food last longer. To harness this antibacterial potential, silver nanoparticles (AgNPs) have been produced for over a century (Nowack et al., 2011). A nanoparticle is defined as a small particle having at least one dimension measuring 100 nm or less (EFSA, 2016). While silver is not directly toxic to humans, ingesting large amounts of it can cause a skin discolouration called argyria, which is caused by the formation of silversulphite (Ag_2SO_3) which is permanently incorporated into the skin (EPA, 1993).

AgNPs are becoming more and more prevalent, for example in clothing, packaging material, medical supplies or even washing machines (Yu et al., 2013), and are therefore increasingly finding their way into the wastewater system. The silver precipitates in the wastewater treatment plant and 96% of it accumulates in the biosolids, however, it is not in the form of AgNPs and rather in the form of silver sulphide (Ag_2S). This is because during the treatment process, AgNPs react with sulphur in the sewage and as well as present organic matter to form Ag_2S (Burkhardt et al., 2010). In the UK, the majority of biosolids are spread onto the field as a fertiliser and scientists have long investigated the potential dangers of the increased amount of AgNPs in the soil (Choi et al., 2008, McGee et al., 2017), but the effects of Ag_2S have so far been largely neglected. One way to investigate the effects of substances on soil, is to conduct experiments using earthworms. Earthworms are an important part of the soil fauna and are a great test organism due to their importance and ease of handling.

There are basically three types of earthworms: epigeic, anecic, and endogeic. With epigeic earthworms living mostly above surface and feeding on leaf litter, anecic earthworms burrowing into

the soil but feeding on the surface, and endogeic earthworms living and feeding in the soil (Bouché, 1977). Experiments concerning the effects of toxic substances on the soil community are often performed on epigeic earthworm species, as recommended by the OECD (OECD, 1984, 2016).

Effects of AgNPs on earthworms have been studied widely, mostly with epigeic earthworm species, but also with endogeics. It is not possible to report a level of AgNPs in soil which is toxic towards earthworms as there are multiple influences towards any toxicity. Not only do different earthworm species react differently, but toxicity is also influenced by the type of AgNP coating, AgNP size, and type of soil used. However, what is certain is that AgNPs can reduce reproduction, cause mortality and loss of mass in earthworms (Shoults-Wilson et al., 2011a, Brami et al., 2017b). Both epigeic and endogeic earthworms have previously been shown to avoid silver-containing soil at 10 mg kg^{-1} (Shoults-Wilson et al., 2011c) and 12.5 mg kg^{-1} (Brami et al., 2017b) silver respectively, showing that earthworms can detect the suboptimal conditions of the soil and avoid it at levels below which negative effects would be recorded.

Due to its antimicrobial properties, silver and especially AgNPs have shown the potential to be used as plant protection products. Multiple studies have shown effectiveness of AgNPs as an anti-fungal agent (Kim et al., 2009b, Min et al., 2009), able to treat pests such as mildew or onion mould. However, there are also negative effects towards plants, as it has been shown that high levels of AgNPs reduce germination (El-Temsah and Joner, 2012, Nair and Chung, 2015).

This thesis investigates the effects of AgNPs, biosolids, and Ag_2S on the soil community by using *Aporrectodea caliginosa*, an endogeic earthworm species which compares all result to each other; and investigates the effects of silver in different forms on plants. These results aim to provide a comprehensive overview over different toxicity levels between different forms of silver and show which areas of this subject should be investigated further.

1.2 Problem statement

Due to convention and ease of purchase, the majority of toxicity studies on earthworms are performed with epigeic earthworms. However, when assessing a toxicant in the soil, endogeic earthworm species are more suitable, as they feed upon and in the soil, rather than searching for food on the soil surface. And while the effects of AgNPs on the environment have been widely studied, it is necessary to take into account the transformation of AgNPs to Ag_2S in the wastewater treatment system. An application

of biosolids will increase occurrence of Ag_2S, not AgNPs and more environmental studies acknowledging that fact should be performed.

In the case of AgNP-containing plant protection products, the main focus is still on the effectiveness of use and not yet on the potential increase of silver in produce consumed by humans or effects on the soil community.

1.3 Research Aims

Aim 1: Assess the effects of AgNPs and Ag_2S in soil on a representative member of the earthworm community, by recording survival, growth, avoidance behaviour, and reproduction of *A. caliginosa* exposed to AgNPs and Ag_2S in soil.

Aim 2: Assess the effects of AgNPs and Ag_2S on plants.

Each of these will be examined through experimental investigation, with detailed objectives given at the start of each Chapter.

1.4 Thesis structure

Chapters one and two of this thesis provide the concept and aims of this research followed by a review of the literature to give further context of AgNPs, biosolids, earthworms, and other relevant topics. Chapter three describes some commonly used methods within this work to prevent unnecessary repetition in following Chapters. This thesis has four experimental Chapters, the first 3 concern the effects of AgNPs, biosolids, and Ag_2S on earthworms. The next Chapter looks at different aspects of silver in the context of plants. Finally, Chapter 8 provides an overall discussion of results from each experimental Chapter in the context of thesis aims and published work. The contributions to knowledge in this thesis, its limitations and further research are then discussed.

2 Literature Review

Ecotoxicology looks at the effects that substances have on the environment. Pollutants enter the environment in different ways ending up in all areas of our ecosystem and in the case of the agricultural ecosystem, pollution may happen through the application of fertiliser, fungicides or pesticides. One substance that can enter the agricultural system both though fertiliser and plant-protection products, is silver nanoparticles (AgNPs).

Effects of AgNPs within the agricultural systems is a wide-reaching topic. It starts with questions relating to what AgNPs are, and from where they originate. There is then a need to discuss how they find their way into agricultural systems and which parts of the system can be directly or indirectly affected. Which effects have already been studied and where gaps in the research remain, and still need to be addressed? The following literature review is designed to provide context to this project and seeks to address some of these points.

2.1 Introduction to silver

Humans have made objects from silver (e.g., jewellery and eating utensils) for millennia and continue to do so today. Silver is a non-essential element and unlike many other non-essential elements it is not considered toxic to humans, except at high concentrations where it has been found to have negative effects such as enzyme inhibition by binding to proteins (Dolara, 2014).

Half a century ago, Hamilton et al. (1973) and the International Commission on Radiological Protection (1975) compiled a comprehensive list of concentrations of different elements present in the human body. They quantified a range of elements in different tissue samples from all over the UK. Due to this work an estimate of silver concentrations in the human body can be presumed (Table 2.1).

Table 2.1: Average silver concentration in different human tissues (sources shown in table)

Organ	Ag concentration [µg g^{-1} ±STDEV wet weight]	
	(Hamilton et al., 1973)	(International Commission on Radiological Protection, 1975)
Blood	0.008 ± 0.0008	0.18
Brain	0.04 ± 0.002	0.9286
Kidney	0.002 ± 0.0002	0.011
Liver	0.006 ± 0.002	0.111
Lung	0.002 ± 0.0001	7.6
Lymph Nodes	0.001 ± 0.0002	
Muscle	0.002 ± 0.0005	0.0121
Testis	0.002 ± 0.0004	0.0089
Ovaries	0.002 ± 0.0005	
Bones	1.1 ± 0.2	

There are two drawbacks with the values seen in Table 2.1: First, the data is nearly 50 years old and might not represent the current silver burden on the human body. Second, silver concentrations in the reference man (International Commission on Radiological Protection, 1975) are much higher than in the 1973 study, the reason for which is unknown. Is it due to the location from where samples were obtained, to different analytical methods used, or potentially another reason? Nevertheless, accurate average silver concentration in the human body remains unknown. In addition, there are also no values that describe acute toxicity in humans. There are also no recorded cases of humans dying from an acute overdose of silver. Miners exposed to high levels of silver in the air have been reported to experience irritation of the upper and lower respiratory system (Rosenman et al., 1979). However, it is not clear whether these symptoms were specifically caused by the presence of silver in the air or by dust in general.

Figure 2.1: Paul Karason and Rosemary Jacobs, argyria sufferers (Telegraph, 2008)

A side effect of chronic silver ingestion is argyria, a permanent discolouration of the skin caused by the formation of silver sulphite which is permanently incorporated into tissues including the skin (EPA, 1993). Figure 2.1 shows two argyria-sufferers: Paul Karason turned blue after ingesting large doses of homemade colloidal silver and Rosemary Jacobs took colloidal silver nose drops for 4 years at age 11 before developing argyria (Telegraph, 2008). Argyria is not considered a toxic endpoint, as it is solely cosmetic, and sufferers have no other physical symptoms.

Since LD_{50} (lethal dose needed to kill 50% of a given population) data for elemental silver is not available, recommended doses are based on values which might cause argyria. The WHO (2004) estimated a no observed adverse effect level (NOAEL) of 10 g silver per human lifetime, from which they concluded that the level of silver in drinking water should not exceed 0.1 mg L^{-1}. Measured silver concentrations in drinking water, however, generally do not exceed 5 µg L^{-1} and daily intake by humans is estimated to be 7 µg (WHO, 2004). In Europe, the silver levels in tap water range from 6.1 to 19.8 ng L^{-1} with no incident of silver toxicity due to tap water ever reported (Peters et al., 2011). Considering

that the bioavailability of silver is estimated to be around 2-20% depending on the animal species (EFSA, 2016), silver in drinking water is unlikely to cause harm to humans. In the USA, the RfDs (oral reference dose) for silver has been set by the U.S. Environmental Protection Agency (EPA) at 0.005 mg per kg bodyweight per day (EPA, 1993).

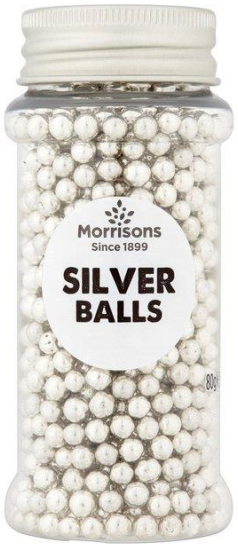

Figure 2.2: Cake decoration containing silver (Morrisons.com)

Silver can be used as a food additive (EFSA, 2016) and is readily found on supermarket shelves. Figure 2.2 shows silver balls which are commonly used in the decoration of cakes, biscuits and cupcakes. Although silver balls always contain silver as a colouring, the exact amount is not stated.

2.2 Introduction to silver nanoparticles (AgNPs)

Nanoparticles are defined as particles of any form where at least one dimension measures 100 nm or less (EFSA, 2016). Their composition is not defined, can vary from metals and carbon to lipids and polymers (Missaoui et al., 2018). Nanoparticle production and use has increased since their first production, and especially since the start of the millennium. Due to their different properties compared to larger particles, such as small size in addition to large surface area, they are used in a variety of application such as drug delivery, diagnostics and in food additives (Missaoui et al., 2018). Their small size means that nanoparticles often have an effect compared with their much different elemental counterparts.

Nanoparticles can form naturally or can be produced in various ways. Silver nanoparticles (AgNPs) have been engineered for over a century mainly due to their antimicrobial properties (Nowack et al., 2011) thought to result from the release of Ag^+ ions and their increased volume to surface area ratio. As early as the 19th century, production of stable silver colloids was reported. And while at that time a size determination was not possible, following this method produces AgNPs of less than 10 nm in diameter (Nowack et al., 2011). Currently, AgNPs can be manufactured accurately to specific sizes and come with a variety of coatings, usually polymers and surfactants. Coatings are added to AgNPs to prevent agglomeration of particles to particle clusters or dissolution into ions. This is especially relevant when the particles are in a liquid medium but can also prevent oxidation when the particles are dried. Some of the most commonly used coatings include citrate, tween, and sodium dedocyl sulfate (SDS), which stabilise the AgNPs in dispersions through electrostatic and steric repulsion (Li et al., 2012).

Examples of commercial products containing AgNPs include, but are not limited to, odour resistant clothing, food packaging material, washing machines, wound dressings and soaps (Yu et al., 2013). These products may be disposed of in landfills or incinerated. In both use and disposal, silver can be released into the environment, either into groundwater by leaching from landfills, into the air through incineration of waste and biosolids (treated sewage sludge (see Section 2.5)) or directly into soil by application of biosolids (as a fertiliser) in agricultural systems (Blaser et al., 2008).

Due to their widespread use in consumer products, AgNPs are one of the most researched nanoparticles. The effects on soil ecosystems and subsequently on human health have been studied but are not yet completely known (Kim et al., 2009a, Wang et al., 2017a). Studies have found that AgNPs have the potential to cause neurotoxicity through oxidative stress and mitochondrial damage (Teleanu et al., 2018) and that high amounts increase production of reactive oxygen species and induce toxicity towards human sperm (Wang et al., 2017a).

The toxicity mechanism of AgNPs is not well understood, but it is known that particle size affects AgNP toxic level (Yin et al., 2011). It is widely suggested that the toxicity of AgNPs is due to the slow release of Ag^+ ions when compared to ionic silver, as the latter is often shown to be more acutely toxic while AgNPs have a longer-term toxicity (Shoults-Wilson et al., 2011a, Brami et al., 2017b).

In a study on the effect of AgNPs, in addition to $AgNO_3$ on single celled algae, Navarro et al. (2008) found that Ag^+ ions were the only cause of toxicity in AgNP solutions. Algae exposed to AgNPs stopped photosynthesis, but algae exposed to AgNPs in combination with cysteine, an amino acid, which binds free ions, were not affected. In contrast, a very similar structured study found that there is more to

AgNP toxicity than just the release of ions. Yin et al. (2011) exposed *Lolium multiflorum* (ryegrass) seeds to different forms of silver with or without cysteine as a silver ion inhibitor. They found that silver ions were not the cause of the AgNP toxicity. Furthermore, exposure to AgNPs had a negative effect on the outer cells of the roots while ionic silver had no effect. So, the question of whether the toxicity of AgNPs stems solely from their release of ions or whether they pose a risk on their own remains open.

2.2.1 Silver Colloids

Colloidal silver is a liquid containing small, suspended silver particles. While their manufacturing process is similar to AgNPs, their size is not monitored as closely as that of AgNPs and therefore they are considered in their own category. For decades, colloidal silver has been used as an antiseptic and also marketed as a dietary supplement (Woods, 2012). It is widely available for purchase online and can easily be prepared at home with instructions available on the internet (see Figure 2.3).

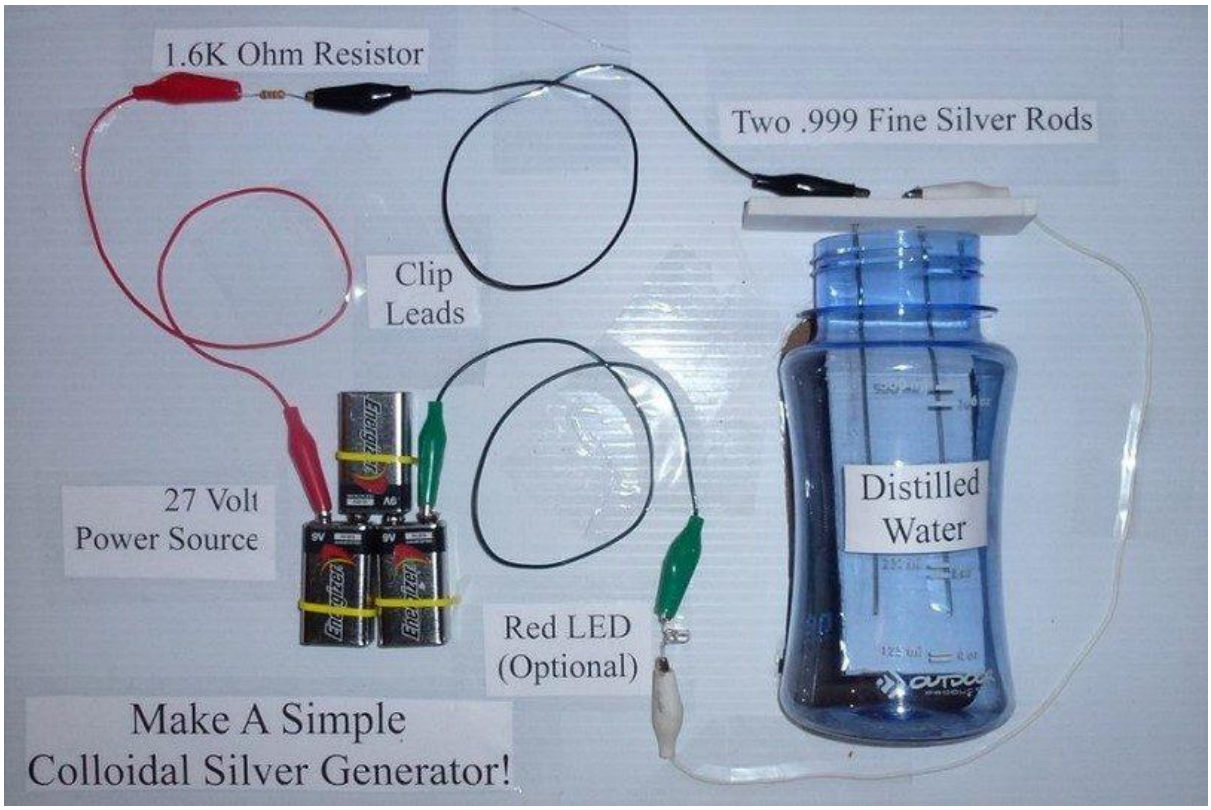

Figure 2.3: Instruction on how to prepare colloidal silver at home (McKay, 2021)

As already established, silver has antimicrobial properties and subsequently, if used in a medically appropriate way, colloidal silver can also be beneficial. For example, it can be used to prevent an eye infection called conjunctivitis in new-borns, in wound dressings and to treat skin infections (Killen Jr., 2017). However, users should be advised to buy their colloidal silver from reputable sources as the

safety and concentration of homemade concoctions cannot be confirmed. Continuous ingestion of highly concentrated colloidal silver is a common cause of Argyria (Telegraph, 2008). This is because when preparing colloidal silver at home, the user has no way to determine the exact concentration and is therefore at risk of ingesting large amounts of silver, often over an extended time period.

2.3 AgNP Analysis

AgNPs are often analysed as total silver content and the presence of particles is not always confirmed. This is partly because of difficulties extracting AgNPs from complex matrices. Analysis of nanoparticles in general is also difficult and not every laboratory has the capabilities to differentiate between dissolved silver and nanoparticles. To quantify total silver concentration, samples are digested using microwave-assisted acid digestion and then measured with atomic absorption spectroscopy (AAS) or inductively coupled plasma (ICP) techniques. Atomic absorption spectroscopy has the highest limit of detection (LOD) followed by inductively coupled plasma optical emission spectroscopy (ICP-OES) with inductively coupled plasma mass spectrometry (ICP-MS) being able to detect the smallest quantities of silver.

AAS is based on the principle of atoms absorbing certain wavelength of light. AAS utilises this phenomenon and scans wavelengths specific to an analyte to quantify the amount present in the sample. The sample must be in aqueous form and is taken up by the instrument, burned and the wavelengths of the resulting flame are analysed. It is a relatively rapid and simple way to analyse a sample, however, it is not appropriate for trace analysis (Robinson, 2014).

Compared to AAS, ICP-OES is a more sensitive method to identify and quantify elements. Helium or Argon gas is used to nebulise the liquid sample and introduce it into the inductively coupled plasma (ICP). This ionises the sample and excites the elements, which emit radiation with distinct wavelengths. These wavelengths and their intensity are measured by a spectrometer (Robinson, 2014). From this, an element can be analysed both qualitatively from the emitted wavelength and quantitatively by measuring the intensity of the wavelength.

The most sensitive method for elemental analysis is ICP-MS, which also ionises the sample using plasma, but the detection method is different from ICP-OES. It uses mass spectrometry to measure ions rather than wavelengths. Its high sensitivity makes it the ideal instrument for quantifying elements at low concentrations. The exceptional sensitivity of the ICP-MS is due to the high ionisation rate of the sample which is achieved by the plasma, having a temperature of 7000K – 10000K

(Langford, 2005). To perform an analysis, the sample needs to be in liquid form without any large particles present to prevent blocking the nebuliser or otherwise obstructing the instrument. After preparing the sample, an autosampler, in combination with a peristaltic pump, is used to introduce the sample into the instrument via a nebulisation system including a spray chamber. The gas flow draws the finely aerolised droplets into the gas stream using the low pressure created by this set up. As the ICP is operated at atmospheric pressure and MS works at high vacuum, the interface needs to build up a vacuum using specialised vacuum pumps.

When analysing nanoparticles; knowing the amount of silver in a sample is insufficient. It is also essential to know in what form the silver is present, whether the silver is solved and therefore present in ionic form, or if it is present in the form of nanoparticles and what size the particles are. In the case of soil analysis, a third factor is the adherence of silver to soil particles. Depending on the type of sample, measuring the total amount of silver might be the only option, as extractions are not always possible.

A rapid method to determine whether a single particle suspension of AgNP has been achieved is to measure the absorbance of the resulting solution. AgNPs in solution have a yellow colour, the exact wavelength is dependent on the size of the particle. Nanoparticles are smaller than the wavelength of visible light and the light exerts an electromagnetic force on them. The particles start oscillating at a resonance frequency, absorbing light of a certain wavelength (Serrano Rubio, 2015). As the resonance frequency is dependent on size, metal and structure, it can be used to roughly establish if singular metal particles are present. Additionally, the wavelength roughly corresponds to the size of the nanoparticle, as seen in Figure 2.4.

Figure 2.4: Dispersion of different sized citrate coated AgNPs (NanoComposix, 2019)

Other factors also influence the absorption values of the nanoparticle dispersion. In Figure 2.4, the AgNPs are dispersed in a 2 mM citrate solution (NanoComposix, 2019). Therefore, the particles are not uncoated but are coated in citrate. Different coatings can cause shifts in absorption and the

amount of coating correlates with the size of the shift (Li et al., 2012). Li et al. (2012) found that citrate coating causes a blue shift while coating with Tween 80 and sodium dodecyl sulphate, two other popular AgNP coatings, cause a red shift. So, while measuring the absorption of a solution is a helpful method to determine size, it is only achievable if the exact properties of the given AgNPs are known.

Another possibility to differentiate between ionic and particulate silver is to quantify the amount of Ag^+ ions present in a sample. For this, an ion selective electrode can be used. These are available for different ions and can measure the conductivity produced by that specific ion (Benn and Westerhoff, 2008). Silver-specific electrodes measure all Ag^+ ions present in the sample. This is a useful method if available, and has been successfully used in soil analysis (Benoit et al., 2013).

In general, particle analysis consists of two parts: 1. Confirming the presence of the analyte in a particular form and, if possible, 2. Estimating the size of the particles present. This can be achieved with electron microscopy techniques, single particle ICP-MS and asymmetric flow field flow fractioning (Mitrano et al., 2012). A selection of techniques is described below.

A relatively popular way to determine the presence, in addition to size of, nanoparticles is electron microscopy such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Both techniques give high resolution images in the nm range meaning single NPs can be identified and measured. Additionally, the elemental composition of the sample can be identified. Both SEM and TEM techniques work by producing an electron beam which is moved across the surface giving information about surface structure and elemental composition (Robinson, 2014). In practice, the difference between SEM and TEM is that SEM only gives the elemental composition of the surface of the sample while TEM can be used to detect elements inside a sample. Electron microscopy is most commonly performed to characterise freshly prepared nanoparticles and is less often used in sample analysis. A low concentration of NPs in environmental samples and high matrix load can make it more difficult to locate the NPs in a sample.

As previously described, AgNPs absorb certain wavelengths specific to their size. Due to this capacity, AgNPs can be detected with ultraviolet/visible light (UV-Vis) detectors. UV-Vis detectors can serve as detectors after several different separation methods. These separation methods need to be capable of separating different sized AgNPs. One possible method is size exclusion chromatography (Pitkänen and Striegel, 2016). The drawback of this method is that the size exclusion column is very specific in terms of sizes and particles it can separate which is limiting in everyday use. A less restrictive separation method is asymmetric flow field-flow fractionation (AF4) which is based on size dependent

diffusion coefficient (Koopmans et al., 2015). It requires an AF4 instrument and has been successfully employed in the determination of AgNP size in soil samples (Delay et al., 2011, Koopmans et al., 2015).

Depending on the type of analysis, a simple digestion is not always an appropriate method of sample preparation. Sometimes extraction is a preferable method of obtaining a liquid sample from soil. There is no fixed method on how to perform soil extraction as it changes depending on the goal of the analysis. There are three main categories of extractions. The first are soft extraction methods using only water or water containing different salts to enable ion exchange. Second is sequential extraction which uses multiple different extraction steps to increase the amount of extracted analyte and is able to define whether and how the analyte is bound to the soil. Lastly, acidic extractions can be used which are very similar to digestions in that the analyte is likely to break down. The difference is that in digestions the sample may be broken down completely and in acidic extractions, the soil remains mostly intact.

2.4 AgNP in Animals

Multiple studies concerning particulate silver in animals have been undertaken. These usually fall into one of two categories: 1. Studying the effects of AgNP on vertebrates and 2. Studying the potential for silver solutions to replace antibiotics in animal husbandry. Consumers are increasingly concerned about antibiotics in meat and animal products, so replacing them with AgNPs or, more generally, colloidal silver solutions, could be beneficial to producers.

Concerns have been raised in replacing antibiotics by AgNPs in live-stock breeding, and the potential increase in silver intake for the consumer. Sergeevna et al. (2018) experimented by feeding colloidal silver to chickens and testing their muscle and body organs for silver. All analysed tissues, including controls, contained low amounts of silver. By feeding the chickens silver in low but effective concentrations, the silver content either did not increase or changed insignificantly. In another study, Ognik et al. (2017) found a correlation between the amount of silver fed to chickens and the amount detected in their tissues, however, this result was not significant.

Ognik et al. (2017) found no toxicity and no significant accumulation of silver in the exposed chickens but found that the ingestion of AgNPs led to a significant decrease in iron, calcium and potassium uptake. While this decreased nutritional uptake does not appear to have harmed the chickens, it might be of interest when considering toxicity to humans. In the experiment, the chickens were reared for

42 days and only received AgNPs after 8 days. This meant that they were only exposed to AgNPs for 34 days, which may not be long enough for nutrient deficiency to have a significant effect on health.

Considering these studies, AgNPs might be a suitable alternative in rearing chickens. Chickens are especially suitable as they grow in a relatively short amount of time and therefore the silver does not have time to accumulate in their tissue where it would be consumed by humans or cause harm to the animal. Larger animals take longer to raise, and the addition of silver to their diet might have a longer-term effect on them.

To gain a better understanding of the toxicity mechanism of AgNPs, multiple studies on rats have been performed. Sizes of 10 nm, 25 nm and 60 nm AgNPs in concentration up to 1 g per kg bodyweight per day for four weeks, have shown no effect on body weight. Interestingly, it was found that male and female rats accumulate silver differently. For example, female rats accumulate more silver in their kidneys compared to males, the reason of which is unknown (Kim et al., 2008, Lee et al., 2013). In another study, it was found that AgNPs caused a dose-dependent increase in micro nucleated polychromatic erythrocytes. However, the results were not statistically significant, so it is not possible to state that AgNPs cause genotoxicity in rats (Kim et al., 2008). These findings are consistent with the general consensus that AgNPs are not acutely toxic and do not cause cancer.

An important question in toxicology is how efficiently a toxicant is eliminated from the body. Lee et al. (2013) investigated this question in rats and found that silver is eliminated from different tissues at different rates. For example, AgNPs are eliminated at a much faster rate from the liver, the spleen or the kidneys than from the brain. Particle size also appears to play a role in determining the rate of elimination, as 10 nm particles are eliminated at a slower rate than 25 nm particles. Whether the silver is present as particulates or in another form is unknown, as this was not investigated by the researchers.

2.5 AgNPs in biosolids

It has been shown that 96% of silver reaching a wastewater treatment plant accumulates in the biosolids rather than the supernatant of wastewater (Tiede et al., 2010, Burkhardt et al., 2010). Consequently, biosolids applied to agricultural land may contain relatively concentrated amounts of silver. However, neither study specified in which form the silver was present. It is unknown whether the AgNPs are still in their original form, have transformed to an inert substance, or are newly formed as the consequence of precipitating Ag^+ ions. While there is general agreement that increased use of

AgNPs leads to an increased amount of silver in the soil, it is not known if the silver is in particulate form. Multiple studies have been performed on the effect on AgNPs on soil (Choi et al., 2008, McGee et al., 2017) without the certainty of the incidence rate of this scenario.

While the exact silver concentrations in biosolids are unknown, it can be assumed that they vary widely. Wastewater treatment plants take in water from their surrounding sewage system. This system can be fed mainly by households or by industry which would alter how much silver accumulates in the wastewater and subsequently in the biosolids.

The increased use of AgNP-containing products has resulted in an increased amount of AgNPs in the environment. AgNP may also have a detrimental effect on the wastewater treatment process as they inhibit nitrifying bacteria (Choi et al., 2008) which are essential to the treatment process. Biosolids from wastewater treatment plants are either incinerated, deposited in landfill or spread onto agricultural fields, where they have long been used as an additional source of organic matter. The application of biosolids represents a direct deposition of silver onto soil, while biosolids disposed in landfill can leach silver into the groundwater and air emissions from incineration eventually settle in the environment (Blaser et al., 2008).

Currently, there is a lack of information on AgNP concentrations in the environment. It is known that silver concentrates in the biosolid fraction of sewage rather than the effluent (Burkhardt et al., 2010). With that knowledge, it is presumed that the application of biosolids onto fields leads to the accumulation of AgNP in agricultural soils. To date, the presence of AgNPs in such soils has not been confirmed.

The issues associated with quantifying AgNP in complex matrices has meant that there is no consensus on the amount of AgNPs present in biosolids or in soil treated with it. Probability modelling has estimated median AgNP content in soil regularly treated with biosolids to be in the range of 0.3 µg Ag kg^{-1} (Sun et al., 2014) to 7.4 µg Ag kg^{-1} (Gottschalk et al., 2009). While there is general agreement that increased use of AgNPs leads to an increased amount of silver in the soil, it is not possible to know if the silver is in particulate form. The AgNPs could remain in their original form, have aggregated to larger particles of silver, have transformed to an inert substance, or are newly formed as the consequence of precipitating Ag^{+} ions. The effect of AgNPs on soil and the soil microbial community has been studied (Choi et al., 2008, McGee et al., 2017, Sillapawattana et al., 2016) without the certainty of the incidence rate of this scenario.

Additionally, the use AgNP-containing fertilisers and pesticides could lead to further accumulation of AgNP in the environment. Accumulation of nanocarriers in soil has been reported if they are not

washed out of the soil (Kah et al., 2013). It is therefore critical to establish the potential toxicity of AgNP to the environment.

In the UK, it is very common to dispose of biosolids by spreading them onto fields, and over time the amount of biosolids has increased. In 1996 and 1997, only 47% of biosolids were applied to agricultural land and in 1999, after disposing of biosolids was banned in surface waters, the percentage rose to 52% (Defra, 2002). In 2006, the UK produced over 1.5 million tons of dry biosolids of which 68% was spread onto fields. In 2008 and 2010, the percentage of biosolids applied to agricultural land in the UK rose to 81% and 79% respectively (Defra, 2012). However, across the whole of Europe, biosolids are only applied to 5% of agricultural land and account for less than 5% of organic matter (Milieu Ltd, 2010).

Strict guidelines exist in the UK to manage the spread of biosolids onto agricultural land. Hereafter, the main regulations will be listed according to SI UK Statutory Intrument (1989). Before application of biosolids, the soil has to be tested to ensure compatibility. For example, biosolids must not be applied if the soil has a pH of 5 or lower, as acidic soil could increase the uptake of unwanted elements by plants. Should the soil already contain elevated levels of potentially toxic elements (PTE), biosolids can only be applied if that would not cause the PTE levels to exceed set limits. There are also regulations on when farmers can spread biosolids onto fields. For example, spreading must not occur on growing fruit and vegetable crops, however there are no restrictions for application on cereal fields. Even untreated biosolids can be applied to fields with the condition that they are worked into the soil and applied at least 10 months before planting of crops, if they are fruits or vegetables with direct contact to the soil. In the case of cereals, sugar beet or fruit trees, there are no time restrictions on application.

In addition to these laws, there are good practices which are followed by biosolid producers and landowners which further guide how biosolids are applied. For example, farmers should only apply 250 kg of total nitrogen per hectare of field per year, which limits the amount of biosolids that can be applied to the land (Defra, 2009). The nitrogen content in biosolids is around 1.5% to 7.5%, with the value largely dependent on the type of treatment (Rigby et al., 2016). Air-dried, lime-treated biosolids having the lowest N content and liquid mesophilic anaerobic digestion resulting in sludge with the highest N content. This means that, depending on the treatment, varying amounts of biosolids can be applied to agricultural land. Assuming that the amount of AgNPs in the influent of the wastewater treatment plant is constant, that would result in different amounts of silver reaching the soil.

There is evidence to suggest that applied nanoparticles do not easily migrate into the soil. Baccaro et al. (2019) posit that silver migration downwards into the soil is mostly due to earthworm activity. In this study, rain did not contribute to the distribution of the silver; however, it increased the silver uptake by earthworms *Lumbricus rubellus*. This lab-based experiment was performed using uncoated Ag_2S nanoparticles rather than pristine AgNPs. While this may be a more realistic depiction of nanoparticles entering the soil, it is possible that the results would differ with pure AgNPs, different coatings, silver ions or even larger particles.

2.6 Silver sulphide

Some studies have shown that in biosolids, AgNPs are most likely to be present in the form of silver sulphide (Ag_2S) (Burkhardt et al., 2010). The toxicity of these nanoparticles is less well studied than the toxicity of pristine AgNPs. Currently, major nanoparticle distributors do not sell Ag_2S particles, so in order to do research, scientists have to synthesis particles.

AgNPs react readily with sulphur to form Ag_2S, a process which can be recreated in the laboratory by adding Na_2S to create a 5 mM sulphide solution. It is necessary that the sulphur is in sulphide (S^{2-}) form rather than sulphate (SO_4^{2-}) or sulphite (SO_3^{2-}), as the latter do not react readily with AgNPs (Liu et al., 2011). Sulphide is mostly present under anaerobic conditions, which is another factor that increases the reaction to Ag_2S particles is the presence of organic matter (Liu et al., 2011). When picturing the reaction of sulphide with AgNPs, one would expect an Ag_2S layer to form on the surface. However, Liu et al. (2011) found that the sulfidation is not dependent on the particle size, meaning that the silver reacts completely forming an Ag_2S particle. This also changes the surface composition of the particle, leading to larger particles with a porous surface. While they do not have as powerful an effect as pristine AgNPs, silver sulphide nanoparticles do have an inhibitory effect against *Escherichia coli* (Sadovnikov et al., 2016).

2.7 AgNPs as a pesticide

Another route whereby AgNPs are introduced into agricultural systems is through the application of plant protection products (pesticides). Due to the antibacterial properties of silver, AgNP-containing formulations have been proposed as an alternative to chemical pesticides. *In-vitro* studies have found AgNP to be an effective antifungal agent (Kim et al., 2009b, Min et al., 2009). Jo et al. (2009) treated

rye grass with silver preparations as a preventative against fungi (*Bipolaris sorokiniana* and *Magnaporthe grisea*) and found AgNPs to be more effective than $AgNO_3$. $AgNO_3$ is used to produce a solution of free Ag^+ ions in water, so the study showed that nanoparticles have a stronger antifungal effect than free ions. This might be due to the nanoparticles ability to continuously release Ag^+ ions over an extended period when compared to $AgNO_3$, which is equivalent to a one-time ion application. Another study used an aerosol containing AgNPs to prevent mildew in cucumbers and pumpkins, and found it nearly as effective as chemical fungicides (Lamsa et al., 2011). Jung et al. (2010) found that onion mould (*Sclerotium cepivorum*) can be prevented by treating the soil in which the plants grow with AgNPs. In both studies there was no recorded reduction in plant growth at effective AgNP concentrations.

AgNPs have been proposed to be a feasible alternative to chemical pesticides, as they protect against fungi without harming the plant. However, *in-vitro* experiments have shown that high concentrations of AgNPs reduce germination of seeds and reduce the growth of new plants (El-Temsah and Joner, 2012, Nair and Chung, 2015). The concentrations used in these experiments (10 mg L^{-1} and 50 mg L^{-1}) are higher than would be expected in the soil. However, the fate of AgNP-containing pesticides in soil has yet to be studied and it is unknown whether they would accumulate in an active form. If that was the case, continued used of the products could have negative effects on plants and/or the soil.

The European Union (EU) regulates pesticides and biocides to protect both humans and the environment. Regulations on nanomaterials in biocides are thorough and any approved substance is only covered in its specified nanoparticular form. If nanoparticles are present in any product, the risks for humans, animals, plants and the environment overall must be assessed separately. In the regulations, nanomaterials are defined as any substance in which over 50% occur in particles, aggregates or agglomerates less than 100 nm in at least one dimension (Regulation (EU) No 528/2012 2012). However, the regulation covering pesticides (here called plant protection products) (Regulation (EC) No 1107/2009, 2009) does not include specific regulations concerning nanomaterials. Active substances must be reviewed and considered safe for use to be used in any pesticide. Due to the lack of regulation on pesticide labelling, the nanoparticle form of an active substance does not have to be declared as such. Therefore, if a substance is cleared to be used, the nanoparticle version of that substance can be used unless it is specified that nanoscale particles are present, in which case the nanomaterials have to be reviewed separately for safety. This grey area of legislation has made silver nanoparticle-containing products freely available on the market.

Silver has been a registered pesticide in the USA since 1954, mostly as a water purifying agent and to a lesser extent as an algicide (EPA, 1993). Currently, 22 silver-containing chemicals are registered with

the EPA of these, 8 are currently used in federally registered products. In 2017, the EPA prevented an AgNP-containing pesticide agent called Nanosilva from entering the US market (Erickson, 2017). This was not because of evidence of it being harmful to humans or the environment but rather the producers could not demonstrate improvements over existing agents. It is entirely possible that such pesticides will be approved in the future and so far, the burden on soil and humans has not been satisfyingly investigated.

2.7.1 Colloidal silver as a plant protection product

A growing online community promotes the use of colloidal silver in home gardening. Colloidal silver is also a dispersion of AgNPs and therefore can be used in a similar way. In one study, two commercially available colloidal silver suspension were tested for their ability to inhibit the growth of fungus found on fruits and vegetables (Venat et al., 2018). While both suspensions claimed to have the same concentration of silver in pure water, they inhibited fungal growth to different degrees. The researchers did not test if this discrepancy was due to the manufacturer being incorrect about the silver concentration in their products. Another possibility could be that the colloidal suspensions had different sized silver particles in them, which was not investigated either. Despite the differences, both colloidal silver suspensions had antimicrobial properties at low silver concentrations. One suspension inhibited the growth of all tested fungi from 41% to 100% at a 15 mg L^{-1} concentration.

2.8 Soil and silver

AgNPs applied to the soil have the potential to react or interact with the soil. Many types of soil exist and exactly how nanoparticles react depends on a multitude of physical and chemical factors, few of which have been studied.

Soil chemistry is complex, and a myriad of tests can be performed to gain information on soil properties. Simple tests, like pH determination, can give important information. For example, it is known that certain toxic metals such as Zn, Cu and Ni are more toxic in acidic conditions which is why the pH of a soil has to be measured before the application of biosolids (SI UK Statutory Intrument, 1989). This increase in toxicity is linked to bioavailability, meaning that the toxic component is more readily taken up by the biological system and thus can cause harm. For example, inert Ag_2S particles might be ingested by earthworms, but due to the silver bonding to sulphide it is not biologically available and therefore will not cause harm.

2.8.1 AgNPs and soil

The interaction between AgNPs and soil is complex and not well understood. Different aspects of the soil such as soil sand/silt/clay composition, organic matter content and pH affects the availability of AgNPs (Cornelis et al., 2012). Soil with a high in organic matter content is more likely to bind AgNPs making them less available and potentially less toxic (Coutris et al., 2012). Another important factor is the amount of clay present in the soil. Soils with a high clay content retain AgNPs and silver ions more effectively than soils with low clay content, lowering the toxic potential of AgNPs (Cornelis et al., 2012). Due to the complexity of the subject, the exact relationship is still largely unknown, so the availability and toxicity of AgNPs cannot yet be predicted even if extensive data on the soil is collected.

In addition to the complex interactions between soil and AgNPs, what is added to the soil can affect the mobility of silver. Navarro et al. (2014) found that thiosulphate remobilised ionic silver, AgNPs with different coatings, plus Ag_2S particles with up to 100% efficiency. In the agricultural environment, sodium thiosulphate is often used as a soil fertiliser and is therefore able to reach the soil and potentially remobilise silver (PubChem, 2019). Sodium thiosulphate is also used in the de-chlorination of water during the treatment process, which would present another way that more available silver could reach the agricultural environment.

2.9 An Introduction to earthworms

Earthworms form a major component of the soil fauna in many temperate and tropical ecosystems and their functions have been extensively studied. Charles Darwin was notably one of the first to realise this and wrote a book on their importance in soil processes (Darwin, 1881). Earthworms can usefully be divided into three ecological groupings, based on size, morphology, and their burrowing and feeding behaviours. These main ecological groupings (epigeic, anecic, and endogeic) have distinct roles in the soil ecosystem, impacting soil functions (Bouché, 1977, Bottinelli et al., 2020). While these are only the main categories, some earthworms fall into sub-categories or exhibit a mixture of traits making them more difficult to categorise.

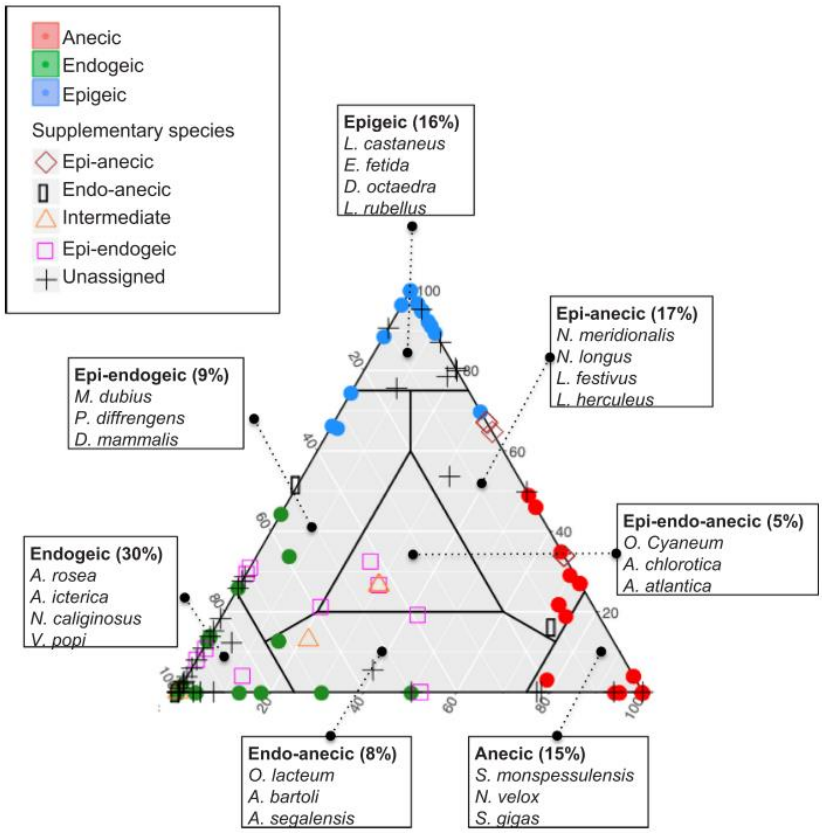

Figure 2.5: Revised Earthworm classification triangle with the three main categories and including 7 subcategory zones, adapted from Bottinelli et al. (2020)

For simplicity, only the main categories of epigeic, anecic, and endogeic will be discussed, as they are widely recognised and used throughout the world (Bottinelli et al., 2020).

Epigeic earthworms, sometimes known as litter dwelling species, primarily live above the mineral soil within dead plant material. Their activities contribute to the decomposition of organic material and in combination with microorganisms, subsequently leads to humus formation. Examples of epigeic earthworms include *Eisenia fetida* (tiger worm) and *Lumbricus rubellus* (red worm). These are often employed in vermicomposting activities (Sherman, 2018) which are of great interest to industry. *Anecic* earthworms, such as *Lumbricus terrestris* (dew worm) and *Aporrectodea longa* (black-headed worm), live in deep, semi-permanent burrows which may extent to a metre or more into the soil, but primarily feed on plant material gathered at the soil surface (Edwards and Bohlen, 1996). These earthworms increase the organic content of the soil by collection of dead plant material into their burrows to feed upon. Through this action and through surface casting, new soil is formed, nutrients are incorporated and made available to other fauna, flora and microbes (Blouin et al., 2013). Examples of *Endogeic* earthworms include *Aporrectodea caliginosa* (grey worm) and *Allolobophora chlorotica* (green worm), both of which live and feed below, but relatively close to the soil surface. Their

burrowing behaviour is more horizontal when compared to anecic species. Endogeic earthworms feed on organic material already partially incorporated into the soil, breaking it down further and homogenising the soil in the process (Bottinelli et al., 2020).

Burrowing activity of both anecic and endogeic earthworms aerates the soil, and the burrows also help with water drainage. The walls of their burrows becomes coated with the mucus that earthworms produce, which can increase microbial activity as well as mineralisation due to the nutrient rich composition (Brown et al., 2000). These physical and chemical properties have an influence on plant growth in the soil, whether in an agricultural, natural, or garden setting. For example, the burrows of earthworms can also serve as a pathway for plant root expansion (Brown et al., 2000).

Earthworms also play an important part in nutrient cycling in general and the nitrogen cycle in particular. Through their feeding behaviour, earthworms increase the decomposition of organic matter and thus make more nitrogen available (Blouin et al., 2013). Especially tougher plant parts are more readily broken down by earthworms and other invertebrates and this recycled organic material can then be transported into deeper parts of the soil where it serves as a source of nutrients for plants and other parts of the soil ecosystem (Blouin et al., 2013).

While there are many positive attributes that earthworms bring to the soil, they also have some detrimental effects. They cause an increased emission of greenhouse gasses, such as N_2O and to a lesser extent CO_2 through their feeding and subsequent metabolism (Lubbers et al., 2013). In their review of 57 published studies, Lubbers et al. (2013) pointed out that while the release of CO_2 decreased in studies longer than 200 days, no studies of that length currently exist for N_2O release, so there is still much that remains unknown about earthworms contribution to the release of greenhouse gasses. While earthworms increase the emission of N_2O from soil, the addition of Ag_2S can decrease that level of emission by having a negative effect on earthworm gut bacteria responsible for nitrification (Wu et al., 2020).

As earthworms are such an integral part of the soil ecosystem, they have been used in research for decades, in numerous areas of ecology including ecotoxicology.

2.10 *Aporrectodea caliginosa*

A. caliginosa (Savigny, 1826) of the oligochaeta: Lumbricidae is a common endogeic species in the UK but can be found in a variety of other countries. Commonly referred to as the grey worm, the colour

of *A. caliginosa* ranges from an unpigmented pink to a pale greyish, with a distinctive three-coloured anterior of medium, light, and dark segments. The clitellum is saddle shaped and extends over at least 6 segments ((XXVII) XXXI-XXXIV (XXXV)) (Sims and Gerard, 1999). They are often found in gardens and other cultivated land with alkaline soil, where they live relatively close to the surface, mostly only burrowing around 7 cm deep.

This three-coloured anterior part (Figure 2.6) is a rapid way to identify adults of this species, from field-collected animals. Nevertheless, segment counting is required for a certain identification (Sims and Gerard, 1999, Sherlock, 2018). By comparison with other earthworm species, *A. caliginosa* can grow to different adult sizes ranging from body masses of 200 mg to over 1200 mg, a function of different phenotypes, as discussed by Sims and Gerard (1999).

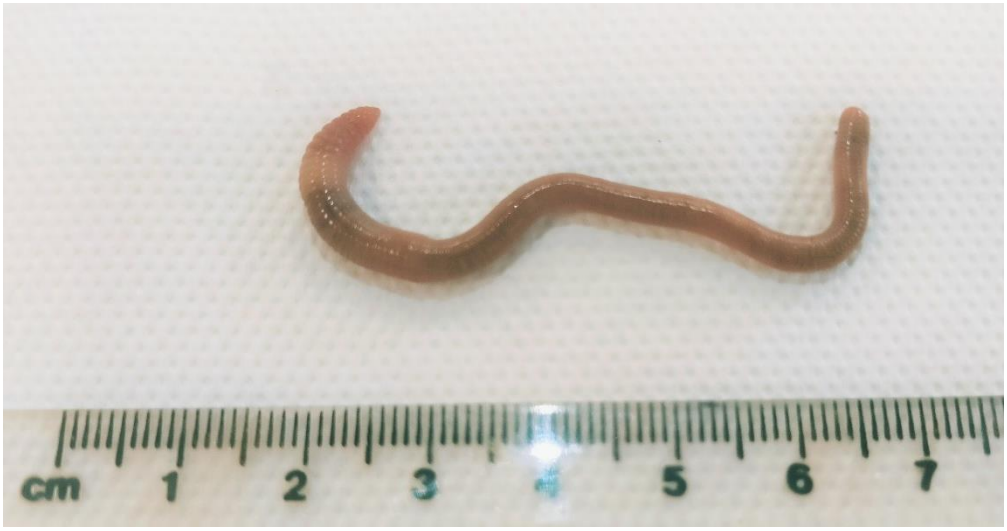

Figure 2.6: Adult A. caliginosa (author's image)

Due to the variety in phenotype, *A. caliginosa* can be divided into 4 sub-species, also called morphs. Those are known as *Aporrectodea caliginosa* s.s., *Aporrectodea tuberculata*, *Aporrectodea nocturna*, and *Aporrectodea trapezoides*. Sims and Gerard (1999) argue that these morphs are mainly phenotypic variations and the differences between them are a result of their size, regional occurrence, or potential pathological conditions. This is why these morphs are not necessarily always differentiated between, especially in the UK. However, genomic testing has shown that there is in fact a genetic difference between these subtypes (Pérez-Losada et al., 2009), and even in the UK, *A. nocturna* is now recognised as a separate species (Sherlock, 2018).

2.10.1 Lifecycle

As with any earthworm, the lifecycle of *A. caliginosa* consists of hatchlings, emerging from cocoons, growing in size and eventually reaching maturity with the ability to produce cocoons. From hatching to reproductive maturity takes around 4-6 months under ideal laboratory conditions (Bart et al., 2018). Maturity is recognised when the earthworm has a distinct clitellum (saddle). Earthworms are hermaphrodites, so have both male and female reproductive organs. *A. caliginosa* is amphimictic, so requires mating with another earthworm of the same species to produce cocoons. Different hatching times have been reported for cocoons produced by *A. caliginosa* both at 15 °C, from 56-63 days in a water-filled Petri dish to 70-84 in soil (Bart et al., 2018).

Growth, reproduction and general survival depend on environmental conditions, whether it is in the field or in the laboratory. As with most earthworms, *A. caliginosa* needs to be housed in sufficiently moist soils, although prescribing a percentage soil moisture content is difficult due to differences in the composition of different soils. What is easier to accurately suggest is the temperature at which *A. caliginosa* should be kept. This is 10-15°C, with 15°C being widely recognised as the preferrable temperature to conduct experiments with soil dwelling earthworms (Butt and Lowe, 2011).

2.11 Effects of AgNPs on earthworm as an indicator for soil health

Earthworms are a widely used organism in soil toxicity studies as they have a widespread distribution, are relatively easy to keep, recommended in standardised tests (OECD, 1984, 2016) and as non-sentient invertebrates do not require ethical clearance. While toxicity experiments often focused on acute end-points (e.g., establishing LC_{50} values), there are chronic and more sensitive tests employing growth rate, fecundity, cocoon viability and avoidance as end-points (Lowe and Butt, 2007). LC_{50} (lethal concentration at which kills 50% of a given population) is used rather than LD_{50}, as the earthworms are not fed nor injected with the substance in question but are exposed to it.

Most earthworm-based toxicity studies are performed on epigeic (surface/litter dwelling) earthworms, as recommended by the International Organisation for Standardisation (ISO) and the Organisation for Economic Co-operation and Development (OECD) (OECD, 1984, OECD, 2016). However, these species do not usually inhabit the mineral soil, therefore their ecological relevance is limited and the use of soil dwelling endogeic species has been advocated (Brami et al., 2017b, Bart et al., 2018).

Multiple studies have been performed on the effect of AgNPs on earthworms, utilising predominantly epigeic species (Heckmann et al., 2011, Shoults-Wilson et al., 2011c, Choi and Park, 2015). Most of these studies assessed short term toxicity, which requires relatively high concentrations of AgNPs to produce an effect. This could lead to the erroneous conclusion that lower concentrations are not harmful to earthworms and/or soil health. Chronic toxicity studies have the potential to show whether lower concentrations would affect the earthworm community, related ecosystem services and therefore the overall condition of the soil.

One of the most sensitive tests which can be performed on earthworms relate to avoidance, as earthworms have been known to avoid contaminated soil. For example, Langdon et al. (2005) found that epigeic and endogeic earthworms avoid Pb-containing soil, even if the Pb concentration was not directly toxic to them. In the field, this would mean that earthworms would avoid certain areas, so the soil no longer receives the ecosystem service benefits associated with the earthworm's presence. *E. fetida* (epigeic) has been shown to avoid soil contaminated with silver in all forms starting at 10 mg kg^{-1} dry soil (Shoults-Wilson et al., 2011c). How earthworms detect the presence of metals is not clear. In a 14-day exposure experiment, avoidance behaviour has also been established in the endogeic species *A. chlorotica* (Brami et al., 2017a).

Makama et al. (2015) studied the uptake of silver and AgNPs in the epigeic *Lumbricus rubellus* and found that exposure to AgNPs led to an accumulation of silver in the earthworm, of which 34% was particulate. Earthworms exposed to $AgNO_3$ still contained particulate silver (<5%) implying that ionic silver either precipitates in the soil or in the earthworm, forming nanoparticles. *L. rubellus* were also used in another study which showed that earthworms take up silver both orally as well as through their skin (Diez-Ortiz et al., 2015).

E. fetida exposed to 100 mg kg^{-1} of both AgNPs and $AgNO_3$ showed a significant, as well as a similar increase in DNA damage as measured with a comet assay (Choi and Park, 2015). A comet assay measures the amount of DNA strand breaks in a nucleus, estimating the genotoxic effects of a substance. Exposure to 100 mg kg^{-1} of AgNPs or $AgNO_3$ increased DNA damage by 300-400% compared to the control group. In the same experiment, $AgNO_3$ was shown to significantly increase the genetic expression of metallothionein, catalase, superoxide dismutase and heat shock protein 70, while AgNPs only increased glutathione-S-transferase. All of these gene expressions are associated with oxidative stress, and an increase in these expressions infers a rise in oxidative stress to the test subject. This suggests, that $AgNO_3$ is more toxic to *E. fetida* than AgNPs, which was also been shown by Shoults-Wilson et al. (2011b). That $AgNO_3$ showed a greater toxicity than AgNPs in earthworm experiments is consistent with the findings of experiments where forms of silver were used as a fungicide (Jo et al.,

2009). Using $AgNO_3$ causes a high, short-term increase in free Ag^+ ions while AgNPs releases them for an extended period. Therefore, $AgNO_3$ might be more likely to cause short-term toxicity, while AgNPs can be used as a long-term antifungal agent.

Shoults-Wilson et al. (2011b) found that the type of soil has an important effect on toxicity. AgNPs inhibited the growth of *E. fetida* in natural sandy soil at 7.41 mg kg^{-1} while the same concentration did not affect earthworms in artificial soil, prepared according to guidelines for an earthworm reproductive test (OECD, 2016). Furthermore, AgNPs caused a decreased hatching rate in sandy loam while the number of cocoons produced remained the same. In artificial soil, however, AgNPs caused a decrease in cocoon production without a change in hatching rate. These differences show the difficulty of comparing results between different studies, even if the same species of earthworm is used. Every researcher has access to different soils and even the preparation of artificial soil has been shown to vary between laboratories (Moser et al., 2009).

Soil is not the only matrix that affects the toxicity of AgNPs. For example, *L. terrestris* exposed to AgNPs had a higher rate of mortality if the AgNPs were dispersed in 0.2% Tween in comparison to a dispersion in water (Lapied et al., 2010). In both cases, the experiment lasted for 24 h with earthworms kept in vials containing the experimental dispersion. However, exposing earthworms to a Tween solution without AgNPs did not cause an increase in cell death.

Two Masters' theses concerning AgNPs and *A. caliginosa* have been produced at Lincoln University in New Zealand. The first by Zhan (2012), performed multiple acute toxicity tests as well as genotoxicity experiments. Acute toxicity tests were conducted in soil and in Petri dishes with citrate-coated AgNPs and $AgNO_3$ as a control, both with concentrations ranging up to 2 mg kg^{-1} soil. Neither of the two forms of silver caused mortality. This might partly be due to the short duration of the experiment, which only lasted for 48 h, while the OECD guideline recommends 14 days. Once the earthworms were exposed to an aqueous solution for 48 h, toxicity was established. For AgNPs, the LC_{50} was 483 mg kg^{-1} and in the case of $AgNO_3$, LC_{50} was 1.64 mg kg^{-1}.

In terms of genotoxicity, Zhan (2012) found that AgNP, in addition to $AgNO_3$ in the concentrations 415 ng kg^{-1}, cause the occurrence of micronuclei in cells. However, no significant increase in DNA strand breakages was found using the comet assay. Micronuclei assay and comet assay do not measure the same thing; micronuclei occur if, during cell division, parts of a chromosome are not included into the new nucleus and comet assay measure the amount of DNA strand breaks. Despite the difference in the methods, one would expect to see some genotoxicity in the comet assay if such high results were found in the micronuclei assay.

In the second Master's thesis, Bate (2015) attempted to measure the effect of AgNPs on the expression of different genes in *A. caliginosa* utilising primers for gene expression in *E. fetida*. However, the primers did not work for *A. caliginosa* and other experimental results were inconclusive.

Biomarkers can be used to establish low level toxicity (e.g., oxidative stress) and metabolism mechanisms. Oxidative stress is measured by an increase in biomarkers which are meant to combat it. In the case of exposure to metals, expression of metallothioneins (MTs) and heat shock proteins (HSPs), both of which play a role in the organisms response to heavy metal exposure and stress in general, is widely measured (Homa et al., 2005). MTs and HSPs are expressed within 3 days of exposure to heavy metals even if the exposed earthworms do not yet show an increase in metals in their tissues (Homa et al., 2005).

Exposure to silver in both particulate and ionic form caused an increase in MT production in a study performed on *E. fetida* (Patricia et al., 2017). After one day, MT production increased but the sharpest rise was after 3 days. While expression was dose-dependent, $AgNO_3$ caused a larger increase compared to AgNPs. After 14 days, expression decreased and at that time the earthworms exposed to AgNPs had a higher MT expression than those exposed to $AgNO_3$ (Patricia et al., 2017). This may give credence to the theory that AgNPs have a longer-term toxicity while ionic silver is a more immediate threat. In another study on *E. fetida* exposed to Zn, Cd and Pb for 3 days, MT expression was found to be independent of dose (Homa et al., 2005). This suggests that either different metals have a different effect on biomarker expression, or expression is independent of dose for a short period of time after which it becomes dose-dependent.

2.11.1 Colloidal silver and earthworms

Lapied et al. (2010) exposed *Lumbricus terrestris* to both AgNPs and colloidal silver. Colloidal silver was found to be ten times more toxic than AgNPs, but reasons for this were not investigated. One of the possibilities was the size of the particles, as the AgNPs had a mean size of 20.2 nm while colloidal silver had an average size of 8.8 nm. However, 45% of the colloidal silver was present in particles with a size of less than 2 nm and this extensive size discrepancy might be related to the difference in toxicity. Additionally, if particle size is so unpredictable there is also a chance that a part of the silver is present in an ionic form.

2.12 Earthworm life stage experiments

Standardised earthworm experiments such as the recommended OECD tests regarding acute toxicity and reproduction test are all performed on adult earthworms (OECD, 1984, 2016). Many researchers adapt these recommendations and tailor their experiments to better answer their research question. Still, these experiments are performed on adult, sexually mature earthworms. Hatchlings are rarely mentioned in scientific studies and if they are, they appear as a by-product of reproduction experiments, for example if the hatching rate of cocoons was determined (Shoults-Wilson et al., 2011b).

A limited amount of studies have been performed where hatchlings are the focus of an experiment. Whalen (2002) studied the uptake of labelled food by hatchlings, juveniles and adult earthworms of the species *Aporrectodea tuberculata*. Growth of one week old *Perionyx excavatus* hatchlings was compared to the growth in *Perionyx sansibaricus* by Suthar (2009). Sauvé and Fournier (2005) studied the immune response as well as lethal effects of methylmercury chloride on hatchling, juvenile and adult *E. fetida*. It was found that while hatchlings had a lower phagocytosis response, they did not show a higher sensitivity to methylmercury chloride.

2.13 Biosolids and earthworms

Multiple studies have looked at the effect of biosolids on earthworms. In general, biosolids can be beneficial to earthworms, as they contain a large amount of organic matter which can serve as a food source for earthworms. Pallant and Hilster (1996) incubated *L. terrestris* in very acidic mine soil with and without addition of biosolids. In the untreated soil, earthworms lost weight over the course of 10 weeks while they gained weight in the soil with biosolid addition. However, no organic matter was added to the mining soil, so it is unclear whether the conditions of the mining soil caused the weight loss, or the earthworms simply starved. In either scenario, the biosolid addition supplied an excess of food and resulted in superior living conditions for the earthworms. Activated biosolids have also been shown to be a good source of nutrition for *E. fetida* (Hartenstein and Neuhauser, 1985).

Treated biosolids are low in bacteria and high in organic matter which can be utilised by earthworms as food. However, there are concerns such as the presence of toxic metals; as their presence could lead to the accumulation of heavy metals in earthworms (Beyer et al., 1982, Brewer and Barrett, 1995). As per the regulation concerning sludge, biosolids have to be tested for certain metals prior to land

application (SI UK Statutory Intrument, 1989). This should prevent excessive amounts of toxic metals being applied to the soil and therefore limits the negative effect on earthworms.

High amounts of biosolids in the soil can be lethal to earthworms (Kinney et al., 2012), however only concentrations over 3% were found to have an effect which is higher than would be found in the field. Biosolids can be applied onto the field or, in liquid form, injected into the soil. A large application would be 2 kg m^{-2}. Presuming the biosolids diffuse only in the upper 10 cm, it would yield a concentration of about 2% dry biosolids per wet soil, so a 3-5% concentration is very unlikely to be achieved.

A proposed cause of the toxicity of biosolids to earthworms is the increase of electrical conductivity. In a study on *A. caliginosas* in soil with or without the addition of biosolids, the earthworms gained significantly less weight in biosolid-amended soil (McDaniel et al., 2013). The soil used had a low organic matter content so the addition of biosolids would have provided an increase in food for the earthworms. McDaniel et al. (2013) found that the main difference between the two soils was the electrical conductivity which increased significantly after biosolids were incorporated into the soil.

Electrical conductivity (EC) is largely caused by salts, so a soil with high electrical conductivity can be assumed to be rather salty. EC is measured in Deci Siemens per metre (dS m^{-1}) and 3.35 dS m^{-1} were found to have negative effects on cocoon production while 7.35 dS m^{-1} caused a decrease of viability in *A. caliginosa* (Jun et al., 2012). In the same study it was found that a low organic matter content increased the negative effects of high EC soil on *Aporrectodea trapezoides*, therefore, it can be suggested that biosolids can be harmful to earthworms if their addition causes a major increase in soil salinity.

Overall, it can be said that AgNPs are one of the most extensively studied type of nanoparticles, but there are major gaps in the literature when it comes to their impact on the environment. Only a few studies acknowledge that AgNPs to a large extent transform to Ag_2S before entering the environment through biosolid application (Kim et al., 2010, Doolette et al., 2013). And while there are numerous studies using earthworms to investigate the impact of AgNPs on soil, the species of earthworm used is not necessarily always the most suitable (Shoults-Wilson et al., 2011c, Li et al., 2015). Further research is certainly warranted and forms the context of the remainder of this thesis.

3 General Methods

This Chapter describes details of the major techniques used within this thesis. These are crucial background details that were necessary to execute experiments, such as information on how earthworms were obtained or kept and specifics about containers used. These methods are based both on findings from the scientific literature and expertise of others who have previously worked in the same (Earthworm Research Group) laboratory.

3.1 Earthworms

3.1.1 Collection

Aporrectodea caliginosa was the only earthworm species used in experimental work. It is an endogeic species, commonly found in the UK (Sims and Gerard, 1999). This research programme focussed on the effects of pollutants on agricultural soils. Therefore, epigeic earthworms are less likely to be present due to the lack of a sufficient amount of decaying surface organic matter. The current choice of earthworm species conflicts with recommendations by the OECD (1984), but it is argued, that using an endogeic species is more realistic. Using the same earthworm species throughout all experiments ensured that results from experiments were comparable. *A. caliginosa* were collected from pasture at Bottoms Farm in Preston (53° 42’ 26’’ N; 2° 40’ 34’’ W). Collections were done manually through digging and hand sorting of the soil. While this may not be the most efficient method of collecting earthworms in soil (Butt and Grigoropoulou, 2010), it was chosen as it caused least damage towards *A. caliginosa*. Vermifuge extraction, e.g., using mustard powder in water, can yield a higher quantity of earthworms, but mustard powder acts as a skin irritant, causing mainly anecic earthworms to exit their burrows. As only *A. caliginosa* were required to be collected and needed to be in the best condition, a vermifuge was not employed.

Field collected *A. caliginosa* were employed in some experiments, and in others, earthworms produced from cocoons, hatched and grown to maturity in the laboratory. These laboratory-reared earthworms were descended from *A. caliginosa* also collected from Bottoms Farm. By sourcing all earthworms from the same area, a similar morphology was ensured, or through use of earthworms descended from this group, a homogenous culture was largely maintained.

3.1.2 Husbandry

Earthworm husbandry of epigeic species is simple and has been used in vermicomposting for decades (Sherman, 2018). However, maintenance and breeding of endogeic species is less straight forward and requires some amount of equipment and techniques, guidelines for which can be learned (e.g. Fründ et al., 2010, Butt and Lowe, 2011).

After collection, earthworms were placed into Kettering Loam (sterilised soil, bought from Boughton Loam Company) moistened to around 25-30%. Sufficient dried horse manure was added to act food, in a ratio of approx. 10:1 (Lowe and Butt, 2005). Earthworms were then left to acclimatise to laboratory conditions for at least two weeks (Fründ et al., 2010). Horse manure was obtained locally from stables in Lancashire, after confirmation that the horses producing said manure had not been recently treated with anti-worming medication, such as ivermectin. Residues of such medication could potentially affect the health and behaviour of earthworms fed with the manure (Madsen et al., 1990). On collection, the manure was dried at 105 ^{o}C for storage and rewetted as required. As *A. caliginosa* is an endogeic earthworm species, the horse manure was distributed throughout the soil in which the earthworms were housed, rather than placed on the soil surface, as would be the practice for anecic earthworms.

The pots used to house the earthworms were 750 mL food storage tubs (Lakeland Plastics), with close fitting, removable lids, pierced to prevent oxygen starvation. Earthworms prefer a moist soil environment, and the living conditions provided for that, however the aim was not a specific soil moisture percentage, but rather a sufficient water content which is achieved based on experience in preparation of soil in the laboratory. All earthworms were kept in temperature-controlled incubators (LMS, Kent) to ensure a consistent temperature. The incubators were maintained at 15°C, with sufficient air circulation to prevent oxygen starvation, and in complete darkness (Butt and Lowe, 2011). Unless used for experimentation, *A. caliginosa* were checked every 2-3 months, the soil was moistened, and they were re-fed as deemed necessary. Most earthworms used for experimentation were adults (fully mature, as shown by a swollen clitellum with no visible ailments) unless otherwise specified. After any experiments, *A. caliginosa* used were either killed for analysis (as per experiment) or released into garden soil. No earthworm was ever used in more than one experiment.

3.2 Soil preparation for experimental use

While there is a standard soil preparation method for earthworm experiments (OECD, 2016), it was not used throughout these experiments, instead Kettering Loam was utilised as a base soil. The Kettering Loam was bought commercially in 25 L bags (Boughton Loam Company). It was used because it was pre-sterilised, so contained no soil macro-organisms, and was consistent in nature. This soil has been used in the Earthworm Research Group Laboratory and was therefore known to be a consistently good substrate in which to cultivate endogeic earthworms. Additional food was added in the form of horse manure. In addition, Brami et al. (2017a) showed in a series of experiments, that Kettering Loam is preferred by both *Allolobophora chlorotica* and *Octolasion cyaneum* (both endogeic) while a standard soil can cause a significant decrease in mass compared to Kettering Loam.

The soil used in all earthworm experiments throughout this thesis was made up of Kettering Loam, with an addition of 5% (dry w/w) Sand and varying (small) percentages of horse manure (HM). Horse manure was obtained as mentioned in Section 3.1.2. Prior to addition, the horse manure was dried, milled and passed through a 2 mm sieve. The sand (Hanson kiln dried sand (lime-free washed silica, average grain size 0.5 mm)), acted as a vehicle to add small amounts of suspected toxicant to the soil. Silver nanoparticles (AgNPs) or silver sulphide (Ag_2S) were added to sand in varying concentrations with a later goal to have specific end concentrations in the final dry soil. For example, preparation of 16 g sand with 4 g AgNPs would result in 1000 mg kg^{-1} AgNPs in the final soil. In any control treatments, plain sand was used to ensure any reaction was due to the additive, not the sand. In the case of a biosolid experiments, no sand was used as the concentrations of biosolids in the soil were sufficiently high not to need a vehicle.

Control soil was moistened using tap water, with the amount of water being objective and determined through previous experience in the laboratory. The final amount of water needed to achieve sufficient moisture was recorded. After the first soil concentration was prepared, the same amount of water was then used to prepare all the other treatment soils, ensuring that all prepared soils had the same amount of water added and there were no major differences in soil moisture within an experiment. Soil samples from all prepared treatment concentration were dried to assess moisture concentration to further rule out inconsistencies in moisture concentration, as these could potentially influence the outcomes of any experiment.

3.3 Experimental Containers

3.3.1 Regular experimental pots

Small disposable pots were used in the experiments, measuring 11.5 cm in diameter and able to hold ca. 250 mL (Figure 3.1), with mounted needle holes pierced into the lid to allow for air circulation. These pots were bought in bulk (cater4you Ltd.). This size of pot was chosen to be suitable to house two adult *A. caliginosa* for extended periods of time, as 750 mL Lakeland pots are commonly used in the Earthworm Research Group laboratory to house 5-6 adults (Lowe and Butt, 2005). Single use pots were employed to prevent any cross contamination from potentially toxic AgNPs or Ag_2S to other experiments performed in the Earthworm Research Group laboratory. For consistency, the same containers were used throughout all experiments unless special containers were needed (e. g. in avoidance experiments).

Figure 3.1: Plastic pot used in the majority of experiments (author's image)

3.3.2 Circular avoidance chambers

Earthworm avoidance experiments can provide important information about sub-acute toxicity not detected in acute avoidance experiments. The recommendation from ISO (2008) is to perform either a two chamber design or a circular 6 chamber design, using epigeic *Eisenia fetida* or *Eisenia andrei*, over the course of 48 h. In the circular 6 chamber design, there is a space in the centre void of any soil, and earthworms can move into any of the 6 compartments containing soil. While they are permanently divided from each other, the dividers have sufficient holes to allow for earthworm

movement. Despite the 6 available compartments, the ISO (2008) design is only intended to analyse avoidance behaviour between 2 concentrations of soil which alternate in the experimental soil compartments.

A different design of circular avoidance chamber had been produced for previous experiments in the Earthworm Research Group laboratory (Brami, 2020), with the intention to negate possible edge effects which could potentially occur in the use of a linear avoidance chamber (Section 3.3.3). The novel design had 5 compartments, and its dividers were completely removable to increase possible earthworm movement. The chamber consisted of a circular HDPE container (ø 30 cm, 12 cm high) with dividers splitting it into 5 segments, one for each treatment concentration. The layout of the chamber with its compartments is depicted in Figure 3.2. The concentrations were allocated to segments to avoid the highest concentration (Concentration 4) from being adjacent to the control. This circular avoidance chamber allowed for multiple concentrations to be tested at the same time and gave free movement to the earthworms between these.

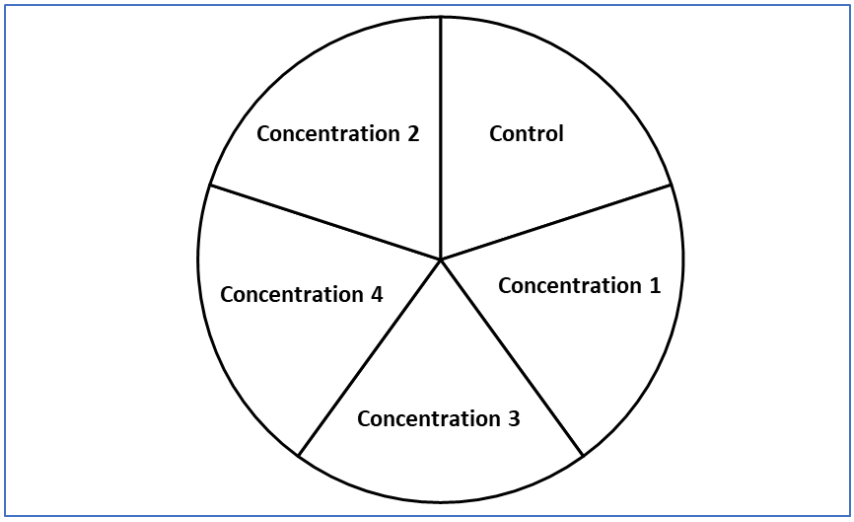

Figure 3.2: Treatment layout in a circular avoidance chamber

Once the chamber was set up, one earthworm was placed at the centre on the surface of each treatment concentration (Figure 3.3). The dividers were removed after the earthworms burrowed down into the soil. A lid (not shown) was then placed onto the chamber, it did not provide an airtight seal, so chambers did not become oxygen deficient during an experiment.

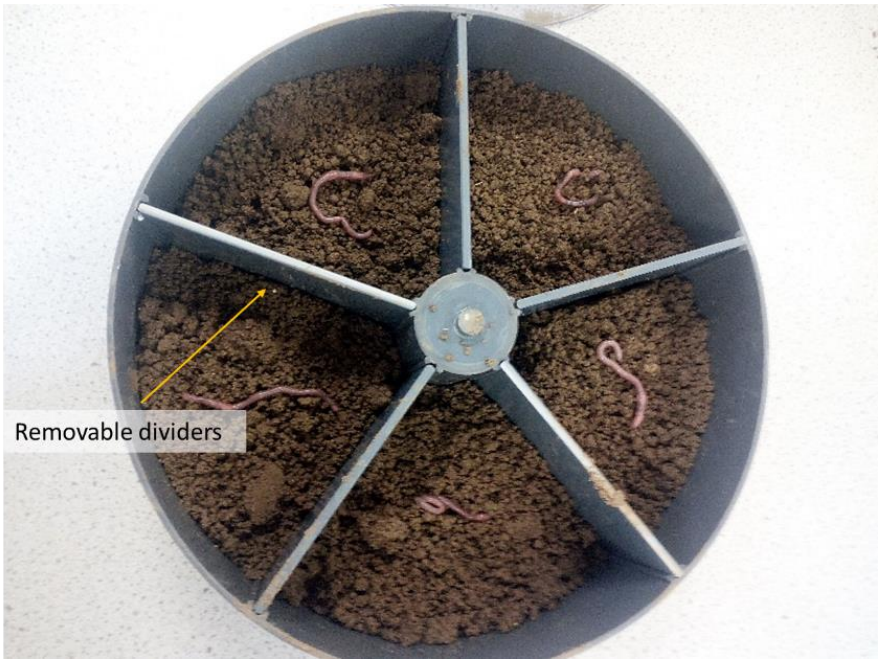

Figure 3.3: A. caliginosa in a circular avoidance chamber before burrowing into the soil (author's image)

3.3.3 Linear avoidance chambers

ISO (2008) does not have a recommendation for a linear avoidance design, only those that are circular or have only two-chambers. The two-chamber design of avoidance experiment is the preferable test if the goal is to establish an LC_{50} factor for avoidance, but a linear gradient approach is a rapid way to test a wide range of concentrations at the same time.

The linear avoidance chamber consisted of a plastic trough planter (0.6 m × 0.13 m × 0.1 m, able to contain 7 L) for which dividers had previously been cut. Dividers had an exact fit to the cross-sectional area to stop any soil from moving between created sections. Dividers were placed to create 5 equally sized compartments, to which 5 soil treatments were added (Figure 3.4). The chambers were set up as a gradient, with the control soil at one end and the highest concentration at the other end. At the start of an experiment, after all earthworms had burrowed into the soil, the dividers were removed, and the chambers were sealed tight with cling film and tape. Air holes were pierced into the clingfilm to allow for air circulation. To assess the outcome of the experiment, the dividers were re-inserted after 14 days, and the location of the earthworms determined within the soil treatments. This type of investigation has previously been used in the ERG laboratory (Lowe et al., 2016, Brami et al., 2017b).

Figure 3.4: A. caliginosa in linear avoidance chamber (author's image)

3.4 Inductively Coupled Plasma-Mass Spectrometry analysis

In some instances, as determined necessary, samples (earthworms and soil) were digested and analysed using an ICP-MS. First, samples of known mass were placed into digestion vessels with 1 mL MQ water and 9 mL 80% nitric acid and digested using a microwave digester (Ethos EZ, Microwave digestion system, Milestone) at 120 °C for 15 min. After digestion, the samples were diluted 100 times with ultra - water. Digested samples were analysed for their silver content using an ICP-MS (X-series II, Thermo Fisher using helium gas). The instrument was calibrated using an elemental standard (Fluka analytical) and calibration curves had a correlation of R=0.999.

3.5 Carbon, Hydrogen, Nitrogen, and Sulphur analysis

Samples (soil, dried lettuce, and dried radish) were analysed for their carbon, hydrogen, nitrogen and sulphur content ratio using a ThermoScientific CHNS Organic Elemental Analyser. Prior to analysis, all samples were dried, ground using an electric coffee grinder (KYG, 300W) and sieved through a 1 mm sieve. Using a microbalance (Mettler Toledo XP6), approximately 2-3 mg of sample and 1 mg of vanadium pentoxide standard were transferred into an aluminium tin foil capsule and folded into a rough dice shape, ready to be analysed. The instrument was calibrated using a 2,5-Bis(5-tert-butyl-2-benzo-oxazol-2-yl)-thiophene (BBOT) standard.

3.6 Scanning electron microscope

Some samples were analysed using a ThermoScientific Quattro S SEM-EDX (Scanning Electron Microscopy (SEM) with Energy Dispersive X-Ray Analysis (EDX)). SEM was used to determine particle size and EDX was used to determine elemental composition. Dried samples were milled and sieved through a 1 mm sieve before they were placed on a 12 mm carbon filament tab which was fixed on an aluminium stub which was loaded into the instrument.

3.7 Preparation of Biosolids

Anaerobically digested biosolids were collected from the United Utilities Blackburn treatment plant and dried in the oven at 60 °C overnight. The solids had a moisture content of 68.2% and an organic content of 53.56%. The silver content was found to be 14.9 mg kg^{-1} Ag in dry biosolids, quantified after digestion using ICP-MS.

3.8 Statistical Analysis

All statistical analysis was done with the SPSS program by IBM (Versions 28.0.0 and earlier). Normal distribution of data was analysed using a Shapiro-Wilk test and further statistical tests were chosen based on the result. When p-values were less than 0.05, results were considered statistically significant.

4 Assessing the effects of silver nanoparticles on earthworms

4.1 Introduction

Silver nanoparticles (AgNPs) are widely studied as they are used in different fields and have effects on the environment. While many studies have been performed on their effects on earthworms, the results have varied widely (Lapied et al., 2010, Choi and Park, 2015, Brami et al., 2017b). This may be due to different laboratory conditions or techniques and the wide variety of earthworm species used. To gain a more comprehensive overview of the toxicity of AgNPs, a series of experiments was performed. These used the same species of earthworm in the same laboratory, to provide comparable toxicity data across multiple experiments. After assessing the toxic impact of AgNPs on *A. caliginosa*, further toxicity experiments using other additives were performed, and the toxic impact can be directly compared.

Experiments described in this Chapter investigated the toxicity of AgNPs on *A. caliginosa*, a representative of the soil community (Aim 1). Objectives to achieve this aim included investigation of the acute toxicity of AgNPs towards both adult *A. caliginosa* and hatchlings using both pristine and aged AgNPs. Thereafter, more sensitive tests were used to study sub-lethal effects such as avoidance and a longer-term reproductive study. These experiments built upon each other, with earlier results influencing the experiments that followed. Each experiment will be discussed separately.

4.2 Experiment to assess toxicity of AgNPs towards adult and hatchling *A. caliginosa*

4.2.1 Introduction

Initially, preliminary experiments were performed using AgNPs previously used in UCLan's Earthworm Research Group laboratory. Experiments were originally designed to be closely related to research undertaken by Brami et al. (2017b), with the main difference being a change in earthworm species from *A. chlorotica* to *A. caliginosa*. This was because *A. caliginosa* was seen as more representative of the agricultural environment, as it is a more commonly found species (Sims and Gerard, 1999).

4.2.2 Materials and Methods

Toxicity experiments were modelled after Brami et al. (2017b), to achieve comparability in results. Kettering Loam containing 0, 1, 10, 100, 250, 500 and 1000 mg AgNPs kg^{-1} was prepared as described in Section 3.2, with the addition of 50 g kg^{-1} horse manure (HM) and filled into single use pots. The highest concentration was chosen at 1000 mg kg^{-1} as recommended by the OECD (1984). Samples of each treatment were analysed for silver content using an ICP-MS (Section 3.4). Adult *A. caliginosa* which were field collected and were acclimated to laboratory conditions for 2 weeks as described by Fründ et al. (2010).

Before the experiment started, each *A. caliginosa* had its mass determined and was examined to ensure good condition. Per treatment concentration, there were 5 replicate pots with 2 adults each. Once in the experimental soil, pots were placed into the incubator at 15°C, in complete darkness (Butt and Lowe, 2011). After 7 days, earthworms were checked for survival and had their mass determined before they were placed back into the soil and returned to the incubator. The toxicity experiment lasted for 14 days and ended with a final mass determination and check for any mortality, as recommended by OECD (1984). A similar set up was used for hatchlings, except that there were 2 replicates with 5 hatchlings in each pot. Hatchlings had been sourced from other experiments using *A. caliginosa* in the same laboratory.

As the mass datapoints of adult *A. caliginosa* were not normally distributed, potential changes were analysed using a Kruskall-Wallis test with pairwise comparison. The hatchling datapoints were normally distributed, so a one-way ANOVA and a repeated measures ANOVA with a Tukey HSD post-hoc were performed.

4.2.3 Results

The mean mass of adult *A. caliginosa* exposed to different concentrations of aged AgNPs for 2 weeks is shown in Figure 4.1. No mortality was recorded, even in the highest concentrations of aged AgNPs.

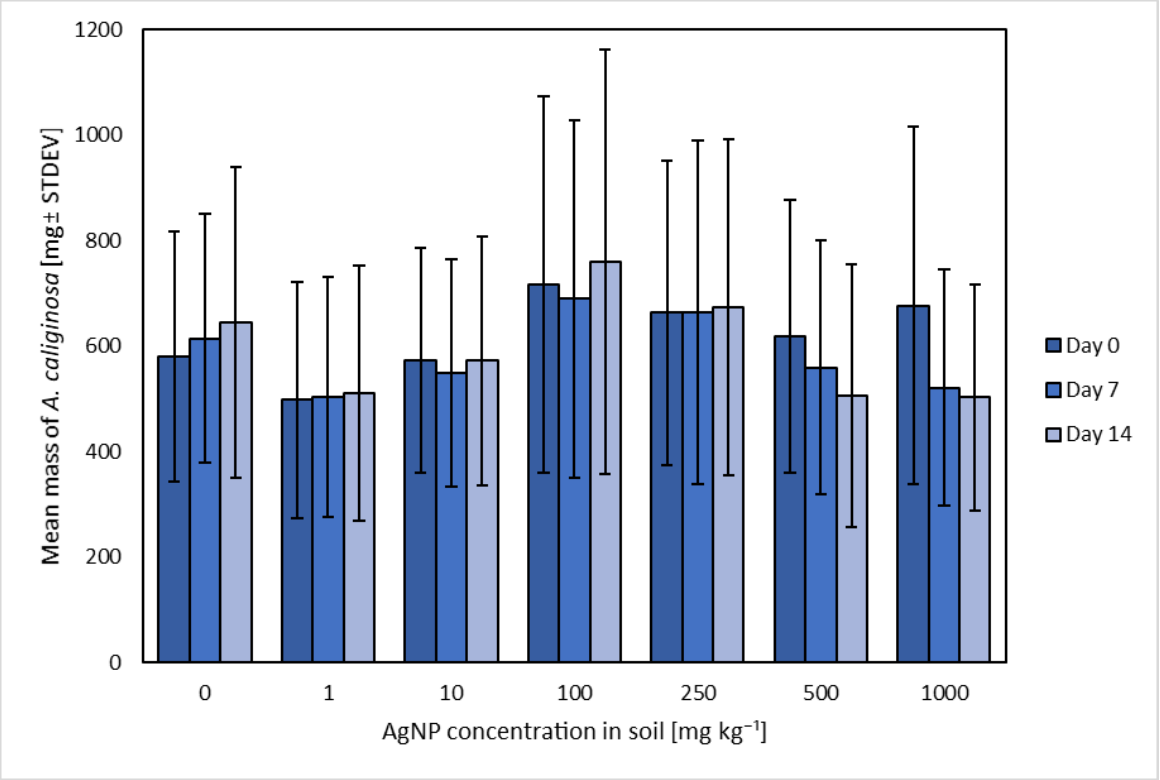

Figure 4.1: Mean mass (± standard deviation) of adult A. caliginosa exposed to aged AgNPs for 14 days

No mortality of *A. caliginosa* was recorded and decrease in mean mass at higher concentrations was not significant. Despite no significant results, a clear trend can be seen where earthworms were exposed to higher concentrations of AgNPs, especially 500 and 1000 mg kg^{-1}, which caused a decrease in mass. To ensure that the lack of observed significant toxicity was not due to an error in soil preparation, the silver content of all soils was analysed and was found to contain silver in the expected concentration or higher (details in Appendix III).

As in the experiment with adults, 100% hatchling survival was recorded in all treatments, but individuals lost mass in soil with higher AgNP concentrations (Figure 4.2).

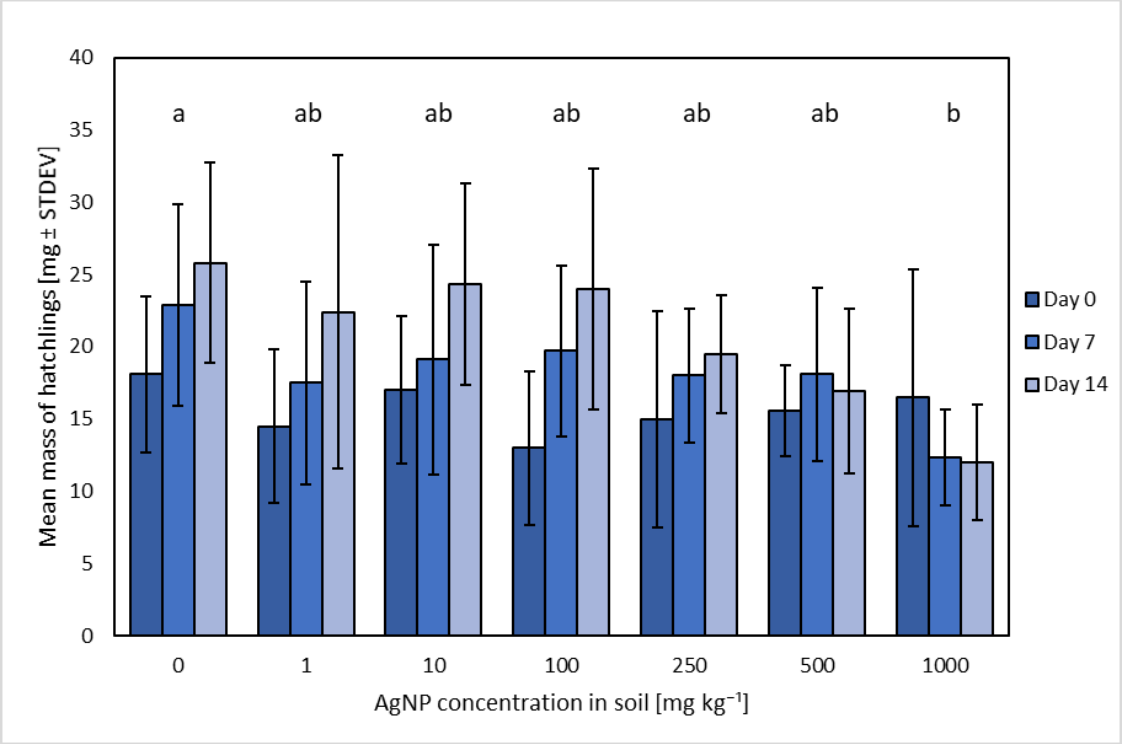

Figure 4.2: Mean mass (± standard deviation) of hatchling A. caliginosa exposed to aged AgNPs for 14 days; different letters denote significant differences (repeated measure ANOVA, p<0.05)

There was a significant difference in mean mass on Day 14 between the control and 1000 mg kg^{-1} (p=0.002, $F_{(6, 64)}$=3.956) when analysed with a one-way ANOVA using a post-hoc Tukey analysis. Using the repeated measure ANOVA with a Tukey post-hoc test, a significant difference between control and 1000 mg kg^{-1} (p=0.027) can be found.

4.2.4 Discussion

Even 1000 mg kg^{-1} AgNPs caused no mortality in *A. caliginosa*, despite the high mortality rate reported in other papers towards *L. terrestris* (Lapied et al., 2010) and *A. chlorotica (Brami et al., 2017b)* for the same concentration range. But considering the AgNP particles used, it is less surprising and even confirms previously formed hypotheses. It is hypothesised that silver has antimicrobial properties due to the release of Ag^+ ions and the surface area to volume ratio of AgNPs increases the ion release. Subsequently, the release of Ag^+ ions is seen as a major cause of AgNP toxicity (Shoults-Wilson et al., 2011b, McShan et al., 2014, Li et al., 2015). The AgNPs used in this experiment were old (3+ years) and were not stored in a protective environment (such as in nitrogen gas) meaning their surface will have oxidised over time. While bonded with oxygen, the surface of the particles would not have been able

to release as many Ag^+ ions as pristine AgNPs. The lack of negative effects on *A. caliginosa* seems to confirm the hypothesis that the release of ions is the main source of AgNP toxicity.

The decrease in hatchling mass differed from the change of mass observed in adults. Under favourable conditions, hatchlings will grow and therefore gain mass, while mass of fully grown adults would remain relatively constant. In these experiments, an excess of organic matter (food) was added to the experimental soil to ensure that no negative effects occurred due to lack of nutrition. Therefore, it is likely that adult earthworms would have gained mass, or their body mass remains constant over the course of the experiment unless the toxic substance negatively affected them. In contrast, hatchlings were expected to grow and gain mass rapidly and a lack of mass gain is a negative effect. So, for the mean mass of hatchlings at a given concentration to remain constant or decrease, was a severe negative toxic effect.

4.3 Acute toxicity of pristine AgNPs towards adult and hatchling *A. caliginosa*

4.3.1 Introduction

After findings that aged AgNPs had reduced toxicity towards *A. caliginosa*, the effects of pristine AgNPs were investigated. Acute toxicity was a great starting point to determine concentrations for later experiments, as more sensitive experiments could then be set up using concentration found to be non-lethal.

4.3.2 Materials and Methods

Using freshly purchased AgNPs (80 nm, uncoated, purchased from GetNanoMaterials), an LC_{50} experiment was repeated with adult and hatchling *A. caliginosa*. While adult earthworms were exposed to the same concentration range as in the previous experiment (0, 1, 10, 100, 250, 500 and 1000 mg AgNPs kg^{-1}), lower concentrations were chosen for hatchlings, namely 0, 10, 50, 100, and 250 mg kg^{-1} AgNP. This was done with an expectation (based on the previous experiment, Section 4.2) that they would be more sensitive compared to their adult counterparts. Due to mortality reducing the number of available mass datapoints on day 14, statistical analysis was difficult. Ultimately, it was decided to use mean mass per container which was then analysed using a Kruskal-Wallis test with pairwise comparison. Hatchling were analysed as single datapoints using the Kruskal-Wallis test.

In addition to monitoring changes in mass and mortality, silver uptake was measured. For this, 4 adult earthworms from each concentration (with the exception of the highest concentration where mortality restricted analysis to 3), were placed into Petri dishes with water and a #1 Whatman filter paper for 24 h to void their gut contents (depuration), had their mass determined, and were killed by freezing (-20 °C). They were then placed into a freeze dryer (Scanvac) for 24 h (-120 °C) and dry mass was assessed. The samples were analysed for their silver content using an ICP-MS (Section 3.4).

4.3.3 Results

Exposure to fresh AgNPs caused mortality in adult earthworms at a rate relating to the silver concentration to which they were exposed. After 2 weeks, 20% mortality was recorded in the 500 mg kg^{-1} AgNP concentration, 60% in 750 mg kg^{-1} and 70% in the highest concentration of 1000 mg kg^{-1}.

In Table 4.1, the mean initial mass of adult *A. caliginosa* exposed to AgNPs can be found. It is noticeable that earthworms of lower mass were more susceptible to the toxic effects of AgNPs compared to larger earthworms. This was known due to the difference in mass of the earthworms on day 0. As only two earthworms were in each pot, a sequential monitoring of their mass allowed for a confident estimate of which of the two earthworms survived and which did not. Table 4.1 is split into columns to show earthworms that survived the experiment and those that did not.

Table 4.1: Mean initial mass (± standard deviation) of A. caliginosa exposed to different AgNP concentrations

AgNP concentration [mg kg^{-1}]	Initial mass of earthworms that survived ±STDEV	Initial mass of earthworms that died ±STDEV
0	710.6 ± 314.2	N/A
1	698.3 ± 325.3	
5	584.5 ± 357.4	
10	683.8 ± 293.9	
50	634.2 ± 199.0	
100	782.2 ± 325.7	
250	713.2 ± 275.0	
500	890.5 ± 258.2	272.0 ± 86
750	826.5 ± 84.6	626.2 ± 218.1
1000	1076.7 ± 61.1	677.3 ± 332.4

Overall, the earthworms lost body mass over the course of the experiment, even if they survived for two weeks. Figure 4.3 shows the cumulative change in mass of adult earthworms exposed to fresh AgNPs, excluding the mass of individuals which had died.

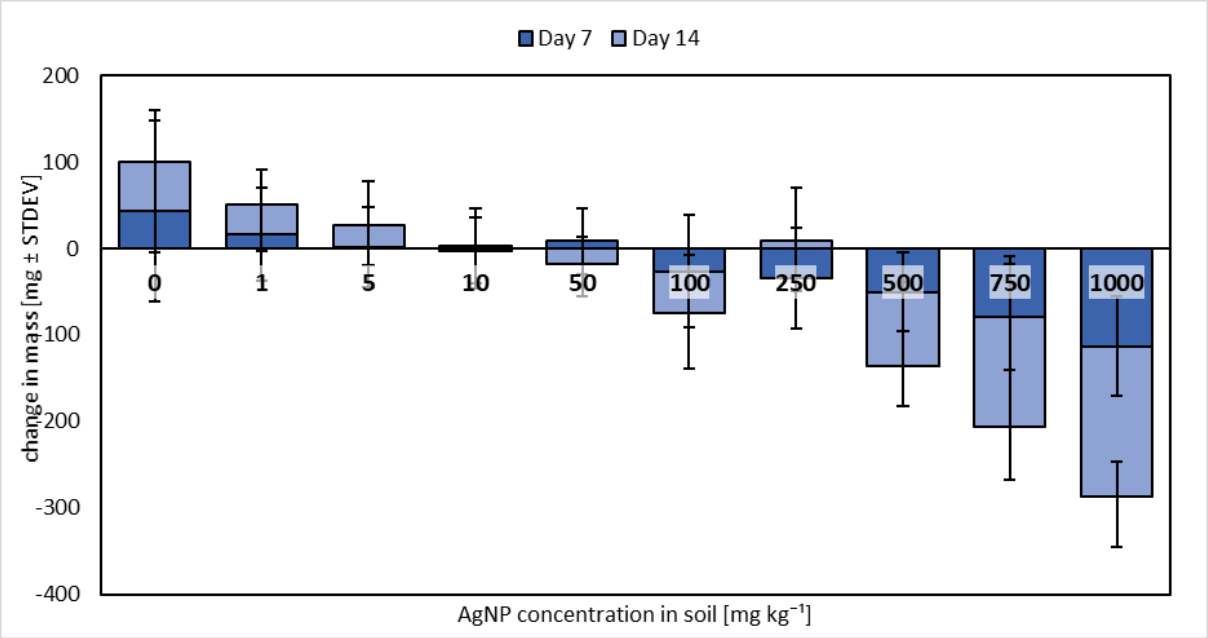

Figure 4.3: Change in mass (± standard deviation) of adult A. caliginosa exposed to fresh AgNPs for 14 days

Figure 4.3 is expressed in change in mass rather than mean mass. Due to smaller earthworms dying rather than larger ones, the mean mass in the higher concentrations increased despite all worms losing mass. This is why this type of figure is depicted.

The mean mass per pot changed significantly between day 0 and 14 (Kruskal-Wallis; H(9)=18.647; p=0.028). Pots in the 1000 mg kg^{-1} concentration had a significantly lower mean mass than those in all other concentrations, and pots with 750 mg kg^{-1} AgNP soil were significantly different to control, 1, 100, and 250 mg kg^{-1} (p=0.004, 0.017, 0.039, 0.030)

The amount of silver taken up by *A. caliginosa* during the experiment is shown in Table 4.2:

Table 4.2: Silver content in adult A. caliginosa after exposure to pristine AgNPs for 14 days [mg kg^{-1} dry mass]

		AgNP in soil [mg kg^{-1}]						
		10	50	100	250	500	750	1000
Replicates	1	3.56	195.75	27.61	74.24	227.36	191.40	242.52
	2		35.56	19.54	48.64	131.84	263.07	102.81
	3	1.11	25.49	5.31	73.94	168.66	339.60	125.75
Mean		2.34	85.60	17.49	65.61	175.95	264.69	157.03
STDEV		1.23	77.99	9.22	11.99	39.34	60.52	61.17

Earthworms exposed to soils containing 0, 1, and 5 mg kg^{-1} of AgNPs, plus one of the earthworms exposed to 10 mg kg^{-1}, had silver values below the limit of quantification of the instrument and were therefore excluded from Table 4.1. As shown, the amount of silver found in earthworms ranged widely with a silver content more than 100 mg kg^{-1} higher in some replicates than others exposed to the same amount of AgNPs in soil.

For hatchling *A. caliginosa*, no significant differences in mass gain between fresh AgNPs treatments were found (Figure 4.4). No mortality recorded during the 2 weeks of the experiment. These results suggest that the chosen concentrations were insufficient to cause toxicity in *A. caliginosa* hatchings.

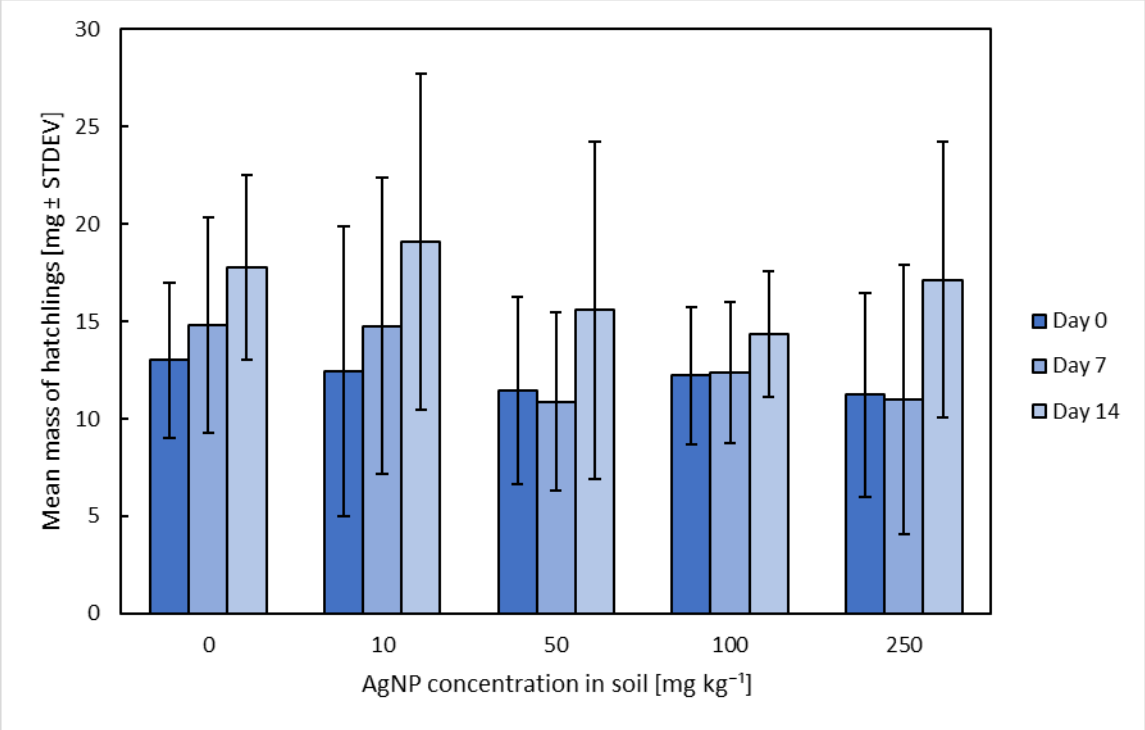

Figure 4.4: Mean mass (± standard deviation) of hatchling A. caliginosa exposed to fresh AgNPs for 14 days

4.3.4 Discussion

Pristine AgNPs were shown to be toxic to adult *A. caliginosa* at concentrations of 500 mg kg^{-1} and higher. This is in line with previous findings of AgNPs being toxic towards earthworms for example *E. fetida* and *A. caliginosa* (Shoults-Wilson et al., 2011b, Zhan, 2012), however it still differs from the results obtained by Brami et al. (2017b) despite many similarities in experimental set up including the use of endogeic earthworms. One main difference is that Brami et al. (2017b) used *A. chlorotica* while the current experiment used *A. caliginosa*. *A. chlorotica*, as a species, was found to be more vulnerable to the toxic effects of AgNPs, possibly as *A. chlorotica* is a much smaller species than *A. caliginosa* with

the mean mass of *A. chlorotica* at 200-300 mg against 300-1200 mg for *A. caliginosa*. This could be the source of the difference in results and is supported by the fact that in this experiment, the smaller *A. caliginosa* were more likely to die from exposure to higher concentrations of AgNPs (see Table 4.1). It is also possible that *A. caliginosa* and *A. chlorotica* vary enough to be affected differently by AgNPs. Silver content in *A. caliginosa* exposed to AgNPs varied widely between individuals. Additionally, silver content measured in *A. chlorotica* by Brami et al. (2017b) exposed to the same type and amount of AgNPs, for the same time period, was lower. Even when correcting the silver concentration from mg kg^{-1} dry mass to living mass, the concentrations were up to 10 times lower. It is not clear whether *A. caliginosa* and *A. chlorotica* take up silver in different amounts, which would explain the difference in toxic response, or if it was an analytical failure to determine the silver uptake.

Concentrations chosen in this experiment were based on the results of the previous experiment which used aged AgNPs. A concentration of 250 mg kg^{-1} pristine AgNP was expected to cause negative effects because hatchlings exposed to 500 mg kg^{-1} of aged AgNPs experienced a slight decrease in mass during the second week of the experiment. Fresh AgNPs were more toxic than aged ones, indeed, 500 mg kg^{-1} fresh AgNPs yielded a 20% mortality rate in adult *A. caliginosa*, however no negative effects towards hatchlings was recorded. The reasons for these findings are unclear but could suggest that generally hatchlings are not a reliable option for toxicity testing.

4.4 Avoidance behaviour of *A. caliginosa* towards AgNPs (circular chamber)

4.4.1 Introduction

Earthworms can be sensitive to unfavourable soil conditions. So even if a substance is not actively harmful to them, they may choose to avoid the area where it is present, if possible. To observe such sublethal effects of a substance, avoidance experiments can be performed. Here, a circular avoidance chamber design was chosen. This was to potentially negate edge effects as may be perceived in some linear gradient chambers. In some cases, earthworms may prefer to burrow in corners of a container, and a circular choice chamber may provide equal conditions to all treatment concentrations. It is not a commonly used methods to determine avoidance behaviour in earthworms; details on how it was chosen in Section 3.3.2.

4.4.2 Materials and Methods

To test avoidance behaviour of earthworms exposed to pristine AgNPs, five treatment concentrations were chosen and prepared. The concentrations were 0, 10, 50, 100, and 250 mg kg^{-1} of pristine AgNPs in Kettering Loam, as those were the 5 highest concentrations which caused no negative effects in the acute toxicity experiment (Section 4.3). For soil preparation method, see Section 3.2, with 10 g kg^{-1} horse manure added due to shorter duration of the experiment. Samples of each soil concentration were taken, and soil moisture was assessed to preclude preferential behaviour of the earthworms due to slight differences. Circular avoidance chambers were divided into 5 equal compartments using dividers and were filled with the experimental soil. The control section was not adjacent to the highest concentration. For details on the avoidance chamber and layout of treatments, see Section 3.3.2.

Five avoidance chambers (replicates) were prepared. Twenty-five adult *A. caliginosa* in good condition had their mass determined. One earthworm was placed onto the middle of the surface of each treatment section. After earthworms burrowed into the soil the dividers were taken out of the soil, a Perspex© lid was placed onto the chamber and it was placed into an incubator (15 °C, 24 h dark cycle) for 7 days. The lids do not provide an airtight seal so that chambers could not become oxygen deficient during the experiment. After 7 days, the dividers were re-inserted, and each concentration (segment) was checked for the number and mass of earthworms present. Any individuals cut in half by re-insertion of the dividers were recorded as 0.5 for each adjacent treatment. Results were analysed using a one-way ANOVA.

4.4.3 Results

There was no detectable avoidance behaviour by *A. caliginosa* to AgNPs in the circular avoidance chamber (see Figure 4.5)

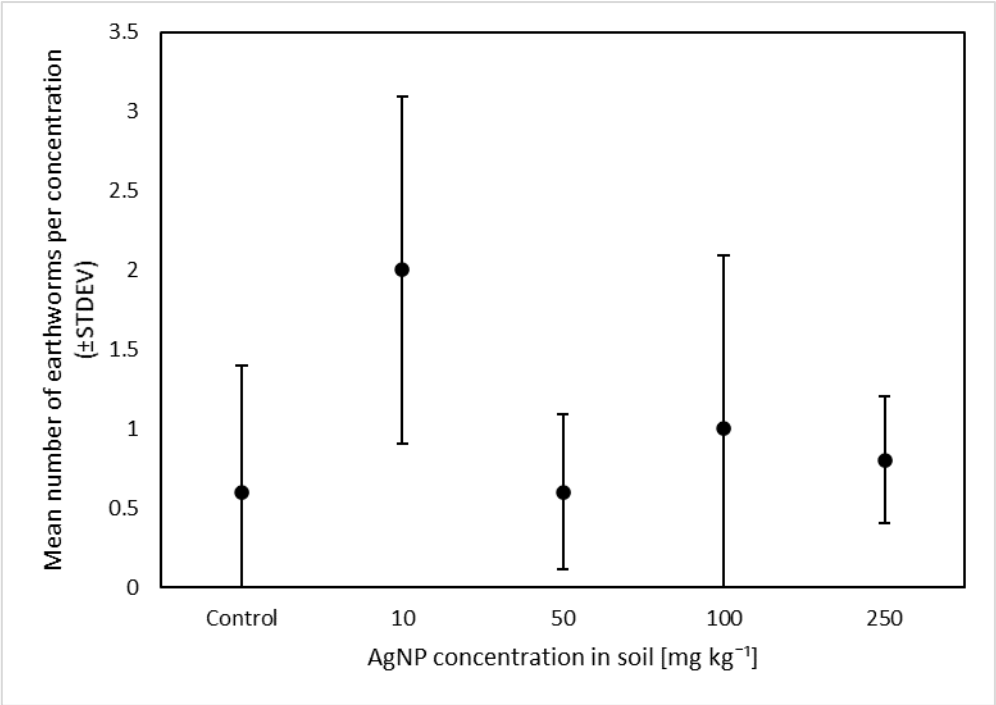

Figure 4.5: Mean number of A. caliginosa located in AgNP-containing soil after 7 days in a circular avoidance chamber

In the acute toxicity experiment (Section 4.3), *A. caliginosa* with a lower mass were more susceptible to the toxicity of AgNPs, but this was not the case in the circular avoidance experiment (Table 4.3).

Table 4.3: Mean mass of A. caliginosa found in different concentrations of the circular AgNP avoidance experiment

Concentration	Control	10 mg kg^{-1}	50 mg kg^{-1}	100 mg kg^{-1}	250 mg kg^{-1}
Mean mass [mg]± STDEV	487.8 ± 232.1	285.6 ± 33.2	660.0 ± 161.5	664.8 ± 218.9	497.5 ± 225.5

4.4.4 Discussion

Avoidance experiments are considered to be more sensitive compared to acute toxicity experiments (Lowe and Butt, 2007). The lack of avoidance in the AgNP experiment was unexpected. A concentration of 250 mg kg^{-1} soil was half the amount of the first concentration that caused mortality in adult *A. caliginosa* (Section 4.3). This result may be associated with the length of the experiment, as the avoidance experiment only lasted for 7 days while the earthworms were exposed to the spiked soil for 14 days in the acute toxicity experiment. *A. chlorotica* were found to avoid soil concentrations as low as 12.5 mg kg^{-1} after 14 days in a linear avoidance experiment (Brami et al., 2017b). Both experiments were performed in the same laboratory, using the same soil, the same type of nanoparticles from the same supplier, and the same source of organic matter. The main difference is

that they used a different avoidance chamber (linear versus circular), different experimental time periods were employed and different species of endogeic earthworm used. *A. chlorotica* died at concentrations of 125 mg kg^{-1} soil, so they were more sensitive than *A. caliginosa*, and this may be partly due to their smaller size. While the experiments were set up to be comparable, the differences are enough for it to be unclear why exactly this is, and it was thought that a linear avoidance experiment might give a better understanding.

4.5 Avoidance behaviour of *A. caliginosa* towards AgNPs (linear chamber)

4.5.1 Introduction

The lack of an avoidance response in the circular avoidance experiment when compared to the results of the acute toxicity experiment and the linear avoidance experiment by Brami et al. (2017b), suggested a systematic problem with the setup and a linear avoidance experiment was conducted.

4.5.2 Materials and Methods

As in the previous experiment, 5 kg of 0, 10, 50, 100, and 250 mg kg^{-1} AgNP containing soils were prepared as described in 3.2 with the addition of 10 g kg^{-1} HM. After placing the dividers into the linear avoidance chamber, there were 5 equally sized compartments to which the 5 soil mixtures were added. The chambers were set up as a gradient, with the control soil on one side and the highest concentration on the other side. For more information about the chambers see Section 3.3.3.

From laboratory stock, 25 adult *A. caliginosa* in good condition were taken and after determining their mass, one was placed onto each treatment section in each replicate container. After all earthworms had burrowed into the soil, the dividers were removed, and the chambers were sealed tight with plastic wrap and packaging tape. Air holes were pierced into the plastic wrap with a mounted needle to allow for air circulation.

The chambers were placed into an incubator at 15 °C in complete darkness for 14 days (Butt and Lowe, 2011). At the end of the experiment, each chamber was sampled separately. After removing the plastic wrap, the dividers were placed back into the soil and the soil in each section was carefully removed. Each earthworm located had its mass determined. Earthworms cut into two parts by the dividers were counted as 0.5 earthworms found in each section. This linear avoidance chamber was previously used by Brami et al. (2017b), although details were changed to adapt to the results of previous experiments.

Results were analysed using a multinomal goodness-of-fit test and as post-hoc analysis the highest count group was compared with every other concentration in a binomal analysis. To counteract errors due to multiple testing, the p-values were adjusted using the Bonferroni correction.

4.5.3 Results

A. caliginosa preferred soil with no or lower concentrations of AgNPs as shown in Figure 4.6. Statistical tests confirmed this (p=0.010), and the post-hoc test showed a significant difference in the number of earthworms found in the control soil vs 100 mg kg^{-1} (p=0.039) and 250 mg kg^{-1} AgNPs (p=0.012).

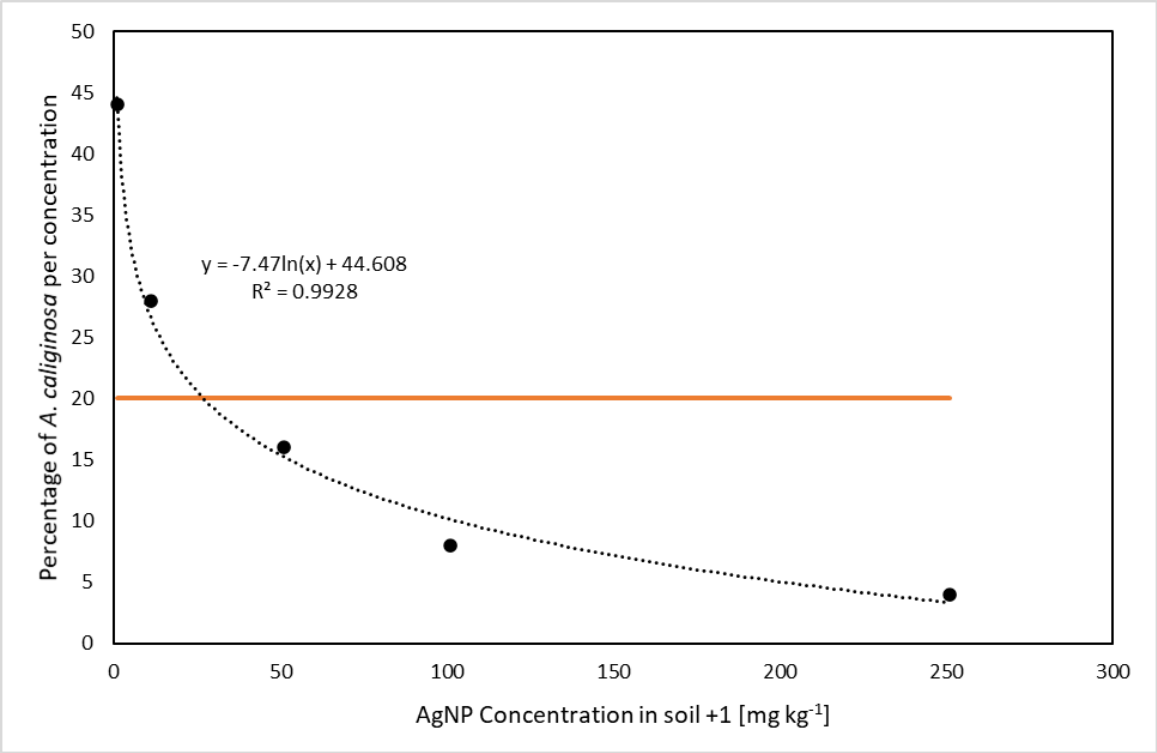

Figure 4.6: Total percentage of A. caliginosa located in different treatment concentrations after a 14-day AgNP linear avoidance experiment; orange line represents theoretical value of equal distribution between concentrations

During the AgNP acute toxicity study, a clear relationship between mass of the earthworms and experimental result was found. In Table 4.4, the mean mass of the earthworms found in the respective soil concentrations is detailed.

Table 4.4: Mean mass of A. caliginosa found in the different concentrations of the linear AgNP avoidance experiment

Concentration	Control	10 mg kg^{-1}	50 mg kg^{-1}	100 mg kg^{-1}	250 mg kg^{-1}
Mean mass [mg] ± STDEV	605.3 ± 144.4	529.7 ± 217.8	727.7 ± 203.0	669.5 ± 71.5	1138 ± 0

While the relationship is not as linear as it was in the acute toxicity experiment, the only earthworm found in the highest concentration of 250 mg kg^{-1} AgNPs, was the largest worm used in this experiment, which started the experiment in the 100 mg kg^{-1} AgNPs concentration section of the gradient.

4.5.4 Discussion

The results of this linear avoidance experiment were more consistent with previous results than those of the circular avoidance experiment. In the acute toxicity experiment, *A. caliginosa* placed in soil with high AgNP concentrations died, especially if they had a lower body mass. 250 mg kg^{-1} AgNPs in soil was the highest used concentration in the acute toxicity experiment which led to no mortality after 14 days. While the circular avoidance experiment used the same concentration range as the linear one, no preference could be found. This could be due to the randomised set up used in the circular avoidance experiments in this particular set of experiments, the shorter time span of the experiment or could be indicative of linear set ups being more sensitive in general.

4.6 Effects of AgNPs on the cocoon production and viability of cocoons

4.6.1 Introduction

A further technique to test sublethal toxic effects on earthworms is the use of a reproduction study (OECD, 2016). Reproductive toxicity (reprotox) is an important toxicological endpoint and can present in the inability to produce offspring or in developmental difficulties of offspring. In the case of earthworm experiments, a reprotoxic substance could lower the number of cocoons produced or decrease the hatching rate of said cocoons.

4.6.2 Materials and Methods

Before the reproductive experiment started, a control experiment was set up with 60 adult *A. caliginosa* which were chosen from the laboratory culture and paired according to morphological similarity. Each of the 30 pairs were placed into a container containing control soil. Control soil consisted of Kettering Loam, 5 g kg^{-1} sand and 100g kg^{-1} dried, milled and sieved horse manure. The pots containing the earthworms were placed into an incubator at 15°C in complete darkness (Butt and Lowe, 2011). These conditions were kept for 4 weeks to assess the capacity of the pairs to live in

experimental conditions without losing mass and to ensure that each pair can produce cocoons in control conditions.

After 4 weeks, 1 kg of each experimental soil containing 0, 10, 50, 100, and 250 mg kg^{-1} pristine AgNPs and 100 kg^{-1} HM was prepared (see 3.2) and 5 replicate single use pots were filled with each treatment concentration. Each earthworm pair had their mass determined and the number of cocoons produced were counted. Afterwards, the pairs were grouped into 5 groups, so that all groups were roughly similar in average mass and cocoon production. This helped ensure that the starting conditions were equal among groups and any pairs that failed to produce cocoons could be excluded before the experiment started. *A. caliginosa* pairs were then placed into their respective experimental concentrations and placed back into the incubator. Every 4 weeks for 5 sampling periods total, fresh treatment soil was prepared as previously described, all earthworms had their mass determined and were placed into their new containers. Afterwards, the soil in the old containers was sieved for cocoons. All cocoons were kept and once per week, the cocoons were observed to see how many had hatched.

During the experiment, the amount of horse manure changed from 100 to 50 g kg^{-1} due to changes in the HM. After the previous stock was exhausted, a new source of HM was found and when milled and sieved, this HM was significantly less dense and to not greatly deviate from the previous volume ratios of soil to HM, the mass added was decreased from that point onwards and kept constant for later experiments.

After establishing, that the data was normally distributed, mass and cocoon production were analysed using both a one-way ANOVA and a repeated measures ANOVA. Viability of cocoons was analysed with the Kaplan-Meier survival analysis with a Breslow post-hoc test, which takes into account both status (did/did not hatch) and time point of status occurring.

4.6.3 Results

The mass of *A. caliginosa* remained constant through the duration of the experiment across all groups, as shown in Figure 4.7 and which was confirmed with a one-way ANOVA and a repeated measures ANOVA.

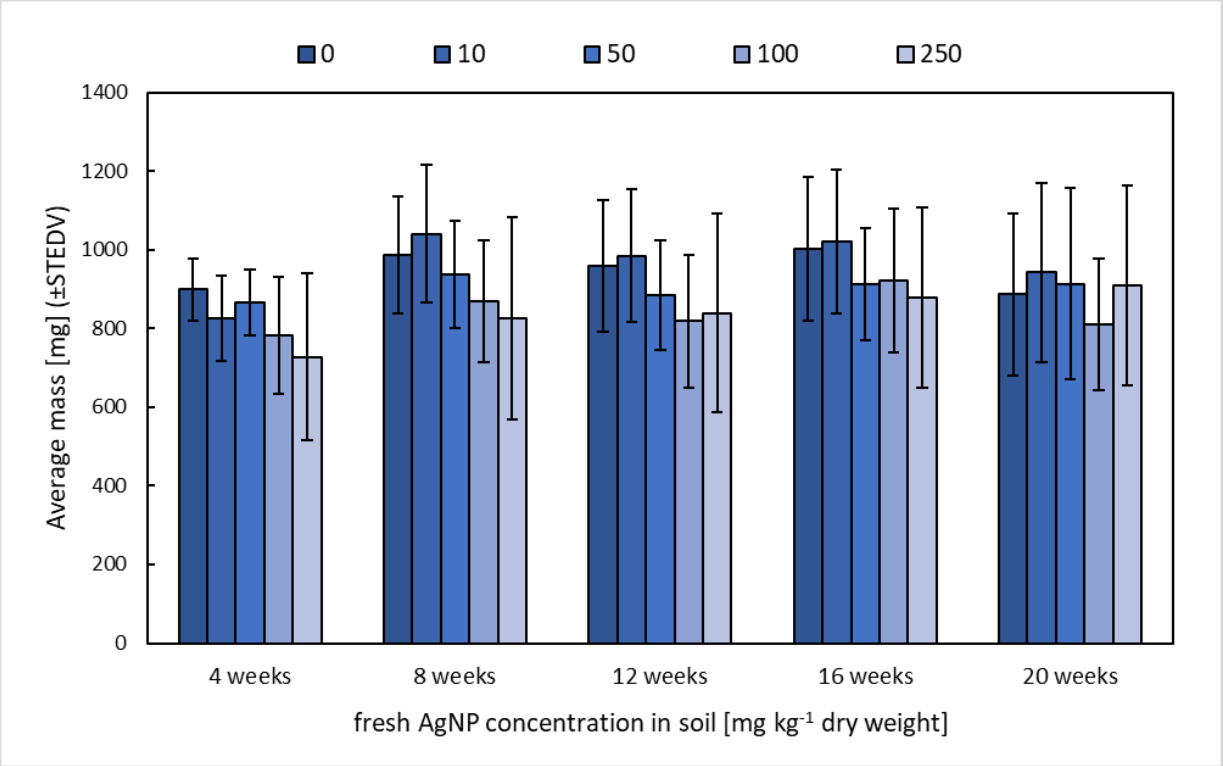

Figure 4.7: Mean mass (± standard deviation) of A. caliginosa at each sampling point in the AgNP reproduction experiment

While cocoon production varied widely between sampling points, there were a few significant differences between the groups (Figure 4.8), as tested by a one-way ANOVA and a repeated measures ANOVA.

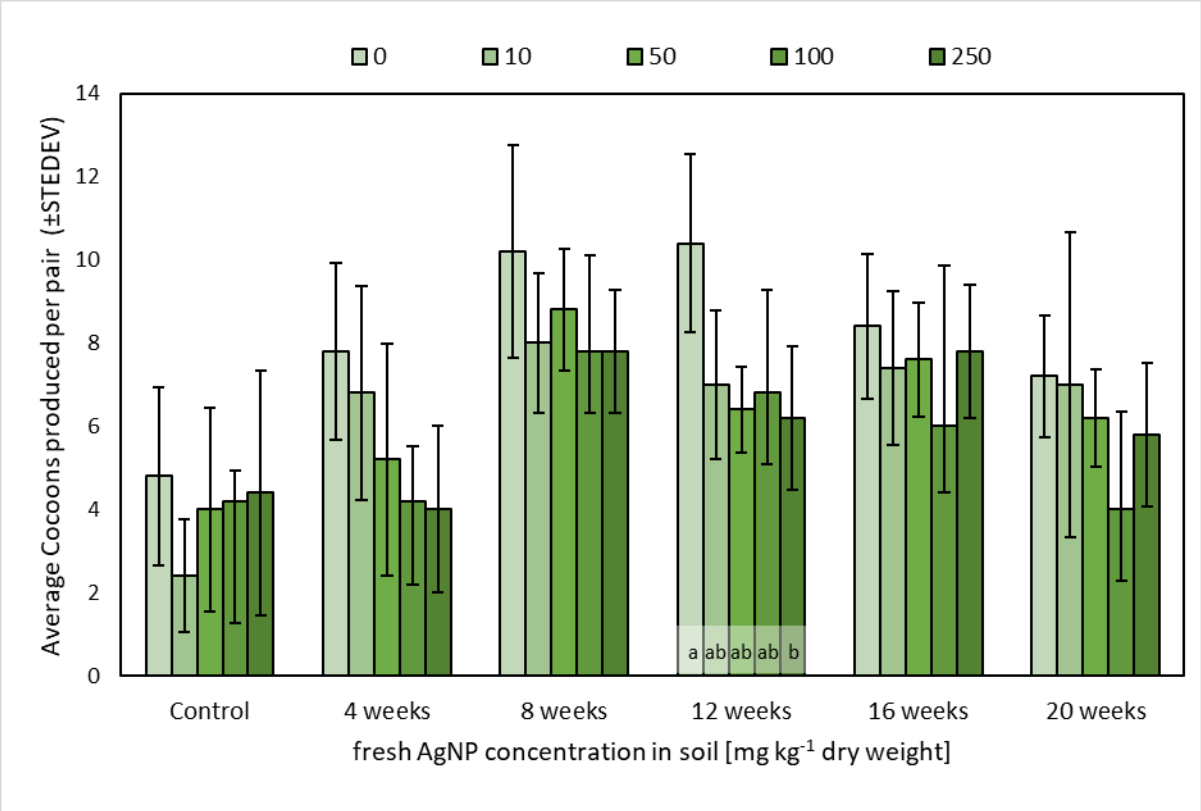

Figure 4.8: Mean number of cocoons (±standard deviation) produced by each pair of A. caliginosa exposed to AgNPs; different letters denote significant differences (ANOVA, p<0.05)

At the 12-week sampling point, there was a significant difference in results (one-way ANOVA $F_{(4, 20)}$=3.320, p=0.031) *A. caliginosa* exposed to 0 mg kg^{-1} AgNP produced significantly more cocoons than those exposed to 250 mg kg^{-1} AgNP (Tukey post hoc: p=0.038). However, a trend in the first 3 sampling points can be seen of *A. caliginosa* in control soil produced more cocoons than those in soil containing AgNPs. Especially at week 4 there appears to be a negative relationship between AgNP exposure and cocoons production, although this is not significant.

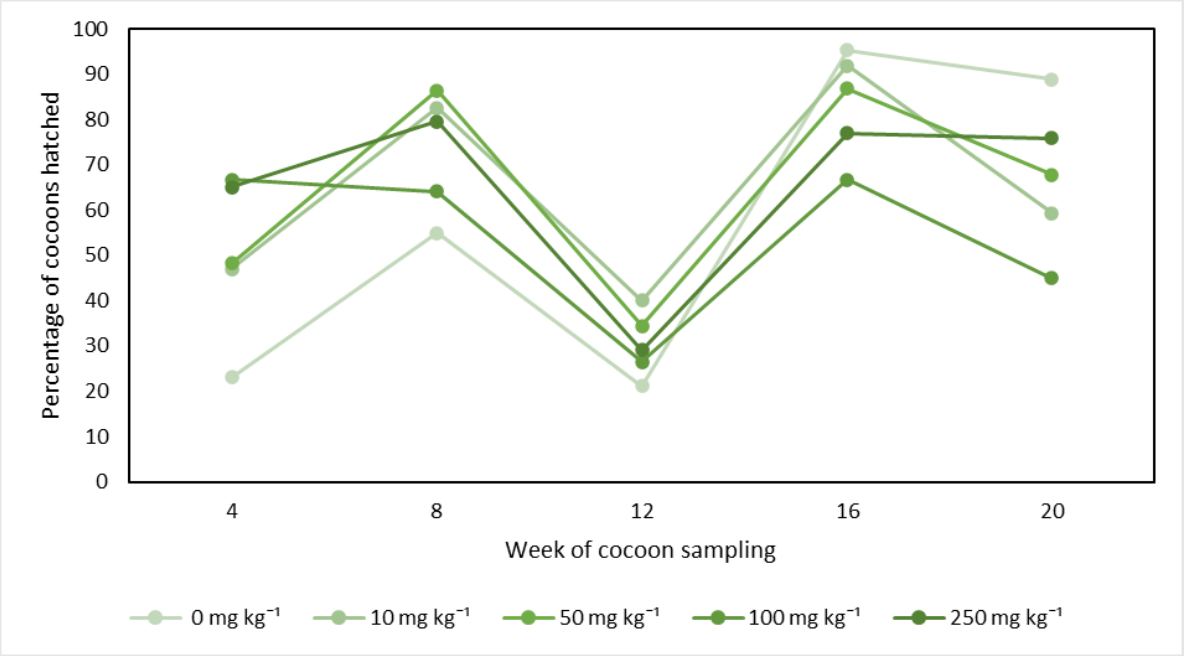

Figure 4.9: Hatching rate of A. caliginosa cocoons collected in the AgNP reproduction experiment

The hatching rate of cocoons produced during this experiment varied widely between sampling points and concentration, as shown in Figure 4.9. Cocoons produced by the control group started out with the lowest hatching percentage and performed significantly worse in statistical survival analysis during the first sampling points. Control group cocoons from the week 4 sampling point had a lower survival performance then 10, 100, and 250 mg kg^{-1} (X^2=6.163, p=0.013; X^2=6.457, p=0.011; X^2=6.161, p=0.013); at week 8 they were outperformed by 10, 50, and 250 mg kg^{-1} (X^2= 5.261, p=0.022; X^2=8.797, p=0.003; X^2=6.235, p=0.013). At week 16, 10 mg kg^{-1} performed significantly better than the 0 and 100 mg kg^{-1} (X^2=4.108, p=0.043; X^2=5.813, p=0.016). And finally, cocoons produced in the control group at week 20 were significantly better than 10 and 100 mg kg^{-1} (X^2=9.578, p=0.002; X^2=8.330, p=0.004).

4.6.4 Discussion

During the first 8 weeks of the experiment, the exposure to AgNPs reduced cocoon production in *A. caliginosa* significantly. However, from weeks 12-20, no such changes were found. Due to the control period where all earthworms were kept in control conditions, it can be assured that the difference in cocoon production is not due to natural differences between individuals. This suggests that exposure to sublethal concentrations of AgNPs reduced the reproductive capacity in *A. caliginosa*. It also appears that *A. caliginosa* adapted to this new environment and regained their ability to produce cocoons with no long-term effects. In a study using *E. fetida*, AgNP concentrations of 727.6 mg kg^{-1} caused a significant decrease in reproduction (Shoults-Wilson et al., 2011a).

Survival rate for cocoons varied widely between concentration groups and sampling days with the control group starting off performing significantly worse. One possible hypothesis could be that due to the significantly higher cocoon production during the first two months by the control group, the viability was reduced. An interesting deviation from the norm was that all hatching percentages dipped at the 12-week sampling point. The only difference between the 12-week sampling point and the rest was the change in HM used. It is unclear whether the two events are related.

In conclusion, the exposure to AgNPs had minimal effect on the reproductive ability of *A. caliginosa* with most negative effects attributed to exposure seemingly adapted to in a short period of time.

4.7 Overall Discussion

This Chapter adequately investigated Aim 1 by quantifying the effects of pristine AgNPs on *A. caliginosa* regarding survival, growth, avoidance, and reproduction. It further investigated some effects of aged AgNPs which helped confirm the toxicity mechanism of AgNPs relying on the ability to release Ag^+ ions.

Overall, AgNPs appear to be somewhat toxic towards *A. caliginosa* in all of the experiments shown in this chapter. The extent varies widely between experiments, but each provided an important element towards understanding of the toxicity. Experiments using aged and oxidised AgNPs showed little toxic effect, affirming the previously held hypothesis that Ag^+ ions are the main cause of the toxicity of AgNPs (Shoults-Wilson et al., 2011b, McShan et al., 2014, Li et al., 2015). While pristine AgNPs can be lethal towards *A. caliginosa* in large quantities, the reproduction experiment showed that earthworms can acclimatise to subtoxic conditions and continue to reproduce without permanent impact. So, if there were a contamination of soil with AgNPs, it appears that no lasting negative effect will persist on *A. caliginosa*, considering that 250 mg kg^{-1} is already a relatively high concentration of fresh particles to appear in the soil. It couldn't be reached via the application of biosolids even if the AgNPs would not largely sulphidise, which is expected (Kaegi et al., 2013). The only negative effect could be *A. caliginosa* avoiding or vacating the affected area, as seen in the linear avoidance experiment.

An unclear effect of AgNPs is that on hatchling *A. caliginosa*. While smaller adults were more affected by the exposure to AgNPs, the same effect did not necessarily apply to the hatchlings despite their much smaller size. One possible reason is the ineffectiveness of using earthworm hatchlings in toxicity testing in general, or the choice of species is not suitable for it, as adult *A. caliginosa* have a wide range of body mass which makes tracking the gain of mass in hatchlings difficult. It is also possible that the

methodology was inappropriate and needs to be improved. It is still an avenue that has the potential to yield valuable results if studied further, as survival of the population is dependent on the survival of the offspring.

5 Establishing effects of biosolids on *A. caliginosa*

5.1 Introduction

Generally seen as the main pathway of silver entering agricultural systems, biosolids play an important role into research of silver nanoparticles (AgNPs) in the environment. Where AgNP-containing products are used in day-to-day life, silver is ultimately released into the wastewater system. This may be through washing AgNP-treated fabrics, use of anti-microbial appliances, AgNP-containing skincare, or effluent from industry, and lead to AgNPs washing down the drain. After treatment, most biosolids produced in the UK are used as a fertiliser on agricultural fields (Defra, 2002), and while there are standards to adhere to including strict guidelines on the upper limit of several metals, silver is not considered a problem and is not regulated (SI UK Statutory Intrument, 1989).

The purpose of this Chapter falls under the wider scope of Aim 1; The effect of Ag_2S on endogeic earthworms. To study potential toxicity of Ag_2S towards the earthworm community, that occur due to the application of biosolids, first, the effects of biosolids need to be established. While this is a topic that has been previously researched, it is important to reconsider with *A. caliginosa* under consistent laboratory conditions, to gain a better understanding of effects and be able to compare results to other experiments. As biosolids contain a variety of components, they could influence any effects later found in experiments focussing on Ag_2S.

Treatment concentrations were selected based on potential application rates. Application onto agricultural fields is regulated based on nitrogen content. One of the highest reported Nitrogen concentrations is 7% (Rigby et al., 2016) which in this model equals 3.57 g kg^{-1} biosolids in dry soil and as per best practices at United Utilities (Waste Water Company in the NW), the maximum amount of biosolids annually applied is 200 kg ha^{-1} which equates to 20 g kg^{-1} in this set of experiments. So overall, the concentrations used experimentally in this Chapter: 3.57, 5, 8.33, and 20 g kg^{-1}, relate to theoretical nitrogen concentrations of 7%, 5%, 3%, and 1% in biosolids. These are high assumed concentrations which equate 1 litre field volume to one kg dry soil.

5.2 An assessment of toxicity of biosolids towards (a) adult and (b) hatchling *A. caliginosa*

5.2.1 Introduction

While previous studies have shown little to no negative effects of biosolids addition in soil towards earthworms (e.g. Hartenstein and Neuhauser, 1985, Pallant and Hilster, 1996, Butt, 1999), good practice still dictates a preliminary test. No amount of biosolids application should cause mortality in earthworms, as this is common practice and strict regulations ensure that the biosolids do not contain excessive amounts of toxic metals (SI UK Statutory Intrument, 1989).

5.2.2 Methods

Kettering Loam was prepared as described in Section 3.2 with the addition of 20 g kg^{-1} HM and biosolids concentrations of 0, 3.57, 5, 8.33, and 20 g kg^{-1}. Plastic pots (250 ml; Section 3.3) were filled with the prepared treatment soils. Adult *A. caliginosa* from laboratory stock were used in this experiment, two were placed in each single-use pot, with 5 replicates per treatment concentration (10 earthworms). A similar set up of treatments was used for laboratory-bred, hatchling *A. caliginosa*, except that here, there were 2 replicates with 5 hatchlings in each pot.

Before use in the experiment, each *A. caliginosa* had its mass determined and was examined to ensure it was in good condition. Once set up, pots were placed into an incubator at 15°C, in complete darkness (Butt and Lowe, 2011). After 7 days, earthworms were examined for survival and had mass recorded before they were placed back into the soil and returned to the incubator. Due to no recorded mortality after 14 days, the experiment was extended to 10 weeks, earthworms continued to have their mass determined every 7 days. No additional organic matter (food source) was added to the pots over the course of the 10 weeks.

Mass datapoints in this experiment were not normally distributed and a Kruskal-Wallis test with pairwise comparison was used to analyse the results of both the adult and hatchling experiment.

5.2.3 Results

Biosolids were found to have no toxic effects on either adult (Figure 5.1) or hatchling (Figure 5.2) *A. caliginosa*. All earthworms survived the whole duration of the experiment.

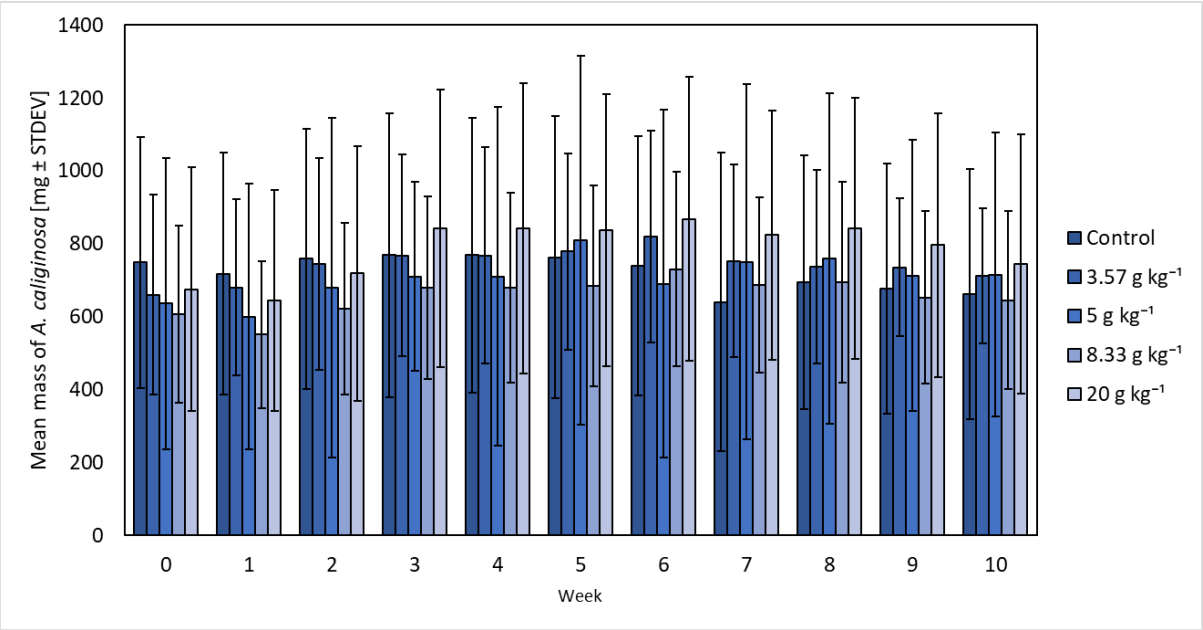

Figure 5.1: Mean mass (± standard deviation) of adult A. caliginosa exposed to biosolids for 10 weeks

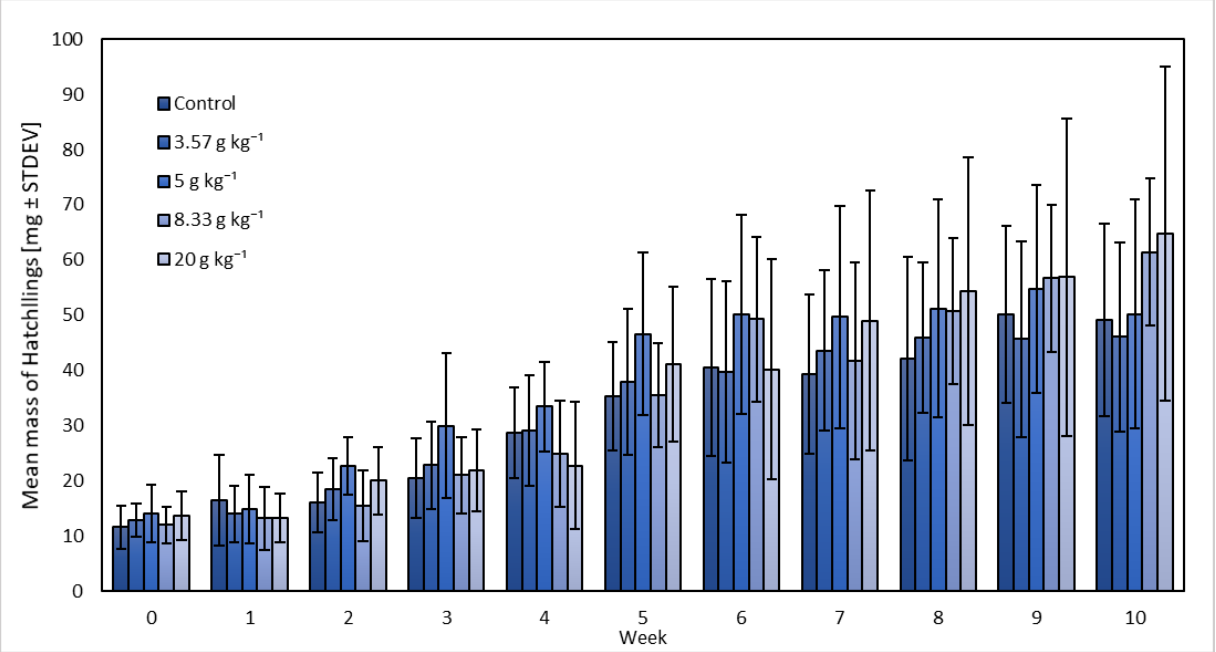

Figure 5.2: Mean mass (± standard deviation) of A. caliginosa hatchlings exposed to biosolids for 10 weeks

In soil with higher concentrations of biosolids, hatchlings appear to have gained more mass when compared to lower biosolids concentrations (Figure 5.2). While this observation was not statistically significant, it was a positive trend.

5.2.4 Discussion

Despite no further organic matter addition to the pots, no loss in mass was recorded in either adult or hatchling *A. caliginosa* over the course of the 10-week experiment. While no significant differences were found in the data, the trend seemed to indicate that earthworms utilised the biosolids as an additional food source. This can be seen especially well in the mass gain of *A. caliginosa* hatchlings exposed to the highest concentration of biosolids. Earthworm use of biosolids as a food source has been shown previously (Hartenstein and Neuhauser, 1985, Pallant and Hilster, 1996). In future experiments, sufficient organic matter would have to be added to all soils to ensure that the addition of biosolids is not an advantage.

5.3 Avoidance behaviour of *A. caliginosa* towards biosolids (circular chamber)

5.3.1 Introduction

An avoidance experiment was conducted as a more sensitive experiment to assess negative effects on *A. caliginosa*. While the acute toxicity test (Section 5.2) showed no toxicity of biosolids towards *A. caliginosa*, it was also necessary to test for sublethal effects. A circular avoidance chamber was chosen to negate any potential edge effect which could occur in a linear avoidance chamber. It is not a commonly used chamber for avoidance experiments and details on why it was chosen are presented in Section 3.3.2.

5.3.2 Methods

To test avoidance behaviour of earthworms to biosolids, five concentrations (as in Section 5.2) were chosen and prepared, namely 0, 3.57, 5, 8.33, and 20 g kg^{-1} dried biosolids in Kettering Loam. As no mortality occurred during the acute toxicity experiment, no adjustment was necessary to the chosen concentrations. For soil preparation method, see Section 3.2, with 10 g kg^{-1} horse manure added due to a short experimental duration (7 days). Soil moisture was assessed to preclude it as an influence on preferential behaviour of earthworms. Circular avoidance chambers were set up with their dividers sectioning each chamber into 5 parts. Each part was filled with one of the treatment soils, ensuring that the control segment was not adjacent to the highest concentration. For details of the avoidance chamber and setup, see Section 3.3.2.

Five avoidance chambers (replicates) were prepared. Twenty-five adult *A. caliginosa* were taken from laboratory stock and had their masses determined. One earthworm was placed at the centre on the surface of each treatment soil segment. After all earthworms burrowed into the soil, the dividers were removed from between the soil treatments, a Perspex© lid was placed onto the circular chamber and each was placed in a temperature-controlled incubator (15 °C, 24 h dark cycle) for 7 days (Butt and Lowe, 2011). The lids do not provide an airtight seal so that chambers could not become oxygen deficient during the experiment. After 7 days, the dividers were re-inserted, and each soil treatment (segment) was checked for the number and mass of earthworms present. Any individuals cut in half by re-insertion of the dividers were recorded as 0.5 for each adjacent treatment. The earthworms were released after the experiment. Statistical difference in number of earthworms in each segment was analysed using a one-way ANOVA.

5.3.3 Results

A. caliginosa did not avoid or prefer any of the given treatment concentrations of biosolids (Figure 5.3) ($p>0.05$).

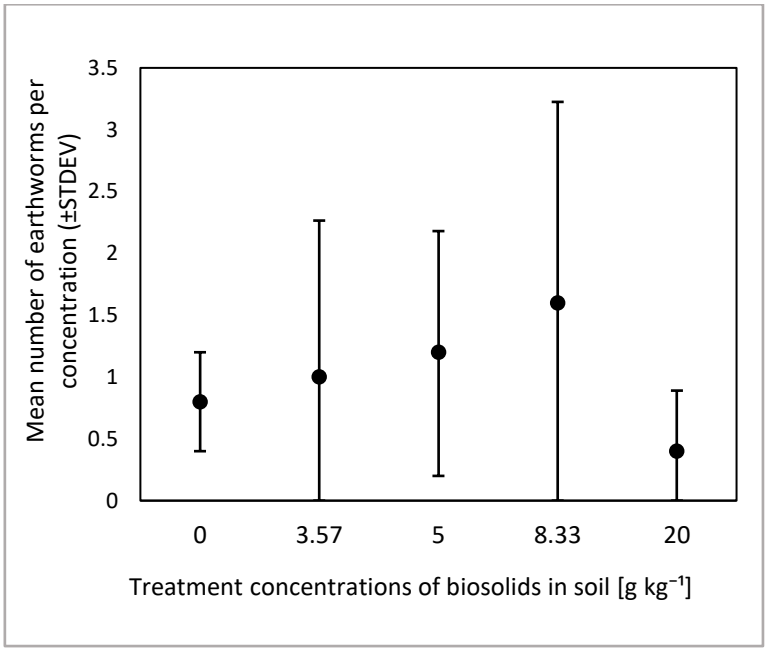

Figure 5.3: Mean number of A. caliginosa in biosolid-containing soil after a 7-day circular avoidance experiment

5.3.4 Discussion

As already established in the previous Chapter, the circular chamber appeared to have severe drawbacks in determining avoidance behaviour of *A. caliginosa*. It could have been due to the chosen

distribution of treatment concentrations, the timeframe chosen, or just a general shortcoming of the circular set up. Therefore, the experiment was repeated with a linear chamber.

5.4 Avoidance behaviour of *A. caliginosa* towards biosolids (linear chamber)

5.4.1 Introduction

In the previous Chapter, a linear avoidance chamber proved itself to be more effective at detecting avoidance behaviour than a circular one. Consequently, the effects of biosolids on potential avoidance behaviour of *A. caliginosa*, was reassessed using a linear avoidance chamber.

5.4.2 Methods

Treatment soils, containing the previously used (Section 5.3) concentrations of biosolids (0, 3.57, 5, 8.33, and 20 g kg^{-1}), were prepared as described in Section 3.2, with 10 g kg^{-1} HM. Dividers were places into a linear avoidance chamber, creating 5 equally sized compartments into which the soil treatments were added. The chambers were set up as a gradient, with the control soil at one end and the highest treatment concentration at the other end. For more detail of the linear chambers see Section 3.3.3.

Once 5 replicated chambers had been prepared, 25 adult *A. caliginosa* had their masses determined and one was placed onto each treatment section in each replicate chamber. After all earthworms had burrowed into the soil, the dividers were removed, and the chambers were tightly sealed with plastic wrap held in place by packing tape. Air holes were pierced into the plastic wrap with a mounted needle to allow for air circulation.

The chambers were placed into an incubator at 15 °C in complete darkness for 14 days (Butt and Lowe, 2011). At the end of the experiment, each chamber was sampled separately. After removal of the plastic wrap, the dividers were reinserted into the soil and the soil in each section was carefully removed and searched. The location of earthworms was recorded. Earthworms split into two parts by the dividers were counted as 0.5 earthworms found in either section. Each earthworm had its mass determined.

Results were analysed using a multinomal goodness-of-fit test and as post-hoc analysis the highest count group was compared with every other concentration in a binomal analysis. To counteract errors due to multiple testing, the p-values were adjusted using the Bonferroni correction.

5.4.3 Results

In the linear avoidance experiment, *A. caliginosa* appeared to prefer soil containing higher concentrations of biosolids (Figure 5.4).

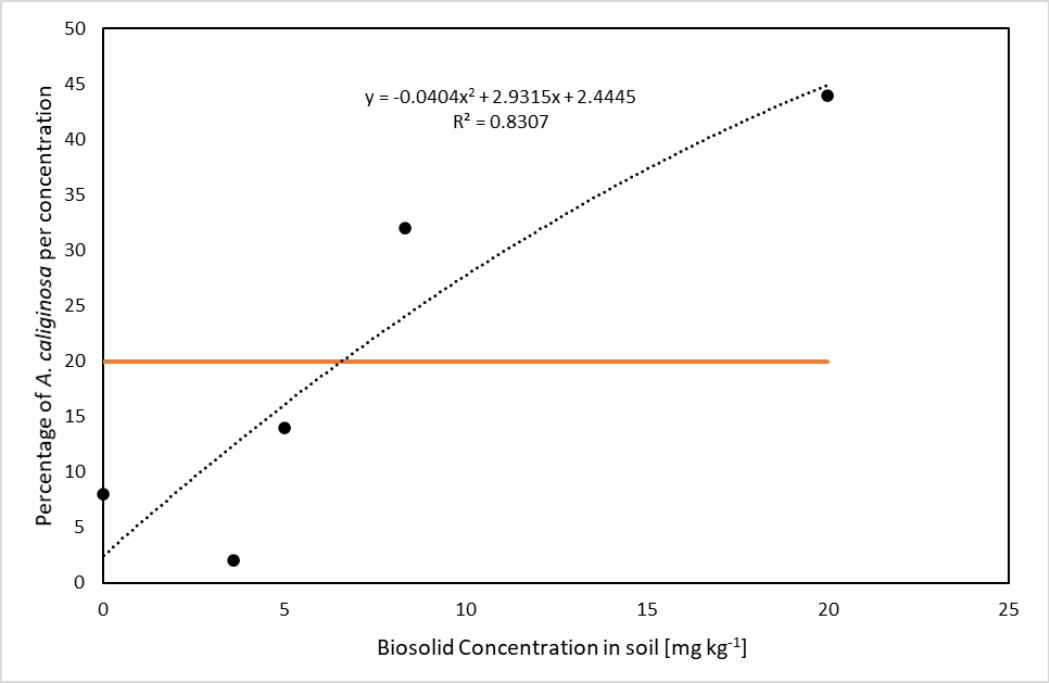

Figure 5.4: Total percentage of A. caliginosa located in different treatment concentrations after a 14-day biosolids linear avoidance experiment; orange line represents theoretical value of equal distribution between concentrations

There was a significant difference in earthworm location at the end of the experiment (p=0.009). *A. caliginosa* preferred the highest concentration to the Control and 3.57 g kg^{-1} (p= 0.022 and 0.006 respectively).

5.4.4 Discussion

The results found in the linear avoidance experiment differ from those from the circular set up, as was also seen in Sections 4.4 and 4.5. After two experiments showing the same type of results, it can be surmised that the linear avoidance chamber is more sensitive than the circular set up.

The preference of *A. caliginosa* for soil with a high biosolids content is consistent with the previous long-term toxicity experiment (Section 5.2) where hatchlings appeared to gain greater mass when exposed to higher biosolid concentrations. This suggests that there are no toxic effects, hence no avoidance, and biosolids provide an additional food source.

5.5 Overall Discussion

Biosolids obtained from United Utilities were not immediately toxic towards adult and hatchling *A. caliginosa*. On the contrary, the earthworms most likely used it as an additional food source as appeared as a trend in the acute toxicity Section 5.2 and preferred biosolid containing soil in the linear avoidance experiment (Section 5.4). These experiments may not have been statistically significant but did show a trend in that direction. These results are contrary to a study of McDaniel et al. (2013), where *A. caliginosa* gained less mass over 12 weeks when exposed to biosolids compared with no exposure in soil free of biosolids. This was a study performed in the USA with anaerobically digested biosolids (as used here) and Colorado topsoil rather than Kettering Loam and *A. caliginosa* were not native to the area. Another factor was the application concentration treatments used in both projects; the Colorado study used a concentration of 5.6 g per litre which is much lower than 20 g kg^{-1}, so the benefits of additional organic matter were likely to have been less than potential contaminants in the biosolids or to overcome the fact that *A. caliginosa* were not native to the area. Exposure to biosolids can lead to the accumulation of toxic metals in earthworms (Beyer et al., 1982, Brewer and Barrett, 1995).

Despite the lack of significances, the linear avoidance experiment was closer to finding a preference than the circular chamber, with similar results found in avoidance experiments using AgNPs (Sections 4.4 and 4.5). While one such occurrence could be considered a coincidence, the fact that it happened twice shows that there is a major drawback to the circular avoidance set up chosen or the chambers used. It might be valuable to determine the exact reason why the circular avoidance chamber failed to produce any results, such as changing the treatment concentrations into a gradient arrangement or adjusting the length of the experiment. Prior to such experiments, it is not possible to conclusively judge the suitability of the circular avoidance chamber.

5.5.1 Difficulties due to Covid-19

Because the purpose of this Chapter may not have been completely clear in the context of this thesis, it has to be explained that the experiments presented were meant to be preliminary and would have led to more complicated investigations. An idea at the beginning of this thesis was to test not only single components (such as fresh and oxidised AgNPs, biosolids, and Ag_2S) but also combinations of those with biosolids. Due to the complex composition of biosolid, they could potentially influence the toxic effect of other substances. In order to test this, baseline data on biosolids had to be established.

In the end, none of the extended experiments were performed, because Covid-19 severely limited the time in which experiments could be conducted.

6 Experimental investigations of silver sulphide (Ag_2S) on *Aporrectodea caliginosa*

6.1 Introduction

As shown in Chapter 4, pristine AgNPs can be toxic to *A. caliginosa* at higher concentrations, although not as toxic as found in experiments with *A. chlorotica*, a smaller endogeic species of earthworm (Brami et al., 2017b). However, the likelihood of pristine AgNPs reaching the soil through the application of biosolids to agricultural fields, is very unlikely. Multiple studies have shown that AgNPs react with different forms of sulphur in the sewage treatment process and are transformed to Ag_2S (Kim et al., 2010, Doolette et al., 2013). Chemically, Ag_2S is less reactive than silver and in previous experiments investigating their toxicity (Section 4.1), aged (oxidised) AgNPs were shown to be less toxic than pristine AgNPs. In general, the effects of Ag_2S on earthworms is not as well studied as the effects of AgNPs. In this Chapter, Aim 1 will be investigated through the following objectives: Determination of whether Ag_2S causes mortality, loss in mass, avoidance of contaminated areas, or changes in reproduction in *A. caliginosa*.

6.2 Preparation of Ag_2S

6.2.1 Introduction

To study nanoparticles, it is beneficial to use particles produced by a commercial supplier to ensure a consistent particle size, elemental composition, and overall quality. It is, however, difficult to obtain Ag_2S in nanoparticle form, potentially due to a lack of research currently undertaken with them. When searching through PubMed®, there were 366 results for silver sulphide and 26,386 results for silver nanoparticle in the twenty years from 2001 to 2021. While this may not be the most accurate data for research interest, it does give an indication of the engagement with each subject. Therefore, a decision was made to synthesise the particles for the current experiments.

6.2.2 Methods

Ag_2S particles were synthesised according to the supplementary information provided by Sadovnikov et al. (2016). Masses of 8.49 g of $AgNO_3$ and 12.9 g of citrate dihydrate were dissolved in 100 mL

Millipore water (ultrapure water) and 6.5 g of sodium sulphide hydrate (60%) was dissolved separately in 100 mL Millipore water. After both solutions were prepared, they were combined into a 250 mL Duran bottle which was vortexed until the previously white solution turned black. The solution was then placed into an ultrasound bath for 30 mins. The solution was kept in the dark at ambient temperature for 3 days, sonicated again for 20 mins and washed with Millipore water before the particles were airdried (Sadovnikov et al., 2016). Particles were analysed using ThermoScientific Quattro S scanning electron microscope (SEM) (detail in Section 3.6).

6.2.3 Particle production results

An analysis using a ThermoScientific Quattro S scanning electron microscope showed that the produced particles consisted mainly of Ag (~70%) and S (~30%). The exact percentages varied depending on the exact point of analysis but not by more than 2 percentage points. As there is more than twice the amount of silver in the sample compared to sulphur, it seemed as if the particle synthesis was not complete. A completely successful synthesis should cause a split of 66% silver to 33% sulphur, as the chemical formula of silver sulphide is Ag_2S.

The size of the particles was estimated to be around 100-300 nm (based on Figure 6.2) with considerable particle agglomeration, as seen in Figure 6.1.

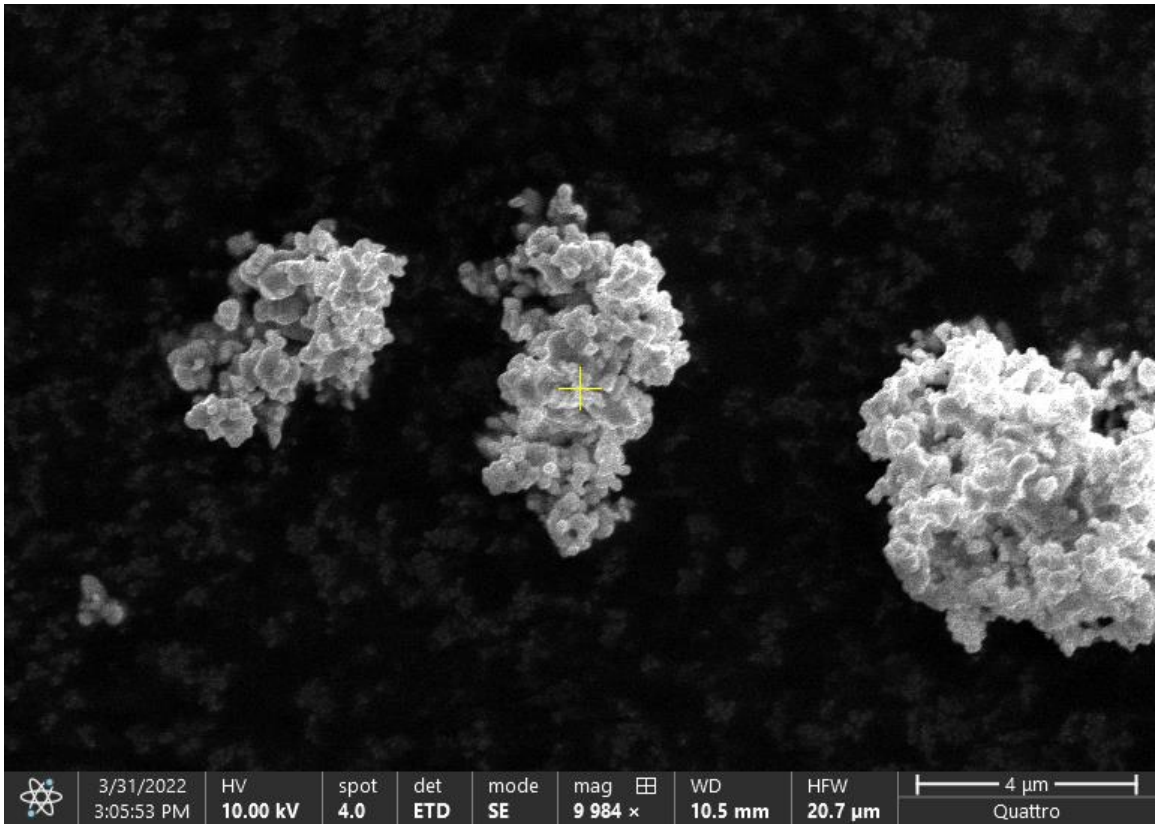

Figure 6.1: Ag_2S particles at 9984x magnification using a Quattro S scanning electron microscope from ThermoScientific (author's image)

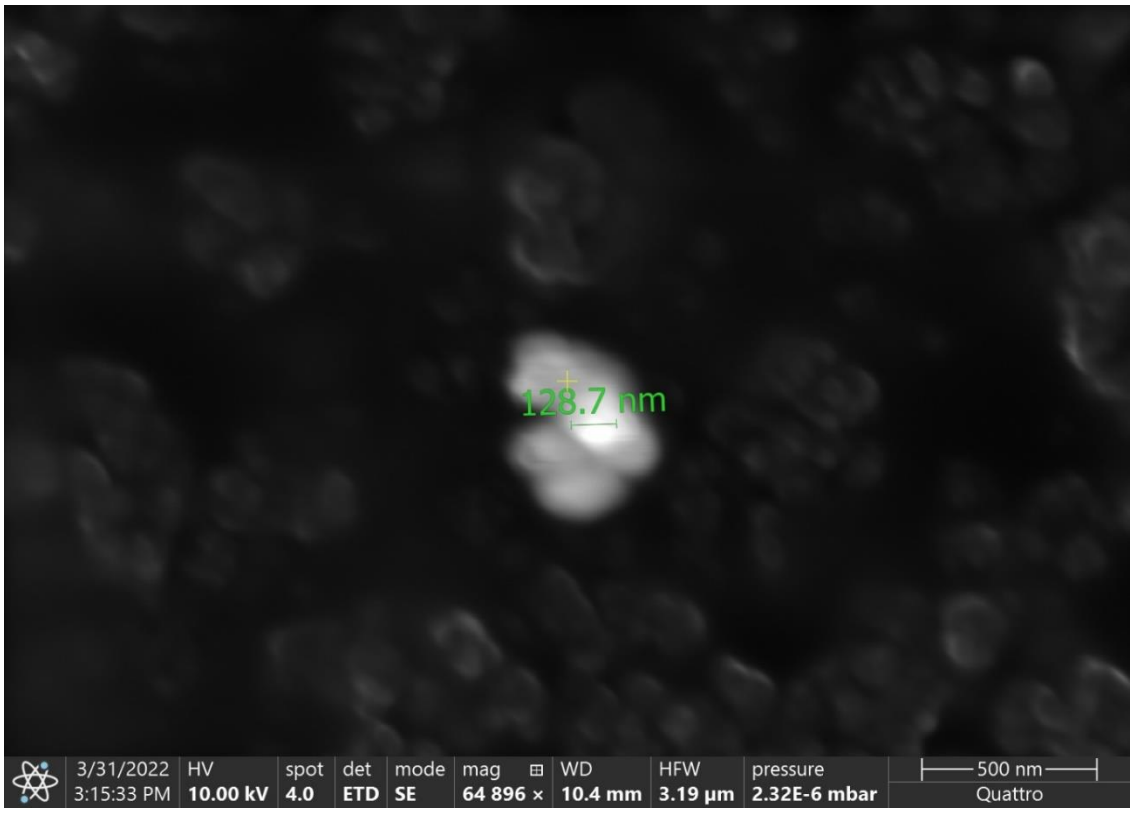

Figure 6.2: Close up (64896x magnification) of a small cluster of Ag_2S particles with size estimation picture taken using a Quattro S SEM by ThermoScientific (author's image)

6.2.4 Discussion

Production of Ag_2S was successful, but the particle size was too large to classify these as nanoparticles, which need to be no more than 100 nm in at least one dimension (ISO, 2015). In addition, there appeared to be a substantial amount of particle aggregation, causing the already larger than nano-sized particles to be even larger. As this could not be helped, the particles were still used experimentally, but are not described as nanoparticles, but rather particles. Sadovnikov et al. (2016) achieved nano-sized particles in the powders produced, and one of the problematic factors could have been the ultrasound bath used at UCLan. A stronger ultrasound bath would result in smaller particles, but no stronger ultrasound bath was available to reattempt the synthesis.

6.3 Experiment to assess toxicology of Ag_2S towards *A. caliginosa*

6.3.1 Introduction

As in Chapters 4 and 5, potential toxicity was tested first. This was to determine at which level *A. caliginosa* might suffer from negative effects such as mortality and loss of mass. Very little research has been undertaken on the toxic effects of Ag_2S on earthworms so there is little information to draw from.

6.3.2 Methods

Kettering Loam was prepared containing 0, 10, 50, 100, 500, and 1000 mg kg^{-1} Ag_2S with 10 g kg^{-1} horse manure (HM) added and filled into single use pots (250 mL). Details of soil preparation are provided in Section 3.2. Adult *A. caliginosa* were collected in the field and were let to acclimate to laboratory conditions for 2 weeks as described by Fründ et al. (2010).

Each Ag_2S treatment concentration consisted of 5 replicate pots with 2 adult *A. caliginosa* that had their mass and condition determined before the experiment and after every 7 days. Between sampling, pots remained in an incubator at 15°C, in complete darkness (Butt and Lowe, 2011). Due to poor consistency in results obtained in previous experiments using hatchling *A. caliginosa* (see Chapter 4), only adults were used to test the acute toxic effects of Ag_2S.

This Ag_2S toxicity experiment was set up to last 2 weeks as it was based upon the AgNP acute toxicity experiment (Section 4.3). However, it was decided to extend the length of the experiment to 4 weeks, due to a lack of mortality (full survivorship) and little change in mass in the earthworms after two

weeks. Due to lack of normality in distribution, differences in mass were analysed using a Kruskal-Wallis test.

6.3.3 Results

All earthworms survived for the full duration of the 4-week experiment and no significant change in mass was observed within or between treatments and all earthworms remained in good condition.

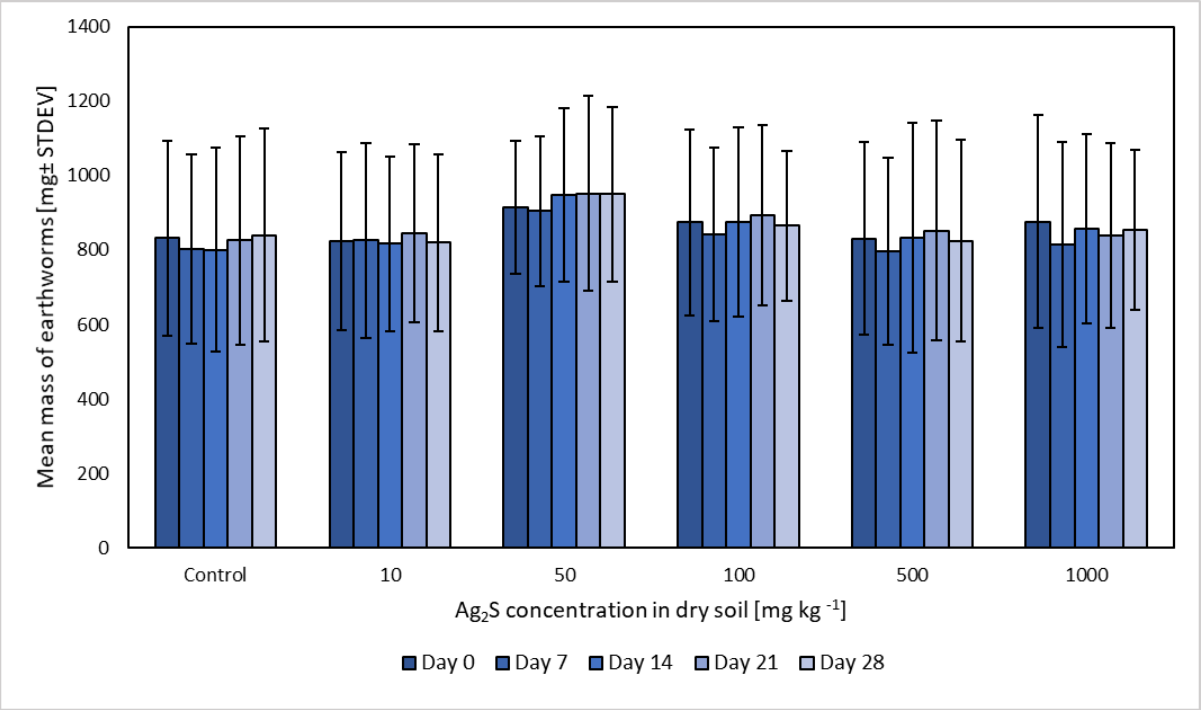

Figure 6.3: Mean mass (± standard deviation) of adult A. caliginosa exposed to Ag_2S in soil for 28 days

As shown in Figure 6.3, the mass did not change over the course of the experiment and statistical analysis confirmed this observation.

6.3.4 Discussion

Ag_2S exposure did not cause death in *A. caliginosa* over the course of the first 2 weeks of the experiment and even when extending the experiment for a further 2 weeks, no mortality and no change in mass was observed. This was different to the results obtained in an experiment examining acute toxicity of pristine AgNP (Section 4.3) where earthworms lost mass over the course of two weeks, dependent on concentration. AgNPs also caused some mortality in concentrations from 250 mg kg^{-1} upwards. The lack of mortality and no loss in mass were more comparable to the results obtained in Section 4.2, were *A. caliginosa* were exposed to aged AgNPs. This showed that the

sulfidation of the AgNPs reduced their ability to release Ag^+ ions which are suspected to be a major factor in the toxicity of AgNPs (Shoults-Wilson et al., 2011b).

While pure silver readily reacts and releases reactive Ag^+ ions, the bond between silver and sulphur is comparatively strong (Doolette et al., 2015) and Ag_2S will be less likely to react or release any ions. This reduced reactive behaviour causes a decline in toxic effects. In addition to that, the Ag_2S particles used in this experiment are not nanoparticle size and therefore have a lower surface area to volume ratio, further hindering their ability to release Ag^+ ions.

6.4 Avoidance behaviour of *A. caliginosa* towards Ag_2S

6.4.1 Introduction

Use of a circular avoidance chamber was shown to be ineffective at discerning *A. caliginosa* avoidance behaviour in previous experiments using AgNPs and biosolids (Chapters 4 and 5). For this reason, a linear avoidance experiment was performed to obtain more reliable results and to establish potential Ag_2S avoidance behaviour by *A. caliginosa*.

6.4.2 Materials and Methods

As no treatments used in the previous experiment caused mortality, avoidance was tested for Ag_2S concentrations of 0, 50, 100, 500, and 1000 mg kg^{-1}. Experimental soil with 10 g kg^{-1} HM was prepared at those concentrations, for details on soil preparation see Section 3.2. Dividers were inserted into 5 linear avoidance chambers (Section 3.3.3) and treatment soil was filled into each section to form a gradient. Samples of each soil concentration had their moisture content assessed to preclude any influence of soil moisture on earthworm avoidance.

Laboratory raised, adult *A. caliginosa* were taken from stock sources and after determining their mass, one was placed onto each section in each container. After all earthworms had burrowed into the soil, the dividers were removed, and the chambers were tightly sealed with plastic wrapping and packaging tape. Air holes were pierced into the plastic wrapping using a mounted needle to allow for air circulation.

The avoidance chambers were placed into an incubator at 15 °C in complete darkness for 14 days (Butt and Lowe, 2011). At the end of the experiment, each chamber was sampled separately. After removing the plastic wrapping, the dividers were replaced into the soil at the predetermined positions and the

soil in each section was carefully removed and searched. Each earthworm found had its mass determined. Earthworms cut into two parts by the dividers were counted as 0.5 earthworms found in each section. Results were analysed using a multinomal goodness-of-fit test and as post-hoc analysis the highest count group was compared with every other concentration in a binomal analysis. To counteract errors due to multiple testing, the p-values were adjusted using the Bonferroni correction.

6.4.3 Results

After 2 weeks, the majority of *A. caliginosa* were located in the control soil and, on average, less than one earthworm was found in soil containing any amount of Ag_2S (see Figure 6.4). This result was significant ($p<0.001$) and the percentage of earthworms present in the control soil was significantly higher than in 100, 500, and 1000 mg kg^{-1} Ag_2S ($p=0.001$, 0.013, and 0.013 respectively). Soil moisture content in each of the sections of the gradient was not significantly different.

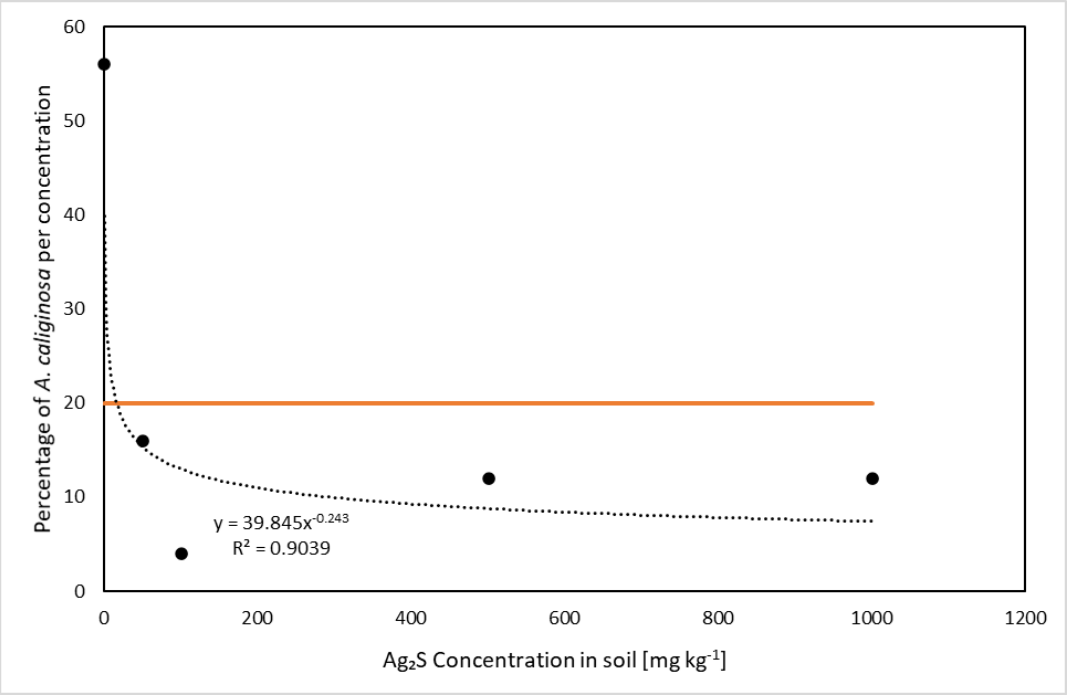

Figure 6.4: Total percentage of A. caliginosa located in different treatment concentrations after a 14-day Ag_2S linear avoidance experiment; orange line represents theoretical value of equal distribution between concentrations

There was no observable gradient reaction, i.e., a greater avoidance of soil containing a higher concentration of Ag_2S and the post-hoc analysis confirmed this. Some of the earthworms lost mass over the course of this experiment, with the mean decreasing by just under 7%.

6.4.4 Discussion

A. caliginosa avoided soil containing Ag_2S, from 100 mg kg^{-1} and higher. While acute toxicity tests did not show Ag_2S to be directly toxic towards *A. caliginosa* and the reproductive study showed no long-term effects, *A. caliginosa* still appear to avoid it. It could be due to the shape of the particles being slightly abrasive towards the earthworms, so they avoid the area out of comfort rather than the substance being necessarily toxic. Another possible explanation is that earthworms avoid soils with a high salt content (Jun et al., 2012) and Ag_2S is, chemically, a salt. Even though it is not a very soluble salt and therefore would not raise the chemical conductivity in the soil substantially. Without further research, the avoidance behaviour cannot be fully explained, and may have an effect on the overall earthworm population in the field if levels of Ag_2S rise.

It was noted that the mean mass of the earthworms in this experiment declined from mean of 686.72 mg before the experiment to 639.28 mg after the experiment. While not ideal, it was less than the 20%, which is generally accepted as the maximum mass earthworms should be allowed to lose during an experiment (Fründ et al., 2010).

6.5 Experiment to assess effects of Ag_2S on *A. caliginosa* reproduction

6.5.1 Introduction

To investigate the effects of Ag_2S on *A. caliginosa* reproduction, a long-term experiment was conducted. Reproductive toxicity experiments are sensitive tests to establish toxicity on endpoints such as cocoon production and hatching rate of cocoons, which, if affected, can have lasting effects on an earthworm population. Different endpoints, such as changes in mass, number of produced cocoons and survival of said cocoons (percentage hatched and length of time span) were all recorded to gain an insight into any potential reprotoxic effects.

6.5.2 Methods

Before the reproductive experiment started, a control experiment was set up with 60 laboratory-raised adult *A. caliginosa*, paired according to morphological similarity, such as size and colour variations. Control soil was prepared, consisting of Kettering Loam, 5 g kg^{-1} sand and 50g kg^{-1} dried, milled and sieved (<1 mm) HM. Each of the 30 pairs were placed into a single-use pot containing control soil. The pots containing the earthworms were placed into an incubator at 15°C in complete

darkness, for 4 weeks to assess the capacity of the pairs to survive in experimental conditions without mass loss and to ensure that each pair could produce cocoons.

After 4 weeks, 1 kg of experimental soil containing each of 0, 50, 100, 500, and 1000 mg kg^{-1} Ag_2S and 50 kg^{-1} HM was prepared (Section 3.2) and 5 single-use pots were filled for each concentration. The earthworm pairs were put into 5 groups, so that all groups were roughly similar in average mass and cocoon production. This helped ensure that the starting conditions were equal among groups and any pairs that failed to produce cocoons could be excluded before the experiment started.

A. caliginosa pairs were then placed into their respective treatment concentrations and returned to the incubator. Every 4 weeks, fresh soil was prepared as previously described, all earthworms had masses determined and were placed into fresh pots. Afterwards, the soil in the pots from which earthworms had been removed was sieved for cocoons. [Note: One week of sampling was missed due to illness which resulted in sampling points at 4, 8, 13, 17, and 21 weeks.] All cocoons were retained and set up in labelled Petri dishes containing a filter paper and tap water and observed weekly to determine hatchability.

Changes in mass and cocoon production were analysed with both a regular one-way ANOVA in addition to a repeated measures ANOVA. Both were chosen because, while the repeated measures ANOVA is the more appropriate analysis for this type of dataset but is relatively uncommon and therefore an additional more common analysis was also performed. The survival rate of the cocoons was analysed with a Kaplan-Meier test.

6.5.3 Results

Mass of *A. caliginosa* in the reproduction experiment remained relatively constant over the duration of the experiment with no statistical differences found between treatments and between sampling points (Figure 6.5).

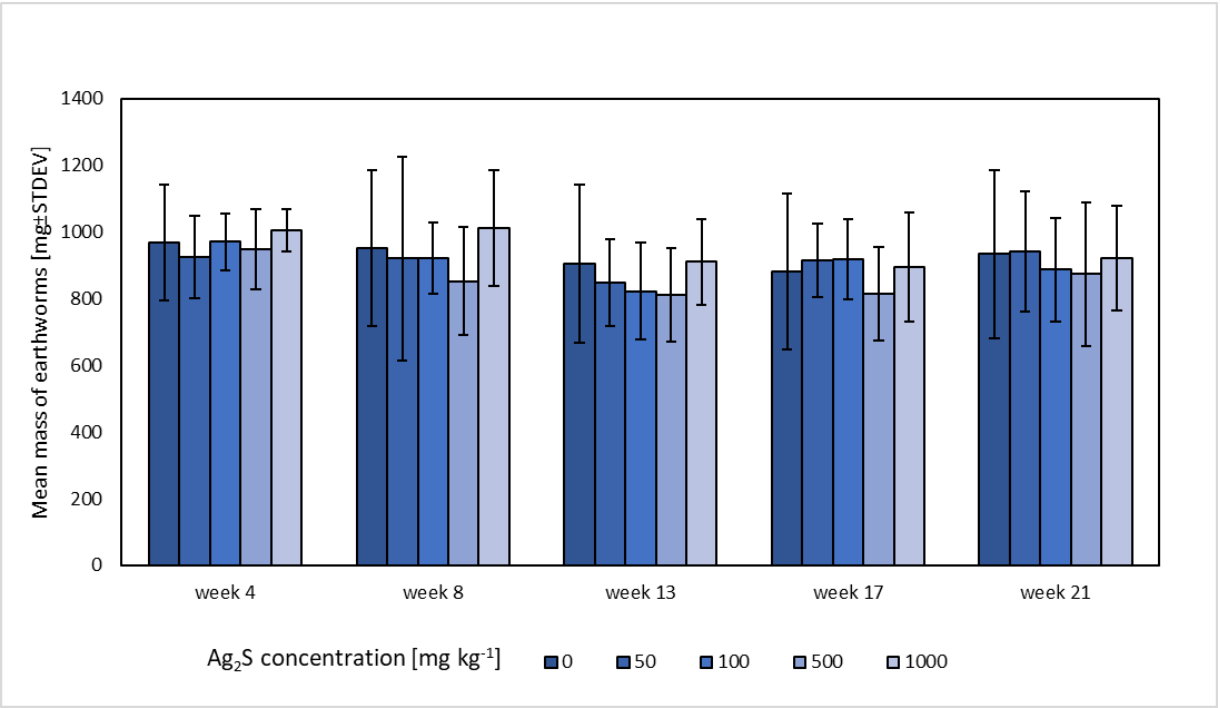

Figure 6.5: Mean mass (± standard deviation) of adult A. caliginosa at each sampling point in the Ag_2S reproduction experiment

Exposure to different concentrations of Ag_2S did not affect the mean number of cocoons produced by *A. caliginosa*.

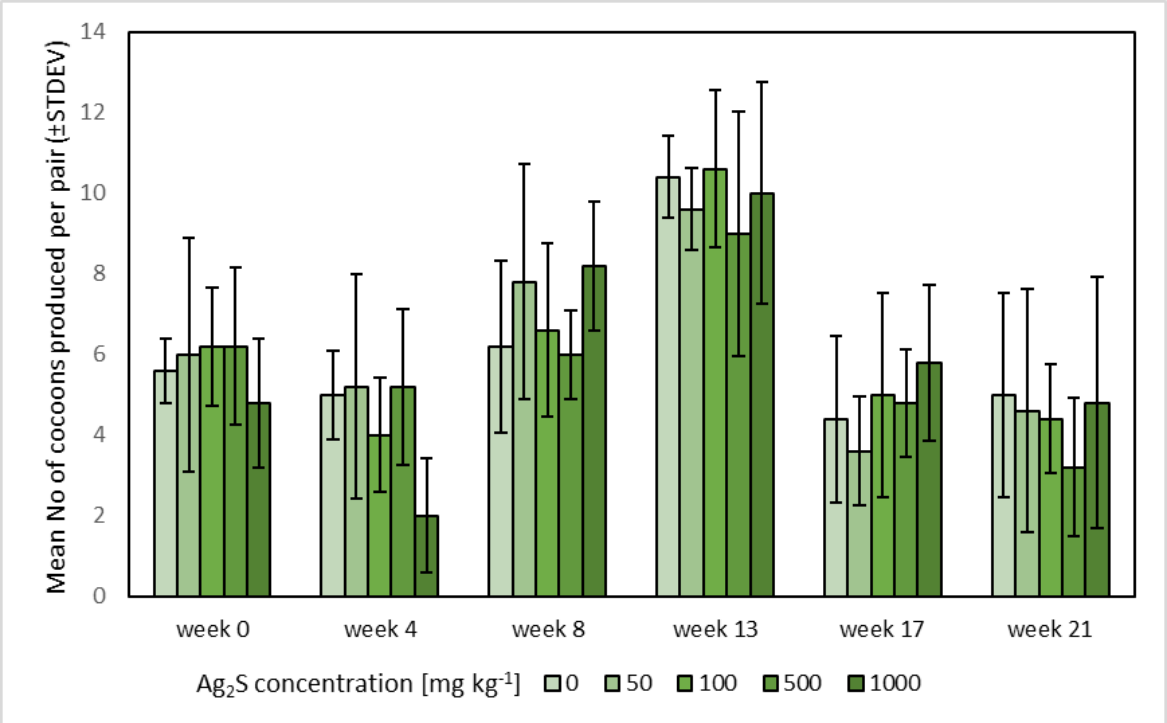

Figure 6.6: Mean cocoon production (± standard deviation) produced by each pair of A. caliginosa exposed to Ag_2S

The earthworm pairs exposed to 1000 mg kg^{-1} had mean cocoon production of 2 per pair at week 4, but differences between the concentrations was not significant at that point (Figure 6.6). At week 13, the cocoon production increased overall due to the missed sampling point at week 12, resulting in the number of cocoons from a 5-week window rather than the usual 4. [To view a Figure where cocoon production at week 13 has been corrected to discount the additional week, see Appendix IV.]

Cocoon survival

The cocoons produced during the experiment were mostly viable and the viability (number of cocoons which hatched) did not differ significantly across treatment groups (Figure 6.7).

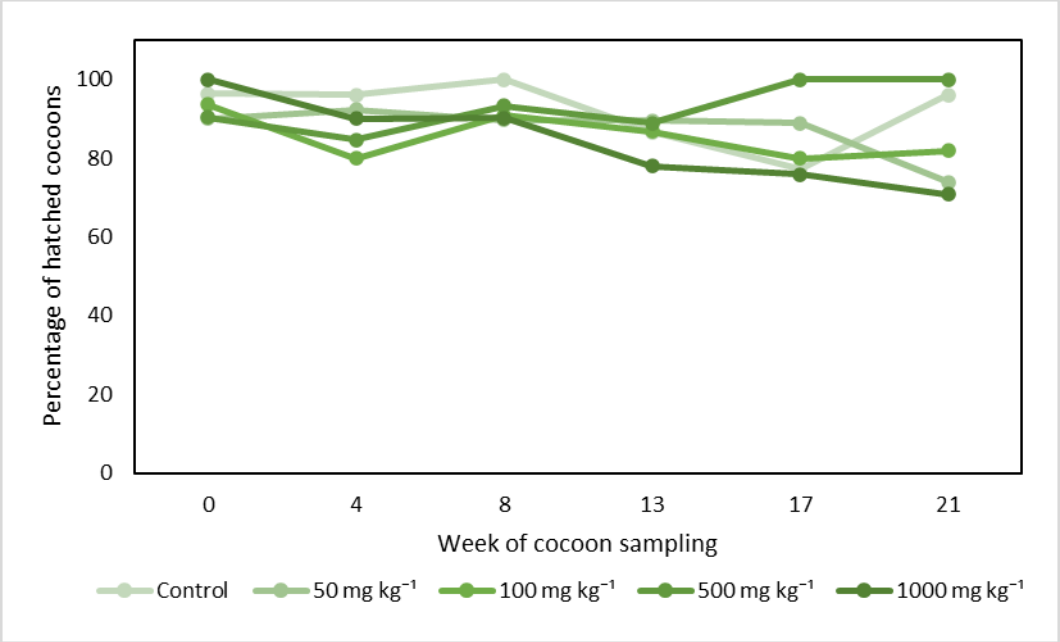

Figure 6.7: Hatching rate of A. caliginosa cocoons collected in the Ag_2S reproduction experiment

The results of the Kaplan-Meier analysis varied widely over the course of the experiment, for example at week 8, 1000 mg kg^{-1} was significantly lower than Control, 100, and 500 mg kg^{-1} (X^2=3.973, p=0.046; X^2=5.500, p=0.019; X^2=5.531, p=0.021). While at week 21, 500 mg kg^{-1} outperformed Control, 50, and 1000 mg kg^{-1} in terms of hatchability (X^2=4.683, p=0.030; X^2=6.801, p=0.009; X^2=10.322, p=0.001). No definite trends were found over the course of the experiment. However, it should be noted that when comparing the mean time for the cocoon hatching (part of the Kaplan-Meier analysis, see Table 6.1), cocoons produced at the highest treatment concentration, were the worst performing group during weeks 4, 8, 17, and 21; and in a central position at week 13. It was noted that cocoons collected at

week 13 showed no significant differences in hatchability, potentially due to 5 weeks' worth of cocoons having been collected rather than 4. This spread the timeframe in which cocoons hatched, potentially equalising minor differences. Cocoons produced by the earthworm pairs which were later in the 1000 mg kg^{-1} group actually were the most rapid to hatch when in control conditions (not significantly).

Table 6.1: Estimated mean time for cocoon hatching ranked from most rapid to slowest, significance chosen from Tarone-Ware post-hoc test (Different letters in the same column denote significance p<0.05)

Hatching rate	week 0		week 4		week 8		week 13		week 17		week 21	
	Ag_2S		Ag_2S		Ag_2S		Ag_2S		Ag_2S		Ag_2S	
Most rapid	1000	a	50	a	0	a	500	a	500	a	500	a
	0	a	0	ab	100	a	50	a	50	ab	0	b
	100	a	500	ab	500	a	1000	a	100	ab	100	bc
	500	a	1000	ab	50	ab	100	a	0	ab	50	bc
slowest	50	a	100	b	1000	b	0	a	1000	b	1000	c

While not consistently significant, there were some trends that showed a worse performance at the highest treatment concentration for cocoon viability.

6.5.4 Discussion

Neither cocoon production nor mean mass showed any significant trends across treatments over the course of the experiment. This aligns with the hypothesis that Ag_2S is less reactive than AgNPs and therefore less toxic, which was also shown in the acute toxicity experiment.

While there were no consistent trends in cocoon hatchability relating to Ag_2S exposure in this experiment, there appeared to be a trend where the cocoons produced in the highest concentration has a slightly lower survival ranking than the rest. Due to cocoons produced in the 500 mg kg^{-1} treatment having consistently good viability, and those produced in control conditions not necessarily having the highest viability, it would be difficult to conclude Ag_2S had a negative effect on cocoon viability. Potentially, a higher concentration would elicit a significant result if the experiment was repeated. However, is there a relevance in testing this, as 1000 mg kg^{-1} Ag_2S is already an unrealistic concentration to achieve in the field with the application of biosolids, even considering long-term application (highest reported amount of silver reported is 7.4 µg Ag kg^{-1} (Gottschalk et al., 2009)). Unless further experiments showed otherwise, the Ag_2S in biosolids probably does not have an effect on the survival of *A. caliginosa* cocoons.

6.6 Overall discussion

Overall, Ag_2S did not have any conclusive toxic effects on *A. caliginosa*. Observed avoidance behaviour did occur, but no negative impact was recorded on earthworm survival, mass, cocoon production or hatchability of cocoons. This aligns with the widely held belief that the toxicity of AgNPs stems from their release of Ag^+ ions (Shoults-Wilson et al., 2011b, McShan et al., 2014, Li et al., 2015). As previously reported (Section 4.2), where the oxidation of AgNPs rendered them less harmful to *A. caliginosa*, the reaction with sulphur to Ag_2S creates a strong enough bond to minimise or completely prevent the formation of Ag^+ ions.

However, while Ag_2S does not negatively affect *A. caliginosa*, the avoidance behaviour could still cause a negative effect on the overall earthworm density of a soil if earthworms avoid the area. If the application of biosolids is a cause for the increase in Ag_2S, it would probably be a widespread application leaving very little option for earthworms to avoid the area, which could potentially minimise the effects of any avoidance behaviour. In addition, the previously performed experiment of linear avoidance to biosolid application (Section 5.4) showed that soil with the addition of biosolids is preferred by *A. caliginosa*, probably due to the high organic matter content, so the effects might negate each other, or in fact the provision of OM may be overriding. Larger scale, field-based experiments would usefully seek to explore this further.

The data found in this project are far from being sufficient to make definitive statements. The research community could usefully further investigate Ag_2S toxicity in general, as it can be hypothesised that Ag_2S presents no acute risk to the soil community. While other routes of silver entering the environment may still be of concern, the hazards of increased AgNPs in the wastewater system and subsequent application onto soil is less of a concern. Biosolids are already screened for specific metals (e.g. copper, cadmium, lead) which should not be applied in larger quantities (SI UK Statutory Intrument, 1989), so addition of silver to this list is not necessary.

7 Effects of different forms of silver on plants

7.1 Introduction

This Chapter assesses the impact of silver in different forms on plants (Aim 2), where various preliminary effects were investigated. AgNPs have antimicrobial properties and therefore have the potential to be used in plant protection products as an antifungal agent (Nowack et al., 2011). While this has been studied and proven an effective alternative to chemical fungicides (Jo et al., 2009, Jung et al., 2010), some aspects are yet to be investigated. These include the effects of different forms of silver on germination of seeds or potential effects on the earthworm community when their food source is treated with AgNP-containing agents. Objectives to achieve this Aim include investigating the effect of silver containing products on the germination and growth of lettuce, effects of Ag_2S on the growth of radishes, and whether the spraying of AgNP-containing products onto plant material has an influence on the feeding behaviour of *A. caliginosa*.

7.2 Effects of different forms of silver on lettuce growth

7.2.1 Introduction

At first, a simple growth experiment was conducted to determine the effects of AgNP-containing fertiliser (Argentum from Plantosys), pure AgNPs, and $AgNO_3$ on lettuce. Lettuce was chosen as an appropriate plant in this experiment as the edible part of the plant in in direct contact with any applied solutions. This enabled the assessment of silver uptake in the plant and therefore also how much silver would be consumed by humans if they ate silver-treated produce.

7.2.2 Methods

Lettuce seeds (*Lactuca sativa*; dobies.co.uk type 437317) were germinated in a shallow tray in John Innes seed sowing compost and after two true leaves emerged (approximately 2 weeks), they were transferred into larger pots. These were 10 x 10 cm square black plastic pots (11 cm high) with drainage holes (Wilko Stores, UK). They were filled with John Innes No. 2 soil, contained 1 lettuce plant each, and were kept in a growth cabinet (Kays Horticulture products, Whitehaven). Appropriate growing lights (Phillips PL-L 55W/65 4P 2G11) were used in the growth cabinet and kept on a 13 hours light, 11 hours darkness cycle to mimic UK spring conditions. Temperature could not be controlled but

was ambient and due to lack of windows and infrequent use of the growth room, the temperature remained relatively constant at approximately 22-25 °C. Each pot was treated with one spray of silver-containing product per week (3 pots per replicate). The amount of liquid per spray was ~ 150 µL, as determined by assessing the mass of 50 spray pumps in 5 instances (using separate small spray bottles). The experiment was planned to run for 8 weeks but lasted for only 4 weeks due to the light system breaking, after which the above ground lettuce was harvested and dried after mass determination at 60°C. Differences in lettuce mass (fresh) were compared with a Kruskal-Wallis test with pairwise comparison.

Several treatment sprays were prepared: 2 mg mL^{-1} AgNPs, 2, 10, and 20 mg mL^{-1} Silver nitrate ($AgNO_3$), and 2, 10, and 20 mg mL^{-1} Argentum (measured in silver mass, not mass of Argentum), with dosage calculated so that one spray per pot corresponded to a specified dosage in the field. A commercial company, Plantosys Biostimulants, provided a sample of Argentum for use in this work. Argentum is a fertiliser containing 1% nanosilver and is produced to be sprayed onto crops. Its suggested dosage is 20 mL Argentum in 100 litres of water, with recommended application rates of 600-1000 litre (diluted product) $hectare^{-1}$ $week^{-1}$. The lowest concentration spray was 2 mg mL^{-1} silver (one pump spray per 100 cm^2 pot per week) which is the equivalent of the highest recommended Argentum dosage of 1000 litres $hectare^{-1}$.

7.2.3 Results

One lettuce plant (treated with 20 mg mL^{-1} $AgNO_3$) died over the course of the experiment while all others survived to four weeks. Overall, results show that no or low concentrations of silver application resulted in a higher plant mass when compared to higher concentrations of the same treatment (Figure 7.1).

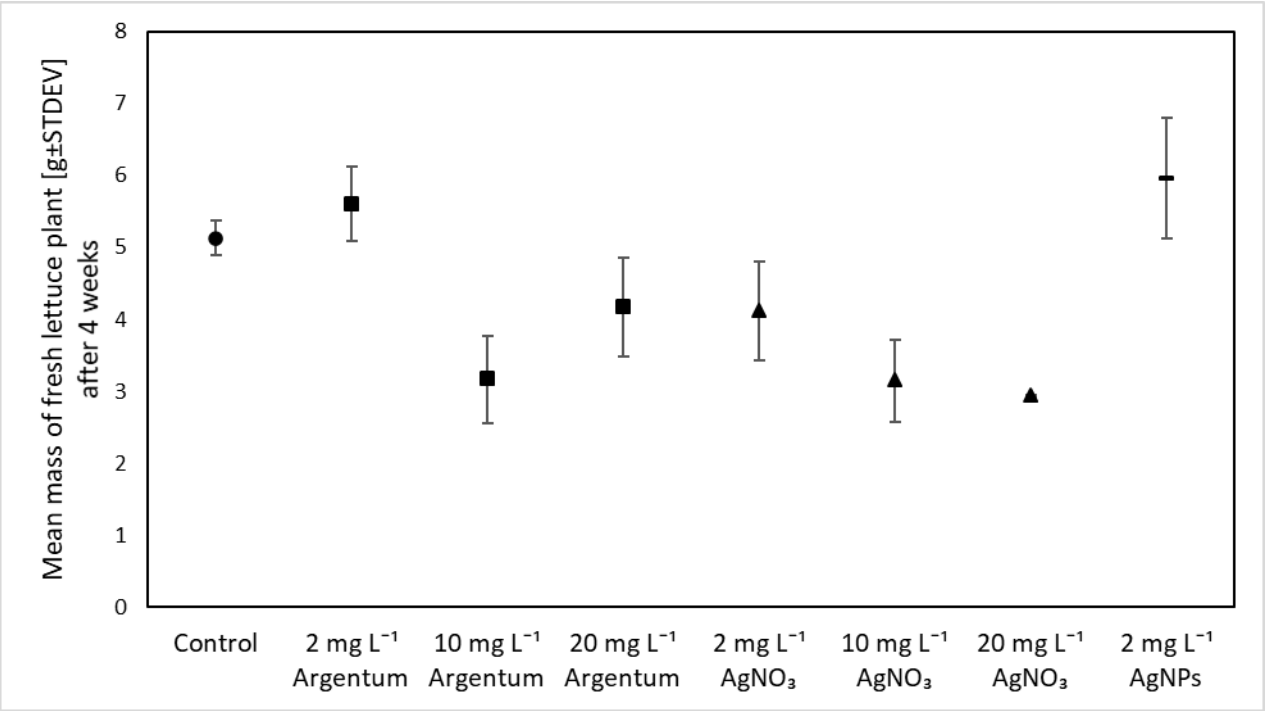

Figure 7.1: Mean mass (± standard deviation) of lettuce plants after treatment with different silver-containing sprays

Fresh lettuce plants treated with water (control), 2 mg mL^{-1} Argentum, and 2 mg mL^{-1} AgNPs had the highest mean mass; while 10 mg mL^{-1} Argentum, 10, and 20 mg mL^{-1} $AgNO_3$ resulted in the 3 lowest mean masses. Some of these results were shown to be significant (Table 7.1).

Table 7.1: Significant differences in mean lettuce weight (Kruskal-Wallis) after 4-week treatment with silver-containing products

Sig. differences using Kruskal-Wallis	Control	2 mg L^{-1} Argentum	10 mg L^{-1} Argentum	20 mg L^{-1} Argentum	2 mg L^{-1} $AgNO_3$	10 mg L^{-1} $AgNO_3$	20 mg L^{-1} $AgNO_3$	2 mg L^{-1} AgNPs
2 mg L^{-1} AgNPs			p=0.004			p=0.047	p=0.007	
20 mg L^{-1} $AgNO_3$	p=0.049	p=0.012						
10 mg L^{-1} $AgNO_3$								
2 mg L^{-1} $AgNO_3$								
20 mg L^{-1} Argentum								
10 mg L^{-1} Argentum	p=0.041	p=0.008						
2 mg L^{-1} Argentum								

While $AgNO_3$ had an increasingly negative effect on lettuce mass with increased concentration, only a treatment of 10 mg mL^{-1} Argentum had a significantly negative effect while 20 mg mL^{-1} Argentum did not.

7.2.4 Discussion

A negative effect on mean mass of lettuce was seen when treated with $AgNO_3$, however, the effects of Argentum were less clear and dosing with AgNPs appeared to have no effect on growth. It is possible that the trends in mass could have become more prominent over time, but with the limited mass of the lettuce collected, the negative effects of $AgNO_3$ were the only ones immediately apparent. Measurement of silver uptake was planned as it would have furthered the objective of estimating silver uptake into plants, however, after digesting the samples, the ICP-MS at UCLan broke down and no analysis could be performed. One potential reason for the decrease in lettuce mass, when treated with Argentum, was that AgNPs have been linked to phytotoxicity in plants, damaging their photosynthesis process leading to a decreased ability to grow (Rastogi et al., 2019).

A previous study found that spraying AgNPs on lettuce increased silver content, but AgNPs in the soil had a stronger negative effect on the plants (Li et al., 2020). In another study, simply washing spinach leaves with water did not remove commercially available AgNPs (Zhang et al., 2016), which does give credence to the hypothesis of an increased use of AgNP-containing fertiliser increasing the human silver burden. However, the spinach in that study was not sprayed with AgNPs during growth, but rather the silver was applied onto the surface of dead leaves. Therefore, more studies should be undertaken to this effect, preferably with commercially available products and within a growth experiment rather than applying the product after harvest.

7.3 Effects of different concentrations of Argentum on the germination of lettuce seeds

7.3.1 Introduction

A germination test (OECD, 2006) is commonly used to test ecotoxic effects of substances. It is a preliminary test to show any potential effects of substances on seedling emergence or growth but cannot investigate any chronic or sub-chronic effects which should not be neglected. As with the previous experiment (Section 7.2), this served Aim 2: Assess the effects of AgNPs and Ag_2S on plants.

This experiment was the final iteration after a line of preliminary germination tests (detailed in Appendix V) to assess and improve the testing conditions and ensure that the most suitable seed was used.

7.3.2 Methods

Petri dishes (9 cm ø) were lined with No. 1 Whatman filter papers and wetted with approximately 5 mL of either distilled water (control), 2 mg mL^{-1} Argentum, 20 mg mL^{-1} Argentum, or 2 mg mL^{-1} $AgNO_3$. Each treatment concentration had two replicates with 20 lettuce seeds placed onto each filter paper. The Petri dishes were kept at ambient temperature in a large plastic tray, covered with aluminium foil and observed every 6 hours. At each time point, the seeds were monitored for the first sign of radicle emergence and for the first sign of plumule emergence. Filter paper was re-wetted as needed, if it became too dry. For statistical analysis of the results, the Kaplan-Meier survival test was chosen (McNair et al., 2012), as it takes into account both survival (germination or plumule emergence) as well as the time of this event occurring. Germination and plumule emergence were analysed separately.

7.3.3 Results

All control seeds germinated and had their plumule emerge, but 2 mg mL^{-1} $AgNO_3$ inhibited all germination. The latter is therefore not shown in the depiction of results (Figure 7.2).

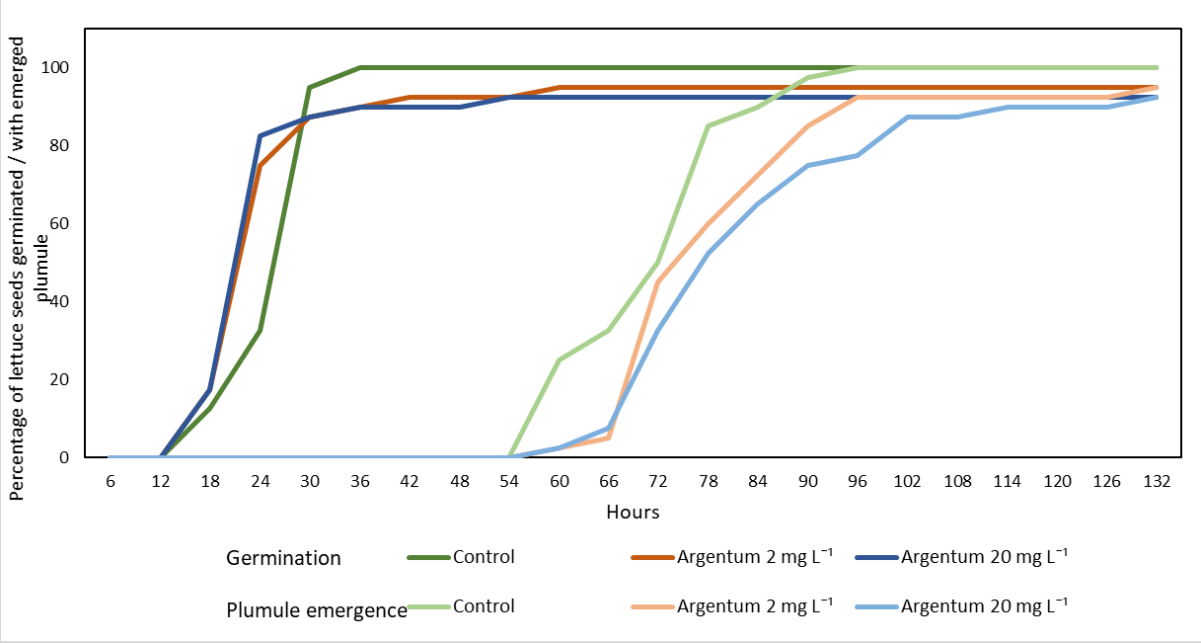

Figure 7.2: Results of a germination experiment exposing lettuce seeds to different forms and concentrations of silver

Kaplan Meier survival analysis with a Breslow post-hoc showed that lettuce seeds were significantly inhibited in germination and plumule emergence when exposed to both 2 mg mL^{-1} and 20 mg mL^{-1} of Argentum (X^2= 6.545, p=0.011; X^2=9.490, p=0.002 respectively). While this is easily visible from Figure

7.2 in the case of plumule emergence, it appeared that the control seeds were slower to germinate. This was the case, but as more seeds exposed to water germinated successfully compared to seeds exposed to Argentum, they statistically performed better overall.

7.3.4 Discussion

While lettuce seeds exposed to 2 and 20 mg mL^{-1} of Argentum at first appeared to germinate earlier than the control group, all seeds in the control group finally germinated, which probably influenced the given statistic sufficiently to make the control group significantly more successful.

Silver and especially AgNPs have been previously shown to inhibit germination of seeds (Wang et al., 2018, Budhani et al., 2019), which is consistent with the results seen in this experiment. It was not expected that 2 mg mL^{-1} $AgNO_3$ would inhibit all germination, as it did not cause a significant effect in a previous, preliminary, but similar experiment (Appendix V). In the previous, less extensive form of this experiment, only the higher concentration of $AgNO_3$ (20 mg mL^{-1}) completely inhibited germination. It is unclear what caused these disparate results, but human error cannot be excluded.

7.4 Radish growth in soil containing silver sulphide

7.4.1 Introduction

The effects of Ag_2S on earthworms was studied in Chapter 6 and it was shown that the negative effects were limited, compared to those of AgNPs. However, in consideration of application of biosolids onto agricultural fields, it is not only the soil community that may be affected, but also potentially the crops. An increase of AgNPs in a wastewater system would inevitably increase the amount of Ag_2S in biosolids deposited onto fields. To determine if this might have an effect on crops, was investigated in an exploratory experiment by growing radish in soil treated with amounts of Ag_2S. Radish was chosen because the edible part of the plant grows in direct contact with the Ag_2S. In addition, radish is a rapidly growing plant, historically used in experiments (OECD, 2006), which makes it a good model plant for experiments.

7.4.2 Methods

After the difficulties of growing lettuce in a growth chamber at UCLan (Section 7.2), an agreement was made to outsource the radish growth to a greenhouse at another University. Under direction from the

author, technical staff at the Berliner Hochschule für Technik (BHT), Germany, set up and monitored an experiment with data and samples sent back to UCLan for analysis.

As per common practice in the BHT greenhouse, a generic sandy topsoil was used, pH ~6.8; as per CHNS analysis; it contains 0.20% C, 3.18% H, and 0.36% N (compared to 0.19% C, 2.77% H, 0.60% N in Kettering Loam). From this, five soil concentrations were prepared, by mixing topsoil with 5 g kg^{-1} sand which contained differing levels of Ag_2S to achieve final treatment concentrations of 0, 50, 100, 500, and 1000 mg kg^{-1} Ag_2S in soil. Four replicate pots (15 x 15 x 5 cm) of each soil Ag_2S concentration were prepared, and 9 radish seeds (*Raphanus sativus L.*) were planted in each. In addition to the radishes, 8 further pots (4 each of control soil and 1000 mg kg^{-1} Ag_2S) were seeded with napa cabbage (*Brassica rapa*) as it is a common indicator for contaminated soil used in the BHT greenhouse. An automatic drip watering system (Gardena, OBI) ensured sufficient soil moisture content. Figure 7.3 shows the complete set up.

After 4 weeks, all radishes and cabbages were visually examined for any malformations and then harvested. Healthy and unhealthy plants were separated and leaves of the former were cut from the root. After mass determination of all plant parts, they were dried at 65 °C for 24 h. For further analysis the root and shoot samples were pooled by treatment, milled using an electric coffee grinder bought for this purpose (KYG, 300 W, Amazon) and sieved using a 1 mm sieve.

a)  b) 

Figure 7.3: a) Experimental set up of radishes growing in the greenhouse; b) Set up with watering system after some leaves emerged.

Samples were analysed for % CHNS using a ThermoScientific CHNS Organic Elemental Analyser (for detailed methods, see section 3.4).

In addition to CHNS analysis, samples of the ground radish roots were also examined using a ThermoScientific Quattro S scanning electron microscope (SEM) (detail in Section 3.6), in an attempt to assess silver and sulphur concentrations in the sample. Both the CHNS analyser and the SEM provide results in terms of percentage of the total sample. Statistical analysis used an ANOVA with a Tukey post-hoc test.

7.4.3 Results

Addition of Ag_2S to the soil caused an increase in the mass of radish plants, both in the mass of the leaves and mass of the radish root (Figure 7.4). Napa cabbages grown in both control soil and soil containing 1000 mg kg^{-1} Ag_2S did not differ significantly in mass (Figure 7.4), however when grown in the Ag_2S containing soil, more cabbages sprouted and grew healthily (there were no malformed plants in those pots).

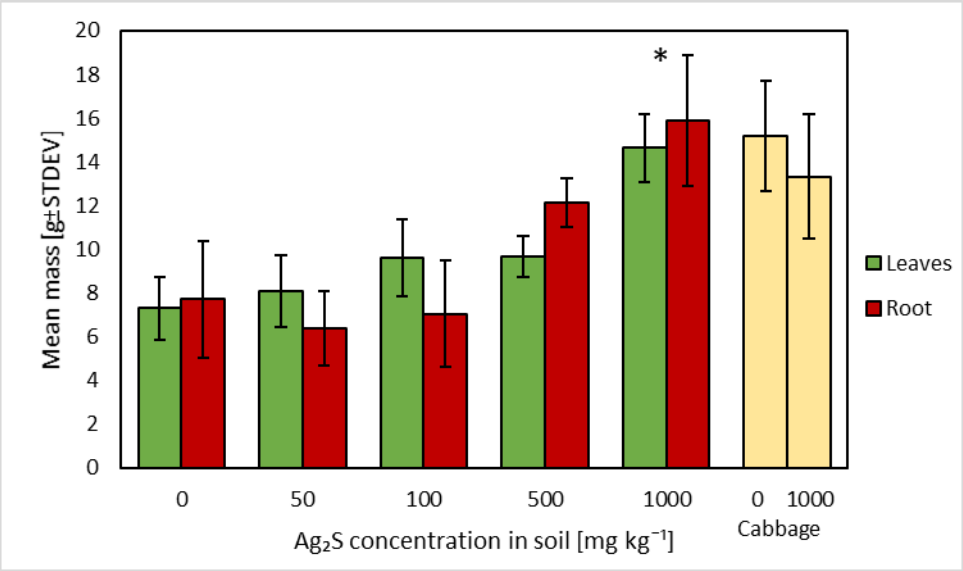

Figure 7.4: Mass of leaves and roots of radish plants grown in soil, with varying concentrations of Ag_2S, asterisk indicates a significant difference (ANOVA $p<0.05$)

Radish plants (leaves in addition to roots) grown in soil with 1000 mg kg^{-1} were significantly larger than those grown in all other conditions (ANOVA $F_{(4, 13)}=8.419$, $p=0.001$, Tukey post-hoc in both cases).

CHNS analysis had less consistent results, with sulphur not detected in all samples and hydrogen and nitrogen not having significant results. However, a trend was seen, in that content of nitrogen decreased and carbon content increases in the roots when grown in soil containing higher amounts of

Ag_2S (Figure 7.5). The carbon content in the 500 mg kg^{-1} Ag_2S-treated radishes differed significantly from 0 and 50 mg kg^{-1} Ag_2S.

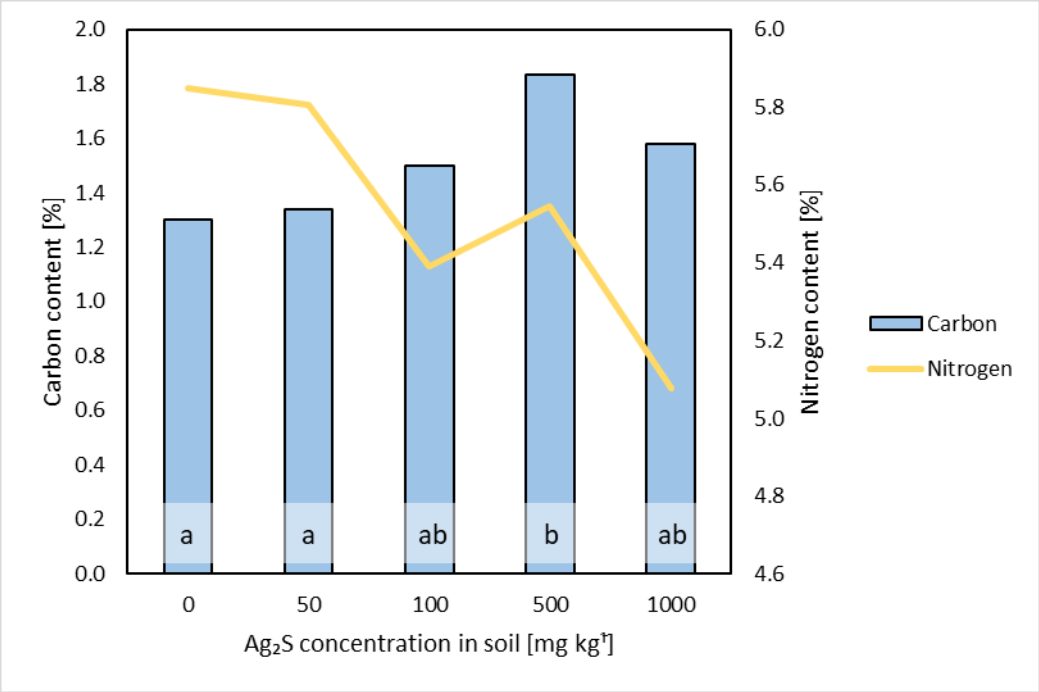

Figure 7.5: Carbon and Nitrogen content in radish roots grown in soil containing Ag_2S; difference in letters denotes a statistically significant difference (ANOVA $p<0.05$)

Measurement of sulphur content in the radishes proved to be a challenge but two data sets were obtained, one measured using CHNS analysis (Figure 7.6) and one from scanning electron microscopy (Figure 7.7).

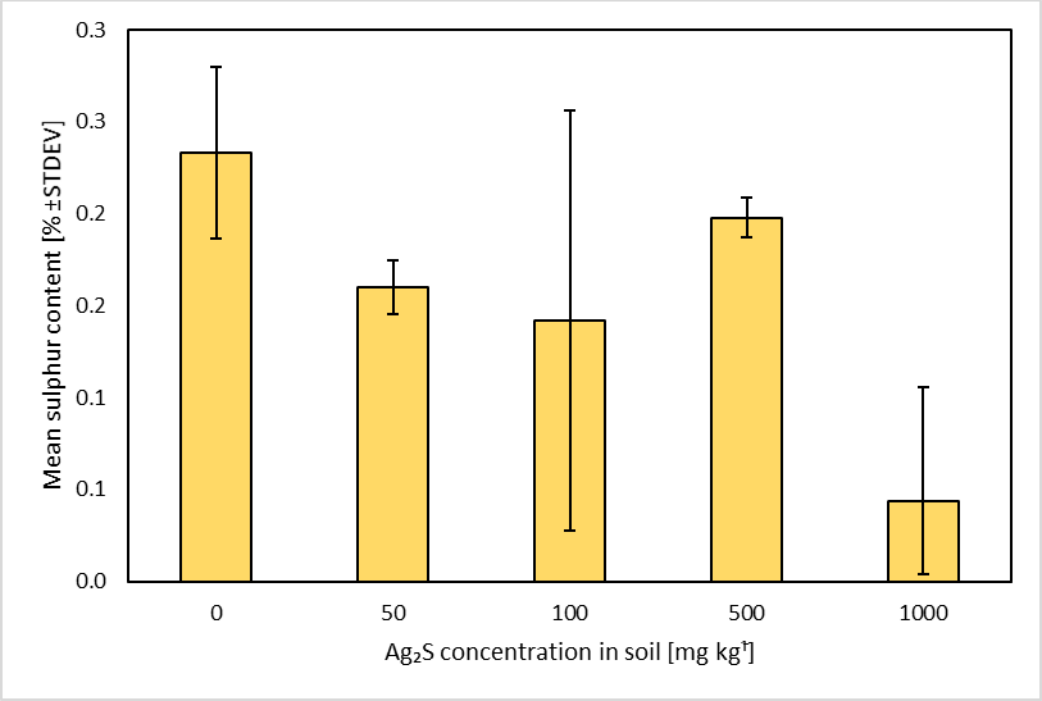

Figure 7.6: Sulphur content in radish roots grown in Ag_2S-containing soil, measured by CHNS analysis

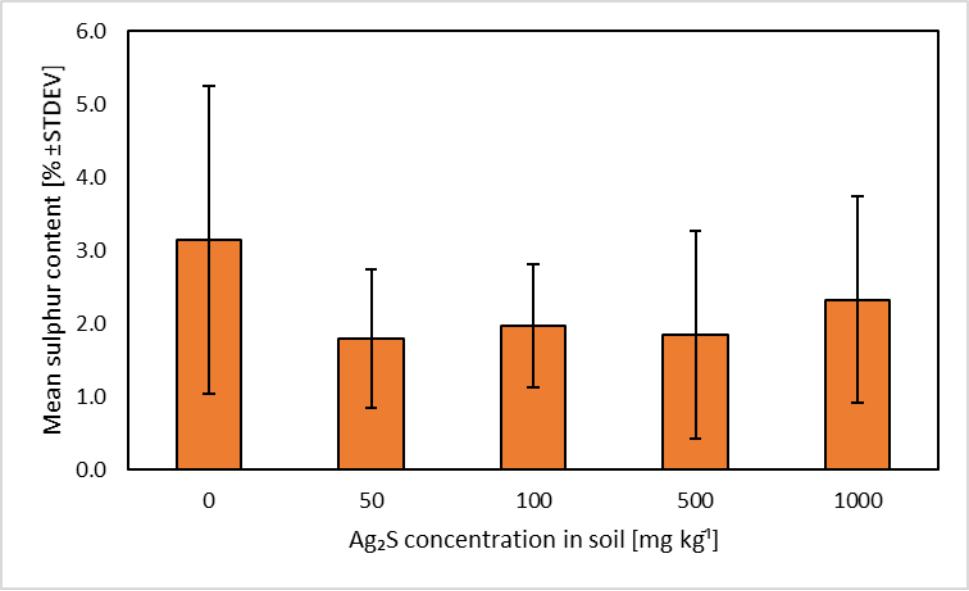

Figure 7.7: Sulphur content in radish roots grown in Ag_2S-containing soil, measured by SEM

While both techniques of measuring sulphur use dried, ground samples and gave results as a percentage, there is a large discrepancy between these results. In both cases, the deviation is too large to result in any statistical significances, but the sulphur content was less than 0.5% with a CHNS

analysis and around or above 2% using an SEM. The only similarity between the two methods appears to be a slight downwards trend in sulphur concentration with an increased exposure to Ag_2S.

The results from an attempt to estimate the silver content of radishes grown in Ag_2S-containing soil, measured with an SEM, are shown in Figure 7.8.

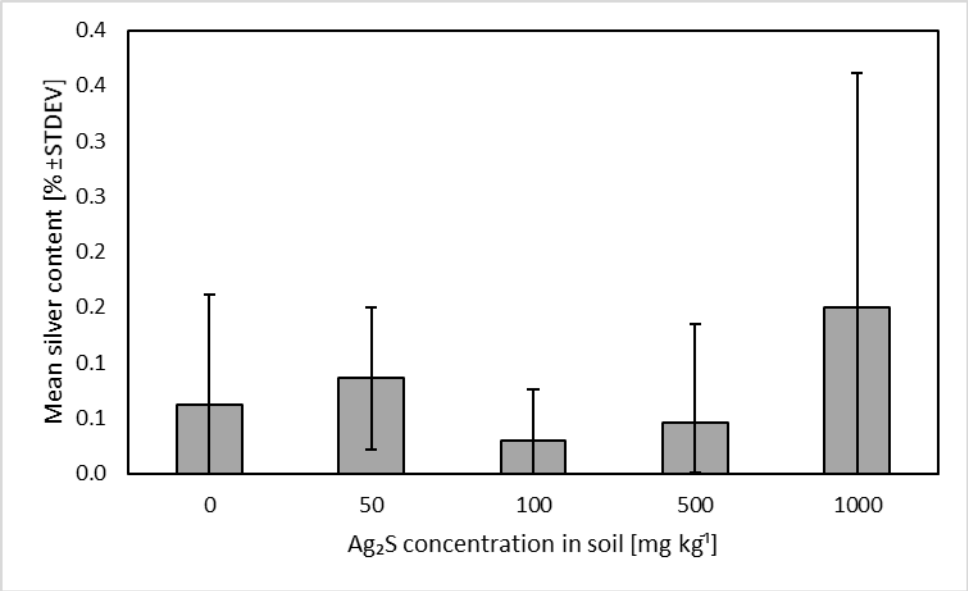

Figure 7.8: Silver content in radish roots grown in Ag_2S-containing soil, measured by SEM

The results varied widely, and no succinct conclusion could be made on whether any silver was taken up due to increased Ag_2S concentration in the soil. A result which matches the insignificant differences in sulphur content.

7.4.4 Discussion

With an increased level of Ag_2S in soil, radish plants appeared to grow larger and the increase in mass became significant at the highest treatment concentration. At the same time, the nitrogen content decreased, while the carbon content increased. A reason for the increase in carbon content could not be found, as it is difficult to know the implications of a higher or lower carbon content. It may be related to increased growth or a change in how the radishes store energy.

The decrease in nitrogen could be caused either by an increase in mass or the increase in Ag_2S concentration in the soil. With respect to Ag_2S content in the soil, one may assume a negative effect, however the total nitrogen content in the topsoil used was so low (less than 0.4%), that it is quite likely the radishes took up the available nitrogen. As the radishes grew larger, more nitrogen was taken up

to achieve this building up of mass, depleting the soil store. Once the soil had less or not enough nitrogen to provide, the plant nitrogen content decreases, which would explain the decrease in nitrogen content seen in Figure 7.5.

Overall, in this simplified experiment, the addition of Ag_2S to the soil did not appear to cause a negative effect on the growth of radishes, rather, the addition of Ag_2S appeared to have somewhat positive effects on both radishes and napa cabbage. In contrast to this, Wang et al. (2017b) found a significant reduction in growth of both cucumber and wheat seeds exposed to Ag_2S. A similar reduction was found in another study on wheat and cowpea (Wang et al., 2015). This study also noted that the effects were significantly weaker than that of AgNPs or $AgNO_3$, which show once again that the inertness of Ag_2S appears to have a decreased toxicity compared to AgNPs.

The main objective of this experiment, to estimate whether plants exposed to Ag_2S might lead to an increased human uptake in silver from food, could not be determined. Silver concentration in the radish was too low to measure accurately with the SEM, which could mean that there was very little present, or that a different method of analysis was needed. Other studies have found an uptake of silver when exposed to Ag_2S (Wang et al., 2017b), so it would have been expected to have taken place. While an analysis of silver content using an ICP-MS was planned, equipment failure (and delay to repair) prevented such an analysis.

Measurement of sulphur content seemed like a logical next step to estimate silver uptake, given that Ag_2S would be taken up into the plant. As described above, a realistic estimate of this also failed. If repeated, a better measurement for sulphur must be found, as it is difficult to quantify using ICP-MS or OES as oxygen causes interference during analysis (May and Wiedmeyer, 1998).

7.5 Food choice experiment by *A. caliginosa* with Argentum-treated birch leaves

7.5.1 Introduction

After an attempt to assess the impact of AgNP-containing fertiliser on the growth of lettuce (Section 7.2) and finding it could potentially affect germination (Section 7.3), the effects of AgNP-containing fertiliser on a selected earthworm species (*A. caliginosa*) was investigated. Earthworms feed on organic matter, including plant material, so if the plant material is treated with AgNPs, it may have effects on the health of the earthworms. A food choice experiment was chosen to investigate potential impacts of treated plant material.

7.5.2 Methods

Food choice chambers were prepared in the ERG laboratory as previously used (Rajapaksha et al., 2013, Ashwood et al., 2017, Butt et al., 2020). They were constructed from circular aluminium foil trays (20 cm in diameter and 2 cm high) to which six 1.5 mL Eppendorf tubes were attached. Prior to attachment, each tube cap was cut off and a hole was drilled through it, which later enabled earthworm entry to the tubes. Six equally spaced holes were cut into the wall of the aluminium tray into which the caps could be inserted, so that the tube on the outside was locked in place by the fenestrated cap on the inside (Figure 7.9).

Figure 7.9: Example of food choice chamber (20 cm ø) with aluminium foil cover removed, (author's image)

This was the second attempt at conducting a food choice experiment with Argentum and food for earthworms. The first attempted to use lettuce leaves grown with consistent dosing of Argentum, but failed due to problems with the growth chamber and shop-bought lettuce was used. During the experiment it was discovered that the lettuce quickly developed mould, and therefore the initial food choice experiment had to be stopped, as the Eppendorf tubes contained more mould than lettuce. Thereafter, dried birch leaves were chosen as representative plant material in this second experiment.

In addition, comparisons could be drawn with previous work as birch leaves had previously been used in the ERG laboratory to feed *A. caliginosa* (Rajapaksha et al., 2013).

Birch leaves, collected the previous autumn (October 2021) and air dried for storage, were treated with water, 2 mg mL^{-1} Argentum and 20 mg mL^{-1} Argentum, (concentrations used in a previous experiment - Section 7.2). After air drying for 24 h (with the treatments applied), the leaves were shuffled to turn them over and the treatment process was repeated twice more. After the final drying step, the leaves were ground into fine powder using an electric coffee grinder (KYG, 300 W, Amazon) and passed through a 1 mm sieve. The bodies of each Eppendorf tube had masses recorded individually and were marked accordingly. For each treatment, 10 Eppendorf tube bodies were filled with the ground leaves which were then wetted and left to soak in the tubes overnight. After draining any excess water by leaving them upside down on absorbent paper for 4 hours, all Eppendorf tubes with birch treatment contents has masses recorded to give time zero mass.

Each of the 5 replicate choice chambers were filled with wetted Kettering Loam (approximately 25-30% moisture content), to which 6 filled Eppendorf tubes were then attached (2 of each treatment, attached sequentially - 0, 2, and 20 mg kg^{-1} twice). These were labelled accordingly. An aluminium foil sheet was then fastened over the trays, secured with a rubber band, and the trays were left to equilibrate for 2 days (Figure 7.10).

Figure 7.10: Example of food choice chamber (20 cm ø) with aluminium foil cover in place, (author's image)

At the beginning of the experiment, 5 adult, laboratory-reared *A. caliginosa* (mean mass: 565.24 mg) were placed into each choice chamber and the aluminium foil was reattached. This marked the start of the experiment and from then on, all Eppendorf tubes had masses determined at regular intervals and the soil in the choice chamber was moistened with a spray of water if it became dry.

7.5.3 Results

Over the course of 40 days, *A. caliginosa* did not prefer or avoid food treated with Argentum (Figure 7.11). Any differences were minor and not statistically significant. One outlier data point was excluded from all data analysis at it was consistently lower than all other data points from that treatment.

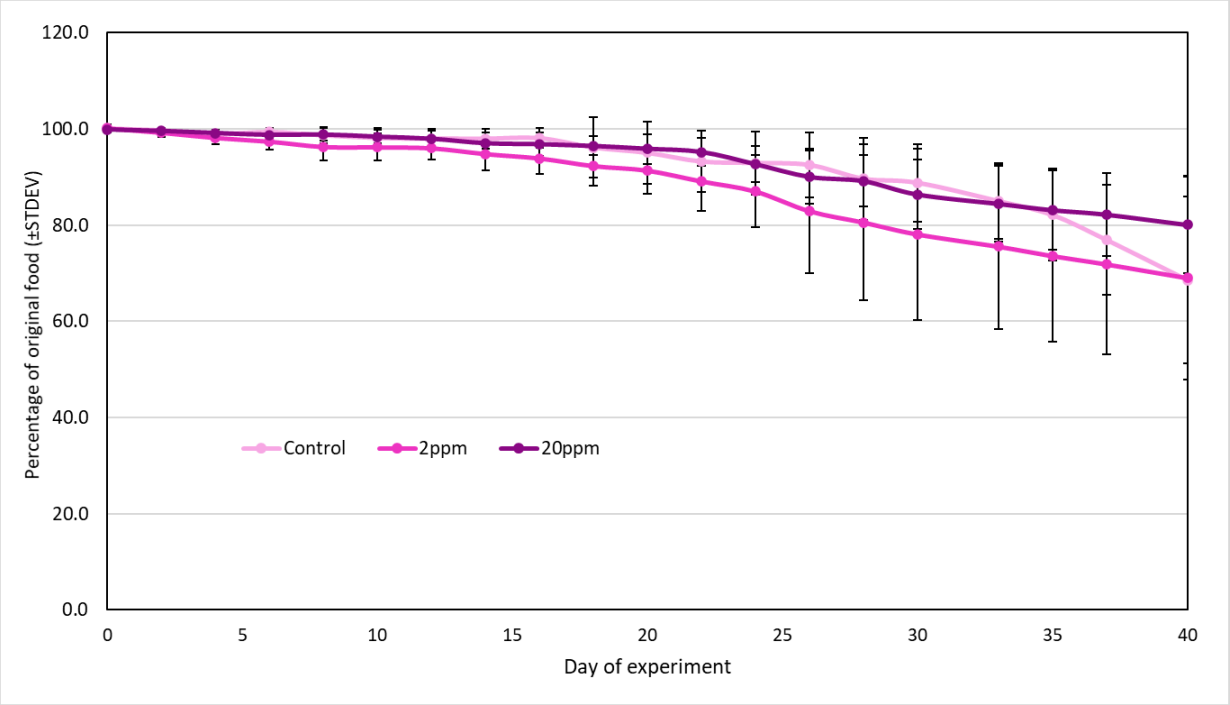

Figure 7.11: Percentage of original food remaining in the food choice experiment using A. caliginosa and Argentum-treated birch leaves

Over the course of the experiment, 2 earthworms died, 3 worms aestivated, and the mean mass declined in each choice chamber. The overall mean mass of surviving earthworms at the end of the experiment was 441.15 mg. The experiment was terminated after 40 days.

7.5.4 Discussion

A lack of preference for a particular food treatment is one of the possible results that can be found in a food choice experiment. However, the earthworms would then be expected to feed on all provided

choices equally for this to be the outcome. Here, this was not the case as little food was taken from any of the treatments. At the outset of the experiment, it was to be expected that the (endogeic) earthworms would feed on the organic matter present in the Kettering Loam rather than foraging for food in the Eppendorf tubes (as might be seen with anecic species). For an experiment to last for 40 days without a substantial decrease in food is unusual, and earthworms should not be losing an average of 20% of their mass. Rajapaksha et al. (2013) used 15 *A. caliginosa* per tray (field collected with a mean starting mass of 430 mg), in a food choice experiment using different tree leaves including birch, where about 50% of the provided birch leaves were consumed within 7 days. Of the offered food sources, 3 were completely consumed after 28 days and while triple the number of earthworms were available to feed, it is a stark contrast to the experiment described here where less than 30% of the leaves were consumed after 40 days.

With the objective to determine whether earthworms avoid food contaminated with silver containing plant protection products, two main decisions were made. First, which earthworm species to use. This type of experiment works particularly well with the feeding pattern exhibited by anecic earthworms, but throughout all other experiments described in this thesis, *A. caliginosa* were used which are an endogeic species. Second was the choice of plant material to be used as a food source. It could have been a leaf treated with plant protection products in a realistic scenario, such as lettuce or a material previously used in food choice experiments such as straw or birch leaves (e.g. Rajapaksha et al. (2013) or a food known to be eaten by the target species, such as horse manure (Butt and Lowe, 2011).

For the choice of earthworm species, it was decided to maintain consistency across experiments by using *A. caliginosa* rather than using another species for one experiment. In the case of plant matter, an initial attempt to use lettuce leaves had failed due to growth of mould. Therefore, birch leaves were chosen, a material which had previously been shown by Rajapaksha et al. (2013) to be a food source accepted by *A. caliginosa*. Laboratory-maintained earthworms, used in this experiment, had been fed horse manure their whole lives so that the sudden change in food choice may have initially been part of the cause for rejection. Another possibility is that the Eppendorf tubes were packed too tightly, and the earthworms had trouble accessing the food materials on offer. In the end, it cannot be determined whether Argentum treatment had any effects on the choice of food materials by *A. caliginosa*.

7.6 Overall Discussion

Experiments detailed in this Chapter are very different from each other and do not aid an overall understanding of a single question. It is, however, important to look at possible effects of AgNPs and subsequently Ag_2S on not only soil fauna but also on flora. In this Chapter, a variety of potential areas of interest were investigated relating to plants including: the effects of AgNP-containing fertiliser; the effects of increased Ag_2S particles in soil; the potential consequences of contaminated food sources for earthworms. While many of these experiments have shown limited results, partially due to time restrictions and partially due to insufficient analytic capabilities, they do provide a starting point for further questions and investigations that can be developed. In addition, some of the experiments undertaken have shown some of the problems that can befall research of this nature. Nevertheless, this is an area where more studies could be done.

8 Discussion

8.1 Introduction

This research aimed to investigate the impact of silver nanoparticles (AgNPs) in the agricultural environment, explored through assessment of effects on a component of the endogeic earthworm community, in addition to various routes through which silver could enter and affect such systems. AgNPs are a well-researched subject in the scientific community but there are some aspects which have received less attention.

The previous experimental Chapters sought to bridge the gap in research with a range of laboratory-based investigations using the endogeic earthworm *Aporrectodea caliginosa* plus experiments with lettuce and radish. This Chapter will provide a wider, more general discussion that brings together all findings. Subsections of this Chapter start with initial research aims and discuss how well these were achieved. The Chapter ends with a discussion of some limitations of the research conducted and an outlook towards further research that could be undertaken.

8.2 Aim 1: Assess the effects of AgNPs and Ag_2S in soil on the earthworm community, by recording survival, growth, avoidance behaviour, and reproduction of *A. caliginosa* exposed to AgNPs and Ag_2S in soil

The main focal point of this thesis, this aim was addressed in most experiments conducted and therefore provided the most results. Utilisation of an endogeic earthworm species plus all experiments undertaken in the same laboratory environment, on a homogenous group of *A. caliginosa* fortified the results. Overall, it was found that high concentrations of pristine AgNPs had a strong negative effect towards *A. caliginosa*, but that these effects waned at lower concentrations (akin to those that might be expected in the environment) or once the AgNPs became less reactive. Both aging through air exposure and reacting with sulphide would make AgNPs less reactive. The latter occurs after traversing the wastewater treatment system and results in biosolids being contaminated with silver sulphide (Ag_2S), rather than AgNPs.

As seen in previous studies, AgNPs were toxic towards *A. caliginosa*. They caused a reduction in body mass and, at concentrations above 500 mg kg^{-1}, started to cause mortality. Earthworms with lower body mass were more susceptible to death from exposure to high concentrations of AgNPs, which suggested that hatchlings might be more vulnerable to AgNP exposure. However, it was shown that

hatchlings were no more susceptive to mortality due to AgNPs exposure than adults. There is still uncertainty as to why this was observed, and it might have been due to the choice of earthworm species not being suitable to hatchling experimentation. The differing rate of mass gain between individuals complicated the experiment and therefore another species, such as *Allolobophora chlorotica*, which grows to a more uniform end mass, might be better suited. It is also possible that toxicity experiments using earthworm hatchlings are not reliable in general.

By performing similar experiments with aged and with pristine particles, it can be infered that AgNP toxicity was caused through the release of Ag^+ ions, because aged (oxidised) AgNPs caused no mortality and only brought about a limited decrease in body mass. *A. caliginosa* sought to avoid areas contaminated with pristine AgNPs, which was only significant from a soil concentration of 100 mg kg^{-1}, but the avoidance behaviour was directly related to treatment concentrations. However, this was only observed when a linear avoidance chamber was used. Results from a circular avoidance chamber were inconclusive and may suggest the design of such an experimental apparatus is fundamentally flawed.

Ag_2S had a lower toxic effect towards *A. caliginosa* compared to AgNPs, with neither mortality nor significant loss in mass recorded throughout experiments. These results indicated that the binding of silver to sulphur is strong enough to inhibit the release of Ag^+ ions, and the results were similar to those in experiments which used oxidised AgNPs. *A. caliginosa* did, however, significantly prefer control soil to soil containing any amount of Ag_2S, which suggested that there was either a low level of toxicity not found in other experiments or that the particles could have been abrasive, and earthworms avoided spiked soil. While an exact reason for this result could not be determined, it could still lead to a negative effect in the field if *A. caliginosa*, or even all earthworm species, avoided any areas where Ag_2S particles were present. Nevertheless, this should be taken in context with experimental results where *A. caliginosa* preferred soil containing biosolids to soil with none. The proposed reason for an increase of Ag_2S in soil is the application of biosolids, therefore, the preference behaviour to the biosolids and Ag_2S linear avoidance experiments conflict with each other. Without further research the extent to which Ag_2S avoidance would affect an agricultural system is difficult to fully speculate upon.

8.3 Aim 2: Assess the effects of AgNPs and Ag_2S on plants and estimate silver uptake by humans after consumption of produce exposed to silver

Due to external circumstances, this aim was addressed less completely than originally planned. It does, however, remain an interesting area for research with further knowledge to be gained.

AgNP-containing plant protection products such as Argentum can inhibit both germination and plant growth at high concentrations, when applied to lettuce seeds or growing plants. However, these results only become apparent at concentrations higher than the recommended application rate, meaning that if used in moderation and as directed by the manufacturer, the benefits are likely to outweigh the negative effects. AgNPs provide a slight antimicrobial effect and therefore could protect lettuce from fungi, potentially increasing the yield, an endpoint that was not studied in the current experiments. Results from the germination experiment should be seen as preliminary and could be followed by a complementary study investigating sprouting success in contaminated soil providing further insight into the results.

To compensate for an inability to perform ICP-MS analysis to determine the concentration of silver in the radish grown in Ag_2S containing-soil, both SEM-EDX and CHNS analysis were employed. Silver content from SEM measurement varied widely despite having multiple sampling points per sample, and the attempt to quantify sulphur was unsuccessful. As sulphur is a component of Ag_2S but is also a micronutrient found in plants to varying degrees, quantification meant relating it solely to Ag_2S uptake, which was difficult to interpret.

Ag_2S in the soil affected radish growth. Increased radish mass was recorded, both above and below ground, which related to the increase in Ag_2S concentration in the soil and was significant at a soil concentration of 1000 mg kg^{-1}. This coincided with an increase in total carbon content and a decrease in total nitrogen content in the radish. The decrease in total nitrogen could be explained by depletion to support the increase in radish mass, the increase in carbon content was less readily explained.

8.4 General discussion

To conclusively assess the effects of an increase in use of AgNPs on agroecosystems is difficult. There are many variables: environmental conditions, different forms of silver, various routes how the silver can reach the soil, and each has different effects on different soil-dwelling species, plus the differing effects within a species. Pristine AgNPs appeared to be the most toxic, so are the primary concern. This means that use of AgNP-containing plant protection products is a larger concern than the increased use of AgNP-containing products by consumers.

Assessing the impact of AgNPs on soil due to the increased use of AgNP-containing consumer products and subsequent application of biosolids appears erroneous. Previous research has shown that this would not increase amounts of AgNPs in soil but rather increase levels of Ag_2S. The work of this thesis has shown that the effects of AgNPs and Ag_2S are not comparable in severity. Ag_2S was significantly

less toxic towards *A. caliginosa* to such a degree that it can be hypothesised that Ag_2S is likely less toxic towards other members of the soil fauna. More research needs to be conducted concerning the increasing presence of Ag_2S in soil, as this relatively small series of experiments is only a beginning. Nevertheless, results suggest that an increase in silver going through the wastewater treatment system is probably not a major concern for the health of earthworms, and perhaps the wider environment.

8.5 Contribution to knowledge

This research has made the following contributions to knowledge:

- Toxic effects of AgNPs on endogeic earthworms are dependent on the size of the earthworm, with lower body mass resulting in a higher susceptibility to negative effects (such as mortality).
- Aged/oxidised AgNPs have drastically reduced toxic effects on *A. caliginosa* (and likely other soil dwelling earthworm species) when compared with pristine AgNPs.
- Negative effects on cocoon production by *A. caliginosa*, caused by low levels of AgNPs, appear short-lived after the earthworms acclimatise to given soil conditions.
- Ag_2S particles have few direct negative effects on mass, mortality, or reproduction of *A. caliginosa*. Avoidance behaviour is nevertheless exhibited by this earthworm to the presence of Ag_2S.

8.6 Limitations

There were many limitations within this research, a majority stemming from lack of time to do further, more specific experiments or repeating experiment which did not go to plan. This was due to a loss of laboratory access because of Covid-19 and could not be adequately rectified. When, in the given timeframe, 'lockdown' started, two experiments had to be aborted without any usable data, and no laboratory access was possible for a full 5 months. Thereafter only 1 day per week was authorised. At this point, the majority of earthworm stock had perished or was in no state to perform experiments with. After another shorter, period of no access from mid-December to mid-January, full laboratory access was only achieved in February 2021. Considering the aborted experiments, the time without any or at least without sufficient access, as well as the loss of earthworm stock, it can be estimated that almost 12 months of experimental time was lost, at a critical phase of the project.

In addition to this, a relatively large amount of analytical work was not possible due to faulty equipment in the UCLan analytical suite, which caused a lack of data such as missing silver concentrations in plant-related experiments. The unavailable instrument was the ICP-MS, and the ICP-OES was not sensitive enough to replace it. Attempts were made to undertake as much work as possible, but results obtained were not ideal.

Despite the adverse circumstances, a large amount of data was collected and while not all research questions could be addressed satisfactory, knowledge was gained.

8.7 Further research

Use of a single earthworm species throughout all experiments in this thesis made the results comparable and is a practice which should be adopted more widely. Repetition of similar experiments in the same laboratory would provide further insight into differences between species, potentially with investigations into why species react differently by performing, for example, biomarker analysis. Another species might also be more appropriate to conduct long-term hatchling-related experiments with. This is a concept that should not be forgotten as it could provide insight into true chronic effects of earthworm population exposure to toxic substances in the environment.

Results in this thesis showed that Ag_2S was significantly less toxic to *A. caliginosa* than AgNPs, which could be investigated further. For example, in biosolids and Ag_2S combination treatments or other mixtures likely to occur in the field. In this thesis, smaller organisms were found to be more susceptible to toxic effects of AgNPs, then smaller members of the soil fauna could also be included in such experiments. These could include earthworm species but extend to other beneficial soil organisms.

No reason was found why *A. caliginosa* avoided areas with Ag_2S, and more experiments could investigate this, and also effects on other earthworm species. It is also possible that preference for biosolids-containing soil negates the avoidance of Ag_2S-containing soil. A series of studies, including some that are field based, could be employed to better understand such avoidance behaviour.

The effects on plants were not well investigated in this thesis due to adverse circumstances but could be a point of interest going forwards. A start would be a further investigation into the differences in germination when lettuce seeds were exposed to different forms of silver by performing in-soil germination experiments. Results from radish growth in Ag_2S-containing soil leave many questions, including carbon and nitrogen content which could affect plant health or quality of produce.

Furthermore, the silver content in crops grown in Ag_2S-containing soil or sprayed with AgNP-containing products should be determined, as it could add to the human silver burden. Such experiments could determine if washing would remove any silver applied to the surface. Effects on fauna should not be neglected, whether it is through feeding or food choice experiments of AgNP-treated leaves to earthworms. Much more could be learned here from further experimentation.

8.7.1 Future research, presented as bullet points

- Repetition of experiments concerning the effects of AgNPs and Ag_2S on different earthworm species and other soil fauna
- Using hatchlings, of endogeic earthworm species with uniform adult mass, to investigate long-term effects
- Combination studies including biosolids and other fertilisers with Ag_2S
- Determine why Ag_2S-containing soil was avoided by *A. caliginosa*
- Effects of AgNP-containing products on seed germination in soil
- Repetition of plant experiments with determination of silver uptake
- Choice chamber experiments to examine effects of AgNP-treated food sources
- Investigation of the effects of Ag_2S on carbon and nitrogen content in radish

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APPENDICES

Appendix I: Ethical approval

27 September 2019

Chris Lowe & Jeannette Kister
School of Forensic and Applied Sciences
University of Central Lancashire

Dear Chris and Jeannette

Re: Science Ethics Review Panel Application
Unique reference Number: Science 0005

On the basis of the information contained in the Research Degrees Application form, the Health Ethics Review Panel does not envisage any insoluble ethical issues arising that might make the proposed project non-viable for ***MPhil/PhD***. The committee therefore has no objection to the project '***Assessing the toxicological effects of very small silver particles on the environment. That includes experiments with soil and earthworms.***' proceeding to research programme approval / registration.

It appears from the Research Degrees Registration application, that the project will not require full ethics committee approval. This is because it does not mention the inclusion of human (or animal) research participants or their data and seems not to have any significant ethical issues. If any phase of the research includes human (or animal) research participants or their data, or significant ethical issues are identified by you or your supervisory team, a full proposal application will need to be submitted to and approved by the Ethics Review Panel. If this occurs, please ensure that you quote the unique reference number (above) on your application form. (You may then also find it convenient to make separate proposal applications for different stages of the project, especially if the design of the later stages is highly dependent on the findings from the earlier stages.)

Yours sincerely

Afzal Ali
Research and Integrity Officer

Science Ethics Panel

NB - Ethical approval is contingent on any health and safety checklists having been completed, and necessary approvals gained as a result.

Appendix Figure 1: Copy of ethical approval for this research project

Appendix II: Long-term toxicity of aged AgNPs towards hatchling *Aporrectodea caliginosa*

Introduction

Most earthworm toxicity experiments are either short, such as acute toxicity or avoidance experiments, or run for a certain number of months such a reproduction experiment. They all require healthy worms being exposed to substances for a set amount of time and a main endpoint is measured, such as mass, avoidance, number of cocoons produces.

Low concentrations of suspected toxicants in the soil might have minute effects on the lifecycle of earthworms which escape other experiments. A long-term experiment was devised to investigate such effects. Currently, there are no guidelines on how to perform such an experiment on earthworms. The experiment was set up to use hatchlings and have them grow in soil containing AgNPs in minute concentrations, and once they were mature, to measure their cocoon production and time for the hatchlings from these produced cocoons to reach maturity. The earthworms were to be exposed to the AgNP-containing soil for 1-1.5 years in different generations with regular monitoring.

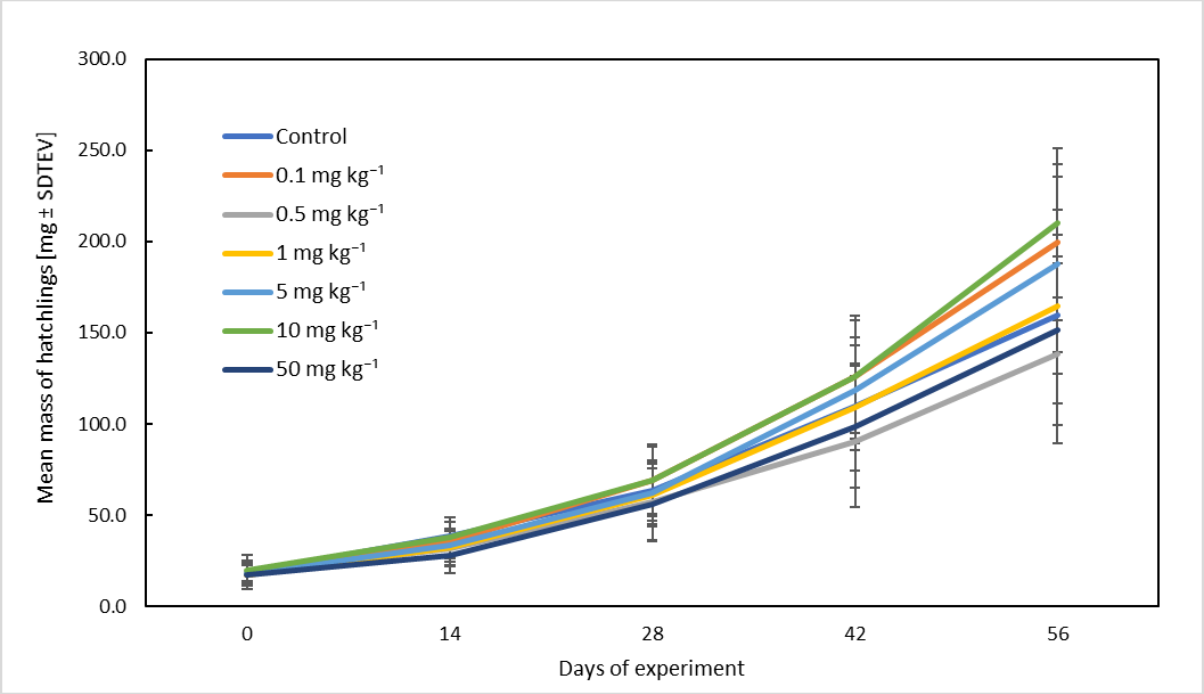
Methods

Aporrectodea caliginosa hatchlings were produced in the Earthworm Research Group laboratory and kept in water and at 5 °C to prevent growth until a sufficient number were collected. Prior to the experiment, hatchlings were acclimated by introduction to moistened Kettering Loam for 7 days (15° C; darkness (Butt and Lowe, 2011)).

For the experiment, soil was prepared using Kettering Loam, 5% horse manure (HM) and aged AgNPs in the concentrations: 0, 0.1, 0.5, 1, 5, 10, and 50 mg kg^{-1}. For details on soil preparation, see Section 3.2. For each soil concentration, 7 single use plastic pots (Section 3.3.1) were filled and to each pot, 2 *A. caliginosa* hatchlings were added. The pots were placed into an incubator at 15° C in complete darkness. Every 14 days, earthworms had their mass determined and their condition was observed. After 8 weeks, it became apparent that the experiment would not give adequate results and was aborted.

Results

The 8-week extended experiment which exposed hatchling *A. caliginosa* to low concentrations of aged AgNPs (Appendix Figure 2) showed no significant difference in mass gain between treatment groups.



Appendix Figure 2: Change in mass (± standard deviation) of hatchling A. caliginosa exposed to aged AgNPs over 8 weeks

After 8 weeks, the mean mass of the hatchling varied widely between 138 mg in the group exposed to 0.5 mg kg^{-1} and 209 mg in those exposed to 10 mg kg^{-1}. This disparity would have likely increased had the experiment gone on for longer and as there did not appear to be a relation between gain in mass and treatment, the experiment was ended.

Discussion

While the concept of exposing hatchlings to low concentrations of a toxin for a long period of time remains relevant, the main problem with the experiment was the species used. Adult *A. caliginosa* vary in body mass, often from around 300 mg up to over 1500 mg. Therefore, any hatchling, despite their original size, can grow to widely different end-mass. As the focus of experiments in this thesis were *A. caliginosa*, it was decided that no more long-term hatchling experiments would be conducted. Overall, it is unlikely hatchling experiments will be conducted widely, despite their potential.

Appendix III: Silver content in soil amended with aged AgNPs

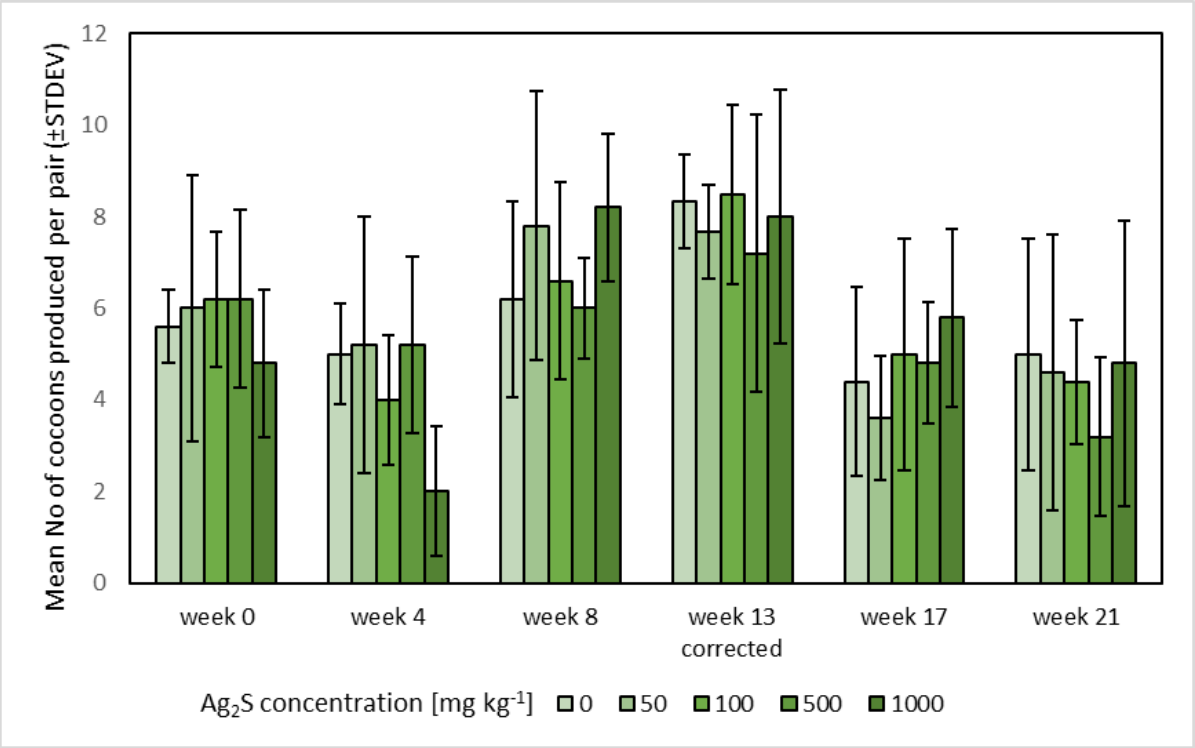
Appendix Table 2: Silver content in soil amended with aged AgNPs, measured with ICP-MS

Expected Ag soil concentration [mg kg^{-1}]	10	100	250	500	1000
Measured Ag concentration [mg kg^{-1}]	14.96	177.89	429.6	705.31	1702.73

The measured silver concentrations are higher than as prepared, but this discrepancy can be explained by sample preparation. When digesting soil for ICP-MS analysis, it has to be sieved through a 0.2 mm sieve. Prior to this, the dry soil is crushed using a pestle and mortar. While this was done thoroughly, it can be expected that smaller parts of the soil, such as the AgNPs and sand with which they were diluted, pass through the sieve more easily and are therefore over-represented in the digested sample.

Appendix IV: Corrected cocoon production figure (Ag_2S reproduction experiment)

Due to a missed sampling week, one sampling point (week 13) collected data of 5 weeks rather than 4. While this had no effect on the mass of *A. caliginosa*, it influenced the cocoon production data, as earthworm pairs had been producing cocoons for an additional week. Therefore, a correction factor was applied with 20% deducted from all cocoon production data points. Results are shown in Appendix Figure 3.



Appendix Figure 3: Mean number of cocoons produced per pair of A. caliginosa exposed to selected concentrations of Ag_2S (corrected)

In the corrected graph, it is apparent that the cocoon production was similar to the previous month.

Appendix V: Prior iteration of a germination experiment

Introduction

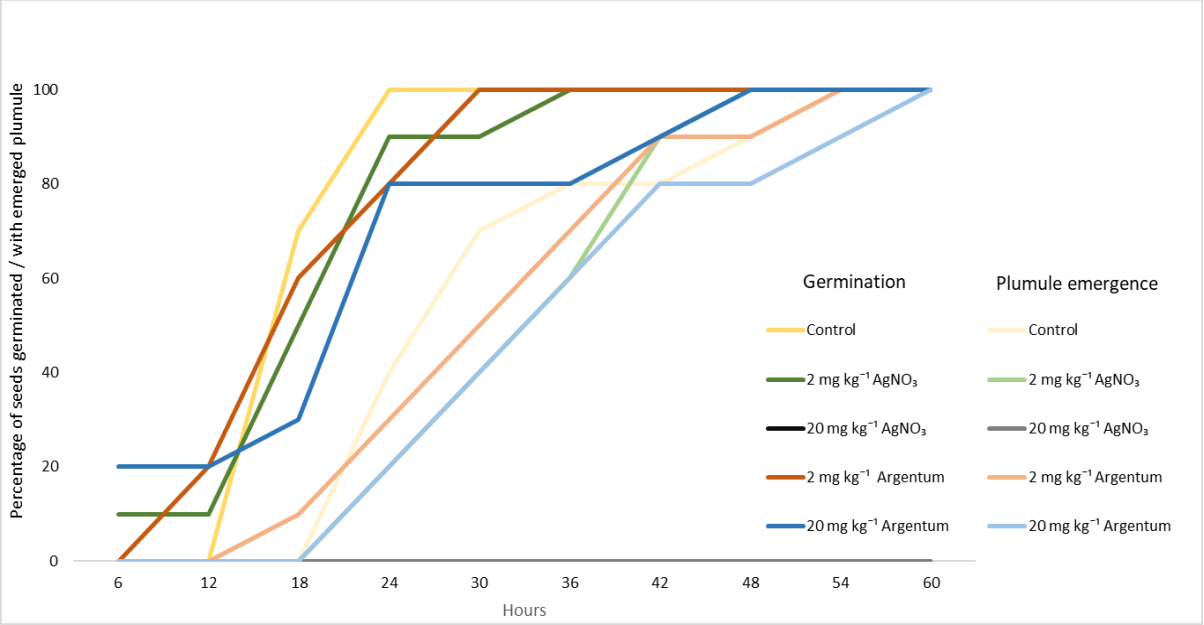
This appendix describes the penultimate iteration of a line of preliminary germination experiment to assess and improve the testing conditions and ensure that the most suitable seed was used. While data trends were visible, the number of seeds used was too low to result in significant differences, which is why the final experiment had only one type of seed and fewer treatment concentrations to permit a greater number of lettuce seeds.

Methods

Petri dishes (9 cm ø) were lined with No. 1 Whatman filter papers and wetted with approximately 5 mL of either distilled water, 2 mg mL^{-1} Argentum, 20 mg mL^{-1} Argentum, 2 mg mL^{-1} $AgNO_3$, or 20 mg mL^{-1} $AgNO_3$. Each treatment concentration had two replicates with 20 lettuce seeds placed onto the filter paper. The Petri dishes were kept at ambient temperature (21-25 ^{o}C) in a large plastic tray, covered with aluminium foil with observations made every 6 hours. At each time point, the seeds were monitored for the first sign of radicle emergence and for the first sign of plumule emergence. Filter papers were re-wetted as needed, if it became too dry. For statistical analysis of the results, the Kaplan-Meier survival test was chosen, as it takes into account both "survival" (germination or plumule emergence) in addition to the time of this event occurring. Germination and plumule emergence were analysed separately.

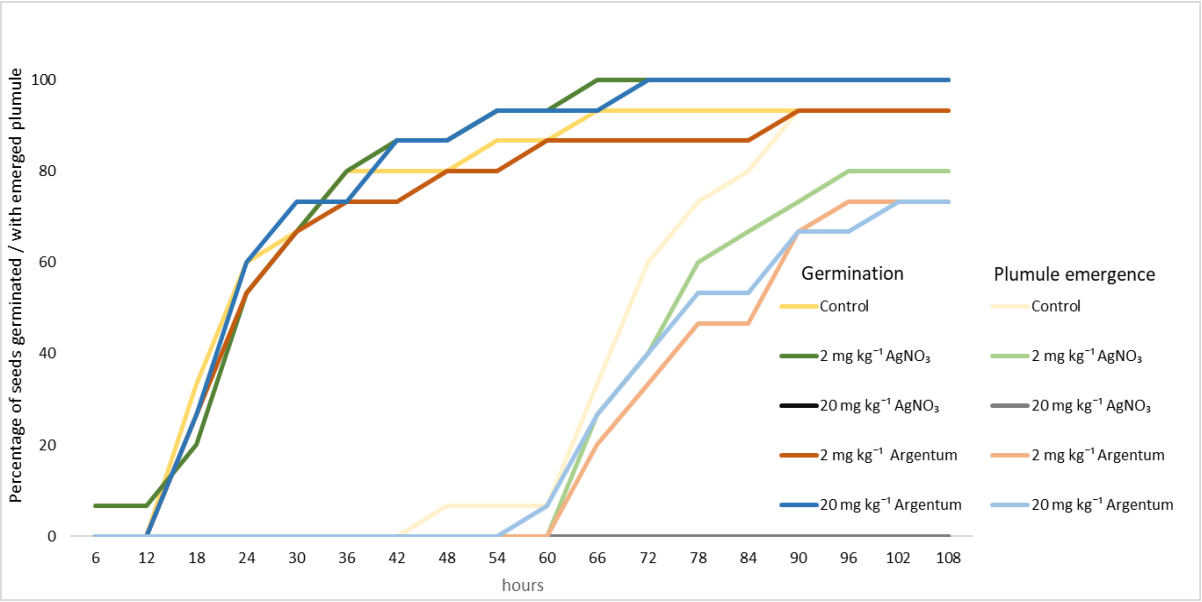
Results

In the case of both lettuce and radish seeds, 20 mg mL^{-1} $AgNO_3$ inhibited germination and therefore also all plumule emergence. Radish seeds rapidly germinated and had their plumule emerge (Appendix Figure 4) and the control treatment appeared to perform best followed by low concentrations of both Argentum and $AgNO_3$. However, these results were not significant.



Appendix Figure 4: Germination and plumule emergence of radish seeds exposed to different forms of silver

Lettuce seeds took longer to germinate than radish, and the trends were less visible and also not significant (Appendix Figure 5)



Appendix Figure 5: Germination and plumule emergence of lettuce seeds exposed to different forms of silver

The results from this experiment suggested that lettuce seeds were better suited for this type of investigation, because they took longer to germinate and therefore differences between treatments were more likely to be observed. In addition, lettuce seeds are smaller than radish seeds and the experiment could be scaled up more easily.