Ecotoxicological effects of deinking biosolids and the cytotoxic potential of microplastics-bound copper on the earthworm *Eisenia fetida*

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A to polyethylene high density, with spectral similarity at the C-H, and the C-C peaks. A shift in transmittance was noted, likely due to comparing samples extracted from deinking biosolids to pristine polymers in the database. This preliminary analysis validated the presence of microplastics in deinking biosolids.

LIST OF ABBREVIATIONS

CFIA	Canadian Food Inspection Agency
EPA	Environmental Protection Agency
OECD	Organisation for Economic Co-operation and Development
ROS	Reactive oxygen species
DAMP	Danger-associated molecular patterns
TLR	Toll-like receptors
DMT1	Divalent metal transporter 1
MT	Metallothionine
SOD	Superoxide dismutase
CAT	Catalase
GPx	Glutathione peroxidase
GSH	Glutathione
MDA	Malondialdehyde
TCEP	Tris(2-chloroethyl) phosphate
CCME	Canadian Council of Ministers of the Environment
MAPAQ	Ministry of Agriculture, Fisheries and Food in Quebec

ABSTRACT

Deinking biosolids are a byproduct of the fine sieving, floatation and clarification processes used to remove ink during paper recycling. They are nutrient-rich organic residual that can be used to fertilize plants. However, they also contain non-nutritive substances, including plastics and trace metals, which are a potential risk to the environment and public health. The aim of this research was to assess the toxicity of deinking biosolids and microplastic-bound copper to earthworms, an indicator species for soil ecotoxicology, to contribute valuable insights that can inform decisions and policies for the management of organic residues. The specific objectives of this thesis were to: (1) investigate the chronic toxicity of deinking biosolids in copper-amended artificial soil to earthworms through growth and reproduction assays; (2) quantify cell viability as the outcome of a size-dependent phagocytic response in earthworm coelomocytes; (3) evaluate how deinking biosolids and aged microplastics-bound copper impact earthworms, specifically in terms of causing oxidative stress and DNA damage. In the chronic toxicity test, earthworm reproduction was inhibited by exposure to ≥ 68 g deinking biosolids or ≥ 410 mg copper compared to controls ($p < 0.05$). However, adding deinking biosolids to copper-spiked soil did not exacerbate copper toxicity or increase copper accumulation in earthworms and subcellular fractions (cytosol and metallothionine) compared to single-treatment exposures. The *in vitro* investigation of the cytotoxicity of polyethylene microplastics in earthworm coelomocytes revealed size-dependent differences in phagocytosis. Coelomocytes engulfed 85% of small polyethylene microplastics (1–10 µm) at a cell: particle ratio of 1:5 through phagocytosis ($p < 0.05$), while phagocytosis of larger polyethylene microplastics (20–27 µm) was negligible. Despite these differences, cell viability was reduced significantly to 6–7% when coelomocytes were exposed to small and large polyethylene microplastics, compared to 94% viability in untreated control. Furthermore,

exposing earthworms to pristine, and aged copper-spiked polyethylene (10–22 and 90–106 μm) and polystyrene (14–20 and 85–105 μm) microplastics for 28 d increased reactive oxygen species levels, altered antioxidant enzyme activities, and induced DNA damage compared to the control ($p < 0.05$). While no size- or type-dependent toxicity was observed in treatments with pristine microplastics, earthworm cells contained significantly higher reactive oxygen species levels when exposed to aged copper-spiked polyethylene microplastics than to pristine polyethylene microplastics. Moreover, greater DNA damage was observed in earthworms exposed to large, aged copper-spiked polyethylene microplastics compared to its pristine states. This research highlights the fact that microplastics can become more toxic when aged and combined with trace metals like copper in organic residues and soil.

RÉSUMÉ

Les biosolides de désencrage sont un sous-produit des processus de tamisage fin, de flottation et de clarification utilisés pour éliminer l'encre lors du recyclage du papier. Ils constituent un résidu organique riche en nutriments pouvant être utilisé pour fertiliser les plantes. Cependant, ils contiennent également des substances non-nutritives, notamment des plastiques et des métaux traces, qui représentent un risque potentiel pour l'environnement et la santé publique. L'objectif de cette recherche était d'évaluer la toxicité des biosolides de désencrage et du cuivre lié aux microplastiques chez les vers de terre, une espèce indicatrice en écotoxicologie des sols, afin d'apporter des informations précieuses pouvant contribuer aux décisions et politiques de la gestion des résidus organiques. Les objectifs spécifiques de cette thèse étaient de : (1) étudier la toxicité aiguë des biosolides de désencrage dans un sol artificiel contaminé par le cuivre chez les vers de terre à travers des tests de croissance et de reproduction ; (2) quantifier la viabilité cellulaire en tant que résultat d'une réponse phagocytaire dépendante de la taille dans les coelomocytes des vers de terre ; (3) évaluer comment les biosolides de désencrage et le cuivre lié aux microplastiques vieillis impactent les vers de terre, notamment en termes de stress oxydatif et de dommages à l'ADN. Lors du test de toxicité aiguë, la reproduction des vers de terre a été inhibée par une exposition à ≥ 68 g de biosolides de désencrage ou ≥ 410 mg de Cu par rapport aux témoins ($p < 0.05$). Cependant, l'ajout de biosolides de désencrage au sol enrichi en cuivre n'a pas exacerbé la toxicité du cuivre ni augmenté l'accumulation de cuivre dans les vers de terre et dans les fractions subcellulaires (cytosol et métallothionine) par rapport aux expositions en traitement unique. L'étude in vitro de la cytotoxicité des microplastiques en polyéthylène sur les coelomocytes des vers de terre a révélé des différences de phagocytose dépendantes de la taille. Les coelomocytes ont englouti 85 % des petits microplastiques en polyéthylène (1–10 µm) avec un rapport cellule :

particule de 1:5 via la phagocytose, tandis que la phagocytose des microplastiques plus grands (20–27 µm) était négligeable. Malgré ces différences, la viabilité cellulaire a été réduite de manière significative à 6–7 % lorsque les coelomocytes étaient exposés aux petits et grands microplastiques en polyéthylène, contre 94 % de viabilité dans le témoin non traité. De plus, l'exposition des vers de terre à des microplastiques en polyéthylène (10–22 et 90–106 µm) et polystyrène (14–20 et 85–105 µm) vierges et vieillis enrichis en cuivre pendant 28 jours a entraîné une augmentation des niveaux d'espèces réactives de l'oxygène, une altération des activités enzymatiques antioxydantes et des dommages à l'ADN par rapport au témoin ($p < 0.05$). Bien qu'aucune toxicité dépendante de la taille ou du type n'ait été observée dans les traitements avec des microplastiques vierges, les cellules des vers de terre contenaient des niveaux significativement plus élevés d'espèces réactives de l'oxygène lorsqu'elles étaient exposées aux microplastiques en polyéthylène vieillis enrichis en cuivre qu'à leurs états vierges. De plus, des dommages plus importants à l'ADN ont été observés chez les vers de terre exposés aux grands microplastiques en polyéthylène vieillis enrichis en cuivre par rapport à leurs états vierges. Cette recherche met en évidence le fait que les métaux traces comme le cuivre peuvent devenir plus toxiques lorsqu'ils sont combinés avec des microplastiques dans les résidus organiques et les sols.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

Earthworms are susceptible to toxic substances, such as metals contained in organic residues such as deinking biosolids, but they may also be impacted by plasticizers and plastic debris in biosolids. Plastics that are degraded into microplastics are potentially toxic to earthworms, based on digestive tract inflammation that produces measurable physiological and cellular responses. While intestinal fibrosis may develop in the presence of foreign bodies like microplastics, my original hypothesis is that microplastics toxicity to earthworms depends on their size, their chemical properties and their association with trace metals, specifically copper (Cu). To the best of my knowledge, my work is the first to examine the effects of aged and trace metal-spiked microplastics to earthworms' cellular response. I found that earthworms exposed to pristine polyethylene and polystyrene microplastics had similar levels of oxidative stress, but that aged Cu-spiked polyethylene microplastics were more toxic than pristine polyethylene microplastics, as they significantly increased reactive oxygen species levels and caused DNA damage.

Another original contribution is my work on the impact of microplastics on earthworm physiology and cellular stress responses. My literature review revealed a knowledge gap about the influence of size-dependent microplastics on the direct cellular immune responses of earthworms, especially in *in vitro* conditions. In this thesis, I quantified cell viability as the outcome of a size-dependent phagocytic response in earthworm coelomocytes. The results demonstrate the phagocytic capacity of *E. fetida* coelomocytes, which can engulf multiple microplastic particles. Interestingly, while coelomocytes internalized significantly more small polyethylene microplastics (1–10 μm) than larger polyethylene microplastic (20–27 μm), cell viability was significantly affected in both treatments. This finding highlights distinct cytotoxic pathways triggered by different microplastic size: small microplastics accumulating in cells, whereas larger microplastics appear to cause frustrated phagocytosis, causing inflammation or cellular damage or death.

PREFACE AND CONTRIBUTION OF AUTHORS

Noura Alsarawi authored the four chapters of this thesis. Her contributions include investigation, methodology, conceptualization, performing the experiments, data collection and interpretation, visualization, and the creation of original drafts. Professor Joann Whalen served as Noura's supervisor and co-author for these four chapters. Professor Whalen played a pivotal role in overseeing the research, ensuring validation, providing advice about the experimental design and data interpretations, securing resources, funding, and providing input during the review and editing stages. Additionally, Dr. Geofferrey Sunahara joined as a co-author for Chapter 2 of this thesis. Dr. Sunahara participated in validating the findings and assisted with the review and editing of the chapter.

The manuscript-based chapters are presented in the following order:

Chapter 2: Alsarawi, N., Whalen, J.K., Sunahara, G., Ecotoxicological effects of deinking biosolids in copper-amended soil on the earthworm *Eisenia fetida*. (In preparation for Applied Soil Ecology).

Chapter 3: Alsarawi, N., Whalen, J.K., Microplastics cytotoxicity and the phagocytic response of earthworm immune cells. (Submitted to Environmental Science and Pollution Research – in review).

Chapter 4: Alsarawi, N., Whalen, J.K., Aged polyethylene microplastics magnifies copper-induced oxidative stress and DNA damage in *Eisenia fetida.* (In preparation for Journal of Hazardous Materials)

GENERAL INTRODUCTION

Papermill biosolids are a valuable soil amendment, containing essential plant nutrients such as nitrogen and phosphorus, and supplying organic matter that improves soil bulk density, soil structure, and water holding capacity (Rashid et al., 2006). However, the deinking fraction of papermill biosolids, which is the by-product of paper recycling, can also contain non-nutritive substances, including plastics and trace metals. Microplastics are in deinking biosolids due to the use of polyethylene-based coatings on paper, and trace metals like Cu originate from Cu phthalocyanine dyes used in the printing industry. Although the Canadian Food Inspection Agency (CFIA) has a regulatory standard for safe application of biosolids to agricultural lands that limits the amounts of chemical contaminants–including trace metals– as well as pathogens, and foreign bodies >25 mm, there is no restriction currently on microplastics. This is a concern because biosolids contains 10^3–10^4 particles kg^{-1} (dry weight) of microplastics (Christian and Köper, 2023). Therefore, the potential toxicity of deinking biosolids and emerging contaminants within them, such as microplastics-bound trace metals, needs to be evaluated on soil invertebrates like earthworms.

One of the ways to assess the potential toxicity of organic residues such as deinking biosolids, is to expose soil organisms like earthworms to the biosolids. The test procedure should follow a comprehensive environmental impact assessment, according to the United States Environmental Protection Agency (EPA), Environment Canada, and the European Chemical Agency. Following the Organisation for Economic Co-operation and Development (OECD) standards, earthworms are exposed to deinking biosolids in a controlled laboratory setting. This approach is particularly necessary when evaluating the toxicity of emerging contaminants within the biosolids, as testing their impacts directly in the field is neither feasible nor environmentally responsible. Besides microplastics, deinking biosolids also contain an appreciable amount of Cu, from 50–100 mg Cu kg^{-1} (Marouani et al., 2021; Price & Voroney, 2008), which is up to 3 times greater than the Cu concentration in unpolluted agricultural soil (i.e., <30 mg Cu kg^{-1}, Lu et al., 2003). While Cu is essential for earthworm metabolic processes, a hormetic effect of Cu on earthworm growth and reproduction is often reported, suggesting a stimulus to earthworm physiology at low concentration (10–55 mg Cu kg^{-1} soil), while there is a 50% inhibition (EC_{50}) of juvenile production at 154 mg Cu kg, cocoon hatchability with exposure to 309 mg Cu kg^{-1}, and adult earthworm biomass with 530 mg Cu kg^{-1} (Kilpi-Koski et al., 2020; Spurgeon et al., 2004). One of the toxicity pathways of Cu salts to earthworms occurs when Cu^{2+} ions transported across cell membranes cause electron loss from the mitochondrial electron transport chain, followed by electron binding to oxygen that creates reactive oxygen species (ROS) (Turrens, 2003). Combined microplastic-Cu exposure is expected to be even more toxic to earthworms because microplastics act as carriers that facilitate the movement of trace metals from deinking biosolids into the earthworm cells and tissues. Earthworms exposed to microplastics, alone or together with Cu, induce an immunity response that prevents the circulation of foreign bodies in tissues. One key aspect of this immune response is phagocytosis, the process where immune cells engulf foreign particles > 0.5 µm. This immune response may not work against microplastics that exceed 10 µm in diameter because they are too large to be engulfed, resulting in frustrated phagocytosis, causing inflammation and cellular damage or death.

The global objective of this research is to assess the potential toxicity of deinking biosolids and microplastic-bound Cu to ecotoxicological indicator species e.g., earthworms, to contribute valuable insights that can inform decisions and policies for the management of organic residues. The thesis specific objectives include: (1) to evaluate the potential toxicity of deinking biosolids in copper-amended soil to earthworms, based on physiological responses, i.e., growth and reproduction, (2): to quantify cell viability as the outcome of a size-dependent phagocytic response in earthworm coelomocytes, (3): to investigate how deinking biosolids and microplastics-bound Cu affect biomolecular responses in earthworms, reflecting a realistic environmental condition, where pollutants often exist in combination rather than isolation. In chapter 1, hypothesized that (1) the growth and reproduction of earthworms exposed to DB at sublethal Cu concentrations is stimulated at low concentrations and is diminished at higher concentrations; and (2) that this biphasic effect is modulated by the presence of growth-enhancing substrates (e.g., nutrients and organic matter contained within the biosolids). In chapter 2, I expected that greater cell death will occur when coelomocytes are exposed to larger (20–27 μm) than smaller (1–10 μm) microplastics, due to the greater likelihood of frustrated phagocytosis with larger microplastics. Finally, in chapter 3, I anticipated that soil amended with aged Cu-spiked polyethylene and polystyrene microplastics will produce more oxidative stress and DNA damage in earthworms than soil containing pristine microplastics because of greater stress from the combined microplastics and Cu exposure.

CHAPTER 1

Literature review

1.1 Physicochemical mechanisms of metal adsorption on microplastics

Metals attach to microplastics via intermolecular binding, surface binding, and diffusion. Specifically, metals bind to microplastics via coordinate interactions with specific functional groups, such as hydroxyl (-OH), carboxyl (-COOH), or amine ($-NH_2$) on or within the microplastics. This chemical adsorption results in a strong attachment via chemical bonding (Liu et al., 2024). In addition, physical adsorption due to electrostatic attraction and weaker reversible interaction such as Van der Waals forces bind the metal ions to the microplastic particle (Maity et al., 2021). Further, metals can diffuse into the microplastic following a concentration gradient influenced by the physicochemical properties of the microplastics and the surrounding environment (Fig. 1.1). Results from sorption dynamic experiments between microplastics; polyethylene, polystyrene, and polyvinyl chloride, and Cd^{2+} revealed that a second-order model is a better fit for these reactions, indicating that the Cd^{2+} ions could be sorbed onto microplastics at different binding sites; a fast-binding stage (0-6 h), followed by slow binding (6-24 h) (Guo et al., 2020). In a two-stage sorption process, the initial rapid binding phase is likely dominated by electrostatic interactions, followed by a second, slower phase that may be driven by metal diffusion into the microplastic matrix (Liu et al., 2021). Unfortunately, this type of sorption dynamic could pose a significant threat to soil organisms in the environment. This is because the two-stage process, involving both rapid surface adsorption followed by slower diffusion, represents a combination of immediate and delayed interactions between metals and microplastics, leading to a more complex pattern of metal release and bioavailability over time. Therefore, microplastics-bonded metals exposure should be considered for its potential toxicity to soil organisms.

Consequently, we need to understand that microplastics characteristics play a key role in transporting metals to earthworms.

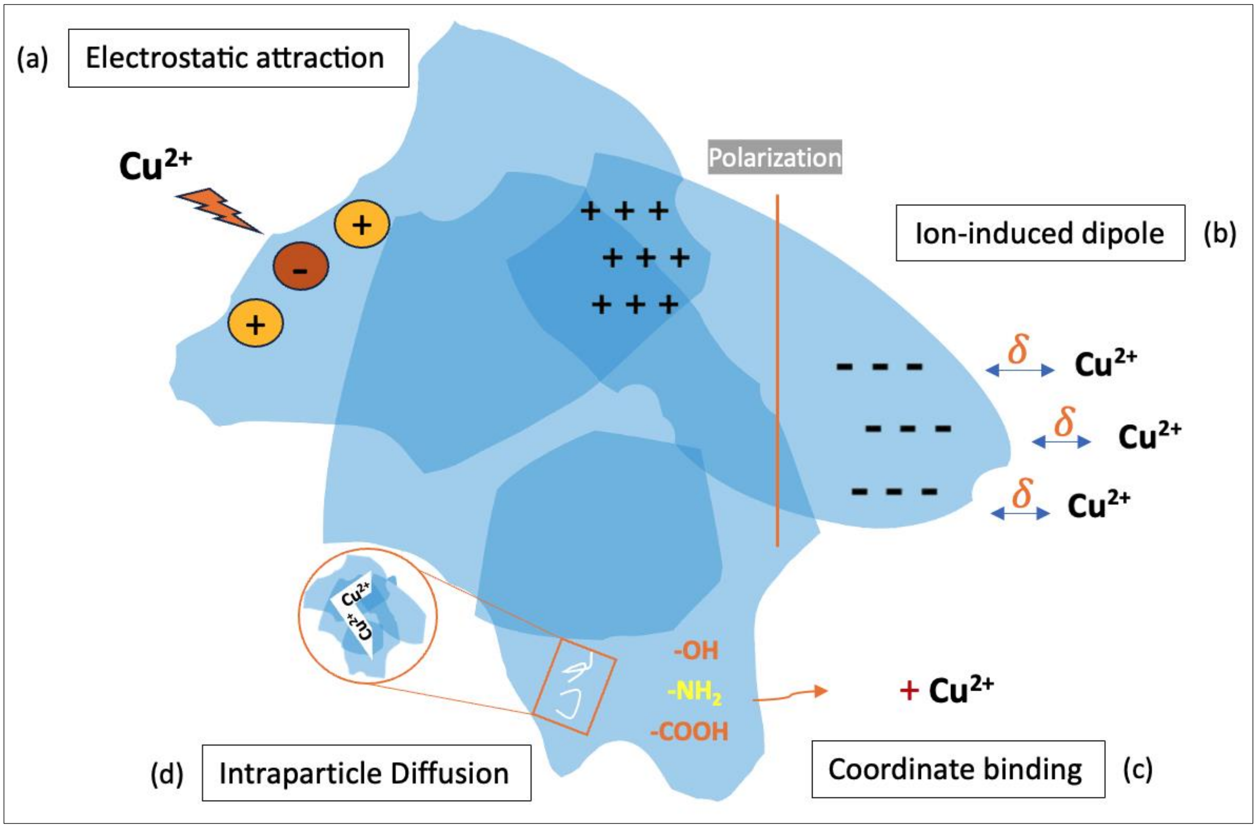

Figure 1.1 A mechanistic concept of Cu ions adsorption to microplastics. (a) In electrostatic attraction, the low ionization energy of copper ions results in a net loss of electrons, which creates an ionic bond with the microplastics surface. (b) Van Der Waals forces cause copper cations to remain near the negatively-charged microplastic surface without exchanging electrons. (c) In coordinate binding, copper ions form inner-sphere complexes with functional groups, such as hydroxyl (-OH), carboxyl (-COOH), or amine ($-NH_2$) present on the microplastic surface. (d) Copper ions can also diffuse slowly into microplastics' pores following a concentration gradient.

1.2 Microplastic-metal ion interactions

Microplastics are generally chemically inert in their manufactured state, but they can develop electrostatic charge through physical interactions. While these charges can influence the microplastics interaction with metal ions, the combination of physical and chemical properties, including surface area, polarity, and any functional groups as a result of weathering effects and environmental exposure determines their efficiency to sorb or adsorb metal ions. Therefore, with the passing of time, environmental processes can alter the surface of microplastics, potentially increasing their chemical reactivity and causing them to interact with a wide range of chemical substances.

1.2.1 The effect of aging on microplastic-metal ion interactions

Aged microplastics have greater adsorption capacity for metals than pristine microplastics. This is because aged microplastics develop cracks and fractures as well as functional groups due to oxidation, which increase their specific surface area, and possibly accumulate minerals and organic matter. This creates more diffusible space and additional binding sites for metal ions. In fact, naturally aged microplastics collected from river sediments in Hunan province, China, adsorbed 13.6 mg Pb^{2+} g^{-1} from a 10 mg l^{-1} $Pb(NO_3)_2$ solution, significantly more than the 5 mg g^{-1} adsorption of pristine microplastics (Fu et al., 2021b). However, aging not only affects the adsorption capacity of metal ions onto microplastics, but it also affects the leaching of metals from microplastics. A study by Bandow et al. (2017) investigated Cu^{2+} release from polyethylene, polystyrene, and polyvinyl chloride recycled microplastics during artificial aging in a water column (UV photooxidative, 25 ± 3 W m^{-2}, 2000 h). The study revealed that photooxidative aging led to the leaching of 0.33 mg Cu^{2+} kg^{-1} from polyethylene microplastics, 0.8 mg Cu^{2+} kg^{-1} from polystyrene microplastics (up to a 4-fold increase) and 0.16 mg Cu^{2+} kg^{-1} from polyvinyl chloride microplastics (up to an 8-fold increase) compared to unexposed samples. Interestingly, the study also demonstrated the formation of new adsorption bands (C=O, C–O and OH) in microplastics due to aging, confirming that aged microplastics possess additional binding sites for metals. Therefore, compared to pristine microplastics, aged microplastics can adsorb and release more metals into the environment.

1.2.2 Microplastics' physiochemical properties affecting metal ion adsorption

While aging increases the specific surface area of microplastics by introducing more cracks and fractures, it is important to note that the specific surface area of microplastics in their pristine state also varies, mainly due to the polymer porosity and crystallinity, additives used and the manufacturing process (Ivleva, 2021). Microplastics specific surface area; polyvinyl chlorine > polystyrene > polypropylene > polyethylene, is linearly proportional to its adsorption capacity, with polyvinyl chlorine adsorbing 151 mg Cd^{2+} kg^{-1}, polystyrene adsorbing 134 mg Cd^{2+} kg^{-1}, polypropylene adsorbing 123 mg Cd^{2+} kg^{-1}, and polyethylene adsorbing 113 mg Cd^{2+} kg^{-1} when exposed to $Cd(NO_3)_2$ solution (Guo et al., 2020). Moreover, the adsorption of metal ions onto microplastics is also influenced by the polymer polarity. Polyvinyl chlorine, polyamides and polycarbonate are polar polymers, while polystyrene, polypropylene and polyethylene are non-polar. Typically, polar polymers have stronger intermolecular attractions with metal ions than non-polar molecules (Figure 1a). This is evident by the higher Cu^{2+} adsorption capacity with polyvinyl chlorine (partitioning coefficient, Kd = 850) than polystyrene having a Kd of 650 (Brennecke et al., 2016), and the higher adsorption capacity of polyvinyl chlorine than polystyrene, polypropylene, and polyethylene to Cd^{2+} as discussed earlier (Guo et al., 2020). Given our current understanding, it is crucial to consider how microplastics impact metal bioavailability due to their role in accumulating and mobilizing metals in the environment.

Plastic pollution gained significant attention due to the magnitude of plastics accumulating in the soil and water environment. To promote sustainability, we started transitioning towards using biodegradable plastics. Although biodegradable plastics are an attractive choice because they are less persistent in the environment compared conventional plastics, it is important to also consider other aspects, besides material longevity, that contributes to their overall environmental impact. Lin et al. (2023) compared conventional polyethylene plastic bags to biodegradable poly(lactic acid) bags and found that the biodegradable bags adsorbed and desorbed significantly more Pb than conventional bags. Specifically, Pb adsorption on the conventional plastic bag was approximately 150 mg kg^{-1}, compared to 900 mg kg^{-1} for the biodegradable bag, probably because of the difference in oxygen-containing functional groups and lower crystallinity of poly(lactic acid) compared to polyethylene (Lin et al., 2023). This study in my opinion highlights the importance of considering broader environmental implications and potential contaminant transfer risks beyond material longevity. Therefore, understanding the specific plastic physical and chemical characteristics is crucial for predicting their metal adsorption capacities (Table 1.1). One research gap in this area is the need for comprehensive studies on the interactions between different types of microplastics and metals under various environmental conditions.

Table 1.1 Pristine microplastics physiochemical characteristics

Type	**Specific surface area (m^2 g^{-1})**	**Porosity (%)**	**Crystallinity (%)**	**Polarity**
Polyvinyl chloride	0.836	27.1	Amorphous	Polar
Polystyrene	0.348	12.6	Amorphous	Non-polar
Polypropylene	0.348	non-porous	50	Non-polar
Polyethylene	0.173	29.9	LDPE: 50 HDPE: 70	Non-polar
References	(Guo et al., 2020)	(Linda et al., 2022)	(Ojeda, 2013)	(Fu et al., 2021a)

1.2.3 Metal properties determine their adsorption efficiency onto microplastics

Metal properties, such as ionic radius, electronegativity, and redox status, are important parameters that determine their adsorption threshold onto microplastics. For example, the ionic radius could be an indicator because smaller metal ions likely bind more efficiently onto microplastics compared to larger ions. In moist and biological environments such as agricultural soil and within cells, the hydrated ionic radius, which is the radius of ions and closely bonded water molecules (Barus, 2021), appears to determine the sorption efficiencies of metals. For example, Pb^{2+} and Cd^{2+} have radiuses of 401 pm and 426 pm, respectively (Table 1.2). Barus (2021) demonstrated that Pb^{2+}, which have a lower hydrated ionic radius, have a higher adsorption efficiency to microplastics than Cd^{2+} in an aqueous solution. Additionally, Pb has a greater electronegativity (1.9) than Cd (1.7) (Table 1.2), which may also contribute to its higher adsorption ability. Further, metals with a higher redox potential (Table 1.2) are more easily reduced (gain electrons), which can increase their reactivity and their ability to form stable complexes with adsorbent surfaces, like microplastics. Therefore, it is necessary to consider that both metal and microplastic properties are responsible for metal bioavailability in microplastic-metal mixtures when conducting ecotoxicology assessments. In summary, the relevant parameters include microplastics size, type, and aging characteristics, and the trace metals physical and chemical characteristics including redox potential, electronegativity, ionic and hydrated ionic radius (Table 1.2).

Table 1.2 Key physicochemical properties of various metal ions relevant to adsorption studies.

Type	Redox potential (V)	Electronegativity (Pauling's)	Ionic radius (pm)	Hydrated ionic radius (pm)
Cu^{2+}	+0.34	1.9	73	419
Zn^{2+}	-0.76	1.6	74	430
Fe^{2+}	-0.44	1.8	70	348

Mn^{2+}	-1.18	1.5	70	400
Pb^{2+}	-0.13	1.9	119	401
Cd^{2+}	-0.40	1.7	95	426
References	(Ball et al., 2011)		(Well, 2012)	(Barus et al., 2021)

1.3 Cellular uptake of microplastics-bound Cu by macrophages

Phagocytosis is the process of engulfing particles > 0.5 μm into a plasma membrane derived vesicle, known as phagosome (Rosales & Uribe-Querol, 2017). Upon the formation of phagosomes, the cell fuses them with lysosomes, which contain digestive and hydrolytic enzymes, reactive oxygen and nitrogen species, and antimicrobial peptides, creating phagolysosomes (Gray et al., 2016). This process of breaking down foreign bodies within cells is called autophagy, and its role is to clear tissues of foreign particles. Research has shown that immune cells can engulf synthetic materials through phagocytosis. For example, a study by Merkley et al. (2022) demonstrated the mechanisms behind the engulfment of synthetic particles by exposing murine macrophages to 10 μm polystyrene microplastics and observed the colocalization of microplastics with LC3 (Microtubule-associated protein 1A/1B-light chain 3), indicating LC3-associated phagocytosis (LAP); a process related to the maturation of phagosomes. Further, Yin et al. (2023) confirmed lysosome activation in macrophages exposed 5 μm polystyrene microplastics for 4 h. However, unlike bacteria and viruses, which have pathogen-associated molecular patterns such as lipopolysaccharides and viral RNA that can be recognized by pattern recognition receptors on macrophages (Mogensen, 2009), synthetic material like microplastics do not.

When synthetic particles enter organisms' tissues, they become coated with proteins; a concept known as protein corona (Breznica et al., 2020). Those proteins include antibodies that can tag the foreign bodies and make them recognizable by macrophages. Few studies investigated the recognition of synthetic particles by immune cells. For instance, Gessner et al. (2003) confirmed the absorption of IgG antibody and albumin to latex beads. Interestingly however, while IgG enhance cell recognition of synthetic particles through Fc receptor, albumin has been linked to mask this recognition by preventing the binding of IgG (Breznica et al., 2020). Thus, the types of protein accumulated in the protein corona layer influence the recognition of synthetic materials by immune cells. In addition to protein corona, immune cells can interact with synthetic material via non-specific binding and processes associated with innate immune response. In this process, oxidative damage induced by synthetic materials lead to the release of danger-associated molecular patterns (DAMPs) which activate toll-like receptors (TLRs) on cells that aid in the recognition and engulfment of foreign bodies (Yang et al., 2022). Therefore, immune cells are capable of recognizing and engulfing microplastics > 0.5 µm via phagocytosis through multiple recognition pathways.

Exocytosis, which follows foreign bodies uptake and digestion by phagolysosomes, is an area that is still not understood for synthetic particles. I believe this is because synthetic particles like conventional plastics are indigestible. It raises the question of what happens when the cell is incapable of digesting the foreign body it engulfed. Merkley et al. (2022) showed that when murine macrophages engulfed 10 µm polystyrene microplastics during a 72-hour exposure period, the microplastics were not degraded, despite triggering the autophagic pathway and LAP. The problem with cellular uptake of synthetic particles is that, because they cannot be digested, microplastics can accumulate in lysosomes, leading to lysosomal instability and damage. Yin et al. (2023) observed that after a 12-hour exposure to polystyrene microplastics (5 µm), macrophages experienced ongoing autophagy and lysosomal activation, resulting in lysosomal rupture.

Therefore, while immune cells can recognize and engulf synthetic materials, recent evidence suggests this immunity response fails to degrade them through the autophagic pathway or LAP. It would be interesting to perform similar studies on biodegradable microplastics and investigate why the macrophages does not preform exocytosis when failing to digest the foreign body.

1.4 Cu desorption from microplastics in cellular compartments: mechanisms of uptake and transport

The mechanisms of cellular uptake of metal ions are well understood, but the cellular response to microplastics-metal complexes are less well described. My research focuses on the potential toxicity of aged microplastics-bound Cu to earthworms. Therefore, my goal is to understand the mechanisms of how metal ions, such as Cu, desorb from microplastics in earthworm tissue and cells. This area of research is unexplored, but I will share some theories based on general knowledge and existing evidence.

Metals desorb from microplastics within earthworms via multiple pathways that breaks the intra- and intermolecular bonds that bind the contaminants. The first possible pathway is for metals to desorb from microplastics via diffusion following a concentration gradient. Then, this could also happen when the metal concentration in the microplastic is higher than the metal concentration in tissue and cells. Further, we know that ROS could participate in redox reaction with Cu ions attached to microplastics, which could alter the Cu ions oxidation state, stability, and binding properties. Moreover, when microplastic-bound Cu enters the cell via phagocytosis, digestive enzymes within lysosomes may alter the surface characteristics of microplastics leading the desorption of Cu ions. However, there is no evidence to support that theory and further investigation is required. Finally, the last proposed pathway of desorption of metal ions from microplastics onto immune cells is that lysosomes are acidic in nature, and Lin et al. (2023) found that lower pH values lead to significant increase in desorption of Pb ions from polyethylene and polylactic acid plastics bags. Thus, I hypothesize that Cu ions would eventually desorb from microplastic when earthworms ingest microplastic-bound metals due to diffusion, redox reactions and the acidic environment within lysosomes.

Cu ions move from the extracellular to the intracellular environment through several mechanisms. These mechanisms include facilitated diffusion, active transport including endocytosis, and through divalent metal transporter 1 (DMT1). In facilitated diffusion, negatively charged regions on the cell membrane, such as the extracellular amino-terminus of Ctr1, generate an electrostatic field that attracts positively charged Cu^{+} ions closer to the cell membrane, where they are transported across Ctr1 pores via ligand exchange reactions between distinct binding sites like methionine and histidine, facilitated by dipole interactions at the carboxyl-terminus, forming an exit pathway for the ions (Ohrvik & Thiele, 2014). In active transport, Cu-transporting ATPases ATP7A and ATP7B proteins use the energy from ATP hydrolysis to actively transport Cu^{+} ions across cellular membranes, maintaining Cu homeostasis (Ohrvik & Thiele, 2014). Another form of active transport is endocytosis. In endocytosis, the cell extends its membrane to engulf Cu^{+} and Cu^{2+} ions bound to carrier proteins or other complexes, such as ceruloplasmin, albumin and histidine (Gioilli et al., 2022). DMT1 transports Cu ions simultaneously with protons, utilizing the proton gradient to facilitate the transport of Cu^{2+} ions into the cell (Wolff et al., 2018 & Arredondo et al., 2003). Although the presence of DMT1 protein specifically in earthworms was not confirmed, a study by Procházková et al. (2014) provides evidence for the presence of DMT1-like proteins with similar structure and function in *Eisenia fetida*. Therefore, understanding these mechanisms is important when interpreting the ecotoxicological effects of Cu on earthworms due to their role in maintaining Cu homeostasis, and that their disruptions can result in toxicity and adverse effects.

Metallothionein (MT), a metal binding protein, play an important role in binding to and transporting free Cu ions inside cells. What is interesting about MT specifically is that many studies use the protein as biomarker in ecotoxicology research, either by examining its expression or by measuring Cu accumulation within it. Upregulation of MT often indicates that the organism is responding to elevated Cu concentration in cells, suggesting potential toxicity. For example, Fisker et al. (2013) demonstrated that the relative expression of the mt2 gene was lower in six different populations of earthworms exposed to highly Cu-contaminated soil 173 μg g^{-1} dw worm, compared to medium and low Cu-contaminated soil: 34 and 20 μg g^{-1} dw worm respectively. When the capacity of the cell to produce sufficient MT is exceeded, Cu can accumulate to toxic levels. This means that free Cu would participate in redox reactions that generates ROS as discussed earlier, causing protein oxidation. Subsequently, the oxidized form of metallothionine (MTox) forms disulfide bonds between cysteine residues, which prevent the protein from effectively binding Cu and other metal ions, resulting in a loss of MT scavenging abilities (Santon et al., 2009). Therefore, when MT is oxidized and loses its ability to bind excess Cu, it is considered a threat because it could lead to increased cellular toxicity.

1.5 Cellular oxidative stress and molecular damage mechanisms

Oxidative stress is the excess of oxidants over antioxidants, resulting in disrupted redox signaling and control or causing molecular damage (Fink & Gale, 2007). ROS are reactive chemicals formed from molecular oxygen that when accumulated, can cause molecular damage. Superoxide dismutase (SOD), catalase (CAT) are antioxidants that scavenge ROS as a defense response. The build-up of ROS in cells is a primary cause of oxidative stress. However, ROS are also formed during normal cell processes, such as aerobic metabolism, where they function as signaling molecules (Forrester et al., 2018), and moderate levels of ROS, e.g., 50-150 OD mg^{-1} protein, from the literature have been considered to be baseline levels. For instance, earthworms exposed to 50 mg CuO kg^{-1} shown to have ROS levels of approximately 300 OD mg^{-1} protein, which correlated with oxidative damage to lipids, compared to 150 OD mg^{-1} protein in control treatments (Li et al., 2020a). This is consistent with a study by Li et al. (2023) which showed that exposing earthworms to 52 mg Zn^{2+} kg^{-1} significantly increased ROS levels to 320 OD mg^{-1} protein compared to 140 OD mg^{-1} protein in unexposed earthworms.

1.5.1 Reactive oxygen species formation and antioxidant enzymes activity

Metals entering tissue can disrupt the electron transport chain in mitochondria, causing electron leakage (Turrens, 2003). Electron leakage refers to the process where electrons escape before they can contribute to the reduction of oxygen to water at the cytochrome c oxidase stage, and instead react with oxygen to produce superoxide ($O_2^-\bullet$) (Jastroch et al., 2010).

$$O_2 + e^- \rightarrow O_2^-\bullet \quad (1)$$

In addition, metals can directly bind with oxygen to generate $O_2^-\bullet$

$$M^{n+} + O_2 \rightarrow M^{(n+1)} + O_2^-\bullet \quad (2)$$

$O_2^-\bullet$, the simplest form of reactive oxygen species, interacts with other chemicals to form different types of ROS, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$). For example, when $O_2^-\bullet$ interact with hydrogen, it generates H_2O_2, which in turn can bind with $O_2^-\bullet$ or metals to form hydroxyl radical ($HO\bullet$).

$$M^{n+} + O_2 \rightarrow M^{(n+1)} + O_2^-\bullet + H^+ \rightarrow HO_2\bullet$$

$$2\,HO_2\bullet \rightarrow H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 \quad + \quad O_2^{-}\bullet \quad \rightarrow \quad O_2 \quad + \quad HO_2^{-} \quad + \quad HO\bullet$$

$$H_2O_2 \quad + \quad M^{n+} \quad \rightarrow \quad M^{(n+1)} \quad + \quad OH^{-} \quad + \quad HO\bullet \tag{4}$$

Secondary ROS are formed during interactions of primary ROS e.g., $O_2^{-}\bullet$, H_2O_2, and singlet oxygen ($^{1}O2$) with other chemicals or each other and form peroxyl radicals (ROO•), HO•, hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{-}$) (Collin, 2019). Peroxyl radicals are formed when a molecular oxygen reacts with carbon-centered radicals (R•), which could be produced when lipids are oxidized (Siraki et al., 2018).

$$R\bullet \quad + \quad O_2 \quad \rightarrow \quad ROO\bullet \tag{5}$$

Further, the enzyme myeloperoxidase catalyzes the reaction of hydrogen peroxide (H_2O_2) with Cl^{-} to form hypochlorous acid (HOCl) (Furtmüller et al., 2000), and peroxynitrite ($ONOO^{-}$) are produced when $O_2^{-}\bullet$ reacts with nitric oxide (NO) (Guzik et al., 2002).

$$H_2O_2 \quad + \quad Cl^{-} \quad + \quad H^{2} \quad \rightarrow \quad HOCl \quad + \quad H_2O \tag{6}$$

$$O_2^{-}\bullet \quad + \quad NO \quad \rightarrow \quad ONOO^{-} \tag{7}$$

Upon ROS generation and build-up, the defense system triggers antioxidants, like SOD, which converts $O_2^{-}\bullet$ to H_2O_2. Then, CAT converts H_2O_2 to oxygen and water.

$$2\ O_2^{-}\bullet \quad + \quad 2\ H^{+} \quad \rightarrow \quad H_2O_2 \quad + \quad O_2 \tag{8}$$

$$2\ H_2O_2 \quad \rightarrow \quad 2\ H_2O \quad + \quad O_2 \tag{9}$$

Similar to CAT, glutathione peroxidase (GPx) is another important antioxidant enzyme that protects cells from oxidative damage by reducing H_2O_2 to oxygen and water, using glutathione (GSH) as a substrate to carry out the reaction (Forrester et al., 2018).

$$2\ GSH \quad + \quad H_2O_2 \quad \rightarrow \quad GSSG \quad + \quad 2\ H_2O \tag{10}$$

Fortunately, the oxidized form of GSH: GSSG, can be reduced back to GSH by GSH reductase (Forman et al., 2010).

$$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+ \quad (11)$$

It is important to note that the relationship between ROS and antioxidant enzymes activity is complex. In other words, when the cell produces antioxidant enzymes against ROS, it does not always result in mitigating oxidative stress. Moderate toxicity might lead to a controlled increase in ROS and a corresponding increase in antioxidants. However, when the organism is experiencing high levels of stress, the ROS production might overwhelm the antioxidant defenses, leading to oxidative damage despite increased antioxidant activities. For example, exposing earthworms to 40 mg Ni^{2+} kg^{-1} significantly increased SOD and CAT activities on day 14, yet malondialdehyde content (MDA); a biomarker indicative of lipid peroxidation, continued to increase until the end of the exposure period on day 21 (Li et al., 2021). Another good example that emphasizes the need to consider variety of biomarkers is a study conducted by Sobhani et al. (2022), which showed that exposing earthworms to 0.1 g kg^{-1} of polyethylene microplastics for 4 wk did not affect reproduction but caused significant DNA damage, averaging 40% tail DNA. Therefore, it is important to consider physiological as well as molecular and biochemical biomarkers in ecotoxicology studies.

1.5.2 Molecular damage induced by oxidative stress

Reactive oxygen species can react with membrane lipids, proteins, and nucleic acids. These reactions can damage the molecule, affecting its ability to participate in biochemical reactions or its function within the cellular structure. Consequently, oxidative reactions may lead to lipid peroxidation, impair enzymatic processes and cause DNA strands to break, resulting in cell death (Wen et al., 2021).

1.5.2.1 Lipid peroxidation and protein degradation

Reactive oxygen species-induced oxidative stress can oxidize lipids and proteins and cause inflammation, which leads to lipid peroxidation and protein degradation. An excess of ROS can react with polyunsaturated fatty acids in the lipid bilayer and initiate lipid peroxidation (Su et al., 2019). There is a positive correlation between elevated ROS levels and lipid peroxidation in numerous ecotoxicology studies. For example, Saint-Denis et al. (2001) showed that exposing earthworms to 30 mg Pb kg^{-1} resulted in significant increase of ROS levels and lipid peroxidase, which is a biomarker of lipid peroxidation. Further, ROS can directly react with amino acids in proteins leading to their degradation or destroy their function. For example, He and colleagues (2024) observed that exposing earthworm immune cells to 20-50 mg l^{-1} of polystyrene nanoplastics lead to a significant increase in the levels of protein carbonyl group: a biomarker of ROS-induced protein oxidation. Inflammation triggered by ROS occurs when excess ROS overwhelms the antioxidant defense mechanisms and as a result causes oxidative cellular damage. It appears that this damage triggers the activation of signaling pathways that promote the expression of pro-inflammatory cytokines. While no studies have been conducted to test the effects of metal exposure on cytokine activation specifically in earthworms, research by Gao et al. (2022) found that exposing murine macrophage RAW264.7 cells to a solution containing 0.095 mg Mg^{2+} mL^{-1}, 0.004 mg Fe^{2+} mL^{-1}, and 0.00128 mg Cu^{2+} mL^{-1} increased expression of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. Therefore, exposure to high concentrations of metals or toxic substances can cause molecular damage such as lipid peroxidation and protein degradation, which possess a threat on cell viability.

1.5.2.2 Mechanical injury and additive leaching

Exposing earthworms to microplastics can lead to oxidative stress. I believe that the two major pathways leading to oxidative stress are mechanical injury and the leaching of additives such as flame retardants, plasticizers and stabilizers from the microplastics. Mechanical injury from microplastics exposure can be tissue damage in the gastrointestinal tract during the digestion process. For instance, exposing earthworms to 500 mg polyethylene microplastics kg^{-1} soil (<300 μm) for 28 d led to visible damage to the digestive tract including signs of detachment of the gut epithelium, thickened enteric walls and constricted intestinal lumen (Cao et al., 2022). Another form of mechanical injury would be when earthworm immune cells engulf or try to engulf microplastics. Detailed discussion of the mechanisms of cell recognition of microplastics and their engulfment via phagocytosis are explained in detail in Chapter 1, Section 3. However, this immunity response could cause harmful effects when the plasma membrane fails to completely isolate the microplastic during phagocytosis. Frustrated phagocytosis happens when macrophages interact with a foreign body coated with opsonins−molecules that tag foreign particles for immune recognition−and spread to try and engulf it, but if it fails it results in phagocytic spreading (Johnson et al., 2012; Mularski et al., 2018). This could happen when the microplastic, for example, is too large to be engulfed by phagocytic cells, causing lysosome organelles to be released to the extracellular environment. In addition to mechanical injury cause by frustrated phagocytosis, lysosomal content contains digestive enzymes capable of degrading proteins, lipids, and DNA (Gray et al., 2016). Thus, exposing phagocytic cells i.e., monocytes, macrophages, and neutrophils, to microplastics greater than the cell diameter could lead to frustrated phagocytosis and cause mechanical injury as well as risk lysosomal contents leakage in extracellular environments.

The leaching of additives from microplastics is a significant concern. Because many studies have examined the additives effect on soil, aquatic and terrestrial organisms, I will focus specifically on the stress mechanisms of plastic additives on soil organisms, particularly earthworms. Tris(2-chloroethyl) phosphate (TCEP) is a plastic additive commonly used as flame retardant. An interesting study conducted by Cao et al. (2022) investigated the effect of 500 mg polyethylene microplastics kg^{-1}; stripped from solvent soluble surface chemicals, TCEP; 1 mg kg^{-1}, and their combined exposure for 28 d and found that co-exposure increased oxidative stress in earthworms, lead to gut microbiota dysbiosis and altered metabolic pathways including significant up-regulation of citrate and down-regulation of L-glutamate affecting nervous, digestive and excretory systems in earthworms. Another 28-d soil exposure study conducted by Yang et al. (2023) showed that although low concentrations of TCEP (5 mg kg^{-1}) did not cause molecular damage in earthworms, it affected 11 pathways including ECM-receptor interactions and protein digestion and absorption. Similarly, exposing earthworms to 300 mg kg^{-1} of the plasticizer Diisobutyl phthalate (DIBP) for 28 d significantly increased ROS and MDA levels, inhibited SOD, CAT and peroxidase activities, and activated glutathione S-transferase activity, indicative of lipid damage and genotoxicity caused by oxidation (Yao et al., 2023). Thus, it is evident that the leaching of additives is another pathway of oxidative stress-induced molecular damage caused by the ingestion of microplastics.

1.5.2.3 DNA damage

DNA damage from oxidative stress can impair cellular function and, in some cases, lead to apoptosis, which is the process of programmed cell death (Kaufmann & Paules, 1996). Glutamine deficiency has been linked to cause DNA damage (Tran et al., 2017), and H_2O_2, can disrupt glutamine synthesis. In addition, glutamine provides nitrogen required for purine and pyrimidine nucleotide synthesis (Fu et al., 2019), which are essential for DNA synthesis. Further, glutamine is involved in the production of GSH, which as discussed earlier in section 3.1 is a major antioxidant contributor that protect cells from oxidative stress. Consequently, inhibiting glutamine synthesis leads to impaired immunity response and DNA damage. In addition, ROS can react directly with DNA bases; guanine, cytosine, thymine, creating oxidative DNA lesions such as 8-oxoguanine (8-oxoG), cytosine glycol, and thymine glycol, which can cause mutations and genomic instability (Huang et al., 2020). Thus, because metals catalyze the production of ROS, metal exposure would increase oxidative stress in the exposed organism. Now we have a better understanding of the mechanisms that cause molecular damage due to oxidative stress from metals and/or microplastics-bound metals exposure (Figure 1.2).

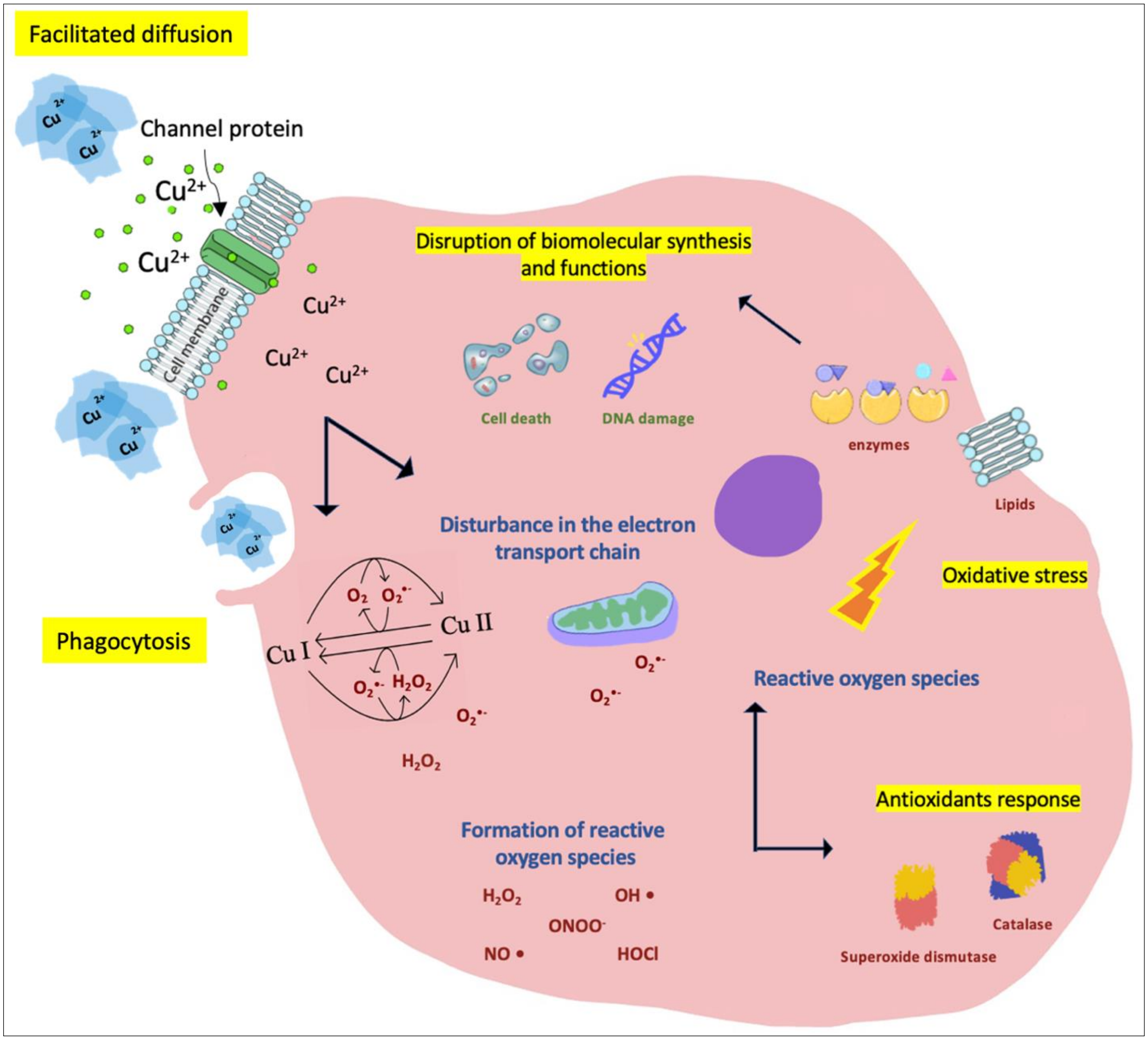

Figure 1.2 A mechanistic concept of oxidative stress due to Cu exposure in earthworms (a) Cu ions enter the cell via facilitated diffusion. At elevated concentrations, Cu ions disturb the electron transport chain in the mitochondria causing electrons to leak and bind with oxygen to generate the simplest form of reactive oxygen species; superoxide. Through a series of enzymatic and non-enzymatic chemical reactions, superoxide generates several reactive oxygen species. Further, the transformation of copper from oxidized (Cu (II)) to reduced (Cu (I)) states generates additional superoxide and hydroxyl radicals. (b) When reactive oxygen species production increases, the cell defends itself by mediating transcriptional activation of genes promoting the generation of

antioxidants enzymes such as, superoxide dismutase and catalase. When reactive oxygen species production rate overpowers the antioxidants scavenging capabilities, the cell will start experiencing harmful effects. (c) Reactive oxygen species can react with membrane lipids, proteins, and nucleic acids. These oxidative reactions lead to lipid peroxidation, impair enzymatic processes and growth functions, and cause DNA strands to break, resulting in cell death.

1.6 Microplastics in agricultural soil

Microplastics are emerging contaminants in terrestrial environments, due to the magnitude and diversity of plastic wastes that end up in the soil. Microplastics are retained in porous matrices, making soil a sink for an increasing amount of microplastics. This is an issue because plastic manufacturing is expected to continue on increasing to up to 33 billion tons by 2050 (Sharma et al., 2020). The accumulation of microplastics in agricultural soil is often traced to practices such as applying organic fertilizers like biosolids, irrigating with wastewater from primary sewage treatment facilities, and covering soil with plastic mulch to control weeds and conserve water. For example, a study in Ontario identified thirteen types of microplastics in agricultural soil treated with biosolids, the most abundant being polyethylene, polypropylene and polystyrene, with an estimated 4–64 kg microplastics ha^{-1} (Crossman et al., 2020). In addition, up to 1.2 g of microplastics were found per kg of compost and up to 125,000 fragments per m^{-3} of agricultural soil when treated wastewater was used for irrigation (Bläsing & Amelung, 2018; Zhao et al., 2022a). The benefits of plastic mulch, including water conservation, enhanced nutrient utilization, weed suppression, and improved crop yield and quality (Surendran et al., 2023), have led to a significant increase in its use by farmers, with China leading global usage on 19.8 million ha of agricultural land (Qi et al., 2018). However, soil covered continuously with plastic mulch for 30 yr accumulated 40 mg of microplastic (0.3–0.8 mm) and 308 mg of mesoplastics (5–25 mm) kg^{-1}

of agricultural soil, emphasizing a noticeable reduction in plastic particle size as the mulching period increased from 5 to 30 yr (Li et al., 2020b). Therefore, organic fertilizer application, wastewater irrigation and plastic mulching all need to be regulated to address microplastics as an emerging contaminant, given the potential of these practices to introduce microplastics to agricultural lands.

It seems inevitable that organic fertilizers, including biosolids and other organic residues from municipalities and industries, will be applied to agricultural lands. There are few options for alternative recycling of biosolids, and landfilling is restricted in many countries. For example, Canada has proposed national organic waste diversion goals of 30% by 2030 and 50% by 2040, with provinces such as Nova Scotia and Prince Edward Island having already banned organic waste from landfills since 2015 (Environment and Climate Change Canada, 2021; GPEI 2022; EREF, 2019). In addition, the European Union has projected that no more than 10% of organic waste will be permitted in landfills by 2030 (Council of the European Union, 2018). In the United States, the State of California aims to reduce organic waste landfilling by 75% by 2025 (California Legislative Information, 2016). Given the current diversion goals and bans of organic waste from landfills, it is important to fund projects to find affordable solutions to control the involuntary input of microplastic into agricultural lands through land spreading of organic residues.

1.7 Canadian regulations and guidelines for land spreading of fertilizing organic residues

Canada's strict landfilling policies encourage the recycling of affordable and locally sourced organic residues as crop fertilizer. In the province of Quebec alone, 1.8 million Mg (wet weight) of fertilizing organic residues are applied annually to croplands, and about 70% of these organic residues are biosolids generated by the pulp and papermill industry (Hébert, 2015). The Canadian Food Inspection Agency (CFIA) sets regulatory standards for the safe application of biosolids to agricultural lands under the authority of the *Canadian Fertilizers Act*. These standards are informed by guidelines from the Canadian Council of Ministers of the Environment (CCME), which incorporates recommendations from the provincial ministries in Ontario, Quebec, and British Columbia, each of which has adopted its own guidelines. These guidelines limit the accumulation of chemical contaminants, pathogens, and foreign bodies such as metal parts, glass, and plastics in agricultural lands (Table 3). However, to prevent the long-term accumulation of harmful levels of trace metals in agricultural soil, the CFIA sets maximum acceptable metal concentrations in biosolids which should not be exceeded by the CCME and the provincial guidelines. These limits are based on the maximum acceptable cumulative metal concentrations in soil over a 45-year period, calculated using the following formula (CFIA, 2023):

$$1{,}000{,}000 \text{ mg kg}^{-1} = \left[\frac{maximum\ acceptable\ cumulative\ metal\ addition\ to\ soil\ over\ 45\ years\ (kg\ metal\ per\ ha)}{45\ years\ x\ annual\ application\ rate\ (kg\ product\ per\ ha\ per\ yr)}\right]$$

Table 1.3 Maximum permissible contaminants in biosolids for agricultural land application in Canada

Chemical contaminant ($mg\ kg^{-1}$)	Quebec		Ontario			British Columbia		CCME		CFIA
	*C1	*C2	*AA	*A	*B	*A	*B	*A	*B	*Safety standards
As	13	41	13	13	75	13	75	13	75	18.75
Co	34	150	34	34	150	34	150	34	150	37.5
Cr	210	1000	210	210	1060	100	1060	210	-	262.5
Cu	400	1000	100	400	760	400	2200	400	-	187.5
Mo	10	20	5	5	20	5	20	5	20	5
Ni	62	180	62	62	180	62	180	62	180	45
Se	2	14	2	2	14	2	14	2	14	3.5
Zn	700	1850	500	700	1850	500	1850	700	1850	462.5
Cd	3	10	3	3	20	3	20	3	20	5
Hg	0.8	4	0.8	0.8	5	2	15	0.8	5	1.25
Pb	120	300	150	150	500	150	500	150	500	125
Dioxins and furans (ng TEQ/kg)	17	50	-			-		-	-	PCDD/Fs: 2.6775 ng TEQ/kg growing media *Maximum acceptable cumulative addition to soil = 5.355 mg

							TEQ/ha over 45 years	
Biological contaminants and parameters								
Pathogens	Salmonella: not detected in at least 2/3 of the samples	Salmonella: < 3 MPN per 4 grams E. coli: < 1000 CFU or (MPN) per gram		Fecal coliform: < 1,000 MPN per gram	Fecal coliform: < 2,000,000 MPN per gram	Fecal coliform: < 1,000 MPN per gram Salmonella: < 3 MPN per 4 grams	Salmonella: Not detectable at detection limit of less than 1 CFU per 25 grams Fecal coliform: < 1,000 MPN per gram	
Impurities								
Foreign bodies	> 25 mm: ≤ 1 per 500 ml	> 25 mm: 0 pieces per 500 ml Plastics (> 3mm): 0.5%		-		> 25 mm: ≤1 piece per 500 ml	> 25 mm: ≤2 pieces per 500 ml	-
Sharp foreign bodies	0 pieces per 500 ml (for at least 2/3 of the samples)	0 pieces in a size and shape that can cause injury	≤ 3 pieces per 500 ml, maximum dimension of 12.5 mm	0 pieces in a size and shape that can cause injury		> 3 mm: 0 pieces per 500 ml	≤ 3 pieces per 500 ml, maximum dimension of 12.5 mm	-
Total foreign bodies	> 2 mm: < 0.5%, excluding plastic beads	> 3mm: 1%	> 3mm: 2%	≤ 1%		-	-	

*C1 criteria, class A, category AA: suitable for unrestricted use on agricultural lands (Hebert, 2015; British Columbia MOE, 2002; Ontario MOE, 2012; CCME, 2005).

*C2 criteria, class B, category A: Can be applied to agricultural lands but with stringent controls and monitoring to ensure safety (Hebert, 2015; British Columbia MOE, 2002; Ontario MOE, 2012; CCME, 2005)

*B category (Ontario): considered “waste”, and is subject to ministry approval for transportation, use, and disposal. Not suitable foe sensitive areas like parks or residential areas. Could be used in agriculture for nutrient purposes only but require ministry approval under stringent controls and regulatory oversight (Ontario MOE, 2012)

*CFIA safety standards retrieved form (CFIA, 2023)

*TEQ: Toxic Equivalency Quotient, check table 3 from (CFIA, 2023) for calculations.

The Canadian Soil Quality Guidelines for agricultural land sets limits on metals, dioxins and furans, and monitors indicator organisms such as pathogens and microbial contaminants to protect the environment and human health (CCME, 2007). The trace metal concentration limits for agricultural land per the Canadian Soil Quality Guidelines are detailed in Table 1.4.

Table 1.4 Canadian soil quality guidelines for the protection of environmental and human health (CCME, 2007)

Chemical contaminant	Canadian soil quality guidelines for agricultural land use (mg kg^{-1})
As	12
Co	40
Cr	64
Cu	63
Mo	5
Ni	50
Se	1
Zn	200
Cd	1.4
Hg	6.6
Pb	70

At this time, there is no regulation to limit the amount of micron-sized plastics that are added to agricultural fields through land-spreading of biosolids. Still, the province of Quebec restricts the presence of foreign bodies > 25 mm in organic residues to 1 piece per 500 ml, while Ontario sets a stricter limit of zero pieces per 500 ml and specifically limits plastics (> 3 mm) to 0.5% (dry matter) (Hébert, 2015; Ontario MOE, 2012). Other regulatory agencies worldwide also impose restrictions on applying organic residues with larger plastic fragments. For example, the Canadian National Standard size restriction of > 3 mm only applies to sharp foreign matter, whereas general foreign matter, including plastic debris, is restricted to one piece not exceeding 25 mm in a 500 ml grab sample (CCME, 2005). In addition, the European Union established the Fertilising Product Regulation as a binding law across all member states to harmonize the requirements for fertilising products. This regulation limits foreign bodies > 2 mm to 3 g kg^{-1} and specifically limits plastics to 2.5 g kg^{-1} in biosolids (Langenkamp & Part, 2001). To this day, the United State EPA does not impose any restrictions on the presence of plastic debris in biosolids intended for agricultural land applications under the 40 CFR Part 503 Rule and its amendments, which governs the use and disposal of biosolids in the United States (US EPA 2023).

The lack of more strict regulation globally does not stem from a lack of awareness. There are many challenges in restricting the involuntarily input of microplastics to agricultural lands. Extracting microplastics from samples with high organic matter content, such as biosolids, is difficult. For example, a study by Wang et al. (2018) showed that only 20% of spherical polystyrene microplastics < 100 μm were successfully extracted from biosolids using conventional methods i.e., density separation. Advanced methods to recover microplastics from organic residues, such as the method developed by Ruffell et al. (2024), are time-consuming, costly, and introduces toxic materials, including hydrogen peroxide to recover the microplastics, making them impractical for the purpose of extracting microplastics and recovering the biosolids for agricultural land applications.

1.8 Papermill deinking biosolids

Papermill biosolids is the by-product of the pulp and paper industry. The composition of papermill biosolids can vary, based on the source of the recycled material. They typically consist of carbohydrates, wood fibers, such as hemi-cellulose, cellulose, and lignins, as well as trace metals, clays, and water (Price & Voroney, 2008). In addition, papermill biosolids contain nutrients known to aid in optimizing plant health including N, P, K, Ca and Mg. The agronomic use of papermill biosolids increases soil organic matter content, which has been shown to improve soil structure, bulk density and water-holding capacity (Rashid et al., 2006). However, the deinking fraction of papermill biosolids can contain non-nutritive substance. Deinking biosolids are produced from the fine sieving, flotation, and clarification sludge from washing filters, which are pressed to form the deinking sludge. This sludge is a complex mixture of organic matter, plastic debris, charged ions and trace metals. Plastics are present in deinking biosolids due to polyethylene-based coatings on paper, and trace metals like Cu originate from Cu phthalocyanine dyes used in the printing industry (Fyberg, 2005).

1.8.1 Effect of deinking biosolids on soil health and earthworm Physiology

Similar to primary papermill biosolids, deinking biosolids have been shown to significantly improve soil structure in a 2-yr field study with applications of 50, 100, and 150 Mg ha^{-1}. The study found that deinking biosolids reduced bulk density and increased hydraulic conductivity while maintaining soil pH and electrical conductivity (Price & Voroney, 2007). However, the same study showed that concentrations of Cu, Cr, and Pb in soil significantly increased with increasing application rates of deinking biosolids. Although Cu is an essential trace metal at lower concentration, elevated concentrations can be toxic to soil organisms, particularly earthworms. Kilpi-Koshi et al. (2020) and Owojori et al. (2009) investigated the effect of Cu on earthworms' physiology and found that exposure to 530 and 309 mg Cu kg^{-1} soil inhibited earthworm growth and cocoon hatchability by 50%, while juvenile production, identified as the most sensitive physiological biomarker in this study, was 50% inhibited at 154 mg Cu kg^{-1} soil. While the organic matter in deinking biosolids is considered a growth substrate for earthworms, it can be involved in interactions that are biologically harmful. For instance, organic matter is expected to bind Cu^{2+} ions to negatively-charged functional groups, such as carboxyl and hydroxyl, and occlude them in nano-sized pores. Specifically, cellulose fibers absorb Cu ions and form Cu^{2+}-d-gluconate complexes, a binding mechanism based on a ligand exchange reaction (Emam et al., 2012). Therefore, while papermill biosolids contain organic matter, nutrients and trace elements essential for maintaining and improving soil health, the deinking fraction of these biosolids should be carefully investigated. This is due to the potential for trace metal accumulation and their interactions with cellulose fibers in organic matter, which could lead to long-term environmental accumulation and possible risks to soil organisms like earthworms.

Although 70% of land applied biosolids in the agricultural sector in Quebec are supplied by the pulp and papermill industry, there is limited ecotoxicology assessment of the impact of deinking biosolids on soil organisms like earthworms. Research by Price and Voroney (2008) examined the response of field earthworms to annual applications of deinking biosolids to field earthworms on three agricultural plots, where four rates of papermill biosolids (0, 50, 100, and 150 ton ha^{-1}) were applied for 3 yr. They concluded that deinking biosolids had no adverse effects on earthworm population and biomass. Yet, investigating the effects of deinking biosolids on earthworms in a controlled laboratory setting using sensitive biomarkers, such as oxidative stress and DNA damage is necessary to sensitively determine their risk potential.

1.9 Effect of microplastics on earthworms

1.9.1 Physiological damage

Earthworms play an essential role in nutrient cycling and decomposition. They convert organic waste and biodegradable materials into nutrients, thus promoting maintenance and development of the nutrient content in the soil. These features make earthworms priority test organisms for monitoring soil contamination and an effective indicator of soil biological health (Han et al., 2014). Studies have shown that earthworms can be sensitive to microplastics exposure at a physiological level. For instance, Rodriguez-Seijo et al. (2017) observed that earthworms exposed to 125 mg polyethylene microplastics (250–1000 μm) kg^{-1} soil displayed grade 2 intestinal fibrosis. However, Mondal et al. (2023) found that exposure to 125 μm of polyethylene microplastics at a concentration of 15 g kg^{-1} soil for 28 d did not affect earthworm growth. On the other hand, when it comes to more sensitive physiological stress indicators, such as reproduction, several studies have found that microplastics exposure affect earthworm reproduction. For example, exposure to 5 g kg^{-1} soil of polyethylene microplastics (250–1000 μm) resulted in 70% inhibition of earthworm reproduction (Sobhani et al., 2021), possibly due to damage of the reproductive organs. This speculation was supported by Kawk and An (2021), who examined the effects of exposing earthworms to soil spiked with 1 g polyethylene microplastics kg^{-1} soil (180–212 and 250–300 μm) for 21 d. They discovered that this exposure caused damage to male reproductive organs while having minimal effects on female reproductive organs. Specifically, microplastics exposure affected the development of seminal vesicles in earthworms and significantly decreased sperm density in both treatments. It is clear that the presence of microplastics in agricultural soils can disrupt earthworm physiology and reduce their populations, which could in turn affect plant nutrient uptake.

1.10 Cytotoxicity

Evidence of microplastics uptake and internalization by macrophages, and the microplastics effect on cell viability are well documented in the literature. For instance, Adolfsson et al. (2018) observed a reduction in the ability of *Drosophila melanogaster* immune cells to phagocytize polystyrene microplastics (10 μm) *in vitro*, which is close to the immune cell diameter, compared to 1–7 μm microplastics exposure. In addition, exposing murine cells *in vitro* to polystyrene microplastics (0.2–6 μm) at a concentration of 250 μg ml^{-1} reduced cellular metabolic activity and provoked the generation of reactive oxygen species (Rudolph et al., 2021). Due to growing concerns about microplastics on human health due to their biomagnification in the environment (Yan et al., 2022), researchers started investigating the presence of microplastics in agricultural soil. This has led to a notable increase in *in vitro* studies exploring the effects of plastic on soil organisms, such as earthworms, at the cellular level; a topic that was not explored before 2023. Zhou et al. (2023) demonstrated that exposing earthworm coelomocytes to polystyrene nanoplastics (50–140 nm) for 2 h at concentrations of 50 and 200 μg ml^{-1} caused significant lysosomal membrane instability and rupture, and eventually cell death. Further, He et al. (2024) exposed earthworm immune cells to polystyrene nanoplastics (100 nm) at concentrations of 10, 20, 30, 40, and 50 mg l^{-1} for 12 h and reported significant cell damage at concentration of 30 mg l^{-1} and higher. In a whole organism study, Kwak et al. (2021) observed cytotoxicity in cells extracted from earthworms that survived exposure to soil spiked with microplastics (180-300 μm) at a concentration of 0.45 mg ml^{-1} for 21 d. Yet, the effects of microplastics that are similar in size to earthworm cells (5 to 30 μm, estimated in microscopic analysis, chapter 3), require further assessment. Exploring this gap is crucial to understanding the mechanisms behind the cytotoxicity of microplastics on earthworms and enhancing current regulations for land-spreading of biosolids.

1.11 Oxidative stress and DNA damage

1.11.1 Size-dependent effect

Numerous studies suggest that microplastics impact earthworms' oxidative stress, including molecular responses like DNA damage and lipid peroxidation, as well as the resulting antioxidant defense mechanisms. For instance, earthworms exposed to 1.25 g of polyethylene microplastics (28–145, 133–415 and 400–1464 μm) and polypropylene microplastics (8–125, 71–383 and 761–1660 μm) kg^{-1} soil significantly altered the activities of SOD, CAT, glutathione S-transferase, and 8-hydroxy-2′-deoxyguanosine levels in earthworms, denoting smaller microplastics showing the greatest effect (Li et al., 2021). This may be due to earthworms selectively ingesting smaller microplastics (< 50 μm) rather than larger ones (Cui et al., 2022). In contrast, Xu et al. (2021) illustrated that larger plastic fragments are more toxic than smaller ones. They found that 10 μm of polystyrene microplastics (10 mg kg^{-1}) caused significantly higher DNA damage in earthworms compared to 100 nm in a 21-d soil-exposure assessment. It appears that smaller microplastics are generally more toxic than larger ones, while overall, microplastics appear to be more toxic than nanoplastics, possibly due to the greater surface area occupied by microplastics in cells compared to nanoplastics and the possibility of microplastics causing frustrated phagocytosis. The theoretical basis of frustrated phagocytosis is explained in section 1.5.2.2 of the Literature review and the possibility is explored experimentally in Chapter 3 of this thesis. However, the comparative size-dependent toxicity seems to disappear when the particle sizes are relatively close. For instance, Jiang et al. (2020) investigated the effects of exposing earthworms to 100 nm and 1.3 μm polystyrene plastic at concentrations of 0.1 and 1 mg kg^{-1} soil for 14 d. They found that, in general, SOD activities were inhibited, and GSH levels increased upon exposure to microplastics. Yet, there was no significant size-dependent effect between treatments when the exposure concentration was the same. Thus, further comparative assessment using plastic particles of the same type and shape but with different size ranges from nanoplastics to microplastics with mechanistic pathway investigation, would reveal more information.

1.11.2 Biodegradability effect

In efforts to reduce plastic pollution, many countries shifted to using biodegradable plastic for food packaging, single use utensils, and grocery bags. This shift sparked interest among researchers about the impact of biodegradable microplastics on soil and aquatic organisms. A study by Ding et al. (2021) investigated the effect of 120 μm non-degradable polyethylene, and biodegradable polylactic acid and polypropylene carbonate microplastics on earthworms in a mesocosm and found significant reduction in cocoons and juvenile production exposed to 53 and 97 g microplastics kg^{-1} respectively, emphasizing no significant difference between biodegradable and non-biodegradable microplastics treatments. However, Shang et al. (2023) investigated the effect of non-degradable and biodegradable microplastics on earthworms, with and without Cd mixed in the soil. They found that in the presence of 20 mg Cd kg^{-1} soil, the integrated biomarkers response index which includes ROS, SOD, CAT, peroxidase, GSH, MDA, and DNA damage data, revealed higher toxicity of biodegradable PLA microplastics compared to non-degradable polystyrene. In short, polymer biodegradability appears to have negligible effect on earthworms. However, when the soil is mixed with trace metals, it appears that biodegradable microplastics can be more toxic than non-degradable microplastic. This finding aligns with my earlier interpolation in section 1.2.2 of the Literature review, suggesting that biodegradable microplastics would induce more toxicity in the presence of metals due to their cracks and fractures, which accumulate and bind with metals because of their reduced rigidity compared to non-degradable plastics.

1.11.3 Type-dependent effect

When it comes to non-degradable plastics, few studies examined the type-dependent toxicity on earthworms. Wang et al. (2019) illustrated that exposing earthworm to polyethylene (300 μm) and polystyrene (250 μm) microplastics at concentrations of 3, 15, 30 and 60 g kg^{-1} soil for 14 d induced stress symptoms in the earthworms in both treatments. Earthworms in the polyethylene treatments exhibited a significant increase in the CAT and peroxidase activities at the highest concentration. In the polystyrene treatment, SOD activities were significantly inhibited at higher concentrations; 60 g kg^{-1}, while peroxidase and CAT activities significantly increased across all concentrations. Further, the study by Li et al. (2021) where earthworms were exposed to polyethylene and polypropylene particles of various size reported statistically significant altercation in antioxidant enzymes activities compared to the control. In general, previous studies suggest minimal type-dependent toxicity of pristine non-degradable microplastics on earthworms. A comparative assessment using exact size ranges for different types of microplastics is needed to accurately evaluate the type-dependent toxicity.

1.11.4 Shape-dependent effect

No comparative assessment was done on the effect of microplastic shape on earthworm responses. However, Prendergast-Miller et al. (2019) illustrated that exposing earthworms to microfibers averaging 361 μm in length and 40 μm in diameter (at concentrations of 1 and 10 g kg^{-1} soil) did not induce avoidance behavior or mortality in earthworms. Nonetheless, the molecular chaperon heat shock protein 70, which helps protect cells from stress, was downregulated. This downregulation suggests a toxic response, as it indicates that the cellular defense mechanisms may be impaired or overwhelmed. Further, in a zebrafish study, 100 mg l^{-1} of microfibers 50 and 200 µm long with a diameter of 20 µm caused significant inflammation, oxidative stress and lipid depletion in a length-dependent manner, with longer fibers causing more toxicity (Zhao et al., 2022b). The only shape-dependent comparative study I found was conducted on the mussel *Mytilus galloprovincialis* (Park et al., 2024). In this study, mussels were exposed to small (20 µm), medium (45–75 µm), and large (>150 µm) polyethylene terephthalate fragmented plastic, and fiber plastic with a diameter of 13 µm and lengths of 200–400 µm (small) and 3000 µm (large) for 21 d. The results showed that both fragmented and fiber plastic caused significant increase in global DNA methylation level in gill tissues and in relative *Mg*TLR mRNA expression, with fiber plastic debris causing greater effect ($p < 0.01$) compared to fragmented plastic ($p < 0.05$). In addition, fiber plastic caused a significant increase in global DNA in digestive gland tissues ($p < 0.01$), whereas fragmented plastic did not cause a significant effect (Park et al., 2024). These observations suggest that fibrous microplastics can induce greater toxicity to the exposed organism. Currently, there is not enough data to determine the shape-dependent effects of microplastics on earthworms due to the lack of comparative studies between spherical microplastics and polymer-based microfibers specifically on earthworms.

1.12 Combined effect of microplastics-bound metals

In addition to their physical toxicity, microplastics also pose a chemical threat. Microplastics in agricultural soil and biosolids are not pristine – many charged substances sorb to the surface and within the molecular structure of plastic polymers. Consequently, and as discussed section 1.2.1 of the Literature review, microplastics also act as metal ion carriers, accumulating and transporting trace metals from soil or biosolids into the earthworms. This is supported by increasing Cd accumulation in *E. fetida*'s body tissues by 10–160% when exposed to a mixture of 300–9000 mg Cd kg^{-1} soil and polypropylene microplastics (< 150 μm), compared to earthworm exposure to an equivalent amount of $CdCl_2$ solution in soil (Zhou et al., 2020). Microplastics could increase metal toxicity to earthworms by exposing them to a medium containing both metals and microplastics. In fact, Li et al. (2021) noted that the addition of 0.5 g kg^{-1} of polyethylene microplastics (30 μm) to 100 mg kg^{-1} of Cu-spiked soil increased MDA content and SOD, peroxidase, and CAT activities in earthworms compared to exposure to an equivalent concentration of Cu alone in soil. Further, because microplastics found in biosolids and agricultural soil are not pristine due to weathering and abrasion effects, few studies investigated the effect of aged microplastics on earthworms with and without trace metals mixed in the soil. For instance, Li at al. (2023) investigated the effects of 100, 1000, and 10,000 mg of aged and pristine polyethylene microplastics (138 μm) per kg of natural soil containing 52 mg kg^{-1} Zn^{-2}, 16 mg kg^{-1} Pb^{-2}, and 0.11 mg kg^{-1} Cd^{-2}. They did not find any statistical significance in the trace metal accumulation in earthworms nor in ROS, SOD, MDA, and heat shock protein 70 expressions between aged and pristine microplastics. However, previous studies that investigated the co-exposure of microplastics and trace metals exposed earthworms to soil mixed with trace metals and added microplastics to the soil. This approach makes it challenging to evaluate how microplastics, acting as carriers for trace metals, affect earthworms. To better understand the oxidative stress response, further research is needed on earthworms exposed to aged microplastics spiked with trace metals then added to the soil as a joint contaminant. This will provide a more comprehensive and accurate assessment of the potential toxicity of microplastics to earthworms.

Summary and Future Directions

There is increasing concern that microplastics, commonly present in agricultural soil, from the use of organic fertilizers, wastewater irrigation and plastic mulching, could have long-term impacts on soil health and the environment. Aging increases the adsorption capacity of microplastics, creating additional binding sites for metals and potentially enhancing the biological risks these contaminants pose to soil organisms like earthworms. In addition, although cellular interactions reveal that macrophages can engulf microplastics, the cellular damage caused by size-dependent mechanisms remains poorly understood. Furthermore, current regulations and guidelines lack stringent control over the input of microplastics into agricultural lands, emphasizing the need for improved monitoring and management practices. Therefore, the objectives of my thesis are to (1) evaluate the potential toxicity of deinking biosolids in Cu-amended soil to earthworms, based on physiological responses, i.e., growth and reproduction, (2) quantify cell viability as the outcome of a size-dependent phagocytic response in earthworm coelomocytes, (3) investigate how deinking biosolids and microplastics-bound Cu affect biomolecular responses in earthworms, reflecting a realistic environmental condition, where pollutants often exist in combination rather than isolation. I hypothesize that:

1. The growth and reproduction of earthworms exposed to deinking biosolids at sublethal Cu concentrations will be stimulated at low concentrations and diminished at higher concentrations, with this biphasic effect modulated by the presence of wellbeing-enhancing substrates (e.g., nutrients and organic matter contained within the biosolids).

2. Greater cell mortality will occur when coelomocytes are exposed to larger (20–27 μm) than smaller (1–10 μm) microplastics, due to the greater likelihood of frustrated phagocytosis with larger microplastics.

3. Soil amended with aged Cu-spiked microplastics will lead to increased oxidative stress and DNA damage in earthworms, more so than soil containing only pristine microplastics, due to greater stress induced by the Cu-microplastic complex.

CHAPTER 2

Physiological effects of deinking biosolids in copper-amended soil on the earthworm Eisenia fetida

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2.1 Abstract

Deinking biosolids (DB) are nutrient-rich organic residues used as a fertilizer to promote plant growth in agriculture and on reclaimed lands. However, DB also contains non-nutritive substances like copper (Cu) in the phthalocyanine dyes used by the printing industry. Consequently, agricultural soils amended with DB could accumulate Cu to levels that threaten soil health and crop production. This study evaluates the effects of DB-amended soil on the earthworm *Eisenia fetida* in Cu-amended soil, focusing on the sublethal effects on growth, reproduction, and Cu accumulation in earthworm homogenate, cytosol, and metallothionine. Following the modified OECD guideline, the earthworms were exposed to artificial soil amended with DB (nominally 0, 34, 68, 136, and 272 g kg^{-1} soil), Cu (nominally 0, 100, 160, 256, and 410 mg kg^{-1} soil), or a combination of DB and Cu. Results indicate that DB and Cu had no significant effects on the 28-d earthworm growth in single-exposure treatments compared to unamended controls ($p > 0.05$), possibly due to growth-enhancing substrates, i.e., nutrients and organic matter contained within the biosolids. However, reproduction was inhibited in earthworms exposed to concentrations ≥ 68 g deinking biosolids or ≥ 410 mg Cu compared to controls ($p < 0.05$). In combined exposures, the addition of DB to Cu-spiked soil did not exacerbate Cu toxicity or increase Cu accumulation in earthworms and subcellular fractions, compared to single-treatment exposures. Thus, earthworm exposure to combined Cu and DB treatment in artificial soil was not more toxic than the individual contaminants.

Keywords: Organic residues, Essential trace metals, Toxicity mixture, Metal bioaccumulation, Dose-response analysis.

2.2 Introduction

Historically, papermill biosolids were disposed of in landfills and through incineration. After the 1990s, several European countries diverted papermill biosolids from landfills to gasification and pyrolysis facilities for energy recovery as part of their sustainability and circular economy initiatives (Monte et al., 2008; CEPI, 2004). However, this was not a cost-effective solution in many jurisdictions because wet biosolids have a low net energy return and are similar to other products (e.g., wastewater biosolids and municipal compost) that are recycled through direct land application. If papermill biosolids are to be recycled through land spreading, this requires a coordinated plan to distribute this organic residual in agricultural and marginal (non-agricultural) lands. Globally, the pulp and paper mill industry produces around 400 million Mg (wet weight) of paper mill biosolids annually (Fauber et al., 2016). Canada is among the top five paper producers in the world and generates 1.4 million Mg of papermill biosolids in the province of Quebec alone (Recyc-Quebec, 2018).

Papermill biosolids can be used as a soil amendment because they contain cellulose and lignin biopolymers and nutrients. Deinking biosolids (DB), a by-product of paper recycling, contain cellulose fibers that improve soil bulk density, soil structure, and water-holding capacity, and it is also rich in essential nutrients like nitrogen and phosphorous that support crop production (Rashid et al., 2006). However, DB also contains copper (Cu), a trace metal in the phthalocyanine dyes (50–100 mg Cu kg^{-1}) used by the printing industry (Marouani et al., 2021; Price & Voroney, 2008). Consequently, the impact of Cu accumulation on soil biota and crop health in soils amended with DB should be evaluated.

The extensive soil burrowing by earthworms, coupled with their ability to decompose organic waste and mineralize nutrients, makes them a useful model organism to assess the ecotoxicity of DB in soil. Stimulatory and hormetic effects of Cu on earthworm growth and reproduction are often reported because Cu is essential for earthworm metabolic processes at tissue concentrations ~ 10-14 ng Cu mg^{-1} wet weight (Mincarelli et al., 2019; Xing et al., 2018). Soil containing < 80 mg Cu kg^{-1} soil can stimulate earthworm metabolic and physiological responses (Pelosi et al., 2024), but higher Cu concentrations can be toxic and inhibit physiological processes. For earthworms exposed to an artificial soil amended with Cu salts, the 50% inhibition effect concentration (EC_{50}) for adult earthworm growth was 530 mg Cu kg^{-1} soil (measured) after 30 d of exposure, 309 mg Cu kg^{-1} soil for cocoon hatchability, and 154 mg Cu kg^{-1} soil (nominal) for juvenile production after 56 d of exposure (Kilpi-Koski et al., 2020; Owojori et al., 2009).

The present study evaluates the effects of DB-amended artificial soil on the earthworm *E. fetida* based on changes in growth and reproduction in Cu-contaminated soil. It is hypothesized that (1) the growth and reproduction of earthworms exposed to DB at sublethal Cu concentrations is stimulated at low concentrations and is diminished at higher concentrations; and (2) that this biphasic effect is modulated by the presence of growth-enhancing substrates (e.g., nutrients and organic matter contained within the biosolids).

2.3 Material and methods

2.3.1 Biosolid collection and preparation of artificial soil

The DB was supplied by a papermill factory in Kingsey Falls, Quebec. The DB samples contained 30 mg total Cu kg^{-1}; other physical and chemical properties are reported in Table 2.1. The DB sample meets the C1-P1-O1 criteria of the Canadian Council of Ministers of the Environment, indicating that the permissible land application rate of 31 Mg ha^{-1} does not pose a significant environmental risk from chemical contaminants, pathogens, and odors (Hébert, 2015).

The artificial soil (used here as the substrate) contained 70% silica sand, 20% colloidal kaolinite clay, and 10% sphagnum peat moss (OECD, 2016). This soil was sieved through < 2 mm mesh, adjusted to pH 6 ± 0.5 using calcium carbonate (15 mg kg^{-1}), moistened with deionized water to reach 70% of its maximum water holding capacity, and stored at 20°C for 10 d to achieve steady-state conditions without the formation of aggregates. Each experimental unit was a 1-L glass jar with a perforated lid containing 500 g (wet weight) of artificial soil. For the treatment groups (4 replicate jars per group), the soil was amended with different concentrations of DB, dissolved $Cu(NO_3)_2$, or a combination of DB and the Cu solution. The negative control group consisted of earthworms in artificial soil (no DB or Cu added). The nominal concentrations were 100, 160, 256, and 410 mg kg^{-1} soil for Cu, and 34, 68, 136, and 272 g kg^{-1} soil for DB, based on our earlier earthworm growth and reproduction range-finding studies (unpublished data). All DB treatments exceeded the nominal permissible land application rate in Quebec (i.e., 31 t ha^{-1} ≈ 16 g kg^{-1} on an equivalent mass basis). For each treatment jar, the Cu solution was mixed into the soil. The DB was mixed thoroughly with the soil. ACS-grade copper II nitrate ($Cu(NO_3)_2$) and Tris/HCl buffer used in this study were obtained from Fisher Scientific (Ottawa, Ontario, Canada). All other chemicals and reagents were at least analytical grade purity and were obtained from commercial sources.

2.3.2 Earthworm growth and reproduction studies

E. fetida were purchased from Merlan Scientific (Toronto, Ontario, Canada). The earthworms were kept in a breeding substrate of peat moss at 20°C for 7 d. During this incubation period, the earthworms were fed Magic Worm Food (Merlan Scientific). The earthworms were then transferred into the artificial soil and acclimatized in the dark for 7−14 d. Adult earthworms (with clitellum present) weighing between 250 to 600 mg were selected for the following growth and reproduction studies. The earthworms were washed with deionized water, placed briefly on filter paper, and weighed. A group of 10 earthworms were assigned to each control and treatment jar. The jars were incubated for 12 hr (light/dark cycle) at 20 ± 2 °C (mean ± SD). Earthworms were fed 2 g of Magic Worm Food once a week, and the moisture content was adjusted to maintain 70% water-holding capacity (OECD, 2016). After 28 d, the soil was removed from the jars, and adult earthworms were collected, washed with deionized water, placed briefly on filter paper to remove excess water, and weighed (g fresh weight). The instantaneous growth rate (IGR, g final mass g^{-1} initial mass d^{-1}) was calculated according to Equation 1 (Brafield and Llewellyn, 1982; Whalen et al., 2012).

$$\mathrm{IGR} = \ln \frac{(Wf - Wi)}{Dt} \qquad (1)$$

where *Wf* and *Wi* are the final and initial earthworm mass (g), respectively, and *Dt* is the growth interval (after 28 d).

The soil (containing cocoons and juveniles) was then returned to the jars, and incubated for an additional 28 d. The number of juvenile worms in each jar was recorded at the end of the exposure period (56 d) and reported per 100 g of soil. Boric acid (3524 mg kg^{-1}) was used as the 14-d positive control, which approximates the 50% lethal concentration (LC_{50}) of boric acid to *E. fetida* (Environment Canada, 2007). This concentration led to a mean mortality of 45 ± 5% (SE, n = 4 replicates) and confirmed the lethality of boric acid on earthworms.

2.3.3 Preparation of subcellular fractions and Cu analysis

In heavy metal invertebrate bioaccumulation studies, the unbound or free metal fraction can be absorbed and accumulated in the whole earthworm (specifically in the cytosol) and is biologically active (Wallance & Louma, 2003). Metallothionein (MT) is a metal-binding cytosolic protein responsible for transporting and sequestering free or loosely bonded Cu ions within cells (Santon et al., 2009), so MT-bound Cu is not considered biologically active. Thus, Cu uptake in whole earthworms, cytosol, and metallothionine fractions was evaluated here to understand the distribution of biologically active and detoxified Cu.

Adult earthworms were weighed and placed on moistened filter paper for 24 h to void their gut contents, before being frozen at -70°C. On the day of analysis, three earthworms from each treatment were pooled, thawed, and homogenized in 3 mL of cold 10 mM Tris/HCl buffer (pH 7.5) using a T10 basic ULTRA-TURRAX (IKA) dispersion device (n=4). The homogenate was centrifuged at 1450 ×g for 15 min at 4°C (Yu et al., 2010). The supernatant was then centrifuged at 100,000 ×g for 1 h at 4°C to separate the cytosol from organelles (Wallance & Luoma, 2003). The resulting supernatant (containing cytosol) was heated to 80°C and centrifuged at 30,000 ×g for 10 min in a pre-heated (40°C) centrifuge to separate the heat-stable proteins (including MT-like proteins) from the precipitated heat-sensitive enzymes (Wallace & Luoma, 2003). The Cu concentrations in the whole homogenate and whole and heat-resistant cytosolic fractions were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS) at A&L Canada Laboratories (London, ON, Canada). Soil Cu concentrations were not measured due to variations in DB application rates in treatments, which could affect the uniformity of Cu distribution in the soil. Since DB was thoroughly mixed with the soil, sampling could unintentionally bias the results by capturing areas with higher or lower concentrations of DB, leading to inconsistent measurements.

2.4 Data analyses

Differences in the mean toxicological responses (earthworm growth and reproduction) and Cu accumulation in earthworms were compared for DB alone, Cu alone, and the combined DB+Cu treatments using ANOVA ($p < 0.05$). The EC_{50} was estimated with a three-parameter logistic regression curve fitting approach described in Supplementary Text S1.

2.5 Results

2.5.1 Effects of Cu exposure on earthworm 28 d-growth, 56 d-reproduction, and Cu uptake

Exposure to Cu (0 to 410 mg Cu kg^{-1} soil) did not significantly affect the growth rate ($p > 0.05$) (Fig. 2.1A). The unbounded NOEC for growth was > 410 mg Cu kg^{-1} soil (highest Cu concentration tested). Earthworm juvenile production was not significantly affected by < 256 mg Cu kg^{-1} soil compared to the control (48 ± 3 worms 100 g^{-1} soil). On the other hand, a significant decrease in juvenile production (10 ± 3 worms 100 g^{-1} soil) was observed in the 410 mg Cu kg^{-1} soil treatment group relative to the control ($p \leq 0.05$) (Fig. 2.1C). Based on three-parameter logistic regression, the EC_{50} and NOEC values for juvenile production were 362 (95% CI 312–423) mg kg^{-1} and 256 mg Cu kg^{-1} soil, respectively. The Cu accumulation in adult *E. fetida* was not significantly affected by Cu concentrations up to 410 mg Cu kg^{-1} compared to the control (Table 2.2). The lack of growth affects and the significant inhibition of juvenile production in the 410 mg Cu kg^{-1} soil treatment suggest that earthworm reproduction response is more sensitive to Cu exposure (in artificial soil) than earthworm growth.

2.5.2 Effects of deinking biosolids and the combined DB-Cu exposure on earthworm 28 d-growth and 56 d-reproduction, and Cu uptake

Exposure to DB did not significantly affect the growth rate and Cu accumulation of adult *E. fetida* in all treatment concentrations ($p > 0.05$) compared to control (Fig. 2.1B). However, the lowest juvenile production of 22 ± 1 worms 100 g^{-1} soil was found in the group spiked with the highest DB application rate of 272 g DB kg^{-1}, which was about 50% lower than the 48 ± 3 juveniles

100 g^{-1} soil produced in the control ($p < 0.05$, Fig. 2.1D). The juvenile production NOEC following DB exposure was estimated at 136 g kg^{-1}. The Cu accumulation in adult *E. fetida* and the subcellular fractions (cytosol and metallothionine) was not significantly affected by these DB treatments compared to the control (Table 2.2). Growth and reproduction studies were then conducted using earthworms exposed to combined Cu and DB treatments. Earthworm growth, reproduction, and Cu accumulation were not significantly affected by the combination of DB and Cu treatments ($p > 0.05$, two-way ANOVA; Table 3).

2.6 Discussion

2.6.1 Effects of DB on earthworm growth and reproduction

This study hypothesizes that the growth and reproduction of earthworms exposed to DB at sublethal Cu concentrations are stimulated at low concentrations and are diminished at higher concentrations and that this biphasic response is modulated by the presence of growth-enhancing substrates (e.g., nutrients and organic matter) contained within the biosolids. However, our findings (Table 2.2) suggest that earthworm growth was not affected by DB exposure ($p > 0.05$). This lack of effect may be attributed to organic matter, and essential nutrients such as N, P, and K in DB (Table 2.1). Similarly, an earlier three-year field study reported no negative significant effect on earthworm biomass in three agricultural fields treated with DB at application rates of up to 150 Mg ha^{-1} (Price and Voroney, 2008), which corresponds to 15 g deinking biosolids kg^{-1} soil in a lab-scale jar assessment (assuming soil bulk density of 1 g cm^{-3}). Further, the present study found no significant correlation between DB exposure and Cu concentrations in earthworm tissue, cytosol, and metallothionine ($p > 0.05$, Table 2.2). Our results suggest that exposure to DB of application rates up to 272 g kg^{-1} does not lead to Cu accumulation in earthworms and that its lack of impact on growth is likely due to the growth-enhancing substrates present in the biosolids.

Earthworm juvenile production was decreased at 68 and 272 g DB kg soil compared to the control ($p < 0.05$, $R^2 = 0.78$). When experiencing environmental stress, earthworms can allocate more energy towards maintaining their growth at the expense of reproduction (Aira et al., 2007). This trade-off may explain the maintenance of earthworm growth but there was an inhibition of juvenile production when adult *E. fetida* were exposed to elevated application rates of DB (Fig. 2.1), particularly at the highest application rate tested. Further, the observed effect of DB on earthworm juvenile production was not associated with a significant increase in Cu concentration in earthworm tissue, cytosol, and metallothionine as we hypothesized ($p > 0.05$). It is believed here that while DB application as a soil amendment seems favorable for earthworms from a nutritional perspective; however, an application of ≥ 68 g DB kg^{-1} soil (equivalent to 133 Mg ha^{-1}) could impair earthworm reproductive functions and potentially reduce their population.

2.6.2 Effects of Cu on earthworm growth and reproduction

Earthworm growth was not affected by Cu exposure, but juvenile production was inhibited at the highest concentration (410 mg Cu kg^{-1} soil (nominal), $p < 0.05$, $R^2 = 0.99$). These findings align with those of Fai et al. (2023), who reported no significant effect on *Alma nilotica* growth at nominal Cu concentrations up to 668 mg Cu kg^{-1} garden soil, despite observing inhibited reproduction ($p < 0.05$). Interestingly, the significant effect of Cu exposure on earthworm reproduction was not associated with a significant increase in Cu concentrations in earthworm homogenate, cytosol, and metallothionine ($p > 0.05$). However, this is consistent with other reports of no correlation between Cu content in soil and whole earthworms (Li et al., 2010; Nannoni et al., 2014). Furthermore, Fai et al. (2023) estimated Cu BAF to be < 1 in a 35-d test, suggesting Cu has a low bioaccumulation potential, and found no correlation between BAF and Cu concentrations in soil or whole earthworms. In our study, we measured Cu uptake in earthworms, including its distribution in subcellular fractions such as the metal-binding protein metallothionine, to gain insight into the efficiency of earthworms in detoxifying internal Cu under stress. In a 28-d exposure assessment at Cu concentrations of up to 410 mg kg^{1}, earthworms appeared capable of detoxifying Cu effectively, as we observed no significant increase in Cu accumulation. This capability could be attributed to earthworms' ability to regulate internal Cu levels through hemostatic mechanisms (Richardson et al., 2020). However, time series analysis of the uptake of Cu and its distribution between cytosol and metallothionine could provide further insights into the regulatory mechanisms that earthworms employ to cope with Cu exposure.

2.6.3 Effects of combined exposure of DB and Cu on earthworm growth and reproduction

We expected that the addition of DB to Cu-spiked soil would increase Cu toxicity to earthworms. However, DB application did not exacerbate Cu toxicity to earthworms, for their growth, reproduction, and Cu uptake. Additionally, there was no pattern between Cu accumulation in earthworm tissues, earthworm growth, and reproduction in the combined treatments. DB is rich in transition metals, including Ni^{2+}, Zn^{2+}, and Hg^{2+}, which can compete with Cu^{2+} for binding sites on transporters that selectively bind metals depending on the cellular metal ion homeostasis, metal intrinsic properties, polarizability of the metal ligating atom, ligand type, and binding site geometry (Dudev & Lim, 2023). Similarly, Vijver et al. (2006) reported that earthworms have a higher affinity for Zn^{2+} than Cu^{2+}, attributing this to the broader role of Zn in metabolic processes, enzyme functions, and protein structure.

2.7 Conclusion

We expected that the growth and reproduction of earthworms exposed to DB at sublethal Cu concentrations would be stimulated at low Cu concentrations and diminish at higher concentrations and that this biphasic effect would be driven by the presence of growth-enhancing substrates (e.g., nutrients and organic matter contained within the biosolids). However, the addition of DB to Cu-amended soil did not affect the growth rate or reproduction of *E. fetida*. Thus, synergistic interactions between Cu and DB were not observed in the present artificial soil studies. The controlled results from this study add to the toxicology exposure database used for ecotoxicological risk assessment by regulatory agencies (USEPA, 2024). Yet, more sensitive biomarkers, such as reactive oxygen species levels, antioxidant enzyme activity, and DNA damage, could provide insight into the subcellular effects of DB exposure, and potentially provide mechanistic explanation for the observed *in vivo* effects.

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Table 2.1 Physicochemical properties of deinking biosolids

Parameters	Value	*Maximum allowable in the C1 category of biosolids
Moisture content (%)	52	- [1]
pH	8.7	- [1]
C/N	164	≥ 70 (O1) [2]
		< 70 (O2) [2]
Organic matter (%)	52	- [1]
Total N (mg kg^{-1})	1591	- [1]
NH_4-N	36	- [1]
Total P_2O_5 (mg kg^{-1})	317	- [1]
Total K_2O (mg kg^{-1})	80	- [1]
Dioxins and furans (ng kg^{-1})	2	17
Arsenic (mg kg^{-1})	0.7	13
Cobalt (mg kg^{-1})	2	34
Chromium (mg kg^{-1})	4	210
Copper (mg kg^{-1})	30	400
Molybdenum (mg kg^{-1})	4	5
Nickel (mg kg^{-1})	2	62
Selenium (mg kg^{-1})	0.5	2
Zinc (mg kg^{-1})	140	700
Cadmium (mg kg^{-1})	0.9	3
Mercury (mg kg^{-1})	0.1	0.8
Lead (mg kg^{-1})	5	150

* Criteria enforced by the Canadian Council of Ministers of the Environment (CCME), to limit chemical contaminants to levels that do not pose a significant environmental risk (Hébert, 2015).

[1] The CCME does not set the required value for the parameter.

[2] O1: odour score (low odour).

[2] O3: odour score (strongly malodorous).

Table 2.2 Toxicological responses and Cu accumulation in the earthworm *E. fetida* exposed to single treatments of Cu and deinking biosolids in artificial soil.

Nominal Cu (mg kg^{-1} soil)	IGR ($\times 10^2$ g g^{-1} d^{-1})	Juveniles (count 100 g^{-1} soil)	Homogenate (μg Cu g^{-1})	Cytosol (μg Cu g^{-1})	Metallothionine (μg Cu g^{-1})
0	4.4 ± 1.44 a	48 ± 3.23 a	1.15 ± 0.22 a	1.14 ± 0.25 a	0.52 ± 0.22 a
100	6.0 ± 2.26 a	51 ± 2.40 a	1.59 ± 0.46 a	1.35 ± 0.60 a	0.36 ± 0.03 a
160	5.4 ± 1.67 a	51 ± 5.83 a	1.77 ± 0.29 a	1.39 ± 0.36 a	0.65 ± 0.10 a
256	5.3 ± 1.55 a	49 ± 4.96 a	1.85 ±0.43 a	0.46 ± 0.36 a	0.90 ± 0.30 a
410	4.3 ± 1.61 a	10 ± 2.67 b	2.87 ± 0.69 a	1.87 ± 0.52 a	1.00 ± 0.34 a
NOEC	410 mg Cu kg^{-1}	256 mg Cu kg^{-1}	-	-	-
EC_{50} (95% CI)	N/A	368 mg Cu kg^{-1} (312–423)	-	-	-
p	0.95	< 0.05	0.14	0.82	0.29
Nominal deinking biosolids (g kg^{-1} soil)					
0	4.4 ± 1.44 a	48 ± 3.23 b	1.15 ± 0.22 a	1.14 ± 0.25 a	0.52 ± 0.22 a
34	6.3 ± 2.35 a	38 ± 4.05 ab	0.80 ± 0.05 a	0.57 ± 0.09 a	0.39 ± 0.02 a
68	4.7 ± 1.58 a	33 ± 3.90 ac	0.89 ± 0.05 a	0.72 ± 0.08 a	0.31 ± 0.06 a
136	5.4 ± 1.24 a	42 ± 4.23 b	0.94 ± 0.03 a	0.66 ± 0.08 a	0.35 ± 0.04 a
272	5.0 ± 1.49 a	22 ± 1.00 c	0.91 ± 0.08 a	1.01 ± 0.42 a	0.32 ± 0.07 a
NOEC	272 g DB kg^{-1}	136 g DB kg^{-1}	-	-	-
EC_{50} (95% CI)	N/A	-[1]	-	-	-
p	0.94	< 0.05	0.28	0.38	0.64

IGR, instantaneous growth rate of adult worms. Cu accumulation; in subcellular fractions (cytosol and metallothionine) and whole earthworm (homogenate) were measured after 28 d of exposure. The number of juveniles was counted after 56 d. Values are mean ± standard error, n=4. *p*-values represent significance determined by One-way ANOVA. EC_{50} (95% CI) values were estimated using a 3-parameter logistic regression curve fitting model (Fig. S2.1).

-[1] The estimated EC_{50} for the effects of DB on earthworm growth of 379 g DB kg^{-1} soil was excluded due to unrealistic estimate of confidence intervals.

Table 2.3 Toxicological responses and Cu accumulation in the earthworm *E. fetida* exposed to combined treatments of Cu and deinking biosolids in artificial soil.

Deinking biosolids (g kg^{-1} soil)	Cu (mg kg^{-1} soil)	Growth ($x10^2$ g g^{-1} d^{-1})	Juveniles (count 100 g^{-1} soil)	Homogenate (μg g^{-1})	Cytosol (μg g^{-1})	Metallothionine (μg g^{-1})
0	0	4.4 ± 1.44	48 ± 3.23	1.15 ± 0.22	1.14 ± 0.25	0.52 ± 0.22
34	100	6.4 ± 2.06	29 ± 9.39	1.58 ± 0.15	1.07 ± 0.18	0.60 ± 0.04
34	160	6.0 ± 2.28	36 ± 8.06	1.96 ± 0.25	1.09 ± 0.19	0.55 ± 0.17
34	256	7.6 ± 1.90	28 ± 7.61	2.06 ± 0.35	1.44 ± 0.19	0.72 ± 0.12
34	410	6.2 ± 1.64	14 ± 4.47	2.70 ± 0.69	1.92 ± 0.66	1.23 ± 0.56
68	100	5.4 ± 1.55	29 ± 4.52	2.10 ± 0.69	1.15 ± 0.17	0.78 ± 0.23
68	160	5.1 ± 2.30	41 ± 6.24	1.85 ± 0.25	1.29 ± 0.09	0.62 ± 0.09
68	256	6.0 ± 2.33	28 ± 8.62	2.0 ± 0.05	1.62 ± 0.10	0.90 ± 0.29
68	410	6.0 ± 1.85	33 ± 6.46	2.49 ± 0.39	1.89 ± 0.25	1.34 ± 0.30
136	100	5.3 ± 1.86	40 ± 8.54	1.46 ± 0.15	1.33 ± 0.13	0.54 ± 0.09
136	160	5.5 ± 1.89	47 ± 7.72	1.62 ± 0.34	1.11 ± 0.20	0.55 ± 0.07
136	256	5.3 ± 2.02	43 ± 10.4	2.13 ± 0.55	1.71 ± 0.35	0.94 ± 0.28
136	410	6.3 ± 1.94	31 ± 7.05	3.26 ± 0.66	2.81 ± 0.39	1.70 ± 0.39
272	100	5.3 ± 1.84	34 ± 7.0	1.60 ± 0.16	1.36 ± 0.22	0.85 ± 0.26
272	160	4.9 ± 1.89	39 ± 9.68	1.64 ± 0.26	1.16 ± 0.27	0.66 ± 0.09
272	256	6.1 ± 2.62	34 ± 6.08	1.96 ± 0.21	1.44 ± 0.08	0.89 ± 0.12
272	410	5.90 ± 2.3	22 ± 6.78	2.92 ± 0.32	2.56 ± 0.26	1.32 ± 0.11
Treatment effects Deinking biosolids: Cu		$p = 0.31$	$p = 0.15$	$p = 0.99$	$p = 0.87$	$p = 0.91$

Instantaneous growth rate (IGR) of adult worms, and Cu accumulation in whole earthworm (homogenate) and subcellular fractions (cytosol and metallothionine) were measured after 28 d of exposure. The number of juveniles was counted after 56 d. Values are mean ± standard error (n = 4). *p*-values represent significance determined by Two-way ANOVA.

FIGURES

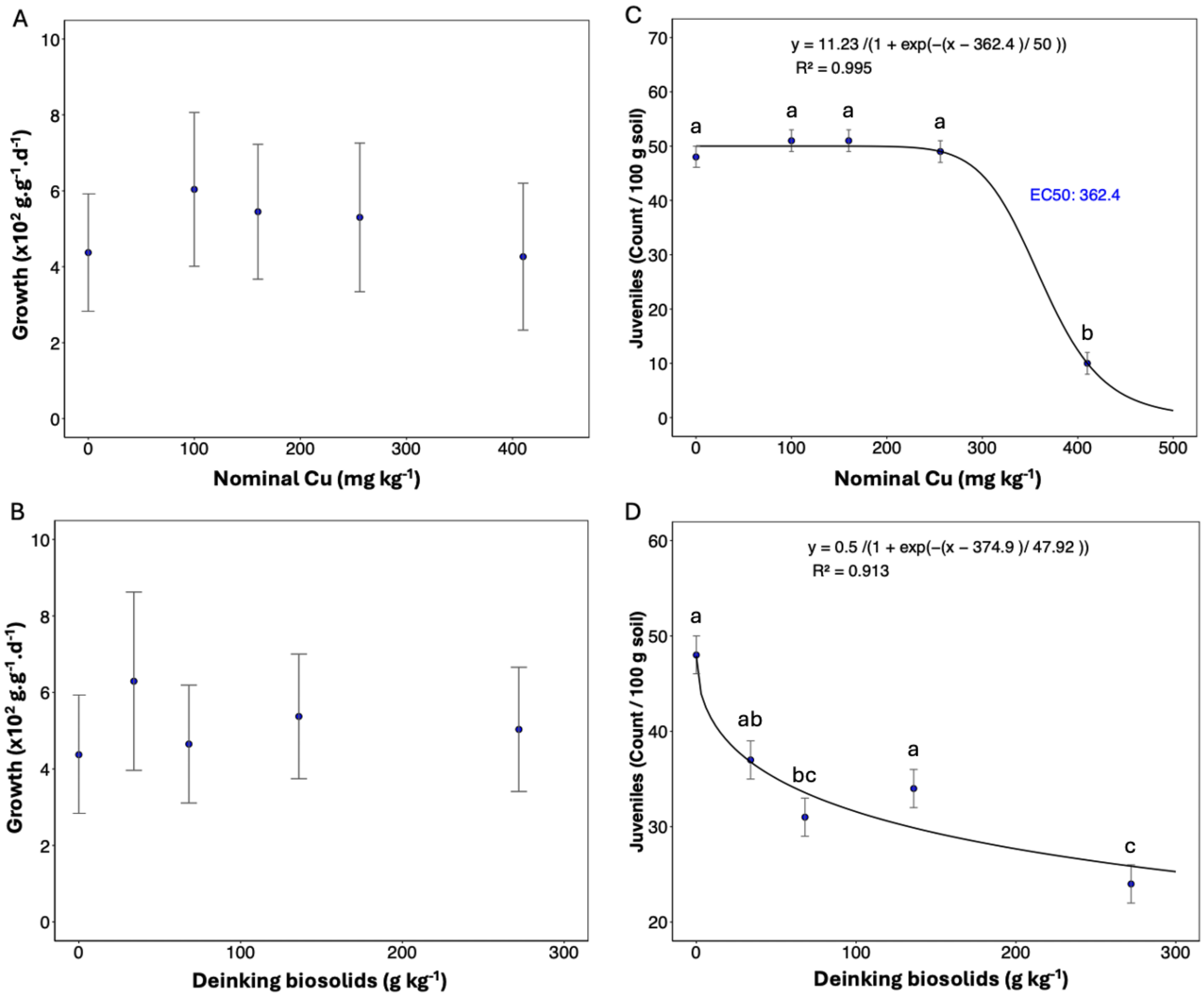

Figure 2.1 The effects of deinking biosolids and nominal Cu exposure on growth and reproduction of the earthworm *Eisenia fetida* in artificial soil. Growth results of adult earthworms were collected after 28 d of exposure (A, B), and the number of juveniles was counted after 56 d of exposure (C, D). Differences in mean toxicological responses were compared using ANOVA followed by Tukey's HSD post-hoc tests (significance at $p < 0.05$).

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SUPPLEMENTARY MATERIALS

Physiological effects of deinking biosolids in copper-amended soil on the earthworm *Eisenia fetida*

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The EC_{50} was estimated using a 3-parameter logistic regression model (Kilpi-Koski et al., 2020) defined by equation S1:

$$Y = \frac{Y_{max}}{1+(\frac{c}{EC_{50}})^{slope}} \qquad \text{(S1)}$$

where *Y* represents the response variable (e.g., adult *E. fetida* growth rate or the juvenile count), Y_{max} is the maximum response observed, *c* is the concentrations of the treatment (e.g., Cu and deinking biosolids), and EC_{50} is the concentration at which the response is reduced to 50% of Y_{max}.

Figure S2.1 displays the data fitted using 3-parameter logistic regression model for the effect of Cu on *E. fetida* growth and juvenile production, and the effect of deinking biosolids on *E. fetida* juvenile production. For the effects of deinking biosolids on *E. fetida* growth, a 4-parameter logistic regression model was used because it provided better R-squared value (better fit to the data). The 4-parameter logistic regression model (Ritz et al., 2020) is defined by equation S2:

$$Y = c + \frac{d-c}{1+(\frac{x}{EC_{50}})^{b}} \qquad \text{(S2)}$$

where *Y* represents the response variable (i.e., adult *E. fetida* growth in deinking biosolids treatment), *c* is the minimum observed growth rate, *d* is the maximum observed growth rate, *x* is the concentration of the treatment (i.e., deinking biosolids), and EC50 is the concentration at which the response reaches 50% of the range between *c* and *d*.

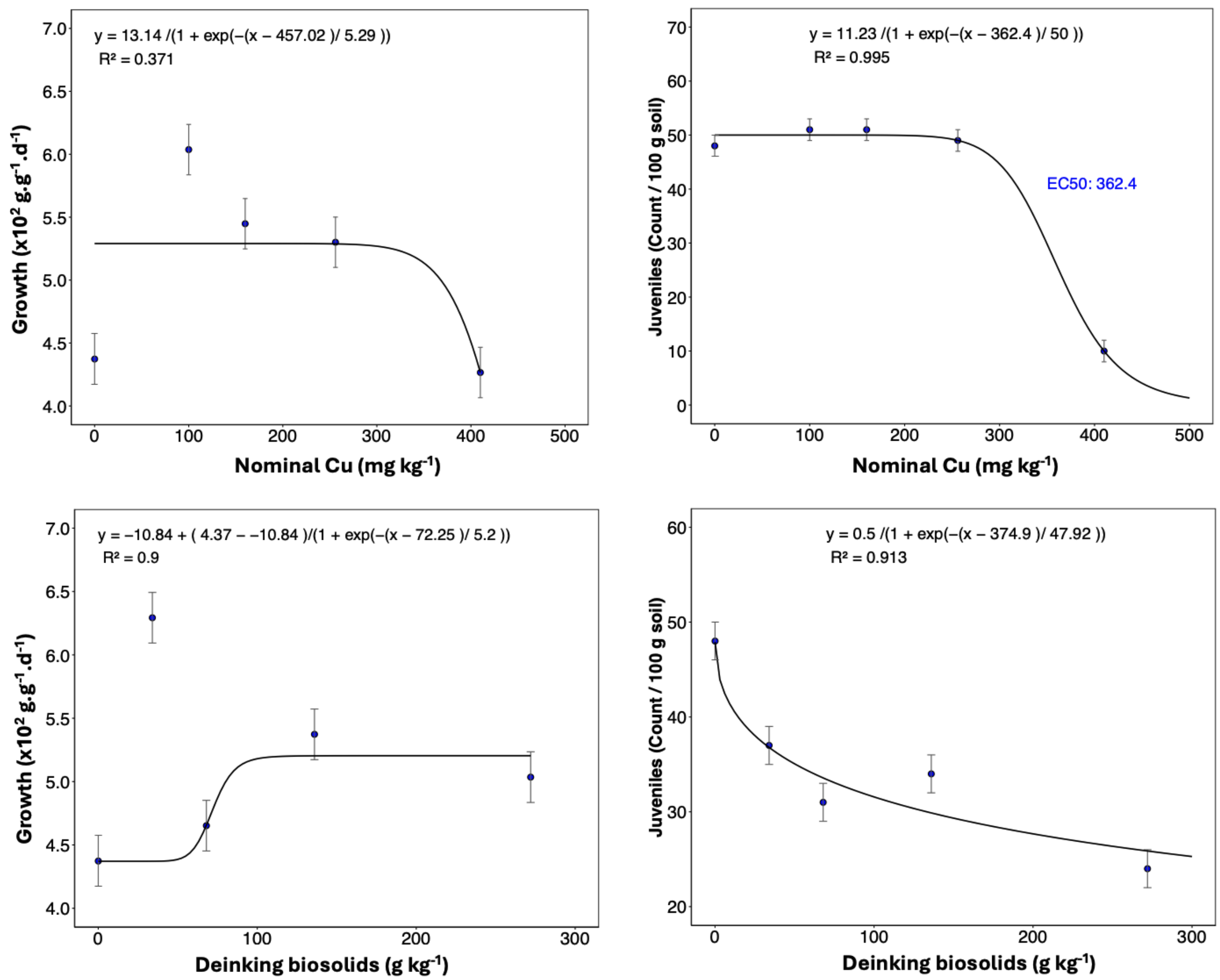

Figure S2.1. Logistic regression analysis of the effects of Cu and deinking biosolids on earthworm growth and reproduction. The instantaneous growth rate (IGR) of adult worms were measured after 28 d of exposure. The number of juveniles were counted after 56 d. The effect of Cu on *E. fetida* growth and reproduction, and the effect of deinking biosolids on *E. fetida* reproduction were fitted using 3-parameter logistic regression. A 4-parameter logistic regression model was used to display the effects of deinking biosolids on *E. fetida* growth.

Table S2.1 Toxicological responses and Cu accumulation in the earthworm *E. fetida* exposed to Cu and deinking biosolids in artificial soil. Instantaneous growth rate (IGR) of adult worms, and Cu bioaccumulation; in subcellular fractions (cytosol metallothionine) and whole earthworm (homogenate), were measured after 28 days of exposure. The number of juveniles were counted after 56 days. Values are mean ± standard error, n=4.

Deinking biosolids (g kg^{-1} soil)	Cu (mg kg^{-1} soil)	Growth ($x10^2$ g g^{-1} d^{-1})	Juveniles (count 100 g^{-1} soil)	Homogenate (µg g^{-1})	Cytosol (µg g^{-1})	Metallothionine (µg g^{-1})
0	0	4.4 ± 1.44	48 ± 3.23	1.15 ± 0.22	1.14 ± 0.25	0.52 ± 0.22
0	100	6.0 ± 2.26	51 ± 2.40	1.59 ± 0.46	1.35 ± 0.60	0.36 ± 0.03
0	160	5.4 ± 1.67	51 ± 5.83	1.77 ± 0.29	1.39 ± 0.36	0.65 ± 0.1
0	256	5.3 ± 1.55	49 ± 4.96	1.85 ±0.43	0.46 ± 0.36	0.90 ± 0.30
0	410	4.3 ± 1.61	10 ± 2.67	2.87 ± 0.69	1.87 ± 0.52	1.00 ± 0.34
34	0	6.3 ± 2.35	38 ± 4.05	0.80 ± 0.05	0.57 ± 0.09	0.39 ± 0.02
34	100	6.4 ± 2.06	29 ± 9.39	1.58 ± 0.15	1.07 ± 0.18	0.60 ± 0.04
34	160	6.0 ± 2.28	36 ± 8.06	1.96 ± 0.25	1.09 ± 0.19	0.55 ± 0.17
34	256	7.6 ± 1.90	28 ± 7.61	2.06 ± 0.35	1.44 ± 0.19	0.72 ± 0.12
34	410	6.2 ± 1.64	14 ± 4.47	2.70 ± 0.69	1.92 ± 0.66	1.23 ± 0.56
68	0	4.7 ± 1.58	33 ± 3.90	0.89 ± 0.05	0.72 ± 0.08	0.31 ± 0.06
68	100	5.4 ± 1.55	29 ± 4.52	2.10 ± 0.69	1.15 ± 0.17	0.78 ± 0.23
68	160	5.1 ± 2.30	41 ± 6.24	1.85 ± 0.25	1.29 ± 0.09	0.62 ± 0.09
68	256	6.0 ± 2.33	28 ± 8.62	2.0 ± 0.05	1.62 ± 0.10	0.90 ± 0.29
68	410	6.0 ± 1.85	33 ± 6.46	2.49 ± 0.39	1.89 ± 0.25	1.34 ± 0.30
136	0	5.4 ± 1.24	42 ± 4.23	0.94 ± 0.03	0.66 ± 0.08	0.35 ± 0.04
136	100	5.3 ± 1.86	40 ± 8.54	1.46 ± 0.15	1.33 ± 0.13	0.54 ± 0.09
136	160	5.5 ± 1.89	47 ± 7.72	1.62 ± 0.34	1.11 ± 0.20	0.55 ± 0.07
136	256	5.3 ± 2.02	43 ± 10.4	2.13 ± 0.55	1.71 ± 0.35	0.94 ± 0.28
136	410	6.3 ± 1.94	31 ± 7.05	3.26 ± 0.66	2.81± 0.39	1.70 ± 0.39
272	0	5.0 ± 1.49	22 ± 1.0	0.91 ± 0.08	1.01 0.42	0.32 ± 0.07
272	100	5.3 ± 1.84	34 ± 7.0	1.60 ± 0.16	1.36 ± 0.22	0.85 ± 0.26
272	160	4.9 ± 1.89	39 ± 9.68	1.64 ± 0.26	1.16 ± 0.27	0.66 ± 0.09
272	256	6.1 ± 2.62	34 ± 6.08	1.96 ± 0.21	1.44 ± 0.08	0.89 ± 0.12
272	410	5.9 ± 2.3	22 ± 6.78	2.92 ± 0.32	2.56 ± 0.26	1.32 ± 0.11

CONNECTING TEXT TO CHAPTER 3

In chapter 2, I investigated the effect of deinking biosolids alone and the combined exposure of deinking biosolids and Cu on earthworm physiological responses, i.e., growth and reproduction. I observed that exposure to deinking biosolids had no effect on earthworm growth but inhibited reproduction at higher concentrations. When deinking biosolids and Cu were combined, no significant effect was observed on Cu accumulation in earthworms and their subcellular fractions, as well as on earthworm growth and reproduction, compared to single exposure treatments. I confirmed that there was neither synergistic nor antagonistic interaction between deinking biosolids and Cu in artificial soil. This study demonstrates that a moderate application of deinking biosolids at 34 g kg^{-1} soil, has no adverse effects on earthworm growth or reproduction. While confirming the potential risk of deinking biosolids to earthworms, this study did not explicitly confirm that other substances in deinking biosolids are non-toxic to earthworms. Among these other substances are plasticizers and plastic debris, which are ubiquitous in most organic residues of municipal and industrial origin. Regulatory agencies worldwide have established standards for plastic fragments ≥ 2 mm in organic soil amendments intended for agricultural use. However, this threshold is too high to account for the potential toxicity of smaller microplastics. In chapter 3, I evaluated the potential toxicity of polyethylene microplastics sized 1–10 and 20–27 µm, which are more likely to interact with earthworm immune cells due to their size similarity, on the direct immune response of earthworms in an *in vitro* setting.

CHAPTER 3

Microplastics cytotoxicity and the phagocytic response of earthworm immune cells

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3.1 Abstract

An *in vitro* investigation of the cytotoxicity of polyethylene microplastics in *Eisenia fetida* coelomocytes revealed size-dependent differences in phagocytosis. Coelomocytes engulfed 85% of small polyethylene microplastics (1–10 µm) through phagocytosis, but there was negligible phagocytosis of larger polyethylene microplastics (20–27 µm). Cell viability was 6–7% when coelomocytes were exposed to 1–10 µm and 20–27 µm sized microplastics, significantly less than the 94% viability of coelomocytes in the untreated control. Polyethylene microplastics are cytotoxic to coelomocytes because their presence may interfere with the functions of phagolysosomes containing 1–10 µm microplastics, and larger microplastics particles (20–27 µm in this study) may cause cell death through frustrated phagocytosis.

Keywords: Cellular lysis, Ecotoxicology, Earthworm immunity, Plastic pollution, Soil invertebrate

3.2 Main text

Agricultural soils contain microplastics that are inadvertently added with sewage sludge, organic residues, and abraded from plastic film mulch. Microplastics exposure can be detrimental to earthworms, keystone soil biota involved in soil fertility and crop nutrition. Earthworms developed grade 2 intestinal fibrosis when exposed to 125 mg microplastics kg^{-1} soil of polyethylene microplastics with 250–1000 μm diameter (Rodriguez-Seijo et al. 2017). Although the relatively unpolluted agricultural soils of Canada contain an estimated 2–30 mg microplastics kg^{-1} (Crossman et al. 2020), the plastic pollution in global soils is much higher, reaching 40,800 microplastics (count) kg^{-1} in plastic-contaminated agricultural soils in Yunnan Province, China (Tang 2023). Regulations limit plastic waste in soil amendments to ≥0.5% by mass for >2 mm plastics in the European Union and ≥3 g kg^{-1} (dry weight) for >3 mm in Ontario, Canada (Langenkamp & Part 2001; MOE 2012). However, these regulations do not address the issue of microplastics, which are several orders of magnitude smaller and thus more likely to be ingested or in contact with the dermis of earthworms. Therefore, we sought to assess the size-dependent toxicity of microplastics on earthworms and understand the cytotoxicity mechanisms.

Earthworms have cellular mechanisms to engulf micron-sized foreign bodies, as part of their immune response, i.e., phagocytosis. This process sequesters particles >0.5 μm in the phagosome, a plasma membrane-derived vesicle, and occurs in coelomocytes, specialized immune cells that serve as the first line of defense in earthworm immunity (Rosales and Uribe-Querol 2017). However, the phagocytic action of coelomocytes depends on the microplastic size. In *Drosophila melanogaster*, the phagocytic response *in vitro* was more effective for polystyrene particles of 1–7 μm than 10 μm, probably because the immune cells were not large enough to engulf the larger microplastics (Adolfsson et al. 2018). This increases the likelihood of frustrated phagocytosis, which damages lysosomal organelles and causes leakage of lysozymes, leading to the degradation of proteins, lipids, and DNA. Thus, frustrated phagocytosis damages the affected cell and is expected to increase the vulnerability of nearby cells to oxidative stress, DNA damage and lipid peroxidation.

The purpose of this study was to quantify cell viability as the outcome of a size-dependent phagocytic mechanism in earthworm coelomocytes. We hypothesized that greater cell death will occur when coelomocytes are exposed to larger (20–27 μm) than smaller (1–10 μm) microplastics, due to the greater likelihood of frustrated phagocytosis with larger microplastics.

The experiment used polyethylene microplastics with size ranges of 1–10 μm and 20–27 μm, both having a density of 0.96 g cm^{-3}, from Cospheric LLC (Santa Barbara, California, United States) because polyethylene fragments ≤1 mm, along with polystyrene particles ≤1 mm, are the most common microplastics in agricultural soils (Crossman et al. 2020; Tang 2023). Small microplastics (1–10 μm) were stained with 4 ml rhodamine B dye (1:1 w/v of Rhodamine B and methanol) for 30 min in a dark glass vial (Tong et al. 2020) in an orbital shaker at 100 rpm. Large microplastics (20–27 μm) were factory stained with green fluorescent protein (GFP) from the supplier. Then microplastics were transferred onto a 0.45 μm filter, washed twice with deionized water to remove excess dye, and dried at 25°C for 16 h in the dark. Microplastics were counted using the spheres per gram calculation provided by the manufacturer. One day before the experiment, we prepared 12 microtubes (2 mL) containing 50,000 microplastic particles of 1–10 μm and 12 microtubes with 50,000 particles sized 20–27 μm.

Earthworm coelomocytes were extracted from *E. fetida* by non-invasive extrusion method (Eyambe et al. 1991). The average cell size was 5 to 20 μm, estimated with an AxioImager Z1 (ZEISS, Oberkochen, Baden-Württemberg, Germany). Each earthworm (n=4) was placed into a test tube (15 mL) containing 1 ml of the extrusion media for 1 min (Supplementary Materials). Coelomocytes were washed three times with 1 mL phosphate buffered saline (pH 7.2) and centrifuged (1000 *g*, 4 °C, 10 min). After decanting the supernatant, the cell pellet was resuspended in 3 mL of culture media (Garcia-Velasco et al. 2019). Subsequently, cells were counted in a hemocytometer and seeded into the microtubes containing the microplastics at a density of 10^4 cells per tube, achieving a cell: particle ratio of 1:5. We prepared additional tubes (n=4) with cell suspension only as a negative control of cell viability.

Microtubes were incubated for 2 h at 100 rpm and 37 °C to allow for phagocytosis, centrifuged (1,000 g, 4°C) to remove non-phagocytized microplastics, then cells were resuspended in 400 µL of phosphate buffered saline. DAPI (4',6-diamidino-2-phenylindole) was used to assess cell vitality. The cell suspension was analyzed via flow cytometry (BD FACSCanto II, BD Biosciences, Franklin Lakes, NJ, USA). Phagocytic ability was the percentage of cells that incorporated microplastics (recorded as fluorescent events) out of the total cell population using FlowJo (BD Life Sciences, 2024):

$$\text{Phagocytic ability} = \frac{\text{Number of cells with phagocytized particles}}{\text{Total number of cells}} \times 100 \qquad (1)$$

Statistical and graphical analysis was done with R software. Independent t-tests were used to compare the percentage of phagocytosis and cell viability between two treatments: 1–10 µm and 20–27 µm microplastics. Data are the mean ± standard error.

Coelomocytes from *E. fetida* were damaged by exposure to polyethylene microplastics, which reduced cell viability to 6% in the presence of 1–10 µm microplastics and to 7% viability with 20–27 µm microplastics, whereas unexposed coelomocytes had 94% viability (p <0.001, Fig. 3.1a). Coelomocytes were damaged by extended exposure to microplastics (180–250 µm) *in vivo* during a 21 d assay (Kwak and An 2021), but the study also reported fragmentation of microplastics to nanoplastics through digestive activity introduced to soil through cast, possibly leading to coelomocyte exposure to a range of particle sizes.

Coelomocytes engulfed 85% of the 1–10 µm microplastics, but there was negligible phagocytic engulfment of 20–27 µm microplastics ($p < 0.001$, Fig. 3.1b). Exposure to 20–27 µm microplastics could result in frustrated phagocytosis because coelomocytes cannot engulf particles that are larger than the cell (Fig. 3.2a). Loss of cell viability by >90% of coelomocytes exposed to 20–27 µm microplastics (Fig. 3.1a) could be the result of frustrated phagocytosis, which causes cellular leakage and eventually cell death (Baranov et al. 2020). Although 1–10 µm microplastics were internalized and accumulated inside coelomocytes (Fig. 3.2b-e) they were still cytotoxic to >90% of coelomocytes (Fig. 3.1a). Phagosomes may experience lysosomal dysfunction when attempting to digest the non-biodegradable microplastics (Hu & Palić 2020; Von Moos et al. 2012), causing inflammation-triggered programmed cell death pathways like apoptosis. One possibility, to be investigated, is that biodegradable microplastics ≤10 µm accumulating in phagosomes do not interfere with the development of a mature phagolysosome (PL) with full degradative capacity (Nguyen & Yates 2021), including the ability to hydrolyze biodegradable microplastics. This could allow earthworms to tolerate and bioremediate soil containing biodegradable plastics.

The phagocytic capacity in *E. fetida* coelomocytes for the engulfment of individual and multiple microplastics particles ≤10 µm offers novel mechanistic insight into how earthworms may tolerate plastic-contaminated agricultural soils. Why phagocytosis failed to protect coelomocytes from the damaging effect of microplastics *in vitro*, possibly due to physical damage, inflammatory responses and oxidative stress in cells, is to be confirmed in whole-organism studies. Our findings reinforce the need for safety thresholds to protect earthworms and other soil biota from the unintended input of microplastics to agricultural fields.

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height vs forward scatter-area to exclude doublets. The resultant single-cell population was then analyzed for the stain used in the bioassay. (c) Representative analysis of rhodamine B-associated fluorescence, indicative of cells that have engulfed small polyethylene microplastics (1–10 μm), (d) and for 4′,6-diamidino-2-phenylindole- stained cells, indicative of compromised cells.

FIGURES

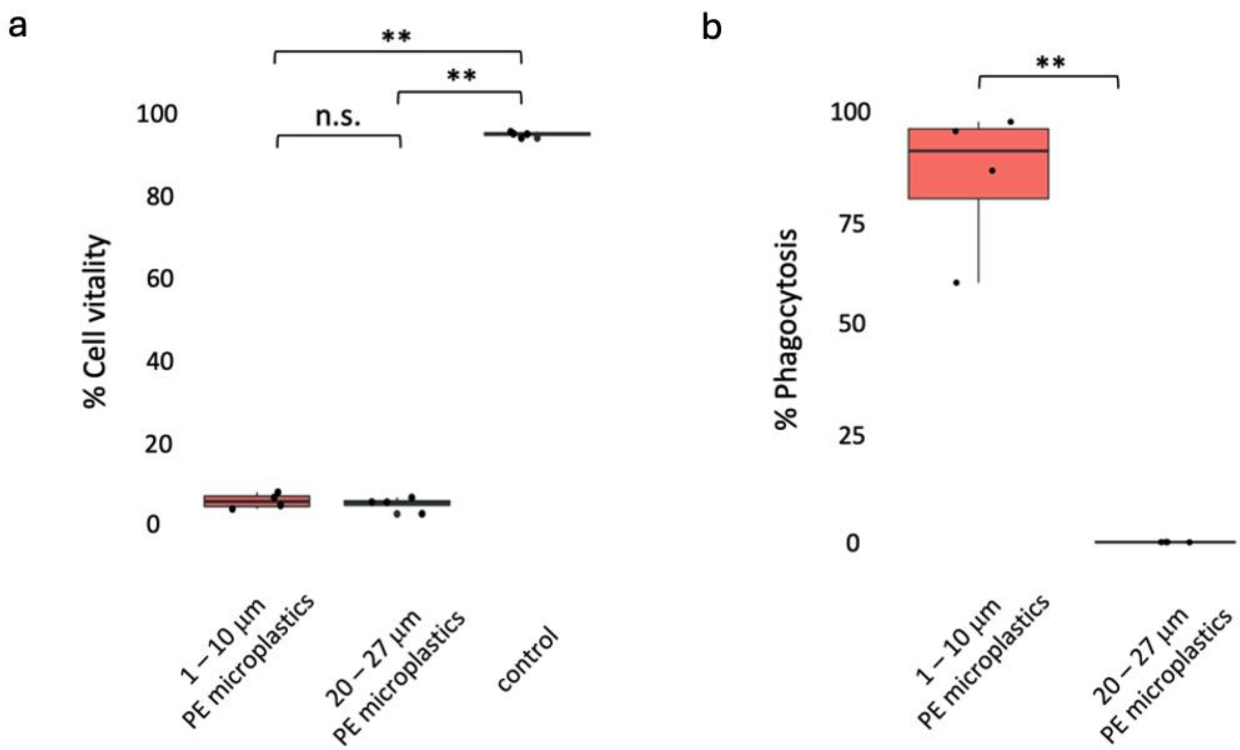

Figure 3.1 Coelomocytes from *E. fetida* had (a) lower cell viability after 2 h exposure to 1–10 µm and 20–27 µm fluorescent polyethylene microplastics, compared to control cells without microplastics, with (b) greater phagocytic ability for 1–10 µm than 20–27 µm fluorescent polyethylene microplastics. Viability and phagocytosis were quantified by flow cytometry on cells stained with 4′,6-diamidino-2-phenylindole. Values are the mean with standard error bars (n=4), and statistical differences were determined using a t-test.

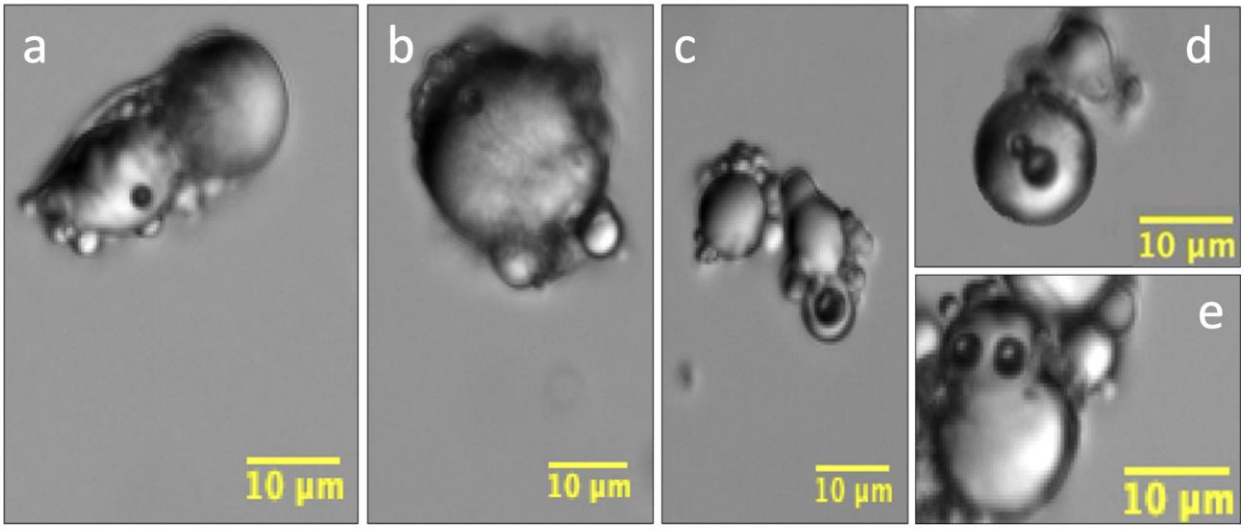

Figure 3.2 Brightfield microscopy images of coelomocytes from *E. fetida* interacting with polyethylene microplastics. (a) An amebocyte binding with a microplastic particle greater than its cell diameter, presumable due to cell-particle recognition process. (b) Pseudopod extension of a coelomocyte as it begins to engulf a 1–10 µm microplastic particle. (c) Closure of a phagosome around a 1–10 µm microplastic particle, indicating the end of the engulfment stage. (d–e) A coelomocyte with multiple internalized microplastic particles of 1–10 µm.

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SUPLEMENTARY MATERIALS

Microplastics cytotoxicity and the phagocytic response of earthworm immune cells

Noura Alsarawi & Joann K. Whalen

Extended materials and methods

Chemicals and materials

E. fetida were purchased from Merlan Scientific (Toronto, Ontario, Canada), and kept in laboratory culture for 2 wk in a breeding substrate composed of peat moss at 20 ± 2 °C. Analytical grade dimethyl sulfoxide, rhodamine B, amphotericin B, gentamicin solution, ethanol, and phosphate buffered saline pH 7.2 (PBS) were purchased from Fisher Scientific (Ottawa, Ontario, Canada). Cell culture medium RPMI-1640 with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), guaiccol glyceryl ether, penicillin/streptomycin, sodium chloride, methanol, 4′,6-diamidino-2-phenylindole (DAPI), and trypan blue were supplied by Sigma-Aldrich (Oakville, Ontario, Canada).

Coelomocytes extraction

Earthworm coelomocytes were extracted from *E. fetida* by non-invasive extrusion method. Replicate earthworms were rinsed in the extrusion media composed of 5% ethanol, 95% saline solution, 2.5 mg mL^{-1} EDTA, and 10 mg ml^{-1} guaiacol glyceryl ether (pH 7.3) Eyambe et al. (1991). Each earthworm (n=4) was placed in a test tube (15 mL) containing 1 mL of the extrusion media for 1 min. Coelomocytes were washed three times with PBS (1 mL) by gently pipetting up and down, and centrifuged (1000 *g*, 4 °C, 10 min). After decanting the supernatant, each cell pellet was resuspended in 3 ml of culture media, RPMI-1640 medium supplemented with several antibiotics: 1% Amphotericin B (250 µg mL^{-1}), 1% Penicillin/Streptomycin (10,000 units penicillin and 10 mg streptomycin mL^{-1} and 0.5% Gentamicin solution (10 mL^{-1}) (Garcia-Velasco et al. 2019). Subsequently, 100 μL of the cell culture suspension was transferred to a microtube containing 100 μL of 0.4% trypan blue and 300 μL of culture media. Cells were counted on a 10 μL aliquot of the culture suspension in a hemocytometer.

Experimental design

One day before the experiment, we prepared 12 microtubes (2 mL) containing 50,000 microplastic particles of 1–10 μm and 12 microtubes with 50,000 particles sized 20–27 μm. In the experiment, each plastic size treatment (2 plastic sizes × 12 replicate tubes = 24 in total) was randomly divided into 4 tubes to assess phagocytosis of coelomocytes, 4 tubes to evaluate cell viability of coelomocytes, and 4 tubes for the gating strategy. We prepared additional 4 tubes that contain cells only as the negative control.

Phagocytosis validation using fluorescent microscopy

After the phagocytosis bioassay, we pipetted two 10 μL aliquots from a well containing small and large microplastics, placed them on slides and evaluated phagocytosis using AxioImager Z1 (ZEISS, Oberkochen, Baden-Württemberg, Germany). The emission and excitation wavelengths were set at 358/461 to visualize DAPI- stained coelomocytes, 555/580 to visualize rhodamine B-stained microplastics (1-10 μm), and 395/509 to visualize green fluorescent protein (GFP)-tagged microplastics (20-27 μm) (Fig. S1).

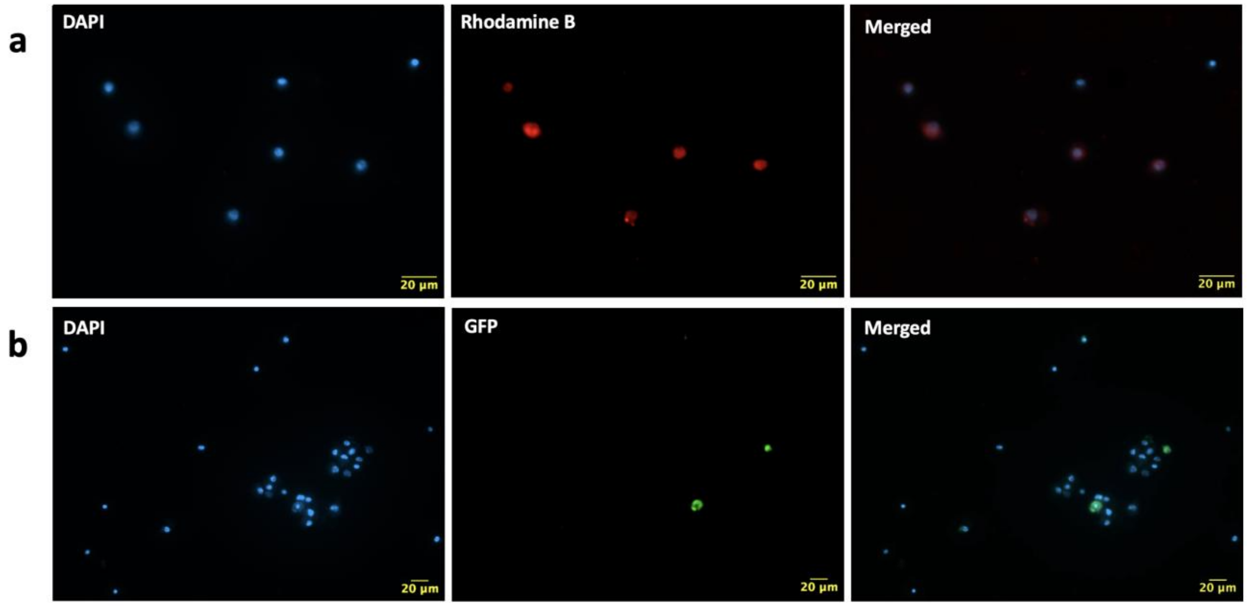

Figure S3.1 Fluorescence microscopy analysis of polyethylene microplastic uptake by *E. fetida* coelomocytes. (a) Representative images displaying coelomocytes after exposure to small polyethylene microplastics (1–10 µm) stained with rhodamine B, with nuclei counterstained using 4′,6-diamidino-2-phenylindole. (b) Representative images showing coelomocytes post incubation with large green fluorescent protein-tagged polyethylene microplastics (20–27 µm). Both treatments were carried out *in vitro* for a duration of two hours. The rightmost images represent the merged channels, highlighting the internalization of microplastics within the coelomocytes.

Extended data analyses

Gating strategy

The acquired data from the flow cytometer were analyzed using FlowJo software (BD Life Sciences, 2024) for initial gating and population identification. Intact cells scatter light differently than debris; debris and dead cells typically exhibit a low level of forward scatter and are commonly located in the bottom-left quadrant of the density plot, whereas intact cells scatter more light, mainly due to the cells' complex internal structure and granularity (Shapiro 2003). This allows us to recognize intact cells as a different population from debris including non-phagocytized microplastics, if present after centrifugation (Fig S3.2-a). To ensure quality control, we also prepared tubes containing microplastics only to establish a baseline for the gating strategy, allowing us to effectively exclude non-phagocytized microplastics from our analysis. We also excluded doublets by plotting forward scatter-height vs forward scatter-area to ensure only single cells were analyzed (Fig. S3.2-b). The analysis of cells that have engulfed stained microplastics is represented in Figure S3.2-c. Compromised cells, indicated by DAPI staining, are shown in Figure S2-d.

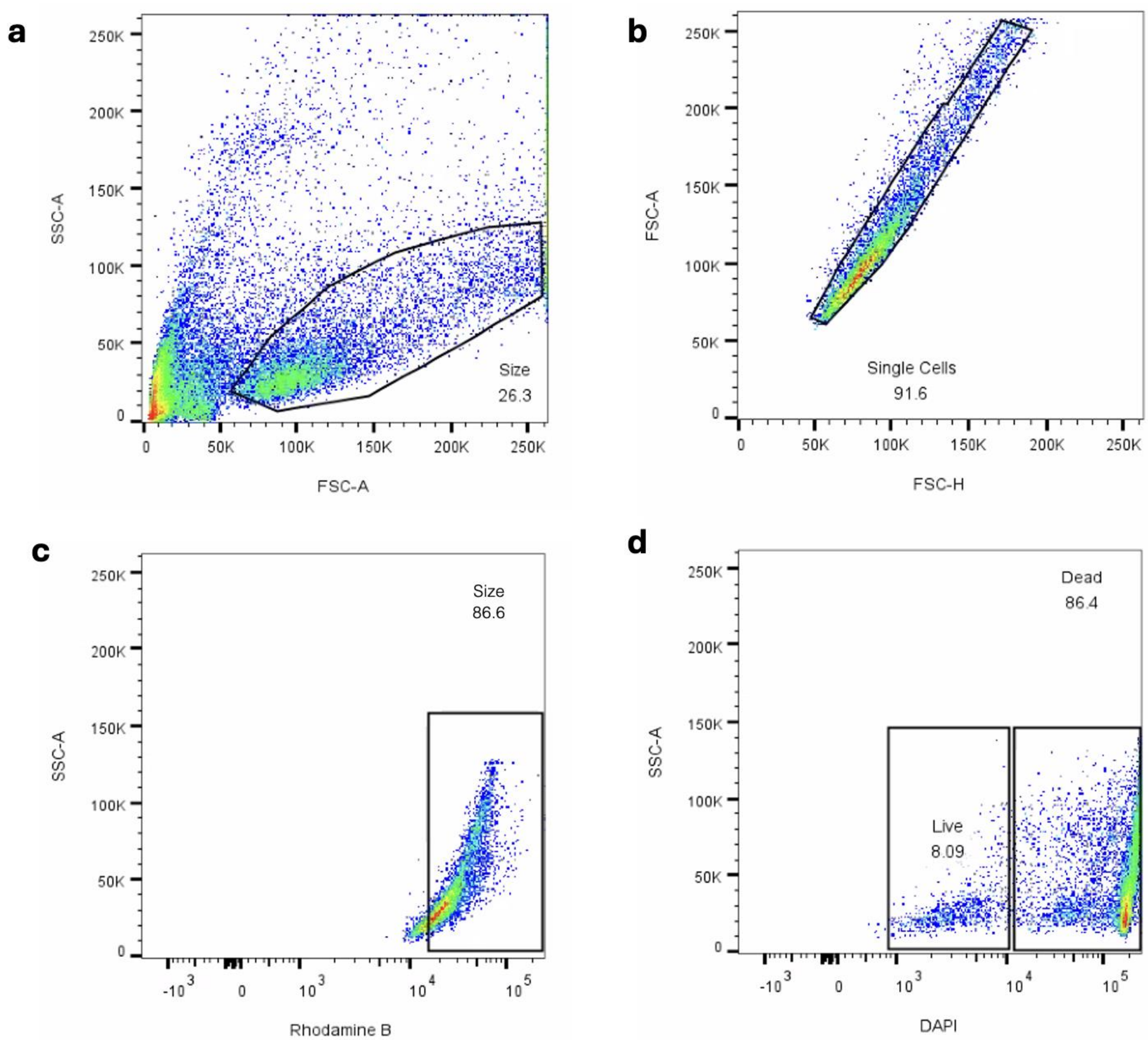

Figure S3.2 Gating strategy for analyzing flow cytometry data. (a) Forward scatter-area vs side scatter-area to exclude debris and non-phagocytized microplastics and identify intact cells with and without microplastics. (b) Single-cell populations were then identified using forward scatter-height vs forward scatter-area to exclude doublets. The resultant single-cell population was then analyzed for the stain used in the bioassay. (c) Representative analysis of rhodamine B-associated fluorescence, indicative of cells that have engulfed small polyethylene microplastics (1–10 µm), (d) and for 4′,6-diamidino-2-phenylindole- stained cells, indicative of compromised cells.

Statistical and graphical analysis were conducted using R software (R Core Team 2023). We performed independent t-tests to compare the percentage of phagocytosis and cell viability between two treatments: 1–10 µm and 20–27 µm microplastics. Data are presented as the mean ± standard error. A summary of the key findings is presented in Table S1.

Table S3.1 Cell vitality and phagocytosis of *E. fetida* immune cells after 2 h exposure to polyethylene microplastics *in vitro*.

	% Cell vitality	% Phagocytosis
Control	93.9 ± 0.31a	N/A
1–10 µm polyethylene microplastics	7.25 ± 0.88b	85.2 ± 8.60a
20–27 µm polyethylene microplastics	6.58 ± 0.83b	0.05 ± 0.01b

Data presented as mean (n=4) ± standard deviation. Significance ($p < 0.05$) was determined using t-test.

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CONNECTING TEXT TO CHAPTER 4

In chapter 3, I investigated the cytotoxic potential of microplastics to earthworm coelomocytes *in vitro* by evaluating the coelomocytes' phagocytic ability to engulf polyethylene microplastics. Earthworm coelomocytes engulf 85% of the small microplastics (1–10 µm), which significantly exceeds the phagocytic engulfment of large microplastics (20–27 µm). However, earthworm coelomocytes exposed to polyethylene microplastics displayed significant cell death through different mechanisms. I deduce that cell death due to small microplastics exposure resulted from the internalization and accumulation of non-degradable microplastics within phagolysosomes, potentially leading to lysosomal dysfunction and causing inflammation-triggered programmed cell death pathways like apoptosis. Cell death following exposure to large microplastics exposure likely stemmed from frustrated phagocytosis that occurred during the engulfment of particles greater than earthworms' coelomocytes probably leading to cellular content leakage in the extracellular environment and eventually cell death. Given the toxicity resulting from deinking biosolids exposure (chapter 2) and the significant response in cellular immunity due to *in vitro* microplastics exposure (chapter 3), I decided to investigate more sensitive biomarkers to understand the mechanisms underlying the toxicity of deinking biosolids and microplastics on earthworms. However, microplastics extracted from deinking biosolids are not pristine. They often have various charged substances adhering to their surfaces and embedded within their molecular structure. Therefore, in chapter 4, I examined the impact of deinking biosolids and aged microplastics bound with Cu on earthworms' molecular processes. This assessment included DNA damage and oxidative stress responses including levels of reactive oxygen species and enzymatic activities.

CHAPTER 4

Aged polyethylene magnifies copper-induced oxidative stress and DNA damage in *Eisenia fetida*

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4.1 Abstract

Agricultural soil is a major sink for microplastics, which accumulate as a consequence of plastic mulching, wastewater irrigation, and the application of organic materials containing plastic residues as soil amendments. Deinking biosolids, a byproduct of paper recycling that contains microplastics, copper and other contaminants, could impact soil biological health. The purpose of this work was to evaluate earthworm oxidative stress and DNA damage following exposure to deinking biosolids and representative constituents (pristine and aged microplastics, with and without copper) in artificial soil. We exposed earthworms for 28 d to deinking biosolids, to pristine microplastics, and to aged copper-spiked microplastics, which were polyethylene (10–22 and 90–106 μm) and polystyrene (14–20 and 85–105 μm). Earthworms DNA damage, reactive oxygen species levels and antioxidant enzyme activities (superoxide dismutase and catalase) were affected by > 68 g deinking biosolids kg^{-1} soil. Earthworms exposed to pristine, and aged copper-spiked microplastics had greater reactive oxygen species levels, altered antioxidant enzyme activities, and more DNA damage than the control ($p < 0.05$). While no size- or type-dependent toxicity was observed with pristine microplastics, earthworm cells contained higher reactive oxygen species levels ($p < 0.05$) when exposed to aged copper-spiked polyethylene than to pristine polyethylene. Moreover, greater DNA damage was observed in earthworms exposed to large, aged copper-spiked polyethylene (90–106 μm) than large pristine polyethylene. This highlights the fact that trace metals like copper can become more toxic when combined with microplastics in organic residues and soil.

Key words: earthworms, microplastics, copper, oxidative stress, DNA damage.

4.2 Introduction

Agricultural soil is a sink for microplastics (plastic fragments <5 mm) because plastics are used deliberately (e.g., plastic film mulching for water conservation and weed control on about 13% of cropland covering ~17 million ha in China; Sun et al., 2020) or incidentally with soil amendments and irrigation water. For instance, nutrient-rich compost contained up to 1,200 mg plastics kg^{-1} and there was as much as 125,000 fragments m^{-3} of treated wastewater used for irrigation in cropland (Bläsing & Amelung, 2018; Zhao et al., 2022a). Soil organisms like earthworms are sensitive to microplastic exposure. When the earthworm *E. fetida* was exposed pristine microplastics, specifically 1.25 g of polyethylene (28–145, 133–415 and 400–1464 μm) and polypropylene (8–125, 71–383 and 761–1660 μm) particles, this significantly altered the activities of superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and 8-hydroxy-2′-deoxyguanosine (8-OHdG), with the greatest impact was from smaller (8–125 μm) polypropylene microplastics (Li et al., 2021).

Microplastics in agricultural soils are not pristine because they sorb charged metals, which could increase their toxicity to earthworms. For example, a dynamic sorption experiment on naturally aged polyethylene (< 5000 μm) revealed that approximately 14 mg Pb g^{-1} was adsorbed to the particles, mainly via electrostatic interactions (Fu et al., 2021b). Consequently, microplastics act as vectors that increase metal transfer from soil to biological organisms. This resulted in 10–160% more Cd accumulation in *E. fetida* body tissues when earthworms were exposed to a mixture of 300–9000 mg kg^{-1} $soil_{dw}$ of polypropylene microplastics (< 150 μm) and $CdCl_2$ solution, compared to exposure to an equivalent amount of $CdCl_2$ solution alone (Zhou et al., 2020). Still, the amount of microplastics-bound metals that are transferred and subsequently accumulated in earthworm tissues, and their potential cytotoxicity, is poorly understood.

Copper (Cu) is a ubiquitous trace metal in soil amendments like compost and biosolids, and it is toxic to earthworms based on dose-response at the organismal level (i.e., reproduction and survival; Owojori et al., 2010). The Cu toxicity in earthworms is associated with an excess of reactive oxygen species (ROS), which manifest damage to cell membranes and components, proteins and DNA in the affected organism (Fink & Gale, 2007). Due to the prevalence of microplastics in soil amendments, they are likely to deliver microplastics-bound metals to soil. This is particularly true of deinking biosolids, which contain 50–100 mg Cu kg^{-1} (Marouani et al., 2021; Price & Voroney, 2008), along with microplastics that are generated from the breakdown of paper coatings during recycling. These paper coatings typically consist of polymeric materials, such as polyethylene, polypropylene or polystyrene, used to provide properties like water resistance, gloss, or durability to paper products (Kunam et al., 2022).

The aim of this study is to evaluate how microplastics-bound Cu impacts earthworms, specifically in terms of causing oxidative stress and DNA damage. Earthworms were exposed to microplastics-bound Cu after mixing Cu salt solution with aged microplastics (polyethylene and polystyrene, with average diameters of about 10 and 100 μm), compared to pristine microplastics. Earthworm oxidative stress and DNA damage resulting from these representative constituents was also compared with the response to deinking biosolids. We hypothesize that soil amended with aged Cu-spiked polyethylene and polystyrene microplastics will produce more oxidative stress and DNA damage in earthworms than soil containing pristine microplastics because of greater stress from the combined microplastics and Cu exposure.

4.3 Materials and Methods

4.3.1 Bioassay materials

The test substrate was artificial soil composed of 70% silica sand, 20% colloidal kaolinite clay, and 10% peat moss (OECD, 2016). *E. fetida* was purchased from Merlan Scientific (Toronto, Ontario, Canada). Spherical microplastics were obtained in two sizes, with polyethylene polymers of 10−22 µm (small) and 90−106 µm (large), while polystyrene polymers were 14−20 µm (small) and 85−105 µm (large), purchased from Cospheric LLC (Santa Barbara, California, United States). Deinking biosolids were supplied by a papermill factory located in Kingsey Falls, Quebec. Copper II nitrate ($Cu(NO_3)_2$), Tris buffer, hydrochloric acid solution (HCl), ethanol, phosphate buffered saline pH 7.2 (PBS), agar, sodium hydroxide (NaOH), 2',7'-dichlorodihydrofluorescein diacetate, 4-Nitro blue tetrazolium chloride, Na-sarcosine, dimethyl sulfoxide, and SYBR green were obtained from Fisher Scientific (Ottawa, Ontario, Canada). Sodium ethylenediaminetetraacetic acid (Na_2EDTA), guaiacol glyceryl ether, sodium chloride (NaCl), Coomassie brilliant blue, hydrogen peroxide, low gelling temperature agar, bovine serum albumin, L-methionine, and triton X-100 were supplied from Sigma-Aldrich (Oakville, Ontario, Canada).

4.3.2 Microplastics aging and copper spiking protocol

Microplastics were aged by exposure to ultra-violet (UV) light in a photoreactor (LZC-ORG, Luzchem Inc, Ottawa, Canada) equipped with 10 UVA and UVB light bulbs (5 per side) interchangeably that deliver 355 and 313 nm respectively. Following 4 wk of UV aging, aged microplastics were placed in glass flasks containing 100 ml of Cu stock solution (100 mg Cu l^{-1} from $Cu(NO_3)_2$). The suspension was shaken at 150 rpm for 4 d at 25 °C, which results in Cu adsorption to microplastics (Chen et al., 2022). Subsequently, the microplastics-spiked Cu were washed with deionised water and filtered through a 0.2 µm filter, in which they were collected and dried at room temperature. Microplastic samples of each type and size were analyzed for Cu by inductively coupled plasma mass spectroscopy (ICP-MS) at A&L Canada Laboratories (London, ON, Canada).

4.3.3 Earthworm culture and artificial soil

Earthworms were kept in a plastic container with perforated lid containing moistened peat moss at 20 ± 2 °C for 4 wk. They were fed every 2 to 3 d with a high protein substrate (Magic Worm Food, Merlan Scientific). Adult earthworms with a weight range of 400 to 500 mg, identifiable by the presence of clitellum, were selected for the experiment. These were washed with deionised water, placed briefly on a filter paper to remove excess water, and weighed. Artificial soil (OECD, 2016) was homogenized by sieving < 2 mm mesh, and the pH was adjusted to 6 ± 0.5 by adding 15 mg kg^{-1} of calcium carbonate. Soil was moistened to 50% moisture content by adding deionised water. The earthworms selected for the experiment were transferred into artificial soil and acclimated in the dark at 20 ºC for 1 wk.

4.3.4 Experimental design

The experiment was a time-series experiment with 10 treatments evaluated at 4 time points in the study; day 7, 14, 21 and 28. The experimental unit was a 1-L glass jar with a perforated lid containing 350 g of artificial soil (control). The treatments include soil amended with deinking biosolids, spiked with Cu, and mixed with small and large, pristine, and aged and Cu-spiked polyethylene and polystyrene microplastics (Table. 4.1). This approach provides insights into the effects of a widely used organic soil amendment containing microplastics and trace metals on earthworms. Deinking biosolids and microplastics were added separately to the assigned jars by mixing them thoroughly with the soil. The jars were maintained at a temperature of 20 ± 2 °C with a 12-hour light/dark cycle. Jars were set up with twelve earthworms for each control and treatment group, 3 were removed for analysis in 4 sampling days (repeated sampling with time) (n=3). Earthworms were fed once a week with a high protein substrate; Magic Worm Food, and soil moisture levels were carefully regulated to maintain a consistent 50% moisture content.

4.3.5 Earthworm sampling

At each time point (days 7, 14, 21, and 28), 3 earthworms per treatment were randomly selected for analysis (n=3). The selected earthworms were washed with deionised water and placed on moistened filter paper for 24 h to void their gut contents. After the rinsing and fasting period, coelomocytes were extracted from one earthworm per replicate (n=3) for DNA damage analysis. The remaining earthworms were individually homogenized in potassium phosphate buffer (1:8 w/v, pH 7) using an ULTRA-TURRAX IKA T10 basic tissue homogenizer. Of these, one homogenate per replicate (n=3) was processed for ROS and protein content, one for enzyme activity assays, and one was stored at -4 °C until the day of analysis for Cu concentration by inductively coupled plasma mass spectroscopy (ICP-MS).

4.3.6 Protein content in earthworm tissue

Protein content of the homogenized tissue was measured with the Bradford method on the day of analysis, which is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford, 1976).

4.3.7 Enzyme activities in earthworm tissue

The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C to obtain the fraction needed for protein, SOD and CAT analysis (Han et al., 2014). SOD activity was assessed by evaluating its ability to obstruct the photochemically induced reduction of nitroblue tetrazolium chloride, following the method of Giannopolitis and Ries (1977) modified by Song et al. (2009). The sample well contained 50 µl enzyme extract and the reaction mixture; 50 mM potassium phosphate buffer (pH 7.8), 100 µM EDTA, 130 mM methionine, 750 µM nitroblue tetrazolium chloride, and 20 µM riboflavin which was added last. The samples were shaken briefly and placed under 4000 lx fluorescent light. After 20 min, the absorbance was measured at 560 nm. CAT activity was determined by observing the reduction in ultraviolet absorption resulting from H_2O_2 decomposition by CAT in the sample. The sample well contained 10 µl enzyme extract, 100 mM potassium phosphate buffer (pH 7), and 10 mM H_2O_2 (Liu et al., 2017). The samples were shaken for 30 s and the ultraviolet absorption was measured at 250 nm.

4.3.8 Quantification of reactive oxygen species level

To obtain the mitochondria suspension, the homogenate was centrifuged twice; once at 3,070 rpm for 10 min at 4 °C, and the supernatant was recentrifuged at 13,720 rpm for 20 min at 4 °C and the pellet was resuspended in 1 ml of potassium phosphate buffer (pH 7.4) (Han et al., 2014 and Zhang et al., 2013). The dichlorohydrofluorescein diacetate solution (5 µM final concentration) was added to the mitochondria suspension, and the samples were shaken for 30 s and placed in a humidified incubator for 15 min at 37°C. The fluorescence was measured at an excitation and emission wavelength of 485 and 530 nm and later adjusted for protein concentration.

4.3.9 DNA damage

Earthworm coelomocytes were extracted from one earthworm per sampling date (n=3) by non-invasive extrusion method (Eyambe et al., 199). Coelomocytes were washed three times with

PBS (1 ml) by gently pipetting up and down, and centrifuged (1000 *g*, 4 °C, 10 min). After decanting the supernatant, each cell pellet was resuspended in 1 ml PBS (pH 7.2). Single cell gel electrophoresis (SCGE) protocol was carried out following the protocol of Singh et al. (1988) with slight modifications. Initially, 1% normal agar coated slides were prepared. We then added 40 µl of the cell suspension to 140 µl of low melting agar (0.7%), which was kept at 37 °C in a water bath. Subsequently, two drops of 75 µL of this mixture was placed onto each slide. Cover slips were immediately applied over the drops and the slides were refrigerated for 5 min to allow the agarose to solidify. The slides were then lysed in 2.5 NaCl, 100 mM Na_2EDTA, 10 mM Tris, 10% DMSO 1% Na-sarcosinate and 1% triton x-100 and incubated for 1 hr at 4 °C. Subsequently, slides were placed in an electrophoresis buffer containing 300 mM NaOH and 1 mM Na_2EDTA (pH 12) for 20 min then the current was switched to 25 V (300 mA) for another 20 min. Following electrophoresis, the slides were neutralized in 0.4 mM Tris (pH 7.5) for 10 min and rinsed twice with water. Slides were stained with SYBR green (75 µl) and placed in the dark for 30 min. Observations were made using AxioImager Z1 (ZEISS, Oberkochen, Baden-Württemberg, Germany) capturing 50 comets per treatment at emission and excitation wavelengths of 395/509. The images were analyzed using Comet Score 2.0 software.

4.4 Data analyses

One-way ANOVA was used to determine if earthworm exposure to the microplastics and deinking biosolids treatments affected the mean ROS levels, antioxidant enzyme activities (SOD and CAT) and DNA damage (expressed as % tail DNA) significantly ($p < 0.05$). Two-way ANOVA with LSD post-hoc tests was used to examine the effects of treatment type, exposure period, and their interactions (treatment type × exposure period) on the biomarkers. The biomarkers with $p >$ 0.05 for the exposure period and in the treatment type × exposure period interaction were pooled by treatment type to increase the number of replicates (time series raw data provided in supplementary text). Due to variations in control readings, CAT activity was reported as relative quantification by normalizing treatment values to their respective control readings at each time point. CometScore software (version 2.0; TriTek Corp, 2017) was used to evaluate and analyze 50 comets per sample for the single-cell gel electrophoresis assay. All statistical analysis were performed using R software (version 2023.09.1+494; R core Team, 2022).

4.5 Results

The deinking biosolids application rates and the microplastics concentrations used in this study were not acutely toxic to earthworms. Further, earthworm weight stayed consistent throughout the exposure period measured as instantaneous growth rate (IGR) for exposure period of 28 d (IGR x exposure period, $p < 0.05$, Table S4.2).

4.5.1 Effects of deinking biosolids, microplastics, and Cu on oxidative stress biomarkers in *E. fetida*

Exposure to 272 g deinking biosolids kg^{-1} soil significantly increased ROS levels in earthworms throughout the exposure period ($p < 0.05$), except on day 7. In addition, ROS levels were elevated in treatments amended with 68 g deinking biosolids kg^{-1} on days 14, 21 and 28 and with 136 g kg^{-1} on days 14 and 28 ($p < 0.05$, Fig. 4.1A). On day 7, SOD activity was stimulated in earthworms exposed to 272 g deinking biosolids kg^{-1} soil, and greater SOD activity was also measured on day 14 in earthworms exposed to 68 g deinking biosolids kg^{-1} soil, and on day 21 when earthworms were exposed to 68, 136, and 272 g deinking biosolids kg^{-1} ($p < 0.05$, Fig. 4.1B). The CAT activity was stimulated in earthworms exposed to 34 and 272 g of deinking biosolids kg^{-1} soil on day 7, and in earthworms exposed to 34 g of deinking biosolids on day 14 ($p < 0.05$), but there was significantly less CAT activity following exposure to 136 and 272 g deinking biosolids kg^{-1} on day 28 (Fig. 4.1C).

Large polystyrene microplastics induced higher ROS levels in earthworms than small polystyrene microplastics ($p < 0.05$, Fig. 4.2A). The small and large microplastics of aged Cu-spiked polyethylene induced significantly higher ROS levels than their pristine states ($p < 0.05$, Fig. 4.2A). More CAT activity on day 7 was measured in earthworms exposed to small pristine polyethylene microplastics ($p < 0.05$, Fig. 4.2C). On day 14, significant effects were detected in the small pristine polystyrene treatment, and on day 21, both large polyethylene and polystyrene microplastics caused significant increase in CAT activity ($p < 0.05$). By day 28, greater CAT activity was recorded in earthworms exposed to large polyethylene, large polystyrene and small polystyrene microplastics ($p < 0.05$). In Cu-spiked microplastics treatments, CAT activity was elevated on day 7 in earthworms exposed to small and large Cu-spiked polyethylene and small Cu-spiked polystyrene microplastics ($p < 0.05$, Fig 4.2D). By day 14, CAT activity was significantly stimulated in earthworms in all treatments with Cu-spiked microplastics ($p < 0.05$). On day 21, earthworms exposed to Cu-spiked small and large polyethylene microplastics responded with significantly higher CAT activity compared to the control ($p < 0.05$). By day 28, only Cu-spiked small polystyrene microplastics remained elevated ($p < 0.05$). Lastly, exposure to Cu alone significantly altered CAT activity in earthworms on day 7, 14, and 21 ($p < 0.05$).

4.5.2 Effects of deinking biosolids, microplastics and copper on DNA damage in *E. fetida*

The comet assay on earthworm coelomocytes revealed a significant increase in % tail DNA in earthworms exposed to 68 and 272 g deinking biosolids kg^{-1} soil compared to the control through the exposure period ($p < 0.05$, Fig. 4.3A). In addition, on days 7, 21 and 28, treatments amended with 136 g of deinking biosolids kg^{-1} soil showed statistically significant differences, while exposure to the lowest application rate of deinking biosolids (34 g kg^{-1} soil) resulted in an increase in % tail DNA on day 7 only ($p < 0.05$). Exposing earthworms to pristine, and aged Cu-spiked microplastics resulted in significantly greater % tail DNA compared to the control ($p < 0.05$, Fig. 4.3B). Comparing differences in DNA damage in earthworms exposed to pristine and to aged and Cu-spiked revealed that % tail DNA was significantly higher in earthworms exposed to large Cu-spiked polyethylene microplastics compared to its pristine state ($p < 0.05$).

4.6 Discussion

4.6.1 Effects of deinking biosolids on oxidative stress and DNA damage in *E. fetida*

Considered the first line of defense against oxidative stress, SOD is responsible for scavenging superoxide anions; the simplest form of ROS (Li et al., 2021). Notably, we observed a temporal relationship between ROS levels and SOD activity in earthworms exposed to deinking biosolids (Fig. 4.1A & B). On day 14, both ROS levels and SOD activity significantly increased compared to the control in earthworms exposed to 68 g deinking biosolids kg^{-1} soil ($p < 0.05$), indicating an acute oxidative response followed by upregulation of antioxidant defenses. However, although ROS levels remained significantly elevated at 136 and 272 g deinking biosolids kg^{-1} soil on days 21 and 28 ($p < 0.05$, Fig. 4.1A & B), SOD activity did not show a corresponding increase. This temporal relationship suggests that while earthworms may initially stimulate an adaptive antioxidant response, prolonged or higher-level exposure to deinking biosolids may impair their ability to sustain SOD activity, potentially due to enzyme inhibition (Li at el., 2021b).

The dismutation of superoxide anions ($O_2^{\bullet-}$) by SOD, generates hydrogen peroxide (H_2O_2), another ROS (Schrader & Fahimi, 2006), which can be broken down into oxygen and water by CAT. In our study, on day 7, CAT activity was significantly stimulated compared to the control at both the lowest (34 g kg^{-1}) and highest (272 g kg^{-1}) deinking biosolids application rates tested (*p*

< 0.05, Fig. 4.1C). However, by day 28, while CAT activity in earthworms exposed to 34 g deinking biosolids kg^{-1} soil returned to baseline levels, it was significantly inhibited in treatments amended with 136 and 272 g deinking biosolids kg^{-1} ($p < 0.05$, Fig. 4.1C). The inhibition of CAT activity at the two highest application rates at the end of the exposure period coupled with the consistent increase in ROS levels ($p <0.05$, Fig. 4.1C) suggest that earthworms exposed to 136 and 272 g deinking biosolids kg^{-1} soil are experiencing oxidative stress, mainly due to the accumulation of H_2O_2 in cells. This is particularly concerning because if H_2O_2 is not broken down by CAT, highly reactive and toxic hydroxyl radicals (• OH) can be generated through the Fe- and Cu-catalyzed decomposition of H_2O_2 (Schrader & Fahimi, 2006). As • OH can react with and oxidize protein, lipids and DNA, higher CAT activity should prevent cellular damage.

The buildup of ROS in cells can trigger multiple damaging pathways, including lipid peroxidation, gene mutations, and DNA damage (Wen et al., 2021). To counter these threats, antioxidant enzymes such as SOD and CAT are produced to neutralize ROS and mitigate their damaging effects. In our study, we observed that earthworms exposed to deinking biosolids experienced DNA damage when ROS levels are significantly elevated compared to the control ($p < 0.05$, Fig. 4.3A & 4.1A), except on day 7 in which DNA damage was significant despite the baseline ROS levels in treatments amended with 34, 68 and 136 g deinking biosolids kg^{-1} soil. Interestingly, moderate oxidative stress can activate cell cycle checkpoints, which temporarily halt cell proliferation to allow DNA repair and prevent the transmission of damaged or incomplete DNA to daughter cell (Viktoria et al., 2024; Gomez & Hergovich, 2016). This may explain the return to baseline DNA damage levels in earthworms exposed to 34 g deinking biosolids kg^{-1} soil on day 14, 21, and 28 following the spike observed on day 7 ($p < 0.05$), while higher treatment levels i.e., 68, 136, and 272 g kg^{-1}, continued to show significant DNA damage over time (p < 0.05).

4.6.2 Effects of pristine and aged Cu-spiked microplastics on oxidative stress and DNA damage in *E. fetida*

Earthworms exposed to pristine microplastics had greater ROS levels, altered antioxidant enzyme activities, and more DNA damage than the control ($p < 0.05$, Fig. 4.2 & 3B). However, there was no size-or type-dependent effects on earthworm oxidative stress and DNA damage (p > 0.05, Fig. 4.2 & 3A). The size-dependent effects of microplastics on earthworms reported in the literature are conflicted, possibly due to the different size-ranges tested between studies. For instance, Li et al. (2021) found that earthworms exposed to 1.25 g of polyethylene microplastics (28–145, 133–415 and 400–1464 μm) and polypropylene microplastics (8–125, 71–383 and 761–1660 μm) kg^{-1} soil significantly altered the activities of SOD, CAT, glutathione S-transferase, and 8-hydroxy-2′-deoxyguanosine levels in earthworms, denoting smaller microplastics showing the greatest effect. In contrast, Xu et al. (2021) reported that larger plastic fragments are more toxic than smaller ones, reporting that 10 mg kg^{-1} of large polystyrene microplastics (10 μm) caused significantly higher DNA damage in earthworms compared to smaller ones (100 nm) in a 21-d soil-exposure assessment. It appears that the size-dependent toxicity of microplastics on earthworms is influenced by the specific comparative assessment of size-ranges. In our experiment, we did not observe a size-dependent effect, likely because the microplastics tested have a relatively small size difference compared to previous studies, resulting in similar toxicity mechanisms across the two treatments.

When comparing pristine and aged Cu-spiked microplastics, we observed higher ROS levels in earthworms exposed to aged Cu-spiked polyethylene microplastics than those exposed to pristine polyethylene ($p < 0.05$, Fig. 4.2A). Further, exposure to large polyethylene microplastics induced greater DNA damage in earthworms compared to their pristine state ($p < 0.05$, Fig. 4.3B). Interestingly, Li at al. (2023) investigated the effects of 100, 1000, and 10,000 mg of aged and pristine polyethylene microplastics (138 µm) per kg of natural soil containing 52 mg kg^{-1} Zn^{-2}, 16 mg kg^{-1} Pb^{-2}, and 0.11 mg kg^{-1} Cd^{-2}. They did not find any statistical significance in ROS levels and SOD activities between aged and pristine microplastics. However, our experimental design enabled us to examine the direct effect of Cu bound to aged microplastics as a combined stressor, as the microplastics were spiked with Cu rather than being mixed with Cu-spiked soil. This approach also allowed us to isolate Cu toxicity from that of the microplastics.

In our study, only polyethylene microplastics spiked with Cu were more toxic to earthworms than their pristine state, corresponding with soil-Cu concentrations, as only polyethylene microplastics showed significantly higher Cu concentrations than the control ($p < 0.05$, Fig. 4.4). This suggests that compared to aged polystyrene, aged polyethylene has a greater potential to accumulate and transport Cu into the soil. Interestingly, in a sorption dynamics experiment with pristine microplastics and $CdCl_2$ solution, polystyrene microplastics adsorbed 134 mg Cd kg^{-1}, while polyethylene microplastics adsorbed 113 mg^{-1} Cd kg^{-1}, indicating that polystyrene has a greater sorption capacity than polyethylene (Guo et al., 2020). However, this may be attributed to the pristine nature of the tested polymers in Guo at al. (2020) study. For instance, Burrows et al. (2024) demonstrated that polyethylene microplastics undergo greater UV-induced aging, evidenced by significantly higher carbonyl index, which indicate extensive surface oxidation compared to polystyrene microplastics ($p < 0.05$). Metal ions bind to microplastics via coordinate interactions with specific functional groups, such as hydroxyl (-OH), carboxyl (-COOH), or amine ($-NH_2$) that form on or within the microplastics. Therefore, the combined effect of UV aging and Cu-binding appear to drive the increased toxicity of Cu-spiked polyethylene microplastics to earthworms.

4.7 Conclusion

This study illustrates that exposing earthworm to ≤ 34 g deinking biosolids kg^{-1} soil did not induce oxidative stress in a controlled laboratory setting. The data collected on the effects of deinking biosolids on earthworms informs ecotoxicology risk assessment models that evaluate the potential impacts of biosolids application on earthworms at larger scale, such as agricultural fields. The intriguing finding of this study was that weathered microplastics can augment the toxicity of the combined microplastics-Cu exposure to earthworms in a type-dependent manner, offering an environmentally relevant method to evaluate their combined effect. The next step in this research will be to investigate the size-dependent toxicity of microplastics on earthworms using consistent size ranges, and to investigate sorption and desorption characteristics of trace metals to and from weathered microplastics with diverse polymer chemistry.

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Figure 4.4 Measured Cu concentrations in microplastics (A), and in artificial soil at the end of exposure period on day 28 (B). The soil was mixed with aged small and large polyethylene (10−22 μm and 90−106 μm) and polystyrene (14−20 μm and 85−105 μm) round polymers (n=3), spiked with 100 mg $Cu(NO_3)_2$ l^{-1} (nominal). Data presented as (mean ± SE). Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

TABLES

Table 4.1 Description of treatments, application rates and Cu concentrations in deinking biosolids and microplastics.

Treatment	Description	Application rates	Nominal Cu concentrations	Measured Cu concentrations
Control	350 g OECD artificial soil	N/A	N/A	0
Deinking biosolids	*C1-P1-O1	34, 68, 136, 272 g deinking biosolids kg^{-1} soil	1, 2, 4, 8 mg kg^{-1}	30 mg kg^{-1}
PE-S	Pristine 10–22 μm	0.75 g microplastics kg^{-1} soil	100 mg l^{-1}	0 μg g^{-1}
PS-S	Pristine 14–20 μm			0 μg g^{-1}
PE-L	Pristine 90–106 μm			0 μg g^{-1}
PS-L	Pristine 85–105 μm			0 μg g^{-1}
Cu-PE-S	Aged 10–22 μm			18 μg g^{-1}
Cu-PS-S	Aged 14–20 μm			10 μg g^{-1}
Cu-PE-L	Aged 90–106 μm			30 μg g^{-1}
Cu-PS-L	Aged 85–105 μm			15 μg g^{-1}

*The deinking biosolids sample meets the chemical contaminants, pathogens and odor criteria of the Canadian Council of Ministers of the Environment, indicating that the permissible land application rate of 31 Mg ha^{-1} does not pose a significant environmental risk from chemical contaminants, pathogens, and odors (Hébert, 2015).

FIGURES

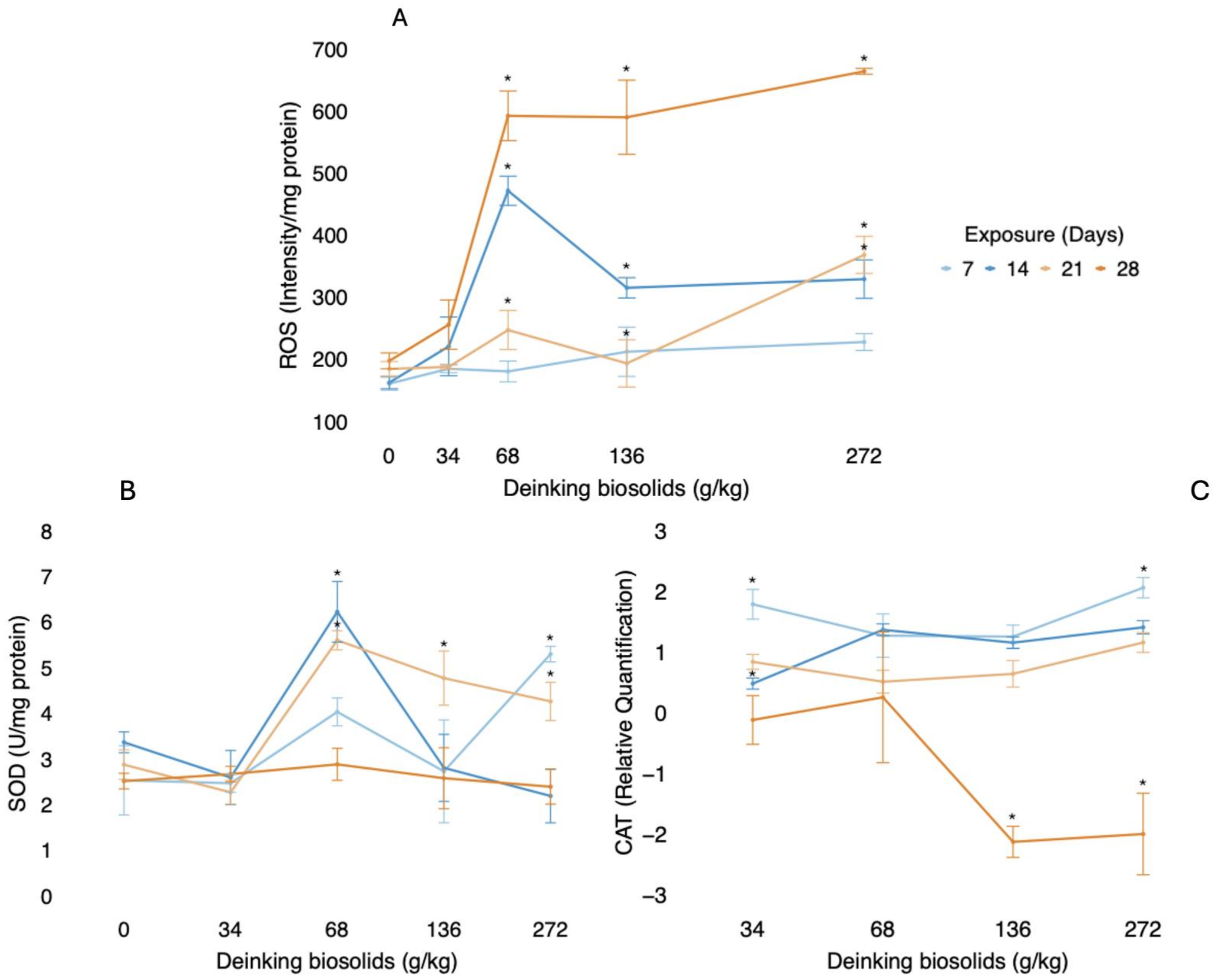

Figure 4.1 The effects of deinking biosolids in artificial soil exposure on reactive oxygen species levels, and superoxide dismutase and catalase activities in the earthworm *E. fetida*. Reactive oxygen species levels and superoxide dismutase activities are presented as means of three replicates ± SE. Catalase activity is presented as relative quantification compared to control ± SE from three replicates. Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

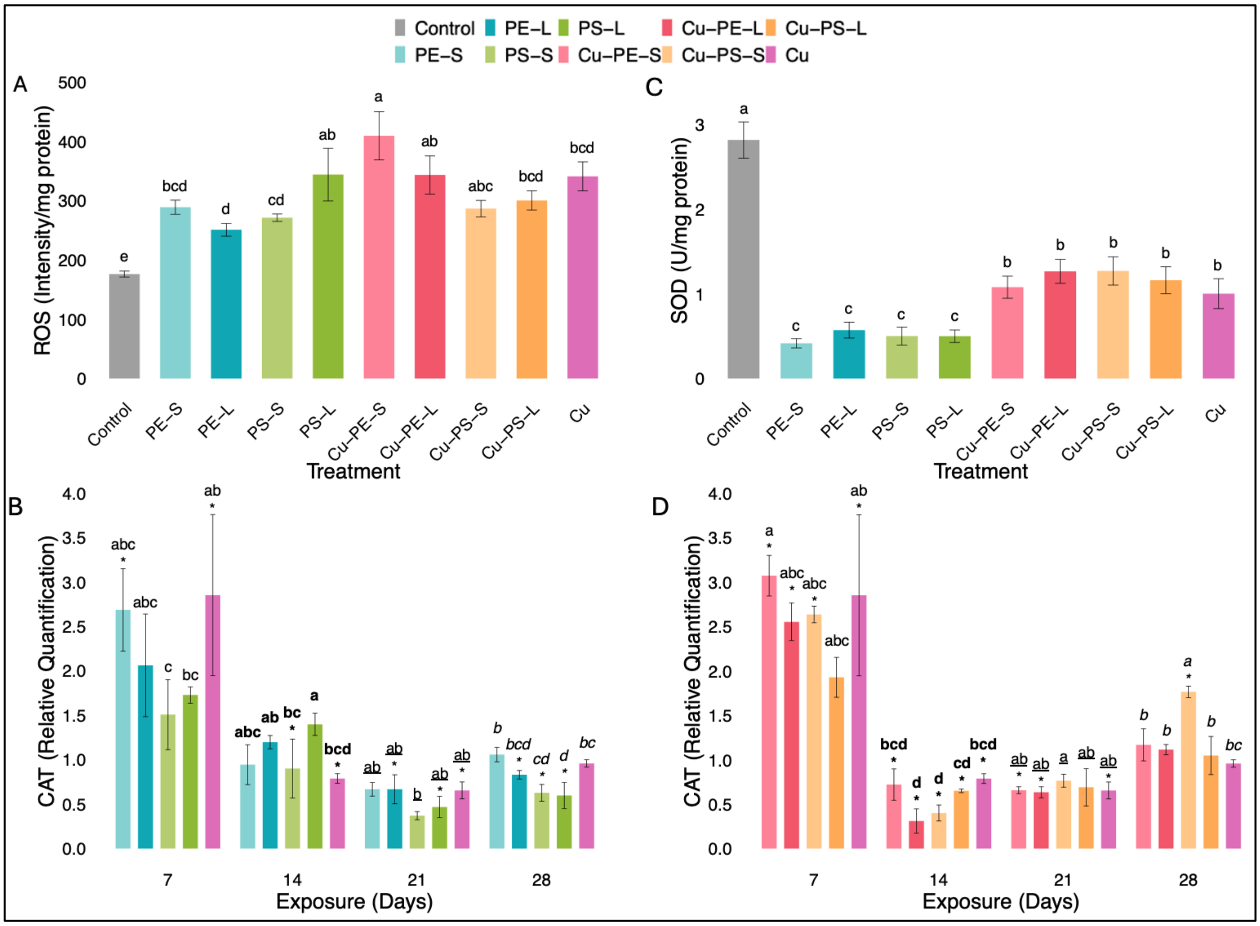

Figure 4.2 The effects of pristine, and aged Cu-spiked microplastics in artificial soil exposure on reactive oxygen species levels, and superoxide dismutase and catalase activities in the earthworms *E. fetida*. Reactive oxygen species levels and superoxide dismutase activities are presented as pooled means from days 7, 14, 21 and 28 of three replicates ± SE. Catalase activity is presented as relative quantification compared to control ± SE from three replicates. Microplastics used in this experiment are small and large polyethylene (10–22 μm and 90–106 μm) and polystyrene (14–20 μm and 85–105 μm) round polymers. Letters indicate significant differences between treatments. For catalase activity the addition of (*) indicate significance compared to the control. Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

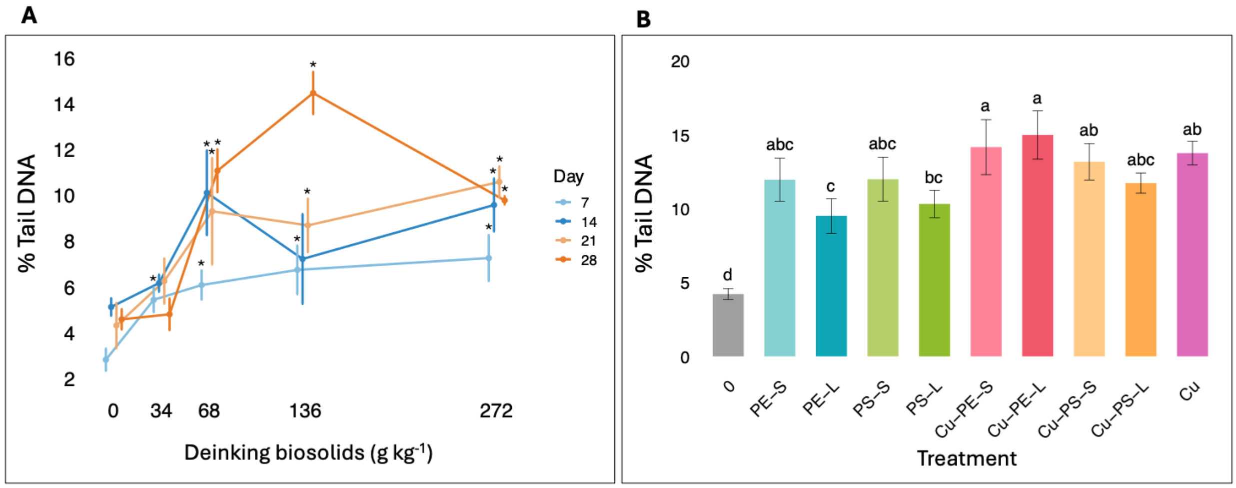

Figure 4.3 The effects of deinking biosolids, pristine, and aged Cu-spiked microplastics in artificial soil exposure on % tail DNA in the earthworm *E. fetida*. Microplastics used in this experiment are small and large polyethylene (10–22 µm and 90–106 µm) and polystyrene (14–20 µm and 85–105 µm) round polymers. DNA damage data from the microplastics treatments are presented as pooled means from days 7, 14, 21 and 28 of three replicates ± SE. Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

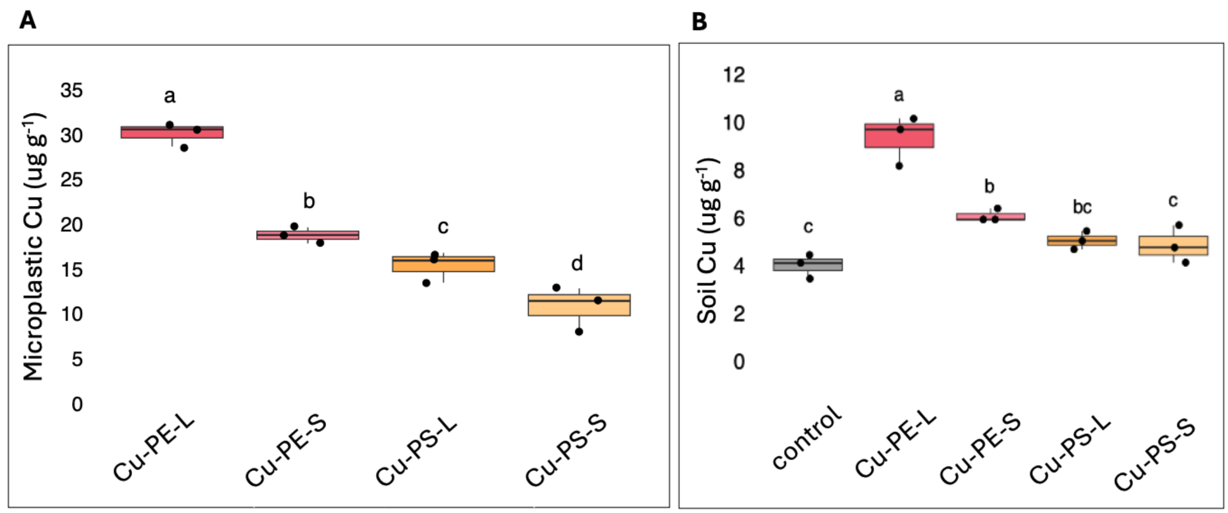

Figure 4.4 Measured Cu concentrations in microplastics (A), and in artificial soil at the end of exposure period on day 28 (B). The soil was mixed with aged small and large polyethylene (10−22 μm and 90−106 μm) and polystyrene (14−20 μm and 85−105 μm) round polymers (n=3), spiked with 100 mg $Cu(NO_3)_2$ l^{-1} (nominal). Data presented as (mean ± SE). Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

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GENERAL DEISCUSSION

In 2011, the Ministry of Environment of Quebec developed and implemented the Quebec Residual Materials Management Policy. The primary goal of this policy is to build a green circular economy by improving waste management practices and sustainability measures within the province. One of the ten proposed strategies is to ban organic residues from disposal sites by 2024 (MELCC, 2011). This strict landfilling policy encouraged the agricultural sector, with oversight from the Ministry of Agriculture, Fisheries and Food in Quebec, to recycle the affordable and locally sourced organic residues of value as crop fertilizer. Due to this policy, 1.8 million Mg (wet weight) of fertilizing organic residues were applied annually to croplands and for land reclamation in Quebec, and about 70% were biosolids generated by the pulp and papermill industry (Hébert, 2015). While papermill biosolids are rich in organic matter that improves soil bulk density and structure, and in essential nutrients such as N, P, and K for crop nutrition (Rashid et al, 2006) the deinking fraction of papermill biosolids contains non-nutritive substances such as plastics, an emerging contaminant, and trace metals. Thus, deinking biosolids and the potential contaminants within them need to be evaluated for their safety concerning soil organisms.

Risk assessment of deinking biosolids and Cu, and the use of toxicity predictive and interaction models

The toxicity of deinking biosolids was evaluated with the earthworm *E. fetida*, based on its growth and reproduction in Cu-amended artificial soil. I specifically chose to test the toxicity of deinking biosolids in a Cu-contaminated medium because (1) the permissible Cu level in deinking biosolids set by the MAPAQ is 400 mg Cu kg^{-1} biosolids, and (2) regulations allow the application of biosolids on agricultural lands if the soil-Cu level is 63 mg Cu kg^{-1}, according to the CCME soil quality guidelines for agricultural land use (Hébert, 2015; CCME, 2007). The Cu limits within these two regulatory standards caught my attention, as the allowable Cu levels in both deinking biosolids and soil could potentially have harmful effects on earthworm physiology and impact their population dynamics. Therefore, I designed the experiment in a setting that allow me to evaluate the possible antagonistic or synergistic interactions between the two substances on earthworm physiology and Cu accumulation.

Despite the significant amount of deinking biosolids from the pulp and papermill industry that are available in Quebec for agricultural purposes, research on its impact on soil organisms is limited. A three-year field study by Price & Voroney (2008) investigated the effects of applying 0, 50, 100, and 150 Mg deinking biosolids ha^{-1} on earthworm biomass and population. The study reported beneficial effects on earthworm population in 2 fields, and beneficial effects on earthworm biomass in 1 out of three fields tested. However, field testing introduces variables that are difficult to control, such as weather, soil type and trace metal variations, which can mask the impacts of contaminants on earthworms. Regulatory agencies, including Environment Canada and the USEPA, recommend testing potential contaminants on organisms in controlled laboratory assays for the purpose of risk assessment and management of potentially harmful substances (Environment Canada, 2013; USEPA, 2012). I believe the difference between this study and Price & Voroney (2008), mainly the observed inhibited reproduction at 68 g deinking biosolids kg^{-1} soil equivalent to 132 Mg ha^{-1}, could be attributed to exposure period and field conditions including temperature, moisture, field soil minerals and metals variations, plant metal uptake, and microbial activity, which promote the degradation of biosolids, that are not considered in my lab-scale study. Additionally, the larger experimental units used in field plots (3 m x 12 m) would allow earthworms more space to move compared to the smaller space of a 1-L jar, where they are in more direct contact with the biosolids.

Comparing my lab-scale dose-response assays endpoints to actual risks in a field exposure is challenging. This is mainly due to difference in exposure space surface area and volume, soil physiochemical parameters, climatic conditions, and the low biological activity in the artificial soil used in my lab-scale study. However, the data collected from these controlled experiments, which captures both isolated and combined toxicity effects, are valuable for predictive and interaction-based numerical toxicity models. Using toxicity endpoints collected from my experiments in models such as Terrestrial Biotic Ligand Model (TBLM) and the geospatial speciation model WHAM allows for contaminant risk assessments under varying soil parameters including pH, moisture content, organic matter, clay content, multiple contaminant concentrations, and metal speciation (Sdepanian, 2011). However, it is important to consider the assumptions used in these models. For instance, these models use metal bioavailability, speciation and their competition with other cations as indicators of toxicity (Qiu, 2014). Still, a ecological risk assessment should consider more than how the contaminants are transformed and their fate, including the amount that leaves soil by uptake through biotic foodwebs or via material transfer to adjacent water and air environments. Consequently, we need information on how model organisms like earthworms respond to contaminant exposure, including bioaccumulation and toxicological outcomes, which is where my work contributes to the body of knowledge. The US EPA Ecotoxicological Assessment and Monitoring (ETAM) Research Area is developing streamlined and integrated modeling strategies to enhance chemical risk assessments that encompasses these key stages of ecological toxicity (U.S. EPA, 2024)

Limitations of assessing Cu bioavailability in organic-rich biosolids

A limitation in Chapter 2 was the inability to measure Cu concentrations in the soil at the end of the incubation period, which prevented estimating Cu bioavailability. This limitation occurred from concerns about potential bias during the collection of representative soil samples from the 4x4 factorial experimental design. This is because varying application rates of biosolids were mixed with the soil and/or Cu-spiked soil. I worried that sampling might unintentionally result in the collection of disproportionate amounts of biosolids or soil from the sample. In my opinion, grinding the biosolids before the incubation period, or grinding the treatments at the end of the incubation period was also not a reliable option, as it risked altering the original interactions between Cu, the soil, and the biosolids. This is because Laurent et al. (2023) demonstrated that Cu bind to dissolved organic matter in dairy manure compost through covalent interactions, while Shi et al. (2018) provided evidence that hydroxyl and carboxyl functional groups play a role in the adsorption of Cu onto particulate organic matter. Thus, to address this limitation, I decided to measure Cu concentrations in earthworm tissue, cytosol, and metallothionine. By conducting this protocol, I was able to measure the detoxified Cu fraction, which accumulates in the metallothionine (Santon et al., 2009), and the toxic Cu fraction, which according to Wallance & Louma (2003) is found in the cytosol.

Microplastics, an emerging contaminant in organic-rich biosolids

An original finding in this study was finding microplastics in deinking biosolids. The microplastics were detected when I conducted Fourier Transform Infrared Spectroscopy analysis on foreign bodies extracted from deinking biosolids using the density separation protocol (Appendix 5.1 & 5.2). However, the amount/mass of microplastics present in deinking biosolids were not quantified. The quantification of microplastics in organic-rich substances like deinking biosolids requires complex methodologies that were beyond the scope of this research. Nonetheless, the screening protocol conducted following the density separation methodology (Han et al., 2019) provided valuable insights for Chapter 3 and 4 experiments, including the type of plastics present and their surface characteristics. I found that deinking biosolids contain high density polyethylene (Appendix 5.2). I believe the presence of polyethylene in deinking biosolids is due to the polymer being used in paper coating. This led me to focus on the effects of exposing earthworms to polyethylene and included polystyrene in Chapter 3 experiments. This decision was supported by Crossman et al. (2020), who identified polyethylene and polystyrene as two of the most common plastic types found in agricultural lands that receive organic fertilizers in Ontario, Canada. In addition, scanning microscopy analysis of the microplastics samples extracted from deinking biosolids exhibited cracks and fractures (Appendix 5.1). Thus, although the protocol I followed have quantitative limitation, the screening was helpful because it supported the experimental design by (1) focusing on the appropriate types of microplastics for the study, and (2) confirming that microplastics were weathered, which led me to add the "aged" treatments to represent the realistic conditions rather than using pristine microplastics particles.

Technical challenges in evaluating earthworm response to microplastics, and alternative approaches

Assessing DNA damage is a critical step in understanding the subcellular effects of contaminants like microplastics on earthworms. Earthworm coelomocytes are prone to DNA damage when interacting with microplastics, mainly due to oxidative stress and mechanical injury, both of which are important for evaluating microplastic-induced toxicity. There are various protocols available to assess DNA damage. From literature review, I observed that the Comet Assay (Single Cell Gel Electrophoresis) and the 8-OHdG Assay (8-Hydroxy-2'-deoxyguanosine) are commonly used methods for detecting DNA damage in earthworms. This could be due to their sensitivity and well-established protocols for earthworms. In my experiment on the effects of microplastics on earthworm oxidative stress and DNA damage (Chapter 4), I selected the Comet Assay over the 8-OHdG Assay, as the Comet Assay specifically detects oxidative DNA damage (Shekaftik & Nasirzadah, 2021). This is because my investigation of the effects of microplastics on earthworm coelomocytes (Chapter 3), I noticed mechanical cell injury. This was deduced from the observation that microplastics with a size close to or larger than the cell diameter could cause cell death through mechanical injury, possibly via frustrated phagocytosis. As a result, I implemented the Comet Assay in Chapter 4 experiment to detect DNA strand breaks. However, the procedure for scanning Comet Assay images uses fluorescent microscopy and is done manually, which was challenging. The experiment involved analyzing samples from a large number of earthworms, with at least 50 comet images required per earthworm. This process was time-consuming, especially given the need to prepare other samples for additional biomarker analysis on the same day. The use of a motorized slide-scanning stage to expedite the process was considered. Unfortunately, some comet images were blurry. Upon inspecting the focal plane, I realized that not all comets i.e., intact DNA or DNA fragments, were at the same Z-axis position. This is expected due to the migration of the DNA during electrophoresis through the agar gel. As a result, I was unable to automate the process. Consequently, in future DNA damage analyses using the Comet Assay, I would optimize the protocol by ensuring the consistency of the thickness of the agar gel layer so I could use the motorized slide-scanning stage to be more efficient.

Technical challenges were also noticed in Chapter 3 when I analyzed the effects of microplastics exposure to earthworm coelomocytes *in vitro* with emphasis on phagocytosis. The flow cytometry analysis revealed that coelomocytes engulfed 85% of small polyethylene microplastics (1–10 µm) through phagocytosis, but there was negligible phagocytosis of larger polyethylene microplastics (20–27 µm). Nonetheless, exposure to both size ranges of microplastics led to significant cell death compared to untreated coelomocytes ($p < 0.001$). I deduced that microplastics are toxic to coelomocytes because the smaller microplastics appear to accumulate within cells, and the larger particles likely caused cell death through frustrated phagocytosis. I supported this conclusion with fluorescent and brightfield microscopy images (before cell fixation) that shows various stages of interaction between coelomocytes and microplastics, including coelomocytes attempting to engulf the synthetic particles larger than their own diameter (Figure 3.2, Chapter 3). This approach differs from previous studies, which typically involved exposing whole earthworms to microplastic-treated soil for extended periods, followed by cell extraction to assess cell vitality and microplastic accumulation (Kwak et al., 2021). My research bridged the knowledge gap regarding the size-dependent cytotoxicity of microplastics in earthworms. It does so by examining the effects on innate cellular immune defense mechanisms, specifically phagocytosis. Other studies have conducted *in vitro* assessments, examining lysosomal integrity after exposing cells to nanoplastics (He et al., 2024). Yet, to the best of my knowledge, this study is the first to quantify phagocytosis as the outcome of cell death and proposed possible toxicity mechanisms for different size ranges of microplastics. However, I believe there is room for improvement in the methodology. The use of micropipette aspiration (YoungSheng et al., 2018) would be ideal for precisely capturing the phagocytic spreading process. This technique involves isolating and manipulating single cells, which could be more precise than evaluating cell suspensions. The main reason is that micropipette aspiration holds the sample in place to prevent movement during the exposure period. Therefore, if I am to refine the methodology, I would have implemented micropipette aspiration by isolating a single cell and a single microplastic particle to visualize their live interaction. Unfortunately, at this time, I lack the technical skills necessary to implement such a protocol.

Research gaps

Exposure to soil spiked with microplastics has no significant impact on earthworm mortality, growth, and reproduction. For instance, Mondal et al. (2023) reported that exposure to up to 3% w/w of polyethylene microplastics (≤ 125) for 28-d did not affect earthworm physiology. However, previous studies that exposed earthworms to different size ranges of microplastics at environmentally relevant concentrations (< 1% w/w) revealed that microplastics exposure can affect sensitive biomarkers, including DNA damage and oxidative stress. For example, exposure to 5 g kg^{-1} soil of polyethylene microplastics (250–1000 μm) caused significant DNA damage to *E. fetida* compared to control (Sobhani et al., 2021). Yet, this is not the only concern regarding earthworms' exposure to microplastics in the soil environment. It was evident that the level of stress in earthworms could be amplified with the addition of trace metals in the same exposure media containing microplastics. Li et al. (2021) found that the addition of 0.5 g kg^{-1} of polyethylene microplastics (30 μm) to 100 mg kg^{-1} of Cu-spiked soil increased MDA contents and SOD, POD, and CAT activities in earthworms compared to exposure to an equivalent concentration of Cu alone in soil. This confirms that microplastics exposure can be toxic even at low concentration (0.5 g microplastics kg^{-1} soil) when trace metals are present in the soil. Yet, relating the increased stress response to microplastics acting as vectors to trace metals is extremely difficult in this experimental setting. This is because the earthworms were exposed to both contaminants (Cu and microplastics) in the same media separately as opposed to joint contaminants exposure. Given the dual threat posed by microplastics; both as physical irritants and chemical carriers, and their impact on earthworm immune and physiological functions, it was vital to establish a novel approach to examine the effects of aged Cu-spiked microplastics to earthworms' cellular response and explore the underlying mechanisms behind this toxicity.

Consequently, in Chapter 4, I evaluated how aged microplastics-bound Cu impact earthworms, specifically in terms of causing oxidative stress and DNA damage.

The size-dependent microplastics effects on earthworms dilemma

In my study, I found no size- or type-dependent effect of pristine microplastics on earthworm ROS levels, SOD and CAT activities, and DNA damage. This conclusion was based on the observation that while exposure to large polystyrene microplastics induced greater ROS levels in earthworms compared to small polystyrene microplastics ($p < 0.05$), this effect was not observed in the polyethylene treatments. In addition, although exposure to small polyethylene microplastics caused a significant stimulation in CAT activity compared to large polyethylene microplastics, there were no significant differences between polystyrene treatments. The size-dependent effects of microplastics on earthworms reported in the literature are conflicted. For instance, Li et al. (2021) found that earthworms exposed to 1.25 g of polyethylene microplastics (28–145, 133–415 and 400–1464 μm) and polypropylene microplastics (8–125, 71–383 and 761–1660 μm) kg^{-1} soil significantly altered the activities of SOD, CAT, glutathione S-transferase, and 8-hydroxy-2′-deoxyguanosine levels in earthworms, denoting smaller microplastics showing the greatest effect. In contrast, Xu et al. (2021) illustrated that larger plastic fragments are more toxic than smaller ones, reporting that 10 μm of polystyrene microplastics (10 mg kg^{-1}) caused significantly higher DNA damage in earthworms compared to 100 nm in a 21-d soil-exposure assessment. This inconsistency may be attributed to differences in the size ranges of microplastics tested in the three studies, mine and that of Li et al. (2021) and Xu et al. (2021). It appears that the size-dependent toxicity of microplastics on earthworms is influenced by the specific comparative assessment of size-ranges. In other words, microplastic size that is labelled small in one study is labelled large in another study. Therefore, and in addition, I believe I did not observe a size-dependent effect in my study because the size gap between the microplastics I tested is relatively small i.e., average particle size of the small microplastics is 16 μm and the large microplastics are 95 μm, which is also not comparable to previous research size-ranges. Based on my own experiments and literature review, I postulate that all microplastic sizes induce toxicity to earthworms compared to control, albeit through different mechanisms.

GENERAL CONCLUSIONS

My work contributes to Canada's mission of achieving zero waste and a circular economy in the agricultural sector by evaluating the potential toxicity of deinking biosolids on earthworms, to encourage the recycling of affordable and locally sources organic residues as soil amendments. I investigated the effects of deinking biosolids and emerging contaminants within them i.e., microplastics-bound Cu on earthworms, both individually and in interaction, in controlled laboratory settings using artificial soil as the test substrate. My findings demonstrate that moderate application of deinking biosolids is safe for earthworms in controlled laboratory conditions. This adds to the toxicology exposure database used for ecotoxicological risk assessment by regulatory agencies (US EPA, 2024). My data could also be validated by measuring the growth, reproduction and stress response of earthworms exposed to field soil collected from areas where deinking biosolids was applied at various rates and frequencies. Furthermore, I discovered that earthworm immune cells can recognize, interact with, and, depending on the particle diameter, engulf multiple microplastics in vitro, causing stress and eventually cell death, albeit through different mechanisms. In addition, I addressed that the aged polymer type influences the toxicity of microplastics on earthworms when mixed with Cu, evident by soil-Cu concentrations, possibly due to differences in Cu sorption efficiency of various microplastic polymers.

My dose-response toxicology data of deinking biosolids, Cu and microplastics collected from the controlled lab-scale studies could be integrated into computational models that integrate the material reactions in the soil environment and organismal responses (exposure, internal dosimetry, metabolism, and toxicology) in a broader environmental risk assessment context. These isolated and combined effect results collected from my study are particularly valuable in network numerical modeling for addressing synergistic and/or antagonistic interactions, specifically concerning complex substance that includes variety of potential contaminants such as deinking biosolids. Furthermore, while I recognized that microplastics exposure induces toxicity in earthworms at both the cellular and whole-organism levels regardless of particle size, the observed polymer type-dependent effect when joined with Cu has the potential for informed material selections for agricultural applications. These findings can guide the choice of materials for agricultural bulk bags, plastics mulches and irrigation pipes towards less absorbent polymers when applicable. This research provides valuable knowledge to the Ministry of Agriculture, Fisheries and Food in Quebec, particularly regarding the potential toxicity of deinking biosolids on earthworms. It also highlights the need to address the toxicity of microplastics-bound Cu as an emerging contaminant within biosolids. Such findings can support enhanced regulations to limit the ecological consequences of the unintentional input of microplastics into agricultural soil.

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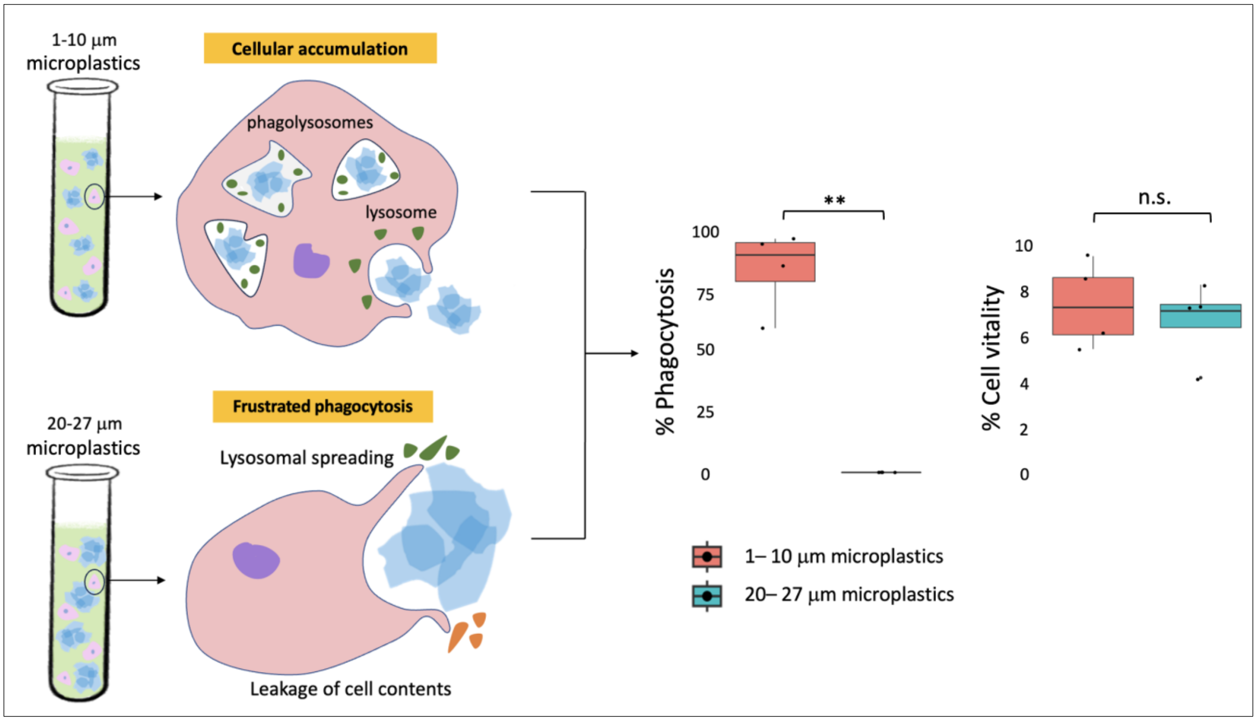
APPENDCIES



Appendix 3.1 Chapter 3 graphical abstract illustrating the *in vitro* experimental setup and the deduced phagocytic mechanisms involved in earthworm coelomocytes death due to microplastics exposure.

Appendix 4.1 Superoxide dismutase and catalase activities in the earthworm *E. fetida* exposed to deinking biosolids in artificial soil. Data collected on day 7, 14, 21, and 28 of exposure. Values are mean ± standard error, n=3.

Exposure period (d)	7		14		21		28	
Deinking biosolids ($g\ kg^{-1}$ soil)	SOD ($U\ mg^{-1}$ protein)	CAT ($U\ mg^{-1}$ protein)	SOD ($U\ mg^{-1}$ protein)	CAT ($U\ mg^{-1}$ protein)	SOD ($U\ mg^{-1}$ protein)	CAT ($U\ mg^{-1}$ protein)	SOD ($U\ mg^{-1}$ protein)	CAT ($U\ mg^{-1}$ protein)
0	2.53 ± 0.76	1.90 ± 0.05	3.36 ± 0.23	5.54 ± 0.13	2.86 ± 0.32	5.41 ± 0.26	2.51 ± 0.17	3.77 ± 0.10
34	2.46 ± 0.20	3.36 ± 0.21	2.58 ± 0.59	2.62 ± 0.24	2.26 ± 0.26	4.40 ± 0.17	2.66 ± 0.17	3.64 ± 0.15
68	4.02 ± 0.30	2.36 ± 0.33	6.21 ± 0.66	7.52 ± 0.18	5.59 ± 0.21	2.60 ± 0.44	2.87 ± 0.35	4.02 ± 0.54
136	2.72 ± 1.13	2.37 ± 0.18	2.80 ± 0.73	6.37 ± 0.34	4.77 ± 0.59	3.53 ± 0.80	2.57 ± 0.67	1.63 ± 0.22
272	5.29 ± 0.17	3.88 ± 0.08	2.18 ± 0.59	7.73 ± 0.23	4.25 ± 0.42	6.10 ± 0.25	2.38 ± 0.38	1.76 ± 0.29

Appendix 4.2 Reactive oxygen species levels and DNA damage, measure as % tail DNA using the comet assay protocol in the earthworm *E. fetida* exposed to deinking biosolids in artificial soil. Data collected on day 7, 14, 21, and 28 of exposure. Values are mean ± standard error, n=3.

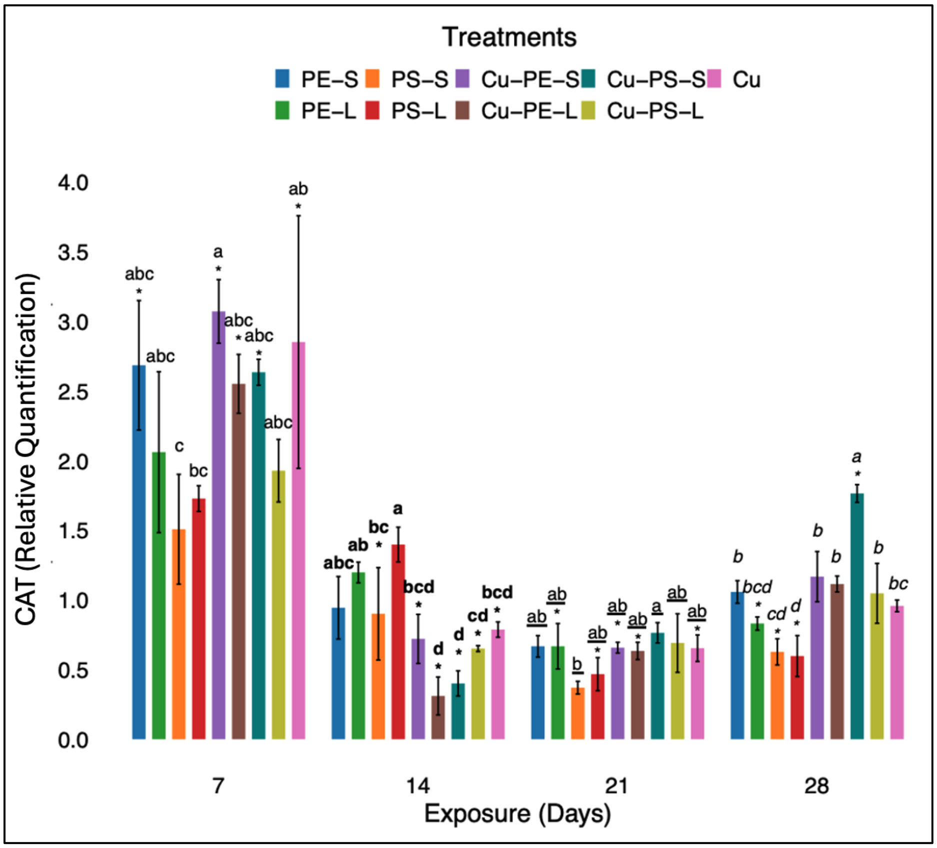
Exposure period (d)	7		14		21		28	
Deinking biosolids ($g\ kg^{-1}$ soil)	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA
0	160 ± 10	2.8 ± 0.48	162 ± 10	5.1 ± 0.38	184 ± 12	4.3 ± 0.98	197 ± 13	4.5 ± 0.44
34	184 ± 6	5.4 ± 0.53	220 ± 47	6.1 ± 0.37	187 ± 4	6.2 ± 0.98	255 ± 40	4.8 ± 0.69
68	180 ± 17	6.0 ± 0.64	471 ± 24	10.1 ± 1.84	246 ± 31	9.3 ± 2.32	592 ± 40	11.0 ± 0.93
136	212 ± 40	6.7 ± 1.06	315 ± 16	7.2 ± 1.95	193 ± 38	8.6 ± 1.16	589 ± 60	14.4 ± 0.92
272	227 ± 14	7.2 ± 1.00	329 ± 31	9.5 ± 1.15	368 ± 30	10.5 ± 0.67	663 ± 5	9.7 ± 0.18

Appendix 4.3 Superoxide dismutase and catalase activities in the earthworm *E. fetida* exposed to pristine and aged copper-spiked polyethylene and polystyrene microplastics (0.75 g kg soil^{-1}, spiked nominally with 100 mg Cu l^{-1}) in artificial soil. Data collected on day 7, 14, 21, and 28 of exposure. Values are mean ± standard error, n=3.

Exposure period (d)	7		14		21		28	
Treatment	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)	SOD (U mg^{-1} protein)	CAT (U mg^{-1} protein)
control	2.53 ± 0.76	1.90 ± 0.05	3.36 ± 0.23	5.54 ± 0.13	2.86 ± 0.32	5.41 ± 0.26	2.51 ± 0.17	3.77 ± 0.10
PE-S	0.41 ± 0.04	5.03 ± 0.37	0.35 ± 0.07	5.32 ± 0.85	0.37 ± 0.04	3.54 ± 0.08	0.54 ± 0.17	3.96 ± 0.12
PS-S	0.65 ± 0.29	4.01 ± 0.73	0.16 ± 0.01	6.65 ± 0.35	0.35 ± 0.07	3.52 ± 0.35	0.84 ± 0.21	3.13 ± 0.14
PE-L	0.90 ± 0.22	2.81 ± 0.35	0.49 ± 0.10	4.98 ± 1.06	0.34 ± 0.12	1.98 ± 0.09	0.55 ± 0.14	2.34 ± 0.20
PS-L	0.57 ± 0.12	3.27 ± 0.06	0.48 ± 0.05	7.80 ± 0.58	0.35 ± 0.16	2.62 ± 0.45	0.59 ± 0.20	2.28 ± 0.37
Cu-PE-S	1.19 ± 0.09	5.81 ± 0.13	1.04 ± 0.29	4.01 ± 0.56	0.57 ± 0.06	3.55 ± 0.22	1.50 ± 0.24	4.37 ± 0.38
Cu-PE-L	1.55 ± 0.17	4.81 ± 0.10	0.93 ± 0.17	1.74 ± 0.44	0.89 ± 0.14	3.46 ± 0.33	1.68 ± 0.23	4.21 ± 0.23
Cu-PS-S	1.64 ± 0.61	3.63 ± 0.17	0.94 ± 0.08	3.60 ± 0.09	0.77 ± 0.11	3.58 ± 0.47	0.65 ± 0.32	3.87 ± 0.35
Cu-PS-L	1.22 ± 0.10	5.32 ± 0.93	1.16 ± 0.08	4.32 ± 0.07	0.97 ± 0.13	3.47 ± 0.22	1.71 ± 0.03	3.59 ± 0.01
Cu	1.52 ± 0.14	5.03 ± 0.22	0.92 ± 0.09	2.24 ± 0.31	0.57 ± 0.17	4.07 ± 0.04	1.63 ± 0.66	6.62 ± 0.11

Appendix 4.4 Reactive oxygen species levels and DNA damage, measure as % tail DNA using the comet assay protocol in the earthworm *E. fetida* exposed to pristine and aged copper-spiked polyethylene and polystyrene microplastics (0.75 g kg soil^{-1}, spiked nominally with 100 mg Cu l^{-1}) in artificial soil. Data collected on day 7, 14, 21, and 28 of exposure. Values are mean ± standard error, n=3.

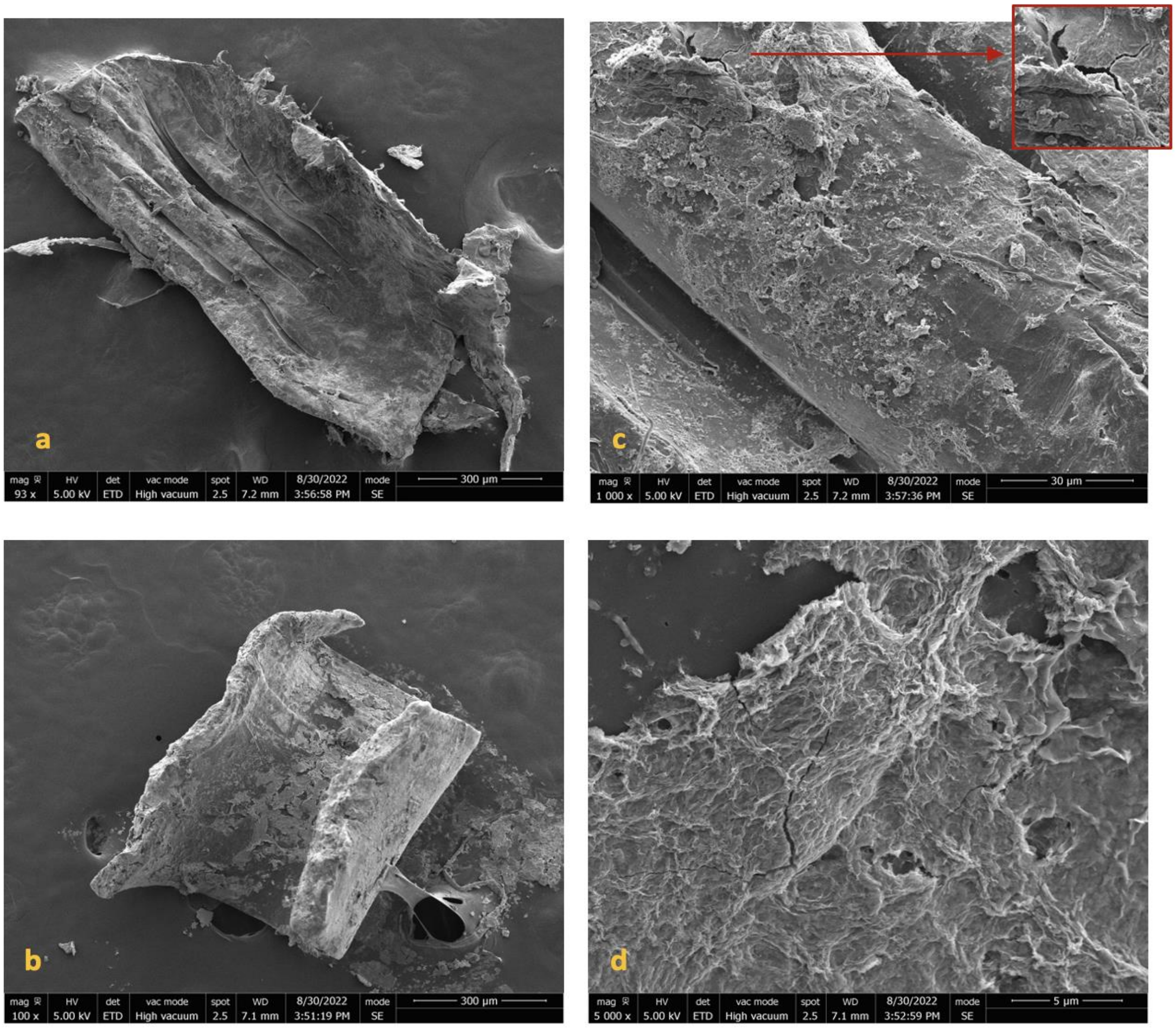
Exposure period (d)	7		14		21		28	
Treatment	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA	ROS (Intensity mg^{-1} protein)	% tail DNA
control	160 ± 10	2.8 ± 0.48	162 ± 10	5.1 ± 0.38	184 ± 12	4.3 ± 0.98	197 ± 13	4.6 ± 0.44
PE-S	299 ± 79	7.4 ± 0.46	378 ± 44	11.0 ± 0.62	194 ± 19	10.9 ± 2.98	284 ± 36	18.5 ± 2.18
PS-S	260 ± 50	6.2 ± 1.56	410 ± 95	11.0 ± 1.34	216 ± 8	13.2 ± 3.02	197 ± 43	17.5 ± 1.40
PE-L	276 ± 57	5.0 ± 0.76	222 ± 15	7.3 ± 0.75	293 ± 6	12.8 ± 0.57	211 ± 15	13.0 ± 2.16
PS-L	404 ± 87	9.4 ± 1.38	253 ± 44	7.2 ± 0.40	334 ± 46	10.8 ± 0.71	384 ± 51	13.9 ± 2.12
Cu-PE-S	411 ± 83	8.1 ± 0.88	433 ± 34	11.9 ± 0.44	457 ± 4	18.7 ± 5.28	335 ± 68	17.9 ± 2.99
Cu-PE-L	343 ± 33	9.6 ± 1.86	390 ± 31	14.2 ± 0.80	298 ± 63	17.6 ± 3.92	341 ± 52	18.5 ± 3.93
Cu-PS-S	297 ± 24	11.5 ± 0.81	306 ± 16	11.8 ± 0.59	347 ± 30	15.9 ± 1.04	250 ± 28	13.5 ± 2.38
Cu-PS-L	320 ± 83	9.3 ± 1.71	257 ± 43	12.8 ± 1.11	494 ± 13	11.8 ± 4.39	291 ± 25	13.0 ± 1.94
Cu	386 ± 48	10.9 ± 1.03	268 ± 16	13.6 ± 1.93	210 ± 10	15.6 ± 0.82	281 ± 14	14.9 ± 0.61



Appendix 4.5 Time-series analysis of catalase activities recorded on day 4, 14, 21, and 28 in earthworm *E. fetida* exposed to pristine and aged copper-spiked polyethylene and polystyrene microplastics (0.75 g kg soil^{-1}, spiked nominally with 100 mg Cu l^{-1}) in artificial soil. Catalase activity is presented as relative quantification compared to control ± SE from three replicates. Letters indicate significant differences between treatments. (*) indicate significance compared to the control. Significant differences were determined using ANOVA followed by LSD post-hoc test ($p < 0.05$).

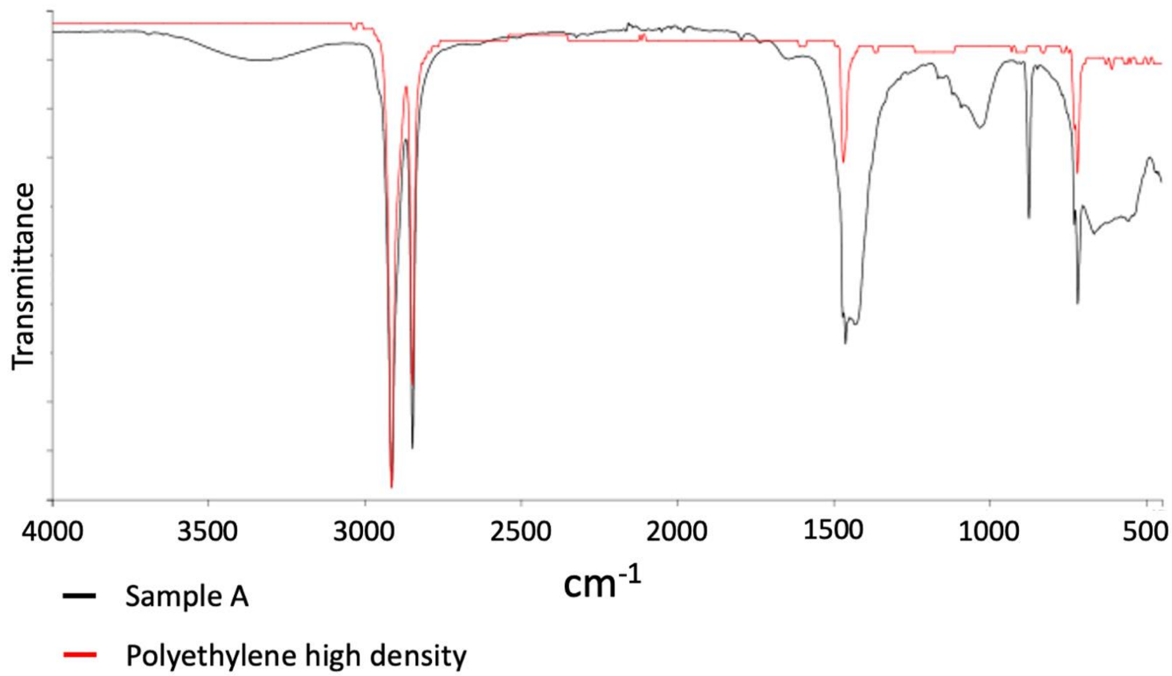
Appendix 4.6 Measured copper concentrations in soil and in the earthworm *E. fetida* exposed to pristine and aged copper-spiked polyethylene and polystyrene microplastics (0.75 g kg soil^{-1}, spiked nominally with 100 mg Cu l^{-1}) in artificial soil. Data presented as mean ± SE (n=3), BDL: below detection limit.

Treatment	**Cu (μg g^{-1}) Whole earthworm**				**Cu (μg g^{-1}) Soil**
	Day 7	Day 14	Day 21	Day 28	Day 28
control	BDL	BDL	BDL	BDL	3.99 ± 0.17
PE-S	BDL	BDL	BDL	BDL	4.17 ± 0.35
PS-S	BDL	BDL	BDL	BDL	4.13 ± 0.21
PE-L	BDL	BDL	BDL	BDL	3.73 ± 0.15
PS-L	BDL	BDL	BDL	BDL	3.89 ± 0.46
Cu-PE-S	BDL	BDL	BDL	BDL	6.07 ± 0.09
Cu-PE-L	BDL	BDL	BDL	BDL	9.33 ± 0.35
Cu-PS-S	BDL	BDL	BDL	BDL	4.84 ± 0.26
Cu-PS-L	BDL	BDL	BDL	BDL	5.04 ± 0.13
Cu	1.52 ± 0.003	2.69 ± 0.10	1.19 ± 0.03	1.56 ± 0.01	75.27 ± 0.93



Appendix 5.1 Surface features of microplastics extracted from deinking biosolids using the density separation protocol and analyzed using scanning electron microscopy (SEM) (a, b) showing ageing characteristics (c, d). The NaCl floatation solution with a density of 1.50 g cm^{-3}, exceeded the density of most common plastic polymers, which typically range from 0.015–1.50 g cm^{-3} (Han et al., 2019). A total of 200 g of biosolids was added to the floatation solution, and the beaker was placed in an orbital shaker at 160 rpm for 1 hr. After shaking, the biosolids were kept overnight to settle at the bottom, and the water layer on top was collected and filtered using 0.45-micron filter. The foreign bodies extracted from the solution were collected. The

microplastics extracted from deinking biosolids were analyzed for surface characteristics using SEM - FEI Quanta 450 (Thermo Fisher Scientific, Waltham, MA, USA).



Appendix 5.2 Fourier Transform Infrared Spectroscopy analysis of a foreign body extracted from deinking biosolids using the density separation protocol. The extracted samples were analyzed using FTIR PE Spectrum II spectrometer (PerkinElmer, Inc., Waltham, MA, USA) and put against an open-source database of spectra to validate the presence and to determine the types of microplastics found in deinking biosolids. The database search revealed 85% similarity of sample A to polyethylene high density, with spectral similarity at the C-H, and the C-C peaks. A shift in transmittance was noted, likely due to comparing samples extracted from deinking biosolids to pristine polymers in the database. This preliminary analysis validated the presence of microplastics in deinking biosolids.