Study on the Influence of Polystyrene Nanoplastics on the Toxicity of Glyphosate in *Eisenia fetida*

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in

Environmental Sciences

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It is to certify that the research work presented in this thesis, entitled **"Study on the Influence of Polystyrene Nanoplastics on the Toxicity of Glyphosate in *Eisenia fetida*"** was conducted by **Ms. Safa Zahoor (Reg. No. 02312013016)** under the supervision of **Dr. Mazhar Iqbal Zafar**. No part of this thesis has been submitted else for any other degree. This thesis is submitted to the Department of Environmental Sciences, in the partial fulfillment of the requirements for the degree of Master of philosophy in the field of Environmental Science, Quaid-i-Azam University, Islamabad.

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DEDICATION

It is my genuine gratefulness and warmest regards that I dedicate this research work to my loving, caring, supporting and hard-working parents, Father Mr. Zahoor Hussain, mother Shahnaz Akhter and my siblings. Their faith, endless love, support and encouragement made me able to complete this study. Without their helping hands, support and encouragement, it was impossible to accomplish my studies.

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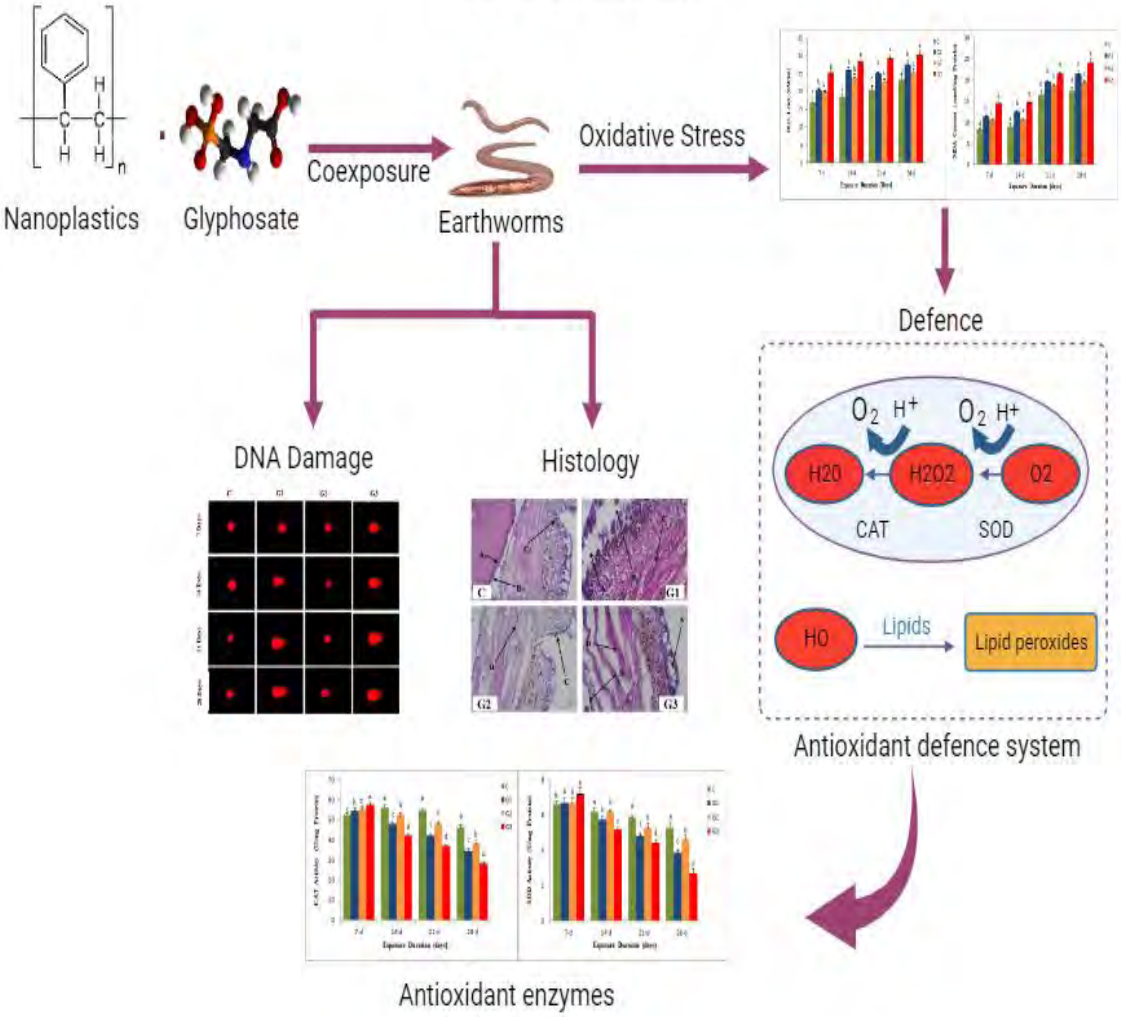
Abbreviations	Stands for
AChE	Acetylcholinesterase
AI	Acetylcholine iodide
AMPA	Aminomethyl phosphoric acid
BSA	Bovine serum albumin
BTCs	Breakthrough curves
CAT	Catalase
CNS	Central nervous system
DEPPD	N-Diethyl-p-phenylenediamine sulphate salt
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPSP	5-enolpyruvylshikimate-3-phosphate
EU	European Union
FAO	Food and agriculture organization
GLY	Glyphosate
H_2O_2	Hydroxyl radicals
ISO	International Organization for Standardization
L×W×H	Length ×Width× Height
LMA	Low melting agrose
LPO	Lipid peroxidation
MDA	Malondialdehyde
MPs	Microplastics
N P K	Nitrogen, phosphorus, potassium
$Na_2H_2PO_4$	Monobasic sat
Na_2HPO_4	Di basic anhydrous salt
NaOH	Sodium hydroxide
NBC	Nitrotetrazolium blue chloride
NMA	Normal melting agrose
OECD	Organization for economic co-operation and development
PAHs	Polycyclic aromatic hydrocarbons

PBS	Phosphate buffer saline
PSNPs	Polystyrene nanoplastics
PVC	Polyvinyl chloride
Radical O^{2-}	Superoxide anion
Radical OH	Hydroxyl radicals
ROS	Reactive oxygen species
SCGE	Single-cell gel electrophoresis
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TBA	Thiobarbituric acid
TM	Tail moment
WHC	Water holding capacity
ZnO NPs	Zinc oxide nanoparticles

HIGHLIGHTS

- The study investigated the separate and combined effects of nanoplatics and glyphosate on earthworms.
- Acetylcholinesterase activity was inhibited in brain tissues of earthworms by combined exposure of nanoplatics and glyphosate.
- Reactive oxygen species and Malondialdehyde content increased with increasing the time period.
- Antioxidant enzymes (SOD and CAT) are significantly reduced after the exposure of nanoplatics and glyphosate.
- Combined exposure of both contaminants showed the increase in DNA damage of earthworms.
- Histopathological alterations included enlarged cell space, cell lysis and irregular nucleus size were observed across all sampling intervals.
- These findings may provide valuable insights for future studies on the toxicological effects of nanoplastics and glyphosate in soil ecosystems, enhancing our understanding of their potential interactions with other contaminants.

Graphical Abstract



ABSTRACT

Background: The widespread presence of microplastics/nanoplastics (MPs/NPs) in both aquatic and terrestrial ecosystems, along with their associated ecological consequences, has gained worldwide attention. Nanoplastics (NPs) have a broad distribution across diverse environments, including soil, and are recognized for their harmful effects on soil organisms. Nanoplastics have garnered worldwide attention owing to their extensive presence in the environment and their capacity to penetrate biological barriers. Glyphosate (GLY) stands out as the most extensively utilized organophosphorus herbicide in agriculture. The simultaneous presence of microplastics/nanoplastics alongside other environmental pollutants has prompted an increased emphasis on their co-occurrence and interactive toxicity. Nanoplastics and glyphosate often coexist within the soil environment. The primary objective of this study was to investigate the extensive toxicological impacts of NPs and GLY on earthworms. **Methodology:** This study attempted to explore the chronic impacts arising from the separate and combined exposures of polystyrene nanoplastics (PSNPs) concentration (12.5 mg/kg) and glyphosate (GLY) concentration (26 mg/kg) on the earthworms over a period of 28 days. Earthworms are highly important for soil health and functioning. In a 28 day chronic toxicity test, 300 earthworms were exposed to different concentrations of nanoplastics and glyphosate over 7, 14, 21, and 28 days, divided into three treatment groups and a control group. Samples were collected at 7-day intervals, with 11 earthworms sacrificed per treatment. Tissues from the brain, intestine, and other organs were stored for analysis, and histopathology was examined using formalin-preserved intestinal tissues. The experiment was conducted in triplicates using natural soil as the substrate. Biochemical parameters, including AChE activity, ROS, MDA, antioxidant enzyme levels, DNA damage, and histopathological changes, were evaluated.

Results: The activity of acetylcholinesterase (AChE) in earthworms was suppressed by both nanoplatics and glyphosate, resulting in an excessive accumulation of acetylcholine within the synaptic space ultimately inducing neurotoxic effects. The combined treatment resulted in a greater inhibition of AChE compared to the individual treatments. Exposure to nanoplatics and glyphosate can initiate a process where the production of reactive oxygen species (ROS) triggers the release of more ROS within the earthworm, and increased malondialdehyde content (MDA). The combined exposure to nanoplatics and glyphosate led to increased levels of ROS and MDA content at all sampling intervals compared to individual exposures. Antioxidant enzymes (SOD and CAT) activity decreased significantly with increasing exposure time. The

combined exposure to nanoplatics and glyphosate significantly reduced the activity of SOD and CAT compared to the individual groups. Nanoplatics and glyphosate caused DNA damage by a substantial increase in tail moment and tail length at all sampling intervals by combined exposure of glyphosate and nanoplatics as compared to individual treatments. Histopathological alterations in intestine were most significant and caused irregular cell shape, enlarge nucleus and cell lysis. The combined exposure to nanoplatics and glyphosate results in more pronounced alterations than the individual treatments.

Conclusion: These pollutants disrupt the integrity of the intestinal cell membrane in earthworms. This study revealed that earthworms exposed to specified doses of nanoplastics and glyphosate exhibit synergistic effects. Furthermore, this study contributes toxicity data relevant to soil invertebrates and nontarget terrestrial organisms.

Keywords: Earthworms, Polystyrene nanoplastics, Glyphosate, Co-occurrence, Coexposure, Oxidative stress, DNA damage

Chapter 1

Introduction and Literature Review

1. INTRODUCTION

1.1. Unveiling the Sources of Plastics

Concern over the prevalence and permanence of plastic contamination in the environment is growing among scientists and the general population. The global production of plastic is projected to reach 33 billion tons by 2050, showing a significant increase from the 359 million tons recorded in 2018 and 2019 (Plastics Europe). Over time, the plastic debris breaks down into microscopic fragments that are typically classed as macroplastics (>2cm), mesoplastics (5-10mm), microplastics (>5 mm), and nano plastics (<1 mm) based on their size (Gigault et al., 2018; Blettler et al., 2017). Plastics' malleability enables the production of solid objects in a variety of forms and dimensions. Different synthetic or partially synthetic organic polymers are used to create them. Plastics have been employed in a variety of products due to their adaptability, strength, and affordability.

Road litter, illegal solid waste dumping, landfills from municipalities and industry, wastewater irrigation, home sewage, and mulching (an agricultural practice) are the main contributors to plastic pollution (Peng et al., 2019). Discharged plastics are continually broken down by mechanical processes, particularly in oceans, until they are reduced to micro and nanoparticle levels (Enfrin et al., 2020). According to nanoplastics (NPs) has a minimum dimension of 100 nm, while microplastics (MPs) have a minimum dimension of less than 5 mm (Costa et al.,2019; NOAA et al., 2015). The landfill's soil can store large amounts of MPs and NPs and act as a long-term sink for plastic (Huang et al., 2019). Soil is especially susceptible to MP/NPs pollution because it is the base of terrestrial ecosystems and one of the most crucial resources for human society (Nielsen et al., 2015). For instance, only 0.4% of polypropylene (PP) deteriorated in soil after one year (Teuten et al., 2009), whereas polyvinyl chloride (PVC) and polystyrene (PS) showed no evident signs of degradation for over 32 years (Zhu et al., 2019).

1.2. Tracing the Sources of Nanoplastics Contamination

Nanoplastics are also described as particles with a size range of 1 to 1000 nm that are created and released during the breakdown of industrial plastics (Gigault et al., 2018). Additionally, items containing NPs, such as nanomedicine and personal care products, have the potential to release NPs directly into the environment (Hernandez et al., 2017).

Nanoplastics has been discovered in the environment recently due to the advancement of innovative detecting technology (Foetisch et al., 2021, Wahl et al., 2021). According to estimates, the environmental level of NPs is 17 orders of magnitude greater than that of microplastics, and it is on the rise over time (Besseling et al., 2019). Due to their high abundance and mobility, smaller plastic particles like MPs and NPs might be more hazardous (Qi et al., 2020; Law and Thompson, 2014). Understanding the fate and effects of MPs/NPs in soil ecosystems with various types of organisms is therefore crucial (Wang et al., 2020a; Caruso, 2019; Horton et al., 2017). Given their size, soil fauna may consume microplastics and nanoplastics while ingesting them (Huerta Lwanga et al., 2016; Sussarellu et al., 2016; Wright et al., 2013; Graham and Thompson 2009; Browne et al., 2008).

As a result, concern over the ecological impact that NPs pose has grown in recent years (Gaylarde et al., 2021). Various studies have shown that due to their high surface-to-volume ratio, nanoplastics may be more harmful than MPs in a number of organisms, powerful penetrating abilities, and inherent hydrophobicity (Yang et al., 2020; Chen et al., 2017). There are only a few studies that have looked at the mobility of microplastics and nanoplastics in natural soil systems (O'Connor et al., 2019; Quevedo and Tufenkji, 2012). Polystyrene nanoplastics (PSNPs) were chosen as a representative model NPs because they are widespread and cover 90% of the demand for plastic (Yang et al., 2016). For instance, the breakthrough curves (BTCs) for polystyrene nanoparticles had the shape typical of "blocking," where the filling of favorable retention sites resulted in a drop in the deposition rate and a rise in the effluent particle concentration with time. For instance, Quevedo and Tufenkji (2012) discovered that certain soil components may help explain why polystyrene nanoparticles are retained more in loamy sand than in quartz sand.

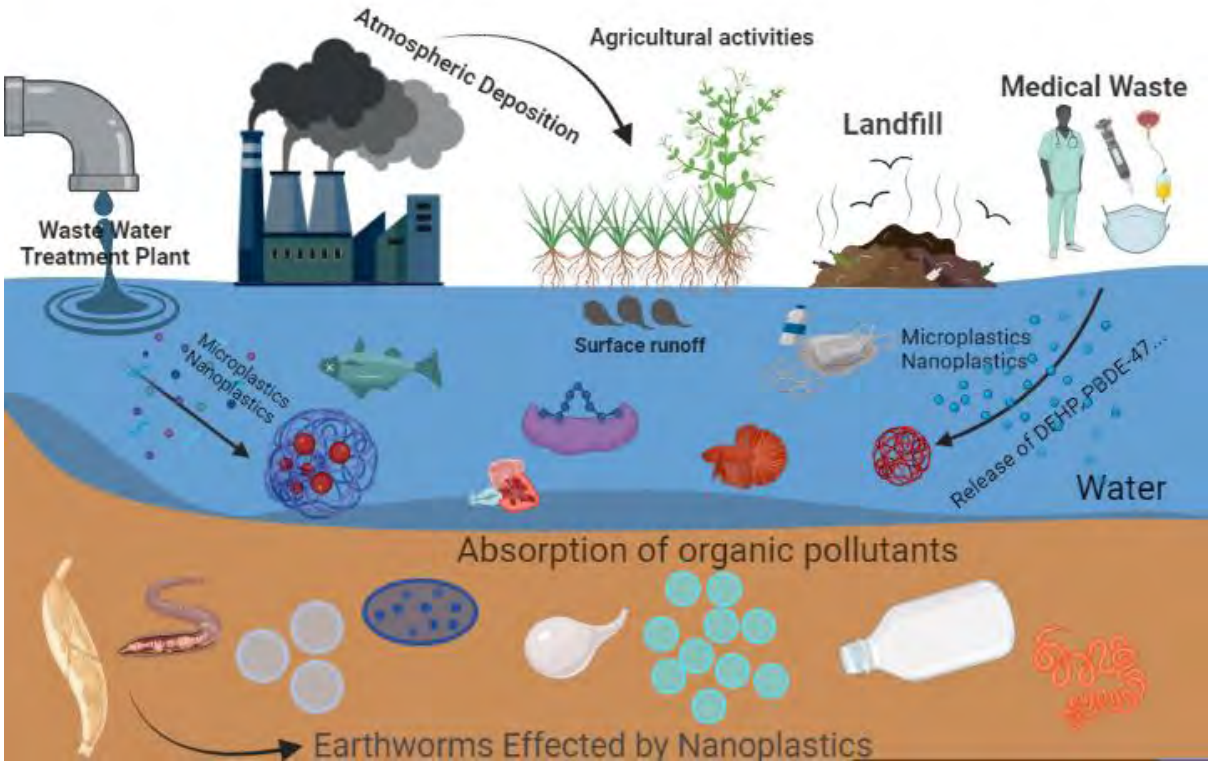


Figure 1: Pathways of Plastic Pollution in the Environment

1.1.1. Synthesis of Polystyrene and their uses

When styrene monomers are polymerized, an aromatic polymer called polystyrene is created. Styrofoam, a substance with limited elasticity that is stretched or melt-formed, is likewise made using polystyrene. It is made by quickly heating polystyrene pellets with a foaming agent. Polystyrene is an aromatic polymer formed as a result of polymerization of styrene monomers. Food containers including trays, plates, and cups are frequently made out of Styrofoam. Additionally, it is employed in the transportation and storage of food, as well as in the manufacture of toys, office supplies, and a variety of packaging materials (Domininghaus et al., 2005; Johannaber and Michaeli, 2004). In addition, in Tamar Estuary in South England, polystyrene was discovered to be the second most prevalent kind (25%) of floating plastic trash (Sadri and Thompson, 2014).

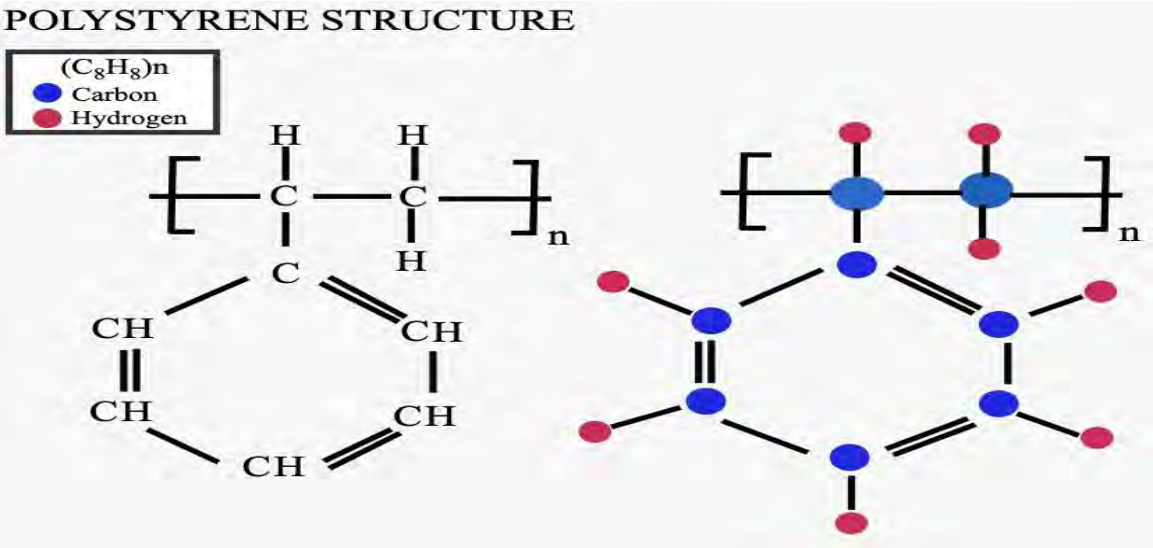


Figure 2: Structure of polystyrene

Table 1: Properties of Polystyrene

Chemical Formula	**Polystyrene $(C_8H_8)_n$**
Molecular weight	104.1 g/mol
Density	1.04 g/cm^3
Solubility in water	Insoluble
Melting Point	Approx. 240 °C

1.1.2. Partitioning of Nanoplastics in soil

When carried both vertically and horizontally into soils, micro/nanoplastics exhibit very dynamic behavior. The soil's characteristics, water infiltration, human activity, and interactions with the soil fauna all have an impact on the dynamic transport of micro- and nanoplatics (Pignattelli et al., 2020; Ng et al., 2018). On the other hand, soil micro/nanoplastics can be divided into several soil matrices in either a solid or liquid phase. These comprise soil fragments, soil organic components that mimic natural fragments, soil fauna, and pore waters for the solid phase and liquid phase, respectively. The amount of organic matter in that specific compartment can be used to understand the relative importance of a certain environmental matrix for the transport of organic molecules, such as hydrophobic organic compounds (Koelmans et al., 2016). Hydrophobic and electrostatic interactions which causes micro/nano

plastics to bind to soil particle surfaces. Due to the high surface-to-volume ratio, surface-to-volume creates as particle size decreases. So, it stands to reason that nanoplastics should adhere to soil particles' surfaces more firmly than microplastics. The rate at which nanoplastics absorb from the surface of soil particles may be slowed by this solid binding (Amity et al., 2022).

1.1.3. Toxicological effects of Nanoplastics

Nanoplatics particles can absorb chemicals from water (heavy metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, etc.), which may exacerbate the negative effects of nanoplastics on living things (Koelmans et al., 2016). Nanoplastics represent potential risks to ecological and human health due to their small size, distinct properties, and persistence in the environment. Marine species can easily consume them because of this (Peng et al., 2020; Shen et al., 2019; Revel et al., 2018; Lu et al., 2016; Cole et al., 2015). Smaller particles can pass through cell membranes, move through tissues, and also pass through trophic levels. Ingesting or breathing nanoplastics (NPs) can clog the digestive tract, cause false satiety, or disrupt the metabolism (Khosrovyan et al., 2020; Liu et al., 2019).

They can permeate biological membranes, cells, or tissue and cause harmful effects such as alterations in membrane permeability, inflammation, oxidative stress, a decline in enzyme activity, etc. (Jeong et al., 2016). More seriously, polystyrene nanoplastics may be able to cross the blood-brain barrier, a highly selective barrier that works through an active transport mechanism mediated by P-glycoprotein to block the entry of potential neurotoxins, and enter the brain tissues, where they could have a profound impact on organisms (Kashiwada, 2006).

Other studies have shown that nanoplastics have the capacity to breach the other tissues in mussels (Farrell and Nelson, 2013) or enter the circulatory system (Browne et al., 2008), opening up further pathways that may result in additional harmful consequences. This data was primarily shown in field and laboratory research that investigated marine ecosystems using experimental models like fish, mollusks, crustaceans, and annelids (Sá et al., 2018).

1.1.4. Effect of Nanoplastics on Soil Organisms

Nanoplastics and microplastics can enter the terrestrial environment directly from sources like polymer-coated fertilizers as primary plastic components (Kah et al., 2019). Examples of sources in agricultural systems include soil conditioners (sludge and composts) that include NPs and MPs as well as the breakdown of plastic mulch films (Frehland et al., 2020; Li et al.,

2019; Liu et al., 2018; Yang et al., 2018). An essential part of nutrient cycling, organic matter breakdown, and soil structure are played by the soil oligochaete, *Enchytraeus crypticus*. *E. crypticus* individuals dramatically lost weight after being exposed to 10% nano-polystyrene (dry weight basis), and oligochaete reproduction was hermetically sealed after being exposed to 5% nano-polystyrene. However, soil oligochaete *E. crypticus* mortality remained the same as when it was exposed to nanoplastics which led to reduced weights and stimulus of cocoon production (Zhu et al., 2018).

Earthworms are frequently utilized as bioindicators to determine the level of soil contamination due to their sensitivity to contaminants. ZnO nanoparticles (NPs) are widely utilized in the manufacturing of cosmetics, coatings, pigments, and electronic equipment (Ma et al., 2013). After being exposed to the environment, excess ZnO NPs might disrupt the ecology. Previous research has demonstrated that ZnO NPs can cause a number of harmful effects on several arms, including oxidative stress, damage to DNA and gut cell mitochondria, and Zn bioaccumulation (Li et al., 2011; Hu et al., 2010). Combining exposure to ZnO NPs and polyethylene microplastics resulted in a higher rate of weight loss and a lower fatality rate at day 28 compared to a single application. The earthworms with the higher Zn concentration (55.6 mg/kg) were also subjected to 1 percent PE and 1000 mg/kg ZnO NPs. In conclusion, co-exposure to PE MPs (Polyethylene Microplastics) and ZnO NPs worsened toxicological reactions and elevated Zn bioaccumulation in earthworms, which results the great ecological issues (Zhang et al., 2022).

1.1.5. Effect of Nanoplastics in Aquatic Organisms

Zebrafish have served as a valuable research model for human contamination, developmental studies, and conventional toxicity testing over the past few years (Lleras-Forero et al., 2020; Pereira et al., 2019; Zang et al., 2018; Deveau et al., 2017; Garcia et al., 2016; Shimada et al., 2014; Dai et al., 2014; Tat et al., 2013;). Consider the specifics: It is projected that 8 million tonnes of plastic trash are discarded into the ocean annually, and by 2030, this amount of plastic will have increased (Choong et al., 2021). Thus, for the normal disposal of micro/nanoplastics, the oceanic system has the potential to be act as significant pollutant sink.

In zebrafish intestines, exposure to PSNPs at environmental relevant doses (1–100μ g/L) led to altered microbiome composition and function as well as immune response dysfunction. The changed neurotransmitter metabolites and the activity of the zebrafish intestinal microbiota in

response to PSNPs make it obvious that the fundamental causes of this toxicity involve a modification of the brain-gut axis interaction (Teng et al., 2022). The central nervous system (CNS) and locomotor activity in zebrafish embryos are negatively impacted by exposure to 50 nm PS NP at a concentration of 1 mg/L (Chen et al., 2017). After a 24-hour exposure, amine-modified 52 nm polystyrene nanoplastics (PS-NH_2) up to a concentration of 25 mg/L were nonlethal to aquatic zooplankton, but at a concentration of 75 mg/L, mortality became apparent within 13 hours (Mattsson et al., 2017).

On the other hand, research on freshwater organisms is sparse and primarily concentrated on crabs of the genus *Daphnia*, which includes species that are model organisms in ecotoxicology (Liu et al., 2020, Zhang et al., 2020). *D. Magna* serves as a vital connection between producers and high-trophic consumers in the aquatic ecosystem (Chae et al., 2018). Previous research has demonstrated that *D. Magna* may consume nano- and microplastic particles with sizes ranging from 20 nm to 5 mm and that *D. Magna* exhibit lower reproduction after 21 days of exposure to 70 nm polystyrene particles (Besseling et al., 2014). *D. Magna's* reproduction and body size were also adversely affected by exposure to 70 nm polystyrene particles at doses of 0.22 and 103 mg/L.

Table 2: Review of Study of Nanoplastics on Terrestrial and Aquatic Organisms.

Type of Nanoplastics	Organisms	Exposed Dose	Toxicological Effects	References
Polystyrene	Earthworms (*Eisenia Fetida*)	10, 100 mg/kg	Severe oxidative damage and disturb immune balance	Xu et al., 2021
Polystyrene Aqueous suspension	Earthworms (*Eisenia Fetida)*	250,500mg/kg 10g/kg	Reduced the weight, effect on cocoon production	Zhu et al.,2018
Fluorescent polystyrene nanoplastics	Zebra Fish (*Danio rerio*)	0.04, 34 ng/L 34µ/L	Genotoxic damage, break DNA strand and mutagenic changes	Besseling et al., 2014
Polystyrene beads Aqueous suspension	*Daphnia magna*	0.22−150 mg nano-PS/L	Reduced weight and severe alteration in reproduction	Besseling et al., 2014
Polystyrene	Zebra Fish (*Danio rerio)*	1, 10, and 100 µg/L PSNPs	Histopathological changes, DNA damage	Torres-Ruiz, et al., 2021

1.2. An Overview of Pesticides

Due to the extensive use of organic pesticides in agriculture, soil pollution has recently increased (Kavitha et al., 2020). More consideration is being given to the extensive application of pesticides, their potential effects on the environment, and the risks they pose to the stability of ecosystems (Bishop et al.2020; Utami et al., 2020; Fang et al. 2019). Particularly in the past decade, the pesticide business has expanded dramatically in tropical areas (Andersson and Isgren, 2021; Goulson, 2020). The soils from conventional agriculture contained more chemicals in higher concentrations when compared to organic agricultural plots. Despite the fact that residue is even quite low, mixtures of insecticides, herbicides, and fungicides were regularly present in soils (Pelosi et al., 2021).

To manage weeds, pests, and illnesses, pesticides are utilized on a global scale. Few insecticides are effective against the targets; the majority sink into the soil. Pesticide contamination leading to soil degradation is once again in the spotlight (Jiang et al., 2016).

1.2.1. Glyphosate: Origins, Common Uses and Applications

Glyphosate, also known as N-(phosphonomethyl) glycine, functions as the primary active ingredient in a popular non-selective, broad-spectrum, systematic herbicide, (Bai and Ogbourne, 2016). Glyphosate, widely recognized as the most prevalent herbicide on a global scale, experienced a remarkable 15-fold increase in usage from 1996 to 2014 (Benbrook, 2016). This substantial rise in adoption can be attributed to its initial introduction in 1974, becoming the herbicide of choice worldwide, as reported by (Székács and Darvas, 2018).

Mode of Action: Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in plants, disrupting the shikimic acid pathway, (CDS Tomlin et al., 2006) The shikimic acid pathway is exclusive to plants and a few microbes. The absence of this route in mammals may explain glyphosate's low toxicity to non-target organisms. (Wu, Jiunn Yih, et al., 2006). According to an ecological risk assessment of Roundup®, the main hazard to arthropods were changed habitat structure and food availability (JP Giesy et al., 2000).

Global agricultural systems are increasingly using chemical pesticides to control weeds, which poses a major risk to the environment and public health. The escalating utilization of this herbicide has led to it becoming a prominent water pollutant, raising concerns about its environmental impact. The surge in its application has been particularly notable in response to the cultivation of genetically modified crops that exhibit resistance to glyphosate. While traditionally used on woody and herbaceous crops, glyphosate's reach has expanded to non-agricultural areas, including industrial zones, civil areas, embankments, and roadsides, as observed (Paris et al., 2016).

During the past decade, growing apprehensions have emerged regarding the environmental and health repercussions associated with the annual utilization of over 800,000 tonnes of this chemical worldwide and the allowance of more than 750 commercial formulations (Kniss, 2017; EPC EU, 2017; Benbrook, 2016; Message et al., 2015).

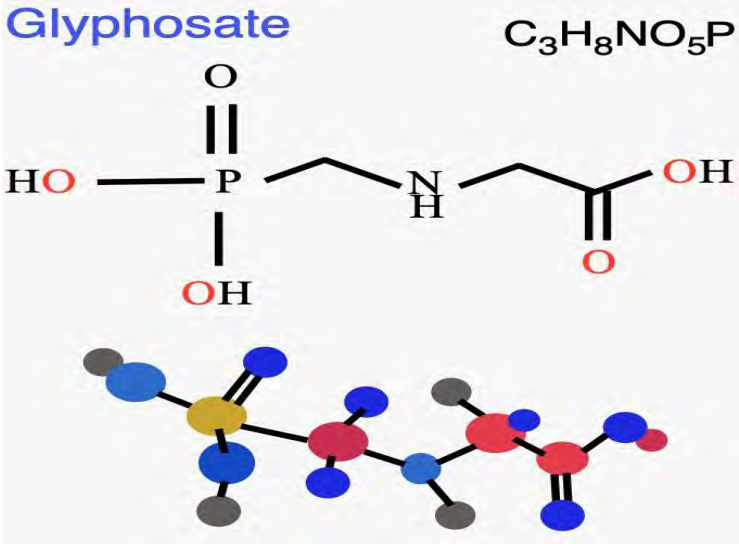


Figure 3: Chemical structure of glyphosate

Aminomethyl phosphoric acid (AMPA)is the main byproduct of the breakdown of glyphosate. As a result, frequent use of glyphosate and glyphosate-based herbicides can strongly spread the active component in the environment and has the potential to contaminate organisms as well as water, soil, and air (Sousa et al., 2019; Sliva et al., 2018; Dollinger et al., 2015). Spray drift allows glyphosate to infiltrate the soil, and heavy rainfall causes it to wash off the surface of the plants. Additionally, it might seep into the soil through glyphosate-sprayed dead and decomposing plants.

Table 3: Properties of Glyphosate

Active Ingredient	**Glyphosate**
Form	Odorless, white solids
Molecular weight (g/mol)	169.07
Half-life in soil (d)	143.3 to 514.9
Solubility in water (mg/L)	pH 1.9: 10,500 pH 7.0: 57,000

1.3.2. Glyphosate Usage Trend in Pakistan

The world's sixth most populous nation is Pakistan. By 2050, Pakistan's population is projected to be 350 million (Planning Commission, 2014; United Nations, 2012). To fulfill the rising demands of Pakistan's expanding population and preserve food security, more food must be produced (Kirby et al., 2017). In order to address this issue, must be boosted beyond what it is now, which will put tremendous strain on the already depleting natural resources (Ali et al., 2017a; Hanif et al., 2017).

In Pakistan, the agro-production industry is heavily infested by parasites and pests, which has a significant negative economic impact. To eliminate pests and parasites and increase crop yield, many corrective procedures have been promoted across the country, including the frequent application of pesticides, insecticides, herbicides, and fungicides (Ghaffar et al., 2020; Hussain et al., 2019).

Effective weed control in Pakistan requires sophisticated weed management methods. Examples of these consist of targeted and site-specific weed management, variable rate soil-applied herbicide treatment (particularly when weed growth is patchy and there is spatial heterogeneity for seed bank and soil qualities), harvest weed seed collection, weed seed destruction by predation and microbial decay, nano-herbicides, and optical spraying technologies (Bajwa et al., 2016; Rashid et al., 2014; Nasim and Shabbir, 2012).Currently, glyphosate-based herbicides are the second most widely used pesticide in urban areas and the most widely used pesticide in agriculture (Battaglin et al., 2014).

People anticipate that the usage of this herbicide will expand further in the approaching years due to the widespread proliferation of glyphosate-resistant weeds and novel pre-harvest, desiccant-use patterns (such as "green burndown") (Myers et al., 2016). The escalating use of glyphosate in the environment exerts a significant impact on plants, animals, and human populations alike. Glyphosate harms humans by interfering with the endocrine system (Van Bruggen et al., 2018). Glyphosate may be cancer-causing in high quantities, according to preliminary research on substances that cause cancer (Andreotti et al., 2018). The earthworm *(Eisenia fetida)* has been demonstrated to be harmed by glyphosate, although its formulation, roundup, did not exhibit any toxicity towards soil invertebrates (Pochron et al., 2020).

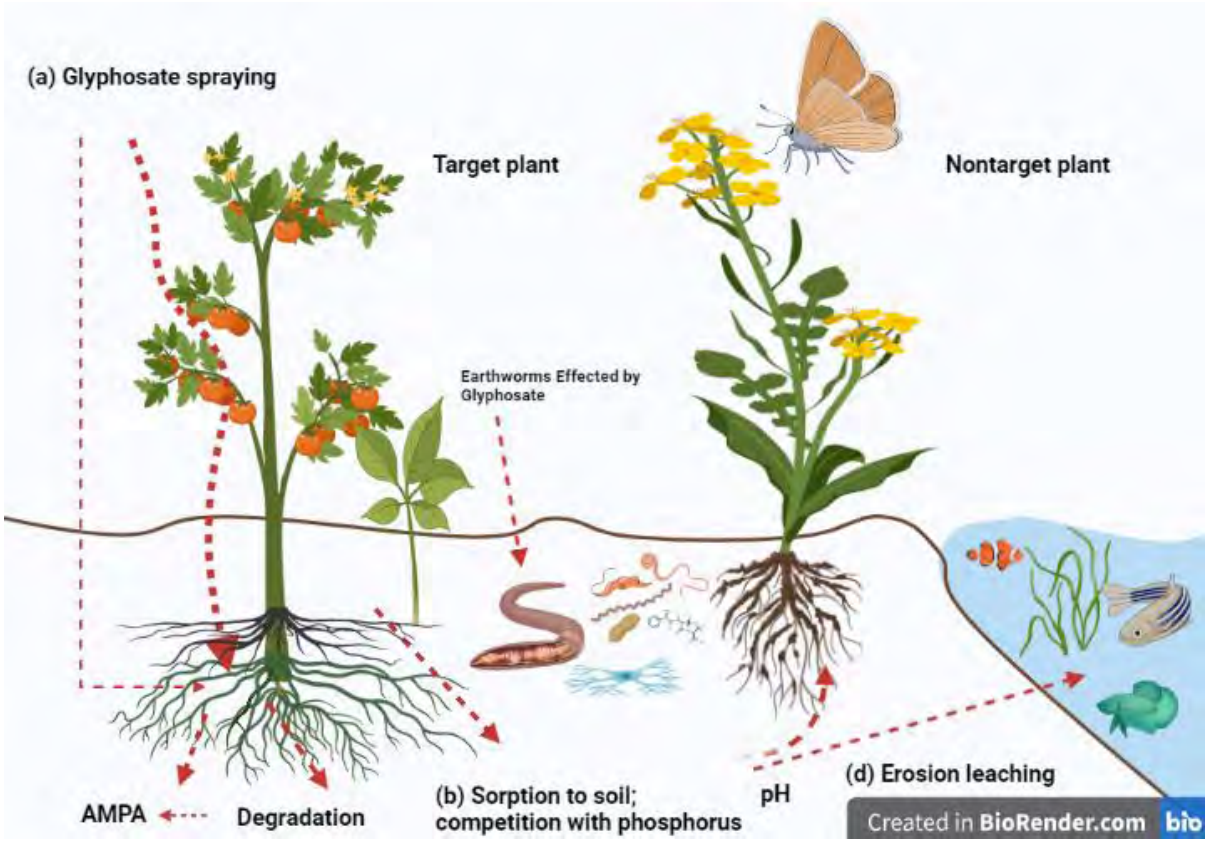


Figure 4: Entry routes of glyphosate in different environmental compartments

1.3.3 Interaction of Glyphosate in Soil

Given the extensive usage of glyphosate, it is imperative to understand how it interacts with soil ecosystem in order to properly apply for its impact on the environment.

Although glyphosatecontaining herbicides are not purposely sprayed into the ground, they may contaminate nearby soils through spray drift during application and after being wiped off of leaf surfaces by rain.

1.3.4 Fate of Glyphosate in Soil

Due to processes like mineralization, degradation, immobilization, and leaching, glyphosate has a complex destiny in soil. In an effort to pinpoint and co-mechanisms that govern how chemicals behave as a source of environmental pollution, particularly in soils and water, numerous research has lately been published. While some experiments employed glyphosate in its acid form, other investigations made use of well-known compounds. This is because glyphosate is discharged into the environment as formed products that also contain co-formulate chemicals (adjuvants) and other additives rather than as pure active components. Recently, analyzed the most prevalent co-formulates in glyphosate-based herbicides that

contain surfactants, and they talked about whether or not the presence of these surfactants, like Triton CG-110, would affect the adsorption, leaching, and mineralization of glyphosate in the soil (Robin et al., 2019).

1.3.5. Half-life in Soil

Depending on the circumstance, glyphosate's half-lives might range from a few days to one or two years (soil, water, and air). Temperature and soil moisture are two edaphic and environmental elements that significantly affect half-life (Silva et al., 2018; Bento et al., 2016; EFSA 2013, 2017). According to the European Food Safety Authority (EFSA), glyphosate and AMPA had half-lives in soils of 143.3 days and 514.9 days, respectively (EFSA, 2013).

1.3.6. Glyphosate Runoff from surface to soil

Direct use of glyphosate results in contamination of the soil and water systems when it spreads (Villamar-Ayala et al., 2019). It comes into contact with the target organism's folio. The weed's broad roots disperse glyphosate deep into the soil. Glyphosate attaches in small amounts to organic matter, clay, and soil particles (Van Bruggen et al., 2018). The widespread use of glyphosate can raise the residual levels in the aquatic environment since it is transmitted into water bodies through runoff. In the United States, the groundwater surface was discovered to have up to 430μg/L of glyphosate at one time (Mahler et al., 2017).

Table 4: Literature Review on the Toxic Effects of Glyphosate on Earthworms

Chemical	Organism	Exposed Dose	NOEC	LC50	Responses	References
Glyphosate	*Eisenia fetida*	1, 10, 100, 500, 1,000 mg/kg	-	-	Decrease in the body weight Loss of cocoons Physical changes was seen in earthworms	(Correia and Moreira, 2010)
Glyphosate	*Eisenia fetida*		-	-	Changes in body weight and their reproduction rate	(Yasmin and D'Souza, 2007)
Glyphosate	*Eisenia fetida*	26.3 mg/kg	-	-	Changes in growth and activity of worms show behavioral alterations	(Verrell and Van Buskirk, 2004)
Glyphosate (Roundup)	*Eisenia fetida*	Glyphosate	>21.3,mg/kg	>5600ppm	-	Pesticides Properties Database (PPDB)
Glyphosate	*Eisenia fetida*		-	>5000ppm	-	Vencill, W. K. (2002). Herbicide handbook (No. Ed. 8). Weed Science Society of America.
Glyphosate	*Eisenia fetida*	>100	-	>5000ppm	Low risk to earthworms	(Hayward and Mallett, 2000)
Glyphosate	*Eisenia fetida*	21.31mg/kg dry soil	-	-	No significant effect on earthworms reproduction	(Hayward and Mallett, 2000)

1.3.7 Toxicity of Glyphosate on soil organisms

Pesticides and other agricultural chemicals, as well as harmful pollutants in fertilizers, can have an impact on earthworms (Yancheng et al., 2016; Nicolas et al., 2014). Studies have shown that glyphosate has harmful consequences for earthworms, some of which include a decrease in sperm quantity *in Lumbricus terrestris* and stunted growth in *Eisenia fetida* (Sherwin et al., 2013).

Nemathelminths (Nematodes) are an important group of lesser invertebrates. Although they are parasitic, their diversity, widespread occurrence, and relationship to soil reveal the health of the ecosystem. Even while some nematodes spread diseases, they also contribute to the diversity of natural ecosystems and the maintenance of the earth's nutrient cycle (Achiorno et al., 2008). The nematodes have also been impacted by the usage of glyphosate in soil systems and cause numerous harmful effects.

Eisenia fetida

Nemathelminths

1.3.8. Harmful effects of Glyphosate on Aquatic Life

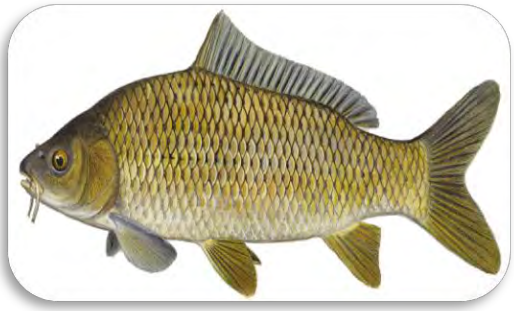
Water sources have been found to contain quantifiable amounts of glyphosate, the herbicide's active ingredient, as a result of surface runoff and careless use (Annett et al., 2014). Fish play a crucial role in preserving the health of the aquatic ecosystem as essential consumers from the start of the aquatic food chain (Liu et al., 2013).

According to Hosseinian et al. (2017), the common carp *(Cyprinus Carpio* L.*)* are an essential freshwater fish that are found in numerous nations. Furthermore, they are most significant aquatic product consumed in China. In aquatic toxicity, *common carp* have served as animal models to assess the state of the environment's health (Ma et al., 2018; Sehonova et al., 2017).

In order to assess the toxicity of the environmental pollutants in aquatic environments, fish have so frequently been utilized as sentinel animals for aquatic toxicity studies (Ma et al., 2018; Mayson et al., 2016; Ma and Li, 2015; Liu et al., 2013). The immunotoxicity of GLY on *Cyprinus Carpio* L, was preliminary assessed in our earlier studies, and the findings demonstrated that GLY-treatment altered complement 3 and immunoglobulin M contents, lysozyme activities, and some cytokine (interleukin (IL)-1, interferon, and tumor necrosis factor (TNF)-) levels in the liver, kidneys, and spleen of *Cyprinus Carpio L*. and caused histopathological damage in organs (Ma and Li, 2015; Ma et al., 2015a, 2015b).

In the Hawaiian Islands, a genetically and geographically distinct subpopulation of green turtles *(Chelonia mydas* Linnaeus*)* has recovered in numbers over the past forty years (Balzac et al., 2015), yet some nearshore feeding regions have lower somatic growth (Balzac and Calpe, 2004). In the Hawaiian Islands, both the public and private sectors commonly use glyphosate-based herbicides (such as Roundup® and Rodeo®) to eliminate undesirable or invasive vegetation from yards, golf courses, fields, and areas close to freshwater, marine, and anchialine shorelines.

Glyphosate may be entering coastal waters and impacting non-target marine animals, for instance, green turtles, their food sources, and/or their (Gastro-Intestinal) GI bacterial ecosystems, even though as far as we are aware, the Hawaiian Islands' coastal waters have not been examined for its presence.



Cyprinus Carpio L

Chelonia mydas

1.3.9. Earthworms and their importance in the Soil

The "ecosystem engineers" known as earthworms are crucial for preserving the fertility and health of the soil as well as for enhancing soil structure (Singh et al., 2016). The well-known

ecosystem engineer is earthworms. In most ago-ecosystems, earthworms are the most numerous soil invertebrates in terms of biomass, and through their activities, they raise soil microporosity, which improves soil nutrient availability and boosts soil water dynamics and aeration (Nico et al., 2020).

They alter soil porosity, bulk density, water infiltration, gas emission, mineralization of nutrients, and plant growth by consuming soil and litter, burrowing both horizontally and vertically through soils, and releasing fecal contents to mix nutrients in soils (Blouin et al., 2013). While earthworms are usually believed to be common in all forest, grassland, agricultural, and garden habitats worldwide, a comprehensive picture of their global distribution is still being put together (Phillips et al., 2019).

The vital role of earthworms in maintaining soil health is acknowledged to such an extent that esteemed organizations like the European Union (EU), the Organization for Economic Co-operation and Development (OECD), the International Organization for Standardization (ISO), and the Food and Agriculture Organization of the United Nations (FAO) have adopted earthworms (*Eisenia fetida*) as indicator organisms for ecotoxicological testing, as evidenced by (Santadino et al., 2014, Piola et al., 2013).

***Eisenia fetida* (Earthworm)**

Table 5: Classification of Earthworms

Kingdom	**Animalia**
Phylum	Annelida
Class	Oligochaeta
Order	Haplotaxiaa
Family	Lumbricidae
Genus	*Eisenia fetida*
Species	*Eisenia fetida*

1.2.2. Significances of Earthworms

By altering soil structures and controlling the structures and activities of the soil's bacterial community, earthworms play a significant part in the cycling of nutrients in the soil (Huang and Xia, 2018; Hoang et al., 2016; Gomez-Brandon et al., 2011). According to research, earthworms were observed to be helpful in the elimination of organic pollutants such as antibiotics, insecticides, and polycyclic aromatic hydrocarbons (PAHs) (Hao et al., 2018; Contreras-Ramos et al., 2008). The ability of soil communities to adapt to the effects of climate warming can also be determined through the presence of earthworms (Lubbers et al., 2013; Eisenhauer et al., 2012).

1.2.3. Ecological divisions/Functional groups

The three ecological groups into which earthworms can be classified are based on their feeding and burrowing activities. While endogenic earthworms live in mineral soils and eat organic matter that has been degraded as well as microbes and minerals, epigenetic earthworms live and genetic surface litter. Anemic earthworms consume leaf litter near the soil's surface despite residing in deep vertical tunnels (Briones et al., 2005). However, as the earthworm invasion advances, the relative importance of various earthworm ecological groups changes (Wicket et al., 2018; Hale et al., 2005a), demonstrating that the soil depth and invasion history play a significant role in the ecosystem repercussions of the invasion.

1.3. Interactive Effect of Nanoplastics and Glyphosate on *Daphnia magna*

Daphnia magna: The toxicity of glyphosate (GLY) and polystyrene nanoplatics (PSNPs) individually and collectively was studied in relation to the multigenerational effects on the freshwater crustacean *Daphnia magna*. When GLY and PSNPs are present together, both their combined and individual toxicities are raised. This suggests a synergistic relationship between the mixture's constituent parts, particularly when GLY and PSNPs are exposed together in higher equitoxic doses. The PSNPs and GLY combination increased immobility and ROS production while lowering swimming activity. It is significant to note that additional research is required to increase our understanding of the impact of these pollutants in the environment, in order to undertake a more accurate assessment, it is necessary to test complicated mixes on non-target creatures, particularly on non-target organisms. This will help us better comprehend the joint toxicity of GLY and PSNPs in a mixture (Diego et al., 2022).

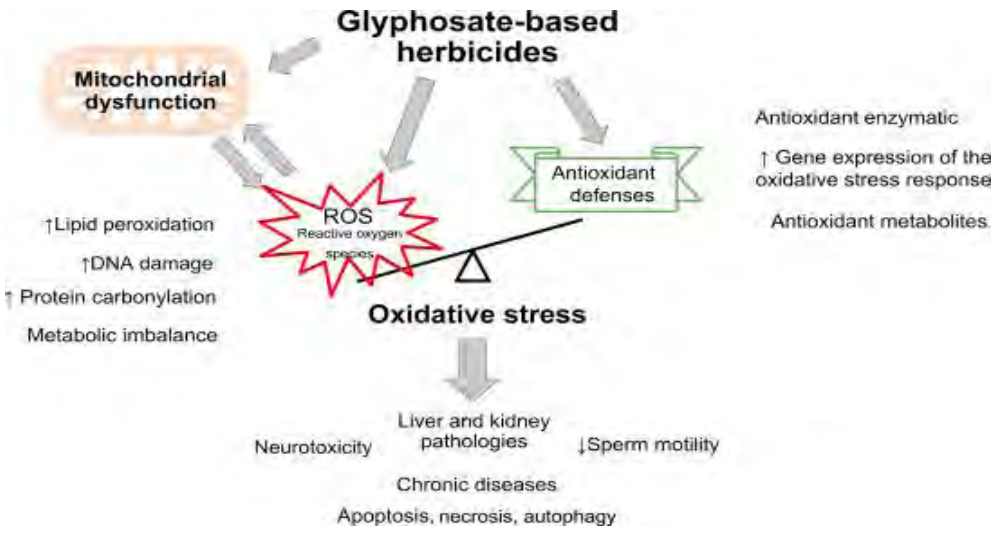


Figure 5: Glyphosate can enter to the cells and cause cellular damage through various pathways (Kronberg et al., 2021).

1.3 Toxicity Biomarkers

1.2.4. Acetylcholinesterase

Acetylcholinesterase (AChE), a critical neurotransmitter, is involved in a variety of central nervous system processes. By catalysing the hydrolysis of acetylcholine in synaptic connections, this enzyme facilitates in the transmission of brain messages from one cholinergic neuron to the next (Soreq and Seidman, 2001). Acetylcholinesterase (AChE), which is involved in neurotransmission and muscular activity (Hackenberger et al., 2018; Wang et al., 2015), is

one of the most investigated earthworm biomarkers in ecotoxicological assessment. Tetania, paralysis, and even death can ensue from AChE inhibition, which alters brain behavior and activates muscle fibers (Kirby et al., 2000).

1.6. Oxidative Stress and Antioxidant Enzymes

Biochemical reactions within organisms frequently serve as early indicators of chemical toxicity and aid in diagnosing soil pollution (Song et al., 2009). Biomarkers encompass quantifiable biochemical reactions in response to pollutants, serving as indicators of both exposure and effects (Lammertyn et al., 2021). They offer a suitable framework to investigate the stress responses of earthworms subjected to foreign substances and to comprehend pollutant mechanisms (Shi et al., 2017). A multitude of these biomarkers have been developed in earthworms across various levels, encompassing parameters such as growth inhibition rate, reproduction rate, levels of reactive oxygen species (ROS), antioxidant enzyme activity, lipid peroxidation levels (Shi et al., 2017).

Commonly recognized as the primary enzyme guarding against reactive oxygen species and lipid peroxidation, SOD plays a pivotal role (Wang et al., 2011). SOD initiates the transformation of the hazardous O_2 free radical into a less detrimental variant by facilitating dismutation, resulting in the formation of H_2O_2 (Halliwell and Gutteridge, 2015). In collaboration with the CAT enzyme, SOD generates H_2O_2, which then undergoes conversion into water and molecular oxygen. It's established that heightened O_2 production leads to a reduction in CAT activity (Kono and Fridovich, 1982).

1.7. DNA Damage

Numerous chemicals found in polluted water possess the potential to disrupt biological processes, thereby affecting specific populations and entire ecosystems. A pivotal aspect of pollution mitigation through environmental monitoring is assessing the genotoxic potential within surface water. In pursuit of this objective, studies related to environmental biomonitoring propose utilizing DNA damage indicators like strand breaks to effectively gauge the genotoxic impacts of environmental pollutants (Everaats et al., 1998; Felder et al., 1998; Theodorakis and Shugart, 1998).

Assessing genotoxicity and oxidative stress caused by pesticides involves employing DNA damage and alterations in earthworm enzyme activities as biomarkers. Consequently, earthworms serve as valuable indicators for investigating the potential impact of pesticides on soil organisms, offering efficient toxicity data. The presence of surplus reactive oxygen species

(ROS) within organism's triggers lipid peroxidation (LPO), which in turn damages cytomembranes, and induces changes in DNA structures, as observed through the work of (Oliveira et al., 2009).

The single cell gel electrophoresis technique, commonly known as the comet assay, represents one of the methodologies employed to evaluate the genotoxic potential of environmental toxins. This approach enables the visualization of DNA damage or repair processes even at the level of individual cells (Olive et al., 1990). The experiment can be conducted using any cell containing a nucleus and requires only a small cell count (a few hundred).

When compared to several other monitoring techniques, the assay's processing time is shorter, and it incurs lower costs. Ultimately, no other conventional method for environmental impact assessment adequately captures the cumulative genetic toxicity resulting from the assortment of genotoxic contaminants that organisms encounter.

1.8. Histological Alterations

Previous research has highlighted histopathological reactions in earthworms as significant indicators of toxicity (KilIç et al., 2011; Giovanetti et al., 2010; Amaral et al., 2006). Changes in tissue histology, capable of inducing notable modifications in tissue and cell functioning, could serve as indicators of detrimental impacts on organisms. These effects might stem from past or ongoing exposure to toxic substances (Reddy and Rao, 2008).

1.9. Problem Statement

Pakistan is the world's sixth most populous nation. By 2050, Pakistan's population is projected to be 350 million. To fulfill the rising demands of Pakistan's expanding population and preserve food security, more agricultural production is required. Pesticides play an important role in protecting the crops from agricultural pests especially of complex mixtures on non-target organisms, in order to conduct a more accurate assessment. However, excessive use of pesticides with other emerging pollutants like nanoplastics may cause ecological hazards for soil biota. Earthworm is a soil annelid which plays a vital role in improving soil health and are one of the significant organic matter decomposers. They improve the nutrient cycle, soil structure, aeration, infiltration, water flow, and plant growth. Unfortunately, excessive use of pesticides has side-effects on non-targeted soil organisms, even on such eminent ones like earthworms. It is necessary to explore the effect of these emerging pollutants on soil microorganisms to design comprehensive solution to improve environmental health.

1.10. Research Gap

Several studies reported the individual effects of glyphosate and polystyrene nanoplastics on earthworms. However, the effect of fluorescent PSNPs has not been documented yet. Additionally, the combined exposure of GLY and fluorescent PSNPs is still unexplored. Consequently, the purpose of the current study is to evaluate the toxicity of fluorescent PSNPs (separate and combined with GLY) on the essential soil macro organisms; earthworms.

1.11. Objectives

The objectives of present study are assessing the potential impacts of nanoplastics and glyphosate on earthworms under separate and combined treatments on earthworms. Neurotoxicity will be assessed the form of Acetylcholine esterase inhibition. DNA damage will be studied to assess genotoxicity and histological alterations will be studied in intestine tissues.

- To assess the impacts of nanoplastics and glyphosate on Acetylcholinesterase (AChE) activity in brain tissues of earthworm.
- Investigating the effects of nanoplastics, glyphosate and its co-exposure on Reactive Oxygen Species and Malondialdehyde content in earthworms.
- To evaluate the impacts of nanoplatics and glyphosate on Antioxidant enzymes.
- To estimate the effects of glyphosate and nanoplastics on histology of intestine of the earthworms.
- To evaluate the DNA damage in response to nanoplastics, glyphosate and its co-exposure in earthworms by comet assay.

Chapter 2
Materials and Methods

2. MATERIALS AND METHODS

2.1. Chemical and Reagents

Glyphosate (CAS; 1071-83-6, purity: 95%) was purchased from Sigma Aldrich® local dealer. The yellow-green fluorescent polystyrene nanoplastics (Product No. L5155-1Ml, 0.03µm mean particle size) were purchased from Sigma Aldrich®, USA. Nitrotetrazolium blue chloride [CAS Number; 298-83-9, purity; 98%] and Acetylthiocholine iodide [CAS; 1866-15-5, purity: 99.0%] were purchased from Sigma Aldrich® (Germany). Ethylenediamine tetra acetic acid (EDTA) [CAS: 60-00-4, purity: 99%] of Sigma Aldrich, Germany. Hydrogen peroxide [CAS: 7722-84-1] Riboflavin [CAS: 83-88-5, purity;98%] of Merck (Darmstadt, Germany) were used. Other substances that were used were all analytical-grade (AG) compounds and solvents.

2.2. Maintenance of Earthworms

All tests are conducted using adult earthworms (*Eisenia fetida*) with clitella well developed and less than 2 months of age. The average weight of each earthworm is between 200-400mg. Prior to the experiment, earthworms were carefully transported in to the laboratory and acclimatized in the plastic container of 25 ×25 ×25cm (L×W×H) for 30 days. Uncontaminated soil used for the acclimatization were taken through garden area (Natural soil) of the Quaid-i-Azam University, Islamabad, Pakistan. Earthworms were fed with cow dung during acclimatization period. The moisture content of the soil (60 to 80%) were maintained by sprinkling the tap water after every 2 days. Specific conditions were maintained with 12-12 h light and dark period, 20-25 °C temperature of the soil. Movement of the earthworms were observed by placing the toothpicks vertically in the soil randomly in evening. Afterwards, in the morning count the toothpicks that were fallen horizontally in soil. In this way, movement was observed regularly in each interval.

2.3. Preparation of the Stock and Dose solution of Nanoplatics and Glyphosate

Stock solutions of both chemicals polystyrene nanoplastics and glyphosate were prepared in distilled water.

2.3.1. Polystyrene Nanoplatics

Carboxylate-modified polystyrene, fluorescent yellow-green nanoplastics was present in aqueous suspension. 500ul was taken from stock suspension of 25 mg/ml and added 100ml to make up a final suspension of 12.5 mg/ml. From this stock, suspension 100ml was transferred

to 1kg of soil in each treatment for nanoplatics exposure to achieve the final exposed concentration of 12.5mg/kg.

2.3.2. Glyphosate

Glyphosate stock solution was made by dissolving 26mg glyphosate in water. From this stock, solution 100ml was added to 1kg of soil to achieve final exposure concentration of 26mg/kg.

2.4. Chronic Toxicity Experimental Setup

The experimental work began with a 28-day chronic toxicity test. For analyzing the toxicity, of 300 earthworms were exposed, with different concentration of nanoplastics and glyphosate for the duration of 7, 14, 21 and 28 days. There were three treatment groups assigned with their replicates. One plastic pot was set aside for the control group. Group 1(NPs) was exposed with the 12.5mg/kg with nanoplastics. Group 2(GLY) was exposed with the 26mg/kg glyphosate. Group 3(NPs + GLY) was exposed with 12.5mg/ml nanoplatics and 26mg/ml with glyphosate. The experimental conditions for the control group were the same but without any stressors. The samples were taken after every 7 days' interval for 28 days. Eleven earthworms were randomly sacrificed from each treatment at each sampling interval. Brain, intestine, tissues were collected in Eppendorf tube and mixed with phosphate buffer saline (PBS) and kept at -80°C for the analysis. For the examination of histopathology, intestinal tissues were combined with formalin and stored at or below 4 °C. Natural soil was used for the entire experiment. All the treatments were performed in triplicates. The schematic representation of experimental outline is provided in Figure 6.

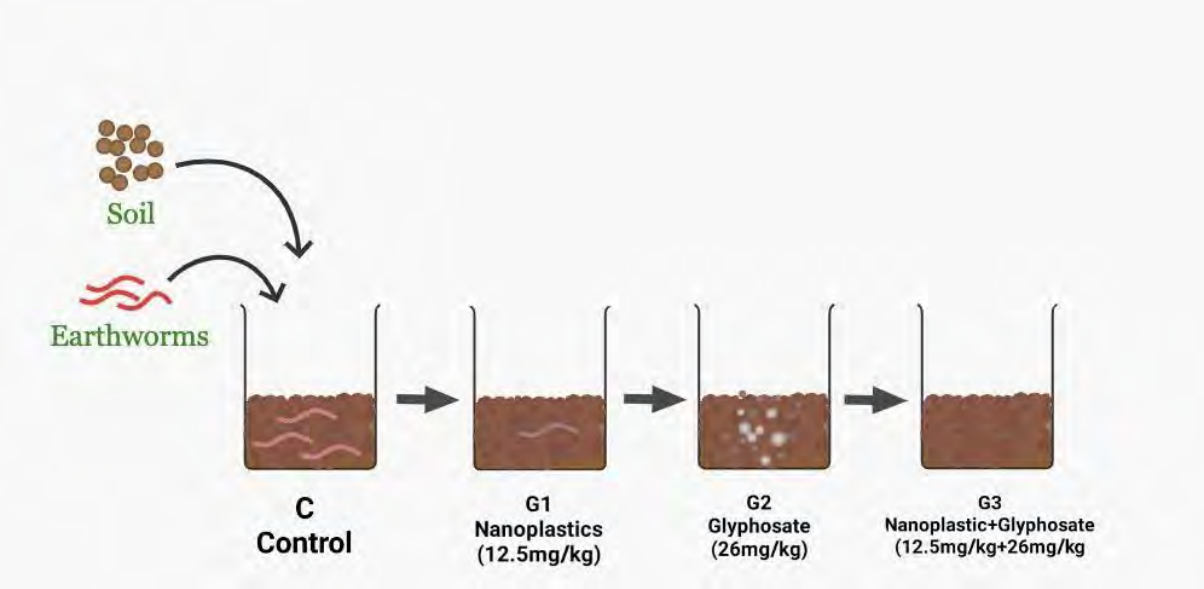


Figure 6: Schematic Representation of Experimental Work of Present Study

2.4. Phosphate Buffer Saline Preparation

Phosphate buffer saline solution (PBS) was made from PBS tablets (CAT No. 2810305) purchased from Bio World. The tablets were dissolved in 1000ml distilled water to make 1Liter of PBS (1 tablet/100ml).

2.5. Buffer Formalin Preparation

The preferred fixative for histopathology was 10 percent natural buffered formalin (NBF). In an aqueous solution, the NBF contains 37 to 40 percent formaldehyde. It is un-buffered solution. From this un-buffered solution, 10% stock formalin was mixed with 90% distilled water, or 1 part NBF and 9 parts of distilled water, to create a histological fixative. 10% formalin solution was prepared in this way. Un-buffered formalin had a pH ranging from 3 to 4. The following procedure was used to adjust the pH of a 10% formalin solution to a neutral level.

1. 100ml formalin (37-40% stock solution)
2. 900ml (H_2O)
3. 4g Monobasic salt (NaH_2PO_4)
4. 6g Di basic anhydrous salt (Na_2HPO_4)

All these salts were mixed and the mixture was stirred for 10 minutes using a magnetic stirrer and the buffer formalin solution was stored in the refrigerator until use.

2.6. Homogenate Preparation

The SilentCrusher M Homogenizer from the Heidolph technology was used to homogenize the brain, intestine tissues individually in 50mM potassium phosphate buffer (pH 7.0) in 2ml eppendorf tubes. The homogenate was centrifuged for 10 minutes at 10,000 rpm. For additional examination, the supernatant was removed and transferred to other labelled eppendorf tubes.

2.7. Acetylcholine Esterase Activity (AChE)

To assess AChE inhibition, the brain tissues of exposed earthworms were dissected on an ice-cold dish. Once dissected, the tissues were promptly transferred to 2 mL Eppendorf tubes and stored at or below -20 °C. To preserve the specimens for further analysis, they were immersed in 1.5 mL of phosphate buffer saline. This method ensured the integrity of the brain tissues and provided an appropriate environment for the accurate determination of AChE inhibition levels in the earthworms.

2.7.1. Determination of the AChE Activity in *Eisenia fetida*

AChE activity was assessed by adapting an Ellman et al. study (1961).

The mixture includes;

1. 1% Sodium Citrate
2. 50µL of 0.5 mM DTNB
3. 200µL 0.5 M Phosphate buffer (pH 8.0)
4. 50µL 10mM acetyl thiocholine iodide
5. 50µL enzyme supernatant
6. 650µL H_2O

Acetyl thiocholine iodide was not present in the control cuvette. By monitoring the variations in absorbance over a 5-minute period at 412 nm, enzyme activity was measured. Inhibition of AChE activity was measured using the following formula:

- **Acetylcholine Esterase activity (AChE) =** $\frac{(\mathbf{T}f-\mathbf{T}i)}{\mathbf{0.01}}$ **= U/min**
- **Whereas:**
- T_f = Final Absorbance
- T_i = Initial Absorbance
- 0.01 = Time Constant (for 1 minute)

2.8. Total Protein Estimation

The total protein content of earthworm tissues was evaluated utilizing bovine serum albumin as the standard (BSA) and the Sigma Bradford Method. The Bradford reagent was bought from Sigma Aldrich. In Bradford Reagent, five BSA dilutions (20, 40, 60, 80, and 100µg/L) were made. UV-Vis Spectrophotometer (T80+ UV/Visible spectrometer) was used to measure the absorbance of BSA dilutions at 595 nm. and standard curve was generated. The BSA curve was used to determine the protein level. The following equation is used to determine total protein;

$$Y = 0.0053X + 0.0306$$

While Y represents the samples' absorbance and X estimates the amount of total protein.

2.9. Reactive Oxygen Species (ROS)

The methodology employed for assessing reactive oxygen species in fish liver tissues was based on the approach outlined by (Hyashi et al., 2007). To make 0.1 molar sodium acetate buffer, 4.1g of sodium acetate was dissolved in 500l of distilled water. To maintain the pH at 4.8, 10mg of N-Diethyl-p-phenylenediamine sulphate salt (DEPPD) was dissolved in 100ml of sodium acetate buffer. Next solution was made by dissolving 50mg of Ferrous Sulphate (FeSO4) in 10ml of sodium acetate buffer. Both solutions were mixed in a 1:25 ratio and incubated in the dark for 20 minutes at room temperature. After that 20µl was extracted from the solution mixture, 1.2ml of buffer, and 20µl of homogenate were added in a cuvette. The absorbance was measured at 505nm using a Smart Spec TM plus Spectrophotometer. Each sample received three readings every 15 seconds and calculated by using formula:

- **Reactive Oxygen Species (ROS) =** $\frac{(\textbf{Sample OD} \times \textbf{Cuvette Vol.} \times 10\text{^}6)}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \Delta\textbf{time} \times (\textbf{Constant 15600})} =$

  Units = nanomoles/mL

  Whereas:

- Sample OD = Sample Absorbance
- Cuvette Vol. = Volume of Cuvette
- Protein = Total protein in sample (mg/mL)
- Sample vol = Volume of Enzyme Supernatant
- Time = Measurement Time
- 15600 = Extinction co-efficient (M^{-1} cm^{-1})

2.10. Malondialdehyde (MDA) / Lipid peroxidation Content Measurement

Malondialdehyde content in earthworm's tissues was measured using the technique described by (Han et al., 2014) utilizing the thiobarbituric acid reactive substance test (TBARS)

Reaction mixture contains:

1. 200µL of 8.1% Sodium Dodecyle Sulfate (SDS)
2. 1500µL of 20% Acetic Acid (pH3.5)
3. 100µL of 1% Thiobarbituric Acid
4. And 1000µL distilled water
5. 200µL of enzyme supernatant

After that, the final product was kept in a water bath at 90°C for one hour. Following that, the solution was chilled and centrifuged for 15 minutes at 3000 rpm. Using a UV-Vis Spectrophotometer, the mixture's absorbance was measured at 532 nm. he mixture that didn't contain tissue homogenate was treated as a blank in the assay. The MDA content was determined to be the ratio of thiobarbituric acid reactive compounds in nanomoles per milligram of protein.

- **Malondialdehyde (MDA)** $= \frac{(\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol.} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times (\text{Constant } 15600)} =$

  Units = (nanomoles/ mg protein)

- Whereas:
- ΔSample = Change in Sample Absorbance
- ΔBlank = Change in Blank Sample Absorbance
- Cuvette Vol. = Volume of Cuvette
- Protein = Total protein in sample (mg/mL)
- Sample vol = Volume of Enzyme Supernatant
- Time = Measurement Time
- 15600 = Extinction co-efficient (M^{-1} cm^{-1})

2.11. Superoxide Dismutase (SOD) Activity

Protocol of Han et al. (2014) and Liu et al. (2017) were followed to measure activity of superoxide dismutase.

Reaction mixture was 3ml that contains;

1. 50mM Phosphate Buffer (pH 7.8)
2. 130mM Methionine
3. 100µM Ethylenediaminetetraacetic acid (EDTA)
4. 20µM Riboflavin
5. 750µM Nitrobluetetrazolium chloride (NBT)
6. And 50µL enzyme supernatant

The resulting mixture was exposed to 4000 1x fluorescent lamp for 20 minutes, and the mixture's absorbance was assessed at 560 nm using a UV-Vis Spectrophotometer (T80+ UV/Visible spectrometer). The evaluation of superoxide dismutase activity was based on the enzyme's ability to cause a 50% inhibition of the Nitrobluetetrazolium chloride photoreduction rate. This inhibition was considered as one unit of superoxide dismutase activity. The obtained results were expressed as U/mg of protein, providing a quantitative measure of the enzyme's activity relative to the protein content. Results obtained were expressed as U/mg of protein.

- **Superoxide Dismutase (SOD)** $= \frac{(\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times (\text{Constant } 6.22)} =$

  Units : (U/mg of protein)
- Whereas:
- ΔSample = Change in Sample Absorbance
- ΔBlank = Change in Blank Sample Absorbance
- Cuvette Vol. = Volume of Cuvette
- Protein = Total protein in sample (mg/mL)
- Sample vol = Volume of Enzyme Supernatant
- Time = Measurement Time
- 6.22 = Extinction co-efficient (M^{-1} cm^{-1})

2.12. Catalase (CAT) Activity

The techniques employed by Greenwald (1987) were utilized to test catalase activity.

Reaction mixture contains:

1. 500µL of 5.9mM H2O2
2. 1mL of 50mM Potassium Phosphate Buffer
3. 100µL of tissue homogenate

The resulting mixture was incubating at 37 °C for 15 minutes. At 240 nm, absorbance was noted. The catalase activity was expressed as U/mg of protein.

- **Catalase (CAT) =** $\frac{(\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times (\text{Constant } 43.1)} =$

  Units : (U/mg of protein)

- Where:
- ΔSample = Change in Sample Absorbance
- ΔBlank = Change in Blank Sample Absorbance
- Cuvette Vol. = Volume of Cuvette
- Protein = Total protein in sample (mg/mL)
- Sample vol = Volume of Enzyme Supernatant
- Time = Measurement Time
- 43.1 = Extinction co-efficient (M^{-1} cm^{-1})

2.13. Comet Assay (Single Cell Gel Electrophoresis)

Single-cell gel electrophoresis (SCGE) was used to assess the DNA damage in the coelomocytes of earthworm. Comet Assay was carried out using a method that was reported by Song et al. (2009).

2.13.1. Reagents Preparations

Reagents for the Comet assay were prepared as follows;

1. Low Melting Agarose (LMA): To make 0.5 percent LMA, 250 mg of LMA were dissolved in 50 ml of 1% PBS and stored at low temperature in the refrigerator. Before use, the temperature was raised to 37 °C.
2. Normal Melting Agarose (NMA): The solution was heated to dissolve 500 mg of normal melting agarose in 50 ml of distilled water. This method was used to prepare 1% of the NMA.
3. 1.46g of NaOH (2.5M), 37.2g of EDTA (100M), and 1.2g of Trizma base (100M) were dissolved in distilled water and the volume was increased to 890ml to create the lysing solution. Following pH maintenance to 10.0 with HCl or NaOH, the final volume was increased to 1000 ml. Final lysing solution was created by mixing suspension that had already been prepared with 1% Triton X and 10% DMSO. Prior to creating slides, the final solution must be chilled for 30 minutes.

4. The electrophoresis buffer consists of 0.5 ml of EDTA (200 mM) and 30 ml of NaOH in 1000 ml of distilled water. The pH was kept basic, or >13.
5. Neutralization buffer; pH of 7.5 (using HCL) was achieved by dissolving 48.5g of 0.4 M tris in 1000ml of distilled water, and the mixture was maintained under room temperature.
6. Staining solution: 50ml of distilled water was used to dissolve 10mg of ethidium bromide in this way staining solution was prepared.
7. PBS buffer: 990mL of phosphate buffer saline were made, and their volume was increased to 1000mL when the pH was raised till 7.4.

2.13.2. Slides Preparations

Slides were first cleaned with methanol and using fire blazes to disinfect them. Onto frosted slides with a coating of NMA, 200µL of LMA and cell suspension were pipetted. As the first LMA layer solidifies, a second one (85µL) was introduced. Slides were immersed in lysis solution and chilled for 120 minutes while being kept in the dark following after the fixation of the second layer.

2.13.3. Electrophoresis

After two hours in the lysis solution, the slides were removed and kept in the horizontal gel apparatus. The freshly made buffer solution was poured over the slides. For 30 minutes, 24 volts of power was applied, then turned off to let the DNA uncoil. The neutralizing buffer was applied to the slides. The same steps were repeated twice. Slides were coated with cover slips before being stained with 80L of 1X ethidium bromide.

2.13.4. Slides Visualization

To evaluate DNA damage, a fluorescent microscope (Nikon, 40X) was used. The software CASP 1.2.3.b was useful in assessing the magnitude of DNA damage. DNA migration from head to tail was assessed in each sample, which contained between 50 and 100 cells. Comparing each cell's DNA transfer and the proportion of cells with a high DNA fragment migration ratio allowed for the mounting of results.

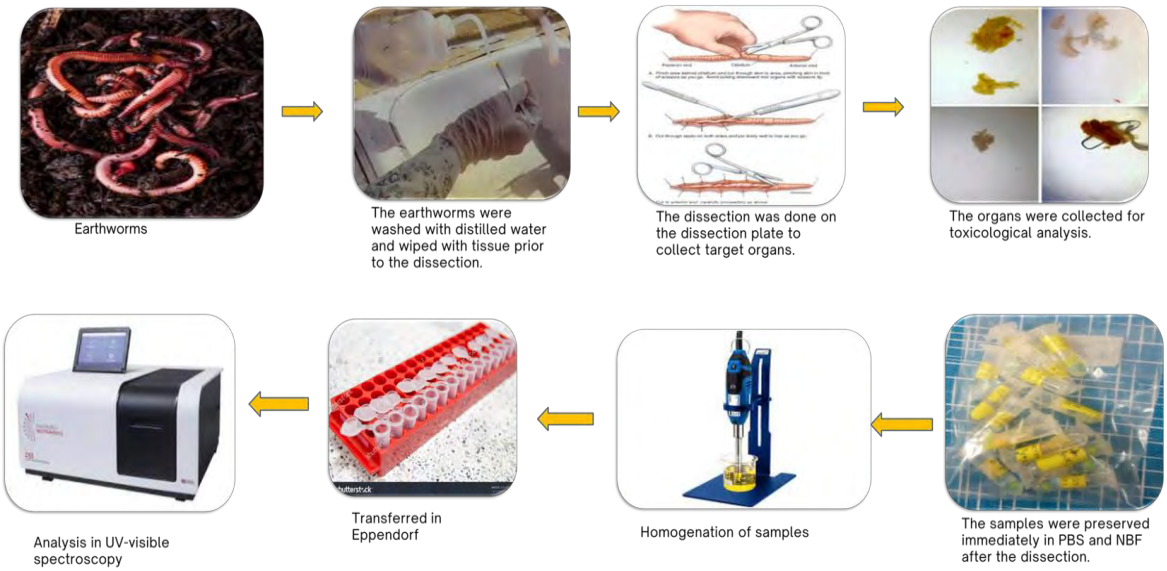
2.14. Tissue Histopathology

The histopathological examination of intestine tissues. At the end of each time interval, the earthworm was dissected, and intestine tissues were collected. These tissues were then

promptly transferred into 2 mL Eppendorf tubes containing 1.5 mL of formalin fixative buffer. For further analysis, the samples were kept at 4°C.

2.14.1. Hematoxylin and Eosin Staining of Intestine

After the exposure to GLY and NPs, the histopathological alterations in the intestinal were evaluated using the Hematoxylin and Eosin staining method. The steps of fixation, dehydration, embedding, slide preparation, staining, and microscopy. The tissues were cleaned and rinsed in a saline solution containing 0.75% NaCl. Section of the tissues were cut at a thickness of 4-6 microns, fixed in aqueous Bouins solution for 24 hours, processed through a graded series of alcohols, cleaned in xylene, and embedded in paraffin wax, then all the parts stain with hematoxylin eosin and dissolved in 70% alcohol (Yang et al., 2018). TUCSEN digital camera (Model: ISH500) was used to take pictures of the intestine tissues after preparing slides and histopathological lesions were studied using an Olympus CX41 light microscope at 400X.



Sampling methodology and different steps used in earthworm dissection.

2.15. Statistical Analysis

Experiments were performed in triplicate for each tested concentration (treatment), and calculations took into account means and standard deviations. Statistical analysis was performed on all experimental data using IBM SPSS Statistics version 21.0. (SPSS Inc., USA). Three replicates of the data (n=3) are used to calculate the means and standard deviation (SD). One-way analysis of variance was used to calculate the statistical differences between control and treatments and means were separated using Duncan Post-Hoc comparison test with significance at $p<0.05$.

Chapter 3

Results

3. RESULTS

The chronic toxicity test was performed for 28 experimental days to evaluate the combined toxicological effects of glyphosate and nanoplastics on earthworms. Prior the experiment was conducted, soil macronutrients (N P K) were measured. MDA, ROS and antioxidant enzymes (SOD, CAT) activities were also assessed in tissues of earthworms. Acetylcholinesterase inhibition were measured in brain tissues of earthworms. Using the comet assay, the DNA damage in earthworm's coelomocytes was also examined. Additionally, histopathological alteration was examined in the intestine of earthworms. In this section, outcomes of all parameters is described.

3.1. Soil Characterization

The results of soil analysis showed that the soil was sandy clay loamy in texture, with an organic matter concentration of 0.93 (Table 6). The soil pH was 7.1, indicating that alkaline in nature, and the EC was 694 µS/m. The soil had a water retention capacity 40% to 60% and a moisture content of 72%. The nutrient level of the soil was N= 0.59mg/g, P= 4mg/kg, and K= 89 mg/kg.

Table 6: Physicochemical Properties of Soil

Parameters	**Value/Units**
Texture	Sandy Clay Loam
Organic Matter	0.93%
pH	7
EC	694 µS/m
Water Holding Capacity (WHC)	40% to 60%
Moisture Content	72%
$NO-^{3}N$	0.83 mg/kg
Phosphate	4.20 mg/kg
Potassium	114mg/g

3.2. Chronic Toxicity Test

The single and combined effects of nanoplastics and glyphosate were assessed in a 28-day exposure period. This section describes the results of all parameters investigated during the experiment.

3.2.1. Acetylcholinesterase Activity

Acetylcholinesterase activity was determined in earthworm brain tissues after 7, 14, 21, and 28 days of exposure to nanoplastics (G1), glyphosate (G2) and coexposure (G3). The treatment groups G1, G2 and G3, showed more neuron damage in the brain of earthworms. The results revealed that AChE activity decreased with increasing duration. The nanoplastics (G1) group considerably reduced from the control after 7 days of exposure, but there was no statically difference between the G1 and G2 groups. The G3 group differed significantly from the control, G1, and G2 groups. The coexposure (G3) group indicated a significant decrease ($p<0.05$) in AChE activity at the first sampling interval after 7 days of exposure. Among all experimental groups, the G3 group exhibited the highest inhibition. After 7 days of exposure, all other treatments, G1, G2 and G3 (14, 21, and 28 days) showed a consistent decrease in AChE activity, indicating a similar trend across the sampling intervals. Significant decrease ($p<0.05$) in AChE activity was observed in all treatment groups G1, G2, and G3, as compared to the control group. The glyphosate (G3) group showed the lowest inhibition, while the G3 group exhibited the maximum inhibition. Result demonstrated that G1, G2 and G3 groups, as well as the duration of exposure time, decreased AChE activity in earthworm brain tissues as shown in Figure 7.

Table 7: Acetylcholinesterase (AChE) activity in brain tissues of *Eisenia fetida* exposed to nanoplastics, glyphosate and coexposure of (nanoplastics + glyphosate).

Treatment Groups	**Exposure Days**			
	7 Day	**14 Day**	**21 Day**	**28 Day**
Control	7.333 ± 0.305^{a}	7.133 ± 0230^{a}	6.233±0.251a	6.8 ± 0.2^{a}
G1(Nanoplastic)	6.7 ± 0.1^{b}	5.86 ± 0.15^{c}	4.6 ± 0.2^{c}	3.96 ± 0.47^{c}
G2 (Glyphosate)	6.98 ± 0.16^{ab}	6.33 ± 0.30^{b}	5.66 ± 0.15^{b}	4.9 ± 0.3^{b}
G3(Coexposure)	5.5 ± 0.4^{c}	4.46 ± 0.35^{d}	3.7 ± 0.2^{d}	2.6 ± 0.52^{d}

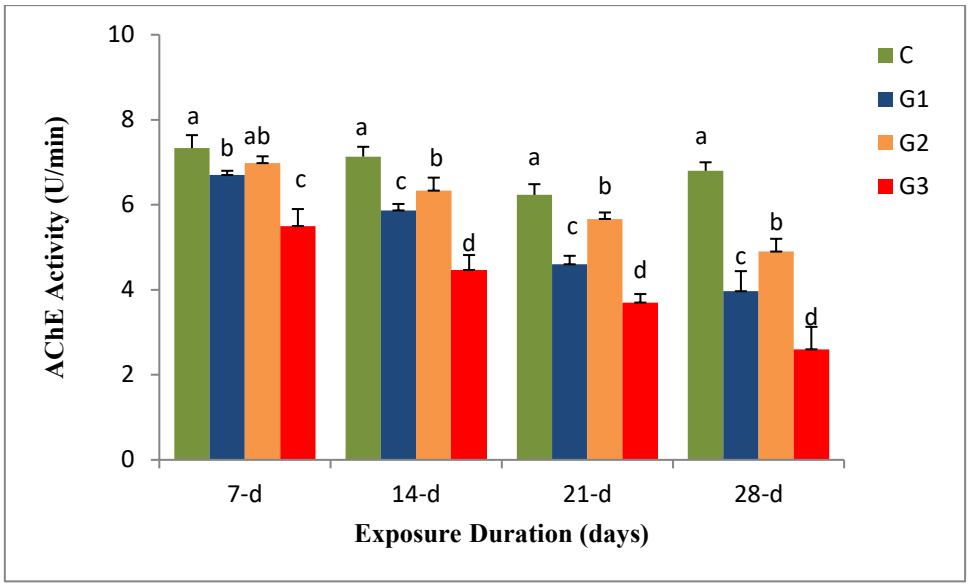


Figure 7: Single and joint effects of nanoplatics and glyphosate on AChE activity in the brain tissue of earthworms. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference ($p<0.05$) among treatment groups.

3.3. Total Protein Estimation

Proteins, being vital macromolecules for the viability of all life forms, play a central role in the formation and progression of tissues and organs within organisms (Guarda et al., 2003). Furthermore, they act as the building blocks for enzymes, hormones, neurotransmitters, and cofactors, contributing to various physiological processes (Qiu et al., 2019).

Table 8: Absorbance of Standard BSA (Bovine Serum Albumin).

Dilutions (µg/L)	Average Absorbance (nm)
0	0
10	0.016
20	0.028
40	0.0485
60	0.069
80	0.0855
100	0.1022

3.3.1. Calibration Curve for Estimating Total Protein in Tissue Samples

Dilution of standard Bovine Albumin solution generates the standard curve for total protein estimation. The absorbance of the entire mixture was measured at 595 nm, and an absorbance calibration curve was plotted to determine the concentration of total protein content in the unknown sample.

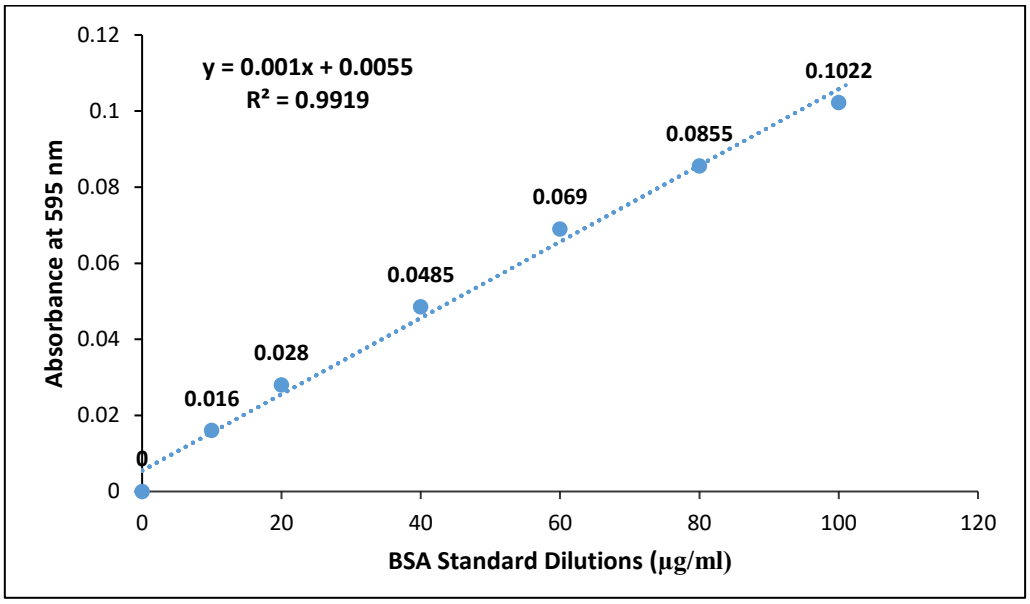


Figure 8: Standard calibration curve of BSA dilutions for total protein estimation at 595nm.

3.3.2. Total Protein Content

The results indicated a significant decline ($p < 0.05$) in the overall protein content present in the body of the earthworms. At the 7 days of exposure, a noticeable distinction emerges between the control group and the exposed group. As time progresses, there is a consistent reduction in the total protein content. By the 14 day of exposure, a more substantial decrease in total protein content becomes apparent. Progressing to the 21 day of exposure, the total protein content in nanoplatics and glyphosate groups decreased as compared to control group. Significant decrease in total protein content is observed, and by the 28 day exposure, an even greater decline in total protein content is evident.

Table 9: Total Protein determination in *Eisenia fetida* exposed to nanoplatics, glyphosate and coexposure of nanoplastics and glyphosate.

Treatment Groups	Exposure Days			
	7 Day	**14 Day**	**21 Day**	**28 Day**
Control	46.75±1.21[a]	43.68±1.11[a]	42.68±1.26[a]	44.9±1.04[a]
G1 (Nanoplastic)	36.64±1.52[b]	28.35±1.11[b]	30.46±0.82[b]	33.90±1.10[b]
G2 (Glyphosate)	30.01±0.53[c]	23.24±0.55[c]	20.79±1.08[c]	24.97±1.04[c]
G3 (Coexposure)	26.5±1.32[d]	20±2[d]	15.66±2.08[d]	13.33±2.51[d]

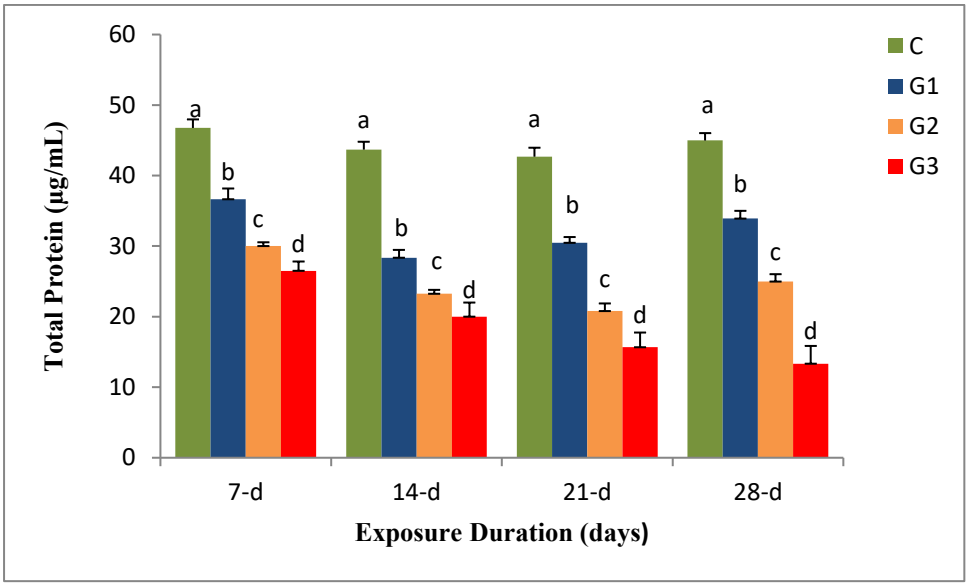


Figure 9: Single and joint effects of nanoplatics and glyphosate on Total Protein level in the whole body tissue of earthworms. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference ($p<0.05$) among treatment groups.

3.1. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) serve to identify oxidative damage caused by xenobiotic. The fundamental function of antioxidant enzymes is to scavenge free radicals, thereby protecting the body from the detrimental effects of harmful substances.

On day 7 there was no significant difference between nanoplatics (G1) and glyphosate (G2). However, ROS level were significantly higher from the control group in both treatments groups G1, and G2. Significant increase in ROS was observed in the coexposure group (G3)

as compared to the control and from G1and G2(nanoplatics and glyphosate). After the exposure of 14 days, there was no significant difference between the G1 and G2 groups. The results were observed to be similar to the first interval. On day 21, G1 group showed the significant increase in ROS as compared to G2 group. G2 showed the significant increase in ROS level from the control but lower than other groups (G1 and G3). After 28 days, there was significant difference between control group and treated groups. The G3 group showed significant increase in ROS from the all other groups ($p<0.05$). All the groups showed the significant increase from the control group and highest significant increase in ROS level of G3 group as shown in Figure 9.

Table 10: Reactive Oxygen Species (ROS) in body tissues of *Eisenia fetida* exposed to nanoplatics, glyphosate and coexposure of nanoplastics and glyphosate.

Treatment Groups	**Exposure Days**			
	7 Day	**14 Day**	**21 Day**	**28 Day**
Control	17.16 ± 18.87^{c}	18.4 ± 0.92^{c}	20.5 ± 1^{d}	23.2 ± 1.5^{d}
G1 (Nanoplastic)	20.51 ± 0.42^{b}	25.9 ± 1^{b}	25.17 ± 0.37^{b}	27.16 ± 0.76^{b}
G2 (Glyphosate)	19.95 ± 0.35^{b}	23.8 ± 1^{b}	22.7 ± 0.86^{c}	25.3 ± 1.25^{c}
G3 (Coexposure)	25.25 ± 1^{a}	28.41 ± 1.32^{a}	29.24 ± 0.73^{a}	30.3 ± 1.03^{a}

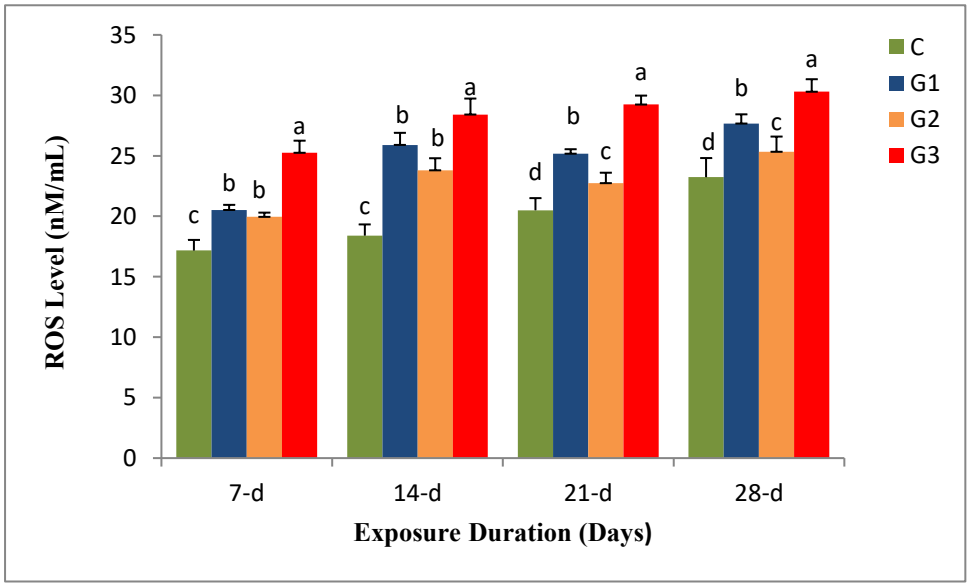


Figure 10: Single and joint effects of nanoplatics and glyphosate on ROS level, in the whole body tissue of earthworms. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three

replicates (n=3). Different lowercase letters indicate significant difference ($p<0.05$) among treatment groups.

3.2. Malondialdehyde Content (MDA)

Following 7 days of exposure, there is significant increase observed in MDA content in all the treated groups G1, G2 and G3 (nanoplastic, glyphosate and coexposure) as compared to the control. The highest MDA content was observed in coexposure group (G3). MDA content increased significantly ($p<0.05$) in the G1 group compared to the G2 group after 14 days of exposure. However, both the treatments, G1 and G2 significantly increased in comparison to the control group whereas, the G3 group exhibited the highest MDA level compared to the other treatments. This sampling interval showed similar effect on MDA content as the first interval. MDA content increased significantly after 21 days of exposure as compared to 7 and 14 days. The nanoplatics (G1) and glyphosate (G2) groups considerably increased from the control throughout 21 day interval, but there was no statistical difference between the G1, G2 groups. As compared to control, the G3 group exhibited the highest MDA content. At last sampling interval after the 28 days G1 and G2 groups showed the significant increase ($p<0.05$) in MDA content as compared to control but lower then G3 group. G2 group showed the lowest elevated MDA level in all of these treatments, yet it was higher than the control.

Table 11: Malondialdehyde (MDA) in body tissues of *Eisenia fetida* exposed to nanoplatics, glyphosate and coexposure of nanoplastics and glyphosate.

Treatment Groups	**Exposure Days**			
	7 Day	**14 Day**	**21 Day**	**28 Day**
Control	8.4 ± 0.2^{d}	8.86 ± 0.9^{d}	16.5 ± 1.1^{d}	17.5 ± 0.6^{d}
G1 (Nanoplastic)	11.4 ± 0.2^{b}	12.5 ± 0.3^{b}	19.6 ± 0.2^{b}	21.5 ± 0.5^{b}
G2 (Glyphosate)	10.5 ± 0.31^{c}	$10.6\pm0.21_{c}$	18.7 ± 0.2^{c}	19.4 ± 0.41^{c}
G3 (Coexposure)	14.3 ± 0.21^{a}	14.8 ± 0.04^{a}	21.5 ± 0.30^{a}	24.1 ± 0.88^{a}

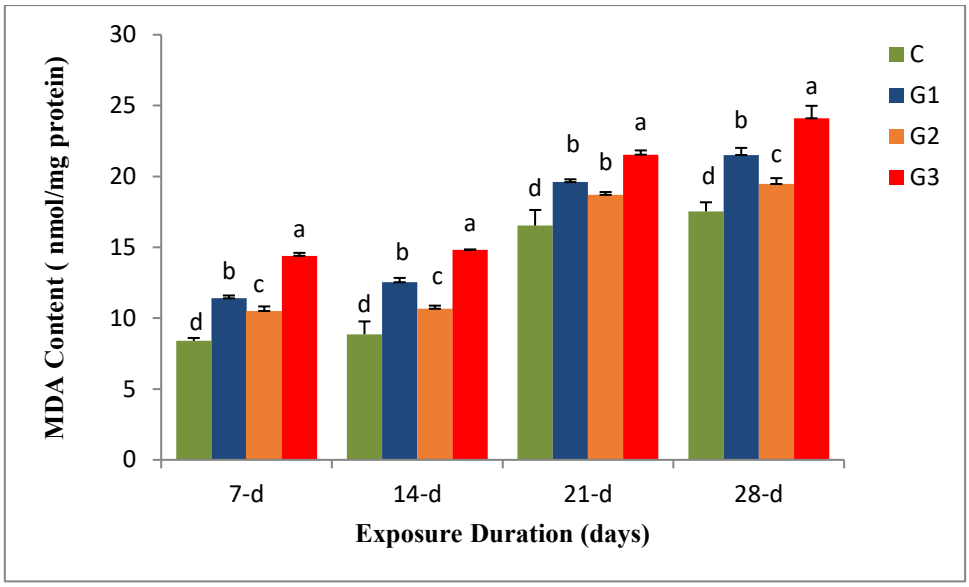


Figure 11: Single and joint effects of nanoplatics and glyphosate on MDA level in the whole body tissue of earthworms. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference ($p<0.05$) among treatment groups.

3.3. Antioxidant Enzymes Activity Parameters

3.3.1. Super Oxide Dismutase (SOD)

During the chronic toxicity test, superoxide dismutase activity was evaluated at four different intervals. SOD enzyme activity increased as concentration increased and decreased as exposure time increased. After the 7days of exposure in G3 group, SOD activity was increased from all other groups ($p<0.05$). There was no statically difference between G1, G2 group and the control group. In G3 group the SOD activity was significantly increased. On day 14 all the groups were showed lower activity from the control. SOD activity significantly decreased in the G3 group compared to the control group. There was no significant difference between the G1 and G2 groups, however both were lower than the control. After 21 days of exposure, SOD activity decreased in all treatments as compared to control. There was significant decrease in SOD activity in G1, and G2 groups from the control ($p<0.05$). G3 group the SOD activity was significantly decreased from the control and other groups. On the last sampling interval after the 28 days, all treatments exhibited lower SOD activity than the control group. The enzyme activity in both groups (G1 and G2) was significantly lower than in the control. The G3 group exhibited the lowest drop compared to the other groups. Overall, SOD activity decreased significantly in this interval and differed significantly from all other intervals.

Table 12: Superoxide Dismutase (SOD) in body tissues of *Eisenia fetida* exposed to nanoplatics, glyphosate and coexposure of nanoplastics and glyphosate.

Treatment Groups	Exposure Days			
	7th Day	**14th Day**	**21st Day**	**28th Day**
Control	6.63 ± 0.1^{b}	6.2 ± 0.2^{a}	5.8 ± 0.11^{a}	5.3 ± 0.26^{a}
G1 (Nanoplastic)	6.66 ± 0.3^{b}	5.7 ± 0.15^{b}	4.8 ± 0.2^{c}	3.8 ± 0.15^{c}
G2 (Glyphosate)	6.66 ± 0.30^{b}	6.16 ± 0.11^{b}	5.26 ± 0.25^{b}	4.6 ± 0.2^{b}
G3 (Coexposure)	7.2 ± 0.3^{a}	5.1 ± 0.11^{c}	4.4 ± 0.2^{d}	2.6 ± 0.30^{d}

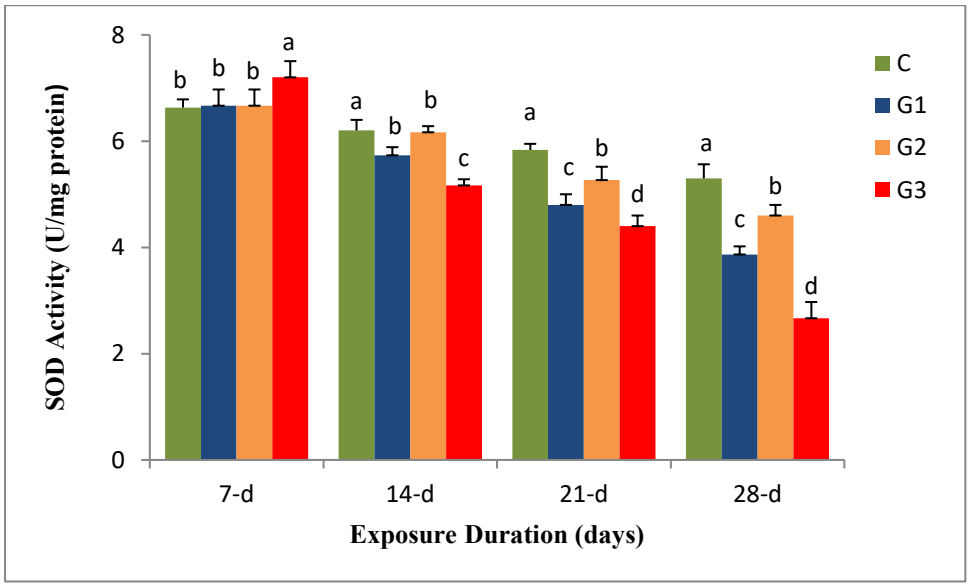


Figure 12: Single and joint effects of nanoplatics and glyphosate on SOD activity in the whole body tissue of earthworms. C shows (control), G1 (Nanoplastics), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference ($p<0.05$) among treatment groups.

3.3.2. Catalase Activity (CAT)

The catalase activity showed significant changes as compared to control. The G1 and G2 group are the same as the control on day 7, there was no statically difference between the (G1, G2) groups. CAT activity in these two groups was the same like the control group. CAT activity in the G3 group was significantly higher from the control group ($p<0.05$). After the 14 days CAT activity was significantly decrease in all groups compared to the control. The significantly lowest decrease of CAT activity in the G3 group whereas, G1 and G2 groups exhibited a significant decrease in CAT activity than the control group. CAT activity was considerably

different in G1 and G2 groups from the control. On day 21, CAT activity trend is similar to the 14 interval. There was a significant difference between the both intervals. At the day 28 sampling interval, there was a substantial decrease of CAT activity in all groups compared to the control. CAT activity in G1 and G2 group significantly decrease from the control group, but G2 slightly increased from G1 and G3. The lowest decrease of CAT activity in G3 group ($p<0.05$). Overall, CAT activity was significantly reduced in G1, G2, and G3 on day 14, 21, and 28 as compared to day 7 sampling interval.

Table 13: Catalase Activity (CAT) in body tissues of *Eisenia fetida* exposed to nanoplatics, glyphosate and coexposure of nanoplastics and glyphosate.

Treatment Groups	**Exposed Days**			
	7 Day	**14 Day**	**21 Day**	**28 Day**
Control	52.3 ± 1.25^{c}	56.0 ± 1.05^{a}	54.7 ± 1^{a}	46.3 ± 1.25^{a}
G1(Nanoplastic)	54.4 ± 1.31^{b}	48.05 ± 1.05^{c}	42.06 ± 1.05^{c}	34.36 ± 1.28^{c}
G2(Glyphosate)	55.15 ± 1.33^{b}	52.3 ± 1.25^{b}	48.05 ± 1.05^{b}	38.1 ± 1.075^{b}
G3(Coexposure)	57.3 ± 1.25^{a}	42.05 ± 1.05^{d}	36.7 ± 1^{d}	28.1 ± 1.075^{d}

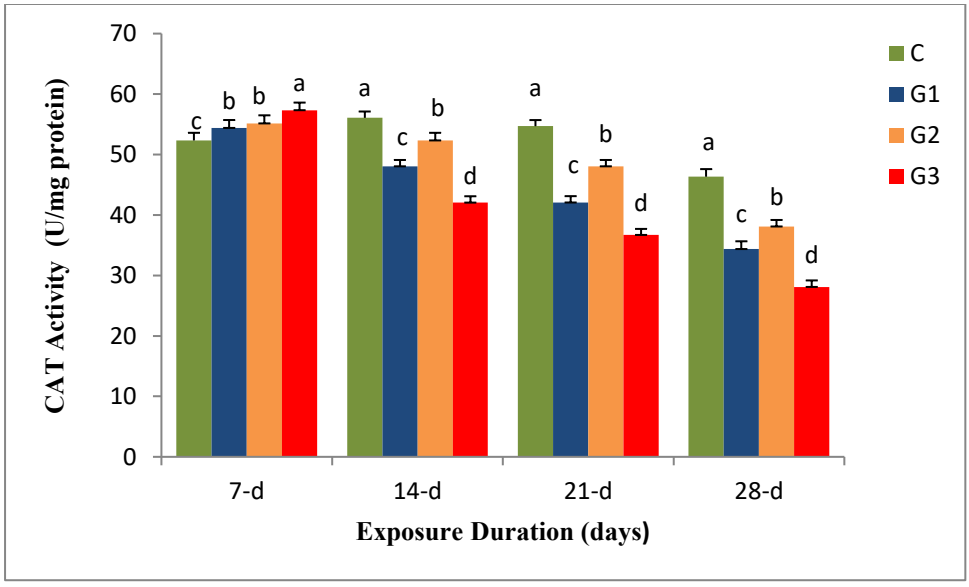


Figure 13: Single and joint effects of nanoplatics and glyphosate on CAT activity in the whole body tissue of earthworms. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference ($P<0.05$) among treatment groups.

3.4. DNA Damage and Tail Moment determined by Comet Assay

The comet assay was used to assess the DNA damage caused by combined nanoplatics and glyphosate exposure. CASPLAB was used to measure comet length. The results revealed DNA changes gradually after exposure. At all sampling intervals (7, 14, 21, and 28 days), head length decreased gradually in all treatments (G1, G2, and G3), whereas tail length increased due to migrating damaged DNA from head to tail. At all sampling intervals in control group the DNA in head remained 84 to 87%. It steadily decreases with increasing exposure time of G1, G2 and G3 group. The percentage of DNA in the tail slowly increased from day 7 to day 28. It was the highest in the coexposure (G3) treatment after 28 days of exposure, compared to 7, 14, 21 and 28 days. In all control groups the %DNA remained 15 to 16%. Continuously comet length also increases at all intervals as compared to control. Apart from treatments of G1, G2 and G3, the time period of exposure plays an important role in the increase of DNA damage (see Table 7 and Figure 13) illustrated all of the parameters that were observed.

Table 14: Genotoxicity Assessment of nanoplastic, glyphosate and nanoplastic+ glyphosate homogenates of earthworms by Comet Parameters

Sample	Comet length (µm)	Head length (µm)	Tail length (µm)	% DNA in head	% DNA in tail	Tail moment (µm)
7 Days Exposure						
C	40.3±2.7	34.1±2.2	6.2±1.1	84.61±3.2	15.38±1.8	0.10±0.03
G1	43.5±2.2	35.6±3.1	7.91±1.3	81.83±3.1	18.16±1.9	0.14±0.04
G2	41.9±3.1	34.1±3.1	7.9±0.6	81.14±3.1	18.85±2.2	0.13±0.03
G3	42.6±3.2	33.4±3.1	9.2±0.9	78.40±3.2	21.59±1.4	0.17±0.02
14 Days Exposure						
C	38.9±2.8	33.1±2.1	5.9±0.5	87.01±3.4	12.99±1.3	0.11±0.03
G1	43.8±2.7	32.1±2.8	11.81±1.6	73.05±3.1	26.94±2.2	0.21±0.02
G2	41.5±3.1	33.2±2.2	8.3±1.4	80.01±3.5	19.99±1.8	0.15±0.03
G3	45.9±3.5	31.7±3.4	14.2±2.7	69.07±2.9	30.93±3.7	0.24±0.01
21 Days Exposure						
C	40.8±3.3	35.5±2.3	5.3±1.9	87.01±3.1	12.99±2.6	0.10±0.03
G1	45.1±3.3	35.2±3.4	9.8±1.9	78.2±3.1	21.77±2.5	0.22±0.04
G2	43.2±2.5	36.1±2.1	7.1±1.5	83.33±3.3	16.55±1.6	0.15±0.04
G3	45.6±3.1	35.1±2.5	10.6±1.5	76.75±3.1	23.24±2.6	0.41±0.03
28 Days Exposure						
C	38.6±3.1	32.3±2.3	6.3±1.1	83.67±3.3	16.32±1.7	0.13±0.05
G1	46.4±3.1	32.1±3.0	14.3±1.4	69.18±4.1	30.81±3.1	0.51±0.3
G2	41.9±3.2	33.1±2.2	8.9±1.3	78.75±3.7	21.24±1.3	0.20±0.02
G3	47.8±3.1	30.2±2.3	17.6±1.9	63.17±3.3	36.82±3.1	0.92±0.02

Control(C): without nanoplatics and glyphosate, G1: nanoplastic, G2: glyphosate, G3: coexposure of nanoplastics + glyphosate. All values are expressed as mean ± SD (n = 3).

3.4.1. Tail Moment (TM)

When compared to control, it was found that all treatments had increased tail moment (TM). The first sample interval revealed significant differences between the treatments (7 days). In contrast to control, a considerable rise in TM was seen in coexposure group (G3) after 14 days. The similar tendency was still seen with higher TM values at the third sampling period (21 days). After 28 days of exposure, when a substantial change in all treatments was seen, the tail moment was at its maximum in G3. Increasing tail moment was significantly influenced by NPs and GLY exposure concentration with the passage of time. When compared to other

treatments, (G1and G2) TM had the higher rise in G3 after 28 days of exposure. Thus NPs and GLY exposure over an extended period of time can cause DNA damage in earthworm's coelomocytes. In the coelomocytes of earthworms, Figure 14, displays TM for all treatments and sampling periods.

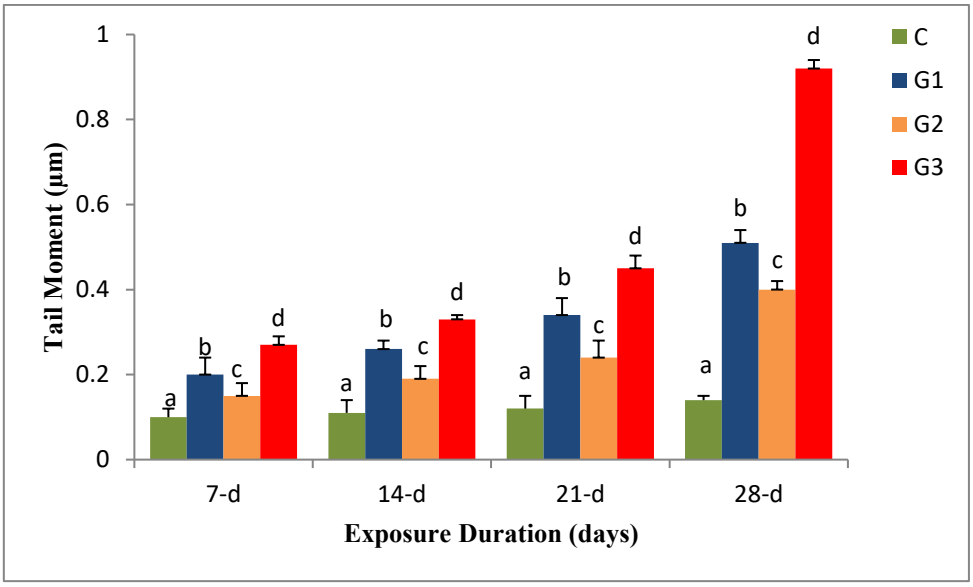


Figure 14: Tail moment in coelomocytes of earthworms by exposure of nanoplastics and glyphosate. C shows (control), G1 (Nanoplastic), G2 (Glyphosate), G3 coexposure (NPs + GLY). Each bar represents the mean ±SD of results obtained from three replicates (n=3). Different lowercase letters indicate significant difference (P<0.05) among treatment groups.

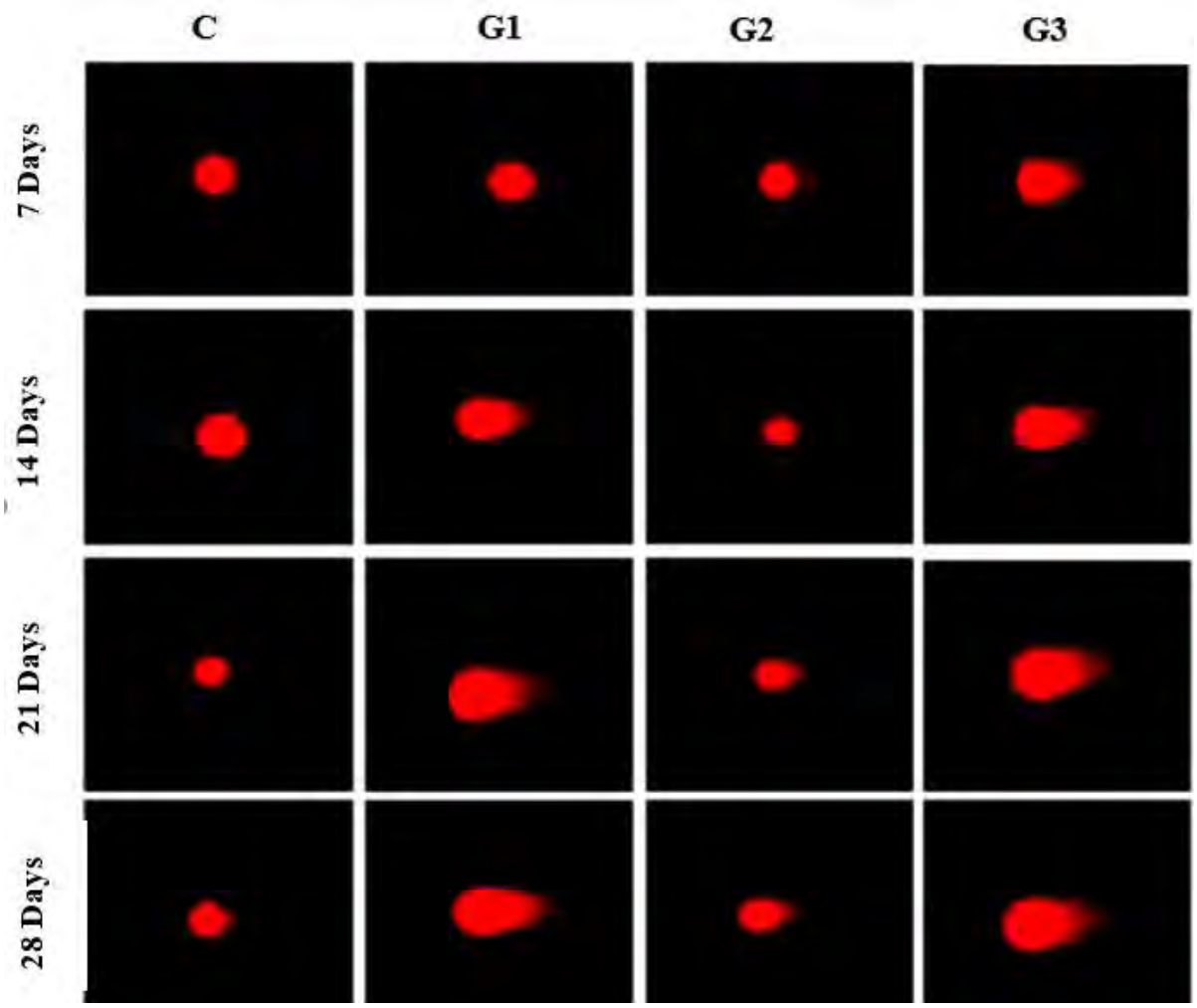


Figure 15: Fluorescence photomicrographs of glyphosate and nanoplatics induced DNA damage in coelomocytes of earthworms, Control(C): without glyphosate and nanoplastic, G1: nanoplastic, G2: glyphosate G3: Coexposure of nanoplastic and Glyphosate (NPs + GLY).

3.4.2. Tissue Histopathology

Histopathology is utilized to assess chemical toxicity and its effects on earthworms. Intestine tissues of earthworm's microscopic examination showed various histopathological changes after individual and combined exposure to the nanoplastics and glyphosate.

3.4.3. Histopathological Alterations in Intestine of Earthworms

Food is taken up and metabolized in the intestine. Food and chemical substances are then broken down and taken up by the blood stream. The intestines provide a route of entry for the harmful substances into the blood stream. The health of the intestine is a useful indicator of environmental contamination and exposure to the earthworms.

We assessed histopathological changes caused by nanoplastics and glyphosate in *E. fetida* intestines after 28 days of exposure. All examined sections in the control group showed normal histology of the earthworm intestines. However, earthworm intestines exposed to NPs and GLY showed enlarged intestinal cells with aberrant shapes and altered cell nuclei size in G1(NPs), G2 (GLY)and G3(NPs + GLY) group. Furthermore, greater intestinal cell lysis was seen in coexposure (G3) group that was exposed with NPs and GLY. After the 28days of exposure intestine cells rupture more severely as compared to first two intervals. As well as, higher concentrations and time period produce greater accumulation and abnormal histopathology in earthworms.

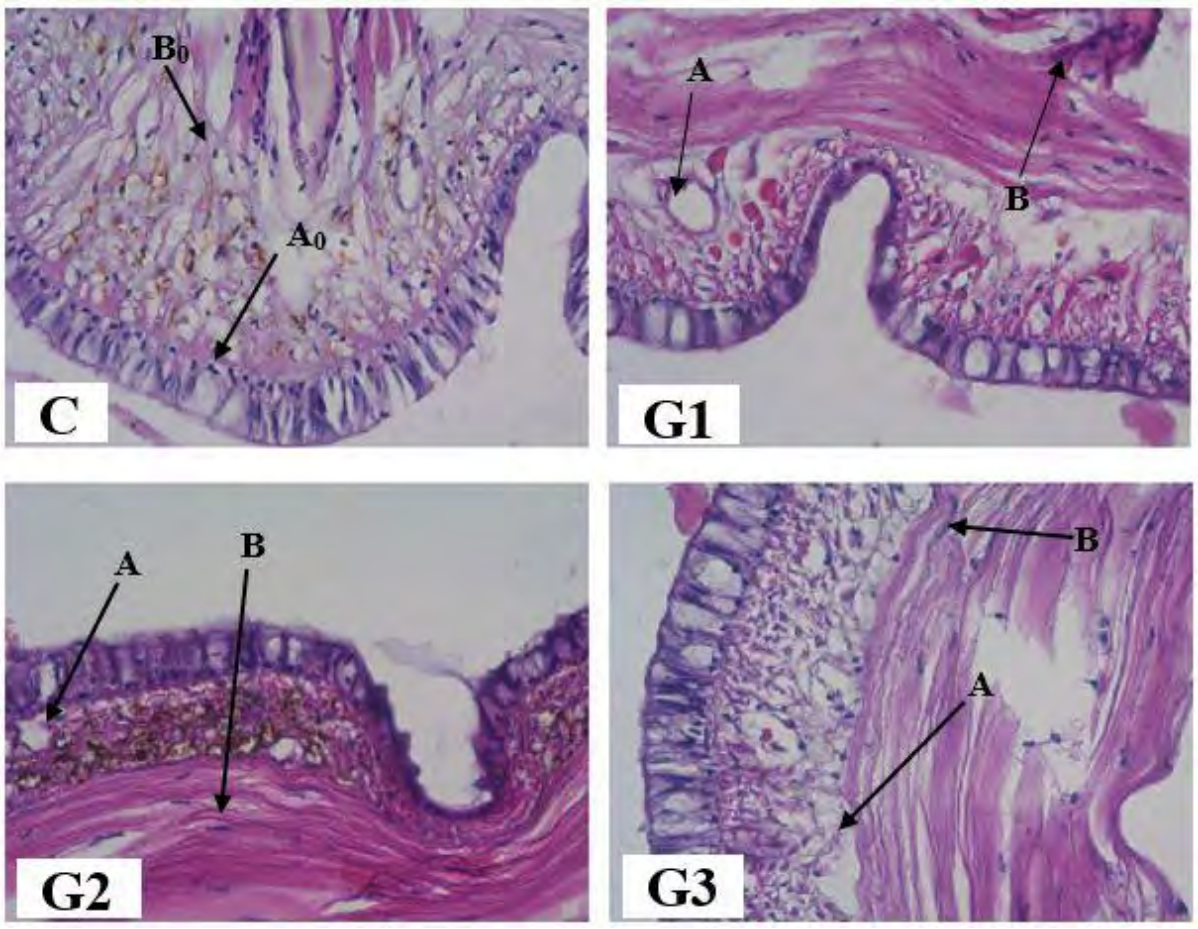


Figure 16: Photomicrographs of intestine of earthworms at 40X magnification, stained with hematoxylin and eosin after the exposure of 7days. The Control group(C) showed normal cell division(A_0), regular cell shape (B_0). While G1 showed Enlarged cell space (A), Irregular cell shape (B). G2 indicating the enlarged cell space(A), irregular cell shape(B). The G3 showed wide enlarge space(A), irregular cell shape(B).

(*C = Control; *G1: Group 1 (Nanoplastic); *G2: Group 2 (Glyphosate); *G3 Group3 (Nanoplatics + Glyphosate)

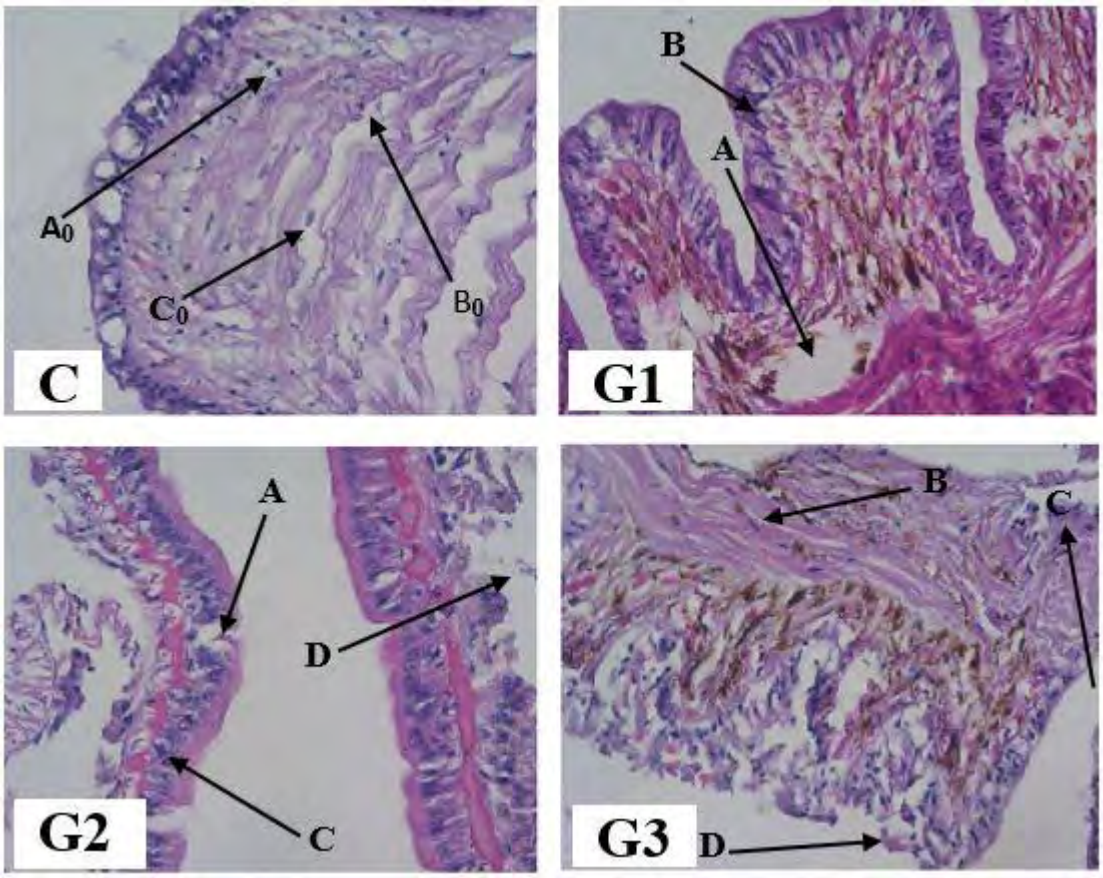


Figure 17: Photomicrographs of intestine of earthworms at 40X magnification, stained with hematoxylin and eosin, after the exposure of 14 days. The Control group (C) showed normal histological cell divisions (A_0), regular cell shape (B_0), and regular nucleus shape(C_0). While G1 showed abnormal cell division (A), irregular cell shape (B). Moreover, the G2 indicating the abnormal cell division(A), irregular nucleus shape(C), and showed the cell lysis into pieces(D). The G3 showed irregular cell shape(B), irregular nucleus shape(C), and cell lysis(D).

(*C Control; *G1: Group 1 (Nanoplastic); *G2: Group 2 (Glyphosate); *G3: Group 3 (Nanoplatics + Glyphosate)

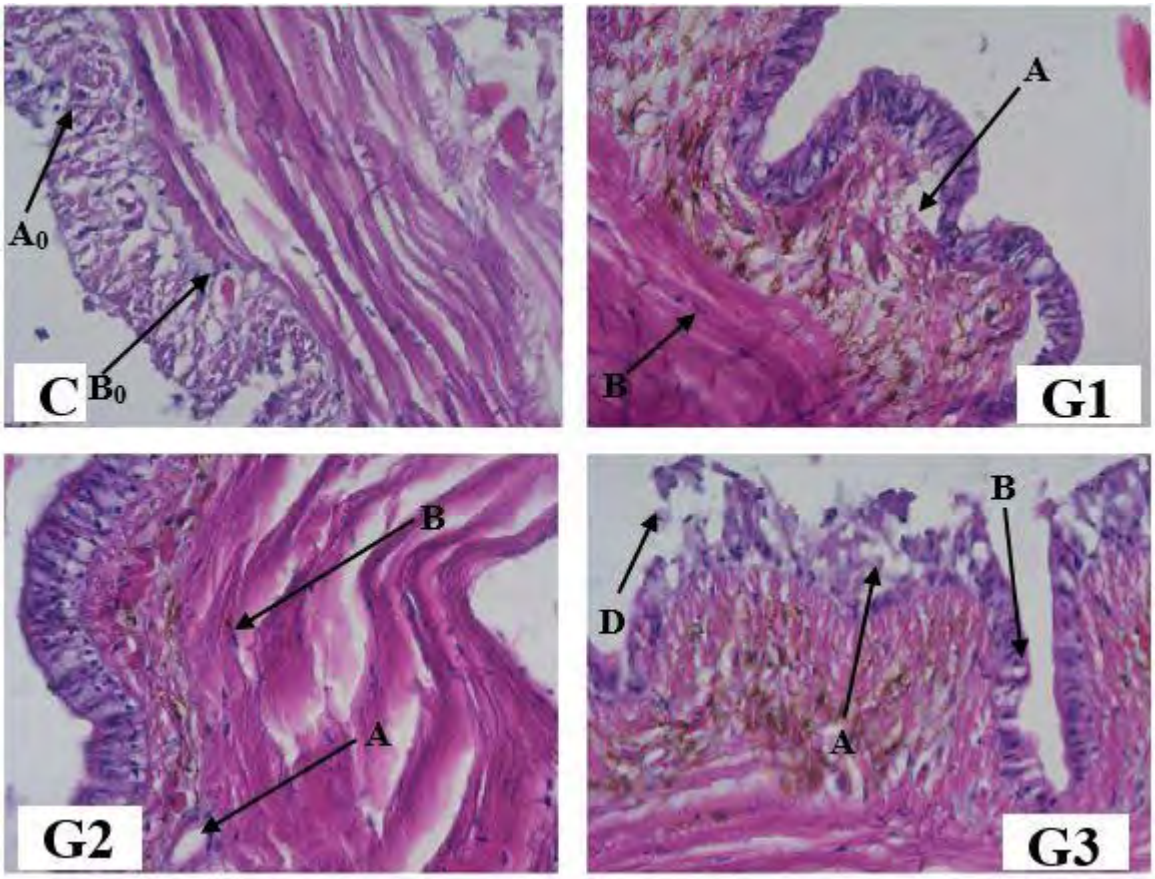


Figure 18: Photomicrographs of intestine of earthworms at 40X magnification, stained with hematoxylin and eosin, after the exposure of 21 days. The Control group (C) showed normal cell division (A_0), regular cell shape (B_0). While G1 showed, large number of cell space(A), indicating cell irregular cell shape (B). Moreover, the G2 showed large space between the cells(A), cell shape was not regular (B). The G3 showed wide enlarge space (A), irregular cell shape (B), and shows the cell lysis(D).

(*C Control; *G1: Group 1 (Nanoplastic); *G2: Group 2 (Glyphosate); *G3: Group 3 (Nanoplatics + Glyphosate)

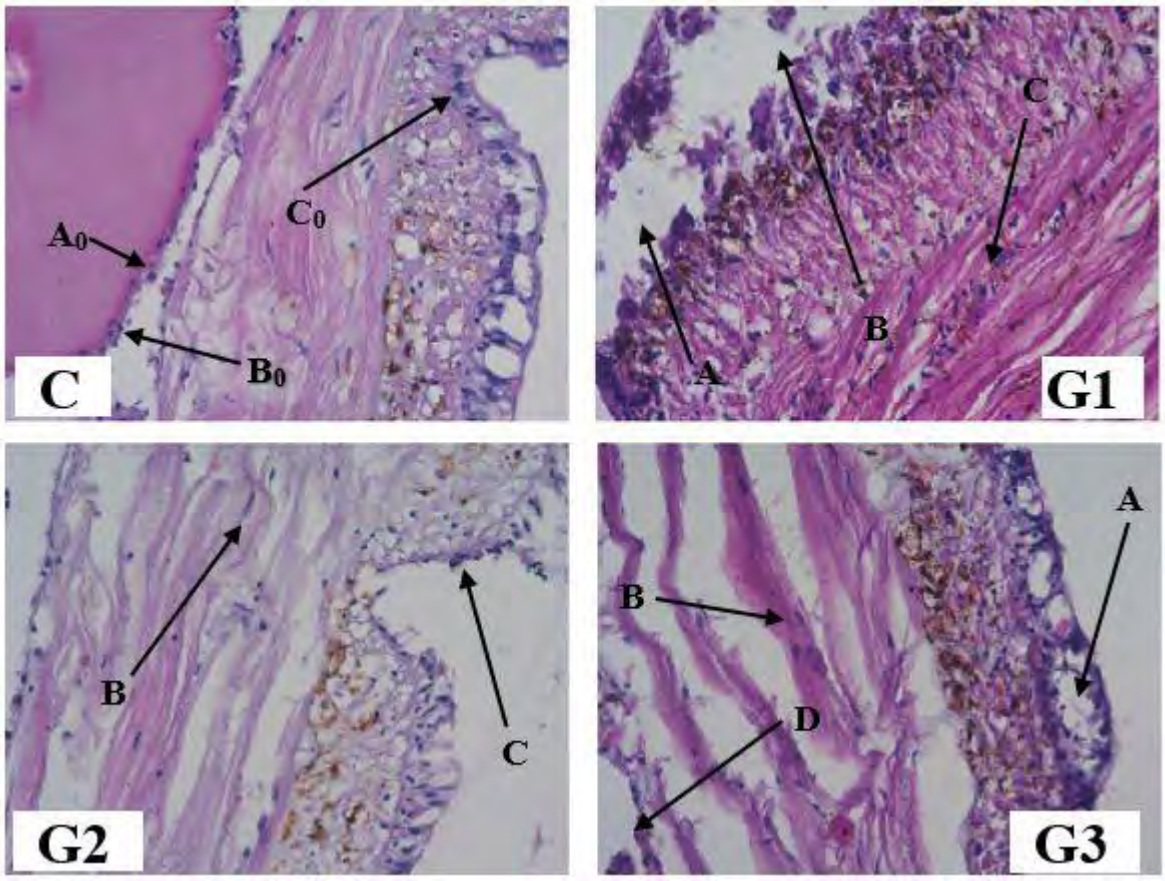


Figure 19: Photomicrographs of intestine of earthworms at 40X magnification, stained with hematoxylin and eosin, after the exposure of 28 days. The Control group (C) showed normal cell division (A_0), regular cell shape (B_0), and normal nucleus size (C_0). While G1 showed enlarged spaces in a wide area (A), total denaturing of the cell shape (B), and Irregular nucleus shape (C). Moreover, the G2 indicating irregular cell shape (B) and abnormal nucleus shape (C). The G3 showed large cell spaces in the intestine (A), denatured cell shapes (B), and all the cells were break down into pieces (D).

(*C Control; *G1: Group 1 (Nanoplastic); *G2: Group 2 (Glyphosate); *G3: Group 3 (Nanoplatics + Glyphosate)

Chapter 4

Discussion

4. DISCUSSION

4.1. Soil Physicochemical Properties

All soil quality parameters were maintained following the OECD guidelines for testing of chemicals (OECD, 1984). Soil quality was maintained throughout the experiment, moisture content, water holding capacity, organic content and pH in accordance with OECD guidelines. Moreover, water moisture content was maintained by spraying the water after every 2 days and soil quality parameters remained within permissible limits as recommended by OECD guidelines. Results for the soil quality parameters were consistent with experimental conditions of Sobhani et al. (2021).

4.2. Acetylcholinesterase Activity

Acetylcholinesterase (AChE) plays a pivotal role in regulation of cholinergic signal transduction within the nervous system (Song et al., 2022; Xu et al., 2020a). To maintain the proper functioning of the nervous system, AChE catalyzes the breakdown of the neurotransmitter acetylcholine (ACh) into choline and acetic acid. The inhibition of AChE indicates neurotoxicity, as it disrupts this process (Palanikumar et al., 2012). The reduction in AChE activity following exposure to pollutants is strongly linked to the decline in the organism's in vivo antioxidant defense capacity (Song et al., 2022). In the current study, a progressive inhibition of AChE was observed by exposure to glyphosate. These findings are consistent with Lin et al. (2019) which reported that polystyrene nanoplastics led to notable decreases in AChE activity, suggesting harmful impacts on cholinergic neurotransmission as well as nervous and neuro-muscular functioning. Inhibited AChE activity leads to a buildup of acetylcholine in the synaptic region and the disruption of nerve function, can be brought on by long-term exposure of *E. fetida* worms to nanoplastics (He at al., 2023). Notably, organophosphorus insecticides are recognized for their ability to suppress AChE activity, whereas GLY has been known to manifest toxicity in manner similar to most organophosphorus herbicide (Roex et al., 2003). AChE activity was also found to be reduced in *Cyprinus carpio* L. following the exposure to glyphosate over 60 days (Chen et al., 2022). Our results also showed the reduction in AChE activity which can be attributed to the inhibition of enzyme function caused by the substantial generation of reactive oxygen species (ROS), resulting in neurotoxic effects (Jiang et al., 2022). AChE activity in *E. fetida* in the combined treatment of (glyphosate + nanoplastic) was lower than that in the individual treatment. These findings were found to be same as Deng et al. (2018) who also reported that combined exposure

with organophosphorus herbicide and nanoplatics led to higher AChE inhibition in earthworm brain as compared to the exposure separately. Likewise, polystyrene nanoparticles (1 mg/L; 50 nm) has been shown to notably reduce the activity of brain AChE in zebrafish (Chen et al., 2017). Our results also indicated a decrease in AChE activity in earthworms following combined exposure of GLY and nanoplastics and individually. Our findings are in accordance with the study of Chen et al. (2017) in which it is reported that smaller-sized nanoplastics could potentially lead to higher inhibition of AChE activity.

4.3. Total Protein determination in earthworms

Proteins are integral to metabolic pathways and serve as a diagnostic tool for evaluating an organism's physiological condition (Prasath and Arivoli, 2008). This study showed the progressive decline in total protein content over an entire experiment period. These findings are consistent with the Lammertyn et al. (2021). The results demonstrate a notable reduction in protein content, under the both single and joint exposure of (nanoplastics + glyphosate) consistent with the findings of Li et al. (2019) which showed that increase in the energy required to detoxify and excrete the harmful compound leading to a degradation in the energy spent for the earthworm growth.

4.4. Reactive Oxygen Species

Reactive oxygen species (ROS) are reactive oxygen-containing molecules, which encompass the superoxide anion (radical O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (radical OH). These species are generated through normal metabolic processes within the body (He et al., 2021b). Elevated ROS within the organism typically indicate an increased oxidative stress. Polystyrene nanoplatics have been shown to trigger the production of ROS in organisms, resulting in the depletion of antioxidants and potentially causing adverse impacts on behavior and survival (Pitt et al., 2018; Wuk et al., 2016; Jeong et al., 2016, 2017). These findings are according to our results and showed increased ROS level during the exposure period. Lin et al. (2019) documented that nanoplastic-triggered ROS production could result in oxidative stress and harm to organisms. When *Cyprinus carpio* L. was exposed to glyphosate, our findings indicated an elevation in ROS levels, comparing with the observations made by Lee et al. (2021). The excessive production of ROS can contribute to cellular oxidative damage, which has been associated with the possibility of mitochondrial dysfunction as indicated by Lee et al. (2021). Moreover, research conducted by Truzzi et al. (2021) revealed that GLY exhibited comparable cytotoxic effects to other organophosphorus pesticides such as chlorpyrifos. Elevated ROS generation subsequent to 30-day exposure to glyphosate has been documented

(Cao et al., 2022). In the combined treatment of nanoplatics and glyphosate, the ROS were observed to be higher than those in the single treatments, supported with the findings reported by Nogueira et al. (2022). This suggests that both GLY and PSNPs, whether individually or when combined, caused mitochondrial dysfunction linked to ROS, (Xie et al., 2007). Similarly, in our results, we also noted an increase in ROS within the combined treatment of earthworms.

4.5. Malondialdehyde (MDA)

Regarded as the principal biomarker of cellular damage in conditions of oxidative stress, MDA emerges as a significant outcome of lipid peroxidation (Araujo et al., 2022). Derived from the oxidation of cellular lipid membranes, MDA can indirectly reflect the extent of oxidative harm inflicted upon organisms due to pollutants (Lin et al., 2012; Saint-Denis et al., 2001). Elevated MDA activities, signifying lipid peroxidation, were noted in reaction to the PSNPs. This also led to the adjustment of antioxidant enzymes, such as SOD on *Daphnia magna* (Lin et al., 2019). Lu et al. (2015) demonstrated in their study, that the affinity binding of NPs to the lipid bilayer depended on interactions involving particle-cell wall interactions, which seemed to possess an electrostatic nature. Our results are in accordance with these findings revealing increased MDA content in both the nanoplatics group and all other exposure groups (Lin et al., 2019). Individual exposure of glyphosate resulted in elevated MDA content, which are agreed with the findings of Cao et al. (2022), where exposure of *Cyprinus carpio* L. to glyphosate similarly led to an increase in MDA content.

When subjected to aryl triazolinones herbicide sulfentrazone exposure to earthworms, there was also a rise in MDA content as demonstrated by Li et al. (2020). Further, the study by Tiwari et al. (2019) which revealed a significant increase in MDA content due to the exposure of organophosphate insecticide chlorpyrifos, cypermethrin, and their combination in earthworms. Similar outcomes are also observed in present study which indicated a notable increase in MDA content in earthworms upon combined exposure to nanoplatics and glyphosate.

4.6. Antioxidant Enzymes

4.6.1. Super Oxide Dismutase (SOD)

SOD serves as a crucial defense mechanism in the body against oxidative damage, actively eliminating free radicals generated within the body as evidenced in following studies (Wang et al., 2020; Cruz-Rojas et al., 2018;). Hydrogen peroxide (H_2O_2) is a potent reactive oxygen species (ROS) that possesses the capacity to inflict extensive cellular harm, contributing to the induction of oxidative stress as well (Liu et al., 2018). Elevated enzyme activities suggest that

SOD aims to effectively eliminate surplus reactive oxygen species ($\bullet O^{2-}$), thus alleviating the oxidative harm inflicted on *E. fetida* due to nanoplatics exposure. Conversely, the reduction in SOD activity could stem from the utilization of exceedingly active $\bullet O^{2-}$, a portion of which is converted into hydrogen peroxide through the actions of SOD (He et al., 2022c; Yao et al.,2020). Our findings demonstrated a comparable pattern, initially observing an increase in SOD due to nanoplatics exposure in the group of earthworms, followed by a notable decline as time progressed. Our outcomes of a significant reduction in SOD activity exposure to the nanoplatics align with the observations (He et al., 2023). In present study, significant decrease in SOD activity by the exposure of glyphosate to the earthworms, these results are consistence with exposure of glyphosate on *Cyprinus carpio* L. for 30 days (Cao et al., 2022). Zheng et al. (2021) also noted a decline in SOD activity as a result of glyphosate (GLY) exposure in the liver of tilapia (*Oreochromis niloticus*). In both individual and combined exposures to the nanoplatics and glyphosate, the current study observed a decrease in SOD activity in all treatments. Likewise, Cheng et al. (2020) demonstrated that exposure of *E. fetida* to soil contaminated with microplastics and atrazine resulted in the inhibition of SOD activity. These findings are similar to the outcomes of Baihetiyaer et al. (2023), who also observed comparable results in earthworms exposed to a combination of microplastics and imidacloprid over a 28-day period a significant decrease in SOD activity.

4.6.2. Catalase Activity (CAT)

CAT stands as another pivotal antioxidant enzyme, responsible for detoxifying H_2O_2 by transforming it into innocuous H_2O and O_2, as elucidated by these studies (Liu et al., 2018a; Zhang et al., 2018). The equilibrium of redox reactions is disrupted due to an excess of free radicals, consequently inhibit the synthesis of CAT (Xu k. et al., 2021). Our results indicated an initial rise in CAT activity, possibly attributed to the excessive production of ROS. However, this activity subsequently experienced a significant decline across all treatments. These findings are consistence with (He et al., 2023). This phenomenon can be elucidated by the notion that stress induced by nanoplatics prompts an overproduction of ROS or other free radicals, surpassing the detoxification capacity of CAT. Consequently, the activity of CAT diminishes, as observed in these studies (Guimarães et al., 2021; Li et al., 2020b). These outcomes are in line with our findings, wherein exposure of earthworms to nanoplatics demonstrated a notable decrease in CAT activity across all treatments. The findings indicated a substantial reduction in CAT activity across all treatments upon glyphosate exposure to earthworms, in agreement with the results reported by Santos et al. (2011). Cheng et al. (2020)

demonstrated the inhibition of CAT activity in *E. fetida* when subjected to soil contamination involving microplastics and atrazine. Our findings are in line with these results, as we observed that the individual as well as combined exposure to nanoplastics and glyphosate led to the inhibition of CAT activity.

4.7. Comet Assay to Analyze DNA Damage

The comet assay stands as a simple approach to evaluate DNA damage provoked by environmental pollutants. Widely recognized as a sensitive biomarker, it effectively determines the genotoxic impact of pollutants on invertebrates, as evidenced by in these studies (Gajski et al., 2019; Saez et al., 2015; Reinecke and Reinecke, 2004). The extent of DNA damage in worm coelomocytes can be evaluated using the tail DNA% metric, which quantifies the ratio of DNA in the tail to the total DNA within the nucleus, following a methodology documented by Anderson et al. (1994). Point toward an elevation in ROS levels within earthworms triggered by the exposure to nanoplastics, ultimately resulting in oxidative stress and subsequent oxidative damage (He et al., 2023). Upon exposure to nanoplastics, there was a decrease in head length and an increase in %DNA in tail length, accompanied by a significant rise in tail moment. A similar pattern of these parameters was evident as exposure time increased, culminating in the highest DNA damage observed after 28 days of exposure. Our findings are in line with He et al. (2023), conducted a study involving the exposure of earthworms to nanoplatics significantly decreased head length and increase in tail length. The outcomes of this study provide compelling evidence that PS NPs possessed the ability to target erythrocyte DNA, leading to both clastogenic and molecular impairments. To be more specific, the observed DNA damage is likely attributed to various mechanisms, including single-strand breaks, double-strand breaks, the formation of DNA adducts, and cross-linking between DNA molecules and proteins. These events are presumed to stem from interactions between pollutants or their metabolites and DNA. All of these mechanisms are consistent with the findings of (Guimaraes et al., 2021). Our results showed that DNA damage was induced by glyphosate exposure on earthworms and slowly increased in tail moment because of migrating DNA from head to tail, likely due to an abundance of ROS and a compromised antioxidant enzyme system. Furthermore, instances of DNA damage triggered by GLY have been recorded in the blood cells of various species, including the Chinese mitten crab (*Eriocheir sinensis*), rainbow trout (*Oncorhynchus mykiss*), and Nile tilapia (*Oreochromis niloticus*). Our findings are in accordance with the results of the following studies (Santos et al., 2019; Hong et al., 2017; Alvarez-Moya et al., 2014). Moreover, the impact on DNA integrity in *Eisenia andrei*

was notable specifically when exposed concurrently to dichlorophenoxy-acetic acid (2,4-D) and microplastic (MP). This outcome can be attributed to the established genotoxic effects associated with both MP and 2,4-D (Boughattas et al., 2022). This is illustrated by the increased MDA levels observed in earthworms exposed to the combination, providing additional insight into the observed genotoxic effect. Our results are in accordance with the study of Huang et al. (2021). Similarly, Huang et al. (2021) unveiled that the exposure to MP and Cd induces significant harm to earthworms. These findings are in accordance with our results. In present study, as a result of coexposure of (nanoplatics + glyphosate), tail length is increased with increasing time in all treatments because, long tail length indicates more DNA damage. These findings align with the investigation conducted by Boughattas et al. (2022) on earthworms, where a combined exposure to microplastics and the herbicide 2,4-D indicate similar results.

4.8. Histopathological Alterations in Intestine of Earthworms

Histopathology stands as a prevalent technique for evaluating the deleterious impacts of diverse stressors on soil invertebrates, as highlighted by He et al. (2022a). Soil-dwelling pollutants can be taken up by earthworms through digestive absorption and or direct contact involving the digestive tract and epidermis, as explained by Yang et al. (2020). Our result showed that within control group, all examined sections exhibited normal histological features of earthworm intestines, characterized by visible cell divisions, consistent cell shapes, and nucleus sizes. Conversely, earthworm intestines exposed to polystyrene nanoplastics displayed enlarged intestinal cells with irregular shapes, these findings are in accordance with the study of Jiang et al. (2020), he conducted research on the toxicity of polystyrene microplastics on earthworms over a duration of 14 days. It is widely recognized that smaller microplastics have a tendency to be more retained within the earthworm intestine owing to their feeding preferences (Rodriguez-Seijo et al., 2017; Huerta Lwanga et al., 2016; Curry and Schmidt, 2007). In this study, we also employed nanoplastics with a size of 30nm, and it was observed that the smaller plastic particles inflicted greater harm on the organisms, leading to the lysis of intestinal cells. Our results are in accordance with (Jiang et al., 2020). The enhanced accumulation and greater occurrence of abnormal histopathological effects on earthworms due to elevated microplastic concentrations were also documented by Rodriguez-Seijo et al. (2017). These findings correspond with the heightened mechanical damage observed in the earthworm intestines resulting from the 30nm nanoplastic. Examination of gut histopathology revealed that glyphosate exposure on *Cyprinus carpio* L. has the potential to induce pathological occurrences such as villi tip shedding, necrosis, and edema. It is postulated that extended exposure to

glyphosate might lead to impairment of the intestinal physical barrier (Ramos et al., 2017). Our findings also indicate that glyphosate exposure leads to phenomena like cell lysis, irregular cell division, and intestinal damage, as observed in the study by (Cao et al., 2022). The coexposure of herbicide penoxsulam (PNX) and sliver nanoparticle (AgNPs) on earthworms for 56 days resulted in alterations to the structural integrity of earthworm tissue, leading to variable degrees of tissue damage. Coexposure induced notable harm to the intestinal tissue, evident as the thinning of the intestinal wall and enlargement of the intestinal space (Le et al., 2022). Our findings demonstrate that the combined exposure to (NPs + GLY) induces greater intestinal damage, accompanied by intestinal enlargement and irregular nucleus sizes, which are in line with the observations made by Le et al. (2022).

Chapter 5

Conclusions and Recommendations

5. CONCULSION AND RECOMMENDATIONS

To our knowledge, this is the first study on the influence of polystyrene nanoplatics and glyphosate using *Eisenia fetida* as the model organism. The findings of present study showed individual and combined exposure to glyphosate and nanoplatics induced toxicity. Various parameters were examined in this study, including oxidative stress indicators such as ROS and MDA content in earthworm tissues, activity of antioxidant enzymes, acetylcholinesterase inhibition in the brain, DNA damage in earthworm coelomocytes, and histopathological alterations in the intestine. Overall, it is clear that acetylcholinesterase activity was consistently inhibited throughout the entire experimental duration. Furthermore, exposure to the pollutants nanoplatics and glyphosate led to elevated ROS and MDA content in comparison to the control group. Moreover, the activities of antioxidant enzymes (SOD and CAT) exhibited a significant decrease as the duration of time increased. Both nanoplastics and glyphosate, as pollutants, lead to a significant increase in DNA damage. This is accompanied by a progressive rise in tail length and tail moment, coupled with a decline in head length. Likewise, both pollutants induced profound histopathological changes within the earthworm intestine, resulting in irregular cell shapes and cell lysis. Toxic contaminants are released in to environment and may harm the terrestrial invertebrates. This information can be helpful in the future for the studies on the toxicological effects of nanoplatics and glyphosate. To date, there has been no research conducted in Pakistan concerning nanoplastic. It is suggested to investigate the nanoplatics toxicity in the soil as well as exposure of contaminants in agriculture soil and their effects on other biota and crops. These findings can provide valuable understanding into the toxicological consequences of nanoplastics and their potential interactions with other coexisting contaminants within soil ecosystems. It is crucial to emphasize that gaining a more comprehensive understanding of the collective toxicity of Glyphosate (GLY) and polystyrene nanoplastics (PSNPs) when present together as a mixture requires additional research. Expanding our knowledge about the environmental impact of these contaminants, particularly their effects on non-target organisms within complex mixtures, is essential for conducting a more precise risk assessment. Furthermore, this research can provide fresh perspectives on how nanoparticles induce toxicity in earthworms and highlights an area that may help in further investigation in future studies.

Bibliography

REFERENCES

Achiorno, C. L., de Villalobos, C., and Ferrari, L. (2008). Toxicity of the herbicide glyphosate to Chordodes nobilii (*Gordiida, nematomorpha*). *Chemosphere*, *71*(10), 1816-1822.

Ahmed, S. T. (2013). The impact of four pesticides on the earthworm *Lumbricus terrestris* (Annelida; Oligochaeta). *International Journal of Current Research and Review*, *5*(21), 1.

Amaral, A., Soto, M., Cunha, R., Marigómez, I., and Rodrigues, A. (2006). Bioavailability and cellular effects of metals on *Lumbricus terrestris* inhabiting volcanic soils. *Environmental Pollution*, *142*(1), 103-108.

Andersson, E., and Isgren, E. (2021). Gambling in the garden: Pesticide use and risk exposure in Ugandan smallholder farming. *Journal of Rural Studies*, *82*, 76-86.

Alvarez-Moya, C., Reynoso Silva, M., Valdez Ramírez, C., Gómez Gallardo, D., León Sánchez, R., Canales Aguirre, A., and Feria Velasco, A. (2014). Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms. *Genetics and Molecular Biology*, 37, 105-110.

Andrady, A. L., and Neal, M. A. (2009). Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 1977-1984.

Anderson, D. Y. T. W., Yu, T. W., Phillips, B. J., and Schmezer, P. (1994). The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 307(1), 261-271.

Andreotti, G., Koutros, S., Hofmann, J. N., Sandler, D. P., Lubin, J. H., Lynch, C. F., ... and Beane Freeman, L. E. (2018). Glyphosate use and cancer incidence in the agricultural health study. *JNCI: Journal of the National Cancer Institute*, *110*(5), 509-516.

Bajwa, A. A., Chauhan, B. S., Farooq, M., Shabbir, A., and Adkins, S. W. (2016). What do we really know about alien plant invasion? A review of the invasion mechanism of one of the world's worst weeds. *Planta*, *244*(1), 39-57.

Balazs, G. H., and Chaloupka, M. (2004). Spatial and temporal variability in somatic growth of green sea turtles (*Chelonia mydas*) resident in the Hawaiian Archipelago. *Marine Biology*, *145*(5), 1043-1059.

Balazs, G. H., Van Houtan, K. S., Hargrove, S. A., Brunson, S. M., and Murakawa, S. K. (2015). A review of the demographic features of Hawaiian green turtles (*Chelonia mydas*). *Chelonian Conservation and Biology*, *14*(2), 119-129.

Battaglin, W. A., Meyer, M. T., Kuivila, K. M., and Dietze, J. E. (2014). Glyphosate and its degradation product AMPA occur frequently and widely in US soils, surface water, groundwater, and precipitation. *JAWRA Journal of the American Water Resources Association*, *50*(2), 275-290.

Benbrook, C. M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe*, *28*(1), 1-15.

Benbrook, C. M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe*, *28*(1), 1-15.

Bento, C. P., Yang, X., Gort, G., Xue, S., van Dam, R., Zomer, P., ... and Geissen, V. (2016). Persistence of glyphosate and aminomethylphosphonic acid in loess soil under different combinations of temperature, soil moisture and light/darkness. *Science of the Total Environment*, *572*, 301-311.

Boughattas, I., Zitouni, N., Hattab, S., Mkhinini, M., Missawi, O., Helaoui, S., ... and Banni, M. (2022). Interactive effects of environmental microplastics and 2, 4 dichlorophenoxyacetic acid (2, 4-D) on the earthworm Eisenia andrei. *Journal of Hazardous Materials,* 424, 127578.

Besseling, E., Redondo-Hasselerharm, P., Foekema, E. M., and Koelmans, A. A. (2019). Quantifying ecological risks of aquatic micro-and nanoplastic. *Critical Reviews in Environmental Science and Technology*, *49*(1), 32-80.

Besseling, E., Wang, B., L. rling, M., and Koelmans, A. A. (2014). Nanoplatics affects growth of S. obliquus and reproduction of *D. magna*. *Environmental Science and Technology*, *48*(20), 12336-12343.

Bishop, C. A., Woundneh, M. B., Maisonneuve, F., Common, J., Elliott, J. E., and Moran, A. J. (2020). Determination of neonicotinoids and butenolide residues in avian and insect pollinators and their ambient environment in Western Canada (2017, 2018). *Science of The Total Environment*, *737*, 139386.

Blettler, M., Ulla, M. A., Rabuffetti, A. P., and Garello, N. (2017). Plastic pollution in freshwater ecosystems: macro-, meso-, and microplastic debris in a floodplain lake. *Environmental Monitoring and Assessment*, *189*(11), 1-13.

Baihetiyaer, B., Jiang, N., Li, X., He, B., Wang, J., Fan, X., ... and Yin, X. (2023). Oxidative stress and gene expression induced by biodegradable microplastics and imidacloprid in earthworms (*Eisenia fetida*) at environmentally relevant concentrations. *Environmental Pollution*, 323, 121285.

Blouin, M., Hodson, M. E., Delgado, E. A., Baker, G., Brussaard, L., Butt, K. R., ... and Brun, J. J. (2013). A review of earthworm impact on soil function and ecosystem services. *European Journal of Soil Science*, *64*(2), 161-182.

Bo-Kai Zhu a, b, Yi-Meng Fang b, Dong Zhu c, d, Peter Christie e, Xin Ke f, Yong-Guan Zhu (2018) Exposure to nanoplastics disturbs the gut microbiome in the soil oligochaete *Enchytraeus crypticus*. *Environmental Pollution*, 239 (2018) 408-415

Brandts, I., Teles, M., Gonçalves, A. P., Barreto, A., Franco-Martinez, L., Tvarijonaviciute, A., ... and Oliveira, M. (2018). Effects of nanoplastics on Mytilus galloprovincialis after individual and combined exposure with carbamazepine. *Science of the Total Environment*, *643*, 775-784.

Briones, M. J. I., Garnett, M. H., and Piearce, T. G. (2005). Earthworm ecological groupings based on 14C analysis. *Soil Biology and Biochemistry*, *37*(11), 2145-2149.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., and Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environmental Science and Technology*, *42*(13), 5026-5031.

Browne, M. A., Dissanayake, A., Galloway, T. S., Lowe, D. M., and Thompson, R. C. (2008). Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environmental Science and Technology*, *42*(13), 5026-5031.

Bai, S. H., and Ogbourne, S. M. (2016). Glyphosate: environmental contamination, toxicity and potential risks to human health via food contamination. *Environmental Science and Pollution Research*, 23, 18988-19001.

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., and Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the total environment*, 584, 1022-1031.

Cao, X., Rao, C., Cui, H., Sun, D., Li, L., Guo, S., ... and Chen, J. (2022). Toxic effects of glyphosate on the intestine, liver, brain of carp and on epithelioma papulosum cyprinid cells: Evidence from in vivo and in vitro research. *Chemosphere*, 302, 134691.

Caruso, G. (2019). Microplastics as vectors of contaminants. *Marine Pollution Bulletin*, *146*, 921-924.

Chae, Y., Kim, D., Kim, S. W., and An, Y. J. (2018). Trophic transfer and individual impact of nano-sized polystyrene in a four-species freshwater food chain. *Scientific Reports*, *8*(1), 1-11.

Curry, J. P., and Schmidt, O. (2007). The feeding ecology of earthworms–a review. *Pedobiologia*, 50(6), 463-477.

Cheng, Y., Zhu, L., Song, W., Jiang, C., Li, B., Du, Z., ... and Zhang, K. (2020). Combined effects of mulch film-derived microplastics and atrazine on oxidative stress and gene expression in earthworm (*Eisenia fetida*). *Science of the Total Environment*, 746, 141280.

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., and Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the Total Environment*, *584*, 1022-1031.

Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., and Hollert, H. (2017). Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. *Science of the Total Environment*, *584*, 1022-1031.

Choong, W. S., Hadibarata, T., and Tang, D. K. H. (2021). Abundance and distribution of microplastics in the water and riverbank sediment in Malaysia—A review. *Biointerface Res. Appl. Chem*, *11*(4), 11700-11712.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., and Galloway, T. S. (2015). The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. *Environmental Science and Technology*, *49*(2), 1130-1137.

Contreras-Ramos, S. M., Alvarez-Bernal, D., and Dendooven, L. (2008). Removal of polycyclic aromatic hydrocarbons from soil amended with biosolid or vermicompost in the presence of earthworms (*Eisenia fetida*). *Soil Biology and Biochemistry*, *40*(7), 1954-1959.

Cortés, C., Domenech, J., Salazar, M., Pastor, S., Marcos, R., and Hernández, A. (2020). Nanoplastics as a potential environmental health factor: effects of polystyrene nanoparticles on human intestinal epithelial Caco-2 cells. *Environmental Science: Nano*, *7*(1), 272-285.

Cui, R., Kim, S. W., and An, Y. J. (2017). Polystyrene nanoplastics inhibit reproduction and induce abnormal embryonic development in the freshwater crustacean *Daphnia galeata*. *Scientific Reports*, *7*(1), 1-10.

Cunha, M. R., Lima, E. C., Lima, D. R., da Silva, R. S., Thue, P. S., Seliem, M. K., ... and Larsson, S. H. (2020). Removal of captopril pharmaceutical from synthetic pharmaceutical-industry wastewaters: Use of activated carbon derived from Butia catarinensis. *Journal of Environmental Chemical Engineering*, *8*(6), 104506.

Cruz-Rojas, C., SanJuan-Reyes, N., Fuentes-Benites, M. P. A. G., Dublan-García, O., Galar-Martínez, M., Islas-Flores, H., and Gómez-Oliván, L. M. (2019). Acesulfame potassium: Its ecotoxicity measured through oxidative stress biomarkers in common carp (*Cyprinus carpio*). *Science of the Total Environment*, 647, 772-784.

Curry, J. P., and Schmidt, O. (2007). The feeding ecology of earthworms–a review. *Pedobiologia*, *50*(6), 463-477.

da Costa, J. P., Paço, A., Santos, P. S., Duarte, A. C., and Rocha-Santos, T. (2018). Microplastics in soils: assessment, analytics and risks. *Environmental Chemistry*, *16*(1), 18-30.

da Costa, J. P., Paço, A., Santos, P. S., Duarte, A. C., and Rocha-Santos, T. (2018). Microplastics in soils: assessment, analytics and risks. *Environmental Chemistry*, *16*(1), 18-30.

de Farias Araujo, G., Soares, L. O. S., Junior, S. F. S., de Carvalho, L. V. B., Rocha, R. C. C., Saint'Pierre, T., ... and Saggioro, E. M. (2022). Oxidative stress and metal homeostasis alterations in *Danio rerio* (zebrafish) under single and combined carbamazepine, acetamiprid and cadmium exposures. *Aquatic Toxicology*, 245, 106122.

Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., ... and Pei, D. S. (2014). Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry*, *33*(1), 11-17.

De F Sousa, M. G., da Silva, A. C., dos Santos Araújo, R., and Rigotto, R. M. (2019). Evaluation of the atmospheric contamination level for the use of herbicide glyphosate in the northeast region of Brazil. *Environmental Monitoring and Assessment*, *191*(10), 1-11.

De Sá, L. C., Oliveira, M., Ribeiro, F., Rocha, T. L., and Futter, M. N. (2018). Studies of the effects of microplastics on aquatic organisms: what do we know and where should we focus our efforts in the future? *Science of the Total Environment*, *645*, 1029-1039.

Deveau, A. P., Bentley, V. L., and Berman, J. N. (2017). Using zebrafish models of leukemia to streamline drug screening and discovery. *Experimental Hematology*, *45*, 1-9.

Diego Jose Nogueira, Aline Conceicao de Oliveira da Silva, Marlon Luiz Neves da Silva, Denice Schulz Vicentini, William Gerson Matias. (2022). Individual and combined multigenerational induced by polystyrene nanoplatics and glyphosate in *Daphnia magna* (Strauss, 1820). *Science of The Total Environment,* 811, 151360.

Dollinger, J., Dagès, C., and Voltz, M. (2015). Glyphosate sorption to soils and sediments predicted by pedotransfer functions. *Environmental Chemistry Letters*, *13*(3), 293-307.

EFSA (2013) European Food Safety Authority (EFSA) glyphosate renewal assessment report of 18 December 2013 Rapporteur Member State (RMS): Germany, Co-RMS: Slovakia

Everaarts, J. M., Den Besten, P. J., Hillebrand, M. T. J., Halbrook, R. S., and Shugart, L. R. (1998). DNA strand breaks, cytochrome P-450-dependent monooxygenase system activity and levels of chlorinated biphenyl congeners in the pyloric caeca of the seastar (Asterias rubens) from the North Sea. Ecotoxicology, 7(2), 69-79.

Deng, Y., Zhang, Y., Qiao, R., Bonilla, M. M., Yang, X., Ren, H., and Lemos, B. (2018). Evidence that microplastics aggravate the toxicity of organophosphorus flame retardants in mice (Mus musculus). Journal of Hazardous Materials, 357, 348-354.

Eisenhauer, N., and Eisenhauer, E. (2020). The "intestines of the soil": the taxonomic and functional diversity of earthworms–a review for young ecologists.

Eisenhauer, N., Fisichelli, N. A., Frelich, L. E., and Reich, P. B. (2012). Interactive effects of global warming and 'global worming'on the initial establishment of native and exotic herbaceous plant species. *Oikos*, *121*(7), 1121-1133.

Enfrin, M., Lee, J., Gibert, Y., Basheer, F., Kong, L., and Dumée, L. F. (2020). Release of hazardous nanoplatics contaminants due to microplastics fragmentation under shear stress forces. *Journal of Hazardous Materials*, *384*, 121393.

European Food Safety Authority (EFSA). (2017). Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate. *EFSA Journal*, *15*(9), e04979.

European Food Safety Authority (EFSA). (2019). The 2017 European Union report on pesticide residues in food. *EFSA Journal*, *17*(6), e05743.

Fang, W., Peng, Y., Muir, D., Lin, J., and Zhang, X. (2019). A critical review of synthetic chemicals in surface waters of the US, the EU and China. *Environment International*, *131*, 104994.

Farrell, P., and Nelson, K. (2013). Trophic level transfer of microplastic: *Mytilus edulis* (L.) to *Carcinus maenas* (L.). *Environmental Pollution*, 177, 1-3.

Foetisch, A., Filella, M., Watts, B., Vinot, L. H., and Bigalke, M. (2022). Identification and characterisation of individual nanoplastics by scanning transmission X-ray microscopy (STXM). *Journal of Hazardous Materials*, *426*, 127804.

Frehland, S., Kaegi, R., Hufenus, R., and Mitrano, D. M. (2020). Long-term assessment of nanoplatics particle and microplastic fiber flux through a pilot wastewater treatment plant using metal-doped plastics. *Water Research*, *182*, 115860.

Felder, D. P., D'surney, S. J., Rodgers, J. H., and Deardorff, T. L. (1998). A comprehensive environmental assessment of a receiving aquatic system near an unbleached kraft mill. Ecotoxicology, 7(6), 313-324.

Frydkjær, C. K., Iversen, N., and Roslev, P. (2017). Ingestion and egestion of microplastics by the cladoceran *Daphnia magna*: effects of regular and irregular shaped plastic and sorbed phenanthrene. *Bulletin of Environmental Contamination and Toxicology*, *99*(6), 655-661.

Guarda, V. L. M., Pereira, M. A., De Simone, C. A., Albuquerque, J. F. C., Galdino, S. L., Chantegrel, J., ... and Luu-Duc, C. (2003). Synthesis and structural study of arylidene thiazolidine and benzothiazine compounds. *Sulfur Letters*, *26*(1), 17-27.

Garcia, G. R., Noyes, P. D., and Tanguay, R. L. (2016). Advancements in zebrafish applications for 21st century toxicology. *Pharmacology and Therapeutics*, *161*, 11-21.

Gaylarde, C. C., Neto, J. A. B., and da Fonseca, E. M. (2021). Nanoplastics in aquatic systems-are they more hazardous than microplastics? *Environmental Pollution*, *272*, 115950.

Ghaffar, A., Hussain, R., Noreen, S., Abbas, G., Chodhary, I. R., Khan, A., ... and Niaz, M. (2020). Dose and time-related pathological and genotoxic studies on thiamethoxam in fresh water fish (*Labeo rohita*) in Pakistan. *Pakistan Veterinary Journal*, *40*(2), 151-156.

Gigault, J., Ter Halle, A., Baudrimont, M., Pascal, P. Y., Gauffre, F., Phi, T. L., ... and Reynaud, S. (2018). Current opinion: what is a nanoplastic? *Environmental Pollution*, *235*, 1030-1034.

Guimarães, A. T. B., Charlie-Silva, I., and Malafaia, G. (2021). Toxic effects of naturally-aged microplastics on zebrafish juveniles: a more realistic approach to plastic pollution in freshwater ecosystems. *Journal of Hazardous Materials*, 407, 124833.

Gigault, J., Ter Halle, A., Baudrimont, M., Pascal, P. Y., Gauffre, F., Phi, T. L., ... and Reynaud, S. (2018). Current opinion: what is a nanoplastic? *Environmental Pollution*, *235*, 1030-1034.

Giesy, J. P., Dobson, S., and Solomon, K. R. (2000). *Ecotoxicological Risk Assessment for Roundup® herbicide* (pp. 35-120). Springer New York.

Givaudan, N., Binet, F., Le Bot, B., and Wiegand, C. (2014). Earthworm tolerance to residual agricultural pesticide contamination: field and experimental assessment of detoxification capabilities. *Environmental Pollution*, *192*, 9-18.

Gomez-Brandon, M., Aira, M., Lores, M., and Domínguez, J. (2011). Epigeic earthworms exert a bottleneck effect on microbial communities through gut associated processes. *PloS one*, *6*(9), e24786.

Goulson, D. (2020). Pesticides, corporate Irresponsibility, and the fate of our Planet. *One Earth*, *2*(4), 302-305.

Graham, E. R., and Thompson, J. T. (2009). Deposit-and suspension-feeding sea cucumbers (*Echinodermata*) ingest plastic fragments. *Journal of Experimental Marine Biology and Ecology*, *368*(1), 22-29.

Guimarães, A. T. B., Estrela, F. N., Pereira, P. S., de Andrade Vieira, J. E., de Lima Rodrigues, A. S., Silva, F. G., and Malafaia, G. (2021). Toxicity of polystyrene nanoplastics in *Ctenopharyngodon idella* juveniles: a genotoxic, mutagenic and cytotoxic perspective. *Science of The Total Environment*, 752, 141937.

Greenwald, R. A. (2018). *Handbook methods for oxygen radical research*. CRC press.

Guo, J. J., Huang, X. P., Xiang, L., Wang, Y. Z., Li, Y. W., Li, H., ... and Wong, M. H. (2020). Source, migration and toxicology of microplastics in soil. *Environment International*, *137*, 105263.

Giovanetti, A., Fesenko, S., Cozzella, M. L., Asencio, L. D., and Sansone, U. (2010). Bioaccumulation and biological effects in the earthworm *Eisenia fetida* exposed to natural and depleted uranium. *Journal of Environmental Radioactivity*, *101*(6), 509-516.

He, F., Yu, H., Shi, H., Li, X., Chu, S., Huo, C., and Liu, R. (2022). Behavioral, histopathological, genetic, and organism-wide responses to phenanthrene-induced

oxidative stress in *Eisenia fetida* earthworms in natural soil microcosms. Environmental Science and Pollution Research, 29(26), 40012-40028.

Hackenberg, S., Scherzed, A., Kessler, M., Hummel, S., Technau, A., Froelich, K., ... and Kleinsasser, N. (2011). Silver nanoparticles: evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. *Toxicology Letters*, *201*(1), 27-33.

Hale, C. M., Frelich, L. E., and Reich, P. B. (2005). Exotic European earthworm invasion dynamics in northern hardwood forests of Minnesota, USA. *Ecological Applications*, *15*(3), 848-860.

Han, Y., Zhu, L., Wang, J., Wang, J., Xie, H., and Zhang, S. (2014). Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. *Ecotoxicology and Environmental Safety*, *107*, 214-219.

Han, Y., Zhu, L., Wang, J., Wang, J., Xie, H., and Zhang, S. (2014). Integrated assessment of oxidative stress and DNA damage in earthworms (*Eisenia fetida*) exposed to azoxystrobin. *Ecotoxicology and Environmental Safety*, *107*, 214-219.

Hao, Y., Zhao, L., Sun, Y., Li, X., Weng, L., Xu, H., and Li, Y. (2018). Enhancement effect of earthworm (*Eisenia fetida*) on acetochlor biodegradation in soil and possible mechanisms. *Environmental Pollution*, *242*, 728-737.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., ... and Geissen, V. (2016). Microplastics in the terrestrial ecosystem: implications for *Lumbricus terrestris* (Oligochaeta, *Lumbricidae*). *Environmental Science and Technology*, *50*(5), 2685-2691.

Hernandez, L. M., Yousefi, N., and Tufenkji, N. (2017). Are there nanoplastics in your personal care products? *Environmental Science and Technology Letters*, *4*(7), 280-285.

Hoang, D. T., Razavi, B. S., Kuzyakov, Y., and Blagodatskaya, E. (2016). Earthworm burrows: kinetics and spatial distribution of enzymes of C-, N-and P-cycles. *Soil Biology and Biochemistry*, *99*, 94-103.

He, F., Li, X., Huo, C., Chu, S., Cui, Z., Li, Y., ... and Liu, R. (2022). Evaluation of fluorene-caused ecotoxicological responses and the mechanism underlying its toxicity in *Eisenia fetida*: Multi-level analysis of biological organization. *Journal of Hazardous Materials*, 437, 129342.

Huang, C., Ge, Y., Yue, S., Zhao, L., and Qiao, Y. (2021). Microplastics aggravate the joint toxicity to earthworm *Eisenia fetida* with cadmium by altering its availability. *Science of the Total Environment*, 753, 142042.

He, F., Shi, H., Liu, R., Tian, G., Qi, Y., and Wang, T. (2023). Randomly-shaped nanoplastics induced stronger biotoxicity targeted to earthworm *Eisenia fetida* species: Differential effects and the underlying mechanisms of realistic and commercial polystyrene nanoplastics. *Science of The Total Environment*, 877, 162854.

Horton, A. A., Walton, A., Spurgeon, D. J., Lahive, E., and Svendsen, C. (2017). Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. *Science of the Total Environment*, *586*, 127-141.

Hong, Y., Yang, X., Yan, G., Huang, Y., Zuo, F., Shen, Y., ... and Cheng, Y. (2017). Effects of glyphosate on immune responses and haemocyte DNA damage of Chinese mitten crab, *Eriocheir sinensis*. *Fish and Shellfish Immunology*, 71, 19-27.

Hu, C. W., Li, M., Cui, Y. B., Li, D. S., Chen, J., and Yang, L. Y. (2010). Toxicological effects of TiO2 and ZnO nanoparticles in soil on earthworm *Eisenia fetida*. *Soil Biology and Biochemistry*, *42*(4), 586-591.

Halliwell, B., and Gutteridge, J. M. (2015). *Free Radicals in Biology and Medicine*. Oxford university press, USA.

Huang, K., and Xia, H. (2018). Role of earthworms' mucus in vermicomposting system: Biodegradation tests based on humification and microbial activity. *Science of the Total Environment*, *610*, 703-708.

Huang, Y., Zhao, Y., Wang, J., Zhang, M., Jia, W., and Qin, X. (2019). LDPE microplastic films alter microbial community composition and enzymatic activities in soil. *Environmental Pollution*, *254*, 112983.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., ... and Geissen, V. (2016). Microplastics in the terrestrial ecosystem: implications for *Lumbricus terrestris* (Oligochaeta: Lumbricidae). *Environmental science and technology*, *50*(5), 2685-2691.

Hackenberger, D. K., Palijan, G., Lončarić, Ž., Glavaš, O. J., and Hackenberger, B. K. (2018). Influence of soil temperature and moisture on biochemical biomarkers in earthworm and microbial activity after exposure to propiconazole and chlorantraniliprole. *Ecotoxicology and Environmental Safety*, *148*, 480-489.

Huerta Lwanga, E., Gertsen, H., Gooren, H., Peters, P., Salánki, T., Van Der Ploeg, M., ... and Geissen, V. (2016). Microplastics in the terrestrial ecosystem: implications for *Lumbricus terrestris* (Oligochaeta: Lumbricidae). *Environmental science and Technology*, *50*(5), 2685-2691.

He, F., Wan, J., Li, X., Chu, S., Sun, N., and Liu, R. (2021). Toxic effects of benzovindiflupyr, a new SDHI-type fungicide on earthworms (Eisenia fetida). *Environmental Science and Pollution Research*, 28(44), 62782-62795.

Hussain, R., Ali, F., Javed, M. T., Jabeen, G., Ghaffar, A., Khan, I., ... and Ghori, M. T. (2021). Clinico-hematological, serum biochemical, genotoxic and histopathological effects of trichlorfon in adult cockerels. *Toxin Reviews*, *40*(4), 1206-1214.

Hayashi, I., Morishita Y., Imai, k., Nakamura, M., Nakachi, K., and Hayashi, T.2007.

He, F., Shi, H., Liu, R., Tian, G., Qi, Y., and Wang, T. (2023). Randomly-shaped nanoplastics induced stronger biotoxicity targeted to earthworm *Eisenia fetida* species: Differential effects and the underlying mechanisms of realistic and commercial polystyrene nanoplastics. *Science of The Total Environment*, 877, 162854.

Jeong, C. B., Kang, H. M., Lee, M. C., Kim, D. H., Han, J., Hwang, D. S., ... and Lee, J. S. (2017). Adverse effects of microplastics and oxidative stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod Paracyclopina nana. Sci. Rep. 7, 41323. doi. org/10.1038/srep41323á, 2.

Jeong, C. B., Won, E. J., Kang, H. M., Lee, M. C., Hwang, D. S., Hwang, U. K., ... and Lee, J. S. (2016). Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK

and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). *Environmental Science and Technology*, *50*(16), 8849-8857.

Jiang, L., Lin, J. L., Jia, L. X., Liu, Y., Pan, B., Yang, Y., and Lin, Y. (2016). Effects of two different organic amendments addition to soil on sorption–desorption, leaching, bioavailability of penconazole and the growth of wheat (*Triticum aestivum* L.). *Journal of Environmental Management*, *167*, 130-138.

Jang, F. H., Wong, C., Choo, J., Sia, E. S. A., Mujahid, A., and Müller, M. (2022). Increased transfer of trace metals and Vibrio sp. from biodegradable microplastics to catfish *Clarias gariepinus*. *Environmental Pollution*, 298, 118850.

Johannaber, F., and Michaeli, W. (2002). *Handbuch Spritzgießen* (p. 319). München: Hanser.

Kah, M., Tufenkji, N., and White, J. C. (2019). Nano-enabled strategies to enhance crop nutrition and protection. *Nature Nanotechnology*, *14*(6), 532-540.

Karlsson, H. L. (2010). The comet assay in nanotoxicology research. *Analytical and Bioanalytical Chemistry*, *398*(2), 651-666.

Kashiwada, S. (2006). Distribution of nanoparticles in the see-through medaka (*Oryzias latipes*). *Environmental Health Perspectives*, *114*(11), 1697-1702.

Kausar, A., Sher, F., Hazafa, A., Javed, A., Sillanpää, M., and Iqbal, M. (2020). Biocomposite of sodium-alginate with acidified clay for wastewater treatment: Kinetic, equilibrium and thermodynamic studies. *International Journal of Biological Macromolecules*, *161*, 1272-1285.

Kılıç, G. A. (2011). Histopathological and biochemical alterations of the earthworm (*Lumbricus terrestris*) as biomarker of soil pollution along Porsuk River Basin (Turkey). *Chemosphere*, *83*(8), 1175-1180.

Kavitha, V., Anandhan, R., Alharbi, N. S., Kadaikunnan, S., Khaled, J. M., Almanaa, T. N., and Govindarajan, M. (2020). Impact of pesticide monocrotophos on microbial populations and histology of intestine in the Indian earthworm *Lampito mauritii* (Kinberg). *Microbial pathogenesis*, *139*, 103893.

Khosrovyan, A., Gabrielyan, B., and Kahru, A. (2020). Ingestion and effects of virgin polyamide microplastics on *Chironomus riparius* adult larvae and adult zebrafish *Danio rerio*. *Chemosphere*, *259*, 127456.

Kirby, M. F., Morris, S., Hurst, M., Kirby, S. J., Neall, P., Tylor, T., and Fagg, A. (2000). The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Marine Pollution Bulletin, 40(9), 780-791.

Kniss, A. R. (2017). Long-term trends in the intensity and relative toxicity of herbicide use. *Nature Communications*, *8*(1), 1-7.

Kono, Y., and Fridovich, I. (1982). Superoxide radical inhibits catalase. *Journal of* Biological *Chemistry*, *257*(10), 5751-5754.

Koelmans, A. A., Bakir, A., Burton, G. A., and Janssen, C. R. (2016). Microplastic as a vector for chemicals in the aquatic environment: critical review and model-supported reinterpretation of empirical studies. *Environmental Science and Technology*, *50*(7), 3315-3326.

Lahive, E., Walton, A., Horton, A. A., Spurgeon, D. J., and Svendsen, C. (2019). Microplastic particles reduce reproduction in the terrestrial worm *Enchytraeus crypticus* in a soil exposure. *Environmental Pollution*, *255*, 113174.

Lajmanovich, R. C., Attademo, A. M., Lener, G., Boccioni, A. P. C., Peltzer, P. M., Martinuzzi, C. S., ... and Repetti, M. R. (2022). Glyphosate and glufosinate ammonium, herbicides commonly used on genetically modified crops, and their interaction with microplastics: Ecotoxicity in anuran tadpoles. *Science of The Total Environment*, *804*, 150177.

Lee, J. W., Kang, H. M., Won, E. J., Hwang, D. S., Kim, D. H., Lee, S. J., and Lee, J. S. (2016). Multi-walled carbon nanotubes (MWCNTs) lead to growth retardation, antioxidant depletion, and activation of the ERK signaling pathway but decrease copper bioavailability in the monogonont rotifer (*Brachionus koreanus*). *Aquatic Toxicology*, 172, 67-79.

Lam, P. Y., and Peterson, R. T. (2019). Developing zebrafish disease models for in vivo small molecule screens. *Current Opinion in Chemical Biology*, *50*, 37-44.

Law, K. L., and Thompson, R. C. (2014). Microplastics in the seas. *Science*, *345*(6193), 144-145.

Li, L. Z., Zhou, D. M., Peijnenburg, W. J., van Gestel, C. A., Jin, S. Y., Wang, Y. J., and Wang, P. (2011). Toxicity of zinc oxide nanoparticles in the earthworm, *Eisenia fetida* and subcellular fractionation of Zn. *Environment International*, *37*(6), 1098-1104.

Liu, J., Dong, C., Zhai, Z., Tang, L., and Wang, L. (2021). Glyphosate-induced lipid metabolism disorder contributes to hepatotoxicity in juvenile common carp. *Environmental Pollution*, 269, 116186.

Li, M., Ma, X., Saleem, M., Wang, X., Sun, L., Yang, Y., and Zhang, Q. (2020). Biochemical response, histopathological change and DNA damage in earthworm (*Eisenia fetida*) exposed to sulfentrazone herbicide. *Ecological Indicators*, 115, 106465.

Li, Q., Wu, J., Zhao, X., Gu, X., and Ji, R. (2019). Separation and identification of microplastics from soil and sewage sludge. *Environmental Pollution*, *254*, 113076.

Li, Y., Tang, H., Hu, Y., Wang, X., Ai, X., Tang, L., ... and Qiu, J. (2016). Enrofloxacin at environmentally relevant concentrations enhances uptake and toxicity of cadmium in the earthworm *Eisenia fetida* in farm soils. *Journal of Hazardous Materials*, *308*, 312-320.

Liu, H., Wu, J., Zhang, X., Xia, Y., Li, Y., and Du, S. (2017). Enantioselective oxidative stress caused by chiral ionic liquids forms of 1-alkyl-3-methyl imidazolium tartrate on *Scenedesmus obliquus*. *Science of the Total Environment*, *595*, 819-827.

Liu, M., Lu, S., Song, Y., Lei, L., Hu, J., Lv, W., ... and He, D. (2018). Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai, China. *Environmental Pollution*, *242*, 855-862.

Li, D., Zhang, J., Cheng, C., Hou, K., Wang, X., Zhu, L., ... and Wang, J. (2022). Effects of ecotoxicity of penoxsulam single and co-exposure with AgNPs on *Eisenia fetida*. *Chemosphere*, 307, 136134.

Li, X., Wang, M., Chen, W., and Jiang, R. (2019). Evaluation of combined toxicity of Siduron and cadmium on earthworm (*Eisenia fetida*) using Biomarker Response Index. *Science of the Total Environment*, 646, 893-901.

Lin, W., Jiang, R., Xiong, Y., Wu, J., Xu, J., Zheng, J., ... and Ouyang, G. (2019). Quantification of the combined toxic effect of polychlorinated biphenyls and nano-sized polystyrene on *Daphnia magna*. *Journal of Hazardous Materials*, 364, 531-536.

Lammertyn, S., Masín, C. E., Zalazar, C. S., and Fernandez, M. E. (2021). Biomarkers response and population biological parameters in the earthworm *Eisenia fetida* after short term exposure to atrazine herbicide. *Ecological Indicators*, 121, 107173.

Liu, T., Zhang, Z., Chen, D., Wang, L., Yao, H., Zhao, F., ... and Xu, S. (2013). Effect of atrazine and chlorpyrifos exposure on heat shock protein response in the brain of common carp (*Cyprinus carpio* L.). *Pesticide Biochemistry and Physiology*, *107*(2), 277-283.

Lin, W., Jiang, R., Hu, S., Xiao, X., Wu, J., Wei, S., ... and Ouyang, G. (2019). Investigating the toxicities of different functionalized polystyrene nanoplastics on *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 180, 509-516.

Liu, Z., Huang, Y., Jiao, Y., Chen, Q., Wu, D., Yu, P., ... and Zhao, Y. (2020). Polystyrene nanoplatics induces ROS production and affects the MAPK-HIF-1/NFkB-mediated antioxidant system in *Daphnia pulex*. *Aquatic Toxicology*, *220*, 105420.

Liu, Z., Yu, P., Cai, M., Wu, D., Zhang, M., Chen, M., and Zhao, Y. (2019). Effects of microplastics on the innate immunity and intestinal microflora of juvenile Eriocheir sinensis. *Science of the Total Environment*, *685*, 836-846.

Lin, W., Jiang, R., Hu, S., Xiao, X., Wu, J., Wei, S., ... and Ouyang, G. (2019). Investigating the toxicities of different functionalized polystyrene nanoplastics on *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 180, 509-516.

Lin, D., Xie, X., Zhou, Q., and Liu, Y. (2012). Biochemical and genotoxic effect of triclosan on earthworms (*Eisenia fetida*) using contact and soil tests. *Environmental Toxicology*, 27(7), 385-392.

Li, M., Ma, X., Saleem, M., Wang, X., Sun, L., Yang, Y., and Zhang, Q. (2020). Biochemical response, histopathological change and DNA damage in earthworm (*Eisenia fetida*) exposed to sulfentrazone herbicide. *Ecological Indicators*, 115, 106465.

Liu, T., Chen, D., Li, Y., Wang, X., and Wang, F. (2018). Enantioselective bioaccumulation and toxicity of the neonicotinoid insecticide dinotefuran in earthworms (*Eisenia fetida*). *Journal of Agricultural and Food Chemistry*, 66(17), 4531-4540.

Lammertyn, S., Masín, C. E., Zalazar, C. S., and Fernandez, M. E. (2021). Biomarkers response and population biological parameters in the earthworm *Eisenia fetida* after short term exposure to atrazine herbicide. *Ecological Indicators*, *121*, 107173.

Lin, D., Xie, X., Zhou, Q., and Liu, Y. (2012). Biochemical and genotoxic effect of triclosan on earthworms (*Eisenia fetida*) using contact and soil tests. *Environmental Toxicology*, 27(7), 385-392.

Lleras-Forero, L., Winkler, C., and Schulte-Merker, S. (2020). Zebrafish and medaka as models for biomedical research of bone diseases. *Developmental biology*, *457*(2), 191-205. Zang, L., Maddison, L. A., and Chen, W. (2018). Zebrafish as a model for obesity and diabetes. *Frontiers in Cell and Developmental Biology*, 91.

Lin, C. X., Yang, S. Y., Gu, J. L., Meng, J., Xu, H. Y., and Cao, J. M. (2017). The acute toxic effects of silver nanoparticles on myocardial transmembrane potential, I Na and I K1 channels and heart rhythm in mice. *Nanotoxicology*, 11(6), 827-837.

Lozano, Y. M., Lehnert, T., Linck, L. T., Lehmann, A., and Rillig, M. C. (2021). Microplastic shape, polymer type, and concentration affect soil properties and plant biomass. *Frontiers in Plant Science*, *12*, 169.

Lubbers, I. M., Van Groenigen, K. J., Fonte, S. J., Six, J., Brussaard, L., and Van Groenigen, J. W. (2013). Greenhouse-gas emissions from soils increased by earthworms. *Nature Climate Change*, *3*(3), 187-194.

Ma, H., Williams, P. L., and Diamond, S. A. (2013). Ecotoxicity of manufactured ZnO nanoparticles–a review. *Environmental Pollution*, *172*, 76-85.

Ma, J., and Li, X. (2015). Alteration in the cytokine levels and histopathological damage in common carp induced by glyphosate. *Chemosphere*, *128*, 293-298.

Ma, J., and Li, X. (2015). Alteration in the cytokine levels and histopathological damage in common carp induced by glyphosate. *Chemosphere*, *128*, 293-298.

Ma, J., Bu, Y., and Li, X. (2015). Immunological and histopathological responses of the kidney of common carp (*Cyprinus carpio* L.) sublethally exposed to glyphosate. *Environmental Toxicology and Pharmacology*, *39*(1), 1-8.

Ma, J., Feng, Y., Xie, W., Yang, L., and Li, X. (2015). Immune response in common carp (*Cyprinus carpio* L.) following glyphosate-exposure. *Toxin reviews*, *34*(4), 184-189.

Ma, J., Li, Y., Li, W., and Li, X. (2018). Hepatotoxicity of paraquat on common carp (*Cyprinus carpio* L.). *Science of the Total Environment*, *616*, 889-898.

Ma, Y., Huang, A., Cao, S., Sun, F., Wang, L., Guo, H., and Ji, R. (2016). Effects of nanoplatics and microplastics on toxicity, bioaccumulation, and environmental fate of phenanthrene in fresh water. *Environmental Pollution*, 219, 166-173.

Mahler, B. J., Van Metre, P. C., Burley, T. E., Loftin, K. A., Meyer, M. T., and Nowell, L. H. (2017). Similarities and differences in occurrence and temporal fluctuations in glyphosate and atrazine in small Midwestern streams (USA) during the 2013 growing season. *Science of the Total Environment*, *579*, 149-158.

Masiol, M., Giannì, B., and Prete, M. (2018). Herbicides in river water across the northeastern Italy: occurrence and spatial patterns of glyphosate, aminomethylphosphonic acid, and glufosinate ammonium. *Environmental Science and Pollution Research*, *25*(24), 24368-24378.

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L. A., and Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific Reports*, *7*(1), 1-7.

Mesnage, R., Benbrook, C., and Antoniou, M. N. (2019). Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. *Food and Chemical Toxicology*, *128*, 137-145.

Mesnage, R., Defarge, N., De Vendômois, J. S., and Séralini, G. E. (2015). Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. *Food and Chemical Toxicology*, *84*, 133-153.

Miaomiao Teng, Xiaoli Zhao*, Chengju Wang, Chen Wang, Jason C. White, Wentian Zhao, Lingfeng Zhou, Manman Duan, and Fengchang Wu. (2022). Polystyrene Nanoplastics Toxicity to Zebrafish: Dysregulation of the Brain–Intestine–Microbiota Axis *American Chemical Society* ***5****, 8190–8204*

Moyson, S., Liew, H. J., Fazio, A., Van Dooren, N., Delcroix, A., Faggio, C., ... and De Boeck, G. (2016). Kidney activity increases in copper exposed goldfish (*Carassius auratus auratus*). *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *190*, 32-37.

Myers, J. P., Antoniou, M. N., Blumberg, B., Carroll, L., Colborn, T., Everett, L. G., ... and Benbrook, C. M. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health*, *15*(1), 1-13.

Nogueira, D. J., da Silva, A. C. D. O., da Silva, M. L. N., Vicentini, D. S., and Matias, W. G. (2022). Individual and combined multigenerational effects induced by polystyrene nanoplatics and glyphosate in *Daphnia magna* (Strauss, 1820). *Science of The Total Environment*, 811, 151360.

Ng, E. L., Lwanga, E. H., Eldridge, S. M., Johnston, P., Hu, H. W., Geissen, V., and Chen, D. (2018). An overview of microplastic and nanoplatics pollution in agroecosystems. *Science of the Total Environment*, 627, 1377-1388.

Nasim, G., and Shabbir, A. (2012). Invasive weed species–a threat to sustainable agriculture. In *Crop production for agricultural improvement* (pp. 523-556). Springer, Dordrecht.

Nations, U. (2013). World Population Prospects The 2012 Revision Volume I: Comprehensive Tables. *Economic and Social Affairs*.

Nielsen, U. N., and Wall, D. H. (2015). Soil biodiversity and the environment.

De Oliveira, A. M., Dos Santos, A. G., Dos Santos, R. A., Csipak, A. R., Olivato, C., da Silva, I. C., ... and Soares, C. P. (2009). Ethanolic extract of Casearia sylvestris and its clerodane diterpen (*caseargrewiin* F) protect against DNA damage at low concentrations and cause DNA damage at high concentrations in mice's blood cells. *Mutagenesis*, *24*(6), 501-506.

Olsen, G. W., Burris, J. M., Mandel, J. H., and Zobel, L. R. (1999). Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in Olive, P. L., Banáth, J. P., and Durand, R. E. (1990). Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the" comet" assay. *Radiation Research*, 122(1), 86-94.

O'Connor, D., Pan, S., Shen, Z., Song, Y., Jin, Y., Wu, W. M., and Hou, D. (2019). Microplastics undergo accelerated vertical migration in sand soil due to small size and wet-dry cycles. *Environmental Pollution*, *249*, 527-534.

Owagboriaye, F., Dedeke, G., Bamidele, J., Aladesida, A., Icebox, P., Feyisola, R., and Adeleke, M. (2020). Biochemical response and vermiremediation assessment of three earthworm species (*Alma millsoni, Eudrilus eugeniae* and *Libyodrilus violaceus*) in soil contaminated with a glyphosate-based herbicide. *Ecological Indicators*, *108*, 105678.

Pitt, J. A., Kozal, J. S., Jayasundara, N., Masaryk, A., Trevisan, R., Geitner, N., ... and Di Giulio, R. T. (2018). Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (Danio rerio). *Aquatic Toxicology*, 194, 185-194.

Pelosi, C., Bertrand, C., Daniele, G., Coeurdassier, M., Benoit, P., Nélieu, S., ... and Fritsch, C. (2021). Residues of currently used pesticides in soils and earthworms: A silent threat? *Agriculture, Ecosystems and Environment*, *305*, 107167.

Piola, L., Fuchs, J., Oneto, M. L., Basack, S., Kesten, E., and Casabé, N. (2013). Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere,* 91(4), 545-551.

Palanikumar, L., Kumaraguru, A. K., Ramakritinan, C. M., and Anand, M. (2012). Biochemical response of anthracene and benzo [a] pyrene in milkfish Chanos. *Ecotoxicology and Environmental Safety*, 75, 187-197.

Peng, L., Fu, D., Qi, H., Lan, C. Q., Yu, H., and Ge, C. (2020). Micro-and nano-plastics in marine environment: Source, distribution and threats—A review. *Science of the Total Environment*, *698*, 134254

Peng, L., Fu, D., Qi, H., Lan, C. Q., Yu, H., and Ge, C. (2020). Micro-and nano-plastics in marine environment: Source, distribution and threats—A review. *Science of the Total Environment*, *698*, 134254.

ParisP, PaceE, Parisi PresicceD, MaschioG, UrsinoS (2016) Rapporto nazionale pesticidi nelle acque. Dati 2013–2014. ISPRA

Prasath, P. M. D., and Arivoli, S. (2008). Biochemical study of freshwater fish Catla catla with reference to mercury chloride. *Journal of Environmental Health Science and Engineering,* 5(2), 109-116.

Pereira, A. C., Gomes, T., Machado, M. R. F., and Rocha, T. L. (2019). The zebrafish embryotoxicity test (ZET) for nanotoxicity assessment: From morphological to molecular approach. *Environmental Pollution*, *252*, 1841-1853.

Phillips, H. R., Guerra, C. A., Bartz, M. L., Briones, M. J., Brown, G., Crowther, T. W., ... and Eisenhauer, N. (2019). Global distribution of earthworm diversity. *Science*, *366*(6464), 480-485.

PlasticsEurope, E. P. R. O. (2016). Plastics—The Facts 2019. An Analysis of European Plastics Production, Demand and Waste Data. *Plast. Eur*.

Pochron, S., Simon, L., Mirza, A., Littleton, A., Sahebzada, F., and Yudell, M. (2020). Glyphosate but not Roundup® harms earthworms (*Eisenia fetida*). *Chemosphere*, *241*, 125017

Qi, R., Jones, D. L., Li, Z., Liu, Q., and Yan, C. (2020). Behavior of microplastics and plastic film residues in the soil environment: A critical review. *Science of the Total Environment*, *703*, 134722.

Qiu, L., Jia, K., Huang, L., Liao, X., Guo, X., and Lu, H. (2019). Hepatotoxicity of tricyclazole in zebrafish (*Danio rerio*). *Chemosphere*, *232*, 171-179.

Quevedo, I. R., and Tufenkji, N. (2012). Mobility of functionalized quantum dots and a model polystyrene nanoparticle in saturated quartz sand and loamy sand. *Environmental Science and Technology*, *46*(8), 4449-4457.

Rashid, M. A. H. R. I. N. E., Abbas, S. H., and Rehman, A. B. D. U. L. (2014). The status of highly alien invasive plants in Pakistan and their impact on the ecosystem: a review. International *Journal of Innovation Agriculture Sciences*, *2*, 1-4.

Rodriguez-Seijo, A., Lourenço, J., Rocha-Santos, T. A. P., Da Costa, J., Duarte, A. C., Vala, H., and Pereira, R. (2017). Histopathological and molecular effects of microplastics in *Eisenia andrei* Bouché. *Environmental Pollution*, 220, 495-503.

Roex, E. W., Keijzers, R., and Van Gestel, C. A. (2003). Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, *Danio rerio*, after chronic exposure to parathion. *Aquatic Toxicology*, 64(4), 451-460.

Reddy, N. C., and Rao, J. V. (2008). Biological response of earthworm, *Eisenia fetida* (Savigny) to an organophosphorous pesticide, profenofos. *Ecotoxicology and Environmental Safety*, *71*(2), 574-582.

Reinecke, S. A., and Reinecke, A. J. (2004). The comet assay as biomarker of heavy metal genotoxicity in earthworms. *Archives of Environmental Contamination and Toxicology*, 46, 208-215.

Rashid, T., Sher, F., Hazafa, A., Hashmi, R. Q., Zafar, A., Rasheed, T., and Hussain, S. (2021). Design and feasibility study of novel paraboloid graphite based microbial fuel cell for bioelectrogenesis and pharmaceutical wastewater treatment. *Journal of Environmental Chemical Engineering*, *9*(1), 104502.

Revel, M., Châtel, A., and Mouneyrac, C. (2018). Micro (nano) plastics: A threat to human health. *Current Opinion in Environmental Science and Health*, *1*, 17-23.

Ramos, M. A., Batista, S., Pires, M. A., Silva, A. P., Pereira, L. F., Saavedra, M. J., ... and Rema, P. (2017). Dietary probiotic supplementation improves growth and the intestinal morphology of Nile tilapia. *Animal*, 11(8), 1259-1269.

Richmond, M. E. (2018). Glyphosate: a review of its global use, environmental impact, and potential health effects on humans and other species. *Journal of Environmental Studies and Sciences*, *8*(4), 416-434.

Rodriguez-Seijo, A., Lourenço, J., Rocha-Santos, T. A. P., Da Costa, J., Duarte, A. C., Vala, H., and Pereira, R. (2017). Histopathological and molecular effects of microplastics in *Eisenia andrei Bouché*. *Environmental Pollution*, *220*, 495-503.

Rojas, E., Lorenzo, Y., Haug, K., Nicolaissen, B., and Valverde, M. (2014). Epithelial cells as alternative human biomatrices for comet assay. *Frontiers in genetics*, *5*, 386.

Sadri, S. S., and Thompson, R. C. (2014). On the quantity and composition of floating plastic debris entering and leaving the Tamar Estuary, Southwest England. *Marine Pollution Bulletin*, *81*(1), 55-60.

Shen, M., Zhang, Y., Zhu, Y., Song, B., Zeng, G., Hu, D., ... and Ren, X. (2019). Recent advances in toxicological research of nanoplastics in the environment: A review. *Environmental Pollution*, *252*, 511-521.

Silva, V., Montanarella, L., Jones, A., Fernández-Ugalde, O., Mol, H. G., Ritsema, C. J., and Geissen, V. (2018). Distribution of glyphosate and aminomethylphosphonic acid (AMPA) in agricultural topsoils of the European Union. *Science of the Total Environment*, *621*, 1352-1359.

Saez, G., Aye, M., De Meo, M., Aimé, A., Bestel, I., Barthélémy, P., and Di Giorgio, C. (2015). Genotoxic and oxidative responses in coelomocytes of *Eisenia fetida* and Hediste diversicolor exposed to lipid-coated CdSe/ZnS quantum dots and CdCl2. *Environmental toxicology*, 30(8), 918-926.

Saint-Denis, M., Narbonne, J. F., Arnaud, C., and Ribera, D. (2001). Biochemical responses of the earthworm *Eisenia fetida* andrei exposed to contaminated artificial soil: effects of lead acetate. *Soil Biology and Biochemistry*, 33(3), 395-404.

Song, P., Jiang, N., Zhang, K., Li, X., Li, N., Zhang, Y., ... and Wang, J. (2022). Ecotoxicological evaluation of zebrafish liver (*Danio rerio*) induced by dibutyl phthalate. *Journal of Hazardous Materials*, 425, 128027.

Santadino, M., Coviella, C., and Momo, F. (2014). Glyphosate sublethal effects on the population dynamics of the earthworm *Eisenia fetida* (Savigny, 1826). *Water, Air, and Soil Pollution,* 225, 1-8.

Silva, V., Montanarella, L., Jones, A., Fernández-Ugalde, O., Mol, H. G., Ritsema, C. J., and Geissen, V. (2018). Distribution of glyphosate and aminomethyl phosphonic acid (AMPA) in agricultural topsoils of the European Union. *Science of the Total Environment*, *621*, 1352-1359.

Singh, S., Singh, J., and Vig, A. P. (2016). Earthworm as ecological engineers to change the physico-chemical properties of soil: soil vs vermicast. *Ecological Engineering*, *90*, 1-5.

Sobhani, Z., Panneerselvan, L., Fang, C., Naidu, R., and Megharaj, M. (2021). Chronic and transgenerational effects of polystyrene microplastics at environmentally relevant concentrations in earthworms (*Eisenia fetida*). *Environmental Toxicology and Chemistry*, 40(8), 2240-2246.

Song, Y., Zhu, L. S., Wang, J., Wang, J. H., Liu, W., and Xie, H. (2009). DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia fetida*) induced by atrazine. *Soil Biology and Biochemistry*, *41*(5), 905-909.

Shi, Z., Tang, Z., and Wang, C. (2017). A brief review and evaluation of earthworm biomarkers in soil pollution assessment. *Environmental Science and Pollution Research*, *24*, 13284-13294.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., ... and Huvet, A. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. *Proceedings of the National Academy of Sciences*, *113*(9), 2430-2435.

Székács, A., and Darvas, B. (2018). Re-registration challenges of glyphosate in the European Union. *Frontiers in Environmental Science*, 78.

Santos, M. J., Morgado, R., Ferreira, N. G. C., Soares, A. M., and Loureiro, S. (2011). Evaluation of the joint effect of glyphosate and dimethoate using a small-scale terrestrial ecosystem. *Ecotoxicology and Environmental Safety*, 74(7), 1994-2001.

Soreq, H., and Seidman, S. (2001). Acetylcholinesterase—new roles for an old actor. *Nature Reviews Neuroscience*, *2*(4), 294-302.

Santos, S. W., Gonzalez, P., Cormier, B., Mazzella, N., Bonnaud, B., Morin, S., ... and Cachot, J. (2019). A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, Oncorhynchus mykiss. *Aquatic Toxicology*, 216, 105291.

Song, Y., Zhu, L. S., Wang, J., Wang, J. H., Liu, W., and Xie, H. (2009). DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia fetida*) induced by atrazine. *Soil Biology and Biochemistry*, *41*(5), 905-909.

Theodorakis, C. W., and Shugart, L. R. (1998). Genetic ecotoxicology III: the relationship between DNA strand breaks and genotype in mosquito fish exposed to radiation. *Ecotoxicology*, 7(4), 227-235.

Tiwari, R. K., Singh, S., and Pandey, R. S. (2019). Assessment of acute toxicity and biochemical responses to chlorpyrifos, cypermethrin and their combination exposed earthworm, *Eudrilus eugeniae*. *Toxicology Reports*, 6, 288-297.

Trestrail, C., Nugegoda, D., and Shimeta, J. (2020). Invertebrate responses to microplastic ingestion: Reviewing the role of the antioxidant system. *Science of The Total Environment*, 734, 138559.

Tomlin, C. D. S. (2006). The Pesticide Manual: A World Compendium. 14th ed British Crop Protection Council. *Hampshire, UK*.

Tat, J., Liu, M., and Wen, X. Y. (2013). Zebrafish cancer and metastasis models for in vivo drug discovery. *Drug Discovery Today: Technologies*, *10*(1), e83-e89.

Teuten, E. L., Saquing, J. M., Knappe, D. R., Barlaz, M. A., Jonsson, S., Björn, A., ... and Takada, H. (2009). Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2027-2045.

Utami, R. R., 151360Geerling, G. W., Salami, I. R., Notodarmojo, S., and Ragas, A. M. (2020). Environmental prioritization of pesticide in the Upper Citarum River Basin, Indonesia, using predicted and measured concentrations. *Science of the Total Environment*, *738*, 140130.

Van Bruggen, A. H., He, M. M., Shin, K., Mai, V., Jeong, K. C., Finckh, M. R., and Morris Jr, J. G. (2018). Environmental and health effects of the herbicide glyphosate. *Science of the Total Environment*, *616*, 255-268.

Villamar-Ayala, C. A., Carrera-Cevallos, J. V., Vasquez-Medrano, R., and Espinoza-Montero, P. J. (2019). Fate, eco-toxicological characteristics, and treatment processes applied to water polluted with glyphosate: A critical review. *Critical Reviews in Environmental Science and Technology*, *49*(16), 1476-1514.

Wackett, A. A., Yoo, K., Olofsson, J., and Klaminder, J. (2018). Human-mediated introduction of geoengineering earthworms in the Fennoscandian arctic. *Biological Invasions*, *20*(6), 1377-1386.

Wahl, A., Le Juge, C., Davranche, M., El Hadri, H., Grassl, B., Reynaud, S., and Gigault, J. (2021). Nanoplatics occurrence in a soil amended with plastic debris. *Chemosphere*, *262*, 127784.

Wang, K., Mu, X., Qi, S., Chai, T., Pang, S., Yang, Y., ... and Jiang, J. (2015). Toxicity of a neonicotinoid insecticide, guadipyr, in earthworm (*Eisenia fetida*). *Ecotoxicology and Environmental Safety*, *114*, 17-22.

Wang, W., Ge, J., and Yu, X. (2020). Bioavailability and toxicity of microplastics to fish species: a review. *Ecotoxicology and Environmental Safety*, *189*, 109913.

Westgate, C. J. (2014). One Language, One Nation, and One Vision. *Contemporary Latina/o media: Production, Circulation, Politics*, 82.

Wright, S. L., Rowe, D., Thompson, R. C., and Galloway, T. S. (2013). Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*, *23*(23), R1031-R1033.

World Health Organization (WHO). (1996). Data sheets on pesticides: glyphosate. *International Programme on Chemical safety*.

Wu, J. Y., Chang, S. S., Tseng, C. P., Deng, J. F., and Lee, C. C. (2006). Parenteral glyphosate-surfactant herbicide intoxication. *American Journal of Emergency Medicine*, *24*(4), 504-506.

Wang, G., Xiong, D., Wu, M., Wang, L., and Yang, J. (2020). Induction of time-and dose-dependent oxidative stress of triazophos to brain and liver in zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology Part C*: *Toxicology and Pharmacology*, 228, 108640.

Wang, H., Zhong, X., Shi, W. Y., and Guo, B. (2011). Study of malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in chickens infected with avian infectious bronchitis virus. *African Journal of Biotechnology*, *10*(45), 9213-9217.

Xu, K., Zhang, Y., Huang, Y., and Wang, J. (2021). Toxicological effects of microplastics and phenanthrene to zebrafish (*Danio rerio*). *Science of The Total Environment*, 757, 143730.

Xu, J., Zhao, S., Zhang, S., Pei, J., Li, Y., Zhang, Y., ... and Hu, L. (2020). Development of a multivalent acetylcholinesterase inhibitor via dynamic combinatorial chemistry. *International Journal of Biological Macromolecules*, 150, 1184-1191.

Xie, F., Lampi, M. A., Dixon, D. G., and Greenberg, B. M. (2007). Assessment of the toxicity of mixtures of nickel or cadmium with 9, 10-phenanthrenequinone to *Daphnia magna*: Impact of a reactive oxygen-mediated mechanism with different redox-active metals. *Environmental Toxicology and Chemistry*: *An International Journal*, 26(7), 1425-1432.

Yang, H., Xiong, H., Mi, K., Xue, W., Wei, W., and Zhang, Y. (2020). Toxicity comparison of nano-sized and micron-sized microplastics to *Goldfish Carassius auratus* Larvae. *Journal of Hazardous Materials*, *388*, 122058.

Yang, X., Bento, C. P., Chen, H., Zhang, H., Xue, S., Lwanga, E. H., ... and Geissen, V. (2018). Influence of microplastic addition on glyphosate decay and soil microbial activities in Chinese loess soil. *Environmental Pollution*, *242*, 338-347.

Yang, Y., Xiao, Y., Chang, Y., Cui, Y., Klobučar, G., and Li, M. (2018). Intestinal damage, neurotoxicity and biochemical responses caused by tris (2-chloroethyl) phosphate and tricresyl phosphate on earthworm. *Ecotoxicology and Environmental Safety*, *158*, 78-86.

Yao, X., Zhang, F., Qiao, Z., Yu, H., Sun, S., Li, X., ... and Jiang, X. (2020). Toxicity of thifluzamide in earthworm (*Eisenia fetida*). *Ecotoxicology and Environmental Safety*, 188, 109880.

Yang, Y., Liu, P., and Li, M. (2020). Tri-n-butyl phosphate induced earthworm intestinal damage by influencing nutrient absorption and energy homeostasis of intestinal epithelial cells. *Journal of Hazardous Materials*, 398, 122850.

Zhang, F., Wang, Z., Song, L., Fang, H., and Wang, D. G. (2020). Aquatic toxicity of iron-oxide-doped microplastics to Chlorella pyrenoidosa and *Daphnia magna*. *Environmental Pollution*, *257*, 113451.

Zhu, F., Zhu, C., Wang, C., and Gu, C. (2019). Occurrence and ecological impacts of microplastics in soil systems: a review. *Bulletin of Environmental Contamination and Toxicology*, *102*(6), 741-749.

Zizioli, D., Mione, M., Varinelli, M., Malagola, M., Bernardi, S., Alghisi, E., ... and Russo, D. (2019). Zebrafish disease models in hematology: highlights on biological and translational impact. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1865*(3), 620-633.

Zheng, T., Jia, R., Cao, L., Du, J., Gu, Z., He, Q., ... and Yin, G. (2021). Effects of chronic glyphosate exposure on antioxdative status, metabolism and immune response in tilapia (GIFT, *Oreochromis niloticus*). Comparative Biochemistry and Physiology Part C: *Toxicology and Pharmacology*, 239, 108878.

Zhang, C., Zhu, L., Wang, J., Wang, J., Du, Z., Li, B., ... and Wang, Z. (2018). Evaluating subchronic toxicity of fluoxastrobin using earthworms (*Eisenia fetida*). *Science of the Total Environment*, 642, 567-573.

ANNEXURES

Annexure 1

Calculations of Nanoplastics

1ml aqueous suspension of nanoplatics contains 2.5% nanoplatics beads = 2.5% nanoplastic beads

So, we have 2.5% w/v solution.

2.5% mean = = $\frac{2.5}{100}$ %

2.5g in 100 ml

= $\frac{2.5g}{100}$ %

= 0.025g/ml

= 0.025×1000mg/ml

= 25mg/ml

Now we have 1.05g density = 1.05 g

So, in case when volume 1ml =1g, then density would be equal to

d = 1g

d = $\frac{m}{v}$

d × v = m

d =1

(1) v = m , If density is equal to one then volume would be equal to mass.

v = m

d × v = m

(1.05) (25) = 26.25mg/ml

m = 26.25mg/L

According to formula:

Given = 26.25 mg/ml

Required = 20 mg/L

$c_1v_1 = c_2v_2$

26.25/1ml ×v1 = $\frac{20\times1000}{1000\times26.25}$

V1 = 0.761ml

So, from aqueous suspension of nanoplastics volume 0.761ml will be taken and 999.239 water will be added which will be equal to 0.761+999.239 = 1000 ml = 1L

1ml nanoplastic suspension contains = 2.5% Nanoplastic beads

So, we have 2.5% w/v Solutions

2.5% means 2.5/100

= 0.025 g/ml

=0.025×1000 mg/ml

= 25 mg/ml

=25 mg/ml ---------- To prepare 20 mg/ L

Applying the formula:

Given = 25 mg/ml

Required = 20 mg/L

C1V1 = C2V2

25×V1=20×1000

V1= 20×1000/25

= 800ml

V1= 800/1000

= 0.8 ml

- ➔ So, from nanoplastic suspension 0.8ml volume will be taken and 999.2 ml water will be added which is equal to 0.8 ml+999.2ml water= 1000 ml = 1L

****

$C_1V_1 = C_2V_2$

25 mg/ml v1= 25mg/kg ×1kg

V1= $\frac{25mg/kg \times 1kg}{25mg/ml}$

V1= 1ml

- ➔ So, if we take 1 ml aqueous suspension of nanoplastic from stock solution vial, then this volume will give us the nominal concentration of 25 mg/Kg.

$\frac{25mg/ml \times 0.5ml}{kg}$ = Concentration

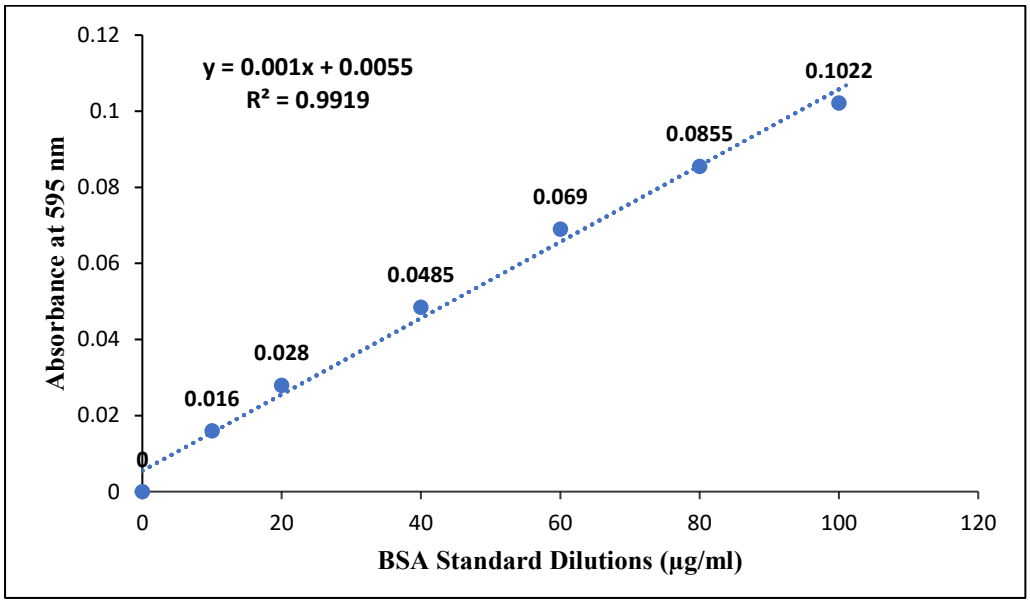
= 12.5mg/kg

- ➔ So, if we take 0.5 ml aqueous suspension of nanoplastic from stock solution vial, then this volume will give us the nominal concentration of 12.5 mg/Kg.
- ➔ We have nanoplatics aqueous suspension containing 1 ml. So, 1 ml NPs aqueous suspension contains 25mg/ml beads and half would be equal to 12.5 mg/ml. Hence, half ml is used for combined exposure and half ml is used for individual.

Annexure 2

- **Formulae for calculations of total protein, oxidative stress and antioxidant enzyme activities by Mr. Muhib**

Formula Total Protein Estimation using calibration curve:



➔ Standard curve of absorbance of BSA dilutions for determination of total protein in unknown sample.

Slope Equation: $Y = 0.001X + 0.0055$

$$X = Y - 0.0055/0.001$$

Whereas:

Y: Absorbance of unknown sample

X: Total protein in unknown sample (µg/ml)

****

Formula for Calculation of ROS:

$$\text{Reactive Oxygen Species (ROS)} = \frac{(\textbf{Sample OD} \times \textbf{Cuvette Vol.} \times \textbf{10\^6})}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \Delta\textbf{time} \times (\textbf{Constant 15600})}$$

Units = U/mg Protein

Whereas:

Sample OD = Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

ΔTime = Measurement Time

15600 = Extinction co-efficient (M^{-1} cm^{-1})

Formula for Calculation of MDA:

Malondialdehyde (MDA) = $\frac{(\textbf{Sample} - \textbf{\Delta Blank}) \times \textbf{Cuvette Vol.} \times \textbf{10\^{}6})}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \textbf{\Delta time} \times (\textbf{Constant 15600})}$ =

Units= U/mg protein

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

ΔTime = Measurement Time

15600 = Extinction co-efficient (M^{-1} cm^{-1})

****

Formula for Calculation of SOD:

Superoxide Dismutase (SOD) = $\frac{(\textbf{\Delta Sample} - \textbf{\Delta Blank}) \times \textbf{Cuvette Vol} \times \textbf{10\^{}6})}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \textbf{\Delta time} \times (\textbf{Constant 6.22})}$ =

Units = U/mg of protein

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

ΔTime = Measurement Time

6.22 = Extinction co-efficient (M^{-1} cm^{-1})

****

Formula for Calculation of CAT:

Catalase (CAT) = $\frac{(\textbf{\Delta Sample} - \textbf{\Delta Blank}) \times \textbf{Cuvette Vol} \times \textbf{10\^{}6})}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \textbf{\Delta time} \times (\textbf{Constant 43.1})}$ = U/mg of protein

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

ΔTime = Measurement Time

43.1 = Extinction co-efficient (M^{-1} cm^{-1})

Annexure 3

- **Formulae for calculations of, AChE, oxidative stress and antioxidant enzyme activities by Dr. Majid**

Acetylcholine Esterase activity (AChE) = $\frac{(\mathbf{T}f-\mathbf{T}i)}{\mathbf{0.01}}$ = U/min

Units = U/min

Whereas:

T_f = Final Absorbance

T_i= Initial Absorbance

0.01= Time Constant (for 1 minute)

Catalase (CAT) $= \frac{\mathbf{Tf}-\mathbf{Ti}}{\mathbf{0.01}}$ = U/min

Units = U/min

Whereas:

T_f = Final Absorbance

T_i= Initial Absorbance

0.01= Time Constant (for 1 minute)

- Reactive Oxygen Species (ROS) $= \frac{(\mathbf{Sample\ OD} \times \mathbf{Cuvette\ Vol} \times \mathbf{Protein})}{(\mathbf{Constant} \times \mathbf{Sample\ Vol.} \times \mathbf{Time})}$ = nM/mL

- Malondialdehyde (MDA) $= \frac{(\mathbf{Sample\ OD} \times \mathbf{Cuvette\ Vol} \times \mathbf{Protein})}{(\mathbf{Constant} \times \mathbf{Sample\ Vol.} \times \mathbf{Time})}$ = nM/mL

- Superoxide Dismutase (SOD) $= \frac{(\mathbf{\Delta Sample} - \mathbf{\Delta Blank}) \times \mathbf{Cuvette\ Vol} \times \mathbf{Protein})}{(\mathbf{Constant} \times \mathbf{Sample\ Vol.} \times \mathbf{Time})}$ = U/min

Annexure 4

- **Calculation for Antioxidants Enzymes Formula Sheet**

Calculate protein content from linear regression equation:

Y= 0.005X + 0.030

Y= absorbance (OD)

X has to find = Protein (Pr)

For all enzymes:

Enzyme activity = $\frac{OD}{t\,(min)} \times \frac{1}{\varepsilon} \times \frac{Total\ vol(ml)}{Volume\ of\ enzyme\ extract(ml)} \times \frac{Total\ enzyme\ extract}{Weight\ of\ tissue\ (mg)} \times \frac{1}{Protein}$

Enzyme activity = µmol/mg/min

Here,

OD = A_S - A_b

OD = Optical Density

As = Absorbance of the Sample

Ab = Absorbance of the Blank

ε = extinction coefficient

ε = CAT = 39.4 Mm^{-1} cm^{-1}

ε = POD = 26.6 Mm^{-1} cm^{-1}

t = incubation time

Total volume = Mixture of substrate, enzyme and buffer

In case of SOD,

$\frac{OD}{t}$ = Y → = $\frac{Ac-At}{Ac} \times 100$ = x%

= $\frac{x}{50}$ = Y ($Umin^{-1}$ mg^{-1} FW).

- ✓ Ac = Absorbance of Control
- ✓ At = Absorbance of the Sample at respective time interval

- **Second Method**

Specific activity of enzyme = $\frac{A}{\varepsilon \times l \times t} \times \frac{Total\ volume\ (ml)}{Volume\ of\ enzyme\ extract\ (ml)} \times \frac{1}{Protein}$

A = absorbance (OD)
ε = Extinction coefficient
l = path length (1cm)
t = Incubation period/time of hydrolysis
Pr = Protein calculated from linear regression equation. x=?

Example:

980 µl = substrate

20 µl = enzyme extract

3 ml = buffer

Total volume = mixture of above all (µml)

5min = t (Incubation time)

e.g. A = 0.35

$\frac{\Delta A}{t} = \frac{0.35}{5}$

$\frac{\Delta A}{t}$ = 0.07 min^{-1}

$\frac{\Delta A}{t \times l \times \varepsilon}$ = 0.07 min^{-1} $(\frac{1}{4600\ Lmol{-}1\ cm{-}1 \times 1cm})$

OR

= 1.52 µmol L^{-1} min^{-1}

It can also be expressed as:

$= \frac{15.2\ \mu mol}{1000\ ml}$ min^{-1}

$\boldsymbol{\frac{A}{t \times l \times \varepsilon} \times \text{total volume} = \frac{15.2\ \mu mol\ min{-}1}{1000\ ml} \times 4\ \cancel{ml}}$

= 0.0609 µmol min^{-1}

$\boldsymbol{\frac{A}{t \times l \times \varepsilon} \times \frac{\text{total volume}}{Vol.of\ Enzyne\ extract} = \frac{0.0609\ \mu mol\ min{-}1}{20\ \mu L}}$

Convert 20µl to ml = $\frac{20}{1000}$ = 0.02 ml

$= \frac{0.006090\ \mu mol\ mim{-}1}{0.02ml}$

= 3.04 µmol min^{-1} ml^{-1}

- Protein = 10 mg ml^{-1}

$\boldsymbol{\frac{A}{t \times l \times \varepsilon} \times \frac{\text{total volume}}{Vol.of\ Enzyne\ extract} \times \frac{1}{Pr} = \frac{3.04\ \mu mol\ min{-}1\ ml{-}1}{10mg\ ml{-}1}}$

A = 0.304 µmol min^{-1} mg^{-1} Pr.

Conversions

U = µmol min^{-1}

U/mg = µml min^{-1} mg^{-1}

U/ml = µmol min^{-1} ml^{-1}

Annexure 5

Activity = (ΔE xVf) / (Δt x € x Vs x d)

Whereas:

ΔE → is change in absorbance,

Vf → final reaction volume

Vs → volume of enzyme extract used.

Δt → is time of hydrolysis.

€ → is extinction coefficient.

d → is diameter of cuvette used.

The final answer is divided by mg or g protein of the tissue.

➔ **You can calculate enzyme activity of enzyme by using this :**

Enzyme activity = change in OD / time taken (min) × 1 / extinction coefficient of enzyme × total reaction volume / volume of enzyme extract taken x total volume of enzyme extract / Fresh weight of tissue (g) × total protein × 1000 nano moles of enzyme present per gram of sample tissue. For catalase extinction coefficient is 39.4 mM^{-1} cm^{-1} and for peroxidases 26.6 mM^{-1} cm^{-1}.

In case of SOD % inhibition = control OD - treatment OD/ control × 100 = X% inhibition.

50% inhibition is equal to 1 unit of enzyme, then X% is equal to 1/50 x X= Y unit.

➔ Calculate activity by inserting value of Y in above formula of activity in place of change in OD w.r.t. time. Rest of formula will be same.

- ✓ SOD → µmol / min / mg protein (µmol min^{-1} mg^{-1} protein)
- ✓ CAT → µmol / min / mg protein (µmol min^{-1} mg^{-1} protein)
- ✓ POD → nmol / min / mg protein (nmol min^{-1} mg^{-1} protein)
- ✓ GR → nmol / min / mg protein (nmol min^{-1} mg^{-1} protein)

Study on the Influence of Polystyrene Nanoplastics on the Toxicity of Glyphosate in *Eisenia fetida*

Authors
Safa Zahoor; Mazhar Iqbal Zafar

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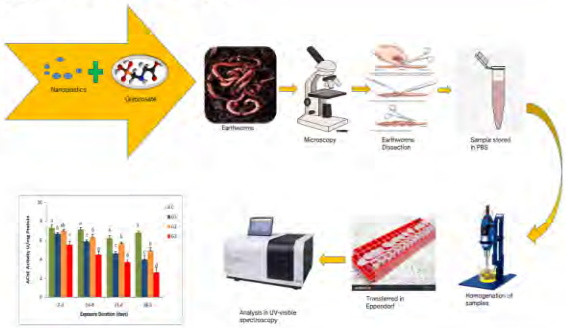
1 Introduction

- Growing concerns surround the escalating presence and long-lasting impact of plastic pollution in the environment. Global plastic production is projected to soar to 33 billion tons by 2050, a stark increase from 359 million tons in 2018 and 2019.
- As plastics degrade over time, they fragment into various sizes, including macroplastics (>2cm), mesoplastics (5-10mm), microplastics (>5mm), and nanoplastics (<1mm), posing diverse ecological challenges.
- Nanoplastics, ranging in size from 1 to 1000 nm, are generated during the degradation of industrial plastics. Moreover, products like nanomedicine and personal care items can directly introduce these nanoparticles (NPs) into the environment, highlighting potential sources of contamination.
- In Pakistan, the agro-production industry grapples with severe pest infestations, causing substantial economic losses. To mitigate these issues and enhance crop productivity, widespread use of pesticides, herbicides. Glyphosate is most widely used for crops in all over Pakistan.
- Pakistan being developing country lacks the literature explaining combined and individual effect of nanoplastics and glyphosate on earthworms.

2 Objective

- To investigate the combined effect of nanoplastics and glyphosate on the AChE activity of earthworms
- Evaluating the effects of nanoplastics and glyphosate on ROS, MDA content and antioxidant enzymes activity.
- Assess the effects on DNA damage and Histopathological alterations in intestine of earthworms

3 Methodology



4 Results

- By the exposure of nanoplastics and glyphosate AChE activity decreased significantly in both nanoplastic and glyphosate group but in coexposure group lowest AChE inhibition observed .
- Due to oxidative stress ROS and da content significantly increased in individual and combined groups. Due to coexpsure of nanoplastic + glyphosate ROS and MDA content significantly highest increased compare to other groups.
- Antioxidant enzymes (SOD&CAT) activity significantly reduced in all groups due to oxidative stress.
- DNA damage was prominent after 28days in coexposure group the head length decreases and tail is significantly increased due to migration of damage DNA from head to tail.
- Histopathological alterations was seen in intestine , irregular cell shape, lysis of cell and enlargement of nucleus size.

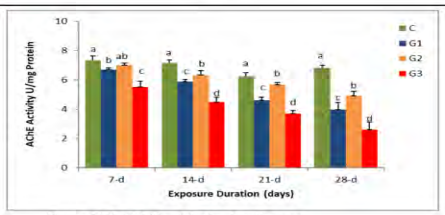
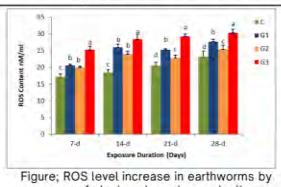


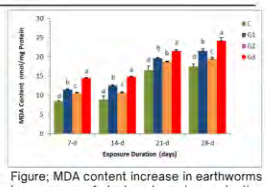
Figure: AChE inhibition in brain of earthworms

5 Conclusion

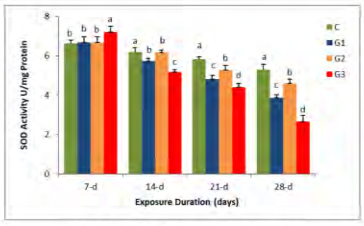
- Main findings of the study describe that nanoplastics and glyphosate induced toxicity to the earthworms.
- Overall both the pollutants inhibited the activity of AChE in earthworms.
- Combine and individual exposure of nanoplastics and glyphosate increase ROS, MDA and antioxidant enzymes SOD, CAT significantly decreased during the experiment.
- Furthermore all over the experimental duration DNA damage migrated from head to tail and histopathological alterations was prominent showed cell lysis and enlargement of cell space .
- This study can be helpful in future of the study of toxicological interactions of nanoplastics and glyphosate on terrestrial organisms.



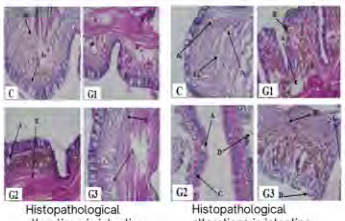
Figure; ROS level increase in earthworms by exposure of glyphosate and nanoplastics



Figure; MDA content increase in earthworms by exposure of glyphosate and nanoplastics

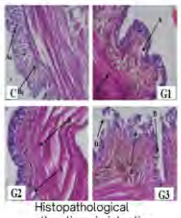


Fig; Antioxidant enzyme activity (SOD)

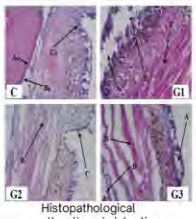


Histopathological alterations in intestine after 7 days

Histopathological alterations in intestine after 14 days



Histopathological alterations in intestine after 21 days



Histopathological alterations in intestine after 28days

6 Acknowledgement

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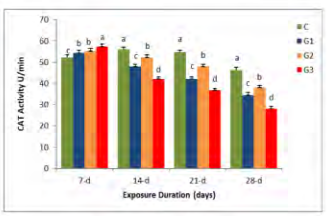
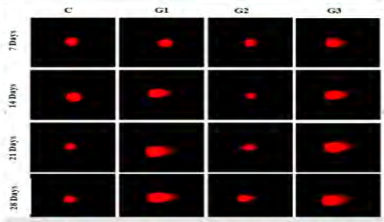


Figure; Antioxidant enzyme (CAT) activity



DNA damage in earthworms by the exposure of glyphosate + nanoplastics

Related Literature

- Hu, B., Li, Y., Jiang, L., Chen, X., Wang, L., An, S., & Zhang, F. (2020). Influence of microplastics occurrence on the adsorption of 17β-estradiol in soil. Journal of Hazardous Materials, 400, 123325.
- Leslie, H. A., Van Velzen, M. J., Brandsma, S. H., Vethaak, A. D., Garcia-Vallejo, J. J., & Lamoree, M. H. (2022). Discovery and quantification of plastic particle pollution in human blood. Environment international, 163, 107199.
- Kinigopoulou, V., Pashalidis, I., Kalderis, D., & Anastopoulos, I. (2022). Microplastics as carriers of inorganic and organic contaminants in the environment: A review of recent progress. Journal of Molecular Liquids, 118580.