MICROPLASTIC DEGRADATION BY EARTHWORM GUT ASSOCIATED BACTERIA AND ITS EFFECT ON THE EARTHWORM GROWTH

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY

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LOVELY PROFESSIONAL UNIVERSITY, PUNJAB
2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled "Microplastic degradation by earthworm gut associated bacteria and its effects on the earthworm growth" in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Prof. (Dr.) Joginder Singh, Department of Microbiology, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, India and Dr. Jaswinder Singh, Associate Professor, Department of Zoology, Khalsa College Amritsar. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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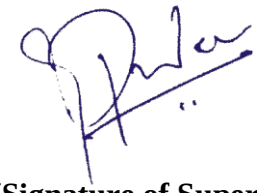
CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "Microplastic degradation by earthworm gut associated bacteria and its effects on the earthworm growth" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Microbiology, School of Bioengineering and Biosciences is a research work carried out by Babita (11919652), is bonafide record of her original work carried out under our supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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ABSTRACT

Microplastics are very small size plastic particle (<5mm) and are widely scattered in ecosystem. These small particles originate from the primary and secondary sources which produce deleterious effects to ecosystem. Microplastics have potential to easily diffuse, assemble and migrate in ecosystem due to their different properties such as hydrophobic nature, constant chemical properties, presence of persistent organic pollutants, highly ductile, and ability to transport several other harmful pollutants. Microplastic consists of different types of harmful additives like plasticizers, highly effective lubricant and different types of flame retardants. Different types of microplastics have widely polluted the soil and terrestrial ecosystem.

Microplastic enters into the soil through activities of human beings, use of plastic mulch, compost, sewage irrigation, domestic waste and atmospheric deposition. Microplastic affects the physical (soil porosity, change in bulk density and aggregates) and chemical properties (pH, temperature and organic matter) of the soil. Microplastics also have the potential to produce toxic effects on the soil macro and micro fauna such as earthworms, arthropods, collembolans and mussels. Soil organisms easily ingest the tiny fragments or particles of plastics which changes the microbial and enzymatic activities.

Earthworms act as ecological engineers; have a significant impact on the agriculture field or soil. Agricultural intensification through the extensive use of plastic mulch, sewage sludge and composting has resulted in sharp decline in the soil biodiversity as well as soil fertility. To overcome this serious issue, it is important to identify different types of microplastics in agriculture soil. The quantities of different types of microplastics in soil were investigated that produce several toxic effects on earthworm. Soil samples were collected from three plastic product manufacturing industries located at Kapurthala, Jalandhar and Amritsar. All the soil samples were analyzed for different physico-chemical parameters. The present study reported different types, shape and size of particles and were identified by using ATR-FTIR spectrophotometer. Microplastics in the agriculture soil such as polyethylene, polypropylene, polystyrene, polybutylene terephthalate, polyethylene terephthalate were separated by using density separation

method. These polypropylene particles were more dominant and polybutylene terephthalate were less dominant in all soil samples. Different types of extracted microplastics were counted under stereomicroscope and then stained with Nile red dye for visualization under fluorescence microscope. Further, the crystalline natures of different types of microplastics were checked through X-Ray Diffraction analysis. SEM analysis showed the surface morphology of different types of extracted microplastics.

Two species of earthworm, exotic (*Eisenia fetida*) and indigenous (*Lampito mauritii)* were selected to check the toxic effects of polypropylene in terms of growth, fecundity rate and antioxidant activity. Polypropylene microplastics were selected on the basis of their abundance as compared to other microplastics in the present study. The results of this study suggested that polypropylene microplastics decreased the activity of earthworm with increase in exposure time and concentrations. The growth rate of earthworms were declined at high concentration, similarly the fecundity rate of earthworms were also declined with increase in concentration. Antioxidant study revealed the oxidative stress produced by the different enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione-S-Transferase (GST) and Guaicol Peroxidase (POD) after exposure of polypropylene. The antioxidant activities of *E. fetida* enzymes i.e. SOD, CAT and GST were initially increased but the activity decreased with increase in time period as well as treatment exposure. POD activity of *E. fetida* showed an increasing trend from intial to final period of polypropylene exposure. Similarly in *Lampito mauritii* the enzymatic activity of SOD, CAT and GST initially increased with increase in exposure time and then slightly reduced with increase in exposure period and polypropylene exposure. POD activity exhibits similar pattern in *E. fetida* and increased with increase in polypropylene exposure.

Molecular docking studies revealed the binding of polypropylene with earthworms enzyme (SOD, CAT, GST and POD) at catalytic and non-catalytic sites. The binding affinity of polypropylene with enzymes was measured by calculating the docking score. SOD has potential to bind with polypropylene at three sites, CAT has four active binding sites with polypropylene, GST has five binding sites and POD has three binding sites with polypropylene.

The earthworm gut microorganisms play an important role in the degradation of organic pollutants such as microplastic polymers. The biodegradation of polymers can be observed by changes in the chemical properties, surface morphology and loss in weight of polymers. The present study was planned to assess the ability of different gut microorganisms of earthworms (*Eisenia fetida* and *Lampito mauritii)* towards degradation of polypropylene because plastic polymers have deleterious or negative effects on environment. It must be eliminated by employing eco-friendly method.The earthworm gut microorganisms study reveals that the polypropylene causes moderate effect on both species of earthworms. Different types of gut microorganisms were identified by 16s rDNA metagenomic sequencing. At phylum level the percentage of bacteria were Tenericutes (0.01-0.06%), Bacteroidetes (0.01-0.10%) Chloroflexi (0.01-0.12%), Cyanobacteria (0.02-0.22%), Acidobacteria (0.6-0.9%) Saccharibacteria–TM7 (0.9-2.1%) Verrucombria (3-3.9%), Gemmatimonadetes (3.3-4.7%), Actinobacteria (5.6-7.1%), Planctomycetes (7.1-9%), TM6 (7.4-11%), Firmicutes (12-14.2%), Chlamydiae (15.4-15.6%) and Proteobacteria (32.6-35.4%). The changes in phylogenetic shift were measured by studying the alpha and beta diversity.

Earthworms gut microorganisms have potential to degrade the polypropylene microplastics. Microplastics degradation rate was determined on the basis of ingestion and egestion of microplastics in the form of cast. The present study observed the changes occur in the peak or functional group of polypropylene. ATR-FTIR analysis predicts the changes in the peak and formation of new groups. In case of *E. fetida* new peaks were observed at wave number 2868.15cm^{-1} due to presence of C-H group similarly in case of *L. mauritii* new peak were formed at 1460.11 and one peak were disappeared from wave number 898.33cm^{-1}. SEM analysis shows the changes in surface morphology of earthworms cast it means polypropylene particles causes changes in the surface morphology of cast. Polypropylene microplastics were recovered from each treatment group and degradation percentage was also measured.The efficiency of earthworms gut microorganisms of *E. fetida* towards degradation of polypropylene was 7.2%, 2.03%, 1.57%, 1.2% and *L. mauritii* 7.19%, 2.23% 2.41% and 1.26%.

Dedicated to

My Dadaji, Late Sh. Ran Singh Thakur
and
Dadiji, Late Smt. Barfi Thakur,
The two most pious souls I have ever seen…
My mom, dad, brother, mother-in-law, father-in-law,
For their support, encouragement and everything under the sun…
My husband Rhishabh Prashar,
For his immeasurable support,
love and effort that keeps me motivate…

Acknowledgement

I would like to thanks the Almighty GOD for giving strength and positive vibes for completing this project and planned everything for me in every step to complete this project.

I express my warm gratitude to my Supervisor, ***Dr. Joginder Singh,*** *Professor, School of Bioengineering and Biosciences, Lovely Professional University, for his proper guidance, support, knowledge and always give positive suggestions to complete this thesis. I am very lucky to have work under a dynamic teacher.*

I seems expressionless to me how to do thanks to my Co-Supervisor, ***Dr. Jaswinder Singh,*** *Associate Professor, Department of Zoology, Khalsa College Amritsar for his unconditional support, valuables comments, time to time supervision of my research work. He encourages me in each step and to solve difficulties of my research work. I feel proud to work under such a delightful guide.*

I also express my thanks to ***Dr. Adarsh Pal Vig,*** *Chairman of Central Pollution Control Board Patiala and Professor in Guru Nanak Dev University Amritsar and Dr. Saroj Arora for allowing me to do research work in his laboratory.*

I would like to express my heartful gratitude to ***Dr. Neeta Raj Sharma,*** *Associate Dean, School of Bioengineering and Biosciences,* ***Dr. Ashish Vyas,*** *Head of the Department of Microbiology. I would also like to do warm thanks,* ***Mrs. Rashmi Mittal****, Pro-Chancellor of university,* ***Dr. Lovi Raj Gupta****, Pro Vice-Chancellor of university and* ***Dr. Monica Gulati*** *Registrar of university for providing me proper facilities and good environment to complete my research work.*

I would like to express thanks to my friend in short words Deachen Angmo, Jaspreet Singh, Dr. Sanjay Sharma, Dr. Muneer Malla, Satveer Singh, Dr. Poonam, Dr. Sharanpreet Singh, Joat Singh, Ali Khan, Bhawna Kaushal, Vaidehi Katoch and Renu Singh for their support during this journey.

I would like to heartful gratitude to my grandparents and my father ***Sh. Sanjeev Thakur*** *and mother* ***Smt. Santosh Thakur*** *for their unconditional love. I would also*

like to do deeply thanks to my parents-in-law ***Sh. Sanjay Prashar*** *and* ***Smt. Sujata Prashar*** *for their unconditional patience, care and time encouragement.*

I have no words to express gratitude thanks to my husband ***Rhishabh Prashar*** *for his everlasting patience, trust, encourage, moral support and love. I always cherish your support and love during my good times and bad times of my research. I think this achievement has been fulfilling your dream that had for me.*

At last I would like thanks to my brother ***Babit Thakur*** *for his care and love during this work. I think my brother dream regarding me has been not been complete without his support.*

Last but not least, I am also thankful to those whose name cannot mention but helped me directly or indirectly at different times in the pursuit of this journey.

Babita

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1. INTRODUCTION

The earth surface is continuously contaminated by different types of organic pollutants. Organic pollutants are also known as persistent organic pollutants (POPs) due to its long time existence in environment (Geetha and Nagarajan, 2021). These pollutants cause various diseases in human being as well in other organisms. Different types of organic pollutants are present in environment in the form of plastics, pesticides, organic solvents, detergent and petroleum hydrocarbons (Alharbi et al., 2018; Tran et al., 2020). These organic pollutants enter into the soil through various direct and indirect pathways such as personal care products, pharmaceuticals products, compost and sewage sludge, agriculture products and industrial wastes (Ohkubo et al., 2012; Song and Guo, 2014; Shen et al., 2022; Zhang et al., 2019a; Rochman, 2018). Out of the above said organic pollutants, the microplastics in these days consider as most harmful and toxic to organisms. They are tiny plastic particles of less than 5mm in diameter and contaminate all the ecosystem includes terrestrial, aquatic, marine and freshwater ecosystem. Due to its tiny size, longer durability and capacity to carry harmful pollutants, which may produce adverse toxic accoutrements to the organisms (Barboza et al., 2020; Chen et al., 2020a; Lei et al., 2018a; Song et al., 2019).

Plastic production reached 368 million tonnes all over the world (Europe, 2018) and in India the production reached 20 million metric tonnes (Bardhan et al., 2024).The different types of plastic polymers enter into the agriculture soil through modern agricultural practices and large amount of plastics utilized in the form of compost, fertilizer mulch and sludge. Out of these, plastic mulch is a major source to contribute the microplastic pollution in agriculture field. Plastic mulch maintains the temperature of agriculture or farmland soil due to this reason the farmers in high cold area widely utilize plastic mulch films to keep the appropriate temperature for the crops (Zhang et al., 2020a; Liu et al., 2021). Previously reported abundance of microplastics in agricultural soil in different cities of China includes Shanghai (vegetable field) 78 itemskg^{-1}, Xinjiang (agriculture field) 40.35 mgkg^{-1} and Wuhan (agriculture field) 12560 itemskg^{-1} (Liu et al., 2018; Li et al., 2020a; Chen et al., 2020b). Apart from mulching, compost is another source of microplastics in agriculture soils. Large

amount of microplastics (approximately 1.20 gkg^{-1} soil) were detected in agriculture soil where compost is utilised to improve the crops (Braun et al., 2021).

Nowadays MPs pollution became a major problem to ecosystem (terrestrial and aquatic) that causes various harmful effects. To overcome this issue different techniques and methods have been developed to extract the MPs from ecosystem like density separation, electrostatic extraction, magnetic, oil and solvent extraction method (Prata et al., 2019; He et al., 2021). Density separation method is mostly employed to extract MPs from soil by using different solutions like sodium chloride (NaCl), calcium chloride ($CaCl_2$), zinc chloride ($ZnCl_2$), sodium bromide (NaBr) and sodium iodide (NaI).The extraction efficiency of NaCl is 1.2gcm^{-3}, $ZnCl_2$ is 1.0gcm^{-3}, $CaCl_2$ is 1.5gcm^{-3} and NaI is 1.8gcm^{-3}(Yadav et al., 2022; Li et al., 2018a). NaCl is most commonly used solution to extract MPs. The main advantage of this solution is cheap and safe use as compared to other solutions (Hurley et al., 2018). Plastic polymers (fibres, films and fragements) have been identified by using attenuated total reflectance through Fourier infrared spectroscopy (ATR-FTIR), Raman spectroscopy, Photoluminescence spectroscopy, Pyrolysis coupled with Gas chromatography-mass spectrometry and electron microscopy (Piehl et al., 2018; Hayany et al., 2020).

MPs have potential to pose effects on the soil properties, plants, soil organisms (earthworms, nematodes, collembolans and arthropods) and soil microorganisms. High concentration of microplastics changes the physical and chemical properties of soil. Physical properties include soil porosity, texture, soil structure and conductivity (Huffer et al., 2019). Soil is a permeable substance consists of macro and microscopic pores in the structure. MPs in soils usually produce changes in soil porosity and these based upon the concentration of microplastics and size present in soil (Wan et al., 2019; Zhang et al., 2019b). MPs also decrease the soil conductivity and cause cracks on soil surface and finally decrease the porosity of soil (Liang et al., 2021; Xing et al., 2021). The decline in pH of MPs contaminated soil leads to discharge of lactic acid from the aliphatic polyester which causes cracks in soil surface through a process known as mineralization (Ainali et al., 2022; Schopfer et al., 2022). Microplastic induces the effects on the bulk density of soil because it plays an important role to maintain the water level, solute movement, soil structure and aeration of soil (Shah et al., 2017). Microplastics in soil decreases the organic matter of soil by reducing the soil aggregate balance or depleting the nutrients content and cause major effects to

soil micro and macro fauna (Obalum et al., 2017).Chemical properties of soil are strongly affected by MPs for example pH, soil electrical conductivity and soil salinity (Lozano at al., 2021).

MPs produce deleterious effects to soil organisms through ingestion or feeding. Its small size appearance in soil helps to transfer the particles to the tissues of organisms. Various studies reported the adverse effects of MPs on aquatic organisms include mortality, reduce reproduction and alter the biological function of organisms (Zitouni et al., 2020, 2021). Moreover, fewer studies reported the effects of microplastics on terrestrial organisms. Earthworm is a widespread model organism of terrestrial ecosystem to study the effects of MPs on soil biota MP particles and debris enters to the earthworm's intestine through the food chain (Zhu et al., 2018a; Rodriguez et al., 2017). They act as a MPs transporter in soil and help to assimilate MPs in soil through casts, egestion and burrows (Rillig et al., 2017). Earthworms play an important role in reconstruction of soil structure; maintain nutrient cycle and degradation of organic matter (Zhou et al., 2020). MPs reduce the growth, survival, reproduction rate and also produce oxidative stress in earthworm. MPs are easily ingested by earthworm and gets accumulate in the intestine of earthworm and results in intestinal damage which affects the feeding behaviour.The presence of microbial community in earthworm gut is directly linked to earthworm health, immunity and uptake of proper nutrition.

Earthworm gut micro biota performs a major role towards pathogenic defence mechanisms and cellulose metabolism. In earthworm, MPs cause imbalance and trigger the alpha and beta diversity of microorganisms through alterations and inflammation of mucus layer (Cheng et al., 2021). Earthworms gut rich with different types of aerobic, anaerobic and facultative anaerobic microorganisms (Sun et al., 2020) and these microorganisms are analyzed by employing latest technique 16s rDNA metagenomic sequence. MPs not only generate oxidative stress but also cause the effect on the gene expression. Oxidative stress leads to increase or decrease the antioxidant biomarkers such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione -S-Transferase (GST) and Guaicol peroxidase (POD) (Cui et al., 2022a). These enzymes acts as free radical scavenger and plays important role in enzymatic defense mechanism.

In addition MPs affect the plants and disregulate the electron transport chain and photosynthetic cycle of plants, this result in decrease the enzymatic activity of plants (Li et al., 2020b). Effects of MPs on varieties of plants (*Lepidium sativum, Arabidopsis thaliana, Triticum aestivum*) depend upon the type, shape and size. MPs decrease the germination rate as well as reduce the roots, shoot traits and leaves etc (Qi et al., 2018; de Souza et al., 2019). Human beings are also affected by these organic pollutants through inhalation or by ingestion of microplastic contaminated food. MPs are also ingested through root vegetables e.g. *Raphanus sativus* (radish), *Allium cepa* (onion) (Yadav et al., 2022). MPs in human beings produce a consequence of disease on body system such as digestive, respiratory, oxidative stress, immune disorders, neurotoxicity, and also change cell viability (Prata et al., 2020).

Microorganisms play an important role in degradation of different types of conventional or non-conventional microplastics and utilize as sole energy source. The degradation rate of MPs totally depends upon the change in chemical properties of polymers i.e functional group and bond strength (Wani et al., 2023).These enzymes breakdown the larger plastic polymers into monomers and oligomers (Lin et al., 2022). Earthworm gut act as crucial component in decomposition and mineralization of microplastics. The earthworm gut epithelium secretes various types of enzymes including chitinase, lipases, eaterases, proteases, cellulase and phosphatase.Gut of earthworms indirectly promotes the production of microbial exoenzymes that degrade the microplastic polymers (Sanchez – Hernandez et al., 2020). Most commonly reported microorganisms in degradation of MPs belong to phylum Proteobacteria, Actinobacteria and Firmicutes (Tareen et al., 2022).

In light of the above mentioned facts, the present study was planned to measure the tolerance potential of earthworm species (*E. fetida* and *L. mauritii*) towards polypropylene in terms of growth and fecundity.To overcome the microplastics pollution from the soil, the role of microplastic degrading bacteria from earthworm gut through metagenomic analysis were also studied.

2. REVIEW OF LITERATURE

2.1 Microplastics

Plastic whose size is less than 5mm in diameter is referred as microplastics (Hidalgo et al., 2012; Mourgkogiannis et al., 2018). Microplastics (MPs) pollution is globally distributed and has become an emerging threat to ecosystems. Microplastic particles are widely conveyed and dispersed in soil, water and sediments due to the excessive utilization in daily life, including household goods, cosmetic products such as face scrubs; face wash etc (Hamidian et al., 2021). Plastic/MPs are organic polymers made from non-renewable sources such as crude oil, natural gas and coal. According to Rahman and Bhoi, (2021) approximately 8660 millions metric ton of microplastics was produced worldwide and 132 millions metric ton of microplastic was produced in Asia in 2018. Microplastic has been categorized into two main types; primary and secondary microplastics. Primary MPs are formed from cosmetics, toothpaste, medical products and clothing fibres (Zhang et al., 2021). Secondary MPs are formed from the breakdown of larger plastic products (Andrady, 2011; Gewert et al., 2015; Salvador et al., 2017; Siegfried et al., 2017; Dalvand and Hamidian, 2022). They are further categorized in two types on the basis of degradation; biodegradable and non-biodegradable (Table 2.1). Biodegradable MPs are eco-friendly and completely degraded by microbes (for example, bacteria, fungi and algae) into carbon dioxide and water (Iwata, 2015; Wei et al., 2021). Non-biodegradable MPs cannot be degraded easily by microbes.

A huge amount of MPs enters in ecosystem through different pathways due to poor management, dumping practices and cause serious pollution obstacles (Zhang et al., 2021). Now there is a dire need to control MPs pollution through different degradation processes such biological, thermal and photo catalytic degradation process (Du et al., 2021). Biological degradation complete by using different types of microorganisms. Microorganisms have potential to degrade different types of organic pollutants without causing harm to environment (Yuan et al., 2020). The degradation efficiency of microorganisms depends on the conditions such as temperature, pH and moisture. Temperature and pH controls the degradation rate of MPs by regulating metabolism of microorganisms (Lin et al., 2022).

MPs enter into the soil through different sources such as sewage sludge, plastic film mulching, irrigation, car tire debris, atmospheric deposition etc. (Li et al., 2020c).

Table 2.1 Different types of biodegradable and non-biodegradable microplastics

Biodegradable/ Non-Biodegradable	**Types of microplastics**	**Applications**	**References**
Biodegradable microplastics	Polylactic acid (PLA)	Use in bottles, plastic film, medical instruments.	Iwata, 2015; Lambert and Wagner, 2017
	Polyhydroxyalkanoates (PHA)	Use in disposal cups, tissues, dipers, bags and fertilizer.	Gonzalez-Pleiter et al., 2019
	Polycaprolactone (PCL)	Use in medical devices and food packaging.	Krueger et al., 2015
Non- biodegradable microplastics	Polyethylene	Use in plastic bottles and can.	Majewsky et al., 2016
	Polypropylene	Used in stoppers and clothes	Zhang et al., 2020b
	Polystyrene	Used in food cans	Zhang et al., 2020b
	Polyethylene terephthalate	Used in water bottles	Wagner et al., 2018
	Polyurethane	Used in tyres, gaskets, furniture cushioning, life jackets and bumpers in refrigerator insulation.	Shah et al., 2008

MPs easily accumulates into the soil due to its small size and cause change in physical and chemical properties of soil like porosity, bulk density and cracks on the soil surface (De Souza et al., 2019; Wan et al., 2019). MPs cause adverse effect on the soil fauna due to the presence of plastic additives or chemicals (Lei et al., 2018b). Soil fauna includes nematodes, amphipods, isopods, collembolans, snails and other invertebrates which are reported to ingest microplastic. The effects depend upon the concentration (reported upto 250g kg^{-1}soil), shape (film, fibers, fragments) and size of MPs (<5mm) (Selonen et al., 2020; Ji et al., 2021). Microplastic remains in the intestine of soil fauna for a longer period, altering gut microbial community and intestinal damages that disturb the feeding behaviour, growth, reproduction and survival rate (Xi et al., 2022; Ding et al., 2022).

2.1.1 Use of plastic mulching in agriculture fields

Farmers use plastic mulch in the agriculture field to improve the quality of crops and yields. Plastic mulch maintains the hydrothermal properties of the soil by a rise in soil temperature and decreasing the soil water evaporation, humidity (Tarara, 2000; Fan et al., 2017; Wu et al., 2017). Mulch provides proper nutrients to the soil to increase the crop yield and reduce nitrogen leaching. Mulch provides support to soil and prevent water erosion (Li et al., 2018b). Plastic mulch is made up by using different types of lightweight plastics, such as low-density polyethylene (Hayes et al., 2012). According to the previous data large amount of plastic mulch is used in Europe and approximately 4270 km area has been covered with plastic mulch (Mugnozza et al., 2012). In China, Japan and South Korea 80% of the agricultural area is covered with plastic mulch (Espi et al., 2006), but in US the PVC containing mulch is totally banned due to its carcinogenic and toxic properties. The use of plastic mulch in different countries is presented in Table 2.2.

2.1.2 Sources of microplastics from wastewater irrigation

Wastewater is used for irrigation purpose in agricultural field and act as a source of microplastic in soil (Table 2.3). Wastewater contains a large number of microplastics from personal care products and plastic fibre from the washing of clothes (Hartline et al., 2016). Plastic enters directly into the agricultural field by using the wastewater and cause changes in soil properties.Wastewater contains several harmful substances such as pharmaceuticals and acts as a source of microplastic in agricultural soil.

Table 2.2 Plastic mulch used by various countries or cities in the agricultural field.

Country/Cities	Year	Use of plastic mulch in Hectares or tons	References
Western Europe	1997	500,000 tons	Hussain and Hamid, 2003
China	1999 2014	10 million hectare 19.8 million hectare	Miles et al., 2012 Liu et al., 2014
USA	1994 1998 2001 2004	519 million lb 85 0 million lb 1,000 million lb 1,30,000 tons	Hussain and Hamid, 2003; Lawrence, 2007 Warnick et al., 2006
US	2006	160,000 hactare	Brodhagen et al., 2015
China Shandong Xinjiang Sichuan	2008	148,100 tons 121,200 tons 71,000 tons	Changrong et al., 2014
Europe	2011	1.3 million hectare	Brodhagen et al., 2015

In Sweden, 15000 items m^{-3} plastic in 2014 were reported in the wastewater, Russia 627000 items m^{-3} plastic were reported in 2014. In the US, 1000 items of plastic were reported in 2016 (Carr et al., 2016; Helcom, 2014; Magnusson and Noren, 2014). Other sources of microplastics in agricultural soil are tyres abrasion and illegal dumping of waste (Fig 2.1). Microplastic directly enters into the soil through wind and is immobilised on the soil surface to cause bioturbation. Due to these, changes occur in the soil properties such as soil structure, vegetation, soil fertility (Sommer et al., 2018: Gonzalez-Pleiter et al., 2019).

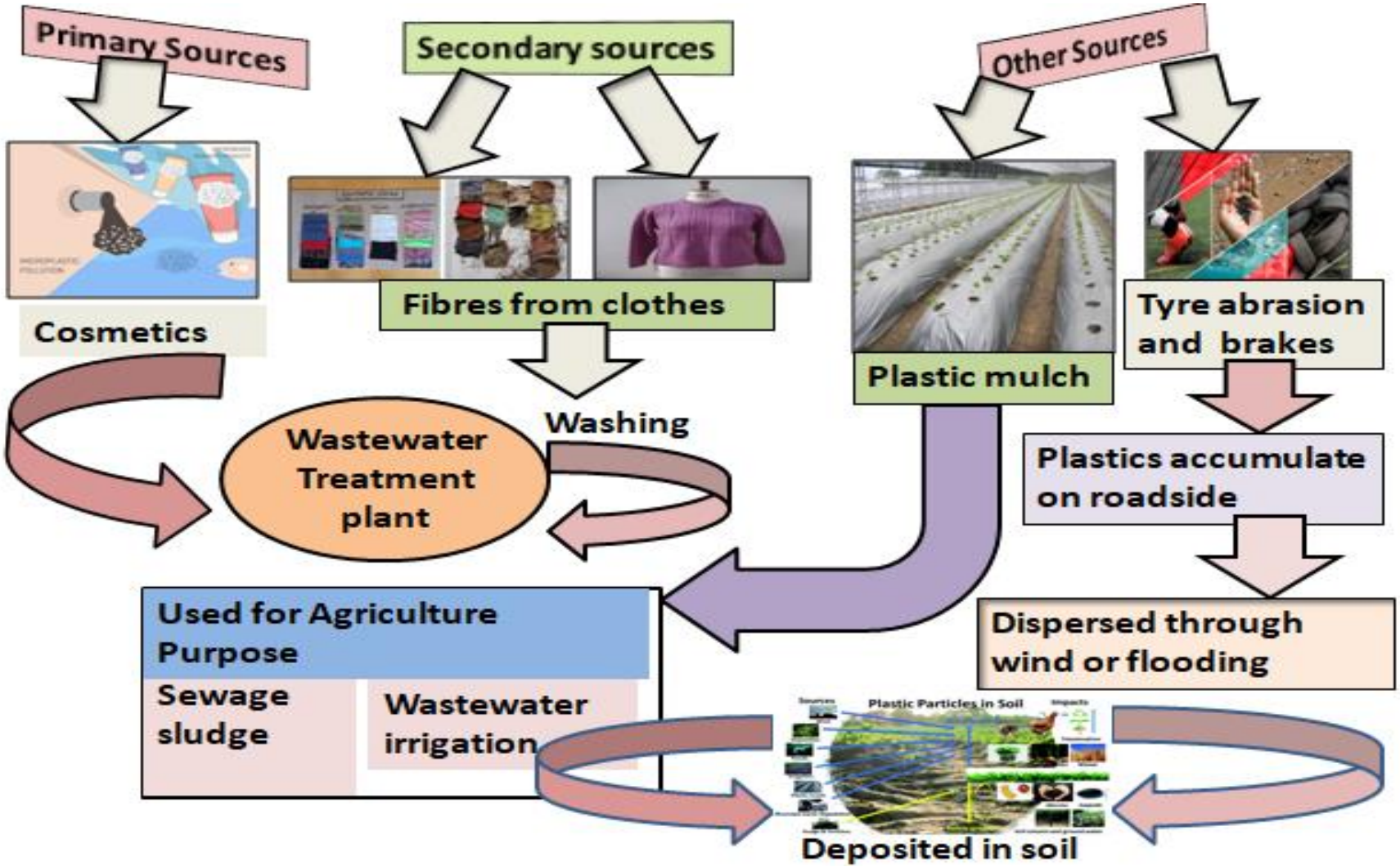

Fig 2.1 Different sources of microplastic and its entry into the agriculture soil through a different ways.

Table 2.3 Occurrence of different types of microplastics in agricultural, industrial soil, wastewater treatment plant and sewage sludge.

Countries	Soil Sampling	Types of microplastic	Microplastic Abundance	References
US	Agricultural soil	Synthetic fibre	1500 – 4000 itemskg^{-1}	Zubris and Richards, 2005
Sweden	Wastewater treatment plant	Plastic fibres	14740-18,660 itemskg^{-1}	Magnusson and Noren, 2014
Sydney	Industrial soil	Polyethylene, Polyvinyl chloride, Polystyrene	300- 67,500 mgkg^{-1}	Fuller and Gautam, 2016
Germany	Sewage sludge	Polypropylene, Polyethylene, Polyvinyl chloride, Polystyrene	1000- 2400 itemskg^{-1}	Mintenig et al., 2017
Ireland	Sewage sludge	Polypropylene, Polyethylene, High-Density Polyethylene	4200- 15800 itemskg^{-1}	Mahon et al., 2017
Sweden	Agricultural soil	Polyethylene	16,700 itemskg^{-1}	Blasing and Amelung, 2018
Canada	Wastewater treatment plant	Plastic fibre	4400-14900 itemskg^{-1}	Gies et al., 2018
Finland	Wastewater and sewage sludge	Polypropylene, Polyester, Polyamide, Polyethylene	2300- 170,000 itemskg^{-1}	Lares et al., 2018
China	Wastewater treatment plant		1565 – 56,386 itemskg^{-1}	Li et al., 2018c
China	Agricultural soil	PE, Polyproylene, Polystyrene	0.54 mgkg^{-1}	Zhang et al., 2018
China	Agricultural soil	PE , PP	78.00 ± 12.91	Liu et al., 2018
Germany	Agricultural soil	PE, PP	0.34±0.36	Piehl et al., 2018
Australia	Agricultural soil	???	1241 – 7170 tonnes/ year	Mohajerani and Karabatak, 2020
China	Agricultural soil	Plastic fibres, fragments, films	13660- 78930 tonnes/ year	Mohajerani and Karabatak, 2020
European Union	Agricultural soil	PE, Low density Polyethylene Polystyrene	26156- 15137 tonness/ year	Mohajerani and Karabatak, 2020
United State	Agricultural soil	PS, PET, PVC	21249- 122780 tonnes/ year	Mohajerani and Karabatak, 2020
Canada	Agricultural soil	Plastics granules, sheet, films, pellets	1581-8770 tonnes/ year	Mohajerani and Karabatak,2020

2.2 Extraction of microplastics from soil

Various types of methods, their advantage and disadvantage to extract microplastics from the soil are given in Table 2.4 and Table 2.5 respectively.

2.2.1 Density Separation

This extraction method is commonly used to extract microplastics from the soil samples on the basis of density (Table 2.4, Fig 2.2).

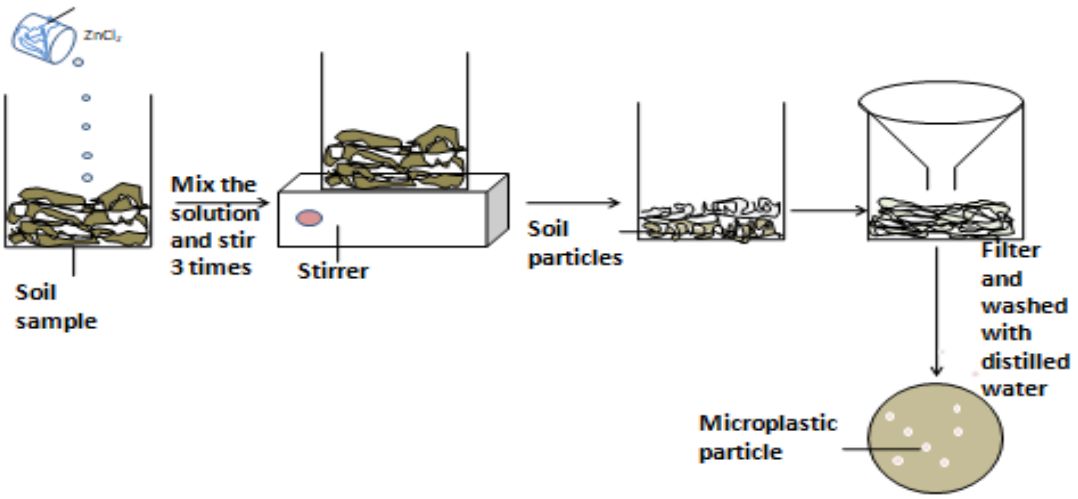

Fig 2.2 Schematic diagram of density separation method for extraction of microplastic from soil.

Principle

This method is based on the density difference of different types of microplastic particles and the soil. Microplastic particles impose the potency in the solution with a higher density of soil particles, and soil particles settle at the surface (Enders et al., 2020; Liu et al., 2020). Microplastic is extracted on the basis of their density because microplastic particles have a low density as compare to the soil, but microplastic particles also have differences in size. Some particles are lower in density, and some are higher (Prata et al., 2019). This method is useful for the extraction of microplastics from different soil samples. Various types of high-density solutes are used for the extraction of microplastics, such as $ZnCl_2$, $CaCl_2$, NaCl, and NaI. In this method, collected soilsamples were mixed with the selected salt after a limited time period, visualised the mixing sample and collected the floating microplastic particles.

Table 2.4 Different types of density solutions used for extraction of microplastics from soil.

Solution	Types of microplastics	Advantages	Disadvantages	References
NaCl	PE, PP, PS	Cheap and non-toxic solution. Due to its low cost and low toxicity, most researchers used this solution for the extraction.	This solution is not used for the extraction of high-density microplastic such as PET or PVC.	Nuelle et al., 2014 ; Liu et al., 2018 ; Scheurer and Bigalke, 2018; Zhou et al., 2018
$CaCl_2$	PE, PP, PS, PET, PVC	Used to extract all types of microplastics.	Ca^{2+} ions easily react with organic residue and cause coagulation in organic residues.	Scheurer and Bigalke, 2018
$ZnCl_2$	PS	-------	Cost is very high and corrosive.	Flury et al., 2019
Sodium Iodide	PE, PP, PS,PVC	--------	Cost is very high.	Huang et al., 2020

Table 2.5 Advantages and disadvantages of different types of extraction methods.

Method	Advantages	Disadvantages	References
Oil Extraction	Rapid Cheap Simple Non-toxic	Low accuracy efficiency rate. Organic residues attach to some microplastics and damage MP in oil-water intermediate.	Mani et al., 2019; Scopetani et al., 2020
Electrostatic Separation	It is a highly convenient and accurate method for the extraction of microplastics. It takes less time for the extraction of microplastics.	Low influence of matrix related variables. Handling should be done by a trained person.	He et al., 2021
Magnetic extraction	Small size MPs can be easily extracted by using this method.	Time-consuming method.	Grbic et al., 2019
Density Separation	A rapid method for the extraction of microplastics. Micro-sized particles are easily extracted. Cost-effective and accurate method.	Various chemicals are used for the extraction of microplastics.	He et al., 2021; Li et al., 2020c
Solvent extraction	A rapid method for the extraction of microplastics. Low cost. High-efficiency rate.	A large amount of solvent required for the extraction.	Wen et al., 2021

2.2.2. Electrostatic Separation

This method is used for the extraction of microplastics from different soil samples, such as industrial and agricultural soil (Rajaonarivony et al., 2017).

Principle

Its principle is based on the conduction properties of soil particles because soil particles are charged due to their conduction characteristics, and microplastics are non-conductive. Soil sample added to the funnel of electrostatic separator instrument and it passes to the corona electrode system with the help of vibrating conveyor. Instrument fitted with high voltage current. At high voltage current soil particles easily charged between the grounded drum and above-fitted electrode of the instrument. Soil particles rapidly discharge due to their conductive property and start jumping from the grounded drum. Microplastic particles discharge slowly and attach to the rotating drum of the instrument. Attach particles of microplastics erase with the help of a scraping plate (Enders et al., 2020).

2.2.3 Oil Extraction

Generally, two types of oil are used for the extraction of microplastics from the soil, such as olive oil and castor oil (Fig 2.3).

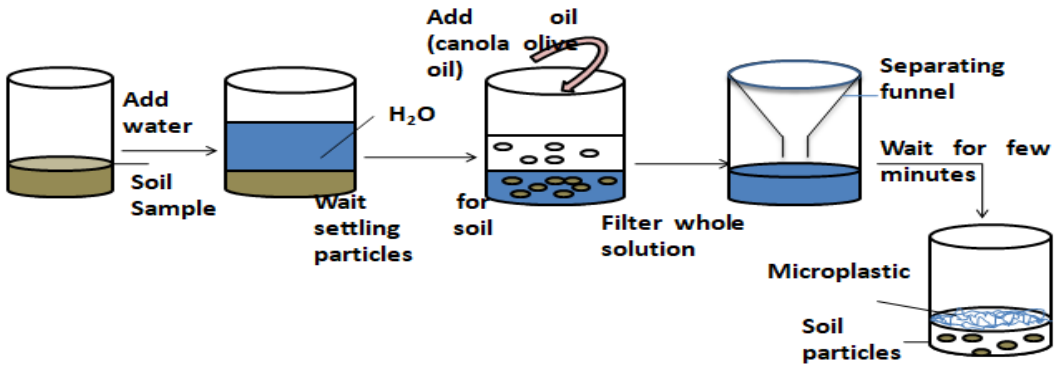

Fig 2.3 Schematic diagram of extraction of microplastic from the soil by using oil.

Principle

Its principle is based on the oleophilic interaction of plastic polymers, which means oil consists of long-chain fatty acids. These long-chain fatty acids rapidly interact with the backbone of plastic polymer.

2.3.4 Magnetic Extraction

The principle of this method is based on the Fe coated nanoparticles easily magnetized with the hydrophobic surface of microplastics (Fig. 2.4). Microplastics from the soil sample are extracted by applying magnetic force on the magnetic field because of the magnetic properties of Fe nanoparticles bound to microplastic and allow microplastics to separate out (He et al., 2021).

2.3.5 Solvent Extraction

Fuller and Gautam, (2016) automatically extract microplastics by using different types of solvents. This method is used to extract the organic pollutants present in the soil (Fig 2.5). Two types of organic solvents are mostly used for the extraction of microplastics, such as dichloromethane and methanol.

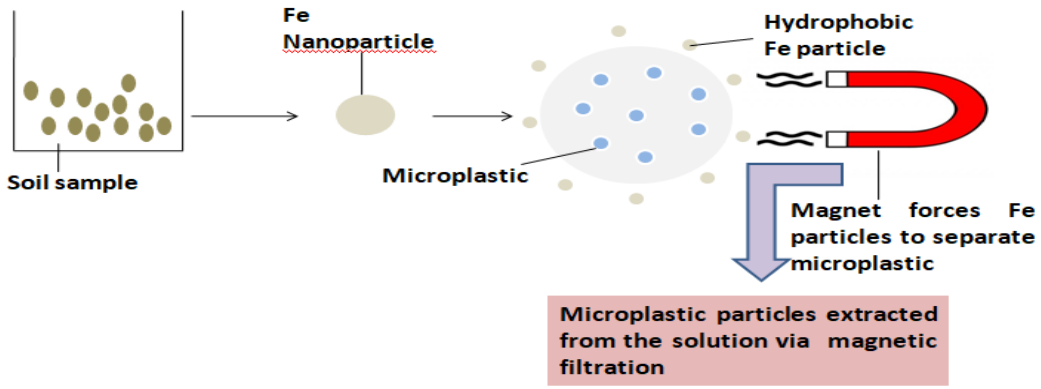

Fig. 2.4 Schematic diagram of extraction of microplastics from soil via magnetic separation.

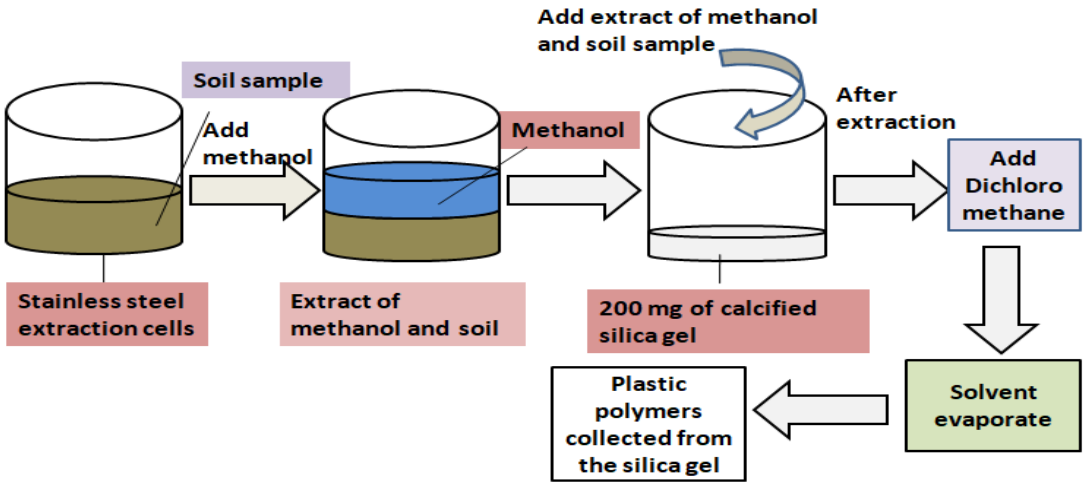

Fig 2.5 Systematic diagram of extraction of microplastics via organic solvent.

2.2.6 Pressurized Fluid Extraction

Principle

This is based on recovering semi-volatile organic pollutants using solvent at critical conditions such as temperature and pressure. By maintaining the temperature and pressure parameters, the microplastics were extracted from the soil. Extraction is based on either stir together or dissolving the sample (Fuller and Gautam, 2016).

2.3 Detection techniques of Microplastics

2.3.1 Vibrational Spectroscopy

It is the most commonly used spectroscopic technique for the detection of microplastic particles. The main principle is based on vibrational microscopy combines with optical microscopy resulting in the determination of the composition of the microplastic particles and the visual identification of the particles (Nguyen et al., 2019).

2.3.2 FTIR Spectroscopy

Fourier transform infrared spectroscopy is a technique used to obtain an infrared spectrum of absorption emission of a solid, liquid and gas. An FTIR spectrophotometer simultaneously collects high- spectral- resolution data over a wide spectral range (Griffiths and De, 2007).

FTIR spectroscopy principle is based on the variation that occurs at the dipole moment of chemical bonds and provides a spectrum of microplastic particle analysis. These variations occur at the dipole moment due to the signals that produce a spectrum, and polar functional groups make the particle more sensitive (Nguyen et al., 2019, Hu et al., 2019). FTIR spectroscopy can easily detect the different types of plastic polymer. Compared to other spectroscopic techniques, FTIR detects a large number of microplastics within a short period of time. FTIR is an adorable technique for detecting the microplastic for various reasons such as ease of use, non-destructive technique, and low cost to the other spectroscopic technique (Chalmers and Everall, 1996; Coates, 2006; Evanson et al., 1991). FTIR spectroscopy generally depends on reflectance and transmittance. A small amount of sample is required for the detection due to its spatial resolution being less than 5μm (Elert et al., 2017, Mallikarjunchari

and Ghosh, 2016). FTIR spectroscopy techniques include Micro FTIR, attenuated total reflectance (ATR- FTIR) and focal plane array (FPA-FTIR).Micro FTIR is used only to detect the small microplastic size, whereas ATR- FTIR technique is used to analyze the asymmetrical microplastics of size 500 micrometer (Prata et al., 2019; Loder and Gerdts, 2015) while FPA-FTIR detects the microplastic of size more than 20µm (Ojeda et al., 2015). Complex microplastics can be analyzed by using the FPA-FTIR and ATR-FTIR. ATR- FTIR spectroscopy uses a chemo-metric method to assess and identifying the antiquated and characterized the surface contaminated with microplastic (da Costa et al., 2019). Microplastic from the wastewater is easily identified and detected by using FPA- FTIR (Ojedha et al., 2015).

2.3.3 Raman Spectroscopy

Raman spectroscopy was first used to detect the microplastic by Signer and Weiler in 1932, in which they attained the polystyrene spectra (Crawford and Quinn, 2017). Raman spectroscopy technique is a very important technique based on the principle that causes the polarization of the scattered light to detect the microplastic particles at a particular range of wavelengths (Lenz et al., 2015). The scattering of lights occurs due to the changes in molecular vibration. Raman spectroscopy is used to detect the microplastic size less than or equal 20 microns. This technique is applicable for the characterization and alteration of several compounds containing different aromatic bonds (Hu et al., 2019). Using this technique, the composition of microplastic polymers can be detected by passing through the irradiating monochromatic beam and providing information about the molecular structure and the composition of the atoms present in the microplastics at different scattering frequencies (Araujo et al., 2018).As a comparison to FTIR organic and inorganic fillers, dyes and microbiological substances can be detected easily by using the Raman spectroscopy (Imhof et al., 2013; Lenz et al., 2015; Kappler et al., 2016). The main drawbacks of this spectroscopic technique are taking a long processing time, heating the microplastic polymer and degradation, as well as fluorescence inference (Strangaru et al., 2019; Ribeiro- Clairo et al., 2017).

2.3.4 Mass Spectrometry

Mass spectroscopy is a very important physio-chemical technique used to identify different types of the compound and provides information about the chemical structure and reactivity of the compounds. This method is based on the detection of microplastics by using a spatial resolution that increases the sensitivity of microplastic particles. These microplastic particles become more sensitive and easily attract to the signal that analyzes the total surface.Mass spectrometry is used to analyze the qualitative information about the mixture of plastic particles and is sometimes used for quantification of the plastic particles (Nguyen et al., 2019).

2.3.5 Pyrolysis Coupled with Gas Chromatography-Mass Spectrometry

This technique is employed to detect various chemicals and organic additives used in plastics (Fries et al., 2013, Kappler et al., 2018). The principle is based on the degradation of microplastic depends on the pyrolysis temperature, results in the degradation of volatile polymers, and traces the microplastic polymer. GC column separates the pyrolysis product of the microplastic, and these pyrolysed polymers can be characterized on the basis of pyrolysis pattern (Kappler et al., 2018). Pyr-GC-MS technique works by the hydrolysis of larger plastic polymers into smaller ones. The volatile component can be detected and separated from the smaller plastic polymers by using Pyr-GC-MS. This technique cannot provide morphological information such as shape and size. Microplastics of larger than 100 µm can be analyzed manually by putting them into a pyrolysis tube (Dekiff et al., 2014). It is a harmful technique for the degradation of microplastics thermally and compares the thermally degraded product with pyrogram of known pure polymers. This technique cannot identify the polymer that forms polar pyrolyzate, which consists of polar subunits, including polyester and polyether (Challinor, 1989; 2001).

2.3.6 TDS-GC-MS (Thermal Desorption Coupled with Gas Chromatography-Mass Spectrometry)

To overcome the shortcomings of Py-GC-MS, another technique is available in which the microplastics are treated thermally at the ambient temperature, e.g. 1000 degrees (Dumichen et al., 2017). In this method, detection and characterization of microplastic are done by absorbing the microplastics on a solid phase. After that, microplastic

particles are transferred to a thermal desorption unit. This is due to the high temperature of microplastic particles. The microplastic particles analyzed by the high degree of temperature can be separated with the help of a chromatographic column and characterized by using mass spectrometry (Dumichen et al., 2014; 2015). The main advantage of this technique is that it can characterize a larger amount of samples in one time compared to PY-GC-MS. It is a quantitative technique for detecting and characterization of a large number of microplastics (Dumichen et al., 2015).

2.3.7 TGA-DSC (Combined Thermogravimetric Analysis- Differential Thermal Calorimetry)

This thermal technology is used to detect microplastics from the environmental samples, mainly for the detection of microplastics from the wastewater. Detection can be done by checking its thermodynamic properties, for example, enthalpies, heat capacities and temperature. If collected samples exhibit an endothermic or exothermic property and increase or decrease, the temperature shows the result in the form of the peak. The peak area is further used for the identification of the microplastic (Penalver et al., 2020).

2.3.8 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Inductively Coupled Plasma-Mass spectrometry principle is based on the single event mode, and this technique is also known as Single Particle (SP- ICP-MS). This single-particle theory was first reported in contrast to detect or identify the colloidal particle in water by Degueldre (Meermann and Nischwitz, 2018). This technique utilises the single event mode to detect microplastics and provides detailed information about the morphological characteristics such as size distribution, chemical composition, and mass concentration of the microplastics (Bolea et al., 2020). When microplastics pass through the ICP, the separate signals provide for screening each spike, and the period of signal for each spike is 0.5 ms (Hineman and Stephan, 2014). When signals reach the surface of the microplastic, it starts differentiating the microplastics on the basis of the signal. This technique is based on the single-particle or single-mode event changes that occur in the signal baseline of blank, and microplastics exhibit the dissolved concentration of the element. The single event mode frequency is directly

proportional to the number of microplastic particles (Hineman and Stephan 2014; Bolea et al., 2020).

2.3.9 Photoluminescence Spectroscopy

Photoluminescence spectroscopy is also used for the detection of microplastics. This technique is based on the principle in which the light emits from the microplastic sample surface and is optically excited from the sample (White and Argauer, 1970). The excitation of light from the sample surface is due to the higher wavelength of photoluminescence emission. Microplastics can be identified by studying the photoluminescence spectra. This analytical technique is not an encouraging technique for the detection of microplastics (Ornik et al., 2020).

2.3.10 Visual Identification

Direct observation of microplastics can be done by naked eyes observation and by using the microscope. The large size of MPs particle was observed using the naked eye, but the small size of microplastic particles was examined using the microscope under different magnification lenses (Lv et al., 2021). Detection is based on the shape and colour of microplastic particles. Microplastic particles are difficult to differentiate (Qui et al., 2016; Song et al., 2015).

2.3.11 Scanning Electron Microscope (SEM)

Scanning Electron microscopy detects microplastics on the basis of individual surface characteristics of microplastics (Kalcikova et al., 2017; Fu et al., 2020). This microscopic technique coupled with Energy-dispersive X-ray spectroscopy detects the morphological characteristics and chemical compositions. The principle is based on characterizing the microplastic element and provides information about the microplastic particle by emitting the electron beam with the help of X-rays (Fu et al., 2020). Characterization can be done by using high magnification power for better image and differentiate the microplastic particle (Copper and Corcoran, 2010). The prospects and consequence of all these microplastic detection techniques are mentioned in Table 2.6.

Table 2.6 Different techniques for detection of microplastic and their merits and demerits.

Techniques	Applications	Merits	Demerits	References
Laser Direct Infrared (LDIR)	This technique is used to detect the size ratio and types of microplastic particles present in the sample.	It is a rapid and automatic technique that provides detailed information about each microplastic particle's spectra with the validation of their identity. This technique is used to detect a large number of microplastics.	A microplastic particle such as a plastic polymer or mixture of polymers cannot be identified.	Scircle et al., 2020; Zhang et al., 2015
Fourier Transform Infrared Spectroscopy (FTIR)	This spectrophotometer can detect smaller (less than 20 µm) and larger particles (greater than 500 µm). This technique is helpful to provide information about the shape and size of the classes of plastic polymer.	It is an easy and rapid method. It can analyze several thousand samples in a short period. This spectroscopic technique is very easy than other techniques.	The cost of the instrument is very high.	Zhang et al., 2015; Crawford and Quinn, 2017; Li et al., 2018a; Fries et al., 2013
Micro- FTIR	Microplastic polymers of regular shapes can be easily detected by using this technique.	Characterize the small size microplastics such as 10 µm.	Time-consuming technique.	Shim et al., 2016; Fu et al., 2020
Attenuated Total Reflections	ATR-FTIR is used for the detection of irregularly shaped microplastics.	Non-destructive analysis. An easy technique, not sample preparation, is required.	Expensive instrument.	Fu et al., 2020
Nuclear Magnetic Resonance Spectroscopy (NMR)	This technique is used to detect the plastic polymer by characterizing the chemical structure of the polymer chain.	It is a very reliable and sensitive technique for the detection of polymer and chemicals.	It is time-consuming and costly.	Crawford and Quinn, 2017

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Techniques	**Applications**	**Merits**	**Demerits**	**References**
Raman Spectroscopy	This technique is applicable to identify small microplastic sizes less than 1µm. This method can characterize microplastic particles on the basis of the difference of interaction of the laser light and the frequency of back scattered light	Easily identify the organic and inorganic substances by the intrusion of fluorescence.	Time-consuming analytical technique. Various parameters can be determined before analysis, such as wavelength, photo bleaching and laser power. It is a slow and automatic method used for the collection of spectra.	Cole et al., 2013; Zhao et al., 2014; Weisheu et al., 2016
Fluorescence Spectroscopy	Semi-quantitative type of spectroscopy detects the microplastics on the basis of physical and chemical mapping of microplastic.	The detection rate is very low and provides spectra of the sample by using single absorption and emission line.	It takes a longer duration for detection due to the sample preparation.	Fu et al., 2020; Towett et al., 2013
Scanning Electron Microscopy (SEM)	This technique is generally used to characterize the whole surface of the sample to identify the microplastics particles and elementcompositionofmicroplastic identified by using SEM-EDS.	It provides clear and highly magnified images of microplastics with high spatial resolution. It is applicable for the characterization of microplastic particles such as fibres and spherule.	SEM-EDS microscopy is more costly than other microscopy and acquires more laborious work to prepare the sample. It takes a long time for the characterization of samples; hence only limited samples can be analyzed.	Shim et al., 2017; Dehghani et al., 2017; Zbyszewski et al., 2014 ; Vianello et al., 2013
Pyrolysis GC-MS (Pyr - GC MS)	It is a technique used to study microplastic polymers' science and characterise the different types of chemical and organic additives with their composition.	This technique is very sensitive in comparison to other techniques to enabling polymer types in microplastics with relatively low masses of microplastics polymer can be identified.	It requires the manual placement of microplastic particles. It can analyze only one microplastic particle from the running sample at one time.	Lusher et al., 2017; Fries et al., 2013; Kappler et al., 2018; Fabbri et al., 2000
Matrix-assisted laser desorption/ionization	This technique is applicable for the detection and characterization	It is a rapid, simple and cheap technique.	Characterization of the microplastic polymers based on	Dimzon and Knepper, 2012; Schirinzi et al., 2019;

Cont...

Techniques	Applications	Merits	Demerits	References
time-of-flight mass spectrometry (MALDI-TOF–MS)	of the high molecular weight of the plastic polymer.	It is easily characterized or detected the microplastic polymers from a whole sample.	the area between the matrix and polymers.	Rizzarelli and Carroccio 2014; Fu et al., 2020
Ambient Ionization Technique	This technique provides information on the basis of the composition of polymer in microplastics.	Easily identify the polymer with a short period.	This technique does not require the pretreatment of the sample to obtain spectra.	Schirinzi et al., 2019
Liquid Chromatography	This chromatographic technique is applicable for the detection of nonvolatile compounds combined with microplastics due to high sensitivity	A small amount of sample (milligram) is required for the identification.	It is unavailable to give the details about the physical parameters includes size details and types of plastic polymer.	Hintersteiner et al., 2015; Elert et al., 2017; Fu et al., 2020
Microscopic Count	It is used for the identification of microplastic particles that are micrometer in size and can be identified with the help of stereomicroscope by direct counting. Only pretreated microplastic particles are recommending for detection.	A large quantity of microplastic can be easily identified. It takes a short time and gives the best result at a low cost.	The quality of microplastic particles cannot be determined.	Li et al., 2018a
Staining Technique	Micrometer plastic particles can be detected by using hydrophobic dye such as Nile red.	This process is less expensive and fast than other techniques.	Plastic debris can be identified by staining with dye.	Shim et al., 2016
Thermogravimetry coupled with Differential Scanning Calorimetry (TGA – DSC)	This thermal technology is used for the detection of primary microplastics and polymer types on the basis of their mass concentration.	For the detection of microplastics very small amount of sample is required.	This thermal technology is coat effective and laborious.	Majewsky et al., 2016 ; Shim et al., 2017; Penalver et al., 2020

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Techniques	**Applications**	**Merits**	**Demerits**	**References**
Differential Scanning Technique (DSC)	This technique is used to detect microplastic from a large number of polymer products.	Simple and fast technique for detection of microplastics.	Destructive technique.	Shim et al., 2017: Tsukame et al., 1997
TGA- GC- MS	This thermal technique detects microplastic on the basis of additives and chemicals.	The major advantage of this method large number of samples can be analyzed.	Time-consuming and laborious technique.	Zhang et al., 2020c; Shim et al., 2016; Penalver et al 2020
TED-GC-MS	This thermal technique is available for the detection of unknown organic microplastic particles.	Fast and easy technique as a comparison to other techniques.	It cannot give information about the size and quantity of microplastic.	Lares et al., 2019

2.4 Effect of microplastics on micro and macro-fauna of soil

Microplastic shows adverse effects on the soil micro and macrofauna. Microplastic ingestion by nematodes (*Caenorhabditis elegans)* cause several changes such as effects on the reproduction and growth rate. It also causes intestinal and oxidative damage (Xu et al., 2020: Lei et al., 2018b). Intestinal and oxidative damages improve by minimization in intestinal calcium level of *C. elegans* and by a rise in the expression of oxidative stress gene (*gst-4*) (He et al., 2018). Microfauna such as snail (*Achatina fulica*) are used as a model to check the toxic effect of microplastic ingestion. The adverse effect includes a decrease in food hold and produces oxidative stress (Song et al., 2019). In isopods (*Proisotoma minuta*), microplastic cause an effect on the food uptake, defecation rate, body weight and leads to death (Kokalj et al., 2018).

Microplastic causes various biological changes such as the growth and reproduction of earthworms due to their small size and easy accumulation in the earthworm body. Earthworm directly takes microplastic from the soil because microplastic used in agriculture field by using wastewater irrigation, etc. Earthworm ingests microplastics that's enters into the intestine and accumulates in the casts cause bioturbation (Lwanga et al., 2016). Mainly microplastic particles attach to the gut and stomach of the earthworm and change the feeding activities, which alter the growth or development of earthworm (Table 2.7; Fig 2.6). Due to the large existence of absorbed organic pollutants in the gut of organisms, which are desorbed by the organisms after ingestion and cause a deleterious effect on earthworms (Bakir et al., 2014). Due to its small size, microplastics attach to the outer surface of the earthworm precisely prohibit the flexibility of the organisms. Normally changes occur, such as false satiation and decrease in the level of carbon biomass leads to death, loss of energy, and effects on the growth and development of the organisms (da Costa et al., 2016; Setala et al., 2016). Other mechanical obstruction includes metabolism disorder, intestinal damage and direct effect of ingested microplastics on the earthworm oesophagus (Lahive et al., 2019; Lonnstedt and Eklov, 2016; Wang et al., 2019a). Immune system of earthworm also effected by ingestion of microplastics because microplastics attach to villi and enters into the different cells of intestine epithelial.

This accumulation of microplastic to the intestine causes inflammation of the gut and also causes various sub-lethal effects on the immune system and growth (Hirt et al., 2020).

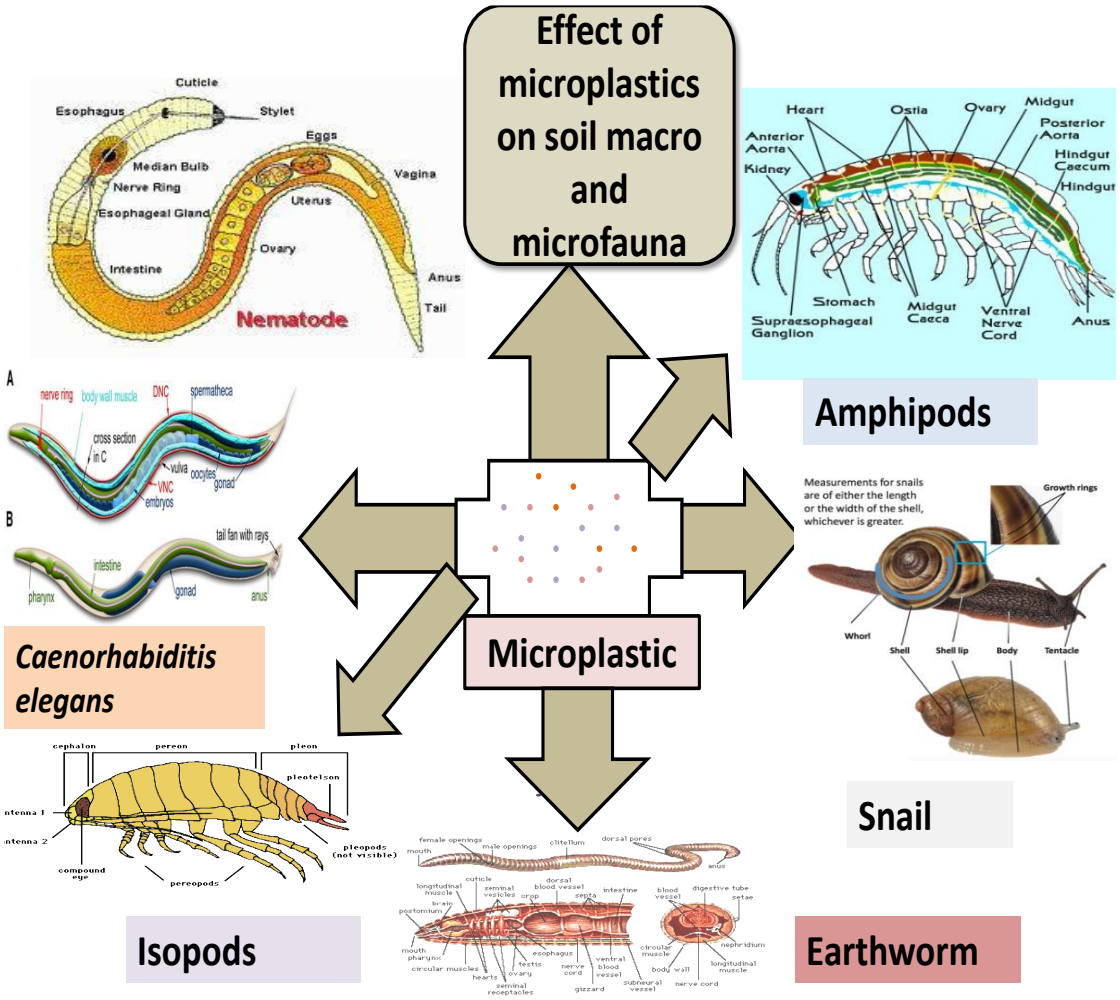

Fig 2.6 Impact of different types of microplastics on soil micro and macro fauna.

Table 2.7 Effects of microplastics on different species of earthworm after exposure.

Species	Type of microplastic	Size range	Concentration	Exposure	Observation	References
Lumbricus terrestris	Polyethylene (PE)	<400 µm	0,7, 28, 45, and 60%	14 and 60 days	After 14 days of exposure, no changes occur in mortality, but after 60 days, exposures show an effect on concentration.	Lwanga et al., 2016
Lumbricus terrestris	Low Density Polyethylene (LDPE)	400 µm	0 (control), 7, 28, 45 and 60% (w/w)	14 days	Changes can be seen in the growth and rapid increase in the mortality of the earthworm.	Lwang et al., 2017
Eisenia fetida	Polystyrene (PS)	58µm	0, 0.25, 0.5,1 and 2%	30 days	Growth of *E. foetida* is greatly affected at concentrations 1% and 2%, as well as lethal effects, can notice.	Cao et al., 2017
Lumbricus terrestris	Polyethylene (PE)	710-2800 µm		21 days	Microplastic attached to the skin mucus of earthworm. This attachment is the major source to carry the microplastic and affect the growth of earthworms.	Rillig et al., 2017
Lumbricus terrestris	High Density Polyethylene (HDPE)	<150 µm		28 days	Ingestion of zinc bearing microplastic affect the growth and the mortality of the earthworms.	Hodson et al., 2017
Eisenia andrei	Polyethylene(PE)	250-1000 µm	0, 62.5, 125, 500, 1000 $mgkg^{-1}$	28 days 56 days	Gastrointestinal tissue of earthworm affected after ingestion of microplastic.	Rodriquez-Seijo et al., 2017
Lumbricus terrestris	Low-Density			60 days	Gut bacteria reduce the size of	Lwanga et al.,

Cont...

Species	Type of microplastic	Size range	Concentration	Exposure	Observation	References
	Polyethylene (LDPE)				LDPE and affected the volatile compounds of microplastics.	2017
Eisenia fetida	Low-Density Polyethylene (LDPE)	250-100 µm	65, 125, 250, 500, 1000 $mgkg^{-1}$	28 days	After the exposure of 28 days of polypropylene microplastic, the toxic effects include oxidative stress and changes occur in the metabolic process of earthworm.	Rodriguez-Seijo et al., 2018
Lumbricus terrestris	Polyester microfibers	0.05-2 mm	0 (Control), 0.1 and 1.0% (w/w)	35 days	After the ingestion of polyester microplastics death rate is very low.	Prendergast-Miller et al., 2019
Lumbricus terrestris	Low-Density Polyethylene (LDPE)	-----	----------	----	Microplastics in earthworms can directly or indirectly enter the tissue, adhere to the tissue, and cause obstructions on the earthworm's gut.	Lu et al., 2019
Eisenia fetida	Low-Density Polyethylene (LDPE)	5 mm and 0.25 µm -1 mm in diameter	----	14 days	After 14 days of exposure, neurological changes can be observed.	Rodriquez-Seijo et al., 2019
Aporrectodea rosea	High-Density Polyethylene (HDPE)	---	-----	30 days	After exposure to microplastics, earthworm loses their weight.	Boots et al., 2019
Eisenia fetida	High-Density Polethylene (HDPE) Polyethylene terephthalate (PET) Polyvinyl Chloride	----	0.1, 0.25, 0.5 and 1% w/w for acute toxicity test 0.01, 0.1, 0.25, 0.5, 1%	28 days for testing acute toxicity 56 days for chronic toxicity	At concentration 0.5%, there are no toxic effects on an earthworm. In other concentrations, slightly acute and chronic changes can be seen.	Judy et al., 2019

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Species	Type of microplastic	Size range	Concentration	Exposure	Observation	References
	(PVC)					
Eisenia fetida	Low-Density polyethylene (LDPE) Polystyrene (PS)	<300 µm <250 µm	0, 1, 5,10 and 20%	14 days	At concentration 20%, mechanical obstructions can be seen includes oxidative stress and damage in earthworm.	Wang et al., 2019b
Enchytraeus crypticus	PVC Nylon	106-150 µm 13-18µm 63-90 µm 90-150 µm	90 gKg^{-1} 20, 50, 90, 120 gkg^{-1}	21 days	At a high concentration of microplastic in the soil, the reproduction rate is reduced in juveniles.	Lahive et al., 2019
Eisenia fetida	Low-density polyethylene (LDPE)	<400 µm	0.1, 0.25, 0.5, 1.0, 1.5 gkg^{-1} dry weight	28 days	The rapid increase in the CAT activity of earthworms. Various mechanical obstructions can be seen, such as ulceration, blockage and skin damages.	Prust et al., 2020
Eisenia fetida	Polypropylene (PP)	150 µm in diameter	0.03, 0.3, 0.6, 0.9%	42 days	A high concentration of microplastics and cadmium leads to an increase in Lipo peroxide level and GSH content and show various harmful effects on an earthworm.	Zhou et al., 2020
Eisenia fetida	Polystyrene (PS)	-----	----	15 days	After 15 days of exposure cause toxic effects such as DNA damage and induce oxidative stress	Jiang et al., 2020

Cont...

Species	**Type of microplastic**	**Size range**	**Concentration**	**Exposure**	**Observation**	**References**
Eisenia fetida	Polystyrene	-----	------	21 days	Microbial diversity of earthworm increase after the exposure of polystyrene microplastics.	Xu et al., 2020
Eisenia fetida	Low Density Polyethylene	550-1000 µm	------	28 days	Induce oxidative stress due to an increase in the activity of the antioxidant enzymes.	Chen et al., 2020d
Eisenia fetida	MP + Dufulin	40- 50 µm	--------	28 days	Microplastic and dufulin cause oxidative damage and direct effect on the metabolic profile of earthworm.	Sun et al., 2021
Eisenia fetida	Low Density Polyethylene Polypropylene	28–145, 133–415, 400-1464 µm 8–125, 71–383 and 761–1660 µm	------ -	28 days	Both types of MPs produce changes in the different types of earthworm's enzymes.	Li et al., 2021

2.5 Effect of microplastics on Antioxidant biomarkers of earthworms

Various types of antioxidant enzymes of earthworms induce various types of oxidative stress and affect the mortality of the earthworm when exposed to different types of microplastics (Rodriquez-Seijo et al., 2018). The digestive system of the earthworm contains various types of highly active antioxidant enzymes such as catalase, phosphatase, protease, polyphenol oxidase (Tikhonov et al., 2011; Frouz et al., 2011). These enzymes play a key role in the breakdown of the peptidic element, and changes occur in the elemental composition. All these enzymes degrade the organic waste with the help of microorganisms. The presence of enzymes in earthworms is due to the microorganism's activity in soil (Shan et al., 2010). Oxidative stress is defined as the "imbalance between the oxidative as well as antioxidant indices in the living systems" (Tiwari et al., 2016). The function and role of different types of antioxidants is given in Table 2.8 and Fig 2.7.

2.5.1 Role of antioxidant biomarkers

Different types of biomarkers catalyze the antioxidant mechanisms and various organic pollutants such as microplastic produces oxidative circumstances and cause disruption in the tissue of organisms. Oxidative challenges occur when ROS concentration increases very fast in the tissue of the organisms. These organic pollutant leads to changes occur in the spike of ROS concentration which regulates physiological pathway. An increase in the ROS concentration plays an important role to produce oxidative damage. In the first stage, a spike occurs in ROS concentration is normal, but in the second stage, excess ROS concentration is not counter balanced by antioxidant biomarkers. In the second stage, all immune able antioxidant biomarkers regulate physiological activity. These antioxidant biomarkers rapidly increase the antioxidant level. An increased level of antioxidant biomarker neutralises the large concentration of ROS. Various types of enzymes take part to maintain ROS concentration. In stage third, disruption occurs due to the late response of antioxidant constituents because cell takes a long time to construct these constituents. These antioxidant constituents normally up-regulated various changes produced in the organism, such as oxidative stress (Sies and Cadenas, 1985).

Table 2.8 Antioxidant enzymes of the earthworm and their functions.

Enzymes	**Functions**	**References**
Catalase (CAT)	Catalase is an enzyme that plays an important role in the breakdown of the free radical of hydrogen peroxide in the form of water and oxygen. It acts as a detoxifying enzyme.	Claiborne, 1985; Zhang et al., 2009; Liu et al., 2011
Glutathione –S-Transferase (GST)	Glutathione-S-Transferase plays an important role in catalysing the conjugation reaction of GSH.	Maity et al., 2008 Jiang et al., 2020
Glutathione	It is an antioxidant molecule that oxidizes glutathione and overcomes the ROS concentration. It acts as a cofactor for another biomarker such as GPx and GST.	Trestrail et al., 2020; Sharma et al., 2004
Lactate dehydrogenase	Lactate dehydrogenase enzymes play an important role to provide the amount of energy if organisms need. It is an anaerobic enzyme.	Diamantino et al., 2001; Tripati et al., 2011
Superoxide dismutase (SOD)	Superoxide dismutase enzyme plays an important role to catalyze the conversion of oxygen into hydrogen peroxide.	Jiang et al., 2020
Malondialdheyde (MDA)	It is an antioxidant biomarker that causes oxidative stress in the organisms and produces after lipid per oxidation	Trestrail et al., 2020

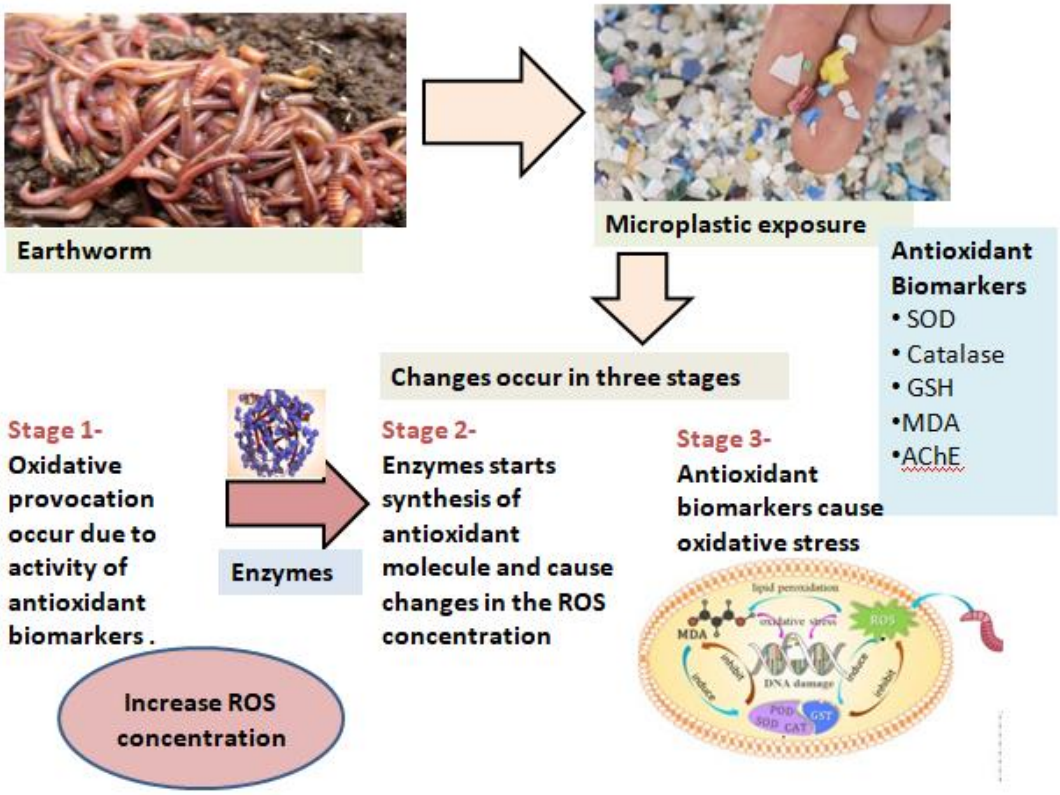


Fig 2.7 Interaction of microplastic to the earthworm and relevant antioxidant biomarkers cause oxidative stress in three stages.

2.6 Different types of microorganisms in gut of earthworm

The rapid use of microplastic in the agricultural field to increase the crop yield and quality of crop cause a threat to terrestrial biota. Earthworm gut provides a microenvironment for the growth of microorganisms such as appropriate moisture content, neutral pH and a large quantity of mucus (secreted from the foregut and absorbed by gut bacteria). The presence of mucus in the earthworm gut consists of a mixture of low molecular weight organic matter and act as a good source for the growth of microorganisms (Du, 2018). Microplastic acts as a sole carbon source for the metabolism of microorganisms in the gut of earthworms (Table 2.9). Gut microorganisms play an important role in the degradation of organic pollutants such as microplastic, chemical pollutants, etc. A large amount of microplastic disturbs the metabolism as well as intestinal diversity of microorganisms.

Table 2.9 Different types of bacteria present in the gut of different species of earthworm.

Species	Microorganisms in Earthworm gut								References Referen
	Proteobacteria	Firmicutes	Actinobacteria	Bacteroidetes	Acidobacteria	Verrucomicrobia	Chloroflexi	Plantomycetes	
Eisenia fetida	44%	31%	6%	-	-	-	6%	-	Hong et al., 2011
	50%	-	9%	7%	-	-	-	-	Wang et al., 2017
	62%	14%	24%	-	-	-	-	-	Wang et al.,2017
	24%	52%	13%	-	-	-	-	-	Yausheva et al., 2016
	10%	21%	10%	3%	-	-	-	6%	Singh et al., 2015
	16%	46%	11%	-	-	1%	-	5%	Schulz et al., 2015
Eudrilus eugeniae	52%	26%	7%	8%	-	-	3%	-	Schulz et al., 2015
	46%	2%	1%	42%	-	-	-	6%	Zhu et al., 2018
Enchytraeus crypticus	9%	12%	48%	1%	-	-	-	-	Zhu et al., 2018
	44%	42%	-	-	1%	9%	-	1%	Zhu et al., 2018
Pheretima carnosa	45%	18%	2%	2%	2%	2%	-	1%	Liu et al., 2011
Perionyx excavates	20%	15%	2%	5%	-	-	-	6%	Singh et al., 2015
Metaphire calfornica	24%	12%	29%	4%	6%	3%	3%	13%	Wang et al., 2019a
Metaphire sieboldi	13%	21%	59%	2%	1%	-	-	1%	Hu et al., 2016
Lumbricus rubellus	36%	20%	3%	17%	-	10%	-	-	Knapp et al., 2009
	50%	30%	-	6%	3%	-	-	-	Pass et al., 2015
Lumbricus terrestris	30%	-	28%	-	4%	9%	4%	7%	Meier et al., 2018

2.7 Degradation of the microplastic by microbes associated enzymes

Generally, the mechanisms of degradation of MPs depend upon the different types of factors such as the chemical structure of plastic polymer, molecular weight of chemical compounds, plasticizer and additives used for making plastics (Alshehrei, 2017b; Yuan et al., 2020). Various biochemical reactions are involved in the degradation of microplastic by microorganisms. Microorganisms (*Bacillus* sp., *Ideonella sakaiensis, Rhodococcus* sp. and *Paenibacillus* sp. etc.) secretes different varieties of enzymes (esterase, urease, lipase, protease, glycoside hydrolases and laccase), these enzymes gets attached on the backbone of long chain plastic polymers and cleave long chain of polymer into monomers units. The first and most important step of degradation is the hydrolysis of microplastic to improve the hydrophobicity by the enzymes by offering the functional group of the microplastic polymers (Iram et al., 2019; Yuan et al., 2020). MPs are not easily utilized and absorbed by microbes due to their high molecular weight. Therefore, intracellular and extracellular enzymes play an important role in the cleavage of microplastic particles. Intracellular degradation engages the breakdown of stored endogenous carbon by accumulating several microorganisms itself. On the other hand, extracellular enzymes use an exogenous carbon reservoir to break down MPs polymers into smaller fragments such as oligomers, dimers and monomers through endo and exo attacks (Wilkes and Aristilde, 2017; Yuan et al., 2020). Microorganisms secret extracellular enzymes to hydrolyze the long-chain polymer into short-chain molecules. The short chain molecules are very small to enter through membrane, and these short chain molecules. Once the MPs are cleaved into small molecules by extracellular enzymes then Intracellular enzymes metabolized these small molecules into carbon dioxide (CO_2) and water (H_2O) which is utilized as a source of energy (Yoon et al., 1996; Gu, 2003; Zhang et al., 2021; Lin et al., 2022).

Enzymatic degradation of MPs is divided in two different groups; hydrolysable (PET) and non-hydrolysable (PE, PS and PP). Enzymes reported for degradation of PET includes cutinase, PETase and MHETase. Cutinase enzyme secreted by *Fusarium solani* degrades the PET through breakdown of both the aliphatic and aromatic ester bonds of polyester (Yoshida et al., 2016). Cutinase enzyme perform biodegradation at optimum pH range 7 and temperature at 50-55˚C. PETase enzyme secreted by microorganism (*Ideonella siakensis*) breaks the aromatic ester bond of polyester and

performs degradation at pH value 7- 9 (Yoshida et al., 2016; Urbanek et al., 2021). This enzyme produces changes in amino acids resulting in inhibiting the thermal balance. MHETase cause nucleophillic attack on the carbon atom of polyester. It requires optimum pH of 6.5 - 9 and a temperature 45˚C (Urbanek et al., 2021). On the other hand, PE degradation is completed by two intracellular enzymes, such as polyethylene glycol (PEG) dehydrogenase and alkane hydrolase. PEG dehydrogenase cleaves the PEG and produces glyoxylic acid while alkane hydrolases cleaves polyethylene microplastic at optimum temperature of 45˚C and pH 4.5 (Wilkes and Aristilde, 2017). Similarly for PS degradation the different enzymes are capable to form single ring aromatic compounds and hydrolyze the C-C bond. *Bacillus subtilis*, *Sphingomomonas paucimobilis, Alcanivorax borkumensis* secretes Cytochrome P450CPX152A1, Cytochrome P450CPX152B1, Cytochrome CPX153s, respectively to catalyze oxidation of styrene and hydroxylation of ethyl benzene. These enzymes play an important role in the conversion of alkane to alcohol (Hou and Majumder, 2021). Hydrolytic enzyme (esterase) was reported for degradation of PS, this enzyme breakdown the polymer into smaller fragments at an optimum temperature of 45˚C at pH 9 (Temporiti et al., 2022).

2.8 Microplastics (MPs) Degradation

MPs are degraded by two main process i.e. biological and oxidation process. Biological degradation of MPs continues by the enzymatic action of microorganisms, resulting in various changes occurring on the microplastic polymer structure (Fig 2.8). Oxidation mechanisms decompose MPs via different processes under the exposure of light and influence of reactive oxygen species (ROS) such as photochemical oxidation, electrochemical oxidation and photo degradation. For the biological degradation of organic pollutants, the factors such as temperature, moisture and pH play a role (Lucas et al., 2008; Shah et al., 2008; Sivan, 2011).

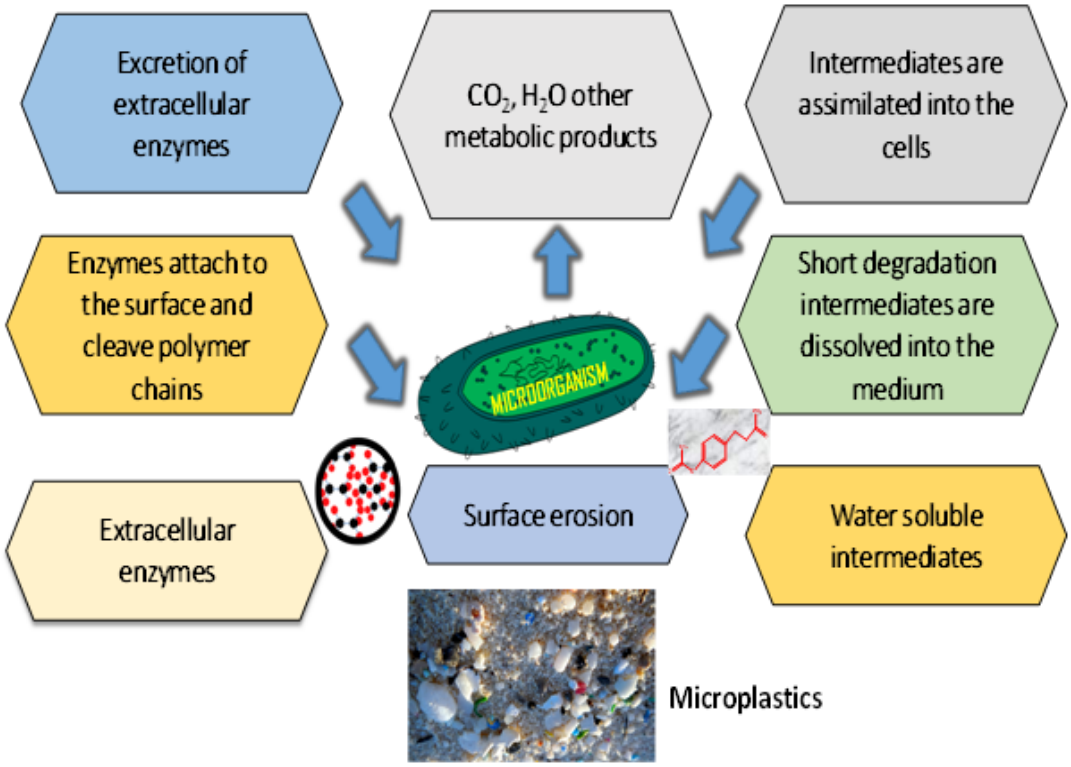


Fig 2.8 Schematic representation of mechanism of microplastics biodegradation

2.8.1 Biological degradation

a) Bio-deterioration

In this phase of degradation, enzymes act on the microplastic and disrupt the microplastic polymer into the monomers. Disruption of microplastic polymer occurs on the inside and outside of the plastic material (Lucas et al., 2008). Enzymatic degradation of microplastics depends upon the types of enzymes secreted by microorganisms, such as urease, protease and lipases (Sivan, 2011).

b) Bio-fragmentation

Bio-fragmentation means the biological cleaving or fragment of the microplastic polymer with the help of enzymes (Kjeldsen et al., 2018; Wagner et al., 2014). In this stage, enzymes such as hydrolase and oxidoreductase hydrolyze the polymeric structure of plastics. While other enzymes catalyze the several oxidation reaction resulting in the production of free radicals. Polarities of the plastic polymer gained by the oxidation occur in the free radicals and formation of carbonyl and hydroxyl functional groups (Luccas et al., 2008; Shah et al., 2008; Laycock et al., 2017; Ali et al., 2021).

c) Assimilation or Mineralization

In this phase, monomers were assimilated by the microorganisms to produce the microbial biomass, CO_2 and CH_4 (Shah et al., 2008). These monomers are used as a carbon source for energy purposes (Kjeldsen et al., 2018) and utilized by the microbial cell for growth. However, due to the semi-permeability of cell membranes, some monomers of plastics are difficult to assimilate. Microbial cell utilize the non-assimilated monomer through the biotransformation process. Nonetheless, the non-assimilated monomers of polymers are used by microorganisms through a process called transformation, i.e. microorganisms secreted enzymes generate the transformation of chemical compounds into an end product that could be assimilated by similar microorganisms or other (Ali et al., 2021).

2.8.2 Oxidation mechanism

This is an efficient process to decompose persistent pollutants. In this process, degradation of organic pollutants is based on the formation of reactive oxygen species (Du et al., 2021). These reactive oxygen species directly activate the degradation process by breaking the long polymer chain and completing the degradation cycle by forming useful products (Kang et al., 2019b).

a) Photocatalytic degradation

Photo catalytic degradation is a green technique to decompose organic pollutants by employing free solar energy. This process is based on the decomposition of semiconductor components of organic pollutant. In this process, decomposition starts when the photon energy of semiconductor components is too much higher than the gap energy of semiconductor substances. The valence band electrons easily transfer to the conduction band and cause a positive hole in the valence band of semiconductor substance resulting in the partition of electron holes (Du et al., 2021). Both electron and positive holes react with free hydroxyl radicals, generating reactive oxygen species. Free reactive oxygen directly initiates degradation of MPs (Tofa et al., 2019).

b) Photochemical degradation

Photo chemical degradation is another method for decomposing organic pollutants. Ultra violet (UV) light plays an important role in photochemical degradation (Gewert et al., 2015). Organic pollutants decomposed through long-time exposure to UV light results in the formation of oxygen free radicals and cross-linked the long chain of

polymers (Song et al., 2017).

c) Electrochemical degradation

This method is based on the anodic and cathodic surface degradation of pollutants. Anodic degradation causes direct oxidation by transferring the charge on the anode surface of pollutants and indirect oxidation by reactive oxygen species and H_2O_2. Cathodic degradation is completed by Electron-Fenton technique and oxygen free radicals. It is generated by Fe^+ and is responsible for degradation of MPs (Du et al., 2021).

2.9 Types of Microplastic degradation by microorganisms

2.9.1 Polyethylene Terephthalate (PET)

The degradation and molecular mechanism of PET by different microbes and their degradation efficiency are compiled in Table 2.10 and Fig. 2.9. *Idonella sakaiensis* and *Thermobifida fusca* reported 97% and 50% degradation rate respectively incubate at a temperature 55˚C for 21 days (Yoshida et al., 2016; Ali et al., 2021). PET consists of an amorphous semi-crystalline structure and linear polymer of repeating units of ethylene glycol or aromatic terephthalic acid (Danso et al., 2019). Two types of enzymes are involved in the degradation of PET that is PETase and MHETase. These two enzymes are used for the rapid degradation of PET and its monomer, such as terephthalic acid and ethylene glycol (TPA and EG) and also help in the bioconversion of high value trace compounds (Taniguchi et al., 2019). PETase converts polyethylene terephthalate into mono terephthalic (2-hyroxyethyl) acid and bis (2- hydroxyethyl) terephthalic acid, and MHETase converts mono-2-hydroxyethyl terephthalate (MHET) to terephthalic acid and ethylene glycol. This intermediate product is also internalized by the cell and breakdown by an organism that uses PET as a major source of carbon and energy source by enzyme PET hydrolase (Othman et al., 2021). The depolymerized products of PET are used by the bacteria for their metabolism (Yoshida et al., 2016).

Table 2.10 Degradation of Polyethylene Terephthalate (PET) by bacteria, fungi and algae.

Name of microplastic	Types of microbes	Species	Conditions	Degradation percentage	Days	References
Polyethylene Terephthalate (PET)	**Bacteria**	*Vibrio*	Nutrient broth Temp- 50˚C±2	35%	60	Sarkhel et al., 2020
		Bacillus cereus	Bushnell Hass Broth Temp- 27˚C±2	1.6%	40	Auta et al., 2017
		Bacillus gottheilii	Nutrient Broth Temp- 37˚C	3.0%	30	Auta et al., 2017
		Pseudomonas sps.	Bushnell Hass Broth Temp- 30˚C±2	5%	----	Taghavi et al., 2021; Wilkes and Aristilde., 2017
		Ideonella sakaiensis	Nutrient Rich Medium Temp-27˚C±2	1%	----	Yoshida et al., 2016 ; Wei et al., 2019
		Thermobifida fusca	Temp- 55˚C±2	54%	21	Wei et al., 2019
	Algae	*Phaeodactylum tricornutum*	Culture Agar media Temp-21˚C±2	-	54	Moog et al., 2019
		Spirulina sps.		-	--	Khoironi et al., 2019
	Fungus	*Penicillium* sps.	Czapek-Doxa Broth medium Temp-50˚C	-	28	Sepperumal et al., 2017
		Penicillium funiculosum	Czapek-Doxa Broth medium Temp-30˚C±2	0.21%	84	Nowak et al., 2011
		Thermomyces sp.		97%	18	Ronkvist et al., 2009
		Pichia pastoris	Buffered Glycerol complex medium Temp-65˚C	-	1	Chen et al., 2020c, Chen et al., 2020e
		Aspergillus sp	Potato Dextrose Agar Medium Temp-37˚C±2	22%	42	Sarkhel et al., 2020
		Fusarium solani	Temp-50˚C	5%	----	Ronkvist et al., 2009

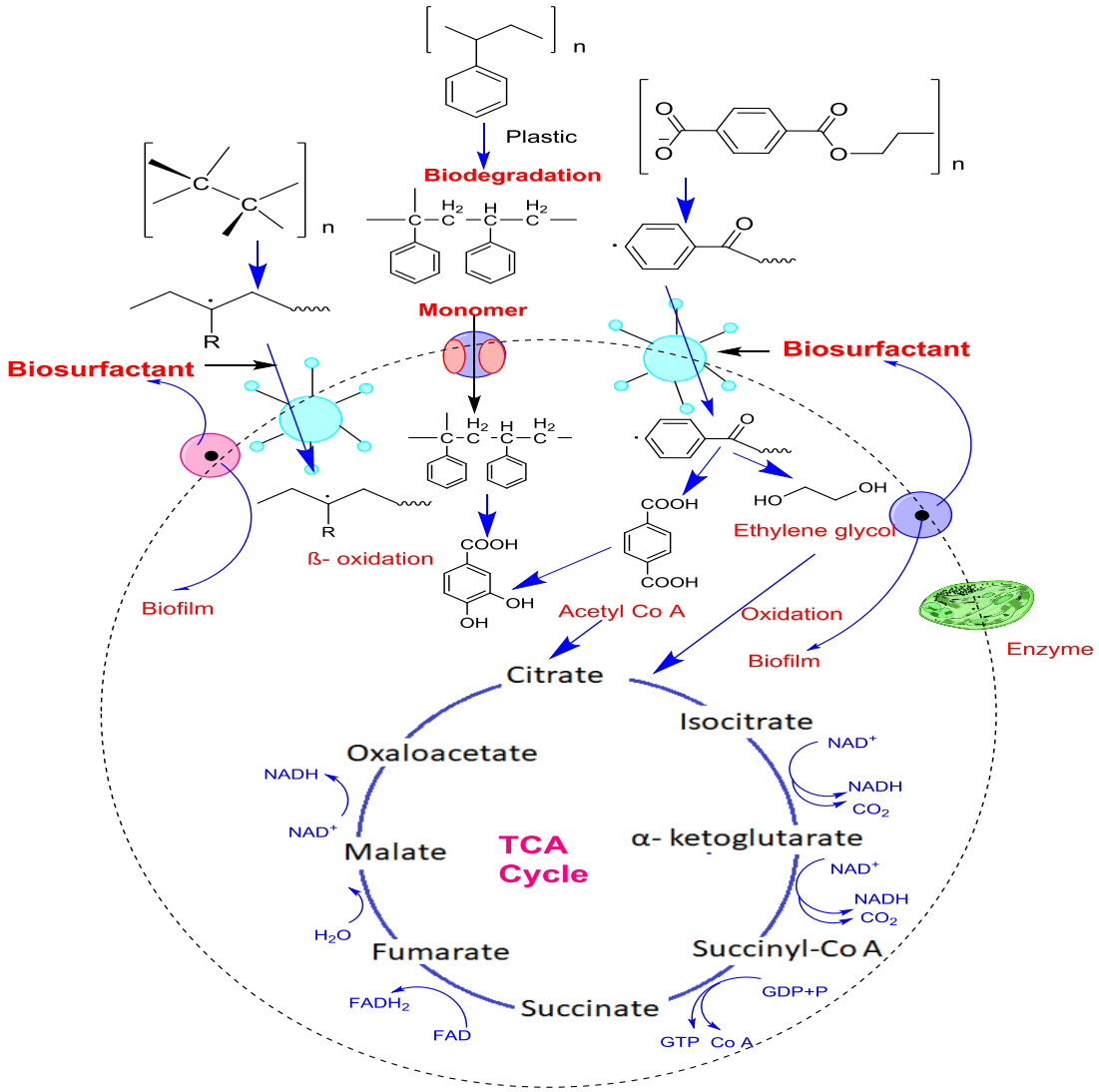


Fig 2.9 Molecular mechanism of degradation of Polyethylene Terephthalate.

2.9.2 Polyethylene degradation (PE)

In a year, all around the world, about 500 billion to one trillion polyethylene bags are used (Sarmah and Rout, 2020). PE extrapolates from petroleum based sources and uses in large quantities by people in the form of plastic bags. PE group of MP are categorized into two groups on the basis of their density; low-density polyethylene (LDPE) and high-density polyethylene (HDPE). It consists of a large linear carbon chain of poly-olefin. Poly-olefin is a polymer formed from the monomer of olefin or alkene. Olefin is an unsaturated hydrocarbon in which carbon and hydrogen are held together by double or triple bonds of one or more carbon (Chen and Marks, 2000). Approximately 63% of polyolefin has been used globally for the production of microplastic polymers (Posch, 2017). Degradation of polyethylene is too difficult due

to the presence of stable long linear carbon and hydrogen chains, and both carbon and hydrogen contain a balanced charge. The molecular mechanism and degradation efficiency of microbes in degradation of PE are given in Table 2.11- 2.13 and Fig. 2.10.

Previous studies reported the various microorganisms for degradation of MPs such as *Bacillus gottheilli, Lysinibacillus fusiformis, Bacillus cereus,* and *Bacillus borstelensis* and have efficiency to degrade 6.2%, 21.9% 36%, 20% MPs respectively at temperature 25˚C (Ali et al., 2021; Muhonja et al., 2018). Microorganisms use various enzymes to diminish the electric charge. With the help of enzymes, microorganism adds oxygen molecules to long linear carbon chains (Krueger et al., 2015). Mono-oxygenases enzyme adds one oxygen atom and di-oxygenases add two oxygen atoms to form alcohol and peroxyl group. Alcohol and peroxyl group act as less recalcitrants for biodegradation. PE oxidation form carboxylic acids, alcohol, ketones and aldehyde (Gewert et al., 2015). PE polymer after oxidation and fragmentation become more hydrophilic. Basically degradation of polyethylene occurs in two stages. The first stage is depolymerization, where different types of extracellular enzymes involve such as laccase and alkane hydroxylase and depolymerise the PE polymer into shorter chains such as oligomers, dimers and monomers. Depolymerization stage mainly aids low-density polyethylene molecules and absorb them into the cell through a permeable lipid membrane (Othman et al., 2021). The second stage is mineralization, in this stage long linear chain of low-density polyethylene mineralized and gives end products such as H_2O, CO_2, and CH_4. Microorganisms and bacteria use these end product as a carbon source (Sen and Raut, 2015; Ghatge et al., 2020).

Table 2.11 Different types of microorganisms degrade polyethylene.

Type of Microplastic	Microorganism	Conditions	Degradation percentage	Days	References
Polyethylene	*Pseudomonas aeruginosa*	Nutrient Broth Culture medium Temp-30˚C	-	5	Yoon et al., 2012; Taghavi et al., 2021; Tribedi and Sil, 2013
	Pseudomonas fluorescens	Nutrient Broth Culture medium Temp-27˚C	18%	270	Thomas et al., 2015 ; Nowak et al., 2011 ; Balasubramanian et al., 2010
	Paenibacillus sps.	-	14.7%	60	Park and Kim, 2019; Nowak et al., 2011
	Rhododcoccus rhodochrous	Culture Medium Temp-27˚C	-	---	Fontanella et al., 2010; Koutny et al., 2006; Bonhomme et al., 2003
	Rhododcoccus ruber	Nutrient Broth	20%	56	Hadar and Sivan, 2004; Sivan et al., 2006
	Bacillus brevies	Mineral Salt Medium Temp-30˚C	-	60	Watanable et al., 2009
	Bacillus cereus	Mineral Salt Medium Temp-37˚C±2	-	90	Sudhakar et al., 2008; Satlewal et al., 2008; Auta et al., 2017
	Bacillus subtilis	Mineral Salt Medium Temp-37˚C±2	1.5 – 1.75%	28	Harshvardhan and Jha, 2013
	Bacillus pumilus	Temp-30˚C	1.5%-1.75%	30	Roy et al., 2008; Nowak et al., 2011; Satlewal et al., 2008; Harshvardhan and Jha., 2013
	Bacillus sp. *BCBT21*	-	44%	---	Dang et al., 2018
	Bacillus cereus strain A5	-	35.72 %	---	Muhonja et al., 2018
	Bacillus vallismortis bt-dsce01	-	75%	----	Skariyachan et al., 2017
	Bacillus siansesis	-	8.46%	90	Maroof et al., 2021
	Streptomyces badius	-	-	20	Pometto et al., 1992
	Staphylocoocus epidermis	-	-	-	Chatterjee et al., 2010
	Microbacterium paraoxydans	-	-	-	Rajandas et al., 2012
	Arthrobacteria paraffineus	-	-	14	Albertsson et al.,1995
	Klebsiella pneumonia CHOO1	-	18.4%	-	Awasthi et al., 2017

Cont...

Type of Microplastic	Microorganism	Conditions	Degradation percentage	Days	References
Low Density Polyethylene	*Phormidium lucidium*	-	30%	-	Sarmah and Rout, 2018
	Aneurini baccilus sps.	Temp-50˚C	58.2%	140	Skariyachan et al., 2018
	Oscillatoria subbrevis	-	30%	-	Sarmah and Rout, 2018
	Brevibaccilus sps.	-	46.6%	30	Skariyachan et al., 2018
	Bacilus cereus	Synthetic Minimal media Temp-25˚C±2	35.72%	90	Muhonja et al., 2018
	Brevibaccilus borstelensis	Mannitol Free VB medium Temp-50˚C	11%	30	Hadad et al., 2005
	Cupriavidus neactor	Temp-25˚C	33.7%	21	Montazer et al., 2019
	Klebsiella pneumonia	Temp- 70˚C	18.4%	60	Kotova et al., 2021
	Microbacterium paraoxydans	Minimal Medium Temp- 50˚C±2	61%	60	Rajandas et al., 2012
	Micrococcus luteus	Minimal Medium	18.9%	21	Montazer et al., 2019
	Pseudomonas citronellolis	Minimal Medium Temp-25˚C	17.8%	----	Bhatia et al., 2014
	Rhodococcus ruber	Minimal Medium	8%	60	Hadar and Sivan., 2004
	Rhodococcous sp.		33%	21	Nanda and Sahu, 2010; Koutny et al., 2009
	Rhodococcus ruber C208	Mineral Medium Temp-35˚C	4%	60	Hadar and Sivan, 2004
	Pseudomonas sp. *AKS2*	Minimal Salt Medium	5%	90	Tribedi and Sil, 2013
	Bacillus subtilis H1584	Minimal salt Medium	1.75%	28	Harshvardhan and Jha, 2013
	Bacillus sphericus	Nutrient Broth culture	2.5- 10%	8	Sudhakar et al., 2008
High Density Polyethylene	*Achromobacter xylosoxidans*	Luria Bertani Broth Medium Temp-27˚C±2	9%	50	Kowalczyk et al., 2016
	Alcanivorax borkumensis	Bushnell Hass Broth Temp-27˚C	3.5%	80	Delacuvellerie et al., 2019
	Aneurinibacillus sps.	Minimal salt media	45.7%	140	Skariyachan et al., 2018
	Brevibacillus sps.	Minimal salt media	37.2%	----	Skariyachan et al., 2018
	Arthrobacter sp. *GMB5*	-----	12- 15%	14	Balasubramanian et al., 2010

Table 2.12 Different types of fungi that degrade various types of polyethylene.

Microplastics	Fungi	Degradation efficiency (%)	References
Polyethylene	*Aspergillus niger*	17.4%	Raghavan and Torma,1992; Nandi and Joshi, 2013
	Aspergillus oryzae strain A5	36.4%	Muhonja et al., 2018
	Aspergillus nomius RH03	6.63%	Munir et al., 2018
	Aspergillus flavus	16.2%	Nandi and Joshi., 2013
	Aspergillus sydowii	37.94%	Sangale et al., 2019
	Aspergillus terreus	4.182%	Sangale et al., 2019
	Zalerion maritimum (ATTC 34329)	56.7%	Paco et al., 2017
	Phanerochaete chrysosporium	-----	Browne et al., 2008
	Trichoderma viride RH03	5.13%	Munir et al., 2018
	Fusarium sp. AF4	-------	Shah et al., 2009
	Aspergillus fumigatus	20.5%	Alshehrei., 2017a
	Penicillium sp.	43.4%	Alshehrei., 2017a
	Aspergillus glaucus	28.8%	Kathiresan, 2003
	F. oxysporum	---	Spina et al., 2021
Low Density Polyethylene	*Aspergillus niger*	5.8%	Manzur et al.,2004; Pramila and Ramesh., 2011a; Jeeva and Kanchana, 2021 ; Raaman et al., 2012
	Aspergillus nominus	6.63%	Munir et al., 2018
	Aspergillus oryzae	36.4%	Muhonja et al., 2018
	Trichoderma viride	5.13%	Munir et al., 2018
	Aspergillus japonicus	11.11%	Raaman et al.,2012
	Aspergillus flavus MMP_{10}	-----	Kunlere et al., 2019
	Aspergillus flavus MCP_5	-----	Kunlere et al., 2019
	Penicillium pinophilum	-----	Manzur et al., 2004; Pramila and Ramesh, 2011a
	Penicillium oxalicum NS4	36.60%	Ojha et al., 2017
	Mucorcicinelloides	----	Pramila and Ramesh, 2011b
	Nodulisporium gregarium	----	Jeeva and Kanchana, 2021
	Xylaria sp.	----	Jeeva and Kanchana, 2021
High Density Polyethylene	*Cephalosporium* sp.	-----	Chaudhary and Vijayakumar, 2020
	Aspergillus tubingensis VRKPT1	6%	Devi et al., 2015
	Aspergillus flavus VRKPT2	8.5%	Devi et al., 2015
	Bjerkandera adusta TBB-03	-	Kang et al., 2019a
	Penicillium oxalicum NS4	55.34%	Ojha et al., 2017
	Aspergillus flavus	5.5%	Kang et al., 2019b ; Taghavi et al., 2021

Table 2.13 Different types of algae degrade polyethylene.

Algal species	**Conditions**	**Degradation percentage**	**Days**	**References**
Navicula popula	Bold Basal Medium Temp-27˚C	4.44%	30	Kumar et al., 2017; Ali et al., 2021
Scenedesmus dimorphous	Diatomic medium Bold Basal medium Temp-27˚C	3.74%	30	Kumar et al., 2017 ; Ali et al., 2021
Anabaena spiroides	Blue green algae 11(BG 11) Medium	8.18%	45	Kumar et al., 2017; Ali et al., 2021
Phormidium lucidium	-	-	-	Sarmah and Raut, 2018
Oscillatoria subbrevis	Temp- 27˚C	30%	42	Sarmah and Raut, 2018
Nostoc carneum	-	-	-	Sarmah and Raut, 2019

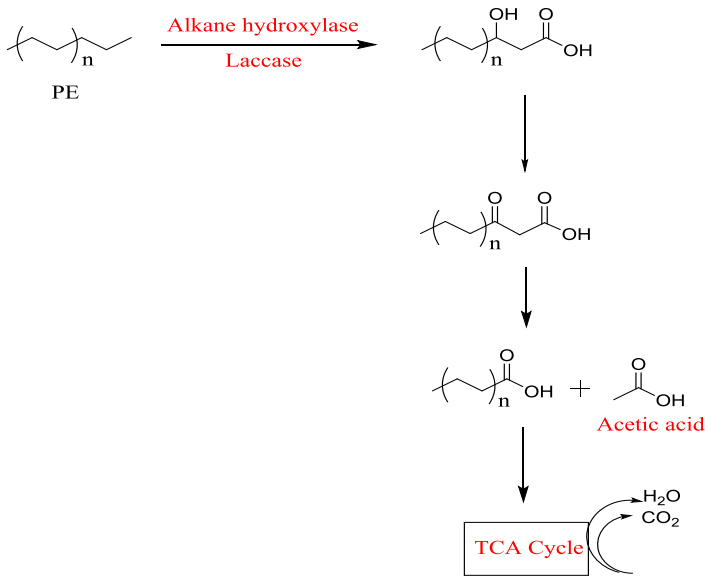


Fig 2.10 Mechanism of polyethylene degradation by microorganisms.

2.9.3 Degradation of polystyrene (PS)

Polystyrene is a type of high molecular-weight synthetic aromatic polymer made from the monomer units of styrene (Sheikh et al., 2013). PS is applicable for making different types of plastic products such as yoghurt containers, cold drink cups and disposable plates or bowls etc. (Krueger et al., 2017). The degradation efficiency and mechanisms of PS are carried out by the different types of bacteria, algae and fungi (Table 2.14 and Fig. 2.11). Various microorganisms have been reported with degradation rate such as *Xanthomonas* sp., *Sphingobacterium* sp., *Enterobacter*, *Bacillus gottheilii* and *Rhodococous ruber* with degradation efficiency 56%, 40% , 12.4%, 5.8% and 0.8% and respectively to degrade MPs (Muhonja et al., 2018; Ali et al., 2021). Microorganisms use styrene as the sole carbon source for their growth. First monomer of styrene breaks into styrene oxide by using a specific enzyme, styrene monooxygenase. Further, the enzyme isomerase breaks styrene oxide into phenylacetaldehyde, and phenylacetaldehyde dehydrogenase is further converted into phenylacetic acid. Finally, phenylacetaldehyde coenzyme A ligase breaks phenylacetic acid into phenyl acetyl coenzyme A. To yield acetyl CoA in Tricarboxylic acid (TCA) cycle, phenyl-acetaldehyde coenzyme A experiences β-oxidation (Ho et al., 2018; Danso et al., 2019; Othman et al., 2021).

2.9.4 Degradation of polypropylene (PP)

Polypropylene (PP) is similar to polyethylene and the most common petroleum-based microplastic comes after polyethylene (Jeya et al., 2013). It consists of a straight chain of a hydrocarbon containing carbon atom in the main ring structure. Due to the presence of individual carbon in ring structure PP becomes more stable in structure. It has a hydrophobic surface because of its arrangement in hydrocarbon and consists of three stereoisomers isotactic, syndiotactic and atactic.

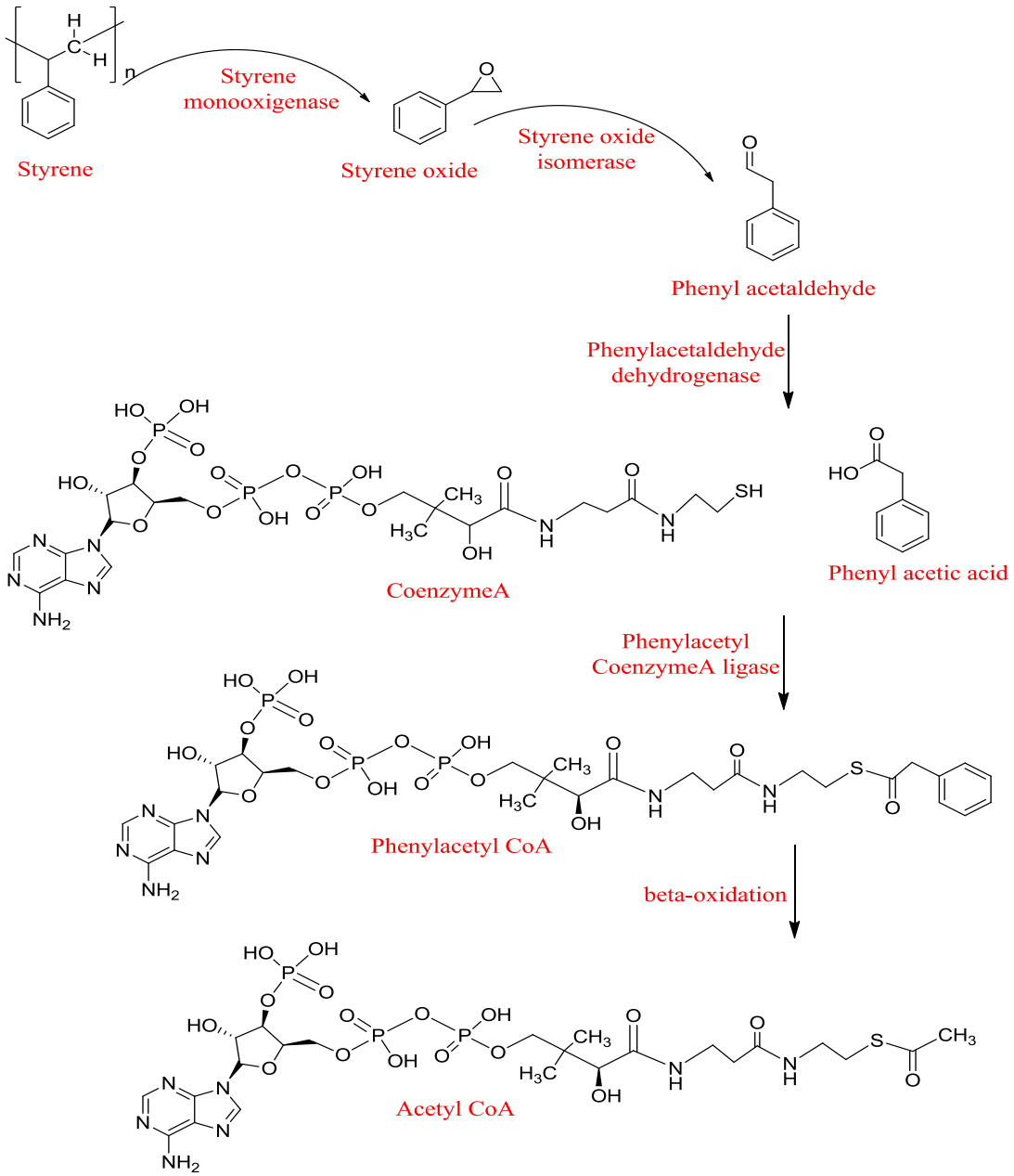


Fig 2.11 Molecular mechanism of degradation of polystyrene.

Table 2.14 Degradation of polystyrene (PS) by algae, fungi and bacteria species.

Name of microplastic	Microorganisms	Species	Conditions	Degradation percentage	Days	References
Polystyrene (PS)	**Fungus**	*Cepahlosporium* sps.	Mineral Salt Medium Temp-28˚C	2.17%	56	Chaudhary and Vijayakumar, 2020
		Penicillium sps.	Mineral Liquid Medium	8.4%	-	Oviedo-Anchundia et al., 2021
		Mucor sps.	Mineral Salt Medium Temp-28˚C	1.81%	56	Chaudhary and Vijayakumar, 2020
		Mortierella sps.	Mineral Salt Medium Temp-18˚C	2.2%	90	Oviedo-Anchundia et al., 2021
		Geomices sps.	Mineral Salt Medium Temp-18˚C	6.8%	90	Oviedo-Anchundia et al., 2021
	Bacteria	*Citrobacter* sps.	Bushnell Hass Broth Temp-30˚C	-		Brandon et al., 2018
		Exguobacterium sps.	Liquid Carbon Free Basal Medium, Mineral Medium	7.4%	28	Yuan et al., 2020
		Serratia sps.	-	-	-	Lou et al., 2020
		Bacillus subtilis	Bushnell Hass Broth, Nutrient Broth Temp- 37˚C	20%	28	Ashimta et al., 2015
		Microbacterium sps. *NA23,*	-	-	-	Oikawa et al., 2003

Cont...

Name of microplastic	Microorganisms	Species	Conditions	Degradation percentage	Days	References
		Rhodococcus ruber C208	Mineral Medium,Synthetic medium Temp- 35˚C	0.8%	56	Mor and Sivan, 2008
		Paenibacillus urinalis NA26	-	22.8	60	Oikawa et al., 2003
		Bacillus sp.	Nutrient Broth	4- 6.4%	40	Auta et al., 2018
		Pseudomonas sp.	Minimal Salt Medium Temp- 20˚C±2	5%	28	Taghavi et al., 2021 ; Oikawa et al., 2003 ;Ward et al., 2006; Ashmita et al., 2015
		Staphylococcus aureus	Bushnell Hass Broth Temp-37˚C	4.7%	30	Ashmita et al., 2015
		Streptococcus. pyogenes	Bushnell Hass Broth Temp-37˚C	8.3%	30	Ashmita et al., 2015
	Algae	*Chlamydomonas reinhardtii*	-	-	-	Barone et al., 2020
		Pseudokirchneriella subcapitata	-	-	-	Padervand et al., 2020

Table 2.15 Degradation of polypropylene by bacteria fungi and algae.

Microplastics	Species	Microrganisms	Conditions	Degradation percentage	Days	References
Polypropylene	Fungi	*Aspergillus fumigatus*	-	18.08%	-	Oliya et al., 2020
		Aspergillus sps.	Rose Bengal Medium Temp-27˚C	12%	90	Raaman et al., 2012
		Lasiodiploida theobromae	Rose Bengal Medium Temp-27˚C	-	90	Sheikh et al., 2015
		Aspergillus niger	Rose Bengal Medium Temp-27˚C	53.09%	90	Williams and Osahon, 2021
		Phanerochaete chrysosporium	Mineral Salt medium Temp-30˚C	4-5%	-	Jeya et al., 2013
		Paecilomyces lilacinus	Rose Bengal Medium	-	90	Sheikh et al., 2015
		Phanerochaete chrysosporium NCIM 1170	Mineral Salt medium Temp-30˚C	18.8%	360	Jeya et al., 2013
		Engyodontium album MTP091	Mineral Salt medium	9.42%	360	Jeya et al., 2013
	Bacteria	*Stenotrophomonas panacihumi PA3-2*	Temp-37˚C ±2	12.8%	90	Jeon and Kim, 2016
		Pseudomonas sps.	Bushnell Hass Broth Temp-25˚C	9%	40	Jeon et al., 2021
		Lysinibacillus sps. *JJY0126*	Minimal medium Temp-30˚C	3%	28	Mukherjee et al., 2016
		Brevibaccilus sps.	-	56%	-	Skariyachan et al., 2018
		Bacillus sps.	Bushnell Hass Broth	6%	40	Auta et al., 2018
		Rhodococcus sps.	Bushnell Hass Broth Temp-25˚C.	6%	40	Auta et al., 2018
		Staphyloococcus sps.	-	9.5%	-	Oliya et al., 2020
		Bacillus cereus	-	0.03% /day	-	Helen et al., 2017
		Sporosarcina globispora	-	0.02% / day	-	Helen et al., 2017
		Actinomycetes	-	0.08%	-	Helen et al., 2017
		Aneurinbaccilus sps.	Minimal Medium	-	140	Skariyachan et al., 2018
	Algae	*Spirulina*	-	-	-	Khoironi et al., 2019

Its hydrophobic property becomes more recalcitrant in the environment (Khoironi et al., 2020; Othman et al., 2021). It is degraded by different types of microorganisms, algae and fungi (Table 2.15). Polypropylene consists of C-C and C-H bond in the chemical structure. These bonds are too much stable in degradation in comparison to the ester bond. During degradation both C-C and C-H are oxidized by different types of microorganism's secreted enzymes. Enzymes such as alkane hydroxylase, alcohol dehydrogenase and aldehyde dehydrogenase get attached to surface of PP and start the decomposition process resulting in reduce the number of the carbonyl group. Further, enzymes convert carbonyl groups into carboxylic acids (Ali et al., 2021) and later the enzymes break down the long carbon chain into small hydrocarbon and release alkane and alkenes. Microorganisms metabolize small hydrocarbons which pass them to cells and convert ultimately into carbon dioxide and water (Zhang et al., 2022).

2.10 Role and mechanisms of different insects gut microflora in in-situ degradation of microplastic

Different types of insects are considered as model organisms to study the in-insitu degradation of microplastic by their gut microflora (Table 2.16). Mechanism of microplastic degradation by insects divides into five different stages; a) Microplastics are actually gnawed by mouthparts and enter the gastrointestinal tract; b) microorganisms in the gastrointestinal tract stick to and deteriorate plastic polymers; c) the plastic polymers is depolymerized into fragments (oligomers) through oxidation or hydrolysis of enzymes offered by the host and intestinal microbiome; d) the host delivers bioemulsifying representatives which elevate the efficiency of microbial and host enzymes in attacking polymers; e) oligomer bonds are broken to form fatty acids; and f) fatty acids are decomposed by insects biological metabolism.

Table 2.16 Insects involved in the in-situ degradation of different types of microplastics.

Insects	Microplastics	Microflora	References
Tenebrio molitor (Yellow worm)	PVC	Firmicutes, Tenericutes, Proteobacteria, Bacteriodetes, Actinobacteria, Chloroflexi, Saccharibacteria, SBR1093	Peng et al., 2020
	PS, PU, PE	Firmicutes, Bacteriodetes, Actinobacteria, Acidobacteria, Chloroflexi, Verrucomicrobiota	Bulak et al., 2021
	PS	Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Acidobacteria, Chloroflexi, Verrucomicrobiota, Myxococcota	Jiang et al., 2021
	PVC	Gammaproteobacteria, Proteobacteria, Enterobacterales, Aquabacterium	Xu and Dong., 2024
Galleria mellonella (Greater waxmoth)	PS	Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Acidobacteria, Chloroflexi, Verrucomicrobiota, Myxococcota	Jiang et al., 2021
Zophobas atratus (Superworm)	PS	Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Acidobacteria, Chloroflexi, Verrucomicrobiota, Myxococcota	Jiang et al., 2021

2.11 Conclusion

MPs pollution has long been a source of consideration in ecology and environmental studies as well as in environmental engineering. Microorganisms associated in MPs degradation also contribute a better compassionate path to overcome microplastic pollution. The main challenges that occur in the degradation of microplastic by microbes also depend on the properties of microplastics. Plastic properties play an important role in the degradation of plastic particles or their colonization on the surface. Different types of microorganisms involved in the biodegradation process and the degradation of plastic depends upon the enzymatic reaction produced by microorganisms. This review briefly highlights the types and extraction techniques of microplastics from the agriculture soil, mechanism of degradation process and recent aspects of the biological degradation of MPs along with the microbes associated for MPs degradation mechanisms. Different strains of bacteria, fungi and algae play a role in breakdown the larger plastic particles into smaller ones. The present study also highlights the role of extracellular and intracellular enzymes produced by microbes for the degradation of microplastics.Further research explore for degradation of different types of plastics polymer by employing different arthropods, isopods, snails, invertebrates, and also study their intestinal microflora that help to degrade to microplastics. Apart from this further research proceed on the ecotoxicological effects of plastic polymers on insects.

3. HYPOTHESIS

Plastic pollution increases day by day due to its low cost, more ductile and high durability. Our society permeates use of plastics product in each and every aspects of life but people do not aware about its harmful effects. Plastic produces various harmful toxic effects to human being as well as terrestrial ecosystem. Nowadays, plastics become a hot topic due to its excess use. Large amount of plastic enters to the agriculture field through sewage sludge, wastewater irrigation and plastic mulching.

Earthworm act as ecological engineer in agriculture field but tiny particles of plastics easily ingested and cause many changes in their body such as oxidative stress, neurotoxic effects, gut micro biome dysbiosis, growth inhibition and tissue damage. Therefore, the present study designed to investigate the effects of polypropylene on earthworm species in terms of biomass, reproduction, oxidative stress, gut micro biota and their potential to degraded polypropylene.

For best of my observation till date there is no study reported on this designed project work on toxic effects and degradation of polypropylene by gut microbiota of two different species of earthworm i.e *Eisenia fetida* and *Lampito mauritii*.

4. OBJECTIVES

1. To identify and quantify the microplastics from the agricultural soil situated near to industrial area.
2. To study the effect of microplastics on the growth and fecundity of Indigenous and Exotic earthworm species.
3. To study the antioxidant activities by performing biochemical assay.
4. To identify microplastic degrading bacteria from earthworm gut by metagenomic analysis.
5. To study the degradation of microplastic in the gut of earthworm by microorganisms through FTIR and SEM techniques.

5. MATERIALS AND METHODS

Experiments were aimed to identify and quantify the microplastics from the agricultural soil situated near to industrial area. Apart from this the tolerance potential of earthworm in terms of growth, fecundity and oxidative stress towards the polypropylene microplastic was assessed. Molecular docking and earthworms gut microrganisms were also studied.

5.1 Earthworm species

5.1.1 *Eisenia fetida*

Healthy and mature well developed clitellated earthworms having weight 200-400 mg were procured from vermicomposting unit of Guru Nanak Dev University, Amritsar.

5.1.2 *Lampito mauritii*

Adult earthworms were collected from Botanical garden of Guru Nanak Dev University, Amritsar and its culture was maintained in the garden soil by maintaining the moisture level at 50 to 60%.

5.2 Microplastics

Polypropylene microplastic was purchase from Indian Oil Corporation Limited, Panipat (India) and Fig 5.1a shows the polypropylene microplastic used for experimental study.

5.3 Artificial soil

Artificial soils were purchased from the Kamal Traders, Preet Nagar, Sodal road, Jalandhar (Punjab) city. Artificial soil consists of mixture of three different soil components (Fig 5.1b).

- Industrial Quatrz Sand (70%)
- Coco peat or sphagnum peat (10%)
- Clay (Kaolinite clay) (20%)

The above content was thoroughly mixed and pH of artificial soil was adjusted (pH-6.0) by adding calcium carbonate ($CaCO_3$). Distilled water was employed to maintain the moisture (35%) of soil according to OECD guidelines (OECD. 1984).In this study artificial soil was used as a control.

Fig 5.1(a) Polypropylene microplastics used for the experimental study b) The mixture of artificial soil (Kaolinite clay, Sand and Coco peat).

5.4 Collection of soil sample

Three plastic industries were selected for sampling i.e. Amritsar (Frontiers Pvt. Ltd.), Jalandhar (Aman Polymers Pvt. Ltd.) and Kapurthala (Gupta Traders Pvt. Ltd.). Soil samples were collected from different sites near plastic product manufacturing industries for extraction of MPs. Twelve soil samples (four from each site) were collected by doing bore hole up to 25 cm with the help of spade. All soil samples were stored in polyethylene zip lock bag and properly labeled. Soil samples were dried, sieve with 2 mm mesh and stored in polyethylene zip lock bags for further physico-chemical analysis and microplastic extraction.

5.5 Extraction of MPs by density floatation method

5.5.1 Preparation of reagent

NaCl was prepared by dissolving 337 g in 1000 ml of distilled water.

Salt	Density of salt (g/cm^{-3})	Weight of NaCl added in 1000 ml
Sodium Chloride (NaCl)	1.2	337 g

5.5.2 Pre-treatment

To achieve the optimal separation of different size of microplastics from soil, the various steps were involved such as removal of organic matter by performing pre digestion before flotation.

5.5.3 Pre digestion (Organic matter removal)

The first step of extraction of microplastics from soil was the removal of organic matter by performing mild digestion. Digestion treatments were conducted to digest

low and high organic matter concentration of soil. Initially 30% hydrogen peroxide (H_2O_2) was employed as reagent for oxidation. Precisely, 10g of soil sample were taken in conical flask for digestion in triplicate manner. Gradually add 200 ml of 30% H_2O_2 with slowly increment in the conical flask containing sample. To avoid the excessive increase in temperature, the conical flasks were placed in cold water bath. When the temperature stays constant, the conical flasks were heated on the hot plate at 70˚C and continuously stirred to avoid the colliding of H_2O_2 and soil. At the time of excessive froth few drops of butyl alcohol was added to the flask. Heat the sample for approximately 12 hrs until its colour turned to greyish and dry. After digestion, flask were allowed to cool down and prepared to carry floatation process.

5.5.4 Density Separation and floatation of Microplastics (MPs)

Density separation method was regularly employed to separate small size of plastics particle present in the soil, due to less density of microplastics. In this method, Sodium chloride (NaCl) was employed as a density separation solution. Prepare NaCl solution by dissolving 300 ml distilled water. After pre digestion of soil sample, add 200 ml of NaCl to each conical flask and flask was tightly sealed for shaking in orbital shaker for 1 hr at 200 rpm. After shaking, allow it to stand for 48 hrs and then decanted 150 ml of supernatant from the conical flask. Again add remaining 100 ml of NaCl solution to conical flask and repeat shaking with decrease in shaking time (30 min) and again allow settle down and collect the supernatant.

5.5.5 Floatation process

For floatation process, supernatant containing microplastics filtered by employing vacuum filtration using Whatman filter paper (pore size 11µm). Floatation process was repeated for 3 times until no plastics particles were seen floated or adhered around the sides of conical flask. After filtration, the filter paper was placed in a cleaned covered petriplates and dry in oven at 60˚C for 1 hr.

5.5.6 Post- oxidation digestion and Purification of MPs

After filtration, the filter paper containing MPs were washed with 30% H_2O_2 in a conical flask. Prior to the qualitative analysis, organic matter was eliminated from soil by performing post- digestion for extraction of microplastics from agriculture soil. Then 30% H_2O_2 was added and flask was sonicated for 30 min and placed flask in ice bath. Filtered the solution by employing filter paper and dried the filter at 50-60˚C.

The extracted microplastics on filter paper were visualize under stereomicroscope and identified the extracted microplastics by Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR-ATR).

5.5.7 Identification, detection and characterization of MPs

The detection of extracted microplastics filters were stained with Nile Red dye (NR). NR dye was prepared by dissolving 1 mg/ml concentration of solvent (methanol). Add 2-3 drops of dye on filter paper and incubate the filter paper for 30 min. Dry the filter paper and examine the NR stained microplastic particles irradiated with wavelength of light employing UV lamp. Microplastics particles fluoresce when irradiate under red light and excitation wavelength varied from 540-580 nm and emission wavelength 600-660 nm. Extracted microplastics particles were observed and counted under fluorescence microscope (Nikon model SMZ 18, Japan). The shape, size and color of microplastics were recorded by Image J processing program. Different types of MPs with different shape include fibres, fragments and other miscellaneous shapes (size range from 100 µm – 1 mm). MPs particles were identified by using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR Shimazdu model IR Tracer 100, Japan) with 64 scans in the spectral range of 4000 – 550 cm^{-1} and at resolution 4 cm^{-1}. All obtained spectra were compared with the Shimadzu libraries database with at least 80% matching score for spectra. X-ray diffraction spectra of different types of microplastics were obtained by X-ray diffractometer (Bruker Company model D8 discover, Germany) and analysis were performed in the range 1˚to 80˚ under 2Θ diffraction angle.

5.5.8 Recovery Test

The recovery test was performed in NaCl solution for different types of microplastics extracted from soil. The soil samples were dry at 72˚C and then all dried soil sample were sieved three-four times to remove all the microplastics particles in soil. For recovery test 10 pieces of each type of microplastics were add in 50 g of clean sieved soil and add 200 ml of saturated NaCl solution and stirred the soil containing MPs particles and NaCl solution at 40˚C. For further floatation, soil sample was stirred at 600 rpm for 5 min. MPs were recovered from soil by using a stainless stell mesh size of 0.1 mm. Different types of microplastics particles were collected from soil by using mesh and place the MPs particles on petriplates. MPs particles were counted and

identified by their shape, size and colors. This test was repeated several times for accurate results.

5.6 Physico-chemical analysis

Physico-chemical investigation of soil samples was done at the beginning of the experiment to measure organic carbon, pH, electric conductivity (EC), nitrogen, total dissolved solids (TDS), phosphorus, potassium, lithium and sodium

5.6.1 pH

5g dried soil sample was dissolved in 50 ml of de-ionized water (1:10 w/v) and vigorously shaken for 40 min on an orbital shaker. Then supernatant was collected and finally the pH was measured by using digital pH meter (PCS Tester 35 series, Eutech Instruments,).

5.6.2 TDS

5g dried soil was dissolved in 50ml of de-ionized water (1:10 w/v) and continuously shake for 40 min on an orbital shaker. TDS was measured from the collected supernatant by using (PCS Tester 35 series, Eutech Instruments).The values were expressed in unit mg/I.

5.6.3 EC

5g dried soil was dissolved in50 ml of de-ionized water (1:10 w/v) and shake for 40 min on an orbital shaker. Then supernatant was collected and measure the EC by using (PCS Tester 35 series, Eutech Instruments). The values were expressed in unit mS/cm.

5.6.4 Organic Carbon

Organic carbon content was estimated by the method of Nelson and Sommers (1996).

Add 1g dried soil sample in pre-weighed crucibles and soil sample was ignited at 550˚C for 60 min. Then allowed to cool down the muffle furnace and weighed the ash content. Calculate total OC in soil sample by using formula.

$$Ash\ (\%) = \frac{Wt.\ of\ sample\ left\ after\ ignition}{Weight\ of\ soil\ sample\ taken}\ \text{X}\ 100$$

5.6.5 Nitrogen

Total Kjeldhal Nitrogen (TKN) was estimated by the method of Bremner and Mulvaney (1982).

(i) Chemicals and reagents

a) Digestion mixture: Selenium dioxide (SeO_2), copper sulphate ($CuSO_4$) and potassium sulphate (K_2SO_4) was mixed in ratio 1:4:10 respectively.

b) Boric acid (H_3BO_3) indicator solution: 20g of H_3BO_3 acid was dissolved in 700 ml of hot ultrapure water. Cooled H_3BO_3 solutions. Indicator solution was prepared by dissolving 100 mg bromocrescol green and 50 mg methyl red in 100 ml of ethanol. Transferred in volumetric flask and add 700ml of H_3BO_3 solution. Comprehensively mix the solution in volumetric flask and made final volume to 1 lt by adding ultrapure water.

c) Sodium Hydroxide (NaOH) 40% solution: Weigh 40g of NaOH and dissolved in 100 ml of ultrapure water to made up 40% NaOH.

d) Titration solution: 0.01N Hydrochloric Acid.

e) Concentrated sulphuric acid (H_2SO_4).

(ii) Digestion of soil

a) 0.5g of dried soil was placed in flask and added 15 ml of digestion acid mixture. Digestion acid mixture was prepared by dissolving 1g digestion mixture in 15 ml concentrated H_2SO_4.

b) The flask containing solution was heated at low temperature until the color of solution and sample turned into light yellow green.

c) Cool digested sample solution and add ultrapure water to raise the volume upto 50 ml.

(iii) Distillation

a) 10 ml aliquot of digested soil sample was reacted with 10 ml of 40% NaOH and run in Kjeldhal apparatus.

b) 5 ml H_3BO_3 acid indicator added in flask and placed beneath the condenser of apparatus, tip of the condenser properly soaked into indicator to ingest the liberate ammonia.

c) Following this, the distillation was started and approximately 50 ml of

condensate was collected in the flask.

d) Remove the flask before stopping the heat to avoid back sucking of liquid.

e) The condensate indicator in the flask titrated with 0.01N HCl until the color turned from greenish-blue to permanent light pink.

(iv) Calculation

$$\text{Nitrogen (\%)} = \frac{(a-b)\times N\times 14\times vol\ made\ of\ digest}{Vol.of\ digested\ aliquot\ taken\times Weight\ of\ soil\ sample\ taken\times 1000}\times 100$$

5.6.6 Phosphorus

Phosphorus content of soil was determined by using the John (1970) method.

(i) Chemicals and reagents

a) Stock solution: 20 g ammonium molybdate was dissolved in 300 ml of de-ionised water. Gently 450 ml of 10N H_2SO_4 was added with proper stirring, 100 ml of 0.5% antimony potassium tartarate ($C_4H_4K_2O_6$) was prepared and added to the solution. Final volume of solution was made up to 1 litre by using de-ionized water and stored in a dark colored bottle.

b) Standard solution: 1000 mg/l standard stock solution was prepared by weighing 0.439 g potassium dihydrogen phosphate (KH_2PO_4), dissolve in 100 ml of de-ionised water and standard curve was prepared in spectrum of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/l.

c) Working Reagent: This reagent was freshly prepared by dissolving 1.5 g ascorbic acid ($C_6H_8O_6$) in 100 ml stock solution.

d) Diacid mixture: Concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) was mixed in ratio 4:1 (v/v) respectively.

(ii) Procedure

a) 0.5g dried soil sample and 15 ml of diacid mixture was added to the digestion flask. Further the whole mixture was digested in digestion chamber until it was colorless. Dilute the content by using 30ml de-ionized water, after filtered the solution by using Whatman filter paper and relocate to a volumetric flask and final was made by adding de-ionised water.

b) 1ml aliquot was taken from each digested sample in volumetric flask of 50 ml and added 5ml freshly prepared working reagent. Then deionised water was added to make final volume 50ml. After placing for 30 min, absorbance was taken at 880 nm by suing a Systronics UV-Visible spectrophotometer model-117.

5.6.7 Potassium, Sodium and Lithium

Potassium (K), Sodium (Na) and Lithium (Li) were estimated according to American Public Health Association Guidelines (APHA guidelines, 1998) by using Digital Flame photometer. Diacid digested soil samples were taken from previously prepared in case of phosphorous.

a) Standard Stock solution for K, Na and Li

1000 ppm (1000mg/l) stock solution of K and Na was prepared by separately dissolved 0.191g of potassium chloride (KCl) and 0.254g of sodium chloride (NaCl), 152.7 mg of lithium chloride (LiCl) in 100 ml of deionised water.0.5g dried soil were dissolved in all solutions. Standard curve was prepared in the range of 20, 40, 60, 80 and 100 mg/l for K, Na and Li.

5.6.8 Heavy metal analysis

The content of Iron (Fe), Zinc (Zn), Chromium (Cr), Cadmium (Cd), Lead (Pb), and Manganese (Mg) in soil samples were determined from the diacid digested samples by using Microwave Plasma Atomic Emission Spectrophotometer (MP-AES 4200, Agilent technologies).Diacid digested soil samples were taken from previously prepared in case of phosphorous.

5.7 Microplastic treatment on earthworm

Healthy and mature earthworms of both species (*Eisenia fetida* and *Lampito mauritii* were collected from the acclimatized culture and earthworms were weighed by using measuring scale prior to the experiment. Ten weighed earthworms were added in each tray having different concentrations of microplastics (1000, 4000, 8000 and 16000 $mgkg^{-1}$) and control (without microplastic) in triplicate manner for 28 days (Fig 5.2). Spread 5gm of partially decomposed cow dung in each tray as nutrient to earthworms. Earthworms from each tray was withdrawn and weighed on day 7, 14, 21 and 28^{th} day. At 28^{th} days the numbers of coccons from each tray were counted and weighed,

again incubate for another 28 days. On 56th day the numbers of earthworm's juveniles were counted from each tray.

Fig 5.2 Experimental setup for study the effects of different concentrations of polypropylene on earthworms.

5.8 Antioxidant enzymatic assay (Biochemical Assay)

- Four antioxidant enzymes Superoxide Dismutase (SOD), Catalase (CAT), Glutathione–S-Transferase (GST), Guaicol Peroxidase (POD) were studied to know the the oxidative stress in earthworm tissues after the treatment with microplastics.

5.8.1 Preparation of reagents

a) 50 mM Sodium Carbonate Buffer (pH 10.0)

Stock solution	Chemical name	Weight of chemical	Volume
Stock A	Sodium carbonate (Na_2CO_3)	0.529 g	100 ml
Stock B	Sodium bicarbonate ($NaHCO_3$)	0.420 g	100 ml

Procedure: Sodium carbonate buffer was prepared by using 55 ml of stock solution A of Na_2C0_3 and 45 ml of stock solution B of $NaHCO_3$ and make final volume 100 ml by mixing both.

b) 20 mM Hydroxylamine Hydrochloride

Chemical name	Weight of chemical	Volume
Hydroxylamine Hydrochloride ($CIH.H_3N0$)	1.38 g	100 ml

Procedure: 1.38 g Hydroxylamine hydrochloride was mixed in 100 ml of buffer (Sodium carbonate) to make stock solution (200 mM). Add 200 µl of stock solution in 2 ml of reaction mixture.

c) 96µM of Nitroblue Tetrazolium

Chemical Name	Weight of chemical	Volume
NBT	0.078 g	100 ml

Procedure: 0.078 g of NBT was mixed in 100 ml of buffer (Phosphate) to make stock solution (960 µM). Add 200 µl of stock solution in 2 ml of reaction mixture.

d) 0.6% Triton X-100

Chemical Name	Quantity of chemical (ml)	Volume
Triton- X-100	6	94 ml

e) 50 mM Phosphate Buffer (pH -7.0)

Stock Solution	Chemical name	Weight of chemicals	Volume
Stock A	Dipotassium hydrogen orthophosphate (K_2HPO_4)	0.684 g	100 ml
Stock B	Potassium dihydrogen orthophosphate (kH_2PO_4)	0.870 g	100 ml

Procedure: 50 mM Phosphate buffer was prepared by mixing 39 ml of stock A of K_2HPO_4 and 61 ml of stock solution B of KH_2PO_4 and make final volume 100 ml. Adjust pH 7.6 by using pH meter (Systronic, model 361).

f) 30 mM Hydrogen Peroxide (H_2O_2)

Chemical name	Quantity of chemical (ml)	Volume
Hydrogen Peroxide	3	100 ml

Procedure: 3 ml H_2O_2 was dissolved in 100ml of buffer (Phosphate) to make stock solution (300 mM). Add 200µl of stock solution in 2ml of reaction mixture to make final concentration of 30mM.

g) 20 mM Guaiacol solution

Chemical Name	Quantity of chemical (ml)	Volume
Guaiacol	2.262	100ml

Procedure: 2.262 ml of Guaiacol was dissolved in 100ml of buffer (Phosphate) to make stock solution (200 mM). Add 200µl of stock solution in 2ml of reaction mixture.

h) 20mM Hydrogen Peroxide (H_2O_2)

Chemical name	Quantity of chemical (µl)	Volume
Hydrogen Peroxide	204	100 ml

Procedure: 204 µl H_2O_2 was mixed in 100 ml of buffer (Phosphate) to make stock solution (200 mM). Add 200 µl of stock solution in 2ml of reaction mixture.

i) Methanol (80%)

Chemical Name	Quantity (ml)	Volume
Methanol	80	100 ml

80% methanol was prepared by dissolving in 20ml of distilled water to make 100ml final volume.

j) 10mM 1-chloro, 2, 4- dinitro benzene (CDNB)

Chemical name	Weight of chemical	Volume
CDNB	2.02 g	100 ml

Procedure: 2.02 g CDNB was mixed in 100 ml of buffer (Phosphate) to make stock solution (100 mM). Add 200 µl of stock solution in 2 ml of reaction mixture.

k) 10 mM Glutathione reduced

Chemical name	Weight of chemical	Volume
Glutathione Reduced	3.073g	100 ml

Procedure: 3.073 g was mixed in 100 ml of buffer (Phosphate) to make stock solution (100 mM). Add 200 µl of stock solution in 2 ml of reaction mixture.

5.8.2 Tissue Extract Preparation

Oxidative stress was measured on day 7, 14, 21 and 28 of polypropylene exposure. On the respective day earthworms were collected from each tray. All the collected earthworms were kept in beakers having moist filter paper for 24 hrs to empty the gut. Homogenize 5 g of earthworm tissue in 5 ml of 50 mM Sodium carbonate buffer. After homogenization, centrifuged the tissue extract at temperature 4˚C for 30 min. Supernatant was collected to estimate the enzymatic activity.

5.8.3 Superoxide Dismutase assay

Superoxide dismutase assay was estimated by the method described by Kono (1978). The ability of the enzyme inhibits the photochemical reduction of nitroblue tetrazolium (NBT) dye to superoxide radicals produced by auto-oxidation of hydroxylamine.

Procedure

Take (300 µl) tissue extract in a cuvette and 50mM Phosphate Buffer (1000 µl) was added, 96 µM NBT (300 µl), 20 mM (200µl) and 0.6% Triton (200 µl) adjust pH 6.0 in a cuvette. The change in absorbance was estimated through UV-Visible spectrophotometer (Shimazdu UV-1800) at 560 nm for interval of 120 sec at temperature 27˚C.

(vi) Calculations of SOD enzyme assay

$$X = \frac{\text{Change in Absorbance min}^{-1}\text{(blank)} - \text{Change in absorbance min}^{-1}\text{(test)}}{\text{Change in absorbance /(blank)}}$$

5.8.4 Catalase (CAT) Assay

Catalase assay was estimated by the describe method of Aebi (1984) with slight modifications. CAT plays an important role to convert H_2O_2 to water (H_2O) and oxygen (O_2). The principle of this biochemical assay is based on the reaction of catalase with H_2O_2.

Procedure

Take (70 µl) tissue extract in a cuvette, add 50 mM Phosphate Buffer (1600 µl) and 30 mM H_2O_2 (330 µl) cuvette. The change in activity and absorbance was recorded at 240 nm for 60 sec interval at temperature 27˚C.

Calculation

CAT enzyme activity was estimated by using molar extinction value 40 $mM^{-1}cm^{-1}$.

$$CAT\ Unit\ Activity(Umin^{-1}g^{-1}FW) = \frac{Change\ in\ Absorbance min^{-1} \times Total\ Volume\ (ml)}{Extinction\ coefficient \times Amount\ of\ Sample\ (ml) \times Fresh\ weight\ of\ tissue\ (g)}$$

5.8.5 Guaiacol Peroxidase (POD)

Guaiacol peroxidase assay was estimated by described method of Xu et al (2013) with slight modifications. The principle is based upon the conversion of lipid hydroperoxidase to alcohols and H_2O_2 to H_2O.

Procedure

Take (70 µl) tissue extract in a cuvette, add 50 mM Phosphate Buffer (1000µl), 20mM Guaiacol (450 µl), 20 mM H_2O_2 (450 µl) in a cuvette. The change in absorbance was recorded at 436 nm for 1 min interval at temperature 27˚C.

Calculations

POD enzyme activity was estimated by using molar extinction coefficient value 26.6mM^{-1} cm^{-1}.

$$POD\ UnitActivity(Umin^{-1}g^{-1}FW) = \frac{Change\ inAbsorbance\ min^{-1} \times Total\ Volume(ml)}{Extinction\ coefficient \times Amount\ ofSample(ml) \times Fresh\ weightoftissue\ (g)}$$

5.8.6 Glutathione-S-Transferase (GST) assay

This assay was estimated by the described method of Habig and Jakoby (1981). The principle of this assay is based on the chemical reaction of 1-chloro, 2, 4- dinitro benzene (CDNB) with another reagent reduced glutathione (GSH).

5.8.6.1 Preparation of reagents

Procedure

Take (70 µl) tissue extract in a cuvette, add 50mM Phosphate Buffer (1300µl) ,10mM CDNB (315µl), 10Mm Glutathione reduced (315µl) in a cuvette. The change in absorbance was recorded at 340 nm for 1 min interval at temperature 27˚C.

Calculations

GST enzyme activity was estimated by using molar extinction coefficient value 9.6 $mM^{-1}cm^{-1}$.

$$GST\ UnitActivity(Umin^{-1}g^{-1}FW) = \frac{Change\ in\ Absorbance\ min^{-1} \times Total\ Volume(ml)}{Extinction\ coefficient \times Amount\ of Sample\ (ml) \times Freshweight of tissue\ (g)}$$

5.8.7 Molecular docking

Molecular interactions between polypropylene and antioxidant enzymes (SOD, POD, CAT and GST) were studied to know the effects of polypropylene on the enzyme activities The Glide module of Schrödinger software (Schrödinger Release 2022-1: Maestro, Schrödinger, LLC, New York, NY) was used to explore different binding sites available on these enzymes. The 2D sketcher of Maestro was employed to draw the structure of Polypropylene, which was then optimized using Lig prep. The structures of SOD (PDB Id: 1CBJ; resolution 1.65 Å), POD (PDB Id: 1GZA; resolution 2.06 Å), CAT (PDB Id: 1TGU; resolution 2.80 Å), and GST (PDB Id: 1M0U; resolution 1.75 Å) were obtained from the Protein data bank (www.rcsb.org) and imported into Maestro. The protein structures were optimized using Protein prep wizard, which involves eliminating water molecules, inserting lost hydrogen atoms, and other pre processing steps to clean up the protein structure. The sitemap tool of Glide were employed to identify the optimal SOD, POD, CAT, and GST binding pockets, and a grid was generated for each binding pocket using the receptor grid generation wizard. The prepared structure of polypropylene was docked into the binding sites obtained from sitemap using Glide docking module in Schrodinger. The binding affinities of the polypropylene at different site were then compared using the Glide-Score provided by docking studies. The 3D enzyme-ligand interactions were visualized using XP-visualizer.

5.9 To identify the microplastic degrading bacteria from earthworm gut.

Before experiment, the artificial soil was sterilized to free from contamination and all the earthworms were kept for 24 hrs in glass beakers with moist filter paper for starvation. Earthworms were washed with distilled water and wipe with 70% ethanol. Ten mature earthworms were placed in each tray without microplastics (Control) and with microplastics concentration (16000 $mgkg^{-1}$) in triplicate (Zhou et al., 2020). These concentrations were selected on the basis of previous reported study to check the effects of microplastic concentration i.e. 9000 mg on gut microorganisms of earthworm. After 28 days the earthworms were collected from each tray to study the intestinal microbial community.

5.9.1 Isolation of earthworms gut to isolate DNA for metagenomic studies to assess microbial diversity.

All earthworms were kept for 24 hrs starvation period on wet filter paper to egest all the ingested particles. Entire body of earthworms were washed with distilled water and again washed with 70% ethanol or 5% Sodium hypochlorite solution. Before the isolation of gut, earthworms were defrosted and kept under low temperature (-16˚C). Earthworms were dissected from clitellum part to end part i.e anus with sterilized scissors.

5.9.2 Gut metagenomic analysis using Amplicon sequencing

The metagenomic DNA was extracted using Blood and Tissue (Qiagen miniprep kit, USA) following manufacturer's instructions. The quality of isolated DNA was assessed on 0.85% agarose gel and the concentration was quantified using nanodrop spectrophotometer. Polymerase Chain Reaction (PCR) amplification of the hypervariable region (V3-V4) of the 16s rDNA gene was performed with forward and reverse primer (5'CCTACGGGNGGCWGCAG3') (5'GACTACHVGGGTATCTAATCC3') respectively. The specific sequences were selected according Klindworth et al (2013). PCR programming was carried as denaturation with following parameters 5min at 95˚C, followed by 35 cycles at 95˚C for 50 sec, annealing and elongation was perform at 52˚C and 72˚C for 40 sec and 60 sec respectively with 72˚C final extension for 10 min.The amplified PCR products were combined in equal fractions and purified using Qiagen gel extraction kit (Qiagen, Germany). Multiplexed pair-end libraries (250×2bp) were prepared using the illumine DNA Prep Kit (Illumina, San Diego, USA), followed by sequencing on the Illumina Novaseq 600 platform (Illumina, San Diego, USA) at Molsys Pvt. Ltd. Bangalore (India). The obtained nucleotide sequences were deposited in NCBI database with accession number (PP815664, PP815665 and PP815665).

5.9.3 Bioinformatic Analysis

The obtained raw sequences (fastq files) were processed using the open-source Quantitative Insights into Microbial Ecology (QIIME2 2020.8) package (Bolyen et al. 2019). Demultiplexing and quality filtering on the rawreads was done using the q2-demux plug-in followed by denoising with divisive amplicon denoising algorithm Divisive Amplicon Denoising Algorithm (DADA2) viaq2-dada2 (Callahan et al., 2016). Amplicon sequence variants (ASV) were aligned by MAFFT via q2-alignment

(Katoh and Standley, 2013) and were used to construct phylogeny with fast-tree (via q2-phylogeny) (Price et al., 2010). Operational Taxonomic Unit (OTU) clustering was performed at 3% divergence (i.e. 97% similarity) using VSEARCH (via q2-vsearch) (Rognes et al., 2016). Taxonomy was assigned using the q2-feature-classifier (Bokulich et al., 2018) against the Greengenes 13_8 at 99% OUT reference sequences (McDonald et al., 2012). To obtain more in-depth detail of different metabolic pathways and enzymes involved, the functional abundances of amplicon sequences were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUSt2 v2.3.0 (Douglas et al., 2018).

5.10 To study the degradation of microplastic in the gut of earthworms by microorganisms

Polypropylene exposure experiment was aimed for 4 weeks (28 days) to know the degradation of microplastic in the gut of earthworm. Prior to the experiment, the earthworms of both species (*E. fetida* and *L. mauritii)* were put 24 hrs for degutting and soil was sterilized by using autoclave. 10 healthy mature well developed clitellated earthworms were added in each tray in triplicate manner containing different concentrations of microplastic viz. 1000, 4000, 8000 and 16000 mg $kg^{-1}$and control (without microplastic). For degradation of PP, particles were extracted from casts, degutting and soil. Everyday ten earthworms cast were collected from each tray and store in petriplates upto 28 days. After 28 days, collected cast were employed for analysis. PP microplastic concentration in earthworms cast was quantified by using the method of Erni-Cassola et al (2017) and Meng et al (2023) with slight modifications. For extraction of microplastic from cast add 30% H_2O_2 in a flask containing casts and heat the flask to digest organic matter for 24 hrs time period at optimum temperature 50˚C. After 24 hrs flasks were placed for desiccation for 48 hrs at 60˚C temperature. 30 ml of saturated NaCl solution was added in the flask containing casts were sonicate for 1 hrs and stirred cast solution for 30 min and then allow settling for 1day (24 hrs). After 24 hrs collect the supernatant in beakers and repeat this step for three times to extract PP microplastic. Supernatant were filtered by using filter paper and dry the filter paper in oven at 40˚C to measure the constant weight of PP in earthworms casts. Similarly microplastics were extracted from soil by using same method. On 28^{th} day all the earthworms were collected from each tray, collect the degutting content and extract PP particles by using similar method.

Extracted microplastic particles from the soil, cast and degutting were employed to know the degradation of PP by ATR-FTIR to study the change in functional groups.

5.11 Statistical Analysis

For statistical analysis different software includes SPSS (version 20), Microsoft Excel (version 14) and GraphPad prism (version 8) was used to done the analysis. Analysis of variance (One way anova) was use to check the significant variations between different concentrations in biomass, fecundity and interaction with earthworm enzymes followed by using Tukey's (HSD). All the values are presented in mean, standard deviation and standard error. All chemical structures and degradation mechanism was draw by using Chem Draw Ultra (version 7.0.4). For microorganisms study all the downstream and statistical analysis was performed in R language (R core team, 2016). The significance level between the control and polypropylene were tested by Wilcoxon Rank Sum Tests at a significant level of less than 5% ($p \leq 0.05$). Alpha diversity was calculated by using R microeco v0.20.0 packages. All the tests were performed using microeco package in R and plots were made using ggplot2 package v 3.4.2. Furthermore Bray-Curtis distance was used to calculate beta-diversity.

6. RESULTS AND DISCUSSION

Punjab is also known as "Food Basket of the Country" or "Granary of India" and 16th largest state according to population. The cultivated land is approx 95.7% (4.023 million hectare out of 5.036 million hectare). Punjab is famous for different types of crops production such as wheat, sugarcane, rice, cotton, maize and different types of fruits. It consists of 23 districts and 237 cities. Out of which Kapurthala, Jalandhar and Amritsar are known for manufacturing of plastic items and these manufacturing industries dispose the waste into rivers and canals etc (Fig 6.1). These wastes have been used for agriculture purpose through wastewater irrigation. Farmers also use plastic mulch and sewage sludge in agriculture fields. Use of plastic through different pathways changes the physical and chemical properties of soil.

6.1 Physico-chemical analysis of agriculture soil near industrial area

Plastic pollution increase day by day and causes several deleterious effects to human beings as well as terrestrial ecosystem. Therefore it is important to study the effects of plastics on soil physical and chemical properties by studying the different parameters such as pH, EC (Electrical Conductivity), TDS (Total Dissolved Solids), OC (Organic Carbon) (Table 6.1) and different elements like Nitrogen, Phosphorus, Potassium, Sodium and Lithium. The heavy metals content were also measured from all soil samples like Iron, Zinc, Chromium, Copper, Cadmium and Lead were reported in soil samples (Table 6.2).

The pH of all soil samples were found to be significantly different ($p< 0.05$). The average pH value at sampling sites A1-A4 was 8.26±0.07, J1-J4 was 8.07±0.07 and K1-K4 was 7.36±0.06. The average EC value of A1-A4 sites were 146.41±1.03, J1-J4 was 136.5±1.19 and K1-K4 was 209.62±1.71.The average value of TDS and OC in all samples were found to be significantly different ($p < 0.05$). Similarly the N, P, K, N, Li and heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) content of all soil samples were found to be significantly different. The availability of different elements in all soil samples were in the order of Na > K > P > Li > N. The Fe content was found maximum at site A4 (331.9±0.68 mg/kg) while minimum in K3 site (138.5±0.34 mg/kg). Cd content was found minimum in all the sampling sites compared to other heavy metals. The

content of availability of different heavy metals in the soil sampling were in the order of Fe > Zn > Cr > Cu > Pb > Cd.

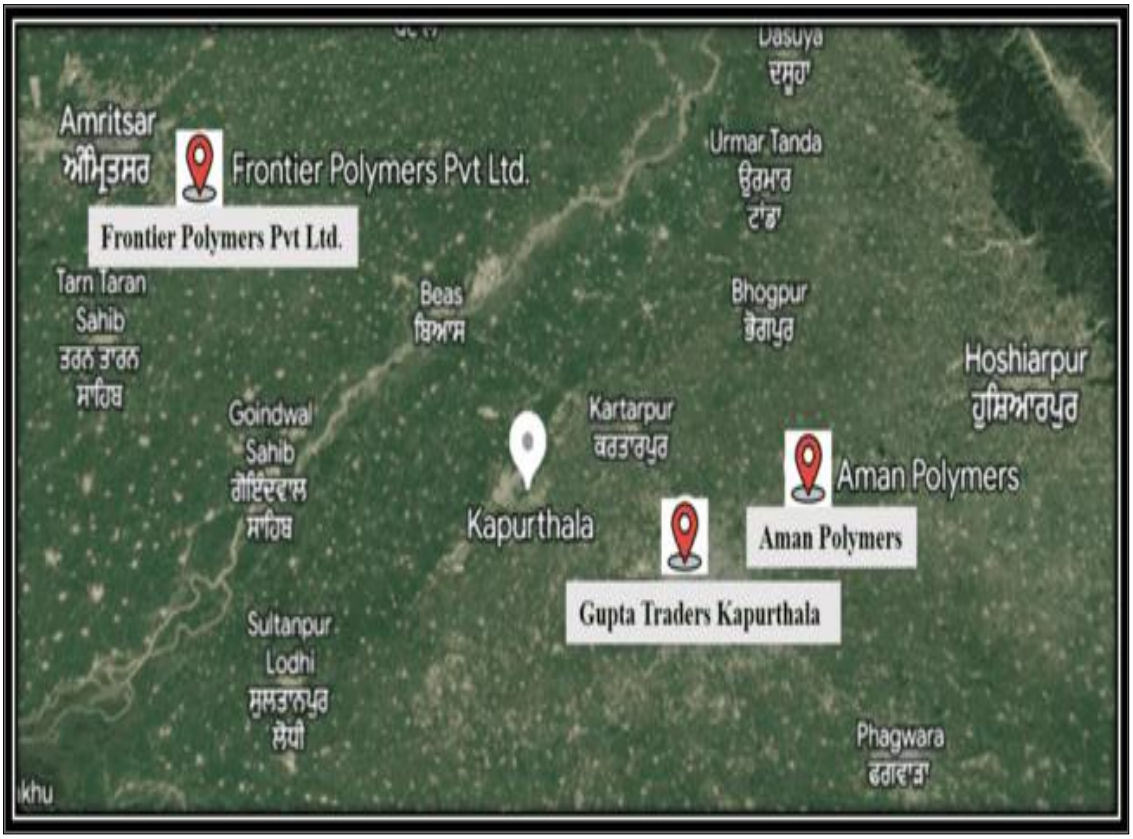


Fig 6.1 Industrial area selected for selection of soil samples.

Table 6.1 Physico-chemical analysis of different soil samples of agriculture field.

City	Sites code	Location	pH	EC (µS/cm)	TDS (mg/l)	OC (%)	N (g/kg)	P (g/kg)	K (g/kg)	Na(g/kg)	Li (g/kg)
Amritsar (Frontiers Polymers Pvt. Ltd.)	A1	N 31˚ 34' 40.19 E 74˚ 59' 42.21	**8.42±0.10**	176.3±0.88	94.9 ±0.39	2.56±0.86	0.25±0.05	0.72±0.03	2±0.05	3.8±0.05	0.36±0.06
	A2	N 31˚ 34' 41.11 E 74˚ 59' 44.96	8.4±0.15	132.3±1.76	71.5±0.26	4.47±0.68	0.18±0.02	**1.42±0.25**	1.8±0.12	1.46±0.84	1.03±0.03
	A3	N 31˚ 34' 41.61 E 74˚ 59' 43.70	8.7±0.03	161.0±1.15	68.5±0.20	3.52±0.50	0.14±0.04	1.23±0.18	1.5 ±0.18	1.83±0.12	0.5±0.15
	A4	N 31˚ 34' 41.68 E 74˚ 59' 43.53	7.53±0.02	115.7±0.33	82.5±0.14	3.96±1.39	0.18±0.05	1.02±0.04	1.9±0.03	1.23±0.08	**1.33±0.06**
	Average		8.26±0.07	146.41±1.03	79.37±0.25	2.76±0.86	0.19±0.04	1.09±0.12	1.83±0.09	2.08±0.09	0.80±0.07
Jalandhar (Aman Polymers Pvt. Ltd.)	J1	N 31˚ 22' 46.43 E 75˚ 35' 31.56	8.10±0.06	116.0±2.52	**124.1±0.08**	4.19±1.09	0.40±0.06	0.25±0.01	3±0.05	3.73±0.12	0.4±0.01
	J2	N 31˚ 22' 45.87 E 75˚ 35' 30.79	7.7±0.09	135.0±0.58	70.2±0.05	2.04±0.50	0.13±0.02	0.92±0.05	1.63±0.08	1.76±0.12	0.56±0.08
	J3	N 31˚ 22' 46.49 E 75˚ 35' 30.44	8.36±0.08	153.7±0.33	121.1±0.31	1.14±0.85	0.11±0.01	0.16±0.01	1.46±0.03	1.7±0.05	0.33±0.05
	J4	N 31˚ 22' 46.00 E 75˚ 35' 30.91	8.13±0.05	141.7±1.33	78.6±0.12	3.95±2.96	0.14±0.03	0.59±0.23	1.56±0.08	1.86±0.03	0.3±0.05
	Average		8.07±0.07	136.5±1.09	98.52±0.14	2.83±1.35	0.19±0.02	0.48±0.07	0.11±0.06	2.26±0.08	0.4±0.08
Kapurthala (Gupta Traders Pvt. Ltd.)	K1	N 31˚ 39' 53.1 E 75˚ 38' 16.2	7.18 ±0.09	184.9±1.80	120±0.57	3.29±2.03	**0.52±0.03**	0.25±0.01	**4.13±2.3**	**5±0.1**	0.63±0.08
	K2	N 31˚23' 51.46 E 75˚ 38' 21.31	7.08±0.04	224.7±1.86	94.1±0.80	**5.65±0.39**	0.46 ±0.03	0.23±0.13	3.23±1.8	4.2±0.08	0.46±0.06
	K3	N 31˚23'53.66 E 75˚23' 16.30	7.1±0.07	198.6±1.75	118.5±0.18	5.05±0.36	0.52±0.08	0.25±0.00	3.7±0.11	5.1±0.1	0.20±0.05
	K4	N 31˚ 23' 53.43 E 75˚ 23' 16.29	8.1±0.03	**230.3±1.45**	121.9±0.11	5.22±0.63	0.35±0.05	0.21±0.00	2.9±0.05	3.43±0.12	0.63±0.03
	Average		7.36±0.06	209.6±1.71	113.64±0.41	4.81±0.85	0.46±0.05	0.23±0.00	3.49±0.08	4.45±0.07	0.48±0.06

Table 6.2 Heavy metal content (mg/kg) in different soil samples of agriculture field.

City	Sites Code	Cd	Cr	Cu	Fe	Pb	Zn
Amritsar (Frontiers Polymers Pvt. Ltd.)	A1	0.03±0.02	25.5±0.62	29.2±0.43	322.1±0.68	0.14±0.04	37.2±1.02
	A2	**0.06±0.24**	22.9±0.34	28.2±0.27	315.9±0.61	0.20±0.05	37.4±1.05
	A3	0.03±0.01	25.2±0.90	29.0±0.44	324.1±0.67	0.43±0.18	41.4±0.58
	A4	0.04±0.01	26.0±0.64	30.1±1.19	**331.9±0.68**	0.26±0.03	34.0±1.21
	Average	0.04±0.01	24.96±0.63	29.1±0.58	323.52±0.66	0.26±0.08	37.52±0.97
Jalandhar (Aman Polymers Pvt. Ltd.)	J1	0.02±0.18	22.9±0.48	27.0±0.89	235.6±1.35	2.76±0.31	43.4±1.72
	J2	0.02±0.01	21.5±0.82	33.0±0.30	264.6±0.51	**3.33±0.28**	**47.1±1.12**
	J3	0.05±0.01	**26.7±0.28**	31.3±0.66	237.9±0.11	3.08±0.27	43.1±1.44
	J4	0.02±0	26.1±1.53	31.2±0.62	236.2±2.82	2.61±0.80	40.8±0.88
	Average	0.03±0.00	24.349±0.78	30.67±0.62	243.58±1.20	2.94±0.23	43.62±1.29
Kapurthala (Gupta Traders Pvt. Ltd.).	K1	0.02±0.18	22.1±1.9	33.3±1.71	140.2±1.05	1.08±0.01	41.7±0.31
	K2	0.02±0.01	24.8±0.75	31.7±0.89	143.2±0.73	1.19±0.41	42.6±0.21
	K3	0.05±0.01	22.5±0.84	**35.9±0.99**	138.5±0.34	0.98±0.06	39.7±0.35
	K4	0.02±0	22.3±0.85	31.2±0.61	141.8±1.18	0.81±0.28	41.3±0.29
	Average	0.05±0	22.96±1.08	33.08±1.05	140.99±0.83	1.02±0.19	41.37±0.19

6.2 Extraction, Identification and characterization of microplastics particles from soil samples.

6.2.1 Shape, size and Color of MPs

Different types of MPs were recovered with different shapes include fibres, fragments and miscellaneous and their size range from 100 µm–1 mm (Table 6.3). Five different colors of microplastics were extracted from agricultural soil including blue, white, light green, dark blue and orange. Blue and white color MPs presiding in all soil samples (Fig 6.2). Out of all blue color is more prevalent (67%) than white color MPs (30%) orange color are present in very low quantity (2%), light brown, light pink and light pink color (1%) are also present. Three different shapes of microplastic were observed i.e fibers, fragments and film. Fragments of different colors of microplastics were present in large quantities in all soil samples. All stained microplastic fibers and particles were observed under fluorescence microscope (Fig 6.3). Li et al, (2018c) also extracted the different shapes and sizes of microplastic by employing NaCl from wastewater treatment plant sludge. In another study Zhang et al., (2018d) extracted low density polyethylene (LDPE) and polypropylene (PP) from soil.

6.2.2 FTIR analysis

Total five different types of microplastics were detected and identified by using ATR-FTIR viz polypropylene (PP), polystyrene (PS) polyethylene terephthalate (PET), polybutylene terephthalate (PBT) and polyethylene (PE). These microplastic polymers were identified on the basis of absorption peak in the spectra. PS were identified by two intense absorption peaks present at wave number 3026.6 cm^{-1} and 2922.2 cm^{-1} due to medium aromatic C-H stretching vibrations, 1938.4 cm^{-1} and 1871.1 cm^{-1} occur due to combination band C=C=C, 1804 cm^{-1} and 1744.4 cm^{-1} appear due to strong **C**=O, 1602.8 cm^{-1} is due to presence of aromatic C=C stretching, 1177.8 cm^{-1} were due to strong C-O stretching, 842.4 cm^{-1} were due to strong C-H bending, 752.9 cm^{-1} and 700.7 cm^{-1} absorption peak observed due to C-H bending and 1-2 di-substituted and presence of benzene derivative. PP polymer absorption peak appears at wavenumber 2922.2 cm^{-1} due to C-H stretching, 1714.6 cm^{-1} were due to strong C=O stretching, 1617.7cm^{-1} due to C=C stretching, 1453.7cm^{-1} occurs due to presence of CH_2 bending 1371.7 cm^{-1} due to CH_3 bending, 1162.9 cm^{-1} and 842.4 cm^{-1} due to C-H bending all these are the identification peak of polypropylene polymer.

Table 6.3 Abundance and characterization of different types of microplastics extracted from agriculture soil near to industries.

Soil Sample	City	Strategy for identification	Microplastic Parameters				Mean abundance in (g)
			MP type	MP shape	MP size	MP color	
A1	**Amritsar**	ATR-FTIR	PS, PP	Fragments	<5 mm	White, Light brown	0.117
A2			PP, PE, PET	Fibres, Fragments, Film	<0.05 mm	Blue, white, White	0.215
A3			PP, PBT	Fragments	<1 mm	White, Light blue, Light Green	0.009
A4			PP	Fibres	<100 µm	Blue	0.007
J1	**Jalandhar**	ATR- FTIR	PP, PET	Miscellaneous, Film	100-5 mm	Light sky blue, Transparent	0.234
J2			PP, PE	Fragments, Fibres	< 2 mm	Blue, Light pink	0.017
J3			PP, PE	Fragments	2 mm-5 mm	Blue, Transparent	0.023
J4			PP, PET, PE	Fibres, Fragments	< 2 mm	White, Dark blue, Green	0.113
K1	**Kapurthala**	ATR-FTIR	PP, PET	Fibres, Fragments, Film	<0.05 mm	Blue, White	0.267
K2			PP, PE	Fragments	<1 mm	Dark blue, Light green, White	0.452
K3			PBT, PP	Fibres, Miscellaneous, Fragments	<100 µm	Orange, Blue	0.309
K4			PS, PP	Fragments, Fibres	100-200 µm	White, Blue	0.209

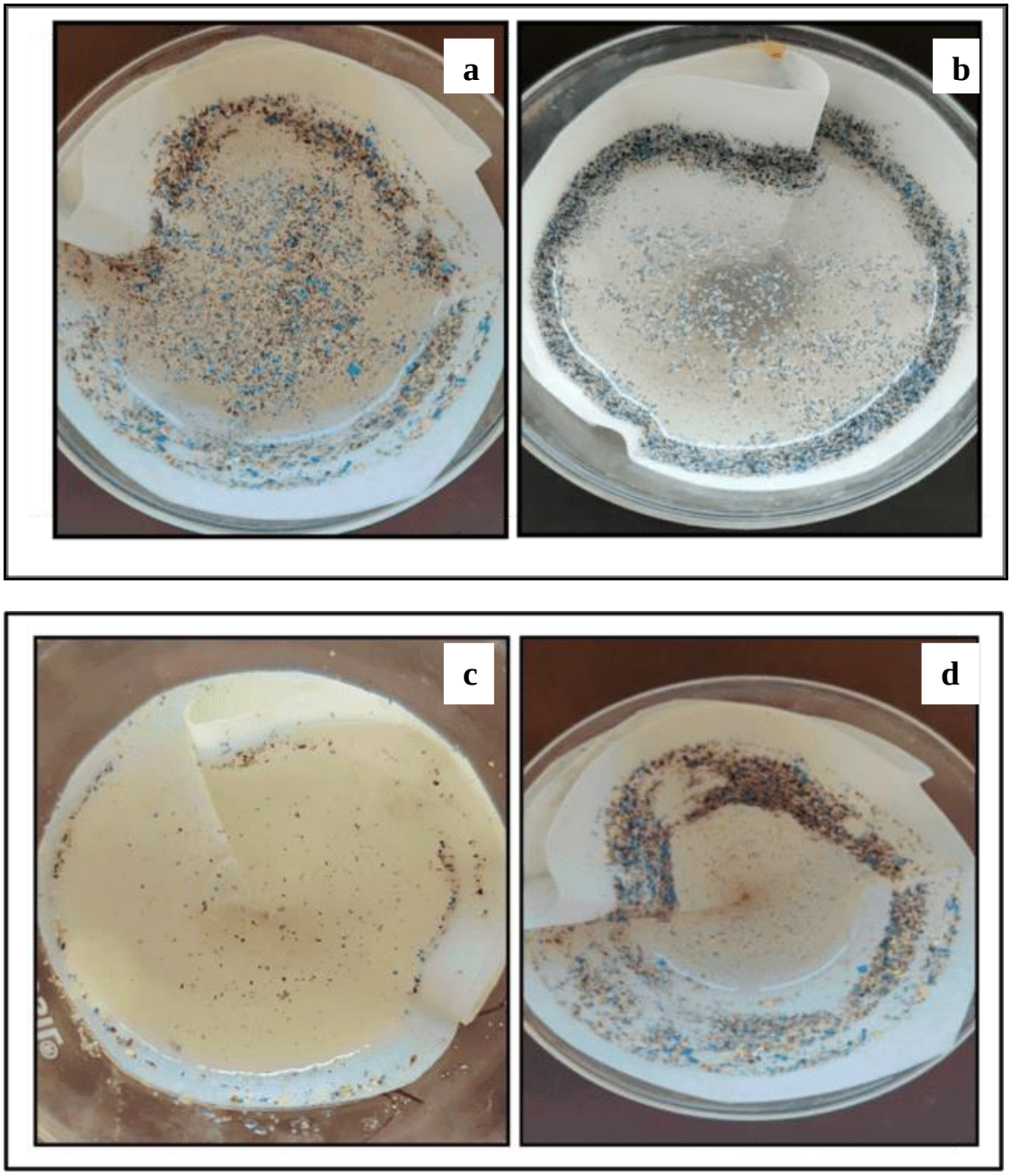


Fig 6.2 Different types of microplastics particles were extracted using density separation and floatation method.

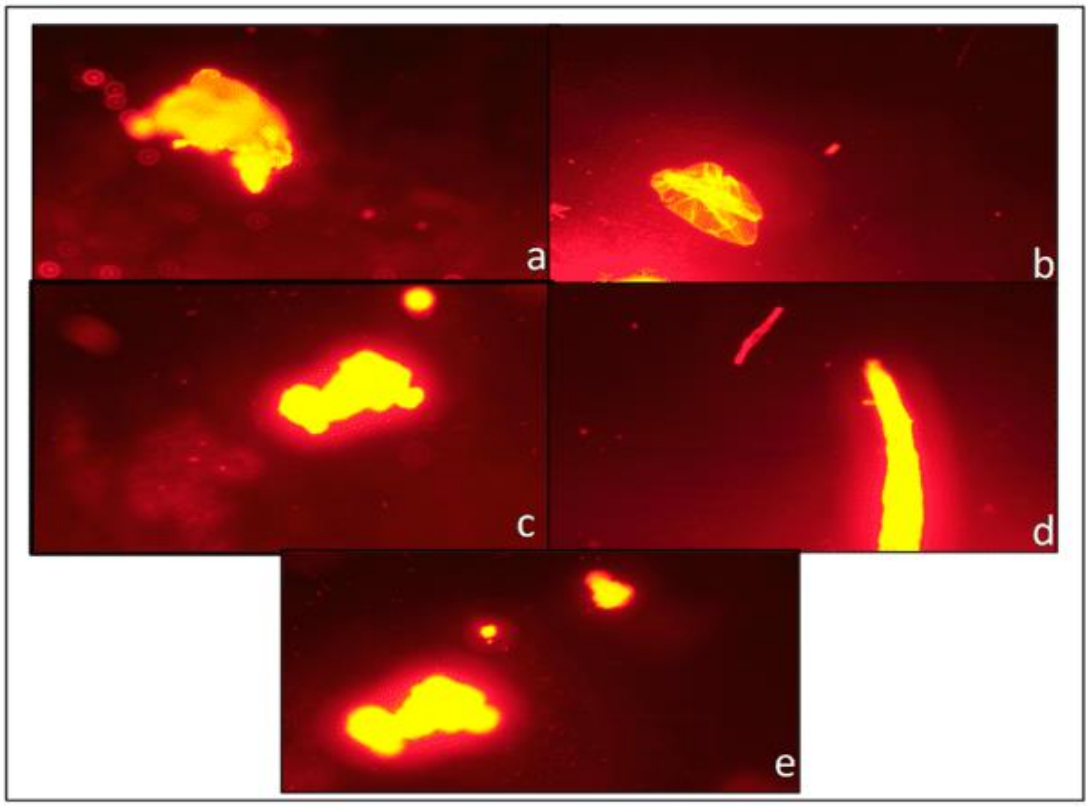


Fig 6.3 Different types of microplastic polymer stained with nile red dye under fluorescence microscope (a) PET (b) PBT (c) PP (d) PE fibre (e) PS.

The absorption peak of PET at wave number 2918.34 $cm^{-1}$is due to C-H stretching, alkane group present, 1714.6 cm^{-1} strong C=0 stretching, 1364.2 cm^{-1} CH_2 bending, 1237.5 cm^{-1} due to strong C-O stretching , 872.2 cm^{-1} due to strong C=C bending, 723.1 cm^{-1} due to aromatic CH bending out of plane. PE polymers were identified by studying the absorption peak at wave number 2914.8 cm^{-1} due to C-H stretching, 1468.6 cm^{-1}due to CH_2 bending, 872.2 cm^{-1} due to strong C=C bending, 775.3 cm^{-1} and 715.6 cm^{-1} is due to CH_2 rocking. Different types of microplastics such as PS, PP, PE and PET spectra were matched with previous reported study (Morgado et al., 2021; Mataji et al., 2020; Mecozzi and Nisini, 2019). PBT was identified by observing absorption peak at 2922.2cm^{-1} due to aromatic C-H stretching, 1714.6 cm^{-1} is due to C=0 stretching, 1267.3cm^{-1} due to the presence of strong C-O stretching , 1013.8 cm^{-1} strong aromatic C-H bending in plane, 797.7cm^{-1} due to C=C bending and tri-substituted and 723.1cm^{-1} due to C=C bending and di-substitute. Fig 6.4 shows the FTIR spectra of different types of microplastics polymers present in agriculture soil. In previous study different types of microplastics were identified on the basis of resulting peak in the FTIR spectra with a familiar microplastic polymer in the spectral

library (Mecozzi et al., 2016; Jung et al., 2018; Veerasingam et al., 2021; Alvim et al., 2020). Different types of microplastic polymer have different chemical structures (Fig. 6.5) and these stretching and bending depends on the presence of carbon, hydrogen and oxygen atoms.

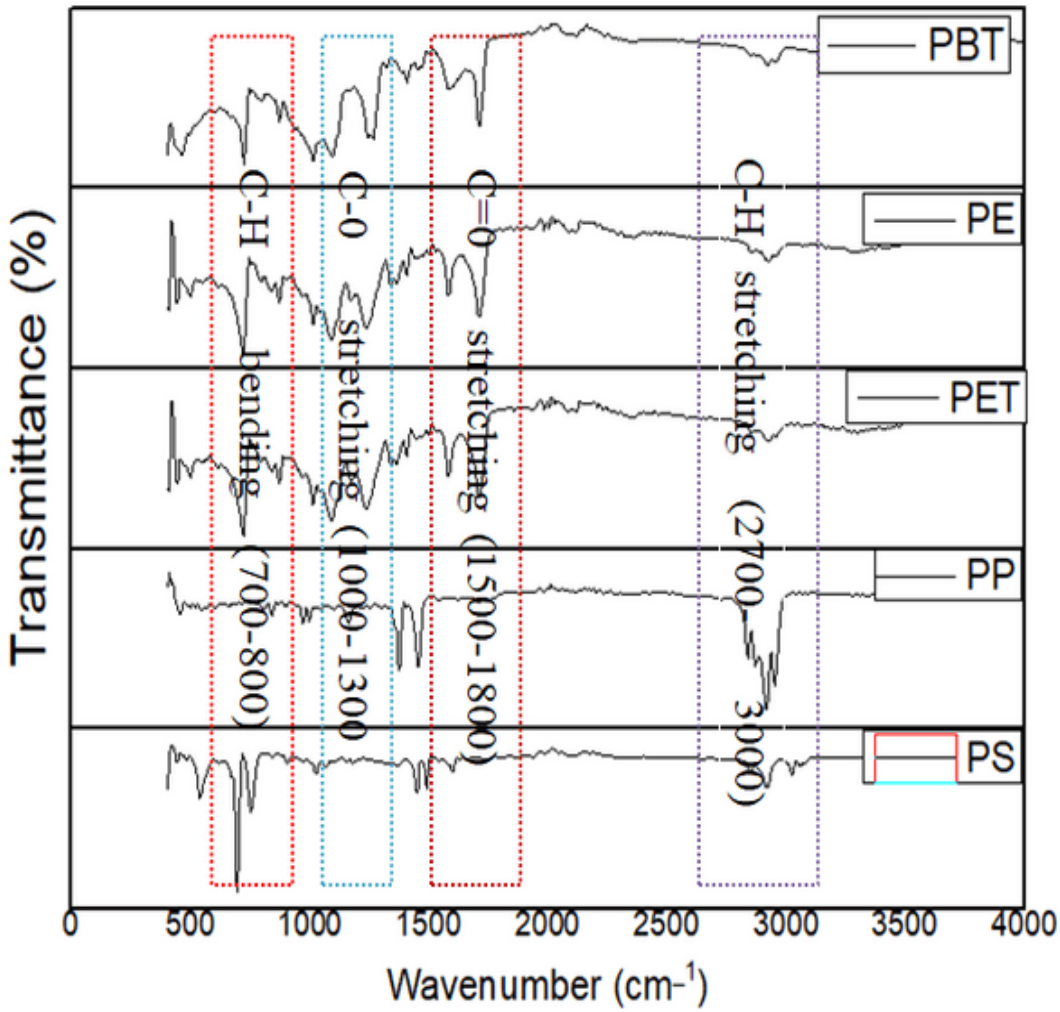


Fig 6.4 ATR-FTIR spectra of different types of microplastic extracted from agriculture soil.

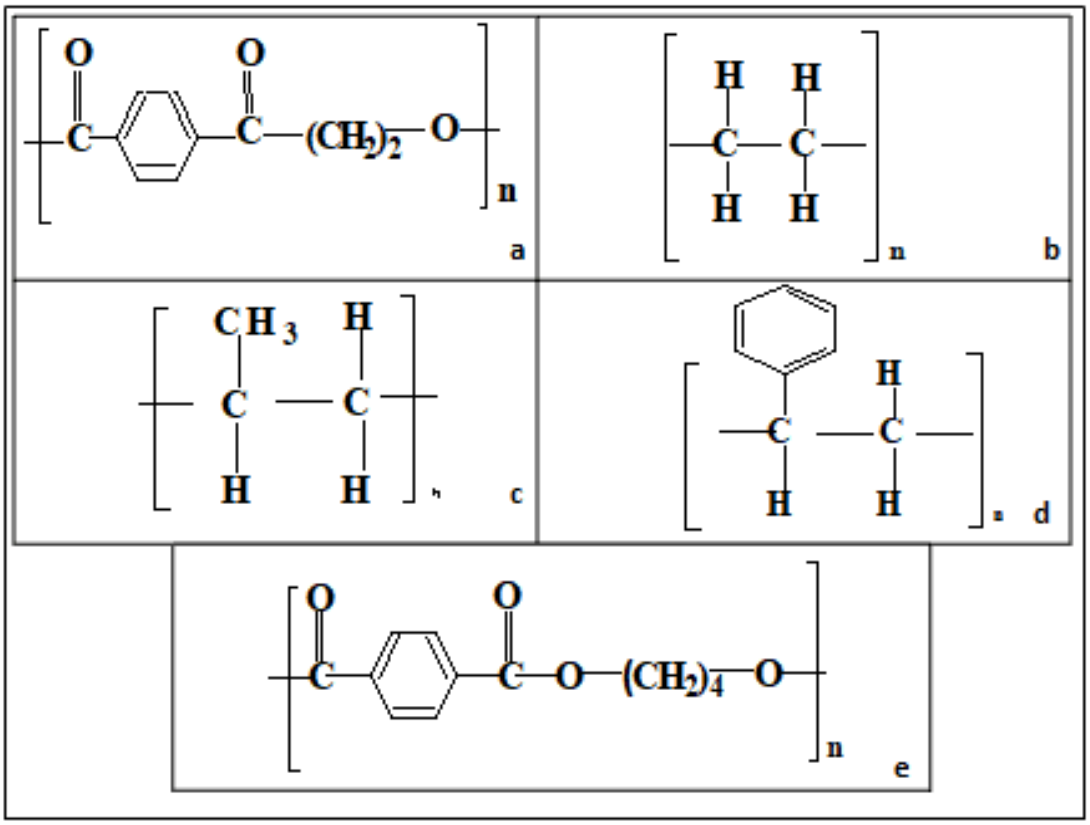


Fig 6.5 Chemical structures of different types of MPs extract from agriculture soil (a) PET (b) PE (c) PS (d) PP (e) PBT.

6.2.3 X-Ray diffraction

Different types of microplastics were studied by XRD patterns.Table 6.4 shows the peak intensity and crystalline percent of different types of polymer. PET is a very poor crystalline in nature and shows broad peak and its maximum peak intensity falls in 2Θ of 25.71˚ (Fig 6.6). PP shows sharp peak due to its strong crystalline nature. It has a maximum diffraction peak decline in 2Θ of 31.15˚. PE is crystalline in nature and its maximum diffraction peak intensity decline in 2Θ of 21.62˚. All these three peaks are very sharp and clearly visualize. PS shows the poor crystalline nature with the presence of broad and wide peaks. PS has maximum peak intensity at 2 Θ of 22.68˚. PBT microplastic polymer is amorphous in nature and has no sharp peak, but the pattern is noisy. XRD pattern reported in the previous study of three types of microplastics (PE, PVC, PS) shows two intense sharp peaks at 2Θ of 21.1˚, 23.4˚ for PE, no sharp peak in PVC and broad peaks in PS described poor crystalline in nature (Liu et al., 2019; Ezeonu et al., 2019; Moura et al., 2023).

Table 6.4 XRD pattern of different microplastics polymer.

Microplastic	2Θ˚value of intense peak	Crystallinity (%)	Crystalline size (Å)
PET	25.71	50.6	3.47
PP	31.15	64.5	4.09
PE	21.62	61.1	2.86
PS	22.68	70.3	3.46
PBT	10.10	60.4	8.78

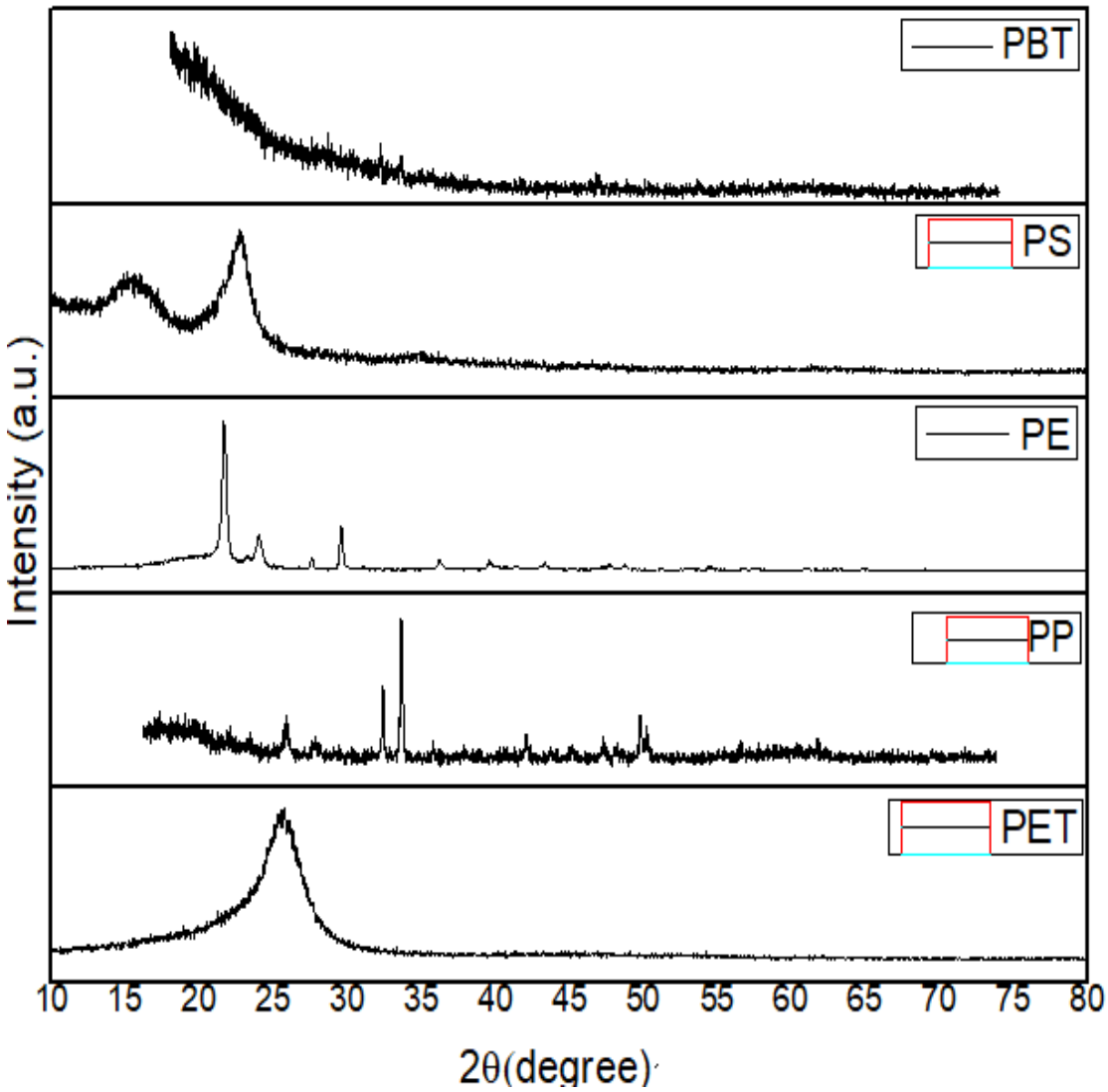


Fig 6.6 X-Ray diffraction pattern of different types of MPs extracted from agriculture soil.

6.2.4 Scanning Electron Microscopy (SEM) analysis

SEM analysis of different types of microplastics (PBT, PE, PET, PP and PS) extracted from agriculture soil revealed the surface morphology of different types of conventional and non-conventional microplastics (Fig 6.7).

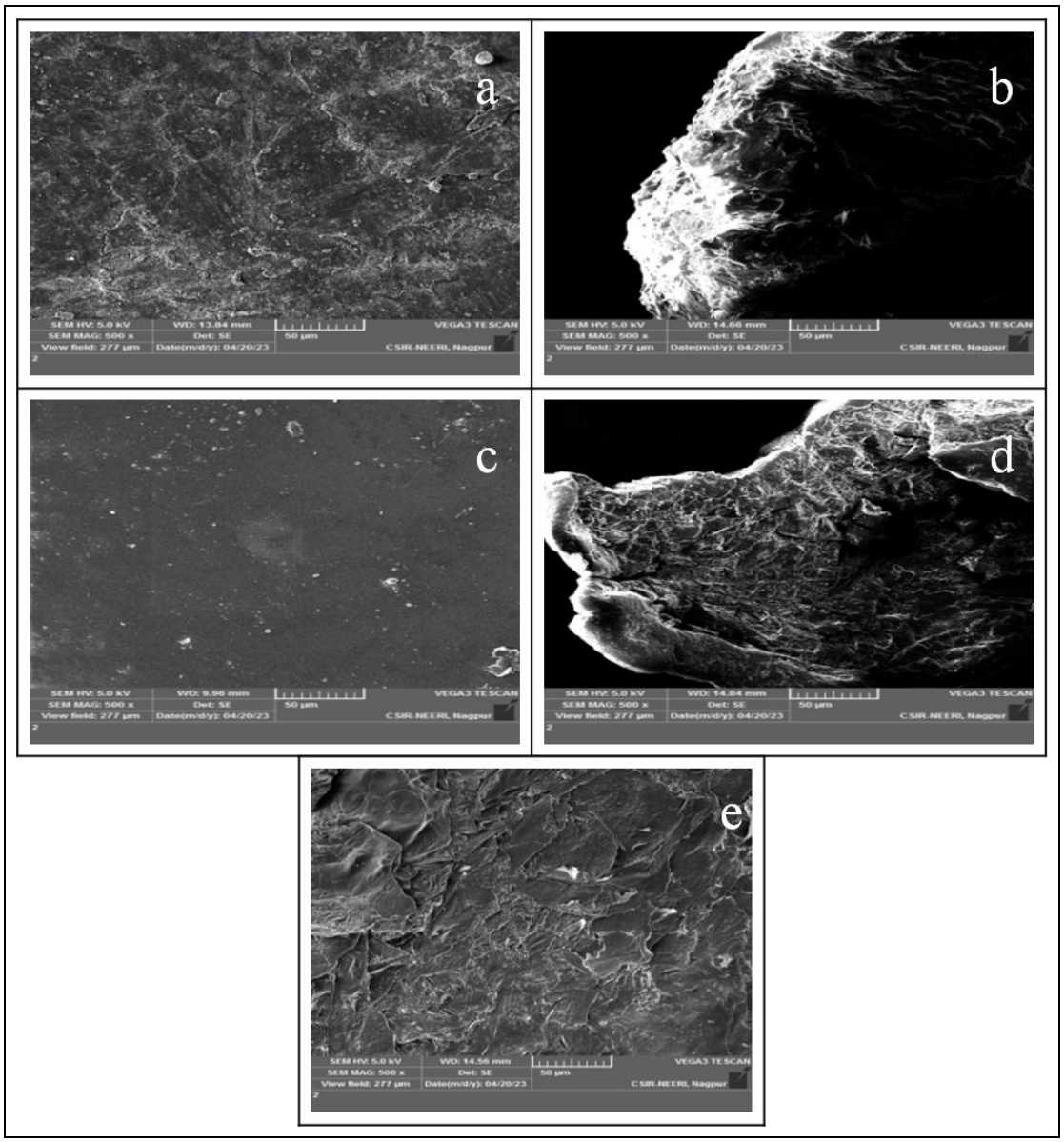


Fig 6.7 SEM micrographs of different types of microplastics extracted from agriculture soil (a) PBT (b) PE (c) PET (d) PP (e) PS.

6.2.5 Validation of recovery rate

The recovery efficiency of different types of microplastics depends upon the types of microplastics and Sodium chloride solution (NaCl) (Table 6.5). PP has the highest recovery efficiency in NaCl solution compared to other microplastics, and PET has

the lowest recovery efficiency in NaCl solution. Remaining microplastic particles cannot recover from the soil due to the high density of solutions. In a previous study, the recovery rates of seven different types of microplastics were extracted from sediments by employing two types of saturated solution, NaCl and Sodium dihydrogen phosphate (NaH_2PO_4). NaCl has poor efficiency in recovering microplastics, but NaH_2PO_4 recovered 93% of microplastics (Zhang et al., 2018; Zhang et al., 2020d).

Table 6.5 Mean productivity of different types of microplastics in NaCl solution.

Microplastics	Mean recovery productivity in NaCl solution
PET	6 ± 0.5
PP	9.3 ±0.3
PE	7.0 ±1.0
PS	8.0 ±1.0
PBT	7.0 ±0.5
Mean productivity	7.6 ±0.5

6.3 Toxic effects of polypropylene on exotic (*E. fetida*) and indigenous (*L. mauritii*) species of earthworm.

Two earthworm species were selected to study the toxic effects of polypropylene in terms of growth and reproduction.

6.3.1 Earthworm (*E. fetida*) biomass and fecundity rate towards polypropylene microplastic exposure

E. fetida were treated with polypropylene microplastic for 28 days and all the earthworms were acclimatized for 7 days in artificial soil (Fig 6.8). Five concentrations (0, 1000, 4000, 8000 and 16000 $mgkg^{-1}$) were selected to study the effects of microplastics on growth and reproduction (Fig 6.9, 6.10). No earthworm mortality was observed during the different treatment of polypropylene microplastics. Earthworm biomass increased significantly from 0 to 28 day in control. At 0 day the data was non significant and p value is 0.63 and on 7 day the earthworm biomass increased significantly (p value is 0.00). On 14^{th} day the high concentration of

polypropylene (4000, 8000, 16000 $mgkg^{-1}$) strongly inhibit the biomass of *E. fetida* earthworm except at 1000 $mgkg^{-1}$. On 21st and 28th day, the biomass decreased significantly (p value is 0.01) in each concentration as compared to control (Table 6.6 and Fig 6.9). Overall, different concentrations of polypropylene MP show significant influence on the biomass of *E. fetida*.

Table 6.6 Effect of polypropylene on biomass of *E. fetida* and all values represents as Mean ± S.E. with different superscripts (a-d) within column shows significance value ($p \leq 0.05$) at different treated concentrations.

Conc ($mgkg^{-1}$)	Treatment Period				
	0th day	7th day	14th day	21st day	28th day
0	4.16 ± 0.00^{a}	5.23 ± 0.00^{d}	5.70 ± 0.12^{c}	5.93 ± 0.05^{d}	6.00 ± 0.03^{d}
1000	4.19 ± 0.04^{a}	4.98 ± 0.02^{cd}	4.99 ± 0.03^{b}	4.87 ± 0.03^{c}	4.76 ± 0.04^{c}
4000	4.17 ± 0.02^{a}	4.76 ± 0.05^{bc}	4.62 ± 0.09^{ab}	4.46 ± 0.04^{bc}	4.34 ± 0.03^{bc}
8000	4.16 ± 0.01^{a}	4.62 ± 0.09^{b}	4.34 ± 0.15^{ab}	4.19 ± 0.06^{ab}	4.09 ± 0.15^{ab}
16000	4.13 ± 0.00^{a}	4.29 ± 0.03^{a}	4.11 ± 0.09^{a}	3.97 ± 0.04^{a}	3.69 ± 0.13^{a}

Reproduction rate was measured on the basis of different criteria like number of cocoons, cocoons biomass and number of hatchlings. On 28th day, the cocoons from each tray were harvested, counted and weighed (Fig 6.9). As compared to control, the higher concentrations (4000, 8000 and 16000 $mgkg^{-1}$) of polypropylene showed more toxicity on cocoon formation as well as cocoons weight (Table 6.7).The cocoons were put back in trays after counting and weighing and incubate for another 28 days. On 56th day of activity, all juvenile earthworms in each tray were counted. The result showed that the number of juvenile earthworms (hatchlings) decreased with increase in concentrations. Overall maximum numbers and weight of *E. fetida* cocoons reported in control (without microplastic) and minimum reported at concentrations 8000, 16000 $mgkg^{-1}$. Low amount of microplastic concentrations and less exposure duration have no deleterious or negative effects on the growth and mortality rate. In the present study, no mortality of *E. fetida* was reported in all treatments which is in accordance with the study of Rodriguez-Seijo et al, (2017) that no negative impact of polyethylene on *E. andrei*.

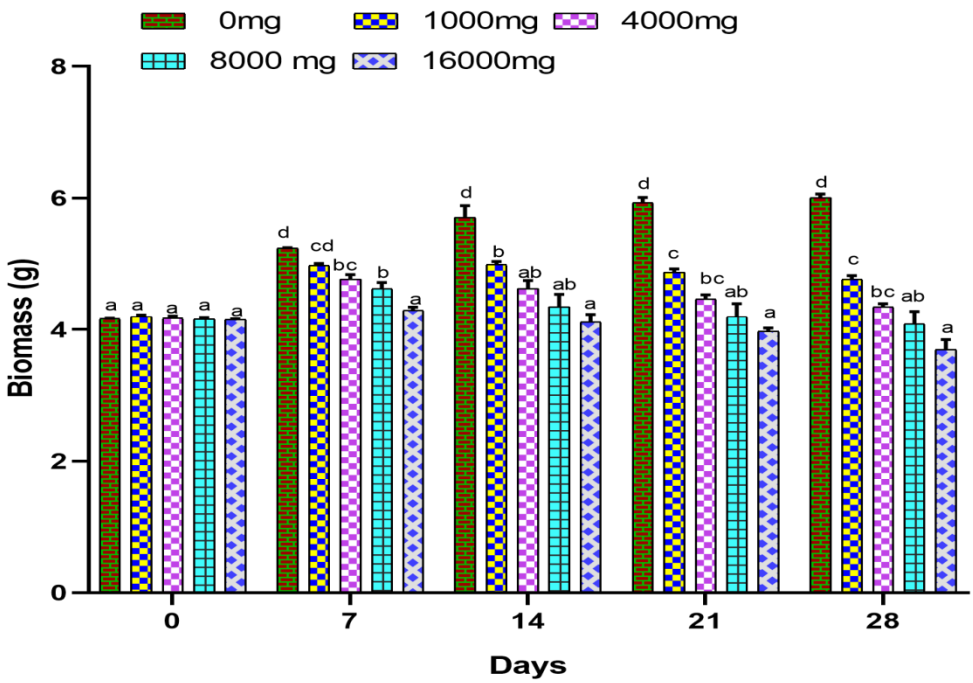


Fig 6.8 Effect of different concentrations of polypropylene on the biomass of *E. fetida* and superscripts (a-d) within column shows significant difference between concentrations.

Fig 6.9 Cocoons of *E. fetida* during 28 days exposure of polypropylene.

Table 6.7 Effect of polypropylene on reproduction rate (Cocoons weigh, cocoon numbers and numbers of hatchlings of *E. fetida* and all values represents as Mean ± S.E. with different superscripts (a-c) within column shows significant difference at $p \leq 0.05$ at different treated concentrations.

Conc. mg kg^{-1}	Reproduction Parameters		
	No of cocoons per earthworm	Weight of cocoons (mg)	No of hatchlings per cocoons
0	5.33 ± 0.33^{c}	15.66 ± 0.88^{b}	4.66 ± 0.33^{b}
1000	4.66 ± 0.33^{c}	15.33 ± 0.33^{b}	4.33 ± 0.33^{b}
4000	4.33 ± 0.33^{bc}	13.00 ± 0.57^{ab}	3.33 ± 0.66^{ab}
8000	2.66 ± 0.33^{ab}	7.66 ± 0.88^{ab}	3.00 ± 0.57^{a}
16000	2 ± 0.57^{a}	5.66 ± 1.76^{a}	1.66 ± 0.33^{a}

Further the high dose of microplastics for long duration cause stomach and intestinal damage to terrestrial organisms (especially earthworms) includes blockage, abrasion in gut and also effects the feeding activity, which may be a major reason to reduce the biomass and growth rate (Lwanga et al., 2017). In this study, *E. fetida* showed significant change in biomass. The biomass of earthworm declined at 4000, 8000, 16000 $mgkg^{-1}$of polypropylene concentration at 28^{th} day. Lwanga et al, (2016) also reported the effects of polyethylene (PE) microplastic on *Lumbricus terrestris* and observed the reduction in growth rate with increase in the concentrations. In this study, fecundity rate (in terms of cocoons formation, biomass and juveniles) of *E. fetida* toward different concentrations of polypropylene shows impact at longer duration and high dose (28 days, conc. 16000 mg kg^{-1}). Previous finding reported that three different types of microplastics (polyethylene, polylactic acid, polypropylene carbonate) at high concentration affect the cocoons biomass and number (Ding et al., 2021). Microplastics have potential to cause disturbance in male reproductive organs and coelomocyte and this may be a major cause to reduce fecundity rate (Kwak and An, 2021).

6.3.2 Earthworm (*L. mauritii*) biomass and fecundity rate towards polypropylene microplastic exposure.

No mortality of earthworm was noticed during 28 days exposure period of polypropylene microplastic. The biomass of *L. mauritii* significantly increased in control group (without microplastic) with increase in exposure time up to 28 days as compared to microplastic treatment. Average biomass of *L. mauritii* in control group at initial day (0 day) were non significant (p value is 0.473). Earthworm's biomass increased upto 984.3mg at final day (28^{th} days). Similarly biomass of *L. mauritii* was increased in concentration 1000 and 4000 $mgkg^{-1}$ concentrations upto 28 days. The biomass of *L. mauritii* was significantly (p value is 0.01) declined on day 14, 21 and 28 day at highest concentration (8000 and 16000 $mgkg^{-1}$). Overall, the biomass reduction was found in high concentration of polypropylene (Table 6.8 and Fig 6.10).

Reproduction test of *L. mauritii* were evaluated on the basis of different parameters such as cocoon numbers, cocoon weight and hatchlings. At 28^{th} day, number of cocoons was decreased with increase in polypropylene concentrations. As compared to control the PP exposure significantly reduced the number of cocoons, cocoons weight and hatchlings with increase in polypropylene concentration (Table 6.9 and Fig 6.11). Similarly on 56^{th} day the hatchlings number was significantly reduced with increase in exposure period as well as PP concentrations.

Table 6.8 Effect of polypropylene on biomass of *L. mauritii* and all values represents as Mean ± S.E. with different superscripts (a-d) within column shows significant difference at $p \leq 0.05$ in concentrations.

Conc ($mg\ kg^{-1}$)	Treatment period				
	0^{th} day	7^{th} day	14^{th} day	21^{st} day	28^{th} day
0	8.55 ± 0.18^{a}	9.50 ± 0.04^{b}	967.3 ± 0.85^{c}	981.6 ± 0.54^{c}	984.3 ± 0.05^{d}
1000	8.58 ± 0.19^{a}	9.20 ± 0.04^{b}	935.3 ± 0.60^{bc}	940.3 ± 0.61^{bc}	9.47 ± 0.25^{cd}
4000	8.29 ± 0.12^{a}	8.61 ± 0.12^{a}	881.3 ± 1.62^{b}	8.87 ± 1.69^{b}	892.6 ± 1.73^{c}
8000	8.33 ± 0.05^{a}	8.54 ± 0.02^{a}	762.0 ± 2.17^{a}	7.49 ± 1.98^{a}	739.6 ± 2.04^{b}
16000	8.30 ± 0.11^{a}	8.33 ± 0.05^{a}	711.6 ± 0.44^{a}	7.00 ± 0.66^{a}	635.6 ± 2.44^{a}

Ju et al., (2023) reported that the earthworm (*Lumbricus terrestris*) biomass and mortality rate were significantly affected by the LDPE microplastics particles but shows no effect on the reproduction. Rashti et al, (2023) also studied the effects of microplastics on *Amynthas gracilis* and *E. fetida*. The biomass of *Amynthas gracilis* was not affected but the body weight of *E. fetida* was strongly affected after 28 days treatment period. The result of our study relates with previously reported study on the effect of polyamides or nylon particles on *Enchytraeus crypticus* reproduction rate and was significantly reduced with increase in polyamide concentration in soil (Lahive et al., 2019). Reproduction rate of different species of earthworm were altered by small size of plastic particles due to the formation of oocytes, follicles and results in the imbalance of germ cells (Kwak and An, 2021).The effect depends upon the size, concentrations and types of microplastics (Mondal et al., 2023).

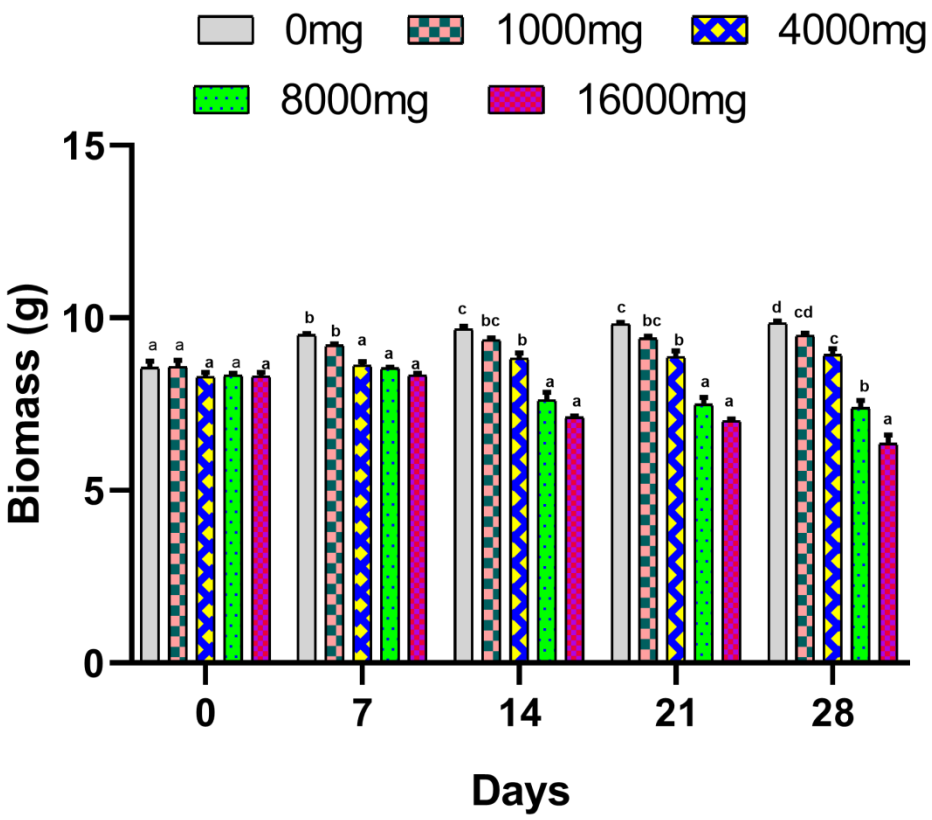


Fig 6.10 Effect of different concentrations of polypropylene on the biomass of *L. mauritii* and superscripts (a-d) shows significant within column difference between concentration.

Fig 6.11 Cocoons of *L. mauritii* during 28 days exposure of polypropylene.

Table 6.9 Effect of polypropylene on reproduction rate (Cocoons weigh, cocoon numbers and number of hatchlings) of *L. mauritii* and all values represent as Mean ± S.E. with different superscripts (a-c) within column shows significance value (p≤0.05) in different treated concentrations .

Conc $mgkg^{-1}$	Reproduction parameters		
	No of cocoons per earthworm	Weight of cocoons (mg)	No of hatchlings per cocoons
0	5.33 ± 0.33^{c}	14.36 ± 0.31^{c}	7.33 ± 0.33^{d}
1000	4.66 ± 0.33^{bc}	13.89 ± 0.47^{c}	6 ± 0.57^{cd}
4000	4 ± 0.57^{abc}	12.40 ± 1.27^{bc}	4.33 ± 0.33^{bc}
8000	3 ± 0.57^{ab}	8.23 ± 1.39^{ab}	2.66 ± 0.88^{ab}
16000	2 ± 0.57^{a}	6.63 ± 1.59^{a}	1.66 ± 0.33^{a}

6.4 Antioxidant activity of exotic (*E. fetida*) and indigenous (*L. mauritii*) species of earthworms.

Different types of antioxidant enzymes (SOD, CAT, GST and POD) of both species of earthworm were studied after exposure of polypropylene at different concentrations on different days.

6.4.1 Oxidative stress towards MPs exposure on exotic species (*E. fetida*) of earthworms

Different enzymes of *E. fetida* produce oxidative stress after exposure of polypropylene. A graphical representation depicts the changes in SOD, CAT, GST

and POD antioxidant enzymes activity in *E. fetida* after exposure of different concentrations of MPs at different days.

6.4.1.1 Effect of polypropylene on SOD

The SOD activity of *E. fetida* increased initially as compared to control with increase in concentration and then decrease in activity were observed with increase in concentrations. At 7th day of experiment, the enzymatic activity increased ($p \leq 0.05$) in all concentrations as compared to control but at 14th day, SOD activity increased in all concentrations except 16000 mgkg^{-1} concentration (Fig 6.12a). On 21st and 28th day, the SOD activity increases as compared to control but decreased at high concentrations i.e. 8000 and 16000 mgkg^{-1}.

6.4.1.2 Effect of polypropylene on CAT

The change in CAT antioxidant enzyme activities at different days and different concentrations showed in (Fig 6.12b). During the initial day of MP exposure the CAT activity of *E. fetida* was increased with increase in concentrations but decreased with concentration and days ($p < 0.05$).

6.4.1.3 Effect of polypropylene on GST

The GST enzyme activities of *E. fetida* at different concentrations were illustrated in Fig 6.12c and significantly ($p<0.05$) increased up to 14th days as compared to control. GST enzymatic activity is greatly influenced at highest concentration (8000 and 16000 mg kg^{-1}) on 21st day. At highest concentration (16000 mgkg^{-1}), the enzymatic activity is lower as compared to control, whereas at 8000 mg kg^{-1} concentration the enzymatic activity increased relatively to control. GST activity level decreased at 16000 mgkg^{-1} concentration as compared to control.

6.4.1.4 Effect of polypropylene on POD

All the changes occur in the POD activity of *E. fetida* shows in Fig 6.12d.The figure depicts the variations occur in the POD activity of *E. fetida* as compared to control and the enzymatic activity significantly increased with increase in concentrations (1000, 4000, 8000, 16000 mg kg^{-1}) up to 28 days.

Antioxidant enzymes (SOD, CAT and POD) and detoxifying enzyme (GST) plays an important role in eradicating the extreme level of reactive oxygen species (ROS) in earthworm and these enzymes recognize as effective signal of microplastics (Liang et al., 2017). SOD plays an important role in antioxidant defence system by scavenging

the superoxide anions; convert O_2^- into H_2O_2 (peroxide), O_2 (oxygen) and produce oxidative stress to the organisms (Liu et al., 2012; Jia et al., 2014). In the present study, SOD activity significantly inhibited with increase in concentrations and exposure time. PP exposure towards *E. fetida* resulted in accumulation of ROS and act as first line of defense opposed to ROS. Excess amount of ROS produced by the earthworm body diminish antioxidant defence system and produce direct influence on structure and synthesis of antioxidant enzymes (Yang et al., 2016). In previous findings, SOD activity of *E. fetida* was significantly ($p<0.05$) enhanced in all polystyrene (PS) microplastic concentration but strongly inhibit the enzymatic activity at 1000µg kg^{-1} concentration (Jiang et al., 2020).

CAT is a tetrameric heme-containing antioxidant enzyme and plays a crucial role in the conversion of H_2O_2 to H_2O (water) and oxygen (O_2) and act as second line of defence against ROS. In this study the CAT activity of *E. fetida* at high concentration (8000, 16000 $mgkg^{-1}$) decreased on 21st and 28th day as compared to control. The decreased in the CAT activity for long exposure may be due to changes in the synthesis and subunits of enzyme (Liu et al., 2011). Chen et al, (2020d) reported the biochemical activity of low density polyethylene (LDPE) on *E. fetida.* CAT activity significantly reduced at 21st day of microplastics exposure and at 28th day the enzymatic activity increased at highest concentrations (0.25, 0.5, 1.0, 1.5 g kg^{-1}). GST is a detoxification enzyme and act as a biomarker to diminish DNA damage and scavenge the activity of lipid peroxidation. In the present study, the enzymatic activity significantly enhanced on 21st day at concentration 4000 and 8000 $mgkg^{-1}$. At 28th day the GST activity of earthworm significantly decreased at highest concentration as compared to control. Similar findings reported by Li et al, (2021) with the treatment of LDPE and PP microplastics resulted in decrease in GST activity of *E. fetida* at 14th and 28th day as compared to control. Another study revealed the decreasing trend in GST activity of *E. fetida* against two different types of microplastics (polystyrene and polyethylene) (Wang et al., 2019b). POD is one of most important enzyme that plays a key role in hydrolysis of compounds by adopting H_2O_2 as a electron acceptor and convert to H_2O. The present study revealed the exposure of polypropylene on earthworm POD activity on 7, 14 21 and 28 days at different concentrations (1000, 4000, 8000, 16000 $mgkg^{-1}$). In comparison to the control, POD activity increases with increasing PP concentration and exposure time. Previous study showed (Wang et al.,

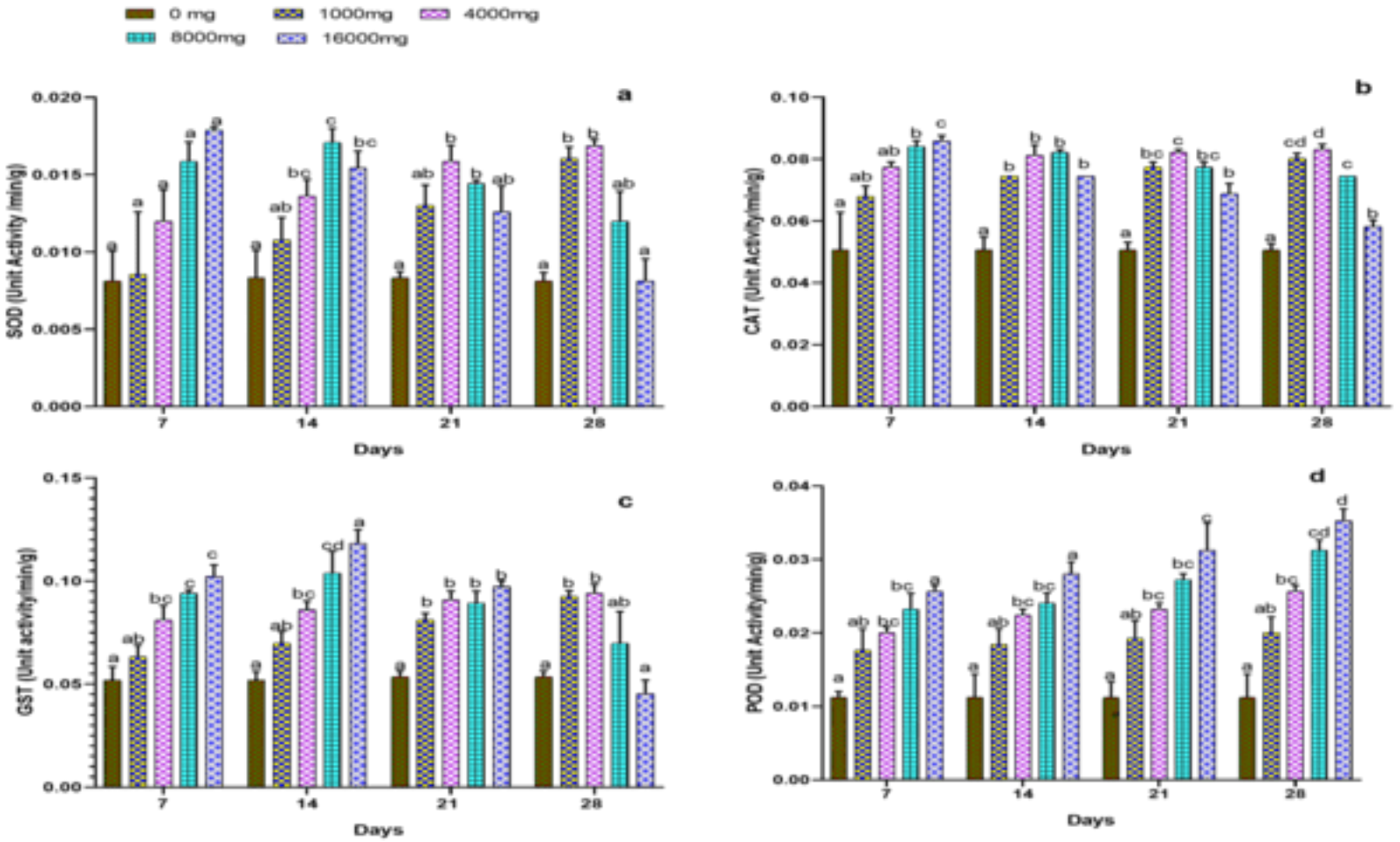


Fig 6.12 Changes in antioxidant enzymes (a) SOD, (b) CAT, (c) GST and (d) POD activities of *E. fetida* towards different polypropylene concentrations at different days and all values are presented as Mean ± S.E. Different superscript (a-d) within column shows significant difference ($p < 0.05$) between different concentration.

2019b) significant increase in trend of POD activity under polystyrene (PS) and polyethylene (PE).

6.4.2 Oxidative stress towards MPs exposure on indigenous species of earthworms *L. mauritii*

The oxidative stress changes occur under the influence of PP microplastic exposure in different oxidative markers such as SOD, CAT, GST and POD.

6.4.2.1 Effect of polypropylene on SOD activity of *L. mauritii*

SOD activity on 7^{th} and 14^{th} day was initially increased with increase in concentration of microplastic (Fig 6.13a) as compared to control but on 21^{st} and 28^{th} day as compared to control (without microplastic), the activity of SOD increased at 1000 and 4000 $mgkg^{-1}$ concentration and significantly decreased ($p<0.05$) at concentration 8000 and16000 $mgKg^{-1}$.

6.4.2.2 Effect of polypropylene on CAT activity of *L. mauritii*

The CAT activity (Fig 6.13b) of *L. mauritii* at different concentrations on day 7^{th} and 14^{th} increased with increase in concentrations with contrast to control but on day 21^{st} and 28^{th} the CAT enzyme shows increment upto concentration 4000 $mgKg^{-1}$ and at concentration 8000 and 16000 $mgKg^{-1}$the activity greatly reduced ($p<0.05$).

6.4.2.3 Effect of polypropylene on GST activity of *L. mauritii*

The GST enzymatic activity (Fig 6.13c) of *L. mauritii* on day 7^{th} significantly increased ($p < 0.05$) with increase in concentrations of PP but on day 14^{th} the enzyme also possess a similar activity as on 7^{th} day. On day 21^{st} and 28^{th}, the GST enzyme activity possess changes at high concentration (8000, 16000 $mgkg^{-1}$) as compared to 7^{th} and 14^{th} day activity. On day 21^{st} and 28^{th} day, the activity greatly increased at concentration (1000, 4000 $mgkg^{-1}$) but PP treatments highly alters the GST activity at high concentrations (8000, 16000 $mgkg^{-1}$) when compared with control.

6.6.2.4 Effect of polypropylene on POD activity of *L. mauritii*

POD level of *L. mauritii* (Fig 6.13d) showed increase pattern with increase in different concentrations as well as exposure period. The trend of the present study was consistent with previously reported effect of polystyrene microplastics on different enzymes of earthworms such as SOD, CAT, GST and POD (Liu et al., 2022). The activity of SOD, CAT and GST enzymes increased upto 14 days but activity

decreased with increase in exposure period and increase in microplastics concentration (Liu et al., 2022). The other studies also reported a similar trend during exposure of conventional and biodegradable microplastics for 28 days in yellow and black soil on the different oxidative enzymes of *E. fetida.* The enzymes exhibit initial increase but on 14^{th} days the enzyme activity starts decreased with increase in exposure period (Yu et al., 2022; Zhao et al., 2023).

6.4.3 Molecular docking and binding mode of PP with SOD, CAT, GST and POD

The molecular docking studies were carried on to predict the binding of polypropylene (PP) with enzymes (SOD, POD, CAT, and GST) at catalytic or non-catalytic sites. The docking scores were used to compare the binding affinities of these enzymes for PP.

SOD is an important antioxidant defense mechanism in cells that converts superoxide radicals to oxygen and hydrogen peroxide (Ighodaro and Akinloye, 2018). The docking analysis indicated that there were three potential binding sites for PP binding on Cu/Zn SOD, as indicated in Fig 6.14(i) (Chowdhary et al., 2022).The interaction behavior of PP with SOD enzyme was studied on all of these three available sites (Table 6.10). The site 1, which is located at the junction of the cavity between two subunits of SOD and surrounded by Val 7, Lys 9, Asn 51, Gly 145, Val 146 of Chain A and Cys 6, Val 7, Lys 9, Asn 51, Cys 144, Gly 145, and Val 146 of Chain B amino acids, was found to be a potential binding site for PP with the docking score of -3.694 **(**Fig 6.14(ii)a). The site 2, located near chain B of SOD enzyme and is surrounded by amino acids Ala 1, Leu 104, Ser 105, Gly 106, Glu 107, Ser 109, Ile 111, Arg 113, Ile 149 of Chain A and Ala 1, Leu 104, Ser 105, Gly 106, Ser 109, Ile 111, Arg 113, Ile 149 of Chain b, showed a docking score of -2.322 with PP (Fig 6.14(ii)b). Site 3, located in subunit A and comprising of amino acids Lys 67, Pro 72, Lys 73, Asp 74, Glu 75, Glu 76, Arg 77, Hie 78, exhibited a docking score of -2.591 with PP **(**Fig 6.14(ii)c). The PP showed good interaction with Cu/Zn SOD enzyme and has the potential to modulate its activity significantly. CAT is an essential tetrameric enzyme found in nearly all organisms and the main functions of enzyme is to prevent cells from oxidative damage by converting hydrogen peroxide into water and oxygen (Kirkman and Gaetani, 1984; Chelikani et al., 2004; Glorieux and Calderon, 2017). The site map calculations showed five potential binding sites available for PP binding as indicated in Fig 6.15(i) and the interaction behavior of PP was studied on all the available sites of CAT (Table 6.10).

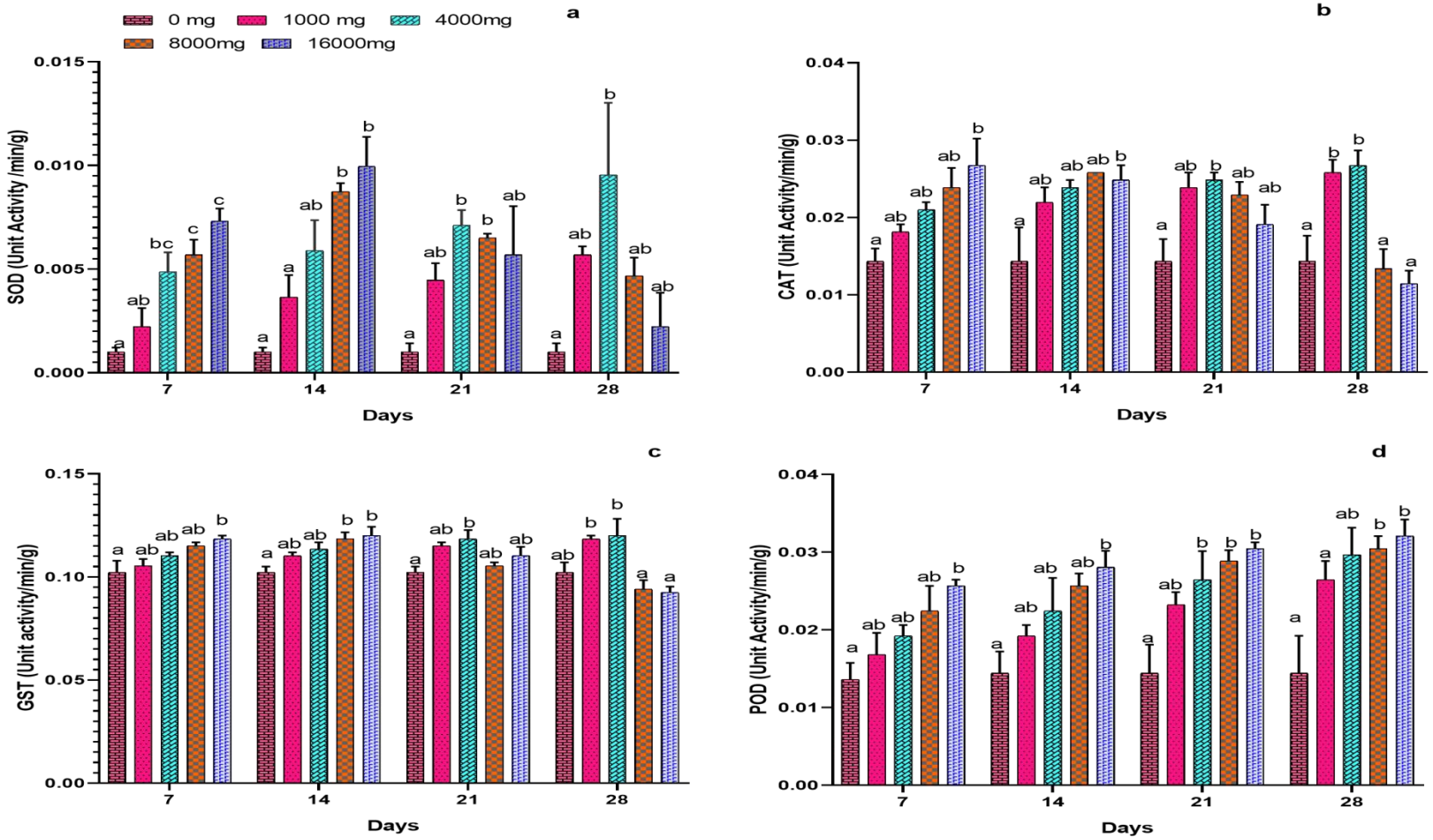


Fig 6.13 Changes in antioxidant enzymes (a) SOD, (b) CAT, (c) GST and (d) POD activities of *L. mauritii* towards different polypropylene concentrations at different days and all values are presented as Mean± S.E. Different superscript (a-c) shows significant difference ($p < 0.05$) between different concentrations.

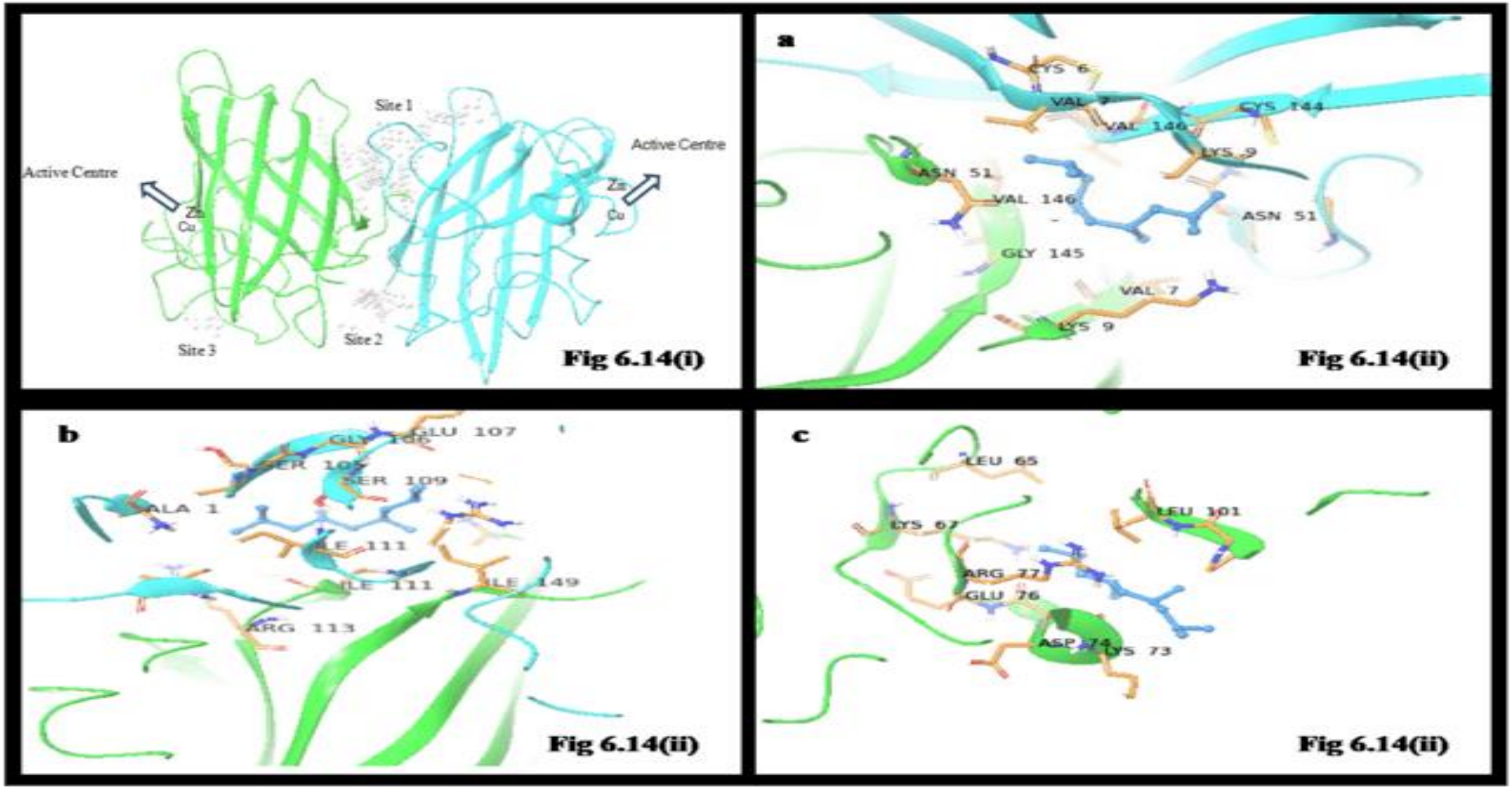


Fig 6.14(i) Potential binding sites available for PP binding in SOD and Fig 6.14(ii) shows the 3D interactions of PP with SOD at (a) Site 1 (b) Site 2 & (c) Site 3.

The Site 1 is surrounded by Asn 268, Pro 367, Leu 370, Gln 371, Val 382, Asn 384, Gln 386, Arg 387, Asp 388, Cys 392, Met 393, Asn 395, Asn 396, and Gln 397 exhibited docking score of -2.735 (Fig 6.15(ii)a).The Site 2 is composed of Thr 114, Ala 116, Gly 117, Ser 121, Ala 122, Val 125, Arg 126, Asp 127, Gln 167, Val 181, Lys 176, Trp 185, Leu 198 and Phe 199 exhibited docking score of -2.964 (Fig 6.15(ii)b). The site 3 flanked by amino acid residues, Val 72, Val 73, Ala 157, Leu 158, Phe 160, His 165, Pro 361, Ile 364, Gly 352, Phe 355, Ala 356, Asp 359, Thr 360 exhibited docking score of -3.239 **(**Fig 6.16(ii)c).The PP displayed highest docking score of -4.00 with site 4 which is surrounded by Ala 7, Ala 78, Gly 79, Ala 80, Asp 258, Leu 261, Arg 262, Leu 264, Phe 265, Asn 320, Pro 321, Tyr 324 and Val 328 (Fig 6.15(ii)d).The site 5 which is near to site 2 is surrounded by Pro 150, Ile 151, His 193, Phe 197, Arg 202, Tyr 214, Lys 236, Thr 444, Phe 445, Val 449 and showed docking score of -3.617 (Fig 6.15(ii)e).

GST enzyme that can combine the tripeptide glutathione with various electrophilic compounds such as carcinogens, xenobiotics and oxidative stress products, resulting in the production of nontoxic compounds (Lushchak, 2012). Site mapping analysis showed that among the five available binding sites on the protein, three of them (Site 2-4) were potential binding sites (Table 6.10 and Fig 6.16(i) (Chowdhary et al., 2022). PP was found to interact with these sites via hydrophobic interactions. Site 1 had a docking score of -2.977 with PP and was surrounded by Asp A143, Leu A146, Ala A149, Lys A147, Val A150, Asp B143, Leu B146, Lys B147 and Val B150 amino acid residues (Fig 6.16(ii)a). Site 2 had a docking score of -4.032 with PP and consisted of Thr 140, Val 137, Ile 136, Ile 134, Gln 133, Lys 178, Leu 179, Thr 182, Asn 186, Leu 190, Ala 191 and Leu 192 amino acid residues in Chain A and Arg 106, Val 107, His 108, Pro 93, and Met 94 in Chain B (Fig 6.16(ii)b). The amino acid residues surrounding Site 3 (Docking score -3.197) were Pro 93, Met 94, Val 107, Hie 108 in Chain A, Leu 132, Gln 133, Ile 136, Val 137, Thr 140, Leu 179, Thr 182, Asn 186, Leu 190, Ala 191 and Leu 192 in Chain B (Fig 6.16(ii)c). Site 4 (Docking score -3.076) was present in subunit B and Site 5 (Docking score -2.411) was present in subunit A, with the lowest binding affinity for PP (Fig 6.16(ii)d-e). Comparing the scores indicated that GST preferentially binds to Site 2 via hydrophobic interactions. POD are enzymes containing heme that are made up of single units and can cause the oxidation of different types of compounds i.e. organic and inorganic by employing hydrogen peroxide (Koua et al., 2009; Demarche et al., 2012).

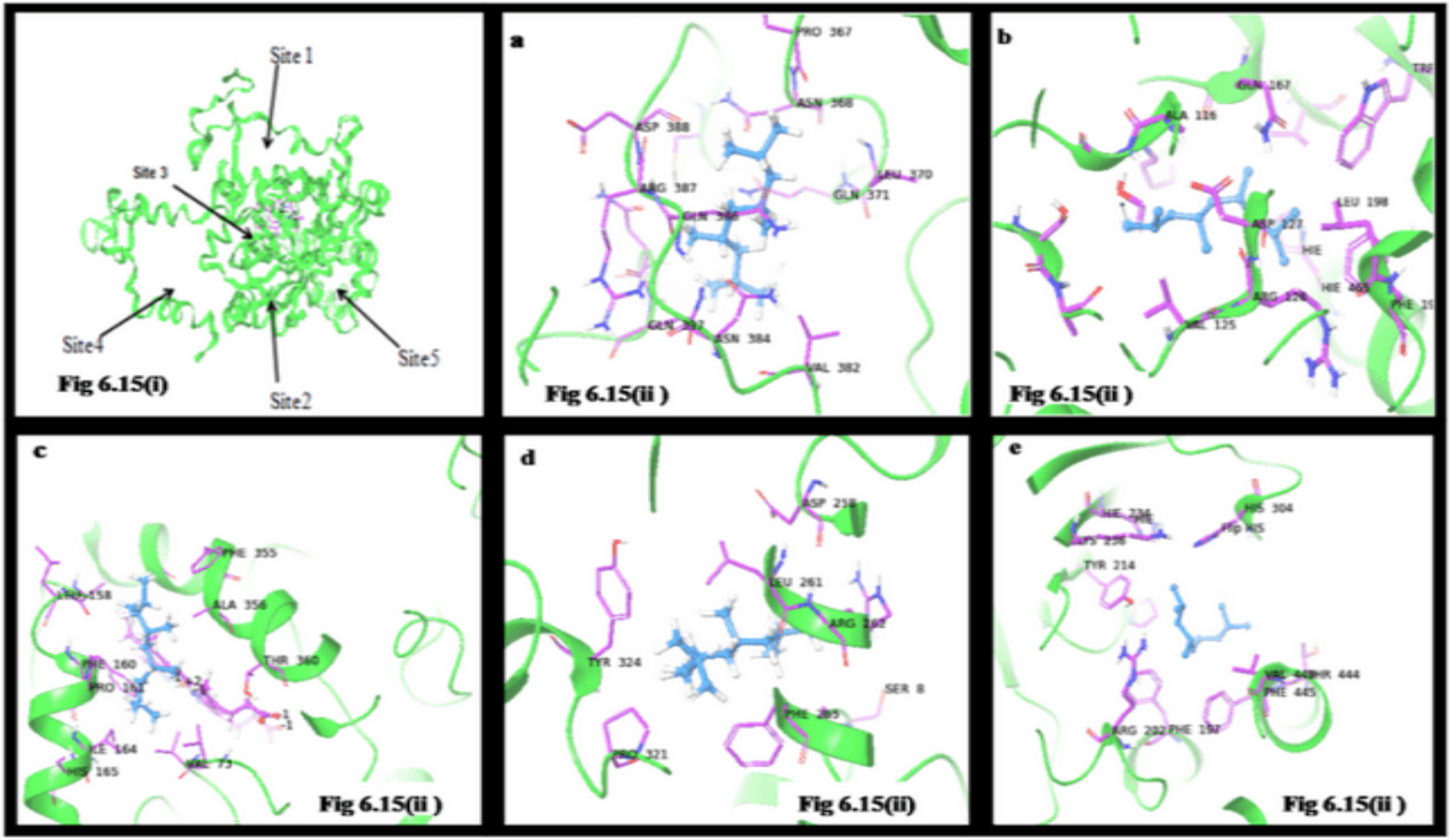


Fig 6.15 (i) Potential binding sites available for PP binding in Catalase and Fig 6.15(ii) shows the 3D interactions of PP with CAT (a) Site 1 (b) Site 2 (c) Site 3 (d) Site 4 (e) Site 5.

Table 6.10 Different molecular interactions of polypropylene microplastic with different enzymes SOD, CAT, GST and POD.

S. No	Protein Target	Site	Dock Score	Backbone Interaction	Aromatic Residues	Side Chain Interactions	Polar Residues	Hydrophobic Residues	Charged Residues
1	SOD	Site 1	-3.694	Val 7, Lys 9, Asn 51, Gly 145, Val 146 of Chain A Cys 6, Val 7, Lys 9, Asn 51, Cys B55, Cys 144, Gly 145, Val 146	Nil	Val A7, Lys A9, Asn A51,Val A146, Val B7,Asn B51,Cys B55, Val B146,Cys B146	Lys A9, Asn A51, Lys B9, Asn B51	Val A7, Val A146,Val B7,Cys B144, Val B146	Lys A9, Asn A51, Lys B9,Asn B51
		Site 2	-2.322	Ala A1, Leu A104, Leu B104, Ser B105, Gly B106, Ser B109	Nil	Ala A1, Ser A109, Ile A111,Ile A149 Ser B105,Arg B113 Ser B109, Ile B149	Ser A109, Ser B105, Ser B109, Arg B113,	Ile A111, Ile B149	ArgB113
		Site 3	-2.591	Pro A72,Lys A73, Asp A74, Glu A75,ARG A77	Nil	Lys A67, Lys A 73 Arg A77,Pro A100,Leu A101	Lys A67, Lys A 73 Arg A77	Pro A100,Leu A101	Lys A67,Lys A73 A75,Arg A77
2	CAT	Site 1	-2.735	Pro A367, Gln A386, Asn A396, Gln A397	Nil	Asn A368, GlnA 371, Asn A 384, Gln A386, Met A 394, Asn A396, Gln A 397	Asn A 368, Gln A371, Asn A384, Gln A386, Asn A396, Gln A 397	Met A 394	Nil
		Site 2	-2.964	Arg A126, Gln A167	Phe A199	Val A125, Asp A127, Gln A167, Trp A185, Phe A199, Hie A 365	Asp A127, Gln A167, Hie A 365	Val A125, Phe A199	Asp A127
		Site 3	-3.239	Ala A157, Pro A161	Phe A160, Phe A355	Ala A157, Phe A160, Pro A161, Phe A355	NIL	Ala A157, Phe A160, Pro A161, Ile A164, Phe A 355	Nil
		Site 4	-4.00	Ala A 78, Gly A79, Phe A265	Phe A265, Tyr A324	Ala A 78, Leu A261, Arg A262, , Phe A265,Pro A321, Tyr A324	Arg A262, Asn A320	Ala A 78, Leu A261, Phe A265,Pro A321, Tyr A324	Asn A262
		Site 5	-3.617	Nil	Tyr A214, Phe A 445	Pro A150, His A193, Phe A197,Tyr A214, Val A 301, His A 304, Phe A445	His A193, His A304	Pro A 150, Tyr A214, Val A301, His A304, Phe A445, Val A 449	Nil
3	GST	Site 1	-2.977	Leu A146,	Nil	Asp A143,Lys A147,	Asp A143,	Leu A146,	Asp A143,

S. No	Protein Target	Site	Dock Score	Backbone Interaction	Aromatic Residues	Side Chain Interactions	Polar Residues	Hydrophobic Residues	Charged Residues
				Lys A147, Asp B143		Leu A146,Val A150, Lys B147, Leu B146,Val B150	Lys A147, Lys B147	Val A150, Leu B146, Val B150	Lys A147, Lys B147
		Site 2	-4.032	Gln A133 Lys A178, Leu A 179 Ala A191 Val A137 Leu A190	Nil	Gln A133,Ile A136, VAl A137, Thr A140, Lys A178, Leu A179, Thr A182, Ala A191, Pro B93, MetB94, Val B107, His B108	Gln A133, Thr A140, Lys A178, Thr A182, His B108	Val A137, Ile A136, Leu A179, Met B94, Val B107	Lys A178
		Site 3	-3.197	Gln B133, Ileu B136, Val B137, Lys B178, Leu B190	Nil	Met A94, Gln B133, Ile B136, Val B137, Lys B178, Leu B179, Thr B182, Asn B186	Gln B133, Thr b140, Lys B178, Thr B182, Asn B186	Ile B136, Val B137, Ala B 191	Lys B178
		Site 4	-3.076	Phe B68, Ala B69, Asn B72, Gln B73, Tyr B75	Tyr B75, Trp B240	Ala B69,Tyr B75, Asp B77, Trp B240, Lys B243	Asp B77, Lys B243	Ala B69, Tyr B75, Trp B240	Asp b77 Lys B
		Site 5	-2.411	Ala A69, Gln A73, Glu A 74, Tyr A75, Asp A77, Pro A236	Tyr A75, Trp A240	Tyr A75, Asp A77 Ala A69, Trp A240 Lys A243	Asp A77, Lys A243	Tyr A75, Ala A69, Trp A240	Nil
4	POD	Site 1	-3.169	Asn A36, PheA37, Pro A46, Asp A98,Thr A99.	Nil	Phe A 37, Gln A39, Pro A46, Ser A45, Lys A49, Asp A98, Thr A99, Ala A102	Gln A39, Lys A 49, Asp A98, Thr A99,	Pro A46, Ala 102	Lys A 49, Asp A98
		Site 2	-2.732	Gln A22, Val A112 ,Ser A113, Asn A143	TRP A26	Gln A22,Val A25,Trp A26, Ser A113, Asp A116,Thr A139, Asn A 143	Gln A22, Ser A113, Asp A116, Thr A139, Asn A 143	Trp A26, VAL A112	Asp A116
		Site 3	-2.859	Gly A131,Ser A132, Pro A133,Asn A266, Glu A267, Gly A270, Gln A271, Ser A 302, Asn A303	Nil	Pro A133, Glu A267, Ser A302, Asn A303	Glu A267, Ser A302	Pro A133	Glu A267

Cont...

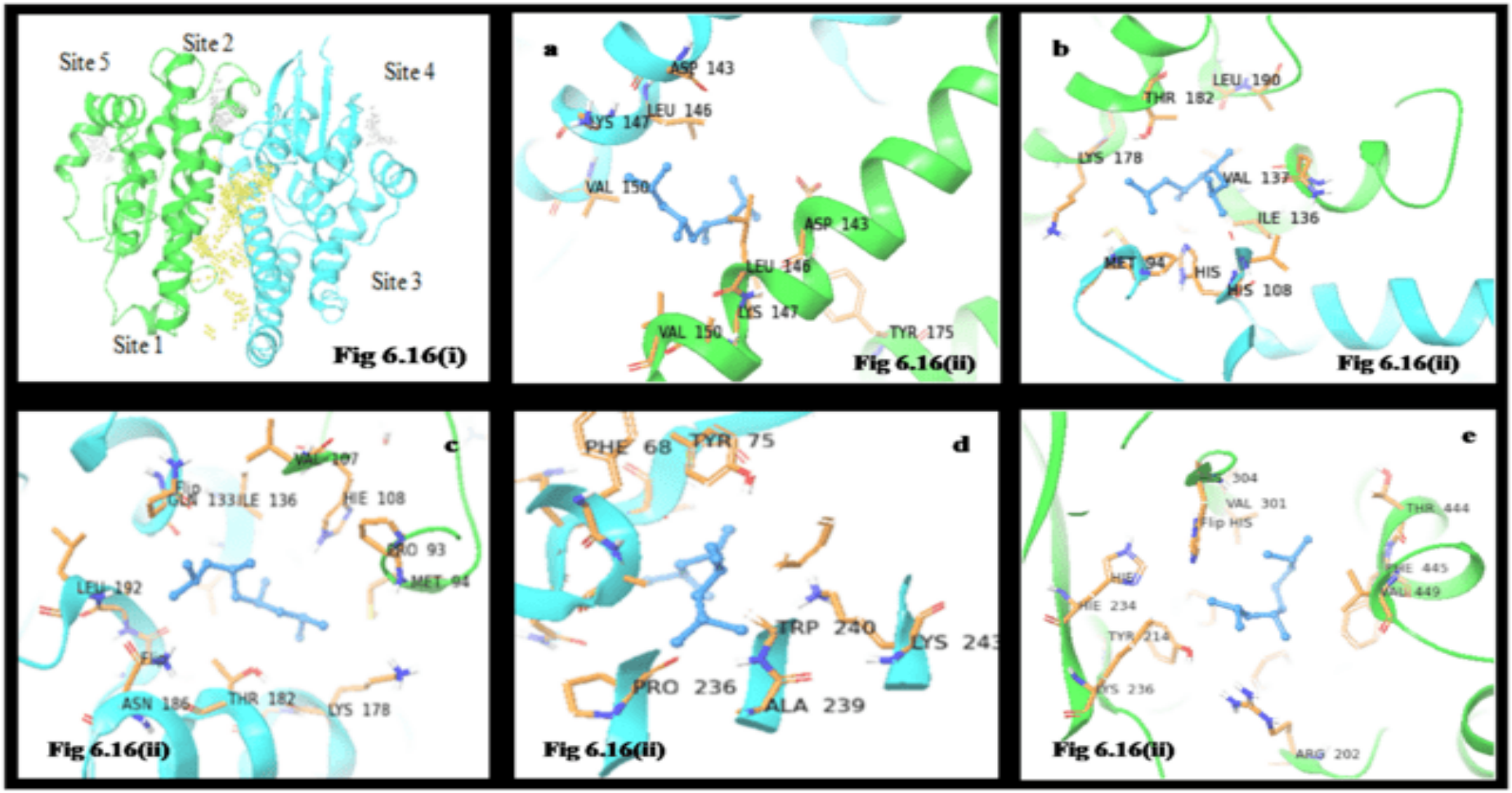


Fig 6.16(i) Potential binding sites available for PP binding in GST and Fig 6.16(ii) shows the 3D interactions of PP at different sites of GST (a) Site 1 (b) Site 2 (c) Site 3 (d) Site 4 (e) Site 5.

Through site mapping calculations Halgren, (2007) reported three potential binding sites for PP binding as seen in Table 6.10 and Fig 6.17(i).The Site 1, which is surrounded by amino acid residues including Asn 36, Phe 37, Gln 39, Ser 45, Pro 46, Lys 49, Asp 98, Thr 99, Ala 102, Arg 198, exhibited a docking score of -3.169 with PP (as shown in Fig 6.17(ii)a). Site 2, located near the heme group of the POD enzyme and surrounded by amino acid residues such as Gln 22, Val 25, Trp 26, Gly 111, Val 112, Ser 113, Asp 116, Thr 139, Gly 140, Arg 141, Ser 142, and Asn 143 displayed a docking score of -2.732 with PP (as displayed in Fig 6.17(ii)b).Site 3, which is surrounded by specific amino acid residues such as Gly 131, Ser 132, Pro 133, Arg 134, Phe 208, Asn 266, Glu 267, Met 269, Gly 270, Gln 271, Arg 274, Ala 300, Val 301, Ser 302, Asn 303, and Asn 304 showed a docking score of -2.859 with PP (Fig 6.17(ii)c).Overall, the docking results indicated that POD has a relatively weak binding affinity for PP.

The molecular docking analyses were conducted to gain insights into the observed experimental activities of PP and their potential binding interactions with SOD, CAT, GST and POD enzymes using Glide suite of Schrodinger software. The docking analysis with SOD enzyme (Omar et al., 1992; Valdivia et al., 2006) revealed that PP exhibited the highest docking score of -3.694 at the junction cavity among two subunits of superoxide dismutase (SOD). This suggests that preferentially binding of PP at the junction cavity either than the active sites or center through hydrophobic interactions, inhibiting the enzymes function. The site map analysis on CAT revealed five potential binding sites for PP with the highest docking score observed at site 4 (docking score -4.00) of CAT and PP stabilized by hydrophobic contacts (Corbo et al., 2022). Further, the docking analysis with GST showed that out of the 5 available binding sites, PP preferably interacts with site 2 at the junction of GST (docking score -4.032) through hydrophobic interactions, indicating excellent binding interactions with GST (Latif et al., 2018). Additionally, the docking studies with POD indicated that PP showed a higher docking score at site 1 and was stabilized by hydrophobic contacts. Moreover, the docking analysis suggested that POD has appreciable affinity for PP. Although PP did not form any hydrogen bond interactions with any of the four studied enzymes, it was found to be stabilized in the cavity of the receptors through hydrophobic interactions. These hydrophobic interactions of PP with amino acid residues of the receptors induced structural changes in all four studied enzymes, resulting in inhibition of their activity. These findings are consistent with our experimental observations.

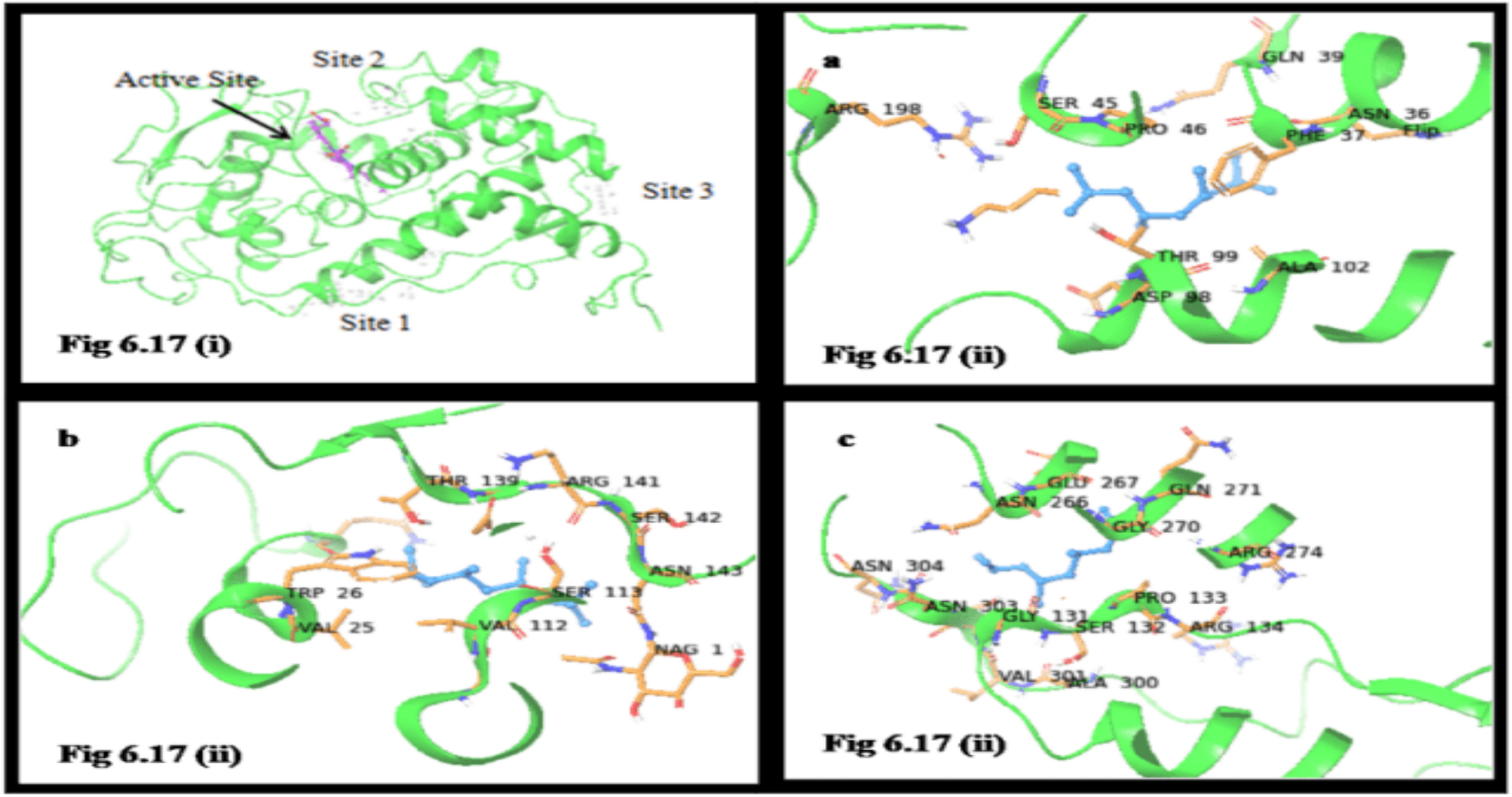


Fig.6.17 (i) Potential binding sites available for PP binding in POD and Fig 6.17 (ii) shows the 3D interactions of PP at (a) Site 1 (b) Site 2& (c) Site 3of POD.

6.5 Identification of different types of degrading microplastic gut microorganisms of *E. fetida* and *L. mauritii*.

6.5.1 Bacterial diversity of earthworms at taxonomic and functional levels in Control and treated group of polypropylene.

Analyzing the gut-microbial communities of control and microplastic exposed earthworms, approximately 2,736,216 high-quality reads were obtained, which resulted in 95,403 OTUs, at 97% similarity thresholds. The observed α-diversity metrics such as Chao1, ACE, Shannon, Simpson, Simpson_e, and Pielous are given in Table 6.11. The Good's coverage was much higher for all the samples (> 0.99), which shows a good sequencing depth and suggests that extreme diversity of earthworms gut was covered. In this study, the most relevant number of earthworms gut OTUs (32, 641) was observed in the control sample, when compared to the treatment groups (*E. fetida* (31,621), *L. mauritii* (31,141) (Table 6.11). Likewise, the maximum abundance of species; Chao1 (Cnt = 3721, *E. fetida* = 4331; *L. mauritii* = 4121); ACE (Cnt = 2971, *E. fetida* = 3611; *L. mauritii* = 3421); species richness in terms of Shannon (Cnt = 2.87, *E. fetida* = 4.02; *L. mauritii* = 3.92) and Simpson (Cnt = 0.96; *E. fetida* =0.97 and *L. maurtii* = 0.97) were measured for both without polypropylene (control) and polypropylene exposure groups (Table 6.11).The results of present study directly indicated that the control samples harbour more diversity in comparison to the treatment groups; moreover the abundance of bacterial species was higher in polypropylene treated groups.The β-diversity between the control and treated groups was measured in terms of Bray-Curtis and Jaccard index and the results showed a clear separation or distance between the control and treated group (Table 6.12 and Table 6.13).The present study reported that the microplastic treated earthworm species have small distance compared to the control (Table 6.12 and 6.13).

Relative abundance and taxonomic distribution of major phyla of earthworm gut are given in (Fig 6.18, 6.19 and 6.20). Earthworms gut microbial community shows a significant difference at phylum level in relative abundance of different gut bacterial groups between control (Cnt) and the treated groups (*E. fetida* and *L. mauritii*) (Fig 6.18).The overall phylogenetic composition of the bacterial communities (top fourteen) at phylum level across all the groups showed a highly relative abundance of different groups such as Proteobacteria (32.6 - 35.4 %), Chlamydiae (15.4-15.6%), Firmicutes (12- 14.2%), TM6 (7.4-11%), Planctomycetes (7.1-9%), Actinobacteria (5.6-7.1%), Gemmatiomonadetes (3.3-4.7%), Veerucomicrobia (3-3.9%),

Saccharibacteria_TM7 (0.9-2.1), Acidobacteria (0.6-0.9%), Cyanobacteria (0.02-0.22%), Chloroflexi (0.01-0.12%), Bacteroidetes (0.01-0.10%), Tenericutes (0.01-0.06%). The dominant classes were Gammaproteobacteria (19.3 -24.4%), Chlamydiae (11.4- 14.7%), TM6 (7.9-15.6%), Bacilli, (7.4-10.9%), Planctomycetia (9.4-10.3%),

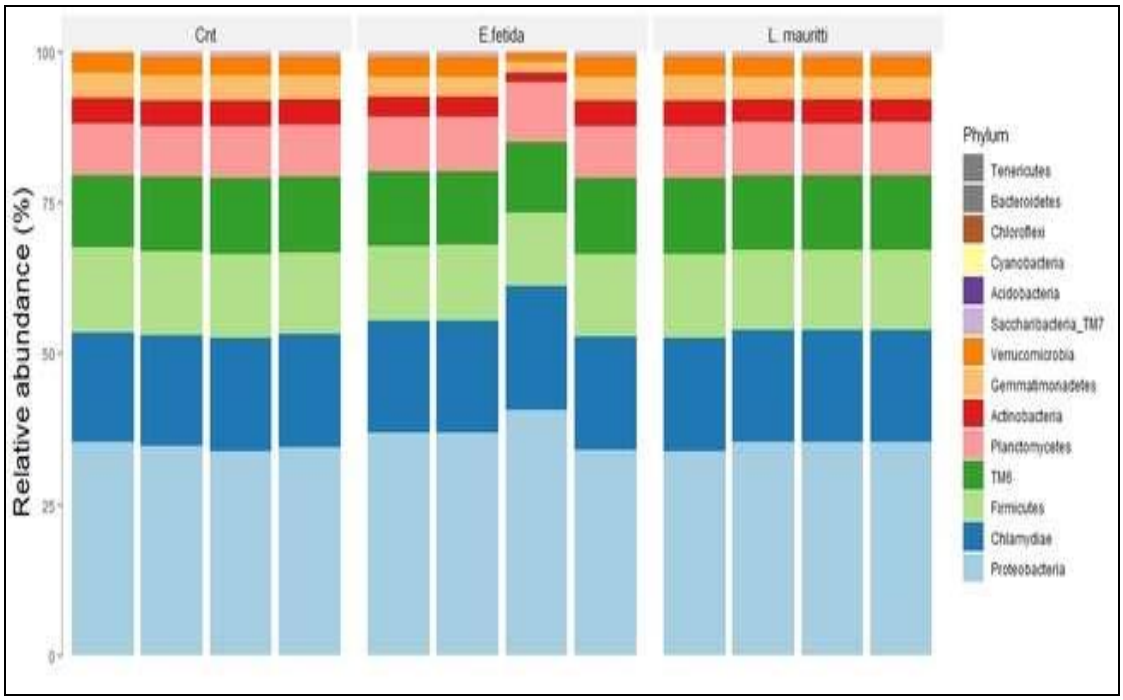


Fig 6.18 Taxonomic distribution and the relative abundance of major phylum after treatment of polypropylene microplastic in *E. fetida and L. mauritii.*

Alphaproteobacteria (6.3-12%), Clostridia (4.5-8.5%), Acidimicrobiia (3.4-8.6%), Longimicrobia (0.6-3.1%), Spartobacteria (0.7-2.3%), Oligoflexia (0.1-0.3%), Saccharimonas (0.04-0.13%), PAC002280_c (0.05-0.10%), Actinobacteria (0.02-0.13%), Vampirovibrioc (0.01-0.13%) and others (0.01-0.12%) (Fig 6.19). The relative division of the expressive bacterial genus was found as *Pantoea* (6.3-8.10%), *Pseudomonas* (2.7-5.99%), *Streptococcus* (2.5-4.89%), *Aquicella* (2.5-3.7%), *Planctomicrobium* (1.7-4.7%), *Protochlamydia* (1.7-3.4%), *Parachlamydiaceae* (2.1-2.98%), DQ129127 (1.5-3.19%), *Alcanivorax* (1.7-2.98%), *Bacillus* (1.5-2.98%), *Hyphomicrobium* (1.5-2.98%), *Catonella* (0.6-3.9%), EU363464 (0.6-3.03%), EF540396 (0.71-2.34%), EF51641 (0.5-2.68%) (Fig 6.20). From the results, it is clear that a change or shift in the relative abundance of bacterial group was measured across the control and polypropylene treatment groups, with relative high distribution and relative abundance of the peculiar group of bacteria in the exposed earthworm species (Table 6.14).

Table 6.11 Estimated alpha-diversity indices of control and microplastic exposed earthworm species (*E. fetida* and *L. mauritii*).

Group	Alpha diversity Indices					
	Observed OTUs	**Chao1**	**Shannon**	**Simpson**	**ACE**	**Goods Coverage**
Control	32641	372	2.87	0.96	297	0.99
E. fetida	31621	433	4.02	0.97	361	0.99
L. mauritii	31141	412	3.92	0.97	342	0.99

Table 6.12 Beta-diversity based on Bray-Curtis dissimilarity distances between the control and treatment groups.

	Control	***E. fetida***	***L. mauritii***
Control	0	0.3	0.79
E. fetida	0.3	0	0.81
L. mauritii	0.79	0.81	0

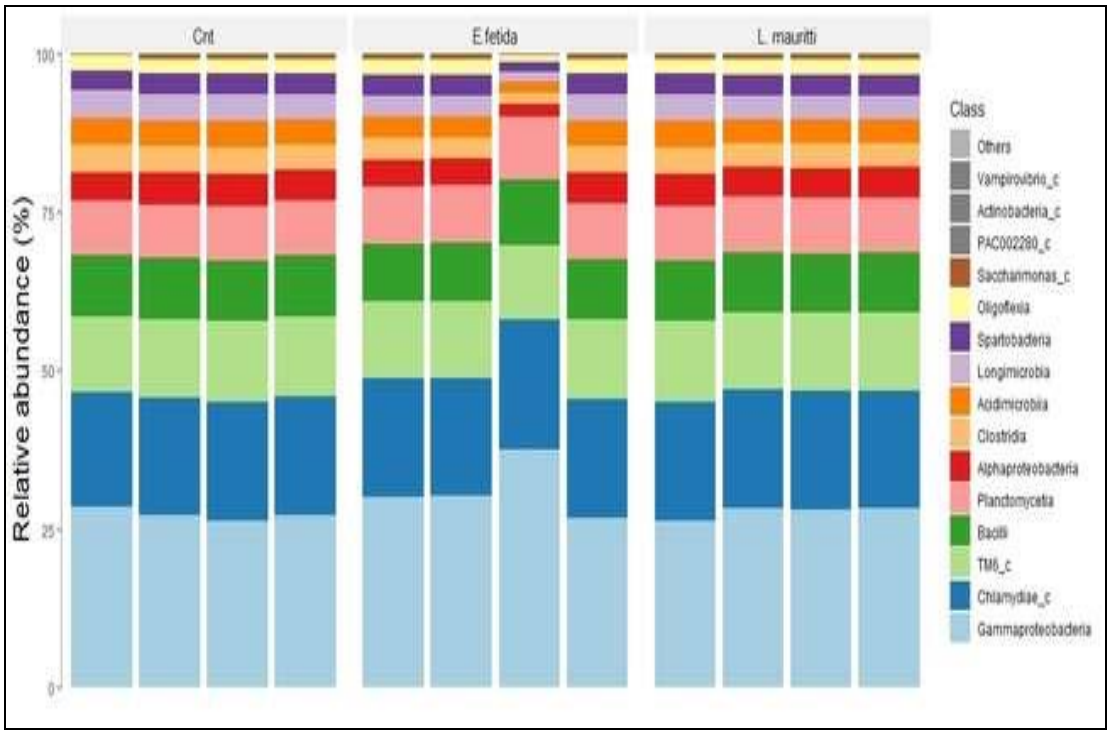


Fig 6.19 Taxonomic distribution and the relative abundance of *E. fetida* and *L. mauritii* bacteria at class level after treatment of polypropylene microplastic.

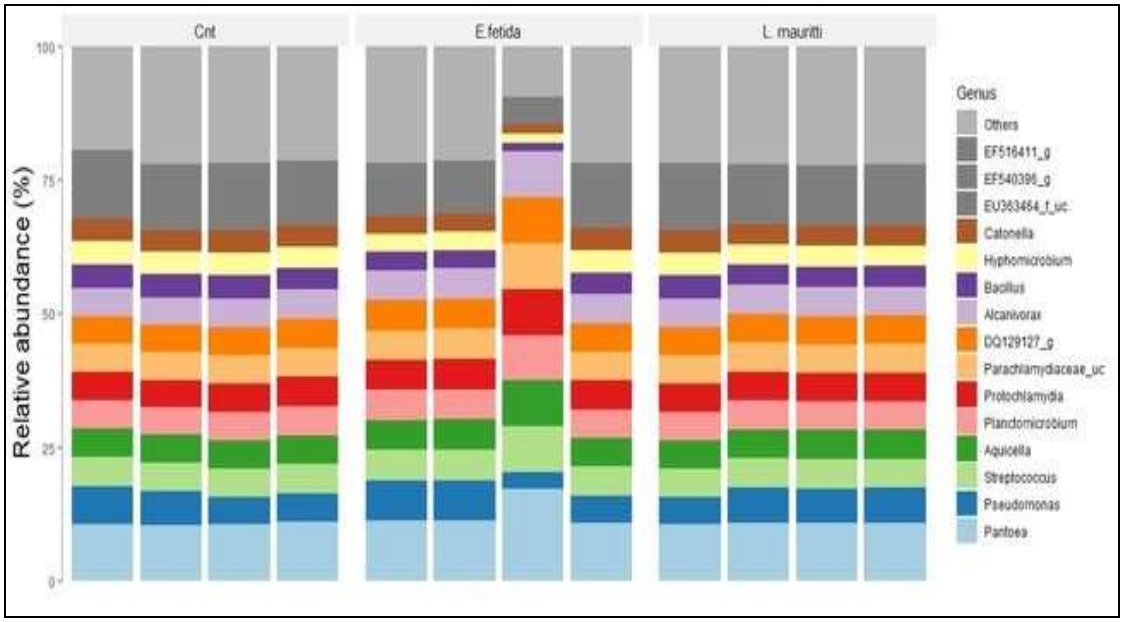


Fig 6.20 Taxonomic distribution and the relative abundance of *E. fetida* and *L. mauritii* bacteria at genus level after treatment of polypropylene microplastic.

Table 6.13 Beta-diversity based on based on Jaccard-dissimilarity distance between the control and treatment groups.

	Control	***E. fetida***	***L. mauritii***
Control	0	0.19	0.2
E.fetida	0.19	0	0.25
L. mauritii	0.2	0.25	0

6.5.2 Predicted pathways of bacteria communities in control and exposed earthworm species.

For functional analysis, the taxonomic delineation obtained from QIIME2 were subjected to Picrust2 (Douglas et al., 2020) investigation for functional predictions to estimate the metabolic potential of the earthworm bacterial communities and to identify the functional characters that were differentially abundant in different microbial communities. The predicted genes were further categorized using KEGG orthologs (KOs) which were obtained from the KEGG (Kyoto Encyclopedia of Genes and Genomes Orthology) database and classified further (Kanehsia et al., 2016). When compared with control group, it was found that the exposed earthworms have a

much larger abundance of genes. For example, metabolism, environmental information processing, genetic information, cellular processes and organismal systems were found higher in exposed worms than in control (Fig 6.21). Moreover, the other relative abundance of genes allied with metabolism of different biological molecules (such as carbohydrate, amino-acids, energy, nucleotides, co-factors and vitamins), xenobiotics biodegradation and metabolism, signalling and interactions, environmental adaptation transport and catabolism, degradation and sorting, etc were found higher in exposed earthworms than in control (Fig 6.22). In exposed worms, the growth and survival of microbial communities are totally dependent upon the expression of genes. Moreover, the gut-microbiota analysis of earthworm exposed to microplastic resulted in the discovery of various genes encoding enzymes involved in the degradative pathway of different pollutants including microplastics (Fig 6.23).

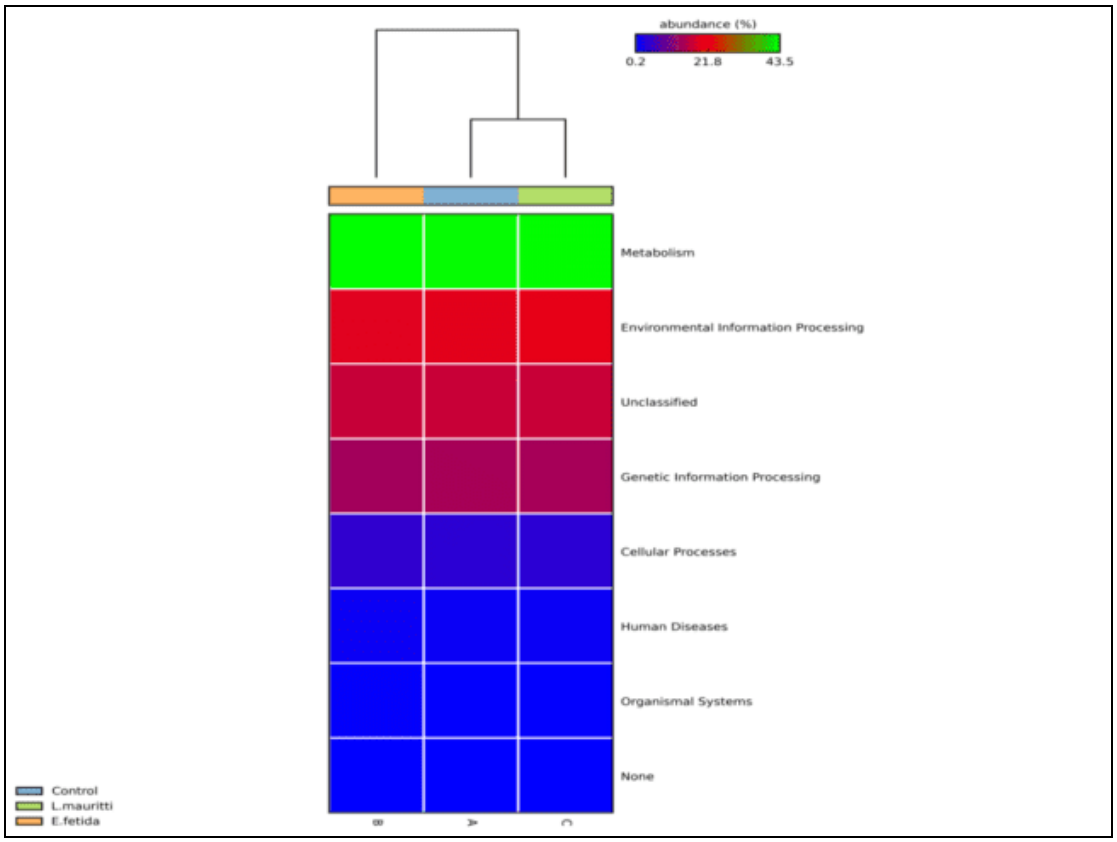


Fig 6.21 Depicts the heat-map of different Clusters of Orthologous Gene (COG) of three analyzed samples (Control, *E.fetida* and *L. mauritii*) and colour strength of each panel represent the abundance or plenitude of degradation pathway.

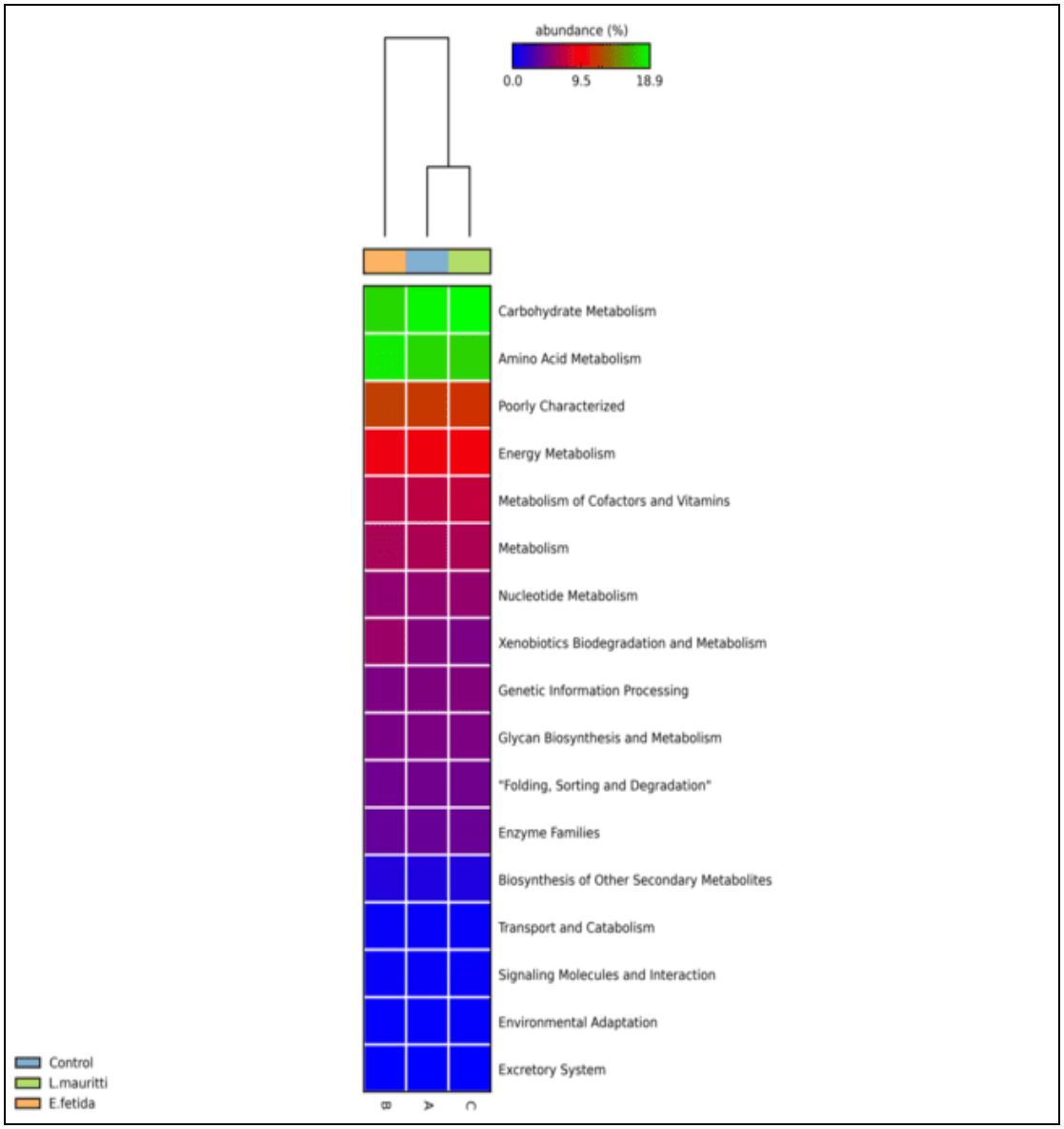


Fig 6.22 Heat-Map of bacterial degradation pathway in all three samples and colour intensity represents the relative abundance of gene associated metabolism in degradation pathway.Top box referring as colour key.

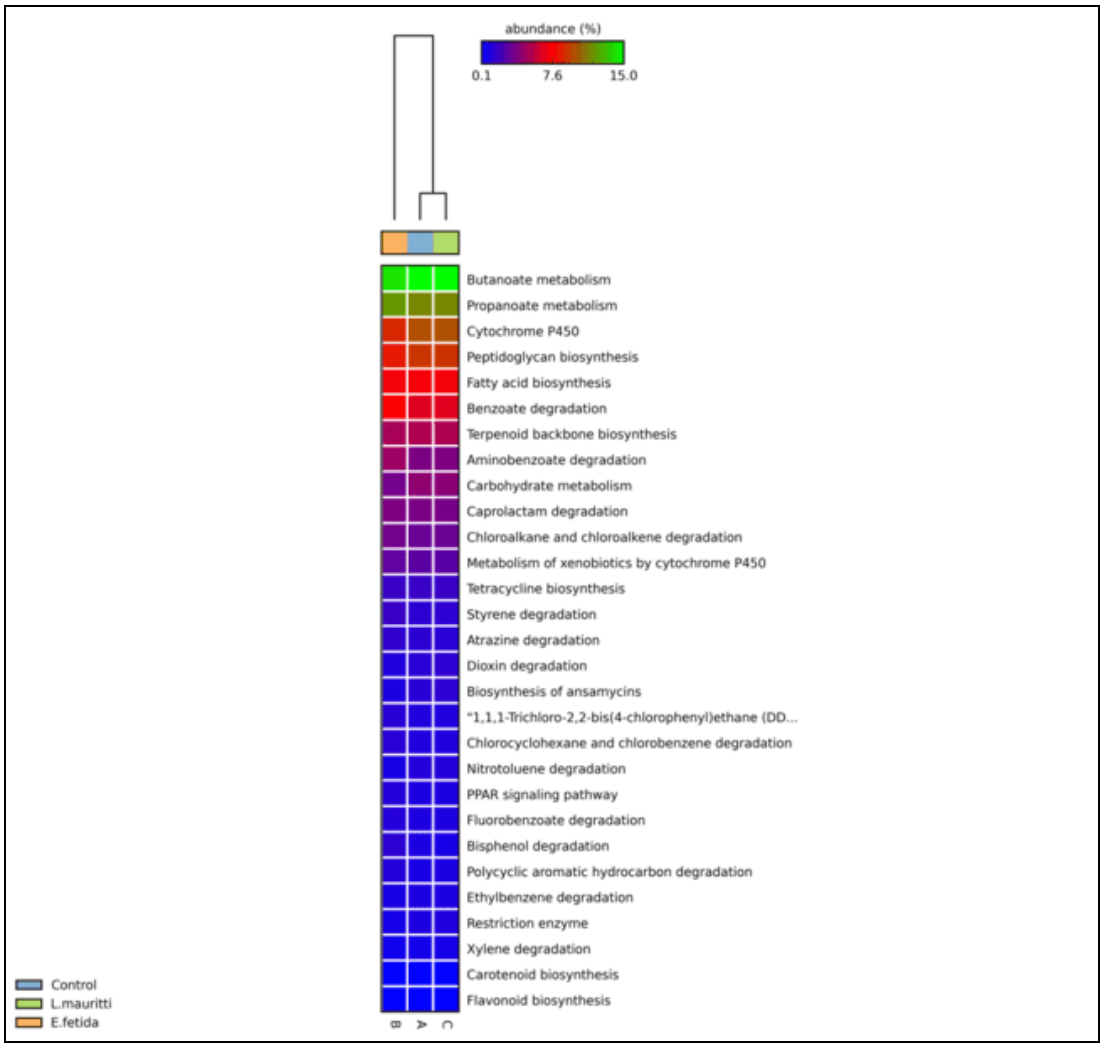


Fig 6.23 Heat-Map of bacterial degradation pathway in all three samples and colour intensity represents the relative abundance of gene associated enzymes metabolism in degradation pathway.Top box referring as colour key.

Table 6.14 Comparison of top fourteen bacterial phylotypes from control and microplastic treated earthworms.

Phylogeny	Relative abundance (%)		
	Control	***E. fetida***	***L. mauritii***
Pantoea	6.3184	7.1126	8.104
Pseudomonas	2.7745	5.5687	5.9953
Streptococcus	3.5384	4.8529	3.9973
Aquicella	2.5438	3.7019	3.0528
Planctomicrobium	1.7245	3.1955	4.7368
Protochlamydia	1.7385	3.4376	3.2986
Parachlamydiaceae_uc	2.1483	2.9191	2.9842
DQ12912	1.5303	2.9476	3.1997
Alcanivorax	1.7451	2.919	2.9842
Bacillus	1.5226	2.919	2.9837
Hyphomicrobium	1.5226	2.9187	2.9836
Catonella	0.6754	2.1164	3.9007
EU363464	0.6759	2.1166	3.0397
EF540396	0.7097	2.1167	2.3405
EF516411	0.5006	2.6874	0.6759

Earthworm gut microorganisms recreate a crucial role in the metabolism (carbohydrate, amino acids etc), immunity and provide defence mechanism to pathogen.The alpha and beta community of different microorganisms in the gut is balanced but after the exposure of organic pollutants such pesticides, microplastics etc the stability of bacterial diversity might be disrupted. The disruption of bacterial diversity might be due to harmful and toxic components such plasticizers, additives etc. Cheng et al, (2021) observed the effects of two different types of microplastics (HDPE and PP) at concentration 0.25% w/w for 28 days on the relative abundance of microorganisms in the *Metaphire guillelmi* gut and surrounding soil. Microplastics were decreased both richness and diversity of earthworms gut as comparison to surrounding soil. Therefore, there was no significant effect of HDPE and PP on gut of *Metaphire guillelmi* at concentration 0.25% as compare to control the result of this study suggesting HDPE and PP did not alter the gut microbiome of *Metaphire guillelmi*. Adhikari et al., (2023) also demonstrate the effect of LDPE and PBAT microplastics on *L. terrestris* gut community and the dominant bacterial taxa at phylum levels in gut includes Acidobacteria, Bacteriodetes, Actinobacteria, Firmicutes, Proteobacteria, Gemmatimonadetes and Verrumicrobia. The findings of study reveals microplastics produce effects on the relative abundance of microrganisms. Other than earthworm, microplastics also change the gut microbial diversity of soil collembolan (*Folsomia candida*). *Folsomia candida* was exposed with PVC concentration 0.1% w/w in soil for 56 days and result suggests that the alpha diversity was increased as comparison to non-treated organisms (Zhu et al., 2018b). On the contrary, the microbial diversity in the gut of *Folsomia candida* was decreased when treated with polyethylene concentration 0.5% w/w for 28 days (Ju et al., 2019). It is cleared from previous reported studies the effect of different types of microplastics on microbial diversity of organisms totally depends on the types, concentration and exposure time. Our results are corroborated with the findings of reported study by Yu et al, (2022) that polyethylene and polylactic acid microplastics showed no effect on dominant microorganisms of gut but change the relative abundance of different microorganisms. In this study polypropylene exposure causes the changes in the relative abundance of different microorganisms and also produces

moderate effect on the earthworm's alpha and β diversity. Microplastics act as a sole carbon source for microorganisms a nd microorganisms supports the degradation process.The magnitude of the taxonomic distribution of gut microorganisms phyla indicated that microplastics caused shifts in gut wall-associated bacterial community.

6.6 Degradation of microplastic in the gut of earthworm by microorganisms

Different types of aerobic and anaerobic microorganisms were identified in the gut of exotic (*E. fetida*) and indigenous (*L. mauritii*) species of earthworm. These different types of gut microorganisms degrade polypropylene microplastics particles.

6.6.1 Extraction of polypropylene from earthworm cast, degutting of *E. fetida, L. mauritii* and soil.

During 28 days treatment period of different concentration (0, 1000, 4000, 8000 and 16000 $mgkg^{-1}$ sterilized soil) of polypropylene microplastic to both species of earthworm. Cast of earthworms were collected from each tray continuously for 28 days (Fig 6.24) and the collected cast of 28 days were incorporated for extraction of polypropylene to know the abundance of ingested and egested polypropylene particles.Table 6.15 shows the quantity of polypropylene microplastics extracted from 28 days cast of *E. fetida.* Large quantity of polypropylene microplastics were extracted from cast collected from different treatments. From lowest concentration of polypropylene i.e 1000 $mgkg^{-1}$, 43.03 mg of polypropylene microplastics were extracted from cast. The average abundance of polypropylene particles in cast collected from 4000 and 8000 were 172 mg, 234.66 mg respectively and 376 mg polypropylene extracted from cast collected from highest concentration (16000 $mgkg^{-1}$) of polypropylene microplastic.

The abundance of polypropylene microplastics extracted from 28 days casts of *L. mauritii* is shown in Table 6.15. Polypropylene microplastics extracted from cast shows significance level ($p< 0.05$). From initial concentration i.e. 1000 $mgkg^{-1}$, the total quantity of polypropylene in cast were 50.03mg and from highest concentration (16000 $mgkg^{-1}$) cast consists of 368.67 mg of polypropylene particles. Appearance of microplastics in the cast (digested material) proves the ingestion behaviour of both species of earthworm (Wang et al., 2022).

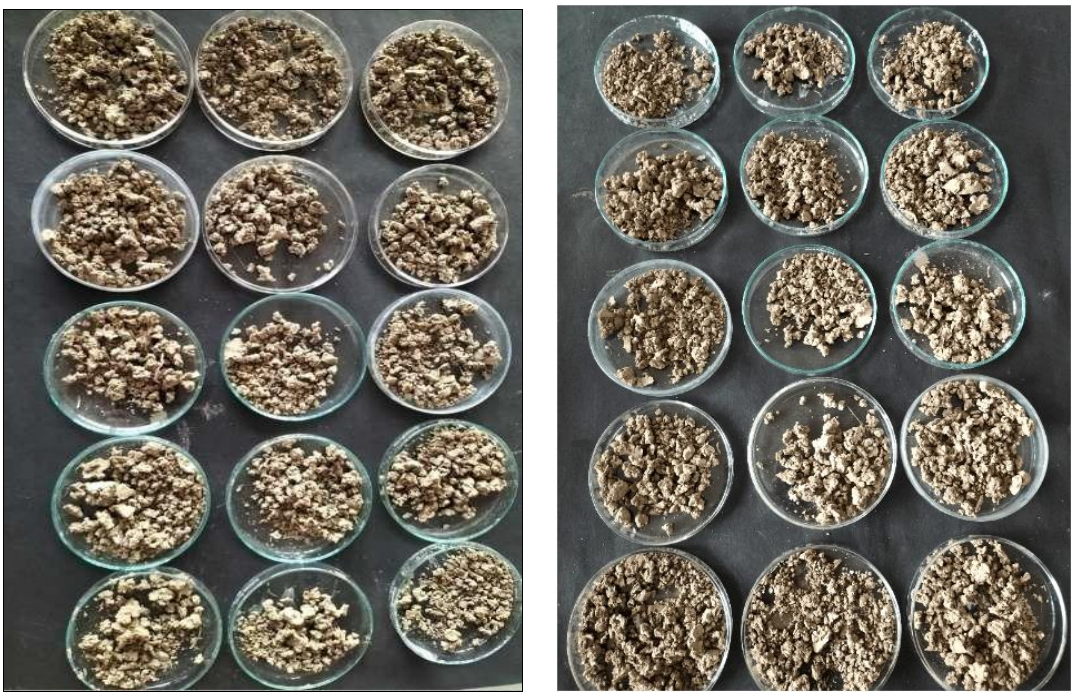


Fig 6.24 Cast collected from both exotic and indigenous species of earthworms during exposure of polypropylene.

Microplastics were also extracted from degutting of both species of earthworms. The degutting content of earthworms was used to estimate the polypropylene microplastics in earthworm gut. Table 6.16 shows the quantity of polypropylene microplastics. Earthworms ingest polypropylene MP together with soil. Similarly polypropylene MP particles also extracted from the soil in which we fed earthworms (*E. fetida* and *L. mauritii*) with PP. Weighs all the extracted MP particles to know the abundance of MP in soil. Table 6.17 shows the abundance of MP extracted from soil treated with earthworm species. This study revealed that the abundance of polypropylene was not high in degutting of both species of earthworms. Our results are corroborated by the findings of Adhikari et al, (2023) and reported that the polypropylene microplastics were ingested through soil and ingested polypropylene microplastics particles passes through the intestine (digestive tract) of earthworm where different gut micro-organisms secrets different types of enzymes to breakdown the microplastic. In comparison with *E. fetida, L. mauritii* ingested or digested large amount of polypropylene microplastics particles in casts.

Table 6.15 Abundance of polypropylene microplastics (mgkg$^{-1)}$ in the casts of both exotic and indigenous species of earthworms. All value represents the Mean ± S.E. with different subscripts (a-c) within column shows significant difference in different concentration of polypropylene.

Conc.(mg kg^{-1})	Abundance of PP in cast (mgkg^{-1}) (Mean ± S.E.)	
	E. fetida	***L. mauritii***
1000	43.03± 15.07^{a}	50.03 ± 11.25^{a}
4000	172 ±81.05^{a}	178.6 ± 75.05^{ab}
8000	234.66 ± 21.86^{ab}	236.33 ± 16.38^{bc}
16000	376 ± 6.11^{b}	368.67 ± 19.88^{c}

Table 6.16 Abundance of polypropylene microplastics in degutting of both exotic and indigenous species of earthworms. All value represents the Mean ± S.E. with different subscripts (a-b) within column shows significant difference ($p<0.05$) in different concentration of polypropylene.

Conc. (mg kg^{-1}	Abundance of PP (mgkg^{-1}) in degutting content (Mean ± S.E)	
	E. fetida	***L. mauritii***
1000	0.03 ± 0.00^{a}	0.07 ± 0.02^{a}
4000	0.06 ± 0.00^{ab}	0.13 ± 0.01^{ab}
8000	0.08 ± 0.01^{ab}	0.16 ± 0.00^{ab}
16000	0.10 ± 0.00^{b}	0.22 ± 0.05^{b}

Table 6.17 Abundance of polypropylene in the soil of both exotic and indigenous species of earthworms. All value represents the Mean ± S.E. with different subscripts (a-b) within column shows significant difference ($p<0.05$) in different concentration of polypropylene.

Conc.(mg kg^{-1})	Abundance of PP (mgkg^{-1}) in culture media (Mean±S.E)	
	E. fetida	***L. mauritii***
1000	885 ± 21.19^{a}	878.1 ± 3.21^{a}
4000	3746.6 ± 60.64^{b}	3732 ± 48.21^{b}
8000	7640 ± 123.60^{c}	7570.1 ± 101.44^{c}
16000	15433 ± 141.65^{d}	15431 ± 92.53^{d}

The total recovery of polypropylene microplastics extracted from different parameters such as cast, degutting of both *E. fetida* and *L. mauritii* and from soil. From each concentration of polypropylene treatment (1000, 4000, 8000 and 16000 mgkg^{-1}). The recovered amount of polypropylene from *E. fetida* were 928.06, 3918.73, 7874.08 and 15808.01 mgkg^{-1}(Fig 6.25 and Table 6.18) and remaining amount of polypropylene were degraded by gut microorganisms. The degradation percentage of *E. fetida* towards polypropylene was 7.2%, 2.03%, 1.57% and 1.2%. Similarly in case of *L. mauritii* total recovered polypropylene microplastic from each concentration were 928.10, 3910.8, 7806.49 and 15798.88 mg respectively and degradation percentage of *L. mauritii* towards microplastics were 7.19%, 2.23%, 2.41% and 1.26% (Fig 6.26 and Table 6.18). Adhikari et al, (2023) also extracted LDPE and PBAT microplastics from earthworm (*L. terrestris*) cast, bulk soil and gut. Twenty five and twenty three particles of LDPE and PBAT respectively extracted from the cast by using stereomicroscope (Meng et al., 2023). Lwanga et al, (2017) degrade 60% LDPE through gut microorganisms of *L. terrestris* under 21 day's exposure period.

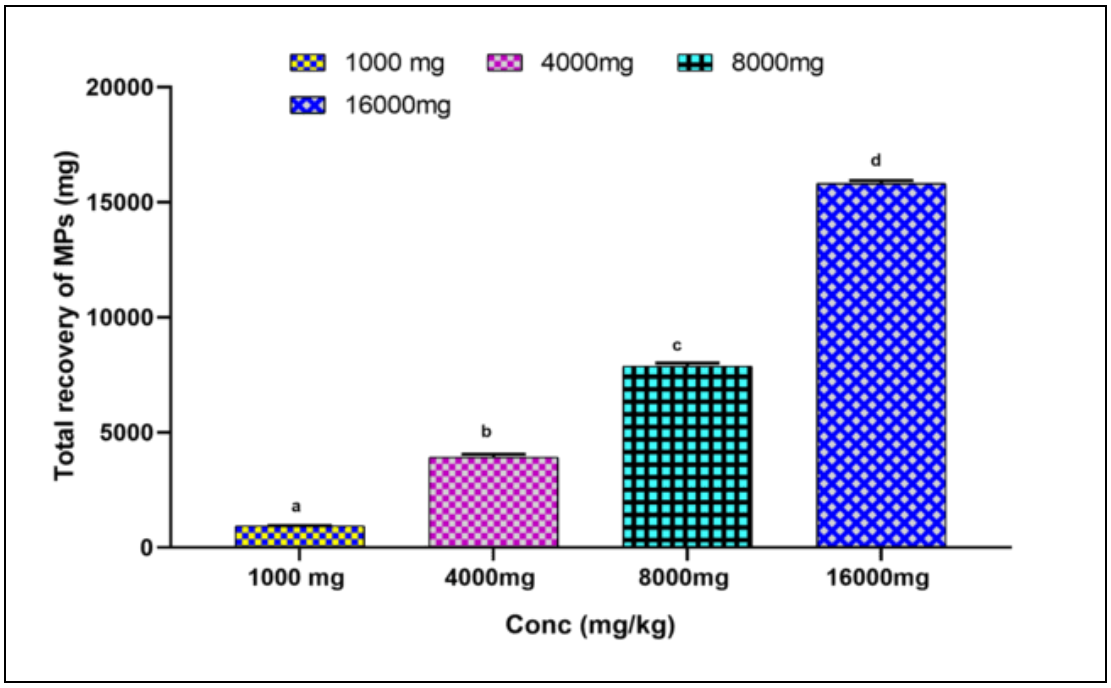


Fig 6.25 Total recovery of polypropylene PP from cast, degutting and soil of *E. fetida*.

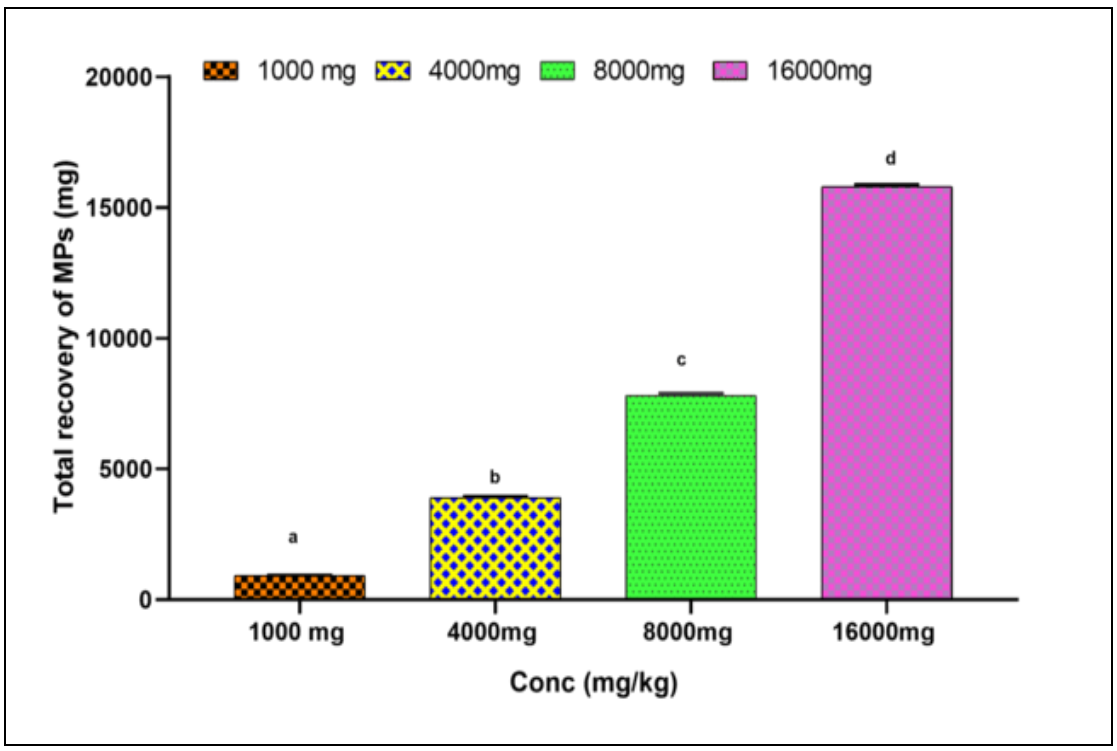


Fig 6.26 Total recovery of polypropylene from cast, degutting and soil of *L. mauritii*.

Table 6.18 Abundance of total recovered polypropylene from the cast, soil and gut of both exotic and indigenous species of earthworms. All value represents the Mean ± S.E. with different subscripts (a-d) within column shows significant difference ($p<0.05$) in different concentration of polypropylene.

Conc. (mg)	Total recovered amount of PP (Mean ± S.E.)	
	E. fetida	***L. mauritii***
1000	928.06 ± 12.09^{a}	928.10 ± 4.83^{a}
4000	3918.73 ± 47.23^{b}	3910.80 ± 41.09^{b}
8000	7874.08 ± 48.49^{c}	7806.49 ± 39.27^{c}
16000	15808.01 ± 49.25^{d}	15798.88 ± 37.48^{d}

6.7 Biodegradation analysis

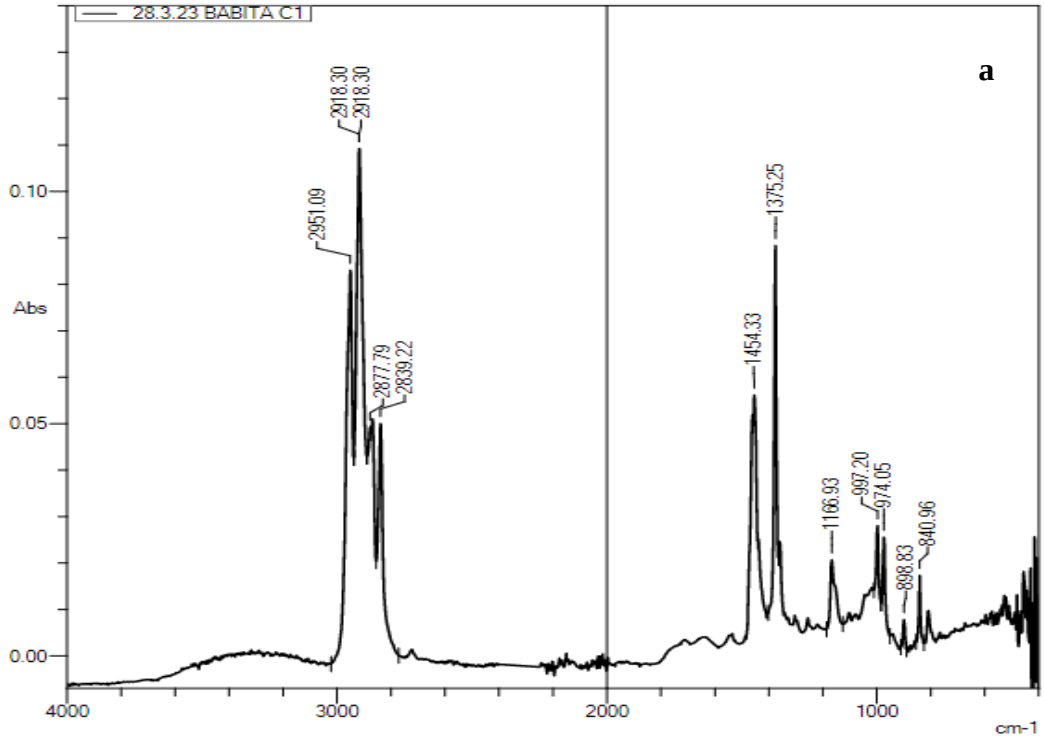
6.7.1 ATR- FTIR spectroscopic analysis of polypropylene microplastic extracted from both *E. fetida* and *L. mauritii* earthworms.

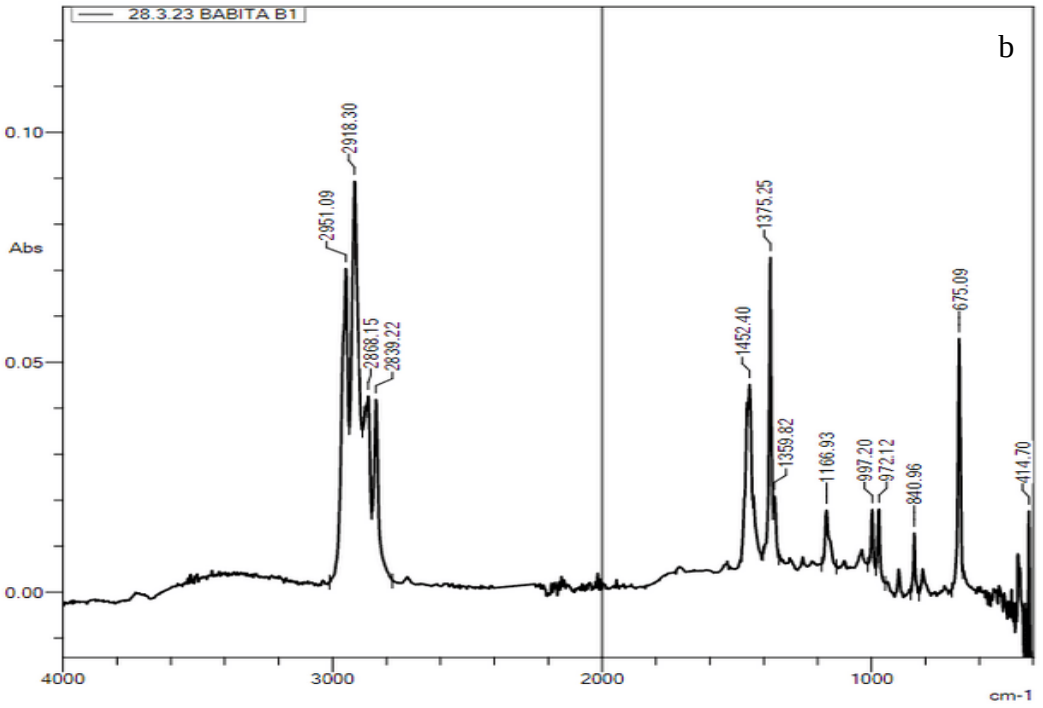
FTIR analysis of extracted polypropylene microplastics were taken after 28 days interval of degradation in the gut of earthworm by different microorganisms. Additionally, the results of FTIR analysis were showed appearance and disappearance of peaks, change in chemical groups and it also offer potential proof for degradation of microplastic particles through different types of microorganisms present in the intestine of earthworms (Cui et al., 2022b).The FTIR spectra of polypropylene without treated with earthworms (Control) (Fig 6.27a) and after 28 days exposure of polypropylene to earthworms to study the degradation of polypropylene in the gut of earthworms through the presence of different intestinal microrganisms that cause change in the chemical groups and peak intensity of polypropylene. The peak of polypropylene without earthworm was formed at 2877.79cm^{-1}(Fig 6.27b) but the peak of polypropylene after 28 days polypropylene exposure period of earthworms (polypropylene microplastics extracted from degutting) were shift at wave number 2868.15cm^{-1}(Fig 6.27b and Table 6.19) and presence of C-H group Another peak was appeared at wave number 1454.40cm^{-1} in case of *E. fetida* but in *L. mauritii* peak was formed at 1460.11 and C-H (methylene) group were present. Due to the formation of

C-H group the peak formed at 1359.82cm^{-1} in *E. fetida*. Due to the presence of C=C group after 28 days exposure in both species *E. fetida* and *L. mauritii* the peak were formed at wavenumber 972.12cm^{-1}. The another peak were formed at wave number 675.09cm^{-1} due to C-H group.The appearance of peak probably represents the oxidation effects of polypropylene due to activity of different functional gut microorganisms. Tziourrou et al, (2021) reported that different types of microorganisms play a role in the degradation of microplastics by enhancing the hydrophilicity of microplastics. The peaks were present at wavenumber 898.33cm^{-1} without earthworm treatment and this peak were disappearing after 28 days exposure in both species of earthworm. Meng et al, (2023) reported the activity of *L. terrestris* gut microorganisms and enzymes that triggered, hydrolyze or breakdown the PBAT and PLA microplastics. The gut of *E. fetida* and *L. mauritii* were more effective to breakdown of microplastic polymers.The ingested polypropylene microplastics may transformed through the digestive tract through the action of gut microorganisms.The breakdown of microplastic relates with the polymers properties. Previously reported study showed that earthworm has higher ability to breakdown low density polyethylene (Lwanga et al., 2016). Polylactic acid microplastics were degraded by the action of different intestinal microorganisms of earthworm and these microorganisms were secreting different enzymes such as carboxylestrase to degrade microplastics (Sanchez-Hermandez et al., 2009; 2014). The main finding of our study revealed that the ingested polypropylene microplastics were mineralized and degraded by different gut microorganisms of both earthworm species.

Table 6.19 ATR-FTIR spectral analysis of extracted polypropylene particles from earthworms gut.

S.NO.	New Peaks Appear		Functional Group	Peaks disappear		Functional Group
	E. fetida	*L. mauritii*		*E. fetida*	*L. mauritii*	
1	2868.15	2868.15	C-H	898.33	898.33	C-H
2	1452.40	1460.11	C-H			
3	1359.82	-----	C-H			
4	972.12	972.12	C=C			
5	675.09	------	C-H			


28.3.23 BABITA C1
a
2918.30
2918.30
2951.09
1375.25
1454.33
2877.79
2839.22
1166.93
997.20
974.05
840.96
898.83
0.10
Abs
0.05
0.00
4000
3000
2000
1000
cm-1


28.3.23 BABITA B1
b
2918.30
2951.09
1375.25
1452.40
675.09
2868.15
2839.22
1359.82
1166.93
997.20
972.12
840.96
414.70
0.10
Abs
0.05
0.00
4000
3000
2000
1000
cm-1

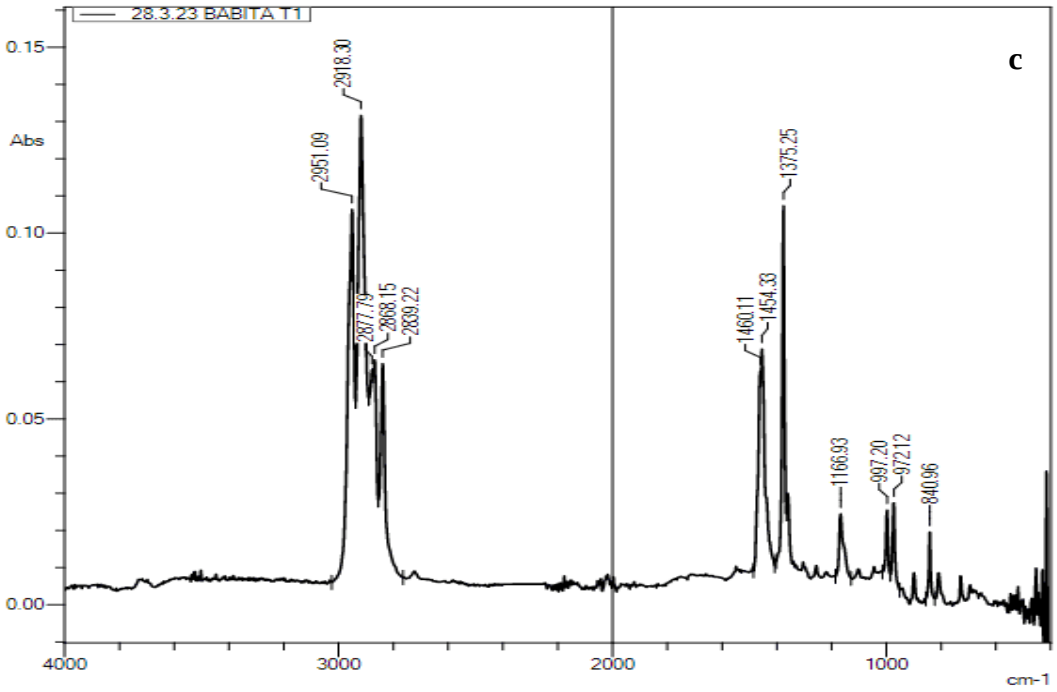


Fig 6.27 FTIR spectra of polypropylene microplastic (a) depicts the FTIR spectra of polypropylene (control) without earthworm (b) FTIR spectra of polypropylene particles extracted after 28 days from the gut of *E. fetida* (c) spectra of polypropylene microplastics extracted from the gut of *L. mauritii.*

6.7.2 Scanning Electron Microscopy (SEM) analysis.

After incubation of 28 days casts from control (without microplastics) and polypropylene treatment group of both *E. fetida* and *L. mauritii* were employed for SEM analysis. SEM analysis was implemented to examine morphological alterations of casts after polypropylene passing through the earthworms gastrointestinal tract (Song et al., 2019). Fig 6.28 showed the micrographs of cast from control (without polypropylene) and with polypropylene microplastics. As compared to control of *E. fetida* and *L. mauritti* polypropylene microplastics changed the surface morphology of cast.

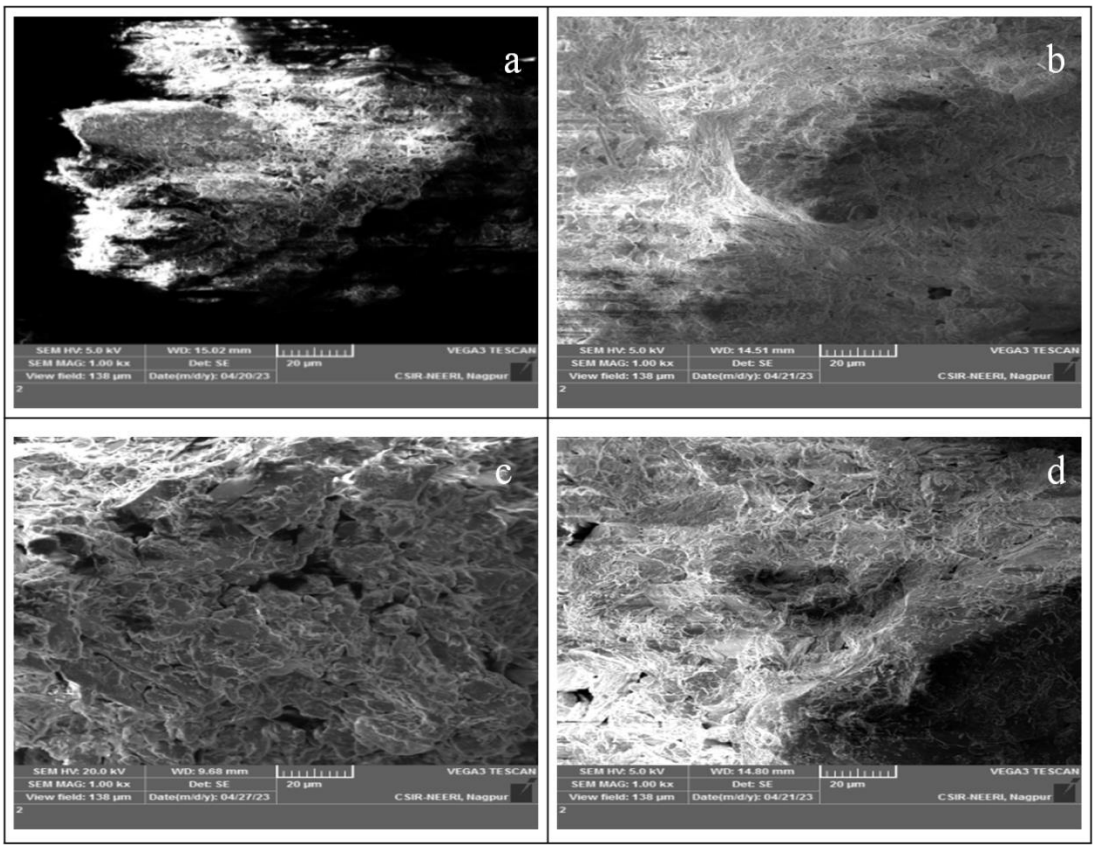


Fig 6.28 SEM micrographs of cast (a-b) collected from *E. fetida* (a) without polypropylene (control) (b) with polypropylene treatment (c-d) shows the micrographs of cast collected from *L. mauritii* without polypropylene (control) (d) after polypropylene treatment.

6.8 Evidences control startegies and policies to mitigate plastic pollution

Government implements different campaigns and policy to reduce MPs pollution from environment. It is important to clear that reducing plastic polymer production and managing microplastics waste must be a priority. The United Nations Environment Assembly implements several aspects to overcome the microplastic pollution so as to reduce plastic use and increase recycling, reuse, and proper disposal (Rochman, 2018). Further, it is also necessary to address the issue of microplastic waste management in developing countries, particularly in Asia and Africa, which account for the largest share of plastic waste to ocean. The use of artificial intelligence technology, improving waste collection systems, implementing public awareness programs and education about the harmful impacts of plastic waste in some aspects help to overcome microplastic pollution.

7. SUMMARY AND CONCLUSIONS

Punjab has very large number of plastic product manufacturing industries. The waste of these industries reached in agricultural field through different sources such as utilization of wastewater for irrigation and sewage sludge. Plastic mulching is one of the major source of microplastic in agriculture field.The use of these sources in the agricultural field no doubt increase the crop quality and productivity but also produces negative effect on the soil micro and macrofauna.Therefore the present work were designed on the basis of plastic pollution in agriculture field and its toxic effects on soil ecological engineer (earthworms).

Soil collected from agriculture field near plastic product manufacturing industries was employed to know the concentration, shapes, size, color and types of microplastics. Various types of microplastics i.e. polyethylene, polypropylene, polystyrene, polyethylene terephthalate, polybutylene terephthalate were extracted by using density separation method. Different shapes (fibres, fragments, miscellaneous, film), size (100 μm-1 mm) of microplastics particles were extracted. These microplastics particles were identified by using ATR-FTIR spectrophotometer on the basis of their identification peak and functional groups. The surface morphology of PE, PP, PS, PET and PBT microplastics was studied by SEM and the nature of these particles was studied through XRD. PE, PS and PET microplastics particles are poorly crystalline in nature where as PP exhibits the strong crystalline nature but PBT are amorphous in nature due to its noisy peak.

Microplastic polypropylene were tested to check the toxic effects in terms of biomass, fecundity rate and antioxidant enzymes (biochemical assay) (Superoxide dismutase, Catalase, Glutathione-S-Transferase, Guiacol Peroxidase**)** on two earthworm's species namely *E. fetida* (exotic species) and *L. mauritii* (indigenous species)**.** Two earthworms species (*E. fetida* and *L. mauritii)* were treated with different concentrations of polypropylene (0, 1000, 4000, 8000, 16000 $mgkg^{-1}$) in artificial soil to know its effect in term of biomass and fecundity rate and oxidative stress earthworm. Artifical soil (OECD soil) was free from any harmful pollutants contaminats so that earthworms test species depicts the real toxic effects toward the microplastic or other chemical compound. Highest concentrations of polypropylene

pose significant negative impact on the biomass and reproduction of *E. fetida* and *L. mauritii*. Different antioxidant enzymes (SOD, CAT, GST and POD) activity increase up to 14 days but inhibit the enzymatic activity at 8000 and 16000 $mgkg^{-1}$ while POD activity increases with increase in exposure time. The SOD, CAT and GST enzyme unit activity of *E. fetida* on 14^{th} day at 16000 $mgkg^{-1}$ was 0.015, 0.074, and 0.097 respectively but in *L. mauritii* the unit activity of SOD, CAT and GST (0.009, 0.024 and 0.012 respectively) at 1600 $mgkg^{-1}$ were increased on 14^{th} day while POD unit activity of *E. fetida* and *L. mauritii* were increased with increase in exposure time. On 28^{th} day in *E. fetida* at similar concentration the unit activity of SOD, CAT and GST were significantly decrease 0.008, 0.058, 0.045 similarly in case of *L. mauritii* the unit activity of SOD, CAT and GST (0.002, 0.011 and 0.09 respectively) at 16000 $mgkg^{-1}$ on 28^{th} day were declined. The unit activity of POD enzymes of *E. fetida* on 7^{th} day at highest concentration (16000 $mgkg^{-1}$) was 0.025 and on 28^{th} day at similar concentration the unit activity is 0.035 but in *L. mauritii* the POD unit activity on 7^{th} day at 16000 $mgkg^{-1}$ was 0.027 and on 28^{th} day at same concentration the unit activity was 0.035. High concentrations of polypropylene produce oxidative stress on different antioxidant enzymes of *E. fetida*. Molecular docking study is a tool to analyze the available binding site of ligand in particular enzymes and its interaction with catalytic and non-catalytic sites at a molecular level that are responsible for the modulation of the activity of that enzyme. Futhermore docking study illustrates the docking score of different enzymes. In this study the docking score was high with CAT and GST indicated that these two enzymes have high binding affinity for polypropylene and have different active binding sites towards polypropylene such as SOD have three, CAT has four, GST has five and POD has three active binding sites.

Earthworms gut provides a stable environment for different types of microorganisms. Organic pollutants includes microplastics, pesticides etc disturb the stability of gut microorganisms. In this study different types of gut microorganisms of earthworms treated with polypropylene were identified by metagenomic sequencing. Study reported that polypropylene affects the alpha and beta diversity of microorganisms. Alpha diversity were measured on the basis of Chao1, ACE, Shanon, Simpson and beta diversity were measured by Bray-curtis dissimilarity, Jaccard dissimilarity distance between control and polypropylene treated group.The measured Operational

Taxonomic Unit (OTUs) was 32641, 31621 and 31141 respectively in control, treated *E. fetida* and *L. mauritii*.This clearly showed that the polypropylene causes moderate effects on the earthworms gut microbiome. At phylum levels different types of microorganisms were identified i.e Tenericutes, Bacteriodetes, Chloroflexi, Cyanobacteria, Acidobacteria, Saccharibacteria-TM7, Verrucomicrobia, Gemmatimonadetes, Actinobacteria, Plantomycetes, TM6, Firmicutes, Chlamydiae and Proteobacteria. At genus level different types microorganisms were identified i.e. *Pantoea, Pseudomonas, Streptococcous, Aquicella, Planctomicrobium, Protochlamydiae, Parachlamydiae-uc, Q129127-g, Alcanivorax, Bacillus, Hyphomicrobium, Cantonella, EUC363464-f-uc, EF516411-g.*These different types of microorganisms play a crucial role in degradation of microplastics. Earthworm gut microorganisms have the ability to degrade or breakdown microplastic particles. In this study earthworms were treated with polypropylene for 28 days to study the degradation of polypropylene in the gut of earthworm. Degradation of polypropylene was studied on the basis of weight loss of particles. Weight loss of polypropylene were analyze on three different parameters i.e polypropylene recovered from cast up to 28 days, degutting of earthworms and soil. Total amount of recovered polypropylene from *E. fetida* and *L. mauritii* was 928.06, 3918.73, 7874.08, 15808 $mgkg^{-1}$ and 928.10, 3910.80, 7806.49 and 15798.01 $mgkg^{-1}$ respectively when earthworms were treated in 1000, 4000, 8000 and 16000 $mgkg^{1}$. Microplastics particles passed through the earthworm intestine and incorporated in the form of cast but different types of microrganisms present in the gut they degrade microplastic particles and cause change in the chemical groups and peak intensity. Hence, the remaining amount of polypropylene may be degraded by earthworm gut microorganisms. The extracted polypropylene particles from degutting and cast were employed for ATR-FTIR analysis to study the appearance of new peaks or group. ATR-FTIR spectral analysis shows the formation of C-H group at wavenumber 2868.15 cm^{-1} and 1460.11cm^{-1},972.12cm^{-1} (C=C group) and one peak disappear from wavenumber 898.33cm^{-1} (C-H group). Scanning Electron Microscopy were employed to study the changes in the surface morphology of treated and without treated cast. SEM micrographs showed changes between treated and without treated group.Our results represents the earthworms gut microrganisms are capable to degrade the

polypropylene.Therefore, further study is needed to investigate or determine the interaction of different insects (nematodes, collembolans isopods and amphipods) and their gut microrganisms to reveal the degradation mechanisms of various types of plastic by insects eating. In terms of molecular docking further it is necessary to illustrate the hydrophobic interaction and catalytic and non-catalytic sites of different types of other insect enzymes with microplastic.

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APPENDIX-I

ABBREVIATIONS

S.No	Abbreviation	Expansion
1.	CDNB	1-chloro, 2, 4- dinitro benzene (CDNB)
2.	Ala	Alanine
3.	APHA	American Public Health Association Guidelines
4.	ANOVA	Analysis of variance
5.	Å	Angstrom
6.	Arg	Arginine
7.	C_6H_8O	Ascorbic acid
8.	Asn	Asparagine
9.	Asp	Aspartic acid
10.	ATR-FTIR	Attenuated total reflectance - Fourier transform infrared spectroscopy
11.	β	Beta
12.	H_3BO_3	Boric acid
13.	Cd	Cadmium
14.	$CaCO_3$	Calcium carbonate
15.	$CaCl_2$	Calcium chloride
16.	Ca^+	Calcium ions
17.	CO_2	Carbon dioxide
18.	Catalase	CAT
19.	C	Celsius
20.	cm	Centimetre
21.	Cr	Chromium
22.	COG	Clusters of Orthologous Gene

S.No	Abbreviation	Expansion
23.	TGA-DSC	Combined Thermogravemetric Analysis - Differential Thermal Calorimetry
24.	$CuSO_4$	Copper sulphate
25.	Cys	Cysteine
26.	˚	Degree
27.	EC	Electrical conductivity
28.	EG	Ethylene glycol
29.	FPA-FTIR	Focal plane array- Fourier transform infrared spectroscopy
30.	Glu	Glutamic acid
31.	GSH	Glutathione
32.	Gly	Glycine
33.	g	Gram
34.	Glutathione-S-Transferase	GST
35.	High Density Polyethylene	High-density polyethylene (HDPE)
36.	His	Histidine
37.	hrs	Hours
38.	H_2O_2	Hydrogen Peroxide
39.	ICP-MS	Inductively Coupled Plasma Mass Spectrometry
40.	Fe	Iron
41.	Kg	Kilogram
42.	Km	Kilometre
43.	KEGG	Kyoto Encylopedia of Genes and Genomes
44.	LDIR	Laser Direct Infrared

S.No	Abbreviation	Expansion
45.	Pb	Lead
46.	<	Less than
47.	Leu	Leucine
48.	Ltd	Limited
49.	Li	Lithium
50.	LiCl	Lithium chloride
51.	l	Litre
52.	LDPE	Low density polyethylene
53.	Lys	Lysine
54.	Mg	Manganese
55.	MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
56.	CH_4	Methane
57.	Met	Methionine
58.	µl	micro litre
59.	µM	Micromolar
60.	µm	Micronmeter
61.	MPs	Microplastics
62.	mg	Milligrams
63.	ml	Millilitre
64.	mm	Millimetre
65.	min	Minute
66.	MHET	Mono-2-hydroxyethyl terephthalate
67.	nm	Nanometre
68.	NR	Nile red

S.No	Abbreviation	Expansion
69.	HNO_3	Nitric acid
70.	NBT	Nitroblue tetrazolium
71.	N	Normality
72.	NMR	Nuclear Magnetic Resonance Spectroscopy.
73.	OTU	Operational Taxonomic Units
74.	OC	Organic carbon
75.	OECD	Organization for Economic Co-operation and Development
76.	O_2	Oxygen
77.	%	Percentage
78.	$HClO_4$	Perchloric acid
79.	POP	Persistent organic pollutions
80.	Phe	Phenyalanine
81.	Guiacol peroxidase	POD
82.	PBT	Polybutylene terephthalate
83.	PCL	Polycaprolactone
84.	PE	Polyethylene
85.	PS	Polyethylene
86.	PET	Polyethylene terephthalate
87.	PHA	Polyhydroxyalkanoates
88.	PLA	Polylactic acid
89.	PCR	Polymerase Chain Reaction
90.	PP	Polypropylene
91.	PVC	Polyvinyl chloride
92.	K	Potassium

S.No	Abbreviation	Expansion
93.	K_2SO_4	Potassium sulphate
94.	Pvt	Private
95.	Pro	Proline
96.	Pyr-GC-MS	Pyrolysis Coupled with Gas Chromatography-Mass Spectrometry
97.	ROS	Reactive Oxygen Species
98.	SEM	Scanning Electron Microscope
99.	SeO_2	Selenium dioxide
100.	Ser	Serine
101.	SP-ICP-MS	Single Particle Inductively Coupled Plasma Mass Spectrometry
102.	Superoxide dismutase	SOD
103.	Na	Sodium
104.	NaBr	Sodium bromide
105.	NaCl	Sodium chloride
106.	NaH_2PO_4	Sodium dihydrogen phosphate
107.	NaOH	Sodium Hydroxide
108.	NaI.	Sodium iodide
109.	SE	Standard Error
110.	H_2SO_4	Sulphuric acid
111.	Temp	Temperature
112.	TPA	Terephthalic acid
113.	TDS-GC-MS	Thermal Desorption Coupled with Gas Chromatography- Mass Spectrometry.
114.	Θ	Theta
115.	Thr	Threonine

S.No	Abbreviation	Expansion
116.	TDS	Total dissolved solids
117.	TKN	Total Kjeldhal Nitrogen
118.	Trp	Tryptophan
119.	Tyr	Tyrosine
120.	UV	Ultra violet
121.	US	United State
122.	Val	Valine
123.	v/v	Volume by volume
124.	H_2O	Water
125.	w/v	Weight by volume
126.	w/w	Weight by weight
127.	XRD	X-Ray diffraction
128.	Zn	Zinc
129.	$ZnCl_2$	Zinc chloride

APPENDIX II

LIST OF SCIENTIFIC NAMES AND THEIR ABBREVIATIONS MENTIONED IN THE THESIS

S.NO	Scientific name	Abbreviated name
1.	*Eisenia fetida*	*E. fetida*
2.	*Lumbricus terrestris*	*L. terrestris*
3.	*Lampito mauritii*	*L. mauritii*
4.	*Caenorhabditis elegans*	*C. elegans*

APPENDIX III

LIST OF PUBLICATIONS

1. **Thakur, B.,** Singh, J., Singh, J., Angmo, D., & Vig, A. P. (2023). Biodegradation of different types of microplastics: Molecular mechanism and degradation efficiency. *Science of The Total Environment*, *877*, 162912.

2. **Thakur, B.,** Singh, J., Singh, J., Angmo, D., & Vig, A. P. (2023). Identification and characterization of extracted microplastics from agricultural soil near industrial area: FTIR and X-ray diffraction method. *Environmental Quality Management*.

3. Angmo, D., Dutta, R., Singh, J., Chowdhary, A. B., Quadar, J., **Thakur, B.,**& Vig, A. P. (2023). Biochemical responses, growth and reproduction of earthworm in low density polyethylene (LDPE). *Environmental Quality Management*.

4. Angmo, D., Singh, J., Dutta, R., Chowdhary, A. B., Quadar, J., Sharma, M., **Thakur, B.,** & Vig, A. P. (2023). Earthworms Modulate the Toxicity Effect of Low-Density Polyethylene on Plant Development. *Journal of Soil Science and Plant Nutrition*, 1-13.

5. Dutta, R., Chowdhary, A. B., Angmo, D., **Thakur, B.,** Quadar, J., Singh, J., & Vig, A. P. (2021). Vermicomposting of Different Organic Wastes into Organic Manure: A Review. *Journal Punjab Academy of Sciences*, *21*(1), 01-14.

6. **B.,** Singh J., & Sing, J. (2022).Source, Extraction and Identification of Microplastics and their effect on earthworm.

7. Angmo, D., Singh, J., Rashid, F., Sharma, P., **Thakur, B**., Singh, S., & Vig, A. P. (2024). Vermiremediation of organic wastes: vermicompost as a powerful plant growth promoter.In *Earthworm Technology in Organic Waste Management* (pp. 59-77). Elsevier.

8. Angmo, D., Singh, J., Bhat, S.A., **Thakur, B.,** & Vig, A. P. (2024).Micro-Nano-Plastics in Sewage Sludge: Sources, Occurrence, and Potential Environmental Risks. In *Management of Micro and Nano-plastics in Soil and*

Biosolids: Fate, Occurrence, Monitoring, and Remedies (pp. 343-363). Cham: Springer Nature Switzerland.

9. **Thakur, B.,** Kaur, P., Kumar, R., Singh, J., Vig, A. P., Angmo, D., & Singh, J. Environmental Risk and Management of Microplastics in Soil. In *Environmental Nexus Approach* (pp. 301-310). CRC Press.

LIST OF CONFERENCES

a. Internationals Conferences

1. Babita Thakur, Jaswinder Singh and Joginder Singh. Extraction and detection of Microplastics from agricultural soil near industrial area of Punjab (Kapurthala). International conference Plastic Pollution from Macro to Nano from 17-18 November, 2022, organized by UNESCO. **(Oral presentation)**

2. Babita Thakur, Jaswinder Singh and Joginder Singh. Exotoxicological effects of polypropylene on earthworm *E. fetida***.**International Conference in recent advances in Biotechnology, Dr. B R Ambedkar National Institute of Technology, Jalandhar (Punjab) from 2-4 December, 2022. **(Oral presentation)**

3. Babita Thakur, Jaswinder Singh and Joginder Singh. Exotoxicological effects of polypropylene on earthworm *L. mauritii***.** 2nd International Conference on Emerging Scenario of Science Technology and Innovation, Carrier Point University Hamirpur (H.P) from 24-25 February, 2023. **(Oral Presentation)**

4.Participate in International conference on Sustainability: Life on Earth, Lovely Professional University, Phagwara (Punjab) from 17-18 December, 2021.

b. National Conferences

1. Babita Thakur, Jaswinder Singh and Joginder Singh. Separation and identifications of different types of microplastics in agriculture soil. National conference on Air Quality and Human Health: Consequences of Remedies, Khalsa College, Amritsar from November 4-5, 2022. **(Poster presentation)**.

LIST OF WORKSHOPS

1. Participate in One-Day Training workshop on "Science Communication and Popular Science Writing" under AWSAR programme organized by Department of Science and Technology, Vigyan Prasar, Guru Nanak Dev University, Amritsar (Punjab), 5 August, 2022.

2. Participate in one day National workshop "Vermicompost: Recycling Waste into wealth" organized by Khalsa College Amritsar (Punjab) in collaboration with Punjab Pollution Control Board, Patiala, 5 November, 2022.

3. Participate in Two- Day workshop on "Training of Dairy Farmers for Management of Cattle Dung through Vermicomposting, organized by Directorate of Environment and Climate Change, Punjab, Guru Nanak Dev University, Amritsar (Punjab) from 13- 14 June, 2023.