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Evaluation of Different Biomolecules in Two Epigeic Earthworms Cultured in Microplastic-Treated Foods

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Abstract: ***Plastic use has increased significantly in terrestrial habitats, mainly microplastics (MPs) like Polyvinyl Chloride (PVC), in agricultural ecosystems for various purposes. Microplastics are known to adversely affect earthworms in soil ecosystems, including growth and reproduction. Here, we used the No Observed Effect Concentration (NOEC) (4 g/100 g of CM) to assess the influence of microplastics (MPs) on the composition of different biomolecules in two epigeic earthworms, Eisenia fetida (EF) and Eudrilus eugeniae (EE). We analysed major biomolecules, including proteins, Carbohydrates (Glucose), and Lipids, in earthworm biomass using standard methods. The results showed that MP treatment increased protein content in earthworm EF (806.18 µg/ml) and decreased it in EE (688.55 µg/ml). In contrast, MP treatment decreased glucose content in both earthworm species (EF-470.01 & EE-515.82 µg/ml) compared with Control-CM (EF-578.50 & EE-540.83 µg/ml), respectively. Total lipid content was higher in EF-MPT (0.2008 g) than in Control-CM (0.1594 g), but lower in EE-MPT (0.1336 g) than in Control-CM (0.1784 g). Saturated lipids were higher in EF-MPT (0.0842 g) and lower in Control-CM (0.065 g), whereas in EE, they were higher in Control-CM (0.0744 g) than in MPT (0.028 g). For unsaturated lipids, EE-MPT showed the maximum (0.1056 g) and Control-CM the minimum (0.104 g), whereas Control-CM showed less (0.0944 g) and MPT more (0.1166 g) in the case of EF. Hence, based on these results, both epigeic earthworm species contain all major biomolecules in various forms. Still, microplastic exposure can positively or negatively influence the composition and percentage of these biomolecules in different earthworm species.***

Keywords: Biomolecules, Epigeic Earthworms and PVC Microplastics.

Nomenclature:
PVC-Polyvinyl Chloride
MPs-Microplastics
CM-Cattle Manure
EF-Eisenia Fetida
EE-Eudrilus Eugeniae
BSA-Bovine Serum Albumin
NOEC: No Observed Effect Concentration

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I. INTRODUCTION

Earthworms are macro-invertebrates in soil ecosystems that maintain soil biota and are important for soil structure, fertility, and the food chain [19]. Earthworms have high reproductive potential and strong adaptive capabilities, although they are susceptible to toxic and detrimental substances. Substrate effectiveness can be evaluated based on earthworm survivability, biomass, growth, and reproduction. Soil organisms, including earthworms, face many problems in nature, and the most significant threat to their survival is increasing plastic pollution. Epigeic earthworms are commonly used in vermicomposting and are often used to evaluate toxicity. Earlier researchers reported toxic effects of soil contaminants on growth [15], body movement [14], and oxidative stress in earthworms [18 & 13]. Studies have also reported that microplastics could cause cytotoxicity, body swelling, and oxidative stress [4], and thus increase the risk of cancer [6].

Biomolecular and physiological changes in animal tissues are better indices of the effect of any type of pollution (Internal stress damage). Various biomolecular studies have been carried out at the tissue and organ levels. After ingestion of these microplastics along with soil, their physiological and biochemical constituents were disrupted. These biomolecular changes offer an interesting field for using novel, sensitive, and non-destructive biomarkers. Earthworm biomass (Vermiprotein) is rich in a wide variety of biomolecules, making it a valuable source for medical and pharmaceutical diagnosis [20]. Biomolecules such as proteins, carbohydrates, and lipids play an important role in earthworm metabolism and physiology. Few studies have examined the effects of polypropylene microplastics or polyethene on earthworm growth and reproduction [21]. However, the literature lacks information on the biomolecular composition of earthworm biomass treated with PVC microplastics.

Therefore, this research was undertaken to determine the influence of microplastics on biomolecular composition, such as Proteins, Carbohydrates, and Lipids, in the biomass of the epigeic earthworms Eisenia fetida and Eudrilus eugeniae cultured on control and MP-treated food.

II. MATERIALS AND METHODS

A. Collection of Earthworms

We acquired juveniles of the epigeic earthworm species E. fetida and E. eugeniae from the stock culture maintained in the Vermi Technology

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Laboratory, Department of Zoology, Karnatak University, Dharwad.

B. Culturing of Earthworms

We cultured juveniles of both E. fetida and E. eugeniae in Control-CM and MP-treated food until they reached maturity, for about 10 weeks.

C. Preparation of Earthworm Powder Sample/Test Sample

We selected and collected healthy adult clitellate earthworms of both E. fetida and E. eugeniae individually from the Control-CM and MP-treated food. Initially, we washed the worms with tap water to remove external debris. Afterwards, we fed them wet blotting paper for about 18-20 hours to clear their gut. We then gently washed the gut-cleared earthworms with tap water and dried them on tissue paper; then, we placed the earthworms in separate Petri plates and exposed them to sunlight for approximately 8-10 days. Soon after drying, we ground the worms into a fine powder using a pestle and mortar. The resulting earthworm powder served as a test sample to evaluate the concentration of different biomolecules.

D. Biomolecular Estimation

The influence of microplastics on the concentration of various biomolecules such as Proteins, carbohydrates, and Lipids content in the biomass of epigeic earthworms, *E. fetida* and *E. eugeniae,* was analysed as per the following standard methods;

i. Protein Estimation

The Lowry method was used to estimate total protein concentration in earthworm test samples. Total protein content was measured at 660nm using Bovine Serum Albumin (BSA) as the standard protein [1].

ii. Carbohydrate Estimation:

Total carbohydrate was estimated using the Anthrone Reagent method, with D-glucose as the standard solution. Carbohydrate was quantified by adding conc. H_2SO_4 and 5% phenol (v/v) to the supernatant fraction in a proportion of 1:1:4. The absorbance at 540 nm (wavelength) was measured after 30 minutes of incubation at room temperature. Glucose was used as the carbohydrate standard [3].

iii. Lipid Estimation:

The Barnes and Blackstock method was used to measure total, saturated, and unsaturated lipid concentrations in the earthworm test samples using fractionation and gravimetric techniques. Total lipids were first extracted using a Chloroform: Methanol solvent system. Then, excess cold acetone was added so saturated lipids would precipitate due to their lower solubility, while unsaturated lipids remained dissolved in the Acetone. The two fractions were then separated, dried, weighed, and expressed as percentages [16].

Percentage Saturated Lipid:

% of saturated lipid = \frac {SL}{TL} \times 100

Where TL = total lipid (g); SL = saturated lipid (g)

Percentage Unsaturated Lipid:

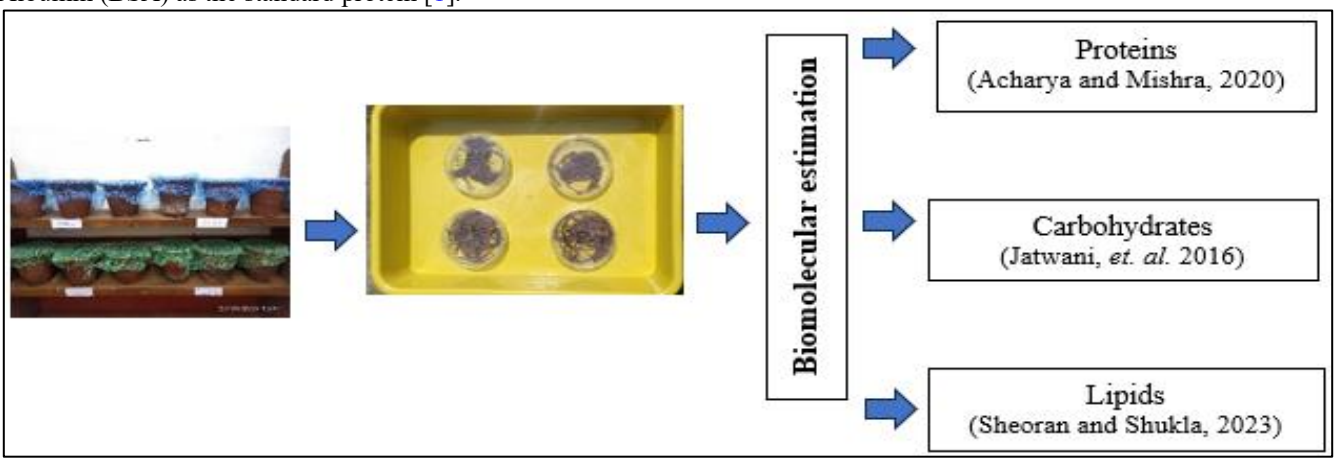
% of saturated lipid = \frac {USL}{TL} \times 100

USL= Unsaturated lipid (g)

E. Statistical Analysis

All experiments were conducted in replicates, and the data were statistically analysed using PAST software. In addition, Levene's test and the Shapiro-Wilk test were used to assess normality and homogeneity for parametric analysis. Significant differences ($P \leq 0.05$) were calculated using a paired t-test.

F. Schematic Diagram of the Experimental set-up



Earthworm Culture Pots **Sun Drying of Both Earthworm Samples**

III. RESULTS AND DISCUSSION

The results of the biomolecular estimation of Proteins, carbohydrates, and lipids in the biomass of E. fetida and E. eugeniae earthworms are presented in Tables I-III, Graphs 1-9, and Figures 1-2.

A. Proteins

Proteins are essential biomolecules composed of different amino acids and play crucial roles in DNA replication, cellular metabolism, and other processes. In this study, BSA was used as a standard solution to determine protein concentration in the earthworm test samples. Protein concentration in earthworms varies by treatment. The highest protein concentration was observed in EE-CM (855 µg/ml), followed by EF-MPT (806 µg/ml) and EF-CM (798 µg/ml), whereas the lowest protein concentration was observed in EE-MPT (688 µg/ml) among all the earthworm test samples (Table I).

Figure 1 shows the test tubes containing blank solution, standard BSA solution [1-5],

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and the earthworm test samples (EF-CM, EF-MPT, EE-CM, and EE-MPT). Graph 1 shows the regression (r) value (0.979) and coefficient of determination (R^2) (0.960) for standard BSA solutions at different concentrations (200, 400, 600, and 1000). Graph 2 shows the protein concentrations of the different test samples (EF-CM, EF-MPT, EE-CM, and EE-MPT). No significant difference was observed between Control-CM and the MP-treated group in E. fetida, whereas a significant difference ($P \leq 0.05$) was observed between Control-CM and the MP-treated group in *E. eugeniae*.

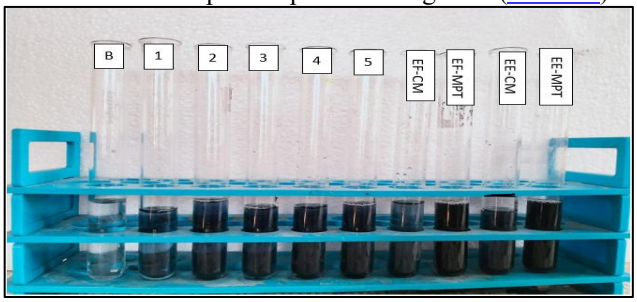
B. Carbohydrates (Glucose)

Carbohydrates are important macromolecules that act as an immediate energy precursor for earthworms exposed to stress conditions. Glucose is a simple sugar and the most fundamental type of carbohydrate. D-glucose is used as a standard solution to determine glucose concentration in the earthworm test samples.

Figure 2 shows the test tubes containing the blank, standard (D-glucose) solution, and earthworm test samples for estimating carbohydrates (Glucose). Graph 3 shows the regression (r) value (0.993) and the coefficient of determination (R2) (0.987) for the standard D-glucose solution at different concentrations (200, 400, 600, and 1000). Graph 4 shows the glucose concentrations in the earthworm test samples (EF-CM, EF-MPT, EE-CM, and EE-MPT). Among all samples, the Control-CM group in both earthworm species showed the highest glucose concentration compared with the treated groups (EF-MPT and EE-MPT; Table II and Graph 4). A significant difference ($P \leq 0.05$) was observed between the Control-CM and MP-treated groups in both earthworm species.

C. Lipids

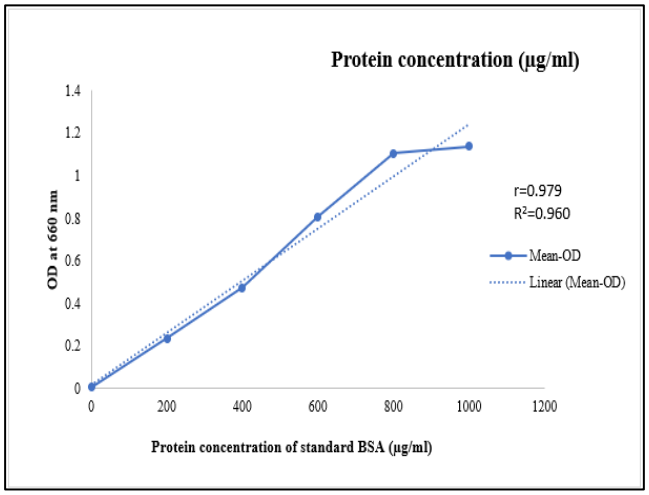
Lipids are essential biomolecules for cell signalling, acting as messengers and energy storage molecules in organisms, and occur as saturated and unsaturated lipids (Fahy et *al.*, 2009). Among all test samples, unsaturated lipids showed the highest percentage (79.041%) in the EE-MPT group, followed by EF-CM and EE-CM, and the lowest in EF-MPT. In contrast, EF-MPT showed the highest percentage of saturated lipid (41.932%), followed by EE-CM (41.704%) and EF-CM (40.772%), and the lowest in EE-MPT (20.958%) (Table III and Graphs 8 & 9). Graphs 5, 6, and 7 show the total, saturated, and unsaturated lipid concentrations (g) in EF-CM, EF-MPT, EE-CM, and EE-MPT samples from epigeic earthworm species (*E. fetida* and *E. eugeniae*). We observed significant differences ($P \leq 0.05$) among all test samples for lipids and saturated and unsaturated lipids, except between saturated lipid samples in E. eugeniae (Table III).



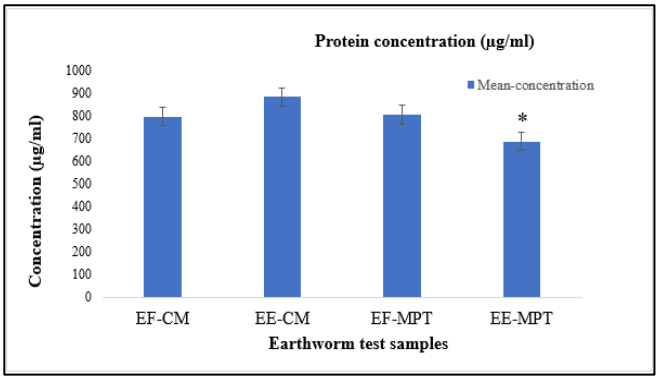
[Fig.1: Test Tubes Containing Blank (B), Standard BSA Solution (1-5) and the Earthworm test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT) for the Estimation of Proteins]

Table I: Protein Concentration (µg/ml) of the Standard BSA Solution and the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM, and EE-MPT). Data are Mean ± SE

Sl. No.	Standard and Earthworm Test Samples	Protein Concentration (µg/ml)
1	Blank (B)	0
2	Standard BSA solution	200
3		400
4		600
5		800
6		1000
7	EF-CM	798.72 ± 2.78
8	EF-MPT	806.18 ± 2.87
9	EE-CM	885.60 ± 3.64
10	EE-MPT	688.55 ± 3.65

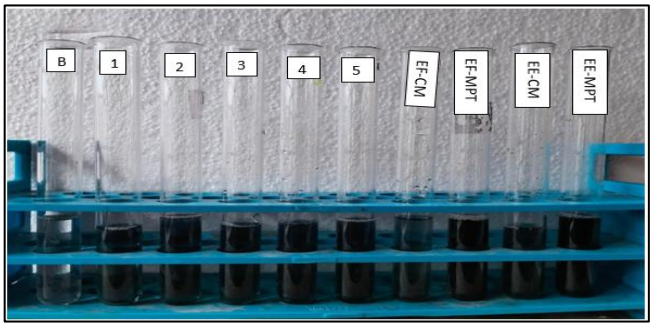


[Graph 1: Standard Protein (BSA) Concentration (µg/ml) at Different Concentrations]



[Graph 2: Protein Concentration (µg/ml) of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT). * Indicates a Significant Difference Between Control-CM and the MP-Treated Group of EE at $P \leq 0.05$]

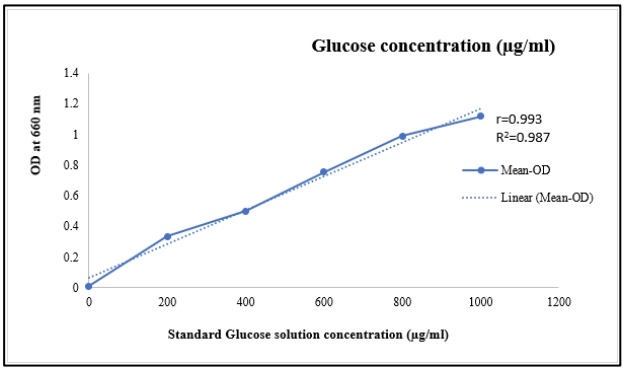
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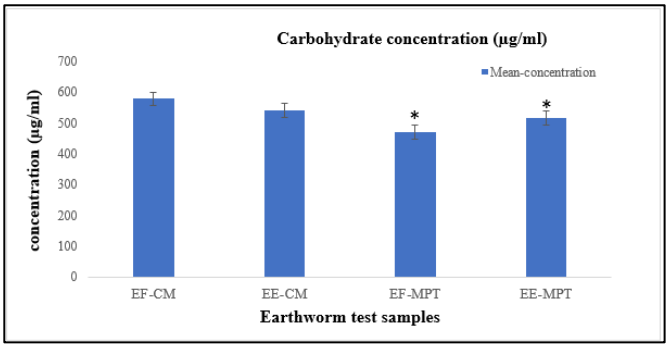
[Fig.2: Test Tubes Containing Blank (B), Standard Glucose Solutions (1- 5) and the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT) for the Estimation of Carbohydrate (Glucose)]

Table II: Glucose Concentration (µg/ml) of the standard D-glucose solution and the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM, and EE-MPT). Data are Mean ± SE

Sl. No.	Standard and Earthworm Test Samples	Glucose Concentration (µg/ml)
1	Blank (B)	0
2	Standard D-Glucose solution	200
3		400
4		600
5		800
6		1000
7	EF-CM	578.50 ± 3.06
8	EF-MPT	470.01 ± 2.83
9	EE-CM	540.83 ± 2.86
10	EE-MPT	515.82 ± 2.74



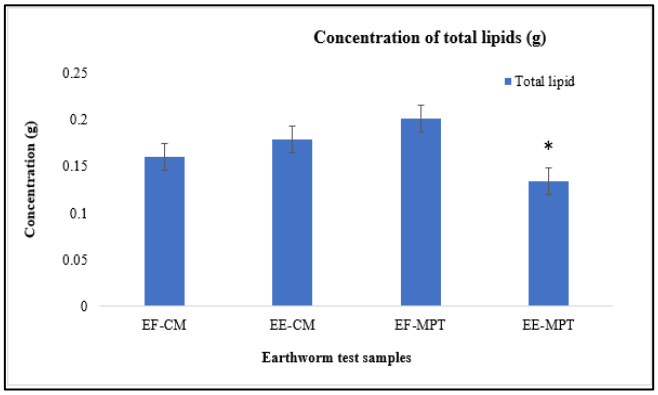
[Graph-3: Standard D-Glucose Concentration (µg/ml) at Different Concentrations]



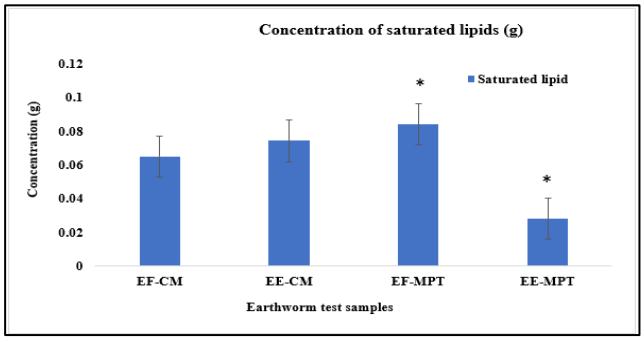
[Graph-4: Glucose Concentration (µg/ml) of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT). * Indicates a Significant Difference Between Control-CM and the MP-Treated Group of EF & EE at P≤0.05]

Table III: Total Lipid, Saturated and Unsaturated Lipid Concentrations (g) of the Earthworm Test Samples (EF-CM, EF-MPT and EE-CM and EE-MPT). Data are Mean ± SE

Sl. No.	Parameters	Samples			
		EF-CM	EF-MPT	EE-CM	EE-MPT
1	Weight of earthworm samples (g)	2g	2g	2g	2g
2	Total Lipids (TL) (g)	0.1594 ± 0.001	0.2008 ± 0.0009	0.1784 ± 0.0008	0.1336 ± 0.0008
3	Saturated Lipids (SL) (g)	0.065 ± 0.0013	0.0842 ± 0.0008	0.0744 ± 0.001	0.028 ± 0.011
4	Unsaturated Lipids (USL) (g)	0.0944 ± 0.0005	0.1166 ± 0.0008	0.104 ± 0.0008	0.1056 ± 0.001
5	Percent of Saturated Lipids	40.777	41.932	41.704	20.958
6	Percent of Unsaturated Lipids	59.222	58.067	58.295	79.041

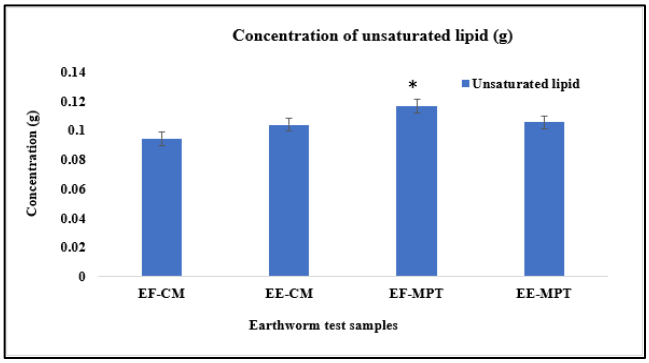


[Graph 5: Total Lipid Concentration (g) of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT). * Indicates a Significant Difference Between Control-CM and the MP-Treated Group of EF & EE at P≤0.05]

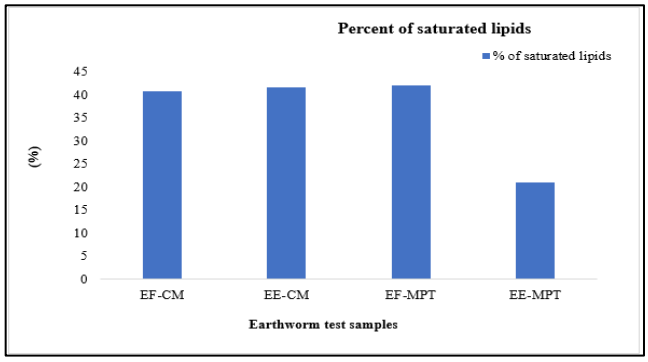


[Graph-6: Saturated Lipid Concentration (g) of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT). * Indicates a Significant Difference Between Control-CM and the MP-Treated Group of EF & EE at P≤0.05]

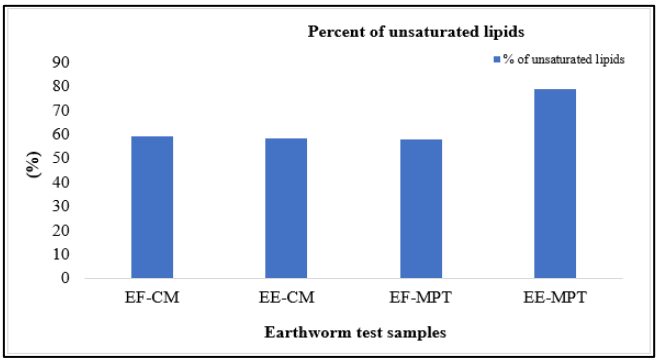
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[Graph-7: Unsaturated Lipid Concentration (g) of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT). * Indicates a Significant Difference Between Control-CM and the MP-Treated Group of EF at P≤0.05]



[Graph-8: Per Cent Saturated Lipid Concentration of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT)]



[Graph-9: Per Cent Unsaturated Lipid Concentration of the Earthworm Test Samples (EF-CM, EF-MPT, EE-CM and EE-MPT)]

Protein determination is a fundamental method in biochemical research. Variations in protein quality and quantity are highly significant for recognising various diseases and evaluating their effects. The protein content of different epigeic earthworm species ranges from 61.9-72.9%, which is comparatively higher than that of conventional protein sources like egg, pork, and salmon. The carbohydrate concentration of the epigeic earthworm species (*E. andrai*) ranges between 23-26%. In terms of lipid content, some invertebrate species have high lipid levels (8.1% to 60%), whereas chicken has the lowest lipid content (about 30.3%). In comparison, pork contains 54.4% lipid among common commodities. Crude fat is much lower in earthworms than in conventional food sources, whereas polyunsaturated lipids are high in earthworms, similar to some fish oils [5].

In the present study, the protein content of the earthworm E. fetida increased when exposed to microplastic (MPT); this may reflect the earthworms' defence response to microplastic stress, which might increase protein content [7]. The decrease in protein content (%) in the earthworm E. eugeniae (EE-MPT) may indicate the impact of microplastic stress in this species [2]. It has been reported that the decrease in protein content was due to oxidative stress; arachidonic acid metabolism and steroid biosynthesis may both contribute to the decrease when food is mixed with microplastics (MP). Disturbance in an organism's osmoregulation may also decrease protein concentration [17].

Carbohydrates are the principal and immediate energy precursors for organisms and are exposed to stress conditions, while proteins are spared during chronic stress. Glycogen content in earthworms indicates the energy reservoir available for metabolic processes such as growth, sexual maturity, and reproduction [5]. The present study observed a decrease in carbohydrates in both epigeic earthworms, E. fetida and E. eugeniae, when exposed to microplastics (MP); this may be due to hypoxia, which suddenly maximises carbohydrate consumption. Similarly [8], decreased levels of lactate and maltose were also observed, indicating disruption of energy metabolism.

Total, saturated, and unsaturated lipids, and the per cent of saturated and unsaturated lipids, vary across groups. All parameters in E. fetida were higher in the MP-treated group than in the Control-CM group, whereas they were higher in Control-CM and lower in the MP-treated group for *E. eugeniae* (Table III). % unsaturated lipids increased drastically in E. eugeniae and less in E. fetida compared with Control-CM [11]. Studies have also observed increased levels of monounsaturated and polyunsaturated lipids when earthworms were exposed to microplastics (MP) [9]. Studies have also reported higher levels of saturated fatty acids such as palmitic acid, stearic acid, and lauric acid in the earthworm *E. Andrei [12]* and reported that polystyrene microplastic exposure to earthworms causes decreased high-density lipoprotein cholesterol (HDL-C) and increased low-density lipoprotein cholesterol (LDL-C) in rats [10] and also reported that under extreme stress conditions, there is an increase in the levels of total Omega-3 fatty acids, which may correspond to the energy requirements of the organisms.

IV. SUMMARY AND CONCLUSION

The present study clearly indicates that microplastics have both negative effects and positive influences on the composition of various biomolecules in the epigeic earthworms, *E. fetida* and *E. eugeniae*. Results showed that MP treatment increased protein content in EF and decreased it in EE, whereas MP treatment decreased glucose content in both earthworm species compared with Control-CM. Total lipid content was higher in EF-MPT than in Control-CM, but lower in EE-MPT than in Control-CM. Saturated lipids were higher in EF-MPT and lower in Control-CM, whereas in EE, they were higher in Control-CM than in MPT. For unsaturated lipids, EE-MPT showed the maximum and Control-CM the minimum, whereas in EF, Control-CM showed less and MPT more.

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Hence, based on the above results, it can be concluded that both epigeic earthworm species contain all major biomolecules (Protein, glucose, and lipid) in various forms. Still, MP exposure influences the composition and percentage of these biomolecules, both positively and negatively, in different earthworm species.

DECLARATION STATEMENT

Some of the cited references are older and are noted explicitly as [4]. However, these works remain significant for the current study, as they are pioneering in their fields.

After aggregating input from all authors, I must verify the accuracy of the following information as the article's author.

- **Conflicts of Interest/ Competing Interests:** Based on my understanding, this article has no conflicts of interest.
- **Funding Support:** This article has not been funded by any organisations or agencies. This independence ensures that the research is conducted objectively and free from external influence.
- **Ethical Approval and Consent to Participate:** The content of this article does not necessitate ethical approval or consent to participate with supporting documentation.
- **Data Access Statement and Material Availability:** The data in this article are publicly accessible.
- **Author's Contributions:** Dr. Pulikeshi M. Biradar designed, supervised, data reviewing and drafting the manuscript. Suhas H. S. conducted all the experimental procedures as well as data compilation and statistical evaluation. Both authors were actively involved in the research and have approved the final version of the manuscript.

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