



On the Novel Process of Pristine Microplastic Bio-fragmentation by Zebrafish (*Danio rerio*)

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Abstract

Microplastics are highly persistent particles that deliberately contaminate our ecosystem. These small-sized particles can pass through filtering systems into the water bodies, affecting various forms of aquatic and terrestrial life. However, little is known about their fragmentation process within the organism's body. In previous studies, commercially available microplastics were used that are rarely found in the environment naturally, hence they cannot mimic the effects on our surroundings. Therefore, using the zebrafish, *Danio rerio* we have evaluated the process of bio-fragmentation of ingested pristine polyethylene microplastics which are widely used in our daily life. We have also examined their faecal pellets through Field Emission Scanning Electron Microscopy (FE-SEM) and Fourier transform infrared spectroscopy (FTIR). Our results show that zebrafish can potentially bio-fragment the pristine microplastic particles into nano-plastic within a short period of 24 h. Additionally, zebrafish cannot recognize the pristine microplastic particles and can ingest them as food. No mortality occurred during the experiment. Thus, we have identified a natural pathway of microplastic bio-fragmentation, introducing an emerging role of zebrafish in biogeochemical cycling and the fate of plastics.

Abbreviations

PEMPs	Pristine polythene microplastics (PEMPs)
mg	Milligram
FE-SEM	Field Emission Scanning Electron microscopy
FTIR	Fourier Transform Infrared Spectroscopy
COVID-19	Coronavirus disease
PPE	Personal Protective Equipment
LDPE	Low Density Polyethylene
PVC	Polyvinyl chloride

Microplastics are a growing global issue. When plastic gets discharged into the environment, it degrades, causing severe problems (Zalasiewicz et al. 2016). Because of their tiny size, a significant number will pass through filtering systems and into water bodies, affecting aquatic life (Wang et al. 2018; Miraj et al. 2019). In the year 2020, The COVID-19 pandemic increased the production and consumption of plastics in the form of facemasks, face shields, and personal protective equipment (PPE) kits, that got degraded,

exacerbating the burden of microplastics on the environment (Dharmaraj et al. 2021).

Fish is an essential component of the food chain, and fish-meal is a potential pathway for microplastic contamination into the aquatic environment (Thiele et al. 2021). The processes of bioaccumulation and biotransformation of microplastics in fishes is well documented (Cedervall et al. 2012; Lu et al. 2016; Limonta et al. 2021). However, no research has been reported on the biological fragmentation of microplastics in fishes which are ingested as food. The biological fragmentation of polyethylene particles in soil is often associated with microbial degradation (Paço et al. 2017; Dey et al. 2020; Khan et al. 2022), which proceeds slowly due to the limited availability of oxygen, light, and microorganisms capable of the polymer degradation (Rogers et al. 2020). Similarly, the bio-fragmentation of microplastics have also been reported for some marine invertebrates such as Antarctic krill (*Euphausia superba*), Amphipod (*Hyaella azteca*) (Dawson et al. 2018; Borges et al. 2022).

In laboratory, researchers have acquired microplastic particles from scientific vendors of precise size and shape (Espinosa et al. 2018; Yang et al. 2019; Malafaia et al. 2020). These microplastic particles have uniform sizes and smooth textures which might pass and unaffected through, or might sufficiently too small to translocate in the organism body (Dawson et al. 2018), whereas the microplastics found in

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environment are degraded plastic polymers having irregular in sizes and rough surfaces which may retain in organism body for longer period and can mimic the natural effects of microplastics in our delicate ecosystem. Therefore, it is necessary to understand the impact of pristine microplastic particles (PEMPs) that are present in the environment.

In the present study, we have focussed on the biological fragmentation of pristine polyethene microplastics (PEMPs) eaten by zebrafish and have performed chemical structure analysis of PEMP before ingestion and of the egested faecal pellets of zebrafish containing PEMP through Fourier Transform Infrared Spectroscopy (FTIR) and morphological studies by Field Emission Scanning Electron microscopy (FE-SEM). The primary objective of this study is to identify the biological fragmentation process of PEMP eaten by zebrafish and to demonstrate that zebrafish can potentially ingest pristine polyethene microplastic particles (PEMPs) and egest them by converting them into fragmented nano-plastics. In the present research, the high concentration of PEMP (20 mg/L) was selected for zebrafish exposure to inculcate the severe response during the experiment. The tested concentrations are equivalent to 9.4×10^7 particles/ m^3 , quantified according to Leusch & Ziajahromi (2021) protocol. In surface water the MPs concentration can range from 1×10^{-2} to 10^8 particles/ m^3 (Koelmans et al. 2019). Therefore, the exposed PEMP concentration was environmentally relevant concentration in the present plastic pollution scenario.

Materials and Methods

Microplastic Characterization and Dispersion

Pristine microplastic particles were collected from polyethene-making industry in Bhopal (M.P). Microplastic particles were confirmed to be polyethene by Fourier Transform Infrared Spectroscopy, FTIR (3000 Hyperion Microscope with Vertex 80 FTIR System). The size and morphology of PEMP were determined through Field Emission Scanning Electron microscopy, FE-SEM (Zeiss ULTRA Plus). For experimentation, pristine microplastic particles were treated with Tween 80, a surfactant used to disperse microplastic particles in an aqueous solution. PEMP were soaked in 0.01% of Tween 80 for 24 h or until equal dispersion, (Lu et al. 2016). Then, PEMP were filtered with Whatman Grade 1 filter paper. Further, microplastic particles were dispersed in sterile aerated water.

Zebrafish Housing and Acclimatization

8–10 months old zebrafish adult of both sexes ($0.6 \text{ g} \pm 0.4 \text{ g}$; $3.5 \text{ cm} \pm 2 \text{ cm}$ total length-mean \pm SD-proportion 1:1) were purchased from commercial dealer in Bhopal, Madhya Pradesh, India, and were acclimatized for 15 days in laboratory conditions, in dechlorinated water providing photoperiod of 14 h light: 10 h dark. Temperature range of $25 \text{ }^\circ\text{C}$ – $27 \text{ }^\circ\text{C}$, pH 7–7.5 and dissolved oxygen of 2–4 ppm were maintained. Fishes were fed micropellets twice a day, (Westerfield et al. 2000; Kim et al. 2017).

Experimental Design

After acclimatization, 12 healthy zebrafish were transferred into ($n=6$, zebrafishes) in control group and ($n=6$, zebrafishes) in experimental group. Both groups were kept in the small tanks ($20 \text{ cm} \times 15 \text{ cm} \times 15 \text{ cm}$) filled with 2 L of water. The experimental group was exposed to water containing pristine polyethene microplastic particles (PEMPs). The fishes of the control group were maintained in PEMP-free aerated water. Upon transfer to the experimentation tank, zebrafish started consuming microplastics immediately present in the aquarium water. Control fishes were fed with commercially available food pellets (betta diet). The egested particles also contained organic matter as they were found into the bottom of the tank. After 24 h, the zebrafishes were removed from the tank and the faecal pellets were collected from both experimental and control tanks and observed under a light microscope with 40X resolution. To confirm microplastic fragmentation, the faecal pellets were washed with distilled water and air dried for 48 h. The dried pellets were observed under light microscope, and no faecal matter was observed with the particles.

Sample Analysis

The ingested and egested PEMP particles were examined through Fourier Transform Infrared Spectroscopy (FTIR) and further analysed using Field Emission Scanning Electron Microscopy (FESEM), (Wang et al. 2017; Truchet et al. 2021). Approximately 100 ± 20 PEMP particles were observed through FESEM from the control (PEMP before ingestion) and experiment (egested PEMP). SEM photomicrographs of PEMP particles were examined with Image J software for particle size measurements. Also, Leusch and Ziajahromi, (2021) method was adopted for PEMP particle counting.

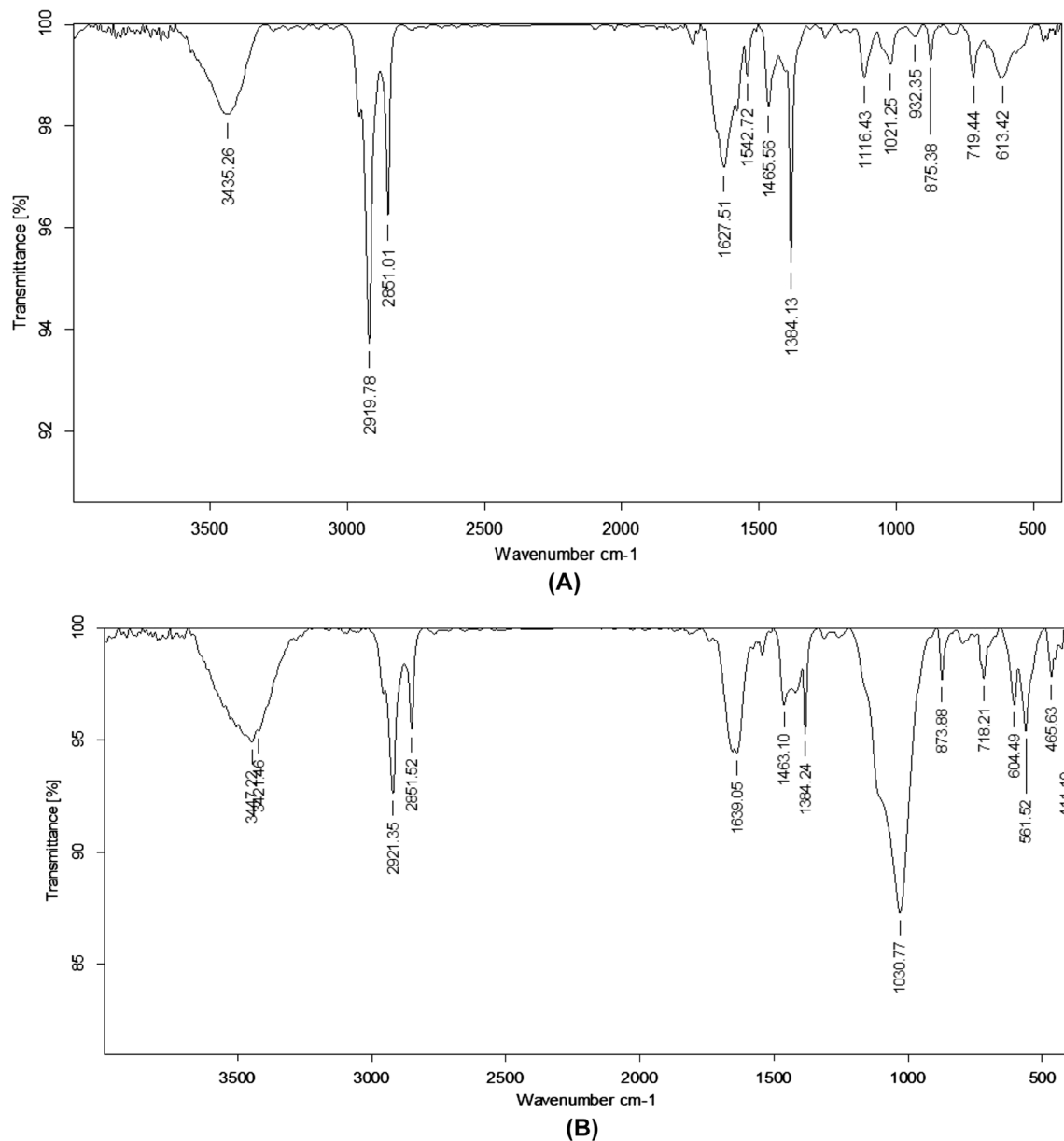


Fig. 1 **A** FTIR spectra of pristine polyethene microplastics (PEMPs) before ingestion (control) by zebrafish. **B** FTIR spectra of pristine polyethene microplastics (PEMPs) egested (Experimental) by zebrafish

Results

In the present study, we have observed the process of biological fragmentation of ingested microplastics by zebrafish, *Danio rerio*. Polymer identification and chemical structure alterations by FTIR spectroscopy. In our experiment, FTIR spectroscopy confirmed the microplastic particles are polyethene particles. FTIR spectra of PEMP before ingestion (control) are illustrated in Fig. 1A depicting peaks that belonged to polyethene at 3435.26 cm^{-1} indicated (O–H stretching), 2919.78 cm^{-1} & 2851 cm^{-1} indicated (C–H bond stretching). The peaks obtained at 1627.51 cm^{-1} (C=C

stretching alkene), 1542.72 cm^{-1} (CH₂ bending vibration), 1485.56 cm^{-1} and 1384.13 cm^{-1} (CH₃ methyl bending deformation) & the peak at 719.44 cm^{-1} were determined to be due to (CH₂ rock vibration) mode.

After 24 h of ingestion of the microplastics, zebrafish egested faecal pellets containing fragmented microplastics which were examined through Fourier Transform Infrared Spectroscopy (FTIR) as illustrated in Fig. 1B depicts the generation of new peaks at 1030.77 cm^{-1} (S–O stretching) and 873.88 cm^{-1} (C–H bending). The reduction in peaks in comparison to control PEMP was also been observed at 1467 cm^{-1} (C–H bending alkane), 718 cm^{-1} (C=C stretching

alkene), 1463.10 cm^{-1} (C–H bending alkane), 2921.35 cm^{-1} & 2851.52 cm^{-1} (C–H bond stretching). The overlapping peaks 3447.22 cm^{-1} and 3421.46 cm^{-1} with higher intensity were observed corresponding to (O–H stretching) alcohols representing the chemical structure alteration on the surface of the egested PEMP. The absorption peaks generated in the fingerprint region (1500 cm^{-1} – 500 cm^{-1}) of control PEMP (microplastic before ingestion) are different from the egested PEMP representing the change in the chemical structure of PEMP. Thus, the FTIR spectroscopy results suggested that the chemical structure of PEMP gets biologically altered after being digested by zebrafish.

We have identified the morphological alterations in the PEMP by Field Emission Scanning Electron microscopy (FE-SEM). We have examined PEMP particles before ingestion (control) and egested PEMP in faecal pellets (experimental) as illustrated in Fig. 2A and B. The surface of egested PEMP becomes rough, and cracks had appeared. Thus, the reduced size and significant alterations in their morphology suggest that the egested PEMP get breakdown into nano-plastic particles. Approximately 100 ± 20 PEMP particles were observed through SEM from the control (PEMP before ingestion) and experiment (egested PEMP). SEM photomicrographs of PEMP particles were examined with ImageJ software for particle size measurements. The observed size range of PEMP before ingestion is between $10\text{ }\mu\text{m}$ and $100\text{ }\mu\text{m}$ with mean diameter of $76.74\text{ }\mu\text{m}$ (± 14.07 standard deviation, S.D), whereas the size range of egested PEMP is between 100 nm and $10\text{ }\mu\text{m}$ with mean diameter of $5.92\text{ }\mu\text{m}$ (± 4.96 standard deviation, S.D) which shows approximately 70% size reduction in PEMP particles after digested by the zebrafishes.

Discussion

The zebrafish do not have a true stomach. Without a stomach, digestion and absorption must begin as early as possible in the limited length of the zebrafish digestive tract. Its digestive system is however segmented into the mouth, oesophagus, three gut segments (anterior, middle, and posterior), and anus. The zebrafish oesophagus is connected with the anterior gut segment, where nutrient absorption predominantly occurs due to the high presence of digestive enzymes. Under normal physiological conditions, the pH of the intestinal lumen of zebrafish is alkaline (> 7.5) (Nalbant et al. 2004; Flores et al. 2020). Ingested food is temporarily stored in the rostral intestinal bulb that bulges like an elastic sac, where food starts to be broken down in the absence of a stomach, and the entire length of the intestine may serve to degrade food (Kapoor et al. 1975; Wallace et al. 2005; Nadal et al. 2020). Our results are inline with these findings, it might be possible that PEMP particles get fragmented in

the zebrafish intestine due to the presence of different digestive enzymes and microbiomes in the digestive system.

Our results clearly demonstrate that zebrafish can significantly alter the structural integrity and cause reduction in the size of microplastics engulfed in feeding by a process called bio-fragmentation. We have identified that zebrafish readily ingested and egested the microplastics within 24 h. In a previous study, Micusik et al. (2021) reported degradation of LDPE which was accidentally found in the stomach of catfish in the Bodrog River, Slovakia. On the contrary, we have performed a series of a controlled experiments which demonstrate the gradual feed intake of pristine polyethylene microplastics particles by the zebrafish which gets bio-fragmented or bio-degraded, followed by their egestion as smaller fragments of microplastics.

During the experiment, we also observed that zebrafish got confused with the microplastic particles as food and consumed all of them, as reported earlier by Ory et al. (2018) & Lusher et al. (2020). After 24 h of ingestion, we have observed the significant colour differences in the faecal pellets of zebrafishes of the control and experimental fishes. The faecal pellets of both control and experimental fishes were analysed under a light microscope ($40\times$ resolution) and found to be microplastic particles in the faecal pellets of PEMP exposed zebrafish, illustrated in Fig. 3. There were no mortalities or any kind of adverse effects on the body condition of fishes during the feeding with PEMP.

Fourier Transform Infrared Spectroscopy (FTIR) has been widely used to identify the polymer and changes in the chemical structure of the polymer (Chalmers and Dent. 2006; Veerasingam et al. 2020). The observed FTIR spectra of PEMP particles before ingestion (control) showed the incorporated carbonyl groups, carboxylic acids, hydroxyl groups and alkene double bonds confirming the microplastics is low density polyethylene (LDPE). In our observations, FTIR spectra have exhibited the generation of peak at 3435.26 cm^{-1} representing O–H bond stretching, while peaks at 2919.78 cm^{-1} and 2851 cm^{-1} representing CH bond stretching. The peak that was also found at 1627.51 cm^{-1} , which representing C=C stretching alkene bonds, and the peak that was found at 1542.72 cm^{-1} , which showed CH_2 bending vibration. In addition, we also identified the peaks at 1485.56 cm^{-1} and 1384.13 cm^{-1} exhibited CH_3 methyl bending deformation. These peaks, along with the peak that was observed at 719.44 cm^{-1} , were a result of the rock vibration mode. Our investigations are in agreement with those of D'Souza et al. (2021), who also found similar peaks in LDPE.

In our study, FTIR spectroscopy also confirmed the level of structural changes in egested PEMP by the zebrafish. During the process of microbial biodegradation microorganisms secrete various extracellular enzymes, that facilitate a number of chemical reactions that may lead to alteration in

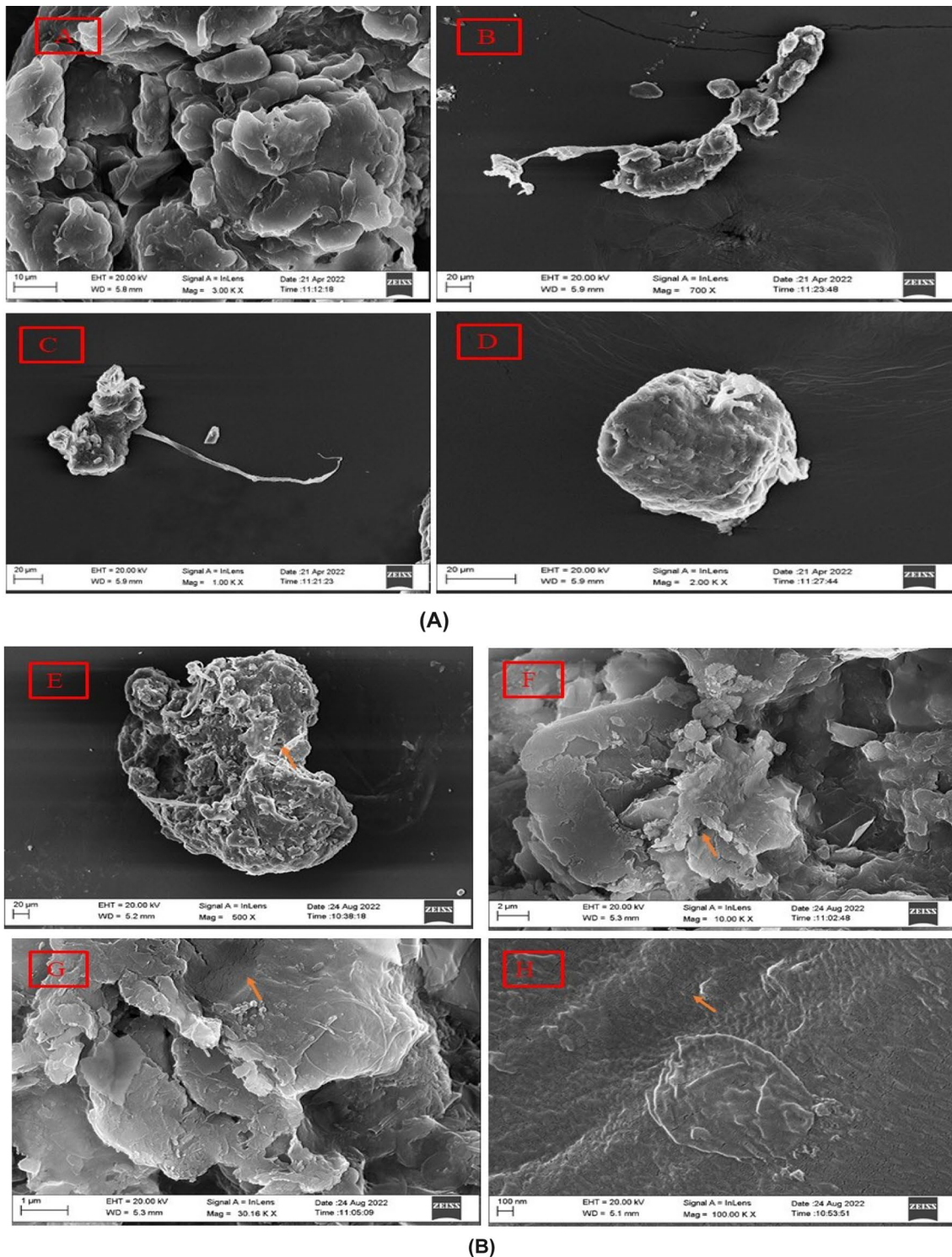
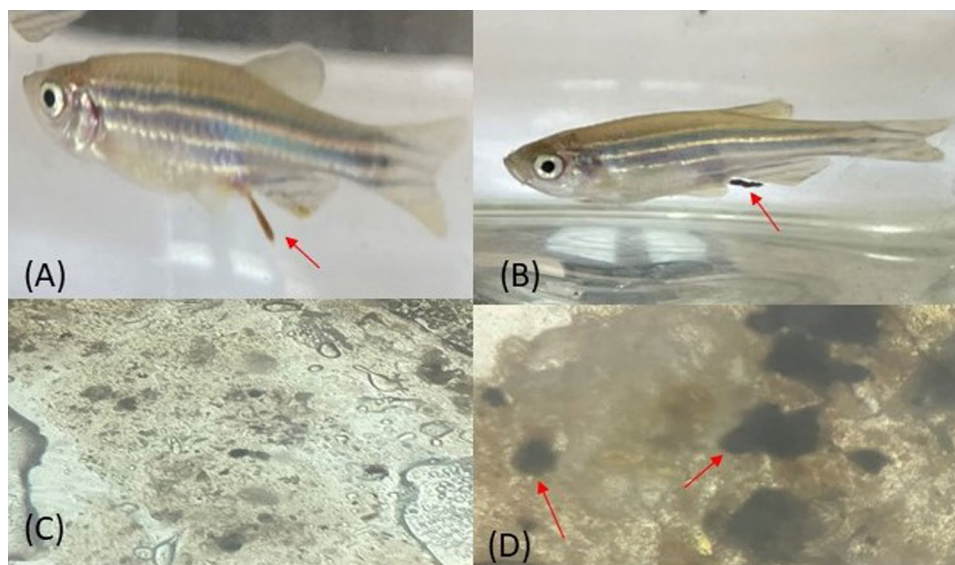


Fig. 2 A FE-SEM images of pristine polyethene microplastics (PEMPs) before ingestion (control) of zebrafish. **B** FE-SEM images of egested pristine polyethene microplastics (PEMPs) by zebrafish. **▶** showing cracks and holes caused by bio fragmentation

chemical conversions, including oxidation, reduction, esterification, hydrolysis, and inner molecular conversion. These chemical reactions reflected the change in the PE carbon

backbone by the formation of various new functional groups and dissolution of older groups, indicating biodegradation process which has also been demonstrated by Muhonja et al.

Fig. 3 Faecal colour changes **A** control and **B** Zebrafish egesting microplastics; Faecal pellets observed under light microscope (40X resolution) **C** Control and **D** Zebrafish faecal pellets containing PEMP



(2018) and Khan et al. (2022). Similarly, the FTIR spectra of egested PEMP showed the variations in older peaks and the formation of new peaks confirming the biological oxidation or degradation of pristine microplastic particles by the zebrafishes.

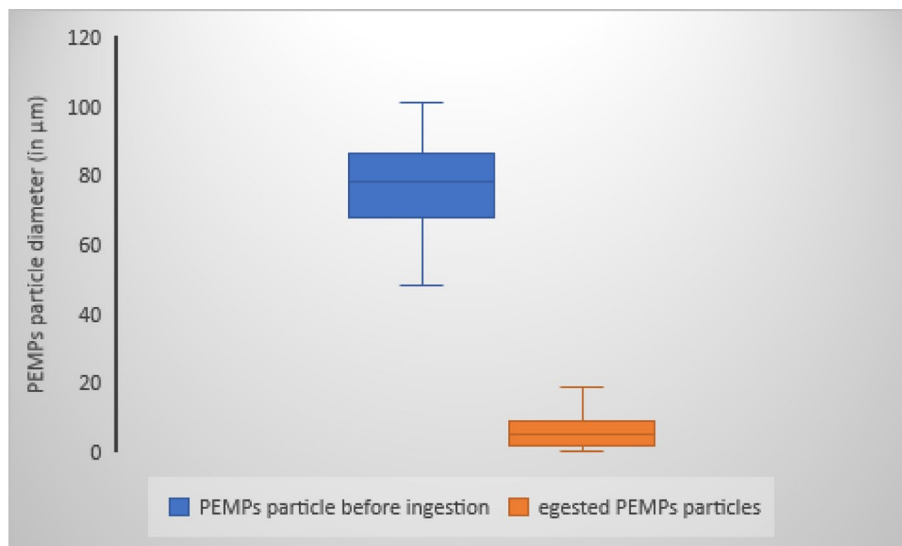
Field Emission Scanning Electron Microscopy (FESEM) allows the determination of biodegradation of polymers by investigating the morphological properties. Using this FESEM, we have found that fish egested PEMP developed cracks and holes at the interface with approximately 70% reduction in the size of PEMP from 100 μm to 100 nm mean diameter of 5.92 μm (± 4.96 standard deviation, S.D) as illustrated in Fig. 4. Surface erosion, cracks and holes have been observed in the photomicrographs of egested PEMP particles. These characteristics of egested PEMP were similar to the previous studies of polyethylene

biodegradation. This confirms the degradation of the microplastics by the zebrafish during feeding and egestion process. This is in conformation of a report by Wang et al. (2017), who also found development of cracks in the PVC particles which were obtained from ocean trawl and fish guts using Field Emission Scanning Electron Microscopy.

Conclusion

The findings of the present study indicate that zebrafish can potentially bio-fragment the pristine microplastic particles, consumed during the process of feeding, within a short period of 24 h. Physical and chemical alterations on the surface of the polymers were the results of bio-fragmentation of

Fig. 4 Difference in particle size of PEMP before ingestion and egested by zebrafishes (Mean \pm S.D)



microplastic particles during the process of feeding, ingestion, digestion and egestion. The present work aspires to constitute a stepping stone towards the natural process of biological mitigation of the microplastics. Thus, we have identified a new natural pathway of microplastic bio-fragmentation, introducing an emerging role of zebrafish in biogeochemical cycling and the fate of plastics. Further investigations are needed to understand the role of metabolic enzymes of zebrafish, which highlight the efficient bio-fragmentation of pristine polyethene microplastics at cellular and molecular levels.

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Author Contributions Both authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Darakhshan Khan and Dr. Sharique A. Ali. Both authors read and approved the final manuscript.

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Declarations

Conflict of interest None.

Ethical Approval The present experimental work was carried out in the laboratory of the Department of Biotechnology, Saifia Science College Bhopal, India, approved by Institutional Animal Ethics Committee (IAEC) under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, Government of India letter No. 2154/PO/Re/S/22/CPCSEA.

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