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Impacts of physical-chemical property of polyethylene on depolymerization and biodegradation in yellow and dark mealworms with high purity microplastics

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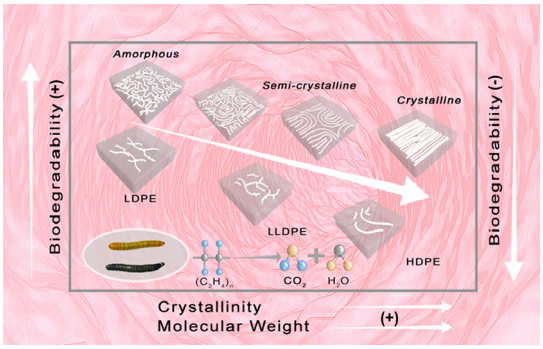
HIGHLIGHTS

- Comparison of biodegrability of LDPE, LLDPE and HDPE by yellow and dark mealworms
- Sequence of biodegradation extent showed LDPE > LLDPE> HDPE
- Low molecular weight, high branching and low crystallinity are positive for biodegradation.
- Molecular weight is a key factor influencing biodegradability.

GRAPHICAL ABSTRACT



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ABSTRACT

Yellow and dark mealworms (*Tenebrio molitor* and *Tenebrio obscurus*) biodegrade commercial polyethylene (PE) materials at a high rate. We examined the impact of physical and chemical properties on biodegradation using high purity microplastics (MPs). These included high-density polyethylene (HDPE), low-density polyethylene (LDPE), and linear low-density polyethylene (LLDPE), all with different weight average molecular weights (M_w) and different crystallinity degrees in *T. molitor* and *T. obscurus* larvae. The biodegradation extent in the two mealworms was similar but strongly depended on the polymer type in sequence, since LDPE > LLDPE> HDPE (with respective M_w of 222.5, 110.5 and 182 kDa). When LDPE MPs with M_w of 0.84, 6.4 and 106.8 kDa and HDPE with M_w of 52, 105 and 132.7 kDa were tested, the PE MPs with lower M_w showed a greater extent of depolymerization. The results of dominance analysis indicated that less branching structure and higher crystallinity degree negatively impacted depolymerization and biodegradation. Py-GC/MS analysis confirmed the breaking of the macromolecule backbone as well as the formation of oxidized functional groups after all the tested PE materials passed through the mealworm intestine. The results demonstrated that molecular weight, PE type, branching, and crystallinity degree significantly affect the biodegradation capability of PE by the mealworms, and possibly by other biological systems as well.

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1. Introduction

The discovery of polymer materials in the early 20th century paved the way for the manufacture of plastics, an innovative material which is now abundant in many industries across the world (Rasmussen, 2021). In 2020, the annual production of plastics was found to be upwards of 367 million tons (PlasticsEurope, 2021). Among the hundreds of different global plastics that are in demand distribution by resin type, no polymers are in as high demand as polyethylene (PE), 31.3%; polypropylene (PP), 19.7%; polyvinylchloride (PVC), 9.6%; polyethylene terephthalate (PET), 8.4%; polyurethane (PUR), 7.8%; and polystyrene (PS), 6.1% (PlasticsEurope, 2021). However, because of the prevalence of plastic in society, plastics waste has become a major environmental concern; contamination by plastics solid wastes, debris, microplastics (MPs), and even nanoplastics (NPs) is all on the rise (Geyer et al., 2017; Wu et al., 2017; L. Wang et al., 2020; Z. Wang et al., 2020).

Among the plastics, PE is the most used polymer around the world, with over one trillion plastic bags made of PE being used every year (Geyer et al., 2017). PE is expressed as "$[CH_2 - CH_2]_n$" and comprises a linear backbone of carbon atoms. Commercial PE polymers include high-density polyethylene (HDPE), linear low-density polyethylene (LLDPE), and low-density polyethylene (LDPE) (Wilkes and Aristilde, 2017). As shown in the Graphic Abstract, HDPE has a high degree of crystallinity and is composed of linear chains which are packed closely together with a very low level of short chain branching. HDPE has little or no branching and the molecules can stack and form strong intermolecular forces (typically <2 CH_3 groups/1000 C atoms). LLDPE is composed of linear molecules with no long chains of middle crystallinity as well as a higher level of short chain branching than HDPE. It contains more short branches (10–30 CH_3/1000 C), which are caused by the introduction of one or more co-monomers such as 1-butene, 1-hexene, and 1-octene (Ojeda et al., 2011). LDPE is characterized by a significant level of long chain branching (typical branch length of several hundred carbon atoms) as well as short chain branching (2 to 6 carbon atoms long). The short branches of LDPE hinder close packing and result in a relatively low crystallinity (45–60%) because branching chains prevents tight packing into a crystalline structure (Wilkes and Aristilde, 2017). HDPE (as well as middle-density PE) contributes 12.9% of total plastics globally while LDPE and LLDPE contribute 17.4% (PlasticsEurope, 2021). The significant differences in their properties result in a diverse application of the polymers in manufactured products, which range from packaging film and toys to pipes and fuel storage tanks (Zhang et al., 2004; Koutny et al., 2006). On the other hand, the differing structures and physical properties of the PE polymers also affects their biodegradability and the efficiencies of microorganisms in attacking them (Gu, 2003; Hadad et al., 2005; Nowak et al., 2011; Restrepo-Flórez et al., 2014).

Since the early 1970s, tests on the biodegradation of PE products, primarily LDPE, have been performed using microorganisms enriched or isolated from various environmental sources which harbor a multitude of diverse microbial communities. These include sources such as soils, seawater, sludge, landfill, and compost (Restrepo-Flórez et al., 2014; Ghatge et al., 2020; Ru et al., 2020; He and Luo, 2020). These studies concluded that either PE is not biodegradable, or that PE degradation occurs at extremely slow rates (Shah et al., 2008). In studies that concluded the latter, the biodegradation of PE in the environment occurred mainly via the biological activity of microorganisms after photo- or thermo- oxidation (Shah et al., 2008; Wagner and Lambert, 2018). Thus, given appropriate conditions, slow PE biodegradation (in periods of weeks/months) by microorganisms (bacteria, fungus etc.) can be observed, (Ghatge et al., 2020; Ru et al., 2020).

The biodegradation of plastics via microbiological and enzymatic approaches has been investigated for decades (Wei et al., 2020; Inderthal et al., 2021). However, to date, research results, primarily on biodegradation of PET, indicate that physical and chemical structure and properties of the polymers impact degradation and biodegradation, with the major influencing factors including surface hydrophobicity, molecular weight distribution (MWD), physical structure, mechanical property, and crystallinity (Restrepo-Flórez et al., 2014; Shah et al., 2008; Ghatge et al., 2020; Min et al., 2020; Wei et al., 2020; Inderthal et al., 2021). The polymer molecular weight is expressed as the number-averaged (M_n), weight-average (M_w), and size-averaged weight (M_z). In general, non-hydrolyzable plastics (PE, PP, PS, PVC, etc.) are more resistant to biodegradation than hydrolyzable polymers (PET, PUR, etc.); degradation of longer chains and polymers with higher molecular weight or higher crystallinity is more difficult than that of shorter chain bonds or lower molecular weight or lower crystallinity polymers. Previous studies on plastics degradation, which were mainly focused on PE degradation, reported different results depending on the PE products tested. Previous research reported that low molecular weight PE or low molecular PE wax (molecular weight around 1.0 kDa or less) could be consumed relatively quickly or degraded by bacterial consortium with a mass reduction of 31.5% in 21 days (Kawai et al., 2004). A *Pseudomonas* sp. degraded PE wax (M_w of 1.7 kDa) in sterilized compost conditions after 80 days (Yoon et al., 2012). Another study on microbial degradation of PE materials of M_w < 1.5 kDa also showed 50% mass reduction in 120 days (Chiellini et al., 2003). For higher molecular weight PE, however, it took more than two months for a PE-degrading *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1 to degrade about 6.5% and 11.3% of commercial LDPE film with a M_w of 88.2 and M_n of 27.7 kDa (Yang et al., 2014). A co-culture of *Acinetobacter* sp. NyZ450 and *Bacillus* sp. NyZ451 could reduce 18% of the weight of PE of mulching film (M_n of 27.5 and M_w of 179.3 kDa) over 30 days (Yin et al., 2020). To date, no report has been published to compare the biodegradation performance of PE materials with different molecular weight and physical properties with either mixed cultures or single microbial cultures. Additionally, the impact of crystallinity on PE biodegradation has not yet been specially addressed. Crystallinity defines the degree of long-range order in a material and the more crystalline a polymer, the more regularly aligned its chains. Increasing the degree of crystallinity increases hardness and density. Enzymatic degradation of PET indicated that increasing the degree of crystallinity increases hardness and density and thus negatively affects enzymatic reactions (Inderthal et al., 2021; Wei et al., 2020).

Recently, the ability to rapidly biodegrade plastics (PE and PS) has been found in insect larvae within the darkling beetle families (Coleoptera: Tenebrionidae), i.e., *Tenebrio molitor*, *Tenebrio obscurus*, *Zophobas atratus*, *Tribolium castaneum*, *Plesiophthalmus davidis*, and honey bee pest *Uloma* sp. (Yang et al., 2015a; Brandon et al., 2018; Peng et al., 2020a, 2020b; Peng et al., 2019; Yang et al., 2020; L. Wang et al., 2020; Z. Wang et al., 2020; Woo et al., 2020; Kundungal et al., 2021), as well as in larvae of pest moths (Lepidoptera: Pyralidae), i.e., Indian mealmoths (*Plodia interpunctella*) (Yang et al., 2014), greater waxworms (*Galleria mellonella*) (Bombelli et al., 2017; Kong et al., 2019), and lesser waxworms (*Achroia grisella*) (Kundungal et al., 2019). Currently, the most studied insect species for plastic biodegradation are yellow mealworms (the larvae of *Tenebrio molitor* Linnaeus 1758) and dark mealworms (the larvae of *Tenebrio obscurus* Fabricius 1792) which belong to Tenebrionidae, the seventh most speciose taxon in Order Coleoptera, which comprises of about 20,000 species in 2300 genera worldwide (Slipinski et al., 2011). It has been observed the yellow and dark mealworms have the novel ability to biodegrade all major polymers, i.e., PS (Yang et al., 2018a, 2018b; Yang et al., 2015a, 2015b; Tsochatzis et al., 2021a, 2021b), LDPE (Brandon et al., 2018; Yang et al., 2021a, 2021c), PVC (Peng et al., 2020a), PP (Yang et al., 2020), and polylactic acid (PLA) (Peng et al., 2021). Up to 50% of mass of the tested plastics polymers can be reduced in less than 24 h after passage through the larval intestine when the larvae are fed with PS or LDPE as their sole diet for 15–21 days (Yang et al., 2015a; Brandon et al., 2018; Yang et al., 2021a, 2021c). Therefore, these insects can serve as an ideal "invertebrate bioreactor" for testing biodegradation of plastics and providing scientific evidence of plastic biodegradation within a relatively short test period compared to laboratory tests with single or mixed microbial cultures.

To date, the results of tests regarding the mealworms demonstrate that high molecular weight commercial plastic polymers are susceptible to rapid biodegradation and mineralization in the mealworms (Yang et al., 2015a,

2015b; Yang et al., 2018a, 2018b; Brandon and Criddle, 2019; Peng et al., 2019). Both *T. molitor* and *T. obscurus* larvae demonstrated the ability of biodegrading expanded PS foam (M_w 345 kDa and M_n 107 kDa) (Peng et al., 2019), while *T. molitor* larvae has shown the capability of degrading LDPE foam (M_w 184.6 kDa and M_n 27.48 kDa) at a comparable rate (Brandon et al., 2018). Similar results were later observed for both *T. molitor* and *T. obscurus* larvae during the biodegradation of two LDPE foams with M_n of 28.9 and 27.30 kDa, and M_w of 342 and 264 kDa (Yang et al., 2021c). Broad depolymerization (i.e., a decrease in both M_n and M_w) was observed in the aforementioned studies. However, different depolymerization patterns were found during the biodegradation of LDPE foam in *T. molitor*, i.e., the M_w of residual LDPE polymer was decreased from 288.7 to 241.5 kDa but M_n increased from 35.8 to 66.8 kDa (Yang et al., 2021a). Commercial LDPE products (e.g., foam, film) are a mixture of LDPE, LLDPE, and HDPE. Therefore, the results obtained with commercial PE materials could be influenced by their polymer components. Moreover, the mass reduction could also be impacted by additives in the PE products such as antioxidants, antiblock compounds, and slip agents (Hahladakis et al., 2018). The research on mechanisms of PE biodegradation using defined polymers rather than commercial mixtures could provide an in-depth mechanism for PE biodegradation. However, as of now, the biodegradation of high purity LDPE, LLDPE, and HDPE with defined physical and chemical properties (e.g., molecular weight and crystallinity) in *Tenebrio* larvae has not been characterized.

In this study, we used two *Tenebrio* species, *i.e*, *T. molitor* and *T. obscurus* larvae, to investigate biodegradation of high purity LDPE, LLDPE, and HDPE microplastics with different physical and chemical properties (molecular weights and crystallinity degree). Subsequently, six high purity PE samples of LDPE of M_w of 0.84, 6.4 and 106.8 kDa, and HDPE with M_w ranging from 52, 105 and 132.7 kDa were tested for biodegradation. The results obtained were then analyzed using a model developed by Budescu's (1993) to analyze the impacts of major variables (PE types, molecular weight change, crystallinity degree, and larval species) on PE depolymerization/biodegradation. The results indicated that the two *Tenebrio* species do degrade all tested PE materials with similar efficiency, but that the extent of PE depolymerization and biodegradation was significantly impacted by their physical and chemical properties, especially their molecular weight and branching structure.

2. Material and methods

2.1. Mealworm species and feedstocks

Tenebrio molitor larvae with an average weight of 27 ± 0.5 mg/larva, the length 1.6 ± 0.1 cm/larva and a growth stage of 4–5 instar were purchased from Dafa Birds & Flower Market in Harbin, China (Graphic abstract, Fig. S1A). *T. obscurus* larvae with 130 ± 5.0 mg/larva, length 2.5 ± 0.2 cm/larva and a growth stage of 5–6 instar were purchased from Zaozhuang Breeding Farm in Shandong, China (Fig. S1B). Prior to the experiments, both *T. molitor* and *T. obscurus* larvae were fed with a normal diet wheat bran for comparison although *T. obscurus* larvae prefer corn flour as primary diet for growth (Peng et al., 2019). According to the suppliers, the larvae were not fed with any antibiotics. They were subjected to a 48-h starvation period to empty their guts before initiating experimental diets.

High purity microplastic resin powders of HDPE, LDPE, and LLDPE manufactured according to ASTM International (ASTM Standard, 2017) were purchased via Shanghai Plastics Trading Co., Ltd., Shanghai, China (Fig. S1C). The powders were high in density, with densities ranging between 0.918 and 0.924 g cm^{-3}, and high molecular weights (Table S1), i.e., M_w of 182, 222.5 and 110.5 kDa; M_n of 59.5, 93.2 and 42.2 kDa; and M_z of 408.8, 456.5, and 216.1 kDa. Comprised of high purity PE of their respective PE types, the powders were different from commercial LDPE or LLDPE film, which are mixtures of LDPE, LLDPE and HDPE with LDPE or LLDPE as dominant component. The purchased resin powders were used as feedstocks to test the impact of the polymer types of HDPE, LDPE, and LLDPE on biodegradation (Fig. S1D). Furthermore, these PE materials did not contain any additives or catalysts.

Six high purity LDPE and HDPE powder samples (size <0.4 mm) with different molecular weights were purchased from American Polymer Standards Corp. (Mentor, Ohio, USA) to test the impact of molecular weight and branching on PE biodegradation. The three LDPE powders had a respective M_w of 0.84, 6.4 and 106.8 kDa corresponding to M_n of 0.72, 3.2 and 12.9 kDa, while the three HDPE had a respective M_w of 52, 105 and 132.7 kDa corresponding to M_n of 18.3, 10.4 and 82.6 kDa, respectively. These six MPs were labeled as $LDPE_{840}$, $LDPE_{6400}$, $LDPE_{102000}$, $HDPE_{52000}$, $HDPE_{105000}$, and $HDPE_{132700}$ (Fig. S1E). The physical properties of the aforementioned high purity PE MPs are summarized in Tables S1 and S2.

Agar was purchased from Beijing Aoboxing Bio-Tech Co., Ltd. Wheat bran was purchased from a farm in Harbin, China. All chemicals used for analyses were analytical grade from Sinopharm Chemical Reagent Co., Ltd., Beijing, China.

2.2. Biodegradation of different PE polymers

The biodegradation of PE was conducted in two tests and in duplicate. The first test was performed to compare three high purity PE polymers. Prior to feeding the larvae, each of the PE MPs (25 g) were mixed in liquid agar (30 mL, 3%, w/w). After it solidified, the PE-agar mixture was fed to the larvae over 21 days. *T. molitor* and *T. obscurus* larvae were divided into two groups, respectively. Each group consisted of three incubators made of food grade PP food containers (1000 mL in volume) with 400 larvae each. The larvae were fed with either HDPE, LDPE, or LLDPE MPs as their primary diet for 21 days in order to evaluate biodegradation performance of HDPE, LDPE, and LLDPE, respectively.

The second test was designed to examine biodegradation of LDPE and HDPE MPs with different molecular weights. Prior to testing, the PE MPs (0.4 g) were mixed with distilled water (0.25 mL) and wheat bran (0.4 g), resulting in a PE-bran mixture. The test matrix consisted of six *T. molitor* incubators and six *T. obscurus* incubators (400 larvae in each incubator) set up according to the same conditions as the first test. The mealworms were fed with the respective PE-bran mixtures (i.e., $LDPE_{840}$, $LDPE_{6400}$, $LDPE_{102000}$, $HDPE_{105000}$, $HDPE_{52000}$, and $HDPE_{132700}$) for 5 to 15 days. The incubation duration ended when all PE MPs were ingested by the larvae. During incubation, dead mealworms were removed immediately to avoid cannibalism. All incubators were maintained in a 250 L artificial climate chamber (Artificial climate incubator, Shanghai Shuli, Shanghai, China) at 25 ± 0.5 °C and 65 ± 5% humidity.

At the end of the test, the larvae were cleaned with compressed air and then transferred to a clean box for 12 and 18 h to collect frass samples. The frass samples from samples of duplicate incubators were combined together and then stored in a refrigerator at −20 °C (BCD-215WTM, Media, Shenzhen, China) for further analysis.

2.3. Characterization of residual PE polymers in frass

All analyses were detailed in supporting information (SI) and employed using established methods for the characterization of plastic biodegradation as described in previous publications (Brandon et al., 2018; Yang et al., 2018a, 2018b; Peng et al., 2019; Yang et al., 2021a, 2021b, 2021c; Wu and Criddle, 2021). All analyses were performed in triplicate.

To characterize the depolymerization of PE polymers in frass, the residual PE in the frass was extracted using 1, 2, 4-trichlorobenzene (1, 2, 4 TCB). M_n, M_w, and M_z and MWD were determined by high-temperature gel permeation chromatography (HT-GPC) (PL-GPC 220, Agilent Technologies, Inc., USA) at 150 °C using 200 μL injection volume with an eluent (1,2,4 TCB) flow rate of 1.0 mL/min. The analysis was performed as described previously (Yang et al., 2021a, 2021c). Polydispersity index (PDI) was calculated as PDI = M_n/M_w, which gives an indication of broadness of the polymer distribution (Wu and Criddle, 2021). The reduction of molecular weight (%) was calculated as.

$$R\ (\%) = (M_o - M_f)/M_o \times 100\% \quad (1)$$

where R is the change (%), M_o is molecular weight of the virgin PE sample, and M_f is the molecular weight of residual PE extracted from frass. When molecular weight decreases, R is expressed as a positive value. When molecular weight increases, the R value becomes negative.

Analyses of the water contact angle (WCA) of the PE powder and frass samples from PE-fed larvae were performed using Dataphysics DCAT21 (Heilna Trade GmbH, Germany).

Characterization of oxidization/biodegradation of PE was performed using Fourier transform infrared (FTIR) spectroscopy (Nicolet iS50 FTIR Spectrometer, Thermo Fisher Scientific, U.S.A.), Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy with a Bruker Avance 600 MHz NMR spectrometer (Bruker Corporation, Germany), an X-ray diffraction instrument (Bruker D8 Advance, Germany), and Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) with an Agilent GC/MS instrument (7890B-5977B GC/MS from Agilent Technologies, USA) combined with a PY-3030D pyrolizer unit (EGA/PY-3030D, Frontier, Japan) from Frontier Laboratories. FTIR was used to characterize chemical modification of the polymer structures and monitor the formation of major oxidized functional groups of the residual PE polymers in the frass (Wu and Criddle, 2021). ^{1}H NMR was conducted to characterize the chemical modification of PE polymers as well as the degraded functional groups in the egested frass (Wu and Criddle, 2021), while spectra were analyzed using MestReNova software (version 6.1.1–6384-Win). Crystallinity degrees of the different PE MP powders were determined using an X-ray diffraction instrument. Py-GC/MS was performed to study the difference in chemical composition between original PE samples and residual PE polymers from the frass.

2.4. Dominance analysis

Dominance analysis (DA) was conducted to determine factors influencing PE biodegradation based on the method originally developed by Budescu's (1993) for the determination of predictor importance. In this study, we replaced the predictors with variables which are considered to have an impact on plastics biodegradation. This method is unique in that (a) it measures relative importance for each variable alone (e.g., R^2), and (b) the two variables (or predictors in the original model) are compared in the context of all $2^{(p-2)}$ models that contain some subset of the other variables, where p is the number of predictors. DA is more sensitive to the various importance patterns that can emerge in these cases and addresses a more general definition of importance. The criterion of general dominance for the ranking and scaling of importance across all the variables is presented for comparing the relative importance of variables (or predictors) in multiple regression (Azen and Budescu, 2003). Mathematically, general dominance weights are equal to the squared semi-partial correlation coefficients for each predictor averaged across all possible subset regression models.

To conduct the DA and relative weights, we firstly specified a multiple linear regression (MLR) model in a certain population with p ($p \geq 1$) variables (X_1, X_2, …, X_p) and one dependent variable Y:

$$Y = \beta_0 + \beta_1 X_1 + \ldots + \beta_p X_p + \varepsilon = \beta_0 + \sum_{j=1}^{p} \beta_j X_j + \varepsilon \quad (2)$$

where β_0 represents the constants, β_j is the population regression coefficient associated with the j_{th} variable (predictor) (X_j), and $\varepsilon \sim N(0, \sigma^2)$. The randomly selected set of n observations from that population will consist of (y_i, x_{i1}, x_{i2}, …, x_{ip}); $i = 1, 2, \ldots, n$. In this study, six variables including the PE type, mealworm species, M_n, M_w, M_z, and crystallinity degree (X_1, X_2, X_3, X_4, X_5, and X_6) describe the biodegradation of PE (Y, M_z reduction (%), M_w reduction (%), and M_n reduction (%), respectively). Using this dataset, unweighted least squares were introduced to obtain the estimated linear model, as shown in Eq. (3):

$$\hat{y} = \widehat{\beta_{0+}} \sum_{j=1}^{p} \widehat{\beta_j}\, x_j \quad (3)$$

where $\widehat{\beta_j}$ is the estimated regression coefficient associated with x_j.

We defined the model's predicted values as shown in Eq. (4):

$$\hat{Y} = \sum_{j=1}^{p} B_j x'_j \quad (4)$$

where B_j is the standardized regression coefficient associated with x'_j.

MLR analysis is a statistical method that uses multiple explanatory variables to predict or explain response variable. The major purposes of MLR analysis are prediction and interpretation. To analyze and interpret the function and significance of each variable on the dependent variable, the relative importance of each variable in the regression model was compared. A central principle of MLR analysis is the partitioning of the sum of squares of deviations (SSY, a measure of the variability in the response) into the regression sum of squares (SSR, the variability explained by the predictor variables x_1, x_2, …, x_p) and the residual (or error) sum of squares (SSE) (Chao et al., 2008). We denoted the squared correlation between the observed (Y) and predicted ($\hat{Y}$) values as the squared multiple correlation ($R^2_Y(x_1, \ldots, x_p)$), which is also known as the coefficient of determination (R^2), as shown in Eq. (5):

$$R^2 = \text{SSR}/\text{SSY} \quad (5)$$

The value of R^2 (= SSR in the unit length standardized regression model) reflects the relative degree of regression contribution, a measure often used to quantify model fit and which is interpreted as the proportion of the variance in Y that can be reproduced, or accounted for, by the p variables (or predictors) (Azen and Budescu, 2003). In this study, variables for PE degradation included molecular weights (i.e., M_n, M_w, and M_z), crystallinity degree, and mealworm species. R statistical software was used for data analysis and model development.

2.5. Statistical analysis

Statistical analyses were performed in IBM SPSS Statistics (version 25). To evaluate the differences in PE consumption as well as in changes in molecular weight. ANOVA was performed on the egested frass, followed by pairwise comparisons using Student's t-test with Tukey's correction to assess the differences between diets and species. All p-values are adjusted p values, and all error values are reported as average ± standard deviation.

3. Results and discussion

3.1. Depolymerization/degradation of LDPE, LLDPE and HDPE in mealworms

The consumption of the LLDPE, LDPE, and HDPE MPs by *T. molitor* and *T. obscurus* larvae was monitored over 21 days (Table 1, Fig. 1A_1–A_3). The larvae consumed 1.8–2.5 g PE/100 larvae. At the end of test, frass samples were collected for HT-GPC analysis. We used M_n, M_w, and M_z to examine the performance of depolymerization as well as biodegradation, as depolymerization is the first and essential step of the biodegradation of plastics (Wu and Criddle, 2021). M_n provides information about the lowest molecular weight portion of the sample, M_w is the average closest to the center of molecular distribution curve, and M_z represents the highest molecular weight portion of the PE sample (Yang et al., 2021a, 2021b, 2021c; Wu and Criddle, 2021).

The LDPE, LLDPE, and HDPE MPs tested had typical M_w (222.5, 110.5 and 182 kDa) for most commercial film products (Table S1). The depolymerization patterns of the three PE materials were different (Fig. 1A_1–A_3).

The molecular weight (M_n and M_w) of residual PE polymers from the frass of *T. molitor* and *T. obscurus* larvae fed with HDPE increased significantly (Table 1, $p < 0.05$), i.e., M_n increased by 30.08 ± 0.37% and 35.80 ± 0.38%, M_w increased by 10.00 ± 0.26% and 10.77 ± 0.17%, but M_z was basically unchanged (it decreased slightly by 1.03 ± 0.21% and increased only by 1.64 ± 0.13%, respectively). This indicated that depolymerization did occur but that it more rapidly reacted with smaller chains of polymers, resulting in the accumulation of longer chain polymers.

Table 1
Molecular weight changes of LLDPE, LDPE, and HDPE microplastics before and after digestion by *T. molitor* and *T. obscurus* larvae.

Polymer	Mealworm	Residual PE after biodegradation						
		M_w/M_n	M_n kDa	M_w kDa	M_z kDa	M_n %	M_w %	M_z, %
LLDPE	*T. molitor*	2.50	71.6 ± 3.0	179.2 ± 6.9	361.1 ± 12.6	−69.67 ± 0.12	−62.17 ± 0.24	−67.10 ± 0.56
	T. obscurus	2.35	82.2 ± 3.1	193.5 ± 7.1	379.1 ± 13.2	−94.79 ± 0.51	−75.11 ± 0.44	−75.43 ± 0.59
LDPE	*T. molitor*	2.35	67.6 ± 3.0	158.9 ± 5.8	306.5 ± 11.3	27.47 ± 0.08	28.58 ± 0.15	32.86 ± 0.14
	T. obscurus	2.35	69.0 ± 2.6	162.4 ± 6.0	315.0 ± 10.2	25.97 ± 0.16	27.01 ± 0.14	31.00 ± 0.30
HDPE	*T. molitor*	2.59	77.4 ± 3.0	200.2 ± 7.7	404.6 ± 15.7	−30.08 ± 0.37	−10.00 ± 0.26	1.03 ± 0.21
	T. obscurus	2.50	80.8 ± 3.1	201.6 ± 8.1	415.5 ± 16.9	−35.80 ± 0.38	−10.77 ± 0.17	−1.64 ± 0.13

Note: LLDPE powders: M_n = 42.2, M_w = 110.5, M_z = 216.1; crystallinity degree 51.9%. LDPE powders: M_n = 93.2, M_w = 222.5, M_z = 456.5, crystallinity degree 48%. HDPE powders: M_n = 59.5, M_w = 182.0, Mz = 408.8, crystallinity degree 55.3%. The initial number: *T. molitor* larvae, 400; *T. obscurus* larvae, 400. Molecular weight in kDa.

The unchanged M_z also indicated no or less breaking down of large molecular chains.

The molecular weights of the residual polymers from frass of *T. molitor* and *T. obscurus* larvae fed with LLDPE also increased significantly (Table 1, $p < 0.05$), i.e., M_n increased by 69.6 7 ± 0.12% and 94.79 ± 0.51%, M_w increased by 62.17 ± 0.24% and 75.11 ± 0.44%, and M_z by 67.10 ± 0.56% and 75.43 ± 0.59%, respectively. This indicated that the larvae of both mealworms performed depolymerization of shorter chain polymers more rapidly, resulting in accumulation of longer chain polymers. The significant increases ($p < 0.05$) in M_n and M_w of residual LLDPE and HDPE in the frass of the larvae of both species confirmed that both performed limited extent depolymerization (increase in M_n and/or M_w). The increase in M_z indicated that the long chain polymers also accumulated in the residual PE, which was different from what was observed during HDPE depolymerization.

The molecular weights of residual polymers from the frass of *T. molitor* and *T. obscurus* larvae fed with LDPE decreased significantly (Table 1), i.e., M_n decreased by 27.47 ± 0.08% and 25.97 ± 0.16%, M_w decreased by 28.58 ± 0.15% and 27.01 ± 0.14%, and M_z decreased by 32.86 ± 0.14% and 31.00 ± 0.30%, respectively. This showed a typical broad depolymerization pattern and indicated that the larvae depolymerized and biodegraded all chains simultaneously.

The depolymerization and biodegradation pattern of LLDPE and HDPE by both *Tenebrio* species demonstrated limited extent pattern (i.e., increase in M_n and M_w) which was similar to what was observed in LDPE biodegradation by *T. molitor* larvae from another source in Beijing, China (Yang et al., 2021a), as well as with biodegradation of PP foam by *T. molitor* in our lab (Yang et al., 2021b), and with PP degradation by a mesophilic bacterium *Stenotrophomonas* strain (Jeon and Kim, 2016). Interestingly, the depolymerization of LLDPE resulted in the increase of M_z, which was not reported previously. In this work, however, noticeable reductions in M_n, M_w, and M_z of LDPE by both larvae of *T. molitor* and *T. obscurus* indicated significant broad depolymerization: M_n, M_w, and M_z of LDPE ($p < 0.05$).

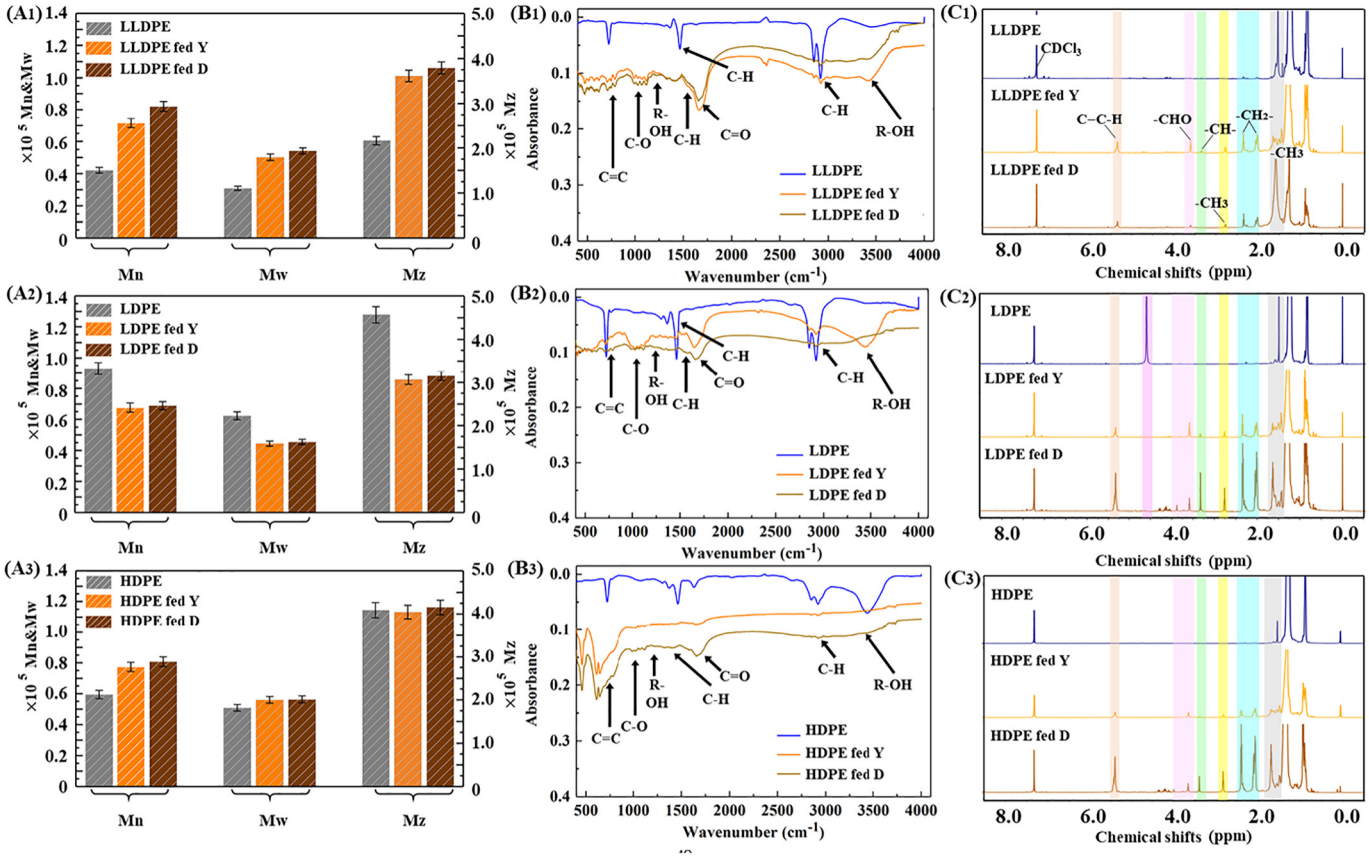

Fig. 1. Changes in M_w and M_n of LLDPE, LDPE and HDPE microplastics before and after biodegradation by *T. molitor* and *T. obscurus* larvae (A_1, A_2, A_3). Comparison of (B_1, B_2, B_3) FTIR spectra of residual PE polymers in the frass of *T. molitor* and *T. obscurus* fed respective LLDPE, LDPE, and HDPE only. (C_1, C_2, C_3) ^{1}H NMR spectra of residual PE polymers from the frass of *T. molitor* and *T. obscurus* fed respective LLDPE, LDPE, and HDPE only. During incubation, PE MPs (25 g) was mixed with agar (30 ml, 3%, *w*/w) during the test. Y = *T. molitor* larvae; D = *T. obscurus* larvae. All samples for analyses were obtained on day 21.

(Table 1, Fig. 1A_2). The limited extent depolymerization was also observed in superworms (*Zophobas atratus* larvae) from Guangzhou, China and Marion, USA, who were fed with LDPE foam with respective M_w of 173.4 and 248.0 kDa and M_n of 40.4 and 36.3 kDa. After one month, M_w was increased by 63.8% and 8.8% and M_n was increased by 26.2% and 44.1%, respectively (Peng et al., 2020b). In addition, a publication on biodegradation of polyurethane (PUR) by a landfill microbial culture reported that initially, the M_w of PUR increased from 208.5 to 229.4 kDa and then decreased to 169.9 kDa (Gaytán et al., 2019). Another report indicated PS degradation in *Galleria mellonlla* larvae (Lou et al., 2020) with an increase in M_n from 132.1 to 146.9 kDa as well as in M_w, from 361.7 to 377.8 kDa. The limited extent depolymerization of LLDPE and HDPE was likely related to the selective depolymerization of lower molecular polymers at a higher rate than longer chain polymers. However, the presence of crosslinking reactions during depolymerization, biodegradation, or both might also occur. Further study is needed to understand whether this mechanism contributes to the formation of longer chain polymers in the frass.

This pattern was observed in the biodegradation of LDPE by both species, which was broad depolymerization (i.e., decrease in M_n, M_w as well as M_z) in agreement with the results of previous research on biodegradation of LDPE and PS in both *Tenebrio* species (Brandon et al., 2018; Yang et al., 2018a, 2018b; Peng et al., 2019; Yang et al., 2021c). The polydispersity index (PDI) of HDPE dropped from 3.06 to 2.59 or 2.50, PDI of LLDPE slightly decreased from 2.62 to 2.50 or 2.35, but PDI of LDPE remained basically unchanged at 2.39, compared to the PDI at 2.35 for both *T. molitor* and *T. obscurus* larvae (Tables S1 and 1).

Analysis of molecular weight distribution (MWD) of LLPE, LDPE, and HDPE before versus after biodegradation by *T. molitor* and *T. obscurus* larvae (Fig. S2) indicated clearly that the MWD of LLDPE and HDPE moved toward high molecular weight while MWD of LDPE moved toward low molecular weight, supporting limited extent depolymerization of LLPE and HDPE and broad depolymerization of LDPE MPs.

Based on depolymerization or changes in M_n, M_w, M_z, MWD, and PDI, the biodegradation of the three PE polymers in *T. molitor* and *T.obscurus larvae* was similar. It was clear that biodegradation of LDPE was performed via broad depolymerization, showing significant reduction of M_n, M_w, and M_z and suggesting more rapid depolymerization of LDPE than LLDPE and HDPE. LLDPE and HDPE polymers showed limited extent depolymerization with increased M_n and M_w as well as increased M_z or unchanged M_z. The LDPE depolymerization/degradation extent appeared to be much greater than that of LLDPE and HDPE.

In this study, the crystallinity degree of LDPE, LLDPE, and HDPE was 48.0%, 51.9%, and 55.3% (Table 1). The biodegradation of LDPE was more pronounced than that of the LLDPE and HDPE MPs, possibly due to the fact that compared to LDPE, HDPE and LLDPE consist of closely packed chains which results in a higher stability and crystallinity degree against degradation (Guadagno et al., 2001; Benítez et al., 2013). In this study, the crystallinity degree of HDP, LLDPE, and HDPE MPs was 48%, 51.9%, and 55.3%. LLDPE and HDPE are composed of linear chains with a very low level of short chain branching, and the molecules can stack and form strong intermolecular forces (Wagner and Lambert, 2018). LDPE, however, is characterized by a significant amount of long chain branching (typical branch length of several hundred carbon atoms) and short chain branching (length of 2 to 6 carbon atoms). These branching chains prevent tight packing into a crystalline structure (Wilkes and Aristilde, 2017), resulting in a lower crystallinity degree. LLDPE is a linear molecule with more short chain branching and less long chains when compared to HDPE (Guadagno et al., 2001). In this study, the crystallinity degree of the three PE did impact depolymerization extent.

Surface hydrophobicity is typically determined by the WCA of the surface (Restrepo-Flórez et al., 2014; Yang et al., 2014). WCA of the residual polymer samples from the frass of *T. molitor* larvae fed with LDPE, LLDPE, and HDPE was 87.7 ± 1.5°, 91.5 ± 1.3°and 99.5 ± 1.3° (n = 3), respectively, while WCA of those from *T. obscurus* larvae fed with LDPE, LLDPE, and HDPE were 91.6 ± 0.0°, 93.9 ± 0.2° and 100.0 ± 1.4° (n = 3), which was significantly lower than that of the 134.1 ± 0.5°, 136.7 ± 0.1° and 133.0 ± 0.0° (n = 3) WCA of LDPE, LLDPE, and HDPE MPs, respectively. This observation indicated that the ingested PE MPs became less hydrophobic after passing through the digestive system of both *Tenebrio* species, and thus less resistant to enzymatic attacks or biodegradation (Sudhakar et al., 2008). Results obtained from the WCA indicated that the sequence of the decrease in WCA of the residual PE polymers was LDPE>LLDPE>HDPE (Fig. S3), demonstrating that the modification of surface property and PE degradation was significantly impacted by the three PE types. This is consistent with the reported results of abiotic degradation treatments, which indicated that the HDPE matrix was less efficiently degraded than the other two PE types (Fontanella et al., 2010).

GPC and WCA analyses demonstrated significant physical and chemical modification and structure change after LDPE, LLDPE, and HDPE MPs passed through the digestive systems of both *Tenebrio* species. The depolymerization and biodegradation extent was impacted by PE polymer types with a sequence of LDPE>LLDPE>HDPE.

3.2. Oxidation and chemical modification of LDPE, LLDPE and HDPE

The oxidation of the three PE types in the two *Tenebrio* species was characterized by FTIR analysis and confirmed biodegradation of the three PE MPs by both mealworms (Fig. 1B_1–B_3). In comparison with their corresponding controls, the intensity increase was found at several low-intensity bands located in the region of 1100–1300 cm^{-1}, which represents stretching of COC groups (Novotný et al., 2018). This indicates the appearance of aldehyde, ketone, ether, or ester groups formed after biodegradation. Specifically, in the region of 1650–1800 cm^{-1}, new peaks appeared in all frass samples, representing the formation of different oxidized products with CO groups, e.g., carboxylic acids (1708–1698 cm^{-1}), ketones (1723–1713 cm^{-1}), aldehydes (1740–1733 cm^{-1}), and lactones (1786–1780 cm^{-1}) (Benítez et al., 2013; Grigoriadou et al., 2018). These bands were probably the low molecular weight degradation products of PE chains which, as reported previously (Albertsson et al., 1993, 1995), were mostly terminated by the carboxylic (COOH) acid group. The spectra of frass samples from HDPE-fed groups showed relatively weak stretching vibrations of CO groups (Fig. 1B_3) compared with those of LDPE and LLDPE-fed groups (Fig. 1B_1 and B_2). Furthermore, in the region of 3200–3500 cm^{-1}, the spectra of all frass samples showed the formation of a new broad peak of OH stretching vibrations of an alcohol or phenol group as well as a decreased intensity of the CH_2 group. This was also observed in PE degradation tests by fungal and bacterial cultures (Asgari et al., 2014; Mukherjee et al., 2018; Kundungal et al., 2019). Previous studies on the characterization of bacterial biodegradation of HDPE using FTIR found additional peaks in the 2100–1600 cm^{-1} range (detection of CO) and 3600–3100 cm^{-1} range (scission of CH bond) with respect to its control HDPE material (Skariyachan et al., 2018). The appearance of the new additional peaks in this study indicated the presence of CO and the scission of CH bonds after HDPE degraded in the mealworms. In particular, for all three PE MPs, the signal at 1470 cm^{-1} (attributed to the polymer backbone) decreased in FTIR spectra of the frass of both species. This observation is consistent with the results showing a decrease of a typical peak at 1470 cm^{-1} in the LLDPE polymer after microbial degradation reported previously (Novotný et al., 2018).

^{1}H NMR analysis of extracts from frass of the larvae fed with the three PE polymers in comparison with the original MPs is shown in Fig. 1C_1–C_3. In the spectra of the residual polymers, new peaks were observed in regions of chemical shifts associated with methyl, carbonyl, aldehyde, aminoic, and unsaturated allylic groups, indicating the breakdown of long chain polymers and the incorporation of oxygen as observed in FTIR analysis. A sharp peak observed at about 4.7 ppm in the LDPE MP sample (Fig. 1C_2) was attributed to contamination of water moisture (Skariyachan et al., 2018). New resonances between 4.0 and 4.3 ppm, which are attributed to CH_2 groups of the linker after digestion, were only present in the ^{1}H NMR spectra of the frass from LDPE-fed and HDPE-fed *T. obscurus* larvae. Furthermore, more additional peaks were observed between 1.4 and 1.7 ppm in the spectra of frass samples from *T. molitor* groups compared to those of the *T. obscurus* groups. These differences suggested that the biodegradation

process of these PE polymers by *T. obscurus* larvae were slightly different from those by *T. molitor* larvae.

For investigations into PE biodegradation, Py-GC/MS analyses can also provide information for degradation products by comparing the frass samples of two *Tenebrio* species fed with LDPE, LLDPE, and HDPE diets versus virgin PE MPs after thermal decomposition at 550 °C (Fig. S4). The different types of PE produced similar programs consisting of regular triplets of terminal alkadienes, terminal alkenes, and *n*-alkanes (Fig. S4), which are formed by random chain scission and back-biting during the pyrolysis process (Duemichen et al., 2019). In comparison with those of the three virgin PE MPs, new signals were observed in frass samples (Fig. 2). Signals of functional groups related to degradation products were detected in frass residues, which belonged to acids, esters, ketones, alcoholic hydroxyl groups, carboxylic acids, phenols, and some hydrocarbons but not in virgin PE MPs (Table S4 and Fig. 2), revealing the cleavage of the macromolecule backbone and the consequent formation of new functional groups through the incorporation of oxygen during biodegradation. The products obtained by Py-GC/MS supported the biodegradation of three PE polymers characterized by FTIR and ^{1}H NMR analyses (Fig. 1). The results also showed that the products of pyrolysis of frass samples of *T. molitor* and *T. obscurus* were different. For example, long chain hydroxide radicals were only observed in the frass samples of *T. molitor* larvae fed with the three PE polymers. They were represented by peaks at residence times (min) of 16.505 (1-pentacontanol, $C_{50}H_{102}O$), 12.195, 14.505 (1-hentetracontanol, $C_{41}H_{84}O$), and 16.720 (*n*-hexadecanoic acid, $C_{16}H_{32}O_2$) (Table S5). In comparison, the formation of long chain fatty acids, such as octadecanoic acid ($C_{18}H_{36}O_2$), 9-hexadecanoic acid ($C_{16}H_{30}O_2$), *n*-hexadecanoic acid ($C_{16}H_{32}O_2$), tetradecanoic acid ($C_{14}H_{28}O_2$), oleic acid ($C_{18}H_{34}O_2$), and 9,12-octadecanoic acid ($C_{18}H_{32}O_2$) was found in the frass of *T. obscurus* groups fed with the three PE polymers (Table S5 and Fig. 2). The source for the formation of phenolic products in frass samples was not clear but it might be induced by the insertion of oxygen in the cyclic scission products due to the random scission and backbiting reactions of PE (Jin et al., 2016), or from the ingestion of larval residues due to cannibalism among the mealworms.

In summary, the FTIR results of this study were consistent with previous research on biodegradation of commercial LDPE materials (foam) by *T. molitor*, *T. obscurus* and *Z. atratus* (Brandon et al., 2018; Peng et al., 2020b; Yang et al., 2021a, 2021c; Lou et al., 2021). Different spectra of ^{1}H NMR and pyrolysis products suggest that the biodegradation pathways in the two *Tenebrio* species are different.

3.3. Depolymerization of LDPE and HDPE with different molecular weights

The second test was conducted to compare depolymerization/biodegradation of LDPE and HDPE using six high purity PE samples with different molecular weights as the sole diet of *T. molitor* and *T. obscurus* larvae (Table 2 and Fig. 3). The larvae of each group were fed with 400.0 ± 20.0 mg of the respective PE MPs. The duration for consumption of individual PE MPs was significantly different, varying from 5 to 15 days (Table 2). *T. molitor* and *T. obscurus* larvae consumed MPs of $LDPE_{840}$ (which belongs to PE wax), $LDPE_{6400}$, and $LDPE_{102000}$ on day 5, day 10, and day 12, respectively, and $HDPE_{52000}$, $HDPE_{105000}$, and $HDPE_{132700}$ on day 12 and day 15, respectively (Table 2). Notably, they consumed $LDPE_{102000}$ and $HDPE_{105000}$ with similar molecular weights on day 12, and took the longest time to consume $HDPE_{132700}$ with the highest molecular weights (15 days). Results indicated that the larvae consumed MPs with lower molecular weight PE, especially PE wax, more rapidly than those with higher molecular weights, and that they consumed LDPE with molecular weight of 102.0 kDa and HDPE with similar molecular weights within the same amount of time. Overall, the consumption rate of both LDPE and HDPE decreased progressively with the increase in molecular weights for both *Tenebrio* species. This observation could be related to the fact that the PE polymers with higher molecular weights have higher rigidity, which makes it difficult for the larvae to chew and ingest them, while lower molecular weight polymers have a wax-like flavor, which appeals to the larval appetite.

Depolymerization/biodegradation of the six PE MPs was characterized using HT-GPC to determine molecular weights of the residual polymers in frass compared to the virgin PE MPs (Fig. 3). For *T. molitor* larvae fed with three LDPE MPs, the residual polymers showed a significant decrease ($p < 0.05$) in M_n (44.44%, 83.13%, and 96.59%), M_w (47.62%, 88.13%, and 52.34%), and M_z (51.00%, 91.16 and 23.52%) compared to the virgin MPs (Table 2 and S3). For *T. obscurus* larvae fed with the three LDPE MPs, similar reduction was observed ($p < 0.05$) in M_n (41.67%, 81.25%, and 97.75%), M_w (39.29%, 87.81%, and 84.08%), and M_z (39.00%, 91.34%, and 11.54%) (Table 2), indicating that significant depolymerization of LDPE occurred within the gut of both species. After biodegradation, the residual polymers of $LDPE_{840}$ and $LDPE_{6400}$ diets in the frass excretion had M_n of 0.42 and 0.6 kDa, and M_w of 0.51 and 0.78 kDa, respectively (Table S3). Furthermore, the PDI (M_w/M_n ratio) of $LDPE_{102000}$ polymers significantly ($p < 0.05$) increased from a high value of 8.28 to 115.68 and 58.62 for frass excretions from *T. molitor* and *T. obscurus* larvae, respectively (Table 2), indicating rapid decomposing polymers with widely distributed molecular weights.

Significant differences ($p < 0.05$) were found between the depolymerization and biodegradation of HDPE versus that of LDPE by *T. molitor* and *T. obscurus* larvae (Fig. 3B_1–B_3). For $HDPE_{52000}$, the reductions in M_n, M_w, and M_z were 35.52%, 66.35%, and 82.25% for *T. molitor*, while similar results were obtained by *T. obscurus* larvae (33.88%, 64.42%, and 80.72%, respectively). For the $HDPE_{105000}$ and $HDPE_{132700}$-fed groups, M_w and M_n of the residual polymers were significantly increased in both *Tenebrio* species (Table 2 and S3). This indicated that these two HDPE MPs were biodegraded via limited extent depolymerization, as observed previously during LDPE degradation in the presence of antibiotic depression (Yang et al., 2021a, 2021c). These results suggested that when HDPE MPs with molecular weights higher than 100.0 kDa were fed to the larvae, the lower molecular weight fractions were degraded more rapidly than the longer chains, resulting in the accumulation of higher molecular weight fractions in the residual polymers. Furthermore, we compared the molecular weight changes of $HDPE_{105000}$ versus $LDPE_{102000}$ and found that the residual fraction of former increased M_n by 407.69% and 432.69%, and M_w by 16.76% and 12.67%, while $LDPE_{102000}$ showed a decrease in M_n by 96.59% and 97.75% and in M_w by 52.34% and 84.08%. This indicated that depolymerization of the HDPE MPs was much less efficient than that of LDPE MPs with a similar molecular weight. Overall, the results of the $HDPE_{105000}$-fed *T. molitor* and *T. obscurus* groups revealed that the lower molecular weight fraction was degraded more rapidly than the longer chain polymers, resulting in an accumulation of residual polymers with higher molecular weight. For example, the M_n of residual HDPE polymers was increased by 407.69% and 432.69% in M_n while M_w was 16.76% and 12.67% in the $HDPE_{105000}$-fed *T. molitor* and *T. obscurus* groups, respectively. Significant decreases ($p < 0.05$) in the M_w/M_n ratio from a value of 10.10 to 2.32 and 2.14 for polymers extracted from frass of *T. molitor* larvae and *T. obscurus* larvae (Table 2 and S2) further demonstrated the presence of low molecular weight oligomers in frass as a result of the biodegradation by gut microbiota in the digestive system of the mealworms. The HT-GPC analysis of the residual polymers from mealworms fed with PE of similar molecular weights but of a different type ($HDPE_{105000}$ and $LDPE_{102000}$) further revealed that LDPE is more readily biodegraded by gut microbes of mealworms than HDPE, which is consistent with the results on biodegradation of different types of PE microplastics observed in Fig. 1A_1–A_3.

3.4. Relative dominance analysis

To examine the biodegradation performance of three PE types with different molecular weights, the variance analysis upon three PE types (LLDPE, LDPE, and HDPE) in M_n reduction, M_w reduction, and M_z reduction was depicted in Fig. 4A. This reduction in M_n was significantly associated with a change in the three PE types ($p = 0.0285 < 0.05$; Fig. 4A_1). Similarly, the reductions in M_w ($p = 0.007 < 0.05$; Fig. 4A_2) and M_z ($p = 0.0014 < 0.05$; Fig. 4A_3) were not significantly associated with a significant

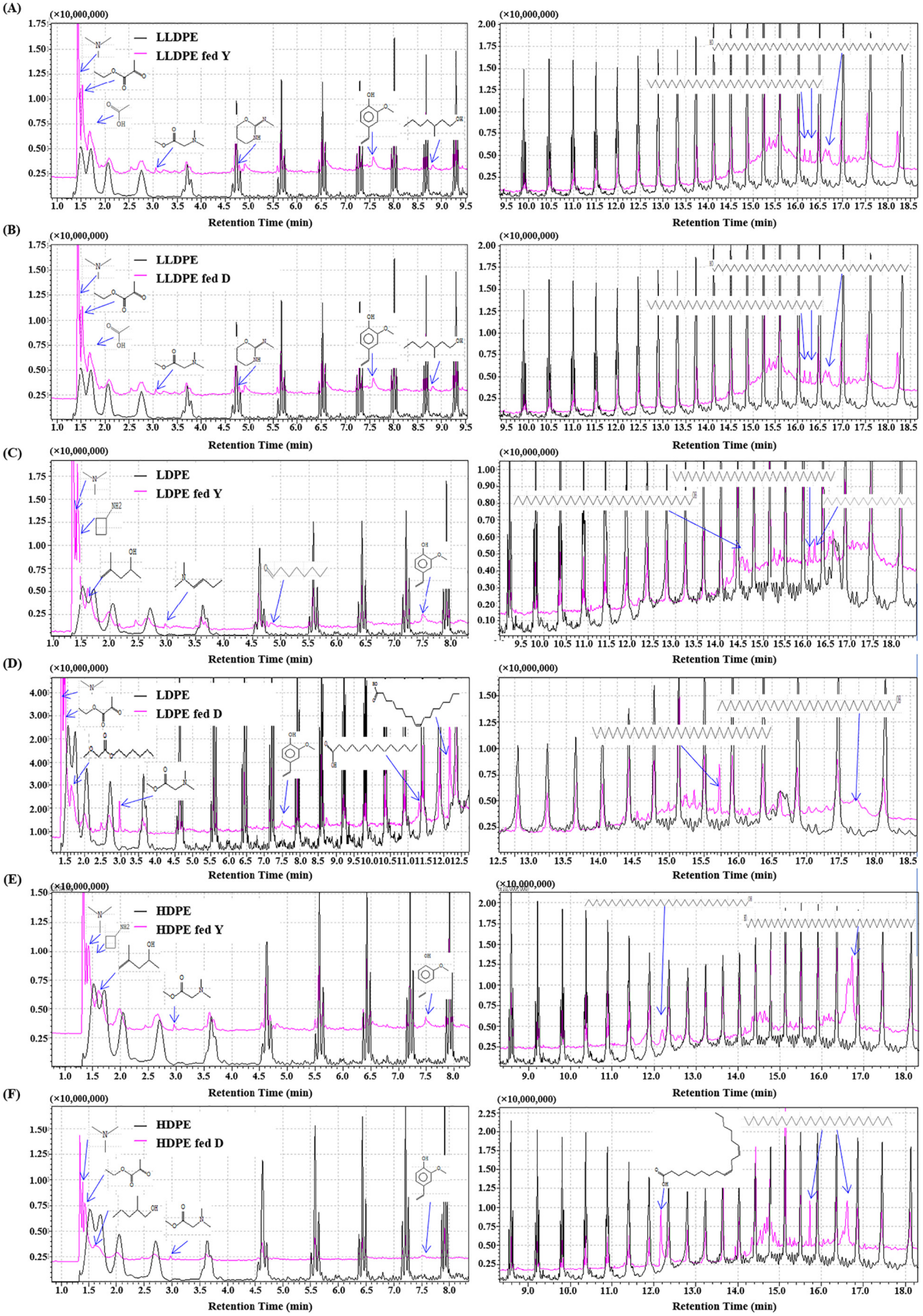
(A)
(×10,000,000)
LLDPE
LLDPE fed Y
Retention Time (min)
(B)
(×10,000,000)
LLDPE
LLDPE fed D
Retention Time (min)
(C)
(×10,000,000)
LDPE
LDPE fed Y
Retention Time (min)
(D)
(×10,000,000)
LDPE
LDPE fed D
Retention Time (min)
(E)
(×10,000,000)
HDPE
HDPE fed Y
Retention Time (min)
(F)
(×10,000,000)
HDPE
HDPE fed D
Retention Time (min)

Table 2
Cumulative consumption of six PE high purity microplastics and complete consumption time by *T. molitor* and *T. obscurus* larvae.

PE	Mealworm	Biodegradation			Residual PE after biodegradation						
		Cumulated PE consumed, mg	Cumulated bran consumed, mg	Test time days	M_n kDa	M_w kDa	M_z kDa	M_w/M_n	M_n %	M_w %	M_z %
$LDPE_{840}$	*T. molitor*	403.7 ± 14.3	407.2 ± 11.7	5	0.40	0.44	0.49	1.10	44.44	47.62	51.00
	T. obscurus	397.5 ± 17.2	392.3 ± 15.9	5	0.42	0.51	0.61	1.21	41.67	39.29	39.00
$LDPE_{6400}$	*T. molitor*	399.0 ± 13.4	395.8 ± 15.2	10	0.54	0.76	0.99	1.41	83.13	88.13	91.16
	T. obscurus	394.8 ± 14.1	391.4 ± 16.3	10	0.60	0.78	0.97	1.30	81.25	87.81	91.34
$LDPE_{102000}$	*T. molitor*	401.8 ± 16.4	403.9 ± 15.5	12	0.44	50.9	277.7	115.68	96.59	52.34	23.52
	T. obscurus	402.1 ± 15.4	404.9 ± 13.2	12	0.29	17.0	321.2	58.62	97.75	84.08	11.54
$HDPE_{52000}$	*T. molitor*	396.2 ± 16.2	394.3 ± 15.9	12	11.8	17.5	24.5	1.48	35.52	66.35	82.25
	T. obscurus	404.4 ± 13.4	402.1 ± 15.8	12	12.1	18.5	26.6	1.53	33.88	64.42	80.72
$HDPE_{105000}$	*T. molitor*	402.2 ± 16.8	400.4 ± 15.5	12	52.8	122.6	236.6	2.32	−407.69	−16.76	50.19
	T. obscurus	403.1 ± 14.3	402.6 ± 15.3	12	55.4	118.3	216.0	2.14	−432.69	−12.67	54.53
$HDPE_{132700}$	*T. molitor*	498.3 ± 16.3	397.6 ± 17.2	15	156.1	279.4	448.8	1.79	−88.98	−110.55	−39.81
	T. obscurus	394.7 ± 15.3	395.9 ± 16.5	15	164.5	277.9	420.9	1.69	−99.15	−109.42	−31.12

Note: The initial larval number: 400. The M_n, M_w and M_w of virgin PE MPs are listed in Table S6.

change in the three PE types, suggesting a significant relationship between PE types and PE biodegradation extent. As for the HDPE biodegradation, the three pictures correspond to the HDPE plastic and are far from the center line of the box picture (Fig. 4A), which has the most prominent effect on molecular weight reduction/biodegradation (M_n reduction, M_w reduction, and M_z reduction).

The obtained dataset to illustrate and compare the *relative importance indices* is presented in Table S6. The dataset contains six input variables including PE type (X_1), mealworm species (X_2), M_n (X_3), M_w (X_4), M_z (X_5), and crystallinity degree (X_6). Three criterions, *Y*, were the percentages on M_z reduction (%), M_w reduction (%), and M_n reduction (%) and consisted of seven items. The six predictors were responses to single items pertaining to biodegradation degree of PE. According to the correlation matrix (Fig. 4B_1), it appears that obvious correlation cannot be established between X_1 (PE type) or X_2 (larval source) and all of the other five predictors; X_3 (M_n) is highly correlated with M_w (X_4) and M_z (X_5), M_w (X_4) is highly correlated with M_n (X_3) and M_z (X_5), M_z (X_5) is highly correlated with M_w (X_4), and X_6 (crystallinity degree) is moderately correlated with M_n (X_3), M_w (X_4), and X_5 (M_z).

Fig. 4B_2 depicts all the commonality coefficients of all possible subsets, which represent the proportion of variance explained by each dependent variable. The dominance percentages of X_3 (M_n) over M_n reduction (%), M_w reduction (%), and M_z reduction (%) are 3.4%, 53.96%, and 35.79%, respectively, indicating that the value of M_n alone explains 3.4%, 53.96%, and 35.79% of the variation in the dependent variable. M_z (X_5) is very highly correlated with M_n reduction (54.15%), while M_w (X_4) is only moderately correlated with M_n reduction (21.43%), M_w reduction (22.65%), and M_z reduction (15.45%). The dominance percentages of X_1 and X_6 over M_n reduction, M_w reduction, and M_z reduction can explain 9.38%, 0.97%, and 30.15% vs. 11.53%, 4.1%, and 7.1%, indicating that both PE type and crystallinity are each only moderately correlated with PE biodegradation degree. Interestingly, the two sources of mealworms tested in this study, *Tenebrio molitor* from Harbin and *Tenebrio obscurus* from Zaozhuang, can explain 0.1%, 0.01%, and 0.16% of the variation in the response variable (M_n reduction, M_w reduction, and M_z reduction), which suggests that there was no significant relationship between mealworm species and PE biodegradation. The various measures indicate the values of M_n (X_3), M_z (X_5), and M_w (X_4) (in that order) to be the three top predictors, with mealworm species (X_2) being the least important variable (or predictor). The ordering of PE type (X_1) and crystallinity degree (X_6) is less clear and varies across the measures of importance. Thus, the overall ordering is M_n > M_z > M_w > PE type > crystallinity degree > mealworm species. Results of this study demonstrated that PE biodegradation degree was more affected by molecular weights than other factors (PE type, crystallinity degree, and mealworm species).

The differences in factors affecting biodegradation between HDPE and LDPE are presented in Fig. 4C_1 and C_2. The predominant factor affecting M_w reduction in the tests of LDPE-fed groups was affiliated with crystallinity degree (43.64%). Crystallinity degree likewise dominated M_w reduction (43.47%) in the HDPE-fed group. Using GPC analysis, M_w is the average closest to the center of the molecular distribution curve, and M_z (the size average molecular weight) represents the highest molecular weight portion of the sample. Crystallinity defines the degree of long-range order in a material, and strongly affects its properties. The more crystalline a polymer, the more regularly aligned its chains. Increasing the degree of crystallinity increases hardness and density. According to the results obtained in this study, depolymerization of PE was likely related to the crystallinity degree, which is supported by the conclusion obtained from this study that the biodegradation degree order of LDPE>LLDPE >HDPE accompanies the order of increasing crystallinity degree. Previous studies also found that the lower crystallinity degree is more susceptible to biotic and abiotic attack (Guadagno et al., 2001; Benítez et al., 2013). Besides crystallinity degree, M_n value of PE, which provides information about the lowest molecular weight portion of the sample, also influenced M_w reduction and M_z reduction (32.97% and 35.65%) in HDPE-fed groups, whereas M_z dominated the M_n reduction (63.31%) in the biodegradation of HDPE test. However, the predominant factor affecting M_n reduction in the tests of LDPE-fed groups was affiliated with M_n (35.14%), followed by crystallinity degree (27.87%), M_w (22.95%), and M_z (13.99%), respectively. The results further indicate that the larvae of the two *Tenebrio* species ingest and metabolize PE from diverse locations, indicating that the PE degrading capacity is independent of *Tenebrio* species, and could be ubiquitous to other members of *Tenebrio* genus, such as larvae of *Zophobas atratus*, *Tribolium castaneum*, *Plesiophthalmus davidis*, and honey bee pest *Uloma* sp., which have all showed plastic-degrading ability (Peng et al., 2020b; Yang et al., 2020; Woo et al., 2020; L. Wang et al., 2020; Z. Wang et al., 2020; Kundungal et al., 2021). Future research efforts could also focus on elucidating the mechanisms of core gut PE-degrading microbes in order to better understand the digestive system in mealworms. Focus on understanding the toxicology associated with PE biodegradation, which could be associated with the polymer types, biodegraded intermediates, gut microbiome and additives, is also needed (Sanchez-Hernandez, 2021).

4. Conclusion

This study is the first to report on the biodegradation of different types of high purity PE MPs (i.e., LLDPE, LDPE, and HDPE) with different

Fig. 2. Py-GC/MS chromatograph of degradation products from the three virgin PE MPs and frass samples of *T. molitor* and *T.obscurus* larvae fed LLDPE, LDPE, and HDPE diets. (A) *T. molitor* larvae fed LLDPE MPs. (B) *T. obscurus* larvae fed LLDPE MPs. (C) *T. molitor* larvae fed LDPE MPs. (D) *T. obscurus* larvae fed LDPE MPs. (E) *T. molitor* larvae fed HDPE MPs. (F) *T. obscurus* larvae fed HDPE MPs. During incubation, PE MPs were mixed with agar (30 ml, 3%, w/w) during 21 days. Samples were taken on day 21. Y = *T. molitor* larvae; D = *T. obscurus* larvae; LLDPE = linear low-density polyethylene; LDPE = low-density polyethylene; HDPE = high-density polyethylene.

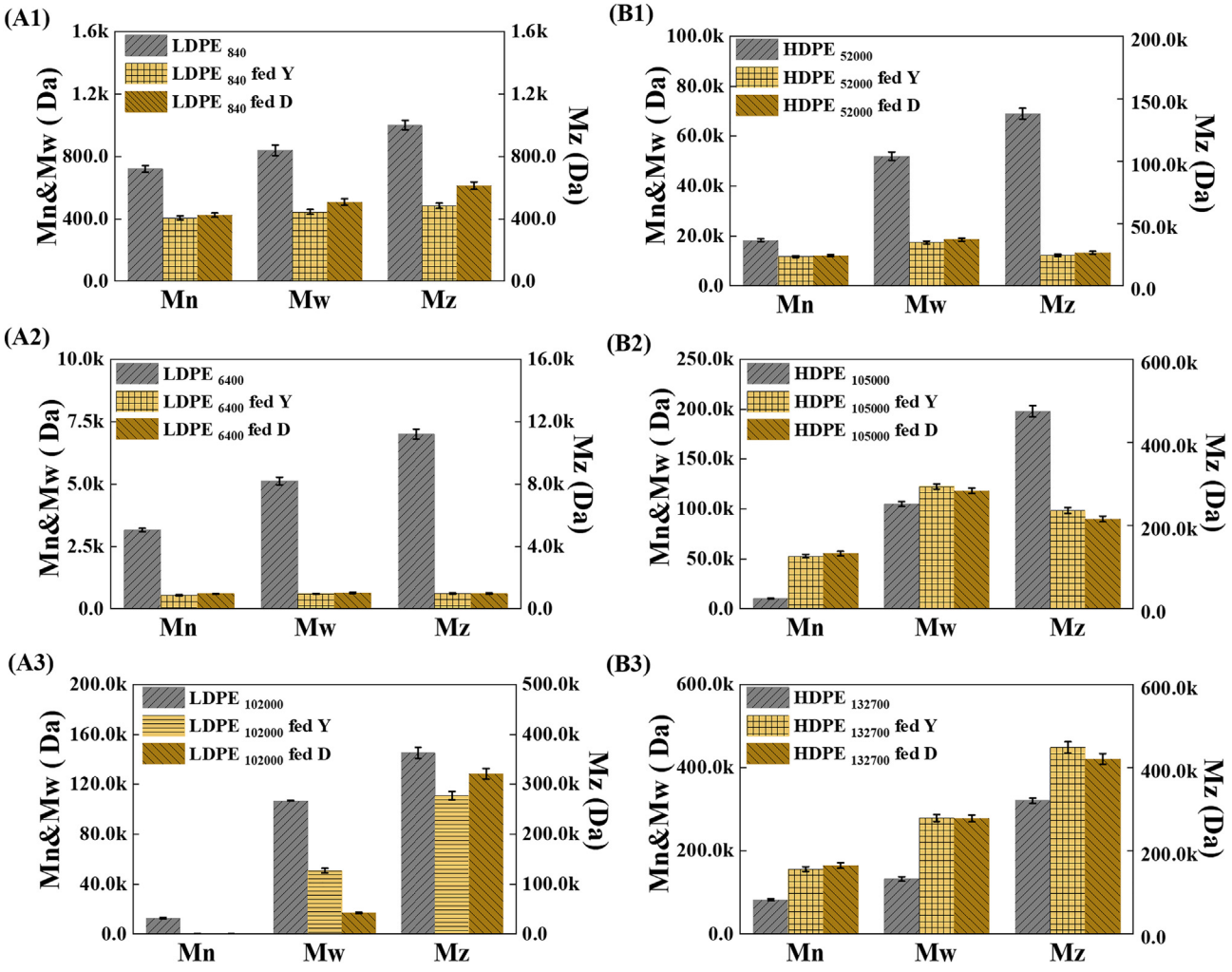

Fig. 3. (A_1, A_2, A_3) The changes in M_w and M_n of $LDPE_{840}$, $LDPE_{6400}$, and $LDPE_{102000}$ high purity MPs versus residual PE polymers extracted from the frass of *T. molitor* and *T. obscurus* fed LDPE MPs-bran mixture. (B_1, B_2, B_3) The changes in M_w and M_n of $HDPE_{52000}$, $HDPE_{105000}$, and $HDPE_{132700}$ high purity MPs versus residual PE polymers extracted from the frass of *T. molitor* and *T. obscurus* fed HDPE MPs-bran mixture. During incubation, PE high purity MPs were mixed with bran at 1:1 (w/w). Y = *T. molitor* larvae; D = *T. obscurus* larvae.

molecular weights, crystallinity degree, and branching structures by two *Tenebrio* species. Our work revealed that the susceptibility of PE polymers for the biodegradation processes decreases in the order: LDPE>LLDPE>HDPE. Changes in M_n, M_w, and M_z of all PE types after passage through the larval guts of the two species revealed different patterns of biodegradation; LDPE was biodegraded via broad depolymerization, while LLDPE and HDPE exhibited limited extent depolymerization patterns. Py-GC/MS analyses confirmed the formation of long chain fatty acids during biodegradation of all three PE-fed *T. obscurus* groups. However, long chain hydroxide radicals were only observed in the frass of *T. molitor* groups, suggesting the possibility of different biodegradation pathways between *T. obscurus* and *T. molitor* larvae.

The results of biodegradation of LDPE and HDPE MPs with different molecular weights ranging from 0.84 to 132.7 kDa demonstrated significant differences in the depolymerization and biodegradation extent between LDPE and HDPE, which was also impacted by different molecular weights. Results indicated that LDPE less than 5.0 kDa were rapidly decomposed. However, considerable increase in M_w and M_n of residual polymers was observed in the residual PE polymers in the frass from both mealworm species when fed with LLDPE and HDPE MPs with high molecular weights. This demonstrated that the polymers with higher molecular weights were degraded at a much slower rate than those with a lower molecular weight fraction. The HT-GPC analysis of the residual polymers from the larvae fed with HDPE of similar molecular weights to LDPE MPs ($HDPE_{105000}$ versus $LDPE_{102000}$) confirmed that LDPE is more conducive than HDPE to being biodegraded by the larvae. DA analysis indicated that molecular weight and PE type are important variables that impact the biodegradation process and reaction mechanism. Overall, no significant difference was observed between *T. molitor* and *T. obscurus* larvae. The results of the study also suggested that the accumulation of higher molecular weight polymer fractions, especially of HDPE, could be a concern for the biodegradation of commercial PE products (e.g., foam, films) which have broad SPI and are commonly comprised of mixtures of LDPE, LLDPE, and HDPE. In this study, we did not measure crystallinity of residual PE in frass due to difficulty in separating it from other organic impurities in excrement. We hypothesized that after depolymerization/biodegradation, the crystallinity of residual PE could be reduced. Technically, the crystallinity of biotreated plastic materials (e.g., film, particles) incubated with microbial culture can be determined after clean-up of biomass from plastic surface with SDS solution or other methods. In future research, we will try to develop reliable methods to separate residual PE polymers from frass in order to verify the change in the crystallinity of the residual PE.

CRediT authorship contribution statement

Shan-Shan Yang: Conceptualization, Methodology, Supervision, Validation, Writing – original draft, review & editing, Funding acquisition. **Meng-Qi Ding:** Methodology, Formal analysis, Investigation, Methodology, Writing-review & editing. **Xin-Ran Ren:** Conceptualization –

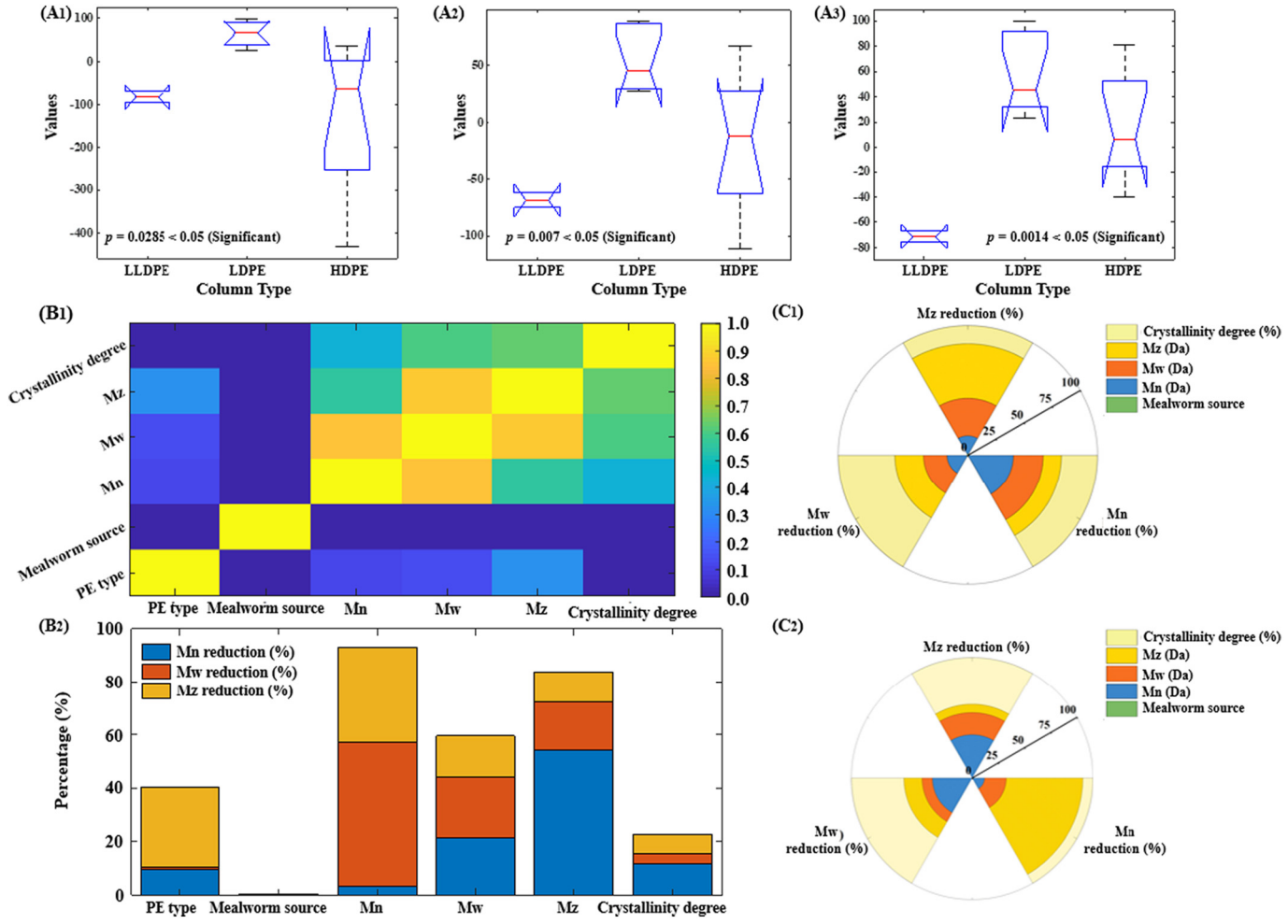

Fig. 4. Variance analysis upon three PE types (LLDPE, LDPE, and HDPE) in M_n reduction (A_1); M_w reduction (A_2); M_z reduction (A_3). (B_1) Correlation matrix for relative dominance analysis with six variables: PE type (X_1), mealworm species (X_2), M_n (X_3), M_w (X_4), M_z (X_5), and crystallinity degree (X_6); (B_2) the commonality coefficients of all possible subsets: M_n reduction (Y_1), M_w reduction (Y_2), and M_w reduction (Y_3); The differences in factors affecting biodegradation between HDPE (C_1) and LDPE (C_2).

experimental design. **Zhi-Rong Zhang:** Conceptualization – experimental design. **Mei-Xi Li:** Conceptualization. **Li-Li Zhang:** Conceptualization. **Ji-Wei Pang:** Conceptualization – experimental design, Dominance analysis. **Cheng-Xin Chen:** Review & editing. **Lei Zhao:** Conceptualization – experimental design. **De-Feng Xing:** Conceptualization – experimental design. **Nan-Qi Ren:** Conceptualization – experimental design. **Jie Ding:** Conceptualization. **Wei-Min Wu:** Conceptualization, Methodology, Supervision, Validation, Writing – drafting, review & editing. All authors contributed to manuscript reviewing & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154458.

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