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Response mechanism of sludge anaerobic fermentation on polyethylene terephthalate microplastic (PET-MPs) particle sizes

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ABSTRACT

Residual microplastics (MPs) in wastewater sludge inevitably enter anaerobic digestion systems. However, information regarding the impact of microplastic particle size on the anaerobic fermentation of waste activated sludge (WAS) is rarely reported. To investigate the impact of MPs particle sizes on the WAS fermentation process, four sizes (0.15, 0.3, 0.6, and 1 mm) of polyethylene terephthalate microplastics (PET-MPs) were added into the sludge fermentation system, and results showed that PET-MPs sped up WAS cell fragmentation and hindered hydrolysis and acidification. This effect increased with smaller particle sizes. The production of volatile fatty acids decreased by 9.12–22.21 % in comparison to the control group. Small-sized PET-MPs did not significantly reduce the relative abundance of functional bacteria such as *Proteobacteria*, *Firmicutes*, and *Bacteroidota*, but reduced their utilization of acidifiable organic matter and limited amino acid and carbohydrate metabolism. Furthermore, small-sized PET-MPs exhibited a stronger inhibitory effect on the expression of key hydrolytic and acidogenic enzymes. This study is of significant importance in elucidating the effect of PET-MPs with varying particle sizes on the anaerobic fermentation process.

1. Introduction

The pollution issue of microplastics (MPs) is becoming a growing concern as a result from the increase in global use of these materials and the inappropriate methods of disposal (Lin et al., 2020). The formation of smaller MPs by plastics is facilitated by physical and chemical interactions, which subsequently result in their dispersion into the human environment, thereby inducing environmental pollution (Thompson et al., 2004; Wang et al., 2025). In recent years, wastewater treatment plants (WWTPs) have garnered increased attention as a significant conduit for MPs into the environment. Though MPs delivered to WWTPs via various pathways are largely removed by the conventional activated sludge process, more than 90 % of MPs are adsorbed on the organic surfaces of sewage sludge during the treatment process as a consequence of their hydrophilicity and high surface area (Dilara Hatinoglu and Dilek Sanin, 2022; Li et al., 2020).

Waste activated sludge (WAS) produced during municipal wastewater treatment is rich in complex organic materials, which are a potential source of renewable energy (Liu et al., 2022). Anaerobic fermentation is a commonly WAS treatment process and capable of converting a substantial quantity of organic matter in WAS into valuable bioproducts (Wang et al., 2024). For instance, hydrolytic and acidogenic bacteria cooperate with each other to degrade large organic molecules to produce volatile fatty acids (VFAs) for their resource utilization during anaerobic fermentation. However, MPs in WAS could release toxic substances during fermentation and inhibit microorganisms and key enzymes. This inhibition affects hydrolysis, acidification, and VFAs accumulation, and associates with the concentration, particle size, and

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type of MPs (Azizi et al., 2021; He et al., 2021). Given the significance of anaerobic fermentation in WAS resource treatment, it is of paramount importance to illuminate the detrimental effects of MPs on this process.

Polyethylene terephthalate microplastics (PET-MPs) represent a significant proportion of the microplastic contamination in wastewater sludge. PET-MPs have been demonstrated to affect anaerobic fermentation (Zhang et al., 2019). Wei et al. (2019b) found that PET-MPs were shown to release reactive oxygen species (ROS) and toxic dibutyl phthalate (DBP) during alkaline anaerobic fermentation to lead to a reduction in the abundance of microorganisms including *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. In turn, the biodegradability of organic compounds decreased and the sludge hydrolysis and acid production processes were inhibited. Besides, PET-MPs led to decreased SCOD through DBP leaching and inhibited the acidification step by reducing the abundances of acidogenic bacteria (such as *Leviloptera* sp.) (Zhang et al., 2020a). However, the impact of PET-MPs of varying particle sizes on anaerobic fermentation of WAS, particularly concerning microbial communities and gene enzymes, remains largely unexplored.

The objective of this paper was to explore the effect of varying particle sizes of PET-MPs during fermentation with WAS. The dissolution of organic matter and inorganic salts was analyzed by setting up five experimental groups, and the influence of varying PET-MPs particle sizes on microbial community structure, diversity, and the abundance of genes and enzymes involved in metabolism was investigated. The response mechanism of WAS anaerobic fermentation to PET-MPs particle size was revealed.

2. Materials and methods

2.1. Sources and characteristics of WAS and PET-MPs

The WAS utilized in the experiment was procured from a sewage treatment plant in Nanjing city, Jiangsu province. It was then filtered through a 10-mesh mesh and stored at 4 °C for subsequent use. Table 1 presents the characteristics of the WAS. The PET-MPs utilized in this experiment were procured from Guangdong Zhonglian Plasticizing Co., Ltd. Prior to utilization, the MPs were divided into distinct particle sizes (0.15, 0.3, 0.6, and 1 mm), subjected to three washes with ultrapure water, and then the purified MPs were stored in sealed brown bottles for backup after drying.

2.2. Fermentation operation

Five groups of experiments were conducted in five 500 mL fermenters. The experimental groups were added equal amounts of PET-MPs with different particle sizes (0.15, 0.3, 0.6, and 1 mm). While maintaining the total suspended solids (TSS) at a constant level, stirring and mixing were employed to ensure sufficient diffusion and reaction of the substances in the reaction system. Every two days, 10 mL of WAS was extracted from the reactor, centrifuged, and the supernatant was collected for the measurement of various indexes, including pH, oxidation reduction potential (ORP), ammonia nitrogen (NH_4^+-N), orthophosphate phosphorus (PO_4^{3-}-P), soluble chemical oxygen demand (SCOD), proteins, polysaccharides, and VFAs.

Table 1
The nature of the WAS used in the experiment.

Index	Unit	Numerical value
pH	–	6.1 ± 0.1
ORP	mV	−69 ± 2
TS	g/L	21.78 ± 0.1
VS	g/L	12.4 ± 0.23
SCOD	mg/L	980 ± 2
Protein	mg/L	39.9 ± 0.16
Polysaccharide	mg/L	10.01 ± 0.3
NH_4^+-N	mg/L	167.8 ± 0.8
PO_4^{3-}-P	mg/L	1080.4 ± 0.1

Note: SCOD, protein, polysaccharides, NH_4^+-N, and PO_4^{3-}-P represent the contents in the supernatant after centrifugation of the WAS.

2.3. Analysis methods

2.3.1. Chemical detection methods

The pH and ORP were recorded with a digital pH meter (HQ11D, HACH, USA), while SCOD, NH_4^+-N, PO_4^{3-}-P, VSS, and TSS were analyzed following standard procedures (APHA, 2000). Gas chromatography (Agilent 7890 A) and FID-bound columns (Agilent, 19091N-133, 30 m × 250 m × 0.25 m) were used for the determination of VFAs concentrations. Lactate dehydrogenase (LDH), α-glucosidase, and protease were processed using assay kits provided by Solarbio, and their release levels were further measured using visible spectrophotometry. The treated WAS samples were measured using Fourier infrared spectroscopy (Bruker ENSOR) in ART mode, with a mechanical scanning range of 500 cm^{-1}∼4000 cm^{-1}. Three measurements were made for the above-mentioned indices, and their mean values were analyzed. The fluorescence char acteristics of organic matter composition and structural changes in the sludge fermentation broth were measured using a fluorescence spec trophotometer (Hitachi F-4500).

2.3.2. Microbial community analysis

Throughout the fermentation cycle, DNA was extracted every two days from raw WAS using the Power Soil DNA kit (Mobio, CA, USA) as an amplification template and stored at −80 °C. Amplification of PCR products was performed using PCR device (9700, GeneAmp Abi, USA). After purification and sequencing in pairs on the Illumina MiSeq platform, equimolar samples were collected for sequencing at Majorbio (Shanghai, China). The samples were subdivided into different operational taxonomic units (OTUs) by setting a threshold (>97 %) within the grouped sequence range, in accordance with the instructions of USEARCH software. Ace, Chao, Simpson, and Shannon metrics were calculated using sparse curves.

2.4. Data analyses

To improve the reliability of the experiment and minimize errors, each sample was tested in triplicate. Data recording and organization were carried out using Excel 2021, while data visualization was performed with the Origin 2023b64Bit system. The metabolic pathway diagram was generated using GraphPad Prism 8, and the heatmap was constructed with Tbtools software.

3. Results and discussion

3.1. Effect of PET-MPs on hydrolysis processes

3.1.1. SCOD

The changes in SCOD during WAS fermentation process with PET-MPs are showed in Fig. 1(a). The SCOD in each reactor exhibited a pattern of initial increases followed by a decline with the time extending. The groups with PET-MPs were found to be slightly higher than the control group, which corresponded to the findings of Wei et al. (2019a), who showed that the increase in SCOD was caused by PE microplastics. Furthermore, Fig. 1(d) illustrates a notable inverted correlation between SCOD concentration and PET-MPs particle size. This implied that the incorporation of PET-MPs expedited the fragmentation of WAS cells and dissolution process, thereby liberating a greater quantity of macromolecule-soluble organic matter. Moreover, the smaller the particles, the greater the impact. The presence of MPs in wastewater inhibited the secretion of extracellular polymers in granular WAS to result in the fragmentation of WAS particles (Zhang et al., 2020a). The total amount of microorganisms and the relative abundance of functional bacteria in the fermentation system reduced and impeded the

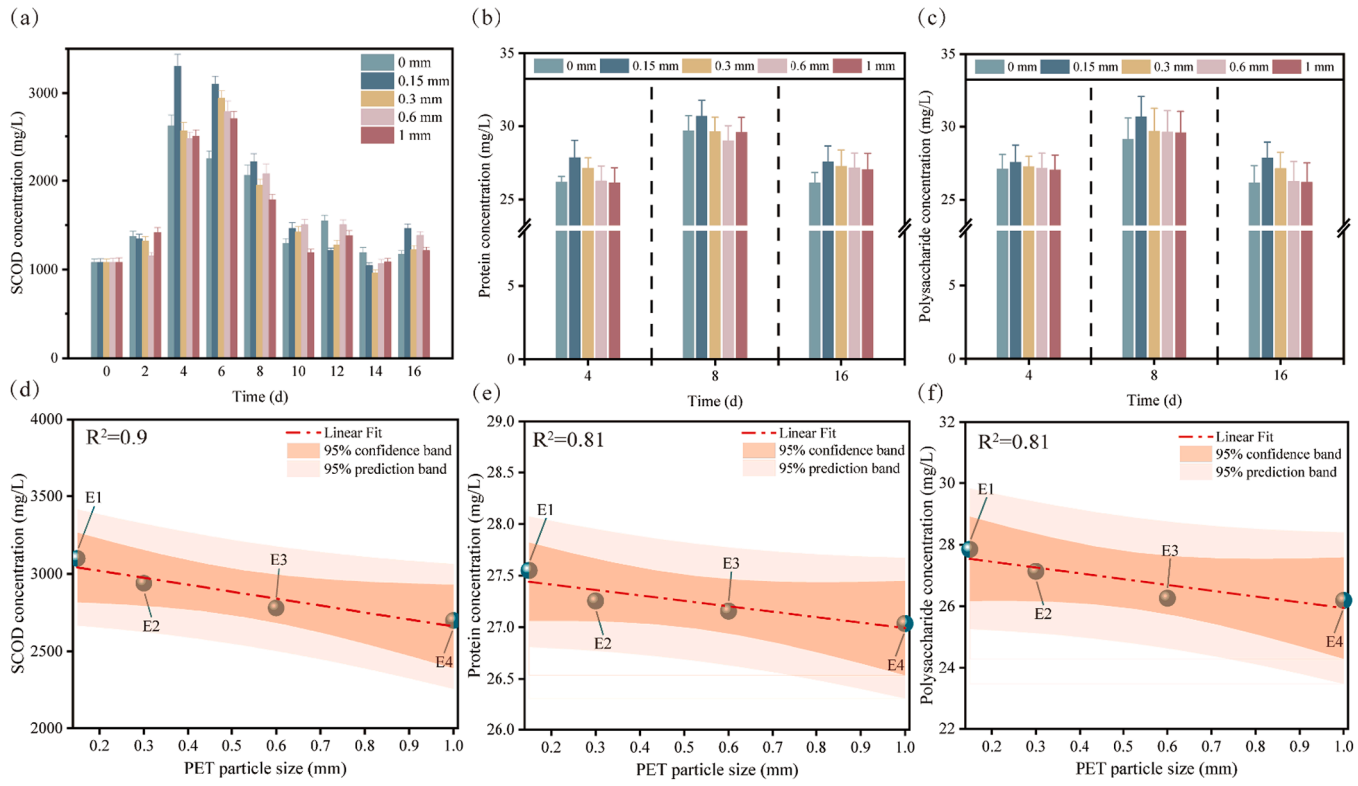

Fig. 1. Effects of PET microplastics with different particle sizes on SCOD (a), proteins (b), and polysaccharides (c), and fitting analysis of SCOD (d), proteins (e), and polysaccharides (f).

further degradation of SCOD (Zhang et al., 2020b). This could also explain the delayed turning point of SCOD concentration in the experimental group in comparison to the control group. Above all, the addition of PET-MPs had the effect of promoting the dissolution process. However, it may slow down the hydrolysis of organic matter by interfering with the biological activity of hydrolytic bacteria (Shen et al., 2022). Furthermore, the effect was more pronounced at smaller particle sizes.

3.1.2. Proteins and polysaccharides

Fig. 1(b) and (c) show the variation of effects of different PET-MPs sizes on proteins and polysaccharides releases. More proteins and polysaccharides were released in the PET-MPs groups, and the smaller PET-MPs particle size enhanced their release. This suggested that the addition of PET-MPs accelerated WAS fragmentation, allowing polysaccharides and proteins to be released into the fermentation broth. However, PET-MPs inhibited the hydrolysis process during fermentation, which made it difficult for macromolecules such as proteins and polysaccharides to be further degraded into small-molecule organic matter (acidified substrates). This was consistent with the results of Zhao et al. (2010), whose study also found that the presence of PET-MPs adversely affected the hydrolysis of proteins and polysaccharides. This further result indicated that the addition of small-sized PET-MPs more readily accelerated the fragmentation of WAS cells but adversely affected the hydrolysis of proteins and polysaccharides.

3.1.3. EEM fluorescence spectra

EEM spectroscopy can be utilized to assess the organic liberation from sludge cells. The organic component reflected in the EEM spectrum is divided into the peak α and peak β fluorescence peaks (Fig. 2). Peak α is the fluorescent response of humic acid-like compounds, whereas peak β is mainly related to fulvic acid-like proteins (Chen et al., 2022). The addition of PET-MPs increased the fluorescence response of these two peaks. This indicated that the addition of PET-MPs resulted in a greater release of soluble organic matter, and smaller particle size promoted the release of soluble organic matter. The findings further demonstrated that PET-MPs facilitated the breakdown of WAS cells, which resulted in increased release of soluble organic matter, with the effect being more pronounced for smaller particle sizes.

3.1.4. Release of PO_4^{3-}-P and NH_4^+-N

From Fig. 3(a) and (b), both PO_4^{3-}-P and NH_4^+-N showed a tendency to first increase and then decrease with the fermentation time extending. In addition, the experimental groups with PET-MPs had slightly higher levels of PO_4^{3-}-P and NH_4^+-N than the control group. It suggested that adding PET-MPs accelerated the breakdown of WAS cells and thus increased the release of PO_4^{3-}-P and NH_4^+-N. Smaller particle size PET-MPs demonstrated high release rate that exceeded the utilization rate and resulted in the accumulation of PO_4^{3-}-P and NH_4^+-N. This result also further illustrated that the addition of PET-MPs had a greater inhibitory effect on the hydrolysis process than a positive effect on promoting WAS fragmentation. In comparison to other MPs of varying particle sizes presented in Table 2, PET-MPs exhibited enhanced efficacy in disrupting WAS cells to result in the liberation of greater quantities of organic matter and the inhibition of subsequent hydrolysis processes. The effect was more pronounced at smaller particle sizes.

Overall, the analysis of SCOD, polysaccharides, proteins, NH_4^+-N, and PO_4^{3-}-P elucidated that the addition of PET-MPs accelerated the destruction of WAS cells and promoted the dissolution process, but inhibited the hydrolysis process. The inhibition performance became more pronounced with decreasing particle size. This could be due to the fact that the small-sized PET-MPs can release more toxic substances to inhibit the abundance and activity of the hydrolytic bacteria with the fermentation time, which needs to be analyzed in further studies.

3.2. Effect of PET-MPs on acidification process

Fig. 3(c) shows the influence of PET-MPs particle sizes on VFAs

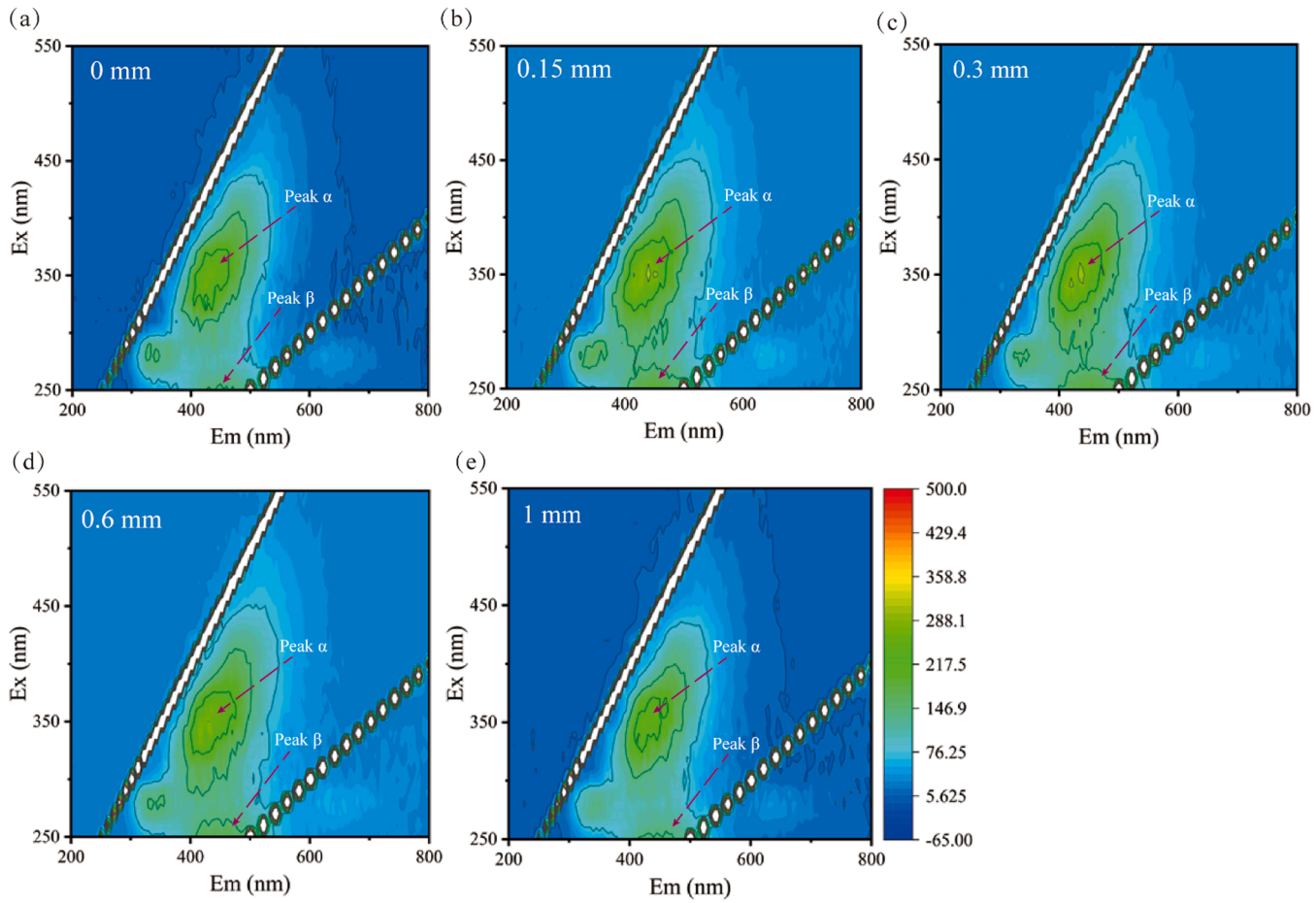

Fig. 2. Effect of PET microplastics with different particle sizes on EEM fluorescence spectra.

production. With the extension of fermentation time, the VFAs showed a tendency to increase and then decrease. At the 6th d, the VFAs in each fermenter reached their peak, and the PET-MPs groups decreased by 9.12–22.21 %. In addition, from Fig. 3(e), the VFAs showed an obvious decrease trend with the decrease in PET-MPs particle size. This may be due to the fact that the addition of PET-MPs promoted WAS rupture and released a large amount of organic matter to promote VFAs production at the early stage of fermentation. With the prolongation of the fermentation time, the toxicity of MPs was gradually released, and the acidogenic bacteria and enzyme activities were inhibited. Ultimately, the production of VFAs in the reactor decreased (Chen et al., 2021). From Fig. 3(d), the addition of PET-MPs inhibited VFAs production. The percentage of acetic acid was the highest in all groups. Notably, it was found from Fig. 3(f) that the inhibition of propionic acid, butyric acid, and valeric acid by PET-MPs with different particle sizes showed a significant linear correlation. The inhibition effect was rather better for larger particle sizes.

Therefore, PET-MPs addition had some inhibitory impact on the acidification stage of WAS anaerobic fermentation, thus inhibiting the production of acidification products such as VFAs. The inhibition performance became more pronounced with decreasing particle size.

3.3. Changes in the fermentation environment and cell structure

3.3.1. Fermentation environment

Fig. 4(a) shows the effect of different particle sizes of PET-MPs on pH. The pH levels in each fermenter exhibited a fluctuating trend, initially declining and subsequently rising throughout the fermentation process. Additionally, the fermenters remained within the alkaline range (7.82–9.06) throughout the fermentation period, with no discernible difference in pH observed between the groups. This finding was in agreement with the result of Wang et al. (2023a). It had been demonstrated that in alkaline fermentation, PET-MPs released toxic DBP. This reduced hydrolytic bacteria and inhibited protein and carbohydrate hydrolysis (Wei et al., 2019b).

Fig. 4(b) illustrates the ORP trend in each fermenter during the fermentation of WAS with PET-MPs of varying particle sizes. Throughout the test, ORP showed an increasing and then decreasing trend, which was opposite to pH. Some studies have demonstrated that the amount of VFAs produced as a product of the acidification stage is directly related to ORP and that low ORP is more likely to produce high levels of VFAs (Luo et al., 2020). The overall ORP in the experimental group was slightly higher than that observed in the control group. Therefore, this conclusion can clarify that PET-MPs has an inhibitory effect on the production of VFAs.

3.3.2. ATR-FTIR

ATR-FTIR scanning of the fermentation broth pretreated in each reactor allowed for exploring the influence of PET-MPs with varying sizes on the functional groups of WAS cells (Fig. S1). Significant spectral peaks were present at 1000–1250, 1600–1700, and 3250–3500 cm^{-1}. Among these, the region of 1000–1250 cm^{-1} was assigned to the C-O bond stretching of polysaccharide (Falk et al., 2015). And the peaks in the region of 1600–1700 cm^{-1} as well as 3250–3500 cm^{-1} were due to the stretching vibration of C═O and O-H bonds. At the beginning of anaerobic fermentation, PET-MPs adsorbed on the surface of WAS particles, which caused changes in the number of C-H bonds. As time progressed, the release of toxic substances from PET-MPs resulted in

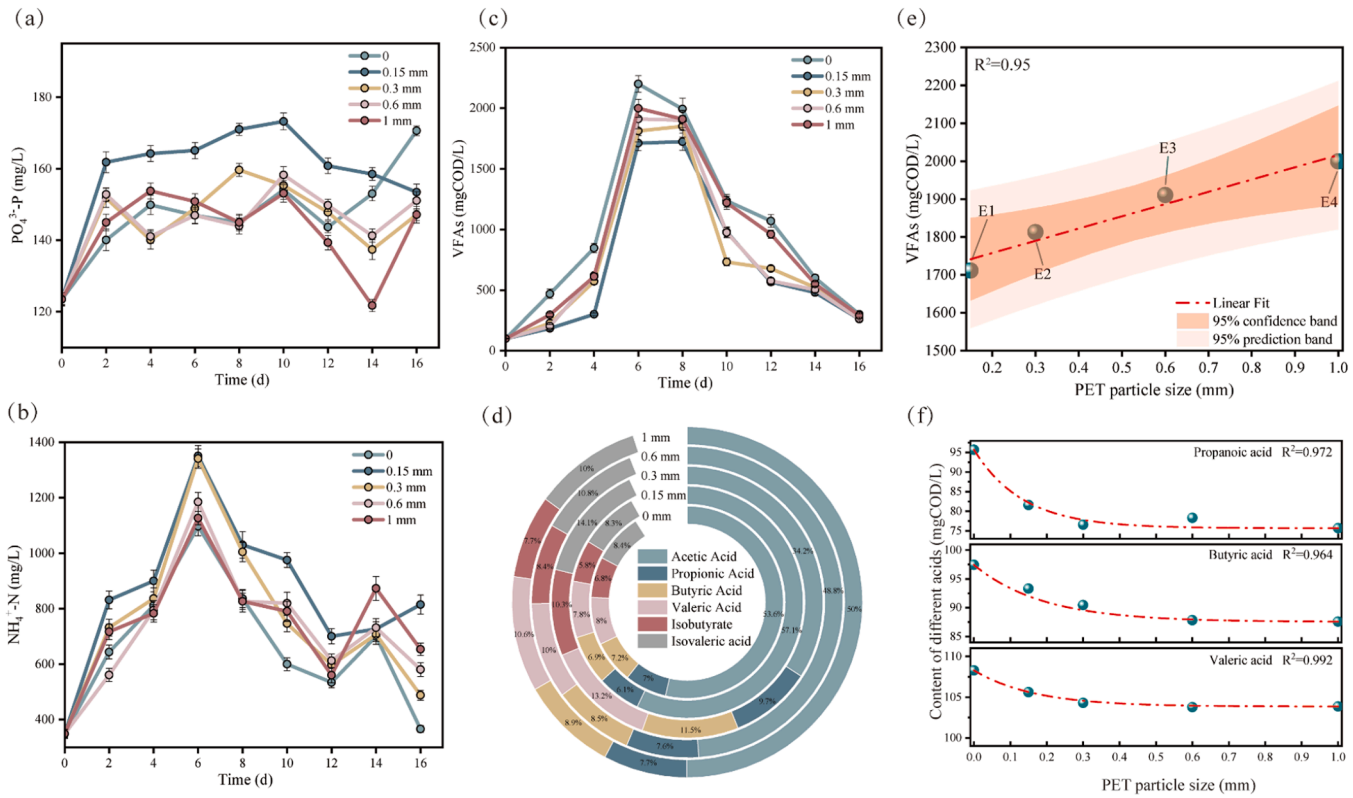

Fig. 3. Effect of PET microplastics with different particle sizes on PO_4^{3-}-P (a), NH_4^+-N (b), VFAs (c), and VFA composition (d), and fitting analysis of VFAs (e) and different acids (f).

alterations to the surface structure of WAS, which affected the dynamics of C-O and C═O bonds, consequently impacting the anaerobic fermentation process of WAS (Tao et al., 2020; Chen et al., 2024). However, the specific effects of PET-MPs with different particle sizes on WAS cell structure and the anaerobic fermentation process need to be further investigated.

3.3.3. Enzyme assays

LDH, a cell membrane integrity marker, indicates the impact of PET-MPs on WAS cell damage, while protease and α-glucosidase activities mirror hydrolysis effects in anaerobic fermentation (Fig. 4(c)-(f)). The influence of PET-MPs exhibiting varying particle sizes on the three enzymes shows a significant linear correlation (Fig. 4(c)). The addition of PET-MPs significantly increased the LDH activity by 34.01–71.5 %. This indicated that PET-MPs greatly enhanced WAS cell fragmentation. However, the relative activities of α-glucosidase and protease were reduced by 11–39 % and 18.18–23.18 %, respectively, in the PET-added reactors. This indicated that the presence of PET-MPs accelerated WAS cell fragmentation but inhibited hydrolysis. This could be due to the fact that PET-MPs disrupted WAS cell integrity and reduced the abundance and activity of hydrolyzed and acidogenic bacteria by leaching toxic substances. In addition, smaller particle sizes generally possess a larger specific surface area, thereby augmenting their available contact area with WAS cells and microbial cells. This could result in heightened adverse impacts on microorganisms (Huang et al., 2023). Consequently, smaller PET-MPs have a greater inhibitory effect on WAS cell integrity and microbial activity, and it can also well explain the effect trends of PET-MPs with different particle sizes on soluble substrates such as SCOD.

3.4. Microbial community structure analysis

3.4.1. Microbial community richness and diversity index

As shown in Table 3, and Fig. 5(a) and (b), the α-diversity indicated that the addition of PET-MPs resulted in a decrease in microbial richness and diversity, but the effect was not significant. Venn and PCA can effectively evaluate the similarities and differences of microbial communities, and 1310–1406 OTUs were identified in five groups of samples. Each group of samples contained unique OTUs, and the differences in these unique OTUs elucidated the distinctions in the microbial communities among different components (Pang et al., 2022). Adding PET-MPs caused the number of unique OTUs to decrease. This indicated that PET-MPs changed the community composition of microorganisms. PCA is a technique that simplifies data analysis by focusing on analyzing the principal components in each sample, which could reflect the differences and relative distances between samples (Awasthi et al., 2017). The PCA plot further illustrated the large difference in microorganisms between the control and experimental groups. This result suggested that the added PET-MPs induced a difference in microbial structure, but the effect between different particle sizes was not significant.

3.4.2. Microbial community composition

3.4.2.1. At the phylum level. Fig. 5(c) displays the phyla of microbial communities in the fermenters dosed with PET-MPs of different particle sizes. *Proteobacteria* (29.73–39.97 %), *Firmicutes* (24.80–31.98 %), and *Bacteroidota* (21.41–25.54 %) were the three most abundant phyla in all reactors. The relative abundance of *Proteobacteria* and *Firmicutes* was not significantly different between groups. The control group exhibited a slightly higher relative abundance of *Bacteroidota* compared to the experimental groups, with a decrease in *Bacteroidota* relative abundance observed as the particle size of PET-MPs decreased. Many microorganisms in these three phyla are involved in the hydrolysis process, which

Table 2
Effects of different microplastics in different particle size ranges on WAS cell fragmentation and the hydrolysis process.

Types of microplastics	Particle size	Effects on the release of organic matter	Reference
PS	1 mm、100 μm、1 μm	The SCOD of the experimental group was higher than that of the control group 1692.50 ± 180.31 mg/L, and the small particle size group was higher than that of the large particle size group under the same dosage.	(Li et al., 2022)
PS	0.5μm	The greatest increase in total organic matter was 113.9 % of the control, and humic acid intensity increased by 63.1 %.	(Wang et al., 2022)
	1 μm	Humic acid strength increased by 58.1 %	
	10 μm	Humic acid strength increased by 56.1 %	
	50 μm	Humic acid content decreased by 22.5 %	
	75 μm	Humic acid content decreased by 22.5 %	
	150 μm	Humic acid content decreased by 22.5 %	
PET	30 μm (0.45、1.35、2.70 mg/g TS)	SCOD content decreased by 7.37–8.82 %	(Wang et al., 2023a)
	250 μm (0.45、1.35、2.70 mg/g TS)	SCOD content decreased by 2.46–6.17 %	
This study (PET)	0.1、0.15、0.3、0.6、1 mm	The contents of SCOD, protein and polysaccharide were increased by 20.05–37.80 %, 3.5–5.47 % and 4.21–6.49 %, respectively, and the smaller the particle size, the greater the increase.	This study

can degrade carbohydrates and proteins into small molecules such as volatile organic acids (Wu et al., 2016). For example, *Bacteroidota* and *Proteobacteria* are able to promote the hydrolysis of proteins and polysaccharides to produce VFAs, and *Firmicutes* secrete extracellular enzymes such as cellulases, proteases, and lipases and can survive harsh environmental conditions (Zeng et al., 2019; Shen et al., 2019). Therefore, this could explain why Fig. 5(c) displays the phyla distribution of microbial communities in the reactors dosed with PET-MPs of different particle sizes. The addition of MPs did not reduce the abundance of *Firmicutes*. It was found that the enrichment of *Proteobacteria*, *Firmicutes*, and *Bacteroidota* during anaerobic fermentation could be involved in the degradation of toxic contaminants in PET-MPs. The larger particle size of PET-MPs can provide more sites for microorganisms, which may be one of the reasons why the microorganisms in the group dosed with larger PET-MPs were slightly higher than those in the control group (Wu et al., 2022; Wang et al., 2023a). Overall, the PET-MPs addition was able to inhibit the hydrolytic acidification of these microorganisms.

3.4.2.2. At the genus level. At the genus level (Fig. 5(d)), the main bacteria are *Soehngenia* (10.14–13 %), *Lentimicrobium* (7.38–10.41 %), *Advenella* (2.52–9.94 %), *Pseudorhodobacter* (4.71–5.68 %), *Thauera* (2.28–5.83 %), *Erysipelothrix* (2.6–4.94 %), *Flavobacterium* (2.16–4.74 %), and *Pseudoxanthomonas* (1.94–3.93 %).

The relative abundances of *Soehngenia*, *Lentimicrobium*, and

Table 3
Effect of PET microplastics of different particle sizes on microbial abundance and diversity.

Sample	shannon	simpson	ace	Chao1	coverage
0 mm	4.733917	0.028882	1636.399	1570.237	0.993554
0.15 mm	4.791441	0.030727	1701.761	1691.786	0.993203
0.3 mm	4.768754	0.032363	1790.577	1749.127	0.994403
0.6 mm	4.703174	0.032846	1788.923	1772.655	0.99383
1 mm	4.476591	0.039204	1591.509	1534.081	0.994192

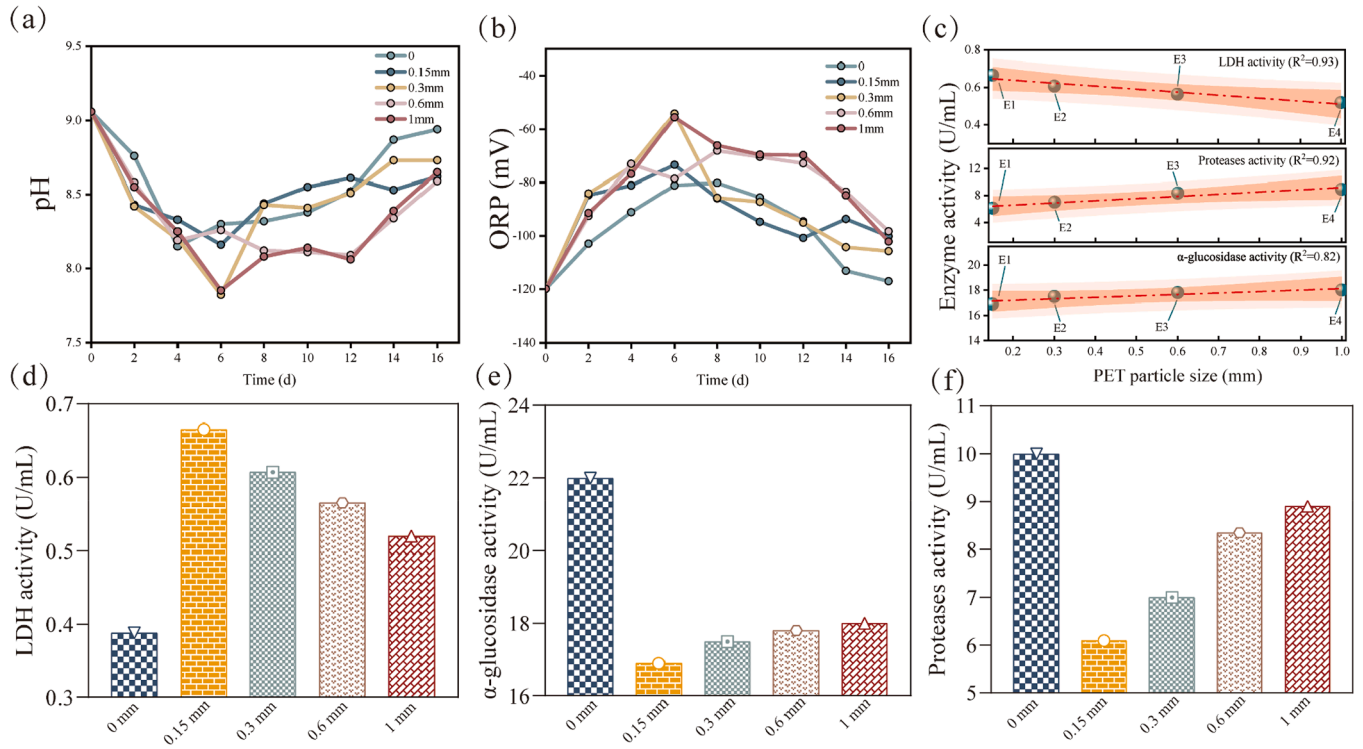

Fig. 4. Effect of PET microplastics with different particle sizes on pH (a) and ORP (b); fitting analysis of PET microplastics with different particle sizes on enzyme (c); effect of PET microplastics of different particle sizes on LDH enzyme (d), α-glucosidase (e), and protease (f).

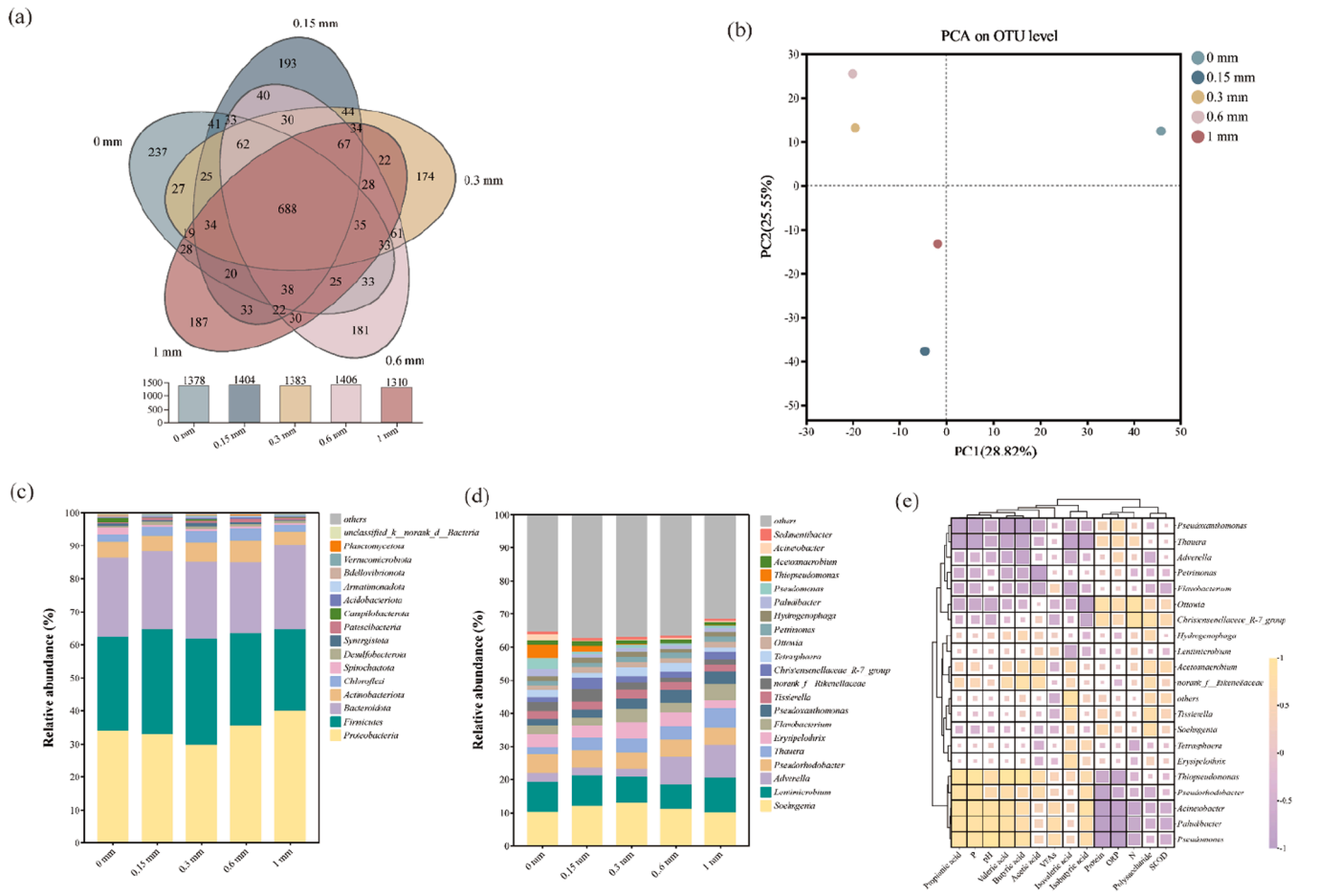

Fig. 5. Venn diagram (a), PCA (b), and effect of PET microplastics with different particle sizes at the level of microbial phylum (c) and genus (d); correlation analysis of the relative abundance of soluble substrates with the level of dominant bacteria (yellow represents positive correlation and purple represents negative correlation) (e).

Erysipelothrix did not differ much among the reactors, which might be due to the fact that these bacteria produced corresponding antibodies and survived with the release of toxicity from PET-MPs. *Soehngenia* and *Erysipelothrix*, both belonging to the phylum *Firmicutes*, are common VFA-producing bacteria, and *Soehngenia* is frequently detected in many toxic pollutant enrichments (Nazina et al., 2020; Xie et al., 2022). *Lentimicrobium* is an anaerobic hydrolytic and acidogenic bacterium capable of degrading carbohydrates into acetic and butyric acids (Xu et al., 2023). The control group exhibited a slightly higher relative abundance of *Pseudorhodobacter* compared to the experimental group, but the difference between the groups was not significant. The relative abundances of *Advenella*, *Thauera*, *Flavobacterium*, and *Pseudoxanthomonas*, on the other hand, had a significant decreasing trend with decreasing PET-MPs particle size in the experimental group. *Advenella*, *Thauera*, and *Flavobacterium* were all able to degrade organic matter into VFAs, and *Pseudoxanthomonas* was mainly involved in the degradation of glucose and transformed into VFAs (Zhang et al., 2023; Muratçobanoğlu et al., 2020). The prolonged exposure to toxic substances in MPs makes it possible that these microorganisms may be able to develop tolerance to the toxic substances and survive in harsh environments with a high degree of adaptability. However, prolonged exposure to toxic substances inhibits the activity and function of the microorganisms and affects their metabolic activities.

According to previous studies, MPs with smaller particle sizes were more likely to impair the activity of certain enzymes and microorganisms, thus negatively affecting microorganisms (Huang et al., 2023). Although the incorporation of PET-MPs did not markedly alter the abundance of some microorganisms, the toxic substances in PET-MPs were released with the fermentation time. This could disrupt the functions of hydrolytic bacteria and acidogenic bacteria. This would prolong the microbial degradation period of organic matter and thus inhibit the accumulation of VFAs (Zhang et al., 2021). To summarize, PET-MPs with small particle sizes can inhibit the hydrolysis and acidification processes of WAS by reducing microbial activity.

3.4.3. *Microbe-environment correlations*

Fig. 5(e) illustrates the correlation between soluble substrate and dominant bacterial abundances in experimental groups with varying particle sizes of PET-MPs at the genus level (yellow represents positive correlation and purple represents negative correlation). The key genera *Soehngenia*, *Advenella*, *Erysipelothrix*, *Thauera*, and *Pseudoxanthomonas* are all common hydrolytic and acidogenic bacteria in anaerobic fermentation (Sitthi et al., 2022; Wang et al., 2023b). Yet under these conditions, they are negatively correlated with VFAs, acetic acid, etc., and positively correlated with macromolecular organic matter such as proteins. This further elucidated the stronger inhibition of hydrolysis and acid production during anaerobic fermentation caused by PET-MPs with smaller particle sizes.

3.5. *KEGG based metabolic pathways*

The functional characteristics of the bacterial community during fermentation were predicted based on the results obtained from the KEGG analysis. The five samples showed small differences in KEGG

pathway abundance at level 1 (Fig. S2(a)). The primary functional category was metabolism, which constituted over 76 %. The incorporation of PET-MPs decreased metabolic abundance, and the effect was more significant at smaller particle sizes. Based on the abundance of the 14 core pathways at level 2, carbohydrate metabolism and amino acid metabolism were the most important pathways (Fig. S2(b)). The metabolic abundance of carbohydrate metabolism and amino acid metabolism was significantly decreased in the experimental group with the addition of small-sized PET-MPs. Therefore, the incorporation of small-sized PET-MPs limited the metabolism of carbohydrates and amino acids.

The abundance of three levels of carbohydrate and amino acid metabolic pathways is shown in Fig. 6(a). When PET-MPs were added, both amino sugar and nucleotide sugar metabolism and the biosynthesis of amino acids were inhibited, while the effect of glycolysis/glucoseogenesis was not significant. Amino sugar and nucleotide sugar metabolism and glycolysis/gluconeogenesis are both central steps in the production of VFAs during hydrolytic acidification (Kong et al., 2022). During anaerobic fermentation, various hydrolytic and acidogenic bacteria converted polysaccharides and proteins to pyruvate and produce intermediates (e.g., acetyl-CoA, NADH, etc.) through various metabolic pathways (Bhatia and Yang., 2017). Pyruvate was then further reacted as a substrate in pyruvate metabolism to produce various VFAs (Shan et al., 2012). Therefore, this further confirmed that PET-MPs dosing inhibited the metabolism of amino acids and carbohydrates during hydrolysis, thus inhibiting the production of acidification products such as VFAs.

3.6. *Fermentation enzyme function is affected by PET-MPs*

From Fig. 6(b), the incorporation of PET-MPs inhibited glycolysis, amino acid, and VFAs metabolism. The key genes implicated in amino acids and VFAs, such as *serB*, *scoA*, *scoB*, *ivd*, and *aldB*, were reduced by 0.78–18.92 % compared with the control group. The inhibition performance became more pronounced as the particle size decreased. Among them, *scoA* and *scoB* are engaged in the transformation of acetoacetate into poly-β-hy-droxybutyrate in the amino acid metabolic pathway, and *ivd* is mainly involved in the accumulation of valeric acid in the VFAs metabolic pathway (Huang et al., 2020; Peng et al., 2023). It was noteworthy that, in the context of glycolysis-related gene expression, while PET-MPs were observed to reduce intracellular metabolism, large-sized PET-MPs demonstrated superior inhibition compared to small-sized PET-MPs.

Anaerobic microorganisms are crucial in anaerobic fermentation, with their metabolic function reliant on key enzyme activity (Yuan et al., 2020). Consequently, the activities of key enzymes associated with the anaerobic fermentation process were analyzed to better understand the influence of PET-MPs on anaerobic fermentation. Fig. 5(c) illustrates the various fermentation pathways associated with the production of VFAs.

During anaerobic fermentation, protease and α-glucosidase catalyze macromolecular proteins and polysaccharides into amino acids and monosaccharides, respectively, and are subsequently degraded to pyruvate (Li et al., 2019). Pyruvate is the key control point and can be converted to acetyl-CoA, which is further transformed into butyric acid and acetic acid by butyric kinase and acetokinase (Chen et al., 2017; Li et al., 2022). An analysis of key enzyme genes, including beta-glucosidase [EC: 3.2.1.21], acetate kinase [EC: 2.7.2.1], and alpha-amylase [EC: 3.2.1.1], showed that PET-MPs hindered hydrolysis and acidification, which resulted in diminished VFAs accumulation.

The examination of microorganisms, metabolic pathways, and key enzymes indicated that PET-MPs did not notably diminish the abundance of crucial microorganisms. Small-sized PET-MPs could reduce the activity of key hydrolytic and acidogenic bacteria by inhibiting key enzymes, thus affecting the metabolic pathways of amino acids and carbohydrates to produce VFAs. However, the investigation of relevant metabolic pathways and associated gene enzymes in this study remains insufficient, and further discussion is needed in future work.

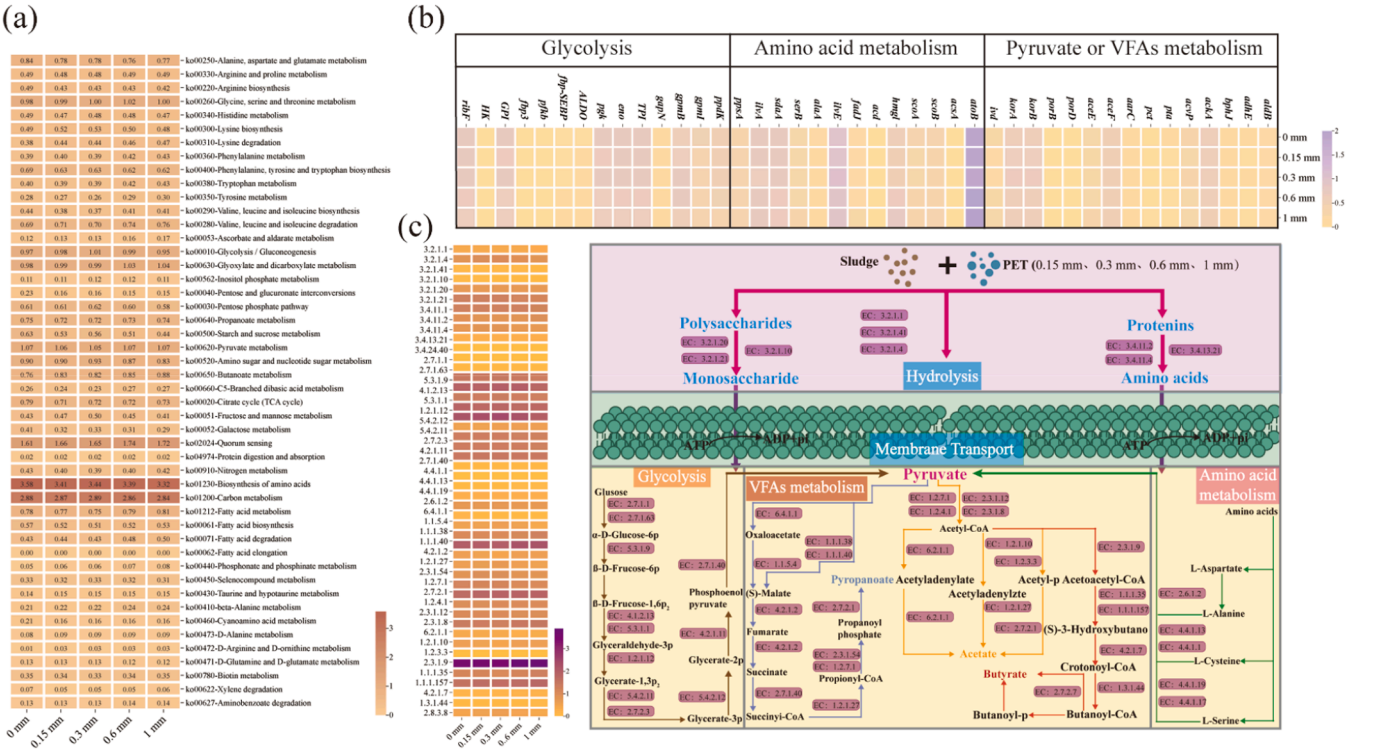

Fig. 6. Effect of PET microplastics with different particle sizes on the abundance of amino acid and carbohydrate metabolism pathways at level 3 (a), functional genes (b), and the schematic of anaerobic pathways associated with VFA production and consumption (c).

4. Conclusion

The effects of different sizes of PET-MPs on the hydrolysis and acid production stages of WAS anaerobic fermentation were revealed. The incorporation of PET-MPs promoted the fragmentation of WAS cells but inhibited the hydrolysis and acidification stages and hindered the formation of VFAs. The effect became more significant as the particle size decreased. In addition, small-sized PET-MPs reduced the activity of functional bacteria and enzymes, thereby inhibiting hydrolysis and acid production.

CRediT authorship contribution statement

Zhihao Zheng: Writing – review & editing. **Jingsi Gao:** Writing – review & editing, Validation, Funding acquisition, Conceptualization. **Guorun Zhou:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Xindong Teng:** Formal analysis. **Jun Wei:** Writing – review & editing. **Shuai Zhang:** Writing – review & editing, Formal analysis, Conceptualization. **Xiao Huang:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psep.2024.12.075.

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