Supplementary Information

Revealing the Mechanisms of Polyethylene Microplastics Affecting Anaerobic Digestion of Waste Activated Sludge

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The following are included as supporting information for this paper:

number of pages: 16
number of tables: 3
number of figures: 4
Determination of PE microplastics in Raw WAS

The studied raw sludge from the local WWTP was collected in three groups, with each at every two weeks. PE microplastics in each group of raw WAS were extracted and quantitated, according to the reported method. Briefly, 20 g of WAS was collected as the sludge sample. 300ml deionized water with 360 mg sodium chloride (NaCl) were added to WAS samples to remove organic matters or inorganic solids in sludge. The mixture was stirred for 15 min and settled for 2 h. The supernate was filtered using a filter with 37 um pore size. The extracts were washed with distilled water and collected in the sieve. Then, the extracts were treated with hydrogen peroxide solution (H₂O₂, 100 ml, 30%) for 12 h. After adding 200 ml of distilled water, microplastics sample were collected gridded petri dishes with sequentially numbered grids followed by filtration, washing and drying procedures. Subsequently, the extract was inspected and counted using a stereomicroscope (Carl Zeiss Jena, Stemi 508), then were further identification by Microscopes Fourier Transform infrared spectrometer (M-FTIR, IR/NicoletiN10 MX, Thermo Fisher Scientific). All tests were performed in triplicate. Recovery experiments were performed using clean sludge and PE microplastics (size 40 μm) standards. Each PE microplastic particle was marked with a blue marker to ensure the accuracy of the measurement. Fifty particles of PE microplastics were added to 20 g of clean sludge, and spiked for 15 min. Then, the number of PE particles separated from the sludge sample was enumerated. Results showed that the percentage efficiency was 87-96% (n = 5).
Illumina Miseq Sequencing Analysis

The genomic DNA in samples was extracted using the Fast DNA Kit (MoBio Laboratories). The quantity and purity of collected DNA was measured using Thermo Scientific Nanodrop spectrophotometer. Nearly complete bacterial 16S rRNA genes were amplified using the primers 338F (5’-ACTCCTACGGGAGGCAGCAG-3’) and 806R (5’-GGACTACHVGGGTWTCT AAT-3’). Archaeal 16S rRNA genes were amplified using the primers 524F10extF (5’-TGTCAGCCGCGCGGTAA-3’) and Arch958RmodR (5’-YCCGGCGTTGAVTCCAATT-3’). All PCR amplifications were performed using the Taq PCR Master Mix Kit (Qiagen, Basel, Switzerland). After the DNA was quantified using a spectrophotometer (Nanodrop Technologies, Rockland, DE, USA), the archaeal and bacterial DNAs from the same sample were mixed and analysed by Illumina miseq sequencing (Illumina Miseq PF300 platform), as detailed in our previous study. The operation taxonomic units (OUTs) used the 97% identity thresholds for the taxonomic classification analysis.

The Tests of Effects of PE Microplastics on Each Step Involved in WAS Anaerobic Digestion

Solubilisation test: The influences of PE microplastics exposure on solubilization were carried out under the same procedure as the BMP tests but with 50 mmol/L of
2-bromoethanesulfonic acid (BESA) addition to eliminate methanogens interference. The rate of sludge solubilization was assessed by measuring the concentrations of soluble COD released in fermentation liquor after fermentation for 3 d.³

Hydrolysis test: The rate of this process was evaluated using model substrates according to the literature.⁴ As the main sludge solubilized products were soluble protein and polysaccharide, bovine serum albumin (BSA, average molecular weight Mw ~67000) and dextran (Mw ~23800) were used as model protein and polysaccharide compound, respectively. The operation was conducted with the same approach described in solubilisation test except that 20 mL WAS was replaced with equivalent volume of synthetic wastewater. 20 mL synthetic wastewater contained KH₂PO₄ 20.00 mg, MgCl₂·6H₂O 12.00 mg, CaCl₂ 8.00 mg, FeCl₃ 2.00 mg, NiCl₂·6H₂O 0.02 mg, CuSO₄·5H₂O 0.01 mg, MnCl₂·4H₂O 0.01 mg, CoCl₂·6H₂O 0.01 mg, ZnSO₄·7H₂O 0.01 mg. Then 0.08 g BSA and 0.02 g dextran were added in synthetic wastewater. The pH in each test was adjusted from 6.3 to 7.0 using 1 mol/L NaOH and HCl. The effects of PE microplastics (10, 30, 60, 100 and 200 particles/g-TS) on hydrolysis were evaluated by analyzing the degradation efficiency of BSA and dextran after fermentation for 3 d.

Acidification test: In this set of tests, glutamate and glucose serve as the model amino acid and monosaccharide compound, respectively. Acidification process was assessed using the same operations as hydrolysis test except that 0.08 g glutamate and 0.02 g
glucose were dissolved in 20 mL synthetic wastewater instead of BSA and dextran.

Methanation test: Sodium acetate (0.048 g) was used as the model short-chain fatty acid (SCFA) compound, which was the main acidification product. All other procedures were the same as described above but without BESA addition. The influences of PE microplastics exposure on this process was explored by the analysis of methane production after fermentation for 15 d.

**Additives Concentrations Measurement**

75 ml n-hexane was added in freeze-dried sludge sample. The mixture was filtered after sonication for 60 min. Then the extract was evaporated to 1 ml by rotary evaporation (30°C), then dissolved with 200mL of deionized water. The extracts in sludge solids were mixed with the filtrate of sludge for further solid-phase extraction (SPE). The samples passed through SPE cartridges (5 mL/min), washed with n-hexane–water (10:90, v/v) solution. After vacuum drying and elution with n-hexane (1-2 mL/min), the eluates were dried with anhydrous sodium sulfate, evaporated to 0.2 ml by rotary evaporation (40°C), then transferred to a test tube and concentrated under a flow of nitrogen. After that, the extract was diluted with n-hexane to 0.2 mL and then analyzed by GC/MS. GC equipped with a splitless injector and a HP-5 capillary column (30 m×0.25 mm i.d., 0.25 um film thickness) was used. Helium was
used as the carrier gas with a flow rate of 1.0 ml/min. The initial column temperature was 60°C and was raised to 220°C at a rate of 20°C/min and then raised to 280°C at a rate of 5°C/min and held for 4 min. For quantitative analysis, a mass spectrometer was used in selected ion monitoring (SIM) mode. Ionization was by EI (70 eV). The ionization current was 40-500 AMU. The ionization chamber temperature was maintained at 150°C. Mass numbers of monitored ions for three additives (i.e., ATBC, DEHP and Irgafos® 168 phosphate) were detailed in Suhrhoff and Scholz-Böttcher.

**Measurement of Reactive Oxygen Species (ROS) Production**

According to Limbach et al., the intracellular ROS production was determined using 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes, Eugene, USA), which can be oxidized to fluorescent dichlorofluorescein (DCF) by intracellular ROS. The digestates were centrifuged at 10000 rpm for 10 min, then the pellets were washed using phosphate buffer (0.1 mol/L, pH 7.4) and resuspended in phosphate buffer with H2DCF-DA (50 μmol/L) and incubated in dark (35 ± 1 °C). After 30 min, the pellets were centrifuged for 20 min and plated into a 96-well plate with phosphate buffer. Fluorescence measurements of DCF were conducted by a fluorescence spectroscopy (Thermo Electron, Vantaa, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

**Cell Viability Analysis**
Cell viability was measured according to Worle-Knirsch et al.\textsuperscript{8} Briefly, 15 mL digestate was washed with phosphate buffer (0.01 mol/L, pH 7.4) and plated into a 96-well plate with 10 uL CCK-8 (Dojindo, Japan), then the plates were incubated at 35 ± 1 °C for 30 min. The absorbance of the plate was obtained using Tecan Sunrise (SHIMADZU, Japan) at 450 nm with a reference wavelength of 600 nm.

**Calculation of Increased Volume for Sludge Disposal by PE Microplastics Exposure**

To derive the increased volume for sludge disposal by PE microplastics exposure, consider the results in VS destruction (%). “a” represents the mass of WAS with inorganic solids representing ~18.2% of the WAS.

In the Control, the average VS destruction is ~30.4%. Thus, the volume of sludge to be disposal of is a×(1-18.2%)×(1-30.4%)+a×18.2%.

For the case of PE microplastics at 200 particles/g TS, the average VS destruction is ~22.1%. Thus, the volume of sludge to be disposal of is a×(1-18.2%)×(1-22.1%)+a×18.2%.

From the above, the PE microplastics exposure with 200 particles/g TS was estimated to increase the volume of sludge to be disposed of by approximately 9.1%.
Table S1. Primary properties of WAS and ADS.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WAS</th>
<th>ADS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS) (g/L)</td>
<td>39.6 ± 0.4\textsuperscript{a}</td>
<td>22.3 ± 0.2</td>
</tr>
<tr>
<td>Volatile solids (VS) (g/L)</td>
<td>32.4 ± 0.2</td>
<td>16.8 ± 0.1</td>
</tr>
<tr>
<td>Total chemical oxygen demand (TCOD) (g/L)</td>
<td>45.2 ± 0.3</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td>Soluble chemical oxygen demand (SCOD) (g/L)</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>pH</td>
<td>6.9 ± 0.1</td>
<td>7.8 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Indicate standard deviations of triplicate measurements.
Table S2. Estimated k, B<sub>0</sub> and Y<sub>0</sub> of WAS with different PE levels based on one-substrate model.

<table>
<thead>
<tr>
<th>PE (particles/g-TS)</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k (d&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>0 (i.e. control)</td>
<td>0.210 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>0.209 ± 0.012</td>
</tr>
<tr>
<td>30</td>
<td>0.208 ± 0.012</td>
</tr>
<tr>
<td>60</td>
<td>0.202 ± 0.009</td>
</tr>
<tr>
<td>100</td>
<td>0.192 ± 0.011</td>
</tr>
<tr>
<td>200</td>
<td>0.178 ± 0.004</td>
</tr>
</tbody>
</table>

<sup>a</sup> Indicate standard deviations.
Table S3. Alpha diversity revealing the community diversity and phylogenetic diversity of microbial community in the two long-term operated reactors (control: raw WAS; experimental: WAS at 200 particles PE microplastics/g-TS).

<table>
<thead>
<tr>
<th></th>
<th>Shannon index(^a)</th>
<th>PD-whole-tree index(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Archaea</td>
</tr>
<tr>
<td>Control</td>
<td>5.57</td>
<td>2.05</td>
</tr>
<tr>
<td>Experimental</td>
<td>5.58</td>
<td>2.04</td>
</tr>
</tbody>
</table>

\(^a\) Response to: community diversity; \(^b\) Response to: phylogenetic diversity.
Figure S1. Photomicrographs of PE microplastics in sludge.
Figure S2. Schematic diagram of the two continuous digester systems.
Figure S3. Effects of different levels of PE microplastics exposure on cumulative methane production from waste activated sludge during anaerobic digestion. Error bars represent standard deviations.
**Figure S4.** The possible mechanisms of ROS generation from PE microplastics that affect WAS anaerobic digestion through reducing cell viability, leading to the negative influences of ROS produced on sludge hydrolysis, acidification and methanation processes in WAS anaerobic digestion.
SUPPLEMENTARY REFERENCES


