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Stress-Responses of Performance and Microbial Community in Anaerobic Digestion System Under Long-Term Enrichment of Phenanthrene

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Abstract. The expanded granular sludge blanket reactor (EGSB) was operated for 198 days to study the long-term effects of phenanthrene (PHE) enrichment on system performance and microbial community. The results showed that the PHE was significantly enriched in the reactor. The final PHE concentration in effluent and sludge reached to 1.764±0.05 mg/L and 12.52±0.42 mg/gTS, respectively. While the average daily methane production was decreased by 5.0%-9.8% under long-term PHE exposure. The 3D-EEM of effluent indicated that PHE stimulated the microbial metabolism with the higher intensity of soluble microbial byproduct-like materials (SMP) and proteins. Moreover, the removal efficiency of soluble chemical oxygen demand (SCOD) and NH4+-N gradually diminished with the enrichment of PHE. PHE shaped the microbial community, and the predominant fermentative bacteria (Mesotoga) was severely inhibited. Contrarily, the bacteria (Syntrophorhabdus, Acinetobacter, Desulfovibrio, Desulfomicrobium) involved in PHE-degradation was enriched at end of Phase V. In addition, the relative abundance (RA) of hydrotrophic methanogens (Methanofastidiosum, Methanolinea, Methanobacterium, Methanomassiliicoccus) increased by 0.96-fold with the long-term enrichment of PHE, while the RA of acetoclastic Methanosaeta obviously decreased.

Keywords. Expanded granular sludge blanket reactor, phenanthrene, anaerobic digestion system

1. Introduction

In recent years, soil contamination caused by the production and transportation of petroleum has attracted increasing attentions. The polycyclic aromatic hydrocarbons (PAHs) are a common class of pollutants at contaminated sites, which has harmful effects on living and non-living taxa due to their recalcitrant and lipophilic nature [1]. Therefore, researchers attached great importance to the studies about PAHs removal [2, 3].

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The biological treatment with the advantages of environmental friendliness, low operational and investment costs has been applied to remove PAHs. Many studies have focused on the aerobic biodegradation of PAHs, and the high removal efficiency has been achieved [4]. However, in the deep soil, the oxygen transfer is limited, which is not conducive to the survival of aerobic microorganisms. Thus, the anaerobic microorganisms play an important role in the attenuation of PAHs. Nevertheless, the anaerobic biodegradation of PAHs still suffers many challenges such as long biodegradation period and high microbial sensitivity [2]. So far, few studies focused on the response of system performance and microbial community in anaerobic environment under the long-term enrichment of PAHs.

In present study, the phenanthrene (PHE) was selected as the model pollutant to investigate the effects of long-term PAH exposure on the anaerobic system. Meanwhile, the carbohydrate (starch) was added to provide the sufficient carbon source for the metabolism of anaerobic microbes. The variations of biomethane production, SCOD, NH_4-N and dissolved organic matter (DOM) in effluent were analyzed. Also, the succession of microbial community under the long-term enrichment of PHE was evaluated.

2. Materials and Methods

2.1. Experimental Design

The PAH of PHE was purchased from the reagent company. The inoculum sludge was taken from an industrial plant which was operated at mesophilic condition in Shandong province. The experiment was conducted in an expanded granular sludge blanket reactor (EGSB) with 6.0 L work volume filled with 2.96 kg sludge. The EGSB was operated with a HRT of 48 h under 35±2℃, and the up-flow rate was constant (0.69 L h^{-1}) with effluent recirculation. The characteristics of influents in different phases are shown in table 1.

Table 1. Characteristics of influents in different phases.

Phases	Duration (in days)	COD (g/L)	NH_4^+-N (mg/L)	C/N	pH	PHE (mg/L)
I	0-8	3	57.6	15	7.6±0.3	0
II	9-80	6	115.2	15	7.6±0.3	0
III	81-122	6	115.2	15	7.6±0.3	1
IV	123-156	6	115.2	15	7.6±0.3	10
V	157-198	6	115.2	15	7.6±0.3	100

2.2. Analytical Methods

2.2.1. Chemical Analysis

The indexes including SCOD, NH_4^+-N, pH, TS and VS were determined according to standard methods [5]. The volumes of biogas and biomethane were measured by a glass injector, and the CH_4 content in biogas was measured via absorbing the CO_2 by saturated sodium hydroxide solution. The concentration of PHE in effluent and sludge was analysed by High Performance Liquid Chromatography (Shimadzu LC-2030) [1].

2.2.2. EEM Analysis

Excitation-emission matrix (EEM) fluorescence spectrum of effluent was determined by a fluorescence spectrophotometer (Hitachi Japan, F-4600). The emission wavelengths from 200 to 550 nm at 5nm increments and the excitation wavelengths from 200 to 500 nm at 5nm increments were set. Milli-Q water was used as reference to eliminate the inner filter effect [6].

2.2.3. DNA Extraction and Sequencing

The sludge samples were washed with phosphate buffer saline (PBS) three times and centrifuged at 10000 G for 2 min. Microbial DNA was directly extracted from 2.0 g sludge of each sample with a MetaVx™ (GENEWIZ, Inc., South Plainfield, NJ, USA) according to manufacturer's instructions. Then, the full length of 16S rRNA was amplified using the primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-GGTTACCTTGTTACGACTT-3') and sequenced using PacBio Sequel system (Pacific Biosciences, USA) at Biomarker Technologies Co, Ltd. (Beijing, China).

3. Results and Discussion

3.1. PHE Impacts on EGSB Treatment Performance

The EGSB was operated for 198 days to evaluate the variations of treatment performance with the PHE enrichment. As shown in figures 1a and 1b, the concentration of PHE in reactor gradually increased at the end of different phases after Phase II. The highest final PHE concentration in effluent and sludge reached to 1.764±0.05 mg/L and 12.52±0.42 mg/g TS at Phase V (influent PHE=100 mg/L), respectively, indicating that the most of PHE was absorbed in sludge due to the hydrophobic property. The enrichment of PHE adversely affected the system performance. At Phase III (influent PHE=1 mg/L), the average daily biomethane yield (DMY) was decreased from 1461.24±151.40 mL/d in Phase II (influent PHE=0) to 1317.74±171.77 mL/d (figure 1c). It is suggested that the inhibition of biomethanation was caused by feeding PHE to anaerobic system. The previous study has reported that the PHE is toxic to methanogens which have low growth rate and are sensitive to changes in the environment [1]. Interestingly, the biomethane production of reactor didn't continue to decrease after Phase III, and the average DMY of Phase IV (influent PHE =10 mg/L) and Phase V with the values of 1351.21±131.33 mL/d and 1388.44±106.79 mL/d were slightly higher than Phase III. It was might ascribed to that the microbial community in reactor changed under the long-term PHE exposure, as discussed in section 3.3. However, they were still lower than that of Phase II, indicating that the biomethane production was suppressed by the PHE enrichment.

Furthermore, the long-term enrichment of PHE also posed negative effects on the removal of SCOD and NH_4^+ -N (figures 1d and 1f). After Phase I, the average influent COD concentration was 6.0 g/L during the whole experimental period. At Phase II (Start-up period) without adding PHE, the highest removal efficiency of SCOD was achieved in Phase II with the value of 65.5%±2.8%. However, when the PHE target pollutant of 1 mg/L was added in Phase III, the microbes in the reactor were sensitive to PHE, thus the SCOD removal efficiency decreased to 62.5%±2.8%. With increasing the influent PHE concentration, the removal efficiency of SCOD gradually decreased, and the lowest SCOD removal efficiency was found in Phase V (55.0%±3.1%). It is might because the

PHE is toxic to anaerobes, which may inhibit the activities of anaerobes. Similar to the removal of SCOD, the NH_4^+ -N removal efficiency continued to decline with the enrichment of PHE. Compared with Phase II (37.3%±8.2%), the removal efficiency of NH_4^+ -N was significantly diminished by 62.7%, indicating that the PHE could adversely affect bio-metabolic activity of NH_4^+ -N.

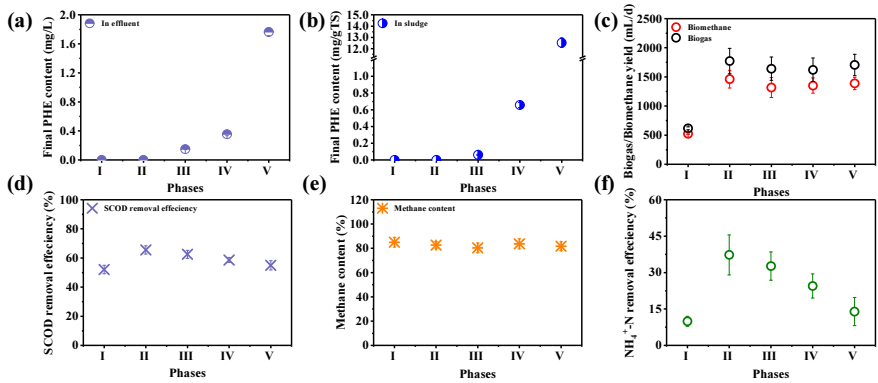

Figure 1. The variation of EGSB treatment under the long-term enrichment of PHE.

3.2. DOM Characteristics of Effluent in Different Phases

Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy of effluent in different phases was obtained, as shown in figure 2. The information from the EEM could provide a high value of reference for the metabolism of microorganisms during anaerobic digestion process [7], as it usually showed the relevant characteristics of dissolved organic matter (DOM) in effluent samples comprehensively, such as the components and source of organics.

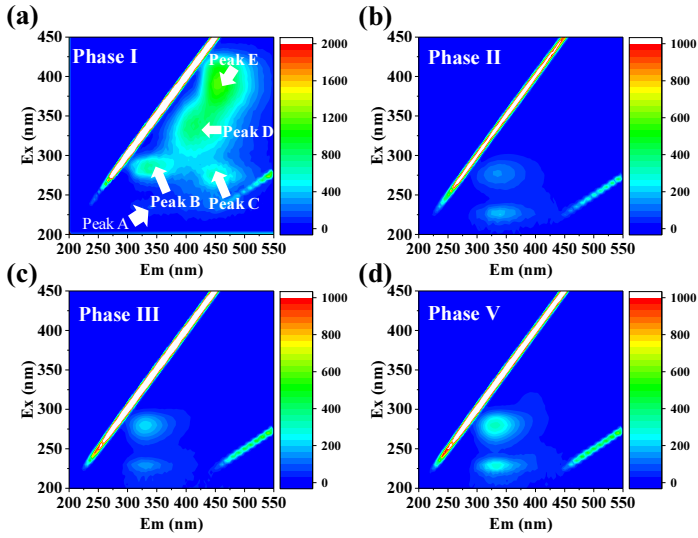

Figure 2. 3D-EEM analysis of DOM in effluents of different phases.

The locations of the DOM fluorescence peaks were identified based on excitation/emission (Ex/Em) (figure 2a), which can be summarized into five peaks, as followed: Peak A: Ex/Em=275-285/325-350 nm; Peak B: Ex/Em=225-240/320-340 nm; Peak C: Ex/Em=275-280/445-450 nm; Peak D: Ex/Em=320-340/410-430 nm; Peak E: Ex/Em=380-400/450-470 nm. Figure 2 showed that the characteristics of DOM in effluents of different phases. The five peaks were all found in the Phase I with the high intensity, but the peak C, peak D and peak E belonged to humic substances [6]. It is indicated that the severe humification was occurred and the system of reactor was unstable. Fortunately, figure 2b showed that only peak A and peak B were detected in the fluorescence spectrum of Phase II, and they represented soluble microbial byproduct-like materials and the component of tryptophan-like protein, respectively [7], indicating that the community structure of microorganisms in reactor achieved a stable state. After Phase II, with the increase of PHE concentration, the fluorescence intensity (FI) of SMP and tryptophan-like protein gradually increased. It is obvious that FI of components in effluent of Phase V was higher than that of Phase II. The possible reason was that the PHE stimulated the bio-metabolism, which leaded to more byproducts from microbial activities including the PHE biodegradation byproducts.

3.3. Succession of Microbial Community

Sludge samples were collected at the end of Phase II and Phase V to identify the structure of microbial community, as shown in figure 3. Eight kinds of bacterial phyla were detected including: *Thermotogae*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi*, *Synergistetes*, *Verrucomicrobia*, *Actinobacteria*. As the most predominant phylum, the *Thermotogae* occupied 87.0% in Phase II. Previous study has reported that members of *Thermotogae* could ferment a various of simple sugars (e.g., glucose) and complex polysaccharides (e.g., xylan and starch) [8]. However, it's relative abundance (RA) significantly reduced to 47.9% in Phase V with the enrichment of PHE, indicating the PHE posed negative effects on fermentation of substrate (starch). The RA of *Proteobacteria* in Phase V (39.3%) was comparatively higher than Phase II (9.3%). And the phyla of *Firmicutes* was enriched by 4.3 times in Phase V compared with Phase II (1.3%). Lee et al. reported that *Proteobacteria* and *Firmicutes* were the dominant phyla in the oil contaminated sediment and potentially participated in the degradation of PAHs [9]. At genus level (figure 3a), eight dominant genera were found, as followed: *Mesotoga*, *Syntrophorhabdus*, *Acinetobacter*, *Clostridium*, *Bacteroides*, *Chryseomicrobium*, *Desulfovibrio*, *Desulfomicrobium*. Among them, the hydrolytic *Mesotoga* is the most predominant genus with the RA of 91.2% in Phase II, but it declined to 58.4% in Phase V. *Clostridium* and *Bacteroides* were responsible for carbohydrate hydrolysis in anaerobic system [10]. Their RA increased from 1.3% and 0.5% in Phase II to 7.3% and 2.9% in Phase V, respectively. Moreover, the abundance of *Syntrophorhabdus and Acinetobacter* which were typical acetogens increased with the enrichment of PHE in Phase V, reaching to 11.8% and 10.8%, separately. Compared with Phase II, *Desulfovibrio* and *Desulfomicrobium* belonging to sulfate-reducing bacteria exhibited higher abundance, which are reported to take part in the anaerobic degradation of PHE [11]. Above results indicated that the enrichment of PHE shaped the bacterial community.

The variations of archaeal community are showed in figure 3c. It was obvious that acetoclastic *Methanosaeta* occupied the highest proportion with the value of 71.1% in the Phase II. However, it's abundance was decreased by 29.8% in Phase V, indicating that the increase of PHE caused suppression on the growth of *Methanosaeta*. Conversely,

the RA of hydrotropic methanogens including *Methanofastidiosum*, *Methanolinea*, *Methanobacterium*, and *Methanomassiliicoccus* was promoted from 28.3% in Phase II to 55.6% in Phase V. It was suggested that the long-term enrichment of PHE affected methanogenic activities of archaea.

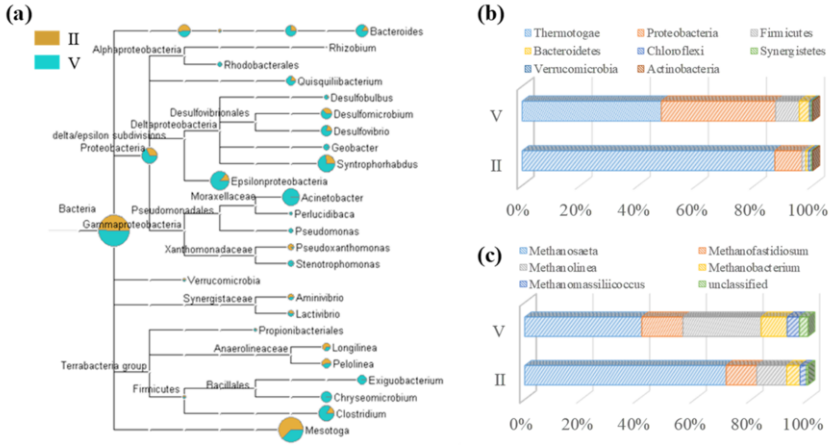

Figure 3. (a) The relative abundance (RA) of bacteria at different levels; (b) the RA of bacteria at phylum level; (c) the RA of methanogenic archaea at genus level.

4. Conclusions

The variations of anaerobic digestion performance and microbial community were investigated in EGSB under the long-term enrichment of PHE. There was slight suppression on biomethane production after the addition of PHE. Compared with Phase II (influent PHE=0), the average daily biomethane yield was diminished by 5.8-9.8% in the next phases. Meanwhile, PHE posed negative effects on the removal of SCOD and NH_4^+-N, and the biggest inhibition ratio (16.0% of SCOD removal and 62.7% of NH_4^+-N removal) in Phase V (influent PHE=100 mg/L). The sequencing results showed that the abundance of predominant fermentative bacteria (*Mesotoga*) significantly decreased under PHE exposure. Conversely, the typical acetogens (*Syntrophorhabdus*, *Acinetobacter*) and sulfate-reducing bacteria (*Desulfovibrio*, *Desulfomicrobium*) which potentially participated in biodegradation of PHE were enriched with increasing PHE concentration. Moreover, PHE also affected the structure of archaeal community. The growth of hydrotropic methanogens (*Methanofastidiosum*, *Methanolinea*, *Methanobacterium*, and *Methanomassiliicoccus*) were promoted, while acetoclastic *Methanosaeta* was inhibited.

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