Influence of n-Hexadecane and Naphthalene on Anaerobic Digestion: Kinetic Simulation, DOM Variation and Microbial Community Assessment

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Abstract. The influences of n-Hexadecane and naphthalene on anaerobic digestion in batch tests were investigated. The batch kinetic analysis showed that the maximum methane accumulations (141.18 mL of n-Hexadecane and 146.76 mL of naphthalene) and production rates (20.48 mL/h of n-Hexadecane and 20.88 mL/h of naphthalene) were obtained while adding with 30 mg/L n-Hexadecane and 2 mg/L naphthalene, respectively. However, a significant inhibition ratio of 10% was observed at 100 mg/L of n-Hexadecane and 20 mg/L of naphthalene, respectively. The 3D-EEM results showed that the naphthalene was degraded by related microorganism as potential carbon source with concentration lower than 10 mg/L. In addition, the microbial community analysis indicated that the abundance of hydrotrophic Methanolinea and Methanobacterium increased from 7.92% to 12.76% as the increase n-Hexadecane, while decreased from 13.01% to 9.07% with increase of naphthalene. Contrastly, the acetoclastic methanogens were increased due to the well synthetic action with syntrophic VFA-oxidizing bacteria.

1. Introduction

The petroleum is a complex mixture containing hydrocarbons and organic matter with significant effects of carcinogenic, teratogenic and mutagenic [1, 2]. The petroleum plays a key role in human life as energy sources. However, there was a phenomenon of oil spills during exploitation, transportation and use. It is inevitable that the damage of human, animals and plants caused by oil spill due to interaction of petroleum and environment. The pollution of petroleum had attracted much attention [3].

Nowadays, the biodegradation was applied to remove petroleum hydrocarbons from polluted soil. High efficiency was observed in the aerobic treatment. However, for the deep soil, many disadvantages exist in aerobic biodegradation. There are many factors that limit the oxygen transport in deep soil [4]. Therefore, more and more researches have shifted the work on anaerobic digestion of petroleum hydrocarbons [5, 6]. The anaerobic digestion plays an important role in the natural attenuation of petroleum hydrocarbons [7]. The degradation of petroleum hydrocarbons based on the microbial interaction not only reduces the cost of anaerobic digestion, but also produces clean energy. So far, there was few related research on the properties and microorganism response of petroleum hydrocarbons anaerobic biodegradation.
To investigate the anaerobic biodegradation in the middle and deep soil contaminated by petroleum hydrocarbons. In this study, the n-Hexadecane and naphthalene were acted as the model compound of alkane and aromatic hydrocarbon. The influences of different concentration on the methane yield were analyzed. The dissolved organic matter (DOM) measured by excitation-emission matrix (EEM) variation response were evaluated. Moreover, the microbial community under the different petroleum hydrocarbons concentration was also assessed.

2. Materials and Methods

2.1. Experimental Design

The petroleum hydrocarbon of n-Hexadecane and naphthalene were purchased from the reagent company. The inoculum sludge was taken from a working UASB reactor. The experiment of anaerobic digestion was performed in serum bottles with 100 mL work volume filled with 10 g sludge (VS: 10.04%; TS: 14.06%) under 35°C, 120 rpm. Different concentrations of petroleum hydrocarbon (n-Hexadecane: 0, 1, 5, 10, 30, 50, 100 mg/L; naphthalene: 0, 0.5, 1, 2, 3, 10, 20 mg/L) were added.

2.2. Analytical Methods

2.2.1. Chemical Analysis. The TS and VS were analyzed according to Standard Methods [8]. The biogas accumulations were measured by a vacuum glass injector. The methane content was measured by capturing the CO2 from saturated NaOH solution.

2.2.2. EEM Analysis. Fluorescence Excitation–emission matrix (EEM) was obtained by an F-4600 spectrophotometer. The dissolved organic matter (DOM) during digestion could be reflected by EEM. EEM spectra was obtained through setting the emission (Em) wavelength, which in the range from 200 to 600 nm in 4 nm steps, while the excitation (Ex) wavelength was increased from 200 to 400 nm in 5 nm steps. Each different fluorophore was characterised by an Ex/Em wavelength pair.

2.2.3. Kinetic Analysis. The Gompertz fitting equation is used for the kinetic fitting of the gas-producing curve, as following equation 1:

\[ PCH_4(t)=P \cdot \exp\left[-\exp\left(K \cdot e^{P(A-t)+1}\right)\right] \]

where, P is the maximum methane potential, mL; K is the maximum methane production rate, mL/h; A is the lag time, h; t is the response time, h.

2.2.4. DNA Extraction and Sequencing. The collected samples were washed with PBS three times and centrifuged at 10000 G for 2 min. Microbial DNA was directly extracted from 2 g sludge of each sample with a MetaVx™ (GENEWIZ, Inc., South Plainfield, NJ, USA) according to the manufacturer's instructions. The primer targeted the V3-V4 region were selected for sequencing as our previous reported [9].

3. Results and Discussion

3.1. Batch Performance and Kinetic Analysis

3.1.1. Methane Production in Hexadecane-Digestion and Naphthalene-Digestion. The batch tests were operated for 142 hours to evaluate digestion performance with n-Hexadecane or naphthalene. The results of biogas and methane were showed in figure 1. The maximum methane accumulation was observed in digester added 30 mg/L n-Hexadecane with 126.75 mL as almost 69.64% of total biogas (figures 1a and 1c). Followed by digester added 10, 50 and 0 mg/L, while the minimum production was found at a100 mg/L of n-Hexadecane. The similar tendency of production rate about biogas and methane was showed in figures 1b and 1d. Figures 1e-1h showed that the accumulation and production
rate of biogas and methane with different concentration of naphthalene. A maximum methane accumulation of 132.25 mL was obtained at digester with 2 mg/L naphthalene, which account for 70.82% biogas. Followed by digester added 1, 3, 0.5 and 0 mg/L naphthalene, which produced 123.00 mL, 111.00 mL, 110.00 mL and 105.50 mL of methane, respectively. The highest production rate of 20.88 mL/h was observed in the digester added 2 mg/L naphthalene at the start phase. The above results indicated that the petroleum hydrocarbons could be used by anaerobic microorganism acted as carbon source, and the driving force for overcoming the mass transfer resistance between solid and liquid phases was provided due to the addition of moderate petroleum hydrocarbons [10]. However, toxic metabolites produced by microorganism with excessive amount of petroleum hydrocarbons resulted in the inhibition of anaerobic digestion process [11, 12].

![Figure 1](https://example.com/figure1.png)

**Figure 1.** The accumulation and production rate of biogas and methane.

### 3.1.2. Kinetic Analysis of Biogas and Methane Production

To further investigate the digestion performance of n-Hexadecane and naphthalene, the kinetic analysis was applied with Gompertz fitting equation. As shown in table 1, the methane accumulation for each batch test could be divided into two phases, the first phase was in accordance with the results fitted by equation (1), and the second phase was simulated by a linear equation with high coefficient of determination. The accumulation and production rate of methane increases first and then decreases with the increase of the n-Hexadecane concentration. The maximum methane production rate was 16.2 mL/h with the digester added 1 mg/L n-Hexadecane while 20.5 mL/h was observed in the digester added 30 mg/L n-Hexadecane. The maximum methane production rate was reduced to 14.6 mL/h in the digester added 100 mg/L n-Hexadecane, which was 28.8% lower than the optimal digester. The excessive concentration of n-Hexadecane could inhibit the degradation. Moreover, the maximum methane production rate reached a peak of 1.1 mL/h with 30 mg/L n-Hexadecane while 0.8 mL/h was observed at a concentration of 100 mg/L. It indicated that the addition of excessive n-hexadecane would seriously inhibit the activity of microorganism.

The tendency of naphthalene was similar to n-Hexadecane, which increase first and then decrease with increasing concentration. The methane production rate was reduced to 9.3 mL/h with 20 mg/L naphthalene added, which was 55.5% lower than the peak value of 2 mg/L naphthalene. It indicated that the activity of anaerobic microorganism was stimulated under the moderate concentration of naphthalene while the activity was inhibited in the digesters added excess naphthalene.

### 3.2. DOM Characteristics of Different Concentrations of Naphthalene

Three-dimensional excitation-emission matrix (3D-EEM) information was obtained by continuously changing the excitation wavelength and specific fluorescent substances. The indicators obtained from the EEM had a high value of reference for anaerobic digestion process because they usually indicated
the relevant characteristics of DOM in sewage comprehensively, such as the source of organic material and the degree of humification.

**Table 1.** Kinetic parameters of naphthalene (Nap) and n-Hexadecane (n-Hex) in methane production.

| P (one) | P (two) | K(one) | K(two) | A(one) | A(two) |
|---------|---------|--------|--------|--------|--------|
| Methane            | SD Methane | Methane | SD Methane | Methane | SD Methane | Methane | SD Methane | SD |
| 0mg/L          | 43.8            | 1      | 113.5                | 3.1 | 14.1            | 1.2 | 0.9              | 0.1 | 8.5  | 0.1 | -27.2 | 3.9 |
| 0.5mg/L        | 46.3            | 0.9    | 122.6                | 4.9 | 14.3            | 1.1 | 1.1              | 0.1 | 8.4  | 0.1 | -27.6 | 5.3 |
| 1mg/L          | 52              | 0.9    | 136.4                | 4.8 | 18.1            | 1.1 | 1.1              | 0.1 | 8.5  | 0.1 | -26.7 | 4.6 |
| Nap 2mg/L       | 58.5            | 1      | 146.8                | 5.3 | 20.9            | 1.2 | 1.1              | 0.1 | 8.6  | 0.1 | -31.2 | 5.1 |
| 3mg/L          | 47.8            | 1.3    | 120.9                | 3.3 | 14.3            | 1.6 | 1.4              | 0.1 | 8.3  | 0.2 | -25   | 4   |
| 10mg/L         | 43              | 1      | 113.4                | 4.2 | 14.2            | 1.2 | 0.9              | 0.1 | 8.3  | 0.1 | -28.4 | 4.8 |
| 20mg/L         | 34.5            | 0.8    | 102.5                | 4.5 | 9.3             | 0.9 | 0.8              | 0.1 | 8    | 0.2 | -22.6 | 4.6 |
| n-Hex 0mg/L     | 43.3            | 0.9    | 120.8                | 3.9 | 17.6            | 1.3 | 0.9              | 0.1 | 8.5  | 0.1 | -27.8 | 3.8 |
| 1mg/L          | 42.5            | 1      | 119.5                | 4.4 | 16.2            | 1.4 | 0.9              | 0.1 | 8.4  | 0.1 | -27.6 | 4.2 |
| 5mg/L          | 43.5            | 1      | 122.7                | 4.6 | 16.3            | 1.4 | 0.9              | 0.1 | 8.3  | 0.1 | -27   | 4.1 |
| 10mg/L         | 49              | 1.2    | 132                  | 4.2 | 18.6            | 1.7 | 1                | 0.1 | 8.3  | 0.1 | -27.8 | 3.8 |
| 30mg/L         | 56.8            | 1.3    | 141.2                | 4.3 | 20.5            | 1.9 | 1.1              | 0.1 | 8.2  | 0.1 | -31.1 | 4.3 |
| 50mg/L         | 45.3            | 1.1    | 129.8                | 4.7 | 16.2            | 1.4 | 0.8              | 0.1 | 8.4  | 0.1 | -33.5 | 3.8 |
| 100mg/L        | 40              | 0.7    | 108.3                | 2.3 | 14.6            | 0.8 | 0.8              | 0.1 | 8.6  | 0.1 | -26.0 | 2.7 |

**Figure 2.** 3D-EEM analysis of different naphthalene concentration tests.

The position of the dissolved organic matter DOM excitation/emission (Ex/Em) fluorescence peak can be summarized as: tryptophan: Ex/Em=275-285/355-365 nm; tyrosine: Ex/Em=270-280/305-320 nm; Naphthalene: Ex/Em=220-230/345-355. Figure 2 showed that Peak A gradually moves from (360, 280) to (305, 275), and the peak intensity first increased and then decreased as the potential degradation process of naphthalene progresses. At the same time, tyrosine was produced, and tyrosine was continuously utilized; Peak B gradually moves from (355, 225) to (310, 220), and the peak
intensity first increases and then decreases. It indicated that naphthalene was continuously degraded to form an intermediate product, and finally degraded to methane.

3.3. Microbial Community Analysis
The abundance of major microorganisms was showed in Table 2. The n-Hex_{30} and n-Hex_{100} were the digesters added 30 and 100 mg/L n-Hexadecane while the Nap_{2} and Nap_{20} of 2 and 20 mg/L naphthalene. The anaerobic degradation of hydrocarbon was connected with methanogenesis related to the use of acetic acid and H_{2}/CO_{2} [13]. The acetic acid and H_{2}/CO_{2} could be associated with many reactions to constructed a complex regulatory network [14]. The methane was produced by the interaction of microorganism during the process of methanogenesis [15]. The abundance of hydrotrophic Methanolinea and Methanobacterium was increased with increase n-hexadecane while decreased with naphthalene increase. Contrarily, the proportion of acetoclastic Methanoseta and Methanomassiliicoccus was decreased with the n-hexadecane increase whereas increase with the naphthalene increase. Syntrophomonas could construct a syntrophic metabolism pathway with methanogens [16]. The proportion of Syntrophomonas was 2.01\%, 1.80\%, 2.07\% and 2.11\% in n-Hex_{30}, n-Hex_{100}, Nap_{2} and Nap_{2}. Besides, Syntrophorhabdus and Syntrophobacter could transform propionate to acetate [17]. Syntrophobacter belong to bacteria of producing acetic acid on digestion, which could transform VFAs to acetic acid and H_{2}/CO_{2}. 8.60\%, 9.69\%, 9.23\% and 10.65\% were observed in n-Hex_{30}, n-Hex_{100}, Nap_{2} and Nap_{2}, respectively. The acetogens were decreased followed the hydrocarbon increase. Song et al. [9] considered that the high abundance of acetogens could maintain the system stability. The proportion of the dominated acetogens were 28.12\%, 23.22\%, 26.69\% and 20.85\% in n-Hex_{30}, n-Hex_{100}, Nap_{2} and Nap_{2}, respectively.

Table 2. Major microorganisms’ abundance in the samples.

| Samples                               | C16-30 | C16-100 | C10-2 | C10-20 |
|---------------------------------------|--------|---------|-------|--------|
| **Methanogens**                       |        |         |       |        |
| Methanobacterium                      | 224    | 268     | 217   | 242    |
| Methanolinea                          | 473    | 848     | 644   | 588    |
| Methanoseta                           | 454    | 724     | 248   | 511    |
| Methanospirillum                      | 232    | 349     | 157   | 185    |
| Methanomassiliicoccus                 | 165    | 199     | 170   | 172    |
| **Syntrophic VFA-oxidizing bacteria**|        |         |       |        |
| f__Synergistaceae_Unclassified        | 2373   | 1919    | 1614  | 2666   |
| Syner-01                              | 1299   | 1205    | 932   | 1568   |
| Syntrophomonas                        | 177    | 157     | 137   | 193    |
| Syntrophobacter                       | 757    | 847     | 611   | 974    |
| Syntrophorhabdus                     | 102    | 102     | 69    | 71     |
| f__Syntrophomonadaceae_Unclassified   | 14     | 27      | 7     | 21     |
| Syntrophus                           | 22     | 21      | 22    | 11     |
| f__Syntrophaceae_Unclassified         | 32     | 47      | 25    | 39     |
| Other dominated Acetogens             |        |         |       |        |
| Acetoanaerobium                       | 263    | 127     | 245   | 81     |
| Smithella                             | 45     | 55      | 38    | 35     |
| Proteiniclasticum                    | 0      | 1       | 0     | 0      |
| Proteiniphilum                       | 2137   | 1802    | 1470  | 1758   |
| Propionicicella                      | 29     | 45      | 14    | 34     |

4. Conclusion
The anaerobic digestion performance was evaluated in batch tests added different naphthalene and n-Hexadecane. The optimal digesters were observed in the digesters added 30 mg/L n-Hexadecane and 2 mg/L naphthalene, respectively. There were significant inhibitions in the digesters added
excess hydrocarbons. The maximum inhibition ratio (9.66% of 20 mg/L naphthalene and 10.35% of 100 mg/L n-Hexadecane) was obtained compared to the control. Moreover, the microbial community analysis showed that the abundance of syntrophic VFA-oxidizing bacteria and the dominated acetogens was decreased with the increase concentration of n-hexadecane while increased with the increase concentration of naphthalene. The abundance hydrotrophic Mhanolinea and Methanobacterium was increased with increase of n-Hexadecane, which was contrast to the trend of naphthalene tests.

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