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Abstract

The wide application of graphene oxide nanoparticles inevitably leads to their discharge into wastewater treatment plants and combination with the activated sludge. However, to date, it is largely unknown if the nano-graphene oxide (NGO) has potential impacts on the anaerobic digestion of waste activated sludge (WAS). Therefore, this work aims to fill the knowledge gap through comprehensively investigating the effects of NGO on carbon

transformation and methane production in the anaerobic digestion of WAS. Biochemical methane potential tests demonstrated the methane production dropped with increasing NGO additions, the cumulative methane production decreasing by 7.6% and 12.6% at the NGO dosing rates of 0.054 mg/mg-VS and 0.108 mg/mg-VS, respectively. Model-based analysis indicated NGO significantly reduced biochemical methane potential, with the highest biochemical methane potential decrease being approximately 10% at the highest NGO dosing rate. Further experimental analysis suggested that the decreased methane production was firstly related to a decrease in soluble organic substrates availability during the process of sludge disintegration, potentially attributing to the strong absorption of organic substrates by NGO. Secondly, NGO significantly inhibited the methanogenesis by negatively affecting the corresponding enzyme activity (i.e. coenzyme F420), which could also resulted in a decreased methane production.

Key words: nano-graphene oxide; waste activated sludge; biochemical methane potential; anaerobic digestion; methane production; methanogenesis.

1. Introduction

Graphene oxide is a class of two-dimensional sp^2 nanocarbon discovered in 2004. Nowadays, it has been widely applied in multiple industrial areas such as electronic industry, semiconductor manufacture, electrocatalyst synthesis and electrochemical biosensors production, due to its excellent physicochemical properties (Ahmed and Rodrigues, 2013; Chen et al., 2012). Because of its wide application, the waste nano-graphene oxide (NGO) is

inevitably discharged into wastewater treatment plants (WWTPs), then entering into waste activated sludge (WAS) stream due to its limited solubility and strong adsorption capacity for organic matters (Barrena et al., 2009; Ramesha et al., 2011; Xing et al., 2014). Therefore, it is necessary to evaluate the impacts of NGO on the processes of WAS treatment.

Anaerobic digestion is a highly effective way for WAS treatment, which possesses many advantages, including low energy consumption, high sludge reduction, effective pathogen killing and substantial biogas production (Chen et al., 2008; Dai et al., 2017; Zhen et al., 2014). Anaerobic digestion is usually divided into four key stages, i.e., disintegration, hydrolysis, acidogenesis, and methanogenesis (Kim et al., 2003). The composite particulate materials in WAS were firstly disintegrated into carbohydrates, proteins and lipids, which were then breakdown by anaerobic bacteria and eventually converted to methane by methanogens (Sun et al., 2014; Sun et al., 2015; Tian et al., 2017). Previous investigations have reported that nano-graphene and Fe_3O_4 nanoparticles induced positive effects on the methane yield (Li et al., 2015; Tian et al., 2017). While, other studies indicated that ZnO nanoparticles and nano zero valent iron posed a negative effect on the methane production (Mu and Chen, 2011; Yang et al., 2013). These results suggested that different nano materials might have different impacts on anaerobic digestion processes, which could depend on their physical and chemical properties.

NGO with its stratified structure, which links carboxyl, hydroxyl and epoxide groups in the surface and edge, has received increasing attentions on its interactions with microorganisms (Wang et al., 2013b). Previous work has showed that NGO is biocompatible with *Escherichia Coli* (*E. Coli*) growth in Luria-Bertan (LB) medium (Luo et al., 2016).

However, NGO showed significant cytotoxicity at the dosage of 50 $\mu\text{g/ml}$, leading to the cell apoptosis (Wang et al., 2011a). The bacterial metabolic activity was also found to be inhibited by the addition of NGO in the activated sludge, with the normal activity of micrograms being hindered (Ahmed and Rodrigues, 2013). However, to date, there is no study to investigate the impacts of NGO on microbial processes in the anaerobic digestion of WAS.

Therefore, the aim of this study is to systematically assess the potential impacts of NGO on carbon transformation and methane production in the anaerobic digestion of WAS. The effects of different NGO levels on methane production from WAS were firstly investigated with biochemical methane potential tests. The impact of NGO on both the hydrolysis rate and the methane production potential were revealed through model-based analysis. Then effect of NGO on WAS characteristics and microbial enzyme activities were evaluated. Finally, batch experiments were conducted to further investigate the comprehensive impacts of NGO on the each step of anaerobic digestion of WAS. The results are expected to provide a new insight for water industries into the management of anaerobic digestion processes with NGO exposure.

2. Materials and methods

2.1 Sludge sources

The WAS used in this study was collected from a WWTP treating domestic wastewater located in Shanghai, China. Its main characteristics were as follows: pH 6.8 ± 0.1 , total solids (TS) 23200 ± 100 mg/L, volatile solids (VS) 14100 ± 100 mg/L, soluble chemical oxygen demand (SCOD) 306 ± 27 mg/L, total chemical oxygen demand (TCOD) 19100 ± 650 mg/L, soluble carbohydrates 20 ± 1 mg-COD/L, and soluble protein 99 ± 4 mg-COD/L. The

concentration of soluble carbohydrates and soluble protein of the WAS were also detected as they were important components of the WAS and could reflect how easy the WAS could be degraded. The sludge collected was stored less than 15 days in 4°C freezer for all experiments to minimize the biotransformation.

For the anaerobic digestion tests further described below, the inoculum was harvested from a laboratory scale semi-continuous anaerobic digester treating the WAS collected from the same WWTP as aforementioned. The reactor had an effective volume of 10 L and a sludge retention time (SRT) of 20 days. The main characteristics of the inoculum are follows: pH 7.1 ± 0.1 , TS 18900 ± 300 mg/L, VS 11800 ± 200 mg/L, SCOD 126 ± 10 mg/L, TCOD 16100 ± 350 mg/L, soluble carbohydrates 10 ± 1 mg-COD/L, and soluble protein 40 ± 4 mg-COD/L. The inoculum was withdrawn immediately before starting each test.

2.2 Nano graphene oxide

The NGO used in this study was purchased from Suzhou TANFENG graphene Tech Co., Ltd, China and its lateral dimensions was down to <10 nm. According to data provided by the manufacturer, the main characteristics of the NGO are as follows: thickness 3.4-8 nm, layer numbers 5-10, specific surface area $100\text{-}300\text{ m}^2/\text{g}$, diameter $10\sim50\text{ }\mu\text{m}$, and purity >98%. A stock suspension of graphene oxide was produced by adding 1g of graphene oxide into 0.1L deionized water, which was sonicated (25°C, 600W, 40kHz) for 30min to obtain an applicable dispersion.

2.3 Anaerobic batch biochemical methane potential tests

The anaerobic batch biochemical methane potential (BMP) tests were carried out in a series of 120 ml serum bottles. Each bottle was filled with 26 ml of inoculum and 52 ml of WAS to reach a VS ratio of 0.4. Before the WAS being added to the bottle, a stock solution of NGO was added to the WAS to achieve a concentration of 0.054, and 0.108g NGO/g VS, respectively. These tested NGO concentrations were in the range of commonly investigated concentrations in wastewater and sludge treatment systems (Suárez-Iglesias et al., 2017). Each serum bottle was flushed with nitrogen gas for 1 min with a flow rate of 1L/min to remove oxygen, and then was sealed with a rubber stopper retained with an aluminum crimp cap. The BMP tests were operated in a shaker-incubator at 100 rpm under mesophilic conditions ($35\pm1^{\circ}\text{C}$). Tests were mixed by inversion prior to each sampling event. Two sets of blanks (Blank I and Blank II) were also set up. Blank I was added with inoculum without WAS to evaluate the biogas production of inoculum. Blank II contained inoculum and WAS without NGO addition to serve as a control group of no NGO addition. All tests were carried out in triplicates. The BMP tests lasted for 55 days, when the biogas production dropped to an insignificant level.

The biogas (CH_4 , CO_2 , and H_2) production was detected with gas chromatograph (GC) on daily basis over first week and every 2-4 days in the following weeks. The detailed methods for biogas collection and detection were further described in Section 2.7. The biogas production from experimental group was obtained by subtracting biogas production from Blank I. The methane production was reported as the volume of methane produced per kilogram of VS added ($\text{mL CH}_4/\text{g VS added}$). The organic matters were monitored simultaneously during anaerobic batch biochemical methane potential tests. Several BMP

bottles were setup in parallel for the sludge sampling purpose. The sludge samples for organic matter analysis were obtained by opening the bottle and withdrawn using a pipette. The SCOD, soluble proteins, soluble carbohydrates, and ammonia nitrogen ($\text{NH}_4^+\text{-N}$) was measured about every ten days during the tests.

2.4 Biochemical methane potential tests modeling

The hydrolysis rate (k) and biochemical methane potential (B_0), two key parameters associated with methane production, were used to compare methane production kinetics and potential of WAS with and without NGO exposure. They were estimated by fitting the methane production data obtained from BMP tests to a first-order kinetic model using a modified version of Aquasim 2.1d with sum of squared errors (J_{opt}) as objective function (Batstone et al., 2009; Sun et al., 2017). The first-order kinetic model was shown in Equation (1):

$$B(t) = B_0(1 - e^{-kt}) \quad \text{Equation (1):}$$

Where $B(t)$ is cumulative methane production at time t (mL methane/g VS added); B_0 is biochemical methane potential (mL CH_4 /g VS added); k is hydrolysis rate (d^{-1}) (hydrolysis is considered as the rate-limiting step in the anaerobic digestion of WAS); t is time (d). The uncertainty surfaces of k and B_0 were also based on a model-validity F-test with 95% confidence interval. The degradation extent (Y) of WAS was determined using Equation (2) (Liu et al., 2015).

$$Y = B_0/380 \times R_{WAS} \quad \text{Equation (2)}$$

Where B_0 is biochemical methane potential (mL CH_4 /g VS added), 380 is theoretical

biochemical methane potential under standard condition (25 °C, 1atm) (mL CH₄/g TCOD) (Eddy, 2003); R_{WAS} is measured ratio of VS to TCOD in the studied WAS (i.e. 0.72 in this study). The organic contents in the WAS used for cell synthesis was not included in the Y and its value could be varied according to the microbial community composition in the inoculum.

2.5 Effect of NGO on WAS characteristics and microbial enzyme activities

Firstly, the WAS and NGO were mixed to assess the effect of NGO on the characteristics of WAS, which was important to the performance of methane production from WAS. Four 250 ml batch reactors were firstly filled with 100 ml WAS for each. A stock solution of NGO was then added to the reactors to reach the NGO levels of 0, 0.027, 0.054, 0.081 g/g VS, respectively. As the WAS characteristics was a key factor affecting the methane production from the anaerobic digestion, the NGO levels applied here were designed with a smaller increment than other tests, which could reflect how sensitive the WAS characteristics changed according to NGO addition. A calculated amount of deionized water was also added to keep the total volume consistent in each reactor. The mixtures of NGO and WAS were then mixed for 10 minutes with magnetic stirrers (Meiyingpu, Shanghai) at a rate of 500 rpm. In each test, the concentration of TCOD, SCOD, TS, VS, NH₄⁺-N, soluble proteins and soluble carbohydrates was measured.

Secondly, the interaction between the NGO and the soluble organic substances in the WAS were also characterized using the Fourier-transform infrared spectroscopy (FTIR) analysis. The WAS of 100ml was firstly centrifuged at a speed of 4000 g for 20 min and then filtered through disposable needle filter (0.45 µm pore size). The filtrate was then added with

different amount of NGO to reach the concentration of 0, 0.054, and 0.108g /g VS. FTIR spectra were obtained used a Nicolet 5700 spectrometer in transmittance mode within the wavenumber range of 400-4000 cm^{-1} (64 scans and 4 cm^{-1} resolutions).

Finally, the effect of NGO addition on the key microbial enzyme activities governing the key processes of the WAS anaerobic digestion was evaluated. The inoculum sludge was taken from a laboratory anaerobic digester treating WAS and was mixed with NGO to reach the NGO dosage levels of 0 g/g VS, 0.054 g/ g VS, and 0.108 g/ g VS, respectively (based on the ratio of NGO to VS in the unfiltered WAS). The mixture obtained was the used to assess enzyme activities of protease, acetate kinase (AK) and coenzyme F420, which were considered as key enzymes in processes of hydrolysis, acidogenesis and methanogenesis, respectively. The detailed analytical procedures of protease, AK and coenzyme F420 activities were described in the Supporting Information (SI).

2.6 Effect of NGO addition on individual steps of WAS anaerobic digestion

There are four steps (i.e., disintegration, hydrolysis, acidogenesis, and methanogenesis) that are generally included in the WAS anaerobic digestion. Disintegration referred to the processes that composite particulate materials were breakdown into carbohydrates, proteins, lipids and other inert materials, while hydrolysis referred to the processes that carbohydrates, proteins and lipids were further degraded into monosaccharides, amino acids and long chain fatty acids. The experiments carried out in this section aimed to provide insights into the impacts of NGO on each step as described above.

The effect of NGO on sludge disintegration is indicated by the release of SCOD in the

anaerobic digestion liquor. In the experiment, 52 ml of WAS and 26 ml of anaerobic inoculum was added into each serum bottle with a volume of 110 ml. Different amounts of NGO were added to the bottles to reach the NGO dosage of 0, 0.054 and 0.108g /g VS, respectively. The serum bottles were flushed with nitrogen gas for 1 min in a flow rate of 1L/min to remove oxygen, and then were sealed with a rubber stopper retained with an aluminum crimp cap. The experiments were operated in a temperature controlled incubator in a mesophilic condition ($35 \pm 1^\circ\text{C}$). The SCOD of sludge was determined every three hours for a 9-hour period.

The effect of NGO on hydrolysis and acidogenesis were evaluated by the degradation of model substrates, namely Bovine serum albumin (BSA) and glucose. For hydrolysis process, 630 ml of BSA solution (3000 mg/L) and 70 ml of anaerobic inoculum were added into an anaerobic batch reactor. Different amount of NGO were also added to the reactor to reach the same NGO dosage as mentioned above. The experiments of were operated in a temperature controlled incubator in a mesophilic condition($35 \pm 1^\circ\text{C}$). The concentration of BSA was analyzed daily for an eight days. The acidogenesis process was evaluated in a similar way. Glucose (3000ml/L) was used as the model substrate in the experiment and its concentration were monitored every 3 hours for a 12-hour period. Here, the volatile fatty acids (VFAs) concentration were not directly used for the assessment of the acidogenesis rate, as the produced VFAs in the tested system could be further degraded for methane production which might induce bias in the assessment.

The effect of NGO on methanogenesis was indicated by cumulative methane production using acetate as the substrate. This was because that acetoclastic methanogenesis was

considered as the predominant pathway of methane generation in the mesophilic anaerobic digestion of WAS (Rivière et al., 2009). In addition, the effect of NGO on the activity of hydrogenotrophic methanogens could also be reflected with this experimental design as acetate could be converted to hydrogen and carbon dioxide by fermentative bacteria and the produced hydrogen could then be used for methanogenesis. A mixture with anaerobic inoculum of 26 ml and acetate (6.8 g/L) of 52 ml was added to serum bottles. Different amount of NGO were also added to the bottles to reach the same NGO dosage as mentioned above. Blanks were setup without acetate solution to evaluate the biogas production of inoculum. The cumulative methane productions in a mesophilic condition ($35 \pm 1^\circ\text{C}$) were measured every two days for six days.

2.7 Analysis

Sludge samples were centrifuged at a speed of 4000g for 20min, and then were filtered through disposable needle filter (0.45 μm pore size) for the measurements of SCOD, $\text{NH}_4^+\text{-N}$, soluble proteins and soluble carbohydrates. The determinations of SCOD, $\text{NH}_4^+\text{-N}$, TS and VS were conducted according to standard methods (Clesceri et al., 1995). The soluble carbohydrate was measured using the Anthrone Method with glucose as standard (Lowry et al., 1951). The soluble protein was determined by Lowry-Folin method with Bovine Serum Albumin (BSA) as the standard (Gao et al., 2012). A 1 ml of the sludge sampled was diluted to 100 ml to assure the TCOD value of the diluted liquor in a measurable range. Then TCOD of diluted liquor was measured according to standard methods (Clesceri et al., 1995).

The cumulative biogas in the headspace of each serum bottle was calculated from the

pressure increase and expressed under standard conditions (25°C, 1atm). For each biogas sample, the gas content (CH₄, CO₂ and H₂) was analyzed using a gas chromatography (Lunan GC 6890, China) equipped with a thermal conductivity detector (TCD). One milliliter of the biogas was withdrawn from the headspace of the bottle using a syringe (SGE analytical science, Trajan Scientific and Medical Corporation) with a needle pierced into the septum. Then the sampled gas was directly injected into the inject port of GC for analysis. The remained biogas in the BMP bottle was released after each measurement.

3. Results

3.1. Biochemical methane production and carbon transformation

The effect of NGO on the biochemical methane production from WAS in anaerobic digestion is shown in Figure 1. Methane production presented a linear growth during initial ten days. Afterwards, the methane production rate slowed down in all cases due to the limited availability of the substrates, and significant differences in cumulative methane production were observed. Overall, an increased NGO level in WAS led to a decreased methane production from WAS during the 50-day test period. Compared to the control test (0g NGO/g VS), the cumulative methane production decreased by 7% and 12.6% at the dosage of 0.054 and 0.108 gNGO/gVS, respectively. This result indicated that NGO addition would induce inhibitory effect on methane production in anaerobic digestion of WAS.

The effect of NGO on the degradation of soluble organic matters is shown in Figure 2. The initial concentration of SCOD, soluble carbohydrates, and soluble proteins were significantly decreased with the increase of NGO dosage (Figures 2A-2C), which could be

related to the reduced biochemical methane production (Figure 1). However, the degradation patterns of these soluble organics were similar in all the tests. The sCOD, soluble proteins and soluble carbohydrates were mostly degraded during first 10 days, corresponding to the linear cumulative methane production during initial 10 days. Specifically, the soluble COD was removed about 55% to 70% during initial 10 days, which was continued to be degraded by about 0 to 5% afterwards. The removal rate of soluble proteins in the WAS reached about 85% to 100% on 10th day. The soluble carbohydrates were degraded by about 60% to 95% during first 10 days, and its degradation rate remained unchanged at the rest of test period. In contrast, the NGO addition under tested conditions had no significant effect on the releasing of soluble ammonium nitrogen (Figure 2D), indicating that NGO did not pose adverse effect on the hydrolysis rate of protein. This result was in accordance with the degradation of protein as shown in Figure 2B, which showed that protein degradation rate was not significantly affected by NGO addition, as indicated by similar protein profiles at different NGO level. The relatively high protein concentration at 10 min without NGO addition was likely due to the potential measurement errors for protein, since further investigation also supported that degradation rate of protein would not affect by NGO addition (Section 3.4).

3.2. Model based analysis of hydrolysis rate and biochemical methane potential

The first-order kinetic model was applied to simulate the methane production in the BMP test and to determine the hydrolysis rate (k) and biochemical methane potential (B_0) at different NGO dosing rates. The model simulated methane productions are illustrated in Figure 3A, which showed satisfactorily fits between experimental measurements and model predicts

($R^2 > 0.99$). The estimated k and B_0 and their 95% confidence regions at different NGO dosing rates are shown in Figure 3B. The result suggested that the biochemical methane potential decreased gradually with the increase of NGO dosage. The highest methane potential decrease was approximately 10% (from 190 to 171 mL CH₄/g VS added) at the NGO dosage of 0.108 NGO/VS, compared with the WAS without NGO exposure. Correspondingly, the sludge degradation extent (Y) also decreased (from 0.36 to 0.32) with increased NGO level. On the other hand, k was not significantly affected when the NGO dosage increased to 0.054 g/gVS and it slightly decreased when the dosage was further increased to 0.108 g/gVS. This result was consistent with the result of enzyme analysis, that NGO inhibited the activity of coenzyme F420, a key enzyme in the process of methanogenesis (Section 3.3).

3.3 Effect of NGO on WAS characteristics and microbial enzyme activities

The changes of substances in WAS after the addition of NGO were presented in Figure 4. Figure 4A shows that an increased NGO level resulted in a decreased SCOD concentration in WAS. The SCOD concentration decreased by 24% in sludge at the NGO dosage of 0.027 g/g VS, compared with the WAS without NGO addition. At the NGO dosage of 0.054 g/g VS, the SCOD concentration in the WAS further decreased by 7%, while this decline became smaller (1%) at the dosage of 0.081 g/g VS. This suggested that the NGO addition in WAS could result in a significant decrease of SCOD concentration, but the decrease became less significant when the NGO level was further increased above 0.054 g/g VS. Similar concentration variation patterns were also observed in the cases of soluble proteins and soluble carbohydrates, with the increased level of NGO resulting in the decline of soluble

proteins and soluble carbohydrates in the WAS. This result indicated that the NGO might bond with these organic substances in WAS through its strong adsorption capacity, which in turn decreased the substance availability for methane production. In contrast, the NGO addition did not induce obvious difference in TS, VS, TCOD and NH_4^+ -N concentrations in WAS.

Figure 5 shows the results of FTIR analysis at different dosages of NGO which were directly added into the WAS supernatant. The main absorption peaks identified according the literature were: i) O-H vibration of carboxylic and alcoholic groups at about 3400cm^{-1} ; ii) C-H stretching of the aliphatic at about 2970 cm^{-1} and 2930 cm^{-1} ; iii) C=O stretching of carboxylic acid at about 1720 cm^{-1} ; iv) -COO^- asymmetric vibration of carboxylic acid at about 1620 cm^{-1} ; v) N-H deformation vibration of amide II at about 1560 cm^{-1} ; vi) C=N stretching of amide III and COO^- stretching of carboxylic acids at about 1415 cm^{-1} ; vii) C-O stretching of carboxylic and C=N stretching of aromatic, secondary amines, or amide III at about 1295 cm^{-1} ; and viii) C-O asymmetric stretching of carbohydrate at about 1100cm^{-1} , 1071cm^{-1} and 1075 cm^{-1} (Abdulla et al., 2010; Gamage et al., 2014). It was observed that the increased NGO resulted in apparent wavenumbers blue shift (wavenumbers shifted to higher wavenumbers). For example, the wavenumbers shifted from 1078cm^{-1} and 1071cm^{-1} at the dosage of 0 g/g VS and 0.054 g/g VS to 1100cm^{-1} at the dosage of 0.108g/g VS. The wavenumbers shifted from 1563cm^{-1} at the dosage of 0 g/g VS to 1575cm^{-1} and 1614cm^{-1} at the dosages of 0.054 and 0.108 g/g VS, respectively. The occurrence of wavenumbers shifting might result from the interaction of the soluble organics (e.g. proteins and carbohydrates) with the hydroxyl on the surface of NGO (Dai et al., 2017).

Figure 6 shows the effect of NGO addition on the relative activities of protease, AK and coenzyme F420, which play critical roles in the processes of hydrolysis, acidogenesis and methanogenesis in WAS anaerobic digestion (Mu and Chen, 2011). Clearly, the activities of protease and AK did not change significantly with the addition of NGO at both levels of 0.108 and 0.054 g / g VS ($P > 0.05$, t-test). However, the activity of coenzyme F420 was decreased by 50% compared to control when the dosage of NGO was 0.054 g / g VS, and it further dropped to 30% of the control as the dosage of NGO was increased to 0.108 g / g VS ($P < 0.05$, t-test).

3.4 Effects of NGO on individual steps of WAS anaerobic digestion

Figure 7 shows the impacts of NGO on each step in methane production from WAS anaerobic digestion including disintegration, hydrolysis, acidogenesis and methanogenesis. As showed in Figure 7A and 7D, the processes of disintegration and methanogenesis were significantly inhibited with the NGO addition in WAS anaerobic digestion. However, there was no significant effect of NGO on hydrolysis and acidogenesis in the anaerobic digestion. Figure 7A shows that increased NGO level resulted in a decrease of SCOD released from the WAS throughout the 9-h tests. The initial value of SCOD concentration also decreased with the increasing dosage of NGO, which was consistent with the effects of NGO addition on the characteristics of WAS (See section 3.3). Figure 7B shows no significant difference was found on the degradation rate of model substrates at different dosages of NGO in the step of hydrolysis. Also, the glucose conversion also did not change significantly with the addition of NGO during the acidogenesis from glucose (Figure 7C). The initial concentration of the

protein and glucose were also not significantly affected by NGO addition in these tests, which could be attributed to the saturation of adsorption by NGO. When the saturation of adsorption was reached, the concentration of adsorbate would not be further decreased. Given the high initial concentration of protein and glucose applied in these tests (about 20-100 times higher than that presented in Figure 2), the effect of adsorption on the concentration decrease could become insignificant. However, the methanogenesis was heavily inhibited by adding the NGO in WAS. The presence of NGO prolonged the lag period of methane production from acetate. During the first 2 days, the methane production rate under NGO free conditions was 14.1 ml/g-acetic acid per day. In contrast, the methane production rate decreased to 2.06 ml/g-acetate per day under the condition of 0.054 g / g VS. And at the dosage of NGO was 0.108 g / g VS, almost no methane was produced during first 2 days. After the 2-d lag period, the methane production rate was about 25 ml/g-acetate per day without NGO, which decreased to 18 ml/g-acetate and 14ml/g-acetate per day when the NGO additions were 0.054 and 0.108 g / g VS, respectively. This result was consistent with the analysis result of the key enzyme for methanogenesis (Section 3.3), that NGO inhibited the activity of coenzyme F420.

4. Discussion

The results of this work clearly demonstrated that the methane production was significantly decreased with the presence of NGO, due to the strong inhibitory impacts of NGO on the processes of sludge disintegration and methanogenesis. In comparison, the NGO would not have significant effects on the processes of hydrolysis and acidogenesis.

Model-based analysis also confirmed that NGO significantly reduced biochemical

methane potential, with the highest methane potential decrease being approximately 10% (from 190 to 171 mL CH₄/g VS added). The decreased methane production potential were highly related to a decrease in soluble organic substrates availability during the process of sludge disintegration, which decreased with increasing NGO dosage, potentially attributing to the strong adsorption of organic substrates by NGO. This strong adsorption capacity of NGO could be resulted from its high specific surface area as well as the linked functional groups (Kuila et al., 2012). In particular, the SCOD, proteins and carbohydrates in WAS decreased with the increasing dosage of NGO. The results of FTIR further confirmed that the absorption peak of proteins and carbohydrates showed a clear shift to higher wavenumbers, indicating the potential bonding between proteins and carbohydrates and NGO with the presence of NGO in WAS due to the strong absorption capacity of NGO (Ramesha et al., 2011; Xing et al., 2014).

Indeed, it has been reported that NGO owned a highly effective ability for adsorption of some organic substances such as proteins (Li et al., 2014; Sun et al., 2012). The amidogen linked to proteins bonding proton is positively charged. NGO is an oxidizing form of graphene, which is decorated with epoxy groups and hydroxyl groups on the surface, and carboxyl groups at the edges. The ionization of carboxyl at the edge of NGO would have carboxylate radical with negative charges. Therefore, NGO in WAS might exert a strong adsorption of soluble organics such as proteins in SCOD due to the electrostatic interactions between carboxyl groups and amidogen in NGO and proteins, respectively.

Furthermore, NGO significantly inhibited the methanogenic process in WAS anaerobic digestion through inhibiting the corresponding enzyme activity (i.e., coenzyme F₄₂₀),

resulting in the decreasing methane production. The antibacterial activity of NGO has been previously reported. For instance, the metabolic activity of *E. Coli* was inhibited in the presence of NGO (Hu et al., 2010). It has been reported that NGO contributed to increases of membrane press due to sharp edges of NGO nanosheets, which might damage the structure of cell membrane, leading to the discharge of intracellular materials such as RNA (Akhavan and Ghaderi, 2010). Oxidizing press of NGO was also regarded as a factor producing antibacterial activity (Liu et al., 2011).

In contrast, the inhibitory effect of NGO on fermentative bacteria governing the hydrolysis and acidogenesis was not observed in this study. The different effect on methanogenic archaea and fermentative bacteria could be due to the differences in cell structures and metabolic pathways of archaea and bacteria (Nanninga, 2009). For example, different compositions of cell membranes might lead to methanogenic archaea being more vulnerable to NGO addition than the fermentative bacteria. In fact, methanogenic archaea also showed a lower tolerance to many other compounds than fermentative bacteria such as oxygen, sulfide and long-chain fatty acids (Appels et al., 2008). Although the metabolic activity of some fermentative bacteria, such as *E.coli* as aforementioned, could be affected by NGO, that study was carried out in pure culture with residual macromolecules and other growth medium constituents all washed out (Hu et al., 2010). Under such conditions, NGO could more easily reach the cell and work on it. However, in this study, mixed cultures in the engineered system were investigated and the microorganisms were aggregated in flocs. Such a system could provide good protect to microorganisms from being affected by environmental changes (Kleerebezem and van Loosdrecht, 2007). As a result, activities of fermentative

bacteria remain unaffected at the NGO dosing range applied in this study.

In fact, NGO also showed toxicity effect on different species including human beings. For example, Liao et al. (2011) found that small-size NGO showed strong hemolytic activity on red blood cells. NGO could induce conformational changes in human serum albumin and malfunction in its binding capacity to bilirubin (Ding et al., 2014). The investigation conducted with human lung cell showed that NGO presented concentration-dependent cytotoxicity and genotoxicity to human lung fibroblast cells, and the genotoxicity induced by NGO was more severe than the cytotoxicity (Wang et al., 2013a). In addition, a more recent study showed that that NGO modulated immune system biomarkers and that these may pose a health risk to individuals exposed to this type of nanoparticle (Lategan et al., 2018).

In consideration of the revealed inhibitory effect of NGO on methane production from WAS anaerobic digestion, effective measures should be taken to alleviate the adverse impact of the NGO on the WAS anaerobic digestion in practical application. For example, thermal treatment of the NGO-WAS mixture could be applied to remove carboxyl groups of NGO to release the absorbed SCOD from NGO (McAllister et al., 2007; Schniepp et al., 2006; Zhu et al., 2017), which would potentially facilitate the carbon transformation and methane production from WAS. Also, chemical reduction of hydrazine vapor could also be tried transfer NGO to the form of graphene (Gao et al., 2010; Park et al., 2012; Ren et al., 2011), which has been demonstrated to be able to facilitate methane production in anaerobic digestion (Tian et al., 2017). In addition, microbial reduction could serve as an environmental-friendly method to replace chemical and thermal reduction of NGO. For example, *Shewanella*, *E. coli* and recently reported *Azotobacter chroococcum* could be used to

reduce NGO to the form of graphene to avoid the inhibitory effect of NGO (Chen et al., 2017; Gurunathan et al., 2013; Wang et al., 2011b). Given that the anaerobic digestion communities had a high biodiversity, further investigation are worthy to be carried out to explore their potential on NGO reduction. In addition, since NGO showed toxicity effect on many species as well as human beings, the disposal of NGO containing wastes should be regulated to avoid its massive release into the environment.

5. Conclusion

This work comprehensively investigated the effect of the NGO on carbon transformation and methane production in anaerobic digestion of WAS. The methane production was significantly decreased with the presence of NGO due to the strong inhibitory impacts of NGO on the processes of sludge disintegration and methanogenesis in WAS anaerobic digestion. The NGO would not have significant effect on the processes of hydrolysis and acidogenesis. The decreased methane production potential was related to a decrease in soluble organic substrates availability during the process of sludge disintegration, attributing to the strong absorption of organic substrates by NGO. The NGO also significantly inhibited the methanogenesis process through inhibiting the corresponding enzyme activity (i.e., coenzyme F420) and thus the metabolic activity of methanogens, resulting in the decreasing methane production.

Acknowledgments

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Figure Legends

Figure 1. Cumulative methane production from waste activated sludge at different dosage of graphene oxide. Error bars represent the standard errors from triplicated experiments.

Figure 2. The profiles of SCOD (A), soluble protein (B), soluble carbohydrates (C) and soluble ammonium nitrogen (D) in the sludge supernatant during BMP tests at the different dosages of NGO. Error bar represent the standard error from triplicated experiments.

Figure 3. (A) Measured and simulated methane production in BMP test (Symbols represent experimental measurements and lines represent model predicts. (B) Confidence regions (95%) of the estimated hydrolysis rate (k) and biochemical methane potential (B_0).

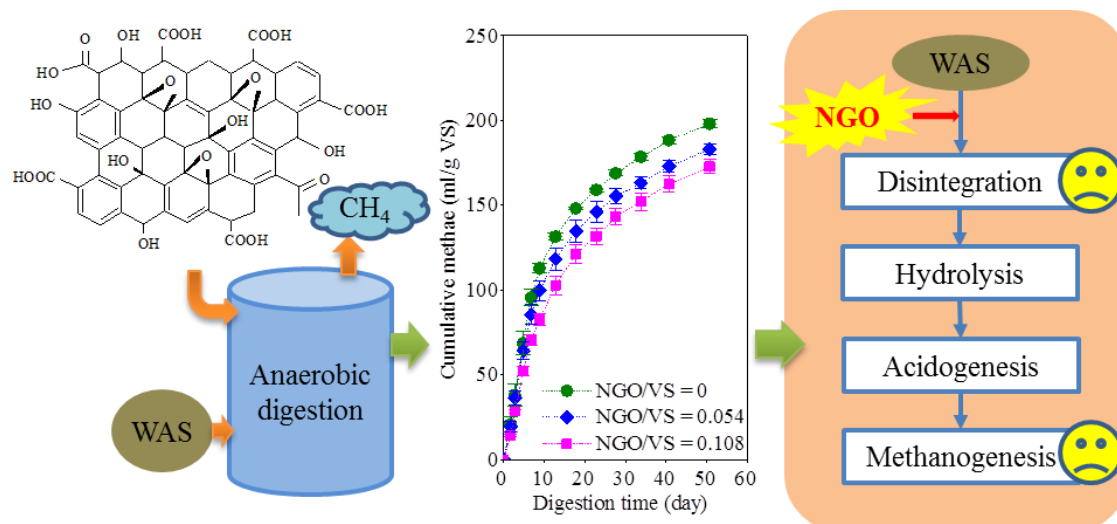
Figure 4. Comparisons of SCOD, soluble protein, soluble carbohydrates, VS, TCOD and NH_4^+ -N after adding different levels of graphene oxide to the WAS. Error bars represent the standard errors from triplicated experiments.

Figure 5. FTIR spectra of sludge supernatant exposed to different dosages of NGO. Sludge represented the sludge with free graphene oxide. NGO represent the FTIR spectrum of pure NGO solution.

Figure 6. The effect of different dosages of NGO (0.054 and 0.108 g / g VS) on the activity of protease, AK, coenzyme F420. Error bars represent the standard errors of triplicated

experiments.

Figure 7. Effects of NGO addition on each step of anaerobic digestion: (A) the concentration of SCOD during the disintegration of solid organic matter; (B) the concentration of protein during hydrolysis; (C) the concentration of glucose during acidogenesis; and (D) the methane production using the acetate as substrate. Error bar represent the data range from triplicated experiments.



Graphical abstract

**The Inhibitory Impacts of Nano-Graphene Oxide on Methane Production from Waste
Activated Sludge in Anaerobic Digestion**

Highlights

The methane production from WAS anaerobic digestion deteriorated with NGO exposure.

NGO decreased the release of soluble organics during WAS disintegration due to adsorption.

NGO negatively affected the methanogenesis through inhibiting coenzyme F420 activities.

Hydrolysis and acidogenesis were not affected by NGO during WAS anaerobic digestion.

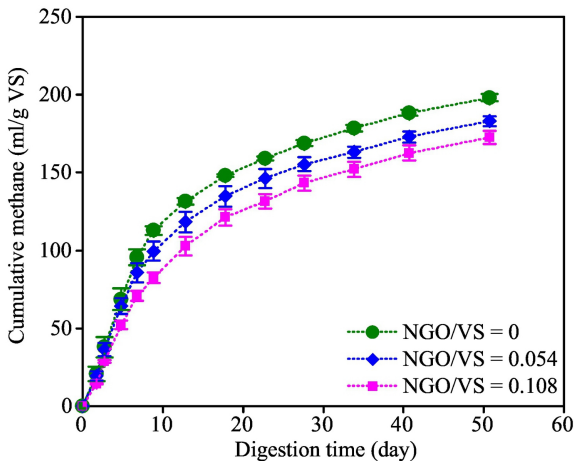


Figure 1

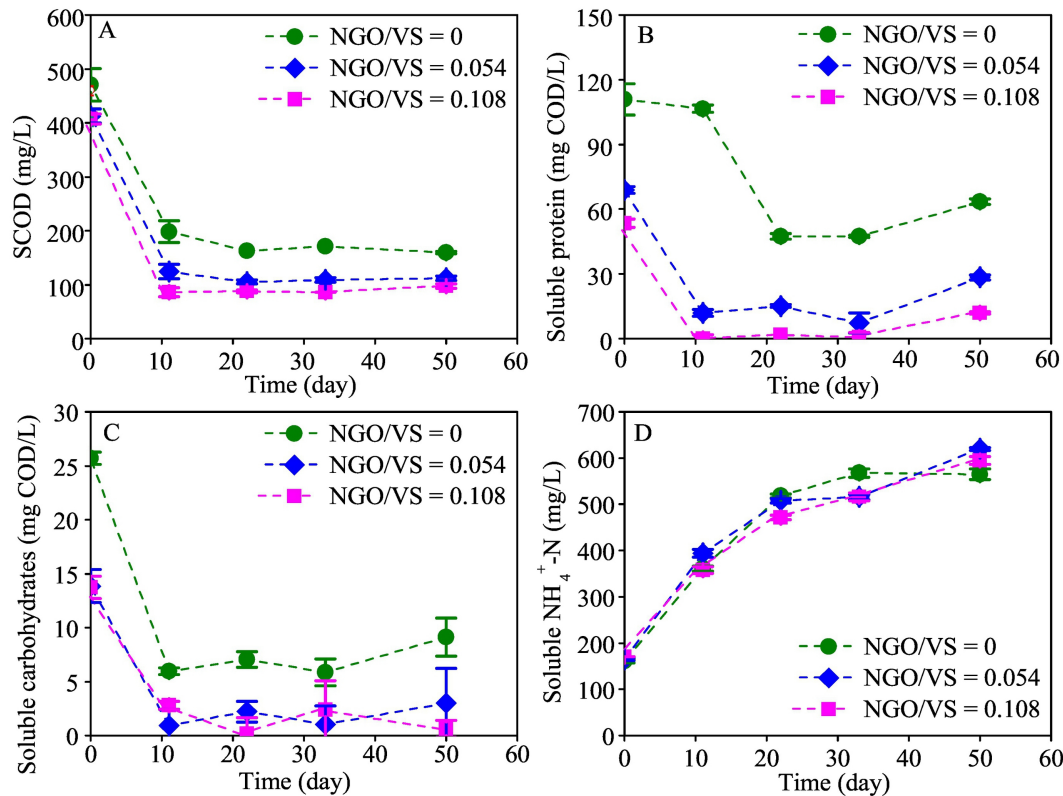


Figure 2

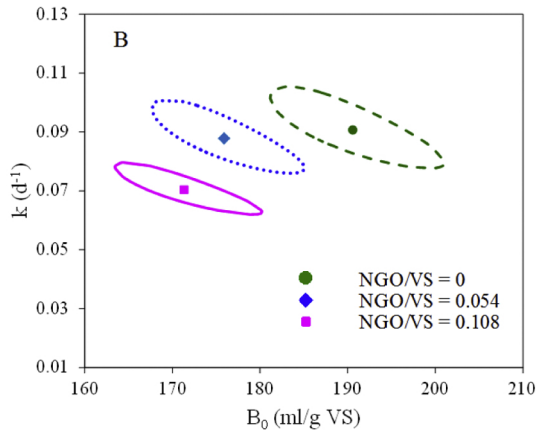
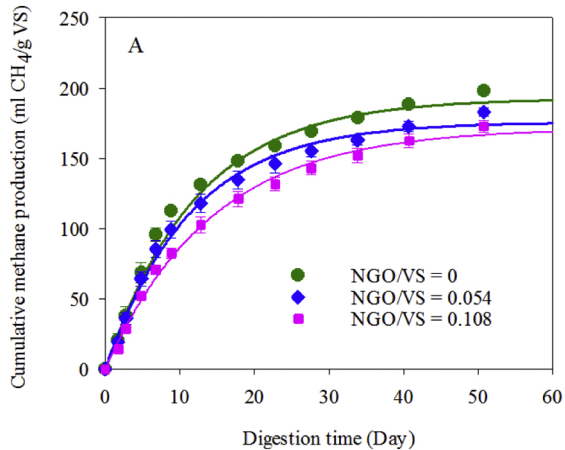


Figure 3

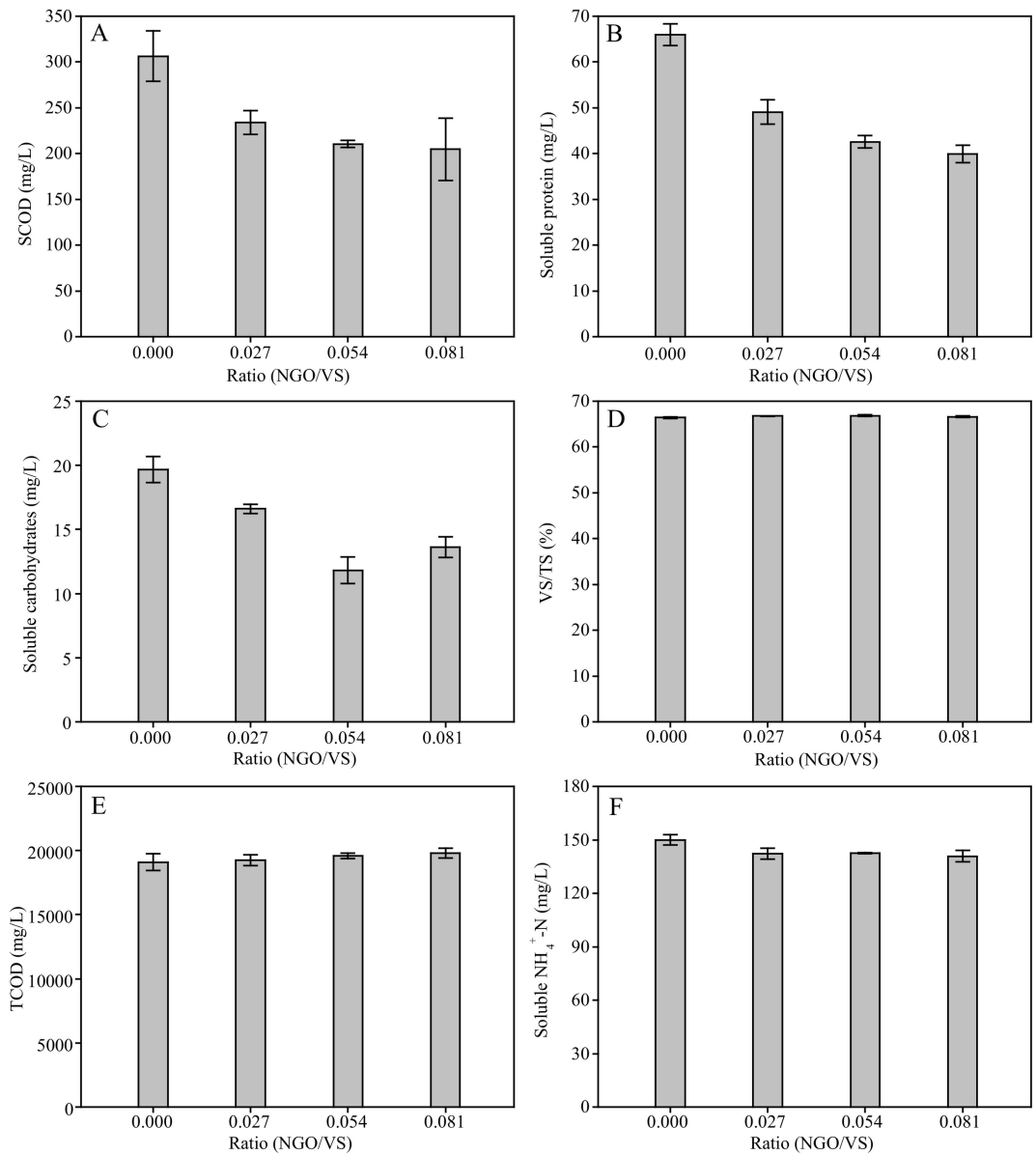


Figure 4

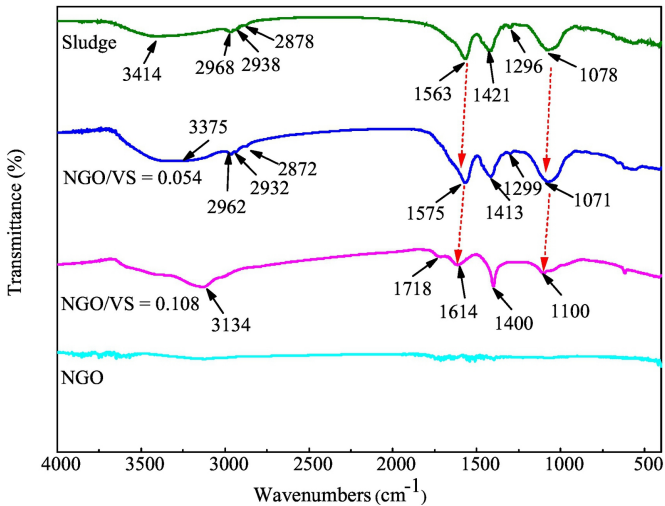


Figure 5

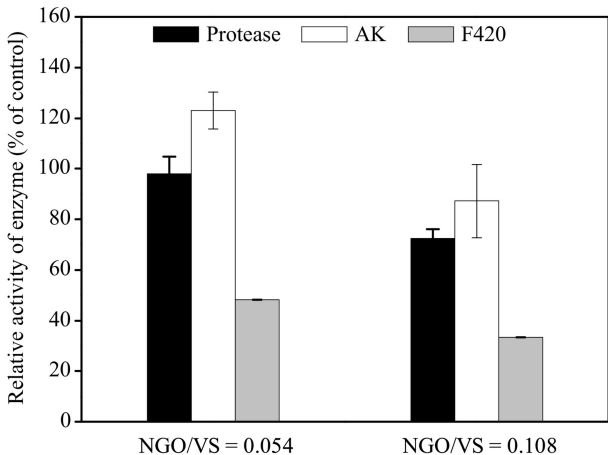


Figure 6

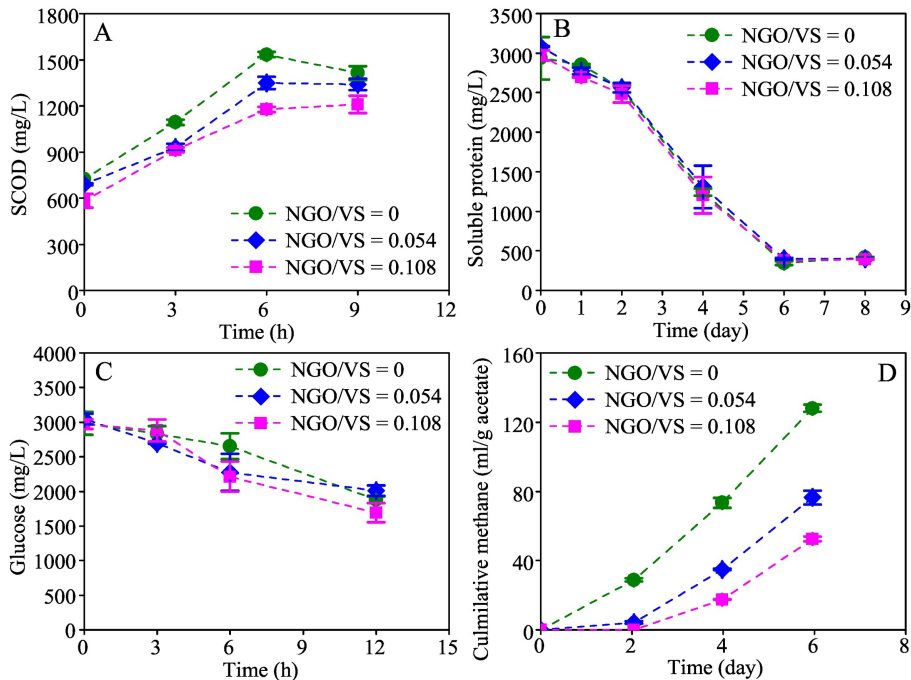


Figure 7