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The interactive effects of ammonia and carbon nanotube on anaerobic digestion

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Abstract

Carbon-based materials are promising in improving the performance and stability of anaerobic digestion (AD). However, it is not clear if carbon-based materials would have any interaction with ammonia on AD performance, especially when ammonia is above inhibition level. This study aims to explore the effects of ammonia on carbon nanotube assisted AD reactors. Methanogenic performance, intracellular energy production, potassium transport activity as well as extracellular polymeric substance (EPS) changes were investigated. The results show that the presence of CNT may mitigate or worsen the ammonia inhibition depending on the total ammonia nitrogen (TAN) concentration. Further investigation found that potassium transportation process of the culture was disturbed with the presence of CNT, which dispatched the proton motive force. As a result, the intracellular energy level reduced, and the microbial detoxification capability decreased. TAN exhibited a strong positive correlation with microbes' detoxification activity, and CNT concentration was positively correlated with polysaccharide which created a physical structure to protect cell membrane.

Keywords: Anaerobic digestion (AD); Carbon nanotube (CNT); Ammonia inhibition; Intracellular ATP; Potassium transport; Extracellular polymeric substance (EPS)

1.Introduction

The performance of anaerobic digestion (AD) is closely related to organic loading rate and the solids content in the feed [1]. To improve the digestibility, many pretreatment methods have been developed to enable higher loading rate and solids removal. However, the pretreated wastes often bring severe inhibition on the down-stream AD process. For example, the ammonia concentration in AD reactors could reach 3000 - 4000 mg N/L after fed with thermal hydrolysis pretreated waste active sludge [2]. Moreover, anaerobic digestion of livestock and food waste are often inhibited by ammonia accumulation as well [3].

Carbon-based materials have been widely applied to environmental biotechnologies [4, 5]. They were employed in both laboratory and engineering-scale for organics removal, toxicity reduction and AD performance improvement [6, 7]. For instance, the methane yield increased by 41% in an activated carbon supplemented pilot-scale AD system (1000 L) compared with control system at the same organic loading rate [8]. In such scenario, activated carbon is used as carrier for microbial immobilization, and it stimulates the synergistic effect of adsorption and biodegradation of organic compounds onsite itself. Other type of carbon materials, which has high conductive properties, e.g. carbon nanotube (CNT), are also very helpful in stimulating AD, especially in converting complex or toxic organics [9, 10]. The mechanism is different from activated carbon. CNT can establish direct interspecies electron transfer (DIET) between syntrophic partners due to its conductive properties [9]. Given the benefits of DIET in AD system, the organic loading rate and the operational stability can be improved significantly after adding such conductive

carbon-based materials. However, to date, it is not clear how conductive materials (CMs) would interact with the inhibition from inorganic compounds, typically ammonia. Volatile fatty acids (VFAs) are the main intermediates accumulated under ammonia inhibition. Considering that DIET enable faster degradation of VFA, it is hypothesized that the inhibition from ammonia could be mitigated effectively after dosing CMs.

DIET is based on applying CMs as electron conduit between syntrophic partners, and the conductivity of the materials is important [11]. Carbon nanotube (CNT) exhibits good conductivity (above 100 S/cm). Previous studies demonstrated that CNT could effectively stimulate AD performance. For example, Li et al. [12] reported 1 g/L CNT induced faster substrate consumption and methane production rate via accelerating syntrophic electron transfer. Another study on methanogens showed 5 g/L CNT could improve 17 times of methane production rate via reducing redox potential in anaerobic microcosms [13]. In addition, our previous study showed CNT was more effective materials in promoting AD performance compared with other carbon-based materials [10, 14]. Therefore, in this study, CNT was chosen as the representative conductive materials to explore its role in anaerobic digestion under ammonia inhibition state.

Bacterial antagonistic action towards ammonia is an ATP consumption process [15]. Free ammonia (FA) rather than ammonium ion is often considered as the main toxic compound causing ammonia inhibition [16]. Hydrophobic ammonia molecular can diffuse passively into bacterial cell and further convert into ammonium ion by absorbing proton and result in intracellular pH disturbance. Then, bacteria and methanogens will activate an energy driven detoxicate effect, during which a K^+/H^+ antiporter like process is stimulated to maintain intracellular pH via pumping out K^+ for exchanging proton [16, 17]. In addition,

studies evidenced CNT could form potassium porin like channel for K^+ transport through cell membrane [18-20]. Therefore, intracellular ATP level and potassium transport activity were also investigated to understand the interaction mechanism.

2. Materials and methods

2.1 Seed sludge and material

Seed sludge for anaerobic reactors was collected from an anaerobic digester in Ulu Pandan Water Reclamation Plant (UPWRP) Singapore. Volatile suspended solids (VSS) was adjusted to 6.5 ± 0.03 g/L for all reactors with nutrients solution.

CNT used in this study has the following characteristics: purity > 98%; length of 1-20 μm , inner diameter of 0.8-1.6 nm, outer diameter of 1-2 nm (Fig. S1); electric conductivity of > 100 S/cm; specific surface area of 407 m^2/g ; true density of 2.1 g/cm^3 ; bulk density of 0.14 g/cm^3 .

2.2 Experimental set-up

2.2.1 The effects of step-wisely increased TAN on CNT assisted anaerobic digestion

The anaerobic reactors were set-up in an automatic methane potential test system (AMPTSII, Bioprocess Control Company, Sweden) to record methane production automatically. Two experimental groups were setup, namely control group and CNT group (1 g/L CNT). Each group was in triplicates with working volume of 360 mL and headspace of 140 mL. Reactors were purged with nitrogen and sealed immediately to ensure anaerobic condition, then operated with continuous stirring at a speed of 80 rpm at 35 °C. The reactors were fed in sequential batch mode, and new feed (1.29 g/L glucose) was added when methane production stopped.

The operation period can be divided into 5 phases according to total ammonia nitrogen concentration (TAN). Specifically, the TAN concentration from Phase I to Phase V were 355.2 mg N/L, 1500 mg N/L, 1500 mg N/L, 2500 mg N/L and 3500 mg N/L, respectively (Table S1 and S2). NH_4Cl was adopted to control the TAN concentration. 0.4 g/L NaHCO_3 was only added from phase III to phase V to control the pH in reactors. To figure out the influence of ammonia on AD at each TAN concentration, each phase contained multiple feeding cycles. Detailed operating information can be found in Table S1 and S2. Methane generation profile was logged by AMPTS and displayed in Fig. 1.

2.2.2 The effects of CNT on ammonia inhibited anaerobic digestion

This separate study aimed to investigate the effects of CNT on methanogenic performance and intracellular ATP when microbes were under ammonia inhibition. Seed sludge, carbon source and operating conditions were similar with the setup described in section 2.2.1. When new batch reactors were set up, CNT was added in with concentration of 0, 1, 3 and 5 g/L of each group, respectively. Each group was performed in triplicates. The reactors were operated for three feeding cycles (6 days) to stabilize the microbial activity before ammonia was added. NH_4Cl at 1500 mg N/L was then added together with buffer (0.4 g/L NaHCO_3) into the reactors during the following cycles. The reason to choose 1500 mg N/L NH_4Cl was due to its moderate inhibitory effect (Fig. S2). The methane production profile was monitored by AMPTS system automatically.

Volate fatty acids (VFA) and ATP samples were taken at the beginning of the cycle, the exponential gas production stage, the stationary gas production stage, and the end of gas production stage. Specifically, samples were taken at 7 h, 22 h and 42 h in no ammonia

addition cycles. At the ammonia addition cycle, samples were taken at 7 h, 22 h, 46 h, 80 h and 180 h, respectively.

2.2.3 The interactive effects of ammonia and CNT on K^+ transport

Separate batch study was conducted in 55 mL serum bottles with 35 mL working volume. CNT concentration of 0, 1, 3 or 5 g/L were applied in 4 groups of reactors, respectively. Each CNT group consisted of nine identical reactors with the same substrate and seed sludge. After the inoculum had been acclimation for 10 days, samples were taken from each reactor for background K^+ check and membrane integrity analysis, the method of which was described in Supplemental materials. Then, the nine replicate reactors of each group were further divided into three sub-groups, of which three TAN levels, i.e. 0, 800, 1500 mg N/L, were achieved by adding NH_4Cl together with the carbon substrate (glucose). For all reactors, samples were then taken for potassium measurement at two time points, i.e. before ammonia addition and 8 min after ammonia addition.

EPS samples were also taken from each reactor to reveal the impact of ammonia and CNT on cell stress level. Baseline EPS content was identified before NH_4Cl addition. The other batch of EPS samples were taken at 200 h after feeding when methane generation stopped.

2.3 Analytical methods

2.3.1. Sample analysis

VFA was quantified by gas chromatograph (GC), equipment with a flame ionization detector, and DB-FFAP column (Phenomene, USA). The VSS was measured in accordance with APHA Standard methods [21]. TAN was analyzed using Nessler's reagent colorimetric method. pH was measured with a pH meter (Mettler-Toledo, model S220).

The concentration of free ammonia (FA) in the reactors was calculated based on the following equation [22]. The FA concentration in all the experiments is displayed in Fig. 2 and S4.

$$\text{NH}_3 = \text{TAN} * \left(1 + \frac{10^{-\text{pH}}}{10^{-\left(0.09018 + \frac{2729.92}{T(K)}\right)}} \right)^{-1} \quad \text{Eq. 1}$$

Where TAN (mg N/L) is the concentration of total ammonia nitrogen; NH_3 (mg N/L) is the concentration of free ammonia; T(k) represents the temperature.

2.3.2 ATP quantification and Potassium analysis

ATP quantification was conducted by using ATP Test Kit (QuenchGone21waster water test Kit-50, Luminultra) according to the protocol. Sludge samples for extracellular K^+ analysis was first centrifuged at 4000 g for 10 mins, and then the supernatant was collected and filtered through 0.45 μm filter. Potassium concentration in the filtrate was measured by using microwave plasma atomic emission spectrometry (MP-AES) (Agilent model 4200). The coefficient of variance for ten identical samples was within $\pm 1\%$ with 0.01 mg/L detection limit.

2.3.3 EPS extraction and analysis

EPS fractions were extracted following the method described in the literature [23]. A liquid chromatography-organic carbon-organic nitrogen detection (LC-OCD-OND) system (DOC-LABOR, Karlsruhe, Germany) was adopted to quantify EPS fractions. The identified total dissolved organic compounds (T-DOC) were divided into two parts, namely, hydrophobic and hydrophilic DOC. The hydrophilic dissolved organic carbon was subdivided into biopolymers, including high molecular weight (HMW) protein and HMW polysaccharides, humic substances, low molecular weight (LMW) neutral and LMW acids.

EPS fractions were quantified by a software (Chrom CALC) with the unit of equivalent carbon per liter (mg-C/L). The net change of EPS, including increase and decrease, was imputed for Spearman correlation analysis.

2.4 Statistical analysis

To quantify the methanogenic performance, methane production profile was fitted to Gompertz three-parameter model using 8.5 OriginPro software.

$$P(t) = P \times e^{\left\{-e \left[\frac{R_{max} \times e}{P} \times (\lambda - t) + 1 \right]\right\}} \quad \text{Eq. 2}$$

$$R = R_{max} \div (VSS \times V) \quad \text{Eq. 3}$$

Where $P(t)$ is the methane yield (mL) at time t (h); P is the maximum CH_4 potential (mL); R_{max} is the maximum CH_4 production rate (mL/h); λ is the lag time (h); e is mathematical constant, approximately equal to 2.71828; t is the time. R is the specific methane production rate (mL/g VSS/h); VSS is the volatile suspended solids (g/L); V is the working volume (L).

A two-way Analysis of Variance (two-way ANOVA) was conducted to identify the influence of ammonia and CNT on the methanogenic performance and K^+ transport using SPSS version 19.0. Spearman ranking correlation was adopted to analyze the correlation between K^+ /EPS fractions and TAN/CNT. p value < 0.05 was considered as statistically significant.

3. Results and discussion

3.1 The effects of step-wisely increased TAN on CNT assisted anaerobic digestion

The specific methane production rate was closely monitored in all the phases. In Phase I (FA: 11.8 ± 0.5 mg $\text{NH}_3\text{-N/L}$), the methanogenic performance did not exhibit statistical difference between CNT group (2.35 ± 0.08 mL/g VSS/h) and control group (2.23 ± 0.06

mL/g VSS/h) (Fig. 1, 2 and S3). In Phase II (TAN: 1500 mg N/L and FA: 49.9 ± 3.9 mg $\text{NH}_3\text{-N/L}$), dramatic inhibition on methane production was observed in both groups and a greater inhibition occurred in CNT group (Fig. 2). In the first feeding cycle, the specific methane production rate declined to 0.41 ± 0.06 mL/g VSS/h in CNT ($82.6 \pm 5.3\%$ reduction) and 1.11 ± 0.03 mL/g VSS/h in control ($50.2 \pm 2.7\%$ reduction) group (Fig. 1 and S3). Such performance reduction was also observed in previous studies when TAN concentration was up to 1500 – 1700 mg N/L [3, 24]. In the second and third feeding cycle, methanogenic activity gradually recovered in CNT group, whereas the corresponding activity continued to decrease in control group. Consequently, better performance was shown in CNT group (0.67 ± 0.07 mL/g VSS/h) than control group (0.41 ± 0.03 mL/g VSS/h) in the third feeding cycle of Phase II. The existence of carbon-based materials could trigger the DIET process, and enhance substrate and fatty acids degradation, which was confirmed by the VFA profile (Fig. S5). It is noted that the pH did not show statistical difference in the first and second cycles of this phase, meaning biomass in the two groups of reactors exposed to similar FA inhibition (Fig. 2). In the third feeding cycle, higher FA was observed in CNT groups. Interestingly, better methanogenic performance also occurred in CNT group. Thus, CNT promoted performance recovery despite higher FA concentration than Control group in this phase.

However, low buffer capacity could greatly reduce bacterial capability to cope with ammonia inhibition. In Phase III, the pH was adjusted from 6.58 ± 0.18 to 7.20 ± 0.02 to avoid the low pH impact on methanogens. The pH was maintained in the range of 7.20 – 7.46 and the FA was in the range of 48.3 – 65.9 mg $\text{NH}_3\text{-N/L}$ during the whole phase. At each monitoring point, pH and FA did not exhibit statistical difference between control and

CNT groups. Methanogenic activity recovered immediately after pH adjustment, and CNT groups resumed the activity to the Phase I level at the end of Phase III. However, control groups still exhibited $27.1 \pm 2.4\%$ lower activity compared to that of CNT group. Similar results were observed in other studies, where CNT promoted VFA degradation and AD recovery [12, 25] as a result of triggering DIET.

TAN increased to 2500 mg N/L in Phase IV. Greater inhibition was also observed in CNT groups with $31.7 \pm 3.7\%$ activity reduction, whereas the corresponding value was $19.2 \pm 2.2\%$ in control group (Fig.1, 2 and S3). Although the activity of both groups could partially recover from the second feeding cycle, the recovery speed was slower than previous phase, especially for CNT group. This fact confirms CNT affected AD performance adversely at FA of 103.7 – 109.6 mg $\text{NH}_3\text{-N/L}$ condition. When the concentration of TAN further increased to 3500 mg N/L (FA: 151.5 ± 7.1 mg $\text{NH}_3\text{-N/L}$) in Phase V (Fig. 2), the performance of both groups was deteriorated. During the four feeding cycles in Phase V, the specific methane production rate in control groups fluctuated around 1.02 ± 0.08 mL/g VSS/h, whereas the corresponding value continuously decreased from 0.90 ± 0.08 mL/g VSS/h to 0.49 ± 0.07 mL/g VSS/h in CNT groups (Fig.1 and S3). It seems methanogenic communities reached a suboptimal state in control group at phase V. On the contrary, CNT group potentially resulted in earlier system failure with continuous deterioration of methanogenic activity.

Although CNT could promote the system recovery at lower ammonia concentration (FA of 48.3 – 65.9 mg $\text{NH}_3\text{-N/L}$), the sudden increase in TAN always posed greater inhibition on CNT group. Moreover, beyond 103.7 – 109.6 mg $\text{NH}_3\text{-N/L}$ the methanogenic activity of CNT group was much lower than that of control group, which was opposite to

the expectation that carbon-based materials would always enhance AD performance. The mechanism behind this phenomenon was further explored in the following sections.

3.2 The effects of CNT on ammonia inhibited anaerobic digestion

3.2.1 Methane generation profile

Before ammonia addition, the methane generation profiles are similar in all groups (Fig. 3a). After 1500 mg N/L TAN was added, inhibition was observed in all groups with greater impact on CNT supplemented groups. Specifically, the specific methane production rate (R) decreased by $52.5 \pm 1.5\%$ in 0 g/L CNT groups, whereas the corresponding reduction were $61.2 \pm 3.2\%$, $72.5 \pm 3.5\%$, $82.2 \pm 2.5\%$ in 1g/L, 3g/L and 5 g/L CNT groups, respectively (Table 1). Spearman rank correlation reveals a strong negative correlation between specific methane production rate and CNT concentration ($R_s = -0.972$, $p < 0.01$). This fact indicates that, with the presence of high TAN, CNT could greatly worsen the shock inhibition of ammonia on AD system, which is also observed in section 3.1.

The accumulation of VFA at the initial stage reduced the pH in all reactors (Fig. S4). With the consumption of VFA, pH gradually resumed to original value in 0 and 1 g/L CNT groups. However, due to the slow VFA consumption rate in 3 and 5 g/L CNT groups, the pH and the concentration of FA were relatively lower in 3 and 5 g/L CNT groups. Thus, with regards to the effects induced by FA, the inhibition from FA should be even higher in 0 and 1 g/L CNT groups. Nevertheless, better performance was observed in 0 and 1 g/L CNT groups. The results clearly demonstrate the CNT worsened the ammonia inhibition on AD system.

3.2.2 VFA profile

The accumulation of VFA reflects a kinetic uncoupling between acids producers and consumers and is considered as typical indicator of AD reaction imbalance [26]. In this short-term study, VFA profile was closely monitored (Fig. 4 and Fig. S6). Before ammonia supplemented, VFA concentration peaked at 4 h after the pulse feeding with glucose (Fig. 4a). At 50 h, VFA concentration was below detectable limitation in all groups (Fig. 4a). With the addition of 1500 mg N/L TAN, VFA peaked time delayed by 5 h - 15h depending on the CNT concentration (Fig. 4b). Comparing to no ammonia addition case, the accumulated VFA concentration increased by $31.2 \pm 1.4\%$, $51.6 \pm 2.5\%$, $36.5 \pm 2.8\%$ and $49.2 \pm 2.9\%$ in 0, 1, 3, and 5 g/L CNT groups, respectively. The delayed peak time and higher VFA accumulation indicate that both acidogenic and methanogenic microflora were inhibited, and the methanogenic archaea was more sensitive to ammonia inhibition. After 90 h, limited VFA was detected in 0 or 1 g/L CNT groups, whereas there were still about 245 ± 11.5 mg/L COD and 315 ± 23.8 mg/L COD remaining in 3 g/L and 5 g/L CNT groups (Fig. 4b). From 180 h onwards, the methane producing rate in 3 g/L and 5 g/L groups was negligible (Fig. 3), while the VFA in the two groups still maintained as high as 184 ± 5.7 mg/L COD and 235 ± 6.3 mg/L COD till the end of the monitoring time (Fig. 4b). In consistent with methane production profile, the accumulation of VFA further confirmed the negative impact of CNT on the AD system under ammonia inhibition state.

3.2.3 Intracellular ATP quantification

ATP is the product of oxidative phosphorylation, in which electrons are transferred from electron donors (substrate) to acceptors. Its production is coupled with catabolism of microbe, and it is a reliable and fast indicator of microbial metabolism activity [27]. Before ammonia addition, the intracellular ATP level was similar in all groups (Fig. 5a). After

1500 mg N/L TAN was supplemented, 25% to 55% less ATP was observed within all experimental group, suggesting that microbial metabolism was repressed (Fig. 5b). Specifically, the 3 or 5 g/L CNT groups exhibited $25 \pm 3.7\%$ lower ATP compared to 0 g/L CNT group at 22 h (Fig. 5b). At 46 h, the difference enlarged to $35 \pm 2.5\%$, which indicates greater inhibition occurred in high CNT supplemented groups. Afterwards, the difference of ATP quantity between control and CNT supplemented groups was narrowed down due to limited substrate available in 0 g/L CNT groups. Bacterial anti-ammonia inhibition is an ATP consumption process. Considering the reduced ATP level in CNT groups, it can be concluded that the adverse effects was associated with the interruption of ATP generation and/or consumption.

3.3 The effects of ammonia and CNT on K⁺ transport

Both modeling and experimental studies evidenced CNTs can create an ideal transmembrane channel due to the graphitic characterization. They can insert into the hydrophilic lipid membrane bilayer [20, 28] and function like biological channels for ions (such as potassium) transport [18, 29]. As shown in Fig. 6, carbon nanotube exhibited nanofibers structure (Fig. 6a) and part of cell surface was occupied by CNT (Fig. 6b). Since CNT obtained graphene shell, its rigid characterization made it possible to insert into cell membrane when cells were encapsulated by CNT [30], which was also observed in this study (Fig. S7). Besides, bacterial cell membrane has the fluidity capability, and membrane curvature can happen when exterior impact exists. As shown in Fig. 6b, many cells exhibited depression deformation with hollow like area on their surface in CNT group. This indirectly demonstrates the formation of CNT channel through cell membrane [18, 19].

Potassium is the major intracellular cation in bacteria as well as archaea [31]. The uptake and release of K^+ is the most universal process in facing the change of external environment [31]. In this study, the concentration of extracellular K^+ could be potentially affected by three factors in CNT group: osmotic driven potassium uptake, efflux of K^+ for anti- ammonia toxicity effect, and CNT channel conducted potassium transport. In order to reveal the mechanism behind the severer inhibition on CNT group, the uptake of potassium under different ammonia/CNT concentration was closely monitored.

As shown in Fig. 7, extracellular K^+ did not exhibit significant difference among all experimental groups ($p > 0.05$, 94.4 ± 3.87 NH_3 -N/L in average) at the end of no ammonia addition cycle. However, the dose of ammonia diverged the quantity of extracellular K^+ among all groups immediately. Among 0 g/L CNT sub-groups, the extracellular K^+ concentration declined in none ammonia addition (new feeding cycles) and 800 mg N/L groups, whereas the corresponding quantity increased remarkably in 1500 mg N/L group (Fig. 7). This could be because the sudden dose of ammonia increased the medium osmolarity, which is the general driven force for K^+ uptake, thus resulted in less extracellular K^+ . On the other hand, when extracellular TAN reached inhibition level of 1500 mg N/L, the process of K^+ efflux for exchanging proton would be activated to maintain the intracellular pH [15-17]. Similar to the result of Sprott and Patel [16], the elevated extracellular K^+ in 1500 mg N/L group was because the potassium efflux caused by antagonistic effects. Such effect may surpass the uptake of K^+ , which is driven by osmotic pressure.

Contrary to the effects of ammonia, the existence of CNT may cause the decrease of extracellular potassium. Among no ammonia addition groups, less K^+ was observed in CNT

groups once COD substrate was added (Fig. 7 – 0 (b)). Typically, extracellular K^+ decreased by 28.8% in 5 g/L CNT group (from 94.9 ± 2.07 to 64.1 ± 3.83 mg/L), whereas only 3.4% decline was shown in 0 g/L CNT group (from 94.3 ± 2.23 to 91.3 ± 1.47 mg/L). Moreover, 5 g/L CNT groups always exhibited 20% to 30% less extracellular potassium than that in 0 g/L CNT groups regardless of the TAN level. These facts evidence that the existence of CNT could greatly stimulate the uptake of K^+ . Given the complexity of the studied mixed culture system, further studies are required to track the responses from individual functional species, in which K^+ flux can be more accurately quantified.

Spearman rank correlation analysis reveals strong negative correlation between CNT and extracellular K^+ ($R_s = -0.807$, $p < 0.01$), while a positive correlation was shown for TAN and extracellular K^+ ($R_s = 0.398$, $p < 0.05$) (Table S4). In addition, two-way ANOVA analysis concludes that a strong interaction existed within the two factors, namely, CNT and TAN, which both affected extracellular K^+ concentration ($p = 0.017$) (Table S4). These facts further indicate that CNT likely acted as channel for potassium influx. Bacterial detoxicate activity may lead to potassium efflux. And the two factors had interactive effects on K^+ transport.

In fact, the influx of K^+ was only observed after adding substrate, indicating that K^+ transportation through cell membrane or CNT channel is an energy consuming process [18, 31]. The influx of potassium and proton through CNT channel would gradually dispatch the proton motive force, consequently less ATP could be generated. This hypothesis was evidenced by lower ATP level in the culture of CNT groups (Fig. 5). As bacterial detoxicate activity is an energy consumption process, it is likely that CNT deteriorated AD

performance via decreasing ATP production, resulting in less effective anti-ammonia inhibition activity.

3.4 EPS fractions influenced by coexistence of ammonia and CNT

The change of EPS constitution is usually considered as the response of microorganism to harsh environmental conditions [32, 33]. Confocal Laser Scanning Microscope images showed the uniformly distributed fractions of bound EPS could form into meshwork-like structure as an adaption to exotic materials [12]. Bound EPS, especially TB-EPS, showed a stronger correlation with the concentration of TAN and CNT than SB-EPS ($p > 0.05$) in term of dissolved organic matters as well as other sub-fractions (Table 2, S5 and S6). This is because bound EPS directly contact with cell surface and may respond to external circumstance changes first [34]. Therefore, analysis on the change of bound EPS can aid in understanding the response of microorganisms to ammonia and CNT exposure.

A strong positive correlation was observed between the total dissolved organic compounds (T-DOC) from TB-EPS and the concentration of TAN ($R_s = 0.648$, $p < 0.01$), which was mainly attributed to the hydrophilic ($R_s = 0.773$, $p < 0.01$) rather than hydrophobic compounds ($R_s = 0.226$, $p > 0.05$) (Table 2 and S5). Further analysis on the subfractions of hydrophilic compounds revealed that HMW protein ($R_s = 0.662$, $p < 0.01$), HS ($R_s = 0.616$, $p < 0.01$), LMW neutral ($R_s = 0.737$, $p < 0.01$) were positively correlated to TAN concentration, while no statistical correlation was observed for HMW polysaccharides and LMW acids ($p > 0.05$) (Table 2 and S5). The positive correlation of HMW protein with TAN concentration was consistent with previous study that bacterial detoxicate activity could enhance protein or enzyme secretion [35]. Humic substances of TB-EPS were mainly derived from the resynthesizes of macromolecular organics. Under

highly stressed condition, e.g. high ammonia in this study, the inhibition state could induce the production of secondary metabolite, thus promote the production of HS [36]. Similar to HS, LMW neutrals were the metabolic by-products under stressful condition [35]. On the contrary, there is no significant correlation observed between T-DOC of LB-EPS and concentration of TAN. As for the subfractions of LB-EPS, only LMW neutral and LMW acids have moderate positive correlation with TAN concentration ($p < 0.05$). Therefore, the change of TB-EPS fractions was the representative response of microbes to ammonia inhibition.

Compared to ammonia, the correlation between CNT and TB-EPS ($p > 0.05$) or LB-EPS ($p > 0.05$) was minor (Table 2, S5 and S6b). Among T-DOC subfractions of TB-EPS, the significant correlation was only observed with HMW polysaccharide ($R_s = 0.773$, $p < 0.01$) and HMW protein ($R_s = -0.586$, $P < 0.01$) (Table 2 and S5). HMW polysaccharide has been visualized as fine strands attached on the bacterial cell surface and forming networks for protecting the cells [37]. CNT are graphic nanoparticles and can physically interact with cell surface. As reported by Li et al. [12] that the excessive production of polysaccharide could be a result of defense against the intrusion of CNT. This explains the positive correlation between CNT and polysaccharide. As for HMW protein, its correlation with CNT depended on its location. Contrary to the negative correlation between CNT and HMW protein from TB-EPS, the corresponding value of HMW protein from LB-EPS was $R_s = 0.677$ ($p < 0.01$) (Table S5). This is probably attributed to the electrostatic attraction between the oppositely charged amino group of proteins and CNT surface, which led to the outward movement of HMW protein from TB-EPS to LB-EPS.

This study clearly demonstrates EPS, especially TB-EPS, was more sensitive to high ammonia inhibition than CNT. The production of HMW protein from TB-EPS could be stimulated under high TAN as a response to its inhibition. In addition, the secondary metabolism related products, i.e. HS and LMW neutrals, also exhibited strong positive correlation with TAN rather than CNT. This phenomenon indicates ammonia was the predominate inhibitor, which is corresponding to the observed methanogenic performance, where the adverse effects of CNT only observed after ammonia addition. The HMW polysaccharide could function in physical protection of bacterial surface. As a response to CNT exposure, its quantity increased greatly to resist CNT physical interruption on cell membrane. Therefore, CNT stimulated the physical protection rather than exerted cytotoxic effects on sludge microbes, which confirms that bacterial integrity would not be affected by its existence (Table S3). However, CNT worsened the ammonia inhibition on AD as it could form ion channel to interrupt potassium transport and ATP formation.

4 Conclusions

The effects of CNT on AD performance varied with TAN concentration. Specifically, CNT accelerated the performance recovery of AD from ammonia inhibition when the TAN concentration was below 1500 mg N/L (FA: 48.3 – 65.9 mg NH₃-N/L). However, CNT could also worsen ammonia inhibition, when TAN concentration was above 2500 mg N/L (FA: 103.7 – 109.6 mg NH₃-N/L). In addition, CNT groups always exhibited greater inhibition upon the shock addition of ammonia, characterized by decreased methane production rate and higher VFA accumulation. The phenomenon was likely due to the formation of transmembrane CNT channel, which interrupted potassium transport, reduced proton motive force and ATP generation. As a result, less ATP was available for microbial

detoxification in CNT group compared to control group. The change of TB-EPS fractions revealed that the stimulated production of HMW protein was associated with high TAN concentration and might attribute to microbial anti-toxicity activity. On the other hand, the production of polysaccharide was promoted by the existence of CNT, which could be related to physical protection of cell membrane from CNT intrusion.

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Fig. 1. The methane generation profiles of experimental data and modeling results.

Fig. 2. Free ammonia and pH concentration at the end of each phase

Fig. 3. Methane production profile before (a) and after (b) 1500 mg N/L ammonia addition

Fig. 4. VFA profile before (a) and after (b) 1500 mg/L-N ammonia addition

Fig. 5. ATP profile before (a) and after (b) 1500 mg N/L ammonia addition

Fig. 6. SEM images of sludge. Bacteria were encapsulated by CNT (a), and hollow-like area on bacterial cell surface (b). The arrow pointed to the hollow-like area.

Fig. 7. Extracellular potassium concentration. 0 (a) means the samples taken before substrate addition. 0(b), 800(b) and 1500 (b) denote the samples taken at substrate/ammonia added cycle and the TAN concentration was 0, 800, 1500 mg N/L, respectively.

Table 1. Methane production parameters before and after 1500 mg N/L ammonia addition.

	CNT Conc.	R (mL/g VSS/h)	Potential CH ₄ yield (mL)	R^2
Before ammonia Addition	0 g/L	2.76 ± 0.09	148.89 ± 1.78	0.990
	1 g/L	2.78 ± 0.12	144.78 ± 1.59	0.982
	3 g/L	2.39 ± 0.07	147.97 ± 2.14	0.991
	5 g/L	2.12 ± 0.07	152.10 ± 3.27	0.987
After ammonia addition	0 g/L	1.31 ± 0.02	143.80 ± 0.89	0.995
	1 g/L	1.08 ± 0.02	127.27 ± 0.83	0.995
	3 g/L	0.66 ± 0.01	101.51 ± 0.32	0.991
	5 g/L	0.38 ± 0.01	98.78 ± 0.80	0.989

Table 2 TB-EPS fraction change after 1500 mg N/L ammonia addition (unit: ug/L- C).

CNT (g/L)	TAN (mg N/L)	T-DOC	Hydrophobic DOC	Hydrophilic DOC	Biopolymers	HMW Protein	HMW Polysaccharide	HS	LMW Neutrals	LMW Acids
0	0	-2499	182	-2735	-1543	969	-2472	-837	-882	504
0	800	21304	4769	16887	1126	4431	-3039	2624	10898	1038
0	1500	6603	874	6056	-443	2512	-2951	563	5375	859
1	0	-7986	-4511	-3335	-1199	-273	-1003	1436	-1044	239
1	800	1565	-2501	4115	-487	533	-1057	535	3300	786
1	1500	5860	-2315	8822	1492	1218	288	854	5343	761
3	0	-8040	-11204	3483	120	105	10	739	1358	1279
3	800	-4800	-11274	6338	360	457	-100	1108	3586	1206
3	1500	-1153	-10715	9054	1495	860	617	1690	4443	1306
5	0	701	237	510	-591	-439	-138	-5	372	704
5	800	4232	484	3721	1259	717	521	814	1132	587
5	1500	6269	575	5343	1046	724	327	1299	2415	676

T-DOC: total dissolved organic carbon; HMW: High molecular weight; LMW: Low molecular weight; SB-EPS: Soluble bound EPS; LB-EPS: Loosely bound EPS; TB-EPS: Tightly bound EPS; HS: Humic substances

Highlights:

- CNT worsened ammonia inhibition when TAN was above 2500 mg N/L.
- Potassium transport was interrupted by the interactive effects of CNT and ammonia.
- CNT formed potassium channel and dispatched proton motivate force.
- CNT affected intercellular ATP level and reduced microbial detoxification activity.
- Enhanced secretion of bound EPS was related to the presence of ammonia and CNT.