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Biogas Emission from an Anaerobic Reactor

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ABSTRACT

Anaerobic denitrification is accompanied by biogas emissions, which may orient greenhouse gases or air pollutants. Ferrous iron play an important role in wastewater treatment. However, the effect of ferrous ion on biogas emission and the relationship between microbial community and nitrogen removal performance are not fully understood, especially in up-flow anaerobic sludge blanket (UASB) reactors. Here, the results revealed that nitrogen gas increased rapidly with Fe(II) addition. And ferrous ions enhanced denitrification and COD removal efficiency with more than 60% and 10%, respectively. Moreover, Ferrous ions addition evidently increased *Hydrogenophaga*, *Methylotenera*, *Zoogloea* and *Fluviicola* during the whole UASB reactor operation progress. The correlation coefficient analysis further confirmed that Fe(II) was positively correlated with the abundances of *Hydrogenophaga*, *Methylotenera* and *Fluviicola*. Based on these results of chemicals transformation kinetics, microbial community and the correlations coefficient analysis, a hypothetical mechanism is proposed: In the UASB system with Fe(II) and NH_4^+, firstly *Paludibacter* made a contribution to NH_4^+ oxidation and generated NO_3^-, and then *Hydrogenophaga*, played an important role in NO_3^- reduction, *Zoogloea* coupled NO_3^- reduction to Fe(II) oxidation, *Fluviicola* combined with *Methylotenera* conducted NO_2^- reduction together with Fe(II) oxidation. This study will improve our understanding of ferrous ion’s influence on biogas emission, denitrification process and corresponding microbial community.

Keywords: Biogas emission; Nitrogen gas; Ferrous iron; Denitrification; Anaerobic reactors.

INTRODUCTION

Up-flow anaerobic sludge blanket (UASB) reactors are commonly used in municipal wastewater treatment, because the reactor with small occupied area, low sludge generation, low cost of investment and maintenance (Powar *et al.*, 2013). The effluent of UASB reactor is required to meet the effluent standards, especially in terms of nutrients. In general, nitrogen removal via aerobic nitrification and anaerobic denitrification by bacteria (Kartal *et al.*, 2010). Generally, nitrogen removal efficiency in UASB reactors does not reach the requirement. In order to develop fast and effective approaches for nitrogen removal, many investigations are performed to unveil the influence of operational parameters on nitrogen removal efficiency and microbial communities (Kim *et al.*, 2011; Reddy *et al.*, 2017). Recently, more and more research points to greenhouse gas emission and benefits of biogas electricity generation during anaerobic sludge digestion (Yang *et al.*, 2017; Gingerich and Mauter, 2018).

Iron is the most abundant transition metal element on the earth (Kappler *et al.*, 2005; Li *et al.*, 2016), and it is also the necessary trace element for microorganisms. In recent years, it was found that iron strongly influence many nutrients and contaminants removal performance and their degradation products (Lalonde *et al.*, 2012; Melton *et al.*, 2014). Ferrous iron could improve chemical oxygen demand (COD) and nonbiodegradable contaminant removal efficiency a stable level by adjusting redox potential. In addition, the settle ability of microorganisms was also enhanced (Chen *et al.*, 2007; Vlyssides *et al.*, 2009). However, previous research has been concentrated on the physicochemical changes of reactor for wastewater treatment after catalysts addition (Li *et al.*, 2017; Liu *et al.*, 2017), but the influence on biogas emission and its mechanism for microorganism changes are not clear. As we known, many gases, such as NO_x, CO_2 and CH_4, are released to the atmosphere by biological processes occurring in anaerobic environments (Rajab *et al.*, 2012). Considering their environmental effects, it is important to investigate underlying mechanisms on these biogas emissions.

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In this study, after the influences of ferrous iron on the stability of COD and nitrogen removal efficiency were evaluated, the roles of ferrous iron on biogas emission and corresponding microbial community were investigated in UASB reactor across different conditions. Then, potential mechanisms were further proposed based on the observed relationships among microorganisms, biogas emission and ferrous iron.

MATERIALS AND METHODS

The UASB Reactor Set up and Operating Condition

The inoculum of UASB reactor was anaerobic sludge obtained from the Wangtang municipal wastewater treatment plant in Anhui province, China. The configuration of UASB reactor was shown in Fig. 1, working volume was 4.5 L, the operating temperature maintained at 35 ± 2°C by a constant temperature chamber. The medium composition was $K_2HPO_4 \cdot 3H_2O$ 155 mg L^{-1}, $CaCl_2$ 50 mg L^{-1}, $MgCl_2 \cdot 6H_2O$ 100 mg L^{-1}, $FeCl_2 \cdot 4H_2O$ 25 mg L^{-1}, NaCl 10 mg L^{-1}, $CoCl_2 \cdot 6H_2O$ 5 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 5 mg L^{-1}, ACl_3 2.5 mg L^{-1}, $(NH_4)_6Mo_7O_{24}$ 15 mg L^{-1}, H_3BO_4 5 mg L^{-1}, $NiCl_2 \cdot 6H_2O$ 5 mg L^{-1}, $CuCl_2 \cdot 5H_2O$ 5 mg L^{-1}, $ZnCl_2$ 5 mg L^{-1}, 2500 mg L^{-1} glucose was fed as carbon source, the medium pH was adjusted to 6.8–7.2 with $NaHCO_3$ or hydrochloric acid. $FeSO_4 \cdot 7H_2O$ with 5 mM concentration was added to the influent for one UASB reactor on Day19 until stop on Day 67 and then recovered Fe(II) addition on Day 105. The other UASB reactor as the control system, the influent composition was not changed during the whole operation period without Fe(II) supplement. The whole operating process of UASB reactor was divided four stages, before Fe(II) addition period was named as Con; after Fe(II) addition was named as AdF; Fe(II) addition stopped and pH decreased to acid condition, this period was named as Aci; Fe(II) addition was recovered period was named as Rev.

Chemical Analytical Methods

During the reactor operation, the concentration of COD, Fe^{2+}, Fe^{3+}, NO_3^-, NO_2^-, NH_4^+ in influent and effluent were measured daily. All chemicals were purchased from Sigma-Aldrich (USA), and their analysis carried out in accordance with standard methods (Clesceri *et al.*, 2012). Samples Liquid sample were sampled from different ports and mixed with equal volume. And after centrifuged at 8500 rpm for 20 min, the samples were filtered with mixed cellulose ester membrane (0.22 µm pore size) to remove suspended solids (Liu *et al.*, 2014). NO_3^- and NO_2^- were analyzed with ion chromatography (Dionex ICS-90), ion column was IonPac AS14A 4 × 250 mm, mobile phase was the mixed liquid 8 mM Na_2CO_3 and 1 mM $NaHCO_3$, the flow rate was 1 mL min^{-1} NH_4^+ was measured with spectrophotometry at 420 nm after a colorimetric reaction with Nessler's reagent (Paul *et al.*, 2007). The concentration of Fe^{2+} was extracted with 0.5 M HCl and analyzed with 1,10-phenanthroline colorimetric assay as previously described (Li *et al.*, 2010).

DNA Extraction and High Throughput Sequencing

The DNA was extracted from the sludge in UASB using a PowerWater™ DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's instructions.

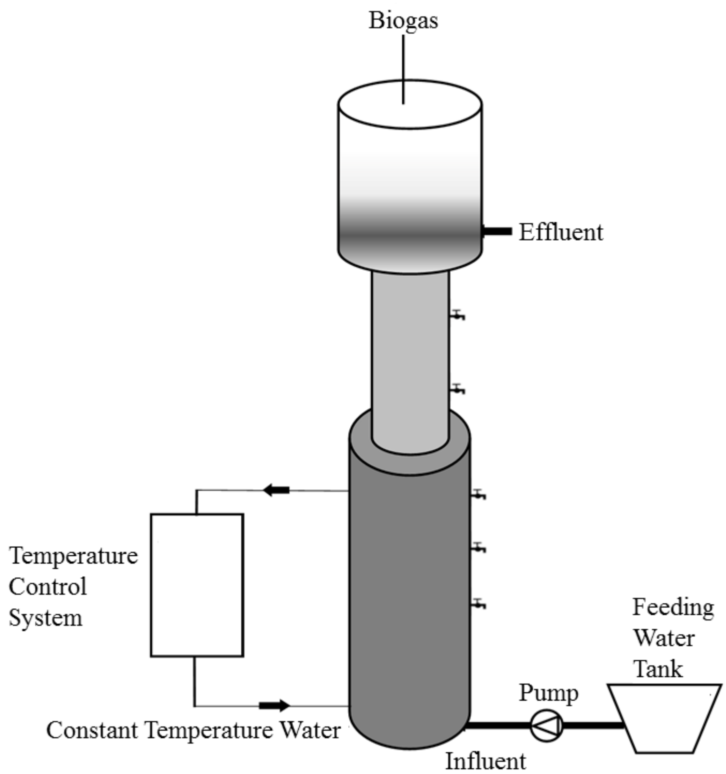

Fig. 1. The configuration of up-flow anaerobic sludge blanket.

Amplicon libraries were prepared as previously reported (Fadrosh *et al.*, 2014; Lax *et al.*, 2014). Briefly, the V3-V4 hypervariable region of bacterial and archaeal 16S rRNA genes was amplified using this primer pair 338F and 806R. The primer sequences are 5'-ACTCCTACGGGAGGCAGCA-3' and 5'-GGACTACVSGGGTATCTAAT-3', respectively. In addition, a sample specific 12-bp barcode was added to the reverse primer. According to a primer coverage test using TestPrime 1.0 (Lee *et al.*, 2011; Fadrosh *et al.*, 2014), the primers have high target coverage (> 90%). Polymerase chain reaction (PCR) was conducted as follows: initial denaturation at 95°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, primer annealing at 61°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Replicate amplicons were pooled for purification with an AMPure®XP PCR purification kit (Agencourt Bioscience Corp., Beverly, MA). The purified PCR products from each sample and then sequencing on an Illumina HiSeq 2500 platform in the Beijing Genomics Institute in China.

Analysis of Sequencing Data

Raw data was firstly removed ambiguous bases, then combined using the Flash software with default parameters to obtain sequences (Magoc *et al.*, 2013). The obtained sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software pipeline, to remove chimeric and low-quality sequences and assign sequences to individual samples (Caporaso *et al.*, 2010). Operational taxonomic units (OTUs) were identified with 97% similarity (Edgar, 2010), and the representative sequence of each OTU was classified with Ribosomal Database Project (RDP) database (Wang *et al.*, 2007). The alpha diversity of samples was also compared with Shannon index, Chao1 index, and Simpson index. The raw sequences of this study have been deposited in Sequence Read Archive database, the under deposited number was PRJNA347292.

Statistical Analysis

All the experiments in this study were conducted in triplicate, and the results were showed as average value added standard deviation. The correlation coefficients were analyzed by SPSS 18.0. Values of P were determined using Student's t-test.

RESULTS AND DISCUSSION

Ferrous iron Enhanced COD Removal and Affected pH of UASB Reactor

Two UASB reactors were set up to investigate the effect of Fe(II) on COD removal performance. In control reactor, the COD of influent was about 500 mg L^{-1}, the COD of effluent gradually increased from 70 mg L^{-1} to 250 mg L^{-1}, the COD removal efficiency decreased from 90% to 45% and stabilized in this level (Fig. 2(A)), accompanied with COD removal, the pH of effluent decreased from 7.5 to 4.5 (Fig. 2(B)). After 5 mM Fe(II) addition on Day 105, COD removal efficiency gradually recovered to 90%. In experiment reactor, the operating condition was same to control reactor (Con period), 5 mM Fe(II) was added on from Day 19 to Day 67 (AdF period), COD removal efficiency slightly increased from 80% to 88% and the pH was stable before and after Fe(II) addition (Figs. 2(C) and 2(D)). After 67 days, Fe(II) no longer added and COD removal efficiency sharply decreased from 88% to 45% companied with pH decreased from 7.2 to 4.9 (Aci stage). Comparable to the results in Rev stage, both the COD removal efficiency and pH gradually increased when Fe(II) addition was recovered.

The operational and environmental variations influence the typical responses include a decrease in performance, the accumulation of volatile fatty acids, and a drop in the pH and alkalinity in anaerobic wastewater treatment systems (Leitão *et al.*, 2006). In this study, the COD removal efficiency declined to below 50% following the pH decrease, especially since pH lower than 6. The lower pH caused microbial activity inhibition and led to bad reactor performance, which was similar to previous studies (Haandel, 1994). The pH could be an indicator of stability or the COD removal efficiency of the UASB.

Ferrous ion could slightly improve COD removal efficiency and maintain the stable reactor performance. Importantly, UASB reactor with low COD removal efficiency and acid pH condition could be recovered to high COD removal performance and normal pH conditions. It has been reported that Fe(II) addition significantly affect reactor performance. Fe(II) addition was an effective method to obtain a high removal rate of nitrobenzene in UASB reactor (Chen *et al.*, 2007). Increasing Fe(II) concentration could decrease the specific activity of sludge granules in UASB reactor (Yu *et al.*, 2000).

Ferrous iron Affected Nitrogen Removal Performance

NO_3^-, NO_2^-, NH_4^+, Fe^{3+} and Fe^{2+} concentrations were analyzed to explore the relationship between iron addition and nitrogen removal performance in experiment reactor (Fig. 3). The 5 mM Fe(II) in influent was partly oxidized to Fe(III), the concentration of Fe(III) and Fe(II) in effluent was about 3.5 mM and 1.5 mM, respectively. In this study, the influent contained NH_4^+ and NO_3^- and NO_2^-, and NH_4^+ was the dominant component with concentration about 14 mM, NO_3^- concentration was less 2 mM and NO_2^- concentration was less 1 mM. In effluent NH_4^+ concentration was below 1 mM, when Fe^{2+} was added to influent, NO_3^- and NO_2^- concentration was close to influent, almost all the NH_4^+ was oxidized to $NO_2^-$and NO_3^- by nitrification process, and then they were removed by denitrification. When Fe^{2+} addition was stopped, NO_3^- and NO_2^- concentration was higher than influent, almost all the NH_4^+ was removed, but a part of NO_2^- (22%) and NO_3^- (61%) was failed to remove by denitrification. Whether Fe(II) added or not the NH_4^+ in effluent was no observed difference, this indicated that Fe(II) addition does not affect nitrification, but seriously affected denitrification.

The concentration of NO_3^- in effluent was about 6 mM, after Fe(II) addition it decreased to 2 mM similar to the influent level, when stopped Fe(II) addition, NO_3^- in effluent gradually increased to 8 mM, Fe(II) was added again on Day 105, and NO_3^- in effluent gradually decreased and recovered to the influent level. The effect of Fe(II) on NO_2^- removal trend was similar to Fe(III). The bad NO_3^- and NO_2^- removal performance was shown after Fe(II) addition stopped, it was not only affected by Fe(II) but also

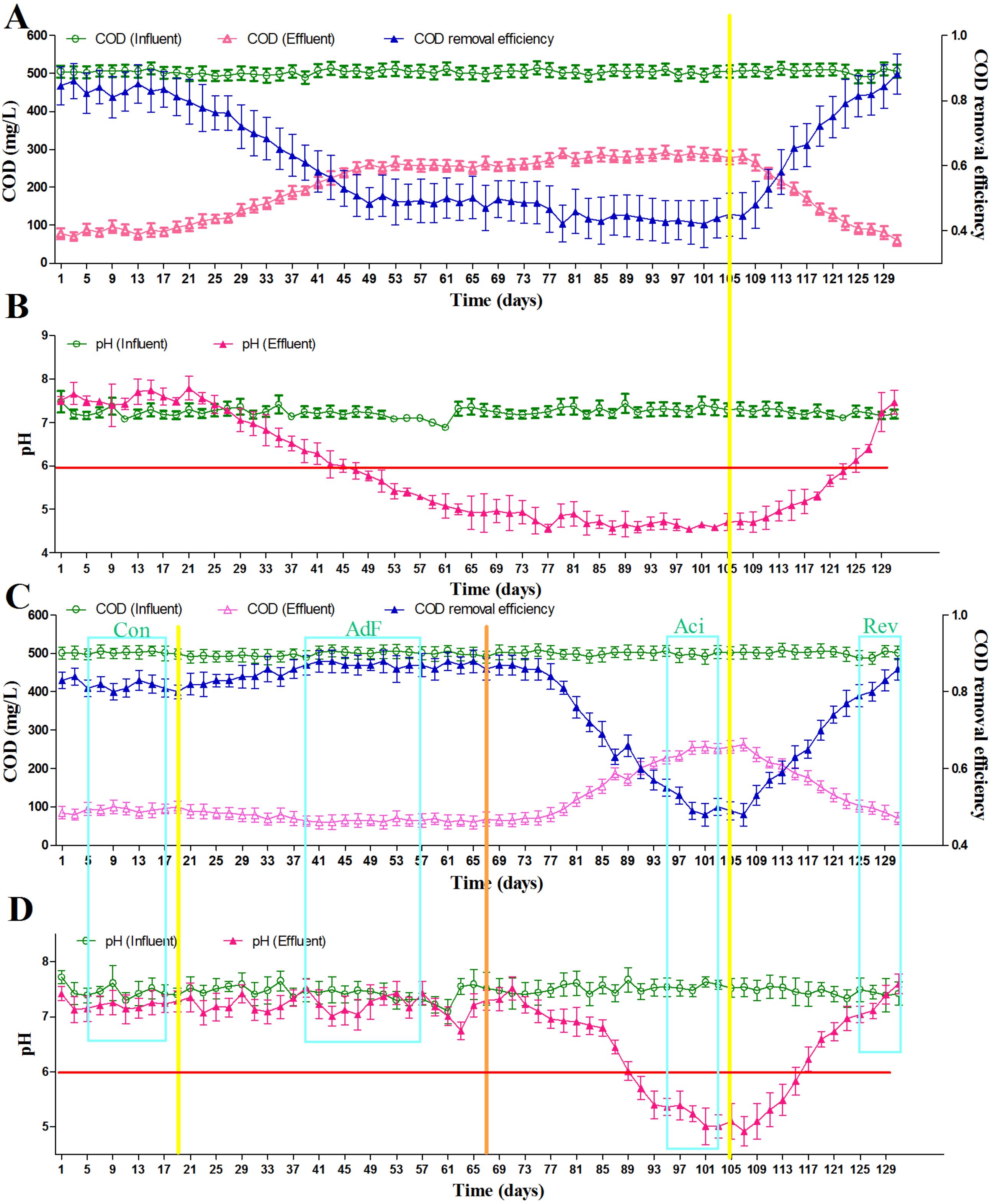

Fig. 2. Profiles of pH and COD removal efficiency with time in different UASB reactors. (A) and (B): control UASB reactor without the addition of Fe(II); (C) and (D): UASB reactor with Fe(II) addition; the yellow vertical solid lines represented the beginning of Fe(II) addition; the orange vertical solid lines represented the stop of Fe(II) addition; the box represented the sample for high-through sequencing.

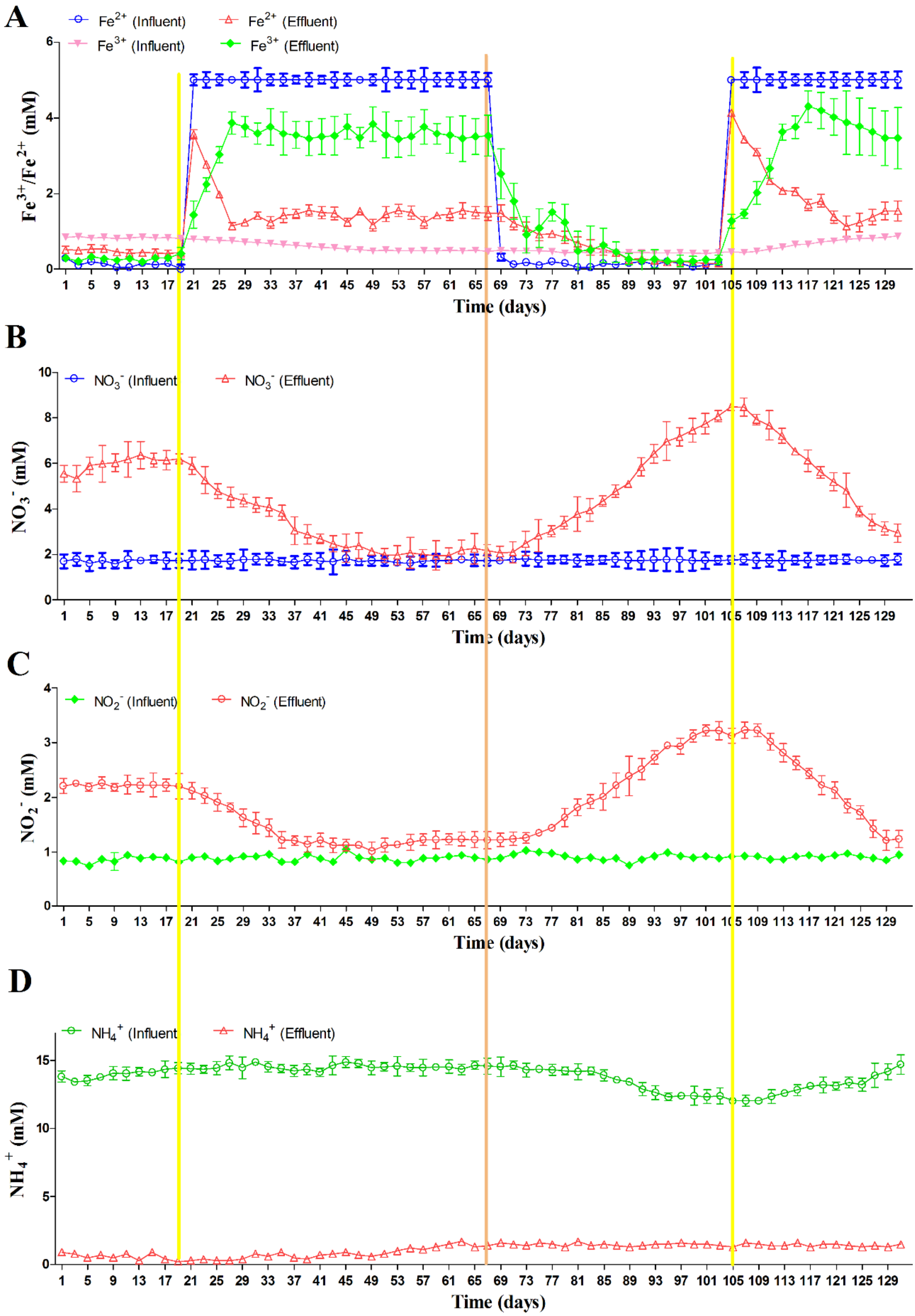

Fig. 3. Concentration of (A) Fe^{3+} and Fe^{2+}, (B) NO_3^-, (C) NO_2^- and (D) NH_4^+, in the UASB reactor. The yellow vertical solid lines represented the beginning of Fe(II) addition; the orange vertical solid lines represented the stop of Fe(II) addition.

related to the low pH, because no Fe(II) addition led to pH decrease to below 6 from Day 89 to Day 105, the acid condition was negative for microbial activity. These results indicated that Fe(II) addition contributed to denitrification.

Ammonium in domestic wastewater was oxidized to nitrite or nitrate via nitrification in aerobic condition, nitrite or nitrate was reduced to nitrogen gas via denitrification in anaerobic condition, and denitrification is usually regarded as one of the determining steps of the nitrification–denitrification process (Mac Conell *et al.*, 2013; Wu *et al.*, 2016). The results revealed that NO_3^- and NO_2^- could be rapidly reduced to nitrogen gas with Fe(II) addition, which was consistent with previous study(Li *et al.*, 2016). Ferrous ions (Fe2+) are required for metalloenzymes such as hydrogenases and ferredoxins. And Fe2+ ions are transported by the FeoAB transporter encoded by genes implicated in ferrous iron uptake (Kammler *et al.*, 1993). The expression *feo* operon was down-regulated under aerobic conditions and up-regulated under anaerobic conditions through transcription factors ArcA and FNR. Feo-mediated import of Fe2+ promotes Fur-Fe2+ occupancy and contributes to Fur regulation under anaerobic conditions (Beauchene *et al.*, 2017). Although many other hidden mechanisms warrant further investigations, our results suggested that Fe(II) addition is an effective method to improve nitrogen removal efficiency and maintain the high reactor performance.

Ferrous iron Affected Microbial Community

To study the effect of Fe(II) on microbial community, the samples of the experiment reactor were analyzed with high throughput sequencing during four different periods, samples were named as Con, AdF, Aci and Rev. The sequence number of these samples were 22,593, 23,908, 26,222 and 23,656, respectively (Table 1). In total, 1412 OTUs were identified in the complete data set, and the average OTUs number of each sample was 718. Over 98.7% of the OTUs was assigned to a taxonomic group (phylum), and over 89.5% was identified at the order level. The Shannon index, ACE index and Chao index for Con sample tended to be higher than other samples, which indicated that bacteria diversity was most abundant in Con sample.

The dominant phyla in Con sample were *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes* and *Verrucomicrobia* (Fig. 4). After the addition of Fe(II), *Proteobacteria*

Table 1. Summary of the 16S rRNA sequences, operational taxonomic units (OTUs), and microbial diversity indices for all examined samples.

Sample	Sequences number	OTUs number	Chao index	Shannon index	Simpson index	ACE index
Con	22593	807	846	5.21	0.014	905
AdF	23908	635	690	4.14	0.058	676
Aci	26222	756	786	4.46	0.056	825
Rev	23656	677	743	4.11	0.058	704

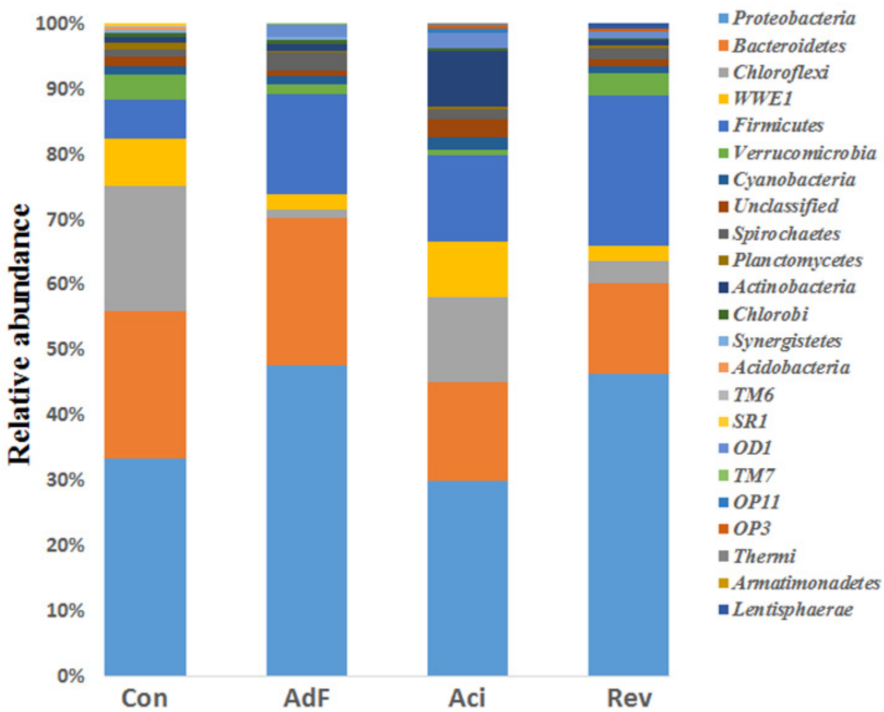

Fig. 4. Relative abundances of microbial community for four samples in phylum level. the. Con: control UASB reactor; AdF: UASB reactor with Fe(II) addition; Aci: UASB reactor under acid condition (pH < 6); Rev: UASB reactor recovered from acid condition.

increased from 32.77% to 47.33% and *Firmicutes* also increased from 5.89% to 15.36%. Fe(II) could stimulate *Proteobacteria* and *Firmicutes* increase, which was consistent with previous studies investigating iron oxidation and NO_3^- reduction (Coby *et al.*, 2011; Melton *et al.*, 2014; Li *et al.*, 2016). However, *Chloroflexi* sharply decreased from 18.86% to 1.36% after Fe(II) addition, which indicated that Fe(II) may down-regulate *Chloroflexi* growth. These results suggested that Fe(II) remarkably affected the microbial community.

In the genus level, the dominant genera in Con sample were *Clostridium*, *Hydrogenophaga*, *Flavobacterium*, *Prevotella*, *Achromobacter*. After Fe(II) addition, the relative abundances of *Hydrogenophaga*, *Methylotenera*, *Zoogloea* and *Fluviicola* evidently increased, while the relative abundances of *Clostridium*, *Flavobacterium*, *Prevotella*, *Achromobacter* and *Paludibacter*, dramatically decreased in AdF sample. When stopped Fe(II) addition, the pH decreased below 6, the relative abundances of *Paludibacter*, *Zoogloea* and *Methylomonas* notably increased in Aci sample. When the pH of the reactor returned to the range from 7 to 8, the relative abundances of *Hydrogenophaga*, *Methylotenera*, *Zoogloea* and *Fluviicola* increased again in Rev sample, which was similar to the observations in AdF (Fig. 5). Interestingly, previous related studies have documented that some denitrifying bacterial strains with high nitrogen removal efficiency were isolated from biofilters and identified as *Pseudomonas aeruginosa* and *Chelatococcus daeguensis* according to 16S rRNA gene homology (Wu *et al.*, 2013; Yang *et al.*, 2013). The results are not agreement with ours. This implies that different strains may acquire similar functions through convergence evolution.

Correlations between Chemicals and Abundances of Genera

The composition of influent and pH significantly affected the UASB reactor performance and stability by influencing the bacterial community and diversity. Therefore, the correlation coefficients between abundant genera and chemicals reaction rate were analyzed (Table 2). The results reconfirmed that the abundances of *Hydrogenophaga*, *Zoogloea*, *Fluviicola* and *Syntrophus* positively correlated with COD removal efficiency; the abundances of *Hydrogenophaga*, *Methylotenera* and *Fluviicola* also positively correlated with Fe(II); the abundances of *Paludibacter* and *Syntrophus* were related to low pH condition.

Paludibacter, *Hydrogenophaga*, *Zoogloea*, *Fluviicola*, and *Methylotenera* were the dominant bacteria in the UASB reactor with Fe(II) addition. When the pH was below 6 and without addition of Fe(II), the relative abundances of *Paludibacter* increased notably in Aci, which was in accordance with a previous study and may result from that

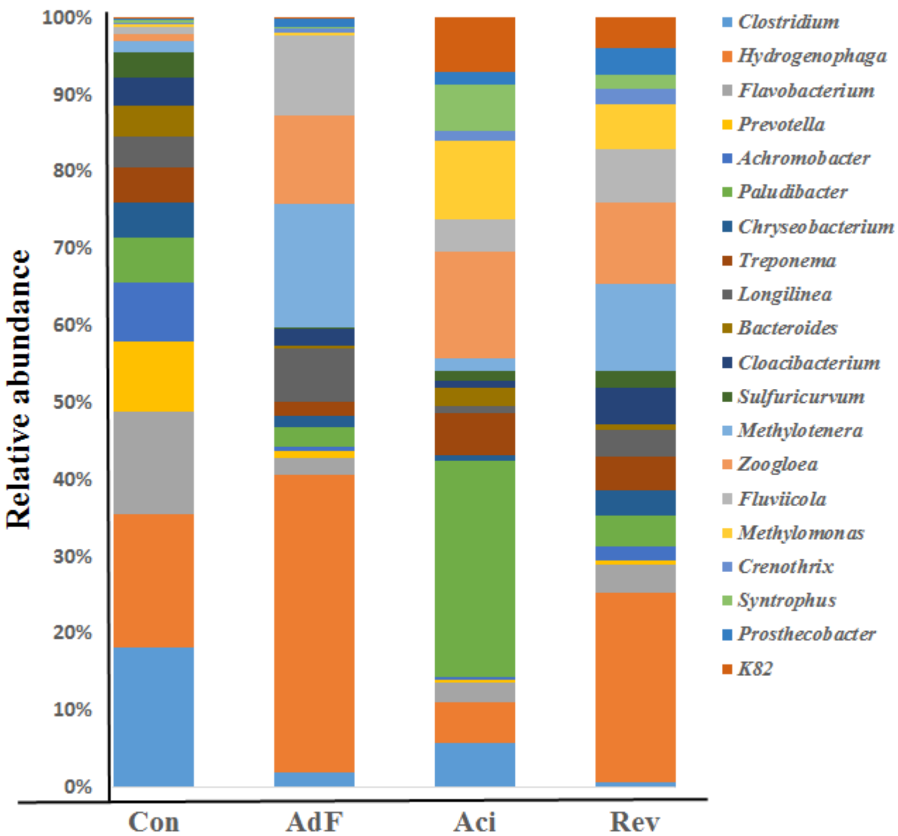

Fig. 5. Relative abundances of microbial community for four samples in genus level. the. Con: control UASB reactor; AdF: UASB reactor with Fe(II) addition; Aci: UASB reactor under acid condition (pH < 6); Rev: UASB reactor recovered from acid condition.

Table 2. Correlation coefficients between chemicals and abundance of genus.

	Clostridium	*Hydrogenophaga*	*Prevotella*	*Paludibacter*	*Treponema*	*Longilinea*	*Bacteroides*	*Cloacibacterium*	*Methylotenera*	*Zoogloea*	*Fluviicola*	*Methylomonas*	*Syntrophus*	*K82*
pH	−0.82*	−0.89*	0.81*	−0.96*	−0.67	0.8	−0.24	0.88*	0.58	0.81*	0.88*	−0.82*	−0.96*	−0.83*
COD	−0.11	0.84*	0.19	0.92*	−0.7	0.81*	−0.36	0.77	0.86*	0.9*	0.89*	−0.77	−0.92*	−0.78
Fe^{2+}	−0.88	0.85*	−0.84*	0.85*	−0.89*	0.65	−0.84*	0.54	0.93*	0.93*	0.8	−0.33	−0.56	−0.35
Fe^{3+}	−0.77	0.54	−0.55	−0.66	−0.7	0.63	−0.91*	0.41	0.96*	−0.86*	0.48	−0.26	−0.47	−0.28
NO_3^-	0.6	0.91*	0.34	−0.81*	0.77	−0.75	0.79	−0.54	−0.94*	−0.14	−0.79	0.46	0.66	0.48
NO_2^-	0.45	−0.63	0.16	0.51	0.79	−0.81*	0.67	−0.62	−0.88*	0.04	−0.68	0.6	0.78	0.62
NH_4^+	0.44	−0.43	0.31	0.91*	0.77	−0.36	0.23	−0.46	−0.26	0.44	−0.41	0.23	0.52	0.34

* Correlation is significant at $P < 0.05$.

Paludibacter produces many organic acids (Ueki *et al.*, 2006). *Hydrogenophaga*, an important bacterium for denitrification, is an autotrophic facultative aerobe genus that uses hydrogen as an electron donor, with the metabolites of N_2 and H_2O, which was consistent with our results that the NO_3^- concentration was decreased after the addition of Fe(II) (Chen *et al.*, 2013). *Zoogloea* has been previously reported to have NO_3^- reduction and nitrogen-fixing capabilities with Fe(II) oxidation (Shao *et al.*, 2009; Oosterkamp *et al.*, 2011). Both *Fluviicola* and *Methylotenera* have been proven to play roles in denitrification with Fe(II) oxidation (Kalyuhznaya *et al.*, 2009; Gonzalez-Martinez *et al.*, 2016).

Based on the correlation coefficient analysis and known function of dominant bacteria, the hypothetical mechanism was proposed in Fig. 6. Firstly, *Paludibacter* made a great contribution to nitrification utilizing COD to oxidize NH_4^+ and generated NO_3^-; secondly, *Hydrogenophaga* and *Zoogloea* reduced NO_3^- to NO_2^- companied with Fe^{2+} oxidized to Fe^{3+}; *Fluviicola* and *Methylotenera* reduced NO_2^-

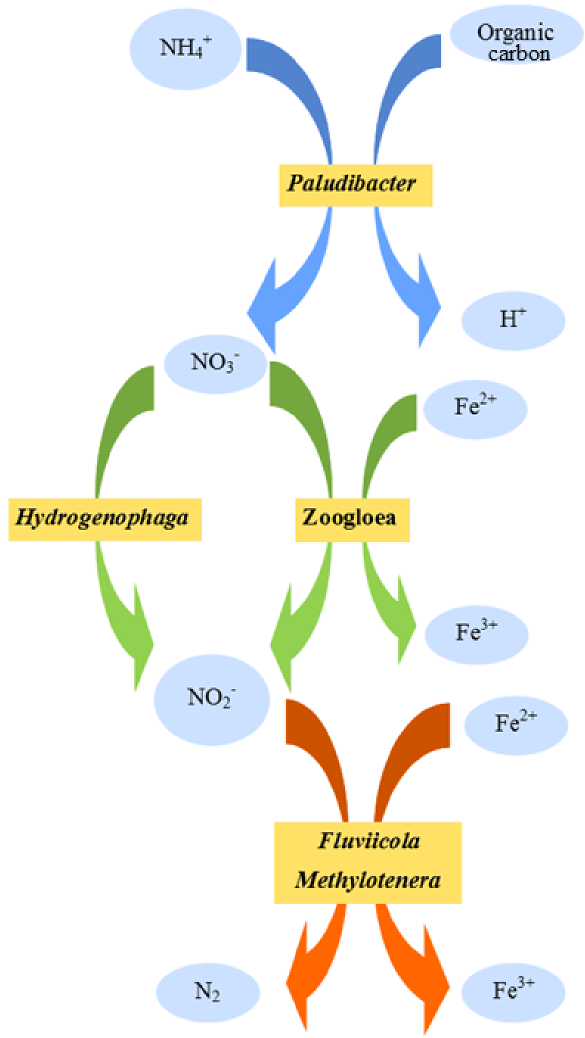

Fig. 6. A proposed mechanism of the accelerated biogas production with Fe(II) addition by the dominant bacteria in UASB reactor.

to N_2 with Fe^{2+} addition. Recently, it was documented that maritime emissions have a notable influence on air pollution over coastal areas, especially in summer (Ding *et al.*, 2018). Apart from VOC control, NO_x control is also critical to reduce peak ozone concentrations (Li *et al.*, 2013). As reported by Liu *et al.* (2017), our study may provide a hint for the design and preparation of Fe catalysts for biologically-derived NO_x control.

CONCLUSION

This study investigated the roles of ferrous ion in UASB reactor. Ferrous ion not only play an important role in maintaining appropriate pH and stable reactor performance, but also significantly influence biogas emission and corresponding microbial community. The findings will improve understanding the mechanisms of ferrous ions on denitrification process and provide hints for biogas emission management.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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