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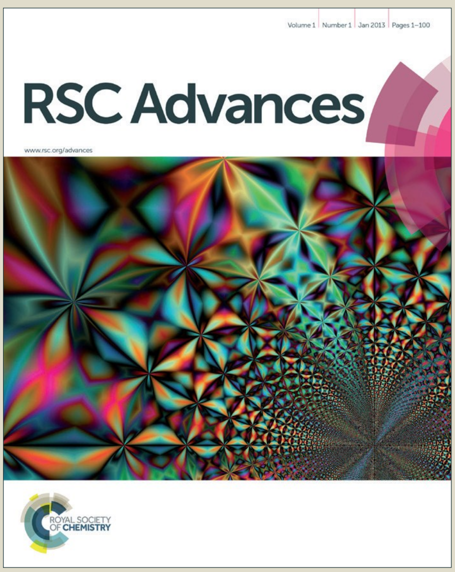

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ARTICLE

Effects of ferric nitrate additions under different pH conditions on autothermal thermophilic aerobic digestion for sewage sludge

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The effects of ferric nitrate additions at different pH values on stabilization of sewage sludge and microbial communities were investigated in autothermal thermophilic aerobic digestion (ATAD). The lowest pH value but highest VS removal had been achieved at optimal pH of 6.5 when $Fe(NO_3)_3$ was added, comparing to other dosing groups, and the stabilization time had been shortened by 7 days. The increased dosing pH reduced the effectiveness of $Fe(NO_3)_3$ on disinhibition of excessive volatile fatty acids, and even caused failure of stabilization. However, a highly basic condition of dosing (such as pH 9.5) could restore the ATAD ability by promoting the processes of hydrolysis and acidification, and then take back control of pH value. Illumina high-throughput sequencing revealed the group of optimal dosing pH significantly enhanced the abundance of phylum *Firmicutes* (from 61.0% to 96.6%), while the rise of dosing pH decreased the richness of phylum *Firmicutes*. The structure of microbial community was even totally changed under strong basic condition of $Fe(NO_3)_3$ addition.

1 Introduction

Biological processes of wastewater will inevitably produce large amounts of sludge, and the price of treatment and disposal of the sludge could account for up to 50% of a wastewater treatment plant's (WWTP) operating cost.[1] Due to the lower investment and simpler control requirements comparing to the technology of anaerobic digestion, aerobic digestion process is much more suitable for small- and medium-sized WWTPs, especially the autothermal thermophilic aerobic digestion (ATAD).[2] ATAD process can meet Class A biosolids demand for a wide range of organic sludge, such as animal sludge, sewage sludge and food processing wastes, and significant advances of optimization and adaptation of ATAD technology have been achieved.[3]

Despite of the superiority presented by ATAD,[3] however, recently, inhibition of excessive volatile fatty acids (VFA) had been found to be a severe issue in the initial stage of ATAD process for sludge with high volatile solid (VS).[4] A chemical method had been devoted to relieving the inhibition caused by over-high VFA concentration in the ATAD system, through complex-precipitation process involved in reactions between sludge components and ferric nitrate.[5] Although some efforts had been made to reveal the influences of the factors related to the agent itself on the ATAD for sludge, for instance, the dosage and the timing of ferric nitrate additions,[6,7] there are still lots of difficulties unsolved, such as effects of pH, temperature and so on. As a chemical reaction, the crucial impact of

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pH on the product and the reaction rate is obvious. In addition, the involvement of hydroxyl in the reaction of complex will make the pH condition much significant as well.[5]

On the other hand, the importance of pH to the biological processes in ATAD is very evident. The most immediate response to the change of pH was the fluctuations of VFA levels in the procedures of hydrolysis and acidification,[8] as well as the variations of dominant VFA species.[9] Especially in thermophilic condition, the VFA concentrations changed drastically at different pH values.[10] Furthermore, the optimal pH was divergent in different systems. It was found that the optimal pH was 10 for VFA production from excess sludge,[11] through the break of sludge matrix which increased the effective contact between extracellular organic matters and enzymes, and created a favourable environment for microbes to accumulate VFA.[12] In the presence of alkyl glycoside, short chain fatty acid productions from membrane bioreactor sludge at initial alkaline pH values were also more efficient than those at acidic and near-neutral pH conditions, but the optimum initial pH was 11.[13] The pH of 12 was adopted to obtained continuous volatile fatty acid production from waste activated sludge as well.[14] In addition, hydrolytic enzymatic assays demonstrated that abiotic effect was responsible for increased solubility of organic matter in sludge under high alkaline condition.[1,12] Nevertheless, VFA accumulation was optimized at pH 8 despite higher solubility at higher pH, and the pH at 9 and above would lead to disruption of biological activities and VFA production eventually.[1] Meanwhile, some research also indicated that the alkaline pH improved the solubility and biodegradation of proteins in the sludge, and significantly influenced the biodiversity and bacterial community in the system.[15] Lastly, the pH level had close relationship with the complexing state of metallorganics, particularly the iron organic complex, as well as their migration and transformation in aerobes.[16]

As we known, the initial stage of ATAD system was much like a facultative anaerobic environment, in view of the limited aeration as well as the low oxidation-reduction potential (ORP).[17] The survival of anaerobic microbes in ATAD also supported this argument.[18] Hence, the stress of pH changes to anaerobic microbial metabolism, especially the hydrogen production, should also be taken into consideration.[19-21] Besides, the pH condition affected the ammonia stripping[22] and phosphorous release[23,24] as well. Therefore, the effects of ferric nitrate additions at different pH values on ATAD performance for sewage sludge were investigated in this study. At the same time, the corresponding microbial communities at different pH values were compared and analysed.

Table 1
Properties of initial sludge employed in simulated one-stage ATAD process.

Parameter	pH	TS (g/L)	VS (g/L)	SCOD (mg/L)	TN (mg/L)	NH_4^+-N (mg/L)	TP (mg/L)
Value	6.5 ± 0.2	54.8 ± 0.4	36.0 ± 0.3	1205 ± 20	221 ± 10	35 ± 1	182 ± 8

SCOD, soluble chemical oxidation demand; TN, total nitrogen in supernatant; TP, total phosphate in supernatant.

2 Experimental

2.1. Start-up of the experiments

Sewage sludge used in this study was secondary sludge collected from a WWTP in Shanghai, China. This WWTP owns an anaerobic-anoxic-oxic process with a capacity of 45,000 m^3 wastewater treatment daily. The sludge sample was screened to remove particles coarser than 0.5mm before going through centrifugation at 2200 g for 3 min to obtain total solid (TS) between 5% and 6%. The main properties of raw sludge were shown in Table 1.

The batch digestions were conducted in five simulated autothermal thermophilic aerobic digesters of 200 mm (D) × 400 mm (H), and the available volume of a cylinder reactor was 4 L. The self-heat process was imitated through a water bath connected to the heating water jacket outside the body of digester, and the temperature of digestion was rising from 35 ℃ to 55 ℃ at a rate of 5℃/d. After temperature reached 55 ℃, the digestion process would stay on such a circumstance until the end. A continuous aeration rate of 0.13 L/min and a constant stirring rate of 120 resolutions was supported[17].

The entire process of digestion last for 21 days. $Fe(NO_3)_3$ was added in reactors on 6th day[7] with dosage of reducing 1000 mg/L of acetic acid,[6] but the control one was not fed. The decreased amount of sludge by sampling before 6th day was taken into consideration when the chemical reagent was added into the reactors on 6th day. $Fe(NO_3)_3$ was put into digester 6 hours before sampling on 6th day, for the sake of adequately reaction between chemical reagent and sludge. The pH of four systems were adjusted to 6.5, 7.5, 8.5 and 9.5 just after the additions of $Fe(NO_3)_3$ by using of NaOH and HCl, in view of the pH of well operation ATAD system is always between 6 and 9, and sometimes can reach up to 9.5,[25] while the control was still not given any treatment. Samples were taken on 4th, 6th, 8th, 11th, 14th, 17th, 21st day and the start of the digestion, and microbial communities were analysed in raw sludge and digestion sludge on 21st day.

2.2. Chemical Analysis

The pH of sludge was measured by a pH meter (pHs-3C, Leici Co. Ltd., Shanghai). VS and TS were determined according to the Standard Methods[26] with values of influence caused by chemical reagents subtracted. The sampled sludge went through centrifugation at 12,000g for 5 min following with filtration through a 0.45 μm mixed cellulose ester membrane to obtain the supernatant. Then the values of NH_4^+-N, SCOD, TN and TP in the supernatant were analysed on the basis of Standard Methods.[26] As for the determination of VFA level in the supernatant, the filtrate was blended well with 3% H_3PO_4 (to keep the pH of the filtrate staying at approximately 4.0) and then injected into a Shimadzu GC-2010 gas chromatograph with a flame ionization detector and DB-FFAP column (30 m × 0.25 mm × 0.25 mm) according with method of Chen et al.[8] The value of VFA was expressed in terms of COD. All of indicators were measured in triplicate and the standard deviations were acquired. The software SPSS version 19.0 for Windows (SPSS, IBM) was applied for statistical analysis and statistically significant correlations were decided at a 95% confidence interval ($p < 0.05$; Tukey's test).

2.3 Molecular biological analysis

The five DNA samples of sludge after 21 days digestion were sent for Illumina miseq sequencing of V3-V5 region of bacterial 16S rRNA gene (Genewiz, South Plainfield, USA). The software of Trimmomatic (v 0.30) was applied to optimizing process of Illumina raw reads. The barcodes and primers were trimmed off. The quality values less than twenty on both ends were removed as well as the bases of average quality less than twenty. Every two sequences were compared and jointed using the tool of pandaseq to filter out the reads containing nitrogenous bases and the sequences shorter than 400 bp or longer than 480 bp in the overlap region of the end. Afterwards these sequences were optimized, removing the sequences outside target region by comparing to the 16s rDNA sequences in Greengene database and wiping off chimera sequences. Taxonomic classification was carried out to classify the optimized sequences into operational taxonomic units (OTUs), with a confidence threshold of 97% using QIIME standard pipeline.[27] To compare the microbial diversity and richness, Shannon rarefaction curves, clustering tree and species distribution diagrams of multi-samples were covered.

3 Results and Discussion

3.1 Change of pH

The changes of pH values in five digestion systems were illustrated in Fig. 1. Few differences of the pH values were observed among all digesters in the first 4 days (*P<0.05*). Then the variations of pH values in ATAD of $Fe(NO_3)_3$ dosed at pH 6.5, 7.5 and the control had more or less the same tendency after the ferric nitrate additions, while the other two were much similar. The former three curves had a sharp increase from 6th day to 14th day before fluctuating moderately until the end, because of the consumption of VFA by precipitation with $Fe(NO_3)_3$ and increase of alkaline substances releasing.[5] However, the other two systems had a fall from 8th day to 12th day after increase on 6th day, and went back to normal as the others afterwards.

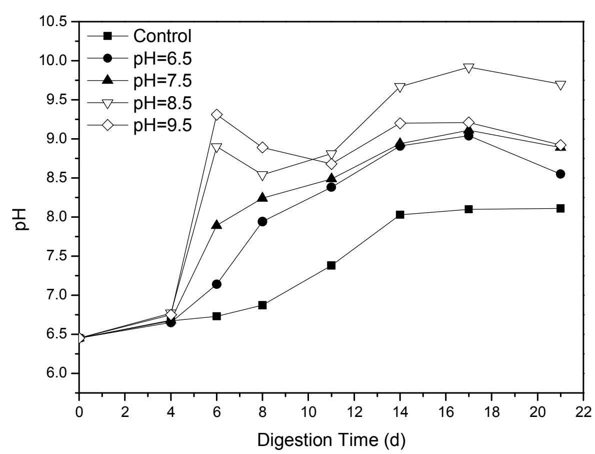


Fig. 1. Changes of pH values in simulated one-stage ATAD systems.

The emergence of reduction in pH should be due to the increase of VFA, NH_4^+ and cations, especially the VFA, which was result from the acceleration of cell rupture and promotion of solubility of organic matters under alkaline condition.[12,28] As for the faster growth of pH 8.5 comparing to pH 9.5 afterwards, it should be attributed to the optimum pH 10 (closer to 9.5) in hydrolysis and acidification, which would make contribution to the control of pH and even the digestion later.[12] On the other side, the out of control in pH would lead to the failure of ATAD system when $Fe(NO_3)_3$ addition at pH 8.5, and would result in the higher level of pH as well. As seen in Fig. 1, the range of pH changes were all between 6 and 9 except the one dosed at pH 8.5, agreeing with the domain of well operation ATAD systems' pH,[25] and also supported the design of pH set in this study. The pH of the control got the lowest level throughout the digestion, while the pH of ATAD process with $Fe(NO_3)_3$ addition at pH 8.5 reached as high as nearly 10 in the end.

3.2 Removal efficiency of VS

VS removal always plays an important role in stabilization of sludge for aerobic digestion. The VS removals of ATAD processes for sewage sludge were shown in Fig. 2. There were few differences of the VS removal values in all reactors in the first 6 days (*P<0.05*), and had a little delay comparing to the changes of pH values. Afterwards, there was a plateau in either curve of pH 8.5 or pH 9.5, while the others increased continuously until the end. As seen in the Fig. 2, the ATAD systems had all obtained stabilization for sewage sludge (>38%) after 21 days digestion except the group of pH 8.5.[29] As for the failure in stabilization

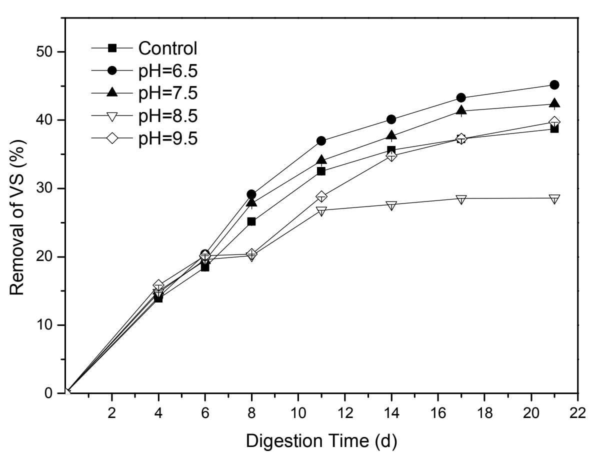


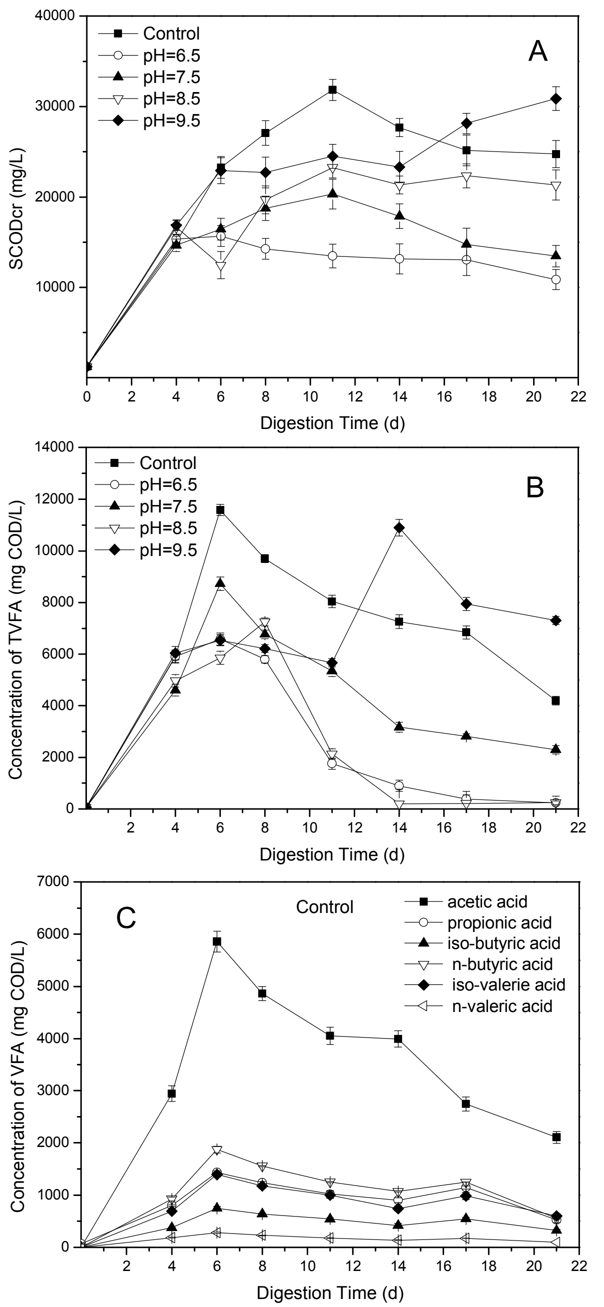
Fig. 2. Variations of VS removals in simulated one-stage ATAD systems.

of ATAD process with $Fe(NO_3)_3$ addition at pH 8.5, the losing control of pH should be the main reason. The most efficient VS removal was obtained in group of pH 6.5, and its final VS removal was 45.16%, which was 6.42% advanced comparing to that of the control. After a lag phase, the VS removal in group of pH 9.5 had sharply increased and exceed that of the control finally, indicating the advantageous pH condition had played a part in the digestion as mentioned above. Nevertheless, the stabilization time obtained by group of pH 9.5 was almost the same as that of the control, but still lagged behind those achieved by groups of pH 6.5 and pH 7.5 (reached removal of VS above 38%), 7 days and 4 days behind, respectively. Given the pH in the control was only a little higher than 6.5 on 6th day, the addition of NaOH or HCl for adjustment of pH was very little. Thus, the pH of 6.5 at which $Fe(NO_3)_3$ dosed was certainly the most proper choice. These results demonstrated that the effect of ferric nitrate played a leading role on enhancement of ATAD performance rather than the pH at which the ferric nitrate added, and the optimal pH was 6.5.

3.3 SCOD and VFA in the supernatant

The variations of soluble chemical oxygen demand (SCOD) in the supernatant of five digesters were presented in Fig. 3A. The change of SCOD concentration in the supernatant of the control was in line with the trend revealed by Liu et al.[2] The lowest SCOD level was obtained by the group of pH 6.5 throughout the digestion. As illustrated in Fig. 3A, the SCOD of all reactors were almost the same in first 4 days (*P<0.05*). Then the SCOD in group of pH 6.5 decreased until the end after a plateau on 6th day. However, the SCOD value in group of pH 8.5 had a reduction when $Fe(NO_3)_3$ added, and then increased to the top on 11th day. Afterwards, it fluctuated moderately to the end, agreeing with the response of VS removal, indicating the microbial activity had been inhibited under the condition of losing control of pH.[1] Unlike the SCOD value in group of pH 8.5, the SCOD concentration in group of pH 9.5 still increased as that in the control on 6th day, though the microbial activity was suppressed (reflected by the plateau of VS removal on 8th day) when the dosing pH adjusted at 9.5,[1] implying the acceleration of hydrolysis and acidification had made up the lost SCOD value by inhibition of microbes. After a fluctuation, the SCOD level in group of pH 9.5 rose again, just like the reflection of VS removal.

The total volatile fatty acids (TVFA) included acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid and iso-valeric acid. As shown in Fig. 3B, the changing trend of TVFA concentration was roughly similar with that of SCOD value, respectively, in accord with the internal relationship between TVFA and SCOD reported by Liu et al.[17] The TVFA level in group of pH



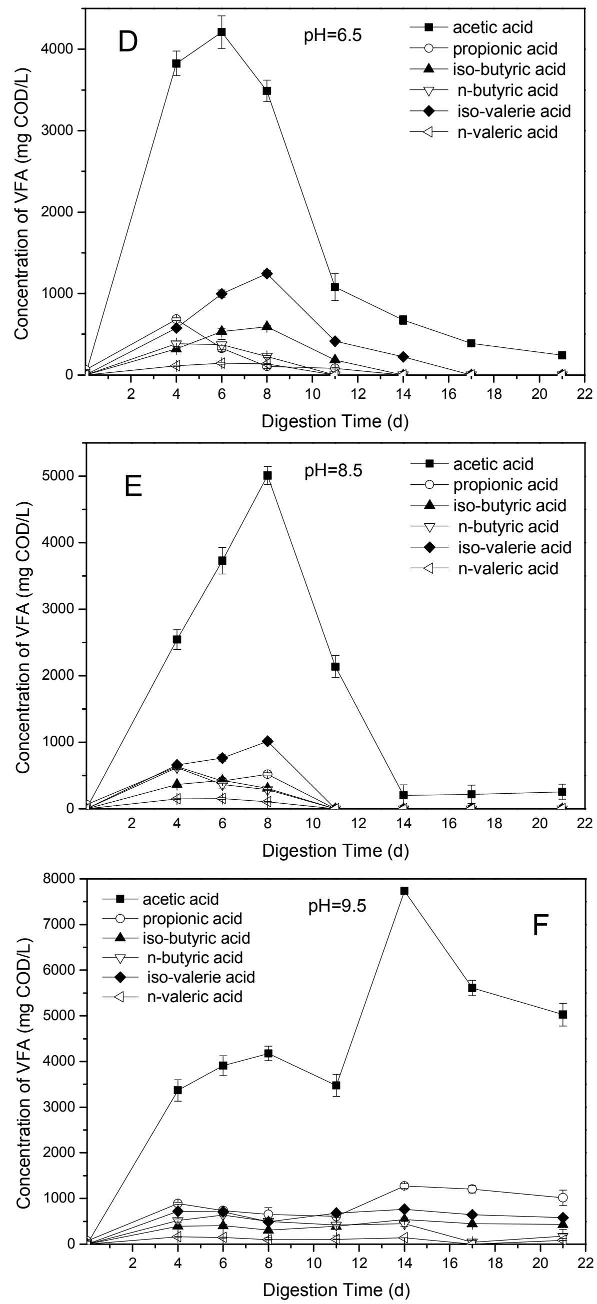


Fig. 3. Changes of (A) SCOD; (B) TVFA {total VFAs}; (C) individual VFA of the control; (D) individual VFA of pH 6.5; (E) individual VFA of pH 8.5; (F) individual VFA of pH 9.5 in the supernatant of simulated one-stage ATAD systems.

6.5 was very low (<1000 mg COD/L) after digestion of 14 days, denoting the stabilization had been achieved in another respect. As for the TVFA value in group of pH 8.5, although the level of TVFA was almost undetected after 14th day, the nearly stopped increase of VS removal and fluctuation of SCOD demonstrated the metabolism of microbes were restrained.

The variations of individual VFA in the supernatant were illustrated from Fig. 3C to Fig. 3F. As seen in Fig. 3C, either the concentration of propionic acid or content of n-butyric acid was larger than that of iso-valeric acid during the whole digestion process. However, the level of iso-valeric acid turned out to be higher after $Fe(NO_3)_3$ addition (as shown from Fig. 3D to Fig. 3E), and the concentration of propionic acid was much less than 1000 mg COD/L, indicating the success had been achieved in disinhibition of propionic acid by relieving the stress of acetic acid through precipitation,[5] and then promoting n-butyric acid degradation and transformation to shorter VFA.[30] On the other hand, the pH increased by $Fe(NO_3)_3$ addition and adjustment of itself also had a strong impact on the distribution of individual VFA, such as increase of iso-valeric acid and decrease of propionic acid.[15] Lower pH condition revealed to be more favourable to accumulation of n-butyric acid as well.[31] Nevertheless, the variations of individual VFA in group of pH 9.5 (as seen in Fig. 3F) demonstrated that the key role played in promotion of microbial metabolism was pH condition in this system, rather than the $Fe(NO_3)_3$ addition, in view of the abovementioned relatively higher concentration of iso-valeric acid and lower content of n-butyric acid as well as the second highest level of propionic acid (>1000 mg COD/L)[5] throughout the entire digestion process.

3.4 NH_4^+ and TN in the supernatant

The change of pH value was a macroscopic phenomenon of variations of acidic substances and alkaline substances. The NH_4^+ concentration in the supernatant of the control was the highest during the whole digestion process (as seen in Fig. 4A), and the TVFA level in the control was also the maximum, both of which contributed to the lowest pH value.[28] On the other hand, the concentration of NH_4^+ had a close relationship with the content of TVFA (as shown in Fig. 3B and Fig. 4A). Because that the main constituents of buffer system in the ATAD were NH_3 - NH_4^+ and NH_3 - VFA, the level of NH_3 in the supernatant would decreased when the TVFA reduced, which would lead to the reduction of NH_4^+ in turn. Therefore, the lowest TVFA concentration in group of pH 8.5 caused the lowest NH_4^+ content after 8th day, and the NH_4^+ level in group of pH 9.5 rose on 11th day when TVFA concentration increased. The other digesters' situations

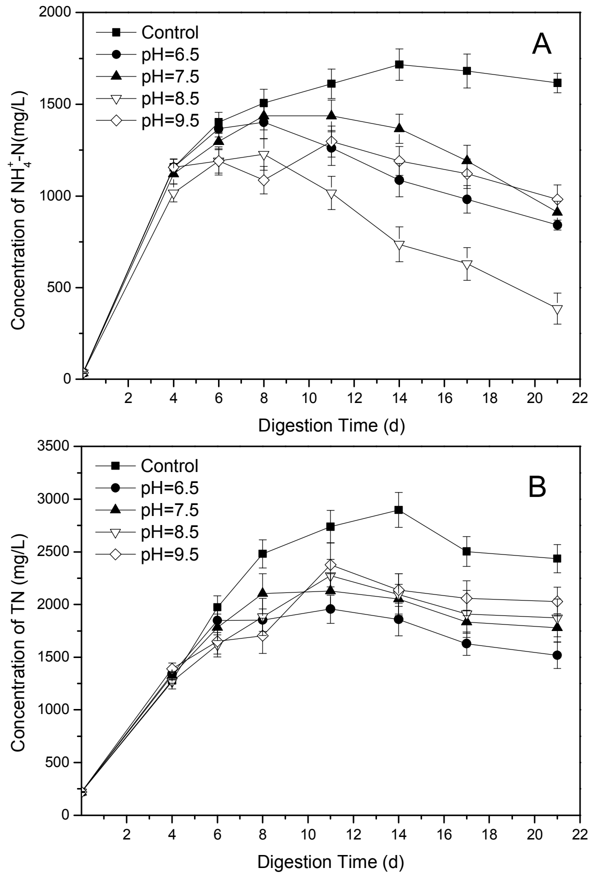


Fig. 4. Variations of (A) NH_4^+ and (B) TN in the supernatant of simulated one-stage ATAD systems.

were very similar. Additionally, the import of OH^- to the systems had also made the NH_4^+ level decreased (as presented in Fig. 4A) because of the reaction in the following: $NH_4^+ + OH^- \leftrightarrow NH_3{\cdot}H_2O$.

The changes of TN in the supernatant of five systems were illustrated in Fig. 4B. As we known, the NH_4^+-N was the dominant component of TN in the supernatant, on account of the nitrification and denitrification were inhibited under thermophilic condition.[17] Hence, the tendency of variation in TN was very similar to that in NH_4^+-N. Nevertheless, the lowest level of TN in the supernatant was obtained by ATAD process of $Fe(NO_3)_3$ dosed at pH 6.5, but not the one of $Fe(NO_3)_3$ added at pH 8.5, which was different to the situation of NH_4^+-N. As seen in Fig. 4B, the concentration of TN in group of pH 8.5 rose continuously to the top on 11th day, while the content of NH_4^+-N started to reduce from 8th day (as shown in Fig. 4A), indicating a large amount of organic amines were not degraded, considering that the microbial activities had been inhibited as mentioned above. The higher TN level in group of pH 9.5

comparing to that in group of pH 7.5, which was contrary to the condition of NH_4^+-N, supported the presence of mass of organic amines in the supernatant as well.

3.5 TP in the supernatant

The variations of TP in the supernatant were shown in Fig. 5. The ATAD system was a closed system for resource of phosphorus,[2] so the TP in the supernatant was derived from the degradation of microbial cells and substances, and reduced by the metabolism of microbes[17], as well as precipitation and adsorption by ferric nitrate.[32] As seen in Fig. 5, the TP level in the control was highest during the digestion except the TP value in group of pH 8.5 after 14th day, as the death and lysis of massive microbial cells under over-high pH condition. The concentration of TP in group of pH 9.5 was fluctuant moderately throughout the entire digestion process, which should be attributed to the acceleration of hydrolysis and acidification as mentioned above. The TP values in group of pH 6.5 and pH 7.5 kept pace with each other, and enlarged constantly until the end after a decline when $Fe(NO_3)_3$ dosed on 6th day, which differed from the results in previous study.[5] This difference should be due to the adjustment of pH condition when $Fe(NO_3)_3$ added as hydroxyl was a strong competitor to phosphate while reacting with ferric.

3.6 Microbial community

After filtration of low-quality reads using the QIIME standard pipeline, a range of 118420 to 264538 high-quality reads (average length of 264 bp) was obtained from six sludge samples, with a high coverage of bacteria sequences (ranging from 0.95 to 0.96). The diversity curve became flat after 10010 reads, and the six samples all had much more sequences than 10010, which could perfectly revealed community diversity (as illustrated in Fig. 6A).The largest total number of operational taxonomic units (OTUs) estimated by Chao1 estimator with infinite sampling was observed in raw sludge (1346), and that of the control on 21st day second (1046), indicating that the addition of ferric nitrate had a negative impact on richness of bacteria phylotypes, though the stress environment in ATAD had the ability as well.[33] The total number of OTUs in group of pH 8.5 was the lowest (414), implying that the least richness of microbes was maintained. The other three

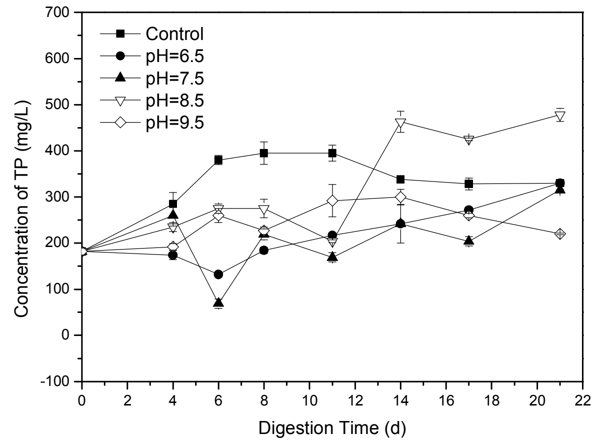


Fig. 5. Changes of TP in the supernatant of simulated one-stage ATAD systems.

were 702 for group of pH 6.5, 637 for group of pH 7.5 and 567 for group of pH 9.5, respectively, denoting the increase of dosing pH also had a reducing effect on abundance of bacteria phylotypes. As seen in Fig. 6A, the raw sludge had the highest diversity (*Shannon = 8.34*), and the control ranked second (6.47) while the group of pH 8.5 ranked third (5.28). The highest diversity but lowest richness achieved in group of pH 8.5 comparing to the other three dosing groups, contributing to the lowest microbial activities as mentioned above. Furthermore, the microbial community in group of pH 8.5 had the closet relationship with that of raw sludge (as shown in Fig. 6B), demonstrating that the level of stabilization in group of pH 8.5 was lowest of all. As for the other three dosing groups, the group of pH 6.5 had the highest diversity among them (5.07), and the one of pH 9.5 had larger Shannon index (4.74) than the group of pH 7.5 (4.29), indicating the strong alkali environment would increase diversity of aerobic microbes in a certain extent, considering that the abundance of anaerobic fermentative microorganisms under alkaline or acidic pH conditions was less than that under neutral pH condition.[15] The microbial community in group of pH 7.5 had a closer relationship with that in group of pH 9.5 than that in group of pH 6.5 (as presented in Fig. 6B), denoting the alkaline pH condition had an influence on structure of microbial community.

The species distribution diagrams of six sludge samples at phylum level were illustrated in Fig. 6C. After a period of 21 days digestion, the abundance of phylum *Firmicutes* increased in all digesters except for

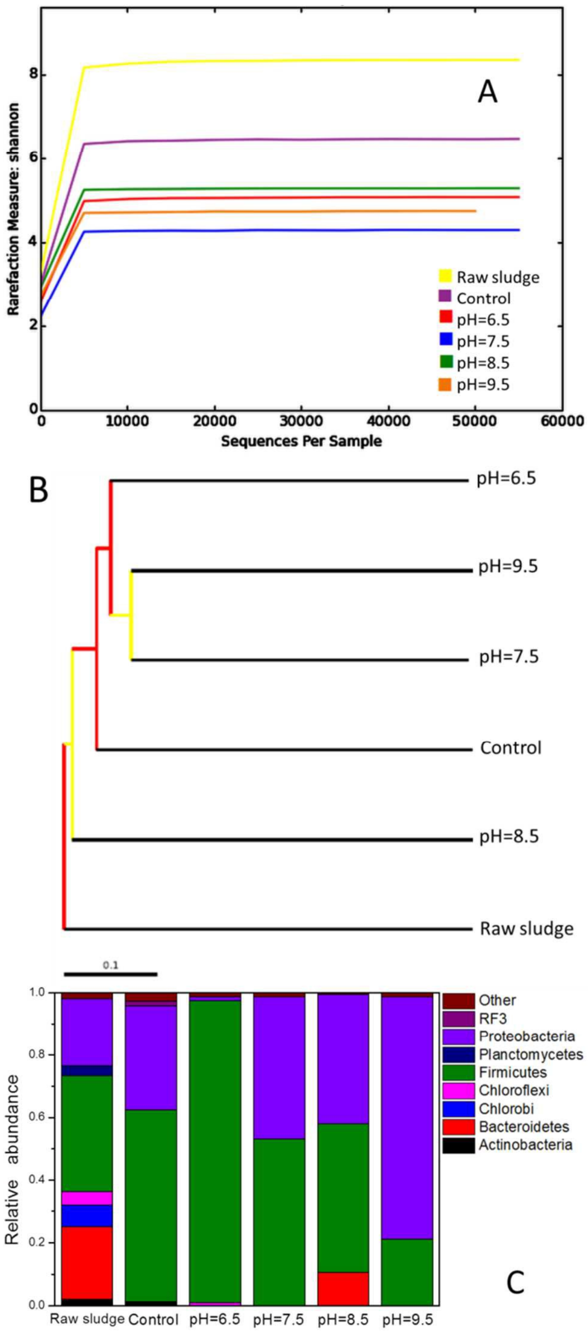


Fig. 6. Results of Illumina high throughput sequencing: (A) Shannon rarefaction curves; (B) clustering tree; (C) taxonomic compositions of bacterial communities at phylum level in the simulated one-stage ATAD systems. Relative abundance was defined as the number of sequences affiliated with that OTU divided by the total number of sequences per sample. The relative abundance of phyla less than 1.0% of total composition in the three libraries was defined as "other".

that in group of pH 9.5. The phylum *Proteobacteria* was the dominant bacteria (77.6%) in group of pH 9.5, indicating the structure of microbial community had been totally changed under strong alkali condition when $Fe(NO_3)_3$ addition. That is why the diversity in group of pH 9.5 was higher comparing to that in group of pH 7.5, while the diversity in group of pH 7.5 was observed reduced comparing to that in the control. As seen in Fig. 6C, the composition of microbial community in group of pH 8.5 was much similar to that in the raw sludge, supporting the closet relationship of microbial communities between them as mentioned above. The abundance of phylum *Firmicutes* in digestion sludge (except for group of pH 9.5) had increased a lot comparing to that in the raw sludge, indicating a restricted phylum distribution into the *Firmicutes*, who played an important role for thermophilic aerobic degradation of waste sludge.[18,33] Especially in the group of pH 6.5, the abundance of phylum *Firmicutes* had reached as high as 96.6%, demonstrating the great impact on selection and reinforcement of bacteria phylotypes by ferric nitrate. Additionally, the increase of pH had an effect of inhibition to the phylum *Firmicutes* as the abundance of these bacteria phylotypes decreased in group of pH 7.5 as well. This found was opposite to the results revealed by Piterina et al.,[18] which should be due to the combined action of ferric nitrate and alkaline environment.

4 Conclusion

The addition of ferric nitrate at different pH values had distinct effects on stabilization of sewage sludge in one-stage ATAD system. The increase of pH when ferric nitrate added had a negative influence on promotion of stabilization by ferric nitrate, while a strong alkaline environment accelerated the hydrolysis and acidification, and even totally altered the structure of microbial community, which restored the digestion process. The optimal pH of ferric nitrate addition was 6.5, at which the abundance of phylum *Firmicutes* was significantly enhanced. However, the moderate increase of pH would inhibit the phylum *Firmicutes* and even caused failure in ATAD performance.

Acknowledgments

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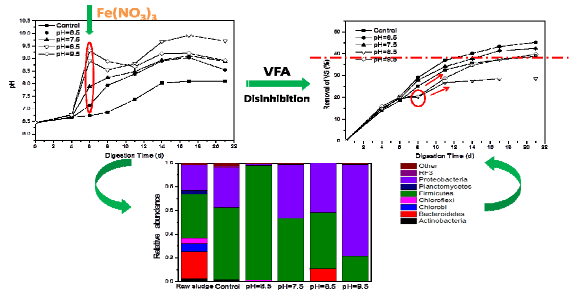
Graphical Abstract



Effects of ferric nitrate additions under different pH conditions on disinhibition of excessive VFAs for enhancement of ATAD performance