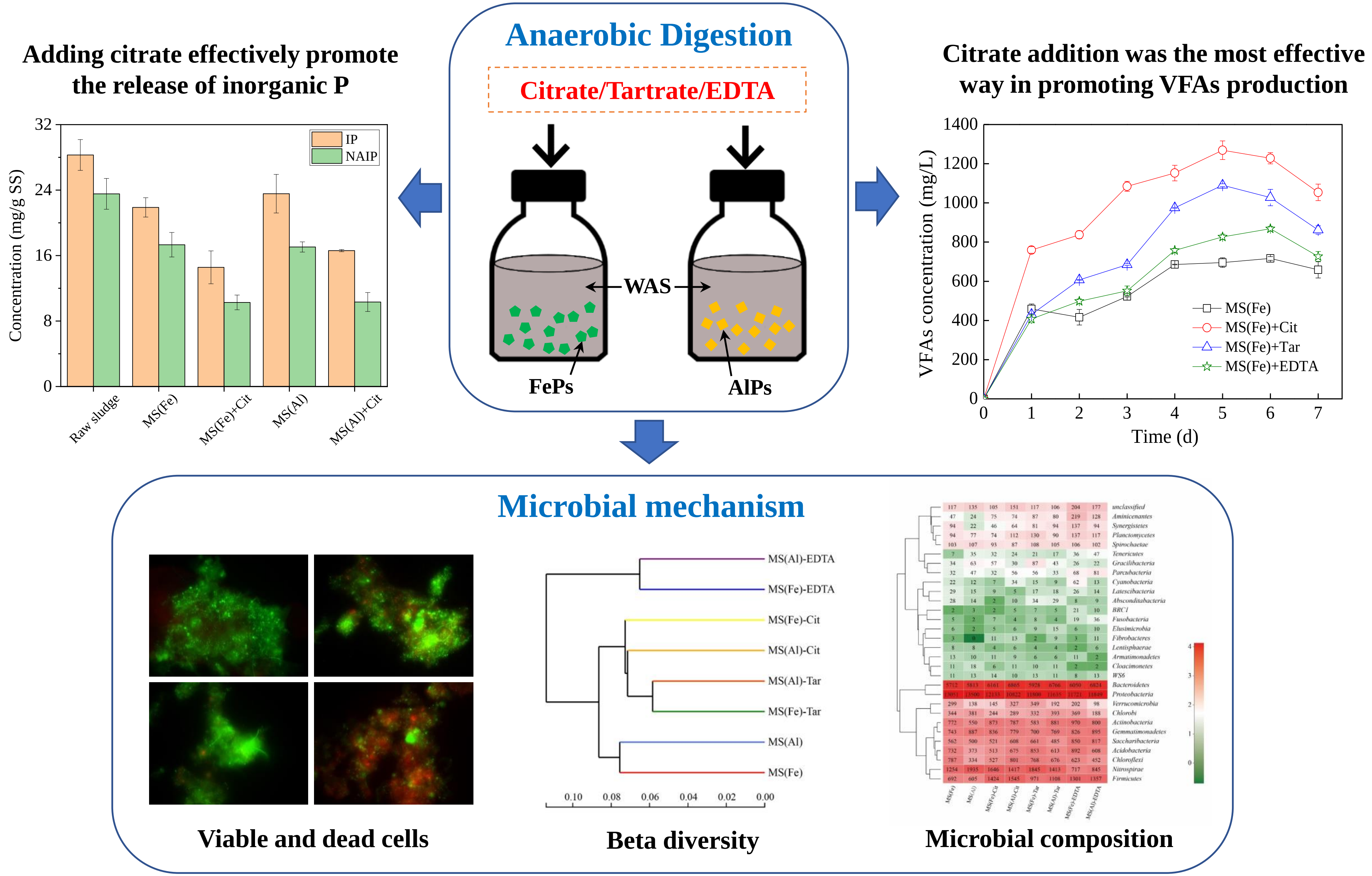
Graphical Abstract (for review)



***Highlights (for review)**

- Citrate addition can enhance P release from FePs and AlPs during fermentation.
- Citrate was most effective for VFAs promotion compared with tartrate and EDTA.
- Equimolar citrate addition to chemical precipitates was the optimal dosage.
- EDTA has the strongest inhibition on microbial activity and community structure.
- Correlations between complexing agents and microbial communities were analyzed.

Effect of complexing agents on phosphorus release from chemical-enhanced phosphorus removal sludge during anaerobic fermentation

Qian Ping [a, 1], Xiao Lu [a, 1], Yongmei Li [a, b, *], Giorgio Mannina [a, c]

[a] State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, P.R. China

[b] Shanghai Institute of Pollution Control and Ecological Security, Shanghai 200092, P.R. China

[c] Engineering Department, Palermo University, Viale delle Scienze, ed.8, 90128, Palermo, ITALY

[*] Corresponding author: E-mail: liyongmei@tongji.edu.cn Tel: +86 21 65982692

[1] These authors contributed equally to this work.

Abstract

Phosphorus (P) release from sludge containing phosphate precipitates (FePs or AlPs) as well as the anaerobic performance with the addition of complexing agents (citric, tartaric and EDTA) during ambient anaerobic fermentation process were investigated. Results showed that citrate addition was the most effective method to enhance P release from inorganic phosphate by chelation and promote volatile fatty acids (VFAs) production simultaneously during anaerobic fermentation. Equimolar citrate addition with chemical precipitates was the optimal dosage. Microbial analysis revealed that EDTA has the strongest inhibitory effect on microbial activity and community structure, while citrate was more effective in enhancing important acidifying microorganisms than tartrate and EDTA. Therefore, citrate addition can be regarded

as an alternative and promising method to recover P and carbon source from sludge containing chemical precipitates. These important discoveries will help to enrich P recovery path from sludge produced in the chemical-enhanced P removal treatment processes.

Keywords: Anaerobic fermentation; Waste activated sludge (WAS); Citrate; Complexing agent; Microbial community

1. Introduction

Phosphorus (P) plays a vital role in human life and industry, and is mainly extracted from nonrenewable rocks which were reported to be used up in the following 50-100 years (Cordell et al., 2009). With the continuous increase of population, the demand of P will be growing in the whole world. However, large amount of P is transferred into water during human activities, which is the main cause of eutrophication. It was reported that nearly all the P in wastewater was eventually transformed to the sludge during wastewater treatment processes (Van Vuuren et al., 2010). Therefore, waste activated sludge (WAS) can be regarded as a valuable P resource. P can be recovered from WAS and recycled as fertilizer or other valuable P products.

Nowadays, chemical-enhanced phosphorus removal (CEPR) treatment is widely used in wastewater treatment plants (WWTPs) (Wang et al., 2009; Wilfert et al., 2015; Wu et al., 2019). By dosing iron- or aluminum-based coagulants before or after biological process, P could be removed stably at a large range of concentration. However, CEPR process produces lots of chemical sludge that is difficult to be recycled. Commonly, P in WAS could be classified into organic phosphorus (OP) and inorganic phosphorus (IP). It was reported that non-apatite inorganic phosphorus (NAIP) was the major part of IP, which accounted for around 80% of IP (Medeiros et al., 2005). In addition, the percentage of IP including iron-phosphorus compounds (FePs) and aluminum-phosphorus compounds (AlPs) would raise up to more than 70% in WAS with CEPR process (Zhang et al., 2019). Therefore, if P in these chemical precipitates in WAS could be recovered, P recovery from WAS would be increased significantly.

Anaerobic fermentation is an environmental friendly method for WAS treatment that can not only effectively release P from biosolids but also covert many organic matters into energy-rich resources. However, it was reported that coagulants used in wastewater treatment made WAS less biodegradable (Dentel & Gossett, 1982). Compared with the sludge mainly containing biosolids, volatile fatty acids (VFAs) and methane produced by sludge containing large amount of FePs or AlPs precipitates during the anaerobic digestion were significantly decreased (Kim & Chung, 2015; Lin et al., 2017). On the other hand, P in FePs and AlPs was very hard to be released in anaerobic fermentation process (Wilfert et al., 2015). Several pretreatment technologies such as acidic treatment (Latif et al., 2015), alkaline treatment (Zhang & Li, 2014), microwave hybrid pretreatment (Wang et al., 2016), ultrasound coupled with oxidation pretreatment (Gong et al., 2015), thermal hydrolysis (Liu et al., 2019; Yu et al., 2017) have been reported to enhance P release effectively from sewage sludge. However, these methods aimed to promote sludge disintegration and P release from biosolids. There is still a lack of economic technology to release P from FePs or AlPs efficiently during anaerobic fermentation process (Liu et al., 2019). Therefore, it is necessary to find out a method that can not only improve the anaerobic performance of sludge but also release P from chemical precipitates.

Complexing agents are usually used in the treatment of solid wastes, sediments and soils contaminated with toxic metals. Yang et al. (2001) reported that the complexing agents can mobilize polyvalent metal ions, particularly Al and Fe from soil, and they can also enhance the release of many organic matters. In the field of alloy colloids, complexing agents can be used to synthesize nanoparticles (Lo et al., 2007; Zhou et al., 2006). Recent researches indicated that complexing agents can also leach P from incinerated sewage sludge ash (Fang et al., 2018) and effectively enhance the removal of bisphenol A in the CaO_2/Fe^{3+} system as well as improve the utilization rate of the Fe-sludge (Zhou et al., 2017). Zou et al. (2017) reported that the addition of EDTA enhanced P release significantly from FePs, AlPs and biosolids during mesophilic anaerobic fermentation. However, EDTA is costly and may cause environmental risk in the further disposal of treated sludge (Falciglia et al., 2016). Therefore, it is imperative to search for an environmentally friendly and economically viable alternative. Citric and tartaric acids are environmentally friendly chelating ligands of polyvalent ions, and they exhibit excellent biodegradability and are harmless to microorganisms (Yang et al., 2001). Furthermore, citrate is also an important intermediate in citric acid cycle in biological metabolism (Milosev & Strehblow, 2015). Therefore, organic acids such as citric and tartaric acids might be the excellent complexing agents to enhance P release and VFAs production during anaerobic fermentation of WAS.

This study presents an innovative technology for P release from phosphate precipitates and promote anaerobic performance of WAS simultaneously. Citric, tartaric acids and EDTA were used to enhance P release from sludge containing phosphate precipitates (FePs and AlPs) as well as the anaerobic performance. Their effects were compared. Especially, beta diversities, microbial community and the influence of environmental factors on microbes were completely identified.

2. Materials and methods

2.1 Chemicals

Trisodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$, analytical reagent, ≥99%), disodium tartrate dihydrate ($C_4H_4O_6Na_2{\cdot}2H_2O$, analytical reagent, ≥99%), ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA-2Na, $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, analytical reagent, ≥99%), $FePO_4{\cdot}4H_2O$ (chemical pure, ≥98.0%), $AlPO_4$ (chemical pure, 85±5%) were all obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Source of WAS

The WAS was acquired from a secondary sedimentation tank in a WWTP located in Shanghai, China. The anaerobic-anoxic-oxic activated sludge process was used to treat municipal wastewater in this plant. Chemical flocculants were not added for P removal during the treatment process. A 1 mm × 1 mm screen was used to filter the retrieved sludge, and then the sludge was concentrated by settling for about 24 h at 4°C. The main characteristics (average data plus standard deviations of triplicate analysis) were as follows: pH 6.7±0.2, total suspended solid (TSS) 14.4±1.3 g/L, volatile suspended solid (VSS) 7.5±0.9 g/L, total phosphorus (TP) 31.8±1.1 mg/g VSS. Raw sludge was denoted as biological sludge (BS), $FePO_4 \cdot 4H_2O$ or $AlPO_4$ was added to BS to form the mixed sludge containing FePs [MS(Fe)] or AlPs [MS(Al)] precipitates.

2.3 Batch anaerobic fermentation experiments

Identical serum bottles (V=600 mL) were used to investigated the effect of complexing agents on P release from FePs, AlPs and biosolids during anaerobic fermentation. 500 mL of WAS was dosed in each bottle, and then $FePO_4 \cdot 4H_2O$ and $AlPO_4$ were added (Zou et al., 2017). The dosage of $FePO_4 \cdot 4H_2O$ in MS(Fe) was 1338 mg/L, while the dosage of $AlPO_4$ in MS(Al) was 732 mg/L. They were all equivalent to 186 mg P/L (6 mM-P). Citrate, tartrate and EDTA were added according to the molar ratio of phosphate precipitation (the molar ratio of complexing agent to phosphate precipitate was 1.5:1), which were 2647 mg/L, 2071 mg/L and 3350 mg/L (equivalent to 9 mM), respectively. The control tests were performed without addition of any complexing agent in each set of tests. In order to further investigate the effect of citrate dosage, 1764 mg/L, 2647 mg/L and 5294 mg/L citrate were added into the sludge, with the molar ratio of citrate to phosphate precipitate being 1:1, 1.5:1 and 3:1, respectively. Nitrogen gas was used to remove oxygen by purging all the serum bottles for 2 min. Then the bottles were immediately capped with rubber stoppers. Then they were placed in an air-bath shaker (120 rpm) under ambient condition (25±1°C) for 7 d anaerobic fermentation. The sludge was sampled every day and centrifuged for 15 min at 8000 rpm to acquire the supernatant. Chemical analysis was conducted after filtering the supernatant using 0.45 μm cellulose membranes. The sludge samples were also saved for microbial community analysis and live/dead staining analysis. The batch experiments were carried out in triplicate and all data were expressed as mean ± standard deviation.

2.3 Analytical methods

TSS, VSS, PO_4^{3-}-P and TP were analyzed according to the Standard Methods (APHA, 2012). The Standards, Measurements and Testing programme (SMT) was used to determine phosphorus fractions of IP and NAIP (Ruban et al., 2001). The extracellular polymeric substances (EPS) was extracted according to the study of Niu et al. (2013), including slime EPS (S-EPS), loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). Polysaccharides were measured using the phenol-sulfuric acid method (Gerhardt et al., 1994), and proteins were determined by the modified Lowry method (Frølund et al., 1996). Liquid chromatography coupled to mass spectrometry (LC-MS, Agilent 1290, USA) was used to measure citrate. Soluble total organic carbon (STOC) was measured using a total organic carbon analyzer (TOC-VCPH, Shimadzu, Japan). The determination of VFAs concentration was according to Li et al. (2015) with a gas chromatograph (Agilent 6890, USA). A LIVE/DEAD® BacLight™ bacterial viability kit (Invitrogen, USA) was used to determine the viable and dead cells in the sludge. The principle and procedure was according to Zou and Li (2016).

P release efficiency (P_e) from FePs or AlPs was calculated according to Eq.(1).

$$P_e=\frac{P_M-P_B}{P_S}\times 100\% \quad (1)$$

where P_M and P_B were the PO_4^{3-}-P concentrations in supernatant of MS samples (MS(Fe) or MS(Al)) and BS sample, respectively; P_S was the initial dosed P content (FePs or AlPs).

2.4 Microbial community characterization

2.4.1 DNA extraction, PCR and Illumina MiSeq sequencing

Microbial DNA was extracted from sludge using the E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, USA) according to manufacturer's protocols, and then pooled together. The primers for bacteria were 338F (5’- ACTCCTACGGGAGGCAGCA-3´) and 806R (5´-GGACTACHVGGGTWTCTAAT-3´). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform according to the standard protocols.

2.4.2 Processing of sequencing data

Operational Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (http://drive5.com/uparse/). The reads which could not be assembled were discarded. The UPGMA (Unweighted Pair-group Method with Arithmetic Mean) was conducted to calculate the distance matrix using QIIME (http://qiime.org) to obtain Beta diversity. The hierarchical clustering analysis was conducted using R software to form the visualized tree structure. The Pearson's correlation coefficient was calculated to investigate the correlation between individual microorganisms and environmental factors according to Ping et al. (2018).

3. Results and discussion

3.1 Effects of different complexing agents

3.1.1 P release during anaerobic fermentation

P release during the anaerobic fermentation process of MS(Fe) or MS(Al) with the addition of different complexing agents was shown in Fig.1. P concentrations were all increased with the addition of complexing agent. However, the improvement of P release was significantly dependent on complexing agent type. It shows a continuous release of P with the addition of citrate or tartrate during the test time, which was similar to the trend in the control tests. The increase in P concentration was minimal with the addition of tartrate, which has average increases of 25.1% and 10.4% during the anaerobic fermentation process of MS(Fe) and MS(Al), respectively, compared with the control tests. Greater improvement was achieved with the addition of citrate than tartrate. Compared with the control tests, the average increases in P concentration in MS(Fe) and MS(Al) were 50.9% and 28.1%, respectively. It has been reported that citrate had good metal ion chelating ability of Fe and Al (Yang et al., 2001), therefore releasing more P from sludge to the supernatant. When adding EDTA, large amount of

P was released from the sludges in the initial two days, and it was essentially unchanged after that. Compared with the control test, the P releases were improved by 497.8% and 627.8% with EDTA addition during the anaerobic fermentation process of MS(Fe) and MS(Al), respectively. This was because EDTA offered greater complex ability than citrate and tartrate (Juang et al., 2003), therefore causing strong complexation of metal ions (Zou et al., 2017). However, it may also greatly damage the anaerobic microorganisms simultaneously, which will be discussed in the following part. In addition, Fig.1(A) and Fig.1(B) show that P concentrations in the anaerobic fermentation process of MS(Fe) were all higher than those in the anaerobic fermentation of MS(Al). This was due to a much stronger bonding of P with the Al-based precipitates than with the Fe-based precipitates (Lin et al., 2017). Moreover, Fe transformation such as reduction of Fe(III) to Fe(II) during anaerobic fermentation would lead to the disintegration of sludge flocs and dissolution of FePs (Johnson et al., 2003; Lin et al., 2017).

It was known that IP could contribute more than 70% of the total phosphorus of activated sludge during chemical enhanced process (Zhang et al., 2019), and NAIP was the main component of IP (Medeiros et al., 2005). Liu et al. (2019) reported that only small increases of P (14.4-17.6 mg/L) were observed during the hydrolysis and acidification stages of WAS containing high NAIP with ultrasound sonication pretreatment, illustrating that IP was difficult to solubilize from the sludge. The concentrations of IP and NAIP in raw sludge and digested sludge (with or without citrate) were shown in Fig.1(C). The concentration of IP in MS(Fe) and MS(Al) decreased by 33% and 29% after adding citrate, respectively; and the decrease of NAIP was more obvious, which were up to 40.7% and 41.2%, respectively. This further proved that adding citrate to the sludge containing phosphate sediment can effectively promote the release of inorganic phosphorus such as FePs and AlPs.

3.1.2 Anaerobic fermentation performance

The variations of STOC and VFAs concentrations with the addition of different complexing agents during the anaerobic fermentation process of MS(Fe) or MS(Al) are shown in Fig.2. After 7-days fermentation of MS(Fe) and MS(Al), the concentration of STOC reached 901 mg/L and 981 mg/L with the addition of citrate, respectively. The theoretical concentration of STOC contributed from citrate was 648 mg/L, while the STOC concentrations of the control tests were 421 mg/L and 448 mg/L on day 7, respectively, during the anaerobic fermentation process of MS(Fe) and MS(Al). Therefore, the theoretical concentrations of STOC with addition of citrate (1069 mg/L and 1096 mg/L for MS(Fe) and MS(Al)) were higher than the experimental values, indicating that about 168 mg/L and 115 mg/L STOC contributed by citrate were utilized as substrate by the microorganisms during anaerobic fermentation. The same phenomenon also occurred with the addition of tartrate, and the STOC contributed by tartrate during the anaerobic fermentation of MS(Fe) and MS(Al) were 216 mg/L and 141 mg/L, respectively. Citrate and tartrate are organic acids with innocuous nature (Fang et al., 2018; Yang et al., 2001), so they can be used as substrate by microorganisms. In addition, metal ion chelation with citrate and tartrate could disrupt organic-mineral linkages, resulting in mobilization of organic compounds so that bacteria can degrade the organics more quickly (Yang et al., 2001). It can be seen from Fig.2(A) and Fig.2(B) that the STOC concentration was the highest with the addition of EDTA. This might because it can not only remove the extracellular polymeric substances (EPS) of activated sludge (Kavitha et al., 2016), but also release the intracellular substances due to the greatly increase of dead cells (Zou et al., 2017).

Although more complex agent was utilized as substrate in tartrate addition tests than in citrate addition tests, the improvement of VFAs was the greatest with the addition of citrate. During the anaerobic fermentation of MS(Fe) and MS(Al) with citrate addition, the highest production of VFAs were 1269mg/L and 1475 mg/L, respectively, which were 1.77 and 1.85 times higher than that in the control tests. The promotion efficiencies were higher than the other treatment methods such as ultrasound sonication and thermal hydrolysis when releasing P from WAS containing high inorganic P content during anaerobic fermentation (Liu et al., 2019). In tartrate addition tests, the highest VFAs concentrations during anaerobic fermentation of MS(Fe) and MS(Al) were 1.52 and 1.51 times greater than the control tests, respectively. Citrate addition could promote fermentation performance of MS(Al) more remarkably than that of MS(Fe). It was reported that the bonding of Al-based sludge was stronger due to charge neutralization and chain-bridging (Lin et al., 2017).

However, citrate can be used as an effective additive chemical for P release and VFAs production simultaneously, especially for sludge containing Al precipitates. In addition, there was only a slight improvement of VFAs production with the addition of EDTA in both sludge samples, which was contradictory with the high concentration of STOC. It was apparent that the conversion of VFAs from STOC during fermentation was significantly inhibited by EDTA addition. VFAs were the intermediary products and were mainly produced by acidogenic and acetogenic bacterial populations (Franke-Whittle et al., 2014; Zhao & Ruan, 2013). Although lots of P and STOC can be released with EDTA addition, the microbial activity was significantly reduced during anaerobic fermentation of the sludge. Therefore, the addition of citrate was the most effective way in promoting the production of VFAs, indicating that the ambient operating condition with citrate addition created an environment that is more favorable to the growth and intense activity of acidogenic and acetogenic microorganisms.

3.2 Effect of citrate dosage on P release and VFAs production

In order to further clarify the effect of citrate on chemical-enhanced sludge, different dosages of citrate were added during the anaerobic fermentation of MS(Fe) and BS. As shown in Fig.3(A), P was mainly released on the first day with slight change in the following days during the anaerobic fermentation process of BS. The addition of citrate has little effect on P release from BS regardless of citrate dosage. With citrate dosage of 0, 6, 9 and 18 mM, P concentration on day 7 were 104, 107, 110 and 118 mg/L, respectively. However, continuous P release was observed during the anaerobic fermentation of MS(Fe), and the effect of citrate addition on P release became obvious. Accordingly, it can be concluded that the improved P release was mainly released from chemical phosphorus precipitates ($FePO_4$) by the chelation of citrate rather than organic phosphorus in microorganisms during the anaerobic fermentation of MS(Fe). In addition, the concentration of P was increased by 17.4% after 7 days fermentation with citrate dosage of 6 mM compared with the control tests. When the citrate dosage was increased to 9 mM and 18 mM, the release efficiency of P was not enhanced as expected. The concentration of P was increased by 17.0% and 22.9% with citrate dosages of 9 mM and 18 mM, respectively. In addition, P release from FePs was not improved with the increase of citrate dosage. P release efficiencies were 33%, 31% and 32%, respectively, with citrate dosages of 6 mM, 9 mM and 18 mM. When citrate dosages were 6 mM, 9 mM and 18 mM, the molar ratios of citrate to $FePO_4$ were 1:1, 1:1.5 and 1:3, respectively. Juang et al. (2003) mentioned that some complexing agents readily form stable complexes with most divalent metal ions in a 1:1 molar ratio. This study illustrated that equimolar citrate addition to FePs was sufficient for the chelating reaction. Accordingly, the optimal dosage of citrate was 6 mM (the molar ratio of citrate to $FePO_4$ was 1:1), and the efficiency of P release can not be further improved with higher dosage of citrate.

The variations of VFAs concentration with different dosages of citrate during the anaerobic fermentation process of BS and MS(Fe) are shown in Fig.3(B). As the dosage of citrate increasing, the VFAs concentration was greatly improved in both BS and MS(Fe) fermentations. Citrate could disrupt the organic-mineral linkages so that bacteria can degrade the organics more quickly (Yang et al., 2001). In addition, about half of citrate can be used as carbon source by microorganisms to improve hydrolysis and acidification. Moreover, it could also effectively enhance the acidogenic and acetogenic bacterial populations, which will be discussed in the following part. Therefore, higher dosage of citrate subsequently improved the production of VFAs. It can be seen from Fig.3(B) that VFAs concentrations in BS sample were all slightly higher than those in MS(Fe) samples with the same dosage of citrate during the anaerobic fermentation process. Citrate could react with the metals in MS(Fe) preferentially, resulting in a reduction amount of citrate which can be utilized by the acidifying microorganisms. However, the VFAs concentrations were still much higher than those in the control test of MS(Fe). At the citrate dosages of 6 mM, 9 mM and 18 mM, VFAs concentrations after 7-day fermentation of MS(Fe) were 1.4, 1.6 and 2.4 times higher than the control test, respectively. As aforementioned, citrate can be degraded by microorganisms as substrate and it is also an important intermediate in citric acid cycle of biological metabolism (Milosev & Strehblow, 2015), while citric acid cycle was closely related to VFAs biosynthesis pathway. In addition, the fraction of polysaccharide and protein in S-EPS and LB-EPS greatly increased with citrate addition (data was shown in Supplementary Material), demonstrating that citrate addition effectively enhanced the release of polysaccharide and protein embedded originally in EPS from the inner fraction to the outer fraction. So the organic-linkages can be disrupt by citrate and the contact between organics and microorganisms was also significantly facilitated. Therefore, higher citrate addition resulted in higher VFAs production during 7-day anaerobic fermentation of MS(Fe).

3.3 Microbial analysis

3.3.1 Viable and dead cells

Viable and dead cells of BS, MS(Fe) and MS(Al) with different complex agents addition were investigated (Figures were shown in Supplementary Material). The percentage of dead cells was 20% in BS, while it increased to 27% and 39% in MS(Fe) and MS(Al), respectively, without addition of any complexing agent. This might due to the inhibition effect of chemical precipitates on microorganisms (Lin et al., 2017). The percentages of dead cells were all increased with the addition of three complexing agents, indicating that the complexing agents could ruin cell structures. However, the destructive effect was obviously different with different kind of complexing agents. The dead cells percentage was in the range of 35%-56% when adding citrate and tartrate in the three sludge samples. It was apparent that the fluorescent area with red color was significantly expanded with the addition of EDTA, and the percentages of dead cells were all around 70% in all sludge samples. This led to the great dissolution of intracellular substances to the supernatant, which was in accordance with the SCOD and VFAs concentrations in the previous section. On the other hand, it also significantly inhibited the activity of microorganisms. Therefore, EDTA has the strongest inhibitory effect on microorganisms.

3.3.2 Diversity of microbial communities

In the microbial community analysis, 292244 high-quality pyrosequencing reads were generated totally, with an average read length of 442 bp. The reads number for bacteria in samples ranged from 30369 to 44562, so 30369 reads were selected randomly in each sample for further analysis during the subsampling. The rarefaction curves (at 97% sequence similarity) from all samples are shown in Supplementary Material. All the curves became plateaus, illustrating that the amount of pyrosequencing reads was sufficient to explain most of OTUs in the sludge samples (Poirier et al., 2017).

In order to study the response of microbial species to environmental heterogeneity, beta diversity analysis was adopted to compare the diversity among different ecosystems. The greater beta diversity occurred with less common species between different communities or different points on an environmental gradient (Liu et al., 2016; Lou, 2007). Beta diversity of microbial structures with the addition of different complexing agents based on OTUs level was presented as a dendogram in Fig.4(A), and their distance matrix is shown in Table 1. Sludge containing different phosphate precipitates with same complexing agent addition was in the same cluster, indicating that their microbial community was relatively similar. In addition, significant difference was observed when adding different complexing agents to the same sludge. Sludge samples with the addition of citrate and tartrate were close in clusters, while they were far from raw sludges (MS(Fe) and MS(Al)), indicating that the microbial structure and communities were changed with citrate or tartrate addition. Furthermore, samples with the addition of EDTA were far from the others, illustrating that the microbial structure were greatly changed. This also suggests a more remarkable influence of EDTA than citrate and tartrate on microbial community structure during the anaerobic fermentation of MS(Fe) and MS(Al).

3.3.3 Microbial composition

The heatmap of microbial relative abundance (top 30) at phylum level of sludge samples after anaerobic fermentation is shown in Fig.4(B). It was apparent that the relative abundances of *Bacteroidetes* and *Firmicutes* were greatly improved with different kind of complexing agents, and promotion efficiency was in the order of citrate > EDTA > tartrate. It was reported that *Bacteroidetes* and *Firmicutes* play important roles in hydrolysis and acidification, for example, degrading various organic matters such as polysaccharides and protein to produce VFAs (Watanabe et al., 2017; Yi et al., 2014). Furthermore, *Firmicutes* was reported to enrich and grow at a rapid multiplication rate in an environment with abundant soluble organic matters (Kabisch et al., 2014). Due to the substrate availability and moderate effect of microorganisms, citrate was the most excellent agent in promoting acidifying microorganisms. In addition, *Proteobacteria* was the most abundant phylum and it widely existed in WWTPs and AD process (Ariesyady et al., 2007; Yang et al., 2014). It was reported that *Proteobacteria* was one of the important consumers of VFAs, such as propionate, butyrate or acetate (Ariesyady et al., 2007). The relative abundance of *Proteobacteria* was greatly decreased with the addition of citrate, therefore more VFAs can be effectively accumulated with citrate addition.

Bacterial communities determined by the family (Fig.5(A)) and genus (Fig.5(B)) levels were further analyzed by comparing the bacterial populations among the samples. The relative abundance of family *Nannocystaceae* and genus *Nannocystis* were significantly increased with the addition of citrate, especially in the sludge containing FePs. It was reported that *Nannocystaceae* could synthesize iron chelating agents (Kunze et al., 1992). This may be a possible mechanism for P release from sludge containing iron phosphate precipitates with citrate addition. It can be seen from Fig.5(A) that *Rhodocyclaceae* was sensitive to the addition of complexing agents, and the relative abundance was decrease obviously regardless of the agent type. It has been reported that genus *Ferribacterium* was affiliated to family *Rhodocyclaceae,* which can oxidize acetate and lactate with ferric iron as the electron acceptor (Brenner et al., 2005; Oren, 2014). The relative abundance of *Ferribacterium* was also greatly decrease with the addition of complexing agent (Fig.5(B)), suggesting that the release of P in sewage sludge containing phosphate precipitates was not accounting on the reduction of ferric precipitates.

In addition, the Pearson's correlation was also conducted between complexing agents and microorganisms at genus level (Fig.6). *Trichococcus and Terrimonas* show significant positive correlation with citrate addition, which means these genera can be effectively enriched after citrate addition. They are all important acidifying bacteria to hydrolyse a wide range of substrates for VFAs production (Holzapfel & Wood, 2014; Scheff et al., 1984). Although citrate and tartrate both had positive correlations with these genera, higher value of the Pearson's correlation coefficient (*Trichococcus*: 0.76 versus 0.25, *Terrimonas:* 0.50 versus 0.38) was found with citrate addition. This means that the addition of citrate was more effective in enhancing important acidifying microorganisms than tartrate. In addition, there were significant negative correlations between genera *Ferruginibacter, Flavobacterium, Terrimonas* and EDTA addition, indicating that acetogenesis process was tremendously inhibited with EDTA addition. Consequently, anaerobic fermentation for VFAs production and P release process could be separated when using EDTA as treatment agent in the future study. Therefore among the three complexing agents, adding citrate was the most effective method to promote acidifying microorganisms in sludges.

4. Conclusions

Citrate addition was the most effectively method to enhance P release from inorganic phosphate by chelation and promote VFAs production simultaneously during ambient anaerobic fermentation of sludge containing FePs and AlPs. Equimolar citrate addition with chemical precipitates was the optimal dosage. EDTA has the strongest inhibitory effect on the microbial activity; it also has a more remarkable influence than citrate and tartrate on the microbial community structure. Citrate was more effective in enhancing important acidifying microorganisms than tartrate and EDTA. Further study should focus on the effect of citrate on CEPR sludges from different WWTPs with economic analysis.

Appendix A. Supplementary data

E-supplementary data of this work can be found in online version of the paper.

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Figure captions

Fig.1 Concentrations of PO_4^{3-}-P in the supernatant during anaerobic fermentation of (A) MS(Fe) and (B) MS(Al) with the addition of different complexing agents as well as concentrations of inorganic phosphorus (IP) and non-apatite inorganic phosphorus (NAIP) in sludges before (Raw sludge) and after (MS(Fe), MS(Fe)+Cit, MS(Al), MS(Al)+Cit) anaerobic fermentation (C)

Fig.2 The variations of STOC (A, B) and VFAs (C, D) concentrations with the addition of different complexing agents during the anaerobic digestion process of MS(Fe) (A, C) and MS(Al) (B, D)

Fig.3 Concentrations of PO_4^{3-}-P (A) and VFAs (B) during the anaerobic fermentation of BS and MS(Fe) with citrate addition at different dosages

Fig.4 Cluster dendrogram based on OTUs level (A) and heatmap of microbial relative abundance (top 30) at phylum level (B) of sludge samples with different complexing agents addition after anaerobic fermentation

Fig.5 Relative abundance of microbial community at family level (A) and genus level (B) among all samples after anaerobic fermentation

Fig.6 Heatmap for the effect of environmental factors on microorganisms at genus level among all samples after anaerobic fermentation

Figure 1
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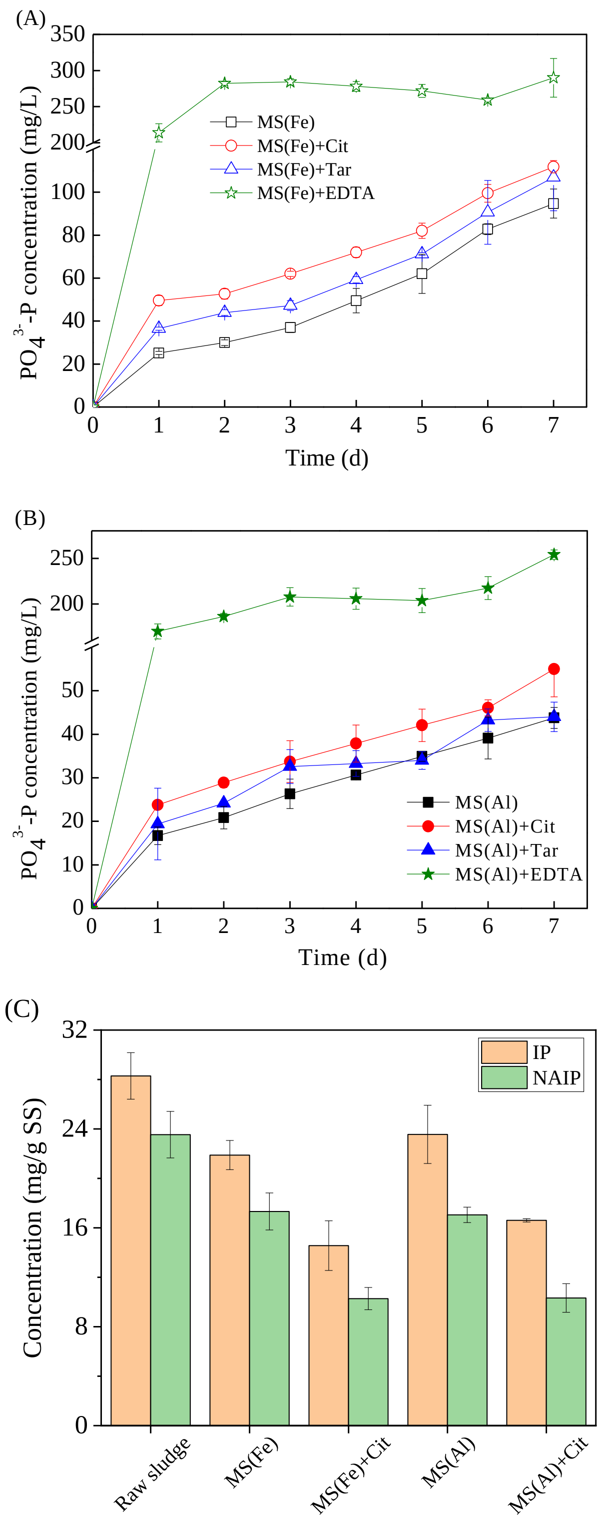

Fig.1 Concentrations of PO_4^{3-}-P in the supernatant during anaerobic fermentation of (A) MS(Fe) and (B) MS(Al) with the addition of different complexing agents as well as concentrations of inorganic phosphorus (IP) and non-apatite inorganic phosphorus (NAIP) in sludges before (Raw sludge) and after (MS(Fe), MS(Fe)+Cit, MS(Al), MS(Al)+Cit) anaerobic fermentation (C)

Figure 2

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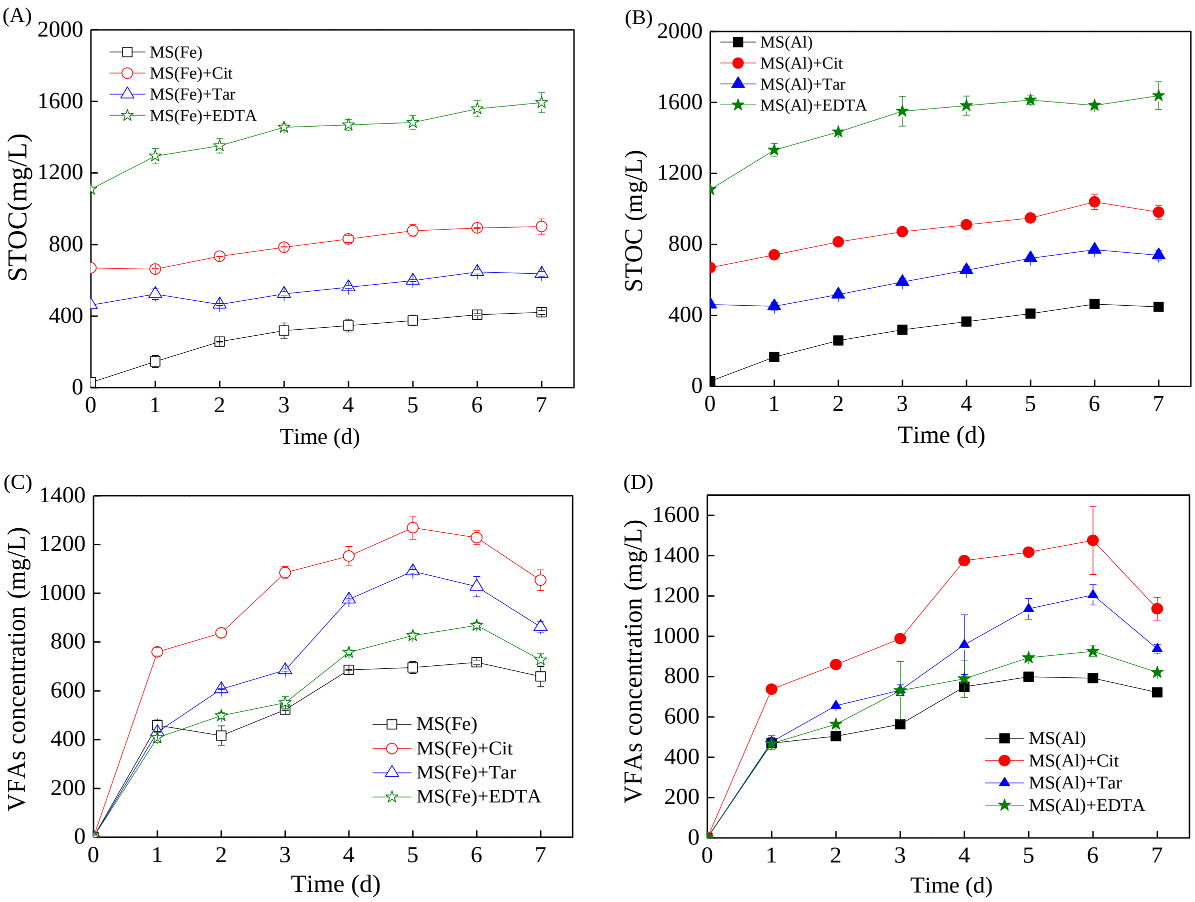

Fig.2 The variations of STOC (A, B) and VFAs (C, D) concentrations with the addition of different complexing agents during the anaerobic digestion process of MS(Fe) (A, C) and MS(Al) (B, D)

Figure 3

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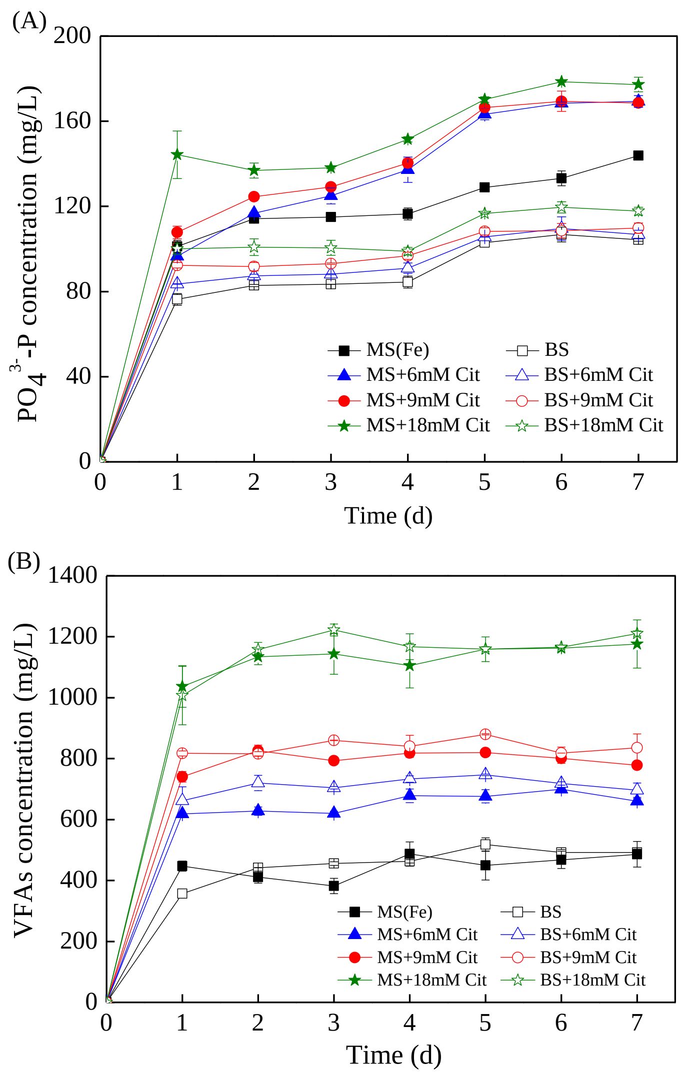

Fig.3 Concentrations of PO_4^{3-}-P (A) and VFAs (B) during the anaerobic fermentation of BS and MS(Fe) with citrate addition at different dosages

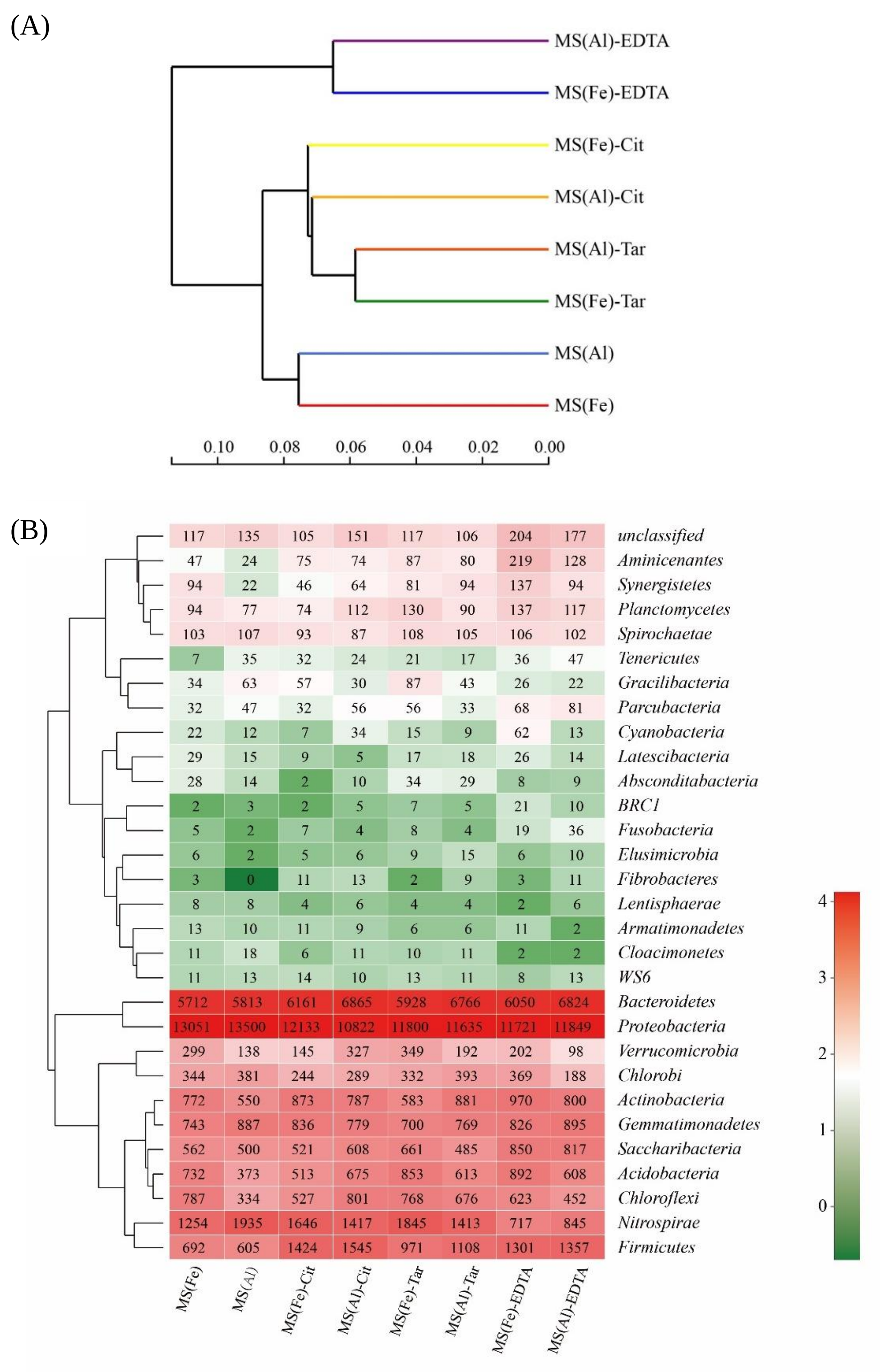

Fig.4 Cluster dendrogram based on OTUs level (A) and heatmap of microbial relative abundance (top 30) at phylum level (B) of sludge samples with different complexing agents addition after anaerobic fermentation

Figure 5
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(A)

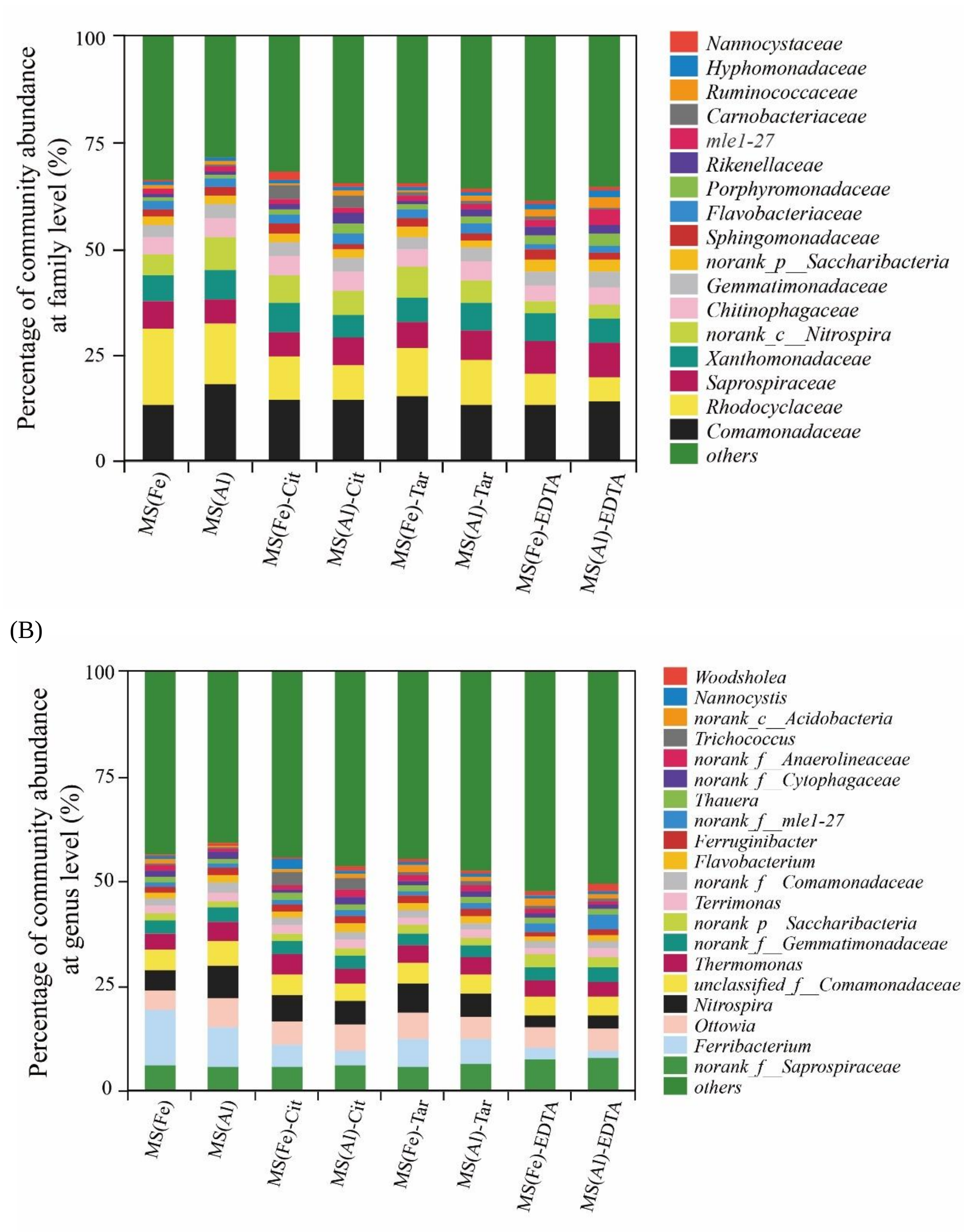

Fig.5 Relative abundance of microbial community at family level (A) and genus level (B) among all samples after anaerobic fermentation

Figure 6

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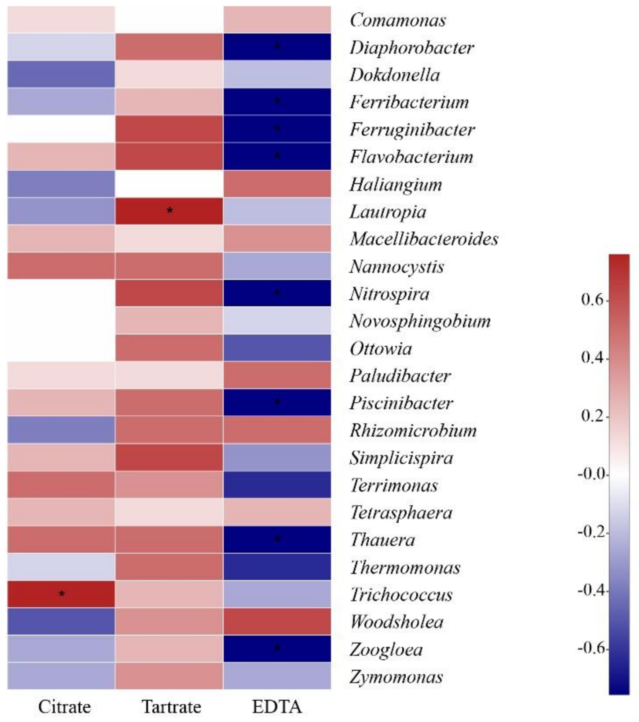

Fig.6 Heatmap for the effect of environmental factors on microorganisms at genus level among all samples after anaerobic fermentation

Table 1

Table 1 Distance matrix of cluster dendrogram based on OTUs level with the addition of different complexing agents after anaerobic fermentation

	MS(Al)	MS(Al)-Tar	MS(Fe)	MS(Al)-EDTA	MS(Al)-Cit	MS(Fe)-EDTA	MS(Fe)-Cit	MS(Fe)-Tar
MS(Al)	0	0.16	0.15	0.27	0.21	0.26	0.19	0.14
MS(Al)-Tar	0.16	0	0.15	0.21	0.13	0.19	0.14	0.12
MS(Fe)	0.15	0.15	0	0.26	0.21	0.23	0.19	0.14
MS(Al)-EDTA	0.27	0.21	0.26	0	0.20	0.13	0.23	0.24
MS(Al)-Cit	0.21	0.13	0.21	0.20	0	0.19	0.15	0.15
MS(Fe)-EDTA	0.26	0.19	0.23	0.13	0.19	0	0.22	0.21
MS(Fe)-Cit	0.19	0.14	0.19	0.23	0.15	0.22	0	0.15
MS(Fe)-Tar	0.14	0.12	0.14	0.24	0.15	0.21	0.15	0

***Author Contributions Section**

CRediT author statement

Qian Ping: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft; **Xiao Lu:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing-Original Draft; **Yongmei Li:** Writing - Reviewing and Editing, Supervision, Project administration, Funding acquisition; **Giorgio Mannina:** Writing - Reviewing and Editing

***Declaration of Interest Statement**

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: