Abstract

BACKGROUND: Three lab-scale sequencing batch reactors were used to investigate the effects of Fe$^{3+}$ on aerobic granular sludge (AGS) formation, nutrient removal, and microbial community.

RESULTS: The addition of 6 and 12 mg Fe$^{3+}$/L could not shorten the granulation time. However, compared to the reactor without Fe$^{3+}$ addition (average sludge volume index at 30 min (SVI$_{30}$): 70.8 mL/g; stable average particle size: 548 μm), the addition of 12 mg Fe$^{3+}$/L helped improve the physical properties of AGS (average SVI$_{30}$: 57.0
mL/g; stable average particle size: 1067 μm). Furthermore, with 12 mg Fe\(^{3+}\)/L addition (Fe\(^{3+}\) to PO\(_4^{3-}\)-P molar ratio = 1.33), good removals of NH\(_4^+\)-N (≤ 0.5 mg/L) and PO\(_4^{3-}\)-P (< 1 mg/L) were achieved. The addition of Fe\(^{3+}\) reduced the microbial diversity and specific activity of heterotrophic bacteria, but promoted the growth of nitrite-oxidizing bacteria. The growth of particle size not only increased the active biomass ratio and microbial diversity, but alleviated the inhibition effect of Fe\(^{3+}\) on the specific heterotrophic bacteria activity.

CONCLUSION: The addition of Fe\(^{3+}\) had both negative and positive effects on the formation and stability of AGS. The results will help enrich the understanding of AGS application in nutrient especially phosphorus removal.

Keywords: Aerobic granular sludge; ferric iron; phosphorus and nitrogen removal; microbial community; microbial activity

INTRODUCTION

Aerobic granular sludge (AGS) is a promising technology that has attracted lots of attention from researchers due to its excellent settling property, high biomass retention, small footprint, and potential to remove nitrogen and phosphorus (P) simultaneously (Wu et al., 2012; Nancharaiah and Reddy, 2018; Zou et al., 2018).\(^1\)\(^-\)\(^3\) Extensive works have been conducted to better understand the mechanisms of AGS formation and stability.\(^3\)\(^-\)\(^6\) The crystalline nuclei and selection pressure are widely regarded as the two main factors affecting sludge granulation.\(^2\)\(^,\)\(^7\) Extracellular polymeric substances (EPS) are also considered to play an important role in AGS formation and stability.\(^8\)\(^,\)\(^9\) In addition, with more and more stringent requirement for
nutrient discharge from wastewater treatment plants (WWTPs), P removal are usually achieved by combining chemical and biological methods. Ferric salt is one of the most common chemical flocculants used in WWTPs to remove P.10 Recently, some full-scale applications of AGS around the world have been reported.11,12 Therefore, it is meaningful to study the effects of ferric ion (Fe$^{3+}$) on AGS formation, nutrient removal, and microbial community.

Generally, Fe$^{3+}$ added to the mixed liquor will react with phosphate and hydroxide ions to form insoluble ferric phosphate precipitates and hydroxide complexes which will subsequently assist in P removal by adsorption and co-precipitation.13 The ferric phosphate precipitates and ferric hydroxide complexes are reported to have poor settleability, which will result in an unfavourable settling velocity for the activated sludge.14,15 The decline of sludge settleability due to the addition of Fe$^{3+}$ may adversely affect the sludge granulation process. However, on the other hand, metal ions can stimulate sludge granulation by neutralizing the negative cell surface charge, polymer bridging of EPS, forming precipitates onto which cells can aggregate.8,16,17 Therefore, the actual effect of Fe$^{3+}$ on AGS formation needs to be studied deeply. Recently, Kończak et al.16 studied the synergetic effect of Ca$^{2+}$ (5 mg/L), Mg$^{2+}$ (3 mg/L) and Fe$^{3+}$ (4.2 mg/L) addition on the formation and structure of AGS, but the effect of Fe$^{3+}$ was not deeply investigated. Their study also indicated that Fe$^{3+}$ promoted the secretion of protein (PN) and could bind with EPS similarly to divalent cations.16 By adding Fe$^{2+}$ (1 and 10 mg/L) to the feed solution and reactor, Yilmaz et al.18 noted that iron ions (Fe$^{2+}$/Fe$^{3+}$) increased the size and stability of AGS, but did
not affect the granulation time. In activated sludge systems, Liu et al.\textsuperscript{19} found that the effects of Fe\textsuperscript{3+} and Fe\textsuperscript{2+} on biological P removal and nitrification were weak, and these iron salts inhibited P removal only when the dose was large enough (> 40mg/L). However, Liu and Horn\textsuperscript{20} reported that nitrogen removal was evidently deteriorated during the deammonification process when Fe\textsuperscript{3+} concentrations were increased from 0.75 to 2.19 mg/L. To the best of our knowledge, studies related the impacts of Fe\textsuperscript{3+} on AGS formation and performance are still very limited.

This study aims to investigate the effects of Fe\textsuperscript{3+} on AGS formation, nutrient removal, and microbial community. Two levels of Fe\textsuperscript{3+} (6 and 12 mg/L) were tested in the sequencing batch reactors (SBRs). Biomass concentration, sludge settleability, particle size, EPS content, chemical precipitates content, reactor performance, microbial activity, and community were monitored during the experimental period. The results of this study will help enrich the understanding of AGS application in nutrient especially P removal.

\textbf{MATERIALS AND METHODS}

\textbf{Reactor setup and operation}

Three identical plexiglas SBRs (R1, R2, and R3) with the internal diameter of 10 cm and the effective height to diameter ratio of 8.9 were used. The effective working volume of each reactor was 7 L. Bottom aeration was supplied with an airflow rate of 7.0 L/min, and the volumetric exchange ratio was 50%. The temperature in the reactors was around 16 ± 2 °C during the experimental period. The reactors were all operated with a 4-h cycle, consisting of feeding (4 min), aeration (180 min), settling
(4-30 min), decanting (2 min), and idling (24-50 min). Thus, the hydraulic retention time was 8 h. In order to investigate the effect of Fe$^{3+}$ on AGS formation and performance, a concentrated FeCl$_3$ solution was added to R2 and R3 to obtain the initial concentrations of 6 and 12 mg Fe$^{3+}$/L, respectively. To completely mix the FeCl$_3$ solution and sludge, the FeCl$_3$ solution was added after 5 min of aeration. The adding time for each cycle was 1 min, and the adding volume was controlled by a peristaltic pump. R1 without extra Fe$^{3+}$ addition served as the control. The Fe$^{3+}$ concentrations selected in this study were based on a preliminary batch test for chemical removal of P (data not shown).

Synthetic wastewater with the following composition was used: 512 mg of sodium acetate (400 mg/L as chemical oxygen demand (COD) basis), 21.9 mg of KH$_2$PO$_4$ and 36.8 mg of K$_2$HPO$_4$·3H$_2$O (10 mg/L as PO$_4^{3-}$-P basis), 172.0 mg of NH$_4$Cl (45 mg/L as NH$_4^+$-N basis), 88.6 mg of MgSO$_4$·7H$_2$O (8.6 mg/L as Mg basis), 111.0 mg of CaCl$_2$ (40 mg/L as Ca basis), and 0.3 mL of trace solution per liter. The trace solution consisted of the following compounds per liter: 1.5 g of FeCl$_3$·6H$_2$O, 0.15 g of H$_3$BO$_3$, 0.03 g of CuSO$_4$·5H$_2$O, 0.18 g of KI, 0.12 g of MnCl$_2$·4H$_2$O, 0.06 g of Na$_2$MoO$_4$·2H$_2$O, 0.12 g of ZnSO$_4$·7H$_2$O, 0.15 g of CoCl$_2$·6H$_2$O, and 10 g of EDTA.

The seed sludge was taken from a municipal WWTP in Shanghai, in which an anaerobic-anoxic-aerobic process was used for nutrient removal. The sludge volume index at 5 min (SVI$_5$) and sludge volume index at 30 min (SVI$_{30}$) for the seed sludge were 210.5, and 191.2 mL/g, respectively. The initial concentration of mixed liquor suspended solid (MLSS) in each reactor was approximately 4655 mg/L. Fe$^{3+}$ was
added in each cycle after one day of cultivation. In order to avoid the excessive biomass wash-out, the settling time decreased stepwise from 30 min to 5 min based on the sludge settleability in each reactor (Fig. 1). During the experimental period, the biomass wastage was not controlled in all the reactors but occurred through sludge washout during the decanting phase.

Additionally, a batch experiment was conducted to evaluate the particle size of the iron precipitates and hydrolyzates. Specifically, a 1-L beaker filled with 0.1 M Tris-HCl buffer (pH 8.1) was used. A concentrated phosphate solution was added to achieve the initial PO₄³⁻-P concentration of 5 mg/L. Then, a concentrated FeCl₃ solution was added to each beaker to achieve the initial Fe³⁺ concentrations of 6 or 12 mg/L. Finally, the particle size was measured after one hour of reaction.

**Analytical methods**

COD, NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, PO₄³⁻-P, total nitrogen (TN), total phosphate, MLSS, mixed liquor volatile suspended solid (MLVSS), pH, SVI₅, and SVI₃₀ were measured according to the Standard Methods. The size of sludge was monitored by laser diffraction (Mastersizer 3000, Malvern, UK) with tap water as the suspension medium and standard optical parameters (main statistical size parameters: D₁₀, D₅₀ and D₉₀ indicate that 10 %, 50 % and 90 % of the total particle volume have a smaller particle size than the D₁₀, D₅₀ and D₉₀, respectively). X-ray diffraction (XRD) analysis was performed using a D8 Advance diffractometer (Bruker, Germany) with a ceramic tube scattering from 10° to 90° in 2°. In order to remove the organic fraction, samples were dried and calcined in an oven at 500 °C for 2 h before XRD
analysis. The AGS morphology was observed via digital camera and scanning electron microscopy (SEM) according to the method described by Li et al.\textsuperscript{24} Energy dispersive X-ray (EDX) combined with SEM was used to analyze the element composition of precipitates adhered to AGS. The contents of P, Ca, Mg and Fe in sludge were analyzed using an inductively coupled plasma emission spectrometer (ICP 720ES, Agilent, USA) after sludge digestion according to the method reported by Ščančar et al.\textsuperscript{25} The EPS of sludge samples was extracted using a heat method.\textsuperscript{23} The polysaccharide (PS) content in EPS was quantified using the anthrone method.\textsuperscript{26} The PN content in EPS was also quantified using a reagent kit (Sangon Biotech, China) based on the bicinchoninic acid method.\textsuperscript{27}

**Measurement of microbial activity and microbial community**

Batch experiments were performed to evaluate the specific oxygen uptake rate (SOUR) of bacteria (including ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and heterotrophic bacteria (HB)), and the maximum specific oxidation rates of NH\textsubscript{4}+-N and NO\textsubscript{2}--N in each reactor. The measurements of SOUR\textsubscript{AOB}, SOUR\textsubscript{NOB}, and SOUR\textsubscript{HB} were according to the method described by Liu et al.\textsuperscript{19} The measurements of maximum specific oxidation rates of NH\textsubscript{4}+-N and NO\textsubscript{2}--N can be found elsewhere.\textsuperscript{23}

Seed sludge and sludge of R1, R2, and R3 on day 80 were used to analyze the characterization of microbial community. DNA was extracted from sludge using the E.Z.N.A.\textsuperscript{®} Tissue DNA Kit (Omega Bio-tek, UAS) according to manufacturer's protocols. The DNA was checked on 1% agarose gel, and DNA quality was
determined through OD260/280. The primes for bacteria were 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The detailed polymerase chain reaction amplification, Illumina Miseq sequencing and processing of sequencing data were conducted according to the reported protocol. The raw sequencing data of bacteria have been deposited in the GenBank database under the accession number SRR3538700.

**Statistical analysis**

An analysis of variation (ANOVA) was used to evaluate the significance of results, and p < 0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Formation and characterization of AGS**

As shown in Fig. 1(a), MLSS and MLVSS sharply decreased on day 1 in all the three reactors due to the reduction of settling time from 30 min to 7-12 min. After that, MLSS and MLVSS in R1 gradually increased, and SVI gradually decreased. On day 20, the SVI5 and SVI30 in R1 decreased to 74.7 and 69.3 mL/g, respectively, and the ratio of SVI30/SVI5 and D50 were 0.93 and 370 μm, respectively (Fig. 1). According to the previous study, granulation is deemed to be completed when the ratio of SVI30/SVI5 is higher than 0.9 and at the same time, a clear outline of sludge is observed. Thus, it is assumed that the granulation time in R1 was 20 days. In R2, the SVI5 and SVI30 were higher than 100 mL/g in the first 32 days, and D50 was lower than 200 μm in the first 55 days. Furthermore, the ratio of SVI30/SVI5 was always
lower than 0.9 during the whole experiment (Fig. S1). These results indicated that the addition of 6 mg Fe\(^{3+}\)/L was adverse to the sludge granulation. In R3, MLSS and MLVSS increased rapidly after 32 days of cultivation. On day 35, the SVI\(_5\) and SVI\(_{30}\) decreased to 63.1 and 60.8 mL/g, respectively, and the ratio of SVI\(_{30}/SVI_5\) and D50 increased to 0.96 and 721 μm, respectively. Therefore, it is assumed that the granulation time in R3 was 35 days. The settling time was longer in R3 (12 min) than in R1 (6-7 min) in the first 43 days (Fig. 1(a)). This is probably the main reason for the longer granulation time in R3 because the longer settling time resulted in a longer granulation time.\(^{30}\) However, the average SVI\(_5\) and SVI\(_{30}\) in R3 after granulation were 60.9 and 57.0 mL/g, respectively, which were lower than those in R1 (SVI\(_5\): 75.4 mL/g; SVI\(_{30}\): 70.8 mL/g). The average particle size in the steady period in R3 was 1067 μm, which was larger than that in R1 (548 μm). These results indicate that although the addition of 12 mg Fe\(^{3+}\)/L could not shorten the granulation time, it helped improve the settleability and particle size of AGS.

It should be noted that D10 in R2 was always very low (< 60 μm) (Fig. 1(b)). Similar phenomenon was also observed in R3 in the first 32 days (< 50 μm). The low value of D10 in R2 and R3 should be attributed to the Fe\(^{3+}\) addition, leading to the formation of iron precipitates and hydrolyzates with small particle size. The particle size determination by a batch experiment without biomass confirmed that the iron precipitates and hydrolyzates had a very small particle size (D50 < 15 μm) (Table S1). The iron precipitates and hydrolyzates also have poor settleability, possibly because PO\(_4^{3-}\)-P is loosely bound to the Fe(OH)\(_3\) flocs.\(^{14}\) Consequently, the addition of Fe\(^{3+}\) in
R2 and R3 adversely affected the sludge settleability due to the formation of iron precipitates and hydrolyzates with poor settleability. Similar phenomenon was also observed in other researches. The results that the effluent SS and VSS in R2 and R3 were still higher than those in R1 even when the settling time was longer in R2 and R3 at the beginning of cultivation (Fig. S2) further supported this conclusion. The poor sludge settleability at the beginning would adversely affect the granulation process. On the other hand, the PS content was almost at the same level in R1 (10.4 ± 1.3 mg/gVSS), R2 (9.1 ± 1.6 mg/gVSS) and R3 (11.8 ± 3.4 mg/gVSS) during the experimental period (Fig. 2). However, the PN content in R2 and R3 was always higher than that in R1 during the whole experiment. The maximum PN contents in R2 and R3 were 132.2 and 109.2 mg/gVSS, respectively, while it was only 70.8 mg/gVSS in R1. Generally, microbial cells in sludge produced more EPS in the presence of toxic substances. The addition of 6 and 12 mg Fe$^{3+}$/L might have some toxicity to the sludge as indicated by the inhibition of microbial activity (Table 1, discussed in section of Microbial community and activity). Therefore, the addition of Fe$^{3+}$ increased the PN production in EPS. The PN in EPS positively affects the cell hydrophobicity and settleability, and high PN content in EPS benefits the formation and stability of AGS. Therefore, the addition of Fe$^{3+}$ has a positive effect on AGS formation and stability by improving the PN content in sludge EPS.

**Variation of chemical precipitates**

The ratio of MLVSS/MLSS in R1 increased from 0.83 (day 1) to 0.93 (day 16), and then stabilized within this range (Fig. S1). The increase in MLVSS/MLSS ratio might
be attributed to the fact that the inorganic salts concentration in the synthetic wastewater was less than that in the real municipal wastewater. However, the ratio of MLVSS/MLSS in R2 rapidly decreased to 0.76 (day 5), and then stabilized at around 0.79. The rapid decrease in MLVSS/MLSS ratio was attributed to the addition of Fe\textsuperscript{3+}, leading to the accumulation of iron precipitates and hydrolyzates on sludge. Additionally, the ratio of MLVSS/MLSS in R3 was lower than that in R2 in the first 32 days. This was mainly due to the higher dose of Fe\textsuperscript{3+} in R3. However, about 35 days later, the ratio of MLVSS/MLSS was higher in R3 than in R2. The increase in the ratio of active biomass in R3 might be attributed to the increase in particle size of AGS.

As shown in Fig. 3 (a), the contents of Ca, Mg, Fe, and P in sludge in R2 were always higher than those in R1 during the whole experiment. Similar phenomenon was also observed in R3 in the first 46 days. These results indicate that the addition of Fe\textsuperscript{3+} increased the contents of inorganic precipitates in sludge. The SEM-EDX analysis also supported this conclusion. Some tiny particles containing metal elements of Ca and Fe were observed in AGS of R2 and R3, but not in AGS of R1 (Fig. S3). XRD analysis showed that the detected minerals in sludge samples of R2 and R3 were hematite (Fe\textsubscript{2}O\textsubscript{3}) and calcium pyrophosphate (Ca\textsubscript{2}P\textsubscript{2}O\textsubscript{7}) (Fig. S4). Moreover, srebrodolskite (Ca\textsubscript{2}Fe\textsubscript{2}O\textsubscript{5}) was found in sludge sample of R2, while iron phosphate hydroxide (Fe\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}(OH)\textsubscript{3}) in sludge sample of R3. Since Fe(OH)\textsubscript{3} can be transformed to Fe\textsubscript{2}O\textsubscript{3} at 500 °C, the main hydrolyzate in the original sludge samples of R2 and R3 should be Fe(OH)\textsubscript{3}. In addition, the contents of Ca, Mg, Fe, and P in
sludge in R3 were much higher than those in R2 at the beginning of cultivation due to the higher dose of Fe$^{3+}$ in R3. The high contents of inorganic precipitates in sludge at the beginning of cultivation could improve the sludge specific density and serve as nuclei, and thereby alleviate the adverse effect of Fe$^{3+}$ on sludge settleability. This might be the main reason that complete granulation was observed in R3 with 12 mg Fe$^{3+}$/L addition, but not in R2 with 6 mg Fe$^{3+}$/L addition. Moreover, the contents of Ca, Mg, Fe, and P in sludge in R3 exhibited a decreasing trend during the granulation process. After 32 days of cultivation, these elements in sludge in R3 were all lower than those in R2. Thus, it indicates that the increase in particle size in R3 helped to increase the active biomass ratio of AGS. The main reason is probably that AGS with large particle size has lower specific surface area, adsorption cumulative volume of pores, and adsorption average pore width in comparison to the AGS with small particle size,$^{26}$ leading to a lower adsorption of chemical precipitates onto the AGS with large particle size.

As shown in Fig. 3 (b), the contents of Ca and Mg in sludge of different size (5.4 ± 0.6 and 3.7 ± 0.1 mg/gVSS) were relatively stable in R1; whereas the contents of Fe and P in sludge gradually decreased as the particle size increased from 50-150 to 300-500 μm (statistical difference as p < 0.05). In R2, the contents of Ca, Fe, and P in sludge decreased significantly as the particle size increased from < 50 to 150-300 μm (statistical difference as p < 0.05). However, when the particle size was greater than 150-300 μm, they became relatively stable. Similarly, a significant decrease in the contents of Ca, Mg, Fe, and P in sludge was observed in R3 when the particle size
increased from < 50 to 300-500 μm (statistical difference as p < 0.05). Nevertheless, they were relatively stable when the particle size was greater than 300-500 μm. These results further confirm that the particle size growth resulted in the decrease in precipitates and hydrolyzates contents of AGS.

Performance of the reactors

The concentrations of COD and NH\textsubscript{4}+-N in the effluent in R1, R2, and R3 during the experiment were 42 ± 11 and 0.5 ± 0.5, 37 ± 10 and 0.3 ± 0.3, 37 ± 10 and 0.5 ± 0.9 mg/L, respectively (Fig. 4). Thus, good removals of COD and NH\textsubscript{4}+-N were achieved in all the reactors during the experimental period. The concentrations of COD and NH\textsubscript{4}+-N in the effluent during the experiment showed no statistical difference in R1, R2, and R3 (p > 0.05), which indicates that the addition of Fe\textsuperscript{3+} had no significant effect on the removal performance of COD and NH\textsubscript{4}+-N. Additionally, the pH value in the effluent fluctuated within the range of 7.7-8.6 during the whole experiment in R1, R2, and R3 (Fig. S5). The highest pH values in R1, R2, and R3 in one typical cycle were 9.2, 8.8, and 9.0, respectively, and the highest concentrations of free ammonia (FA) were 7.2, 3.2, and 5.2 mg/L, respectively (Fig. S5). It is generally accepted that both AOB and NOB are inhibited by FA, but the NOB is more sensitive to FA than AOB, giving the inhibition range of 0.1-1.0 mg/L and 10-150 mg/L, respectively.\textsuperscript{33} Thus, nitrite accumulation was observed in all the reactors. The occurrence of partial nitrification in AGS system resulting from the high pH value and FA concentration was also reported by Li et al.\textsuperscript{24} However, nitrite accumulation completely disappeared in R2 and R3 in 60 days. This indicates that the addition of
Fe$^{3+}$ was detrimental to the partial nitrification in AGS system. During the experimental period, the effluent pH value in R1 was in the range of 8.1-8.6, which was higher than that in R2 (7.8-8.3) and R3 (7.7-8.3) (Fig. S5). Meanwhile, the maximum concentration of FA in one typical cycle in R1 was 7.2 mg/L, which was higher than that in R2 (3.2 mg/L) and R3 (5.2 mg/L) (Fig. S5). Therefore, the inhibition of FA on NOB activity was weaker in R2 and R3 than in R1. Thus, partial nitrification gradually disappeared in R2 and R3. At the steady period (day 62 to day 83), the average removals of TN in R1, R2, and R3 were 42.5%, 30.3%, and 51.1%, respectively. These values were lower than the results in our previous studies, which was probably due to the lower COD concentration in the influent. Moreover, the removed nitrogen amount via simultaneous nitrification-denitrification in the aeration phase in R1, R2, and R3 were 11.4, 0.0, and 32.3 mg, respectively, which accounted for 19.5%, 0.0%, and 32.3% of the total removed nitrogen in a typical cycle (Table S2). Therefore, the higher TN removal in R3 may be attributed to the larger particle size, leading to more anoxic zones for denitrification during the aeration phase.

As shown in Fig. 4(b), the concentration of PO$_4^{3-}$-P in the effluent significantly decreased with the addition of Fe$^{3+}$ (statistical difference as $p < 0.05$). The concentrations of PO$_4^{3-}$-P in the effluent in R1, R2, and R3 during the experiment were 8.6 ± 0.7, 3.5 ± 0.4 and 0.9 ± 0.1 mg/L, respectively, and the average removal efficiencies were 17.8%, 66.5% and 91.0%, respectively. Considering the volumetric exchange ratio (50%) in this study, the actual molar ratios of Fe$^{3+}$ to PO$_4^{3-}$-P in R2 and
R3 were 0.66 and 1.33, respectively. Therefore, good removal of PO$_4^{3-}$-P ($< 1$ mg/L) was achieved in AGS system when the molar ratio of Fe$^{3+}$ to PO$_4^{3-}$-P was greater than 1.33. This is consistent with the study in activated sludge systems.$^{35}$

**Microbial community and activity**

The high-throughput sequencing was used to investigate the microbial community in seed sludge, sludge of R1, R2, and R3. The coverage in all the sludge samples was greater than 99%, indicating that microbial community could be well represented by the collected gene sequences. The indexes of Ace, Chao and Shannon in seed sludge (274, 274, and 4.48) were higher than those in R1 (222, 233, and 3.31), R2 (200, 213, and 1.79), and R3 (217, 216, and 2.85), and the index of Simpson in seed sludge (0.0294) was lower than that in R1 (0.0828), R2 (0.4790), and R3 (0.1176). This indicates that the microbial richness and diversity decreased after the formation of AGS. This result is in agreement with the previous study.$^{36}$ Moreover, the indexes of Ace, Chao, and Shannon in all the sludge samples were ranked as follows: R1 $>$ R3 $>$ R2. The index of Simpson was ranked as R1$<$ R3$<$ R2. Consequently, the addition of Fe$^{3+}$ reduced the microbial richness and diversity, but the growth of particle size increased the microbial richness and diversity.

At the phylum level, *Proteobacteria* was the most abundant phylum in seed sludge, sludge of R1, R2, and R3, accounting for 41.7%, 77.7%, 85.3%, and 66.3%, respectively (Fig. 5(a)). *Bacteroidetes* was the second dominant phylum in the four sludge samples (39.1%, 19.7%, 12.8%, and 31.0%, respectively). This is consistent with our previous study stating that *Proteobacteria* and *Bacteroidetes* are the
commonly predominant phyla in AGS system. At the genus level, the most abundant genus in seed sludge was *Saprospiraceae Uncultured* (23.6%), followed by *Comamonadaceae Unclassified* (15.3%) and *Dokdonella* (6.0%) (Fig. 5(b)). However, they all showed a declining trend in sludge of R1, R2, and R3. The differences of microbial community between the seed sludge and sludge of R1 were probably due to the formation of AGS and the use of synthetic wastewater and aerobic SBR in this study. In addition, the main genera in R1 were *DB1-14 Norank* (20.8%), *Azoarcus* (15.9%), *Zoogloea* (15.3%), and *Cytophagaceae Uncultured* (7.0%). However, they became *Zoogloea* (68.8%) and *Saprospiraceae Uncultured* (6.0%) in R2, and *Zoogloea* (20.2%), *Thauera* (20.1%), *Saprospiraceae Uncultured* (13.5%), *Alcaligenaceae Uncultured* (10.6%), *Flavobacterium* (7.5%), and *Cytophagaceae Uncultured* (5.3%) in R3. Therefore, the addition of Fe³⁺ influenced the structure of microbial community, which was also reported by Ren et al. As shown in Fig. 5(c), the relative abundances of AOB in R2 (0.3%) and R3 (0.4%) were lower than that in R1 (0.9%). Nevertheless, the relative abundances of NOB in R2 (1.1%) and R3 (4.3%) were higher than that in R1 (0.2%). Thus, the addition of Fe³⁺ gradually inhibited the growth of AOB, but stimulated the growth of NOB (mainly *Candidatus Nitrotoga*). Similar phenomenon was also observed in other study with the addition of 10 mg Fe²⁺/L. The inhibition of Fe³⁺ on AOB growth was one of the reasons that partial nitrification gradually disappeared in R2 and R3.

As shown in Table 1, the SOURAOB and maximum specific oxidation rate of NH₄⁺-N in R3 (8.5 ± 0.6 mgO₂/(gVSS·h) and 2.0 ± 0.3 mgN/(gVSS·h)) were lower
than those in R1 (10.4 ± 0.8 mgO₂/(gVSS·h) and 2.5 ± 0.2 mgN/(gVSS·h)) (statistical difference as p < 0.05). However, they showed no statistical difference in R2 and R1 (p > 0.05). This result indicates that the significant decrease in the specific activity of AOB occurred when the long-term addition of Fe³⁺ was 12 mg/L. The SOURNOB and maximum specific oxidation rate of NO₂⁻-N in R2 (9.9 ± 0.3 mgO₂/(gVSS·h) and 1.9 ± 0.2 mgN/(gVSS·h)) and R3 (10.0 ± 0.2 mgO₂/(gVSS·h) and 2.1 ± 0.2 mgN/(gVSS·h)) were higher than those in R1 (2.3 ± 0.4 mgO₂/(gVSS·h) and 0.5 ± 0.1 mgN/(gVSS·h)) (statistical difference as p < 0.05). This was probably related to the relatively weaker inhibition of FA on NOB activity when 6 or 12 mg Fe³⁺/L was added, resulting in the growth of NOB in R2 and R3 in the long run (Fig. 5(c)). Additionally, the SOURHB in R3 (34.7 ± 1.0 mgO₂/(gVSS·h)) was lower than that in R1 (52.5 ± 0.9 mgO₂/(gVSS·h)), but higher than that in R2 (24.2 ± 0.4 mgO₂/(gVSS·h)) (statistical difference as p < 0.05). This indicates that the long-term addition of Fe³⁺ might reduce the specific activity of HB, but the growth of particle size could help alleviate the inhibition. Generally, AOB mainly reside in the outer layer of AGS, while some heterotrophic denitrifying bacteria usually reside in the inner layer of AGS. Therefore, the inhibition effect of Fe³⁺ on the specific activity of HB could be alleviated due to the substrate limitation resulting from the large particle size of AGS. Although the specific activity of AOB and HB in R2 and R3 decreased due to the long-term addition of Fe³⁺, the removal performance of COD and NH₄⁺-N was not affected during the experimental period.

CONCLUSION
The addition of Fe\(^{3+}\) had both negative and positive effects on the formation and stability of AGS. On the one hand, the addition of Fe\(^{3+}\) adversely affected sludge granulation due to the formation of iron precipitates and hydrolyzates with poor settleability. On the other hand, the addition of Fe\(^{3+}\) increased the contents of precipitates as crystalline nuclei in sludge at the beginning of cultivation, and the PN content in EPS, which were beneficial to the formation and stability of AGS.

Granulation time was not shortened with the addition of 6 and 12 mg Fe\(^{3+}\)/L, but the addition of 12 mg Fe\(^{3+}\)/L helped improve the settleability and particle size of AGS (average SVI\(_{30}\): 57.0 mL/g; stable average particle size: 1067 μm) compared to the reactor without Fe\(^{3+}\) addition (average SVI\(_{30}\): 70.8 mL/g; stable average particle size: 548 μm). Additionally, good removals of NH\(_4^+\)-N (≤ 0.5 mg/L) and PO\(_4^{3-}\)-P (< 1 mg/L) were achieved after granulation with the addition of 12 mg Fe\(^{3+}\)/L (Fe\(^{3+}\) to PO\(_4^{3-}\)-P molar ratio=1.33). The addition of Fe\(^{3+}\) reduced the microbial diversity and specific activity of HB, but promoted the growth of NOB. The particle size growth not only increased the active biomass ratio and microbial diversity, but alleviated the inhibition effect of Fe\(^{3+}\) on the specific HB activity.

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REFERENCES


39 Luecker S, Schwarz J, Gruber-Dorninger C, Spieck E, Wagner M, Daims H,


Table 1 Specific oxygen uptake rates (SOUR) and maximum specific oxidation rates of NH$_4^+$-N and NO$_2^-$-N in R1, R2, and R3 on day 76

<table>
<thead>
<tr>
<th>Reactor</th>
<th>SOUR (mgO$_2$/(gVSS·h))</th>
<th>Oxidation rates (mgN/(gVSS·h))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AOB</td>
<td>NOB</td>
</tr>
<tr>
<td>R1</td>
<td>10.4 ± 0.8</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>R2</td>
<td>9.9 ± 0.3</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td>R3</td>
<td>8.5 ± 0.6</td>
<td>10.0 ± 0.2</td>
</tr>
</tbody>
</table>

*ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, heterotrophic bacteria*
Fig. 1 Variations of MLSS, MLVSS, SVI, settling time (a), and particle size (b) in R1, R2, and R3 during the experimental period.
Fig. 2 Extracellular polymeric substances (EPS) variation in R1, R2, and R3 during the experimental period (PS: polysaccharides contents in EPS; PN: proteins content in EPS); error bars indicate the standard error of individual measurements (n=3)
Fig. 3 Variations of Ca, Mg, Fe and P contents in sludge in R1, R2, and R3 during the experimental period (a), and in sludge of different size in R1, R2, and R3 on day 81 (b); error bars indicate the standard error of individual measurements (n=3)
Fig. 4 Variations of NH$_4^+$-N, NO$_2^-$-N, NO$_3^-$-N, TN (a), COD and PO$_4^{3-}$-P (b) concentrations in R1, R2, and R3 during the experimental period.
Fig. 5 Microbial community characterization in seed sludge, sludge of R1, R2, and R3 on day 80: (a) relative abundance of community at the phylum level (> 0.1% in at least one sample are listed); (b) relative abundance of community at the genus level (>1.0% in at least one sample are listed) (c) relative abundance of identified AOB and NOB)