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# Size-dependent calcium carbonate precipitation induced microbiologically in aerobic granules

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### Abstract

Calcium and ash accumulation in aerobic granules treating organic wastewater was investigated systematically in this study. It was found that ash content of granular sludge reached 43% at stable state of reactor operation with calcium carbonate precipitation as the main cause. Since granular sludge in the reactor at the steady state was the mixture of granules with different sizes, granules in the reactor on operation days 90, 120 and 150, respectively, were sorted into 10 categories by size to study the inorganic substances. It was found that calcium was selectively enriched in granules and the precipitation of calcium carbonate was just dependent on granule size instead of operation duration at the steady state. More calcium carbonate was precipitated in bigger aerobic granules, but both calcium and ash contents reached stable values when granule size was bigger than 700µm. Analysis by X-ray Diffractometer disclosed that aragonite was the only polymorph of calcium carbonate detected in aerobic granules with size bigger than 300µm. Scanning electron microscope revealed clearly for the first time that bundles of needle shaped aragonite was embedded in extracellular polymeric substances of aerobic granules. A mechanism on calcium carbonate precipitation was proposed to deepen the understanding of biomineralization.

Keywords: aerobic granules, precipitation, calcium carbonate, aragonite, extracellular

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polymeric substances (EPS), size, wastewater treatment

### 1. Introduction

Aerobic granule is a kind of unique self-immobilized microbial aggregates without carrier material, which is distinctly different from activated sludge and conventional biofilm. Because of good biomass settleability, high biomass retention in bioreactor, and ability to sustain high organic loading rate, aerobic granulation as a novel technology for biological wastewater treatment has attracted considerable attention recently as a hot research topic.

Metal ions such as Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Al<sup>3+</sup> were believed to play an important role for granulation and they have been purposely dosed to stimulate and enhance the formation of aerobic granules, anaerobic granules, and biofilms [1, 3]. Basically there is no doubt that Ca<sup>2+</sup>, Mg<sup>2+</sup>, or Al<sup>3+</sup> augmentation is beneficial to microbial aggregation. The mechanism of this metal ion augmentation is not fully understood, but it has been accepted that nucleus formation of inorganic precipitates could be the main reason as metal precipitates were observed in the enhanced granular sludge. In addition, it has been evidenced that inorganic precipitates in granular sludge could improve the structure stability and strength of aerobic granules [4-6]. Meanwhile, however, a very high ash content in aerobic granules up to 50-84% [7, 12] was reported at some circumstances, in which calcium, magnesium, or iron precipitation was observed [14-16]. Although these metal precipitates in granules are favourable in terms of granule structure and stability, the negative impact was also reported. For example, bioactivity of granule sludge was reduced by Ca accumulation in granules [7], which could result in the incompetence with suspended sludge for grabbing substrate and the instability of granular sludge reactor operation [8]. In addition, the accumulation of inorganic precipitates and excessive high ash content in sludge mean that effective biomass concentration is low. This might pose challenge to meet the reactor design criteria from the aspect of effective biomass concentration. At the same time, it also might require a higher

aeration rate for fluidization of granules or a complete mixing of granular sludge. Given both positive and negative effects that inorganic precipitates on aerobic granular sludge, it is very necessary to investigate the mechanism of inorganic precipitation and develop strategies to employ the advantages but prevent the disadvantages of precipitates in granules.

So far, there are some studies on inorganic precipitates in aerobic granules, but they mainly focused on the augmentation of aerobic granulation [1, 3], identification of main inorganic precipitates in granules [16] and the relationship between inorganic precipitates with granule structure [4-6]. The mechanism of inorganic precipitation in granules and how granule size affects inorganic precipitation especially at steady state are still not clear. Therefore, this paper aims to identify metal precipitation in aerobic granules in a sequencing batch reactor without an excessive Ca<sup>2+</sup> and Mg<sup>2+</sup> addition in the wastewater and investigate the main factors leading to metal precipitation in aerobic granules at steady state. A relationship between granule size and calcium precipitation was established to explore the mechanism of carbonate precipitation.

### 2. Materials and Methods

2.1. Experimental setup

An internal loop airlift reactor with a working volume of 4.75 liters was used to cultivated aerobic granules in this study. The internal diameter of the reactor column was 8 cm with a ratio of working height to the internal diameter of column of 12. The draft tubes had an internal diameter of 5 cm. The reactor was operated sequentially with a cycle time of 4 h, which included 5 min of influent filling, 215-227 min of aeration, 3-15 min of settling and 5 min of effluent discharging from the middle port of the reactor with a volumetric exchange ratio of 50%. This operation resulted in a 8 h hydraulic retention time (HRT). Fine air bubbles for aeration were supplied through a ceramic dispenser at the reactor bottom with an airflow rate of 300 L/h. A time controller was installed to achieve the reactor automatic operation. The reactor was placed into a temperature controlled room at 25 °C. Influent wastewater was stored in a refrigerator at 4 °C to avoid the deterioration of water quality.

Thus, the water temperature in the cycle ranged from around 15 to 25 °C. Meanwhile, influent wastewater was prepared every two days to avoid degradation.

#### 2.2. Sorting of granules

After the reactor reached stable state in terms of biomass concentrating and the average granule size, aerobic granules were taken from the reactor on days 90, 120 and 150, respectively. The granules were washed three times with  $1 \times$  phosphate buffered saline (PBS) before the wet-sieving was employed for granule sorting by size. Granules with a diameter range of 106-212µm, 212-355µm, 355-425µm, 425-500µm, 500-600µm, 600-710µm, 710-850µm, 850-1000µm, 1000-2000µm, and 2000-2800µm were sorted using metal sieves, with a corresponding pore size in diameter.

### 2.3. Medium and Inoculum

The synthetic wastewater was prepared to simulate the real wastewater in Jurong Water Reclamation Plant [40] without considering excessive ammonium for nitrification and denitrification. It consisted of: NaAc (C source) 1665 mg/L, NH<sub>4</sub>Cl (N source) 150 mg/L, K<sub>2</sub>HPO<sub>4</sub> (P source) 45 mg/L, CaCl<sub>2</sub>·2H<sub>2</sub>O 30 mg/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 25 mg/L, FeSO<sub>4</sub>·7H<sub>2</sub>O 20 mg/L, and trace elements which were same with those used. This wastewater quality gave the reactor an organic loading rate (OLR) of 6 g/L<sup>-</sup>d with a cycle time of 4 h. Concentrations of Ca<sup>2+</sup>, Mg<sup>2+</sup> and Fe<sup>2+</sup> in this study were totally same with those used by Liu and Tay [17], Qin et al. [14], and Liu et al.[8] . Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations in tap water used for the preparation of synthetic wastewater were 11 mg/L and 8 mg/L, respectively, leading to 19 mg/L total Ca<sup>2+</sup> and 11 mg/L total Mg<sup>2+</sup> concentrations in the synthetic wastewater. pH of the synthetic wastewater was not adjusted with an average value around 8.4 due to the alkaline nature of sodium acetate. All chemicals used were as analytical grades.

4750 ml of raw activated sludge from a local municipal wastewater treatment plant was

acclimated with synthetic wastewater used in this study in the sequencing batch reactor (SBR) at the similar conditions described above for reactor operation except that settling time was set at 30 min. After three-day acclimation, the settling time was changed to 15 min and the reactor was started up for granulation. Over the operation time, settling time was shortened gradually to 3 min, which was kept constant for long term operation.

2.4. Analytical methods

Chemical oxygen demand (COD), sludge volume index (SVI), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS), and the specific oxygen utilization rate (SOUR) were analyzed by standard methods [18].

COD concentration and MLVSS was set at around 300 mg/L and 100 mg/L, respectively, for SOUR measurement, which was based on the report by Liu et al. [36]. Dissolved oxygen concentration for SOUR was measured with DO-electrode (YSI 5000). Ash content of reactor sludge was obtained by calculation based on the equation below:

$$Ash \ content = \frac{MLSS - MLVSS}{MLSS} \times 100\%$$

Ash content of sorted granules with different sizes was measured by drying at 105 °C for 24 hours and followed ignition at 550 °C for half hour. Ash content was then calculated by granule dry weight at 550°C/granule dry weight at 105 °C.

Granules were sampled and granule size was measured immediately by a laser particle size analysis system with a stirring speed of 100 rpm (Malvern MasterSizer Series 2600) when size was smaller than 2000µm, or an image analysis system (Image-Pro Plus, V4.0, Media Cybernetics, Maryland, USA) with an Olympus SZX9 microscope (Olympus, Token, Japan) when size was bigger than 2000µm [38]. Microscopic observation was carried out to make sure that the stirring speed used in the size analysis does not damage granules.

Microbial composition of granules was observed qualitatively with scanning electron

microscope (SEM) (Stereosan 420, Leica Cambridge Instruments) after fixing with 2.5% glutaraldehyde for 2 h, dehydration via successive passages through 30%, 50%, 75%, 85%, 90%, 95% and 100% ethanol, followed by critical drying in a critical point dryer (HCP-2) (Hitachi Ltd., Tokyo, Japan). To observe the granule surface and the granule interior, respectively, a few of granules were halved by a sterile surgical blade before fixation. Mineralogical analyses of granules were carried out by X-ray diffraction (XRD) with Bruker D8 Advance X-ray Diffractometer equipped with a Cu Kα radiation source (Bruker AXS GmbH, Karlsruhe, Germany) after freeze drying followed by pulvarization. For the analysis of element concentration in granules, granules were first digested according to standard methods [18] and then concentrations of soluble elements such as Na, K, Ca, Mg, Fe and P, mainly contained in synthetic wastewater, were analyzed using an Inductively Coupled Plasma Emission Spectrometer (ICP) (Perkin-Elmer P400, Perkin-Elmer Corporation). The element analysis of C, H, N and S was performed in a Leco TruSpec CHNS analyzer (USA) and O was obtained by weight difference.

# 2.5. Models for simulating the dependence of ash content, calcium content and SOUR on granule size

A modified logistic model (sigmoidal model) [24]shown below was employed to describe the changes of ash content and calcium content in granules with granule diameter.

$$y = \frac{y_{\max}}{1 + e^{(-k \cdot (D - D_0))}}$$
(1)

Where y is the ash content or calcium content of aerobic granules with the diameter of D (µm),  $y_{max}$  is the asymptote of the curve, i.e., the maximum ash content or calcium content in aerobic granules,  $D_0$  is the granule size (µm) from which ash or calcium to increase sharply, and k is the specific increasing rate of ash or calcium content by diameter. For the relationship between SOUR and granule size, equation 2 was used:

$$y = a_0 + y_0 \cdot \exp(-kD) \tag{2}$$

Where y is SOUR of aerobic granules with the diameter of D ( $\mu$ m),  $a_0$  is the minimum SOUR of aerobic granules,  $y_0$  is the difference between the maximum SOUR and the

minimum SOUR, and k is the specific decreasing rate of SOUR by diameter. Simulation is based on experiment data with parameters in the models obtained at maximum and minimum values.

### **3** Results and Discussion

### 3.1. Granulation and precipitation of inorganic substance in granular sludge

The initial sludge in the reactor after 3-day acclimation by synthetic wastewater had a mean particle size of  $130\mu$ m, which is a typical value for suspended sludge. The initial MLSS concentration in the reactor was 5 g /L with ash content of 12%. Since short settling time i.e. 3-15 min was used, most of biomass was washed out of the reactor with MLSS decreased to 2 g /L within 10 days and then MLSS gradually increased to around 5 g /L on day 60.

As shown in Figure 1, the average particle size of sludge increased almost linearly in the first 50 days and then slowly reached a plateau on day 80. After 80 days, the mean sludge size was stable at around 1200 $\mu$ m. Sludge ash content increased linearly during the granulation period correspondingly with a rapid increase in granule size within the first 80 days. From operational day 80 onwards, ash content of granular sludge stabilized at around 43%. It needs to be pointed out that almost half of granular sludge was inert at the steady state due to the ash accumulation although we did not provide inorganic suspended solid in the wastewater and Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations in the water were only 19 mg/L and 11 mg/L, respectively.

The cycle analysis at the stable state in Figure 2 shows that DO concentration was around 5.3 mg/L during COD removal process. Once COD decreased to less than 30 mg/L, DO increased immediately to the saturated value i.e. around 7.2 mg/L. This is in line with the simultaneous complete COD removal and sharp rise of DO reported by Liu and Tay [19]. Although 4 hour cycle was used in this study, COD in the water was removed within 20 minutes. In addition,

it was noticed that pH in the cycle increased from 8.41 at the beginning of the cycle to 9.16 when COD was reduced from 1000 mg/L to less than 30 mg/L, suggesting alkalinity production from acetate metabolism within the first 20 minutes of the cycle. After acetate removal, pH dropped from 9.16 to 8.29 at 90 min. This indicates there were still other chemical reactions occurring in granular sludge although external carbon had been depleted.

Acetate metabolism by microorganisms is an alkalinity producing process. The overall metabolic reaction is based on the combination of three semi-reactors 1) C-source oxidation 2) cell material synthesis and 3) electron acceptor i.e. oxygen reduction [20].

The electron donor half reaction for the oxidation of acetate is shown in equation 3

$$\frac{1}{8}CH_3COO^- + \frac{3}{8}H_2O \rightarrow \frac{1}{8}CO_2 + \frac{1}{8}HCO_3^- + H^+ + e^-$$
(3)

The reaction of cell synthesis, assuming that  $C_5H_7O_2N$  is the average typical formula for cell biomass and ammonium is provided as nitrogen source, is described by equation 4

$$\frac{1}{5}CO_2 + \frac{1}{20}HCO_3^- + \frac{1}{20}NH_4^+ + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7O_2N + \frac{9}{20}H_2O \quad (4)$$

For aerobic growth, the oxygen reduction half-reaction is given by equation 5

$$\frac{1}{4}O_2 + H^+ + e^- \to \frac{1}{2}H_2O$$
 (5)

For aerobic granular sludge with acetate as sole carbon source in SBR, the biomass yield is 0.12 g biomass per g of acetate [21]. So the overall reaction with cell synthesis is able to be expressed as equation 6.

 $CH_{3}COO^{-} + 1.69O_{2} + 0.062NH_{4}^{+} \rightarrow 0.062C_{5}H_{7}O_{2}N + 0.752CO_{2} + 0.938H_{2}O + 0.938HCO_{3}^{-}$ (6)

It can be seen from equation 6 that each mole of acetate produces 0.938 mole of  $HCO_3^-$ , which is higher than 0.783 mole of  $HCO_3^-$  with a biomass yield of 0.42 [22]. The lower biomass yield of aerobic granule means more acetate is oxidized to produce alkalinity instead of biomass cells, leading to a higher pH. In addition, the dissolved  $CO_2$  from acetate metabolism exists in the species of  $HCO_3^-$  at neutral conditions and  $CO_3^{-2-}$  at alkaline condition (equation 7).

$$\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-}$$
 (7)

Therefore, a more alkaline condition as shown in Figure 2 with a high pH due to acetate metabolism created more  $CO_3^{2^-}$  in granules, in which local microenvironment i.e.  $CO_3^{2^-}$  and pH, should be more favourable than bulk solution for carbonate precipitation due to mass transfer resistance and concentration gradient Higher or lower pH in compact structure such as biofilm than bulk solution due to mass transfer resistance have been evidenced by pH measurement in biofilm with pH microsensor [36-37].

### 3.2. Dependence of calcium and ash accumulations on granule size

Mature granular sludge from days 90, 120 and 150, respectively, were sieved into different size categories to study the relationship between granule size and ash content. It can be seen from Figure 3 that ash content increased linearly with granule size when granule size was smaller than 550  $\mu$ m and kept stable after granule size was larger than 700  $\mu$ m. Metal analysis revealed that calcium was the dominant metal while all the other metal contents were little in the granules regardless of granule size (Table 1). Calcium accumulation became more serious in bigger granules, which increased with granule size in a similar manner of ash content increase over granule size (Figures 3a and 3b). This suggests that calcium was selectively enriched in granules especially when granules increased their sizes. So it is reasonable to believe that ash content increases beyond 700  $\mu$ m, calcium content in granule reached a steady state, i.e. 90 mg/g MLSS. This value is very close to that reported by Qin et al. [14] without excessive Ca<sup>2+</sup> dosage.

Figure 3c showed the dependence of SOUR of granules on size. Granules with the smallest size had the highest SOUR, which reached 120 mg  $O_2/g$  MLVSS·h. However, SOUR of granules dropped dramatically to around 50 mg  $O_2/g$  MLVSS·h with the increase of granule size to 700  $\mu$ m. With the further increase of granule size from 700  $\mu$ m to 2400  $\mu$ m, SOUR of granules

decreased slowly to around 30 mg  $O_2/g$  MLVSS h. SOUR is the specific oxygen utilization rate of biomass, which is usually used to indicate the bioactivity of bacteria. The decrease in granule SOUR with granule size means the bioactivity loss of aerobic granules. This could be caused by either the increased mass transfer of oxygen and other chemicals in the bigger granules [25] or the increased ash content which could also result in the reduced mass transfer rate in granules as well as potential negative effects on microbial activity because of the precipitation of inorganics on bacteria surface. To differentiate the influence from granule size or ash content on granule SOUR, granules with size of 1500 μm on days 90, 120 and 150, respectively, were grinded to suspended sludge in a mortar [23] with a mean particle size around 160 µm. It was found that SOUR was improved significantly after size reduction by grinding, which reached around 95 mg O2/g MLVSS h. Obviously, the higher SOUR of grinded sludge is theoretically due to the reduction of mass transfer resistance caused by big size and compact structure. However, SOUR of grinded sludge was still 25 mg  $O_2/g$  MLVSS h lower than granules with the same size sorted directly from the reactor. It was noted that the ash content in the grinded sludge was same with granules before grinding, i.e. around 45%, while the ash content in small granules sorted directly from the rector was only 9%. It is thus speculated that the lower SOUR of grinded granules was caused by high ash content. In comparison with 35 mg O<sub>2</sub>/g MLVSS h of the intact granules with a size of 1500  $\mu m$  and 120 mg O\_2/g MLVSS h of granules with size of around 160  $\mu m$  sorted directly from the reactor, we can calculate that granule size at 1500 µm contributed to 50% reduction in SOUR ((SOUR of grinded granule with size of 160 µm -SOUR of intact granules with size of 1500  $\mu$ m)/SOUR of granules with size of 160  $\mu$ m sorted from the reactor) while ash content of 45% played a relatively slight role for SOUR reduction i.e. 20% reduction in SOUR ((SOUR of granules with size of 160  $\mu$ m sorted from the reactor- SOUR of grinded granule with size of 160  $\mu$ m)/ SOUR of granules with size of 160  $\mu$ m sorted from the reactor). Obviously, both granule size and ash content had negative impact on sludge bioactivity although the influence from granule size was much serious. The bioactivity loss due to high ash content were also reported in bigger aerobic granules with a size range from 2.0 mm to 8.0mm [4] and anaerobic granules [10]

The relationships between ash content and granule size, calcium content and granule size, and SOUR and granule size from different operation periods at the steady state were simulated by a modified logistic model [24] and a power function as shown in equations 1 and 2. At the 0.05 significance level, the datasets from days 90, 120 and 150, respectively, were not statistically different at F-test with Prob>F values much higher than 0.05, indicating that ash content, calcium content and SOUR of granular sludge were mainly determined by granule size instead of operation duration. It could thus be understood that the increase of sludge ash content in the reactor before day 80 in Figure 1 was due to the increase of granule size and the stable ash content after day 80 was due to the stabilized granule size. In addition,  $D_0$  in logistic models for ash accumulation and calcium accumulation (equation 1) representing the granule size ( $\mu$ m) from which point ash or calcium increases sharply are very close , further suggesting the ash accumulation in granules could be mainly caused by calcium carbonate precipitation.

Hammes et al. [9] reported that high pH, presence of carbonate at minimal concentration, concentrated source of calcium and availability of nucleation sites are four key factors for calcium carbonate precipitation. For granular sludge, it is the mixture of granules with a wide or narrow size distribution [39]. In SBR, the environmental conditionals such as pH, carbonate and calcium concentrations in bulk solution are same for all granules with different sizes. Thus, it is not environmental condition in bulk solution which caused the different levels of ash contents in granules. On the contrary, granular sludge likely creates different local microenvironments in the interior of granules with different sizes. For example, the reduced SOUR with granule size in Figure 3 partially reflected a more serious mass transfer resistance in bigger granules. ., Therefore, based on the phenomenon that more calcium precipitated in bigger granules, it is hypothesized that pH and carbonate concentration from acetate metabolism in granule interior are much higher than bulk solution and the areas close to the granule surface due to mass transfer resistance. The

localized favourable conditions for calcium carbonate precipitation especially in granule interior are thus created. This also explains the increase in ash content and calcium accumulation with granule size. With the further increase of granules size over 700  $\mu$ m, however, calcium carbonate precipitation did not occur further. This is mainly because that diffusion would be a limiting factor over biological reaction in aerobic granules with a size larger than 700  $\mu$ m [25], which leads to an anaerobic layer and a dead cell layer [26] carrying out different and much slower metabolism with unfavourable micro-environments for CaCO<sub>3</sub> precipitation.

# 3.3. Element distribution in granules with different sizes and mineral morphology of calcium carbonate

The elements carbon (C), hydrogen (H), nitrogen (N) and oxygen (O) of granules with different sizes were measured and granule C/N, C/H and C/N ratios are shown in Figure 4 while element contents of Na, K, Ca, Mg, Fe and P of different granules are listed in Table 1. Since the element contents at the steady state over operation time (from 90 days to 150 days) were similar, only the data on day 120 were shown in Table 1. It can be seen from Figure 4 that C/H and C/N increased significantly with granule size while C/O decreased greatly when granule size was smaller than 700  $\mu$ m. Since granular sludge in this study contains both biomass and carbonate precipitates, the measured C is from both organic matters such as bacteria and inorganic carbonate. But elements H and N are most likely only from biomass. If granular sludge only comprises of biomass cells with a formula of C<sub>3</sub>H<sub>7</sub>O<sub>2</sub>N, C/H, C/N and C/O would be 8.6, 4.3 and 1.9 while C/O would be 0.25 if sludge only contains caCO<sub>3</sub>. It could be seen from Figure 4 that C/N and C/H of smaller granules were close to pure biomass cells of C<sub>3</sub>H<sub>7</sub>O<sub>2</sub>N while C/O ratio increased with granule size and ranged between pure biomass cells and CaCO<sub>3</sub>. This implied further that the main inorganic precipitate in granules was CaCO<sub>3</sub>.

In addition, it was noted that calcium content rose significantly with the increase of granule size while Na, K, Ca, Mg, Fe and P contents remained same regardless of granule size.

When granule size was bigger than 700  $\mu$ m, all elements reached relatively stable values. The changing trend of oxygen and calcium elements with granule size is highly in agreement with that of ash content. Therefore, it validated again that ash accumulation in big granules is due to the precipitation of calcium carbonate. The constant P contents in granules with different sizes indicated that no calcium phosphate was formed. Further analysis of mineral in granules with different sizes by XRD showed that calcium existed in the form of calcium carbonate in aerobic granules and aragonite was the only detected mineral in aerobic granule with size bigger than 300  $\mu$ m as shown in Figure 5. It also needs to be pointed out that XRD is unable to detect amorphous inorganic precipitates, so there is possibility that calcium carbonate could be formed in granules with both crystalized and amorphous forms.

To observe inorganic substances in granules, granules with size of 1200  $\mu$ m were halved and the interior of granule was observed by SEM. Figure 6 shows clearly the crystals of bundles of aragonite with needle shapes embedded in some thin and amorphous extracellular polymeric substances (EPS) sheets, which has been reported to have Ca<sup>2+</sup> binding capacity [4]. For needles shown in Figure 6, they had around 2  $\mu$ m width with 20  $\mu$ m length, which are much bigger than those with a length of 1  $\mu$ m observed around the suspended cell [27]. Needle shape of aragonite crystal has been widely reported. In the clusters of crystals of aragonite, few cells were observed indicating that the nucleation and growth of aragonite crystals do not need the direct participation of bacteria, but bacteria metabolism may provide the favourable micro-environments such as carbonate supersaturation for crystallization. To the best of our knowledge, this is the first time that clear crystals of calcium carbonate were observed in granular sludge treating wastewater.

Microbiologically-induced CaCO<sub>3</sub> precipitation is recognized as a far more complicated and faster process than chemically-induced precipitation [28]. Therefore, simply meeting the chemical requirement of calcium carbonate precipitation may not lead to much precipitation. Nucleation site is a crucial factor for crystallization as nucleation is often energetically the most difficult process in the sequence of precipitation events [29]. The bacterial cell surface

with a variety of ions was thought to non-specifically induce mineral deposition by providing a nucleation site [30], while mechanism of EPS secreted by bacteria as nucleation sites gets experimental dada support too [2, 31, 32]. In Figure 6, only EPS was observed around bundles of aragonite needles suggests that EPS should be nucleation sites for crystallization in aerobic granules. Meanwhile, EPS might also play a role to concentrate Ca ions [2] as the smallest granules still had a much higher Ca concentration compared with other metals (Table 1). Therefore, it is believed that in aerobic granule that mechanism of calcium carbonate precipitation could be 1) bacterial metabolism may serve a role to provide favourable pH and produce enough carbonate 2) granule with mass transfer resistance further creates the localized micro-environments with much higher pH and supersaturated carbonate 3) EPS combined with concentrated Ca ions in the localized micro-environments from step 2) as nucleation sites induces calcium carbonate precipitation.

COD/N ratio used in this study is only 100/3, which is higher than that for the traditional cultivation of heterotrophic bacteria with COD/N ratio as 100/5. In addition, it was found that SOUR of ammonium oxidizing bacteria in granules was negligible and at the same time no nitrite and nitrate were detected. Thus, it is believed that granular sludge in this study did not contain nitrifying or denitrifying bacteria or there was no nitrifying or denitrifying metabolism involved in granules. The image on EPS and needle shaped crystals in granules shown in Figure 6 is in good agreement with the lithified layers of marine stromatolites consisting of abundant aragonite needles embedded within a matrix of extracellular polymeric secretions (EPS) by cyanobacteria, Schizothrix sp., one of photosynthetic autotrophs [31]. The similar findings of aragonite needles in different microbial communities developed by this study (only heterotrophs) and by Reid et al. (photosynthetic bacteria) [31] indicate that calcium carbonate precipitation and its crystallization are not species specific processes. As long as the above mentioned three conditions in the proposed mechanism of calcium carbonate precipitation are satisfied, precipitation could occur. But what kind of conditions are favourable for crystallization of CaCO<sub>3</sub> is still unclear, which needs further exploration.

Based on the compositions of synthetic wastewater used in this study, magnesium and iron are another two divalent ions which could be co-precipitated with calcium carbonate. Juang et al. [15] reported both calcium and iron precipitates while Zhou et al. [16] observed both calcium and magnesium precipitation in aerobic granules. However, in this study, only calcium carbonate was formed at studied conditions with magnesium and iron at very low concentration as shown in Table 1. It has been reported that biochemical compositions in EPS influence the precipitation, compositions, and CaCO<sub>3</sub> polymorphism [11, 32]. For example, acidic polysaccharides isolated from calcareous algae induced Mg-rich calcite precipitation [33] and inhibited the growth of aragonite crystals [33] while acidic proteins isolated from aragonitic biominerals induce aragonite formation [34]. Thus, it is believed that EPS compositions in aerobic granules determined the aragonite formation in this study, which is very hard to allow Mg<sup>2+</sup> enter the aragonite lattice [35] while the calcite crystal structure readily accommodates Mg<sup>2+</sup> into its lattice with magnesium concentration up to 30% [13].

### 4. Conclusions

Calcium was the only metal precipitated in aerobic granules at the studied conditions although other metal ions provided in the wastewater too. In addition, calcium carbonate precipitation affected bioactivity of aerobic granules negatively. Strong relationship was found between calcium carbonate precipitation and granule size, from which mass transfer resistance is the crucial factor to create favourable pH and carbonate concentration in localized micro-environments of big granules for precipitation. The size effects on calcium carbonate disappeared when granules were bigger than 700 µm. Therefore, a precise pH control in bulk solution is suggested to adjust pH and carbonate concentration in micro-environment of granules to alleviate calcium carbonate precipitation and ash accumulation when required. The polymorph of calcium carbonate identified in aerobic granule is only aragonite, which was embedded in EPS matrix with EPS as nucleation sites.

The excessive EPS secretion in aerobic granules contributes to the calcium carbonate precipitation.

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Figure 1 Evolutions of ash content and average particle size of sludge in SBR over operation time



Figure 2 The changes of DO, COD and pH in one cycle at the stable state of the reactor operation



Figure 3 The changes of ash content, calcium content and SOUR over granule size on operation days 90, 120 and 150, respectively, and their respective simulation curves



Figure 4 The changes of carbon/hydrogen (C/H), carbon/nitrogen (C/N) and carbon/oxygen (C/O) contents over granules size at stable state of reactor operation







Figure 6 Needle shaped aragonite embedded in EPS matrix of the interior of aerobic granule disclosed by scanning electron microscope

Na      K      Ca      Mg      Fe      P        106-212      2.6      2.9      16.7      2.1      5.1      7.1        212-355      2.0      3.3      18.2      2.0      1.9      6.8        355-425      1.8      2.7      68.6      1.9      2.4      5.1        425-500      2.6      2.4      7.4      1.8      1.7      5.3        500-600      2.1      1.9      72.4      1.7      3.3      4.8        600-710      2.4      2.1      74.0      1.7      4.1      4.7        710-850      2.3      1.4      104.9      1.5      1.5      3.8        1000-2000      2.1      1.5      84.2      1.9      1.9      4.3        2000-2800      2.5      1.8      88.7      2.2      5.1      5.1		<b>C</b> :	Size (um) mg/g MLSS						
106-212    2.6    2.9    16.7    2.1    5.1    7.1      212-355    2.0    3.3    18.2    2.0    1.9    6.8      355-425    1.8    2.7    68.6    1.9    2.4    5.1      425-500    2.6    2.4    74.4    1.8    1.7    5.3      500-600    2.1    1.9    72.4    1.7    3.3    4.8      600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.4    104.9    1.5    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		Na	к	Са	Mg	Fe	Р		
212-355    2.0    3.3    18.2    2.0    1.9    6.8      355-425    1.8    2.7    68.6    1.9    2.4    5.1      425-500    2.6    2.4    74.4    1.8    1.7    5.3      500-600    2.1    1.9    72.4    1.7    3.3    4.8      600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.6    97.3    1.9    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		106-212	2.6	2.9	16.7	2.1	5.1	7.1	
355-425    1.8    2.7    68.6    1.9    2.4    5.1      425-500    2.6    2.4    74.4    1.8    1.7    5.3      500-600    2.1    1.9    72.4    1.7    3.3    4.8      600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.4    104.9    1.5    3.8      850-1000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		212-355	2.0	3.3	18.2	2.0	1.9	6.8	
425-500    2.6    2.4    74.4    1.8    1.7    5.3      500-600    2.1    1.9    72.4    1.7    3.3    4.8      600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.4    104.9    1.5    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		355-425	1.8	2.7	68.6	1.9	2.4	5.1	
500-600    2.1    1.9    72.4    1.7    3.3    4.8      600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.4    104.9    1.5    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		425-500	2.6	2.4	74.4	1.8	1.7	5.3	
600-710    2.4    2.1    74.0    1.7    4.1    4.7      710-850    2.3    1.4    104.9    1.5    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		500-600	2.1	1.9	72.4	1.7	3.3	4.8	
710-850    2.3    1.4    104.9    1.5    1.5    3.8      850-1000    2.3    1.6    97.3    1.9    1.5    3.8      1000-2000    2.1    1.5    84.2    1.9    1.9    4.3      2000-2800    2.5    1.8    88.7    2.2    5.1    5.1		600-710	2.4	2.1	74.0	1.7	4.1	4.7	
850-1000 2.3 1.6 97.3 1.9 1.5 3.8 1000-2000 2.1 1.5 84.2 1.9 1.9 4.3 2000-2800 2.5 1.8 88.7 2.2 5.1 5.1		710-850	2.3	1.4	104.9	1.5	1.5	3.8	
		850-1000	2.3	1.6	97.3	1.9	1.5	3.8	
		1000-2000	2.1	1.5	84.2	1.9	1.9	4.3	
		2000-2800	2.5	1.8	88.7	2.2	5.1	5.1	
						k.			

Table 1 Element contents in sorted granules with different sizes

### Highlights

- Calcium carbonate precipitated in aerobic granules ٠
- Calcium carbonate precipitation was significantly dependent on granule size
- Aragonite is the only polymorph of carbonate in granules ٠
- Needle shaped aragonite was embedded in EPS matrix of granule
- Acceleration