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Effect of Mg/Ca molar ratios on characteristics of anaerobic-anoxic denitrifying dephosphatation



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HIGHLIGHTS

- Complete phosphate removal achieved at influent Mg/Ca molar ratio of 3.8.
- Mg/Ca molar ratios influence phosphate removal.
- Mg/Ca molar ratios have significant impact on the EPS of biomass.
- A higher Mg/Ca molar ratio could induce deterioration of anaerobic-anoxic EBPR.

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ABSTRACT

In this study, the effect of three Mg/Ca molar ratios (5.0, 3.8 and 1.7) on denitrifying phosphate removal performance, biomass morphology, and Extracellular Polymeric Substances (EPS) were examined. Results showed that when the influent Mg/Ca molar ratio was 3.8, the anaerobic-anoxic EBPR performed complete phosphate removal. The microbial bacterial population was a mixed culture comprised of $81 \pm 3\%$ DPAO and $13 \pm 2\%$ denitrifying glycogen accumulating organisms (DGAO). A higher influent Mg/Ca molar ratio (5.0) had a distinct impact on phosphate removal, biomass morphology, and EPS. This probably induced the deterioration of the anaerobic-anoxic Enhanced Biological Phosphorus Removal (EBPR). The results of this study may inform the proper operation of an anaerobic-anoxic EBPR, and contribute to its application in the real world.

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1. Introduction

In the early 1990s, it was first found that PAOs can use oxygen and nitrate as electron acceptors to complete denitrification in a anaerobic/anoxic/aerobic reactor; the subset of PAOs with denitrification capacity were identified as DPAOs (Kern-Jespersen and Henze, 1993). Subsequently, a lab-scale anaerobic-anoxic SBR (A_2 SBR) was created to further study DPAO characteristics. It was concluded that DPAO could use nitrate as the sole electron acceptor, achieving the same phosphorus removal efficiency as in an anaerobic-aerobic reactor (Kuba et al., 1993). Additionally, DPAO

produced less sludge compared with common PAOs (Kuba et al., 1994), and saved carbon sources. This is because it simultaneously considers both denitrification and phosphorus removal (Kuba et al., 1997).

More recently, researchers have conducted a large number of studies on PAO and DPAO, focusing particularly on their classification. PAO has three classifications, based on the final electron acceptor of polyphosphate: the first can only use oxygen as an electron acceptor; the second can only use oxygen and nitrate as electron acceptors; and the third can only use nitrate, nitrite and oxygen as electron acceptors (Hu et al., 2003). Other research has reported that one kind of PAO can only use nitrite, but not nitrate, as an electron acceptor (Guisasola et al., 2009). PAOs have since been divided into two main types, using the polyphosphate kinase

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(ppk1) as a genetic marker: *Accumulibacter* I and II (hereafter referred to as PAO I and PAO II) (He et al., 2007).

Then, these two types were classified in terms of their denitrifying capabilities: PAO I is able to use nitrate and/or nitrite as an electron acceptor; PAO II is only able to use nitrite for anoxic P-removal (Flowers et al., 2009). DPAO is comprised of both PAO I and II, and they are the main clades of PAO. Nevertheless, there have been limited studies on PAO I and/or PAO II culture in anaerobic-anoxic reactors. Most of these applied an anaerobic-aerobic or anaerobic/anoxic/aerobic reactor. Consequently, anaerobic-anoxic denitrifying phosphate removal has been poor in these reactors and few related studies have been published in the last decade.

Reviewing studies on different kinds of EBPR reactors has provided insights about the effect of different parameters on PAO I and/or PAO II enrichment in EBPR; these study parameters have included temperature, pH, SRT, DO, carbon substrate and electron acceptor (Oehmen et al., 2007). Metal cations (calcium, magnesium and potassium) have also been explored (Barat et al., 2006). Choi et al. (2011) found that only changes in the concentrations of soluble Mg²⁺ and K⁺ resulted in increases and decreases in phosphorus release and phosphorus uptake, which were apparently unaffected by the Ca²⁺ concentration. However, Zhang et al. (2015) demonstrated that the PAO metabolic pathway could shift between the dominance of polyphosphate-accumulating metabolism (PAM) and glycogen-accumulating metabolism (GAM) along with the variation of the influent Ca²⁺ concentration. Furthermore, it was found that a clear increase in floc size and higher MW EPS and formation of larger flocs at higher magnesium concentrations (at the highest Mg:Ca ratio (5:1)), due to divalent cation binding (Arabi and Nakhla, 2009a, 2009b). Despite this work, no research has been conducted about the effect of the molar ratio (e.g., Mg/Ca molar ratio) of metal cations on phosphate removal in EBPR.

Given this background, the objective of this study is to investigate the Mg/Ca molar ratio on denitrifying phosphate removal performance, biomass morphology and EPS, to find the reasons of the deterioration of a denitrifying EBPR.

2. Materials and methods

2.1. Operations of the sequencing batch reactor

A lab-scale anaerobic-anoxic sequence batch reactor (A₂SBR) with a working volume of 2 L (Tian et al., 2013) was inoculated with the parent PAO reactor at ambient temperature. The SBR cycle consisted of three phases: a 2.5 h anaerobic phase, a 3.0 h anoxic phase, and a 0.5 h settling phase. The SBR was operated with a hydraulic retention time of 12 h and a solids retention time of 20 days. The SBR was constantly mixed at 500 rpm; the pH was maintained at 7.0 ± 0.1. To supply nitrate as an electron acceptor for denitrifying phosphorus removal, 0.1 L (45 mg NO₃⁻/L) nitrate was injected into the reactor at a constant flow rate (1 mL/min) in each anoxic phase. Further details about the A₂SBR operation are provided in a previous study (Kuba et al., 1997).

2.2. Synthetic media

The synthetic media feed was composed of two solutions (A and B) mixed in a 1:1 ratio. Solution A contained the carbon source; solution B contained the minerals and trace elements. After mixing the solutions, the synthetic feed contained, per litre: 850 mg/L NaAc·3H₂O, 107 mg NH₄Cl, 49 mg K₂HPO₄, 28 mg KH₂PO₄, 90 mg MgSO₄·7H₂O, 14 mg CaCl₂·2H₂O, 1 mg yeast extract, and 10 mL trace mineral. Trace mineral was prepared as described previously (Smolders et al., 1994).

2.3. Analyses

SBR performance was regularly monitored, and the following parameters assessed: acetate (HAc), orthophosphate ³⁻(PO₄³⁻-P), mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS). During cycle measurements, orthophosphate (PO₄³⁻-P), acetate (HAc), MLSS, MLVSS, polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), glycogen concentrations were measured. Mg and Ca were measured using Standard Methods (APHA, 2005). The PHB and PHV were extracted based on a previous study (Johnson et al., 2009), as was glycogen content (Lanham et al., 2012).

2.4. Microbial characterisation and sludge morphology

Fluorescence *in situ* Hybridization (FISH) was applied using rRNA targeted oligonucleotide probes (Amann, 1995). The detail information of the related oligonucleotide probes and quantification methods were as described by Tian et al. (2013). The morphology of the biomass flocs/granules was examined using a Leica DFC420 imaging system.

2.5. Extracellular Polymeric Substances (EPS)

EPS were extracted from the biomass based on methods in a previous study (Lin et al., 2010). Dried biomass (0.5 g) was extracted in 0.2 M Na₂CO₃ at 80 °C. After centrifuging the sample at 15,000 rpm for 20 min, the pellet was discarded. The supernatant pH was adjusted to 2 by adding 0.1 M HCl. The precipitate was collected by centrifugation (15,000 rpm, 30 min), washed using di-deionized water until the effluent pH reached 7, and dissolved in 0.1 M NaOH. The EPS in the supernatant was precipitated by adding cold absolute ethanol to a final concentration of 80% (vol/vol). The precipitate was collected using a centrifuge (15,000 rpm, 30 min), washed three times in absolute ethanol, and lyophilized. Ash content of the extracted exopolysaccharides was measured using standard methods (APHA, 2005).

2.6. FT-IR spectra

A Bruker Ten 37 FTIR Spectrometer (Bruker Optics Inc., Billerica, MA, USA) was used to investigate the major organic functional groups of freeze-dried sludge and to determine the composition of EPS (especially carbohydrate). First, the sludge samples were freeze-dried at -35°C for one week prior to the analysis. Then, dried KBr powder was uniformly mixed with the sludge sample and subsequently pressed for 2 min at 145,000 psi, generating clear pellets. The FT-IR spectra were conducted over a 4000–400 cm⁻¹ range in the transmittance mode at a spectral resolution of 4 cm⁻¹.

3. Results and discussion

3.1. Performance of phosphate removal

After 250 cycles at a Mg/Ca molar ratio of 3.8, the A₂SBR achieved a steady-state, displaying a good performance of denitrifying dephosphatation. Fig. 1 shows the evolution of C, N, and P during a typical cycle at a steady-state period. Anaerobically, HAc was completely taken up within 0.5 h; the glycogen consumption was 3.44 C-mmol/L, and the released phosphate concentration of 2.92 P-mmol/L. The anaerobic P released/HAc uptake ratio was 0.46 P-mol/C-mol. PHB and PHV production were 7.34 C-mmol/L and 1.78 C-mmol/L, respectively. In the anoxic stage, PHB and PHV consumption were 7.13 C-mmol/L and 1.7 C-mmol/L, respec-

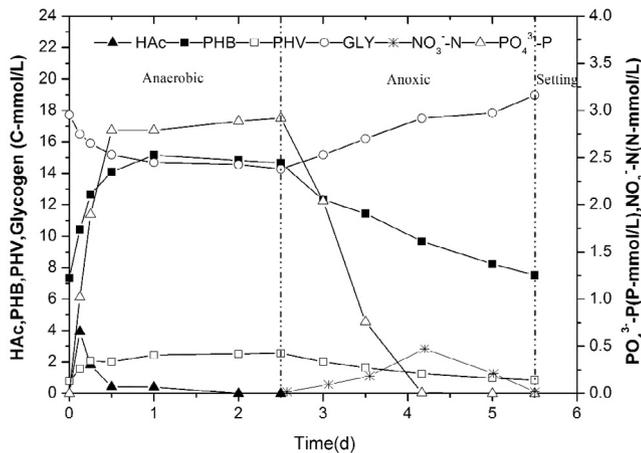


Fig. 1. Typical cycle of evolution of C, N and P during steady-state periods (Mg/Ca = 3.8).

tively. All the PHA produced in the anaerobic stage were depleted within 1.5 h. Glycogen was synthesized at 4.69 C-mmol/L, which was more than anaerobic glycogen consumption. This indicates the glycogen recovery was active in A₂SBR.

Nitrate slowly accumulated and was then completely depleted in the anoxic stage. No nitrite accumulation was detected during the cycle test; this suggests that nitrite may be the intermediate and/or final electron acceptor, and can be easily consumed in denitrifying dephosphatation. Denitrifying phosphorus removal was not inhibited by excessive nitrite accumulation when controlled at a proper influent nitrite concentration (Kuba et al., 1993, 1997).

3.2. Microbial community structure population distribution

With a stable influent Mg/Ca molar ratio of 3.8, The microbial community was comprised of 81 ± 3% of PAO and 13 ± 2% of DGAO, PAO I and PAO II occupied 43 ± 1% and 52 ± 3% of PAO, respectively (Supplementary data Fig. S1). Generally, PAO II can only use nitrite and oxygen as an electron acceptor, whereas PAO I can use nitrite, nitrate and oxygen as an electron acceptor (Flowers et al., 2009). Therefore, the nitrate and nitrite were the electron acceptor in the anaerobic- anoxic system.

In this study, there were no ordinary denitrifying bacteria, but PAO I and DGAO had denitrifying ability, reducing nitrate to nitrite. Therefore, PAO I and PAO II competed for nitrite as an electron acceptor, but cooperated in removing biological phosphate. The coexistence of a high fraction of PAO II with PAO I shows that PAO II has a stronger affinity for nitrite than PAO I. This knowledge can help inform an understanding of biological phosphate removal mechanisms involving different kinds of PAO clades and their relationships.

3.3. Effect of Mg/Ca molar ratio on performance of phosphate removal

Three different Mg/Ca molar ratios (5.0, 3.8, and 1.7) were selected to study the effect of the Mg/Ca molar ratio on phosphate removal. Fig 2 shows phosphorus removal at different Mg/Ca molar ratios. The anaerobic phosphate release remained at 60–64 mg P/L and the average effluent phosphate concentration was below 0.3 mg/L, resulting in a removal efficiency of 98% at Mg/Ca molar ratio of 3.8. Subsequently, the Mg/Ca molar ratio was increased to 5.0 for 80 running cycles. At that level, the anaerobic phosphate release significantly decreased, and the mean effluent phosphate concentration climbed to 3.27 mg/L. The corresponding removal efficiency dropped to 78%.

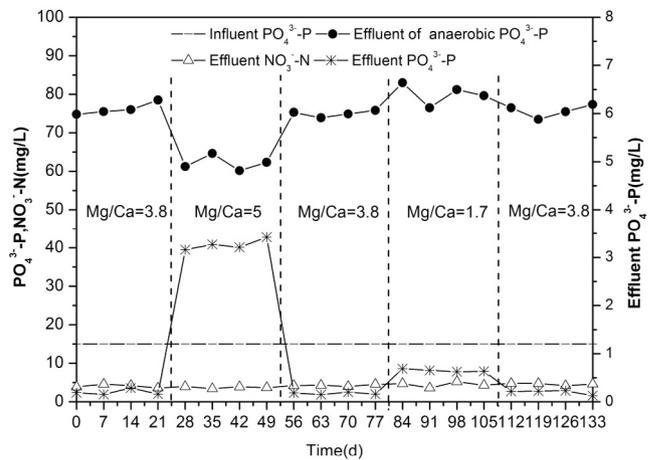


Fig. 2. Phosphate removal performance at different Mg/Ca molar ratios.

Then, the Mg/Ca molar ratio was decreased to 3.8, and phosphate removal soon recovered to previous levels. After that, the Mg/Ca molar ratio was decreased to 1.7. The anaerobic phosphate release was not affected, but the full P-removal could not be achieved. The average effluent phosphate concentration was 0.76 mg/L. Finally, once the Mg/Ca molar ratio was again changed to 3.8, the system recovered as it had before. A similar result was seen in the anaerobic-aerobic EBPR system (the data is not shown here). This suggests that the Mg/Ca molar ratio has a significant impact on phosphate removal of anaerobic-anoxic SBR-EBPR, or other types of EBPR.

The biomass morphology under different Mg/Ca molar ratios is shown in Supplementary data Fig. S2. The biomass appears as scattered flocculent at a Mg/Ca molar ratio of 5.0; the biomass was a relatively regular fine granule when the Mg/Ca molar ratio was 3.8 or 1.7. There was a much finer granule at a Mg/Ca molar ratio of 1.7 than when the Mg/Ca molar ratio was 3.8. This information suggests that the Mg/Ca molar ratio significantly impacts the biomass morphology in the anaerobic-anoxic SBR-EBPR system.

3.4. Effect of Mg/Ca molar ratio on biomass EPS

The FT-IR spectra of EPS under different Mg/Ca molar ratios were analyzed (Supplementary data Fig. S3a). Peaks at 3300 cm⁻¹ and 2928 cm⁻¹ show the stretching vibrations of —OH and C—H of methyl. The peak at 3070 reflects a C=C double bond stretch. Peaks at wave numbers of 1655 cm⁻¹ and 1411 cm⁻¹ were reflecting symmetric and asymmetric stretching vibrations of —COO—, respectively. Peaks near 1535 cm⁻¹ indicated the stretching vibration of C=C and the deformation vibration of the —COCH₃ group. Peaks at 1000 and 1200 cm⁻¹ contributed to the stretching vibration of C—O—C and C—O. The peak observed at 828 cm⁻¹ was associated with the C—H equatorial peaks of α-type end group in α-L type guluronic acid.

The typical peaks of EPS at the three Mg/Ca molar ratios were very similar (Supplementary data Fig. S3b), the sludge EPS should therefore theoretically be the same kind of polysaccharide. The wavelengths between 1050 cm⁻¹ and 750 cm⁻¹ indicate the fingerprint region for polysaccharide in the FT-IR spectra; the second derivative spectrum could increase the spectral band of overlapping apparent resolution. This ensured that few substances would be disturbed by a large amount of matter, determining the wave bands and wavelengths. This allowed the carbohydrates to be distinguished under the wide background (Duarte et al., 2002). The second derivative spectra of sludge polysaccharide fingerprint region at the different Mg/Ca molar ratios show that the

signal amounts were greater than those in the same region of the normal sludge FT-IR spectra (Supplementary data Fig. S3b); the signals were similar to those detected at the three different Mg/Ca molar ratios. Thus, the sludge EPS at the three different Mg/Ca molar ratios could be considered to be the same kind of polysaccharide.

Two kinds of artificial sewage recipes are widely used in lab-scale EBPR research. One recipe was used in the anaerobic/anoxic system (Kuba et al., 1993); another one used in the anaerobic/aerobic system (Smolders et al., 1994). By changing the Mg ion concentration with fixed Ca ions to obtain Mg/Ca molar ratios of 5.0, 3.8 and 1.7, this study was able to investigate the effect of different Mg/Ca molar ratios on the EPS of DPAO sludge. Two sewage recipes with different concentrations of Mg and Ca ions at different Mg/Ca molar ratios influenced EPS gel formation; a good EPS gel could not be formed under a Mg/Ca molar ratio of 5 (Supplementary data Fig. S4). However, when Mg/Ca molar ratios were 3.8 and 1.7, a better gel was formed. This indicates that the main reason for the degenerated removal efficiency of phosphorus was that the EPS gel was damaged, and could not supply a better carrier for DPAO/PAO growth. This led to the deterioration of phosphorus removal. The results seemingly explained the inoculation with the conditional activated sludge, using the sewage recipe of Mg/Ca molar ratio of 5.0 in accordance with previous reported one in a similar study (Kuba et al., 1993). The system did not adsorb phosphorus during the long term operation. However, when the system changed to use the Smolders' sewage recipe (Smolders et al., 1994) (Mg/Ca molar ratio of 3.8). At this ratio, the A₂-SBR denitrifying phosphorus removal system was successfully initiated and remained stable. This was similar to our results.

Previous studies found that anaerobic/anoxic SBR achieved better denitrifying phosphate removal when the Mg/Ca molar ratio of the sewage recipe was 5 (Kuba et al., 1993). This may be because tap water was used by Kuba (Kuba et al., 1993) to prepare the raw water; the average Mg and Ca concentration in the tap water from Delft University of Technology was 8.0 mg/L and 55.7 mg/L (data not shown), respectively. This suggests that the correct Mg/Ca molar ratio entering the reactor was 1.0. The earliest and most authoritative articles about anaerobic/anoxic denitrifying phosphorus removal process originated from this research (Kuba et al., 1993), and many laboratories have applied Demi water rather than tap water to prepare synthetic feed water. This has resulted in a higher Mg/Ca ratio in the substrate. Therefore, this research indicates that the anaerobic/anoxic denitrifying phosphorus removal process remains stagnant for a long time, indirectly restricting its application in practical engineering.

4. Conclusions

This study investigated the effect of influent Mg/Ca molar ratios on denitrifying phosphate removal performance, biomass morphology and EPS. The following conclusions can be drawn: complete phosphate removal achieved with Mg/Ca molar ratio of 3.8, the morphology of biomass behaved compact granule. Mg/Ca molar ratio had significant impact on the phosphate removal, morphology of biomass and EPS, a higher Mg/Ca molar ratio (5.0) could induce deterioration of anaerobic-anoxic EBPR system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.01.063>.

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