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The effect of seed sludge type on aerobic granulation via anoxic–aerobic operation

Yusuf Çağatay Erşan[a,b] and Tuba Hande Erguder[a*]

[a]*Environmental Engineering Department, Middle East Technical University, Ankara, Turkey;* [b]*Environmental Engineering Department, Abdullah Gul University, Kayseri, Turkey*

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The effects of two seed sludge types, namely conventional activated sludge (CAS) and membrane bioreactor sludge (MBS), on aerobic granulation were investigated. The treatment performances of the reactors were monitored during and after the granulation. Operational period of 37 days was described in three phases; Phase 1 corresponds to Days 1–10, Phase 2 (overloading conditions) to Days 11–27 and Phase 3 (recovery) to Days 28–37. Aerobic granules of 0.56 ± 0.23 to 2.48 ± 1.28 mm were successfully developed from both MBS and CAS. First granules appeared on Day 9 in both reactors, indicating that there was no difference between two seed sludge types in terms of the time period for granulation initiation. The results revealed that the granules developed from MBS performed better than CAS in terms of settleability, stability, biomass retention, adaptation, protection of granular structure at high loading rates (0.86 g N/L d and 3.92 g COD/L d) and low COD/TAN ratio (5). Granules of MBS were also found to be capable of providing better protection for nitrifiers at toxic free-ammonia concentrations (38–46 mg/L NH_3–N), thus showing better treatment recovery than those of CAS.

Keywords: denitrification; extracellular polymeric substance (EPS); free-ammonia (NH_3) inhibition; nitrification; recovery

Introduction

A basic concept behind the granular sludge formation (development of granules from suspended culture) is microbial aggregation in which cells cluster and form stable, concentrate microbial community under stressful operational conditions.[1] Granular sludge contains various types of species and numbers of microorganisms per gram biomass in a compact form. The compactness and high amount of microorganisms make the granular sludge advantageous over suspended sludge in terms of their density, structural properties, settling velocities, resistance to toxicity and capability to treat high strength wastewaters.[2,3] Furthermore, since they are easily separable from water media and contain large amount of microorganisms in smaller volumes, it is possible to save 75% from construction site and in turn 20% from total cost.[4]

Granular sludge can be categorized as aerobic and anaerobic granules. The feasibility and efficiency of anaerobic granular sludge's applications and its modifications had been proven via treating various types of wastewater by industrial-scale systems.[5–7] On the other hand, pilot-scale installations for aerobic granular sludge development started a decade ago. These successful installations revealed that wastewater treatment with aerobic granular sludge is a promising technology providing efficient organic and nutrient removal.[8–10] Although there are many industrial pilot-scale plants and some full-scale applications of aerobic granular systems (such as Nereda™),[10] low stability of aerobic granules due to high growth rates of heterotrophic microorganisms and the growth competition between flocculent sludge and aerobic granules remains to be a drawback.[11,12] Therefore, to avoid weaknesses and improve sustainability of aerobic granular systems, studies continue to investigate the problems encountered with stability.

The strategies to improve the stability of the aerobic granules developed in sequencing batch reactors (SBRs) are well reviewed by Adav et al. [11], Lee et al. [13] and Nor Anuar et al.[14] One of the decisive parameters for stability is the flocculating capability of strains in seed sludge.[13] The importance is relevant to the morphological properties and extracellular polymeric substance (EPS) content of seed sludge for the cultivation of granules. Seed sludge type can, therefore, affect the development of aerobic granular sludge from suspended sludge and their nutrient (N, P) removal efficiency. Seed sludge type is known as a decisive parameter in formation and stability of granules in anaerobic granulation.[2] In aerobic granulation concept, it is thought to be affecting the settleability, microbial activity, hydrophobicity and other macroscopic characteristics of granular sludge.[2]

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*Corresponding author. Email etubahan@metu.edu.tr

The common trait of the researches on seed sludge effect is to investigate the physical properties of the seed sludge.[15,16] Wilen et al. [17] investigated the microbial community structure in seed sludge and concluded that the higher the number of floc-formers is, the earlier the cultivation of the granular sludge with higher settleability will be. Sheng et al. [16] investigated the effect of small-loose flocs and large-dense flocs on aerobic granulation in SBRs. Granular sludge development by selective discharge of slow settling biomass was reported for both the flocs. No significant difference in microbial community was observed between the granules obtained from large-dense flocs and small-loose flocs, indicating that granule-forming microorganisms existed in both large-dense flocs and small-loose flocs. It was concluded that effective granulation can be achieved by selection from the community. Xu et al. [18] investigated two different sludge forms, namely activated sludge flocs and sludge pellets (achieved from sludge flocs). It was found that sludge pellets responded better than the sludge flocs and granulation occurred earlier with sludge pellets. Better chemical oxygen demand (COD) removal, larger diameter, higher hydrophobicity and Mg^{2+} content were reported for granules developed from sludge pellets compared with those achieved from activated sludge flocs. It was suggested for pilot-scale or full-scale applications that sludge pellets would be better choice for inoculation than sludge flocs. Verawaty et al. [19] investigated the effect of seed sludge on granule cultivation time. They seeded an SBR with a mixture of floccular sludge and crashed granules. It was stated that crashed granular sludge acted like a packing material and flocs directly attached to them, hence the use of the crashed granules significantly decreased the time required to reach steady-state granular system. Seeding with crashed granules was reported to be an effective strategy to decrease the start-up period and achieve better biological nutrient removal by improving biomass holding capacity.

In studies investigating the effects of seed sludge, conventional activated sludge (CAS) is usually used as seed sludge [15–17] and there is still a gap in literature about the possibility of aerobic granule cultivation by using seed sludge different than CAS. Membrane bioreactor sludge (MBS) differs from CAS in terms of sludge morphology, EPS content and bacterial composition. MBS has a mean floc size of 100–240 µm and CAS has a mean floc size of 70–160 µm.[20,21] Furthermore, EPS amount of MBS flocs is higher than that of CAS flocs, thus they are good at holding bounded EPS.[20,21] It is also stated that MBS contains denser flocs and fewer amounts of long filamentous bacteria than CAS sludge.[21,22] Therefore, this study was conducted to investigate the effects of seed sludge type, namely CAS and MBS, on granulation and treatment efficiency of granules. The results are expected to fill the gaps in the aerobic granulation field about the effects of seed sludge type, thus contributing to the stable granular sludge cultivation and enhancing treatment performances.

Materials and methods

Seed sludge and sequencing batch reactors (SBRs)

The CAS was obtained from the return activated sludge line of secondary clarifier of the Greater Municipality of Ankara Domestic Wastewater Treatment Plant. The MBS, which was also used as seed sludge in experiments, was obtained from membrane unit of METU-Vacuum Rotation Biomembrane Plant. The properties of the seed sludge types are given in Table 1. Before seeding the reactors, both the sludge types were concentrated or diluted (if necessary) to achieve 5000 mg/L initial mixed liquor volatile suspended solids (MLVSS) concentration.

Two identical Plexiglas cylindrical SBRs (R1 and R2) each with a height of 60 cm, inlet diameter of 8 cm, effective volume of 2.45 L and exchange ratio (ratio of discharged effluent volume to the working volume) of 50% were used in the experiments. R1 was seeded with MBS, whereas R2 was seeded with CAS.

Wastewater composition

The synthetic wastewater content was as follows: COD 2000 mg/L (as acetic acid, 1.78 mL/L); NH_4–N 400 mg/L; NO_3–N 40 mg/L; PO_4–P 10 mg/L; $NaHCO_3$ 3000 mg/L; $MgSO_4 \cdot 7H_2O$ 180 mg/L; $CaCl_2 \cdot 2H_2O$ 160 mg/L; $Na_3PO_4 \cdot 12H_2O$ 244 mg/L; yeast extract 2 mg/L and micro nutrients 0.6 mL/L.[23–25]

Experimental procedure

The operational conditions of SBRs were identical during the experiments. Both the seed sludge types were initially acclimated to SBR conditions and synthetic wastewater for 2 days. During the acclimation period, one cycle (6 h) consisted of 5 min of feeding, 120 min of anoxic period, 198 min of aerobic period, 35 min of settling period and 2 min of withdrawal period. Acclimation period, duration of which was decided by monitoring the settling properties of sludge, was conducted for 2 days. Therefore, the possibility to lose sludge content of the reactors was avoided. After 2 days of acclimation, anoxic, aerobic and settling periods were changed in time as given in Table 2 to promote granulation and improve treatment efficiency. Both the reactors were operated for 37 days (Table 2). In the beginning of the operational period of 37 days, MLVSS concentrations

Table 1. The properties of seed sludge used in the study.

Sludge properties[a]	Membrane bioreactor sludge (MBS) (R1)	Conventional activated sludge (CAS) (R2)
MLSS (mg/L)	8560 ± 226	4640 ± 289
MLVSS (mg/L)	5740 ± 113	3860 ± 158
SVI_{30} (mL/g)	103	134

[a] MLSS, mixed liquor suspended solids; MLVSS, mixed liquor suspended solids; SVI_{30}, Sludge Volume Index (30 min).

Table 2. Cycle details during operational period of 37 days.

Periods (min)	Operation days 1–5	6–7	8–13	14–20	21–24	25–26	27–37
Feeding period	5	5	5	5	5	5	5
Anoxic period	120	120	120	90	90	90	90
Aerobic period	208	213	218	248	253	255	258
Settling period	25	20	15	15	10	8	5
Withdrawal period	2	2	2	2	2	2	2

of R1 and R2 were 7670 ± 325 and 4570 ± 183 mg/L, respectively.

Both SBRs had a hydraulic retention time (HRT) of 12.3 h. Solid retention time, which does not influence the granulation process significantly [26], was not controlled during the study. In other words, the withdrawal of the solids was accomplished by selective discharge naturally occurring during the granulation process. During the aerobic periods of the SBR operations, air was supplied by using aeration pumps with a capacity of 180 L air/h, which also provided hydraulic shear force (1 cm/s superficial air velocity). Steel mixers (48 rpm) were used to mix reactors' contents (both in anoxic and aerobic periods). Theoretical organic and total nitrogen (TN) loading rates of the reactors were 3.92 g COD/L d and 0.86 g N/L d, respectively, during the entire operation.

Analytical methods

The pH and DO values of reactor contents were measured daily in the middle of anoxic and aerobic periods. Samples were collected in the beginning of the cycles (after feeding period at $t = 5$ min; as the initial sample) and at the end of the anoxic and aerobic periods of the same cycles (as the effluents). Samples were filtered through 0.45-µm filter paper for further analyses of soluble COD (sCOD), total ammonium nitrogen (TAN: NH_4^+–N+ NH_3–N), NO_2 and NO_3. sCOD was measured by using EPA approved digestion method. Cadmium reduction (high range) and ferrous sulphate (high range) methods were used for the determination of NO_3–N and NO_2, respectively.[27] TAN, mixed liquor suspended solids (MLSS) and MLVSS were analysed according to Standard Methods.[28] The possible free-ammonia (FA) concentrations that microorganisms might be exposed to during the aerobic periods of each cycle were calculated according to Anthonisen et al. [29] via using the monitored pH, temperature and measured TAN concentrations.

Granulation process was monitored by conducting particle size, settling velocity, SVI and EPS measurements. Samples for particle size, settling velocity and SVI measurements were collected on days 0 (seed sludge), 14, 28 and 37. Particle sizes of randomly selected granules (24 granules for each sampling) were measured via ocular micrometer and light microscope (Leitz Wetzlar Microscope), and the average values were calculated for each sampling.[24] Granule pictures were taken by 3.2 Megapixel camera. SVI_5 and SVI_{30} values were measured following the Standard Methods [28] and used to determine the percent of granulation (i.e. $(SVI_{30}/SVI_5) \times 100$) in the sludge.[30] Settling velocities of the granules were measured as defined by Etterer and Wilderer [31]. EPS analyses were performed for weekly collected samples. For EPS extraction, the method (ultrasound → Formamide → NaOH → Centrifuge) suggested as the best EPS extraction method for aerobic granules was used.[32] To avoid any interference coming from the supernatant, prior to EPS extraction, the samples were washed three times with phosphate-buffered saline (PBS) by centrifuging at 3500 rpm and re-suspending in PBS.[33] After extraction, protein and polysaccharide amounts of EPS were measured as defined by Lowry et al. [34] and Dubois et al. [35], respectively.

Results

The effects of seed sludge types on granulation and treatment efficiency were investigated and assessed in three different phases; Phase 1 for Days 1–10, Phase 2 (overloading conditions) for Days 11–27 and Phase 3 (recovery) for Days 28–37.

The observations and results obtained for pH and DO were similar for both reactors. The anoxic and aerobic conditions obtained during the anoxic and aerobic periods of cycles, respectively, were verified by DO concentrations. During the operation period of 37 days, the average DO concentrations recorded in anoxic and aerobic periods were 0.3 ± 0.1 and 8.5 ± 0.4 mg/L in R1, and 0.3 ± 0.2 and 8.5 ± 0.3 mg/L in R2, respectively. During 37 days of operation, the average pH value of feed solution was 7 ± 0.2, while average pH values in anoxic periods were 8.2 ± 0.4 and 8.3 ± 0.2 for R1 and R2, respectively (graphical representation in SI). The average pH values for 37 days in aerobic periods were 8.5 ± 0.3 and 8.7 ± 0.1 for R1 and R2, respectively (see Supplementary data).

Treatment performances

Nitrogen removal

Nitrogen removal performances of reactors were investigated in terms of TAN removal (TAN oxidation), total oxidized nitrogen (TON) removal and TN removal (N loss).

Analyses revealed that the average initial TAN concentrations of R1 during Phases 1, 2 and 3 were 263 ± 73, 343 ± 25 and 250 ± 44 mg/L, respectively, and of R2 were 240 ± 73, 327 ± 21 and 248 ± 45 mg/L for Phases 1, 2 and 3, respectively. Figure 1(a) and 1(b) shows the profiles of TAN loading rate, TAN oxidation efficiencies and effluent TAN concentrations of anoxic and aerobic periods of monitored cycles. TAN loading rates of both the reactors were 0.8 g N/L d during the experiments (Figure 1(a) and 1(b)).

As seen in Figure 1(c), during the operation period, TAN removal efficiencies of reactors were low (5–45%) compared with those obtained (96–99%) in other granulation studies.[36–38] Considering the initial and effluent TAN concentrations of the anoxic periods of the monitored cycles, there was no or negligible TAN oxidation during anoxic periods of both the reactors, as expected (Figure 1). TAN oxidation was mainly obtained during the aerobic periods of the cycles despite the low efficiencies.

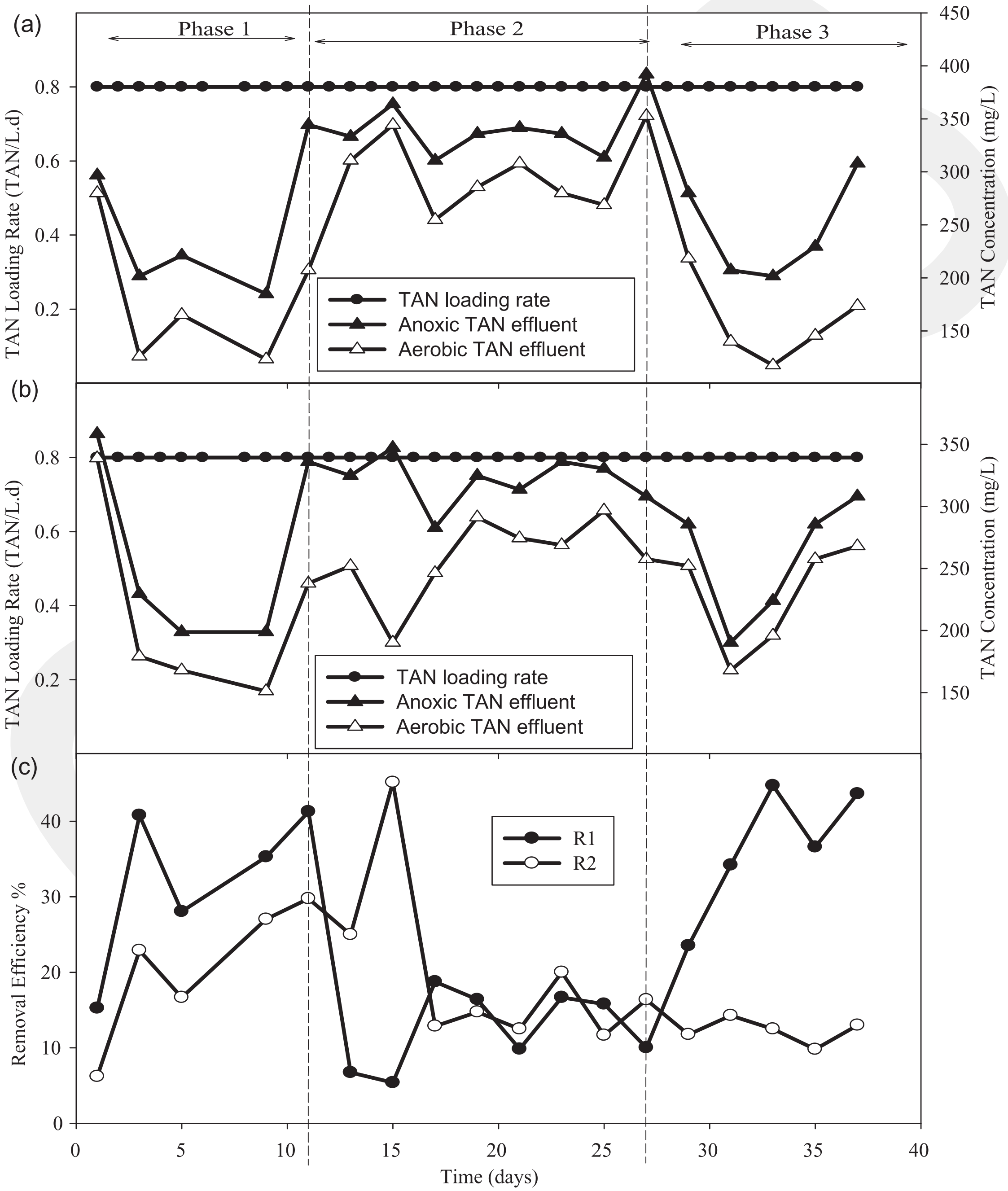

Figure 1. TAN overview of reactors during operation (R1: MBS as seed and R2: CAS as seed). (a) Effluent concentrations and loading rate of R1 (LR), (b) effluent concentrations and loading rate of R2 and (c) removal efficiencies (i.e. cyclic efficiencies obtained considering the concentrations of the samples taken initially and at the end of the cycles).

During aerobic periods of Phase 1, TAN oxidation efficiencies of R1 and R2 fluctuated in the range of 25–40% and 10–30%, respectively (Figure 1(c)). Despite the fluctuations, TAN removal efficiencies generally displayed an increasing trend for both reactors during Phase 1. Average TAN oxidation efficiencies of R1 and R2 were 32 ± 11% and 20 ± 8% in Phase 1, respectively (Table 3). The low treatment efficiencies resulted in TAN accumulation in the reactors and, in turn, further increase in FA concentration to inhibitory levels (38 ± 8 mg/L NH_3–N in R1 and 46 ± 6 mg/L NH_3–N in R2, Table 3).

In the following 16 days (Phase 2), TAN removal efficiency of R1 decreased due to FA inhibition, fluctuated around 10–20% and averaged at 13 ± 5% (Figure 1(c) and Table 3). TAN removal efficiency of R2, on the other hand, reached its peak value (45%) in the beginning of Phase 2 (Day 15, Figure 1(c)). Yet, following Day 15, TAN removal efficiency of R2 also drastically decreased to 10–15%.

Due to TAN accumulation and mentioned FA inhibition, the reactors' contents were washed with water on Day 27 (end of Phase 2). Thereafter, FA concentration in both the reactors was measured as 16 mg/L NH_3-N in the beginning of Phase 3 (Day 28). During Phase 3, TAN removal (oxidation) efficiency of R1 gradually recovered and increased to 40–45% (Figure 1(c)). Yet, the TAN oxidation efficiency of R2 remained same as in Phase 2 and the accumulation of TAN was observed again (Figure 1(b) and 1(c)). The difference in nitrification efficiencies of the reactors was also reflected by the effluent TON concentrations, which were higher in R1. The average TON concentrations measured at the end of aerobic periods of Phase 3 were 12 ± 5 mg/L NO_2–N and 10 ± 3 mg/L NO_3–N in R1, and 4 ± 1 mg/L NO_2–N and 4 ± 5 mg/L NO_3–N in R2. The high effluent TON concentrations of R1 indicate its better TAN oxidation/nitrification efficiency. It was seen that the self-recovery of nitrifiers in CAS floc-granule mixture (R2) was not possible under the high TAN loading rate of 0.8 g TAN/L d investigated in this study. On the other hand, MBS floc–granule mixture (R1) showed better performance in terms of self-recovery of nitrifiers under similar operational conditions.

During 37 days of operation, denitrification was achieved during the anoxic periods of the cycles for both reactors (Table 3). Denitrification efficiencies fluctuated between 60% and 90% (anoxic periods, Table 3). TON removal during aerobic periods (simultaneous nitrification denitrification (SNDN)) is also possible with aerobic granules, since in addition to aerobic zones, anaerobic and anoxic layers (zones) also exist in granular structure due to limited DO diffusion.[9,37,39,40] To speculate for the SNDN performance of reactors, N losses in aerobic periods were calculated according to the N mass balance (Table 3). N losses ranging from 8 ± 1 to 18 ± 11 mg/L were obtained, being much higher for R1 during Phase 3 (Table 3). However, considering the potential FA stripping and its interference with N loss during the aerobic periods, N losses could not be directly linked to the SNDN activity. Thus, SNDN performances of reactors, in particular during Phase 2, could not be assessed exactly.

Organic removal

Organic removal performances of reactors were investigated in terms of sCOD removal. Average initial sCOD values of R1 were 1252 ± 92, 1411 ± 311 and 1154 ± 73 mg/L and of R2 were 1243 ± 97, 1226 ± 145 and 1187 ± 161 mg/L in Phases 1, 2 and 3, respectively. Figure 2(a) and 2(b) shows the profiles of organic loading rate (OLR), sCOD removal efficiencies and effluent concentrations of anoxic and aerobic periods of monitored cycles. As represented in Figure 2(c), during the operation, sCOD removal efficiencies of reactors were low (45–80%) compared with those obtained in literature (85–97%).[36–38] However, R1 performed slightly better sCOD treatment than R2.

Both reactors, each seeded with different sludge types, displayed similar sCOD treatment performances during Phase 1. Total sCOD removal efficiencies of R1 and R2 gradually increased from 47% to 85% and from 46% to 77%, respectively, following the adaptation of both MBS and CAS to the reactor operation, during Phase 1. However, during Phase 2, the removal efficiencies of both the

Table 3. Average cyclic removal efficiencies of each operational period.

	R1[b]			R2[b]		
Parameter[a]	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
TAN RE (%)	32 ± 11	13 ± 5	37 ± 8	20 ± 8	16 ± 11	12 ± 2
TN RE (%)	34 ± 10	14 ± 3	38 ± 8	26 ± 8	19 ± 11	20 ± 3
Denitrification (%)	77 ± 30	87 ± 17	70 ± 8	82 ± 20	88 ± 8	74 ± 10
N loss in aerobic period (mg/L)	9 ± 1	–	18 ± 11	10 ± 3	–	8 ± 1
FA concentration (mg NH_3–N/L)	16 ± 4	38 ± 8	24 ± 8	23 ± 6	46 ± 6	24 ± 7
sCOD RE (%)	70 ± 13	39 ± 12	70 ± 16	67 ± 11	54 ± 15	36 ± 4

[a] RE, removal efficiency; FA, free-ammonia concentration of the initial samples; TAN RE-during aerobic periods, TN RE and sCOD-cyclic removal, Denitrification data for anoxic periods, N loss in aerobic period, SNDN-related removed nitrogen.

[b] Phase 1: Days 1–10, Phase 2: Days 11–27, Phase 3: Days 28–37.

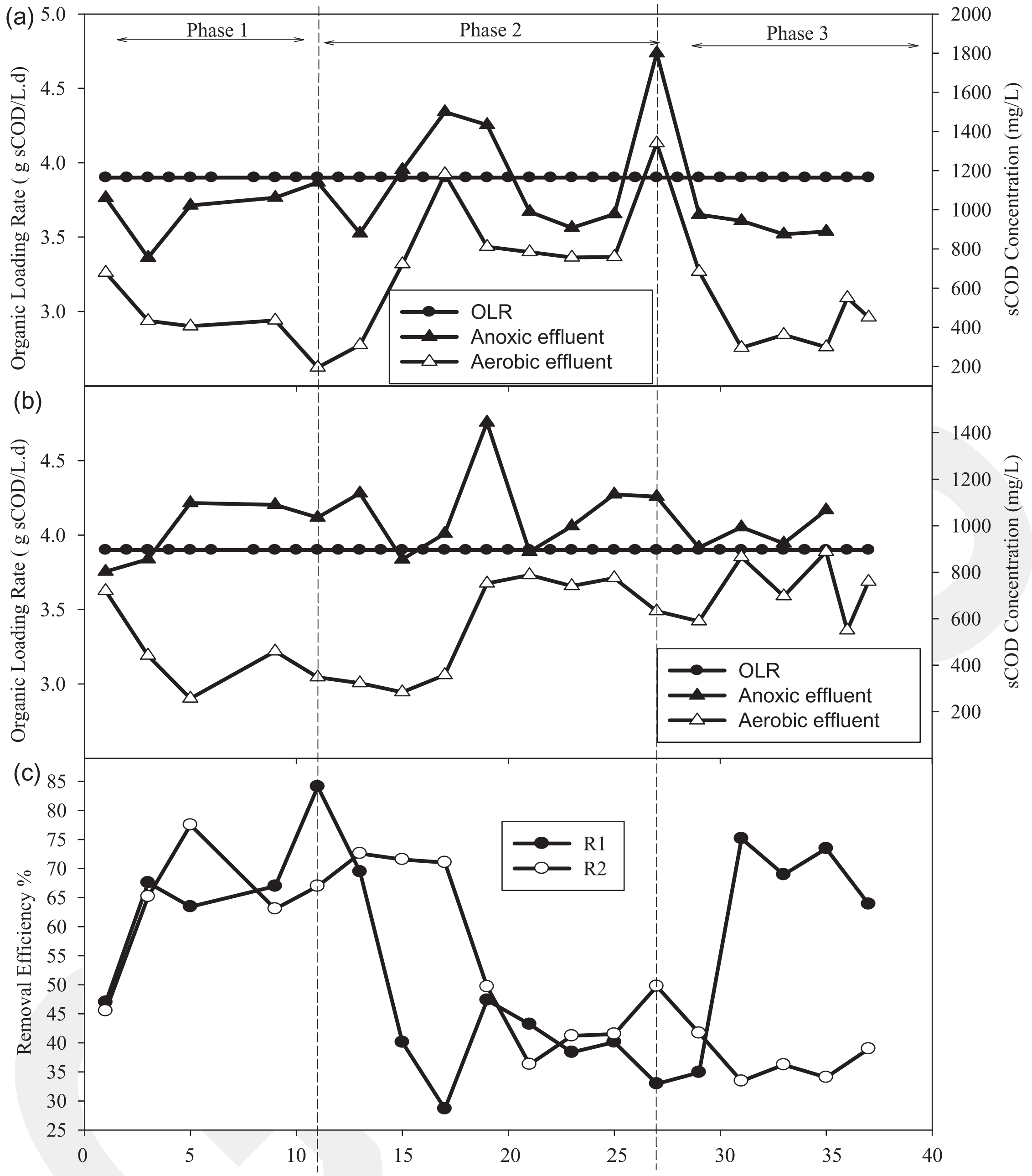

Figure 2. sCOD overview of reactors during operation (R1: MBS as seed and R2: CAS as seed). (a) Effluent concentrations and loading rate of R1 (LR), (b) effluent concentrations and loading rate of R2 and (c) removal efficiencies (i.e. cyclic efficiencies obtained considering the concentrations of the samples taken initially and at the end of the cycles).

reactors decreased to 30–35% (Figure 2(c)). Yang et al. [41] stated that 2.5–39.6 mg/L NH_3–N can inhibit specific oxygen uptake rate (SOUR) of heterotrophs five times. As given in Table 3, the average initial FA concentrations in R1 and R2 increased up to 38 ± 8 mg/L NH_3–N and 46 ± 6 mg/L NH_3–N, respectively. Therefore, the decrease in sCOD removal was attributed to the aforementioned FA inhibition.

During Phase 3, sCOD removal efficiency of R1 gradually increased; however, the low sCOD removal performance of R2 remained still (Figure 2(c)). The average sCOD removal efficiencies of R1 and R2 during Phase 3 were calculated as 70 ± 16% and 36 ± 4%, respectively (Table 3).

Granulation performance

Granulation process was observed by taking samples periodically from the reactors. Evolutions of two seed sludge types were depicted in Figure 3. Granular sludge was developed in both reactors and first appeared on Day 9. However,

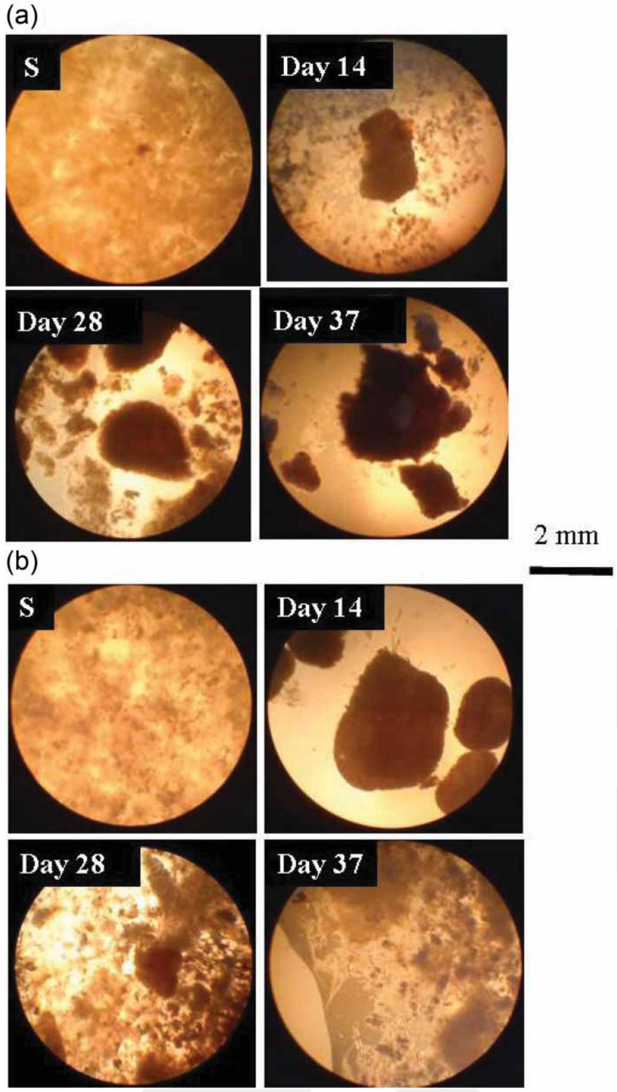

Figure 3. Evolution of the sludge in reactors. (a) R1-MBS as seed and (b) R2-CAS as seed (S: seed sludge, Day 14: representing Phase 1, Day 28: representing Phase 2, Day 37: representing Phase 3).

full granulation (granule percentage of 80–90% [42]) could not be achieved in both reactors till the end of the operation, and the sludge in both reactors was granule–floc mixture dominated.

As seen in Figure 3, the seed sludge of both the reactors was in suspended and floccular form, and they had no granular sludge portion in the beginning. The colours of the seeds were reddish brown and dark brown for R1 and R2, respectively. The colour of active aerobic granules is known to be yellow-brown.[43] In this study, the colour of the sludge in both the reactors also turned from dark reddish-brown to light yellow-brown during granulation. Yet, the colour of the sludge in R2 was lighter than that in R1.

Settling velocities of the granules developed in R1 and R2 were 36–39 and 19–20 m/h, respectively (Table 4). Granular sludge can have settling velocities varying from 18 m/h to more than 91 m/h.[9,36,38,44,45] Although settling velocities of R1 granules were higher than that of R2 granules, they were even not in the mid-range of the literature values.

Microscopic analyses of sludge were conducted on Days 14, 28 and 37. As mentioned before, first granules appeared in both reactors on Day 9. Deterioration of the granular properties (in terms of stability and settleability) was observed for both reactors after Day 15 (corresponding to Phase 2). Therefore, the granules sampled on Day 14 had close similarity to the granules of Phase 1 and thus accepted to represent Phase 1. Similarly, considering the delayed effects of the operational conditions on the physical properties of granules of high strength, the granules sampled on Days 28 and 37 were accepted to represent Phases 2 and 3, respectively. On Day 14, granules with an average size of 1.6 ± 0.8 mm, dense non-filamentous structure and irregular shape were observed in R1 (Figure 3(a) and Table 4). On the other hand, granules in R2 had an average size of 2.2 ± 0.8 mm, filamentous structure and spherical shape (Figure 3(b) and Table 4).

During Phase 2 (Days 11–27), particularly after Day 15, the aforementioned deteriorations in granular properties of both reactors were observed. Disintegrated granules were washed out from the system and caused a decrease in MLVSS concentrations in both the reactors (Table 4). This led to a slight increase in granular sludge percentage (i.e. (SVI30/SVI5)*100), for the ratio values were improved (Table 4). The microscopic analyses performed on Day 28 (at the end of Phase 2) revealed that the granules of R2 decreased in size (1.1 ± 0.5 mm), became loose and

Table 4. Granular sludge properties of each reactor.

	R1[a]			R2[a]		
Parameter[a]	Phase 1	Phase 2	Phase 3	Phase 1	Phase 2	Phase 3
Settling velocity (m/h)	–	36.0	39.6	–	19.1	20.2
Average size (mm)	1.6 ± 0.8	2.5 ± 1.3	1.0 ± 0.5	2.2 ± 0.8	1.1 ± 0.5	0.6 ± 0.2
MLVSS[b] (g/L)	4.6–2.4	2.4–1.5	1.5–3.0	4.5–2.4	2.4–1.4	1.4–1.7
MLVSS/MLSS	0.71	0.84	0.75	0.90	0.77	0.52
Granule[c] (%)	34	37	32	33	37	22

[a] Phase 1: Days 1–10, Phase 2: Days 11–27, Phase 3: Days 28–37.
[b] MLVSS values were analysed at the beginning and end of each phase.
[c] Granule (%): granular sludge percentage, $(SVI_{30}/SVI_5) \times 100$).

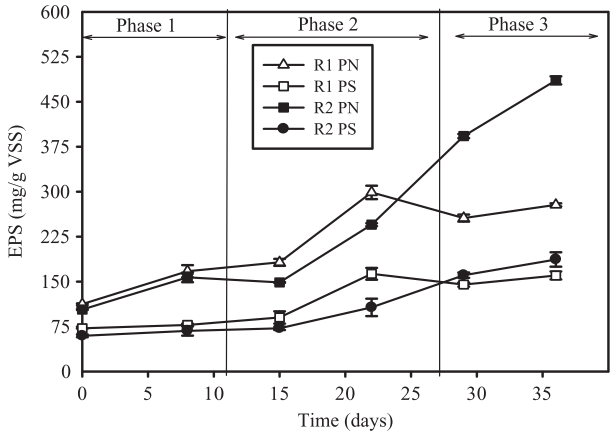

Figure 4. Variations in the EPS contents of the granules developed during operation (R1: MBS as seed, R2: CAS as seed, PN: protein, PS: polysaccharides).

were dominated with filamentous structure (Figure 3(b) and Table 4). A portion of the granules in R1 also disintegrated at the end of Phase 2. However, the average granule sizes increased to 2.5 ± 1.3 mm. This observation revealed that the intact R1 granules kept growing in size and were more dominant than the disintegrated and floccular forms in the reactor by the end of Phase 2 (Figure 3(a) and Table 4).

During Phase 3, R1 granules, despite the decrease in size, remained in their intact structure, whereas R2 granules decreased in size and also developed a fluffy structure. The average particle sizes of the granules sampled from R1 and R2 at the end of the experiment (Day 37) were measured as 1 ± 0.5 and 0.6 ± 0.2 mm, respectively (Table 4). MLVSS/MLSS ratio was more or less constant in R1, while it decreased from 0.9 to 0.52 in R2 (Table 4).

Granulation and disintegration patterns were also followed by EPS of the granules developed (Figure 4). Protein (PN)-EPS and polysaccharide (PS)-EPS contents of the seed sludge of both reactors were around 100 and 75 mg/g VSS, respectively. During Phase 1, PN-EPS increased to 180 and 160 mg/g VSS for R1 and R2 sludge, respectively. PS-EPS was almost stable during granulation in Phase 1. PN/PS ratio was reported to increase sharply during granulation.[46,47] Therefore, the increase in PN amount was attributed to the granule formation in Phase 1. In Phase 2, both protein and polysaccharide contents of EPS significantly increased from 180 to 300 mg/g VSS and from 160 to 250 mg/g VSS in R1 and R2, respectively. PN and PS contents of granules in R1 almost stabilized in Phase 3. However, PN increase in R2 continued significantly up to a concentration of 500 mg/g VSS, while PS increased to a value of 186 mg/g VSS.

Discussion

SBRs were operated for 37 days (12.3 h HRT) at high TAN loading rates (0.8 g N/L d) and low COD/TAN ratio of 5. Partial granulation was achieved under these stressful conditions in both reactors seeded with different sludge types. Yet, the responses of the reactors and the properties of the sludge content of the reactors differed. The effect of the seed sludge type was evaluated and discussed herein with respect to the properties of granular-floc sludge developed in SBRs, their responses to the varying operational conditions and removal efficiencies.

TAN removal efficiencies of both reactors were in an increasing trend in Phase 1 (Figure 1(c)). Figure 2(c) reveals the similarity between the trends of R1 and R2 in organic removal efficiencies as well. Yet, R1 achieved slightly better sCOD and TAN removal performances than R2 during Phase 1 (Figures 1(c) and 2(c)). However, this difference was still not significant enough to discriminate between the seed sludge types during Phase 1.

TAN removal efficiencies on Day 11 were three times higher than that in Day 1 and organic removal efficiencies increased up to one and a half times of the initial in both reactors by Day 9 (Figures 1(c) and 2(c)). These observations indicated the enrichment of nitrifying and heterotrophic bacteria in both sludge types. Despite the mentioned enrichment, TAN removal efficiencies in Phase 1 were far less than the existing literature data (around 96–99%) and organic removal efficiencies were around the lower limit of the existing literature data (85–97%).[36–38] The initial reason of low TAN removal efficiency in Phase 1 might be the pH values obtained during aerobic periods which were at the upper limit (pH ∼ 8.6) of the optimum pH range defined for nitrification as pH 7.5–8.6.[48] The low TAN treatment efficiency and, in turn, TAN accumulation due to cyclic nature of SBR operation and related operational parameters caused more severe problems in further stages of the study. One of the important operational parameters of SBR is the volumetric exchange ratio. In most of the studies, 50–60% volumetric exchange ratio was suggested for aerobic granulation.[2,11,44] Granulation-oriented SBR systems, which are operated at low volumetric exchange ratios to increase the selection pressure on microorganisms, are very prone to accumulation and overloading in case of low treatment performance conditions. As summarized in Table 3, the average TAN removal efficiencies of R1 and R2 were 32 ± 11% and 20 ± 8%, respectively, in Phase 1. The high TAN loading rate (0.8 g N/L d) and the mentioned low removal efficiencies in Phase 1 caused TAN accumulation in the reactors, increasing by each cycle (up to the influent feed concentrations) and led to the overloading conditions (defined as Phase 2, Days 11–27). TAN accumulation could easily cause FA inhibition for nitrifiers under the pH conditions (∼8.6) and loading rates experienced in this study. Anthonisen et al. [29] reported that 10–150 mg/L FA (8.2–123 mg/L NH_3–N) concentration inhibited the activity of nitrifiers in suspended sludge. Additionally, Yang et al. [41] reported that 2.5–39.6 mg/L NH_3–N inhibited the microbial activity (respirometric activity) of nitrifiers and aerobic heterotrophs in a nitrifying granular sludge system by two and a half and five times, respectively. The average FA concentrations for each phase are calculated and given in Table 3. During Phase 2, FA concentrations in the reactors were around aforementioned toxic levels (38 ± 8 mg/L NH_3–N in R1 and 46 ± 6 mg/L NH_3–N in R2, Table 3) with peak values of 47 and 55 mg/L NH_3–N for R1 and R2, respectively. Therefore, the high FA portion of TAN under the studied loading rates, experienced pH and temperature conditions resulted in severe inhibition of ammonia-oxidizers. Negligible or no TON production during aerobic periods in Phase 2 was a strong indication for inhibition of ammonia-oxidizers in both reactors. Thus, the removed TAN concentrations observed in Phase 2 were mostly attributed to the FA stripping rather than TAN oxidation and hence not taken into account during the assessment of SNDN performances (Figure 1 and Table 3).

Results also indicated that FA inhibition severely affected the organic removal in both reactors. Yet, there was 4 days of delay in inhibition of organic removal performance of R2 compared with R1 (Phase 2, Figure 2(c)). The delay was attributed to the granule size, shell structure and further abundant microbial group of the granules. At the end of Phase 1, granules developed in R2 had greater sizes than those of R1 (Table 4). Gao et al. [9] state that granules have shell structure and microorganisms are systematically located. The outmost, the inner and the innermost shells are composed of aerobic heterotrophs, nitrifiers and denitrifiers/anaerobic autotrophs, respectively.[9] Even if the certain granular properties (compactness and biomass content) are similar, the granules of greater sizes might resist to high loading rates much better than the smaller-sized granules.[49] Considering the similar MLVSS concentrations in both reactors (during Phase 1) and greater-sized granules in R2 (Table 4), it was concluded that R2 granules might have contained more aerobic heterotrophs than that of R1 granules. This might explain the higher tolerance in R2 and in turn the 4 days (16 cycles) of delay in organic removal inhibition in R2 (Figure 2(c)). The possibility of having different bacterial abundance in granular structure of MBS and CAS might also be explained by the developed granules of different colours. The colour of the sludge is generally an indicator of the predominant microorganisms or the chemical composition of the content in reactors.[9] In this study, the colour of the sludge in R2 was lighter than that in R1. Luxmy et al. [50] stated that bacterial community in MBS is significantly different than that of CAS. Therefore, the colour difference observed in reactors might be due to the predominance of the different bacterial communities. The different bacterial abundances in granular structure of MBS and CAS might result in different responses to certain conditions, which is consistent with the findings of Gao et al. [9] and Luxmy et al. [50].

Microscopic analyses indicated the difference between the granules developed in R1 and R2, in terms of their physical appearance (Phase 1, Figure 3). The granules obtained from CAS sludge (R2) had filamentous structure while granules developed from MBS (R1) were non-filamentous (Day 14, Figure 3). Cicek et al. [22] also observed that filamentous microorganisms were in greater amounts in CAS compared with that in MBS (and mostly placed inside the flocs). Janczukowicz et al. [51] reported that filamentous microorganisms are the indicators of sludge bulking, which enriches at low F/M conditions, low or high DO concentrations or due to inappropriate reactor configuration, and have adverse effects on settling properties. Therefore, non-filamentous structure of granules in R1 might indicate the successful adaptation of MBS to the stressful conditions applied for granulation in Phase 1. Yet in R2, granular sludge having filamentous structure revealed that adaptation of CAS sludge to the stressful conditions was still not successfully completed and delayed. The granules with filamentous structure as observed in R2 might also result in poor settling properties and stability of the granules.[9,51] Settleability of R1 granules was better than that of R2 granules in Phase 2 (Table 4), which can be attributed to the better structural properties and adaptation capability of the former.

According to the microscopic analysis results of Phase 2, developed granules started to disintegrate in both reactors, disintegration being more severe in R2. The reason behind severe disintegration of granules in R2 might be due to the FA inhibition in Phase 2. Yang et al. [41] indicated that FA concentration higher than 23.5 mg/L prevents granule formation. Aforementioned FA concentrations during Phase 2 could easily result in disintegration of granules and decrease in MLVSS concentrations (Figure 3 and Tables 3 and 4). Disintegration/degradation of the granules in R2 was also confirmed by the decreasing trends of MLVSS/MLSS ratio values and the granular sludge percentages of the total biomass content through Phases 1 to 3 (Table 4). In contrast to R2, R1 had more or less constant MLVSS/MLSS ratio (Table 4). MLVSS/MLSS ratio gives an approximate idea about the biologically active biomass content of the sludge.[52] Therefore, continuous decrease in MLVSS/MLSS ratio indicated that the amount of active microorganisms was decreasing in R2. Based on these results, it can be claimed that granule–floc mixture of R1 was more resistant to the studied high loading rates and potential toxicity (occurred during Phase 2) than that of R2.

EPS analyses could also explain the behaviour of sludge at different conditions (Figure 4). The sharp EPS increase observed for both reactors during Phase 1 was attributed to the granulation.[46,47] The increase was more severe for both reactors during Phase 2. It was revealed that the increase in OLR and stressful conditions caused an increase in EPS concentration,[53,54] in particular after Day 15. If it is considered that the granules were partially disintegrating and granulation was halted during Phase 2, the FA toxicity (stressful conditions) and high initial (TAN) concentrations could be the main reason of the sharp EPS increase in both reactors.

The performances of the reactors were not promising for the self-recovery during Phase 2. Therefore, the reactor contents were treated and regulated externally. The accumulation inside the reactor had been removed by washing (diluting) the reactor content and afterwards recovery performances of the reactors were investigated (Phase 3). Ammonia oxidation and organic removal performance of MBS granules (R1) rapidly recovered to some extent after the inhibitory conditions were minimized. However, no recovery was observed for CAS granules (R2) in terms of ammonia oxidation and organic removal performance (Figure 1(c) and 2(c)). Manser et al. [55] stated that there is no difference between MBS and CAS systems in terms of nitrifier diversity and treatment performances. It was also mentioned that nitrifiers were located in the MBS flocs much deeper than those located in CAS flocs. The TAN oxidation recovery performances observed in Phase 3 indicated that MBS flocs and thereby developed granules had more appropriate structure for conservation of ammonia-oxidizers and nitrite-oxidizers from toxic environment. This appropriate structure and better protection of nitrifiers might have resulted in better TAN oxidation recovery of MBS flocs and granules than CAS flocs and granules. Since CAS sludge and granules were poor in recovery performance (Phase 3) in terms of TAN oxidation, TAN accumulation and high pH related FA inhibition still remained (Table 3). Therefore, observing no sign of recovery for organic removal efficiency and the on-going decrease in granule size (by disintegration) in R2 were attributed to the existing FA inhibition on aerobic heterotrophs [41] during Phase 3. On the other hand, rapid TAN oxidation recovery of MBS improved the conditions to the appropriate FA levels for the recovery of organic removal. It was stated that optimum granule size for effective nutrient removal is 1.2–1.4 mm.[40] At the end of Phase 3, granules of R1 were close to the size range given, but granules of R2 were smaller in size for effective nutrient removal (Table 4).

EPS analyses also well reflected the effect of Phase 3 and related operational conditions on granules. The on-going increase in EPS concentration of R1 observed during Phase 2 (due to stressful conditions) ended in Phase 3, while it was still continuing in R2. The continuous increase of EPS in R2 could be an indication of on-going stress and toxicity in Phase 3, while stable EPS production of R1 indicated the successful recovery (Figures 1(c) 2(c) and Figure 4). Mu et al. [56] revealed that high levels of EPS clogged the pores of granular sludge and decreased the substrate gradient, which would lead to the granule disintegration. Durmaz and Sanin [33] also stated that the PN content of EPS increased if cell lysis occurred. Therefore, continuous increase of EPS content, in particular PN-EPS, observed in R2 during Phases 2 and 3 might have negatively affected the granules and caused disintegration of granules and cell lysis, which led to more EPS production.

During the entire operation, denitrification performances of both reactors in anoxic periods were between 60% and 90% and showed similar trends. Denitrification performances were also confirmed with pH increase during anoxic periods (Graphical representation in SI) as a result of OH^- production. The pH increase (0.3–0.4 units) was also observed during aerobic periods in both reactors. This increase was attributed to SNDN-related OH^- production. Under oxygen-limited or oxygen-lacking conditions (non-aerated periods), if NO_x ($NO_3 + NO_2$) exists, heterotrophs use NO_x as the electron acceptor and denitrification occurs during which OH^- ion is produced.[57] Both oxidation of TAN and reduction of TON during aerobic periods (i.e. SNDN) can be achieved with aerobic granules despite the high DO level.[9,37,39,40] Yoo et al. [48] also achieved significant SNDN performance with suspended sludge while DO level was around 2–2.5 mg/L, which is likely due to floc nature of the sludge and limitation of DO diffusion. Therefore, SNDN might have occurred during the aeration periods and led to pH increase. As previously mentioned, N losses observed during aerobic periods indicate the potential SNDN (Table 3). The low amounts of N losses in Phases 1 and 3 might be due to the inhibition-related low ammonia oxidation efficiencies and in turn low amount of TON production. On the other hand, no SNDN activity and TAN removal through FA stripping at high pH were likely the cases observed during Phase 2.

Conclusion

Granules were developed from both sludge types at the same time. Yet, the ones cultivated from MBS were found to be more advantageous. The granules developed from MBS had greater size and showed higher tolerance to FA toxicity (38–46 mg/L NH_3–N), which enabled well protection of the granule structure and the nitrifiers, thus increased stability and eased the recovery, respectively. It was shown that FA concentrations of 38–46 mg/L totally inhibited TAN oxidation and decreased sCOD oxidation by 55–60%. However, MBS granules performed better TAN oxidation efficiency (37 ± 8%) and sCOD removal efficiency (70 ± 16%) than CAS granules (TAN oxidation 12 ± 2% and sCOD removal 36 ± 4%) after recovery period. Higher settling velocities (36–39 m/h MBS and 19–20 m/h CAS) were obtained with the MBS granules; thus, one can expect easier separation from the effluent. Additionally, they had higher biomass retention (2.8 g/L MBS granules and 1.5 g/L CAS granules), which facilitated the withstanding to high loading rates (0.8 g TAN/L d and 3.92 g COD/L d). In other words, using MBS instead of CAS as inoculum can decrease the start-up duration of the reactors.

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Supplemental data

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