Bioresource Technology 148 (2013) 149–156

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

The effects of aerobic/anoxic period sequence on aerobic granulation and COD/N treatment efficiency

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HIGHLIGHTS

- Aerobic granules were developed by both anoxic–aerobic and aerobic–anoxic periods.
- More stable granules of greater sizes were developed at anoxic–aerobic period.
- Post-anoxic application resulted in granule disintegration due to lack of carbon.
- Sulfide and NH_3–N inhibited both COD and TAN removal efficiency.
- Sulfide-related inhibition is more severe for TAN oxidation.

ARTICLE INFO

Article history:
Received 8 May 2013
Received in revised form 13 August 2013
Accepted 17 August 2013
Available online 29 August 2013

Keywords:
Granule
Inhibition
Simultaneous nitrification–denitrification
Extracellular polymeric substance (EPS)

ABSTRACT

The effects of period sequence (anoxic–aerobic and aerobic–anoxic) on aerobic granulation from suspended seed sludge, and COD, N removal efficiencies were investigated in two sequencing batch reactors. More stable granules with greater sizes (1.8–3.5 mm) were developed in R1 (anoxic–aerobic sequence). Yet, no significant difference was observed between the reactors in terms of removal efficiencies. Under optimum operational conditions, 92–95% COD, 89–90% TAN and 38–46% total nitrogen removal efficiencies were achieved. The anoxic–aerobic period sequence (R1) resulted in almost complete denitrification during anoxic periods while aerobic–anoxic sequence (R2) led to nitrate accumulation due to limited-carbon source and further granule disintegration. NH_3–N concentration of 15–28 mg/L was found to inhibit COD removal up to 30%. This study also revealed the inhibitory sulfide production during anoxic periods. Sulfate concentration of 52.6–70.2 mg/L was found to promote sulfate reduction and sulfide generation (0.24–0.62 mg/L) which, together with free-ammonia, inhibited TAN oxidation by 10–50%.

1. Introduction

Aerobic granules contain high amount of microorganisms in dense form, have high settling velocities and high resistance to toxic compounds and fluctuating loading rates (Liu and Tay, 2004). Therefore they are more advantageous compared to suspended sludge (Liu and Tay, 2004). Due to their phosphorus and nitrogen removal capacities, they are also superior to anaerobic granules (Liu and Tay, 2004). Wastewater treatment with aerobic granules in sequencing batch reactors (SBRs) is thus considered as an efficient technology in the last decade (Liu and Tay, 2004). Yet, there are still some aspects to be improved and thus widely researched. One of these aspects is the problems encountered with the low stability of aerobic granules due to high growth rates of heterotrophic microorganisms (Adav et al., 2008a). In addition, there are various parameters affecting the stability such as extracellular polymeric substance (EPS), predominant EPS content, starvation and flocculating capability of strains in seed (Adav et al., 2008a; Liu and Tay, 2004, 2008). A new parameter, i.e. non-aerated periods, came into consideration with the studies researching nutrient removal (Jang et al., 2003; Wan et al., 2009; Yilmaz et al., 2008). It is proved that the presence of anoxic and/or anaerobic periods in an SBR cycle is beneficial to improve granular density, compactness and microbial diversity (Su et al., 2012; Wan et al., 2009). Anoxic period stimulates the growth of denitrifiers. Denitrifiers locate in the deeper parts while the slow-growing nitrifiers locate close to the surface of the granules. This systematic placement avoids competition for space and increases granular stability (Wan et al., 2009). The competition for carbon between heterotrophic denitrifiers and aerobic carbon removers also stimulates the growth of nitrifiers, even at high organic loading rates, leading to better granulation, higher stability and diversity (Wan et al., 2009). Zhang et al. (2011) stated that long-term stable

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http://dx.doi.org/10.1016/j.biortech.2013.08.096

granules could be achieved with the sequence of anaerobic–aerobic–anoxic periods in an SBR operation. Most of the organic matter is used during anaerobic or anoxic period and famine phase occurs in the subsequent aerobic period, which enhances microbial auto-aggregation (de Kreuk and van Loosdrecht, 2006).

The other advantage of adding an anoxic period in an aerobic SBR operation is the improvement of simultaneous nitrification–denitrification (SNDN) (Adav et al., 2009a; Jang et al., 2003; Zhang et al., 2011). Anoxic periods of SBR cycles help the enrichment of nitrate-reducers in the oxygen-limited core of the granules during cultivation (Wan et al., 2009) and therefore enable SNDN even during aerobic periods (Jang et al., 2003). Adav et al. (2009a) reported enhanced SNDN and almost complete nitrogen removal with 2 h of aerobic and anoxic operations, while they observed nitrate accumulation under fully aerobic conditions.

The researches on granulation usually integrate the anoxic or anaerobic periods randomly in an SBR cycle (Adav et al., 2009a; Jang et al., 2003; Su et al., 2012; Wan et al., 2009; Yilmaz et al., 2008). To the authors' knowledge, there is no detailed research that compares and investigates the effect of period sequence (aerobic–anoxic sequence to anoxic–aerobic sequence) on granulation process and treatment efficiency. Therefore, this study was conducted to investigate and compare the effects of different period sequencing (anoxic–aerobic and aerobic–anoxic) on aerobic granulation and removal of COD and nitrogen. The effect of influent sulfate concentration on treatment performance of granules was also investigated.

2. Methods

2.1. Wastewater composition and seed sludge

The synthetic wastewater content was as follows: COD 1500 mg/L (as acetic acid); NH_4-N 200 mg/L; NO_3-N 40 mg/L; PO_4–P 10 mg/L; $NaHCO_3$ 1500 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; micro-nutrients 0.6 mL/L (Erguder and Demirer, 2005; Shi et al., 2010; Smolders et al., 1994). $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were used as Mg^{2+} and Ca^{2+} sources for granulation, respectively (Gao et al., 2011).

Suspended seed sludge was obtained from membrane bioreactor of METU Wastewater Treatment Plant. The seed sludge was initially acclimated to the SBR operation and synthetic wastewater. To this purpose, one SBR was seeded with suspended sludge of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations of 7300 and 5000 mg/L, respectively. An acclimation protocol was further applied as shown in Table 1.

2.2. Experimental procedure

2.2.1. The effect of period sequence

Two similar SBRs (R1 and R2), each with a height of 60 cm, inlet diameter of 8 cm, total working volume of 2.45 L and exchange ratio of 50%, were operated under the same operational conditions for 63 days (Table 2). The reactors differed only in period sequence; R1 was operated with anoxic–aerobic sequence, while R2 was operated with aerobic–anoxic sequence. Both reactors were seeded with almost equal amounts of acclimated suspended sludge. The initial MLVSS concentrations of R1 and R2 at the beginning of the operational period were 1180 ± 118 and 1230 ± 102 mg/L, respectively. Both reactors were operated at 6 h-cycles composed of feeding (10 min), anoxic (45 min), aerobic (288–302 min), settling (gradually decreasing from 15 to 1 min) and withdrawal (2 min) periods (Table 2).

Table 1
Operational conditions during acclimation period of 14 days.

Operational conditions	Acclimation period (days)					
	1	2–4	5	6–9	10	11–14
Number of cycles/day	1	1	2	2	2	4
Organic loading rate (g COD/L.d)	0.19	0.39	0.78	1.18	1.95	2.94
Total N loading rate (g N/L.d)	0.024	0.048	0.094	0.14	0.24	0.47

In order to achieve almost the same F/M ratio (1.2 g COD/g d and 0.19 g N/g d) as that of the final conditions of the acclimation period (Table 1, Days 11–14), synthetic wastewater content was diluted by half. Thus influent COD:TAN concentrations were 750:100 (mg/L:mg/L) for both reactors for the first 9 days of the operation period. Following the doubling of the MLVSS concentrations in both reactors (>2000 mg/L, Day 10), influent COD:TAN concentrations were set as 1500:200 (mg/L:mg/L). Therefore, theoretical organic loading rate (OLR) and nitrogen loading rate (NLR) were 2.94 g COD/Ld and 0.47 g N/Ld, respectively (Table 2). After the development of more than 65% granular sludge in the reactors (Day 15), influent NO_3–N was decreased from 40 to 20 mg/L, leading to an NLR of 0.43 g N/Ld (Day 17). In addition, the influent sulfate concentration of 70.2 mg/L ($MgSO_4 \cdot 7H_2O$ of 180 mg/L) was decreased by half on Day 42 in order to investigate the effect of sulfate concentration on treatment performance.

Reactors were operated at an upflow air velocity of 1–2 cm/s (during aerobic periods) and a hydraulic retention time (HRT) of 12.3 h through the study. The reactor contents were mixed with 48 rpm-mixers. After almost full granulation was achieved (Day 22), mixers were removed (Day 25) to prevent mechanical destruction of the granules (Nor Anuar et al., 2012). Following the removal of mixers, in order to protect the hydraulic selection provided by the settling time in R2 (aerobic–anoxic sequence), reactor content was mixed by 1 min of aeration before the start of the settling period.

Influent pH was set as 6.9 ± 0.2 throughout the study. The pH of the reactors was not controlled. The daily recorded pH values of R1 were ranging between 7–8.5 and 8.0–8.8 during anoxic and aerobic periods, respectively, while those of R2 were 8.0–8.5 and 8.5–9.0 during aerobic and anoxic periods, respectively (data not shown). DO concentrations of both reactors were less than 0.5 mg/L during anoxic periods and more than 6 mg/L during aerobic periods (data not shown).

2.2.2. The effect of influent sulfate concentration

The results of the study on period sequence effect (Section 2.2.1) indicated that influent sulfate concentration might have negatively affected the treatment efficiency of the developed granules. In order to investigate the effect of sulfate in detail, an SBR was operated with wastewater of same influent characteristics as that of R1 (Section 2.2.1) except the influent SO_4 concentration. The SBR, initially exposed to influent SO_4 concentration of 35.1 mg/L, was further fed with higher influent sulfate doses such as 46.8 mg/L, 52.6 mg/L, 58.5 mg/L and 70.2 mg/L (SO_4 source; $MgSO_4 \cdot 7H_2O$). The variations in the treatment efficiencies, granule size and properties with respect to the varied influent SO_4 concentration were investigated. As seed sludge, granular sludge obtained from R1 (Section 2.2.1) was used (after operated for 67 days of steady-state conditions, see Supplementary Information (SI)). The SBR operational conditions were same as that of R1 (Table 2, Days 49–63).

2.3. Analytical methods

The pH and DO values of reactor contents were measured daily in the middle of anoxic and aerobic periods. Samples were

Table 2
Cycle details during operational period of 63 days.[a]

Operational conditions	Operation (days)							
	1–9	10–13	14	15–16	17–25	26–39	40–48	49–63
Modifications	LR × 2	→I						
	Influent NO_3 halved – 40 mg/L				→II (20 mg/L)			
	Mixers removed					→III		
	Aeration doubled – 3 L/min						→IV (6 L/min)	
	Influent SO_4 halved – 70.2 mg/L							→V (35.1 mg/L)
Number of cycles/day	4	4	4	4	4	4	4	4
OLR (g COD/Ld)	1.47	2.94	2.94	2.94	2.94	2.94	2.94	2.94
Total NLR (g N/Ld)	0.24	0.47	0.47	0.47	0.43	0.43	0.43	0.43
Periods and durations in one cycle								
Feeding period (min)	10	10	10	10	10	10	10	10
Anoxic period (min)	45	45	45	45	45	45	45	45
Aerobic period (min)	288	298	299	301	301	298	301	302
Settling period (min)	15	5	4	2	2	5	2	1
Withdrawal period (min)	2	2	2	2	2	2	2	2

[a] LR: Loading Rate, OLR: Organic Loading Rate, Total NLR: Total nitrogen loading rate (Nitrate-N + TAN in feed). Periods in R1: feeding, anoxic, aerobic, settling and withdrawal. Periods in R2: feeding, aerobic, anoxic, settling and withdrawal.

collected at the beginning of the cycles (after feeding period at $t = 10$ 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 sCOD, Total Ammonium Nitrogen (TAN: NH_4^+-N + NH_3-N), NO_2, NO_3, sulfate and total soluble sulfide analyses. sCOD, NO_3, NO_2, SO_4 and total soluble sulfide were analyzed spectrophotometrically by using EPA approved digestion method (for sCOD), ferric sulfate method (for NO_3-N), cadmium reduction methods (for NO_2), SulfaVer 4 turbidity method (for SO_4) and methylene blue method (for total soluble sulfide) (Hach Water Analysis Handbook, 2012). COD values shown in the text, figures and tables refer to the sCOD values. TAN, MLSS and MLVSS analyses were done according to Standard Methods (APHA, AWWA, WEF, 2005). 'Theoretical initial value' term used in the text (especially for Days 50–63) indicates the exact concentration expected after feeding, when biochemical reaction during feeding period was neglected. Theoretical initial values were calculated using the concentrations of influent and the previous cycles' effluent, and exchange ratio (i.e., (influent concentration + previous cycle's effluent concentration) × 0.50). 'Initial measured value' indicates the concentration measured at the end of the feeding period ($t = 10$ min).

Granulation process was followed by weekly sampling. Particle sizes of randomly selected granules (24 granules for each sampling) were measured via ocular micrometer and light microscope (Leitz Wetzlar Microscope) and average values were calculated for each weekly sampling (Erguder and Demirer, 2005). Granule pictures were taken by 3.2 Megapixel camera. SVI_5 and SVI_{30} values were measured following the Standard Methods (APHA, AWWA, WEF, 2005) and used to determine the percent of granulation (i.e., $(SVI_{30}/SVI_5) \times 100$) in the sludge (Liu et al., 2010). Settling velocities of the granules were measured as defined by Etterer and Wilderer (2001). EPS concentrations of weekly samples were also measured. For EPS extraction, the method (Pre-ultrasound + Formamide + NaOH + Centrifuge) suggested as the best EPS extraction method for aerobic granules was used (Adav and Lee, 2008). In order to avoid any interference coming from supernatant, prior to EPS extraction, the samples were washed 3 times with phosphate buffer saline (PBS) solution by centrifuging at 3500 rpm and resuspended in PBS solution (Durmaz and Sanin, 2001). After extraction, protein and polysaccharide amounts of EPS were measured as defined by Lowry et al. (1951) and Dubois et al. (1956), respectively.

3. Results and discussion

3.1. The effect of period sequence

3.1.1. Treatment performance

Fig. 1 indicates the COD and TAN removal efficiencies of both reactors. As previously mentioned (Section 2.2.1), COD and TAN concentrations of feed solution were adjusted to 1500 and 200 mg/L, respectively, on Day 10 (Modification I), and remained constant till Day 63. The initial average COD concentrations (measured after feeding periods, $t = 10$ min) were 803 ± 66 and 808 ± 65 mg/L for R1 and R2, respectively (Days 10–63). The average initial TAN concentrations measured ($t = 10$ min) were 131 ± 17 and 139 ± 18 mg/L for R1 and R2, respectively (Days 10–63).

3.1.1.1. COD removal efficiency. COD removal efficiencies of both reactors were less than 80% and displayed fluctuating trend during the first 42 days of operation (Fig. 1a). In order to improve the treatment efficiencies and achieve steady performance, operational modifications such as decreasing the influent NO_3-N concentration to 20 mg/L (Modification II), plugging out the mixers (Modification III) and the doubling of the aeration rate to 6 L/min (Modification IV) were performed. However, the low treatment performances and unsteady conditions did not recover.

The pH analyses interestingly indicated the sudden pH increase from 7.5 to more than 8.5 within the first 15 min of the aerobic periods in both reactors. This was attributed to the alkalinity production in the preceding (idle/non-mixing) anoxic periods and its further immediate distribution in the reactor with the start of the aeration during aerobic periods. Alkalinity production during anoxic conditions, further pH increase and related dominance of NH_3–N content of TAN might have led to the inhibition of both TAN and COD removal due to free-ammonia toxicity, concentration of which were around 15 mg/L and 18 mg/L NH_3–N for R1 and R2, respectively. It was reported that the specific oxygen uptake rate of aerobic heterotrophs was inhibited by 5 times at 2.5–39.6 mg/L NH_3–N (Yang et al., 2004). In order to decrease the level of pH increase, the influent nitrate concentration was decreased by half (Modification II) as an attempt to decrease the alkalinity amount produced by denitrification, as previously mentioned. However, system performances did not recover and similar level of pH increase was still observed at the beginning of the aerobic periods.

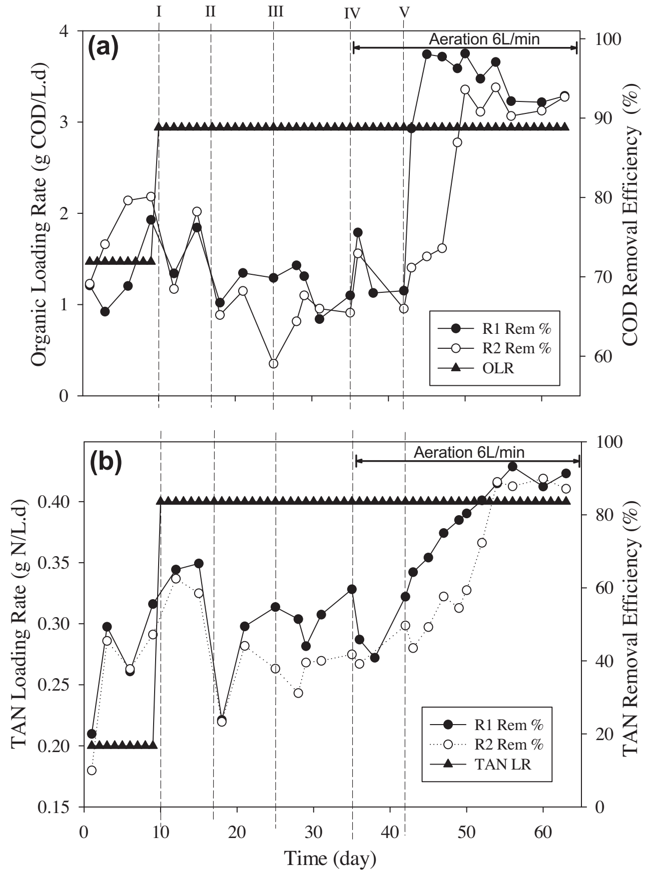

Fig. 1. (a) COD, and (b) TAN removal efficiency of reactors (Modifications: I – COD:TAN increase to 1500:200 mg/L, II – influent NO_3-N decrease to 20 mg/L, III – mixers plugged out, IV – aeration increase to 6 L/min and V – influent $MgSO_4{\cdot}7H_2O$ decrease to 90 mg/L).

A further attempt of increasing aeration rate (Modification IV) to increase nitrification efficiency and hydrogen ion production and, in turn, decrease the level of pH increase was not useful either. The other hypothesis for pH increase (alkalinity production) is sulfate reduction that is possible to occur in the core of the granules. Therefore, the influent SO_4 concentration (in the form of $MgSO_4{\cdot}7H_2O$ used as Mg^{2+} source for granulation) was decreased by half to 35.1 mg/L SO_4 on Day 42 (Modification V). Following this modification, COD removal efficiency of both reactors significantly increased (Fig. 1). COD removal efficiencies of 93–98% and effluent COD concentrations of less than 50 mg/L were obtained for both reactors between Days 49–63 (Table 3, Fig. 1a). As seen in Fig. 1a, both reactors displayed similar COD treatment trend towards the end of operation with optimized influent composition. These results indicated that the difference in period sequence (anoxic–aerobic or aerobic–anoxic) had no effect on COD removal efficiencies of aerobic granules under the studied conditions.

3.1.1.2. Nitrification efficiency. Nitrification was only observed during the aerobic periods of both reactors. During the first 42 days, 51 ± 11% and 43 ± 11% TAN oxidation efficiencies were obtained in R1 and R2, respectively (Fig. 1b). The effluent total oxidized nitrogen (TON) concentrations of both reactors were less than 10 mg/L due to low TAN oxidation efficiencies (Days 1–42). Yet, after Modification V on Day 42, nitrification efficiency of both reactors significantly increased. Sulfide, produced via sulfate reduction, might also have been one of the inhibition sources of TAN oxidation (Sears et al., 2004) as well as high pH and related free-ammonia concentration. TAN removal efficiencies of both reactors reached a steady level by Day 54 and remained around 90% till the end of the study (Fig. 1b, Table 3). Accordingly, the effluent NO_3-N concentrations of aerobic periods also increased and recorded as 73 ± 8 and 71 ± 9 mg/L for R1 and R2, respectively (Table 3). The effluent nitrite concentrations of both reactors were negligible (0.1–4 mg/L NO_2-N).

3.1.1.3. Denitrification efficiency. Denitrification can simultaneously occur during aerobic periods in the inner part of the granules where DO diffusion is limited (Tay et al., 2002; de Kreuk et al., 2005). In the first 42 days, the average granule sizes were 3 ± 0.5 and 2.3 ± 0.3 mm, which were appropriate to prevent DO diffusion to inner parts of the granules and form anoxic zones (Tay et al., 2002; de Kreuk et al., 2005). Therefore, the average total *N* losses of 63 ± 15 and 70 ± 19 mg/L observed during aerobic periods in R1 and R2, respectively, might have been attributed to SNDN process. Yet, high pH values (pH > 8.5) observed during aerobic periods might lead to free-ammonia dominance (theoretically 16–19 mg/L NH_3-N, (Anthonisen et al., 1976)) (Days 10–42). Therefore, *N* loss might also have been due to ammonia stripping as well as SNDN. With the decrease in the influent sulfate concentration and further recovery of pH to lower values (pH < 8) in both reactors after Day 42 (Modification V), potential free-ammonia concentration was minimized (~5 mg/L) and ammonia stripping effect was neglected. The *N* balance analyses indicated the loss of 44 ± 12 and 25 ± 8 mg/L TON during aerobic periods in R1 and R2, respectively. The SNDN efficiencies of R1 and R2 between Days 54–63 were 34 ± 10% and 30 ± 4%, respectively (Table 3).

Denitrification performance was also investigated for anoxic periods. The initial NO_3-N amount (measured after feeding, *t* = 10 min) was denitrified by 93 ± 7% and 80 ± 14% in R1 and R2, respectively, for the first 42 days. Although the denitrification

Table 3
Performances of reactors after Day 54.

Parameter[a]	COD		TAN		TON[b]	
	R1	R2	R1	R2	R1	R2
Influent (mg/L)	1458 ± 43	1458 ± 43	231 ± 14	231 ± 14	16 ± 6	16 ± 6
Theoretical initial (mg/L)	754 ± 9	757 ± 16	121 ± 19	124 ± 15	42 ± 8	40 ± 16
Effluent (mg/L)	39 ± 19	60 ± 12	12 ± 2	15 ± 3	73 ± 8	71 ± 9
Removal efficiency (%)	95 ± 2	92 ± 1	90 ± 3	89 ± 1	–	–
Denitrification in anoxic periods (%)	–	–	–	–	95 ± 7	37 ± 15
SNDN in aerobic periods (%)	–	–	–	–	34 ± 10	30 ± 4
TN removal efficiency (%)	–	–	–	–	46 ± 9	38 ± 11

[a] Influent: feed solution – measured values, effluent: sample taken at the end of the cycle, theoretical initial: the exact concentration expected after feeding, when biochemical reaction during feeding period was neglected (i.e., influent concentration + previous cycle's effluent concentration) × 0.50), TN: total nitrogen, TAN: total ammonia nitrogen, SNDN: Simultaneous nitrification–denitrification (TN removal in aerobic periods).

[b] TON ≈ NO_3-N (NO_2-N was negligible).

performance of R1 remained constant after Day 42, there was a decreasing trend in R2. Under the operational conditions applied for Days 54–63, the average denitrification efficiencies (in anoxic periods) were recorded as 95 ± 7% in R1 and 37 ± 15% in R2 (Table 3). In order to determine the reason of such a difference in denitrification performances, one cycle (Day 50-cycle 197) was investigated in detail.

3.1.1.4. Cycle performance. Fig. 2 represents the change of COD, NH_4-N, NO_2-N and NO_3-N concentrations in cycle 197 (Day 50). In R1 (anoxic–aerobic sequence), 30% of the theoretical initial COD was removed in anoxic period (45 min) and 75% of the remaining COD was removed in the first 2 h of the aerobic period. Therefore, there was enough carbon source (752 mg/L COD) at the beginning of the cycle in R1 for heterotrophic denitrification to occur (Fig. 2a). In R2 (aerobic–anoxic sequence), almost 50% of the theoretical initial COD was consumed in the first 75 min of aerobic period and the COD remained at the beginning of the anoxic period was only 94 mg/L (Fig. 2b). This amount of COD was not enough for complete denitrification of 65.2 mg/L TON (measured at the end of aerobic period of R2) which stoichiometrically requires 186 mg/L COD. Therefore, the poor denitrification performance of R2 was attributed to the limited electron donor (COD) concentration resultant of its aerobic–anoxic periodic sequence. Eventually, the denitrification efficiencies of R1 and R2 during the anoxic period of the cycle were 99% and 54%, respectively, which were verifying the stoichiometric calculations. The results of batch test 1 also verified this finding (details in SI). Cycle performance study also indicated that, considering the COD and TON amounts consumed during anoxic period of R2, an additional COD source (rather than acetate) might have been used for reduction of 35.1 mg/L TON, which might be attributed to EPS (Gao et al., 2011).

Significant TAN oxidation was observed in both reactors during the aerobic period only after almost 70% of the theoretical initial COD was depleted (Fig. 2). The main reason of this delay was the competition between aerobic heterotrophs and nitrifiers, particularly for oxygen. Since the pH was in the range of 7.7–8.1, 98% of the theoretical initial TAN was in ammonium ion form and ammonia stripping was negligible. Therefore, *N* losses of 25% and 18% recorded during aerobic period in R1 and R2, respectively, were resultant of SNDN.

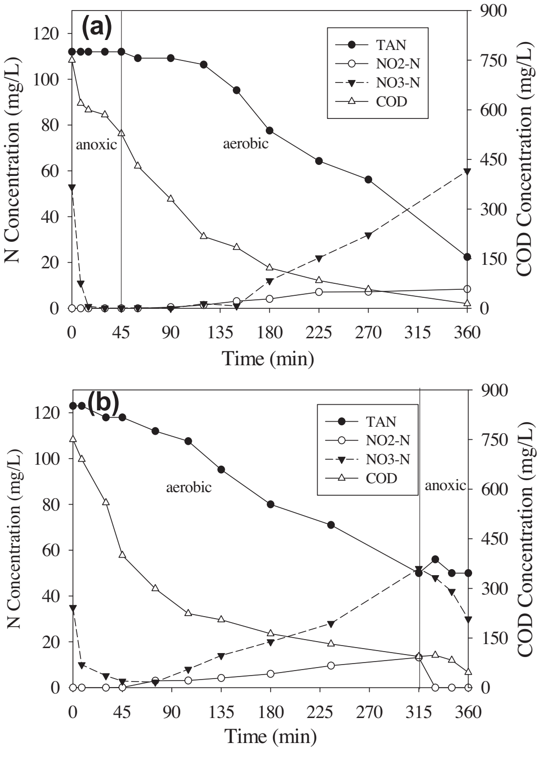

Fig. 2. Cycle performance of (a) R1 and (b) R2.

It was also observed in Fig. 2 that almost 90% and 50% of the theoretical initial NO_3-N was consumed during feeding period (first 10 min of the cycle) in R1 and R2, respectively. In order to investigate whether the first 10-min of NO_3-N removal was due to denitrification or any abiotic process, batch test 2 was conducted by simulating the feeding period in R1 (details in SI). It was seen that there was no NO_3-N consumption under abiotic conditions, in contrast to the consumption of 83% of the theoretical initial NO_3-N under biotic conditions. This result also reveals that, once granular sludge is developed, the anoxic period might be shortened or even removed considering the available carbon source amount and denitrification rate, for nitrate removal occurs even during feeding period.

3.1.2. Development of granular sludge

The evolution of flocs and granules in R1 (anoxic–aerobic) and R2 (aerobic–anoxic) during 63 days of operation were investigated (related figure in SI). Acclimated seed sludge was composed of flocs, had a loose/irregular structure and no filamentous growth (see SI). The first granules, which had small particle sizes, round shape and dense structure, were observed on Day 5 in R1 and on Day 9 in R2. Average sizes of the granules (developed in the first week) in R1 and R2 were 1.8 ± 0.6 and 0.4 ± 0.05 mm, respectively (Fig. 3a). Following the decrease in the settling period from 15 to 5 min, a sharp increase in the average sizes was observed for both reactors. Yet, the average granule sizes were always higher in R1. The granulation rate was also higher in R1 (Fig. 3b). The percent of granular sludge in R1 and R2 reached 90% and 82%, respectively, by Day 22 (Fig. 3b). After almost full granulation was achieved by Day 22, the average sizes of the granules reached 3.2 ± 0.9 mm in R1 and 2.6 ± 0.6 mm in R2 (Fig. 3a). Since the system was granular sludge dominated after Day 22, the mixers were removed from the system (Day 25) to avoid any possible granule deterioration. Immediately after the mixers were removed, settling time was increased to 5 min (Days 26–39) to increase and get a more or less constant MLVSS level in the reactors (around 5–6 g/L). As seen in Fig. 3b, these changes had no significant effect on EPS release and granule percentages in both reactors. The removal of mixers as precaution, suggested by Nor Anuar et al. (2012) to protect the stability of granular system, worked well. Adjusting the settling time to 5 min, which was stated to promote granulation (Adav et al., 2009b), had no negative effect as well (Fig. 3, Days 26–35). The increase in granular biomass (5–6 g/L) after the settling time adjustment resulted in increased oxygen consumption during aerobic periods and DO levels in both reactors decreased (6.5 mg/L on Day 26 to 3.2 mg/L on Day 35 in R1, 6.2 mg/L on Day 26 to 4.1 mg/L on Day 35 in R2). In order to maintain a DO level similar to those of previous periods (around 6 mg/L) and to avoid potential negative effects on treatment, the aeration rate was doubled on Day 36. This further led to a decrease in granule sizes in both reactors which was potentially aeration-related mechanical decrease (Fig. 3a, Day 36). Despite the following decrease in granule sizes, granular sludge percentage and EPS compositions did not significantly change and remained more or less constant in both reactors till Day 42. After Day 42, the granular sludge percentage of both reactors displayed a decreasing trend, which slowly increased after Day 49 in R1 and reached a steady level at 88%. However, the percent of granular sludge in R2 continued to decrease after Day 49 and reached 57% at the end of the study, during which granule

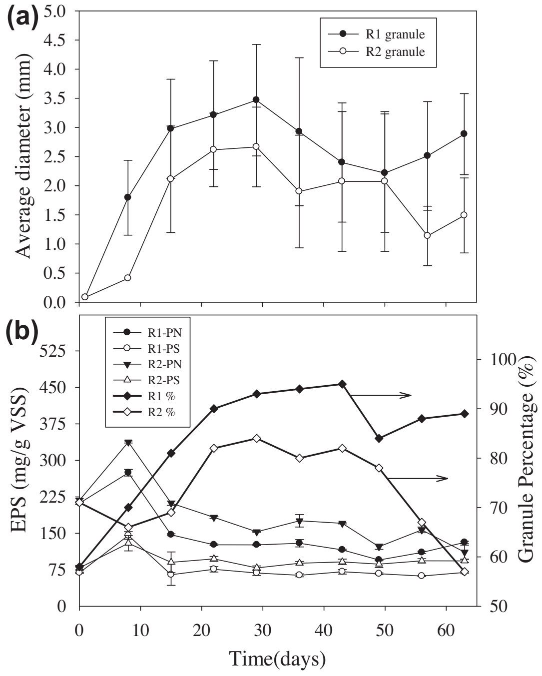

Fig. 3. (a) Average granule sizes and (b) variations of EPS and granule percentage. (PN: protein content of EPS, PS: polysaccharide content of EPS.)

disintegration was also observed (Fig. 3, see also SI). By the end of operation (Day 63), the granules in R2 (1.5 ± 0.6 mm) were smaller in size and loose while the ones in R1 (2.9 ± 0.7 mm) were still compact and dense. The first decrease in granule percentages observed in both reactors after Day 42 might have been due to the change in the influent SO_4 concentration (Fig. 1, Day 42). The halving of SO_4 concentration might have caused the starvation of anaerobic SRB located in the core of the granules. However, the granule percentage in R1 increased after Day 49 while it continued to decrease in R2, which was also the case for granule sizes. The further disintegration observed in R2 was attributed to the starvation of heterotrophic denitrifiers being dominant in the core of the granules. R2 was operated in a post-anoxic mode, where carbon-limiting conditions occurred during anoxic periods (details in Section 3.1.1). Thus, long-term starvation might have resulted in the corruption of the core and further granule disintegration (Adav et al., 2009a). This further decrease observed in R2 (after Day 49) was not attributed to the decrease in settling time (from 2 to 1 min) on Day 49, which was performed in both reactors and did not affect R1. Despite the disintegration, the granules of R2 had settling velocities (36 m/h) comparable to the literature data given as 18 m/h to more than 91 m/h (Adav et al., 2009a; Gao et al., 2011; Jang et al., 2003). Yet, as expected, the settling velocities of the granules of R1 (72 m/h) were greater than those of R2, due to higher particle sizes, compact structure and potential higher densities.

EPS analyses and protein/polysaccharide (PN/PS) ratios of EPS well indicated the granulation and disintegration processes (Fig. 3b). PN and PN/PS ratio of EPS were reported to increase sharply during granulation (Zhang et al., 2007). Similar sharp increase in PN and PS amounts during the first 10 days, where the first granules were developed in both reactors, was thus attributed to the granulation start-up (granular sludge view in SI). After Day 15, PN and PS amounts and PN/PS ratio (1.9) remained almost constant in R1, despite the decrease in PN/PS ratio (1.4) on Day 49 which further recovered to 1.9. Yet, PN content of granules in R2 displayed a decreasing trend leading to a decrease in PN/PS ratio from 1.9 to 1.2 between Days 42 and 63, indicating the disrupted stability. Although some researchers indicate the granular skeleton to be composed of β-polysaccharide, some others state that PN plays the major role in core stability and thus long-term granule stability is dependent on PN which increases sharply during granulation (Adav et al., 2008b; Zhang et al., 2007). Zhu et al. (2012) also stated that decreasing PN caused disintegration of the granules since hydrophobicity also decreased. Similar PN decrease and disintegration observed in R2 support the idea that PN is the major component of core stability. EPS results also indicated that anoxic-aerobic operation (R1) resulted in more stable granules as well as a stable granulation process.

Despite the lower PN/PS ratio values, EPS amount of the granules (both PS and PN amounts) in R2 was always higher than that of R1 (Fig. 3b). Flocculent sludge was reported to have higher EPS release than granular sludge under similar stressful conditions (Xuan et al., 2010). As seen in Fig. 3b, the percent of granular sludge in R2 was always lower than R1, indicating the higher fluffy-like flocculent sludge content of R2. Therefore, the higher EPS amounts obtained in R2 might be explained by its higher flocculent sludge percentage, which remains to be researched. The other possible reason of higher EPS amount of R2 was attributed to starvation, which was reported to increase EPS production (Zhang et al., 2007). The aerobic–anoxic period sequence of R2 led to carbon-lacking situation during anoxic periods, because most of the carbon was already consumed during aerobic periods of the cycles (details in Section 3.1.1). This might have resulted in long-term starvation of denitrifiers and, in turn, more stressful conditions and more EPS release in R2 (Gao et al., 2012).

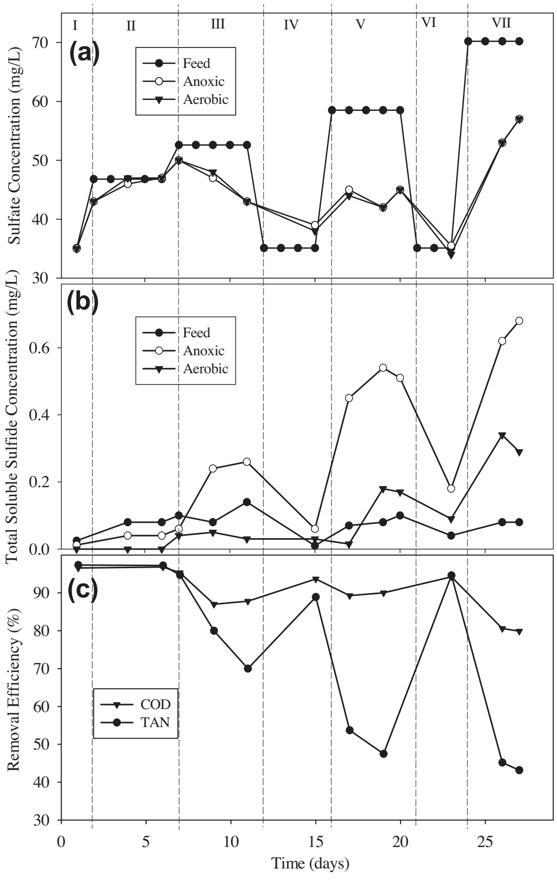

Fig. 4. Changes in (a) SO_4 concentration, (b) total soluble sulfide concentration and (c) COD and TAN removal efficiencies (I – 35.1 mg/L, II – 46.8 mg/L, III –52.6 mg/L, IV – 35.1 mg/L, V – 58.5 mg/L, VI – 35.1 mg/L, VII – 70.2 mg/L influent SO_4 concentration).

3.2. The effect of influent sulfate concentration

The SBR (anoxic–aerobic sequence) and granular sludge being operated at an influent SO_4 concentration of 35.1 mg/L for 67 days of steady conditions, was used for this study. The steady-state average COD, TAN and TN removal efficiencies recorded were 94 ± 3%, 90 ± 4% and 43 ± 10%, respectively (data in SI). In order to investigate the effect of sulfate on treatment efficiency of granules, influent SO_4 concentrations of 35.1, 46.8, 52.6, 58.5 and 70.2 mg/L (i.e. from 90 to 180 mg/L $MgSO_4{\cdot}7H_2O$) were applied during the operational period of 28 days (Fig. 4). Results indicated that, at 35.1 and 46.8 mg/L influent SO_4 concentrations, COD and TAN oxidation efficiencies were not negatively affected and both remained at 97% (Days 1–6). When the influent SO_4 concentration was increased to 52.6 mg/L, noticeable sulfate consumption and sulfide generation were obtained in anoxic periods (Table 4, Fig. 4a and b). TAN and COD oxidation efficiencies decreased by 20% and 7%, respectively, and the pH of reactor content increased during aerobic periods (Table 4, Fig. 4c). Reactor was further fed with 35.1 mg/L SO_4 concentration (Days 13–18) to decrease the possible stress and provide recovery of the treatment performance. After nitrification efficiency recovered back to 90%, the influent SO_4 concentration was increased to the next dose of 58.5 mg/L. Following that, sulfide generation in anoxic periods doubled and TAN oxidation decreased down to 45%. Although COD removal efficiency did not significantly decrease with 58.5 mg/L influent SO_4, it started to fluctuate indicating the risky conditions for steady organic removal. After repetition of the recovery session with 35.1 mg/L of SO_4 (Days 20–23), the reactor was exposed to the final dose of 70.2 mg/L SO_4 (180 mg/L $MgSO_4{\cdot}7H_2O$). TAN oxidation efficiency decreased to 44 ± 2%, and COD removal efficiency also decreased from 92 ± 2% to 80 ± 1%. As previously mentioned, the increase in pH was observed during the first 15 min of aerobic periods (with the start of aeration). The level of pH increase was found to increase with the increasing influent SO_4 doses, especially for the doses promoting sulfide production (from 46.8 to 70.2 mg/L SO_4) (Table 4).

Fig. 4 and Table 4 revealed that increasing the influent sulfate concentrations resulted in increases in the amount of sulfate consumed and sulfide produced during anoxic periods and in turn pH value. This was attributed to the presence of sulfate-reducing bacteria (SRB) in the inner cores of the granules. The total soluble sulfide concentration of 0.24–0.62 mg/L S^{2-} produced in this study, which is close or greater than 0.25 mg/L S^{2-} value reported to completely inhibit ammonia oxidation (Sears et al., 2004), inhibited TAN removal. It should also be noted that the increase in pH observed with the increasing SO_4 dose results in the increase (dominancy) in free-ammonia concentration. Yang et al. (2004) stated that 2.5–39.6 mg/L NH_3–N concentration inhibited specific oxygen uptake rate of nitrifiers and aerobic heterotrophs by 2.5 and 5 times, respectively. It was also stated that 10–150 mg/L NH_3–N was toxic for nitrifiers (Anthonisen et al., 1976). Therefore, in this study, not only sulfide concentrations of 0.24–0.62 mg/L S^{2-}, but also free-ammonia concentrations of 15.2–27.8 mg/L NH_3–N might have been inhibitory for TAN oxidation (Table 4). The slight decrease in COD removal efficiency (7–12%) was not attributed to the sulfide inhibition. It is stated that sulfide and existence of SRB in aerobic systems have negligible effects on COD removal (Lens et al., 1995). Therefore, free-ammonia concentrations of inhibitory levels that were produced in line with the increased sulfide production and pH were likely to cause the decrease in COD removal efficiency.

Table 4
Influent SO_4 concentrations and corresponding sulfide concentrations produced (anoxic periods), pH values and free-ammonia concentrations.

Influent SO_4 (mg/L)	Total soluble sulfide produced (mg/L)	pH at the end of anoxic period	pH in aerobic period (after 15 min- to - the end of period)	Theoretical NH_3–N (mg/L)
35.1	<0.1	8.5	8.5–7.7	2.06
46.8	<0.1	8.3	8.7–8.1	4.8
52.6	0.26	8.4	9.1–8.6	15.2
58.5	0.54	8.5	9.1–8.8	27.8
70.2	0.62	8.4	8.9–8.6	18.9

Granular sludge is more resistant to toxic effects than suspended sludge (Liu and Tay, 2004). Similarly, the percent of TAN and COD removal inhibition observed in this study was less than the values given in the literature for suspended cultures (such as 100% TAN oxidation inhibition at 0.25 mg/L of sulfide, (Sears et al., 2004)). Rapid recovery was also observed for the inhibitory sulfide and ammonia doses (Fig. 4c), which signifies the advantages of aerobic granular sludge over suspended sludge.

The analyses performed to determine the effect of influent SO_4 (i.e. $MgSO_4{\cdot}7H_2O$) concentration on granule properties indicated that granular compactness, outer surface properties and shape did not change during the operational period (data in SI). The initial sizes of the granules (3.2 ± 0.8 mm) remained more or less constant (final size as 3.6 ± 1 mm) for all $MgSO_4{\cdot}7H_2O$ concentrations studied (data in SI). The granular sludge percentage in the reactor slightly decreased (from 94% to 90%) during the operation. However, SVI_{30} values of the reactor sludge remained almost same (34–37 mL/g) which indicated that settleability of granules was not deteriorated either.

4. Conclusions

Aerobic granules could be developed by both anoxic–aerobic and aerobic–anoxic period sequences. Yet, the former provides the development of granules with higher stability. In case of aerobic–anoxic sequence application, adequate carbon source is needed to protect the granule stability and improve total nitrogen removal. Influent sulfate concentration is of significance for triggering the inhibitory sulfide production under anoxic conditions (within the granules) and further reduction of removal efficiencies. In aerobic granular sludge applications, the wastewater content in particular the sulfate concentrations should be therefore carefully considered for better control and achievement of stable treatment efficiency.

Acknowledgement

This study was funded by The Scientific and Technological Research Council of Turkey (TUBITAK), Project No. 111Y176.

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.2013.08.096.

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