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LONG-TERM CULTIVATION OF AN AEROBIC GRANULAR ACTIVATED SLUDGE

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ABSTRACT

Aerobic granules were cultivated in a sequencing batch reactor (SBR) fed with sucrose as a sole carbon source at superficial upflow air velocity of 1.9 cm/s and under an organic loading rate (OLR) of 2 g COD/L.d. This study shows that cultivated granules during 1 year were stable and had capability in simultaneous removal of carbon, nitrogen and phosphorus from wastewater. At a 6 h SBR cycle removal efficiency of chemical oxygen demand (COD), $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$ were 93%, 66% and 83%, respectively. The value of sludge volume index (SVI) was between 90-110 mL/g and biomass concentration reached up to 8.0 g/L. With the granulation, the specific gravity and surface hydrophobicity of sludge increased and aerobic granules mean diameter was 4.9 mm.

***Key words*:** sequencing batch reactor (SBR), microbial granules, nitrification, denitrification, microbial activity.

INTRODUCTION

Microorganisms can exist as free or sessile state in biological wastewater treatment systems. The most recognized sessile states of microorganisms are biofilm and biogranule. Biofilm is a kind of microorganisms attached to the inert support whereas a biogranule is completely self-immobilization of microorganisms without any carrier. Biogranules cannot occur in the natural environment and strong selective pressure is required to trigger biogranulation in reactors [1-3]. In conventional activated sludge, microorganisms accommodated in wastewater would exist in the form of desultory flocs. The flocs are composed of filamentous matrixes and zoogloea, thus the structure of activated sludge has low biomass concentration, bad settling ability and poor ability against impact [4]. Granular sludge was first described in strictly anaerobic systems, such as upflow anaerobic sludge blanket reactors (UASB), biofilm airlift reactors [5] and anaerobic sequencing batch reactors. The anaerobic granulation technology exhibits several drawbacks that include a long start-up period, a relatively high operating temperature, unsuitability for low strength organic wastewater, and low efficiency in the removal of nutrients (N and P) from wastewater [4]. This fact prompted the development of aerobic granular technology, which became a popular topic of discussion for

environmental engineers. Compact structured, biologically efficient aerobic sludge granules with diverse microbial species and excellent settling capabilities have been developed in sequencing batch reactors [6]. Microscopic observations show that the formation of aerobic granules is a gradual process from seed sludge to compact aggregates, further to granular sludge and finally to mature granules [7]. It has been reported that mature granules arrange their structure to protect against environmental attacks. For instance, mature granules have special heterogeneous structure with compact outer layer and loose inner layer [8]. Granule structure is similar to biofilm in mass transferring, with aerobic zone, anoxic zone and anaerobic zone along the direction of mass transfer [9,10], which provides favorable environment for growth of facultative and aerobic bacteria, such as ammonia oxidizing bacteria, denitrifying phosphate accumulating bacteria (DPB), denitrifying glycogen-accumulating bacteria and phosphate-accumulating organisms (PAOs). Therefore, aerobic granule can remove carbon, nitrogen and phosphorus simultaneously [11]. Heterotrophic and nitrifying bacteria could coexist in microbial granules [12]. An increased substrate N/COD ratio results in a signification shift among the three populations within granules [13]. Aerobic granular sludge features a number of advantages, such as a denser and stronger microbial structure, better settling ability - it has a settling velocity greater than 10 m/h and sludge volume index of up to 30 mL/g. Moreover it has facility of effluent separation, greater biomass retention (as high as 10 g/L of MLSS) and a much improved capability to withstand high strength wastewater and shock loadings [14]. Furthermore, the granulation system can operate at high organic loading rate of up to 15 g COD/L.d [15]. To date, researches were focused on the main parameters influencing formation of aerobic granular sludge: settling time, substrate, hydrodynamic conditions, etc. A first driving force is the selection of granules by applying extreme settling conditions for the sludge separation, i.e. low settling time, high volume exchange ratio and low discharge time [16-18]. The second aspect is the influence of substrate: type, concentration and feeding pattern. Previous experiments showed that aerobic granular sludge could be formed with a wide variety of substrates including glucose, acetate, ethanol, phenol, synthetic wastewater and special industrial wastewater [19, 20-22]. Moreover, intermittent feeding strategy definitively enhances aerobic granulation [23]. In other words, the feast–famine cycle of the SBR favored the formation of compact and dense aerobic granule [24]. The last major aspect is the effect of aeration flow rate, which plays two important roles in the formation of granules: firstly it imposes the dissolved oxygen (DO) concentration, and secondly it determines the hydrodynamic shear forces [25]. In the work of Mosquera-Corral [26], it was difficult to obtain stable granular sludge when the oxygen saturation was reduced to 40%, whereas it was possible at high level (100%). Unless the gradients concentration of oxygen and substrate are not high enough, filamentous structures develop on the surface of the granule. It is now commonly postulated that dense and smooth aggregates would develop if high substrate (and electron acceptor) gradients are maintained and if diffusion and transport are facilitated by high mixing. As a consequence, it was found that aerobic granules could be formed above a threshold value in terms of superficial upflow air velocity above 0.8 cm/s in a column SBR [25]. Aerobic granular system is much easier to startup comparing with anaerobic granular technique. However, instability in the former has been reported in literature [27]. Fast growing and filamentous microorganisms have frequently led to disintegration of aerobic granules [28]. From an engineering and economic point of view, aerobic sludge granulation is a promising process that has the potential to lead the next generation of biological wastewater treatment technologies. Despite the advantages and potential of the aerobic sludge granulation process, the mechanism of aerobic granulation is not well understood.

MATERIALS AND METHODS

Experimental set-up and the SBR operation

Aerobic granular activated sludge was cultivated in the reactor with a working volume of 3 L (volumetric exchange ratio was 50%). Reactor was operated sequentially in a 6-h cycle with 5 min of feeding, 345 min of aeration, 5 min of settling and 5 min of effluent withdrawal. Air was supplied during the aeration phase with an airflow rate of 450 L/h equivalent to a superficial upflow air velocity of 1.9 cm/s. Sludge retention time (SRT) was 8 days and dissolved oxygen concentration in the reactor was near the saturation value. The pH in reactor was about 7.2 and hydraulic retention time (HRT) was 12 h. The experiment was performed at room temperature.

Medium

The composition of the synthetic wastewater was as follows (mg/L): sucrose 892; NH_4Cl 195, KH_2PO_4 44, Ca^{2+} 128, Mg^{2+} 20 and trace element solution 1.0 ml/L. This gave a total chemical oxygen demand of 500 mg/L in the reactor. The trace element solution contained (50 mg/L): H_3BO_3, $ZnCl_2$, $CuCl_2$, $MnSO_4{\cdot}H_2O$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, $AlCl_3$, $CoCl_2{\cdot}6H_2O$ and $NiCl_2$.

Seeding

The reactor was started up with 500 mL of activated sludge from the local municipal wastewater treatment plant with mixed liquid suspended solids being 1.8-2.0 g/L and sludge volume index being 150-160 mL/g.

Analytical methods

Soluble COD (filtering through 0.45 μm membrane filters), $P\text{-}PO_4^{3-}$, $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, total hardness (TH), total alkalinity (TA), biomass as mixed liquid suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) was measured according to Standard Methods [29]. $N\text{-}NO_3^-$ in the bulk liquid was determined by use of nitrate cell test method (Merck). Specific gravity was measured according to [30]. Total organic carbon (TOC) and total nitrogen (TN) was estimated by using a TOC analyzer (IL 550 TOC-TN Analyser). Settling velocity of sludge was estimated by measuring the time taken for individual granules to drop from height of 40 cm in a measuring cylinder. Dissolved oxygen and pH were measured by multifunction meter CX-401. Sludge volume index (SVI) was monitored at the end of aeration phase. This was done by determinating the volume of the granules after 30 min of settling and dry weight of the separated biomass was measured after drying at 105°C for 24 h. The evolution of aerobic granules in the reactor was observed under an optical microscope XSZ-21, equipped with a digital camera Moticam 1000 connected online to a computer. Photographs of aerobic granules were also taken with a camera (Benq DCC 850). Hydrophobicity of the granules was measured as adherence to hexadecane as described by Rosenberg [31]. Hexadecane (2.5 mL) was used as the hydrophobic phase. Hydrophobicity was expressed as the percentage of cells adhering to the hexadecane after 15 min of partitioning. Heterotrophic specific oxygen uptake rate (SOUR) was measured by a batch respirometer (at 20°C) with sucrose as the substrate; the relation COD/MLVSS was 0.4. The sludge retention time was controlled by discarding a certain volume of mixed liquor at the end of the aeration phase and the effluent suspended sludge (ESS). Thus, the SRT was the ratio of the sum of discarded SS during one day to the total SS in the reactor.

RESULTS AND DISCUSSION

Start up and formation of granules

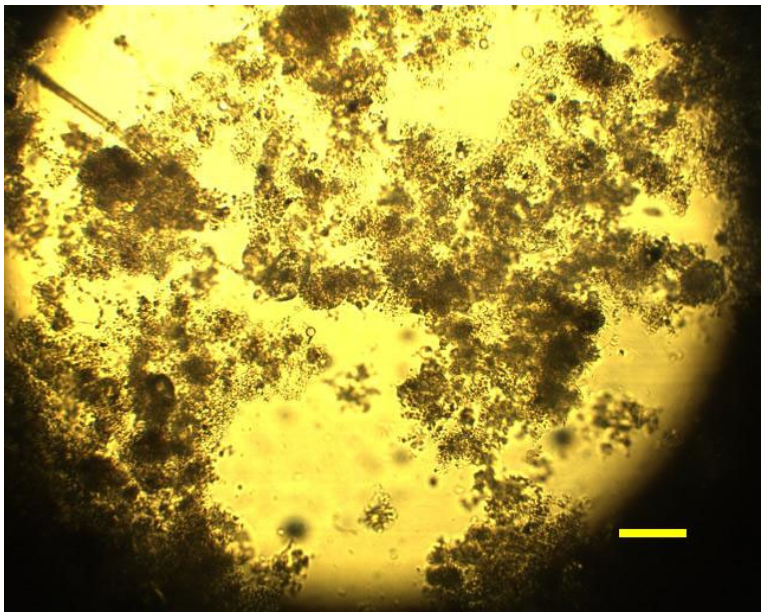
The granulation is a competitive process between loose bioflocs and compact aggregates under certain selective pressures, which is affected by many operation conditions [16,18,32]. In this study the aerobic granular sludge were successfully cultivated during a period of over 12 months maintained at a loading rate of 2 g COD/L.d. The SBR was started-up with the inoculum which was a mixture of filamentous sludge with a fluffy, irregular and loose-structure morphology, as shown in Fig. 1A. The startup strategy was designed to gradually decrease the settling time to promote granule formation, i.e. a settling period of 30 min was initially imposed to avoid excessive washout of the acclimated biomass, and subsequently reduced to 5 min on day 15. Since the settling time was kept short, selected biomass with enhanced settling properties remained in the rector, i.e. light and dispersed sludge was washed out, while heavier components with a settling velocity greater than 10 m/h were effectively retained. During this period there was no significant change of the sludge morphology and the flocculent sludge was dominant form in the reactor. Tiny irregular aggregates became visible in the reactor during the following days and their color changed from grayish brown to black gradually with the progress of the experiment. In the next weeks the floc-like sludge gradually changed to granular and more granules appeared in the reactor. Granules increased diameter quickly with time and after one month of operation black granules were the dominant form of biomass in the SBR. After 12 months of operation, the reactor was still working and the granules were stable. No visible signs of deterioration in granule physiology were observed throughout the experiment.

Granule characterization

As literature shows granulation is not restricted to certain microbiological groups, but related to the way reactors are operated [7]. By preventing the accumulation of suspended cells (by adjusting the HRT) or flocs (by the settling velocity) proper granules could be formed [17,18]. The excellent settleability of the sludge enabled aerobic granules to be successfully retained within a reactor, which allowed a greater biomass accumulation. The settling velocity of individual mature granules after 250 days of operation was in the range of 20–90.0 m/h. It was observed that after 4 min of settling, the mature granules were well settled leaving a clear supernatant in the reactor. Its high settling velocity was caused by big sizes of the granules, as it is shown in Fig. 2. A wide range of sizes of approximately 0.5–9.0 mm in diameter granules coexisted during the time of operation and strong shearing force produced by aeration could not prevent the development of high diameter granules. The granules size distributions was measured by an image analysis method with the sample from the reactor at 280 day of operation Fig. 1B. Microscopic examination also showed that the shape of the granules was round with a cauliflower like aspect. In the environmental engineering field, specific gravity has been commonly used to describe the structural compactness and stability of a microbial community [13]. The average specific gravity of the granules was 1.008 g/cm^3 compared with 1.004 g/cm^3 for the flocculating sludge. Its low value might be related with the low density of the granules, due to high void space inside the granules, which in turn increase the granules sizes [33]. Low densities of aerobic granules might be attributed to the existence of anaerobic cultures in the inner parts. The direct relationship between the mean diameter and specific gravity of aerobic granules is further shown in Fig. 3. Liu and Tay [34] reported that the microstructure and species diversity of the granules may be related to the type of carbon source. Glucose-fed aerobic granules have a loose microbial structure dominated by filamentous bacteria and developed an irregular

appearance characterized by folds, crevices and depressions [15]. These irregularities allowed for shorter diffusion distances and better penetration of nutrients into the granule interior compared to spherical-shaped granules [34]. Diffusion was also enhanced by the high substrate concentration that existed in the bulk solution. Moreover, filamentous microbes played a crucial role in the granulation process, because they acted as the structural backbone existed in the interior of granules [35]. Sludge volume index [36] is one of the important parameters in the conventional environmental engineering to assess settleability, stability as well as compactness of sludge in any aerobic suspended growth system. Along with the formation of granules the SVI decreased and stabilized between 90-110 mL/g, what confirmed that the granules had better SVI values than the seed flocs. With the retention of the heavy biomass fraction, the biomass concentration in the reactor started to increase and the average MLSS concentration of aerobic granules in the reactor was around 8 g/L; which is in agreement with the findings on bacterial granules in SBRs. The ratio of MLVSS/MLSS was of around 0.9, which indicated an increase of active biomass in the sludge in comparison to 0.75 of inoculum. The ESS in the effluent was constituted by the presence of very small flocs, which were always observed in the reactor. The average effluent suspended solids concentration was about 0.25 g ESS/L.

A



B

Fig. 1. Images of bioflocs on day 2 (A), bar 2 μm and granules on day 260 (B), bar 10 mm

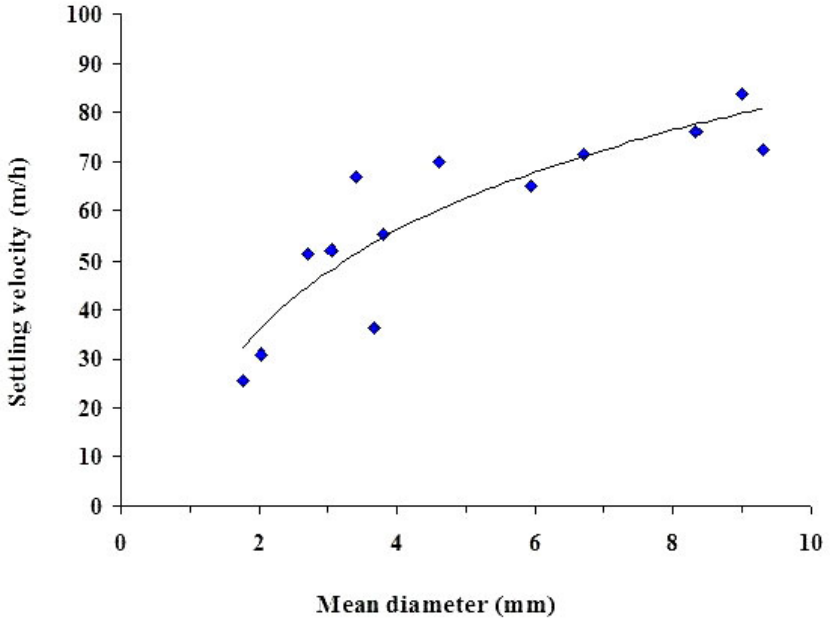


Fig. 2. Settling velocity versus mean granules diameter

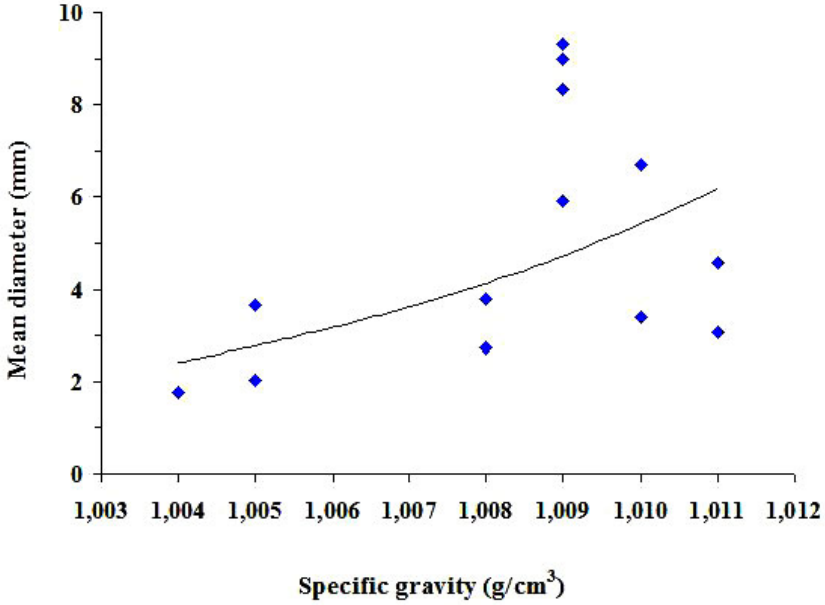


Fig. 3. Mean granules diameter versus specific gravity

Nutrient removal

To date, aerobic granulation phenomena have been only observed in SBR, while no successful example of aerobic granulation has been reported in continuous culture [37]. Compared to a continuous culture, the unique feature of SBR is its cycle operation. As a result, microorganisms are subject to a periodical feasting and famining [24,38]. The feast phase means that the exogenous substrate is available, while the famine phase represents a period in which there is no longer exogenous substrate. The degradation of different substrates, such as carbohydrates, ammonium nitrogen and phosphates involves great diversity of microorganisms in granule microstructures which imply that aerobic granules consist of a layered structure where aerobic, anoxic and anaerobic zones would co-exist [39]. The outer layer [9] of granule probably is aerobic, containing heterotrophic organisms, in the deeper layers existing autotrophic organisms, while its inner part would be subject to anoxic and anaerobic conditions. Coexistence of heterotrophic, nitrifying, denitrifying and phosphors accumulating organisms populations with different specific biochemical functions in the microbial granules provide an efficient way for organic carbon, nitrogen and phosphorus removal [40]. Typical nutrient concentration profiles during a single cycle when the reactor was operating under stable conditions (day 200) are shown in Figs. 4A and 4B. During the operational time almost all

biodegradable COD (TOC) was in a linear way reducing in the first 20 min, which indicated a long starvation phase occurred during the aeration. A high amount of soluble biodegradable COD available during feeding phase led to greater transport by diffusion of this organic matter fraction inside the granules than of oxygen. This is advantageous, because most of denitrifying bacteria populations under anoxic conditions use nitrate and nitrite as terminal electron acceptor and exogenous organic carbon as the electron donor for energy generation and growth. Usually denitrification efficiency is mainly determined by the availability of external organic carbon source and in conventional denitrification process various organic carbons is added e.g. methanol, acetate and ethanol [41]. The fact that the granules exhibited denitrifying activities under aerobic conditions may be confirmed by low levels of nitrate and nitrite during the cycle. The amount of nitrite did not exceed 0.5 mg/L and nitrate concentration in the influent decreased from 3 mg/L to 0.5 during 1 h and slightly increased to 5 mg/L at the end of the cycle. Since after aeration phase no sufficient mixing power is provided microbial granules settled down to the bottom of the reactor and contact between granules and nitrate was extremely poor caused inefficient denitrification. During famine phase nitrates could be also simultaneously denitrified inside the granule using the stored materials as electron donor. In the absence of external carbon source PHB is supposed to serve as alternative carbon source for denitrification [42]. It has been reported that storage and subsequent degradation of PHB would benefit the denitrification, especially PHB was found to be stored in bacteria situated in deeper layers of aerobic granules [43]. Qin et al. [44] investigated the potential role of PHB for denitrification in aerobic granular sludge SBR, and found that stored PHB can be used as energy and carbon source for denitrification when external carbon is no available. Moreover, extracellular polysaccharide in the centre of granule would be anaerobically degraded as potential energy source and the biomass in the granule centre would undergo anaerobic decay. It has been reported that a significant portion of extracellular polymeric substances (EPS) produced by aerobic granules can be degraded by their own producers [45]. These in turn would lead to a porous and weak structure of aerobic granule [9]. Ammonium concentrations decreased during the cycle, but removal rates are clearly higher during the feast period. It should be pointed out that ammonium nitrogen removal in the first hour could be removed by heterotrophic assimilation rather than nitrification, because no nitrite or nitrate is generated in this period. Orthophosphates concentration in the influent was about 5 mg/L, and after 10 min it decreased to values below 0.5 mg/L (its removal efficiency was more than 90%). This implies that microbial granules developed in this study have a capability of biological phosphorus accumulation. It should be pointed out that soluble phosphorus may be removed through biological accumulation in the form of poly-phosphate by PAO under the aerobic condition or as a chemical precipitates. Some multivalent metal ions, such as Ca^{2+} and Mg^{2+}, can also bond with soluble phosphate to form of poly-phosphate precipitates. But it is clear that chemical precipitation cannot be attributed to biological phosphorus removal [46]. During the 365 days of operation reactor demonstrated excellent treatment performance in nutrient removal. COD, N-NH_4^+ and P-PO_4^{3-} average removal efficiency was 93%, 66% and 83% respectively. Table 1 shows mean influent and effluent concentrations of the COD in the reactor during the entire operation time. Influent COD slightly fluctuated around 500 mg/L and the residual concentration was always lower than 50 mg/L. Concentrations of influent N-NH_4^+ decreased from about 30 to 10 mg/L, which resulted in good ammonium removal performance. The concentration of N-NO_3^- in the influent was below 5 mg/L and maintained at a level of about 2 mg/L at the end of the cycle. Nitrite concentration was mostly below 0.5 mg/L and P-PO_4^{3-} remained at average effluent concentration 1 mg/L. Thus indicate that, aerobic granule may have a great potential for efficient simultaneous nutrient removal.

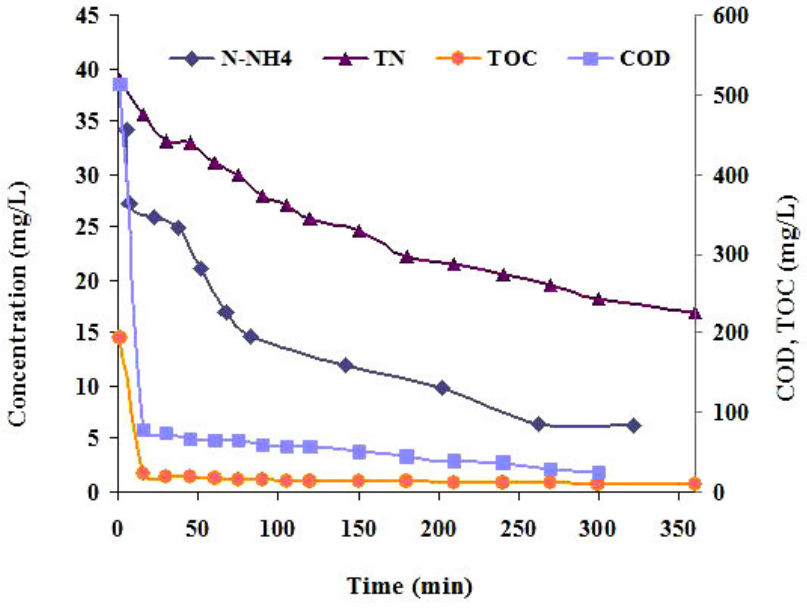


Fig. 4A. Carbon, ammonium and total nitrogen profiles observed at a one cycle

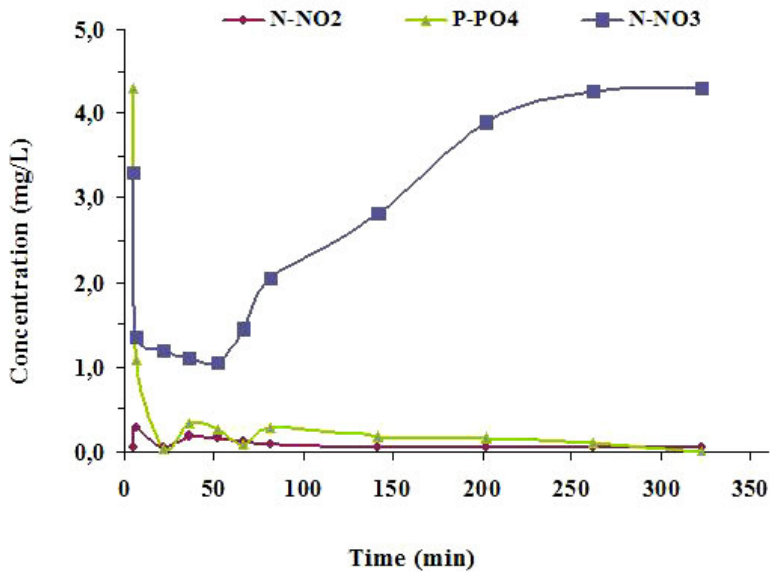


Fig. 4B. Nitrification and phosphate profiles observed at a one cycle

n=50	Influent	Effluent	Removal efficiency (%)	n=30	
COD mg/L	523±40,4	33±14,3	93,55	ESS g/L	0,25±0,14
$N\text{-}NH_4^+$ mg/L	27,±3,7	9±2,5	66,99		
$N\text{-}NO_2^-$ mg/L	0,2±0,2	0,3±0,31		MLSS g/L	7,5±1,8
$N\text{-}NO_3^-$ mg/L	2,7±0,9	1,3±1,3			
$P\text{-}PO_4^{3-}$ mg/L	6±1,1	1±0,6	83,68		
TA meq/L	3,3±1,2	1,8±1,4	45,58	SVI mL/g	104±18,4
TH meq/L	4,7±0,3	4,6±0,3	2,05		

Table 1. A comparison of operational parameters during 1 year of granules cultivation

Monitoring biomass activity

In the environmental engineering literature microbial activity of activated sludge is usually characterized by the specific oxygen uptake rate (SOUR), in terms of milligrams of oxygen consumed by a gram of sludge per hour. In this study, respirometric assays were performed in a 576-mL jacketed plexi glass vessel, which was completely filled with the mixed liquor withdrawn from the SBR. The respirometric vessel was mixed using magnetic stir-bar. A decrease in the DO level in the vessel attributed to substrate oxidation was measured by the DO probe and data were continuously recorded. Aerobic granules show changes in specific oxygen utilization rate (SOUR) during the respirometric experiment; maximum SOUR was associated with a quick reduction in the external carbon concentration and low values were supposed to be related to the degradation of internal storage compounds after depletion of the external carbon materials. The biological treatment activity of aerobic granular sludge was 30 mg O_2/g MLVSS.h which was much more lower than typical values described in literature (greater than 100 mg O_2/g MLVSS.h). Fig. 5 illustrates the SOUR profile during the batch experiment.

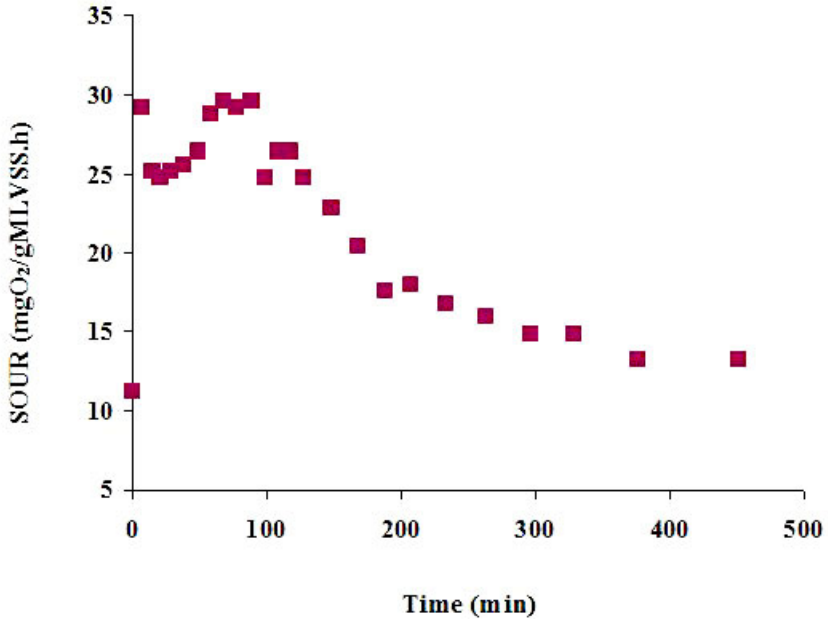


Fig. 5. Evolution of respirometric activities

Hydrophobicity

Hydrophobicity and hydrophilicity are usually used to describe a molecule or a structure possessing the feature or being rejected from an aqueous medium (i.e. hydrophobicity), or being positively attracted (i.e. hydrophilicity). It is believed that microbial cells would prefer a dispersed, rather aggregated state under normal culture conditions (bacterial surface is negatively charged at usual pH). Microbial granulation might be the result of cell response to the stressful environments that lead to changes of surface characteristics of bacteria [47]. It was reported that cell hydrophobicity was inversely correlated to the quantity of surface charge of microorganisms. The sludge surface charge, in terms of zeta potential tended to decrease with the granulation, which implies that with the increase of cell hydrophobicity the electrostatic repulsion between the cells will be weaker. It is suggested that the sludge surface charge and hydrophobicity might be the sum effect of exopolymers (EPS) interactions and is related to the proportion of EPS components (proteins/carbohydrate). Some evidences suggest that the protein and amino acids are the hydrophobic components of the EPS, while carbohydrates are hydrophilic. The higher PN/PS value might correlate with the less surface negative charges since electrostatic bonds with multivalent cations are more likely to be created with proteins (high content of negative charges) rather than sugars. Decreasing the negative surface charge density surrounding the cell might favor the aerobic granulation. Calcium ions were suggested to stimulate granulation by neutralizing negative charges by forming cationic bridges (EPS–Ca^{2+}–EPS) on bacterial surfaces [48]. Also some studies showed that the periodical starvation conditions could induce cell surface hydrophobicity, which in turn facilitated microbial adhesion and aggregation [49]. Cell surface hydrophobicity, which in terms of adherence to hydrocarbons, was found to increase the initial value of 42% to about 78% with the complete granulation of sludge in the reactor. It indicated that the cell hydrophobicity of aerobic granules was nearly two times higher than that of the flocculent seed sludge.

CONCLUSIONS

In this study, the aerobic granular sludge reactors had been run stably over a period of 1 year before the experiment was terminated. These results seem to indicate that the use of aerobic granules for upgrading of the existing wastewater treatment plants towards simultaneous C, N, P removal may be feasible and beneficial. The mature granules in the SBR had high settling velocities leading to good solid-liquid separation, high biomass retention and low heterotrophic activity. Analysis of typical cycle shows that granule has good ability of simultaneous carbon, nitrogen and phosphorus removal. The SBR maintains stable removal performances for COD, ammonium nitrogen and orthophosphate at average removal efficiencies of 93%, 66% and 83%, respectively.

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