ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Improved Phosphate Removal by Selective Sludge Discharge in Aerobic Granular Sludge Reactors

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ABSTRACT: Two lab-scale aerobic granular sludge sequencing batch reactors were operated at 20 and 30°C and compared for phosphorus (P) removal efficiency and microbial community composition. P-removal efficiency was higher at 20°C (>90%) than at 30°C (60%) when the sludge retention time (SRT) was controlled at 30 days by removing excess sludge equally throughout the sludge bed. Samples analyzed by fluorescent in situ hybridization (FISH) indicated a segregation of biomass over the sludge bed: in the upper part, *Candidatus* Competibacter phosphatis (glycogen-accumulating organisms—GAOs) were dominant while in the bottom, *Candidatus* Accumulibacter phosphatis (polyphosphate-accumulating organisms—PAOs) dominated. In order to favour PAOs over GAOs and hence improve P-removal at 30°C, the SRT was controlled by discharging biomass mainly from the top of the sludge bed (80% of the excess sludge), while bottom granules were removed in minor proportions (20% of the excess sludge). With the selective sludge removal proposed, 100% P-removal efficiency was obtained in the reactor operated at 30°C. In the meantime, the biomass in the 30°C reactor changed in color from brownish-black to white. Big white granules appeared in this system and were completely dominated by PAOs (more than 90% of the microbial population), showing relatively high ash content compared to other granules. In the reactor operated at 20°C, P-removal efficiency remained stable above 90% regardless of the sludge removal procedure for SRT control. The results obtained in this study stress the importance of sludge discharge mainly from the top as well as in minor proportions from the bottom of the sludge bed to control the SRT in order to prevent significant growth of GAOs and remove enough accumulated P from the system, particularly at high temperatures (e.g., 30°C).

Correspondence to: M.C.M. van Loosdrecht
Received: 13 October 2011; Revision received: 20 January 2012;
Accepted: 26 January 2012
Accepted manuscript online 13 February 2012;
Article first published online 22 February 2012 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.24457/abstract)
DOI 10.1002/bit.24457

Biotechnol. Bioeng. 2012;109: 1919–1928.

KEYWORDS: phosphate removal; PAO; GAO; granular sludge; biomass segregation; SRT

Introduction

Enhanced biological phosphate removal (EBPR) processes are widely applied for phosphorus (P) removal from domestic and industrial wastewaters due to their economic and environmental advantages compared to physical–chemical processes. EBPR systems rely on the activity of organisms capable of storing P-intracellularly as polyphosphate. These organisms known as polyphosphate-accumulating organisms (PAOs), can be enriched by submitting the sludge to alternating anerobic and aerobic/anoxic conditions (Mino et al., 1998). However, these operating conditions can also favor the growth of other microorganisms which perform similar carbon transformations of that of PAOs (Satoh et al., 1994). These organisms are referred to glycogen-accumulating organisms (GAOs), which compete with PAOs for available organic carbon substrates but not contribute for P-removal (Oehmen et al., 2006). Therefore, they are undesired organisms generally associated with the deterioration of bio-P-removal systems.

Several factors influencing the PAO–GAO competition are reported in literature: pH (Felipe et al., 2001), P/C ratio (Liu et al., 1997; Schuler and Jenkins, 2003), SRT (Whang and Park, 2006), organic carbon sources (Oehmen et al., 2007), and influent phosphate/volatile fatty acids (P/VFA) ratio (Liu et al., 1997; Schuler and Jenkins, 2003). Besides all aforementioned factors, other important parameter influencing the PAO–GAO competition is the temperature. In general, temperatures higher than 20°C are reported to cause a deterioration of EBPR processes because GAOs become the dominant microorganisms (Barnard and Steichen, 2006; Gu et al., 2005; Panswad et al., 2003; Whang and Park, 2002).

PAOs were found to dominate the microbial community at lower temperatures, such as 10°C (De Kreuk et al., 2005; Lopez-Vazquez et al., 2009).

In order to obtain stable P-removal at high temperatures, such as 30°C, several operational strategies have been reported in literature. The application of a short cycle length in sequencing-batch reactors or low sludge retention time (SRT) are examples (Freitas et al., 2009; Whang and Park, 2006). In previous research (Winkler et al., 2011) on PAO–GAO competition in an aerobic granular sludge reactor operated at 30°C, segregation of biomass over the height of the sludge bed was observed. FISH analysis showed that the granules at the bottom of the sludge bed were dominated by PAOs, whereas GAOs were dominant at the top of the sludge blanket. This different distribution of PAOs and GAOs over the sludge bed was used to control the SRT of the individual populations by selective removing sludge only from the GAO-rich part of the sludge blanket, while no bottom PAO-dominated sludge was removed. Indeed, the specific sludge removal strategy enabled a high P-removal efficiency at 30°C. Several open questions, however, still remained. In the previous study, it was pointed out that eventually it would be necessary to remove part of the bottom sludge to renew that specific biomass in which PAOs are dominant in order to remove P from the system and allow PAOs to grow. Moreover, the selective sludge removal from specific parts of the sludge bed was not studied for lower temperatures. Therefore, a comparative study on the performance, in terms of P-removal, of two aerobic granular sludge reactors operated at different temperatures (20°C and 30°C) was conducted. The selective sludge removal for SRT control was modified compared to the previous study in order to improve reactor performance and stability.

Materials and Methods

Experimental Set-Up

Two lab-scale aerobic granular sludge sequencing batch reactors, designated as SBR_{20} and SBR_{30}, were operated in parallel at 20°C and 30°C, respectively. The volume of both systems was 2.6 L. For the SBR_{30}, the temperature was maintained constant at 30°C by pumping water with the desired temperature through tubes which were placed around the reactor. A thermal insulation surrounded the whole reactor to prevent cooling. Aeration and mixing were supplied through an air diffuser placed in the bottom of the reactors (airflow rate of 4 L/min). The pH was controlled during the aeration phase at 7.0 ± 0.2 by dosing 1 M NaOH or 1 M HCl. Both reactors were operated using a 3-h cycle under alternating anerobic and aerobic conditions. The SBR cycle consisted of an anerobic-feeding phase of 60 min from the bottom of the reactor in a plug-flow regime through the settled bed, 112 min aeration, 3 min settling, and 5 min effluent withdrawal. The volume exchange ratio was 57%, resulting in a hydraulic retention time (HRT) of 5.2 h. A bio controller (Braun DCU4 coupled with mass flow control system and data acquisition software) was used to control and operate the SBRs. The dissolved oxygen (DO) concentration was maintained at 1.8 mgO_2/L by using two mass flow controllers (one for air and other for nitrogen gas).

Operating Conditions and Calculation Procedures

SBR_{20} was inoculated with granules from a pilot-scale aerobic granular sludge reactor treating municipal wastewater (Nereda®; EPE, The Netherlands). When a stable operational performance was established in SBR_{20} (full P-removal), half of the sludge was removed from that system to inoculate SBR_{30}. From this point henceforth, the operation of the aerobic granular sludge reactors was divided in different experimental phases, as shown in Table I. The SRT was determined according to Winkler et al. (2011) and was maintained at around 30 days by periodically removing sludge from the reactor (excess sludge) every 2 days throughout the experimental phases. During Phase I, excess sludge for SRT control was removed during aeration (mixed sample). In Phase II, excess sludge was removed during anerobic feeding, when the granular sludge was settled. In this phase, most of the sludge was removed from the top of sludge bed to favor PAOs over GAOs. In general, 80% of the wasting sludge (in volume basis) was removed from the top and 20% from the bottom of sludge blanket. During Phases I and II, the synthetic feeding medium consisted of two solutions: (A) $NaCH_3COO{\cdot}3H_2O$ 63 mM, $MgSO_4{\cdot}7H_2O$ 3.6 mM, and KCl 4.7 mM and (B) NH_4Cl 35.4 mM, K_2HPO_4 4.2 mM, KH_2PO_4 2.1 mM, and 10 mL/L trace element solution (Vishniac and Santer, 1957). Per cycle, 150 mL was dosed from both media together with 1,200 mL of tap water

Table I. Experimental phases of both aerobic granular sludge sequencing batch reactors.

Experimental phase[a]	Excess sludge for SRT control	Influent phosphate concentration (mgP/L)	Operational time (days)
Phase I	Mixed sample	20	39
Phase II	80% Top[b] 20% Bottom[b]	20	131
Phase III	100% Bottom	2	50

[a]The operational time of SBR_{20} before the inoculation of SBR_{30} was not taken into account here. Instead, it was considered a start-up phase prior to Phase I.
[b]Top and bottom refers to the upper and down part of sludge bed, respectively.

in order to achieve influent chemical oxygen demand (COD), ammonium, and phosphate concentrations of 400 mg/L, 60 mgNH_4-N/L, and 20 mgP-PO_4/L, respectively.

The strategy of selective removing sludge mainly from the top of the sludge bed performed in Phase II, was changed in Phase III. In order to observe the link between the key factors influencing biomass segregation within the reactor sludge bed (i.e., chemical precipitation, poly-P and ash content of biomass), sludge was removed mainly from the PAO-rich bottom of the sludge bed and P-influent concentration was decreased from 20 to 2 mgP/L to provoke the washout of PAOs. Moreover, the supernatant concentrated in P, released by PAOs during the anerobic feeding, was replaced by a mineral solution (similar to the reactor influent but without P) immediately after the start of the aeration phase. This procedure was repeated for several days in order to speed up the washout of PAOs which would not be able to recover the poly-P content of cells. Nitrite, which was reported to inhibit PAOs (Saito et al., 2004), was also added in the beginning of aeration in some cycles in order to reach a concentration of 20 mgNO_2-N/L. P-removal efficiency along the operation of the reactors was determined on influent–effluent basis (100% P-removal was achieved when no P was detectable in the effluent). Cycle tests were conducted in the steady-state operation of Phases I and II in order to obtain the biomass-specific P-uptake rates. Samples were collected every 10–20 min only during aeration phase when the reactor content was mixed. Sampling during anerobic feeding was not possible due to the plug-flow operation. Specific P rates were obtained by linear regression of the concentration over time divided by the amount of volatile suspended solids (VSS) in the reactor.

Determination of Granules Physical Properties and Analytical Measurements

Particle size distribution of the granules from both top and bottom of the sludge bed (here further referred as top and bottom granules, respectively) was determined by using an image analyzer. Biomass density was measured with a pycnometer. The procedures used to calculate the reactor biomass concentration and excess sludge are described by Winkler et al. (2011). Phosphate (PO_4-P) was determined spectrophotometrically by Hach Lange cuvette tests.

In order to calculate theoretical settling behavior, the average density and diameter of both top and bottom granules were taken into account. If the Reynolds number of the particle was lower than 1, Stokes' law was used to calculate the settling velocity, $v_s = (g/18) \times (\rho_p - \rho_w/\rho_w) \times (d_p^2/\nu_w)$. The settling velocity for particles with Reynolds number higher than 1 were determined by calculating the Archimedes number ($Ar = (\rho_p - \rho_w/\rho_w) \times d_p^3(g/\nu_w)$) and hence defining the omega number from an Omega-Archimedes diagram. The settling velocity was then calculated by using the equation, $\Omega = (v_s^3 \times \rho_w)/\nu_w \times g(\rho_p - \rho_w)$. Definitions for equations are given in the Appendix.

Fluorescent In Situ Hybridization (FISH)

To assess the microbial populations, FISH was conducted on crushed granules. Crushed granules were washed twice with 1× phosphate-buffered saline (PBS) and immediately fixed with 4% (w/v) paraformaldehyde in PBS solution for 3 h at 4°C. After fixation, cells were centrifuged at 13,000*g* for 1 min, washed twice in 1× PBS, and re-suspended in an ethanol/PBS solution (1:1) for storage at −20°C. The hybridization step was performed according to Bassin et al. (2012). Fixed samples were spread on microscope slides and incubated at 46°C for drying. The cover slips with the dried cells were dehydrated in three steps with 50, 80, and 96% (v/v) ethanol. After dehydration, 10 μl of a hybridization buffer solution containing of 0.9 M NaCl, 0.02 M Tris/HCl, 35% (v/v) formamide for all probes, 0.02% (w/v) sodium dodecylsulfate (SDS), and including fluorescently labeled oligonucleotide probes (0.5 pmol for Cy3/Cy5 and 0.83 pmol for fluorescein-labeled probes) were added to the different slide wells. Hybridization was carried out in a humid chamber for 1.5 h at 46°C. A subsequent washing step to remove unbound oligonucleotides was carried out by immersing the gelatin-coated slides in a buffer containing 20 mM Tris–HCl (pH 8), 0.01% (w/v) SDS, 0.08 mM NaCl, and 0.005 mM EDTA for 10 min at 48°C. The slide wells were rinsed with Milli-Q water, dried by compressed air, and embedded in 2 μl of Vectashield H-1000 mounting oil for fluorescence (Vector Laboratories, Burlingame, CA). An epifluorescence microscope (Axioplan 2, Zeiss, Weesp, the Netherlands) was used for observation of the slides and image acquisition was performed with a Leica D350F camera. The hybridization experiments were performed using different fluorochromes for each probe to make sure that the results were reproducible. The images were exported as jpg format from the Zeiss microscopy imaging software (AxioVision version 4.7). The rRNA-targeted oligonucleotide probes labeled with three different fluorescent dyes (Cy3, Fluos, and Cy5) are listed in Table II. The PAOmix combination (PAO462, PAO651, and PAO846) was used to target *Candidatus* Accumulibacter phosphatis. GAO phenotype bacteria was also targeted by combinations of the probes GAOQ431 and GAOQ989 (GAOmix).

Sampling for FISH analysis was conducted by selectively removing sludge from either top or bottom of the sludge bed. Since our columns are made from a transparent material, removal a certain fraction of biomass can be easily controlled. Biomass samples were regularly taken from both SBRs over the whole experimental period.

Results

Long-Term Operation of the Aerobic Granular Sludge Reactors: Reactor Performance and Microbial Diversity

The operation of both reactors was divided in three experimental phases, which lasted in total 220 days. Before

Table II. Oligonucleotides probes and their targeted microbial groups.

Probe	Sequence (5′–3′)	Target group	Refs.
PAO 462	CCGTCATCTACWCAGGGTATTAAC	PAO cluster[a]	Crocetti et al. (2000)
PAO 651	CCCTCTGCCAAACTCCAG	PAO cluster[a]	Crocetti et al. (2000)
PAO 846	GTTAGCTACGGCACTAAAAGG	PAO cluster[a]	Crocetti et al. (2000)
GAO Q431	TCCCCGCCTAAAGGGCTT	Competibacter phosphatis[b]	Crocetti et al. (2002)
GAO Q989	TTCCCCGGATGTCAAGGC	Competibacter phosphatis[b]	Crocetti et al. (2002)
EUB 338 I	GCTGCCTCCCGTAGGAGT	Most bacteria	Amann et al. (1990)
EUB 338 II	GCAGCCACCCGTAGGTGT	*Planctomycetes*	Daims et al. (1999)
EUB 338 III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	Daims et al. (1999)

[a]Closely related to Rhodocyclus (*Candidatus* Accumulibacter phosphatis).
[b]*Candidatus* Competibacter phosphatis.

the start of Phase I, SBR_{20} was running stable. P-removal was around 90% and P-released/COD uptake ratio was 0.37 ± 0.05 P-mol/C-mol (data not shown). In the beginning of Phase I, when half of the biomass was removed from SBR_{20} to inoculate SBR_{30}, P-removal and P-release were not affected in SBR_{20} (Fig. 1). Conversely, the temperature shock severely affected the biomass in SBR_{30}. A significant amount of gelatinous compounds were found in the wall of the reactor, probably as a response of the biomass to the sudden change in the operating temperature from 20 to 30°C. Even though the SBR_{30} was inoculated with granules from SBR_{20}, which was achieving good P-removal, the start-up period of SBR_{30} (Phase I) was characterized by low P-removal efficiency (around 50%). Moreover, P-released/COD uptake ratio was only around 0.22 ± 0.08 P-mol/C-mol.

Samples were collected from the top and bottom of the sludge bed of the reactors for FISH analysis in order to observe the microbial community composition. FISH results indicated that PAOs and GAOs formed the majority of the microbial population. Moreover, a stratification of microbial community structure over the sludge bed was observed during Phase I. In the top of the sludge bed of both SBRs, more GAOs (Competibacter phosphatis) than PAOs (Accumulibacter phosphatis) were present. On the other hand, in the bottom of the sludge bed, PAOs were dominant. The biomass segregation was more noticeable in the reactor operated at 30°C, where the difference between microbial composition in the bottom and top of the sludge bed was even higher. A similar trend was observed in previous research at 30°C (Winkler et al., 2011).

In order to favor PAOs over GAOs and achieve better and stable P-removal, particularly in the SBR operated at 30°C, excess sludge for SRT control started to be removed mainly from the top and in minor proportions from the bottom of the sludge bed in Phase II. In this way, the SRT of top granules (GAO-rich biomass) would be reduced compared to the bottom biomass (PAO-rich biomass). In order to have the same operational conditions in both reactors, the selective sludge removal mainly of the upper part of the sludge bed was also implemented in SBR_{20}, although P-removal efficiency in this reactor was around 100% in the end of Phase I. The sludge removal in both reactors was

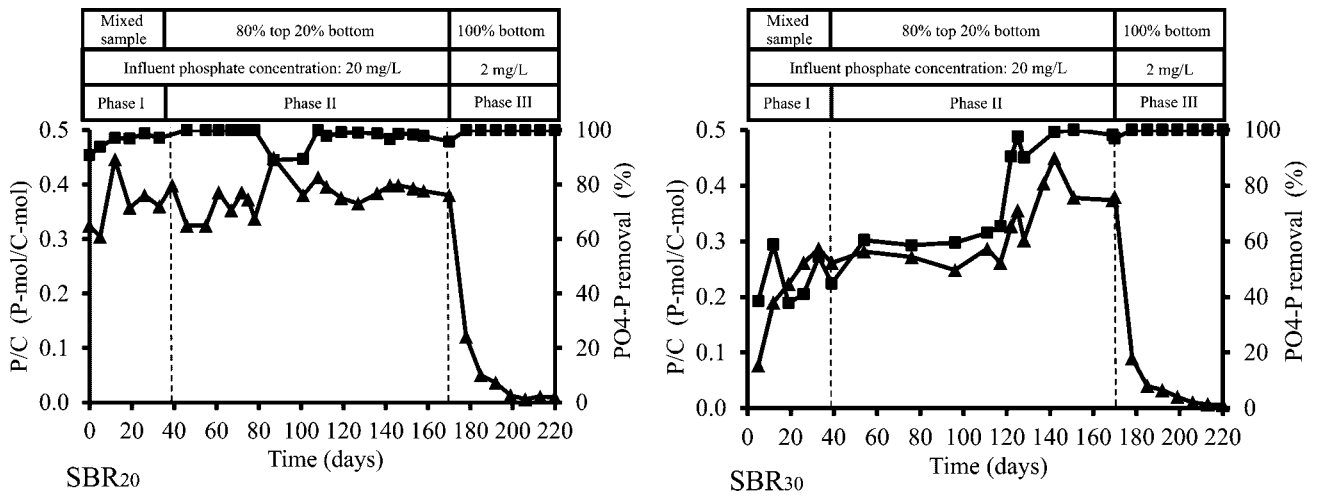

Figure 1. Phosphorus (P)-release/COD uptake ratio (▲) and P-removal (■) in the long-term operation of the SBR_{20} (**a**) and SBR_{30} (**b**). Removal of excess sludge for controlling SRT was performed in three different ways: mixed sample during aeration (Phase I), 80% from top and 20% from bottom of the sludge bed (Phase II), and 100% from bottom (Phase III). P-influent concentration was 20 mgP/L in the first two experimental phases and 2 mgP/L in the last phase.

performed every 2 days and the SRT was kept at around 30 days.

In SBR_{20}, the strategy adopted in Phase II for sludge removal did not change the performance of the reactor, which continued to achieve stable P-removal close to 100% (Fig. 1). P-release slightly increased indicating a preferential selection of PAO biomass, as shown in the cycle test (Fig. 2a). In the SBR_{30}, no immediate improvement in terms of P-removal was observed in the beginning of Phase II, when excess sludge was mainly removed from the top of the sludge bed. P-release/COD uptake ratio and P-removal remained practically constant (around 0.3 P-mol/C-mol and 60%, respectively). However, more than 90% P-removal was achieved after 80 days of operation in Phase II. P-release/COD uptake also increased during this phase, amounting around 0.4 P-mol/C-mol at the steady-state condition (Fig. 1). The increase in the P-release from Phases I to II can also be observed in the cycle tests of SBR_{30} (Fig. 2b).

The specific P-uptake rate in both reactors at the end of operation of Phases I and II was obtained from the cycle tests. The results are displayed in Figure 2a (SBR_{20}) and 2b (SBR_{30}). During anerobic feeding from the bottom of the reactors, all influent acetate (COD of 400 mg/L) was taken up (data not shown) and P was released by PAOs. P was removed from the bulk liquid by PAO both aerobically (by aerobic PAOs) and anoxically (by denitrifying PAOs (DPAOs)) in the aerobic and anoxic zones within the granules, respectively. Ammonium and nitrite were nitrified in the outer aerobic layer by ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), respectively, and nitrite/nitrate was simultaneously denitrified in the anoxic zone of the granules by denitrifying organisms. Since all acetate was consumed in the anerobic phase (feast phase) by PAOs and GAOs, no external carbon source was available during aeration period for heterotrophic denitrifiers. Therefore, the presence of ordinary denitrifying heterotrophs was minimized and denitrification was performed by DPAOs or denitrifying GAOs (DGAOs) in the inside of the granules, which used intracellular PHB as electron donor for this process during the starvation period (famine phase). The activity of DPAOs was confirmed by conducting anoxic cycle tests (continuous supply of nitrite or nitrate and nitrogen gas instead of air) in the reactor (data not shown).

In Phase I, specific P-uptake rates observed in SBR_{20} and SBR_{30} were 3.5 and 2.3 $mgPO_4$-P/(gVSS · h), respectively. The lower P-uptake rate obtained in SBR_{30} reflects the results obtained in the long-term operation of this system, which showed that the maximum P-removal obtained during Phase I was around 60%. The results obtained in SBR_{30} clearly show that the growth and activity of PAOs were severely affected by temperature increase from 20 to 30°C. With the strategy in which excess sludge for SRT control was mainly removed from the top of sludge bed to favor PAOs over GAOs during Phase II, the specific P-uptake rate in SBR_{20} slightly increased from 3.5 to 3.9 $mgPO_4$-P/(gVSS · h). On the other hand, specific P-uptake significantly increased in SBR_{30}, reaching 5.9 $mgPO_4$-P/(gVSS · h).

The stratification in the microbial population over the sludge bed observed in Phase I was maintained in both reactors in Phase II. Moreover, during the course of Phase II in SBR_{30}, biomass gradually changed in color compared to the original biomass collected from SBR_{20} (Fig. 3a), and some big white granules appeared (Fig. 3b). FISH analysis showed that these white granules were strongly dominated by PAOs, while GAOs were present in minor proportions (Fig. 4b). Some black granules (shown in detail in Fig. 3a) were also separately analyzed by FISH for comparison. As shown in Figure 4a, these specific granules were composed

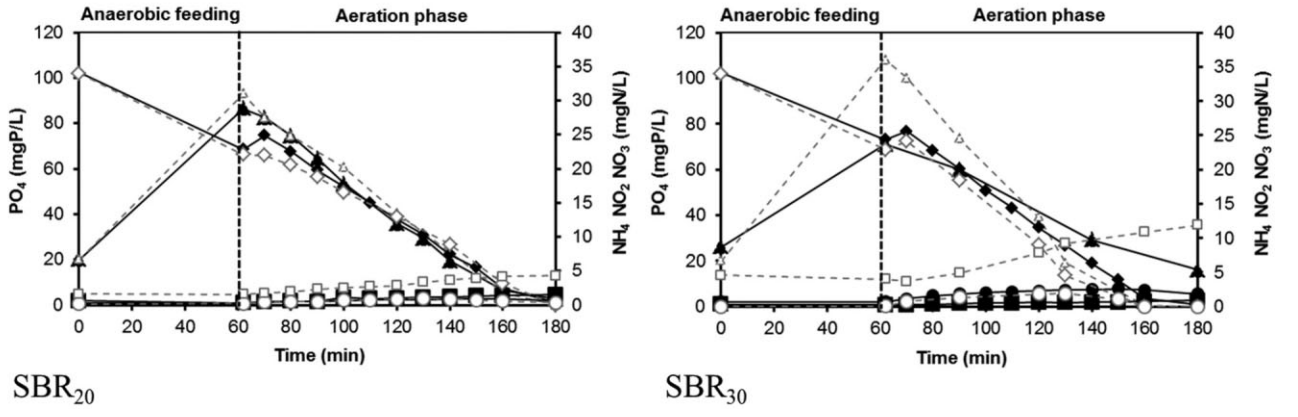

Figure 2. Cycle tests performed in the SBR_{20} and SBR_{30} during different operational phases: phosphate (▲), ammonium (◆), nitrite (●), and nitrate (■) in Phase I; phosphate (Δ), ammonium (◇), nitrite (○), and nitrate (□) in Phase II. The starting ammonium and phosphate concentrations depicted at time 0 were calculated based on the influent concentration (60 $mgNH_4$-N/L and 20 $mgPO_4$-P/L) and the dilution in the reactor. Nitrite and nitrate concentrations at time 0 were calculated based on their concentrations in the end of the cycle and the dilution in the reactor.

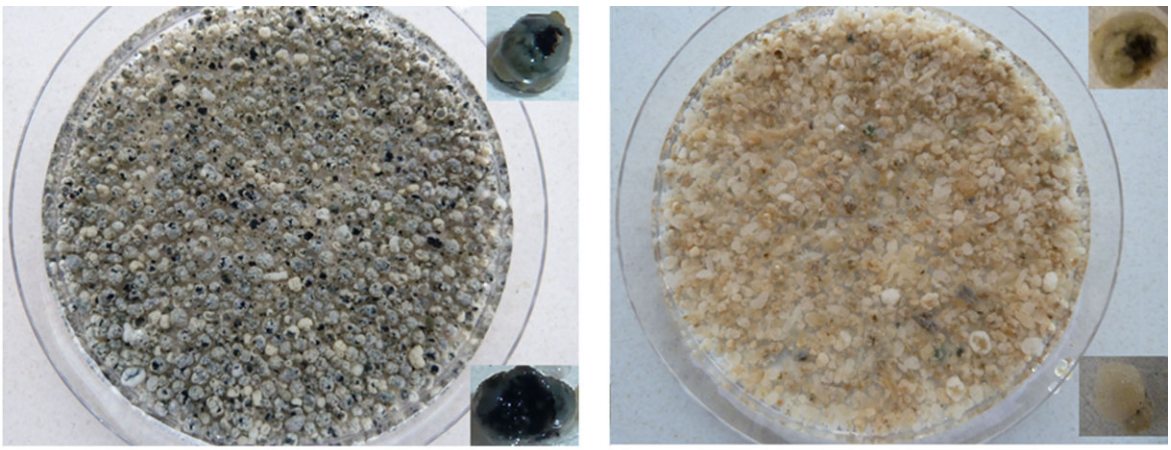

Figure 3. Granules from SBR_{20} and SBR_{30} during operation in Phase II. The core of big granules is shown in detail in the right side of each picture. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

mainly by GAOs. The ash content of the white granules was around 40%, which is higher compared to other granule types in the reactor (presenting around 20% ash content). In general, the ash content of mixed biomass of SBR_{30} considerably increased from around 15% to almost 30% along the operation in Phase II (Fig. 5). In SBR_{20}, biomass ash content remained constant in the first two experimental phases.

In Phase III, excess sludge for SRT control started to be removed only from the bottom of the sludge bed and the influent phosphate concentration was decrease from 20 to 2 mgP-PO_4/L. This experimental procedure was adopted to provoke the washout of PAOs and hence have a dominance of GAOs in order to clarify the link between the biomass ash content, poly-P content of the granules and chemical precipitation. The resulted biomass density and settling velocity were also investigated. With the low influent phosphate concentration in Phase III (2 mgP-PO_4/L), P-removal efficiency was always 100% in both SBRs. However, the P-release/COD uptake ratio decreased gradually and after 20 days of operation in Phase III, almost no P-release was observed in both reactors (Fig. 1). In the meantime, FISH pictures showed that the PAO population gradually disappeared from the granular sludge, and after 1 month of operation in Phase III, only few cells of PAOs were still detected in a GAO-dominated culture in

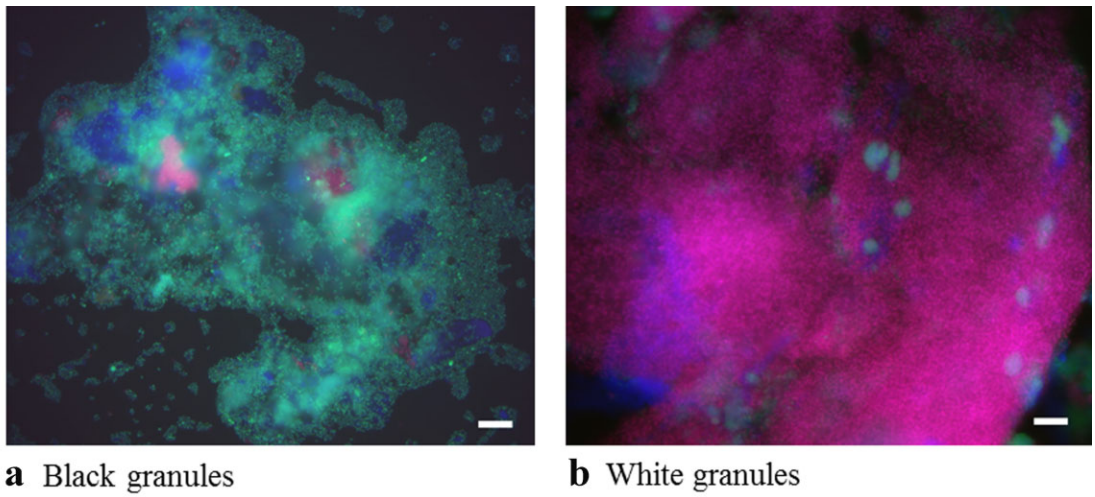

Figure 4. Fluorescent in situ analysis of the PAO/GAO populations among all the bacteria present in the black (**a**) and white (**b**) granules from SBR_{30} (Phase II). Combinations of specific probes for PAO (PAO462, PAO462, and PAO846, shown in red), GAO (GAOQ431 and GAOQ989, shown in green), and general bacteria EUB338 (EUB338I, EUB338II, and EUB338III, shown in blue) were used. Scale bar indicates 20 μm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

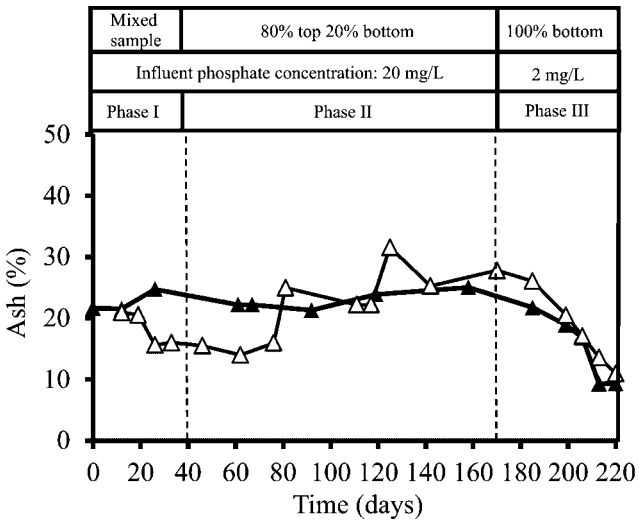

Figure 5. Ash content of biomass (mixed top and bottom samples) from both reactors during operation in Phases I–III.

both SBRs (GAOs amounted to more than 95% of the total microbial community). In SBR_{30}, the color of the granules changed back to its original color (brownish-black) and the ash content of the biomass in both reactors gradually decreased from 30% to 10% (Fig. 5).

Biomass Concentration and Physical Properties of Top and Bottom Granules

The biomass concentration in both aerobic granular sludge SBRs was kept in the range between 10 and 14 gVSS/L. Density, average diameter, and settling velocity of both top and bottom granules are displayed in Table III. In general, both the diameter and the density of top and bottom granules increased from Phases I to II. The increase of these parameters was more noticeable in the bottom biomass. However, in Phase III, the density of both top and bottom granules considerably decreased. Moreover, since the granules were composed mainly by GAOs in the last experimental phase, the difference in density of bottom and top granules was marginal. The calculated settling velocity (based either on Stokes law or Archimedes' number) of bottom granules was considerably higher than top granules due to their higher diameter and density. Ash content of the biomass (mixed top and bottom sample) during the whole operational period is shown in Figure 5.

Discussion

Operational Factors Influencing PAO/GAO Competition and P-Removal Performance

Two aerobic granular sludge reactors were operated at 20°C and 30°C. Stable P-removal was readily established at 20°C but found to be more troublesome at 30°C. This is in line with results described in literature where higher temperatures (>20°C) are reported to cause the deterioration of bio-P-removal systems (Barnard and Steichen, 2006; Gu et al., 2005; Panswad et al., 2003; Whang and Park, 2002). Indeed, during operation in Phase I, P-removal observed in SBR_{30}, operated at 30°C, was considerably lower than that observed in SBR_{20}, operated at 20°C.

The fraction of PAOs and GAOs present in the biomass was not evaluated by quantitative FISH. This technique is more suitable when individual (free) cells are present, whereas clusters of biomass (as in our case) are difficult to be quantified by image analysis software. Instead, we used the ratio between P-release and COD (in our case acetate) uptake (P-release/COD uptake), which is a typical parameter used to estimate the amount of organic carbon (COD) taken either by PAOs or GAOs under anerobic conditions (Schuler and Jenkins, 2003). This ratio is expected to be around 0.5 P-mol/C-mol in a highly enriched PAO culture and zero in a pure GAO culture (Brdjanovic et al., 1997).

Table III. Density, average diameter, and settling velocity of both top and bottom granules.

	SBR_{20}						SBR_{30}					
	Phase I		Phase II		Phase III		Phase I		Phase II		Phase III	
Parameter	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom	Top	Bottom
Density (g/L)	1003 ± 2	1008 ± 4	1004 ± 3	1010 ± 5	1001 ± 2	1004 ± 5	1004 ± 2	1005 ± 3	1004 ± 1	1008 ± 4	1001 ± 2	1003 ± 2
Average diameter (mm)	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	1.0 ± 0.3	0.6 ± 0.3	0.9 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 0.3	0.5 ± 0.1	0.8 ± 0.1
Reynolds[a]	0.6	1.8	1.1	6.3	0.3	2.3	2.4	2.7	1.5	7.6	0.6	3.2
Archimedes' number	n.c.	32.4	19.1	114.0	n.c.	41	40.5	49.0	17.4	137.4	n.c.	57.3
Calculated settling velocity (m/h)	3.3	20.2	15.1	50.6	1.9	37.1	39.4	41.6	15.4	48.4	3.2	39.0

n.c., Not calculated.

[a]For $Re < 1$, Stokes law was considered to calculate the settling velocity; for $Re > 1$, settling velocity was calculated from the Archimedes' number, as described in the Materials and Methods Section.

During Phase I, we observed that the P-release/COD uptake ratio was indeed higher in SBR_{20} (0.37 ± 0.05) compared to that of SBR_{30} (0.22 ± 0.08). This result suggests that a higher amount of active PAOs was present in SBR_{20} in comparison to that of SBR_{30} in the first experimental phase. However, the P-release/COD uptake ratio observed in both reactors indicates that a significant amount of acetate was taken up by non-PAO bacteria.

The segregation of the biomass over the sludge bed (PAOs concentrated at the bottom and GAOs at the top) observed during the operation of both reactors in Phase I opened a possibility to influence the PAO–GAO competition. This trend was already observed in our previous recent research (Winkler et al., 2011), in which we operated an aerobic granular sludge reactor at 30°C. In order to obtain better P-removal, particularly at 30°C (SBR_{30}), we decided to remove excess sludge mainly from the top of the sludge bed (dominated by GAOs) during the feeding period. A similar procedure for excess sludge removal was also conducted in previous work (Winkler et al., 2011). Indeed, the selective removal of sludge mainly from the GAO-rich upper part of the sludge bed decreased the SRT of GAOs compared to PAOs allowing for complete P-removal in SBR_{30} after 80 days under these operating conditions. Moreover, the gradual increase of P-release/COD uptake ratio (up to 0.4) suggested that the percentage of active PAOs increased during the course of Phase II. Also the appearance of white granules in SBR_{30} in which PAOs corresponded to more than 90% of the microbial population may also explain why higher P-uptake rates and P-release/COD uptake ratio started to be obtained in SBR_{30} than in SBR_{20}.

The strategy of selective removal of sludge from specific parts of the sludge bed, already suggested in our previous research (Winkler et al., 2011) and adopted again in this study, confirmed to be a good method to favor the development and growth of PAOs at unfavorable high temperatures and therefore improve P-removal capability. Our strategy also enhances PAO activity since the feeding is performed from the bottom of the reactor, where PAOs are located. Consequently, these organisms get more substrate than GAOs. However, in our previous study no sludge was removed from the PAO-rich bottom of the sludge bed for SRT control. This led to a very high SRT of bottom granules and PAOs will get completely filled with polyphosphate due to the decreased effective sludge yield, limiting further P-removal. In the research, we conducted previously this fact was neglected leading to an open question for implementation of the strategy applied. We pointed out that eventually it would be necessary to remove part of the bottom sludge to avoid excessively high SRT values and to remove poly-P from the system. High SRT of bottom granules will cause a big fraction of inert material (ash) in this particular biomass, which is not desirable. First of all, high ash content would lead to significantly heavy granules and probably cause mixing problems during aeration. It is also well possible that the granules containing high ash would become very big and the surface area available for aerobic organisms (e.g., aerobic PAOs, AOB, and NOB) would decrease.

Taking into account all these factors, the selective removal of sludge from specific parts of the sludge bed was modified in the current research from that applied in our previous work (Winkler et al., 2011). Therefore, even though sludge was removed mainly from the GAO-rich top of the sludge bed, in this study we also removed a small fraction (20%) of the excess sludge from the PAO-rich bottom sludge. This procedure of renewal of bottom biomass, besides not causing a negative impact on P-removal, allowed obtaining lower amount of ash in the biomass compared to our previous study and avoided the deterioration of P-removal in the long-term operation of both SBRs.

Even though the importance of selective sludge removal was confirmed in the current research to be a good method to improve and obtain stable P-removal at 30°C, we observed that at lower temperatures, such as 20°C, the selective removal sludge from specific parts of the sludge blanket to favor PAOs over GAOs is not crucial, as indicated by the results obtained during the operation of SBR_{20}. In that system, full P-removal was obtained even when excess sludge was removed during aeration phase, in which biomass was equally distributed throughout the reactor. Therefore, the strategy adopted to control microbial population in our study is especially relevant under operational conditions in which the development of GAOs is favored compared to that of PAOs, such as high temperatures. It is important to remark that we have chosen to remove 80% of the excess sludge from the top of the sludge bed and 20% from the bottom of the sludge blanket to favor PAOs over GAOs. For full-scale applications it is likely that the ratio of top and bottom sludge has to be controlled based on process performance. This ratio might depend for instance on seasonal variations. This will have to be further tested in full-scale and pilot-scale research.

Understanding the Link Between the Main Factors Influencing Biomass Segregation Within the Reactor Sludge Bed

A possible explanation for the occurrence of biomass segregation can be the different settling velocities shown by bottom (dominated by PAOs) and top granules (dominated by GAOs). In the granules in which PAOs are dominant, higher poly-P would be present in the biomass compared to the GAO-dominated granules, since no P-uptake or P-release is involved in the metabolism of GAOs. In a first attempt, the significant amount of poly-P accumulated by PAO-rich bottom granules during the aerobic phase was assumed to be responsible for their higher ash content and density compared to GAO-rich granules, making those granules heavier and hence contributing to improve their settling properties. As a consequence, PAO-dominated granules settled faster than GAO-rich granules, and thereby accumulate in the bottom fraction of the sludge bed

(Winkler et al., 2011). Additionally, heavier poly-P containing granules, present at the bottom of the sludge bed, were more exposed to substrate (acetate) fed from the bottom of the reactor. This can also enhance the dominance of PAOs over GAOs, driven by the selective sludge removal mainly from the top of the sludge bed.

The appearance of complete PAO-dominated white granules in the reactor operated at 30°C was also associated to the amount of poly-P present in the biomass. The development of these granules is possibly a consequence of the sludge removal procedure adopted. Since most of the sludge was removed from the GAO-rich upper part of sludge bed, bottom sludge could be highly enriched for PAOs. Eventually, in some of the granules, the fraction of PAOs and therefore the amount of poly-P was higher than in others. This could enhance chemical precipitation of PO_4^{3-} within the granules (Carlsson et al., 1997; Maurer et al., 1999). As a consequence of chemical precipitation, the color of the granules may have turned white and may have increased the ash content in this specific biomass (to around 40%). However, the color changes of the granules observed in our study (from dark color to white) is not a proof, although it gives an indication of higher precipitation in some granules. The substantial increase in P-release observed during the operation of SBR_{30} in Phase II could possibly enhance chemical precipitation within the granules and therefore contributed for the appearance of white granules.

In order to better understand the link between the main factors influencing biomass segregation and therefore explain why PAOs and GAOs occupy different positions within the sludge bed, we decided to perform extra experiments to support the observations pointed out in this work and in our previous research (Winkler et al., 2011). By provoking the washout of PAOs in both reactors, we could observe how the ash content, poly-P content and chemical precipitation within the granules change from an operational stage in which PAOs and GAOs coexisted to other when GAOs are prevalent (corresponding to more than 95% of the whole bacterial community). In the GAO-dominated systems, we observed that not only the ash but also the density of the granules considerably decreased. As a consequence, the settling velocity of the granules was substantially reduced when PAOs were removed from the system. This implies that PAO/GAO segregation over the sludge bed is primarily associated with the different poly-P and hence different ash content/density of the PAO- and GAO-dominated granules, confirming our previous expectations.

The biomass segregation observed in this study seems to be a trend in sequencing-batch reactors operated in a similar way. When a plug-flow feeding regime from the bottom of the reactor is applied, bottom biomass would have more availability of substrate and the chance of having this type of segregation (more PAOs at the bottom and more GAOs at the top of the sludge bed) is even higher. The probability of having the segregation is especially relevant at high temperatures (e.g., 30°C).

Conclusions

We have shown that segregation of biomass in sequencing-batch aerobic granular sludge reactors accomplishing P-removal offers a possibility to control PAO–GAO competition. The selective removal of biomass mainly from the GAO-rich part of the sludge bed is especially relevant at high temperatures, such as 30°C, unfavorable for the P-removal process. At 20°C, this procedure is not crucial and biomass can be removed equally throughout the sludge bed. The removal of sludge from the PAO-rich part of the sludge bed in minor proportions did not negatively affect P-removal and allowed to obtain biomass with a lower ash content. Our results indicate that high ash content and density positively correlated with the presence of PAO-dominated granules and therefore with high biomass poly-P content and P-removal efficiency. Higher poly-P content and higher temperatures would possibly lead to higher chemical precipitation within the granules, which eventually can provoke changes in biomass color.

Appendix

$$Ar = \frac{\rho_p - \rho_w}{\rho_w} \times d_p^3 \times \frac{g}{\nu_w}$$

$$\Omega = \frac{v_s^3 \times \rho_w}{\nu_w \times g(\rho_p - \rho_w)}$$

$$v_s = \frac{g}{18} \times \frac{\rho_p - \rho_w}{\rho_w} \times \frac{d_p^2}{\nu_w}$$

v_s sedimentation velocity of a single particle (m/s)
d_p particle diameter (m)
ρ_p density of particle (kg/m^3)
ρ_w density of the fluid (kg/m^3)
g gravitational constant 9.81 (m/s^2)
ν_w kinematic viscosity water (m^2/s)

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