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# Selective sludge removal in a segregated aerobic granular biomass system as a strategy to control PAO–GAO competition at high temperatures

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ABSTRACT

An aerobic granular sludge (AGS) reactor was run for 280 days to study the competition between Phosphate and Glycogen Accumulating Organisms (PAOs and GAOs) at high temperatures. Numerous researches have proven that in suspended sludge systems PAOs are outcompeted by GAOs at higher temperatures. In the following study a reactor was operated at 30 °C in which the P-removal efficiency declined from 79% to 32% after 69 days of operation when biomass removal for sludge retention time (SRT) control was established by effluent withdrawal. In a second attempt at 24 °C, efficiency of P-removal remained on average at 71 ± 5% for 76 days. Samples taken from different depths of the sludge bed analysed using *Fluorescent in situ hybridization* (FISH) microscopy techniques revealed a distinctive microbial community structure: bottom granules contained considerably more *Accumulibacter* (PAOs) compared to top granules that were dominated by *Competibacter* (GAOs). In a third phase the SRT was controlled by discharging biomass exclusively from the top of the sludge bed. The application of this method increased the P-removal efficiency up to 100% for 88 days at 30 °C. Granules selected near the bottom of the sludge bed increased in volume, density and overall ash content; resulting in significantly higher settling velocities. With the removal of exclusively bottom biomass in phase four, P-removal efficiency decreased to 36% within 3 weeks. This study shows that biomass segregation in aerobic granular sludge systems offers an extra possibility to influence microbial competition in order to obtain a desired population.



## 1. Introduction

Aerobic granular sludge (AGS) reactors are based on the same principle as upflow anaerobic sludge blanket (UASB) reactors in which particles are freely suspended in an upward flow of air and liquid. Contrary to flocculent sludge processes, the biomass in these reactors is not homogeneously mixed. Mixing with gas yields a higher concentration of biomass at the bottom of the reactor than the top. Granules do not have identical physical characteristics and therefore there is a segregation of granules. Granules with larger radius or higher specific density will develop more rapidly settling characteristics, are therefore

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often at the bottom of the sludge bed. This segregation is viewed as a disadvantage in the particulate biofilm reactors because they lead to instabilities (Ro and Neethling, 1994; Safferman and Bishop, 1996; Trinet et al., 1991). These instabilities are attributed to a lower shear stress in the top of the sludge bed due to lower density (Gjaltema et al., 1997). The first attempts to numerically-model segregation of biomass in dependency of particle density and diameter caused by outgrowth were made by DiFelice et al., 1997 for fluidized bed reactors. Selection properties can be applied to prevent uncontrolled outgrowth of biomass by using batch wise feeding in granular sludge reactors (Beun et al., 2001). This generates a microbial population with a lower growth rate and hence smoother granules, which makes shear less important for a selection of well settling particles (de Kreuk and van Loosdrecht, 2004; Van Loosdrecht and Heijnen, 1993). Nicolella and colleagues wrote a review concerning the strength of particle-based biofilm reactors and their potential to develop compact and high rate nutrient removal processes. Nevertheless, for this particular technology segregation of granules is acknowledged to be a difficult process due to, for instance, clogging. However, researchers have neglected that segregation of differently sized granules may in fact offer different biological niches for bacteria (Nicolella et al., 2000). In UASB reactors as well as in AGS reactors segregation of microbial communities can occur as a function of depth within the different layers of the granules (Macleod et al., 1990; Tsuneda et al., 2003; Xavier et al., 2007). However, it has been neglected that segregation might also occur over the settled sludge bed due to differently sized granules. Circumstances like shear stress or substrate concentrations are different at certain depths. As a result, distinct biological niches can be generated within one reactor, by which one organism can be favoured over others. For instance, it might be possible to influence the SRT of certain organisms independent of other bacteria depending on the place of excess sludge withdrawal. The importance of controlled biomass removal in biofilm systems has already been experimentally and mathematically discussed earlier (Morgenroth and Wilderer, 1999).

Previous research has demonstrated that PAOs were prevalent at 10 °C regardless of the specific carbon source or pH (de Kreuk et al., 2005; Lopez-Vazquez et al., 2009b). At temperatures between 20 and 30 °C GAOs are expected to dominate the culture, while at increasing temperatures common heterotrophs dominate the system (Erdal et al., 2003; Lopez-Vazquez et al., 2009a; Panswad et al., 2003; Whang et al., 2007). In our research we hypothesise that if PAOs and GAOs would be differentially distributed over the sludge bed, then selective removal of the GAO dominated section of the settled sludge bed would reduce the SRT for GAOs relative to PAOs. This would in turn make it feasible to obtain good biological phosphate removal at temperatures above 20 °C.

## 2. Material and methods

### 2.1. Cycle operation and measurements

The granular sludge reactor operation was similar to that described by de Kreuk and van Loosdrecht (de Kreuk and van Loosdrecht, 2004). It consisted of a 60 min anaerobic feeding period from bottom of the reactor in a plug flow regime followed by a 111 min period of aeration, 3 min settling, 5 min effluent withdrawal and a 1 min idle period. In the aeration period the dissolved oxygen (DO) concentration and pH were controlled at 20% air saturation and 7 ± 0.2 pH units, respectively. Temperature was held constant at 30 °C with a thermocycler and was protected against cooling with a cellular isolation placed around the reactor. The feed medium consisted of 3.1 mM $NaCH_3COO \cdot 3H_2O$ (400 mg COD/L), 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM KCl, 2.1 mM $NH_4Cl$ (60 mg N/L), 0.2 mM $K_2HPO_4$ and 0.1 mM $KH_2PO_4$ (20 mg P/L). A 'Vishniac and Santer' solution was used to provide trace elements (Vishniac and Santer, 1957). A cycle measurement was conducted and samples were taken during the aerated mixing period to measure system performance of P- and COD- removal during one cycle of operation. Phosphate was analysed spectrophotometrically by the use of standard test kits (Hach-Lange). Calculations for P-removal efficiency are based on influent–effluent basis and 100% efficiency was hence reached when no P was detectable in effluent.

### 2.2. Long term reactor operation

The reactor operation can be divided into 4 phases. Firstly, the reactor was run for 69 days at 30 °C and was inoculated with granules from an aerobic granular sludge pilot plant in Epe, The Netherlands, treating municipal wastewater and showing excellent N- and P- removal efficiencies. In the second phase, half of the granules were removed and replaced with granular sludge from a lab reactor that was operating at 20 °C with an excellent P-removal efficiency. Temperature was decreased to 24 °C, to favour PAOs, and the reactor was operated from day 69 until day 150 under the set conditions. During phase one and two the SRT was controlled by the sludge washed out with the effluent withdrawal. In the third phase which lasted from day 150 until day 240, sludge was manually withdrawn from the upper part of the sludge bed and the temperature was raised to 30 °C. In the final and fourth phase, sludge was removed from the bottom to provoke the washout of PAOs. In the final two phases, the SRT was controlled at approximately 21 days by removing every third day 15% (phase 3 and 4a) or every sixth day 30% (phase 4b) of the settled sludge bed. The SRT was calculated taking the volatile suspended solids (VSS) from the reactor, effluent, and excess sludge into consideration. A sample was taken and transferred into a small measuring cylinder for determination of the VSS from the reactor and excess sludge. The volume of settled biomass in the small cylinder was used to calculate the ash and dry weight of the biomass. The VSS of settled biomass within the cylinder was then related back to the volume occupied by the settled sludge bed in the reactor as well as to the volume taken out as excess sludge by recording the height of settled sludge in the reactor and the height of excess sludge removed. The VSS was calculated on a mass basis by the following equation $VSS_{r,ex} = DW_{set.\ r,ex} - ash_{set.\ r,ex}$[gVSS]. The SRT was calculated on the basis of the change in height of the sludge bed occurred due to growth ($VSS_r$), the amount of excess sludge removed for manual SRT control ($Q_{ex,VSS}$) and by the sludge washed out with effluent withdrawal ($Q_{eff,VSS}$). The calculation was conducted according to the following equation

$SRT = V_r \times VSS_r/Q_{eff,VSS} + Q_{ex,VSS}$[day]. Please refer to Appendix 1 for the definitions and side calculations.

### 2.3. Density and size distribution measurements of top and bottom granules

Bottom and top granules were sampled for measurements of particle size distribution, dry weight, ash content and granule density. Specific biomass density was measured with a pycnometer and size distribution measurements were conducted by the means of an image-analyser. Stokes law for laminar flow was used to calculate settling velocities by applying the equation $SV = g/18 \cdot \rho_p - \rho_w/\rho_w \cdot d_p^2/\nu_w$ for $Re_{particle} \leq 1$ and compared to measured settling velocities which were recorded as the time that granules settled in a 30 cm volumetric cylinder. Definitions for Stokes law are given in Appendix 2. In phase four, the removed bottom biomass was sampled on a weekly basis to measure a change in settling behaviour and physical properties caused by selective sludge removal over time.

### 2.4. Fluorescent in situ hybridization (FISH)

Bottom and top samples were taken over time for FISH analysis in order to assess microbiological properties. FISH was performed on crushed mixed (Fig. 3.1a and 1b), top (Fig. 3.2a–4a) and bottom granules (Fig. 3.2b–4b) in order to determine GAO and PAO microbial populations. Different probes were tested to ensure a good representation of PAO and GAO population and the resulting probes and sequences are listed in Table 1. Crushing was accomplished on 10 ml granules by the means of a glass mortar (Glas-Col). From this suspension 500 μl were fixed in 4% paraformaldehyde and incubated for 120 min at room temperature. After fixation, samples were centrifuged for 2 min at 16,000 rpm, washed twice in 1x Phosphate buffer saline (PBS), and re-suspended in volume of 1:1 Ethanol/PBS buffer for storage at −20 °C. For hybridization, the fixed samples were dried on a hybridization slide with 6 wells preventing mixing of probe in adjacent wells and dehydrated by incubating the microscope slides in 50%, 80% and 100% ethanol for 3 min in each solution. After dehydration, the hybridization solution (10 μl) and 25 ng of oligonucleotide probe tagged with a fluorescent label (Fluos, Cy5 or Cy3) was added to each well, and the samples were incubated for 2 h in a humid chamber at 46 °C. The hybridization buffer consisted of a mixture of 360 μl of 5 M NaCl, 40 μl of 1 M Tris (pH 8), 10 μl of a 10% (w/v) sodium dodecylsulfate buffer (SDS), 700 μl of formamide, and 900 μl of MilliQ water (Amann et al., 1990; Crocetti et al., 2002, 2000; Daims et al., 1999). After hybridization, the microscope slides were washed at 48 °C for 15 min by immersing them into 50 ml of washing solution consisting of 800 μl of 5 M NaCl, 500 μl of 0.5 M EDTA, 1000 μl of 1 M Tris (pH 8), and 50 μl of 10% SDS (w/v). The samples were dried and prepared with 2 μl antifade fluorescent mounting oil and analysed with an epifluorescence microscope (Axioplan 2, Zeiss). Ratios between PAOs and GAOs were roughly estimated based on visual determination.

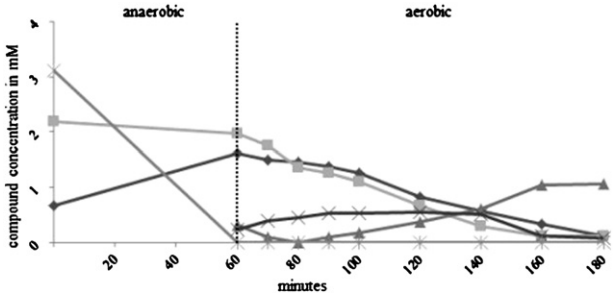


**Fig. 1 – Typical concentration patterns of phosphate (◆), nitrate (▲) nitrite (X) and ammonium (■) during a cycle in an aerobic granular sludge reactor. COD (✳) is completely consumed in the anaerobic period. Note that measurement during anaerobic phase is not possible due to a strict anaerobic plug flow operation without mixing.**

## 3. Results

### 3.1. Cycle operation

A cycle measurement was conducted in phase three to show a typical reactor performance during one cycle of operation (Fig. 1). A classical graph for N- and P- removal behaviour in aerobic granular sludge based systems is depicted as described by de Kreuk et al. (2005); nitrification occurs on the outer layers and denitrification and phosphate uptake in the core of the granules. One cycle lasted 3 h starting with 60 min of anaerobic feeding during which all incoming ammonium, phosphate and acetate were fed from the bottom of the reactor in a plug flow regime. All acetate was taken up during the anaerobic feeding period and phosphate was released due to the activity of PAOs. Samples for the cycle measurement were collected only during the aerobic mixing period since sampling is not possible during feeding due to the plug flow regime, which should not be disturbed.

### 3.2. Long term operation

The reactor was operated in four phases over a time period of 280 days. The reactor initially established a P-removal efficiency of 79% when the reactor was run in phase one at 30 °C. However, after 69 days of operation P-removal efficiency dropped to 32% (Fig. 2A phase 1). In order to enhance P-removal efficiencies in phase two, half of the sludge was discharged and the reactor was inoculated with new granular sludge from a lab-scale AGS reactor run at 20 °C showing 100% P-removal efficiencies. Following this inoculation, the operational temperature was decreased to 24 °C (phase 2) and the P-removal efficiency remained on average at 71 ± 5% for 76 days (Fig. 2A). During the first two phases, the SRT was defined by washout of sludge during effluent withdrawal.

In the second phase, FISH results illustrated that sludge samples taken from the top of the sludge bed consisted of more GAOs (Fig. 3.2a) whereas the bottom sludge contained more PAOs (Fig. 3.2b). In order to selectively remove GAOs (*Competibacter*) from the system and hence keep their SRT lower in respect to PAOs (*Accumulibacter*) a third phase was initiated in which biomass was withdrawn from the upper part of sludge bed. The amount of biomass withdrawn was established as such that an average SRT of 25 ± 15 days was

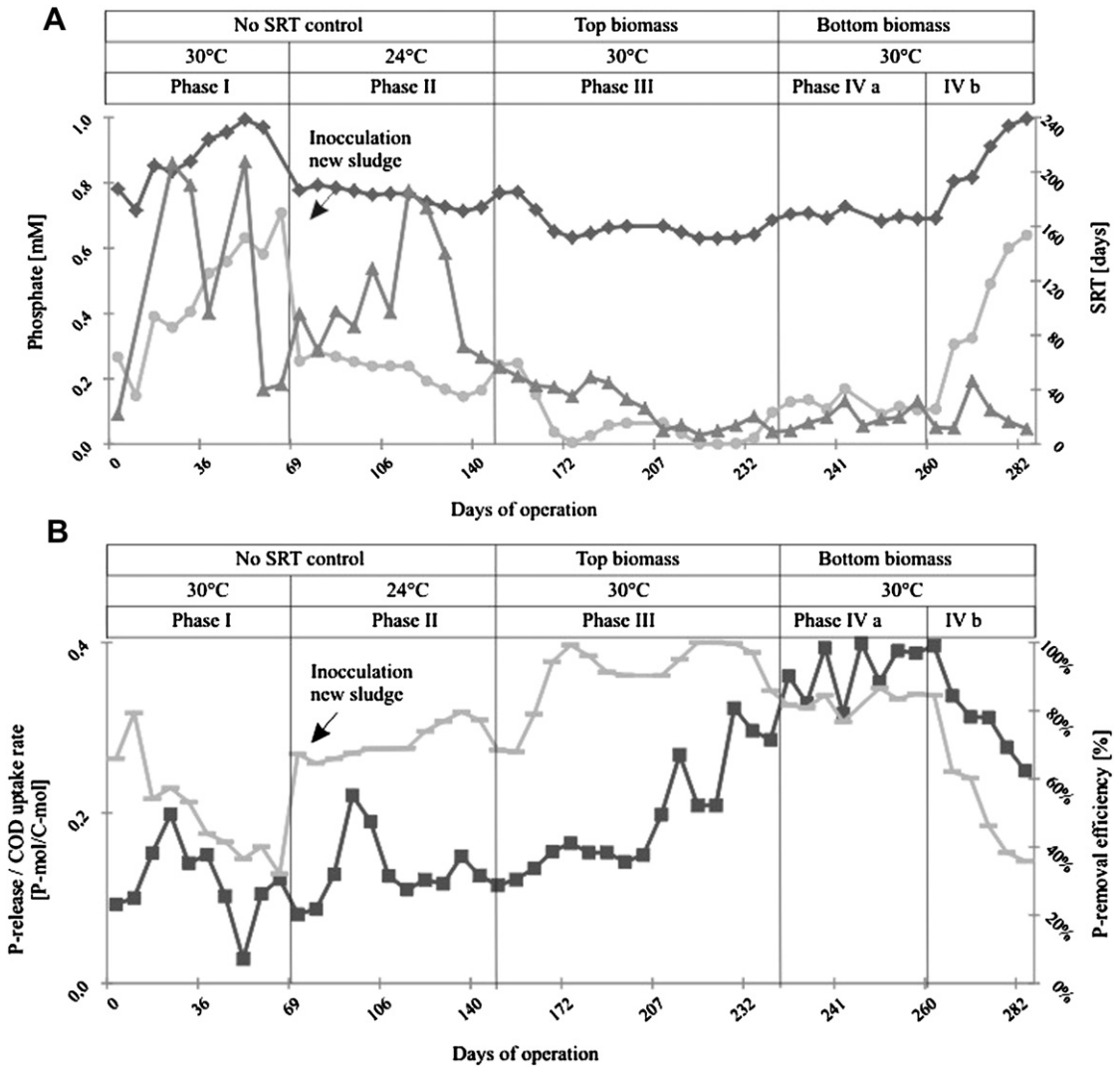


**Fig. 2 – (A) Phosphate effluent (●) and influent (◆) concentration in mM $PO_4$–P as well as SRT (▲) in days. (B) shows the P release/COD uptake ratio (■) and the P-removal efficiency (-) over time. Experimental setup was divided in four phases. In phase one the reactor was run at 30 °C when removal efficiency dropped to 30%, a second phase started and the system was inoculated with new granular sludge with excellent P-removal capacity, after which P-removal efficiency remained 50%. In phase three, granules were manually withdrawn from the upper part of the sludge bed resulting in 100% P-removal efficiency. In phase four, granules were discarded from the bottom of sludge bed resulting in a collapse in removal efficiency.**

achieved according to the SRT calculation as given in the material and methods section. Furthermore, the temperature was raised to 30 °C to disfavour PAOs and in order to measure the effect of sludge control on the PAO–GAO competition. Within 3 weeks the P-removal efficiency improved to up to 100% and remained on average at 92 ± 7% for an additional 67 days (Fig. 2A phase 3). In the fourth phase sludge was removed from the bottom to determine whether higher P-removal efficiencies from phase 3 are indeed due to selective removal of top sludge or solely an effect of lower SRT. Selective removal of the bottom PAO dominated sludge eventually resulted in a decrease in P-removal efficiency to 36% within 6 weeks (Fig. 2A phase 4). Phase four consisted of two sub phases: in phase 4a sludge was removed in the same manner as it was accomplished for removal of top sludge which was based on removing approximately 15% of the settled sludge bed every three days, achieving on average an SRT of 21 ± 7 days. During this phase removal efficiency remained on average at 83 ± 8%. In phase 4b the selective removal was changed to see the effect of removing a bigger PAO fraction. Here the SRT was kept constant, however, instead of removing 15% of the settled sludge bed every three days, 30% was removed every six days. During this time the P-removal efficiency dropped from 85% to 36% stressing the importance of proper sludge control.

The P release/COD uptake ratio is depicted in Fig. 2B. In a highly enriched PAO culture a P release/COD uptake ratio of about 0.5 P-mol/C-mol can be expected in contrast to a pure GAO culture in which this ratio would decline to zero (Brdjanovic et al., 1997; Smolders et al., 1994). Ratios of about

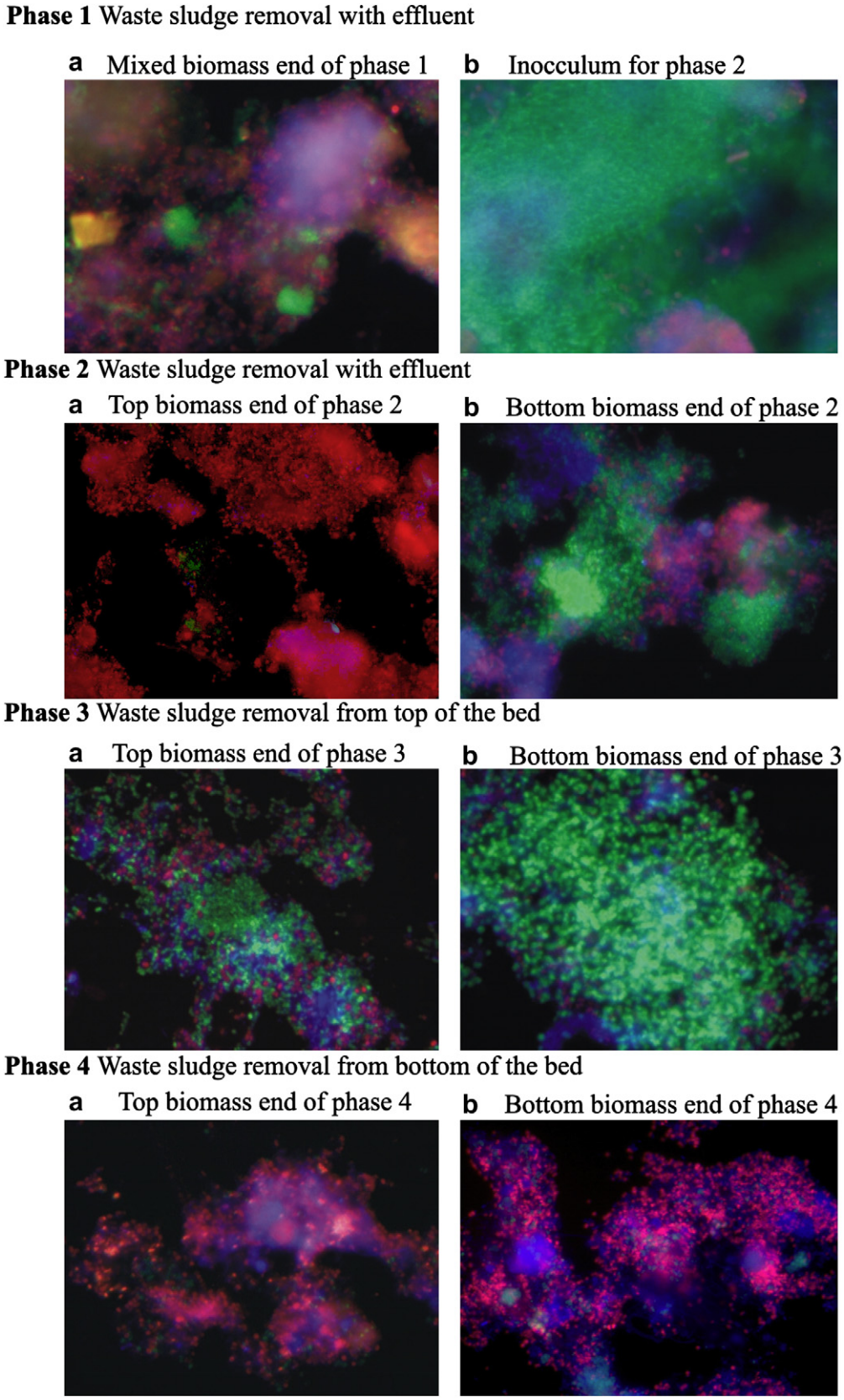


**Fig. 3 – Hybridization with Cy3-red (GAO-*Competibacter*), Cy5-blue (Eub) and Fluos-green (PAO-*Accumulibacter*)-labelled probes. Epifluorescence photomicrographs are shown for top and bottom biomass at different time points during reactor operation. Picture 3.1a FISH image of mixed biomass at the end of phase 1, Picture 3.1b population used for inocculum to start up phase 2, Picture 3.2 segregation of PAOs and GAOs at a) top and b) bottom of the reactor at the end of phase 2 as well as same relation shown for phase 3 when top sludge was removed and phase 4 when bottom sludge was removed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

**Table 1 – Oligonucleotide probes, target microorganisms, and references used in this study.**

| Probe | Sequence (from 5′ to 3′) | Specificity | Reference |
|---|---|---|---|
| PAO 462 | CCGTCATCTACWCAGGGTATTAAC | Most *Accumulibacter* | (Crocetti et al., 2000) |
| PAO 651 | CCCTCTGCCAAACTCCAG | Most *Accumulibacter* | (Crocetti et al., 2000) |
| PAO 846 | GTTAGCTACGGACTAAAAGG | Most *Accumulibacter* | (Crocetti et al., 2000) |
| GAO Q989 | TTCCCCGGATGTCAAGGC | Some *Competibacter* | (Crocetti et al., 2000) |
| GAO Q431 | TCCCCGCCTAAAGGGCTT | Some *Competibacter* | (Crocetti et al., 2002) |
| EUB 338 | GCTGCCTCCCGTAGGAGT | Most *bacteria* | (Amann et al., 1990) |
| EUB 338 II | GCAGCCACCCGTAGGTGT | *Planctomycetales* | (Daims et al., 1999) |
| EUB 338 III | GCTGCCACCCGTAGGTGT | *Verrucomicrobiales* | (Daims et al., 1999) |

Probes PAOs were tagged with the fluorescent dye Fluos (green) GAOS with Cy3 (red) and Eub with Cy5 (blue). For analysis probes of one target group were mixed.

0.15 P-mol/C-mol were observed in phase 1 and 2 in which removal efficiencies were low and GAOs dominated the system, as indicated by FISH (Fig. 3). However, in phase three when SRT was controlled by selectively removing top granules the ratio gradually increased in correlation with removal efficiencies to 0.34 P-mol/C-mol. In phase four the GAOs became prevalent again and the ratio decreased in correlation with the decreasing P-removal efficiencies.

### 3.3. *FISH analysis of sludge*

During the experiment, samples of the sludge were regularly subjected to analysis by FISH. Virtually all cells in the sludge where stained by either *Accumulibacter* (PAO) or *Competibacter* (GAO) probes, indicating that these formed the large majority of the microbial population in the sludge. Fig. 3 gives an overview of the most relevant samples. Firstly, the reactor was run at 30 °C in which a mixed sludge sample, taken at the end of phase one (no distinction between bottom/top), revealed a higher dominance of GAOs (Fig. 3.1a). At the same time the removal efficiency was also low (32%, day 66). For phase two, half of the reactor sludge was discarded and inoculated with new granular sludge containing mainly PAOs (Fig. 3.1b) to ensure an equal starting point for competition of PAOs and GAOs. P-removal efficiency increased instantaneously after inoculation but declined over time. At day 140 bottom and top sludge were checked separately for their microbial community composition. FISH analysis of bottom and top sludge was conducted because stratification of biomass was visually observed in both phases. During the aerobic mixing period biomass density was higher at the bottom. Following the settling period, large, heavy granules remained closer to the bottom whereas smaller granules were concentrated at the top portion of the expanded sludge bed. FISH results revealed that the top sludge contained considerably more GAOs (Fig. 3.2a) whereas the bottom sludge were enriched by PAOs (Fig. 3.2b), overall indicating a vertical segregation of microorganisms over the sludge bed. Based on these observations a third phase was initiated in which top sludge was removed to favour PAOs over GAOs. P-removal efficiencies increased during this phase to 100% and FISH results of top sludge illustrated an increase in the PAO populations (Fig. 3.3a) and a dominance of PAOs in the bottom sludge (Fig. 3.3b).

A fourth phase was conducted in order to show that the segregation of community composition over the sludge bed was indeed an effect of sludge control from a specific height of the settled bed. During this phase, sludge was removed from the bottom while keeping the same SRT. P-removal efficiency dropped to 36% and the bottom and top microbial populations were dominated by GAOs (Fig. 3.4a and 4b).

### 3.4. *Density and size distribution measurements of top and bottom granules*

The physical properties of top and bottom granules during phase three have been evaluated and are given in Table 2. During phase three, the sludge age was manually controlled by sludge removal from the top of the sludge blanket. There was a clear difference in ash percentage between top and bottom sludge. The higher ash content was also reflected in a higher density of the bottom granular sludge. The measured density of 1018 ± 13 g/l for bottom granules versus 1004 ± 4 g/l for top granules contributed to the higher settling rates of bottom granules. The diameter derived from the average surface area of bottom granules was also larger. The obtained physical parameters were used to estimate the settling velocity applying Stokes law as given in the material and methods section. There was an estimated 3–4 factor difference in settling velocities between top and bottom granules. Furthermore, calculations revealed that differences in settling velocities were influenced equally due to both changes in the radius and the density of the granules. The estimated velocities were similar to the measured velocities. Additionally, the settling properties of removed bottom sludge were measured during phase 4 (see Fig. 4). The settling rate of the bottom sludge decreased rapidly and continuously over time. Results revealed that in phase 4a, settling velocities of bottom sludge decreased from 80 m/h to 50 m/h when 15% of the settled

**Table 2 – Physical properties of bottom and top granules during sludge control of top biomass (Phase 2).**

| Parameter | Top | Bottom |
|---|---|---|
| Settling velocity calculated m/h | 20 ± 5 | 80 ± 9 |
| Settling velocity measured m/h | nm | 66 ± 9 |
| Ash content % | 15 ± 0.1 | 34 ± 0.1 |
| Density g/l | 1004 ± 4 | 1018 ± 13 |
| Average diameter (mm) | 0.8 ± 0.1 | 1.1 ± 0.2 |

Measurements were conducted during phase 3 in which SRT was controlled by discarding top biomass. nm: not measured.

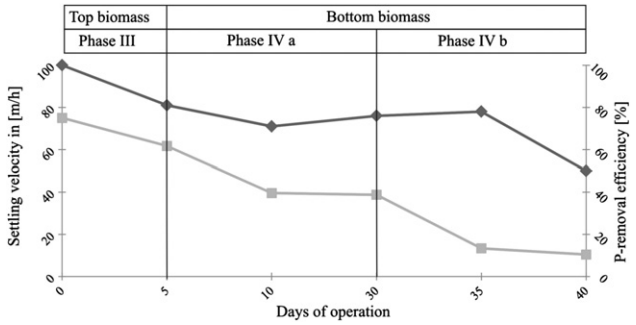


**Fig. 4 – P-removal efficiency (◆) and settling velocity in m/h (■) of bottom granules in phase three (top sludge removal) and in phase four (bottom sludge removal). Settling velocities were calculated by Stokes law based on data obtained from density and size distribution measurements.**

sludge bed was removed every third day. However, 30% of the settled sludge bed was removed every six days in phase 4b. As a consequence, P-removal efficiency dropped to 36% while settling velocities of bottom sludge declined to 12 m/h.

## 4. Discussion

In granular sludge systems segregation of biomass can easily occur due to slight variations in density and diameter of the particles. The opportunity to select for specific microbial groups at different heights within the column, allows for imposing additional selective pressure in granular sludge systems compared to traditional activated sludge systems. This is delineated by the ability of the granular sludge to maintain PAOs as the dominant group at 30 °C by selectively removing top biomass dominated by GAOs and thus keeping their SRT low. Studies which were carried out at higher temperatures have shown that a very short cycle length or a low sludge age (3 days) can also lead to a stable P-removal efficiency at 30 °C (Freitas et al., 2009; Whang and Park, 2006). Without sludge control, flocculent sludge systems have been shown to favour the enrichment of GAOs over PAOs (Lopez-Vazquez et al., 2009a). The results obtained in these experiments revealed that granules dominated by PAOs (*Accumulibacter*) were bigger, more dense and thus have the advantage to remain at the bottom of the reactor due to faster settling velocities. Since the reactor is fed in a plug flow regime from the bottom, it is evident that bottom granules have more substrate available, leading to a niche where PAOs were exposed to a higher percentage of the available substrate as compared to GAOs. Since sludge withdrawal is accomplished from the top of the sludge bed the SRT of the GAO population is effectively lower than for PAO dominated granules leading to a washout of GAOs over time. The main heterotrophic microbial population consisted of PAOs (*Accumulibacter*) or GAOs (*Competibacter*) since all acetate was always taken up before the aerobic period started leaving no more organic carbon available for normal heterotrophs. Moreover, the P release/COD uptake ratio positively correlated with removal efficiencies and data derived from FISH analysis. For example, when P-removal efficiencies and PAO content were high within the reactor, the P release/COD uptake ratios were additionally high.

P-removal deteriorated in phase four when the required SRT was established by extracting bottom biomass instead of top biomass as performed in phase 3, demonstrating that the P-removal efficiencies were improved due to selective sludge removal and not only due to the lower SRT values. The difference in P-removal between phase 4a and b demonstrate that selective sludge control is strongly dependent on the amount of biomass extracted. The high values for the P release/COD uptake ratios in phase 4a suggest that although P-removal dropped, bottom biomass removal also stimulates new growth of PAOs. In phase 4b, when a larger proportion of bottom granules was removed, P-removal declined and at the same time also the settling velocities of bottom granules (due to a decrease in diameter and density), which minimizes segregation and herewith the advantage of bigger PAO dominated granules to always settle first to the bottom. Again, this is of advantage because bottom granules have more substrate available due to the plug flow feeding regime. This highlights the importance in obtaining knowledge concerning mechanisms leading to segregation of biomass, particularly how selective pressure of certain organisms over others can be influenced by removal of sludge from a specific depth within the reactor. In addition it is also significant to gain more knowledge about how selective sludge removal from the top or bottom of the sludge bed is influencing the SRT distribution of different granules in the reactor. For the renewal of bottom biomass and hence the growth of PAOs, sludge removal of bottom biomass could facilitate in avoiding the deterioration in P-removal over time. In order to better understand the effect of sludge removal it would be necessary in future studies to determine the P content of removed sludge and to make a proper P-mass balance.

An explanation of how segregation occurs is that in PAO dominated granules the $PO_4$ released per unit of acetate removed is higher than in GAO dominated granules, which is attributed to a lack of an active P-uptake/release metabolism in GAOs. Since the settling of biomass occurs after the aeration period PAO dominated granules have accumulated high amounts of poly-P, which will improve their settling properties in comparison to GAO dominated granules. The higher ash content of bottom PAO dominated granules might hence be due to higher poly-P content. Since chemical precipitation is strongly dependent on $PO_4$ concentrations (Carlsson et al., 1997; Maurer et al., 1999) and only PAOs excrete phosphate, chemical precipitation in a PAO dominated granule might enhance this effect. This way the system has a self-enhancing effect on P-removal efficiencies since PAOs can remain in the system despite the high temperatures, which are known to be favourable for GAOs (Lopez-Vazquez et al., 2009b).

Selective sludge removal at different heights in a granular sludge bed might offer a good opportunity to conduct microbial population engineering in AGS and UASB bed technology. Recent research has demonstrated the existence of segregation in other systems. For example, Volcke et al. (2010), illustrated that in a granular sludge nitritation/anammox system nitrite oxidising bacteria accumulate preferentially in smaller

granules, again allowing a method for controlling nitrite oxidising bacteria by selective sludge withdrawal. Additionally, selective sludge removal has been shown to enhance granulation processes (Li and Li, 2009). It is thus of interest to evaluate the activity of microorganisms in relation to the depth within the sludge bed and to design a sludge extraction protocol based on the specific population one wants to select for.

## 5. Conclusions

In this work it was investigated whether segregation of biomass occurs along the sludge bed. PAOs were prevalent at the bottom, whereas GAOs dominated at the top of the sludge bed. By selective removal of GAO dominated sludge from the top of the sludge bed 100% P-removal efficiencies were achieved at 30 °C. This study also shows that selective sludge withdrawal in granular sludge reactors can be used as an extra operational parameter to engineer the microbial population in the reactor.

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## Appendix 1

Equations for calculating VSS of reactor and excess sludge as well as sludge retention time (SRT) during sludge control.

$$SRT = \frac{V_r \times VSS_r}{Q_{eff,VSS} + Q_{ex,VSS}} \text{ [day]}$$

$$V_{set.\ r,ex} = \pi r^2 \times h_{set.\ r,ex} \ [m^3]$$

$$DW_{set.} = \frac{DW}{V_{set.\ cyl}} \times V_{set.\ r,ex} \text{ [g dry weight]}$$

$$ash_{set.} = \frac{ash}{V_{set.\ cl}} \times V_{set.\ r,ex} \text{ [g ash]}$$

$$VSS_{r,ex} = DW_{set.\ r,ex} - ash_{set.\ r,ex} \text{ [gVSS]}$$

$V_r$ = volume reactor [$m^3$]
$VSS_{r,ex}$ = volatile suspended solids reactor or excess [gVSS]
$Q_{eff,VSS}$ = outflow VSS effluent [$gVSS_{eff} \times m^3$/day]
$Q_{ex,VSS}$ = outflow VSS of bottom or top sludge control [$gVSS_{ex} \times m^3$/day]
$h_{set.\ r,ex}$ = bed height settled sludge reactor and removed for excess [m]
$V_{set.\ r,ex}$ = volume settled sludge bed in reactor or excess sludge [$m^3$]
$V_{set.\ cyl}$ = volume settled sludge bed in measuring cylinder [$m^3$]
SRT = solid retention time [days]

## Appendix 2

Equations for calculation of settling velocity by Stokes law

$$SV = \frac{g}{18} \cdot \frac{\rho_p - \rho_w}{\rho_w} \cdot \frac{d_p^2}{\nu_w} \text{ for } Re_{particle} \leq 1$$

SV = sedimentation velocity of a single particle [m/s]
$d_p$ = particle diameter [m]
$\rho_p$ = density of particle [$kg/m^3$]
$\rho_w$ = density of the fluid [$kg/m^3$]
g = gravitational constant 9,81 [$m/s^2$]
$\nu_w$ = kinematic viscosity water [$m^2/s$]
Re = Reynolds number of a particle [–]

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