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Evaluating the solid retention time of bacteria in flocculent and granular sludge

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ABSTRACT

The specific solid retention time for different bacteria within flocculent and granular sludge was determined. Samples were collected from reactor and effluent sludge and the number of a specific bacterial group was evaluated in respect to the total bacterial community with quantitative polymerase chain reaction (qPCR). The ratio of the relative presence of a specific bacterial group in the reactor sludge and wasted sludge was established to observe if preferential wash-out occurred. From the data also the solid retention time for different microbial groups can be estimated. Using this tool, we were able to show that the SRT of populations found on the exterior of granules is slightly lower than the SRT for population in the interior. Archaea were not found in the flocculent system but were present in small amounts within the granular system. It was further observed that protozoa were grazing on the bacterial community within the system indicating that they have the potential to shorten the specific SRT of bacteria.

1. Introduction

Microbial population engineering for the purpose of developing a bacterial community that performs a specific function requires the presence of niches that select for the retention of the desired populations (Grady and Filipe, 2000). This can be accomplished by manipulating reactor conditions with the intention of influencing community assembly. Although we currently cannot confidently predict community assembly at the species level as it appears to be a chaotic event (Curtis et al., 2003), intuition gathered from years of operating and studying mixed communities gives us a sense of what factors are important. For example, we know that process loading rates (F/M), solids retention time (SRT) and shifts in redox conditions can influence the development of a functionally stable microbial community (Lee et al., 2003; Li et al., 2008; Saikaly et al., 2005).

Of the multitude of operational parameters that can be used to constrain niche development, SRT (also known as sludge age and mean cell retention time) is one of the most powerful. This parameter is traditionally used in the design and operation of biological nutrient removal (BNR) systems (Ekama, 2010) to control for the presence or absence of desired microbial populations (e.g. nitrifiers). Practically, this is achieved by removing biomass at specific intervals and assuming a homogenous distribution of bacterial populations within the

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sludge. This assumption is not always valid as has been demonstrated in biofilm systems and granular sludge (Bassin et al., 2012; Winkler et al., 2011a,b) where stratification of populations (over the depth of the biofilm as well as over the height of reactor/sludge bed) results in non-homogenous biomass distribution. Moreover, in granules bacteria are not equally distributed since, the outer oxygen penetrated layer selects for the growth of aerobic bacteria (nitrifiers), whereas the microaerobic/anoxic conditions of the interior allows for the growth of denitrifying phosphate accumulating (PAO) and glycogen accumulating (GAO) organisms (De Kreuk et al., 2005). Since it is clear that an uneven distribution of bacteria within the sludge is more the norm than exception, there is a need to properly assess the total bacterial SRT as well as the SRT for individual functional groups. From a practical perspective, knowledge of these parameters can help engineers operate systems to preferentially enrich or select against bacteria with a desired function.

The emergence of molecular methods like pyrosequencing and quantitative polymerase chain reaction (qPCR) gives us an unprecedented ability to characterize species level shifts in stable and unstable communities (Werner et al., 2011). This data has allowed to examine how community resistance and resilience is impacted by microbial diversity (Briones and Raskin, 2003); however, translation of these findings towards defining guidelines for reactor design and operation are lacking. From this perspective, we studied flocculent and granular sludge with the intent to understand how molecular tools can be used to derive essential engineering parameters. Specifically, we use qPCR to derive the SRT for different microbial populations in the different sludges. We then used this approach to test the hypothesis that the SRTs of microbial groups in flocculent sludge and aerobic granules performing the same function are different due to biomass stratification.

2. Material and methods

2.1. Reactor operation and sample collection

Samples were taken from an aerobic granular pilot plant and activated sludge plant from Epe the Netherlands over a period of 1 year. Both treatment systems were operated at the same site treating the same wastewater. The conventional plant removed phosphate chemically whereas the granular system was based on biological phosphate removal. Further description of these reactors and the wastewater composition are provided elsewhere (van der Roest et al., 2011). The reactors were operated under steady state conditions and both reactors removed COD, N and P according to the effluent guidelines of the European water frame work directive (European-Water-Framework-Directive, 2000). From both reactors, biomass samples were collected (discharged reactor effluent and mixed liquor from the aerated period) for molecular analyses as described below. In this study, 6 sampling events were conducted. For the granular sludge reactor samples were also taken during the anaerobic feeding period from the top and bottom of the settled sludge bed.

2.2. qPCR procedure

DNA extraction was conducted according to the manufacturer's recommendation using an UltraClean™ Microbial DNA Isolation Kit. qPCR was then performed to monitor the community composition. Primer specificity was checked *in silico* using the SILVA database (Pruesse et al., 2007) and the Ribosomal Database Project ProbeMatch tool (Cole et al., 2009). All primers were optimized using temperature gradient qPCR. The resulting conditions, primer concentrations as well as DNA used as a standard for the qPCR calibration are listed in Table 1. A picogreen protocol was used to determine the amount of DNA template in order to normalize all C_T values to 5 ng DNA. All qPCR assays were performed in triplicate.

2.3. Combination of molecular data with operational data

All functional primers were adapted for their copy number. ΔC_T was calculated by following equation $\Delta C_T = C_{T(Eub)} - C_{T(A)}$ (Zhang et al., 2009). For determination of the proportion of one species (denoted as A) to the total Eubacterial community (denoted as Eub) the following equation was applied: $p_A = 2^{\Delta C}{}_T$. This approach is based on the $2^{-\Delta\Delta CT}$ method for quantifying gene expression (Livak and Schmittgen, 2001). Species proportion ratios (SPR) were calculated for samples obtained from the effluent and mixed liquor of both reactors. SPRs were also calculated for samples obtained at the top (p_{Atop}) and the bottom (p_{Abot}) of the granular reactor. Ratios between top and bottom samples were calculated ($ratio_{A^*} = p_{A\_bot}/p_{A\_top}$). A comparison of SPRs for the same species in the effluent (p_{Aout}) versus mixed liquor (p_{Ain}) was also performed ($ratio_A = p_{A\_in}/p_{A\_out}$). Using the overall SRT Θ_{total} ($\Theta_{total} = V_R \times x_R/(Q \times x_{runoff} + x_{ex} \times Q_{ex})$[day]) of the system derived from mixed liquor suspended solid concentrations, the SRT of specific microbial groups/species (Θ_A) was calculated:$\Theta_A = \Theta_{total} \cdot ratio_A$[day].

2.4. Clone library

Since only the primer Arc934R but not Arc915R resulted in a positive outcome in the qPCR protocol, a clone library was constructed using Univ518f and Arc934R to confirm the presence of Archaea (Gábor et al., 2006). The reaction conditions were similar to those described in Table 1 except 36 cycles were used. 16 clones were picked and sequenced.

2.5. FISH

Samples from the granular treatment plant in Epe were fixed in 4% paraformaldehyde. Granules were embedded in a tissue freezing medium (Leica Microsystems), hardened by freezing (−20 °C) and cut in the frozen state with a microtome-cryostat (Leica CM1900-Cryostat) into 25 μm thin slices. Dried slices were kept on a microscopic glass slide and FISH was performed to detect the presence of nitrifiers (Cy3), PAO (Cy5), and GAO (Fluos) microbial populations using protocols previously described (Winkler et al., 2011b) FISH probes used in this study are described in Table 2.

Table 1 – Primers, qPCR conditions and primer concentrations used in this study.

Primer	Con. [μM]	qPCR conditions	Standard	Reference
CTO189F A/B & CTO189F C CTO654R	0.2	94°/2′ [94°/0.30′ 61°/1′ 72°/0.45′] × 30 52°/10′ 10°/∞	*Nitrosomonas europaea*	(Kowalchuk et al., 1997)
AmoA1R/ AmoA 2F	0.2	94/4 m, (94/60 s, 57/45 s, 72/60 s, 80/25 s)*40, 72/10 min, 12/∞	*Nitrosomonas europaea*	(Rotthauwe et al., 1997)
NTSPA1026F NTSPA1026R	0.2	94°/1′ [94°/0.5′ 45°/0.2′ 72°/0.3′80°/0.25′] × 40 72°/10′10°/∞	*Nitrospira defluvii*	(Juretschko et al., 1998)
NTS232F/ NTS 1200R	0.2	95/5 m, (95/40 s, 55–48/30 s, 72/60 s)*35, 72/10 min, 12/∞	Nitrospira	(Lim et al., 2008)
NxrB F706/ NxrB R1431	0.2	95/5 m, (95/40 s, 56/30 s, 72/30 s)*35, 72/10 min, 12/∞	Nitrobacter	(Degrange and Bardin, 1995)
FGPS872/ FGPS 1269	0.2	95/3 m, (95/60 s, 50/60 s, 72/60 s)*35, 72/3 min, 12/∞	Nitrobacter	(Degrange and Bardin, 1995)
Bac341F	0.35	95°/6′ [95°/0.30′ 55°/0.4′ 52°/0.4′80°/0.25′] × 40	Sludge from wwtp[b]	(Muyzer et al., 1993)
Bac905r	0.35	52°/5′10°/∞	Sludge from wwtp[b]	(Weisburg et al., 1991)
ARC519fc & ARC519fa	0.2	95°/5′ [95°/0.30′ 62.5°/0.4′	Nitrosopumilus maritimus	(Øvreås et al., 1997)
ARC934r	0.2	72°/0.4′/80°/0.25′] × 40	Nitrosopumilus maritimus	(DeLong et al., 1989)
ARC915r	0.2	72°/7′ 10°/∞		(Raskin et al., 1994)
cren_amoAf	0.2	95°/5′ [95°/0.30′ 62.5°/0.4′ 72°/0.4′/80°/0.25′] × 40	Nitrosopumilus maritimus	(Hallam et al., 2006)
amoAr	0.2	72°/7′ 10°/∞		(Francis et al., 2005)
SRB-Dsr4R	0.2	95°/5′ [95°/0.40′ 55°/0.4′ 72°/0.6′/72°/0.6′ 80°/0.25′]	Sludge from wwtp[b]	(Wagner et al., 1996a)
SRB-Dsr2060F	0.2	×35 72°/10′ 10°/∞	Sludge from wwtp[b]	(Geets et al., 2006)
Univ518f	0.2		Sludge from wwtp[b]	(He et al., 2007)
PAO-846r	0.2	94°/5′ [94°/0.15′ 57°/0.20′ 68°/0.15′68°/0.15′ 80°/0.25′]	EBPR sludge[a]	(He et al., 2007)
PAO-651f	0.2	×38 68°/10′ 10°/∞	EBPR sludge[a]	(Fukushima et al., 2007)
APAO-184f	0.2	95°/2′ [95°/0.1′ 64°/0.1′ 72°/0.2′ 80°/0.25′]	EBPR sludge[a]	(Okunuki et al., 2007)
A445r	0.2	×50 72°/10’ 10°/∞	EBPR sludge[a]	(Okunuki et al., 2007)
GAO-Gbf	0.2	94°/2′ [94°/0.15′ 57°/0.15′ 68°/0.25′ 80°/0.25′]	EBPR sludge[a]	(Fukushima et al., 2007)
GAO-Gbr	0.2	×38 68°/10′ 10°/∞	EBPR sludge[a]	(Fukushima et al., 2007)

Primers used and their concentrations as well as the standard used. qPCR conditions show temperature and cycle length for denaturing, annealing, elongation and cooling steps.
a Sludge from the aerobic granular sludge pilot plant in Epe, The Netherlands.
b Sludge from the conventional wastewater treatment plant in Epe, The Netherlands.

3. Results

3.1. *Structure and composition of aerobic granules and flocculent sludge*

FISH analyses on sliced granules showed the typical spatial distribution of nitrifiers in the oxygen penetrated outer layer (Cy3) as well as PAOs (Cy5) and GAOs (Fluos) in the outer as well as interior layers (Fig. 1C). Sulphate reducing bacteria and ammonium oxidizing archaea were not detected in these granules. Archaea were only present in aerobic granular sludge as confirmed by qPCR and a clone library. One out of sixteen picked clones resulted in a positive result for an uncultured crenarchaeote clone. Only the primer Arc934R but not Arc915R resulted in a positive outcome (qPCR signal). Ciliated protozoa (*Vorticella*-like) were also identified on the outer layer of the granules through light microscopy

Table 2 – Oligonucleotide probes, target organisms, 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	CCC TCTGCCAAACTCCAG	Most *Accumulibacter*	(Crocetti et al., 2000)
PAO 846	GTTAGCTACGGACTAAAAGG	Most *Accumulibacter*	(Crocetti et al., 2000)
GAO Q989	TTCCCCGGATGTCAAGGC	Some *Competibacter*	(Crocetti et al., 2002)
GAO Q431	TCCCCGCCTAAAGGGCTT	Some *Competibacter*	(Crocetti et al., 2002)
Ntspa662	GGAATTCCGCGCTCCTCT	*Nitrospira like organisms*	(Daims et al., 2001)
NIT1035	CCTGTGCTCCATGCTCCG	*Nitrobacter*	(Wagner et al., 1996b)
NSO190	CGATCCCCTGCTTTTCTCC	All AOB	(Mobarry et al., 1996)
NSO1225	CGCCATTGTATTACGTGTGA	All AOB	(Mobarry et al., 1996)

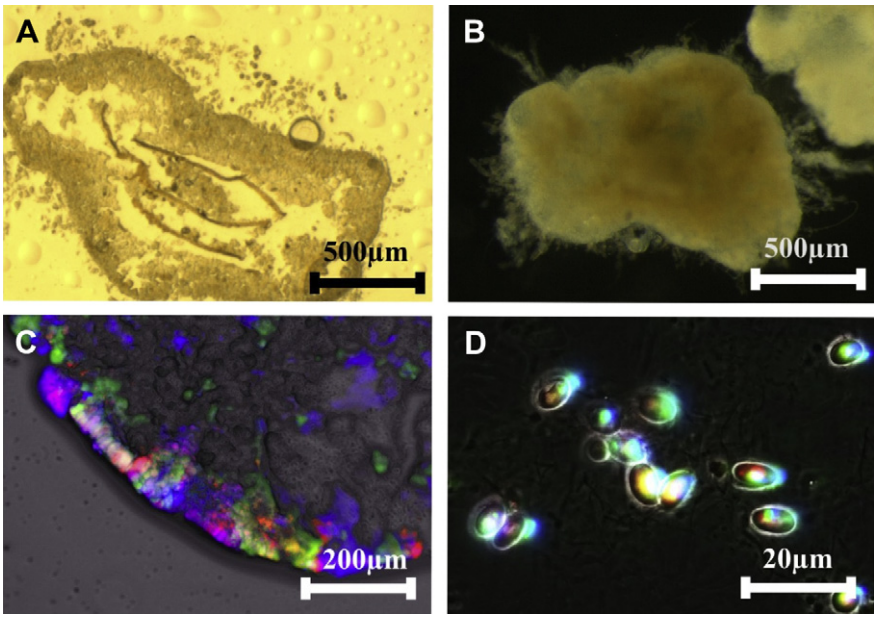

Fig. 1 – FISH and light microscopy images. Image A–B shows a light microscopy image of a granule with Protozoa on outer layers. Images C-D shows the FISH image of C) PAO (blue) GAO (green) and nitrifiers (red) on a sliced granule as well as D) the presence of GAO (red) nitrifiers (green) PAO (blue) within protozoa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 1A–B). FISH images indicated the simultaneous presence of GAO (red), PAO (blue) and nitrifiers (green) within these cells, indicating active grazing (Fig. 1D).

3.2. *Microbial population distribution and corresponding SRT values*

Total bacterial SRT for the flocculent sludge and aerobic granular sludge systems were 11 and 20 days, respectively. To determine the effective SRT of the individual microbial populations, species proportion ratios (SPR) were calculated for both granular and conventional systems (Fig. 2). An SPR smaller than one indicates a relative short SRT for the group of organisms, whereas an SPR higher than 1 a longer SRT for the concerned bacterium. Results from granular sludge samples indicated that the SRT of nitrifying bacteria (exterior layer of granules; 11 ± 3 days) was slightly lower than the SRT for bacteria present within the interior of the granules (PAO, GAO, actinoPAO, archaea) (13 ± 4 days; Table 3). For the conventional systems, the SRT of nitrifying bacteria ranged from 7 to 15 days, with the average SRT of AOB being 15 ± 4 days versus 8 ± 2 days for NOB (average from *Nitrobacter* and *Nitrospira*). For

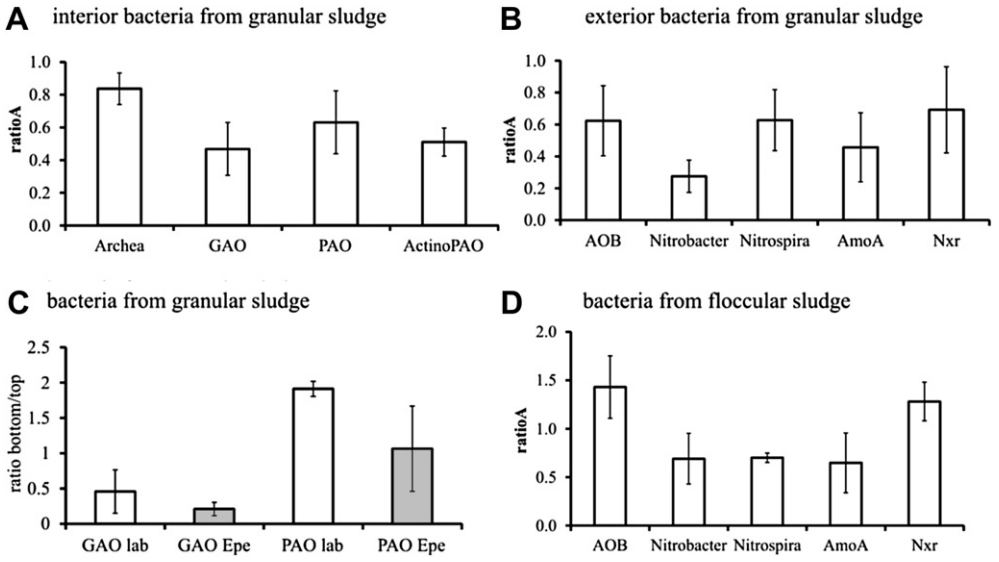

Fig. 2 – Calculated species proportion ratios (SPR) for different microbial populations in granules (A–B) and flocculent sludge (D) as well as bottom/top ratios of GAOs and PAOs from aerobic granular sludge samples (C) collected from the laboratory ▭ and the pilot scale plant in Epe/The Netherlands ▭.

Table 3 – SRT of bacteria based on calculated wash-out ratios as given in Fig. 2 and the SRT of the conventional system and the granular sludge system.

Bacterium	SRT [days]	Bacterium	SRT [days]	SRT [days]
	Interior layer		Exterior layer	Flocs
Archea	17 ± 2	**AOB**	12 ± 4	15 ± 4
GAO	9 ± 3	***Nitrobacter***	6 ± 2	8 ± 3
PAO	13 ± 4	***Nitrospira***	12 ± 4	8 ± 1
ActinoPAO	10 ± 2	**AmoA**	11 ± 5	7 ± 3
		Nxr	14 ± 5	14 ± 2
Average	13 ± 4		11 ± 3	11 ± 4

the granular systems, the SRT of nitrifying bacteria ranged from 6 to 14 days, with the average SRT of AOB being 12 ± 4 days versus 9 ± 3 days for NOB.

SPRs for PAOs and GAOs present in the bottom versus top sludge of the granular sludge reactor suggested that PAOs were circa 1.5–2 times more abundant in the bottom sludge. In contrast, GAOs were twice as abundant in the top sludge. These results were consistent within lab reactor and pilot scale reactors in Epe/Netherlands (Fig. 2C). The reactor configuration of the lab scale system is described in detail in earlier research (Winkler et al., 2011a).

4. Discussion

In this study, we examined granular and flocculent sludge from a full-scale reactor as case studies for demonstrating the use of molecular tools for calculating key engineering parameters. We succeeded in linking operational data (SRT from suspended solids) with a wash-out ratio derived from qPCR and enabled herewith a direct combination of traditional engineering with classical molecular tools. Studies connecting microbial and operational data within full-scale systems are sparse (Wells et al., 2011) but are of importance to better understand reactor performance. Here we present a new powerful approach to combine these two fields enabling a direct determination of the specific SRT of bacteria and hence define the wash-out rate of distinct bacterial groups. The techniques presented here are relatively easy to use and interpret and could allow for the utilization of this approach at utilities to more carefully manage specific solids inventory. This is particularly important at facilities that encounter frequent weather events that compromise aerobic SRT control, which leads to the loss of nitrification (Giokas et al., 2002). This tool could also be used to define design criteria (e.g. Aerobic SRT and Anoxic SRT) that are needed for operation and management of facilities intentionally performing simultaneous nitrification and denitrification through the use of dynamic aeration control strategies (Thauré et al., 2008).

We initially hypothesized that bacterial populations (nitrifiers) that colonize the exterior of aerobic granules are more rapidly eroded and subsequently washed out in higher numbers than biomass on the interior of the granule (PAO, GAO, Archaea) (Fig. 3A). Thus, the SRT of these exterior populations would be lower in respect to the interior populations. For flocculent sludge we expect that the SRT of the individual populations is similar to the SRT of the total solids since there will be hardly stratification. When granule breakage (Fig. 3B) dominates over erosion there will also be equal SRT for total granular sludge as well as individual populations.

From an engineering point of view it is appealing to correlate microbial shifts to system performance by the usage of molecular tools. Studies have already attempted to quantify community shifts over time and partly correlated these changes to system performance (Ayala-Del-Río et al., 2004; Cai-Yun et al., 2011; Pholchan et al., 2010; van Nostrand et al., 2011). Instead of correlating operational data with molecular data in this study we directly linked molecular techniques with operational system performance to investigate the SRT of district bacterial groups. Specifically, we calculated wash-out ratios for microbial populations in granular and conventional system using qPCR data. These ratios allowed us to describe how an individual microbial population SRT is related to the overall system SRT. The results obtained here show that in aerobic

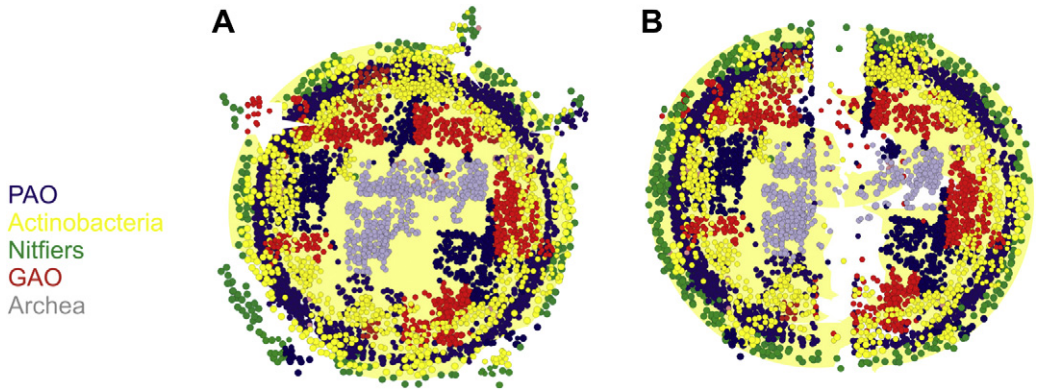

Fig. 3 – Schematic view of granules with a typical multi structural layer with nitrifies in the outer oxygen penetrated layer followed by PAOs (blue) ActinoPAOs (yellow) GAOs (red) and with Archea (grey) in the interior anaerobic core. The graphical model shows A) breakage of mainly the outer layers hence decreasing the SRT of nitrifiers and B) granule breakage in the middle leading to an equal wash-out of bacteria and hence to the same SRT for the whole bacterial population. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

granular sludge, the SRT of the nitrifiers growing on the oxygen penetrated outer layer was 4 days shorter (Fig. 1B) than the SRT of bacteria growing in the interior of the granule (11 ± 3 days versus 14 ± 4 days) (Table 3). Although the differences are not very strong, this difference suggests that granular disassembly occurs from outer layer erosion (Fig. 3A) versus intra-granule rupture (Fig. 3B). Due to the fast settling properties of granular sludge, small fragments and flocculent material are removed preferential over large granules, which might additionally influence the results obtained in this study. Further work is needed to confirm this finding.

It should be mentioned that the technical replicates (3 per sampling day) had a very small standard deviations for the ΔC_T-values of all primer sets (0.9 ± 0.2% for granules, 2 ± 1.3% for flocs). The relatively big standard deviation as shown in the results (Fig. 1) were due to different wash-out ratios ($ratio_A$) between the samples from different sampling days (6 samples per 1 calculated SRT of one bacterium). Therefore, we suggest that future investigations perform extensive sampling across multiple days to further characterize inherent biological variations that exist. It should also be noted that even if a certain fraction of the population is underestimated (e.g. due to primer unspecificity etc.), the proposed method will not be affected since the calculation is based on a ratio between sludge samples (which are all exposed to the same bias). Thus, this proposed method is robust for calculating population SRTs (Θ_A) if enough sampling days are used.

In this study, we also investigated the segregation of biomass in a granular sludge bed. Our results confirmed earlier reports (Winkler et al., 2011a) that glycogen accumulating organisms dominated the slower settling granules and the preferred phosphate accumulating bacteria dominated the fast settling granules. Thus, PAOs would preferentially benefit from substrate supply since the reactor was fed in a plug-flow regime from the bottom. These results are consistent with our prior findings, whereby we used FISH to study biomass stratification (Bassin et al., 2012; Winkler et al., 2011a).

The effect of protozoa grazing on microbial population SRT has not been extensively studied to date (Huws et al., 2005; Moussa et al., 2005; Van Loosdrecht and Henze, 1999). In this study, we were able to identify that *Vorticella*-like ciliated protozoa were actively grazing on bacteria in the aerobic granules (Fig. 1D). While it may be tempting to hypothesize that the exterior populations (e.g. nitrifiers) are more prone to grazing events, our current results give no clear indication that this is the case. Instead, FISH analyses suggested that protozoa incorporated all bacteria and not only nitrifiers. Additional work is needed to understand whether protozoa and metazoa can selectively impact population dynamics of functional groups. We also identified that archaea were present within granular sludge but not within flocculent sludge. Archaea comprised ca 5% of the total population detected by qPCR, as well as with a clone library. It is possible that granules offer better growing conditions for archaea (e.g. continuous anaerobic conditions in the interior) as opposed to a sludge floc. This work represents a successful example in which molecular techniques are used to improve our understanding of how to engineer mixed cultures with multiple functional identities.

5. Conclusions

In this study, we showed that molecular tools can be used to effectively calculate key engineering parameters e.g. specific retention time of bacteria. A dimensionless wash-out ratio from reactor and effluent sludge was determined for one specific bacterium by the means of qPCR. This ratio was then coupled with the total SRT derived from the suspended solids [days] to calculate the specific retention time of one functional group [days]. Using this tool, we are able to confirm that the SRT of populations found on the exterior of granules is lower than the SRT for population in the interior. Further we found evidence that protozoa can influence microbial populations numbers, however, it is unclear whether individual populations are preferentially consumed.

6. Calculations

$$\Theta_{total} = \frac{V_R \times x_R}{Q \times x_{runoff} + x_{ex} \times Q_{ex}} \quad [day]$$

V_R = Volume reactor $[m^3]$

x_R = Biomass reactor $[kg/m^3]$

Q = Flow rate $[m^3/day]$

Q_{ex} = Flow rate $[m^3/day]$

x_{runoff} = Sludge in runoff $[kg/m^3]$

x_{ex} = Sludge in effluent $[kg/m^3]$

Θ_{total} = SRT of all bacteria [day]

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