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# Importance of Species Sorting and Immigration on the Bacterial Assembly of Different-Sized Aggregates in a Full-Scale Aerobic Granular Sludge Plant

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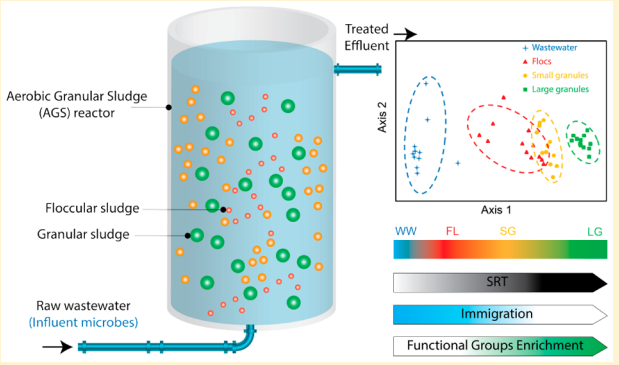
Supporting Information

**ABSTRACT:** In aerobic granular sludge (AGS) systems, different-sized microbial aggregates having different solids retention time (SRT) coexist in the same reactor compartment and are subjected to the same influent wastewater. Thus, the AGS system provides a unique ecosystem to study the importance of local (species sorting) and regional (immigration) processes in bacterial community assembly. The microbial communities of different-sized aggregates (flocs <0.2 mm, small granules (0.2−1.0 mm) and large granules >1.0 mm), influent wastewater, excess sludge and effluent of a full-scale AGS plant were characterized over a steady-state operation period of 6 months. Amplicon sequencing was integrated with mass balance to determine the SRT and net growth rate of operational taxonomic units (OTUs). We found strong evidence of species sorting as opposed to immigration, which was significantly higher at short SRT (i.e., flocs and small granules) than that at long SRT (large granules). Rare OTUs in wastewater belonging to putative functional groups responsible for nitrogen and phosphorus removal were progressively enriched with an increase in microbial aggregates size. In contrast, fecal- and sewage infrastructure-derived microbes progressively decreased in relative abundance with increase in microbial aggregate size. These findings highlight the importance of AGS as a unique model ecosystem to study fundamental microbial ecology concepts.






## INTRODUCTION

Mixed-culture flocculent aggregates, also known as flocs, in engineered environments such as the activated sludge system, are regarded as an important component of modern sanitation systems used to treat domestic and industrial wastewaters.[1] Despite serving society for 105 years, there is growing awareness that the activated sludge system cannot meet the current demands of a rapidly growing human population and urbanization because of its high capital and operational costs, and large land area requirements.[2] An emerging biological wastewater treatment system that holds great promise to replace the activated sludge system is the aerobic granular sludge (AGS) system, where bacteria asssemble predominantly as suspended granules instead of flocs. The AGS system can greatly reduce the footprint by 75% and by up to 60% of the capital and operational costs, with the effluent quality being comparable to or even better than that of the conventional activated sludge process.[3,4] These features illustrate that the AGS system could soon become the standard for biological wastewater treatment, with more than 40 full-scale AGS plants already in operation or under construction.[5] Despite the importance of the AGS system in modern and future sanitation, studies on the underlying mechanisms of microbial community assembly in full-scale AGS systems are lacking. A recent study on a lab-scale AGS reactor showed that biotic interactions (i.e., local factors) played a significant role in the assembly of the local granule communities.[6]

It is always questionable as to what extent local factors (so-called species sorting) or regional factors related to dispersal, which determine rates of immigration from the source habitat to the local community (sink habitat), regulate the assembly of local communities in engineered systems.[7,8] Metacommunity theory provides a framework to incorporate both local and regional processes to obtain a mechanistic understanding of local microbial community assembly patterns in natural and

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engineered ecosystems.[7,8] The framework comprises four paradigms (patch dynamics, species sorting, mass effects, and neutral), with species sorting[9−18] and mass effects[19−23] as the two most commonly tested and supported paradigms.[13] In general local factors are easier to measure than regional factors, and in many studies geographic distance was used as a proxy for dispersal[7] instead of real estimates of dispersal rates,[7,12] defined as the number of cells entering an ecosystem per unit of time.[24] Studies lacking real estimates of dispersal rates cannot distinguish between effects of dispersal limitation (low dispersal rates) and mass effects (high dispersal rates).

Realizing the importance of regional factors, a few studies have made explicit attempts to estimate the effect of immigration from the regional pool of species on bacterial community dynamics in natural water ecosystems.[12−15,25] In contrast, the effect of immigration from the influent wastewater on the bacterial community dynamics in engineered microbial ecosystems such as the activated sludge system, is often overlooked,[17,18,26,27] and in some cases geographic distance is used as a proxy for dispersal in variation partitioning analysis.[18] Very few have studied the taxonomic identity and relative abundance of immigrants to a full-scale activated sludge system.[28,29] However, these studies did not investigate the mechanisms driving bacterial community assembly in activated sludge. Also, the explicit attempt to study both mechanisms (i.e., species sorting and immigration) at the same time is lacking in engineered microbial systems.

In addition to its importance in protecting the environment and public health, full-scale AGS systems can provide a unique microbial ecosystem to test the effect of immigration because of the presence of habitat patches with different environmental conditions in the same reactor compartment, and with immigrants from the regional pool of species (i.e., influent wastewater) arriving to all habitat patches. In a full-scale AGS system different-sized microbial aggregates (flocs, small granules, and large granules) coexist in the same reactor compartment with granules representing the majority of the biomass.[3] This results in biomass not homogeneously distributed over the height of the reactor with large granules often more present at the bottom and flocs at the top during nonaeration phase. This spatial segregation of biomass can result in different biological niches because of differences in substrate availability at certain depths.[30] This contrasts with the flocculent sludge system where biomass is homogeneously distributed, and all bacteria are subjected to the same substrate supply. More importantly, due to biomass segregation and selective excess sludge withdrawal, flocs have shorter solids retention time (SRT), the average time that microorganisms reside in a bioreactor, than granules because flocs are wasted more during the excess sludge removal phase.[31] The SRT is a powerful operational parameter that can control the presence or absence of microbial populations based on their net growth rate. For a particular organism to be maintained in the bioreactor, its net growth rate should be $\geq 1/\mathrm{SRT}$; otherwise, it will be washed out from the bioreactor,[32] unless it is added with the influent stream at high dispersal rate. Thus, the different-sized microbial aggregates represent different sink habitats having different SRT, as a key local factor, and subjected to the same bacterial dispersal rate from the source habitat (influent wastewater). Thus, in the context of the metacommunity framework,[8] the AGS system provides a unique ecosystem to study biogeographic patterns (beta diversity) at a small spatial scale. Another unique feature of the AGS system is that it can be regarded as a biofilm process because granules are subjected to mass transfer limitation similar to surface-attached biofilms. Also, due to their large size and density, granules obey biofilm kinetics more closely than flocs. Likewise, the flow rates around granules are relatively low (granules move with the flow), making mass transfer from the bulk liquid to the granule surface relatively slow, another characteristic of biofilm processes.[33]

The primary objective of this study was to investigate, for the first time, the relative importance of species sorting as opposed to immigration in structuring bacterial community assemblages of different sized-aggregates (flocs, small granules, and large granules) in a full-scale AGS system. Species sorting is important from the perspective of biological wastewater treatment, as it allows the selection of functional groups that can drive specific ecosystem processes.[34] We characterized the microbial community of influent, effluent, excess sludge, and the bulk (mixed liquor) along with different-sized microbial aggregates of a full-scale AGS plant in The Netherlands over a period of six months. A simple mass balance approach was combined with 16S rRNA gene amplicon sequencing to estimate the SRT and net growth rates of operational taxonomic units (OTUs) in the AGS plant. This approach was previously applied to a flocculant sludge (i.e., activated sludge) system[29] and anaerobic digestion plants.[35] However, the main motivation of these previous studies was not to identify the underlying mechanisms (i.e., local vs regional factors) causing differences in community composition (beta diversity) among the different plants. The calculated net growth rates of the different OTUs were used to evaluate if incoming microbes from the influent wastewater are putatively growing (with net growth rate $\geq 0 d^{-1}$) in the receiving microbial aggregates or are present simply due to their constant and high rate of dispersal.[29]

Our results showed that species sorting effect was stronger than immigration for the different-sized microbial aggregates. The immigration effect was higher in smaller and less settling microbial aggregates because of their lower SRT limiting the influence of environmental filtering or species-sorting. In addition, we observed that, similar to surface-attached biofilms in natural[13,14] and engineered water ecosystems,[13−15] granules assemble from the suspended microbial community (i.e., flocs), and stochastic dispersal from flocs is unlikely to shape the community composition of granules.

## MATERIALS AND METHODS

**AGS Treatment Plant and Sampling.** The full-scale AGS treatment plant in Garmerwolde, The Netherlands (Supporting Information, SI, Figure S1) started operation in July 2013, and details on its operating parameters, environmental conditions, and ecosystem function are presented elsewhere.[3] It should be noted that during the sampling period, ecosystem functions in terms of 5-day biochemical oxygen demand, chemical oxygen demand, and nutrient removal were stable (Pronk et al.[3] and Table S1).

Influent wastewater, mixed liquor, excess or surplus sludge, and effluent samples were collected on a biweekly basis for a period of six months (August 2014−February 2015). All samples were transported on ice. The sampling locations for the microbial community analysis are presented in Figure S1C. Mixed liquor samples were collected from Nereda reactor 1 as 0.5 L grab samples during the aeration phase when the reactor was well mixed. The influent wastewater, mixed liquor, excess


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or surplus sludge, and effluent samples were centrifuged at 5000 rpm for 10 min to get the pellets. In parallel, separate mixed liquor samples were washed and segregated by wet sieving into three fractions: flocs (<0.2 mm), small granules (0.2 to 1.0 mm), and large granules (>1.0 mm). The fractions of the different-sized aggregates in the sludge of the Garmerwolde AGS plant was previously determined: flocs (20%), small granules (20%) and large granules (60%).[3] Similarly, the fraction distribution of flocs (60%), small granules (33%) and large granules (7%) in the excess sludge line was also adopted from a previous study of the same AGS reactor.[36] The sieved granules and pellets (influent, mixed liquor, excess sludge, and effluent) were then stored at −80 °C until DNA extraction.

**DNA Extraction and 16S rRNA Gene Sequencing.** Genomic DNA was extracted from 0.2 g of biomass using the PowerBiofilm DNA Isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, U.S.A.) following manufacturer's protocol with a modified bead-beating time of 2 min. The DNA concentration was measured with Quant-iT Broad-Range dsDNA Assay kit (Q33130, Life Technologies, Carlsbad, CA, U.S.A.) using the manufacturer's protocol. The 16S rRNA genes were amplified with forward primer Pro341F (5′-CCTACGGGNBGCAS-CAG-3′) and reverse primer Pro805R (5′-GACTACNVGGG-TATCTAATCC-3′)[37] targeting the V3−V4 region. The V3−V4 16S rRNA sequencing libraries were prepared by a custom protocol based on Illumina (for details, see SI Methods). The purified sequencing libraries were pooled in equimolar concentrations and diluted to 4 nM. The samples were paired-end sequenced (2 × 301bp) on a MiSeq (Illumina, Carlsbad, CA, U.S.A.) using a MiSeq Reagent kit v3, 600 cycles (Illumina) following the standard guidelines for preparing and loading samples on MiSeq. Raw sequencing data were deposited at the National Center for Biotechnology (NCBI) Sequence Read Archive (SRA) under accession number SRP115069.

**Sequence Processing and Microbial Diversity Data Analysis.** Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32[38] with the settings SLIDINGWINDOW:5:3 and MINLEN:275. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7[39] with the settings −m 25 −M 200. The merged reads were dereplicated and formatted for use in the UPARSE workflow.[40] The dereplicated reads were clustered, using the usearch v. 7.0.1090-cluster_otus command with default settings. Chimeras were filtered by cluster_otus command. Operational taxonomic unit (OTU) abundances were estimated using the usearch v. 7.0.1090-usearch_global command with 97% sequence identity threshold.[41] Taxonomy was assigned using the RDP classifier[42] as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME,[43] using the MiDAS database v.1.20.[44] The MiDAS database is a maunal curation of the SILVA database.

Comparison of beta diversity between samples was made after data sets rarefied to the minimum number of reads (~33 000) per sample using QIIME. Beta diversity metrics (Bray−Curtis and Unweighted Unifrac) were derived from the rarefied OTU table using QIIME. These metrics were used for permutational multivariate analysis of variance (MANOVA or ADONIS) with 999 permutations to test for significant differences in community composition between influent and different-sized aggregates. Nonmetric multidimensional scaling (NMDS) was performed in R[45] through the Rstudio IDE using the vegan package 2.4.3[46] and ampvis package v.1.9.1.[47] Network-based analysis and visualization were performed with an open source software Cytoscape v.3.5.1[48] and Venn diagram was prepared using Venn and Euler Diagrams app in the Cytoscape environment.

To determine the probability that the granular sludge community (i.e., small and large granules) represented a random assembly of the flocculent sludge community, a random resampling procedure on the floc community was performed using functions of the R-packages vegan, ecodist, and gdata as described elsewhere.[14−16] Individual reads were sampled from the floc community (the probability of each OTU to be sampled being its relative abundance) with replacement until the number of OTUs in this randomly assembled community equaled the richness of the respective small and large granule community. This procedure was repeated to yield 1000 random assemblages of the flocculent sludge community. The probability of the small or large granule community to fall within the distribution of these random assemblages was calculated as the percentage of the distances of the random assemblages to their centroid. The results of the random subsampling procedure were visualized in NMDS using Rstudio.

**Calculation of SRT and Net Growth Rates from Amplicon Data.** The solids retention time ($\theta_x$) and the net growth rate ($\mu_x$) of an organism ($x$) or species-level OTU in the AGS plant were calculated using a recently proposed mass balance approach.[29] We assumed that the AGS system was at steady-state (i.e., there was no net change in the number of cells in the AGS system ($N_{x,\mathrm{AGS}}$). At steady-state, $\theta_x$ was calculated using eq 1. More detailed explanation of the calculations and assumptions are presented in the SI Methods.

$$\theta_x = \frac{N_{x,\mathrm{AGS}}}{n_{x,\mathrm{ES}} + n_{x,\mathrm{EF}} - n_{x,\mathrm{WW}}} \quad (1)$$

where: $\theta_x$ is the solids retention time of organism $x$ in the AGS plant; $N_{x,\mathrm{AGS}}$ is the number of cells of organism $x$ in the AGS plant; $n_{x,\mathrm{WW}}$ is the number of cells of organism $x$ entering with influent wastewater per day; $n_{x,\mathrm{ES}}$ is the number of cells of organism $x$ removed with excess sludge per day; and $n_{x,\mathrm{EF}}$ is the number of cells of organism $x$ removed with the effluent per day.

## RESULTS

**Microbial Community Composition Was Different between Influent Wastewater and Different-Sized Microbial Aggregates.** A total of 95 samples from the AGS plant were sequenced corresponding to 7 types of samples including influent wastewater (WW), flocs (FL), small granules (SG), large granules (LG), mixed liquor from AGS reactor 1 (AGS1), excess sludge (ES), and effluent (EF). Of the 95 samples, 10 samples produced very low number of reads (<5000 reads per sample) and were discarded from subsequent analysis (Table S2). The minimum and maximum number of nonchimeric, quality-filtered reads were 33 057 and 83 373, respectively, with more than four million reads in total (Table S2). The nonchimeric, quality-filtered reads were clustered into 2095 OTUs at 97% identity.

In the current study, dominant OTUs were defined as those that are present at a relative read abundance ≥0.1% in a certain type of sample. After rarefaction, the number of dominant OTUs with their total relative abundance in the different-sized


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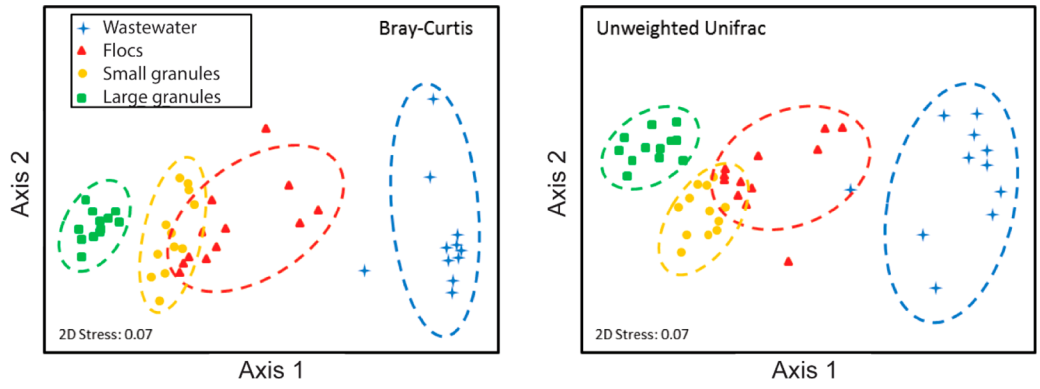


**Figure 1.** Nonmetric multidimensional scaling (NMDS) plot of influent wastewater, flocs, small granules, and large granules based on Bray−Curtis (A) and unweighted UniFrac distance (B). The ellipse indicates that a random assemblage of the corresponding samples falls within at a confidence of 0.95.

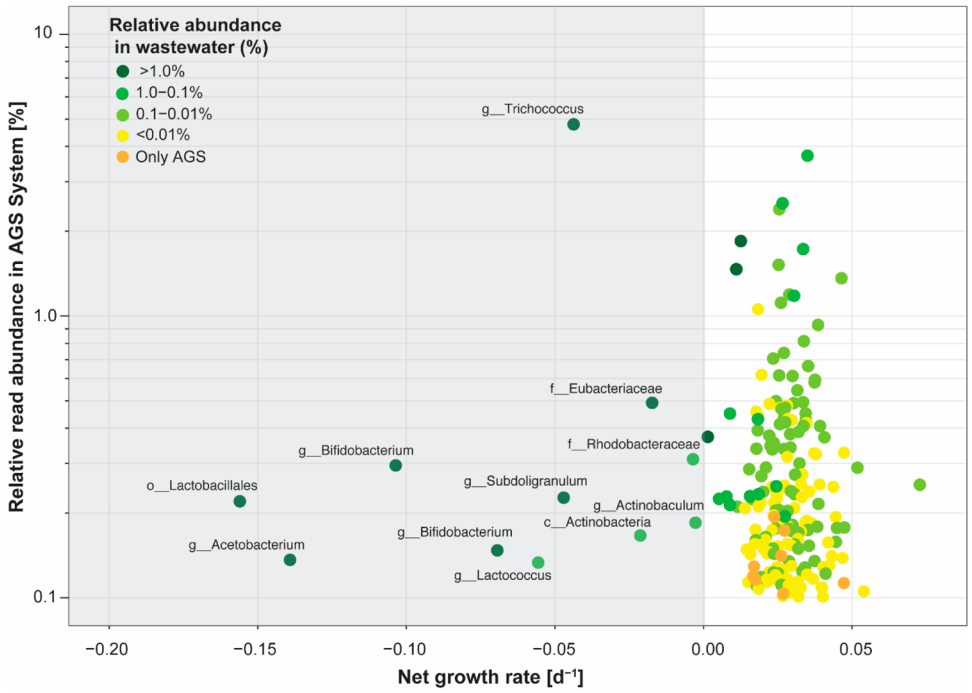


**Figure 2.** Comparison of relative read abundance (%) and net growth rate of dominant species-level OTUs detected in the aerobic granular sludge (AGS) plant. Dominant OTUs are defined as OTUs with relative read abundance ≥0.1%. The circles are colored based on their relative read abundance (%) in the influent wastewater. OTUs with negative net growth rate (gray-shaded) indicate putatively inactive OTUs in the AGS plant and their presence was likely due to immigration. One OTU (g_Blautia with net growth rates −0.410 $d^{-1}$) was not plotted for better visualization of the graph.

aggregates was: FL (195 OTUs, 76%), SG (235 OTUs, 73%), and LG (170 OTUs, 79%). Of these dominant OTUs, 94 were shared between the three communities, corresponding to total relative read abundance of 52, 44, and 62% in FL, SG, and LG, respectively. The number of shared dominant OTUs was higher between FL and SG (165) than FL and LG (96) (Figure S2A). Similarly, the number of shared rare OTUs, which were defined as taxa having relative read abundance <0.1%, was higher between FL and SG (1602) than FL and LG (1365) (Figure S2B).

The NMDS analysis using both taxonomic (Bray−Curtis) and phylogenetic (Unweighted UniFrac) metrics revealed differences in community composition (i.e., beta-diversity) between the different-sized aggregates, and between WW and different aggregates (Figure 1). The FL samples were located closer to WW samples followed by SG and LG. This suggests that FL samples had the highest similarity with WW samples, followed by SG and LG. Next, permutational multivariate analysis of variance (ADONIS) was used to determine if bacterial community composition between the influent WW and different-sized aggregates and between the different-sized aggregates was statistically different. The bacterial communities of different-sized aggregates were significantly different from influent WW. However, for both metrics (Bray−Curtis and Unweighted UniFrac), the value of $R^2_{ADONIS}$ statistic decreased with decrease in microbial aggregate size, which indicates a moderate increase in similarity with the influent WW (Table S3). Also, the dissimilarity among the different-sized aggregates communities increased with the increase in microbial aggregate size from FL to LG. Taken together, these results support the assumption that species sorting has a role in bacterial community assembly in AGS system.



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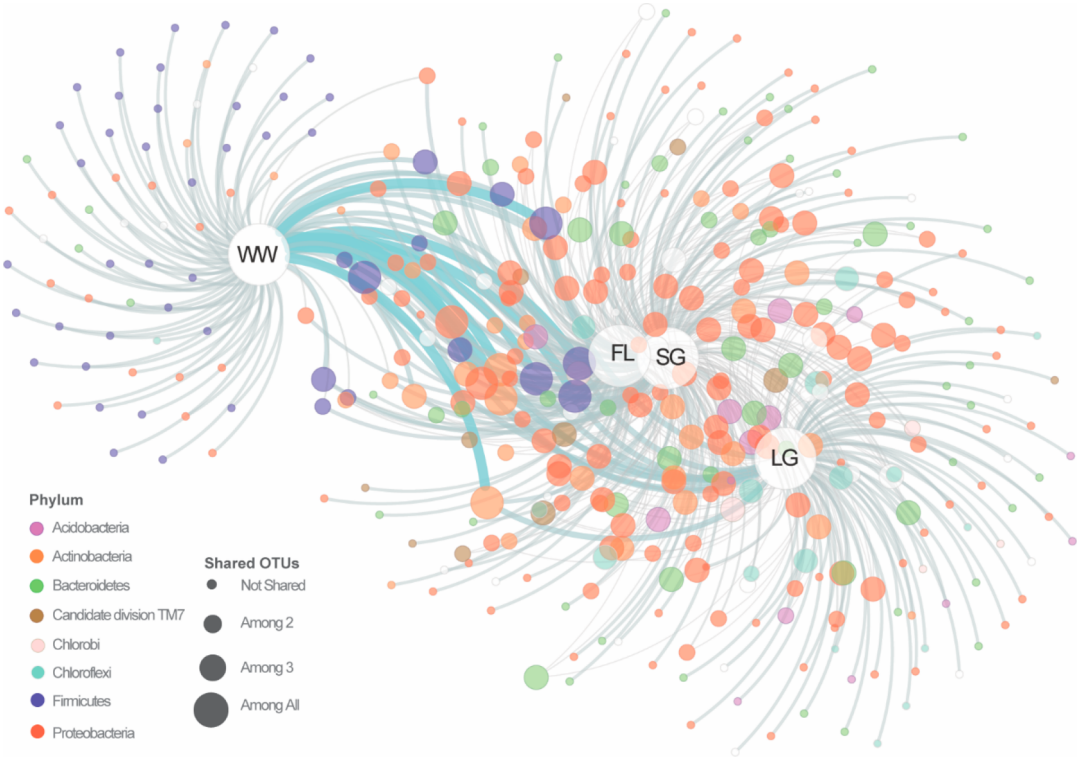


**Figure 3.** Co-occurrence network analysis of bacterial communities of influent wastewater (WW), flocs (FL), small granules (SG), and large granules (LG) of the full-scale aerobic granular sludge plant in Garmerwolde, The Netherlands. The network was created using OTUs with relative read abundance ≥0.1%. The nodes represent OTUs colored based on phylum-level of classification. The node size represents co-occurrence of OTUs among the different samples. The edge thickness is proportional to the relative read abundance of the respective OTU in the connected samples.

**Different-Sized Microbial Aggregates Have Different SRTs Calculated by Combining Amplicon Sequencing Data with Mass Balance.** We combined amplicon sequencing data with simple mass balancing to estimate the SRT of the dominant OTUs in the AGS system and different-sized microbial aggregates (see SI Methods). The observed read abundance of each species-level OTU was assumed to be equal to the actual abundance of those organisms in the system.[29] Pronk et al.[3] indicated that protozoa, mainly ciliated, were associated with granules. Therefore, we conducted quantitative polymerase chain reaction (see SI Methods) for WW, AGS, FL, SG, and LG samples over time, and the results confirmed that bacterial 16S rRNA gene copy number per ng DNA was similar for the different samples (Figure S3). To estimate the SRT of the whole AGS system, we applied the mass balance model schematically described in Figure S4. The dominant OTUs observed in AGS1 were used for estimating the SRT of the whole AGS plant. The calculated SRT was 26.0 ± 6.5 days, which was within the actual SRT (20−38 days) of the same AGS plant[3] measured during the same sampling period as the current study. Next, the same mass balance approach was applied to determine the SRT of the different-sized microbial aggregates in the AGS system using the mass balance model schematically described in Figure S5. As expected, FL had the lowest SRT of 6.2 ± 2.2 days, followed by SG with SRT of 7.7 ± 0.5 days. The calculated SRT of LG was 142.6 ± 14.9 days, which was significantly longer than SG and FL (student's *t* test, $P < 0.01$ for FL vs LG, and SG vs LG).

**Immigration from the Source Community Was Higher to Flocculent than Granular Sludge.** In this work the influence of immigration from the source habitat (influent wastewater) into an ecosystem (AGS system) was estimated by calculating the net growth rate of the incoming species-level OTUs in the AGS system using amplicon sequencing data coupled with mass balancing (see SI Methods). The use of mass balance-based calculation of the net growth rate of each dominant species-level OTU showed that 12 OTUs (representing 7.5 ± 4.0% of the OTUs; $n = 9$), with relative read abundance of 9.7 ± 4.9%, were putatively not growing in the system, with a net growth rate <0 $d^{-1}$ (Figure 2). Eleven out of the 12 OTUs belong to the phylum Firmicutes and Actinobacteria, which contain members such as *Trichococuss*, *Blautia*, *Lactobacillales*, *Lactococcus*, *Subdoligranulum*, *Acetobacterium* (order Clostridiales), *Eubacteriaceae* (order Clostridiales), *Actinobaculum*, and *Bifidobacterium*, which are known human fecal- and sewage infrastructure-derived microbes.[49] These 12 OTUs were highly abundant in WW representing more than 51.0 ± 13.1% of the reads, and constantly detected in the AGS plant.

The effect of immigration was then individually analyzed for different-sized microbial aggregates. In FL, dominant OTUs (13.6 ± 4.4%, $n = 8$) that were putatively not growing (Figure S6), had a relative read abundance of 52.6 ± 11.4% in WW and were detected in FL at significantly lower relative read abundance (11.6 ± 6.1%) (Table S4). Likewise, SG contained dominant OTUs (6.1 ± 1.3%, $n = 9$) that were putatively not growing (Figure S7) with relative read abundance of 6.7 ± 1.8% and these OTUs had significantly higher relative read abundance (48.6 ± 10.0%) in WW (Table S4). While LG had five putatively nongrowing dominant OTUs (3.7 ± 1.5%, $n = 9$) (Figure S8) with relative read abundance of 1.8 ± 0.8%, and these OTUs had significantly higher relative read abundance


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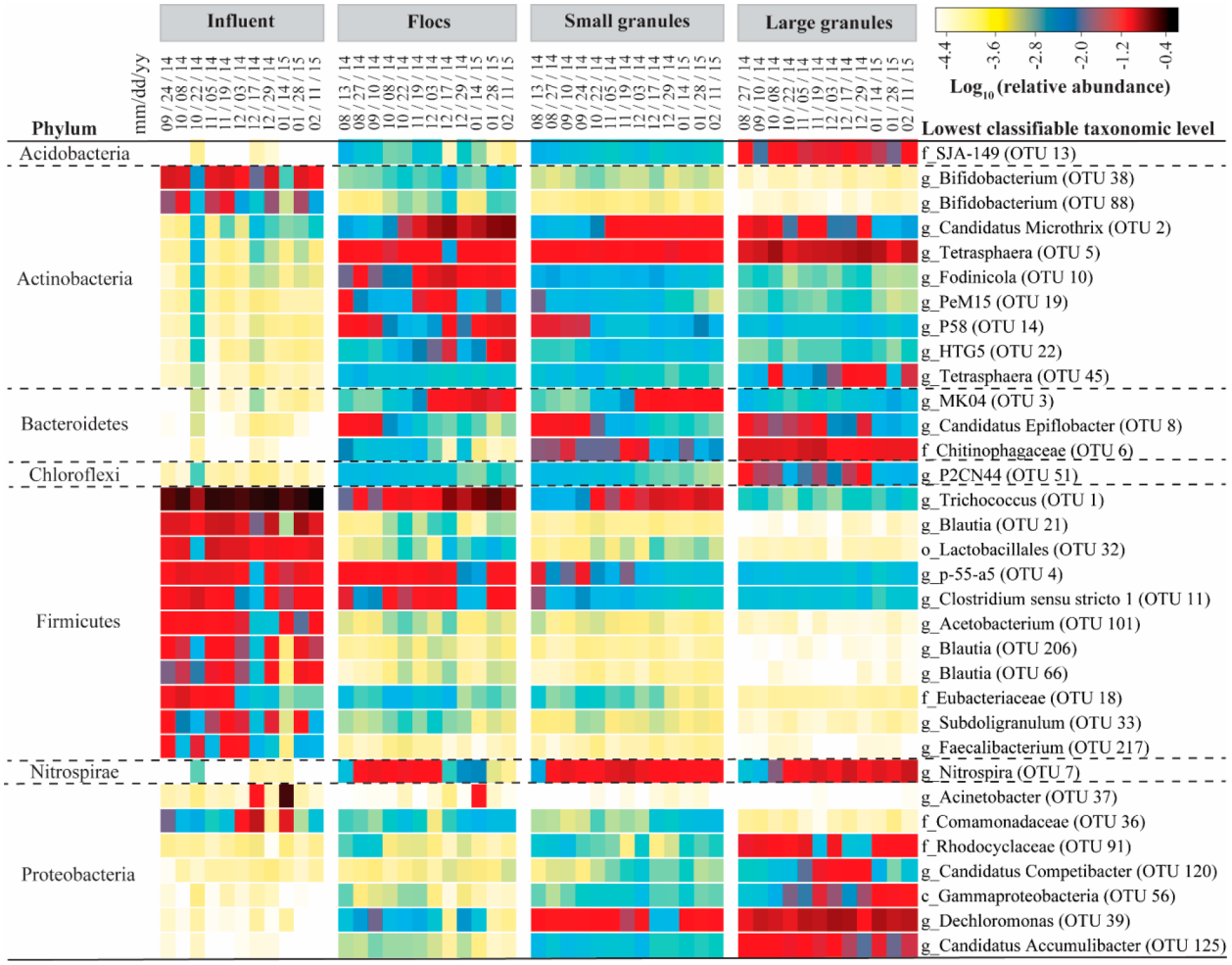


**Figure 4.** Heatmap distribution of the most dominant OTUs (≥1.0%) classified down to the lowest classifiable taxonomic level (g, f, o, c, and p represent genus, family, order, class, and phylum, respectively) for the influent wastewater, flocs, small granules, and large granules.

(37.9 ± 11.1%) in WW (Table S4). It should be noted that the relative abundance of putatively nongrowing OTUs, belonging to human fecal- and sewage infrastructure-derived microbes,[49] progressively decreased with the increase in aggregate size (Figures S6, S7, and S8) and eventually became rare or not detected in LG. In total, FL had more (dominant and rare) putatively nongrowing OTUs (17.7 ± 3.1%) compared to SG (15.0 ± 2.8%) and LG (12.6 ± 2.5%) (Table S5). Taken together, these results suggest that flocs and small granules were more susceptible to immigration from the source habitat than large granules (student's *t* test, $P < 0.05$ for SG vs LG and $P < 0.01$ for FL vs LG).

A shared OTU network analysis of the dominant OTUs was constructed to further illustrate the impact of immigration on different-sized microbial aggregates in the AGS system (Figure 3). In the network, the different sample types (i.e., WW, FL, SG, and LG) are shown as white large nodes. Samples (i.e., white nodes) that are located close to each other have more OTUs shared between them. The OTU nodes were colored based on phylum, and the size of the OTU nodes represents the number of sample types sharing that specific OTU. Edges radiate from the respective sample type to their OTUs, and the edge width corresponds to the relative read abundance of the respective OTU in the radiating sample. The largest distance between sample nodes was observed between WW and LG samples, and the shortest distance was between SG and FL. The distance between WW and FL was shorter than the distance between WW and SG. The network analysis depicted that the most abundant OTUs within the immigrant community (thick edges radiating from WW) had a greater probability of invading the receiving ecosystem, with FL showing the highest number of shared OTUs with WW and LG showing the lowest number of shared OTUs with WW. The OTUs that were shared the most among WW and the other samples belong to the phylum Firmicutes, followed by Actinobacteria, Proteobacteria, and Bacteroidetes (Figure 3).

Venn diagram (Figure S2A) further supports OTU network analysis results by showing dominant OTUs in WW were mostly shared with FL (37 OTUs corresponding to 65% of the dominant reads in WW), and then gradually decreased in SG (23 OTUs corresponding to 51% of the dominant reads in WW) and LG (10 OTUs corresponding to 36% of the dominant reads in WW). Similar results were obtained with rare OTUs, where rare OTUs in WW were more shared with FL (1034 OTUs), and then gradually decreased in SG (1002 OTUs) and LG (886 OTUs) (Figure S2B).

**Putative Functional Groups Progressively Enriched with Increase in Aggregate Size.** Heatmap of the most dominant OTUs, with relative read abundance ≥1%, classified down to the lowest classifiable taxonomic level was plotted to visualize the variation in time of individual taxa in WW, FL, SG, and LG (Figure 4). Taxa that were highly abundant in



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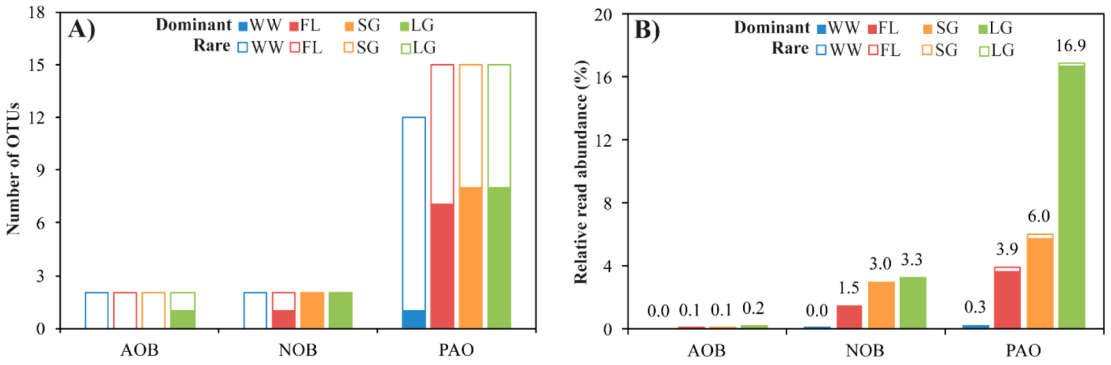


**Figure 5.** Number of operational taxonomic units (OTUs) (A) and mean relative read abundance (%) (B) of putative functional bacterial groups detected in the influent wastewater (WW), flocs (FL), small granules (SG), and large granules (LG). AOB: ammonium oxidizing bacteria; NOB: nitrite oxidizing bacteria; PAO: polyphosphate-accumulating organisms.

WW, such as those belonging to Firmicutes, *Bifidobacterium* (affiliated to phylum Actinobacteria), and *Comamonadaceae* (affiliated to phylum Proteobacteria), progressively decreased in relative abundance with increase in microbial aggregate size to become rare in LG. The fecal- and sewage infrastructure-derived microbes (*Trichococuss*, *Blautia*, *Lactobacillales*, *Clostridium*, *Acetobacterium* (order Clostridiales), *Eubacteraiceae* (order Clostridiales), p-55-a5 (family Peptostreptococcaceae), *Subdoligranulum*, and *Bifidobacterium*)[49] belonging to the phylum Firmicutes and Actinobacteria (Figure 4) had a negative growth rate (Figures S6, S7, and S8), and their presence in the AGS system was mainly due to immigration. Interestingly, several important putative functional genera for nitrogen and phosphorus removal (such as *Nitrospira*, *Candidatus Accumulibacter*, *Tetrasphaera*, and *Dechloromonas*) that were considered rare in the WW samples where progressively enriched with increase in microbial aggregate size (i.e., from FL to LG) (Figure 4).

Species-level OTUs belonging to potential functional groups (functions obtained from the MiDAS field guide)[44] for nutrient removal in WW, FL, SG, and LG is shown in Figure 5A. Fifteen OTUs belonging to polyphosphate-accumulating organisms (PAO) were detected in FL, SG, and LG samples, and 12 out of these 15 OTUs were also detected in WW samples. Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) each contained only two OTUs for all type of samples, e.g., AOB (OTU 497 and 541) and NOB (OTU 7 and 597). The putative OTUs belonging to AOB, NOB, and PAO represented rare members of the WW community and became dominant within the AGS system. For example, the rare OTUs belonging to NOB (i.e., OTU 7 and OTU 597), became dominant in SG and LG (Figure 5A). Similarly, OTU 497 belonging to AOB became dominant in LG, and 7 out of the 12 OTUs in WW belonging to PAO became dominant in SG and LG. A closer look at the relative abundance of these putative functional groups suggest that LG provided a suitable niche for the enrichment of these relatively slow-growing bacteria.[1] For example, the relative read abundance of PAO was almost 3 times higher in LG (16.9%) than SG (6.0%) and FL (3.9%) (Figure 5B). It should be noted that glycogen-accumulating organisms (GAO), which can compete with PAOs for organic carbon sources, were also enriched with increase in microbial aggregate size, with relative read abundance 3 times higher in LG (2.6%) than SG (0.9%) and FL (0.5%) (data not shown). *Nitrosomonas* (OTU 497 and 541) was the only detected genus of AOB, with relative abundance of 0.2% and 0.1% in LG and SG, respectively. *Nitrospira* sublineage I (OTU 7) was the dominant genus of NOB with relative abundance of 3.1% (LG), 2.9% (SG) and 1.4% (FL). The NOB *Ca. Nitrotoga* (OTU 597) was also detected in several samples but with very low relative read abundance (<0.2%).

The selection and enrichment of certain bacteria in granular sludge (SG and LG) and exclusion of others (Figures 4 and 5) suggest that the assembly of flocculent sludge community into highly structured granular sludge community is not a random process. This was further confirmed by viewing granules as biofilms and adopting metacommunity ecology as a framework. Here, the microbial community in the granules (SG or LG) was statistically compared with the random assemblies of FL community to determine whether SG and LG communities were a product of purely stochastic immigration from FL community. The SG and LG communities differed significantly from the random assemblages produced (probability of the SG or LG community to fall within the distribution of the random assemblages of FL community, $P < 0.001$; Figure S9). These results indicate that stochastic immigration from the FL community was unlikely to shape the composition of SG and LG communities, thus supporting the assumption that species sorting has a role in granule community assembly.

## DISCUSSION

The main aim of this study was to investigate the relative importance of local (species sorting) versus regional factors (immigration) in structuring the bacterial community assemblages of different-sized microbial aggregates in a full-scale AGS system. We found indications on the effect of both factors, but signature of species sorting was stronger, particularly for SG and LG. This was suggested by the fact that sink communities (i.e., FL, SG, and LG) were significantly different from their source community (i.e., WW) using both taxonomic (Bray–Curtis) and phylogenetic (unweighted UniFrac) distance (Table S3, Figure 1). Also, OTUs that were highly abundant in WW gradually decreased in abundance with increase in aggregate size (Figure 4), and hence increase in SRT. Our results support previous studies showing strong indications of species sorting in natural[12−15,50] and full-scale engineered[16−18,34] water ecosystems.

Although community composition varied between the different-sized microbial aggregates, there was a lack of a strong species sorting between FL and SG (Table S3, Figure 1), and this could be because of the fact that the SRT was not


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significantly different between FL (6 days) and SG (8 days). Due to biomass segregation and selective excess sludge withdrawal, smaller and less settling microbial aggregates were removed with the excess sludge, while larger granules were maintained in the reactor.[3] Based on the microbial-aggregate size distribution in the excess sludge, aggregates with size <0.2 mm (i.e., FL) and 0.2−1.0 mm (i.e., SG) represented on average 33% and 60% of the excess sludge, respectively, whereas LGs represented 7% of surplus sludge.[3,36] This could explain why the SRT of FL and SG was not significantly different, and for LG to have a high SRT (143 days).

In the current study, dominant OTUs were defined as those that are present with relative read abundance ≥0.1%. Although, the selection of this cutoff between dominant and rare OTUs is arbitrary, as is the case in several previous studies related to wastewater treatment plants,[16,17,27] the dominant OTUs in the AGS represented ∼76% of sequence reads. This result is in line with Saunders et al.,[29] where they defined dominant OTUs as those that represent the top 80% of sequence reads obtained by amplicon sequencing, as these account for most (∼80%) of the carbon turnover in biological wastewater treatment systems. In ecosystems undergoing large disturbance events, the conditionally rare taxa (CRT) approach might be more appropriate to define rare taxa.[51] In the current study, the AGS system was operating under stable conditions. Using this cutoff threshold, only 11.6% of the dominant OTUs (37 out of the 319 dominant OTUs) in the AGS system was shared with the influent wastewater (Figure S2A). These could represent OTUs that were actively growing or were present in the system due to high dispersal from the influent wastewater. Combining amplicon sequencing with mass balance, Saunders et al.[29] estimated that 10% of the total observed reads in full-scale flocculent sludge wastewater treatment systems belong to putatively slow- or nongrowing OTUs, and their presence in the sludge was due to high immigration from the influent wastewater. Using a similar approach, we estimated that immigration had a modest effect on the observed community in the AGS system, with only 8% of the dominant OTUs in the AGS system were putatively nongrowing (Table S5), corresponding to 10% of the total reads in the AGS system (51% of total reads in the influent wastewater) (Table S4). Their consistent presence as dominant microbial community in the AGS system was due to high immigration from the influent wastewater. Organisms that are highly abundant in WW have sufficient cells entering the plant with the influent, and at high influent flow rate (i.e., low hydraulic retention time), these organisms can be detected in the system even if they are inactive (net growth rate <0 $d^{-1}$) due to immigration. The OTUs under question had high relative read abundance (>1% in most cases) in the influent wastewater (Figure 2). For example, one of the dominant OTUs (33% of sequence reads) in wastewater belonging to the genus *Trichococcus* was constantly present as dominant OTU in the AGS system despite having a negative net growth rate.

In microbial metacommunity ecology mass effects are predicted to dominate in inland natural water ecosystems with short water retention time, such as streams, estuaries, and lakes.[19] Several previous studies observed signature of mass effects in inland natural water ecosystems, however, there seems to be no universal cutoff point for water retention time to differentiate between mass effects and species sorting, with <69−100 days suggested for lakes[20,23] and lower (<2−3 days) for estuaries and streams.[21,22] In engineered wastewater treatment systems, the water or hydraulic retention time is separated from the SRT through the formation of surface associated biofilms or surface-independent microbial aggregates (flocs or granules). The formation of microbial aggregates or biofilms reduces the constraint of short hydraulic retention time and increases their residence time relative to free-living cells in the influent wastewater. Nevertheless, this does not exclude the possibility that at short SRT, the effect of mass effect could be higher, as organisms will have less exposure time to environmental filtering or species sorting. Also, it has been suggested that short-SRT sludge communities are more subjected to neutral factors than long-SRT sludge communities, such as oscillations in species abundances at shorter SRT due to the high specific growth rate,[52] or to continuous random colonization by new species from the influent wastewater.[53] Our estimation of the net growth rate (Table S4) suggests that immigration from the influent wastewater was higher at short SRT (FL and SG) than long SRT (LG). This was further supported by Vuono et al.[28] who studied the effect of immigration in a full-scale activated sludge system by temporally partitioning the activated sludge metacommunity by successively decreasing the SRT from 30 to 3 days (i.e., wasting more biomass). They observed higher effect of immigration at SRT of 3 days, manifested by an increase in taxonomic and phylogenetic similarity between sludge and influent wastewater, and increase in the number of shared OTUs. The opposite trend was observed (i.e., decrease in similarity and shared OTUs between sludge and influent) when the SRT was increased to 30 days, and they interpreted the results under the specie sorting metacommunity paradigm. Since LG represent the majority of the biomass in the AGS system,[3] our results suggest that the effect of immigration is expected to be lower in AGS system than that in activated sludge system where biomass is mainly present as flocs.

Despite the high number of sequence reads and OTUs shared between FL and granules (SG and LG) (Figure S2), our results suggest that the granular sludge community was not a mere reflection of the flocculent sludge community (Figure S9), and species sorting was the likely mechanism for their assembly. This species sorting resulted in the presence of unique dominant OTUs in the SG and LG (Figure S2) and in different relative abundance of shared putative functional OTUs (Figure 5). Our results were supported by previous studies in natural[14,15] and engineered[16] water ecosystems. Wilhelm et al.[15] and Besemer et al.[14] showed that stochastic dispersal from the suspended community was unlikely to shape the biofilm community in streams, and suggested species sorting as the likely mechanism for biofilm assembly, whereas, the suspended community was likely shaped by mass effects.[15] Similarly, Matar et al.[16] found that stochastic dispersal from flocculent sludge was unlikely to shape the biofilm community on membrane surfaces of full-scale activated sludge membrane bioreactors. Taken together, these results indicate that species sorting is the likely mechanism for biofilm assembly in natural and engineered microbial ecosystems.

So far, the discussion above has mainly focused on the effect of dominant OTUs in wastewater on immigration. Interestingly, our results showed that some rare OTUs in wastewater belonging to putative functional groups responsible for nutrient removal, were progressively enriched with increase in microbial aggregates size (Figures 4 and 5). These results suggest the potential importance of rare organisms, especially habitat specialists, in the influent wastewater, which in the


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frame of species sorting were selected and became dominant members in the treatment plant. These results support previous indications that species sorting would be more important for habitat specialists than generalists.[7,25,54] Also, these rare OTUs in wastewater can be regarded as a seed bank, that can recolonize and become abundant following disturbance, thus maintaining ecosystem function.[55]

The AGS system represents an ecosystem with a gradient of selection pressure of local habitat condition (i.e., local factor), represented here by SRT.[56] Due to biomass segregation and selective excess sludge withdrawal, relatively slow-growing bacteria such as AOB, NOB, and PAOs were enriched more in large granules due to their high SRT. The presence of these slow-growing bacteria contributed to the stable nutrient removal performance of the AGS reactor (Table S1). Similar findings were reported where larger microbial aggregates harbored slow-growing organisms due to higher SRT.[56,57] Similarly, Vuono et al.[58] reported loss of ecosystem function (nitrification, denitrification, and biological phosphorus removal), when a full-scale activated sludge system was disturbed by decreasing the SRT to ≤12 days. Upon return to the condition before disturbance (i.e., 30-day SRT), complete functional recovery of performance was observed. Typically, flocculent sludge wastewater treatment plants are operated at long SRT as 30 days to avoid washout at low temperatures.[29] Low temperatures reduce bacterial growth rates resulting in the washout of slow-growing bacteria at low SRT. Griffin and Wells[17] reported 70% decrease in the abundance of *Nitrospira* from October to April before recovering in the summer in full-scale activated sludge wastewater treatment plants operated at SRT between 6 and 14 days, whereas the treatment plant operated at higher SRT (17 days) did not experience washout of *Nitrospira*. Our results showed that slow-growing bacteria were enriched more in granules than flocs due their high SRT. In this regard, growing microbes in granules might be advantageous compared to flocs especially in response to seasonal changes. However, there are limited studies on the effect of low temperature on slow-growing organisms such as AOB, NOB and PAOs in an AGS system.

Substrate availability and anaerobic feeding also resulted in the selection of slow-growing bacteria such as PAO in LG. Due to the difference in settling velocity among FL, SG, and LG, the sludge bed was segregated after settling phase where LG were located at the bottom of the AGS reactor followed by SG and FL, which were located at the top. In the Garmerwolde AGS plant, the influent WW was fed anaerobically in a plug flow regime from the bottom of the reactor, and hence LG were exposed more to readily biodegradable chemical oxygen demand, which is suitable for the enrichment of PAO.[1]

It should be noted that AOB were underrepresented in the studied AGS plant, despite low effluent $NH_4^+$−N concentration (Table S1). Similar results were obtained by Ju and Zhang[41] who observed poor representation of AOB in a full-scale flocculent sludge wastewater treatment plant despite low effluent $NH_4^+$−N. It is not clear why AOB were present at low abundance, most likely justifying previous findings that AOBs have high specific activity in these types of engineered ecosystems.[59] It is also likely that there are some undiscovered or unidentified AOB. Future studies should elucidate possible reasons for the low abundance of AOB in full-scale flocculent and granular sludge wastewater treatment systems.

It should be noted, for simplicity we assumed that the observed read abundance of each species-level OTU is equal to the actual abundance of that organism in the different samples (i.e., influent wastewater, flocs, small granules, large granules, excess sludge, and effluent).[29] This assumption has some limitations due to (i) variation in the 16S rRNA gene copy number per genome for the different species; (ii) differences in the specificity of the primers used in this study; and (iii) biases due to PCR and DNA extraction. However, these limitations are universal for amplicon sequencing. The correction for the different 16S rRNA gene copy number per genome is not possible due to lack of reference genomes.[60] Nevertheless, this issue can be resolved as more reference genomes become available in the future. Further, the variation in the 16S rRNA gene copy number per genome is uniform in all the different samples, which eventually would normalize in our mass balance model. Further, the cell concentration values in the influent, effluent and sludge, were obtained from a previous study.[61] These values were within the range found in other geographically distant wastewater treatment plants.[1,62,63] To obtain more accurate values, the actual cell numbers in the influent, effluent, and sludge can be measured using fluorescence in situ hybridization[64] or flow cytometry.[1]

Our findings indicate that species sorting is an important mechanism in the assembly of granules in AGS system. In contrast, we found indications of a modest effect of immigration from influent wastewater. The finding that spatially segregated sludge having different SRT responded differently to immigration from continuously shared influent is rather novel. It is pertinent to mention that to differentiate between growing and nongrowing OTUs, we integrated 16S rRNA gene sequencing with mass balance to estimate the net growth rate of individual OTUs. Amplicon DNA sequencing technique is not yet considered as a standard technique for studying the active members in the microbial community.[65] Methods such as reverse-transcribed rRNA,[13,14] or propidium monoazide[66] can be applied, for identifying active populations. It should be noted that our model was not intended to yield absolute growth rates or SRTs, but rather simulate an overall trend of the effect of immigration on different-sized microbial aggregates. The findings in this study highlight the importance of aerobic granular sludge system as a unique model ecosystem to study fundamental microbial ecology concepts and call for more research such as exploring several geographically distributed full-scale AGS treatment plants, to determine whether a core community of abundant organisms exits in AGS similar to activated sludge.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b07303.

Additional materials and methods (1.1−1.3), supplementary figures (Figures S1−S9), and tables (Tables S1−S5) (PDF)

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### Notes

The authors declare no competing financial interest.

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