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Nonrandom Assembly of Bacterial Populations in Activated Sludge Flocs

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Abstract The aim of this work was to investigate the dynamics of assembly of bacterial populations in activated sludge flocs. We approached this question by following the development of active bacterial populations during floc development in four replicated lab-scale activated sludge reactors, in which solid retention time (SRT) was set at 4 days. The null hypothesis was that the similarities in community composition could be accounted for by the probability that the same organisms occur in more than one replicated reactor. Microscopic imaging showed that the size of flocs in reactors with biomass retention increased during the first few days until a steady-state size was reached. The diversity and community structure of the sludge in all reactors were analyzed during a period of up to ten SRT, using denaturing gradient gel electrophoresis (DGGE) of reverse-transcription polymerase chain reaction-amplified 16S rRNA. High rates of change in DGGE profiles from consecutive sampling points suggested a high level of dynamics in all reactors. This conclusion was confirmed by the application of the Raup and Crick probability-based similarity index (S_{RC}) for the comparison of rRNA-based fingerprinting patterns, which indicated that bacterial communities within reactors were not significantly similar after three SRT ($0.05 < S_{RC} < 0.95$) and became significantly dissimilar after five SRT ($S_{RC}<0.05$). More importantly, significant similarity between replicate reactors was observed at all times analyzed ($S_{RC}>0.95$). The fact that the patterns between replicates were more reproducible than expected by chance under highly dynamic conditions allowed us to reject the null hypothesis that activated sludge floc communities assemble randomly from the available source pool of bacteria. We suggest that communities progressively recruit from the available pool of bacterial species, each with particular ecological requirements that determine their time of emergence into the community.

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Introduction

Most biological wastewater treatment systems have the ability to exhibit stable performance in the context of a variable microbial community structure. Research from several laboratories showed that bacterial communities in bioreactors are highly dynamic and may diverge with time in replicated systems, even in the absence of external disturbances [11, 12, 15, 21, 22, 24, 28, 37]. The overall process performance appears to be maintained by numerous coexisting species performing similar functions [29]. In principle, the existence of functional redundancy could allow the assembly of differently composed communities in replicated systems. Lab-scale reactors, too, contain a high diversity of species, which assemble to form a functional community. Thus, the issue of reproducibility of microbial community dynamics in replicated systems relates to the way in which microbial communities assemble. Accordingly, examining the degree of determinism is important for elucidating the underlying mechanisms of microbial community assembly.

Efficient aggregation is essential to the activated sludge process since the operational success depends largely on good settling sludge [5]. The continuous recycle in conventional activated sludge, as well as the repetition of the settling process in sequencing batch reactors, selects for

microorganisms living in flocs. The aim of this work was to investigate whether the selected populations in actively growing activated sludge flocs are stochastically recruited from a pool of redundant species in the community or whether there are assembly rules, which albeit unknown, determine the observed patterns. We approached this question by following the evolution of active bacterial populations during floc development in four replicate lab-scale activated sludge reactors. The null hypothesis was that the similarities in community composition could be accounted for by the chance that the same organisms occur in more than one reactor. Using appropriate statistical tests, we concluded that the observed assembled communities in all replicates were significantly more similar than expected by random collection of species from the source community.

Materials and Methods

Seed Preparation and Reactor Setup

Sludge was collected from the aeration basin of a small full-scale domestic wastewater treatment plant in the Province of Buenos Aires (3,500 p.e.). In order to study the dynamics of flocculation, it was necessary to disrupt the activated sludge flocs into smaller particles and to enrich the biomass in planktonic populations. After vigorous vortexing, samples were incubated at 20°C for 7 days, with daily addition of sterile 20-fold concentrated synthetic sewage (3.2 g/l peptone, 2.2 g/l yeast extract, 0.6 g/l urea, 0.18 g/l NaCl, 0.08 g/l $CaCl_2 \cdot 2H_2O$, 0.04 g/l $MgSO_4 \cdot 7H_2O$, 0.56 g/l K_2HPO_4) and a corresponding amount of dilution water, without biomass wasting. Synthetic sewage was used to avoid community shifts due to changes in feed composition. Its composition is similar to that of domestic sewage. After dilution, this synthetic sewage contains approximately 105 mg/l of C, 46 mg/l of N, and 5 mg/l of P, and the pH was between 7.0 and 7.5 [20]. The nonflocculent biomass suspension (800 ml) was then diluted fourfold with synthetic sewage and used to seed four replicates of lab-scale bioreactors. All reactors were fed with sterile synthetic medium. Therefore, the components of the bacterial community detected throughout the time course of polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) experiments were already present in the seed inoculum.

The reactors (800 ml of working volume) were placed inside a temperature-controlled cabinet (Aqualytic) at a constant temperature of 20±1°C and operated for 40 days in a sequencing batch mode with biomass retention. Aeration and stirring were disconnected for 30 min everyday to allow sludge to settle, and 150 ml of clarified supernatant was decanted manually. Once mixing and aeration was resumed, each reactor was fed separately with 350 ml of sterilized synthetic sewage at a flow rate of 0.3 ml/min, using a peristaltic pump. At the end of the feeding plus aeration phase, 200 ml of sludge was wasted, resulting in a hydraulic retention time of 54 h and a solid retention time (SRT) of 4 days. The samples were taken at this time, centrifuged, and stored at −70°C until analysis.

Microscopy

Flocs were visualized in an epifluorescence microscope BX41 (Olympus) using the LIVE/DEAD BacLight bacterial viability staining kit (Invitrogen). For each sample, ten microscopic fields were examined at random. Floc area was determined by outlining the floc manually using the *Freehand* tool in the ImageJ software (rsbweb.nih.gov/ij). Areas were subsequently converted to average floc diameter, as if they were approximately spherical.

RNA Extraction

All materials and solutions used were RNAse free. Pellets from 1,000-µl sludge were resuspended in 500 µl of Tris–EDTA (TE) buffer (pH 8.0) and transferred to 2-ml screw-capped tubes with 0.5-g zirconia silica beads (Biospec Products, OK, USA). Fifty microliters of 10% sodium dodecyl sulfate was added, and the tube was immediately filled with 250 µl of RNAse-free phenol equilibrated with buffer TE, pH 8.0 and chloroform–isoamyl alcohol (24:1). Cells were physically disrupted by shaking for 30 s in a bead beater (Biospec Products, OK, USA) at 2,500 rpm. The aqueous phase was transferred to a clean tube and re-extracted twice with chloroform–isoamyl alcohol (24:1). Nucleic acids were precipitated with two volumes of ethanol 100% and 0.1 volumes of 3 M sodium acetate, pH 5.2, and washed twice with 70% ethanol. The pellet was resuspended in 50 µl of RNAse-free buffer and treated with RNAse-free DNAse (Promega).

RT-PCR Assay of 16S rRNA

The reverse-transcription (RT) reaction contained 4 µl of 5× buffer (Promega), 20 µM of dNTPs, 0.6 µM of primer 534r [30], and RNAse-free water to a volume of 20 µl. The mix was preincubated for 10 min at 37°C, after which 200 U of RT MMLV (Promega) was added, and the reaction was incubated for an additional 50 min at 37°C. Control RT reaction mixtures included no-template reactions with or without RT and purified RNA templates with or without RT.

PCR–DGGE

Each PCR contained 2 ng of cDNA template. DGGE was performed as described [30]. DGGE system (CBS Scientific, DelMar, CA) was used as specified by the manufacturer. After electrophoresis, gels were soaked for 30 min in SYBR Gold nucleic acid stain (Invitrogen, 1:10,000 dilution in Tris–acetic acid–EDTA; pH=8.0). The stained gels were immediately photographed on an UV transillumination table combined with a camera module and imaging system (Syngene).

Fingerprinting Analysis

The obtained DGGE patterns were analyzed using Bionumerics software version 2.0 (Applied Maths, Sint-Martens-Latem, Belgium, licensed to Mario Aguilar, Universidad Nacional de La Plata).

The diversity of the bacterial community was examined by the Richness, the Shannon index of general diversity, and the Evenness. Richness (R) referred to the number of bands in each DGGE lane. The Shannon diversity index (H') was calculated as: $H' = -\sum_{i=1}^{n} pi.\ln pi$, where pi is the proportion of the total intensity for each band in a track. It was calculated from $pi=n_i/N$ where n_i is the area of a peak, and N is the sum of all peak areas in the densitometric curve. Evenness (E) was calculated as: $E = \frac{H'}{\ln R}$.

Moving-window analysis was performed to reflect the dynamics of bacterial community structure [27]. The analysis consisted of plotting the change in time of the Bray–Curtis similarity index between two consecutive dates [32].

Statistically significant similarity and dissimilarity at the 95% confidence level in species composition was measured using the probabilistic Raup–Crick index (S_{RC}) for absence–presence of data [34, 36], followed by clustering using the unweighted pair group method with arithmetic mean. The S_{RC} similarity index uses a Monte Carlo procedure to compare the observed number of species common to two assemblages and the probability distribution of the expected number of common species as a measure of the similarity between the two assemblages [34]. The probability of seeing c species in common between two samples was calculated based on the global diversity in all samples. Assuming that the total number of species (bands in the DGGE gel) for each sample is indicated by n_1 and n_2, c ranges from 0 to the number of bands in the sample with the lower richness. By comparing the observed number of species occurring in both assemblages with the distribution of co-occurrences from 200 random replicates, one obtains the probability that the observed number of species in common was a result of random sampling from the global observed diversity. S_{RC} values above 0.95 indicate significant differences with random assortments of the same species, i.e., the two assemblages are significantly similar. S_{RC} values below 0.05 indicate that samples were significantly dissimilar. The S_{RC} was calculated with the PAST program (Palaeontological Statistics, version 1.85) available from the University of Oslo web site link (http://folk.uio.no/ohammer/past).

Results

Floc Development

Four replicate lab-scale reactors were seeded with an activated sludge inoculum, which had been previously enriched in planktonic populations. Figure 1 shows the changes in floc size observed in the four replicate reactors immediately after the operation was switched to the sequencing batch mode with biomass retention. The floc particles at the time of startup had an average diameter, considering spherical shape, of 80 μm. During the first 10 days, the average diameter of flocs increased monotonically, reaching their mature stage of about 200 μm. As expected, a large range of sizes was observed comparing floc sizes within replicated reactors as well as between reactors. Sizeable changes in settling properties were not observed, although the actual sludge volumetric indices were not measured. In all cases, a clear supernatant was formed after 30 min of settling.

Population Dynamics During Floc Development

The diversity and community structure of the sludge in all reactors were analyzed during a period of up to ten SRT using DGGE of RT-PCR-amplified 16S rRNA (Fig. 2). Analysis of rRNA was selected in order to reduce the interference from nonactively growing bacteria in the community.

Moving-window analysis of RNA-based DGGE profiles of the bacterial communities of all reactors showed a similar shifting pattern in rRNA community structure throughout the 40 days of reactor operation (Fig. 3). The average degree of change between consecutive DGGE profiles of the same community over a time interval of 3 days was 36.6±6%. A moderate decreasing trend was observed along the timescale, from a maximum of 45±5% during the first days of operation, down to a minimum of 27±6% at the end of the experiment. In every case, these rates of change were indicative of a very high level of dynamics.

A complementary view of community dynamics was obtained by application of the Raup and Crick probability-based similarity index (S_{RC}). Figure 4 shows the pairwise comparison of community profiles between day 4 and the following dates for each individual reactor. Despite the

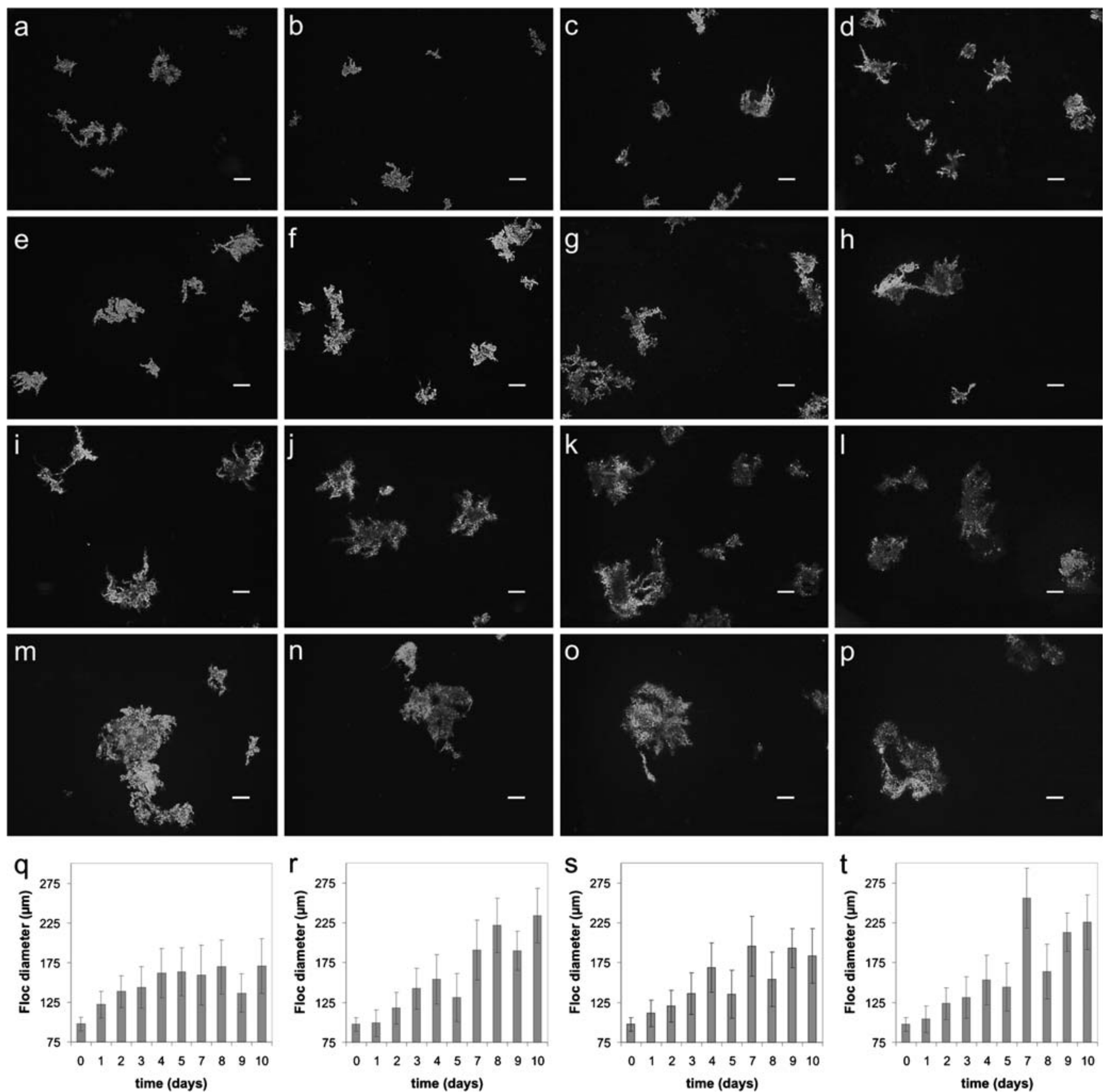

Figure 1 Photomicrographies showing activated sludge flocs from reactors R1 (**a**, **e**, **i**, **m**), R2 (**b**, **f**, **j**, **n**), R3 (**c**, **g**, **k**, **o**), and R4 (**d**, **h**, **l**, **p**) at 1 day (**a**–**d**), 4 day (**e**–**h**), 7 days (**i**–**l**), and 10 days (**m**–**p**) after startup. Flocs were visualized in an epifluorescence microscope using the LIVE/DEAD BacLight bacterial viability staining kit. *Scale bar* 100 µm. **q**–**t** Average diameter of flocs as a function of operation time. *Bars* are standard error of the mean

small offset between curves, DGGE profiles of all reactors showed an almost identical trend. After three SRTs, the bacterial communities were no longer significantly similar ($0.05 < S_{RC} < 0.95$), and they become significantly dissimilar after five SRTs ($S_{RC}<0.05$). Similar results were obtained when other dates were used for pairwise comparison of similarity between bacterial communities within each reactor (data not shown).

Comparison of Community Structure Between Reactors

Despite the dramatically high temporal changes in species composition, measures of richness, evenness, and Shannon indices showed little variation (i.e., small standard deviation) when they were averaged over the total time of the run (Table 1). These results indicate that the detectable bacterial diversity remained approximately constant throughout the

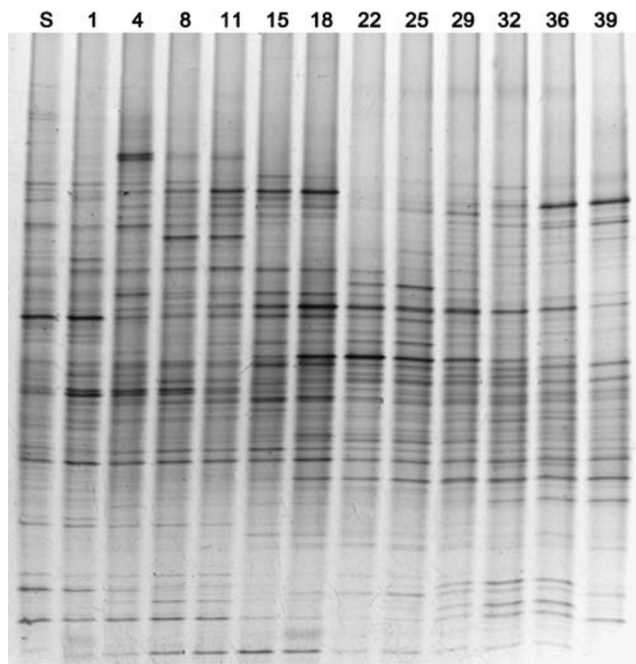

Figure 2 Representative DGGE profiles of 16S rRNA fragment amplified by RT-PCR amplification, showing shifts in bacterial communities during activated sludge floc formation in reactor R1. *S* is the acclimated seed used as inoculum to start up all reactors (time 0). The *numbers on top* of the lanes indicate the time of operation after startup

period. Pairwise comparison of evenness and Shannon diversity indices did not reveal significant differences between reactors. Richness was similar between reactors, yet reactors 1 and 4 contained significantly more bands than reactors 2 and 3 (Table 1, average pairwise comparison between reactors 1 and 2, 1 and 3, 2 and 4, and 3 and 4, $p=0.05$, as determined by Student's *t* test).

PCR-amplified ribosomal cDNAs from the four replicated reactors taken at days 4, 22, and 39 of operation,

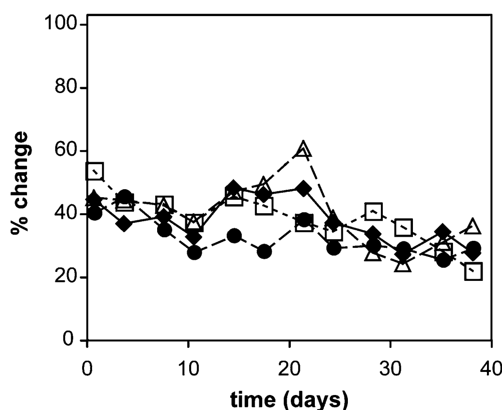

Figure 3 Moving-window analysis based on pairwise similarity Bray–Curtis coefficients from DGGE data to evaluate the dynamics of bacterial communities during floc development in activated sludge reactors R1 (*filled circles*), R2 (*empty squares*), R3 (*empty triangles*), R4 (*filled diamonds*). Percent change is defined as 100 − %similarity [27]

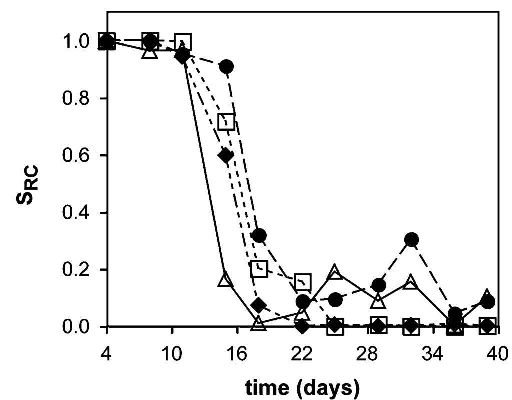

Figure 4 Raup and Crick similarity data (S_{RC}) from DGGE profiles. Pairwise similarity between bacterial communities of day 4, and the following dates were calculated separately for each reactor. R1 (*filled circles*), R2 (*empty squares*), R3 (*empty triangles*), R4 (*filled diamonds*)

corresponding to one, 5.5, and ten SRTs, were loaded into a DGGE for similarity analysis (Fig. 5). The dendrogram generated using the Raup and Crick probability-based similarity index (S_{RC}) for the comparison of rRNA-based fingerprinting indicated that replicate reactors clustered together at all times, with significant similarities (S_{RC}>0.95).

Discussion

The assembly of microorganisms within the activated sludge may be regarded according to two contrasting theories put forward to describe the abundance and distribution of species in any environment. Niche-based theories focus primarily on tradeoffs, such as competitive ability, implying a deterministic selection of specific organisms that are best adapted to the environment [9]. In opposition, neutral theories rely on stochastic processes of births, deaths, and immigration of species, which are considered functionally equivalent [4, 19]. The application of neutral community models in microbial ecology has attracted considerable attention lately for their success in providing a quantitative description of bacterial community assembly [33, 40, 41]. However, it is generally recognized

Table 1 Diversity indices for the bacterial communities in four replicate activated sludge reactors

Reactor	Richness	Evenness	Shannon (H)
R1	41.6±4.4	0.72±0.06	3.39±0.16
R2	37.4±4.6	0.76±0.05	3.34±0.15
R3	37.2±3.7	0.73±0.10	3.29±0.20
R4	40.1±4.0	0.73±0.06	3.37±0.14

The results are expressed as mean ± SD deviation of the mean of the index averaged over the 40-day study

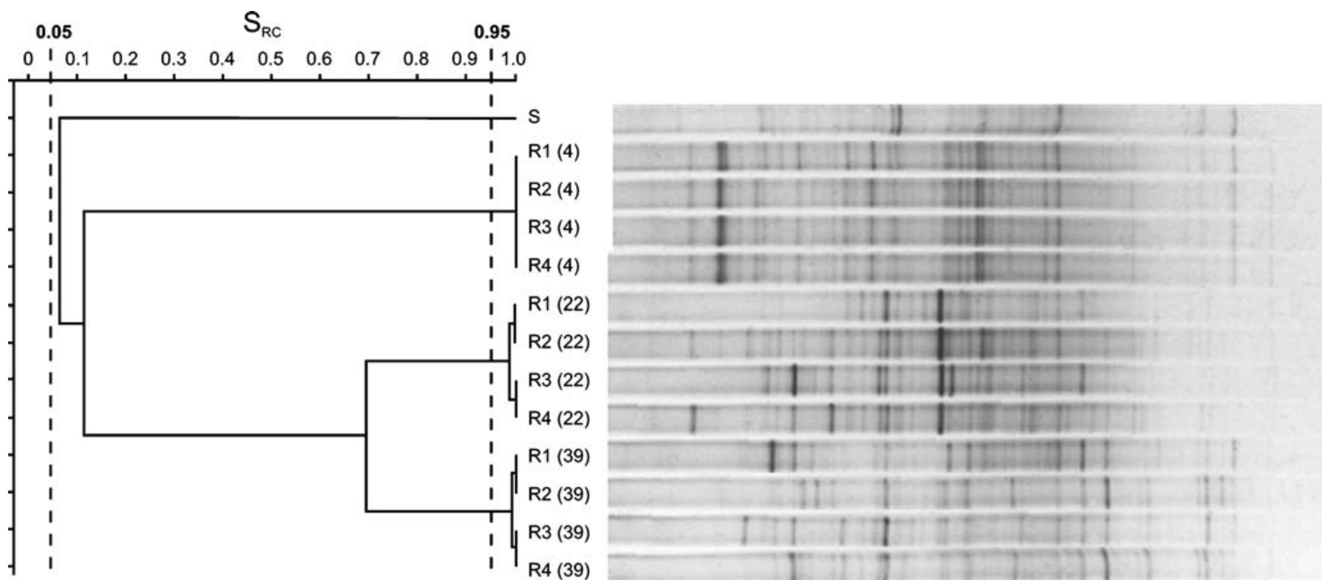

Figure 5 16S rRNA-based fingerprinting of samples from reactors 1 to 4, taken at days 4, 22, and 39 of reactor operation. The *dendrogram* is based on the Raup and Crick similarity analysis. $S_{RC}<0.95$ and $S_{RC}>0.05$: similarity no greater than expected by chance (null hypothesis holds), $S_{RC}<0.05$: significant dissimilarity and $S_{RC}>0.95$ significant similarity (null hypothesis is rejected)

that the underlying biological activity of wastewater treatment may be deterministic [10]. A considerable effort is currently being given to the search of a combination of both theories that can fit and predict the observed species abundance patterns [1, 18]. This work provides indirect evidence for an ordered path during floc formation, which constrains floc assemblage to certain structural patterns of the microbial community.

The acclimation period was designed with the aim of enriching the planktonic populations, while maintaining the richness of the original activated sludge community. In this way, the original inoculum constituted the source community from which any “invader” species were derived. Successful colonization occurred when a given species became detectable with the fingerprinting technique used. Thereof, we followed the definition by Marzorati et al., interpreting dynamics as the number of species that on average come to significant dominance (above the detection limit of the technique) during a defined time interval [27].

It is generally believed that a high degree of diversity confers stability to wastewater treatment reactors [6]. A highly diverse community would guarantee the presence of several different organisms, which are capable of carrying out the same function in the ecosystem, presumably across a wide range of environmental conditions [29]. Despite the high dynamics of bacterial communities, the individual mean indices of diversity were remarkably similar over time and across reactors. This may likely be an indication of functional redundancy. When one organism is lost from the system, another organism may occupy the open niche within the system to allow the maintenance of the system function.

Quantitatively, dynamics of microbial communities in the activated sludge reactors were monitored using a moving-window analysis [32]. A constantly drifting pattern of bacterial communities was observed in the absence of external perturbations. This result is in agreement with previous work showing the dynamic character bacterial community structures in functionally stable reactors [11, 12, 15, 21, 24, 28, 37]. High dynamics of bacterial communities at startup was also observed in sequencing bath reactors and membrane bioreactors (MBR) and has been attributed to the change of the food to microorganism ratio (F/M) upon biomass splitting [11]. Nonetheless, the rates of changes of the bacterial communities observed in this study were higher than those reported in comparable experiments performed by others [2, 11]. A number of reasons can be advanced to account for these differences. Firstly, in this study, a period of acclimation to the sequencing batch mode was purposefully avoided, in order to follow the dynamics of floc development. Therefore, high bacterial turnover was expected, as the reactors operated under non-steady-state conditions. Because the bacteria in the outer parts of the flocs are loosely bound to the floc matrix [39], it is conceivable to infer that floc growth involves repeated changes in bacterial community structure. Aggregation dynamics, as well as floc morphology, are related to sludge age. Figure 1 shows that newly formed flocs exhibited the typical irregular shape of young flocs, ascribed to a cluster–cluster reversible aggregation, rather

than the round and compact older flocs that reflect a low or no aggregation dynamics [8]. In agreement with this conjecture, activated sludge community of a sequencing batch reactor operated under stable conditions exhibited a very high rate of changes during reflocculation [16], although lack of quantification of the level of dynamics did not allow for a direct comparison with our results. Additionally, we have monitored the dynamics of operating replicate bench-scale reactors by looking at the rRNA, rather than the rRNA genes. This is important as staining with the bacterial viability staining kit indicated that the centers of the flocs contain a number of dead cells (Fig. 1), which may contribute significant amounts of DNA to the total DNA pool, leading to the underestimation of the actual dynamics.

Interestingly, as shown in Fig. 3, all reactors followed similar dynamics. Yet parallel courses of consecutive pairwise similarities do not necessarily imply identical trajectories of species turning into dominance and species becoming undetectable. In order to gain insight into the routes of assembly, DGGE band profiles were compared along the timescale and across replicated reactors using the Raup–Crick index as a measure of similarity. The Raup–Crick index is a probabilistic measure, which distinguishes similarities in band matching between samples, at a greater or lesser level than expected by chance [34, 36]. When all reactors were evaluated independently, it was observed that changes in S_{RC} index followed a similar course but showed a small offset between each other. However, the fact that the community fingerprinting between replicates was more reproducible than expected by chance under highly dynamic conditions allowed us to reject the null hypothesis that activated sludge floc communities assemble randomly from the available source pool of bacteria [3, 35]. Instead, we suggest a succession process, in which bacteria are progressively recruited from the available pool of bacterial species, each with particular ecological requirements that determine their time of emergence into the community. Successful invaders must be able to survive within the biomass and become transiently dominant, displacing the established species, when their fitness are higher relative to those of established species.

Although the data did not allow for a rigorous test of community assembly models, the results of this work add to the empirical evidence obtained previously by us and other researchers, pointing to a niche-oriented character of the assembly of bacterial communities: the increase in the proportion of nonculturable types of bacteria with succession has previously been attributed to the selection for organisms with greater niche specialization [14]. Competition experiments with two phenol-degrading bacteria showed that the fitness prevailed over the manipulation of immigration rates [26]. In this work, low invasion rate was expected, as all reactors were fed with sterile medium. Therefore, the effects of selection from the inocula community were likely more prevalent than neutral dynamics driven by immigration. It has been shown that environmental factors such as wastewater composition play a major role in structuring bacterial communities in activated sludge [13], influencing also the taxa turnover of the taxa–time relationship along a gradient of increasing industrial wastewater concentrations [38]. Interestingly, van der Gast et al. demonstrated that the temporal turnover, defined as the number of bacterial species eliminated and replaced per unit time, in a range of wastewater treatment reactors, was maximum when the incoming wastewater was municipal wastewater. They also suggested that the community assembly switched to a more deterministic or niche-based character with increasing industrial wastewater concentration [38]. The composition of the synthetic sewage chosen in this study mimicked the nutrient composition usually found in domestic sewage but is markedly less heterogeneous. Therefore, it would be expected that the synthetic sewage used would drive selection more than real municipal wastewater. In addition, neutral dynamics would be reduced because of the low rate of invasion from the sterilized feed compared to real wastewater which would contain high number of bacterial cells.

Results from a high-throughput environmental tag sequencing study of an extreme natural environment suggested that the distribution of the most abundant microbial taxa was dictated by niche differentiation [31], in an analogous way of an estuarine fish community, where the core community was accompanied by a large number of transient rare species, whose occurrence depended on immigration [25]. The possibility that neutral changes in community composition might only be evident in low abundance populations that are not detected by conventional analyses was also suggested by Akarsubasi et al. [2].

This study shows that it is possible to attain reproducible changes in microbial communities in controlled laboratory experiments. Similar observations have been made under comparable experimental setups, such as activated sludge operating at different SRT [37], denitrifying bioreactors [28], activated sludge systems fed with nonionic surfactants [24], and MBR previously acclimated to laboratory conditions [11]. Yet, bacterial communities of replicate reactors did not evolve identically over time, and it is possible that the trajectories of the replicate reactors would have continued to diverge further if the experiments had been run in excess of ten SRTs. The increase in variance in DGGE profiles in time was apparent from the decrease in the mean and the increase in the variance of the Bray–Curtis similarities of day 4 (0.83 ± 0.05), day22 (0.60 ± 0.06), and day 39 (0.54 ± 0.09).

This suggests a small role for ecological drift due to stochastic forces acting on the systems that overprint the deterministic signal, albeit in this experiment the signal

from deterministic selection is stronger than the influence of neutral dynamics. Others have observed that replicated communities developed differently in terms of species richness and community composition [7, 15, 23, 35]. As originally suggested [21], divergent trajectories of replicated communities can also be due to chaotic behavior of bacterial communities. Chaos, which results from a deterministic process, was already demonstrated during biological nitrification [17].

In conclusion, we have shown that active communities display significant changes in their bacterial constituents during the development of the activated sludge floc and that those changes are subjected to strong deterministic constraints. Further work on these ecologically relevant mixed communities can help to advance the search of the mechanisms that underlie community assembly.

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