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Balance of Neutral and Deterministic Components in the Dynamics of Activated Sludge Floc Assembly

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Abstract Understanding the processes that generate patterns of community structure is a central focus of ecological research. With that aim, we manipulated the structure of bacterial activated sludge to test the influence of the species richness and composition of bacterial communities on the dynamics of activated sludge floc assembly in lab-scale bioreactors. Bacterial community structure was analyzed using denaturing gradient gel electrophoresis of RT-PCR amplified 16S rRNA. Fingerprinting of four parallel reactors, started with the same source communities added in different proportions, converged to patterns that were more similar than expected by chance, suggesting a deterministic selection in floc development. Evidence for neutral dynamics was suggested by the dependence of the rate of replacement of species (bacterial taxa–time relationships) on the number of available species in the source community. Further indication of stochastic dynamics was obtained by the application of the Sloan neutral model for prokaryotes. The fitting of the observed data to the model predictions revealed that the importance of the stochastic component increased with the size of the reservoir of species richness from which the community is drawn. Taken together, the results illustrate how both neutral and deterministic dynamics operate simultaneously in the assembly of the bacterial floc and show that the balance of the two depends on the richness of the source community.

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Introduction

Detecting patterns in community assembly is required for gaining insight into the processes driving the community structure [23, 31, 41]. Ecosystems involved in biological wastewater treatment are particularly appealing targets for studies of community assembly, inasmuch as the success of the treatment relies on the multiple microbial activities and interactions occurring in the context of highly diverse microbial communities [13, 55]. Yet the communities are not deliberately assembled from individual species with known functions but remain the result of selection [10, 27]. It is generally recognized that the underlying biological activity of wastewater treatment may be deterministic [11]. Accordingly, results from several laboratories reveal that certain environmental conditions exert a distinctive selective pressure, indicating an important contribution of niche-oriented components to the assembly dynamics of bacterial communities [2, 9, 14, 15, 24, 32–35, 48, 57].

Despite these considerations, for many biological wastewater treatment systems stable performance does not necessarily imply a matching stability in the structure of the microbial community. The finding that replicated communities undergo erratic changes in time [7, 16, 18, 19, 26], and the fact that different communities develop under similar habitat conditions [4, 29, 45] have stimulated the development of stochastic approaches in microbial ecology. Stochastic community models provide a theory for biodiversity, assuming neutral dynamics and a progression of continuous cycles of immigration, births and death [5, 25]. Neutral models are based on the concept of the metacommunity, which comprises a pool of individuals from which local communities are assembled at random. Sloan, Curtis and coworkers have proposed a stochastic neutral community model to describe the patterns of

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microbial communities at the scale at which they are typically observed [49, 58]. According to this model, the bacterial composition of a local community is influenced by (1) the total diversity and the species abundance distribution in the metacommunity, (2) the probability of immigration, and (3) the spatial structure of the community [12, 39, 47]. Consequently, it is expected that smaller communities will exhibit less diversity as well as greater reproducibility between replicates [12, 39, 47].

It is currently understood that ecological communities are subject to both niche-oriented and stochastic dynamics [1, 8, 20, 38]. In a recent study on the dynamics of activated sludge assembly, we observed that a deterministic selection pressure was superseded by an increasing variation with time, indicative of some underlying neutral dynamics [3]. In the current study, we have manipulated the size and composition of bacterial communities to detect shifts in the balance between stochastic and deterministic components during the assembly dynamics of activated sludge floc. We hypothesized that niche-oriented assembly of activated sludge is sufficiently strong to cause bacterial communities containing approximately the same number of available species but differing in their relative abundance, to converge in composition in time. Additionally, we tested the hypothesis that communities containing a larger reservoir of species (i.e., larger metacommunities) display a higher stochastic component.

Materials and Methods

Seed Preparation and Reactors Set-up

Sludge samples were collected from the aeration basin of three industrial and one domestic full-scale wastewater treatment plants (WWTP) in the Province of Buenos Aires, Argentina (see Electronic Supplementary Table S1 for operational parameters).

Three experiments, referred to as experiments A, B, and C throughout the text, were designed (1) to test whether bacterial communities containing approximately the same number of available species but differing in their relative abundance converged in composition in time; (2) to compare the fitting to the neutral model according to the richness of the bacterial communities. In experiment A, each of the four laboratory-scale reactors contained 70% (in terms of mixed liquor suspended solids (MLSS)) of one of the four full-scale activated sludge systems (Electronic Supplementary Table S1) and 10% of sludge from the remaining three wastewater treatment plants (Table 1). The actual richness of the resultant mixtures of activated sludge cannot be measured. However, we assume that adding sludge from three WWTPs to a single WWTP will increase richness, as long as the species pool of each reactor is not entirely the same. This assumption is supported by recent high throughput studies, which showed that only a limited number of operational taxonomic units (OTUs) are shared among anaerobic sludge digesters [44], and the intestine of several individuals [53]. In experiment B, four laboratory-scale reactors were replicates of a mixture of sludge from the four full-scale wastewater plants, added in equal proportions (Table 1).

For the second objective, data from experiments A and B were compared with the data from [3], referred to as experiment C (Table 1), where sludge from a single full-scale activated sludge had been used as inoculum (domestic WWTP in Electronic Supplementary Table S1).

Activated sludge flocs disruption and enrichment of the biomass in planktonic populations were performed as

Table 1 Origin and proportion of sludge in the mixtures used to start-up experiments A, B, and C

Experiment	Reactor	Proportion (%)			
		Domestic	Pet food processing	Textile dye and finishing	Petroleum refinery
A	A1	70	10	10	10
	A2	10	70	10	10
	A3	10	10	70	10
	A4	10	10	10	70
B	B1	25	25	25	25
	B2	25	25	25	25
	B3	25	25	25	25
	B4	25	25	25	25
C	C1	100	0	0	0
	C2	100	0	0	0
	C3	100	0	0	0
	C4	100	0	0	0

Each experiment comprised four laboratory-scale reactors, containing a mixture of sludge obtained from the aeration basins of full-scale wastewater plants, added in the proportion indicated in the table

described in [3]. The biomass suspensions (800 mL) were diluted fourfold with synthetic sewage and used to seed four replicates of laboratory-scale bioreactors. All reactors were fed with sterile synthetic medium. The composition of the synthetic sewage used was K_2HPO_4, 28 mgl^{-1}; $MgSO_4$, 2 mgl^{-1}; NaCl, 7 mgl^{-1}; $CaCl_2.2H_2O$, 4 mgl^{-1}; peptone, 160 mgl^{-1}; yeast extract, 110 mgl^{-1}; urea 30 mgl^{-1}, pH= 7.3 [3]. Synthetic sewage was used to avoid community shifts due to changes in feed composition.

The reactors (800-mL of working volume) were placed inside a temperature-controlled cabinet at a constant temperature of 20±1°C, and operated for 40 days in a sequencing batch reactor. After aeration was disconnected for 30 min for settling, 150 mL of clarified supernatant was decanted manually. Next, aeration was resumed, and each reactor was fed separately with 350 mL of sterilized synthetic sewage with six pulses of 30 min, at a flow rate of 2 mL/min. Feeding pulses were separated by intervals of 3 h. The solid retention time (SRT) was set at 4 days by wasting 200 mL of sludge at the end of the feeding plus aeration phase. Sampling was carried out at this time. Sludge samples were centrifuged and the pellets were frozen in liquid nitrogen and stored at −80°C until analysis.

RNA Extraction, RT-PCR Assay of 16S rRNA and DGGE

The procedure for RNA extraction from fresh sludge samples, and the conditions for reverse transcription and denaturing gradient gel electrophoresis (DGGE) were given in [3].

Fingerprinting Analysis

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

Statistically significant similarities at the 95% confidence level in species composition between pairs of DGGE lanes were measured using the probabilistic Raup–Crick index (S_{RC}) for absence-presence data [42, 47]. S_{RC}, which gives the probability that two samples share fewer species than expected under a null model, was calculated using the PAST program (Palaeontological statistics, version 1.85, http://folk.uio.no/ohammer/past).

Species–Time Relationship

In order to characterize the temporal variability of the bacterial communities in the bioreactors, the species–time relationship was used to describe how the species of bioreactor communities were eliminated and replaced along the time span T over which the communities were observed [46]. Species-time relationship (STR) was fitted to the power function:

$$S = cT^{w},$$

where S is the "cumulative" species richness and c is an empirically derived taxon- and location-specific constant. The taxa–time relationship exponent w was estimated from the slope of the linear regression line fitted to the log–log plots. STR has also been fit in the literature using a logarithmic functions such as $S=c+w \log T$ [56]. However, the power function is used more often than the logarithmic function to describe the species–time relationship of both microbial and macrobial communities, providing a simple measure of relative species turnover [43, 54, 56]. The influence of richness on the taxa turnover rate was determined by Student's t test.

Sloan Neutral Model for Prokaryotes

We applied the method developed by Sloan et al. [49, 50, 58] for the analysis of stochastically assembled communities. The model takes the observed taxa abundance data (p_i) and, employs the least-squares method to generate the best-fit distribution curve with associated values for the distribution parameters α and β [49]. These parameters are functions of p_i and N_Tm (where N_T is the total number of individuals in the local community and m is the immigration rate of the individuals from the source community into the local community). We have adapted the model to compare the DGGE data with the relationship generated by the model between the frequency at which taxa are detected in the samples and the relative abundance of individual taxa across the time course. [4]. Each band in the DGGE gel was assumed to be an OTU. Band relative abundance (p_i) was derived from the band intensity data. Band frequency was determined as the number of samples in which the band was found divided by the total number of samples. Model calculations were performed using *Mathematica* (Wolfram Research, Inc., IL, USA).

Results

Reactor Performance

The reactors were operated at a SRT of 4 days for a total period of ten SRT. The effluent chemical oxygen demand, turbidity, pH, and MLSS from the reactors in experiments A were measured periodically throughout the sampling period (Fig. 1). Despite the fact that the four bioreactors were seeded at the start-up with different proportions of sludge from four wastewater treatment plants, the effluent COD, turbidity, pH, and MLSS were similar throughout the

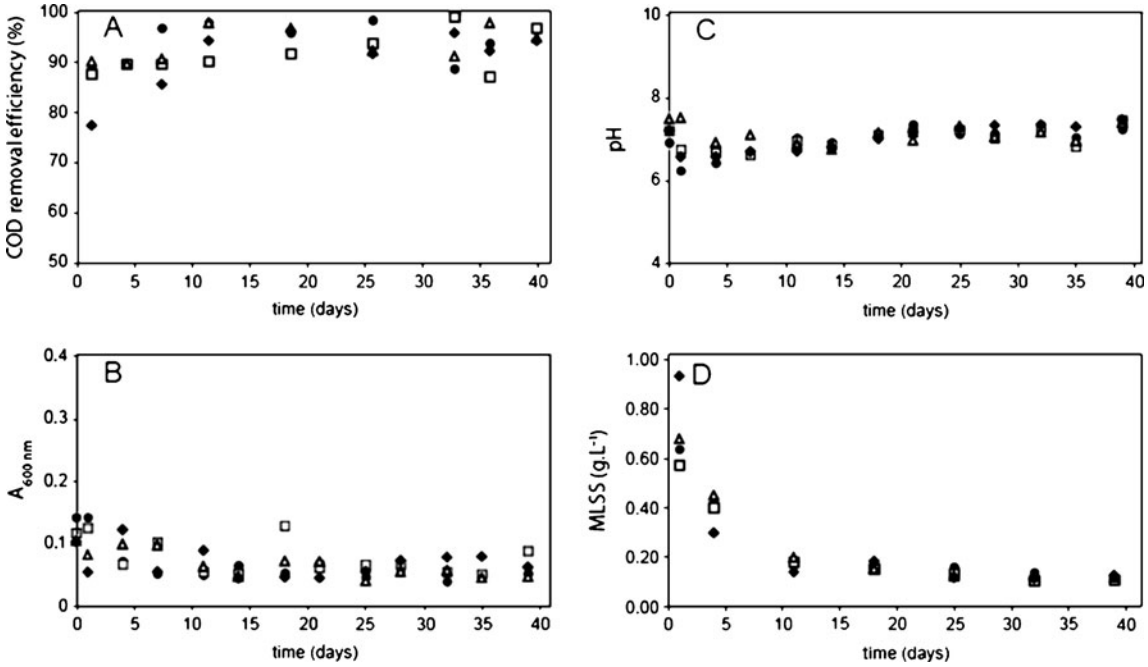

Figure 1 Variation in the effluent **a** chemical oxygen demand (*COD*), **b** turbidity, **c** pH and **d** mixed liquor suspended solids (*MLSS*) in the four bench-scale reactors, initially seeded with activated sludge taken from four full-scale wastewater treatment plants added in proportions 7:1:1:1 (experiment A). *Filled circles* A1, *open squares* A2, *open triangles* A3, *filled diamonds* A4

period studied in the four reactors. These results indicate that the bacterial communities were functionally stable. Similar results were obtained for the replicated bioreactors in experiment B. The deviations observed in COD removal amongst the identical replicates from experiment B (Electronic Supplementary Fig. S1) are of the same magnitude as those observed for the reactors in experiment A, suggesting that the temporal variations were random. Therefore, we assume that the differences in the composition of the inocula used to start-up the reactors in experiment A have not detectable effect on the reactors performance

Community Structure Dynamics

Denaturing gradient gel electrophoresis of polymerase chain reaction (PCR) amplified ribosomal cDNA was used to evaluate the community structure of the activated sludge reactors. By performing the analysis on the ribosomal cDNA, rather than on the ribosomal RNA gene, it was guaranteed that the interference from non-actively growing bacteria in the community was minimized.

Figure 2 shows the DGGE profiles and similarity analysis obtained for the two sets of four reactors, corresponding to experiments A and B, taken at days 0 (inoculum), 4, 18, and 39 of operation. Dendrograms generated using Dice similarity index showed that the four reactors in both experiments clustered together according to the time of sampling, rather than by the bioreactor identity (Fig. 2).

Reactors in experiment A were not identical replicates, but combination of four inocula mixed in different proportions (Table 1). Although it is expected that they contained initially approximately the same species richness and composition, because only bacterial taxa above the detection limit are observed, each reactor showed a different DGGE pattern at time 0 (Fig. 2a). The application of the Raup and Crick probability-based similarity index (S_{RC}) for the pair-wise comparison of the rRNA-based bacterial fingerprinting community profiles in bioreactors A showed that only two of six pair-wise comparisons of DGGE profiles between the four reactors were significantly similar (Table 2). Yet all the bacterial communities became significantly similar at day 18 (S_{RC}>0.95). At ten SRT (day 39), the communities in all reactors were still more similar than if they had been randomly assembled from the common source community. Replicated reactors B, which were initially identical, maintained significant similarity between replicates throughout the experiment (Table 2).

Bacterial Turnover Rates

To analyze the effect of the size of the source community on the bacterial turnover rate, the bacterial taxa–time relationships for the four bioreactors were examined using the power law equation $S = cT^w$, and displayed in a log–log space plot (Fig. 3). The calculated average exponent w of the experiment B (0.31±0.05) was significantly higher

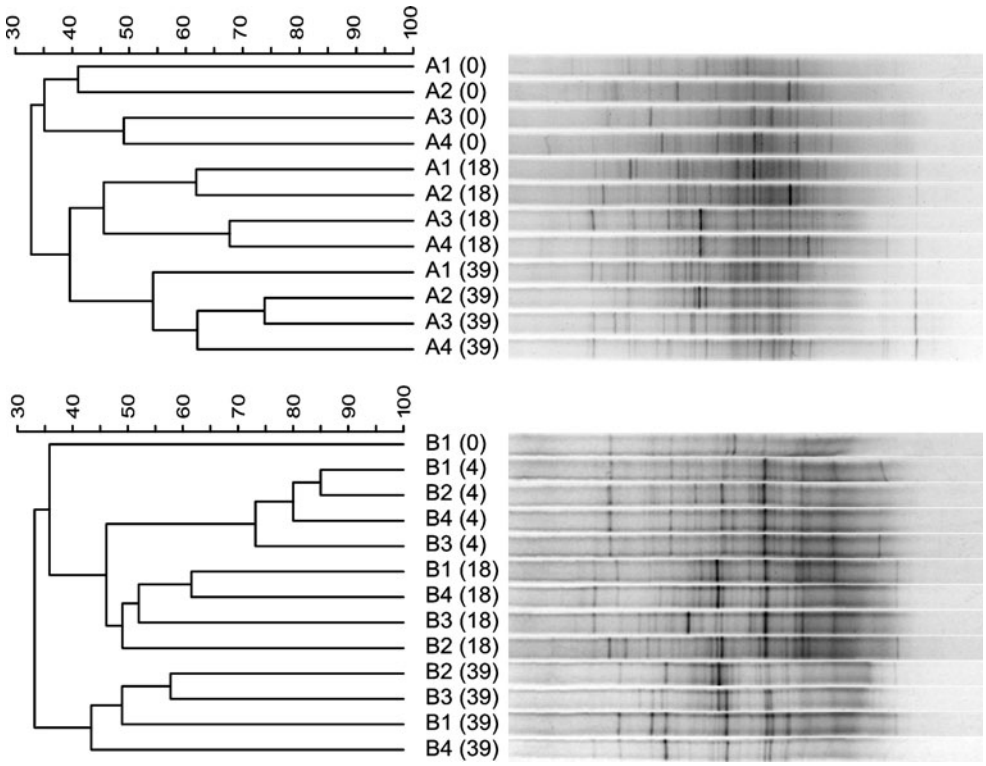

Figure 2 16S rRNA-based fingerprinting of samples from bench-scale activated reactors in experiments A and B. Sampling days are indicated between brackets. The dendrograms display the result of cluster analysis using Dice coefficient as a measure of similarity among samples. The scale represents percent similarity values

from the *w* determined for experiment C (0.16±0.02) ($P<$ 0.01, Student's *t* test).

As the four reactors in experiment A contained different combinations of the same four inocula, they could not be considered independent replicates. Therefore they were not included in the previous statistical analysis. Nevertheless, the average exponent *w* was calculated for the four reactors of experiment A, yielding a value of 0.32±0.05 (Fig. 4), which was remarkably close to the average value obtained for the reactors of experiment B.

Taken together, these results demonstrate that increasing the number of available species in the reservoir of species from which the community is drawn, produced a significant increase in cumulative taxa richness.

Table 2 Results of pair-wise comparison of the Raup and Crick similarity of bacterial communities of bench-scale activated sludge reactors against all other reactors in the same experimental set

		A1	A2	A3	A4	B1	B2	B3	B4
Day 0	A1		0.55	*0.95*[a]	0.56				
	A2			0.72	0.63				
	A3				*1.00*				
Day 18	A1		*1.00*	*0.99*	*0.94*				
	A2			*1.00*	*0.98*				
	A3				*1.00*				
Day 39	A1		*1.00*	*0.99*	*0.94*				
	A2			*1.00*	*0.95*				
	A3				*1.00*				
Day 4	B1						*1.00*	*1.00*	*1.00*
	B2							*1.00*	*1.00*
	B3								*1.00*
Day 18	B1						*1.00*	0.76	*0.99*
	B2							*1.00*	*1.00*
	B3								*0.99*
Day 39	B1						*1.00*	*0.98*	*1.00*
	B2							*1.00*	*0.99*
	B3								*1.00*

[a] Data set in italics indicate significant similarity

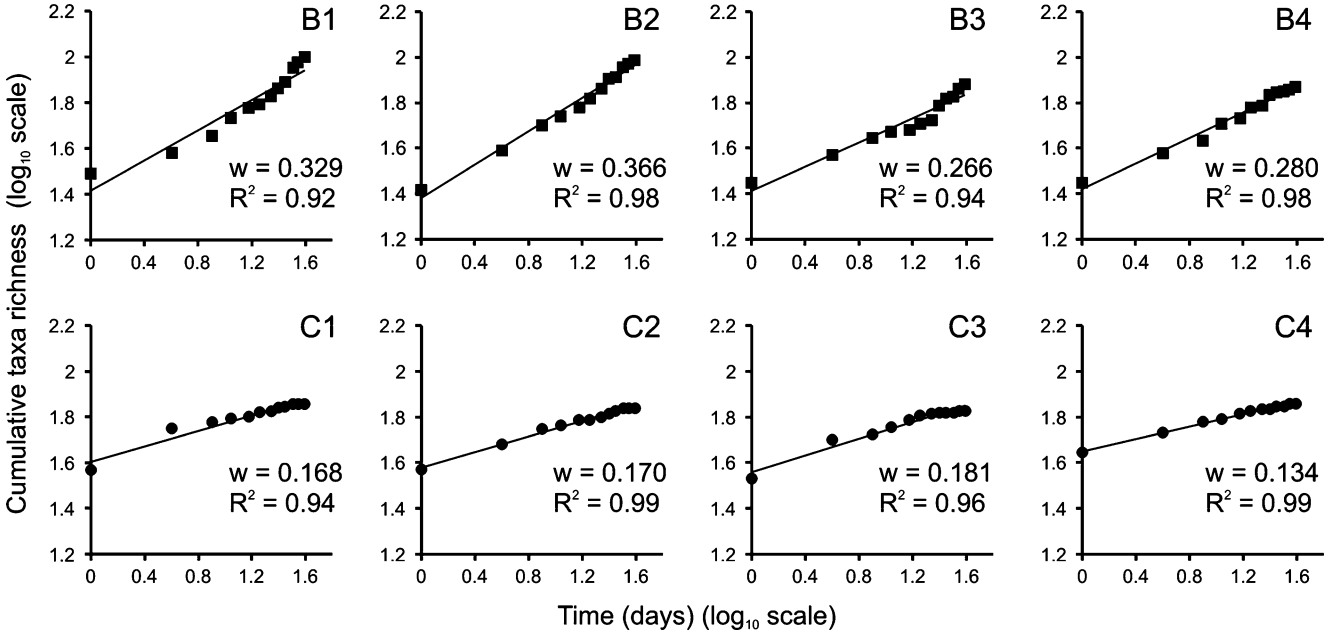

Figure 3 Bacterial taxa–time relationships for the two sets of four replicate reactors in experiments B and C (the latter data taken from [3]. The numbers inside the boxes indicate the taxa–time power law equation $(S = cT^w)$ and the coefficient of correlation (R^2) of the linear regression

Application of the Neutral Model for Prokaryotes

Further evidence for stochastic dynamics in activated sludge floc assembly was obtained by the application of the neutral model for prokaryotes, put forward by Sloan and coworkers [49]. The results are shown in Fig. 5. The relationship between frequency and relative abundance generated by the model of Sloan et al. is for a microbial community whose assembly has been driven by stochastic processes. The data represent the frequency at which individual bands were detected in the DGGE during the sampling period as a function of their mean relative abundance. The solid line is the best-fit (least square error) theoretical description of the data set for each bioreactor. The values of N_Tm for the three experiments were different but of the same order of magnitude. Because DGGE was preceded by PCR, the total number of individuals in DGGE sample (N_T) could not be determined accurately, and therefore attempts to estimate m were not undertaken. More importantly, it is clearly evident that the fitting of the observed data to the model predictions was considerably better for experiments A and B, supporting the hypothesis that the higher the number of species in the reservoir from which the community is drawn, the more important is the stochastic component in the formation of activated sludge floc communities.

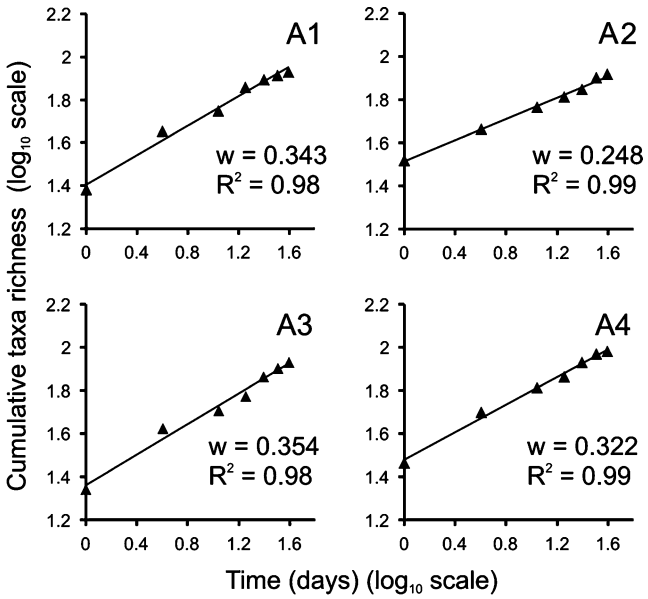

Figure 4 Bacterial taxa–time relationships for set of four reactors in experiment A. Details as in Fig. 3

Discussion

By manipulating the structure of activated sludge communities we have been able to detect, using three independent methods of analysis, a strong selection of bacterial assembly in the activated sludge floc overlaying a stochastic process.

The concept of metacommunity [30] has incorporated the interactions between local community members and immigrating individuals to address the influence of regional factors on the assembly of communities [1, 6, 17, 38]. A

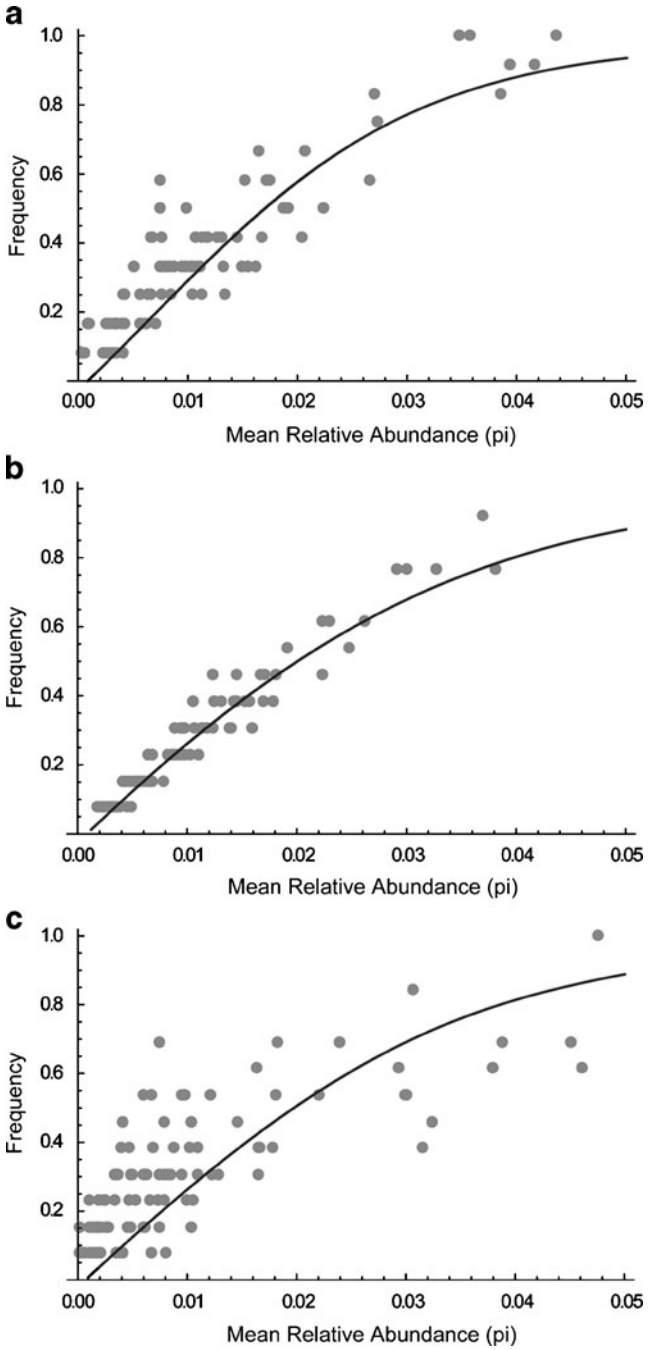

Figure 5 DGGE gel band frequency versus relative abundance for the bacterial populations in the set of reactors A, B, and C. Each graph included all observed data (*circles*) of the corresponding experiment throughout the investigated period. The predictions (*solid lines*) were performed using the model for the analysis of stochastically assembled prokaryotic communities developed by [49, 50, 58]

metacommunity has been defined as a set of local communities that are linked by dispersal of multiple potentially interacting species [30]. Although this definition implies some degree of isolation between the set of local communities, the concept of metacommunity still provides a convenient framework to get insight into the assembly dynamics of activated sludge flocs structure in manipulated bioreactors. It is known that stochastic processes, such as mutation, dispersal and extinction may contribute to the diversity patterns. The reactors are open systems with no physical barriers to species dispersal. While immigration from external sources was essentially prevented by the use of sterilized media, in this work each reactor was considered to act as a region supporting a metacommunity, supplying immigrants to the activated sludge flocs. In that context, we analyzed the dynamics by which emergent community structures were generated by individuals entering the system chiefly through immigration from the source community. Accordingly, the high dynamics detected through changes in DGGE patterns originated from the recruitment of bacterial species from the available pool of species in the reservoir. It follows that the relative effect of the deterministic and stochastic components depended on the richness in the source community.

DGGE band number was used in this work as a surrogate for bacterial richness [6, 54] of communities. DGGE is a very popular molecular fingerprinting method, yet suffers from several shortcomings, as do most techniques used to analyze complex biological ecosystems. For the most part, DGGE targets only the most abundant members of the community [51]. It can be biased by the sampling and recovery of DNA [21], by the differential selectivity of PCR [52], and by some artifacts that may occur during electrophoresis [22]. Therefore, it has been recommended that the parameters calculated from DGGE should not be taken as absolute measures of the degree of diversity in a bacterial community [57].

Nonetheless, although the number of DGGE bands in a sample may be biased, it can still serve as a practical way of monitoring the predominant members of a community for the testing of relevant hypothesis or models in microbial ecology [4, 6, 17, 26, 28, 37, 40, 45, 54, 57]. Rather than using DGGE as a proxy for species richness, in this work we sought to capture the dynamics of active bacterial communities emerging from the reservoir communities in the reactors, by focusing on the of changes of RT-PCR amplified rRNA profiles.

Communities were assembled under the assumption that the mixture of sludge from four wastewater treatment plants, as was performed in reactors A and B, possess higher richness that any of the individual sludge from which the mixture derive. Although it cannot be tested directly, all present evidence supports this assumption, including recent high throughput studies of other complex microbial systems, such as anaerobic sludge digesters [44], and the human intestine [53].

The results of the Dice analysis of DGGE patterns revealed that reactors clustered together according to sampling date rather than reactor identity. Reproducible

patterns in replicated samples within highly variable microbial communities have been reported previously for several SBR systems and taken as evidence of deterministic operating factors [3, 33, 57]. A more conclusive indication of deterministic behavior was obtained using the Raup–Crick similarity index, a probabilistic measure which distinguishes similarities in band matching between samples taken pair-wise appearing at a greater or lesser level than expected by chance [3, 4, 42, 47].

It might be argued that 40 days, corresponding to ten solid retention times, was too short a time to detect a large degree of divergence in the communities present in replicate reactors. However, this should not be a matter of concern for experiment A, in which each reactor was seeded in different proportions with the same source communities. Based on the fact that only the most dominant bacterial community members were detected by DGGE [36], the initial patterns of all four bioreactors were not significantly similar. Notably, selection was unmistakably observed through convergence of bacterial patterns after several days of reactor operation. Yet we cannot be certain which environmental factors, other that the selection of floc forming bacteria [3], drive the niche differentiation leading to the observed composition of the most abundant member of the community.

We have applied the STR to relate the temporal variability of the bacterial communities with the stochastic character of floc assembly. STR is an extension of the species–area power law that can be considered a measure of temporal turnover [46]. It was observed that the clustering of species–time relationship exponents within and between taxa demonstrated the ubiquity of the relationship and suggested that the STR was a fundamental ecological pattern [56]. After van der Gast and colleagues incorporated the species–time relationship for bacterial communities [54], Redford et al. have subsequently reported that it was similar, albeit on a shorter time scale, to that observed in plant and invertebrate communities [43].

It has been suggested that in many systems external factors influence richness and turnover in opposite directions [56]. Because only the dominant members of the community are detected in PCR-DGGE of RT amplified rRNA, the increase in cumulative species richness observed in the DGGE experiments originated from the emergence of species in the available pool in the reactors that became active during the time course of the study, and not to the increased sampling from a static pool of species. In other words, external factors may have influenced the turnover rate, but it is not expected that they would have influenced richness.

The steeper slopes of STRs indicate more rapid taxa turnover in time for reactors containing higher number of available species (reactors A and B). Analogous findings were reported by van der Gast and colleagues, who examined the STR relationship upon testing bioreactors of fixed volume size under increasing selective pressure exerted by a gradient of increasing industrial wastewater concentrations and decreasing municipal wastewater [54]. They observed that the taxa turnover of the bacterial STR decreased as selective pressure (industrial wastewater concentration) increased, associating lower slopes with an increase in niche-related selection within bacterial communities. The values of the slopes calculated in this work were within the range of the slopes found in the bioreactor bacterial communities shifting from deterministic to more stochastic community pattern [54].

To test the hypothesis that stochastic processes were more dominant as the number of available species increased, we analyzed the data using the near-neutral model for prokaryotes developed by Sloan and coworkers [49, 58]. Species departing from the best-fit neutral model in reactors C were mostly to the left of the theoretical predicted abundance curve, meaning a higher frequency than their abundance would dictate or a lower abundance than that suggested by their high frequency, for which they could be assigned a positive advantage parameter (α_i in the definition of [49, 58]). As expected from the existence of higher functional redundancy for the assembly of bacterial communities, the fits to the neutral model improved greatly for the systems containing higher number of species.

In summary, taken together our results illustrate that both neutral and deterministic dynamics operate together and that the balance of the two depends on the available number of species in the source community. We envision that the analysis of bacterial communities in laboratory-controlled environments could offer a convenient means to explore other factors that drive community assembly.

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