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Use of 16S rRNA Gene Terminal Restriction Fragment Analysis To Assess the Impact of Solids Retention Time on the Bacterial Diversity of Activated Sludge

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Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes was used to investigate the reproducibility and stability in the bacterial community structure of laboratory-scale sequencing batch bioreactors (SBR) and to assess the impact of solids retention time (SRT) on bacterial diversity. Two experiments were performed. In each experiment two sets of replicate SBRs were operated for a periods of three times the SRT. One set was operated at an SRT of 2 days and another set was operated at an SRT of 8 days. Samples for T-RFLP analysis were collected from the two sets of replicate reactors. HhaI, MspI, and RsaI T-RFLP profiles were analyzed using cluster analysis and diversity statistics. Cluster analysis with Ward's method using Jaccard distance and Hellinger distance showed that the bacterial community structure in both sets of reactors from both experimental runs was dynamic and that replicate reactors were clustered together and evolved similarly from startup. Richness (*S*), evenness (*E*), the Shannon-Weaver index (*H*), and the reciprocal of Simpson's index (1/*D*) were calculated, and the values were compared between the two sets of reactors. Evenness values were higher for reactors operated at an SRT of 2 days. Statistically significant differences in diversity (*H* and *D*) between the two sets of reactors were tested using a randomization procedure, and the results showed that reactors from both experimental runs that were operated at an SRT of 2 days had higher diversity (*H* and *D*) at the 5% level. T-RFLP analysis with diversity indices proved to be a powerful tool to analyze changes in the bacterial community diversity in response to changes in the operational parameters of activated-sludge systems.

Studies of biodiversity (species richness and evenness) are important for macro- and microecology due to its potential correlation to ecosystem function (27, 33, 39). Increasingly, microbial biodiversity has become a research theme for understanding engineered ecosystems such as bioreactors. Several studies have reported the importance of measuring diversity in bioreactors to test the efficacy of bioaugmentation (12), to assess site and temporal variations in microbial diversity (5, 7, 14, 38), to assess microbial diversity in laboratory bioreactors (3, 15, 24), to provide insight on the correlation between diversity and operational parameters of treatment plants (4), and to link biodiversity to bioreactor stability (41).

Activated-sludge sewage treatment systems are engineered bioreactors used to remove organic substances and nutrients (nitrogen and phosphorous) from municipal wastewater. A consortium of bacterial species is required to achieve the desired biological conversions, and the performance of these reactors largely depends on the bacterial diversity present. The vast majority of bacteria present in activated sludge cannot be isolated using traditional culture-dependent techniques (1). However, with the advent of small-subunit rRNA-based molecular fingerprinting techniques, including ribosomal DNA restriction analysis (36), denaturing gradient gel electrophoresis (28), thermal gradient gel electrophoresis (12), length heterogeneity PCR (31), automated ribosomal intergenic spacer analysis (13), and terminal restriction fragment length polymorphism (T-RFLP) (25), it became possible for environmental engineers and scientists to assess bacterial diversity in activated-sludge systems.

Most of these methods use PCR to amplify small-subunit rRNA genes, in particular the 16S rRNA gene, and then PCR amplicons are separated based on differences in DNA sequences of the 16S rRNA genes. Although sequence analysis of 16S rRNA gene clone libraries provides the most detailed information on the microbial community structure, this method is time-consuming and costly, especially when many samples are to be analyzed. T-RFLP is popular for its rapid production and analysis of data, and it has been shown to be an effective method for discriminating microbial communities in a wide range of environmental samples (11, 21). T-RFLP provides several advantages over other fingerprinting methods, as it is highly reproducible (30) and has greater resolution and sensitivity than denaturing gradient gel electrophoresis (25, 26).

In T-RFLP, one of the primers, usually the forward primer, used for PCR has a fluorescent molecule attached to it. The PCR amplicons, which are of equal size, are then subjected to enzymatic digestion with restriction endonucleases. The digested fragments are then separated by polyacrylamide gel or capillary gel electrophoresis and visualized by an automated DNA sequencer, which can only detect the fluorescently

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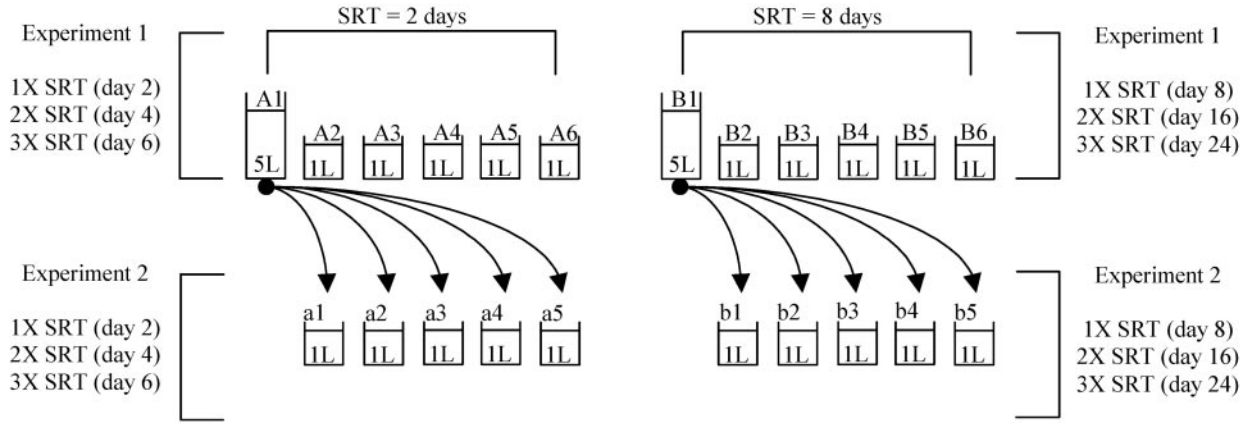

FIG. 1. Schematic of reactor setup.

labeled fragments or terminal restriction fragments (T-RFs). The unique T-RFs or operational taxonomic units is used as the measured unit of diversity in a community. Given the widespread use of T-RFLP, it is anticipated that this method will be applied more frequently for community analysis of activated sludge.

Despite the importance of activated-sludge systems, knowledge of the correlation of operational parameters or plant configuration with the degree of bacterial diversity is scarce. Most of the experimental studies using molecular fingerprinting of bacterial community structure in activated-sludge system have focused on studying the spatial and temporal changes in microbial diversity (4, 7, 14, 20), assessing microbial diversity in activated sludge (3), studying the impact of certain toxins on microbial diversity (5), and studying the efficacy of bioaugmentation (12). Recently, Saikaly and Oerther (32) developed an ecology-based mechanistic model predicting the impact of bioreactor operating conditions on the diversity of bacterial species in activated-sludge system. The study reported a systematic examination of the usefulness of varying the solids retention time (SRT) to enhance the biodiversity of the bacterial community. The model results suggested that bioreactors operated at an intermediate SRT (2.28 to 5.66 days) contained a greater number of different bacteria than bioreactors operated at an SRT of >5.66 days.

The main objective of the current study was to use T-RFLP to investigate the reproducibility and stability of the bacterial community structure in a laboratory-scale activated-sludge system and to experimentally test model predictions that SRT impacts bacterial diversity.

MATERIALS AND METHODS

Laboratory-scale bioreactors. Two experiments, referred to as experiments 1 and 2 throughout the text, were designed to (i) investigate the reproducibility and stability in the bacterial community structure of replicate laboratory-scale activated-sludge sequencing batch reactor operated under steady environmental conditions and seeded with the same inocula and (ii) assess the impact of SRT on bacterial diversity in activated-sludge systems. In experiment 1, two sets of six laboratory-scale activated-sludge sequencing batch bioreactors were operated for a period of three SRTs (Fig. 1). The inocula for each bioreactor were obtained from the municipal Mill Creek activated-sludge wastewater treatment plant in Cincinnati, OH. Six bioreactors were operated with an SRT of 2 days (reactors A1 to A6), and six bioreactors were operated with an SRT of 8 days (reactors B1 to B6). Reactors A1 and B1 had working volumes of 5 liters, while reactors A2 through A6 and B2 through B6 had working volumes of 1 liter each. After three SRTs, the contents of reactors A2 to A6 and B2 to B6 were emptied.

In experiment 2, two sets of five laboratory-scale activated-sludge sequencing batch bioreactors were operated for a period of three SRTs. Five bioreactors were operated with an SRT of 2 days (reactors a2 to a6), and five bioreactors were operated with an SRT of 8 days (reactors b2 to b6) (Fig. 1). Reactors a2 through a6 and b2 through b6 had working volumes of 1 liter each. The inocula for reactors a2 through a6 were obtained from reactor A1 (three times the SRT), and the inocula for reactors b2 through b6 were obtained from reactor B1 (three times the SRT). All reactors (experiments 1 and 2) were operated with a hydraulic retention time of 12 h with three cycles per day. Each cycle consisted of a fill phase of 10 min, a reaction phase of 7 h, and a settling and decanting phase of 1 h. The bioreactors treated a synthetic wastewater containing (per liter of water) 512 mg $NaCH_3COO$, 166 mg $NaHCO_3$, 107 mg NH_4Cl, 75.5 mg $NaH_2PO_4 \cdot 2H_2O$, 90 mg $MgSO_4 \cdot 7H_2O$, 36 mg KCl, 14 mg $CaCl_2 \cdot 2H_2O$, 18 mg EDTA, 1 mg yeast extract, 1.5 mg $FeSO_4 \cdot 7H_2O$, 0.0015 mg H_3BO_3, 0.48 mg $CuSO_4 \cdot 5H_2O$, 0.003 mg KI, 1.5 mg $MnCl_2 \cdot 4H_2O$, 0.33 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.66 mg $ZnSO_4 \cdot 7H_2O$, and 0.015 mg $CoCl_2 \cdot 6H_2O$.

Analytical methods. Grab samples of mixed liquor were collected after each SRT. Performance measures included determination of total and soluble chemical oxygen demand, nitrite-nitrogen, nitrate-nitrogen, and orthophosphate using Hach Test 'N Tube reagents (catalog numbers 2415815, 26083-45, 26053-45, and 21060-46, respectively). An ion-specific electrode was used to measure ammonia-nitrogen and pH. The levels of mixed liquor suspended solids and volatile suspended solids were determined according to Standard Methods for the Examination of Water and Wastewater (2). Sludge settling was measured using a modified 30-minute sludge volume index where the standard 1-liter graduated cylinder was replaced with a 100-ml graduated cylinder and quiescent settling was allowed to occur for 30 min.

DNA extraction and PCR conditions. For bacterial community analysis, samples of mixed liquor from each bioreactor were collected after each SRT in 2-ml centrifuge tubes and centrifuged at 10,000 × *g* for 10 min, the supernatant was decanted, and the samples were stored in −80°C for later analysis by T-RFLP. Genomic DNA was extracted from each sample of mixed liquor using the Ultraclean soil DNA extraction kit (Mo Bio Laboratories, Inc.) according to the manufacturer's instructions. The genomic DNA isolated was used as template material for the PCR.

PCR was performed in 50-μl reaction volume using a reaction mixture of 1X PCR buffer, 200 μM each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.025U of *Taq* DNA polymerase/μl (QIAGEN), and 0.3 μM of each primer. The primers used were specific for conserved bacterial 16S rDNA sequences, 8-27f (AGAGTTTGATCCTGGCTCAG) and 906-926r (CCGTCAATTCCTTTR AGTTT) (24) (manufactured by the University of Cincinnati DNA Core laboratory). The forward primer was labeled at the 5′ end with 6-carboxyfluorescein.

Optimization of PCR was done by adjusting the volume of DNA (0.8 to 2 μl) for each sample used in the PCR to obtain a single strong band of equal concentration of DNA on an agarose gel. This method was shown to be more efficient than quantification of DNA using a spectrophotometer. Amplification of DNA was performed in a GeneAmp PCR system 2700 (Perkin Elmer) by using the following program: an initial denaturing step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 65°C for 1 min, extension

at 72°C for 1.5 min, and final extension at 72°C for 10 min. PCR tubes were placed in the thermocycler when the block temperature reached 94°C. Three replicate PCRs were performed for each sample and the products were pooled and verified visually (5 μl) using 1% agarose gel electrophoresis in 1X Tris-borate-EDTA and SYBR Green I staining (Molecular Probes).

T-RFLP. Amplicons (145 μl) were purified using Wizard PCR Preps DNA purification system (Promega, Madison, Wis.) as directed by the supplier, and eluted with 50 μl sterile water. Purified PCR products (approximately 200 ng) were digested separately with 5 U of tetrameric restriction endonucleases HhaI, MspI, and RsaI (Promega, Madison, Wis.) in a 20-μl reaction volume. Restriction digests were incubated at 37°C for 4 h. Aliquots (8 μl) of restriction digests were examined by 2.5% agarose gel electrophoresis using SYBR Green I staining. To analyze the terminal restriction fragments (T-RF), 1 μl of digested samples was mixed with 1 μl of formamide (contains loading buffer and DNA fragment length standard [Rox 2500, ABI]). The mixture was denatured at 94°C for 5 min and snap-cooled on ice before electrophoresis on 7% polyacrylamide gel for 10 h at 2,250 V on an ABI 377 automated DNA sequencer (Applied Biosystems Instruments). T-RFLP profiles were analyzed using Genescan software (version 3.7, Applied Biosystems).

Analysis of T-RFLP profiles from activated-sludge bioreactors. T-RFLP profiles were analyzed as follows. First, only profiles with a cumulative peak height ≥5,000 fluorescence units were used in the analysis. Second, peaks with peak height <50 fluorescent units were excluded from the analysis. Third, profiles from different environmental samples were manually aligned by visual inspection of the size of peaks in bases. Fourth, T-RFLP profiles were standardized based on peak height to account for variations in DNA loading between samples using the procedure suggested by Dunbar et al. (11). Simply, total fluorescent units in each profile was calculated after excluding peaks with peak height <50 fluorescent units. T-RF profiles were then compared and standardized to the profile with the smallest total fluorescent units. The range of total fluorescent unit in the collection of samples was between 5,097 and 7,471 fluorescent units. This procedure was repeated until the cumulative peak height in all the samples was the same. After standardization, T-RFLP profiles were normalized so that the cumulative peak height in each profile was 10,000 fluorescent units. This allowed for comparison of profiles based on relative peak heights (peak height divided by the cumulative peak height for a profile). Normalized data were then subjected to statistical analysis.

Two distance metrics were used to analyze T-RFLP profiles based on presence/absence of T-RFs and their relative abundance. These include the Jaccard distance (1 − Jaccard coefficient) and the Hellinger distance. The Jaccard coefficient considers the presence/absence of T-RFs and is equal to the ratio of the number of T-RFs in common between two profiles to the total number of T-RFs present in both profiles. Hellinger distance is equal to the Euclidean distance after taking the square root of the relative peak heights (23). The agglomerative hierarchical clustering Ward (43) was applied to obtain a dendrograms for each distance metrics using the Community Analysis Package software 3.0 (Pisces Conservation Ltd.).

To evaluate structural diversity between samples, the Shannon-Weaver diversity index (H), richness (S), evenness (E), and the reciprocal of Simson's index ($1/D$) were used. The Shannon-Weaver diversity index (34) was calculated as follows: $H = -\Sigma(p_i)\ (\log_2 p_i)$, where the summation is over all unique fragments i and p_i is the relative abundance of fragment i. The abundance of a particular fragment can be determined by using the peak height intensity in fluorescent units. Evenness was measured as follows: $E = H/(\log_2[S])$. The reciprocal of Simpson's index of diversity ($1/D$) was calculated as follows: $1/D = 1/(\Sigma p_i^2)$ (35). Richness (S) was defined as the number of unique T-RFs or operational taxonomic units in a profile. Statistically significant difference in H and D among the two sets of reactors was tested by a randomization procedure as described by Solow (37) using the Species Diversity and Richness software 3.0 (Pisces Conservation Ltd.).

RESULTS AND DISCUSSION

Reactor performance. The effluent chemical oxygen demand from the reactors in experiments 1 and 2 was measured after each SRT. The chemical oxygen demand removed was between 90.02 and 96.55% for reactors that were operated at an SRT of 8 days and between 91.60 and 97.12% for reactors that were operated at an SRT of 2 days. Variability in the effluent chemical oxygen demand after each SRT for the two sets of reactors (SRT of 2 days and 8 days) was minimal in both experiments. This suggests that the bacterial community was functionally stable and has acclimated to the new operational changes (adapting domestic sludge to synthetic wastewater [experiment 1], and reactor subsampling after the sludge had acclimated to synthetic wastewater [experiment 2]).

Reproducibility and stability of the bacterial community of activated sludge. To investigate the reproducibility and stability in the bacterial community structure of replicate sequencing batch bioreactors operated under steady environmental conditions and seeded with the same inocula (replicate reactors in experiment 1 were seeded with sludge from a municipal activated-sludge wastewater treatment plant, while replicate reactors in experiment 2 were seeded with sludge from a laboratory-scale activated-sludge sequencing batch bioreactor that was acclimated to synthetic wastewater [see Fig. 1]), samples of activated sludge from experiments 1 and 2 were collected from each reactor at start-up and after each SRT and analyzed with T-RFLP. T-RFLP profiles from three separate restriction digests, HhaI, MspI, and RsaI, were processed using two distance metrics, the Jaccard distance and the Hellinger distance. A dendrogram was constructed from these distance metrics using Ward's method.

Figure 2 presents the dendrogram constructed for HhaI digestion. The dendrogram clearly shows that samples from replicate reactors were clustered together and evolved similarly from start-up in both experiments. This reproducibility in the bacterial community structure was also shown using the average similarity and dissimilarity (distance) between samples digested with HhaI from replicate sequencing batch bioreactors after each SRT. The average similarity between replicate reactors ranged between 72 and 90% for SRT of 8 days and between 75 and 87% for SRT of 2 days when using the Jaccard coefficient, but when comparison was done using the Hellinger distance, replicate reactors were less similar (data not shown).

Several studies showed that despite the fact that replicate laboratory-scale reactors were operated under identical conditions, the bacterial community structure was not reproducible and might diverge over time (5, 14, 20). In the current study, we could not tell whether the bacterial community structure of replicate sequencing batch bioreactors would continue to evolve similarly or diverge with time because both experiments were terminated after three SRTs. A possible explanation for the difference in the bacterial community structure of replicate laboratory-scale reactors was offered by Kaewpipat and Grady (20) and Curtis et al. (9). Kaewpipat and Grady (20) speculated that the structure of the bacterial community structure might display characteristics of chaotic systems and thus might respond differently to perturbations, like exposing the bacterial community of laboratory-scale activated-sludge reactors to new operating conditions (feed composition, SRT, hydraulic retention time, etc.) that are different from that present in the actual wastewater treatment plants. The authors also suggest adapting the biomass to the new operational conditions before running replicate reactors. Curtis et al. (9) also attributed the difference in the bacterial community structure of replicate reactors to the presence of chaotic dynamics in the bacterial community and that these dynamics in the bacterial community are present in small-scale biological treatment plants but are not seen in large-scale biological treatment plants. This difference between laboratory-scale reactors and full-scale biologi-

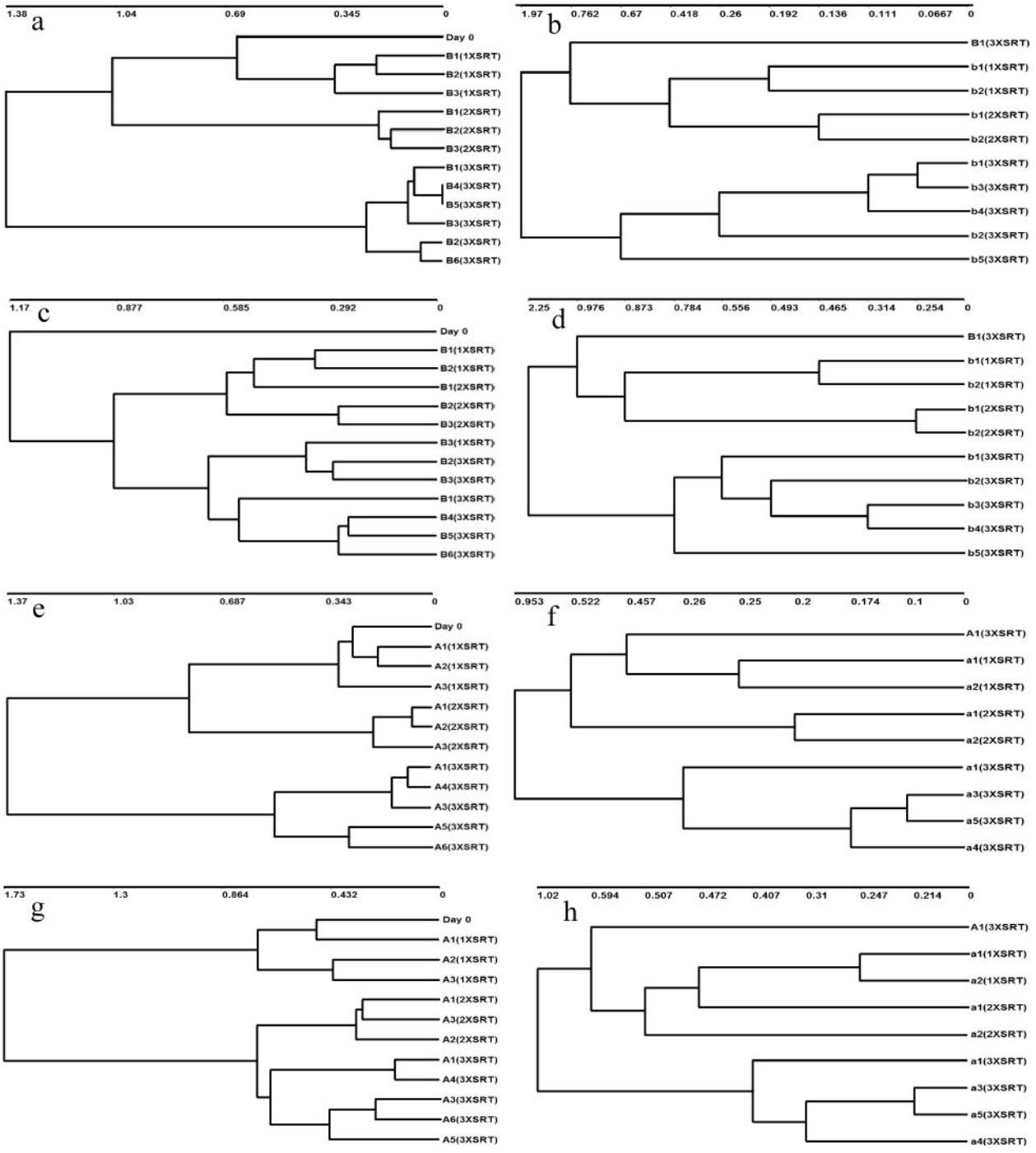

FIG. 2. (a to d) Dendrogram constructed with Ward's method for samples collected from replicate reactors operated at an SRT of 8 days using (a) Jaccard distance (experiment 1); (b) Jaccard distance (experiment 2); (c) Hellinger distance (experiment 1); and (d) Hellinger distance (experiment 2). (e to h) Dendrogram constructed for samples collected from replicate reactors operated at an SRT of 2 days using (e) Jaccard distance (experiment 1); (f) Jaccard distance (experiment 2); (g) Hellinger distance (experiment 1); and (h) Hellinger distance (experiment 2).

cal treatment plants could be explained by the equilibrium model of island biogeography, which predicts that larger islands, such as full-scale wastewater treatment plants, have higher biodiversity and stabler community structure (8).

Another significant finding that can be concluded from the dendrogram in Fig. 2 is that at each sampling event the bacterial community structure was more closely related to the previous sampling event but more distinct from the seeding sludge. This suggests that the bacterial community structure was dynamic and constantly changing despite the fact that operating conditions and reactor performance as measured by chemical oxygen demand were constant. Several investigators

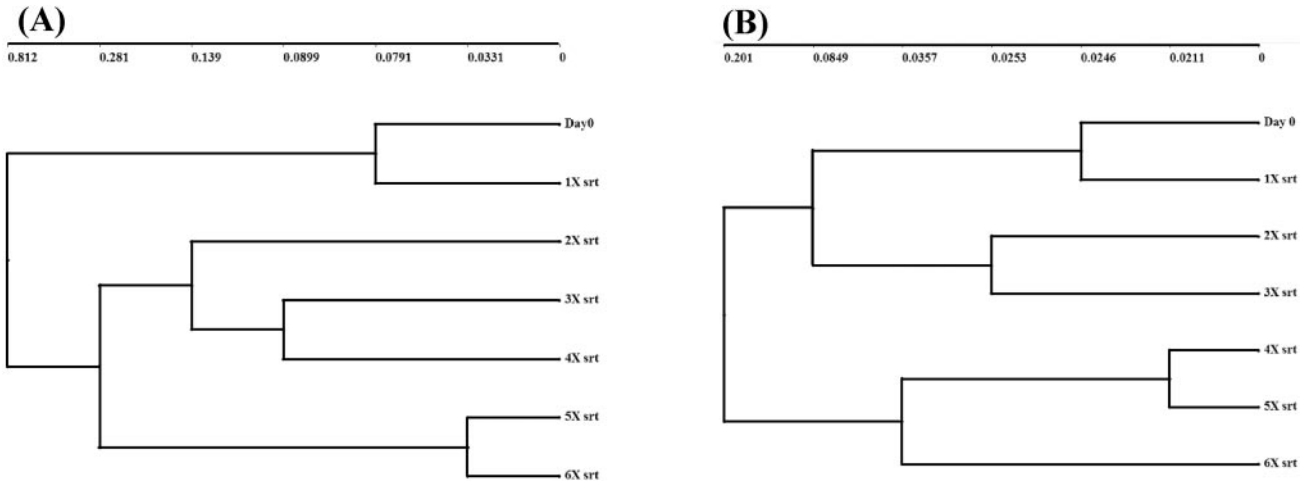

FIG. 3. (A) Dendrogram constructed using the algorithm of Ward and Hellinger distance for model predictions at an SRT of 8 days. (B) Dendrogram constructed using the algorithm of Ward and Hellinger distance for model predictions at an SRT of 2.5 days.

(5, 12, 14, 20) have reported that the bacterial community structure of laboratory-scale activated-sludge reactors seeded with sludge from domestic wastewater treatment plants was not static but constantly changing. Interestingly, this dynamic behavior in the bacterial community structure that was observed in laboratory-scale bioreactors operated under constant conditions was not observed in full-scale biological treatment plants.

LaPara and colleagues (22) examined the bacterial community structure of seven full-scale biological treatment plants treating pharmaceutical wastewater and their results showed that the bacterial community structure was stable under normal operating conditions. As mentioned above, a stabler community structure in full-scale biological treatment plants compared to laboratory-scale activated-sludge reactors could be explained by the equilibrium model of island biogeography (8).

This dynamic behavior in the bacterial community structure in laboratory-scale activated-sludge reactors could be attributed to several biotic and abiotic factors such as resource competition (17, 18), predation, and new selective pressure imposed on domestic sludge (14). It is already recognized in ecology that competition for three or more growth-limiting resources may generate oscillations and chaotic fluctuations in species abundances (17–19).

Recently, Saikaly and Oerther (32) developed an ecology-based mathematical model describing the mechanism behind these chaotic dynamics in the bacterial community in activated-sludge system. The model describes the competition of six aerobic heterotrophic bacterial species on three essential resources using the continuous stirred tank reactor with biomass capture as the model activated-sludge system. Essential resources fulfill metabolically independent requirement for growth. For example, ammonia and orthophosphate are examples of essential resources because they meet the requirement for nitrogen and phosphorous.

In developing the model, the following assumptions were made: (i) readily biodegradable substrates (i.e., sources of carbon and energy) are not limiting; (ii) oxygen is present in excess; (iii) the limiting resources are consumed by all of the different heterotrophic bacteria; (iv) competition is exploitative; (v) the hydraulic retention time is kept constant at 0.6 days; and (vi) an ideal clarifier with assumed zero volume is present. It is to be noted that in the current study, the main sources of carbon and energy (acetate) and oxygen were not limiting. Also, nitrogen, phosphorus, and sulfur that were present in the synthetic wastewater could be considered three essential resources since they are required for growth by the bacteria and have different metabolic routes.

The model simulations showed that for a certain range of SRTs (2.28 to 5.66 days) the competition of six species on three essential resources produces oscillations within the structure of the bacterial community and these oscillations in species abundances allowed the coexistence of more species than there are limiting resources (17, 18, 32). This outcome is a direct contradiction of an existing activated-sludge steady-state competition theory, the principle of competitive exclusion, which states that the competition process proceeds to equilibrium. The model also predicts that at higher values of SRT (e.g., greater than 5.66 days), the bacterial community structure reached a steady state where competitive exclusion occurred, resulting in reduced diversity. For more details on the model, refer to Saikaly and Oerther (32).

For the sake of comparison only, a dendrogram was constructed using the biological data (relative abundance of each species) from the model predictions at 0, 1, 2, 3, 4, 5, and 6 times the SRT for SRTs of 8 and 2.5 days (Fig. 3). The data in Fig. 3 were not presented in the original paper. The conclusion from Fig. 3 is that the bacterial community is dynamic and constantly changing. It is to be noted that this dynamic in the bacterial community persists indefinitely for SRT of 2.5 days, whereas for the SRT of 8 days competitive exclusion dominated after a period of 600 days.

In the current study, the two experimental runs were terminated after three SRTs. Therefore we could not predict if the dynamics in the bacterial community structure would persist for longer periods or if the constant operating conditions of laboratory-scale bioreactors would eventually lead to stable community structure for reactors operated at an SRT of 8 days.

Despite this limitation, the experimental design in the current study is unique since it involves many replicate reactors (a total of 12 reactors in experiment 1 and a total of 10 reactors in experiment 2), whereas similar studies of stability and reproducibility of bacterial community in laboratory-scale bioreactors were performed using only two reactors (5, 12, 14, 20). Another unique aspect of the experimental design is that experiments 1 and 2 could be considered two separate studies that aim to investigate the same concepts in activated-sludge systems, mainly stability, reproducibility, and the impact of SRT on diversity. The only difference between the two experimental runs is that experiment 1 was started with a seed that was not acclimated to synthetic wastewater, while experiment 2 was started with a seed that was already acclimated to synthetic wastewater.

The theoretical results of the model developed by Saikaly and Oerther (32) and the results from the current experiment using T-RFLP and the experimental studies discussed above collectively show that the bacterial community structure of activated-sludge system is dynamic. This suggests that the bacterial community in these systems could be innately dynamic and that the process of competition for essential resources could be responsible for generating these dynamics. Despite the similarity between the model and the experiment, the results should not be used to generalize or formulate a hypothesis on the dynamics and stability of bacterial communities in laboratory-scale activated-sludge reactors since several limitations exist in the model and the experiment that further hinder the generalization of the results. These observations, however, should be taken as a platform for further research. It is to be noted here that in the current study, analysis of T-RFLP profiles using MspI or RsaI gave similar results to analysis with HhaI (data not shown).

Impact of solid retention time on diversity indices. Microbial diversity is an important concept in ecology (27). Its calculation reflects key phenomena such as competition, succession, predation, ecosystem stability, response to perturbations, and, in the current study, to assess the impact of operational parameters of activated-sludge systems, in particular the impact of SRT on microbial diversity. While it is simple to determine diversity (richness and evenness) in macroecology, the situation is complicated in microecology because of limitations in the methods available to assess diversity. Microbial diversity in environmental samples is normally determined using PCR-based molecular fingerprinting of small-subunit rRNA, e.g., ribosomal DNA restriction analysis, denaturing gradient gel electrophoresis/thermal gradient gel electrophoresis, length heterogeneity PCR, automated ribosomal intergenic spacer analysis, and T-RFLP. Thus, T-RFLP, like other molecular fingerprinting techniques, is subject to the caveats of PCR-based techniques (e.g., differential cell lyses, PCR amplification biases, and formation of PCR artifacts such as chimeric sequences and heteroduplex fragments) (42).

In addition, some organisms may produce more than one T-RF because of *rrn* operon copy number heterogeneity (6). On the other hand, multiple phylogenetically related organisms could be represented by a single T-RF and therefore may not represent a true operational taxonomic unit (24). Additionally, current molecular fingerprinting techniques are unable to detect populations that are present in low abundance and hence T-RFLP profiles reflect the most abundant species (40). Because of these shortcomings, diversity measures using T-RFLP should be interpreted as a reflection of the PCR product pool rather than the absolute bacterial community diversity. Nonetheless, T-RFLP profiles do provide some means of assessing apparent diversity and in the current study the number and peak heights of T-RFs were used to determine diversity indices.

Diversity indices have been used in microbial ecology for various purposes that are of theoretical and practical nature. In a theoretical context, diversity indices have been used to compare different communities (10), to compare the same community at different times (38), and to determine if there is a correlation between operational parameters of wastewater treatment plants and diversity (4). In a more practical sense, diversity indices have been used to test the efficiency of bioaugmentation for bioprotection from pollutant shocks (12). In the current study, diversity indices were used to assess the effect of SRT on the bacterial diversity of laboratory-scale activated-sludge reactors.

Bacterial community diversity of samples taken from the two sets of reactors in experiments 1 and 2 was assessed using the Shannon-Weaver index of diversity (H), evenness (E), richness (S), and the reciprocal of Simpson's index of diversity ($1/D$). Diversity indices summarize both species richness and relative abundance using a single number and thus they are useful as a first approach to estimate the diversity of bacterial species. For example, communities with more species and even distribution of abundance will have higher values of H than communities with fewer species or uneven distribution of abundance. Standardized T-RFLP data from replicate reactors were combined at one, two, and three times the SRT and the combined data were used to determine richness and diversity indices values.

Diversity indices values for the three restriction enzymes HhaI, MspI, and RsaI are presented in Table 1. The choice of the restriction enzymes used in this study was based on recommendations found in the literature using primers 8-27f and 906-926r (16, 24). No trend was observed in richness values for the three restriction enzymes used in this study. For example, the richness value at two times the SRT (experiment 1) was higher for an SRT of 2 days ($S = 14$) than an SRT of 8 days ($S = 11$) using MspI but lower when HhaI was used ($S = 16$ versus $S = 19$). Similarly, the richness value at two times the SRT (experiment 2) was higher for an SRT of 2 days ($S = 22$) than an SRT of 8 days ($S = 18$) using HhaI but lower when RsaI was used ($S = 16$ versus $S = 17$).

Because of the high variability in the results, we could not determine which enzyme gave the best resolution (e.g., the greatest number of T-RFs). This inconsistency in the richness results obtained in the current study was not unique. Two other studies using T-RFLP to assess microbial diversity in soil samples (10) and marine samples (26) have reported variability in richness values between different restriction endonucleases. Combined, these findings show the high degree of variability in the resolving power of restriction endonucleases to reveal small-subunit rRNA gene sequence variants, especially in complex microbial communities such as those found in soil, marine environments, and activated sludge. Dunbar et al. (10) revealed that comparison of richness in complex communities such as soil using T-RFLP was ineffective and recommended

TABLE 1. Comparison of richness, evenness, and diversity values for reactors operated at SRTs of 2 days and 8 days

Endonuclease	Expt and SRT	SRT = 8 days				SRT = 2 days			
		S	*H*	*E*	1/*D*	*S*	*H*	*E*	1/*D*
HhaI	Expt 1								
	1XSRT	21	1.723[a]	0.566	2.725[a]	24	2.535[a]	0.798	8.098[a]
	2XSRT	19	1.812[a]	0.615	2.905[a]	16	1.983[a]	0.715	4.836[a]
	3XSRT	16	1.907[a]	0.688	3.514[a]	16	2.004[a]	0.723	4.869[a]
	Expt 2								
	1XSRT	26	2.889[b]	0.887	12.320[b]	20	2.186[b]	0.730	6.083[b]
	2XSRT	22	2.514[b]	0.814	9.063[b]	18	2.434[b]	0.812	8.320[b]
	3XSRT	20	1.923[a]	0.642	3.535[a]	22	2.114[a]	0.684	4.144[a]
MspI	Expt 1								
	1XSRT	14	1.248[a]	0.473	1.978[a]	15	1.696[a]	0.626	3.523[a]
	2XSRT	11	1.159[a]	0.483	1.860[a]	14	1.824[a]	0.691	4.211[a]
	3XSRT	23	1.614[a]	0.515	2.388[a]	19	2.006[a]	0.682	4.731[a]
	Expt 2								
	1XSRT	22	2.631[b]	0.851	9.538[b]	16	1.889[b]	0.681	4.618[b]
	2XSRT	23	2.333[c]	0.744	5.896[a]	21	2.330[c]	0.765	7.123[a]
	3XSRT	23	1.630[a]	0.520	2.341[a]	21	2.332[a]	0.766	6.444[a]
RsaI	Expt 1								
	1XSRT	14	1.774[a]	0.672	4.246[a]	20	2.149[a]	0.717	5.065[a]
	2XSRT	15	1.912[a]	0.706	4.613[a]	17	2.191[a]	0.773	6.848[a]
	3XSRT	24	2.003[a]	0.630	4.053[a]	26	2.134[a]	0.655	4.713[a]
	Expt 2								
	1XSRT	19	2.498[b]	0.849	8.864[b]	17	2.044[b]	0.721	5.730[b]
	2XSRT	16	2.058[a]	0.742	5.448[a]	17	2.367[a]	0.835	8.261[a]
	3XSRT	27	1.992[a]	0.604	3.278[a]	21	2.132[a]	0.700	4.386[a]
Model predictions	1XSRT	6	1.772	0.989	6.066	6	1.783	0.995	6.195
	2XSRT	6	1.489	0.831	3.452	6	1.775	0.991	6.089
	3XSRT	6	1.432	0.799	3.220	6	1.768	0.987	6.000
	1XSRT	6	1.464	0.817	3.365	6	1.753	0.979	5.817
	2XSRT	6	1.525	0.851	3.702	6	1.746	0.975	5.732
	3XSRT	6	1.558	0.869	3.919	6	1.740	0.971	5.667

[a] Randomization test using the Shannon-Weaver and the reciprocal of Simson's index with 10,000 random partitions. Sample SRT = 2 is more diverse than sample SRT = 8 at the 5% level.
[b] Randomization test using the Shannon-Weaver and the reciprocal of Simson's index with 10,000 random partitions. Sample SRT = 8 is more diverse than sample SRT = 2 at the 5% level.
[c] Randomization test using the Shannon-Weaver and the reciprocal of Simson's index with 10,000 random partitions. Sample SRT = 8 has the same diversity as sample SRT = 2 at the 5% level. 1XSRT, 2XSRT, and 3XSRT, one, two, and three times the SRT, respectively.

that the method be used to assess richness from simple communities. The authors also recommended using group- or subgroup-specific primers to better assess differences in richness of complex communities.

Contrary to what was found for richness, there was a trend in the results obtained using the Shannon-Weaver index, evenness, and the reciprocal of Simpson's index. For the three enzymes, community evenness were higher for reactors operated at an SRT of 2 days than reactors operated at an SRT of 8 days for samples collected from experiment 1 at one, two, and three times the SRT and from experiment 2 at two and three times the SRT (Table 1). These results suggest that bacterial communities in the reactors operated at an SRT of 2 days have a more even distribution of abundance than the bacterial community in reactors operated at an SRT of 8 days. Similar results were observed with the Shannon-Weaver index and the reciprocal of Simpson's index, where diversity index values were higher for reactors operated at an SRT of 2 days than reactors operated at an SRT of 8 days for samples collected from experiment 1 at one, two, and three times the SRT and from experiment 2 at two and three times the SRT. The only time where we observed a higher diversity for reactors operated at an SRT of 8 days was at one times the SRT (experiment 2).

A somewhat greater difference in diversity between the two sets of reactors was observed when the reciprocal of Simpson's index was used. This may be due to the fact that Simpson's index is more sensitive to abundant species than rare species and hence is more reflective of predominant species (37). In the current study, plotting rank-abundance curves for the two sets of reactors revealed that dominance was higher in reactors operated at an SRT of 2 days than SRT of 8 days (data not shown).

To assess if the differences in the observed diversity indices between the two sets of reactors were significant we applied a randomization test as described by Solow (37) using the Species Diversity and Richness software 3.0 (Pisces Conservation Ltd.). This test resamples 10,000 times from a distribution of species abundances produced by a summation of the two samples. The estimated *P* values for a one-sided test (against the alternative that sample [SRT = 2 days] is more diverse than sample [SRT = 8 days]) were calculated for the Shannon-

Weaver index and the reciprocal of Simpson's index. The test results showed that reactors from experiments 1 and 2 that were operated at an SRT of 2 days were more diverse than reactors operated at an SRT of 8 days at the 5% level (Table 1). The above results were further independently supported using the biological data obtained from the ecology-based mechanistic model developed by Saikaly and Oerther (32). Diversity indices from model predictions are presented in Table 1. The model results showed higher diversity values at an SRT of 2.5 days than an SRT of 8 days at one, two, three, four, five, and six times the SRT (data not shown in the original paper).

Collectively, the results from T-RFLP analysis and model predictions show that SRT impacts species diversity. However, it is still unclear what causes reactors operated at an SRT of 2 days to have higher diversity than reactors operated at an SRT of 8 days. Several biotic (predation) or abiotic (competition) factors (17, 18, 32) could be responsible for the differences in diversity. It is already recognized in ecology that nonequilibrium dynamics and oscillations in species abundances favor species coexistence (17, 18). Saikaly and Oerther (32) showed theoretically that in activated-sludge systems, resource competition at intermediate SRT (2.28 to 5.66 days) resulted in oscillations in species abundances and that these oscillations enhanced species diversity. However, at an SRT of >5.66 days competitive exclusion dominated and diversity was reduced. The above model results could be a potential mechanism to explain the observed difference in diversity between the two sets of reactors.

In the current study, T-RFLP analysis with diversity indices proved to be a sensitive tool to analyze changes in the bacterial community diversity in response to changes in operational parameters of activated-sludge systems. A significant practical application of the above results is that environmental engineers could use SRT as a design tool to enhance bacterial diversity in activated-sludge systems. This is important because both laboratory and field studies showed that diversity is positively related to ecosystem stability (29, 39). Stability can refer to resistance to disturbance and resilience (rate of recovery after disturbance) (39).

If the diversity-stability hypothesis developed in these studies of macroecological systems applies to activated-sludge systems, then we expect systems with higher diversity to better maintain performance when exposed to environmental perturbations (e.g., toxic shock loads). The importance of species diversity was shown in a recent study examining toxic loads of mercury in bioreactors (41). The results of the study showed that diverse biofilm communities demonstrated enhanced resistance to mercury toxicity compared to monoculture biofilms. Thus, an increase in species diversity may increase the chance of obtaining species with different complementary physiological traits that are better adapted to handle specific environmental perturbations. Therefore, future work will focus on investigating the relationship between diversity and ecosystem stability of activated-sludge systems.

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