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Structural and Functional Responses of a Sewage Microbial Community to Dilution-Induced Reductions in Diversity

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Abstract

The relationship between functional redundancy and microbial community structure–diversity was examined using laboratory incubations to ensure constant environmental conditions. Serial dilutions of a sewage microbial community were prepared, used to inoculate sterile sewage, and maintained in batch culture. Probability suggests that dilution of the initial community should remove rare organism types, creating mixtures of cells differing in diversity. Regrowth of the diluted mixtures generated communities similar in abundance but differing in community structure and relative diversity (as determined using two DNA fingerprinting techniques and dilution-to-extinction analysis of community-level physiological profiles). The *in situ* function of each regrown community was examined by monitoring the short-term uptake of five different ^{14}C-labeled compounds (glucose, acetate, citrate, palmitic acid, and an amino acid mixture). No significant differences were detected between treatments in either the rate of uptake of a substrate or the efficiency with which each community assimilated each compound. The fact that the activity of the original community was the same as that of a community regrown from an inoculum containing fewer that 100 cells (10^{-6} dilution) indicates that functional redundancy was quite high in this system. For each organism type eliminated during the dilution process, at least one of the remaining types was able to provide the same function at the same level as the lost one. Further research is necessary to determine what impact this functional redundancy may have on overall ecosystem function and stability.

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Introduction

Over the past 50 years, there has been intense research and debate in the field of ecology about the significance of biodiversity in ecosystem function. Many ecological theories, developed from the study of plant and animal communities, propose that there is a relationship between diversity and important ecosystem processes such as resource partitioning, succession, productivity, community function, and ecosystem stability [9, 25, 32, 40, 41]. Although there is research to support these relationships in some systems [38, 39, 47, 48, 50], there is considerable debate over what aspects of a community and what particular features of an ecosystem should be compared [4, 21, 23, 49, 53]. These theories have been tentatively applied to the study of bacterial communities, but methodological limitations have made rigorous hypothesis testing especially difficult. Because of the limitations of culture methods and the extreme diversity and abundance of microbial communities, it is not reasonable to use ecological approaches that rely on determining the distribution of different types of organisms as a means of testing hypotheses about microbial community dynamics. Although the use of molecular techniques and community-level analyses has greatly increased scientists' ability to monitor the distribution of bacteria in the environment, it is still not possible to completely categorize the constituents of a community or to thoroughly understand the functional abilities of each individual organism type.

In addition to the taxonomic and methodological difficulties associated with monitoring the hyperdiversity of microbial communities, there are several conceptual issues, unique to the field of microbial ecology, that suggest that the use of diversity as a indicator of ecosystem performance may not be appropriate in these communities. For example, Tate [46] questioned whether it is even useful to distinguish between "higher" and

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"lower" diversity in natural microbial communities, considering their tremendous richness (even for "low" diversity communities) and the fact that these communities may contain numerous dormant populations. The physiological versatility of many microorganisms presents another difficulty in determining the nature of any relationship between microbial community structure and function. A number of studies have shown that the broad-scale functional ability of a microbial community is often not controlled by organism diversity [1, 5, 10, 28], and it has been suggested that redundancy of function may be much more important for understanding the stability of microbial communities, and of the ecosystem functions they perform, than traditional diversity measures [2, 3, 11, 27, 55, 60].

Although it is a potentially important ecosystem performance parameter, the functional redundancy of microbial communities has not been well studied. Most recent work has focused on manipulating microbial diversity in the laboratory while monitoring the associated change in function [18, 19, 35, 36, 38, 43]. Another set of studies has focused on field comparisons, where differences in microbial diversity were predicted/inferred based on a historical knowledge of the communities' development (e.g., along a soil reclamation gradient [58], in association with different agricultural land uses [8], or in response to a metal contamination [42, 54]). Some of these studies have defined functional redundancy as simply the presence of multiple species that are *capable* of performing each function (i.e., redundancy of *functional potential*) and have attempted to quantify the extent of this redundancy (e.g., how many species may be found within each functional group or guild [58]). However, studies that consider how *in situ* function is affected by changes in community diversity are also needed, as there are several reasons why overall ecosystem functioning may not be maintained even in a community that is redundant with respect to each individual function. For example, although multiple populations may be capable of performing a function, they may not all perform it with the same efficiency, or they may not generate the same metabolic by-products. Similarly, a "replacement" species may not have the same growth rate or competitive ability as the original community member. Changes such as this could influence the activity of other populations in the community and indirectly cause a change in overall ecosystem function despite the fact that the original function of interest has been maintained [4]. More research is needed to determine whether the presence of multiple taxa/species, with overlapping functional abilities, actually results in functional stability.

The purpose of the research presented here was to examine the relationship between functional redundancy and microbial community structure–diversity using laboratory microcosms to ensure constant environmental conditions. Serial dilutions of a sewage microbial community were prepared, used to inoculate sterile sewage, and maintained in batch culture. Probability suggests that dilution of a relatively diverse community will remove rare organism types, creating mixtures of cells differing in species richness. Regrowth of the diluted mixtures can then be used to produce communities with roughly the same biomass but differing in overall diversity. Previous work in our laboratory has demonstrated that this approach is an effective means of generating communities differing in structure as determined using genetic, physiological, and culture-based techniques [12, 14]. In the present study, several different methods were used to characterize the microbial communities, and the results indicate that the dilution/regrowth procedure did produce microbial communities that differed in overall structure and diversity. However, there were no significant differences between treatments in *in situ* function (short-term uptake of five different ^{14}C-labeled compounds), which suggests that functional redundancy was quite high within the microbial consortium and conferred some stability on the community—at least for the processes we considered.

Materials and Methods

Microcosm Setup. A single large sample (approximately 20 L) of sewage was collected from the aeration basin of the Rivanna Wastewater Treatment Plant in Charlottesville, Virginia. After heat sterilization (autoclaving for 2 h at 120°C and 15 psi), the sample was allowed to settle for 72 h at 4°C. The clarified supernatant liquid was then used as the growth medium for the flask experiments. Microcosms were prepared by adding 60 mL of autoclaved sewage supernatant to a 125-mL Erlenmeyer flask capped with a sterile foam plug.

At the start of the experiment, a fresh sample of sewage was collected from the same aeration basin. From this sample, serial dilutions were prepared, using sterile sewage as the diluent, and each of these dilutions was used as an inoculum in the flask experiments. Seven treatments were established by adding 1 mL of sewage ("10^0 treatment") or diluted sewage (10^{-1} through 10^{-6}) to each microcosm; three flasks were inoculated for each treatment. After inoculation, flasks were placed on a shaker table (150 rpm) and incubated at 23°C. Each day, 20 mL of fluid was removed from each microcosm and replaced with 20 mL of sterile sewage. The cultures were maintained in this fashion for 9 days, which corresponds to three retention times [12].

At the end of the experiment, several attributes of the regrown communities were measured using samples collected from the bulk solution of each microcosm.

Because of the amount of laboratory effort required, final analysis of the communities took place over a 2-day period. On the first day (day 8), acridine orange direct counts (AODC [22]) were performed, the heterotrophic uptake of three different ^{14}C-labeled substrates (glucose, an amino acid mixture, and citric acid) was measured, and a sample was preserved for molecular genetic analyses. On the second day (day 9), samples were again collected for AODC and genetic analyses, as well as for cultural counts on R2A agar (spread plates incubated for 48 h at 23°C). In addition, the heterotrophic uptake of two other ^{14}C-labeled substrates (acetate and palmitic acid) was measured, and dilution–extinction analysis of community-level physiological profiling (CLPP) was performed [13].

Determination of Relative Structural Diversity. Dilution–extinction analysis of CLPP was performed on a subset of the regrown communities (two flasks from each treatment) to determine the relationship between cell density (I) and functional richness [number of positive wells (R)] for each treatment [13]. This information was then used to make inferences about the relative structural diversity of each set of communities. At the end of the experiment, serial dilutions of the regrown microbial communities were prepared (10^{-1} through 10^{-8}), and inoculated into Biolog GN2 microplates (Biolog, Inc., Hayward, CA, USA). Plates were incubated at 23°C for 6 days, at which time the absorbance (590 nm) in each well of each plate was measured using a Labsystems Multiskan RC plate reader. A positive response was defined as any value greater than 0.25 absorbance units, after correction for the control well.

Data for each treatment were pooled and plots were made of the number of positive responses (R) in each plate versus the number of cells in each well (I) as determined using R2A counts. A hyperbolic model, $R = R_{max}I/(K_I + I)$, was then fit to the data; nonlinear regressions were performed using SigmaPlot (Version 5.0), and the parameters R_{max}, which equals the maximum (asymptotic level) of R, and K_I, which is the value of I when R is 1/2 of R_{max}, were determined.

Molecular Genetic Analyses

DNA Extraction and Quantification. Samples (20 mL) were collected from each of the three replicate flasks for each treatment at the end of the experiment (day 9) and from one flask of each treatment on day 8. The suspended microbial community was concentrated from this solution by centrifugation (23,000 × g for 20 min). The resultant cell pellet was resuspended in 200 μL of phosphate-buffered saline (pH 7.4) and stored at −20°C. Whole-community DNA was later extracted using the High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN, USA) following the manufacturer's instructions for bacterial cultures. DNA concentration was determined using the PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR, USA).

Comparison of Overall Community Structure Using AFLP. Amplified fragment length polymorphism (AFLP) analysis was completed using the primers and protocols described in Franklin *et al.* [12] and the PerkinElmer Microbial Fingerprinting Kit (PE Applied Biosystems, Foster City, CA, USA). Data were analyzed using the Genotyper software (PE Applied Biosystems), and the presence or absence of each fragment/peak in each sample was coded as 1 or 0. This type of data matrix was prepared for each primer pair, and the information from the three matrices was pooled into a single large data set. This resulted in a total of 114 bands, all of which were polymorphic.

The Jaccard coefficient was used to calculate the relative similarity between each sample pair [44], and a Mantel test [33, 45] was used to evaluate whether overall microbial community structure was significantly different among the treatments. In general, a Mantel test determines the amount of correlation between two matrices, and a permutation procedure is used to assess the significance of this correlation. In this application, the two matrices being compared were 1) the observed similarity matrix calculated from the AFLP data and 2) a conceptual model matrix that defined group identity (dilution treatment). Data were analyzed using the Mantel–Struct program [37] to test the null hypothesis that there was no difference in within- and between-group genetic similarities of the communities. A Monte Carlo procedure (using 5000 permutations) was employed to assess the significance of the test statistic, and a Bonferroni-type correction was made to adjust the α level, depending on the number of comparisons.

A principal components analysis (PCA) was performed on the original pooled data matrix (SPSS 10.0), and plots of the first two principal components were made. PCA of binary data positions objects in multidimensional space at distances that are the square roots of complements of simple matching coefficients [17, 30].

Comparing Community Composition Using T-RFLP. Terminal restriction fragment length polymorphism (T-RFLP) was performed as described in Liu *et al.* [31], with slight modification. The eubacterial primers 27 forward [5′-AGA GTT TGA TCC TGG CTC AG-3′; fluorescently labeled with 5[6]-carboxyfluorescein (6-FAM)] and 1492 reverse (5′-GGT TAC CTT GTT ACG ACT T-3′) (Operon Technologies, Alameda, CA, USA) were used. The polymerase chain reaction (PCR) mixture included 1× PCR buffer, 200 mM of each dNTP, 2.0 mM $MgCl_2$,

1.0 μM of each primer, 0.4 μg μL^{-1} bovine serum albumin (BSA), and 1.25 units of Ampli *Taq* DNA polymerase (PE Applied Biosystems) per 50-μL reaction. The thermal cycling conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 0.5 min, 58°C for 1 min, 72°C for 2 min, with a final elongation at 72°C for 10 min. PCR products were purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI, USA) and eluted in a final volume of 50 μL. Separate portions (10 μL) of the purified PCR product were then digested with either the *Hha*I and *Rsa*I restriction enzymes (New England Biolabs, Beverly, MA, USA).

Data were collected using an ABI Prism 310 Genetic analyzer (Applied Biosystems). The presence or absence of each terminal restriction fragment (T-RF) in each sample was determined and recorded as a matrix of 1's and 0's. The data from the two restriction digests were pooled into a single large data set and analyzed using a Mantel test and a PCA as described for the AFLP analyses.

Short-term Heterotrophic Uptake Assay. The following substrates, labeled with ^{14}C, were used: (a) D-[^{14}C(U)]glucose (specific activity, 340 mCi $mmol^{-1}$), (b) [1,5-^{14}C]citric acid (specific activity, 83.8 mCi $mmol^{-1}$), (c) [2-^{14}C]acetate (sodium salt, specific activity, 51.2 mCi $mmol^{-1}$), (d) [1-^{14}C]palmitic acid (specific activity, 52 mCi $mmol^{-1}$), and (e) L-amino acid mixture (specific activity, 2.23 μCi μg C^{-1}, which is equivalent to 334 μCi μmol^{-1} assuming an average molecular weight of amino acids as 150 g mol^{-1}). Glucose and palmitic acid were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), and all other substrates were obtained from NEN Life Sciences Products (Boston, MA, USA). An additional set of experiments was performed using a sixth substrate, radiolabeled benzene, but the results are not reported here as it was later determined that the benzene stock solution had been contaminated.

Two of the replicate flasks from each treatment were randomly selected for analysis of heterotrophic activity [29, 56]. From each flask, 5 mL of the culture were removed and mixed with 5 mL of sterile sewage and 0.1 μCi of radiolabeled substrate in a 25-mL Erlenmeyer flask. At the end of a 2-h incubation period, the amount of ^{14}C-labeled CO_2 produced in each flask was determined and represented the portion of substrate taken up by the community and respired (mineralization). The amount of substrate taken up and accumulated into biomass (assimilation) was estimated by filtering the sewage through a 0.4-μm filter to trap the microbial cells and then quantifying the amount of radiolabel associated with the filter. Radioactivity was measured using a Beckman LS 6500 liquid scintillation counter. Total uptake was calculated as the sum of mineralization and assimilation, and standardized to a per cell value using the AODC; assimilation efficiency was calculated as the portion of the total label uptake incorporated into biomass. For each substrate, each of these parameters was compared across treatments using an analysis of variance (ANOVA).

Results

Community Size. No significant differences in the total concentration of cells (AODC) were observed across the treatments for either day 8 or 9 of the experiment ($df = 27$, $F = 0.498$, $p = 0.492$). The average concentration of cells based upon these two sets of measurements was 3.5×10^8 cells mL^{-1} [$\pm 3.3 \times 10^7$ (SEM)]; the concentration of cells in the original undiluted inoculum (at the start of the experiment) was 2.2×10^8 cells mL^{-1}.

The number of colonies able to grow on R2A agar was significantly different across treatments ($df = 18$, $F = 4.39$, $p = 0.014$). *Post hoc* pairwise comparisons (Tukey HSD) indicated that the community regrown from the 10^{-5} dilution inocula was significantly different from all of the others. The average concentration for the 10^{-5} treatment was 1.9×10^8 colony-forming units (CFU) mL^{-1}, and the average for all of the other treatments combined was only 2.4×10^7 CFU mL^{-1} [$\pm 8.1 \times 10^6$ (SEM)]. The concentration of cells in the original inoculum that were capable of growing on R2A agar was 2.3×10^6 CFU mL^{-1}.

Relative Structural Diversity. The data from the dilution–extinction analysis were fit with a rectangular hyperbola to estimate the parameters K_I and R_{max} for each treatment (Table 1). Because of an error made when inoculating the plates from the 10^{-1} treatment, no values are available for that set of communities. K_I, the cell density at which R (functional richness) = $1/2(R_{max})$, decreased as expected in the communities that were predicted to have lower diversity based upon extent of dilution. R_{max} did not change significantly along the dilution/diversity gradient.

Overall Community Genetic Structure (AFLP). A Mantel test was performed on the AFLP data to test

Table 1. Results of dilution–extinction CLPP analysis (mean ± 1 SD)]

Treatment	R_{max}	K_I	R^2*
Undiluted (10^0)	88 ± 5	135 ± 47	0.94
10^{-2}	88 ± 3	74 ± 23	0.94
10^{-3}	93 ± 2	24 ± 5	0.94
10^{-4}	88 ± 4	47 ± 17	0.90
10^{-5}	91 ± 1	35 ± 3	0.98
10^{-6}	92 ± 4	36 ± 14	0.86

*All associated p values <0.0001.

whether the communities from the different dilution/diversity treatments were significantly different. After applying a Bonferroni procedure to correct the significance level for multiple comparisons (initial α = 0.05), three significantly different subsets were established: undiluted (10^0) with 10^{-1}, 10^{-2} through 10^{-5}, and 10^{-6}. These results are consistent with those obtained from the PCA (Fig. 1A). In that analysis, PC1 explained 17% of the variance in overall community structure, and PC2 explained 12%. No significant differences were observed for the community profiles obtained on separate sampling days.

T-RFLP Analysis of Dominant Community Members. In the T-RFLP analysis, 38 T-RFs were observed across all treatments (pooled results for two separate enzymes); only 7 of these bands were common to all of the communities, 5 were found only in the low-dilution treatments (10^0–10^{-4}), and 16 were found only in high-dilution treatments (10^{-5} and/or 10^{-6}). The number of bands found for each treatment did not change significantly or consistently across the dilution series. As with the AFLP data, there were no significant differences in the samples collected on separate days.

Using a Mantel test, the communities from the low-dilution treatments [undiluted (10^0) through 10^{-4}] were found to be distinct from both the 10^{-5} and the 10^{-6} dilution treatments. The two high-dilution treatments (10^{-5} and 10^{-6}) were nearly significantly different from one another (p = 0.03). These differences were also manifest in the PCA (Fig. 1B), and the first two principal components explained nearly 50% of the total variance in community structure (PC1, 33%; PC2, 15%).

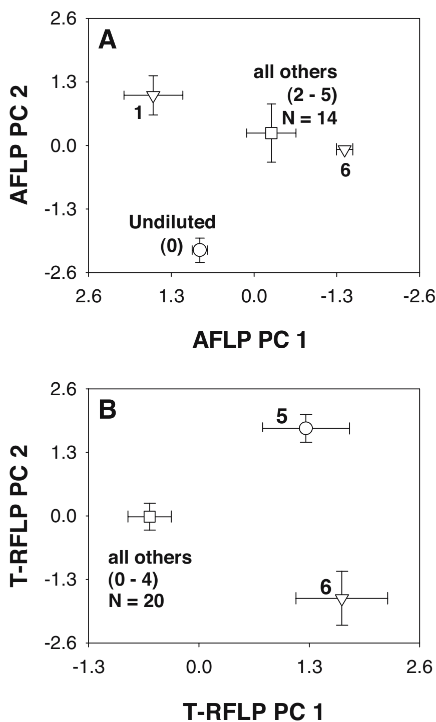

Figure 1. Principal components analysis of the AFLP (A) and T-RFLP (B) DNA fingerprinting data (mean ± 1 SD). The symbols used in the figure correspond to the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution treatment).

Short-term Heterotrophic Uptake Assays. For each substrate, total uptake (Fig. 2) and assimilation efficiency (Fig. 3) were calculated for each treatment and expressed per AODC cell. No statistically significant differences were detected between any of the treatments for any of the substrates (ANOVA). For palmitic acid, assimilation could not be measured because the substrate adhered to the cells and/or filters, so instead, respiration (normalized per cell) was compared across treatments using an ANOVA (Fig. 2E). As with the other substrates, no significant differences were observed. Because the assimilation of palmitic acid could not be measured, the efficiency was not calculated.

Discussion

The dilution/regrowth approach described here has been used a number of times as a means of manipulating diversity in microbial systems [12–14, 18, 34, 43]. These studies have shown that regrown communities may differ in overall community structure, relative diversity, functional potential, and metabolic redundancy, and that more diverse communities, those regrown from less dilute mixtures, may be more stable (e.g., better able to withstand an invasion attempt [14, 34]). The purpose of the present study was to use the dilution/regrowth procedure to generate a set of communities, similar in composition but systematically differing in diversity, and to monitor any associated changes in community function. In addition to monitoring the short-term *in situ* uptake of several substrates, community structure was analyzed to confirm that the procedure actually generated communities with different structure and diversity.

Microbial community structure was analyzed using both genetic and physiological methods. The genetic techniques (T-RFLP and AFLP) demonstrated that the different dilution/diversity treatments were quite different in overall community structure, whereas the CLPP assay confirmed that the communities regrown from the very dilute inocula were less diverse. Despite the fact that microbial community structure and diversity changed along the dilution gradient, there were no significant differences in either the uptake rate (how rapidly the communities were able to process a given substrate) or the efficiency of the communities (how they partition the substrate between biomass and loss to respiration) for

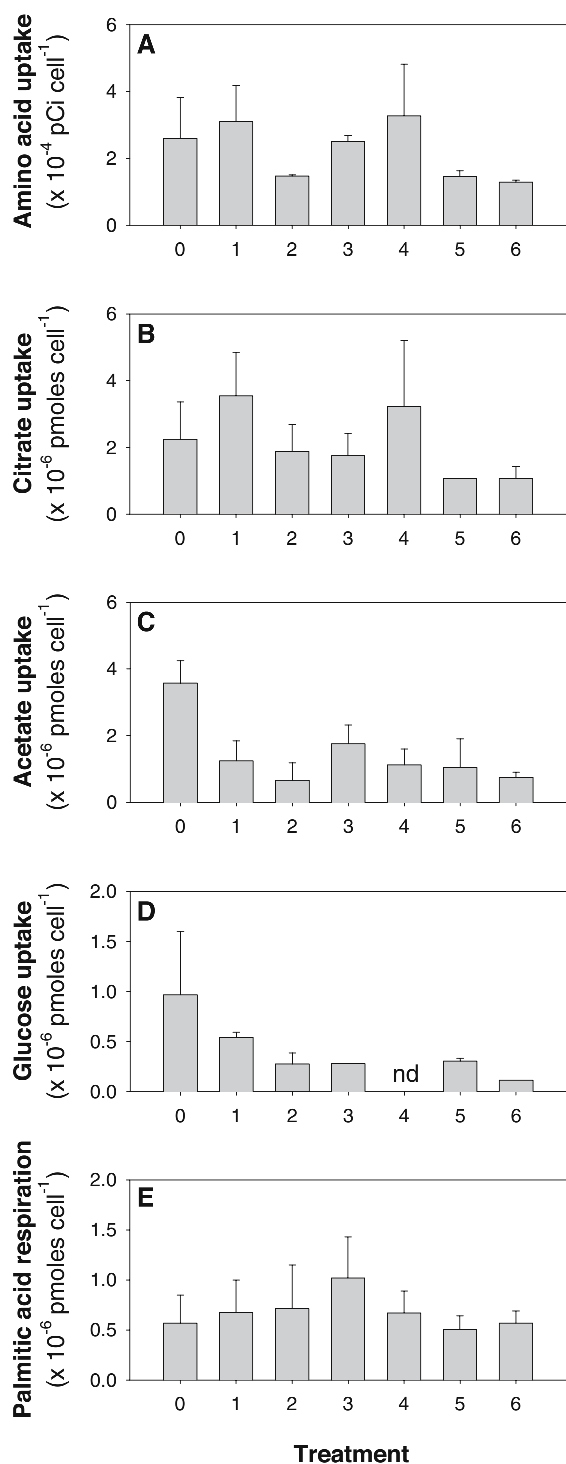

Figure 2. Total uptake of ^{14}C-labeled substrate (mean ± 1 SD) for the amino acid mixture (A), citrate (B), acetate (C), and glucose (D), and respiration of palmitic acid (E). The *x* axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution treatment). ND: not determined.

glucose, acetate, citrate, or the amino acid mixture (Figs. 2 and 3). Similarly, community respiration of palmitic acid did not vary significantly across treatments (Fig. 2E).

In this study, all of the communities were incubated in the same environment, supplied with the same growth medium, and inoculated with a similar group of organisms. Therefore, it was not necessarily surprising to discover that they performed several functions to the same extent, given how tightly coupled microbial activities are to physical and chemical surroundings. The fact that the activity of the communities regrown from the very dilute inocula (10^{-6}) could be the same as the communities regrown from the undiluted one (10^{0}) suggests that functional redundancy was quite high in the original community. Whereas the undiluted (10^{0}) treatment was inoculated with approximately 10^{8} cells, the 10^{-6} treatment was inoculated with only 100 cells (AODC); yet, for all of the substrates considered, the activity of the regrown communities was the same.

Another possible interpretation of these results is that the similarity in function across treatments was not the result of functional redundancy but simply reflected a similarity in community composition; it is possible that the subset of organisms common to all treatments/dilutions happened to be responsible for the particular functions examined. However, this is unlikely given the variety of substrates used, the wide range of treatments/dilutions studied, and the results of the various structural assays (AFLP, T-RFLP, and CLPP). Each of these structural assays showed a large difference in community structure among treatments, particularly when comparing the two ends of the dilution series (10^0 and 10^{-6}). For example, using AFLP, the average relative similarity calculated among communities within these treatments was high (10^0: 0.50, 10^{-6}: 0.63) compared to the relative similarity between treatments (10^0 to 10^{-6}: 0.13).

The results of our study are similar to the findings of Salonius [43], who used dilution to reduce diversity of a soil microbial community. After inoculating the dilutions into sterile soil, he monitored net respiration (O_2 uptake) for 5 months. In general, respiration was the same for each of the regrown communities, except when richness was decreased below a critical level; in treatments with a very low diversity (just above the extinction point), respiration was severely impaired. Griffiths *et al.* [20] reported similar results for a set of batch culture experiments. In that case, changing the evenness of a community had no impact on function, which was assessed by comparing the spectra of volatile organic compounds produced by the microorganisms. Several additional studies have been performed that used chloroform fumigation for increasing periods of time as a way to progressively destroy species and manipulate microbial diversity in soil microbial communities [5, 7, 19, 20, 61]. That work also demonstrated that there can be changes in microbial community structure with no

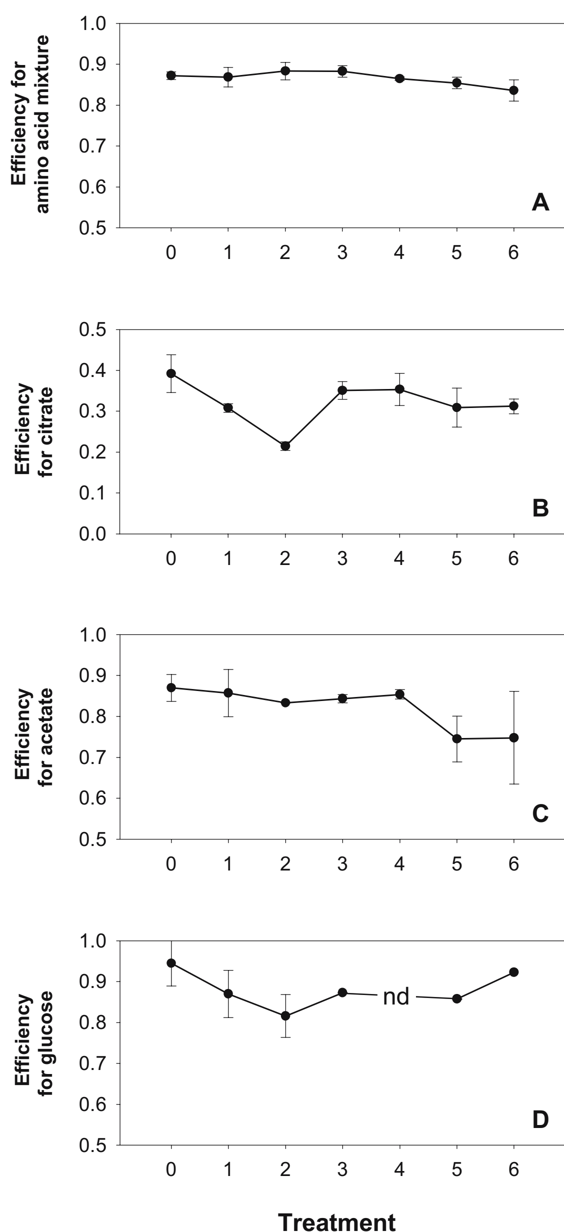

Figure 3. Assimilation efficiency of each ^{14}C-labeled substrate (mean ± 1 SD) for the amino acid mixture (A), citrate (B), acetate (C), and glucose (D). The *x* axis in each of these graphs represents the negative exponent of the dilution factor used to create the original inoculum (e.g., "4" corresponds to a 10^{-4} dilution treatment). ND: not determined.

change in function [6], but that function can be affected below certain levels of species richness [19, 20]. A similar phenomenon might have been observed in the present study if even more dilute treatments had been maintained (e.g., 10^{-7} and 10^{-8}, which would have been inoculated with 10 cells and 1 cell respectively).

The two DNA fingerprinting techniques (T-RFLP and AFLP) used to characterize microbial community structure in this study produced slightly different results and seemed to differ in their resolution and sensitivity to different aspects of community structure (e.g., richness and evenness). T-RFLP was used to analyze the microbial communities based on variability in the 16S ribosomal RNA gene, and showed a major difference in community structure between the very dilute (10^{-5} and 10^{-6}) treatments (Fig. 1B) and all of the others. The T-RFLP profiles for the 10^{-5} and 10^{-6} communities contained several unique T-RFs, which were responsible for the separation of these treatments from the others in the PCA. The organisms corresponding to these T-RFs were likely present in all of the treatments, but were too rare in the less dilute/more diverse communities to be detected with the T-RFLP procedure; it is well known that populations that are not numerically dominant are not represented in the T-RFLP profiles if their template DNA is too small a fraction of the total community DNA pool [8, 31]. The detection of the unique T-RFs in the high-dilution treatments suggests that *evenness* increased in the communities regrown from the more dilute inocula, which is consistent with previous numerical simulations [12].

AFLP is a technique in which a restriction digest is performed on a DNA sample (similar to RFLP), and then a set of primer-recognition sequences (adaptors) is used to amplify the restriction fragments using PCR [59]. In contrast to T-RFLP, the primers and restriction enzymes used are not specific for a given gene or group of genes but can, theoretically, interact in numerous random places throughout a genome. This makes AFLP a particularly useful technique for analysis of *overall* differences between communities, including strain- or species-level changes. In general, the results from T-RFLP and AFLP were similar, except that the separation of the low-dilution communities (10^{0} with 10^{-1}) was not reflected in the T-RFLP profiles. The separation of these low-dilution communities in the AFLP analysis was likely related to the large decrease in *richness* of the inoculum that occurred as a result of the first few dilutions, and is consistent with previous analysis of community structure in dilution/regrowth experiments [12]. However, since T-RFLP primarily detects dominant organisms, that method did not distinguish any differences in community structure for the less dilute/higher diversity treatments. The fact that the AFLP technique detected differences in community structure that were not just due to changes in the dominant organisms is particularly important, considering the fact that so many of the methods currently used to assay microbial communities focus solely on the characterization of these most abundant organism types. Those organisms may actually represent a very small portion of the total microbial community; for example, in water only about 10% of the simultaneously coexisting species are dominant [51]. Further development of techniques that better consider the contribution of rare organism types

to overall community structure and function is an important area for future study.

There is considerable debate among ecologists as to what processes should to be chosen to best characterize ecosystem or community functioning [15, 16], and the study reported here only addresses the metabolic uptake of a relatively small number of substrates. Although it is certainly possible that differences in function might have been found if the communities had been presented with different compounds, the substrates we considered were chosen to represent a range of different types of chemical groups: amino acids, carboxylic acids [short- (acetate and citrate) and long-chained (palmitic acid)], and carbohydrates (glucose). Ideally, a more complete analysis of the function of microbial communities would include measures of other compounds and processes and would compare the assemblages based on both general (e.g., overall decomposition rates) and specific functions. There is some evidence that suggests that well-defined microbial functions such as nitrification and methane oxidation, which are carried out by a limited microbial subset, may be more sensitive to changes in diversity than broader-scale functions such as respiration or decomposition [19, 52, 57]. Furthermore, Kandeler *et al.* [26] also showed that carbon cycling may be less sensitive to changes in microbial community composition than nitrogen and phosphorus dynamics.

Despite growing knowledge of the magnitude of prokaryotic diversity, most of the organisms in natural environments are uncultivated, and their functional roles and abilities remain unknown. The fact that most questions about the structure and function of microbial communities require relative comparisons, which can be made at the community level, helps to overcome this problem, particularly if multiple analytical techniques are employed to increase the robustness of the relative comparisons [24]. Most of the interest in studying the relationship between microbial diversity and function is based on the assumption that diversity may influence ecosystem stability and productivity and could help mediate a community's response to stress and perturbation. In the study presented here, we examined the relationship between microbial diversity and function for a sewage microbial community and found that functional redundancy may play an important role in the stability of microbial communities, even for communities with a relatively low diversity such as sewage.

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