Microbial Ecology

Effect of Microbial Species Richness on Community Stability and Community Function in a Model Plant-Based Wastewater Processing System

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Abstract

Microorganisms will be an integral part of biologically based waste processing systems used for water purification or nutrient recycling on long-term space missions planned by the National Aeronautics and Space Administration. In this study, the function and stability of microbial inocula of different diversities were evaluated after inoculation into plant-based waste processing systems. The microbial inocula were from a constructed community of plant rhizosphere-associated bacteria and a complexity gradient of communities derived from industrial wastewater treatment plant-activated sludge. Community stability and community function were defined as the ability of the community to resist invasion by a competitor (*Pseudomonas fluorescens* 5RL) and the ability to degrade surfactant, respectively. Carbon source utilization was evaluated by measuring surfactant degradation and through Biolog and BD oxygen biosensor community level physiological profiling. Community profiles were obtained from a 16S–23S rDNA intergenic spacer region array. A wastewater treatment plant-derived community with the greatest species richness was the least susceptible to invasion and was able to degrade surfactant to a greater extent than the other complexity gradient communities. All communities resisted invasion by a competitor to a greater extent than the plant rhizosphere isolate constructed community. However, the constructed community degraded surfactant to a greater extent than any of the other communities and utilized the same number of carbon sources as many of the other communities. These results demonstrate that community function (carbon source utilization) and community stability (resistance to invasion) are a function of the structural composition of the community irrespective of species richness or functional richness.

Introduction

The National Aeronautics and Space Administration's (NASA) new human exploration initiative calls for manned missions to the moon and Mars in the next several decades. Microorganisms will be the numerically dominant and most diverse biological component within such systems, including those associated with human and biotechnological approaches to waste treatment [33]. Defined or constructed microbial communities will likely be used to inoculate these waste processing systems (WPS) because these communities would provide a standardized means for conferring specific properties to the system. A significant concern of this approach, however, is the establishment of robust and reliable microbial communities to accomplish wastewater treatment as well as to ward off invasive species and microbial pathogens. Therefore, the ability of inoculants to survive and form a cohesive community in the system will be vital to ensure reproducible, reliable, and resilient system function.

The effect of structural diversity on community function is of interest not only to NASA, but also for application in ecological, microbiological, and biotechnological enterprises. The significance of biological diversity for

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ecosystem function is highly debated; however, greater species diversity is generally associated with improved community function and stability [26, 29, 39]. Alternative explanations have been put forth to explain the effect of diversity on ecosystem function including: (1) more efficient utilization of resources due to increased competition, niche differentiation, and resource exploitation or (2) greater likelihood that individual species with crucial functional characteristics will be present [6, 17, 25]. Community structural and functional characteristics may, however, be as important as overall diversity [8, 18]. Symstad *et al.* [37] evaluated the effect of loss of plant species on community and ecosystem properties and found that decreases in species richness resulted in concomitant losses in productivity. However, changes in plant community composition had similar significant effects on biomass, independent of diversity. Community function and stability may, therefore, be as closely related to the individual species within the community as to the overall species diversity [2, 39].

Experiments evaluating the role of individual species within a community or ecosystem normally involve manipulation of community composition by creating defined mixtures of organisms [29, 37, 38]. Defined communities allow the researcher to manipulate specific factors such as the degree of functional redundancy among individuals, and provide greater confidence that potentially deleterious organisms are not being introduced into the system. However, this macroecological approach is complicated in microbial ecology studies because only a small portion of microorganisms in the environment can be isolated and manipulated in this way [30]. Therefore, microbial communities assembled by combining different numbers of known isolates may be poor analogs of diverse natural assemblages comprising both culturable and nonculturable types. One approach for creating gradients in community diversity without relying on culturing involves dilution to extinction [10, 13, 17, 25, 35]. This approach is based on the concept that dilution of a diverse microbial community will remove rare organism types so that regrowth of dilutions results in mixtures of cells differing in structural diversity, but with similar cell density. The complexity gradient communities that develop from the series of dilutions are introduced into a new system of interest, and these communities are treated as different levels of a diversity treatment.

The objective of this research was to investigate how differences in species richness affect community function and stability within inoculated plant-growth systems. Communities of varying structural diversities were created by combining rhizosphere isolates to obtain an artificial or constructed community and by using dilution to extinction of an undefined community from industrial wastewater treatment plant (WWTP)-activated sludge to obtain a gradient of microbial diversity. Inocula were placed into the rhizosphere of wheat plants used as part of a model plant-based WPS. A 16S–23S rDNA intergenic spacer region (ISR) array [5] and community level physiological profiling (CLPP) [11, 15] were used to assess community structural and functional differences. Two measurable parameters, surfactant degradation and invasion by a competitor (*Pseudomonas fluorescens* 5RL), were used as performance criteria to evaluate community function and community stability.

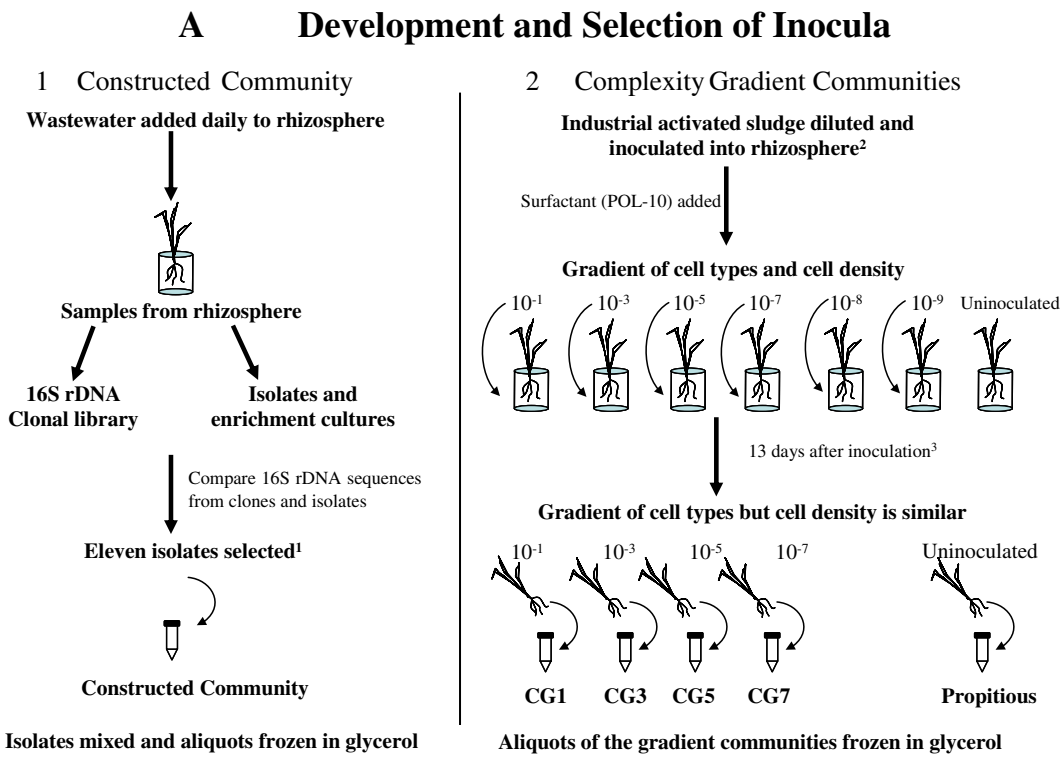
Materials and Methods

Environmental Conditions and Plant Cultural Techniques.

Wheat (*Triticum aestivum* L. cv. Apogee) was grown in a 1.8 × 2.4 m walk-in growth chamber (EGC, Inc., Chargin Falls, OH, USa) located at Kennedy Space Center, FL, USA. Lighting was provided by very high output daylight fluorescent lamps (Lucolux; General Electric Co., Cleveland, OH, USA) set for a 20-h light/4-h dark photoperiod, and a constant temperature of 23°C. Relative humidity was maintained at 70% for the duration of the study. Atmospheric CO_2 was maintained at 1200 ppm with an infrared gas analyzer (LiCor Model 6252; LiCor Corp., Lincoln, NE, USA). Wheat seeds were sterilized by using a combination of mercuric chloride and hydroxylamine hydrochloride as previously described by Barber [1]. After 8 days, seedlings were randomly selected and aseptically placed into slits in autoclaved foam plugs and transferred into sterile wide-mouthed vessels containing approximately 500 mL of filter-sterilized, modified 1/2 strength Hoagland's nutrient solution [14] with 5 mM MES (Sigma, St. Louis, MO, USA) [28] added to maintain pH at or about 5.5 for the duration of the experiment. Nylon wicks were used to conduct nutrient solution to the seedlings until the roots reached the solution. Plant growth vessels were covered with plastic to prevent light penetration to roots, and vessels were placed on a rotating shaker table (75 rpm) to provide aeration.

Development and Selection of Inocula

Constructed community. Isolates to be included in the constructed community were chosen as outlined in Fig. 1A and selected isolates are identified in Table 1. For more detail concerning methods used for selection of isolates, see [4]. Ten of the isolates from the constructed community were grown separately in R2A broth [32] for 18 h. *Cytophaga hutchinsonii* was grown for 4 days on Dubos media [4] with sterile filter paper as a source of cellulose. Each isolate was centrifuged at 16,000 × *g* for 15 min to pellet, supernatant was removed and cells were resuspended in 1/2 strength Hoagland's nutrient solution. To obtain cultures of similar cell density each culture was grown to an OD 600 nm of 1 and cell concentration was evaluated using a modification of the acridine orange



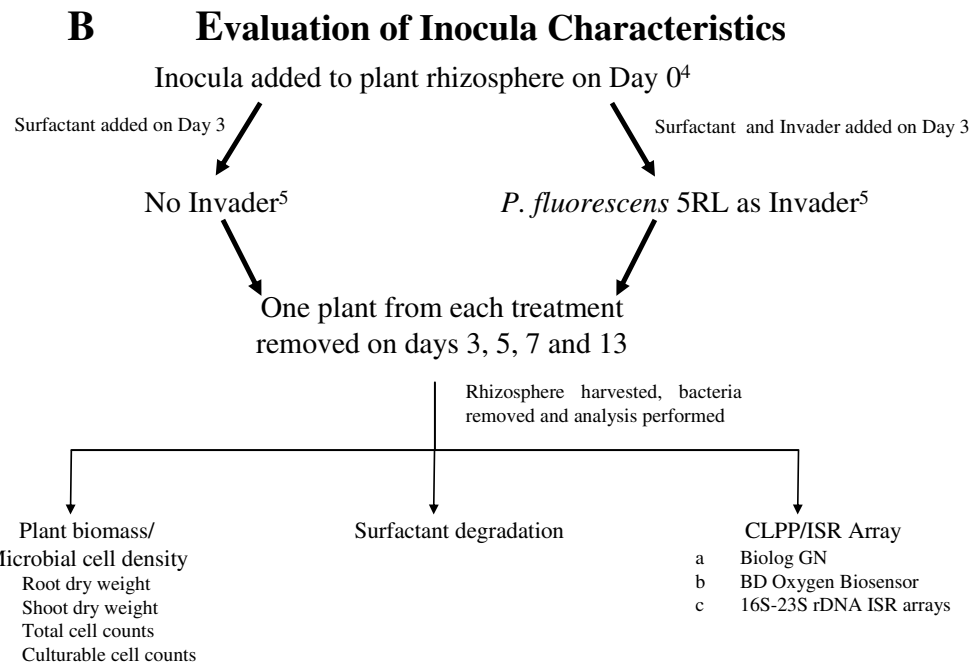


Figure 1. (A) Schematic showing procedure used to develop and select the constructed community (1) and the complexity gradient communities (2) used for community studies. (B) Schematic showing procedure used for analysis of inocula community characteristics. [1]Selection criteria for inclusion of isolates in the construed community are described in results; for additional details, see Cook et al. [4]. [2]Triplicate plant rhizospheres were inoculated with 1 mL of the appropriate sludge dilution. [3]After 13 days of growth, rhizosphere samples were taken, rhizosphere-associated organisms were removed and analyzed as described in materials and methods. [4]Plant rhizospheres were inoculated with 1 mL of the appropriate inoculum. [5]Experiments were performed in triplicate.

Table 1. Constructed community members and persistence in rhizosphere treatments

		Persistence[a]			
		Day 5	*Day 5*	*Day 13*	*Day 13*
Consortium identification	*Phylogenetic placement*	*Not invaded*	*Invaded*	*Not invaded*	*Invaded*
Rhizobium leguminosarum (AF041443) (99%)	Proteobacteria, Alpha subdivision	+	+	+	+
Ralstonia eutropha (AB015605) (99%)	Proteobacteria, Beta subdivision	+	+	+	+
Burkholderia cepacia (U96927) (98%)	Proteobacteria, Beta subdivision	+	+	+	-
Aquaspirillum metamorphum (AMY18618) (98%)	Proteobacteria, Beta subdivision	+	+	+	+
Frateuria aerantia (AJ010481) (95%)	Proteobacteria, Gamma subdivision	+	+	+	+
Xanthomonas axonopodis (AF123090) (99%)	Proteobacteria, Gamma subdivision	+	+	+	+
Pseudomonas putida (AE016774) (99%)	Proteobacteria, Gamma subdivision	+	+	+	-
Chryseobacterium joosteii (AJ271010) (97%)	Flavobacteriaceae, Chryseobacterium	-	+	+	+
Flexibacter sp. (AF361187) (94%)	Flexibacteraceae, Flexibacter	-	-	-	-
Cytophaga hutchinsonii (M58768) (98%)	Flexibacteraceae, Cytophaga	-	-	-	-
Paenibacillus amylolyticus (D85396) (100%)	Firmicutes, Bacillales	+	+	+	-

[a]Persistence as measured by positive response on 16–23S rDNA ISR array.
Positive response (+) indicates majority (two of three replicates) positive;
Negative response (-) indicates majority (two or three replicates) negative

(AO) direct count method of Hobbie *et al.* [20] as previously described [12]. OD 600 nm of 1 corresponded to AO total cell counts of 2×10^8 to 5×10^{10} cells. Each culture was mixed in equal portions (10 mL each) to form the constructed community mixture. This mixture was dispensed in 1-mL aliquots and frozen at -80°C with a final concentration of 12% (v/v) sterile glycerol.

Complexity gradient communities. The complexity gradient (CG) communities were prepared from an industrial WWTP-activated sludge sample as shown in Fig. 1A. The rhizosphere of sterile wheat plants was inoculated with 1 mL of dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-8}, or 10^{-9}) of the sludge prepared in filter-sterilized plant nutrient solution. Three other sterile plants were prepared and left uninoculated. However, organisms surviving the surface sterilization procedure were likely present. Surfactant (15 ppm) was also added to each treatment to enrich for surfactant degraders. On day 13, microbial suspensions were prepared from each treatment in the complexity gradient by placing the plant root into a 25-mL filter-sterilized nutrient solution containing glass beads and hand shaking for 3 min. Rhizosphere community suspensions were frozen at -80°C in 1-mL aliquots with a final concentration of 12% (v/v) sterile glycerol. Rhizosphere samples were taken for microbiological analysis. Functional potential was determined from the number of carbon sources used by each community upon inoculation into Biolog Ecoplates (Biolog, Hayward, CA, USA). Mean number of positive responses was determined after 4 days of incubation at room temperature.

Evaluation of Inocula Characteristics

Plant growth studies. A total of six plant growth studies were performed. On day 0 of each experiment, inocula (1.0 mL) were added from the preserved samples, directly into the root zone of four separate plant growth vessels. Initial cell concentrations in inocula were the same as those reported in Table 2. The cell concentration in the constructed community mixed inocula was higher than that of the other communities (Table 2); therefore, it was diluted 1:100 before adding 1 mL to each of the four plant growth vessels to obtain a final cell density similar to that of the other treatments. Each experiment lasted 13 days. On days 3, 5, 7, and 13, one plant from each

Table 2. Evaluation of complexity gradient communities

Treatment	*Sludge dilution*	*Total cells* (10^6 *cells* mL^{-1})	*Culturable cells* (10^6 *cells* mL^{-1})	*Biolog Ecoplates*[a]	*Root dry weight (mg)*	*Shoot dry weight (mg)*
CG1	10^{-1} dilution	22.3 ± 3.2	21.4 ± 0.21	30	57.6 ± 2.62	136.2 ± 12.6
CG2	10^{-3} dilution	13.6 ± 4.8	24.7 ± 5.20	29	73.7 ± 30.5	160.1 ± 48.7
CG3	10^{-5} dilution	46.0 ± 1.52	41.9 ± 33.0	21	108.1 ± 31.1	156.8 ± 13.8
CG4	10^{-7} dilution	13.6 ± 11.2	8.70 ± 8.37	18	43.1 ± 3.9	115.5 ± 17.3
N/A	10^{-8} dilution	11.7 ± 12.6	9.47 ± 9.08	16	85.9 ± 51.6	135.1 ± 40.9
N/A	10^{-9} dilution	12.2 ± 8.68	19.1 ± 22.7	17	65.5 ± 31.5	128.0 ± 41.4
Propitious[b]	N/A	2.7 ± 1.93	1.7 ± 1.62	16	45.0 ± 10.2	103.2 ± 26.6
Constructed	N/A	1630	7300	N/A	N/A	N/A

N/A = not applicable.
[a]Mean number of carbon sources utilized (of triplicate samples).
[b]Community composed of contaminant species that survived the surface sterilization procedure or entered from the environment.

treatment was removed for plant and microbiological analyses (Fig. 1B). The plant growth experiments were repeated three times with no invader added. The invasibility of the respective inocula was tested in three other plant growth experiments.

(1) Plant measurements, total and culturable cell counts. Suspensions of rhizosphere organisms from each treatment were prepared by placing the plant root into filter-sterilized nutrient solution (20 mL) containing glass beads and hand shaking for 3 min. A portion of the resulting liquid cell suspensions (5 mL) was fixed with formalin and refrigerated until analysis by AO staining followed by epifluorescent microscopy [12, 20]. Culturable cell density was analyzed by serial dilution and spread plating onto R2A media (Difco, Detroit, MI, USA). Colony forming units were enumerated following incubation at 25°C for 3 days. Wheat root and shoot dry weight was determined after plant material was dried at 70°C for 72 h.

(2) Surfactant analysis. Wastewater was simulated by the addition of the nonionic surfactant polyoxyethylene 10 lauryl ether (POL-10) (Sigma-Aldrich, St. Louis, MO, USA) to plant nutrient solution. Surfactant was added to simulate wastewater because it represents the major source of carbon input into the system from addition of human wastewater (i.e., shower, sink, laundry, and dishwater) [14]. POL-10 was added to the plant nutrient solution on day 3 of each experiment at a final concentration of between 7 and 11 ppm. Plant nutrient solution amended with surfactant was sampled on day 3 (10 min after POL-10 addition) and on days 5, 7, and 13. Samples (1 mL) were filtered through a 0.2-μm nylon Acrodisc high-performance liquid chromatography (HPLC) syringe filter (Gelman Sciences, Ann Arbor, MI, USA) into an HPLC autosampler vial and stored at -25°C until analysis. Surfactant concentration was determined by using reversed-phase gradient HPLC in conjunction with electrospray ionization quadrupole ion trap mass spectrometry as described by Levine *et al.* [22]. Surfactant data were normalized to day 3 concentrations to account for variability in the concentration of surfactant added to each treatment.

(3) Detection of invader by culturable cell counts. *P. fluorescens* 5RL (5RL), was added to the rhizosphere of plants on day 3 of relevant experiments. This organism contains the *luxCDABE* gene cassette with a naphthalene-inducible promoter [21]. Culturable 5RL cells were readily differentiated by counting bioluminescent colonies on selective media by plating dilutions of rhizosphere suspensions on Luria–Bertani agar with tetracycline (15 μg mL^{-1}) and salicylate (50 μg mL^{-1}), an intermediate in naphthalene degradation.

(4) Community level physiological profiling. (a) Biolog GN2 plates. For each sample, a 10^{-1} dilution of the rhizosphere suspension was prepared in 0.85% NaCl and inoculated into Biolog GN2 microplates (Biolog). Color formation in each of the 96 wells was read and data were analyzed as previously described [11, 15]. (b) BD Oxygen Biosensor System. The BD Oxygen Biosensor System (BD Biosciences, Bedford, MA, USA) was used to detect shifts in substrate utilization by the rhizosphere communities by incubation of mixed community samples in the presence of 12 different carbon sources. Carbon sources tested included four sugars (glucose, rhamnose, arabinose, and xylose), four amino acids (lysine, alanine, aspartic acid, and glycine), two organic acids (sodium acetate and propronic acid), polyethylene glycol, and an unamended control. These substrates are broad-based compounds that are used by a wide range of microorganisms. Substrate solutions were prepared, plates were inoculated, and the response to oxygen depletion (i.e., respiration of the substrate) was measured with an automated fluorescence plate reader as described by Garland *et al.* [16]. Time to peak responses and normalized data were used for subsequent analyses.

(5) Community analysis using 16S–23S rDNA ISR array. A plasmid-based 16S–23S rDNA ISR array was prepared as previously described [5]. Briefly, the ISR array was composed of probes containing plasmid with 16S–23S rDNA ISR inserts spotted in 50% dimethyl sulfoxide (DMSO; Sigma) at a final concentration between 400 and 550 ng μL^{-1}. The ISR array contains 160 plasmids from an industrial WWTP 16S–23S rDNA ISR library produced from the same activated sludge used to generate the CG treatments. Each member of the constructed community as well as isolates from rhizosphere samples, and selected clones from a 16S–23S rDNA ISR library from the propitious community were also included on the array. Each glass slide contained triplicate ISR arrays. TA vector with no insert, dimethyl sulfoxide, and water blanks served as negative controls. Labeled 16S–23S rDNA ISR sequences from mixed community rhizosphere samples (days 5 and 13) from each treatment were hybridized to the ISR array. 16S–23S rDNA ISR sequences from mixed community DNA samples (2–5 ng) were amplified and labeled with Cy3 labeled dCTP (Amersham Pharmacia, Peapack, NJ, USA) using techniques described by Cook *et al.* [5]. The kanamycin gene from the pCR®2.1-topo vector was used as reference DNA by PCR incorporation of Cy5-dCTP (Amersham Pharmacia) into a portion of the gene [5]. The Cy3 labeled target and the Cy5 labeled reference standard were mixed in equal portions and hybridized to the array simultaneously. The arrays were hybridized, washed, and analyzed as described previously [5]. Signal intensity and local median background for each slide were determined by using Imagene 4.0 (BioDiscovery, Inc., Los Angeles, CA, USA) analysis software. Signal intensities were corrected for background and negative control fluorescence to obtain the final hybridization

intensity (FHI). Normalized signal intensity for each sample spot was calculated by dividing the FHI intensity of the sample spot by the corresponding intensity of the reference DNA spot ($Cy3\text{-}Sample_{FHI}/Cy5\text{-}Reference_{FHI}$). Points were excluded from the analysis if they possessed a Cy-3/Cy-5 ratio greater than 2.2 (values with Cy3/Cy5 more than 1 standard deviation from the mean). Values with final FHI ratios over 0.2 were set to positive (1). Values with negative normalized intensities or with FHI ratios below 0.2 were set to negative (0) signifying the absence of hybridization.

Extraction of DNA for Molecular Analysis. Rhizosphere suspensions (10–15 mL) for molecular analysis were filtered onto Supor filters (Gelman Sciences) and stored at -80°C until analysis. Mixed-community DNA was extracted using the Ultra Clean™– Soil DNA Isolation kit (MoBio Laboratories, Solana Beach, CA, USA). Extractions were carried out according to the manufacturer's instructions, except that bead tubes were placed in a Fast Prep FP120 (QBiogene, Carlsbad, CA, USA) for 30 s at a speed of 5.5 m s^{-1} instead of vortexing. The concentration of extracted DNA was quantified by using the PicoGreen ds DNA quantification kit (Molecular Probes, Eugene, OR, USA).

Statistical Analysis. Analysis of variance (ANOVA) was performed with SPSS version 11.0 (SPSS, Chicago, IL, USA) with mean differences significant at the 0.05 level. Tukey's *post-hoc* tests were used for multiple comparisons between groups. Data were normally distributed, and transformations were used when the assumption of equality of variance was not met with untransformed data. Differences in CLPP among communities were evaluated by using principal component analysis (PCA) and data were further analyzed by comparing principal component scores among treatments using ANOVA. Presence/absence data from array studies were analyzed via categorical principal component analysis (CatPCA). CatPCA is a technique similar to standard PCA, in that a large number of variables can be reduced to a smaller number of factors based on which original variables have the highest correlations with the principal component factors; however, CatPCA permits modeling of nonlinear, categorical relationships between variables. Differences among communities were further evaluated by ANOVA analysis of CatPCA object scores.

Results

Development and Selection of Inocula. The six communities used for evaluation in these studies were produced by either (1) combining isolates to form a defined or constructed community or (2) using a dilution to extinction approach to form a complexity gradient of communities. The constructed community consisted of 11 isolates mixed in equal portions (Table 1 and Fig. 1A) [4]. Selection of isolates for inclusion in the constructed community was based on: (a) similarity to clones from a 16S rDNA library from a hydroponic system receiving a daily influx of wastewater, (b) fit into similar physiological groups as common clones, (c) similarity to commonly isolated rhizosphere organisms, and (d) ability to degrade surfactant.

The CG communities were prepared from industrial WWTP-activated sludge (Fig. 1A). To obtain a gradient in microbial complexity (i.e., a gradient in the number of different types of species present) without a gradient in overall density, surface sterilized wheat plants were inoculated with dilutions of industrial WWTP sludge and allowed to regrow. After 13 days of growth, cell density was similar in all treatments (Table 2). Carbon source utilization, as determined on Biolog Ecoplates, was reduced in the high dilution treatments (10^{-8} and 10^{-9}) to a level similar to the uninoculated plants, indicating that these treatments had a rhizosphere community structure similar to the contaminant community. Based on this analysis, communities from the 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} treatments (designated CG1, CG3, CG5, and CG7; Table 2) were chosen for use as rhizosphere inocula in subsequent experiments.

The microbial community resulting from growth of contaminant species in the rhizosphere of uninoculated plants was designated the propitious community (i.e., community of potential or chance). After 13 days of growth in the plant root zone, this community had a cell density similar to that found in the other treatments (Table 2). Sequence analysis of clones from a 16S rDNA clonal library prepared from total genomic DNA extracted from the propitious community inoculum suggests that there were only three types, *Enterobacter* sp. (four clones), *Pseudomonas* sp. (two clones), and *Burkholderia* sp. (four clones). This is supported by species diversity analysis on R2A media that showed a predominance of only one type with only two to three morphotypes in total. Because culturability was high in this treatment (86%), these results, in addition to the genetic analysis, would suggest that this population was dominated by only those three to four morphotypes.

Evaluation of Inocula Characteristics. The six inocula were added as separate treatments into the rhizosphere of surface sterilized wheat and community characteristics were evaluated 3, 5, 7, and 13 days following inoculation (Fig. 1B).

(1) Plant biomass and microbial cell density. Neither total and culturable cell density nor shoot and root dry weight were significantly different among experiments with or without invader added ($P = 0.05$); therefore, data for

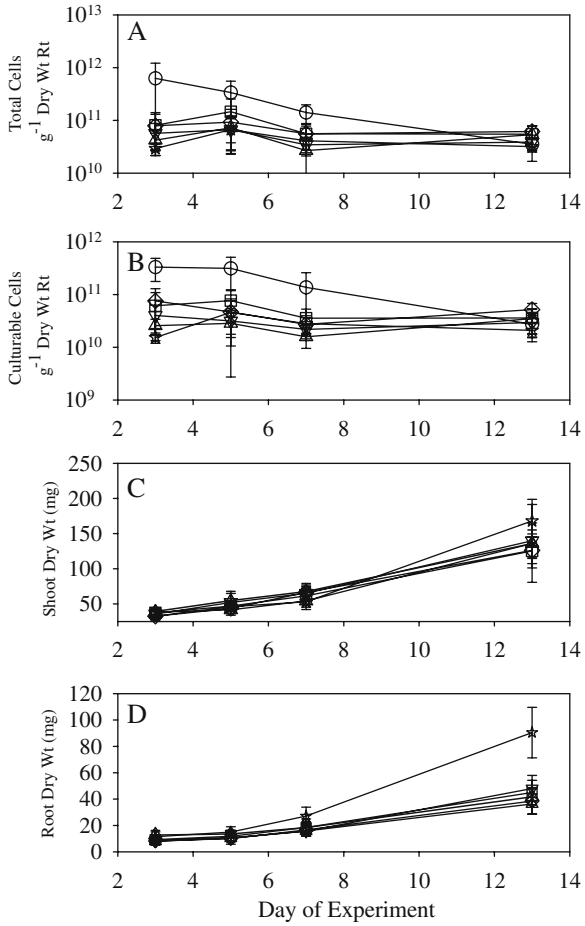


Figure 2. Total (A) and culturable (B) cell counts for each treatment. Shoot dry weight (C) and root dry weight (D) for plants from each treatment. Samples were taken on days 3, 5, 7, and 13. All values are reported as the average of six replicate experiments. Bars represent standard deviation. Constructed community (☆), CG1 (□), CG3 (△), CG5 (▽), CG7 (◊), and propitious community (○).

each treatment from each of the six experiments (three with invader and three without invader) were averaged. There were no significant differences in culturable or total cell density among inocula from the constructed, CG1, CG3, CG5, and CG7 communities ($P = 0.05$) (Fig. 2A, B). However, total and culturable cell densities for the propitious community were between 15% and 40% higher than the next highest treatment until the final day of sampling. Cell density in the propitious community inoculum, added on day 0 of the experiment, was at least 18% lower than were inocula for the other treatments (Table 2). Therefore, the increase in cell density occurred after inoculation. On the final day of sampling (day 13), however, there were no differences in culturable or total cell density among any of the treatments. Culturability (the ratio of culturable to total cells) was 86% (±7.0%), 64% (±12%), 63% (±4%), 71% (±11%), 71% (±7%), and 77% (±15%) for the propitious, constructed, CG1, CG3, CG5, and CG7 communities, respectively.

There were no significant differences ($P = 0.05$) in shoot dry weight for any of the treatments at any of the sample points (Fig. 2C). Root dry weight was the same for all treatments on days 3 and 5 (Fig. 2D). By day 7, the root dry weight of plants inoculated with the constructed community was higher than that of plants inoculated with all other treatments except CG1 ($P < 0.05$). On day 13, root dry weight was significantly higher for plants inoculated with the constructed community than any of the other treatments (Fig. 2D).

(2) Surfactant degradation. There were no significant differences in surfactant degradation among experiments with or without invader added ($P = 0.05$), therefore data for each treatment from each of the six experiments (three with invader and three without invader) were averaged (Fig. 3). Surfactant was degraded more rapidly and more completely in the rhizosphere of plants inoculated with the constructed community than in the rhizosphere of plants inoculated with other communities (Fig. 3). However, surfactant degradation had increased in plants inoculated with the CG1 treatment by the end of the experiments (Fig. 3).

(3) Detection of invader. To evaluate susceptibility of the communities to invasion, the invader species *P. fluorescens* 5RL (5RL) was added to the root zone of plants on day 3 of three of the six experiments. Although background growth was observed on the selective media used for isolation, invader colonies were readily detected via light production. The detection limit on plates was 10^2 cells mL^{-1} or 10^3 cells g^{-1} dry weight root. No

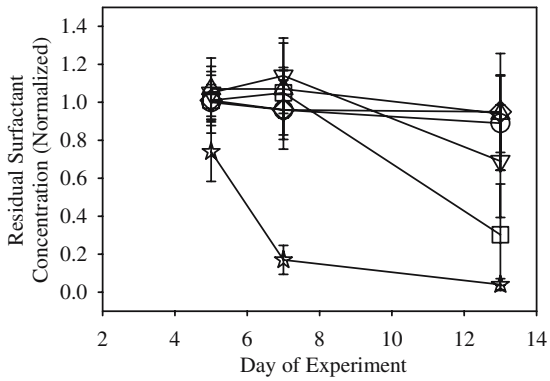


Figure 3. Residual surfactant concentration in each treatment. Surfactant degradation was normalized to the surfactant concentration as measured on day 3 of each experiment. Samples were taken on days 3, 5, 7, and 13. All values are reported as the average of six replicate experiments. Bars represent standard deviation. Constructed community (☆), CG1 (□), CG3 (△), CG5 (▽), CG7 (◊), and propitious community (○).

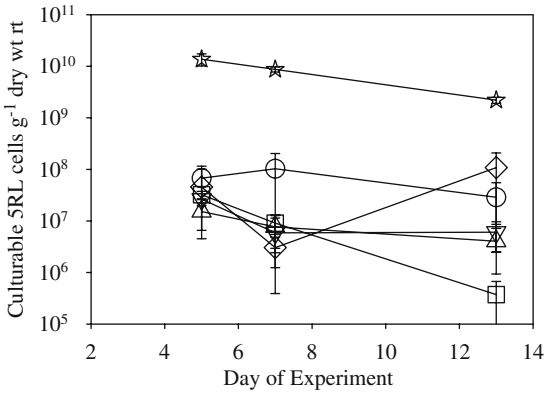


Figure 4. Invader culturable cell numbers. Samples were taken on days 3, 5, 7, and 13. Culturable cells numbers are reported as the average of triplicate experiments. Bars represent standard deviation. Constructed community (☆), CG1 (□), CG3 (△), CG5 (▽), CG7 (◊), and propitious community (○).

bioluminescent cells were observed in any treatment before invader was added. Within 4 days of inoculation with 5RL, the concentration of the invader was significantly higher in the rhizosphere of plants inoculated with the constructed community than in all other treatments, and it remained significantly higher throughout the course of the 13-day experiments ($P < 0.001$; Fig. 4). In contrast, the concentration of the invader in the rhizosphere of plants inoculated with CG1 (the most diverse treatment) was significantly lower than that of the constructed, CG5, and CG7 treatments ($P < 0.05$), and less than CG3 at the $P = 0.06$ level on the final day of sampling.

(4) Community level physiological profiling. PCA analysis of Biolog GN2 (GN2) and BD oxygen biosensor plate (BD) CLPP data showed that the most significant effect of the data was the separation of the constructed community from all of the other treatments regardless of day of experiment, invasion, or surfactant addition (Fig. 5A, B). The first principal component (PC1), reflecting the primary effect in the data (61% and 30% of the variance for the GN2 and BD data, respectively), separated the constructed community from the other communities ($P < 0.001$). The first PC of the BD data also separated CG1 from the other treatments ($P < 0.005$) (Fig. 5B). Analysis of BD data from only the CG treatments separated CG1 from all other treatments ($P < 0.05$) and CG7 from all other treatments ($P < 0.01$) on PC1. Correlation of the PCs to specific loading factors (i.e., carbon sources) indicated that the CG communities (particularly CG1) more strongly utilized carbohydrates (specifically, arabinose, xylose, and rhamnose). In contrast, the constructed community pattern was negatively correlated with carbohydrate utilization and more positively correlated with utilization of amino acids (specifically, glutamic acid, asparagine, and aspartic acid). The total number of carbon sources utilized was obtained by counting the number of positive GN2 responses after 48 h of incubation at room temperature. The number of positive responses for the constructed community was intermediate to those for the mid- to high-diversity (CG1, CG3, and CG5) and the low-diversity (propitious community and CG7) treatments (Table 3).

(5) Community analysis using a 16S–23S rDNA ISR array. The array was used in a fingerprinting capacity in these studies, owing to the finding of high levels of cross-hybridization. Cross-hybridization was unexpected because hybridization had been very specific in preliminary tests using this array [5]. Therefore, array data were converted to presence/absence data based on the Cy3-Sample$_{FHI}$/Cy5-Reference$_{FHI}$ ratio and the resulting data matrices were analyzed by CatPCA. Differences among communities were further evaluated by ANOVA analysis of CatPCA object scores. Separation of communities by pattern analysis showed that the constructed community (day 5 after invasion) was significantly dif-

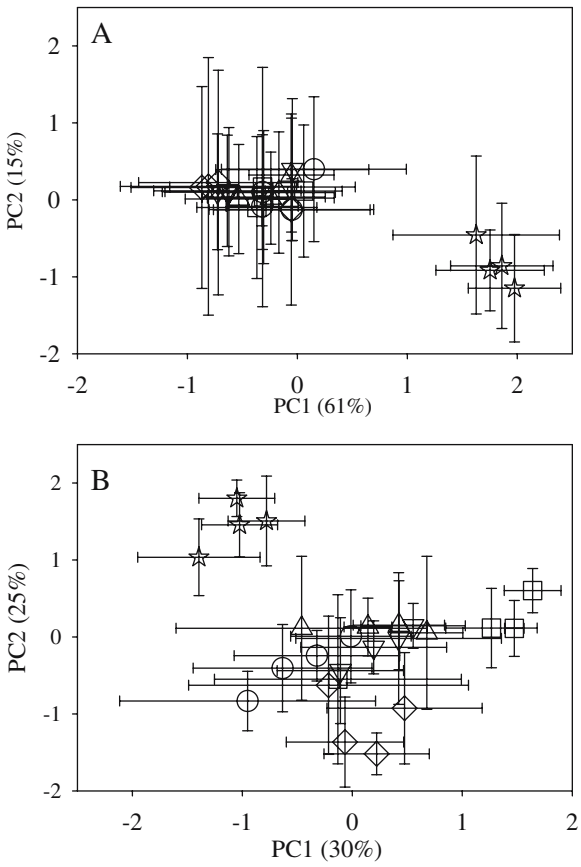


Figure 5. Principal component analysis of Biolog data (A) and BD Oxygen Biosensor data (B) for all treatments. Samples were taken on days 3, 5, 7, and 13. Points represent the average of six experiments. Bars represent standard deviation. Constructed community (☆), CG1 (□), CG3 (△), CG5 (▽), CG7 (◊), and propitious community (○).

Table 3. Percentage of positive responses from Biolog CLPP analysis

	Day 5[a]		*Day 13*[a]	
	Not invaded	*Invaded*	*Not invaded*	*Invaded*
CG1	88 (05)	76 (08)	86 (10)	83 (10)
CG2	81 (06)	73 (06)	88 (01)	70 (09)
CG3	81 (03)	74 (02)	83 (10)	75 (06)
CG4	68 (15)	44 (12)	75 (10)	65(19)
Propitious	67 (07)	51 (03)	69 (01)	68 (09)
Constructed	75 (09)	59 (17)	67 (13)	73 (04)

[a]Average percent positive (standard deviation).

ferent from CG3, CG5, CG7, and the propitious community ($P < 0.01$; Fig. 6B). This effect was not statistically significant in day 5 uninvaded samples, although there is a clear separation of the constructed community from the CG communities (Fig. 6A). By day 13, the distinction between the constructed community and the CG communities was no longer evident (Figs. 6C, D). Similar analysis of array data from CG communities showed that CG1 was significantly different than CG5 and CG7 ($P < 0.05$), but not CG3.

Qualitative analysis of the presence/absence of specific organisms was used to evaluate the persistence of members of the constructed community 5 and 13 days postinoculation (Table 1). Two members of the constructed community, *C. hutchinsonii* and *Flexibacter* sp., both members of the *Cytophaga–Flavobacterium* (CF) cluster of the *Cytophaga–Flavobacterium–Bacteriodes* division, were not detected in any rhizosphere samples by the ISR array. Nine other members of the constructed community were consistently detected in both day 5 and day 13 samples (Table 1). Three members of the constructed community (*Pseudomonas putida*, *Burkholderia cepacia*, and *Paenibacillus amylolyticus*) were present in day 5 and day 13 samples that were not invaded, and in day 5 samples that were invaded, but were not detected in day 13 samples that were invaded. These results suggest that most

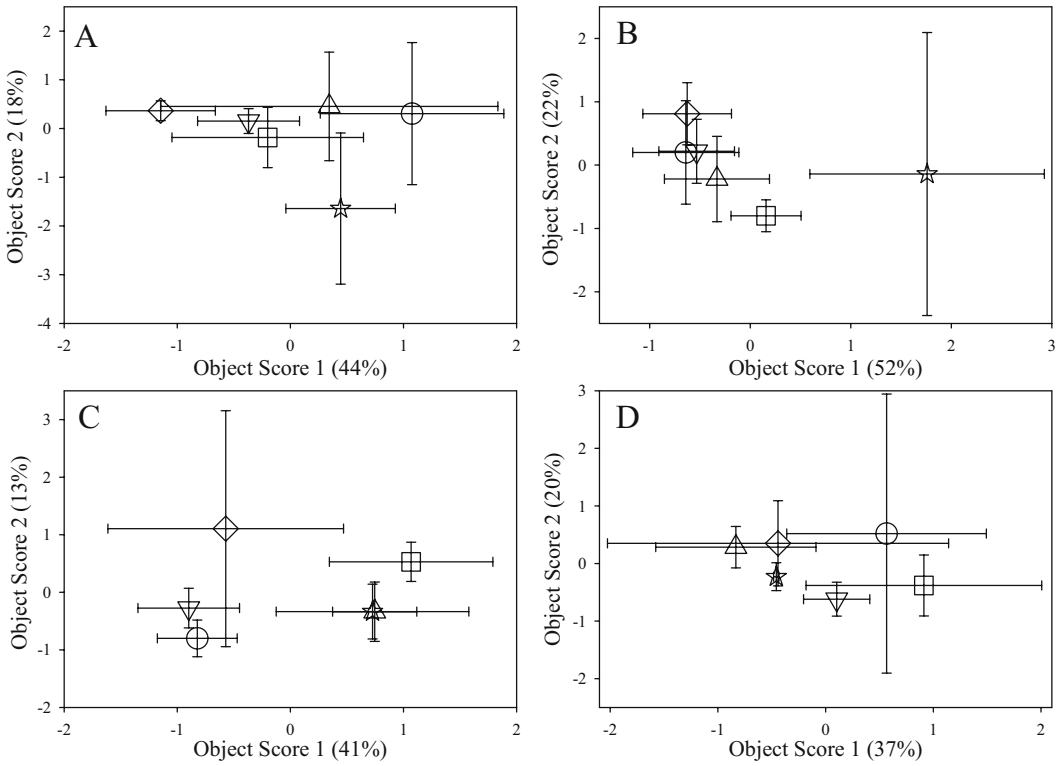


Figure 6. Categorical Principal Component Analysis (CATPCA) of array data from day 5 samples, not invaded (A); day 5 samples, invaded (B); day 13 samples, not invaded (C); day 13 samples, invaded (D). Error bars represent standard deviation of triplicate samples. Constructed community (☆), CGI (□), CG3 (△), CG5 (▽), CG7 (◊), and Propitious community (○).

members of the constructed community remained in the rhizosphere for the duration of the experiments, but invasion did have a measurable effect on the structure of the constructed community.

Discussion

In these studies, we found that greater species richness resulted in better community function (degradation of surfactant) and community stability (resistance to invasion) in a complexity gradient of microbial communities derived from a WWTP sludge population. However, the constructed community of isolates from a plant rhizosphere-based WPS persisted at the same levels and over the same duration as the communities from the complexity gradient, utilized a similar number and range of carbon sources, and degraded surfactant to a greater degree than the other treatments, but this community was invaded to a greater extent than any of the other communities. These results suggest that community function and community stability, as determined by carbon source utilization and resistance to invasion, are influenced as much by the structural composition of the community as by the overall diversity within the community.

The four communities in the complexity gradient (CG1, CG3, CG5, and CG7), created using a dilution to extinction approach, had similar cell densities, but differed in species richness. Other studies have shown that the dilution to extinction approach produces communities that differ in structural diversity as measured by terminal-restriction fragment length polymorphism, amplified fragment length polymorphism, Shannon's diversity index, and CLPP analysis [10, 13, 25]. In this study, measures of functional and structural differences among treatments suggest that CG1 was distinct from the rest of the CG treatments, CG3 and CG5 were intermediate, and CG7 exhibited lower overall richness. Separation of the highest diversity treatment from the other CG treatments suggests that the initial sludge community was unevenly distributed with some highly dominant species. Franklin *et al.* [10] used numerical simulations and batch bioreactor cultures to show that in unevenly distributed communities, the first dilution along a gradient resulted in the removal of a large number of types. The communities resulting from the initial dilution were more evenly distributed, and differences among subsequent dilutions were small until the dilution factor exceeded the original number of types of organisms in the community [10]. In this study, the CG1 community was able to degrade diverse carbon sources including surfactant and was able to resist invasion from an outside species. Subsequent dilutions resulted in no consistent differences in system function or stability; however, the lowest diversity CG treatment (CG7) was different based on structural and functional analyses, was invaded to a greater extent than the other CG treatments, and lacked the ability to degrade surfactant.

Differences in patterns of carbon source utilization, structural analysis obtained using the 16S–23S rDNA ISR array, and susceptibility to invasion suggest that there were key structural differences between the constructed community and the complexity gradient communities. Both Biolog GN2 and BD CLPP methods separated the constructed community from all of the other communities. The difference between treatments was stronger than the effect of invasion, time, or surfactant degradation. However, the constructed community was very similar to the other communities in the ratio of culturable to total cells, in the level of persistence of the population, and was more effective than the other communities in degradation of surfactant. All communities utilized approximately the same number of carbon sources (Table 3). Additionally, the time to peak measure for BD response, used by Garland *et al.* [16], was not effective for discrimination of these communities, because all of the communities had similar rates of response. Therefore, the difference between the constructed community and the other communities was not correlated to a diminished functional response, but instead reflected differences in the extent and type of carbon sources utilized; the constructed community preferably degraded amino acids, whereas the other communities more readily degraded sugars. This difference in carbon utilization strategy between the constructed community and the other communities may reflect a physiological difference resulting from the constructed community's greater ability to degrade surfactant, or it may reflect a fundamental difference between the culturable organisms in the constructed community and the mixture of organisms in the other treatments. This difference in carbon source utilization suggests that differences in microbial community structure may be more important to community function than are differences in species richness.

Within 3 days of inoculation, significantly higher cell density was detected in the rhizosphere of plants inoculated with the propitious treatment than in the rhizosphere of plants inoculated with the other treatments. This increase in cell density occurred despite the fact that initial cell density of inocula was the same in all treatments when added to the rhizosphere (Table 2). The observed increase in cell density was unexpected, but has been observed in other studies [8, 10, 18]. Alternative explanations for this phenomenon included the following: (1) differential grazing pressure among treatments; (2) release of the population from competition due to low diversity; and (3) domination of the populations by early colonizers that are generally fast growing, generalist species with a broad-niche width, and high investment of energy in reproduction [10, 23]. Low diversity in this treatment was supported by the phylogenetic data suggesting that the community was dominated by a limited number of

phylotypes (*Enterobacter* sp., *Burkholderia* sp., and *Pseudomonas* sp.). These phylotypes have been shown to be early rhizosphere colonizers with *Enterobacter* sp. often dominating in the endorhiza [24]. These results suggest that some portion of the contaminant species may have been present inside the seed cover and unaffected by surface sterilization of seeds.

Surfactant was degraded more completely in the rhizosphere of plants inoculated with the constructed community than in the rhizosphere of plants inoculated with the other communities. Six different isolates included in the constructed community were capable of growing on the surfactant [4]. Based on array analysis, five of the six organisms known to degrade the surfactant were detected over the course of the 13-day experiments. These results agree with those of other researchers who suggest that assembly of artificial communities of organisms predisposed to degradation of targeted compounds for bioremediation [9, 19] and biocontrol [3, 31, 41] in rhizosphere systems is often successful when competition from indigenous organisms is low.

Surfactant addition had a negative impact on the root dry weight of plants inoculated with organisms that did not completely degrade the surfactant. This may be attributable to the fact that surfactants are surface-active agents that increase the permeability of the plant cell wall causing root leakage. We hypothesized that an increase in total microbial biomass should be observed in treatments with low degradation of surfactant (CG3, CG7 and propitious treatments especially); however, no increase was detected, suggesting that either the root leakage was not pronounced or that the additional carbon was respired rather than assimilated into biomass. Failure of most of the CG communities to degrade surfactant suggests that the sludge communities were not enriched for surfactant degraders despite the addition of 15 ppm POL-10 to the rhizosphere of plants used to regrow each CG community in the dilution/extinction experiment. Stoffels *et al.* [36] also found that enrichment for organisms predisposed to utilization of a particular compound fails if the culture has to be reinoculated into a new system. This loss in specific functions as a result of manipulation of the community has also been reported by other researchers, supporting the idea that processes carried out by a limited number of species are more susceptible to perturbations than are those carried out by diverse species [8, 17, 18].

P. fluorescens 5RL was chosen as an invader of the rhizosphere communities to evaluate the stability of the inocula. This organism was chosen because (1) this strain survives in the rhizosphere [7], (2) *P. fluorescens* strains have plant growth promoting properties [41], (3) the presence of the *lux* cassette allows monitoring of the organism in the rhizosphere through detection of light production on plates [40], and (4) the *lux* cassette is inducible, suggesting that its presence will not produce an additional metabolic drain on the cell while it was in the rhizosphere [21]. Resistance to invasion was used as a model for ecosystem stability because resistance to invasion is a fundamental measure of community in transience [34]. Additionally, resistance to invasion (i.e., competition among species) is of special importance in environmental systems where inoculants have limited success in competition with indigenous flora [27, 42].

Complexity gradient communities and even the propitious community resisted invasion to a greater extent than the constructed community. The invader quickly died off in the highest diversity CG treatment (CG1), whereas the CG7 treatment was the only one to have a net increase in invader concentration over time (42%). This trend was the same as that observed by Matos *et al.* [25]. They found that higher complexity treatments were more resistant to invasion than lower complexity treatments. However, their constructed or gnotobiotic community was invaded to the same extent as the lowest complexity treatment, although molecular and functional analysis suggested that the community richness was more similar to the intermediate diversity treatment. Similarly, our data suggest that the constructed community was as structurally and functionally diverse as several of the CG treatments (CG5 and CG7) and the propitious community, but it was invaded to a significantly greater extent. In fact, three members of the constructed community were displaced or disappeared in response to invasion by 5RL suggesting that invasion had structural, physiological, and/or environmental effects that resulted in significant changes in the composition of the constructed community. Two alternative explanations can be presented for this finding: (1) the absence of key process species left these constructed communities open to invasion or (2) lower overall biodiversity presented open niches for incorporation of the invader. It should also be noted that success/failure of the invader depends heavily on properties of the invader itself. Once again, however, these results point to the importance of species composition in determining community performance. Mechanisms of resistance to invasion were not explored in these studies, but are warranted in light of these results and those of other recent studies [17, 25].

For application to NASA's long-term space missions and for other ecological and biotechnological applications, it is important to better understand the mechanisms that affect community function and stability. In many respects, the constructed community was very successful. Specific functions, in this case surfactant degradation, were successfully "designed" into the constructed community. The functional potential of the community was broad and was unaffected by time or invasion. However, the extreme susceptibility of the community to invasion has negative implications for application to closed systems used by NASA and for other environmental or industrial applications; loss of ecosystem function through removal or replacement of key process species and susceptibility to

invasion by potential human- or plant-associated pathogenic organisms. Data from these studies suggest that although the constructed community was functionally similar to the other communities, structural differences seem to be responsible for the two major differences between the constructed community and the other communities: (1) differences in patterns and types of carbon sources utilized and (2) differences in susceptibility to invasion. Future work should focus on evaluating the relative importance of species composition versus species richness in development of stable inocula for use in environmental and/or industrial applications.

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