Replicability of Bacterial Communities in Denitrifying Bioreactors as Measured by PCR/T-RFLP Analysis

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Bioreactors hold great promise for treating graywater in an advanced life support system for space applications. However, questions remain regarding the reproducibility and reliability of biological systems for long-term use. Although there have been numerous studies on ground-based biological systems, most studies focus on a single reactor or a simple (single carbon) waste stream. There have been very few studies on microbial communities in replicate reactors using a nonsterile, complex waste stream. In this report, we describe the characterization of five replicate denitrifying reactors receiving a complex feed, including urine and limb washes from donors at Johnson Space Center over a 100-day period. Denitrifying conditions were employed because of the ease in adding a terminal electron acceptor to the bioreactor. Bacterial populations were tracked by 16S rRNA and nosZ genes T-RFLP analysis to target the total and denitrifying microbial communities. The results demonstrated reproducible biological communities with nearly identical performance that slowly changed with time and exhibited low variability with respect to the bacterial community (T-RFLP peak area) in all reactors. These results suggest that, when designed for replication, bioreactors are not stochastic systems exhibiting chaotic behavior, but are biological systems that can be highly reproducible and reliable.

1. Introduction

Bioregenerative wastewater processing will be an important technology for long-term, manned space missions where constraints of mass and energy restrict the types of systems that can be economically placed outside Earth’s gravity well. Biological reactors have an inherent advantage over physical/chemical approaches by efficiently providing elemental recycling (e.g., carbon, nitrogen, etc.) at ambient temperatures, low pressures, and low energy inputs (I). However, the performance of such systems also needs to be reliable, stable, and robust. Fundamental questions remain about the reproducibility of the microbial community structure within a bioreactor and how that bacterial population composition relates to performance. For example, does the same inoculum lead to the same population and reactor activity in replicate reactors, or is the system fundamentally chaotic? Unfortunately, there is little information in the literature on the study of microbial populations in replicate reactors. Fernandez et al. (2, 3) examined the replicability of microbial communities in two sets of methanogenic reactors operating with a simple feed of glucose. Other studies of biological waste treatment have considered the population shifts within a single reactor (4–6) or several reactors operating under different conditions through time (7–9). No studies have investigated the question of replication in the microbial community using uniform inocula and operating conditions with a complex feed that would be typical of an advanced life support (ALS) system. Understanding the development of the microbial community within a bioreactor degrading a complex waste stream and how that community structure relates to its function is vitally important in designing any biological waste treatment that is intended for space applications.

This investigation was undertaken to determine whether replicate denitrifying waste-processing reactors produced replicate microbial communities. Denitrifying conditions were chosen because of the ease of terminal electron addition compared to molecular oxygen, especially when considering a microgravity environment. Five identical reactors were inoculated with the same inoculum and maintained under replicate conditions for several months. Each reactor received a simulated wastewater composed of limb washes and urine from Johnson Space Center (JSC) donors from a common influent tank. Throughout the process, samples were collected for chemical- and nucleic-acid-based analyses to determine the functional replicability of the reactors. A DNA approach was used to characterize the bacteria inhabiting the five denitrifying reactors, as many bacteria from complex systems (including bioreactors) are widely believed to be “as-yet-unculturable” using traditional microbiological approaches (10, 11). Both total microbial population and denitrifying bacteria were characterized by targeting the 16S rRNA and the nitrous oxide reductase (nosZ) genes. The results suggested that highly reproducible microbial populations and biogeochemical transformations can be achieved when bioreactors are designed to create similar conditions. Additionally, the temporal variability of the microbial population within all of the reactors was found to change in a well-ordered and predictable manner. The data underscore the concept that microbial populations and reactor performance in advanced life support systems do not exhibit chaotic behavior, and efforts to improve biological reactor design and performance can focus on the construction of specific microbial communities with specific metabolic capabilities.

2. Methodology

Wastewater Treatment Reactors. The bioreactors consisted of five approximately 0.5-L packed-bed reactors with 12 ports (I). The ports allowed for sampling of biomass through septa located around the circumference and at approximately four heights from top to bottom in each reactor (Figure 1). All reactors were inoculated at the same time with the same inoculum, DBC Plus, a commercial product from Enviroflow Inc. (Manassas, VA). The packing material for the reactors was Biobale (CPR Aquatic, Inc., Arcata, CA), a high-surface-area packing material made out of thin strips of poly(vinyl chloride). The influent flow was 1.5 mL/min, with a recycle
flow rate of 45 mL/min and a hydraulic retention time of 6 h. All reactors were plumbed from a common influent tank to minimize variability in the feedstock of simulated wastewater.

The ersatz waste stream consisted of limb washes using NASA whole-body shower soap, urine from male and female donors from JSC at a 1:1 ratio, and a simulated humidity condensate solution. Nitrate additions were made to provide the terminal electron acceptor needed for denitrification. Initially, the nitrate was added directly to the influent tank as potassium nitrate (KNO₃), but 1 month into the study, a syringe pump was added that dispensed a nitric acid (HNO₃) / potassium nitrate solution directly into the feed stream for the reactors to control the pH and to provide a terminal electron acceptor. Nitrate additions ranged from 899 to 1348 mg per reactor per day during the course of the experiment.

**Sampling, DNA Extraction, and PCR Amplification.**
Wastewater samples of the reactor influent and effluent were collected twice per week for chemical analysis in the advanced water recovery system development facility at Johnson Space Center. An Orion 266 pH meter was used to determine the pH of the influent and effluent samples. Total organic carbon (TOC) was analyzed using a Shimadzu TOC-V TOC analyzer (Kyoto, Japan). Ions, including nitrate (NO₃⁻) and nitrite (NO₂⁻), were analyzed using a Metrohm 780 series ion chromatograph (Houston, TX). Biomass samples for community analysis were collected from the five reactors at four depths and on four different days (27, 66, 80, and 94 days after inoculation) by syringe. The syringe was inserted into the Biobale packing material to withdraw a sample of the biofilm and reactor fluid. The sample was filtered onto a 0.2-µm Supor filter and stored frozen until processed in the laboratory. Total genomic DNA was extracted from samples using a modified phenol/chloroform procedure described in ref 5 with five freeze/thaw cycles of freezing with liquid nitrogen and a 37 °C thaw. For 16S rRNA gene amplification, genomic DNA (<10 ng) was amplified in 50-µL reaction mixtures with 20 pmol of the universal primers 27 Forward (5’ AGA GTT TGA TCC TGG CTC AG3’) and 519 Reverse (5’ ATT ACC GCG GCT GCT G3’) per reaction. The PCR amplification parameters were as follows: 94 °C for 5 min and then 25 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 50 s, with a final extension at 72 °C for 7 min. The nosZ gene was amplified using a newly designed forward primer from the putative Cu/Zn active site 752F (ACC GAY GGS ACC TAY GAY GG) and 1773R (ATR TCG ATC ATC TGB TCG TT) (12) with thermocycling of 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. Both forward primers were labeled with 6-carboxyfluorescien (6-FAM; Applied Biosystems, Foster City, CA) on the 5’ end.

**Terminal Restriction Fragment Length Polymorphism Analysis.**
Fluorescently labeled PCR product was run on a 1% agarose gel, and the product was quantified by image analysis as described previously (13). Fifteen nanograms of PCR product was digested with MnlI endonuclease (New England Biolab, Beverly, MA) for both 16S rRNA and nosZ amplicons. All digests were in 20-µL volumes for 6 h at 37 °C. DNA was precipitated by adding 2.3 µL of 0.75 M sodium acetate and 5 µg of glycogen with 37 µL of 95% ethanol. The precipitated DNA was washed with 70% ethanol and dried briefly. The dried DNA was resuspended in 19.7 µL of deionized formamide and 0.3 µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 min before analysis. T-RFLP fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software. Peak detection was set at 25 arbitrary fluorescent units. For comparative analysis, all peaks within a fingerprint were normalized to the total area for that sample. Peaks <1% of the total area were excluded from further analysis. The presence or absence of a T-RFLP peak was used to compare any two samples with a Bray–Curtis similarity index:

\[
SIM_{ij} = \frac{2 \sum_{k=1}^{S} \min(x_{ik}, x_{jk})}{\sum_{k=1}^{S} (x_{ik} + x_{jk})}
\]

where \( S \) is the number of terminal restriction fragments (T-RFs) and \( x_{ik} \) is the abundance (peak area) of the T-RF (\( k \)) in sample \( i \). The comparative Bray–Curtis index was calculated for all sample pairs of the normalized profiles using the COMbinatorial Polythentic Agglomerative Hierarchical clustering package (http://alpha.es.umb.edu/faculty/edg/files/edgwebp.htm#COMPAH).

**Results**
**Reactor Performance (Organic Carbon and Inorganic Nitrogen Concentrations).** The bioreactors employed in this study were designed to remove organic carbon (OC) under denitrifying conditions with nitrate as a terminal electron acceptor. A comparison of the organic carbon concentrations of the feedwater and the effluent during the first 100 days of operation is shown in Figure 2.

The influent concentration is indicated with a solid line, and the mean effluent concentrations with standard deviations from the replicate reactors are illustrated by the symbols and error bars. The arrows indicate the days on which biomass samples were collected for microbial population analysis. The OC concentration of the feedwater fluctuated from day to day, as would be expected in a reactor deployed to treat real human graywater waste. The average OC in the feed over 100 days was 526 ± 80.9 mg/L. In the effluent, the average of the OC concentrations at day 4 of the study was 571 ± 29.7 mg/L OC, and at day 97, the average was 145 ± 8.1 mg/L OC. The OC removal efficiency was initially around 19% but
increased to 75% by day 97. The variability of TOC removal in all reactors was greatly reduced on days 50–100, as evidenced by the small standard deviations (Figure 2). On days 80 and 90, there were spikes in the influent TOC that corresponded to higher effluent carbon concentrations, reflecting the inherent variability of a complex waste stream from human donors. The carbon removal efficiency of the reactors generally returned to prespike levels within 24 h. For example, on day 94 (a sample date for molecular analysis), there was a sharp increase in effluent carbon to 330 mg/L, with the level returning to 177 mg/L by day 95.

Error bars represent one standard deviation. Inorganic nitrogen (N) in the form of nitrate and nitrite was also measured in the effluent on a twice-weekly basis (Figure 3). Nitrate additions were 899 mg of NO₃⁻–N per reactor per day from day 28 to day 60. On day 61, the nitrate additions were increased to 1348 mg per day. Finally, on day 91, the nitrate was decreased to 1252 mg per day. The changes in the nitrate additions were related to shifts in maintaining a pH of around 7–8. Nitrate concentrations were generally low in the effluent, around 46 mg per day from day 30 to day 61 (data not shown), but nitrite concentrations were generally >550 mg per day until day 38, leading to the high sum of nitrogen in the effluent. These results suggest that the microbial populations were merely reducing nitrate to nitrite for an electron acceptor and not completely denitrifying. On day 39, the reactors were placed in recirculation mode, and the nitrite levels dropped considerably. The removal of nitrate and nitrite reached >90% by this day and averaged around 75% removal until day 61, when the nitrate addition was increased. Nitrogen removal fell to about 40% after this until day 90, when the nitrate addition to the influent was decreased. The nitrate/nitrite removal returned to >80% but dropped to >40% by day 100.

The pH of the reactors was highly variable initially until day 38 and ranged from 6.7 to 9.1 (data not shown). From day 41 to day 59, the variability between reactors decreased, but the average pH was high (around 9.0). On day 61, when the nitric acid additions were increased, the pH declined to 7.3 and continued at this level until 80. The variability of the pH between reactors continued to decline throughout this period. Other than a spike in pH on day 94, the pH remained around 7.5 through the remainder of the experiment.

16S rRNA/nosZ Gene Analysis. To gauge the replicability of the microbial communities in the five reactors, the microbial populations were characterized by similarity analysis of T-RFLP profiles using both 16S rRNA and nitrous oxide reductase (nosZ) genes. A total of 86 samples were analyzed for the 16S rRNA gene. The samples were taken from four days at four depths per reactor for all five reactors, and on day 66, there were eight samples from reactors 1, 3, and 5, and 5 processed with two samples from each depth (see Figure 1). Fourteen of the samples were not processed because of poor extractions, loss of sample, or PCR inhibitors. An example of the 16S rRNA gene T-RFLP profiles generated from the reactors is shown in Figure 4.

For the entire microbial community, a total of 68 different 16S rRNA T-RFs were observed across all fingerprints. The mean number of T-RFs in all of the T-RFLP profiles was 14.2 ± 3.0. Overall, six peaks occurred in >90% of the reactors. Peaks 214, 273, 277, and 282 were observed in all of the T-RFLP fingerprints, and peaks 74 and 122 were in detected in 84 and 85 of the total of 86 T-RFLPs, respectively. These common peaks were also generally the largest peaks. Peaks 214, 273, 277, and 282 accounted for >40% of the total peak area in any profile. The six 16S rRNA gene peaks found in 90% or more of the samples accounted for more than 55% of the total area of any one T-RFLP. Highly similar 16S rRNA gene fingerprints were observed from day 66 to day 94, suggesting that the same population of bacteria have colonized each

FIGURE 2. Concentrations of organic carbon in the feed (−) and averages of the five reactors (■). Error bars are one standard deviation.

FIGURE 3. Nitrogen concentrations in the feed tank (−) and averages of the five reactors (●). Feed is nitrate nitrogen only.

FIGURE 4. 16S rRNA T-RFLP profiles for sample days 27 and 94.
reactor, and there are only slight differences in peak areas that might represent differences in bacterial abundance between the reactors or variability in sampling the biofilm.

In contrast to the ubiquitous peaks, the majority of the 16S rRNA gene T-RFs were observed only during specific times of the bioreactor run. For example, peak 96 was found frequently in samples at day 27, but it disappeared by day 66 and was not detected in day 80 or 94 samples. Other peaks such as 234 were found in 75% or more of the samples on day 27 but declined to 50% of the profiles by day 80 and 27% by day 94. A significant proportion of the different T-RFLP peaks were observed only once in all of the T-RFLP fingerprints during the experiment (13 peaks, or 19% of all peaks detected).

Because these reactors are designed to use nitrate as an oxidant to burn organic carbon, we also fingerprinted the denitrifier population using the nitrous oxide reductase (nosZ) genes. An example of the nosZ gene profiles within the bioreactors is shown in Figure 5. A total of 67 different terminal restriction fragments were observed for the nosZ gene in this analysis across all fingerprints. The mean number of peaks in all of the T-RFLP profiles was 9.0 ± 1.9. There were two peaks that appeared in 90% or more of the samples (173 and 438 bp). At day 27, peak 173 was usually the largest of the peaks, comprising >20 of the total area in any profile. At day 66, the dominant peak was 438, with an area of >20% of total profile, and on day 94, 425 was the largest peak with more than 45% of the total area in the fingerprints. As with the 16S rRNA gene T-RFLP, there were peaks that were prominent on some days and less abundant on others. Peak 283 was found in all samples on days 27 and 80, but it was in only 26% and 54% of the samples on days 66 and 94, respectively. Peak 395 was not detected on day 27, but it was found in 100% of the samples on day 66 and then declined in prominence by day 94. In the nosZ T-RFLP, 27 of the 67 peaks occurred in only one sample, that is, slightly more than 40% of the detected peaks occurred only once.

To ascertain whether the profiles from reactors were more similar within individual reactors than between all reactors,

![FIGURE 5. nosZ gene T-RFLP profiles for sample days 27 and 94.](image)

![FIGURE 6. COMPAH analysis on 16S rRNA T-RFLP fingerprints from all reactor samples. The date of sampling for the various nodes is indicated by the dark stripe.](image)

![FIGURE 7. COMPAH analysis on nosZ T-RFLP fingerprints from all reactor samples.](image)
FIGURE 8. Mean peak area vs coefficient of variation for 16s rRNA (□) and nosZ (△) for all samples.

samples by depth, suggesting little spatial variability during the course of this experiment. The variability between reactors was assessed by calculating the variance between ranked means of similarities within a reactor versus similarities between reactors (Kruskal–Wallis nonparametric analysis of variance). The results showed significant differences in population for the 16S T-RFLPs on days 27 ($P < 0.001$) and 66 ($P < 0.05$). By day 94, there were no significant differences within and between all reactors ($P < 0.5$ for all reactors); Bray–Curtis COMPAH analysis of the nosZ T-RFLP profiles also demonstrated a clear grouping by date similar to the 16S rRNA gene data (Figure 7), with days 27 and 94 each clustering distinctly from the other sample times.

The average similarity within a reactor ranged from 0.39 to 0.82. As with the 16S rRNA gene fingerprints, there was no clear clustering by depth in the nosZ profiles. However, the limited number of nosZ amplifications for some reactors prevented the determination of the statistical significance of mean similarities for all sampling days. Interestingly, on day 66, the overall similarity decreased compared to that of all other days ($P < 0.1$), which coincided with an increase in nitrogen additions as well as decreased nitrogen utilization and pH levels.

The COMPAH analyses implied that 75% of the community was roughly similar across all reactors and that 25% of the population differed. To determine whether small peaks or large peaks were responsible for the differences in the community fingerprints, we calculated the mean peak areas and the coefficients of variation for all of the peaks detected on a particular day. (If the peak was not detected in a particular fingerprint, it was assigned a peak area of zero for the analysis.) The results are shown in Figure 8.

The data set included samples collected throughout the 100-day run at all levels in the bioreactors ($n = 86$ fingerprints; 255 data points). Absence of a particular peak in a number of profiles or a large variation in the area of the particular peak throughout the profiles would contribute to a high coefficient of variation (CV) in the mean peak area. These results demonstrate that most of the differences between the bacterial community profiles in all reactors were associated with the smaller peaks and that the larger T-RFLP peaks were very uniform throughout the reactors when uniform inocula and operating conditions were employed. A power function could be fitted to both the 16S rRNA and the nosZ gene peak area data with $r^2$ values of 0.92 and 0.79, respectively. An overall fit to the data set yielded $r^2 = 0.85$.

**Discussion**

This study demonstrates that reactors with complex waste streams run under replicate conditions produce highly similar populations. The cluster analyses of 16S rRNA and nosZ indicate that the populations showed compositional shifts through time, but that all of the reactors showed a similar succession. As the performance of the reactors became more stable, indicated by the decreased variability of OC and nitrogen utilization, it can be seen that the community also achieved more stability. However, even at day 94, there were noticeable changes in population from day 80, particularly in the 16S rRNA gene profiles, probably reflecting the spike in TOC experienced on day 90. Our findings are in contrast to those of other studies that concluded that community structure could be extremely variable even as the performance of the reactors was very stable. For example, Fernandez et al. (3) found that methanogenic reactors had stable functioning without replication of community structure, with the most diverse system being the least reproducible. One factor that might have contributed to the variability in the methanogenic reactors could be that their substrate was glucose, which is easily utilized by any number of organisms. It is not surprising that a large number of microorganisms could metabolize the glucose in a similar manner to yield comparable performances with large shifts in the microbial community. In contrast, in this report, a very complex waste stream using urine and limb washes from JSC donors is being degraded by the microbial community and contains multiple carbon sources in variable concentrations. In our data, changes in reactor performance mirror changes in the community. This is seen in the increasing population similarity with decreasing variability in the performance among the reactors. These results are surprising in that a complex stream should provide a diverse suite of carbon compounds that could presumably promote the growth of a large number of specialized individuals. Here, we find that the numbers of T-RFLP peaks decrease during the course of operation. Clearly, carbon limitation is not the only selective force being exerted on the microbial community within our experiment, and there are other factors such as surface adhesion or nitrate utilization that play a significant role in structuring the bacterial community.

Although the replicate reactors were found to be very similar, they were not identical. There are three reasons that might contribute to the replicate populations not producing identical fingerprints. Primarily, the differences in fingerprints might result from a small inherent spatial variability within the reactors or a potential sampling artifact. Previous research has demonstrated spatial heterogeneity in various bioreactors (15–18) that was not detected in this study. In the prior work, samples were obtained on ceramic saddles, by backwash, or by manual excavation. In this study, the reactors were enclosed cylinders with blind septa from which the sample was drawn by syringe. Every attempt was made to withdraw the sample from the same depth from within the reactor, but that was difficult to achieve in practice. The differences in the community composition could reflect the spatial variability that sampling by chance encountered. Some of the variability seen in our fingerprints could simply reflect differences in biomass sample prior to processing. The second contribution to variability is the replicability of the T-RFLP method used to generate the fingerprints. The reproducibility of the T-RFLP analysis and the ability of the method to describe changes or differences in community structure have been areas of intense study (for reviews, see refs 19–21). Previously, we have shown that highly reproducible fingerprints can be obtained from complex samples (22, 23). In
our current study, we tested the methodological reproducibility of T-RFLP profiles for three separate PCR reactions from the same bioreactor sample. Our results demonstrated 97–100% similarity based on presence or absence (data not shown) for replicate samples. To assess whether the variability in the fingerprints resulted from the molecular methods, we calculated the coefficient of variance (CV) vs peak area in our replicate fingerprints. The replicates exhibit coefficients of variation from 1% to 10% across a range of peak areas similar to the range of the data in Figure 8 (data not shown), as seen in other comparisons of replicate T-RFLP analyses (24, 25). There was no correlation of variance to peak area. Because each data point from the bioreactors in Figure 8 shows 10–100-fold greater variability than the replicate fingerprints, it seems unlikely that the differences in peak areas result from methodological artifacts. Furthermore, if we remove the peak areas of zero corresponding to a profile lacking a particular peak from the CV analysis of the reactor samples, we observe coefficients of variation from 1% to 10% for all peaks within the data set, in good agreement with our replicates. This variability analysis within the reactors demonstrates that, when a peak is seen in multiple fingerprints, it is roughly the same size. Finally, a third factor might contribute to the variability seen in T-FLP fingerprints, resulting from the analysis software. Figure 9 shows a peak from two of the three replicate T-RFLP’s mentioned above where it can clearly be seen that the overall fluorescent traces are very similar. The software, however, designated one sample (in dark gray) as containing two peaks (113 and 115 base pairs) while listing the other sample (light gray) as one peak (113 base pairs). This type of error could introduce dissimilarity into the analysis that is not truly present by merging peaks within a fingerprint. Such errors probably contribute to the <100% similarity seen in replicate fingerprints when calculating similarity indices.

In conclusion, replicate bioreactors can produce replicate microbial populations with replicate performances. Our studies demonstrate that most of the community-level variability was associated with smaller T-RFLP peaks representing minor contributors to the overall reactor biomass. The T-RFLP analysis detected four types of peaks in the fingerprints: (1) small peaks generally occurring only once in the run, (2) peak of all sizes that declined in area during the course of the experiment, (3) peaks of all sizes that increased in area during the experiment, and (4) large peaks that became established early in the run and maintained large areas throughout the experiment. When each of these types of T-RFLP peaks were averaged and the CVs calculated, all peaks were found to reasonably fit a mathematical model (Figure 8), suggesting that each microbial group represented by the different T-RFLP peaks was responding in a consistent manner. These results highlight how biological wastewater treatment systems need not be chaotic systems and can be reliable and robust for an advanced life support system for exploration of space environments.

Acknowledgments

This research was supported by a NASA grant (NAG 9-1283) for advanced life support research to L.J.K. and K.D.P.

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Received for review May 11, 2005. Revised manuscript received October 7, 2005. Accepted October 11, 2005.

ES050900L