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# Survival of Human-Associated Bacteria in Prototype Advanced Life Support Systems

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ABSTRACT

The inclusion of bioregenerative life support elements (i.e., plant growth systems and bioreactors) will significantly increase the total abundance of microorganisms in extraterrestrial facilities. If the microbial communities associated with these systems (e.g., biofilms attached to plant roots or hardware surfaces) serve as reservoirs for potentially pathogenic human-associated bacteria, then bioregenerative systems may represent a human health risk. Research at the Kennedy Space Center during the past several years has attempted to quantify this risk by assessing the capacity of different human-associated bacteria to survive in prototype ALS systems. Preliminary, short-term studies indicated that many potentially pathogenic human-associated bacterial species identified from past space missions (*Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) have the capacity to grow on the roots of plants, one of the largest potential sites of microbial activity in bioregenerative life support systems. However, only *P. aeruginosa* could persist at detectable levels when competition from typical root-associated bacteria was present. Subsequent long-term plant growth experiments have confirmed the greater capacity of *P. aeruginosa* to persist in plant growth systems, although no human-associated bacteria tested to date have proliferated in the systems. Rather, relative success is measured by the rate at which bacterial numbers decrease following introduction. Recent and current studies have focused on the influence of community richness (i.e., the number of microbial species) on the ability of introduced human-associated bacteria to persist within prototype systems. Richness may be manipulated in a bioregenerative system; a stringent decontamination approach could lead to very low richness, but specific inoculation with either defined bacterial isolates or undefined mixtures of microbial communities would increase richness.

INTRODUCTION

Although microbes are capable of growing anywhere even a small amount of liquid water exists, habitats that contain organic carbon that can be used as an energy source by the microorganisms will support abundant microbial populations. The inclusion of bioregenerative life support (BLS) elements (i.e., plant growth systems and bioreactors for waste processing) will significantly increase the total abundance of microbes in extraterrestrial facilities. For example, bacterial numbers on the roots of plants within prototype hydroponic systems can be as high as $10^{11}$ cells $g^{-1}$ dry wt (3). The potential exists for the communities associated with these systems (e.g., biofilms attached to plant roots or hardware surfaces, or mixed populations in the suspended phase of reactors) to harbor microbes pathogenic to humans or to the plants (12). Management of microbial communities to minimize the potential for risk to the crew and to the plants to be used for supporting the crew is an essential component of successful BLS systems.

Recommended approaches to the management of microbial communities in ALS range from strict decontamination and control to "seeding" the system with a diverse group of microorganisms (Figure 1). One extreme would be the use of axenic plants in subsystems "bio-isolated" from the human habitat module in order to prevent the proliferation of human-associated organisms (including

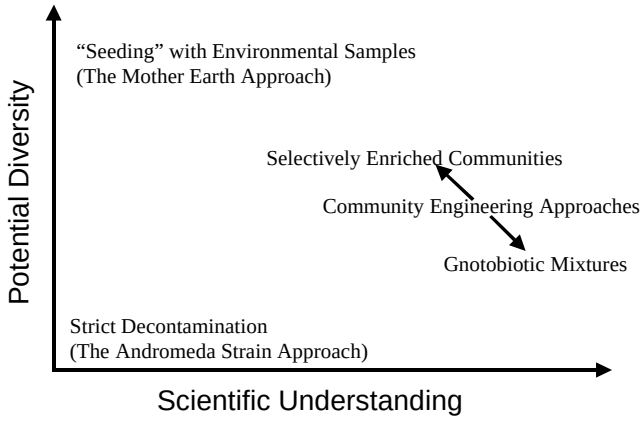


Figure 1. Approaches to inoculum preparation for hydroponic plant growth systems and bioreactors for long term space habitation. The community engineering approaches should provide substantially greater protection of the communities against invasion by human associated bacteria or plant pathogen as compared with the strict decontamination approach (which cannot be reliably maintained for extended periods), while reducing the threat of inclusion of potentially harmful microbes as part of the inoculum.

opportunistic human and plant pathogens) in plant growth systems. Alternatively, inoculation of plant systems with soil from fertile, disease-suppressive soils has been proposed to provide a stable microbial population that reproduces the balance in population dynamics found on Earth (1). An integrated approach involving quarantine, sanitation, compartmentalization, and construction of microbial communities has also been recommended (10). Another approach to community management would be to use gnotobiotic communities, defined assemblages in which all of the members are known. Generation of defined mixed cultures to carry out all of the essential processes necessary for operation of BLS systems is a daunting task, and maintenance of such defined cultures without contamination from the crew or from other systems is likely impossible. Regardless of the starting mixture, evolution of the community will eventually produce something quite different from the initial assemblage. The alternative to the gnotobiotic approach invokes the ecological paradigm that diverse communities tend to be more stable and more resistant to invasion than counterparts with few types of organisms present (e.g., 2, 5, 6, 8, 9, 14). Selection of highly diverse communities should result in narrower niches which will be difficult for pathogens or other unwanted microbes to fill. Although this concept is generally accepted by ecologists (the current emphasis on biodiversity in terrestrial ecosystems is a direct result of that acceptance), it has never been explicitly examined for microbial (i.e., bacterial) communities.

We have examined the relationship of survival of pathogens and organisms commonly used to indicate human contamination to a variety of factors including diversity of the indigenous community. The present study concentrated exclusively on human associated microbes, although the general principles derived from the results would be expected to hold true for plant pathogens as well as for human pathogens.

## METHODS

ROOT COLONIZATION – Inoculum preparation – Inocula used to compare three relative levels of diversity were prepared. A low diversity inoculum was made by rolling a sterile cotton swab over a 100 $cm^2$ wall surface of the clean room at the KSC animal handling facility. The clean room wall was presumed to contain fewer types (i.e., a lower diversity) of bacteria than the other inoculum sources used. The swab was then placed in 50 mL of R2A broth and incubated at 25°C with agitation for 55 hours. 1-mL samples from the resulting culture were frozen at -70°C. for later use. A wheat rhizosphere inoculum representing intermediate diversity (i.e., more bacterial types than the clean room wall, but fewer than soil) was prepared by cutting roots from 68-day-old wheat grown in a controlled growth chamber at the KSC ALS facility. The roots were shaken with glass beads in sterile 0.1% sodium pyrophosphate solution to suspend the bacteria. A 5-mL sample from the suspension was grown in R2A broth and stored as described above. The high diversity inoculum was prepared by placing 1 g of surface soil in 100 mL of R2A broth and incubating and storing the suspension as described above. Full details of these procedures and justification for the assumptions of diversity were presented by Morales et al. (7).

Effect of Competition – To examine the potential invasibility of plant roots in hydroponic culture by potentially pathogenic human associated bacteria, 5 strains of organisms were investigated: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas cepacia*. These strains were described in more detail by Morales, et al. (7). Wheat (*Triticum aestivium* L. cv. Yecora rojo) seeds were sterilized and seedlings prepared as described by Morales et al. (7). Roots of sterile, 5-day-old seedlings were soaked in suspensions of each of the bacteria and transferred to the slits of autoclaved foam plugs which were inserted into wide-mouth glass jars containing 1/4 strength sterile Hoaglund's nutrient solution either unamended or amended with an inoculum prepared from the rhizosphere of wheat, as described above. Plants were grown for 7 days in a plant growth chamber. (For details, see (7))

Effect of diversity and density of indigenous rhizosphere community – The effect of community diversity and density on survivability by *P. aeruginosa* was examined by inoculating wheat roots with the organism as described above, then incubating the inoculated seedlings in nutrient solution containing a microbial community of high, medium, or low diversity, prepared from soil, ALS-grown wheat rhizosphere, or clean room swab, respectively. The communities were added to the incubation vessels at two cell densities, either $10^1$ or $10^6$ CFU $mL^{-1}$ (determined on R2A medium). The plants were then grown for 7 days in the plant growth chamber.

For all experiments involving bacterial survivability on plant roots, enumerations were made initially and at day 7 of the incubation by plating methods using appropriate media. First order growth of the organism of interest was assumed during this period, and the growth rate constant ($\mu$) was determined as:

$$m = \frac{1}{t} \cdot \ln\left(\frac{N_t}{N_0}\right)$$

where t is the incubation time, and $N_0$ and $N_t$ are the number of colonies formed at time 0 and 7, respectively.

The ability of *P. aeruginosa* to invade intact rhizosphere communities was tested by growing plants with a what rhizosphere inoculum prepared as described above at three diversity levels plus a constructed gnotobiotic community. For these particular experiments, the wheat rhizosphere suspension was serially diluted and selected dilutions were added to reservoirs containing sterile 5-day-old wheat seedlings. No bacterial inoculum was added to the control plants ("uninoculated" treatment). Bacteria growing in this treatment represented the low level of contamination present in the study. After 14 days, the bacterial communities colonizing the wheat roots were harvested. Three levels of microbial community diversities (High = Full Strength; Medium = $10^{-4}$ dilution; and Low = Uninoculated) were used to inoculate the nutrient solutions of 24 sterile five-day-old seedlings at a level of $10^8$ CFU $mL^{-1}$ wheat rhizobacteria. Work in this laboratory has demonstrated that the extinction of rare types through dilution results in communities of different diversity, depending on the degree of dilution. In addition, a gnotobiotic inoculum was prepared from the high diversity treatment. After a 5-day incubation, each unique colony type (10 total) was picked from the plates and streaked for isolation. When inocula for the other treatments were prepared, the 10 strains were recombined and used to inoculate a series of seedlings as had been done for each rhizosphere dilution. The plants for all treatments were grown for 7 days, at which time a $10^7$ CFU $mL^{-1}$ *P. aeruginosa* cell suspension (450 µL into 450 mL nutrient solution) was introduced into the nutrient solution of 12 plants. The plants were grown for an additional seven days. On day 14, enumeration of *P. aeruginosa* and wheat rhizobacteria cells was performed following the methods described above. At each sampling time, portions of the suspensions prepared for plate-count enumeration were also fixed and counted directly by epifluorescence microscopy after acridine orange staining (4).

Colonies appearing on the plates used to enumerate the general heterotrophic community were also analyzed for community diversity. 25 colonies grown on R2A agar plates were randomly chosen for determination of colony morphology. Each colony was classified based on the following criteria: colony size, pigmentation, form, elevation, margin, and surface. The Shannon-Wiener index (H´) was used to calculate diversity (11, 13):

$$H' = \sum_{1}^{i} p_i \ln p_i$$

where $p_i$ is the fraction of the total individuals contained in a single type.

WASTEWATER BIOREACTORS – Reactor operation – Reactor vessels consisted of stoppered 1000-mL Erlenmeyer flasks fitted with a sampling port and gas inlet and outlet (Figure 2). Filtered (0.1 µm) air entered the flask through an air stone to diffuse the bubbles, and exited through a port in the top. Exit air passed through a column containing $CaSO_4$ (DriRite) and a 0.2 µm pore size filter and then to an infrared gas analyzer (IRGA) in which the $CO_2$ concentration was measured. A series of 6 such flasks was connected to the IRGA through a multiplexor. The gas flow through each flask was controlled at 100 mL $min^{-1}$. Each of the flasks was filled with 500 mL of sterile sewage. The flasks were mounted to the table of water bath shakers operated at 30 oscillations per min and (initially) at 30°C.

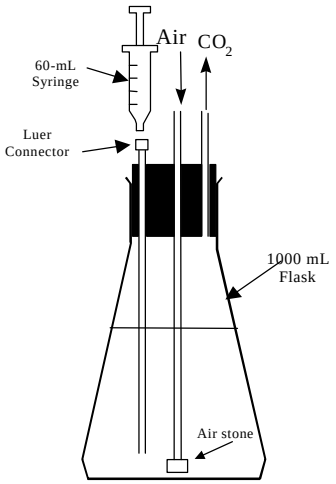


Figure 2 – Construction of the individual bioreactors.

For the experiment, a single large batch (20 L) of sewage was collected from the equilibration basin at the Cape Canaveral Air Station Wastewater Treatment Facility. The entire volume was autoclaved at 121°C for 90 min. The sterile sewage was placed in a refrigerator at 4°C where the solids settled to the bottom of the container, and the clear liquid was decanted from the top for use in the reactors.

The reactors were operated in a pulse-fed mode. At 24-hr intervals, 100 mL of suspension was removed from each flask and replaced with 100 mL of sterile, settled sewage. Gas drying tubes were exchanged for fresh ones every 48 hr.

Reactor inocula – Sewage microbial communities of different diversity were established prior to starting the bioreactors by the dilution-extinction approach. A $10^{-6}$ dilution of freshly collected sewage was made using sterile sewage as the diluent. This dilution and undiluted sewage were inoculated into a larger volume of sterile sewage (60 mL in a 125-mL Erlenmeyer flask). The flasks were incubated for 5 days on a shaker table (150 rpm). Each day, one half the liquid volume was removed and replaced with 50 mL of sterile sewage. This procedure resulted in cultures of roughly the same biomass but with substantially different diversity due to the reduced richness in the flasks receiving the diluted inoculum (Dilution causes loss of many of the less abundant strains). The 5-day-old cultures were then used to inoculate the bioreactors.

Experimental procedures – The 6 reactors were used to run 6 different treatments (Table 1) examining the effect of microbial diversity on the ability of the community to withstand perturbation by physical disturbance (temporary temperature increase) followed by invasion with a human-associated microbe (*P. aeruginosa*). The reactors were inoculated with either the high-diversity or low-diversity community and allowed to operate for 10 days with daily replacement of 20% of the reactor volume. At day 10, the

temperature in the water bath containing 4 of the flasks was raised from 30°C to 40°C and the flasks were incubated for two days, when the temperature was lowered to 30°C. On day 15, four of the flasks were inoculated with 1 mL of a culture of *P. aeruginosa* strain UG2LR (obtained from J. Trevors, University of Guelph). This organism is resistant to rifampicin, a characteristic that was used to track its abundance during the course of the experiment. Samples were

Table 1 – Arrangement of treatments in the 6 bioreactors.

| Diversity | Temperature Perturbation | Invasion with *P. aeruginosa* |
|---|---|---|
| High | yes | yes |
| High | yes | no |
| High | no | yes |
| Low | yes | yes |
| Low | yes | no |
| Low | no | yes |

taken periodically and the numbers of *P. aeruginosa* counted in the various treatments by the spread plate method with Cetrimide agar amended with 50 µg of rifampicin $mL^{-1}$.

RESULTS AND DISCUSSION

SURVIVAL AND INVASION IN RHIZOSPHERE COMMUNITIES – *S. pyogenes* failed to grow in the root mats under any circumstances (Figure3); its numbers declined rapidly during the 7-day incubation period in either the presence or absence of the added wheat rhizosphere community. Three of the 5 strains grew in the roots without

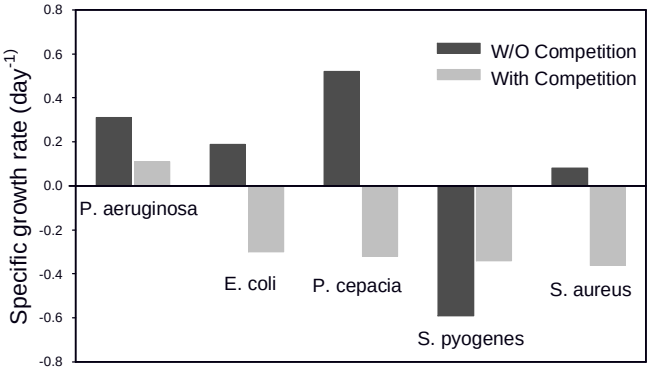


Figure 3 – Growth of human-associated bacteria in axenic plant roots (without competition) or in plant roots inoculated with microbes from a wheat rhizosphere.

the added community, but their numbers declined in the presence of competition. *P. aeruginosa*, on the other hand, grew in the rhizosphere whether in monoculture or when competitors normally associated with wheat roots were present. Competition slowed the growth of *P. aeruginosa* as compared with its growth in the axenic treatments. These results suggest that strict decontamination approaches to microbiological security in plant growth systems have little internal resistance to colonization or invasion from a contamination event. On the other hand, inclusion of a moderately diverse community prevented growth of the invaders at levels in excess of the inoculum. Longer term experiments could help determine if these organisms might persist at low levels in the systems.

When *P. aeruginosa* was introduced to the plant roots simultaneously with mixed cultures representing different degrees of diversity, the strain grew in all of the treatments except that containing the most diverse community and inoculated at the higher density (Figure 4). Estimation of the Shannon-Wiener index (H´) for each of the mixtures yielded results consistent with the predicted diversity of the source community. The community derived from the clean room

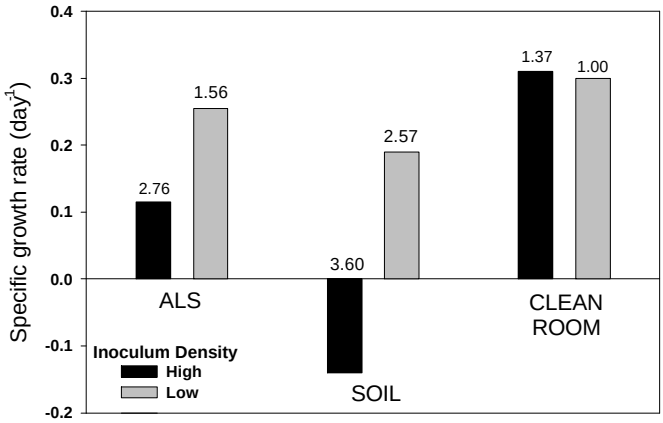


Figure 4 – Growth of *Pseudomonas aeruginosa* on plant roots inoculated with two densities of microbial communities from different environments. The number associated with each bar is the Shannon-Wiener index (H´) for each community.

swab was the lowest diversity, and growth of *P. aeruginosa* was the greatest in that treatment. There was little difference in growth of *P. aeruginosa* between the low- and high-density inoculum for the low diversity community. The intermediate diversity community (from the rhizosphere of wheat grown in the ALS) inhibited growth of *P. aeruginosa* as compared with the effect of the low-diversity, clean room-derived inoculum. In this case the higher density inoculum had a greater effect on the growth of *P. aeruginosa* as compared with the lowest diversity community. The diversity (H´) measured by enumerating colony types from the plates was also greater for the denser inoculum. In the high-diversity treatment, the low-density inoculum inhibited growth of *P. aeruginosa* almost as well as the high-density inoculum of the ALS-derived community, and the diversity was nearly as great as well. The high-density inoculum derived from soil had the highest measured diversity, and *P. aeruginosa* did not grow, but actually declined, in treatments containing this competitive inoculum. The competitiveness of the various inocula (as indicated by µ for the introduced *P. aeruginosa*) correlated well with the measured H´ in each community ($r^2$ = -0.86).

Additional community parameters were determined in the flasks that were not inoculated with *P. aeruginosa* at day 14 after inoculation with the community. Richness is the total number of colony types present on the R2A plates at the dilution used for heterotrophic enumeration. Colonies were classified as described above. Diversity refers to the value of H´ determined from the 25 random colonies. Culturability is the ratio of heterotrophic plate counts (R2A) to total counts (acridine orange direct counts). As expected, the highest richness and diversity were obtained in the treatments receiving the high diversity (undiluted) inoculum (Figure 5).

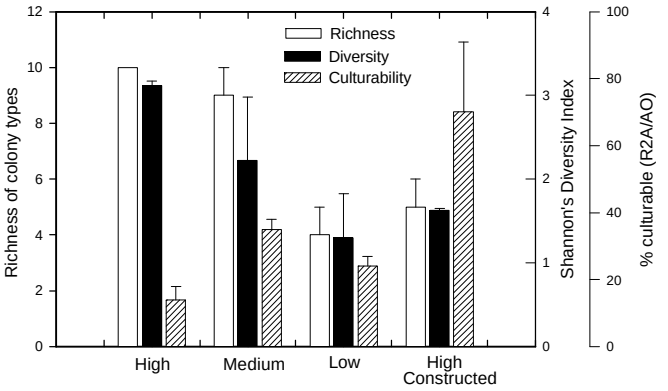


Figure 5 - Changes in rhizosphere community parameters during plant growth and invasion by *P. aeruginosa*.

The medium diversity treatment was nearly as rich as the high diversity treatment, although the mean H´ was substantially lower. This suggests that while dilution removes many of the rare strains, change in diversity reflected on the R2A plates is small, since the plates capture only a few of the most abundant microbes. Indeed, it is interesting that the plates show any difference in diversity at all, but it is encouraging that major overall changes in diversity can be followed with classical culture techniques. The low diversity (uninoculated) community, which comprised any routine lab contaminants, was lowest in both richness and H´. An average of 4 types of colonies were found in each plant root system. Although the constructed community initially contained all of the cultured members in the high diversity inoculum, only an average of 5 of them could be recovered. That the richness was maintained in the high diversity treatment, while lost in the community constructed of the abundant strains from the same source indicates an important role for the non-cultured organisms in the rhizosphere community in maintaining overall community structure, including support of the survival of strains commonly encountered in the undiluted rhizosphere inoculum. The inability to maintain all the strains in the mixed culture implies that the constructed community approach may be difficult to apply successfully in ALS systems.

The percentage culturability, the ratio of the total number of CFUs to acridine orange direct counts, was low (20%-40%) for all treatments in the diversity gradient. For the gnotobiotic community, however, the recovery of total cells as colonies on R2A medium was about 70%. Although several of the strains added to the community at inoculation did not appear on the plates, the recovery of the cultured strains was much higher than in the natural assemblages.

The invasibility of the rhizosphere communities by *P. aeruginosa* was inversely related to the diversity. That is, as the diversity decreased, the degree to which *P. aeruginosa* invaded during the 7-day period increased (Figure 6). This finding is consistent with earlier survival studies and with general ecological paradigms. In the gnotobiotic community, invasion was nearly as high as in the low diversity community, even though the original inoculum contained all the cultured types present in the high diversity treatment. This observation reinforces the earlier assertion that the uncultured fraction of organisms plays a critical role in the organization and maintenance of community structure and stability. Cultural methods can reflect large changes in the overall community structure, but they cannot explain all the manifestations of those changes. It also supports the conclusion that it may be difficult to achieve the same protective effects from invasion seen in the high diversity naturally selected communities using gnotobiotic mixtures of microorganisms.

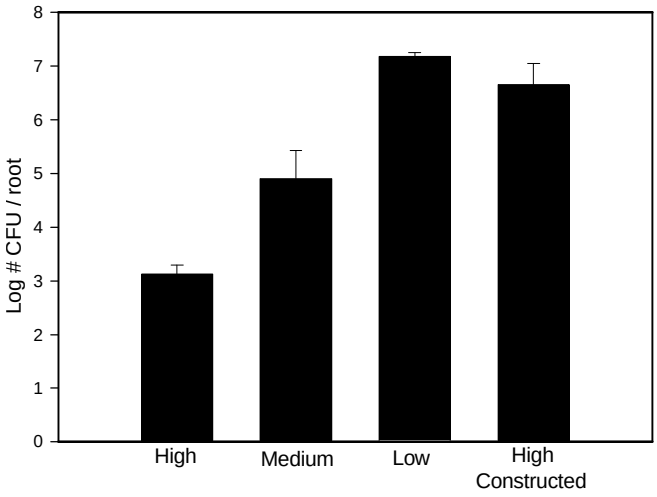


Figure 6 - Numbers of *P. aeruginosa* after 7 days of incubation in plant roots containing rhizosphere communities of different diversity. The High Constructed treatment refers to the gnotobiotic community comprising all the isolates that could be cultured from the undiluted community.

INVASIBILITY AND COMMUNITY DIVERSITY IN SEWAGE BIOREACTORS – There was a clear effect of community diversity on the ability of the bioreactor communities to withstand invasion by *P. aeruginosa*. *P. aeruginosa* did not survive well in high diversity communities whether or not the reactor had been thermally stressed. About 99% of the cells had been lost after the first 3 days of incubation whereas dilution alone would have resulted in a reduction in numbers of 50% (Fig 7). At the end of the incubation (14 days), the number of *P. aeruginosa* in the high diversity treatments had declined to $2.2 \times 10^2$ CFU $mL^{-1}$. (The detection limit for the dilutions used was about 10 CFU $mL^{-1}$.) In the low diversity flask which was held at constant temperature prior to inoculation the number of *P. aeruginosa* at the end of the experiment (day 14) was the same as in the high diversity treatments. At days 3 and 5, however, the numbers were substantially higher than for either of the high diversity treatments. *P. aeruginosa* persisted in the low diversity, thermally stressed community at 100% of its inoculation density (after accounting for dilution) for the first 5 days, and at the end of 14 days, the numbers were still over an order of magnitude higher than any of the other treatments.

SUMMARY

In these experiments, resistance to colonization of the rhizosphere and of bioreactors by human-associated bacteria was best conferred to the systems by inoculation with a diverse community of indigenous microorganisms. Such an inoculum precluded growth of even the most invasive of the

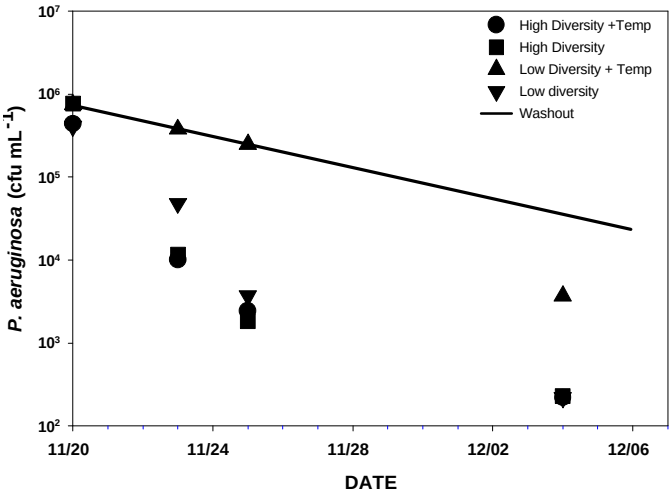


Figure 7. Survival of *P. aeruginosa* in bioreactors with communities of different diversity and subjected to different thermal regimes.

organisms tested, *P. aeruginosa*. Furthermore, a high density of indigenous microbes was best at protecting the systems from invasion. Such a density might be achieved through use of a heavy inoculum to accelerate the establishment of a diverse community in the BLS system of interest. Concern over the potential for inclusion of potential plant pathogens in soil implies that use of an undefined soil inoculum may not be advisable, although that approach offered the best protection against invasion by human associated bacteria in this study. The gnotobiotic approach was not especially successful at protecting the systems in these experiments, leaving the question open of just how to formulate an inoculum with sufficient diversity to afford optimal protection of the system without itself introducing potentially harmful microorganisms. Whereas, the use of fresh soil as an inoculum might raise the probability of growth of undesirable organisms present in the soil to an unacceptable level, selection of a diverse community from soil through growth in ground-based hydroponic systems might provide an optimized inoculum.

Because they cannot be made "germ-free," human crews moving into long-term space missions will be a source of inocula for all of these systems. It is not clear at present whether the crew's microbiota can provide a diverse inoculum to the systems to allow protection from invasion by harmful organisms, or whether they will be a source of invaders that will inhabit the BLS systems in the absence of an appropriately protective indigenous community. In the absence of information to the contrary, the latter would seem to be the more likely scenario.

Finally, the low culturability observed in the rhizosphere systems invokes additional concern over the ability of the communities to maintain a highly diverse, protective composition over a long period of time. It may be necessary to reinoculate the systems periodically to maintain the intended functions and to maintain the protective status from invasion conferred by the community. If reinoculation proves to be necessary, or even desirable, the question of inoculum source becomes an important element in survival and success of the mission.

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