Journal of Industrial Microbiology & Biotechnology (2002) 29, 286–291

www.nature.com/jim

Effect of starvation length upon microbial activity in a biomass recycle reactor

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The kinetics of substrate degradation and bacterial growth was determined in a microbial community from a biomass recycle reactor that had been deprived of substrate feed for 0–32 days. Starvation caused changes in bacterial numbers, community composition, and physiological state. Substrate starvation for less than 1 day resulted in modest (less than threefold) changes in endogenous respiration rate, ATP content, and biomass level. During a starvation period of 32 days, there were substantial changes in microbial community composition, as assessed by denaturing gradient gel electrophoresis (DGGE) fingerprinting of PCR amplicons of a portion of the 16*S* rDNA or by phospholipid fatty acid (PLFA) analysis. When the starved communities were stimulated with organic nutrients, the growth kinetics was a function of the length of the starvation period. For starvation periods of 2–8 days prior to nutrient addition, there was a phase of suboptimal exponential growth (S-phase) in which the exponential growth rate was about 30% of the ultimate unrestricted growth rate. S-phase lasted for 2–8 h and then unrestricted growth occurred at rates of 0.3–0.4 h^{-1}. At starvation times of 12 and 20 days, a lag phase preceded S-phase and the unrestricted growth phase.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 286–291 doi:10.1038/sj.jim.7000322

Keywords: biodegradation; biomass recycle reactor; starvation; growth kinetics

Introduction

Bioreactors often contain complex microbial communities that catalyze the degradation of organic waste materials. These systems have been used for a number of decades. With the appropriate microbial species and engineering design, they have functioned effectively to intensify and control mineralization of a broad variety of organic molecules.

Many systems operate as continuous flow reactors, with a relatively continuous supply of organic waste material as substrate for microbial growth. However, there are applications in which other factors provide unique problems. One example is the shipboard treatment of graywater [12]. The size of shipboard systems is highly constrained; therefore, a system in which bioreactor biomass is retained while cell-free effluent is wasted (biomass recycle reactor) has advantages in terms of the rapidity and efficiency of organic treatment. A second problem is that shipboard treatment systems may not have a continuous supply of wastewater available. These periods may last up to approximately 30 days (while in port). At issue is the impact of extended starvation upon the microbial community, when the bioreactor system is restarted. That is, how long will it take for the system to meet performance criteria? This issue is the central focus of the work described here.

In addition to the practical implications of the microbial response to substrate inputs, fundamental questions arise regarding the microbial physiology of starvation recovery. Many studies have investigated changes in gene expression and physiological properties that occur in microbes after starvation is imposed [9]. There have been relatively few studies on how microbes recover from starvation conditions. A theoretical analysis of the ecological tradeoffs involved in the physiological adaptations to starvation conditions identified the rate of recovery from the dormant state as a key factor [10]. In this study, variations in recovery dynamics were evaluated as starvation time of the mixed microbial community was varied.

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Received 4 January 2002; accepted 8 August 2002

Methods

Biomass recycle cultures

The original inoculum for biomass recycle reactors was activated sludge from the aerator of the West Lafayette, IN, municipal treatment plant. Biomass recycle reactors were run as described by Konopka *et al* [11]. The medium feed contained: 9.3 mM NH_4Cl, 3 mM NaCl, 2 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.25 mM $MgSO_4$, 5 μM $CaCl_2$, 14 μM $FeCl_3$, 23 μM disodium ethylenediaminetetraacetate, and 26 μM sodium citrate. The pH was 7.5. The medium contained 87 mg of commercial laundry detergent and 1 ml l^{-1} SL7 trace element solution [11]. The organic substrates per liter of medium were 400 mg of starch (Argo, Englewood Cliffs, NJ), 150 mg of gelatin (Difco, Detroit, MI), and 17 mg of linear alkylbenzene sulfonate (LAS, 80% by weight; Sigma, St. Louis, MO). The commercial laundry detergent (Dutch brand; Dial, Phoenix, AZ) contained 10% LAS by weight. Therefore, the actual amount of LAS in the medium was 21.5 mg l^{-1}.

The reactors were run under biomass recycle mode for at least 15 days before starvation experiments were run. Although biomass recycle systems do not attain a true steady state, previous results [12,13] showed that the biochemical composition and metabolic potential of the microbial community reached a stable state after

this period. This physiological state was maintained for up to 74 days.

Nutrient shift-up experiments were performed by inoculating samples from the bioreactor into batch cultures (50 ml of medium in a 250-ml flask). The medium contained all of the inorganic components of the reactor feed medium plus 800 mg of maltose, 300 mg of gelatin, and 10 mg l^{-1} LAS. Maltose was substituted for starch to prevent interference with biomass (turbidity) measurements. The culture was incubated on a shaker water bath (30°C) at 125 rpm to provide aeration. The initial turbidity (optical density, OD, 600 nm) of the culture was approximately 0.05. Samples were periodically removed from the batch culture, and turbidity (microbial biomass) and residual substrate concentrations were measured. For substrate measurements, samples were centrifuged at 7500×*g* for 10 min, and the supernatant was frozen at −20° C until analysis.

Analytical methods

The method of Lowry *et al* [15] was used to measure soluble protein. LAS was extracted and assayed by HPLC chromatography as described by Matthijs and DeHenau [16]. The amount of maltose was quantified by the dinitrosalicylic acid method [1]. All values presented are means calculated from analysis of triplicate samples. The coefficients of variation for these samples were typically <15%.

Carbon dioxide gas in the off-gas from the reactor was measured with an Anarad AR600-R infrared gas analyzer (Anarad, Santa Barbara, CA). Gas from the reactor was dried by passage through anhydrous $CaSO_4$, and entered the instrument at a flow rate of 2 l min^{-1}.

For analyses of dry weight and organic carbon content of biomass, 10 ml of culture was centrifuged in dried, tared centrifuge tubes at 7000×*g* for 10 min, and the cell pellet was lyophilized on a Lyph-Lock 12 freeze drier (Labconco, Kansas City, MO). The dried pellets were weighed to determine dry weight. Lyophilized cells were then analyzed in a NA1500 Series 2 CNS analyzer (Fisons Instruments, Beverly, CA) to determine the fraction of organic C in biomass.

Microbial biomass in the reactors was monitored by measuring the OD in a 1-cm cell at 600 nm. If the OD of a sample was >0.4, the sample was diluted with a solution containing the mineral salts components of the medium so that the measured value was between 0.1 and 0.4. The measured OD was divided by the dilution factor to produce an estimated biomass value for the culture. Turbidity values were converted to biomass units using the following experimentally determined conversion factors. An OD of 1.0 corresponded to 260 mg l^{-1} bacterial protein, 625 mg l^{-1} dry weight, and 23 mmol l^{-1} C.

Direct microscopic counts were made by making appropriate dilutions of samples in 0.85% NaCl solution. Direct microscopic counts were made using a Petroff–Hauser counting chamber (Hausser Scientific, Horsham, PA), with a Zeiss standard research microscope at a magnification of 400× with phase contrast optics. The proportion of cells with intact cytoplasmic membranes was determined by addition of LIVE/DEAD BacLight stain (Molecular Probes, Eugene, OR). Samples were incubated for 30 min at room temperature in the dark, and then examined by epifluorescence microscopy with a broad band filter set [14].

Cellular protein and total carbohydrate were measured as described by Herbert *et al* [8]. ATP was extracted from cells and analyzed as described in Cook *et al* [2]. The reported values are means calculated from analysis of triplicate samples. The coefficients of variation were usually <15%. Maltose and casamino acids were used as lower-molecular-weight surrogates for the presumed products of exoenzyme hydrolysis of the starch and protein macromolecules that were in the nutrient feed.

Oxygen consumption by washed resting cell suspensions was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). A total volume of 1.5 ml of washed resting cells (approximately 3×10^8 cells ml^{-1}) was incubated at 28°C, and the rate of endogenous respiration was monitored. An individual test substrate was added and oxygen consumption was monitored over the next 5 min. To measure respiratory potential (the rate achieved when cells were provided a nonlimiting substrate concentration), the concentrations of added test substrates were either 2 mM maltose, 3 mM glucose, 100 μM LAS, or 2 g l^{-1} casamino acids. These concentrations were sufficient to produce the maximum (saturated) rates of oxygen consumption.

Community analysis

Fingerprints of 16*S* rRNA gene diversity were produced by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified gene fragments [17]. DNA was extracted from bioreactor samples using the Fast DNA Extraction Kit (QBIOgene, Carlsbad, CA) in duplicate. PCR was carried out on the DNA total extract, and the products were separated by DGGE using previously described conditions [5,18]. The gel was stained with SYBR Green I (1:10,000 dilution; Molecular Probes), visualized on a UV transilluminator, and photographed.

Phospholipid fatty acids (PLFAs) were also used as biomarkers of community diversity. Phospholipids were extracted from bioreactor biomass using the method of Findlay *et al* [6]. Total lipid was extracted with methanol/chloroform/phosphate buffer (1:2:0.8) and phospholipids were fractionated using column chromatography. Phospholipid extract was esterified by mild alkaline methanolysis and the resulting fatty acid methyl esters (FAMEs) were analyzed by gas chromatography. FAMEs were determined using a Hewlett Packard Model 5890 GC equipped with a capillary column (DB-5, 60 m×0.25 mm, 0.25 μ film; J&W Scientific, Folsom, CA) and flame ionization detector [21], and data collected with Varian Star Workstation software. Identification of fatty acids was based on comparison of retention times to purchased standards (Matreya, Pleasant Gap, PA; Supelco, Bellefonte, PA) and quantified using an internal standard (nonadecanoic methyl ester).

Results and discussion

Short-term perturbations

The impact of organic substrate deprivation upon the physiological state of the microbes was determined by stopping the medium flow into the reactor. In short-term studies (<24 h), changes in biomass and the rate of C mineralization within the reactor were monitored. During biomass recycle operation, the microbial community oxidized >90% of the organic C input to CO_2 [13]. Upon cessation of substrate feed, there was an immediate decrease in the flux of CO_2. However, the rate of endogenous respiration stabilized within 1–2 h. Even after 24 h of starvation, the median rate of CO_2 evolution was 39% (range 30–50%) of the prestarvation rate in five independent experiments. Based upon measurements of bacterial dry mass and the C content of the biomass (45% of dry

weight), this rate of CO_2 evolution consumed 0.08% of biomass C per hour.

Microbial biomass (measured as particulate protein) was relatively constant for approximately the first 8 h of starvation (Figure 1). However, decreases were measured after longer incubation periods. ATP content (normalized to cell protein) was used as an indicator of the energetic status of the biomass. One hour after substrate flow was stopped, ATP content had declined by 40%. However, it subsequently recovered to levels near those found in the active biomass recycle reactor and, even after 24 h of starvation, had a value 63% of that found prestarvation.

Long-term perturbation experiments

The short-term experiments suggested that the physiological state of the bioreactor community had not significantly deteriorated over 24 h. To determine the resiliency of the community, the capacity of starved populations to reinitiate active metabolism and growth was tested in systems that were starved over 32 days.

The biomass recycle reactors were run for 16 days at a hydraulic residence time of 11 h and temperature of 30°C. Previous work [13] demonstrated that the bioreactor population attained a stable physiological state at this time, characterized by levels of RNA and respiratory potential that were approximately twofold lower than those found under chemostat operation. At this time, the biomass density was 2.1 g l^{-1} dry weight. The medium feed was stopped, but aeration of the reactor fluid was continued to maintain aerobic conditions. Samples were taken for analysis of microbial community composition and physiological state just before medium flow was stopped (day 0) and at the end of the starvation period (day 32).

The diversity of the microbial community was determined by DGGE analysis of PCR products of a portion of the 16*S* rDNA (Figure 2). The banding patterns do not necessarily represent the composition of the entire community, but do indicate the dominant phylotypes in samples, and changes in individual community members can be monitored. Microbial diversity in the biomass recycle reactor (lane 1) was highly reduced compared to that found in the original inoculum for the system, a sample from an aerobic municipal wastewater treatment reactor (lane 3). Fifteen bands

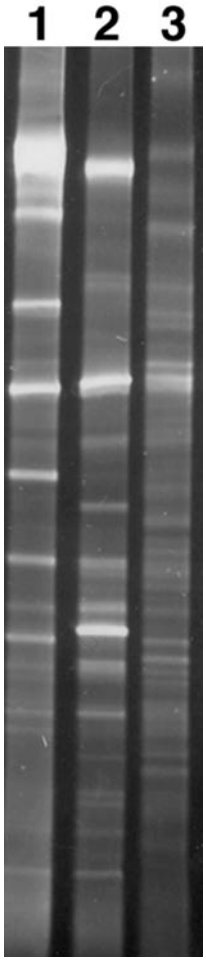

Figure 2 DGGE of bacterial 16*S* rDNA PCR products (bases 338–518 relative to the *Escherichia coli* rRNA sequence) amplified from bioreactor communities. Lane 1: Active biomass recycle reactor community, prior to starvation. Lane 2: The biomass recycle reactor population after 32 days of starvation. Lane 3: Community diversity in the aerobic reactor of a municipal wastewater treatment facility, the inoculum for the biomass recycle reactor.

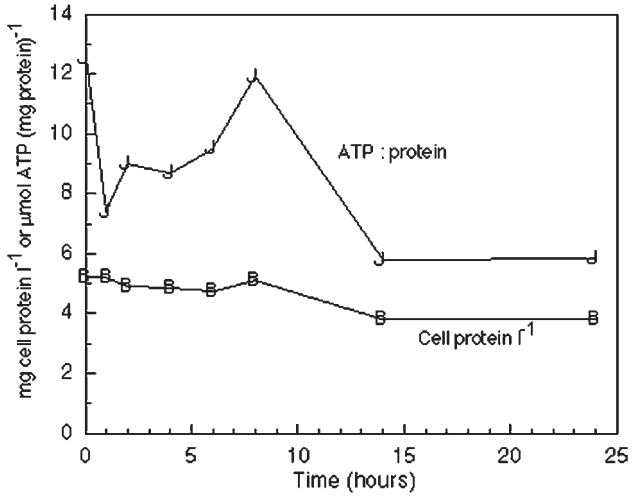

Figure 1 Changes in (●) ATP content per unit of bacterial protein and (■) bacterial biomass (measured as cell protein) after substrate flow into a biomass recycle reactor was stopped.

were observed in the active reactor (day 0, lane 1), and 16 in the population after 32 days of starvation (lane 2). Eight bands were common to the two samples. Therefore, there were substantial phylogenetic changes in the community due to starvation. This implies that species were present at low numbers in the active community (and hence undetectable by DGGE analysis) and had a selective advantage under starvation conditions, and became prominent members of the community. However, these changes are smaller than those observed in active biomass recycle reactors. A series of successional changes occurred over the first 30 days of operation, so that the proportion of shared bands between community samples taken more than 20 days apart was <30% (Konopka *et al*, in preparation).

Changes in the PLFA composition of the bioreactor population were also used to characterize the microbial population. Analysis of PLFA represents a more global view of community changes than does DGGE of 16*S* rDNA, and also permits some insights into microbial physiological state [22]. The amounts of the 19 fatty acids, which comprised >0.75 mol% of total fatty acids, are presented in Figure 3, ordered by abundance in the nonstarved

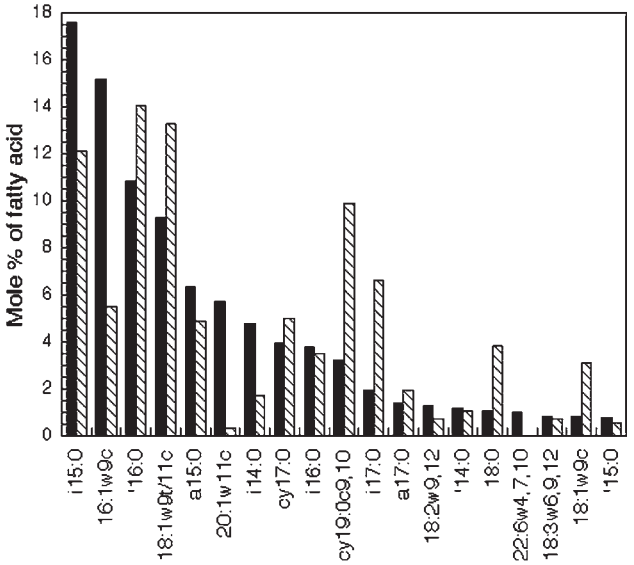

Figure 3 Histogram of the mole percent of PLFAs in the active biomass recycle reactor population and after 32 days of starvation. The fatty acids are ordered by quantitative importance in the active reactor.

community. Although the relative abundance of most PLFA did not change substantially during starvation, there were decreases in the two most abundant fatty acids (i15:0 and 16:1ω9c), as well as in 20:1ω11c, i14:0, 18:2ω9, 12, and i14:0. In contrast, there were significant increases in the following fatty acids during the 32 days of starvation: cy19:0c9,10, i17:0, 18:1ω9t/11c, 16:0, 18:1ω9c, and 18:0.

Changes in fatty acid composition can reflect changes in either community species composition or physiological state [22]. The proportion of Gram-positive fatty acid biomarkers (branched, saturated PLFAs) decreased from 35% to 31% during the starvation period. Despite the relatively small shift in total Gram-positive PLFAs, there was a substantial change in composition; i15:0 had a large decrease and i17:0 increased substantially. The proportion of monoenoic and cyclopropane unsaturated PLFAs, characteristic of Gram-negative bacteria, increased from 32% to 37%. Thus, there was some shift in the proportion of these major bacterial groups.

The major interpretable change from a physiological perspective was the decrease in monoenoic *cis* fatty acids, and an increase in *trans*-saturated or cyclopropyl fatty acids. These changes have been associated with bacterial stress responses to starvation [7]. The data do not show the quantitative specific changes of individual fatty acids that might be expected (e.g., 16:1ω9c to 16:1ω9t or cy17:0), but it is important to remember that in a complex microbial community, there are changes in species composition in addition to physiological changes in individual microbes. In general, the PLFA pattern shifted to fatty acids associated with stress responses to starvation.

Starvation caused a decrease in cell number within the reactor. However, cell number declined by only a factor of 5 over 32 days, from 4.2 to 0.8×10^9 cells ml^{-1}. This decrease in cell number was paralleled by a similar decrease in turbidity of the starved culture. To determine the number of potentially growth-competent cells, the proportion of cells with intact cytoplasmic membranes was measured. Membrane integrity was assayed *via* a membrane-impermeant fluorescent dye. Permeability to these dyes has been correlated with cell viability [14]. By this analysis, there was less than a twofold decrease (from 90% to 50%) in potentially active cells over the 32-day starvation period. Analysis of survival dynamics in the bioreactor community is complex because the death rates of individual species can vary substantially [4]. In addition, lysis of some cells would provide nutrients to sustain other cells (cryptic growth).

Kinetics of unrestricted growth

The capacity of the bioreactor population to resume active biodegradation after periods of starvation was tested in batch growth experiments. Organic substrates were provided in amounts sufficient for a 20-fold increase in biomass. In samples from an active community (Figure 4A), there was instantaneous exponential growth, at a specific growth rate of 0.41 h^{-1} (equivalent to a generation time of 1.7 h). The major organic C sources in the medium (maltose and protein) were consumed simultaneously, with kinetics typical of batch cultures. The specific rates of substrate consumption were 0.48 and 0.13 mg (mg dry weight)$^{-1}$ h^{-1} for maltose and protein, respectively. Bacteria reached stationary phase after 9–10 h. Essentially all of the maltose and most of the

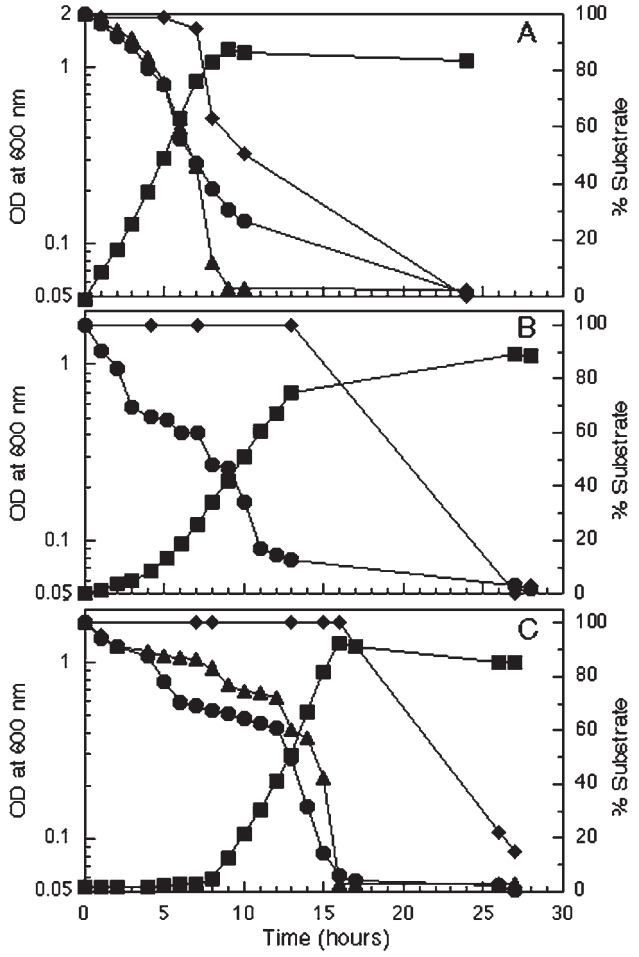

Figure 4 Batch culture growth kinetics of the biomass recycle reactor community after (A) 0, (B) 8, or (C) 32 days of starvation. (■) Bacterial turbidity (OD 600 nm). The percentage of remaining substrate is plotted for (●) protein, (▲) maltose, and (◆) LAS. At the start of the experiment, the culture contained 800 mg of maltose, 300 mg of gelatin, and 10 mg l^{-1} LAS.

protein had been degraded at this time. Note that LAS was degraded more slowly than the other substrates. Some reduction in LAS concentration was found near the end of exponential growth phase (8 h), but complete removal of LAS was not found (in this and most other experiments) until approximately 24 h.

Kinetics of growth and substrate utilization was determined for samples starved for 2, 4, 8, 12, 20, and 32 days. We identified several distinct growth phases during the experiments: (a) lag phase, (b) exponential growth at a submaximal rate (S-phase), (c) phase of maximum exponential growth rate (log phase), and (d) stationary phase, in which organic substrates had been consumed and biomass reached a plateau.

After short periods of starvation (2–8 days), there was no true lag (defined as no increase in biomass), but rather a period (termed S-phase) of exponential growth (defined as a linear increase in log biomass versus time) in which the rate was 25–35% of the succeeding log phase growth rate (Figure 4B, Table 1). These types of kinetics have not been reported very often, but were similar to what we have previously observed in experiments with samples from biomass recycle reactors operated for extended periods [13], and in experiments with *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Arthrobacter globiformis* [20].

As the period of starvation lengthened, the populations exhibited lag phase and S-phase (12 and 20 days of starvation), and finally at 32 days, only a lag phase (Figure 4C, Table 1). The log phase growth rate was greatest when the active bioreactor population was used, but the log phase growth rates of the starved populations did not systematically slow as the starvation period increased, and ranged from 70% to 85% of the day 0 value.

Starved microbial populations did not immediately respond to nutrient inputs by growth, but from a biotechnological perspective were as effective in degrading the pulse of organic substrates as the unstarved bioreactor population. This was due to their capacity to metabolize organic substrates at relatively high rates during lag- and S-phases. Specific rates of maltose and gelatin consumption during lag phases ranged from 0.4 to 0.6 mg (mg dry weight)$^{-1}$ h^{-1}. As a result, it took only a few additional hours for the major organic components (maltose and gelatin) to be consumed by populations starved for 32 days when compared to the unstarved population, despite the 8-h lag in growth.

Physiological mechanisms responsible for elevated substrate consumption under these circumstances are unclear. In general, microbial adaptations to low-nutrient conditions (as experienced in the active biomass recycle reactor and during starvation) appear aimed at maintaining high metabolic fluxes. An inevitable consequence is that catabolism is uncoupled from growth in the period following a nutrient upshift [3].

Because we normalized the inoculum for each batch nutrient upshift experiment to the same amount of total biomass, the lag- and S-phases could be artifacts due to the heterogeneous nature of the bacterial populations. If the population contained a high proportion of dead cells (unable to grow, but particles that contribute to turbidity), an increase in culture turbidity would not occur until the subpopulation of living cells proliferated. A simple mathematical model of batch growth kinetics was evaluated for different ratios of live:dead cells. The model had no physiological lag; for a short S-phase to be observed, 70–90% of the cells in the initial inoculum must be dead, and 99% dead cells were necessary for an apparent "lag phase" of 6 h to be observed. The proportion of membrane-intact cells is much higher than this; thus, we conclude that the observed lag- and S-phases are physiologically meaningful.

The above experiments were designed to determine the resiliency of a bioreactor community, i.e., its ability to recover from starvation and reinitiate active biodegradation. If the bioreactor system were to fail (due either to extended starvation during down time, or during operation), it would be necessary to restart the system as quickly as possible. Methods to restart a failed bioreactor rapidly were evaluated. For this purpose, growth and substrate degradation kinetics was determined for several inocula. (1) An active bioreactor culture was lyophilized. (2) An active bioreactor culture was quick-frozen (in a dry ice–ethanol bath) and stored at -20°C for 30 days. Ten percent glycerol was added to these preparations as cryo-protectants. Note that glycerol could then serve as an additional C source for bacteria during subsequent cultivation. For comparative purposes, (3) a generalist microbial community (a sample of activated sludge from the municipal wastewater treatment plant in West Lafayette, IN) was tested. The experimental design was identical to that used for the starved bioreactor samples.

The kinetics of growth and substrate utilization by the frozen inoculum was similar to those observed for material taken directly from a biomass recycle reactor (Table 1). However, the lyophilized inoculum had a long lag (>12 h) before net growth was observed. With the activated sludge inoculum (unadapted to the specific substrates in the feed medium), there was rapid growth after a lag period of 4 h. Although the substrate maltose was consumed within 14 h, the rate of protein degradation was very slow, and about 50% of the LAS remained. Presumably, there were no large populations capable of degrading specific components in the simulated graywater feed. A frozen inoculum appeared to be the best means of reseeding a bioreactor, if appropriate storage conditions were available.

From an ecological perspective [19], the bioreactor community exhibited several properties that are important for maintenance of a functionally stable system. It was very resistant to the environmental stress of energy substrate starvation; although there were substantial changes in dominant phylotypes, their richness was similar before and after 32 days of starvation. The community was very resilient; substrate consumption was detected soon after organic substrates were added. Lastly, the community remained very reactive. Extended periods of starvation resulted in lag- and S-phases upon nutrient readdition, but the exponential phase growth rates were 70–90% of those found in unstarved communities.

Table 1 Kinetics of growth in batch cultures for inocula from starved biomass recycle reactors (BRR), preserved communities from the reactors, or municipal activated sludge

Inoculum	Lag (h)	S-phase period (h)	S-phase rate (h^{-1})	Exponential phase rate (h^{-1})
Starved 0 days	0	none	–	0.41
Starved 2 days	0	0–2	0.12	0.35
Starved 4 days	0	0–2	0.13	0.31
Starved 8 days	0	0–4	0.07	0.29
Starved 12 days	4	4–8	0.12	0.32
Starved 20 days	4	4–8	0.1	0.32
Starved 32 days	8	none	–	0.38
BRR frozen	1	none	–	0.41
BRR lyophilized	13	13–19	0.16	0.3
Municipal activated sludge	3	none	–	0.43

Acknowledgements

We thank Christine Morgan for DGGE analysis of 16*S* rRNA phylotypes, and Ron Turco and Marianne Bischoff for analysis of PLFAs. This research was supported by a grant from the Office of Naval Research N00014-94-1-0318.

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