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Bacterial function and community structure in reactors treating biopolymers and surfactants at mesophilic and thermophilic temperatures

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Microbial communities capable of degrading biopolymers and surfactants typically found in graywater were selected in continuous-flow bioreactors operated at 30, 44, 53, or 62°C. The effect of temperature upon microbial activity and community composition was determined. Microbial respiration of the organic components of the medium (including linear alkylbenzene sulfonate) was detected in samples from each reactor. The microbial community in each reactor was adapted to the operating temperature. Nucleic acid-based analyses of community composition showed that distinct consortia were present at each temperature. Community complexity was inversely related to temperature. The specific maintenance rate was twofold higher at 62°C than at the lower temperatures. Under starvation conditions, microbes in the 62°C system lost membrane integrity 30- to 100-fold faster than microbes at lower temperatures.

Keywords: biodegradation; temperature selection; graywater; community composition

Introduction

Aerobic biological wastewater treatment systems use mixed microbial communities to catabolize organic substrates to innocuous gaseous products (CO_2). We have investigated the use of bioreactors (both with and without biomass recycle [9,10]) for the treatment of graywater (wastewaters produced from galley, scullery, laundry, shower and lavatory sinks). The primary organic components of these waters are biopolymers (proteins and polysaccharides) and such surfactants as linear alkylbenzenesulfonates (LAS). Previous work explored physiological changes in the microbial community that occur as substrate flux to the microbes is altered, and the impact this has upon process rates of microbial catabolism. Here, the impact of temperature upon reactor functionality, microbial physiology and community composition is investigated.

Most wastewater treatment bioreactors are operated at temperatures of 5–35°C, the temperature of the ambient environment. However, many industrial waste streams are produced at high temperatures or have an organic content that results in reactor auto-heating [11]. Although there is abundant precedent for degradation of biopolymers at temperatures exceeding 45°C [4], the fate of the surfactant LAS at high temperatures is unclear.

The impact of temperature on the growth and activity rates of pure cultures is well understood. These rates increase as a function of temperature over 30–40°C from a species-specific minimum to an optimum level; further increases in temperature cause a rapid decline until growth ceases. However, the outcome in systems populated by complex mixtures of microbial species is less clear. Selection for different mixtures of species as temperature is varied can occur; this selective force might impact not only the identity of individual species but also the diversity of the microbial community. These changes in community composition can impact steady-state process rates and also community stability to perturbations.

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Methods

Continuous cultures

Continuous-flow bioreactors were maintained in 580-ml vessels (Cytolift glass airlift bioreactor, Kontes, Vineland, NJ, USA). The reactor was maintained at the desired temperature by circulating water through the water jacket of the reactor. The rate of sterile medium addition was controlled by a Gilson Minipuls 2 peristaltic pump (Gilson Inc, Middleton, WI, USA). Aeration and agitation occurred by passage of 2 L min^{-1} of sterile, humidified air through the culture.

Medium feed to the continuous culture simulated the composition of graywater [10] and contained the following components: 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 of SL7 trace element solution [10] per L. The organic substrates per L of medium were 400 mg starch, 150 mg gelatin and 18 mg of linear alkylbenzene sulfonate (LAS) (Sigma Chemicals, St Louis, MO, USA, 80% by weight).

The culture turbidity at 600 nm was monitored to recognize steady-state. After the turbidity had reached a steady

value, the culture was maintained for an additional three hydraulic residence times, to ensure that a physiological steady state was achieved, prior to removing samples for analyses.

Four series of chemostats were run. Each series was incubated at a different temperature, ranging from 30°C to 62°C. For each temperature series, an identical inoculum was added: a sample from the activated sludge reactor of the West Lafayette, IN, municipal wastewater treatment plant that had been preserved by quick freezing it in 10% glycerol and storage at –20°C.

For each temperature series, a steady state was obtained at an identical series of dilution rates (D). The reactors were first run at $D = 0.056\ h^{-1}$. After a steady state was achieved and analyses made, the reactors were sequentially shifted to $D = 0.11$, 0.22, and $0.33\ h^{-1}$. Analyses were made at each steady state, and then the reactor was shifted to a higher D.

Steady-state substrate levels were not measured in these experiments, but in earlier studies run at mesophilic temperatures, residual substrate concentrations were <1% of that in the medium feed over the range of D tested in these experiments [10]. This assumption and the steady-state biomass values at different D were used to calculate a specific maintenance rate [19]. The feed medium contained 20 mmol C L^{-1}. The protein : dry weight ratio was determined at some of the dilution rates at each temperature, and these values were used to convert protein biomass into dry weight biomass. The ratios were 0.46, 0.35, 0.33, and 0.29 for reactors operated at 30, 44, 53 and 62°C, respectively. A linear regression of the inverse yield *vs* the inverse D was used to calculate the maintenance energy coefficient and the maximum growth yield. The product of these two values is the specific maintenance rate.

Community analysis

DNA was extracted from concentrated samples of cells that were frozen, and subsequently incubated for 90 min at 70°C in 5% sodium dodecyl sulfate before they were subjected to two freeze-thaw cycles. The 338–534 bp region (relative to *E. coli*) of the 16S rRNA gene was amplified using the forward primer (5′-AC TCC TAC GGG AGG CAG CAG-3′) with a GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) attached to the 5′ end, and reverse primer (5′-ATT ACC GCG GCT GCT GG-3′) [16]. Polymerase chain reaction (PCR) amplification mixtures had a final volume of 50 μl and contained 25 pmoles of each primer, 100 mM dNTPs, 2% BSA, 1× Promega PCR buffer and 2 units of Taq polymerase. PCR amplification was performed in an automated thermal cycler (PTC-100, MJ Research, Watertown, MA, USA) using an initial 94°C denaturation for 9 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, then a final extension at 72°C for 7 min. PCR products were confirmed on 1% agarose gels stained with ethidium bromide.

The mixture of PCR products was separated by denaturing gradient gel electrophoresis (DGGE), carried out in a D-Gene apparatus (BioRad, Hercules, CA, USA) with slight modifications to the method described in [15]. Briefly, PCR products were resolved on 8% (wt/vol) polyacrylamide gels in 0.5 × TAE (20 mM Tris-Cl, 10 mM acetate, 0.5 mM Na_2EDTA) using denaturing gradients ranging from 30% to 55% (where 100% denaturant contains 7 M urea and 40% formamide). Equivalent concentrations of PCR products were loaded into each gel. Electrophoresis was carried out at a constant voltage of 20 volts for 10 min then 200 volts for 4.5 h. Electrophoresis buffer (0.5× TAE) was maintained at 60°C. Gels were then stained with SYBR Green I (1:10 000 dilution, Molecular Probes, Eugene, OR, USA), and visualized on an UV transilluminator and photographed (Polaroid MP4 Land Camera). Polaroid photographs were analyzed directly, by pairwise comparisons of band sharing between samples. Sorenson's index was used to compute a similarity coefficient, $C_s = 2j/(a + b)$ in which j is the number of bands common to two samples, a and b are the number of bands in samples A and B, respectively [21].

Analytical methods

Microbial biomass in the reactors was monitored by measuring the optical density (OD) in a 1-cm cell at 600 nm on a Gilford 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, USA). Cellular protein was measured as described by [8]. Dry weight was also measured in some samples, by centrifuging a known volume of culture in a tared, dried centrifuge tube ($7500 \times g$ for 10 min) and then drying the pellet at 105°C overnight. The pellet was then weighed on a Mettler AG204 analytical balance. The reported values are the means of triplicate determinations.

Oxygen consumption by washed resting cells was measured with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). A total volume of 1.5 ml of washed resting cells (approximately $OD_{600} = 0.8$) was added to a water jacketed cell set to the appropriate temperature, and the rate of endogenous respiration was monitored. Test substrate was added and oxygen consumption was monitored over the next 5 min. For the measurement of potential respiration rates, the concentrations of added substrates were 4 mM maltose, 3 mM glucose, or 2 g casamino acids L^{-1}. These concentrations were sufficient to produce the maximum (saturated) rates of oxygen consumption. For LAS, it was necessary to determine the rate-saturating concentration for individual experiments; this concentration varied between 60 and 150 μM. The reported values are the means of triplicate analyses of subsamples.

The proportion of cells with intact cytoplasmic membranes was determined by addition of LIVE/DEAD BacLight stain (Molecular Probes). Samples were incubated for 30 min at room temperature in the dark, and then examined by epifluorescence microscopy with a broad band filter set [6].

Results

Steady-state biomass levels (measured as cell protein) had dissimilar patterns at different temperatures (Figure 1). At 30°C, biomass level increased 20% as D increased from 0.06 to $0.33\ h^{-1}$. At 44°C and 53°C, biomass yields were relatively constant at $D \geq 0.11\ h^{-1}$, but declined approximately 33% at $D = 0.055\ h^{-1}$. The response at the highest temperature, 62°C, was different; there was a linear correlation between biomass level and D, and biomass increased

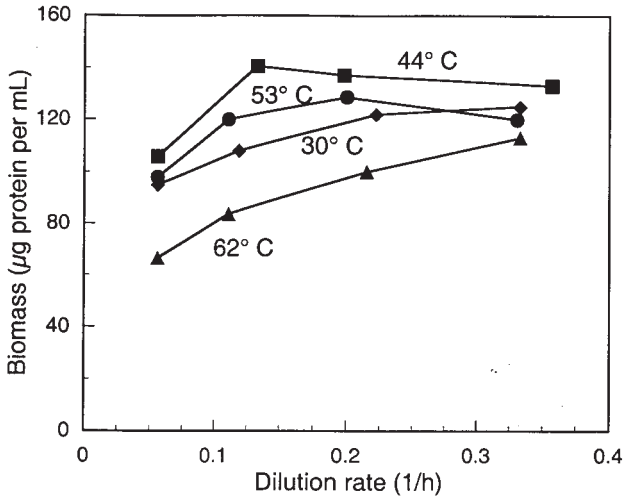

Figure 1 Steady-state biomass levels (expressed as particulate protein) for chemostats run at a series of dilution rates. Chemostats were operated at (◆) 30, (■) 44, (●) 53, or (▲) 62°C.

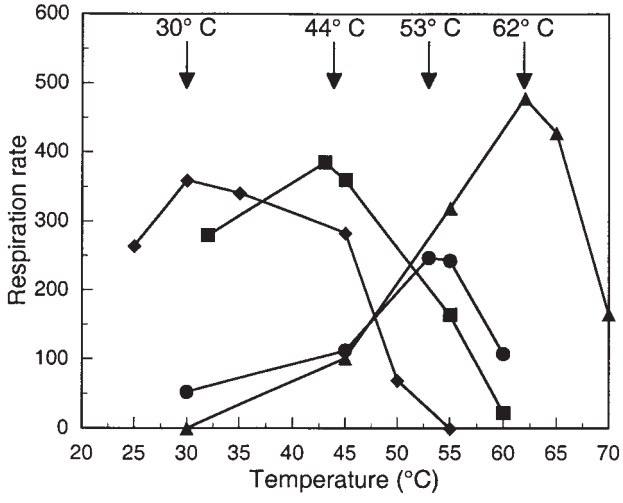

Figure 2 Temperature-dependence of microbial respiration rates. Resting cells were prepared from steady-state chemostat cultures ($D = 0.22\ h^{-1}$) operated at (◆) 30, (■) 44, (●) 53, or (▲) 62°C. The net respiration rates (μg oxygen per mg cell protein per h) were determined after the addition of 4 mM maltose to the suspension.

by 70% over the range of D tested. The decrease in biomass levels at low D were interpreted as due to maintenance energy effects in the C-limited conditions [10] in the reactors. The specific maintenance rates were similar at 30°C, 44°C, and 53°C (0.020–0.022 h^{-1}), but increased to 0.050 h^{-1} at 62°C.

The microbial communities selected at each temperature could metabolize the organic components of the medium. Extensive analyses were made upon the populations at $D = 0.33\ h^{-1}$ at each temperature. Of special note is that LAS was respired by resting cells from the reactors at all temperatures. In the 62°C reactor, the V_{max} of LAS degradation (obtained at 37–111 μM LAS) was 160 μg O_2 consumed mg $protein^{-1}\ h^{-1}$. For comparison, the V_{max} for LAS respiration for the 30°C reactor was 75 μg O_2 consumed mg $protein^{-1}\ h^{-1}$.

One reactor was also run at 70°C ($D = 0.22\ h^{-1}$) for the sole purpose of measuring respiratory potentials on key substrates. The monomers (casamino acids and maltose) derived from the biopolymers (protein and starch) in the feed medium were respired at high rates (570 and 610 μg oxygen mg cell $protein^{-1}\ h^{-1}$, respectively). In addition, the net rate of oxygen consumption stimulated by 50 μM LAS was 200 μg oxygen mg cell $protein^{-1}\ h^{-1}$, a rate higher than that observed in the 62°C reactor.

The temperature optimum for microbial catabolism was determined using resting cells obtained from reactors with $D = 0.33\ h^{-1}$ (Figure 2). The data (V_{max}) for maltose catabolism (a product of starch hydrolysis) are presented. The population in each reactor was optimally adapted to its temperature of operation. For microbes selected at 53°C and 62°C, increases or decreases of 10°C resulted in 2-fold decreases in respiration rate. For the 44°C population, a severe drop was seen at higher temperatures, but activity at 30°C was 75% of the optimal value. The 30°C reactor population was not tested at temperatures lower than 5°C below its operating temperature; a 25% decline in activity was found. This level of activity was retained at temperatures up to 45°C; thus, this mesophilic community appeared to have the broadest temperature range for activity.

The specific adaptation of each bioreactor population to its operation temperature suggested selection of different bacterial species in each reactor. This was confirmed via DGGE analysis of PCR products of the variable V3 region of the 16S rDNA gene (Figure 3). Analyses were performed on samples from the lowest and highest D run at each temperature. The complexity of the microbial communities decreased substantially at higher temperatures. Whereas 17 and 13 bands were detected at 30°C for D = 0.055 and

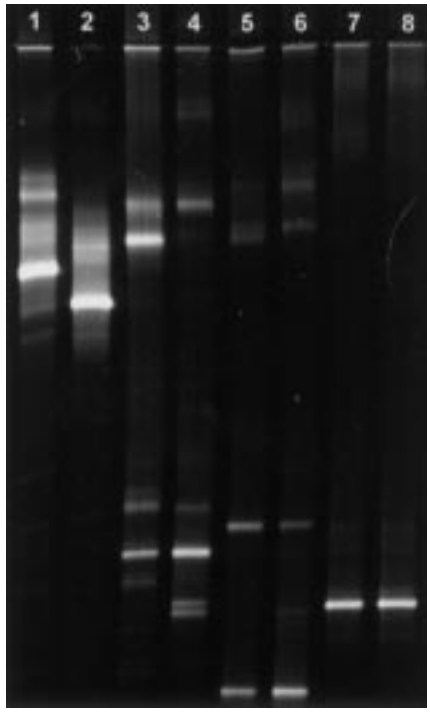

Figure 3 Denaturing gradient gel electrophoresis of bacterial communities from bioreactors. Lanes 1–2, 30°C ($D = 0.055$ and $0.33\ h^{-1}$, respectively); lanes 3–4, 44°C ($D = 0.055$ and $0.33\ h^{-1}$); lanes 5–6, 53°C ($D = 0.055$ and $0.33\ h^{-1}$); and lanes 7–8, 62°C ($D = 0.055$ and $0.33\ h^{-1}$).

0.33 h^{-1}, respectively, only five and four bands were detected (at each D) in the samples from 53°C and 62°C. The 44°C reactors had 10 and 13 bands at D = 0.055 h^{-1} and 0.33 h^{-1}. The microbial populations were also substantially different at each temperature. In the eight bioreactor samples, we identified 44 unique band positions. Only six of these occurred at more than one temperature. Thus, the frequency of band sharing was very low between samples from different temperatures. C_s ranged from 0 to <0.1 for all comparisons between pairs of reactors at different temperatures. For example, no bands were shared between the 53°C and 62°C reactors. The frequency of band sharing was also computed between the two dilution rates analyzed at each temperature, to determine the impact of growth rate upon community composition. At low temperatures, there were substantial differences in the communities (C_s were 0.33 and 0.61 for the 30°C and 44°C reactors), but in the high temperature reactors, the relatively simple communities were very similar at the two D (C_s of 0.8 and 1.0 for 53°C and 62°C, respectively).

In this study and others [11], maintenance requirements were greater at high temperatures. Thermophilic microbes might have more difficulty than mesophilic microbes, maintaining cell integrity during severe energy limitation. The proportion of bacterial cells with intact cytoplasmic membranes was determined at each steady state; lower D imposed a more stringent energy limitation than high D. At all temperatures, the proportion of intact cells was directly correlated with D (Figure 4). However, whereas at 30°C, virtually all cells were intact even at the lowest D, at each successively higher temperature fewer intact cells were found at a specific D, and there was a steeper change in the proportion of intact cells as a function of D. As a result, <50% of cells were intact in the 62°C reactors at D <0.25 h^{-1}.

The capacity to withstand starvation conditions was also tested. Samples taken from reactors at D = 0.11 h^{-1} were resuspended in the mineral salts components of the feed medium (that is, with no organic C source) and incubated at the temperature of the reactor. The proportion of intact cells changed very little over the next 45 h for the 30°C community, but a dramatic loss in membrane integrity occurred in cells from the 62°C reactor (Figure 5). If the loss of intact cells is modeled as an exponential decay process, the rate constants at 30°C, 53°C and 62°C were –0.003, –0.008, and –0.28 h^{-1}; there was almost a 100-fold difference in rate between 30°C and 62°C.

Discussion

The applied aspect of this research was to examine the temperature limits to biodegradation of organic substrates typically found in graywater. However, the results are relevant to basic issues in the microbial ecology of engineered biotreatment systems. The operation of bioreactors at higher temperatures has two potential engineering advantages: higher biodegradation rates (due to the effect of temperature on reaction rates) and reduced biomass accumulation (due to increased consumption of energy for maintenance processes rather than growth) [11]. These engineering considerations are expressed through the physiological characteristics of the microbial species selected at each temperature.

Temperature did operate as a selective force upon the microbial community, because microbial respiratory activity was optimally adapted to the operating temperature. At high temperatures (53°C and 62°C), high activity was restricted to a narrow temperature range (activity was <50% of maximal at temperatures ± 7°C from the optimum), whereas at 30°C and 44°C, high activity extended over temperatures ± 12–15°C from the optimum. Selection for microbes that function optimally at the habitat temperature has also been found in natural ecosystems for photosynthetic microbes [5,17], autotrophic microbes [14], and heterotrophic microbes [1].

The analyses of substrate consumption rates did not fully confirm the principle that higher biodegradation rates occur at thermophilic temperatures. Higher maximum respiration rates (obtained at the operating temperature of the reactor) were measured at 62°C (and 70°C) than at 30°C and 44°C, but the 53°C reactor had a lower rate. A confounding factor in making these comparisons is that changes in bioreactor

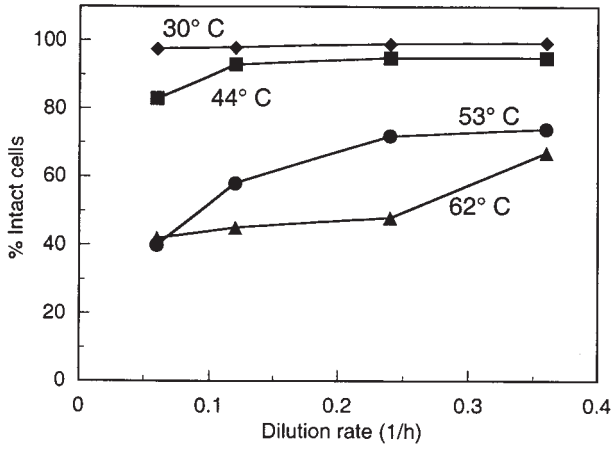

Figure 4 Proportion of cells with intact cytoplasmic membranes in steady state chemostat cultures operated at (◆) 30, (■) 44, (●) 53, or (▲) 62°C.

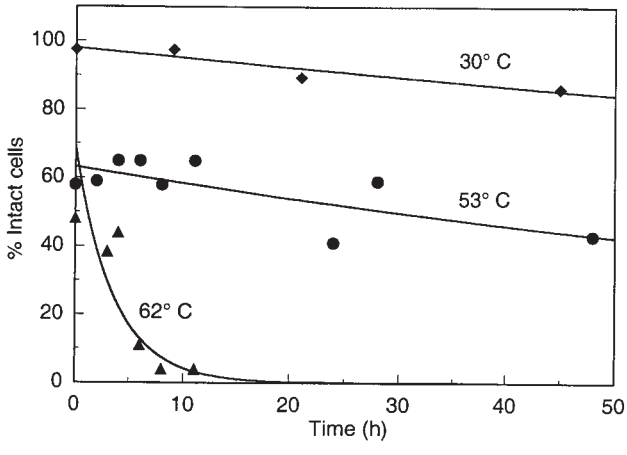

Figure 5 Decline in the proportion of cells with intact cytoplasmic membranes after incubation under C-starvation conditions for cells from reactors at (◆) 30, (●) 53, or (▲) 62°C. Samples were removed from steady-state chemostat cultures at D = 0.11 h^{-1}.

performance reflect not only the direct effects of temperature, but also the specific physiological characteristics of the microbes selected at that temperature.

Of special interest in graywater systems is biodegradation of surfactants such as LAS, because these surfactants are toxic to many microbes in the bioreactors. LAS-degrading microbes are essential to system function, to maintain a low residual LAS concentration [10]. LAS was respired by the bioreactor communities, even at 62°C and 70°C. The specific respiration rates found at these temperatures were approximately two-fold higher than in the mesophilic bioreactor community. Biodegradation of LAS in a broad variety of habitats has been reported [12], but LAS biodegradation at the elevated temperatures used here has not been reported.

Biomass yields on organic substrates are often lower at high temperatures, presumably due to energy consumption for nongrowth processes (called maintenance energy or endogenous decay). Increased maintenance rates at higher temperatures have been found for both mixed [11] and pure cultures [7,18,20]. In the graywater treatment reactors, the maintenance rate was not a linear function of temperature, although it did increase two-fold at 62°C. Thus, microbial populations were active at all temperatures tested, but only operation at 62°C imposed higher energetic costs on the microbes.

Additional insights into the stresses imposed by growth at elevated temperatures were gained by analyzing cellular integrity. Membrane-impermeable fluorescent dyes have been used to determine the permeability of the cytoplasmic membrane. Permeability to these dyes has been correlated with cell viability [13]. Significant proportions of cells not only at 62°C but also at 53°C had leaky membranes. The high maintenance coefficient calculated for the populations at 62°C may be consistent with a significant fraction of energetically stressed cells, but there was no indication from the kinetic data that the populations at 53°C were under substantial energetic stress.

Carbon starvation provided additional evidence that the community at 62°C had higher energetic costs than at other temperatures. Upon starvation, cells lost membrane integrity at a highly accelerated rate compared to populations at lower temperatures. From an applied perspective, intermittent substrate additions at these temperatures would lead to decreases in catalytic activity during the starvation periods between substrate pulses.

In addition to physiological analyses as a function of temperature, the diversity of the microbial community was determined. Biotreatment systems contain a mixture of microbial species [2]. In these experiments, initiated by inoculation with activated sludge, there was a selection pressure for the subset of bacterial species that could function effectively at the imposed temperature.

Community composition was assessed by the banding pattern on DGGE gels. These patterns do not necessarily represent the composition of the entire community, but do indicate the dominant phylotypes in the sample. Ecological theory suggests that: (a) different species will be selected at different temperatures; and (b) 'extreme' environmental conditions (eg, high temperatures) will reduce diversity (although explicit proof of this point is lacking) [3]. The DGGE analyses support both of these points. Very few bands were shared between samples obtained from different temperatures. This is in contrast to results that examined variability of bacteria in aquatic habitats, in which significant sharing of phylotypes occurred as a function of depth in lake, time, and geographical location (Konopka *et al*, submitted).

The complexity (number of bands) of the bioreactor community was severely reduced at higher temperatures. However, this may merely be a consequence of the inoculum's complexity. DGGE analysis of activated sludge samples indicated approximately 50 discrete bands, and low-level fluorescence that suggests the presence of many more minor species in the community (Konopka *et al*, unpublished observations). It is likely that fewer of these microbes could grow at temperatures >50°C than at <50°C.

The distribution of temperature strains in the original inoculum could also explain why the communities at severe *vs* modest nutrient limitation (low *vs* high D) were dissimilar at 30°C, but virtually unchanged at the higher temperatures. Low D selects for bacteria efficient at transporting low substrate concentrations, whereas high D selects for species capable of rapid growth. At 30°C, the pool of potential competitors was large enough that the most competitive species at low D were not the best competitors at high D. In contrast, there was no difference in community compositions at high temperatures because there was almost no other bacteria in the inoculum that served as competitors to the selected community.

Thus, high temperatures did impact the microbial ecology of this biotreatment system. There was a reduced complexity of the microbial community (at temperatures of 53°C and above). This could have many consequences; one was a narrowed temperature range for high (50% maximum) catabolic activity. Thermophilic communities are more dependent upon continuous supplies of energy (at least at 62°C), because maintenance costs are higher and there was a rapid loss of cell integrity under energy starvation conditions. However, high temperatures did not prevent catabolism of an aromatic surfactant, LAS.

Further work should investigate whether thermophilic communities inherently are less complex (for example, by using an inoculum from a thermophilic habitat). In addition, the mechanism for the rapid loss of cell integrity under starvation conditions would be of interest.

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