Microbial population dynamics in laboratory-scale activated sludge reactors

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Abstract As a first step in understanding nonlinear dynamics in activated sludge systems, two laboratory-scale sequencing batch reactors were operated under identical conditions and changes in their microbial communities were followed through microscopic examination, macroscopic observation, and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments from the prokaryotic community. Two experiments were performed. The first used activated sludge from a local wastewater treatment plant to start the replicate reactors. The second used the biomass from the first experiment as a source by intermixing the two and equally redistributing the biomass into the two replicate reactors. For both experiments, the two reactors behaved fairly similarly and had similar microbial communities for a period of 60 days following start-up. Beyond that, the microbial communities in the two reactors in the first experiment diverged in composition, while those in the second experiment remained fairly similar. This suggests that the degree of change occurring in replicate reactors depends upon the severity of perturbation to which they are exposed. The DGGE data showed that the bacterial communities in both experiments were highly dynamic, even though the system performance of the replicate reactors were very similar, suggesting that dynamics within the prokaryotic community is not necessarily reflected in system performance. Moreover, a significant finding from this study is that replicate activated sludge systems are not identical, although they can be very similar if started appropriately.

Keywords Activated sludge; denaturing gradient gel electrophoresis; population dynamics; wastewater

Introduction

Activated sludge bioreactors contain complex microbial ecosystems in which bacteria play a key role. Although operational parameters such as solids retention time (SRT) and food to microorganism (F/M) ratio have given good control of overall system performance, they have not been adequate in stabilizing the microbial communities in ways that engineers desire. This is probably because of the nonlinear nature of complex microbial communities, which causes them to respond to small changes in ways that might be considered chaotic (Kooi et al., 1997). One consequence of chaotic behavior is that two initially identical systems operated in identical ways can diverge in character in response to a perturbation, ultimately exhibiting different characteristics. In this study, microscopic examination, macroscopic examination, and denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified segments of the 16S rRNA gene were used to study population dynamics of two sequencing batch reactors operated under identical conditions.

DGGE has been used extensively with the 16s rRNA gene for genetic fingerprinting of mixed microbial communities (Muyzer et al., 1993). DGGE can separate DNA samples of the same length based on differences in their base-pair sequences. Separation in the gels is based on the electroforetic mobility of partially melted double-stranded DNA molecules in polyacrylamide gel containing a linearly increasing gradient of DNA denaturant. The electroforetic mobility of a partially melted double stranded DNA molecule is slower than that of the completely helical form of the molecule. Different species have different base-pair sequences and will become partially denatured at different
concentrations of denaturant, and therefore will be stopped at different positions in the gel. Consequently, the number of bands theoretically represents the number of species present in the sample. Although the primers used to amplify a portion of the 16S rRNA gene produce a quantitative relationship between gene copy number and PCR-DGGE band intensity (Bruggemann et al., 2000), many other artifacts can prevent the number and intensity of bands in a DGGE gel from representing exactly the number and abundance of species in a microbial community (Eichner et al., 1999; LaPara et al., 2000). Nevertheless, PCR-DGGE is a very sensitive and rapid technique that can detect most single-base variations when a G-C clamp is added to one primer in the PCR amplification process (Muyzer et al., 1998). Thus, it is capable of providing a reasonable picture of changes within a microbial community or of differences between microbial communities and is finding increased use in the analysis of bioreactors used in wastewater treatment. Consequently, it was used herein to investigate the stability of the bacterial communities in activated sludge systems.

Materials and methods

Activated sludge reactors

Activated sludge from a local wastewater treatment plant (Seneca, SC) was used to start two identical sequencing batch reactors (SBRs), each operated with an effective SRT of 10 days, an effective hydraulic retention time of 20.6 hr, and an instantaneous F/M ratio of 6 kg COD/ (kg MLSS-day). Each SBR consisted of a 14 litre glass New Brunswick Microferm (Edison, NJ, USA) fermenter operated with an 8 litre working volume. The reactors were maintained at room temperature, 25°C, which was controlled by the building HVAC system. The pH of the reactors was kept neutral (≥ 6.8) by a pH controller. The dissolved oxygen concentration was maintained above 2 mg/L by supplying a total airflow rate of 6 L/min through two air diffuser stones. The reactors were run with two cycles per day and biomass was wasted directly from the reactors to maintain the desired SRT. Total suspended solids in the wastage, in the reactor, and in the effluent were determined daily (Standard Method 2540D, Clesceri et al., 1998) and used for adjusting the wastage rate to maintain a constant SRT. Two experiments were performed. In the first, the reactors were operated identically for 180 days after being started with the activated sludge from the wastewater treatment plant. After further experimentation, the biomass from the two reactors was combined and redistributed equally to start the second experiment. The reactors were then operated identically for 150 days. Changes in color and other macroscopic parameters, such as sludge volume index (SVI) and effluent total suspended solids (ETSS) concentration, were observed on a daily basis. Performance of the reactors was monitored by soluble COD analysis (Bioscience Accu-Test Low range twist cap vials, Bethlehem, PA, USA) of the effluent on a regular basis. Biomass samples were collected every 10 days for DNA extraction and microscopic examination. Those samples were taken from each reactor during the wasting period. Three replicate 25 mL samples from each reactor were centrifuged at 12,000 × g for 10 min at 4°C. After supernatant was decanted, the biomass samples were resuspended with phosphate buffer (0.12 M, pH 8.0) at a ratio of 500 µL phosphate buffer per 0.2 g of wet biomass pellet. The samples were then ready for DNA extraction.

Synthetic wastewater

The synthetic wastewater consisted of biogenic compounds and inorganic nutrients. It had a total soluble COD of 900 mg/L. Its biogenic components were 350 mg COD/L of bactopeptone, 350 mg COD/L of proteose-peptone, 164 mg COD/L of soluble starch, and 36 mg COD/L of methyl pyruvate. There was enough nitrogen to meet the requirements for cell
growth. However, a phosphate solution of 35 mg/L \(K_2HPO_4\) and 30 mg/L \(KH_2PO_4\), was supplied to meet the growth requirement and provide a buffering capacity. Higgins and Novak (1997) showed that to ensure good sludge settling characteristics, feed to activated sludge systems should contain at least 2 meq/L each of \(Ca^{2+}\) and \(Mg^{2+}\) and a ratio of \(Na^+\) to divalent cations of less than 2. The synthetic wastewater was designed to meet these criteria and contained the following inorganic components: 367 mg/L \(CaCl_2.2H_2O\), 127 mg/l \(NaCl\), 0.265 mg/L \(CoCl_2.6H_2O\), 615 mg/L \(MgSO_4.7H_2O\), 1.08 mg/L \(ZnSO_4.7H_2O\), 0.265 mg/L \(Na_2MoO_4.2H_2O\), 0.04 mg/L \(H_3BO_3\), and 6.86 mg/L \(C_{10}H_{12}FeN_2O_8Na\).

**DNA extraction**

Five hundred and fifty microlitres of resuspended biomass was transferred to a screw-cap tube containing 0.5 g sterile glass beads (Biospec, Bartlesville, OK, USA). Then 500 µL of phenol:chloroform:isoamyl-alcohol (25:24:1) solution was added to the tube. The sample was lysed in a mini-beadbeater (Biospec) at 5,000 rpm, for 30 sec, followed by centrifugation at 11,000 × g for 5 min at 4°C. The upper aqueous layer was transferred to a new snap top microtube, to which 500 µL of phenol:chloroform:isoamylalcohol (25:24:1) solution was added. The mixture was mixed briefly by inverting the microtube several times, centrifuging as before, and the aqueous layer transferred to a new microtube. To remove any RNA present, 0.1 × volume of RnaseA (10 mg/mL) (from beef pancreas, ICN, Costa Mesa, CA) was added to the microtube, which was then incubated in a 37°C water-bath for 30 min. The phenol:chloroform:isoamyl-alcohol extraction was repeated twice more, followed by the addition of 800 µL of chloroform:isoamyl-alcohol (24:1) solution to remove any remaining phenol. The aqueous layer was then transferred to a new microtube. Cold 3M sodium acetate (pH 5.2) was added at 0.1 × volume and mixed briefly by inverting the tube 5 times, followed by the addition of 2 × volume of cold absolute ethanol (−20°C) to precipitate DNA. The sample was allowed to precipitate overnight at −20°C. After precipitation, the sample was centrifuged at 11,000 × g for 10 min at 4°C. The ethanol solution was removed and 200 µL of 70% ethanol solution was added to the sample, followed by centrifugation as before. Finally, the ethanol solution was removed and the sample was allowed to dry in a laminar flow hood for 15 min. The DNA pellet was resuspended in 200 µL TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and stored at −80°C until analysis by PCR-DGGE. A 1:100 dilution of the DNA sample was used to quantify its concentration by spectrophotometric determination (Beckman DU-600, Fullerton, CA, USA). An optical density of 1 at a wavelength of 260 nm corresponds to approximately 50 µg/mL of double-stranded DNA (Sambrook _et al._, 1989).

**Polymerase chain reaction (PCR)**

PCR primers were chosen to amplify the highly variable region of the 16s rRNA gene. The chosen primers were 341f and 907r (Muyzer _et al._, 1998), and were targeted against regions of highly conserved sequence in a wide range of bacterial species. Their nucleotide sequences are as follows: 341f (GC-clamp): 5′ CGC CCG CCG CGC GCG GGC GGG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3′ and 907R: 5′ CCG TCA ATT CCT TTR AGT TT 3′, corresponding respectively to positions (in _Escherichia coli_) 341–358 and 887–907. PCR amplification was performed with PCR Ready-to-go Beads (Pharmacia Biotech, Piscataway, NJ, USA). Each bead has the following components for a 25 µL reaction volume: 50 mM KCl, 10 mM EDTA, pH 8.0) and stored at −80°C until analysis by PCR-DGGE. A 1:100 dilution of the DNA sample was used to quantify its concentration by spectrophotometric determination (Beckman DU-600, Fullerton, CA, USA). An optical density of 1 at a wavelength of 260 nm corresponds to approximately 50 µg/mL of double-stranded DNA (Sambrook _et al._, 1989).
with a denaturation step at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 3 min, after which the annealing temperature was reduced by one degree on successive cycles until it reached 55°C. Next, 15 more cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min was followed, ending with a final elongation step of 72°C for 10 min. The PCR products were kept at 4°C until analysis by DGGE. The PCR products were checked for contamination by running 2% agarose gel electrophoresis. The obtained PCR products were about 550 base pair in length.

DGGE
All DGGE gels (19 × 19 × 0.1 cm) were poured with 6% polyacrylamide (acrylamide:bis-acrylamide at 37.5:1) in 1× TAE buffer (0.04 M Tris base, 0.02 M sodium acetate, 1.0 mM EDTA, pH 8.0), mixed with varying (gradient-dependent) concentrations of urea and formamide. The gels were polymerized by the addition of 1% ammonium persulfate and 0.1% TEMED. The denaturing gradient was formed by linearly increasing the concentration of urea and formamide (0.07 M urea and 0.4% formamide per % denaturant) from 0% to 100% using a model GM-40 gradient maker (CBS Scientific, Delmar, CA, USA). The DGGE system was adapted from a regular vertical polyacrylamide gel electrophoresis unit, which contained 8 litres of 1× TAE buffer at pH 8.0 and 60°C. All DGGE gels were run with the denaturing gradient parallel to the direction of electrophoresis for 18 hours at a constant 100 V at 60°C, as described in the protocol by Muyzer et al. (1998). Gels were stained with Sybr Gold (Molecular Probe, Eugene, OR, USA) for 30 min and photographed under UV Tran illumination. All reagents were of molecular biology grade.

Analysis of band patterns
DGGE gel photographs were scanned and analyzed by a densitometer and its corresponding software (GS-710 Biorad, Hercules, CA). Each gel was normalized by two internal standards to eliminate the difference from gel to gel, in a manner similar to that used by Eichner et al. (1999). Plots of DGGE band pattern were then obtained, with “normalized relative front” representing the positions of the bands in the DGGE gel. A low normalized relative front value represents a position on the gel at the lower end of the denaturant concentration. Since the G-C content of a DNA molecule is related to its strength, bands representing bacteria having low G-C content in their 16S rDNA will stop at lower denaturant concentrations in the gel, therefore giving low relative front values.

Results and discussion
The DGGE patterns of the PCR-amplified 16S rRNA gene segments from the first experiment are shown in Figure 1. Each peak in the pattern represents a different bacterial species and the intensity of the peak represents its relative importance in the community. The patterns represent only the bacterial contribution to the community because of the primer used in the PCR reaction. The activated sludge system from which the biomass for experiment 1 was obtained performs carbon oxidation, nitrification, and denitrification on a domestic wastewater with a moderate industrial component. The experimental reactors, on the other hand, were totally aerobic and received only biogenic organic compounds. As a consequence of those differences, the activated sludge biomass was highly perturbed, causing the composition of the bacterial communities to change markedly, as can be seen in Figure 1. In spite of those changes, however, the communities in the two reactors remained very similar for the first 58 days. After day 58, however, the bacterial communities in the two reactors diverged greatly from each other. From day 58 to day 109, divergence in the low G-C organisms was particularly noticeable, whereas the higher G-C organisms
remained more similar. However from day 109 to day 170, changes throughout the whole community were apparent. Others (Boon et al., 2000; Eichner et al., 1999) have observed large changes in activated sludge community structure upon bringing sludge from the field to the lab. Indeed, investigators have long recognized the need for an adaptation period upon starting experimental activated sludge systems. It is now apparent, however, that the community structure continues to be dynamic. Furthermore, analysis of the data presented by Boon et al. (2000) reveals that they also observed divergences in community structure in identical reactors after a period of similarity following startup. This suggests that our results are not unique and that consideration of such changes in identically operated activated sludge reactors must be considered during experimental design.

The changes in color, SVI, ETSS concentration, and effluent soluble COD are shown in Figure 2. Color is on a relative scale with higher numbers representing darker brown colors and low numbers lighter yellow colors. The changes in bacterial community composition over the first 58 days were reflected in changes in all of the parameters shown in Figure 2, with the two reactors responding similarly. On day 90, the ETSS concentration was increasing in both reactors. Microscopic examination revealed that there were only a few filamentous bacteria in both systems, as well as low numbers of stalked ciliates. By day 100, the number of filamentous bacteria and stalked ciliates had increased and the ETSS concentration had begun to decrease in reactor A, and began to do so shortly thereafter in reactor B. Furthermore, reactor A contained a large number of rotifers. By day 130, the SVI in reactor B had increased and the activated sludge consisted of small-diffused floc with a large number of filamentous bacteria. Even though nothing apparent in the prokaryotic
community changes correlated with the high ETSS concentrations, color changes were reflected in part by community changes, particularly during the first 40 days and near day 120. No differences in substrate removal were evident between the two reactors until day 120, when reactor A consistently had about 10 mg/L and reactor B 15 mg/L. Nevertheless, throughout the entire experiment, COD removal in both reactors was excellent, particularly considering that the influent COD was 900 mg/L. This confirms the observation of Fernandez et al. (1999) that functionally stable bioreactors can be maintained even though their community structures are dynamic. Finally, it is interesting to note that although bulking incidents occurred in both reactors (on day 175 in reactor A and day 140 in reactor B), the densitograms on those days did not show any new high intensity peaks, suggesting that the mass of filamentous bacteria need not be large to introduce a bulking problem.

In the second experiment, the biomass was already adapted to the substrates and operational conditions imposed, and thus the degree of perturbation experienced by the biomass was small. In spite of that, the bacterial community composition continued to be highly dynamic, as shown in Figure 3. However, in this case the bacterial communities in the two reactors remained much more similar, although differences were evident, particularly after day 62. Even then, the community compositions again exhibited a high degree of similarity after day 112. When considered in light of the first experiment, these results suggest that to achieve replicate activated sludge reactors that are as similar as possible, biomass brought into the lab from the field should be adapted to the lab conditions before being split into parallel systems. Even then, however, the bacterial communities will not be identical, although they may behave similarly.

Figure 3 Normalized densitogram of Sybr Gold-stained DGGE patterns containing PCR-amplified segments of 16S rDNA. The DNA samples were collected over the 150-day period of the second experiment.

Figure 4 Color pattern, effluent total suspended solids (ETSS) concentration, sludge volume index (SVI), and effluent COD of both reactors over the 150-day period of the second experiment.
The similarity in behavior of the two reactors in the second experiment can be seen in Figure 4. Again they behaved similarly in terms of color and DGGE pattern for the first 60 days, after which the color in Reactor A started to change, becoming dark brown. Furthermore, the sludge contained large numbers of higher life forms, such as protozoa, rotifers, and water mites. During this time the mixed liquor suspended solid (MLSS) concentration dropped and the ETSS concentration increased. White mucous foam also appeared on the reactor walls. This condition lasted about 30 days, after which Reactor A returned to its previous condition and the mucous foam disappeared. Reactor B experienced a similar episode, although it did not start until day 83 and lasted about 20 days. By day 100, both reactors had returned to a normal condition, but with Reactor B having a slightly darker color than Reactor A. There were still some higher life forms present in both reactors, although the floc structures for the two reactors were different. In Reactor A, the floc consisted of microcolonies arranged loosely into large-size floc, whereas in Reactor B the overall floc size was smaller and consisted of denuded microcolonies around a filamentous backbone. The experiment was terminated when an outbreak of filamentous bacteria in Reactor B resulted in severe bulking.

The most significant finding of this study is that the bacterial communities in identically operated activated sludge reactors became significantly different over time, even though they started from a common community. Thus, they exhibit one characteristic of chaotic systems, although further study is required before it can be concluded that they are indeed chaotic. The change was particularly large in the first experiment, in which the activated sludge was subjected to severe operational changes on being brought into the lab. The changes observed in the second study were much smaller and of shorter duration, reflecting the fact that the biomass had been grown for an extended period under the imposed environment before being intermixed and redistributed to the bioreactors. This suggests that replicate reactors subjected to large operational changes during start-up are likely to experience large divergences in bacterial community structure. However, divergences are much smaller when operational changes are minimized. Therefore, where it is desirable to run replicate reactors, the best strategy would be to adapt the biomass to the new operational conditions and then use it to start replicate reactors.

In spite of the differences in community composition, the COD removal efficiencies in the two reactors were similar, demonstrating that different microbial communities can be functionally similar. However, other aspects of system performance, particularly color and ETSS concentration, were influenced strongly by the eukaryotic community and its interaction with the prokaryotic community. In long SRT systems, which tend to promote growth of higher life forms, the bacterial community can be affected significantly as a result of grazing selection and preferences. Such changes cannot be adequately explained by examining only changes in the prokaryotic community, suggesting that a full understanding of community structure in activated sludge systems will also require investigations of the eukaryotic community.

In spite of their large differences from activated sludge systems, the microbial communities in anaerobic bioreactors are also highly dynamic, suggesting that dynamic behavior in complex microbial communities is normal, even when they are operated under stable conditions. Fernandez et al. (1999) studied the population dynamics in a functionally stable methanogenic reactor over a 605-day period. They found that the bacterial community structure was highly dynamic, and even suggested that it followed a chaotic pattern, whereas the archaeal community had less diversity. Similar results were obtained by Zumstein et al. (2000), who monitored population dynamics in both the bacterial and archaeal communities in an anaerobic digester running under constant environmental conditions over a 2-year period. They observed that there were rapid shifts in the species composition of the...
bacterial community, whereas the archaeal community remained relatively stable. They suggested that the evolution of the microbial community seemed to be induced by inherent parameters, which could be biotic, such as phages, predation, etc.

The feed provided to the bioreactors in this study was sterile, and thus one must ask whether that contributed to the dynamic nature of the communities. Curtis and Craine (1998) observed the presence of influent bacteria in the microbial communities of full-scale activated sludge systems, but were unable to deduce their contribution to the functioning of the system. In fact, it would be difficult to answer the question by studying full-scale systems because the variable nature of the influent would affect the results. Consequently, the question must remain unanswered for the time being and our results must be interpreted in light of that uncertainty.

Finally, it must be recognized that the dynamic nature of activated sludge communities imposes considerable difficulty on those wishing to compare the communities in different systems. Because the community is continually changing, single samples provide only a snapshot of its composition. This suggests that when two systems are being compared, sufficient samples should be analyzed over time to give a representative picture of their dynamic character. Only then can general conclusions be made.

**Conclusion**

Prokaryotic communities in activated sludge systems are highly dynamic. However, overall system performance is not impacted greatly by changes in the prokaryotic community structure, but is influenced by changes in the eukaryotic community structure. Replicate systems are not identical, either in actual composition or in temporal variation, but they can be very similar, provided that they are started in a manner that fosters similarity. The degree of change occurring in a system depends upon how much the initial operating conditions vary from the period prior to start-up. During the first experiment, the biomass had to adapt to a feed comprised of constituents that were different from those at the wastewater treatment plant, as well as to different operational conditions. As a result, after an initial period in which the communities in the two reactors changed similarly, they diverged in composition. However, during the second experiment, in which the microbial community had already adapted to the feed and operating conditions, thereby reducing the magnitude of the perturbations experienced, the communities retained a greater similarity while maintaining their dynamic character. Thus, the development of replicate cultures in the lab requires that the community be adapted to the new condition prior to the start of the replicate reactors.

**References**


