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Application of DNA amplification fingerprinting (DAF) to mixed culture bioreactors

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SUMMARY

The use of DNA amplification fingerprinting (DAF) as a tool for monitoring mixed microbial populations in bioreactors was evaluated. Short (8-mer or 10-mer) oligonucleotides were used to prime DNA extracts from various biological reactors during polymerase chain reaction (PCR) amplification. The reactors examined in this study included two sets of anaerobic stirred tank continuous flow bioreactors. One set of anaerobic reactors was operated under methanogenic conditions and one set was operated under sulfate-reducing conditions. The anaerobic reactor communities in the methanol-fed reactors showed extensive DAF homology. DAF was also applied to a fixed-film azo dye degrading reactor to examine the degree of uniformity of colonization of the substratum in representative regions of the reactor. This method is a quick and relatively inexpensive means of monitoring microbial community structure during biological processes. Since no cultivation of the sample is involved, the genetic profile of the community is not biased by outgrowth conditions. DAF profiles may be useful for comparisons of population changes over time or of bench-scale vs pilot-scale reactors but not adequate for assessing community diversity.

INTRODUCTION

Community structure and stability may dictate the efficiency and reliability of biological treatment processes. Traditional methods of monitoring microbial communities, such as plate counts, direct counts and most probable number (MPN) procedures, have provided the bulk of information pertinent to monitoring bioprocesses but these methods have certain limitations. Specifically, they do not necessarily track the organisms of interest and can be both cost- and labor-intensive [4]. Newer methods, such as lipid analysis, are now being employed to help rectify some of the shortcomings of the older methods [17,30]. More recently, these methods have been augmented by nucleic acid-based analyses [2,7,12,19,20,23]. The latter methods address such issues as type and abundance of specific organisms and detection of the presence of genes mediating specific functions, without subculturing of the indigenous microbial population. However, the use of homologous gene probes is limited by the availability of known DNA sequences. The objective of this study was to evaluate the use of DNA amplification fingerprinting (DAF – also referred to as randomly amplified polymorphic DNA – RAPD) as a means of monitoring mixed microbial communities.

The use of DAF requires no culturing of the sample and no knowledge of specific DNA sequences [27]. DAF has been used extensively in a number of fields for the genomic analysis of single organisms, including bacterial and yeast human pathogens [1,6,15,28]. These methods were sufficiently specific to distinguish serogroups of the nitrogen-fixing bacteria *Bradyrhizobium japonicum* and *Rhizobium meliloti* [5,10,14,27]. Using very short oligonucleotide primers (8-mers) in the analysis of complex DNAs, Caetano-Anolles et al. [3] were able to distingush among soy bean cultivars as well as among DNA samples from human individuals. We have adapted this method to the analysis of uncharacterized mixed microbial populations in bioreactors. Two different types of reactor communities were used to assess the utility of DAF; stirred tank continuous culture anaerobic reactors and a fixed film azo dye mineralizing reactor. The approach offers a means of comparing different reactor communities or of monitoring population changes in one reactor over time.

MATERIALS AND METHODS

Bacterial strains

The strains used in this study are described in Table 1.

Reactor samples

Samples were obtained from different reactors at the College of Engineering, University of Cincinnati, OH, USA.

Azo dye reactor

This rotating drum reactor provided a completely submerged, fixed biofilm grown in continuous culture [9]. This reactor and the anaerobic reactors described below were inocu-

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TABLE 1

Bacterial strains used in this study

Organism	Source	Reference
Pseudomonas putida F1	D.T. Gibson	[31]
P. cepacia G4	M.S. Shields	[21]
P. putida PpG7	D.T. Gibson	[30]
Pseudomonas sp. JS150	C.A. Pettigrew	[22]
P. putida mt-2	ATCC	[29]
P. aeruginosa PAO1c	R. Olsen	[13]

lated with activated sludge. The feed solution for the fixed film system was a synthetic wastewater described by Tabak and Hannah [24]. After the biofilm reached pseudo-steady steady state (defined by constant average film thickness), an azo dye, Acid Orange (AO-7), was added to the feed. The system was operated for six months using flow rates, levels of chemical oxygen demand (COD), pH, and dissolved oxygen concentrations typically found in municipal wastewaters. Concentrations of AO-7 were varied during the experiment [9]. Biofilm samples were taken by removing a strip of plastic film from the reactor. Duplicate biofilm samples were removed from regions representing the top, midsection and bottom of the reactor.

Anaerobic reactors

Six stirred tank continuous flow anaerobic bioreactors were sampled [8]. A nutrient solution containing the necessary inorganic salts, vitamins and buffers was delivered to the reactors. The reactors were operated at a 3 g per day COD loading of the appropriate substrate. Three reactors were maintained under methanogenic conditions (no sulfate in the feedstock) and fed either acetic acid, methanol or formic acid, and three were maintained under sulfate-reducing conditions (4.5 g L^{-1} sulfate added to the feedstock) and fed either acetic acid, methanol or formic acid [8].

DNA isolation and PCR amplification protocol

The method of Tsai and Olsen [25] was used to extract DNA from environmental samples. Lysis of reactor biomass was achieved by lysozyme digestion followed by detergent treatment and three freeze/thaw cycles (−80 °C/65 °C). The lysate was then extracted with phenol:chloroform and chloroform followed by isopropanol precipitation. The DNA was redissolved then processed through GeneClean (BioCheck 101, La Jolla, CA, USA) before PCR amplification. This final step was required for amplifiable DNA.

PCR was carried out using the Stoffel fragment DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) and an MJ Research (Watertown, MA, USA) programmable thermocycler for amplification. The cycling program [see reference 1] was four cycles of : 94 °C, 5 min; 36 °C, 5 min; 72 °C, 5 min; then 30 cycles of: 94 °C, 1 min; 36 °C, 1 min; 72 °C, 2 min; and then 72 °C, 10 min. Three primers were used during this study: I – 5′GTAACGCC 3′, II – 5′GATCGCAG 3′ and III – 5′CCGCAGCCAA 3′. Primers were selected on the basis of ability to produce clear, distinguishable patterns. Reactions were carried out in a 102-μl volume and 20 μl was loaded on 2% agarose gels. Metaphor agarose (FMC Bioproducts, Rockland, ME, USA) was used for resolution of PCR products.

Gene probe isolation, labeling and southern blotting

DNA labeling with ^{32}P-dATP was carried out by random priming using a kit purchased from GIBCO BRL (Gaithersburg, MD, USA). DAF products were Southern blotted according to the method of Maniatis et al. [18] using MSI nylon membranes. Hybridization was carried out overnight in the following solution; 0.5 M NaH_2PO_4 pH 7.0, 1.0 mM EDTA, 7.0% SDS. Membranes were washed for 3-h long washes at 65 °C in the buffer: 10 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 0.5% SDS. Hybridization was detected by autoradiography.

RESULTS

Figure 1(A) shows DAF patterns generated from six characterized bacterial strains using the decaoligonucleotide, primer III. Figure 1(B) shows the results of DAF carried out on mixed cultures reconstructed using the same strains. These templates for PCR amplification were generated by combining equal amounts of DNA from each of the six strains or by omitting one of the strains in succession as described in the legend to Fig. 1(B). The patterns generated are not additive, i.e. complexity of the profile does not necessarily increase when more organisms are present; however, some major products appear to be diagnostic for certain organisms. Two specific examples obtained with primer III are shown here. An approximately 400-bp product from *Pseudomonas* strain JS150 (Fig. 1(A), lane 6), can be seen in all mixed culture DAFs where JS150 genomic DNA has been included (for Fig. 1(B) only lane 4 did not receive JS150 DNA). Similarly, an approximately 350-bp product, from *P. putida* PpG7, obtained with primer III, can be seen in all mixed culture DAFs where DNA from this strain was added (i.e. not seen in Fig. 1(B), lane 5). As would be expected, using the same organisms, diagnostic products are obtained with different primers. Primer I revealed a characteristic *P. putida* F1 fingerprint when that organism was examined in comparable mixtures and against a background of DNA from an experimental biofilm (data not shown).

Samples from an azo dye mineralizing rotating drum reactor were prepared by cutting pieces of the removable support and placing them directly into a lysis solution. Biofilm support strips from the top, middle and bottom of the reactor were sampled in duplicate and analyzed by DAF. The data appear in Fig. 2(A,B). Although the patterns are not totally reproducible, they clearly show a recurring pattern of major products using both of the primers tested. This indicated that generally uniform colonization of the reactor had taken place.

Two sets of three anaerobic reactors were compared using DAF analysis. Three reactors were maintained under methanogenic conditions and fed either acetic acid, methanol or formic acid. The three remaining reactors were maintained under sulfate-reducing conditions and fed either acetic acid, meth-

Fig. 1. DAF profiles from pure cultures and defined mixed cultures generated with primer III. (A) Lane 1, 123 base pair (bp) molecular weight standard; lane 2, negative control; lane 3, *Pseudomonas putida* F1; lane 4, *P. cepacia* G4; lane 5, *P. putida* PpG7; lane 6, *Pseudomonas* strain JS150; lane 7, *P. putida* mt-2; lane 8, *P. aeruginosa* PAO1C. (B) Defined mixed culture DAFs; lane 1, 123 bp standard; lane 2, all strains listed above excluding *P. aeruginosa* O1c; lane 3, all strains listed above excluding *P. putida* mt-2; lane 4, all cultures listed above excluding *Pseudomonas* strain JS150; lane 5, all strains listed above excluding *P. putida* G7; lane 6, all strains listed above excluding *P. cepacia* G4; lane 7, all strains listed above excluding *P. putida* F1; lane 8, all strains.

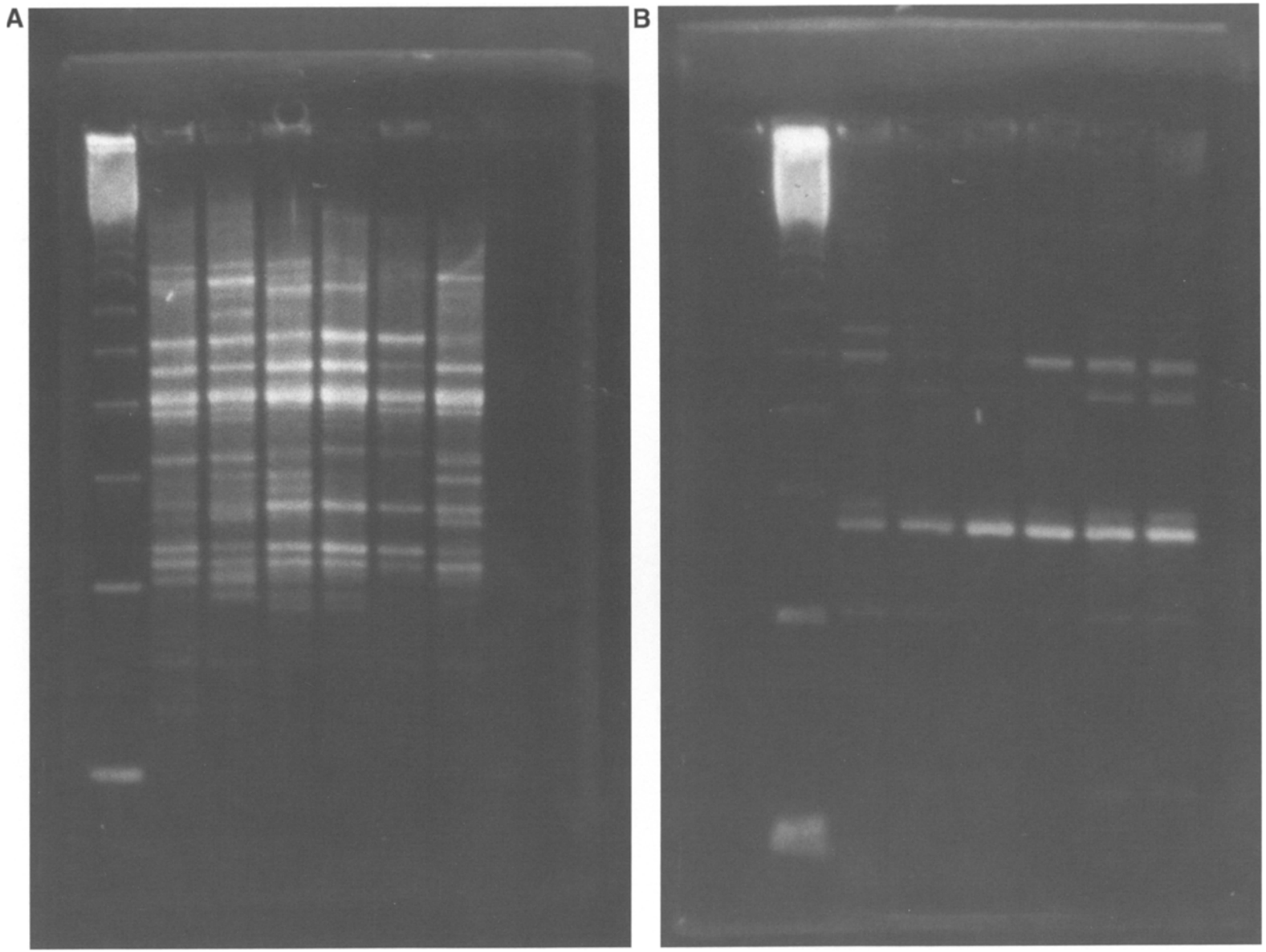

Fig. 2. DAF analysis of fixed film azo dye catabolizing reactor. (A) Primer I. Lane 1, 123 bp standard; lanes 2 and 3, replicate samples from the upper region of the reactor; lanes 4 and 5, replicate samples from the middle of the reactor; lanes 6 and 7, replicate samples from the lower region of the reactor. (B) Same as above, amplified with primer II.

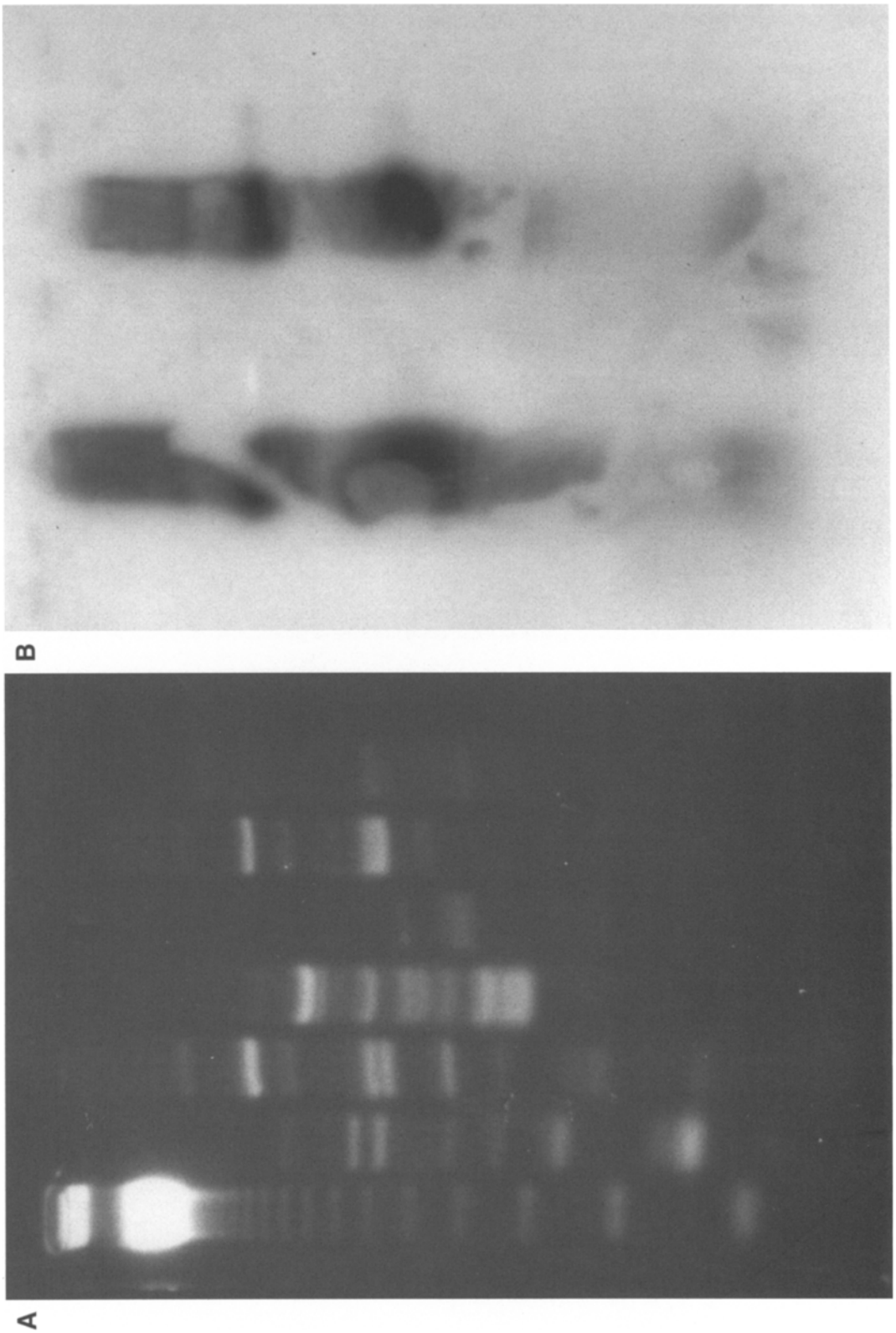

Fig. 3. DAF analysis of anaerobic bioreactors operated under methanogenic or sulfate-reducing conditions. (A) Profiles generated with primer II. Lane 1, 123 bp standard; lanes 2–4, methanogenic reactors (lane 2 acetic acid feed, lane 3 methanol feed, lane 4 formic acid feed). Lanes 5–7, sulfate-reducing reactors (lane 5 acetic acid feed, lane 6 methanol feed, lane 7 formic acid feed). (B) Southern blot of A probed with DAF products from methanol-fed/sulfate-reducing reactor.

anol or formic acid. DAF profiles obtained using primer II revealed common amplification products (Fig. 3(A)). Unique patterns from reactors suggest different populations in the acetic acid and formic acid-fed cultures. However, the two methanol-fed reactors (which were both generating methane) showed similar profiles suggesting common amplification products. The gel used for Fig. 3(A) was then examined by Southern blot using ^{32}P-labeled AF products from the 'sulfate-reducing/methanol-fed' reactor. Blot results, shown in Fig. 3(B), revealed that common PRC products were generated from the methanol-fed rectors but not in either of the acetic acid or formic acid-fed reactors (lanes 3 and 6).

DISCUSSION

DAF analysis has been helpful for genetic analysis in other systems; this study was designed to examine mixed populations of increasing complexity. First a set of well characterized volatile organic carbon catabolic bacteria was examined singly and in reconstructed mixtures. Complex populations from a fixed film bioreactor designed to facilitate biofilm sampling were also examined. In such systems representative sampling and analysis can be difficult. Finally a set of anaerobic reactors, where available microbiological techniques are more complex and time consuming, was examined. DNA fingerprinting in each case made use of short, 8- or 10-mer, oligonucleotide primers. These fingerprints show that DAF profiles from monocultures are not necessarily less complex than profiles generated by mixed cultures. Increased complexity of a DNA sample did not necessarily result in increased multiplicity of bands. Thus DAF is not appropriate for assessing community diversity.

DAF was used as a method to rapidly assess uniformity of biofilm development in a fixed-film azo dye-degrading bioreactor. The degradation of azo dyes is, in general, believed to be a two-stage process. The fixed-film reactor allows the initial cleavage of the azo bond to occur within O_2 depleted regions of the biofilm and subsequent degradation to occur in aerobic regions of the reactor [11,16]. Kinetic models for the degradation process assume that the reactor liquor is completely mixed and therefore that the biofilm is colonized uniformly. Parallel samples of intact biofilm were taken from representative regions in the reactor and subjected to DNA extraction PCR amplification. Our results (Fig. 2) demonstrated a high level of profile similarity. Although reproducibility among duplicates was not absolute, the same prominent bands were present across the three biofilm locations sampled. Thus DAF provided a clear and easy indication of considerable uniformity across the biofilm. DAF may be comparably useful in examining patterns of stratification in microbial samples taken at different depths in a biofilm.

The objective of anaerobic reactor DAF analysis was to assess the effect of different carbon sources on anaerobic community structure. Other investigators had observed that the sulfidogenic reactor/methanol-fed reactor, at the time of sampling, was generating methane [8]. DAF profiling with primer II indicated common amplification products for the methanogenic/methanol-fed reactor and the sulfidogenic/methanol-fed reactor (Fig. 3(A)). This was tested further by Southern blotting. The results revealed a dramatic carbon source effect in these reactor systems. The total DAF products from the sulfidogenic/methanol-fed reactor cross hybridized extensively with the DAF products from the methanogenic/methanol-fed reactor but showed little to no homology with DAF products from the formic acid-fed or acetic-fed reactors. This hybridization data supports the reliability of DAF profiles for comparison of complex populations.

DAF is limited by some factors inherent to the PCR process. Some sequences are amplified preferentially, hence banding intensities cannot be extrapolated to sequence abundance in a particular environment. Indications of this are evident in the profiles obtained from known mixtures shown in Fig. 1. This property limits the utility of DAF for assessing the diversity of microorganisms in complex communities. It would be interesting to compare DAF and lipid analysis for their relative capacities to profile complex microbial communities.

The strengths of DAF are the ease and rapidity with which samples can be analyzed. This method is not subject to the biases inherent in the sub-culturing for enumeration of environmental microorganisms. Our data reveal several areas where DAF analysis can be useful; using controlled mixtures, signature DAF products can be used to track individual organisms; DAF served as a convenient tool for assessing uniformity of population structure in a fixed-film bioreactor, and DAF profiles showed nutrient-dependent differences in anaerobic cultures. Monitoring reactor systems in which standard benchtop microbiology is difficult and time consuming (e.g. systems such as the anaerobic reactors) may be the area where DAF has its greatest utility.

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REFERENCES

1 Akopyanz, N., N.O. Bukanov, T.U. Westblom, S. Kresovich and D.E. Berg. 1992. DNA diversity among clinical isolates of *Heliobacter pylori* detected by PCR-based fingerprinting. Nucl. Acid Res. 20: 5137–5142.

2 Barkay, T., C. Liebert and M. Gillman. 1989. Hybridization of DNA probes with whole-community genomes for the detection of genes that encode microbial responses to pollutants, *mer* genes and HG^{2+} resistance. Appl. Environ. Microbiol. 55: 1574–1577.

3 Caetano-Anolles, G., B.J. Basam and P.M. Greshoff. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Bio/Tech. 9: 553–557.

4 Colewell, R.R., M.A. Levin and N. Palleroni. 1992. Overview: historical perspective, present status and future direction. In: Microbial Ecology: Principles, Methods and Applications (M.A.

Levin, R.J. Seidler and M. Rogul, eds), pp. 11–28, McGraw Hill, New York, NY.

5 DeBruijn, F.J. 1993. Use of repetitive (repetitive extrapalindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58: 2180–2187.

6 Fekete, A., J.M. Bantle, S.M. Halling and R.W. Stich. 1992. Amplification length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. J. Bact. 174: 7778–7783.

7 Govindaswami, M., T.M. Schmidt, D.C. White and J.C. Loper. 1993. Phylogenetic analysis of a bacterial aerobic degrader of azo dyes. J. Bact. 175: 6062–6066.

8 Gupta, A., J.R.V. Flora, G.D. Sayles and M.T. Suidan. 1993. A fundamental kinetic study of the anaerobic biodegradation of chloroform and its products with various cosubstrates. Eighteenth Annual Risk Reduction Engineering Symposium. Abstract Proceedings, US EPA Cincinnati, OH.

9 Harmer, C. and P.L. Bishop. 1992. Transformation of azo dye AO-7 by wastewater biofilms. J. Wat. Sci. Technol. 26: 627–636.

10 Harrison, S.P., L.R. Mytton, L. Skot, M. Dye and A. Cresswell. 1992. Characterization of *Rhizobium* isolates by amplfication of DNA polymorphisms using random primers. Can. J. Microbiol. 38: 1009–1015.

11 Haug, W., A. Schmidt, B. Nortemann, D.C. Hempel, A. Stolz and H.-J. Knackmuss. 1991. Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. Appl. Environ. Microbiol. 57: 3144–3149.

12 Holben, W.E., J.K. Jansson, B.K. Chelm and J.M. Tiedje. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54: 703–711.

13 Holloway, B.W., V. Krshnapilli and A.F. Morgan. 1979. Microbiol Rev. 43: 73–102.

14 Judd, A.K., M. Schneider, M.J. Sadowsky and F.J. De Bruijn. 1993. Use of repetitive sequences and the polymerase chain reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. 59: 1702–1708.

15 Lehman, P.F., D. Lin and B.A. Lasker. 1992. Genotypic identification and characterization of species and strains within the genus *Candida* by using randomly amplified polymorphic DNA. J. Clin. Microbiol. 30: 3249–3254.

16 Leson, G. and A.M. Winer. 1991. Biofiltration: an innovative air pollution control technology for VOC emissions. J. Air Waste Manag. Assoc. 41: 1045–1054.

17 Lipski, A., S. Klatte, B. Bedinger and K. Altendorf. 1992. Differentiation of Gram-negative, nonfermentative bacteria isolated from biofilters on the basis of fatty acid composition, quinone system and physiological reaction profiles. Appl. Environ. Microbiol. 58: 2053–2065.

18 Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning. Cold Spring Harbor Press, New York.

19 Ogram, A.V., G.S. Sayler and T. Barkay. 1987. The extraction and purification of microbial DNA from sediments. Microbiol. Meth. 7: 57–66.

20 Schmidt, T.M., E.F. DeLong and N.R. Pace 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bact. 173: 4371–4378.

21 Shields, M.S., S.O. Montgomery, P.J. Chapman, S.M. Cuskey and P.H. Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene degrading bacterium G4. Appl. Environ. Microbiol. 55: 1624–1629.

22 Spain, J.C. and S.F. Nishino. 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl. Environ. Microbiol. 53: 1010–1019.

23 Stahl, D.A., B. Flesher, H.R. Mansfield and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies ruminal microbial ecology. Appl. Environ. Microbiol. 54: 1079–1084.

24 Tabak, H. and S. Hannah. 1979. Continuous flow procedure for biodegradability determination. Internal EPA report, MERL/ORD US EPA. Cincinnati, OH.

25 Tsai, Y.-L. and B.H. Olsen. Rapid method for the direct extraction of DNA from soils and sediments. Appl. Environ. Microbiol. 57: 1070–1074.

26 Vestal, J.R. and D.C. White. 1989. Lipid analysis in microbial ecology. BioScience 39: 535–541.

27 Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. 18: 7213–7218.

28 Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18: 6531–6535.

29 Williams, P.A. and K. Murray. 1974. Metabolism of benzoate by *Pseudomonas putida* (arvilla) mt-2: evidence for existence of a TOL plasmid. J. Bacteriol. 120: 416–423.

30 Yen, K.-M. and I.C. Gunsalus. 1982. Plasmid gene organization: naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA 79: 874–878.

31 Zylstra, G.J. and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1. J. Biol. Chem. 264: 14940–14946.