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MICROBIAL BIOFILM COMMUNITIES IN GRANULAR ACTIVATED CARBON FLUIDIZED BED REACTORS FOR TREATMENT OF GROUNDWATER CONTAMINATED WITH AROMATIC HYDROCARBONS

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ARTURO ANDRES MASSOL-DEYA

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By

Arturo Andrés Massol-Deyá

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ABSTRACT

MICROBIAL BIOFILM COMMUNITIES IN GRANULAR ACTIVATED CARBON FLUIDIZED BED REACTORS FOR TREATMENT OF GROUNDWATER CONTAMINATED WITH AROMATIC HYDROCARBONS

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Arturo Andrés Massol-Deyá

The role and stability of non-indigenous populations on the structure and composition of biofilms in aerobic granular activated carbon fluidized bed reactors (GAC-FBR) treating groundwater contaminated with monoaromatic hydrocarbons were examined. The biofilm was composed of approximately 10^9 to 10^{10} total cells per gram of GAC. Comparison of community fingerprints of amplified 16S rDNA obtained by restriction endonuclease analysis and fatty acid methyl ester profiles (FAME) of seeded community with GAC colonized by the indigenous aquifer community suggested there was little influence of the non-indigenous populations on community structure. The biofilm community was composed of at least 12 different coexisting bacterial populations. Estimates of their maximum specific growth rate (μ_{max}) half-saturation constant (K_s) and maximum substrate utilization rates (k) obtained by fitting substrate depletion curves to the integrated Monod equation revealed a high diversity of toluene growth kinetics by these microbial populations. Partial 16S ribosomal RNA sequences were determined for each of the three co-dominant toluene degrading strains found among all laboratory operated FBRs studied with the aim of establishing phylogenetic affinities of the biofilm community. Similarity and evolutionary distance matrix analysis suggest closed affiliation of these strains to three subclasses of the *Proteobacteria*, the alpha, beta and gamma subdivision respectively. In addition, scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) were used to study the development, organization, and structure of

the biofilm community. During the early stages of colonization, microcolonies were primarily observed in crevices and other regions sheltered from hydraulic shear forces. Eventually, these microcolonies grew over the entire surface of the GAC. This lead to the development of discrete discontinuous multilayer biofilm structures. Cell free channel-like structures of variable sizes were observed to interconnect the surface film with the deep inner layers. This appeared to increase the biological surface area per unit volume ratio, which may facilitate transport of substrates in and wastes products out of deep regions of the biofilm at rates greater than possible due to diffusion alone. These architectural features were also observed in biofilms from four field scale GAC reactors that were in commercial operation treating petroleum contaminated groundwaters. This suggests that formation of cell-free channel structures and their maintenance may be a general microbial strategy to deal with the problem of limiting diffusive transport in thick biofilms typical of fluidized bed reactors. Understanding of the forces and principles which regulate biofilm development will help in efforts to model, control, and exploit biofilm related processes of industrial, medical, and environmental importance.

This dissertation is dedicated to

my parents Alexis and Tinti,

my wife Vicky,

and my daughter Corali

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Chapter I

AN INTRODUCTION

OVERVIEW

Bacteria in nature have a marked tendency to colonized solid-liquid interfaces (Fletcher, 1979; Costerton et al., 1987; Kolenbrander et al., 1990). The immobilized cells grow, divide, and produce extracellular polymers which frequently form a framework of cells embedded in an organic matrix of microbial origin (Lappin-Scott and Costerton, 1989). Biofilms form on virtually all surfaces immersed in natural aqueous systems including biological surfaces (e.g., aquatic plants) or abiological (e.g., metals). In natural environments, the surface accumulation is not necessarily uniform in time or space. For example, in the case of algal mats found in hot springs or modern lagoonal environments, a thick biofilm (approximately 300 - 400 mm) can contain both aerobic and anaerobic zones due to oxygen diffusion limitations (Ward et al., 1989). Furthermore, diurnal changes in the different compartments are affected by the production of oxygen in photosynthetic processes during daylight hours.

The formation of biofilm communities on surfaces has some very important economic and ecological implications. Biofilm processes are studied because of the scientific and technological applications of these systems. These systems are stable biological units that can transform a remarkably wide range of chemical substrates (Holm et al., 1992; Wolfaardt et al., 1994). These characteristics have been exploited in sewage and wastewater management systems (e.g., trickling filters) and in the development of new bioremediation technologies for detoxification of contaminated sites (e.g., biological activated carbon fluidized bed reactors). However, little information is available on the possible roles of microbial diversity, population interactions, and abiotic factors on the maintenance of structure and function within these communities. Due to the strong interrelation between the microbial processes, substrate removal, community structure and

composition, a practice which neglects these facts is deemed to cause erroneous design and improper operation of biological waste water treatment facilities and/or bioreclamation of contaminated sites. The understanding of these interactive processes will allow us to narrow the differences between laboratory models and actual processes in nature. Without a rigorous and systematic understanding of these ecological relationships then accurate mathematical models can not be formulated. These models will be extremely valuable for engineering biofilm processes. Our knowledge of the forces and principles involved in regulation of biofilm development will help in efforts to manipulate and exploit these processes for technological and ecological advantages.

Biofilms are significant in bioengineering and fundamental microbiology. Thus, conceptual and methodological approaches are required which will aid to better describe and control biofilm processes. The effects of many biological and abiotic factors on structuring of biofilms are still to be determined. Studies are needed on the impact of environmental conditions on microbial community structure. At the same time, we need to evaluate the impact of population interactions (e.g., extinction vs. coexistence; allochthonous populations vs. indigenous populations), and how such interactions dictate community structure. These principles which regulate biofilm development should help in efforts to model, control, and exploit biofilm related processes of industrial, medical, and environmental importance.

This study was organized around the following objectives:

i. to examine the fate of allochthonous populations in preemptive colonization of a porous media and after being challenged by indigenous groundwater bacteria in an aerobic GAC-FBR
ii. to examine the role and stability of seeded pioneer populations
iii. to examine the role and stability of secondary colonizers
iv. to evaluate the effects of substrate composition changes on community structure
v. to isolate and characterize BTX degrading groundwater populations

BIOFILMS

Surface colonization and biofilm formation -- Biofilm develops as a result of several concurrent processes: (i) transport of cells to the substratum, (ii) adsorption of cells, (iii) growth and metabolic processes at the colonized surface, (iv) detachment of portions of the film, and (v) recolonization (Figure 1).

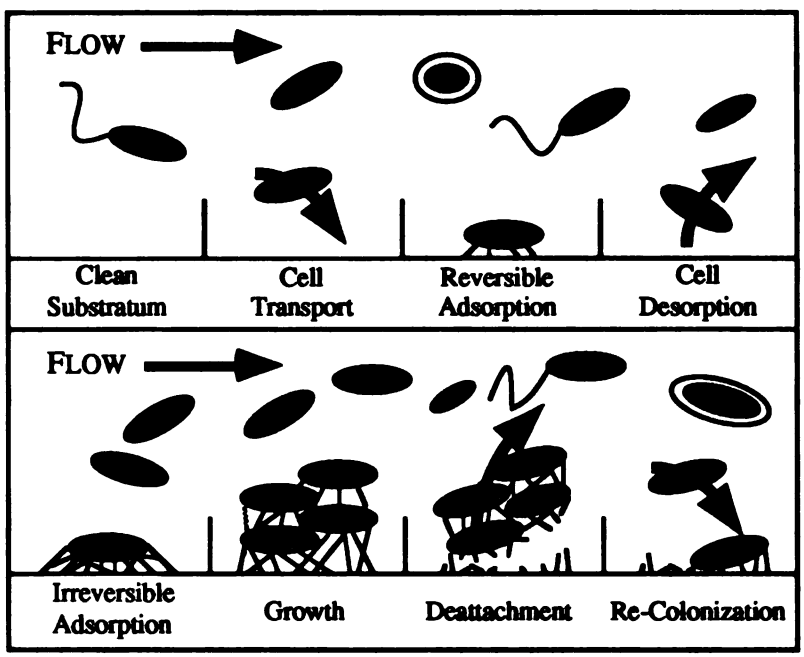

Figure 1. Processes in biofilm accumulation: cells transport to the substratum, reversible cell adsorption in equilibrium with desorption, irreversible cell attachment by secretion of biological active adhesives or by special cell structures, growth and biofilm accumulation, sloughing of fragments of the film, and recolonization of open surfaces.

In general, microbial colonization begins as planktonic cells approache a substratum. These processes may occur by either passive transport (diffusion or advective transport, Brownian motion), and/or active biologically controlled transport (flagella mediated motility, and/or swarming motility) (Criddle et al., 1991). Active transport of motile cells can also be affected by positive or negative chemotactic responses to gradients

of some biologically relevant compounds. As the bacterium meets the surface, reversible adhesion of cells to the substratum initially occurs via physicochemical processes. Surface properties, liquid phase and environmental conditions such as temperature or pH influence the degree of reversible sorption (Fletcher, 1979; Mills and Maubery, 1981; Fletcher and McEldowney, 1983; McEldowney and Fletcher, 1987; Van Loosdrecht et al., 1990). In addition, biological factors such as species composition and interspecific interactions, and their cell surface properties have also been shown to affect levels of adhesion (Warren et al., 1992). For example, the ecological relevance of biological interactions among genetically distinct cell types during colonization and coaggregation of oral bacteria have been reviewed (Kolendrander et al., 1990).

During the reversible adhesion phase, organisms might become detached by moderate shear forces and still capable of exhibiting Brownian motion and/or flagellar motility (Harkes et al., 1992). Meanwhile, irreversible adhesion is characterized by active microbial processes in which production of extracellular polymers, fibrils, and adhesives mediate cell-substratum interactions (Beachey, 1988; Vandevivere and Kirchman, 1993). These include special cellular structures such as pili found on members of the *Enterobacteriaceae* and *Pseudomonadaceae* and many others, and holdfasts which occur on some prosthecate bacteria. Subsequent cell binary fission leads to the formation of microcolonies and eventual biofilm formation. Such biofilms consist of bacterial cells embedded in a matrix of their own exopolysaccharide glycocalyces which helps to maintain the film structure.

A biofilm consists of microorganisms immobilized at a surface and an overlaying liquid or gas layer. Sometimes the substratum can also serves as nutrient rate-limiting step for microbial growth as illustrated in wood decomposition. On the other hand, a continuous liquid phase which may fill and interconnect fraction of the biofilm volume with the bulk liquid solution, contain different dissolved and suspended particulate materials and clearly influence diffusion processes of substrates, nutrients, waste products, and gases

into the film matrix. Thus, the liquid compartment can directly affect biofilm behavior (e.g., microbial activity) primarily as a result of the mixing patterns (fluid dynamics), and system geometry. The gas phase may be absent in some biofilm systems. Oxygen can in these systems be provide via aeration. Mixing patterns may also facilitate removal of gaseous microbial products such as CO_2 and CH_4 from inside the biofilm compartment of anaerobic biofilms.

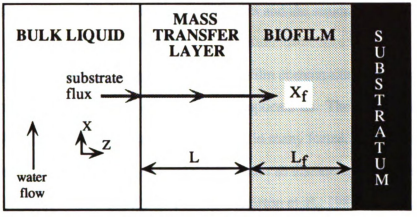

Figure 2. Classical biofilm model illustrating substrates flux from the liquid phase through a mass transfer layer with width L and into the biofilm matrix with width L_f and density X_f (after Criddle et al., 1991).

Descriptions of substrate utilization processes in biofilms have been based on idealized biofilm models consisting of an homogenous matrix of bacteria and their polymeric materials with uniform density, X_f (mg/cm^3), uniform thickness, L_f (cm), and a laminar transfer layer, L (cm) through which substrate is transported from the bulk liquid phase (Figure 2). Within the biofilm, substrate moves by molecular diffusion and is consumed locally. This model has been used for derivation of differential equations to describe substrate removal kinetics in biofilms (Williamson and McCarty, 1976; Rittmann and McCarty, 1980; Criddle et al., 1991). This provides the methodological tools for determining rate limiting processes and prediction power.

In order to address biodegradation issues in fixed-film systems, better knowledge of biological reaction kinetics and improved structural models are required. Christensen and co-workers (1989) have shown that the structure of a biological film used for wastewater treatment will to a large extent be determined by the transportation of substrate and reaction products inside the film. A description of the system based on substrate removal kinetics alone will fail since the reaction kinetics causes inevitable changes in the biofilm structure. Design rules for substrate removal in biological films therefore must include correlations between substrate removal kinetics and the structure and development of the film.

Relevance of biofilms -- The formation of biofilm communities on surfaces has some important economic, technical and ecological implications. The importance of attached microbial community processes are manifest in many forms, from beneficial processes in the natural environment to undesirable roles as disease agents (Characklis and Wilderer, 1989; Characklis and Marshall, 1989; Costerton et al., 1987). The fouling of heat exchanger tubes, cooling towers, and wastewater pipes by biofilms increases heat transfer resistance, energy losses, fluid frictional resistance and will reduce the overall performance eventually rendering these systems almost inoperative . The resultant energy loss and reduced performance, along with the need for regular cleaning, cost hundreds of millions of dollars per year on a worldwide scale (Marshall, 1992). In addition, microbial processes can accelerate deterioration of metallic substrata wherein sulfate-reducing and acidogenic bacteria in the anaerobic zone at the biofilm-metal interface can initiate corrosion by causing cathodic depolarization at the metal surface, ultimately reducing the equipment lifetime. Health risks associated with biofilm accumulation on teeth and gums, urinary tract, intestines, and after insertion of prosthetic devices into the human body have been shown (Costerton et al., 1987).

Many transformation processes in nature are accomplished most efficiently by mixtures of populations. Biofilms in natural aquatic ecosystems frequently influence water

quality by affecting dissolved oxygen content, nutrient fluxes, and serving as sink for many toxic and hazardous materials. For years, engineers have exploited these characteristics in fixed-film systems for extraction and biological oxidation of organic and inorganic contaminants found in domestic, agricultural and industrial wastewaters (e.g., fluidized bed reactors). These systems are based on particles that provide a large surface area for biofilm accumulation and the ability of those established populations to transform a wide range of chemicals. Biotechnological applications of biofilms include the extraction of minerals (biohydrometallurgy), while in other industrial uses the productivity (yield) and stability of the biotransformation processes are improved.

Biofilm ecophysiology -- Attached microbial cells can play important and not well understood roles in the maintenance of ecosystem stability. For example, biofilm communities can act as microbial reservoirs, aiding in the repopulation of surrounding waters when favorable conditions for growth are present (Fletcher, 1979). The predominance of attached growth within a broad range of environmental conditions suggests that this mode of growth must provide an important selective advantage. Possible advantages associated with attachment and biofilm formation have been reviewed (Breznak et al., 1984). First, fixation and preservation of position in preferential zones while preventing extinction by eluding washout near food sources. Accumulation of substrates at the solid surface and their subsequent availability for microbial uptake may also be an influencing factor for cells attaching to surfaces. Another possible advantage can be the optimization of substrate and waste transport from the attached cells by either diffusive and/or advective transport. Planktonic cells will tend to be transported within their locus of surrounding liquid. Therefore suspended cells will benefit mainly from diffusion alone. Protection against predators has been observed in biofilm systems. This is probably the result of sequestering of prey and diminished of area exposure to attack. Finally, attached communities can create and maintain microenvironmental conditions which may be advantageous for growth or survival but which are not achievable under suspended

planktonic states (for example, maintenance of anaerobic zones, optimal pH, as well as avoidance of toxic or temperature shocks, and desiccation).

Biofilm formation is limited mainly by the availability and transport of nutrients from the surrounding aqueous phase (Marshall, 1992). During early stages of biofilm formation however, oxygen and nutrient diffusive processes are normally not limiting factors. Once multilayers of bacterial cells become sequestered in their own polymeric matrix, diffusion becomes a major factor in determining the microbial structure at the colonized surface. Microsensor technology applied to relatively thick aerobic biofilms has shown the existence of steep oxygen and nitrate gradients demonstrating the importance of diffusive transport processes (Dalsgaard and Revsbach, 1992). These gradients develop as a result of rapid oxygen, nitrate, and nutrient consumption by aerobic bacteria, at or near the water-biofilm interface, and the comparatively low rate of diffusion of oxygen and nitrate through the biofilm matrix.

The chemical and physical activities of these microbial communities produce a heterogeneous system at the colonized surface (Lappin-Scott and Costerton, 1989). Organisms living within the biofilm have a tendency to be more stable on a temporal and spatial basis than planktonic populations. A general observation has been the resilience of such communities against perturbations or potentially lethal factors such as biocides in the nearby liquid phase. This is particularly important in the biomedical treatment of biofilm diseases (Blenkinsopp et al., 1992; Brown and Gauthier, 1993).

Microorganisms are distinguished from multicellular organisms by their ability to exist as isolated unicellular forms at least in part of their life cycle. The study of microbes can thus contribute to our understanding of intrinsic cell properties (e.g., control of gene expression). The influence of interspecific interactions on community structuring is a continuing problem in microbial ecology. Current ecological theories are based heavily on the concept of competitive exclusion and the assumption that communities exist at competitive equilibrium. Therefore, success or survival is based mainly on the

physiological fitness of each competitor in a stable uniform environment. Since competitive equilibrium requires that the rates of change of all competitors be zero, and since the physical environment, predation and other factors are changing constantly, it seems likely that true equilibrium rarely occurs in nature (MacArthur and Levins, 1967; Gorden et al., 1969; Lewin). There is increasing evidence that competitive exclusion occurs rarely in natural systems (McNaughton, 1978; Dykhuizen et al., 1983; Wiens, 1983; McCormick et al., 1991). Even in kinetically distinct bi-species competition experiments using steady-state chemostats, the exclusion of one species is rare, perhaps due to either resource or space partitioning (Levin, 1972; Veldkamp et al., 1983).

Dworkin (1991), has suggested that horizontal interactions among microbial populations are an integral feature of the multicellular nature of biofilms. These interactions take place when: (i) a multicellular structure has to be established, (ii) a multicellular activity has to be accomplished, or (iii) information has to be distributed among the populations. While a rich body of mathematical equilibrium theories has been developed, they are not necessarily valid for solutions to natural communities (Fredrickson and Stephanopoulos, 1981; Veldkamp et al., 1983). The effects of many abiotic and biotic factors on community structure still needs to be determined and more information is needed on horizontal control levels on community structuring.

BIOFILMS IN POROUS MEDIA

Bioremediation of benzene, toluene, and xylenes contaminated groundwaters -- Groundwater is one of the principal water sources for domestic, industrial, and irrigation uses. Its contamination by aromatic and alkyl substituted aromatic hydrocarbons such as benzene, toluene and xylenes (BTX) has become a serious threat to human health. Some pathologies have been attributed to BTX contaminated groundwater including benzene-induced carcinogenesis (e.g., leukaemogenesis) and non-specific effects on the central nervous system of vertebrates (Dean, 1978, 1985). Leakage from underground gasoline

and petroleum storage tanks, and distributions systems are the major causes of this pollution. Release of such compounds to the environment, either naturally or accidentally, can severely deteriorate water quality and have adverse effects on biological systems. These effects (Moore et al., 1974) include lethal toxicity, which is ascribed to impairment of essential cellular and subcellular processes (e.g., modified membrane integrity and transport systems) as well as alterations of physiological or behavioral activities (e.g., abnormal responses to physical and chemical stimuli).

The fate of the trace levels of these organics in aquifer systems is governed by physical (solubility, adsorption, hydrogeology, volatilization), chemical and biological factors. On a long term basis, biodegradation in aquatic environments appears to be the major attenuation mechanism (Atlas, 1981; Davis and Carpenter, 1990; Leahy and Colwell, 1990; Konopka and Turco, 1991). A diverse group of heterotrophic bacteria are known to posses the metabolic ability to utilize BTX compounds as their sources of carbon and energy (Olsen et al., 1989; Ridgway et al., 1990; Young, 1990; Fredrickson et al., 1991). Several metabolic pathways, either aerobic or anaerobic, have been described (Evans et al., 1991; Hutchins, 1991; Edwards et al., 1992). For toluene alone, five different aerobic pathways are currently known (Zylstra and Gibson, 1991). In general, the different pathways for aromatic oxidation have initial conversion steps that are carried out by different enzymes. Such enzymes activate the ring structure by the introduction of oxygen atoms to form a limited number of common intermediates; protocatechuate, cathecols and substituted cathecols. These are the substrates for ring cleavage and further metabolism to TCA cycle intermediates (Van der Meer, et al., 1992).

The rates of microbial transformation are generally governed by physical processes (mass transfer and diffusion) as well as the biological process itself (substrate transport and enzymatic machinery). Mass transfer limitations or bioavailability of substrates (both electron donor and electron acceptor) or nutrients however, may limited removal of low concentrations of BTX by suspended bacteria in aquifers. At substrate concentrations at

which growth and decay rates are equal, net growth will not occur. In the absence of an alternative energy source, establishment of degradative populations will not take place (Rittmann and McCarty, 1980; Criddle et al., 1991). This threshold substrate concentration may be in excess of the recommended standard for potable water. At the moment, we lack complete understanding of the factors that promote recalcitrance or biodegradability in nature. In order to gain that knowledge it is necessary to evaluate closer the interaction of abiotic factors with biological processes.

The costs to execute a remedial program at groundwater contaminated sites can be staggering and funds available to clean up the number of seriously contaminated areas are insufficient. The costs of physical and chemical remedial programs for the average site can range from several thousand to millions of dollars per site (Hickey et al., 1991; Evans, 1994). There are only two courses of action: clean up fewer sites, or develop more economical technologies that significantly reduce the cost of reclamation. One attractive alternative is the use of biological treatment, which is generally more economical and may significantly reduce the cost of clean up.

The success or failure of bioremediation technology is determined by a variety of environmental factors including temperature, pH, nutrients, trace elements, electron acceptors, and substrate composition and bioavailability. The interactions of these factors are better understood and can be manipulated in order to optimize microbial growth, survival and performance. Aerobic granular activated carbon fluidized bed reactors (GAC-FBR) have been used for the reclamation of BTX contaminated groundwater in laboratory and field scale systems (Ridgway et al., 1990; Hickey et al., 1991; Jing et al., 1992). Low effluent concentrations of these compounds are achieved in cost effective pump and treat or enhanced in situ systems. The GAC-FBR process is a fixed-film biological treatment which destroys the contaminant, coupled with the adsorption properties of the activated carbon, for ensuring robust high capacity treatment. High surface area per unit volume provides for bacterial colonization to ensure a high-rate biological treatment. Other

advantages include, the ability to achieve low-ppb BTX effluents in short detention times (4-10 minutes); no off-gas production; ability to treat low-concentration waste waters; and biological reduction and adsorption in a single reactor. The biological factors involved however, are poorly understood. An improved understanding of these factors will help to define microbial behavior for a wider range of conditions.

Effect of non-native populations on community structure -- When indigenous populations with the appropriate catabolic pathways are present, environmental restoration may consist of only optimizing growth conditions for the selected group of microbes. However, reliance on these populations may be inadequate, especially in sites where environmental conditions may not be suitable for growth or survival of those microorganisms. Degradation of specific compounds can be accomplished or enhanced by introducing organisms with unique physiologic traits at contaminated sites or in bioreactors. Moreover, the current understanding of the mechanisms involved in xenobiotic degradation at the enzyme and genetic level (Kukor and Olsen, 1990; Zylstra and Gibson, 1991) have led research on the construction of recombinant organisms with novel routes of degradation with the use of cloned genes. Many microbial strains have been genetically manipulated by incorporation of extra catabolic genes or by controlling their regulatory mechanism (Lehrbach and Timmis, 1983). *In situ* experiments have shown that such microbes can be successfully used for site remediation under selective pressure (Chakrabarty, 1986). Nonetheless, biosafety concerns persist with respect to the deliberate release of genetically manipulated populations to the environment and this remains an area of debate (Tiedje et al., 1989; Dunster, 1992).

Regardless of the possible short-term beneficial effects of using transgenic organisms to bioremediate contaminated sites, important ecological issues must first be considered. These include persistence and reproduction of the introduced strain, interactions with other organisms and with its environment, and possible effects of the introduced population upon ecosystem function and stability. Our current insufficient

understanding of survival strategies of microorganisms in natural systems (Roberson and Firestone, 1992; Pipke et al., 1992; Siegele and Kolter, 1992; Smets et al., 1993), increases risks associated with release of transgenic microbes. Although many of these recombinant microorganisms will probably be less fit than indigenous populations, important exceptions may arise and many generations may pass before the organisms disappear completely. Furthermore, the observed ubiquity of biofilms, resilience to perturbations, high cell density and close proximity to one another, and the creation and maintenance of unique microenvironmental conditions give rise to important ecological considerations since these communities may serve as potential refuges for non-native organisms (Wagner-Döbler et al., 1992; Warren et al., 1992).

Although natural selection will tend to increase fitness of the released transgenic microbial population, genetic exchange can certainly be an alternative pathway for the fixation of a novel trait in a community (Orvos et al., 1990; Stewart and Sinigalliano, 1990; Ramos-González et al., 1991; Romanowski et al., 1991, 1992; Barkay et al., 1993). Exchange of genetic material among microbial populations has been known to occur for more than 50 years (Avery et al., 1944). Bacterial genomic plasticity and gene transfer mechanisms have been described: (i) transfer by direct cell to cell contact between a donor and a recipient cell (conjugation) (Freter, 1983; Ippen-Ihler, 1989), (ii) uptake of naked DNA (transformation) (Stewart, 1989), and (iii) phage-mediated transfer (transduction) (Kokjohn, 1989). The role of gene transfer in the ecology of microbial communities and promoting physiological diversity are not known. Conjugal gene transfer in a wide range of natural environmental conditions, and subsequent generation of new novel phenotypes has been noticed in aquatic environments (Paul et al., 1991; Barkay et al., 1993). While the occurrence of plasmid transfer between marine bacteria in aqueous environments has been observed, there is evidence showing significantly higher transfer frequencies among cells attached to bead surfaces in biofilm communities (Angles et al., 1993). Also, when naked nucleic acids are adsorbed onto surfaces, resistance against endonuclease

degradation and subsequent bacterial transformation has been reported (Romanowski et al., 1991). Thus, the influence of non-indigenous populations and their genes on function and structure of surface colonized communities is an important aspect of ecological risk assessment of deliberate release of transgenic organisms to the environment.

Methodological approaches in biofilm community studies -- Methodologies must be preceded by a framework of critical scientific questions for which answers are required in order to gain understanding of relevant ecological and practical issues. The selection of analytical methods will also depend upon a desired level or scale of resolution (molecular-, cellular-, population-, community-, or system-level). Furthermore, the level of observations can also be subdivided into properties, processes, and biotic-abiotic components.

A multiplicity of traditional microbiological culture-dependent techniques, molecular-based methods, and microscopical procedures are currently available for ecological studies of biofilm systems (Costerton, 1983; Marshall, 1986; Characklis and Wilderer, 1989; Lad and Costerton, 1990; Ney et al., 1990; Caldwell et al., 1992). A qualitative and quantitative description of the microbial composition can partially be assessed by using traditional enrichment-isolation procedures, plate counts, or liquid culture techniques. A small fraction of the total diversity however, seems to be represented on those bacterial inventories (Wayne et al., 1987; Torsvik et al., 1990; Ward et al., 1992). Thus, success may be limited by the failure to perceive the niches of many microorganisms or to reproduce these niches in artificial culture environments. In addition, culturing bacteria from biofilms requires dissociation of the cells by potentially harmful procedures. The application of gradient chambers for cell isolation and characterization represents an important improvement for culturing previously described unculturable organisms (Wolfaardt et al., 1993; Emerson et al., 1994). Multidimensional gradients can be build to cover a broad range of physic and chemical parameters creating heterogeneous environments for better simulation of natural conditions.

Molecular methodologies based in the use of biochemical markers allow the study of natural communities free of the bias inherent in cultured dependent methods. Molecular probes targeted to specific biomarkers (DNA, ribosomal RNA, proteins, lipids) are useful tools for the characterization of phylogenetic, enzymatic, and cell surface composition of microbial populations (Adachi et al., 1993; McSweeney et al., 1993). In particular, the extensive phylogenetic work on ribosomal RNA championed by Woese (1987, 1993, 1994) has spanned new frontiers for microbial community analysis. Ribosomal RNA based methods for analysis of community structure and composition have been successfully applied to the study of natural systems without the need of culturing the members of the community (Weller et al., 1991; Spring et al., 1992; Arnosti and Repeta, 1994). They have also led to the development of nucleic acid hybridization probes suitable for spatial analysis of specific populations in nature (Amman et al., 1990; Wagner et al., 1994). In addition to direct comparison of the nucleic acid sequences (Weller and Ward, 1989; Britschgi and Giovannoni, 1991), numerous groups have used the rapid method of PCR amplification of this gene as well as the complete rRNA locus as a simple method for identification of bacterial species and genera (Böttger, 1989; Reysenbach et al., 1992; Muyzer et al., 1993) . This technique allows a rapid synthesis of desired sequences from genomic DNA by DNA chain extension from two opposing primers catalyzed by a DNA polymerase (Mullis and Faloona, 1987; Keohavong and Thilly, 1989; Steffan and Atlas, 1991). In this latter procedure, the amplified DNA (rDNA) is also subjected to restriction analysis (Moody and Tyler, 1990; Hook et al., 1991; Vaneechoutte et al., 1992; Massol-Deyá et al., in press; see Appendix A). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes or as quick assessment of genotypic changes of community samples, over time or between different locations. The procedures have the potential to differentiate between closely related organisms. Nonetheless, differential rRNA recoveries from microbial populations of a natural systems can introduce bias to the community analysis. The potential bias introduced by sampling

problems, cell lysis, or nucleic acids recovery efficiency need to be examined on an individual basis.

Fatty acids derived from phospholipid components of the cellular membranes of microorganisms have been used to estimate microbial biomass and provide insight into the taxonomic and functional diversity of natural microbial communities (Vestal and White, 1989; Rajendran et al., 1992; Frostegard et al., 1993). Community-level fatty acid analysis basically entails the extraction of fatty acids from a sample with organic solvents followed by analysis of certain fractions of the extracted material. This approach can generate simple and reproducible indicators of the relative similarities between microbial communities. Interpretations of community-level fatty acid profiles have been based on two general assumptions: (i) that phospholipids comprise a relatively constant proportion of cell biomass, and (ii) fatty acid composition between taxonomic groups results in "signatures" which can be detected and interpreted (Vestal and White, 1989). Phospholipid contents however, varies across taxa (Haack et al., submitted), and lipid and fatty acid profiles are known to vary quantitatively with changes in the growth environment (Kamimura et al., 1993; Nordström and Laakso, 1992; Nordström, 1993). Based on these factors, interpretation of community fatty acid profiles in terms of biomass or taxonomic composition, must be viewed with caution until knowledge of the quantitative and qualitative distribution of fatty acids over a wide variety of taxa, and effects of growth conditions on fatty acid profiles, is more extensive.

Microbial activity measurements can also be defined at different levels of resolution dependent upon the questions posed. Furthermore, these measurements can be categorized as either *in situ* activity and potential activity. Direct *in situ* measurements of microbial activity offers the advantage of obtaining dynamic information of the system. Some of the available techniques include radiolabeling/autoradiography, radiorespirometry, use of dyes or inhibitors, and chemical analysis of transformation products. These direct activity measurements nonetheless have their own problems including penetration of substrates,

dyes and reagents through the film matrix. This is particularly true when molecular probes are use for *in situ* measurements. Microsensor technology has been one of the most powerful analytical techniques for characterizing chemical and metabolic activity gradients in microbial films (Binnerup et al., 1992; Dalsgaard and Revsbach, 1992; Kühl and Jørgensen, 1992; De Beer et al., 1993). It can be used to measure high resolution depth profiles of chemical species of biological relevance such as O_2 (oxygenic respiration, or gross photosynthesis), N_2O (denitrification), H^+ (proton production/consumption), NO_3^- (net nitrate production/consumption), H_2S (sulfidogenesis, or anoxygenic photosynthesis), NH_4^+ (ammonia production/consumption), and H_2 (hydrogen production/consumption). Complications however occur due to the diffusive nature of surface films, leading to fluctuations in water flows, and three dimensional related problems. The microbial potential activity in biofilm systems can be measured by the use of confocal scanning laser microscopy coupled to fluorescently labeled probes, chemical or electrode analysis of transformation products by microsectioning the biofilm, and immunological and nucleic acid probes (Amman et al., 1990; Wagner et al., 1994). Nevertheless, these approaches present a rather static picture of the metabolic processes at a single moment in time.

Finally, microscopy is one of the best ways to understand the spatial cell organization in a biofilm. Microscopic techniques are relatively easy to understand and highly informative. Observations can answer questions about morphology, cell arrangements, surface and attachment structures, extracellular materials, and when combined with specific probes or dyes, identity and activity of individual cells. Advance methods currently used for direct visualization of biofilm structures includes X-ray microanalysis (e.g., location of elements in the biofilm), and scanning electron microscopy (e.g., distribution and morphology of cells at surfaces). Although electron microscopy offers superb resolution of ultrastructure details (Marcelino and Bensosn, 1992), it is damaging to specimens and suffers from fixation and sectioning artifacts.

Nondestructive experimental techniques must be used to examine biological samples in their natural state. Confocal scanning laser microscopy (CSLM) has recently emerged as the solution to this requirement. CSLM is a rapidly advancing technology which offers detailed visualization of thick biological samples in which out-of-focus blur is essentially absent (Wilson, 1989; Wright et al., 1993). First, the shallow depth of the field (0.5-1.5 μm) of confocal microscopes allows information to be collected from a well defined optical section rather than from most of the specimen as in the conventional light microscope. Second, the CSLM optically sections specimens so that physical sectioning artifacts observed with light and electron microscopy are eliminated. Because optical sectioning is noninvasive, living as well as fixed cells can be observed with greater clarity. Examination of specimens can be accomplished while maintaining the integrity of the three-dimensional matrix and with minimum sample processing. Therefore, it is no longer necessary to disrupt the community superstructure when studying the molecular or behavioral aspects of their ecology. Another advantage of CSLM is that specimens can be optically sectioned not only perpendicular to the optical axis of the microscope (x, y), but also vertically (parallel to the optical axis of the microscope) (Carlsson and Liljeborg, 1989; Shotton, 1989). Stacks of optical sections taken from successive focal planes can be reconstructed to produce a three-dimensional view of the sample. Thus, CSLM not only bridges the gap between light and electron microscopy, but provides a means to observe structural components and ionic fluctuations of living cells and tissues in three dimensions.

Chapter II

SUCCESSION AND CONVERGENCE OF BIOFILM COMMUNITIES IN FIXED-FILM REACTORS TREATING BENZENE, TOLUENE, AND p-XYLENE CONTAMINATED GROUNDWATER

BACKGROUND

Reclamation of contaminated subsurface resources have focused on the use of microorganisms. The catabolic potential of multiple bacterial populations to utilize aromatic and alkyl substituted aromatic hydrocarbons either aerobically or anaerobically as carbon and energy source has been described (Leahy and Colwell, 1990; Ridgway et al., 1990; Aelion and Bradley, 1991, Hutchins, 1991). When indigenous populations with the appropriate catabolic pathways are present, environmental restoration may consist of only optimizing growth conditions for this selected group of microbes. However, reliance on these populations may be inadequate, especially in sites where environmental conditions may not be suitable for growth or survival of these microorganisms. Degradation of contaminating compounds can also be accomplished by introducing and selecting for organisms with unique physiologic traits at contaminated sites or in bioreactors where groundwater extraction and treatment is required to prevent migration of the contaminant plume.

Assessment of the fate and influence of introduced populations into heterogeneous microbial ecosystems has ecological and practical significance. Although the processes of species succession has been studied intensively in ecology (Gorden et al., 1969; Simberloff and Wilson, 1969; Andrews et al., 1987; Kinkel et al., 1989; McCormick et al., 1991), little is known about contributing processes on pathways of community development within microbial populations in natural habitats. There is direct experimental evidence suggesting that interspecific competition within a given trophic level may be an important

determinant in structuring microbial communities (McArthur and Levins, 1967; McCormick et al., 1991).

We studied microbial succession in aerobic fluidized bed reactors (FBR) using granular activated carbon (GAC) as the biomass carrier. The surface adhesion of bacterial populations with subsequent cell binary fission and exopolymer production, leads to the formation of biofilms (Costerton et al., 1987; Warren et al., 1992). FBRs are now in commercial operation for bioremediation of groundwater contaminated with benzene, toluene, ethylbenzene, xylenes (BTEX) and other substituted aromatic hydrocarbons (Hickey et al., 1991). In turn, our ability to understand the function and behavior of natural and introduced populations in these reactors is of particular relevance since they are key environmental mediators of the transformations of these chemicals.

Microbial succession was examined using a reactor initially colonized with three well-studied toluene degrading bacterial strains which were then challenged by endemic aquifer organisms residing in the groundwater. Mature biofilm communities from this reactor were then compared to communities of reactors colonized directly by aquifer organisms. In addition, the influence of changes in carbon source on community structure was evaluated. Classical growth dependent microbiological methods in combination with genomic community fingerprints and community-level fatty acid analysis were used to describe the relative genetic and physiologic relatedness among these biofilm communities. We found convergence of populations in the seeded and the natural aquifer colonized GAC communities when toluene was used as sole carbon source. Although all inoculated strains were outcompeted by the indigenous groundwater populations, survival of one of the inoculated strains in a mature 113-day-old biofilm community was observed. This demonstrated the potential of biofilms as refuges for allochthonous populations. In addition, our studies showed that the available carbon sources are important factors in structuring of the community.

MATERIAL AND METHODS

Granular activated carbon fluidized bed reactors (GAC-FBR) -- Bioreactors consisted of 1-L sterile glass columns (2.54 cm diameter x 195 cm long) and were operated as a one-pass upflow system without recycle at a flow rate of 0.2 l/min (Figure 1). Groundwater from a deep aquifer underlying the MSU campus was amended with toluene or a mixture of benzene, toluene and p-xylene (BTX 1:1:1) at a organic loading rate of 5.4 kg COD/m^3-day. In addition, the influent was supplemented with pure oxygen (11 - 13 mg/l inlet concentration), and a nutrient solution (NH_4Cl & KH_2PO_4) to achieve a ratio of 30:5:1 (carbon : nitrogen : phosphorous). Granular activated carbon (GAC; Calgon Filtrasorb 400, Calgon Company, Pittsburgh, PA) was used as the carrier matrix. Approximately 100 g dry weight of GAC per reactor was washed with deionized water to remove carbon fines, and then sterilized by autoclaving (2 h at 121°C) before use. The GAC had a geometric mean diameter of 0.7 mm and an overall density of 1.6. The following FBR systems were operated in parallel for this study:

(i) Reactor colonized by indigenous aquifer microbial populations with toluene as the sole carbon source (referred to as TOL.NS reactor [toluene.non-sterile]).

(ii) Reactor colonized by indigenous aquifer microbial populations with BTX added as the sole carbon sources (referred to as BTX.NS reactor).

(iii) Reactor seeded with three toluene degrading species (initially referred to as TOL.S). This bioreactor was operated in sequential stages: (a) day 0 to 53, filter-sterilized groundwater with toluene as the sole carbon source, (b) day 53 to 124, the filter was removed and groundwater with toluene continued to feed the reactor, and (c) day 124 to 175, no filtered groundwater was fed but with BTX rather than toluene as the sole carbon sources. This latter configuration is referred to as the BTX.S reactor [S, seeded]. The serial filter-sterilization system consisted of a 0.30 μm fiber filter, an activated carbon filter and two 0.45 μm membrane filters [Polycap 75 ASW/Bell].

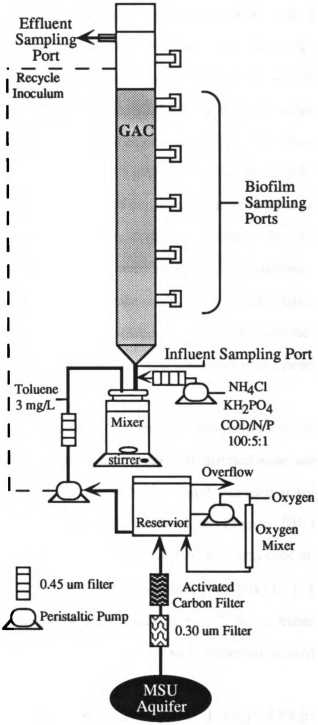

Figure 1. Schematic diagram showing setup conditions of FBRs used for the experiments. Filter system was absent in control FBR (TOL.NS and BTX.NS) as well as in the inoculated FBR (TOL.S) after day 53. Illustration not drawn to scale.

Bacterial strains and growth conditions -- Initial colonization of the filter-sterilized FBR (TOL.S reactor) used three well characterized toluene degrading strains: *Pseudomonas cepacia*, strain G4 which carries the toluene ortho-hydroxylase pathway (Folsom et al., 1990); *P. pickettii*, strain PKO1 which carries the toluene meta-hydroxylase pathway (Kaphammer et al., 1991); and *P. putida*, strain PaW1 which carries the toluene methyl-hydroxylase pathway on plasmid pWW1 (Franklin et al., 1981; Harayama et al., 1989). These strains were grown to mid exponential phase in basal salt medium (BSM) (Owens and Keddie, 1969) with toluene as the sole carbon source. Cultures were enumerated by acridine orange direct counts, mixed in equal numbers (approximately 10^9 cells per culture), and used to inoculate the sterile GAC reactor. After recirculation of the inoculum for 2 h, cell adsorption to the carbon was apparent since the liquid phase change from turbid to clear. At this time continuous feeding of toluene amended filter-sterilized groundwater was started.

Selective media were used to determine the population sizes of inoculant strains in the filter-sterilized FBR. All media were made with distilled water and solidified with 2.2% (w/v) Nobel agar. For strain G4, 1% (w/v) d-sorbitol was used as sole carbon source in basal salt media (BSM) (Owens and Keddie, 1969; see Chapter III); for strain PKO1, 10 μg/ml of kanamycin and γ-hydroxybutyric acid (1% w/v) was used in BSM; and for strain PaW1 the medium was composed of (g/l): sucrose, 10; $NaHCO_3$, 1; $MgSO_4 \cdot 7H_2O$, 5; K_2HPO_4, 2.3; N-laurylsarcosine sodium salt (SLS), 1.2; 20 mg trimethoprim (after sterilization); 10 ml glycerol. Biofilm samples were dispersed, diluted and plated on these media and incubated at 25°C.

Analytical procedures -- Physicochemical analyses of all FBR, including measurements of toluene or BTX, oxygen consumption, temperature, and pH, were generally conducted at 2 to 3 day intervals. For BTX determination, 5 ml samples of influent and effluent groundwater was collected in 10 ml vials, supplemented with approximately 0.10 ml of 6 N HCl, sealed with Teflon-coated butyl rubber septa and

aluminum crimps, and stored at 4°C until use. BTX compounds were analyzed using a gas chromatograph (Varian 3700) equipped with a flame ionization detector (GC/FID), a DB-624 capillary column (J&W Scientific, Folson, CA) and a head space sampler. The vials were equilibrated at 40°C, the column at 90°C and the injector and detector at 200°C. Helium was the carrier gas. For dissolved oxygen (DO) and pH measurements, samples collected from overflow funnel with built in stirring bar were immediately analyzed with a polarographic electrode coupled to an Orion digital pH/millivolt meter (Orion Model 611). Dissolved oxygen consumption was calculated as the difference between the influent and effluent quantities.

Sampling, biofilm homogenization and bacterial counts -- Biofilm samples were collected aseptically from column sampling ports every 2 to 7 days. Cells were removed and homogenized from the activated carbon (approximately 0.2 g wet weight) by repetitive vortexing (three times for 45 s) in 1 ml of cell extraction buffer (Warren et al., 1992). Viable bacterial numbers from GAC samples were determined using medium R2A (Difco, Detroit, MI). R2A plates were incubated at 25°C for 3 to 4 days and total counts and relative densities of different colony types were recorded. For determining cells per GAC, the extracted GAC sample was desiccated at 195°C for a week or until repetitive weight readings were obtained in an analytical balance. Isolation of the major BTX degrading populations from the GAC-FBRs was done as described elsewhere (see Chapter III). Total bacterial cell counts were also determined by acridine orange (AO) epiflourescence microscopy using the method of Zimmerman et al. (1978) as modified as follows. Biofilm homogenates, were fixed in 0.1% (v/v) formaldehyde (final concentration) and then stored at 4°C until processed. Dilutions were made and stained with 0.01% (w/v) AO [final concentration] for at least 10 min. Appropriate volumes were filtered through black polycarbonate filters of 0.2 μm pore size and 47 mm in diameter (Nucleopore Corp., Pleasanton, CA). Filters were pre-wetted by passing approximately 1 ml of a 0.1% (v/v) Tween 80 solution. Excess stain was removed by a last filtration of 3 ml of 25% (v/v)

isopropanol. The dry filters were mounted on glass slides. A drop of immersion oil was placed on top of the filters, followed by examination under epifluorescent illumination. A minimum of 10 randomly selected fields with 30 to 75 cells each were counted and averaged for each subsample. A Leitz phase microscope, equipped with epifluorescent condensers for reflected light excitation was used for the observation and enumeration of microbial cells.

Nucleic acid fingerprint analysis -- Nucleic acid extraction (Maniatis et al., 1982) was performed from cell pellets of bacterial isolates or GAC communities that were resuspended in 100 µl of 25% (w/v) sucrose-10 mM EDTA-50 mM Tris (pH 8.0). TE buffer (40 µl) containing lysozyme (5 mg/ml) was added alone with 40 µl of 250 mM EDTA (pH 8.2). After incubation at 37°C for 10 min, the cells were lysed with the addition of 250 µl of 2% (w/v) sodium dodecyl sulfate (SDS), and treated with RNase A [5 µl of 2 mg/ml stock] and pronase E [10 µl of 10 mg/ml in distilled water] for 60 min at 37°C. Samples were then amended with 120 µl NaCl (5 M), 85 µl TE (pH 8.0), and 80 µl of 10% (w/v) CTAB solution (cetyltrimethylammonium bromide) and incubated at 65°C for 20 min. The cell lysate was then extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), twice with chloroform-isoamyl alcohol (24:1, v/v) followed by a final extraction with chloroform. The nucleic acid was precipitated by the addition of 2.5 volumes of 97% (v/v) ethanol, washed with 70% (v/v) ethanol, dried under vacuum, resuspended in TE buffer and quantified by measuring the optical density at 260 nm (Maniatis et al., 1982).

Extracted DNA was subjected to amplification of the ribosomal RNA genetic loci by the polymerase chain reaction (PCR). Universal primers were used to amplify an approximately 1,500 bp fragment using a forward primer which corresponds to nucleotide positions 49 to 68 (5'-AGAGTTTGATCCTGGCTCAG-3'; primer A) of *Escherichia coli* 16S rRNA and a reverse primer corresponding to the complement of positions 1541 to 1518 (5-AAGGAGGTGATCCAGCCGCA-3'; primer H) (Ulrike et al., 1989). All primers were

synthesized with an Applied Biosystem DNA synthesizer at the Macromolecular Structure and Sequencing facility at Michigan State University. In general, amplification was done in 100 µl total reaction volume containing 100 ng of total community extracted DNA or bacterial isolates as template, 1 µM of each primer, 250 µM of each deoxynucleoside triphosphate, 10 µl of 10X Taq buffer, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) was used. PCR amplification was performed in an automated thermal cycler (9600 Perkin-Elmer Cetus, Norwalk, CT) with an initial denaturation [92°C, 130 s], followed by 30 cycles of denaturation [92°C, 70 s], annealing [48°C, 30 s], and extension [72°C, 130 s], and a single final extension [72°C, 6 min]. An aliquot of 5 µl was run in 0.7% agarose gel to evaluate the quality of the amplified fragment. In general, amplification yielded greater than 10 µg of PCR product.

Community (8 µl, 5X concentrated by ethanol precipitation) or bacterial (13 µl) amplified DNA was digested at 37°C for at least 3 h in 15 µl final volume with tetrameric site-specific restriction endonucleases (*Hae*III, *Hpa*II, *Mse*1, or *Hin*f1; Boehringer Mannheim, Indianapolis, IN). The 16S rDNA restriction fragments were then separated by electrophoresis on 4% (w/v) NuSieve 3:1 agarose gel (FMC, Indianapolis, IN) in 0.5X TAE [Tris acetate-EDTA] at 7 volts per cm, and stained in 0.5 µg per ml ethidium bromide solution. The resulting restriction fragment patterns were then used as a fingerprint to detect similar communities.

Species-specific primers complementary to the V3 region of the 16S ribosomal RNA gene of strains G4 and PKO1 were constructed for a more sensitive and specific detection of these strains by PCR amplification (see Appendix B). PCR was performed as described above but at an annealing temperature of 55°C in 50 µl reaction volumes. After amplification, 10 µl of the PCR reaction was separated by electrophoresis on 0.7% (w/v) agarose gel in 1X TAE buffer.

Community fatty acid methyl ester (FAME) analysis -- Total fatty acids of microbial communities were analyzed using the Microbial ID, Inc. (MIDI; Newark, DE). Duplicate

samples of cells collected from fresh GAC samples (approximately 0.1 g wet weight) were treated as follows: (i) whole community cell preparations were saponified at 100°C with 1 ml methanolic NaOH (15% (w/v) NaOH in 50% (v/v) methanol), (ii) esterification of the fatty acids at 80°C with 2 ml 3.25 N HCl in 46% (v/v) methanol, (iii) extraction of the fatty acid methyl esters (FAME) into 1.25 ml 1:1 (v/v) methyl-*tert*-butyl ether/hexane, (iv) aqueous wash of the organic extract with 3 ml 1.2 % (w/v) NaOH, and (v) analysis of the washed extract by gas chromatography equipped with a flame ionization detector.

The mean concentration and standard deviations as well as principal components analysis for FAME profiles were determined by using SYSTAT™ version 5.1 (SYSTAT Inc., Evanston, IL).

RESULTS

Groundwater -- The influent cell concentration of the groundwater in the open granular activated carbon-fluidized bed reactors (GAC-FBR) was approximately 10^7 total cell counts per liter. These microbial populations were a natural and continuous inoculum source for the colonization of the no seed GAC of the toluene and BTX fed reactors (TOL.NS and BTX.NS FBRs, respectively), as well as a challenge to the seeded toluene reactor (TOL.S) after removal of the filter-sterilization system. The groundwater at the bioreactor influent was 13°C (± 1) and a pH of 7.1 (± 0.3) throughout the course of the experiments.

Non-seeded reactors -- Toluene (TOL.NS reactor) and BTX (BTX.NS reactor) fed reactors were operated in parallel at the same organic loading rate. A description of the startup conditions and overall performance is given in Table 1. After a 5 to 7 day lag phase, dissolved oxygen (DO) consumption increased steadily as did biomass accumulation on the GAC particles. Steady-state conditions were assumed to have been attained when DO consumption was constant. This value was also commensurate with the oxygen required for the complete biodegradation of the added toluene. Steady-state conditions were generally achieved after 15 days, at this time substrate removal was 98 to 99% . A typical

profile of DO and toluene concentration with depth in the reactor treatment column is shown in Figure 2. As can be seen, more than 80% of the toluene was consumed in the first 30% of GAC bed height. Similar results were obtained with the column fed BTX as substrate (data not shown).

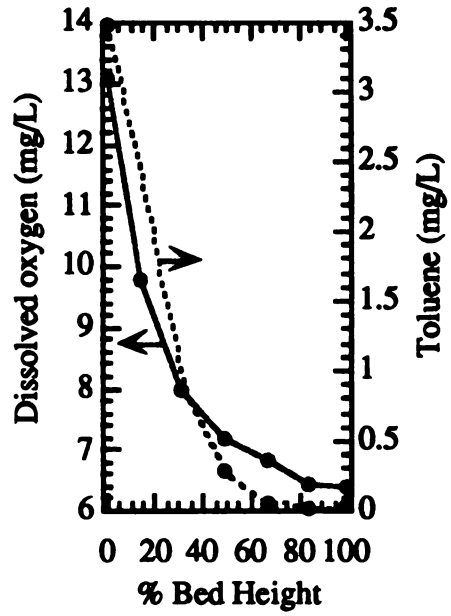

Figure 2. Typical profile of dissolved oxygen and toluene concentration with depth in the bioreactor column.

Seeded reactor -- To determine the influences of non-indigenous populations on community structure, equal numbers (2 x 10^7 cells/g of GAC) of *Pseudomonas cepacia* (strain G4), *P. pickettii* (strain PKO1), and *P. putida* (strain PaW1) preinduced for toluene metabolism were initially inoculated into the sterilized FBR. The reactor was fed filter-sterilized groundwater with toluene to allow the establishment of the seeded populations (TOL.S). A lag phase of 14 days was observed before significant oxygen consumption or biomass accumulation occurred (Figure 3). A phase of transient toluene acclimation occurred from day 14 to 18. This was followed by a 10 day growth phase.

Table 1. GAC-FBR startup phase and performance at steady-state conditions.

Reactor Designation	Inoculum source	Length of operation (days)	Carbon Source	Lag (days)	Dissolved oxygen consumption [mg/L] Mean (± SE)[1]	Toluene or BTX[2]	
						Effluent [mg/L] Mean (± SE)[1]	Removal Rate [%]
NS.TOL	Aquifer	22	Toluene	6 - 7	4.13 (0.82)	0.066 (0.051)	98
NS.BTX	Aquifer	98	BTX	5 - 6	6.09 (2.57)	0.040 (0.035)	99
TOL.S	G4, PKO1, PaW1	0 - 53	Toluene	13 - 14	5.70 (0.73)	0.140 (0.031)	96
	+ aquifer[3]	53 - 129	Toluene	0	4.90 (0.74)	0.063 (0.041)	98
	+ aquifer[4]	129 - 175	BTX	0	4.80 (1.17)	0.094 (0.053)	98

[1] Mean (and standard error of the mean) based on n = 7 to 20.
[2] BTX; benzene, toluene, and p-xylene.
[3] The filter-sterilization system was removed 53 days after inoculation.
[4] The carbon source was shifted to BTX after 129 days

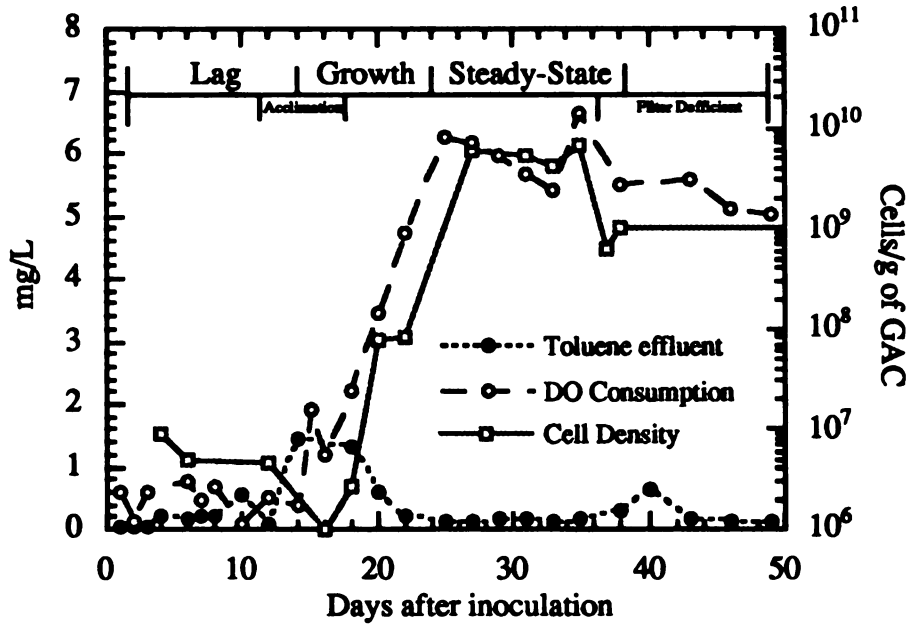

Figure 3. Observed dissolved oxygen consumption (mg/L), toluene effluent concentration (mg/L), and cell density (cells/g of GAC) in the TOL.S reactor inoculated with three different bacterial strains. The bioreactor was fed amended fiter-sterilized groundwater for 53 days to allow the establishment and stabilization of the inoculum members.

Analysis of specific population types by selective plating during the lag phase (Figure 4), showed a decrease in the densities of strains G4 and PKO1 but growth of strain PaW1. During the pseudo steady-state phase strain PaW1 was the sole recoverable culture. The increase in cell density of PaW1 corresponded to an increase in DO uptake, which confirmed that toluene oxidation was occurring. At steady-state, the average DO consumed was 5.70 mg/l and toluene effluent concentration was 0.140 mg/l for a 96% removal efficiency (Table 1). After 35 days, however, new bacterial types appeared on R2A plates, which suggested that groundwater strains may have breached the filter. On day 53, the filtration system was removed from the TOL.S reactor to allow aquifer populations to freely enter the already colonized GAC. A lower steady-state effluent toluene concentration was noted, declining from 0.140 to 0.063 mg/l (Table 1) after the filter was removed. The DO consumption remained at the previous steady-state level. From these mature biofilm

communities, 12 different toluene-degrading cultures were isolated. Inoculum strain PaW1 was recovered from a 113-day-old GAC sample by enrichment for *p*-xylene degradation.

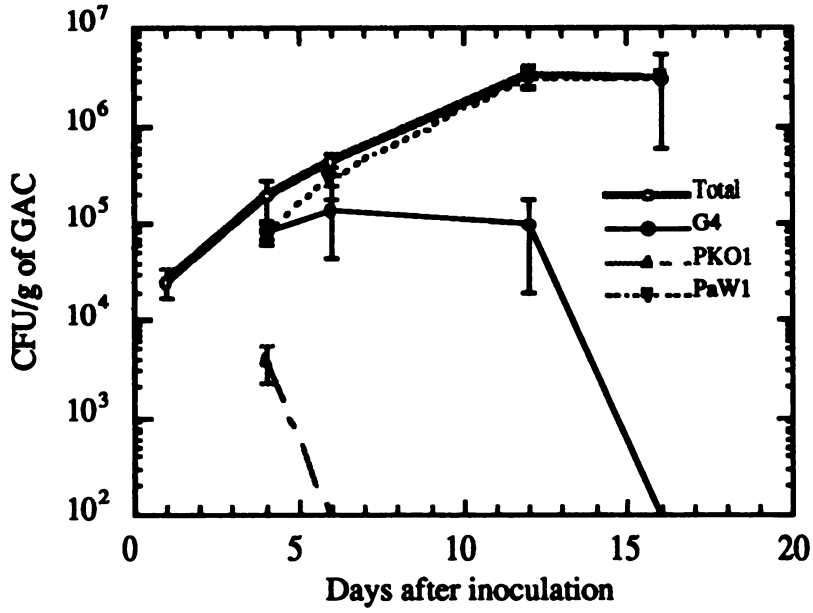

Figure 4. Densities of inoculated strains as determined by selective plate counts over time in the TOL.S reactor. CFU, colony forming units.

To evaluate the effects of carbon sources on community structure, the toluene selected community (TOL.S reactor) was switched from groundwater amended with toluene to groundwater amended with an equal mixture of BTX compounds (BTX.S reactor, Table 1) at the same organic loading rate. No changes in the carbon removal rate or DO uptake were observed. Although similar bacterial types to those isolated from toluene fed reactors were obtained, changes in isolation frequency of particular types were noted (see Chapter III).

Bacterial and community fingerprints -- Characterization of bacterial isolates and community composition was performed by restriction fragment length patterns of amplified rRNA genes from bacterial or community DNA. Greater than 65% of the cells were lysed as determined by direct fluorescent cell counts. This method yielded approximately 60 μg

DNA/g of GAC. The DNA was of high molecular weight (>23.1 kb) and could be digested by restriction endonucleases. Each of the inoculated strains showed a distinct restriction fragment pattern with the amplified rDNA when digested by *Hpa*II (Figure 5, lanes G4, PKO1, and PaW1). These three patterns could also be discerned in mixed samples taken after inoculation (Figure 5, Day 0). By day 22 however, DNA isolated from GAC biofilm showed only a PaW1-like pattern. This finding is consistent with the results obtained from

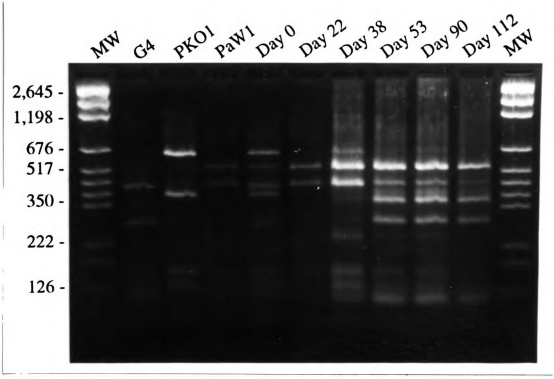

Figure 5. Community fingerprint of bioreactor seeded with bacterial strains G4, PKO1, and PaW1. The filtration system was removed on Day 53. Shown is a *Hpa*II digestion of the amplified 16S rDNA from reference cultures (Lanes G4, PKO1, and PaW1), and GAC community (Lanes Day 0, 22, 38, 53, 90, and 112). The sizes of marker DNA fragments (lanes SIZE) (in base pairs) are indicated on the left.

selective plating (see Chapter III). Further changes in band pattern profiles can be seen over time, reflecting further changes in community composition. An intermediate band pattern was observed at day 38 before the community profiles remained stable (Figure 5, lanes Day 53, 90, and 112). Interestingly, band patterns similar to those generated from G4

and PKO1 can be seen at these later time points. Because of the pure culture results (Figure 4), we suspected that they were from different populations that shared restriction sites with G4 and PKO1 and successfully made it through the filter barrier. In order to examine whether these strains were present, we used the species-specific oligonucleotide primers for these strains for PCR amplification. Samples from time zero showed amplification products for these species specific primers, but bands for G4 and PKO1 were not detected at days 22, 38, 53, 90, or 112, confirming that the inoculated strains were not successful colonizers at these stages.

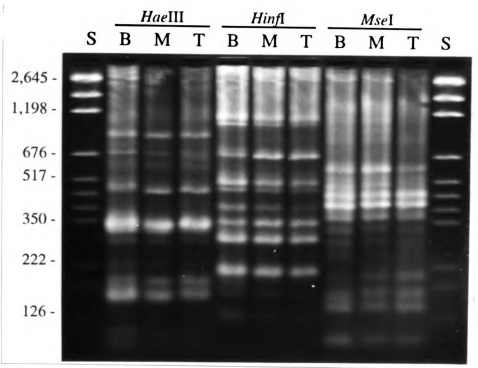

Figure 6. Restriction fragment length polymorphisms of amplified 16S rDNA from S.BTX reactor at day 159. Samples correspond to the 20, 60, and 90% reactor column bed height (lanes B, M, and T, respectively) digested with *Hae*III, *Hinf*I, and *Mse*I. The sizes of marker DNA fragments (lanes S) (in base pairs) are indicated on the left.

To determine whether position within the bioreactor treatment column lead to differences in patterns of bacterial composition, samples collected from the bottom (B), middle (M) and top (T) of the GAC bed height were subjected to RFLP analyses (Figure

6). Identical RFLP patterns were observed at all of these heights with all of the three endonucleases tested.

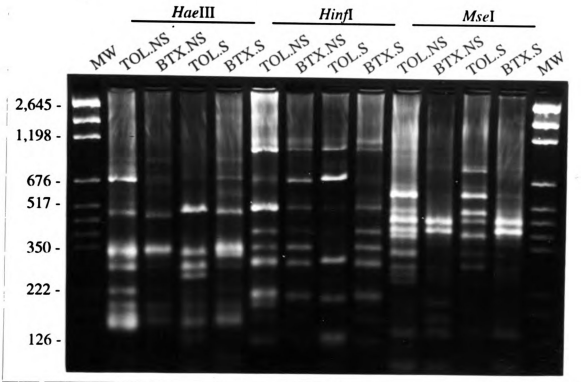

Figure 7. Restriction fragment length polymorphisms of amplified 16S rDNA from mature GAC communities digested with *Hae*III, *Hin*fI, and *Mse*I. The lane designations correspond to the bioreactor treatments (see Materials and Methods). The sizes of marker DNA fragments (lanes SIZE) (in base pairs) are indicated on the left.

The composition of mature GAC communities in all FBRs was also compared by RFLP analysis (Figure 7). Similar DNA patterns were observed between the seeded (TOL.S) and naturally colonized (TOL.NS) toluene fed reactors and between the seeded (BTX.S) and naturally colonized (BTX.NS) BTX fed reactors with each of the three endonucleases. The toluene and BTX fed communities seem to show some differences from each other as particularly noted with the *Mse*I restriction endonuclease digestion in which two signature fragments of approximately 460 and 410 base pairs were more apparent in the two BTX fed reactors. A thorough evaluation of faint bands as well as the

comparison by other enzymes, however, showed no band pattern differences between the systems fed the two substrates. Identical RFLP fingerprints were also observed for strains isolated from the two GAC communities (data not shown). Band patterns of these isolates correspond with most of the bands observed in the community fingerprints (Figure 8).

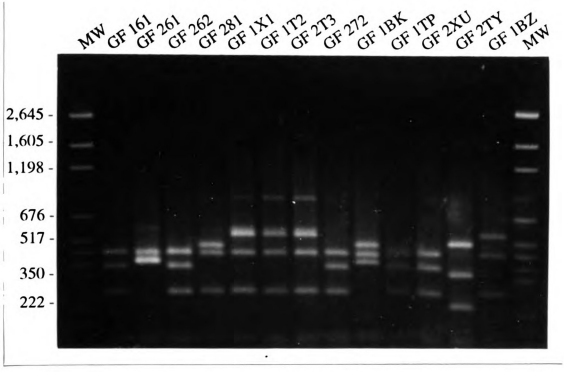

Figure 8. Genomic fingerprints of bacterial isolated from GAC communities. Shown is a *MseI* digestion of the amplified 16S rDNA. Lanes SIZE show the DNA size markers, indicated (in base pairs) on the left.

FAME community analysis -- Reactor community composition was also compared by fatty acid methyl ester (FAME) profile analysis (Table 2). The coefficient of variation for most fatty acids was generally below 10% for duplicate analyses. At least 22 fatty acids were identified among the different bioreactor treatments including saturated, unsaturated, and methyl and hydroxyl substituted fatty acids. Fatty acids 16:1 ω7c and 18:1 ω7c/ω9t/ω12t have been reported to be characteristic of the genus *Pseudomonas* (Haack et al., submitted).

Table 2. Relative abundance (% of total) of fatty acids of the GAC-FBR communities.

Fatty Acid[1]	TOL.NS Day 48	TOL.NS Day 98	TOL.S Day 53	TOL.S Day 129	BTX.NS Day 22	BTX.S Day 151
10:0	0	2.31	0	1.69	0	2.49
10:0 3OH	3.28	5.94	2.84	1.64	6.37	7.75
12:0	3.91	21.62	1.59	21.11	4.35	25.07
12:0 2OH	0.75	1.61	1.91	0.85	0	5.12
12:0 3OH	0.91	1.00	1.42	0.65	1.99	3.99
14:0	2.20	8.24	1.02	9.43	0	7.68
i 15:0	0	1.39	1.24	0	2.55	0
15:0	0	0.53	0	0.69	0	0
14:0 2OH	2.45	0.82	5.31	2.93	0	0
16:1 ω7c	44.21	28.24	30.37	23.68	47.52	21.34
*i 15:0 2OH, 16:1 ω7t	0	0	0	0	14.04	8.76
16:0	15.60	11.74	7.12	9.64	17.43	9.85
17:1 ω6c	0	0	2.64	0.50	0	0
17:0 CYCLO	0	1.51	0	0	0	2.37
i 16:0 3OH	0	0	1.13	0.85	0	0
*18:2 ω6,9c, a18:0	0	1.94	0	0.55	0	0
18:1 ω9c	0	1.11	0	0	0	0
*18:1 ω7c,ω9t,ω12t	25.66	8.58	41.22	22.63	5.74	5.57
18:0	0	1.49	0	1.85	0	0
*20:4 ω6,ω9,ω12,ω15c	0	1.24	0	0	0	0
20:0	1.03	0	2.18	0.69	0	0

[1] Fatty acids are designated as the number of carbon atoms, number of double bonds and position relative to the aliphatic (ω) end of the molecule; prefixes i and a, refer to iso and anteiso branching respectively; CYCLO and OH indicates cyclopropane and hydroxyl substitutions respectively. Data are means of replicate samples.

* a group that MIDI-FAME does not reliably separate.

Significant amounts of both of these fatty acids were observed in all FBR systems. Some fatty acids were detectable in only a particular treatment, for example, 14:0 2OH in toluene fed systems, and i15:0 2OH/16:1 ω7t in BTX amended reactors. There is also a trend of a higher percentage of fatty acid 12:0 in older samples regardless of carbon source. Although this fatty acid can be found at low levels in some *Pseudomonas* species (Haack et al., submitted), it has been generally associated with microeukaryotic organisms (Shaw, 1966). Yeasts and protozoans were observed in the older and more mature biofilms (see Chapter IV).

Table 3. Relative abundance (% of total) of fatty acids from samples collected at the bottom and top of the column bed height level of TOL.S reactor at day 101.

Fatty Acid[1]	Bottom 20%	Top 80%	% Variation
10:0 3OH	2.10	0	2.20
12:0	3.95	2.97	0.48
14:0	2.14	1.98	0.01
14:0 2OH	3.83	5.92	2.18
16:1 ω7c	41.20	35.04	18.97
16:0	11.60	8.36	5.25
17:1 ω6c	1.69	0	1.43
18:1 ω7c,ω9t,ω12t	31.58	41.84	52.63
19:0 CYCLO ω8c	0	1.54	1.19
20:0	1.92	2.34	0.09

[1] For fatty acid description, see Table 2.

MIDI-FAME profiles obtained from the bottom 20% and top 80% GAC bed height of the TOL.S reactor system at day 101 (Table 3) shows higher variation (greater than 15%) relative to the mean for fatty acids 16:1 ω7c and 18:1 ω7c/ω9t/ω12t than between replicate subsamples (less than 5%). Although the relative abundance of the FAMEs is

somewhat different between samples obtained from different positions within the GAC column, the fatty acids present are virtually identical.

In addition to providing some information on taxonomic structure, community-level FAME profiles were used to distinguished temporal and treatment variation using multivariate statistics. Principal component (PC) analysis was conducted using percentages for each fatty acid which appeared in any of the communities. PC scores obtained by a correlation matrix analysis are presented in Figure 9 in three dimensions, each axis corresponding to the first three PCs. The first PC accounted for 38% of the variance with high scores on fatty acids 10:0, 12:0, and 14:0. Separation into two classes of communities can be observed, old GAC samples (above 0) and relatively young communities composed mainly of bacteria (below 0).

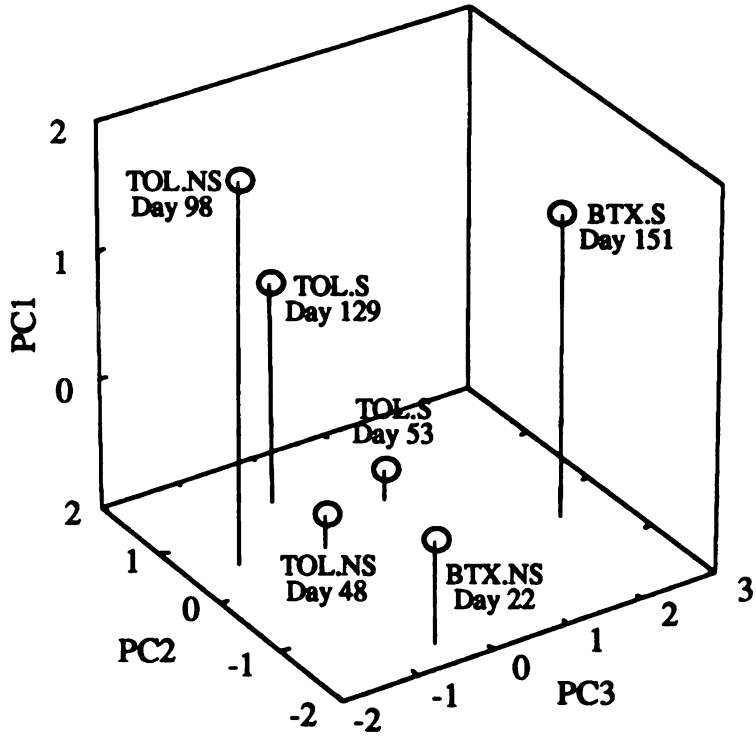

Figure 9. Principal components analysis showing temporal variations of FAME profiles for GAC communities. Reactors: toluene or benzene, toluene, and p-xylene fed systems of seeded (TOL.S and BTX.S) or aquifer colonized (TOL.NS and BTX.NS) biofilm communities. Parameters are listed in Table 2.

The second PC accounted for 26% of the variance with high scores on fatty acids i15:0 2OH/16:1 ω7t and 16:0. Finally, the third PC accounted for 15% of the variation in the data for a total cumulative of 79% with high scores on fatty acid 12:0 2OH.

DISCUSSION

Indicators that reflect changes in phenotype and genotype are important for describing succession, response to disturbances, and dynamics of natural communities. We examined the microbial structure and composition of GAC communities by restriction endonuclease analysis of amplified 16S ribosomal RNA gene fragments and by community-level FAME analysis. These fingerprinting analysis are simple, rapid and efficient methods to quickly describe bacterial assemblages and interrelationships between co-ocurring populations. While RFLP analyses were used as measurements of similarity among communities and with isolates (Moody and Tyler, 1990; Hook et al., 1991; Adachi et al, 1993), fatty acid analyses provided us with insights into the taxonomic composition (Federle et al., 1990; Frostegard et al., 1993a, 1993b).

The RFLP approach greatly simplifies the characterization of communities over the previous approach obtained analyzing several of individual samples of cloned 16S rRNA libraries (Schmidt et al., 1991), because the information is obtained in one or a few reactions. Disadvantages of our method, compared with analysis of individual cloned samples, are potential bias during the amplification reaction, limitation of resolving power on agarose gels for distribution of complex genotype mixtures in the community, and the information is not quantitative. From a practical point of view, however, the complexity of the community-level pattern can be controlled by the cutting frequency of the restriction endonuclease used (Bickle and Krüger, 1993). This feature allowed us to quickly screen and characterize cultures at different time points as well as to identify not yet isolated cultures. The correlation between fingerprints determined from GAC samples and those determined for isolated strains was acceptable. We do recognize , however, that

considerable phenotypic diversity could still be represented by different organisms giving the same bands.

To determine the influence of non-indigenous populations on community structure, three different toluene degrading strains were seeded to a sterile reactor fed toluene amended, filter-sterilized groundwater. After a prolonged lag phase successful colonization was accomplished by only strain PaW1. The growth phase of strain PaW1 in the filter-sterilized reactor was immediately followed by colonization of some groundwater populations which eventually made it through the filtration system as noted by changes in community fingerprints as well as by isolation of toluene degrading populations that were not in the inoculum. To determine whether PaW1 was a dominant population after the secondary colonization by native aquifer populations, Southern blots of total community DNA from the TOL.S reactor samples were hybridized to the *xyl E* gene probe which is specific for the tol plasmid encoded pathway (Harayama et al., 1989). No cross hybridization was observed at low stringency (Jizhong and Tiedje, personal communication), suggesting that PaW1 was below the sensitivity for the hybridization assay and therefore not a dominant member of the mature community structure. Thus, PaW1 a non-native population that was present as single dominant member after the controlled pioneer colonization phase of the GAC, was quickly outcompeted by the endemic aquifer populations. The presence of strain PaW1 in a 113-day-old GAC sample nonetheless, demonstrates that an allochthonous population can survive and avoid exclusion from the biofilm community.

The DNA fingerprints of the stable biofilm community (TOL.S) and the non-seeded cummunity (TOl.NS) converged. In addition, identical bacterial populations were isolated from the preinoculated GAC reactor after colonization by the aquifer populations (TOL.S) and from the TOL.NS systems (see Chapter III) further confirming convergence of populations in both reactor systems. These results suggest little influence of the pioneer colonist as a determinant of community structure. Possible explanations for these

observations are the functional equivalency of strain PaW1 as a primary colonist, and/or independence over short and long term community structure from pioneer colonizers. Since pioneer populations are directly interacting with the substratum, and since the mature biofilm colonizable surface is cells, superior and better adapted species can compete and establish themselves even in GAC-surface limiting conditions. These results contrast with those observed in microbial primary succession of planktonic communities in aquatic microcosms in which there was directional replacement of species and direct effects of changes in the order of colonizers on the outcome of community structure (McCormick et al., 1991).

Our results showed that community-level RFLP profiles reached band pattern stability indicating that bacterial genetic stability was achieved and maintained on mature colonized GAC communities. Furthermore, the bacterial composition of GAC particles throughout the treatment column appears to be similar as noted by RFLP analysis. Switching from toluene as the main carbon source to BTX at the same organic loading rate however, appears to be an important driving factor in influencing community structure. Our RFLP analysis shows transition of the toluene selected community (TOL.S) to a BTX structure (BTX.S) similar to that observed in the BTX.NS control reactor thus, demonstrating the flexibility of the biofilm community to quickly adapt to new environmental conditions.

The FAME community-level profiles from particles collected at different time points and from different sections within the treatment column revealed differences in types and abundance of fatty acids. Since the genomic fingerprints of the microbial community suggested stable bacterial composition on temporal and spatial basis within the bioreactor, changes in abundance of fatty acid appears to be influenced by differences in abundance of the fatty acid-contributing microbial populations and/or physiological status rather than changes in the basic bacterial composition. Generalizations about the distribution of certain fatty acids across microbial taxa have been used for analysis of whole community fatty acid

profiles (Federle et al., 1990; Frostegard et al., 1993a, 1993b; Haack et al., submitted). Fatty acids generally associated with pseudomonads species were found in abundant quantities in the GAC communities. These results are in agreement with preliminary characterization of some of the dominant GAC isolated strains (see Chapter III). There also appeared to be a positive correlation between a signature fatty acid of microeukaryotes and age of the biofilm. This relationship can be attributed to the observed secondary colonization by yeast and protozoa species which require primary surface modifications and longer reproduction cycles. Furthermore, the proportions of the monoenoic fatty acid 16:1 ω7c, which is highly recognized as being primarily associated with prokaryotes (Vestal and White, 1989), decreased in older GAC samples. The RFLP method used is sensitive to only prokaryotes.

Current ecological theories are based heavily on the concept of competitive exclusion and the assumption that communities exist at competitive equilibrium (Fredrickson and Stephanopoulos, 1981). Therefore, success or survival is based mainly on the physiological fitness of each competitor in a stable uniform environment. Since competitive equilibrium requires that the rates of change of all competitors be zero, and since the physical environment, predation and other factors are changing constantly, it seems likely that equilibrium rarely occurs in nature (Levin, 1972; Wiens, 1983). In FBRs, biofilm-GAC particles are being continuously exposed to different gradients. Since the overall density of the biofilm matrix is less than the density of the GAC, microbial growth will tend to reduce the overall density of the biofilm-GAC particle. The particles, accordingly slowly migrate up the profile of the FBR as the biofilm continues to grow. When excess biomass is sheared from the biofilm, the particles become more dense and migrate to the lower portion of the bed. The continuous process of growth and biomass loss (e.g., shearing and sloughing of dead cells) produces a situation where the GAC particles slowly migrate up and down the bioreactor treatment column. The net effect is a gradual exposure, on a temporal basis, of each GAC-biofilm complex to changes in

substrate concentration. A change in location will represent a mild disturbance or regeneration of a niche which may be advantageous for a given group. Therefore, GAC-biofilm particles which are localized at the bottom of the bioreactor will be temporary living at a richer energy zone compared to particles at the middle or top of the reactor. The constancy of fluctuation in the physical factors due to biological events will result in a state of non-equilibrium, therefore, competitive equilibrium can never be reached, and coexistence of trophic equivalent species is maintained.

In this study we combined community-level fingerprints to describe the relative genetic and physiologic relatedness of biofilm community structure in GAC-FBRs. Using restriction endonuclease analyses of community-level PCR amplified 16S rDNA and analysis of isolates, we found convergence of the populations of the inoculated system and the natural aquifer colonized GAC community. Although all the introduced strains were outcompeted by the indigenous populations, survival of strain PaW1 at low density demonstrates how biofilm systems can serve as refuges for allochthonous populations. Useful indices can be obtained to distinguish communities and to characterize their responses to environmental perturbations. It seems certain that by complementation with other recently developed techniques such as *in situ* whole cell hybridization using fluorescent targeted hybridization probes (DeLong et al., 1989; Amman et al., 1992) and confocal microscopy (Caldwell et al., 1992) better estimates of physiological states, species interactions, phylogenetic relationships and spatial cell distribution at various levels of development will be possible. Understanding of microbial interactions in biofilm communities are needed to improve bioremediation techniques and to better assess influences of introduced non-native populations on community structure and performance.

Chapter III

ISOLATION AND CHARACTERIZATION OF BENZENE, TOLUENE, AND P-XYLENE DEGRADERS FROM AEROBIC BIOFILMS IN GRANULAR ACTIVATED CARBON FLUIDIZED BED REACTORS

BACKGROUND

Biological activated carbon fluidized bed reactors (FBR) have been used for remediation treatment of groundwater contaminated with monoaromatic hydrocarbons (Hickey et al., 1991; Voice et al., 1994). This cleanup approach relies primarily on biologically mediated transformation of the contaminants. Bacterial populations will colonize the surfaces of granular activated carbon (GAC) particles resulting in the formation of a complex microbial layer or biofilm communities. The use of microorganisms for bioreclamation has recently become the focus of attention because of their ability for restoring such sites at considerable cost savings compared to technologies such as chemical adsorption or incineration (Kobayashi and Rittmann, 1982). Although the indigenous microbial populations may be adapted to the existing site conditions, reliance on their catabolic potential to transform a wide range of pollutants may be inadequate. For example, the optimum strains capable of quick degradation at high or at low substrate concentrations may be absent. Inoculation of contaminated sites or treatment systems with pollutant-degrading strains may be an alternative in sites where environmental conditions may not be suitable for growth, and groundwater extraction and treatment is required to prevent migration of the contaminant plume. The objectives of this study were to isolate and characterize BTX-degrading bacteria from mature biofilms to assess the inherent substrate utilization kinetics diversity and the phylogenetic affinities among these naturally occurring populations and introduced populations in GAC-FBRs operated under aerobic conditions.

MATERIALS AND METHODS

Isolation of BTX-degrading bacteria from granular activated carbon-fluidized bed reactors (GAC-FBR) -- A description of the different GAC-FBRs used in this study is presented in Table 1. In general, bioreactors consisted of 1-L sterile glass columns (2.54 cm diameter x 195 cm long), operated as a one-pass upflow systems without recycle at a flow rate of 0.2 L/min. Groundwater from a deep aquifer underlying the MSU campus was amended with toluene or a mixture of benzene, toluene and p-xylene (BTX 1:1:1) at a organic loading rate of 5.4 kg COD/m^3-day. The selection strategy employed for isolation of BTX degrading populations from GAC particles included direct bacterial isolation of homogenized GAC-biofilm samples by plating on non selective medium. The bacteria were first dispersed and separated from the GAC particles (~0.2 g wet) by repetitive vortexing (3 times for 45 seconds) in 1 ml of cell extraction buffer. This contained 0.001 M EGTA (ethylene glycol-tetraacetate [Sigma, St. Louis, MO]), 0.0004 M Tween 20 (Sigma, St. Louis, MO), 0.01% (wt/vol) peptone and 0.007% (wt/vol) yeast extract in phosphate buffer (Warren et al., 1992). Bacteria were isolated by diluting biofilm homogenized samples on R2A media plates for oligotrophic bacteria (Difco, Detroit, MI). Plates were prepared in duplicate, maintained at room temperature (about 24°C) in sealed plastic bags, and examined at intervals for colony formation.

Table 1. Start-up conditions of fluidized bed reactors used in this study.

Reactor designation	Inoculum source[1]	Length of operation (days)	Carbon sources
TOL.S	G4, PKO1, PaW1 and aquifer populations	0 - 124	toluene
TOL.NS	aquifer populations	0 - 98	toluene
BTX.NS	aquifer populations	0 - 25	BTX[2]

[1] G4, *Pseudomonas cepacia* (Folsom et al., 1990); PKO1, *P. pickettii* (Kaphammer et al., 1991); PaW1, *P. putida* (Harayama et al., 1989).

[2] BTX; benzene, toluene, and p-xylene.

In addition, parallel liquid enrichments in Teflon coated serum bottles containing amended benzene, toluene, or p-xylene in basal salt media (BSM) under aerobic conditions were used, followed by a secondary transfer and plating onto R2A plates. The BSM consisted of 15 mM NH_4Cl, 5.98 mM K_2HPO_4, 5.51 mM KH_2PO_4, 0.23 mM $CaCl_2$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1.71 mM NaCl with a mixture of trace metals (3.0 ml/L) composed of (g/L): EDTA, 5.0; Zn^+, 0.5; Mn^{2+}, 0.14; Fe^{2+}, 0.1; Co^{2+}, 0.04; Cu^{2+}, 0.04; Mo, 0.06; adjusted to pH 6.0 in destilled water (Owens and Keddie, 1969). The final pH of the media was 7.1. The medium (75 ml) was added to serum bottles, sealed with Teflon-coated butyl rubber stoppers and aluminum crimps. Toluene was added with microliter syringes to the sterilized medium (50 mg/L final), then aseptically inoculated with two to three GAC-biofilm coated particles from each reactor system and incubated in an orbital shaker at 25°C.

Bacterial colonies obtained from direct or indirect isolation protocols were screened for differences in colony morphology, size, and pigmentation and were then purified by repetitive plating (at least three times) and production of discrete, isolated colonies on solid medium.

Chromosomal fingerprint of bacterial isolates -- We used repetitive extragenic palindromic (REP) sequences and the polymerase chain reaction (PCR) to fingerprint bacterial genomes. REP oligonucleotides produced clearly reproducible and resolvable DNA bands by agarose gel electrophoresis following PCR amplification (de Bruijn, 1992). These DNA band patterns generate fingerprints for typing of bacterial cultures. REP-PCR amplification was performed essentially as described previously by Versalovic et al. (1991). The DNA sequence of the REP primers are: 5' - IIIICGICGICATCIGGC - 3', primer REP1R-I, and 5' - ICGICTTATCIGGCCTAC - 3', primer REP2-I. Template DNA (50 ng) was used per reaction in a 25-µl volume containing 50 pmol each of two opposing primers, 1.25 mM deoxynucleosides triphosphate, and 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The amplifications were performed with an automated

thermal cycler (model 9600 Perkin-Elmer Cetus, Norwalk, CT) with an initial denaturation [95°C, 6 min], followed by 30 cycles of denaturation [94°C, 1 min], annealing [40°C, 1 min], and extension [65°C, 8 min], and a single final extension [65°C, 16 min]. After amplification, 10 µl of the REP products were separated on 1% (w/v) agarose gel containing 1X TAE (40 mM Tris-HCl, pH 7.5, 5 mM sodium acetate, 1 mM EDTA [disodium ethylenediaminetetraacetate]), stained in 0.5 µg/ml ethidium bromide solution, and then photographed in UV light with Polaroid Type 55 film.

Catabolic screening of biofilm community isolates -- Axenic cultures were tested for their ability to utilize various monoaromatic hydrocarbons as the sole carbon and energy source in aerobic liquid media. Fresh bacterial colonies grown on R2A plates were resuspended and washed in BSM, and transfer to 10-ml sterile vials containing 5 ml of BSM with 10 mg of toluene, benzene, or *p*-xylene per liter (final concentration) in triplicate sets. The inoculated vials were sealed with Teflon-coated septa, and aluminum crimp sealed, and incubated stationary for 2 weeks at 25°C. Substrate removal analysis was performed using a gas chromatograph equipped with a flame ionization detector (GC/FID), a DB-624 capillary column (J&W Scientific, Folson, CA) and a head space sampler. The vials were equilibrated at 40°C, the column at 90°C and the injector and detector at 200°C. Helium was the carrier gas. Positive results were interpreted when more than 80% removal relative to the control was observed.

Growth parameter estimation of toluene degrading populations -- The dominant biofilm populations were further characterized based on their ability to utilize toluene. Duplicate sets of exponentially growing cultures in BSM containing 50 mg of toluene per liter were used to determine maximum growth rates by optical density measurements (OD_{550}). Further, the Monod growth kinetic parameters, half-velocity coefficient (K_s) and maximum specific rate of substrate utilization (k), were estimated by fitting progress curve data to the integrated Monod equation, using a non-linear analysis. Both kinetic parameters were calculated from a single substrate depletion curve as a function of time (t). There is a

direct relationship in the Monod equation (equation 1), between the number of catalytic units (biomass, X) and the rate of substrate utilization (dS/dt):

$$\frac{dS}{dt} = -\frac{kXS}{Ks + S} \qquad \text{(equation 1)}$$

If biomass is constant, the Monod equation can be simplified to the integrated form for parameter estimation by non-linear regression:

$$\int_0^t X dt = -\int_{So}^{S} \frac{K_s + S}{kS} dS$$

$$\frac{K_s}{k} \ln \frac{S}{So} + \frac{1}{k}(S - So) = -Xt \qquad \text{(equation 2)}$$

$$t = \frac{K_s}{Xk} \ln \frac{So}{S} + \frac{1}{Xk}(So - S)$$

Exponentially growing cells in BSM with toluene as sole carbon source (50 mg/L final) were used for the kinetic assays. A cell suspension of axenic harvested cultures (approximately 10^{10} cells/L) was transferred to fresh preoxygenated media with 3 mg/L toluene (final concentration) and immediately drawn into a 50-ml gastight syringe containing a small magnetic stir bar. The biomass concentration was determined empirically for each culture (high enough not to increase significantly with the amount of substrate added and low enough to allow collection of samples at reasonable time intervals). All assays were performed at room temperature (24 to 25°C). Samples of 1 ml were collected in 10-ml sterile vials every 5 to 15 min and fixed with approximately 0.05 ml of 7N HCl, sealed with Teflon-coated septa, aluminum crimp sealed and stored at 4°C until head space analysis was performed (generally within a week). Cell numbers were determined before and after the assay by direct plating on R2A medium. No significant increased in the cell number was observed. Three of the cultures were independently assayed twice; the obtained results for k and K_s fell within the 95% confidence interval.

Determination and analyses of partial 16S rRNA gene sequence -- The 16S ribosomal RNA gene was amplified from genomic DNA using the polymerase chain reaction and cloned into a plasmid vector as described previously (Zhou et al., unpublished). The 16S rRNA gene was sequenced from both directions using an automated fluorescent sequencer with the forward and reverse primers, which span *Escherichia coli* 16S rRNA gene positions of 785 to 805 and 1115 to 1100, respectively. The DNA sequences were analyzed using the programs in Genetics Computer Group software package (Deveraux et al., 1984) and in PHYLIP phylogeny inference package (Felsenstein, 1989).

RESULTS

Isolation of BTX degrading microorganisms -- To isolate numerically dominant bacteria from GAC-biofilm communities in fluidized bed reactor systems, the end point dilution scheme was carried out using separated and dispersed biofilm bacteria from GAC particles and plated on R2A solid media. Aerobic heterotrophs were observed at a concentration of 10^9-10^{10} CFU/g of GAC. Bacterial populations from dispersed biofilms were highly recoverable on R2A medium as noted by a high plating efficiency as compared to total direct counts (20-45% plate counts/direct counts). Successful enrichments for aerobic BTX degraders were routinely obtained within 24 h from GAC-bacterial coated particles as determined by increased culture turbidity and substrate disappearance (higher than 95% of total added substrate). Isolates were subcultured three additional times to ensure culture purity. Several clones were retrieved that were able to grow aerobically on at least one of the BTX compounds as sole source of carbon and energy. Isolates GF161, GF261, GF262, and GF281 were numerically dominant among all the reactor treatments constituting more than 90% of the total biomass at fluctuating ratios on a temporal and spatial basis. Five of one hundred twenty recovered isolates from the different reactor systems failed to transform any of the BTX substrates in batch culture conditions.

Genomic fingerprints -- Purified bacterial clones were further characterized by the REP-PCR fingerprinting technique. The REP-PCR analysis revealed distinct DNA band patterns which clearly aided in the identification and differentiation at a high level of resolution of diverse bacterial strains (Figure 1). PCR with the REP primer set and chromosomal template DNA from the different cultures yielded multiple distinct DNA products of sizes ranging from approximately 300 to 6,000 base pairs. The patterns generated were found to be very different for several strains examined from the same system. However, similar amplified genomic band patterns were observed among dominant isolates recovered from seeded (TOL.S) and non-seeded (TOL.NS and BTX.NS) reactor treatments fed toluene or BTX compounds (e.g., Figure 1, lanes GF1T2 and GF2T3). This result is consistent with those results obtained by community-level fingerprints in which convergence of seeded and non-seeded communities were observed. Thus, 12 bacterial clones were identified that differed from one another in REP fingerprints, and in other morphological and physiological properties. Only isolate GF2XU had a band pattern profile similar to seeded strain PaW1. This clone was recovered from a secondary transfer of a p-xylene enrichment culture from a 113-day-old GAC sample of the seeded toluene fed reactor system. Furthermore, clone GF2XU has the same aromatic substrate range, similar distribution of contributing cellular fatty acids, and a large plasmid of the same size of strain PaW1 (Massol-Deyá et al., unpublished). From the 12 different GAC isolates, seven were selected for more intensive investigation in addition to the three pseudomonads strains used to seed the sterile GAC-FBR.

Toluene growth kinetics -- The maximum substrate degradation rate (k) and half velocity coefficient (K_s) for toluene utilization were determined by measuring toluene disappearance rates of 5 mg of toluene per liter. The patterns of toluene utilization by the different degrading populations were S-shaped and the data were analyzed by using regression fits to the integrated form of the Monod equation (equation 2). Description and comparison of the kinetic coefficients for toluene utilization by the inoculated strains and

aquifer indigenous GAC colonizers are presented in Table 2. The apparent values for k ranged from 0.061 to 2.061 mg toluene/10^{10} cells·h for strains G4 and GF272 respectively. Observed K_s values ranged from a low of 0.074 mg/L for strain GF161 to a high observed value of 1.701 mg/L toluene for strain GF272. In general, all GAC isolated strains examined showed a lower K_s and/or higher k values than the seeded populations. Although strain GF272 has a relatively high K_s constant, as compared to the rest of the toluene degrading populations, it also has the highest substrate utilization rate constant. The majority of the isolates showed the catabolic ability to utilize more than one of the monoaromatic compounds tested. Estimates of μ_{max} as determined by turbidity measurements on toluene ranged from 0.10 h^{-1} (doubling time T_d = 6.9 h) to 0.52 h^{-1} (T_d = 0.93 h) (Table 2).

Phylogenetic analysis of toluene degrading bacteria -- Approximately 280 nucleotide bases, corresponding to *E. coli* 16S rRNA gene sequence positions of 810 to 1090, were determined for each of the three dominant GAC isolates. Similarity and evolutionary distance matrix analysis suggest closed affiliation to three subclasses of the *Proteobacteria*, strain GF261 to the beta subgroup bacteria *Comomonas testosteroni* with an actual similarity coefficient of 0.929, strain GF262 to the gamma subgroup bacteria *Pseudomonas putida* with similarity of 0.989, and strain GF281 to the alpha subgroup bacteria *Zymomonas mobilis* with actual similarity of 0.897. A phylogenetic tree constructed using neighbor-joining distance method shows this three-way relationship (Figure 2). Very similar tree topologies were also obtained using maximum parsimony and maximum likelihood methods (data not shown).

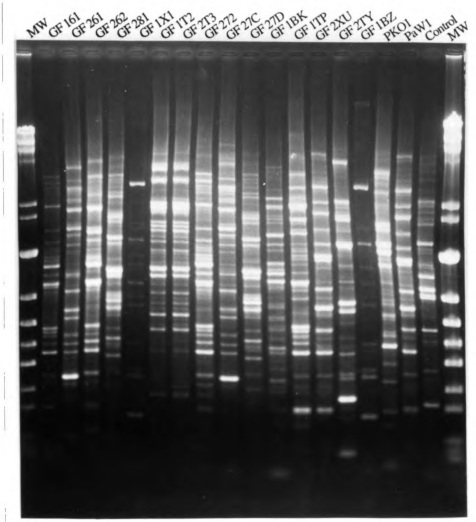

Figure 1. REP fingerprint patterns of bacterial isolates from GAC-biofilm communities. DNA molecular weight standards (lanes MW) are λ/HindIII, pUC19/Taq-I-pUC19/Sau3AI (Stratagene, La Jolla, CA). The pattern of PCR products generated of chromosomal DNA from inoculum strains PKO1 and PaW1 as well as a positive control (*P. aeruginosa*) are included.

Table 2. Description and comparison of toluene growth kinetic coefficients of inoculum strains and dominant GAC-FBR isolates.

Isolate	Description	Substrate range[1]	μ max (hr^{-1}) MEAN (SD)	k (mg Tol/10^{10} cells.hr^{-1}) MEAN (CI 95%)	K_s (mg Tol/L) MEAN (CI 95%)	k/Ks
G4	*P. cepacia*	B, T	0.22 (0.04)	0.061 (0.030)	0.436 (0.178)	0.140
PKO1	*P. pickettii*	B, T	0.16 (0.03)	0.096 (0.031)	0.197 (0.118)	0.487
PaW1	*P. putida*	T, X	0.40 (0.08)	0.515 (0.580)	0.128 (0.045)	4.023
GF161	*Pseudomonas* spp.	B, T, X	0.33 (0.07)	0.717 (0.273)	0.074 (0.137)	9.689
GF261	β subdivision[2]	B, T, X	0.10 (0.02)	0.399 (0.069)	0.098 (0.109)	4.071
GF262	γ subdivision[2]	B, T, X	0.25 (0.05)	1.585 (0.182)	0.089 (0.072)	17.809
GF281	α subdivision[2]	B, T, X	-	0.260 (0.020)	0.098 (0.033)	2.653
GF272	*Pseudomonas* spp.	B, T	0.52 (0.10)	2.061 (1.797)	1.701 (1.691)	1.212
GF1TP	*Pseudomonas* spp.	T, X	0.38 (0.08)	1.189 (0.635)	0.190 (0.239)	6.258
GF2TY	Aeromonas spp.	B, T	0.44 (0.09)	-	-	-

[1] B, benzene; T, toluene; X, para xylene

[2] Based on partial 16S ribosomal RNA sequences analysis, subdivisions of the *Proteobacteria*.

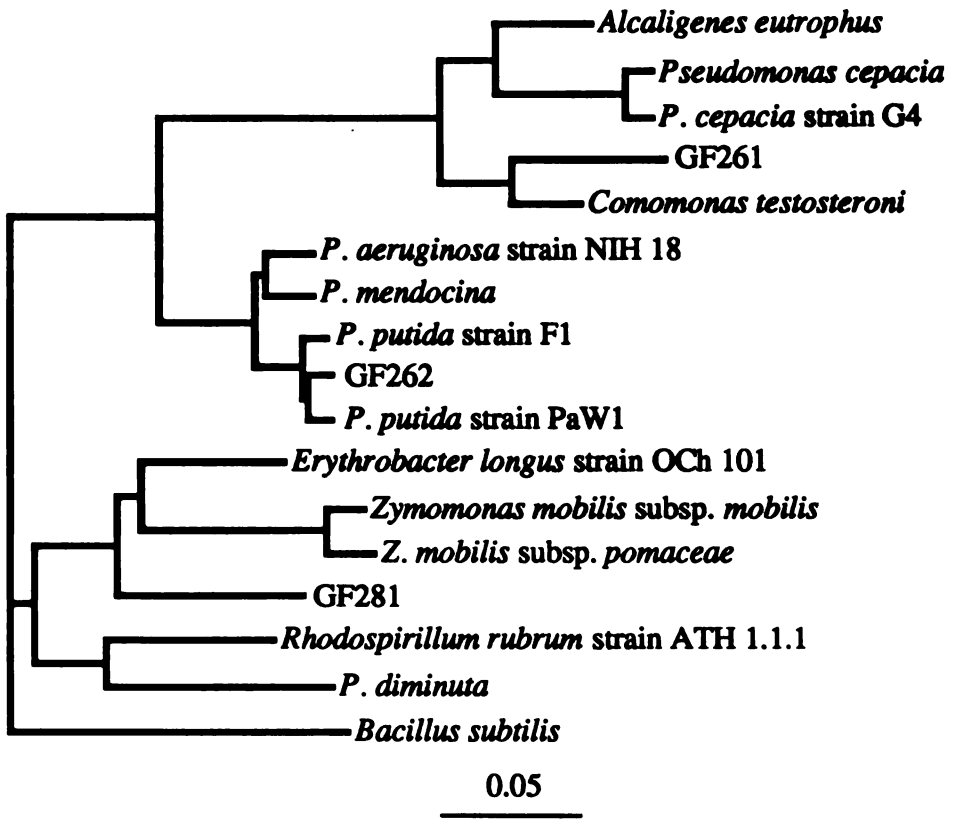

Figure 2. Phylogenetic distance tree produced from partial 16S rRNA sequences (810-1090 positions). Representative members of the various subgroups of the *Proteobacteria* and the dominant toluene degrading bacterial populations from GAC biofilm communities are shown. 16S rRNA sequence of *Bacillus subtilis* was used as an outgroup.

DISCUSSION

Degradative biofilms in FBRs are complex communities composed of a number of coexisting bacterial species. Twelve different toluene degrading populations were recovered from these reactor systems. The same strains were also isolated from the reactor seeded with three *Pseudomonas* spp. and from the BTX groundwater fed reactor. These results complement the previous observation of community convergence of the different reactor treatments as determined by community-level fingerprints and fatty acid methyl ester analysis (see Chapter II).

The successful use of microorganisms for bioremediation purposes requires the selection of populations able to effectively degrade environmental pollutants over a wide range of concentrations. The integrated Monod equation in conjunction with nonlinear regression procedures has been previously used to obtain estimates of k and K_s for pure cultures of organisms (Counotte and Prins, 1979; Greer et al., 1992; Robinson, 1985; Robinson and Tiedje, 1983; Simkins and Alexander, 1984). This technique has several advantages over estimation parameters from chemostat data, in that it is considerably faster since estimates can be derived from a single depletion curve, and volatile and low water soluble compounds can be feasible tested. Our data suggest that, in fluidized bed reactors, a kinetic diversity for toluene utilization exists among naturally coexisting microbial populations in GAC-biofilm communities. The k/K_s ratio, which is the slope of the Monod equation at lowest substrate concentration, offers a simple way of emphasizing both half-saturation value and maximum rates (Healey, 1980). This ratio increases as k increases and K_s decreases, thus a higher ratio indicates a higher rate at lowest nutrient concentrations, and therefore competitive advantage in the process and conditions being considered as competition drives nutrient concentrations down. Despite the selective opportunity of the introduced populations to colonized the GAC particles, they all were outcompeted by the indigenous aquifer populations. The dominant aquifer populations

examined to date showed better ratios of k/K_s than the introduced strains. However, PaW1 which was the only introduced population to successfully remain in the system (as a minor member of the mature community), has a relatively high k/K_s ratio. This results also suggest that endemic populations are better adapted to the local environmental conditions and thus, competitively superior than the introduced populations. Maintenance of functionally redundant species in the biofilm community appears to be favored by both biological and physical factors. The known development of substrate gradients through the treatment column and biofilm matrix appears to sustain the establishment and coexistence of kinetically diverse bacterial populations, possibly by niche partitioning.

Partial 16S rRNA sequences were determined for each of the three dominant toluene degrading strains with the aim of establishing phylogenetic affinities of the biofilm community. The full sequence of this gene is not necessary for phylogenetic assignment and it has been shown previously that phylogenetic trees constructed by using different and limited portions of the 16S rRNA sequence are the same as those generated by using full sequences (Lane et al., 1985; Schmidt et al., 1991). We focused on a portion of the 16S rRNA gene that corresponds to nucleotides 810 to 1090 in the *E. coli* rRNA which contains substantial sequence variability in conserved secondary structure for rapid sequence comparisons. The rDNA sequences examined were affiliated with or closely related to three groups of the *Proteobacteria*, the alpha, beta and gamma subdivisions. Members of the beta and gamma subdivision such as many *Pseudomonas* spp. are common aerobic toluene degraders (Zhou et al., unpublished). The alpha subdivision of *Proteobacteria* however, contains many genera of oligotrophic bacteria including *Caulobacter* and *Hyphomicrobium* but none of them have been previously described to grow aerobically on aromatic hydrocarbons such as BTX. Thus, strain GF281 appears to be a phylogenetically novel BTX degrading population.

Five pathways have been described for the metabolism of toluene by oxygen-dependent reactions (Van der Meer et at., 1992; Zylstra and Gibson, 1991). Nucleic acid

probes for the first steps of all five aerobic degradation pathways were used in Dot blot hybridization assays to determine if any gene homology exist between the GAC isolates and the known pathways. None of the GAC cultures tested to date however, showed any hybridization signal even though low stringency conditions were used (Marcos and Tiedje, personal communication). These results suggests that more diversity exist at the gene sequence of toluene oxidation pathways than what is currently known. In addition, GAC isolate GF262 a *Pseudomonas putida* strain as determined by physiological and partial 16S ribosomal RNA gene sequence analysis, appears to differs from those previously described pathways in *P. putida* strain PpF1 (meta ring cleavage) (Zylstra and Gibson, 1989) or strain PaW1 (methyl group oxidation tol plasmid encoded pathway) (Harayama et al., 1989).

In the present study we have shown the diversity of multiple toluene degrading populations in aerobic biofilms of fluidized bed reactors. The observed coexistence of these populations in such communities can be attributed to their growth kinetic diversity and to the presence of substrate gradients through the treatment column and biofilm matrix. It is certainly possible to complement the information obtained from the phylogenetic analysis of axenic cultures with community-level analysis by selecting molecular based probes for the different subclasses of the *Proteobacteria* (Manz et al., 1992) with *in situ* whole cell hybridization (Amann et al., 1990) and confocal microscopy (Caldwell et al., 1992) to specifically follow, enumerate and study the distribution of these different populations in the biofilm community. In order to address such important questions however, non-destructive fixation and hybridization procedures must first be developed for biofilm studies.

Chapter IV

CHANNEL STRUCTURES IN AEROBIC BIOFILMS FROM FIXED-FILM REACTORS TREATING CONTAMINATED GROUNDWATER

BACKGROUND

The contamination of groundwater resources with aromatic and alkyl substituted aromatic hydrocarbons has become a serious threat to human health (Dean, 1985). Leakage from underground gasoline and petroleum storage tanks, and distribution systems represent a major source of this pollution. Pump and treat remediation techniques have often been implemented to control contaminate plumes (Hickey et al., 1991; Voice et al., 1994). Fixed-film biological systems developed on porous media have recently been employed for the treatment phase. Either through attachment, or agglomeration, bacteria may remain in place within the porous medium forming biofilm communities. The granular activated carbon-fluidized bed reactor (GAC-FBR) couples the absorptive and high surface area per unit volume properties of activated carbon with biological treatment to obtain robust high-capacity treatment (Ehrdardt and Rehm, 1989; Speitel et al., 1989; Hickey et al., 1991; Voice et al., 1994). Under optimum conditions greater than 99% of the total applied organic load can be removed using 5 to 10 min hydraulic retention times.

Past structural studies of aerobic biofilms by using traditional light microscopy, transmission and scanning electron microscopy, show randomly distributed cells. This has led to process models which assume generally homogenous biofilms (Williamson and McCarty, 1976; Criddle et al., 1991). Bacterial cells living in a dense multilayer matrix however, face the problem of limited transport of substrate and nutrients into the film inner layers as well as export of waste products out of these deep regions. The use of microsensor technology has shown the existence of steep oxygen gradients within aerobic biofilms, thus demonstrating the importance of diffusion processes in biofilm systems (Dalsgaard and Revsbech, 1992; Kühl and Jørgensen, 1992; Ramsing et al., 1993).

Temporal and spatial stability of biofilm communities against disturbances or lethal factors have been demonstrated (Blenkinsopp et al., 1992; Brown and Gauthier, 1993). The resilience of such systems to environmental changes has been largely attributed to the interdependency of the community members for nutrient exchange, and to the creation and maintenance of microenvironmental conditions which can be completely different from the adjacent liquid phase (Brown et al., 1988; Criddle et al., 1991).

With the use of confocal scanning laser microscopy (CSLM), detailed examination of microbial biofilms in nondestructive states has begun. Advantages of CSLM to study biofilm structures were described by Caldwell et al. (1992). Careful examination by CSLM of laboratory biofilms has revealed the presence of specific cell distribution patterns and large internal void spaces (Lawrence et al., 1991; Wolfaardt et al., 1994). In view of these findings and the importance of efficient fixed-film processes, we investigated whether there were any structural traits in biofilms that might be important to biofilm functions. The specific objectives were to describe structural patterns and properties of newly colonized GAC in a laboratory reactor operated at an organic loading rate typical of full-scale reactors, and to assess whether or not common spatial arrangement and cell distribution strategies exist regardless of carbon sources or microbial composition in commercially operating field reactors.

MATERIALS AND METHODS

Granular activated carbon fluidized bed reactor (GAC-FBR) and support media -- A one-liter working volume sterile glass column reactor (2.54 cm diameter x 195 cm long) was used in this study. The bioreactor was operated as a one-pass up-flow system without recycle at a flow rate of 0.2 liter per min. Groundwater from a deep aquifer underlying the MSU campus was amended with 32 μmoles toluene per liter (3 ppm). In addition, the influent was supplemented with pure oxygen (approximately 406 μM final concentration), and a nutrient solution (NH_4Cl & KH_2PO_4) to achieve a ratio of 30:5:1

(carbon:nitrogen:phosphorous). About 100 g of sterile GAC (Calgon Filtrasorb 400, Calgon Company, Pittsburgh, PA) was added to the reactor as adsorbent/biomass carrier. The geometric mean diameter was approximately 0.7 mm with an average density of 1.6. The bed height was controlled at the desired level (maximum working height of 180 cm) by a shock brushing procedure to particles near the top of the bed to remove excess biomass from the GAC. The GAC, now with a thinner biofilm returned by gravity to near the bottom of the reactor column (because of the increase in particle density due to removal of most of the biomass) while the sheared biomass was carried away in the effluent stream.

In addition, we examined field-scale GAC-FBRs currently used for the treatment of groundwater contaminated with complex mixtures of hydrocarbon compounds at different sites in Michigan (Table 1). Samples were collected from mature biofilm communities and stored at 4°C (generally less than 24 hours) until analysis was performed.

Table 1. Description of GAC-FBRs used in this study.

Bioreactor	Location[1]	Flow rate (L/min)	Carbon sources	Length of operation at sampling (month)
TOL	MSU, East Lansing, MI	0.2	Toluene	6
BTEX	MBI, Lansing, MI	1.0	Benzene, toluene, xylenes, & ethylbenzene	1.5
TC	Traverse City, MI	60 - 80	Gasoline residues	1
PW	MBI, Lansing, MI	1 - 2	Complex mixture of aromatic hydrocarbons[2]	12
JEN	Grand Rapids, MI	80 - 100	Gasoline residues	6

[1] MSU, Michigan State University; MBI, Michigan Biotechnology Institute.
[2] This waste stream contains 7% salt.

Analytical procedures -- Analyses of physicochemical parameters of the toluene fed GAC-FBR were conducted at 2 to 3 day time intervals. Analyses included toluene

disappearance, oxygen consumption, temperature, and pH. For toluene determination, 5 ml samples of influent and effluent groundwater were collected in 10 ml vials. In order to stop any further biodegradation or loss of volatiles, approximately 0.10 ml of 6 N HCl was added to each liquid sample. Tubes were sealed with Teflon-coated butyl rubber septa and aluminum crimp seals, and stored at 4°C. Analysis was generally performed within a week. Toluene was analyzed using a *Varian 3700* gas chromatograph equipped with an automatic headspace sampler and a flame ionization detector (FID). Sample quantification was performed using external standards. A polarographic electrode (overflow funnel with built-in stirring bar) coupled to an Orion digital pH/millivolt meter (Orion Model 611) was used to measured dissolved oxygen (DO) concentration and pH. Dissolved oxygen consumption is reported as the difference between the influent and effluent readings.

Microbiological procedures -- Microbial cells were removed from the activated carbon (~0.2 g wet) and homogenized by repetitive vortexing (three times for 45 seconds) in 1 ml of cell extraction buffer. The extraction buffer contained 0.001 M EGTA (ethylene glycol-tetraacetate [Sigma]), 0.0004 M Tween 20 (Sigma), 0.01% (w/v) peptone and 0.007% (w/v) yeast extract in phosphate buffer (Warren et al., 1992). Bacterial numbers from GAC samples were determined by acridine orange (AO) epiflourescence microscopy using the method of Zimmerman et al. (1978). Biofilm homogenates (1 ml), were fixed in 10 ml of Milli Q filter-sterilized H_2O with 0.1% (v/v) formaldehyde (final concentration) and then stored at 4°C until processed. A Leitz microscope, equipped with an epifluorescent condenser and a KP 490 blue excitation filter was used for the observation and enumeration of microbial cells.

Microbial activity of biofilm homogenates was determined by measuring rates of degradation of toluene. Duplicate samples were incubated in basal salt medium (Owens and Keddie, 1969) containing 109 µmol/L of toluene. The inoculated medium (5 ml) was added to 10 ml vials, sealed with Teflon-coated butyl rubber stoppers and aluminum crimp seals. Samples were incubated at 25°C, fixed at different time intervals with approximately 0.10

ml of 6 N HCl, and then head space analyzed for toluene disappearance; subsamples were amended with 0.1% (v/v) formaldehyde for AO direct cell counts.

Scanning electron microscopy -- Samples for scanning electron microscopy (SEM) were fixed with 0.1 M phosphate buffer (pH 7.0) containing 4% glutaraldehyde for 1 h, washed with 0.1 M phosphate buffer for 10 min, and then dehydrated through a graded series of ethanol solutions (25, 50, 75, and 100% ethanol). The samples were then critical-point dried and coated with gold. SEM micrographs were taken with a JSM 35C SEM (JEOL, Ltd., Tokyo, Japan).

Confocal scanning laser microscopy -- A Zeiss 210 laser scanning confocal microscope (LSM) (Carl Zeiss Inc., Thornwood, NY) was used in this study. Dark field images, for the purpose of measuring biofilm thickness, were acquired in laser scanning transmitted mode using a Phase 3 condenser with a 10X (non-phase) objective. The samples, which were stained with acridine orange (0.005% final concentration) and mounted in 0.1% agarose beds, were examined using the 488 line of a dual line argon ion laser with an LP520 barrier filter. The samples consisted of 10 to 15 biofilm-coated GAC particles freshly collected at different times from the bottom 20% of the bed height. Images were recorded on Kodak Tmax 100 film using a Matrix Multicolor computerized camera unit (Agfa Matrix, Orangeburg, NY) which received signals directly from the LSM.

Community fatty acid methyl ester (FAME) analysis -- Total fatty acids of microbial communities were analyzed as previously described using the Microbial ID analytical system (MIDI; Newark, DE) (Sasser, 1990; Haack et al., submitted). Duplicate samples of cells collected from fresh GAC samples (approximately 0.1 g wet weight) were treated as follows: (i) whole community cell preparations were saponified at 100°C with 1 ml methanolic NaOH (15% (w/v) NaOH in 50% (v/v) methanol), (ii) esterification of the fatty acids at 80°C with 2 ml 3.25 N HCl in 46% (v/v) methanol, (iii) extraction of the fatty acid methyl esters (FAME) into 1.25 ml 1:1 (v/v) methyl-*tert*-butyl ether/hexane, (iv) aqueous

wash of the organic extract with 3 ml 1.2 % (w/v) NaOH, and (v) analysis of the washed extract by gas chromatography.

The mean concentrations and standard deviations as well as principal components analysis for FAME profiles were determined by using SYSTAT™ version 5.1 (SYSTAT Inc., Evanston, IL).

RESULTS

Laboratory-scale granular activated carbon fluidized bed reactor (GAC-FBR) -- The concentration of bacterial cells in the influent water fed to the toluene amended FBR system (TOL) was approximately 10^7 cells/L. These microbial populations were used as a natural and continuous inoculum for the colonization of the virgin GAC. The inlet water temperature was approximately 13°C (± 1) throughout the course of the experiment. The pH of the bioreactor influent and effluent was neutral.

After an initial 5 to 7 day lag phase, the dissolved oxygen (DO) consumption increased steadily as biomass accumulation began (Figure 1). Steady-state conditions were assumed to have been attained when constant DO consumption occurred; this amount was also commensurate with that required for complete oxidation of the added toluene. These conditions were achieved after 15 days. The average DO consumed was of 129 µmol/L. The effluent toluene concentration was 0.72 µmol/L. A 98% toluene removal efficiency was observed.

Several observations suggest that toluene biodegradation commenced once significant colonization of the GAC occurred. (i) Cell density as observed by bed height increased (Figure 1) and direct cell counts (data not shown) increased coincident with oxygen consumption, indicating that growth had occurred. (ii) Microbial populations capable of utilizing toluene as sole carbon and energy source were readily isolated from the colonized GAC. (iii) On average, the specific activity of toluene oxidation by these communities was 9.9 ± 0.4 µmol/10^9 cells·d, sufficient to account for total transformation

of added substrate. The initial lag phase was likely due to toluene adsorption by the GAC and the relatively cool temperature.

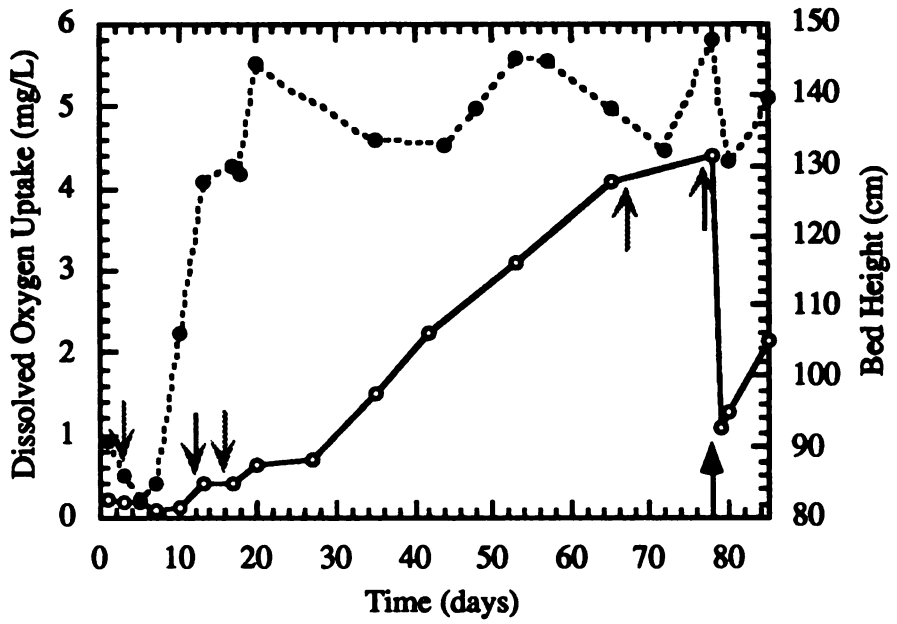

Figure 1. Biofilm development in the toluene-fed reactor. The bed height reflects overall biofilm thickness (open circles); dissolved oxygen uptake is a measurement of biological activity (black circles). Gray arrows are the times at which the microscopic image shown were taken. Black arrow shows the first time at which biomass was controlled by application of shear forces.

Scanning electron microscopy (SEM) -- The surface of the GAC particles was highly irregular (Figure 2). Close examination of the surface revealed the presence of smooth open regions, large rough zones, and open cavities. The latter two provide larger surface areas of sheltered regions and were the regions where microbial growth was initially observed (Figure 3). After 12 days of continuous growth, the GAC surfaces were completely covered with a layer of contiguous bacterial cells embedded within a polymeric matrix (Figure 4). The bacteria present were mainly rod shaped (0.3 to 0.6 by 1 to 3 μm) and many of them were dividing. At this stage, the film depth reached 20 μm in some

areas. Thicker biofilms were particularly difficult to study by SEM because the extracellular structures and bacterial cells collapsed during sample preparation.

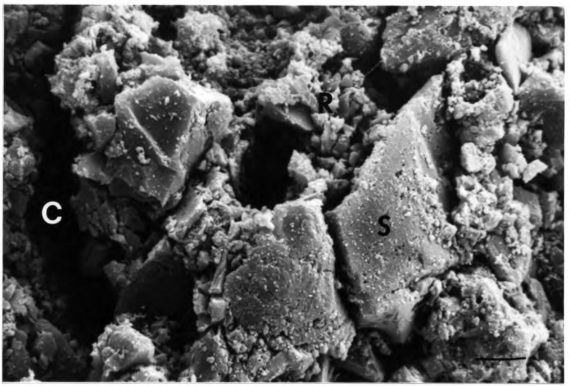

Figure 2. SEM micrograph of sterile GAC particle showing different surface zones: S, smooth; R, rough; C, cavity. Bar, 5 μm.

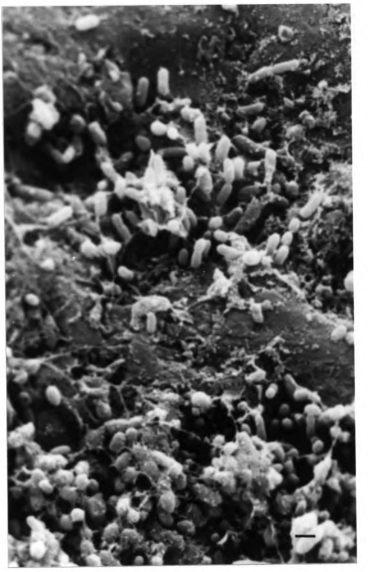

Figure 3. SEM micrograph of a 3-day-old microcolony mainly growing over rough and within cavity areas of the GAC. Bar, 1 μm.

Figure 4. SEM micrograph of a 12-day-old colonized granule from the toluene fed reactor. Most of the surface has been colonized with actively dividing rod cells. However, there is smooth zone (arrow) yet to be colonized. Bar, 5 μm.

Confocal scanning laser microscopy (CSLM) of mature biofilms -- For biofilms greater than 15 μm thick CSLM was preferred to study the development of the biofilm superstructure on GAC particles. The following patterns are derived from observing 30 to 50 images from 10 to 15 granules examined at each of the sampled stages. Initially the entire surface was colonized by a thin film (10 to 20 μm) which exhibited no structural patterns (Figure 5a). However, after the biofilm had grown much thicker (63-days), a mosaic pattern made up of separate lobes of cells was dominant (Figure 5b). At 77 days this lobbed structure was not only maintained but even more pronounced showing that further growth did not obliterate the channels separating the cell communities (Figure 5c). These channels were typically 2 to 25 μm wide. Close examination of some of these open spaces revealed that they can extend from the top of the film to deep inner regions to form a

channel-like network (Figure 6). This overall biofilm structure was prominent after four to six weeks of operation, when biofilm function had stabilized (Figure 1). Secondary colonizers such as yeast and thereafter protozoa, as well as shearing of the excess biomass eventually appears to disturb the integrity of the organized biofilm structure described above. The Protozoan feeding on bacterial cells appeared to recreate void spaces that resulted in conservation of channel-like structures (Figure 7).

Biofilms obtained from field GAC-FBRs also shared common organizational structures with the laboratory biofilms. Bacterial density ranged from 10^9 to 10^{11} cells/g of GAC in all biofilm communities studied. Mature biofilms exhibited patterns in which internal contiguous cell-free spaces were commonly found in all four reactors studied (Table 1; Figure 8). For example, the biofilm superstructure of the Traverse City (TC) field-scale FBR system exhibited contiguous void spaces opportunistically colonized by cocci shaped cells (Figure 8a). In contrast, the benzene, toluene, ethylbenzene, and xylenes (BTEX) fed system showed a 'coral-reef' like structure, where each finger of the biofilm formed of similarly shaped cells has a particularly well defined boundary and distribution (Figure 8b). The dimensions of these fingers varies from 35 to 200 μm long by 20 to 40 μm thick. They all appear to develop from a contiguous base film which completely covers the GAC surface. Biofilm samples examined from two other treatment sites (PW and JEN) also displayed unique spatial cell arrangements and contiguous internal void spaces (Figures 8c-d).

All thick biofilms appear to have a contiguous (e.g., TOL) or semicontiguous (e.g., TC) base layer, which is recognized by the absence of channels or other void space. In the 30-day-old biofilm sample from the TC site, a discontinuous bacterial base film of 10- to 25-μm-deep was observed to cover the substratum from which cell-free spaces and channels appear to develop (Figure 9). Biofilms from the other reactor systems also showed a base film which ranged from 10- to 60-μm-thick.

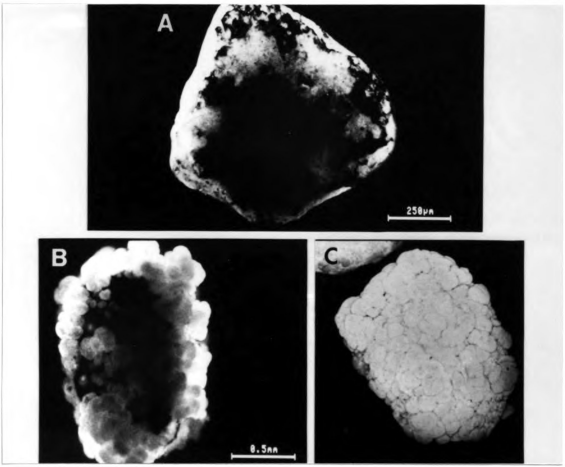

Figure 5. Series of confocal micrographs showing biofilm formation on GAC particles by indigenous aquifer populations growing on toluene as sole source of carbon. Each extended focus view was constructed by computer overlay of the optical sections in a z series. (A) 16-day-old; (B) 63-day-old; and (C) 77-day-old biofilm structure. The biofilm thickness increased from 15 to 300 µm between 16 and 77 days as determined by dark field microscopy.

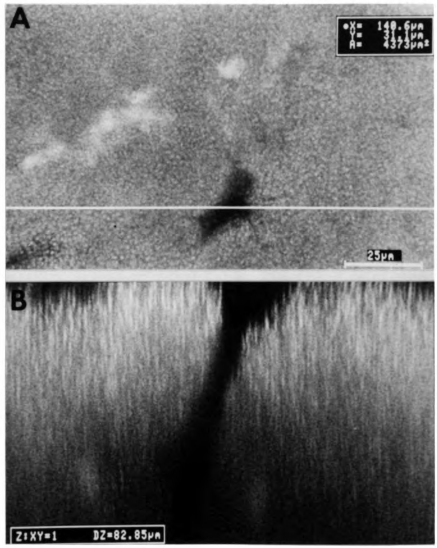

Figure 6. Confocal micrographs of a 77-day-old biofilm showing the nature of the channel structures in the toluene fed reactor. (A) horizontal (x, y) optical thin section, 82.6 µm from the top of the biofilm (the horizontal line indicates the optical cutting position for the sagittal section); and (B) sagittal (x, z) view showing the vertical cell distribution and the cell-free channel reaching up to the surface of the film.

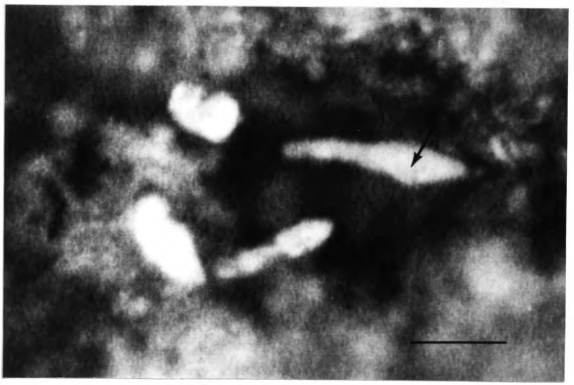

Figure 7. Confocal micrograph of the void spaces created by protozoa (arrow) grazing on bacteria in a 153-day-old biofilm community. At this point, the bioreactor had gone through three shearing treatments for biomass removal and the biofilm structure displays none of the distinctive vertical cell arrangement pattern observed in the early stage. Bar, 5 μm.

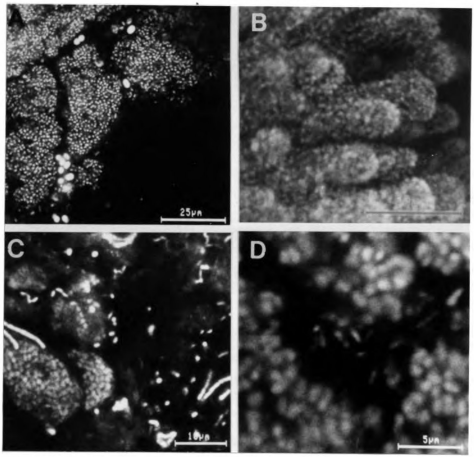

Figure 8. Horizontal (*x*, *y*) optical sections of biofilm communities from different field-scale FBRs. Note the spacing between cell aggregates and cell arrangements. (A) TC reactor system, note also differentially packed cell aggregates and channel-like structure partially colonized by cocci-shaped cells. (B) BTEX fed system showing a 'coral-reef' appearance of bacterial growth. (C) Biofilm structure of JEN system showing bacterial aggregates separated by well-defined void space boundaries (arrow). (D) Micrograph from the PW system showing bacterial aggregates and partitioning by cell-free spaces.

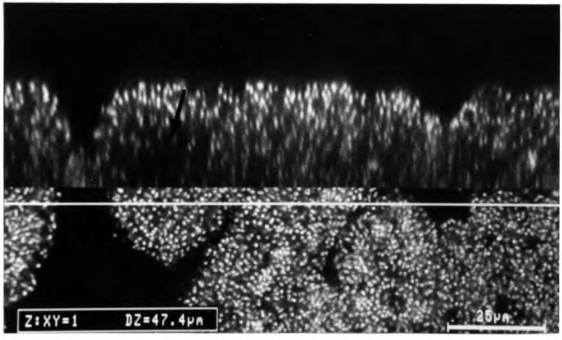

Figure 9. Horizontal (x, y) optical section (lower half of image) and sagittal (*x*, *z*) optical section (upper half of image) of a 30-day-old field reactor site (TC) showing a fully hydrated biofilm with cell-free scpaces and a discontinuous base film (arrow).

Figure 10. Laser scanning dark field photomicrograph of a 77-day-old bacterial GAC coated particle showing variation of the biofilm thickness within the same colonized surface. Bar, 30 μm.

The vertical dimensions of the different biofilm structures were examined by using laser scanning dark field microscopy. We typically saw a high variation in biofilm thickness even within the same colonized granule (Figure 10), e.g. 30 to 200 μm.

Community fatty acid methyl ester (FAME) analysis of mature biofilms -- Since these biofilm communities had similar structural features, we sought to evaluate how similar they might be in microbial composition by FAME analysis. The relative abundance of FAMEs from the different GAC-FBR is presented in Table 2. A total of at least 47 fatty acids, including saturated, unsaturated, and methyl and hydroxyl substituted fatty acids were identified among the samples. The greatest number and diversity of fatty acids were found for the communities in the FBR treating production water brines from an oil producing field heavily contaminated with aromatic hydrocarbons (PW), and from the aquifer contaminated with gasoline residues (TC) at Traverse City. The most abundant fatty acids were 12:0, 14:0, 16:0, and 16:1 ω7c at these two sites. By contrast, the community with the least fatty acid diversity was the laboratory system fed only toluene (TOL), with 16:1 ω7c, 18:1 ω7c/ω9c/ω12t, and 16:0 as dominant fatty acids.

Principal component (PC) analysis of FAME profiles showed that the five GAC communities were different (Figure 11). The first PC accounted for 41%, the second PC for 25%, and the third PC for 14% of the variation in the data, for a cumulative total of 80%. PC scores obtained after analysis of the correlation matrix showed high scoring (higher than 0.90) on fatty acids 13:0, a15:1, 15:0, i16:1, i16:0, 16:1 ω9c, 17:1 ω8c, 17:0, and 18:1 ω9c for PC1; 14:0, i15:0, and 16:1 ω5c for PC2; and 10:0, i14:0, and 16:0 for PC3.

Table 2. Relative abundance (% of total) of fatty acids in the microbial communities of five different GAC-FBR systems.

Fatty acid[1]	Reactor[2]				
	TOL	BTEX	TC	PW	JEN
10:0	0	6.30	0.95	1.12	1.88
i11:0	0	0	0	0	1.77
10:0 3OH	3.28	7.40	3.40	0.39	3.27
12:0	3.91	43.65	14.20	12.46	25.91
i11:0 3OH	0	0	0	0	1.68
13:0	0	0	0	0.45	0
12:0 2OH	0.75	0	0	0	0
12:0 3OH	0.91	1.07	0.14	3.32	0
i14:0	0	2.83	0	0.60	0
14:0	2.20	8.78	6.88	5.96	14.68
i15:1	0	0	1.20	0.42	0
a15:1	0	0	0	0.31	0
i15:0	0	1.93	1.03	1.80	3.44
a15:0	0	1.40	0.16	0.35	0.73
15:1 ω8c	0	0	0	0.41	0
15:1 ω6c	0	0	0.23	0.57	0
15:0	0	0	0.34	3.06	0
14:0 2OH	2.45	0	0	0	0
i14:0 3OH	0	0	0.20	0	0
16:1 ω7c alcohol	0	0	0.25	0	0
i16:1	0	0	0	1.40	0
i16:0	0	0	0	4.89	0
16:1 ω9c	0	0	0	6.33	0
16:1 ω7c	44.21	18.43	39.87	8.80	11.52
*i 15:0 2OH, 16:1 ω7t	0	0	4.03	1.49	2.76
16:1 ω5c	0	1.65	1.00	0.15	3.41
16:0	15.60	4.61	12.46	9.49	11.70
16:0 10 methyl	0	0	0	4.74	0
i15:0 3OH	0	0	0.19	0	0
a17:0	0	0	0	0.38	0
17:1 ω8c	0	0	0.37	4.58	0

17:1 ω6c	0	0	0	1.46	0
17:0 CYCLO	0	0	0.14	0	0
17:0	0	0	0.13	1.14	0
i17:0	0	0	0.50	0.50	1.09
17:0 10 methyl	0	0	0.40	3.03	0
17:1 ω7c	0	0	0	0	0.98
16:0 3OH	0	0	0	0.28	1.33
*18:3 ω6c/ω9c/ω12c	0	0	0.10	0.47	0
18:1 ω9c	0	0	1.14	6.01	2.66
*18:1 ω7c/ω9t/ω12t	25.66	1.96	7.89	1.40	4.93
18:0	0	0	1.15	0.82	4.09
i17:0 3OH	0	0	0.22	0.09	0
19:0 CYCLO ω8c	0	0	0	0.66	0.95
*20:4 ω6c/ω9c/ω12c/ω15c	0	0	0.16	0.61	0
20:0	1.03	0	0	0	0

[1] Fatty acids are designated as the number of carbon atoms, number of double bonds and position relative to the aliphatic (ω) end of the molecule. The prefixes, i and a, refer to iso and anteiso branching respectively; CYCLO indicates cyclopropane substitutions; OH indicates hydroxyl substitutions; and other methyl branched fatty acids are designated upon the methyl group position relative to the carboxylic acid.

[2] The coefficient of variation for most fatty acids was generally below 10% for replicate analyses.

* A group that MIDI-FAME does not reliably separate.

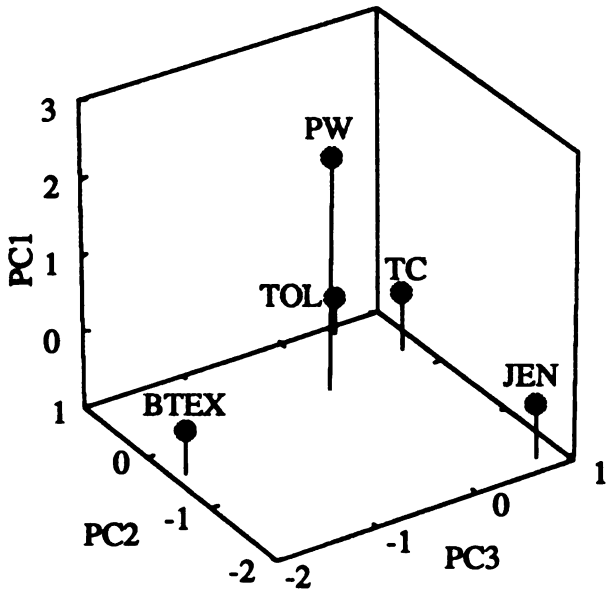

Figure 11. Principal components analysis showing variation in FAME patterns in five different GAC-FBR systems: TOL, BTEX, TC, PW, and JEN.

DISCUSSION

Image analysis is potentially a powerful tool to understand microbial communities at an organizational level, and CSLM and other new methods are beginning to allow this potential to be realized. By using CSLM in this study of petroleum degrading films we found that the classical model of random growth patterns leading to a uniform biofilm structure with active cells at the surface was not supported. In contrast, large interaggregate channels that extend well into the biofilm appear to be maintained for long periods of time. If this model generally proves to be true, which seems to be the case in at least a few other types of biofilms examined (Lawrence et al., 1991; Wolfaardt et al., 1994), then assumptions about uniformity of diffusion within biofilms and where active cells reside should be reevaluated.

The high resolution and magnification power of SEM allowed detailed studies of the early events of preemptive surface colonization. However, sample preparation (fixation, dehydration, and embedding) introduces morphological artifacts for multilayer biofilm

analysis imposing intrinsic limitations on the film thicknesses which can be precisely studied. CSLM however, allows the study of relatively thick film communities because of its ability to obtain clear optical sections free of defocus interference from underlying or overlying materials (Carlsson and Liljeborg, 1989; Carlsson et al., 1989; Caldwell et al., 1992). The spatial relationship of cell arrangements, extracellular polymers, and spaces are, therefore, unaffected.

The GAC of the laboratory scale FBR system fed groundwater amended with toluene was rapidly colonized by the indigenous aquifer populations. SEM analysis showed that colonization initially occurred in sheltered regions, e.g. on rough surfaces and in cavities of the virgin GAC. This pattern of colonization is probably the result of the adsorption properties of the GAC, fluid dynamics, and system geometry. Geesey et al. (1979) have shown differential bacterial colonization in the crevices of suspended detrital material in fast-moving rivers. Planar surfaces of suspended particles in fluidized bed reactors are exposed to greater surface abrasion generated by shear forces and particle collisions. Eventually, however, continuous microbial growth resulted in contiguous coverage of the entire surface.

Once the particle was fully colonized, and the biofilm grew from 20 μm to 300 μm in thickness, we noted that a large number of vertical and horizontal channels permeated the film. These channels extended from the surfaces into at least one half the depth of the film. The structured, more porous film was built on a based film of 10- to 75-μm. To see if this architectural feature was the general case, especially outside of laboratory controlled systems, we examined four field-scale fluidized bed reactors treating petroleum contaminated groundwaters. While the types of structures seen were more varied, the common feature was that channels were permeating the biofilms in all reactors.

All GAC-FBR biofilms exhibited distinctive horizontal and vertical arrangements with the following features: (i) complete GAC surface colonization and base films, (ii) irregular film edges, (iii) variable film thickness within the same colonized granule, and (iv)

contiguous cell-free spaces. Open channels of variable size connected the outside film surface and bulk solution with the deeper inner layers. This biofilm structure, which increases the biological surface area per unit volume, may facilitate transport of substrates, nutrients, and gases deeper into the matrix than is possible due to diffusion alone. Active bacterial cells in deep inner layers may help to keep the basal film firmly attached to the substratum and, therefore, to be more resistant against uncontrolled sloughing of the biofilm caused by shear stress or the rapid stream flow and particle collisions. Although this was not as extensively studied, late secondary colonization by yeast, fungi, and protozoa, and mechanical biomass control (to keep the biofilm thickness from exceeding a certain depth) clearly affected the integrity of these structures in the bacterial biofilms. While we do not know the impact of these structures on biofilm performance, we do know that the reactor performance, as measured by oxygen consumption remained constant.

It might be argued that this more porous structure is simply a result of random growth, but there are important features that suggest that this is not likely to be the entire explanation. First, the channels are maintained for many weeks, well beyond the time when confluent growth should have obliterated the channels. Secondly, there are a diversity of channel types which appears to be influenced by growth conditions and microbial composition. Finally, the hydraulic stress of these systems will tend to select structures in which the base film can be more active to maintain the GAC-film interaction thus, avoiding destruction of itself. This suggests that some cell-cell communication occurs in these mixed communities to form and maintain this architecture.

Community structure would be expected to be influenced by community composition. We used FAME analysis of the community to determine whether the communities of the one laboratory and four field reactors had different composition and whether some were more closely related. This method has been successfully applied by several groups (Federle et al., 1990; Peltonen et al., 1992; Rajendran et al., 1992; Frostegard et al., 1993; Haack et al., submitted) to examine microbial community

composition free of the bias inherent in culture dependent procedures (Wayne et al., 1987). Using principal component analysis we determined that each community had a distinct composition. The TOL (laboratory) and TC reactors were the most similar. This may be due to the fact that the TOL reactor had the simplest substrate feed and, while the TC reactor had a more complex feed, it was in operation only one month and may not have completed its succession to a more complex community. Reactor PW was the only system that was distinguished by PC1; this reactor was the only one with a high salt waste stream (approximately 7% w/v). In spite of the differences in microbial composition among the reactors, the channeled architecture was conserved.

Studies on anaerobic fixed-bed reactor community previously revealed integrative networks of channel-like structures throughout the microbial granules which has been interpreted as playing a role in transport of cooperative substrates, nutrients and gases (Robinson et al., 1984; MacLeod et al., 1990; Wu et al., 1991). In the anaerobic food web it is more clear why interspecific interactions between complementary trophic groups may result in a structure that facilitates molecule transport. It is interesting however, that even though aerobic oxidation of hydrocarbons might require few if any interspecific interactions, that microbe-microbe communication and growth coordination seems to play a key role during progressive biofilm establishment. Our CSLM results suggest conservation of common architectural strategies that appear to be biologically controlled perhaps to deal with diffusion limiting processes and shear stress. This architectural arrangement of a biofilm may represent a more optimal arrangement for influx of substrate, nutrients, and waste transfer, and if true would have important implications on traditional conceptual biofilm models in which microbial growth and horizontal and vertical distribution was assumed to be randomly determined. Further testing of this model and understanding of the forces and principles which regulate biofilm development will help in efforts to model, control, and exploit biofilm related processes of industrial, medical, and environmental importance.

APPENDIX A

APPENDIX A

BACTERIAL COMMUNITY FINGERPRINTING OF AMPLIFIED 16S AND 16-23S RIBOSOMAL DNA GENE SEQUENCES AND RESTRICTION ENDONUCLEASE ANALYSIS (ARDRA)

The 16S and 23S rRNA genes have been utilized for phylogenetic analysis of both prokaryotes and eucaryotes organisms (Woese, 1987). In addition to direct comparison of the nucleic acid sequences, numerous groups have used the rapid method of polymerase chain reaction (PCR) amplification of this gene (Muyzer et al., 1993) as well as the complete rRNA locus (Jayarao et al., 1991; Vaneechoutte et al., 1992; Jensen et al., 1993) for a simple method for identification of bacterial genera and species. In these latter procedures, the amplified DNA (rDNA) is subjected to restriction endonuclease analysis; this has been termed ARDRA (Amplified Ribosomal DNA Restriction Analysis) (Vaneechoutte et al., 1992). The resulting restriction fragment pattern is then used as a fingerprint for the identification of bacterial genomes. This method is used on the principle that the restriction sites on the RNA operon are conserved according to phylogenetic patterns.

Although ARDRA has been used for the characterization of bacterial isolates, in theory this method can also be used for analyzing mixed bacterial populations. If the rDNA fingerprint for individual bacteria in a mixture, e.g. community, were sufficiently different, then one could examine the amplified product for a series of distinct pattern resulting from the different populations that make up the community. Ribosomal DNA fingerprinting of these communities could be used for a quick assessment of genotypic changes, possibly over time or between different locations reflecting different environmental conditions. This method involves the use of a pair of universal priming sequences for the PCR amplification of either the 16S rRNA genetic loci or the intergenic regions of the 16S and 23S rRNA

genes. The latter regions exhibit a large degree of sequence, length and frequency variation because the space region is not well conserved. In general, ARDRA using the 16S rRNA genes will result in a simpler pattern (3-5 bands per genome when 4 base site-specific restriction endonucleases are used) than ARDRA using the 16-23S region. In this case different but related organisms may share common restriction fragments. Some fragments may be unique for a specific organism and would be diagnostic for a given group of microbes. ARDRA using the 16-23S rDNA region will provide a more diverse substrate for restriction analysis resulting in more complex band patterns and potentially higher resolution of community members. This version of ARDRA may be useful in more simple communities which are composed of closely related populations. In highly diverse microbial communities, both of these methods may either yield too complex a pattern or too little resolution of distinct bands. If this is the case, an alternative may be to use group specific primers for a given bacterial group or to carry out the DNA amplification under discriminatory conditions (e.g., increasing stringency conditions during the annealing step of PCR). Further, resolution could also be obtained by hybridization with DNA probes specific for the target groups. Finally, one may be able to dilute DNA samples from communities so that only the more dominant member's genomes are present for ARDRA. The level of resolution (band pattern complexity) is ultimate controlled by the frequency of restriction sites and restriction endonucleases used.

This PCR based technique will allows for assessment of microbial structure and composition at the microbial scale. As little as picograms quantities of DNA (1-10 cells) can be used in this method. In turn, changes or responses of the microbial system can be monitored at the genotypic level without drastically disturbing the system by sampling. Additionally this method is bias free of culture dependent methods, although it can be applicable for characterization of axenic cultures as well as to identify non yet isolated members.

EXPERIMENTAL APPROACH

A pair of highly conserved flanking sequences are used for primer binding sites to amplify the 16S ribosomal genes from microbial systems (Figure 1). The intragenic PCR product (~1500 bp) is then used as substrate for restriction endonuclease digestion follow by gel electrophoresis. In order to facilitate analysis and the resolution (ability to distinguish two different populations) of the assay after the PCR amplification, more than one separate restriction enzyme digestion should be used. Since community amplified rDNA is an indirect indicator of the diversity of sequences, sensitivity is maximized (ability to avoid false negative) by concentrating the amplified rDNA or digested rDNA product before electrophoresis. For the 16-23S rDNA intergenic region, amplification is carried out by using a pair of opposite highly conserved 16S and 23S ribosomal primers (Figure 1) followed by restriction enzyme digestion and gel electrophoresis.

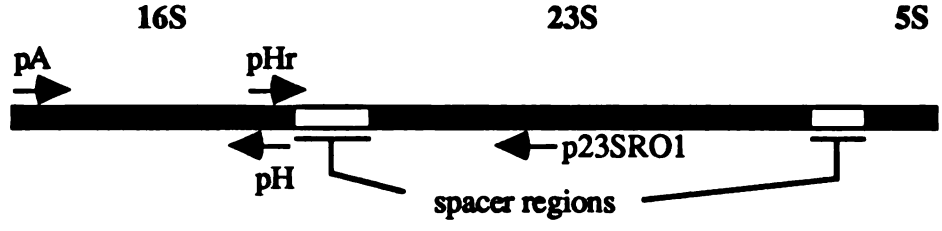

Figure 1. General schematic representation of the ribosomal RNA operon showing approximate localization of priming binding sites for PCR amplification. The spacer region varies in length (e.g., number of tRNA's), and sequences among the different operons within a single organism (Brosius et al., 1981).

Primer sequences for the 16S rRNA gene amplification:

Primer Designation	Position (E.coli 16S rRNA)	Sequence (5' - 3') (Ulrike et al., 1989)
pA	49-68	AGA GTT TGA TCC TGG CTC AG
pH	1541-1518	AAG GAG GTG ATC CAG CCG CA

Primer sequences for the 16-23S rRNA intergenic amplification:

Primer Designation	Position (E.coli 16-23S rRNA)	Sequence (5' - 3')
pHr (reverse)	1518-1541	TGC GGC TGG ATC ACC TCC TT
p23SRO1	1069-1052	GGC TGC TTC TAA GCC AAC

PROCEDURES

Template for PCR amplification -- For mixed microbial systems, it is important to maximize cell lysis before amplification since lysis efficiency is unequal for different bacterial types and physiological stages. Direct lysis using repetitive (5X) freeze (dry ice-ethanol bath) and thaw (80°C water bath) cycles of the bacterial cells suspension can be used for PCR. As an alternative, the use of community DNA (Maniatis et al., 1982) as template for the reaction is suggested. This will ensure a more representative, reproducible and controllable method to successfully carry out the amplification step.

Steps in the PCR amplification:

1. Prepare Master solution containing:

ddH_2O	86.5 µl
10X PCR Buffer	10.0 µl
100X dNTP's	1.0 µl
100X primer *x*	1.0 µl
100X primer *y*	1.0 µl
Taq DNA polymerase (5 U/µl)	0.5 µl

2. Add 1 µl (approximately 100 ng) of bacterial or community DNA, or cell suspension (approximately 10 to 10^4 cells). Total reaction volume is 100 µl.

3. Amplification with the following temperature profile:

	Temperature	*Time*
Denature	92°C	2 min, 10 sec

Melt	92°C	1 min, 10 sec	
Anneal	48°C	30 sec	(30-35 cycles)
Extend	72°C	2 min 10 sec	
Final extend	72°C	6 min 10 sec	
Soak	4°C	hold	

4. After amplification, 5 to 10 µl is electrophoresed on 0.7% agarose gel made in TAE buffer to examine the amplified products (Figure 2).

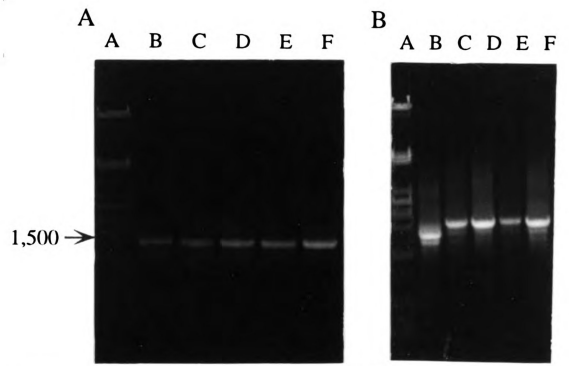

Figure 2. Products of PCR amplification from microbial communities samples. (A) The 16S rDNA amplification yielded a 1,500 bp fragment from axenic culture (lane B) as well as bioreactor samples (lanes C to F). (B) Results of 16-23S rDNA intergenic amplification of the same samples yielded various fragments of different sizes. Phage lambda (λ) DNA digested with *Pst*I was used as marker (lanes A), indicated (in base pairs) on the left.

Notes:

1a. Maintain all solution on ice.

1b. Reagents should be added in that order.

1c. For *n* number of reactions, each solution volume has to be multiplied by *n*, and aliquot it in *n* PCR vials. Always include a non-DNA control (e.g., water).

1d. Primers *x* & *y* are pA & pH for 16S rDNA amplification or pHr & p23SRO1 for 16-23S rDNA amplification.

1e. Taq DNA polymerase from different sources may behave differently because of different formulations, assay conditions, or unit definitions.

2. When cells are used as templates, they can be pre-treat (e.g., freeze & thaw) directly in the PCR vials containing 86.5 µl of ddH_2O with the PCR buffer. The master solution of nucleotides, primers and polymerase are then added before amplification.

3a. Optimal cycle temperatures may vary within different thermal cycler models. Given set of temperatures and times were determined empirically for the Perkin Elmer thermal cycler model 9600.

3b. When cells are used as templates, melting steps should be performed at 94°C for 3 minutes. In addition, annealing should be done at 52°C.

3c. When DNA is used as template, 48°C provides low stringent annealing conditions, but discriminatory enough to avoid non specific amplification.

Solutions:

- 10X PCR buffer
    - 500 mM KCl
    - 100 mM Tris-HCl, pH 8.3
    - 15 mM $MgCl_2$
- 100X dNTP's solution
    - 25 mM of each nucleotide (ATP, TTP, CTP, and GTP)

- Primers
    - 100 μM stock solution in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8)
- AmpliTaq DNA polymerase
    - 5 Units/μl (Perkin-Elmer Cetus, Norwalk, CT)
- 1X TAE electrophoresis buffer (pH 7.5)
    - 40 mM Tris-HCl
    - 5 mM sodium acetate
    - 1 mM EDTA (disodium ethylenediaminetetraacetate)

Restriction endonuclease digestion and gel electrophoresis -- Digestion of the amplified rDNA product with tandem tetrameric site-specific restriction endonucleases (e.g., *Hae*III, *Mse*1, *Hinf*1, *Sau*3A, *Hpa*II; Boehringer Mannheim, Indianapolis, IN) should yield a restriction enzyme digestion pattern which can be discerned by gel electrophoresis. An aliquot of the PCR product can be directly used for each digestion. Digest in the appropriate restriction buffer (generally provided by all commercial companies in 10X concentration when enzymes are purchased) at optimal temperature for 2-3 hours. As noted above with community level amplification it is useful to concentrate the amplified product. This can be accomplished by ethanol precipitation (1/2 volume 3 M sodium acetate with 2 volumes of cold 100% ethanol at -20°C for 30 min to overnight) (Maniatis et al., 1982) before or after digestion. The amount of DNA to be loaded per well is dependent of the limiting bands which need to be resolved. The least amount of DNA that can be consistenly detected with ethidium bromide staining is about 10 ng. DNA amounts greater than 100 ng will not be resolved as a sharp clean band. The rDNA restriction fragments are then electrophoresed on 4% (w/v) NuSieve agarose gels (FMC, Rockland, ME) at 7 volts/cm containing 0.5X TAE, and stained in 0.5 μg/ml ethidium bromide solution for 20 min (Figure 3). Finally, DNA banding patterns database and numerical analysis can be performed with analytical hardware/software package such as AMBIS MicroPM$_{TM}$ based system (Microbiology Pattern Matching) (Hook et al., 1991).

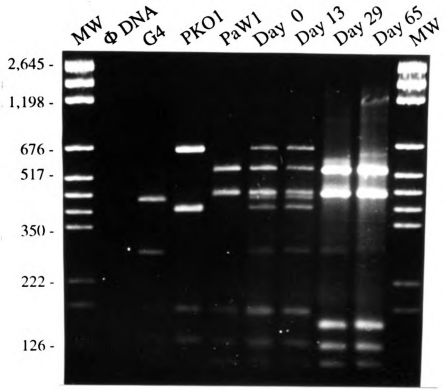

Figure 3. Photograph of ethidium bromide stained agarose gel. Lane MW: molecular weight DNA markers, indicated (in base pairs) on the left. Lane ϕ DNA: PCR amplification of water. All other lanes are *Hpa*II restriction endonuclease digestion of PCR amplified 16S rDNA from: G4, *Pseudomonas cepacia* ; PKO1, *P. pikettii* ; and PaW1, *P. putida* ; Day 0, 13, 29, and 65 are bioreactor samples inoculated with the same strains of bacteria at Day 0 and challenged by aquifer populations after day 19. One interpretation of the results is that PaW1 becomes the dominant population after Day 29. However, this erroneous interpretation is the result of multiple groundwater populations that happen to have the same restriction fragments of PaW1 when *Hpa*II is used. Differences will be more readily detectable when a second or third restriction endonuclease is put to use.

APPENDIX B

APPENDIX B

DIFFERENTIAL AMPLIFICATION OF rRNA GENES FOR THE DETECTION AND IDENTIFICATION OF *PSEUDOMONAS CEPACIA* (G4) AND *P. PICKETTII* (PKO1)

Assessment of the fate of introduced populations on natural ecosystems has ecological and practical significance. For example, in the area of bioremediation technology, several studies have suggested that by introducing and selecting organisms with unique physiologic traits at contaminated sites or in bioreactors may stimulate biodegradation (Chakrabarty, 1986). *Pseudomonas cepacia* (G4), and *P. pickettii* (PKO1) are two well characterized benzene, toluene, and xylene degrading populations which can also cometabolize other toxic recalcitrant compounds such as trichloroethylene (TCE) from the environment (Shields et al., 1989; Kukor and Olsen, 1990). Recently, we have examined the use of these microorganisms in treatment of toluene contaminated groundwater on biological activated carbon fluidized bed reactors and their successes against indigenous aquifer populations.

Molecular-based methods can be faster, more sensitive and accurate than classical plating techniques (Johnson, 1989). For example, molecular phylogeny of comparative analysis of 16S rRNA genes (Gray et al., 1984) has been used to determine the species composition of microbial communities, and several approaches have been used to obtain rRNA sequences from natural systems free of bias inherent in cultivation procedures (Lane et al., 1985; Schmidt et al., 1991; Weller et al., 1992). In one of these methods, the polymerase chain reaction (PCR) is used for the synthesis and recovery of rRNA genes from natural systems (Reysenbach et al., 1992; Muyzer et al., 1993) or axenic cultures (Jayarao et al., 1991; Ward et al., 1992). We developed a differential amplification procedure for the detection and rapid identification of strains G4 and PKO1 that had been released to a natural environment. This amplification was based on alignment of the 16S

rRNA sequence within a specific variable region (Figure 1). Results presented here illustrate the specificity and sensitivity of these amplification probes.

P. cepacia (strain G4)	GGU CGG AAU CCC GAA GAG AU
P. pickettii (strain PKO1)	GCC ACU AAC GAA GCA GAG AU
E. coli	CCA CGG AAG UUU UCA GAG AU
P. cepacia	GGU CGG AAU CCU GCU GAG AG
P. pickettii	GCC ACU AAC GAA GCA GAG AU
P. aeruginosa	GCU GAG AAC UUU CCA GAG AU
P. testosteroni	GGC AGG AAC UUA CCA GAG AU
P. mendocina	GCU GAG AAC UUU CCA GAG AU

Figure 1. Ribosomal RNA sequence alignments (corresponding to positions in the *Escherichia coli* 16S rRNA, 998 to 1017) showing target primer binding sites for strains G4 and PKO1. Sequences are either results from Jizhong et al., unpublished or taken from the ribosomal data base.

Table 1 shows the list of bacterial strains used in this study. Axenic cultures were grown overnight on Luria broth (Difco, Detroit, MI) and harvested by centrifugation. Genomic DNA (RNA-free) was isolated and purified as previously described (Maniatis et al., 1982). Intragenic regions of the small-subunit rRNA was selectively amplified by PCR using a forward primer complementary to a universally conserved region which correspond to nucleotide positions 49 to 68 (5' - AGAGTTTGA TCCTGGCTCAG - 3', noted as pA) (Ulrike et al., 1989) of *Escherichia coli* 16S rRNA and reverse species-specific primers corresponding to the complement of positions 1017 to 998 (5' - GGUC GGAAUCCCGAA GAGAU - 3', noted as pG4V3; and 5' - GCCACUAACGAAGCAGA GAU - 3', noted as pPKV3 specific for strains G4 and PKO1, respectively) (Jizhong et al., unpublished). As a positive control, a universal conserved reverse primer binding site (5' - AAGGAGGTGATCCAGCCGCA - 3', noted as pH; positions 1541 to 1518) (Ulrike et al., 1989) was also used. All primers were synthesized with an Applied Biosystem DNA synthesizer at the Macromolecular and Structure facilities at Michigan State

University. Template DNA (50 ng) was used in a 50-µl reaction volume containing: 5 µl of 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$ [Perkin-Elmer Cetus, Norwalk, CT]), 100 µM each of two opposing primers, 250 µM of each deoxynucleoside trisphosphates (Perkin-Elmer Cetus, Norwalk, CT), and 1.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The amplification cycles used were as follows: denaturation at 94°C for 1 min 30 sec; followed by 30 cycles of denaturation at 92°C for 1 min 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 1 min 30 sec; an extension at 72°C for 6 min; and termination at 4°C. Amplification products were resolved by agarose gel electrophoresis in a 0.8% (w/v) agarose gel containing 1X TAE (40 mM Tris-HCl, pH 7.5, 5 mM sodium acetate, 1 mM EDTA [disodium ethylene-diaminetetraacetate]), and stained in 0.5 µg/ml ethidium bromide solution, and were photographed in UV light with Polaroid Type 55 film.

Table 1. Bacterial strains studied and reaction of specie-specific PCR amplification.

Bacterial strain or isolate	Description[1]	Lab. source or ref.	Reaction with probe: pG4V3	pPKV3
Pseudomonas cepacia G4	beta	Shields et al., 1989	+	-
P. pickettii PKO1	beta	Kukor et al., 1990	-	+
P. mendocina KR	gamma	M. DeFlaun; Yen et al., 1991	-	-
P. putida PaW1	gamma	Harayama et al., 1989	-	-
Azoarcus sp. Tol 4	gamma	Fries et al., submitted	-	-
P. fluorescens	gamma	Bill Holben, Mich. St. Univ.	-	-
P. aeruginosa	gamma	Bill Holben	-	-
Escherichia coli W3110	gamma	Bill Holben	-	-
Enterobacter sp.	gamma	Bill Holben	-	-
Agrobacterium radiobacter	alpha	Bill Holben	-	-
Ps. sp. gf 261	beta	Lab. collection, Chaper III	-	-
Strain gf 281	alpha	Lab. collection, Chaper III	-	-

[1] Subdivision of the *Proteobacteria*.

In standard reactions in which PCR was carried out with universal eubacterial primers, an approximately 1,500-bp amplified fragment was obtained for all the tested strains (Figure 2, lanes 3-8). However, differential amplification with the pPKV3 specific primer only showed an amplified product of the predicted size (approximately 1,000-bp) for strain PKO1, while non of the other species showed product (Table 1; Figure 2, lanes 10-15). The addition of non-specific DNA has no major effects on the reaction, even when the target DNA was 1% of the total (Figure 2, lanes 16-18). Similar results were obtained with the pG4V3 primer for differential amplification of strain G4 (Table 1).

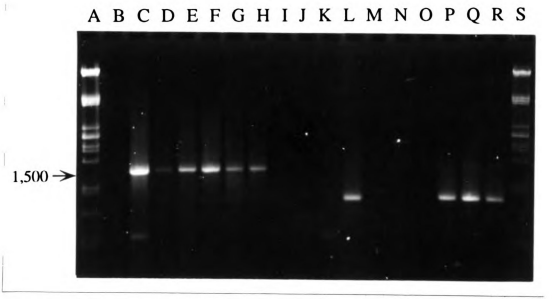

Figure 2. Differential amplification of bacterial 16S rRNA genes. Lanes A and S, λ *Pst* I standards (in base pairs); lanes B and I, PCR without template; lanes C to H, and J to O are PCR amplification of *Escherichia coli*, strain G4, strain PKO1, strain PaW1, *P. fluorescens*, and *P. aeruginosa* using universal primers pA and pH, and using the universal primer pA combined to the specie-specific oligonucleotide primer pPKV3 respectively; and lanes P to R are PCR amplification using pA and pPKV3 primers of 1:1, 1:10, and 1:100 PKO1 template DNA in the presence of non-specific DNA (PaW1).

We also used this procedure for examining a biofilm community pre-seeded with *P. cepacia* (G4) (see Chapter II). Figure 3 shows differential amplification of the 16S rDNA

intragenic region of strain G4. According to the test, strain G4 or closely related populations were below detection limits in the mature biofilm community suggesting that G4 probably was out competed during earlier events of community establishment. Our observations were further supported by traditional microbiological methods and DNA-DNA hybridization techniques with a catabolic gene probe derived from strain G4 (data not shown).

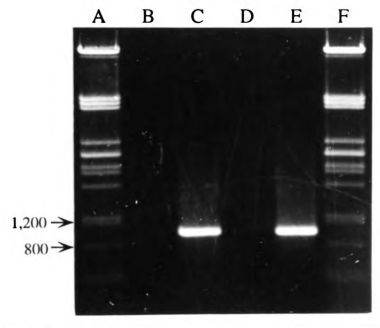

Figure 3. Differential PCR amplification of *P. cepacia* (G4) from a toluene treating bioreactor community inoculated with the same strain of bacteria using primers pA and pG4V3. Lanes A and F, λ *Pst* I standards (in base pairs); lane B, PCR without template; lanes C, strain G4; lane D, bioreactor community, day 63; and lane E, 1:1 of the same community DNA and G4.

In conclusion, we developed a rapid PCR-based method as a useful presumptive test for the detection of toluene degrading strains G4 and PKO1. Lack of amplification would then imply that the template concentration of the target strain or phylogenetically closed relatives were below the limit of sensitivity or that the template was absent, as was the case for our DNA-negative controls. If positive results are obtained by PCR, then other

viability testing using cell culture techniques or DNA-DNA hybridization procedures are recommended.

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