

**THE EMERGENCE OF DEGRADATIVE BIOFILMS**

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**by**

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## ABSTRACT

The aim of this work was to determine the role of cellular recombination and positioning in the emergence of biofilm communities. This involved monitoring a biofilm community cultivated in a flow cell and subjected to cyclic environmental transitions from labile to refractory substrates. Laser microscopy of flow cells revealed that transitions from labile to refractory substrates resulted in a decrease in the thickness and percent area coverage of the biofilm communities. Repeated inoculation of flow cell communities with a composite inoculum resulted in an increase in the number and diversity of emigrants as well as greater thickness and area coverage of the biofilms than when the communities were inoculated only at the beginning of the experiment. This suggested that juxtapositioning through organismal recombination enhanced the growth of biofilm communities subjected to environmental stresses. Repeated environmental cycling also enhanced the growth of biofilm communities, suggesting that the functionality of cellular positioning may have accrued from one cycle to another, representing a form of heritable community-level information. Patterns of emigration that emerged during adaptation of biofilm communities to substrate transitions showed the appearance and disappearance of discrete sets of organisms. In addition, when biofilm communities cultivated using tryptic soy broth (TSB) were plated on benzoate-containing agar, higher plating efficiencies were detected at lower dilutions. This was concomitant with the formation of satellite colonies around primary colonies of benzoate-resistant bacteria. Plating assays and radiolabeled-benzoate mineralization experiments revealed that efficient benzoate degradation by the primary colonies protected the satellite strains against inhibitory concentrations of sodium benzoate. This protection also occurred during batch and flow cell cultivation. When a microbial community derived from a pristine soil environment was subjected to a substrate shift from TSB to benzoate, inclusion of a benzoate-degrading strain of *Pseudomonas fluorescens* (BD1) enhanced community stability. This suggested that bacteria may respond to environmental stresses as sets of spatially-related organisms as opposed to functioning exclusively as individuals. The emergence of protective synergisms also suggests that associative strategies between microorganisms should be a consideration in food preservation, human health as well as in biodegradation.

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## 1 INTRODUCTION

Various microbiological processes in the environment, agriculture and industry are mediated by the coordinated activity of a number of different, often genetically unrelated microorganisms endowed with diverse metabolic capabilities. The associative strategies involved in these processes combine the adaptive potential of individuals (gene mutations and recombination) through spatial and temporal interactions resulting in the formation of diverse multi-species networks such as communities, consortia and microecosystems. Interactions and networking among microorganisms complement the metabolic capabilities of individuals and thus extend their habitat range by creating favorable microenvironments within unfavorable macroenvironments. This enhances the survival and reproductive success of the individuals and of the association as a whole. Although beneficial to the organisms involved, the formation of microbial associations can be either beneficial or detrimental from the human perspective. Beneficial processes mediated through community-level microbial activities include biodegradation (Burkhardt et al., 1993; Wolfaardt et al., 1994b, 1994c; Shen and Bartha, 1996; Holden and Firestone, 1997; Olivera et al, 1997), industrial and municipal wastewater treatment (Eriksson and Weisner, 1997; Amann et al., 1998; Schneider et al., 1998) as well as production of fermented foods such as kefir and yoghurt (Rea et al., 1996). Microbial communities have also been implicated in detrimental activities such as the formation of dental plaque (Kinniment et al., 1996; Yamaguchi et al., 1998), microbial corrosion (Santo et al., 1998), biofouling (McLean et al., 1997), food spoilage (Gardini et al., 1998), mixed bacterial infections (Chen et al., 1996), etc. Consequently, there has been a growing interest in the study of microbial communities with a view to optimize community-mediated beneficial processes as well as to prevent economic losses resulting from their deleterious processes.

Understanding microbial communities as causative agents and their utilization or control requires a fundamental understanding of their formation, structure and spatial organization. Species composition within a microbial community, also referred to as community structure (Caldwell et al., 1997b), determines the community's overall genetic potential for survival and reproductive success under a given set of environmental conditions. Spatial organization within a community determines the

ability of individuals to interact genetically and physiologically. Thus, the study of community-level microbial organization requires that the communities be cultivated under defined environmental conditions, and analyzed by non-destructive methods (thereby conserving their spatial integrity). Systems that offer defined environmental conditions for the cultivation of microbial communities include nutritats, gradostats, chemostats, microstats, flow cells, dual-dilution continuous culture, etc. (Caldwell et al., 1997b, 1997c). Among these, the flow cell allows instantaneous control of the concentration and flux of the growth substrate and facilitates attached growth of microorganisms as biofilms. The structural and spatial organization of biofilm communities developing in flow cells can be periodically monitored and non-destructively analyzed using scanning confocal laser microscopy (SCLM) in conjunction with fluorescent molecular probes. Spatial positioning of specific organisms within multi-species biofilms can also be determined by using species-specific oligonucleotide or antibody probes, or by inserting GFP (Green Fluorescent Protein)-variant genes into specific biofilm members.

Conceiving of microbial communities as units of adaptation and propagation also requires a conceptual basis founded on non-reductive scientific thought. Although widely known, and frequently applied to explain the evolution of life on earth, the theory of natural selection and survival of the fittest species (Darwin, 1859) is unnecessarily complex in that it conceptualizes selection, competition, fitness and survival as mechanisms of evolution rather than as emergent properties. More recent approaches such as group selection theory (Wilson, 1987; Goodnight et al., 1992; Goodnight and Stevens, 1997; Wilson and Dugatkin, 1997), kin selection theory (Kelly, 1992), community-level selection theory (Goodnight, 1990; Wilson, 1997), niche construction theory (Odling-Smee et al., 1996) and game theory (Dugatkin and Reeve, 1998) compound these unnecessary complexities when used to explain the formation and functioning of multi-species microbial communities in terms of selection theory. For example, community-level selection theory envisions natural selection as a mechanism acting at the community-level to select fit communities against unfit communities (Wilson, 1997). Niche construction theory proposed by Odling-Smee et al. (1996), suggests that the process of creation or modification of niches by ancestral organisms serves as a parentally modified source of natural selection to the offspring. However, in natural ecosystems, when microbial communities are subjected to environmental perturbations they undergo spatial restructuring as a mechanism of adaptation that may include microbial interactions and recombination events at the organismal level as well as

at the genetic level and often extending beyond community boundaries, thus precluding the possibility of 'selection' on any single spatial scale, or by any single property (such as a constructed niche). This makes it difficult or impossible to define fitness (Hayward, 1999), and hence to make use of the concept of natural selection.

Recognizing the emergence of reproductive success at multiple levels of biological organization (plasmids, cells, organisms, communities and ecosystems), Caldwell et al. (1997b, 1999) proposed the proliferation theory. According to proliferation theory, the functionality for the spatial recombination of organisms is directly expressed prior to being coded genetically or being coded in any other form of information other than the positioning of the organisms themselves. Thus functionality and spatial coding emerge simultaneously in terms of recombination of cell positioning, while functionality is delayed by gene expression in terms of genetic recombination. The lack of delay in the expression of organismal positioning information during organismal recombination does not make it any less significant than base pair positioning information during genetic recombination. In both cases, the emergence of information results from the coincidental emergence of functional positioning for physical, chemical and biological objects. Thus the generation of positioning information must always precede or occur simultaneously with the generation of functionality involved in the propagation of evolving space-time relationships. Hence, based on proliferation theory, it can be hypothesized that synergisms between organisms (e.g. functional positioning of organisms within a community) may be required for the optimal proliferation of communities, just as synergisms between molecules (e.g. functional positioning of base pairs in DNA) are required for the optimal proliferation of cells. This would mean that the emergence of multi-species microbial communities would involve not only genetic recombination of base pairs in nucleic acids, but also the recombination of organisms within and among communities. Thus the process of recombination at multiple spatial scales (gene, organism, community, ecosystem etc.) optimizes the nested structures and hence the growth of microbial communities. Furthermore, if proliferation occurs at multiple spatial levels including the community-level, an environmental shift should elicit coordinated responses among the community members and the changes in community structure profile in response to substrate transitions should show sets of inflections in addition to members responding individually (individual inflections). Consequently, testing the existence of community-level proliferation strategies involves examining the role of external organismal recombination, i.e. the effects of immigration and emigration, during the process of adaptation of bacterial communities to environmental stresses, as



well as testing the stability of community networks when subjected to sudden or gradual environmental transitions.

Thus, during the present study, the existence of community-level proliferation strategies, as postulated by the proliferation theory, was examined by monitoring the emergence of community structure and other community-level properties during environmental transitions from labile to refractory substrates. In general, properties of biofilm communities, such as community structure, architecture, diversity etc. arise unpredictably in response to an evolutionary process and hence can be considered as emergent properties. During the environmental transitions, repeated inoculation of microbial communities with a composite inoculum allowed examining the role of external organismal recombination in enhancing the propagation of the community. Degradative communities were chosen as model systems because of the well-documented role of community-level physiological, genetic and spatial interactions that emerge during biodegradation.

## **2 LITERATURE REVIEW**

### **2.1 Community approach in microbial ecology**

The current understanding of the genetic makeup and physiological functioning of microorganisms has extensively depended on the study of isolated cell lines or pure cultures of microorganisms. When these studies were extended to understand the nature and extent of microbial interactions in mixed cultures and the effect of environmental variables on mixed-culture population dynamics, information useful to enhance or control the abundance and activities of specific microorganisms in both industrial and environmental settings could be gathered. Examples include screening for antibiotic production based in interactions between presumptive antibiotic producing and antibiotic-sensitive strains, and development of strategies to eliminate or reduce contamination during industrial fermentations. However, an understanding of the microbial processes will be inadequate if efforts focus solely on pure cultures or mixtures of pure cultures after isolation from their native community-networks. This is because isolated cell lines often fail to carry out certain microbial processes (Wolfaardt et al., 1994b), or carry them out less efficiently even when individual organisms are recombined in mixed culture (Jiminez et al., 1991). Thus an understanding of microbially-mediated environmental processes requires a holistic approach not exclusively reliant on pure culture or mixtures of pure cultures.

#### **2.1.1 Examples of community-level microbial processes**

Association of microorganisms as consortia and communities has been shown to be responsible for various environmental and industrial processes. For example, degradation of chlorinated hydrocarbons is commonly mediated by community-level microbial processes (Lackey et al., 1994; Bagley and Gossett, 1995; Thakur, 1995; Wolfaardt et al., 1998). During ruminant digestion, plant cell wall polysaccharides including celluloses and hemicelluloses are degraded by a community of interacting microorganisms composed of bacterial, fungal and algal populations (Forano, 1998; White et al., 1999). Microbial communities have also been shown to be responsible for spoilage of foods such as sandwiches (Gardini et al., 1998), lamb carcasses (Prieto et al., 1993), fish (Wheeler and Hocking, 1993), and fruits (Faid et al., 1994). Microbial

associations have frequently been implicated in animal infections. Scanlan and Edwards (1990) isolated a mixed microbial population consisting of a variety of gram-positive and gram-negative, facultatively- as well as obligately-anaerobic bacteria from lesions of the liver in sheep. Certain types of burn wound infections have been shown to result from bacterial-fungal associations (Mousa, 1997). Complex microbial communities have been shown to be responsible for the formation of dental plaque. Bradshaw et al. (1998) demonstrated that coaggregation-mediated interactions between *Fusobacterium nucleatum* and other species facilitated the survival of obligate anaerobes in oral microbial communities during aeration. Microbial communities are also integral components of the environmental cycling of carbon, nitrogen and other major nutrients (De Boer et al., 1996; Stark and Firestone, 1996). Although individuals are often able to carry out certain processes (e.g., conversion of ammonia to nitrite or nitrite to nitrate during the nitrogen cycle), completion of the cycle requires the activity of various organisms with complementary metabolic capabilities. Industrial and municipal wastewater treatment (activated sludge, anaerobic digester granules) is another example, wherein community-level microbial processes are pivotal to the process efficiency (Bidault et al., 1997).

### **2.1.2 Utility of various theories in understanding the formation and functioning of microbial communities**

Caldwell et al. (1997b) defined a microbial community as an interactive network of associated microorganisms. Recent methodological developments permit laboratory cultivation and non-reductive analysis of intact microbial communities. Typically, application of confocal scanning laser microscopy in conjunction with a consortium of fluorescent probes allows non-destructive analysis of the structural and functional organization of biofilm communities, and approaches based on fatty acid, DNA and RNA analysis are useful in elucidation of community structure without having to rely on traditional culture-based techniques. In spite of these methodological developments, conceptual barriers for community-level thought and experimentation still persist (Caldwell et al., 1997a, 1997b, 1997c). For example, undue adherence to the germ theory (Koch's postulates) will be unproductive if the causative agent of a disease is an interactive community rather than a pure culture. Though a number of theories have been proposed to explain the evolution and existence of various life forms on earth, most are limited in their application or become unnecessarily complex when modified to explain the emergence of microbial communities, particularly in understanding the

existence of microorganisms as interactive networks in the environment (e.g., corrosion biofilms, degradative consortia etc.) or in association with higher animals (e.g., rumen communities, plaque biofilms etc.). Some of these ecological theories are discussed below.

#### **2.1.2.1 Natural selection theory**

Darwin's (1859) approach to the evolution and existence of life, as explained in his monograph, "The Origin of Species by Means of Natural Selection or the Preservation of Favored Races in the Struggle for Life", is based on the selection of fit races or lineages over less-fit lineages. However, this approach is limited by the unnecessary assumption that selection, rather than recombination, is a mechanism of evolution (Caldwell, 1999). This becomes especially evident when analyzing the gregarious existence of microorganisms, which often occur as interactive community networks. These associations propagate through beneficial or synergistic relationships among individuals rather than through competitive interactions. This propagation is simultaneously collective and individual (Caldwell et al., 1997b). Factors such as the small size of microorganisms (which limits their overall genetic capacity or the possession of genetic information that is redundant or non-essential), and the inability of individual microorganisms to control their microenvironment effectively (by preventing the formation of diffusion gradients) necessitates their association with other organisms in order to proliferate effectively (Caldwell et al., 1997b).

#### **2.1.2.2 Group selection and community-level selection**

Wilson and Dugatkin (1997) suggested that natural selection at any level (individual, single-species social group and community levels) requires heritable phenotypic variations at that level. For example, group selection requires a process of natural selection at the group level (Wilson and Sober, 1994). Group selection has been demonstrated for characters such as reduced virulence in parasites (Frank, 1996) and female-biased sex ratios (Colwell, 1981). Group-level trait has also been shown to be more heritable than individual trait (Goodnight and Stevens, 1997).

Goodnight (1990) defined community-level selection as the differential proliferation and/or extinction of communities which can bring about a response that may be qualitatively different from the response to selection acting at lower levels. This theory was based on the assumption that community-level functional organization results from a process of natural selection among communities that vary in their

composition and hence vary in their relative fitness (Wilson, 1997). It was also suggested that selection at the community-level results in the selection of multi-species assemblages containing individuals which interact to produce the selected phenotype. Some phoretic communities that both feed and protect their insect carrier (Wilson, 1983; Brown and Wilson, 1992) are considered to have evolved through the community-level selection process. However, the major drawback of the multi-level selection concept is that, though single-species groups and communities are considered to be units of evolution, it ignores the possibility of recombination events that are not merely limited within community boundaries (e.g., immigration and emigration of microorganisms facilitating organismal recombination within and among communities).

### **2.1.2.3 Niche construction theory**

More recently, Odling-Smee et al. (1996) proposed the theory of niche construction which suggests that evolution occurs in organisms not only by the inheritance of fit or unfit genes, but also by the inheritance of environmental modifications (e.g., niche construction). Niche construction was defined as a phenomenon during which organisms create or modify their own niches through their metabolism or other activities such as creation of artifacts (e.g., construction of dams, nests, holes, burrows and webs by various animal species). They suggested that ancestral niche constructing organisms transfer the modified natural selection pressures to their descendents. It was also suggested that constructed niches have a lifetime longer than that of the niche-constructing organisms, and thus are heritable (Odling-Smee et al., 1996; Odling-Smee and Laland, 1999). The major drawback of this concept (from the perspective of proliferation theory) as explained below, is that a constructed niche is thought of as a heritable source of environmental selection rather than as heritable information accrued at the community-level and passed on to offspring.

### **2.1.2.4 Proliferation theory**

Proliferation theory (Caldwell et al., 1997b) is based on the assumption that propagation and reproductive success occurs at multiple levels of biological recombination (genes, plasmids, cells, organisms, communities, ecosystems etc.) simultaneously precluding the possibility of optimizing at any one level of organization. Individual cells proliferate more effectively through gene mutations and genetic recombination, as well as through association with other organisms by forming interactive networks such as communities and consortia. Caldwell (1999) argued that

both cooperation and competition are emergent properties that arise directly through the coincidental geometric recombination of organisms, rather than being mechanisms of evolution in themselves. This is in contrast to Darwinian selection, group selection and community-level selection approaches that envision individual and collective self-interest as mechanisms of evolution. Though the concept of niche construction as proposed by Odling-Smee et al. (1996) is limited by its Darwinian slant, the phenomenon of niche construction itself is significant from the perspective of the proliferation theory. This is because, constructed niches represent a form of community-level information that is heritable as a whole (and without replication), as opposed to representing a heritable source of natural selection as proposed by the niche-construction approach, or even the genetic coding within organisms being considered as the only form of heritable information (based on Mendelian inheritance). In addition, based on the proliferation approach, constructed niches may be thought of as evolutionary units capable of propagating through the activities of niche-constructing organisms, just as DNA propagates through cell divisions or organismal reproduction. This suggests the possibility of proliferation or propagation strategies that arise at multiple levels of organization, that are not restricted to a single recombinational level. Thus, among the several approaches offered to explain evolution, the proliferation approach may have greater economy and generality, being applicable to all life forms including microorganisms and to the emergent properties of physical and chemical objects as well.

## **2.2 Biodegradation as a community-level process**

### **2.2.1 Significance of biodegradation**

There is a growing concern over the widespread and unmitigated use of organic chemicals such as pesticides, solvents, detergents, dyes and plastics as well as the inadvertent release and spill of toxic chemicals into terrestrial and aquatic ecosystems. These chemicals often persist in the environment, degraded very slowly or converted into even less desirable products (Atlas and Bartha, 1998). Though observed concentrations of these chemicals are below the toxicity thresholds for many organisms, long term exposure and bioaccumulation could pose serious environmental and public health concerns (Bouwer, 1989). These concerns have resulted in governmental bans and controls on a number of environmentally unfriendly chemicals, as well as scientific interest in finding measures to prevent contamination and also to remediate the polluted environments using physical, chemical and biological means. Environmental cleanup

through chemical and physical processes is expensive, and in many cases, has unwanted side effects. Hence, the emphasis has shifted to microorganisms as potential agents of remediation. Madsen (1991) indicated that biodegradation is a subset of biotransformations which cause simplification of an organic compound's structure by breaking intramolecular bonds. The sequential events of biodegradation frequently lead to complete mineralization of the compound.

Biodegradation pathways may sometimes involve non-enzymatic reactions such as production of substances that interact with the organochemical resulting in photochemical degradation (Crosby, 1976). Enzymatic reactions are more common and may include acylation (Kaufman and Kearney, 1976; Haggblom, 1990), alkylation (Haggblom, 1990), dealkylation (Yasir et al., 1998; Zablotowicz et al., 1998), hydrolysis (Tchelet et al., 1993; Liu et al., 1995; Pelletier and Harwood, 1998; Poelarends et al., 1998), dehalogenation (Townsend et al., 1997; Bedard et al., 1998; Magli et al., 1998; Stuart and Woods., 1998), aromatic ring cleavage (Crestini and Sermanni, 1995; Daly et al., 1997; Hermann et al., 1997), decarboxylation (Dutton et al., 1995; Phelps and Young, 1997), hydroxylation (Knight et al., 1990; Liu et al., 1995; Johansen et al., 1997; Kadiyala and Spain, 1998), nitroreduction (Le et al., 1998) or condensate-conjugate formation (Kaufman and Kearney, 1976).

Microbially mediated degradation of environmental contaminants has been shown to occur in a wide range of conditions, including aerobic (Coleman et al., 1998; Fava et al., 1998; Fetzner, 1998; Yerushalmi and Guiot, 1998), and anaerobic (Barrio et al., 1990; Knezovich et al., 1990; Knight et al., 1990; Boucquey et al., 1995; Von Wintzingerode et al., 1999), as well as under specialized conditions such as methanogenic (Ramanand et al., 1993; Chang et al., 1998; Fetzner, 1998; Pareek et al., 1998) and sulphate reducing (De Best et al., 1997; Kim et al., 1997; Chang et al., 1998; Klecka et al., 1998) conditions.

### **2.2.2 Biodegradation by isolated cell lines**

Initial research on biodegradation focused mainly on pure cultures or isolated cell lines of microorganisms. A number of studies have indicated the usefulness of single cultures in complete degradation or partial transformation of environmental contaminants in laboratory flask or batch cultures (Roberts et al., 1998) as well as in soil ecosystems (Golovleva et al., 1988). Roberts et al. (1998) isolated pure cultures of bacteria from a field soil that were able to use isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) as sole carbon and nitrogen source. Rabus and Widdel (1996)

demonstrated the growth of a number of denitrifying bacterial isolates on crude oil provided as the only source of organic substrates. These organisms selectively utilized toluene, ethylbenzene and m-xylene from the organic mixture contained in the crude oil.

Though many of the environmental contaminants are mineralizable, they persist in various environments before a population of microorganisms becomes sufficiently large or adapts to degrade these chemicals. Biodegradation can be enhanced by a process of bioaugmentation, during which the environment is inoculated with microorganisms known to readily and efficiently metabolize the contaminants (Goldstein et al., 1985). For example, inoculation of a pyrene-degrading bacterial strain resulted in 55% mineralization of pyrene contained in a soil obtained from an abandoned coal gasification plant within two days, whereas indigenous populations were able to mineralize only 1% of the pyrene during the same period (Grosser et al., 1991). When the same soil was inoculated with a carbazole degrading strain, it resulted in 45% mineralization of soil carbazole within seven days, whereas indigenous microflora did not result in any detectable mineralization during this period. More recently, bioaugmentation with specialized bacterial strains was proposed as a means of enhancing the efficacy of wastewater treatment processes (Fujita and Ike, 1994). Although bioaugmentation is often beneficial, microorganisms having the capacity to degrade organic pollutants in pure cultures can sometimes fail to degrade the same compound when inoculated into natural ecosystems (MacRae and Alexander, 1965; Anderson et al., 1970). Goldstein et al. (1985) suggested that this could be due to the susceptibility of organisms to the toxic substances in nature, predation or their preference to other easily metabolizable compounds. Pipke et al. (1992) also indicated that the survival, growth and activity of the inoculated bacterium will depend on its interactions with the indigenous microflora as well as on the physicochemical characteristics (i.e. temperature, oxygen, available substrates) of the environment. Thus intact microbial communities adapted to survive and propagate as units, which are also more efficient in degradation due to the complementary metabolic capabilities of component individuals, are now more frequently examined as alternative agents of bioremediation.

### **2.2.3 Biodegradation by microbial associations**

Lappin et al. (1985) suggested that synergistic microbial communities are active agents of biodegradation and undue adherence to pure culture techniques can severely jeopardize the chances of demonstrating biodegradation. The better efficiency of biodegradative consortia, as opposed to isolated cell lines is due in part to the more



diverse metabolic capabilities of the consortia in terms of the number and variety of the chemical bonds on which they can act. Sometimes, within degradative communities, cometabolism of a compound by an organism can provide the second species with an easily metabolizable compound (degraded by a thermodynamically favorable reaction). In addition to metabolic interactions, genetic interactions resulting from events such as the horizontal transfer of degradative plasmids among genetically unrelated cell lines within a microbial community also play a major role in biodegradation.

### **2.2.3.1 Metabolic interactions**

The involvement of bacterial associations has been well demonstrated in biodegradation of many chlorinated hydrocarbons (Rajagopal et al., 1984; Madsen and Aamand, 1992; Wolfaardt et al., 1994a, 1994b, 1994c; Bagley and Gossett, 1995). Microbial communities respond to chlorinated hydrocarbons by forming degradative consortia consisting of populations which are unable to degrade the hydrocarbon as isolated cell lines (Caldwell et al., 1997b). For example, a heterogeneous microbial consortium consisting of nine bacterial species and one algal species was able to completely degrade diclofop-methyl, whereas none of the individual members of the consortium were able to use diclofop as sole carbon source (Wolfaardt et al., 1994b). In addition, distinct spatial arrangements consisting of conical and grape-like cell clusters developed within biofilm communities cultivated using diclofop. These spatial features are emergent properties which did not arise during growth of the community on a labile substrate such as TSB (Wolfaardt et al., 1994c). It was also noted that the biofilm mode of growth enhanced diclofop mineralization (Wolfaardt et al., 1994a, 1994b). These results suggest that the spatial positioning of various microorganisms within the community is also likely to influence the extent of interactions among community members, and hence the degradative efficiency of the community. Møller et al. (1998) demonstrated that metabolic interactions between *Acinetobacter* species and *Pseudomonas putida* was responsible for the induction of the meta-pathway promoter in *P. putida* cells within a biofilm community during the degradation of toluene.

Knackmuss (1996) suggested that syntrophic interactions (based on cross-feeding) between microorganisms can lead to complete mineralization of even complex xenobiotic compounds. Feigel and Knackmuss (1993) demonstrated syntrophic interactions between strains of *Hydrogenophaga palleronii* and *Agrobacterium radiobacter* during the degradation of 4-aminobenzenesulfonic acid (4-ABS). Similarly, anaerobic degradation of benzoate, under conditions of high chemical oxygen demand,

required the syntrophic association between hydrogen-producing acetogens such as *Syntrophus buswellii* and hydrogen-consuming methanogens (Li et al., 1996). Perkins et al. (1994) reported the reductive dechlorination of 2,4,6-trichlorophenol (2,4,6-TCP) to 2,4-dichlorophenol and to 4-chlorophenol in mixed methanogenic cultures required syntrophic interactions between acetoclastic methanogens and dehalogenating bacteria, when acetate was used as an electron donor. Auburger and Winter (1996) demonstrated that an obligately anaerobic, benzoate-degrading bacterium, *Syntrophus buswellii* strain GA, was able to degrade benzoate or 3-phenylpropionate to acetate, CO<sub>2</sub> and H<sub>2</sub>, if the partial pressure of hydrogen was maintained sufficiently low by syntrophic co-culture with a strain of *Methanospirillum hungatei* or *Desulfovibrio* sp.. Harmsen et al. (1998) isolated a strain of *Syntrophobacter fumaroxidans* sp. nov. from an anaerobic granular sludge, which was able to oxidize propionate syntrophically in co-culture with the hydrogen- and formate-utilizing *M. hungatei*.

Due to their complexity of structure, many organic compounds are useless as the sole source of carbon and energy for individual microorganisms. These compounds may, however, be degraded in the soil through cometabolism if there is an acceptable source of carbon and energy available for the soil microorganisms (Kilpi, 1980). Madsen (1991) defined cometabolism as the fortuitous modification of one molecule by an enzyme which routinely acts on another molecule (Madsen, 1991). Cometabolic interactions between bacteria have been shown to be responsible for the degradation a number of organic compounds. For example, Shih et al. (1996) demonstrated the cometabolic degradation of trichloroethylene (TCE) by pulse addition of phenol as a co-substrate. Toluene has also been used as co-substrate for the degradation of TCE by soil microbial communities (Mu and Scow, 1994).

Complex metabolic interactions among community members also lead to the sequential degradation of aromatic hydrocarbons (Miller et al., 1988). Jimenez et al. (1991) reported the sequential degradation of linear alkylbenzene sulphonates (LAS) by a four-member bacterial consortium. There was no mineralization of LAS when individual cultures or combinations of two or three members from this consortium were inoculated into the medium suggesting that a given bacterial component may lack the full range of catabolic diversity needed for ultimate oxidation of LAS (Jimenez et al., 1991). Gerritse et al. (1995) demonstrated that combining anaerobic dechlorinating and aerobic methanotrophic enrichment cultures can lead to complete degradation of tetrachloroethene (perchloroethylene, PCE). The anaerobic enrichments led to the

reductive dechlorination of PCE to form less-chlorinated ethenes, which in turn, were cometabolically mineralized by aerobic methanotrophic bacteria.

### 2.2.3.2 Genetic interactions

The catabolic genes are frequently located in extrachromosomal DNA elements called plasmids (Sayler et al., 1990). Fulthorpe and Wyndham (1991) demonstrated the movement of degradative plasmids among genetically unrelated bacterial cell lines when cultivated using 3-chlorobenzoate as a carbon source. Highly mobile catabolic plasmids survive even in conditions under which their parental cells die out, by moving to other community members (Fulthorpe and Wyndham, 1989). Reineke and Knackmuss (1979) demonstrated the transfer of the TOL plasmid from *P. putida* strain MT2 to *Pseudomonas* sp. strain B13 enabling novel strains to be isolated which can degrade various chlorosubstituted benzoates as sole source of carbon and energy. Smets et al. (1993) studied the kinetics of conjugal TOL plasmid transfer from *P. putida* PAW1 to *Pseudomonas aeruginosa* PAA1162 and pointed out that the specific growth rate of the donor strain could influence the conjugal transfer rate. Kinkle et al. (1993) demonstrated the transfer of plasmids pJP4 and r68.45 between populations of bradyrhizobia in non-sterile soil and observed higher transfer rates of plasmid r68.45 in bulk and rhizosphere soils than the previously reported *in vitro* transfer rates.

Some of the well-characterized degradative plasmids include pJP4 involved in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and methylchlorophenoxyacetic acid (MCPA) (Perkins and Lurquin, 1988), pAC27 involved in the catabolism of chlorocatechols (Perkins et al., 1990) and pWR1 involved in chloroaniline and chlorophenol degradation. Transposons, which are highly mobile DNA elements often harboring copies of plasmid encoded genes (Head and Cain, 1991), have also been implicated in the biodegradation of a number of compounds. Transposable elements such as Tn5271 encoding 4-chlorobenzoate metabolism, Tn4371 encoding 4-chlorobiphenyl metabolism, IS931 encoding 2,4,5-trichlorophenoxy acetic acid metabolism and Tn5280 associated with chlorobenzoate metabolism have been well described (Merlin et al., 1997; Tan, 1999).

Glassman and McNicol (1981) suggested that the bacteria from cleaner sites had small plasmids (3 Md) whereas those from a more polluted site carried larger (30 Md) and multiple plasmids. Burton et al. (1982) screened 400 heterotrophic bacterial isolates from the sediments of polluted and unpolluted sites of the Wales river for plasmids and suggested that the predominantly large size of these plasmids may enable conjugal

transfer in this environment. In general, a higher incidence of plasmids is noted in isolates obtained from hydrocarbon-polluted environments (Hada and Sizemore, 1981).

#### **2.2.4 Microbial adaptation to utilization of refractory compounds**

Adaptation of microbial populations to the use of persistent compounds as their primary carbon and energy sources has been well demonstrated (Spain et al., 1980; Spain and Van Veld, 1983; Aelion et al., 1987; Barkay and Pritchard, 1988). Spain and Van Veld (1983) defined adaptation as a change in the microbial community that increases the rate of transformation of a test compound as a result of a prior exposure to the test compound. Using p-nitrophenol degrading communities they demonstrated that the extent of adaptation by a microbial community is not proportional to the pre-exposure concentration of the substrate. Spain et al. (1980) suggested three mechanisms for the adaptation of a microbial community to a new compound. These are: 1) induction or derepression of specific enzymes not present or present at low levels within the community before exposure, 2) genetic changes leading to new metabolic capabilities, and 3) increase in the number of organisms able to catalyze a particular transformation. Van der Meer (1994) suggested that adaptation may occur through the selection of mutants with novel metabolic activities and altered enzyme specificities. Adaptability may also be enhanced by the genetic rearrangements within and among catabolic plasmids involved in degradation, or by the rearrangements between these plasmids and the chromosomes (Wyndham et al., 1988). The genes in plasmids are often not needed for bacterial growth except under certain selective conditions, which enable them to undergo mutations and major structural rearrangements without affecting the cell's central metabolic functions (Chatterjee and Chakrabarty, 1982). Very often, horizontal gene exchange processes such as conjugation, transduction and transformation also play a significant role in the adaptation of microorganisms to xenobiotic compounds (Van der Meer et al., 1992).

### **2.3 Biofilm communities as models for community-level studies**

#### **2.3.1 Microbial organization within biofilms**

Microbial organization within a biofilm community can be thought of as the product of species composition and spatial positioning of individuals within the biofilm matrix. Species composition within a microbial community, also referred to as community structure (Caldwell et al., 1997b), determines the community's overall genetic potential for survival and reproductive success under various environmental

conditions. Spatial positioning allows individuals to interact physiologically and genetically. Community-level spatial arrangements that have a lifetime longer than the life of an individual organism may also be a primary form of heritable coded information in the same sense that the arrangement of the nucleotides in DNA could be thought of as a heritable internal niche construction. However, the positioning of organisms is a heritable external niche construction (Caldwell, 1999; Odling-Smee and Laland, 1999). It also allows the creation of favorable microbial microenvironments within hostile macroenvironments. When a biofilm community is subjected to an environmental perturbation (i.e., an introduction of a pollutant or antimicrobial compound), continued reproductive success may be facilitated by a process of reorganization consisting of changes in composition and spatial arrangement of individuals within the community. Thus, the structural and spatial organization of a biofilm community, and its functional significance, should be a consideration when controlling or enhancing the activities of biofilm communities in industrial or environmental settings.

### **2.3.2 Biofilm formation**

Abiotic events that precede surface colonization include the formation of a preconditioning film composed of proteins, glycoproteins and inorganic nutrients on the attachment surface rapidly after immersion (Marshall et al, 1971; Baier, 1980; Korber et al., 1995). Microbial adhesion to preconditioned surfaces is then facilitated by Van der Waals forces, electrostatic interactions and specific interactions or by a combination of these, depending on the proximity of the organism to the attachment surface (Zottola and Sasahara, 1994). Initial attachment is followed by a consolidation phase during which production of bacterial exopolysaccharides (EPS) results in a more stable attachment by forming organic bridges between the cells and the substratum (Notermans et al., 1991). Subsequently, growth and multiplication of firmly attached primary colonizing organisms lead to the formation of microcolonies. Cells which are loosely attached may detach and these cells together with offspring of other sessile cells may recolonize previously uncolonized surfaces (Korber et al., 1989), thereby extending the spatial boundaries of the biofilm.

Interaction and networking of community members may indeed be the most important determinants of biofilm organization. Interactive behavior complements the functional capabilities of individuals (Wolfaardt et al., 1994b, 1994c; Zottola and Sasahara, 1994; Kinniment et al., 1996), thereby allowing microbial colonization of environments otherwise adverse or unfavorable. The sequence of colonization impacts

the structure of a biofilm community, as the primary colonizers often predispose the surface environment for subsequent colonization by specific organisms. A good example is the succession of events associated with the formation of dental plaque. Specific protein-protein or carbohydrate-protein molecular interactions between primary and secondary colonizers determine the pattern of bacterial colonization and succession within dental plaques (Marsh and Bradshaw, 1995), as well as the patterns of coaggregation whereby specific pairs of bacteria closely interact (Nesbitt et al., 1993; Holmes et al., 1995; Kolenbrander et al., 1995; Bradshaw et al., 1998). The events of coaggregation are highly specific. During the interaction between *Prevotella intermedia* and an *Actinomyces* sp., coaggregation could occur only between specific strains of these two genera (Nesbitt et al., 1993). The enzymatic activities of early colonizers of the tooth surface, such as *Streptococcus oralis*, might also influence ecological succession (Lo and Hughes, 1996). Sometimes, succession is mediated through modifications in the physical characteristics of the attachment surface by early colonizers. For example, development of saucer-shaped cavitations on tooth surfaces by the wearing-away of dentin previously made necrotic by the root lesion microflora, favors the subsequent colonization by aciduric flora (Schupbach et al., 1996). In the rumen ecosystem, the exopolymeric glycocalyx produced by primary colonizers has been shown to facilitate the attachment of secondary colonizers (McAllister et al., 1994). The beneficial role of exopolymers has also been demonstrated in laboratory model systems. The colonization of *Listeria monocytogenes* on glass coverslips was enhanced by mixing the *L. monocytogenes* culture with an exopolymer-producing strain of *Pseudomonas fragi* (Sasahara and Zottola, 1993). Thus, spatial organization within a biofilm community may be influenced by the specific order in which various bacteria colonize the surface.

As biofilm development proceeds, cellular events of attachment and detachment, also referred to as immigration and emigration respectively, continuously alter the biofilm community structure. This process optimizes cell-cell arrangements and interactions in response to changes in the physical or chemical environment, and may be thought of as a process of self-regulation. Notably, pure culture biofilms also often show a high degree of organization (Lawrence et al., 1991; Korber et al., 1994a), suggesting that in addition to these biotic interactions, there are various other non-biotic factors (e.g. characteristics of the attachment surface, physicochemical environment etc.) which control the ultimate architecture, and therefore organization, in biofilms.

The characteristics of the attachment surface are important determinants during biofilm formation. In general, materials that have low free-surface energies and low

negative surface charges favor the formation and stability of biofilms (Teixeira and Oliveira, 1998). However, variation in cell surface hydrophobicity and charge among various strains of microorganisms (Millsap et al., 1997) may actually translate into differences in their ability to attach to surfaces, thereby modulating the final organization of the biofilm community. The organization of the biofilm matrix is also influenced by the nature of the physicochemical environment, including the type, concentration and flux of nutrients or antimicrobial substances, as well as by the gradients of pH, Eh and other factors (Speers and Gilmour, 1985; Wolfaardt et al., 1994b; Brading et al., 1995; Møller et al., 1997).

Overall, microbial organization within biofilm communities may be influenced by a number of factors, including: 1) the numbers and types of colonizing cells, 2) characteristics of the bacterial and colonization surfaces, 3) characteristics of the physicochemical environment, 4) microbial succession and coaggregation, and 5) the nature and extent of microbial interactions. Structural and spatial reorganization in response to these factors enable biofilm communities to continuously optimize their reproductive success under both steady-state and changing environmental conditions. However, the factors which govern the emergence of these community-level properties are poorly understood, and thus require further study. There is consequently an ongoing need to develop or refine techniques which permit the study of these factors in real time within fully hydrated biofilm communities.

#### **2.4 Methods for the cultivation of microbial communities**

Marshall (1994) has suggested that it is important to focus on community-level microbial processes and interactions, including: 1) defining the population dynamics in living communities, 2) defining the physicochemical characteristics of the microbial microenvironment, and 3) understanding the metabolic processes carried out by the individual bacteria. However, the inherent complexity and temporal variability of the physicochemical environment found in natural ecosystems poses many challenges to the study of microbial communities under *in situ* conditions (Caldwell et al., 1997b, 1997c). In the case of biofilm communities, this problem can be resolved by allowing biofilm formation on artificial surfaces such as stainless steel (Lutterbach and Franca, 1997) under *in situ* conditions, and study them *ex situ* under laboratory conditions. In general, monitoring and analysis of these systems are less difficult if the microbial communities are cultivated in the laboratory. Furthermore, interpretation of the structural and functional relationships among microorganisms is somewhat simplified when these

communities are cultivated using model systems under defined environmental conditions. Examples of typical model systems include the chemostat (Angell et al., 1997), flow cell culture (Korber et al., 1994a; Wolfaardt et al., 1994b), dual-dilution continuous culture (DDCC) (Korber et al., 1994b), and the rotatorque annular bioreactor (Characklis et al., 1990; Neu and Lawrence, 1997).

Senior et al. (1976) noted that the chemostat continuous culture systems provide an input and output sufficient to maintain a dynamic network of organisms in a quasi steady-state condition over prolonged time periods. A bidirectionally linked chemostat, commonly referred to as a gradostat has also been used in cultivation of microbial associations (Wimpenny, 1988, 1992). A disadvantage of using chemostats is that those organisms unable to grow at or above the chemostat dilution rate are gradually lost as a consequence of dilution, and those which grow rapidly at the supplied substrate concentration gradually displace others, thereby decreasing the species diversity within the system (Caldwell, 1995).

Caldwell (1995) described the continuous-flow slide culture systems (flow cells) as one of the simplest and most effective methods for studying biofilm communities non-destructively *in situ* and during time courses of colonization. One advantage of flow cell culture is that it permits instantaneous changes in the flux and concentration of growth substrates. Secondly, microbial communities predominantly grow as biofilms, and the biofilm formation on a glass coverslip facilitates the application of various forms of microscopy (Korber et al., 1989; Lawrence et al., 1989) including SCLM for the non-destructive analysis of the fully hydrated communities (Lawrence et al., 1991; Wolfaardt et al., 1994b).

Korber et al. (1994b) used dual-dilution continuous culture (DDCC) in their bacterial motility studies. DDCC allows independent dilution of attached and planktonic bacteria within a flowing system, and is therefore useful in correlating the behavioral or functional characteristics of a bacterium to surface colonization.

The design of rotatorque annular bioreactors generally consists of two concentrically placed cylinders, one fixed and one rotatable, thereby allowing for the study of friction and shear effects on biofilm formation and organization. Characklis et al. (1990) described a rotatorque annular bioreactor system used to study substrate removal, biocide efficiency, etc. (Peyton and Characklis, 1993). Systems such as rotatorque annular bioreactors and chemostats can be fitted with removable coupons for colonization and analysis of biofilms. Angell et al. (1997) used a chemostat to study effect of biofilm formation on the corrosion of removable coupons. The chemostat was



operated at high dilution rate to select for bacteria adapted to sessile growth and was fed by three separate chemostats containing steady-state populations of *Pseudomonas aeruginosa*, *Thiobacillus ferrooxidans* and *Desulfovibrio vulgaris*. However, systems which involve the physical removal of beads (such as DDCC) or coupons for subsequent *ex situ* analysis may potentially introduce shear effects and hence change biofilm organization.

Methods based on gel-diffusion, although frequently used in antibiotic-sensitivity testing and immunodiffusion (Wimpenny et al., 1995), are yet to find application in other areas. These include the auxanography (Beijerinck, 1889), wedge plate technique (Szybalski, 1952), two dimensional steady-state diffusion systems (Caldwell et al., 1973, Caldwell and Hirsch, 1973; Emerson et al., 1994), and microstats (Wolfaardt et al., 1993). Among these, the microstat provides two dimensional concentration gradients developed through diffusion of the substrates through an agar gel matrix, and provides a constant steady-state environment to the organisms colonized on the solid liquid interface resulting in a constant physicochemistry of the microenvironment (Caldwell, 1995).

## **2.5 Methods for the analysis of microbial communities**

The analysis of microbial organization has been limited by the inherent complexity and temporal variability found in natural ecosystems. Even in communities cultivated in laboratory model systems under defined culture conditions, the comprehensive *in situ* analysis and monitoring of structural and spatial organization may be hampered by conceptual and methodological limitations. Despite efforts to develop methodology suitable for the analysis of microbial communities, relatively few approaches (for example, fluorescence *in situ* hybridization and fluorescent antibody techniques) are useful in conjunction with epifluorescence or scanning confocal laser microscopy (SCLM) for the direct analysis of both structural and spatial organization within intact, fully hydrated biofilm communities. New approaches include the insertion of various GFP (Green Fluorescent Protein)-variant genes into specific biofilm members (Møller et al., 1998; Stretton et al., 1998), thus permitting the direct analysis of the abundance and distribution of these organisms within multi-species systems over time. Other techniques involving fluorescent molecular probes such as fluor-conjugated lectins and dextrans are useful to elucidate the physicochemical heterogeneity (charge distribution, diffusion characteristics, exopolymer chemistry etc.) within intact biofilm communities, however these techniques are limited in their ability to delineate the

structural organization within these communities. Application of most other techniques requires that biofilms be removed from their substratum and disrupted, resulting in the destruction of spatial cellular arrangements (architecture) within the community being analyzed. However, these techniques are useful to study planktonic communities or biofilm communities if the analysis of spatial organization is not of interest.

## **2.5.1 Analysis of intact biofilm communities**

### **2.5.1.1 Analysis of overall biofilm architecture**

The initial stages of biofilm development can be examined by phase contrast microscopy (Caldwell and Lawrence., 1986; O'Toole and Kolter, 1998). Caldwell and Lawrence (1986) used high magnification phase microscopy (100X) to analyze the growth kinetics of *Pseudomonas fluorescens* microcolonies. Cell monolayers can readily be digitized and analyzed using phase-contrast microscopy in conjunction with digital image analysis, however, this approach is not suitable for analysis of thicker or complex biofilms (Caldwell and Lawrence, 1986). Images of high contrast and enhanced depth of field offered by low magnification darkfield microscopy also favors the analysis of adhesion and biofilm formation (Korber et al., 1989; Lawrence et al., 1989). Palmer and Caldwell (1995) used a combination of microscopic techniques, including low-magnification darkfield microscopy, to analyze the regrowth of plaque biofilms developed in flow cells. However, a drawback of darkfield microscopy is that each cell is represented as a point light source, and thus cells appear larger than they are. SCLM is often the most useful technique for the temporal analysis of the three-dimensional architecture of a biofilm community in its fully hydrated state, as it provides optical thin sections of high contrast and resolution. A detailed discussion of the hardware, setup, operation of SCLM as well as application of image analysis techniques in conjunction with laser microscopy can be found in a recent review by Lawrence et al. (1997).

Spatial architecture within intact, fully hydrated biofilm communities can readily be analyzed by fluorescence exclusion in conjunction with SCLM, a technique in which the biofilm is negatively stained by applying fluorescein to the bulk liquid solution (Caldwell et al., 1992a). This technique is based on the principle that the brightness of fluorescein is proportional to the pH of the liquid phase, and at an ambient pH greater than that of the intracellular pH, the cells appear as dark objects in a bright background. During most biofilm studies, a higher extracellular fluorescein concentration results in the bacteria appearing darker than the brighter background. The fully hydrated nature of EPS similarly results in an almost equal fluorescein concentration within EPS when

compared to the bulk fluid phase, thus EPS does not usually interfere with negative staining of bacteria. Negative staining is especially useful to study the initial stages of biofilm formation, although this technique can also be used to study subsequent attachment, recolonization, emigration and immigration (Caldwell et al., 1992a; Wolfaardt et al., 1994b).

The non-toxic nature of fluorescein also permits real time analysis of temporal changes in the spatial organization of the same biofilm community, without having to sacrifice a flow cell channel following each observation. Other compounds, such as resazurin and fluor-dextran conjugates, may also be useful for negatively staining biofilm communities. Negative staining in conjunction with SCLM, may also be used to provide data on biofilm area, thickness and biomass, all of which are necessary for understanding the effects which the physicochemical environment and biological interactions have on the formation and organization within biofilm communities. Positive staining has also been extensively applied for the study of pure culture biofilms and biofilm communities. Acridine orange, Nile red, Texas red isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) can be used for this purpose (Caldwell et al., 1992b; Hood and Zottola, 1997; Tang and Cooney, 1998).

#### **2.5.1.2 Analysis of structural and spatial organization**

In order to study the effect of various physical, chemical and biological factors on the structural and spatial organization of biofilm communities, it is necessary to determine the relative abundance of single species or groups of bacteria, and also track the fate of specific individuals over time. While negative staining in conjunction with SCLM serves as a non-destructive approach for the analysis of overall biofilm architecture, structural and spatial organization (relative abundance and positioning of various bacteria) within intact biofilm communities can be best achieved by using a combination of either epifluorescence or SCLM and one or more fluorescently-labeled oligonucleotide probes or antibodies targeted against specific community members.

Oligonucleotide probes target gene sequences usually within conserved regions of 16S or 23S rRNA of individual species or of broader taxonomic groups within the biofilm community (Amann et al., 1992; Manz et al., 1995; Mobarry et al., 1996; Rothmund et al., 1996; Poulsen et al., 1997). Fluorescence *in situ* hybridization (FISH), when applied in conjunction with SCLM, has been shown to provide valuable data on the three-dimensional distribution of specific organisms within biofilm communities (Møller et al., 1996). Furthermore, FISH provides meaningful inferences of

the structure-function relationships within the microbial community when combined with microelectrode analysis (Lewandowski et al., 1993; De Beer et al., 1994) of the physicochemical environment or with indicators of cell viability, growth or gene expression (Møller et al., 1996, 1998; Schramm et al., 1997). Schramm et al. (1997) used microelectrodes for O<sub>2</sub> and NO<sub>2</sub>/NO<sub>3</sub> in conjunction with fluor-conjugated 16S rRNA-targeted probes to determine the activity and spatial distribution of nitrifiers within a trickling filter biofilm community. Using these techniques, they showed that closer spatial positioning of *Nitrosomonas* spp. and *Nitrobacter* spp. within the trickling filter biofilm community facilitated the complete conversion of ammonia into nitrate. Møller et al. (1996) correlated the rRNA content and size of *P. putida* cells within a toluene-degrading biofilter community to rates of toluene degradation. A 16S rRNA-targeting probe was used to detect *P. putida* cells. Although the application of FISH does not destroy biofilm architecture, it results in cell death precluding the possibility of repeated analysis of the same biofilm.

Fluorescent antibody probes have also proven valuable for the identification of target organisms from within complex microbial communities. Stewart et al. (1997) used fluorescently-labeled monoclonal antibodies to examine the spatial distribution of *Klebsiella pneumoniae* and *P. aeruginosa* within laboratory-grown binary biofilms. Alternatively, immunogold labeling and fluorescein immunolabeling were used to visualize *Legionella pneumophila* within aquatic biofilms developed on glass and polystyrene surfaces (Roberts and Keevil, 1992). Interference contrast microscopy was used in an episcopic mode for simultaneous visualization of the immunogold-labeled *L. pneumophila* as well as total biofilm.

More recently, bacteria tagged with GFP as a conservative cellular marker have seen application in biofilm studies. Organisms genetically tagged in this way may be identified by their green fluorescence (~514 nm) using SCLM or epifluorescence microscopy. This approach has seen usage for the study of structural and spatial organization within biofilm communities (Stretton et al., 1998). The GFP gene, originally derived from the jellyfish *Aequoria victoria*, can easily be introduced into gram-negative target strains using plasmid vectors (Møller et al., 1998). Cloning a gene encoding the GFP behind a promoter responsible for the expression of a specific functional gene can then be used to monitor *in situ* gene expression (Chalfie et al., 1994). Møller et al. (1998) used this approach in conjunction with 16S rRNA oligonucleotide probes to examine *tol* gene expression by specific members of a mixed-species biofilm community. Tombolini et al. (1997) demonstrated that GFP expression was

independent of the growth stage of the bacterial strain used, and was detectable even under nutrient-limiting conditions. However, the formation of fluorophore within the molecular structure of GFP, and hence its fluorescence, requires molecular oxygen (Heim et al., 1994). Thus, GFP may not be suitable for use in oxygen-limited or anaerobic systems. The main advantage of GFP is that it allows direct visualization of specific organisms without involving long staining procedures as required in FISH or fluorescent antibody techniques. It is also noteworthy that a number of GFP variants are now available ([www.clontech.com](http://www.clontech.com)) that have excitation or emission wavelengths different from that of the wild-type gene. Used in conjunction with a multi-line laser excitation source, multiple organisms may simultaneously be tracked within a complex biofilm system.

Fluorescent gram stains have been used as a non-specific probe to determine the overall spatial distribution of gram-negative and gram-positive organisms within biofilm communities. For example, Wolfaardt et al. (1998) applied a fluorescent gram stain to detect regions within a diclofop-degrading biofilm community associated with a *Bacillus coagulans* strain. However, these and other fluorescently-labeled probes often result in the death of bound cells and hence are not suitable for continuous monitoring of the same biofilm community.

In addition to the species-specific or group-specific probes described above, fluorescent probes are also available for the determination of the physicochemical heterogeneity of biofilms in terms of exopolymer chemistry (fluor-conjugated lectins), charge distribution (fluor-conjugated anionic, cationic and neutral dextrans), diffusion (fluor-conjugated size-fractionated dextrans) and hydrogen ion distribution (carboxyfluorescein) (Caldwell et al., 1992b; Lawrence et al., 1994; Møller et al., 1997; Wolfaardt et al., 1998). Fluorescent probes are also available for determination of metabolic activity or viability of the biofilm cells based on the cytoplasmic redox potential (resorufin), electron transport chain activity (cyanoditolyl-tetrazolium chloride) and cell membrane integrity (propidium iodide, ethidium bromide and BacLight™ viability stain). More recently, Korber et al. (1996) used the ability of bacteria to undergo plasmolysis as a physical indicator of viability. When subjected to an osmotic shock, only viable cells containing an intact semi-permeable membrane became plasmolyzed. A detailed discussion of the utility and limitations of these and other methods can be found in the recent reviews by Caldwell et al. (1997b, 1997c) and Lawrence et al. (1997). Overall, the information derived from application of fluorescent molecular probes will facilitate the elucidation of structure-function relationships within

biofilm communities. It should be noted that such approaches are best applied in combination with other analytical methods and also that a number of probes have not been applied under community culture (i.e., BacLight™ viability stain).

Although scanning and transmission electron microscopy have been frequently applied in biofilm research, specimen preparation often results in shrinkage and dehydration artifacts that alter the biofilm spatial organization. For a more detailed discussion of various microscope systems utilized for biofilm research, including environmental scanning electron microscopy, episcopic differential interference contrast microscopy, Hoffman modulation contrast microscopy and atomic force microscopy, the reader is referred to a recent review by Surman et al. (1996).

### **2.5.2 Analysis of planktonic communities and biofilms after disruption**

The structural organization of planktonic as well as sessile communities (after removal from their substratum) can be elucidated by either culture-based or DNA-based methods. Traditional plating techniques are limited by the unknown species composition of natural systems that precludes the design of a plating medium capable of detecting all or most of the diversity present. Thus, the species composition of a community delineated in this manner represents the "culturable structure" of the community. However, there are several DNA-based techniques available for the determination of structure and diversity of microbial communities obtained from various environments. These techniques can also be applied to biofilm communities when the analysis of spatial organization is not of interest. For example, quantitative fluorescent oligonucleotide hybridization of biofilm cells smeared on glass slides can be performed and subsequently analyzed using epifluorescence or laser microscopy (Møller et al., 1996), allowing the simultaneous detection and quantification of a specific organism within a biofilm community.

The presence of a specific organism, or a group of bacteria, within an environmental sample can also be detected by fixation of the DNA or RNA extracted from the sample to nylon or nitrocellulose membranes, followed by hybridization to an oligonucleotide probe containing a sequence complementary to a known target sequence. Probes are commonly labeled with <sup>32</sup>P-nucleotide analogs as well as biotin or digoxigenin containing analogs (Raskin et al., 1994; Wang and Wang, 1995). Detection of hybridized probes usually involves colorimetric, bioluminescent or chemiluminescent reactions. Fluorescently-labeled probes can be directly detected using epifluorescence microscopy.

Reverse sample genome probing (RSGP) has also been used to detect the presence of specific bacteria in environmental samples, and involves spotting DNA obtained from a target organism onto a membrane filter, followed by the hybridization of labeled environmental bacteria. Shen et al. (1998) applied this technique to detect the enrichment of hydrocarbon-degrading bacteria in soil following the addition of easily degradable and refractory hydrocarbons.

The polymerization chain reaction (PCR) can be applied to detect and amplify specific sequences present at a very low level within an environmental sample (Schneegurt and Kulpa., 1998). Nested PCR entails a preliminary amplification step to amplify a larger gene sequence from the sample, and subsequent PCR reactions on the preliminary PCR products to amplify more specific sequences. For example, Hastings et al. (1998) were able to successfully detect *Nitrosospira* 16S rDNA in samples derived from a eutrophic freshwater lake using nested PCR. Most probable number-PCR (MPN-PCR) allows the quantification of specific gene sequences present in an environmental sample by serially diluting the whole-cell DNA or RNA extracts prior to the PCR reaction. Mantynen et al. (1997) applied this technique for the detection and enumeration of enterotoxin C-producing *Staphylococcus aureus* NCTC 10655 from fresh cheese. This method was highly sensitive, and *S. aureus* numbers as low as 20 CFU (colony forming units) g<sup>-1</sup> could be detected. Degrange et al. (1998) also applied this method to quantify *Nitrobacter* spp. in coniferous forest soils.

Restriction digestion of products obtained from PCR of repetitive genome sequences (REP-PCR) (Del Vecchio et al., 1995; Sarand et al., 1998) or randomly amplified polymorphic DNA (RAPD) provides organism-specific restriction patterns which can be used to determine community structure (Segonds et al., 1997; Morea et al., 1998). Density-gradient gel electrophoresis (DGGE) of PCR amplified rDNA products can also be applied to resolve differences in community structure (Gillan et al., 1998; Santegoeds et al., 1998). Entire rDNA sequences can be amplified using PCR and sequenced to identify organisms within mixed microbial populations. This wide array of PCR techniques has yet to see extensive usage for community-level studies.

Apart from nucleic acid sequence variations, differences in the phospholipid fatty acid (PLFA) content of microorganisms has seen use for the analysis of community structure. For example, Guezennec et al. (1998) used signature polar fatty acid profiles of biofilm communities obtained from hydrothermal vent areas to detect the presence of both sulfate-reducing and sulfur-oxidizing bacteria. Neef et al. (1996) also applied fatty acid analysis to detect the presence of *Paracoccus* spp. within a

denitrifying sand filter used in wastewater treatment. Scholz and Boon (1993) used PLFA to analyze the effect of various light regimes on the structure of biofilm communities developing on wooden slides submerged in billabongs. Although fatty acid profiles are useful in comparing microbial communities (Haack et al., 1994), the potential of this approach for the elucidation of structural organization within a biofilm community may be limited by culture-dependent differences in the fatty acid content of community members.

### **2.5.3 Monitoring organizational relationships of functional significance**

Structural and spatial organization within a biofilm community are best interpreted when the organizational relationships can be correlated with a specific function of the community. As previously indicated, this can be achieved via a combination of structural gene probes to detect specific members, microelectrode analysis of the ambient environment, or indicators of gene expression, such as GFP or bioluminescence. These methods used in combination often reveal the functional role of specific structural and spatial patterns detected within biofilm communities (Møller et al., 1997, 1998; Schramm et al., 1997).

Determination of community-level sole carbon source (SCS) utilization patterns, in combination with multivariate data analysis, is a relatively new approach to elucidate overall functional capabilities of microbial communities (Haack et al., 1995; Degens and Harris, 1997; Staddon et al., 1997). Staddon et al. (1997) and Haack et al. (1995) used principle component analysis (PCA) to resolve sole-carbon utilization (functional) differences between soil microbial communities. Degens and Harris (1997) similarly used PCA to identify carbon substrates that differentiated five soil communities based on substrate-induced respiration profiles. The extent to which specific substrates were oxidized resulted in SCS utilization patterns allowing for the discrimination of various communities (Haack et al., 1995). Thus this approach may also help to elucidate organizational relationships of functional significance within biofilm communities.

## **2.6 Conclusions**

Microbial communities have been shown to be the causative agents of various ecological processes of economic significance. An understanding of the formation and organization of microbial communities is necessary in order to enhance community-mediated beneficial processes as well as to control their deleterious activities. Evolutionary theories based on selection that are normally used to explain the nature of



existence of higher animals and plants invariably fail to explain the existence of microorganisms as associative networks. The proliferation approach, based on coincidental recombination events that result in the emergence of biological properties at various spatial scales of organization (molecules, plasmids, cells, communities, etc.) during the evolution of all forms of life (including microorganisms) has been discussed as a more parsimonious alternative. For testing the existence of community-level proliferation strategies (as proposed in the proliferation theory), degradative communities were chosen as model systems. This was due to the well established role of microbial communities in biodegradation. Biofilm communities were preferred, because they have well defined structure and architecture, which are amenable to analysis using scanning confocal laser microscopy and the many other analytical tools discussed in this section.

### **3 RESULTS AND DISCUSSION**

#### **3.1 Functional and structural responses of a degradative microbial community to substrates with varying degrees of complexity in chemical structure**

##### **3.1.1 Introduction**

Substantial quantities of chlorinated hydrocarbons are released into the environment. Many of them are refractory, often persist in the environment, and therefore have undesired environmental impacts. There is a growing interest in the application of natural microbial communities for the attenuation of these contaminants. However, the process of bioremediation has met with only limited success due to the xenobiotic nature of many of the compounds involved (Goldstein et al., 1985). The refractoriness of these synthetic molecules has often been attributed to unusual substitutions (with chlorine and other halogens), unusual bonds and bond sequences such as tertiary and quaternary carbon atoms, highly condensed aromatic rings, excessive molecular size, excessive toxicity of the parent molecule or its metabolic products, and unavailability due to adsorption or insolubility (Alexander and Aleem, 1961; Knackmuss, 1984, Haggblom, 1990; Chaudhry and Chapalamadugu, 1991; Mohn and Tiedje, 1992). The reaction kinetics of the carbon halogen bond are thermodynamically unfavorable and hence pose special challenges to biodegradation (Atlas and Bartha, 1998). Although some exceptions exist, chlorines in 1-2 ring compounds are typically lost after ring cleavage, whereas hydrolytic, oxygenolytic, and reductive dechlorination reactions are involved in the dechlorination of highly chlorinated, and more complex hydrocarbons. When a xenobiotic compound is introduced to a natural microbial community, adaptive changes in community structure and functional capability may be required in order to tolerate or degrade the compound. The nature of such a response is influenced by the chemical structure of the compound and the culture conditions.

Although widely used in recent years, interpretation and ecological relevance of community-level carbon source utilization patterns are still in a developmental phase. Nevertheless, this approach may help to explain the evolution of functional relationships among communities, and thereby contribute to our current understanding of the complex interactions that form the basis of co-existence in a communal context. The objective of this study was to determine the functional and structural responses of a

degradative microbial community to substrates of varying complexity and chlorination, as well as to evaluate methods for the characterization of microbial communities. The functional evolution of the community over time was also investigated. As suggested previously, testing the existence of community-level proliferation strategies would involve testing the stability of community-networks when subjected to environmental transitions and the role of external organismal recombination in enhancing the growth of microbial communities during these transitions. Thus, community-level structural and functional responses to growth on substrates of varying complexity and chlorination as determined from the present study may subsequently be used to formulate strategies for substrate transition experiments aimed at testing the existence of community-level synergisms and hence the utility of proliferation theory (Caldwell and Costerton, 1996; Caldwell et al., 1997b; Caldwell, 1999).

### **3.1.2 Materials and Methods**

#### **3.1.2.1 Degradative community and culture conditions**

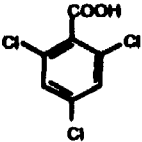
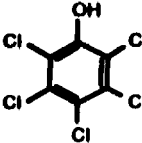
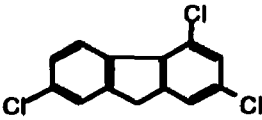
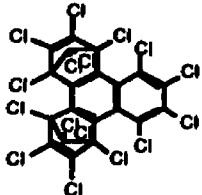
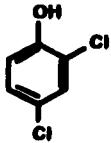
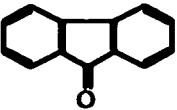
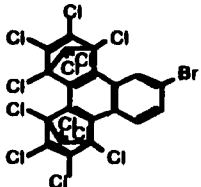
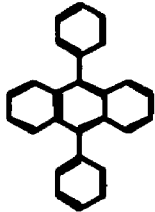
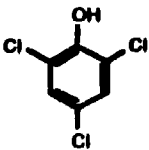
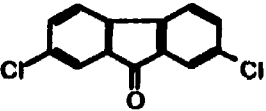
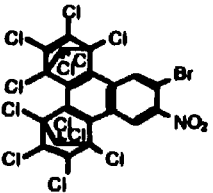
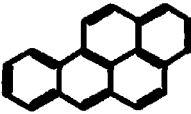
A microbial community (hereafter referred to as 2,4,6-T community) was enriched from oil-contaminated soils collected at an abandoned petroleum refinery site (Northern Petroleum Refinery, Kamsack, Saskatchewan, Canada) (Møller et al., 1997), and maintained in a multi-channel flow cell (Wolfaardt et al., 1994b). The flow cell channels (channel dimensions: 1 mm deep, 3 mm wide, and 40 mm long) were continuously irrigated with a minimal salts (M-salts) medium supplemented with 2,4,6-trichlorobenzoic acid (2,4,6-T) at a concentration of 25 mg l<sup>-1</sup> as sole carbon source at a laminar flow velocity of 0.2 mm s<sup>-1</sup>. M-salts consisted of equal volumes of Solution A and Solution B. Solution A consisted of (per liter) 2 g NaCl, 1 g NH<sub>4</sub>Cl, 0.12 g MgSO<sub>4</sub>·7H<sub>2</sub>O and 1 ml of a trace element solution containing (per liter) 4 g EDTA, 1.5 g CaCl<sub>2</sub>, 1 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.35g MnSO<sub>4</sub>·2H<sub>2</sub>O and 0.5g NaMoO<sub>4</sub>·2H<sub>2</sub>O. Solution B consisted of (per liter) 4.24 g Na<sub>2</sub>HPO<sub>4</sub> and 2.7 g KH<sub>2</sub>PO<sub>4</sub>. Solution A and B were autoclaved separately, cooled, then mixed, and the required quantity of carbon source(s) were added aseptically. After being maintained under these conditions for 5 months, the community was used to inoculate both batch and continuous flow culture systems (flow cells).

### 3.1.2.2 Screening for refractory substrates

Initially, thirty hydrocarbon substrates with varying degrees of chlorination and complexity were tested for utilization by the 2,4,6-T community in batch enrichments. This screening involved inoculation of 1 ml of flow cell effluent (2,4,6-T community) in 50 ml of M-salts medium supplemented with a test substrate (see below) at a concentration of 50 mg l<sup>-1</sup>. Flasks were incubated at 23±2°C on a rotary shaker. These enrichments were plated on 10% tryptic soy agar (TSA) at daily intervals for a period of 13 days, and the maximum cell number supported by each compound was determined. The compounds were then ranked based on a descending order of maximum cell numbers, and the 13 compounds which supported the least growth, as well as glucose and 2,4,6-T were used for further studies. The selected compounds were: i) compounds with no ring structure: trichloroethylene, 1,1,1,2-tetrachloroethane, ii) compounds with a single ring: 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol (PCP), iii) three-ring compounds: 9-fluorenone, 2,7-dichloro-9-fluorenone, 2,4,7-trichlorofluorenone, iv) four-ring compounds: 2-bromonaphthalene-bis (hexachlorocyclopentadiene) adduct, 2-bromo-3-nitronaphthalene-bis (hexachlorocyclopentadiene) adduct, 1,2,3,4-tetrachloronaphthalene-bis (hexachlorocyclopentadiene) adduct, and v) five-ring compounds: diphenylanthracene, benzo(a)pyrene (Figure 3.1.1). All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) except the biphenyls which were purchased from Ultra Scientific (Hope, RI). All subsequent batch enrichments were incubated at 23±2°C on a rotary shaker and plating for cell counts was done at 3-day intervals over a 12 day period. In flow cell cultures, the conditions were as described for the long-term maintenance of the 2,4,6-T community. Flow cells were incubated using each of the 15 substrates as sole carbon source for 2 weeks before analysis.

### 3.1.2.3 Microscopy

Scanning Confocal Laser Microscopy (SCLM) was used to determine biofilm thickness and percent biofilm coverage of the 2,4,6-T community when cultivated in flow cells with the different compounds provided as the sole carbon source. Biofilms negatively stained with 0.1% w/v fluorescein (Sigma Chemical Co., St. Louis, Mo.), were scanned and digitized by using Bio-Rad MRC-600 LaserSharp fluorescence Scanning Confocal Laser System (Bio-Rad Microscience, Mississauga, ON) mounted on a Nikon FXA microscope, under 60X, 1.4 NA oil immersion (Nikon Corporation, Chiyoda-ku, Tokyo) objective. An argon laser operated at 25mW power and 1% beam

<p>glucose</p> <p><math>C_6H_{12}O_6</math></p> <p><b>N</b></p>	<p>2,4,6 - trichloro benzoic acid</p>  <p><b>O</b></p>	<p>pentachloro phenol</p>  <p><b>C</b></p>	<p>2,4,7 - trichlorofluorene</p>  <p><b>K</b></p>	<p>1,2,3,4-tetrachloronaphthalene (bis hexachlorocyclopentadiene) adduct</p>  <p><b>F</b></p>
<p>tetrachloroethylene</p> <p><math>C_2H_2Cl_4</math></p> <p><b>I</b></p>	<p>2,4 - dichloro phenol</p>  <p><b>D</b></p>	<p>9 - fluorenone</p>  <p><b>B</b></p>	<p>2-bromonaphthalene (bis hexachlorocyclopentadiene) adduct</p>  <p><b>G</b></p>	<p>diphenyl anthracene</p>  <p><b>H</b></p>
<p>trichloroethylene</p> <p><math>C_2HCl_3</math></p> <p><b>J</b></p>	<p>2,4,6 - trichloro phenol</p>  <p><b>A</b></p>	<p>2, 7 - dichloro 9 - fluorenone</p>  <p><b>L</b></p>	<p>2-bromo-3-nitronaphthalene (bis hexachlorocyclopentadiene) adduct</p>  <p><b>E</b></p>	<p>benzo(a)pyrene</p>  <p><b>M</b></p>

**Figure 3.1.1.** Chemical structures of the hydrocarbons used as carbon sources for the degradative microbial community. The compounds are shown with increases in ring number from left to right.

transmission (maximum excitation at 488 and 514 nm) was used in the excitation of the fluorophore. The images were collected using a Kalman filter. A Northgate 80846 host computer was used to display the images. The focusing and stage control were computerized. The images were acquired and saved in an optical disc using Corel-940 optical drive (WORM). The primary images were analyzed using the Bio-Rad software in the Northgate host computer. The *BacLight*<sup>TM</sup> gram stain (Molecular Probes Inc., Eugene, OR) was prepared as per the manufacturer's protocol, and used to measure the ratio of gram positive to gram negative bacteria within biofilm communities. Protistan predators feeding on the biofilms were also enumerated using light microscopy. The average number of predators counted in 10 random fields was used to assign a predation index (as indicated in parentheses): no predators (-); 1 predator/field (+); > 1 and ≤ 3 predators/field (++); > 3 and ≤ 10 predators/field (+++), and >10 predators/field (++++). The field areas were 13,500 μm<sup>2</sup> (150x90 μm).

#### **3.1.2.4 Functional fingerprinting of communities**

Whole-community carbon source utilization profiles were determined for the communities grown on each of the 15 different carbon sources in batch or flow cell cultures using Biolog GN plates (Biolog Inc., Hayward, CA). Ten ml aliquots of culture suspension, obtained from either 12-day old batch enrichments or effluent collected from flow cell communities, were washed twice and resuspended in M-salts medium. Washed cell suspensions were then used to inoculate the 96 wells (150 μl per well) of the Biolog plates in duplicate. Plates were incubated 23±2°C for 24 hours before image analysis was used to quantify color development in the wells. The image analysis procedure involved acquiring images using a Sanyo Camcorder model VM - H100 (Sanyo Canada Inc., Toronto, ON) before analysis of the intensity of color development using Scion Image<sup>TM</sup> (National Institutes of Health, Bethesda, MD) software. The images were converted into gray scale, and the pixel values (ranging from 0 = white to 255 = black) for positive wells were defined by using the value of the control well as threshold. Wells with mean gray levels greater than this threshold were interpreted as positive. Systat software (Systat Inc., Evanston, IL) was used to cluster the metabolic fingerprints of communities based on their metabolic relatedness using an average linkage method. Principal Component Analysis (PCA) was performed using Minitab software (Minitab Inc., State College, PA).

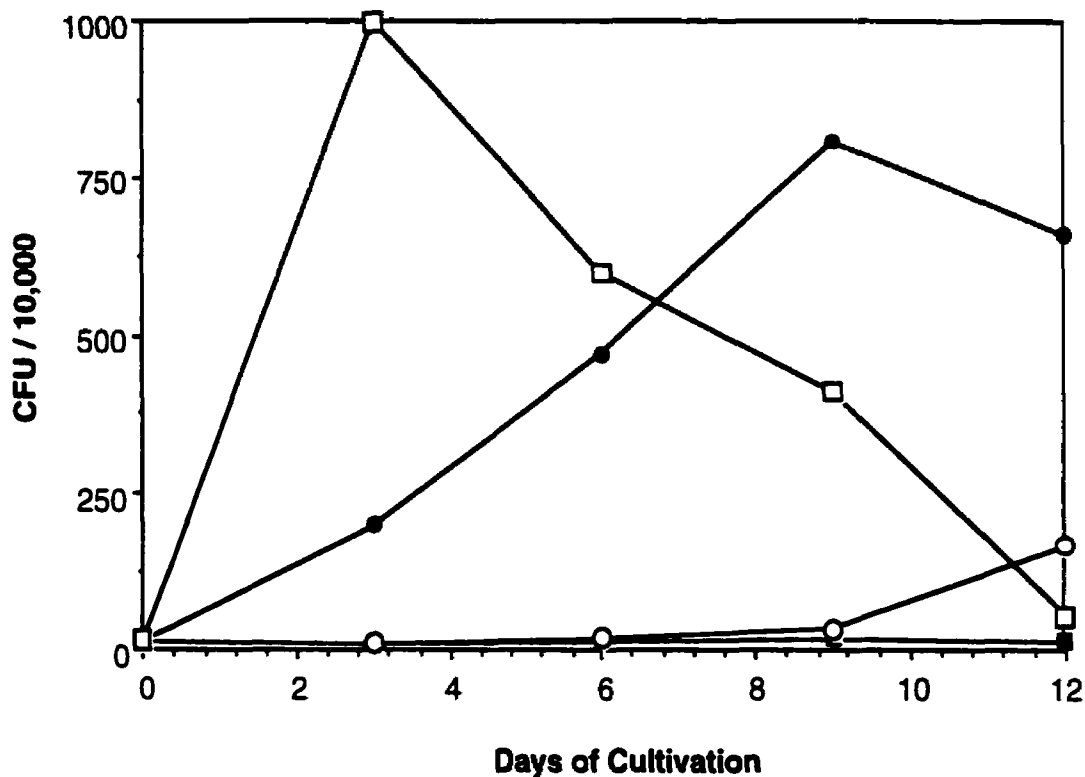
### 3.1.3 Results

#### 3.1.3.1 Effect of substrate chemistry on community structure

Significant differences in community structure (i.e., the ratio of gram positive to negative cells within biofilms), architecture and function were noted when the 2,4,6-T community was cultivated using hydrocarbon substrates of varying degrees of chlorination and structural complexity. The number of colony forming units per milliliter (CFU ml<sup>-1</sup>) revealed differences among the batch cultivations in terms of the acclimation periods required before the onset of exponential growth on the test substrates. To demonstrate this, the growth curves on four substrates are shown in Figure 3.1.2. Little or no acclimation period was required for the community to grow on 2,4,6-T, whereas growth on 2,4,7-trichlorofluorenone was relatively slower as indicated by the slope of the growth curve for this compound. The 2-bromo-3-nitronaphthalene (bis hexachlorocyclopentadiene) adduct required an acclimation period of ~9 days, whereas a slight decrease in cell numbers was observed on PCP (Figure 3.1.2) during the 12 day incubation.

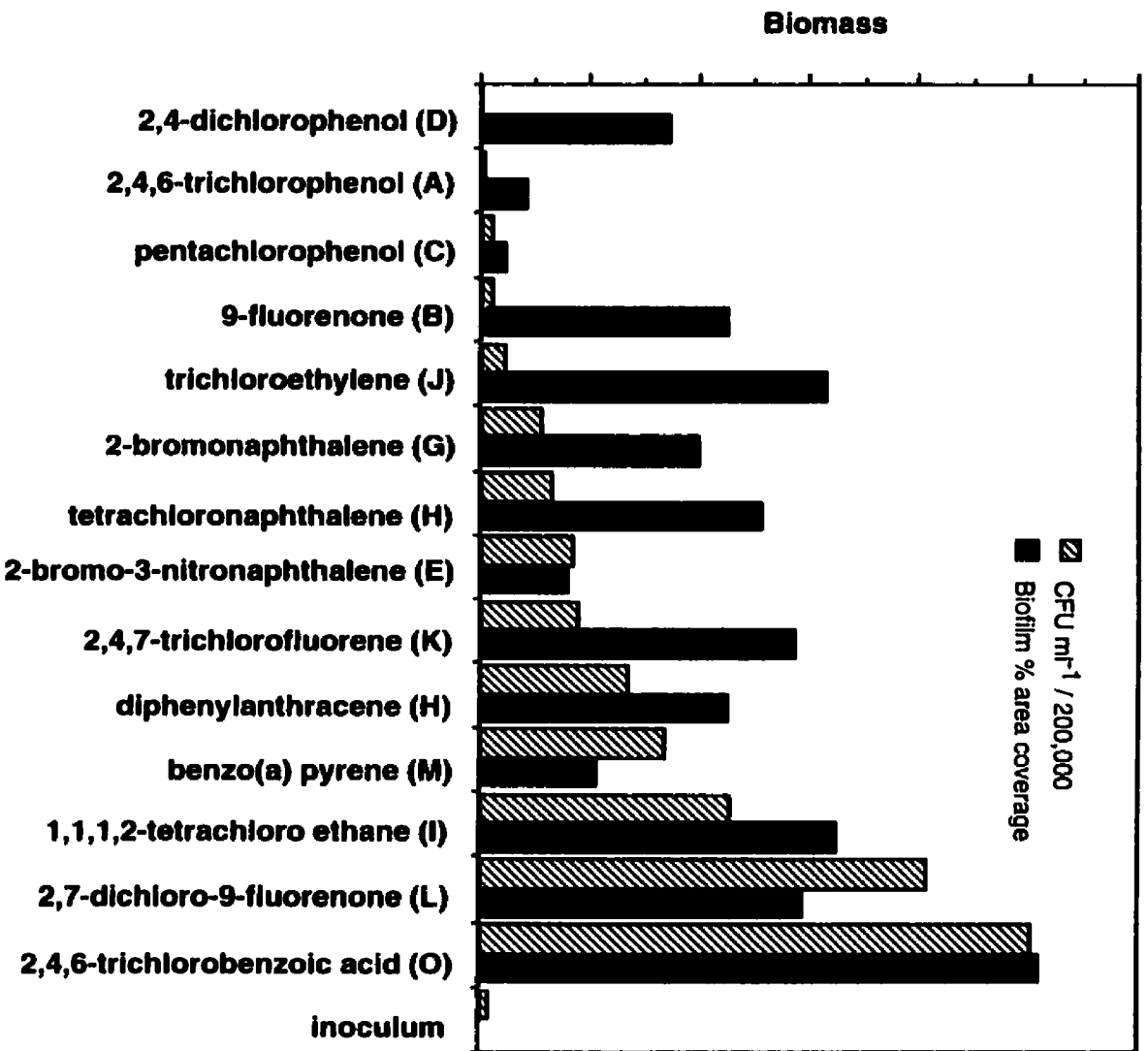
There was no significant correlation ( $r^2 = 0.47$ ) between planktonic (batch) or sessile (flow cell) growth by the 2,4,6-T community on the different substrates (Figure 3.1.3). In Figure 3.1.3, the substrates are ranked on the x-axis in ascending order of measured growth in batch (number of CFU), and in most instances the extent of biofilm development on the respective substrates did not follow the same order. Also, using PCA, neither the degree of chlorination nor complexity of the substrate could be correlated with either the biomass in planktonic enrichments or the thickness and percent area coverage of biofilms. There was no growth in batch cultures and relatively little biofilm formation when phenols were provided as the sole carbon source (Figures 3.1.3 and 3.1.4). 2,4,6-T resulted in the highest amount of biomass under both batch and flowing conditions. When the 15 compounds were ranked in ascending order of measured biofilm development and compared with the biofilm thickness:biofilm area ratio in flow cells, an inverse logarithmic relationship ( $r^2 = 0.88$ ) was observed (Figure 3.1.5).

There was a positive correlation ( $r^2 = 0.75$ ) between the degree of chlorination and the gram ratio (the numbers of gram positive: numbers of gram negative) during sessile growth. Highest ratios (> 5:1) were obtained for some of the highly chlorinated compounds such as pentachlorophenol and hexachlorocyclopentadienes ( $\geq 12$  chlorines per molecule). Biofilms cultivated on phenols did not support any predators whereas growth on 2,4,7-trichlorofluorenone and trichloroethylene supported maximum number

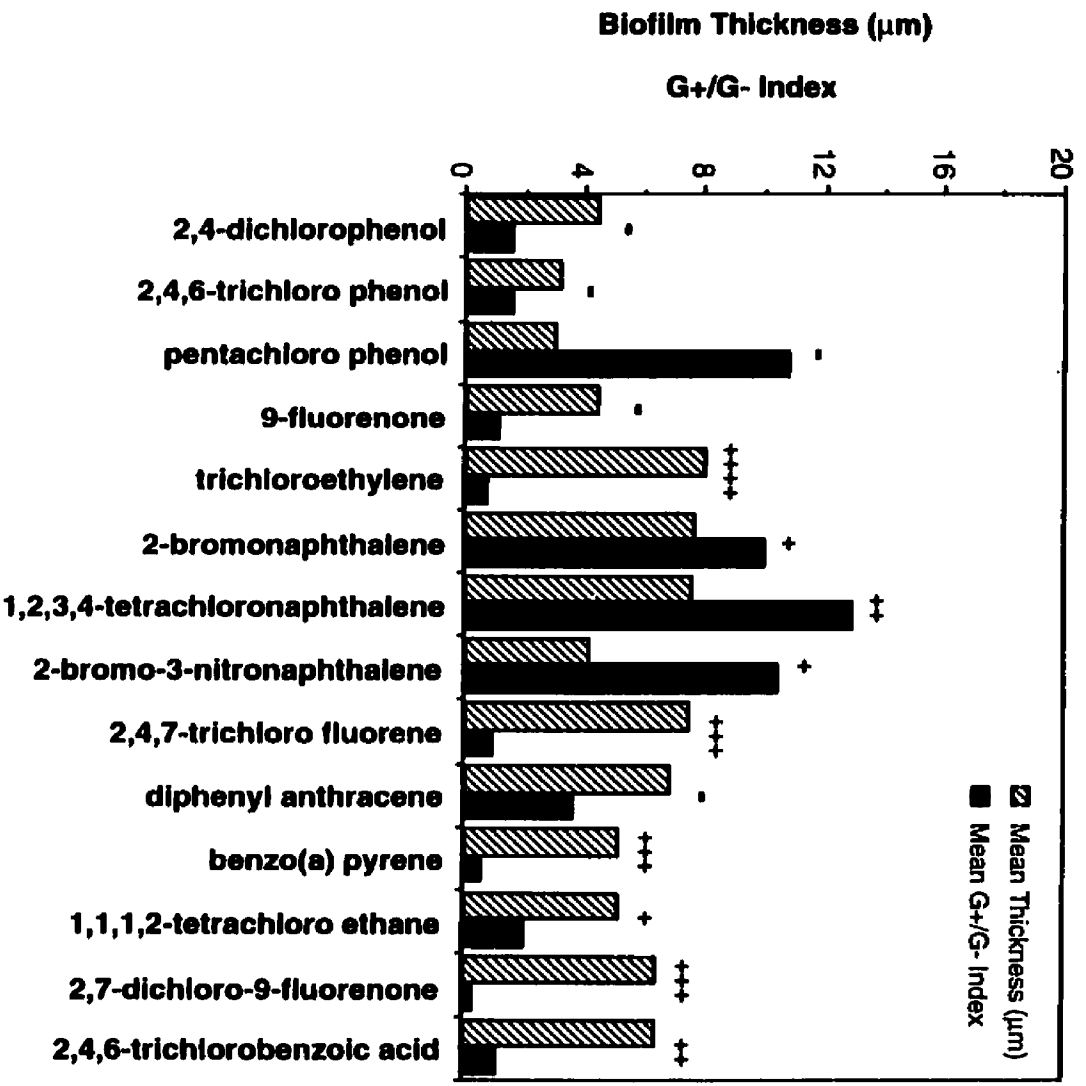


**Figure 3.1.2.** Number of CFU ml<sup>-1</sup> formed by the 2,4,6-T community when cultivated in batch suspension on four selected carbon sources. The community did not show any growth on pentachlorophenol (■) over the 12 day period. In contrast, there was apparently little or no acclimation period required for growth on 2,4,6-trichlorobenzoic acid (2,4,6-T) (□) and 2,7 dichloro - 9 fluorenone (●), whereas relatively longer acclimation period was required for growth on the highly chlorinated and brominated 2-bromo-3 nitronaphthalene (bis hexachloro cyclopentadiene) adduct (○).

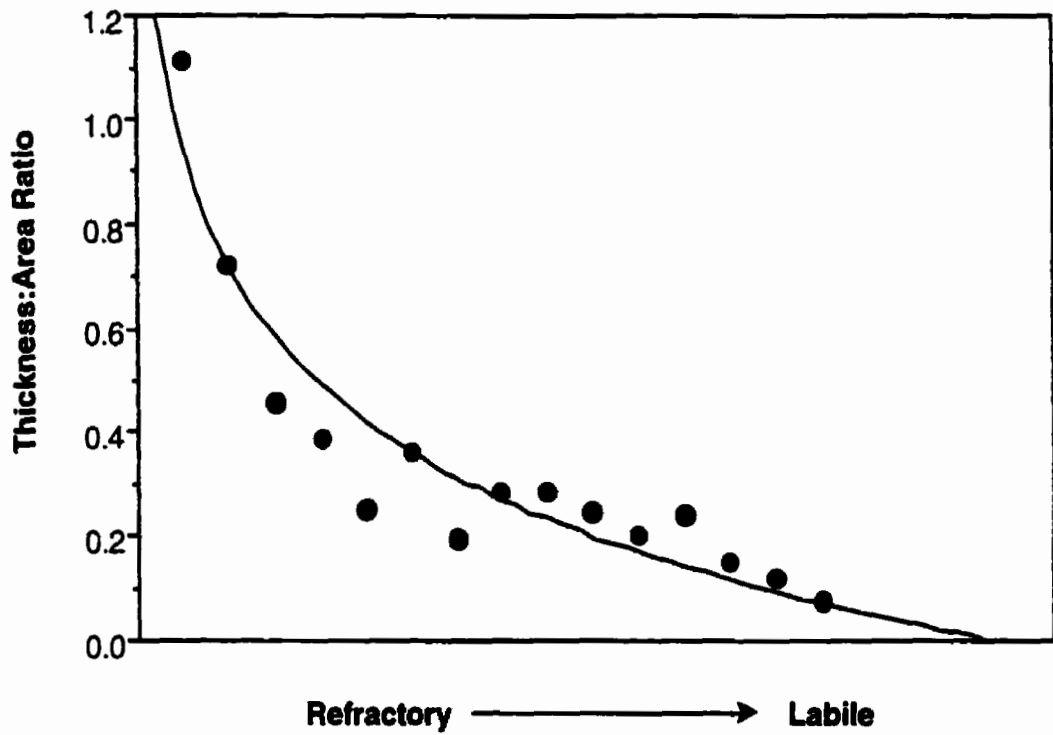




**Figure 3.1.3.** Growth of the 2,4,6-T community in batch (CFU ml<sup>-1</sup>; ranked in ascending order from left to right) and flow cell cultures (percent area coverage) when provided with various chemicals as sole carbon source. Note the extensive biofilm development and planktonic biomass when 2,4,6-T was supplied as the carbon source (the same substrate used to isolate and maintain the community). The data for glucose are not shown.



**Figure 3.1.4.** Thickness (µm), gram ratio (gram positive: gram negative bacteria) and predator counts (number of predators per field; see materials) of two-week-old biofilms. There was a positive correlation ( $r^2 = 0.75$ ) between the gram ratio and degree of chlorination of the substrates. Mean predator numbers per microscope field are indicated as follows: no predators (-); 1 predator/field (+); > 1 and ≤ 3 predators/field (++); > 3 and ≤ 10 predators/field (+++), and >10 predators/field (++++).



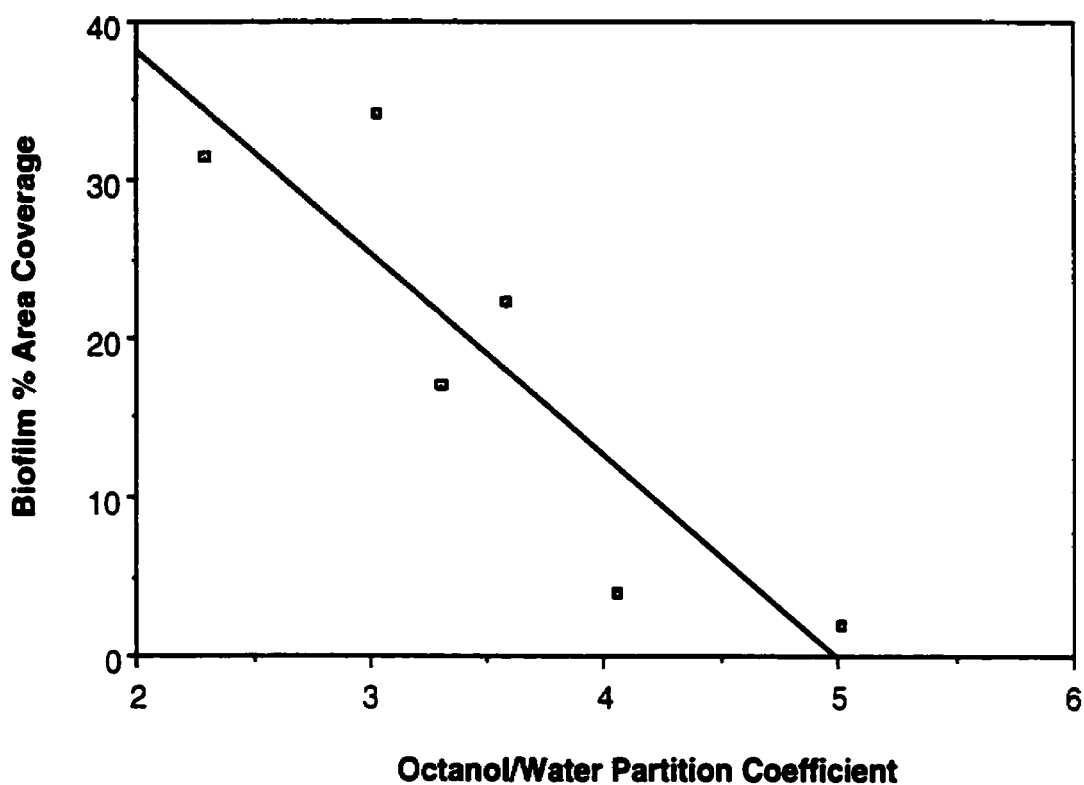
**Figure 3.1.5.** Relationship between the ratio, biofilm thickness:area coverage, when the 15 substrates were arranged in descending order of refractoriness (as deduced from the bacterial growth on the substrates). An  $r^2$  value of 0.88 was calculated using a logarithmic regression.

of predators per field (>10 predators/field). In general there was no correlation between numbers of predators and the average percent area coverage ( $r^2 = 0.15$ ) or average thickness ( $r^2 = 0.33$ ) of the biofilms. Positive correlations were noted between published (Hansch and Leo, 1979; Muller and Klein, 1992) log octanol/water (o/w) partition coefficients for trichloroethylene ( $P_{ow} = 2.29$ ), 1,1,1,2-tetrachloroethane (3.03), dichlorophenol (3.30), trichlorophenol (4.05), pentachlorophenol (5.01) and 9-fluorenone (3.58), and the thickness ( $r^2 = 0.78$ ) or percent area coverage ( $r^2 = 0.80$ ) of biofilms formed by the 2,4,6-T community on these substrates (Figure 3.1.6). In contrast, these o/w partition coefficients did not correlate with growth during batch cultivations.

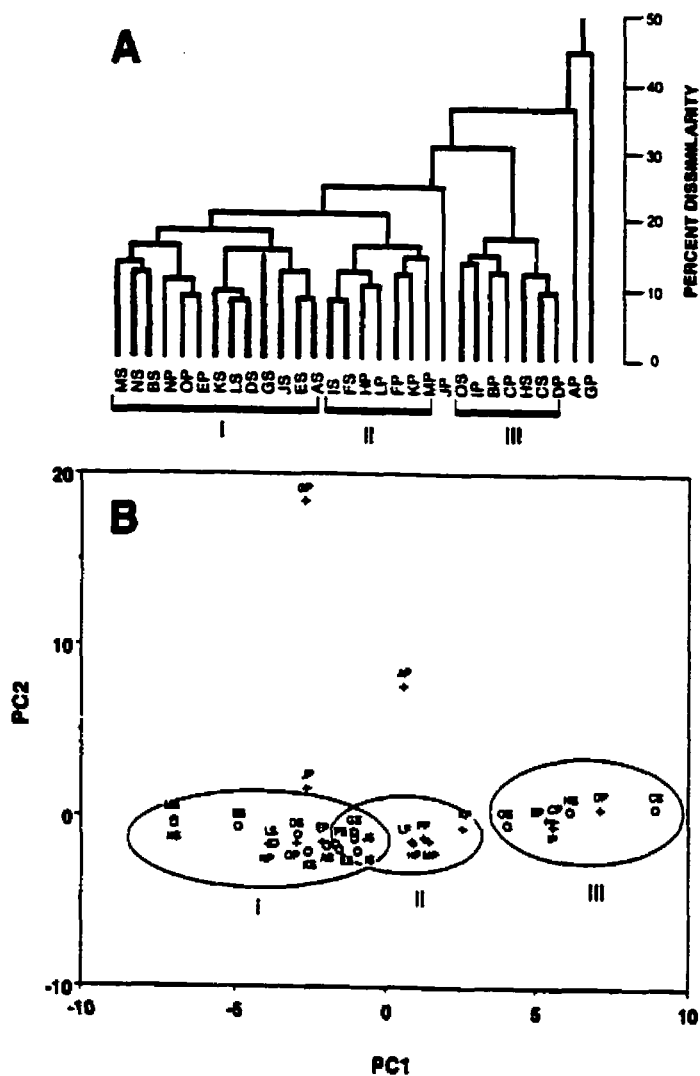
### 3.1.3.2 Effect of chemical structure on whole-community metabolic capability

Cluster analysis of the Biolog sole carbon utilization profiles of the communities which formed following planktonic or sessile cultivations of the 2,4,6-T degrading community on various substrates resulted in three major clusters (Figure 3.1.7 A). Clusters I and II were 80% similar whereas cluster III was 70% similar to clusters I and II. Cluster I consisted of mostly sessile communities (10 sessile to 3 planktonic) whereas more planktonic (5) than sessile (2) communities were grouped in cluster II. Cluster III consisted of almost equal numbers of planktonic and sessile communities (4 planktonic and 3 sessile).

PCA of the sole carbon utilization profiles verified the groupings as determined by cluster analysis (Figure 3.1.7 A,B). Clusters I and III were separated by PC1 which accounted for 22.4% (highest variation accounted for among all the principal components) of the total variation (Fig 3.1.7B). The variation reflected by PC1 primarily resulted from the differences among communities in the utilization of certain carbon sources as indicated by their high loadings (the relative contribution of a particular carbon source on the component, shown below in parenthesis). These carbon substrates included polymers such as dextrin (0.163) and glycogen (0.167), the carbohydrates D-arabitol (0.177) and cellobiose (0.184), the carboxylic acids monomethyl succinate (0.172) (ester), acetic acid (0.172), and formic acid (0.171), and the amino acids succinamic acid (0.174), alaninamide (0.164) (amides), hydroxy-L-proline (0.174) and L-leucine (0.180). PC2 was primarily influenced by the carbon substrates L-rhamnose (0.178), citric acid (0.227), D glucuronic acid (0.178),  $\alpha$ -keto-glutaric acid (0.227), quinic acid (0.227), D-saccharic acid (0.185), L-asparagine and L-



**Figure 3.1.6.** Correlation between published octanol/water partition coefficients of trichloroethylene, tetrachloroethane, dichlorophenol, trichlorophenol, pentachlorophenol and 9-fluorenone, and the extent of biofilm development when the 2,4,6-T community was cultivated using these compounds. A high, negative correlation ( $r^2 = 0.80$ ) suggests that higher partition coefficients might have led to higher intracellular accumulation of the test compounds and consequent reduction in growth rates due to substrate toxicity.



**Figure 3.1.7.** Multivariate analysis of the substrate utilization profiles of planktonic and sessile communities. A) Whole-community metabolic profiles of the 2,4,6-T community when grown on various substrates in batch culture (primarily planktonic population) and in flow cells (primarily sessile population). The first letter in the 2 letter designation (A, B, C, etc.) refers to the substrate (N refers to glucose; for other substrates see Figure 3.1.3); the second letter refers to either planktonic (P) or sessile (S) growth. Three distinct metabolic clusters developed, cluster I consisted mainly of the 2,4,6-T community cultivated in flow cells, the majority in cluster II were the same community cultivated in batch, while almost equal numbers of batch and flow cell cultivations grouped in cluster III. B) Principal component analysis confirmed these groupings and permitted the determination of underlying sources of variation among the communities.

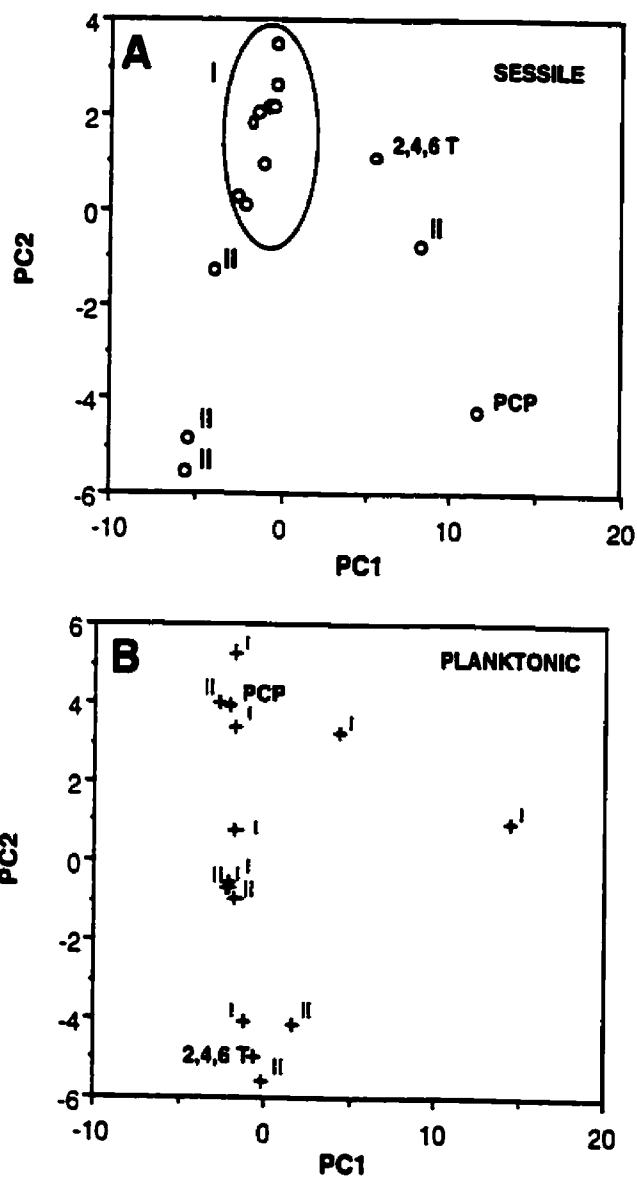
glutamic acid (both 0.227), L-histidine (0.231), L-serine (0.227),  $\gamma$ -aminobutyric acid (0.227), putrescine (0.231), and glycerol (0.231). When sessile communities alone were analyzed using PCA, a distinct grouping of communities cultivated using chlorinated compounds could be noted, with the exception of 2,4,6-T and PCP. In Figure 3.1.8 A, PC1 and PC2 accounted for 40.7% and 14.1% of the total variation, respectively. The BIOLOG carbon substrates that had higher loadings on the PC1 axis were polymers such as  $\alpha$ -cyclodextrin (0.173) and dextrin (both 0.173), carbohydrates such as cellobiose (0.173), maltose (0.188),  $\beta$ -methyl-D- glucoside (0.178) and sucrose (0.188), esters such as methyl pyruvate and monomethyl succinate (both 0.173), carboxylic acids such as acetic acid (0.173), formic acid (0.188), malonic acid (0.188), amino acids such as L-alanine (0.188), and L-leucine (0.188). PC2 was influenced by carbohydrates such as psicose and xylitol, carboxylic acids such as  $\alpha$ -keto-butyric acid (0.262), and other carbohydrates such as L-fucose (0.208), D-galactose (0.194), lactulose (0.203) and m-inositol (0.193). PCA did not discriminate between batch communities cultivated in chlorinated and non-chlorinated compounds (Figure 3.1.8 B).

### **3.1.3.3 Time-dependent changes in community metabolic fingerprints**

Biolog substrate utilization profiles indicated that continuous metabolic changes occurred when the 2,4,6-T community was cultivated in a flow cell irrigated with 2,4,6-T over 12 months. For instance, cluster analysis showed that the profile obtained after one month differed from the six month profile by ~25%, while the profile obtained after 12 months of cultivation differed >50% from the one month or six month profiles. Based on little inter-sample variability between Biolog replicates, it is safe to assume that the changes observed over 12 months were significant, suggesting a continued metabolic evolution over time.

### **3.1.4 Discussion**

The results of this study indicated that both the chemical nature of the substrate and the method of cultivation had a significant impact on the growth potential and metabolic capability of a microbial community. Batch cultivation in 2,4,6-T required the shortest adaptation period of all substrates used (Figure 3.1.2), most likely because of the six-month prior exposure of the inoculum to 2,4,6-T in flow cell cultivation. Batch growth using 2,4,7-trichlorofluorenone occurred more slowly than when 2,4,6-T was provided as substrate, possibly due to its more complex ring structure. Neither the



**Figure 3.1.8.** Principal component analysis of sessile and planktonic communities. A) Sessile growth of the 2,4,6-T community cultivated on various chlorinated compounds formed a distinct cluster (I), away from the same community when cultivated on non-chlorinated compounds (II), with the exception of cultivation on 2,4,6-T (in which the parent community was originally maintained) and PCP. B) When cultivated in batch culture, there was no distinct grouping based on chlorinated (I) or non-chlorinated (II) substrates. The cluster in (A) and not in (B) suggests that the surface allows the cells to form a distinct functional organization based on spatial information that did not form when there was no surface to allow positioning.



degree of chlorination (number of chlorine substitutions in the aromatic nucleus) nor complexity (number and arrangement of rings) of the hydrocarbon compounds used as sole carbon source could be correlated with the extent of growth in batch culture. The next series of experiments was aimed at determining whether the same trends would be observed during biofilm growth. As with the batch cultivations, neither the degree of chlorination nor complexity of the hydrocarbon compounds could be correlated with the extent of growth. However, the flow cell cultivations clustered separately based on growth on chlorinated and non-chlorinated compounds. In addition, the relative abundance of the gram positive and gram negative bacteria in the sessile communities were positively correlated with the degree of chlorination. Previous studies have indicated that disturbances and the presence of contaminants may result in changes in the relative numbers of gram positive and gram negative bacteria. Zelles et al. (1995) found higher numbers of gram positive bacteria in non-cultivated forest soils than in cultivated soils, while Zelles and Bai (1994) found that the upper layers of a soil contaminated with tetrachlorobenzene and pentachlorobenzene had relatively more gram positive bacteria than in the lower, uncontaminated horizons.

In contrast with previous reports that the biodegradability of a compound decreases with increasing number of halogen substitutions (Wackett, 1997), and especially the presence of meta-substituted halogens (Alexander and Aleem, 1961), the above results suggest that other factors than substrate complexity and the degree of halogenation are also involved in determining the capability of microbial communities to degrade xenobiotic compounds provided as sole sources of carbon. Other factors which may affect microbial responses to chlorinated and polycyclic hydrocarbons include the physicochemical characteristics of the compound, such as solubility and hence its availability to microbial degradation (Grimberg et al., 1996; Jimenez and Bartha, 1996; Zhang et al., 1997). Acclimation, the physiological modification of a microbial community that results from long-term exposure to a xenobiotic compound, may also affect the overall metabolic capability of a microbial community. It has been shown that the adaptation of freshwater communities to *p*-nitrophenol (PNP) required three to 10 days, during which the degradation of PNP increased by 1000 fold (Spain, 1990). Even the strains capable of PNP degradation required several days to adapt to use PNP efficiently. Prior acclimation has also been shown to be responsible for enhanced PCP degradation (Nicholson et al., 1992; Watson, 1993; Yu and Ward, 1994; Bhattacharya et al., 1996). Heterogeneous microbial populations growing on glucose under batch activated sludge systems could be acclimated to high PCP concentrations (250 mg l<sup>-1</sup>)

without reduction in substrate removal efficiency. However, much lower concentrations of PCP were toxic to unacclimated sludge communities (Heidman et al., 1967).

Alexander and Aleem (1961) also observed that prior exposure of the community to the test compound resulted in faster degradation of additional quantities of the same compound added to initial enrichments. The growth responses of the 2,4,6-T community in both batch (Figures 3.1.2 and 3.1.3) and flow cell cultivations (Figure 3.1.3) strongly suggest that this community has been adapted to 2,4,6-T.

In spite of their relatively simple chemical structure, phenols supported the least growth in batch cultures, and to some extent in flow cells. This apparent toxicity of phenols may be related to their high aqueous solubility, and subsequently high bioavailability (Lang and Viedt, 1994). Gaudy and Gaudy (1980) found that exposure to phenols resulted in large reductions in cell numbers and inhibition of substrate removal before the microbial population became adapted. Similarly, although numerous workers reported degradation of 2,4-dichlorophenol by pure cultures as well as microbial communities, this compound may also inhibit microorganisms. Tokuz (1991) demonstrated that 3 mg l<sup>-1</sup> 2,4-dinitrophenol inhibited microbial activity in activated sludge. Various studies have demonstrated the inhibitory effects of PCP. Bhattacharya et al. (1996) showed that 15 mg l<sup>-1</sup> PCP could inhibit batch and continuous anaerobic systems, while Chaudri et al. (1996) found that 75 mg PCP kg<sup>-1</sup> soil caused a >99% decrease in the numbers of indigenous soil populations of *Rhizobium leguminosarum*. During nutrient studies, Rutgers et al. (1996) observed a reduction in the specific growth rate of a PCP-degrading bacterium at a concentration >168 µM PCP (44.8 mg l<sup>-1</sup>). The concentration of phenols used in our study (50 mg l<sup>-1</sup>) thus might have been toxic to some or most members of the 2,4,6-T community. The inhibitory effect of phenols was more pronounced in the batch than in flow cell cultivations. Indeed, Figure 3.1.3 shows that although the phenols inhibited growth in batch culture, one of them (2,4-dichlorophenol) supported significant biofilm development when compared with the other substrates (17.1% biofilm coverage compared to a mean value of 21.69% for all substrates). Surface-associated growth and biofilm formation have been described as adaptive mechanisms which render microbial communities more resistant to toxic compounds and improve their overall metabolic capability, including nutrient utilization and waste cycling (Boult et al., 1997). These factors are likely responsible for the observations in the current study that the 2,4,6-T community could form biofilms when provided with substrates such as 2,4-dichlorophenol, 2,4,6-trichlorophenol, PCP, and 9-fluorenone, while these chemicals did not support growth in batch culture.

It was shown previously that biofilm architecture is influenced by, among other factors, the nature of the carbon source (Wolfaardt et al., 1994b; Møller et al., 1997). The differences in area (Figure 3.1.3) and thickness (Figure 3.1.4) of biofilms formed by the 2,4,6-T community on the different substrates are thus not surprising. When supplied with a labile carbon source, biofilm cells do not rely on one another to metabolize the substrate. Close positioning of cells would rather be a disadvantage as the cells will compete for the carbon source. Therefore, an even distribution of cells in biofilms cultivated on labile carbon sources typically occurs. In contrast, when challenged with a refractory substrate as the sole carbon source, the cells form tight clusters, typically with void space between the clusters (Wolfaardt et al., 1994c). As a result, the ratio of biofilm thickness to area covered by biofilm should increase with an increase in substrate refractoriness. Figure 3.1.5 shows that this was indeed the case in the present study and an inverse logarithmic relationship ( $r^2 = 0.88$ ) was observed between the biofilm thickness:biofilm area ratio when the 15 compounds were ranked in ascending order of measured biofilm development.

Pesticides such as dimethoate and pirimicarb have been shown to negatively impact protozoan colonization of sterile soil (Ekelund et al., 1994). Steinberg et al. (1990) noted that pyralene, a mixture of PCBs in trichlorobenzene, resulted in the death of active forms of amoebae and the inhibition of cyst formation when introduced into a soil system. Selective inhibition of microbial growth by these chemicals may have ecological consequences. For example, in the study by Steinberg et al. (1990) pyralene prevented regulation of *Azospirillum lipoferum* numbers by amoebae. In the present study, predators were absent in the biofilms cultivated on the substrates which inhibited bacterial growth in batch culture, suggesting that: 1) these chemicals were also toxic to the predators, and 2) the presence of degradative bacterial biofilms did not alleviate this toxicity to predators. Erb et al. (1997) reported that mixtures of phenols completely eliminated protozoa and metazoa from a laboratory-scale sewage plant. However, in contrast to the study by Erb et al. (1997) in which inclusion of a degradative strain of *Pseudomonas* sp. protected the protozoa and metazoa against inhibition by phenols, these results fail to show such protective interactions. In general, little is known of the effect of substrate toxicity and simultaneous reduction of predator and prey populations. In fact, the presence of these chemicals may greatly influence the predator - prey dynamics within natural microbial communities, including attachment to surfaces and aggregate formation through the production of exopolysaccharides (Deleo and Baveye, 1997), the natural balance between bacterial growth rates and the predation

pressure (Pernthaler et al., 1997), formation of grazing resistant cell structures such as filaments (Simek et al., 1997), and selective predation by protozoans (Pernthaler et al., 1997; Simek et al., 1997).

Octanol/water partition coefficients ( $P_{ow}$ ) have been used to determine the uptake and bioconcentration of hydrocarbons at various trophic levels (Schwarzenbach et al., 1993; Clarkson, 1995). Bryant and Schultz (1994) suggested that the types and positions of substituents in an organic molecule influence the mechanism and ease of microbial degradation as well as its toxicity. They also suggested that narcosis, a state of arrested cytoplasmic activity, is associated with partitioning of the toxicant molecule into the organic phase of the organism, although the overall toxicity is a conjoint effect of hydrophobicity and reactivity. The mode of action of phenols and simple chlorinated phenols on microorganisms has been suggested to be through narcissism, i.e., the arrest of cytoplasmic activity (Bradbury and Lipnick, 1990). In contrast, tetra and pentachlorophenol act as respiratory uncouplers, unlinking respiration from ATP synthesis (Cajina and Schultz, 1990). Siera Alvarez and Lettinga (1991) showed that the log  $P_{ow}$  was positively correlated with methanogenic inhibition. In the present study, high inverse correlations were noted between the octanol/water partition coefficients and biofilm parameters such as area coverage and thickness ( $r^2 = 0.78$  and  $0.80$ , respectively) suggesting that higher log  $P_{ow}$  values resulted in greater intracellular accumulation and thus inhibition of the biofilms.

A number of research groups have used community-level carbon source utilization patterns in combination with multivariate data analysis to elucidate overall functional capabilities of microbial communities (e.g., Haack et al., 1995; Degens and Harris, 1997; Staddon et al., 1997). An objective of the present study was to determine whether cultivation of a degradative community on substrates with varying degrees of chlorination and complexity in chemical structure, as well as cultivation in batch and flow cell culture, would alter the community's functional capability. Ten of the 15 flow cell cultivations (67%) grouped together in cluster I at a similarity of >80% (Figure 3.1.7 A,B). In contrast, there was no distinct grouping of the batch cultivations: 20%, 33% and 27% of the planktonic communities grouped in clusters I, II and III, respectively, while 20% did not group in any cluster. These results suggest that the biofilm mode of growth associated with flow cells strongly influenced the metabolic characteristics of the communities which developed on the different substrates, rendering them more similar than their planktonic counterparts. and that growth in biofilms resulted in 1) the preservation of a number of key surface-associated members less influenced by the

nature of the chemical environment than planktonic communities, and/or 2) the preservation of common metabolic traits. However, PCA revealed that the flow cell communities cultivated on chlorinated substrates, with the exception of PCP and 2,4,6-T, grouped together while chlorination had no effect on the grouping of communities cultivated in batch (Figure 3.1.8 A,B). Thus, biofilm growth was not the sole determinant of metabolic characteristics, although it is possible that the spatial organization and close associations between cells in biofilms lead to a more unified response to the chemical environment that was not possible when there was no surface to allow positioning. These close associations enable biofilm cells to more effectively share resources and information (Caldwell et al., 1997b). Confer and Logan (1998) found that more than 97% of the total hydrolytic activity in biofilm cultures occurred in contact with the cells and not in the bulk solution, and that in trickling filter effluents hydrolysis rates were five times higher in contact with cells and sloughed biofilm pieces than in cell-free solution. Exchanges of genetic material also occur more efficiently in biofilms (Lorenz et al., 1988; Lebaron et al., 1997). Such exchanges may have contributed to the development of metabolic traits in the biofilm communities which were more similar than those between the planktonic communities where sharing of the larger genetic pool would be less likely to occur. Based on their respective loadings and spatial distribution as determined by PCA, the number of rings in the chemical structure of the substrates and the degree of chlorination had no significant effect on the metabolic profiles of either planktonic or sessile communities. Furthermore, no correlation was found between CFU ml<sup>-1</sup> and the number of positive wells in the BIOLOG plates ( $r^2 = 0.19$ ). Therefore, the observed differences in metabolic profiles are due not only to differences in CFU, but also to other factors such as changes in community composition.

The wide array of growth responses of the microbial community to various substrates is an indication of the metabolic diversity found among microbial communities. Furthermore, the results show the inherent complexity of community-level studies and the need to consider these factors when addressing questions related to practical applications, for example, when testing for toxicity or for degradation. Clear separation of sessile communities between growth on chlorinated compounds and non-chlorinated compounds, when analyzed by PCA, suggests that substrate chlorination had an impact on sessile communities. Finally, it is clear from these observations that the functional characterization of microbial communities would be difficult, if not impossible, if only one type of analysis were applied.

### 3.1.5 Summary

The objective of this preliminary study was to determine whether cultivation of a degradative community on substrates with varying degrees of chlorination and complexity in chemical structure, as well as cultivation in batch and flow cell culture, would alter the community's structural and functional characteristics. The community was isolated from oil-contaminated soil and maintained in the laboratory on 2,4,6-T for five months before its ability to grow on 15 different chemicals as sole carbon source was evaluated in batch and flow cell systems. While the community could grow and develop biofilms in flow cells on all the substrates, only eleven of the fifteen substrates could support growth in batch culture. Although biofilm development was less extensive on chemicals such as pentachlorophenol (2.09% average area covered by biofilm; average biofilm depth = 3  $\mu\text{m}$ ) than on 2,4,6-T (50.84% area covered; biofilm depth = 6.4  $\mu\text{m}$ ), no correlation was observed between the degree of chlorination, or number of rings, and the number of planktonic cells or biofilm biomass. In contrast, physicochemical characteristics such as the octanol/water partition coefficient had a significant effect on the development of biofilm biomass. In the case of planktonic communities, the degree of chlorination and ring number also had no effect on the BIOLOG carbon utilization profiles of the resulting communities. Although the sessile communities generally clustered separately from their planktonic counterparts, principal component analysis of carbon utilization profiles of the sessile communities differentiated between those growing on chlorinated versus non-chlorinated substrates. Analysis of the degradative community maintained on 2,4,6-T over an extended period further showed that metabolic adaptation to a new chemical environment is a rather slow process, since the substrate utilization profiles did not stabilize even after 12 months. These results demonstrate the emergence of various metabolic and structural properties within evolving microbial communities.

## **3.2 The effect of coincidental recombination on the emergence of benzoate-degrading biofilm communities**

### **3.2.1 Introduction**

The role of community-level bacterial strategies in biodegradation has been well established (Rajagopal et al., 1984; Madsen and Aamand, 1992; Wolfaardt et al., 1994b, 1994c; Bagley and Gossett, 1995). In degradative microbial communities, the capacity for breakdown of refractory organic compounds arises from physiological (Jimenez et al., 1991; Wolfaardt et al., 1994b) and genetic (Fulthorpe and Wyndham, 1991) interactions among community members. Physiological co-operativity within a community results from the metabolic diversity of its members, in terms of the number and variety of chemical bonds on which they can act. This physiological co-operativity is often responsible for the sequential degradation of complex organic chemicals (Jimenez et al., 1991). Genetic interactions, characterized by "horizontal" movement and transfer of degradative plasmids among genetically unrelated bacterial cell lines (Fulthorpe and Wyndham, 1991), also result in an enhanced reproductive capability in degradative communities by increasing the proportion of degradative phenotypes within the community. Thus, these strategies combine the adaptive potential of cells (spatial recombination of molecules within cells) with the adaptive potential of communities (spatial recombination of cells within communities).

According to proliferation theory (Caldwell and Costerton, 1996; Caldwell et al., 1997a, 1997b, 1997c), synergisms between organisms may be required for the optimal proliferation of communities, just as synergisms between molecules are required for the for the optimal proliferation of cells. Thus the restructuring of communities not only involves gene mutations and recombination at the cellular level, but also recombination of cells and organisms within and among communities. However, the role of external organismal recombination (i.e. the effects of immigration and emigration) in the process of adaptation of bacterial communities to environmental stresses has not been studied from this perspective. Furthermore, if the process of adaptation to a stress occurs at multiple spatial levels of organization, including the community level, then an environmental shift should elicit co-ordinated responses among the community members, and changes in community structure profile in response to substrate transitions should show sets of inflections in addition to members responding individually.

The results discussed in the previous section (section 3.2.1) demonstrated the significant impact of substrate chlorination on the structural and functional

characteristics of sessile communities (as elucidated by substrate utilization profiles). Thus, during the present study, existence of community-level adaptation strategies was tested by subjecting a microbial community to cyclic environmental transitions, from TSB (labile) to benzoate (ring structured) to 2,4,6-T (ring structured and chlorinated) as a model of transition from labile to refractory substrates, and to starvation (no carbon substrate), and by examining the role of coincidental recombination (both cellular and genetic recombination) in enhancing the growth of biofilm communities at various stages of environmental transitions.

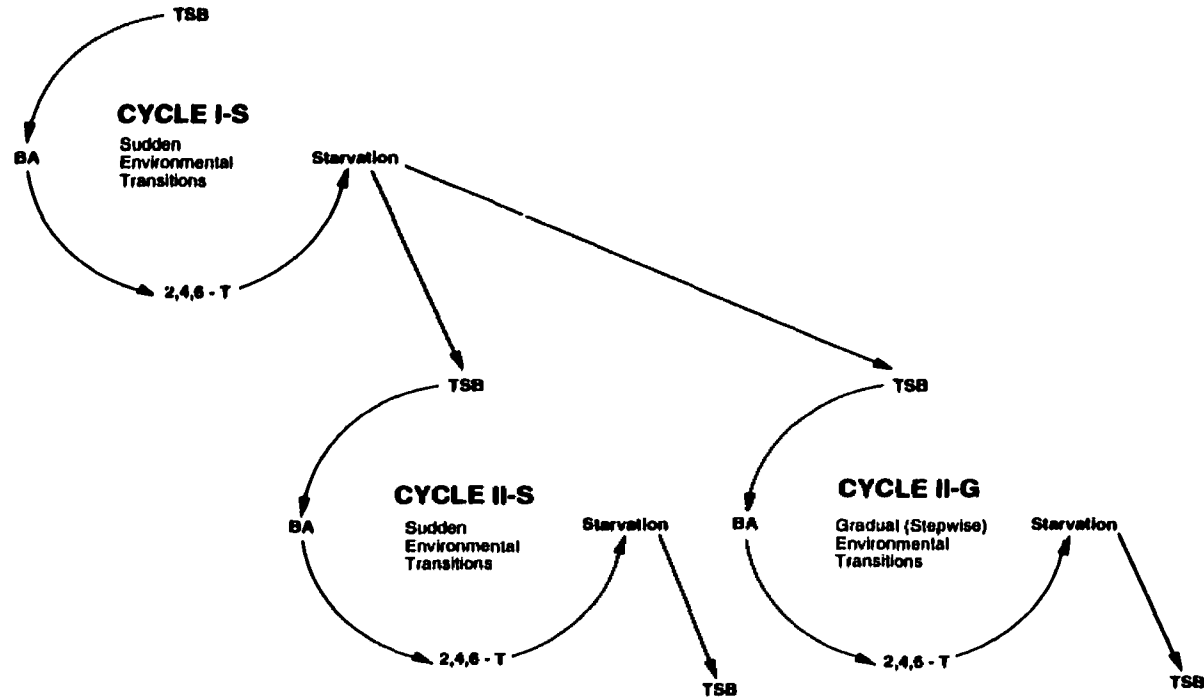
### **3.2.2 Materials and Methods**

#### **3.2.2.1 Bacterial communities and culture conditions**

A composite inoculum was prepared by mixing 0.5 g each of two oil-sludge samples (Kamsack and Baidon sites, Saskatchewan, Canada), a pristine soil sample (Matador grasslands, Saskatchewan, Canada) and a pesticide disposal-pit soil sample (Iowa State University, Iowa, USA), in 50 ml of M-salts medium (see section 3.1.2.1 for composition). This was used to inoculate a flow cell (Wolfaardt et al., 1994b) (channel dimensions: 1 mm by 3 mm by 40 mm) continuously irrigated with 10% (v/v) tryptic soy broth (TSB) at a laminar flow velocity of  $0.2 \text{ mm s}^{-1}$ . A quasi steady-state condition of the biofilms developing on the solid-liquid interface was determined based on the stability of its thickness, area coverage, overall architecture as well as the number and diversity of emigrants (see below). The quasi steady-state community was then subjected to a sudden environmental transition (to sodium benzoate as sole carbon source). Subsequent transitions consisted of a shift to 2,4,6-T as sole carbon substrate and then to a state of starvation (no carbon). The substrate shifts were accomplished by changing the flow cell irrigation medium from 10% (v/v) TSB to M-salts supplemented with 0.15% (w/v) sodium benzoate or 0.05% (w/v) 2,4,6-T or no carbon (when subjected to starvation) depending on the stage of the cycle. All chemicals were procured from Sigma Chemical Co., St. Louis, MO.

In the first cycle (Cycle I - S), the communities were subjected to sudden environmental transitions. The second set of cycles involved sudden as well as gradual (stepwise) environmental transitions (Cycle II - S and Cycle II - G, respectively) (Figure 3.2.1). Sudden transitions consisted of instantaneous shifts in the cultivation medium. During gradual transitions, substrate-shift was stepwise and each shift extended over a period of 30 days. Each shift was accomplished in 10 steps (of 3 days each). Each step consisted of a 0.1 fold decrease in the concentration of the first





**Figure 3.2.1.** A flow-cell community cultivated on tryptic soy broth (TSB, 10% v/v) was subjected to three environmental cycles. A cycle of transition consisted of shifts in carbon substrate from TSB to benzoic acid (BA, 0.15% w/v), to 2,4,6-T (0.05% w/v) (labile to refractory), followed by a period of starvation (30 days). In the first cycle, the communities were subjected to sudden environmental transitions (Cycle I - S). The second set of cycles involved either sudden or gradual (stepwise) environmental transitions (Cycle II - S and Cycle II - G). In all the transitions, the effect of repeated inoculation was compared with a single, initial inoculation (only at the beginning of the experiment).

substrate (10% decrease from the maximum concentration) accompanied by a 0.1 fold supply (10% of the maximum concentration) of the second substrate. At the end of 30 days, when the concentration of the first substrate in the irrigation medium was zero, the concentration of the second was at its maximum.

Both sudden and gradual transition cycles involved parallel studies comparing biofilm communities which were repeatedly inoculated, with the communities inoculated only at the beginning of the experiment (initially inoculated). Repeated inoculation consisted of inoculation at 12 h intervals, using 0.5 ml of a freshly prepared composite inoculum.

### **3.2.2.2 Monitoring biofilm reorganization**

During Cycle I - S and Cycle II - S (Figure 3.2.1), the thickness, percent area coverage and overall architecture of biofilm communities were determined by SCLM. In Cycle I - S, the measurements were made at 12 h intervals until the community reached a quasi steady-state (determined based on stability of biofilm thickness and area coverage), and at longer intervals on the stable biofilm community. In Cycle II - S, SCLM was applied less frequently (once before the transition and twice after the community stabilized in the new environment). Biofilms negatively stained with 0.1% w/v fluorescein were scanned and digitized as described in section 3.1.2.3. The percent biofilm area and the biofilm thickness at each observation period, were respectively the averages of 10 and 20 replicate measurements. Communities subjected to gradual transitions (Cycle II - G) were not examined by SCLM.

### **3.2.2.3 Monitoring the diversity of emigrant populations**

The diversity of the emigrants (as measured by richness and Shannon indices) was monitored at 12 h intervals until the communities reached a quasi steady-state and at less frequent intervals within stable biofilm communities. These determinations were done prior to the laser microscopy of biofilm architecture in Cycle I - S and Cycle II - S. In Cycle II - G, which consisted of stepwise transitions, the emigrants were enumerated just before each stepwise transition. For this purpose, the flow cell effluent was collected for 1 h, and plated on 0.3% (w/v) TSA in triplicate. The plates were incubated at  $23\pm 2^{\circ}\text{C}$  for seven days and the number of various colony morphotypes that appeared on countable plates of highest dilution, usually containing 30 - 100 colonies, were counted. Colony morphotypes were determined based on the descriptions of Benson (1990) and Eklund and Lankford (1967). Richness was based on the number of distinct

colony morphotypes observed. Shannon index of diversity  $H' = -\sum (p_i \cdot \log_2 p_i)$ , was then calculated. The factor  $p_i$  is the number of isolates classified as a certain morphotype divided by the total number of isolates in the sample being analyzed.

#### **3.2.2.4 Plasmid profile analysis**

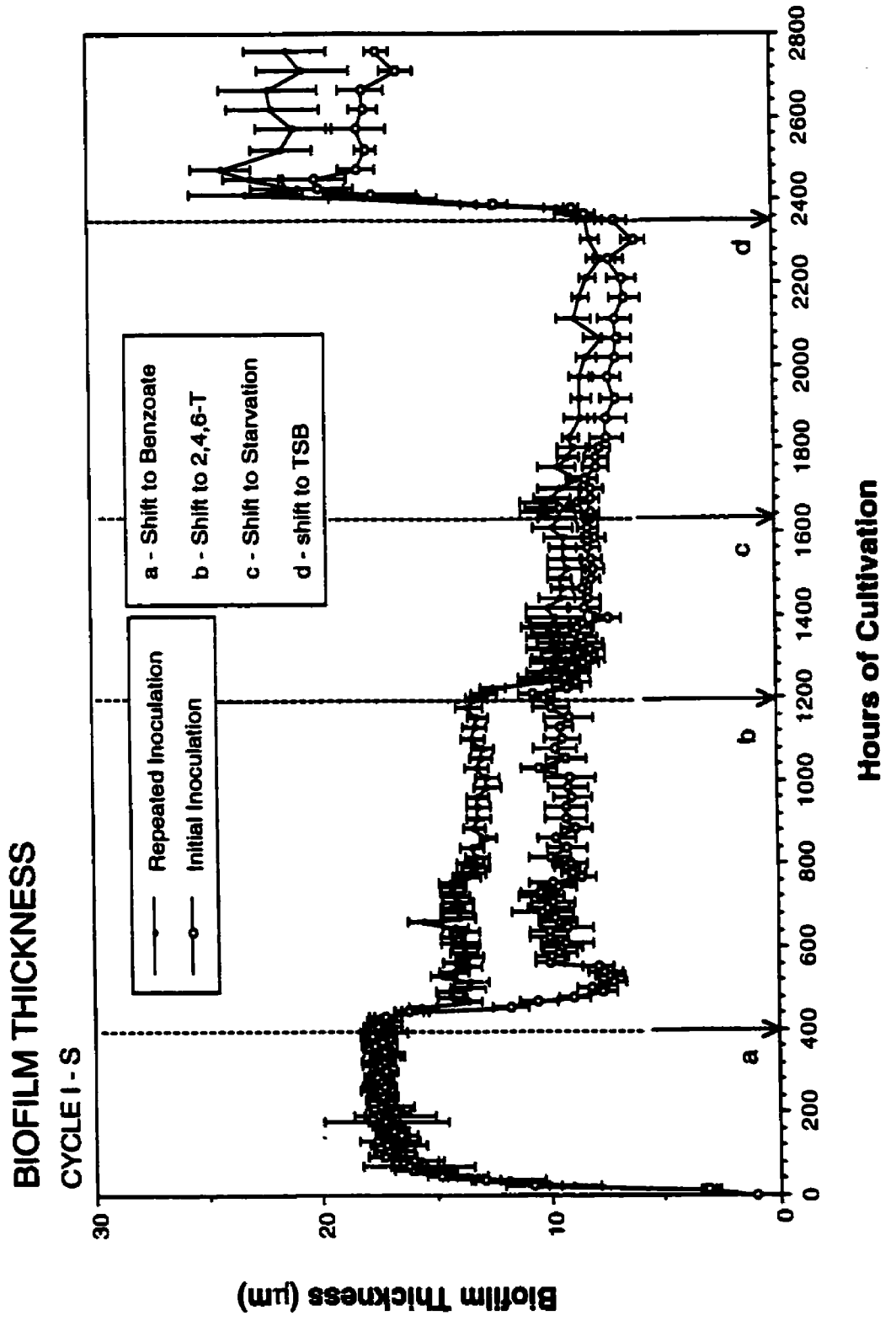
Plasmid content of the emigrant populations of biofilm communities subjected to sudden environmental transitions (Cycle I-S) were analyzed using an alkaline lysis protocol (Kado and Liu, 1981). Frozen aliquots of flow cell effluent collected from stable biofilm communities at various stages of transition were used for this purpose. These aliquots were thawed and used to inoculate 50 ml of culture media. TSB and starvation communities were enriched using TSB (10% v/v), whereas benzoate and 2,4,6-T communities were enriched using M-salts containing 0.15% w/v benzoate and 0.05% w/v 2,4,6-T respectively. The enrichments were done at  $23 \pm 2^\circ\text{C}$  to an optical density of 1.0, and 3 ml aliquots of these enrichments were then used in plasmid isolation. Individual colony morphotypes were analyzed for plasmid content using the same procedure. Electrophoresis of plasmids was performed in a horizontal gel apparatus (Gibco BRL Life Technologies, Rockville, MD) as described previously (Crosa et al., 1994).

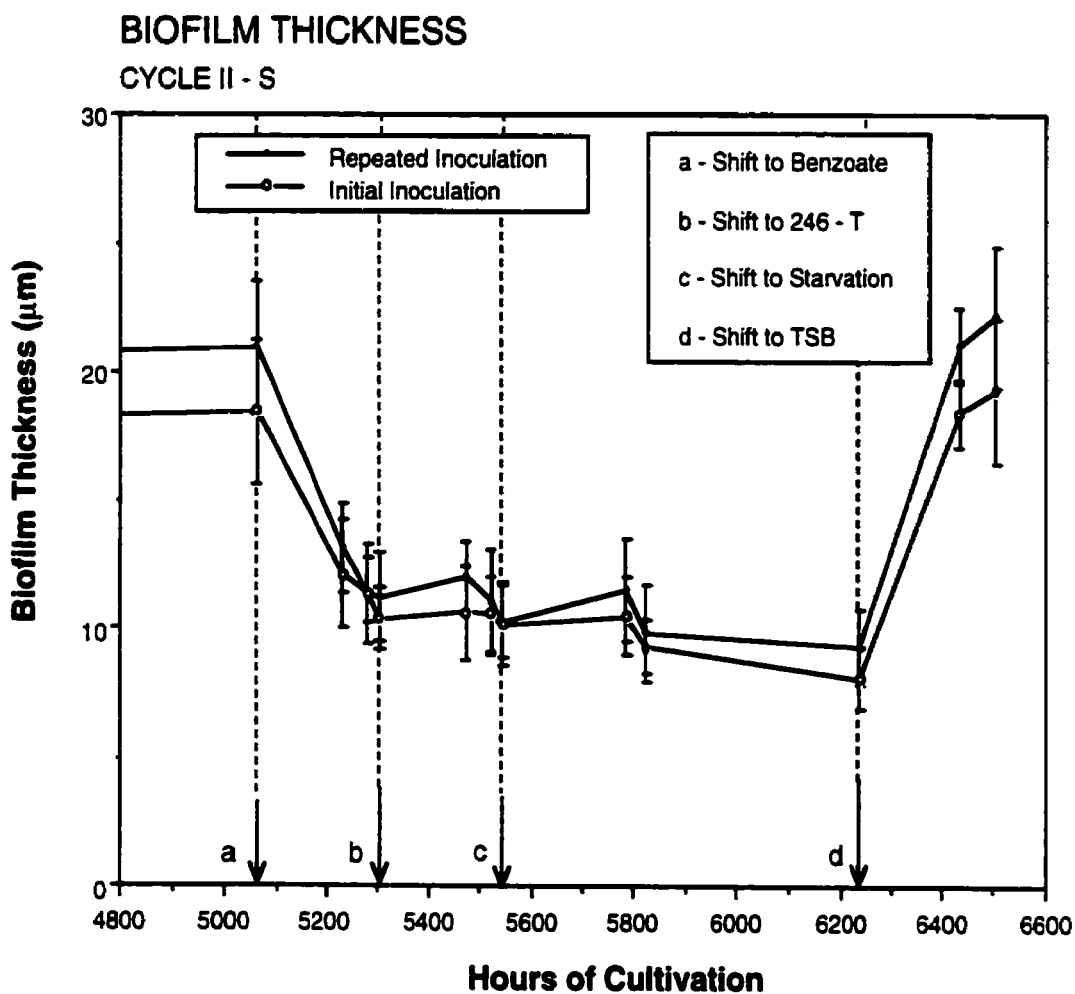
### **3.2.3 Results**

#### **3.2.3.1 Biofilm thickness and percent area coverage**

Shifts in carbon substrates, from TSB to benzoate to 2,4,6-T (labile to refractory substrates), resulted in a general reduction in the thickness and percent area coverage of biofilm communities (Fig 3.2.2, 3.2.3, 3.2.4, and 3.2.5). In cycle I-S, when cultivated using TSB, the thickness (Figure 3.2.2) and percent area coverage (Figure 3.2.4) were not significantly ( $p < 0.05$ ) different between repeatedly inoculated and initially inoculated biofilm communities. When subjected to a substrate shift to sodium benzoate, the thickness and area coverage of both repeatedly and initially inoculated biofilm communities significantly ( $p < 0.01$ ) decreased. However, during cultivation using benzoate, repeatedly inoculated biofilm communities had a significantly ( $p < 0.01$ ) higher thickness (13.1  $\mu\text{m}$ ) and percent biofilm area coverage (14.3%) than communities inoculated only at the beginning of the experiment (10.1  $\mu\text{m}$  and 8.1% respectively). Further decreases in biofilm thickness and coverage were noted upon subsequent

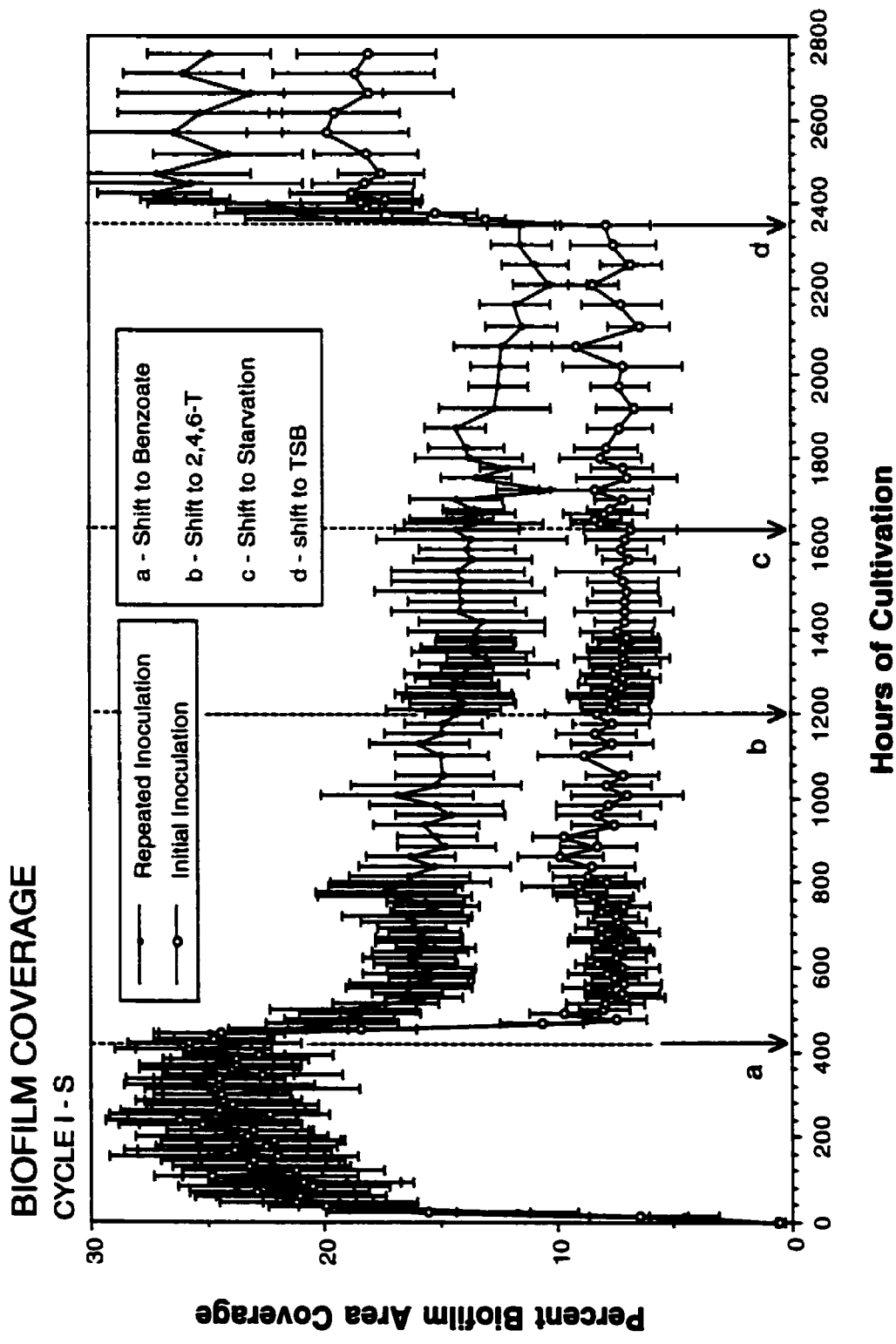
**Figure 3.2.2.** (Facing page) Effect of environmental transitions on the thickness of biofilm communities during the first cycle of sudden environmental transitions (Cycle I-S). There was an overall reduction in the thickness of both repeatedly inoculated and initially inoculated biofilm communities upon successive transitions. In general, repeated inoculation resulted in an enhanced thickness of biofilm communities than when the communities were inoculated only at the beginning of the experiment.



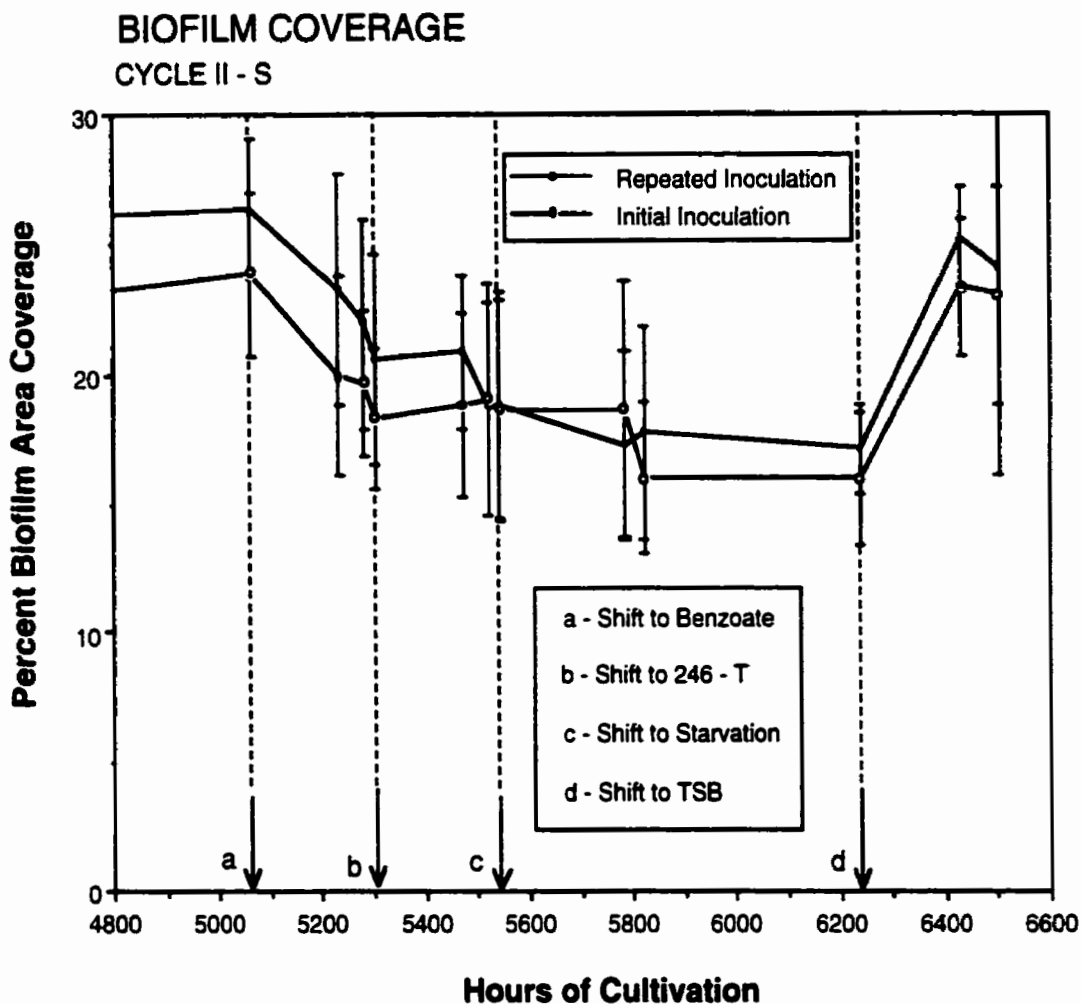


**Figure 3.2.3.** Thickness of repeatedly inoculated and initially inoculated biofilm communities subjected to a second cycle of sudden environmental transitions (Cycle II - S). As also noted in Cycle I - S, during Cycle II - S, there was a general reduction in the thickness of biofilm communities upon successive substrate shifts and repeated inoculation as mechanism of recombination, enhanced the thickness of biofilm communities.

**Figure 3.2.4.** (Facing page) Effect of substrate transitions and starvation on the percent area coverage of biofilm communities during the first cycle of sudden environmental transitions (Cycle I-S). There was a significant reduction in the thickness of both repeatedly inoculated and initially inoculated biofilm communities upon a substrate shift from TSB to sodium benzoate. In general, repeatedly inoculated biofilm communities exhibited a higher percent biofilm coverage than initially inoculated communities (inoculated only at the beginning of the experiment and thus no external recombination was allowed).







**Figure 3.2.5.** Percent area coverage of repeatedly inoculated and initially inoculated biofilm communities during a second cycle of sudden environmental transitions (Cycle II - S). The general trends in biofilm coverage were similar to those of Cycle I - S, with reductions in biofilm coverage in response to successive substrate shifts.

substrate shifts (to 2,4,6-T as sole carbon substrate and to a carbon starvation condition), although they were not significantly different between transitions. During these substrate shifts also, repeated inoculation resulted in biofilm communities having greater thickness and area coverage than when the communities were inoculated only at the beginning of the experiment. At the end of cycle I - S, an environmental shift from a state of starvation (no carbon) to growth on TSB, resulted in significant increases ( $p < 0.01$ ) in the thickness and percent area coverage of both repeatedly inoculated and initially inoculated biofilm communities. In contrast to the TSB communities of Cycle I - S, significant differences ( $p < 0.01$ ) due to inoculation can be noted in the TSB communities of Cycle II - S, with repeatedly inoculated biofilm communities having greater thickness and area coverage (20.9  $\mu\text{m}$  and 26.34% respectively) than initially inoculated (18.5  $\mu\text{m}$  and 23.87% respectively) communities (Figures 3.2.3 and 3.2.5). The overall trends in biofilm thickness and coverage during Cycle II - S were similar to those of Cycle I - S. In general, biofilms were more robust (greater thickness and area coverage) during the second cycle of adaptation (Cycle II - S) than they were during the first cycle (Cycle I - S).

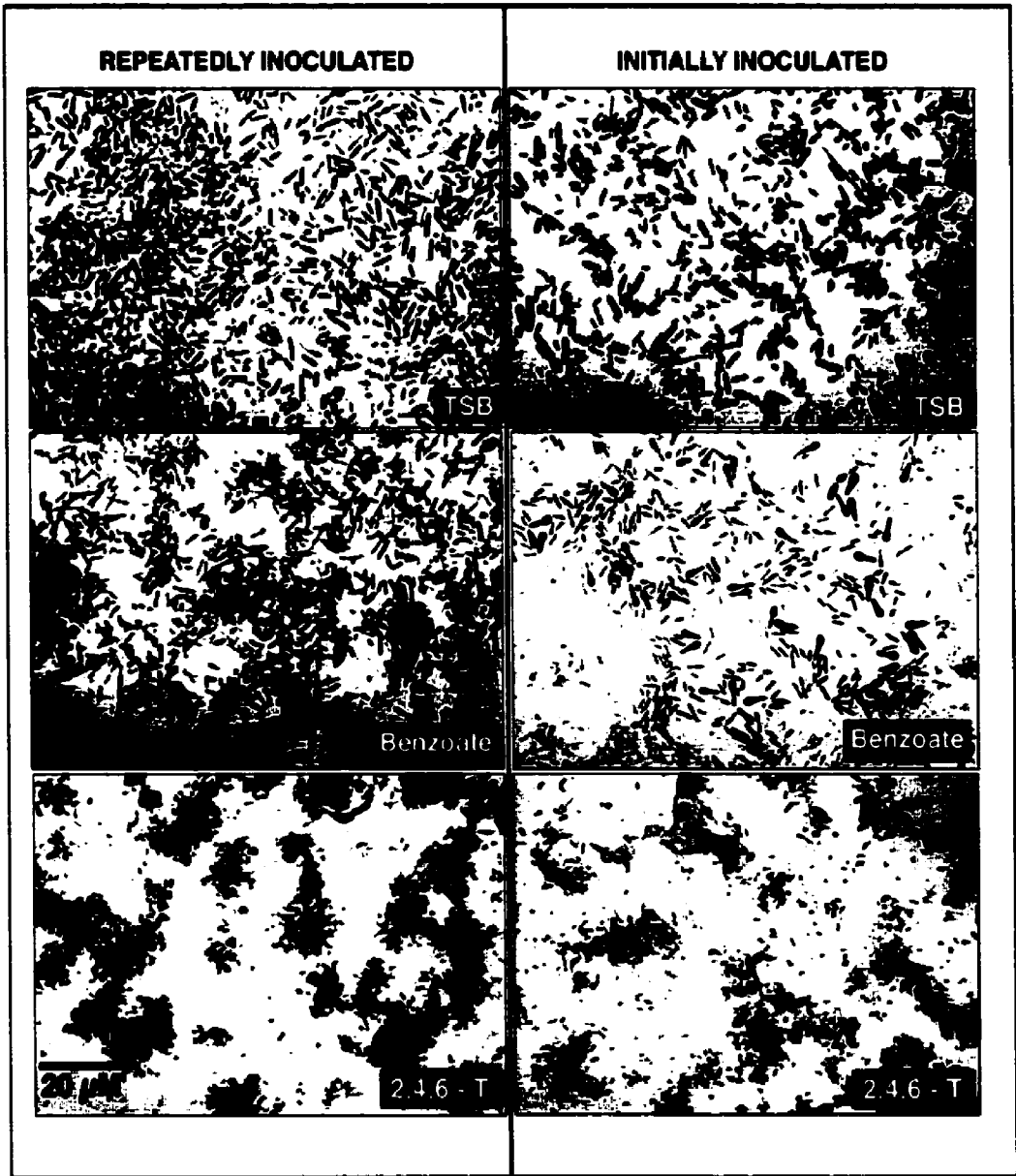
### **3.2.3.2 Biofilm architecture**

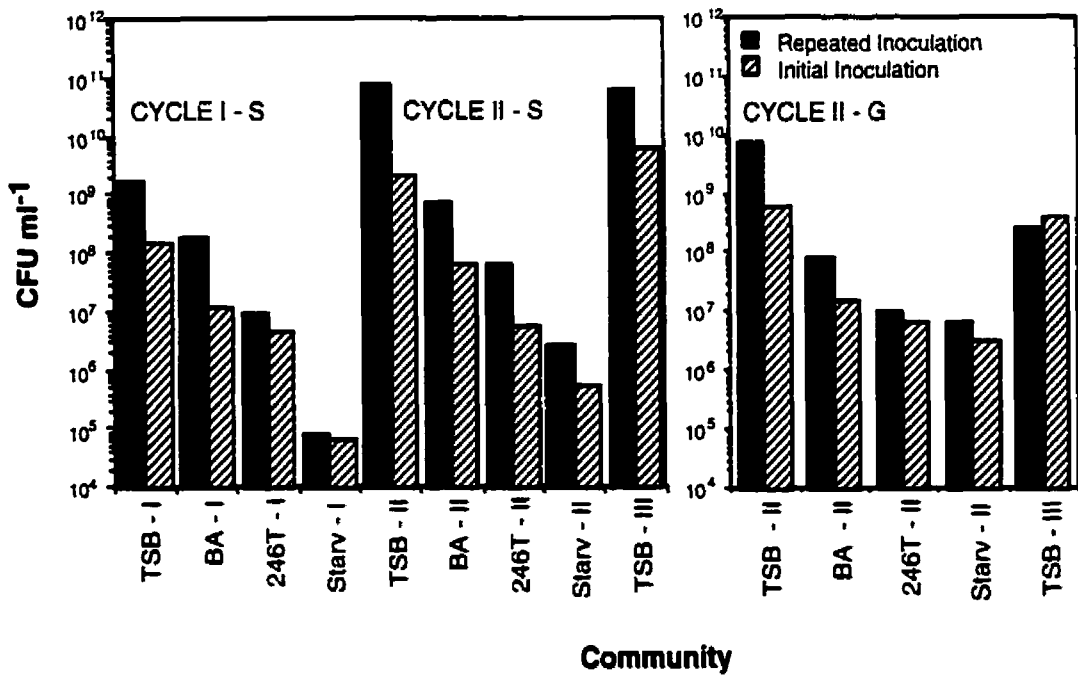
Substrate shifts also resulted in changes in the architecture of the biofilm communities (Figure 3.2.6). Cultivation using a labile carbon substrate such as TSB resulted in randomly distributed cells, whereas cultivation using a more refractory, ring structured compound such as sodium benzoate resulted in the formation of more organized cell clusters. Cultivation using 2,4,6-T resulted in the formation of distinct tree-like arrangement of cells within biofilm communities. Similar architectural features were noted in both repeatedly inoculated and initially inoculated biofilm communities cultivated using the same substrate. The changes in biofilm architecture followed the same trend (from less organized to more organized) during the second cycle of sudden environmental transitions (Cycle II - S) also.

### **3.2.3.3 Emigrant cell numbers**

Irrespective of the frequency of inoculation (single or repeated), the number of emigrants decreased in response to substrate refractoriness in both cycles of sudden environmental transitions (Cycle I - S and Cycle II - S) (Figure 3.2.7). Starvation (no carbon) resulted in the lowest CFU  $\text{ml}^{-1}$ . Similar trends in the number of emigrants were noted during the cycle of gradual environmental transitions (Cycle II - G) also. The

**Figure 3.2.6.** (Facing page) Confocal scanning laser micrographs of repeatedly inoculated and initially inoculated biofilm communities subjected to sudden environmental transitions from TSB to benzoate to 2,4,6-T during Cycle I - S. Note the changes in the overall spatial architecture of biofilm communities in response to substrate shifts. The spatial organization of the biofilm communities changed from a random distribution of cells when cultivated using TSB to a more specialized arrangement to form tree-like cell clusters when cultivated using 2,4,6-T.





**Figure 3.2.7.** Relationship between degree of refractoriness of the substrate and cell counts of biofilm communities. Note the reduction in the number of biofilm community emigrants in response to substrate refractoriness. CFU ml<sup>-1</sup> of emigrants was higher in the second reorganizational cycle (Cycle II - S and Cycle II - G) which may have resulted from pre-exposure of the communities to refractory substrates and the consequent restructuring process.

number of emigrants during the second cycle of sudden transitions (Cycle II - S) were generally higher than the numbers of Cycle I - S. At all stages of Cycle I - S, Cycle II - S and Cycle II - G, the number (CFU ml<sup>-1</sup>) of emigrants was significantly higher ( $p < 0.01$ ) in the repeatedly inoculated communities than in the communities inoculated only at the beginning of the experiment.

#### **3.2.3.4 Structure and diversity of emigrant populations**

Substrate shifts during both sudden and gradual environmental cycles resulted in the reshuffling of members within biofilm communities, as revealed by the changes in the number of colony morphotypes detected when flow cell emigrants were plated. Various colony morphotypes detected during sudden and gradual environmental transitions are shown in Table 3.2.1. Figure 3.2.8 shows the process of restructuring that occurred within repeatedly and initially inoculated communities of cycle I – S, in response to an environmental transition from sodium benzoate to 2,4,6-T as sole carbon source. When the initially inoculated community was subjected to the substrate shift, the patterns of restructuring of emigrant populations showed the disappearance of one morphotype (mpt - 6) and appearance of four new morphotypes (mpt - 11, 14, 15 and 16) in response to the transition. More significant changes occurred in the repeatedly inoculated biofilm community, which lost three of its members (morphotypes) and gained seven new members upon transition. During these transitions, the appearance, propagation and disappearance of some of the emigrants occurred as sets. For example, the morphotypes 7 and 8 were present throughout the transition from benzoate to 2,4,6-T, and propagated as a set (Set 1). This set was present in both repeatedly inoculated and initially inoculated communities. Similarly, during adaptation of the initially inoculated biofilm community to 2,4,6-T, two distinct sets consisting of morphotypes 11 and 16 (Set 2), and morphotypes 14 and 15 (Set 3) appeared. When repeatedly inoculated biofilm communities were subjected to the substrate shift, morphotypes 11, 14 and 16 and morphotypes 9, 12, 13 and 15 appeared as two distinct sets (Set 2 and 3 respectively), whereas morphotypes 6, 9 and 10 disappeared as a set (Set 4). During most transitions, repeatedly inoculated communities adapted to substrate shifts more rapidly than the communities inoculated only at the beginning of the experiment. For example, when subjected to a substrate shift from benzoate to 2,4,6-T, repeatedly inoculated biofilm communities required a shorter period (140 h) for restructuring and stabilization than initially inoculated communities (175 h) (Figure 3.2.8).

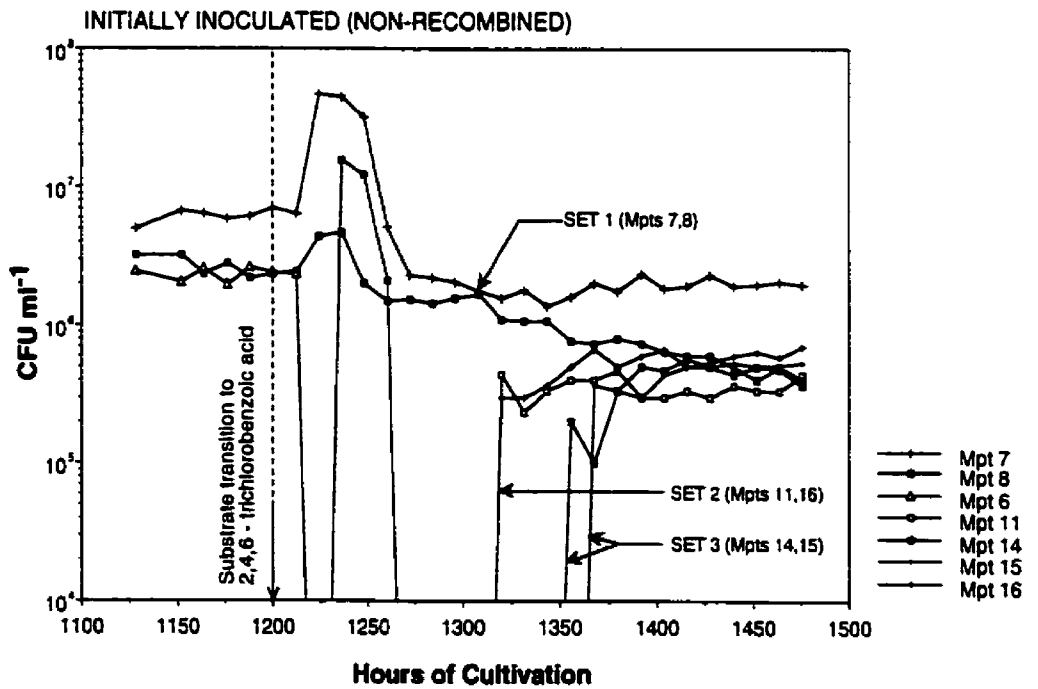
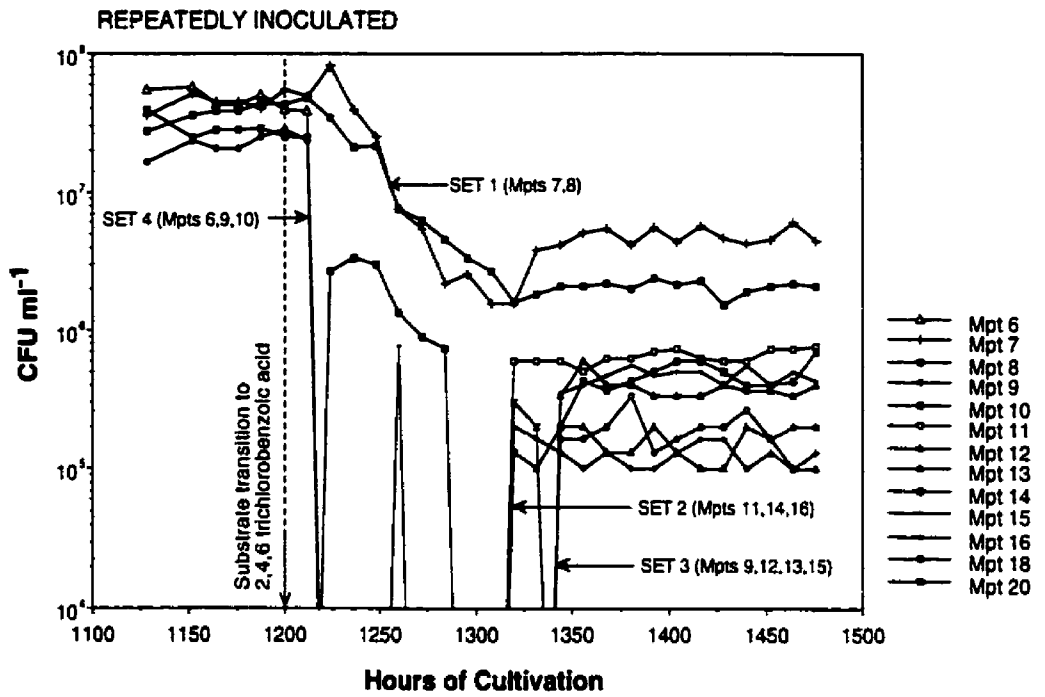
**Table 3.2.1.** Morphological characteristics of various colony morphotypes obtained during the emergence of biofilm communities in response to sudden and gradual environmental transitions

Colony morphology*								
Morphotype	Form	Elevation	Margin	Surface	Optical Characters		Color	Size (mm)
Mpt1	Ir	Ra	Ud	Ru	Tl	Gl	Np	2
Mpt2	Ci	Cv	En	Co	Tl	Gl	Np	2
Mpt3	Ci	Cv	En	Sm	Op	Gl	Np	3
Mpt4	Ir	Ub	Ud	Ru	Op	Du	Np	1
Mpt5	Ci	Cv	En	Sm	Tl	Gl	Yellow	1
Mpt6	Ci	Pu	En	Co	Op	Gl	Yellow	1-2
Mpt7	Ci	Cv	Ud	Sm	Op	Gl	Brown	1
Mpt8	Pc	Cv	En	Ru	Tl	Gl	Np	1
Mpt9	Pc	Pu	En	Sm	Tl	Gl	Np	1
Mpt10	Ci	Ra	Ud	Co	Tl	Gl	Np	1-3
Mpt11	Ci	Ra	En	Sm	Tl	Gl	Np	1-2
Mpt12	Ci	Ub	En	Sm	Tl	Du	Np	1-2
Mpt13	Ci	Pu	En	Ct	Op	Gl	Brown	1-2
Mpt14	Ir	Cv	Ud	Ra	Op	Gl	Np	1-2
Mpt15	Ci	Ub	En	Sm	Op	Du	Np	2-3
Mpt16	Ir	Cv	Ud	Co	Op	Du	Brown	1-2
Mpt17	Ir	Ub	Ud	Sm	Tl	Gl	Orange	2-3
Mpt18	Pc	Cv	En	Sm	Op	Gl	Brown	1-2
Mpt19	Ir	Ra	En	Co	Op	Gl	Np	1-2
Mpt20	Ir	Ub	Ud	Ra	Op	Gl	Np	2-3
Mpt21	Ir	Ub	En	Co	Op	Gl	Np	2-3
Mpt22	Ir	Ub	En	Co	Tl	Du	Brown	2-3

**Colony morphology codes - Form:** Pc - Punctiform; Ir-Irregular ; Ci-Circular;  
**Elevation:** Ra-Raised, Cv-Convex, ; Pu-Pulvinate; Ub-Umbonate ; Fl-Flat;  
**Pigmentation:** Np-non-pigmented; **Margin:** En-Entire; Ud-Undulate; **Surface:** Sm-Smooth; Ct-contoured; Rd-Radiate; Co-Concentric; Ru-Rugose; **Optical characters:** Op-Opaque; Tl-translucent; Du -Dull; Gl - Glistening

**Figure 3.2.8.** (Facing page) Effect of community restructuring on the emigration patterns detected when repeatedly inoculated and initially inoculated biofilm communities were subjected to a substrate transition from benzoate to 2,4,6-T. Repeated inoculation decreased the time required for the biofilm community to restructure and stabilize. It also resulted in higher number, richness and diversity of emigrants than when no external source of recombination was permitted (when communities were inoculated only at the beginning of the experiment). Note that during the adaptation of the repeatedly inoculated biofilm community to a substrate transition from benzoate to 2,4,6-T, sets of morphotypes appeared (Set 2 and 3), disappeared (Set 4) or stably remained throughout the transition (Set 1). The appearance and disappearance of bacteria as sets of organisms suggest that bacterial adaptation to environmental stresses may occur as functional sets of interacting organisms.





Organizational timelines were used to represent the patterns of emigration (presence and absence of specific morphotypes within the emigrant population structure) that appeared in response to sudden (Figure 3.2.9) and gradual environmental transitions (Figure 3.2.10). In the communities subjected to sudden transitions, the patterns of emigration that arose in response to a specific substrate shift were not the same in the first and second cycles of transitions (Figure 3.2.9). However, restructuring through internal recombination (within initially inoculated communities) and restructuring through both internal and external recombination (within repeatedly inoculated communities) may have permitted some members of the biofilm communities to be present in both Cycle I – S and Cycle II – S during cultivation using the same carbon substrate. When communities were subjected to gradual, stepwise environmental transitions also, morphotypes 7 and 8 seem to be present as a set throughout most of the transitions, except for a brief period during transition from 2,4,6-T to starvation and from starvation to TSB (Figure 3.2.10). Similarly, morphotypes 19 and 20, and morphotypes 13,15 and 18 appeared and disappeared as sets during stepwise transitions from benzoate to 2,4,6-T to starvation.

Recoverable diversity of the emigrants determined based on colony morphotypes (Figure 3.2.11 A and B - Richness or the number of colony morphotypes; Figure 3.2.11 C and D - Shannon indices  $H'$ ) was generally higher in repeatedly inoculated communities of both Cycle I-S and Cycle II - S than in the initially inoculated communities cultivated using the same substrate. However, Shannon diversity indices did not show any specific trend based on substrate refractoriness or the nature of transition (sudden or gradual) or cycle of adaptation (I or II).

### **3.2.3.5 Community-level plasmid profiles**

Biofilm communities cultivated using sodium benzoate as sole carbon source, harbored two plasmids of sizes 33 and 29 kb respectively (Figure 3.2.12) as determined from the plasmid profiles of emigrant populations (representing the emigrant portion of the biofilm community). Two plasmids were also detected within 2,4,6-T-cultivated communities and were of the same sizes as those found in benzoate communities. No plasmids could be detected in the TSB or starvation communities, when they were enriched using tryptic soy broth. However, when either of these communities were enriched using sodium benzoate or 2,4,6-T as sole carbon source, two plasmids of sizes similar to those found in the original benzoate or 2,4,6-T communities could be detected. When the individual morphotypes isolated from the emigrant communities were

### REPEATED INOCULATION

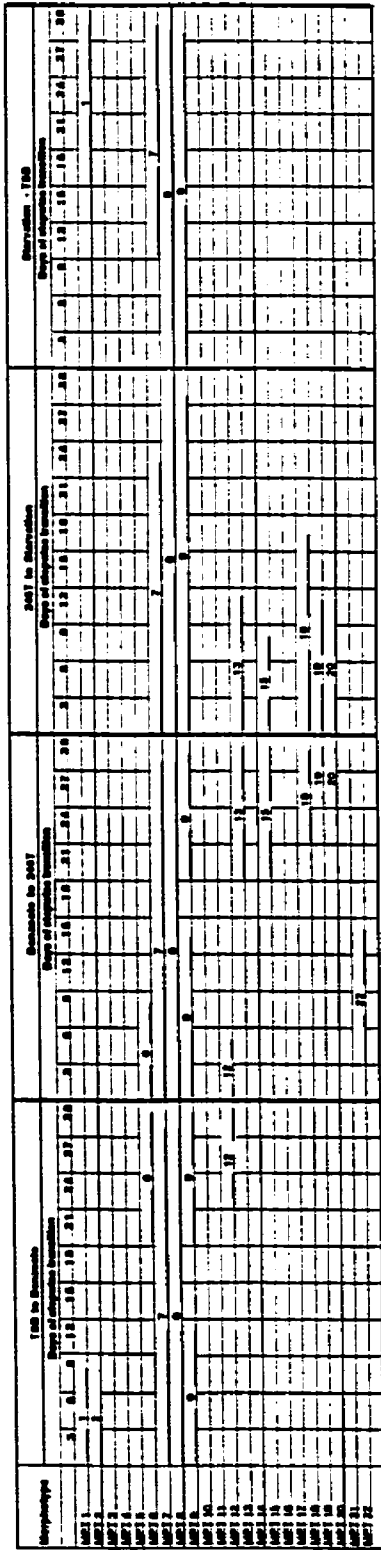
Morphotype	CYCLE I - S				CYCLE II - S				TSB
	TSB	Benzoate	246T	Starvation	TSB	Benzoate	246T	Starvation	
MPT 1	1				1				1
MPT 2	2				2				2
MPT 3	3								
MPT 4	4								
MPT 5	5								
MPT 6	6								
MPT 7				7					
MPT 8				8					
MPT 9			9			9			
MPT 10		10							
MPT 11				11					
MPT 12			12			12			
MPT 13			13						
MPT 14			14						
MPT 15			15						
MPT 16			16				16		
MPT 17									
MPT 18			18						
MPT 19							19		
MPT 20			20						
MPT 21							21		

### INITIAL INOCULATION

Morphotype	CYCLE I - S				CYCLE II - S				TSB
	TSB	Benzoate	246T	Starvation	TSB	Benzoate	246T	Starvation	
MPT 1									
MPT 2									
MPT 3	3								
MPT 4									
MPT 5	5								5
MPT 6	6				6				
MPT 7				7					
MPT 8				8					
MPT 9						9			
MPT 10									
MPT 11				11			11		
MPT 12									
MPT 13									
MPT 14			14						
MPT 15			15						
MPT 16			16						
MPT 17							17		
MPT 18									
MPT 19									
MPT 20									
MPT 21									

**Figure 3.2.9.** Organizational timelines showing the emigration patterns of repeatedly inoculated and initially inoculated biofilm communities in response to cycles of sudden environmental transitions (Cycle I - S). Note that some morphotypes were present throughout the entire cycle of transitions in both repeatedly inoculated and initially inoculated biofilm communities, whereas others appeared and disappeared at various stages of the environmental cycle. However, both internal and external processes of recombination might have permitted some members of the communities of cycle I - S to be present in the communities of cycle II - S also.

Cycle # - G - REPEATED INOCULATION



Cycle # - G - INITIAL INOCULATION

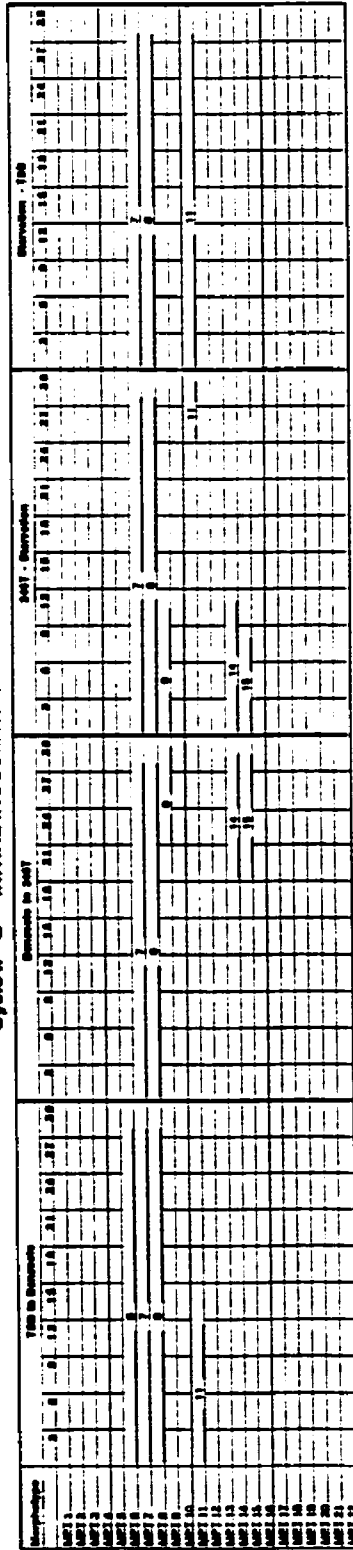
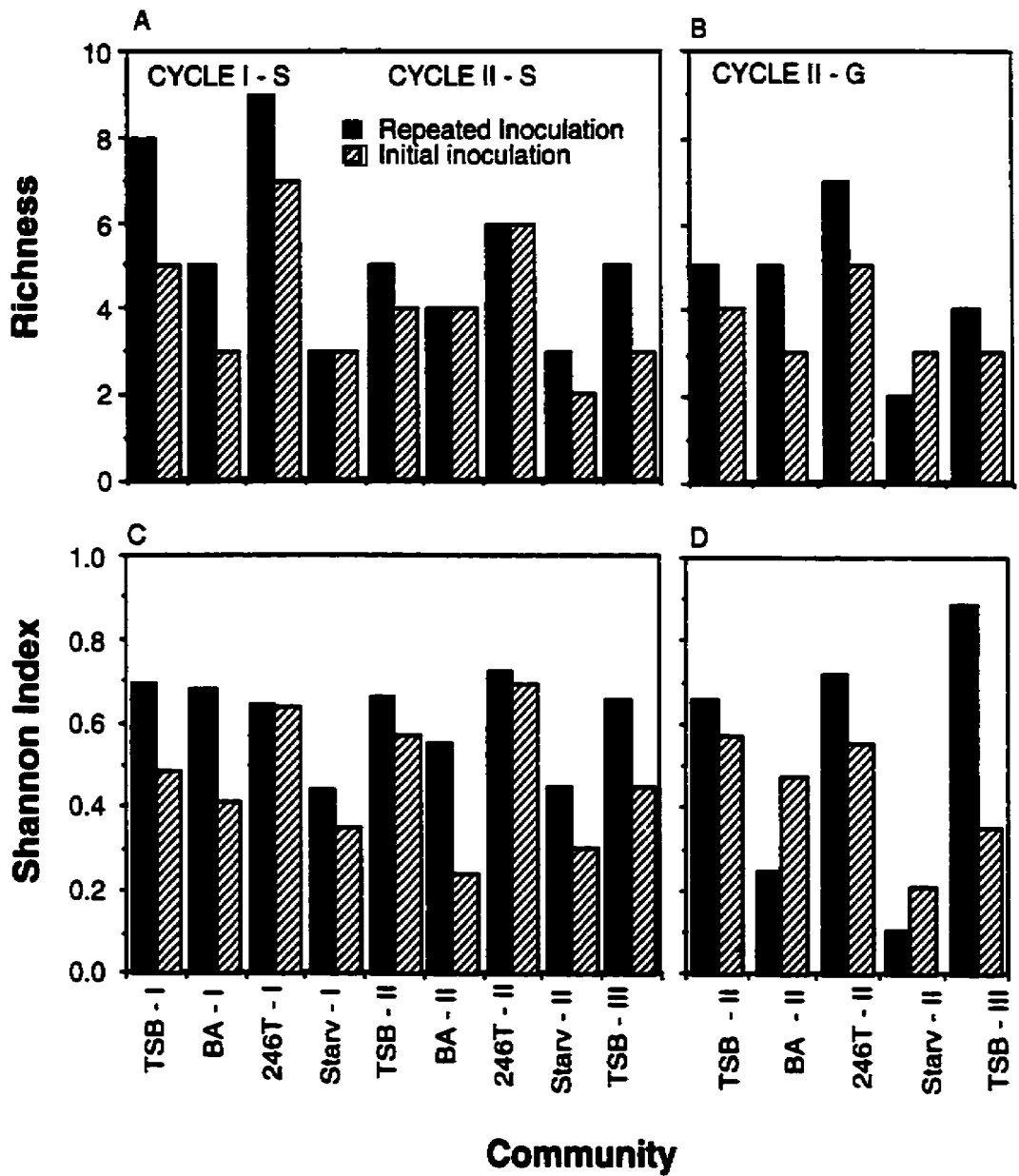
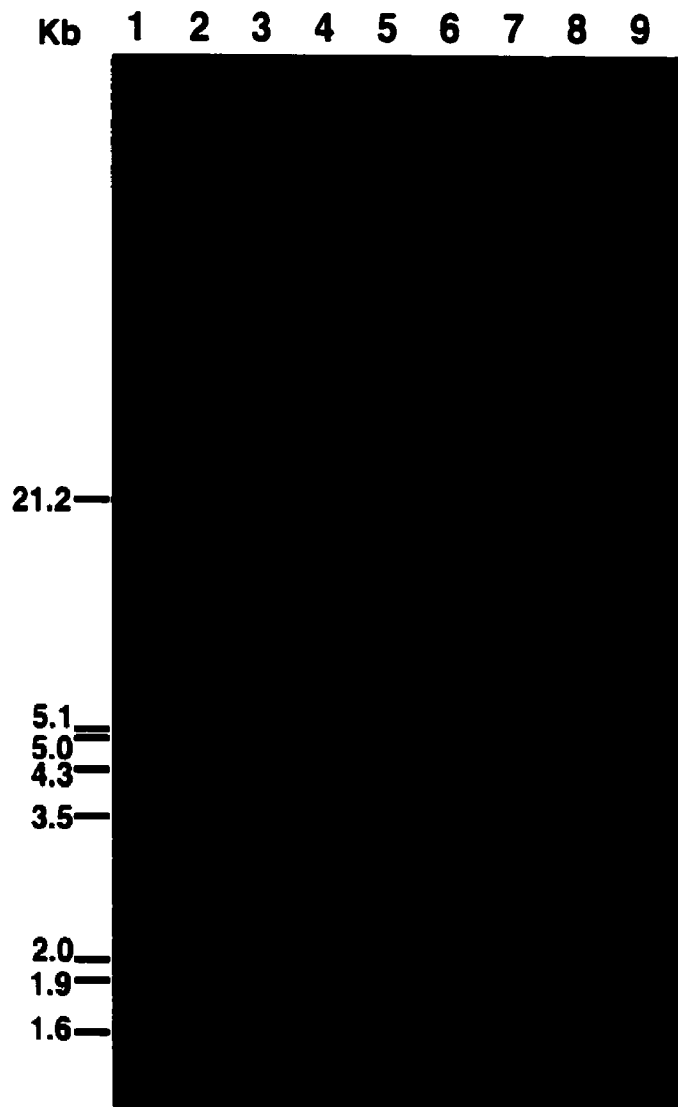


Figure 3.2.10. An organizational timeline showing the patterns of emigration when communities were subjected to gradual or stepwise environmental transitions. Note that some morphotypes appeared and disappeared as sets in response to the transitions.



**Figure 3.2.11.** Recoverable diversity (A and B. Richness or the number of colony morphotypes, C and D. Shannon indices  $H'$ ) of biofilm emigrants. The diversity was generally higher in the repeatedly inoculated biofilm communities of Cycle I-S and Cycle II - S, than in communities inoculated only at the beginning of the experiment. Repeated inoculation thus might have offered a wider gene pool from which useful interactions could be selected, in turn enhancing the growth and adaptation of the biofilm communities subjected to environmental transitions.

**Figure 3.2.12.** (Facing page) Plasmid profiles of microbial communities (subjected to sudden environmental transitions) and of control strains. Lanes: 1. Lambda X Hind III; 2. TSB community; 3. Benzoate community; 4. 2,4,6-T community; 5. Starvation community; 6. TSB community enriched using benzoate 7. Starvation community enriched using benzoate; 8. pAW708 of *E.coli* strain DH5; 9. pCR2.1 of *E.coli* strain TOPO. Note that benzoate and 2,4,6-T communities harbor 2 plasmids each. These plasmids can be detected in TSB and starvation communities, when they were enriched using benzoate (lanes 6 and 7) or 2,4,6-T (not shown).



analyzed for their plasmid content, a morphotype (Mpt-8) that harbored these two plasmids was detected. This strain was detected only in low numbers within TSB and starvation communities, and found to be enriched when these communities were cultivated using sodium benzoate or 2,4,6-T as sole carbon source. This enabled detection of the plasmids harbored by this morphotype.

### **3.2.4 Discussion**

Earlier studies concerning the effects of environmental perturbations on microbial communities have focused mainly on the impacts of organic carbon amendments by pulse feed, step feed or periodic feed patterns (Cohen et al., 1981; Smith and McCarty, 1989; Hickey and Switzenbaum, 1991; Gupta et al., 1994). These substrate perturbations led to changes in the structure or the species composition of microbial communities as a mechanism of adaptation. However, in nature, the factors implicated in environmental perturbations are much more complex. Microbial communities are perturbed by an interplay of physicochemical (changes in the environmental concentrations and gradients of growth substrates, pH, redox potential, etc.) and biotic (effects of micro and macrobiota sharing the same ecosystem) factors. This is especially evident in aquatic ecosystems, where flow influences the flux of nutrients as well as influx and efflux of other microflora. In such systems, the planktonic bacteria can be considered as dynamic 'gene pools', which are able to colonize new environmental niches, and their significance in the new environment might be reflected by a significant shift in the structure and metabolic capability of the native community. If proliferation occurs at multiple levels of biological organization (i.e., at the level of communities as well as individuals), then repeated inoculation with a diverse inoculum should optimize community-level adaptation in terms of the time required for the community to restructure and stabilize as well as the diversity and productivity of the resulting assemblage of organisms. Hence, in the current studies, the effect of repeated inoculation was examined as an external source of organismal recombination, under conditions of cyclic environmental shifts from labile to refractory carbon substrates and to a condition of starvation (no carbon).

The results showed that there was a decline in the thickness and percent area coverage of biofilm communities as well as the number of emigrants upon successive shifts from TSB (a labile carbon substrate) to benzoate (as a model of somewhat refractory, ring-structured compound), to 2,4,6-T (as a model of more refractory, chlorinated and ring-structured compound) to starvation (carbon limitation). Thus, these



shifts constituted a sequence of incremental level of stresses on the communities. As hypothesized previously, repeated inoculation of biofilm communities resulted in an enhanced adaptation (greater thickness and percent area coverage of biofilms as well the number of the emigrants), than when the communities were inoculated only at the beginning of the experiment. This suggested that community-level synergisms (resulting from both internal and external recombination processes) are required for optimal growth of communities just as molecular and cellular synergisms are required for the optimal growth of individuals. James et al. (1995) indicated that interspecies interactions influence the structure and physiology of biofilm communities during the course of their formation. During the present study, repeated inoculation may have permitted a larger genetic pool from which active interactions were selected. This is consistent with the proliferation theory (Caldwell et al., 1997b; Caldwell, 1999), which suggests that the process of bacterial self-organization not only involves mutation and recombination of genes, but also the recombination of organisms in communities (immigration and emigration) as well as the spatial repositioning of organisms within geographic boundaries (of the flow cell and its surface environments).

A number of techniques have been developed for the qualitative analysis of microbial community structure. These include ribosomal RNA (rRNA) analysis using taxon specific probes (Fry et al., 1997), rRNA sequence comparisons (Devereux et al., 1996), whole community DNA hybridization (Griffiths et al., 1996), whole community phospholipid fatty acid profiles (Frostegard et al., 1996; Sundh et al., 1997) and carbon source utilization profile analysis using Biolog<sup>TM</sup> microtiter plates (Bossio and Scow, 1995; Garland, 1996). Some of these methods can only account for the overall changes in community structure, whereas, others are limited in application when the actual process of restructuring (immigration, emigration and reshuffling of members within a community) has to be described. In the current studies, colony morphotypes that appeared when the effluent obtained from flow cells was plated on TSA were used to quantify emigration (the number and diversity of emigrant populations) from the community. Haldeman and Amy (1993), based on their studies on the colony morphotypes of bacterial isolates obtained from various rock samples, concluded that the colony morphotype can be considered as a biotype and could provide an accurate basis to define recoverable diversity. Colony development has previously been used to characterize bacterial communities on wheat roots (De Leij et al., 1994). The selectivity of plating media and cultural methods was unavoidable in this process. During the present study, specific morphotypes were followed through each transition using

countable plates of lowest dilution containing 30-100 colonies and incubated for the same duration. This resulted in colony sizes and morphologies that were comparable between two sampling periods. In addition, colonies of the same morphotype obtained at two different sampling periods resulted in identical substrate utilization patterns using BIOLOG™ microtitre plates, providing evidence that colony morphotypes served as a reliable tool for monitoring the emergence of community structure.

Atlas (1984) suggested that diversity measurements would be useful in monitoring changes due to ecosystem perturbations. It was also indicated that the diversity is less in communities which are under stress (Atlas, 1984). In the case of cometabolic interactions and sequential degradation of pesticides, species diversity is crucial in enabling breakdown of complex bond structures. This may seem to imply that communities under stress (conditions such as chemical complexity of the growth substrate) will be diverse, or at least best benefit from increased diversity. During the present study, the changes in diversity in response to substrate stress did not follow a specific trend. However, emigrants of repeatedly inoculated biofilm communities, in general, had a higher richness and diversity than emigrants of initially inoculated communities. Higher richness and diversity of the repeatedly inoculated communities might have resulted from immigration of new members into the community, which was not permitted in communities inoculated only at the beginning of the experiment. In both cycles of sudden environmental transitions, a shift from TSB to sodium benzoate as sole carbon source decreased the richness of emigrants, whereas a shift from benzoate to 2,4,6-T resulted in an enhanced richness. The concentration of sodium benzoate used in the current studies was similar to its concentration used for food preservation (Chipley, 1983). Thus the reduction in species richness upon a substrate shift from TSB to benzoate might be a direct consequence of the toxicity of benzoate causing a decline in the number of surviving species. However, the increase in richness and diversity noted when the community was shifted to 2,4,6-T may have resulted from a requirement for co-ordinated responses by a diverse group of bacteria for its breakdown and utilization.

At the end of the first cycle of sudden shifts (Cycle I - S), the starvation community was then subjected to a second cycle of sudden environmental transitions. Repeated environmental cycling was used to determine whether pre-exposure of a microbial community to a refractory organic compound would enhance the community's ability to utilize the same compound upon subsequent exposures. Adaptation of microbial populations to use refractory or toxic compounds as carbon and energy

sources has been well demonstrated (Spain and Van Veld, 1983; Aelion et al., 1987; Barkay and Pritchard, 1988; Van der Meer, 1994). Adaptation of microorganisms to high toxicant concentrations would play an important role in biodegradation (Spain et al., 1980). Spain and Van Veld (1983) defined adaptation as a change in the microbial community that increases the rate of transformation of a test compound as a result of a prior exposure to that compound. In the current studies, higher CFU ml<sup>-1</sup> of emigrants observed during the second set of reorganizational cycles (Cycle II - S and Cycle II - G) might have resulted from pre-exposure (acclimation) of the communities to these substrates during Cycle I - S. Enhanced growth of biofilm communities due to repeated environmental cycling also suggests that the cellular positioning information accrued from one cycle to the next and may thus have been heritable just as DNA is heritable at the cellular level. Thus the process of acclimation would involve not only heritable changes in the DNA, but also heritable changes in the composition and spatial positioning of individuals within communities.

A closer analysis of the emigration patterns that resulted from sudden environmental transition (Figure 3.2.8) indicated that substrate perturbations led to the collapse of existing community structures. However, the continuous process of reorganization resulting from both external and internal sources of recombination (in the case of repeatedly inoculated communities) and from the internal recombination only (in the case of communities inoculated only at the beginning of the experiment) probably led to the development of new, stable sets of emigrant populations. Repeated inoculation also decreased the time required for the community to restructure and stabilize in response to most transitions (detected based on the stability of emigration patterns). The emigration patterns of both repeatedly and initially inoculated biofilm communities showed the appearance or disappearance of sets of organisms during bacterial adaptation to refractory substrates. During a sudden environmental transition from benzoate to 2,4,6-T, the appearance (Sets 2 and 3), disappearance (Set 4) and propagation (Set 1) of stable sets of organisms suggests the possibility that the organisms within these sets may be related and that bacterial adaptation to environmental transitions may occur as functional sets of interacting organisms. The patterns of emigration detected during the entire course of Cycle I - S have been represented by an organizational timeline as shown in Figure 3.2.9. These patterns were not the same for communities of Cycle I - S and Cycle II - S, even when they were cultivated using the same substrate. This shows the inherent flexibility of community structure. This also shows the predominant roles played by the pre-existing community structure and recombination processes (internal

and external) in defining the final structure of a microbial community cultivated in a particular environment.

After completion of the first cycle, in order to sharply define the process of restructuring in response to environmental transitions, communities were subjected to gradual environmental transitions. Difficulties encountered in developing smooth temporal concentration gradients of two substrates, led to the application of stepwise concentration gradients. Although the stepwise transitions were not useful in defining the community boundaries or detecting the presence of an ecotone (where one set of community-level relationships disappear and new set of relationships appear), they were just as useful as sudden environmental transitions in detecting groups of organisms that appeared or disappeared as sets. This again suggested that the biofilm communities adapted to environmental stresses as sets of interacting organisms, as opposed to functioning exclusively as unrelated individuals.

Substrate shifts also led to changes in the plasmid profiles of the emigrant communities. Two plasmids that appeared when the biofilm communities were cultivated and subsequently enriched using refractory substrates such as sodium benzoate and 2,4,6-T could not be detected within the TSB and starvation communities when enriched using TSB. However, enrichment of TSB and starvation communities using benzoate or 2,4,6-T as sole carbon source resulted in the detection of two plasmids similar to those obtained from original benzoate and 2,4,6-T communities. Plasmids often carry genes responsible for the catabolism of various organic compounds including benzoic acid (Perkins et al., 1990). Enrichment of the plasmid-containing morphotype (MPT - 8) during cultivation of biofilm communities using either sodium benzoate or 2,4,6 - T as sole carbon source, suggests that these plasmids may have a degradative function. This also suggested that community-level restructuring processes affected the frequency of occurrence of specific plasmids, although no evidence for the horizontal transfer of plasmids or plasmid reshuffling among the community members could be detected.

Overall, the results of the present study suggested that bacteria may respond to environmental stresses as sets of interacting organisms (as opposed to functioning exclusively as unrelated individuals) as revealed by the appearance and disappearance of sets of organisms during adaptation of flow cell communities to sudden or gradual substrate shifts. Repeated inoculation as a mechanism of coincidental organismal recombination led to more rapid adaptation and enhanced growth of microbial communities when subjected to environmental transitions from labile to refractory

substrates. These results suggested that proliferation results from recombination events that occur at multiple levels of biological organization, including at the community-level, and hence supported the utility of proliferation theory.

### **3.2.5 Summary**

The aim of this work was to describe and better understand the emergence of bacterial communities in response to environmental transitions from labile to refractory substrates. This involved testing the hypothesis that bacteria self-organize and propagate as functional sets of interacting organisms as well as individual cellular systems. A biofilm community was cultivated in a flow cell irrigated with TSB and then subjected to a cyclic series of environmental transitions. Each cycle of transition consisted of shifts in carbon substrate from TSB (10% v/v) to benzoic acid (BA) (0.15% w/v), to 2,4,6-T (0.05% w/v) (as a model of transition from labile to refractory substrates), followed by a period of starvation (30 days). The appearance and disappearance of specific colony morphotypes when the emigrants were plated on TSA, was used to monitor the process of community-level restructuring during adaptation to environmental transitions. Confocal laser microscopy of flow cells showed that transitions from labile to refractory substrates resulted in a decrease in biofilm thickness and coverage. Substrate shifts also led to changes in the architecture of the biofilm communities. Repeated inoculation of flow cell communities with a composite inoculum resulted in an increase in the number and diversity of emigrants as well as greater thickness and percent area coverage of the biofilms than when the communities were inoculated only at the beginning of the experiment. It also decreased the time required for the community to restructure and stabilize during most transitions. This suggested that organismal recombination, as a mechanism of adaptation, enhanced the growth of microbial communities exposed to environmental stresses. Changes in the profiles of emigrants during adaptation of biofilm communities to sudden and gradual environmental transitions, showed the appearance and disappearance of discrete sets of organisms. This suggested that the biofilm communities responded to environmental stresses as sets of interacting organisms, as opposed to functioning exclusively as unrelated individuals. Although no evidence for horizontal plasmid transfer between morphotypes could be detected, certain plasmids could be enriched during cultivation using refractory carbon substrates such as benzoate or 2,4,6-T, suggesting the possibility of community adaptation at multiple levels of organization including the number and distribution of both cells and plasmids. Enhanced growth of biofilm communities due to repeated

environmental cycling, suggests that the functionality of cellular positioning accrued from one cycle to the next and was thus heritable although it was not necessarily genetically encoded.

### **3.3 Digital image analysis of satellite colonies involved in benzoate resistance and degradation**

#### **3.3.1 Introduction**

According to proliferation theory (Caldwell and Costerton, 1996; Caldwell et al., 1997a, 1997b, 1997c; Caldwell, 1999), synergisms between organisms may be required for the optimal proliferation of communities, just as synergisms between molecules are required for the optimal proliferation of cells. Assuming that the spatial positioning of nucleic acids is the only significant form of biological information (although there are others), that the bacterial genome contains only two different nucleotides (although there are actually four), and that the bacterial genome is only 9 nucleotides long (although the actual length for *E. coli* is  $4.7 \times 10^6$ ), then the maximum number of different bacteria that could be created using the information capacity (potential for variation) residing within a single bacterium would be  $2^9$  or 512. However, the number of different communities that could be created from 3 such bacteria in all 512 forms of each, would be  $512^3$  or 134,220,000. Correcting for redundancy due to the lack of spatial order in communities, the number of combinations would be reduced but much larger than the potential for variation among 3 isolated individuals. Thus the information capacity residing within isolated individuals is always a small fraction of the information potential residing within a community of associated individuals. Most potential thus resides at the community-level of organization and more effort is required to study community-level microbial synergisms (communality) if the significance of this information is to be understood and effectively applied (Caldwell et al., 1997b).

Enhanced growth of biofilm communities in response to coincidental organismal recombination, as well as the appearance and disappearance of sets of organisms during sudden and stepwise environmental transitions as demonstrated during the previous study (section 3.2), suggested the existence of community-level proliferation strategies as postulated by proliferation theory. Another indication of community-level synergisms is the density-dependence of plating efficiency that sometimes occurs when isolating or enumerating microorganisms using plating media. This was first noted by Stevens and Holbert (1990) when plating bacterial communities from subsurface terrestrial environments. Caldwell et al. (1997a) subsequently observed increased plating efficiency at lower dilutions only when plating microbial communities at high concentrations of benzoate, but not at low concentrations. Enhanced plating efficiencies were detected during the present study also, when biofilm communities cultivated using

TSB were assayed for communality by plating them on M-salts medium containing sodium benzoate as sole carbon source. In the present study, digital image analysis was used to quantify the effect of spatial positioning on the growth and survival of colonies during the plating process. Sodium benzoate was used in these studies due to its importance as a model compound in the degradation of organic toxicants and in the preservation of various food products (Bowen et al., 1953; Chipley, 1983; Pylypiw, 1994; Kasrazadeh and Genigeorgis, 1995). Benzoate is also significant due to its inhibitory effect on the bacteria which cause dental plaque (Ostergaard, 1994), food spoilage (Wind and Restaino, 1995; Efiuvwevwere and Akoma, 1997), and foodborne illness (El-Shenawy and Marth, 1988, 1989; Kasrazadeh and Genigeorgis, 1995; Fang et al., 1997).

The results of the present study showed that during the plating process the primary benzoate-degrading organisms of the community protected the other organisms with which they were associated. The protected organisms appeared as satellite colonies located within the vicinity of the primary benzoate-degrading organisms.

### **3.3.2 Materials and Methods**

#### **3.3.2.1 Bacterial communities, strains and media**

The quasi steady-state biofilm community cultivated using TSB described in section 3.2.2.1 was used in the dilution-dependent plating efficiency assay.

The satellite isolates described in this study were maintained on TSA and the primary colony isolates were maintained on benzoate agar described below. Two bacterial strains Mpt4 and Mpt5 (see Table 3.2.1), which were different from all the primary and satellite isolates (based on colony morphotypes on 0.3% (w/v) TSA), were isolated from the TSB community and maintained on TSA. These two bacterial strains, as well as a culture collection strain of *Escherichia coli* (ATCC 25922) were used in the specificity studies.

Benzoate agar and glucose agar, were prepared by addition of appropriate carbon sources to the M-salts medium. Unless specified otherwise, benzoate agar contained 0.15% (w/v) benzoic acid (sodium salt) (Sigma Chemical Co., St. Louis, MO) and glucose agar contained 0.1% (w/v) glucose (BDH Inc., Toronto, ON). The benzoate agar plates were poured using an equal quantity of the medium in each petri dish. This was to prevent any volume-dependent differences in the concentration of growth factors or degradation products that might be released by the primary colonies into benzoate agar.



### **3.3.2.2 Plating assays**

This study involved various plating procedures (as described in sections 3.3.2.2.1 - 3.3.2.2.9) including an initial assay for dilution-dependent plating efficiency of the TSB community on benzoate agar. When satellite colonies were observed on benzoate agar, putative satellite and primary colonies were isolated. Cross-streak and spot inoculation assays were used to confirm association between individual primary and satellite isolates. Additional assays were used to examine synergisms based on interactions among multiple primary and satellite isolates, to quantify interactions between primary and satellite isolates, to identify the mechanisms of their association and to examine the specificity of the associations. Preparation of standard cell suspensions for various plating assays involved growing cells to log phase in TSB (10% v/v), centrifugation at 7800 x g for 10 min. followed by resuspension of cells in physiological saline (0.85%) and adjusting to a turbidity standard to give an approximate cell number of  $10^8$  CFU ml<sup>-1</sup>. Plate incubations were performed at 23±2°C for 7 days.

#### **3.3.2.2.1 Determination of dilution-dependent and propinquity-dependent plating efficiency**

Ten ml of flow cell effluent was collected over a 5 h period, from the flow cell containing the TSB community. The effluent was serially diluted and plated on benzoate agar. The dilution scheme consisted of ten-fold dilutions ( $1 \times 10^{-1}$ ,  $1 \times 10^{-2}$ ,  $1 \times 10^{-3}$  etc.) and subdilutions between two major dilutions (e.g. from  $0.1 \times 10^{-2}$ ,  $0.2 \times 10^{-2}$  ... to  $0.9 \times 10^{-2}$ ). The plates were incubated and the plating efficiency (CFU ml<sup>-1</sup>) was determined at various dilutions. Plating efficiency was also calculated based on spatial propinquity or spatial separation between cells. Determination of propinquity-dependent plating efficiency involved calculation of mean separation distance between cells when they were plated at various dilutions. A procedure similar to that of Thomas et al. (1997) was used for this purpose. The number of cells inoculated per plate (n) at various dilutions was calculated from the cell number in the inoculum (maximum CFU ml<sup>-1</sup> was used in this case) and the corresponding dilution factor. Area of the plate divided by n gives the mean area available to a single cell. Approximating this area to that of a circle ( $\pi r^2$ ), the radius of each circle was determined. Twice this radius was the mean cell separation distance. The cell separation distances (as a measure of propinquity) were then correlated with the plating efficiency.

#### **3.3.2.2.2 Cross-streak assay**

Cell suspensions of putative satellites and primary colonies were streaked perpendicularly against each other on benzoate agar. After incubation for 7 days, the growth patterns of the primary and satellite strains were observed and documented.

#### **3.3.2.2.3 Spot-inoculation assay**

The spot inoculation assay was performed by serially diluting the putative satellites and spreading them on benzoate agar before spotting a 5  $\mu$ l aliquot of a primary isolate cell suspension at the center. After incubation, plates were observed for the appearance of satellite colonies around the spot of primary isolate. As a control to examine any effect of the satellite isolates on the primary colonies, a 5  $\mu$ l aliquot of satellite cell suspension was spot inoculated on a lawn of primary isolate spread on benzoate agar.

#### **3.3.2.2.4 Assay for the detection of synergistic effects involving three or more organisms**

The primary isolates (BD1 and BD2) were streaked on 0.15% benzoate agar containing a lawn of a satellite strain and a second satellite isolate was then streaked perpendicular to BD1 and BD2. Plates were incubated for 7 days and examined for synergistic growth effects resulting from interactions between three or more members.

#### **3.3.2.2.5 Quantitative analysis of interactions between satellite and primary colonies**

Appropriate dilutions of the standard cell suspensions of satellite isolates were spread as a lawn on benzoate agar (each plate received approximately  $10^3$  cells). A 2  $\mu$ l aliquot of standard cell suspension of a primary colony was then spot-inoculated at the center of the plate. Satellite colonies appearing around primary colonies were analyzed using the digital image analysis procedure described below. When the spatial distribution of the satellites was plotted with respect to the location of primary colonies, the slopes were calculated from peak of the curves to the right (Figures 3.3.6 and 3.3.7). This was necessary because of the reduction in the number of satellites  $\text{cm}^{-2}$  adjacent to the primary colony due to the sharing of limiting substrate concentration. Including this effect in the calculation would mask the satellite effect (dependency of satellites on primary colony) quantified from the slope.

### **3.3.2.2.6 Analysis of the effect of satellite cell concentration on primary colony - satellite interactions**

Three 10-fold dilutions of a standard cell suspension of the satellite isolate BS2 were spread in triplicate on plates containing benzoate agar (at satellite concentrations of  $2.2 \times 10^4$ ,  $2.2 \times 10^3$ ,  $2.2 \times 10^2$  satellites  $\text{cm}^{-2}$ , respectively). Two  $\mu\text{l}$  of the standard cell suspension of a primary colony (BD2) was then spot-inoculated at the center of satellite lawns, plates were incubated and analyzed using the satellite analysis procedure as described in section 3.3.2.3.

### **3.3.2.2.7 Analysis of the influence of satellites on primary colonies**

Satellite and primary isolates were spotted on benzoate agar plates in the form of a rectangular 3 X 3 matrix. Three spots of primary isolate (aliquots of 2  $\mu\text{l}$  each) formed the central row, whereas, a suspension of the satellite isolate (aliquots of 2  $\mu\text{l}$  each) was spotted in top and bottom rows. Following incubation, the growth of primary and satellite isolates was digitized as described in the image acquisition part of the digital image analysis procedure. Subsequently, the sizes of primary colony spots were determined using the following procedure: NIH image software (National Institutes of Health, Bethesda, Maryland; <http://rsb.info.nih.gov/nih-image>) was used to invert and to redefine the image by setting a threshold. The mean size of primary colonies (BD1 and BD2), with and without adjacent spot inoculation of satellites was then determined using the particle analysis function of NIH image.

### **3.3.2.2.8 Analysis of the mechanism of satellite associations**

Serial dilutions of satellite cell suspensions were spread-plated on benzoate agar with 9 different concentrations (0.015%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, 0.5%, 1.0%, 1.5%) of benzoic acid, with and without added glucose ( $1\text{g l}^{-1}$ ). Primary isolates were then spot-inoculated at the center of satellite lawns. Controls with no inoculation of primary isolate were also maintained. Growth of satellite colonies on these plates was then compared with the growth on plates containing glucose as sole carbon source. In another set of experiments, 10 mm size agar plugs removed from areas adjacent to 7 day old, spot-growth of primary isolates, were placed on 0.15% benzoate agar plates previously inoculated with a lawn of satellite colonies. The plates were examined for the development of satellite colonies around agar plugs. 10  $\mu\text{l}$  aliquots of homogenates obtained by homogenizing an agar plug with 1 ml quantity of M-salts solution were also

used to spot on lawns of satellite isolates and after 7 days incubation, the plates were examined for the development of satellite colonies around the spots.

### **3.3.2.2.9 Analysis of the specificity of satellite associations**

The specificity of the two sets of bacterial interactions (BD1 association and BD2 association) was tested by cross-streak assays on benzoate agar with or without added glucose ( $1\text{g l}^{-1}$ ). The standard cell suspensions of the primary colonies BD1 and BD2 were streaked perpendicularly against standard cell suspensions of *E. coli* (ATCC 25922) and two bacterial strains (MPT1 and MPT2) from the TSB community. The plates were incubated and examined.

### **3.3.2.3 Satellite analysis procedure**

Analysis of size and spatial distribution of satellite colonies around primary colonies involved image acquisition and digital image analysis, as detailed below.

#### **3.3.2.3.1 Image acquisition**

The satellite associations which developed on benzoate agar plates were digitized using a Scion video board and NIH image software in conjunction with a Tamron SP - CF macro lens (Tamron Co. Ltd., Japan) mounted on a Cohu high performance CCD camera (Cohu Inc., Electronics Division, San Diego, CA). The lens was stopped down ( $f32$ ) to reduce spherical aberration and to increase the depth of field. The petri dish with satellite colonies to be digitized was placed on top of an inverted petri dish containing agar (1.5% w/v) with 0.12% (w/v) brilliant black food colorant (Sigma Chemical Company, St. Louis, MO) in order to reduce the background brightness to as close to zero as possible (Wivcharuk, 1998). The electronic gain was manually set from within the NIH image software to produce high contrast images for analysis purposes. For each plate, 20 images were digitized at video rate and averaged to minimize electronic interference.

#### **3.3.2.3.2 Digital image analysis**

Analysis of the size and number of satellites, as a function of the distance from the primary colony, involved the use of Superpaint (Silicon Beach Software, Inc. San Diego, CA), Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA), and NIH image applications. A mask of nested circular rings with diameters ranging from 42 pixels to 462 pixels was created using Superpaint. The camera height and magnification

were set such that the width of each analysis ring corresponded to a distance of 1.5 mm (a distance of 21 pixels). This mask was used to create a template in Adobe Photoshop with each ring in a separate layer, so that if one layer was made transparent the colonies in the image appearing through the transparent ring could be analyzed. For analysis, the image showing the transparent ring with colonies, was copied to NIH image, inverted, thresholded (pixels above a certain brightness or gray level set to white and those below this brightness set to black, resulting in a binary image), and the number of colonies and colony sizes were measured within each ring. Since each ring was placed at a known distance from the primary colony, the number and size of satellites (within that ring) as a function of distance could be determined.

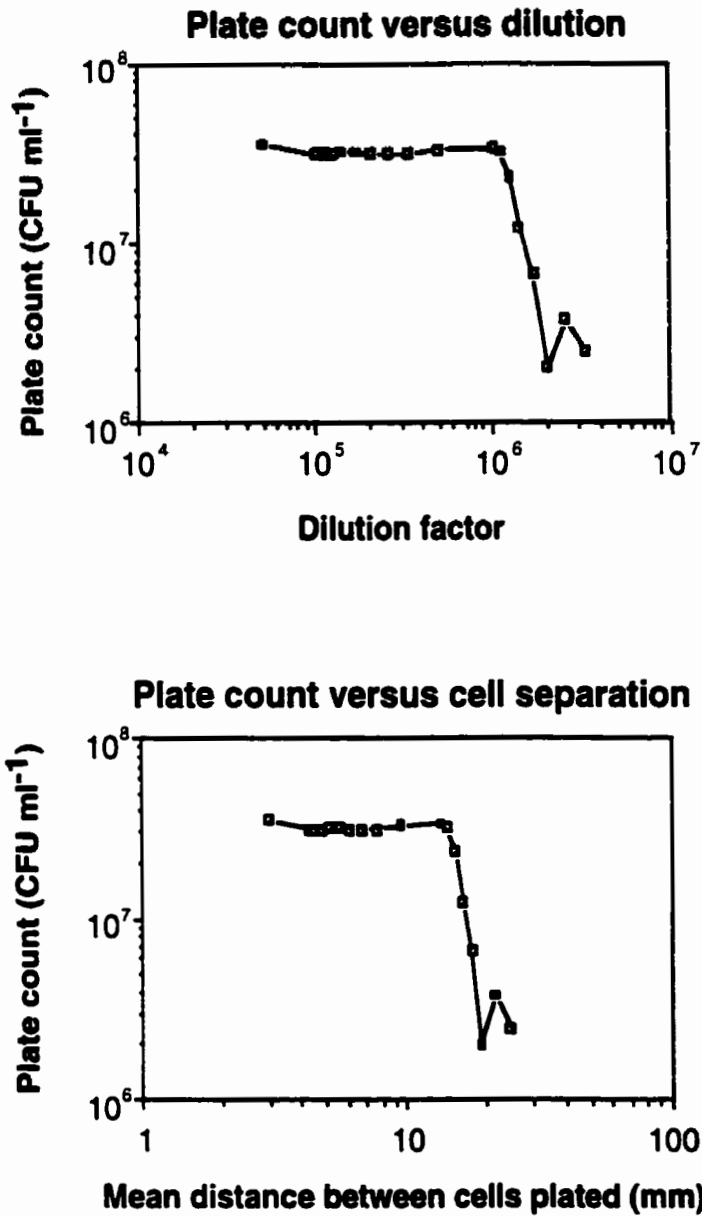
#### **3.3.2.4 Characterization of primary and satellite colonies**

Primary and satellite isolates were streaked on TSA and incubated at  $23\pm 2^{\circ}\text{C}$  for 7 days. The morphotypes of isolated colonies were determined using the descriptions of Benson (1990) and Eklund and Lankford (1967). Gram reaction and cell morphologies were determined using light microscopy. Tentative identification of the isolates was based on API-NFT (BioMerieux Vitek, Inc., Hazelwood, MO) identification profiles (following manufacturer's instructions) and confirmation was based on Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). This included the tests of motility, production of diffusible fluorescent pigments, oxygen requirement, oxidase and other biochemical characters (nitrate reduction, arginine dihydrolase, gelatinase, esculin hydrolase, urease activities, indole production, glucose fermentation) and assimilation of various sugars and organic acids (glucose, arabinose, mannose, mannitol, N-acetyl glucosamine, maltose, gluconic acid, capric acid, adipic acid, malate and citrate).

### **3.3.3 Results**

#### **3.3.3.1 Effect of dilution on the plating efficiency**

When the TSB community was plated on benzoate agar, there was an inverse relationship between plating efficiency and dilution (Figure 3.3.1). The CFU  $\text{ml}^{-1}$  were higher at lower dilutions (at lower cell separation distances) and vice versa. A maximum CFU of  $6.0 \times 10^6 \pm 5.5 \times 10^5 \text{ SD ml}^{-1}$  and a minimum of  $3.7 \times 10^5 \pm 6.4 \times 10^4 \text{ SD ml}^{-1}$  were obtained for the same biofilm community at the various dilutions counted.



**Figure 3.3.1.** Dilution-dependent plating efficiency observed when biofilm communities were plated on a synthetic medium containing 0.15% sodium benzoate as sole carbon source. Note the increase in the colony counts at lower dilutions (top plot). A similar relationship was detected between mean separation distance between cells plated and the plating efficiency (bottom plot). The increased proliferation (higher colony counts) at lower dilutions was concomitant with the formation of satellite colonies around primary colonies.

### 3.3.3.2 Screening for satellite associations

When the TSB community was plated at lower dilutions on benzoate agar plates, satellite colonies appeared around two types of primary colonies (Figure 3.3.2). When the total number of primary colonies on a plate was high, the satellite effect was diminished and the satellites tended to grow across the entire plate, as they did when the concentration of benzoate in the plating medium was reduced to less than 0.15%. The irregular distribution of both primary colonies and satellite colonies also tended to obscure the satellite effect, which was most clearly seen when a primary colony was inoculated upon a diffuse lawn of satellite colonies.

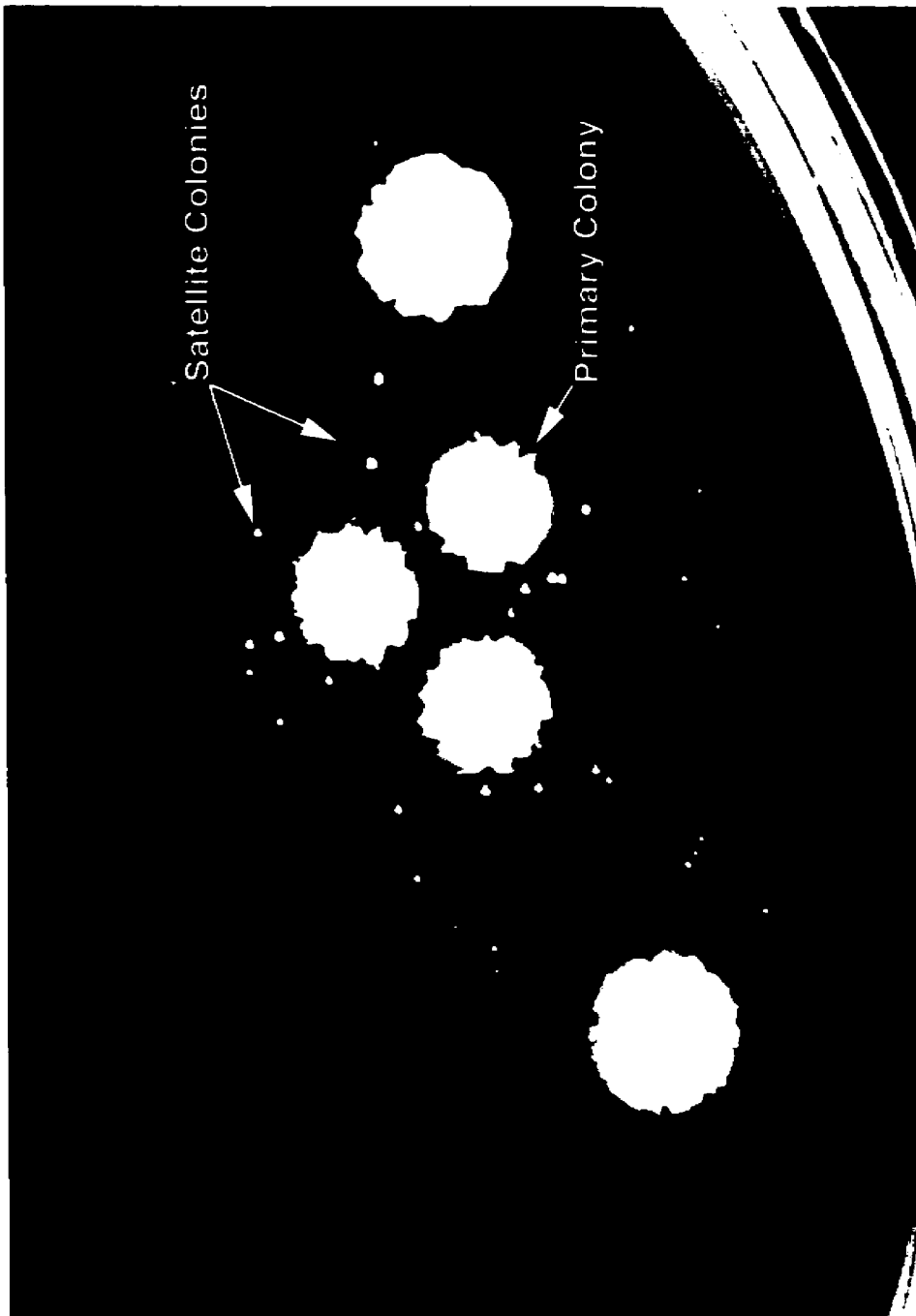
Each satellite was isolated and tested for interaction with the two primary colonies on 0.15% benzoate agar. Two associations were found, each involving a set of 7 satellite colonies. These were designated as the BD1 association (benzoate degrading association 1) and BD2 association (benzoate degrading association 2), based on the primary colonies (strains BD1 and BD2) which allowed satellite colony development. Both primary colonies, BD1 and BD2 were identified as *Pseudomonas fluorescens* strains. Based on API and biochemical tests, all of the satellites were members of the same genus as the primary colony (*Pseudomonas*) but only one fell in the same species (*fluorescens*). The satellite colonies were *Pseudomonas* sp. strain BS1 (benzoate satellite 1), *Pseudomonas* sp. strain BS2, *Pseudomonas* sp. strain BS3, *Pseudomonas fluorescens* strain BS4, *Pseudomonas viridiflava* strain BS5, *Pseudomonas* sp. strain BS6 and *Pseudomonas* sp. strain BS7. Both BD1 and BD2 supported the 7 satellites BS1, BS2, BS3, BS4, BS5, BS6 and BS7. Positive interaction between primary colonies and satellites of each association was confirmed by cross-streak assays, in which the growth of primary isolates promoted the growth of cross-streaked satellite strains (Figure 3.3.3). However, no synergistic growth effect due to interactions requiring three or more isolates could be detected. In addition, the satellites were unable to grow independently on 0.15% benzoate agar.

### 3.3.3.3 Size and spatial distribution of satellites

The size and spatial distribution of the satellites were analyzed using digital image analysis. Within both sets of interaction (BD1 and BD2 associations), the satellite colonies decreased in size with distance from the primary colony (Table 3.3.1). The mean satellite concentration (satellites mm<sup>-2</sup>) also decreased with distance from the

**Figure 3.3.2.** (Facing page) Formation of satellite colonies around primary colonies when biofilm communities were plated on a synthetic medium containing 0.15% sodium benzoate as sole carbon source. Note that the satellite effect is somewhat obscured by the random distribution of both the satellites and primary colonies. Screening for associations between presumptive satellite colonies and presumptive primary colonies on these plates, detected two benzoate degrading associations (BD1 association and BD2 association).





**Figure 3.3.3.** (Facing page) Cross-streak assay shows the interaction effect between an individual satellite and a primary colony but is difficult to quantify. Shown is BS1 streaked against BD1. Note the growth of BS1 closer to the intersection of the streaks, indicating its dependence on BD1 for growth on benzoate agar.



**Table 3.3.1.** Effects of association between primary and satellite colonies as determined by satellite analysis procedure

Primary colony	Satellite colony	Slopes of satellite analysis curves	
		Mean satellite diameter (mm) versus distance (mm) (growth)	Mean satellite concentration (numbers/mm <sup>2</sup> ) versus distance (mm) (survival)
BD1	BS1	-0.0555 (0.940) <sup>a</sup>	-0.0373 (0.962)
	BS2	-0.0893 (0.901)	-0.0293 (0.967)
	BS3	-0.0510 (0.920)	-0.0285 (0.976)
	BS4	-0.0627 (0.856)	-0.0282 (0.964)
	BS5	-0.0323 (0.851)	-0.0444 (0.989)
	BS6	-0.0195 (0.899)	-0.0242 (0.970)
	BS7	-0.0192 (0.861)	-0.0556 (0.856)
BD2	BS1	-0.0680 (0.984)	-0.0145 (0.944)
	BS2	-0.0401 (0.918)	-0.0114 (0.924)
	BS3	-0.0254 (0.938)	-0.0490 (0.933)
	BS4	-0.0269 (0.933)	-0.0179 (0.955)
	BS5	-0.0801 (0.907)	-0.0194 (0.952)
	BS6	-0.0440 (0.848)	-0.0238 (0.931)
	BS7	-0.0509 (0.790)	-0.0324 (0.886)

<sup>a</sup> Correlation coefficients given in parentheses.

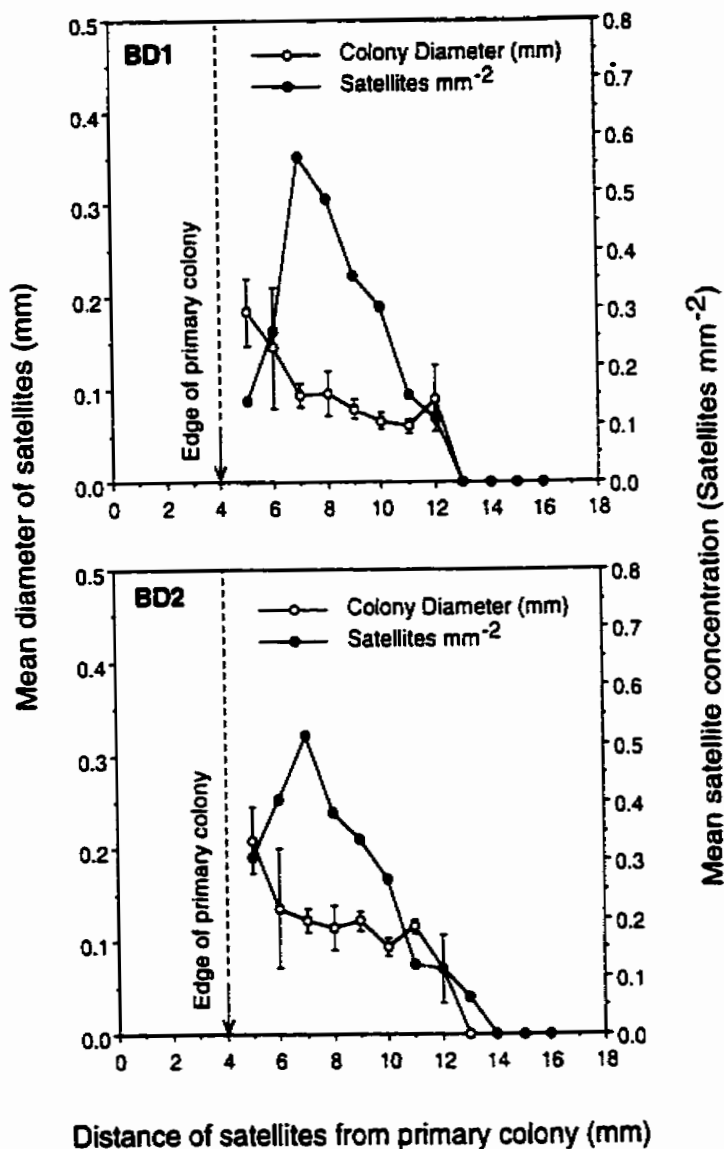
primary colony within both associations. Mean satellite concentrations were maximum, at a distance of approximately 7 mm from the center of the primary colonies. However, closer to the edge of the primary colony (3 - 7 mm from the center of the colony), the number of satellites decreased with decreasing distance (Figure 3.3.4). The size and frequency of individual satellites was determined as a function of distance from the primary colony (Figures 3.3.5, 3.3.6 and 3.3.7). Each satellite showed a positive interaction with both primary colonies (Figures 3.3.6 and 3.3.7). The slopes ranged from -0.0192 to -0.0893 (colony size in mm/distance from primary colony in mm) for size distribution and from -0.0242 to -0.0556 (number of satellites per mm<sup>2</sup>/distance from primary colony in mm) for mean satellite concentration in the BD1 association (Table 3.3.1). The values ranged from -0.0254 to -0.0801 for size distribution and from -0.0114 to -0.0490 for mean satellite concentration in the case of BD2 association. High correlation coefficients ( $r^2 > 0.80$ ) were noted between the mean sizes of satellites and their distance from the primary colony in all the pairs analyzed.

#### **3.3.3.4 Effect of the concentration of satellites on the strength of association**

The size and spatial distribution of satellites varied inversely with distance from the primary colony at the three dilutions tested (Figure 3.3.8). The maximum increase in colony size (growth of satellites) as a function of distance was obtained at the highest dilution (when the lowest number of cells were inoculated; corresponding to an inoculated mean satellite concentration of  $2.2 \times 10^2$  satellites cm<sup>-2</sup>). The minimum increase was obtained at the lowest dilution ( $2.2 \times 10^4$  satellites cm<sup>-2</sup>). In contrast, the maximum increase in observed mean satellite concentration (survival of the satellites) as a function of distance was obtained at the lowest dilution and the minimum increase was obtained for the highest dilution. At the lowest dilution, ( $2.2 \times 10^4$  satellites cm<sup>-2</sup>) the primary colony supported satellite development only to a distance of 25 mm (Figure 3.3.8 A, B). The distance from the primary colony to which satellite colonies could form, was extended at higher dilutions ( $2.2 \times 10^2$  and  $2.2 \times 10^3$  satellites cm<sup>-2</sup>) (Figure 3.3.8 C).

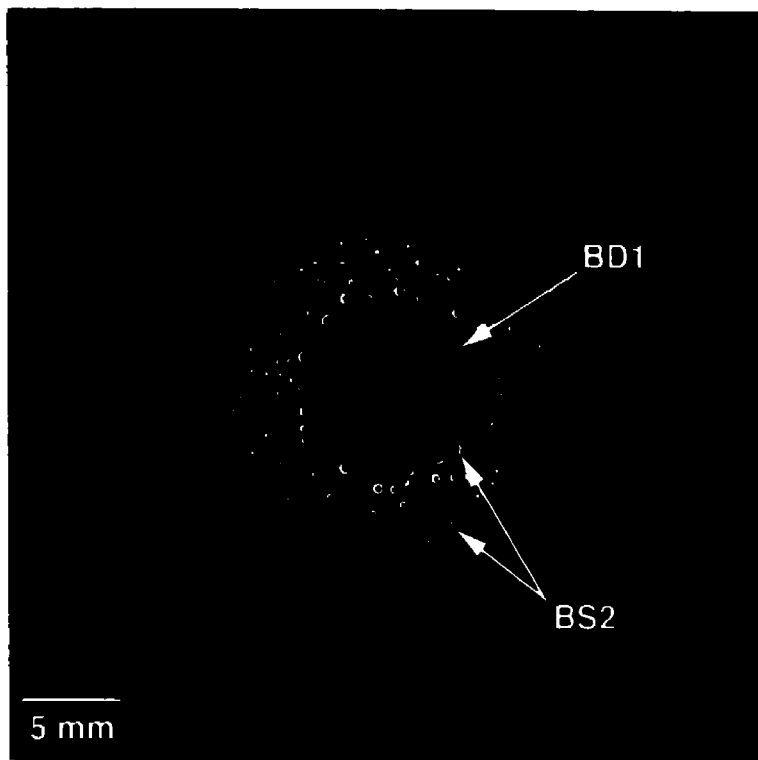
#### **3.3.3.5 Influence of satellites on the primary colonies**

Table 3.3.2 shows the average sizes of primary colonies with and without adjacent inoculation of the satellite strains. There was a significant reduction ( $p < 0.01$ ) in the size of BD1 when cultivated in association with satellite colonies. Significant reductions in the size of BD2 were also noted, when associated with all satellite strains

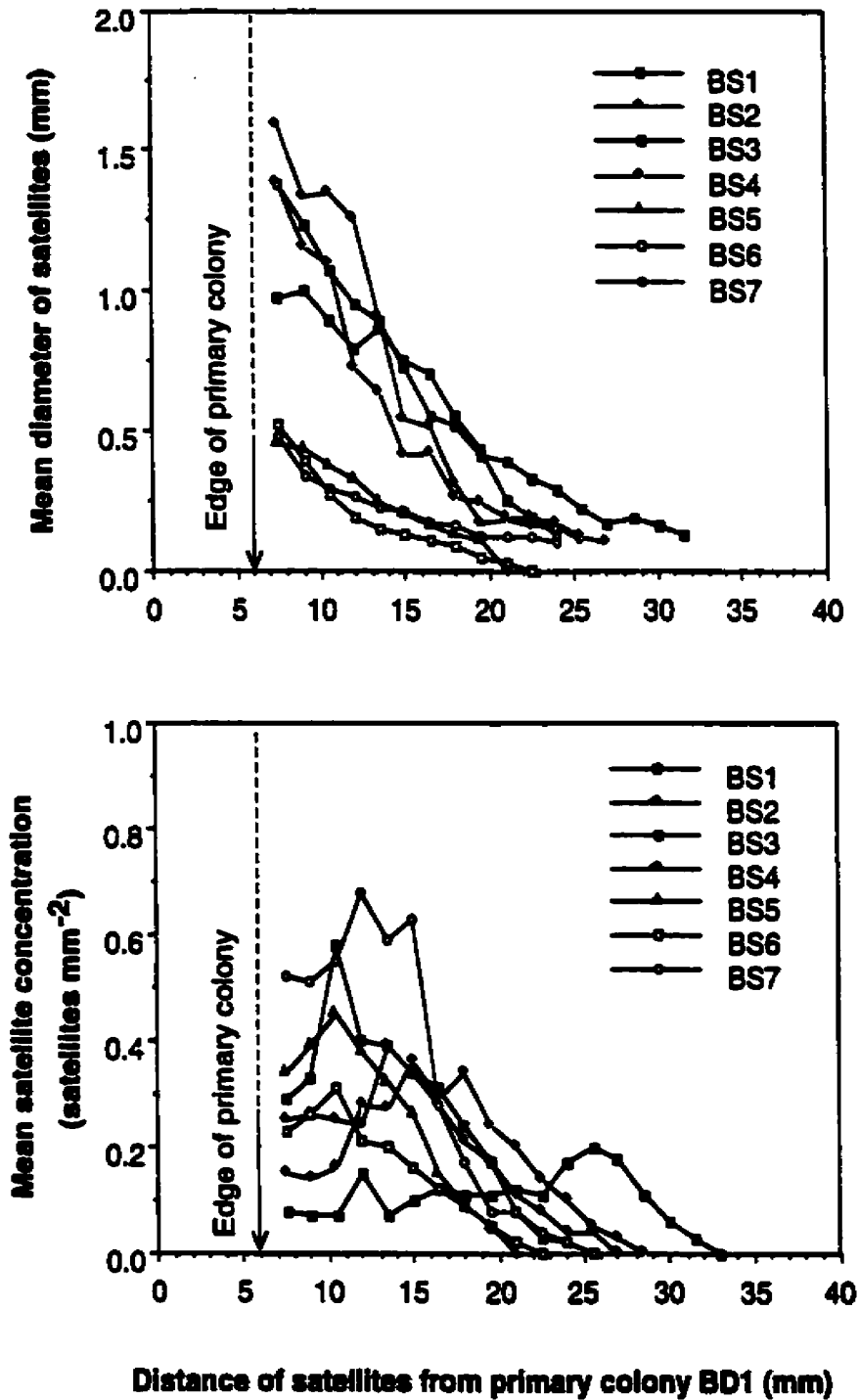


**Figure 3.3.4.** Quantification of the interaction effects between primary and satellite colonies during community plating. However, the satellite effect was obscured by the random distribution of primary and satellite colonies (see Figure 3.3.2). At certain low dilutions, the primary colonies appeared farther apart enabling digital image analysis of the primary colony - satellite interactions. In these plates, the size and spatial distribution of the satellites varied inversely with distance from either BD1 or BD2, suggesting the existence of positive synergisms within the association as a whole. Error bars are 95% confidence intervals based on the number of colonies present at each distance from the primary colony. Distances were measured from the center of the primary colony.

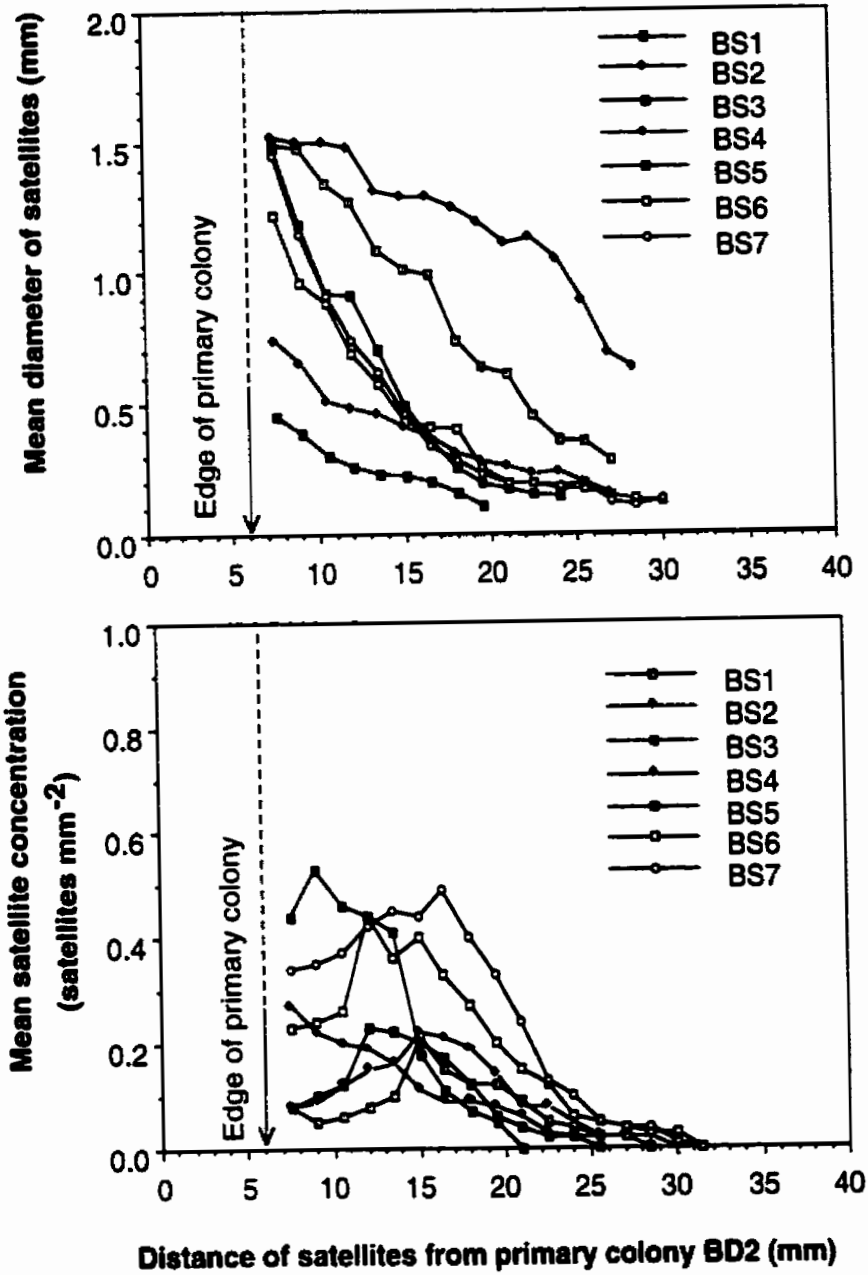
**Figure 3.3.5.** (Facing page) An example of satellite plating procedure for the quantitative analysis of spatial interactions between individual primary and satellite colonies. Image shows BD2 spot-inoculated on a lawn of BS2. Note that the satellite effect is not obscured by the irregular distribution of primary and satellite colonies as it is in Fig. 3.3.2 and is more amenable to quantification by image analysis.





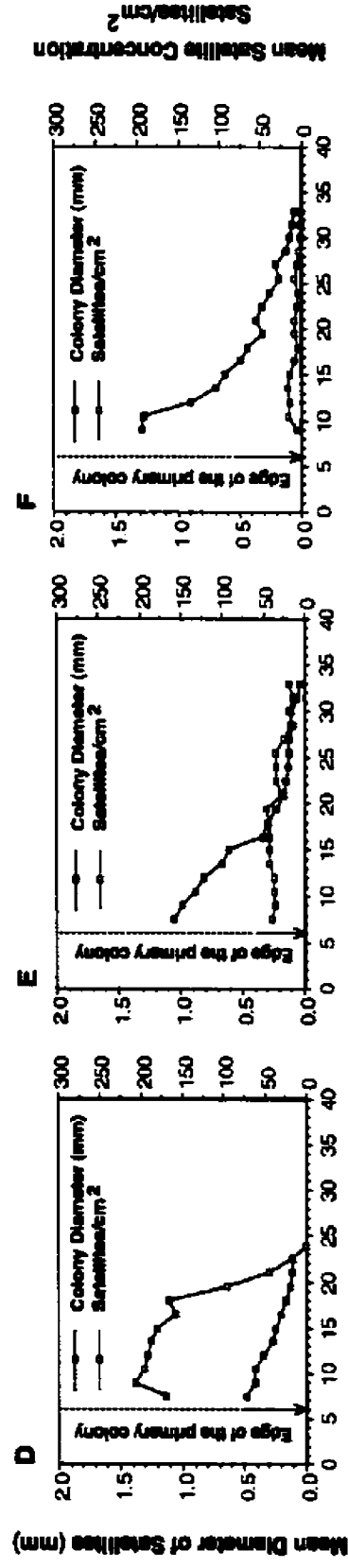
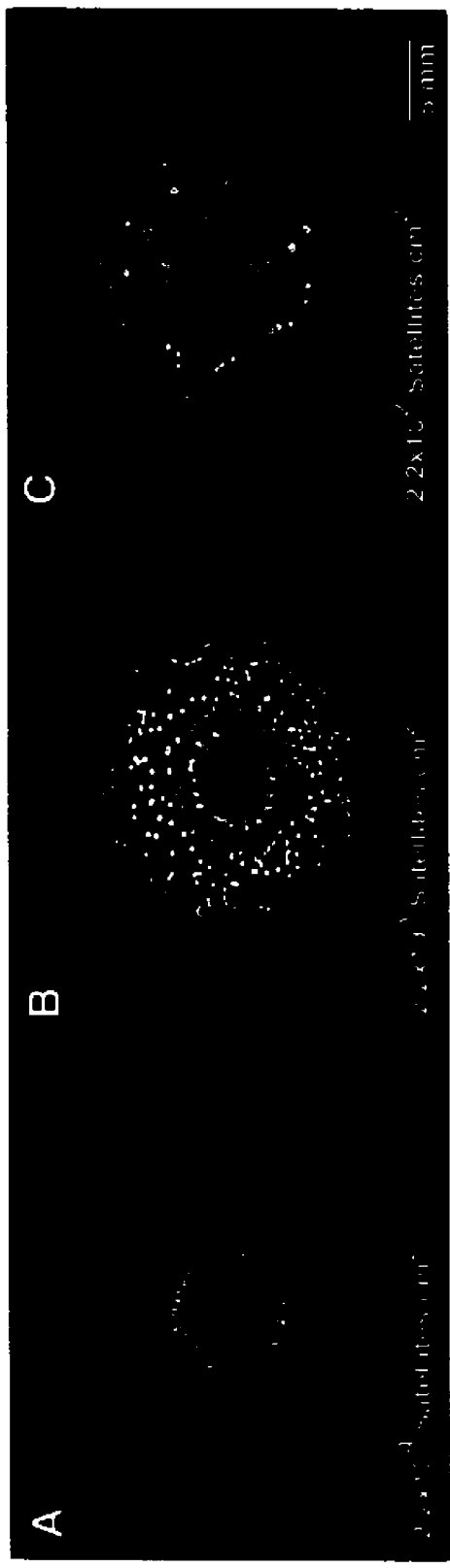


**Figure 3.3.6.** Satellite analysis curves showing the size and spatial distribution of individual satellites as a function of distance from the primary colonies of BD1. Negative slopes of these curves indicate positive synergisms between individual satellites and the primary colony.



**Figure 3.3.7.** Satellite analysis curves showing the size and spatial distribution of individual satellites as a function of distance from the primary colonies of BD2.

**Figure 3.3.8.** (Facing page) Effect of satellite concentration (satellites  $\text{cm}^{-2}$ ) on the association between primary and satellite colonies determined by plating various dilutions of the log phase cells of BS2 on 0.15% benzoate agar. BD2 was spot-inoculated at the center. A, B and C correspond to satellite concentrations of  $2.2 \times 10^4$ ,  $2.2 \times 10^3$  and  $2.2 \times 10^2$  satellites  $\text{cm}^{-2}$  respectively. Note that the positive effect of the primary colony tends to be masked by the self-inhibition of satellites when used at high satellite concentrations. Satellite analysis curves (D, E and F) show the size and spatial distribution of satellite morphotype BS2 as a function of distance from the primary colony BD2, at the three satellite concentrations indicated.



Distance of Satellites from Primary Colony (mm)

**Table 3.3.2.** Influence of satellites on the growth of the primary colony

Primary colony	Satellite colony	Size of primary colony spots (mm <sup>2</sup> ) <sup>a</sup>	
		<sup>b</sup> Sat +	<sup>c</sup> Sat -
BD1	BS1	32.88 <sup>d</sup> (1.63)	40.68 (2.55)
	BS2	27.90 <sup>d</sup> (2.48)	
	BS3	33.70 <sup>d</sup> (3.25)	
	BS4	30.83 <sup>d</sup> (0.83)	
	BS5	34.24 <sup>d</sup> (0.64)	
	BS6	31.64 <sup>d</sup> (1.24)	
	BS7	31.14 <sup>d</sup> (1.62)	
BD2	BS1	59.61 <sup>d</sup> (5.60)	87.68 (6.49)
	BS2	55.51 <sup>d</sup> (7.95)	
	BS3	80.27 <sup>d</sup> (3.50)	
	BS4	79.80 (5.51)	
	BS5	89.26 (5.41)	
	BS6	76.09 <sup>d</sup> (7.32)	
	BS7	62.73 <sup>d</sup> (7.15)	

<sup>a</sup> Values are means of 9 replicated spots (standard deviation in parentheses)

<sup>b</sup> sat+ With adjacent spot inoculation of satellite colonies

<sup>c</sup> sat- Without satellite colony inoculation

<sup>d</sup> Significant difference between sat+ and sat- treatments ( $p < 0.01$ )

except BS4 and BS5. Both primary colonies (BD1 and BD2) produced a brown compound which resulted in the general browning of the agar on extended periods of incubation (over 14 days).

### **3.3.3.6 Mechanism of satellite associations**

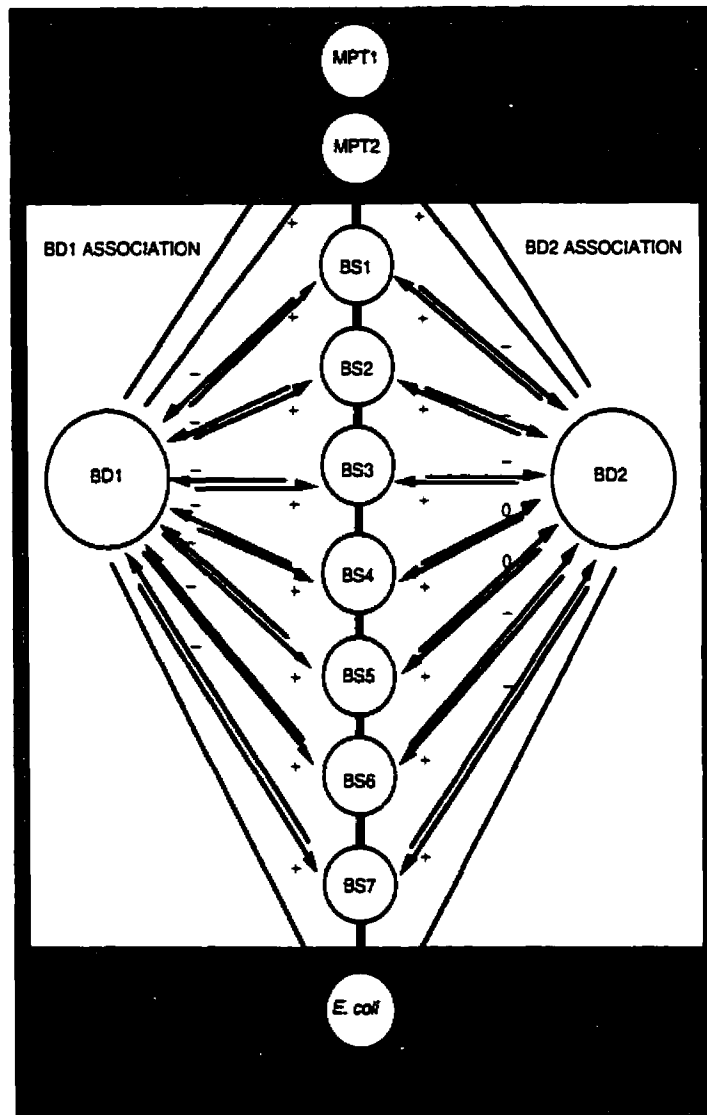
The effect of benzoate concentration on the development of associations between individual primary and satellite colonies was examined to determine if benzoate toxicity was the basis for the formation of such associations. The growth of primary colonies themselves, was inhibited at 1.0 and 1.5% benzoate concentrations. At lower concentrations (< 0.25% benzoate) satellite colonies could form, in association with the primary colony. However, all satellite morphotypes could develop independent of the primary colony only at much lower benzoate concentrations (0.015 - 0.05%). Even when (0.1%) glucose was added to the benzoate medium, satellite isolates were not able to grow independent of the primary colonies at higher benzoate concentrations (>0.05%). All the satellite isolates were able to grow well, independent of the primary isolates, on M-salts medium supplemented with glucose (no benzoate added). Agar plugs removed from areas adjacent to the spot-growth of primary isolates, or homogenates prepared from these plugs did not support the growth of satellite strains on 0.15% benzoate agar.

### **3.3.3.7 Specificity of the BD1 and BD2 associations**

Cross-streak assays showed that both BD1 and BD2 were able to support the growth of organisms unrelated to the BD1 and BD2 associations on benzoate agar supplemented with glucose (Figure 3.3.9). These included a culture collection strain of *E. coli* (ATCC 25923) as well as MPT1 and MPT2, two other members of the TSB community from which the BD1 and BD2 associations were originally obtained.

### **3.3.4 Discussion**

The antimicrobial effectiveness of sodium benzoate has been widely recognized (Ostergaard, 1994; Wind and Restaino, 1995; Kasrazadeh and Genigeorgis, 1995; Efiuvwevwere and Akoma, 1997; Fang et al., 1997). However, the present study demonstrates the formation of protective synergisms involved in benzoate resistance and degradation leading to the reduced antimicrobial effectiveness of sodium benzoate. The protective synergisms were evident from increased plating efficiencies (higher CFU ml<sup>-1</sup> determined based on plate counts) at the lower dilutions, when a TSB community was



**Figure 3.3.9.** Diagram showing the organizational relationships detected within TSB communities. According to the postulates of self-organization, bacterial autopoiesis or self-organization should result in bacterial associations which are optimized for resource use. The existence of BD1 and BD2 benzoate associations, suggest the possibility that there are at least two sets of bacteria optimized for benzoate degradation. In the diagram, the rectangles represent the two associations (BD1 association and BD2 association). The arrows indicate the direction of the effect of one individual on another and the sign indicates whether the influence was positive or negative. The size of the primary colonies was significantly reduced by the adjacent growth of satellites except in the case of BD1 plated with BS4 or BS5 (indicated by '0'). The ability of the primary colonies to support the control strains (*E. coli*, MPT1 and MPT2), which were unrelated to BD1 and BD2 associations, shows the non-specific nature of the protective interactions.

plated on 0.15% benzoate agar (Figures 3.3.1 and 3.3.2) and from the formation of satellite associations in which the primary colony enhanced the growth of the benzoate-sensitive satellite strains (Figures 3.3.2, 3.3.3, 3.3.4, 3.3.5, 3.3.6 and 3.3.7).

Analysis of propinquity-dependant plating efficiency enabled determination of the maximum cell separation distance that still permitted positive interactions between the benzoate sensitive and resistant members of the microbial community. In general, the CFU ml<sup>-1</sup> of the bacterial community was maximum when the mean distance between cells plated was less than 12 mm (Figure 3.3.1). Positive associations within benzoate-degrading biofilms that resulted in an enhanced plating efficiency when lower dilutions were plated on 0.15% benzoate agar have previously been reported (Caldwell et al., 1997a). It was suggested that the closer spatial positioning of the individuals at lower dilutions could facilitate associations and enhance the survival of the benzoate-sensitive members of the community. Density-dependent plating effects have also been reported with bacteria isolated from subsurface environments (Stevens and Holbert, 1990). Thomas et al. (1997) showed the effect of spatial propinquity on the inhibition of subsurface colonies of *Listeria monocytogenes* by a nisin-producing lactic acid bacterium. A separation distance of less than 100 µm from the lactic bacteria resulted in a 3.5 log reduction in the cell numbers of subsurface colonies of *Listeria monocytogenes*, as opposed to only a 0.9 log reduction in cell numbers at a greater separation distance of 3000 µm. As suggested previously (Wimpenny et al., 1995), the propinquity (or spatial separation between cells) could influence the outcome of interactions among the bacteria in natural environments and model systems. Hence, an analysis of the effects of spatial separation should be an important consideration in screening for associative effects among bacteria as well as in the development of efficient methods for food preservation or biodegradation using degradative consortia and communities.

Positive associations among the members of the biofilm community also resulted in the formation of satellite colonies around larger, central colonies on 0.15% benzoate agar. However, the satellite effect was detected only when lower dilutions of the microbial community were plated on a high, inhibitory concentration of sodium benzoate (Figure 3.3.2). This was presumably due to the closer spatial positioning of bacteria at lower dilutions which facilitated interactions among the members of the microbial community. When benzoate satellites and primary isolates were screened, two sets of interacting organisms (BD1 and BD2 associations) were identified. Each primary colony was found to support a set of seven satellite strains. Screening was performed by spot-inoculating individual presumptive primary isolates at the center of a lawn of a



presumptive satellite isolate. A similar procedure has previously been used to screen for antibiotic tolerant variants of several gram-positive bacterial isolates (Traub, 1982). Positive growth effect of each primary colony upon individual satellite strains were further confirmed using a cross-streak assay procedure (Figure 3.3.3). Although cross-streaking provided qualitative evidence of positive interactions between the primary and satellite isolates, it did not permit quantification of the relative effects of the association on the growth and survival of the organisms involved. Consequently, a satellite analysis procedure based on digital imaging was developed to determine the size and spatial distribution of satellite colonies, as a function of the distance from the primary colonies.

Quantitative analysis using the digital imaging procedure revealed that the size and number of satellite colonies decreased as a function of distance from the primary colony, indicating the synergistic nature of the associations (correlation coefficient  $>0.80$ ). This occurred when BD1 or BD2 associations which appeared on the plating-efficiency assay plates (Fig 3.3.2) were analyzed as a whole or as binary pairs involving individual primary colony-satellite strains. During the analyses, the size of satellite colonies served as a measure of growth, and the number of satellite colonies detected per unit area served as a measure of the survival of benzoate-sensitive satellite isolates in association with a primary colony. Decreases in numbers and sizes of satellites as a function of distance resulted in negative slopes (Table 3.3.1). Lower values of slopes suggested greater dependency of the satellite organism on the primary colony for growth on 0.15% benzoate agar. Differences could be noted in the growth and survival of the same satellite as influenced by BD1 or BD2 (Fig 3.3.6 and 3.3.7; Table 3.3.1) meaning that the two primary isolates differed in their capacity to support the growth of various satellites.

Although the growth enhancing effect of the primary colonies upon the satellite isolates was evident from an analysis of size and spatial distribution of satellites around the primary colonies, the mechanism of the association was not clear. One of the requirements for understanding communities as causative agents of various biological processes is that the interactions between community members be understood (Caldwell et al., 1997b). Hence, the next set of experiments was aimed at determining the mechanism for the formation of satellite associations. Many documented satellite effects have been shown to be based on nutritional symbioses (Frenkel and Hirsch, 1961; Cayeux et al., 1971; George, 1974; Carey et al., 1975; Bouvet et al., 1981; Bouvet, 1995). For example, two species of streptococci, *Streptococcus adjacens* and *S. defectivus* (responsible for human endocarditis) were characterized by their growth as

small satellite colonies nutritionally supported by primary colonies (Bouvet, 1995). Similar nutritionally variant streptococci (NVS) have previously been characterized as fastidious viridans group streptococci which require supplemental cysteine (Frenkel and Hirsch, 1961; Cayeux et al., 1971) or vitamin B6 (pyridoxal phosphate) (Frenkel and Hirsch, 1961; Carey et al., 1975) in complex media for growth and form satellite colonies around several gram positive and gram negative bacterial strains (Bouvet et al., 1981). Osawa et al. (1992) isolated three strains which showed concomitant satellite growth around isolates of *E. coli* (due to succinate production). Protection afforded by primary colonies against antibiotics has also been implicated in the formation of other satellite effects (Traub, 1982). Because the carbon substrate (sodium benzoate) used in the present study was rather refractory and because the degradation of benzoate by many pseudomonads has been well demonstrated (Dagley, 1986), it was also possible that the satellites lacked the ability to degrade benzoic acid and depended on the primary colony for easily utilizable degradation intermediates. Thus, three mechanisms were hypothesized for the formation of the primary colony-satellite associations on benzoate agar. These were: 1) primary colonies supplied one or more nutritional factors that the satellite strains were unable to synthesize, 2) satellite strains lacked the ability to degrade sodium benzoate and hence were dependent on the primary colonies for a supply of benzoate degradation intermediates, 3) primary colonies protected the benzoate-sensitive satellites against inhibitory concentrations of benzoate by either reducing the concentration of sodium benzoate to non-inhibitory levels or by the production of specific or non-specific protective factors.

During the present study, the growth of satellites at high concentrations of benzoate (0.10 - 0.25%) was dependent on spatial propinquity with the primary colonies, even in the presence of an additional labile carbon source such as glucose. However, the satellite isolates were able to form colonies independent of the primary isolates at lower benzoate concentrations (0.015 - 0.05%). Satellite isolates were also able to grow on M-salts medium supplemented with glucose as sole carbon source in the absence of primary isolates. This suggested that the satellites were not dependent on a supply of growth factors from the primary colony. The ability of satellite isolates to grow independently of the primary colony at low concentrations of benzoate also suggested that satellite strains were able to utilize benzoate as sole carbon source, and the supply of the benzoate degradation intermediates by the primary colonies might not be the basis for the formation of the satellite associations. The growth of the satellite strains was inhibited at high benzoate concentrations even when provided with an

additional, labile carbon source. Consequently, the most parsimonious explanation for these experimental results addressing the primary colony-satellite interactions is that the primary colonies played a protective role, allowing the development of satellite colonies at inhibitory concentrations of benzoate. The observation that the primary colonies were able to support satellite development to extended distances, when lower numbers of the satellite isolate was plated (at higher dilutions of satellites) (Figure 3.3.8), suggested the possibility of a diffusible protective factor that diffused to extended distances at lower satellite concentrations. The brown pigmentation that appeared around primary colonies (but not satellite colonies) on benzoate agar might not have a protective role, due to the appearance of pigmentation long after the satellites have developed. Production of diffusible brown pigments by bacteria is common (Basu and Wallen, 1967; Elston, 1968; Yabuuchi and Ohyama, 1972) and in some cases, these pigments have been shown to be autooxidation products of bacterial metabolites (Goodwin and Sopher, 1993; Fava et al., 1994). Further, this protection may not involve any other protective factors which might be produced by the primary colonies. This was evident from the observation that benzoate-agar plugs removed from areas adjacent to primary colonies, or the homogenates prepared from these plugs, did not support growth of satellite strains when placed on a lawn of satellite cells on 0.15% benzoate agar. Thus general toxicity reduction is the most probable explanation for the satellite effect.

Clearing of the brown pigment by satellite colonies suggested that the growth enhancement of satellites might be due in part to the availability of these and related degradation intermediates. The possible sharing of the degradation intermediates between primary and satellite colonies is evident from the decreased mean satellite concentration immediately adjacent to the edge of the primary colony (Figures 3.3.4, 3.3.6, 3.3.7 and 3.3.8) and significant ( $p < 0.01$ ) reduction of the size of primary colonies, upon adjacent inoculation with satellite isolates (Table 3.3.2). No reduction in size of BD2 in association with BS4 and BS5, suggests the possibility of mutualistic interactions between these satellites and BD2.

The existence of BD1 and BD2 benzoate associations, suggest the possibility that there are at least two sets of interactions capable of benzoate resistance and degradation. The organizational relationships for the two sets are shown in Figure 3.3.9. The ability of the primary colonies to support the growth of unrelated organisms, as evidenced from cross-streak assays, indicates that the protection afforded by the primary colonies was not specific for satellites and confirms general toxicity reduction as

a mechanism for the satellite effect. The fact that no synergistic effects could be detected due to interactions among multiple primary and satellite isolates also suggests that toxicity reduction, as opposed to the production of any protective factors, was primarily responsible for the formation of satellite associations.

Enhanced plating efficiencies detected when biofilm communities cultivated using TSB were plated on benzoate agar, and concomitant formation of protective associations between benzoate-resistant and benzoate-sensitive bacterial strains, reaffirmed the existence of community-level proliferation strategies as discussed previously (section 3.2.4). The formation of protective synergisms, as demonstrated during the present study, suggests that proliferation strategies based on association should be an important consideration, in order to fully understand and develop applications in the disciplines of food preservation, and other environmental processes such as biodegradation. The results also suggest that density-dependent plate counts and digital satellite analysis can be useful in screening for community-level synergisms within complex microbial communities.

### 3.3.5 Summary

Plating bacterial communities on benzoate-containing agar sometimes results in higher plating efficiencies at lower dilutions. This effect has been attributed to unknown synergistic interactions that depend on the close spacing of community members. In the present study digital image analysis showed that this was due to community protection by two benzoate-degrading strains of *Pseudomonas fluorescens*. Communities were cultivated in flow cells irrigated with TSB. When the effluent from these flow cells was plated on 0.15% benzoic acid, satellite colonies formed in the vicinity of primary colonies. Two of these primary colonies, *Pseudomonas fluorescens* strains BD1 and BD2, were isolated. Seven different satellite colonies were dependent upon the primary colonies. All satellites were *Pseudomonas* spp. but only one was a strain of *Pseudomonas fluorescens*. A digital image analysis procedure was developed to measure the size and spatial distribution of satellite colonies as a function of distance from the primary colony. When these measurements were plotted, the slope(s) were used to quantify the effect of bacterial association on the survivability (mean colony concentration versus distance) and growth (mean colony size versus distance) of the satellite strains. Survivability was optimal 3 to 10 mm from the primary colony, while growth was optimal at or near (within 1 mm) of the primary colony. When in the absence of the primary isolates, individual satellites could grow in pure culture at low

benzoate concentrations (0.015 and 0.05%) but not at higher benzoate concentrations (0.1 - 0.25%). This suggested that the primary colony provided the satellites with concentration-dependent protection from benzoate, as opposed to partially degrading the benzoate and allowing satellite growth by cross feeding the resulting degradation products. The protective effect was non-specific and extended to other benzoate-sensitive control organisms including non-benzoate degraders from the original community as well as a culture collection strain of *Escherichia coli*. The results show that density-dependent plate counts and digital satellite analysis can be useful in screening for community-level synergisms when communal interactions make it difficult to directly plate one or more community members. Overall, the results reaffirmed the existence of community-level proliferation strategies as discussed previously (section 3.2.4).

### **3.4 Protective interactions between benzoate-resistant and benzoate-sensitive bacteria within binary cultures and within a microbial community**

#### **3.4.1 Introduction**

The results of section 3.3 demonstrated the formation of protective associations between benzoate-resistant and -sensitive bacterial strains when a biofilm community cultivated using TSB was plated on M-salts medium containing sodium benzoate as sole carbon source. Sodium benzoate is an important preservative in the food industry (Pylypiw, 1994; Kasrazadeh and Genigeorgis, 1995; Wind and Restaino, 1995), and its inhibitory effect on bacteria which cause food spoilage (Wind and Restaino, 1995; Efiuvwevwere and Akoma, 1997), foodborne illness (Kasrazadeh and Genigeorgis, 1995; Fang et al., 1997) or dental plaque (Ozanich et al., 1993; Ostergaard, 1994) has been demonstrated. Consequently, microbial adaptation which leads to increased resistance against sodium benzoate is of practical interest. Although relatively little is known about associations between bacteria which result in the protection of sensitive strains against antimicrobial agents, community-level microbial interactions have been implicated in other microbial processes such as biodegradation (Wolfaardt et al., 1994b), formation of anaerobic digester granules (Macleod et al., 1990) and dental plaques (Kinniment et al., 1996), as well as in food spoilage (Zottola and Sasahara, 1994).

Despite the use of various methods to eliminate bacteria from food products (e.g., addition of preservatives such as benzoate) and from food processing equipment (treatment with hypochlorite, polyphosphates, heat, quaternary ammonium compounds treatments, trisodium phosphate etc.), food spoilage organisms and pathogens often persist in foods (Smith and Fratamico, 1995; Korber et al., 1997). This persistence in food-processing environments can be attributed, not only to an adequate supply of nutrients, but also to an abundance of surfaces that allow the development of heterogeneous biofilm communities. Incorporation of organisms capable of inactivating antimicrobial agents may enhance the reproductive success of such communities and present a challenge to the food industry when such protection is extended to a food spoilage organism or pathogen. While microbial growth as attached biofilms impart capacity for efficient nutrient scavenging, resistance to removal by shear forces and to chemical agents through structural and diffusional barriers, yet planktonic growth might be microbially preferred in stored food products. Irrespective of the advantages conferred by either biofilm or planktonic modes, growth as communities that might include a species that can degrade or detoxify an antimicrobial agent might permit the growth of other sensitive members and enhance the reproductive success of the

community as a whole. When the protection is extended to a food spoilage organism or a pathogen which is usually sensitive to the antimicrobial, the consequences of this protective interaction can clearly be viewed as deleterious.

In food systems protected by antimicrobial agents, when microbial existence is dependent on a primary organism capable of detoxifying the environment, it might be relevant to examine the interactions as binary associations between the primary organism and individual strains or the community as a whole. Although the study of binary associations may not permit a complete functional understanding of the community, it might help to understand some of the more crucial interactions between individuals.

Results of the previous studies demonstrated: 1) enhanced growth of biofilm communities due to repeated inoculation (as a mechanism of coincidental organismal recombination, 2) increased plating efficiencies when lower dilutions of communities were plated on benzoate agar, and 3) formation of protective synergisms when biofilm communities were plated on benzoate agar (sections 3.2 and 3.3). During the present study, the formation of protective synergisms was further examined in both static (batch) and flowing environments. Also examined was whether the inclusion of a benzoate-resistant strain, *Pseudomonas fluorescens* BD1 (derived from a satellite association described in section 3.3.3), would have a noticeable effect on the growth and stability of a heterogeneous microbial community when exposed to sodium benzoate. Initially, the protective interaction between BD1 and a benzoate-sensitive *Pseudomonas sp.* strain BS2 was examined under both batch and continuous-flow conditions. Subsequently, the community-level beneficial effect of BD1 inclusion was examined. The criteria for these evaluations included determinations of cell numbers, biofilm formation, spatial positioning of *gfp*-labeled strains in biofilms, and mineralization of <sup>14</sup>C benzoate.

### **3.4.2 Materials and Methods**

#### **3.4.2.1 Bacterial strains and community**

Two bacterial strains, *Pseudomonas fluorescens* strain BD1 and *Pseudomonas sp.* strain BS2, involved in a protective association when plated on M-salts medium containing sodium benzoate as sole carbon source (see section 3.3.3), were used in the present study. The microbial community used in the present study was obtained from a pristine soil environment (Matador grasslands, Saskatchewan, Canada). The community inoculum was prepared by shaking one gram of the above soil sample in M-salts solution for two hours.

### 3.4.2.2 GFP labeling of bacterial strains

*Pseudomonas* strains BD1 and BS2 were labeled with GFP using a triparental mating procedure involving a donor strain of *Escherichia coli* JB 127 (CC118-l-pir + pJBA33(Amp<sup>+</sup>, Kana<sup>+</sup>, l-pir, gfp (mut.3)+)), a helper strain, *E. coli* SM 1279 (HB 101 + pRK600 (Cam<sup>+</sup>)) and the recipient strain, *Pseudomonas* strain BD1 or BS2.

Overnight Luria broth cultures were washed twice and resuspended in phosphate-buffered saline (PBS) (pH 7.2), mixed and spotted on 10% Luria agar (Difco Laboratories, Detroit, MI) plates. After incubation for 24h, the resultant colonies were resuspended in PBS, and serial dilutions plated on citrate agar (Difco Laboratories, Detroit, MI) containing kanamycin ( $50 \mu\text{g ml}^{-1}$ ) to select against *E. coli*. The exconjugates were screened for GFP insertion behind strong promoters using the epifluorescence assembly of a Zeiss Photomicroscope III (Oberkochen, Germany) in conjunction with a 10X objective and an FITC filter set (Figure 3.4.1). The growth rate, biochemical characteristics, and carbon substrate utilization patterns of the wild types and the corresponding GFP-labeled strains were compared to confirm that the GFP insertion did not affect either the growth rate or the metabolic characteristics of either BD1 or BS2. API (NFT) strips (BioMerieux Vitek, Inc., Hazelwood, MO) and BIOLOG - GN (Biolog Inc., Hayward, CA) plates were used for these comparisons.

### 3.4.2.3 Binary interaction studies

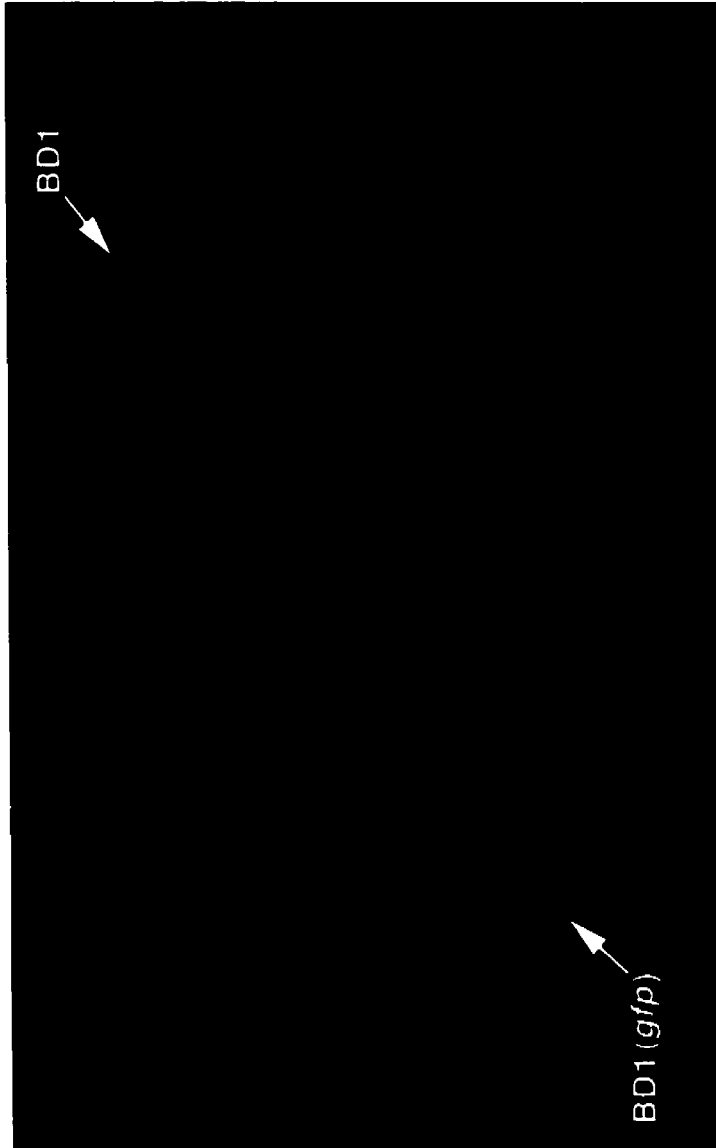
BD1 and BS2 (*gfp*) were grown to log phase in either M-salts medium containing 0.15% benzoate as sole carbon source or 10% v/v TSB, washed twice and resuspended in PBS. The interactions between these two strains were then assayed using batch enrichment, and flow cells at three different concentrations of benzoate. In the batch and flow-through systems, pure and binary cultures were enumerated using 10% (v/v) TSA plates. TSA plates containing  $50 \mu\text{g ml}^{-1}$  kanamycin were used for the selective enumeration of BS2 (*gfp*) within binary cultures.

#### 3.4.2.3.1 Batch enrichments

BS2 (*gfp*) and BD1 strains were either inoculated individually, or co-inoculated in 50ml of M-salts medium containing 0.15%, 0.05%, or 0.015% benzoate as sole carbon source. CFU  $\text{ml}^{-1}$  of the broth cultures were determined at 4, 8, 16, 24, 72, 96, 144, 216 and 264 h after inoculation.



**Figure 3.4.1.** (Facing page) Image showing colonies of *gfp*-tagged (designated as BD1-*gfp*) and non-tagged wild type *Pseudomonas fluorescens* strain BD1. Under blue wavelength excitation, colonies of the *gfp*-tagged bacteria fluoresce green, permitting their differentiation from colonies of the wild type strain.



#### **3.4.2.3.2 Flow cell studies**

Multi-channel flow cells (Wolfaardt et al., 1994b) were used for the cultivation of BD1 and BS2 (*gfp*), either individually or as binary pairs. The flow cell system was continuously irrigated with M-salts containing 0.15, 0.05 or 0.015% benzoate as sole carbon source, at a flow rate of 0.2 mm s<sup>-1</sup>. Effluent collected from the flow cells was plated at daily intervals to determine the CFU ml<sup>-1</sup> of both strains. In a separate multi-channel flow cell, the percent surface area coverage of pure culture and binary culture biofilms were continually monitored using SCLM. While negative staining with 0.1% fluorescein was used to quantify pure culture and binary biofilms (as described in section 3.2.1.1), GFP fluorescence permitted selective measurement of BS2(*gfp*) within binary biofilms. All reported values were the average of 10 (biofilm coverage) random measurements.

#### **3.4.2.4 Community-level interaction studies**

A log phase cell suspension of BD1 was added to the microbial community so that BD1 constituted ~25% of the total cell numbers of the final inoculum (determined by plating procedures). Strain BD1 was previously cultivated in M-salts medium containing 0.15% benzoate as sole carbon source, pelleted by centrifugation and resuspended in M-salts before mixing with the community inoculum.

##### **3.4.2.4.1 Flow cell cultivation**

Biofilm communities were cultivated in flow cells using the community inoculum with and without amended BD1. These flow cells were either continuously irrigated with TSB (1% v/v) for a period of 14 days or were cultivated on TSB for seven days followed by seven days incubation on the M-salts solution containing 0.15% (w/v) benzoate as sole carbon source. The depth and percent area coverage of the resultant biofilms were monitored by laser microscopy. All reported values were the average of 10 measurements.

##### **3.4.2.4.2 Community structure and diversity measurement**

The diversity of the flow cell communities was determined by plating serial dilutions of the flow cell effluent on 10% TSA and counting the numbers of various colony morphotypes (as described in section 3.2.2.3). Colony morphotypes were determined based on the descriptions of Benson (1990) and Eklund and Lankford

(1967). Richness and Shannon diversity indices (De Leij et al., 1994) were determined based on the numbers of colony morphotypes.

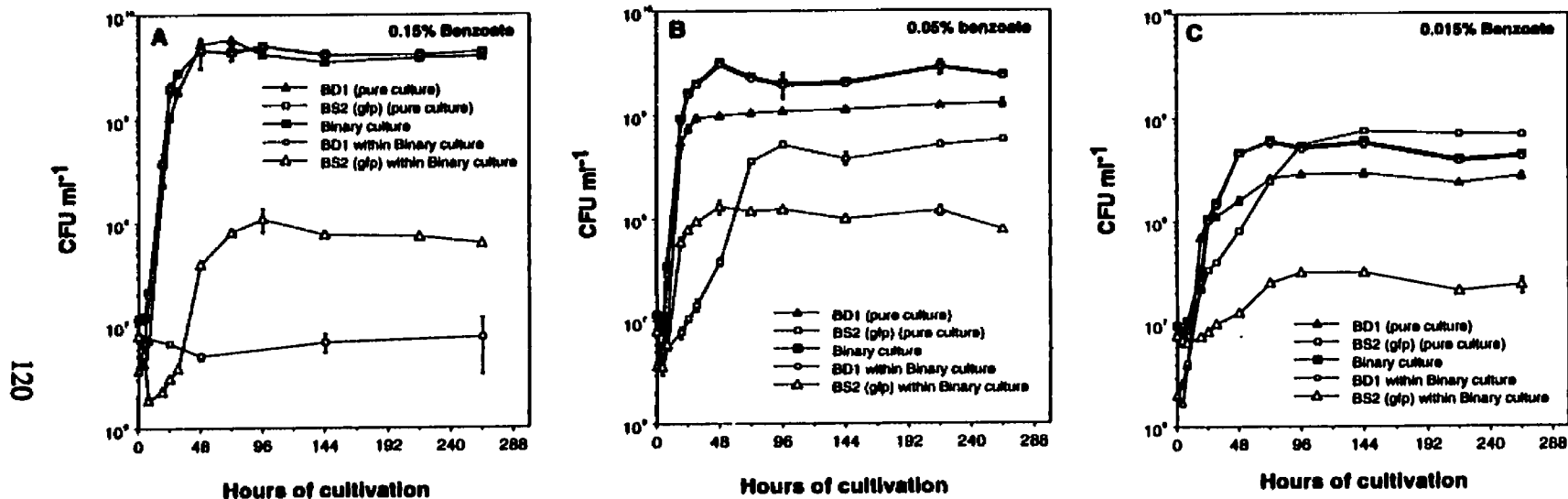
#### **3.4.2.5 Radioisotopic measurement of benzoate mineralization**

Triplicates of BD1, BS2, a binary combination of both, and the community amended and not amended with BD1 were tested for their ability to degrade sodium benzoate provided as sole carbon source at 0.15% and 0.015% concentrations. Twenty-ml scintillation vials served as reactor vials. These vials were filled with five ml of M-salts medium containing sodium benzoate as sole carbon source at either 0.015% or 0.15% concentration. One hundred  $\mu$ l aliquots of PBS-suspended pure cultures or community inocula were inoculated into each vial, the caps tightly closed and incubated at room temperature for a period of seven days. At the end of the incubation period, 10 ml of counting fluid (Aquasol-2, Du Pont-NEN, Boston, MA) was added to the culture fluid contained in the scintillation vials, and the label remaining in the vial, corrected for sample quenching and machine efficiency using an external standard, was counted in a Packard Tri-Carb 1900CA Scintillation Counter. Counting was done in triplicate. Parallel experiments using a non-labeled benzoate were performed to determine changes in cell numbers over the same incubation period.

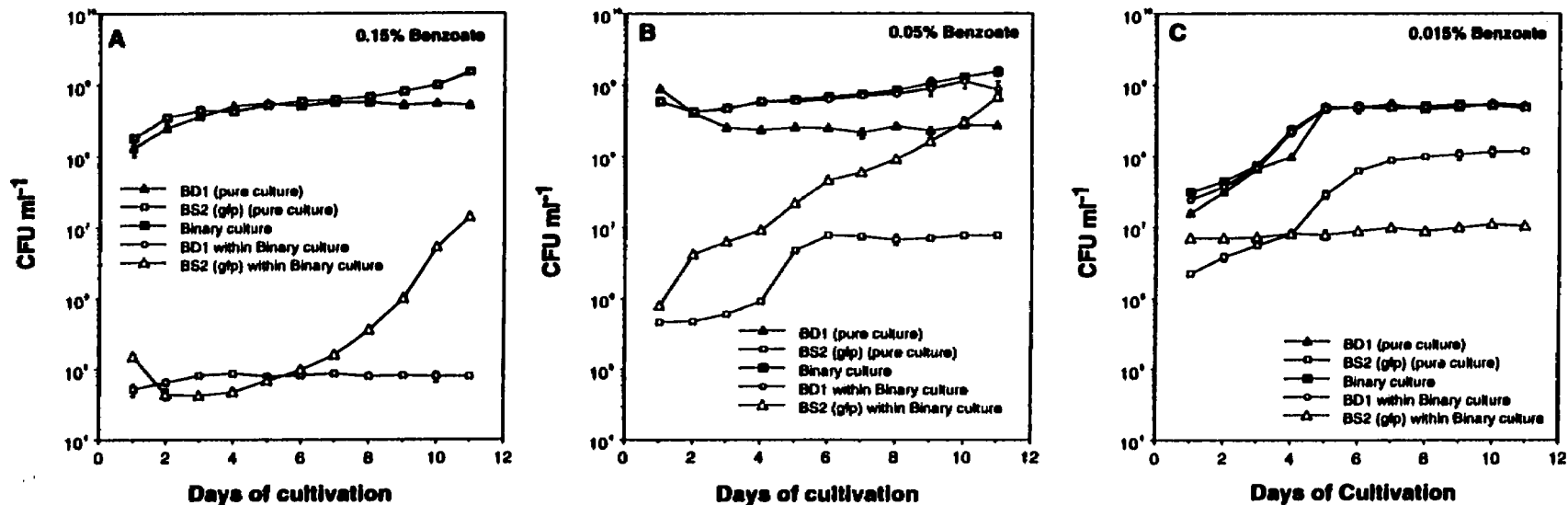
#### **3.4.3 Results**

*Pseudomonas fluorescens* strain BS2 could not grow in pure culture on 0.15% benzoate (Figure 3.4.2 A) for the entire cultivation period of 14 days, however when cultivated as a binary pair along with BD1, though the numbers of BS2 declined initially, there was an approximately two orders of magnitude increase in the numbers of colony forming units afterwards. At lower benzoate concentrations (0.05 and 0.015%), BS2 exhibited more abundant growth as a pure culture than in association with BD1 (Figure 3.4.2 B and C). At these lower concentrations, the ability of BS2 to grow using benzoate was independent of BD1 and no benzoate inhibition of BS2 was observed.

Though the protective effect was also noted during flow cell cultivation, the population dynamics of BD1 and BS2 either as pure cultures or as a binary pair differed from that noted during batch growth. When BS2 was cultivated using 0.15% benzoate as a binary pair with BD1, even in association with the benzoate resistant strain, a lag period of approximately four days was noted during which the numbers of BS2 did not significantly increase (Figure 3.4.3 A). After this period, the numbers of BS2 began to increase, and at the end of 11 days, the planktonic cell numbers of BS2 were



**Figure 3.4.2.** Interaction between BS2 (*gfp*) and BD1 in batch culture at three different concentrations of sodium benzoate. A beneficial interaction was noted only at higher benzoate concentrations (A). For example, when 0.15% benzoate was provided, an increase in the numbers of BS2 (*gfp*) cells was observed in the presence of BD1. At lower benzoate concentrations (0.05% and 0.015%) no benefit was observed during co-culture (see B and C). Rather, lower numbers of BS2 (*gfp*) cells were observed when grown in the presence of BD1 than when BS2 (*gfp*) was grown in pure culture. These results are in accordance with the observations on benzoate agar plates (as a model of static systems) where primary colony-independent growth of BS2 (*gfp*) was noted at low concentrations, whereas primary colony-dependent growth of BS2 (*gfp*) was observed at high benzoate concentration.



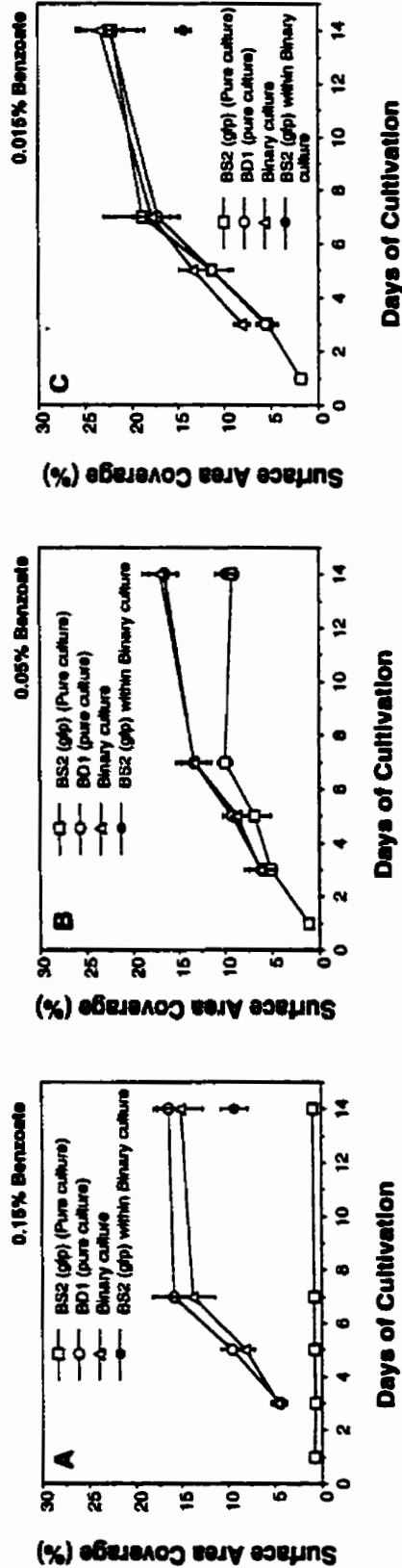
**Figure 3.4.3. Interaction between *BS2 (gfp)* and *BD1* during flow cell cultivation using three different concentrations of benzoate. The growth of *BS2 (gfp)* at 0.15% benzoate was enhanced by its association with *BD1* (A). When cultivated alone, low numbers of *BS2 (gfp)* were observed throughout the period of observation. In contrast, at the lower benzoate concentrations (B and C) the growth of *BS2 (gfp)* was not dependent on association with strain *BD1*.**

approximately three orders of magnitude greater than when it was cultivated as a pure culture. When supplied with the lowest concentration of benzoate (0.015%), BS2 by itself resulted in greater planktonic cell numbers than during binary growth with BD1 (Figure 3.4.3 C), a phenomenon also noted during batch cultivation (Figure 3.4.2).

Consistent with the enhanced planktonic numbers of BS2, its surface area coverage was also greater in association with BD1 ( $9.28 \pm 1.40\%$ ) than when cultivated alone ( $0.91 \pm 0.13\%$ ) in flow cells using 0.15% benzoate (Figure 3.4.4 A). However, the area coverage of BS2 in binary combination and as a pure culture were equal at a lower benzoate concentration (0.05%) (Figure 3.4.4 B). At the lowest concentration of benzoate (Figure 3.4.4 C), the area covered by BS2 was higher ( $22.14 \pm 3.55\%$ ) when cultivated alone than during its binary culture ( $14.44 \pm 0.83\%$ ).

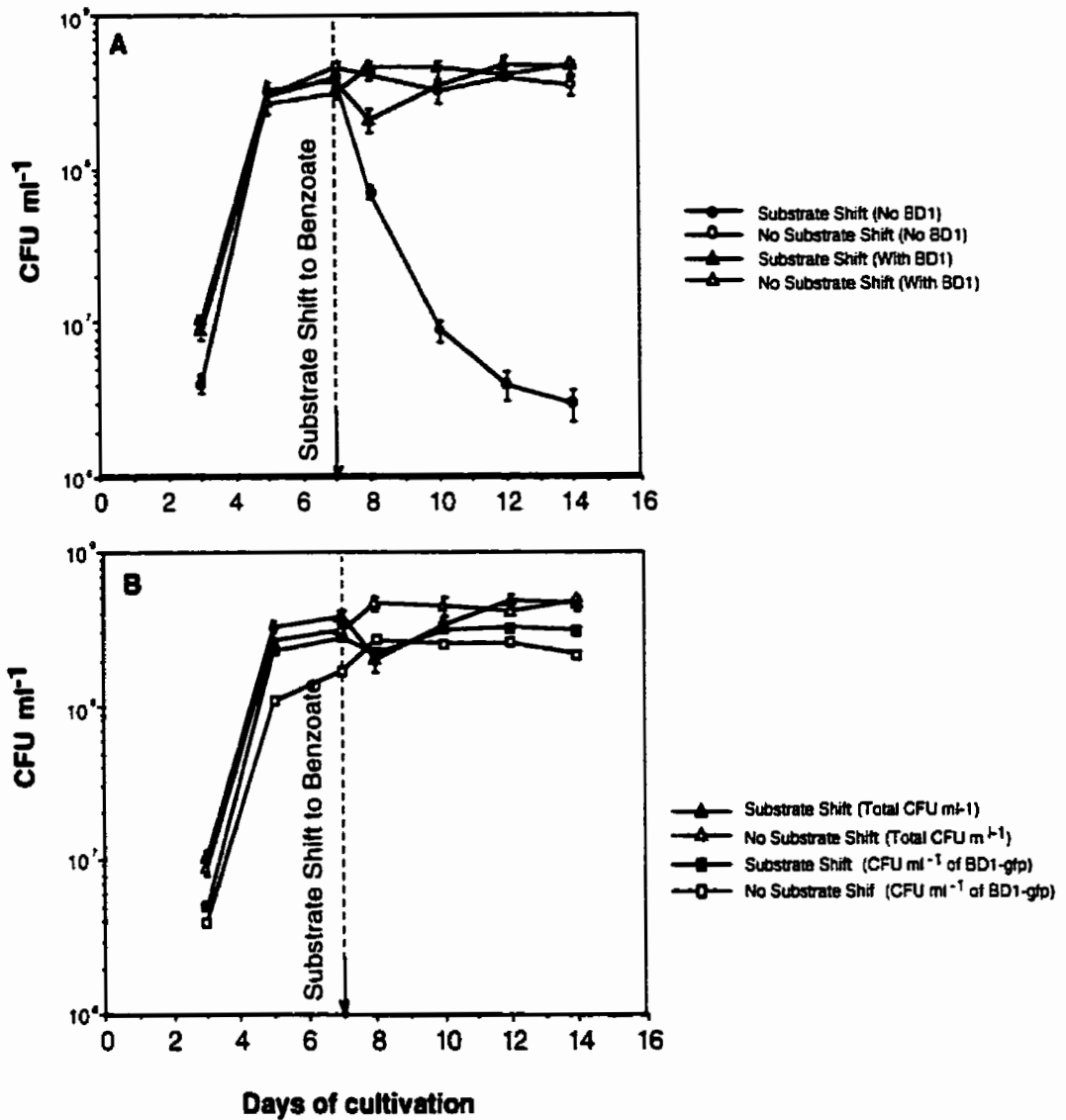
The protective effect of the benzoate resistant strain of *Pseudomonas* upon a microbial community obtained from a pristine soil environment was also studied. When the community not amended with BD1 was subjected to a substrate shift from TSB to 0.15% sodium benzoate as sole carbon source, there was an approximately three orders of magnitude reduction in planktonic cell numbers (from  $\sim 4.00 \times 10^8 \pm 3.54 \times 10^7$  to  $3.00 \times 10^6 \pm 7.07 \times 10^5$  CFU ml<sup>-1</sup>) (Figure 3.4.5). However, when the same community was amended with the benzoate-resistant *Pseudomonas* strain BD1, and subjected to the substrate shift, there was no significant reduction in the total cell numbers due to the shift. The numbers after the shift were also not significantly different from the planktonic cell numbers of the communities that were not subjected to substrate shift. The numbers of BD1 itself, though slightly declined initially, did not change significantly in response to the shift (Figure 3.4.5 B).

The beneficial interaction between BD1 and other members of the pristine community also resulted in more stable biofilms in terms of the overall thickness and surface area coverage occupied by the members of the original community, when the community amended with BD1 was cultivated in flow cells and subjected to the substrate shift (Figures 3.4.6 and 3.4.7). Communities not amended with BD1 responded to the substrate shift by a decline in biofilm thickness from  $22.55 \pm 1.95$   $\mu\text{m}$  before the shift, to  $8.21 \pm 3.85$   $\mu\text{m}$  within seven days after the shift (Fig 3.4.6). When TSB was replaced by benzoate as sole carbon source, the community amended with BD1 did not show any significant reduction in biofilm thickness and the values were not also significantly different from the values obtained when communities were continuously cultivated in TSB and not subjected to a substrate shift. The substrate shift also resulted in a significant reduction in the surface area coverage of biofilm

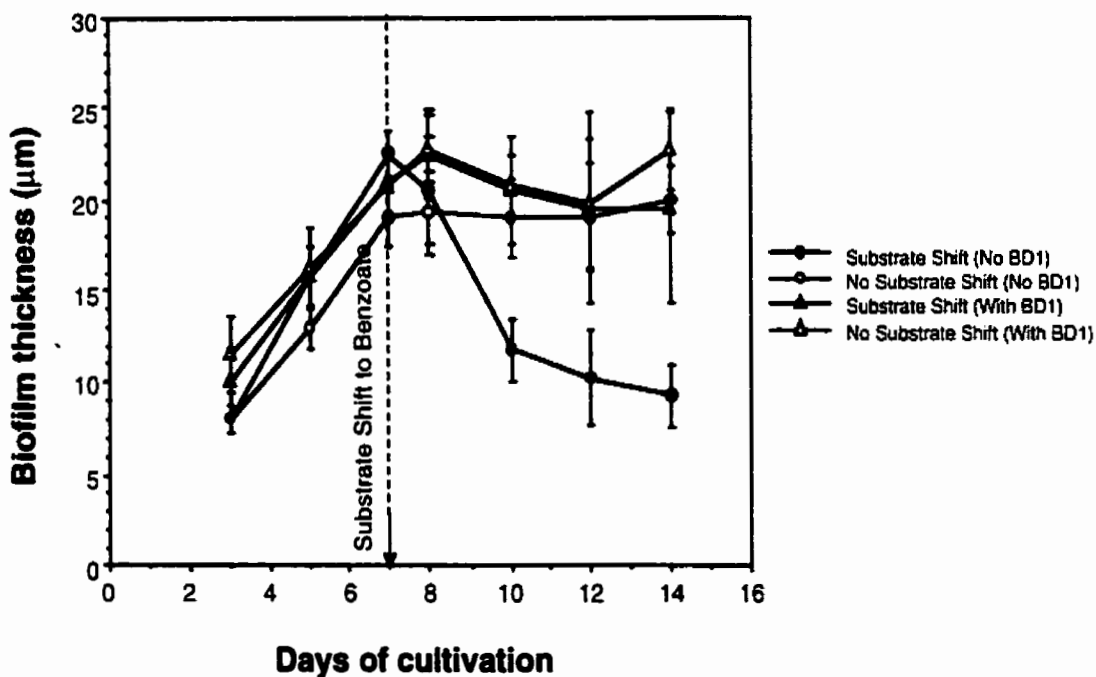


**Figure 3.4.4.** Surface area coverage of pure culture (BD1 or BS2 (*gfp*)) and binary culture (BD1+ BS2 (*gfp*)) biofilms cultivated in flow cells using 0.15 (A), 0.05 (B) and 0.015% (C) sodium benzoate. In general, the surface area coverage at all concentrations increased with time. At 0.15% benzoate concentration, <1% of the area was occupied by the BS2 (*gfp*) throughout the time course of observations. However, when cultivated in association with BD1, there was a significant increase in the area coverage of BS2 (*gfp*) ( $9.5 \pm 2.5\%$ ) in the 14 day old biofilms. These area measurements were also in agreement with the numbers of viable BS2 (*gfp*) cells obtained from the flow cell effluent (see figure 3.4.3). At low benzoate concentrations (figures B and C) the surface area coverage of BS2 (*gfp*) increased with time independently from strain BD1.

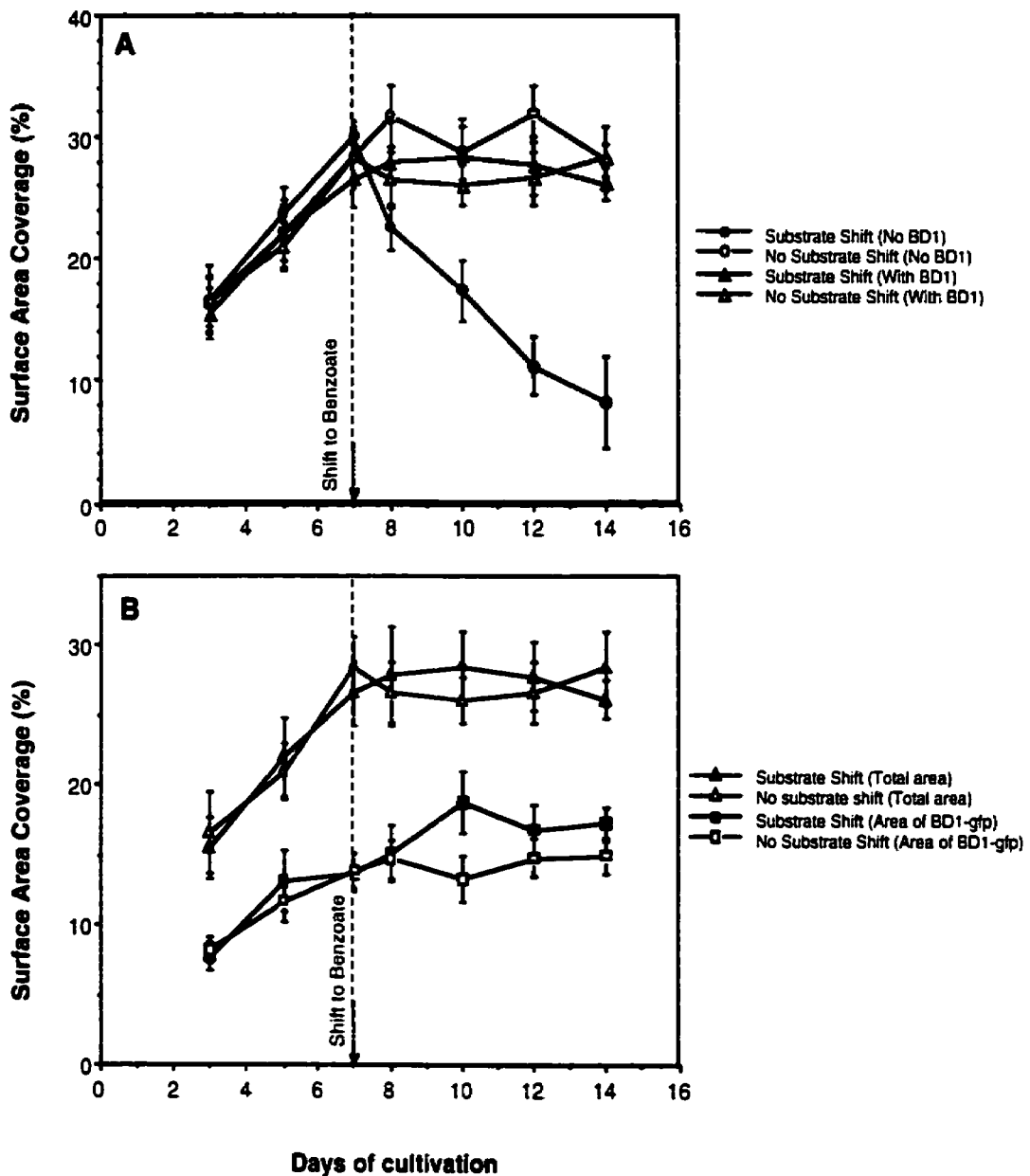




**Figure 3.4.5.** Effect of BD1-amendment on the number of emigrants when a flow cell community was subjected to a substrate shift from TSB to sodium benzoate as sole carbon source. A. Note the sharp decline in cell numbers in response to the shift when BD1(*gfp*) was not present. However, when BD1(*gfp*) was present as a member of the community, substrate shift did not affect the overall numbers. B. Changes in the numbers of BD1(*gfp*) within the amended community in response to the substrate shift. A comparison of the total numbers and the numbers of BD1 shows that presence of BD1(*gfp*) in the biofilm enhanced the growth of other members of the biofilm community also.



**Figure 3.4.6.** Effect of substrate shift from TSB to benzoate on the thickness of biofilm communities. Shift to benzoate resulted in a significant ( $p < 0.01$ ) decrease in biofilm thickness when BD1(*gfp*) (benzoate degrader) was not present. After the shift, thickness of BD1-amended community did not differ significantly from communities which were continuously cultivated in TSB. However, the formation of spatial structures (see Figure 3.4.9) resulted in a greater variability in biofilm thickness upon substrate shift.



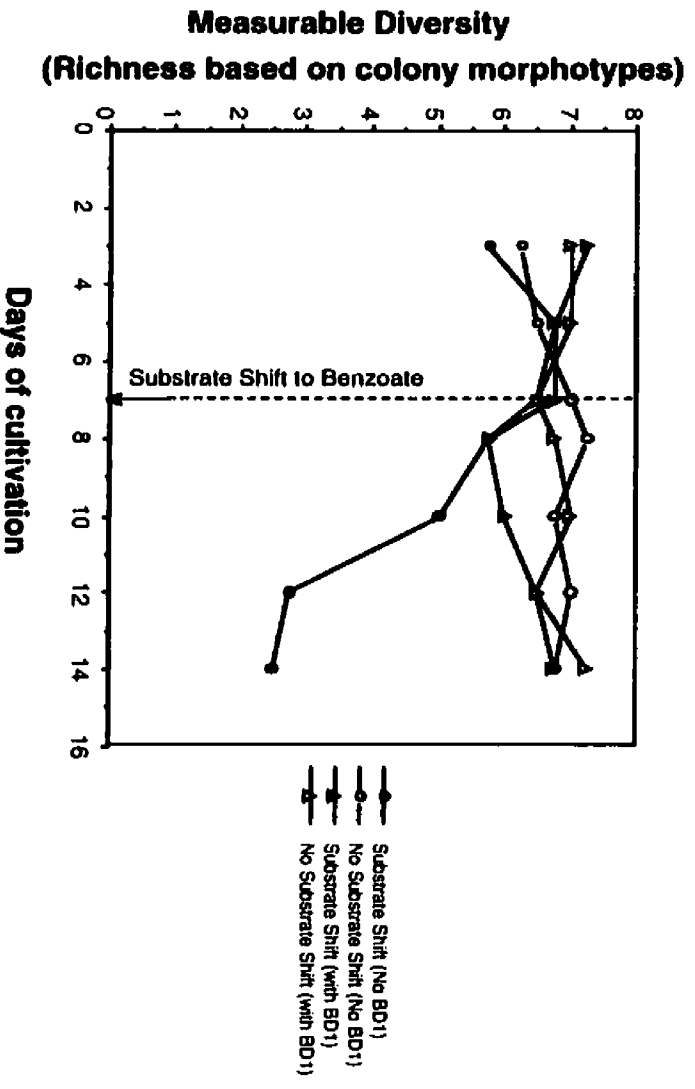
**Figure 3.4.7.** Effect of substrate shift on the surface area coverage of biofilm communities either amended or not with BD1. A. The substrate shift also resulted in a significant ( $p < 0.01$ ) decrease in biofilm area coverage when the community was not amended with BD1 (*gfp*). Note that the area coverage of BD1-amended community did not differ from communities that were continuously cultivated (no shift) on TSB. B. Numbers of BD1 within the BD1-amended communities suggesting that the presence of BD1(*gfp*) in the biofilm enabled the continued survival and proliferation of other members of the community even after the shift.

communities not amended with BD1 (from  $22.50 \pm 1.95\%$  to  $8.21 \pm 3.85\%$ ) (Figure 3.4.7 A). In contrast, when subjected to a substrate shift, the area coverage of the BD1-amended community ( $26.11 \pm 1.33\%$ ) did not significantly differ from that of communities continuously cultivated (no shift) on TSB (Figure 3.4.7 A) ( $28.30 \pm 2.64\%$  and  $28.08 \pm 1.41\%$  at the end of 14 days in the presence and absence of BD1 respectively). The substrate shift also adversely affected the measurable diversity of the pristine community as determined based on colony morphotypes. Richness of the non-amended community declined from  $5.75 \pm 0.96$  to  $2.50 \pm 0.58$  following the shift, whereas the richness of the BD1-amended community, in fact increased from  $5.75 \pm 0.96$  to  $6.75 \pm 0.50$  in response to the shift. Shannon diversity index declined considerably from 1.831 to 1.142 in the absence of BD1, and in the presence of BD1 it decreased only to a lesser extent (from 2.30 to 1.95) (Figure 3.4.8). Laser microscopy of the flow cell communities showed a random distribution of BD1 and other members of the microbial community when cultivated using TSB. However, when subjected to a substrate shift to benzoate as sole carbon source, BD1 developed cell clusters which were surrounded by other members of the community (Figure 3.4.9).

When supplied with 0.15% benzoate, the strain BD1 resulted in a significant reduction (57.31%) in the  $^{14}\text{C}$  label remaining in the medium after seven days of incubation (Figure 3.4.10). In contrast, neither BS2 nor the non-BD1 amended community alone was able to significantly degrade benzoate when supplied at 0.15% ( $94.40 \pm 4.02\%$  and  $99.07 \pm 1.07\%$  of the label remaining in the medium, respectively). However, when either BS2 or the community was amended with BD1, the resulting associations significantly reduced benzoate concentrations, as indicated by the percentage of label remaining in the medium ( $43.84 \pm 2.42$  and  $40.14 \pm 2.10\%$  respectively). At the lower benzoate concentration (0.015%), BS2 as well as the pristine community were able to cause ~60% reduction in the concentrations of benzoate even in the absence of BD1.

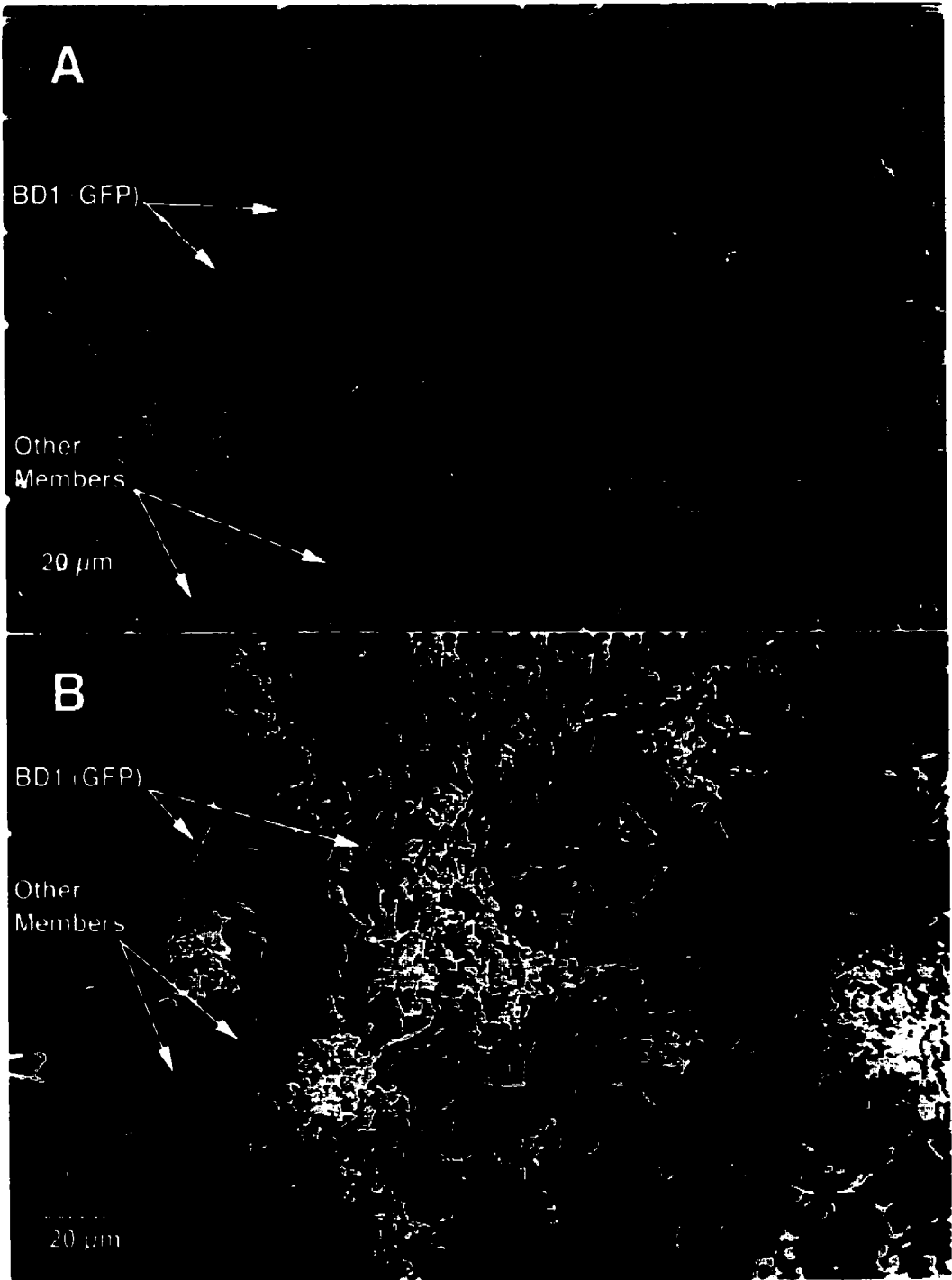
#### **3.4.4 Discussion**

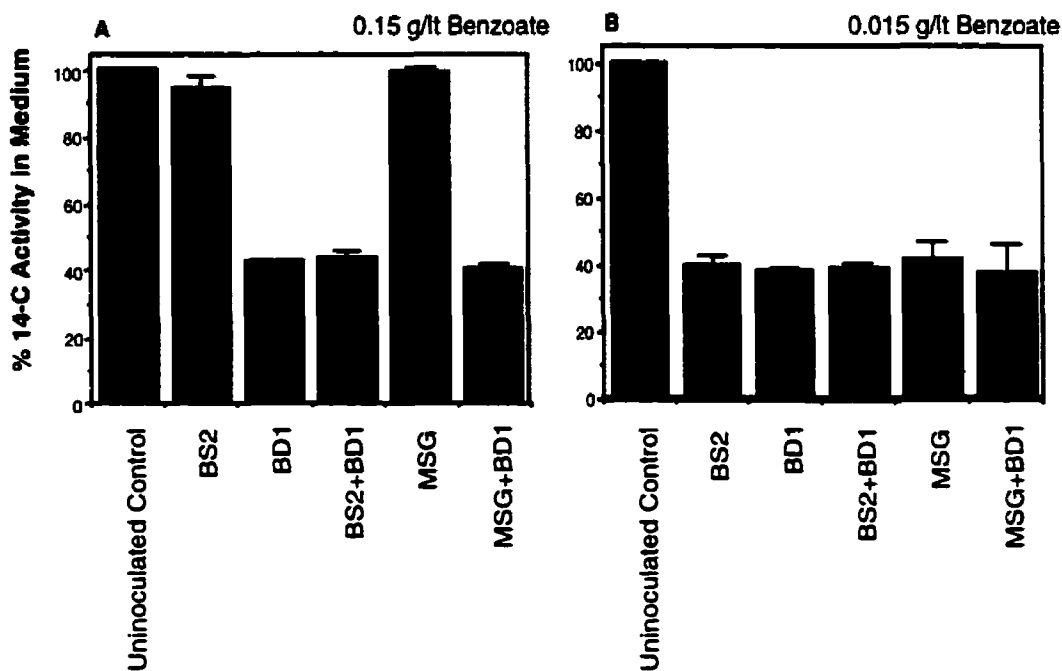
A number of studies have examined the interactions among various physical factors in determining the effectiveness of preservatives used in food products. For example, interactions between factors such as pH, temperature, time and different concentrations of various preservatives have been examined in the control or elimination of *Listeria monocytogenes* (Buchanan and Golden, 1994; Poysky et al., 1997),



**Figure 3.4.8.** Effect of substrate shift on the diversity of biofilm communities. Measurable diversity based on colony morphotypes (and hence, the community structure) remained stable when amended with BD1 (*gfp*). The diversity was decreased in the absence of BD1 (*gfp*). Higher diversity of BD1-amended community, suggested that BD1 (*gfp*) permitted the development of a diverse community network.

**Figure 3.4.9.** (Facing page) Confocal laser micrographs of the BD1-amended biofilm community prior to (A) and after (B) a substrate shift from TSB to sodium benzoate as sole carbon source. (A) Prior to the shift, cells of BD1(*gfp*) and other members of the biofilm community were randomly distributed. (B) After the shift, BD1(*gfp*) cells appeared to develop cell clusters surrounded by other members of the community. This spatial organization indicated that BD1(*gfp*) provides either: 1) metabolites which serve as nutrients to non-benzoate degraders, or 2) microniches of low benzoate concentration within the biofilm where benzoate-sensitive members can grow.





**Figure 3.4.10.** Degradative ability of pure and binary cultures as well as a microbial community either amended or not with BD1, at benzoate concentrations of 0.15% (A) and 0.015% (B). BD1 by itself, resulted in a significant reduction in the concentration of sodium benzoate as opposed to no reduction by BS2 or the community alone. The binary culture, as well as the microbial community could cause significant reduction in benzoate concentration in association with BD1 as indicated by the quantity of the <sup>14</sup>C label remaining in the culture fluid (A). In contrast, when low concentration of benzoate was provided, there was significant reduction in the label by BS2 or by the community, indicating that utilization of benzoate and hence reduction of concentration-dependent inhibition was the basis for the interaction between BD1 and BS2 or BD1 and the community.



*Salmonella enteritidis* (Teo et al., 1996), as well as *E. coli* (O157: H7) and *Salmonella typhimurium* (Kameswar et al., 1998). Models using Gompertz equation and response-surface methodology have been proposed to predict the effects of such interactions upon preservative effectiveness (Linton et al., 1996; Eifert et al., 1996). However, almost all of natural and processed food products are able to support and usually harbor more than one type of microbial species (Bennett et al., 1996; Van Campenhout et al., 1998) that are often engaged in beneficial or harmful interactions (Kim, 1990; Figueroa et al., 1997). Sometimes, these organisms are able to resist antimicrobials or other chemical preservatives. For example, adaptive resistance of bacterial strains against sodium benzoate that led to a lower preservative effectiveness in pharmaceutical products has been demonstrated (Zani et al., 1997). Thus, some of the more important interactions that occur among microorganisms may potentially reduce the effectiveness of antimicrobials, as has been demonstrated previously (section 3.3) as well as during the present study.

As discussed in section 3.3, when microbial communities cultivated using a labile carbon substrate such as TSB were plated on benzoate-containing agar, it resulted in the formation of satellite colonies in the vicinity of benzoate-resistant primary colonies. This effect also resulted in a dilution-dependent increase in the CFU ml<sup>-1</sup> of the community. The ability of the satellite isolates to grow on M-salts medium at low benzoate concentrations (0.015% - 0.05%), but not at higher concentrations (0.1% - 0.25%) suggested that primary colonies provided a concentration-dependent protection against sodium benzoate. The results of the present study, based on growth in batch and flow cell systems, also demonstrated a protective interaction between benzoate-resistant and -sensitive bacteria. This was evident from 1) the ability of BS2 to grow independently at the lowest benzoate concentration (0.015%) in both batch and flow-through systems, and 2) the removal of the inhibitory effect of 0.15% benzoate, when BS2 was cultivated in binary association with BD1.

While the growth enhancement of BS2 during binary growth was noted only at the maximum benzoate concentration (0.15%) in batch cultures, the protective effect was also noted at a lower benzoate concentration (0.05%) when cultivated using flow cells. This may be attributed to the continuous supply of benzoate in flow cells, maintaining its concentration and flux throughout the period of cultivation. Though sessile growth has been shown to offer some degree of protection against antimicrobial agents (Larsen and Fiehn, 1996; Ali-Vehmas et al, 1997), the flux of benzoate resulting from its continuous supply and flow conditions, might have been sufficient to cause

inhibition of BS2 biofilms. It has been previously shown that high fluxes of trisodium phosphate resulting from conditions of high flow were more effective in controlling *S. enteritidis* biofilms (Korber et al, 1997).

At the highest benzoate concentration, the surface area coverage of BS2 was significantly higher ( $p < 0.01$ ) during binary culture with BD1 than when it was cultivated as a pure culture. In contrast, at the lowest benzoate concentration, the BS2 coverage was higher when cultivated alone, than during binary culture. The explanation is possible competition between the benzoate-resistant and -sensitive strains for substrates and/or available functional niches.

The protective effect was also noted when the BD1-amended pristine microbial community was subjected to a substrate shift from TSB (a complex, labile carbon source) to M-salts medium containing a high, inhibitory concentration (0.15%) of sodium benzoate as sole carbon source. As a consequence of association with BD1, the biomass (CFU ml<sup>-1</sup> of emigrants, biofilm thickness and overall surface area coverage) and measurable morphotype diversity of the resultant biofilms were stably maintained even after the shift. This is evident from an analysis of the numbers of the original members of the biofilm community in the presence and absence of BD1, seven days after the shift to benzoate ( $3 \times 10^6 \pm 7.07 \times 10^5$  CFU ml<sup>-1</sup> in the absence of BD1, and  $1.55 \times 10^8 \pm 7.07 \times 10^5$  CFU ml<sup>-1</sup> when amended with BD1). Substrate shift also resulted in significant changes in the spatial distribution of BD1 (*gfp*) within the biofilm communities. Prior to the shift, BD1 cells were randomly distributed. However, after the shift, BD1 cells developed cell clusters which were surrounded by other members of the community. This spatial organization indicated that BD1 provides either: 1) metabolites which serve as nutrients to non-benzoate degraders, or 2) microniches of low benzoate concentration within the biofilm where benzoate-sensitive members can grow. This probably also resulted in higher cell numbers, thickness, percent area coverage and diversity of biofilm communities in the presence of BD1(*gfp*) than in its absence.

Microbial degradation of sodium benzoate has been well demonstrated (Battersby and Wilson, 1989; Malachowsky et al., 1994). Microorganisms with the capacity to degrade benzoates and other aromatic molecules are prevalent in many natural ecosystems (Dunbar et al., 1996; Fang et al., 1997, Crawford et al., 1998). To evaluate whether the protective effect of BD1 was due to the reduction of benzoate concentration, a radiolabeled benzoate mineralization experiment was conducted. This revealed that when supplied with 0.15% benzoate, BD1 was able to reduce the label by approximately 60% within 7 days. In contrast, BS2 or the pristine community alone

were not able to significantly degrade benzoate at this concentration. This might be due to the inhibition of BS2 or the pristine community at high benzoate concentration as revealed by the batch and flow cell cultures of BS2 or the community. Thus, these results confirmed that BD1 played a protective role in both binary and community cultures by reducing benzoate concentration to levels that are non-inhibitory to BS2 or other community members.

Although BD1 protected BS2 and the pristine community members against generally high toxic concentrations of benzoate, by degrading it to lower levels, the growth of the sensitive strains might also have been benefited by a supply of degradation intermediates released during active benzoate degradation by BD1. Such nutritionally dependent microbial associations are often noted in various environments. For example, during the production of sour cassava starch, non-amylolytic *L. plantarum* strains were able to thrive on starch through an association with amylolytic *L. plantarum* strains (Figuerola et al., 1997). Pathogenesis of Legionnaire's disease and Pittsburgh pneumonia was also proposed to depend on the nutritional interaction between pathogens and the normal microflora of the respiratory tract (Stout et al., 1986). Janzen et al. (1995) suggested a possible role of commensalistic interactions in extending the habitat range of soil microflora, and showed that addition of sterile compost extract containing soluble microbial products could mediate commensalistic interactions among populations involved in various mineral cycling processes. In the present study, the habitat ranges of the benzoate-sensitive *Pseudomonas* strain and the pristine community have been shown to be enhanced during growth in association with BD1. However, growth of BS2 or members of the pristine community might not have exclusively dependent on the supply of benzoate metabolites, as BS2 and the members of the pristine community were able to degrade and utilize sodium benzoate when provided at low, non-inhibitory concentrations.

GFP has been used as an *in situ* probe for the visualization of the expression of genes involved in the biodegradation of toluene and some other related compounds (Møller et al., 1997). Stretton et al. (1998) used GFP to visualize the spatial niches of a *Pseudoalteromonas* strain within biofilm communities. Christensen et al. (1998) used GFP expression as a means of detecting horizontal gene transfer within synthetic biofilm communities. GFP-tagged TOL plasmids were used for this purpose. The results of the present study also demonstrated the usefulness of GFP as a stable fluorescent marker for *in situ* visualization and quantification of members within binary culture and

community biofilms. Chromosomal insertion of the gene encoding GFP allowed stable maintenance of the character without maintaining a selective pressure.

The findings of the present study suggests that an understanding of the role of microbial interactions in biodegradation, food spoilage, food-borne pathogenesis, wastewater treatment, and human health is instrumental in developing systems that efficiently utilize microbial associations or control microbial growth. Van Ginkel (1996) described a bacterial consortium that resulted in complete degradation of linear alkyl benzene sulfonate, and suggested that this could be utilized in wastewater treatment plants without toxic metabolite accumulation as long as the sludge retention times were appropriate and would permit maintenance of all members of the consortium. Tartakovsky et al. (1998) developed a commensal system of the aerobic methanotroph *Methylosinus sporium* with anaerobic methanogens, that could efficiently mineralize tetrachloroethylene under coupled anaerobic/aerobic conditions using immobilized systems. Adriaens and Focht (1990) found that a binary-culture biofilm containing two *Acinetobacter* sp. developed in a continuous aerobic fixed-bed bioreactor system was able to cometabolize polychlorinated biphenyls using 500 ppm sodium benzoate as the primary substrate, resulting in chlorobenzoates and chlorides as PCB degradation metabolites.

While the identification of protective or synergistic interactions is advantageous to develop applications in bioremediation and wastewater treatment, identification of such interactions is crucial in the choice and administration of methods to the treatment of human illnesses. For example, Koenig et al. (1998) found that commensalistic penicillin-resistant streptococci could serve as a reservoir of beta-lactam resistance in *S. pneumoniae* that could cause more serious illnesses. It has also been suggested that the primary infection of the lower respiratory tract with *Mycoplasma ovipneumoniae* and *Bordetella parapertussis* in sheep and goats, can increase their susceptibility to secondary *Pasteurella haemolytica*-based pneumonial infections (Brogden et al., 1998). This was primarily due to the breakdown of antimicrobial barriers that are usually found in the respiratory tract, by the primary colonizing organisms. Dykhuizen et al. (1996) reported that failure of co-amoxycylav or penicillin in the treatment of Group A streptococcal (GAS) pharyngitis and its recurrence is associated with the presence of beta-lactamase activity in commensal flora. Saez-Nieto et al. (1990) were able to obtain penicillin-resistant *Neisseria* strains by genetic transformation of penicillin-sensitive strain using DNA from a penicillin resistant strains. This suggested that commensal *Neisseria* spp. could be a source of antibiotic resistance genes in meningococci. Stout et

al. (1986), demonstrated the dependence of *Legionella pneumophila* and *Tatlockia micdadei*, the causative agents of Legionnaire's disease and Pittsburgh pneumonia respectively, on *Haemophilus influenzae* and *Neisseria meningitidis*, for cysteine in a nutritionally deficient medium. They concluded that pathogenesis of Legionnaire's disease and Pittsburgh pneumonia might depend on the interaction between pathogens and the normal microflora of the respiratory tract. As suggested by Brannan (1995), testing the susceptibility of pure-cultured microorganisms to antimicrobials can sometimes give false-positive results. This occurs if the organism is susceptible to an antimicrobial agent when grown in pure culture, but not when grown in community culture if synergistic mechanisms provide resistance (Brannan, 1995; Caldwell and Costerton, 1996).

The results of this study reaffirmed the significance of microbial associative strategies in various environmental processes, including resistance to antimicrobial agents. As discussed in section 3.3.4, proliferation strategies based on association should thus be an important consideration, in order to fully understand and develop applications in the disciplines of food preservation, human health, and environmental processes such as biodegradation. These results also suggest that community-level synergisms may be required for the optimal proliferation of communities, just as molecular synergisms have been optimized for the proliferation of cells. Enhanced homeostasis of BD1 amended biofilm communities when subjected to a substrate shift from TSB to benzoate, reaffirmed the role of organismal or cellular recombination in enhancing the reproductive success of microbial communities under conditions of stress.

### **3.4.5 Summary**

The objective of the present study was to evaluate the protective interactions between benzoate-degrading and -sensitive bacteria in binary culture and in community settings, as well as in batch and flow environments. Initially, the interactions between the benzoate-degrading *Pseudomonas* strain BD1 and the benzoate-sensitive *Pseudomonas* strain BS2, were investigated. When cultivated in flow cells using M-salts solution supplemented with 0.15% benzoate as sole carbon source, the presence of BD1 resulted in an approximately three orders of magnitude increase in the planktonic numbers of BS2 and a 10 fold increase in the surface area covered by BS2 within biofilms. The community-level beneficial effect of BD1 was investigated using a microbial community obtained from a pristine soil environment. When amended with BD1, there was no significant ( $p < 0.01$ ) change in the numbers (CFU ml<sup>-1</sup>) or diversity

of suspended cells associated with the biofilm community following a shift from TSB to benzoate. In contrast, when TSB was replaced by 0.15% benzoate as sole carbon source, the community not amended with BD1 showed an approximately two orders of magnitude reduction in cell numbers as well as a decrease in species diversity (based on Shannon indices and richness measurements). Analysis of the non-amended biofilm community also revealed that there was a significant ( $p < 0.01$ ) reduction in thickness (from  $22 \pm 2.4 \mu\text{m}$  to  $9.2 \pm 1.3 \mu\text{m}$ ) and percent surface area coverage (from  $35 \pm 5.2\%$  to  $8.2 \pm 3.9\%$ ) in response to the substrate shift. In the BD1-amended community, the shift from TSB to benzoate resulted in the spatial repositioning of GFP-labeled BD1 within the community, from randomly distributed cells of BD1 when cultivated in TSB, to growth in the form of cell clusters surrounded by other community members when cultivated using benzoate. Using  $^{14}\text{C}$ -labeled benzoate, it was demonstrated that the benzoate-sensitive strain BS2, as well as the non-amended community were capable of degrading sodium benzoate when provided at a low concentration (0.015%). However, neither BS2 nor the non-amended community could degrade 0.15% benzoate, while BD1 plus BS2, and the BD1-amended community removed 50% of the label at this higher concentration in seven days. These results demonstrated that BD1 played a protective role against high benzoate concentrations both in its association with BS2 and with the community, and hence supported the existence of community-level proliferation strategies. Similar protective associations are probably widespread in industrial and environmental settings, and should therefore be considered during the evaluation of antimicrobial agents.

#### **4 CONCLUSIONS**

Interactive microbial associations have been shown to be responsible for various environmental and industrial processes, as has been discussed in the literature review. Evolutionary theories based on selection (including individual, group or community-level selection and niche construction theories) are unnecessarily complex. They conceptualize selection as a mechanism of evolution rather than an outcome, and hence fail to efficiently explain the emergence of associative networks of microorganisms. Based on Occam's Razor, inefficient concepts must be rejected in favor of more efficient concepts. A more efficient and comprehensive explanation is the proliferation approach, based on recombination events that occur at various spatial scales of organization (molecules, plasmids, cells, communities, etc.) during the evolution of all forms of life, including microorganisms. During the present study, the utility of this approach was tested by subjecting a microbial community to environmental transitions (with and without organismal recombination), and by following the emergence of community-level properties in response to these transitions.

A preliminary study was done to examine the growth responses of a benzoate-degrading biofilm community to substrates with varying degrees of complexity in chemical structure. The results suggested that other factors besides substrate complexity and degree of halogenation were involved in determining the refractoriness of xenobiotic compounds to degradation by microbial communities. These factors include the physicochemical characteristics of the compound (such as octanol/water partition coefficient, and toxicity), as well as community-characteristics (such as prior exposure or acclimation to the test compound). Principal component analysis of metabolic fingerprints of the flow cell communities revealed a clear distinction between growth on chlorinated compounds and on non-chlorinated compounds. This significant impact of substrate chlorination on the emergent metabolic characteristics of the sessile communities led to the application of a model of substrate transition involving chlorinated and non-chlorinated substrates during subsequent studies aimed at testing the existence of community-level proliferation strategies. Overall, results of this preliminary study demonstrated the inherent flexibility of microbial communities permitting the

emergence of various structural and metabolic properties in response to growth in varied physicochemical environments.

Studying the emergence of community-level proliferation strategies with and without external organismal recombination, and thus testing the utility of the proliferation theory, involved subjecting biofilm communities to sudden and gradual environmental transitions and examining the emergence of community structure (based on the patterns of emigration) and other community-level properties (such as biofilm biomass, architecture, community diversity, speed of adaptation and plasmid content) in response to these transitions. Repeated inoculation of flow cell communities with a composite inoculum (organismal recombination) resulted in an increase in the number and diversity of emigrants as well as greater thickness and percent area coverage of the biofilms than when the communities were inoculated only at the beginning of the experiment. It also resulted in more rapid adaptation of biofilm communities during most transitions. These results suggested that organismal recombination (recombination of organisms in a way analogous to genetic recombination at the cellular level) as a mechanism of adaptation enhanced the growth of microbial communities exposed to environmental stresses. Patterns of emigration, detected during the adaptation of biofilm communities to sudden and gradual environmental transitions, showed the appearance and disappearance of discrete sets of organisms. This suggested that the biofilm communities responded to environmental stresses as sets of interacting organisms, as opposed to functioning exclusively as unrelated individuals. Repeated cycling of biofilm communities enhanced the growth of biofilm communities (as revealed by higher cell numbers during the second cycle of environmental transitions), suggesting the possibility that structural and spatial positioning information within these communities accrued from one cycle to the next and was thus heritable, although it was not necessarily genetically encoded. This is consistent with the postulation that heritable information occurs at multiple spatial levels of organization (community, cellular and other levels) and is recombined simultaneously at all levels, as proposed by the proliferation theory.

Additional evidence for the existence of community-level proliferation strategies was obtained when biofilm communities that emerged in response to sudden environmental transitions were tested for dilution-dependent plating efficiency. Increased plating efficiencies were observed at lower dilutions when biofilm communities cultivated using TSB were plated on a medium containing sodium benzoate as sole carbon source. Digital image analysis revealed that higher plating efficiencies were concomitant with the formation of satellite colonies around benzoate-resistant primary



colonies. Subsequent plating assays suggested that primary colonies provided the satellites with a concentration-dependent protection from sodium benzoate. Although this protective effect was non-specific (protection being extended to other control organisms), the formation of protective interactions based on benzoate resistance and higher plating efficiencies at lower dilutions are enough evidence to suggest that community-level synergisms may be required for the optimal proliferation of communities, just as cellular and molecular synergisms have been optimized for the proliferation of cells and molecules. The results also demonstrated that density-dependent plate counts and digital satellite analysis can be useful in screening for synergistic interactions within microbial communities when community-level interactions are crucial to the survival of individuals and hence preclude the possibility of isolation of individuals and subsequent screening for their interactions.

The protective synergisms involved in benzoate resistance which resulted in the formation of satellite colonies on benzoate agar were also noted when benzoate-resistant and benzoate-sensitive bacterial strains (*Pseudomonas fluorescens* strains BD1 and BS2 respectively) were cultivated as binary pairs either in batch or flow cell systems. When a microbial community obtained from a pristine soil environment was amended with a benzoate-resistant *Pseudomonas fluorescens* strain BD1, and subjected to a substrate shift from TSB to benzoate, it exhibited a greater degree of homeostasis (in terms of biofilm thickness and coverage as well as the number and diversity of emigrants) than the non-amended community. This enhanced stability of the BD1-amended communities reaffirms our previous finding that external organismal recombination plays a significant role in the proliferation of microbial communities under conditions of stress. Results of radiolabeled benzoate mineralization experiments confirmed that BD1 played a protective role against high benzoate concentrations both during binary association with benzoate-sensitive BS2, as well as during its association with the pristine community. The primary isolate (BD1) by virtue of its degradative ability reduced benzoate concentration in its vicinity to non-inhibitory levels, thereby permitting the growth of sensitive strains.

Overall, the results of the present study supported the postulation that proliferation occurs at multiple spatial scales of organization including the community-level, and hence supported the utility of the proliferation theory in understanding the formation and functioning of microbial communities. More specifically, the body of evidence to support bacterial proliferation as functional sets of interacting organisms consisted of: 1) patterns of emigration showing the appearance and disappearance of

discrete sets of organisms when biofilm communities were subjected to sudden and gradual environmental transitions, 2) higher plating efficiencies of microbial communities obtained at lower plating dilutions, and 3) formation of satellite associations due to the protective interactions between benzoate-resistant and benzoate-sensitive bacterial strains. Furthermore, the existence of community-level synergisms also was evident from observations showing the utility of organismal recombination as a mechanism of adaptation. These include 1) enhanced biofilm thickness, area coverage, emigrant cell numbers as well as more rapid adaptation of biofilm communities repeatedly inoculated with a composite inoculum, and 2) enhanced homeostasis of BD1-amended biofilm communities when subjected to a substrate shift from TSB to benzoate, resulting in high cell numbers, biofilm thickness, coverage and community diversity. Enhanced growth of biofilm communities due to repeated environmental cycling suggested that biofilms represent a form of constructed niche that is heritable as a whole (replication of community structure not being needed for inheritance during each cell cycle because the external niches are shared by the progeny), in the same sense as the genetic coding contained in the individuals is heritable but must be replicated because it is not immediately shared with other organisms or offspring. Finally, the formation of protective associations between community members involved in benzoate resistance suggest that proliferation strategies based on association should be an important consideration during efforts to control or enhance the activities of microbial communities in industrial or environmental settings.

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