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INFLUENCES OF SESSILE PRIMARY PRODUCTIVITY AND METHANOL ON MICROBIAL COMMUNITY STRUCTURE AND SECONDARY METABOLISM IN AN INDUSTRIAL GROUNDWATER RECHARGE POND

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Timothy P. Laatsch

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By

Timothy P. Laatsch

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ABSTRACT

INFLUENCES OF SESSILE PRIMARY PRODUCTIVITY AND METHANOL ON MICROBIAL COMMUNITY STRUCTURE AND SECONDARY METABOLISM IN AN INDUSTRIAL GROUNDWATER RECHARGE POND

By

Timothy P. Laatsch

The Pharmacia/Upjohn Pond receives $3x10^7$ L day^{-1} of non-contact cooling water containing mg L^{-1} concentrations of methanol and μg L^{-1} concentrations of several organic solvents. Methanol was previously shown to be biologically oxidized by biofilm communities in the system; however, metabolism of other solvents is less well understood. The objective of this study was to determine the influences of sessile primary productivity and methanol on metabolism of other solvents. Attached microbial communities were allowed to develop on PVC surfaces *in situ* under LIGHT and DARK treatment regimes. Mature biofilms were harvested for microbial community structural analyses and aerobic metabolic studies with methanol as a primary growth substrate and several secondary non-growth substrates: dichloromethane (DCM), toluene (TOL), and chlorobenzene (CB). Direct microscopic counts did not vary significantly between treatments, but fatty acid methyl ester (FAME) profiles revealed differences in microbial community structure. TOL and CB were secondarily transformed at a significantly higher rate in communities colonized in the dark. Secondary transformation of DCM was observed only in biofilms that were pre-incubated with a constant source of methanol. Sessile photosynthesis did not stimulate heterotrophic bioremediation activity on a primary or secondary metabolic level. Primary metabolism of methanol enhanced secondary metabolism of DCM, but had no effect on secondary metabolism of other solvents.

To my beautiful wife Jane, whose love, support, and self-sacrifice have allowed me to realize my dreams

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LITERATURE REVIEW

Large quantities of a variety of xenobiotic compounds are synthesized annually for use as solvents, refrigerants, cleaning agents, fire retardants, chemical synthesis intermediates, and pesticides (Janssen *et al.*, 1990). These compounds have become pervasive environmental contaminants through point-source and dispersed discharge in industrial and municipal wastewater, agricultural runoff, and landfill leachate (Chaudry and Chapalamadugu, 1991; United States Evironmental Protection Agency, 1983). Contamination of surface and subsurface drinking water resources presents a particularly complex environmental problem and poses a substantial public health concern.

Xenobiotic compounds are generally toxic and many are suspected carcinogens, mutagens, or terratogens having potentially long-term and adverse health effects. These compounds are often persistent in the environment due to the existence of few natural analogs or naturally-evolved mechanisms for degradation. Halogenation renders these contaminants even more recalcitrant, because removing multiple halogen molecules can be energetically costly and the large physical nature of the halogen molecule can block the active sites of degradation enzymes (Sayler *et al.*, 1984; Neilson *et al.*, 1985; Boyle, 1989; Hardman, 1991). Moreover, wastewater discharges often consist of a complex mixture of compounds of various types and concentrations, rendering a single remediation technology ineffective (Bitzi *et al.*, 1990). Remediation of contaminated

water resources can also be extremely expensive and time-consuming (Amman and Koch, 1994). As such, considerable research effort has been dedicated to discovery and understanding of technology and mechanisms for cost-effective removal of complex mixtures of trace-level contaminants from water resources (Rittman *et al.*, 1980; Bouwer and McCarty, 1985; Henson *et al.*, 1989; Hwang *et al.*, 1989a; Hwang *et al.*, 1989b; Bitzi *et al.*, 1990).

Bioremediation represents one particularly promising approach to treatment of contaminated water resources. Bioremediation exploits the immensely diverse metabolic capabilities of microorganisms to effect a biological transformation of environmental contaminants into harmless end-products. During the course of evolutionary history, microorganisms have developed a wide array of enzymatic pathways for degradation of naturally-occurring compounds (Alexander, 1965), the structures of which are often analagous to many modern xenobiotic compounds. Some of these naturally-evolved enzymes are capable of breaking the chemical bonds within synthetic analog compounds, resulting in biodegradation. Ideally, the microorgansisms completely metabolize the contaminants into the harmless end-products of carbon dioxide and water, releasing energy for growth and generating microbial biomass.

Contaminants that can be completely oxidized by the microorganism and are present in sufficient concentrations to support microbial growth are referred to as primary growth substrates (Alexander, 1981; Rittman and McCarty, 1980). When concentrations fall below the threshhold to support microbial growth, the compounds are known as secondary growth substrates. Secondary substrates can be metabolized in the presence of

a primary growth substrate or when the combined concentrations of multiple secondary substrates is sufficiently high to support microbial growth (McCarty *et al.*, 1981; Bouwer and McCarty 1982; Bouwer and McCarty, 1983). Cometabolism is the apparently fortuitous, partial metabolism of a compound through non-specific enzyme activity (Jensen, 1963). Cometabolism results in no energy gain for the organism and generates potentially harmful intermediate compounds, which can accumulate to toxic levels or be used for energy by other opportunistic microorganisms in a consortium. Throughout this current study, true secondary metabolism is not distinguished from cometabolism and both are collectively referred to as secondary transformation. Primary and secondary metabolic transformations are the principal mechanisms underlying bioremediation as a treatment technology.

Bioremediation is often based upon a consortia of microorganisms growing within a polysaccharide matrix attached to some physical substrate, such as soil, sediment, plants, glass, or plastic. The importance of attached growth has long been recognized (Zobell, 1943) and attached microorganisms are numerically predominant in natural environments, particularly oligotrophic aquatic systems (Geesey *et al.*, 1978; Costerton and Geesey, 1979; Ladd *et al.*, 1979). These attached microbial communities are termed *biofilms* and presumably provide the organisms living within a number of system-specific advantages (Breznak *et al.*, 1984; Hamilton, 1987). Attachment prevents the organism from being washed out of lotic systems and thereby maintains proximity to resources for growth (Jones *et al.*, 1969; Fletcher and Floodgate, 1973; Paerl, 1975; Geesey *et al.*, 1978). The polysaccharide matrix may act as a nutrient exchange reservoir, concentrating

critical nutrients and carbon sources in the proximity of the cell (Costerton and Lashen, 1984). In the case of epiphytic (plant-associated) biofilms, the organism may benefit from substrates exuded from the living attachment surface. Attached growth may prevent or retard bacterial predation by protozoa (Costerton *et al.*, 1985). Perhaps most importantly, attached growth fosters beneficial microbial interactions that contribute to maintaining a favorable microenvironment around the cell (Haack and McFeters, 1982; Wimpenny *et al.*, 1983; Hamilton, 1985; Jeffrey and Paul, 1986; Murray *et al.*, 1986; Hamilton, 1987; Characklis *et al.*, 1990). Microbial biofilms are commonly used among the wide array of bioremediation technologies in practice.

Biodegradation is dependent upon the presence of microorganisms with appropriate metabolic pathways and environmental conditions that favor the desired microbial activity. Bioaugmentation attempts to correct for a deficiency in metabolic pathways by directly inoculating the contaminated site with microorganisms possessing the desired metabolic properties. However, transplanted and engineered microorganisms frequently do not survive in the environment because they lack the necessary adaptations to make them competitively fit (Acea and Alexander, 1988; Goldstein *et al.*, 1985; Pahm and Alexander, 1993; Zaidi, 1988; Zaidi, 1989; Pritchard *et al.*, 1992). In fact, bioaugmentation has never been successfully demonstrated on a significant scale in the scientific literature (Pritchard *et al.*, 1996).

In situ bioremediation relies upon the adapted metabolic activity of microorganisms inherent to the contaminated site. During the course of several years of exposure to the contaminants, natural selection fosters development of a microbial

community with the ability to exploit these compounds as growth and energy subtrates. The high diversity associated with natural environments increases the likelihood of functional redundancy, promotes interspecies interactions, and allows the microbial community to tolerate a broad range of environmental conditions (Atlas and Bartha, 1993). The inherent organisms are also more well-adapted to the conditions of the site and are more likely to survive in highly defined niches than are introduced organisms. Multiple food-chain levels in natural environments may help to preserve balance and maintain active microbial communities. Community diversity is often associated with improved community stability and increases the chances of maintaining desired bioremediation activity under a more broad subset of environmental conditions (Atlas and Bartha, 1993).

Intrinsic or passive bioremediation, also known as natural attenuation, involves allowing the contaminated site to self-remediate while carefully monitoring the water quality and environmental conditions at the site (Hinchee *et al.*, 1995). Passive bioremediation is becoming increasingly accepted by regulatory agencies as a low-cost remediation alternative, particularly groundwater contamination. However, the applicability of passive bioremediation is site-specific, depending upon the nature of the contamination, the proximity to drinking water sources, and the existing environmental conditions. Site-specific applied research is necessary to evaluate the potential applicability of passive bioremediation and to completely understand the mechanisms of remediation therein (Pritchard *et al.*, 1996).

Even if the necessary metabolic pathways may be present, environmental conditions within a contaminated site often need to be manipulated to elicit the desired remediation in a timely fashion. Nutrients may be limiting, electron acceptors may be deficient, a primary growth substrate may be lacking, pH may be too acidic or alkaline, or surface area for attachment may be insufficient. Manipulation of the existing microbiota and/or environmental conditions to optimize bioremediation activity will be referred to in this paper as biomanagement. Biomanagement generally involves extensive site-specific research to identify the important system parameters and to determine the optimal configuration of those parameters. Only through a holistic and multidisciplinary research approach can each system be manipulated in a manner that optimizes remediation activity and thereby minimizes public health risk. This thesis will detail the research efforts to determine the mechanistic basis of *in situ* bioremediation and the feasibility of biomanagement in the Pharmacia/Upjohn Pond system in Portage, Michigan.

The Pond is a 34-ha industrial groundwater recharge basin at the Pharmacia/Upjohn main pharmaceutical manufacturing facility (Figure 1.1). The Pond receives approximately 30 million L day^{-1} non-contact cooling water pumped directly from groundwater resources at the site. This groundwater has been historically contaminated with a host of secondary compounds, which sporadically appear in the Pond influent at $\mu g\ L^{-1}$ concentrations. Water entering the Pond is also chronically contaminated with mg L^{-1} concentrations of methanol, due to the use of methanol brines within the cooling system. Approximately 25-40% of water leaves the system as surface discharge, while the remainder recharges local shallow aquifers. All organic contaminants fall below detection limits in water discharged from the system, suggesting

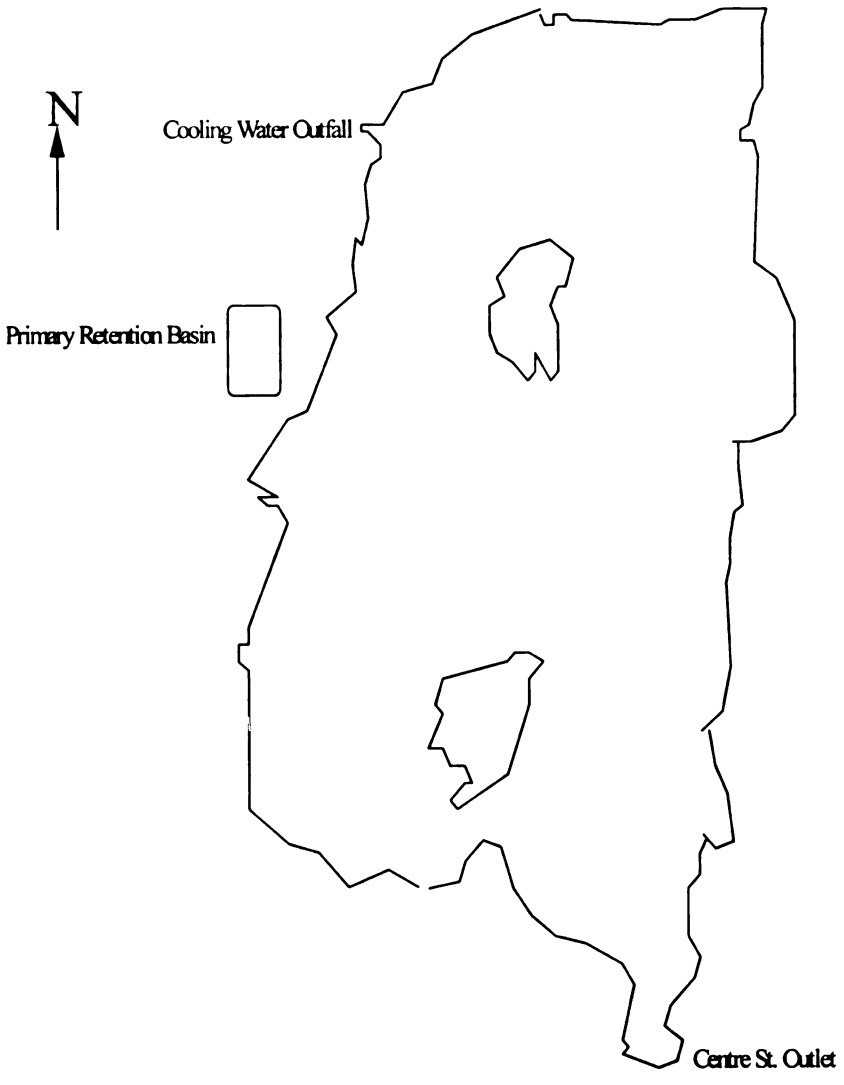

Figure 1.1 Map of Pharmacia/Upjohn Pond (September 1992).

that the pond is functioning as an effective large-scale natural bioreactor. One thrust of this research was to determine the biological mechanisms responsible for contaminant removal and the factors influencing those mechanisms.

Methanol comprises a significant fraction of the dissolved organic carbon entering the highly oligotrophic Pharmacia/Upjohn Pond system (Klug *et al.*, 1996) and is likely serving as a growth substrate for biofilm bacteria in the Pond. Because one-carbon (C_1) compounds such as methane and methanol occur abundantly throughout nature (Hanson, 1992), organisms that utlize C_1 compounds as sole carbon and energy sources (methylotrophs) are also ubiquitously distributed and have been isolated from nearly all types of environmental samples (Corpe, 1985; Hanson, 1980; Heyer, 1977; Hutton and Zobell, 1949; Whittenbury *et al.*, 1970; Sieburth *et al.*, 1987; Strand and Lidstrom, 1984). Expectedly, previous research (Klug *et al.*, 1996) and the present Pond studies have revealed substantial methanol oxidation activity in epiphytic biofilms and sediments. Epiphytic methanol-oxidizing bacteria have also been isolated from Pond samples.

Methylotrophs are a metabolically diverse group of organisms, including both the methane and methanol utilizers. However, the term methylotroph is more commonly used to distinguish the subset of methanol utilizers unable to use methane from the methanotrophs and will be used as such throughout this paper. During primary methanol metabolism, all methylotrophs express the enzyme methanol dehydrogenase to oxidize methanol to formaldehyde, a key intermediate which is then either respired to carbon dioxide to produce energy or assimilated into cellular biomass (Bratina and Hanson, 1992). However, the methylotrophs are a physiologically diverse group or organisms and

include a number of cosmopolitan heterotrophs with a wide array of ancillary enzymatic pathways for metabolism of other carbon compounds (Green, 1992). A vast number of methylotrophs, other bacteria, and various mixed cultures are reported in the literature as being capable of growth on many of the secondary contaminants entering the Pharmacia/Upjohn Pond, a brief summary of which is presented in Table 1.1.

Methylotrophs have diverse bioremediation potential, but would be particularly well-suited to metabolize the spectrum of contaminants entering the Pharmacia/Upjohn Pond. The nature of the contaminant load, the confirmed presence of methanol oxidizers, and the metabolic diversity of methylotrophs as a group led to development of my first series of hypotheses:

Hypothesis 1a: Methanol is serving as a primary growth substrate in the biofilms, fueling secondary metabolism of the trace-level organic compounds entering the system.

Hypothesis 1b: Methanol is serving as a primary growth substrate in the biofilm, naturally selecting methylotrophs and driving changes in microbial community structure.

The second aspect of this research focuses upon determining the feasibility of managing the biofilm communities to optimize bioremediation activity. The predominant macrophyte in the Pharmacia/Upjohn Pond is a submerged, rhizoid-rooted algal macrostructure of a species of *Chara vulgaris* (Klug *et al.*, 1996). *C. vulgaris* is common to hardwater aquatic systems and exhibits a highly dissected growth habit that offers a large surface area for epiphyte colonization (Sculthorpe, 1985). During the warm summer season, *C. vulgaris* provides more than adequate surface area for biofilm

colonization, but senesces in the fall and completely disappears during the cold winter months before reappearing in the spring.

Because *C. vulgaris* is seasonally variable, surface area for attachment represents one important control point in the system. Attachment surfaces in aquatic bioremediation systems can be artificially augmented through the installation of commercially available polyvinylchloride (PVC) biological media. However, artificial substrata raise questions about the influence of the attachment surface itself on microbial community structure and remediative function, especially given that the natural physical substrate is alive. The interior surfaces of the artificial media are also completely devoid of natural light, inspiring questions about the influence of sessile primary productivity on bioremediation activity.

The inherent variability in natural substrata often presents difficulties with uniform sampling and quantification for studies of attached microbial growth. Artificial substrata simplify sampling and processing, reduce heterogeneity, and allow standardized comparisons across habitats or sites. From the earliest work on attached growth (Zobell, 1943) to the present, many biofilm studies have employed artificial attachment surfaces. Artificial materials have included glass, ceramic, brick, aluminum, activated carbon, nylon, plexiglass, polyethylene, polystyrene, and others (Tuchman and Blinn, 1979; Roemer *et al.*, 1984; La Motta and Hickey, 1980; Aloi, 1990; Shreve *et al.*, 1990; Cattaneo and Amireault, 1992; Liu *et al.*, 1993; Haack, 1995). Accordingly, little consensus has been reached on the most appropriate materials, but plastics in many forms now seem to be the preferred substrata for biofilm research (Cattaneo and Amireault,

1992). Hydrophobic plastics have been shown to strongly support biofilm accumulation in pure culture experiments (Shreve *et al.*, 1990). Because most commercially available biological media is constructed of polyvinylchloride and due to supporting evidence in the literature, polyvinylchloride was deemed a suitable prototype substrata for the purposes of this research.

Periphyton communities are the predominant source of primary productivity in many aquatic systems (Kevern *et al.*, 1966; McMahon *et al.*, 1974; Roemer *et al.*, 1984; Geesey *et al.*, 1978). Sessile primary productivity is known to stimulate heterotrophic activity within biofilm communities. Geesey *et al.* (1978) observed that concentrations of sessile bacteria on the upper surfaces of submerged rocks in a small mountain stream coincided with fluctuations in epilithic algae, and that some bacteria grew directly atttached to sessile algal cells. Haack and McFeters (1982) demonstrated a direct flux of algal exudates to sessile bacteria in oligotrophic stream biofilms, with little to no heterotrophic utilization of other sources of dissolved organic carbon. Murray *et al.* (1986) found that a simple biofilm consisting of a diatom and a bacterium expressed greater metabolic activity than did either organism individually.

The precise nature of the stimulated heterotrophic activity and the organisms affected are not well understood. Generic bacterial activity in the experiments above is often estimated by thymidine incorporation, which fails to identify specific ecologically significant changes in function. Haack *et al.* (1994) observed light-induced shifts in bacterial community structure and carbon metabolism profiles in simulated stream biofilms, but distinguishing between changes in species, metabolic function, or both is

difficult. Very few researchers have examined the effect of sessile primary productivity on bioremediation. Bender *et al.* (1995) have examined the biodegradation of chlorinated organics by microbial mats, consortia of bacteria and photosynthetic cyanobacteria. Photosynthetic and heterotrophic activity within the mat leads to adjacent oxic and anoxic microenvironments, which are thought to sequentially facilitate reductive dechlorination and aerobic cleavage of aromatic rings. These results again suggest a mutualistic or synergistic relationship between photosynthetic and heterotrophic attached organisms. The second series of hypotheses is based on this attached mutualism:

Hypothesis 2a: Sessile primary productivity will generate oxygen and carbon exudates, supporting a metabolically active heterotrophic community and stimulating the metabolism of organic contaminants.

Hypothesis 2b: Sessile primary productivity will generate oxygen and carbon exudates, increasing bacterial numbers and altering heterotrophic community structure.

The remainder of this thesis will detail the research work done to test the aforementioned hypotheses. Chapter 2 explores the background research necessary to further define and characterize the naturally-occurring epiphytic biofilms in the Pharmacia/Upjohn Pond. This initial study provided a framework for examining primary and secondary bioremediation activity in the context of biomanagement of naturally-selected biofilms, as detailed in Chapter 3.

Table 1.1 Summary of literature references to biodegradation of compounds of interest in the Pharmacia/Upjohn Pond.

Contaminant	Organisms	References
methanol	Multiple genera: *Methylobacillus, Methylophilus, Methylobacterium, Ancylobacter, Hyphomicrobium, Xanthobacter, Acidomonas* *Paracoccus denitrificans* *Thiobacillus novellus* *Paracoccus alcaliphilus* *Methyhlophaga* *Nocardia* sp. 239	Multiple references: Komogata, 1990. Bratina and Hanson, 1992. deBoer *et al.*, 1990.
dichloromethane	*Pseudomonas* sp. DM1 *Xanthobacter autotrophicus* *Methylococcus capsulatas* *Methylosinus trichosporium* OB3b *Nitrosomonas europea* mixed soil consortia	Brunner *et al.*, 1980. Colby *et al.*, 1977. Oldenhuis *et al.*, 1989. Vanelli *et al.*, 1990. Davis and Madsen, 1991. Henson *et al.*, 1988.
chloroform	*Nitrosomonas europea* *Methylosinus trichosporium* OB3b mixed soil consortia mixed sludge/activated sludge	Vanelli *et al.*, 1990. Alvarez-Cohen *et al.*, 1992. Oldenhuis *et al.*, 1989. Speitel and Leonard, 1992. Strand and Shippert, 1986. Fathepure and Vogel, 1991.
1,2-dichloroethane	mixed aerobic consortia *Ancylobacter aquaticus* *Methylosinus trichosporium* OB3b *Arthrobacter* *Pseudomonas fluorescens* mixed methane-utilizing consortia *Xanthobacter autotrophicus* *Xanthobacter autotrophicus* GJ10 methanogenic consortia	Lanzarone and McCarty, 1990. Van Den Wijngaard, 1992. Oldenhuis *et al.*, 1989. Riebeth *et al.*, 1992. Scholtz *et al.*, 1987. Vandenbergh and Kunka, 1988. Yokata *et al.*, 1986. Janssen *et al.*, 1985. Tardif *et al.*, 1991. Bouwer and McCarty, 1983.
acetone	*Mycobacterium* spp. unknown soil isolates, possibly *Corynebacterium* spp.	Lukins and Foster, 1963. Taylor *et al.*, 1980.
toluene	*Pseudomonas* sp. JS6 *Xanthobacter autotrophicus* GJ10	Pettigrew *et al.*, 1991. Janssen *et al.*, 1985.
chlorobenzene	*Pseudomonas* sp. JS6 *Alcaligenes* spp. *Pseudomonas* spp.	Pettigrew *et al.*, 1991. Oltmanns *et al.*, 1988. Reineke and Knackmuss, 1984. Reineke, 1988.

Chapter 2

OLIGOTROPHIC COMPARISON STUDY

2.1 Introduction

Previous research on the Pharmacia/Upjohn Pond established baseline information about the system (Klug *et al.*, 1996). Among the findings were that methanol and secondary contaminant concentrations decreased along a gradient from Pond inlet to outlet. A corresponding change in methanol metabolism rate was similarly noted, with biofilm communities near the pond inlet showing a greater capacity for methanol degradation than those near the pond outlet. This evidence suggested that the methanol was driving some change in the microbial community function and/or structure, as put forth in Hypotheses 1a and 1b above. A preliminary study was designed to further characterize the Pond biofilm communities and to test Hypothesis 1b, by comparing epiphytic microbial communities in the Pond against those found in a regional class of similar natural, hard-water, oligotrophic systems. The rationale for conducting such a study is expressed in the following research objectives:

1. Determine if the epiphytic communities near the pond inlet are uniquely selected and structured to degrade methanol.

2. Determine if the region of the pond near the outlet is converging chemically and biologically toward regional oligotrophic systems.

3. Collect and characterize methanol-degrading isolates that may serve important functional roles in the epiphytic communities near the pond inlet.

2.2 Experimental Design and Approach

2.2.1 Sampling Locations

In addition to three sampling locations within the pond, three regional lakes were selected for sampling, the selection criteria being that they closely resemble the pond in trophic status and in the predominance of *Chara* among submerged macrophytes. The chosen sites were Lawrence Lake (designated L; 42° 28' latitude, 85° 19' longitude), Hamilton Lake (designated H; 42° 24' latitude, 85° 21' longitude), and Palmetier Lake (designated P; 42° 35' latitude, 85° 25' longitude). Sites within the Pharmacia/Upjohn Pond (42° 12' latitude, 85° 34' longitude) were chosen to reflect the concentration gradient from system inlet in the north to the outlet in the south. These were designated south (S), north (N), and retention basin (RB). Duplicate water samples and triplicate samples of *Chara* with intact biofilms were collected at each site. Due to the difficulty of randomly sampling lake macrophytes, three equidistant sampling locations were selected along the periphery of each site and representative macrophyte samples harvested at random within the chosen sampling areas.

2.2.2 Water Sampling and Analyses

All chemicals used in the present study, unless otherwise noted, were obtained from Fisher Scientific (Pittsburgh, PA). Water samples were collected in proximity to the biological samples with a Van Dorn water sampler deployed to a depth of 0.5 m. Opaque brown 1-L acid-washed Nalgene bottles (Nalge Co., Rochester, NY) were triple-rinsed with sample, filled to overflowing, capped, and stored on ice for transport back to the lab.

Water samples were immediately analyzed for pH, conductivity, alkalinity, and total phosphorus as surrogate indicators of trophic status. Water pH was measured on an Orion model number SA520 pH meter (Orion Research, Inc., Boston, MA) equipped with a combined single probe electrode and 2-point calibrated with buffers at pH 7 and pH 10. Specific conductivity was measured with a YSI model 32 conductance cell (Yellow Springs Instrument Co., Yellow Springs, OH). Alkalinity was determined by titration of a 50-mL sample to a purple endpoint with 0.02 N sulfuric acid in the presence of 4 drops of methyl purple indicator.

Total phosphorus was determined by digesting a 50-mL subsample with 0.5 g potassium persulfate in an autoclave for 30 minutes (APHA, 1992a). The digest was allowed to cool and treated with 5 mL of combined reagent (100 mL ammonium molybdate, 250 ml 2.42 M sulfuric acid, 100 mL ascorbic acid, 50 mL potassium anitmonyl-tartrate). Color development proceded for a period of one hour, after which absorbance at 885 nm was measured for each sample using a Perkin/Elmer Lambda 6 UV/VIS spectrophotometer (Perkin/Elmer Corp., San Jose, CA). Concentration was determined by linear interpolation of a standard curve.

2.2.3 Biological Sampling and Processing

A flow diagram of biological sample processing and analysis is presented in Figure 2.1. Biological samples consisted of whole *Chara* shoots with attached biofilms. Biological analyses of the biofilm communities included direct microscopic counts, total

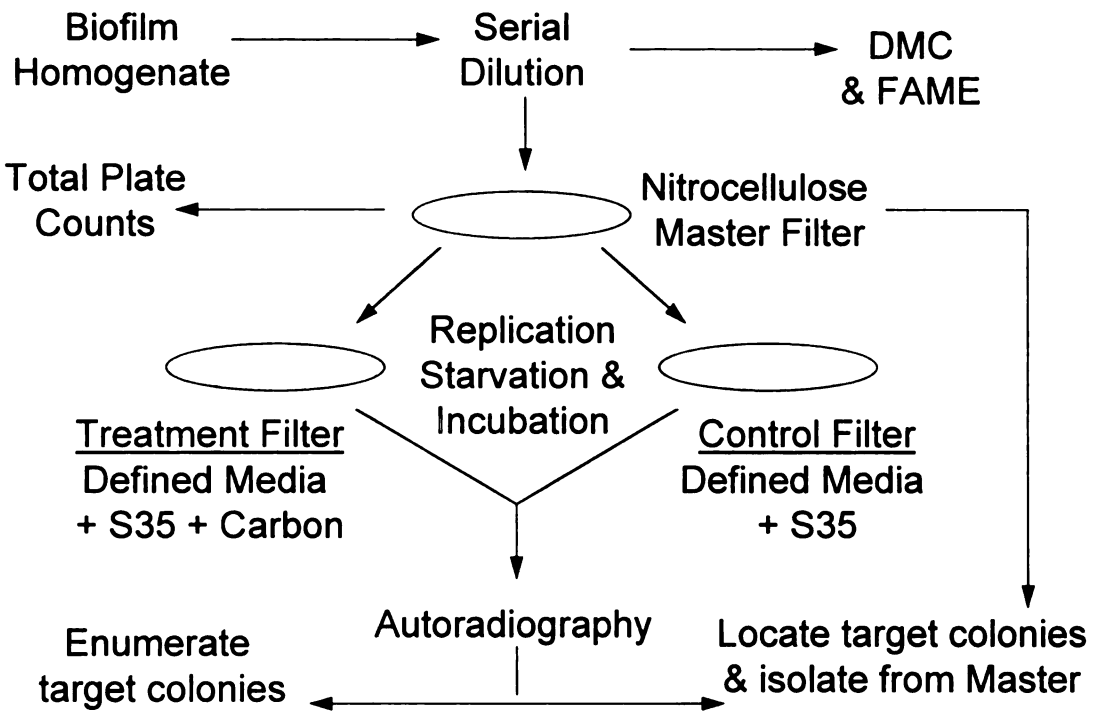

Figure 2.1 Experimental flow diagram for microbiological analyses in the oligotrophic comparison study.

plate counts, methanol degrader counts, colony diversity assessment, whole community fatty acid methyl ester (FAME) profiles, and isolation and preliminary characterization of methanol degrading microbial populations.

Samples were manually harvested with a 4-prong rake. Rootlets and sediments were removed with ethanol-sanitized scissors. Trimmed samples were placed in sterile Whirlpak bags, stored on ice for transport, and processed in triplicate for analytical replication immediately upon return to the lab. A 10-g wet weight subsample was added to 90 mL of sterile 0.01 M $NaHPO_4$ buffer (pH 7.2). The mixture was homogenized in a stainless steel Eberbach tissue grinder (Eberbach Corp., Ann Arbor, MI) on a Waring industrial blender base (Waring Products Division, New Hartford, CT). Sample was homogenized on the low setting for 30 seconds, cooled in an ice bath for 30 seconds to prevent cell damage due to overheating, and homogenized for an additional 30-second interval. Biofilm homogenate was transferred to a sterile screw-cap vial. The tissue grinder was rinsed with an additional 100 mL of sterile buffer. Grinder rinsate was consolidated with the biofilm homogenate, which was collectively subsampled for a series of analyses and serially diluted in sterile buffer.

2.2.4 Direct Microscopic Counts

Triplicate 1-mL subsamples of biofilm homogenate were transferred to screwcap test tubes, each containing 9 mL of sterile phosphate buffer treated with 1% formaldehyde to preserve samples for direct microscopic observation. Bacteria were enumerated by acridine orange (AO) direct microscopic counts (ASTM, 1987). Preserved samples were further diluted on a sample-dependent basis with filter-sterilized phosphate buffer to

achieve a countable number of cells per field. Residual biofilm matrix resulted in clumping on the slide, necessitating sonication of each sample with a Branson Sonic Power model W140D Sonifier Cell Disruptor (Branson Instruments, Inc., Danbury, CT) equipped with a microprobe and operating at 30% power. A 0.9-mL aliquot of diluted sampled was added to 0.1 mL of 0.1% acridine orange solution in a 13x100 mm test tube. The subsample was stained for a period of 1 minute and then vacuum-filtered onto a Nucleopore 0.2 μm polycarbonate filter (Costar Corp., Cambridge, MA). The filter was mounted on a microscope slide with cover slip for microscopic observation on a Zeiss epifluorescent microscope (Carl Zeiss, Oberkochen, West Germany). Twenty-two fields were counted per slide, the highest and lowest field for each slide was discarded, and the mean calculated for the remaining twenty fields. Numbers of bacteria in the original sample were back-calculated according to the field size, mean number of cells per field, and the cumulative dilution factor.

2.2.5 Methanol Oxidizer Counts

Methanol degraders were enumerated and isolated using direct-plating autoradiography (Dunbar *et al.*, 1996). The technique employs radiolabelled sulfur-35 as an indirect indicator of growth on a target compound, such as methanol. Serially diluted cell suspensions of *C. vulgaris* homogenate were inoculated onto Millipore HATF nitrocellulose filters (Millipore, Bedford, MA) placed directly atop general-purpose BBL R2A media (18.1 g L^{-1}; Becton Dickinson Microbiology Systems, Cockeysville, MD). After five days incubation, the filters were replicated, producing two identical mirror-image copies of the original master filter. The master filters were photographed and

stored at 4° C for later use. The replicate filters were incubated on fresh R2A media overnight and then transferred to defined agarose basal (DAB) starvation media (per L of media: 2.68 g $Na_2HPO_4 \cdot 7H_2O$, 1.74 g KH_2PO_4, 1.06 g NH_4Cl, 140 mg Na_2SO_4, 102 mg $MgCl_2 \cdot 6H_2O$, 87 mg $CaCl_2$, 5 mg $Na_2EDTA \cdot 2H_2O$, 2 mg $FeSO_4 \cdot 7H_2O$, 1.86 mg $MnCl_2 \cdot 2H_2O$, 1.53 mg $Na_2MoO_4 \cdot 2H_2O$, 0.3 mg H_3BO_3, 0.32 mg $CaCl_2 \cdot 6H_2O$, 0.16 mg $ZnSO_4 \cdot 7H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.01 mg $CuCl_2 \cdot 2H_2O$, 1.5% high-strength electrophoresis-grade agarose [Bio-Rad Laboratories, Hercules, CA]) to deplete colonies of intracellular carbon reserves.

Following the starvation period, the "treatment" filter was placed upon DAB media containing methanol at 1000 mg L^{-1} as the sole source of carbon and energy, as well as 350 μCi radiolabelled sulfur-35 L^{-1}. Stock radiolabelled sulfur-35 was obtained from New England Nuclear (Boston, MA) in the form of sodium sulfate with a specific activity of 1000 mCi/mmol. The "control" filter was placed atop a similar DAB media, except without added methanol. Both filters were incubated 7 days and then processed for autoradiography. Excess radioisotope was washed from each filter by a 1-hour incubation on wash media (per L of media: 28.408 g Na_2SO_4, 10 g Bacto agar [Difco Laboratories, Detroit, MI]). Filters were removed from the agar to air-dry for 10 minutes prior to mounting with transparent tape on a sheet of 8x10 plain white paper. The paper was covered with a single layer of plastic wrap and placed in an autoradiography exposure cassette. In a darkroom under red light, each cassette was fitted with an unexposed sheet of Kodak X5 X-ray film (Eastman Kodak Co., Rochester, NY). Cassettes were placed in a press to ensure good contact with film and the press was

wrapped in a black plastic bag. The exposure period extended 12 hours at -70° C, after which the films were processed manually with Kodak chemicals according to the recommended Kodak protocol for x-ray development.

Those colonies on the treatment filter that utilized the target compound presumably also incorporated S-35, resulting in dark spots on X-ray film which had been exposed to the colonies. Degraders of the target compound were enumerated by pseudo-plate counts of x-ray films and isolated from the non-radiolabelled master filter. The technique also facilitated total plate counts and assessment of colony morphological diversity (Shannon-Weaver index).

2.2.6 Fatty Acid Methyl Ester (FAME) Processing and Extraction

Undiluted biofilm homogenate was subsampled in triplicate 10-mL aliquots for microbial community structure analysis by FAME profiles. Each subsample was placed in a 15 x 100 mm ashed glass test tube with teflon-lined screwcap. Subsamples were centrifuged at approximately 3400 rpm (setting 6) for 10 minutes on an IEC model CL clinical centrifuge (International Equipment Corp., Needham, MA). The overlaying liquid layer was decanted and the cell pellets were stored at -20° C until extraction and analysis. Bacterial isolates were cultured on non-selective R2A media, harvested directly into the test tubes described above using flamed Pasteur pipette tips, and stored at -20° C until extraction and analysis.

Extraction was performed according to commercial guidelines for preparing pure culture extracts as published in the Microbial Identification System operating manual (Microbial ID, Inc., 1991). Cell pellets were saponified by adding 1.0 mL of a strong

methanolic base solution (45 g NaOH pellets, 150 mL methanol, 150 mL deionized water) to each test tube, vortexing the tube 10 sec, holding the tube in a 100° C water bath for 5 min, vortexing an additional 10 sec, and holding an additional 25 min at 100° C. Fatty acids were methylated by adding 2.0 mL of methylation reagent (325 ml 6.0 N HCl, 275 mL methanol) to each tube, vortexing the tube contents for 10 seconds, holding the samples at 80° C for 10 min, and rapidly cooling the tubes in a water bath. FAMEs were extracted from the acidic aqueous phase by adding 1.25 mL of extraction solvent (1:1 v/v hexane/methyl-t-butyl-ether) to each tube, mixing the tubes end-over-end for 10 min, and removing the upper organic phase to a fresh tube. The extracts were washed by adding 3.0 mL of a weakly basic solution (10.8 g NaOH, 900 mL deionized water) to each tube, rotating the tubes end-over-end for 5 additional min, and removing the upper organic phase to a fresh tube. The washed extracts were dried under a stream of nitrogen, sealed, and stored at -20° C prior to analysis, at which time the dried FAMEs were reconstituted in 200 μL of extraction reagent and transferred to autosampler vials fitted with flat-bottomed glass inserts and sealed with teflon-lined crimp caps (vials, insert, and crimp caps obtained from Sun Brokers, Inc., Wilmington, NC).

FAME analysis by gas chromatography immediately followed reconstitution of dried extracts. FAME extracts were analyzed on a Hewlett Packard (HP) 5890 Series II gas chromatograph equipped with a 7673A autosampling device and flame ionization detector (Hewlett Packard, Wilmington, DE). The GC was fitted with an HP Ultra 2 fused silica capillary column (25 m length, 0.2 mm ID, 0.33 μm layer thickness) with a cross-linked phenyl-methyl-siloxane active matrix. The GC was operated under the

following conditions: detector temperature, 300° C; injector temperature, 250° C; initial column temperature, 170° C; temperature ramp, 5° C min^{-1}; final temperature, 270° C; H_2 combustion gas 30 mL min^{-1}; dry air combustion gas 400 mL min^{-1}; N_2 auxiliary gas, 30 mL min^{-1}; split flow, 50 mL min^{-1}; purge flow, 5 mL min^{-1}; split ratio, 50:1. Raw analytical data was processed by the Microbial Identification System proprietary software to generate FAME profiles for each sample.

2.2.7 FAME Data Analysis

FAME datasets are typically analyzed by a complex multivariate technique known as principal components analysis (PCA; SAS Institute, 1991; Cavigelli *et al.*, 1995). The FAME variables are orthogonally transformed into a set of uncorrelated variables known as principal components (PCs), which are each linear combinations of all the variables; i.e. every variable is represented in each PC. Eigenvector loadings are the extent to which the variables affect each PC. Each principal component in order explains a decreasing percentage of the total variance in the dataset, such that the first few PCs typically account for greater than 80% of the variance in the original dataset.

Because of the constrained nature of the dataset, the covariance matrix was used in the PCA (Aitchison, 1986). Data transformation was required to force the dataset to meet the criteria of SAS-PCA with the covariance matrix. Resultant FAME peak areas were hand-entered into spreadsheet format, eliminating all unknowns and non-fatty acid peaks. All samples were normalized to the lowest total peak area for biomass correction. All those peaks were eliminated that totaled less than 10% of the most abundant peak across all samples. A negligibly small value (less than detection limit) was added to each

peak to ensure a complete non-zero dataset, new total peak areas were calculated, and samples were re-normalized to the new lowest total peak area. Peak area was then converted to relative abundance and logratio transformed to generate a normally distributed dataset meeting the PCA criteria (Aitchison, 1986).

The transformed dataset was subjected to SAS-PCA and the resultant first two PCs of each sample were plotted on opposing axes. Scaled eigenvectors for the ten most heavily weighted fatty acids were then superimposed on the original PC plot. These "biplots" allow a researcher to examine separation between samples while simultaneously determining which acids were most prevalent in determining resolution between samples. All FAME data analysis was standardized for the experiments presented in this work.

2.3 Results and Discussion

2.3.1 Water Chemistry

Table 2.1 presents the combined results of all water chemistry analyses. Water pH ranged between 7.5 and 8.5 for all sites, which is typical for the hard-water, calcerous, alkaline class of lakes. Alkalinity was 3-5 meq L^{-1}, quite high for even this class of lakes, but not substantially different between lakes. Conductivity ranged between 350 and 450 μmhos cm^{-1} for the natural systems, and between 600 and 650 μmhos cm^{-1} in the pond sites, the latter of which is possibly a reflection of iron concentrations entering the pond. Total phosphorus ranged from 8-15 μg L^{-1} in the natural sites and the south-end pond site to 22-26 μg L^{-1} in the pond's north end and retention basin. Natural surface waters typically fall in the range of 10-50 μg L^{-1} for total phosphorus, with concentrations between 10-30 μg L^{-1} indicative of meso-eutrophic status. Less than 5% of total

phosphorus is normally available as orthophosphate. Thus, although water near the system inlet seems to have elevated total phosphorus levels, the concentrations are not beyond the expected range for this class of lakes. Overall, the water chemistry data verifies that all the sites are generally comparable with respect to trophic status.

2.3.2 Microbial Count Data

Means of microbial count data are presented in Table 2.2, with different letters designating significant differences (SAS t-test, alpha=0.05). Total direct microscopic counts were in the range of $1x10^{10}$ cells g^{-1} dry weight *Chara* at all sites, with slightly elevated levels near the system inlet (p=0.0077). Culturable plate counts reflected the same general pattern, but about 2 orders of magnitude less in abundance (p=0.0048). Density of methanol degraders was very significantly higher near the pond inlet, with both the north-end and retention basin samples having nearly 3 orders of magnitude more methanol degraders than the south-end or natural samples (p=0.0001). A SAS overall multivariate linear model of count data was highly significant (p=0.0001) for both Hotelling's T and Wilks' Lambda tests. Methanol degraders comprised approximately 15% of total culturable organisms in the north-end and retention basin communities. This data clearly supports the hypothesis that methanol is exerting selective pressure and driving changes in microbial community structure near the system inlet along the most concentrated portion of the contaminant gradient.

The calculated Shannon-Weaver morphological diversity index revealed depressed diversity near the system inlet. Lowered diversity is common at sites of high selective pressure (Atlas, 1991). Diversity in the south end of the pond is comparable to

Table 2.1 Inorganic water chemistry parameters for three natural lake systems and the Pharmacia/Upjohn Pond.

Sampling Location	pH	Conductivity$_{25}$ (μmhos cm^{-1})	Alkalinity (meq L^{-1})	Total Phosphorus (μg L^{-1})
Palmetier	8.15	351	3.40	14.3
Hamilton	8.43	388	3.80	11.5
Lawrence	8.19	460	4.18	9.0
UJ South	8.00	602	3.92	9.7
UJ North	7.64	648	4.74	22.7
UJ Basin	7.57	653	4.62	25.8

Table 2.2 Microbiological count data and diversity indices for three natural lake systems and the Pharmacia/Upjohn Pond.

Sampling Location	Direct Counts (cells g^{-1})	Plate Counts (cfu g^{-1})	Methanol Oxidizers (cfu g^{-1})	Shannon-Weaver Diversity Index
Palmetier	B 9.17×10^{9}	B 7.20×10^{7}	B 1.08×10^{5}	1.45
Hamilton	B 8.38×10^{9}	B 8.06×10^{7}	B 9.67×10^{4}	1.64
Lawrence	B 9.37×10^{9}	B 2.87×10^{7}	B 1.26×10^{5}	1.35
UJ South	B 1.47×10^{10}	B 7.80×10^{7}	B 2.18×10^{5}	1.34
UJ North	A 3.78×10^{10}	A 2.59×10^{8}	A 4.07×10^{7}	0.79
UJ Basin	B 2.07×10^{10}	A 2.19×10^{8}	A 3.51×10^{7}	0.83

that calculated for the natural sites, further supporting the hypothesis of south-end convergence toward typical systems.

2.3.3 Microbial Community Structure (FAME)

Fatty acids extracted from cell wall lipid components can be used as phenotypic "fingerprints" for isolates and for entire microbial communities. Figure 2.2 shows the FAME biplot for the means of all field replicates in the Oligotrophic Comparison Study. The resulting pattern is quite difficult to interpret, although a few conclusions can be drawn. First, the absolute magnitudes of the PC axes are small, indicating small perceptible differences between samples. The Lawrence Lake communities are greatly separated from all other samples along the PC1 axis, indicating the least similarity to other samples. The fatty acids corresponding to the separation of the Lawrence Lake samples are the fully saturated acids, 14:0, 16:0, 18:0. These fatty acids are ubiquitously distributed in nature and provide little insight into the practical reason for the Lawrence Lake samples being so highly resolved from other samples.

The remaining samples demonstrate a slight gradient separation along axis PC2 from the region of highest contamination in the pond (retention basin) to the region of lowest contamination (south), indicative of a convergence of communities in the south end of the pond toward those associated with natural systems. However, these patterns are not strong and little conclusive information can be gleaned from the corresponding fatty acid weightings.

As an analytical tool, whole community FAME profiles present distinct limitations. Because of the redundant and ubiquitous distribution of many fatty acids

among even widely varying individual microbial species, whole community FAME profiles appear to be useful primarily as a preliminary screening tool to detect large differences between complex microbial communities. Even when large-scale differences are detected, interpretation often requires further investigation using more targeted metabolic assays. The broad-level resolution inherent to whole community FAME profiles severely limits detection and interpretation of small differences between similar microbial communities.

Even those organisms with unique signature fatty acids are often non-detectable in whole community FAME profiles unless they represent a large proportion of the biomass. Many other pure culture strains, such as Methylotrophs, exhibit fingerprint profiles only because of uniquely proportioned ubiquitous fatty acids. The fatty acid ratios become distorted in a complex whole community signal with potentially hundreds of microbial species contributing the same fatty acids but in different proportions. Therefore, even when Methylotrophs are present in large numbers, they become undetectable in a community profile. As an illustration, Methylotrophs were not evident in whole community FAME profiles from the pond, but were detected, isolated, and identified by coupling direct-plating autoradiography and pure culture FAME profiles.

FAME profiles were also generated for the methanol degrading isolates collected during preliminary experiments from communities near the system inlet. The profiles were run against the Microbial Identification System searchable library of profiles for known organisms. All methanol-utilizing isolates appear to be of 3 main types: relatives of *Xanthobacter*, relatives of *Methylobacterium*, and members of an unidentified group of organisms. Both *Xanthobacter* and *Methylobacterium* are known methanol-degrading

organisms capable of growth on a variety of substrates including several common organic contaminants. Such opportunistic and cosmopolitan organisms are often associated with complex trace-level waste streams. The presence of these groups of bacteria holds promise for secondary contaminant transformation in the Pharmacia/Upjohn Pond system.

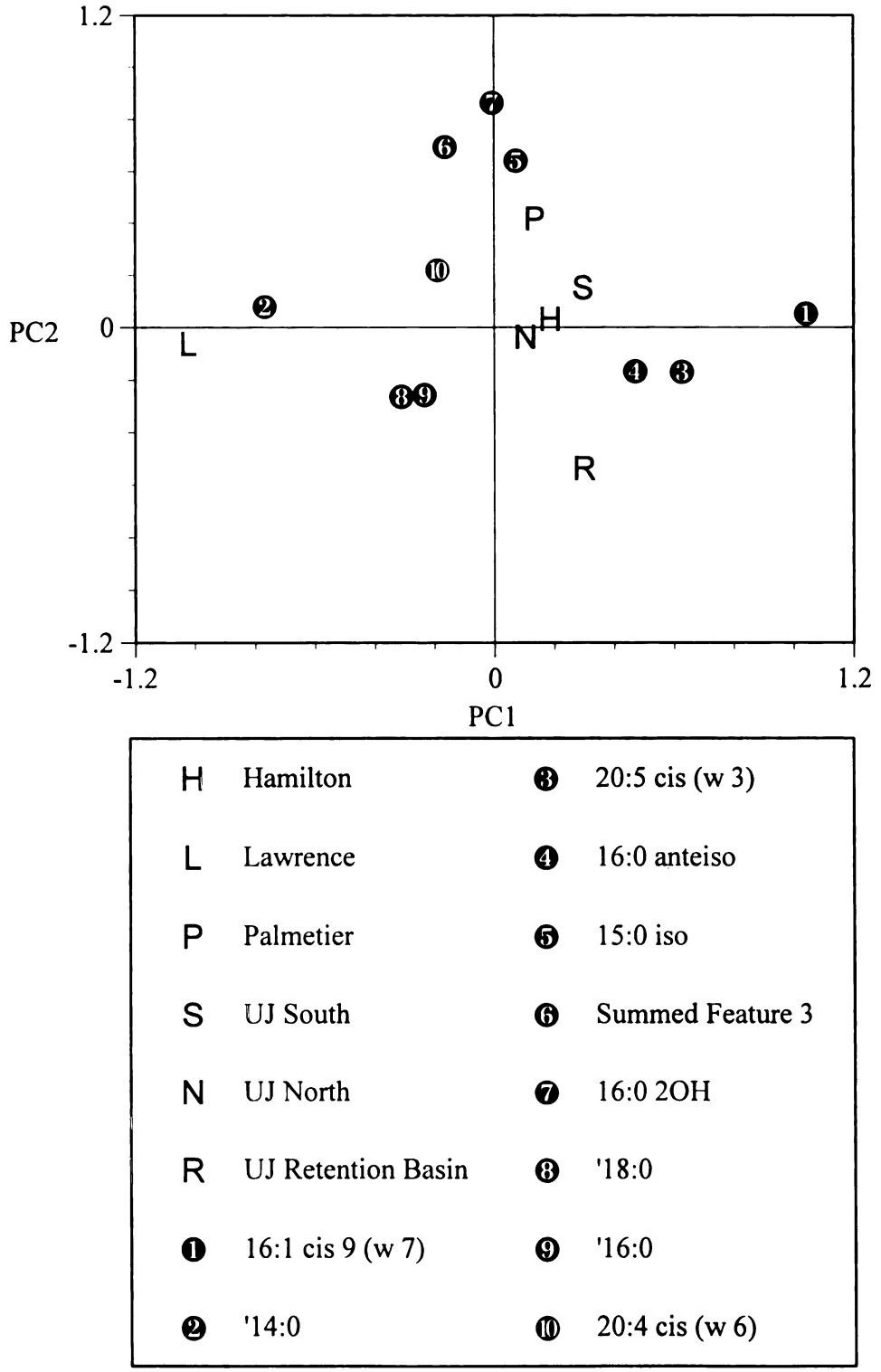

Figure 2.2 Fatty acid methyl ester (FAME) biplots for epiphytic microbial communities from three natural oligotrophic systems and the Pharmacia/Upjohn Pond.

Chapter 3

SECONDARY METABOLISM STUDIES

3.1 Introduction

Methanol comprises a significant proportion of the dissolved organic carbon (DOC) entering the Pharmacia/Upjohn Pond system (Klug *et al.*, 1996), is a labile growth substrate for a diverse array of heterotrophic organisms, and is present in sufficient concentrations (mg L^{-1} range) to support microbial growth. Therefore, methanol is likely to be a primary growth substrate for attached microorganisms near the system inlet. Furthermore, some methylotrophic organisms, such as those identified in the Oligotrophic Comparison Study, have been shown to express alternate and auxiliary enzymatic pathways for the degradation of other carbon compounds, such as toluene and dichloromethane, which are among the suite of "secondary" contaminants periodically entering the system. However, these secondary compounds are typically present in the system at concentrations too low (μg L^{-1} range) to support microbial growth or induce enzymatic pathways. In this case, the secondary compounds are likely to be metabolized in conjunction with primary metabolism of a growth substrate, such as methanol.

Sessile primary productivity is thought to be important for stimulating bacterial secondary productivity in naturally-occurring aquatic biofilms such as those found in the Pharmacia/Upjohn Pond. A small amount of evidence has been collected to suggest that primary producers can have a synergistic relationship with the desired metabolic

remediation activity of heterotrophs in microbial mats (Bender *et al.*, 1995). However, questions remain regarding how applicable these concepts are to aquatic biofilms and the extent to which sessile primary productivity influences biodegradation of organics.

Potential management of the microbiota through manipulation of surface area for attachment mandates answering fundamental questions about the bioremediation capabilities of the biofilm communities and how these communities will be affected by artificial media and light depravation. Thus, the objectives of the secondary metabolism experiments were as follows:

1. Determine the extent of primary methanol utilization and secondary substrate utilization by attached microorganisms near the inlet of the Pharmacia/Upjohn Pond

2. Determine if methanol is linked to or driving secondary substrate utilization by attached microorganisms near the inlet of the Pharmacia/Upjohn Pond

3. Determine the influence of sessile primary productivity on primary methanol utilization and secondary substrate utilization by attached microorganisms in the Pharmacia/Upjohn Pond

4. Determine the feasibility of surface area augmentation

3.2 Field Experimental Approach

In order to examine the feasibility of surface area augmentation, we established a light exclusion experiment in the far north end of the pond near the system inlet. Removeable Sintra PVC attachment surfaces (Commercial Plastics and Supply Corp., Kalamazoo, MI) were installed on 1/16/95 in treatment cells designed to manipulate light intensity. The installation allowed microbial communities to develop *in situ* and simulated the various light regimes that these communities would encounter if surface

area in the pond were augmented by the implementation of a product such as Biomedia (Brentwood Industries, Inc., Reading, PA). Treatment cells consisted of plastic crates suspended in the water column and wrapped in screening materials to manipulate light intensity. Flow vents were placed in the coverings to allow normal water movement within the crates.

The three treatment designations of LIGHT, MEDIUM, and DARK correspond to natural unattenuated diurnal light, moderately attenuated light (approximately 50%), and fully-attenuated light, respectively. However, in response to results from methanol oxidation experiments and periphyton productivity estimates, the MEDIUM treatment was later eliminated in lieu of additional field replications for the DARK and LIGHT treatments. Greater statistical rigor and confirmational analyses were warranted in subsequent experiments due to high variability among field replications.

Sintra PVC was the chosen material for an attachment surface due to its dark color, hydrophobic surface, ready availability, and low price. Sintra PVC tiles of uniform surface area (2.54 cm^2) were attached to unglazed ceramic tiles (anchors) and placed in each crate for colonization. Although effective, this tiling system was later modified to 0.64 cm thick x 7.6 cm wide x 17.8 cm long rectangular-shaped Sintra PVC tiles mounted within the crate interior using nylon hardware.

Periphyton productivity was measured *in situ* as an indicator of the effectiveness of light manipulation treatments on controlling primary productivity. After communities had fully developed, two tiles were harvested from each treatment cell on each of several dates and returned to the lab for a series of analyses that included the following: direct

microscopic counts, fatty acid methyl ester (FAME) community profiles, methanol oxidation and assimilation rate, and secondary metabolism shakeflask experiments.

Tiles were harvested by removing the nylon fastening hardware and carefully manuevering the tiles into sterile Whirlpak bags (Nasco, Fort Atkinson, WI), which were stored on ice for transport to the lab. Each bag received 100 mL of 0.22-μm filter-sterilized pond water for processing purposes. Manual agitation and rubbing was applied to the tiles and sterile pond water in the transport bags to remove biofilm material from the surface of the tiles. Homogenization of the biofilm suspension was necessary to disperse the polysaccharide matrix within biofilms and to distribute the cellular material for uniform subsampling. Although homogenization perturbed the biofilm community, diminished the mass transfer limitation presented by the polysaccharide matrix, and potentially overestimated *in situ* transformation rates, the processing was deemed necessary for accomodation of the destructive subsampling regime established in the laboratory experimental design. The polysaccharide matrix was most prominent in the DARK treatments. Biofilm suspensions were homogenized for 30 seconds on the low setting in a sterile stainless steel Eberbach tissue grinder on a Waring industrial blender base. The tissue grinder was rinsed with an experiment-dependent volume of sterile pond water, the rinsate from which was consolidated with the homogenized suspension. The tissue grinder was sanitized between samples with a triple rinse of 10% sodium-hypochlorite (Clorox Co., Oakland, CA), followed by liberal rinsing with tap water, then distilled water, and finally a triple-rinse with sterile pond water.

The consolidated biofilm suspension was subsampled for a variety of analyses, depending upon the nature of the experiment. Triplicate 10-mL subsamples for FAME analysis and triplicate 1-mL subsamples for direct microscopic counts were collected and analyzed according to the protocols described in Chapter 1. Additional subsamples of experiment-dependent volume were collected and distributed to 20 mm, 135-mL serum vials sealed with teflon-lined septa for an array of metabolic experiments. Processing variations are noted for each individual experiment below.

3.3 Sessile Primary Productivity

Standing water sessile primary productivity was determined according to the standard protocol as defined by Standard Methods for the Examination of Water and Wastewater (APHA, 1992b). PVC tiles with intact communities were placed in light and dark bottles and were incubated for one half the photoperiod. Bottles were subsampled for dissolved oxygen (DO) concentration before and after the incubation. DO samples were chemically fixed in the field for preservation, followed by iodometric titration in the laboratory. Sessile productivity and respiration rates were also corrected for suspended phytoplankton activity by establishing similar bottles without attached communities.

Non-replicated 12-hour sessile productivity rates and 24-hour respiration rates for the three treaments are presented in Figure 3.1. Productivity rates confirmed that the treatments were effective at controlling primary productivity within the biofilms. Interestingly, the respiration rates were nearly the same for all three treatments, a result that will be discussed with the methanol oxidation data. The presented rates of primary

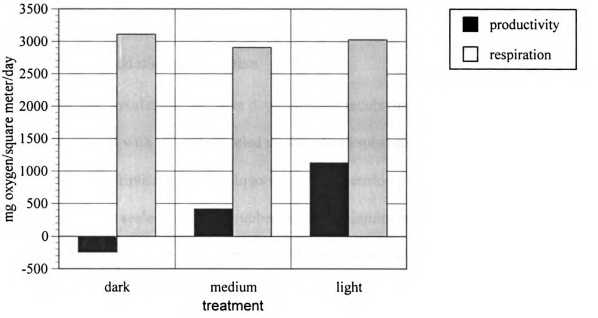

Figure 3.1 Primary productivity and respiration rates (mg O_2 m^{-2} d^{-1}) for attached microbial communities from three light treatment regimes in the Pharmacia/Upjohn Pond.

productivity and respiration are similar to those found in the periphyton literature (Wetzel, 1983).

3.4 Methanol Oxidation/Assimilation

Methanol oxidation rates were determined by incubating cell suspensions in sealed serum vials with ^{14}C-radiolabeled methanol and subsequently trapping ^{14}C-labeled carbon dioxide. Triplicate 10-mL aliquots of cell suspension per sample were placed in 37-ml serum vials sealed with butyl rubber septa and aluminum crimp caps. Two additional flasks per sample were established for a t=0 estimate and biological control. Biological controls were autoclaved for 25 minutes and cooled prior to incubation. A concentrated stock solution of ^{14}C-methanol with specific activity of 5 mCi/mmol was obtained from New England Nuclear (Boston, MA). Each flask received an injection of diluted ^{14}C-methanol via syringe to a final concentration of 0.028 μCi mL^{-1} (4 mg methanol L^{-1} suspension). Injections were staggered to allow immediate flushing and trapping of headspace gases. Samples were incubated at 25° C for 3 hours, except t=0 samples, which were halted immediately upon injection of label.

Methanol oxidation was halted in all flasks by injecting 300 μL of 6 N HCl, which also forces $^{14}CO_2$ in solution to be evolved into the headspace. Upon acidification, each flask was then placed into a separate channel on a closed circuit N_2 headspace flushing apparatus. Each channel is fitted with a series of 3 scintillation vials, each containing 4 mL of 3:5 ethanolamine:methanol trapping reagent for trapping $^{14}CO_2$. Each vial was flushed for 10 minutes at a rate of approximately 60 ml N_2 min^{-1}, after which time the scintillation vials were removed from the apparatus, treated with 15 mL of Safety-Solve

liquid scintillation cocktail (Research Products International Corp., Mount Prospect, IL), and capped. Radioactivity in each vial was analyzed with a Wallac model 1409 Liquid Scintillation Counter (Wallac Inc., Gaithersburg, MD).

Remaining cell suspensions were filtered through Whatman 0.2 μm nitrocellulose filters (Whatman Ltd., Maidstone, England) and the filtrate collected. Biomass recovered on the filter and 10 mL filtrate were each transferred to separate scintillation vials with 15 mL Safety Solve each and counted with the LSC to estimate bacterial assimilation and residual ^{14}C-methanol in solution, respectively.

Methanol oxidation and assimilation rates are presented in Figure 3.2. Rates have been adjusted to a per-million-cell basis by direct microscopic counts, which accurately reflects the community-wide oxidation and assimilation rates. Although the relationship between direct microscopic counts and the number of culturable methanol oxidizers remains to be determined, approximately 10% of the total culturable population were identified as methanol oxidizers by direct-plating autoradiography. Therefore, oxidation and assimilation rates for the populations of interest are most likely underestimated.

Error bars throughout the present study represent plus or minus one standard error of the mean. Although a trend is apparent toward higher oxidation rates in the DARK treatment, an ANCOVA (SAS, alpha=0.05) revealed no significant differences in methanol oxidation or assimilation rates between treatments; i.e. given the high level of inherent variability, our experiment was not designed with sufficient statistical power to recognize the apparent trends in the data.

Despite the inherent variability in field samples, the DARK treatment exhibited a higher per-cell oxidation rate than the other two treatments. Thus, biofilms growing in the dark and without access to a diverse photosynthetically-produced carbon pool appear to be more well-adapted to using methanol as a primary carbon source. In conjunction with the gross respiration rate data, these results also suggest that methanol oxidation may be a larger component of total respiration in the DARK treatment. However, the ratio of mineralization to assimilation was substantially higher in the DARK treatment (approximately 2:1) than in the other treatments (approximately 1:1), suggesting that communities growing without light incorporate less methanol into biomass and divert more to energy production. The practical implication is that Biomedia augmentation would not only be feasible, but may be preferable, since the majority of colonization on artificial media would be in the dark.

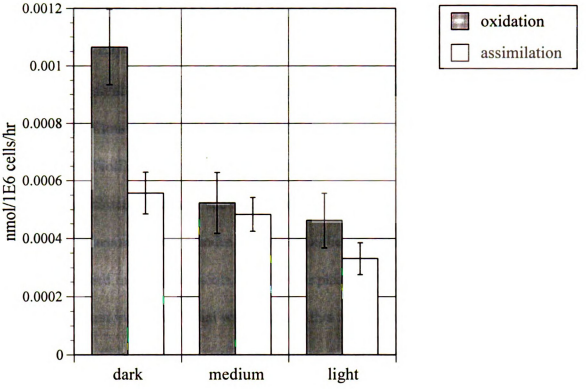

Figure 3.2 Methanol oxidation and assimilation rates (nmol [$1x10^6$]$cells^{-1}$ hr^{-1}) for attached microbial communities from three light treatment regimes in the Pharmacia/Upjohn Pond.

3.5 Secondary Metabolism Shakeflask Experiments

3.5.1 General Experimental Approach

The general experimental approach to studying secondary metabolism involved harvesting biofilms from the artificial attachment surfaces in the light exclusion chambers and preparing biofilm suspensions in the laboratory, as explained in section 2.2 above. In sediment experiments, surficial sediments were Eckman dredged to a depth of 10 cm and a similar suspension prepared under micro-aerophilic conditions. Biofilm suspensions were subsampled in 50-mL aliquots, which were placed in 20 mm, 135-mL acid-washed and sterile serum vials. Each vial was sealed with a teflon-lined septum and aluminum crimp cap. Biofilm suspensions were pre-incubated overnight to deplete any labile DOC prior to initiating secondary metabolism incubations. All biofilm subsamples from both LIGHT and DARK field treatments were incubated in the absence of light to strictly isolate heterotrophic activity and eliminate the confounding influence of simultaneous photosynthetic activity. All laboratory incubations and pre-incubations throughout the present study were conducted at 25° C.

Biological controls (designated KILL) were introduced to establish a cause and effect relationship between biologically active samples and transformation activity. The biological control also allowed isolation and quantification of biological matrix effects (such as sorption) on transformation activity. Biological control vials were prepared as described for biologically active samples, then autoclaved for 20 min and allowed to cool prior to incubation. Experiments #1 through #3 revealed that a 20-min autoclave period was not sufficient to control activity beyond 24 hours, necessitating an extension of the

autoclave sterilization time to 45 min in subsequent experiments. The protective properties of the biofilm matrix may have allowed bacterial spores to survive the 20-min sterilization period, after which spore germination could have led to biological activity in the sterilized controls. Alternately, biological controls may have been cross-contaminated during sampling.

A reagent control (designated RBLK) was introduced to isolate non-biological effects (such as volatilization and chemical interactions) on apparent transformation activity. Reagent control vials were established as described previously, but substituting 50 mL of filter-sterilized Pond water for the biofilm suspension. Reagent controls were treated exactly as biologically active samples throughout the remainder of the experiment.

Following pre-incubation, the sealed suspensions were spiked with 100 mg L^{-1} methanol (or no methanol) and a mixture of secondary compounds at $\mu g\ L^{-1}$ concentrations and varying composition, depending upon the nature of the experiment. Vials were destructively sampled in 6-mL aliquots through time to establish rates of loss for methanol and secondary compounds. During experiments #1 through #3, each subsample was collected in a single 10-mL gastight syringe that was rinsed 3 times with sterile water and fitted with a new sterile 18 ga needle between samples. This technique may have resulted in some contamination of the reagent blanks or biological controls. Subsamples in remaining experiments were each collected with a separate sterile syringe and needle. An equal volume of atmospheric air was injected into each vial following subsampling to replace the lost volume and to replenish oxygen to maintain aerobic

conditions. Anaerobic studies received an equal volume of oxygen-free high purity nitrogen.

Methanol subsamples were filtered through 0.2 μm Millex disposable filtration units (Millipore Products Division, Bedford, MA) to remove active bacterial biomass and thereby stop biological transformation of methanol. Filtrate was collected in GC autosampler vials fitted with glass inserts, which were then sealed with teflon-lined crimp caps and stored at 4°C prior to analysis. Methanol subsamples were analyzed by direct 2-μL aqueous injection and flame-ionization detection on a Hewlett Packard 5890 Series II fitted with a 7673A autosampler and a Restek RTX-200 capillary column (Restek Corporation, Bellefonte, PA) with a crossbonded trifluoropropylmethly polysiloxane active matrix (30 m x 0.53 mm ID, 1.0 μm film thickness) fitted with a Restek 5 m x 0.53 mm ID inert guard column. Concentrations were determined by applying the resultant formula from a linear regression of the methanol external standard curve.

Subsamples for secondary compounds (volatile organic compounds, or VOCs) were biologically stabilized by adjusting to pH 2.0 with 1.0 N HCl. Subsamples were collected in Micro-mate 10 mL glass-barreled/glass-plungered syringes (Popper and Sons, Inc., New Hyde Park, NY), sealed with custom-fabricated teflon-lined Luer caps, and stored at 4°C for no more than 4 days prior to analysis. Although this sample storage protocol is not recommended in EPA Method 8260A (USEPA, 1994), a series of preliminary tests were executed to determine the suitability of the storage protocol and no significant deterioration was detected for the analytes of interest. Samples were then analyzed according to EPA Method 8260A (USEPA, 1994) using a Hewlett Packard

purge-and-trap/gas chromatograph/mass spectrometer system. The HP purge-and-trap concentrator was equipped with a Tekmar VOCARB 3000 trap (Tekmar Co., Cincinatti, OH). The HP 5890 Series II EPC gas chromatograph was equipped with an HP-VOC column (30 m x 0.2mm ID, 1.1 μm film thickness) and a 5972 mass selective detector. Reconstructed ion chromatographs and mass spectra were analyzed with HP Chemstation and Enviroquant software to determine analyte concentrations based upon calibration curves and internal standards.

Plots were prepared of analyte concentration against time and, when applicable, rates were calculated based on linear regression of the transformation plots. Treatment effects were then statistically evaluated using a SAS analysis of covariance for rates and direct microscopic count data. Specific methods and resulting raw transformation plots for individual secondary metabolism experiments are presented in sections 3.5.2 through 3.5.7. Tabular summaries of count data, rate estimates, statistical tests, and experiment-wide means for all secondary metabolism experiments are presented in section 3.5.8. Discussion of secondary metabolism results follows in section 3.6.

3.5.2 Secondary Metabolism Experiment #1: Aromatic/Aliphatic Screen

The objective of experiment #1 was to survey biofilm secondary metabolic activity on dichloromethane (DCM) and toluene (TOL). These compounds were selected as representative secondary contaminants from the aliphatic and aromatic classes of compounds and are both reported to be degraded by methylotrophs (see Table 1.1). Methanol served as the primary growth substrate at 100 mg L^{-1}, while DCM and TOL were spiked at initial concentrations of 480 μg L^{-1} and 260 μg L^{-1}, respectively. Because

this experiment was initiated before the subsample storage protocol was developed, subsamples were collected at incubation times 0.5, 8.0, and 28.0 hours. These time intervals allowed samples to be placed immediately on the purge-and-trap autosampler for analysis.

Primary and secondary transformation plots for experiment #1 are presented in Figures 3.3-3.5. Rapid transformation rates of methanol and toluene were observed in all biologically active biofilm samples, with dark communities exhibiting higher per-cell rates. However, differences in transformation rates were not statistically significant (α=0.05) due to high variance within treatments. Direct microscopic counts were not significantly different between treatments, as shown in Tables 3.1 and 3.2.

Unexpectedly, no biofilm secondary activity was observed with DCM. Some methylotrophs are known to express dichloromethane dehalogenase, an enzyme that dechlorinates DCM to form formaldehyde, which is then shunted directly into the methanol oxidation and assimilation pathways. The failure to express this aerobic enzymatic pathway could be due to absence of the appropriate genetic code, the sporadic nature of DCM contamination, and/or concentrations below the threshhold level for expression. Interestingly, DCM transformation was later observed in aerobic biofilms after extended periods of methanol exposure, as well as in sediment studies under anaerobic conditions (Sections 3.5.6 and 3.7.4).

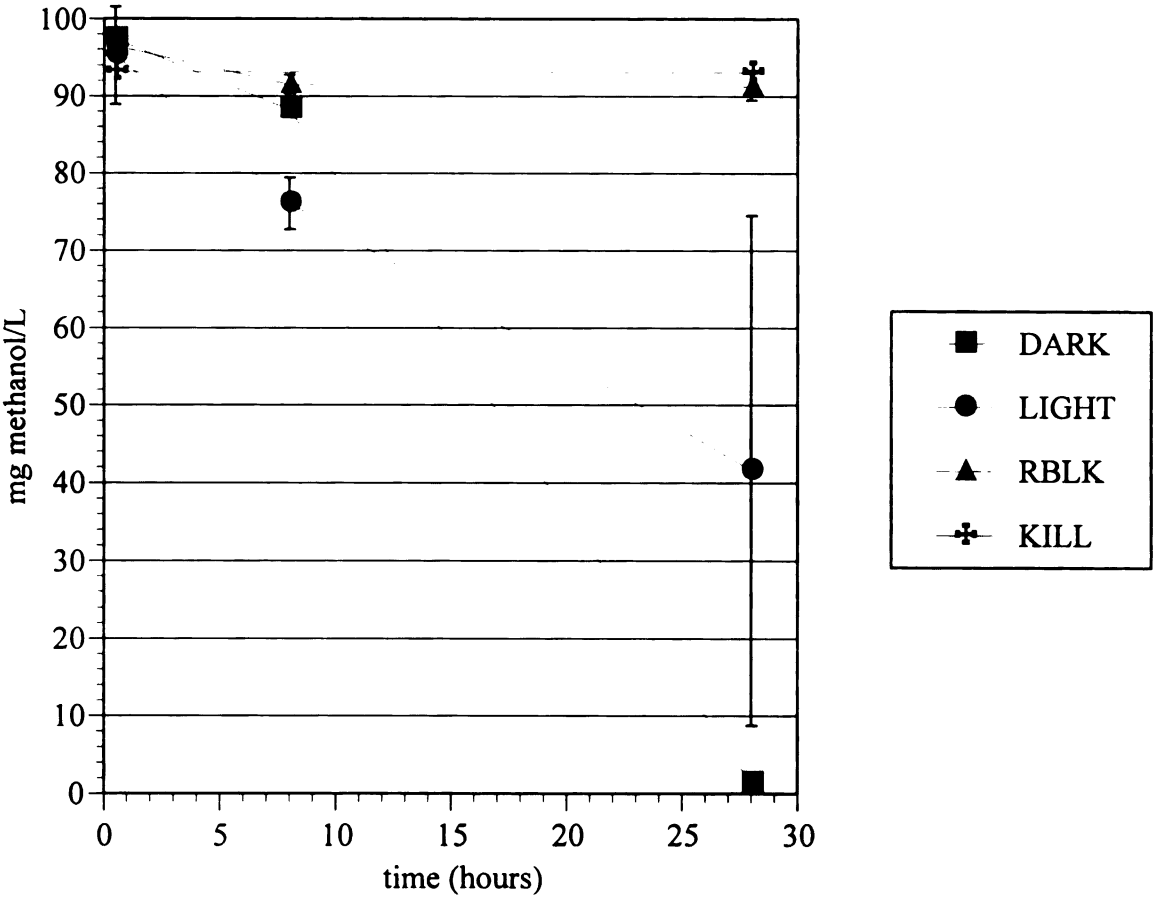

Figure 3.3 Methanol transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #1 (7/13/95).

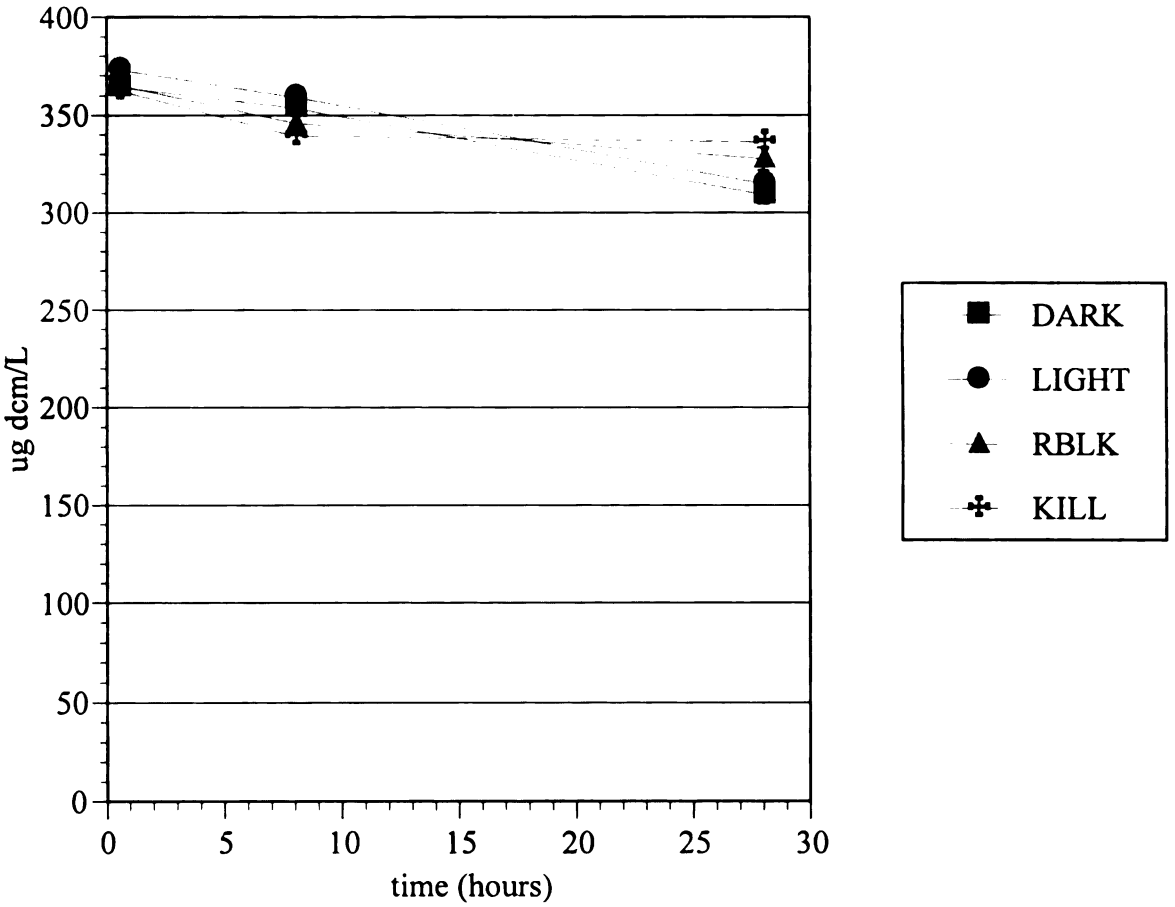

Figure 3.4 Dichloromethane (DCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #1 (7/13/95).

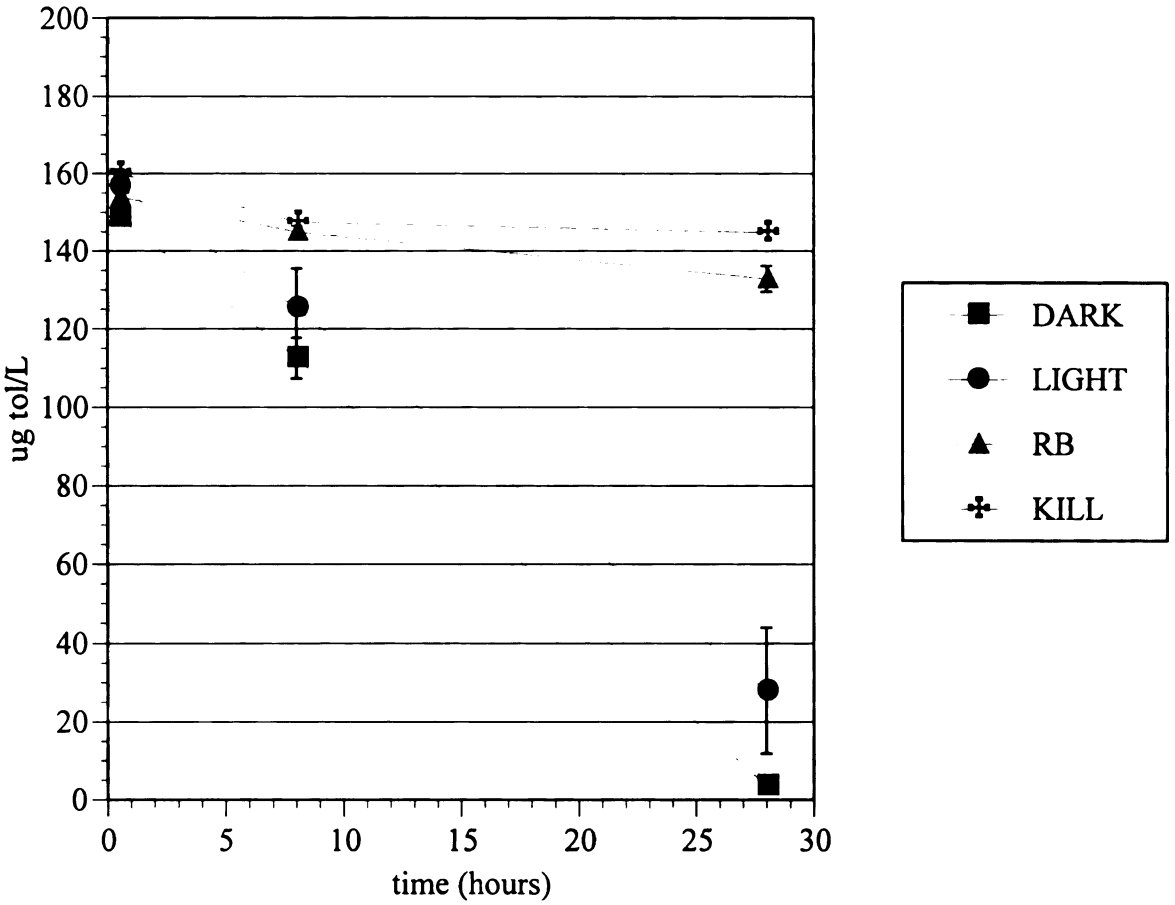

Figure 3.5 Toluene (TOL) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #1 (7/13/95).

3.5.3 Secondary Metabolism Experiment #2: Time-Intensive Subsampling

Experiment #2 was designed as a follow-up study to experiment #1, but with freshly harvested biofilms and a more intensive subsampling schedule (0.5, 9.0, 22.0, and 50.0 hours) for more accurate estimation of transformation rates. Initial concentrations of DCM and TOL were reduced to 300 $\mu g\ L^{-1}$ and 170 $\mu g\ L^{-1}$ respectively to closer emulate *in situ* concentrations (see Table 3.12). The subsample storage protocol was first implemented during this experiment. Subsamples for direct microscopic counts were not collected, necessitating the use of counts from experiment #1 for rate calculation comparisons.

Primary and secondary transformation plots for experiment #2 are presented in Figures 3.6-3.8. Results were similar to experiment #1, but with lower variance. Methanol and TOL were rapidly transformed in both light treatments. DCM was not aerobically transformed throughout the duration of this experiment. No significant differences in primary or secondary transformation rates were observed between treatments. The biological control (KILL) for TOL transformation exhibited substantial activity at the t=50 h sampling, indicating that the 20-min autoclave sterilization period was not sufficient to control biological activity in these samples. The activity in the biological control at 50 h suggests the presence of spore-forming bacteria or possible cross-contamination during sampling.

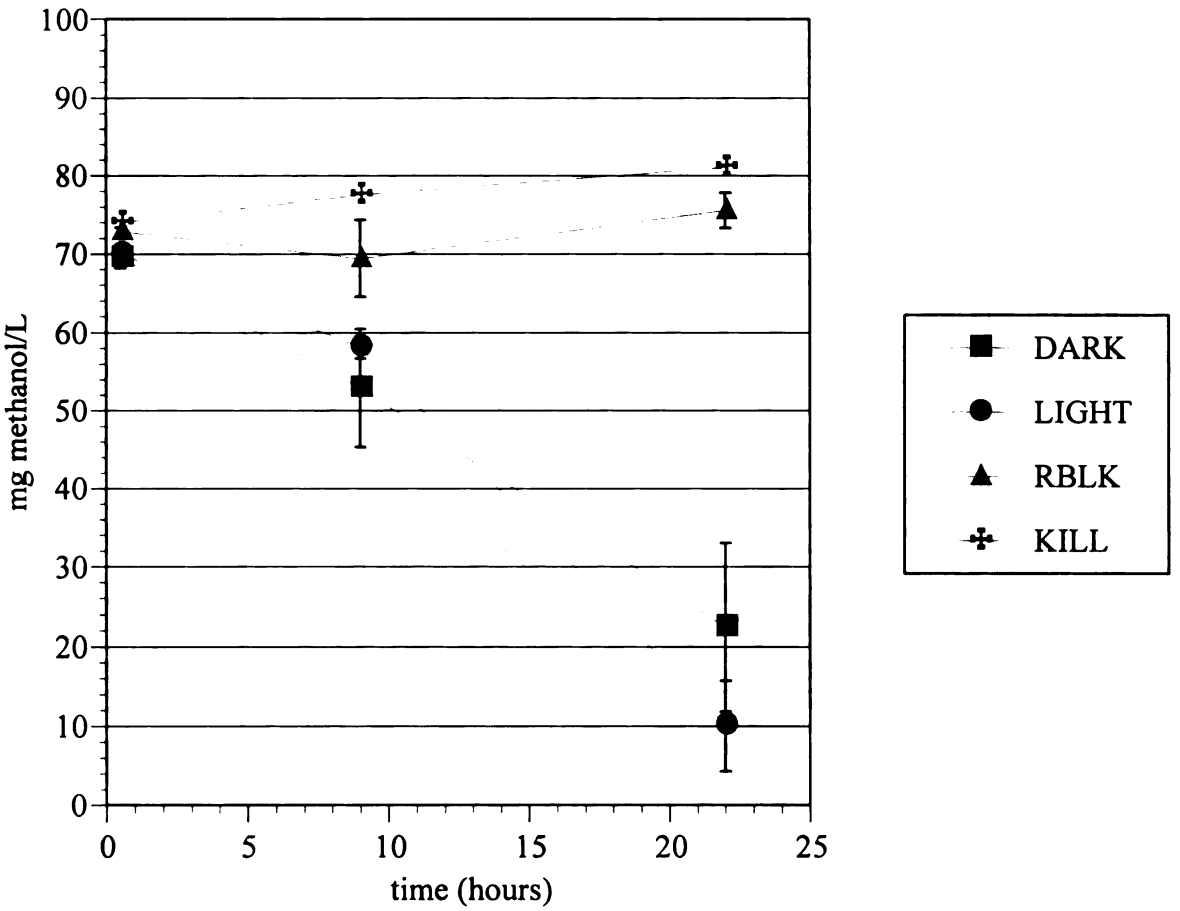

Figure 3.6 Methanol transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #2 (7/26/95).

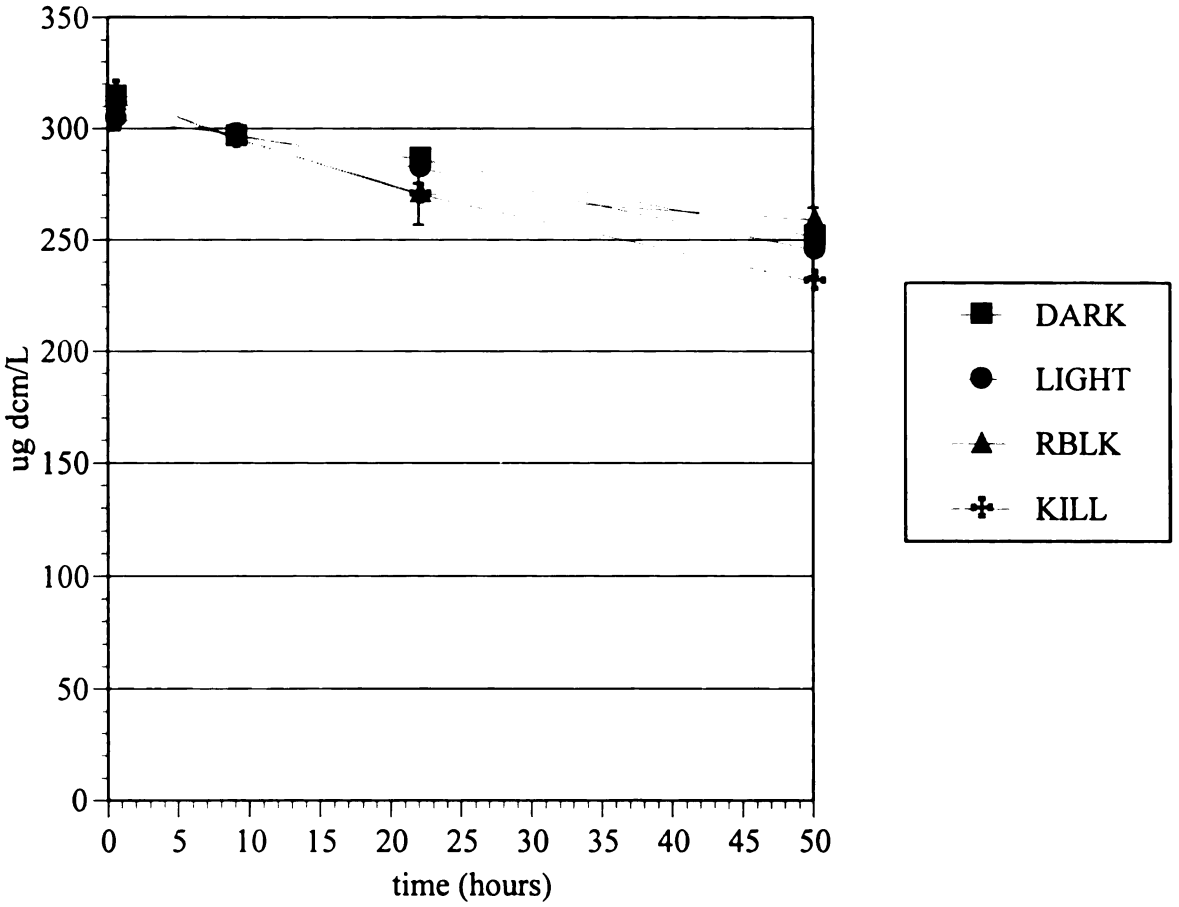

Figure 3.7 Dichloromethane (DCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #2 (7/26/95).

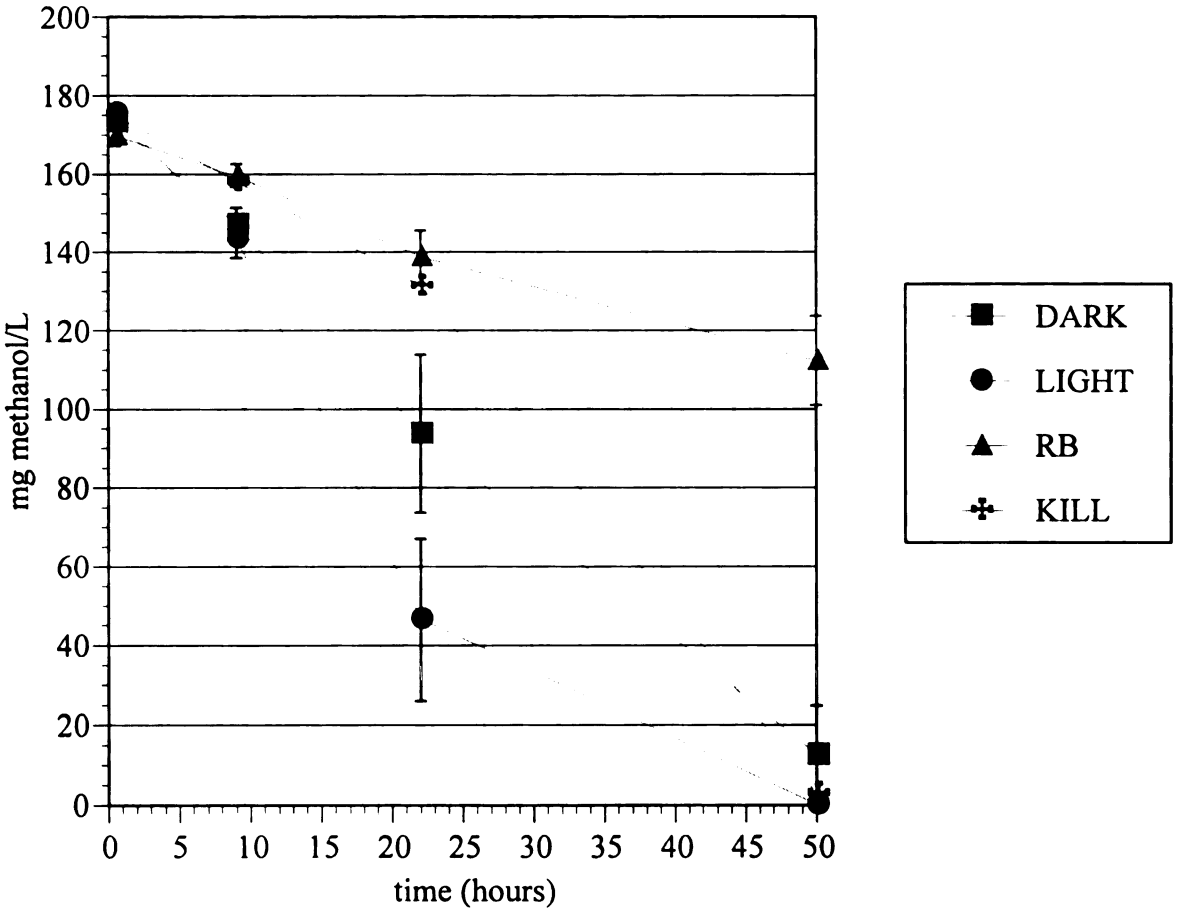

Figure 3.8 Toluene (TOL) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #2 (7/26/95).

3.5.4 Secondary Metabolism Experiment #3: 5 Simultaneous Substrates

Experiment #3 was designed to simultaneously evaluate transformation rates for five secondary substrates, all of which are occasional pond contaminants. Methanol was added at an initial concentration of 68 mg L^{-1}. The following secondary substrates were added at the approximate initial equilibrium concentrations indicated in parentheses: DCM (70 μg L^{-1}), DCA (50 μg L^{-1}), BDCM (40 μg L^{-1}), CB (45 μg L^{-1}), and TOL (30 μg L^{-1}). Subsamples were collected at 0.5, 14, and 72 hours.

Direct microscopic counts were comparable to experiment #1, but the decreased variance in experiment #3 allowed statistical detection of significantly higher cell numbers in the DARK treatment. The decreased variance may have been a function of a fully-matured developmental state within the biofilm. Primary and secondary transformation plots are presented in Figures 3.9 through 3.13. Results for the biological controls were comparable to experiments #1 and #2, but they were omitted from transformation plots due to the previously discussed problem of ineffective autoclave sterilization over long term experiments. Methanol was transformed rapidly and at rates comparable to experiments #1 and #2 (see Table 3.3). Methanol transformation rates were again not significantly different between light treatments (see Table 3.4).

None of the aliphatic secondary compounds were biologically transformed in this experiment. Although all vials exhibited loss of DCM, DCA, and BDCM over time, the rates of loss did not differ from those of the reagent controls (RBLK), indicating that non-biological factors were responsible for these losses. TOL and CB were both secondarily transformed at comparably rapid rates, with DARK communities exhibiting significantly

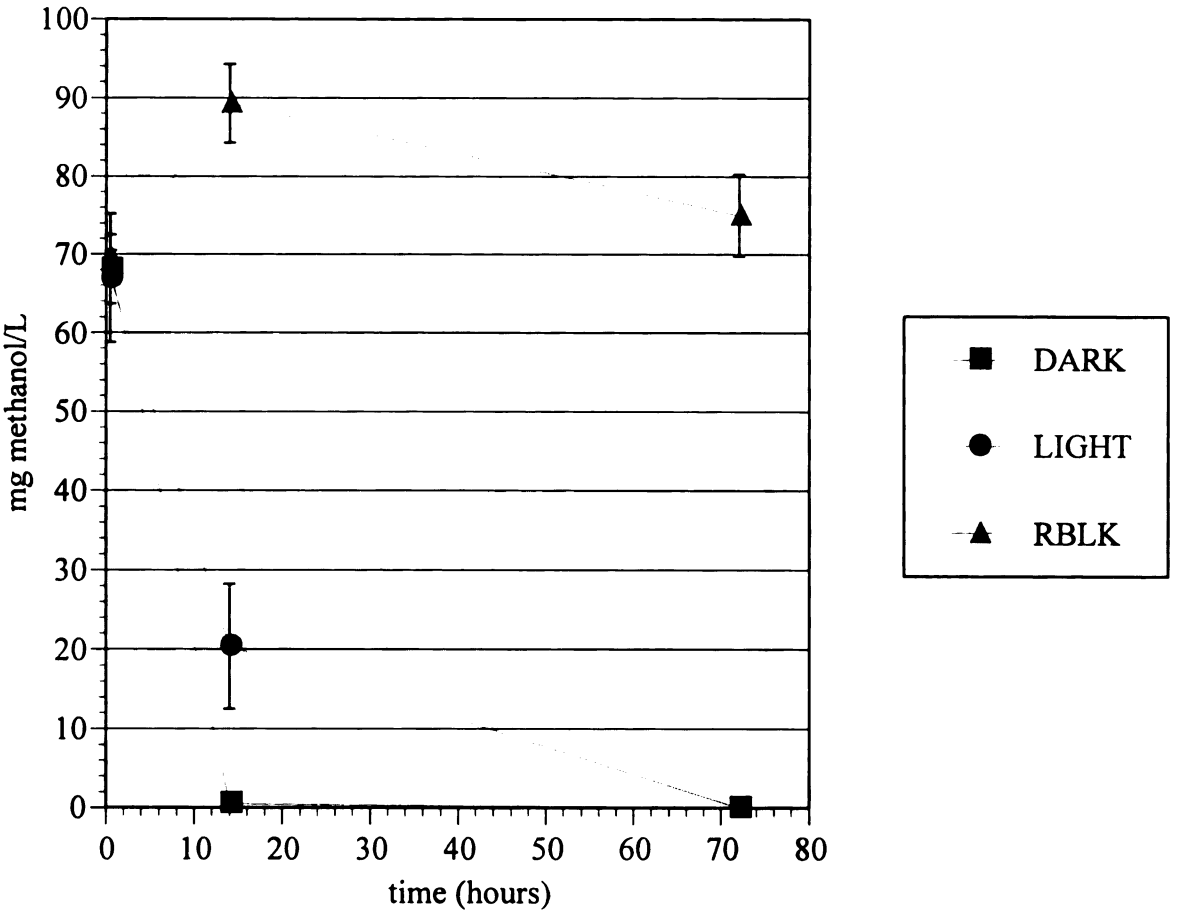

Figure 3.9 Methanol transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

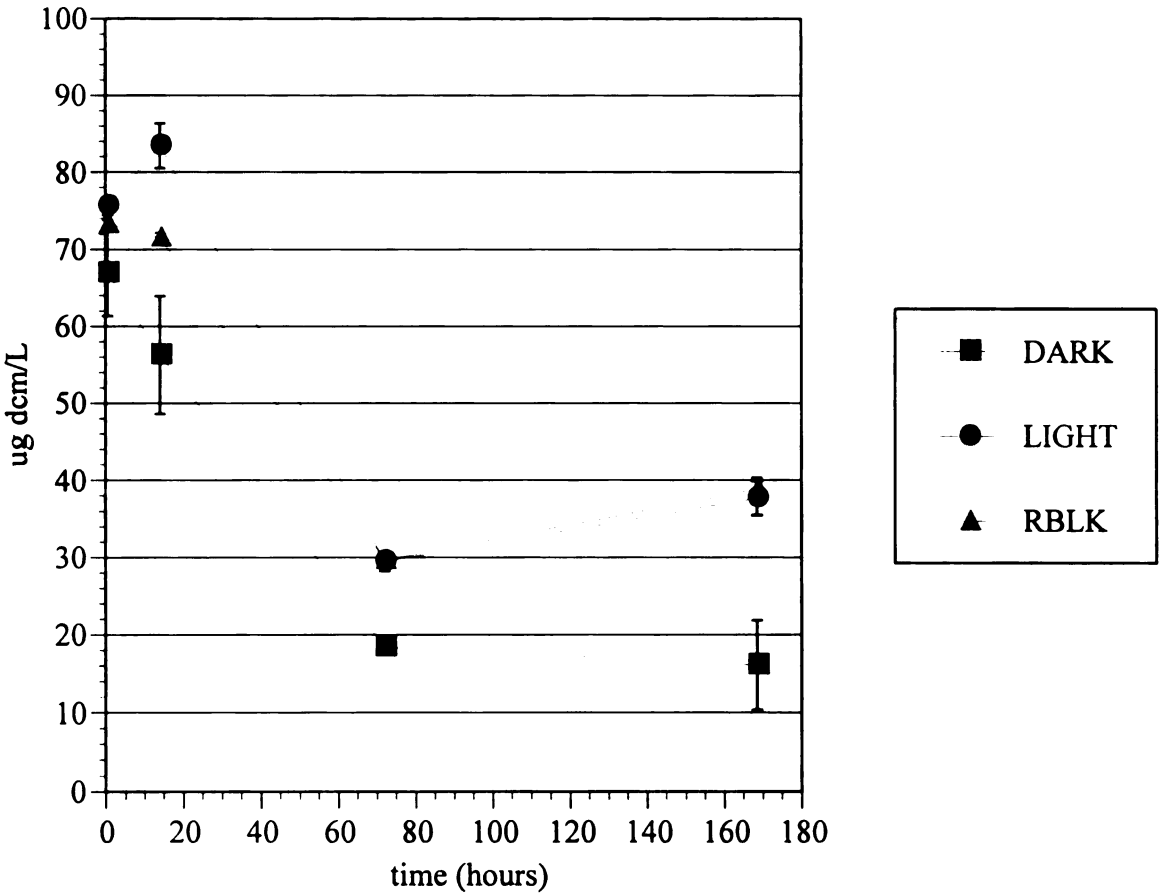

Figure 3.10 Dichloromethane (DCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

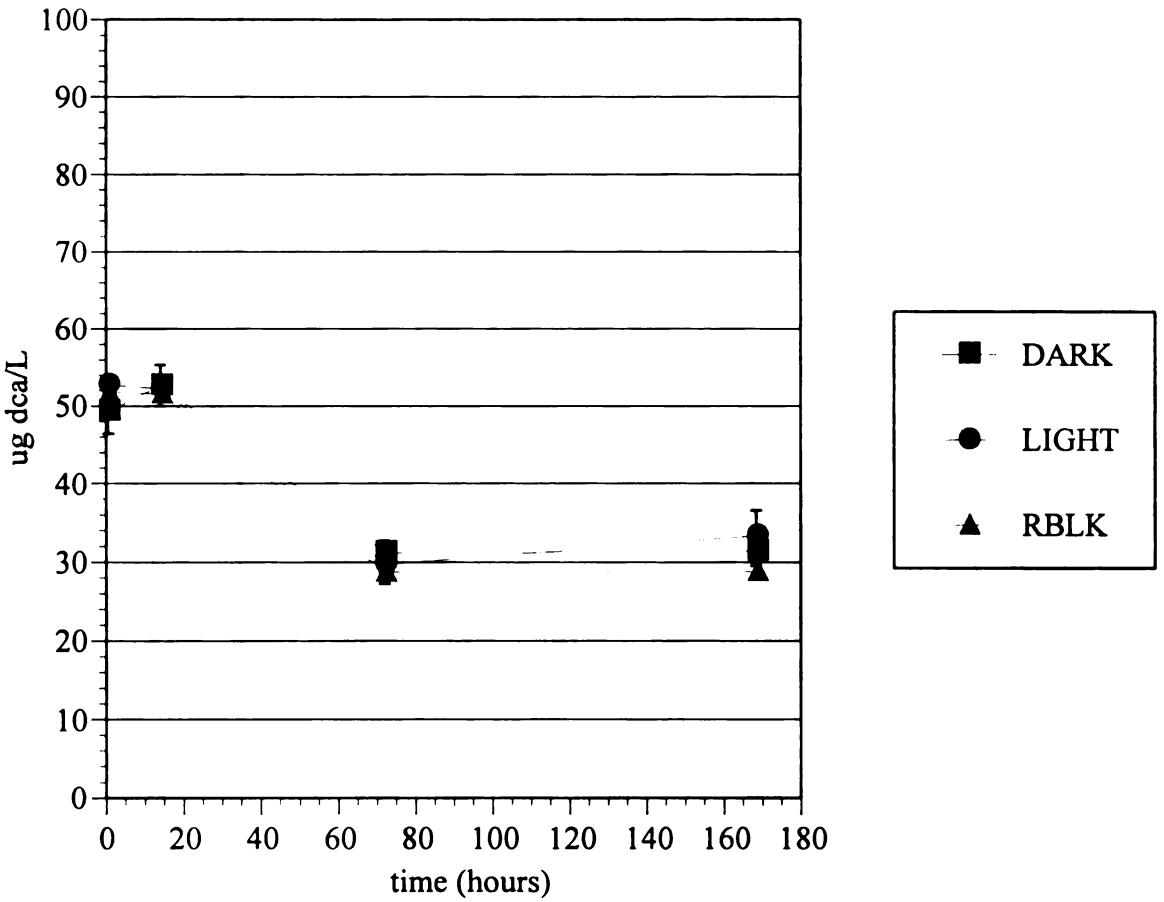

Figure 3.11 1,2-dichloroethane (DCA) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

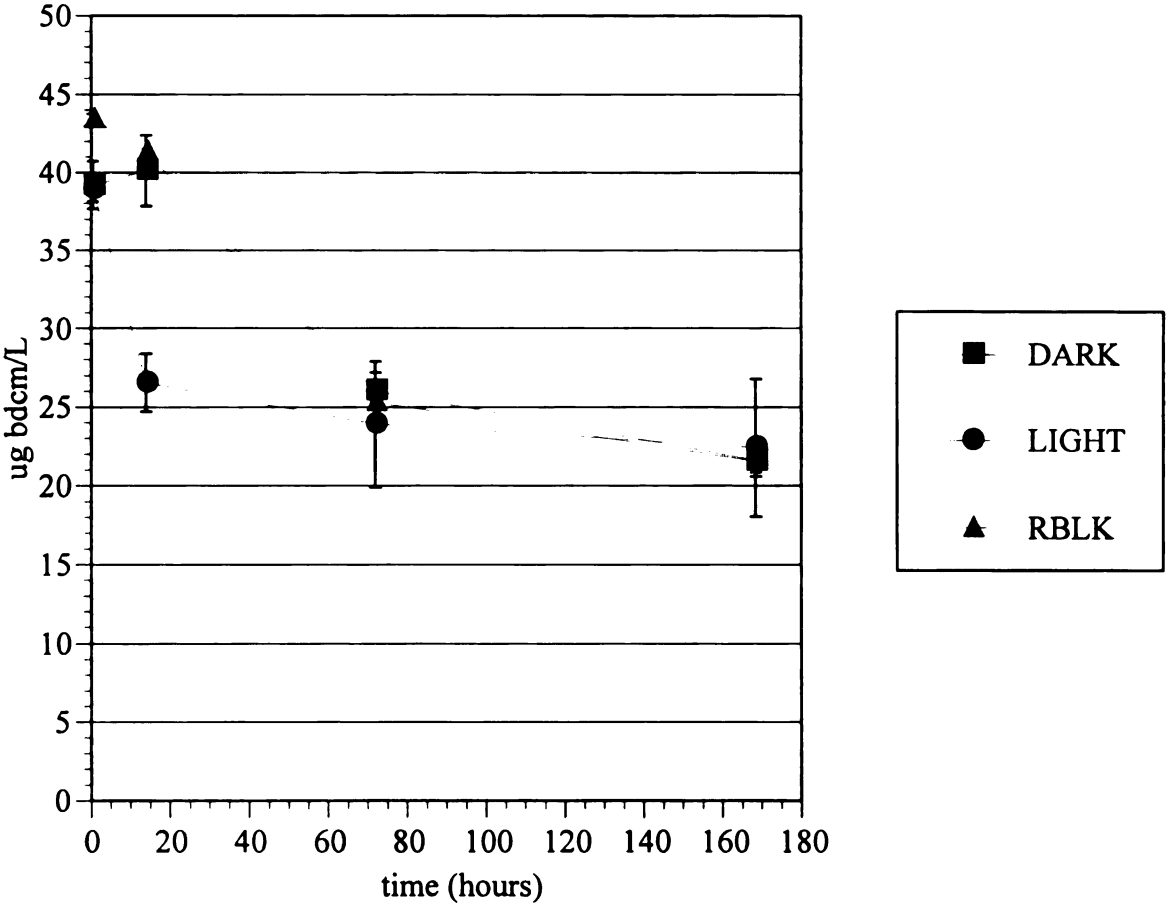

Figure 3.12 Bromodichloromethane (BDCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

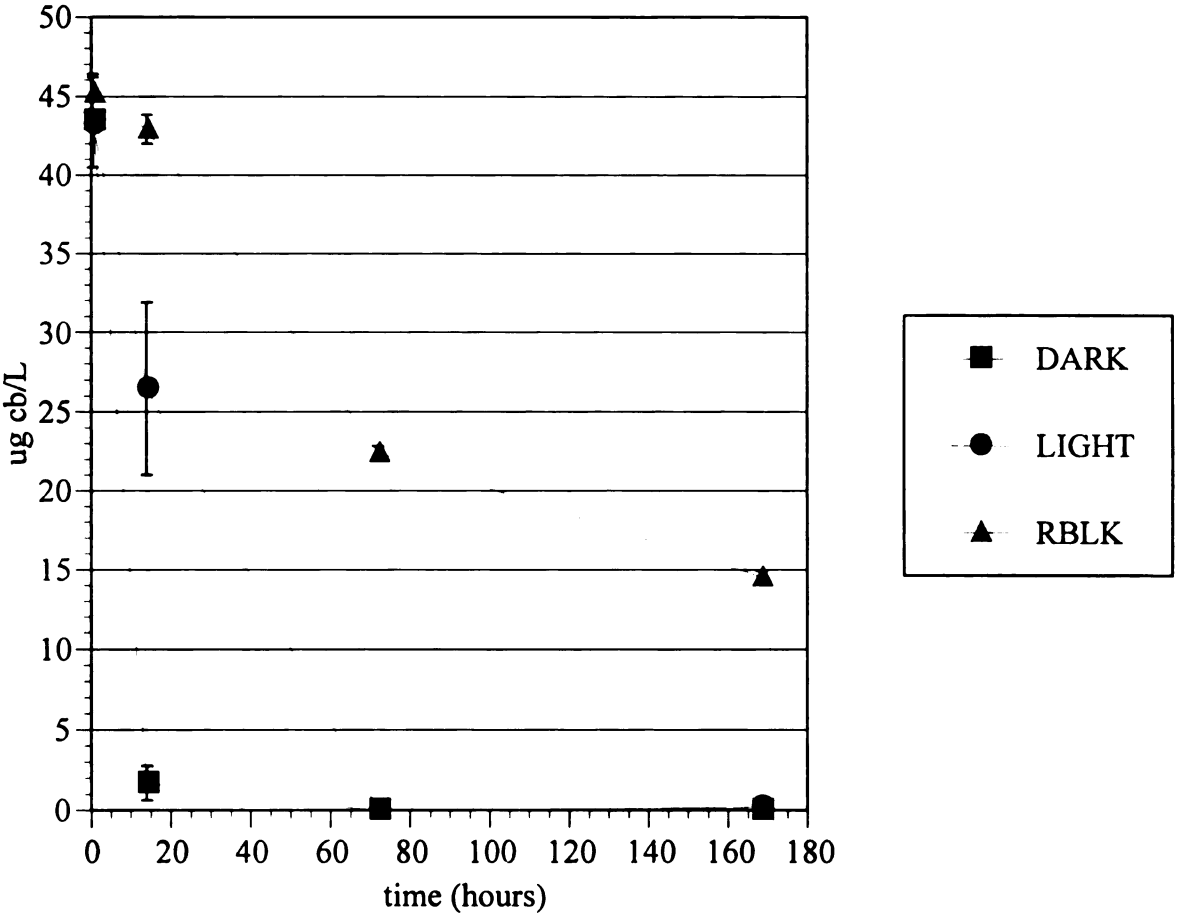

Figure 3.13 Chlorobenzene (CB) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

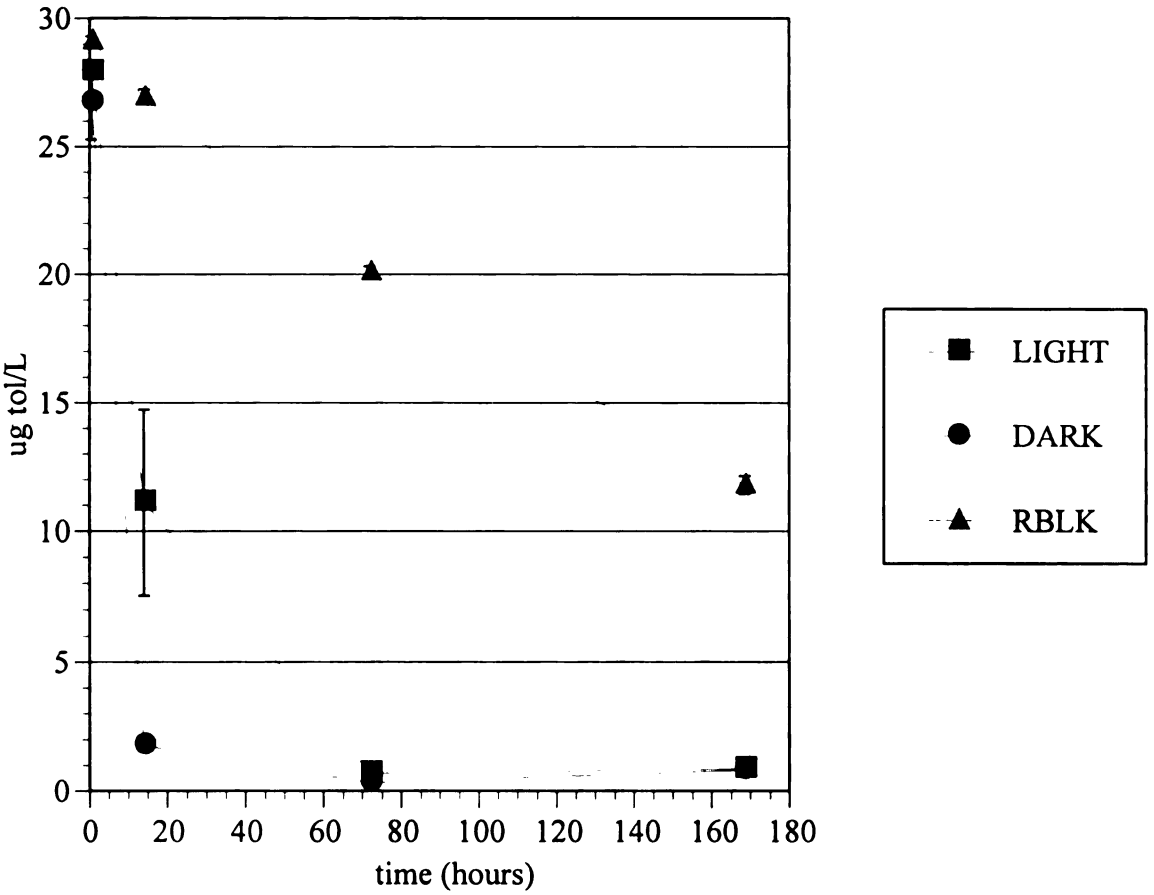

Figure 3.14 Toluene (|TOL) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3 (8/11/95).

higher CB transformation. Again, the reagent control (RBLK) exhibited losses, but the loss rate was significantly greater in the biologically active samples. Changes in the vial headspace volume due to destructive sampling likely disturbed the equilibrium concentration of volatile organics in solution, resulting in apparent losses in the reagent controls.

3.5.5 Secondary Metabolism Experiment #4: Primary/Secondary Link

Experiment #4 was designed to determine the link between primary methanol metabolism and secondary substrate utilization. Incubation flasks in the lab were divided between two treatments: no methanol and 100 mg L^{-1} methanol. Flasks receiving methanol (designated +MeOH) were injected with a mixture of secondary substrates dissolved in methanol to the following initial concentrations: DCM (50 μg L^{-1}), DCA (60 μg L^{-1}), BDCM (50 μg L^{-1}), CB (40 μg L^{-1}), and TOL (40 μg L^{-1}). Flasks not receiving methanol (designated -MeOH) were treated with a water-based solution of the same secondary substrates. DCM and BDCM were apparently immediately volatilized from the water-based stock solution and were not found in any subsamples. Final initial concentrations for the remaining secondary compounds in the water-based solution were as follows: DCA (80 μg L^{-1}), CB (40 μg L^{-1}), and TOL (40 μg L^{-1}). A unreplicated sediment sample was collected and processed according to the method described in section 3.5.6 and injected with the methanol-based stock.

Direct microscopic counts are summarized in Tables 3.1 and 3.2. Total numbers of bacteria in DARK biofilms were comparable to results from experiments #1-#3. However, LIGHT biofilms exhibited approximately 5 times higher total numbers of

bacteria than were observed in previous experiments. Bacteria were significantly more numerous in the LIGHT treatment, perhaps a reflection of the increased temperature and light intensity of late summer.

Primary and secondary transformation plots are presented in Figures 3.15 through 3.20. Figure 3.15 demonstrates that methanol transformation rates (in those flasks receiving methanol) were again comparable to previous experiments and were not significantly different between light treatments. The presence of methanol also had no significant effect on the transformation of any secondary compound.

DCM, DCA, and BDCM were not biologically transformed in the course of this experiment. No data is presented in Figures 3.16 or 3.17 for the methanol-negative vials, due to volatilization and solubility problems with DCM and BDCM in the water-based stock. Although no direct comparison could be made between methanol-positive and methanol-negative transformation rates for DCM or BDCM, methanol clearly did not stimulate secondary transformation of these compounds. Figure 3.17 demonstrates the lack of aerobic DCA transformation activity in either the presence of absence of methanol. Transformation rates of TOL and CB were again comparable to previous experiments and were significantly higher in the DARK treatments. The presence of methanol did not stimulate secondary transformation of either TOL or CB.

As shown in Figure 3.21, the anaerobic sediment slurry exhibited rapid transformation of methanol, DCM, and TOL. Because this was the first evidence of secondary DCM transformation, a replicated sediment experiment was designed and implemented next. BDCM was immediately undetectable in the sediment slurry,

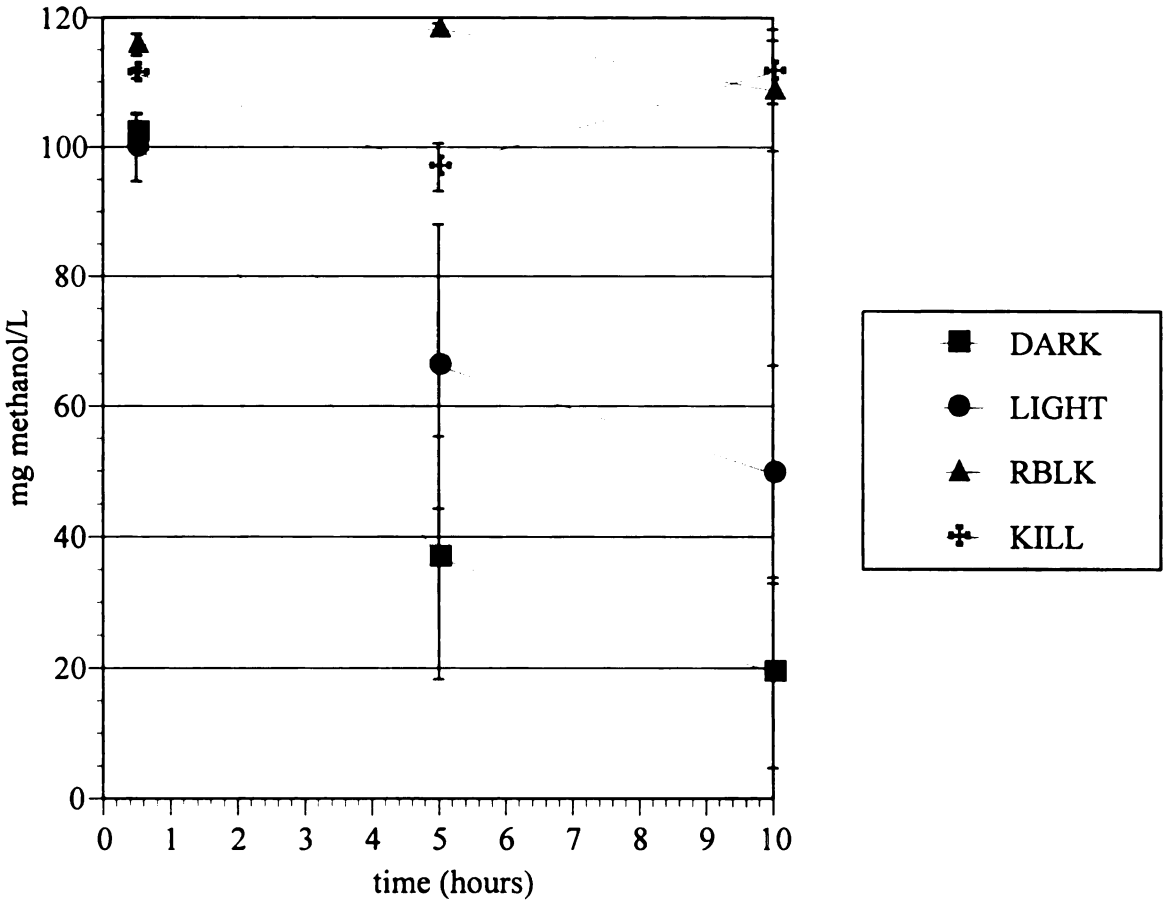

Figure 3.15 Methanol transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #4 (9/13/95).

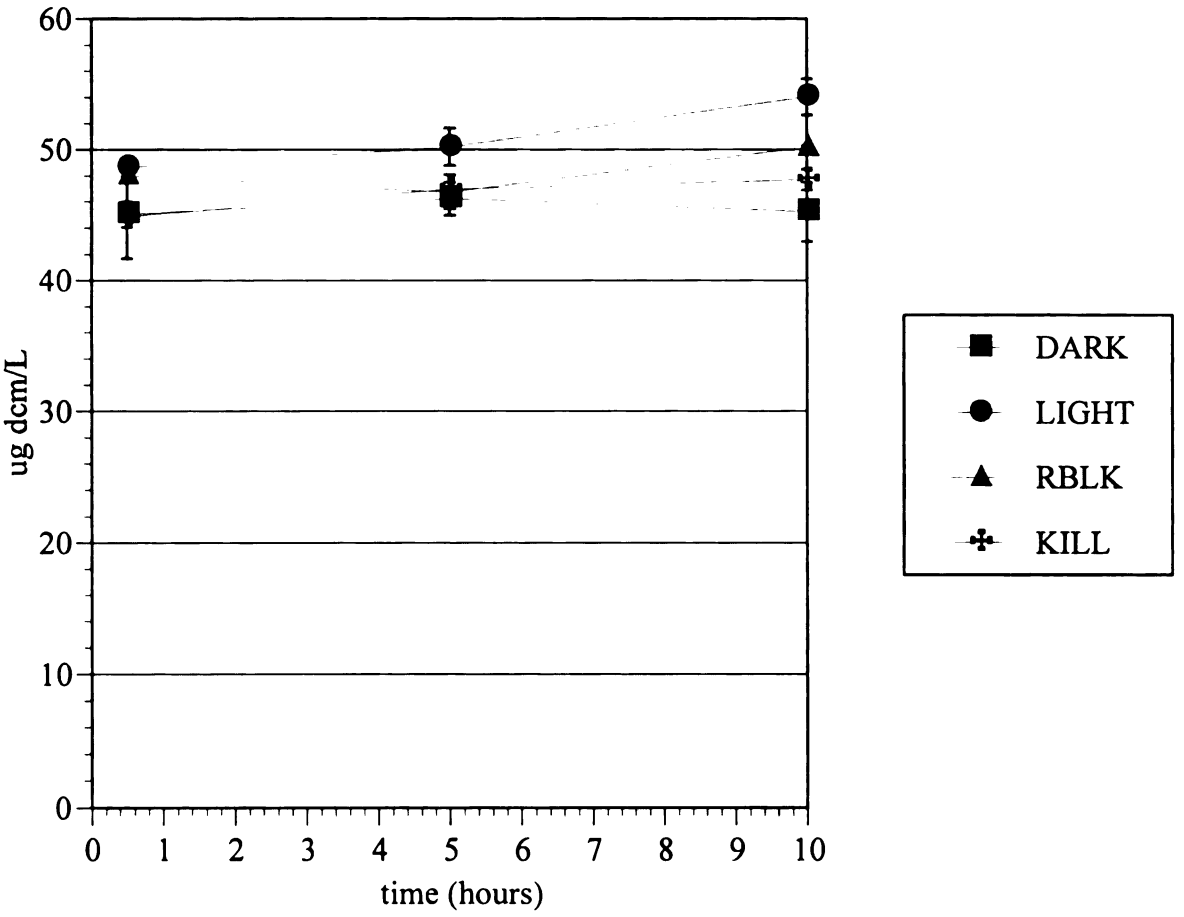

Figure 3.16 Dichloromethane (DCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #4 (9/13/95).

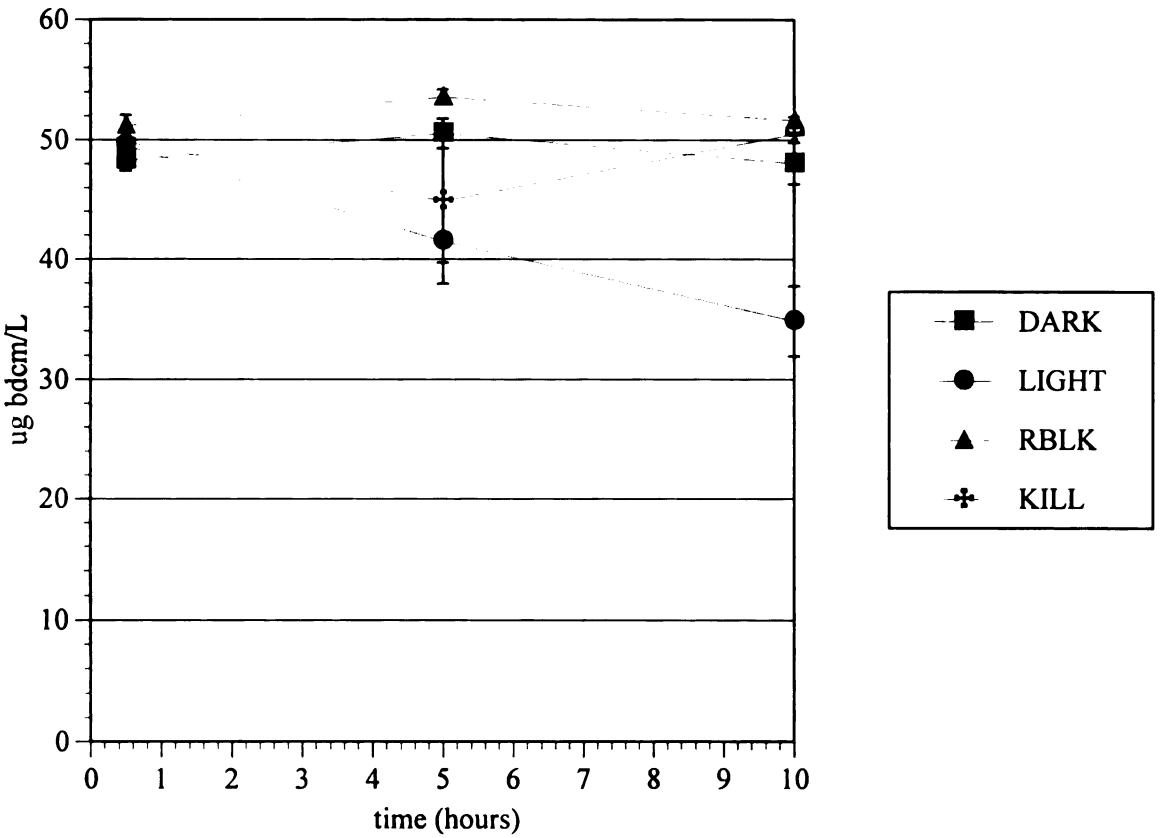

Figure 3.17 Bromodichloromethane (BDCM) transformation plot for laboratory incubations of attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #4 (9/13/95).

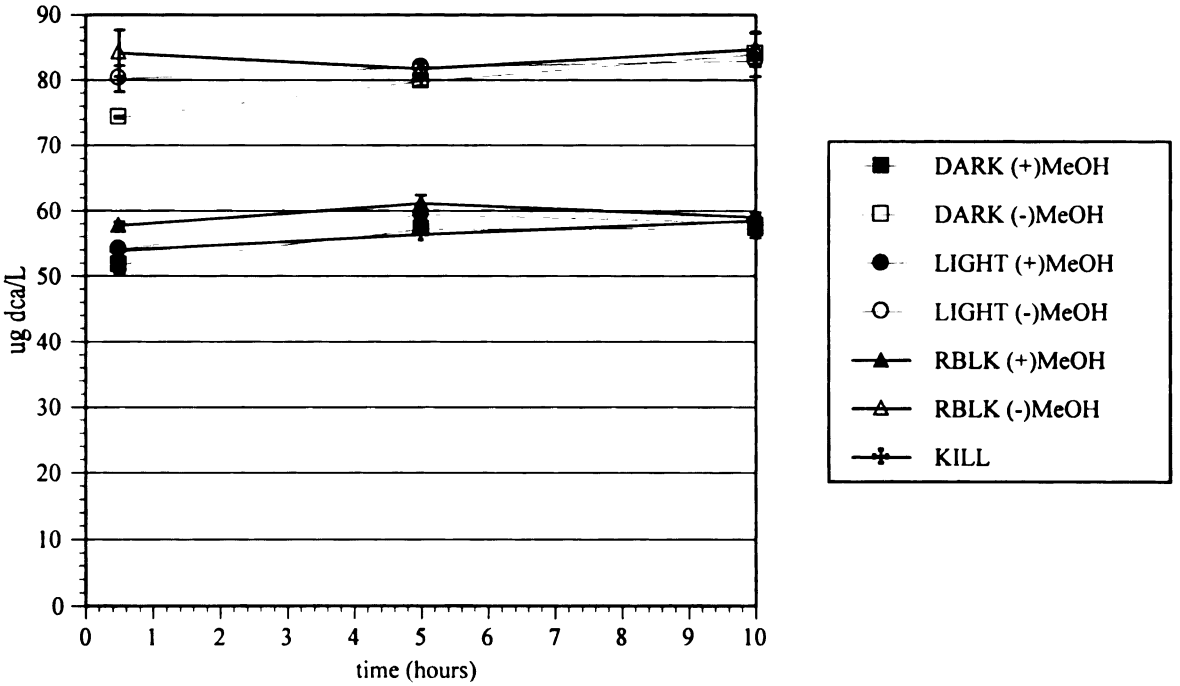

Figure 3.18 1,2-dichloroethane (DCA) transformation plot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, incubated in the laboratory in the presence or absence of methanol, Experiment #4 (9/13/95).

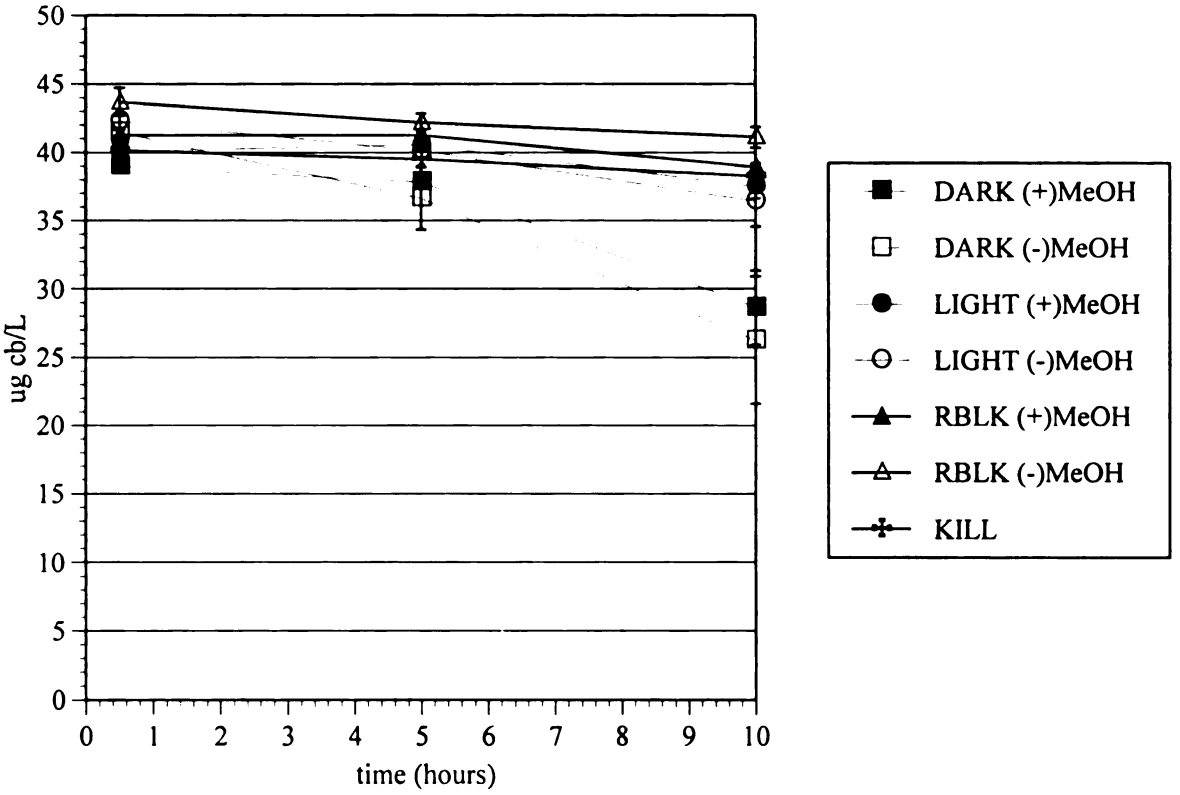

Figure 3.19 Chlorobenzene (CB) transformation plot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, incubated in the laboratory in the presence or absence of methanol, Experiment #4 (9/13/95).

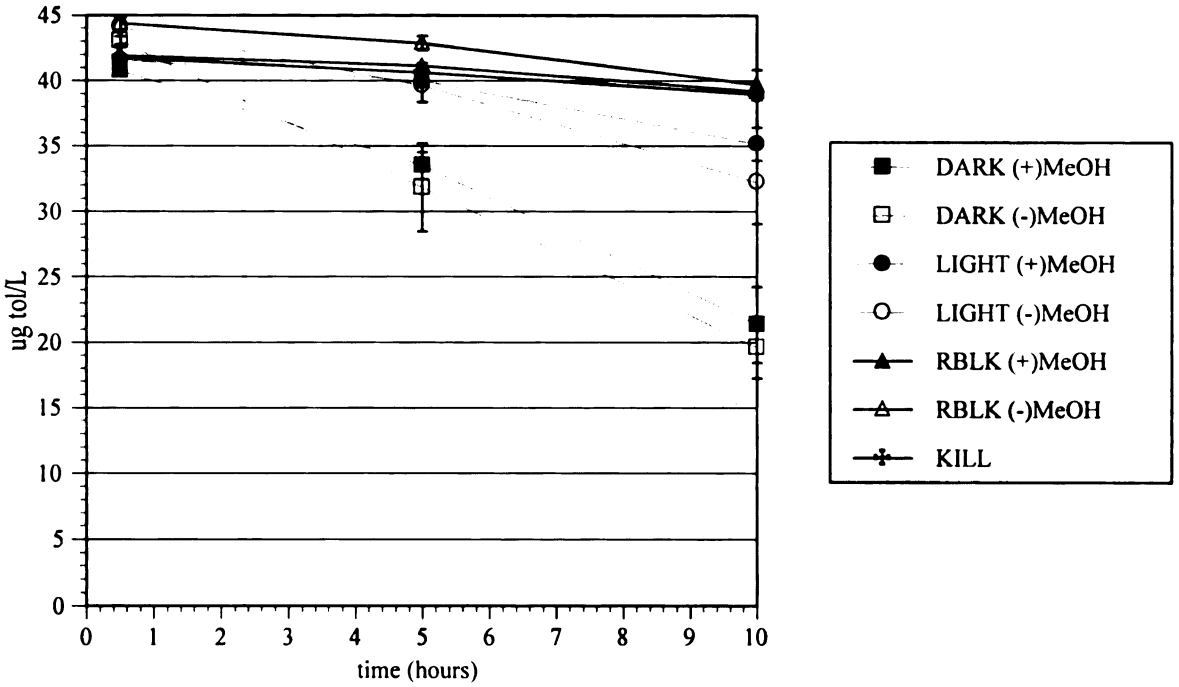

Figure 3.20 Toluene (TOL) transformation plot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, incubated in the laboratory in the presence or absence of methanol, Experiment #4 (9/13/95).

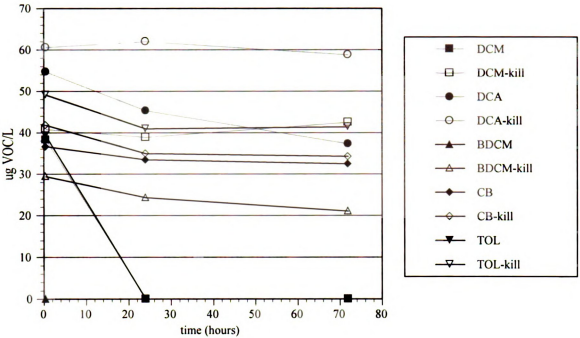

Figure 3.21 Multiple compound transformation plots for laboratory incubations of sediment slurry from the Pharmacia/Upjohn Pond, Experiment #4 (9/13/95)

indicating either very rapid uptake of BDCM by the sediment microorganisms or a strong sorption affinity for the compounds by sediment particles.

3.5.6 Secondary Metabolism Experiment #5: Anaerobic Sediment Study

Experiment #5 was designed to develop rate estimates for sediment-associated anaerobic secondary activity and to determine the associated role of sulfate reduction. Surficial sediments were sampled in triplicate from the north end of the Pond with an Eckman dredge operated to a depth of 10 cm. Sediment slurries were prepared in the lab by diluting 1:1 (v:v) with filter-sterilized and pre-boiled Pond water while working under a stream of oxygen-free high purity nitrogen. Slurry subsamples of 50-mL volume were placed in 20 mm, 135-mL serum vials, which were sealed with teflon-lined septa and purged for 10 minutes prior to incubation with high purity nitrogen gas passed through a metallic copper oxygen-stripping column operating at 250 C. Slurry was also subsampled in 10-ml aliquots for sediment dry-weight determination. Dry weights were measured after oven-drying the slurry 10 days at 105° C. Other incubation methods were similar to experiments #3 and #4, with the exception that each subsample was replaced with an equal volume of oxygen-free high purity nitrogen gas to prevent oxygen infiltration into the flasks.

Sulfate reduction was shown to have a significant role in anaerobic respiration of methanol in the Pharmacia/Upjohn Pond sediments (Klug *et al.*, 1996). Because sulfate reduction significantly impacts primary methanol metabolism, we hypothesized that sulfate reduction would have a similar impact on secondary metabolism, particularly if the secondary activity is linked to primary methanol metabolism. Half the samples were

treated with a 50 mM sodium molybdate solution to block sulfate reduction. A stock solution of substrates was injected in each flask to achieve the initial equilibrium concentrations indicated in parentheses for each compound: MeOH (100 mg L^{-1}), DCM (40 μg L^{-1}), DCA (50 μg L^{-1}), CB (30 μg L^{-1}), and TOL (25 μg L^{-1}). Subsamples were collected at 0.2, 6.5, 12.5, and 18.0 hours.

Several subsamples for methanol analysis were inadvertently destroyed prior to analysis. Therefore, methanol transformation for sediment incubations was not plotted and rate statistics were not calculated. Secondary transformation plots are presented in Figures 3.22-3.25. DCM and TOL transformation (Figures 3.22 and 3.25, respectively) was extremely rapid under sulfate-reducing conditions and was nearly 100% inhibited in the presence of the sulfate reduction block. DCA and CB (Figures 3.23 and 3.24, respectively) were not anaerobically transformed in the course of this experiment and showed no differing response when sulfate reduction was blocked.

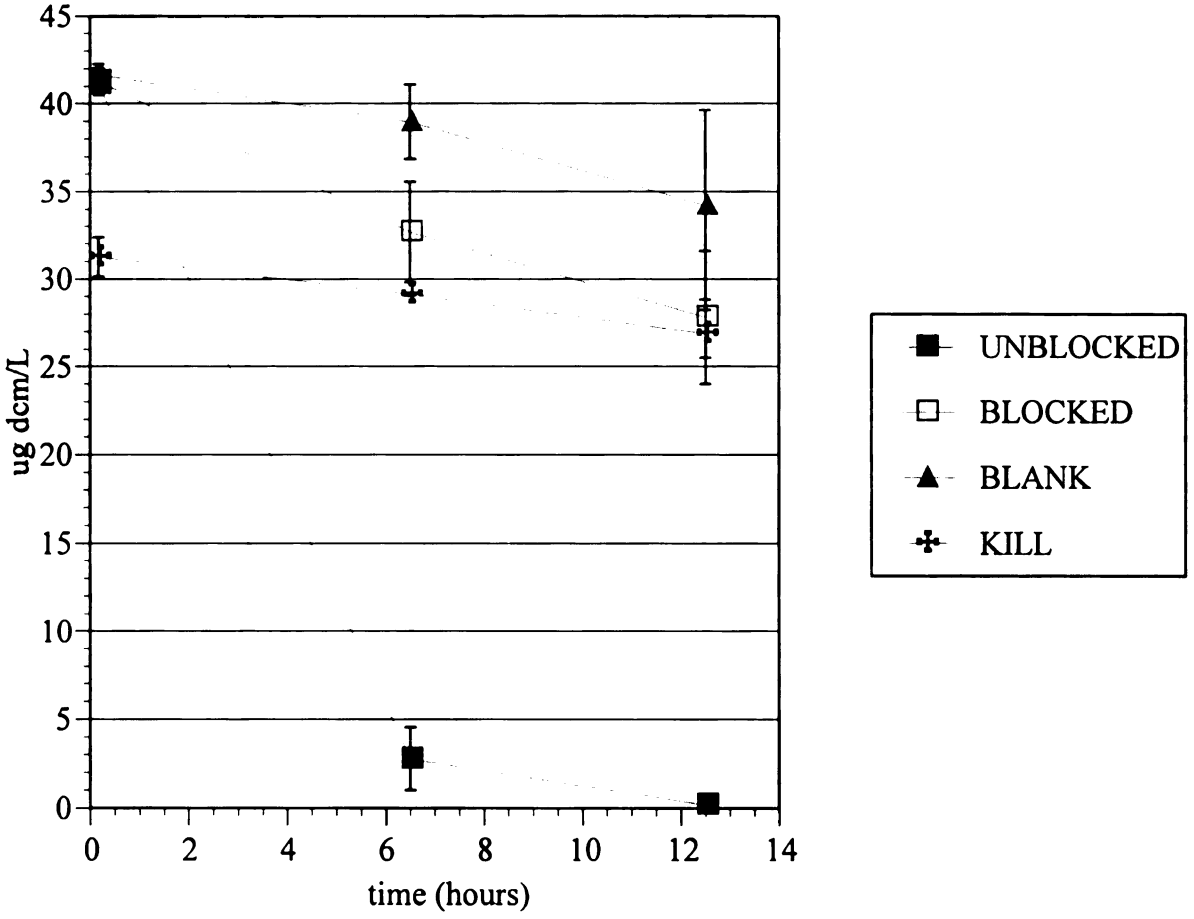

Figure 3.22 Dichloromethane (DCM) transformation plot for laboratory incubations of sediment slurry from the Pharmacia/Upjohn Pond in the presence or absence of a sulfate reduction inhibitor, Experiment #5.

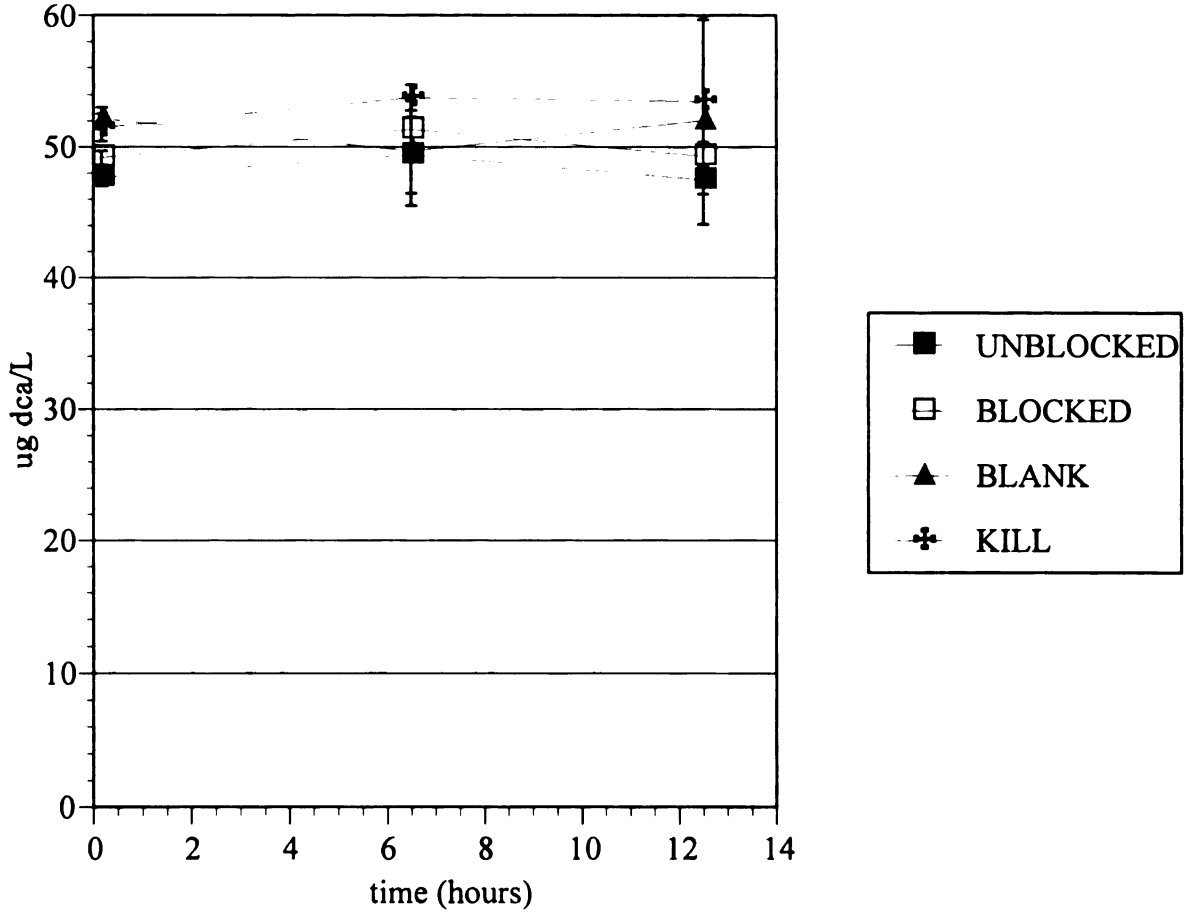

Figure 3.23 1,2-dichloroethane (DCA) transformation plot for laboratory incubations of sediment slurry from the Pharmacia/Upjohn Pond in the presence or absence of a sulfate reduction inhibitor, Experiment #5.

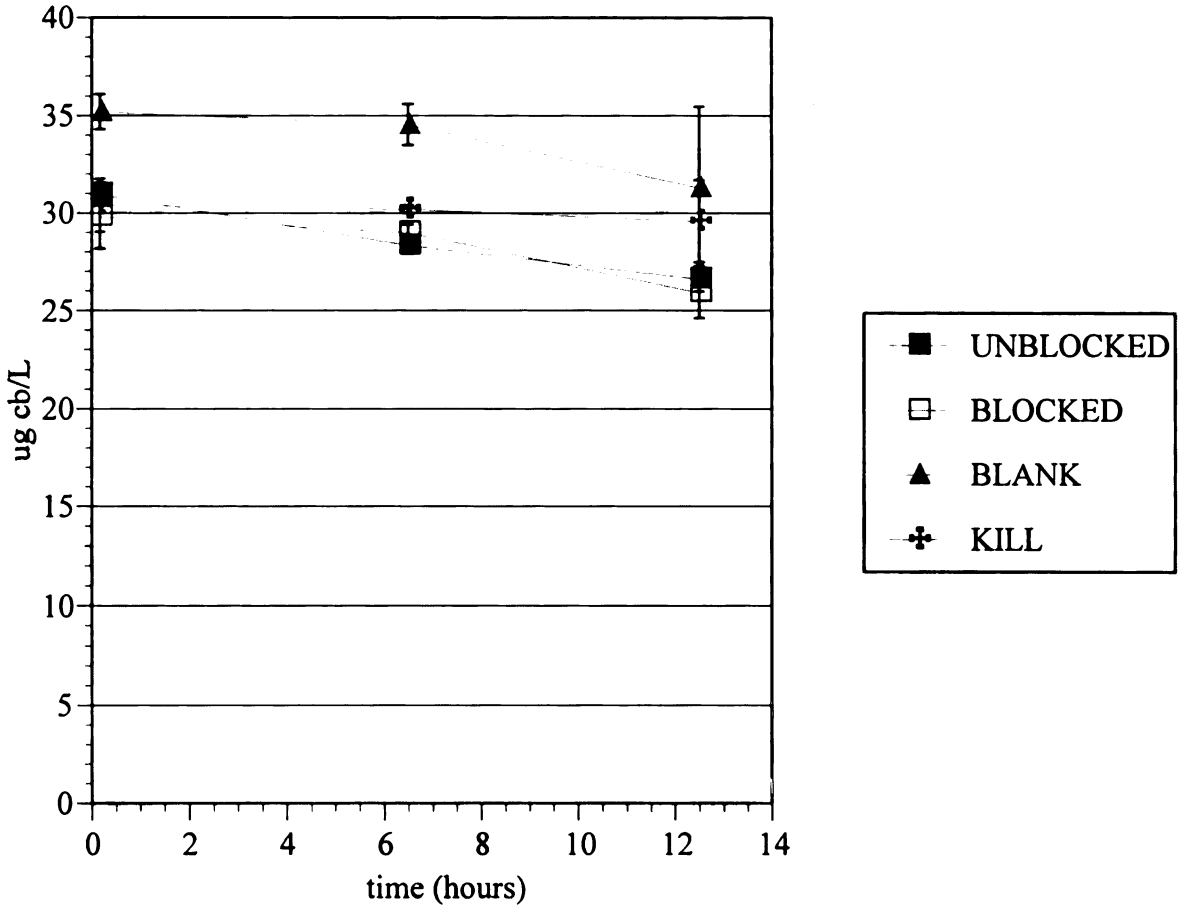

Figure 3.24 Chlorobenzene (CB) transformation plot for laboratory incubations of sediment slurry from the Pharmacia/Upjohn Pond in the presence or absence of sulfate reduction inhibitors, Experiment #5.

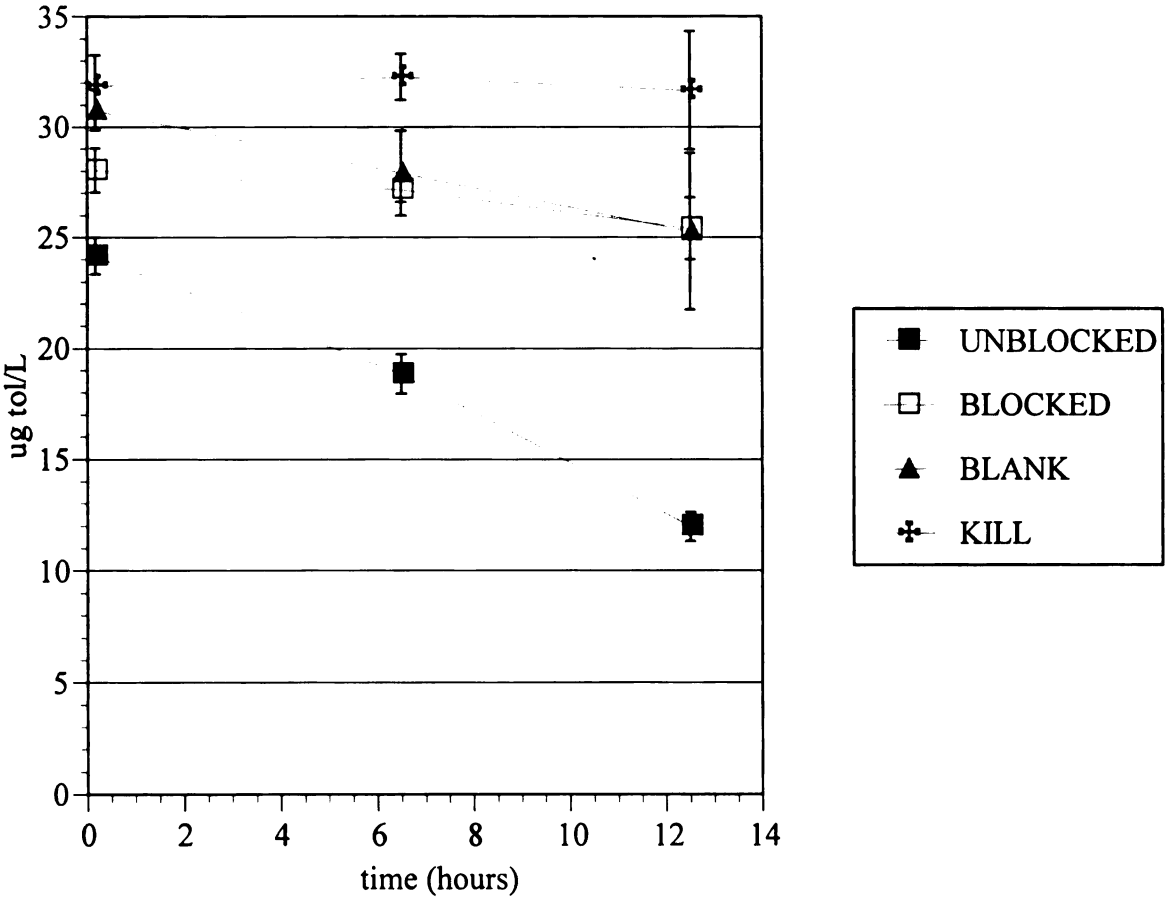

Figure 3.25 Toluene (TOL) transformation plot for laboratory incubations of sediment slurry from the Pharmacia/Upjohn Pond in the presence or absence of sulfate reduction inhibitors, Experiment #5.

3.5.7 Secondary Metabolism Experiment #6: Methanol Exclusion

Experiment #6 was designed to test the hypothesis that primary methanol metabolism supports secondary substrate utilization. Biofilm communities were pre-incubated 3 weeks *in vitro* in the presence or absence of methanol as the primary growth substrate. Assuming that primary metabolism of methanol is linked to secondary compound transformation, continuous exposure to methanol would hypothetically induce or enhance enzyme systems required for metabolism of secondary non-growth substrates. Conversely, biofilms growing in the absence of methanol would hypothetically express these secondary enzyme systems to a lesser degree. Subsequent secondary-level incubations (as described in previous experiments) following the pre-incubation period facilitated detection of any relationship between primary methanol metabolism and induction of secondary enzyme systems.

Biofilms from DARK treatments only (4 replications) were harvested and slurries prepared as in previous experiments. Subsamples of the pre-incubation slurry were collected for direct microscopic count and FAME analysis. Aliquots (100-mL) of biofilm slurry were transferred to sterile 500-ml sidearm Erlenmeyer flasks in a continuous flow *in vitro* incubation system consisting of two treatments: 100 mg L^{-1} methanol (designated +MeOH) and 0 mg L^{-1} methanol (designated -MeOH). Each treatment consisted of a total of 6 flasks: 5 flasks with biologically active biofilms (designated BIOFILM) and 1 groundwater reagent control flask (designated GW).

The majority of cooling water entering the Pond originates from groundwater on the Pharmacia/Upjohn site. The development of microbial communities in the Pond is

likely to have been impacted by continuous inoculation with groundwater microorgansisms. Furthermore, the inorganic chemistry of this groundwater is comparable to the Pond water, but the groundwater is free of methanol. For these reasons, groundwater from the Pharmacia/Upjohn site was deemed the most appropriate media for continuous flow incubations. The basal media for the *in vitro* system consisted of unfiltered groundwater collected directly from Pharmacia/Upjohn wellhead #23, a major source of cooling water for the pharmaceutical plant.

A diagram of the *in vitro* system is presented in Figure 3.26. Fresh basal media was added twice daily to each of 2 bulk media tanks, one for each laboratory treatment. The bulk tank for the +MeOH treatment was treated with methanol following each infusion of fresh groundwater to achieve a final concentration of 100 mg methanol L^{-1}. Media was delivered to each treatment flask by Teel 1/170 hp magnetic drive pumps (Dayton Electric Manufacturing Co., Chicago, IL) and a manual screw-clamp flow-control system. Outflow from each flask was regulated by gravity overflow through the flask sidearm. Flow rates were controlled at 3.33 L $flask^{1}$ day^{-1}, which equates to a residence time in each flask of 3.6 hours. The low residence time ensured that those biofilm communities receiving methanol did not deplete the carbon resources in any given residence cycle.

Following a 3-week incubation, biofilms were destructively subsampled for direct microscopic counts, FAMEs, and secondary metabolism incubations according to the protocols described for previous experiments. One flask per treatment was sacrificed for an autoclave-sterilized biological control (designated KILL) in the secondary incubations.

Reagent control vials (designated RBLK) for secondary incubations were also prepared for each treatment.

All secondary incubation serum vials were spiked with a water-based stock substrate solution to achieve the initial equilibrium concentrations indicated in parentheses for each of the following compounds: DCM (60 $\mu g\ L^{-1}$), TOL (25 $\mu g\ L^{-1}$), and CB (35 $\mu g\ L^{-1}$). Methanol was added as a neat solution at a final concentration of 100 $mg\ L^{-1}$ only to those biofilms that had previously received methanol. Incubations were conducted as in previous experiments with subsampling at 0.5, 5.0, 10.0, 15.0, 20.0, and 25.0 hours.

Direct microscopic counts prior to incubation were comparable to previous DARK biofilms from the Pond, expectedly increasing after pre-incubation with methanol and decreasing after methanol starvation. These differences in post-incubation direct microscopic counts were statistically significant.

A methanol transformation plot for the +MeOH biofilms is presented in figure 3.27. Methanol transformation rates were comparable to and slightly higher than those observed with DARK biofilms in previous experiments. The groundwater-only biofilms (GW) also transformed methanol, but at about 20% of the rate observed with DARK biofilms.

Secondary transformation plots are presented in Figures 3.28-3.30. Although DCM transformation (Figure 3.28) had never been observed in previous aerobic experiments, the +MeOH samples exhibited rapid DCM transformation and at a highly significantly greater rate than that observed in the -MeOH samples.

The DCM transformation plot for +MeOH biofilms exhibits a shape classically associated with first order kinetics. However, closer examination of the data reveals that the deflection point in the curve at 15 hours coincides with depletion of the primary substrate methanol. Therefore, conclusive determination of zero- versus first-order secondary kinetics would only have been possible by mid-incubation replenishment of the primary substrate and subsequent continued monitoring of secondary transformation. Because primary substrate was not replenished and the order of kinetics for secondary transformation could not be deciphered, calculation of DCM transformation rates was based strictly on the zero-order portion of the curve.

CB (Figure 3.29) and TOL (Figure 3.30) were rapidly transformed in both treatments and did not show significant differences between treatments. Neither transformation plot shows a deflection at the time point coinciding with methanol depletion, suggesting that transformations of CB and TOL are not directly associated with primary methanol metabolism.

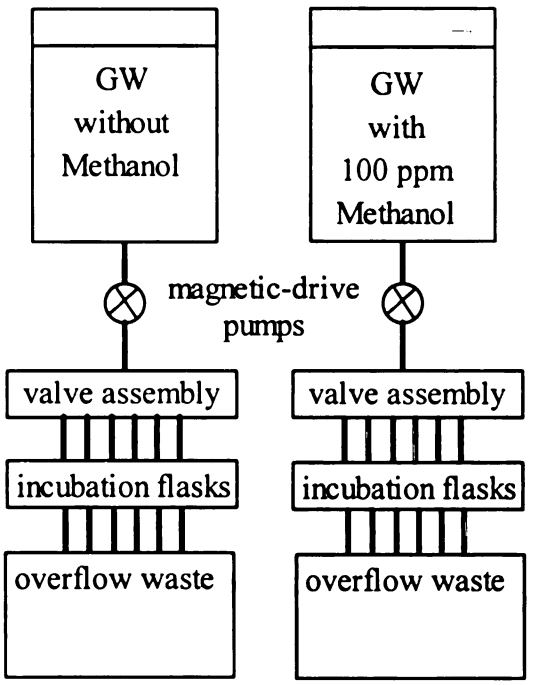

Figure 3.26 Diagram of *in vitro* system for pre-incubation of attached microbial communities from the Pharmacia/Upjohn Pond, Experiment #6.

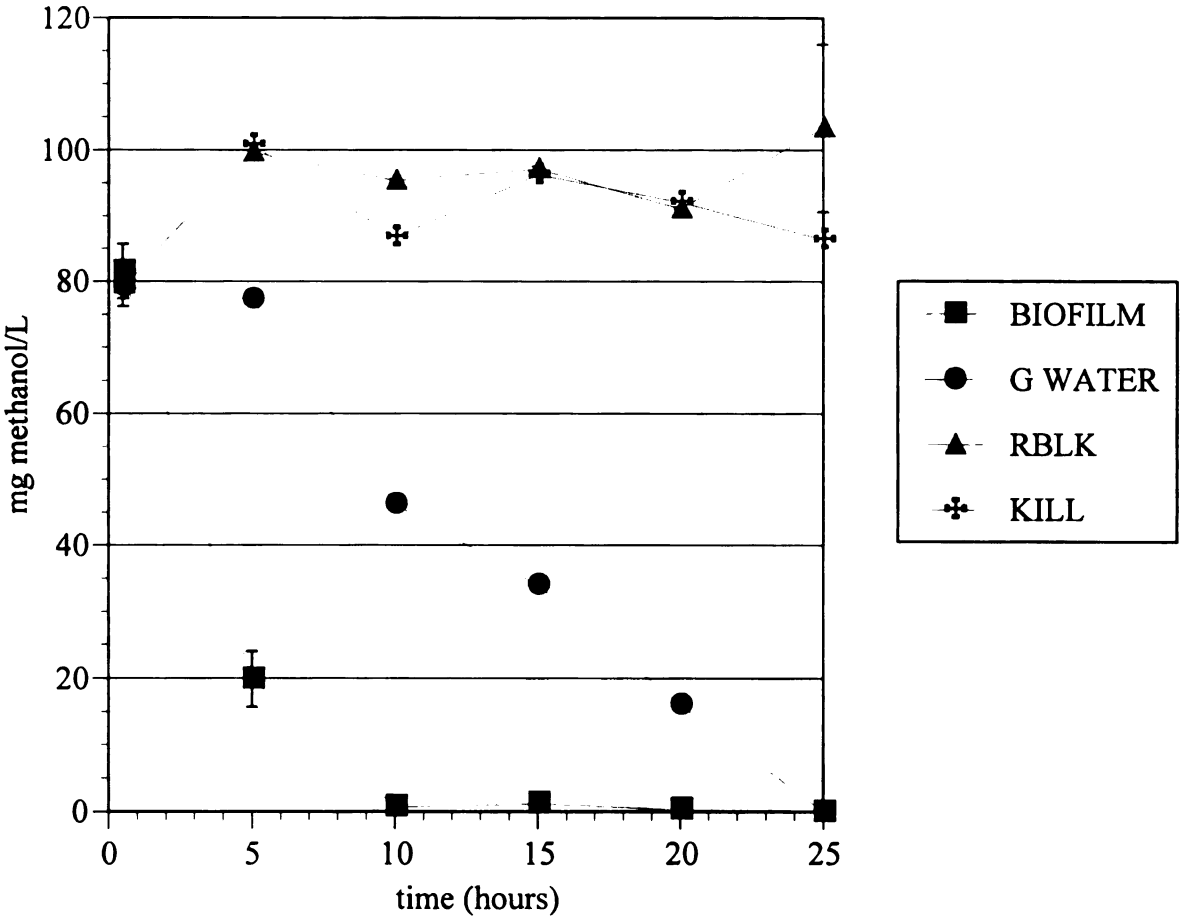

Figure 3.27 Methanol transformation plot for laboratory incubations of attached microbial communities developed under dark conditions in the Pharmacia/Upjohn Pond and pre-incubated for 3 weeks in the presence or absence of methanol, Experiment #6 (12/18/95).

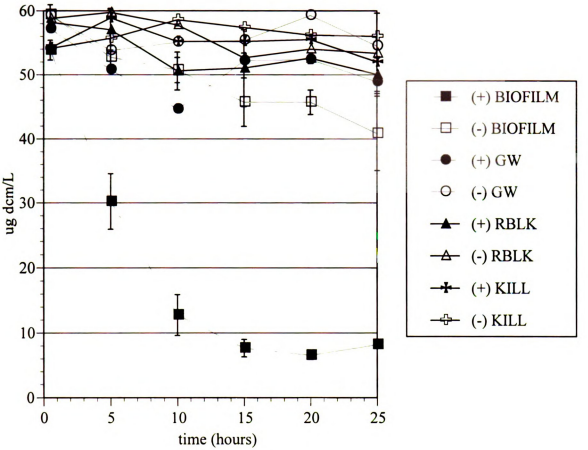

Figure 3.28 Dichloromethane (DCM) transformation plot for laboratory incubations of attached microbial communities developed under dark conditions in the Pharmacia/Upjohn Pond and pre-incubated for 3 weeks in the presence or absence of methanol, Experiment #6 (12/18/95).

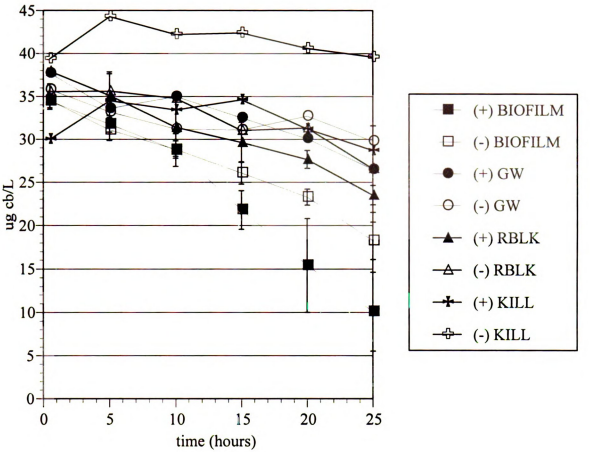

Figure 3.29 Chlorobenzene (CB) transformation plot for laboratory incubations of attached microbial communities developed under dark conditions in the Pharmacia/Upjohn Pond and pre-incubated for 3 weeks in the presence or absence of methanol. Experiment #6 (12/18/95).

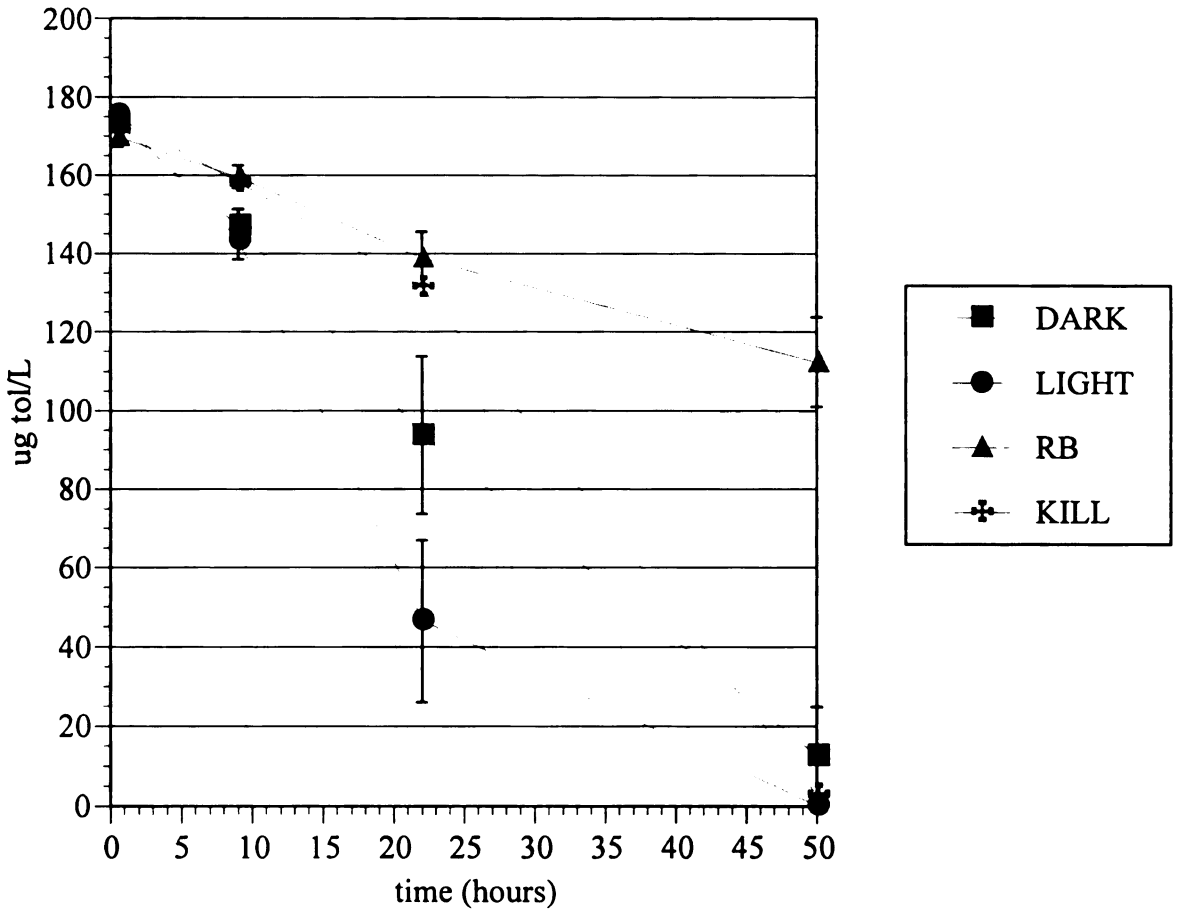

Figure 3.30 Toluene (TOL) transformation plot for laboratory incubations of attached microbial communities developed under dark conditions in the Pharmacia/Upjohn Pond and pre-incubated for 3 weeks in the presence or absence of methanol, Experiment #6 (12/18/95).

3.5.8 Secondary Metabolism Results Summary

Table 3.1 Summary of direct microscopic count data for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Treatment	cells ml^{-1}	Standard Error
1	Light	1.15×10^8	2.61×10^7
	Dark	7.20×10^7	2.72×10^7
2	Light	*no data*	*na*
	Dark	*no data*	*na*
3	Light	1.45×10^8	1.62×10^7
	Dark	2.65×10^8	2.07×10^7
4	Light	5.57×10^8	1.14×10^8
	Dark	2.84×10^8	5.85×10^7
5 (sed)	SO_4^{2-} Block	*no data*	*na*
	Unblocked	*no data*	*na*
6	pre-incubation	9.43×10^7	8.03×10^6
	post-inc (+MeOH)	3.18×10^8	1.62×10^7
	post-inc (- MeOH)	5.94×10^7	1.11×10^7
mean	Light	2.72×10^8	1.79×10^8
	Dark	1.79×10^8	6.42×10^7

Table 3.2 Summary of ANOVA statistics and interpretation for direct microscopic counts for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Effect Test	ANOVA p-value	Interpretation
1	Light/Dark	0.2369	Light = Dark
2	*no data*	*na*	*na*
3	Light/Dark	0.0018	Light < Dark
4	Light/Dark	0.0502	Light > Dark
5 (sed)	*no data*	*na*	*na*
6	+MeOH/-MeOH	0.0001	+ MeOH > - MeOH

Table 3.3 Summary of methanol transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Treatment	mg MeOH $(1x10^6 \text{ cells})^{-1} \text{ hr}^{-1}$	Standard Error
1	Light	1.70×10^{-5}	1.29×10^{-5}
	Dark	1.06×10^{-4}	8.98×10^{-5}
2	Light	2.80×10^{-5}	6.57×10^{-6}
	Dark	3.70×10^{-5}	1.13×10^{-5}
3	Light	2.45×10^{-5}	6.44×10^{-6}
	Dark	1.94×10^{-5}	2.80×10^{-6}
4	Light (+ MeOH)	7.36×10^{-6}	2.08×10^{-6}
	Dark (+ MeOH)	3.69×10^{-5}	1.19×10^{-5}
	Light (No MeOH)	*na*	*na*
	Dark (No MeOH)	*na*	*na*
5 (sed)	SO_4^{2-} Block	*no data*	*na*
	Unblocked	*no data*	*na*
6	+ MeOH	5.14×10^{-4}	6.04×10^{-5}
	- MeOH	*na*	*na*
mean	Light	1.92×10^{-5}	5.28×10^{-6}
	Dark	4.73×10^{-5}	2.30×10^{-5}

Table 3.4 Summary of ANCOVA statistics and interpretation for methanol transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Effect	ANCOVA p-value	Interpretation
1	Light/Dark	0.3308	No Difference
2	Light/Dark	0.0705	No Difference
3	Light/Dark	0.1062	No Difference
4	Light/Dark	0.1563	No Difference
5 (sed)	SO_4^{2-} Block	*no data*	*na*
6	+/- MeOH	*na*	*na*

Tab

Exp					
1					
2					
3					
4					
5					
6					

†S

Table 3.5 Summary of dichloromethane transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Treatment	μg DCM $(1x10^6 \text{ cells})^{-1} \text{ hr}^{-1}$	Standard Error
1	Light	*no transformation*	*na*
	Dark	*no transformation*	*na*
2	Light	*no transformation*	*na*
	Dark	*no transformation*	*na*
3	Light	*no transformation*	*na*
	Dark	*no transformation*	*na*
4	Light (+ MeOH)	*no transformation*	*na*
	Dark (+ MeOH)	*no transformation*	*na*
	Light (No MeOH)	*no transformation*	*na*
	Dark (No MeOH)	*no transformation*	*na*
5 (sed)	SO_4^{2-} Block	9.55×10^{-3} †	4.71×10^{-3}
	Unblocked	5.63×10^{-2} †	5.66×10^{-3}
6	+ MeOH	1.17×10^{-5}	1.25×10^{-6}
	No MeOH	6.72×10^{-6}	2.95×10^{-6}

† Sediment transformation rates expressed as μg DCM $(\text{g dry weight})^{-1} \text{ hr}^{-1}$

Table 3.6 Summary of ANCOVA statistics and interpretation for dichloromethane transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Effect	ANCOVA p-value	Interpretation
1	Light/Dark	*no transformation*	*na*
2	Light/Dark	*no transformation*	*na*
3	Light/Dark	*no transformation*	*na*
4	Light/Dark	*no transformation*	*na*
	+/- MeOH	*no transformation*	*na*
5 (sed)	SO_4^{2-} Block	0.0020	unblocked > blocked (link to SO_4^{2-} reduction)
6	+/- MeOH	0.0003	+ MeOH > - MeOH (link to MeOH metab.)

Table 3.7 Summary of toluene transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Treatment	μg TOL $(1x10^6 \text{ cells})^{-1} \text{ hr}^{-1}$	Standard Error
1	Light	3.40×10^{-5}	1.41×10^{-5}
	Dark	1.41×10^{-4}	1.22×10^{-4}
2	Light	5.81×10^{-5}	1.24×10^{-5}
	Dark	1.42×10^{-4}	1.28×10^{-4}
3	Light	8.42×10^{-6}	2.47×10^{-6}
	Dark	6.62×10^{-6}	3.30×10^{-7}
4	Light (+ MeOH)	6.57×10^{-7}	1.75×10^{-7}
	Dark (+ MeOH)	6.21×10^{-6}	3.24×10^{-7}
	Light (No MeOH)	1.32×10^{-6}	3.46×10^{-7}
	Dark (No MeOH)	7.13×10^{-6}	5.42×10^{-7}
5 (sed)	SO_4^{2-} Block	1.76×10^{-3} †	5.01×10^{-4}
	Unblocked	9.90×10^{-3} †	6.08×10^{-4}
6	+ MeOH	1.38×10^{-6}	2.26×10^{-7}
	- MeOH	7.45×10^{-6}	1.07×10^{-6}
mean	Light	2.05×10^{-5}	1.25×10^{-5}
	Dark	6.06×10^{-5}	3.69×10^{-5}

† Sediment transformation rates expressed as μg TOL $(\text{g dry weight})^{-1} \text{ hr}^{-1}$

Table 3.8 Summary of ANCOVA statistics and interpretation for toluene transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Effect	ANCOVA p-value	Interpretation
1	Light/Dark	0.6386	No Difference
2	Light/Dark	0.1653	No Difference
3	Light/Dark	0.1022	No Difference
4	Light/Dark	0.0001	Light < Dark
	+/- MeOH	0.4984	No Difference
5 (sed)	SO_4^{2-} Block	0.0007	unblocked > blocked (link to SO_4^{2-} reduction)
6	+/- MeOH	0.8249	No Difference

Table 3.9 Summary of chlorobenzene transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Treatment	μg CB $(1x10^6 \text{ cells})^{-1} \text{ hr}^{-1}$	Standard Error
1	Light	*na*	*na*
	Dark	*na*	*na*
2	Light	*na*	*na*
	Dark	*na*	*na*
3	Light	7.90×10^{-6}	3.76×10^{-6}
	Dark	1.09×10^{-5}	6.91×10^{-7}
4	Light (+ MeOH)	1.28×10^{-7}	8.30×10^{-8}
	Dark (+ MeOH)	2.77×10^{-6}	5.29×10^{-7}
	Light (No MeOH)	5.94×10^{-7}	2.62×10^{-6}
	Dark (No MeOH)	4.17×10^{-6}	1.10×10^{-6}
5	SO_4^{2-} Block	*no transformation*	*na*
	Unblocked	*no transformation*	*na*
6	+ MeOH	1.45×10^{-6}	6.48×10^{-7}
	No MeOH	4.00×10^{-6}	1.10×10^{-6}
mean	Light	2.87×10^{-6}	2.18×10^{-6}
	Dark	5.95×10^{-6}	2.17×10^{-6}

Table 3.10 Summary of ANCOVA statistics and interpretation for chlorobenzene transformation rates for all secondary metabolism experiments with attached microbial communities from the Pharmacia/Upjohn Pond.

Expt. #	Effect	ANCOVA p-value	Interpretation
1	Light/Dark	*na*	*na*
2	Light/Dark	*na*	*na*
3	Light/Dark	0.0202	Light < Dark
4	Light/Dark	0.0054	Light < Dark
	+/- MeOH	0.6144	No Difference
5 (sed)	SO_4^{2-} Block	*no transformation*	*na*
6	+/- MeOH	0.2009	No Difference

3.6 Microbial Community Structure (FAME Analysis)

Microbial community structure was analyzed for secondary metabolism experiments #2 through #4 according to the FAME technique described in section 2.2. Resulting FAME biplots (see section 2.2.7) are presented in Figures 3.31-3.33. LIGHT and DARK treatments are clearly well-resolved in all experiments, with approximately 80% of variance explained in the first 3 dimensions in each experiment and a low degree of variability between replications. Resolution of treatments is based primarily on fatty acids of known bacterial origin; i.e. the heterotrophic portion of the microbial community.

Interpretation of which fatty acids are most important in each treatment is more difficult. Temporal variation was large and no consistent or distinguishable patterns are evident in degree of saturation or chain-length with respect to treatment or biofilm developmental state. The single exception may be longer chain fatty acids, which tend to be found in greater abundance in the LIGHT treatment, expectedly indicating a stronger presence of eukaryotes.

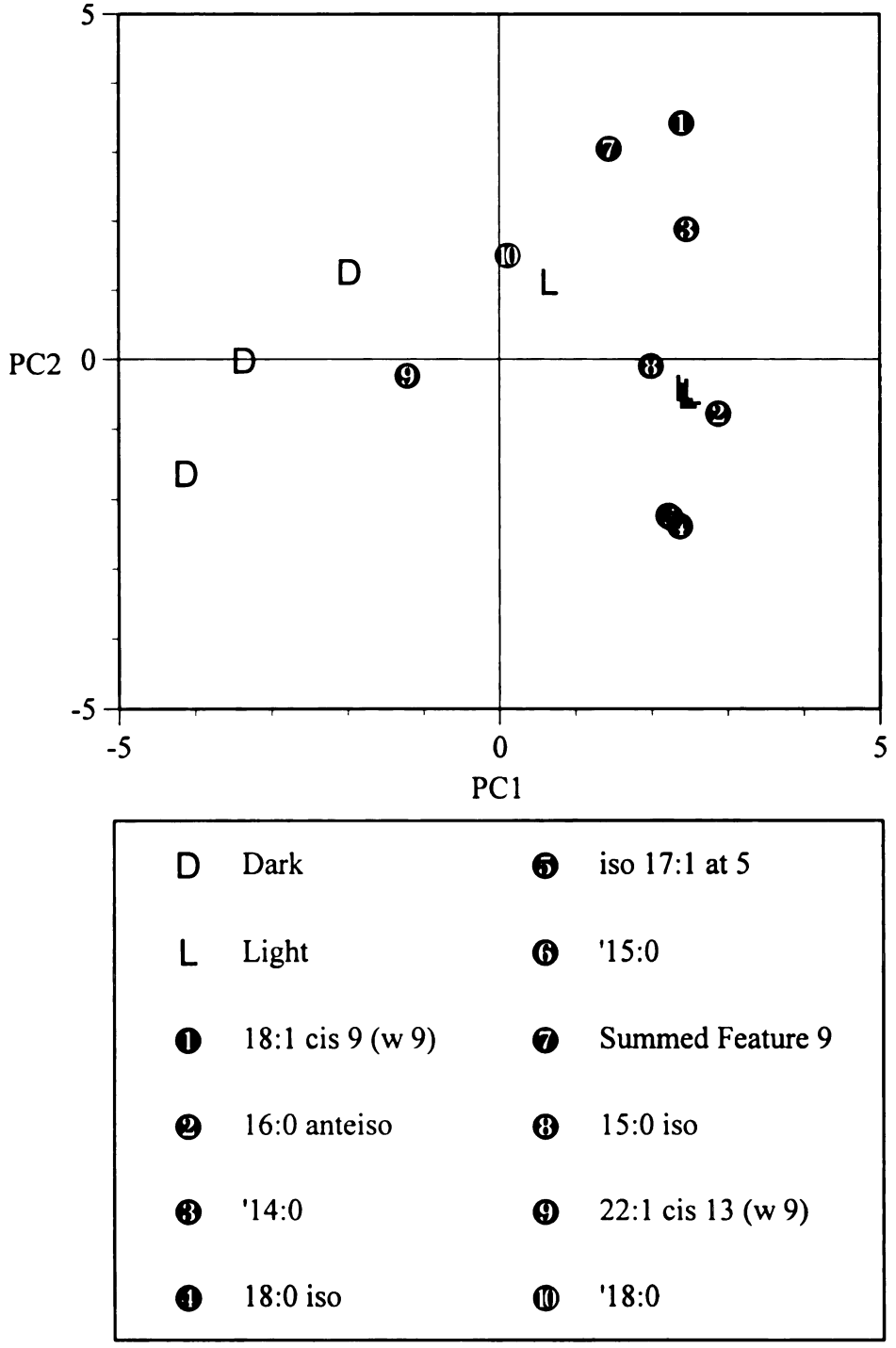

Figure 3.31 Fatty acid methyl ester (FAME) biplot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #2.

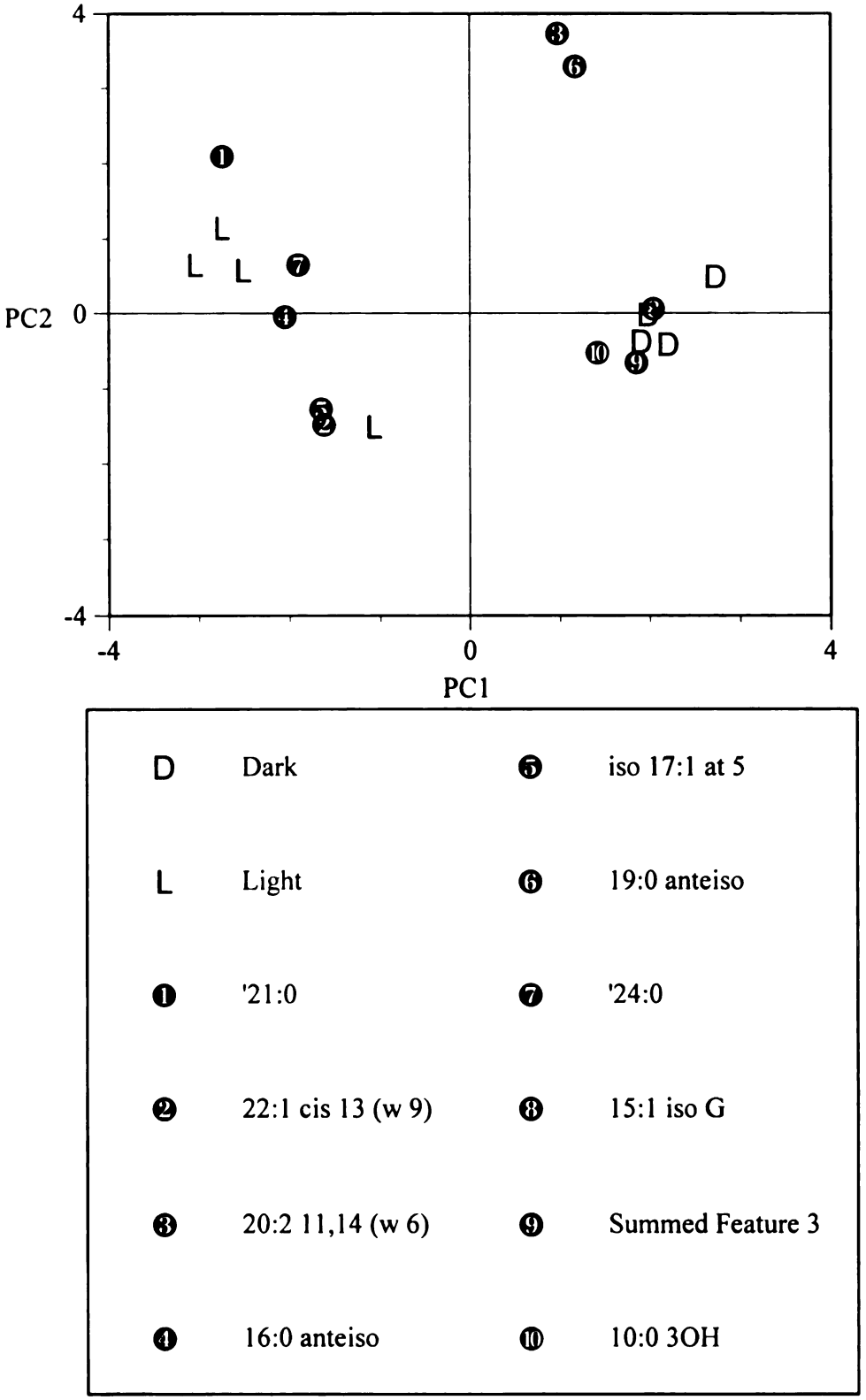

Figure 3.32 Fatty acid methyl ester (FAME) biplot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #3.

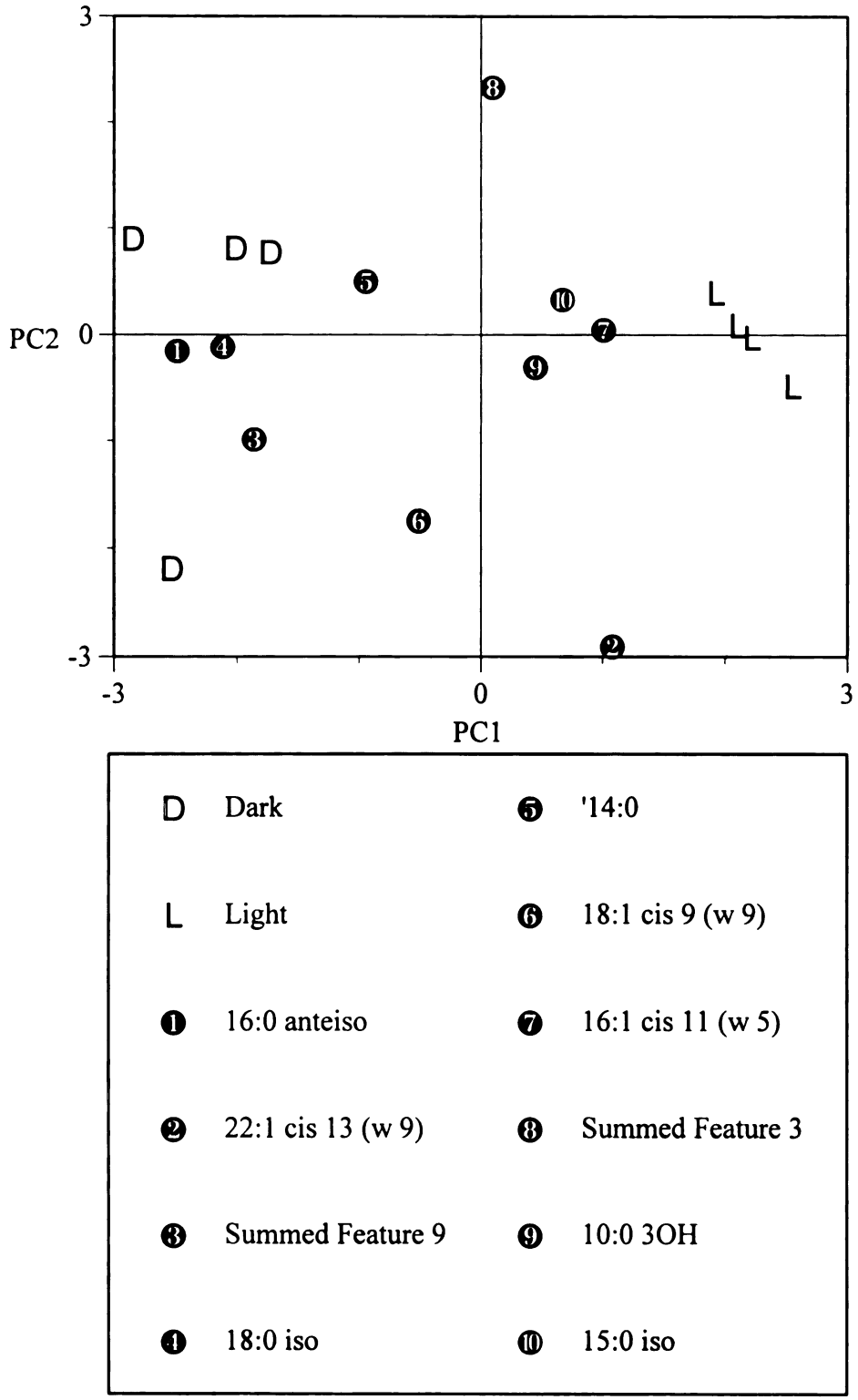

Figure 3.33 Fatty acid methyl ester (FAME) biplot for attached microbial communities from two light treatment regimes in the Pharmacia/Upjohn Pond, Experiment #4.

3.7 Discussion

3.7.1 Microbial Community Structure

Results for direct microscopic counts in the light manipulation experiment were highly temporally variable, with statistical interpretation ranging the extremes: no difference between treatments, DARK significantly more populated than LIGHT, and LIGHT significantly more populated than DARK. Examining the trends in the data from experiments #1 through #4 reveals that direct microscopic counts increase in both treatments with increasing time of biofilm development. The heterotrophic populations of both biofilms increased at an equal rate during early development. As the biofilms matured through early summer, the DARK bacterial populations increased more quickly than the LIGHT populations. As the summer light intensity increased and temperatures became elevated, the productivity in the LIGHT biofilms resulted in rapid increases in heterotrophic population numbers.

Although the trends in the data suggest that sessile primary productivity increases heterotrophic population numbers, the effect is likely seasonal and dependent upon biofilm developmental state. Thus, the overall interpretation on an experiment-wide basis would be a lack of significant difference between treatments, and no support for the hypothesis that sessile primary productivity stimulates heterotrophic population numbers.

Even though the treatments did not vary significantly in overall bacterial numbers, the FAME analyses demonstrated that the heterotrophic communities are indeed structurally or phenotypically different between treatments. Although FAME analysis is

often associated with community structure, interpretation is actually based on a set of phenotypic markers. These phenotypic markers are produced as part of the basic physiology of the microbial species in a given sample. Differences between whole-community microbial FAME profiles may be due to either a true structural difference in species distribution or a difference in physiology among similar species distributions, or some combination of the two. Therein lies a paradox in FAME interpretation; purely structural differences are confounded by physiological differences induced by the very treatments being resolved.

Therefore, the strong resolution between LIGHT and DARK treatments may be due to differences in species distribution, physiology, or both. The secondary metabolism studies demonstrated physiological differences in bioremediation activity between LIGHT and DARK biofilm communities, supporting the hypothesis that sessile primary productivity influences bioremediation activity (physiology). However, differences in eukaryotic species distribution (the presence of algae and diatoms in the LIGHT communities) were also verified by visual microscopic observation and demonstrated by the strong weighting of several eukaryotic marker acids in the LIGHT samples. Several heavily weighted fatty acids are known prokaryotic markers as well, but interpretation is confounded regarding whether these differences among heterotrophs are due to changes in physiology or species distribution. Whole-community FAME interpretation is inherently restricted to concluding that either the communities are different or similar, necessitating the use of complementary physiological, microscopic, and genetic analyses to draw more detailed conclusions.

3.7.2 Primary Methanol Metabolism

LIGHT and DARK communities were not significantly different in their ability to oxidize methanol in the ^{14}C-methanol experiment or in any of the four non-radiolabelled methanol metabolism studies. Because the communities were shown to be structurally different by FAME analysis, one conclusion is that some degree of functional redundancy exists with respect to methanol oxidation. Since the prevailing contaminant loading conditions dictate that methanol comprises a significant fraction of total DOC in the Pond, functional redundancy would be expected for methanol oxidation activity.

A wide range of cosmopolitan and specialized methanol degraders are reported in the literature (see Table 1.1) and would be expected to occur in a heterogeneous system such as the Pharmacia/Upjohn Pond. Functional redundancy is thought to be a stabilizing feature among microbial communities, in this case ensuring that methanol-oxidizing populations are present under a host of temporally variable environmental conditions. The current study resulted in isolation of several different types of methanol-oxidizing bacterial isolates from epiphytic biofilms in the Pond, demonstrated a significantly greater abundance of methylotrophic organisms in these biofilms, and recorded intense methanol oxidation activity in the biofilms and sediment. These results collectively suggest that methanol is a dominant primary growth substrate.

3.7.3 Secondary Metabolism

Biofilms from both light manipulation treatments rapidly transformed the aromatic secondary compounds toluene (TOL) and chlorobenzene (CB). Because several cosmopolitan organisms are known to metabolize TOL, secondary transformation activity was expected in both light treatments, but the original hypothesis predicted that sessile primary productivity would stimulate heterotrophic activity and result in more rapid secondary transformation of TOL in the LIGHT treatment. Contrary to the original hypothesis, no statistically significant differences were detected between LIGHT and DARK treatments for secondary transformation of TOL, leading to several possible conclusions.

One or more cosmopolitan microbial species may be ubiquitously and evenly distributed between treatments and functioning equally well for TOL transformation in either light regime, in which case the differences detected by FAME profiles are due to some other fraction of the community. Alternately, the communities in each treatment may have different microbial species distributions, but the various species are functioning equally in total, in which case the differences detected by FAME profiles are due in part to differences in structure among the TOL-oxidizing communities. In either case, definitive conclusions cannot be made without additional research, perhaps involving the use of genetic probes for different groups of TOL oxidizers.

CB transformation was consistently and significantly higher in the DARK communities, again contradicting the hypothesis that sessile primary productivity promotes bioremediation activity. Given that halogenation generally renders a compound

more recalictrant, only specialized microbial populations have the enzymatic capability to compensate for the chloro-substituent on the aromatic ring. Generalist heterotrophs stimulated by sessile primary productivity in the LIGHT treatments may be less likely to express energetically unfavorable dechlorination activity in lieu of large pools of readily labile carbon exudates. Conversely, microorganisms growing in the DARK treatment without the luxury of labile carbon exudates may be forced to express more exotic enzymatic pathways to extract energy for growth from less labile substrates, such as CB.

Three aliphatic secondary compounds known to sporadically appear in the pond influent (dichloromethane [DCM], 1,2-dichloroethane [DCA], and bromodichloromethane [BDCM]) were not transformed under aerobic conditions during the course of the initial studies. In fact, DCA could potentially present a surface or groundwater quality problem due to a complete lack of aerobic transformation and only very slow anaerobic transformation. BDCM was not transformed, but its sorption affinity to sediments and biofilm matrixes made analyses problematic. Further experimentation is warranted to determine the sorption affinities for BDCM, the adsorption capacities of the matrixes, and the availability of adsorbed BDCM. Sorption of organics is thought to increase their availability to microorganisms and lead to accumulation in the matrixes, which can elevate concentrations from the level of secondary substrates to above the primary substrate threshhold.

The lack of secondary aerobic DCM transformation was unexpected, because some methylotrophs are known to express dichloromethane dehalogenase acitivity. This enzyme dechlorinates DCM to form formaldehyde, which is then shunted directly into the

methanol oxidation and assimilation pathways. DCM transformation was only observed in pre-incubated DARK biofilms that had been exposed to constant methanol concentrations of 100 mg L^{-1} for a 3-week period. Several explanations are possible for this phenomenon, but selection or induction are the most likely scenarios.

The selective pressure of the laboratory incubation conditions may have increased the numbers of a specific population to a level where activity was measureable. Alternately, the constant influx of methanol could have induced auxiliary enzymatic pathways that are not normally expressed under the pulse-loading conditions of the Pharmacia/Upjohn Pond, resulting in a functional shift in the microbial community rather than a structural shift. Poor FAME data for the methanol exculsion experiment precluded us from conclusively determining whether the cause of the induced DCM activity was due to structural changes in the communities during incubation. Further explanation of the relationship between primary methanol metabolism and secondary DCM transformation is given in section 3.7.5

3.7.4 Anaerobic Metabolism

The energetically preferred order of electron acceptors for microbial respiration is oxygen, nitrate, iron/manganese, sulfate, and carbon dioxide. Oxygen is depleted rapidly at or above the sediment water interface in the Pharmacia/Upjohn Pond, necessitating the use of alternate electron acceptors for microbial respiration in the surficial sediments. Nitrate concentrations in the Pond influent are too low (less than 10 $\mu g\ L^{-1}$) to contribute substantially to the pool of alternate electron acceptors. Iron concentrations are elevated, but the fraction of iron in the Pond that is microbially reducible has not been conclusively

determined and appears to be relatively low. Thus, the availability of alternate electron acceptors in the sediment is restricted primarily to sulfate.

Concentrations of sulfate in the Pond influent (60 mg L^{-1}) are sufficient to contribute significantly to the pool of alternate electron acceptors in the Pond. The high volume of flow through the system constantly replenishes the sulfate pool and results in advective flow of sulfate into the sediments via high infiltration rates. New research also suggests that sulfates may be regenerated in sediments by oxidation of reduced sulfides, further contributing to the available sulfate pool. Iron is hypothesized to synergistically contribute to the rate of sulfate reduction via the intermediary binding of iron to reduced sulfides, pulling equilibrium towards sulfate reduction. The elevated iron concentrations in the Pond and the release of hydrogen sulfide from sediments treated with 6 N hydrochloric acid both provide evidence of this intermediary binding phenomenon. For these reasons, sulfate is hypothesized to be the principal alternate electron accepting process in the Pharmacia/Upjohn Pond sediments. Previous research has conclusively validated this hypothesis for anaerobic oxidation of methanol (Klug *et al.*, 1996).

Methanol, dichloromethane (DCM) and toluene (TOL) were rapidly transformed anaerobically in the present studies. Anaerobic secondary transformation of DCM and TOL was significantly linked to sulfate reduction, suggesting that secondary metabolic processes may also be governed by sulfate reduction in the sediments. Very slow anaerobic transformation was noted for 1,2-dichloroethane (DCA) and no activity was observed for chlorobenzene (CB). Sorption to sediment made analysis of bromodichloromethane (BDCM) problematic. The aerobic and anaerobic biofilms act in

concert to provide complete transformation of the methanol, DCM, CB, and TOL entering the system.

3.7.5 Link Between Methanol and Secondary Metabolism

Methanol is a principal component of influent DOC in the Pharmacia/Upjohn Pond system. Evidence from previous research (Klug *et al.*, 1996) and the present studies suggests that methanol is an important primary growth substrate for aerobic biofilms in the Pond. A key hypothesis was that primary metabolism of methanol was sustaining microbial growth in the biofilms and driving secondary substrate utilization. The inability to detect a link between primary methanol metabolism and secondary substrate utilization in experiment #4 was likely due to the confounding influence of *in situ* enzyme expression; i.e. active biofilms continued to express secondary metabolic pathways even after methanol was removed in the laboratory. Thus, the methanol exclusion pre-incubation experiment was devised to force deactivation and/or induction of methanol-linked enzymatic pathways.

The results from experiment #6 support the hypothesis of primary methanol oxidation driving secondary transformation of TOL and DCM. The induction of DCM activity by pre-incubating with a constant source of methanol suggests that the sporadic nature of methanol loading in the Pond may negatively impact DCM secondary activity. A constant source of methanol may have led to preferable population selection, induction of desired secondary metabolic pathways, or a combination of both. Unfortunately, poor FAME data prevented a decisive conclusion regarding whether the induced DCM activity was associated with a shift in bacterial species distribution. Again, genetic probes may be

a more valuable tool for detecting changes in microbial community structure, particularly in the case of methylotrophs, for which an extensive library of probes has been developed.

Secondary transformation of CB was not shown to be linked to methanol oxidation, indicating that either an unrelated (non-methylotrophic) population is responsible for the activity or that the enzymatic pathways for CB transformation are constituitively induced in cosmopolitan heterotrophs also capable of growth on methanol.

3.8 Summary and Conclusions

The oligotrophic comparison study demonstrated that the basic inorganic water chemistry of the Pharmacia/Upjohn Pond is comparable to other natural oligotrophic lake systems in the region. The surface water outlet of the Pond system seems to be converging chemically and biologically toward natural lake systems. The high proportion of methanol in the influent DOC has impacted microbial community structure of epiphytic biofilms near the pond inlet by increasing methylotrophic organisms by more than two orders of magnitude above natural lake systems. However, FAME profiles revealed that the specialized epiphytic biofilms near the Pond inlet are similar in overall microbial community structure to those biofilms found in natural oligotrophic lakes.

High methanol oxidation rates were found in aerobic plant-associated biofilms and in anaerobic sediments. Methanol metabolism in sediments was also linked to sulfate reduction. The present study strengthened the rate estimates for methanol metabolism (Klug *et al.*, 1996), surveyed the extent of secondary substrate transformation, examined the link between primary methanol utilization and secondary transformation, developed

rate estimates for secondary transformations under aerobic and anaerobic conditions, and explored the feasibility of artificially augmenting surface area for attached microbial growth.

Biofilms that developed in the absence of light on artificial surfaces were equally adept at transforming methanol and several secondary substrates (dichloromethane, toluene, and chlorobenzene) as those biofilms grown under natural diurnal light. DARK biofilms exhibited significantly higher secondary transformation rates for chlorobenzene. These results suggest that surface area augmentation, in which most interior surfaces are devoid of light, may be a feasible option for improving the bioremediation capacity of the system.

Dichloromethane was aerobically transformed only after a 3-week pre-incubation with methanol, either due to stimulation of enzymatic pathways or selection of desired microbial populations. Primary methanol metabolism was significantly linked to secondary transformation of dichloromethane, but did not directly impact secondary transformation of toluene or chlorobenzene. Secondary transformation of dichloromethane gradually diminished upon depletion of the primary substrate. Future experiments should examine this relationship in greater detail through a series of methanol additions and depletions to determine if the pattern is repeatable.

Direct microscopic counts of bacterial numbers varied seasonally and with biofilm developmental state, but were not significantly different overall between treatments. Microbial community structure as determined by fatty acid methyl ester (FAME) profiles was well-resolved between treatments, but the precise nature of the differences remains to

be determined. More detailed microbial community structural analysis is warranted, given the lack of sensitivity and inconclusive results with FAME profiles. Genetic probes may provide greater insights into the various types of methylotrophic organisms responsible for primary and secondary bioremediation activity in the system.

Previous and current data collectively suggest that the Pharmacia/Upjohn Pond system is a feasible site for *in situ* bioremediation. Attached microbial communities rapidly metabolize methanol and several secondary contaminants in the cooling water discharge to the pond. Biofilm communities near the pond inlet are clearly well-adapted to primary methanol metabolism, as evidenced by significantly elevated numbers of methylotrophic organisms and rapid methanol oxidation rates. Secondary transformation has been linked to primary methanol metabolism, suggesting that a continuous influx of methanol at part-per-million levels may actually be beneficial to the system by maintaining elevated numbers of adapted organisms while simultaneously stimulating secondary enzyme activity. Anaerobic pond foster extensive attached methylotrophic populations that further contribute to overall system transformation capacity. Anaerobic dechlorination activity may synergistically promote further aerobic transformation of specific halogenated secondary compounds such as CB among spatially heterogeneous biofilm and sediment communities. The capability of methylotrophic populations to function effectively under non-oxygenic conditions on plastic surfaces suggests that artificial surface area augmentation may be feasible as a means to increase system capacity.

Future research to further parameterize the system would be a critical step toward developing a mathematical dispersion model. Considerations in such a model would be

seasonal variation in temperature and oxygen, mass transfer to air and sediment, advective forces, and verification of the kinetic order and rates of metabolic reactions. When a complete array of system parameters has been established, elemental mass balance equations would allow prediction of system capacities under virtually any loading and environmental scenario. Only then would more definitive conclusions regarding system efficacy be possible.

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