PERFORMANCE, MICROBIAL ECOLOGY, AND LIFE CYCLE ASSESSMENT OF AN ACTIVATED CARBON BIOFILTER FOR METHANOL REMOVAL

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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To my grandmother, Mary Lou Whitfield, who inspires my love of learning and appreciation of the past; and to my daughter Nora Caroline Babbitt, who inspires me to do my part to create a more sustainable world for future generations.

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The forest products industry is responsible for producing valuable industrial chemicals, wood products, and consumer goods. However, processes involved in creating these materials at pulp, paper, and paperboard mills also generate hazardous air pollutants (HAPs), such as methanol, that are released during wood pulp production. With increasingly stringent regulations on methanol emissions, mills are continually seeking effective and cost efficient ways to control its release. Motivated by the need to study economical and environmentally friendly methanol control technologies, a bench-scale activated carbon biofiltration system was developed and evaluated for its ability to remove methanol from an artificially contaminated air stream. The biofilter contained a novel packing mixture of activated carbon, perlite, slow release nutrient pellets, and water retaining crystals, and showed excellent biofilm growth and close to 100% biological methanol removal, both with and without addition of an inoculum containing enriched methanol-degrading bacteria.

Design of the biofilter using an inoculum enriched for methanol-degrading bacteria also facilitated characterization of biofilm samples from a pulp and paper mill on the basis of selecting a biofilter inoculum and optimizing growth and activity in mixed culture. Studies of

enriched cultures from the biofilm samples showed higher bacterial community diversity and methanol removal when using nitrate as the nitrogen source for enrichment, rather than ammonium.

Design and operation of this bench-scale system also enabled further investigation with microbial ecology and molecular techniques to characterize diversity of bacterial communities colonizing the biofilter over different points in time and under varied operational conditions. Amplification and separation of DNA from biofilter samples, using polymerase chain reaction (PCR) and denaturing gel gradient electrophoresis (DGGE), indicated that although bacterial diversity and abundance varied over the length of the biofilter, the populations rapidly formed a stable community that was maintained over the entire 138 days of operation and in variable operating conditions. Phylogenetic reconstruction of bands excised from DGGE gels indicated that the biofilter supported a diverse community of methanol-degrading bacteria.

Finally, the design and operation of the bench-scale biofilter provided parameters for use in a life cycle assessment (LCA) that compared raw materials and energy required and emissions and environmental impacts produced by construction and operation of a proposed photocatalytic oxidation (PCO)-biofilter system, to those associated with treatment using a more traditional regenerative thermal oxidizer (RTO). LCA results indicated that environmental impacts associated with construction of a RTO far outweighed infrastructure requirements of the PCO-biofilter system. However, the operating impacts to global warming and human toxicity for the PCO-biofilter system were higher than for the RTO, because of the replacement requirements of packing for the PCO reactor and biofilter, as well as the electricity requirement to operate the PCO reactor.

CHAPTER 1
INTRODUCTION

Background and Significance

The forest products industry, specifically pulp and paper mills, produces a variety of industrial and consumer products from wood cellulose, including paper, paperboard, infant, female, and adult hygiene products, high porosity filters, food casings, rayon filament, and chemicals such as ethers and acetate. Pulp required for these products is produced by physical processes that convert whole trees to wood chips and chemical processes that digest the chips to produce either a brownstock or bleached final pulp. Throughout many of these processes, organic compounds that are naturally present in wood or produced during the degradation of cellulose are released to the environment.

In 1998, the U.S. Environmental Protection Agency (U.S. EPA) passed a regulation known as the "Cluster Rule," which regulates the release of these compounds to both the air and the water (U.S. EPA 1998). Air emissions included in the Cluster Rule that present established or potential impacts to human or environmental health are known as hazardous air pollutants (HAPs). The HAP of greatest concern is methanol, which represents over 70% of the total release of HAPs from this industry, amounting to over 44,000 tons each year. (U.S. EPA 2004).

Released in this quantity, methanol can contribute to human health impacts, such as cancer, respiratory irritation, and damage to the nervous system (U.S. EPA, 1998). Other emissions include acetaldehyde, formaldehyde, methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), α- and β-pinene, and reduced sulfur species including dimethyl sulfide, dimethyl disulfide, and methyl mercaptan, which are collectively termed total reduced sulfur (TRS). To prevent potential environmental and health impacts from these emissions, the Cluster

Rule includes a section specifying the maximum available control technology (MACT) required to limit the amount of HAPs emitted.

MACT regulations require that pulp and paper mills collect and treat non-condensable gas from high volume low concentration sources (HVLC). HVLC gases are those with large volumes (typically 10,000-30,000 acfm for an entire mill) and relatively dilute compound concentrations (below the lower explosion limit of the gas mixture) and are emitted from pulp washing systems, oxygen delignification systems, deckers, knotters, and black liquor storage tanks, etc. (Varma 2003). MACT regulations give pulp and paper mills flexibility of complying with emission limits with any technology proven to perform to at least the minimum requirements of the emission standards (98% methanol removal) (U.S. EPA 1998). Most mills plan to comply with MACT by collecting the gases in vent hoods, using fans or blowers to transport the gases, and eliminating the HAPs by combustion in either an existing power boiler or lime kiln or in new stand-alone thermal oxidizers (Varma 2003). Albeit effective, thermal oxidation has the drawbacks of requiring a constant input of natural gas or other fuel supply to support incineration and increasing the emissions of carbon dioxide (CO_2), sulfur dioxide (SO_2), and nitrogen oxides (NO_x). This technology also has very high capital costs, because of the need to install ductwork and other infrastructure for gas collection and transport. Therefore, a more environmental and economical solution is desired for HAPs control for pulp and paper industry.

One technology proposed as an alternative to thermal oxidation is a photocatalytic oxidation (PCO) reactor containing a packed bed of a composite material of silica and the photocatalyst titanium dioxide (TiO_2) (Stokke et al. 2006), in which the silica provides a catalyst support and adsorbs HAPs from the air stream while the photocatalyst promotes destruction of these compounds when exposed to UV light. However, when the reactor is not functioning with

maximum efficiency or if it is designed to remove less than 100% of incoming contaminant, the HAPs will not be completely eliminated from the gas stream, necessitating a secondary or "polishing" treatment step. This secondary system would consist of a fixed bed granular activated carbon (GAC) biofilter, although such a biofilter may also serve as a primary treatment system.

GAC is a highly porous adsorbent material that removes contaminants by nature of van der Waals interactions between the contaminants and the carbon surface when they are in intimate contact (Dabrowski 2000). Activated carbon is produced from a carbonaceous material (typically wood, coal, or biomass residue) that is carbonized in the absence of oxygen and activated by either a chemical or physical process that increases the porosity and changes the surface chemistry to enable high levels of adsorption of target contaminants (Menéndes-Diaz and Martin-Gullón 2006). Adsorption is a finite process, however, with a physical limit to the extent activated carbon can be used before its capacity is exhausted. One means of extending the GAC service life and the economic advantage of this technology is the incorporation of biological activity to create a synergistic process by which the GAC adsorbs VOCs from the air stream, creating a favorable environment for the formation and maintenance of a stable microbial biofilm that subsequently degrades adsorbed and incoming organics (Aizpuru et al. 2003; Chang and Rittman 1987; Herzberg et al. 2003; Hodge and Devinny 1994; Weber and Hartmans 1995; Zhang et al. 1991). Considering the nature of the pulp and paper mill system, specifically warm temperatures, high humidity, and predominance of one-carbon compounds, biofilm formation, growth, and activity are expected to be inherent to the system.

Based on the design objective to treat low or fluctuating concentrations of VOCs in large volumes of gas (Kennes and Veiga 2001) and the expected availability of biological inoculum

inherent to the pulp mill environment, an activated carbon biofilter would be an ideal solution for the polishing step of the methanol removal train. A biofilter has traditionally been characterized as having a fixed-bed containing inert organic packing that serves as a carrier for biomass and a nutrient source, where contaminants in polluted air are degraded by the active biomass and where no mobile liquid phase is present (Devinny et al. 1999; Kennes and Thalasso 1998; Kennes and Veiga 2001). This definition has been expanded in recent research to include surface-active packing that provides adsorption capacity (e.g., for buffering peak loads or process instabilities) as well as inorganic packing with discontinuous aqueous nutrient addition (Aizpuru et al. 2003; Prado et al. 2002; Teran-Perez et al. 2002; Yang et al. 2002). Use of activated carbon as the primary biofilter packing material (not just as inorganic support for compost or other organic packing) falls into both of the latter categories.

Research Context

A great deal of literature exists that addresses the design, operation, and performance of biofilters for removal of contaminants from gaseous effluent streams (e.g., Devinny et al. 1999; Kennes and Thalasso 1998; Kennes and Veiga 2001). In many of such studies, a drop in a biofilter's performance has been usually hypothesized to be due to factors such as excess or limited moisture, nutrients, or substrate, pH excursions, or biomass buildup or clogging (e.g., Gribbins and Loehr 1998; Jin-Ying et al. 2005; Teran-Perez et al. 2002; Yang et al. 2002). However, a disconnect exists in the literature between hypotheses about bacterial causes of reduced performance and actual observations of the bacterial systems or measurements of bacterial abundance or diversity. In addition, when inorganic material such as activated carbon is used for biofilter packing, it is common to inoculate the system with biological material able to grow and degrade contaminants under the conditions expected in the treatment train, including

particular pH, temperature, and substrate concentration values (Devinny et al. 1999) Often, these criteria aren't explicitly investigated, but, rather, inoculum is derived from enrichment of available activated sludge or other waste materials (Aizpuru et al. 2003; Moe and Qi 2005), obtained from prior lab studies (Herzberg et al. 2003; Thalasso et al. 2000), or added as pure cultures (Speitel and McLay 1993). There is little known about how the microbial populations used as inoculum actually influence the biofilter performance or the ultimate structure of the biofilm colonizing the packing media.

Fortunately, more recent work has begun to focus on the relationships between biofilter performance and biofilm properties, including distribution, activity, and kinetics of the attached biofilm (Song and Kinney 2000; Veiga et al. 1999). These studies and others focusing on the bacterial abundance and activity in biofilters (Acuna et al. 1999; Aizpuru et al. 2003) used traditional culture-dependent techniques to enumerate bacteria present in the biofilm and correlate those results with phases of operation and performance. The major limitation to such culture-dependent techniques is their potential bias to those organisms which grow fastest under lab conditions and their inability to adequately represent all microorganisms present (Hugenholz 2002).

Therefore, molecular techniques have been increasingly used due to more readily available 16s rRNA sequences for comparison purposes (Clarridge 2004) and ever-improving methods for extracting, amplifying, and sequencing DNA directly from an environmental sample with no culturing required (Torsvik and Ovreas 2002). Examples of these methods include phospholipids fatty acid (PLFA) analysis, denaturing (or temperature) gradient gel electrophoresis (DGGE/TGGE), terminal restriction length polymorphism (TRFLP), or ribosomal intergenic spacer analysis (RISA) (Torsvik and Ovreas 2002).

DGGE in particular is a molecular method that is becoming very popular for investigating bacterial diversity and community structure in environmental and applied biological systems. DGGE is an approach used to determine the genetic diversity of mixed microbial populations by electrophoresis of PCR-amplified DNA in a polyacrylamide gel with a linear gradient of denaturant (Muyzer et al. 1993). This approach has been popularized recently due to the reported ability to separate DNA fragments of the same length but slightly different sequences (e.g., single base changes, Myers et al. 1987), as based on the relative electrophoretic mobility associated with the melting point of a given DNA sequence (Muyzer and Smalla 1998). The resulting banding pattern, when the gel is visualized using UV light, provides an illustrative comparison of frequency and presence or absence of banding patterns for different conditions investigated. DNA fragments associated with a banding pattern of interest can be excised from this gel, re-amplified, and sequenced to assess mutations or presence of specific bacterial strains, or the banding patterns can be mathematically assessed to determine relative changes in the diversity or "phylogenetic richness" (Ogram and Sharma 2002) to increase understanding of the microbial ecology of a system. For example, Li and Moe (2004) used DGGE to evaluate the spatial structure throughout the length of two types of biofilters treating methyl ethyl ketone to explain the superior performance of one configuration over another. Others apply this method to determine the acclimation or stability of cultures in biological treatment systems over time (Labbé et al. 2003; Rombaut et al. 2001). Another approach has been to compare banding patterns generated from amplified sequences from functional genes to determine the relative abundance or dominance of specific types of bacteria, such as different types of methanotrophs (Fjellbirkeland et al. 2001; Henckel et al. 1999) or denitrifiers (Goregues et al. 2005). Takaku et al. (2006) were able to relate significant shifts in bacterial community structure, as assessed by

DGGE, to distinct temperature changes in their system, while Sercu et al. (2005) compared attached biofilm diversity in a biotrickling filter to bacterial communities in the planktonic state.

Even with these successes, there are also several limitations to the use of DGGE as a microbial ecology tool, namely the limitation to DNA fragments of about 500 bp or less, the production of anomalous PCR products from incorporating a GC clamp into primers, and potential difficulty interpreting or comparing results (Gilbride et al. 2006; Bruns et al. 1991; Muyzer and Smalla 1998). In addition, the most common approach is to use 16S rRNA gene sequences for amplification, giving an idea of diversity of all bacteria present. Other than a small amount of work done to target specific bacterial groups, as discussed above, the use of functional genes has been much more limited for a combined PCR-DGGE approach. Nevertheless, use of molecular methods such as DGGE can potentially offer a more detailed insight into the microbial ecology of biological systems, such as biofilters, than possible with performance measurements or culture-dependent studies alone.

Research Goals and Objectives

This research was motivated by the need to investigate a more economical and environmentally friendly methanol control technology for the pulp and paper industry, such as an activated carbon biofiltration system. With this goal in mind, the research reported herein focused on developing and testing a bench-scale activated carbon biofiltration system capable of removing methanol from an artificially contaminated air stream in concentrations representative of industrial processes. Operation of this bench-scale system enabled further investigation with four specific objectives:

1. Collect and characterize biological samples from the pulp and paper industry on the basis of selecting a biofilter inoculum and optimizing their growth and biodegradation in mixed culture using different nitrogen sources and concentrations (Chapters 2 and 3);

2. Measure the methanol removal efficiency of the bench-scale biofilter containing a novel heterogeneous packing material comprised of granular activated carbon, perlite, slow-release nutrient pellets, and water-retaining crystals (Chapter 4);

3. Use selected culture-dependent and independent microbial ecology and molecular techniques, such as DGGE, to characterize the diversity of bacterial communities colonizing the biofilter over different points in time, under varied operational conditions, and at different spatial points in the biofilter (Chapter 4); and

4. Perform a life cycle assessment (LCA) to compare raw material and energy requirements and emissions and environmental impacts of the proposed photocatalytic oxidation reactor and biofiltration system to those associated with traditional treatment systems, specifically regenerative thermal oxidation and wet scrubbing (Chapter 5).

A more thorough understanding of the biofilter technology as part of a novel methanol treatment train promises to yield significant environmental and economic savings for the pulp and paper industry, as well as other industry sectors that are challenged with controlling HAPs and other volatile organic compounds (VOCs) in their process streams.

CHAPTER 2
INITIAL PHENOTYPIC CHARACTERIZATION OF METHYLOTROPHIC MIXED CULTURES FROM PULP AND PAPER MILL BIOFILMS

Introduction

Use of a biological system, such as a biofilter, for treating air pollutants requires the presence of active microbial consortia that are capable of degrading contaminants of interest. When inorganic material such as activated carbon is used, inoculation with enrichments targeted for degradation of known concentrations of contaminants under specific temperatures and pH, may be necessary (Devinny et al. 1999). Often, these criteria are not explicitly investigated, but, rather, inoculum is derived from enrichment of available activated sludge or other waste materials (Aizpuru et al. 2003; Moe and Qi 2005), obtained from prior lab studies (Herzberg et al. 2003; Thalasso et al. 2000), or added as pure cultures (Speitel et al. 1993).

Pulp and paper mills possess environments expected to be favorable for microbial growth, due to warm, moist conditions and the presence of readily degradable organic substrates. In fact, overabundance of biofilms or "slimes" at these mills often creates operational problems (Lahtinen et al. 2006), yet such biofilms could make ideal inocula for a biological treatment system. This chapter reports an initial characterization of biofilm samples collected from various locations at a southeast pulp, paper, and paperboard mill that uses a biological wastewater treatment system. Traditional culture-dependent characterization methods were used to evaluate and select potential inocula for subsequent biofiltration treatment for methanol removal from air.

Methods

Sample Collection

Seven grab samples of biofilm samples collected from this mill were collected during June 2004, from locations believed by mill staff to be representative of methanol-degrading

consortia or having a high number of bacteria present and stored on ice in sterilized Teflon collection vessels until they could be processed in the lab or stored over a longer term at 4 deg. C. The locations of these samples, identified as Sample A-G (SA-SG) are as follows:

Sample A (SA): Vent tubes of an oxygen activated sludge "UNOX" reactor
Sample B (SB): Return activated sludge (exiting secondary clarifiers)
Sample C (SC): Wet material from cooling tower baffles
Sample D (SD): Partially dry material from cooling tower baffles
Sample E (SE): Wood wall outside of the cooling tower
Sample F (SF): Secondary clarifier weirs
Sample G (SG): Mixed liquor exiting the UNOX reactor

The wastewater treatment system and selected sites are shown in Figure 2-1.

Mixed Culture Enrichment

The samples collected were enriched in the laboratory in batch culture in a modified nitrate mineral salts medium (NMS) containing 0.2% methanol (vol/vol) as recommended by Hanson (1998). The basal medium contained, on a g/L basis: $MgSO_4*7H_2O$, 1.0; KNO_3, 1.0; $CaCl_2$, 0.2; KH_2PO_4, 0.026; Na_2HPO_4, 0.033. Trace elements were added, on a mg/L basis: $FeSO_4*7H_2O$, 0.5; $ZnSO_4*H_2O$, 0.4; EDTA disodium salt, 0.25; $CoCl_2*6H_2O$, 0.05; $MnCl_2*4H_2O$, 0.02; H_3BO_3, 0.015; $NiCl_2*6H_2O$, 0.01; $Na_2MoO_4*4H_2O$, 0.005; and FeEDTA, 0.0038. Vitamins added, on a mg/L basis, were the following: biotin, 0.02; folic acid, 0.02; thiamin*HCl, 0.05; calcium pantothenate, 0.05; riboflavin, 0.05; nicotinamide, 0.05; and B12, 0.001. All chemicals used were obtained from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Aldrich (St. Louis, MO, USA).

Cultures were maintained in a 1:10 ratio of inoculum:medium to a total volume of 55 mL in 250 mL Erlenmeyer flasks at 30 ^oC on a rotary shaker at 250 rpm. Initial enrichment cultures were incubated for one month, while subsequent transfers to fresh media were prepared on a bimonthly basis. Two cultures, derived from the samples taken from the secondary clarifier (SF)

and the mixed liquor exiting the UNOX reactor (SG), were not easily maintained in batch culture, due to continual creation of pellicles in liquid solution and lack of turbidity formation. For that reason, these two cultures were not compared with the others for many of the characterization tests.

Colony Observations

Serially diluted samples from the five remaining cultures were spread-plated on NMS agar plates, two months after initial enrichment, and incubated at 30 ^{o}C with methanol present in the vapor phase. Colonies appearing to be morphologically unique from each other by inspection under light microscope were transferred by streaking onto new NMS plates in an attempt to obtain relatively pure isolates. These transfers were conducted so that morphological distinctions between isolates could be observed, with the goal of determining how many potentially different strains might be present in the cultures. Observations included color, shape, transparency, and edge of the colony, as well as Gram stain, and motility. Colonies were also streaked onto tryptic soy and nutrient agar plates and incubated at 30 ^{o}C with no methanol present, to determine the ability of the isolates to grow on multiple carbon sources.

Growth of Mixed Cultures in Liquid Medium with Methanol as the Carbon Source

In addition to morphological distinctions among the cultures, Samples A-E cultures were also observed on their ability to grow in liquid media with methanol as the sole carbon and energy source. Enriched mixed cultures were grown to ¾ log phase, harvested by centrifugation in a J2-HS Beckman floor model centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA), then twice washed with phosphate buffer and recentrifuged to remove residual methanol. Cells were resuspended in the NMS medium to obtain an optical density of 0.1 at 600 nm, and aliquotted in triplicate into side-arm flasks to a final ratio of 1:10 cells to medium. Liquid methanol was

added to the mixture to a final concentration of 0.2% by volume, and the cultures were incubated for nine days on a rotary shaker at 250 rpm and 30 ^{o}C. Growth, assayed by optical density, was measured using a spectrophotometer at 600nm, directly from the sample in the glass vial.

Results and Discussion

Colony Observations

Isolated strains from the five mixed methylotrophic cultures (SA-SE) were grown on solid agar plates with methanol in the vapor phase and observed based on their morphology, motility, and Gram stain (on NMS plates), as well as their growth on multi-carbon tryptic soy agar (TSA) and nutrient agar plates, with results shown in Table 2-1. Cultures from samples SA and SB appear to have the most distinct isolates as cultured under these lab conditions, which possibly may represent higher diversity in their natural environment, an ideal characteristic of bacterial consortia used for biological treatment systems. All but one of the isolates was Gram negative, and the isolate (number 1 from SB) that tested Gram positive showed inconclusive results on repeated testing. Only one of the isolates, number 6 from SB, showed clear signs of motility. Lack of motility may be related to the enrichment of samples collected from a biofilm environment, which, in the mill environment, may cause selection for bacteria that can attach to surfaces, rather than for those with appendages required for motility in a suspended state (Dunne 2002). Colonies obtained from all five samples showed a variety of morphological differences, with colors ranging from clear to beige and opacity from transparent to semi-opaque. The various colonies were both irregular and circular in shape and were observed to have edge margins including lobate (irregular lobes), erose (serrated), undulate (wavy), curled, filamentous, and entire (smooth). Colony elevations ranged from completely flat to convex, to pulvinate (completely rounded). All colonies except three (number 4 in SA and numbers 2 and 3 in SB)

were observed to show at least some growth on TSA and nutrient plates. The ability for growth on multi-carbon source plates indicates that the bacterial cultures enriched from the biofilms likely contain facultative or restricted facultative methylotrophs, those bacteria capable of using methanol or other compounds with carbon-carbon bonds as a carbon source (Lidstrom 2001).

Growth of Mixed Cultures in Liquid Medium with Methanol as the Carbon Source

Growth rates for the five mixed methylotrophic cultures are shown in Figure 2-2, with error bars representing the standard error for optical density measurements in triplicate. In many cases, the error is relatively high because of interference from the suspended and attached pellicles formed by the bacteria in the growth flask. Additional parameters extracted from this growth curve are given in Table 2-2, including the lag time, growth rate, and generation (or doubling) time. Based on these results, cultures from SB and SC exhibited the fastest acclimation and highest biomass production within the batch culture. Cultures from SA and SB showed the shortest lag time from inoculation until growth commenced, while cultures from SA, SB, and SC exhibited the fastest growth rate

Conclusions

Phenotypic observations from this initial characterization indicated that the mill biofilms may be host to highly diverse populations of bacteria, which would make for straightforward provision of an inoculum culture if a biofilter or other biological treatment system were to be implemented in the mill environment. Based on potential high diversity (as observed by the number of distinct colony isolates), ability for isolates to grow on multiple carbon sources, and minimal lag time and rapid growth rate in liquid culture, SA and SB were selected as the best potential biofilter inocula, and were further characterized on the basis of their methanol degradation potential using different nitrogen sources, as reported in Chapter 3.

Table 2-1. Observations of mixed methylotrophic culture isolates grown on nitrate mineral salts agar plates

Sample	Isolate	Color	Transparency	Form	Elevation	Margin	Growth on Methanol	Growth on Tryptic Soy Agar	Growth on Nutrient Agar	Gram Stain	Motility
A	1	Clear	Transparent	Irregular	Flat	Lobate	++	+++	+++	(-)	(-)
	2	Pale Yellow	Transparent	Circular	Pulvinate	Lobate	++	+++	+++	(-)	(-)
	3	Pale Yellow	Transparent	Irregular	Pulvinate	Lobate	++	+++	+++	(-)	(-)
	4	Yellow	Semi-Opaque	Circular	Umbonate	Erose	++		+	(-)	(-)
	5	Pale Yellow	Transparent	Irregular	Pulvinate	Undulate	+	+++	++	(-)	(-)
	6	Clear	Transparent	Irregular	Flat	Undulate	+++	+++	++	(-)	(-)
	7	Clear	Transparent	Irregular	Flat	Undulate	++	++	+++	(-)	(-)
B	1	Beige	Transparent	Irregular	Umbonate	Curled	+++	+	+	(-)	(+/-)
	2	Clear	Transparent	Circular	Convex	Entire	+			(-)	(-)
	3	Clear	Transparent	Circular	Convex	Entire	++			(-)	(-)
	4	Beige	Semi-Opaque	Filamentous	Convex	Filametous	++	++	++	(-)	(-)
	5	Yellow	Semi-Opaque	Circular	Convex	Undulate	+	++	++	(-)	(-)
	6	Yellow	Semi-Opaque	Circular	Convex	Entire	+++	++	++	(+)	(-)
	7	Beige	Transparent	Circular	Umbonate	Curled	+++	++	++	(-)	(-)
C	1	Clear	Transparent	Circular	Convex	Entire	++	+	+	(-)	(-)
D	1	Pale Yellow	Semi-Opaque	Circular	Umbonate	Entire	++	++	+	(-)	(-)
	2	Clear	Transparent	Circular	Flat	Erose	+	++	+++	(-)	(-)
	3	Pale Yellow	Semi-Opaque	Circular	Convex	Undulate	++	++	+	(-)	(-)
E	1	Clear	Transparent	Irregular	Flat	Lobate	+++	+	+	(-)	(-)
	2	Yellow	Semi-Opaque	Circular	Umbonate	Erose	+++	++	+	(-)	(-)
	3	Clear	Transparent	Irregular	Umbonate	Lobate	++	++	+	(-)	(-)
	4	Clear	Transparent	Circular	Umbonate	Undulate	++	++	++	(-)	(-)
	5	Clear	Transparent	Irregular	Flat	Curled	+++	++	++	(-)	(-)

For growth measurements, no sign indicates no growth; + indicates minimal growth; ++ indicates growth over at least 50% of the plate; +++ indicates excellent growth over at least 75% of the plate. For motility and gram stain, (+) indicates a positive result, (-) indicates a negative result.

Table 2-2. Growth characteristics of mixed methylotrophic cultures

Culture	Source of Inoculum	Lag Phase (hr)	Growth Rate (hr-1)	Generation Time (hr)
A	Enclosed Aeration Basin Vent Tube	21.0	0.111	6.26
B	Return activated sludge	17.5	0.132	5.24
C	WWT cooling tower baffle (wet)	31.0	0.172	4.04
D	WWT cooling tower baffle (dry)	25.7	0.093	7.49
E	WWT cooling tower wall	25.7	0.062	11.2

Biofilm samples collected from the secondary clarifier weirs (F) and from mixed liquors (G) were not able to be enriched in the lab. All samples were obtained from the wastewater treatment (WWT) system of a southeast paper and paperboard mill. Growth parameters are based on liquid culture containing initial methanol concentrations of 0.2% by volume.

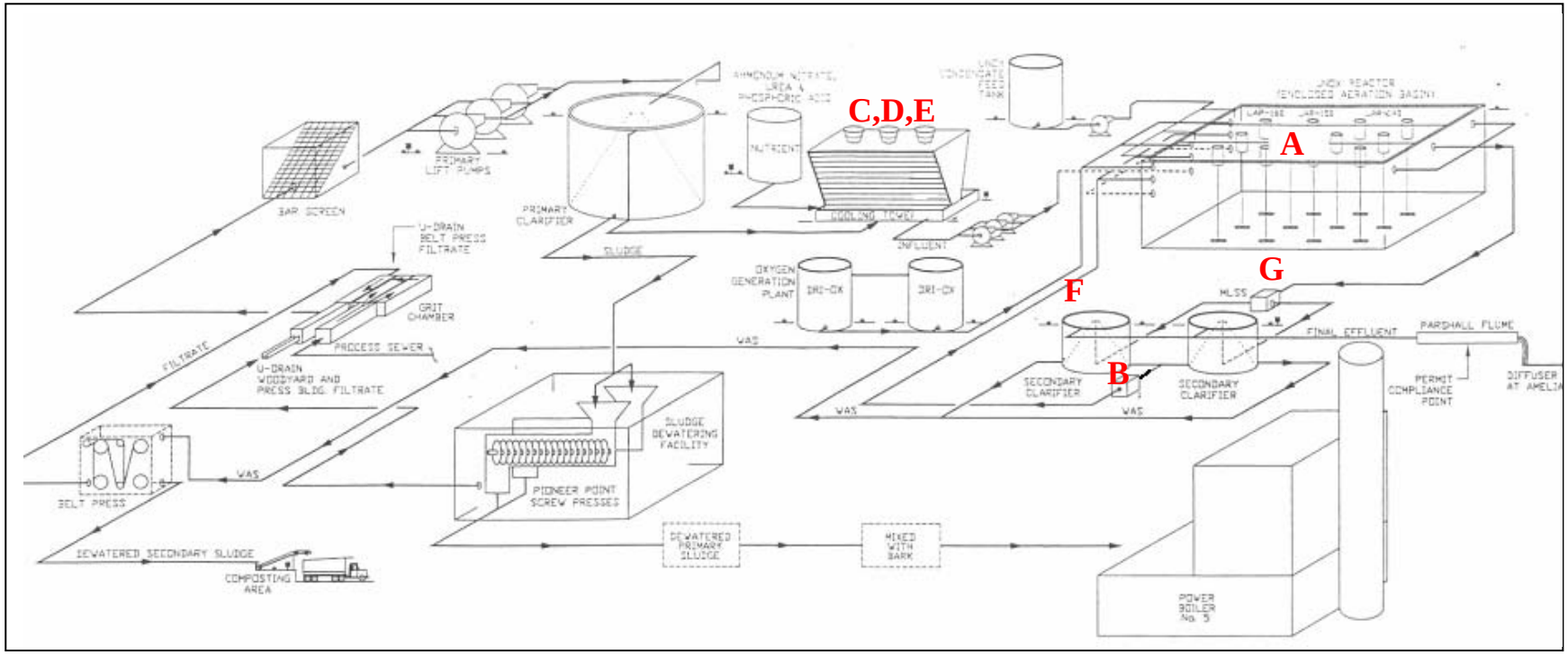

Figure 2-1. Schematic of sampling sites in the pulp and paper mill wastewater treatment system: A: Vent tubes of an oxygen activated sludge “UNOX” reactor; B: Return activated sludge (exiting secondary clarifiers); C: Wet material from cooling tower baffles; D: Partially dry material from cooling tower baffles; E: Wood wall outside of the cooling tower; F: Secondary clarifier weirs; G: Mixed liquor exiting the UNOX reactor.

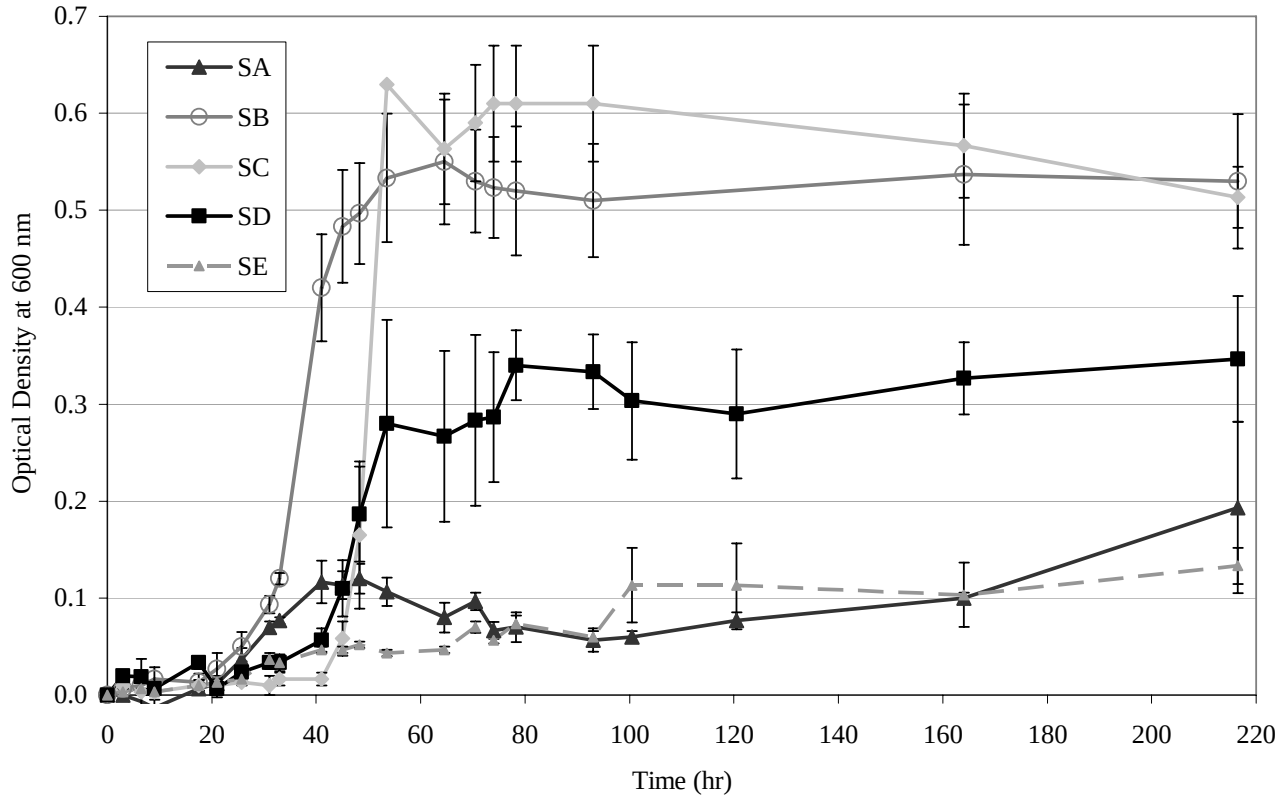

Figure 2-2. Growth in liquid culture over time for five biofilm enrichment cultures (SA-SE) with initial methanol concentrations of 0.2% by volume.

CHAPTER 3
EFFECT OF ENRICHMENT NITROGEN SOURCE ON THE GENETIC DIVERSITY AND METHANOL OXIDATION POTENTIAL OF TWO METHYLOTROPHIC MIXED CULTURES FROM PULP AND PAPER MILL BIOFILMS

Introduction

Methylotrophic bacteria are ubiquitous in many aquatic and terrestrial environments and play an important role in the carbon cycle because of their ability to oxidize methane, methanol, and other reduced carbon substrates. Many previous studies on methylotrophs have focused on methanotrophs, a functional group of methylotrophs able to utilize methane as their sole carbon source. Within this body of work (e.g., Conrad 1996; Hanson and Hanson 1996; King 1992), interest has been directed towards the role of methanotrophs in oxidizing methane, a greenhouse gas, andingand cometabolizing toxic hydrocarbons. However, less attention has been given to the potential use of methylotrophs in removing methanol, a common pollutant in aqueous or gaseous industrial effluents.

The paper and allied products industry is a major contributor of methanol emissions, where it is produced during wood pulping and released to the air and discharged in the mill wastewater (Someshwar and Pinkerton 1992). For example, in 2002, the top three facilities reporting the largest methanol emissions through the Toxic Release Inventory (approximately 10.8 million pounds) were paper and allied products companies (Scorecard 2007). While most pulp mills comply with regulations to control these methanol emissions by incinerating the methanol in thermal oxidation systems (Varma 2003), environmental and economic advantages potentially are achieved by using a biological treatment system that takes advantage of the natural methanol degradation ability of the diverse bacteria classified as methylotrophs.

One biological system of interest is a granular activated carbon (GAC) biofilter, in which the activated carbon provides an adsorptive fixed bed where degrading microorganisms are immobilized. Organic contaminants present in the air flowing through the bed ideally are removed by a synergistic mechanism of adsorption by the GAC and biodegradation by the microorganisms. As such, this biological system requires the presence of active microbial consortia that are capable of degrading contaminants of interest under the conditions expected to be present in the treatment train, including specific pH, temperature, and substrate concentration values (Devinny et al. 1999) When considering treatment of gaseous methanol from a pulp and paper mill, using as an inoculum mixed methylotrophic bacterial cultures enriched from samples obtained from the mill has been demonstrated to influence the ultimate community structure of bacteria colonizing GAC biofilter, while the specific role of the biological community in the biofilter in removing methanol and the effects of conditions in the biofilter on the populations have not been entirely identified (Babbitt et al. 2007).

An important criterion that has not been fully investigated is the importance of the form and concentration of the nitrogen source used in enriching for methylotrophs or supporting their growth and activity in liquid culture or in applications such as a biofilter. The intermittent addition of nitrogen in a mineral salts mixture has been demonstrated to improve the capacity of biofilters in removing ethanol (Terán Perez et al. 2002) and toluene (Prado et al. 2002). Addition of greater concentration of nutrients appears to be most important when the biofilter is subjected to high mass loading rates (Gribbins and Loehr 1998). When comparing nitrogen added as either nitrate or ammonium, Yang et al. (2002) demonstrated that ammonium resulted in higher elimination capacities in a methanol biofilter but that, at high nitrogen-to-carbon ratios, the ammonium could also inhibit methanol removal. The added nitrate did not show this inhibitory

effect (Yang et al. 2002). Despite the benefit of these studies, it is still unclear how the type and concentration of nitrogen used directly affects the methanol degradation potential of mixed methylotrophic cultures a biofilter application.

When considering batch cultures, extensive work has been conducted to select and optimize nutrients, growth factors, trace elements, and substrate concentrations for methanotrophs (Bowman and Sayler 1994; Park et al. 1992; Park et al. 1991). Less specific attention has been focused on enrichment of the more general group of methylotrophs. For example, use of specific nutrient sources, particularly nitrate or ammonium as a nitrogen source, is not consistently reported in studies involving methylotrophs. In part, this inconsistency appears to be due to early work showing better growth of methanotrophic bacteria when using nitrate (Whittenbury et al. 1970), which has been more recently been associated with the possibility that ammonium inhibits methane oxidation in these bacteria (Boiesen et al. 1993; De Visscher and Van Cleemput 2003; Higgins et al. 1991). Therefore, some recommendations for growth of restricted or facultative methylotrophic bacteria follow those for methanotrophs (e.g., use of nitrate as nitrogen source) (Hanson 1998), whereas other studies report use of ammonium as the nitrogen source, in varied concentrations (Patt et al. 1974, El-Nawawy et al. 1990). The effect of form and concentration of nitrogen on methanol degradation potential of methylotrophs is also not clearly understood, nor are the effects on population diversity and stability. Selection and enrichment of an appropriate inoculum for biological treatment systems, such as GAC biofilters, may be greatly improved with additional knowledge of optimum nutrient requirements and concentrations. As an initial step towards this goal, this paper reports the effect of the form and concentration of nitrogen in batch enrichment cultures on the growth and methanol removal

potentials and the genetic diversity of methylotrophic cultures enriched from biofilm samples taken from a Kraft pulp mill.

Methods

Sample Collection

To study the effect of nitrogen source and concentration on methylotrophic cultures that could be used as biofilter inoculum, sampling and analysis was focused on biofilms and other biological cultures obtained directly from a pulp mill environment. These biological samples were obtained from a pulp and paperboard company located in the Southeast and with a biological waste water treatment system. Seven grab samples of biofilm were collected during June 2004 from locations believed by mill staff to be representative of methanol-degrading consortia or having a high number of bacteria present, and stored on ice in sterilized Teflon collection vessels until they could be processed in the lab or stored over a longer term at 4 ^{o}C. Initial culture-dependent growth and isolation techniques demonstrated that two of the seven samples could be good candidates for inocula in a methanol treatment system, based on their superior growth and methanol degradation rates and morphologically diverse, culturable community (determined by identification of visibly distinct colonies on agar plates) (as discussed in Chapter 2). These samples, A and B, were named “SA” and “SB” and described as follows. The SA biofilm was obtained directly from the vent tubes of a pure oxygen activated sludge “UNOX” (Union Carbide Oxidation) reactor, where the conditions would be expected to include temperatures between 32-36 ^{o}C, methanol concentrations between 1,000-5,000 mg/L, and nitrogen as ammonium in concentrations between 20-140 mg/L (ammonium is added to the reactor to improve performance). SB biofilm was collected from the return activated sludge

system, with conditions expected to include ambient outdoor temperatures (26-30 ^{o}C), and low methanol (<10-100 mg/L) concentrations.

Enrichment for Methylotrophic Bacteria

Subsamples (10 mL each) from SA and SB were first homogenized in 90 mL of sterile phosphate-buffered solution for one hour on a rotary shaker at 30 ^{o}C at 250 rpm, then this mixture was used to inoculate batch cultures in both modified nitrate mineral salts (NMS) and ammonium mineral salts (AMS) media, containing 0.2% methanol (vol/vol) as recommended by Hanson (1998). The basal medium contained, on a g/L basis: $MgSO_4*7H_2O$, 1.0; $CaCl_2$, 0.2; KH_2PO_4, 0.026; Na_2HPO_4, 0.033. Trace elements were added, on a mg/L basis: $FeSO_4*7H_2O$, 0.5; $ZnSO_4*H_2O$, 0.4; EDTA disodium salt, 0.25; $CoCl_2*6H_2O$, 0.05; $MnCl_2*4H_2O$, 0.02; H_3BO_3, 0.015; $NiCl_2*6H_2O$, 0.01; $Na_2MoO_4*4H_2O$, 0.005; and FeEDTA, 0.0038. Vitamins were added, on a mg/L basis: biotin, 0.02; folic acid, 0.02; thiamin*HCl, 0.05; calcium pantothenate, 0.05; riboflavin, 0.05; nicotinamide, 0.05; and B12, 0.001. The nitrogen source was added to the medium as nitrate (1.0 g/L KNO_3) or ammonium (0.5 g/L NH_4Cl). All chemicals used were obtained from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Aldrich (St. Louis, MO, USA) and were of the highest purity available. The cultures were maintained in a 1:10 ratio of inoculum to medium to a total volume of 55 mL in 250 mL Erlenmeyer flasks at 30 ^{o}C on a rotary shaker at 250 rpm. Initial enrichment cultures were incubated for one month, while subsequent transfers to fresh medium were made twice, with a two-week period between transfers.

Comparison of Growth and Methanol Degradation Using Two Types of Nitrogen Source

To compare methanol degradation by batch mixed methylotrophic cultures with two potential nitrogen sources, a factorial (3^2) design was used. This design included either nitrate

added as KNO_3 at levels of 0, 1.0, and 2.0 g/L or ammonium added as NH_4Cl at levels of 0, 0.5, and 1.0 g/L (these represent 0, 0.13, and 0.26 g N/L), and methanol added at 10, 100, and 1000 mg/L in the liquid phase. These nitrogen levels reflect common ranges used in both batch and in biofilter applications (Yang et al. 2002; Gribbins and Loehr 1998). Concentrations of 0 g N/L were also included to assess whether the cultures could degrade methanol with only soluble cell nitrogen or atmospheric N_2 present, as such a condition might be expected if nutrients become exhausted in a biofilter or even in a batch culture.

The last transfer of the enrichment cultures was made to a 2400 mL flask, in which 500 mL of the culture was grown to ¾ log phase, harvested by centrifugation in a J2-HS Beckman floor model centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA), then twice washed with phosphate buffer and recentrifuged to remove residual nitrogen and methanol. Cells were resuspended in the appropriate mineral salts medium with no added nitrogen to obtain an optical density of 0.1 at 600nm. For each combination of nitrogen and carbon concentrations, a master mixture of cells and medium in a 1:10 ratio was prepared. Liquid methanol was added to the mixture to the desired concentration, and 4 mL from each master mixture was aliquotted into 20 mL glass vials and sealed with crimp top Teflon-lined septa. Three identical cultures were prepared for each of the nine nitrogen and methanol combinations. Control vials were prepared with killed cells and with no cells, to account for any methanol that might be removed by physical adsorption to the cells or volatilized during the handling and analysis process. The cultures were incubated for 48 hours on a rotary shaker at 250 rpm and 30 ^{o}C. Every 4-6 hours during this incubation, growth, assayed by optical density, was measured using a spectrophotometer at 600 nm, directly from the sample in the glass vial. At 48 hours, the cells were pelleted using the floor centrifuge, and 2 mL of the liquid supernatant were collected and

analyzed for final methanol concentration. Aqueous methanol concentrations were analyzed by GC/FID using a Clarus 500 (PerkinElmer, Wellesley, MA, USA), with helium at 31.3 psig as the carrier gas, and hydrogen and air at 45 mL/min and 450 mL/min, respectively, as combustion gases. Cyclohexanol was used as the internal standard.

Diversity of Microbial Populations in Cultures Enriched with Different Nitrogen Sources

To determine the genetic diversity of the bacterial populations enriched from both samples with both nitrogen sources, denaturing gradient gel electrophoresis (DGGE) was performed using the polymerase chain reaction (PCR)-amplified DNA extracted from the enriched cultures and from the original biofilm samples. Genomic DNA was extracted from the same SA and SB cultures in both AMS and NMS that were used to initiate the methanol oxidation study, using UltraClean Microbial DNA kits (MO BIO Laboratories, Carlsbad, CA, USA) and the accompanying protocol for DNA extraction and purification from microbial samples. In addition, DNA was extracted from the original SA and SB biofilm samples using UltraClean Soil DNA kits (MO BIO Laboratories, Carlsbad, CA, USA) and the accompanying protocol for DNA extraction.

The polymerase chain reaction (PCR) was used to amplify specific DNA sequences found in expected methylotrophic (methanol-oxidizing) populations in the biofilm. In all known gram-negative methylotrophic bacteria, methanol oxidation is catalyzed by the enzyme methanol dehydrogenase (MDH), the large subunit of which is encoded by the highly conserved functional gene *mxaF* (Barta and Hanson, 1993; McDonald and Murrell, 1997). Therefore, *mxaF*-specific primers f1003 (5'-3' GCC CGC CGC GCC CCG CGC CCG TCC CGC CGC CCC CGC CCG GCG GCA CCA ACT GGG GCT GGT), which includes a 39-bp GC-clamp at the 5' end, and r1561 (5'-3' GGG CAG CAT GAA GGG CTC CC) were used to detect methylotrophs as

described by McDonald and Murrell (1997) and McDonald et al. (1995). The 16S rRNA sequences were amplified using primers f27 (5'-3' CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC MTG GCT CAG), which includes a 40-bp GC clamp at the 5' end, and r534 (5'-3' ATT ACC GCG GCT GCT GC).

Initial PCR and DGGE conditions were based on Henckel et al. (1999), Fjellbirkeland et al. (2001), McDonald et al. (1995), and McDonald and Murrell (1997), but optimized for this specific system and primer set. The PCR reaction mixture was prepared in 0.2 mL thin-walled PCR tubes and contained 1X $MgCl_2$-free PCR buffer, 1.5 mM $MgCl_2$, 100 uM of each dNTP,1U *Taq* polymerase (all from Invitrogen, Carlsbad, CA, USA), 0.5µM of each primer (Integrated DNA Technologies, Inc, Coralville, IA, USA.), 1-2 µL of template DNA (50-100 ng), and sterile water to a final volume of 50 µL. Amplifications with *mxaF* primers were carried out using a Mastercycler Personal 5332 thermocycler (Eppendorf North America, Westbury, NY, USA) with the block preheated to 92 ^{o}C, using a reaction program of initial denaturation at 92 ^{o}C for 3 minutes, a total of 30 cycles of denaturation (30 seconds at 92 ^{o}C), annealing using a touchdown program (30 seconds per cycle from 60 to 50 ^{o}C at -0.5 degrees/cycle for the first 20 cycles and 50 ^{o}C for the last 10 cycles), and extension (45 seconds at 72 ^{o}C), and a final extension at 72 ^{o}C for four minutes. The same reaction setup was used for the 16s rRNA primers, but with an annealing touchdown temperature profile of the first 10 cycles from 55 to 50 ^{o}C at -0.5 degree/ cycle and the last 20 cycles at 50 ^{o}C. The touchdown program was used because it increased yield and number of bands observed on subsequent DGGE gels, over a set annealing temperature. PCR products were verified on a 1.2% agarose gel, photographed, and their yield estimated using ImageJ software (Rasband, 2006) calibrated with a low DNA marker (50-2,000 bp, BioNexus, Inc, Oakland, CA, USA.).

DNA Separation using DGGE

DNA fragments were separated using denaturing gel gradient electrophoresis (DGGE) with a 16x16 cm, 1 mm thick gel containing 6% acrylamide, 1X TAE, and a linear gradient of 35-65% denaturant (100% denaturant is equivalent to 7 M urea and 40% formamide), cast for 90 minutes. Approximately 500 ng of PCR product was mixed with 10-20 µL of 2X gel loading dye (70% glycerol, 0.05% Bromophenol Blue, 2mM EDTA), loaded on the gel, and electrophoresed at 60 ^{o}C for 5 hours at 150V in 1X TAE, using a DCode Universal Mutation Detection System Model 475 Gradient Delivery System (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with 50 µg/mL ethidium bromide in 1X TAE for 15 minutes and destained in 1X TAE for 10 minutes. Bands were visualized and photographed using a Fisher Biotech Model 88A variable UV intensity Transilluminator and DCode DocIt software system (Bio-Rad Laboratories, Hercules, CA, USA).

DGGE Image Analysis

The digitized gel images were analyzed using ImageJ (Rasband 2006). The background was subtracted using a rolling ball radius of 50. Bands in each lane were automatically detected and plotted. Peak area and relative intensity of each band was measured, and bands contributing less than 1% to the total intensity within one lane were omitted from subsequent analysis.

Diversity Measurements

Diversity in each sample was estimated using measurements of species richness (S), diversity (H), and evenness (E). S was determined by simply counting the bands in each lane, with the assumption that a single species would migrate to each unique location. Shannon's H (Hayek and Buzas 1997) was used as a diversity index (Equation 3-1).

$$H = -\sum_{i} p_i \ln(p_i) \qquad (3\text{-}1)$$

where p_i is the relative intensity of the ith band compared to the total intensity of all bands in that lane. E was calculated from Pielou's evenness (Equation 3-2) (Hayek and Buzas 1997).

$$E = \frac{H}{\ln(S)} \tag{3-2}$$

DNA Sequencing and Phylogenetic Analysis

To further characterize the bacteria under both nitrogen use profiles, selected bands from the *mxa*F and 16s rRNA DGGE gels were excised for sequencing. Bands were chosen from DNA that showed the highest intensity when visualized on the UV transilluminator and were excised using a sterile pipet tip and scalpel. The gel fragments were eluted overnight at 30 ^{o}C at 250 rpm in 30 µL of an elution buffer containing 10mM Tris-Cl (pH 7.5), 50 mM NaCl, and 1mM EDTA (pH 8.0) (Chory and Pollard 1999). Gel fragments were removed, and DNA was precipitated from the liquid by adding 50 µL of 95% cold ethanol, chilling 30 minutes at -40 ^{o}C, and pelleting the DNA by centrifuging 10 minutes at 10,000xg. After pouring off the ethanol supernatant, the pellet was dried at 40 ^{o}C for 4-5 hours and resuspended in 30 µL of TE buffer (Chory and Pollard 1999). This template was reamplified using the same methods as described previously and checked on a DGGE gel for purity and for migration to the same gradient position as in the original sample. Sequencing was performed at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) using the fluorescent dideoxy terminator method of cycle sequencing on either a Perkin Elmer Applied Biosystems Division (PE/ABD) 373A or 377 automated DNA sequencer, following ABD protocols, with consensus sequences generated using the Sequencher Software from Gene Codes. Sequences of partial *mxa*F and 16s rRNA gene fragments have been deposited in the GenBank database. Fragments M1, M3, and M6 were identical to bands sequenced from biofilter samples (Babbitt et al. 2007), which have previously been submitted under accession numbers EU099402, EU099404, and

EU099407, respectively. For this study, *mxa*F fragments M2, M4, and M5 were submitted under accession numbers EU138867, EU138868, and EU 138869, respectively; and 16s rRNA fragments U1-U7 were submitted under accession numbers EU138870-EU138876, respectively.

Published sequences with high similarity to sample sequences were obtained by performing a nucleotide-nucleotide BLAST (NCBI) search. The 10 most similar sequences of known species with E scores lower than 1E-20 were chosen for each sample, with duplicates removed. Sequences were aligned using ClustalW, with default gap penalties, and manual inspection and refinement of alignments. A phylogenetic tree was constructed using the Neighbor Joining method and bootstrapped with 1,000 replicates. Because all known γ-proteobacteria clustered into a distinct branch, this group was selected as the out-group. All phylogenetic and molecular evolution analyses were conducted using MEGA version 3.1 (Kumar et al. 2004).

Results and Discussion

Comparison of Growth and Methanol Degradation Using Two Types of Nitrogen Source

The two pulp mill biofilm samples (SA and SB), enriched using different nitrogen sources, were compared on the basis of methanol degradation under different methanol and nitrogen concentrations. Both samples, regardless of nitrogen source or concentration, showed 100% methanol removal for initial methanol concentrations of 10 and 100 mg/L. Differences among the cultures became apparent when methanol was introduced at concentrations of 1,000 mg/L. The percent of methanol removed, based on an initial 1,000 mg/L concentration, is shown in Figure 3-1. For all of the cultures, the percent of methanol removed from the liquid phase increased with increasing nitrogen concentration. In addition, for all of the cultures assessed in medium with added nitrogen, a higher methanol removal was achieved when nitrate served as

the nitrogen source. In fact, the SB culture enriched in NMS medium with the highest concentration of nitrate (0.26 g N/L) showed 100% removal. On the other hand, after transfer to medium with no added nitrogen, both SA cultures showed significantly higher methanol removal than SB, regardless of the original enrichment N-source.

A slightly different trend was observed when comparing growth rate with an initial 1,000 mg/L methanol concentration and varied nitrogen sources and concentrations (Figure 3-2). These results showed that the mixed cultures grew almost equally as fast with either ammonium or nitrate present at the high (0.26 g N/L) or medium (0.13 g N/L) concentrations tested. Growth rate slightly increased when NMS was used, but the trends were not as dramatic as when comparing methanol removal. For example, for nitrogen levels of 0.26 g N/L, SA exhibited growth at 0.061 hr^{-1} in AMS and 0.068 hr^{-1} in NMS; and SB grew at a rate of 0.062 hr^{-1} in AMS and 0.074 hr^{-1} in NMS. However, the growth rate was significantly lower when the cultures were cultured in the presence of no added nitrogen in either form (Figure 3-2).

Diversity of Microbial Populations in Cultures Enriched with Different Nitrogen Sources

To expand growth and activity comparisons to the community level of the enriched samples, DGGE was used to separate DNA fragments amplified for methylotrophs and all bacteria from the original biofilm samples and their enrichments, as shown in Figure 3-3. Quantitative estimates of diversity, based on banding patterns in the DGGE gels, are provided in Table 3-1. Results obtained for methylotrophs and universal bacteria were not compared directly, because different primer sets can amplify entirely different populations; however, both sets of results were used for determining trends in the population changes for the nitrogen sources used. The results in Table 3-1 showed consistent trends among the different enrichment and molecular methods, except for the methylotrophs enriched from SA, where regardless of

which nitrogen source was used, the diversity of this type of bacteria dropped to zero, with potentially only one dominant methylotrophic species present. When comparing methylotrophs in SB and all bacteria in SA, species richness, diversity, and evenness generally showed a smaller decrease from the quantities observed in the original biofilm culture to the levels shown in the enriched culture. It was interesting to note that in all the cases, the diversity metrics were greater for the mixed cultures enriched using nitrate, as compared to ammonium, as the nitrogen source. This result could potentially correspond to the observation that cultures enriched in nitrate also showed higher methanol removal and growth rate.

Phylogenetic Analysis of Dominant Species

The genetic comparisons among the cultures were expanded by selecting dominant species within each culture and determining their *mxa*-F or 16s rRNA sequence and phylogenetic relationship to other closely related known bacteria. All excised and sequenced bands are denoted in Figures 3-3A and 3-3B, as indicated by circles placed adjacent to sequenced bands. The phylogenic relationships among the species dominating the cultures in these experiments and known bacteria are shown in Figures 3-4 and 3-5.

Figure 3-4 shows the distribution of selected and recovered dominant bands that were produced by amplifying the functional gene for methanol dehydrogenase. Bands from the six samples show similarity to sequences found in the alpha-, beta-, and gamma-proteobacteria. Dominant bands from the original SA biofilm species are labeled as M1, which appears closely related to beta-proteobacteria *Methylophilus methylotrophus*, a ribulose monophosphate (RuMP) cycle restricted facultative methylotroph; and M6, which shows the greatest genetic similarity to a dominant species from the SB biofilm (M5), and both are grouped with other alpha-proteobacteria in the order Rhizobiales. Interestingly, enriching SA in AMS or NMS produced

similar *mxaF* profiles that appear to be dominated by a single methylotroph, band M3, which is not visible in the original culture, possibly because its DNA was present in too small a concentration to be amplified sufficiently for visualization in the DGGE gel. Band M3 showed high sequence similarity to one cluster within the genus *Hyphomicrobium*, bacteria also classified as non-N_2 fixing, restrictive facultative methylotrophs, but which use the serine pathway and are members of the alpha-Proteobacteria (Lidstrom 2006; Rainey et al. 1998). The community shift observed when enriching SB in AMS or NMS was not as consistent as with SA. M2, a strong band from enrichments of SB in AMS, appeared to be closely related to a species within the genera of *Methylovorus*, an RuMP pathway, restrictive facultative methylotrophs classified as beta-Proteobacteria (Doronina et al. 2005). However, M3 was also present for SB enriched in NMS, as is band M4, which has a highly similar *mxaF* sequence.

As shown in Figure 3-5, dominant bands obtained from 16s rRNA of the samples also showed wide distribution among the alpha-, beta-, and gamma-proteobacteria and no consistent community structure under either enrichment profile. The original SA biofilm sample had three sequenced dominant bands: U3, U4, and U7. Band U3 was most similar to three *Pseudomonas* species (gamma-Proteobacteria); band U4 was grouped with a *Bradyrhizobium* species (alpha-Proteobacteria); and band U7 was highly similar to several *Thiobacillus* species (sulfur oxidizing chemolithoautotrophic beta-Proteobacteria). On enrichment in AMS, one dominant band was shown, U5, which was grouped with one cluster of the *Hyphomicrobium*, a similar result to what was observed when using *mxaF* primers. Although U5 also appeared to be present in the SA NMS enrichment, that was not confirmed by sequencing. However, the dominant SA NMS bands were U2 and U6, which were most similar in 16s rRNA sequence to methylotrophs found in the beta-Proteobacteria, including serine cycle methylotrophs in the family *Rhodocyclaceae*

and RuMP cycle methylotrophs in the family *Methylophilaceae*, respectively. The original SB biofilm sample was dominated by one band, U7, which was also found in SA and described above. SB enriched in AMS produced an intense band, U1, with a 16s rRNA sequence similar to several *Methylobacillus* species. SB enriched in NMS produced multiple strong bands, although only one was able to be recovered and sequenced, U2, which was also found in SA-NMS and shown to be similar to methylotrophic beta-Proteobacteria.

Conclusions

Results from this study illustrated that the type and concentration of nitrogen source used when enriching mixed methylotrophic cultures from samples removed from a pulp mill does influence the growth and methanol degradation ability of these bacteria. Generally, the cultures enriched with nitrate showed faster growth and higher methanol removal than those enriched with ammonium. Higher concentrations of nitrogen in either form also resulted in greater methanol removal in all cases. The form of nitrogen used also affected the diversity and community structure of the methylotrophic populations present in each of the final cultures. Although the enrichment process did decrease the overall diversity from the original samples, the cultures grown with nitrate as nitrogen source preserved a higher level of species number, diversity, and evenness than those grown with ammonium. Based on the results reported herein, nitrate is recommended to be used as the nitrogen source when enriching or working with methylotrophic cultures from pulp and paper mills, and should be investigated further as a nutrient addition to biofilters.

Table 3-1. Bacterial species, diversity, and evenness for SA and SB cultures in both AMS and NMS media

	Methylotrophs (*mxa*F)			Universal (16s rRNA)		
Sample	S	H	E	S	H	E
SA	4	1.04	0.75	9	2.02	0.92
SA-AMS	1	0	0	3	1.06	0.96
SA-NMS	1	0	0	7	1.74	0.89
SB	5	1.56	0.97	10	2.09	0.91
SB-AMS	2	0.34	0.49	8	1.48	0.71
SB-NMS	6	1.39	0.78	9	1.90	0.87

Species richness is indicated by "S," diversity is indicated by "H," and evenness is indicated by "E."

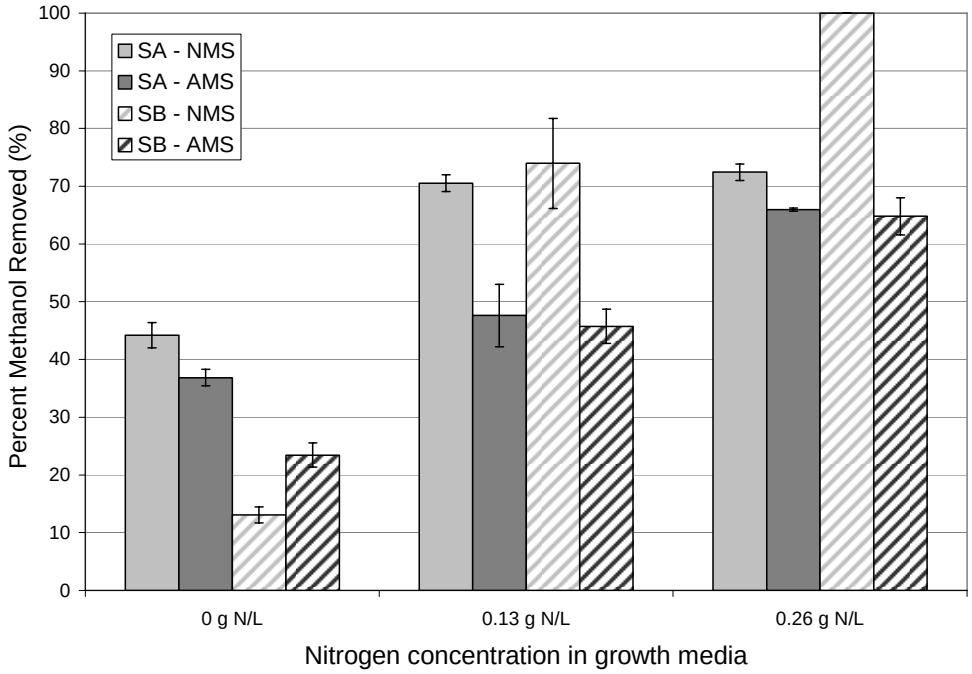

Figure 3-1. Comparison of methanol removal by SA and SB cultures in both AMS and NMS medium with an initial methanol concentration of 1,000 mg/L.

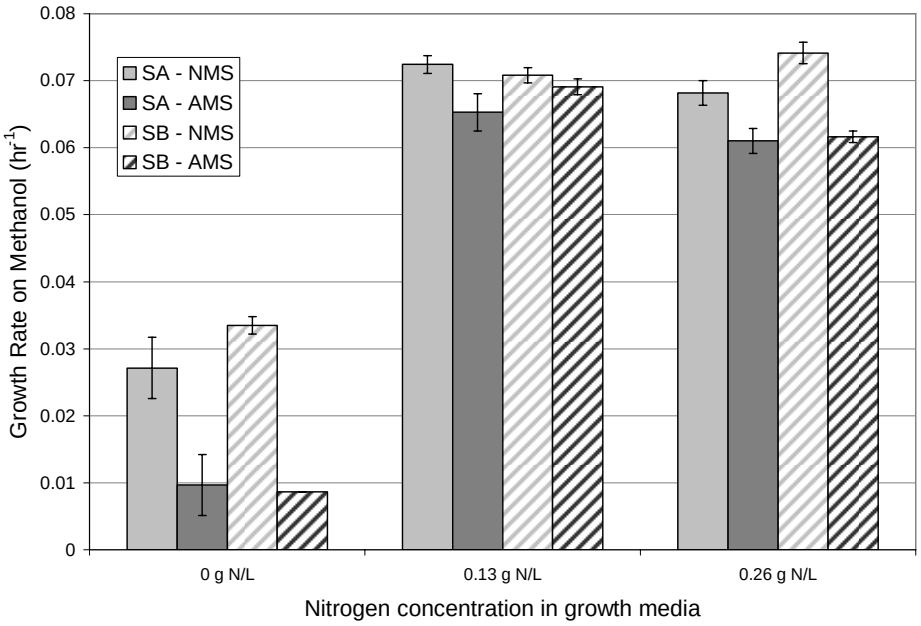

Figure 3-2. Comparison of batch growth rates in SA and SB cultures in both AMS and NMS medium with an initial methanol concentration of 1,000 mg/L.

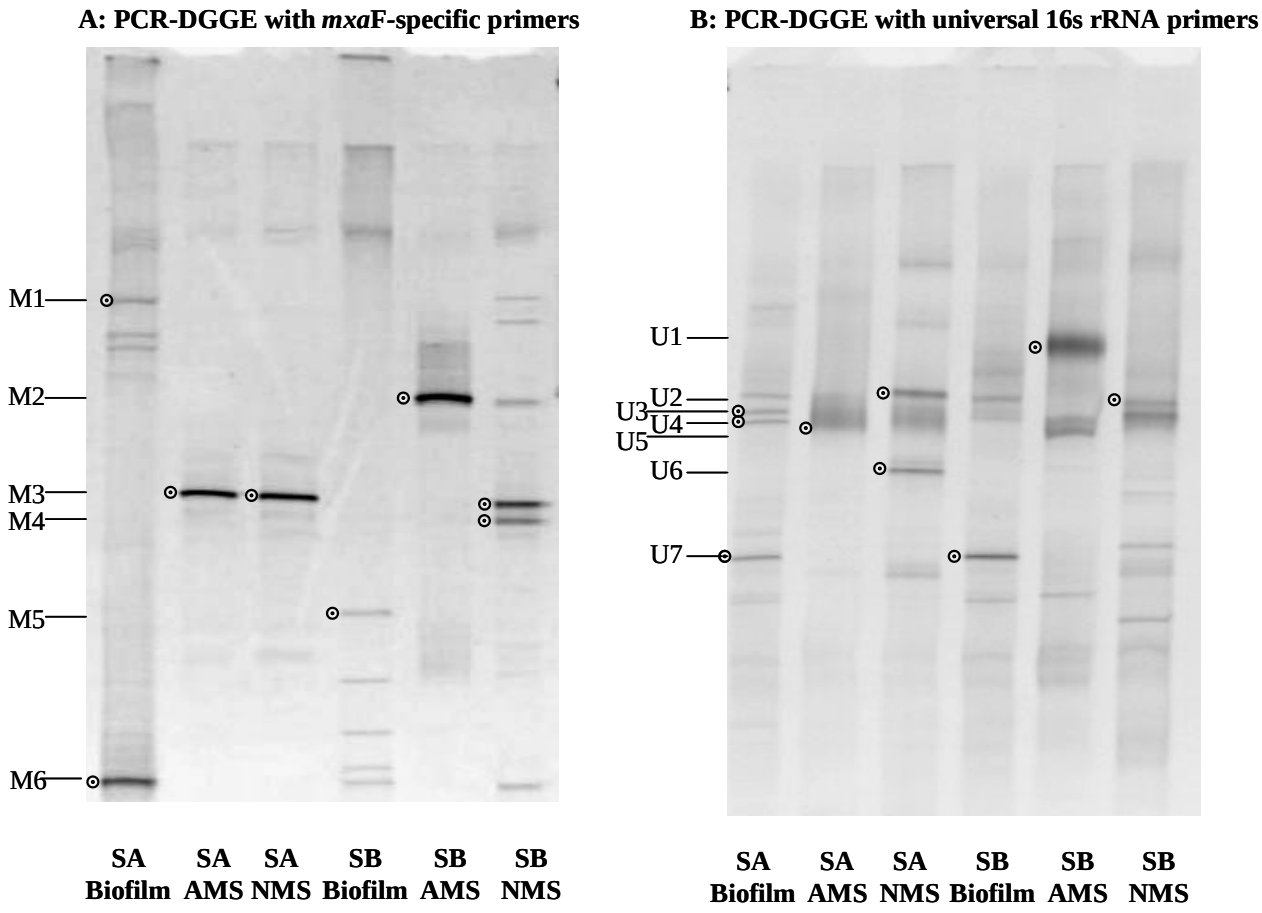

Figure 3-3. Bacterial diversity measured using PCR-DGGE analysis. A) Using *mxa*F primers. B) Using 16s rRNA primers. Bands marked with a circle were excised and sequenced, and all marked bands on a vertical gradient labeled by M (methylotrophs) or U (universal) were confirmed as identical in sequence.

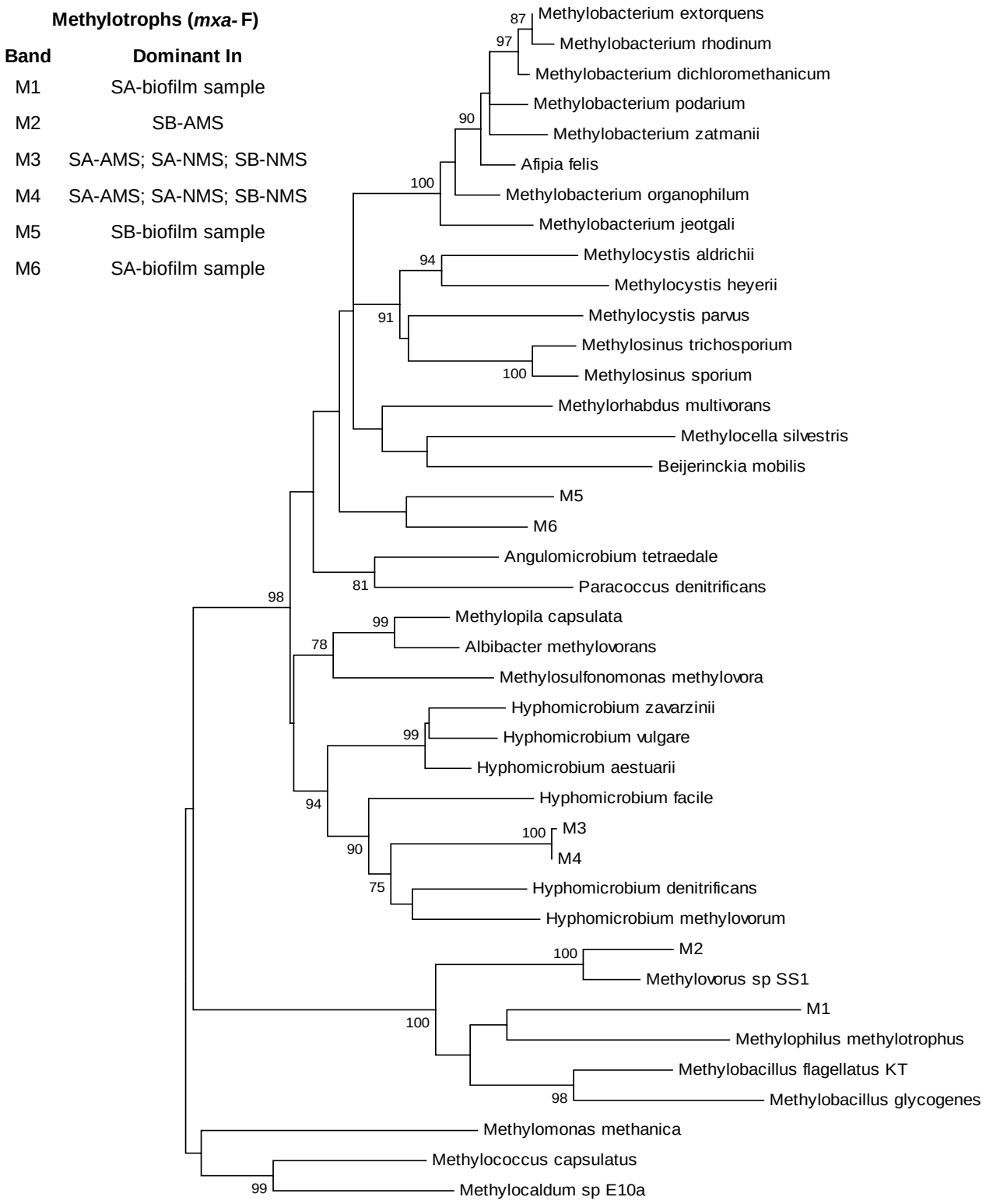

Figure 3-4. Phylogenetic reconstruction of known methylotrophic bacteria and unknown culture strains using *mxa*F gene sequences (Bootstrap values represent 1,000 replicates, and values greater than 75% are shown).

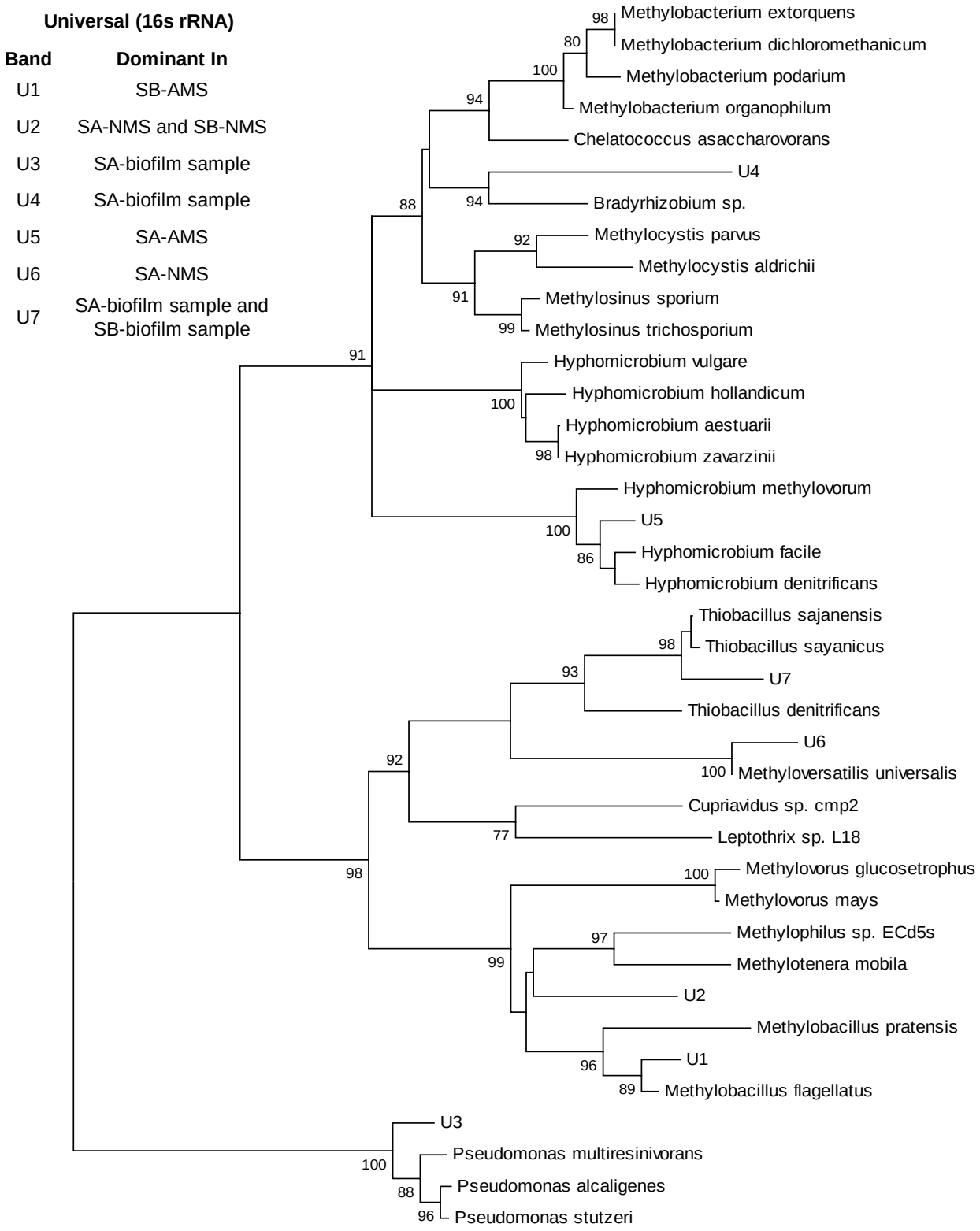

Figure 3-5. Phylogenetic reconstruction of known bacteria and unknown culture strains using 16s rRNA gene sequences (Bootstrap values represent 1,000 replicates, and values greater than 75% are shown).

CHAPTER 4
METHANOL REMOVAL EFFICIENCY AND BACTERIAL DIVERSITY OF AN ACTIVATED CARBON BIOFILTER

Introduction

The forest products industry produces valuable industrial and consumer products from wood cellulose, including paper, paperboard, hygiene products, high porosity filters, food casings, rayon filament, and chemicals such as ethers and acetate. During the chemical processes that convert wood chips to cellulose pulp, organic compounds naturally present in wood or produced during pulping are released to air and water (Someshwar and Pinkerton 1992). In 1998, the U.S. Environmental Protection Agency (U.S. EPA) passed the "Cluster Rule" to regulate the release of these compounds, including hazardous air pollutants (HAPs), chemicals that pose great risk to human or environmental health (U.S. EPA 1998). Methanol is the primary focus of these regulations, as it is released in quantities of over 44,000 tons per year, over 70% of the total HAPs emitted by this industry (U.S. EPA 2004), and can contribute to human health impacts such as cancer, respiratory irritation, and damage to the nervous system (U.S. EPA 1998).

To prevent potential environmental and health impacts from methanol emissions, the Cluster Rule requires implementation of maximum available control technology (MACT) to collect and treat non-condensable gas from high-volume (10,000-30,000 acfm), low-concentration (below the lower explosion limit of the gas mixture) (HVLC) sources, including pulp washing and screening, oxygen delignification, and weak black liquor storage tanks (Varma 2003). Most mills comply with MACT regulations by collection of HAPs in vent hoods and subsequent oxidation in power boilers, stand-alone thermal oxidizers, or recovery furnaces (Varma 2003).

Despite the effectiveness of oxidation in destroying HAPs such as methanol, the drawbacks of requiring natural gas to support incineration, increased production of CO_2, SO_2, and NO_x emissions, and high capital costs for installation and infrastructure have motivated a desire for more environmentally and economically beneficial technologies (Mycock et al. 1995; Schnelle and Brown 2002). One potential alternative to thermal oxidation is the use of a granular activated carbon (GAC) biofilter as a polishing step in combination with a primary treatment system using photocatalytic oxidation (Stokke et al. 2006; Tao et al. 2006). Although biofilters traditionally have contained a fixed bed of inert organic packing that serves as biomass support and nutrient support (Devinny et al. 1999; Kennes and Thalasso 1998; Kennes and Veiga 2001), more recent work has shown the potential advantages of using surface active packing such as GAC (Aizpuru et al. 2003; Prado et al. 2002; Teran-Perez et al. 2002; Yang et al. 2002). The activated carbon adsorbs VOCs from the process stream, creating a favorable environment for a stable microbial biofilm that subsequently degrades the contaminants (Aizpuru et al. 2003; Chang and Rittman 1987; Herzberg et al. 2003; Hodge and Devinny 1994; Weber and Hartmans 1995; Zhang et al. 1991).

While recent studies (e.g., Aizpuru et al. 2003; Chung 2007; Liang et al. 2007) have demonstrated excellent performance of activated carbon biofilters for contaminant removal, a potential challenge to their use is the lack of inherent nutrients, moisture, and microorganisms that would typically be found in an organic packing such as compost (Devinny et al. 1999). Furthermore, little is known about the structure and dynamics of bacterial communities that colonize GAC biofilters. Therefore, the overall goal of this work was to develop and characterize a bench-scale activated carbon biofilter in removing methanol at concentrations typically observed in pulp mill air effluents and to observe the bacterial diversity during the

biofilter operation. This bench-scale biofilter used a novel heterogeneous packing blend containing granular activated carbon as the primary filter medium, mixed with perlite, slow-release ammonium nitrate pellets, and water-retaining crystals, which were added to minimize pressure drop, provide continuous nutrient input, and minimize biofilter drying, respectively. Studies on this biofilter system were carried out to meet three primary objectives: 1) to demonstrate the methanol removal efficiency of the bench-scale activated carbon biofilter, 2) to use denaturing gel gradient electrophoresis (DGGE) methods, in addition to culture-dependent methods, to characterize the abundance, diversity, and spatial distribution of bacteria colonizing the novel packing media; and 3) to use DGGE methods to assess similarities and differences in stable biofilm communities in biofilters with and without an inoculum specifically cultured for methanol-degrading bacteria. The results of this work are reported herein and show promise for the development of activated carbon biofiltration systems as an effective option for methanol control for the pulp and paper industry.

Materials and Methods

Selection of Biological Inoculum

Potential biofilter inocula were obtained from a pulp and paperboard company located in the Southeast that uses a biological waste water treatment system. Seven grab samples of biofilm were collected during June 2004 from locations at this mill suspected to foster conditions for enrichment of bacteria, including methanol-degrading consortia, and stored on ice in sterilized Teflon collection vessels until they could be processed in the lab or stored over a longer term at 4 ^{o}C. The collection locations of these samples were cooling tower baffles, an oxygen activated sludge reactor (UNOX reactor, or Union Carbide pure oxygen reactor), a secondary clarifier, mixed liquor, and return activated sludge from the clarifier.

From each sample, 10 mL or 10 g was homogenized with a phosphate buffer on a rotary shaker at 30 ^{o}C and 250 rpm for one hour. A 5 mL sample of the homogenized mixture was used to start a batch enrichment culture in a modified nitrate mineral salts medium (NMS) containing 0.2% methanol (vol/vol) as recommended by Hanson (1998). The basal medium contained (on a g/L basis $MgSO_4*7H_2O$, 1.0; KNO_3, 1.0; $CaCl_2$, 0.2; KH_2PO_4, 0.026; Na_2HPO_4, 0.033. Trace elements were added, on a mg/L basis: $FeSO_4*7H_2O$, 0.5; $ZnSO_4*H_2O$, 0.4; EDTA disodium salt, 0.25; $CoCl_2*6H_2O$, 0.05; $MnCl_2*4H_2O$, 0.02; H_3BO_3, 0.015; $NiCl_2*6H_2O$, 0.01; $Na_2MoO_4*4H_2O$, 0.005; and FeEDTA, 0.0038. Vitamins were added, on a mg/L basis: biotin, 0.02; folic acid, 0.02; thiamin*HCl, 0.05; calcium pantothenate, 0.05; riboflavin, 0.05; nicotinamide, 0.05; and B12, 0.001. All chemicals used were obtained from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Aldrich (St. Louis, MO, USA) and were of analytical grade or higher.

Cultures were maintained in a 1:10 ratio of inoculum:medium in a total volume of 55 mL in 250 mL Erlenmeyer flasks at 30 ^{o}C on a rotary shaker at 250 rpm. Initial cultures were incubated for one month, and subsequent transfers to fresh medium were made bimonthly. In order to determine the most suitable biofilter inoculum, a series of culture-dependent growth, methanol depletion, and characterization studies were performed on these cultures (Chapters 2 and 3). Based on superior growth and methanol degradation rates and morphologically diverse, culturable community (determined by identification of visibly distinct colonies on agar plates), the cultures derived from biofilm in the aerated activated sludge UNOX reactor (SA) and from the return activated sludge (SB) were selected as potential biofilter inoculums. Despite the higher methanol removal rate of SB demonstrated in Chapter 3, initial trials using this sample as an inoculum resulted in excessive biomass formation, plugging, and high pressure drop in a

preliminary biofilter trial (data not shown). Therefore, SA was selected as the inoculum for the final biofilter experiment reported in this chapter.

Selection of Packing Material

Two types of GAC packing, MeadWestvaco Bionuchar 120 (MeadWestvaco, Richmond, VA, USA) and Calgon F400 (Calgon Carbon Corporation, Pittsburgh, PA, USA), were compared to determine which would better foster the growth of biofilter bacteria. . These two types of GAC were selected because they are commonly available and are recommended by their manufacturers for the removal of organic pollutants (MeadWestvaco 2002; Calgon 1996). Bionuchar, a wood-based, chemically activated GAC with 1.1-1.3 mm particle size, is specifically marketed as having physical characteristics that allow for maximum fixation of beneficial biomass (MeadWestvaco 2002). F400 is a bituminous coal-based, physically activated carbon with a 0.55-0.75 mm particle size (Calgon 1996).

The GACs were compared on the basis of immobilization and growth of the biofilter inoculum on the carbon surface, measured first by enumerating bacteria in the liquid culture inoculum and then by enumerating bacteria recovered from the GAC surface after inoculation. Two inoculation methods were tested. One method involved suspending the biofilter inoculum in the mineral medium and continuously circulating the inoculum through the packed bed for seven days. The other method involved flooding the column with the suspended inoculums incubating the column at 30 ^{o}C for four days, followed by draining the column and circulating fresh medium with methanol for three days. In both cases, a sterile glass column, measuring 10 mm in diameter and 100 mm in length, was used. The inoculum and/or the mineral medium was delivered to the column from sterile flasks using a peristaltic pump and Teflon tubing.

After both inoculation methods were complete, 5 g of GAC was removed from each column and rinsed gently with sterile distilled water to remove particles and non-attached bacteria. The sample was mixed with 5 mL of sterile phosphate-buffered saline (PBS) and vortexed in 10-second pulses at 2500 rpm for two minutes to transfer biomass from the packing surface to a liquid suspension. To measure cultivable bacteria, 1 mL aliquots from both the biofilter inoculum and from the liquid suspension of biomass from the packing were serially diluted (ten-fold) and spread plated in triplicate on NMS plates containing 0.2% methanol. Plates were incubated at 30^{o}C for seven days, at which time the total colony forming units (CFUs) were quantified. The carbon was dried at 45^{o}C for 14 days and then weighed to obtain an air-dry weight.

Biofilter Design

The biofilter housing consisted of a clear PVC column with 2-inch diameter, 24-inch length, and 1,235 mL empty bed volume. The packing was added to a final volume of 1,100 mL dry packing material, composed of a 4:2:1:1 mixture of GAC, bulking agent (Perlite, Miracle-Gro, Marysville, OH, USA), slow-release ammonium-nitrate fertilizer (Osmocote, Scotts-Sierra Horticultural Products, Marysville, OH, USA), and water-retaining crystals (Agrosoke International, Arlington, TX, USA). This mixture had a bulk density of 412 mg/mL. To determine the ability of this material to adsorb methanol with no bacteria present, batch isotherms were performed on small (1-2 g) samples of the packing, added to a 26 mL vial along with a smaller vial containing 10, 50, or 100 µL of methanol. The change in weight of the activated carbon was measured at 24, 48, and 72 hours and at seven days. No change between 48 hours and seven days was observed, and the system was assumed to be at equilibrium. The adsorption capacity was determined by plotting the ratio of adsorbed methanol mass to the GAC

mass as a function of equilibrium concentration, according to the Freundlich isotherm. Results of this analysis (data not shown) indicated that the adsorptive capacity of the packing material would be exhausted in approximately 20 hours and that methanol removal after that time would likely be attributed to the bacterial activity in the biofilter.

Biofilter Operation and Performance Measurements

The GAC biofilter was inoculated with the selected biofilm culture enriched in a mixed medium of ammonia and nitrate (composition of NMS described previously, but amended with 0.5 g/L NH_4Cl), with methanol added to a concentration of 0.2% by volume. A second column, identical to the GAC biofilter but without the inoculum, was used in parallel to the biofilter to demonstrate effects, if any, of using a specifically enriched inoculum. The inoculated packed column will be referred to as "BB" (biologically-inoculated biofilter), and the non-inoculated packed column will be referred to as "NB" (non-inoculated biofilter) throughout the text. Sterile technique was used in working with the inoculum cultures and in preparing the columns, and all tubing, glassware, and the GAC were sterilized by autoclave before use; however, the biofilters were operated in a non-sterile environment and were periodically exposed to the ambient lab air for sampling or maintenance purposes.

The biofilters were operated in up-flow mode with an air stream artificially contaminated with varied methanol concentrations and 90-95% relative humidity (Figure 4-1). The air stream was generated by splitting a stream from a compressed zero-grade air cylinder, with a small fraction of air diverted through a methanol bubbler (about 1-5% by volume, depending on the desired concentration) and the remaining fraction passing through a water bubbler to add humidity. Upflow mode was selected based on the possible need to add liquid nutrients without

creating a leachate stream and to limit potential acidic byproduct buildup from using an ammonium based nutrient to the lower fraction of the bed (Devinny et al. 1999).

Biofilter operation was characterized by three consecutive sets of operating conditions, described here and summarized in Table 4-1. First, the column was operated at a high methanol loading concentration (10,000 ppmv) and high gas retention time (5 minutes) for 46 days to allow for bacterial colonization of the activated carbon and acclimatization to high concentrations. Subsequently, for days 47-108, the operating conditions included methanol concentrations below 100 ppmv and a retention time of one minute. Finally, for the last 30 days, the concentration was increased to 1,000 ppmv while maintaining a retention time of one minute. The stepwise changes in concentration between each set of operating conditions were used to replicate a wide variability of conditions that could be expected in an industrial setting.

Column inlet and outlet gas concentrations were determined by the NCASI chilled impinger method (NCASI, 1998), with two midget impingers (ARS, Gainesville, FL, USA) in series, each containing 20 mL of nanopure water in an ice bath. Methanol concentrations were analyzed by GC/FID using a Clarus 500 (PerkinElmer, Wellesley, MA, USA), with helium at 31.3 psig as the carrier gas, and hydrogen and air at 45 mL/min and 450 mL/min, respectively, as combustion gases. Cyclohexanol was used as the internal standard.

Abundance and Diversity of Microbial Populations in the Biofilter

Samples were collected from both biofilters by removing about 5 g (wet weight) of packing material from the middle of the column using sterile instruments on days 22, 46, 77, 102, 125, and 138, which provided two samples during each of the three operating conditions described previously**.** The non-inoculated column was not sampled on day 22 because visible growth of biofilm on the carbon surface had not yet formed. At the conclusion of the

experiment, packing material was also sampled from the inlet and outlet of the column. Each packing sample was first rinsed gently with sterile distilled water to remove particles and non-attached bacteria. Then the sample was mixed with 5 mL of sterile phosphate-buffered saline (PBS) and vortexed in 10-second pulses at 2500 rpm for a total of two minutes to transfer biomass from the packing surface to liquid suspension.

To determine bacterial abundance as a function of length along the column, the liquid suspension from samples at the top, middle, and bottom of the biofilters from day 138 were serially diluted eight-fold, and 100 µL aliquots were spread on agar plates. Three types of agar plates were used, nitrate mineral salts and ammonium mineral salts with methanol added in the vapor phase and nutrient agar with mixed N- and C-sources and no methanol added. Plates were incubated at 30 ^{o}C for seven days. Results were compared using one-way ANOVA and *post-hoc* comparisons among all groups, using SPSS 8.0 (Chicago, IL, USA).

DNA Extraction and Amplification

DNA was extracted from the liquid suspension from column samples using UltraClean Microbial DNA kits (MO BIO Laboratories, Carlsbad, CA, USA) and the accompanying protocol. It has been reported that results obtained from this type of indirect approach are comparable to those obtained from direct lysis from cells attached to the packing (Li and Moe 2004). The polymerase chain reaction (PCR) was used to amplify specific DNA sequences found in expected methylotrophic (methanol-oxidizing) populations in the biofilm. In all known gram-negative methylotrophic bacteria, methanol oxidation is catalyzed by the enzyme methanol dehydrogenase (MDH), the large subunit of which is encoded by the highly conserved functional gene *mxaF* (Barta and Hanson 1993; McDonald and Murrell 1997). Therefore, *mxaF*-specific primers f1003 (5'-3' GCC CGC CGC GCC CCG CGC CCG TCC CGC CGC CCC CGC CCG

GCG GCA CCA ACT GGG GCT GGT), which includes a 39-bp GC-clamp at the 5' end, and r1561 (5'-3' GGG CAG CAT GAA GGG CTC CC) were used to detect methylotrophs as described by McDonald and Murrell (1997) and McDonald et al. (1995). The 16S rRNA sequences were amplified using primers f27 (5'-3' CGC CCG CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC MTG GCT CAG), which includes a 40-bp GC clamp at the 5' end, and r518 (5'-3' ATT ACC GCG GCT GCT GC).

Initial PCR and DGGE conditions were based on Henckel et al. (1999), Fjellbirkeland et al. (2001), McDonald et al. (1995), McDonald and Murrell (1997), but optimized for this specific system and primer set. The PCR reaction mixture was prepared in 0.2 mL thin-walled PCR tubes and contained 1X $MgCl_2$-free PCR buffer, 1.5 mM $MgCl_2$, 100 uM of each dNTP,1U *Taq* polymerase (all from Invitrogen, Carlsbad, CA, USA), 0.5µM of each primer (Integrated DNA Technologies, Inc, Coralville, IA, USA), 1-2 µL of template DNA (50-100 ng), and sterile water to a final volume of 50 µL. Amplifications with *mxaF* primers were carried out using a Mastercycler Personal 5332 thermocycler (Eppendorf North America, Westbury, NY, USA) with the block preheated to 92 ^{o}C, using a reaction program of initial denaturation at 92 ^{o}C for 3 minutes, a total of 30 cycles of denaturation (30 seconds at 92 ^{o}C), annealing using a touchdown program (30 seconds per cycle from 60 to 50 ^{o}C at -0.5 degree/cycle for the first 20 cycles and 50 ^{o}C for the last 10 cycles), and extension (45 seconds at 72 ^{o}C), and a final extension at 72 ^{o}C for four minutes. The same reaction setup was used for the 16s rRNA primers, but with an annealing touchdown temperature profile of the first 10 cycles from 55 to 50 ^{o}C at -0.5 deg/cycle and the last 20 cycles at 50 ^{o}C. The touchdown program was used because it increased yield and number of bands observed on subsequent DGGE gels, over a set annealing temperature. PCR products were verified on a 1.2% agarose gel, photographed, and their yield estimated using

ImageJ software (Rasband, 2006) calibrated with a low DNA marker (50-2,000 bp, BioNexus, Inc, Oakland, CA, USA.).

DNA Separation and Analysis

DNA fragments were separated using denaturing gel gradient electrophoresis (DGGE) with a 16x16 cm, 1 mm thick gel containing 6% acrylamide, 1X TAE, and a linear gradient of 35-65% denaturant (100% denaturant is equivalent to 7 M urea and 40% formamide), cast for 90 minutes. Approximately 500 ng of PCR product was mixed with 10-20 µL of 2X gel loading dye (70% glycerol, 0.05% Bromophenol Blue, 2mM EDTA), loaded on the gel, and electrophoresed at 60 ^{o}C for 5 hours at 150V in 1X TAE, using a DCode Universal Mutation Detection System Model 475 Gradient Delivery System (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained with 50 µg/mL ethidium bromide in 1X TAE for 15 minutes and destained in 1X TAE for 10 minutes. Bands were visualized and photographed using a Fisher Biotech Model 88A variable UV intensity Transilluminator and DCode DocIt software system (Bio-Rad Laboratories, Hercules, CA, USA).

DGGE Image Analysis

The digitized gel images were analyzed using ImageJ (Rasband 2006). The background was subtracted using a rolling ball radius of 50. Bands in each lane were automatically detected and plotted. Peak area and relative intensity of each band was measured, and bands contributing less than 1% to the total intensity within one lane were omitted from subsequent analysis. No comparisons were made between different gels, because no internal standard was used. No comparisons were made between gels from the two primer sets, although the two were compared based on trends in banding patterns among the samples. A distance matrix for pairwise comparisons between lanes was generated by the unweighted Gower's distance (Equation 4-1),

$$D = \frac{1}{P}\sum_{j=1}^{P}\frac{\left|y_{1,j} - y_{2,j}\right|}{R_j} \quad , \qquad (4\text{-}1)$$

where P is the total number of bands being compared, y_1 and y_2 are the band intensities for the two lanes being compared, and R is the largest difference found across all bands in the gel (Legendre and Legendre 1998). This matrix, generated with Mathcad 13 (Mathsoft, Parametric Technologies Corporation, Needham, MA, USA), was used for hierarchical clustering with the unweighted pair group method with arithmetic mean (UPGMA), constructed using MEGA version 3.1 (Kumar et al. 2004).

DNA Sequencing and Phylogenetic Analysis

To further characterize the community of methanol-degrading bacteria present during biofilter operation and in the original inoculum, selected bands from *mxa*F-specific DGGE gels were excised for sequencing. Bands were chosen from mxaF-amplified DNA that showed the highest intensity when visualized on the UV transilluminator and were excised using a sterile pipet tip and scalpel. The gel fragments were eluted overnight at 30 ^{o}C at 250 rpm in 30 µL of an elution buffer containing 10mM Tris-Cl (pH 7.5), 50 mM NaCl, and 1mM EDTA (pH 8.0) (Chory and Pollard 1999). Gel fragments were removed, and DNA was precipitated from the liquid by adding 50 µL of 95% cold ethanol, chilling 30 minutes at -40 ^{o}C, and pelleting the DNA by centrifuging 10 minutes at 10,000xg. After pouring off the ethanol supernatant, the pellet was dried at 40 ^{o}C for 4-5 hours and resuspended in 30 µL of TE buffer (Chory and Pollard 1999). This template was reamplified using the same methods as described previously and checked on a DGGE gel for purity and for migration to the same gradient position as in the original sample. Sequencing was performed at the University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) using the fluorescent dideoxy terminator method of cycle sequencing on either a Perkin Elmer Applied Biosystems Division (PE/ABD) 373A or 377

automated DNA sequencer, following ABD protocols, with consensus sequences generated using the Sequencher Software from Gene Codes. Sequences of partial *mxa*F gene fragments have been deposited in the GenBank database under accession numbers EU099402 through EU099407.

Published sequences with high similarity to sample sequences were obtained by performing a nucleotide-nucleotide BLAST (NCBI) search. The 10 most similar sequences of known species with E scores lower than 1E-20 were chosen for each sample, with duplicates removed. Sequences were aligned using ClustalW, with default gap penalties, and manual inspection and refinement of alignments. A phylogenetic tree was constructed using the Neighbor Joining method and bootstrapped with 1,000 replicates. Because all known γ-proteobacteria clustered into a distinct branch, this group was selected as the out-group. All phylogenetic and molecular evolution analyses were conducted using MEGA version 3.1 (Kumar et al. 2004).

Results and Discussion

Biofilter Design

Results of the comparisons of granular activated carbon (GAC), Westvaco Bionuchar and Calgon F400, using two inoculation methods are shown in Table 4-2. Both inoculation methods worked well for colonizing the biofilter with an active microbial population. However, the Bionuchar GAC supported a significantly higher number of and increase in colonies and a visible biofilm, as compared to the Calgon GAC. The larger particle size of the Bionuchar likely created larger void spaces within the packed bed, allowing for better transport of oxygen through the biofilter for maintenance of aerobic inoculum cultures, less plugging with excess biomass, and increased nutrient availability to attached bacteria.

Based on these results, the Bionuchar GAC was used for all subsequent biofilter experiments. No apparent difference was observed between the two inoculation methods in their ability to adequately introduce the microbial inoculum to the GAC packing. Simply for ease of setup and maintenance, the flood and drain method was selected for subsequent biofilter experiments.

Biofilter Performance

The performance of the biofilter for methanol removal is shown in Figure 4-2, where removal efficiency (%) of both the biologically inoculated biofilter (BB) and non-inoculated biofilter (NB) is plotted as a function of time, along with the methanol loading rate to the biofilters. Each of the points plotted in Figure 4-2 represents an average for samples collected in duplicate over the 138-day operation of the biofilters. During the first 46 days of operation (at 10,000 ppmv MeOH and 5 min gas retention time), both biofilters showed excellent removal of methanol, after a lag time of about four days to achieve 100% methanol removal. The BB showed lower removal over those first four days, which is probably a result of pre-saturation of the packing material with methanol-laden medium during inoculation. When the methanol loading rate was rapidly decreased on day 47 (to 100 ppmv MeOH, with 1 min residence time), the performance of the NB also fell dramatically, with an almost 10-day period before it returned to 100% removal. When the methanol loading rate was increased to 5 g/m^3 packing/hr at day 109 (corresponding to 1,000 ppmv MeOH, with 1 min residence time), both biofilters continued to perform well. However, the NB showed another decrease in methanol removal at the conclusion of the trial, between days 133 and 138.

Although the NB did not receive the specifically enriched methanol degrading bacterial inoculum, its packing material showed a visible biofilm by day 25 (compared to a biofilm

observed on the NB on day 6), likely due to colonization by opportunistic methanol-degrading bacteria introduced by operation in non sterile conditions. In addition, the NB performed with almost equivalent removal efficiency to the BB, which suggests that the novel packing mixture used in the biofilters provided an excellent support medium for the immobilization of bacteria and subsequent bacterially mediated methanol removal, even without addition of a cultured inoculum. Bacterial growth in non-inoculated systems is not uncommon, for example, Dusenbury and Canon (2004) reported bacterial growth and contaminant removal in a non-inoculated activated carbon biofilter, even with periodic additions of chlorine bleach solution to deter biological growth. It is believed that, despite the lack of introduced inoculum to the NB, the methanol removal was biological in nature, based on results of adsorption isotherms, discussed previously.

Bacterial Counts over the Length of the Biofilters

Bacterial counts at the inlet, middle, and outlet of both biofilters at the end of the operating period were compared for three types of mineral medium, with results shown in Figure 4-3 as colony forming units (CFU) per gram of packing (with standard error from triplicate measurements). The different types of agar media were used to determine if culturable bacteria in different regions of the biofilters could be differentiated based on their nitrogen and carbon requirements. With the exception of the BB inlet and NB outlet, more bacteria were enumerated on the rich nutrient medium in samples removed from all regions of the columns. For all types of media, the distribution of bacterial abundance varied significantly among the regions of both biofilters ($F=80.4$, $p<0.001$), with significantly higher abundance in the inlet section for the BB (*post-hoc* comparison, all $p<0.001$), and significantly lower abundance in the inlet section of the NB (*post-hoc* comparison, all $p<0.012$). High abundance in the BB inlet (as compared to its

middle or outlet), where methanol concentrations are expected to be highest, was possibly due to a population from the inoculum used in the BB already being acclimated to high substrate and nutrient concentration, from the original mill source and in the inoculum culture enrichment. High colonization per gram of packing material in the BB may also have contributed to its continual high methanol removal efficiency (Figure 4-2), which would follow the observations by Song and Kinney (2000) that microbial respiratory activity in a toluene biofilter was directly proportional to the number of culturable colonies from the packing material, and that activity and CFUs were higher at the inlet of the biofilter, where the pollutant concentration was the highest.

Bacterial Diversity Comparisons

The PCR-DGGE approach was used to characterize bacterial diversity for (1) different operating conditions over time for both the BB and NB and (2) different spatial portions of the BB and NB at the end of operation. Both assessments were performed using *mxa*F and 16s rRNA primers to target methanol-degrading bacteria and all bacteria present, respectively. Results of these comparisons include gel images and the UPGMA clusters and are shown in Figures 4-4 and 4-5.

Figure 4-4A shows bacterial diversity in both biofilters from the initial inoculum through the six sampling times (the day the sample was taken is denoted by the corresponding number at the bottom of each gel column) that span the three operating conditions (Table 4-1). Considering methylotrophs, the BB showed a visibly higher bacterial diversity than the NB, which appeared to contain about half as many species. Increased diversity may relate to observations that the BB was the more reliable system (Figure 4-2). However, only a single band was observed in common among the inoculum, BB, and NB, indicating that dominant populations amplified from the inoculum culture were not dominant in the bacterial community that was observed to rapidly

colonize and be maintained in both biofilters. A potential explanation for this observation was two-fold: 1) the source of bacterial growth in the NB originated from the BB, as the two were operated in a connected air flow system and in close proximity to each other, and 2) populations present in the BB represented such a small fraction of the mixed inoculum culture that they were not sufficiently amplified by PCR for detection using DGGE. It is not unexpected that a change in conditions from the enriched culture to the biofilter environment could drastically alter the bacterial community (Devinny et al. 1999) or that PCR-DGGE methods are unable to detect bacteria comprising less than ~1% of the total bacterial community (Sercu et al. 2006). Alternatively, all of the bacterial populations in both the NB and BB could have arisen from sources outside the inoculum, by operating in non sterile conditions. Either of these possibilities supported the premise that the biofilters' performance could be attributed to use of the novel GAC mixture as biofilter packing, rather than to a specifically cultured inoculum, and coincided with the excellent removal efficiency observed for both the NB and the BB (Figure 4-2).

Banding patterns generated by amplifying 16s rRNA from both biofilters were much more varied and numerous than *mxa*F (Fig. 4-4A), which was likely due to the presence of non-methylotrophic bacteria in the biofilters. This result was consistent with the observation in Figure 4-3 that more CFUs per gram of packing were counted on heterotrophic plates than on methylotroph-specific plates. However, similar trends were observed for both primer sets, in that both biofilters appeared to be colonized by some bacterial populations that were dominant in the inoculum, as well as others that were not amplified from the inoculum, and that the community structure generally persisted over time and despite varied operating conditions.

Visual observations were supported by cluster analyses (Figure 4-4B) that took into account the relative intensity of each band, which corresponded to relative abundance of that

band's DNA in the PCR-amplified sample. UPGMA clustering results can be interpreted as the similarity between two samples based on similarities in their banding patterns. Although BB and NB shared some of the same species (Figure 4-4A), bacterial communities in each biofilter differed based on which populations were most dominant. As shown in Figure 4-4B, the greatest similarity was among populations within each biofilter, rather than within each time period or each operating condition or between the biofilters. Because there was no shift in populations over time or in different operating conditions, it was expected that acclimation or succession of bacteria in the columns occurred quickly, within in the first 22 days of operation (before the first DNA sample was extracted). Rapid acclimation could have been due to start up of the column under very high methanol loading concentrations, which may have created selective pressure for methylotrophic bacteria that became robust colonizers. Although it is uncertain what role, if any, the specifically enriched inoculum played in the BB performance, the clustering results suggest that addition of this culture did somehow influence the diversity and community structure of the consortium of bacteria that ultimately colonize the BB, possibly by changes to the biofilter microenvironment, such as surface conditioning or facilitating primary adhesion (Dunne 2002).

Figure 4-5A illustrates the bacterial diversity over three spatial regions in both biofilters, and Figure 4-5B shows the similarity of banding patterns among these regions. For the inoculated biofilter, results from PCR-DGGE with *mxaF*-specific primers and with 16S rRNA primers show opposing trends in diversity over length. The methylotrophic population appeared to be most diverse at the BB outlet, where methanol concentration is expected to be the lowest, whereas populations were more diverse at the inlet when assessed using the universal bacterial primer. Distributional patterns were much less consistent when using 16s rRNA, which showed more distinct changes among the three areas sampled in the biofilter, a similar result to the

observation by Li and Moe (2004) that relative abundance of species in a methyl ethyl ketone biofilter differed significantly as a function of height along the biofilter, likely due to selective pressures of the changing concentration gradient of introduced pollutant over the biofilter length. No such trend was clearly evident for the NB system. It was also interesting to compare these results with counts of culturable bacteria in Figure 4-3 and comparisons of the inoculum and the BB in Figure 4-4A. For methylotrophs, highest counts of CFUs were at the BB inlet, which, according to Figure 4-5A was primarily dominated by a single species. In addition, this band was also shown as being dominant in the inoculum (Figure 4-4A), which supports the possibility put forth earlier that the ability of this species to thrive in the areas expected to have the highest methanol concentration may be due to its prior acclimation to these conditions.

Figure 4-5B shows hierarchical cluster analysis for the spatial comparisons, and, for both amplification methods, indicates that the outlet and middle sections of the BB were very similar in composition, as compared to the inlet region. Despite the comparable abundance results between the two biofilters, the similarity-based clustering results indicated that the actual populations that comprise the total bacterial community at the inlet, middle, or outlet were actually quite different between the BB and the NB. Although a direct correlation cannot be made with the data available, the results suggest that growth within each column did vary based on the type of bacteria present and the means in which they were introduced to the biofilter. However, this variation did not appear to create any significant differences to observed methanol removal efficiency (Figure 4-3).

Phylogenetic Analysis of Methylotrophic Bacteria

Selected bands, corresponding to the highest relative intensity, or highest DNA concentration, amplified with *mxa*F primers, were excised, reamplified, purified, and sequenced

to determine the phylogeny of selected methylotrophic bacteria in the inoculated biofilter. A representation of phylogenetic relationship among these samples and known methylotrophic bacteria is shown in Figure 4-6. Samples are identified by their source (inoculum or BB) and numbered as shown in Figure 4-4, with bands marked with an X corresponding to those unable to be reamplified or purified. Limited sequencing for bands excised for different days in the BB and also in the NB indicated that bands occurring at the same vertical gradient had identical sequences.

Figure 4-6 shows that species obtained from both the inoculum and the biofilter were widely distributed across known types of methylotrophs. Inoculum 1 and BB1, corresponding to inoculum band 1 and BB band 1 (Figure 4-4), appeared to be closely related to species within the genera of *Methylophilus*, *Methylovorus*, and *Methylobacillus*, non-N_2 fixing, restrictive facultative methylotrophs that follow the ribulose monophosphate (RuMP) pathway for formaldehyde fixation and are classified as beta-Proteobacteria (Doronina et al. 2005; Lidstrom 2001). BB2, corresponding to BB band 2 (Figure 4-4), was grouped with several *Hyphomicrobium* species, which are also non-N_2 fixing, restrictive facultative methylotrophs, but follow the serine pathway and are members of the alpha-Proteobacteria (Lidstrom 2006; Rainey et al. 1998). BB3 and BB4, denoting BB bands 3 and 4 (Figure 4-4), were also classified with the alpha-Proteobacteria, but not clustered closely with specific known sequences. However, BB5 and Inoculum 2, corresponding to BB band 5 and inoculum band 2 (Figure 4-4) and having identical sequences, appeared to be closely related to *Beijerinckia mobilis* and *Methylocella silvest*ris, both alpha-Proteobacteria. *B. mobilis* is known to be heterotrophic, N_2-fixing, and use the ribulose bisphosphate (RuBP) pathway (Dedysh et al. 2005a), while *M. silvestris* is known to be facultatively methanotrophic, moderately N_2-fixing, and use the serine pathway (Dedysh et al.

2005b). Interestingly, both of these species are acidophilic and grow in media as low as pH 3 (Dedysh et al. 2005; Dunfield et al. 2003), and, as observed in Figures 4-4 and 4-5, the band correlating to this species increased in relative intensity over operation time and at the inlet of the BB, a region potentially growing more acidic due to operation in upflow mode and some drainage of liquid in the biofilter, although this observation could not be corroborated with actual biofilter pH measurements.

In addition to specific characteristics about the inoculum and biofilter bacterial species that are hypothesized based on this phylogenetic reconstruction, a more important observation is that the biofilters were colonized by a genetically, and likely phenotypically, diverse population of bacteria, expected to thrive in varied C- and N-usage niches. For example, the presence of expected N_2-fixing bacteria would have allowed continued growth and methanol removal even if localized nutrient supply was diminished. The observed diversity may have contributed to the continual high performance of the biofilters over time and in varied operating conditions.

Conclusions

A bench-scale inoculated GAC biofilter (BB) system was demonstrated for the removal of methanol from an artificially contaminated air stream. The methanol removal efficiency for this system and an identical, non-inoculated biofilter (NB) were similarly high (~100%) for both biofilters over the majority of operating time. Whereas the performance and abundance results would indicate that the two biofilters were very similar, an examination of the underlying microbiology using molecular methods shows that, in fact, they were colonized by very different populations of bacteria that were distributed differently throughout the length of the biofilters. Unfortunately, we cannot conclusively relate performance data to specific populations observed. However, from a broader perspective, the results underscore the need to examine microbial

diversity as part of overall design and operation strategies and performance measurements for biofilters, rather than looking at removal efficiency alone.

This work also reports the successful use of a novel heterogeneous biofilter packing material. The mixture of activated carbon, which contributed to initial adsorption of the methanol and may have helped to buffer concentration changes, combined with perlite, slow release nutrient pellets, and water retaining crystals, provided excellent support for the growth and activity of methanol degrading bacteria over time and during high variability in operating conditions. These results show the potential for developing activated carbon biofiltrations systems as potential technological solutions for methanol control in the pulp and paper industry.

Table 4-1. Summary of biofilter operating conditions

Condition	Days	Average Methanol Concentration	Average Methanol Loading Rate	Gas Retention Time
I	1-46	10,000 ppmv	17 g/m^3 packing/hr	5 min
II	47-109	100 ppmv	1 g/m^3 packing/hr	1 min
III	110-138	1,000 ppmv	5 g/m^3 packing/hr	1 min

Table 4-2. Comparison of activated carbons and inoculation methods

Method:	Circulation	Circulation	Flood/Drain	Flood/Drain
Carbon:	Calgon	Bionuchar	Calgon	Bionuchar
Initial Count: (CFU/g carbon)	1.18E+05 (2.58E+04)[1]	2.33E+05 (5.13E+04)	1.04E+06 (3.30E+05)	1.79E+06 (5.67E+05)
Final Count: (CFU/g carbon)	8.14E+06 (6.79E+05)	5.23E+07 (8.94E+06)	2.12E+07 (2.84E+06)	4.80E+08 (2.31E+07)
Factor of Increase (%)	69.2	224.3	20.3	268.1

[1]Parenthetical value is standard error for measurement in triplicate.

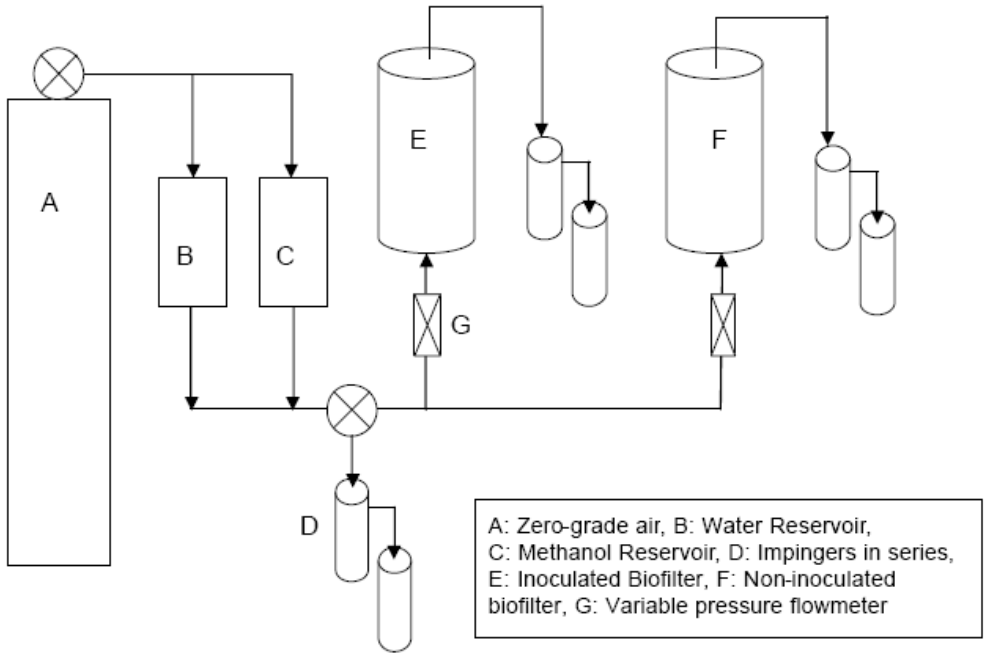

Figure 4-1. Biofilter operation schematic

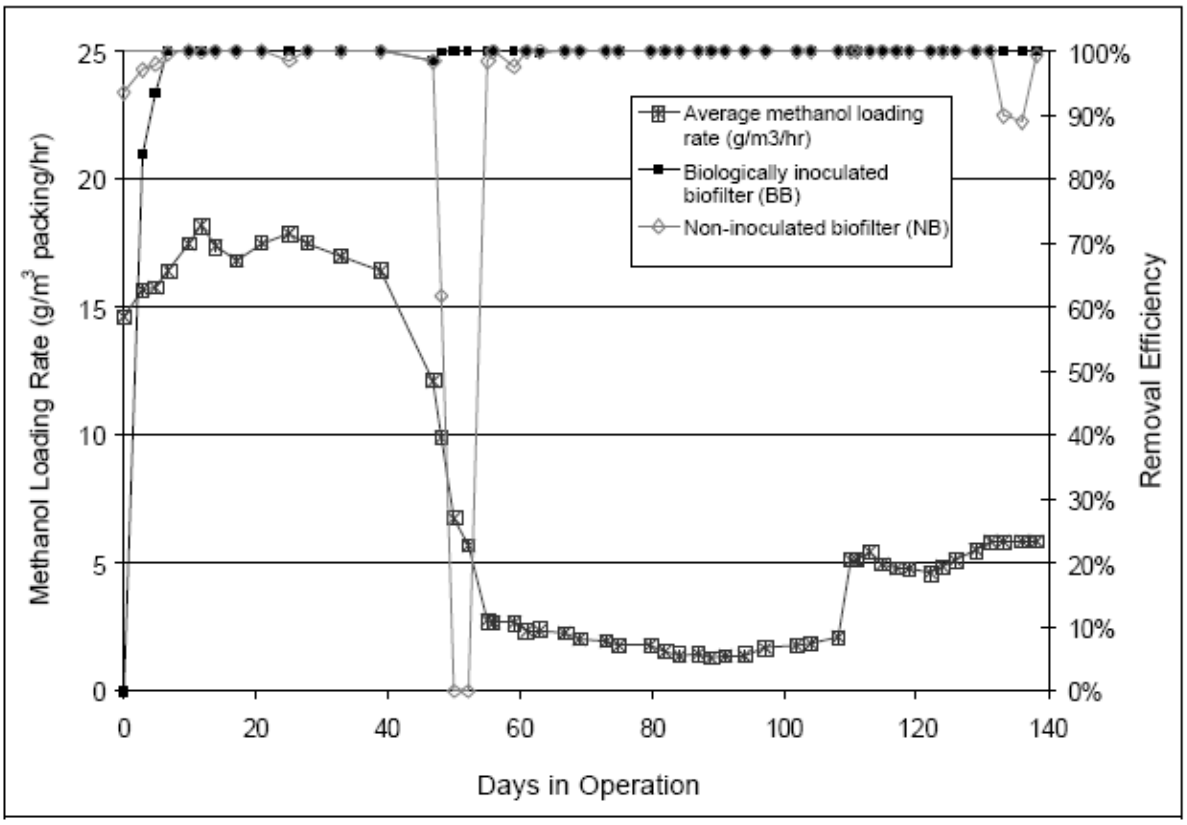

Figure 4-2. Methanol removal efficiency in the biologically inoculated and non-inoculated biofilters as a function of time and methanol loading rate. Each point is the average of two replicates collected consecutively. Standard error was less than 1% for the BB data, less than 5% for the NB data, and less than 10% for the methanol loading rate.

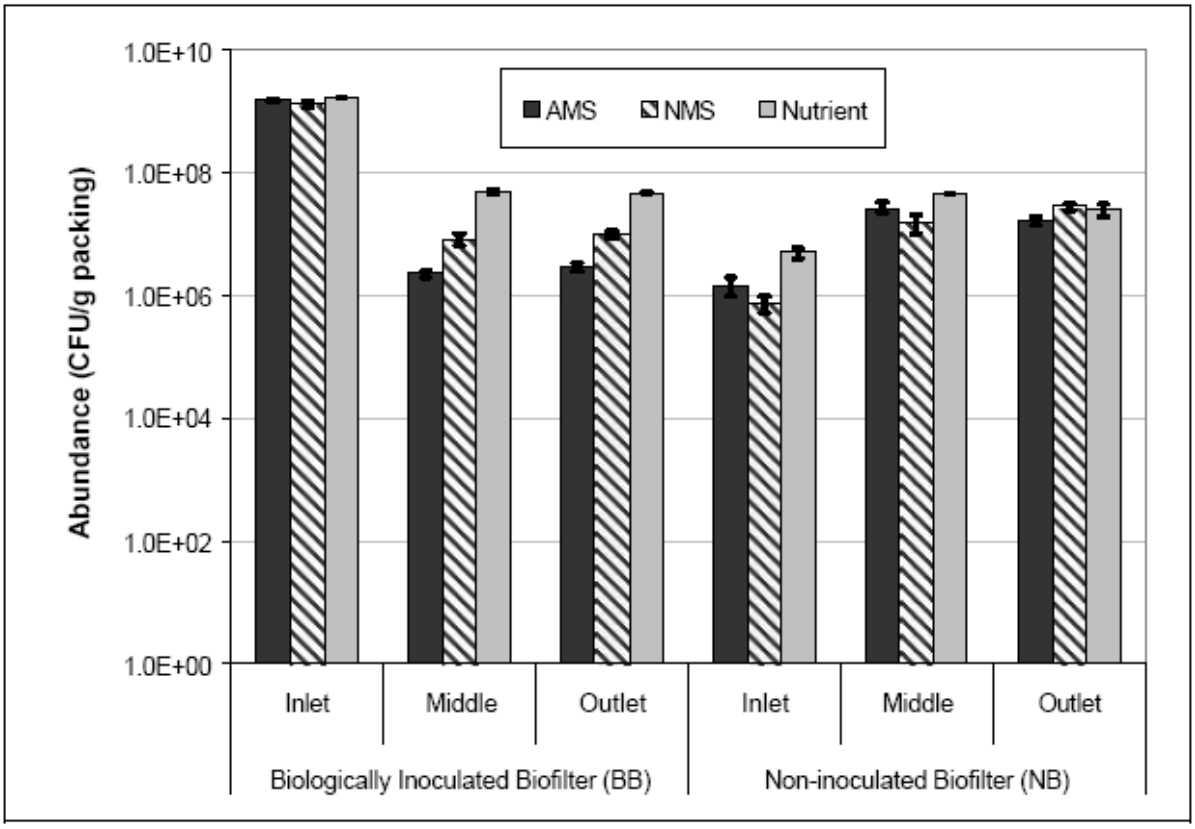

Figure 4-3. Abundance of cultivable bacteria in three spatial regions of the biofilters using three types of culture media. Error bars represent the standard error for measurements made in triplicate.

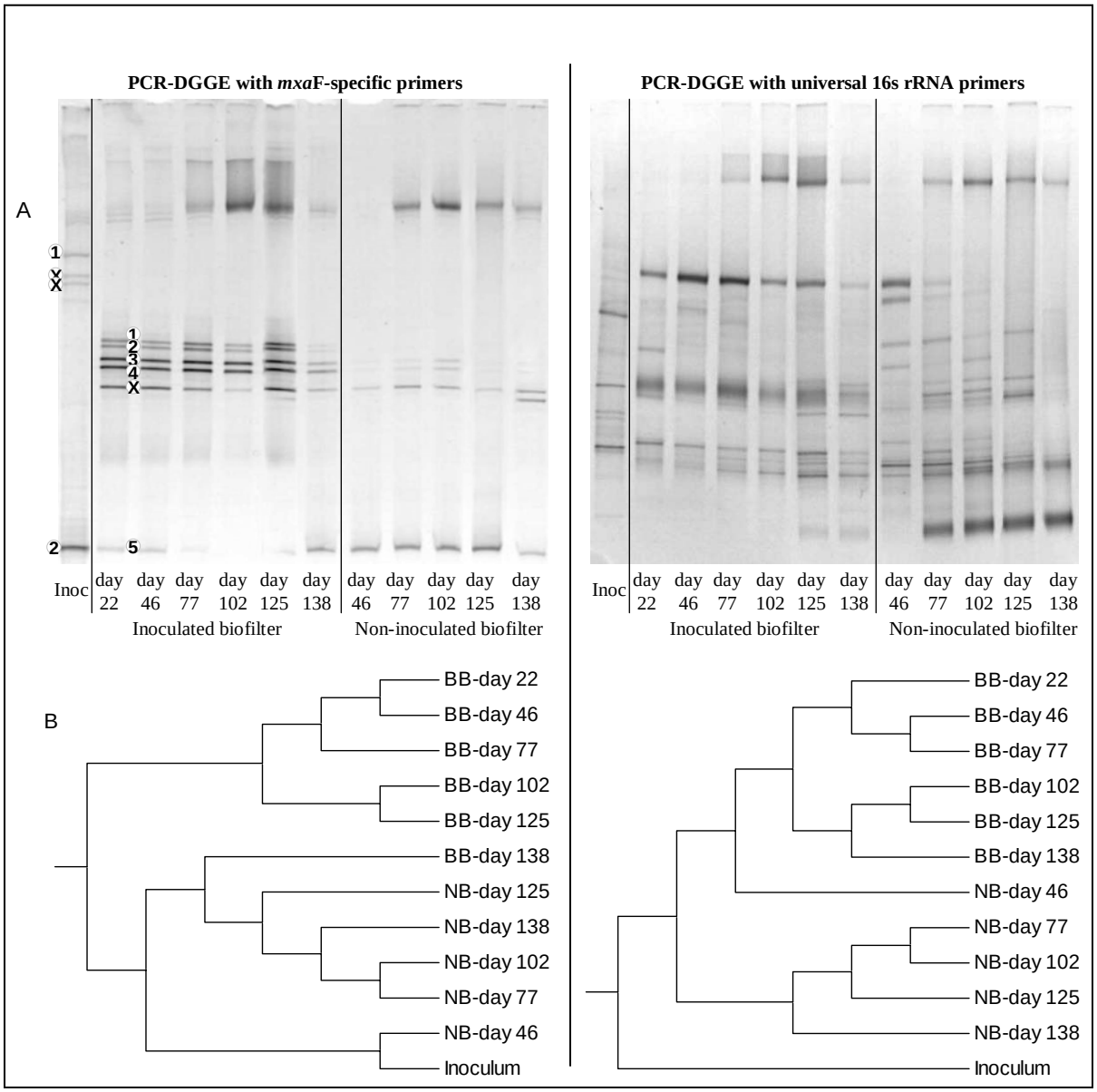

Figure 4-4. Bacterial diversity of the biofilters over time, measured using PCR-DGGE. A) Images of DGGE separated DNA fragments sampled from the biological inoculum and from the biofilters on six days during operation. B) UPGMA cluster analysis of the relatedness of PCR-DGGE banding patterns of the inoculum and the biofilters on six days during operation. Numbers at the bottom of each gel lane correspond to the day on which the packing sample was extracted from the column and used for DNA extraction and amplification. Bands marked with a circle, were excised, re-amplified, and purified for sequencing. Numbers in the circles correspond to the numbers used in phylogenetic analysis, while X in the circles corresponds to bands that were extracted, but unable to be re-amplified or purified.

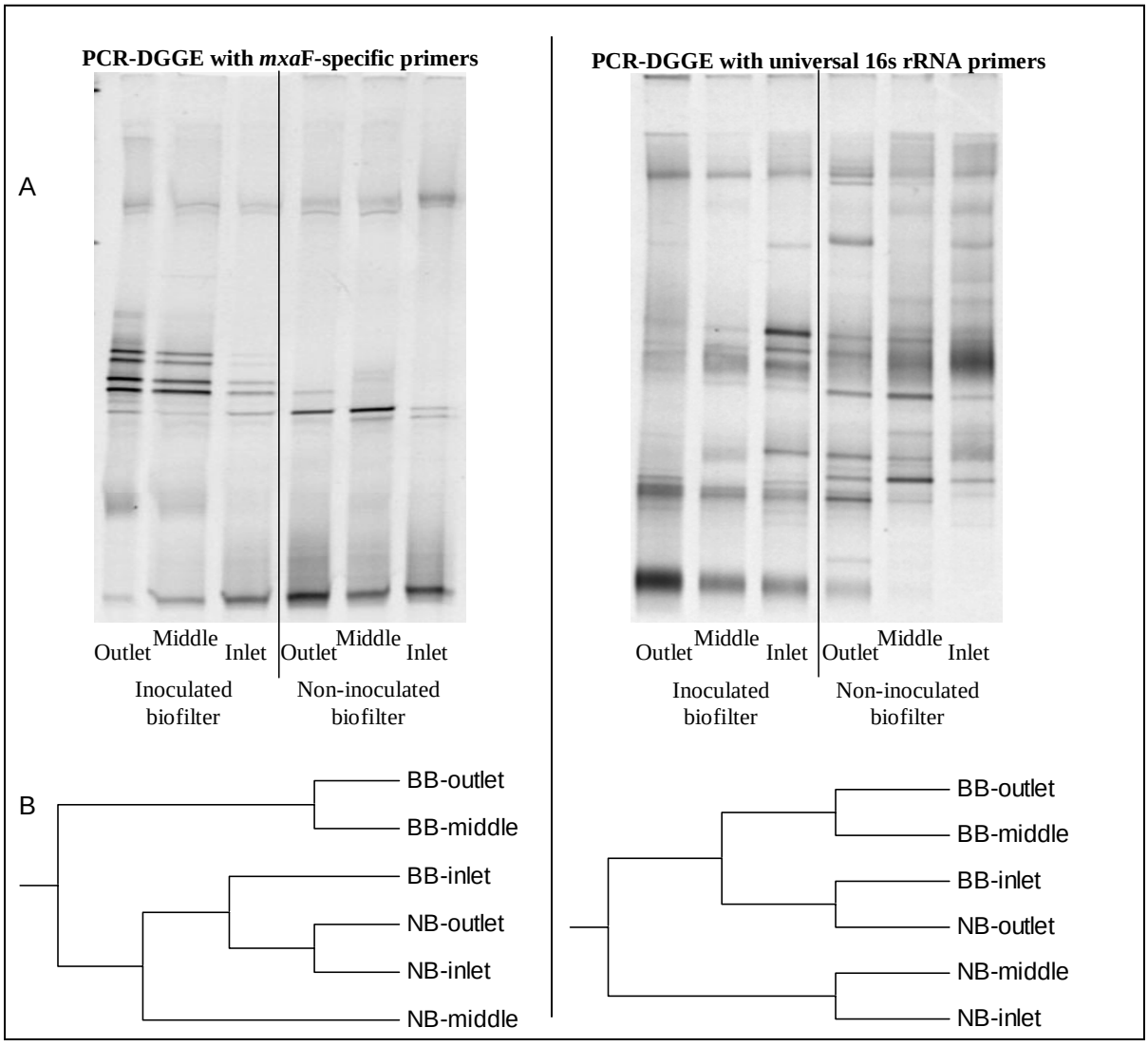

Figure 4-5. Bacterial diversity of the biofilters in different spatial regions. A) Images of DGGE separated DNA fragments sampled from three points along the length of the biofilters at the end of the operating period. B) UPGMA cluster analysis of the relatedness of PCR-DGGE banding patterns from three points along the length of the biofilters at the end of the operating period.

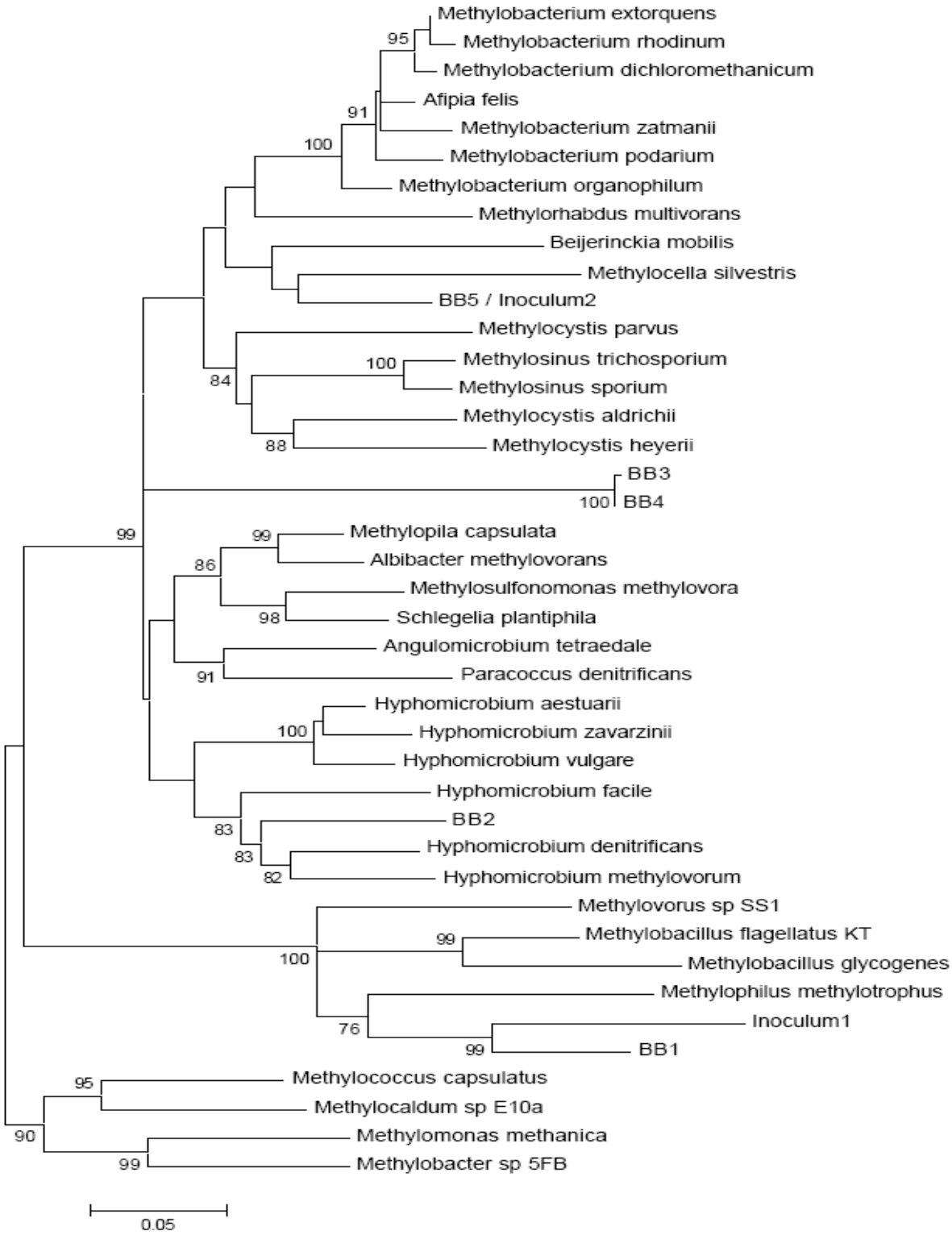

Figure 4-6. Phylogenetic reconstruction of known methylotrophic bacteria and unknown biofilter and inoculum strains using Neighbor Joining method. The inferred phylogeny was bootstrapped with 1,000 replicates, and bootstrap values greater than 75% are shown on corresponding branches

CHAPTER 5
LIFE CYCLE ASSESSMENT OF TWO OPTIONS FOR CONTROLLING HAZARDOUS AIR POLLUTANTS AT PULP AND PAPER MILLS: A COMPARISON OF THERMAL OXIDATION WITH A NOVEL PHOTOCATALYTIC OXIDATION AND BIOFILTRATION SYSTEM

Introduction

The forest products industry, including pulp, paper, and paperboard mills, is one of many industries faced with increasingly stringent regulations on allowable emissions to air and water. In 1998, the U.S. Environmental Protection Agency (U.S. EPA) promulgated guidelines and emissions standards, collectively known as the "Cluster Rule," intended to reduce the discharge of toxic pollutants in wastewaters and emissions of hazardous air pollutants (HAPs) for the forest products industry (U.S. EPA 1998). To this end, the Cluster Rule requires implementation of maximum available control technology (MACT) to limit the amount of HAPs emitted from a variety of mill processes, including chemical pulping (MACT I), papermaking and mechanical pulping (MACT II), and chemical recovery (MACT III) (EPA 2002). Specifically, MACT I regulations require pulp and paper mills to collect and treat non-condensable gas from high volume low concentration (HVLC) sources, which are defined as those producing large volumes of gas (10,000-30,000 acfm for an entire mill) and dilute concentrations (below the gas mixture's lower explosion limit) (Varma 2003). These sources include pulp washing, deckers, knotters, oxygen delignification, and chemical storage tanks. Of the HVLC gases emitted, methanol is a primary focus of MACT I standards because it is emitted in such high quantities from pulping processes (over 44,000 tons from the entire forest products industry in 2004) (EPA 2004).

The most common method for MACT I compliance adopted by pulp and paper mills is the incineration of methanol and other HVLC gases in an existing power boiler or lime kiln or in a new stand-alone device, such as a regenerative thermal oxidizer (RTO) (Varma 2003). While

effective for destroying HAPs, use of an RTO has the potential to create environmental impacts associated with the combustion of natural gas and production of nitrogen oxides (NO_x), the oxidation of carbon and reduced sulfur species to carbon dioxide (CO_2) and sulfur dioxide (SO_2), the amount of raw materials required to construct ductwork and other infrastructure for gas collection and transport, and the need to construct and operate a wet scrubber to remove SO_2 from the RTO exhaust (Mycock et al. 1995; Schnelle and Brown 2002). Recently, however, other options for HAP control have been investigated, including a treatment system using a photocatalytic oxidation (PCO) reactor for primary methanol removal (Stokke et al. 2006) and an activated carbon biofilter for secondary polishing of the air stream (Babbitt et al. 2007).

Although these new technologies show promise for methanol removal to meet MACT standards while decreasing energy requirements, how they compare to thermal oxidation in terms of life cycle environmental impacts is not known. Therefore, the goal of this work was to use a life cycle assessment (LCA) approach to compare the novel PCO-biofilter system with the more traditional RTO technology. A better understanding of potential cradle-to-grave environmental impacts associated with these technologies will not only assist industrial and regulatory communities in selecting the most environmentally friendly option but will also reveal opportunities for environmental improvement in the construction and operation of either system. This paper reports the results of a life cycle inventory, impact assessment, and sensitivity analysis, for the purpose of comparing a PCO-biofilter and a RTO-scrubber system.

Methods

Life cycle assessment (LCA) is the systematic inventory and analysis of environmental impacts for the entire life of a process or product for comparison purposes. This LCA was structured in accordance with ISO 14044:2006 guidelines (ISO 2006), which call for four phases:

1) definition of the assessment goals and scope, 2) inventory of all material and energy inputs and outputs from the system, 3) assessment of environmental impacts associated with the inventoried system inputs and outputs, and 4) interpretation of the impacts according to the defined goal and scope. Each of these phases is described in more detail below.

Goal and Scope Definition

The goal of this LCA was to compare a two-step photocatalytic oxidation and biofiltration system with a thermal oxidation system for methanol removal, to determine the environmental impacts over their entire life cycles. Results from this LCA are intended to be used in comparative assertions intended to be disclosed to the public. Unlike many recent LCA studies of treatment technologies (e.g., Jorgensen et al. 2004; Munoz et al. 2007; Sauer et al. 2002), this comparative LCA includes not only the impacts of operating the PCO-biofiltration and RTO-scrubber systems over their expected lifetimes but also of producing the required infrastructure for these systems. Inclusion of the infrastructure production-related raw materials, energy, emissions, and impacts will yield a more complete understanding of the benefits or drawbacks of both systems that are being compared.

Functional unit

The functional unit for this study was "the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal." This volumetric flow rate and methanol concentration were selected based on recommendations by industry representatives consulted for this project and the subsequent design of a pilot-scale PCO system with these specifications. The specified methanol removal rate of 98% is based on the MACT compliance options, which require thermal oxidation at 1600 ^{o}F with a 0.75 second retention

time (necessary for 98% methanol removal) or use of other control technologies provided that a 98% methanol removal is achieved (U.S. EPA 1998).

System boundaries

The system considered for this LCA was the production and operation of two air pollution control systems for removing HVLC methanol emissions generated from unit processes involved in the production of brownstock (unbleached) pulp at Kraft pulp mills. These processes include brownstock pulp washers, screens, knotters, deckers, and weak black liquor storage tanks. As previously described and as shown in Figures 5-1 and 5-2, the two treatment options considered are 1) an RTO for methanol removal, followed by a caustic wet scrubber for SO_2 control and 2) a photocatalytic oxidation (PCO) reactor for primary methanol removal followed by an activated carbon biofilter for secondary methanol removal. Life cycle inputs of raw material and energy and outputs of emissions to air were included for both control systems for three stages: 1) processing resources extracted from nature to produce primary raw materials; 2) secondary material processing to convert primary raw materials into chemicals, materials, and energy directly used for infrastructure or operation of the control systems; and 3) construction and operation of each control system for an expected 20-year lifetime.

Stage 1, "Primary Raw Material Processing," does not include the direct extraction or mining of ores or other materials from nature, but does include the processing required to convert these resources, or "inputs from nature," into useful raw materials. Stage 2, "Secondary Material Processing," includes the processes required to convert raw materials from Stage 1 directly into feedstocks or electric energy required for the construction or operation of the control equipment. Outputs from the Secondary Material Processing stage are also termed "inputs from the technosphere" in this study. In the case of the PCO-biofilter system, Stage 3 includes the

production and replacement of packing materials for the reactor and the biofilter as individual contributing categories to the total PCO-biofilter impact (Figure 5-2). These materials could not be classified distinctly as either construction or operation, but were rather major material inputs that had several replacement cycles over the lifetime of the project, as described in the assumptions detailed below. For both systems, Stage 3 also includes the emission of treated HVLC gases, as the two technologies are expected to have slightly different removal rates for VOCs other than methanol (as described in the assumptions). The total inputs and outputs from all three stages are aggregated into categories of "construction" and "operation" for comparison purposes. Transportation of materials from their point of production to the mill for installation or use was not included, due to lack of information about this stage.

Assumptions

In addition to the scope and system boundaries defined above, several assumptions about the system parameters are necessary to facilitate data collection and calculations.

1. Except where specified differently, all capital goods and infrastructure are expected to have a 20-year lifetime. Where shorter lifetimes are anticipated, total material and energy flows were adjusted to reflect replacements required to achieve 20 years of service.

2. VOCs in addition to methanol are present in the effluent air stream (NCASI 2003) and are included, to the extent known from literature, in estimates of control technology operation and performance.

3. A ratio of 100 scfm contaminated air generated from brownstock washing processes for every 1 air dry (short) ton unbleached pulp per day production rate (U.S. EPA 1979) was used to normalize inventory data given in mass per ton pulp format.

4. The RTO was sized to operate at 1600 ^{o}F, with a residence time of 0.75 seconds, for a corresponding 98% removal of methanol. All VOCs present in the contaminated air stream are converted to CO_2 according to stoichiometric ratios and 98% destruction efficiency. The regenerator beds are packed with ceramic saddles and sized for 95% thermal efficiency. Natural gas is added to support combustion of the VOCs, based on an energy balance around the RTO.

5. The RTO is located 200 ft from the brownstock washer HVLC collection point, and connected with a 3.5 ft diameter stainless steel duct.

6. The photocatalytic oxidation (PCO) reactor was designed for 90% removal of methanol, followed by an activated carbon biofilter, designed for 80% removal of remaining methanol, to achieve a total of 98% as required by regulation.

7. While the biofilter was designed for methanol removal, it also contributes 65-80% removal of other VOCs present, based on performance of pilot-scale biofilters reported in Devinny et al. (1999). This removal rate is a conservative estimate, as the VOCs may actually be more biodegradable after UV photooxidation than would be expected in their natural state (Koh et al. 2004; Moussavi and Mohseni 2007). Of the carbon in VOCs entering the biofilter, 40% was assimilated into biomass, and 60% was converted to CO_2.

8. The PCO reactor contains a fixed bed of silica-titania composite (STC) pellets, expected to have a 5 year lifetime; and uses 75W UV bulbs with a one-year lifetime for photocatalytic methanol destruction.

9. The biofilter contains a novel packing mixture of Bionuchar granular activated carbon (a wood-based carbon chemically activated with phosphoric acid), mixed in a 4:2:1 volume ratio with perlite spheres and granules of an ammonium nitrate slow release fertilizer, and this mixture is expected to have a five-year lifetime.

10. The treatment trains operate continuously, or 8,760 hours per year.

Inventory

All life cycle inventory (LCI) calculations were performed using SimaPro 5.1 (PRe Consultants, Amersfoort, The Netherlands). However, all data process modules were created specifically for this life cycle, rather than using existing databases in SimaPro (except where noted), to maintain a high level of transparency and specificity to the system of interest as well as consistent data quality levels. Inventory data were collected from interviews of environmental managers at pulp and paper mills participating in this project and engineers involved with creating HVLC control solutions for pulp and paper mills, design and operational specifications of the PCO system, bench- and pilot-scale results for the PCO reactor, bench-scale results for the biofilter, technical documents provided by the National Council for Air and Stream Improvement (NCASI), published literature, regulatory agencies, theoretical calculations, and, on a very

limited basis, from the published database by Franklin Associates (Prairie Village, Kansas, USA), provided in the SimaPro databases. A list of specific data sources is provided in Table 5-1 for each major process included.

As noted in Table 5-1, different emission factors were used for electricity production by pulp and paper mills as compared to the U.S. average production by electric generating facilities. This distinction is made because it is common practice for pulp mills to self-generate most of the electricity required on-site using waste products (e.g., bark boilers) and as part of the chemical recovery process (e.g., recovery boilers for black liquor solids) (Smook 1992). Therefore, air emissions resulting from energy production on- or off-site are significantly different. Differences in the U.S. average and mill average fuel mixes for electricity production are shown in Table 5-2. Assumptions made in this regard included the following: 1) any equipment (fans, blowers, etc.) required for operating the control systems is powered by electricity generated on site, 2) any electricity used for primary or secondary material processing is generated according to the U.S. average fuel mix, and 3) no net CO_2 is produced by combustion of bark or other wood wastes, as these biomass materials contain 'biogenic' carbon that is part of the natural carbon cycle and that does not contribute to atmospheric concentrations of carbon dioxide.

Impact Assessment

Life cycle impact assessment (LCIA) was performed to connect the mass and energy input and emission output results from the LCI to broader indicators or categories of impact to the environment or human health. For this LCIA, categories were selected based on their relevance to this study's focal areas (raw material requirements, energy use, and emissions to air, including greenhouse gases, criteria pollutants, and hazardous air pollutants (HAPs)). Therefore, categories selected include abiotic resource depletion potential (ADP), global warming potential

(GWP), photochemical oxidation potential (POP), acidification potential (AP), and human toxicity potential (HTP). Impacts were calculated based on the published impact factors of CML 2 (The Institute of Environmental Sciences, Leiden University, Leiden, The Netherlands, 2001), provided in SimaPro 5.1. This method was selected based on its widespread use and because its problem-oriented (midpoint) approach could be applied to directly relate LCI flows with the environmental areas to which they contribute impacts. In applying the CML 2 method to this LCA, characterization factors for methane and nitrous oxide global warming potential were modified slightly from published values to reflect the updated 100-year global warming potentials of the Interngovernmental Panel on Climate Change (IPCC) Third Assessment Report (IPCC 2001). No normalization or weighting of impact assessment values was performed, in accordance with ISO 14044:2006 guidelines for a LCA intended for comparative assertion intended to be disclosed to the public (ISO 2006).

Interpretation and Sensitivity Analysis

LCI and LCIA results were interpreted based on the stated goal and scope of the study to compare the two air emissions control technologies using the environmental and human health indicators of interest. In addition to this direct interpretation, a sensitivity analysis was performed on the assumptions and system boundaries used in creating the study's scope. The assumptions tested are described below.

1. **Use of a wood-based activated carbon.** The Bionuchar GAC is chemically activated from wood-based precursors such as sawdust, although many other activated carbons are produced from coal and other nonrenewable resources and activated using steam. This analysis will compare LCIA results for use of a bituminous coal-based, steam-activated GAC (such as Filtrasorb 400, Calgon Corporation, Pittsburgh, PA, USA).

2. **Lifetime of STC pellets and biofilter packing**. Both of these components for the PCO-biofilter system are assumed to have a five-year lifetime. However, it is impossible to determine if this assumption is realistic without full-scale operation in a specific pulp mill

environment. Therefore, this analysis will address possible shorter or longer lifetimes for these packing materials and the effect of their lifetime on LCIA results.

3. **UV bulb energy requirements**. The PCO reactor specifications call for 216 UV bulbs with an energy input of 75 Watts. Given the continuous operation of these bulbs, the material and energy required and resulting air emissions generated are expected to be substantial for their use. Therefore, this sensitivity analyses will examine the effect of using lower wattage bulbs, assuming that photocatalytic methanol removal could be maintained at required levels.

Results

Inventory

The results of the material and energy inventory, normalized per functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal, are shown both as inputs from the technosphere to construction and operation of the control equipment (Table 5-3) and as inputs from nature to the entire life cycle (Table 5-4). This distinction between inputs was made to illustrate various processes and materials ultimately required for both systems. Total solid waste generated is also included in Table 5-3. Emissions to air per functional unit for greenhouse gases, criteria pollutants, and other air pollutants are shown in Table 5-5. Raw materials from nature and air emissions generated were categorized for both the construction and operation phases and aggregated for both control technologies. For example, air emissions from construction of the RTO-scrubber system represented the total emissions for constructing the RTO, the scrubber, and all other infrastructure required for this system, such as duct work. In addition to the complete life cycle air emissions, the inventory also included comparative emissions of the compounds contributing 99.9% by mass to the total HAP concentration in effluent gas from HVLC sources before and after treatment by either system (Table 5-6). In all aspects of the LCI, results per functional unit represented the total input or emission over the 20-year life cycle divided by the number of

functional units occurring in the same 20-year period, to obtain results on the basis of "per functional unit," or "per 350 scfm air containing 50 ppmv methanol, treated to remove 98% of methanol."

Impact Assessment

Figure 5-3 shows comparative environmental impacts per functional unit for both technologies for the five impact categories assessed. Units shown are kg antimony (Sb) equivalent (abiotic resource depletion potential); kg carbon dioxide (CO_2) equivalent (global warming potential); kg ethylene (C_2H_2) equivalent (photochemical oxidation potential; kg sulfur dioxide (SO_2) equivalent (acidification potential); and kg 1,4-dichlorobenzene (1,4-DB) equivalent (human toxicity potential).

Sensitivity Analysis

Results of the sensitivity analysis on coal- versus wood-based granular activated carbon precursor, lifetime of biofilter packing and STC pellets, and the wattage of UV bulbs in the PCO reactor are shown in Figures 5-4, 5-5, and 5-6, respectively. All results are presented as a relative ratio of total impact of the system of interest divided by total impact of the RTO-scrubber system. Presenting relative results allowed for direct comparison of trends among the sensitivity analyses and the impact categories and eliminates the variability in units and scale that would be observed when using absolute results.

Discussion

Inventory

Based on results of the inventory of inputs from the "technosphere" into the construction and operation of the two systems (Table 5-3), it is evident that the stainless steel required to construct the RTO, scrubber, and associated ductwork (1.44E-3 kg/functional unit) and the

energy required (5.59E-2 MJ/functional unit) to produce this and other infrastructure material were substantially higher than any other material or energy input required to produce infrastructure for the PCO-biofilter system. However, there are 11 materials, in addition to water and energy, that were unique to the PCO-biofilter system because of the need to produce STC pellets (including required equipment) and biofilter packing material, both of which were expected to have a five-year lifetime. In terms of operations, the RTO-scrubber system required high volumes of water (1.08E-3 m^3/functional unit) and natural gas (1.76E-2 m^3/functional unit) to operate the wet scrubber and support VOC incineration, respectively. However, because of the use of 216 75-W UV bulbs in the PCO reactor, this system required almost an order of magnitude greater amount of electricity for operations than the RTO system (1.12 as compared to 0.224 MJ/functional unit). In terms of solid waste, the PCO-biofilter system was also the greatest contributor, due in large part to the needed replacement of biofilter packing and STC pellets every five years and the disposal of UV bulbs annually, since there was no assumption included here for recycle or reuse of any of this material. This solid waste could potentially be minimized if these materials' useful life was extended or recycling or reactivation opportunities were pursued.

Raw material inputs from nature required to produce material and energy for the entire life cycle (including the secondary materials described in Table 5-3) are shown on a per functional unit basis in Table 5-4. Trends in raw material usage were similar to what was described in Table 5-3; that is, the construction of the RTO, scrubber, and ductwork system (1.81E-1 kg/functional unit total) and the operation of the PCO-biofilter system (7.51E-1 kg/functional unit total) created the highest demands for resources. Resources for construction of both systems were distributed relatively evenly across materials required for producing

stainless steel and other metals and materials for infrastructure. On the other hand, resources required for operating the systems were those needed for producing electricity, such as wood waste and spent black liquor solids used to produce electricity on-site at the mills, as well as coal and natural gas used to produce electricity purchased by the mills to supplement their own supply. Natural gas requirements were higher for operating the RTO-scrubber system, as compared to the alternate technology, because of the need to add natural gas to support oxidation of VOCs in the HVLC gas stream. Even with energy recovered by the regenerative capacity of the system, there is still an energy deficit in the RTO, primarily because the concentration of VOCs was so low compared to the total volume of gas, that energy produced from combusting the VOCs was not sufficient to sustain required operating temperatures.

Similar trends were observed for the comparison of air emissions produced over the life cycle of both control options, per functional unit, as shown in Table 5-5. Total life cycle air emissions for most of the pollutants considered were slightly higher for the PCO-biofilter system, again due to the energy required to operate UV bulbs and the processes and chemicals used to produce the STC pellets and the activated carbon, nutrient, and perlite used as biofilter packing. Some air emissions, such as vinyl chloride, hydrofluoric acid, and ammonia, were unique to the PCO-biofilter system, and could be attributed to the secondary material processing steps used to produce feedstocks and equipment needed for STC pellet and biofilter packing production. In addition to the life cycle air emissions inventoried, the VOC emissions from HVLC sources before and after the treatment systems were also estimated per functional unit (Table 5-6). Regardless of the system used, there was expected to be a significant reduction in all VOCs present. Although the impact of this reduction was not assessed here, it is expected to be important for photochemical oxidant production and human toxicity potential. Many of these

compounds have high photochemical oxidant potential factors (e.g., the alcohols, ketones, and aldehydes) and human toxicity factors (e.g., formaldehyde), in addition to the nuisance odors associated with the reduced sulfur species, all of which would be significantly reduced by either of the technologies adopted.

Impact Assessment

As is often the case with LCA studies, the impact assessment results did not point to a clear winner between the two air emission control systems. Instead, the impact assessment results in Figure 5-3 illustrate potential environmental tradeoffs of adopting one or the other of the two options. Expected impacts of the PCO-biofilter system were approximately 20% less than impacts of the RTO-scrubber system when considering categories of abiotic resource depletion, photochemical oxidant formation, and acidification. On the other hand, the PCO-biofilter system results were 25% higher for global warming and over 50% higher for human toxicity, as compared to the RTO-scrubber. One clear trend that can be observed, however, is that in all of the impact categories considered, the construction stage of the RTO-scrubber life cycle contributed to at least 25% of the total impact. If this stage had been excluded from the system boundaries, the LCA results would have been drastically skewed toward favoring the RTO system. However, in all but the human toxicity category for the RTO, the operation phase of both systems' life cycles did contribute the highest impact. This result was due primarily to the combustion of fossil, biomass, and waste fuels to produce electricity at the pulp and paper mills that is required to operate fans and pumps required by both systems and the UV bulbs required by the PCO reactor. Electricity generation not only required the input of nonrenewable abiotic resources such as coal and crude oil, but also produced greenhouse gases that contribute to global warming and pollutants such as SO_2, which contributes to photochemical oxidation and

acidification. In all but the human toxicity category, the production of STC pellets and biofilter packing only contributed about 10% to the total impact. The increased contribution to human toxicity is a result of processing hazardous compounds, such as vinyl chloride and hydrofluoric acid, to produce materials needed for the STC pellets (such as HF) or to produce the equipment used to produce the pellets (such as PTFE or PVC).

Interpretation

Given the goal of this LCA to compare two methanol control systems based on raw material and energy inputs, and air emission and solid waste outputs, as well as the resulting environmental impact, the results shown in Figure 5-3 demonstrated that both control systems have advantages and disadvantages, depending on the impact category considered. However, these results and the sensitivity analyses shown in Figures 5-4 through 5-6 could be used to determine opportunities for minimizing environmental impact for both systems.

Sensitivity analysis

Although this LCA was performed under the assumption that GAC produced from a wood waste material would be used as the primary biofilter packing material, mills may reasonably be expected to use other forms of GAC, based on availability, different process requirements, or economics. Therefore, impacts for the entire LCA of PCO-biofilter system were compared for both types of GAC precursor, and results are shown in Figure 5-4, normalized to the impact results for the RTO-scrubber system for each category. Based on the inventory information available for this study, the resulting impact assessment showed no real variation in results for the biofilter packing stage itself or the total life cycle compared to the RTO-scrubber system. The slight increase for the coal-based carbon in the ADP category was due to the use of a nonrenewable fossil fuel rather than a wood waste as precursor. In the other

categories, wood-based GAC impacts were slightly higher, as the wood-based process was more energy intensive, based on the data available. The difference between wood- and coal-based GAC results showed no overall impact on the LCA results.

Regardless of the precursor used, requirements for the GAC and other packing material depended directly on their useful service life and required replacement. For this LCA, the biofilter packing and the STC pellets were assumed to be used for five years, thus requiring three sets of replacement material be produced to achieve a total 20-year system lifetime. However, there are numerous variables related to the actual full-scale use of these systems that may affect their lifetime. For example, process changes in the mill could reduce HVLC concentrations, thus extending the service life of all equipment. Alternatively, the presence of particulate matter or corrosive compounds in the gas stream may dramatically reduce the service life. To this end, a sensitivity analysis was performed to compare one-, five- (baseline), and ten-year lifetimes for both biofilter packing material and STC pellets, with results shown in Figure 5-5. For all of the impact categories, there was a 50% or less reduction in impacts when extending the lifetime of these materials to 10 years. The lower reductions were due to the equipment required for producing the STC pellets that were included in this life cycle system, as the materials and emissions associated with equipment production did not change regardless of pellet production rate. On the other hand, reducing lifetime to one year showed a much higher increase in all impacts, and, for every category considered, the shorter lifetime resulted in impacts higher than the total life cycle of the RTO system. For the categories of global warming, photochemical oxidant formation, and acidification potential, the increase in impact was mostly due to the biofilter packing material demand, based on the energy required for activation of the carbon and production of the ammonium nitrate nutrient. However, for the category of human toxicity, the

increased impact for a one-year packing lifetime was attributable to the use of hazardous chemicals described earlier for the production of materials and chemicals needed to manufacture the STC pellets. Ultimately, the environmental impacts of the PCO-biofilter system considered in this LCA appeared to be highly dependent on the lifetime of the packing materials. Therefore, as this technology continues development towards full-scale application, design criteria should be established to ensure extended life of all packing materials.

Another consideration in development of this innovative system is the continued use of UV bulbs and their associated raw materials and electricity requirements (thus resulting in additional emissions). Results of the sensitivity analysis on bulb energy use are shown in Figure 5-6. These results indicated that, if lower watt bulbs were able to be substituted in the PCO reactor without compromising methanol destruction, the PCO-bioreactor system would be environmentally favorable to the traditional RTO system in all impact categories except human toxicity. Use of 50W bulbs in this LCA, resulted in impacts for the PCO-biofilter system that were 10-40% less than those for the RTO-scrubber in the categories of resource depletion, global warming, acidification, and photochemical oxidation potential. These results illustrated an opportunity to not only minimize the environmental impact associated with this technology but also reduce the economic cost of operating the PCO reactor, a major incentive for the pulp and paper industry.

Conclusions

The use of life cycle assessment (LCA) for this comparative study provided a valuable way to compare two potential technologies that have been proposed as methanol control systems at pulp and paper mills. Although neither option consistently yielded lower impacts, the PCO-biofilter system showed lower environmental impacts to resource depletion, photochemical

oxidant production, and acidification over its entire life cycle, compared to the traditional approach of using a regenerative thermal oxidizer followed by a caustic scrubber. However, the RTO-scrubber system consistently showed lower life cycle impacts to global warming. With the recent increase of public attention to issues of climate change and potential future regulation on greenhouse gases, careful attention to decreasing greenhouse gas emissions associated with operational energy requirements of the PCO-biofilter system is recommended. Fortunately, unlike the more established RTO systems, this innovative methanol control system is still in development, with numerous opportunities for minimizing resource use, emissions, and life cycle environmental and human health impacts. Some of these opportunities have been demonstrated in the sensitivity analysis, particularly the need to minimize electricity use by UV bulbs in the PCO reactor and to maximize the service life of biofilter packing media and STC pellets.

Results of this LCA also showed that the impacts of producing infrastructure required for the RTO and scrubber contributed up to 25% of the total life cycle impacts for this system. From an environmental perspective, these impacts potentially could be reduced if the amount of steel required for constructing ducts were minimized by locating the RTO closer to the HVLC source (although this may not be a feasible solution given limited area near the emissions source). From a methodological perspective, however, results showing the importance of infrastructure impacts helped to demonstrate the importance of including this stage in the system boundaries. Furthermore, results of this LCA can be used to illustrate the benefits of adopting a life cycle perspective and considering environmental impact of all stages of a process when selecting and implementing industrial technology.

Table 5-1. List of data sources used for major processes in compiling the life cycle inventory of two alternative technologies for methanol control

Inventory Data	Data Source
VOC production and control	
VOC emissions from HVLC sources	NCASI 2003
Typical VOC control approaches	Varma 2003; Potlatch Corporation Air Operating Permit (ADEQ 1999); Banks 1998; Santos et al. 1992
RTO and Scrubber Construction and Operation	
RTO design and sizing	Cooper and Alley 2002; Lewandowski 2000
Scrubber design and sizing	Cooper and Alley 2002 Schifftner and Hesketh 1996
Stainless steel production	World Stainless Steel LCI (ISSF, 2006)
Caustic (NaOH) production	AP 42 Emission Factors (U.S. EPA, 1995)
PCO and Biofilter Construction and Operation	
PCO design and sizing	Stokke et al. 2006; other data provided based on design of pilot scale unit
Aluminum, copper, carbon steel, acrylic, and glass production	AP 42 Emission Factors (U.S. EPA, 1995)
Biofilter design and sizing	Babbitt et al. 2007; Devinny et al. 1999
STC pellets	
Pellet production equipment and pellet formulation	Stokke et al. 2006; other data provided based on design of pilot scale unit
PVC, CPVC, PTFE production (for equipment)	AP 42 Emission Factors (U.S. EPA, 1995)
HF, HNO_3, TEOS production	AP 42 Emission Factors (U.S. EPA, 1995)
TiO_2 production	Morters et al. 2001; Reck and Richards 1999
Biofilter packing	
Bionuchar activated carbon production	Menendez-Diaz and Martin-Gullon 2006; MeadWestvaco Virginia Corporation Draft Title V permit (KDEP 2005b)
Perlite and ammonium nitrate fertilizer production	AP 42 Emission Factors (U.S. EPA, 1995)
Energy	
Pulp and paper mill electricity production and emission factors	E-Grid (U.S. EPA 2006); NCASI GHG spreadsheet (NCASI 2004); Smook 1992
U.S. average electricity production and process specific emission factors	E-Grid (U.S. EPA 2006)
Life cycle inputs to U.S. average electricity production	Franklin Associates LCI Database (in SimaPro)

Table 5-2. Average fuel mix for electricity production in the United States and at an average pulp and paper mill

Percent of total (%)	U.S. Average	Pulp Mill Average
Coal	50.2	13.8
Natural gas	17.4	16.4
Fuel oil	3	6.4
Nuclear	20	---
Hydropower	6.6	---
Non-hydro renewables	2.6	---
Black liquor solids	---	40
Bark/wood waste	---	16
Purchased electricity (assume U.S. average)	---	7.4
Reference	U.S. EPA 2006	Smook 1992

Table 5-3. Material and energy inputs from the "technosphere" directly to and solid waste outputs from construction and operation of two alternative technologies for methanol control

Inputs (per functional unit)[1]	RTO and Scrubber	PCO Reactor and Biofilter
Infrastructure		
Stainless steel (kg)	1.44E-03	1.97E-04
Carbon Steel (kg)	---	2.12E-05
Glass (kg)	---	2.53E-04
PVC/CPVC (kg)	---	4.99E-05
Ceramic/Refractory (kg)	2.43E-04	---
Aluminum (kg)	---	4.24E-06
Copper (kg)	---	3.66E-06
Water (m^3)	2.25E-05	3.10E-06
Energy (MJ)	5.59E-02	9.36E-03
STC Pellet Production		
Stainless steel (kg)	---	2.74E-05
PVC/CPVC (kg)	---	9.09E-05
PTFE (kg)	---	3.38E-06
TEOS (kg)	---	3.22E-04
Ethanol (kg)	---	1.52E-05
TiO_2 (kg)	---	1.35E-05
HNO_3 (kg)	---	2.42E-06
HF (kg)	---	2.88E-07
Water (m^3)	---	8.11E-07
Energy (MJ)	---	1.03E-02
Biofilter Packing Production		
Nutrient (kg)	---	8.37E-04
Granular activated carbon (kg)	---	6.83E-04
Perlite (kg)	---	2.24E-04
Water (m^3)	---	9.20E-07
Energy (MJ)	---	3.94E-02
Operations		
NaOH (kg)	3.74E-04	---
Process Water (m^3)	1.08E-03	4.35E-04
Natural gas (m^3)	1.76E-02	---
Electricity (MJ)	2.24E-01	1.12E+00
Solid waste generated (per functional unit)		
Total solid waste (kg)	7.28E-04	1.66E-03

[1]All values are normalized to the functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal

Table 5-4. Raw material inputs, in kg per functional unit[1], from nature into the total life cycle of construction and operation of two alternative technologies for methanol control

	RTO and Scrubber			PCO Reactor and Biofilter				
Raw Material (kg)	Construction	Operation	Total	Construction	Operation	STC Pellets	Biofilter packing	Total
bauxite	2.27E-04	---	2.27E-04	1.56E-05	---	---	---	1.56E-05
chromium	1.17E-04	---	1.17E-04	1.60E-05	---	2.22E-06	---	1.82E-05
coal	5.33E-03	2.47E-03	7.80E-03	8.53E-04	1.13E-02	8.06E-04	1.27E-04	1.31E-02
copper (in ore)	---	---	---	3.84E-06	---	---	---	3.84E-06
crude oil	3.56E-03	1.53E-03	5.09E-03	6.18E-04	6.41E-03	8.68E-04	1.03E-04	7.99E-03
dolomite	7.52E-05	---	7.52E-05	1.02E-05	---	1.43E-06	---	1.17E-05
feldspar	---	---	---	1.95E-05	---	---	---	1.95E-05
fluorspar	---	---	---	---	---	5.84E-06	---	5.84E-06
iron (in ore)	4.22E-04	---	4.22E-04	7.97E-05	---	7.99E-06	---	8.77E-05
lignite	7.54E-05	---	7.54E-05	1.02E-05	---	1.43E-06	---	1.17E-05
limestone	6.16E-04	1.43E-04	7.58E-04	1.39E-04	6.50E-04	5.23E-05	7.32E-06	8.49E-04
manganese	2.82E-05	---	2.82E-05	3.83E-06	---	5.34E-07	---	4.36E-06
molybdenum	4.03E-05	---	4.03E-05	5.47E-06	---	7.63E-07	---	6.23E-06
NaCl	9.34E-05	3.94E-04	4.87E-04	9.97E-05	---	1.44E-04	---	2.44E-04
natural gas	3.60E-03	1.72E-02	2.08E-02	5.71E-04	8.81E-03	6.07E-04	1.22E-03	1.12E-02
nickel	2.86E-05	---	2.86E-05	3.88E-06	---	5.41E-07	---	4.42E-06
perlite	---	---	---	---	---	---	2.35E-04	2.35E-04
phosphorous	6.51E-07	---	6.51E-07	8.85E-08	---	1.23E-08	6.46E-04	6.46E-04
rutile	---	---	---	---	---	3.36E-05	---	3.36E-05
sand	---	---	---	1.90E-04	---	---	---	1.90E-04
silica	1.30E-04	---	1.30E-04	---	---	---	---	---
silicon	1.08E-05	---	1.08E-05	1.47E-06	---	4.36E-05	---	4.51E-05
soda ash	---	---	---	5.93E-05	---	---	---	5.93E-05
spent liquor solids	---	6.55E-03	6.55E-03	---	3.32E-02	---	---	3.32E-02
water	2.25E-02	1.51E-01	1.74E-01	3.10E-03	6.80E-01	8.11E-04	9.20E-04	6.85E-01
wood/wood wastes	5.68E-06	2.11E-03	2.12E-03	9.15E-07	1.07E-02	1.05E-06	1.37E-03	1.20E-02
Total	*3.69E-02*	*1.81E-01*	*2.18E-01*	*5.80E-03*	*7.51E-01*	*3.39E-03*	*4.63E-03*	*7.65E-01*

[1]All values are normalized to the functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal.

Table 5-5. Emissions to air in kg per functional unit[1], for construction and operation of two alternative technologies for methanol control

	RTO and Scrubber			PCO Reactor and Biofilter				
	Construction	Operation	Total	Construction	Operation	STC Pellets	Biofilter Packing	Total
Greenhouse Gases								
carbon dioxide (CO_2)	1.98E-02	5.46E-02	7.44E-02	3.07E-03	8.55E-02	2.33E-03	3.12E-03	9.41E-02
methane (CH_4)	3.93E-05	1.24E-04	1.64E-04	6.38E-06	1.10E-04	7.26E-06	7.13E-06	1.30E-04
nitrous oxide (N_2O)	6.50E-07	6.98E-07	1.35E-06	1.05E-07	3.16E-06	1.24E-07	1.06E-06	4.45E-06
Criteria Pollutants								
carbon monoxide (CO)	3.12E-05	8.85E-05	1.20E-04	5.24E-06	1.32E-04	3.16E-06	1.42E-05	1.55E-04
nitrogen oxides (NO_x)	4.44E-05	1.00E-04	1.44E-04	8.71E-06	1.53E-04	5.23E-06	1.30E-05	1.80E-04
sulfur dioxide (SO_2)	1.91E-04	6.62E-04	8.53E-04	3.33E-05	5.88E-04	3.04E-05	3.55E-05	6.87E-04
particulate matter (PM)	2.37E-05	9.01E-06	3.27E-05	4.86E-06	3.62E-05	4.23E-06	3.88E-06	4.92E-05
Other Air Emissions								
ammonia (NH_3)	---	---	---	5.76E-10	---	2.88E-09	1.16E-06	1.16E-06
chlorine (Cl_2)	7.70E-07	3.25E-06	4.02E-06	8.21E-07	---	1.18E-06	---	2.00E-06
hydrochloric acid (HCl)	---	1.20E-06	1.20E-06	---	---	---	---	---
hydrofluoric acid (HF)	---	---	---	---	---	3.75E-08	---	3.75E-08
mercury (Hg)	1.89E-07	5.64E-08	2.46E-07	3.03E-08	2.63E-07	3.49E-08	5.27E-09	3.33E-07
vinyl chloride (VC)	---	---	---	2.48E-07	---	8.01E-07	---	1.05E-06
non methane volatile organic compounds (NMVOC)	4.92E-05	1.65E-04	2.14E-04	7.93E-06	1.19E-04	9.08E-06	1.49E-05	1.51E-04

[1]All values are normalized to the functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal.

Table 5-6. VOC emissions, in kg per functional unit[1], from HVLC sources in the brownstock pulp washing process before treatment and estimated VOC emissions resulting from the two alternative technologies for methanol control

Emission to Air (kg)	Before Treatment	RTO and Scrubber	PCO Reactor and Biofilter
methanol	6.48E-04	1.30E-05	1.30E-05
dimethyl sulfide	2.49E-04	4.97E-06	4.97E-06
dimethyl disulfide	9.06E-05	1.81E-06	1.81E-06
Alpha-pinene	4.25E-05	8.50E-07	8.50E-06
acetone	4.18E-05	8.35E-07	8.35E-06
beta-pinene	2.50E-05	5.00E-07	5.00E-06
acetaldehyde	1.51E-05	3.02E-07	5.29E-06
o-cresol	1.30E-05	2.60E-07	4.55E-06
methyl mercaptane	1.18E-05	2.37E-07	2.37E-07
ethanol	1.17E-05	2.35E-07	2.35E-06
methyl ethyl ketone	8.97E-06	1.79E-07	1.79E-06
m-cresol	6.81E-06	1.36E-07	2.38E-06
formaldehyde	4.52E-06	9.04E-08	9.23E-07
1,2,4-trichlorobenzene	2.03E-06	4.06E-08	7.10E-07
p-cymene	1.99E-06	3.97E-08	6.96E-07
propionaldehyde	1.65E-06	3.29E-08	5.76E-07
Total VOC emissions	*1.17E-03*	*2.35E-05*	*6.11E-05*

[1]All values are normalized to the functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal.

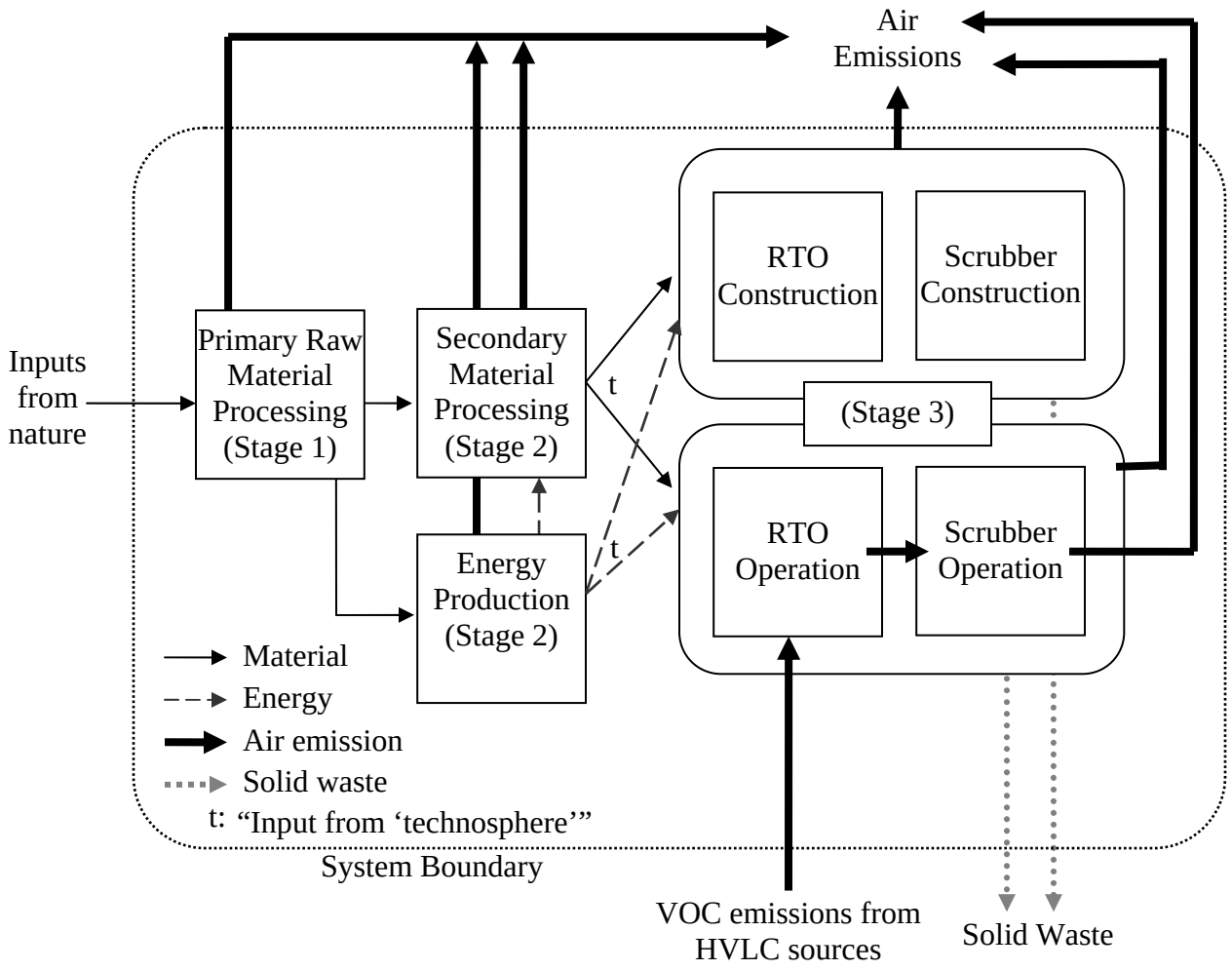

Figure 5-1. System boundaries for the life cycle of the construction and operation of a regenerative thermal oxidizer (RTO) and caustic scrubber for the treatment of methanol

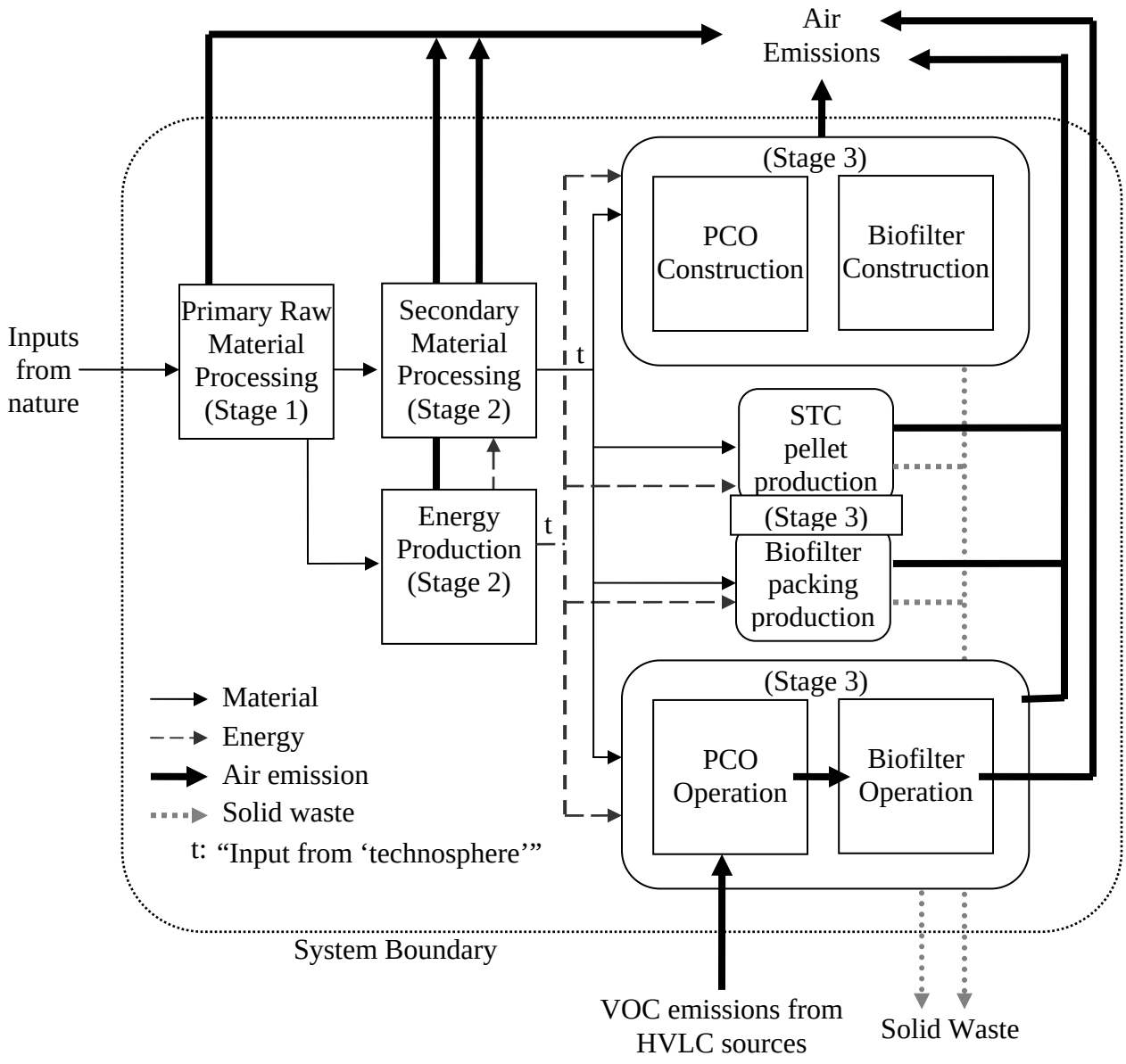

Figure 5-2. System boundaries for the life cycle of the construction and operation of a photocatalytic oxidation (PCO) reactor and biofilter for the treatment of methanol

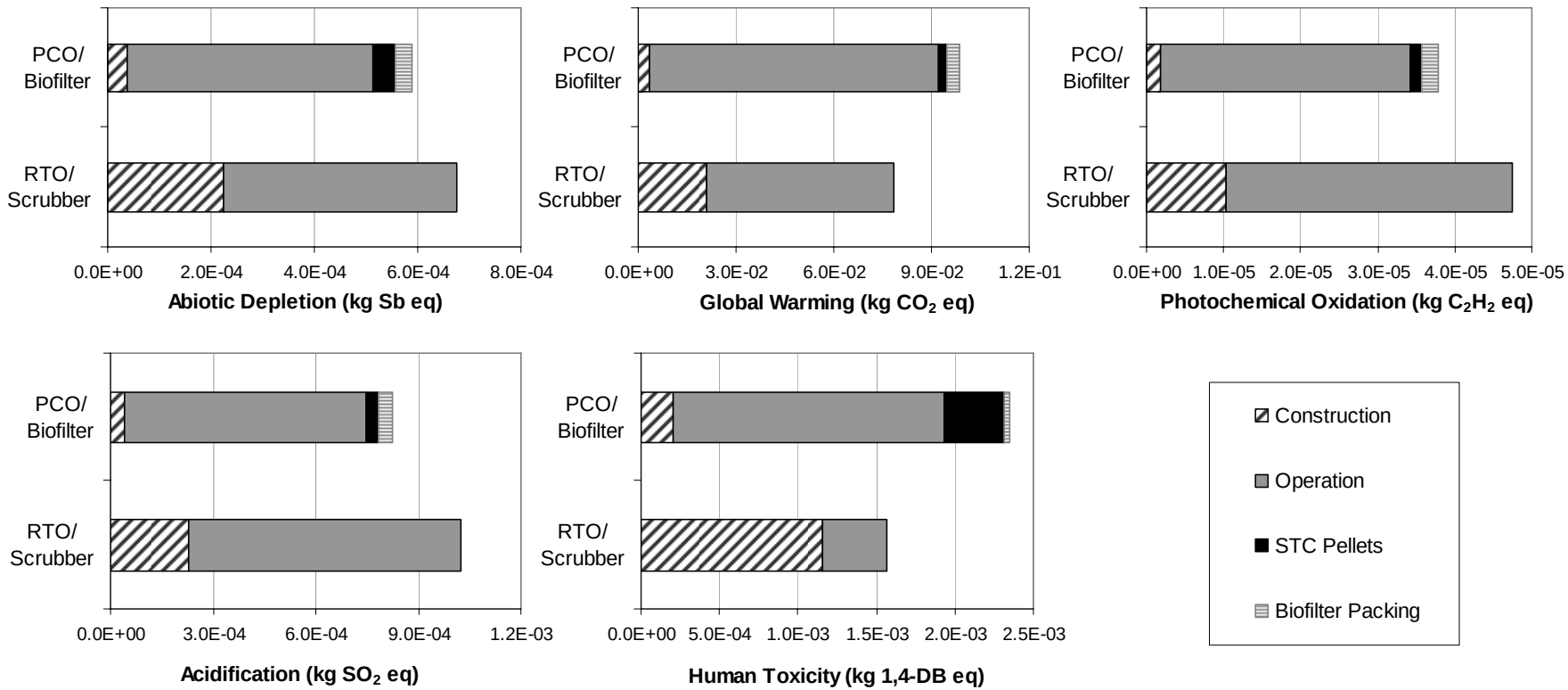

Figure 5-3. Total comparative life cycle impacts, per functional unit[1], of the construction and operation of two alternative technologies for methanol control

[1]All impacts are normalized to the functional unit of the treatment of 350 scfm of air with a methanol concentration of 50 ppmv, in order to achieve 98% methanol removal.

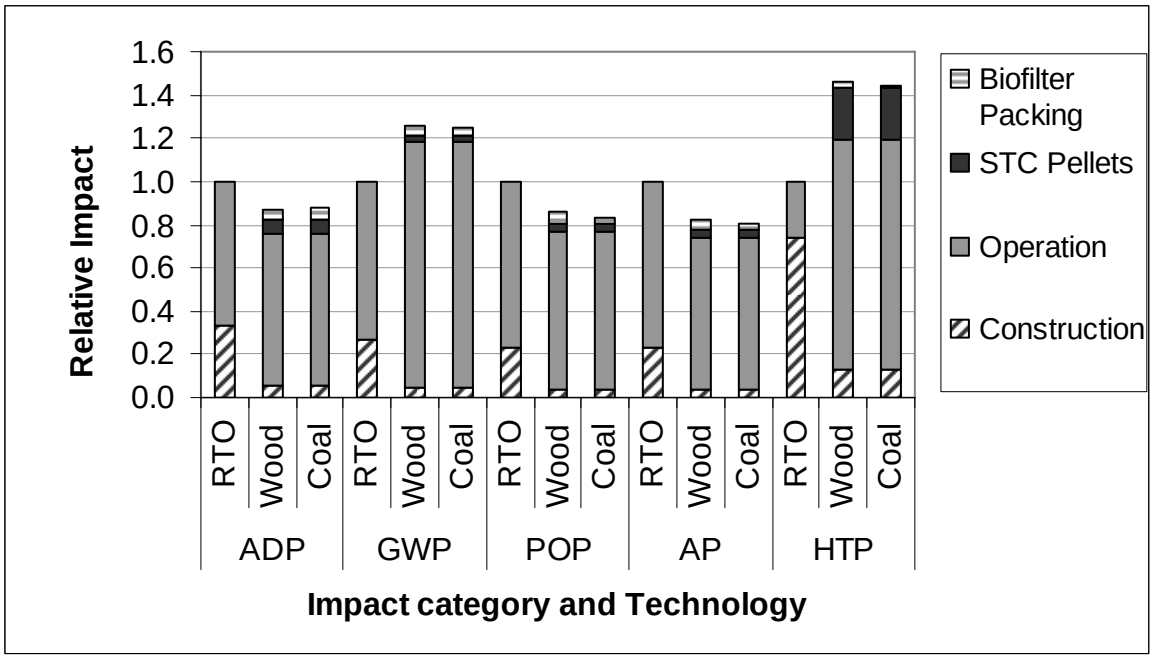

Figure 5-4. Sensitivity analysis of wood or coal used as precursor material for granular activated carbon production, based on relative impact of the PCO-biofilter system as compared to the RTO-scrubber system

All results are divided by the total impact of the RTO-scrubber system for each impact category to provide a relative impact per functional unit compared to the RTO-scrubber (relative RTO impacts are equal to 1). RTO=RTO-scrubber system; Wood=PCO-biofilter system using wood precursor for GAC production; and Coal=PCO-biofilter system using bituminous coal precursor for GAC production. discreet

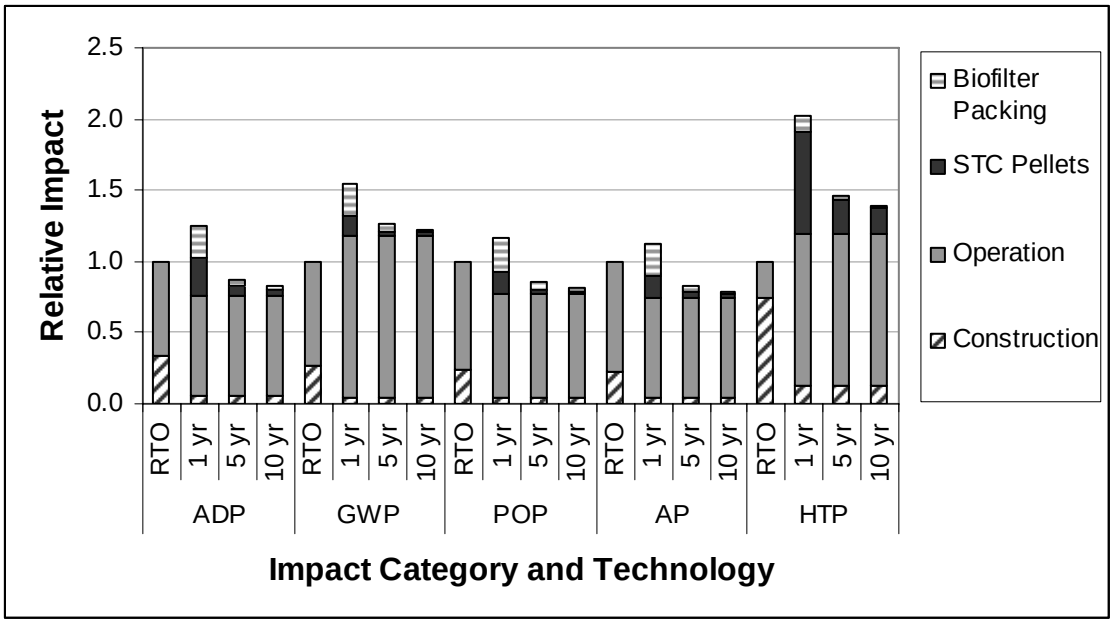

Figure 5-5. Sensitivity analysis of the lifetime of biofilter packing media and STC pellets, based on relative impact of the PCO-biofilter system as compared to the RTO-scrubber system All results are divided by the total impact of the RTO-scrubber system for each impact category to provide a relative impact per functional unit compared to the RTO-scrubber (relative RTO impacts are equal to 1). RTO=RTO-scrubber system; 1 yr, 5 yr, and 10 yr=PCO-biofilter system with a 1, 5, or 10 year (respectively) lifetime of both the biofilter packing media and the STC pellets.

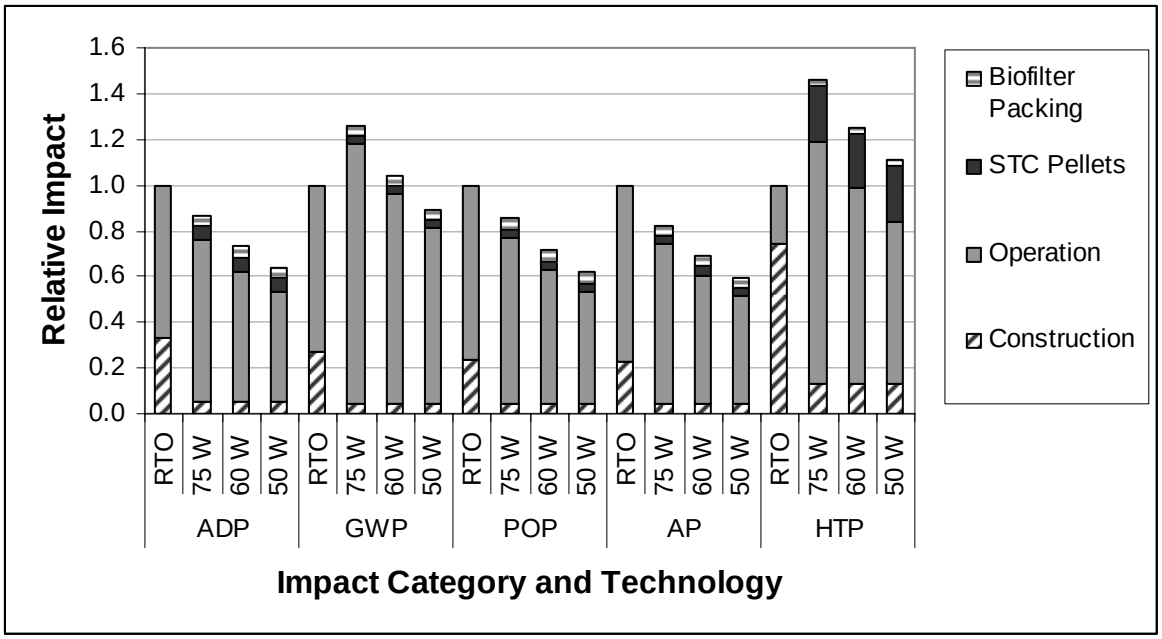

Figure 5-6. Sensitivity analysis of the lifetime energy requirement of UV bulbs in the PCO reactor, based on relative impact of the PCO-biofilter system as compared to the RTO-scrubber system

All results are divided by the total impact of the RTO-scrubber system for each impact category to provide a relative impact per functional unit compared to the RTO-scrubber (relative RTO impacts are equal to 1). RTO=RTO-scrubber system; 75W, 60W, and 50W =PCO-biofilter system with 75, 60, or 50 W (respectively) UV bulbs used in the PCO reactor. For Figures 5-4 through 5-6, ADP= abiotic resource depletion potential; GWP=global warming potential; POP=photochemical oxidation potential; AP=acidification potential; and HTP=human toxicity potential.

CHAPTER 6
SUMMARY, CONCLUSIONS, RECOMMENDATIONS AND BROADER IMPACTS

Summary

This research was conducted to investigate an activated carbon biofiltration system for methanol control in the pulp and paper industry, as a more economical and environmentally friendly alternative to thermal oxidation. This investigation focused on developing and testing a bench-scale activated carbon biofiltration system containing a novel packing mixture and capable of removing methanol from an artificially contaminated air stream in concentrations representative of industrial processes. In addition, design and operation of this biofilter enabled a more thorough study of the technology at a variety of scales and from both a theoretical and practical perspective. These studies included 1) culture-dependent and independent microbial ecology and molecular techniques to characterize biological samples from the pulp and paper industry on the basis of selecting a biofilter inoculum and optimizing their growth and biodegradation in mixed culture; 2) characterization of the activity and diversity of bacterial communities derived from the biofilter under varied operating conditions over different points in time; and 3) life cycle assessment to compare global environmental impacts of the proposed system to those associated with thermal oxidation.

Conclusions

The results of this work have shown the following:

- Biofilms obtained from a Kraft pulp mill could be enriched to support methanol degradation in the batch culture as well as to be used as inoculum for an activated carbon biofilter, as shown in Chapter 2.

- Initial phenotypic characterization of the mixed methylotrophic cultures indicated that the mill biofilms were host to highly diverse populations of bacteria able to rapidly degrade methanol, which would make for straightforward provision of an inoculum culture if a biofilter or other biological treatment system is implemented in the mill environment (Chapter 2).

- The type of nitrogen source used when enriching mixed methylotrophic cultures as potential biofilter inoculums influenced the growth and methanol degradation ability of these bacteria, with faster growth and higher methanol removal in cultures enriched with nitrate as compared to those enriched with ammonium, as shown in Chapter 3.

- Higher concentrations of the nitrogen source also resulted in greater methanol removal in batch cultures (Chapter 3), although increasing the concentration did not significantly affect growth rate of the mixed cultures. These results were promising for applications, such as a biofilter, where adding nitrogen could enhance methanol removal without causing biomass overgrowth that could lead to clogging and minimized performance.

- The form of nitrogen used also affected the diversity and community structure of the methylotrophic populations present in each of the final cultures (Chapter 3), as cultures grown on nitrate maintained a higher diversity, as measured by species richness and evenness observed from DGGE gels, compared to cultures enriched with ammonium.

- A bench-scale inoculated GAC biofilter (BB) system was successfully demonstrated for the removal of methanol from an artificially contaminated air stream (Chapter 4). The methanol removal efficiency for this system and an identical, non-inoculated biofilter (NB) were similarly high (~100%) for both biofilters over the majority of operating time.

- The excellent methanol removal was believed to be attributable to use of novel heterogeneous biofilter packing material containing a mixture of activated carbon, perlite, slow release nutrient pellets, and water-retaining crystals. This packing material provided excellent support for the growth and activity of methanol degrading bacteria over time and during high variability in operating conditions.

- Although performance results for the two biofilters were similarly high, an examination of the underlying microbiology using molecular methods showed that, in fact, they were colonized by different populations of bacteria that were distributed differently throughout the length of the biofilters. Bacterial abundance and diversity were both higher in the inoculated biofilter, which may have contributed to the robust performance of the BB over all operating conditions, even when the NB removal efficiency dropped periodically.

- The use of life cycle assessment (LCA) in Chapter 5 showed that a proposed novel treatment system consisting a photocatalytic oxidation (PCO) reactor and activated carbon biofilter had lower environmental impacts to resource depletion, photochemical oxidant production, and acidification associated with its construction and operation, as compared with a more traditional technology of a regenerative thermal oxidation (RTO) and wet scrubber system.

- The PCO-biofilter system had higher life cycle impacts to global warming and human toxicity (Chapter 5), because of the continual electricity input required to operate the UV bulbs with the PCO reactor and the materials needed to produce the STC pellets. A sensitivity analysis showed that minimizing electricity requirements for the PCO reactor

and maximizing the service life of biofilter packing media and STC pellets would greatly reduce environmental impacts for the PCO-biofilter system.

- Results of the LCA (Chapter 5) also showed that the impacts of producing infrastructure required for the RTO and scrubber contributed up to 25% of the total life cycle impacts for this system, which demonstrated the importance of including infrastructure impacts in the LCA methodological framework.

This research demonstrated that an activated carbon biofilter system is an effective and environmentally friendly option that could be developed for methanol control for the pulp and paper industry, as well as other industry sectors that are challenged with controlling HAPs and other volatile organic compounds (VOCs) in their process streams.

Recommendations and Broader Impacts

Based on results reported throughout this study, an activated carbon biofilter is a promising technology for meeting methanol control requirements for pulp and paper mills. However, limitations and additional questions that arose in this work have provoked new questions that guide recommendations for extension of this investigation. For example, the results that both an inoculated and non-inoculated biofilter provided excellent methanol removal, despite different colonizing populations, can naturally be extended into a study that focuses on the specific roles of the packing and the bacterial community. Additional study on the interactions of the introduced and colonizing bacteria with the packing and with other types of packing material could address this question. From a broader perspective, the results underscore the need to examine microbial diversity as part of overall design and operation strategies and performance measurements for biofilters, rather than focusing on removal efficiency alone.

This type of study involving batch culture characterization is important as a first step towards a better understanding of the ecology of methylotrophic bacteria and their potential use in biological treatment systems. Results indicating a preference by the batch cultures for nitrate

as the nitrogen source should be extended through additional bench- and pilot-scale observations of the methanol removal ability and underlying ecology and diversity of methylotrophic bacteria in biological treatment applications using these and other nitrogen sources and concentrations.

Finally, the results of the LCA illustrated both the environmental and human health benefits and disadvantages associated with a PCO-biofilter system, and these results can guide specific modifications to this developing technology, such as reducing operational energy requirements and increasing service life of the packing materials. Furthermore, this study illustrates the benefits of adopting a life cycle perspective and considering environmental impact for all stages of a process when selecting and implementing industrial technology. It is recommended that this perspective be continually implemented throughout future work on these systems, beginning at the lab and bench-scale and transcending to full scale technologies.

APPENDIX:
ADDITIONAL FIGURES

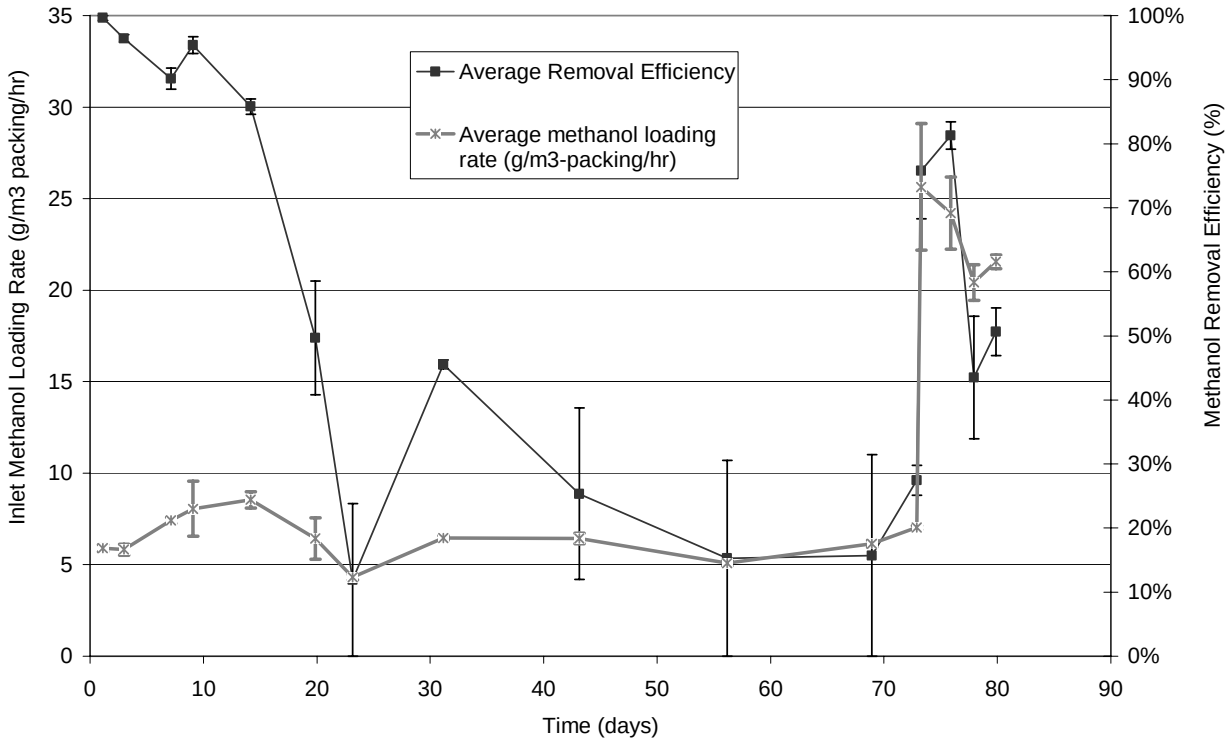

Figure A-1. Methanol removal efficiency as a function of time and methanol loading rate, in a preliminary trial of a biologically inoculated biofilters using SB as inoculum. Excessive biomass formation and clogging was observed by day 20, which correlated with loss of methanol removal performance.

Figure A-2. Photograph of preliminary biofilter, clogged by excess biomass when using SB as inoculum.

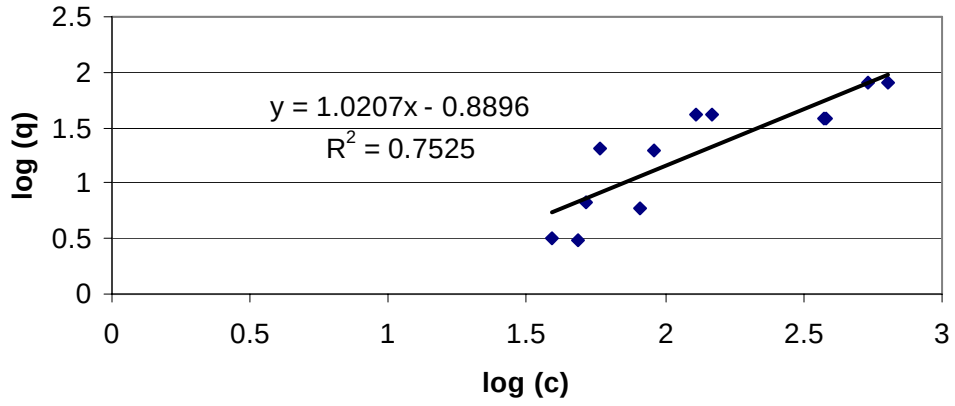

Figure A-3. Freundlich isotherm plot of batch isotherm results for the activated carbon biofilter packing material.

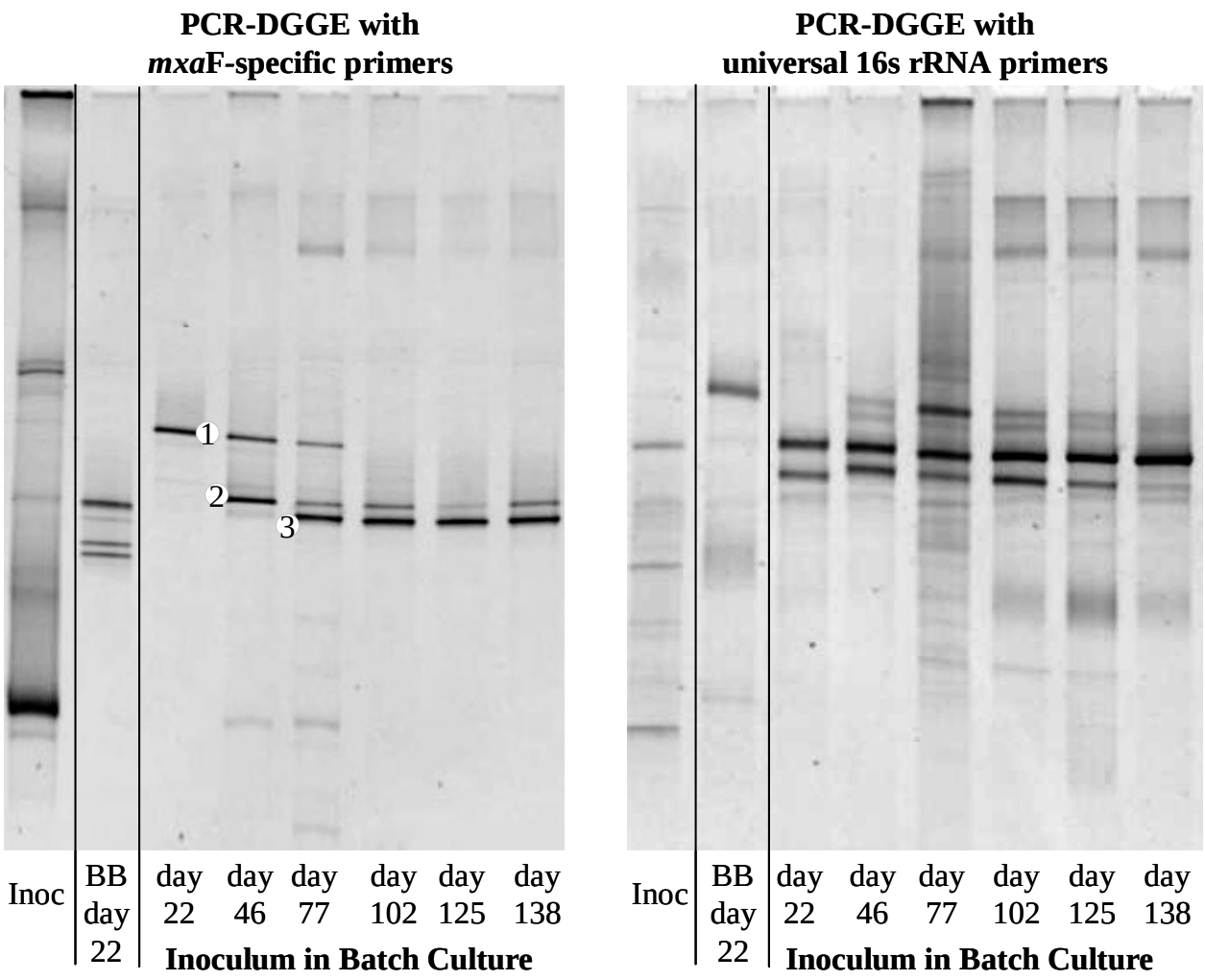

Figure A-4. DGGE analysis of mxaF and 16s rRNA gene sequences from a long-term batch culture created using the same biofilter inoculum derived from SA as used to inoculate the activate carbon biofilter "BB."

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BIOGRAPHICAL SKETCH

Callie Whitfield Babbitt received her Bachelor of Science in chemical engineering from the Georgia Institute of Technology in 2001 and her Master of Engineering in environmental engineering sciences from the University of Florida (UF) in 2003. Her master's research focused on production, disposal, and beneficial use of coal combustion byproducts created during electricity generation. Callie's current interests are in pollution prevention, life cycle assessment, industrial ecology, and microbial ecology, specifically addressing the pulp and paper industry. These topics stem from Callie's experience in the pulp and paper industry, where she first became interested in pollution prevention. She previously worked for Georgia Pacific and Buckeye Technologies, a specialty cellulose company. More recently, she worked as an assistant on air emissions and permitting projects at Golder Associates in Gainesville, FL. Callie has been a research assistant and teaching assistant for Dr. Angela S. Lindner in environmental engineering sciences at UF for 5 years. She was also a research assistant with the UF Office of Sustainability and the M.E. Rinker School of Building Construction.

These experiences shaped Callie's interest in creating environmentally and economically sustainable solutions for industry and academic institutions. Her recent work as program coordinator for Office of Sustainability Initiatives at Arizona State University focused on examining campus practices to reduce environmental impact and initiating programs to increase environmental literacy and stewardship on the ASU campus. She recently completed a university-wide greenhouse gas inventory and assisted in preparing the ASU Strategic Vision for Sustainability. These projects sparked Callie's interest in social aspects of sustainability, and she has taken a role as program coordinator for an interdisciplinary research project entitled "Late Lessons from Early History" in the School of Human Evolution and Social Change at ASU.