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Molecular Characterization of Dechlorination Potential in Kraft Pulp Mill Effluent Treatment Systems

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment for the degree of Masters of Science.

Abstract

Many low molecular weight chlorinated organic compounds produced by the pulp and paper industry during kraft bleaching of the wood pulp are toxic. Mass balance studies suggest that mineralization of chlorinated organics is occuring in pulp and paper mill wastewater treatment systems. To understand the nature of dechlorination activity, molecular tools such as oligonucleotide primers and corresponding DNA probes were developed to monitor the presence of microorganisms possessing key genes (dehalogenases) responsible for the degradation of chloroaliphatic organics in kraft pulp mill effluent treatment systems. Oligonucleotide primers designed from the coding sequence of known dehalogenases and a methane monooxygenase gene, which is known to catalyze dehalogenation reactions, were used for polymerase chain reaction (PCR) analysis, using genomic DNA extracted from dehalogenating bacterial isolates and total community DNA extracted from water and sediments of lagoon treatment systems. PCR amplification with *dhlB* oligonucleotide primers, designed from the haloacid dehalogenase of *Xanthobacter autotrophicus*, revealed the presence of dehalogenase genes in both aerated lagoons and stabilization basins. Similar results were obtained with *mmoX* primers designed from the methane monooxygenase gene of *Methylococcus capsulatus*. DNA sequence analysis of several PCR fragments showed significant similarity to known dehalogenase genes. The molecular tools developed in this study revealed the presence of different types of microorganisms with dechlorination potential in the microbial community of pulp mill effluents.

Résumé

Plusieurs des composés organochlorés créés lors du blanchiment du papier sont toxiques. Des études de bilan masse ont démontré que les composés organochlorés sont minéralisés dans les étangs d'aération et de stabilisation des usines de pâte. Des amorçes et sondes d'ADN ont été conçues à partir de la séquence codante de gènes connus (deshalogénases), afin de détecter des microorganismes ayant la capacité de minéraliser ces composés. Des analyses en chaîne par polymérase (PCR) et de séquençage ont révélé la présence de microorganismes possédant des deshalogénases similaires au gène *dhlB*, codant pour la deshalogénase haloalkanoique de *Xanthobacter autotrophicus*, dans les étangs d'aération et de stabilisation des usines de pâte. Des gènes similaires à *mmoX* codant pour la méthane monooxygénase soluble de *Methylococcus capsulatus* ont également été identifiés dans les échantillons d'eau et de sédiments provenant des mêmes étangs. Les outils moléculaires développés dans cette étude ont révélé la présence de différents types de microorganismes ayant la capacité de décomposer les produits organochlorés créés lors du blanchiment du papier, dans les étangs d'aération et de stabilisation des usines de pâte.

ACKNOWLEDGEMENTS

I wish to thank Dr. Charles Greer for teaching me so much more microbiology that I ever could have extracted from a text book. I also thank Charles for his unlimited advice on how to present my work, and for sharing his "tricks of the trade" regarding figure making on the computer.

I also wish to thank the late Dr. Jordan Ingram for having the confidence in my abilities, and giving me the opportunity to do graduate studies.

I would like to thank the Pulp and Paper Research Centre of the University of Toronto and the National Research Council of Canada for financial support during this project, and also Drs. Grant Allen and Robertha Fulthorpe of the University of Toronto for their support and encouragement.

I thank my colleagues in the lab: the microbiology crew; Judith Godbout, Anca Mihoc, Danielle Beaumier, Ina Wisse, Suzanne Labelle, Claude Masson, Maurice Cattaneo, and Barbara Zilber, for all their encouragement and good cheer, and; Lyle White, David Juck, Pedro Cortez, Luc Bourbonnière, Marc Berthelet, Marc Péloquin, Steve Hosein and Louise Laramée for providing such a stimulating and fun environment to work in. A special thanks to Louise for her friendship and company during our regular excursions to MacDonald college. I also want to thank Kim Hibbeln for her "joie de vivre", encouragement and for helping me with this manuscript.

I wish to thank my piano teacher, Mme Louisette Duchaine for being so patient and understanding when I cancelled my classes or when I simply disappeared while writing this manuscript. Thank you for always letting me believe that if it does not work out for me in science, I always have the talent for a career in music.

I want to thank my friends Nathalie Boucher, and Carole Cinq-Mars, for their support and for "forcing" me once in a while to relax and simply enjoy a nice dinner and a bottle (or two) of wine. I want also to thank Sylvie Lahaie for her frienship and for not giving up inviting me to wonderful cultural activities, art exibits and concerts.

A special thanks to my sister Marjorie and brother Jean-François for their encouragement. I also wish to thank my mom and dad for all their support and for being so proud and curious about what I do. This thesis is dedicated to them.

Finally, I want to thank Terry Roemer for his love and support. The fact that Terry understood the importance for me to do graduate studies, accepted living apart for 2 years, took those miserable train and bus rides or drove 1218 km once a month to see me, is a clear testament of his love. I feel like the luckiest woman in the world.

Contribution of co-authors to manuscript(s) for publication

Fortin, N., J. H. Ingram, R. R. Fulthorpe, D. G. Allen and C. W. Greer. Molecular characterization of the microbial dechlorination potential in pulp and paper mill effluent treatment systems. *In preparation.*

Dr. Charles Greer is my research supervisor at the Biotechnology Research Institute, and will critically be reviewing the manuscript before publication. Dr. Jordan Ingram was my academic supervisor at MacDonald college. Dr. Robertha Fulthorpe provided the bacterial dehalogenating strains and helful discussions pertaining to this project. Dr. Grant Allen is the associate director of the Pulp and Paper Centre in Toronto which provided financial support for this project.

Table of Contents

Chapter 1. Literature Review

Chapter 2. Materials and Methods

Chapter 3. Results

Chapter 4. Discussion

List of Tables

List of Figures

List of Abbreviations

AOX	adsorbable organic halogen
BOD	biological oxygen demand
bp	base pair
COD	chemical oxygen demand
CPFP	Canadian Pacific Forest Products
DBD	dibenzodioxin
DBF	dibenzofuran
DNA	deoxyribonucleic acid
ECF	elemental chlorine-free
EPA	Environmental Protection Agency
EROD	ethoxyresorufin-O-deethylase
kDa	kilodalton
kg	kilogram
MCC	modified continuous cooking
MFO	mixed-function oxidase
MSM	mineral salt medium
ng	nanogram
PCR	polymerase chain reaction
RDH	rapid displacement heating
TCDD	tetrachlorodibenzodioxin
TCDF	tetrachlorodibenzofuran
TCF	total chlorine-free
TSS	total suspended solids
YTS	yeast tryptone starch

Bleaching sequence

C	chlorination stage
D	chlorine dioxide stage
E	extraction stage
H	hypochlorite stage
P	hydrogen peroxide stage
R	reducing stage
Z	ozone stage

Chapter 1 Literature Review

1.1 Introduction

The annual world production of wood pulp for paper production is estimated at more than 160 million metric tons. The use of chlorine and chlorine dioxide for bleaching of wood pulp is the most important and dominant bleaching technique in the pulp and paper industry. In a softwood kraft mill using a conventional bleaching sequence, approximately 5 kg of total organically bound chlorine (measured as adsorbable organic halogen (AOX)) is discharged per ton of bleached pulp (Eriksson 1991). Chlorine-based bleaching has led to the identification of 200 chlorinated organic compounds along with a small quantity of highly toxic dioxins in kraft pulp mill effluents (McKague and Kringstad 1989; Sunito *et al.* 1988). The discovery in 1985 of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in fish downstream of a paper mill resulted in growing public concern over cancer-producing agents in paper products and pulp mill effluents. In the late 1980's pulp producers were voluntarily investing millions of dollars in alternative bleaching processes to reduce dependence on molecular chlorine. Extended kraft cooking and oxygen delignification techniques, as well as substantial replacement of elemental chlorine with chlorine dioxide have significantly reduced the release of chlorinated organic compounds to receiving waters (McDonough 1992; Forbes 1992).

After enormous efforts to upgrade and modernize its operations, the pulp and paper industry still finds itself the focus of a debate over potential negative environmental impact from pulp bleaching processes. Growing environmental awareness has become a major driving force for stricter government regulations of chlorinated organic compounds in mill effluents. Heavy legislation and taxes on the content of mill effluents have thus created a real incentive toward the development of bleaching alternatives that reduce or totally eliminate the use of elemental chlorine in pulp bleaching. New bleaching technologies such as peroxide and ozone-based processes as well as enzyme treatment are currently being tested.

Many Canadian mills use biological treatment facilities such as aerated stabilization basins to meet effluent requirements regarding the levels of suspended solids and biological oxygen demand (BOD). Several studies have established that 10 to 60% of the organically bound chlorine can also be removed in these treatment systems (Aprahamian and Stevens 1990; Bryant *et al.* 1987; Gergov *et al.* 1988;

Lindstrom and Mohamed 1988; Tomar and Allen 1991). Mass balance studies suggest that anaerobic degradation and dehalogenation in the benthic layer may significantly contribute to the removal of chlorinated organic compounds in the wastewater (Bryant *et al.* 1988; Chernysh *et al.* 1993; Tomar and Allen 1991). Very little is known about the bacterial community operating in these systems.

The key steps in the microbial degradation and detoxification of chlorinated compounds are enzymatic dehalogenation reactions. Several dehalogenating enzymes have been identified and grouped on the basis of dehalogenation mechanisms, substrate affinities and gene sequences. These include hydrolytic, haloalcohol, co-factor-dependent, oxidative, and reductive dehalogenases. Microorganisms have had relatively little time to evolve specific enzymes to degrade anthropogenic halogenated compounds. The variety of dehalogenating enzymes however, suggest the existence of a prevalent microbial potential for dechlorination of natural and xenobiotic halogenated compounds in the environment.

1.2 Conventional Bleaching Processes

Wood is by far the most important raw material for the production of chemical pulp. Its main component groups are cellulose, hemicelluloses, lignin and extractives (Sjöström 1981). Cellulose is a linear polysaccharide consisting of β-D-glucopyranose units which are linked by 1-4-glucosidic bonds. Wood hemicelluloses are composed of different carbohydrate units. Unlike cellulose, hemicelluloses are branched to various extents. Xylan is the predominant hemicellulose in hardwood kraft pulp and is a significant component of softwood kraft pulp hemicellulose (50%). Lignin is essentially an aromatic polymer. It is formed through the dehydrogenative polymerization of three cinnamyl alcohol derivatives: ρ-coumaryl, coniferyl, and sinapyl alcohols. The proportions of these alcohols vary with different wood species (Sjöström 1981). Softwood lignin is largely a polymerization product of coniferyl alcohol. It is a branched molecule in which the phenylpropane-based units are linked by different types of bonds. These include ether bonds of alkyl-aryl, alkyl-alkyl, and aryl-aryl configurations. Various types of carbon-carbon bonds are also found. In hardwoods, lignin is formed by the co-polymerization of coniferyl and sinapyl acohol. The ratio between the two may vary from 4:1 to 1:2. In hardwoods, the bonding between lignin and hemicellulose is primarily between lignin and xylan, possibly through arabinose side chains (Eriksson and Lindgren 1977). Finally, extractives include a variety of compounds that can be subdivided into different classes: aliphatic

extractives consist essentially of fat and waxes; phenolic extractives include hydrolyzable tannins, flavonoids, lignans, stilbenes, and tropolines; and terpenoid compounds which are present only in softwood, include a number of mono-sesqui-and diterpenes as well as various resin acids. The total content of wood extractives in wood varies greatly (1.5-5%), depending on the species, place of growth, and the age of the tree. The content of extractives can also vary considerably within the same stem.

1.2.1 Kraft and Sulfite Processes

Most chemical pulping is carried out according to either the kraft (sulfate) process or the sulfite process (Rydholm 1965). In chemical pulping processes, cellulosic fibres are separated from other wood components by chemically solubilizing and modifying lignin. During the kraft process, wood chips are treated at 160-180°C with a liquor that contains sodium hydroxide and sodium sulfide, which promote cleavage of the various ether bonds in lignin (Rydholm 1965). The resulting lignin degradation products dissolve in the alkaline pulping liquor. In addition, portions of the wood polysaccharides, especially those of the hemicellulose, are dissolved during the pulping operation (Rydholm 1965). By comparison, the sulfite process solubilizes lignin through sulfonation at elevated temperatures. The pulping liquor contains sulfur dioxide and an alkaline oxide of sodium, magnesium or calcium. Neither the kraft nor the sulfite process removes all lignin. About 5-10% of the original lignin remains in the pulp. Residual lignin cannot be removed by extended pulping without seriously degrading the polysaccharide fraction. Consequently, removal of the residual lignin, which is responsible for the dark color of kraft pulps, requires a multistage bleaching process.

In conventional bleaching, much of the lignin is depolymerized through substitution and oxidative reactions with chlorine during the chlorination (C) stage (Kringstad and Lindström 1984). After washing, the C stage is typically followed by a caustic extraction (E) stage during which alkaline soluble compounds are removed. Subsequent oxidation, usually with a combination of chlorine dioxide (D) or hypochlorite (H) stages and extraction stages are used to brighten the pulp to the required level.

1.2.2 Formation and Toxicity of Chlorinated Organic Compounds.

Chlorolignin is a major byproduct in the bleaching of wood pulp with chlorine (Kringstad and Lindström 1984). Chlorolignins have molecular weights over 1000, are rich in carboxyl and hydroxyl groups and are consequently hydrophilic (Sagfors and Starck 1988). A variety of other compounds with a relative molecular mass lower than 1000, have been detected in chlorination liquor (Kringstad and Lindström 1984). Aromatic acids are formed from residual lignin by oxidation of the α-carbon in the phenylpropane unit. Like the phenolic compounds, three kinds of aromatic acids are formed: those with one hydroxyl group (phenolic), those with two hydroxyl groups (catecholic) and those with one hydroxyl group and one methoxyl group (guaiacolic). Phenolic compounds like chlorinated catechols, chlorinated guaiacols and vanillin are also formed from residual lignin. Those phenolic compounds are presented in Figure 1. Finally, methanol (neutral compound) which is produced in abundance in the kraft pulping process (Rydholm 1965) is derived from the methoxyl groups present in lignin.

The introduction of chlorine substituents makes organic compounds more lipophilic, facilitating interactions with hydrophobic sites, for example, in enzymes, and promoting enzymatic biotransformation in general. Bioassay studies with fish have demonstrated that some of the low molecular weight chlorinated compounds, especially the phenols and guaiacols, tend to bioaccumulate (Bjorseth *et al.* 1981; McKague *et al.* 1989; Renberg *et al.* 1980). High molecular weight chlorinated organic compounds are generally considered inactive but are still of environmental concern since they can potentially degrade to lower molecular weight compounds. Neilson *et al.* (1990) demonstrated that bacteria isolated downstream of a paper mill are capable of transforming high molecular weight chlorolignins, as well as monomeric chlorinated phenols, to chlorinated veratroles of various types in surprisingly high yields. Some chlorinated veratroles are highly lipophilic and may bioaccumulate in the tissues of higher organisms such as fish.

Since the discovery in 1985 of 2,3,7,8-TCDD, in fish downstream of a pulp and paper mill, the formation of chlorinated organic compounds, in particular dioxins, has been the subject of growing concern among the general public, environmentalists and governments. The National Council for Air and Stream Improvement showed in 1987, that TCDD and tetrachlorodibenzofuran (TCDF) were formed in bleaching operations (United States Environmental Protection Agency, 1987). Additional studies

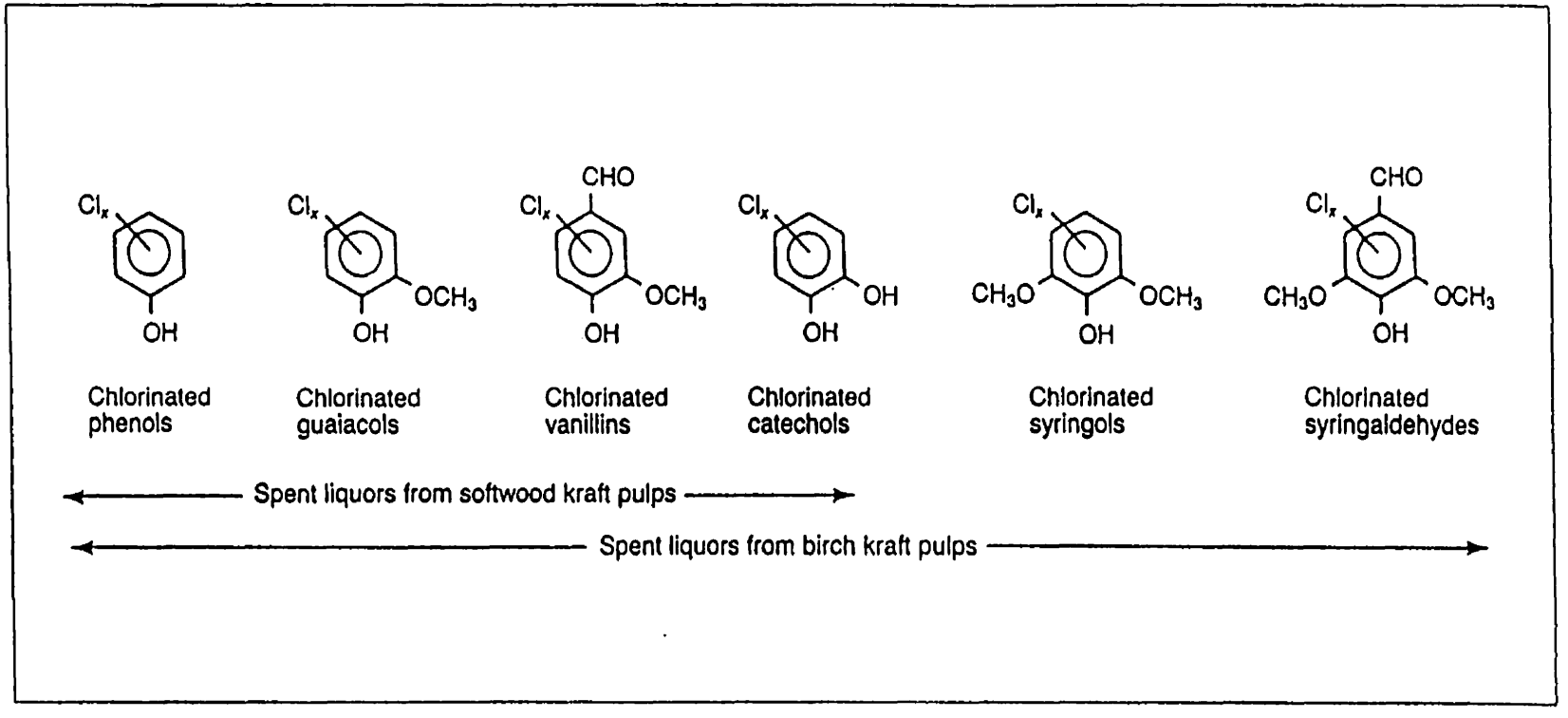

Figure 1. Phenolic compounds most frequently identified in chlorination liquors.
Adapted from Kringstad and Lindström 1984.

by the Pulp and Paper Research Institute of Canada showed that the principal cause of dioxin formation during bleaching was the reaction of elemental chlorine with precursors dibenzofuran (DBF) and dibenzodioxin (DBD) found in defoamers and oils (Allen *et al.* 1988).

The compound 2,3,7,8-TCDD is the most toxic and most extensively studied representative of polychlorinated DBF and DBD. It was shown to cause disturbances to vital biochemical and physiological functions in fish exposed to pulp mill effluents. Typical symptoms were reduced gonadal growth, liver enlargement, strong induction of mixed-function oxygenase (MFO) enzyme system, such as liver EROD (ethoxyresorufin-O-deethylase) activity, altered carbohydrate metabolism, disturbed plasma ion balance, stimulated red blood cell production, and an altered white blood cell picture indicating a weakened immune system (Förlin *et al.* 1995 (references within)). Increasing evidence suggests however, that something other than chlorinated dioxins and furans may be responsible for the effects noted in fish exposed to pulp mill effluents. Andersson (1992) showed that significant reductions in the use of chlorine between 1988 and 1990 at the Norrsundet pulp and paper mill on the Baltic Sea did not result in corresponding reductions in symptoms of increased liver size, blood lactate, or MFO enzyme activity in exposed fish. Similarly, responses such as strong induction of EROD activity and depression of plasma sex steroid levels during early gonadal growth were also seen downstream of mills that did not use chlorine (Munkittrick and Van der Kraak 1994). These results suggest that the factors responsible for the increased activity of the MFO enzymes do not appear to be related to the use of chlorine chemicals to bleach pulp.

The observed changes in biochemical parameters such as MFO enzyme system induction and changes in carbohydrate metabolism are difficult to interpret. For example, the activity of the MFO enzyme system undergoes normal fluctuations associated with reproductive cycling, seasons, temperature, and other environmental factors. The activities of these enzymes can also be affected by a variety of polycyclic aromatic chemicals, including nonchlorinated polynuclear aromatic hydrocarbons, flavones, phytosterols, and perhaps other unidentified chemicals that may or not be present in mill effluents. Virtually, all of these known MFO inducers occur naturally and are also associated with effluents and emissions from a number of anthropogenic sources (Munro *et al.* 1994).

Many chlorinated organic compounds are metabolized by MFO enzymes into more water-soluble forms, thereby facilitating excretion (Matthews and Dedrick 1984). This enzyme system is inherent in mammals, birds, aquatic species and some

invertebrates (Environment Canada 1991). The occurence of chlorinated organic compunds in the environment and their presence in diverse groups of mammalian and nonmammalian species demonstrate that organisms are capable of existing in the presence of certain concentrations of these chemicals with no apparent adverse effects.

Campaigning against existing bleaching technology seriously began when trace amounts of dioxin were discovered in products such as milk carton board, toilet tissue and coffee filters. In Sweden, both the media and the environmental pressure group Greenpeace responded by launching attacks on the use of chlorine for bleaching pulp. They were followed by an advertising campaign against baby diapers which rapidly gained momentum and spread throughout Europe.

Unlike earlier changes, which were made to reduce costs and/or improve pulp quality, many pulp producers began changing their bleaching processes in response to the overwhelming public demand for an improvement in the quality of paper products and pulp mill effluents.

1.2.3 Extended Cooking and Oxygen Delignification

One of the most attractive routes to reduced bleach plant discharges of chlorinated organic compounds and dioxins has been through modification of the pulping process to allow reduction of the residual lignin content of the unbleached pulp. New cooking methods such as modified continuous cooking (MCC), super-batch cooking, and rapid displacement heating (RDH) in extended cooking, have been developed, resulting in lower kappa numbers (a measure indicative of the lignin content in the pulp) (Andrews 1989).

The first commercial use of oxygen delignification in 1970, was undertaken to reduce water consumption, improve effluent water quality, and improve the economics of operation. Today, oxygen delignification is a well-established method for removing a substantial portion of the residual lignin in unbleached pulp. A reduction in kappa number of about 50% can be achieved using oxygen, with a relatively low loss of the carbohydrate yield and without impairing the strength properties of wood pulp (Sjöström 1993).

1.2.4 Chlorine Dioxide Substitution

As the pulp and paper industry moves toward technology that reduces chlorinated organic compounds in bleach plant effluent, a growing tendency has been

toward the use of higher percentages of chlorine dioxide substitution. High chlorine dioxide substitution has been shown to dramatically reduce the formation of AOX in bleach plant wastewater (Axegard 1986; Heimberger *et al.* 1988). Indeed, chlorine dioxide decreases the amount of AOX to one-fifth of that produced by chlorine gas. A recent study demonstrated that complete substitution of chlorine dioxide for chlorine in the first bleaching stage (elemental chlorine free (ECF) bleaching) reduced chlorinated dioxins and polychlorinated phenols to nondetectable levels in the bleach plant effluent (Stinchfield and Woods 1995).

1.3 Exotic Bleaching Processes

The environmental impact of dioxins and AOX was considerably reduced with the installations of extended kraft cooking, oxygen delignification, and substantial replacement of elemental chlorine with chlorine dioxide. However due to growing public concern for the environment in general, increasingly stringent government regulations concerning pulp and paper effluents are being introduced. Many bleach kraft pulp mills responded to those demands by experimenting with and using peroxide, ozone and enzymes to further reduce or eliminate the use of chlorine-containing compounds.

1.3.1 Peroxide Bleaching

A lot of attention has been focused on totally chlorine-free (TCF) bleaching of chemical pulp. Hydrogen peroxide (H_2O_2) is one of the key chemicals in TCF bleaching (Van Lierop *et al.* 1994). H_2O_2 under alkaline conditions can both delignify and brighten kraft pulps.

Untreated pulp contains high levels of metals such as manganese, iron and copper which catalyze the decomposition of H_2O_2. Decomposition of H_2O_2 is necessary to delignify pulp (Backman and Gellerstedt 1993), but the rate of decomposition must be carefully controlled. If H_2O_2 decomposes too fast, selectivity towards lignin is lost and cellulose degradation occurs as well as lignin degradation. Lignin degradation increases hydrophilicity which assists pulp delignification, while cellulose degradation decreases the degree of polymerization and eventually weakens the pulp. Two methods to control the metal content in kraft pulp prior to H_2O_2 bleaching have proven successful: 1) metal chelation at pH 4-7, followed by a standard wash (Basta *et al.* 1991); and 2) thorough acid wash at pH 1.5-3.0 followed by

replenishment of magnesium ions (Bouchard *et al.* 1995). However, because the bonding of metal ions is pulp specific, the optimal pH for an effective acid treatment has to be determined for each pulp.

1.3.2 Ozone Bleaching

Ozone is a bleaching chemical that can provide at least some of the delignification and brightening effects as does chlorine dioxide. One key to the successful operation of an ozone (Z) stage is an incoming pulp with a low kappa number. Consequently, ozone is often used after an oxygen delignification stage. Ozone is a strong oxidizing agent that reacts readily with almost any organic material. Ozone reactions are believed to be selective toward lignin but some of the hydroxyl radicals and carbonyl groups created during ozone bleaching also promote the direct depolymerisation of cellulose (Chirat *et al.* 1994; Chirat and Lachenal 1995). Softwood kraft pulps have been reported to be more sensitive to cellulose degradation (Patt *et al.* 1991; Soteland and Carlberg 1987). In TCF bleaching, the presence of hydroxyl radicals and carbonyl groups in the ozonated pulp contributes to further cellulose degradation during subsequent alkaline treatment. This results in a strong decrease in strength properties of the fully bleached softwood pulps. It was shown that the degradation of cellulose can be minimized by eliminating the carbonyl groups from the pulp before entering the peroxide (P) stage (Chirat *et al.* 1994; Chirat and Lachenal 1995). This can be done by subjecting the ozonated pulp to a reducing stage with sodium borohydride (R stage).

1.3.3 Enzyme Bleaching

The first significant progress in the use of enzymes for kraft pulp bleaching was reported in 1986 (Viikari *et al.* 1986). Viikari and co-workers demonstrated that pretreatment of kraft pulps with xylanases was effective at reducing the amount of chlorine needed in bleaching.

Xylan and glucomannan form the basic backbone polymers of wood hemicelluloses. A number of theories have been developed to explain why xylan hydrolysis reduces the demand for chlorine in the bleaching process. One theory proposes that enzyme treatment hydrolyzes the xylan into smaller fragments, resulting in an increased permeability to bleaching agents and improved extraction of residual lignin and lignocarbohydrates (Garg *et al.* 1996; Kantelinen *et al.* 1993). A second

theory is based on studies showing that parts of xylan initially dissolved in the kraft cooking liquor can be readsorbed or reprecipitated on and within the pulp fibers (Clayton and Stone 1967). The reprecipitation of xylan is followed by the reprecipitation of dissolved lignin during kraft pulping. It was later suggested that these redeposited polymers can be chemically linked to each other (Iversen and Wännström 1986; Jansson and Palenius 1972). The reprecipitated xylan forms a barrier for the extraction of lignin both in hardwood and softwood pulps. Xylanases are believed to act on the surface layers of the cellulosic fibres, making the pulp more permeable for the subsequent chemical extraction of the residual lignin (Kantelinen *et al.* 1993).

It seems unlikely that complete replacement of chlorine-based chemicals can be achieved by xylanase treatment alone. To obtain total chlorine-free bleaching, it would be necessary to develop enzymes capable of degrading the residual lignin.

The main goal in the first industrial application of enzymes in bleaching of kraft pulps was to reduce the consumption of chlorine chemicals. The savings typically amount to 10-15% for softwood pulps and 20-25% for hardwood pulps in both chlorine and chlorine dioxide based sequences (Senior *et al.* 1992). When combined with TCF bleaching, enzymes can also be used to increase the final brightness of pulps (Nelson *et al.* 1995; Suurnäkki *et al.* 1994). The benefits obtained by using enzymes are dependent on the chemical bleaching sequence used as well as the residual lignin content of the pulp.

The incorporation of xylanases into a pulp mill bleach sequence has proved to be relatively simple, requiring only standard equipment (Turner *et al.* 1992). However, xylanases are not gaining general use in bleaching processes because most xylanase preparations are contaminated with cellulases causing loss of yield and pulp viscosity (Puls *et al.* 1990). In addition, the pH and temperature optima of the available enzymes are often incompatible with conditions in the mill.

1.4 Government Regulations

1.4.1 Canada and United States

As of January 1, 1994, Canadian mills are forbidden to discharge "detectable" levels of 2,3,7,8-TCDD and 2,3,7,8-TCDF (Canadian Department of Environment 1992). The allowable liquid effluent concentration is equivalent to 2ppt dioxin in pulp.

The national regulations in Canada have no defined AOX limit. Provincial regulations vary widely. The province of Quebec has called for a reduction in AOX

discharge, while the provinces of British Columbia and Ontario have established that mills must virtually eliminate AOX produced in the bleach plant by 2005 (Evans *et al.* 1994).

In the United States, the proposed guidelines for regulation of pulp mill emissions by the U.S. Environmental Protection Agency (EPA) include both air and water standards in a single set of rules. The regulation is called "Cluster Rule". and mills must comply by the end of 1998. The requirements for TCDD are to be nondetectable and TCDF to be present at a daily average level no greater than 359 ng/ton.

Besides the limitations on specific compounds in the effluent from the bleach plant, Canadian provinces and the United States proposed new limits applying to the whole mill effluent at the final discharge point. They govern the levels of AOX, biochemical oxygen demand (BOD), total suspended solids (TSS), chemical oxygen demand (COD) and color. The proposed regulatory legislation is presented in Table 1. The proposed EPA limits, especially those of AOX and COD, are very demanding.

1.4.2 European Regulations

The pulp and paper industry is of major importance in Austria, Portugal, Spain, and the Scandinavian countries (Sweden, Finland and Norway). Europe and especially the German speaking countries are providing the greatest demand for chlorine-free pulp and paper products. The German market and "Green movement" has practically dictated a move toward TCF bleaching. European consumers accept lower-brightness TCF pulp over high-brightness ECF pulp especially in personal products.

Sweden has been recognized as a world leader in environmental progress. Approximately 30% of all TCF mills are located in Sweden. The operating performance of Swedish mills often exceeds the government regulations regarding AOX discharge. Swedish EPA regulations permit an AOX level of approximately 2kg/ton while the typical Swedish mill effluent averages 0.5kg/ton (Clark 1993).

Finland has effluent discharge regulations similar to those in Sweden. The AOX levels of the wastewater must be approximately 1.4kg/ton. Because pulp and paper mills in Finland are heavily dependent on export for their viability, they are supplying both ECF and TCF pulp. Approximately 12% of all TCF mills are located in Finland.

Table 1. Proposed regulatory legislation on the whole mill effluent at the final discharge point.

Year	Regulatory body	AOX kg/ton	BOD kg/ton	TSS kg/ton	COD kg/ton	Color
1993	British Columbia	2.5	7.5	11.55		
	Alberta	1.3	3.0	5.0		
	Alberta	0.3[a]				
	Ontario	2.5	5.0	4.6		
	Quebec	1.5[b]	5.0	8.0		
	Quebec	2.5[c]	5.0	8.0		
1995	British Columbia	1.5				
	Ontario	1.5				
	Quebec	1.0[b]	5.0	8.0		
	Quebec	2.0[c]				
1998	U. S. EPA					
	Max. daily	0.267	4.26	8.75	35.7	120
	Min. monthly	0.156	2.19	3.89	25.4	76.3
1999	Ontario	0.8				
2005	British Columbia	0				
	Ontario	0				
	Quebec	0.8				

a. applicable to a specific, recently constructed mill

b. hardwood

c. softwood

1.4.3 Elemental Chlorine Free or Total Chlorine Free

To date, the ECF process has the most extensive scientific data to demonstrate its environmental performance. Elemental chlorine free bleaching technologies are rapidly being selected by pulp producers throughout the world. Elemental chlorine free pulp now represents more than 25% of the total production in the United States. In British Columbia, there has been strong market pressure toward ECF pulp since most of the bleached kraft pulp is sold to Europe. However, about two-thirds of the bleached kraft mills in the world today still produce pulp with the use of elemental chlorine as a bleaching agent. One of the main attractions of chlorine is its high brightness yield. Most chemical market pulp has a brightness within the range of 90-92% ISO, a difficult level to achieve without the use of moderate levels of chlorine.

The market has a significant influence on the choice between ECF and TCF processes for pulp bleaching. The overwhelming public demand in Europe for environmentally friendly bleached products has practically dictated a move toward TCF bleaching. Virtually all TCF production is in Europe. The only U.S. producer, Louisiana Pacific in California, has experienced a rather weak demand for TCF pulp.

Extended delignification, oxygen delignification, hydrogen peroxide and ozone are all capital-intensive technologies for replacing chlorine. Historical differences in bleaching practices, market pressure and varying regulatory requirements mean that solutions must be tailored to specific regions and countries to ensure that existing mills maintain their competitiveness.

1.5 Biological Wastewater Treatment Systems

Many Canadian mills use biological treatment facilities to meet effluent requirements for biochemical oxygen demand (BOD), total suspended solids (TSS) and toxicity. One of the most common effluent treatment systems is the aerated stabilization basin or lagoon. It consists of a vigourously aerated water column and an anoxic sludge layer. These systems perform well with respect to suspended solids and BOD, and studies have shown that they can also remove between 10 and 60% of chlorinated organic compounds (Aprahamian and Stevens 1990; Bryant *et al.* 1987; Gergov *et al.* 1988; Lindström and Mohamed 1988; Tomar and Allen 1991). Several studies suggested that the major pathways for the removal of AOX in the aerated lagoon include biosorption onto biomass in the aerobic zone followed by deposition into the benthal layer, and anaerobic degradation and dehalogenation within the anaerobic zone

in the lagoon (Amy *et al.* 1988; Bryant *et al.* 1987; Bryant *et al.* 1988; Chernysh *et al.* 1992; Tomar and Allen 1991). Surveys of different treatment systems showed that aerobic and facultative anaerobic heterotrophs dominate the culturable bacterial community of the lagoons (Chernysh *et al.* 1992; Liss and Allen 1992). An understanding of the bacterial population that makes up the bulk of the culturable heterotrophic community is therefore important, since it can potentially lead to improvements in the reduction of toxicity and the removal of chlorinated organics from bleach kraft pulp mill effluents.

1.5.1 Microbiological Profile of Wastewater Treatment Systems

It was demonstrated that the culturable heterotrophic community of a bleach kraft pulp and paper mill in Ontario was dominated by populations of *Ancylobacter aquaticus*, a group of unidentified pleomorphic methylotrophic rods (*Methylobacterium* species), *Klebsiella* and *Pseudomonas* species (Fulthorpe *et al.* 1993). *Pseudomonas* species were found to be dominant in the initial clarification stage while *Ancylobacter aquaticus* and *Methylobacterium* species dominated in the aerated lagoon and settling pond. Further investigation suggested that *Methylobacterium* species may be superior to *Ancylobacter aquaticus* and *Pseudomonas* species in removing total and recalcitrant AOX from both softwood and hardwood effluents (Fulthorpe and Allen 1995).

1.6 Genetics and Biochemistry of Dehalogenating Enzymes

Historically, the first enzymes involved in dehalogenation reactions were defined as dehalogenases (Jensen 1951). The name started being applied to enzymes involved in hydrolytic reactions for the dehalogenation of 2-haloalkanoic acids. Initially, specificity in naming dehalogenases came by either making reference to different substrates (for example, aliphatic dehalogenase, haloalkane dehalogenase) or early mechanistic classifications (for example, halidohydrolase). Various attempts have been made to group dehalogenases on the basis of substrate affinities, reaction kinetics, detailed molecular mechanisms and sensitivities to inhibitory compounds (Hardman 1991; Little and Williams 1971; Slater 1994). The most recent effort in classifying dehalogenases is based on dehalogenation mechanisms (Slater *et al.* 1995).

1.6.1 Hydrolytic Dehalogenases

The most common dehalogenation mechanism appears to be a hydrolysis reaction. Hydrolytic dehalogenases were first identified in organisms growing on chloroacetic acid, but were subsequently detected in organisms growing on other halocarboxylic acids and haloalkanes. Hydrolytic dehalogenases catalyze the cleavage of carbon-halogen bonds through a nucleophilic substitution by water to yield alcohol. Two sub-groups reflecting substrate specificities were proposed: the first group includes a variety of haloalkanoic acid dehalogenases and the second group is composed of haloalkane dehalogenases (Table 2).

1.6.1.1 2-Haloalkanoic Acid Hydrolytic Dehalogenases

The significant number of 2-haloalkanoic acid dehalogenases that have been identified to date, have been divided into four classes (Table 2). Class 1L hydrolytic dehalogenases remove halides from L-2-haloalkanoic acids inverting the product configuration with respect to the substrate. Nine members of this class have now been identified and they share a high degree of sequence similarity. *Pseudomonas* sp. strain CBS3 produces two dehalogenases of this class (Schneider *et al.* 1991). *Pseudomonas* CBS3 is a bacterial strain that utilizes 4-chlorobenzoate as the sole carbon and energy source (Klages *et al.* 1980). 4-chlorobenzoate was found as an intermediate in the degradation of polychlorinated biphenyls (Abramowicz 1990). In addition to the enzyme that dehalogenates 4-chlorobenzoate to 4-hydroxybenzoate (Thiele *et al.* 1987), this strain was shown to contain several dehalogenating enzymes. A two-component enzyme system termed 4-chlorophenylacetic acid 3,4-dioxygenase dehalogenated 4-chlorophenylacetic acid to 3,4-dihydroxyphenylacetate (Klages and Lingens 1981). The two 2-haloalkanoic acid dehalogenase genes *dehC1* and *dehC2* catalyze hydrolytic dehalogenation of monochloroacetate and L 2-chloropropionate to D-lactate.

The other enzymes were found in *Xanthobacter autotrophicus* GJ10, *Moraxella* species strain B, *Rhizobium* species, and various *Pseudomonas* species. *Xanthobacter autotrophicus* is a nitrogen fixing hydrogen bacterium that produces two different hydrolytic dehalogenases: a haloalkane dehalogenase and a haloalkanoic acid dehalogenase (Janssen *et al.* 1985; van der Ploeg *et al.* 1991). In conjunction with two dehydrogenases, these two enzymes enable the bacterium to grow on 1,2-dichloroethane as a sole carbon and energy source (Janssen *et al.* 1989). The pathway for 1,2-dichloroethane degradation is presented in Figure 2. The first step in the

Table 2. Classification of two groups of dehalogenases involved in the dehalogenation of haloaliphatic compounds. Adapted from Slater *et al.* 1995.

Group	Sub-group	Class	Characteristics	Example	Reference
Hydrolytic dehalogenase	2-haloalkanoic acid hydrolytic dehalogenase	1D	D-isomer specific-	*hadD-P. putida* AJ1	Barth *et al.* 1992 Smith *et al.* 1990
			Inverts substrate product configuration	*hadD-Rhizobium*	Cairns 1994
		1L	L-isomer specific-	*hadL-P. putida* AJ1	Jones *et al.* 1992
			Inverts substrate product configuration	*dehCI-Pseudomonas* sp. CBS3	Schneider *et al.* 1991
				dehCII-Pseudomonas sp. CBS3	Schneider *et al.* 1991
				dhlB-X. autotrophicus GJ10	van der Ploeg *et al.*1991
				deh109 -P. putida 109	Kawasaki *et al.* 1994
				hdlIVa-P. cepacia MBA4	Murdiyatmo *et al.* 1992
				dehH2-Moraxella sp. B	Kawasaki *et al.* 1992
				hadL-Rhizobium	Cairns 1994
		2I	D-and L-isomers as substrates	*dehII--P. putida* PP3	Weightman *et al.* 1982 Topping 1992
			Inverts substrate-product configuration		
		2R	D-and L-isomers as substrates	*dehI-P. putida* PP3	Weightman *et al.* 1982 Topping 1992
			Retains substrate-product configuration	*hlC-A. xylosooxidans*	Brokamp 1991

Table 2. Continued.

Group	Sub-group	Class	Characteristics	Example	Reference
Hydrolytic dehalogenase	Haloalkane hydrolytic dehalogenase	3R	restricted range of substrates	*dhlA-X. autotrophicus* GJ10	Janssen *et al.* 1989
				Ancylobacter aquaticus AD20 and AD25	van den Wijngaard 1992
				X. autotrophicus GJ11	van den Wijngaard 1992
		3B	broad substrate specificity	*R. erythropolis* Y2	Sallis *et al.* 1990
				Arthrobacter sp. HA1	Scholtz *et al.* 1987
				Corynebacterium sp. m15-3	Yokata *et al.* 1987
				X. autotrophicus GJ70	Janssen *et al.* 1988
Haloalcohol dehalogenase		4S	broad substrate specificities	*Corynebacterium* sp. N-1074	Nakamura et al. 1994
			simple multimeric proteins	*Arthrobacter* sp. AD2	van den Wijngaard 1991
		4C	narrow substrate specificities	*Corynebacterium* sp. N-1074	Nakamura *et al.* 1994
			complex multimeric proteins		

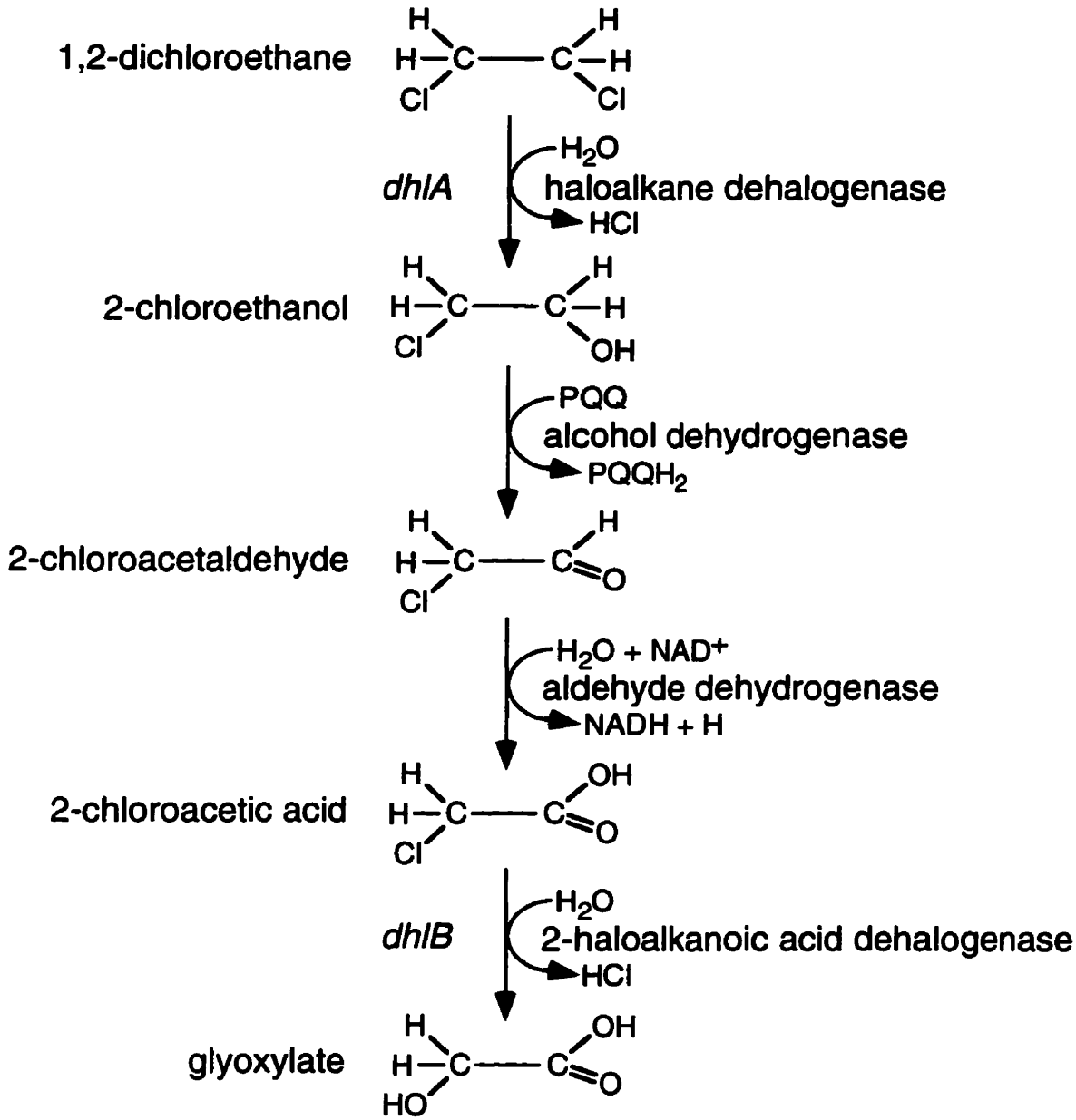

Figure 2. Degradation of 1,2-dichloroethane by *Xanthobacter autotrophicus*.

catabolic sequence is the replacement of one halogen substituent by a hydroxyl group. The product, chloroethanol, is oxidized via the aldehyde to chloroacetate. The haloalkanoic acid dehalogenase gene *dhlB* catalyzes the conversion of chloroacetic acid to glycolate (Fig. 2). The *dhlB* gene encodes a 253 amino acid protein that shows considerable homology with the two haloacid dehalogenase genes from *Pseudomonas* sp. strain CBS3 but not with the haloalkane dehalogenase gene *dhlA*.

Moraxella species strain B, was isolated as a fluoroacetate utilizer from industrial wastewater, and has two haloacetate dehalogenases, H-1 and H-2. H-1 acts better on monofluoroacetate than on monochloro- or monobromoacetate, but has little activity against monoiodoacetate. H-2 acts on monochloro-, monobromo- and monoiodoacetate, but not on monofluoroacetate. Fluoroacetate is known to inhibit the tricarboxylic acid cycle and iodo-and bromoacetate inhibit many enzymes, especially those containing essential thiol groups. The genes for H-1 and H-2 designated *dehH1* and *dehH2* encode proteins of 295 and 225 amino acids respectively, and were found to be closely linked on a plasmid (Kawasaki *et al.* 1981). Sequence analysis revealed no homology between the two genes (Kawasaki *et al.* 1992).

Two haloalkanoic acid hydrolytic dehalogenases have been characterized from *Pseudomonas putida* strain AJ1 and a *Rhizobium* species. *Pseudomonas putida* AJ1 was isolated from soil samples exposed to 2-chloropropionate and shown to contain both D- and L-stereospecific 2-haloalkanoic acid dehalogenases (*hadD* and *hadL*) (Barth *et al.* 1992). The *hadD* gene is highly specific for the D-isomer of 2-chloropropionic acid and dehalogenates the substrate with inversion of configuration at the chiral carbon atom (Smith *et al.* 1990). Finally, Class 2 enzymes (Table 2) have the ability to dehalogenate both D- and L-isomers of 2-haloalkanoic acids with either retention (Class 2R) or inversion (Class 2I) of product configuration with respect to the substrate. Data for haloalkanoic acid dehalogenase I (*dehHI*) from *Pseudomonas putida* strain PP3 shows that it is unrelated to any known dehalogenase (Topping 1992).

1.6.1.2 Haloalkane Hydrolytic Dehalogenases

On the basis of substrate specificity the haloalkane hydrolytic dehalogenases can be divided into two classes: the first class is made of Gram-negative bacteria showing a fairly restricted range of substrate specificities (Class 3R). The second class of haloalkane hydrolytic dehalogenases is represented by enzymes isolated from various *rhodococci* and closely related Gram-positive bacteria that show much broader substrate specificities (Class 3B).

Direct hydrolytic dehalogenation of a chlorinated hydrocarbon was first demonstrated with the haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. The haloalkane dehalogenase gene *dhlA* was identified and found to encode a soluble protein of 310 amino acids (Janssen *et al.* 1989). A number of other aerobic microorganisms possessing haloalkane hydrolytic dehalogenases have been identified, such as *Ancylobacter aquaticus* strains (van den Wijngaard *et al.* 1992), *Pseudomonas* strain UT26 active against g-hexachlorocyclohexane (Nagata *et al.* 1993), various *Corynebacterium* species (Yokota *et al.* 1987), *Rhodococcus* (Sallis *et al.* 1990), and *Arthrobacter* species (Scholtz *et al.* 1988).

1.6.2 Haloalcohol Dehalogenases

Haloalcohol dehalogenases are active towards haloalcohols where the halogen is proximal to the hydroxyl group. The dehalogenation reactions involve the internal rearrangement of the molecule with the simultaneous elimination of a proton with the halide and the formation of an epoxide. The epoxide is then hydrolysed by an epoxide hydrolase. Two classes of alcohol dehalogenase have been identified. Class 4S enzymes show much broader substrate specificities, and are structurally simpler than the Class 4C enzymes (Table 2).

1.6.3 Co-Factor-Dependent Dehalogenases

Glutathione transferases play a key role in the degradation of halogenated compounds. They were shown to be involved in either nucleophilic displacement or substitution of a halogen molecule. The first evidence for the involvement of a glutathione transferase was obtained with facultative and obligate methylotrophs of *Methylobacterium* and *Hyphomicrobium* species. Methylotrophs use dichloromethane as the sole carbon and energy source. The conversion of dichloromethane is mediated by a glutathione dependent enzyme that dechlorinates dichloromethane to formaldehyde and inorganic chloride. Dichloromethane dehalogenases have been purified from five facultative methylotrophs (Kohler-Staub *et al.* 1986; Scholtz *et al.* 1988). Characterization of the structural and kinetic properties of the dichloromethane dehalogenase from *Methylophilus* species strain DM11 suggested that there are two groups of dichloromethane dehalogenases which are immunologically distinct, show different reaction rates and have different N-terminal amino acid sequences. Group A enzymes include dehalogenases from *Hyphomicrobium* strain DM2 and GJ21 and

Methylobacterium strain DM4 (Kohler-Staub *et al.* 1986). The other group is represented by dichloromethane dehalogenase from *Methylophilus* DM11.

Glutathione substitution was shown to be a key step in the metabolism of pentachlorophenol (Xun *et al.* 1992) by *Flavobacterium* species (Orser *et al.* 1993a). Oxidative dehalogenation of pentachlorophenol to tetrachloro-p-hydroquinone is followed by the elimination of a halogen through substitution with glutathione. Glutathione is then removed by displacement of the aromatic moiety by a second glutathione molecule, producing oxidized glutathione (Fig. 3). This reaction sequence occurs three times, leading to the formation of monochlorohydroquinone.

1.6.4 Oxidative Dehalogenases

Several highly chlorinated aliphatics and aromatic compounds such as pentachlorophenol, polychlorinated biphenyls (PCBs), and chlorinated dibenzodioxins and furans may be degraded by oxidative reactions. Dioxygenases and monooxygenases are involved in the dehalogenation of aromatic compounds. Examples of dioxygenation-mediated dehalogenation are the conversion of 2-chlorobenzoate by *Pseudomonas cepacia* 2CBS and 4-chlorophenylacetate by *Pseudomonas* species CBS3 (Fetzner *et al.* 1992). In both cases, a flavin-containing iron sulfur protein of about 37 kDa and a larger dioxygenase component are involved (Fetzner *et al.* 1992).

Detailed biochemical evidence for monooxygenase-mediated dehalogenation comes from biochemical and genetic studies on the degradation of pentachlorophenol by a *Flavobacterium* species. The catabolic step that precedes the gluthatione transferase-mediated dehalogenation (mentioned above) is the conversion of pentachlorophenol to tetrachloro-p-hydroquinone (Fig. 3). The reaction is catalyzed by a 60kDa pentachlorophenol 4-monooxygenase and requires two NADH molecules per turnover if an electronegative substituent such as chlorine is removed (Orser *et al.* 1993b).

Oxygenase-catalyzed dehalogenation reactions of haliphatic compounds such as halogenated methanes, ethanes, and ethylenes are due to multifunctional enzymes with broad substrate specificity like methane monooxygenases or involve enzymes from aromatic degradative pathways. Oxidation can lead to dehalogenation as a result of the formation of chemically unstable products that decompose to produce aldehydes. Methane monooxygenases (MMO) are unique since a single microorganism can possess the mutually exclusive soluble and particulate form of the enzyme (Stanley *et al.* 1983; Tsien *et al.* 1989). Only the soluble form of MMO has been purified to

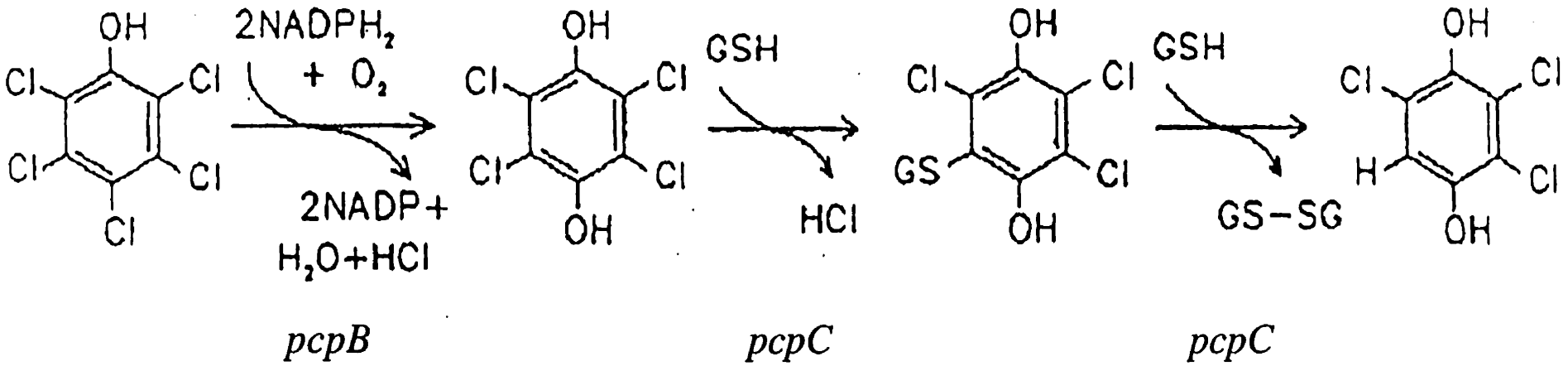

Figure 3. Catabolic route for pentachlorophenol. Dechlorination occurs by a monooxygenase encoded by *pcpB*, and a glutathione transferase encoded by *pcpC*.

homogeneity (for a recent review see Lipscomb 1994). All the soluble MMO characterized to date except for *Methylobacterium* species CRL-26, consist of three protein components: Hydroxylase (MMOH), a dimer of α, β, γ, subunit types (Fox *et al.* 1989; Woodland and Dalton 1984) containing nonheme iron, component B, and a reductase containing FAD and an iron-sulfur cluster (Fox *et al.* 1989). All the components are necessary for the conversion of methane to methanol. The soluble MMO from *Methylococcus capsulatus* is presented in Figure 6 .

Soluble methane monooxygenases from *Methylococcus capsulatus* Bath (Green and Dalton 1989; Stirling and Dalton 1980), *Methylosinus trichosporium* OB3b (Fox *et al.* 1990; Newman and Wackett 1991; Oldenhuis *et al.* 1989), and *Methylobacterium* species strain CRL-26 (Patel *et al.* 1982), where shown to catalyze the oxidation of a variety of hydrocarbons including a number of halogenated short-chain alkanes and alkenes and even aromatic compounds such as benzene, toluene, styrene, 3-chlorophenol and 3-chlorotoluene. The ability of *Methylosinus trichosporium* OB3b to dehalogenate trichloroethylene has led scientists to explore the possibility of using methanotrophs for the controlled biodegradation of trichloroethylene in contaminated groundwater and soils.

1.6.5 Reductive Dehalogenases

Reductive dehalogenation is a two-electron transfer reaction which involves the release of the halogen as a halogenide ion and its replacement by hydrogen. Reductive dehalogenation was found in both aerobic and anaerobic microorganisms. There is evidence for reductive dehalogenation of a number of haloaromatic compounds (Cozza and Woods 1992), under methanogenic, sulfidogenic, and even denitrifying (Häggblom *et al.* 1989) conditions.

However, aerobic microorganisms often fail to metabolize the more heavily halogenated compounds. For example, reductive dehalogenation of highly chlorinated biphenyls, hexachlorobenzene, and tetrachloroethylene has been successful only under anaerobic conditions (for recent reviews see Lowe *et al.* 1993; Mohn and Tiedje 1992). Under anaerobic conditions halogenated aromatic compounds can serve as terminal electron acceptors as a result of anaerobic growth on simple organic molecules. The halide is thus eliminated from the aromatic compound. The first indication of reductive dehalogenation coupled to energy metabolism was with the bacterium *Desulfomonile tiedje* (Mohn and Tiedje 1990).

There is also evidence for non-specific reductive dehalogenation reactions. For example, respiratory c-type cytochromes were shown to be responsible for the reductive dehalogenation of tetrachloromethane to trichloromethane in *Shewanella putrefaciens* 200, an obligate respiratory bacterium that can use a variety of terminal electron acceptors. The reductive dehalogenation reaction did not generate ATP and did not proceed further (Picardal *et al.* 1993).

1.7 Molecular Biological Detection Techniques

Over the last decade, interest in using nucleic acid hybridization techniques and the polymerase chain reaction (PCR) for the detection of microorganisms in the environment has escalated dramatically (For a comprehensive review of PCR and nucleic acid hybridization theory, see Sayler and Layton 1990). These techniques offer high specificity and sensitivity and were proven very successful for the detection of single organisms in clinical, environmental, water, food and air samples (for recent reviews see Bej and Mahbubani 1992; Bej *et al.* 1991; Wolcott 1992). In addition to specificity and sensitivity, nucleic acid hybridization techniques allow the detection of microorganisms that are not culturable under normal conditions.

1.7.1 Nucleic Acid Hybridization

Environmental applications of DNA or RNA hybridization use a variety of target nucleic acid samples ranging from mixed nucleic acids extracted directly from an environmental source, to purified and restricted chromosomal or plasmid DNA recovered from pure bacterial cultures. Detection of target nucleic acid samples is achieved using DNA probes designed to detect a specific organism (species or strain specific) or a specific gene function found in the organism of interest. DNA probes can also be designed to study the survival of particular recombinant bacteria in soil and water. A number of nucleic acid hybridization techniques have been developed: colony or plaque hybridization, nucleic acid extraction and blot hybridization, and Southern hybridization.

Colony hybridization has been the most widely used hybridization protocol in environmental studies, for the detection, enumeration, and isolation of bacteria with specific genotypes and/or phenotypes and for the development of gene probes. Nucleic acid extraction and hybridization can offer a more representative sampling of the genetic diversity in a given environment. Nucleic acid hybridization is also used extensively for

phylogenetic and evolutionary studies (Amann *et al.* 1995; Tsien *et al.* 1990; Giovannoni *et al.* 1988; Woese 1987). The advantage of isolating total genomic DNA from environmental samples is that DNA from both culturable and nonculturable organisms can be assayed. The major problem however, in extracting bacterial DNA from soils and sediments is obtaining DNA with high purity. Both the clay and organic fractions of soil affect DNA isolation and purification (Ogram *et al.* 1987). Clay has a tendency to adsorb DNA (Ogram *et al.* 1987) whereas humic acids found in the organic fraction tend to copurify with DNA during the extraction procedure (Ogram *et al.* 1988). Phenolic or humic acids have been shown to reduce the efficiency of restriction or modification enzymes and even the specificity of hybridization (Steffan and Atlas 1988). In addition, methods developed for soils of a particular clay:organic ratio often need to be modified for soils with different clay:organic ratios (Holben *et al.* 1988; Steffan *et al.* 1988). Finally, direct DNA extraction followed by Southern hybridization can be use to detect changes in natural communities such as rearrangement, deletions, and gene transfer.

1.7.2 Polymerase Chain Reaction (PCR) Amplification

DNA amplification by the polymerase chain reaction (PCR) (Saiki *et al.* 1985; Mullis and Faloona 1987) has facilitated the experimental manipulation of extremely small quantities of DNA. Furthermore, PCR can be used to directly amplify target sequences without having to culture the organisms. The technique has proven applicable to disease diagnosis (Kellog and Knok 1990; Wilson *et al.* 1993), forensic analysis, fingerprinting (Jeffrey *et al.* 1989), cloning, DNA-protein interactions studies, and site directed mutagenesis. In addition, PCR is routinely being used in food testing (Bej *et al.* 1994; Doran *et al.* 1993; Kapperud *et al.* 1993), and water quality control (Juck *et al.* 1996; Oshiro *et al.* 1994; Toranzos *et al.* 1993). Finally, PCR amplification has been of particular value for the characterization of microbial diversity in environmental samples (Muyzer and de Wall 1994; Muyzer *et al.* 1993; Wawer and Muyzer 1995), and in environmental monitoring (Bej and Mahbubani 1992; Greer *et al.* 1993; Steffan and Atlas 1988).

1.8 Outline of Experimental Approach.

Optimizing wastewater treatment systems is a practical alternative to environmental control of chlorinated organic compounds. The development of oligonucleotide primers and corresponding DNA probes to monitor the presence of microorganisms possessing specific dehalogenase genes is an important step in applying a molecular approach to assaying and better understanding the microbial diversity required for the degradation of chloroorganics in bleached kraft pulp mill effluent treatment systems. Molecular tools can also be used to evaluate the microbial response to drastic changes often encountered in wastewater treatment systems. A better understanding of microbial responses to environmental stresses, such as seasonal variations, and changing composition of the effluents, will help design optimal bleaching schedules that will ensure stability and performance of biological treatment systems, resulting in better removal of chlorinated organic compounds in the effluents.

Oligonucleotide primers and corresponding DNA probes for dechlorination activity were designed based on the DNA sequence of known dehalogenases. DNA recovery techniques were developed to directly analyse water and sediment samples from pulp and paper mill effluent treatment systems. The variability of probe positive organisms within and among pulp mill treatment systems was then assessed by PCR analysis using oligonucleotide primers from dehalogenating genes, bacterial enumeration and colony hybridization. PCR analysis was also conducted on the bacterial culture collection that was previously isolated from an aerated stabilization basin by Fulthorpe *et al.* (1993). Several PCR fragments generated during amplification of genomic DNA from dehalogenating bacterial isolates and total community DNA from various environmental sources were cloned and sequenced to permit comparison with genetically characterized degradation systems.

Chapter 2 Materials and Methods

2.1 Bacterial Strains, Media and Methods

The bacterial strains used in this study are listed in Table 3. Sterile YTS-250 (250 mg/l Yeast extract, Bacto-Tryptone, Starch, pH 7.0) with 15g/l agar was used as a solid medium for bacterial growth. The plates were incubated at 25°C for 1 week. Bacterial colonies were grown in sterile liquid LB medium modified with half the amount of NaCl. Cultures were grown for several days at 25°C, with agitation.

Table 3. Bacterial strains used in this study.

Organism	Strain	ATCC	Reference
Xanthobacter autotrophicus	GJ10	32050	Janssen *et al.* 1985
Moraxella species	B		Kawasaki *et al.* 1981
Methylococcus capsulatus		33009	Foster *et al.* 1966
Ancylobacter aquaticus	A7		Fulthorpe et *al.* 1993
Ancylobacter aquaticus	CN13		Fulthorpe et *al.* 1993
Ancylobacter aquaticus	SP6		Fulthorpe et *al.* 1993
Methylobacterium	CP13		Fulthorpe et *al.* 1993
Methylobacterium	SP17		Fulthorpe et *al.* 1993
Pseudomonas	P1		Fulthorpe et *al.* 1993
Pseudomonas	CBS3		Fulthorpe et *al.* 1993
Moraxella sp.			Fulthorpe et *al.* 1993

2.2 Genomic DNA Extraction Procedure

The genomic DNA extraction protocol used in this study was adapted from the method of Ausubel *et al.* (1990). Thirty ml cultures were centrifuged at 6311 x g for 15 min. at 4°C. The pellet was resuspended in 5.67 ml of TE pH 8.0. Prior to lysis

treatment, 50 units of mutanolysin (Sigma Chemicals) were added to help disrupt the cell wall. Samples were incubated at room temperature for 45 min. Then, 300 ul of 10% SDS, and 30 ul of 20 mg/ml proteinase K were added and samples were incubated for 1 hour at 37°C. The lysis treatment was completed with the addition of 800 ul CTAB/NaCl solution and a 15 min. incubation at 65°C. Samples were treated with equal volumes of chloroform/isoamyl alcohol (24:1) and centrifuged for 15 min. at 4°C (4636 x g). Supernatants were treated with 50 ul of 10 mg/ml RNaseA and incubated for 30 min. at 37°C. The RNaseA treatment was followed by a phenol/chloroform/isoamyl alcohol (25:24:1) and a chloroform/isoamyl alcohol extraction. The DNA was precipitated overnight at -20°C with 0.6 volume of cold 2-propanol. Samples were centrifuged 30 min. at 4°C (41,731 x g). Pellets were washed with 70% cold ethanol and air dried. The DNA was resuspended in 500 ul to 1 ml of TE pH 8.0. DNA concentrations were estimated by running dilution series on a 1% agarose gel. Approximately 10 ng of bacterial genomic DNA was used for PCR amplification.

2.3 Total Community DNA Extraction Procedure

The DNA recovery technique used in this study is a modified version of Flemming's method (Flemming *et al.* 1994). Fifteen ml of water and sediment material was extracted. Prior to lysis treatment, 4.5 ml of distilled water was added to sediment samples. Five hundred ul of 250 mM Tris-HCl pH 8.0 and 10 mg/ml lysozyme were added and samples were incubated 30 min. at 30°C, with agitation (295 rpm), followed by a 30 min. incubation at 37°C. Proteinase K was added to a final concentration of 100 ug/ml and samples were incubated for 1 hour at 37°C. The lysis treatment was completed with the addition of 500 ul 20% sodium dodecyl sulfate solution and 30 min. incubation at 85°C. Samples were centrifuged at room temperature, for 10 min. (16,000 x g). Supernatants were treated with one-half volume of 7.5 M ammonium acetate, incubated on ice 15 min. to precipitate proteins and humic acids, and centrifuged 5 min. at 4°C (16,000 x g). The DNA was precipitated overnight at -20°C with one volume of cold 2-propanol. Samples were centrifuged at 4°C, for 20 min. (27,216 x g). Pellets were washed with 70% cold ethanol and air dried. The DNA from water and sediment samples was resuspended into 200 and 400 ul TE pH 8.0 respectively. 50 ul of DNA was purified with Sephacryl-400 Hr MicroSpin column (Pharmacia Biotech). DNA concentrations were estimated by running 5 ul of purified material against the 1 kb

DNA ladder on a 1% agarose gel. Approximately 100 ng of DNA was used for PCR amplification.

2.4 Polymerase Chain Reaction Amplification

For PCR amplification, 2ul of each oligonucleotide primer (0.4-0.8 uM), approximately 10 ng of bacterial genomic DNA, or 100 ng of total community DNA, 5.0 units of Taq DNA polymerase (Pharmacia Biotech), 5 ul of 10X Taq polymerase buffer (100 mM Tris-HCl pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 4 ul of 25 mM $MgCl_2$ (5.5mM), and 8 ul of 1.25 mM deoxynucleoside triphosphates (200 uM [each] dATP, dGTP, dCTP and dTTP) were combined in a final reaction volume of 50 ul. Prior to the addition of Taq DNA polymerase, the samples were boiled for two minutes then transferred to ice. Amplification was carried out for 30 cycles, with each cycle consisting of 1 min. 94°C, 1 min. at 60°C and 1 min. at 72°C, followed by a final extension at 72°C for 4 min. PCR products were loaded on a 1.2% agarose gel together with 100 bp DNA marker (Gibco BRL) which contains fragments of 2072 and 1500 bp to 100 bp in 100 bp increments. The PCR products were visualized with ethidium bromide staining and UV transillumination at 254 nm.

2.5 Amplification, Purification and Radioactive Labelling of Gene Probes

PCR amplification of *Xanthobacter autotrophicus*, *Moraxella* and *Methylococcus capsulatus* genomic DNA with their corresponding oligonucleotide primers was carried out for 30 cycles during which DNA was denatured for 1 min. at 94°C and primers were annealled and extended for 1 min. at 72°C. PCR fragments were purified with Gene Clean (Bio101) as described by the manufacturer. Radioactive labelling of the resulting gene probes was performed using the Amersham Multiprime Kit (Amersham). The reaction consisted of 25 to 30 ng of purified PCR fragment, 5 ul of each dCTP, dGTP and dTTP, and ^{32}P-dATP (4500 Ci/mmole), 5 ul primer/BSA, 5 ul 10X buffer and 2 units of Klenow fragment (Pharmacia Biotech). One unit of Klenow fragment was added at the beginning of the reaction, and a second time 1.5 hours into the incubation. The total incubation was 3 hours at 37°C. The unincorporated nucleotides were removed with Biospin 6 chromatography columns (BioRad) following the instructions of the manufacturer.

2.6 Southern Blotting and Hybridization

Southern blotting of DNA agarose gels was performed with the LKB 2016 VacuGene vacuum blotting system from Pharmacia Biotech. Slight modifications to the protocol proposed by the manufacturer were incorporated. Depurination (0.2 N HCl), denaturation (0.5 M NaOH, 0.5 M NaCl) and neutralization (1M Tris-HCl pH 7.5, 1.5 M NaCl) solutions were applied only to the upper surface of the gel for 15-30 minutes under a vacuum of 50-55 cm.H_2O. The gel was then submerged in a 20X SSC (3M NaCl, 0.2 M sodium citrate pH 7.0) or 10X SSC (1.5 M NaCl, 0.1 M sodium citrate, pH 7.0) transfer solution for 2-3 hours to ensure complete transfer of the DNA onto the Zeta-probe membrane (BioRad). The DNA was cross-linked onto the nylon membrane with ultraviolet light (Stratalinker ultraviolet crosslinker). Membranes were air dried, and stored at -20°C until probed. Membranes were incubated for 1 hour at 65°C in prehybridization solution (1 mM EDTA, 0.5M NaH_2PO_4 pH 7.2, 7% SDS). Membranes were then transferred to fresh prehybridization solution and hybridized overnight at 65°C. The unincorporated radiolabelled nucleotides were removed by washing the membranes twice with Wash solution 1 (1 mM EDTA, 40 mM NaH_2PO4 pH 7.2, 5% SDS) for 30-60 min. at 65°C and twice with Wash solution 2 (1 mMEDTA, 40 mM NaH_2PO4 pH7.2, 1% SDS) under the same conditions. Membranes were exposed to X-ray films (Kodak X-Omat) at -80°C for 1-5 days.

2.7 Bacterial Enumeration and Colony Hybridization

Water and sediment samples collected in biological treatment systems were serially diluted in 0.1% pyrophosphate solution and plated in triplicates on YTS-250, pH 7.0, and a mineral salt medium (MSM) (Greer *et al.* 1990) with 1/20 the amount of 1M $NaH_2PO_4 \cdot H_2O$ and 1M K_2HPO_4 solutions. The MSM medium was supplied with 2mM chloroacetate and dichloroacetate, 50 mg/ml Yeast extract and 75 mg/ml bromothymol blue, pH 7.0. Bromothymol blue is a pH indicator dye that changes from green to yellow as chloride ions are liberated as HCl, reducing the pH of the solid medium. This color change, when related to the metabolism of a selective chlorinated compound indicates the presence of microorganisms with dehalogenating capacities. The plates were incubated at room temperature (21-24°C) for 4 weeks. Colony hybridization was performed as described by Greer *et al.* (1993).

2.8 Cloning of PCR Fragments

PCR fragments were cloned into PTZ/PC, a modified PTZ19R *E. coli* plasmid (D. Tessier, personal communication) which is left with a single T residue on its 3' ends following cleavage with the appropriate restriction endonuclease. This significantly enhances the efficiency of cloning PCR fragments since Taq DNA polymerase will catalyze the preferential addition of a single dATP residue on the 3' ends of blunt-ended double-stranded molecules.

2.8.1 Plasmid DNA Preparation

One nanogram of PTZ/PC DNA was transformed into 10 ul *E. coli* NM522 competent cells (Stratagene) following the instructions of the manufacturer. Large scale cesium chloride plasmid DNA was prepared as described by Sambrook *et al.* (1989). PTZ/PC DNA was linearized with the restriction endonuclease *XcmI*, aliquoted and stored at -80°C.

2.8.2 Ligation and Transformation of PCR Fragments

PCR fragments were purified with Gene Clean (Bio101) according to the instructions of the manufacturer. Typically, 0.1 ug of PTZ/PC-XcmI was ligated with 0.5 ug of purified PCR fragment at 18°C overnight in a final volume of 20 ul containing 4 ul 5X T4 DNA ligase ligation buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000) and 0.5 units of T4 DNA ligase (Pharmacia Biotech). Ligation reactions were stored at -20°C. One half of the ligation mixture was used to transform 100 ul of competent *E. coli* strain DH10B cells. The ligation mixture was transformed following the method described by Ausubel *et al.* (1990). Transformation mixtures were plated on LB plates containing 100 ug/ml Ampicillin, 20 ug/ml 5-bromo-4-chloro-3 indolyl-β-D-galactoside (X-Gal) and 0.25 mM isopropyl-β-D-thiogalactoside (IPTG) for selection of positive clones by the insertional inactivation of the lacZ (α) gene segment of *E. coli*.

DH10B competent cells were prepared following a modified version of the method developed by Hanahan (1983). *Escherichia coli* strain DH10B (BRL-Gibco) was inoculated onto YA plates (5g/l Yeast extract, 20g/l Bacto-Tryptone, 5g/l $MgSO_4$, 12g/l agar, pH 7.6) and incubated overnight at 37°C. Six well-isolated colonies were transferred into 7 ml liquid YA and grown for 2 hours at 37°C (OD $_{550}$ 0.30). Five ml

of the culture was transferred into 100 ml liquid YA and grown for another 2 hours at 37°C (OD_{550} 0.28). The cultures were placed on ice for 10 min. then transferred into ice-cold 50-ml polypropylene tubes. The cells were recovered by centrifugation at 4000 rpm for 10 min. at 4°C in a Centra MP4R benchtop centrifuge (International Equipment Company), and resuspended in 40 ml TFbI solution (30 mM KAc, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8). The resuspended cells were placed on ice for 10 min. and recovered as before. The cells were then resuspended in 4 ml TFbII solution (10 mM 1,4-Piperazine-diethanesulfonic acid (PIPES), 75 mM $CaCl_2$, 10 mM KCl, 15 % (v/v) glycerol, pH 6.5) and incubated on ice for 15 min. The cells were aliquoted and immediately transferred in dry ice and kept at -80°C. The efficiency of competent cells was determined by transforming 1 ng of *E. coli* plasmid PTZ19R (Pharmacia Biotech) into 100 ul of freshly made DH10B competent cells using the protocol described by Sambrook *et al.* (1989).

2.8.3 Recombinant DNA Purification and Characterization

Recombinant DNA was purified according to the modified alkaline-lysis/PEG precipitation procedure developed by Applied Biosystems, Inc. (ABI). The PEG precipitation was done for 45 minutes on ice instead of 15 minutes. Every subclone was linearized with the restriction endonuclease *NcoI* to verify the presence of an insert. In the case of PCR fragments generated from total community DNA, subclones were subjected to restriction endonuclease analysis in order to determine whether the cloned fragments originated from different bacteria. Enzymes were purchased from either New England Biolabs or Pharmacia Biotech and were used according to the instructions of the manufacturer.

2.8.4 Sequencing Analysis of PCR Fragments

Sequencing reactions were prepared with ABI PRISM Dye Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer) in a final volume of 20 ul containing 0.5 ug of double-stranded DNA, 3.2 pmole of PTZ19R universal or reverse primers (Pharmacia Biotech), 8 ul of Terminator Ready Reaction Mix, and 60 ul of light mineral oil. Cycle sequencing was done in a DNA Thermal Cycler model 480 (Perkin Elmer) under the following conditions: the initial denaturation was done at 94°C for 2 min. and was followed by 25 cycles of 30 seconds at 96°C, 15 seconds at 50°C and 4 min. at 60°C. The sequencing reactions were stored at 4°C until purification. Traces of

unincorporated dye terminators were removed using G-50 Sephadex Quick Spin Columns (Boehringer Mannheim). The columns were used as recommended by the manufacturer. Thirty ul of 10 mM EDTA pH 8.0 was added to the oil free sequencing reactions before elution. The DNA was precipitated 30 min. on ice with 1 ul glycogen and 85 ul 100% ethanol. Samples were centrifuged in a microcentrifuge for 15 min. at 4°C at 13,000 rpm. Pellets were washed with 70% ethanol and air dried. Pellets were resuspended in 4 ul deionized formamide and EDTA (8mM pH 8.0). Complete nucleotide sequence of both strands was obtained with an automated sequencing machine (ABI 373). Due to the relatively high rate of misincorporation of nucleotides by Taq DNA polymerase during PCR amplification, a three times redundancy sequencing analysis was performed on each subclone. In the case of PCR fragments generated from total community DNA, this was done by sequencing different subclones that generated the same restriction digest pattern. Sequence analysis was performed using SeqEd 1.0.3 of Applied Biosystems (Perkin Elmer). Computer alignment was created using GENE WORKS (Intelligenetics, Inc. CA).

2.9 Homology Search

Nucleotide and amino acid sequences of subclones generated from the cloning of PCR products were compared with all entries in GenBank non-redundant nucleotide sequence database Release 48 (September 1996) and protein sequence database Release 33 (February 1996) (Altschul *et al.* 1990) using both FASTA (Pearson and Lipman 1988) and BLITZ (Sturrock and Collins 1993) search programs.

Chapter 3 Results

3.1 Molecular Characterization of a Pulp and Paper Mill Biological Treatment System

3.1.2 Oligonucleotide Primer Design

To evaluate the evolutionary relationships between known dehalogenase genes, a DNA sequence alignment was performed using the Unweighted Pair Group Matrix Average (UPGMA) program from GENEWORKS (IntelliGenetics Inc. CA). The alignment gave rise to three different clusters (Fig. 4). The first cluster includes co-factor-dependent dehalogenase genes (*dcmA* and *dcmAA*) from two different methylotrophs and the *dhlA* gene encoding haloalkane dehalogenase from *Xanthobacter autotrophicus*. The second cluster consists of various haloalkanoic acid hydrolytic dehalogenases and a co-factor-dependent dehalogenase gene, *pcpB*, from a *Flavobacterium* species. Finally, the third cluster contains the dehalogenase gene *dehH1* from a *Moraxella* species.

A collection of primers and corresponding DNA probes were designed from the DNA sequences of three known dehalogenases: *dhlB* encoding haloacid dehalogenase (van der Ploeg *et al.* 1991), and *dhlA* encoding haloalkane dehalogenase (Janssen *et al.* 1989) from *Xanthobacter autotrophicus*, and *dehH2* encoding haloacetate dehalogenase (Kawasaki *et al.* 1981b) from *Moraxella*. The primer sequences are presented in Table 4. Particular interest was given to *Xanthobacter autotrophicus* and *Moraxella* species because of their ability to grow on chloroacetate as a sole carbon source (Figs. 2 and 5). Chlorinated acetic acids are the major compounds produced during ECF bleaching of wood pulp (McKague 1995).

Oligonucleotide primers were also designed from *mmoX* encoding the soluble methane monooxygenase from *Methylococcus capsulatus* Bath (Stainthorpe *et al.* 1990), an enzyme known to perform dehalogenation reactions. The *mmoX* gene encodes the alpha-subunit of component A, the site of hydrocarbon oxygenation (Fig. 6). The primer sequences were compared with all the sequences in the EMBL database by using the FASTA search program (Pearson and Lipman 1988). Significant similarity values were found only with dehalogenase and methane monooxygenase sequences.

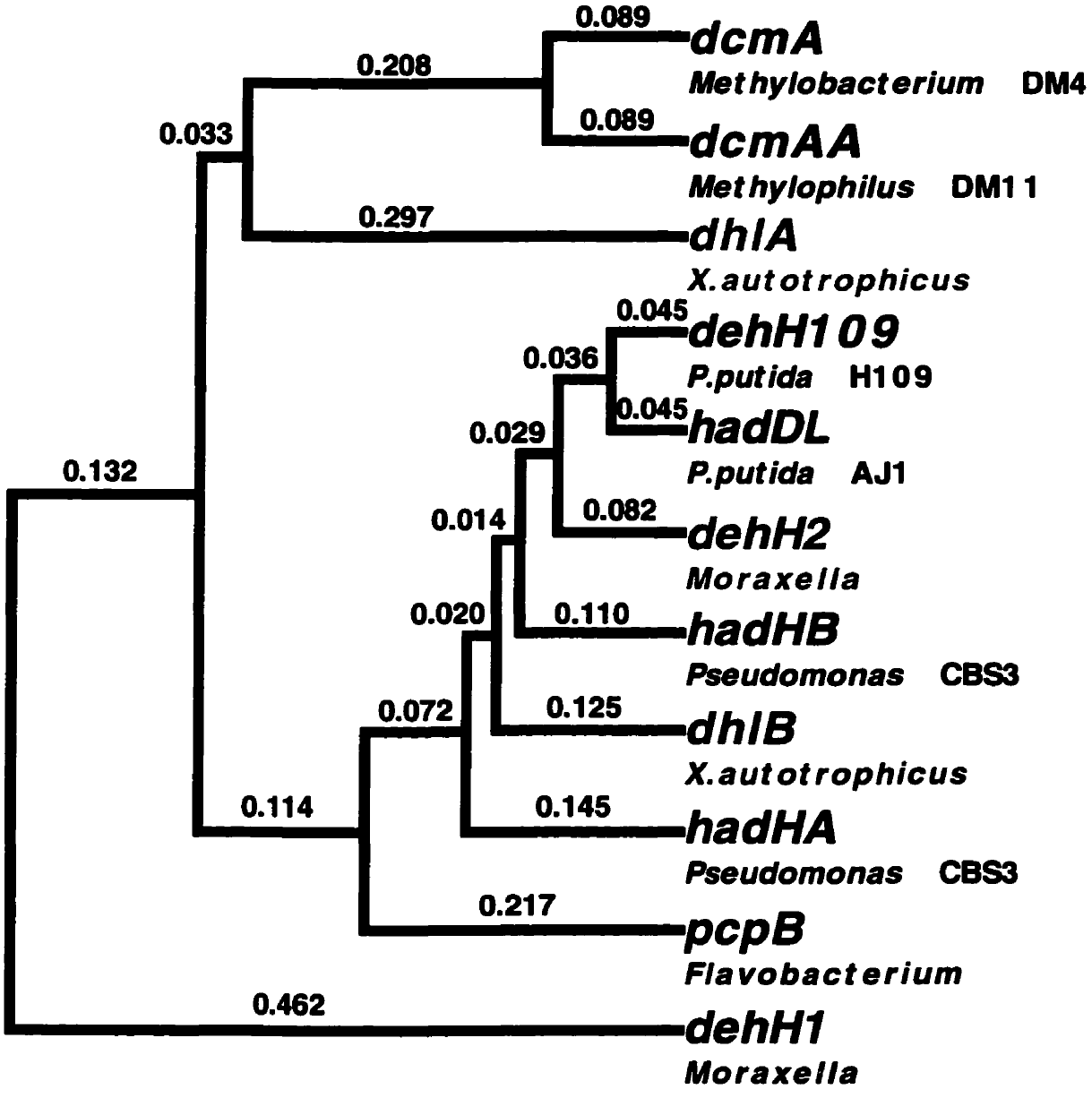

Figure 4. DNA sequence dendrogram of known dehalogenase genes.
The alignment was performed using an Unweighted Pair Matrix Average (UPGMA) program.

Table 4. Oligonucleotide primer sequences.

Organisms	Primer sequence	Fragment (bp)	Reference
Xanthobacter autotrophicus	*dhlA* 1272: 5'-GTAGACGAAGAAGACTACACCTTCG-3'		
	dhlA 1746: 5'-CATTCCAGTCGTTCTGCCAG-3'	475	van der Ploeg *et al.* 1991
Xanthobacter autotrophicus	*dhlB* 314: 5'-TCTGGCGGCAGAAGCAGCTGG-3'		
	dhlB 637: 5'-CGCGCTTGGCATCGACGCTGATG-3'	330	Janssen *et al.* 1989
Moraxella sp.	*dehH2* 1157: 5'-CGGCACCCTCTACGATGTGCATTCGG-3'		
	dehH2 1662: 5'-CATCCCATGGATTCGACGATACAAAGA-3'	506	Kawasaki *et al.* 1981b
Methylococcus capsulatus	*mmoX* 2008: 5'-CGGTCCGCTGTGGAAGGGCATGAAGCGCGT-3'		
	mmoX 2376: 5'-GGCTCGACCTTGAACTTGGAGCCATACTCG-3'	369	Stainthorpe *et al.* 1990

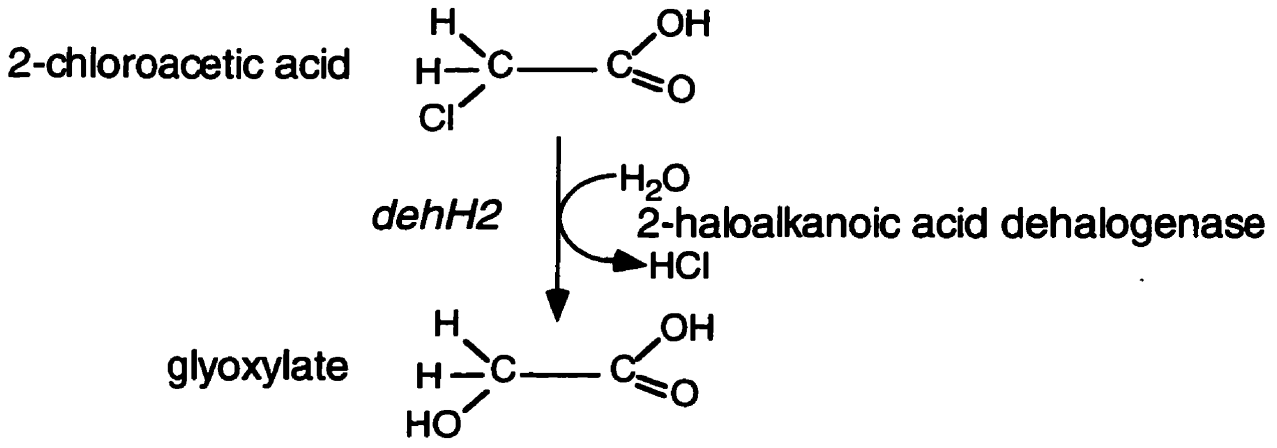

Figure 5. Degradation of 2-chloroacetic acid by *Moraxella* species.

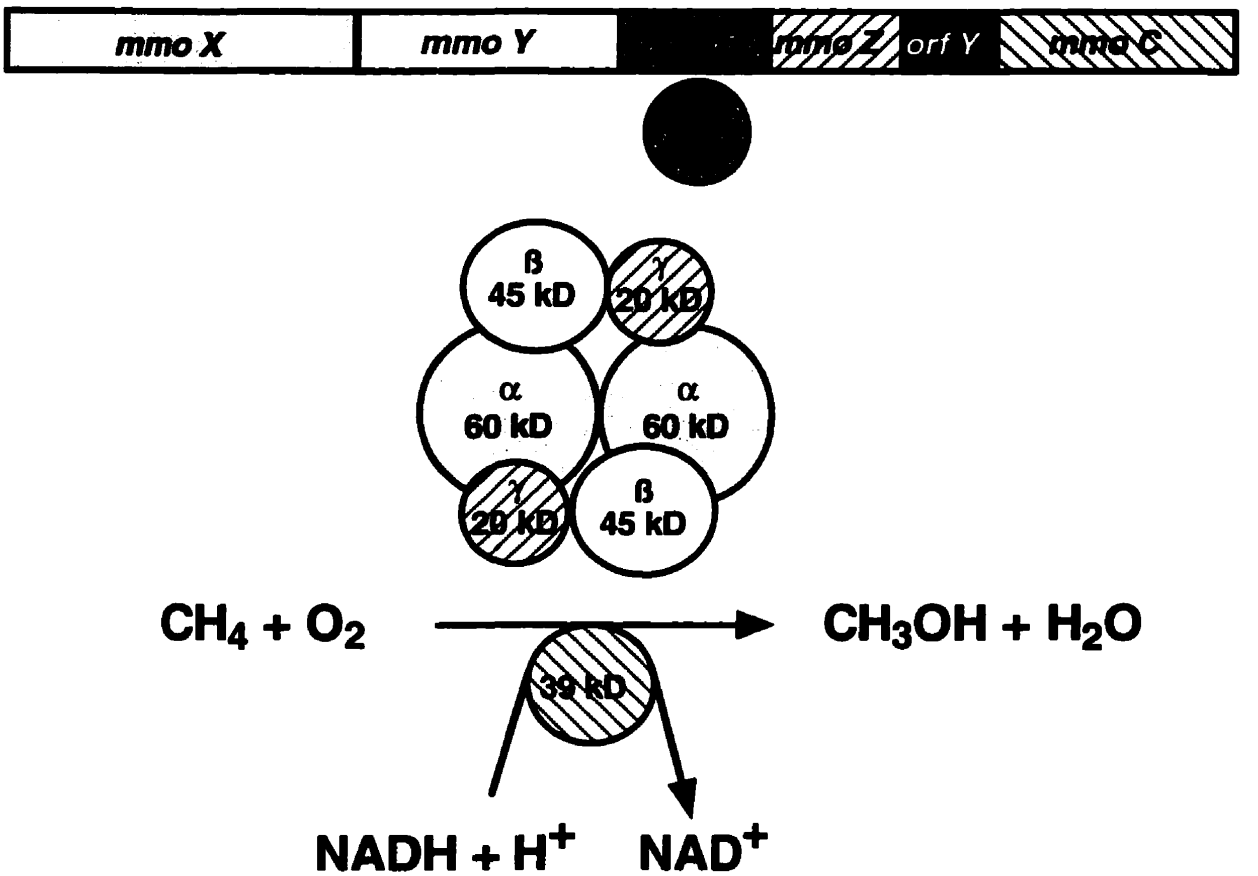

Figure 6. sMMO enzyme complex from *Methylococcus capsulatus* Bath.

3.1.3 Description of a Pulp and Paper Mill Effluent Treatment System.

Individual dehalogenating bacteria were isolated by Fulthorpe *et al.* (1993) from the biological wastewater treatment facility at the Canadian Pacific Forest Products mill in Dryden, Ontario. The mill bleaches approximately 1000 air-dry tonnes of pulp per day with elemental chlorine and 5% chlorine dioxide. Combined wastewater from the acid, alkaline and neutral sewer goes through a clarifier and then flows to the treatment facility at a rate of about 100,000 m^3/day. The aerated lagoon has a hydraulic retention time of about 7 days and is followed by a two-day settling pond where accumulated biomass is allowed to settle.

3.1.4 Isolation of Individual Dehalogenating Bacteria

Samples were collected from the upstream portion of the Wabigoon River, the mill clarifier, aerated stabilization basin (ASB), and the settling pond. The majority of bacterial strains isolated from these samples did not match known fingerprints using the Biolog GN microplate. Results of the Biolog fingerprints as well as other taxonomic tests were used to calculate a matrix of phenotypic similarity coefficients. An average linkage cluster analysis of these coefficients yielded a dendogram depicting the degree of phenotypic relatedness between the isolates (Fulthorpe *et al.* 1993). Forty-one percent of the strains isolated from the aerated stabilization basin were either *Klebsiella* species, *Ancylobacter aquaticus* strains, or members of an unknown group of pleomorphic methylotrophs referred as the cluster C group. Thirty-seven percent of strains isolated from the settling pond were either *Ancylobacter aquaticus* or cluster C organisms. In addition, several *Pseudomonas* strains were isolated from the mill clarifier. The bacterial strains used in this study are presented in Table 3 (Chapter 2). All strains except *Ancylobacter aquaticus* SP6 exhibited growth and released chloride ions from chloroethanol or chloroacetate in liquid media (Fulthorpe *et al.* 1993).

3.1.5 PCR Analysis of Dehalogenating Bacterial Isolates

Oligonucleotide primers derived from dehalogenases or the soluble methane monooxygenase were used to perform PCR amplification using genomic DNA extracted from dehalogenating bacterial isolates. PCR fragments of the expected size (330 bp) were generated with essentially every bacterial isolate using the *dhlB* primers

(Figs. 7, 8, 9, and 10). A similar size PCR product (369 bp) was also detected in *Methylobacterium* SP17 using the *mmoX* primers (Fig. 8). Interestingly, almost identical banding patterns were generated during PCR amplification of genomic DNA from *Ancylobacter aquaticus* A7 and *Methylobacterium* SP17 with both the *dhlB* and *mmoX* primers (Fig. 8). None of the bacterial isolates contained a detectable fragment that would indicate the presence of the *dehH2* haloacetate dehalogenase.

A PCR product of the predicted size (330 bp) was generated during amplification of genomic DNA extracted from the bacterial isolate *Ancylobacter aquaticus* (CN13), with the *dhlB* oligonucleotide primers designed from the coding region of the haloacid dehalogenase gene of *Xanthobacter autotrophicus* (Fig. 7). This result was confirmed by Southern hybridization with the corresponding *dhlB* DNA probe from *Xanthobacter autotrophicus* (data not shown). The 330 bp fragment was cloned into *E. coli* plasmid PTZ/PC. The complete DNA sequence was obtained on both strands with an automated sequencer (ABI 373). Because of the relatively high rate of misincorporation of nucleotides by Taq DNA polymerase during PCR amplification, a three times redundancy sequencing analysis was performed. The nucleotide and amino acid sequence was analyzed against EMBL and SWISSPROT databases, respectively. A FASTA search revealed that the PCR fragment was 88% homologous to the known coding region of the haloacid dehalogenase from *Xanthobacter autotrophicus* (Fig. 11). The PCR product also had homology with a variety of other haloalkanoic acid dehalogenases such as the *hadL* dehalogenase from *Pseudomonas putida* AJ1 (63%), the *dehH109* dehalogenase from *Pseudomonas putida* H109 (61%), the *hdlIVa* dehalogenase from *Pseudomonas cepacia* MBA4 (60%), and the *dehH2* haloacetate dehalogenase from *Moraxella* (58%). Southern hybridization with the *dhlB* DNA probe indicated that this novel dehalogenase is located on the chromosome of *Ancylobacter aquaticus* (data not shown).

PCR fragments of the expected size (330 bp) generated during amplification of genomic DNA from *Pseudomonas* P1 (Fig. 10) and *Methylobacterium* CP13 (Fig. 8) with the *dhlB* oligonucleotide primers were cloned and sequenced. GeneBank searches revealed no homology with any known dehalogenases. Similarly, the PCR product generated during amplification of genomic DNA from *Methylobacterium* SP17 with *mmoX* primers (Fig. 8) did not show any similarity to known methane monooxygenases.

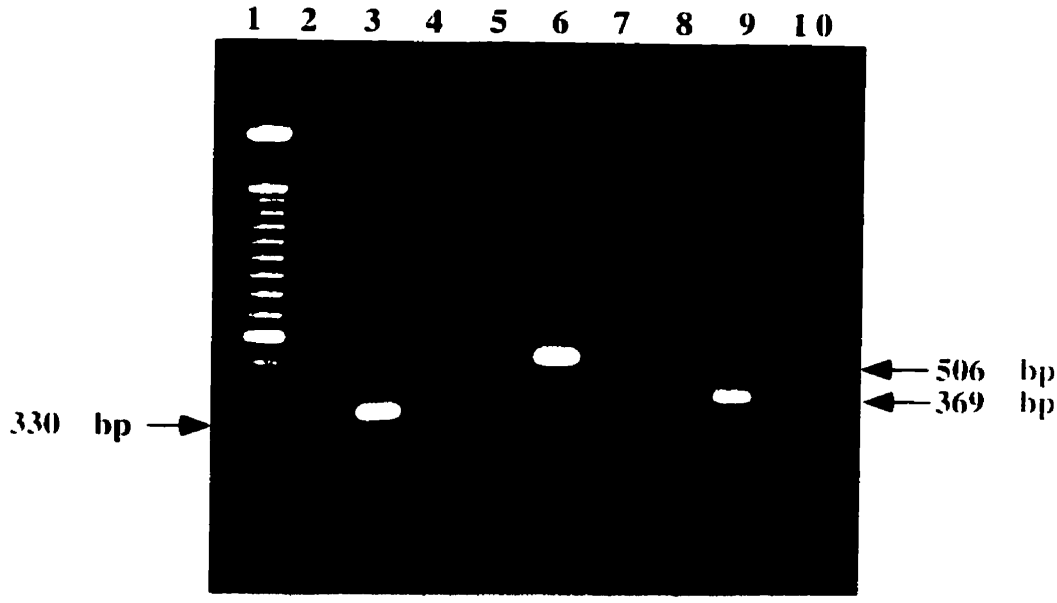

Figure 7. PCR analysis of isolate CN13, belonging to the *Ancylobacter* cluster isolated from a pulp and paper mill effluent treatment system. The expected PCR fragment (330 bp) was generated with oligonucleotide primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus* (lane 4). Genomic DNA from isolate CN13 was also analyzed using primers for the *dehH2* gene, encoding a haloalkanoic acid dehalogenase from *Moraxella* sp. (lane 7) and primers for the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus* (lane 10). Negative and positive controls for *dhlB* (330 bp), *dehH2* (506 bp), and *mmoX* (369 bp) are presented in lanes 2, 5, and 8, and lanes 3, 6, and 9, respectively. Lane 1 indicates the 100 bp DNA marker.

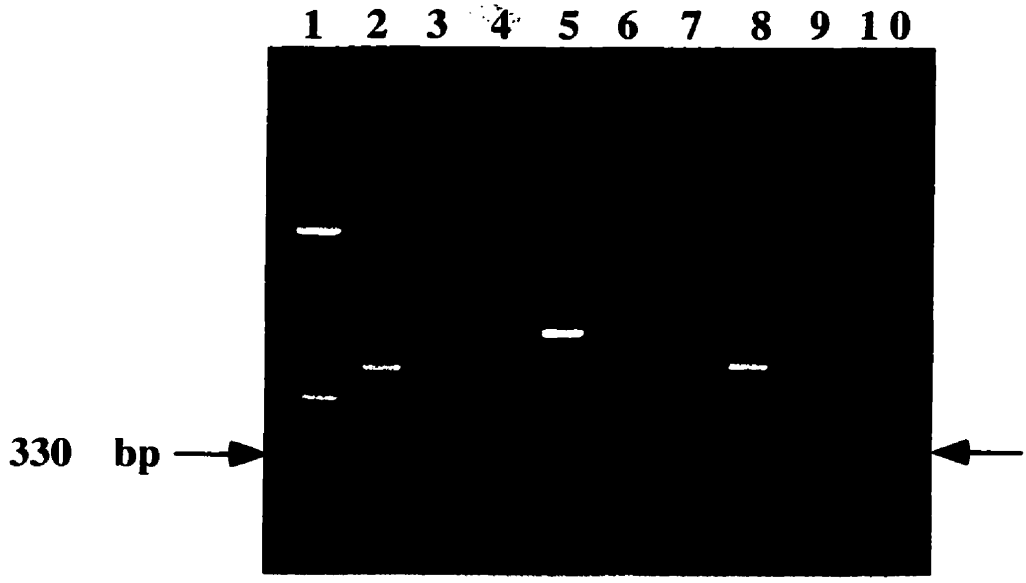

Figure 8. PCR analysis of isolates A7 (*Ancylobacter* cluster), CP13, and SP17 (cluster C), isolated from a pulp and paper mill effluent treatment system. PCR fragments of the predicted size (330 bp) were generated with *Ancylobacter aquaticus* A7 (lanes 2), *Methylobacterium* CP13 (lane 5), and SP17 (lane 8) using primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. Genomic DNA was also analyzed using oligonucleotide primers for the *dehH2* gene, encoding a haloalkanoic acid dehalogenase from *Moraxella* sp., and primers for the *mmoX* gene encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. The arrows indicate the expected PCR fragment (330 bp) for the *dhlB* gene, and a similar size fragment obtained for the *mmoX* gene during amplification of genomic DNA from *Methylobacterium* SP17 (lane 10). Lane 1 indicates the 100 bp DNA marker, Lane 2, A7 (*dhlB*); lane 3, A7 (*dehH2*); lane 4, A7 (*mmoX*); lane 5, CP13 (*dhlB*); lane 6, CP13 (*dehH2*); lane 7, CP13 (*mmoX*); lane 8, SP17 (*dhlB*); lane 9, SP17 (*dehH2*); and lane 10, SP17 (*mmoX*).

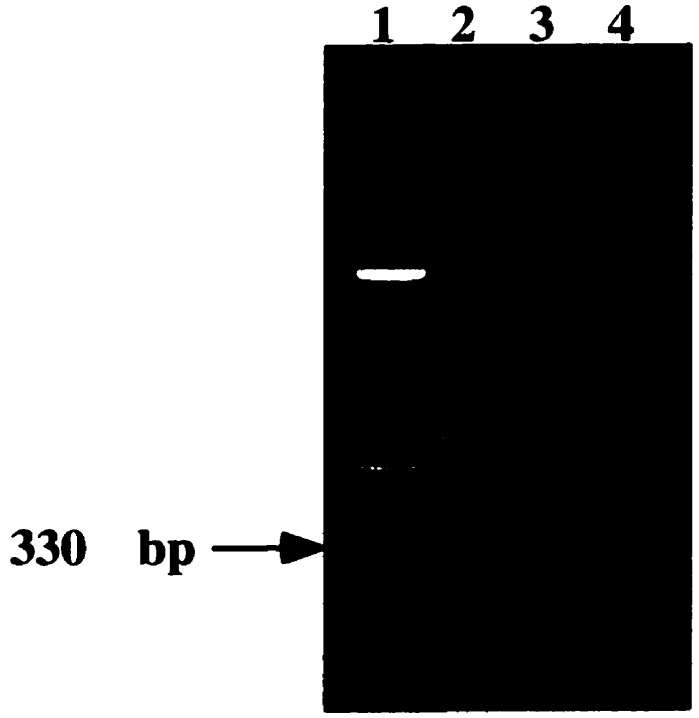

Figure 9. PCR analysis of isolate SP6, belonging to the *Ancylobacter* cluster, isolated from a pulp and paper mill effluent treatmnt system. The expected PCR fragment (330 bp) was generated with oligonucleotide primers for the *dhlB* gene (lane 2), encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. Genomic DNA from SP6 was also analyzed using oligonucleotide primers for the *dehH2* gene (lane 3), encoding a haloalkanoic acid dehalogenase from *Moraxella* sp., and primers for the *mmoX* gene (lanes 4), encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. The arrow indicates the expected PCR fragment (330 bp) for the *dhlB* gene. The 100 bp DNA marker is presented in lane 1.

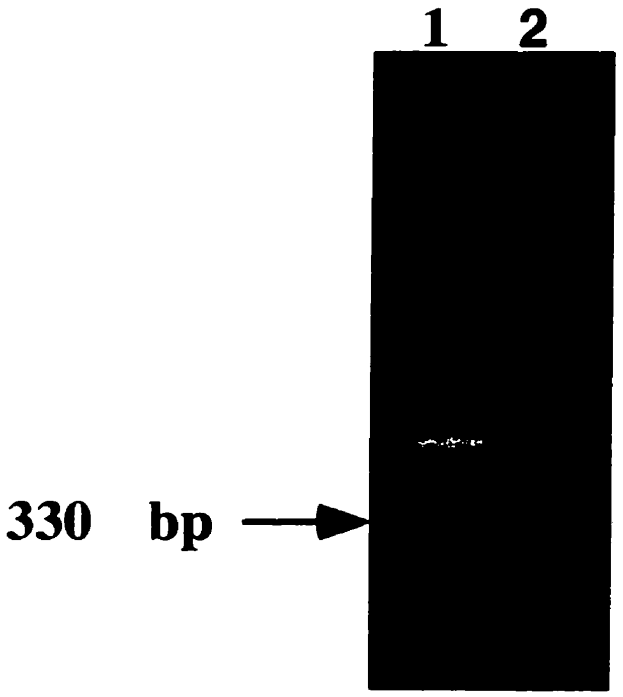

Figure 10. PCR analysis of isolate P1 and CBS3, belonging to the *Pseudomonas* cluster, isolated from a pulp and paper mill effluent treatment system. Genomic DNA from P1 (lane 1), and CBS3 (lane 2) was analyzed using oligonucleotide primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. The arrow indicates the expected PCR fragment (330 bp) for the *dhlB* gene.

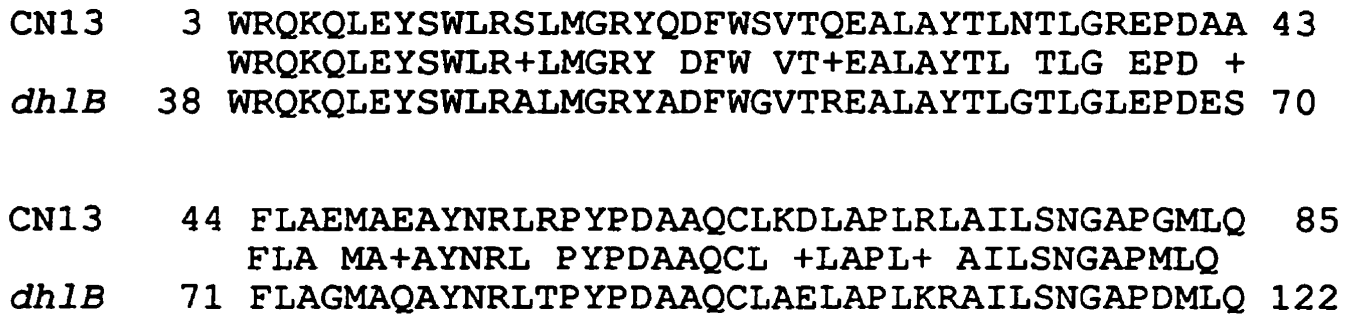

Figure 11. CN13/*dhlB* shares homology with a known dehalogenase gene. CN13/*dhlB* PCR product is 88% homologous to the *dhlB* gene, encoding haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. Conserved amino acid residues are indicated by (+). The accession number for *dhlB* is M81691.

3.1.6 PCR Analysis of Total Community DNA

3.1.6.1 Total Community DNA from an Aerated Stabilization Basin

Prior to PCR analysis, the DNA previously extracted from samples collected in January 1991 (ASB91) and July 1992 (ASB92) from an aerated stabilization basin in the Canadian Pacific Forest Products (CPFP) kraft mill treatment system at Dryden, was purified with Sephacryl-400 Hr MicroSpin columns (Pharmacia Biotech) to remove visible traces of humic acids. PCR analysis was then conducted using oligonucleotide primers from dehalogenating genes. The predicted size fragment (330 bp) was generated during PCR amplification of total community DNA ASB92 with the *dhlB* primers, but not from total community DNA ASB91. Similarly, the predicted size PCR fragment (369 bp) was generated from ASB92 with the *mmoX* oligonucleotide primers but not from ASB91 (Fig. 12).

The 369 bp PCR fragment generated from total community DNA with *mmoX* primers was isolated and cloned into *E. coli*, and the subclones subjected to a *NcoI* restriction endonuclease analysis to verify the presence of an insert. Subclones were then subjected to an *AvaII* restriction endonuclease analysis to determine whether the cloned fragments originated from different bacteria. A total of twenty nine subclones were screened in this experiment. Several different restriction patterns were generated with the *Ava II* enzyme (Figs. 13 and 14). Whenever possible, a three time redundancy sequencing was performed, with the various subclones to minimize errors incorporated by Taq DNA polymerase during PCR amplification. This was done by sequencing three different subclones generating the same restriction pattern.

For example, subclones 4, 5, and 7 (Fig. 13), and subclones 19, 23, 24 (Fig. 14) represented two different restriction patterns. The subclones were also different in size (349bp and 369 bp respectively). DNA sequence alignments revealed however, that the 349 bp subclone was a truncated version of the 369 bp subclone. Only one *AvaII* site was identified in subclones 4, 5 and 7, at position 278 whereas two sites were found in subclones 19, 23 and 24 at positions 3 and 298. The amino acid sequence was sent to GenBank through a FASTA search. The results are presented in Table 5. The 369 bp subclones showed strong similarity over their entire length with the coding region of two known methane monooxygenase genes. The sequence homologies were: 100% to the soluble methane monooxygenase from *Methylococcus capsulatus* and 95% to the methane monooxygenase from *Methylosinus trichosporium.* The nucleotide and protein alignments are presented in Figures 15 and 16.

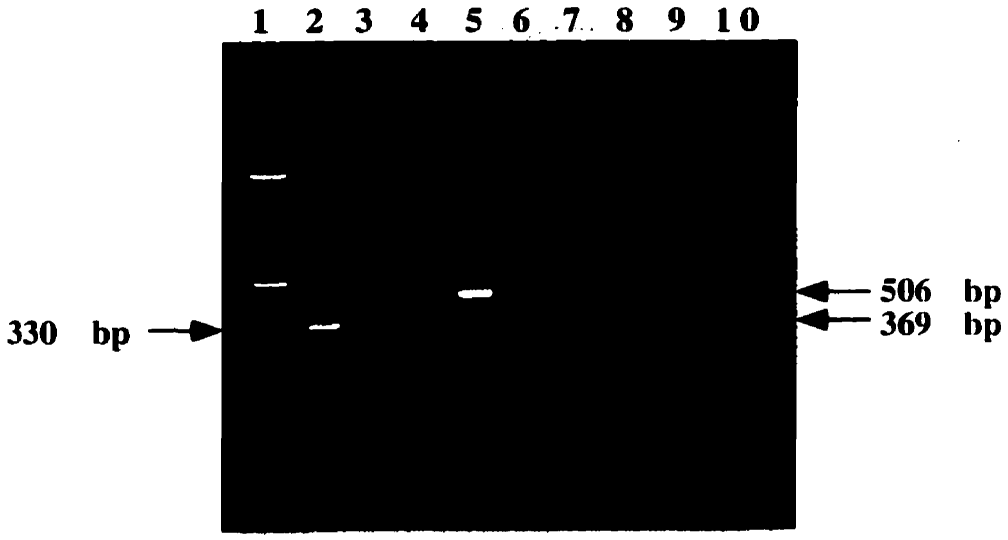

Figure 12. PCR analysis of total community DNA extracted from a pulp and paper mill effluent treatment system. PCR fragments of the expected sizes were generated with a sample collected in the summer of 1992 and oligonucleotide primers for both the *dhlB* gene (lane 4), encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus* and the *mmoX* gene (lane 10), encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Lanes 3, 6, and 9 correspond to a sample collected in the winter of 1991, and lanes 4, 7, and 10 correspond to a sample collected in the summer of 1992. The PCR analysis was also performed using oligonucleotide primers for the *dehH2* (lanes 5, 6, and 7) gene, encoding a haloalkanoic acid dehalogenase from *Moraxella* sp. Positive controls for the *dhlB* (330 bp), *dehH2* (506 bp), and *mmoX* (369 bp) primers are presented in lanes 2, 5, and 8, respectively. The 100 bp DNA marker is presented in lane 1.

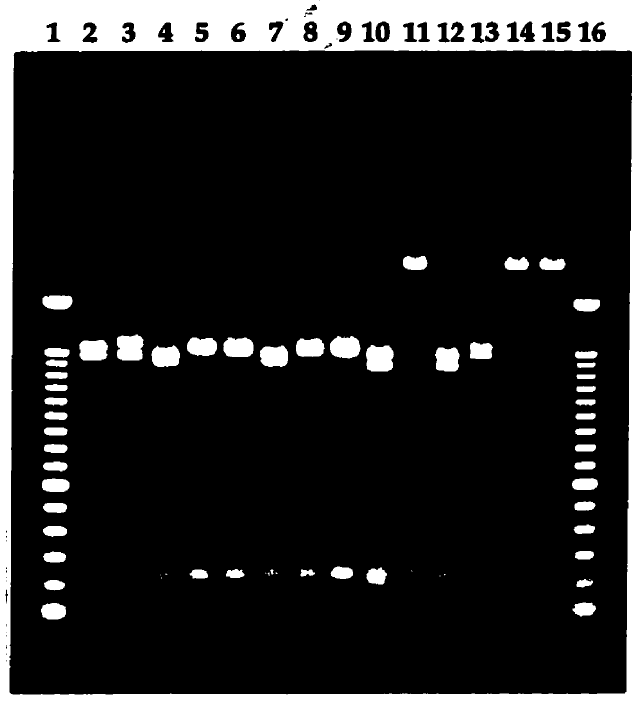

Figure 13. Restriction endonuclease analysis of ASB92/*mmoX* subclones using the *Ava II* enzyme. Subclones 1 to 13 are shown in lanes 2 to 14. The *E. coli* PTZ/PC plasmid is presented in lane 15. The 100 bp DNA marker is presented in lanes 1 and 16.

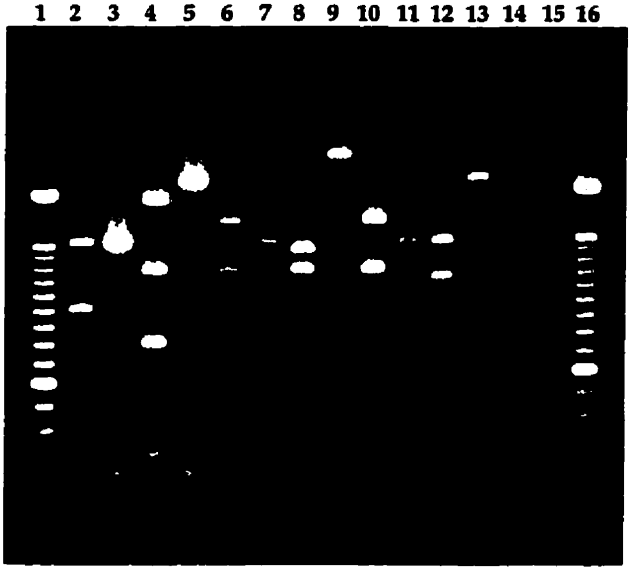

Figure 14. Restriction endonuclease analysis of ASB92/*mmoX* subclones using the *Ava II* enzyme. Subclones 14 to 27 are shown in lanes 2 to 15. The 100 bp marker is presented in lane 1 and 16.

Table 5. Sequence analysis of PCR fragments generated during amplification of total community DNA and dehalogenating bacterial isolates.

DNA source	Oligonucleotide primers	Homology %	Organisms
Total community DNA from natural sources			
Aerated stabilization basin (ASB92-19/23/24)	*mmoX*	100 95	*Methylococcus capsulatus*, *Methylosinus trichosporium*
Aerated stabilization basin (ASB92-3/6)	*mmoX*	64 62	*Methylococcus capsulatus*, *Methylosinus trichosporium*
Bioaugmentation experiments (B3-2/3/6)	*dehH2*	100	*Moraxella sp.*
Dehalogenating bacterial isolates			
Ancylobacter aquaticus (CN13)	*dhlB*	88	*Xanthobacter autotrophicus*

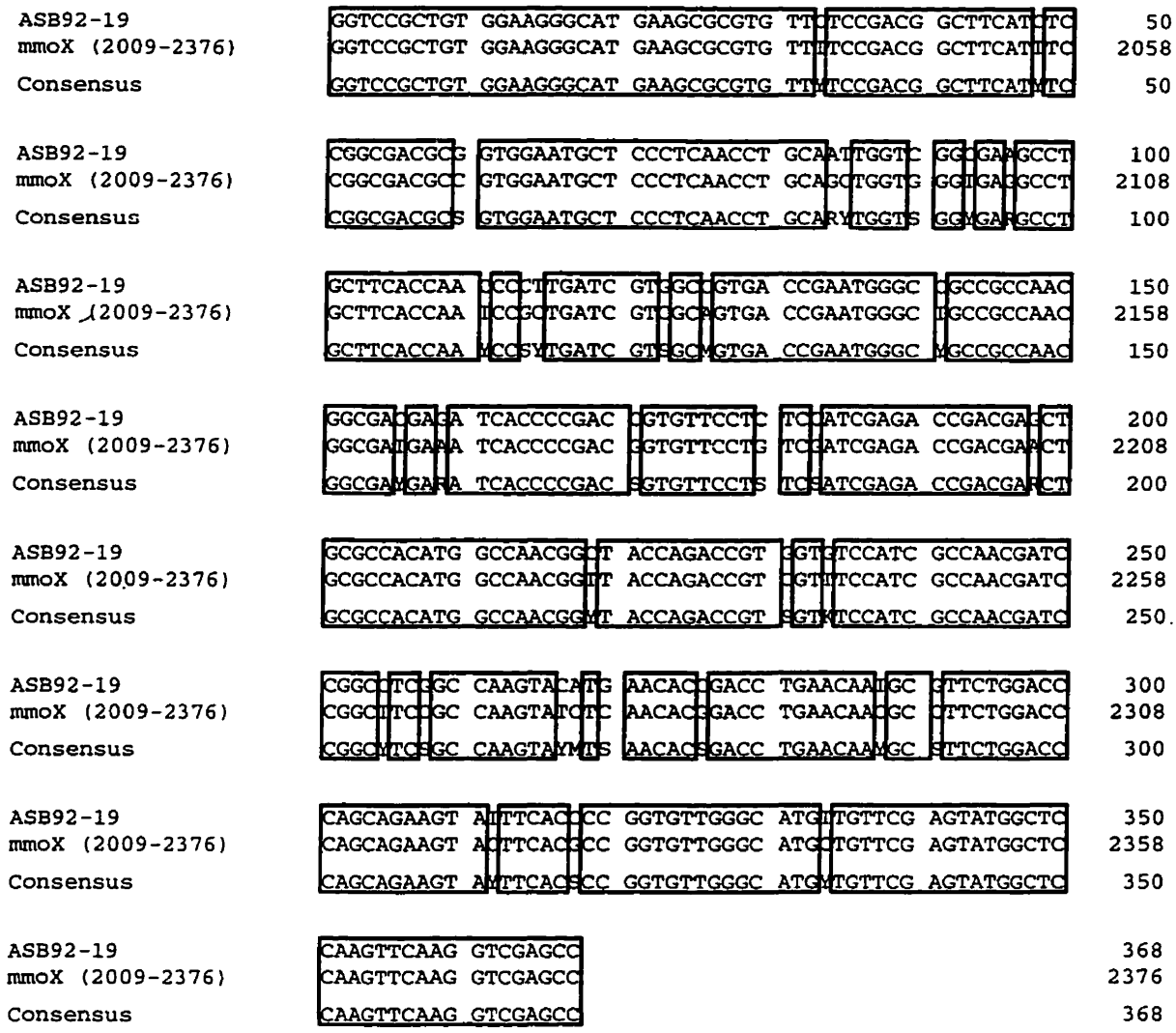

Figure 15. Nucleotide sequence alignment of ASB92/*mmoX* with a known oxidative dehalogenase gene. Similarity of ASB92/*mmoX* PCR product to the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Identical nucleotides are boxed. The accession number for *mmoX* is M90050.

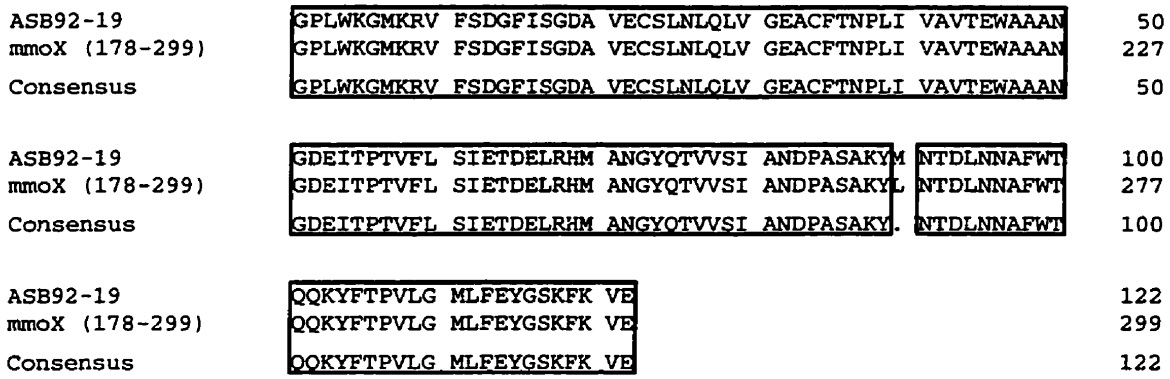

Figure 16. ASB92/*mmoX* shares homology with a known oxidative dehalogenase gene. ASB92/*mmoX* PCR product is 100% homologous to the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Identical amino acid residues are boxed. The accession number for *mmoX* is M90050.

Interestingly, several sequence variations were uncovered by DNA sequence analysis of the 369 bp subclone series (Fig. 15). However, these sequence differences resulted in only one conservative change at the protein level, at amino acid position 90, which substituted a leucine to a methionine residue (Fig. 16).

In addition, subclones 3 and 6 (Fig. 13) were sequenced and found to represent a separate group of dehalogenating bacteria. These subclones showed 64% and 62% homology, to the coding region of the methane monooxygenase found in *Methylococcus capsulatus* and *Methylosinus trichosporium*, respectively (Fig. 17).

Similarity to known methane monooxygenase genes was detected only from the primer sequences of subclones 1 and 12, and subclone 2 (Fig. 13). No open reading frames were detected from subclones 9 and 11 (Fig. 13).

Sequencing analysis and DNA alignments revealed that subclone 22 (one *Ava II* site) (Fig. 14) was in the opposite orientation of subclones 4, 5 and 7 (Fig. 13). Similarly, subclone 20 (two *AvaII* sites) (Fig. 14) was found to be the inverse complement of subclones 19, 23 and 24 (Fig. 14). The presence of shorter PCR fragments in both orientations suggests that the deletion may be due to unwanted primer hybridization at a second site on the template during PCR amplification. However, the 20 bp deletion could also be related to the primer's ability to form a hairpin structure. The potential for such a hairpin structure exists with 4 out of 6 nucleotides at the 3' end of the truncated portion of primer *mmoX* 2008 and its 5' end (Section 3.1.2). The inability of Taq DNA polymerase to correctly read across the base of the hairpin, could have resulted in deletion of the nucleotide sequence in the hairpin.

3.1.6.2 Total Community DNA from Bioaugmentation Experiments.

Total community DNA was extracted from two biomass samples (Biomass#2 and Biomass#3) previously grown in a bench scale continuous cultivation apparatus (aerated). The inoculum for the cultivation apparatus was from the aerated stabilization basin in the CPFP kraft mill treatment system at Dryden. Biomass #3 was exposed to a methanol concentration of 1.0 mM. Total community DNA from these biomass samples was screened with the oligonucleotide primers from dehalogenating genes. Both biomass samples were positive for the *dhlB* gene (Fig. 18). The results with *mmoX* primers were less conclusive but a very faint band of the predicted size (369 bp) could also be detected in biomass #3 (data not shown). The predicted PCR fragment (506 bp) was also generated with Biomass #3 and the *dehH2* oligonucleotide primers, designed from the coding region of the haloacetate dehalogenase in *Moraxella* (Fig. 18). The

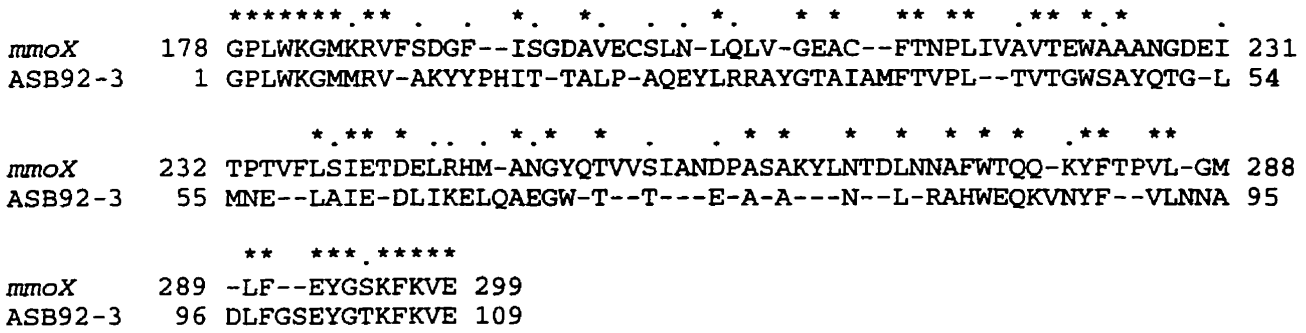

Figure 17. ASB92/*mmoX* shares homology with a known oxidative dehalogenase gene. ASB92/*mmoX* PCR product is 64% homologous to the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Identical amino acid residues are identified with asterisks. Conserved amino acids are marked with dots. The accession number for *mmoX* is M90050.

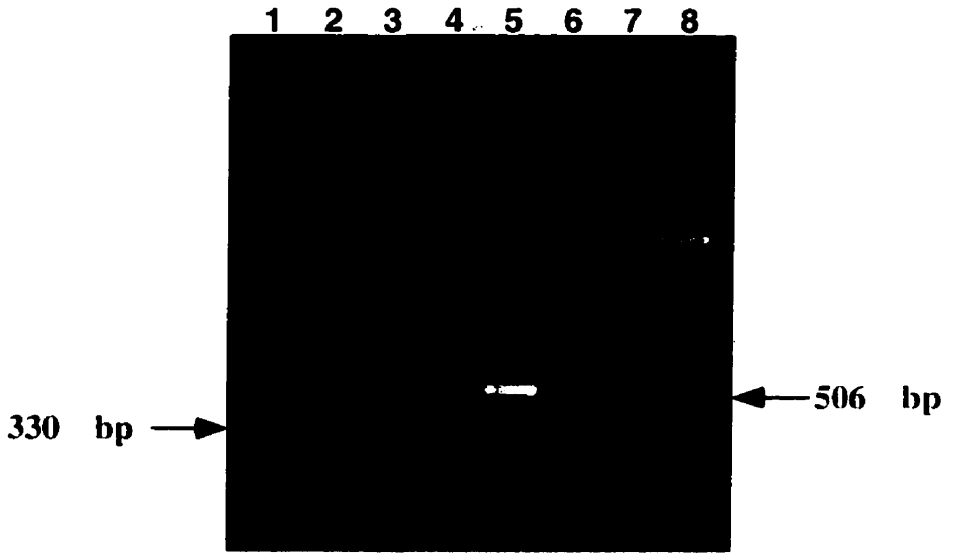

Figure 18. PCR analysis of total community DNA from a bioaugmentation experiment. PCR analysis of total community DNA extracted from biomass samples not exposed to methanol are shown in lanes 3, and 6. PCR analysis of biomass samples exposed to a methanol concentration of 1.0 mM are presented in lanes 4, and 7. PCR fragments of the predicted size (330 bp) were generated with both biomass samples with primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus* (lane 3, and 4). A PCR fragment of the predicted size (506 bp) was also generated from the biomass sample exposed to methanol and primers for the *dehH2* gene (lane 7), encoding a haloalkanoic acid dehalogenase from *Moraxella* sp. Positive controls for the *dhlB* (330 bp), and *dehH2* (506 bp) are presented in lanes 2, and 5. The 100 bp DNA marker is shown in lanes 1, and 8.

PCR fragment generated with the *dehH2 primers* was cloned into *E. coli* PTZ/PC plasmid and the individual subclones subjected to a *NcoI* restriction endonuclease analysis to verify the presence of an insert. The subclones were then subjected to an *AvaI/BglI* restriction endonuclease analysis in order to determine whether the cloned fragments were of different origin. A total of 50 subclones were screened in this experiment. The three different restriction patterns generated with *AvaI* and *BglI* enzymes are shown in Figures 19, 20, and 21.

Sequencing analysis and DNA alignments showed that subclones 2, 3, and 6 (Fig. 19) and subclones 4, 7, (Fig. 19) and 21 (Fig. 21) were in opposite orientations. A search through GenBank revealed 100% homology with the haloacetate dehalogenase gene, *dehH2*, from *Moraxella* (Fig. 22).

Subclones 1 (Fig. 19) and 17 (Fig. 20) also showed striking homology with the haloacetate dehalogenase from *Moraxella*. The protein sequence was over 99% identical to the *dehH2* gene (Fig. 24). Three sequence variations were uncovered by DNA sequence analysis of subclones 1 and 17 (Fig. 23). A T→C change in position 223 allows for a conservative change from a leucine to a phenylalanine residue. Similarly, a C→T change in position 376 substituted a phenylalanine to a leucine residue. The C→T change also created an additional *AvaI* site at nucleotide position 376. A third sequence difference was identified at nucleotide position 186, that did not result in any codon substitution at the protein level. These subclones were found only twice during the screening procedure. Additional sequencing analysis would be required to elucidate the difference between these subclones and the subclones mentioned above. The sequence differences may represent minor variations within the same species, but could also be the result of misincorporation by Taq DNA polymerase during PCR amplification.

A more detailed restriction analysis of subclones 5, 8 (Fig. 19) and 24 (Fig. 21) demonstrated that they were concatemers originating from molecular cloning.

3.2 Microbial and Molecular Characterization of a Biological Effluent Treatment System

The molecular tools developed in this study were used to characterize the microbial dechlorination potential within lagoons of a pulp and paper mill wastewater treatment system. The wastewater treatment system consisted of a primary treatment of alkaline effluent to remove fibers, neutralisation of acidic effluent, mixing of all effluent lines, biological treatment of all wastewater and treatment of sludge from the clarifier.

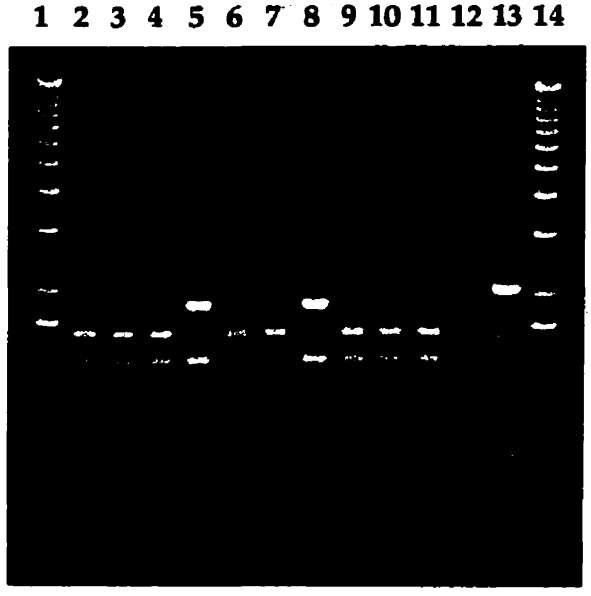

Figure 19. Restriction endonuclease analysis of B3/*dehH2* subclones using *Ava I* and *BglI* enzymes. Subclones 1 to 10 are shown in lanes 2 to 11. The 1 kb DNA marker is shown in lanes 1 and 14. The 100 bp DNA marker is presented in lane 13.

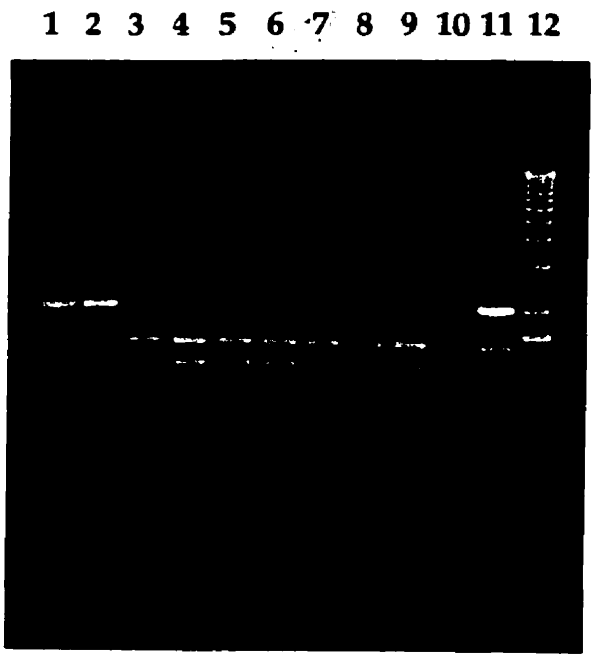

Figure 20. Restriction endonuclease analysis of B3/*dehH2* subclones using *Ava I* and *BglI* enzymes. Subclones 11 to 18 are shown in lanes 2 to 9. The 100 bp DNA marker is shown in lanes 1 and 11. The 1 kb DNA marker is presented in lane 12.

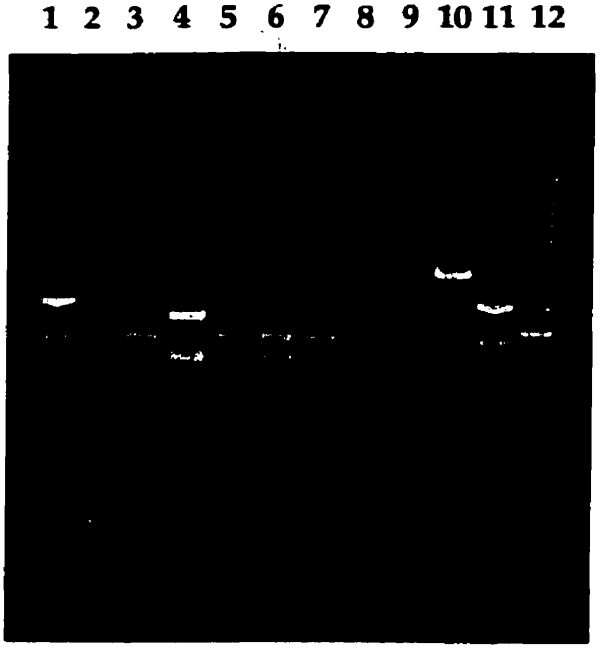

Figure 21. Restriction endonuclease analysis of B3/*dehH2* subclones using *Ava I* and *BglI* enzymes. Subclones 19 to 25 are shown in lanes 2 to 8. Lane 10 represents the *E. coli* PTZ/PC plasmid. The 100 bp DNA marker is shown in lanes 1 and 11. The 1 kb DNA marker is presented in lane 12.

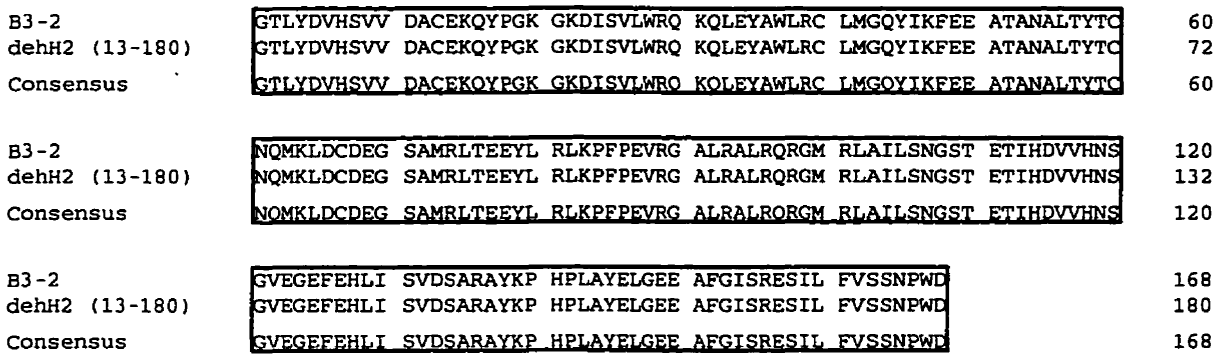

Figure 22. B3/*dehH2* shares homology with a known dehalogenase gene. B3/*dehH2* PCR product is 100% identical to the *dehH2* gene, encoding haloalkanoic acid dehalogenase from *Moraxella* sp.. Identical amino acid residues are boxed. The accession number for *dehH2* is D90423.

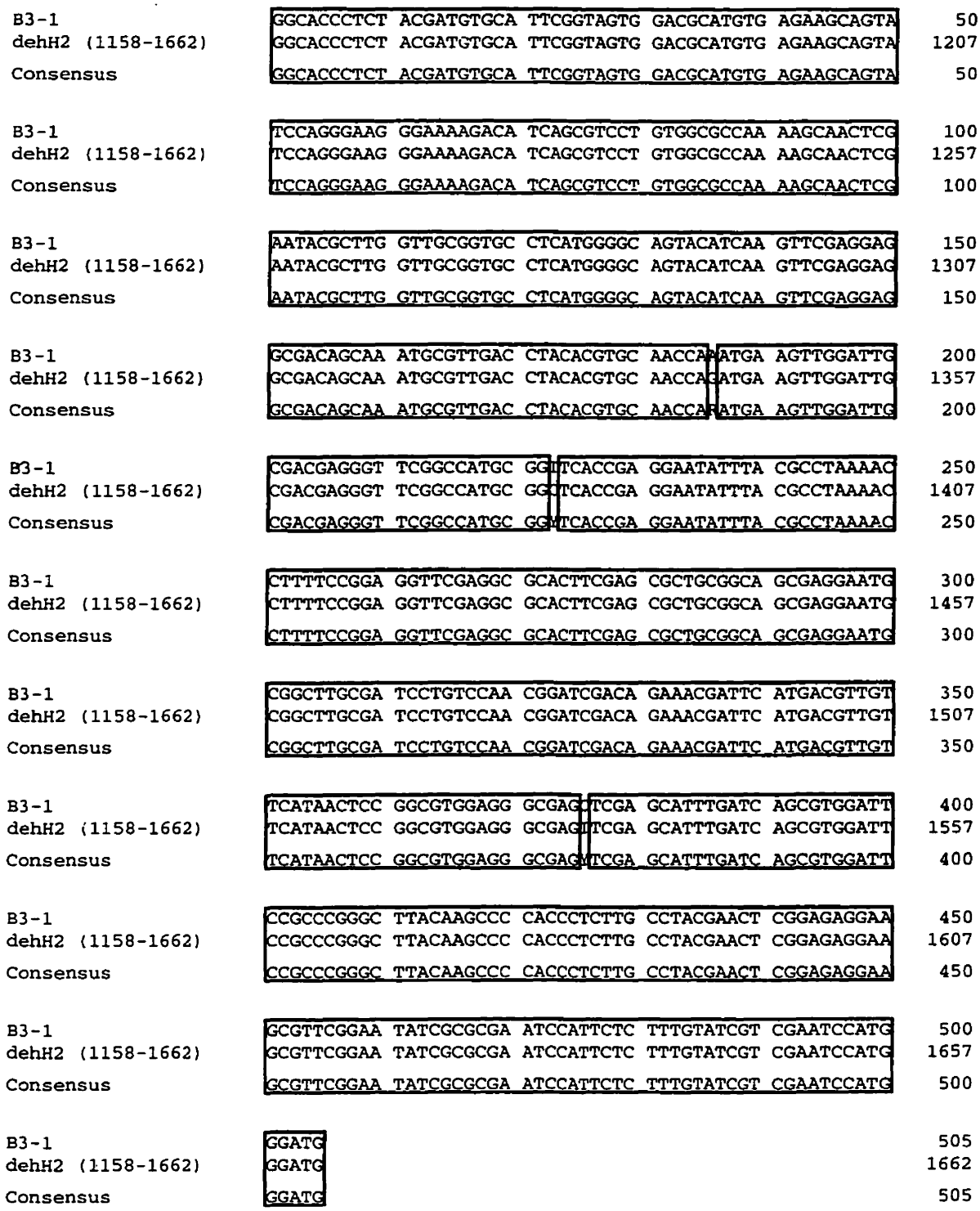

Figure 23. Nucleotide sequence alignment of B3/*dehH2* with a known dehalogenase gene. B3/*dehH2* PCR product is over 99% identical to the *dehH2* gene, encoding haloalkanoic acid dehalogenase from *Moraxella* sp.. Identical nucleotides are boxed. *AvaI* restriction sites (C↓(C,T)CG(A,G)G) are present at nucleotide positions 377 and 405. The accession number for *dehH2* is D90423.

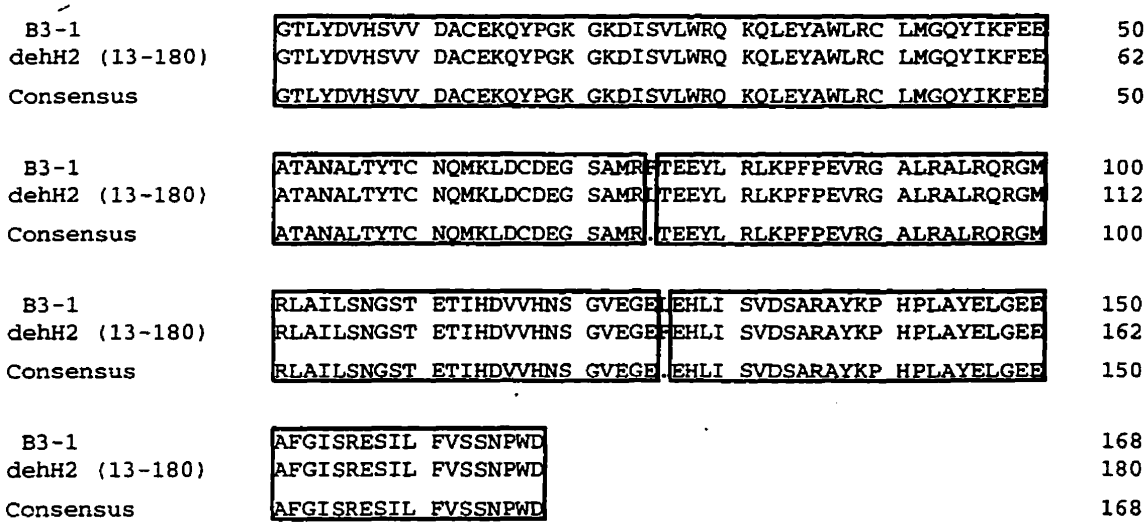

Figure 24. B3/*dehH2* shares homology with a known dehalogenase gene. B3/*dehH2* PCR product is over 99% identical to the *dehH2* gene, encoding haloalkanoic acid dehalogenase from *Moraxella* sp.. Identical amino acid residues are boxed. The accession number for *dehH2* is D90423.

Chemical pulping was carried out according to the kraft process. The effluents from four simultaneous bleaching lines (Standard, ECF, ECF, TCF) were combined and discharged to the aerated lagoons for biological treatment.

The biological treatment system was composed of six lagoons in series (Fig. 25). The retention time was approximately six days, one day per lagoon. The first four lagoons were aerated and the last two lagoons were for settling of the biomass. Part of the effluents was also discharged to a pilot plant. The Pilot Plant Effluent (PPE) duplicated the full scale treatment system using six 250 L tanks connected in series, with a retention time of one day per tank. The first four tanks were aerated to achieve a dissolved oxygen concentration of 2 mg/l.

The sampling points from the wastewater treatment system and the pilot plant are shown in Figure 25. Samples were collected from the pilot plant at the influent to tank 1 and from the effluent of tank 6. Samples from the lagoon treatment system were collected from the influent to lagoon 1 (first aerated lagoon), the effluent from lagoon 4 (last aerated lagoon) and from the effluent of lagoon 6 (last settling pond). Samples were allowed to settle overnight at 4°C, in order to be able to separate the water from the sediments. The water phase was separated from sediments under sterile conditions. No sediments were detected in sample 9804DV05 collected in pilot plant tank #6 (Table 6).

Table 6. Identification of water and sediment phases at different stages of the pulp mill treatment system.

Sample	Water	Sediments
9804DV01	LAG_1W	LAG_1S
9804DV02	LAG_4W	LAG_4S
9804DV03	LAG_6W	LAG_6S
9804DV04	PPE_1W	PPE_1S
9804DV05	PPE_6W	—

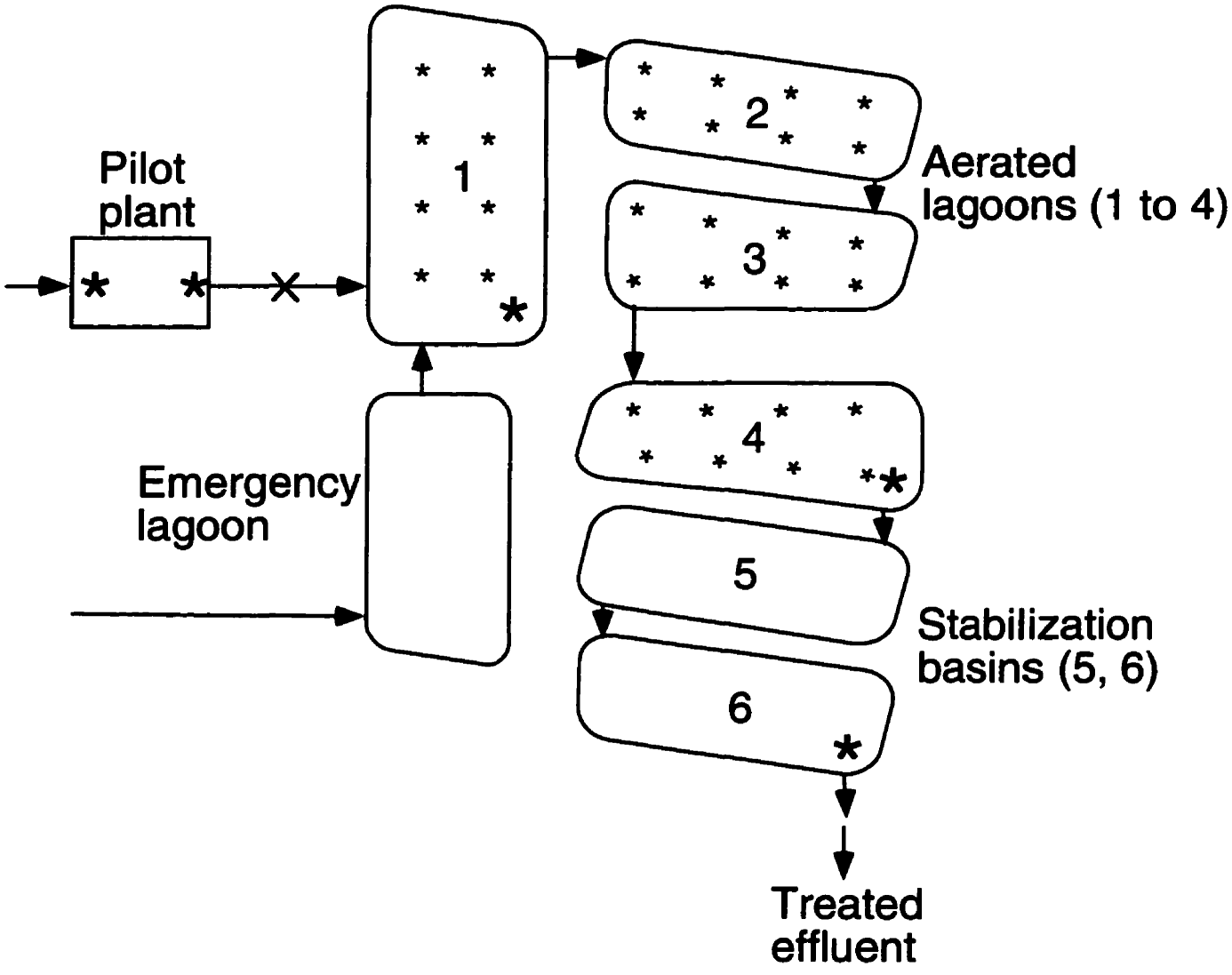

Figure 25. Schematic of a kraft pulp and paper mill effluent treatment system and a pilot plant. The stars indicate aeraters(*) or the sites from which water and sediment samples were collected (✱).

3.2.1 Bacterial Enumeration and Colony Hybridization

Samples were diluted and plated on YTS-250, a non-selective medium and a selective mineral salt medium (MSM), containing 2 mM chloroacetate and dichloroacetate and 50 mg/ml Yeast extract as sole carbon sources, and 75 mg/ml bromothymol blue. The indicator plates changed from green to yellow after only one week incubation in water and sediment samples collected from the pilot plant (Fig. 26). Color shifts were also observed in water and sediments collected from lagoon #6 as well as sediments from lagoon #4 after 3 weeks incubation. The total culturable counts, on each medium after 4 weeks incubation are presented in Table 7.

An increase in colony forming units was observed throughout the lagoon treatment system on both the selective and non-selective media. In general, counts on the selective medium were somewhat lower than counts on the non-selective low nutrient medium (YTS-250). The exception to this was the lag-1-w and lag-1-s, where the counts were slightly higher on the selective medium. This may be related to the presence of more organic matter (BOD) in the initial effluent, which may have contributed some nutrients to the solid medium.

Colony hybridization was performed with the *dhlB*, *dehH2* and *mmoX* gene probes. Probe positive organisms were detected only with the *dhlB* probe (Table 7). An increase in percentage of *dhlB* probe-positive colonies was observed throughout the lagoon treatment system and the pilot plant. The fact that there were no *mmoX*-positive colonies may indicate that sMMO-expressing organisms in pulp and paper effluent treatment systems, are non-facultative methylotrophs incapable to grow under our culturing conditions.

It is not clear, whether wastewater constituents have toxic or inhibiting effects on the bacterial community of biological treatment systems. One can speculate however, that as long as no wastewater constituents reach toxic or inhibiting concentrations for bacteria, bacterial growth may be expected to increase with decreasing toxicity of the effluent.

3.2.2 PCR Analysis of Total Community DNA

Total community DNA was extracted from water and sediment samples following the modified version of Flemming's method (Flemming *et al.* 1994). Potential dehalogenase and soluble methane monooxygenase encoding genes were detected essentially in every sample during PCR amplification of total community DNA

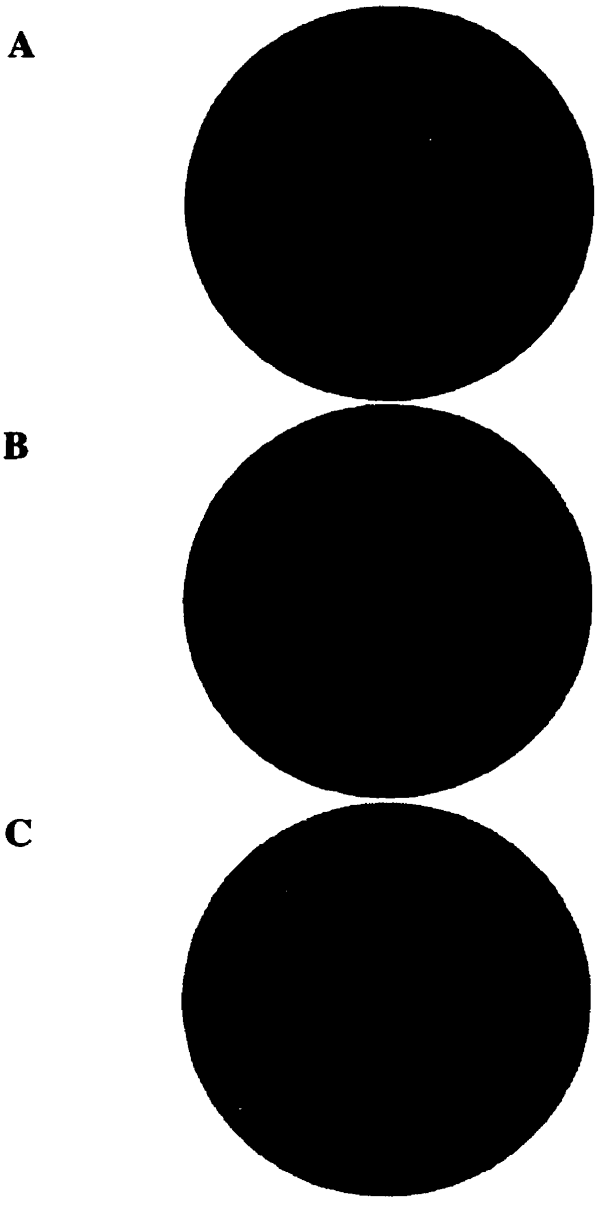

Figure 26. Bacterial growth on 2 mM chloroacetate and dichloroacetate.
Panel A shows a MSM plate supplemented with 2mM chloroacetate and dichloroacetate, streaked with water collected from a pilot plant, after 3 days incubation. Panel B and panel C show the same plate after 1 week and 2 weeks incubation, respectively. The color change is due to the presence of a pH indicator dye (bromothymol blue) that changes from green to yellow as chloride ions are liberated as HCl, reducing the pH of the solid medium. This color change, when related to the metabolism of a selective chlorinated compound indicates the presence of microorganisms with dehalogenating capacities.

Table 7. Microbial characterization of a pulp and paper mill effluent treatment system.

Sample	Viable bacteria (cfu[a]/ml or g X 10^5)		*% dhlB* (YTS)
	YTS	CAA	
Lag-1-w	10	21	1.3
Lag-1-s	58	120	1.1
Lag-4-w	20	7.1	4.6
Lag-4-s	250	200	8.9
Lag-6-w	11	7.2	5.1
Lag-6-s	1000	440	14.0
PPE-1w	230	TNTC[b]	1.0
PPE-1-s	20000	TNTC[b]	3.2
PPE-6-w	32	14	9.9

a. Colony-forming units.

b. Too numerous to count.

with *dhlB* and *mmoX* oligonucleotide primers. The results are presented in Figures 27, 28, 29, and 30.

Analysis of the lagoon samples using the *dhlB* oligonucleotide primers is shown in Figure 27. The samples were split to reflect analysis of the water fraction and the sediment fraction separately. DNA bands corresponding to the size of the *dhlB* fragment were detected in all but sample lag-1-s (sediment from lagoon 1). The inability to detect *dhlB*-positive DNA in lag-1-s may have resulted from a high concentration of humic materials in the sediment that interfered with total DNA extraction or subsequent PCR analysis. The results of the same analysis using DNA extracted from the pilot plant were less conclusive, but faint bands of the predicted size for the *dhlB* fragment were observed (Fig. 28).

Analysis of total community DNA from the various samples using the *dehH2* primers failed to detect any positives at the stringencies usually employed, suggesting that this dehalogenase gene may not be a predominant genotype in the bacteria from this treatment system (data not shown).

Similarly, none of the DNA extracts contained a detectable fragment that would indicate the presence of the *dhlA* gene encoding haloalkane dehalogenase (data not shown).

Analysis of the DNA extracts using the *mmoX* oligonucleotide primers successfully detected the predicted sized fragment in all the mill lagoon samples (Fig. 29). Again, the lag-1-s sample gave a weak and inconclusive result, likely due to the presence of humic acids in the sediment. The correct DNA fragment was also detected in total community DNA extracted from the pilot plant (Fig. 30).

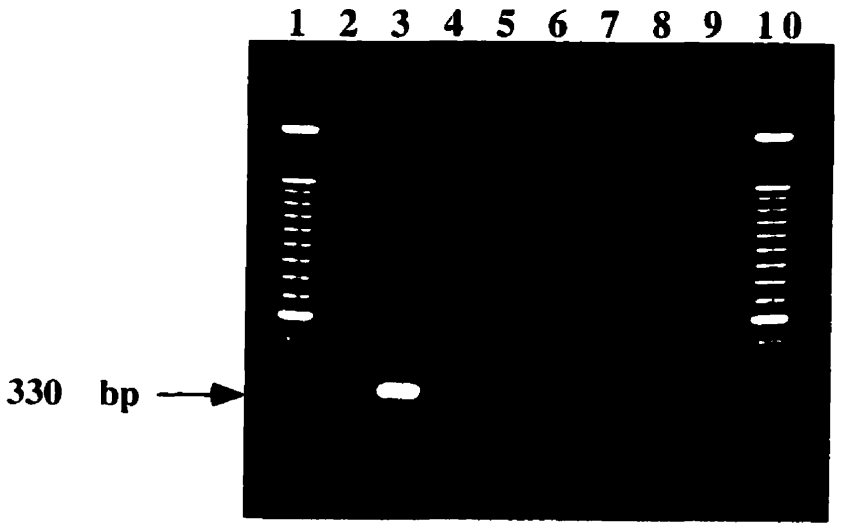

Figure 27. PCR analysis of total community DNA extracted from lagoons in a pulp and paper mill effluent treatment system. Lanes 4, 6, and 8, correspond to water samples collected from aerated lagoons 1 and 4, and settling lagoon 6, respectively. Lanes 5, 7, and 9 correspond to sediment samples collected from the same lagoons. The PCR analysis was performed using oligonucleotide primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. Negative and positive (330 bp) controls for the *dhlB* gene are shown in lanes 2 and 3, respectively. The 100 bp DNA marker is presented in lanes 1 and 10.

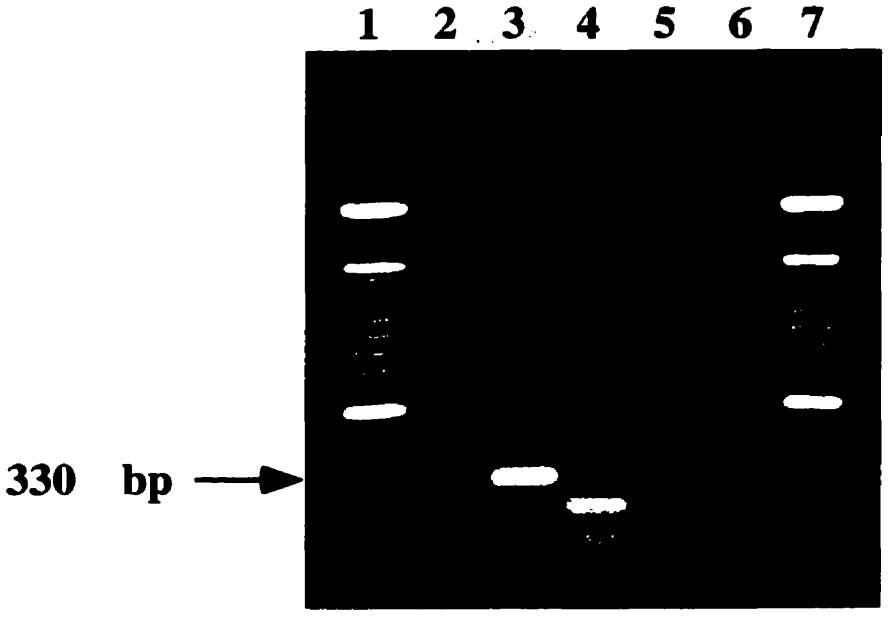

Figure 28. PCR analysis of total community DNA extracted from lagoons in a pulp and paper mill effluent treatment system. Lane 4 and 6 correspond to water samples collected from the pilot plant aerated tank 1, and settling tank 6, respectively. Lane 5 corresponds to sediment samples collected from settling tank 1. The PCR analysis was performed using oligonucleotide primers for the *dhlB* gene, encoding the haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus*. Negative and positive (330 bp) controls for the *dhlB* gene are shown in lanes 2 and 3, respectively. The 100 bp DNA marker is presented in lanes 1 and 7.

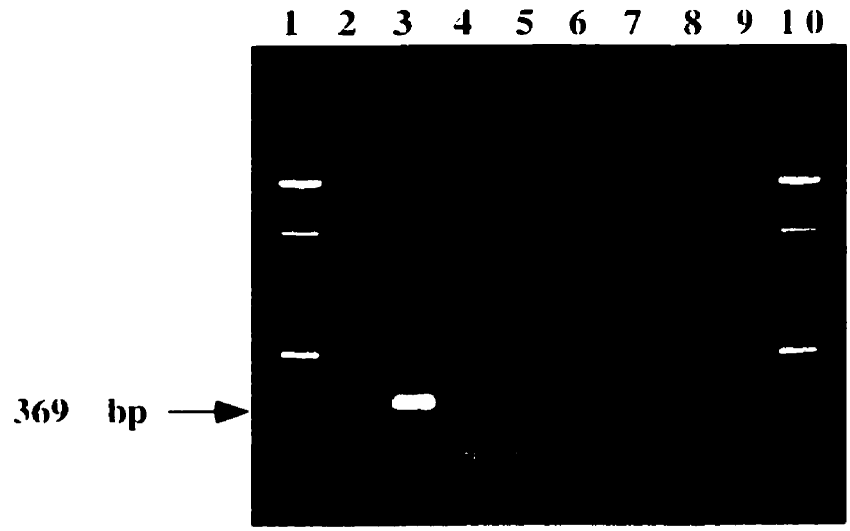

Figure 29. PCR analysis of total community DNA extracted from lagoons in a pulp and paper mill effluent treatment system. Lanes 4, 6, and 8 correspond to water samples collected from aerated lagoons 1 and 4, and settling lagoon 6, respectively. Lanes 5, 7, and 9 correspond to sediment samples collected from the same lagoons. The PCR analysis was performed using oligonucleotide primers for the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Negative and positive (369 bp) controls for the *mmoX* gene are shown in lanes 2 and 3, respectively. The 100 bp DNA marker is presented in lanes 1 and 10.

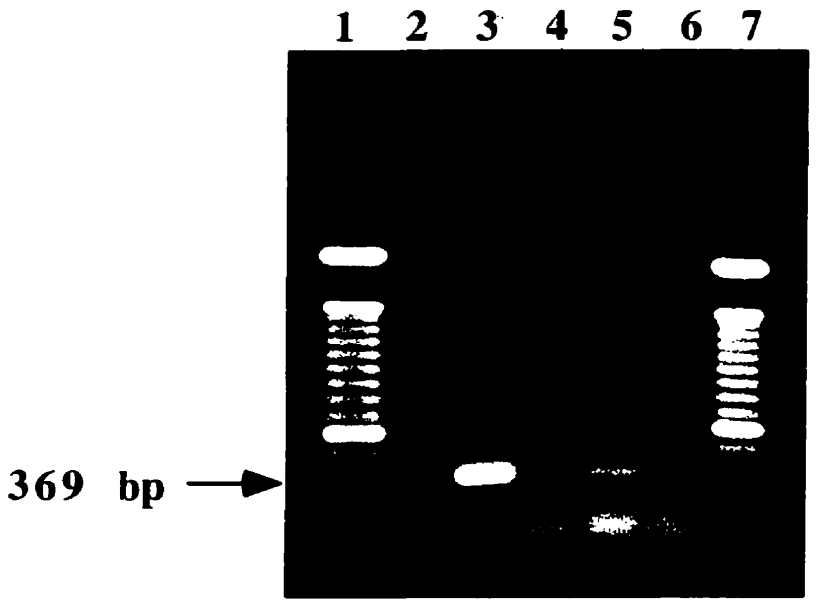

Figure 30. PCR analysis of total community DNA extracted from lagoons in a pulp and paper mill effluent treatment system. Lane 4 and 6 correspond to water samples collected from the pilot plant aerated tank 1, and settling tank 6, respectively. Lane 5 corresponds to sediment samples collected from settling tank 1. The PCR analysis was performed using oligonucleotide primers for the *mmoX* gene, encoding the soluble methane monooxygenase from *Methylococcus capsulatus*. Negative and positive (369 bp) controls for the *mmoX* gene are shown in lanes 2 and 3, respectively. The 100 bp DNA marker is presented in lanes 1 and 7.

Chapter 4 Discussion

In order to minimize the formation of chlorinated organic material and to comply with the various dioxin and AOX regulations, the producers of bleach kraft pulp have made substantial changes in pulp manufacturing technology. These changes include the addition of pre-bleaching steps such as extended cooking of the wood chips and oxygen delignification which result in a lower residual lignin content of the pulp entering the bleach plant. In the bleach plant, the major change has been the replacement of the chlorine used in the first bleaching stage with increasing proportions of chlorine dioxide. In Europe, primarily in response to market pressures, the bleaching of wood pulp without the use of chlorine and chlorine dioxide has been initiated. TCF bleaching technologies such as hydrogen peroxide and ozone-based processes as well as enzyme treatment are becoming popular alternatives for the bleaching of wood pulp.

In Canada, chlorine dioxide substitution has led to a shift towards less highly chlorinated material. Less highly chlorinated organic compounds are generally less toxic, less readily bioaccumulable and less persistent in the environment. In addition to the implementation of in-plant processes, an increasing number of Canadian pulp producers have adopted external control measures such as the installation of secondary biological treatment systems. The less highly chlorinated mono- and dichlorinated phenolics and chlorinated acetic acids produced from the bleaching process have been found to be readily removed from pulp mill effluent by secondary treatment.

Biosorption onto biomass followed by deposition into the benthal layer were found to be the key mechanisms in initial removal of AOX in biological treatment systems (Amy *et al.* 1988; Bryant *et al.* 1987, 1988). These mechanisms probably contribute to a considerable extent in reducing the toxicity of the effluents. It is not clear whether wastewater constituents have toxic or inhibiting effects on the bacterial community of biological treatment systems. For example, chlorinated acetic acids produced in abundance in the chlorine-based bleaching processes can be toxic to many organisms and inhibit their growth. However, several chloroacetate-utilizing bacteria were isolated from the Canadian Pacific Forest Products biological treatment system in Dryden, Ontario. These bacteria may contribute significantly to the removal of chlorinated organic compounds in the pulp and paper mill effluents. One can speculate that initial biosorption and settling of the biomass on the bottom of the lagoon may provide the necessary reduction in toxicity along with potential inhibitory compounds to permit microbial dehalogenation in the benthal layer throughout the treatment system.

A better understanding of the microbial populations operating in these systems can potentially lead to improvements in the reduction of toxicity and the removal of chlorinated organic compounds from bleach kraft pulp mill effluents, and help existing mills meet varying regulatory requirements and maintain their competitiveness.

The molecular tools developed in this study were successful at detecting the presence of dehalogenase genes in both dehalogenating bacterial isolates and in total community DNA extracted directly from water and sediment samples collected from secondary biological treatment systems. The PCR results show that there are bacteria present in effluent treatment systems that can degrade low molecular weight chlorinated organic compounds by two completely distinct mechanisms: by hydrolytic dehalogenation and oxidative dehalogenation.

Potential haloalkanoic acid dehalogenases were detected with the *dhlB* oligonucleotide primers in sediment samples collected in July 1992, from the wastewater treatment facility at the Canadian Pacific Forest Products mill in Dryden, Ontario. Potential *dhlB* encoding haloalkanoic acid dehalogenases were also detected in practically every water and sediment sample collected from three lagoons of a biological treatment system and 2 tanks of a pilot plant. Only one of the sediment samples (lag-1-s) failed to reveal the *dhlB* gene by our PCR detection approach. The inability to detect *dhlB*-positive DNA in this sample may have resulted from a high concentration of humic acid materials in the sediment that interfered with total DNA extraction or subsequent PCR analysis. Such high concentrations of humic acids are expected in wastewater created from cooking and bleaching of wood pulp. The incorporation of a second ammonium acetate precipitation or extraction with polyvinylpolypyrrolidone (PVPP) spin columns in the total community DNA recovery procedure (Berthelet *et al.* 1996), is recommended for the removal of humic acids in sediment samples collected from lagoon treatment systems and pilot plants.

Potential *dhlB* haloalkanoic acid dehalogenases were detected in essentially all individual dehalogenating bacterial isolates previously isolated from the wastewater treatment facility at the Canadian Pacific Forest Products mill in Dryden, Ontario. Furthermore, the *dhlB* oligonucleotide primers were capable of detecting a novel dehalogenase gene very similar to the known haloalkanoic acid dehalogenase from *Xanthobacter autotrophicus* in *Ancylobacter aquaticus* strain CN13. *Ancylobacter aquaticus* has been characterized as an oligotrophic methylotroph because it can fix carbon dioxide under low nutrient conditions in reduced environments (Raj 1989). However, several studies indicate the affinity of this organism for carbon-rich wastewaters. *Ancylobacter aquaticus* strains have been isolated from wood-lake ponds,

the surface of granular activated carbon in a water treatment facility, and from an activated sludge reactor used to treat spent cutting fluids, which included chlorinated paraffins (Raj 1989). *Ancylobacter aquaticus* strains were also isolated from the aerated lagoons of a Weyerhauser pulp mill (Raj 1989), and from the influent and effluents of an aerobic wastewater treatment pond at a Pacific Northwest kraft paper mill (Strand *et al.* 1984). The abundance of *A. aquaticus* in pulp mills is attributable to the fact that they can use a variety of compounds that are found in the wastewaters of bleached kraft pulp mills such as galactose, glucose, arabinose, acetate, formate, and methanol as well as simple chlorinated substrates such as chloroacetate and chloroethanol (McKague *et al.* 1989; Fulthorpe *et al.* 1993). The abundance of various *Ancylobacter aquaticus* strains in secondary biological treatment systems combined with their ability to use a variety of chlorinated compounds make them suitable candidates for *in situ* bioremediation of pulp and paper mill effluents.

Similar banding patterns were generated during PCR amplification of genomic DNA from *Ancylobacter aquaticus* A7 and *Methylobacterium* SP17 with both the *dhlB* and *mmoX* primers. These results suggest that two phenotypically dissimilar species may have comparable dehalogenation mechanisms for the dechlorination of chlorinated organic compounds in pulp and paper mill effluents. It is tempting to speculate that dehalogenase genes in these species may be plasmid borne and derived from horizontal gene transfer. Fulthorpe *et al.* (1993) demonstrated that plasmid incidence levels in culturable bacteria from CPFP pulp and paper mill treatment system was higher than in the bacterial community of the influent river water. Interestingly, the high plasmid incidence levels in the kraft mill treatment system was due to the dominance of *Ancylobacter aquaticus* and *Methylobacterium* strains. The majority of isolated strains that were capable of dechlorinating simple aliphatic molecules also belong to these two groups, and all carried plasmids. Four plasmids were identified in *Ancylobacter aquaticus* strain A7 and several plasmids were found in *Methylobacterium* SP17. Both species were isolated from the aerated stabilization basin of CPFP kraft mill effluent treatment system.

A second possibility is that dehalogenase genes in these species are located on a transposon that can move throughout the genome. Such a transposon was identified in *Pseudomonas putida* PP3, an organism that can grow on 2,2-dichloropropionic acid owing to the production of two different dehalogenase genes (Senior *et al.* 1976). One of the dehalogenase gene, *dehI* is located on a mobile genetic element (DEH) (Thomas *et al.* 1992). In *Moraxella* sp. strain B, the genes *dehH1* and *dehH2* are closely linked on a plasmid pUO1. This plasmid was found to be structurally unstable, with a 5.8 kb

sequence apparently capable of spontaneous excision (Kawasaki *et al.* 1981). Concomitant with the loss of this 5.8 kb sequence was the loss of the *dehH2* enzyme. The *dehH2* sequence was flanked by two repeated sequences (Kawasaki *et al.* 1992), which may play a part in the frequent spontaneous deletion of *dehH2* from the plasmid, suggesting that the fragment may be a transposon. The isolation, sequencing, and localization of dehalogenase genes in *Ancylobacter aquaticus* strain A7 and *Methylobacterium* strain SP17 will help determine whether dechlorination activity is a) plasmid encoded and derived from horizontal gene transfer and b) whether the dehalogenase gene is genetically linked to a transposable element.

PCR fragments of the expected size generated during amplification of genomic DNA from *Pseudomonas* P1 and *Methylobacterium* CP13 with the *dhlB* primers revealed no homology with any known dehalogenases. Additional sequence analysis of PCR fragments of expected or similar sizes, is necessary to determine whether the various dehalogenating bacterial isolates possess dehalogenation systems similar to the *Xanthobacter autotrophicus* haloalkanoic acid dehalogenase. Special attention should be given to *Methylobacterium* strain CP13 since it appeared to be very efficient in removing total and recalcitrant AOX from both softwood and hardwood effluents (Fulthorpe and Allen 1995).

The results for the *mmoX* gene indicate that methylotrophic bacteria appear to be present throughout effluent treatment systems. Bacteria that possess genes similar to the *Methylococcus capsulatus* soluble methane monooxygenase gene *mmoX*, were repeatedly detected in water and sediment samples collected from three lagoons of a biological wastewater treatment system and 2 tanks of the pilot plant effluent. Similar to PCR analyses using the *dhlB* primers, only sample lag-1-s failed to reveal the methane monooxygenase gene by our PCR detection approach. The presence of humic acids in the DNA extract may have reduced the specificity of hybridization during PCR amplification.

Oligonucleotide primers for the *mmoX* gene were capable of detecting two novel methane monooxygenase genes in the aerated stabilization basin in CPFP kraft mill in Dryden, that are 100% and 64% homologous to *Methylococcus capsulatus* and *Methylosinus trichosporium* methane monooxygenases, respectively. The occurence of two different types of oxidative dehalogenation systems in the effluent indicates some level of diversity for methylotrophic bacteria present in the same sampling site. In addition, the presence of several sequence variations in the DNA sequence of one of the methane monooxygenase genes, but only one conservative change at the protein level, suggests that methylotrophs possess some level of heterogeneity in this gene. This

heterogeneity may reflect a polymorphism in this region, suggesting that methylotrophic bacteria may possess specific monooxygenase-mediated dehalogenation mechanisms in wastewater treatment systems capable of degrading a variety of low molecular weight chlorinated organic compounds.

PCR amplification of DNA extracted from a bioaugmentation experiment with the *dehH2* oligonucleotide primers revealed a dehalogenase identical to the known haloacetate dehalogenase from *Moraxella.* The *dehH2* primers also detected a dehalogenase that was over 99% identical both at the nucleotide and protein level to the *dehH2* gene from *Moraxella.* This dehalogenase gene was detected during restriction endonuclease analysis because of a C→T change in the nucleotide sequence that resulted into the incorporation of an additional *Ava I* site.

Additional sequencing information would be necessary to determine whether the sequence variations observed between the dehalogenase gene detected in biomass samples and the *dehH2* encoding haloacetate dehalogenase represent variations within the same species or simply originate from misincorporations by Taq DNA polymerase during PCR amplification. The rate of misincorporation by Taq DNA polymerase during PCR amplification is estimated at 2 $X10^{-4}$ nucleotides/cycle for a 30 cycle amplification (Sambrook *et al.* 1989). Taq DNA polymerase normally exhibits high specificity and favors extension of matched over mismatched base pairs (Creighton *et al.* 1992). However, several factors such as reaction pH (Sowers *et al.* 1989), annealing temperature, and the presence of divalent cations other than Mg^{2+} (Goodman *et al.* 1982) can alter the fidelity of insertion and extension. Relative efficiencies for extending transversion mispairs at template-primer 3'-termini were found to be 10^{-4} to 10^{-5} for T→C and T→T, about 10^{-6} for A→A, and less than 10^{-6} for G→A, A→G, G→G and C→C. However, the C(primer)→T transversion mispair was shown to be extended with exceptionally high efficiency by Taq DNA polymerase (roughly 1/50 the rate of extension of the corresponding correct A·T basepair) (Huang *et al.* 1992). In this study, it is reasonable to think that the additional *AvaI* site detected during restriction endonuclease analysis of biomass subclones may have been created from a C→T transversion mispair. However the possibility remains, that sequence variations and C→T differences between subclones may instead reflect a genetic polymorphism at this locus creating distinct dehalogenase isoforms within this species.

Genetic variation within bacterial strains living in the same geographical site was demonstrated by Masters *et al.* (1991) analysis of nineteen new strains of *Deinococcus radiopugnans* isolated from three 2g soil samples taken from adjacent sites close to a Nottingham (UK) lake. By standard morphological and physiological

criteria, these strains were identical, but restriction fragment length polymorphism (RFLP) analysis with three different probes revealed 17 different groupings with no overlap of RFLP types between the groups.

The genetic diversity of bacterial strains present in the same sampling site, could also be demonstrated with denaturing gradient gel electrophoresis (DGGE). In DGGE, DNA fragments of the same length but with different base pair sequences, such as the PCR fragments obtained from total community DNA, can be separated. This technique was sucessfully applied to discriminate between different *Desulfovibrio* species by analyzing sequence variations in [NiFe] hydrogenase gene fragments (Wawer and Muyzer 1995). This approach could greatly facilitate sequence analysis of the various dehalogenating systems identified in pulp and paper mill effluents by providing the exact number of species that generated a given PCR fragment. This would in turn help during sequence analysis, and discriminate between misincorporations of nucleotides by Taq DNA polymerase.

Interestingly, bacteria possessing genes identical or very similar to the *dehH2* dehalogenase from *Moraxella* were identified only from a bioaugmentation experiment but not from any of the samples collected from the lagoon treatment systems, or the pilot plant. Contrary to methylotrophs, *Moraxella* species, may have adapted to a very specific environment where they can optimize their degradative capabilities, and may be sensitive to drastic changes in conditions often encountered in biological treatment systems.

Biological wastewater treatment systems are constantly subject to perturbations due to the changing composition of the effluent and variations in climate. For example, changing composition of the mill effluent as a result of shifts from processing softwood to processing hardwood and vice versa can result in changes in the microbial population. The numbers of aerobic and anaerobic bacteria in water columns obtained from the aerated stabilization basin in CPFP kraft mill in Dryden, were found to be significantly higher when hardwood was processed (Liss and Allen 1992). Viable counts for the aerobic population during the processing of softwood were about 65% lower than those obtained during hardwood runs. Hardwoods contain significantly less lignin than softwoods and require less chlorine during bleaching. The processing of softwood may generate greater levels of chlorinated organic compounds and extractives such as resins and fatty acids, which may inhibit the growth of many bacteria present in the lagoon. Continuous lab-scale and batch studies of AOX removal in simulated lagoon conditions, indicate that the removal of AOX takes place within the first few days of treatment under both aerobic and anaerobic conditions (Chernysh *et al.* 1992;

Collins 1991). The increase in viable bacterial counts within the lagoon occurred within 3 to 5 days after the processing of hardwood began and would decline within the same amount of time after the beginning of a softwood run (Liss and Allen 1992). Molecular tools are appropriately suited to study the effect of wood type on dehalogenating bacterial populations. A better understanding of dehalogenating bacterial response to such changes could help determine an optimal schedule for the processing of hardwood and softwood that would impose minimum stresses on the dehalogenating bacterial population resulting in more efficient removal of chlorinated compounds in the treatment system.

In this study, an increase in viable bacterial counts as well as *dhlB* probe-positive microorganisms was observed throughout a lagoon treatment system in both water and sediment samples. This trend cannot be attributed to stresses on the bacterial population created from changes in wood type since the mill only processes hardwood. An interesting feature that may have important repercussions on the bacterial populations operating in these systems however, is the combination of effluents from different bleaching lines before treatment in the lagoon system. Particular bleaching combinations may create higher levels of chlorinated organic compounds in the effluents which might impose stresses on microorganisms present in the lagoons. For example, microbial populations may be slow to adapt to Standard, ECF, ECF, TCF bleaching sequences if they were previously subjected to a Standard, Standard, Standard, ECF combination. The molecular approach taken in this study, may similarly be applied to assess the presence of dehalogenating bacterial populations in pulp and paper mill effluents from different bleaching sequences. A better understanding of the microbial response to various bleaching liquors could lead to the optimization of the wastewater treatment system by allowing microorganisms to adapt gradually to different bleaching combinations. A progressive change in toxicity of bleaching liquors should help maintain stable microbial populations resulting in better removal of chlorinated organic material in pulp effluents.

In addition, a molecular detection approach is suitable for detecting the response of bacterial populations to seasonal variations in temperature. Studies by Liss and Allen (1992) demonstrated that bacterial populations found in the lagoon receiving effluent from Canadian Pacific Forest Products bleached kraft pulp mill are affected by seasonal variations. Lagoon effluents were found to be toxic during January, February and March. The occurence of lagoon effluent toxicity during the winter months corresponded with declining temperature and numbers of viable bacteria in the aerated stabilization basin. In this study, the *dhlB* primers failed to detect the haloalkanoic acid

dehalogenase in the sediment sample (ASB91) collected from the aerated stabilization basin in CPFP kraft mill in January 1991. The predicted size PCR fragment was detected however, from sediment samples (ASB92) collected in July 1992. The same results were obtained with the *mmoX* primers. These results may be interpreted as meaning that bacteria possessing the haloalkanoic acid dehalogenase or oxygen-mediated dehalogenation mechanisms may be vulnerable to perturbations created by variations in climate and be below detection limits during the winter months.

The oligonucleotide primers and corresponding DNA probes developed in this study, constitute "first generation" probes to study the microbial dechlorination potential of pulp and paper biological treatment systems. These probes allowed the detection of a novel dehalogenase gene and two methane monooxygenases in an aerated stabilization basin in CPFP kraft mill at Dryden, Ontario that can provide useful information on bacterial populations operating in this system. The novel dehalogenase gene identified from *Ancylobacter aquaticus* can now be isolated and characterized. The ability of *mmoX* primers to detect novel methane monooxygenases can be exploited further by trying to isolate the microorganisms using the corresponding DNA probe. Isolation of novel dehalogenase genes and additional sequence information will help improve the quality of probes for subsequent analysis. Furthermore, additional sequence information from novel dehalogenase genes may serve to provide a better understanding of the structural and biochemical properties of haloalkanoic acid dehalogenases.

Additional sequence information from novel dehalogenation systems may also help elucidate both transport and regulation mechanisms involved in the dechlorination activity observed in pulp and paper mill effluents. For example, sequence analysis of the upstream region of the *dhlB* haloalkanoic acid dehalogenase of *Xanthobacter autotrophicus* revealed the presence of two open reading frames (ORF) (Van der Ploeg and Janssen 1995). The first ORF with two potential translation initiation sites, ended 30 bp upstream of *dhlB* and was designated *dhlC*. The *dhlC* gene encodes a protein similar to the family of Na^+-dependent transport proteins and possibly serves as a carrier of the negatively charged haloalkanoic acids through the cellular membrane. The second ORF designated *dhlR*, was situated in the opposite direction of transcription, upstream of *dhlC*, and was preceeded by a putative ribosome binding site. The *dhlR* gene encodes a protein of 464 amino acids sharing significant similarity with the family of transcriptional activators which activate expression in concert with the alternative sigma factor σ^{54} (Morett and Segovia 1993). The genetic organization of the *dhlB* region, suggests that the expression of *dhlC* and possibly *dhlB* may be under positive

regulatory control by *dhlR* and dependent on both *dhlR* and σ^{54}. This kind of sequencing information provided useful information on the mode of regulation of *Xanthobacter autotrophicus* haloalkanoic acid dehalogenase. Additional sequence information from novel dehalogenation systems in pulp and paper mill effluents, could reveal similar regulatory mechanisms and may provide clues on how to stimulate chloroorganic degradation activity.

Optimizing wastewater treatment systems is a practical alternative to environmental control of chlorinated organic compounds. The molecular tools developed in this study can be used to monitor the presence of microorganisms possessing specific dehalogenase genes and therefore provide a better understanding of the microbial populations operating in these systems. Molecular tools can also be used to evaluate the microbial response to environmental perturbations often encountered in biological treatment systems. A better understanding of the microbial response to seasonal variations, and changing composition of the effluent as a result of shifts from processing softwood to processing hardwood and vice versa, and shifts in bleaching combinations will help design better bleaching schedules that should ensure stability and performance of biological treatment systems. Optimizing wastewater treatment systems will result in better removal of chlorinated organic compounds, and help pulp and paper producers meet effluent requirements and maintain their competitiveness.

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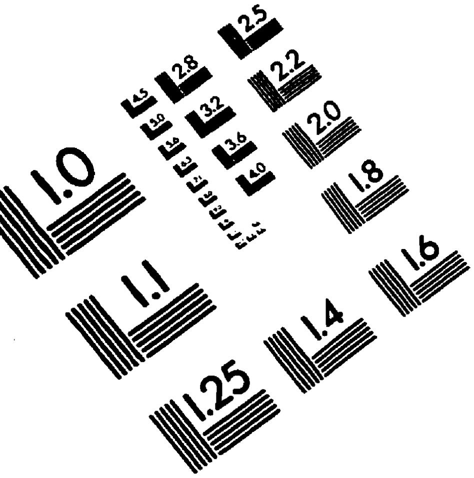
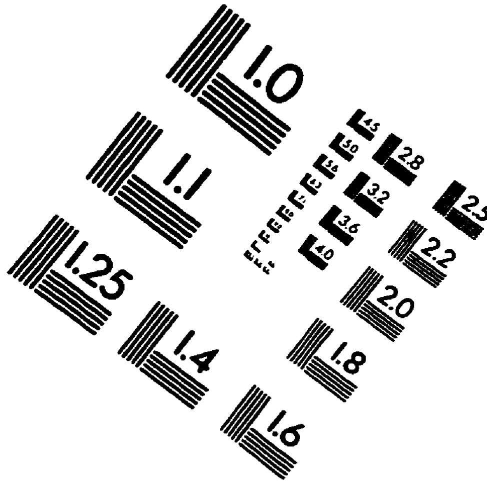
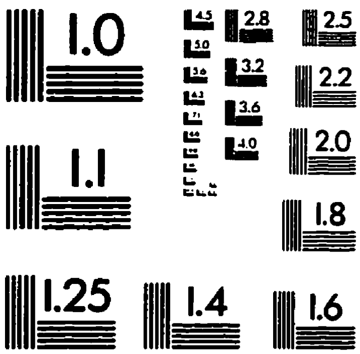
IMAGE EVALUATION TEST TARGET (QA-3)







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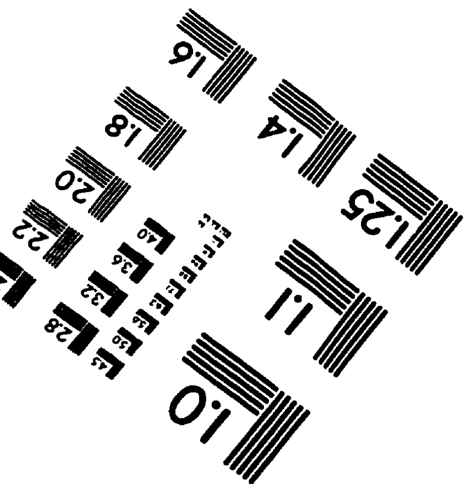

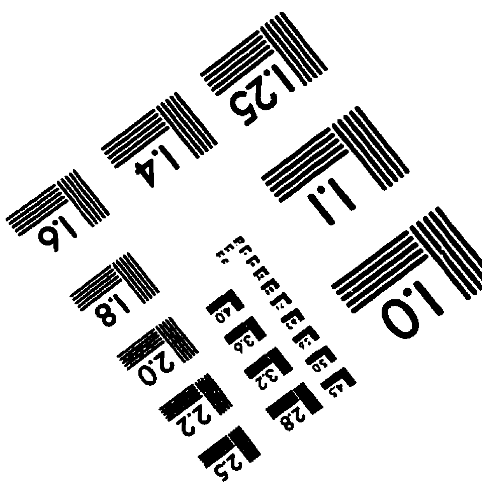

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