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**CATABOLIC GENOTYPE DISTRIBUTIONS IN THE NIAGARA
WATERSHED**

**by
MICHELLE C. PEEL, B.Sc.**

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of**

Doctor of Philosophy

**Department of Biology
Ottawa-Carleton Institute of Biology**

**Carleton University
Ottawa, Ontario
September 1996**

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CATABOLIC GENOTYPE DISTRIBUTIONS IN THE NIAGARA WATERSHED

submitted by
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in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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ABSTRACT:

The distribution of chlorobenzoate and biphenyl catabolic genotypes in a chloroaromatic contaminated environment was studied over an 18-month period. The Niagara watershed was selected as the study site due to the long history of chemical contamination in this area, including the Hyde Park landfill site.

The degradation potentials of 3-chloro-, 4-chloro- and 3,4-dichlorobenzoate were determined by batch culture enrichments at 32°C, 22°C and 4°C. These results showed that contamination from the Hyde Park landfill site had a significant effect on the chlorobenzoate degradation potential in the Niagara watershed. 4-Chlorobenzoate had the highest degradation potential. While seasonal variations were noted for 3-chlorobenzoate degradation, yearly variations were noted for 4-chloro- and 3,4-dichlorobenzoate degradation. Complete chlorobenzoate mineralization by the 4°C enrichments suggested that the culturable microbial consortia contained facultative psychrophiles.

Hybridization results for chlorobenzoate catabolic isolates revealed that four known catabolic genotypes: chlorocatechol 1,2-dioxygenase (*cicABD*), 3-chlorobenzoate 3,4-(4,5)-dioxygenase (*cbaAB*), 4-chlorobenzoate dehalogenase (*fcbB*) and biphenyl dioxygenase (*bphA*) were present in less than 5% of the isolates screened. A continuous culture enrichment of a chloroaromatic contaminated sample from Hyde Park revealed that the *cicABD* and *cbaAB* genotypes co-existed. Likely the hosts harbouring these genotypes metabolized alternative carbon sources and established themselves in different niches, including biofilms.

The probe positive isolates could be placed into five phenotypically distinct groups. Restriction fragment length polymorphism (RFLP) patterns suggested that the genes examined in this study evolved from multiple sources. Two independently evolved copies of the transposon, Tn5271, were found in *cbaAB* probe positive isolates. As well, many of the probe positive isolates harboured IS1071, confirming previous observations of the association of this insertion sequence with nonhomologous pollutant degradation genes.

A total environmental DNA extraction method was optimized for the sediment and water samples that yielded high molecular weight DNA with limited humic acid contamination. Hybridization results of PCR amplification products showed that both the *bphA* and *chaA* genes were detected in all environments, including samples from the Hamilton Harbour. The *bphA* gene was distributed equally in contaminated and non-contaminated sites. In comparison, the *chaA* gene was detected in higher target copy numbers at chloroaromatic contaminated sites.

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LIST OF ABBREVIATIONS:

2,4-D:	2,4-dichlorophenoxyacetic acid
3-Cba:	3-chlorobenzoate
4-Cba:	4-chlorobenzoate
3,4-dCba:	3,4-dichlorobenzoate
3CBP:	3-chlorobiphenyl
BP:	biphenyl
<i>bph</i>:	biphenyl dioxygenase
BRC:	Bloody Run Creek
<i>cba</i>:	3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway genes
<i>clc</i>:	chlorocatechol modified <i>ortho</i>-ring fission pathway genes
CPS:	casitone, peptone, starch general growth medium
<i>fc</i>:	4-chlorobenzoate dehalogenase pathway genes
DHC:	Devil's Hole Creek
ERIC:	enterobacterial repetitive intergenic consensus
FC:	Fish Creek
fg:	femtogram
GC-MS:	gas chromatography-mass spectrometry
GEM:	genetically engineered microorganism
HPLC:	high performance liquid chromatography
kbp:	kilobase pair
kg:	kilogram
mM:	millimolar
MR:	Moses Road
MW:	molecular weight
ng:	nanogram
PCB:	polychlorinated biphenyl

pg:	picogram
RAPD:	random amplified polymorphic DNA
REP:	repetitive extragenic palindromic
RFLP:	restriction fragment length polymorphism
SBR:	sequencing batch reactor
TE:	Tris-EDTA buffer
TYE:	tryptone-yeast extract, bacterial growth medium

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CHAPTER 1:

An Overview of Microbial Degradation of Chlorinated Aromatic Contaminants

1.1 Introduction

Halogenated aromatic compounds have been increasingly detected in the environment as a result of industrial and agricultural activities during the last few decades. Despite the fact that halogenated compounds are produced naturally in the environment (Klages and Lingens 1979, Fetzner and Lingens 1994), their presence is now at higher than natural concentrations making them a significant environmental concern. Although abiotic degradation of halogenated compounds is possible, for instance photochemical degradation, nonenzymatic reactions rarely lead to a substantial alteration of chemical structure (Reineke 1984). Microorganisms play a central role in the complete degradation of organic compounds and therefore are very important for the detoxification or mineralization of chlorinated aromatic compounds to Cl^- ions, CO_2 , and H_2O (van der Meer 1994).

Many physical and chemical properties of the environment influence the microbial degradation of pollutants. These factors include availability of other nutrients, pH, soil structure and texture, moisture content, redox potential, a suitable electron acceptor, and the presence of toxic compounds or more readily utilizable carbon sources (Bewley 1992). Additionally, the concentration of halogenated compounds is critical during microbial degradation. Halogenated compounds may be toxic even to microorganisms capable of metabolizing them if the concentration is too high. Conversely, it has been shown that for the metabolism of a number of chlorinated aromatics there exists a threshold concentration below which rates of biodegradation are extremely slow or non-existent (Neilson 1990).

1.2 Polychlorinated Biphenyl Degradation

One group of halogenated compounds are polychlorinated biphenyls (PCBs). The desirable physical and chemical nature of PCBs (excellent dielectric and flame resistance properties, chemical and thermal stability) led to their extensive industrial use as heat transfer fluids, solvent extenders, plasticizers, flame retardants, organic diluents and dielectric fluids. PCBs are synthesized by direct chlorination of biphenyl (BP) so that, theoretically, 209 different compounds containing 1 to 10 chlorines per BP molecule can be produced. However, the majority of PCBs in the environment are derived from commercial mixtures (Arochlor, Phenoclor, Clophen and Kanechlor) which contain 60 to 80 different congeners. Extensive application of PCBs has resulted in widespread contamination. From 1929 to 1978, approximately 635 million kilograms of these chemically and thermally stable compounds were produced. It is estimated that tens of millions of kilograms of PCBs have subsequently been released into the environment (Bopp 1986). PCBs are very poorly water soluble. In aquatic systems they partition into more hydrophobic environments like sediments (Higson 1992).

Both aerobic and facultative anaerobic bacteria involved in PCB degradation have been isolated using batch and continuous culture enrichment techniques. PCB biodegradation is greatly influenced by the number and position of chlorine substituents on the biphenyl molecules. Anaerobic bacteria attack highly chlorinated PCB congeners through reductive dechlorination. In general, this microbial process preferentially removes *meta*- and *para*- substituted chlorines, resulting in the depletion of highly chlorinated PCB congeners with corresponding increases in lower chlorinated, *ortho*-substituted PCB congeners (Abramowicz 1990). Conversely, aerobic microorganisms target lightly chlorinated PCB congeners for degradation, often the products of anaerobic dechlorination. These organisms attack PCB via the well-known 2,3-dioxygenase pathway, converting PCB congeners to the

corresponding chlorobenzoates. With the exception of a few pure strains that mineralize 4-chlorobiphenyl (4CBP) (Layton *et al.* 1992, Furukawa and Chakrabarty 1982, Shields *et al.* 1985, Barton and Crawford 1988), PCB degradation occurs via consortia or co-metabolism (Sylvestre *et al.* 1985, Adriaens *et al.* 1989).

Recombinant microorganisms capable of PCB mineralization have been isolated following continuous culture enrichments (Adams *et al.* 1992) or matings (Mokross *et al.* 1990) of PCB and chlorobenzoate degrading microorganisms. The known mechanisms involved in PCB and chlorobenzoate degradation are summarized in Figure 1.1.

1.3 Chlorobenzoate Degradation

Besides entering the environment as microbial bioconversion products of PCBs, chlorobenzoates are introduced into the environment directly as herbicides (2,3,6-trichlorobenzoate, Dicamba). Not only are chlorobenzoates environmental contaminants in their own right but they may inhibit PCB metabolism through the build-up of chlorobenzoate metabolites like chlorocatechols and chloromuconate semialdehydes. When chlorobenzoates are produced from PCBs, they accumulate in the growth medium and are converted into unproductive metabolites that inhibit biphenyl (BP) and PCB degradation pathways (Sondossi *et al.* 1992). It has been shown that chlorobenzoate degradation is the rate limiting step in the overall PCB degradation process (Adriaens and Focht 1990). Chlorobenzoates are relatively hydrophilic and therefore partition into the water fraction rather than the sediment fraction of aquatic systems. There are many examples of microbial isolates capable of complete chlorobenzoate degradation.

Four mechanisms have been characterized for haloaromatic biodegradation by prokaryotes and these have been reviewed by Reineke and Knackmuss (1988)

Figure 1.1 A Summary of Microbial Aerobic and Anaerobic Degradation of PCBs and 3-Chlorobenzoate.

Highly chlorinated PCBs are preferentially converted to less chlorinated PCB congeners anaerobically via reductive dehalogenation. The lightly chlorinated PCBs are subsequently converted to chlorobenzoates through aerobic metabolism (primarily biphenyl dioxygenases).

Chlorobenzoates entering the environment either through aerobic conversion of PCBs or as herbicides can be mineralized by three aerobic mechanisms: proximal ring-fission (dioxygenase), distal ring-fission (dioxygenase) or hydrolytic dehalogenation (dehalogenase). Chlorobenzoates can also be mineralized anaerobically via reductive dehalogenation.

Adapted from Reineke and Knackmuss (1988) and Krooneman *et al.* (1996).

PCB Contamination

Less chlorinated PCB congeners

+

Highly chlorinated PCB congeners

Herbicides

Aerobic Degradation
(biphenyl dioxygenase)

Anaerobic Reductive
Dehalogenation

Chlorobenzoate
Contamination

Aerobic Processes

Anaerobic
Reductive Dehalogenation

[Proximal ring-fission]
Dioxygenase

[Distal ring-fission]
Dioxygenase

Hydrolytic
Dehalogenation

3-chlorocatechol

ring-fission

ortho

meta

TCA

suicidal
products

protocatechuate
meta-ring-fission

TCA

3-hydroxybenzoate

gentisate or protocatechuate
ring-fission

TCA

CH₄ + CO₂

Examples of all four mechanisms have been reported in indigenous microorganisms involved in chlorobenzoate mineralization. The first mechanism is reductive dehalogenation whereby a chlorine substituent is replaced with a hydrogen atom. This is an anaerobic process. *Desulfomonile tiedjei* DCB-1 converts 3-chlorobenzoate (3-Cba) to benzoate via this reductive route (Mohn and Tiedje 1992). The second mechanism characterized involves the displacement of the chlorine substituent via a hydroxyl group to hydroxybenzoate intermediates. This hydrolytic dehalogenation process has been reported in the degradation of 3-Cba via a *Pseudomonas* sp. (Johnston *et al.* 1972) and in 4-chlorobenzoate (4-Cba) mineralization by *Pseudomonas* sp. CBS3 (Löffler *et al.* 1991). A third mechanism of biodegradation is oxygenolytic halogen-carbon bond cleavage. One example of this process has been reported in 3-Cba and 3,4-dichlorobenzoate (3,4-dCba) degradation via a 3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway in *Alcaligenes* sp. BR60 (pBRC60) (Nakatsu and Wyndham 1993). This dioxygenase introduces molecular oxygen at the distal *meta*- and *para*-positions and the organism subsequently mineralizes protocatechuate and chloroprotocatechuate using a *meta*-ring-fission route. Finally, chloride elimination from non-aromatic intermediates is the best characterized pathway of chlorobenzoate degradation by microorganisms. The modified *ortho* cleavage pathway converts chlorobenzoates to chloro-substituted catechols that are subsequently metabolized via oxygenolytic intradiol or *ortho*-ring-fission to non-aromatic products. Bacteria encoding this pathway have been isolated worldwide and include *Pseudomonas putida* AC858 (pAC25) from sewage in New York (Chatterjee *et al.* 1981) and *Pseudomonas* sp. strain B13 (pWR1) from sewage in Germany (Dorn *et al.* 1974).

1.4 Evolution of PCB and Chlorobenzoate Degradative Pathways

Most of the characterized genotypes encoding the partial catabolism of PCB and complete chlorobenzoate mineralization have been found on broad host range conjugative plasmids and in some cases are encoded within transposons. Plasmid transfer and transposition are considered to be important driving forces for genetic adaptation in bacterial communities (Asturias *et al.* 1995). Gene transfer also broadens bacterial diversity. It has been assumed that an increased incidence of plasmids can be an indicator of a polluted environment. It was shown that plasmid-bearing bacteria were abundant (20-80%) in polluted aquatic habitats. By contrast the occurrence of plasmids in pristine groundwater was limited (2-8%) (Schutt 1990). The natural distribution of different plasmids and transposons in bacteria in natural ecosystems is not well-known (Wyndham *et al.* 1994a, Dahlberg and Hermansson 1995). Recent evidence by Blázquez *et al.* (1996) suggests that plasmid conjugation, rather than transposition between replicons is the main factor responsible for the dissemination of the transposon Tn5 in the environment.

Halogenated compounds are not uncommon in nature and might be important in the genetic adaptation of microbial communities to halogenated xenobiotic mineralization. More than 700 halogenated natural products have been identified in a number of organisms, including bacteria, fungi, algae, ferns, and higher plants, in low concentrations (Klages and Lingens 1979, Fetzner and Lingens 1994). 2,4-Dichlorophenol, which is produced by a soil *Penicillium*, is one example of a naturally occurring chlorinated compound. Recently, it was reported that microbial enrichments collected from six locations around the world, with no known previous haloaromatic contamination, were capable of 3-Cba mineralization without a lag period. These results suggest that 3-Cba degradative genotypes may have evolved to degrade naturally occurring chloroaromatics (Fulthorpe *et al.* 1996).

An alternative view of the evolution of chloroaromatic degradative genotypes is that certain enzymes involved in the degradation of haloaromatic compounds may have evolved from enzymes that function in the degradation of similarly configured nonhalogenated aromatic compounds (Reineke 1984, Ahmad *et al.* 1990). For example, it has been proposed that exposure of microorganisms to natural sources of aromatic hydrocarbons including the structural plant polymer, lignin, may have provided selective pressure for microorganisms to develop the potential for biphenyl (BP) metabolism (Higson 1992). The ubiquitous distribution of bacteria capable of BP metabolism in environmental samples supports this proposal. PCB catabolic genotypes have evolved by recruitment and subsequent divergence of genes that specify BP degradation to include PCB degradation. One microorganism, *Burkholderia* sp. LB400 (formerly *Pseudomonas* sp.) harbours BP and PCB catabolic genes that are similar to other PCB catabolic genes in *Comamonas testosteroni* (formerly *Pseudomonas testosteroni*) strain B-356, *Corynebacterium* A5, *Alcaligenes eutrophus* H850, *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas paucimobilis* Q1. All of these microorganisms are capable of BP mineralization and bioconversion of some PCB congeners to chlorobenzoate intermediates. These similarities suggest that the multicomponent BP-PCB dioxygenases in these microorganisms share a common ancestral pathway (Ahmad *et al.* 1990). The toluene degrading microorganism, *Pseudomonas putida* F1, which harbours a multi-component toluene dioxygenase, has conserved amino acid sequences with the BP-PCB dioxygenases of the above mentioned microorganisms and may also have the same ancestral pathway (Furukawa *et al.* 1992, Erickson and Mondello 1992).

When microorganisms encounter new organic chemicals in their environment, such as PCBs, they may modify existing genes through mutational processes to expand or modify their substrate range (Chaudry and Chapalamadugu 1991). There are many recorded differences in congener specificity between *Burkholderia* sp. LB400, and *Pseudomonas pseudoalcaligenes* KF707 despite the fact that the dioxygenase

sequences in these organisms have 97% identical nucleotides. While KF707 only has a 2,3-biphenyl dioxygenase, LB400 possesses both 2,3- and 3,4-biphenyl dioxygenase activities. Of the 19 PCB congeners tested for degradation, 17 were degraded by LB400 while only 8 were degraded by KF707. The congeners degraded by KF707 were more lightly chlorinated; none had more than four chlorine substituents. However, against the double-*para*-substituted PCBs 4,4'-CBP and 2,4,4'-CBP, KF707 had greater activity than LB400. These substrate specificity differences between LB400 and KF707 biphenyl dioxygenases were due to structural differences in the biphenyl dioxygenases rather than strain background. Using site directed mutagenesis the PCB congener specificity of the LB400 biphenyl dioxygenase was modified. The novel dioxygenase combined the broad substrate specificity of LB400 with the superior ability of KF707 to degrade some double-*para*-substituted congeners (Erickson and Mondello 1993).

1.5 Environmental Limitations of PCB and Chlorobenzoate Degradation

Recipients of catabolic genes through conjugation, transposition, transformation or transduction must be able to integrate incoming gene function with conserved metabolic pathways that are already expressed, or that may be induced in the host organisms. During aerobic chlorinated aromatic degradation, including chlorobenzoate degradation, there is a channeling of substrates into several common metabolites prior to aromatic ring-fission. These include catechol, protocatechuate and gentisate or chloro-substituted versions of each. Mis-channeling of substrates can lead to dead-end pathways and the accumulation of potentially toxic substrates (Sayler *et al.* 1990). Catechol and substituted catechols are enzymatically cleaved in two ways, between the two hydroxyl groups (intradiol or *ortho*-cleavage) or immediately adjacent to one of them (extradiol or *meta*-cleavage). The toxicity of 3-chlorocatechol

has been demonstrated in a toluene-utilizing pseudomonad that metabolized 3-Cba to this intermediate (Bartels *et al.* 1984). *Meta*-cleavage transformed 3-chlorocatechol into a highly reactive acyl halide, 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid, which quickly and irreversibly bound to and inactivated macromolecules including the *meta*-cleavage enzyme itself. 3-Chlorocatechol has also been implicated in the inactivation of 2,3-dihydroxybiphenyl dioxygenase, a key enzyme in PCB metabolism (Arendsdorf and Focht 1994). The complexities associated with converging metabolic pathways combined with the regulatory conflicts of incoming catabolic genes and host genes limit the functional host-range of catabolic plasmids or transposons (Nakatsu *et al.* 1995a).

There are several limitations in the employment of naturally-occurring or genetically engineered microorganisms (GEMs) in chloroaromatic degradation. Real contamination differs significantly from most model wastes used for bacterial selection in the laboratory. Low concentrations of chloroaromatic substrates in the environment may not support the growth of engineered strains. By contrast, hydrophobic contaminants like PCBs are often associated with high-molecular-weight humic substances in localized high concentrations. Additionally, introduced organisms may abandon the metabolism of the contaminant in favour of other, more readily accessible substrates. There is evidence that in natural environments heterotrophic microbes do not utilize only one carbon substrate for growth (such as a specific pollutant). Rather they are simultaneously utilizing as many natural carbon sources as possible. This behaviour has been described as ‘mixed substrate growth’ (Egli 1995). Second, the introduced bacteria may not be physiologically adapted to the environment and may encounter adverse growth conditions such as the presence of toxins, predators, interspecific competition for nutrients or temperature variations. As well, natural environments undergo seasonal fluctuations that cause additional stress to introduced microorganisms. Third, from a practical point of view some degradative reactions have such a low specific activity that they are not economically feasible. Finally, the

release of modified or enriched microorganisms into the environment has only limited government approval (Sayler *et al.* 1990).

1.6 The Scope of this Study

Almost all knowledge about the microbial physiology and genetics of environmental pollutant degrading strains has resulted from the study of pure laboratory enriched cultures. There is very little known about how significant these microorganisms and their catabolic pathways are for pollutant degradation under natural conditions, how selective pressure influences pathway diversity, what role geographic heterogeneity plays, and how fast gene transfer and other evolutionary mechanisms work in nature. The Niagara watershed provides a good study location as it is a site of long-term chloroaromatic contamination. There are numerous chemical plants in this area involved in the production of herbicides, pesticides, and formerly PCBs.

The Great Lakes Basin encompasses the largest freshwater system in the world. The Niagara River connects Lakes Ontario and Erie. The Hyde Park chemical landfill site located in this region covers 6.1 hectares and contains an estimated 73,000 metric tons of chemical waste, including high concentrations of chlorobenzoates and phenols (Wagner and Collin 1984). Chemical contaminants travel in non aqueous and aqueous plumes through the groundwater around this site. The Hyde Park site has been capped with clay, and a series of three perimeter trenches collect groundwater leachate. Since 1990, Occidental Chemical Corp. has operated a groundwater bioremediation system on site consisting of pH adjustment, precipitation and sedimentation, sequencing batch reactor (SBR) biotreatment and carbon adsorption. The SBR biotreatment process uses indigenous microbial consortia from the

groundwater. Nitrogen and phosphorus supplementation in this process results in a 90% reduction of total organic carbon (TOC) (Ying *et al.* 1992).

In 1985 a 3-Cba degrading bacterial strain, *Alcaligenes* sp. BR60, was isolated from a continuous culture enrichment inoculated with sediment and water from runoff waters adjacent to the Hyde Park landfill. Further studies of this isolate revealed that the genetic information for 3-Cba degradation was encoded on a 88 kbp conjugative, broad-host-range plasmid pBRC60 (Wyndham *et al.* 1988a) within a transposon, Tn5271 (Nakatsu *et al.* 1991). Subsequent cloning and expression studies revealed the presence of chlorobenzoate 3,4-(4,5)-dioxygenase genes, *cbaAB*, that route 3-Cba and 3,4-dCba degradation through a *meta*-ring-fission pathway (Nakatsu and Wyndham 1993). Fulthorpe and Wyndham (1991) determined the fate of the catabolic genetic element Tn5271 introduced in *Alcaligenes* sp. BR60 into 3-Cba dosed freshwater microcosms. They observed that after several weeks the incidence of Tn5271 was up to five orders of magnitude higher than the incidence of the original host microorganism *Alcaligenes* sp. BR60. From this study it was concluded that 3-Cba degradation was correlated with the incidence of the genetic element Tn5271, and not with the host *Alcaligenes* sp. BR60. This group also detected more copies of the catabolic plasmid pBRC60 in a chloroaromatic contaminated creek compared to a more pristine creek in the Niagara watershed (Fulthorpe *et al.* 1989). Other studies have confirmed the presence of the Tn5271 genotype in the Niagara watershed (Wyndham *et al.* 1994b, Wyndham *et al.* 1995).

The research in this Thesis was undertaken in an effort to characterize the distribution of genotypes involved in chlorobenzoate and PCB degradation in a region of chloroaromatic contamination. Knowledge about the local and global distribution of catabolic genotypes and their hosts was limited at the time of this investigation. My hypothesis is that because catabolic genotypes have frequently been characterized on mobile genetic elements and because the Niagara watershed contamination provides a strong selection force, then some previously characterized chloroaromatic catabolic

genotypes are present in this region and are involved in the adaptation of bacteria to these contaminants.

1.7 Objectives

Due to the extensive chloroaromatic contamination in the Niagara watershed this area was selected as a study site to investigate the following:

1. The effect of Hyde Park contamination, season, enrichment temperature, sample year and carbon source on the degradation potential of 3-Cba, 4-Cba and 3,4-dCba in water/sediment batch culture enrichments from the Niagara watershed.
2. The incidence and distribution of four previously characterized catabolic genotypes (*clcABD*, *cbaAB*, *fcB* and *bphA*) in the culturable population of the Niagara watershed over an 18-month sampling period.
3. A detailed phenotypic and genetic analysis of all probe positive isolates from the batch culture enrichments in order to see how they resemble or differ from the archetypal microorganisms or type strain harbouring each genotype.
4. To detect the prevalence of *cbaA* and *bphA* genes in total DNA isolated from the sampling sites in the Niagara watershed creeks, the Hyde Park landfill and in Hamilton Harbour, Lake Ontario.
5. To elucidate how the *cbaA* and *clcA* genes co-exist in a continuous culture enrichment of chlorobenzoate contaminated groundwater from the Hyde Park landfill.

CHAPTER 2:

Microbial Chlorobenzoate Degradation Potential in Batch Culture Enrichments

2.1 INTRODUCTION

Due to the complex nature of most contaminant mixtures, and numerous environmental restrictions; including nutrient limitations, temperature fluctuations, predation, unutilizable or toxic substrates, and pH, it is not realistic to assume that species act in isolation to degrade chlorinated compounds. Mixed microbial communities or consortia degrade complex chlorinated chemical mixtures *in situ* through the exchange of specific nutrients, removal of growth-inhibitory products and co-metabolism. For example, a natural mixed aerobic bacteria culture designated MIXE1 was isolated from PCB-contaminated soil (Fava *et al.* 1993). Different isolates within the MIXE1 culture specialized in the degradation of specific chlorinated compounds. In this consortium, the pseudomonad CPE2 degraded 2-chloro- and 2,5-dichlorobenzoate, while *Alcaligenes* strain CPE3 mineralized 3-Cba, 4-Cba and 3,4-dCba. Pettigrew *et al.* (1990) described a consortium, LPS10, that mediated PCB mineralization. This consortium was obtained by both batch and continuous cultivation and had the following members: a *Comamonas testosteroni* strain that converted 4-chlorobiphenyl to 4-Cba, an *Arthrobacter* strain that mineralized 4-Cba to CO₂, and a *Pseudomonas putida* strain that contributed an unknown factor. Finally, a mixed culture was isolated from PCB contaminated soil which was able to degrade highly chlorinated PCBs. This culture was composed of *Pseudomonas* sp. strain KKS102 and *Pseudomonas fluorescens* KKL101 (Kimbara *et al.* 1988).

Numerous studies have been conducted to ascertain the feasibility of bioremediating contaminated sites by assessing the biodegradation potential. These

investigations have focused on point sources of contamination where the concentration of chemicals was high, such as landfills or spill sites, rather than dispersed pollution sites where the contaminant concentration was lower. In general, biodegradation potential studies have been limited to laboratory studies. For example, laboratory based degradation studies were conducted to test the feasibility of biotreating chlorinated ethenes aerobically at a Naval Air Station (Roberts *et al.* 1990). No attempts to monitor the biodegradation potential at this site or other environmental sites over an extended period of time have been made.

In this study the microbial degradation potential of 3-Cba, 4-Cba and 3,4-dCba in water and sediment samples from the Niagara watershed was assessed. The microaerophilic top layers of sediments in rivers and creeks are highly diverse and metabolically very active microbial ecosystems. The specific objectives were to:

1. Establish which sampled creeks received chloroaromatic contamination.
2. Demonstrate the effect, *in situ*, of chlorobenzoate pollutant stress on the microbial chlorobenzoate degradation potential of streams contaminated by Hyde Park and non-contaminated sampling sites.
3. Evaluate the effects of batch culture enrichment temperature, and seasonal and yearly sampling variations, on the microbial chlorobenzoate degradation potential.

2.2 MATERIALS AND METHODS

2.2.1 Description of Study Sites and Sampling Procedures

The creeks sampled were all tributaries of the Niagara River located near the Hyde Park Landfill site in Niagara Falls, New York (43°, 0.8', 10"N, 79°, 0.2', 30"W). Figure 2.1 shows a map of the Niagara watershed including Hyde Park and the sampled creeks. Bloody Run Creek (BRC) is a small tributary of the Niagara River. It is a site of historical contamination from Hyde Park through surface water and groundwater (Moses Road site). It also receives surface run-off from residential and scrub land. Devil's Hole Creek (DHC) was suspected to be contaminated by Hyde Park through groundwater infiltration as its groundwater sources lie within the mapped underground plume of contaminants originating from Hyde Park (Wagner and Collin 1984). Fish Creek (FC), which receives surface runoff from largely rural residential land and a golf course, was chosen for control sampling. It is separated from the Hyde Park site by a hydroelectric dam and reservoir that runs deeper than the groundwater level and that is flushed continuously by water taken from the upper Niagara river.

Samples from the water-sediment interface were collected from five to seven sites in each creek during May and November 1992 and May, August and November 1993. During May and November 1992 and May, August and November 1993, the ambient air temperatures during the sampling period were 18°C, -2°C, 25°C, 25°C and 10°C, respectively. All samples were collected in sterile 50 ml screw-top polypropylene tubes and consisted of approximately 80% sediment and 20% water. Samples were stored on ice for transport back to the laboratory.

The Hyde Park (Niagara Falls, New York) chemical landfill site was sampled during the May 1993 trip. This was coordinated by Dr. Ying of the Occidental Chemical Corporation Technology Center in Grand Island, New York. Samples of groundwater

Figure 2.1: Location of Study Area and Sampling Sites in the Niagara Watershed.

The dashed perimeter represents the groundwater aqueous and non-aqueous chloroaromatic-contaminant plume from the Hyde Park site. The Niagara river tributaries, Bloody Run Creek (BRC), and Devil's Hole Creek (DHC) receive chloroaromatic contamination from Hyde Park through this plume. Moses Road (MR) is a Hyde Park contaminated groundwater source that runs into BRC. Fish Creek (FC) is a non-contaminated site in this region. It is separated from Hyde Park by a hydroelectric reservoir that extends beneath the contaminant plume.

were collected from Wells A, C, and D, which pump the perimeter trench of the landfill site. Groundwater samples had previously been shown to contain average 3-Cba and 4-Cba concentrations of 61 mg/l and 40 mg/l, respectively (Ying *et al.* 1992). Samples were also obtained from the sequencing batch reactors SBR1, SBR2 and SBR3 which are involved in fill-and-draw activated sludge processes for aerobic pollutant biodegradation of the contaminated groundwater.

During the sampling trip in August 1993, a 6 l. sample of water from each creek was collected for chlorinated compound analysis by gas chromatography-mass spectrometry (GC-MS). 1% sodium azide was added to these samples to kill bacteria and prevent further contaminant degradation. Additionally, water samples (500 ml) were collected for inorganic and organic nitrogen, phosphorus and alkalinity analyses during the sampling trip in November 1993. These analyses were conducted by Don Galloway at the Provincial Ministry of the Environment Laboratories in Kingston, Ontario.

2.2.2 Batch culture enrichments

All batch culture enrichments were set-up within 24 hours of sample collection. Half of each sample (25 ml), was passed through sterile 18 gauge needles using a 10 ml syringe 3 times in order to break up bacterial clumps. The remaining 25 ml from each site was frozen at -70°C for total DNA extraction (discussed in Chapter 5). Triplicate enrichment cultures in 20-fold dilute medium A+N pH 8.0 (Wyndham, 1986) and a final chlorobenzoate concentration of 10 µM were prepared by pipetting 950 µl of the environmental samples and 50 µl of 200 mM 3-Cba, 4-Cba or 3,4-dCba (Aldrich chemicals) solutions in full strength medium A+N into 1.5 ml sterile microtubes. The enrichment cultures were shaken at 200 rpm at 32°C, 22°C or 4°C. Cultures incubated at 32°C were subsequently enriched to chlorobenzoate concentrations of 100 µM, 600 µM

and 1.5 mM over 8-day intervals using 500 μ l of the previous enrichment and 500 μ l of the appropriate chlorobenzoate stock solution (200 μ M, 1.2 mM or 3 mM) in full strength medium A + N. The 1.5 mM cultures were harvested 8 days after the last enrichment stage. The 22°C and 4°C cultures followed a 10-day and 14-day enrichment period, respectively, enriching with the same concentrations of chlorobenzoate isomers. Following centrifugation of the 1.5 mM enrichment cultures, the supernatants were frozen at -20°C for subsequent HPLC analysis. Sterile chlorobenzoate controls in medium A + N were prepared along with the batch culture enrichments.

Batch culture enrichments using biphenyl (BP) (Sigma) crystals dissolved in dimethyl formamide (DMF) and 3-chlorobiphenyl (3CBP) (Aldrich) as sole carbon sources were prepared and sub-cultured following the same enrichment schedule as the chlorobenzoate enrichments. As BP and CBP concentrations can only be accurately measured by GC-MS due to the volatility of these compounds, these enrichment cultures were set-up to isolate bacteria only (described in Chapter 3). Prior to harvesting the 3CBP enrichments, 500 μ l was added to 500 μ l 3 mM 3-Cba and incubated for 8, 10 and 14 days for the 32°C, 22°C and 4°C enrichment cultures, respectively. These are referred to as the 3-Cba(3CBP) enrichment cultures. The purpose of the 3-Cba(3CBP) cultures was to select bacteria capable of either complete or partial 3CBP mineralization for Chapter 3 and to see if there was a difference between the 3-Cba degradation potential in 3-Cba and 3-Cba(3CBP) batch culture enrichments.

2.2.3 Analytical Procedures

The supernatants from all chlorobenzoate enrichments, including uninoculated control tubes, were analyzed using a Waters 501 high performance liquid chromatography (HPLC) system with a Novo-pak C-18 reverse-phase column (15 cm, 4 μ m packing)

combined with a Millipore Waters Lambda-Max Model 481 LC UV detector set at 254 nm. The flow rate was set at 1.0 ml min^{-1} . The solvent used was 50% methanol, 0.5% acetic acid (degassed).

Water samples that were collected in November 1993 (6l at each creek), acidified with hydrochloric acid to pH 2.5, were extracted 10 times with a total of 650 ml ethyl acetate. The pooled extracts were dried with 1 g anhydrous sodium sulfate and evaporated with a Büchi 461 rotary evaporating unit (Brinkmann Instruments). Final volumes were reduced to 1 ml by passing dry N_2 gas over the samples. Samples were methylated with an excess of fresh diazomethane generated from Diazald (Aldrich) according to the manufacturers instructions. All samples were analyzed with a Hewlett Packard 5890 GC linked to a Hewlett Packard 5971A quadrupole MS detector. The initial oven temperature was 110°C . The temperature was increased by $16^\circ\text{C min}^{-1}$ to a final temperature of 300°C . Helium was used as the carrier gas.

2.2.4 Data Analysis

HPLC peak areas for each chlorobenzoate isomer from the final 1.5 mM enrichment cultures were expressed as a fraction of the peak areas for each control (peak area of enrichment culture/peak area of chlorobenzoate control). These fractional degradation results were grouped into 5 ranges: 0 to 0.25; 0.25 to 0.50; 0.5 to 0.75; 0.75 to > 1.0 ; and 1.0. The first and last ranges were designated as complete degradation and no degradation of the chlorobenzoate isomers, respectively. The three middle ranges were pooled and represented partial degradation of the chlorobenzoate isomers (0.25 to < 1.0). Therefore, based on the HPLC data, values were entered into a contingency table as no, partial, or complete chlorobenzoate degradation. The degradation data represented either mineralization or conversion of the chlorobenzoates to metabolites. May 1992 and 1993

results were combined in some analyses as spring results. Similarly, November 1992 and 1993 results were combined for fall analyses. August 1993 data represented summer results. Chi-square analysis, or the Fisher Exact test if the frequency of the cells in the contingency table was less than 5, were applied to the values in each contingency table. The software used was BioStatistics Version 3.0 (McGraw-Hill Inc., 1992). Significance levels were set at $p < 0.05$ for all analyses. Graphs were generated based on complete degradation results only (fractional degradation range 0 to 0.25). Appendix 1 shows a sample of the 3-Cba enrichment results including HPLC peak areas, fractional degradation results, a contingency table and Chi-square analysis.

2.3 RESULTS

2.3.1 Field conditions and elemental water analysis

The water chemistry results from a single sample collected in November 1993 in Table 2.1 revealed that BRC was phosphate limited. This is not surprising as phosphate limitations have previously been noted in this tributary and in the groundwater beneath Hyde Park (Wyndham and Straus 1988a, Ying *et al.* 1992). DHC and FC were also phosphate limited. There was over a 3-fold difference in total nitrogen levels between FC (low) and DHC (high). However, the organic or usable nitrogen levels, measured by total Kjeldahl, were more similar. The conductivity measure gives some indication of the proportion of groundwater in each creek (DHC showed the highest conductivity and FC showed the lowest).

2.3.2 Water analysis by GC-MS

The mass spectra results revealed multiply chlorinated aromatic compounds in BRC and DHC water samples as evidenced by the characteristic m , $m+2$, $m+4$, $m+6$ ion ratios of selected GC-resolved peaks (Silverstein and Bassler 1967). One GC-resolved compound from BRC showed a molecular ion peak distribution indicative of a hexachlorinated compound (Figure 2.2b, Inset). A compound with a slightly lower retention time selected from the DHC chromatogram showed a molecular ion peak distribution consistent with a pentachlorobiphenyl (parent ion $M/e = 318$) (Figure 2.2c, Inset). The chromatograms of water extracted from FC showed no significant contaminants (Figure 2.2a). The only discernible peak was a solvent peak.

Table 2.1 **Elemental Analysis of Surface Water of Creeks in the Niagara Watershed during November 1993**

Parameter	Bloody Run	Devil's Hole	Fish
	Creek	Creek	Creek
Ammonia as N (mg/L)	0.01	0.01	0.01
Total Kjeldahl as N (mg/L)	0.41	0.19	0.27
Nitrite as N (mg/L)	0.06	0.02	0.04
Nitrate as N (mg/L)	0.93	2.5	0.54
Total N (TKN + NO ₃ + NO ₂) (mg/L)	1.40	2.71	0.85
Total Phosphorus as P (mg/L)	0.051	0.068	0.060
Dissolved Reactive Phosphorus as P (mg/L)	0.041	0.018	0.02
Conductivity (µmhos/cm)	1545	3140	634

Figure 2.2: Total Ion Chromatograms and Mass Spectra of Ethyl Acetate Extracted Water Samples from the Niagara Watershed.

- A.** Total ion chromatogram of Fish Creek water. The only peak discernible is a solvent peak.
- B.** Total ion chromatogram of Bloody Run Creek water. Inset: parent ion mass spectrum of the peak marked by an arrow. The characteristic peak ratio $m/m+2/m+4/m+6/m+8/m+10$ identifies the largest peak as a hexa-chlorinated compound.
- C.** Total ion chromatogram of Devil's Hole Creek water. Inset: parent ion mass spectrum of the peak marked by an arrow. The characteristic peak ratio $m/m+2/m+4/m+6/m+8$ and the parent ion weight ($M/e = 318$) identifies the peak in the inset as a pentachlorobiphenyl. The largest peak in DHC, as in BRC, is a hexa-chlorinated compound.

2.3.3 Microbial chlorobenzoate degradation potential under different batch culture enrichment conditions determined by HPLC analysis

The chlorobenzoate degradation potential results presented in Figures 2.3-2.11 are based on the HPLC peak areas at 254 nm for each chlorobenzoate. The peak areas with a fractional degradation result <0.25 compared to an uninoculated control were considered to show complete degradation. Fractional degradation results between 0.25 and 1.0 were considered to exhibit no degradation potential in these graph results. The comments that follow refer to these graphically presented results. Comments on the statistical significance of these results will follow in section 2.3.4.

The potential for 4-Cba degradation by aquatic sediment enrichments of Niagara watershed creek enrichments was greater than for 3-Cba and 3,4-dCba under all conditions (Figures 2.3-2.6).

BRC exhibited a higher frequency of 4-Cba and 3-Cba degradation during all seasons and more frequent 3,4-dCba degradation during spring and summer enrichments than DHC (Figures 2.3-2.5). DHC showed slightly more frequent 3,4-dCba degradation during fall sample enrichments (Figures 2.3, 2.4). The chloroaromatic-contaminated creeks, BRC and DHC, showed more frequent degradation of all chlorobenzoates compared to a non-contaminated creek in this region, FC (Figures 2.3, 2.4, 2.6). This was especially evident for monochlorobenzoate degradation in the spring enrichments (Figures 2.3-2.5).

32°C and 22°C enrichments generally showed similar degradative capabilities (Figure 2.5). 4°C enrichments consistently showed low frequencies of degradation of all chlorobenzoates during all seasons. The best 3,4-dCba degradation potential was observed in the enrichment cultures incubated at 22°C. 3-Cba and 4-Cba degradation was slightly more successful in the 32°C enrichment cultures.

Figure 2.3 The Seasonal Chlorobenzoate Degradation Potential of Microbial Consortia in Batch Culture Enrichments from Chloroaromatic-Contaminated (BRC, DHC) and Non-Contaminated (FC) Sites in the Niagara Watershed.

Samples were collected during three different seasons (spring, summer and fall). Results from the same season were combined for 1992 and 1993 samples. 32°C and 22°C enrichment results have been combined. 4°C results are not presented.

Sites with fractional chlorobenzoate degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A. 4-Chlorobenzoate enrichment results.**
- B. 3-Chlorobenzoate enrichment results.**
- C. 3,4-Dichlorobenzoate enrichment results.**

A. 4-Chlorobenzoate

B. 3-Chlorobenzoate

C. 3,4-Dichlorobenzoate

Figure 2.4 The Effect of Sampling Season on the Microbial Chlorobenzoate Degradation Potential in 32°C and 22°C Batch Culture Enrichments of Chloroaromatic-Contaminated (BRC/DHC) and Non-Contaminated (FC) Sites in the Niagara Watershed.

Sites with fractional degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A. Spring 1992 and 1993 enrichment results.**
- B. Summer 1993 enrichment results.**
- C. Fall 1992 and 1993 enrichments results.**

A. Spring

B. Summer

C. Fall

Figure 2.5 The Effect of Batch Culture Enrichment Temperature on the Chlorobenzoate Degradation Potential of Microbial Consortia Collected from Chloroaromatic-Contaminated Sites (BRC, DHC) in the Niagara Watershed During Different Seasons.

Samples were collected during three different seasons (spring, summer and fall). Results from the same season were combined for 1992 and 1993 samples. The BRC and DHC sites have been combined.

Sites with fractional degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A.** 32°C Enrichment results.
- B.** 22°C Enrichment results.
- C.** 4°C Enrichment results.

A. 32°C Enrichments

B. 22°C Enrichments

C. 4°C Enrichments

Figure 2.6 The Effect of Sampling Year on the Chlorobenzoate Degradation Potential of Microbial Consortia in Batch Culture Enrichments from Chloroaromatic-Contaminated (BRC and DHC) and Non-Contaminated (FC) Sites in the Niagara Watershed.

32°C and 22°C enrichment results have been combined. 4°C enrichments results have not been included.

Sites with fractional degradation results < 0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A. 1992 Enrichment results (spring and fall results combined).**
- B. 1993 Enrichment results (spring, summer and fall results combined).**

A. 1992 Results

B. 1993 Results

Overall, 3-Cba degradation occurred more frequently in the 1992 enrichment samples (Figure 2.6). Conversely, 4-Cba and 3,4-dCba microbial degradation potentials were higher in the 1993 enrichments.

The microbial 3-Cba degradation potential of the 3-Cba(3CBP) enrichments was compared to the 3-Cba enrichment results (Figures 2.7-2.10). Aside from the observation that both groups of enrichment cultures showed superior degradation in BRC samples and minimal 3-Cba degradation by the FC sites, there were different responses in these two enrichment cultures. 3-Cba enrichment cultures showed good degradation of 3-Cba during the spring enrichments. By contrast, the 3-Cba(3CBP) enrichments showed greater 3-Cba degradation during the summer and fall enrichments (Figures 2.7, 2.8, 2.9). As noted in the other chlorobenzoate batch cultures, the 32°C and 22°C enrichments were better at 3-Cba degradation than the 4°C enrichments (Figure 2.9). Greater 3-Cba degradation by the 3-Cba batch cultures during 1992 was noted. Conversely, superior 3-Cba degradation by the 3-Cba(3CBP) cultures occurred during 1993 enrichments (Figure 2.10).

The enrichment cultures from the Hyde Park samples showed that the isomer with the best degradation potential from the Well (groundwater) and SBR sites was 4-Cba. Very poor 3,4-dCba degradation was observed. None of the Well A enrichment cultures showed evidence of chlorobenzoate mineralization. The Well C and D sites showed identical degradation potentials for all chlorobenzoate isomers at all enrichment temperatures. Superior degradation of all chlorobenzoate isomers was noted in the 22°C and 32°C enrichments. The SBR1 enrichments exhibited greater monochlorobenzoate degradation than the SBR2 and SBR3 enrichments. None of the SBR sites exhibited a potential for 3,4-dCba degradation (Figure 2.11).

Figure 2.7 A Comparison of the 3-Chlorobenzoate Degradation Potential in 3-Chlorobenzoate and 3-Chlorobenzoate (3-Chlorobiphenyl) Batch Culture Enrichments of Microbial Consortia from Chloroaromatic-Contaminated (BRC, DHC) and Non-Contaminated (FC) Sites in the Niagara watershed.

Samples were collected during three different seasons (spring, summer and fall). Results from the same season were combined for 1992 and 1993 samples.

Sites with fractional degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

A. 3-Chlorobenzoate enrichment results.

B. 3-Cba (3CBP) enrichment results.

A. 3-Chlorobenzoate

B. 3-Cba (3CBP)

Figure 2.8 The Effect of Sampling Season on the Microbial 3-Chlorobenzoate Degradation Potential in 32°C and 22°C 3-Chlorobenzoate and 3-Chlorobenzoate (3-Chlorobiphenyl) Batch Culture Enrichments of Chloroaromatic-Contaminated (BRC/DHC) and Non-Contaminated (FC) Sites in the Niagara Watershed.

Sites with fractional degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A.** Spring 1992 and 1993 enrichment results.
- B.** Summer 1993 enrichment results.
- C.** Fall 1992 and 1993 enrichments results.

Figure 2.9 The Effect of Batch Culture Enrichment Temperature on the 3-Chlorobenzoate Degradation Potential of Microbial Consortia Collected from Chloroaromatic-Contaminated Sites (BRC, DHC) in the Niagara Watershed During Different Seasons.

Samples were collected during three different seasons (spring, summer and fall). Results from the same season were combined for 1992 and 1993 samples. The BRC and DHC sites have been combined.

Sites with fractional degradation results < 0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

A. 32°C Enrichment results.

B. 22°C Enrichment results.

C. 4°C Enrichment results.

A. 32°C Enrichments

B. 22°C Enrichments

C. 4°C Enrichments

Figure 2.10 The Effect of Sampling Year on the 3-Chlorobenzoate Degradation Potential of Microbial Consortia in 3-Chlorobenzoate and 3-Chlorobenzoate (3-Chlorobiphenyl) Batch Culture Enrichments from Chloroaromatic-Contaminated (BRC and DHC) and Non-Contaminated (FC) Sites in the Niagara Watershed.

32°C and 22°C enrichment results have been combined. 4°C enrichments results have not been included.

Sites with fractional degradation results <0.25 as determined by HPLC analysis were considered to exhibit complete degradation of the chlorobenzoate isomer.

- A.** 1992 Enrichment results (spring and fall results combined).
- B.** 1993 Enrichment results (spring, summer and fall results combined).

A. 1992 Results

B. 1993 Results

Figure 2.11 The Chlorobenzoate Degradation Potential of Microbial Consortia in Batch Culture Enrichments (32°C, 22°C, 4°C) of Hyde Park Groundwater and Sequencing Batch Reactor Samples.

These results show the percentage degradation of each site compared to the uninoculated controls.

- A. 4-Chlorobenzoate enrichment results.
- B. 3-Chlorobenzoate enrichment results.
- C. 3,4-Dichlorobenzoate enrichment results.

A. Hyde Park 4-Chlorobenzoate Enrichments

B. Hyde Park 3-Chlorobenzoate Enrichments

C. Hyde Park 3,4-Dichlorobenzoate Enrichments

2.3.4 Chi-square analysis to determine the effect of Hyde Park contamination on microbial chlorobenzoate degradation

The influence of BRC and DHC contamination by Hyde Park on chlorobenzoate degradation potentials (assessed as complete, partial or none) in batch culture enrichments was tested statistically using chi-square analysis. When the degradation potentials for all seasons and all enrichment temperatures were combined (Table 2.2, n = 279) there was a significant effect of Hyde Park contamination on the degradation of 3,4-dCba ($p < 0.05$). A further sub-division of the data into different enrichment temperatures and seasons showed that this effect was not statistically significant. 4-Cba degradation was effected by Hyde Park contamination for all seasons and specific seasons in 32°C and 22°C enrichment cultures ($p < 0.05$). Thus, the contaminated sites exhibited superior 4-Cba degradation potentials over the non-contaminated sites. 3-Cba degradation was influenced by Hyde Park contamination during the spring seasons for 32°C and 22°C enrichment cultures but was independent of this contamination during the fall enrichments.

The 3-Cba degradation potential at chloroaromatic contaminated sites (BRC and DHC) was dependent of the sample season ($p < 0.05$) (Table 2.3). 4-Cba and 3,4-dCba degradation was generally independent of the sample season ($p > 0.05$). The one exception was the combined 32°C and 22°C enrichment results for 4-Cba. 3-Cba degradation in enrichment cultures from contaminated sites was independent of sample year ($p > 0.05$) despite the apparent superior degradation of 3-Cba in 1992 noted in Figure 2.6 and Table 2.4. Conversely, 4-Cba and 3,4-dCba degradation in enrichment cultures from contaminated sites was dependent on sample year ($p < 0.05$). The 4-Cba and 3,4-dCba degradation potential was better in 1993.

Unlike the 3-Cba results, the 3-Cba(3CBP) enrichments showed that 3-Cba degradation was dependent on sampling season for the fall samples and not the spring samples (Table 2.2). Also when the different enrichment temperatures were considered

the sampling season had no effect on 3-Cba degradation in the 3-Cba(3CBP) enrichments but did in the 3-Cba enrichments (Table 2.3). Finally, unlike the 3-Cba enrichments, the sample year did effect the 3-Cba degradation potential in the 3-Cba(3CBP) enrichments (Table 2.4).

Table 2.2 Chi-Square Analysis of the Effect of Hyde Park Contamination on Microbial Chlorobenzoate Degradation in Batch Culture Enrichments

H₀: (Degradation × Site)

Conditions	Chlorobenzoate in Enrichment Cultures			
	3-Cba	4-Cba	3,4-dCba	3-Cba(3CBP)
All Enrichment Temperatures.				
All Seasons n = 279	p<0.001	p<0.001	p=0.035	p<0.001
22°C, 32°C Enrichments.				
All Seasons n = 186	p<0.001	p<0.001	p = 0.107	p<0.001
All Seasons				
32°C Enrichments n = 93	p<0.001	p<0.001	p = 0.284	p=0.003
22°C Enrichments n = 93	p=0.010	p<0.001	p = 0.283	p=0.004
4°C Enrichments n = 93	p = 0.114	p=0.002	p = 0.128	p = 0.288
Spring Sampling				
22°C, 32°C Enrichments n = 72	p<0.001	p<0.001	p = 0.186	p = 0.071
32°C Enrichments n = 36	p=0.002	p=0.041	p = 0.349	p = 0.104
22°C Enrichments n = 36	p=0.002	p=0.001	p = 0.216	p = 0.556
4°C Enrichments n = 36	p=0.007	p = 0.099	p = 0.532	p = 0.533
Fall Sampling				
22°C, 32°C Enrichments n = 74	p = 0.190	p<0.001	p = 0.487	p<0.001
32°C Enrichments n = 37	p = 0.343	p=0.047	p = 0.321	p=0.012
22°C Enrichments n = 37	p = 0.546	p=0.002	p = 0.597	p=0.015
4°C Enrichments n = 37	p *	p = 0.105	p = 0.236	p *

* unable to compute due to too many zeros in the contingency table.

The hypothesis was rejected for $p < 0.05$. The bold characters identify dependent events

n = number of batch culture enrichment results in the analysis

Table 2.3 Chi-Square Analysis of the Effect of Sampling Season on Chlorobenzoate Degradation in Batch Culture Enrichments of Contaminated Sites (Bloody Run Creek and Devil's Hole Creek)

H_0 : (Degradation x Season)

Condition	Chlorobenzoate in Enrichment Cultures			
	3-Cba	4-Cba	3,4-dCba	3-Cba(3CBP)
All Contaminated Sites,				
22°C, 32°C Enrichments n=134	p<0.001	p<0.001	p=0.957	p=0.028
32°C Enrichments n=67	p<0.001	p=0.684	p=0.173	p=0.111
22°C Enrichments n=67	p=0.021	p=0.510	p=0.775	p=0.206
4°C Enrichments n=67	p<0.001	p=0.495	p=0.917	p=0.813

The hypothesis can be rejected for $p<0.05$. The bold characters identify dependent events.

Table 2.4 Chi-Square Analysis of the Effect of Sample Year (1992 vs 1993) on Chlorobenzoate Degradation in Batch Culture Enrichments of Contaminated Sites (Bloody Run Creek and Devil's Hole Creek).

H₀: (Degradation x Year)

Condition	Chlorobenzoate in Enrichment Cultures			
	3-Cba	4-Cba	3,4-dCba	3-Cba(3CBP)
All Contaminated Sites,				
All Enrichment Temperatures n=201	p=0.138	p=0.002	p=0.007	p<0.001
All Contaminated Sites, 22°C and 32°C				
Enrichments n=134	p=0.129	p=0.008	p=0.008	p<0.001

The hypothesis can be rejected for $p < 0.05$. The bold characters identify dependent events
n = number of batch culture enrichment results in the analysis.

2.4 DISCUSSION

In this chapter the chlorobenzoate degradation potential of microbial communities in the Niagara watershed was studied using batch culture enrichments. GC-MS analysis indicated the presence of multiply chlorinated compounds in a DHC water sample including a pentachlorobiphenyl compound (Figure 2.2c). Based on these mass spectra results, it was inferred that DHC was contaminated with chlorinated compounds by Hyde Park through the groundwater plume. Other GC-MS results showed that BRC still contained chlorinated compounds despite on-going bioremediation activities at Hyde Park. Figure 2.2b shows the mass spectra for a hexa-chlorinated compound from a groundwater seep that feeds into BRC. Similar GC-MS analysis revealed that FC was a suitable non-contaminated creek for this study as it contained no detectable chlorinated contaminants. Due to these mass spectra results, the batch culture enrichment results for BRC and DHC were combined for analyses of the significance of Hyde Park contamination.

This is the first known study to use repeated samplings to compare the microbial degradation potential in creeks contaminated by an industrial site with a pristine or non-contaminated creek. The contaminated creeks, BRC and DHC, consistently showed a greater over-all degradation potential of 3-Cba and 4-Cba than a non-contaminated creek, FC. Prolonged exposure of the indigenous sediment and water microbial populations of BRC and DHC to chlorinated contaminants from Hyde Park has provided a strong selection for monochlorobenzoate degradation. It was evident that chloroaromatic contaminated groundwater originating from Hyde Park in the Niagara watershed had an impact on the microbial degradation potential of two contaminated creeks (Figures 2.3, 2.4, 2.6, 2.7, 2.8, 2.10, Table 2.2).

Overall, the degradation potential of 3,4-dCba at BRC and DHC was statistically greater than the FC degradation potential (Table 2.2, all enrichment temperatures and all seasons combined). However, the results at different enrichment temperatures suggested

that, unlike 3-Cba and 4-Cba mineralization, 3,4-dCba degradation was independent of Hyde Park contamination. This discrepancy may be caused by members of the 3,4-dCba degradative consortia that were not selected for in batch culture enrichments due to culturability limitations. Batch culture enrichments select only the culturable portion of a microbial community. It has been estimated that only 0.01-22% of naturally occurring bacteria are culturable (Roszak and Colwell 1987, Brockman 1995). There are only two known examples in the literature of single isolates capable of 3,4-dCba mineralization (Fava *et al.* 1993, Nakatsu and Wyndham 1993) and one example of 3,4-dCba degradation by co-metabolism (Adriaens and Focht 1991) in contrast to the numerous examples of microbial isolates capable of 3-Cba and 4-Cba degradation. It is possible that 3,4-dCba mineralization is more dependent on microbial consortia than mono-chlorobenzoate degradation is.

4-Cba degradation occurred more frequently than 3-Cba and 3,4-dCba degradation under all enrichment conditions (Figures 2.3-2.11). Although most enrichments of creek sediments contaminated by Hyde Park completely degraded 4-Cba, noticeable 4-Cba degradation was also observed in FC enrichments (Figures 2.3, 2.4, 2.6). One possible explanation may be that this chlorobenzoate isomer has a similar configuration to naturally occurring compounds or synthetic compounds found in FC. Although mass spectra results did not specifically detect chlorinated compounds in FC water, similarly configured compounds may exist. Degradative genotypes often evolve by recruitment and subsequent divergence of genes that specify degradation of analogous naturally occurring compounds. This has been demonstrated by the homologies observed between evolved and ancestral genes, including similarities in their organization and regulation (Rothmel *et al.* 1989)

An alternative explanation is that the significant 4-Cba degradation observed in FC is due to successful horizontal gene transfer or dissemination of catabolic genotypes from contaminated sites. The high 4-Cba degradative potential observed in the contaminated creeks suggests that degradative genotypes are very common in the vicinity of Hyde Park

There is no apparent selective force to keep mobile genetic elements responsible for 4-Cba degradation at high frequencies in FC. However, low frequency genotypes would have been selected during enrichment in 4-Cba. Microbial consortia were able to rapidly evolve the ability to utilize 4-Cba when this was the only carbon source available to them in continuous culture enrichments (Hartmann *et al.* 1979, Chatterjee and Chakrabarty 1982).

3-Cba and 3,4-dCba were degraded poorly in the enrichment cultures from FC. The negligible degradation potential of these two compounds may be due to the fact that these two chlorobenzoate isomers do not have natural or synthetic analogs in this creek from which degradative genes can arise. The placement of the chlorine substituents plays a critical role in the degradation of chlorobenzoates by different enzymes. Even though 3-Cba and 4-Cba differ only in the placement of the chlorine atom, the regiochemistry of these two isomers is quite different. It was recently reported that a high number of 3-Cba catabolic isolates were recovered from pristine soils with no recorded chloroaromatic contamination (Fulthorpe *et al.* 1996). The most likely explanation for the widespread 3-Cba degradation that they observed was that 3-Cba may be an analog of chlorinated or non-chlorinated naturally occurring compounds found in the biomes that were examined.

Improved 3-Cba degradation was observed in the spring enrichments of the contaminated sites (Figure 2.4, Table 2.3). This may be due to elevated 3-Cba contamination from spring surface run-off in the groundwater resulting in a positive selection for bacteria capable of degrading this congener. The selective force of a chemical contaminant on a mobile genetic element has been shown in simulated natural environments (Fulthorpe and Wyndham 1991, Fulthorpe *et al.* 1989, Fulthorpe and Wyndham 1992). However, the 3-Cba degradation potential results for the 3-Cba(3CBP) enrichments contradict these findings. These batch cultures exhibited superior 3-Cba degradation during the summer and fall enrichments (Figures 2.7-2.9). The greater 3-Cba degradation potential by the 3-Cba enrichments in the spring samples may also be a result of seasonal nutrient fluctuations. The water chemistry results for November 1993 (Table

2.1) and earlier studies of this creek revealed that BRC is phosphate limited (Wyndham and Straus 1988a). Analysis of water collected from DHC and FC showed that these creeks were also phosphate limited during November 1993. Analyses of additional water samples collected over different seasons would be required to conclude that these creeks were nutrient limited. It is possible that nutrient surpluses in the spring support better microbial growth overall and fitness of 3-Cba catabolic isolates. Under phosphate limited conditions in continuous culture enrichments with 3-Cba as a sole carbon source, *Pseudomonas fluorescens* NR 52 (unable to mineralize 3-Cba) effectively scavenged available phosphate when it was present at a ratio of 1 cell to 20 of *Alcaligenes* sp. BR60 (a species capable of 3-Cba mineralization) (Wyndham and Straus, 1988a).

The improved 3-Cba degradation by the 3-Cba(3CBP) enrichment cultures during the summer and fall samplings suggests that a different group of microorganisms were selected in these batch cultures. The 3-Cba catabolic members of these consortia may represent a different phylogenetic groups of microorganisms.

With the exception of the slight improvement in 3,4-dCba degradation during the fall enrichments, there was no seasonal variation observed in 4-Cba and 3,4-dCba degradative capabilities. This may be due to a lack of seasonal variation in the concentration of these chlorobenzoate isomers

Continued chlorobenzoate degradation in the fall enrichment samples when the ambient air temperature was -2 and 10°C, in 1992 and 1993, respectively, suggested that some members of the microbial consortia involved in chlorobenzoate degradation were facultatively psychrophilic (can grow well at 0°C but have an optimum growth temperature at or above 20°C) (Inniss 1975). 4°C enrichments showed statistically less frequent partial or complete degradation potentials of all chlorobenzoates over all seasons (Figure 2.5, Tables 2.2, 2.3). This is not surprising as facultative psychrophilic microbial populations show better activity at 20°C than at 4°C (Inniss 1975). Due to the lower

temperature in the Niagara watershed during the fall and winter months, the catabolism of chlorobenzoates would occur at a decreased rate during these seasons.

Although the 3-Cba degradation potential by the microbial community from the contaminated sites enriched in 3-Cba appeared greater in 1992 than 1993, this difference was not statistically significant ($p > 0.05$) (Figures 2.6, 2.10, Table 2.4). Conversely, the 4-Cba and 3,4-dCba degradation potentials were statistically greater in 1993 at all sites ($p < 0.05$). The concentrations of these latter chlorobenzoate isomers may have increased in the water contaminated by Hyde Park between the 1992 and 1993 sampling seasons resulting in microbial consortia with better degradative potentials. Alternatively, the dissemination of chlorobenzoate catabolic genotypes may have increased in the Niagara watershed between 1992 and 1993. The statistically improved 3-Cba degradation potential in the 3-Cba(3CBP) enrichment cultures during 1993 also strongly suggests that a different 3-Cba catabolic microbial population was present in these cultures compared to the 3-Cba enrichment cultures.

Hyde Park groundwater (Well) and SBR sample batch cultures showed similar degradation potentials over-all for the three chlorobenzoate isomers compared to the Hyde Park contaminated creek results (Figure 2.11). There were some differences observed between the groundwater and SBR enrichments. None of the Well A chlorobenzoate enrichment cultures showed evidence of chlorobenzoate degradation. This sample appeared much more contaminated than the other Well samples. It was dark brown, sticky and had a distinctive strong smell of phenol. It is possible that this sample was too toxic to support the growth of chloroaromatic catabolic microorganisms. It is also possible that this sample was from a more oxygen limited environment and that the batch culture enrichments would not select for these catabolic isolates. Finally, there may have been more predators in this groundwater sample that grazed on the chlorobenzoate catabolic microorganisms. The fact that the other two Well samples, C and D, exhibited a high degradation potential for all chlorobenzoate isomers provides evidence that there

were chloroaromatic catabolic bacteria in the Hyde Park groundwater. SBR1 samples showed the greatest degradation potential for 3- and 4-Cba. Whereas the SBR3 32°C enrichment culture exhibited complete 3-Cba mineralization, the SBR3 22°C enrichment culture showed no 3-Cba degradation potential. It is more likely that the this latter culture received less inoculum or the microbial grazing populations were more active in this culture rather than the possibility that the 3-Cba catabolic consortia in SBR3 could mineralize 3-Cba at 32°C but not 22°C. None of the SBR enrichment cultures were capable of 3,4-dCba mineralization. This suggests either that 3,4-dCba in the groundwater is degraded in the groundwater prior to entering the SBRs, or the conditions in the SBRs are not suitable to support the growth of 3,4-dCba catabolic microorganisms.

The *in situ* microbial degradation potential of selected chlorobenzoates in the Niagara watershed was examined. Batch culture enrichments in this study encouraged microbial community growth based on combined (concerted) metabolic attack as well as the growth of microbes capable of complete biodegradation of chlorobenzoates on their own (see Chapter 3). Numerous variables influencing the chlorobenzoate degradation potential in the Niagara watershed were studied including the effect of chlorinated contamination from Hyde Park, degradation of different chlorobenzoate isomers, enrichment temperature (to account for temperature fluctuations in this region) and seasonal variations and yearly variations. In subsequent chapters, results for the isolation of chlorobenzoate degrading and putative BP and 3CBP catabolic bacteria from these enrichment cultures are presented.

CHAPTER 3:

Screening of Microorganisms from Enrichment Cultures for the *cbaAB*, *clcABD*, *fcB* and *bphA* Genotypes

3.1 INTRODUCTION

Chlorobenzoate degradation via different pathways has been elucidated in numerous microorganisms. 3-Cba mineralization can proceed via the chlorocatechol *ortho*-ring-fission pathway. This is the best studied pathway for 3-Cba degradation. The chlorocatechol *ortho*-ring-fission genes have been found to be distributed worldwide. The *clcABD* pathway was genetically characterized in the organism *Pseudomonas putida* AC858 isolated from sewage in Niskayuna, New York. The catabolic genes were found to be localized on a 117 kbp plasmid pAC25 (Chatterjee *et al.* 1981). *Alcaligenes eutrophus* JMP134 from soil in Australia (Don and Pemberton 1985) and *Pseudomonas putida* B13 isolated from sewage in Göttingen, Germany (Dorn *et al.* 1974) were found to be capable of 3-Cba degradation using the same pathway. These catabolic genes were also found to be encoded on high molecular weight plasmids pJP4 (80 kbp) and pB13 (111 kbp), respectively. All three plasmids are highly homologous (Chatterjee and Chakrabarty 1983). Kasberg *et al.* (1995) have recently identified a fourth enzyme in the chlorocatechol modified *ortho* pathway of *Pseudomonas* sp. strain B13. A maleylacetate reductase has been identified downstream of the *clcD* gene. This gene has been named *clcE*. Homologous genes *tfdF* and *tcbF* have been identified downstream from the homologous chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and dienelactone hydrolase operons *tfdCDE* of pJP4 in *Alcaligenes eutrophus* JMP134 and *tcbCDE* of pP51 in *Pseudomonas* sp. strain P51, respectively. The products from these

operons converge at maleylacetate. The maleylacetate reductase products are precursors for the TCA cycle.

Growth in a chemostat of 3-Cba catabolic *Pseudomonas putida* AC858 cells harbouring the plasmid pAC25, in the presence of *Pseudomonas putida* mt-2, resulted in the emergence of recombinant cells capable of 4-Cba mineralization. Strain *Pseudomonas putida* mt-2 contains a 115 kb plasmid pWWO which encodes the catabolic enzymes required for the oxidation of toluene and xylene (Williams and Murray 1974). The recombinant cells that emerged had an increased substrate range that included 4-Cba mineralization. A new plasmid in the recombinant, designated pAC27, revealed a deletion of 11 kbp from the pAC25 plasmid. A 41.5 kbp portion of pWWO was also found to be transposed on to the chromosome of recipient cells. The ability of this lab-engineered microbial strain to mineralize 4-Cba was attributed to the acquisition of the pWWO encoded toluate dioxygenase, having relaxed substrate specificity that enabled the host cells to convert 4-Cba to 4-chlorocatechol (Chatterjee and Chakrabarty 1982). The *clcABD* genotype harboured on pAC27 in *Pseudomonas putida* AC866 is presented in Figure 3.1.

Recently, Ogawa and Miyashita (1995) reported the presence of a set of insertion sequence-like elements associated with the chlorocatechol *ortho*-ring-fission pathway in *Alcaligenes eutrophus* NH9 on the plasmid pENH91. Although transposition was not detected, the composite transposon-like structure suggested that the genes encoding this pathway might be spread by transposition as well as by transmissible plasmids.

The 3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway is capable of 3-Cba and 3,4-dCba mineralization via a *meta*-ring-fission route (Nakatsu and Wyndham 1993) (Figure 3.2). This pathway was first detected on the plasmid pBRC60 in the organism *Alcaligenes* sp. BR60 (Wyndham and Straus 1988a) and was later found to be further encoded within a 17 kb transposable element, Tn5271 on pBRC60 (Nakatsu *et al.* 1991). Selection on 4-Cba as a sole source of carbon and energy resulted in the isolation of mutant colonies of *Alcaligenes* sp. BR60 capable of 4-Cba mineralization (Wyndham *et al.* 1988b). Although this pathway is known to be transposon encoded, until recently its known natural distribution was limited to the Niagara watershed (Wyndham *et al.* 1994a). A related

transposon has been found in *Alcaligenes* sp. strain CPE3 from PCB contaminated soil in Italy (Di Gioia *et al.* pers. comm.). A third ORF, tentatively designated *cbaC* has been identified from DNA sequence data extending beyond the *cbaB* gene. Preliminary results suggest this region encodes a 3-chlorobenzoate 3,4-dihydrodiol dehydrogenase (Nakatsu *et al.* 1995b).

The transformation of 3-Cba to 3-hydroxybenzoate by the direct replacement of the chlorine substituent has also been observed (Johnston *et al.* 1972). No further genetic characterization of this pathway has been reported.

4-Cba degradation has been shown to proceed through 4-chlorocatechol *ortho*- (Hernandez *et al.* 1991) and *meta*- (Arensdorf and Focht 1995) cleavage pathways in addition to a hydrolytic process. *Pseudomonas* sp. CBS3, isolated by selective enrichment from agricultural soil in Stuttgart Germany, converts 4-Cba to 4-hydroxybenzoate via a hydrolytic process involving CoA-ligated intermediates, as opposed to an oxidative pathway (Scholten *et al.* 1991). 4-Hydroxybenzoate is oxidized to protocatechuate and then to carboxymuconate via an *ortho*-cleavage pathway (Liang *et al.* 1993). Other Gram-negative strains *Acinetobacter* sp. 4-CB1 (Crooks and Copley 1994) and *Alcaligenes demitrificans* NTB-1 (van den Tweel *et al.* 1986) employ a similar mechanism of hydrolytic dechlorination for 4-Cba degradation. This dehalogenase pathway (*fc*ABC) (Figure 3.3) has also been detected and genetically characterized in the Gram-positive microorganisms *Arthrobacter globiformis* KZT1 isolated from soil in Russia (Tsoi *et al.* 1991) and *Arthrobacter* sp. SU from soil in Germany (Schmitz *et al.* 1992). The hydrolytic dehalogenation step that converts 4-Cba to 4-hydroxybenzoate is comprised of 3 enzymes: a 4-Cba-CoA-ligase, a 4-Cba-CoA-dehalogenase and a thioesterase. The ligase (*fc*A) and thioesterase (*fc*C) genes are involved only in the formation and removal of the CoA thioester (Liang *et al.* 1993). Although all enzymes are required for 4-Cba degradation, the *fc*B gene dictates congener specificity. It catalyzes an aromatic substitution reaction involving the replacement of the chloride substituent with the hydroxyl group from a molecule of water (Löffler *et al.* 1991). *Arthrobacter globiformis* KZT1, *Arthrobacter* sp. SU and *Pseudomonas* CBS3 all have highly homologous *fc*B genotypes (Schmitz *et al.* 1992). There is an 81% DNA sequence homology between the

pcbB gene in *Arthrobacter* sp. SU and *Pseudomonas* CBS3. The dehalogenase pathway has been reported to be encoded on high molecular weight plasmids in *Arthrobacter globiformis* KZT1 (pBS1502) (Zaitsev *et al.* 1991, Tsoi *et al.* 1991) and *Arthrobacter* sp. SU (pASU1) (Schmitz *et al.* 1992) but not in *Pseudomonas* sp. CBS3. The *pcbABC* pathway is encoded within a 4.5 kb chromosomal DNA fragment in *Pseudomonas* sp. CBS3 and is specific for 4-Cba mineralization.

Hydrolytic dehalogenation of halogenated aromatic compounds is of great interest as these enzymes yield a hydroxylated aromatic compound from a haloaromatic. Many hydroxylated aromatic compounds have applications in the pharmaceutical and petrochemical industries. As there are no readily available preparative chemical reactions for direct, specific aromatic hydroxylation on an industrial scale, the hydroxyl group must be introduced through an expensive series of reactions. There are now many attempts to produce hydroxylated aromatic compounds, including 4-hydroxybenzoate, in a biological system by using either enzymes or whole cells (van den Tweel *et al.* 1986).

Burkholderia sp. LB400 is able to oxidize a wide variety of PCB congeners to chlorobenzoate intermediates up to and including hexachlorobiphenyls. This micro-organism was isolated from PCB-contaminated Hudson River sediments in upstate New York using batch culture enrichments containing BP (Bopp 1986). The pathway for complete BP degradation and PCB conversion to chlorobenzoate intermediates by *Burkholderia* sp. LB400 is a four-step process initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 by a biphenyl dioxygenase (Figure 3.4). Unlike nearly all other PCB-degrading bacteria, *Burkholderia* sp. LB400 also exhibits 3,4-biphenyl dioxygenase activity (pathway not shown). The biphenyl dioxygenase is a multicomponent enzyme which consists of a large (*bphA*) and a small subunit (*bphE*) of iron-sulfur proteins, a ferredoxin (*bphF*) and a ferredoxin reductase (*bphG*). The *bphA* enzyme specifies the regiospecificity of O₂ insertion to yield 2,3-dioxygenase and possibly the 3,4-dioxygenase activities in *Burkholderia* sp. LB400 and therefore determines congener specificity (Erickson and Mondello 1993).

No plasmids have been detected in *Burkholderia* sp. LB400 despite repeated attempts using a wide range of protocols (Bopp 1986). Other BP/PCB catabolic

genotypes, however, have been found to be plasmid encoded (Layton *et al.* 1992, Shields *et al.* 1985, Furukawa and Chakrabarty 1982). There is one reported case where the *bph* operon is encoded within the transposable element, Tn4371 (Springael *et al.* 1993). *bph* Operons show a high degree of homology. All have homologous 2,3-biphenyl dioxygenase genes and these genes have been detected in isolates from different parts of the world (Furukawa *et al.* 1989). This strongly suggests a common ancestral pathway and horizontal gene transmission of this pathway.

Due to chronic chloroaromatic contamination in the Niagara watershed and numerous reports of mobile chloroaromatic catabolic genetic elements it was postulated that previously characterized genotypes may be present in this area. The purpose of this investigation was to:

1. Collect microorganisms from batch culture enrichments capable of 3-Cba, 4-Cba, 3,4-dCba, BP and 3-CBP mineralization.
2. Determine the incidence of catabolic genotypes *clcABD*, *cbaAB*, *fcbb* and *bphA* in the culturable population recovered by batch culture enrichments from Niagara watershed samples.

Figure 3.1 3-Chlorobenzoate Degradation by the Chlorocatechol *ortho*-ring-fission Pathway Encoded in *Pseudomonas putida* AC866 (pAC27).

Compounds: (I) 3-chlorobenzoate, (II) 3-chlorocatechol, (III) 3-chloro-*cis*, *cis*-muconate, (IV) dienelactone, (V) maleylacetate.

Enzymes: (*clcA*) chlorocatechol 1,2-dioxygenase, (*clcB*) chloromuconate cycloisomerase, (*clcD*) dienelactone hydrolase.

Benzoate (toluate) 1,2 dioxygenases (*benABC* and *xy/XYZ*) are responsible for the conversion of 3-Cba to 3-chlorocatechol and are the same enzymes used for the conversion of benzoate to catechol. The *benABC* genes are chromosomally encoded in *Pseudomonas putida* AC866 and have a narrow substrate specificity (Neidle *et al.* 1987). The *xy/XYZ* genes with broad substrate specificity are encoded on plasmid pWWO in *Pseudomonas putida* (Harayama *et al.* 1991).

Adapted from Frantz and Chakrabarty 1987.

clc A	clc B	clc D
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Figure 3.2 3-Chlorobenzoate Degradation by the 3-Chlorobenzoate 3,4-(4,5)-Dioxygenase Pathway Encoded by Tn5271 in *Alcaligenes* sp. BR60 (pBRC60).

Compounds: (I) 3-chlorobenzoate, (II) 3-chlorobenzoate 3,4-dihydrodiol, (III) protocatechuate, (IV) 2-hydroxy-4-carboxy-*cis, cis*-muconate semialdehyde, (V) 3-chlorobenzoate 4,5-dihydrodiol, (VI) 5-chloroprotocatechuate, (VII) enzyme-bound chloroacyl intermediate.

Enzymes: (*cbaAB*) 3-chlorobenzoate-3,4-(4,5)-dioxygenase and dioxygenase reductase.

From Nakatsu and Wyndham 1993 and Nakatsu *et al.* 1995b.

Figure 3.3 Hydrolytic Dehalogenation of 4-Chlorobenzoate by *Pseudomonas* sp. CBS3.

Compounds (I) 4-chlorobenzoate, (II) 4-chlorobenzoate:CoA, (III) 4-hydroxybenzoate:CoA, (IV) 4-hydroxybenzoate.

Enzymes (*pcbA*) 4-chlorobenzoate-CoA ligase, (*pcbB*) 4-chlorobenzoate-CoA dehalogenase, (*pcbC*) 4-hydroxybenzoate-CoA thioesterase.

Adapted from Liang *et al.* 1993 and Schmitz *et al.* 1992.

<i>fc</i> <i>b</i> A	<i>fc</i> <i>b</i> B	<i>fc</i> <i>b</i> C
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Figure 3.4 Biphenyl-PCB Degradative Pathway in *Burkholderia* sp. LB400.

Compounds (I) 3-chlorobiphenyl, (II) 2',3'-dihydro-2',3'-dihydroxy-3-chlorobiphenyl, (III) 2',3'-dihydroxy-3-chlorobiphenyl, (IV) 2-hydroxy-6-oxo-6-(3'-chlorophenyl)-hexa-2,4-dienoic acid, (V) 2-hydroxypenta-2,4-dienoic acid, (VI) 3-chlorobenzoate, (VII) 2-hydroxypenta-2,4-dienoic acid.

Enzymes (*bphAEFG*) biphenyl 2,3-dioxygenase ferredoxin and ferredoxin reductase

(*bphB*) 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase

dihydroxybiphenyl-1,2-dioxygenase, (*bphD*) 2-hydroxy-6-oxo-6-(3'-chlorophenyl)-hexa-2,4-dienoate

hydrolase

From Erickson and Mondello 1992.

<i>bph</i> AEFG	<i>bph</i> B	<i>bph</i> C	<i>bph</i> D
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3.2 MATERIALS AND METHODS

3.2.1 Isolation of Microorganisms from Enrichment Cultures

1.5 mM enrichment cultures, previously described in Chapter 2, were serially diluted from 10^{-2} to 10^{-6} in 1/10th strength medium A+N. Dilutions from the chlorobenzoate enrichments were spread onto medium A+N plates containing 1.5 mM of the appropriate chlorobenzoate, 1.6% agar (Difco Bacto-Agar) and 100 µg/ml cycloheximide (Sigma) to prevent fungal growth. A groundwater sample from Hyde Park collected in August 1990 and refrigerated for one year was serially diluted and plated onto 1.5 mM 3-Cba, 4-Cba and 3,4-dCba selection plates at the same time. The BP and 3CBP carbon sources were dissolved in DMF to final concentrations of 400 mM and approximately 50 µl was dropped onto the underside of the lids of petri plates in order to provide these volatile carbon sources in the vapour state. The plates contained medium A+N and received dilutions of the BP and 3CBP enrichment cultures. The lids containing the drops were left to dry in the sterile cabinet prior to incubation. All enrichment culture serial dilutions were also plated onto medium A+N plates without a carbon source. This was done in order to distinguish false positive growth (microbial growth from agar impurities) from real growth on the selection plates. Replicate plates were incubated for 8, 10 and 14 days at 32°C, 22°C or 4°C respectively.

Isolated colonies were patched onto a second selection plate to ensure purity. Microorganisms from these plates were confirmed to degrade 3-Cba, 4-Cba or 3,4-dCba by HPLC analysis (see Chapter 2) following growth in 1.5 mM chlorobenzoate broth (under the same incubation temperature and length of incubation as with the plates). BP and 3CBP mineralization could not be determined via HPLC analysis. Only isolates exhibiting good growth following transfer on these carbon sources were selected for further analysis. These were considered "putative" BP and 3CBP degraders.

All confirmed and putative degraders were named according to the creek and the location along the creek that they were isolated from. For instance, the first confirmed 3-Cba degrader from DHC site 3 during the second sampling trip was given the name DHC3-1-2 (DHC third sampling site along the creek, first isolate, second sampling trip). A 20% glycerol stock suspension of each of these microorganisms was prepared in duplicate and stored at -70°C for further analysis.

3.2.2 Bacteria and Plasmids used in this Study and Screening Regime

Table 3.1 lists the *Escherichia coli* strains and plasmid clones carrying genotypes used as probes in this study. The table also lists the archetypal microorganisms harbouring each of the catabolic genotypes and their sources. These microorganisms were used as positive controls during the screening process.

Table 3.2 shows the catabolic genotype screening design for the chlorobenzoate, BP and 3CBP degradative isolates from the Niagara watershed. The testing design was based on previous knowledge of the substrate range of each archetypal microorganism. 4-Cba catabolic isolates were screened with the *cbaAB* genotype based on the previous observation of mutant colonies arising on 4-Cba selection plates. All 3,4-dCba isolates were checked for the *clcABD* genotype as bacteria harbouring this genotype are known to degrade the dichlorobenzoate 3,5-dCba (Ghosal *et al.* 1985).

Table 3.1 Bacterial Strains and Plasmids Used in this Study

Strain	Plasmid and Phenotypes	Genotype	References
<i>E. coli</i>			
DH1	pDC100, Ap ^r	<i>cleABD</i>	Frantz and Chakrabarty 1987
JM109	pBRH2, Ap ^r	<i>cbaAB</i>	Wyndham <i>et al.</i> 1988b
JM JM105	pHUG01, Ap ^r , Sm ^r , Su ^r	<i>fcbaABC</i>	Savard <i>et al.</i> 1992
<i>Pseudomonas</i> sp.			
CBS3	-, 4-Cba ⁺	<i>fcbaABC</i>	Keil <i>et al.</i> 1981
AC866	pAC27, 3-Cba ⁺ , 4-Cba ⁺	<i>cleABD</i>	Chatterjee <i>et al.</i> 1982
<i>Burkholderia</i> sp.			
LB400	-, BP ⁺	<i>bphAEFG</i>	Bopp 1986
<i>Alcaligenes</i> sp.			
BR60	pBRC60, 3-Cba ⁺	<i>cbaAB</i>	Wyndham and Strauss 1988a

- , no plasmid; Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Su^r, sulfathiazole resistance.

Table 3.2 Genotype Screening of Chloroaromatic Catabolic Isolates

Carbon Source	<i>cbaAB</i>	<i>clcABD</i>	<i>fcxB</i>	<i>bphA</i>
3-C'ba	+	+		
4-C'ba	+	+	+	
3,4-dC'ba	+	+		
BP				+
3-C''-(3CBP)	+	+		+
3CBP	+	+		+

3.2.3 DNA Isolation

All confirmed chlorobenzoate catabolic isolates and all putative BP and 3CBP isolates were subjected to pooled total genomic DNA isolations. A single colony from a selection plate of each confirmed or putative catabolic isolate was resuspended in 5 ml tryptone-yeast extract (TYE) broth (1% tryptone, 1% yeast extract, 0.5% NaCl) along with a single colony from 9 other isolates and grown overnight in the same tube on a shaker/incubator at the original isolation temperature (32°C, 22°C or 4°C). Total DNA was extracted from these samples using a scaled-up protocol of Ausubel *et al.* (1989). Pure culture total DNA isolations were prepared using the same protocol for the archetypal microorganisms and for isolates from pooled genomics that hybridized to one of the probes tested.

Plasmid DNA for probe preparation was isolated using the alkaline lysis method (Birnboim and Doly 1979).

3.2.4 Probe Preparation

The plasmids pDC100 and pBRH2 were both digested with the restriction endonuclease *EcoRI* resulting in a 4.2 kbp fragment encompassing the *chlABD* genes, and 1.1 and 1.4 kbp fragments (E13 and E14) containing a portion of the *chaAB* genes, respectively. The plasmid pHUG01 contained the 3 genes required for dehalogenase activity of *Pseudomonas* sp. CBS3 (*jcbABC*). Due to the broad substrate specificity of the 4-Cba-CoA-ligase (*jcbA*) and thioesterase (*jcbC*), only the 4-Cba-CoA dehalogenase gene (*jcbB*) was used as a probe. This gene is located on a 1.6 kb fragment of pHUG01 and was isolated following a double digest with *SacI* and *SalI* restriction endonucleases. A region of the *bphA* gene from *Burkholderia* sp. 1B400 was amplified by PCR resulting

in a 805 bp probe. The primers selected using the Oligo Program version 4.0 (National Biosciences) were *bphA1778* (5'→3') AAC GCC AAG GCT TTC AC and *bphA2566* (5'→3') CGC CTG CGG AGA AGT TG and were synthesized using an Applied Biosystem PCR-Mate DNA Synthesizer. The primers were deprotected from the DNA synthesis columns using concentrated ammonium acetate and following the manufacturer's instructions (Applied Biosystems). A portion of each primer concentrated stock solution was diluted in sterile water to 10 mM working stock concentrations. A 100 µl PCR reaction was set-up containing 10 µl 10X Vent buffer (New England Biolabs), 1.2 mM dNTPs (New England Biolabs), 0.5 mM each primer, 10 ng template *bphA* DNA and 1U Vent DNA polymerase (New England Biolabs). PCR reactions were over-layed with 100 µl mineral oil. The template DNA was denatured for 5 min at 95°C. After 4 min Vent DNA Polymerase was added. Amplification was carried out for 29 cycles with denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min using a Perkin Elmer Cetus 480 Thermocycler. The final cycle was followed by a 10 min extension period at 72°C and an indefinite 4°C soak program.

The digested or amplified probe preparations were separated by electrophoresis on 1.0% (1.5% for *bphA* PCR product) low melting temperature agarose (FMC Corp.) gels submerged in Tris-acetate buffer (TAE). The fragment sizes based on electrophoretic motility and relationship to a *HindIII* digested lambda DNA marker were calculated using the IBM PC Bandsize program (University of Toronto). Each band that corresponded to the size of a probe was cut from the gel and purified using glassmilk according to the protocol supplied by the manufacturer (Gene Clean, Bio 101 Inc.). All probes were labelled with a nonradioactive label, digoxigenin-11-dUTP, for 16 to 20 hours under reaction conditions recommended by the manufacturer (Boehringer Mannheim).

3.2.5 Hybridizations with Catabolic Genes Probes

Restriction endonucleases (New England Biolabs) known to digest each catabolic gene of interest were selected for DNA digestion. Previous studies revealed that the restriction endonuclease *NdeI* cut within the *clcABD* region of pAC27 (Bower 1993). All 3-Cba, 4-Cba, 3,4-dCba, 3-Cba(3CBP) and 3CBP pooled genomic DNA isolations and *Pseudomonas putida* AC866 (pAC27) DNA were digested with *NdeI*. *EcoRI* was known to result in multiple fragments within the *cbaAB* genes of *Alcaligenes* sp. BR60 (pBRC60) (Nakatsu *et al.* 1991). This enzyme was used to digest all 3-Cba, 3,4-dCba, 3-Cba(3CBP) and 3CBP pooled genomic DNA isolations and *Alcaligenes* sp. BR60 (pBRC60) DNA.

No sequence information was available at the time of this investigation for the *tcbB* or *bphA* genotypes. As a result, *Burkholderia* sp. 1 B400 and *Pseudomonas* sp. CBS3 DNAs were digested with multiple restriction endonucleases and subsequently hybridized with the respective probes in order to ascertain which enzymes cut within the catabolic genes. DNA was digested with the restriction endonucleases *BamHI*, *HindIII*, *BglII*, *EcoRI*, *SacI*, *SalI* and *NarI*. *EcoRI* and *HindIII* revealed two bands following Southern hybridization of *Pseudomonas* sp. CBS3 DNA with the *tcbB* probe. All other enzymes resulted in only one probe positive band. *EcoRI* was selected as the enzyme to digest all the pooled DNA from all 4-Cba degradative isolates and *Pseudomonas* sp. CBS3 DNA. *Burkholderia* sp. 1 B400 DNA digested with *SalI* resulted in two *bphA* probe positive bands. All other enzymes resulted in only one probe positive band. Therefore *SalI* was selected to digest all putative BP 3-Cba(3CBP) and 3CBP pooled genomics and *Burkholderia* 1 B400 DNA.

Digests were set-up in 20 µl reaction volumes using 5 µl of DNA and following the manufacturer's instructions (New England Biolabs). Following digestion, DNA was separated on a 0.7% agarose gel (Bio Rad) by electrophoresis at 30V overnight. The

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PM-1 3" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

DNA in the gels was denatured and neutralized following the protocol of Boehringer Mannheim and was transferred to nylon membranes (Boehringer Mannheim) overnight by capillary action using the method of Southern (1975). These blots were fixed under UV light for 5 min prior to hybridization with the digoxigenin labelled probes. The blots were pre-hybridized at 65°C for 2 hours and hybridized at 65°C overnight (low stringency) in 5X sodium chloride-sodium citrate (SSC) (Sambrook *et al.* 1989), 0.1% (w/v) N-laurylsarcosine (Na salt), 0.02% (w/v) sodium dodecyl sulphate (SDS) and 1% (w/v) skim milk powder. The hybridized membranes were washed and developed using the colour or chemiluminescent reaction as per the manufacturer's guidelines (Boehringer Mannheim). The membranes developed using the colour reaction were photographed with Polaroid 55 film. Chemiluminescence developed membranes were exposed to x-ray film (XAR-5, Kodak). Some membranes were stripped of reaction products and probe (Boehringer Mannheim protocol) and re-hybridized with a different probe. Membranes were stripped and re-probed no more than twice. Total genomic DNA from individual isolates of the combined genomic pools that showed positive genotype hybridizations was extracted and the Southern blotting and hybridization protocol was repeated as described above in order to determine which isolate harboured the genotype. All membranes were dried and stored at 4°C.

Originally, various colony hybridization protocols were used to detect the four genotypes. These results were inconsistent and confusing due to the heterogeneous colony morphology of the different isolates. The colour, texture, growth rate and tendency of selected isolates to excrete mucoid substances all interfered with the colony hybridization results.

3.3 RESULTS

3.3.1 Chlorobenzoate Catabolic Isolates

Over 700 microorganisms were isolated from chlorobenzoate selection plates and screened for chlorobenzoate degradation. Only microorganisms on the selection plates that were larger than the pinpoint colonies on the medium A - N control plates with no carbon source were selected for screening. A total of 464 microorganisms from the Niagara watershed batch culture enrichments were confirmed by HPLC analysis to degrade 3-Cba, 4-Cba or 3,4-dCba. As well, 354 putative BP and 3CBP catabolic microorganisms were collected (Table 3.3). The greatest proportion of chlorobenzoate catabolic isolates obtained were 4-Cba degradative microorganisms. Additionally, more microorganisms from all chlorobenzoate enrichments were collected from the 32°C and 22°C enrichments than from the 4°C enrichments. The collection of chlorobenzoate catabolic isolates was representative of the sites, temperatures and sampling trips that generated the enrichment results presented in Chapter 2. Appendix 2 shows the distribution of catabolic isolates collected from different sampling trips and enrichment temperatures.

Table 3.3 A Summary of the Chloroaromatic Catabolic Isolate Collection from the Niagara Watershed

Carbon Source	32°C	22°C	4°C	TOTAL
3-Cba	54	51	22	127
4-Cba	91	75	48	214
3,4-dCba	15	10	6	31
BP	114	86	53	253
3-Cba(3CBP)	45	31	16	92
3CBP	31	49	21	101
TOTAL	350	302	166	818

3.3.2 Genotype Screening of Catabolic Isolates

All four degradative genotypes used as probes in this study were detected in the Niagara watershed chloroaromatic catabolic isolate collection (Tables 3.4-3.8). An alternative, more extensive labelling method, was employed to differentiate probe positive isolates with the same name that were collected from different carbon sources and enrichment temperatures. The superscript letters a to f were added to the front of the isolate name and represented the carbon sources, 3-Cba, 4-Cba, 3,4-dCba, BP, 3-Cba(3CBP) and 3CBP, respectively. Similarly, superscript numbers 1 to 3 were placed after the isolate name to show the enrichment temperature (4°C, 22°C and 32°C, respectively). None of the genotypes in this study dominated as degradative genotypes. The *chaAB* and *clcABD* genotypes were both found in microbial isolates from the 3-Cba and 3-Cba(3CBP) enrichment cultures. The *chaAB* genotype was also detected in 3CBP and 3,4-dCba catabolic isolates. The *clcABD* and *fcbb* genotypes were detected in 4-Cba catabolic isolates in almost equal proportions. No 4-Cba catabolic isolates harboured the *chaAB* genotype. Finally, the *bphA* genotype was found in putative BP catabolic isolates but not in any putative 3CBP catabolic isolates. The restriction fragment length polymorphism (RFLP) patterns of the catabolic genotypes in these probe positive isolates will be presented in Chapter 4.

It was noted that a significant proportion of the *clcABD* probe positive isolates (40%) were collected from the 4°C enrichment cultures. By contrast, none of the *chaAB* probe positive isolates were collected from enrichment cultures incubated at 4°C. Two of the four *fcbb* probe positive isolates were obtained from 4°C enrichment cultures. Finally, one of the four *bphA* probe positive isolates was obtained from a 4°C enrichment culture.

Table 3.4 Genotype Distribution Within the Chloroaromatic Catabolic Isolate Collection

Carbon Source	# Isolates	# <i>cha</i> AB (%)	# <i>clc</i> ABD (%)	# <i>fcb</i> B (%)	# <i>bph</i> A (%)
3-Cba	127	3 (2.4)	9 (7.1)	-	-
4-Cba	214	-	3 (1.4)	4 (1.9)	-
3,4-dCba	31	2 (6.5)	0 (0)	-	-
BP	253	-	-	-	4 (1.6)
3-Cba(3CBP)	92	3 (3.3)	3 (3.3)	-	0 (0)
3CBP	101	1 (1.0)	0 (0)	-	0 (0)

Table 3.5 A Summary of *c/c*ABD Probe Positive Isolates from the Niagara Watershed

Isolate	Enrichment Carbon Source	Enrichment Temperature	Detailed Isolate Name
pAC27	3-Cba	Positive Control	pAC27
LB400	PCB	Positive Control	LB400
GW4-16	3-Cba	32 °C	^a GW4-16 ³
GW6-1	3-Cba	32 °C	^a GW6-1 ³
GW6-3	3-Cba	32 °C	^a GW6-3 ⁴
GW6-9	3-Cba	32 °C	^a GW6-9 ³
SBR1-2-3	4-Cba	32 °C	^b SBR1-2-3 ³
SBR3-3-3	4-Cba	22 °C	^b SBR3-3-3 ²
WellD-1-3	3-Cba	4 °C	^a WellD-1-3 ¹
WellD-1-3	3-Cba	22 °C	^a WellD-1-3 ²
WellD-3-3	4-Cba	4 °C	^b WellD-3-3 ¹
BRC3-1-3	3-Cba(3CBP)	4 °C	^c BRC3-1-3 ¹
BRC4-4-3	3-Cba(CBP)	4 °C	^c BRC4-4-3 ¹
BRC3-2-3	3-Cba	22 °C	^a BRC3-2-3 ²
BRC5-1-3	3-Cba	4 °C	^a BRC5-1-3 ¹
BRC4-3-4	3-Cba	22 °C	^a BRC4-3-4 ²
BRC3-3-5	3-Cba(CBP)	4 °C	^c BRC3-3-5 ¹

Superscript letters a to f represent the enrichment carbon sources, 3-Cba, 4-Cba, 3,4-dCba, BP, 3-Cba(3CBP) and 3CBP, respectively. Superscript numbers 1 to 3 represent the enrichment temperatures 4°C, 22°C and 32°C respectively.

Table 3.6 A Summary of *chaAB* Probe Positive Isolates from the Niagara Watershed

Isolate	Enrichment Carbon Source	Enrichment Temperature	Detailed Isolate Name
BR60	3-Cba	Positive Control	BR60
MR1-8-1	3-Cba	32 °C	'MR1-8-1'
DHC1-2-2	3CBP	32 °C	'DHC1-2-1'
DHC2-17-2	3,4-dCba	32 °C	'DHC2-17-2'
BRC3-8-3	3-Cba(3CBP)	22 °C	'BRC3-8-3'
WellD-1-3	3-Cba	32 °C	'WellD-1-3'
BRC2-7-4	3-Cba(CBP)	22 °C	'BRC2-7-4'
BRC3-2-5	3,4-dCba	32 °C	'BRC3-2-5'
BRC3-2-5	3-Cba(CBP)	32 °C	'BRC3-2-5'
BRC5-2-5	3-Cba	32 °C	'BRC5-2-5'

Superscript letters a to f represent the enrichment carbon sources, 3-Cba, 4-Cba, 3,4-dCba, BP, 3-Cba(3CBP) and 3CBP, respectively. Superscript numbers 1 to 3 represent the enrichment temperatures 4 °C, 22 °C and 32 °C respectively.

Table 3.7 A Summary of *fcbB* Probe Positive Isolates from the Niagara Watershed

Isolate	Enrichment Carbon Source	Enrichment Temperature	Detailed Isolate Name
CBS3	4-Cba	Positive Control	CBS3
MR2-1-1	4-Cba	4 C	^b MR2-1-1 ¹
BRC3-4-1	4-Cba	4 C	^b BRC3-4-1 ¹
BRC3-2-3	4-Cba	22 C	^b BRC3-2-3 ²
BRC5-1-4	4-Cba	22 C	^b BRC5-1-4 ²

Superscript letters a to f represent the enrichment carbon sources, 3-Cba, 4-Cba, 3,4-dCba, BP, 3-Cba(3CBP) and 3CBP, respectively. Superscript numbers 1 to 3 represent the enrichment temperatures 4 C, 22 C and 32 C respectively.

Table 3.8 A Summary of *bphA* Probe Positive Isolates from the Niagara Watershed

Isolate	Enrichment Carbon Source	Enrichment Temperature	Detailed Isolate Name
LB400	PCB	Positive Control	LB400
DHC2-1-2	BP	22 °C	^d DHC2-1-2 ^{a2}
DHC5-11-2	BP	22 °C	^d DHC5-11-2 ^{a2}
BRC4-1-3	BP	4 °C	^d BRC4-1-3 ^{a1}
BRC6-3-4	BP	32 °C	^d BRC6-3-4 ^{a3}

Superscript letters a to f represent the enrichment carbon sources, 3-Cba, 4-Cba, 3,4-dCba, BP, 3-Cba(3CBP) and 3CBP, respectively. Superscript numbers 1 to 3 represent the enrichment temperatures 4 °C, 22 °C and 32 °C respectively.

3.4 DISCUSSION

A collection of 464 chlorobenzoate catabolic microorganisms was obtained from Niagara watershed batch culture enrichments (Table 3.4). The members of this collection were representative of the chlorobenzoate degradation potential results presented in Chapter 2. The chlorobenzoate isomer with the greatest degradation potential was 4-Cba. It was also the isomer with the greatest number of catabolic isolates recovered (214). As well, the majority of catabolic isolates were collected from the 32 °C and 22 °C enrichments that previously showed higher frequencies of complete degradation compared to the 4 °C enrichment cultures. BP and 3CBP putative catabolic isolates (354) were also collected from Niagara watershed batch culture enrichments. The preponderance of BP catabolic isolates (253) collected reflects the ubiquitous nature of BP degradation in natural environments (Higson 1992).

Not all microorganisms suspected of chlorobenzoate degradation due to their apparent good growth on selection plates were found to actually mineralize the chlorobenzoate isomer when the medium was subjected to HPLC analysis. This was also evident from the ample growth observed on the negative control selection plates. The presence of false positive colonies, unable to utilize the carbon source they were selected on, has been demonstrated previously. For example it was shown that many environmental bacteria, which were not hydrocarbon degraders, could form colonies on mineral agar plates in the presence of hydrocarbons. Of the colonies growing on minimal medium with benzene, toluene, and xylene as the sole carbon sources 98% were later shown not to degrade any of these three compounds (Randall and Hemmingsen 1994). Agar contains organic impurities and it is likely that many of the bacteria that formed colonies on the control and chlorobenzoate plates derived sufficient energy from these impurities to sustain their growth. The microorganisms responsible for the false positive growth are possibly oligotrophic. Oligotrophic bacteria are common in aquatic

environments that are low in essential nutrients and these bacteria are known to be good scavengers (Atlas and Bartha 1987).

Previously, the only characterized catabolic genotype in the Niagara watershed was the *chaAB* genotype. This study revealed that the *clcABD*, *chaAB*, *fcxB* and *hphA* genotypes were all present in this region (Tables 3.4-3.8). However, none dominated as a degradative genotype in the isolate collection. Less than 5% of the isolates hybridized to any one of the 4 genotypes screened (Table 3.4). As well, none of the catabolic isolates collected from the FC enrichment cultures hybridized to any of the gene probes in this study. These results show that different chlorobenzoate catabolic genotypes with the same substrate range can co-exist. The *clcABD*, *chaAB* and *fcxB* genotypes are all capable of 4-Cba mineralization. The non probe positive isolates in the collection harbour either alternative chlorobenzoate catabolic genes that have not yet been genetically characterized or homologous genes with DNA similarities lower than could be detected in this study. The *clcABD* genotype is known to be transmissible by conjugative plasmids and in some cases is capable of transposition. It has been detected worldwide. Therefore it was not surprising that this genotype was present in the Niagara watershed. It was detected in microorganisms from the 3-Cba, 4-Cba and 3-Cba(3CBP) enrichment cultures (Table 3.5). The *clcABD* genotype was detected in microorganisms from BRC and DHC enrichment cultures only over a 6 month period (May - November 1993).

It was recently shown that in oxic-anoxic interfaces such as the top layers of aquatic sediments, where the oxygen concentration is generally low, 3-Cba metabolism proceeded via the gentisate or protocatechuate pathway rather than via the catechol route (determined by O₂-uptake) (Krooneman *et al.* 1996). Chlorocatechols are formed by the initial action of dioxygenases on chlorobenzoates and the availability of oxygen plays a significant role in the early steps of degradation of 3-Cba to 3-chlorocatechol. Oxygen is needed not only as an electron acceptor in the respiratory electron transfer chain but also as a substrate for dioxygenases. Over half of the *clcABD* probe positive isolates were

from the Hyde Park site, collected during 1990 and 1993. By contrast, the SBRs at the Hyde Park bioremediation facility are mechanically aerated. Thus it is likely that the O_2 content is sufficiently high to support the growth of microorganisms harbouring the chlorocatechol 1,2-dioxygenase pathway in this environment. The success of this genotype in groundwater has been noted previously. The *clc* ABD harbouring isolate *Pseudomonas* sp. strain B13 was injected into a sand and gravel aquifer containing a plume of contaminated groundwater that resulted from secondary sewage disposal. This site had no evidence of 3-Cba contamination and prior to inoculation the *clc* ABD genotype was not detected following PCR amplification of environmental DNA from this site. The *clcD* gene was still detectable by PCR amplification 14.5 months after the release of *Pseudomonas* sp. strain B13. 3-Cba enrichment cultures were used to isolate the host bacteria of the *clc* ABD genotype. RFLP analysis revealed that the *clc* ABD genotype had undergone horizontal gene transfer to establish itself in the natural aquifer microbial community (Thiem *et al.* 1994).

Of the other genotypes screened only one *cba* AB probe positive isolate was recovered from the Hyde Park site. Wyndham *et al.* (1994b) found following PCR amplification that the *cbaA* gene was present in about 1 of 10 culturable bacteria in a continuous enrichment culture inoculated with a sample from one of three Hyde Park bioremediation SBRs. The higher proportion of chlorobenzoate catabolic isolates with the *clc* ABD genotype in my collection may be due to preferential selection using the batch culture technique of hosts containing the *clc* ABD genotype over hosts harbouring the *cba* AB genotype.

The known host ranges of the *clc* ABD and *cba* AB genotypes differ. The natural recipients of the *clc* ABD genotype are the Γ -subclass of Proteobacteria (ie. fluorescent pseudomonads) and the *Burkholderia* species from within the β -Proteobacteria. Enrichment techniques have been optimized for "true" pseudomonads which are fast growing, opportunistic microorganisms and include the Γ -Proteobacteria. Conversely, the

host range of the *cbaAB* genotype is generally restricted to the β -Proteobacteria (ie true *Alcaligenes* and *Comamonas* sp.). The β -Proteobacteria do not respond to nutrient enrichment as effectively as the Γ -Proteobacteria and grow slower in batch culture enrichments (Wagner *et al.* 1993, Andrews and Harris 1986)

Although the *clcABD* and *cbaAB* genotypes are encoded on broad host range IncP plasmids (pAC27 and pBRC60, respectively) and can theoretically conjugate into a wide range of hosts, the effective host range of these genotypes is limited due to complex regulatory problems or metabolite inhibition. For example, the protocatechuate *ortho*-ring-fission pathway in most Γ -Proteobacteria is substrate specific and cannot substitute in the metabolism of 5-chloroprotocatechuate, an intermediate in 3-Cba degradation via the *cbaAB* pathway. By contrast the β -Proteobacteria have a non-specific protocatechuate 4,5-dioxygenase that can effectively metabolize the 5-chloroprotocatechuate intermediate through a *meta*-ring-fission pathway. This biochemical constraint limits the natural host range of the *cbaAB* genotype largely to the β -Proteobacteria (Nakatsu *et al.* 1995a).

The *cbaAB* genotype is stable in the Niagara watershed. It was first isolated from BRC in January 1985 (Wyndham and Stauss 1988a) and was detected in this creek in the intervening years (Fulthorpe and Wyndham 1989) until the most recent sampling in November 1993. Although the *cbaAB* genotype was detected in fewer 3-Cba isolates than the *clcABD* genotype, some of the 3,4-dCba catabolic isolates (6.5%) also harboured the *cbaAB* genotype (Table 3.5). There are very few reported cases of 3,4-dCba mineralization by pure cultures. The two well-characterized 3,4-dCba degradative microorganisms both harbour the *cbaAB* genotype (*Alcaligenes* sp. BR60 and *Alcaligenes* sp. CPE3). It is likely that the chlorine-substitution pattern of 3,4-dCba requires distal dioxygenase attack followed by protocatechuate 4,5-dioxygenase *meta*-ring-fission. The only genetically characterized chlorobenzoate catabolic genotype with this type of dioxygenase attack is the *cbaAB* genotype.

It was noteworthy that almost half of the *clcABD* probe positive microorganisms were isolated from the 4°C enrichment cultures (Table 3.5). The isolates from this enrichment temperature represented a small proportion (20%) of the total isolates (Table 3.3). In comparison none of the microbial isolates from this enrichment temperature harboured the *cbaAB* genotype. One possible explanation for this phenomenon may be that there are fewer different bacterial species capable of growth at this enrichment temperature and that a higher proportion of this limited number of species encodes the *clcABD* genotype. This genotype may be harboured in psychrophilic microorganisms and exhibit a superior ability to degrade chlorobenzoates during the fall and winter months compared to the *cbaAB* genotype, which may be restricted to growth in enrichment cultures at 22°C and 32°C. Psychrophilic microorganisms are mainly Gram-negative aerobic non-sporeforming rods. Most psychrophilic strains belong to the genus *Pseudomonas* and to a lesser extent *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Aeromonas*, *Xanthomonas*, *Serratia*, *Escherichia*, *Proteus* (Margesin and Schinner 1994). With the exception of *Alcaligenes* and *Flavobacterium*, these genera are all members of the Γ -Proteobacteria, and are therefore expected to be most compatible with the *clcABD* genotype.

Only 1.9% of the 4-Cba catabolic isolates hybridized with the *fcB* gene probe. Despite this low number, microbial isolates collected over a 15-month sampling period hybridized to this genotype. The dehalogenase pathway has a wide host range that includes Gram-negative and Gram-positive microorganisms. Although it was reported to be chromosomally encoded in the archetypal microorganism *Pseudomonas* sp. CBS3, there have been reports of homologous *fcB* genes being plasmid encoded (Schmitz *et al.* 1992, Zaitsev *et al.* 1991). The presence of the *fcB* genotype in the Niagara watershed may be due to horizontal transfer mediated by plasmids and transposons.

The extensive host range of this dehalogenase pathway may also include psychrophilic microorganisms as 50% of the *fcB* isolates were recovered from 4°C

enrichment cultures. The isolates from the 4-Cba enrichment cultures that hybridized to the *clcABD* gene probe were all from the Hyde Park enrichment cultures. By contrast, all isolates that hybridized to the *fcbb* gene probe were from either BRC or DHC. It is tempting to speculate that these genotypes have different niches. Although the low number of probe positive species in the isolate collection makes this type of speculation difficult to substantiate, the fact that the *fcbb* genotype encodes a hydrolytic process rather than an oxygenolytic one suggests that hosts in oxygen-limited environments, such as creek sediments, may preferentially harbour the *fcbb*, O₂-dependent genotype over the *clcABD* genotype.

The 2,3-biphenyl dioxygenase of *Burkholderia* sp. LB400 can potentially oxidize the non-chlorinated ring of 3CBP. However, none of the putative 3CBP degraders hybridized to the *bphA* genotype. Only 1.6% of the putative BP catabolic isolates hybridized to the *bphA* genotype. The probe positive isolates represented a 9-month sampling period. High stringency hybridizations of biphenyl-selected isolates from four distinct industrially contaminated soil enrichments with the biphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 (*bphA1*) revealed that 12% of the isolates hybridized significantly with the *bphA1* genotype. However, all of the probe positive isolates originated from the same contaminated site. The remaining three sites had no *bphA1* probe positive isolates (Pellizari *et al.* 1996). The diversity of biphenyl dioxygenases was also noted by Asturias and Timmis (1993) who detected the presence of three nonhomologous 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the PCB-degrading strain *Rhodococcus globerulus* P6.

It is not known how many of the "putative degraders" actually were capable of mineralizing BP or 3CBP. In this study many of these isolates may have been utilizing organic impurities in the selection medium as carbon sources. It was surprising that the archetypal isolate for the *bphA* genotype, *Burkholderia* sp. LB400, also hybridized to the *clcABD* gene probe from *Pseudomonas putida* AC866 (pAC27) (Table 3.5). Both of

these microorganisms have been the focus of much research over the last 10 years. However, this is the first report of this homology. These two pathways may not be co-expressed to enable complete PCB metabolism in *Burkholderia* sp. LB400 due to genetic regulatory restrictions. Chlorobenzoate metabolites such as chlorocatechols are known to inhibit PCB degradation to chlorobenzoates (Sondossi *et al.* 1992). Therefore it is possible that PCB and chlorobenzoate degradation do not occur at the same time in *Burkholderia* sp. LB400. Microorganisms capable of metabolizing more than one pollutant via different genetic pathways have been noted. For instance *Pseudomonas* sp. CBS3 in addition to dehalogenating 4-Cba to 4-hydroxybenzoate contains two isofunctional 2-haloalkanoic acid dehalogenases which catalyze the hydrolytic dehalogenation of monochloroacetate and 2-monochloropropionate (Schneider *et al.* 1991) and a two component enzyme system (*dehCI* and *dehCII*) which oxidizes 4-chlorophenylacetate to 3,4-dihydroxyphenylacetate (Klages *et al.* 1981, Markus *et al.* 1984). It was suggested that microorganisms that express two or more independently regulated dehalogenases have growth advantages under fluctuating environmental conditions (Schneider *et al.* 1991).

The results of this investigation prompted further studies on the phenotypic and genetic diversity in the isolates that hybridized to the *clcABD*, *cbaAB*, *fcB* and *bphA* gene probes. These results will be discussed in the following chapter. The limitations associated with using batch culture enrichments alone to assess genotype distribution were addressed by performing total DNA extractions followed by PCR amplification of selected catabolic genotypes. These results are presented in Chapter 5. Finally, as the *clcABD* and *cbaAB* genotypes were both detected in this region, have a similar chlorobenzoate substrate range (3-Cba) and are known to be harboured in different host strains, competition between these two genotypes was studied. These results are reported in Chapter 6.

CHAPTER 4:

Genetic and Phenotypic Characterization of *clcABD*, *cbaAB*, *fcbb* and *bphA* Probe Positive Isolates

4.1 INTRODUCTION

Most information about chloroaromatic catabolic genotypes has come from batch or continuous culture enrichments and isolation of environmental microorganisms from contaminated environments. Extensive genetic and phenotypic information can be gleaned from environmental isolates that are amenable to lab culturing.

There are numerous techniques available to distinguish between clonal and genetically distinct environmental microorganisms. Genetic fingerprinting techniques have proved to be more informative than traditional serological, phenotypic or biochemical techniques for the characterization of microorganisms. Not only are genetic fingerprinting methods faster but they also effectively differentiate closely related bacterial strains, which can be problematic using traditional techniques. The most common genetic fingerprinting technique is random amplified polymorphic DNA (RAPD). This technique uses short, arbitrarily chosen oligonucleotide primers (approximately 10-mers) to amplify random regions of the bacterial genome. A strain-specific DNA fragment pattern is generated that is characteristic for each strain of bacterial species (Hadrys *et al.* 1992). Other methods have been developed to identify clonal microorganisms including differential amplification of rRNA genes by PCR (Reysenbach *et al.* 1992) and amplification of spacer regions between the 16S and 23S genes in prokaryotic rRNA genetic loci (Jensen *et al.* 1993). Dispersed repetitive DNA sequences have been described recently in eubacteria. ERIC (enterobacterial repetitive intergenic consensus), REP (repetitive extragenic palindromic) and BOX sequences are 35-155 bp long and appear to be restricted to transcribed regions of the genome, within intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. They are highly conserved at the nucleotide sequence level but their chromosomal locations differ between species

(Hulton *et al.* 1991). Multiple functions have been proposed for these highly conserved, dispersed ERIC, REP and BOX elements including roles in transcription termination, mRNA stability, and chromosomal domain organization *in vivo* (de Bruijn 1992). Primers for these DNA sequences have been generated to yield species-specific banding patterns (Versalovic *et al.* 1991).

As mentioned in Chapter 3, converging metabolic pathways combined with possible regulatory conflicts of newly acquired catabolic genes and host genes may limit the functional host-range of chloroaromatic catabolic genotypes (Nakatsu *et al.* 1995b). Reports of the host range of the *cbaAB* genotype shows it to be primarily limited to species of the β -Proteobacteria (44/48 isolates) although it has been detected occasionally in members of the Γ -Proteobacteria (4/48) (Nakatsu *et al.* 1995a). In a study of selected Γ -Proteobacteria hosts carrying the *cbaAB* genotype, 3-Cba was degraded at micromolar concentrations but 4-chlorophenol and chlorocatechol intermediates were produced when these strains were exposed to millimolar concentrations of 3-Cba (Gray 1995). Factors that may limit the natural host range of the *cbaAB* genotype include competition for the chlorobenzoate substrate by other aromatic ring-oxidizing enzymes in certain host strains, for example the benzoate (toluate) 1,2-dioxygenases found in fluorescent *Pseudomonads* (Γ -Proteobacteria). These enzymes are able to oxidize 3-Cba to chlorocatechol metabolites that accumulate in the absence of specialized chlorocatechol 1,2-dioxygenases. The toxicity of these metabolites may outweigh the selective advantages the organism has in expressing the *cbaAB* dioxygenase (Nakatsu *et al.* 1995a).

The host range of the *clcABD* genotype appears to be limited to the Γ -Proteobacteria and several genera in the *Burkholderiaceae* of the β -Proteobacteria. Members of these subclasses are characterized by convergent catabolic pathways for benzoate and *para*-hydroxybenzoate leading, via *ortho*-ring-fission, to the common intermediate β -ketoadipate (Stanier and Ornston 1973). It has been postulated that this is the pathway that the chlorocatechol modified *ortho*-ring-fission pathway evolved from. This was elucidated from the homology observed between evolved and ancestral genes. Two of the three enzymes involved in chlorocatechol degradation in *Pseudomonas putida* (chlorocatechol 1,2-dioxygenase and chloromuconate cycloisomerase) share

approximately 40% amino acid sequence homology with the corresponding enzymes involved in benzoate degradation (catechol 1,2-dioxygenase and muconate cycloisomerase) in the microorganism *Acinetobacter calcoaceticus* (Aldrich *et al.* 1987, Rothmel *et al.* 1989 and Neidle *et al.* 1988).

The known host range of the *fcB* genotype extends to both Gram-negative and Gram-positive microorganisms. In anaerobic systems the degradation of aromatic acids via the formation of CoA-esters is well characterized. It has been suggested that the dehalogenase from *Pseudomonas* sp. CBS3 was derived from these anaerobic enzymes. The fact that the 4-chlorobenzoate dehalogenase pathway proceeds without the involvement of molecular oxygen supports this hypothesis (Löffler *et al.* 1991).

The host range of the *bphA* genotype is phylogenetically extensive including members of the Γ - and β -Proteobacteria. The *bph* operon from *Comamonas testosteroni* B356 hybridized with the *bph* operons of *Burkholderia* sp. LB400, *Alcaligenes* sp. A5 and *Alcaligenes eutrophus* H850 (Ahmad *et al.* 1990).

The *clcABD* genotypes have been found to be highly conserved despite their worldwide distribution. Independently isolated plasmids pAC25, pWR1 and pB13 all harbour the *clcABD* genotype. Not only are the catabolic genes highly homologous but with the exception of a 6 kbp deletion, pB13 and pWR1 had the same restriction endonuclease digestion pattern as pAC25 (Chatterjee and Chakrabarty 1983). pB13 and pWR1 were determined to be homologous plasmids.

Plasmids influence the physiological functions of bacterial communities and plasmid transfer is considered to be one of the driving forces for bacterial genetic adaptation. As mentioned in Chapter 3, all of the genotypes screened in this study have been detected in some instances on high molecular weight conjugative plasmids. Inter-species DNA transfer *in situ* has been shown to occur with the 2,4-dichlorophenoxyacetic acid (2,4-D) degradative plasmid pKA2 (Ka and Tiedje 1994) and with the 3-Cba and 3,4-dCba degradative plasmid pBRC60, harbouring the *cbaAB* genes (Wyndham *et al.* 1994b). Another study revealed that *Pseudomonas putida* PaW1, a mesophilic microbe harbouring the degradative TOL plasmid, pWW0, was able to transfer pWW0 by

conjugation to a psychrotrophic bacterium *Pseudomonas putida* Q5 without a reduction in the physiological activity of the transconjugant (Kolenc *et al.* 1988).

There is no doubt that transposons are also involved in the rapid adaptation of bacterial communities. The *bphA*, *cbaAB* and *clcABD* genotypes have all been found to be transposon encoded in some isolates. The transposase gene (*tnpA*) of Tn21 was detected in 3 of 40 isolates from the Mediterranean sea and in an estimated 1 of 1,000 to 10,000 bacteria from total DNA extractions of marine samples near the Swedish coast. Tn21 mediates resistance to mercury and carries antibiotic resistance genes (*sul* *aadA*). The authors suggest that this element may be more closely associated with the culturable fraction of marine bacteria or that it may be more common in the geographical area where the isolates were collected (Dahlberg and Hermansson 1995).

In a second study the incidence of Tn5 was characterized in 730 clinical and sewage bacterial isolates in Spain. Blázquez *et al.* (1996) found that 25 of these isolates harboured Tn5. In all cases this genetic element was encoded within Inc11 plasmids. The authors concluded that Tn5 dissemination may be dependent on the conjugative ability of these Inc11 plasmids.

Tn5271 is a 17 kbp transposon that is flanked by a directly repeated sequence of 3.2 kbp, which in turn is flanked by 110-bp inverted repeats. The 3.2 kbp repeated sequence, designated IS1071, is involved in recombination of the catabolic genes into the chromosome of this strain. Sequence analysis revealed that the inverted repeat of IS1071 and the derived amino acid sequence of the single open reading frame within IS1071 are related to the inverted repeats and transposase (TnpA) proteins of the class II (Tn3 family) transposable elements. However, the absence of a resolvase gene within IS1071 suggested that this element is capable of determining the first step in class II transposition (cointegrate formation) only (Nakatsu *et al.* 1991). The transposase gene *tnpA*₁₀₇₁ is 2910 bp in length and occupies all but 69 bp of the DNA within the inverted repeats of IS1071. The distribution of *tnpA*₁₀₇₁ is not limited to *Alcaligenes* sp. BR60 (pBRC60). It was detected in a microbial isolate from a chloroaniline dosed microcosm (Fulthorpe and Wyndham 1992). As well, homologous IS1071 elements have been found associated with other contaminant catabolic genes isolated worldwide including *Moraxella* sp. strain B

(pUO1) (Japan) encoding a chloroacetate dehalogenase gene, *dehH2* (two copies of IS1071 on pUO1) (Kawasaki 1994), *Comamonas testosteroni* strain T2 (Switzerland) encoding a 4-sulfobenzoate 3,4-dioxygenase (two plasmid-encoded copies of IS1071) and *Pseudomonas putida* strain PP3 (United Kingdom) involved in chloropropionate and dalapon degradation (one chromosomal copy of IS1071) (Cordeiro 1995).

The purpose of this chapter was to further characterize the *clcABD*, *cbaAB*, *fcbb* and *bphA* probe positive isolates detected in the batch culture enrichments. These investigations included:

1. Identification of unique or clonal isolates using ERIC PCR
2. Elucidation of the chlorobenzoate substrate range of all isolates.
3. Determination of the phenotypic relationship of hosts based on carbon source utilization.
4. Genetic analysis including screening for high molecular weight plasmids, intra-gene (restriction fragment length polymorphism) RFLP mapping of target genotypes and detection of IS1071.
5. Detailed RFLP analysis of *cbaAB* probe positive isolates using multiple Tn5271 probes

4.2 MATERIALS AND METHODS

4.2.1 Elimination of identical microorganisms using ERIC PCR

Genomic fingerprints were generated from 50 ng purified genomic DNA of each probe positive isolate following the ERIC-PCR conditions outlined in Louws *et al.* 1994. Briefly, total genomic DNA was isolated from all probe positive isolates as well as the archetypal microorganisms using a 5 ml scaled-up version from Ausubel *et al.* 1989. The oligonucleotide primers were synthesized and deprotected as described in Chapter 3. The ERIC primers were: ERIC1R 3'-CAC TTA GGG GTC CTC GAA TGT A -5' and ERIC2 3'-GCG AGT GGG GTC AGT GAA TGA A -5'. The PCR reactions were carried out as described by Versalovic *et al.* 1991. A 25 µl reaction contained 5 µl Gitschier Buffer (83 mM (NH₄)₂SO₄, 335 mM Tris HCl, pH 8.8, 33.5 mM MgCl₂, 33.5 mM EDTA, 150 mM β-mercaptoethanol), 0.2 µl BSA (20 mg/ml), 2.5 µl DMSO, 1.25 µl 25 mM dNTP-Mix (1:1:1:1), 1.25 µl each primer (stock 0.3 mg / ml), 0.4 µl Vent polymerase and 50 ng template DNA. The PCR amplification was carried out at 95°C for 7 min, followed by 35 cycles at 94°C for 1 min, 52°C for 1 min and 65°C for 8 min. This was followed by an incubation at 68°C for 16 min and an indefinite 4°C soak program. After amplification, 10 µl of the ERIC PCR products were separated on 1.5% agarose gels (0.5 x TAE) at 105V for 8 hours, stained in ethidium bromide and photographed. All isolates with different ERIC-PCR fingerprinting patterns were studied further. Genomic DNAs from all isolates were amplified more than once to ensure a reproducible banding pattern.

4.2.2 Carbon source substrate range

As the *fcbB* and *bphA* genotypes have been previously detected in both Gram-negative and Gram-positive microorganisms, all *bphA* and *fcbB* probe positive isolates in this study were screened using a Gram stain kit (Difco). The carbon substrate utilization

range of each unique probe positive and archetypal microorganism was determined in a 96-well BIOLOG GN (gram negative) plate (BIOLOG Inc. Hayward California) following the suggestions by Kidd-Haack *et al.* (1995) for the analysis of environmental isolates. As seen in Appendix IV, the carbon sources in the BIOLOG GN plates are primarily sugars and organic acids. The BIOLOG assay colorimetrically measures a tetrazolium dye reduction that is coupled to substrate oxidation. All probe positive isolates were resuspended in 20 ml 50 mM phosphate buffer (pH 7) to a turbidity that matched the BIOLOG low turbidity standard. After 1 h, 150 µl of this suspension was inoculated into each well of the BIOLOG GN microtiter plates. These plates were incubated at the original temperature of isolation for 24 to 48 h. The cluster analysis program from the IBM compatible SSPC for MS Windows (version 6.0) was used to show the phenotypic relationship between the probe positive and archetypal isolates and *Comamonas testosteroni*, *Serratia ficaria*, *Burkholderia pickettii*, and *Pseudomonas tolaasii* (closely related to *Pseudomonas syringae*) based on carbon utilization range.

The ability of each probe positive isolate and archetypal microorganism to mineralize 3-Cba, 4-Cba and 3,4-dCba was also elucidated. One isolated colony was transferred from a selection plate to an eppendorf tube containing 1 ml TYE. These tubes were incubated overnight at the original temperature of isolation. The following morning all cells were spun down, washed and resuspended in 100 µl medium A. 25 µl of this cell suspension was added to 975 µl 1.5 mM broth of 3-Cba, 4-Cba and 3,4-dCba and incubated at the original temperature of isolation for the same length of time as described in Chapter 2. Chlorobenzoate disappearance was measured by HPLC as previously described.

Fluorescence on King's B medium (King *et al.* 1954) was tested as a quick means of determining if the isolate was a member of the fluorescent pseudomonads. *Pseudomonas putida* AC866 (pAC27) was used as a positive control for fluorescent pigment production.

4.2.3 Screening for the detection of high molecular weight plasmids

A modified protocol of plasmid alkaline extraction was performed to screen microorganisms for high molecular weight plasmids (Birnboim and Doly 1979). Bacterial culture from the selection medium (250 μ l) was pipetted into 50 ml of TYE medium. The cells were shaken at the original temperature of isolation and were harvested at mid logarithmic phase (A_{260} optical density between 0.6 and 0.7). Solution I (1.5 ml), solution II (3.0) and solution III (1.87 ml) were employed. The remaining protocol was performed in the stated manner (Birnboim and Doly 1979). An alternative high molecular weight plasmid extraction method, based on lysis at a high pH (12.4) was also performed on selected isolates (Crosa and Falkow 1981). Plasmid DNA was separated by electrophoresis on 0.65% agarose gels at 80V. High molecular weight plasmid isolations were also performed with a positive control such as pBRC60 from *Alcaligenes* BR60.

4.2.4 RFLP analysis of amplified target genes

Portions of the target genes were amplified and subsequently restriction endonuclease digested to detect intra-gene differences in targeted genotypes. The primers for the *clcA* (chlorocatechol-1,2-dioxygenase) gene amplified a 362 bp region (C. Greer, pers. comm.). The nucleotide sequences of the primers were: *clcA*-666 3'- CTC AAG ACC TAC GAT ACC GAC GAC CAC AAA CC -5' and *clcA*-996 3' CCT TCG AAG TAG TAT TGC GTG GTC AAC GGT TC -5'. Due to the length of these primers, they were further purified on an OPC column following the manufacturer's instructions (Applied Biosystems) after deprotection (previously described). The *cbaA* primers amplified a 1058 bp fragment of the *cbaA* (chlorobenzoate-3,4 (4,5)- terminal dioxygenase) gene. The 17-mer sequences of these primers were determined using the Oligo Program version 4.0 (National Biosciences) from the known sequence (Nakatsu *et al* 1995b). The primer sequences were: *cbaA*-4815 3'- TCG GCG TGA TGG ACA GC -5' and *cbaA*-5856 3'- CGG CGT GAG GTA ATA GC -5'. No further purification of

these primers was required after deprotection. A 100 μ l PCR reaction mixture contained 10 μ l of 10X Vent buffer supplied with Vent polymerase (New England Biolabs), 1.2 mM dNTPs, 0.5 mM of each primer, 10 ng template DNA, and 1U of Vent DNA polymerase (New England Biolabs) and was over-layed with 100 μ l mineral oil. The template DNA was denatured for 5 minutes at 95°C. Vent polymerase (New England Biolabs) was added to each reaction tube after 4 minutes. Amplification was carried out using a Perkin Elmer Cetus 480 Thermocycler for 29 cycles with denaturation for 1 minute at 94°C, annealing for 1 minute at 59°C (*clcA*) or 52°C (*cbaA*) and elongation for 2 minutes at 72°C. The final cycle was followed by 10 minutes at 72°C and an indefinite 4°C soak program. The annealing temperature was lowered to 55°C for *clcABD* probe positive isolates that did not amplify at 59°C. Preparation and amplification of the *bphA* gene was performed as described in Chapter 3.

PCR product size was confirmed by electrophoresis of a 10 μ l sample from each reaction on 1.5% agarose gels. If the amplification product was the correct size, the products were further purified by chloroform extraction (equal volume) of the mineral oil. The DNA was ethanol precipitated and resuspended in 50 μ l TE (pH 8.0).

DNA digestions were performed using restriction endonucleases that cut within the amplified regions of the catabolic genes. The DNA sequences of all amplified regions were known. The *clcA* PCR products were digested with *Bam*HI. Analysis of the *clcA* genotype sequence in *Pseudomonas putida* AC866 (pAC27) revealed that digestion with this restriction endonuclease resulted in two fragments; 146 and 216 bp. The *cbaA* amplification products were digested with the restriction endonuclease *Eco*RI. Two fragments, 142 bp and 900 bp were known to result from the digestion of the *cbaA* gene in *Alcaligenes* sp. BR60 (pBRC60). Finally, the *bphA* PCR products were digested with *Sal*I. This resulted in two fragments in the archetypal gene, a 500 bp and a 305 bp fragment. At the time of this investigation the DNA sequence for the *fcbB* gene was not available for primer design. All DNA digests were run on 2% agarose gels.

4.2.5 RFLP analysis of *cbaAB* probe positive isolates using multiple Tn5271 probes

Total genomic DNA from all *cbaAB* probe positive isolates was digested with the restriction endonuclease *NheI* and hybridized with the E5 fragment of Tn5271. Differences were observed in the size of probe positive *NheI* fragments. These differences were divided into two groups, those resembling the 10.6 kbp *NheI* fragment of *Alcaligenes* sp. BR60 (within IS1071) and the remainder with a smaller fragment similar to *Alcaligenes* sp. OCC43. In order to better recognize these differences, genomic DNA from *Alcaligenes* sp. BR60 and OCC43 was digested with the restriction endonucleases *SalI* and *EcoRI* and hybridized with the probes E11, E13/14, E5 and H2 which represent different regions of Tn5271. Probes outside of Tn5271 were also hybridized with BR60 and OCC43 DNA to see if the plasmid backbones of pBRC60 and pOCC43 were similar. The E4 and E7/8 probes are 8.5 and 9.9 kbp in size, respectively (Nakatsu 1992). Southern blot hybridizations and probe labellings were performed as described in Chapter 3.

4.2.6 Detection of IS1071

Total genomic DNA from all probe positive isolates in this collection, archetypal isolates, other *bphA* probe positive isolates including the biphenyl catabolic isolates *Alcaligenes eutrophus* H850 (from D. Bedard) and *Comamonas testosteroni* B356 (from M. Sylvestre) and a collection of *cbaAB* harbouring isolates from SBR2 that have been previously described (Wyndham *et al.* 1994b) were digested with the restriction endonuclease *NheI*. This enzyme cuts 48 bp inside the ends of IS1071 within the inverted repeat. In *Alcaligenes* BR60 (pBRC60) this digest reduces all 7 copies of the IS1071 element to a single 3.1 kbp fragment that spans most of IS1071 and encompasses the *mpA₁₀₇₁* gene (Nakatsu *et al.* 1993). These digestions were Southern blotted and

hybridized at 68°C with a labelled probe, 114, from pBR114 (Nakatsu *et al.* 1991). This probe covers a 1.2 kbp region of IS1071 within the *mpA*₁₀₇₁ gene.

4.3 RESULTS

4.3.1 Elimination of sibling microorganisms by ERIC PCR

Figure 4.1 shows the ERIC PCR result for all *cbaAB* probe positive microorganisms. All isolates were re-amplified to confirm the RFLP banding pattern. Those isolates with ambiguous high or low molecular weight bands (as in lanes 4 and 10) were re-amplified. All *cbaAB*, *fcB* and *bphA* probe positive isolates were non clonal or phylogenetically distinct. Some *clcABD* probe positive isolates were found to be siblings. Each of the following pairs of isolates: ^a BRC5-1-3 ¹ and ^c BRC3-1-3 ¹; ^a BRC3-2-3 ² and ^d BRC4-4-3 ¹; groundwater isolates ^a GW4-16 ³ and ^a GW6-3 ³; ^b SBR1-2-3 ³ and ^b SBR3-3-3 ² had identical banding patterns. Only unique isolates were studied further.

4.3.2 Carbon source substrate range

Cluster analysis of the probe positive isolate collection based on the ability to utilize 95 carbon sources in BIOLOG GN microtiter plates is presented in Figure 4.2. The expected BIOLOG results of phylogenetically diverse common environmental microorganisms were included in this dendrogram as reference strains. Five major phenotypic groups labelled 1-5 were identified. The *cbaAB* harbouring isolates were predominately in group 1, the largest group as well as groups II and III. The *clcABD* probe positive isolates were distributed in all five groups. The *bphA* harbouring isolates were members of groups 1,2 and 5. Finally, hosts of the *fcB* genotype were limited to groups 2 and 4. Generally, microorganisms in groups I and II utilized only sugars in the BIOLOG plates. Conversely, microorganisms in groups III, IV and V mineralized both sugar and organic acid carbon sources.

Figure 4.1 ERIC PCR Results for *cbaAB* Probe Positive Isolates.

Lanes: 1: 1-kbp ladder, 2: *HindIII*-digested lambda DNA, 3: *Alcaligenes* sp. BR60 (pBRC60), 4: ^a MR1-2-1 ³, 5: negative control (no template DNA) 6: ^f DHC1-2-2 ¹, 7: ^c DHC2-17-2 ³, 8: ^c BRC3-8-3 ², 9: ^a WellD-1-3 ³, 10: ^c BRC2-7-4 ², 11: ^c BRC3-2-5 ², 12: ^c BRC5-2-5 ³, 13: ^a BRC5-2-5 ³.

ERIC PCR products were run on a 1.5 % agarose electrophoresis gel (0.5% TAE) at 105V for 8 hours.

All probe positive isolates were amplified more than once to confirm the RFLP banding patterns.

^a: 3-Cba, ^c: 3,4-dCba, ^e: 3-Cba(3CBP), ^f: 3CBP

¹: 4°C, ²: 22°C, ³: 32°C

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 4.2 Phenotypic Cluster Analysis of All Probe Positive Isolates and Common Environmental Isolates Based on Carbon Substrate Utilization.

This dendrogram was generated from 96-well BIOLOG GN microtiter plates using average linkage (between groups) (SPSS for MS Windows release 6.0). The carbon sources in BIOLOG GN plates are presented in Appendix IV.

Five distinct phenotypic groups for the probe positive isolates are shown. The phenotypic relationship of archetypal microorganisms: *Alcaligenes* sp. BR60, *Pseudomonas putida* AC866, *Pseudomonas* sp. CBS3, *Burkholderia* sp. LB400, other probe positive isolates from various locations: *Alcaligenes* sp. OCC43 (*cba*⁺ from SBR2 in Hyde Park), *Alcaligenes* sp. CPE3 (*cba*⁺ from Italy), *Comamonas testosteroni* B356 (*bphA*⁺ from Quebec), *Alcaligenes eutrophus* H850 (*bphA*⁺ from New York), and widespread, phylogenetically diverse environmental strains: *Comamonas testosteroni* (β -Proteobacterium), *Burkholderia pickettii* (β -Proteobacterium), *Pseudomonas tolaasii* (γ -Proteobacterium) and *Serratia ficaria* (α -Proteobacterium) have been included.

^a: 3-Cba, ^b: 4-Cba, ^c: 3,4-dCba, ^d: BP, ^e: 3-Cba(3CBP), ^f: 3CBP

¹: 4°C, ²: 22°C, ³: 32°C

Phenotypic Distance

Tables 4.1-4.4 show the chlorobenzoate mineralization range of all unique probe positive isolates. The only isolates that were fluorescent on King's B medium were 3,4-dCba BRC3-2-5 32°C (*cbaAB*+) and the positive control *Pseudomonas putida* AC866 (pAC27). The *clcABD* harbouring isolates had three chlorobenzoate substrate ranges. They degraded 3-Cba, 4-Cba or 3-and 4-Cba. None mineralized 3,4-dCba. The *cbaAB* probe positive isolates showed four chlorobenzoate substrate ranges. Some isolates harbouring this genotype mineralized all three chlorobenzoate isomers. The chlorobenzoate substrate range of the *fcB*B probe positive isolate was limited to 4-Cba. *BphA* containing isolates degraded 3- and 4-Cba. Some of the *bphA* containing isolates converted 3-Cba to a dead-end metabolite. This compound was yellow in colour and eluted after 1.8 min with the HPLC conditions used in this study. A peak at the same time was noted for some *cbaAB* probe positive isolates that were subjected to HPLC analysis prior to complete 3-Cba metabolism.

4.3.3 High molecular weight plasmid screening

All microorganisms were screened for the presence of high molecular weight plasmids. The isolates that did not show the presence of high molecular weight plasmids cannot conclusively be said not to have a plasmid. These results are shown in Tables 4.1-4.4.

Table 4.1 A Summary of RFLP Patterns, Chlorobenzoate Substrate Range, Presence of High Molecular Weight Plasmids and *IS1071* in *clcABD* Probe Positive Microorganisms

Isolate	<i>Nde</i> I Probe Positive Fragments (kbp)	3-Cba	4-Cba	3,4-dCba	High MW Plasmid(s)	<i>IS1071</i>
pAC27	19.5, 9.0, 1.6	+	+	-	+	+
LB400	7.1	+	+	-		+
^a GW4-16 ³	13.3, 10.3, 5.7	+	-	-		+
^a GW6-1 ³	13.3, 10.3, 5.7	+	+	-		-
^a GW6-9 ³	13.3, 10.3, 5.7	+	+	-	+	-
^b SBR3-3-3 ²	10.3, 5.7	-	+	-		-
^a WellD-1-3 ¹	10.3, 5.7	+	-	-		-
^a WellD-1-3 ²	9.5, 5.7	+	-	-	+	-
^b WellD-3-3 ¹	7.1	-	+	-	+	+
^c BRC4-4-3 ¹	13.3, 5.7	+	-	-	+	+
^a BRC5-1-3 ¹	10.3, 5.7	+	-	-	+	-
^a BRC4-3-4 ²	7.4	+	+	-		-
^c BRC3-3-5 ¹	10.3, 5.7	+	-	-	+	-

^a: 3-Cba, ^b: 4-Cba, ^c: 3-Cba(3CBP)

¹: 4°C, ²: 22°C, ³: 32°C

Table 4.2 A Summary of RFLP Patterns, Chlorobenzoate Substrate Range, Presence of High Molecular Weight Plasmids and IS1071 in *cbaAB* Probe Positive Microorganisms

Isolate	<i>EcoRI</i> Probe Positive Fragments (kbp)	3- Cba	4- Cba	3,4-dCba	High MW Plasmid(s)	IS1071	Tn5271 map
BR60	1.1, 1.4	+	-	+	+	+	BR60-like
OCC43	1.1, 1.4	+	+	+	+	+	OCC43-like
^a MR1-8-1 ³	1.1, 1.4	+	+	+	+	+	OCC43-like
^f DHC1-2-2 ³	1.1, 1.4	+	-	+	+	+	BR60-like
^e DHC2-17-2 ³	1.1, 1.4	-	-	+		+	OCC43-like
^e BRC3-8-3 ²	1.1, 1.4	+	-	+		+	BR60-like
^a WellD-1-3 ³	1.1, 1.4	+	-	+	+	+	nd
^e BRC2-7-4 ²	1.1, 1.4	+	-	-		+	OCC43-like
^e BRC3-2-5 ³	1.1, 1.4	+	-	+		+	BR60-like
^e BRC3-2-5 ²	1.1, 1.4	+	+	+		+	BR60-like
^e BRC5-2-5 ³	1.1, 1.4	+	+	+	+	+	BR60-like

^a: 3-Cba, ^e: 3,4-dCba, ^f: 3-Cba(3CBP), ¹: 3CBP

¹: 4°C, ²: 22°C, ³: 32°C

nd: not determined

Table 4.3 A Summary of RFLP Patterns, Chlorobenzoate Substrate Range, Presence of High Molecular Weight Plasmids and *IS1071* in *fcbB* Probe Positive Microorganisms

Isolate	<i>Eco</i> RI Probe Positive Fragments (kbp)	3-Cba	4-Cba	3,4-dCba	High MW Plasmid(s)	<i>IS1071</i>
CBS3	2.0, 1.6	-	+	-		-
^a MR2-1-1 ¹	4.9	-	+	-	+	+
^a BRC3-4-1 ¹	11.2	-	+	-	+	-
^a BRC3-2-3 ¹	3.4	-	+	-		+ *
^a BRC5-1-4 ²	3.1	-	+	-		+

* smaller fragment, only 2.9 kbp

^a 4-Cba

¹ 4°C, ² 22°C, ³ 32°C

Table 4.4 A Summary of RFLP Patterns, Chlorobenzoate Substrate Range, Presence of High Molecular Weight Plasmids and *IS1071* in *bphA* Probe Positive Microorganisms

Isolate	<i>Sa</i> I Probe Positive Fragments (kbp)	3-Cba	4-Cba	3,4-dCba	High MW Plasmid(s)	<i>IS1071</i>
LB400	1 1, 2 9	+	+	-		+
H850	nd	+ *	-	-		+
^d DHC2-1-2 ²	1 1, 2 9	+ *	-	-		-
^d DHC5-11-2 ²	1 1, 2 9	+ *	+	-	+	-
^d BRC4-1-3 ¹	4 0	+ *	-	-	+	-
^d BRC6-3-4 ³	1 1, 2 9	+	+	-	+	+
B356	3 7	nd	nd	nd		-

* 3-chlorobenzoate was converted to a new peak with the same area under the curve. This is suspected to be a dead-end metabolite. It eluted at 1.8 min and was yellow.

nd; not determined.

^d: BP

¹: 4°C, ²: 22°C, ³: 32°C

4.3.4 RFLP patterns of target genes

The Southern blot results of all probe positive isolates digested with restriction endonucleases that had restriction sites within the catabolic genotypes, as described in Chapter 3, are summarized in Figure 4.3 (A) and Tables 4.1-4.4. The RFLP patterns of the *clcABD* harbouring isolates following *Nde*I digestion and hybridization with the *clcABD* gene probe were all different than the RFLP pattern for the archetypal microorganism *Pseudomonas putida* AC866 (pAC27). Most of these isolates (9/11) contained a 5.7 kbp band. This group also has fainter, larger bands that were 13.3, 10.3 and 9.5 kbp in size. One isolate, ^b WellD-3-3 ¹ had the same sized fragment as *Burkholderia* sp. LB400 (7.1 kbp). The isolate ^a BRC4-3-4 ² had a unique 7.4 kbp fragment. All *cbaAB* containing isolates had the same RFLP pattern as *Alcaligenes* sp. BR60 (pBRC60) following *Eco*RI digestion and hybridization with the *cbaAB* gene probe. The RFLP maps of the *fcB* probe positive isolates were all unique from each other and from *Pseudomonas* sp. CBS3. Finally, three of the four *bphA* containing isolates had the same RFLP pattern as *Burkholderia* sp. LB400 following *Sal*I digestion and hybridization with the *bphA* gene probe. The fourth isolate, ^d BRC4-1-3 ¹, had a unique RFLP pattern for this gene.

Intra-genotype variations following PCR amplification and electrophoresis of PCR products were not observed. All restriction endonuclease digests of *clcA*, *cbaA* and *bphA* PCR products resulted in fragments that were the same size as the archetypal genotype fragments. The *clcA* probe positive isolates ^b Well D-3-3 ¹, ^b SBR3-3-3 ², ^a BRC5-1-3 ¹, ^a BRC4-4-3 ², ^a BRC4-3-4 ², and ^a Well D-1-3 ² did not amplify at an annealing temperature of 59°C. When the annealing temperature was lowered to 55°C, all of these isolates had a distinct amplification product that was the expected size. Following digestion with the restriction endonuclease *Bam*HI the resulting fragments were the anticipated size.

Figure 4.3 RFLP Southern Blot Results for *clcABD*, *cbaAB*, *fcB*B and *bphA* Probe Positive Genes.

- A.** Southern blot of total genomic DNA from *clcABD* probe positive isolates digested with *Nde*I and hybridized with the labelled 4.2 kbp *clcABD* gene probe. There were 5 RFLP patterns determined that were all different than the archetypal isolate *Pseudomonas. putida* AC866 (pAC27). One isolate had the same RFLP pattern as *Burkholderia* sp. LB400 (lane 5). 9 of the 11 probe positive isolates showed a common 5.7 kbp band (lanes 2,3,4). Lane 1 showed a unique probe positive band (7.4 kb).
- B.** Southern blot of total genomic DNA from *cbaAB* probe positive isolates digested with *Eco*RI and hybridized with the 2.5 kbp *cbaAB* gene probe. The RFLP patterns of the isolates were all the same (9/9) as the archetypal isolate *Alcaligenes* sp. BR60 (pBRC60) and the previously characterized isolate *Alcaligenes* sp. OCC43.
- C.** Southern blot of total genomic DNA from *fcB*B probe positive isolates digested with *Eco*RI and hybridized with the 1.6 kbp *fcB*B gene probe. There were 4 RFLP patterns determined that were all unique and different than the archetypal isolate *Pseudomonas* sp. CBS3.
- D.** Southern blot of total genomic DNA from *bphA* probe positive isolates digested with *Sal*I and hybridized with the 800 bp *bphA* gene probe. The isolates showed 2 different RFLP patterns. Three of the four isolates had the same RFLP pattern as the archetypal isolate *Burkholderia* sp. LB400. The remaining isolate had a different RFLP pattern that differed from all other *bphA* probe positive isolates including *Comamonas testosteroni* sp. B356.

A.

B.

C.

D.

4.3.5 RFLP analysis of *cbaAB* probe positive isolates within Tn5271

Preliminary Southern blot results of the *cbaAB* harbouring isolates showed that all of these isolates had the *cbaAB* genotype encoded within Tn5271. However, there were two different RFLP maps of Tn5271 identified using a combination of restriction endonucleases and gene probes. These two maps were characterized as pBR60-like and pOCC43-like (Fig 4.4). The two maps were identical with the exception of approximately an 800 bp region that was missing from the right side of the transposon in pOCC43. This was shown with *EcoRI* and *SaII* digested DNA hybridized with E5 and E13/14 probes from pBRC60, respectively (Figure 4.5). Table 4.2 outlines which *cbaAB* probe positive isolates had BR60- and OCC43-like Tn5271 genetic elements. Digested DNA from BR60 and OCC43 was hybridized with two probes, E4 and E7/8 from pBRC60 outside of the region of Tn5271 to compare the two plasmid backbones for additional homologous regions. OCC43 DNA did not hybridize with these probes (results not shown).

Figure 4.4 Southern Blot Results for the *cbaAB* Probe Positive Isolates, *Alcaligenes* sp. BR60 and OCC43 Hybridized with Probes in the Region of Tn5271.

- A.** 0.7% agarose electrophoresis gels of *Eco*R1 and *Sal*I digested total genomic DNA from *Alcaligenes* sp. BR60 and OCC43. Lanes 1: *Hind*III-digested lambda DNA, 2: *Eco*R1 digested BR60, 3: *Eco*R1 digested OCC43, 4: blank, 5: *Sal*I digested BR60, 6: *Sal*I digested OCC43.
- B.** Southern blot results of *Eco*R1 digested total genomic DNA (lanes 2,3) hybridized with the E13, E14 and E5 Digoxigenin-labelled probes.
These results show that the *cbaAB* genes are a similar length but the E5 region (see Figure 4.5) to the right of these genes is shorter in OCC43 indicating a deletion (Δ E5).
- C.** Southern blot results of *Sal*I digested total genomic DNA (lanes 5,6) hybridized with the E13,E14 Digoxigenin-labelled probes.
These results confirm that the region of Tn5271 from the *cbaAB* genes to the left side copy of IS1071 are a similar size.

Figure 4.5 A Comparison of Tn5271 on plasmids pBRC60 and pOCC43.

These RFLP patterns were generated from *Sal*I and *Eco*R1 endonuclease restriction digests of total genomic DNA isolations from *Alcaligenes* sp. BR60 and OCC43. Southern blots were hybridized with Digoxigenin-labelled probes E5 and E13/E14.

The results revealed an approximate 800 bp deletion on the right side of Tn5271 in pOCC43. The H4 probe used for the detection of IS1071 is also shown. The Italian *cha*AB probe positive isolate, *Alcaligenes* sp. CPE3, harbours a similar sized deletion in this region of Tn5271 (Di Gioia, pers. comm.). Sequence data would need to be collected in order to better compare these deletions.

Tn5271 on pBRC60

cbaAB

Tn5271 on pOCC43

cbaAB

4.3.6 Detection of IS1071

The results of the DNA digestion with *NheI* and Southern hybridization with the H4 probe from IS1071 showed that this element was not limited to the *cbaAB* probe positive isolates (Tables 4.1-4.4). Some of the host genomes harbouring the *clcABD*, *fcB* or *bphA* genotypes hybridized with the H4 probe. With the exception of ^b BRC3-2-3², all IS1071 probe positive fragments were the same size as the 3.1 kb *NheI* fragment of *Alcaligenes* sp. BR60 (pBR60). The ^b BRC3-2-3² *NheI* fragment was 2.9 kbp. A Southern blot of *fcB* and *bphA* probe positive host genomic DNA digested with *NheI* and hybridized with the H4 probe is shown in Figure 4.6. All of the *cbaAB* probe positive isolates in this chlorobenzoate catabolic isolate collection and previously identified *cbaAB* probe positive isolates from SBR2 at Hyde Park contained IS1071. In addition, Figure 4.7 shows that many of the archetypal isolates were found to harbour this element. These were *Pseudomonas putida* sp. AC866 (pAC27) (a derivative of an isolate from Niskayuna, New York), *Burkholderia* sp. LB400 (Hudson River, New York), and *Alcaligenes eutrophus* sp. H850 (Hudson River, New York).

Figure 4.6 IS1071 Distribution in *bphA* and *fcB* Probe Positive Isolates

- A.** 0.7% agarose electrophoresis gel of *NheI* digested total genomic DNA from *fcB* and *bphA* probe positive isolates.

Lanes 1: *HindIII* digested lambda DNA, 2: *Alcaligenes* sp. BR60 (positive control), 3: ^d DHC5-11-2² (*bphA*+), 4: ^d BRC4-1-3¹ (*bphA*+), 5: ^d BRC6-3-4¹ (*bphA*+), 6: *Pseudomonas* sp. CBS3, 7: ^b MR2-1-1¹ (*fcB*+), 8: ^b BRC3-4-1¹ (*fcB*+), 9: ^b BRC3-2-3¹ (*fcB*+), 10: ^b BRC5-1-4² (*fcB*+).

- B.** Southern blot results of the agarose gel shown in A. hybridized with the a Digoxigenin-labelled probe of the H4 region of IS1071.

Probe positive isolates were *Alcaligenes* sp. BR60, ^d BRC4-1-3¹ (*bphA*+), ^b MR2-1-1¹ (*fcB*+) ^b BRC3-2-3¹ (*fcB*+) and ^b BRC5-1-4² (*fcB*+). All isolates that hybridized to the H4 probe had the same 3.2 kbp *NheI* fragment as BR60 with the exception of ^b BRC3-2-3¹ (*fcB*+) . This latter isolate hybridized to a 2.9 kbp *NheI* fragment.

Figure 4.7 Screening Selected Catabolic Archetypal Strains for the Presence of IS1071

- A.** 0.7% agarose eletrophoresis gel of *NheI* digested total genomic DNA from catabolic archetypal strains 1: *Alcaligenes* sp. BR60 (positive control), 2: *Pseudomonas putida* sp. AC866 (*clcABD*⁺), 3: *Burkholderia* sp. LB400 (*bphA*⁺), 4: *Alcaligenes eutrophus* sp. H850 (*bphA*⁺), 5: *Comamonas testosteroni* sp. B356 (*bphA*⁺), 6: *HindIII*-digested lambda DNA.
- B.** Southern blot results of the agarose gel shown in A. hybridized with a Digoxigenin-labelled probe of the H4 region of IS1071. Probe positive isolates were 1: *Alcaligenes* sp. BR60 (IS1071 positive control), 2: *Pseudomonas putida* sp. AC866 (*clcABD*⁺), 3: *Burkholderia* sp. LB400 (*bphA*⁺) and 4: *Alcaligenes eutrophus* sp. H850 (*bphA*⁺). All isolates that hybridized to the H4 probe had the same 3.2 kbp *NheI* fragment as BR60.

A.

1 2 3 4 5 6

B.

1 2 3 4 5 6

3.2 →

4.4 DISCUSSION

Probe positive members of the catabolic isolate collection were further characterized genetically and phenotypically. ERIC PCR provides a genetic fingerprint of microbial isolates so that clonal or sibling strains can be identified. This technique is much simpler and quicker than the use of genera or species-specific probes (de Bruijn 1992). Figure 4.1 shows an example of these genetic fingerprints. Sibling microorganisms were only detected in *c/c*ABD probe positive isolates from the chlorobenzoate catabolic isolate collection. They were from the same sampling trip and creek but from different sites and enrichment temperatures. Interestingly, one groundwater site in Hyde Park had three genetically distinct hosts harbouring the *c/c*ABD genotype in the same enrichment sample (‘GW 4-16’, ‘GW 6-1’ and ‘GW 6-9’). The *c/c*ABD genotypes have previously been reported to be plasmid- or transposon-encoded. Thus this genotype might be expected to have multiple hosts in the Hyde Park groundwater site.

In comparison, a study of 2,4-D degrading microorganisms isolated worldwide and subjected to REP PCR, found sibling isolates from various geographical locations including Michigan, Oregon, Quebec and Saskatchewan (Fulthorpe *et al.* 1995). Unlike the transposon- and plasmid-encoded *c/c*ABD genotype, the 2,4-D catabolic sibling isolates may have chromosomally-encoded 2,4-D degradative genes. This would lead to the dissemination of the host organism to 2,4-D polluted environments rather than mobilization of only the 2,4-D catabolic genes. The 2,4-D catabolic isolate *Burkholderia* sp. RASC has been found to have a chromosomally-encoded degradative (*tfdA*) gene (Suwa *et al.* 1996).

The objective of determining the carbon source substrate range using BIOLOG[®] GN plates was not to identify the microorganism to the species level, but to establish a phenotypic relationship between the hosts of the catabolic genotypes, archetypal catabolic isolates and reference strains. For phylogenetic classification of microbial isolates, genotypic techniques including 16S rRNA sequencing are employed. The microorganism collection was clustered into 5 phenotypically distinct groups (Figure 4.2). Group 1 contained representatives of the β -Proteobacteria, as evidenced by the presence of

Comamonas testosteroni and *Alcaligenes eutrophus*. Hosts harbouring the *cbaAB* genotype were the dominant members of this group. This is not unexpected as the β -Proteobacteria are the most frequent reported hosts of this genotype (Nakatsu *et al.* 1995a). The *cbaAB* genotype was also found in hosts represented in groups II and III. The results of this survey suggest that the hosts of this catabolic genotype have high phenotypic variation. A 3-Cba catabolic isolate collection from a 3-Cba-dosed microcosm was found to represent 4 phenotypic groups. Although the majority of the pBRC60-harbouring strains were closely related to *Alcaligenes* sp. BR60, this plasmid was detected in all 4 groups (Fulthorpe and Wyndham 1992).

The fluorescent Pseudomonads, including *Pseudomonas putida* AC866 (pAC27) and 'BRC3-2-5' (*cbaAB*+) were the only members of group III, the smallest of the five groups. Many of the archetypal catabolic microorganisms were identified in the 1970's and 1980's, using a limited range of phenotypic traits, as *Pseudomonas* species. This gave the false impression that these Γ -Proteobacteria were the dominant environmental species involved in pollutant biodegradation. Less biased isolation protocols and superior taxonomic methods have shown that Gram-positive microorganisms, notably *Rhodococcus*, *Mycobacterium* and Actinomycete species and members of the α - and β -subgroup of the Proteobacteria (Gram-negative species) are also significant bacteria in the environmental biodegradative community.

The *clcABD* probe positive isolates were found in all groups, but primarily in Group IV. Three of the four *fcB* probe positive isolates, the archetypal strain *Pseudomonas* sp. CBS3 and the reference strain *Pseudomonas tolaasii* (a Γ -Proteobacterium, phylogenetically similar to *Pseudomonas syringae*) were also present in this group. This contradicts the conventional view that the *fcB* genotype is not host dependent. Gram-positive isolates harbouring this genotype may not have been isolated because of the choice of sampling sites and the enrichment method. Gram-positive bacteria predominate in soil environments and are less common in freshwater/sediment environments. Also the small number of *fcB* probe positive isolates (4) may make any definitive conclusions concerning the host range of this genotype too speculative.

The *bphA* harbouring isolates were phenotypically widespread and included members of groups I (close to *Alcaligenes eutrophus* sp. H850 and *Comamonas testosteroni* sp. B356), II (close to *Burkholderia* sp. LB400) and V.

The ability of all probe positive isolates to mineralize the three chlorobenzoate isomers in this study was determined and compared to the chlorobenzoate substrate range of the archetypal isolates. The chlorobenzoate substrate range of the *clcABD* harbouring isolates included 3-Cba and 4-Cba and can be divided into 3 groups. The first group degraded 3-Cba only. This group resembles *Pseudomonas putida* AC858 (pAC25) which was originally isolated in Niskayuna, New York. The second group mineralized 3- and 4-Cba. The substrate range of these isolates was similar to the extended substrate range of *Pseudomonas putida* AC866 (pAC27) which evolved in a continuous culture enrichment by the recombination of pAC25 and the TOL plasmid (Chatterjee and Chakrabarty 1982). The final group of isolates had a chlorobenzoate substrate range limited to 4-Cba degradation. There have been no previous reports of isolates harbouring this genotype having a chlorobenzoate substrate range limited to 4-Cba degradation. As expected, no *clcABD* harbouring strains catabolized 3,4-dCba, probably due to the absence of a distal dioxygenase. The ability to mineralize chlorobenzoates other than 3-Cba, 4-Cba and 3,4-dCba was not investigated in this study. However, one *clcABD* probe positive isolate, *Pseudomonas putida* P111, has been reported to encode two separate benzoate dioxygenases. The chromosomally encoded benzoate dioxygenase (showing a transposon-like character) converted 3-Cba and 4-Cba to 3- and 4-chlorocatechol, respectively. A second chlorobenzoate 1,2-dioxygenase converted *ortho*-chlorobenzoates, including 2-Cba, 2,3-dCba, 2,4-dCba, 2,5-dCba and 2,3,5-trichlorobenzoate, to the corresponding chlorocatechols and was determined to be encoded on a 75 kb plasmid, pPB111 (Brenner *et al.* 1993).

The majority of the *clcABD* positive isolates had similar RFLP patterns which included a 5.7 kbp fragment (9/11). The 13.3, 10.3 and 9.5 kbp fragments associated with the 5.7 kbp fragment in some of these isolates likely represented flanking regions outside of the *clcABD* target sequence. The fact that the RFLP patterns were all different from *Pseudomonas putida* AC866 (pAC27), suggests that the *clcABD* genotype in the Niagara

watershed had a high genetic diversity and may have been introduced via horizontal transfer from a source other than the archetypal population. The frequency of isolation of microorganisms with this 5.7 kbp fragment suggests that this genotype had a clonal distribution. However, further RFLP mapping and DNA sequencing would be required to confirm this.

In a recent study a 373 bp portion of the naphthalene dioxygenase gene (*nahAc*) from 9 naphthalene degrading bacteria was sequenced. The results indicated that 7 of the 9 strains harboured identical *nahAc* genes despite the fact that 7 of the strains were taxonomically diverse as evidenced by 16S rRNA sequencing. It was concluded that the horizontal transfer of naphthalene catabolic genes at the site was extensive (Stuart-Keil *et al.* 1996). *Pseudomonas putida* AC866 (pAC27) is a derivative of *Pseudomonas putida* AC858 (pAC25) which was originally isolated from sewage in Niskayuna, New York. Although *Burkholderia* sp. LB400 was also isolated from New York state (Hudson River sediments), the *clcABD* genes in these microorganisms have different RFLP patterns. This genetic diversity may be due to independent evolution of these strains. This would also have to be confirmed by DNA sequencing. It is also possible that point mutations have altered the RFLP pattern without significantly reducing sequence identity. As the *clcABD* genotype has been detected virtually worldwide it is not implausible that this genotype entered the Niagara watershed from multiple sources (at least 3 different sources). Similarly, this genotype may have originated in the Niagara watershed and was mobilized to other locations. One organism, 'WellD3-3' had the same *clcABD* RFLP pattern as *Burkholderia* sp. LB400 and may have originated from the same source as this archetypal isolate. There was no clear relationship between the chlorobenzoate substrate range and the RFLP pattern of the *clcABD* genes.

The portion of the *clcA* gene that was amplified by PCR (362 bp) showed the expected fragment sizes following restriction endonuclease digestion with *Bam*HI. The region that was amplified was highly conserved. The *clcA* primers annealed to template DNA only at a lower temperature for some of the isolates. This may be due to DNA sequence differences in the annealing region of these primers. The primers were very long (32-mers) and base pair differences may have prevented complete annealing in these

isolates. The chlorobenzoate substrate range of those isolates that had *clcA* primers annealing at a lower temperature was varied. Therefore possible nucleotide differences in this region cannot be associated with substrate specificity differences. Further studies with these isolates should include digestion with other restriction endonucleases or cloning and sequencing of the amplified fragments. The conversion of 3- and 4-Cba to chlorocatechol intermediates is dependent on non-specific benzoate (toluate) 1,2-(1,6)-dioxygenases generally unlinked to the *clcABD* genes. The observed variations in chlorobenzoate substrate range are likely a result of differences in the host's non-specific dioxygenases rather than differences in the *clcABD* genotype.

Microorganisms harbouring the *cbaAB* genotype, had 4 different chlorobenzoate substrate ranges. The *Alcaligenes* sp. BR60-like isolates degraded 3-Cba and 3,4-dCba. The *Alcaligenes* sp. CPE3-like isolates (Fava *et al.* 1993) completely degraded all three chlorobenzoate congeners in this study. Microorganisms in the third category degraded 3-Cba only. Finally, members in the fourth category degraded 3,4-dCba only. No clear relationship was established between the phenotypes of the hosts and the chlorobenzoate substrate range of these isolates. Following *EcoRI* digestion and hybridization with a portion of the *cbaAB* genes the RFLP patterns of *cbaAB* harbouring isolates were all identical to the archetypal microorganism, *Alcaligenes* sp. BR60 (Table 4.2). The results showed that the *EcoRI* restriction sites in this region of Tn5271 were conserved. Following restriction endonuclease digestion of the *cbaA* amplified region (1058 bp) there were no differences in the sizes of the resulting DNA fragments. In a related study, a cloned portion of the *cbaA* gene of *Alcaligenes* sp. CPE3 was sequenced. This analysis revealed a 99% conservation of DNA sequence in comparison with the same region of the *cbaA* gene from *Alcaligenes* sp. BR60 (Di Gioia, pers. comm.). Therefore the ability to degrade 4-Cba and superior mineralization of 3,4-dCba by *Alcaligenes* sp. CPE3 compared to *Alcaligenes* BR60 may be a host-specific trait.

There were differences noted in regions downstream of the *cbaAB* genes within Tn5271. Isolates with *Alcaligenes* sp. OCC43-like Tn5271 genetic elements were missing approximately an 800 bp fragment from the right side of Tn5271 (Figure 4.5). *Alcaligenes* sp. CPE3 is also missing a similar sized region of this transposon (Di Gioia,

pers. comm.). There was no correlation between the type of Tn5271 element and the phenotypic characteristics of the host organisms, nor the chlorobenzoate substrate range. The missing region on Tn5271 in OCC43 corresponds to most of ORF8 (1.1 kbp) on Tn5271 in *Alcaligenes* sp. BR60 (Wyndham *et al.* unpublished results). Sequence analysis has shown that this may have originally encoded an acyl-coenzyme A ligase but the gene is truncated in both BR60 and CPE3 and is likely nonfunctional. There is no clear understanding from these results as to what is responsible for the observed chlorobenzoate substrate differences in the *cbaAB* probe positive strains. It is possible that the host of this genotype contributes to the chlorobenzoate substrate range, perhaps by the permeability of the cell membrane to different chlorobenzoate isomers.

All *fcB* probe positive isolates had a RFLP pattern that differed from this gene in the archetypal microorganism *Pseudomonas* sp. CBS3 and from each other (Table 4.3). These isolates all had a chlorobenzoate substrate range limited to 4-Cba. Gram staining results showed that the *fcB* hosts were all Gram-negative microorganisms. The unique RFLP patterns following *EcoRI* digestion and hybridization with the *fcB* gene probe suggests that homologous genes have considerable genetic diversity. These results, and the high 4-Cba degradation potential in the Niagara watershed described in Chapter 2 may reflect a strong selective force for the 4-Cba catabolic genotype in diverse niches. DNA sequence comparison of the 4-Cba dehalogenase region in *Arthrobacter* sp. SU and *Pseudomonas* sp. CBS3 revealed that these two operons evolved independently. The order of the dehalogenation genes (*fcBABC*) varied. In *Arthrobacter* sp. SU the order was *fcBABC* (ligase-dehalogenase-thioesterase). In contrast, in *Pseudomonas* sp. CBS3, the order of these genes was *fcBCA* (thioesterase-dehalogenase-ligase). Additionally, the *fcC* genes were not homologous in these two isolates (Schmitz *et al.* 1992).

Finally, 3 of the 4 *bphA* probe positive isolates had the same RFLP pattern as the archetypal microorganism *Burkholderia* sp. LB400 following *SalI* digestion and hybridization with the *bphA* probe. The *bphA* gene in the Niagara watershed may have the same origin as this archetypal microorganism. Yates and Mondello (1989) found that *Burkholderia* sp. LB400 and *Alcaligenes eutrophus* H850 had *bph* operons that were highly homologous and likely from the same origin. *Alcaligenes eutrophus* H850 was

also originally isolated from Hudson River sediments in New York state. Not only did the Niagara isolate ^dBRC4-1-3¹ have a different RFLP pattern than *Burkholderia* sp. LB400 but it was also unique from the *bphA* gene in *Comomonas testosteroni* B356. This latter isolate was originally isolated from activated sludge in Québec, (Ahmad *et al.* 1990). An 800 bp region amplified from the *bphA* gene showed no changes in any of the isolates when digested with *SalI*. All *bphA* harbouring isolates could completely or partially mineralize at least one chlorobenzoate.

The *bphA* hosts in phenotypic group II could mineralize 3- and 4-Cba. By contrast the *bphA* hosts in groups I and V converted 3-Cba to a dead-end metabolite that was yellow in colour (Figure 4.2, Table 4.4). This metabolite is possibly a *meta*-fission intermediate. It eluted at the same time by HPLC as the metabolite 2-pyrone-4,6-dicarboxylate (also yellow in colour) that is observed during 3-Cba degradation by *cbaAB* harbouring hosts. 2-Pyrone-4,6-dicarboxylate is a catabolite of the *meta*-fission pathways of protocatechuate, 5-chloroprotocatechuate and 5-methoxyprotocatechuate. This compound arises from either of two types of reactions, both involving protocatechuate 4,5-dioxygenase with elimination of chloride or methanol. 2-Pyrone-4,6-dicarboxylate hydrolase, encoded on the host's chromosome, subsequently converts 2-pyrone-4,6-dicarboxylate to precursors of pyruvate and oxaloacetate (Kersten *et al.* 1985). It is possible that the hosts of the *bphA* genotype do not have a chromosomally-encoded 2-pyrone-4,6-dicarboxylate hydrolase to metabolize 2-pyrone-4,6-dicarboxylate. If this is the case, then it is still unclear why the *bphA* harbouring host ^dBRC4-1-3¹ was closely clustered with the *cbaAB* harbouring hosts in group I yet it could not metabolize this intermediate. Alternatively, the dead-end product may be a chlorocatechol *meta*-ring-fission intermediate. GC-MS and NMR analyses would have to be conducted on this intermediate in order to determine its structure.

None of the isolates in the Niagara watershed collection hybridized to more than one catabolic gene probe. *Burkholderia* sp. LB400 hybridized to the *clcABD* and *bphA* genotypes. The fact that some of the *bphA* containing hosts (^dBRC6-3-4³ and ^dDHC5-11-2²) could also mineralize chlorobenzoates strongly implicates the presence of alternative chlorobenzoate catabolic genotypes.

The Niagara catabolic isolate collection was screened for the presence of high molecular weight plasmids as all of the genotypes used for screening in this study have been detected on high molecular weight plasmids. High molecular weight plasmids were detected in approximately half of the hosts from each genotype group. This is likely an under-estimation as it can only be concluded that these isolates had high molecular weight plasmids. The isolates where plasmids were not detected may still have one or more, since the results of these plasmid isolation techniques are often host dependent. The use of pulsed field gel electrophoresis (PFGE) to achieve better separation of high molecular weight DNA, combined with alternative methods of plasmid isolation, may have resulted in more conclusive results. To date all reports of hosts harbouring the *cbaAB* genotype (*Alcaligenes* sp. BR60 and *Alcaligenes* sp. CPE3) and the *clcABD* genotype found that these genotypes were encoded on high molecular weight plasmids. The extensive global distribution of the *clcABD*, *fcB* and *bphA* genotypes strongly suggests that they are plasmid or transposon encoded.

All *cbaAB* probe positive isolates had either BR60-like or OCC43-like Tn5271 genetic elements flanked by IS1071. In BR60, Tn5271 is flanked by two copies of IS1071. It has been hypothesized that Tn5271 was mobilized to BR60 through sequential intermolecular and intramolecular transpositions. This model is presented in Figure 4.8. The isolates harbouring OCC43-like Tn5271 elements likely evolved independently of those isolates with BR60-like Tn5271 elements since random intramolecular transposition at Target 2 (Figure 4.6) would be expected to yield different sizes of DNA inserts within the final composite structure. If the BR60 and OCC43 composite transposons were from the same ancestral microorganism, then the sequences flanked by IS1071 would be of equal length. The isolate *Alcaligenes* sp. CPE3 is missing a 41 bp region on the left hand side of Tn5271 (Di Gioia, pers. comm.). The RFLP mapping in Figure 4.5 was not detailed enough to identify if OCC43 is also missing this small region. Sequence analysis of the OCC43-Tn5271 would show if this genetic element evolved in a similar manner as *Alcaligenes* sp. CPE3. The results of the Tn5271 mapping revealed that the composite transposons on pBRC60 and pOCC43 likely evolved independently.

Figure 4.8 Hypothesis for the Formation of IS1071-Flanked Transposons Involving Sequential Intermolecular and Intramolecular Transpositions. Screening Selected Catabolic Archetypal Strains for the Presence of IS1071

1. A plasmid like pBRC40 with a single copy of IS1071 is nicked at the 3'-ends of the insertion sequence (white arrows) by the action of the *tnpA* gene product (direction of transcription indicated by short black arrow).
2. The target site (Target 1) adjacent to chromosomal catabolic genes (*cbaAB*) is nicked, and the IS1071 element is inserted and replicated by the usual Class II mechanism, in the process replicating the 5 bp between the target site nicks (black ellipse).
3. The integrated plasmid (thick line), flanked by direct repeats of IS1071 (IS) and the 5 bp target site sequence (black ellipse). Intramolecular transposition (curved arrow) to a target site (white ellipse) on the other side of the catabolic genes (*cbaAB*) results in deletion of the plasmid and the catabolic genes.
4. The chromosome is left with one copy of IS1071 and a deletion of the catabolic genes (arrow-head).
5. The deleted plasmid carries a composite transposon structure with direct repeats of IS1071 flanking the catabolic genes (*cbaAB*). This structure is analogous to Tn5271 in *Alcaligenes* sp BR60. Note that the 5 bp duplications created by the two transposition events (black and white ellipses) are separated on the plasmid and chromosome in the final products.

From Wyndham *et al.* 1994a.

Intermolecular Transposition

Intramolecular Transposition

A recent survey of the scientific literature and experimental work on hybridization of *IS1071* probes with unrelated catabolic isolates from various locations revealed that the genetic element *IS1071* has a worldwide distribution and flanks a variety of nonhomologous catabolic genotypes (Wyndham pers. comm.). These observations were further substantiated by the observation that the majority of the archetypal strains in this study harboured *IS1071*. This list includes *Pseudomonas putida* AC866 (pAC27), *Burkholderia* sp. LB400 and *Alcaligenes eutrophus* H850 (Figure 4.7). However the homologous *bphA* isolate, *Comamonas testosteroni* B356, did not have a copy of *IS1071*. A large proportion of probe positive isolates containing one of the four catabolic genotypes in this study also hybridized with the *IS1071* genotype (Figure 4.6). Furthermore, the host range of *IS1071* was extensive and covered all five phenotypic groups in Figure 4.3. It was not established if this genetic element was associated with the catabolic genes in this study or was simply present in the genome. However, the association of *IS1071* with nonhomologous catabolic genotypes and the mobile nature of IS-elements strongly implicates these elements in the worldwide dissemination of catabolic genotypes.

One isolate, ^b BRC3-2-3² (*fcB*), hybridized to a smaller *IS1071* *NheI* fragment of 2.9 kbp. One possible explanation of this size difference may be a deletion event which commonly occurs adjacent to IS elements. It is possible that there were initially two copies of *IS1071* and that one copy and half of a second copy were deleted by intramolecular transposition or some other process. If this is the case then the second *NheI* site is actually outside of the remaining truncated *IS1071* element. In this scenario, the *IS1071* fragment left in this isolate is less than 2.9 kbp.

The results of Chapter 4 showed that there is considerable diversity in the chloroaromatic degradative genotypes isolated from the Niagara watershed. Probes made from catabolic gene fragments are probably too specific as predictors of environmental biodegradation potential. The distribution of *IS1071*, which has been identified in numerous nonhomologous catabolic genotypes, may be a more valuable indicator of microbial adaptation to environmental contamination.

The next two chapters examine the results of total DNA isolations from the Niagara watershed and amplification of the *cbaA*, *clcA* and *bphA* genes. These methods avoid the culturability limitations that are associated with enrichment techniques.

CHAPTER 5:

Detection of *cbaA* and *bphA* Genes in Total DNA Extracted from the Niagara Watershed and Hamilton Harbour

5.1 INTRODUCTION

It has been estimated that only 0.01-22% of the microbial community in soil and aquatic environments is culturable (Brockman 1995, Roszak and Colwell 1987). Therefore when examining the culturable bacteria of a natural environment it must be assumed that only a fraction of the original microbial community is being studied. Torsvik *et al.* (1990) determined environmental bacterial community diversity by measuring community DNA complexity through reannealing experiments. They showed that approximately 10^9 bacteria are present in a gram of sediment or surface soil and this may represent 10^3 - 10^4 different genome equivalents.

One disadvantage of nucleic acid hybridization to DNA isolated directly from the environment is that the low-end sensitivity is generally equivalent to 10^4 - 10^6 gene copies/g soil or sediment (Brockman 1995). PCR amplification of DNA allows a 10^4 -fold increase in sensitivity compared with non-amplified samples (Bej *et al.* 1991). Thus PCR amplification is well suited for the study of natural microbial diversity. A survey of PCR amplification of target gene sequences in environmental DNA samples showed significant variations with regards to the optimum concentration of template DNA, the number of PCR amplification cycles, primer length and in the interpretation of the amplification results (McDonald *et al.* 1995, Bruce *et al.* 1992, Holben *et al.* 1992).

There are many methods reported for environmental DNA extraction and amplification of target genotypes. This variation in protocols can largely be

attributed to the heterogeneous nature of environmental samples. The methods for DNA extraction from sediment and water environmental samples can be either cell separation followed by DNA extraction (cell extraction) or direct lysis of microbial cells in the environmental matrix followed by nucleic acid purification (direct extraction). The cell separation protocols can result in a lower diversity and quantity of extracted DNA, due to biases in cell separation protocols yielding only a portion of total cellular populations before lysis. The direct extraction approach offers greater diversity of DNA. However, limitations of these methods include the large sample size and amounts of reagents that are required for extraction, the long extraction time, the potential for shearing the DNA and increased humic acid and tannic acid contamination. If the genes to be detected are sheared during DNA isolation, they will not provide good templates for PCR and thus the sensitivity of detection will be significantly reduced. Humic and tannic acids contaminants, even in very low concentrations, interfere with enzyme activity during DNA digestion and amplification (Steffan *et al.* 1988).

Leff *et al.* 1995 compared published DNA extraction protocols from sediment and soil samples using the cell and direct extraction approaches. Three DNA extraction techniques were compared on the basis of DNA yield, purity, quality and taxonomic composition. DNA yield was greatest for the Ogram direct extraction method but this DNA was highly sheared and contained a smaller proportion of eubacterial DNA. The second direct extraction protocol, the Tsai method, yielded DNA with the least fragmentation. This was also the shortest protocol but provided DNA of the lowest purity due to high humic acid contamination. The Jacobsen method of cell extraction followed by lysis gave the lowest DNA yield and diversity but the highest purity. The authors concluded that the end use of the environmental DNA (direct DNA-DNA hybridizations, Southern blotting, PCR amplification) should dictate the extraction protocol used. DNA isolation from aquatic environmental samples usually involves filtration of large volumes of water (1L) followed by cell lysis and DNA extraction.

PCR amplified catabolic target genes from environmental template DNA have been found to be highly conserved. Template DNA from various environmental locations in Germany was amplified with primers to detect a portion of the *bphC* gene (2,3-

dihydroxybiphenyl dioxygenase). PCR amplification products of template DNA from numerous PCB contaminated and more pristine environments were digested with seven restriction endonucleases that did not cut within the DNA sequence of 5 known homologous *bphC* sequences. All enzymes failed to cut the amplified PCR products (Erb and Wagner-Döbler 1993). Similarly, digestion of the PCR products following amplification of environmental DNA from various geographical locations with *tfdA* (2,4-D monooxygenase) and *tfdB* (2,4-dichlorophenol hydroxylase) primers showed that the amplified genes were highly conserved (Holben *et al.* 1992). High conservation of *cbaA* and *bphA* genotypes in culturable microorganisms following PCR amplification was noted in Chapter 4. Restriction endonuclease digestion of amplified PCR products resulted in fragments that were the same size as the target genes in the archetypal isolates.

The intent of this chapter was to detect the *cbaA* and *bphA* catabolic genotypes in total DNA isolated from different environmental sites over an 18-month sampling period. All of the sites examined were part of the Great Lakes system. Hyde Park, contaminated creeks BRC and DHC and a noncontaminated creek, FC in the Niagara watershed were sampled. These results were subsequently compared to the results obtained in Chapter 2 and 3 for the genotype distribution in culturable microorganisms from the same samples. The Niagara river flows from Lake Erie to Lake Ontario. Hamilton Harbour, in Lake Ontario, was also sampled over a 12-month period as a comparison site with known PCB exposure from different industrial sources. Most experiments using total DNA extraction, amplification and hybridization are employed to detect populations that have been added to natural microbial communities (usually for tracking GEMs) rather than indigenous populations. As well these studies have monitored the presence of target genotypes at a single time point or directly following experimental treatment (such as pollutant application).

The objectives of this investigation were as follows:

1. To develop a rapid, total DNA extraction method for these samples that minimized DNA shearing.

2. To optimize PCR amplification conditions for the detection of target DNA sequences using *chaA* and *bphA* primers.

3 To detect target genes in Niagara watershed, Hyde Park and Hamilton Harbour samples.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

The remaining 25 ml samples from Bloody Run Creek (BRC), Devil's Hole Creek (DHC) and Fish Creek (FC) that were frozen at -70°C upon collection (May and November 1992 and May, August and November 1993), were used for total DNA isolation. Frozen samples collected from the Hyde Park groundwater and SBRs in May 1993 were also extracted. Finally, water/sediment samples from PCB contaminated ($\text{PCB} > 0.5 \mu\text{g/g}$) and non-contaminated sites ($\text{PCB} < 0.2 \mu\text{g}$) in the Hamilton Harbour, kindly provided during June 1992 and 1993 by Dr. Inniss at the University of Waterloo, were subjected to total DNA extraction.

5.2.2 Development of a total DNA extraction method

In order to develop a total DNA extraction method for samples that were a combination of sediment and water, a review of existing methods for soil/sediment and aquatic samples that looked suitable for the generation of template DNA of high molecular weight and relatively free of humic acid contamination was conducted. These protocols included the Ansari *et al.* method (1992) to extract total nucleic acids directly from samples of wastewater, sludge and pond water and methods from Porteous and Armstrong (pers. comm.) and Bruce *et al.* (1992) to extract DNA directly from soil. A cell extraction method was not considered because the samples had been frozen at -70°C , resulting in cell loss due to lysis.

Some preliminary experiments were conducted using the above mentioned protocols. In order to minimize the losses of Niagara Watershed and Hamilton Harbour samples, Rideau river water samples were tested. A 60 ml sediment/water sample was collected from a storm water outlet emptying into the Rideau river. After thorough mixing the sample was frozen in 20 ml aliquots at -70°C for 2 months.

After 2 months at -70°C , DNA was isolated at the same time from three 20 ml samples using the three methods mentioned above. It was important to thaw the samples on ice and to work quickly in order to limit the activity of nucleases that may have been released from lysed cells. The method of Ansari *et al.* (1992) was performed as stated, scaled-up to a 20 ml volume. Filtration resulted in approximately 100 mg wet weight of sediment. The direct DNA extraction methods were based on this weight. As the filtrates may have contained DNA from the cells that lysed during storage at -70°C , 1/10th ammonium acetate and an equal volume of isopropanol was added to precipitate this DNA. These filtrates were stored at -20°C overnight. The method of Bruce *et al.* (1992) was performed as stated with the exception of the cesium chloride centrifugation and subsequent dialysis. The method of Porteous and Armstrong was an adaptation of an earlier publication (Porteous and Armstrong, 1991). Briefly, 1 ml of an extraction buffer containing 200 mM Tris pH 7.5, 0.5% SDS, 25 mM EDTA and 250 mM NaCl was added to the filter. This was mixed for 1 min and incubated at 65°C for 20 min, inverting every 5 min. The sample was centrifuged for 10 min at $10,000 \times g$. DNA in the supernatant was precipitated overnight at -20°C with an equal volume of isopropanol. The sample was centrifuged the next morning for 20 min at $10,000 \times g$. The final DNA pellets from the filter and filtrate extractions were resuspended in a total volume of 200 μl distilled water. 15 μl of each sample was run on a 0.65% agarose gel.

5.2.3 Total DNA isolation from Niagara watershed and Hamilton Harbour samples

The method of Porteous and Armstrong was further adapted and used to extract total DNA from the Niagara watershed samples that were more resistant to filtration due to varying sediment content. An equal volume of a 2-times concentrated buffer (10 ml) was added to a 10 ml sample from each site during the first sampling trip (May 1992). The final concentration of reagents in the extraction buffer was the same as listed above (200 mM Tris (pH 7.5), 0.5% SDS, 25 mM EDTA (pH 8.0), 250 mM NaCl). The extraction

procedure was followed as described above. In order to investigate the efficiency of the 20 min 65°C incubation in DNA extraction buffer, the pellet following centrifugation was resuspended in 10 ml of fresh extraction buffer (regular strength) and reincubated at 65°C for 20 min. Following centrifugation and removal of the supernatant, the pellet was resuspended a third time in 10 ml extraction buffer at 65°C for 20 min. The precipitated DNA from these additional extractions was resuspended in 200 µl TE buffer. The results of the first, second and third extraction from one Niagara watershed sample are presented in Figure 5.1. As there was very little DNA following the second and third extractions, these subsequent extractions were eliminated from the total DNA extraction protocol.

Preliminary PCR amplification results of DNA from samples collected during May 1992 showed that sites along the same creek had comparable amounts of the *chaA* genotype. As well, the degradation potential results in Appendix 1 showed that different sites in the same creek during the same sampling trips had similar degradation potentials. Therefore, considering resources and available time, I decided to combine an equal volume from each site along the creeks and perform triplicate extractions for each creek. For example, if I sampled 5 sites along FC during one trip, I combined a representative 6 ml from each site, mixed the total 30 ml thoroughly, divided this volume into three 10 ml aliquots and performed triplicate 10 ml DNA extractions. MR and BRC samples were combined and extracted as a single site. These two sites receive the same contaminated groundwater from the Hyde Park site. This total DNA extraction method was performed for the samples collected from the second to fifth sampling trip. As most of the samples were used to perform individual extractions from sites collected during the first sampling trip, only enough samples remained to perform one total DNA extraction per creek.

5.2.4 Selection of a total DNA purification method

The colour of the DNA solution following total DNA extraction using all of the above methods was light brown. This was contamination from humic acid and tannic acid compounds that are known to interfere with DNA amplification. There are many reported

protocols for the removal of humic acid compounds from DNA samples. These include polyvinyl polypyrrolidone (PVPP) adsorption, cesium chloride-ethidium bromide density gradient centrifugation, hydroxyapatite columns and separation of DNA and humic acid compounds by electrophoresis on an agarose gel. I chose the latter option as it enabled me to physically see the separation of the DNA and humic acid contamination and confirmed that the DNA was high molecular weight and unsheared. Total extracted DNA (60 μ l) was combined with 20 μ l of glycerol and loaded onto a 0.65% NuSieve agarose (Mandel) gel. The gel was run at 60V for 4 h, stained in ethidium bromide and viewed under UV light. Figure 5.1 shows humic acid contamination near the bottom of the gel (low molecular weight), well separated from the high molecular weight DNA. High molecular weight DNA (>23 kbp) was collected from these gels by removing gel plugs with the wide end of a sterile 1 ml pipet tip. An equal volume of sterile distilled water was added to these plugs. This was done in order to dilute any remaining humic acid contaminants and the agarose, both of which may have otherwise interfered with PCR amplification. Purified DNA was stored at -20°C.

Figure 5.1 Separation of Humic Acid Contaminants from Large Molecular Weight DNA

0.65% NuSieve agarose ethidium bromide stained gel for triplicate total DNA extractions from a Niagara watershed sample using a revised method of Porteous and Armstrong (pers. comm.) described in the Methods section. Lanes 1-3 triplicate total DNA extractions of the first extraction of the pellet (60 μ l samples); Lane 4: *Hind*III-digested Lambda DNA; Lanes 5-7 triplicate total DNA extractions of the second extraction of the pellet (60 μ l samples); Lanes 8-10 triplicate total DNA extractions of the third extraction of the pellet (60 μ l samples). Based on these results only single extractions were used to obtain high molecular weight DNA. UV absorbing, low molecular weight humic acid contamination is noted in Lanes 1-3 and 5-7 at the lower end of the gel.

First Extraction

λ

Second Extraction

Third Extraction

High MW
DNA

Humic Acid
Contamination

5.2.5 Selection of a total DNA quantitation method

Previous studies have revealed limitations associated with spectrophotometric (OD_{260}) and fluorimetric (Hochst's fluorochrome) DNA quantitation (Smalla *et al.* 1993). Spectrophotometric DNA quantification at OD_{260} was not possible as humic acid contaminants from soil that co-purify with DNA also absorb at this wavelength, making precise quantitation of DNA impossible. Agarose is also known to interfere with absorbance readings.

A negative image of the NuSieve agarose gels prior to DNA plug removal was produced with a Polaroid MP4 Land camera using Polaroid type 55 film. These negatives were scanned with a Bio-Rad Imaging Densitometer model GS-670 (Molecular Analyst Ver. 1.1.1). A uniform box, equivalent in size to the 1 ml pipet tip plug was used to generate the area under the peak for DNA >23 kb. The total extracted DNA was quantified by interpolation from a calibration curve prepared from the densities of Lambda-*Hind*III-cut DNA standards (800 ng, 400 ng, 200 ng and 50 ng). SigmaPlot (version 1.02 for Windows) was used to calculate the linear regression of the calibration curves. Most DNA samples were run on two agarose gels. The negative film with the highest r^2 value (linear regression) for the standard curve was used to quantitate the DNA samples. The r^2 values ranged from 0.824-0.998. SigmaPlot was also used to calculate the standard error of the mean for the average DNA quantitation values.

5.2.6 The effect of the number of amplification cycles on target DNA amplification

Alcaligenes sp. BR60 (pBRC60) template DNA (10 ng) was subjected to PCR amplification using a variety of amplification cycles (0, 10, 20, 30, 40, 50 and 60). A second round of amplification cycles (40, 50, 60) was performed by re-amplifying 10 μ l of first round 30 cycle amplification products for an additional 10, 20 or 30 cycles. All final PCR products were diluted in a 5-fold series and applied to a nylon membrane as described below for the environmental samples.

5.2.7 PCR amplification of DNA isolated from environmental samples

Genomic template DNA (1 ng) was amplified with *bphA* and *cbaA* primers as described in Chapters 3 and 4, respectively. Template DNA was heated at 65°C to fully dissolve the NuSieve agarose before adding the samples to the PCR tubes. For those samples where 10 µl of template DNA resulted in a final quantity of DNA lower than 1 ng, a maximum sample volume of 10 µl was used. The sites where all three total DNA extractions were too dilute included FC and BRC samples from November 1992 and May 1993, respectively. Template DNA (10 µl) for these samples amounted to approximately 0.25-0.7 ng total DNA. Following the first amplification, 10 µl of each product was re-amplified under the same conditions. Positive and negative control tubes containing 10 ng of target genomic DNA and water respectively, were amplified alongside the environmental DNA samples.

The twice-amplified PCR products were chloroform-extracted to remove the mineral oil from the 100 µl amplification product. DNA was ethanol precipitated and resuspended in 60 µl sterile deionized and distilled water. Control and random tubes (2 µl) of amplified environmental DNA were subjected to agarose electrophoresis (1.5%), Southern blotted and hybridized with the appropriate gene probe in order to confirm that the PCR products were the expected size (1058 bp and 800 bp for the *cbaA* and *bphA* amplification products, respectively).

12 µl of amplified DNA was 5-fold serially diluted 8 times in 100 µl volumes (12 µl of DNA was added to 88 µl of distilled water in an eppendorf tube, this dilution was mixed and 20 µl was added to 80 µl distilled water, mixed and further diluted). Thus the first dilution represented 1/6th of the original amplified DNA. Non-amplified *Alcaligenes* sp. BR60 (pBRC60) (*cbaA* positive control) and *Burkholderia* sp. LB400 (*bphA* positive control) genomic DNA was 10-fold serially diluted 8 times in 100 µl volumes commencing with 90 µg of DNA.

All dilutions were boiled 10 min before pipetting onto a nylon membrane in a Bio-Rad Bio-Dot filter manifold. The membrane was equilibrated in TE buffer, applied to the manifold under vacuum and further washed with 100 µl TE. After the DNA was bound to

the membrane, the membrane was washed with 100 µl TE, removed under vacuum, air-dried and the bound DNA was fixed to the membrane under UV light. All membranes were hybridized with the *cbaA* or *bphA* probes. The hybridized membranes were developed using chemiluminescence detection as described previously. A minimum of two of the triplicate total DNA extractions were subjected to PCR amplification. The x-ray films were scanned with a ScanJet IICX (Hewlett Packer) scanner using the DeskScan II program (for Windows). A uniform brightness and contrast setting were used when scanning the x-ray films.

5.2.8 Minimal level of detection of the *cbaA* and *bphA* genes

A total of 10 ng template DNA was amplified with different concentrations of target *cbaA* or *bphA* DNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg) in order to determine the minimal amount of target DNA that could be detected using the previously mentioned PCR amplification conditions and dot blotting method. Non-target template DNA was generated from total DNA extractions (Ausubel *et al.* 1989) from a collection of 10 isolates (*clcABD* and *fcBb* probe positive) described in Chapter 3 that did not cross-hybridize with the *cbaA* or *bphA* genotypes. Total DNA extracted directly from the environment could not be used as non-target template. The dot blot results (see below) revealed that all sites tested contained detectable copies of the *cbaA* or *bphA* genotype. Amplified DNA was diluted and applied to a nylon membrane as described above

5.3 RESULTS

5.3.1 Selection of a total DNA extraction method

A comparison of the DNA integrity following DNA extraction of Rideau river samples is shown in Figure 5.2. The methods of Bruce *et al.* (1992) and Porteous and Armstrong showed the best results for subsequent PCR amplification. The DNA was of high molecular weight and had minimal shearing. The method of Ansari *et al.* (1992) yielded less DNA which was uniformly sheared. I decided to focus on the Porteous and Armstrong method as it was quicker and did not involve a phenol/chloroform purification step that was in the Bruce *et al.* (1992) method.

Figure 5.3 shows the average high molecular weight DNA recovered from triplicate DNA extractions from each creek during the 4 latter sampling trips and the DNA recovered from a single DNA extraction from the first sampling trip. High molecular weight DNA was quantitated based on a Lambda-*HindIII*-digested DNA standard curve. The amount of high molecular weight DNA varied from creek to creek during the same sampling trip and within the same creek over different sampling trips. This range extended from approximately 25 to 250 ng/ml. The total high molecular weight DNA extracted from the groundwater and SBRs at the Hyde Park site also had DNA concentrations in this range (see Appendix 3). The high molecular weight total DNA concentration for all Hamilton Harbour samples ranged from 191 ng/ml to 1.85 µg/ml (see Appendix 3). None of these DNA concentrations can be directly compared to other total DNA extraction protocols as the samples were a variable mixture of water and sediment. As well, only the high molecular weight DNA was quantitated as it was essential to maximize the PCR efficiency by using relatively unsheared DNA. As shown in Figure 5.1 lower molecular weight sheared DNA made up a significant portion of the total DNA extracted. This DNA was deemed unsuitable for PCR amplification.

Figure 5.2 A Comparison of Three Total DNA Extraction Methods for Rideau River Water and Sediment Samples

Lane 1: Total DNA extraction using the method of Ansari *et al.* 1992.

Lane 2: Lambda *HindIII*-digested DNA.

Lane 3: Total DNA extraction using the method of Bruce *et al.* 1992.

Lane 4: Total DNA extraction using an adapted method of Porteous and Armstrong (pers. comm.).

Lane 5: Lambda *HindIII*-digested DNA.

Note that the quality of this scan is low because of the small size of the original print.

The method of Porteous and Armstrong yielded the most high molecular weight DNA for these samples.

1 2 3 4 5

Figure 5.3 Total High Molecular Weight DNA Extracted from Niagara Creek Samples Over an 18-Month Period

The average total high molecular weight DNA values for triplicate DNA extractions from four sampling trips (Nov 92 - Nov 93) including the standard error of the mean and the total high molecular weight DNA values from single DNA extractions from one sampling trip (May 92) are shown. Total high molecular weight DNA values were calculated from agarose gels using an Imaging Densitometer.

BRC and MR samples were combined for this analysis.

5.3.2 Determination of PCR amplification conditions

A range of PCR amplification cycles with 10 ng template DNA (*Alcaligenes* sp. BR60 (pBRC60)) was used in order to decide on the optimal number of amplification cycles to employ for environmental DNA amplification. As seen in Figure 5.4, no DNA was detected until 30 amplification cycles. Following 50 and 60 cycles of amplification (20 and 30 cycles of re-amplification of 10 μ l of the first amplification product), the detectable signal had stabilized at a 5^5 dilution level (ie. a $5 \times 5 \times 5 \times 5 \times 5$ dilution). The scanned results include the same number of dots that were visible on the x-ray film. Any differences in the background can be attributed to differences in the dot blot developing conditions.

5.3.3 The effect of amplification on target DNA

By varying the amount of *cbaA* target template DNA the minimal level of detection of the *cbaA* gene was 10 pg of *Alcaligenes* sp. BR60 (pBRC60) template DNA in the presence of 10 ng non-target template DNA (Figure 5.5A). Assuming an approximate genome size of 5×10^6 bp (Holloway *et al.* 1990), one copy of target gene per cell and a PCR product size of 1058 bp for the amplified *cbaA* product, this amounts to 2.1 fg of target *cbaA* DNA sequence. This is equivalent to approximately 17 cells of *Alcaligenes* sp. BR60 and 17 copies of the *cbaA* gene. The same number of dots (5) was detected for 10 ng and 1 ng amplified *Alcaligenes* sp. BR60 (pBRC60) template DNA (2.1 pg or 210 fg target DNA).

By varying the amount of *bphA* target template DNA (*Burkholderia* sp. LB400), and following the same PCR amplification and dot blot conditions, the minimal level of detection of the *bphA* gene was also 10 pg of *Burkholderia* sp. LB400 DNA (Figure 5.5B). This is equivalent to approximately 1.6 fg of target *bphA* DNA sequence assuming an approximate genome size of 5×10^6 bp, one copy of target gene per cell and a PCR product size of 805 bp. The same number of dots (5) was detected with 10 ng and 1 ng

amplified *Burkholderia* sp. LB400 template DNA (1.6 pg or 160 fg). This is approximately 17 cells of *Burkholderia* sp. LB400 and 17 copies of the *bphA* gene.

Figure 5.4 Effect of PCR Amplification Cycles on Target Gene Detection

A minimum number of 30 amplification cycles was required to detect the *chaA* gene using 10 ng of *Alcaligenes* sp. BR60 (pBRC60) template DNA for the dot blot hybridization method described in the Methods section. Following 50 and 60 cycles of amplification (20 and 30 cycles of re-amplification of 10 µl of the first amplification product), the detectable signal had stabilized at a 5^s dilution level.

Cycles

Figure 5.5 Minimal Level of Detection of *cbaA* and *bphA* Target Template DNA

A. Amount of target *cbaA* DNA detectable

Using variable amounts of *cbaA* target template DNA, 60 cycles of PCR amplification, 5^5 dilutions, dot blot and hybridization conditions in the Methods section, the minimal level of detection of the *cbaA* gene was 10 pg of *Alcaligenes* sp. BR60 (pBRC60) template DNA in the presence of 10 ng non-target template DNA (one dot). Assuming an approximate genome size of 5×10^6 bp and an amplified fragment of 1058 bp for the *cbaA* gene this amounts to 2.1 fg of target *cbaA* sequence. The same number of dots (5) were detected with 10 ng and 1 ng *Alcaligenes* sp. BR60 (pBRC60) template DNA.

B. Amount of target *bphA* DNA detectable

Using variable amounts of *bphA* target template DNA, 60 cycles of PCR amplification, 5^5 dilutions, dot blot and hybridization conditions in the Methods section, the minimal level of detection of the *bphA* gene was 10 pg of *Burkholderia* sp. LB400 template DNA in the presence of 10 ng non-target template DNA (one dot). This is approximately equivalent to 1.6 fg of target *bphA* sequence assuming a genome size of 5×10^6 bp and an amplification product of 800 bp for the *bphA* gene. The same number of dots (5) were detected with 10 ng and 1 ng *Burkholderia* sp. LB400 template DNA.

A.

<i>cbaA</i> Target DNA	BR60 Genomic DNA	5-fold dilutions
2.1 pg	10 ng	● ● ● ●
210 fg	1 ng	● ● ● ●
21 fg	100 pg	● ●
2.1 fg	10 pg	*
210 ag	1 pg	

B.

<i>bphA</i> Target DNA	LB400 Genomic DNA	5-fold dilutions
1.6pg	10 ng	● ● ● ●
160 fg	1 ng	● ● ● ●
16 fg	100 pg	● ● ●
1.6 fg	10 pg	●
160 ag	1 pg	

5.3.4 PCR amplification of Niagara watershed creek samples

Figure 5.6 shows the dot blot hybridization results for the *cbaA* amplification products of the samples collected over an 18-month period from BRC, DHC and FC. For all positive controls, the limit of detection for non-amplified *Alcaligenes* sp. BR60 (pBRC60) DNA was 9 ng. This is 1.9 pg of target DNA sequence assuming a fixed genome size of 5×10^6 bp, that there is one gene copy per cell and that the length of the amplification product is 1058 bp. All sites during the three seasons sampled showed detectable levels of the *cbaA* gene (minimum one dot). On the basis of the results presented in Figure 5.5A it can be assumed that all environmental samples contained at least 2.1 fg target *cbaA* DNA in 1 ng template DNA before amplification. FC samples yielded the lowest number of positive signals with the possible exception of the May and August 1993 data. On these two occasions contaminated creeks equaled this low response; for DHC in May 1993, and BRC in August 1993. August 1993 samples harboured less *cbaA* DNA in all creeks compared to the spring and fall samples even when the poor quality of the image (high background) is considered. No other seasonal or yearly variations in this gene were observed.

Figure 5.7 shows the dot blot hybridization results for the *bphA* amplification products of the samples collected over an 18-month period from BRC, DHC and FC. For all positive controls, the limit of detection was 9 ng non-amplified *Burkholderia* sp. LB400 DNA (1.4 pg target DNA sequence). On the basis of the results presented in Figure 5.5B it can be assumed that all environmental samples contained at least 16 fg target *bphA* DNA in 1 ng template DNA before amplification as all sites showed at least 3 dots. The same creek samples appear to have contained more copies of the *bphA* gene sequence than the *cbaA* gene sequence. There was no apparent difference between the level of *bphA* target DNA in any of the creeks. As well, there was no seasonal or yearly variation noted for this gene.

Figure 5.6 Detection of the *cbaA* Gene in PCR Amplified Total DNA Extracted from Niagara Creek Samples by Dot Blot Hybridizations.

Amplified PCR products were 5-fold serially diluted and hybridized with the *cbaA* gene probe. The results from combined spring, fall (1992 and 1993) and summer (1993) samples are shown. With the exception of the May and August 1993 samples, FC, the noncontaminated creek had less *cbaA* genotype than the chloroaromatic contaminated BRC and DHC samples.

The *cbaA* genotype was detected at all sample sites. Thus there was a minimum of 2.1 fg target DNA sequence in 1 ng template DNA. A maximum of 210 fg target DNA was detected in some of the contaminated sites (BRC, November 1992 and May 1993).

The minimal level of detection of non-amplified *Alcaligenes* sp. BR60 genomic DNA was 9 ng or 1.9 pg target DNA sequence assuming there is one copy of the genotype per cell.

5-fold dilutions

Figure 5.7 Detection of the *bphA* Gene in PCR Amplified Total DNA Extracted from Niagara Creek Samples by Dot Blot Hybridizations.

Amplified PCR products were 5-fold serially diluted and hybridized with the *bphA* gene. The results from combined spring, fall (1992 and 1993) and summer (1993) samples are shown.

The *bphA* gene was detected at all sample sites. The noncontaminated creek, FC, had a similar level of target DNA as the contaminated creeks, BRC and DHC. The minimum amount of target DNA was close to 16 fg as all sites had at least 3 dots.

The minimal level of detection of non-amplified *Burkholderia* sp. LB400 genomic DNA was 9 ng or 1.4 pg target DNA sequence assuming there is one copy of the gene per cell.

5.3.5 PCR amplification of Hyde Park groundwater and SBR samples

Figure 5.8 A shows the dot blot hybridization results for the *cbaA* amplification products of the samples collected from groundwater (Wells A,B,D) and SBR (1,2,3) sites at Hyde Park during May 1993. For all positive controls, the limit of detection was 9 ng *Alcaligenes* sp. BR60 (pBRC60) or *Burkholderia* sp. LB400 DNA. Comparable to the creek results, all samples showed detectable levels of both genotypes. Similarly, the occurrence of the *cbaA* gene was not as prominent as the *bphA* gene. All Hyde Park samples had at least 2.1 fg and 16 fg of target *cbaA* and *bphA* DNA in 1 ng environmental template DNA.

5.3.6 PCR amplification of Hamilton Harbour samples

Relatively pristine and PCB-contaminated samples from the Hamilton Harbour were subjected to total DNA extraction, PCR amplification and dot blot hybridization (Figure 5.9). Both the *cbaA* and *bphA* genotypes were detected in these samples. The PCB-contaminated samples from both years seem to have contained noticeably more of the *cbaA* gene than the non-PCB-contaminated environment (21 fg versus 2.1 fg target DNA). There was very little difference in the proportion of the *bphA* gene in any of the samples. There were more copies of the *bphA* gene compared to the *cbaA* gene in all samples (approximately 16 to 160 fg).

5.3.7 Southern hybridization detection of amplification products

Samples (2 µl) of positive and negative controls and random environmental DNA samples from all sites were detected by Southern hybridization to ensure that the dots detected by dot blot hybridization were the expected amplification product. PCR products of the expected size hybridized to the gene probes, however there was some smearing of the signal, in the area of the expected product and near the bottom of the gel (signifying very low molecular

weight products). This is typical for re-amplified PCR products and is likely due to primer dimer formation (Erb and Wagner-Döbler 1993).

Figure 5.8 Detection of the *cbaA* and *bphA* Genes in PCR Amplified Total DNA Extracted from Hyde Park Samples by Dot Blot Hybridizations.

A. *cbaA* Gene detection

The *cbaA* gene was detected at all sample sites (at least three dots). The target DNA in 1 ng template DNA ranged from 21 to 210 fg.

The minimal level of detection of non-amplified *Alcaligenes* sp. BR60 genomic DNA was 9 ng or 1.9 pg target DNA sequence assuming there is one copy of the gene per cell.

B. *bphA* Gene detection

Amplified PCR products were 5-fold serially diluted and hybridized with the *bphA* gene

The *bphA* gene was detected at all sample sites (at least 4 dots). The target DNA in 1 ng template DNA was in the range of 160 fg

The minimal level of detection of non-amplified *Burkholderia* sp. LB400 genomic DNA was 9 ng or 1.4 pg target DNA sequence assuming there is one copy of the gene per cell.

Samples were collected in May 1993.

A.

Well Samples

SBR Samples

B.

Well Samples

SBR Samples

Figure 5.9 Detection of the *cbaA* and *bphA* Genes in PCR Amplified Total DNA Extracted from Hamilton Harbour Samples by Dot Blot Hybridizations.

A. *cbaA* Gene detection

The *cbaA* gene was detected at all sample sites. There was approximately 2.1 fg per 1 ng template DNA in the non-contaminated (cl) samples (one dot). The PCB-contaminated (ct) samples had approximately 21 fg target sequence.

The minimal level of detection of non-amplified *Alcaligenes* sp. BR60 genomic DNA was 9 ng or 1.9 pg target DNA sequence assuming there is one copy of the gene per cell.

B. *bphA* Gene detection

Amplified PCR products were 5-fold serially diluted and hybridized with the *bphA* gene

The *bphA* gene was detected at all sample sites in approximately an equal amount (3 to 4 dots). The amount of target DNA in 1 ng template DNA ranged from approximately 16 to 160 fg.

The minimal level of detection of non-amplified *Burkholderia* sp. LB400 genomic DNA was 9 ng or 1.4 pg target DNA sequence assuming there is one copy of the gene per cell

Samples were collected in June 1992 and 1993

cl PCB-contaminated sites

ct non PCB-contaminated sites

A.

B.

OF / DE

3

PRECISIONSM RESOLUTION TARGETS

5.4 DISCUSSION

In this chapter a total DNA extraction method for water sediment environmental samples that yielded DNA that was minimally sheared and was sufficiently free of humic and tannic acid contamination for PCR amplification was sought. For practical reasons the method also had to be relatively rapid due to the large number of samples to process. In order to meet these requirements, I reviewed a number of existing protocols for both water and soil samples and developed a total DNA extraction method that best suited the Niagara watershed environmental samples for PCR amplification. A comparison of three total DNA extraction protocols using Rideau river samples is shown in Figure 5.2.

The revised method of Porteous and Armstrong yielded suitable high molecular weight DNA. The recovery of high molecular weight total DNA ranged from 2.55 ng/ml to 24.5 ng/ml as determined by an imaging densitometer (Figure 5.3). The efficiency of extraction using this method cannot be directly compared to other total DNA extraction protocols as the samples contained a variable mixture of water and sediment. Previously it was found that total culturable cell counts were one order of magnitude greater in sediment samples than in water samples from the Niagara watershed (Fulthorpe *et al* 1989). More DNA was recovered from the Hamilton Harbour sites, possibly because these samples contained a higher proportion of very fine grain sediments and therefore harboured more bacteria.

In this investigation only the high molecular weight DNA was quantitated as it was essential to maximize the PCR efficiency by using relatively unsheared DNA. As can be seen in Figure 5.1, lower molecular weight DNA that had been sheared during the extraction procedure was also present. This low molecular weight DNA was not well-suited for PCR amplification and was not quantified. Ansari *et al* (1992) reported a recovery of 1-2 mg DNA/L water. Bruce *et al.* (1992) estimated that they had a yield of 2-21 µg/g soil. Finally, Porteous and Armstrong (1991) claimed to have recovered up to 25 µg total DNA/g soil.

Direct extraction of total DNA from environmental samples enables microbial ecologists to obtain genetic information without the need to isolate microorganisms by

cultivation. This extraction, however, always results in co-extraction of other soil components, mainly humic acids, which interfere with DNA transforming enzymes and gene detection methods (restriction endonucleases or polymerases are inhibited) (Steffen and Atlas 1991). Sufficient removal of humic acid contamination from high molecular weight DNA was achieved by agarose gel electrophoresis as seen in Figure 5.1. Following the first DNA extraction the DNA was sufficiently pure for PCR amplification. It was noted that additional humic acid contamination was removed following a second extraction. The fact that PCR amplification of environmental template DNA was possible after only one extraction step and the observed poor additional DNA recovery following the second and third extraction steps warranted elimination of these additional extraction steps. The success of PCR amplification may be due to the re-amplification of the PCR product following the first 30 amplification cycles. Re-amplification of the PCR products may serve to dilute the initial humic acid contamination. This may be one factor explaining why so many cycles (30) were required to detect significant amounts of the target DNA (Figure 5.4). Reports of PCR amplification of environmental DNA range from 25 cycles (only one round of amplification) (Holben *et al.* 1992) to 70 cycles (35 cycles during the first round and 35 cycles of re-amplification) (Picard *et al.* 1992, Erb and Wagner-Döbler 1993).

The documented amount of template environmental DNA used in PCR reactions is similarly diverse. McDonald *et al.* (1995) reported using up to 50 ng of template DNA while Bruce *et al.* 1992 used as little as 0.1 ng template DNA per 100 µl PCR reaction. Others have used a fixed volume of extracted DNA (often 1 µl). An optimal balance between the concentration of target DNA and residual PCR-inhibitory substances should be considered. Minimizing the amount of template DNA also dilutes co-extracted contaminants (Knaebel and Crawford 1995). The concentration of high molecular weight DNA in my Niagara watershed extractions also limited the total template available for PCR amplification to 1 ng. The hybridization results in Figure 5.5 showed the same number of dots (5) for 10 ng and 1 ng template DNA. It is possible that the limit of detection of target DNA using these PCR amplification and hybridization conditions was between 210 fg and 2.1 pg for the *cbaA* and 160 fg and 1.6 pg for the *bphA* genes.

The minimum amount of non-amplified genomic DNA that was detectable with the *chaA* and *bphA* gene probes was 9 ng of *Alcaligenes* sp. BR60 (pBRC60) DNA and *Burkholderia* sp. LB400 DNA. This represents approximately 1.9 pg of *chaA* target DNA and 1.4 pg *bphA* target DNA assuming a genome size of 5×10^6 bp (Holloway *et al.* 1990) and that there is one copy of the target gene per cell (Figures 5.6-5.9). The assumption that there is one copy of the *bphA* gene per genome is accurate for *Burkholderia* sp. LB400 as this gene is chromosomally-encoded. In the case of *Alcaligenes* sp. BR60, the number of copies of pBRC60 in one BR60 bacterium is not known and likely is dependent on the physiological state of the host bacterium. Although I assumed a *chaA* gene copy number of one per genome, the number of copies of high molecular weight plasmids (such as pBRC60) can range from 1-10 copies per bacterium (Couturier *et al.* 1988). Known amounts of non-amplified plasmid pJP4 (85 kbp) DNA were included as a positive control for PCR amplification of the *tfdA* gene and for comparative quantitation of amplification products (Holben *et al.* 1992). Assuming a copy number of one target sequence per cell the minimum level of detection was 1.2 ng of non-amplified pJP4 DNA using a 801 bp *tfdA* gene probe. This is approximately 11.3 pg of target *tfdA* sequence. Pichard and Paul (1993) detected 50 pg of *xyfE* (catechol-2,3-dioxygenase) target DNA sequence (1567 bp) using dot blot hybridization. Finally, Bruce *et al.* (1992) had a minimum detection level of 10 pg target sequence for DNA extracted from seeded sterile water samples.

The limit of detection of *chaA* and *bphA* target DNA in 10 ng total template DNA following PCR amplification and dot blot hybridization was 2.1 fg and 1.6 fg, respectively (Figure 5.5). This is based on the assumption of that there is one copy of these genes per cell. It was observed in Chapter 4 that many of the *chaAB* probe positive isolates and 75% of the *bphA* probe positive isolates harboured high molecular weight plasmids. Although it was not confirmed that the catabolic genotypes were encoded on these high molecular weight plasmids, there is a high probability that they were. Previous reports of *chaAB* probe positive isolates determined that this genotype was located on high molecular weight plasmids (Wyndham *et al.* 1988b, Di Gioia pers. comm.).

PCR amplification and hybridization results showed that the *chaA* and *bphA* target sequences were present in detectable levels in all Niagara watershed and Hamilton Harbour

samples (Figures 5.6-5.9). This suggests that at least 2.1 fg of *chaA* and 1.6 fg of *bphA* target DNA was present in 1 ng of environmental DNA. Amplification of DNA from the creek samples showed that Hyde Park contaminated creeks, BRC and DHC often had more copies of the *chaA* gene detected than the non-contaminated creek, FC. The Hamilton Harbour results indicated that there was 10-fold more (21 pg) target *chaA* template DNA in the PCB-contaminated sites compared to the non-contaminated sites. By contrast, the *bphA* gene appeared to be uniformly distributed in all creeks, at Hyde Park and in all Hamilton Harbour sites. It was also present in a higher copy number than *chaA* target DNA in the same environmental samples. Although the summer creek samples amplified with the *chaA* primers contained fewer dots compared to the spring and fall creek samples, no other seasonal or yearly variations in target DNA detection were noted.

The widespread distribution of the *bphA* gene observed in this study has been noted for other chloroaromatic catabolic genes. The 2,4-dichlorophenoxyacetic acid (2,4-D) catabolic genes *tfdA* (2,4-dichlorophenoxyacetate monooxygenase) and *tfdB* (2,4-dichlorophenol hydroxylase) have been found by total DNA extraction and subsequent PCR amplification to have a ubiquitous distribution. Several months after a field application of 2,4-D (1 ppm), to soils with a 42-year history of 2,4-D contamination and soils with no prior exposure to 2,4-D, PCR amplification of total DNA revealed that the soils contained the same levels of both genes (Holben *et al.* 1992). These results showed that these genes were present initially in the 2,4-D contaminated and non-contaminated soils and had been enriched by 2,4-D treatment.

In a similar investigation examining the distribution of the *bphC* (2,3-dihydroxybiphenyl dioxygenase) gene from *Burkholderia* sp. LB400 in PCB-contaminated and non-contaminated sediments, the occurrence of the *bphC* gene sequence was not correlated with the level of PCB contamination at the time of sampling (Erb and Wagner-Döbler 1993). However, all sites exhibiting the presence of this gene had received PCB-contamination at some time and had presumably adapted to PCB-contamination. Unlike the *tfdA* and *tfdB* gene distribution, DNA extracted from sites that had no history of PCB-contamination showed no evidence of the *bphC* gene following amplification and hybridization. It is possible that very low, undetectable levels of this gene were present

and would only have been detected following treatment of this soil with biphenyl which would have selected for and enhanced the copy numbers of the *bphA* gene

The presence of *chaA* and *bphA* genes in all samples from the Niagara watershed and Hamilton Harbour suggests prior exposure to contaminants that selected for these genes at all sites. Herbicide application to the golf course adjacent to FC may account for the presence of the *chaA* gene in this creek. Residual copies of the *chaA* gene may still exist and could be detected following PCR amplification and product hybridization. The lower levels of detection of the *chaA* gene in the current non-chloroaromatic contaminated sites (FC and Hamilton Harbour control sites) may be a result of lower gene selection pressures. The *chaA* gene detection results in this chapter support the batch culture enrichment results described in Chapter 2 that showed that the FC samples had a lower 3-Cba degradation potential than the BRC and DHC samples. However, the fact that the *cfcABD* genes and many other non-identified chlorobenzoate catabolic genes were common in the Niagara watershed means the level of *chaA* gene and the 3-Cba degradation potential cannot be directly compared

PCR amplification of continuously cultured samples from Hyde Park (SBR2) showed that approximately 1 in 10 culturable bacteria harboured the *chaA* gene. This showed the importance of the *chaAB* genotype in chlorobenzoate mineralization (Wyndham *et al.* 1994b). The results in Figure 5.8 A show that the *chaA* gene remains a significant chlorobenzoate catabolic gene in this environment

Equivalent levels of the *bphA* gene were detected in most samples, regardless of the current chloroaromatic contamination status of the sites. The continued high levels of the *bphA* gene may be due to continued non-anthropogenic selection. Natural compounds may be continually selecting for the *bphA* gene. It is known that BP catabolic isolates are widespread, possibly due to structurally similar natural aromatic hydrocarbons such as lignin (Higson 1992). It was observed in Chapter 2 that the biphenyl batch culture enrichments from all sites appeared to be utilizing BP as a sole carbon source. The high number of putative BP catabolic isolates recovered in Chapter 3 further supports these results.

The following chapter will investigate the co-existence of the *chaA* and *cicA* catabolic genes in a continuous culture enrichment of a Hyde Park sample

Co-existence of *chaA* and *clcA* Genes in a Continuous Culture Enrichment

6.1 INTRODUCTION

Natural aquatic systems are usually nutrient limited, or oligotrophic. Carbon availability can affect microbial nutrient uptake and growth rates (Braddock and Brown 1994). Limiting substrates including carbon, nitrogen and phosphorous may induce changes in nutrient uptake systems and alter the activities and regulation of enzymes in microbial cells (Duetz *et al.* 1994, Braddock and Brown 1994). Bacteria that are adapted to oligotrophic conditions have physiological properties that permit them to make use of limiting resources more efficiently (Atlas and Bartha 1987). Competition for limiting nutrients determines total biomass and the structure of microbial communities.

In oligotrophic aquatic environments, a large proportion of bacteria may be associated with surfaces in a biofilm (Holm *et al.* 1992). Exopolymers in aquatic biofilms can trap nutrients flowing past thereby replenishing scarce resources. This enables the cells in biofilms to compensate for the nutrient poor conditions (Marshall 1992). In a river receiving organic waste, slow-growing bacteria can compete with fast-growing bacteria if they are attached to stones (epilithic), plants (epiphytic) or sediments (benthic) locations where nutrients are available at concentrations sufficient for growth.

It was noted in Chapter 3 that both the *clcABD* and *chaAB* genotypes were present in the Niagara watershed. Isolates harbouring these genotypes had the same chlorobenzoate substrate range including 3-Cba and 4-Cba. Furthermore, batch culture enrichments from the same sites, but at different enrichment temperatures, contained both genotypes (* BRC3-3-5¹ (*clcABD*⁺) and * BRC5-2-5¹ (*chaAB*⁺) from 3-Cba(3CBP) enrichments and * WellD-1-3¹ (*clcABD*⁺), * WellD-1-3² (*clcABD*⁺) and * WellD-1-3³ (*chaAB*⁺) from 3-Cba enrichments). The host ranges for these two mobile genotypes

differ (Nakatsu *et al.* 1995b) and this may influence competition for limiting nutrients.

The phenotypic distribution in Chapter 4 confirmed that the Γ -Proteobacteria are the usual hosts for the *clcABD* genotype in the Niagara isolates and the β -Proteobacteria are most often the natural hosts for the *chaAB* genotype.

Unlike batch culture enrichments there is a continuous flow of nutrients in chemostat or continuous culture enrichments. This type of enrichment enables researchers to maintain cultures indefinitely under conditions of constant growth rate and nutrient availability. Competition for nutrients in a chemostat will select from the original mixed inoculum those organisms showing the greatest affinity for the limiting nutrient at the dilution rate chosen. Organisms unable to compete are washed out, or they may adhere to the vessel walls. Chemostat enrichments have been used to broaden the biodegradative potential of bacteria or to "naturally engineer" strains capable of mineralizing recalcitrant pollutants through strong selection. An example of the forced evolution of an environmental isolate was presented by Chatterjee and Chakrabarty (1982). These researchers grew the 3-Cba catabolic isolate *Pseudomonas putida* strain AC858 harbouring the plasmid pAC25 and cells harbouring the TOL plasmid in a chemostat with 4-Cba as the sole carbon source. These conditions allowed the emergence of cells that could also utilize 4-Cba (*Pseudomonas putida* AC866 (pAC27)). A more forceful example was the selection under chemostat cultivation for 8 to 10 months in the presence of a pool of plasmid genes of *Pseudomonas cepacia* strain AC1100 that could not only utilize 2,4,5-trichlorobenzoate but could also completely dechlorinate chlorophenols such as 2,4,5-trichlorophenol and pentachlorophenol (Kams *et al.* 1983).

A continuous culture enrichment inoculated with a sample from one of the sequencing batch reactors and receiving a simulated Hyde Park toxic waste feed was used to isolate chlorobenzoate catabolic genotypes. The enrichment culture was screened for the *chaA* gene by PCR amplification, and was used as an inoculum to isolate *chaAB* harbouring microorganisms (Wyndham *et al.* 1994b). The same culture conditions were used to examine the co-existence of the *chaA* and *clcA* genes in a mixed flow-through microbial community. This culture was monitored for the two genotypes over a period of

84 days through total DNA extraction and subsequent amplification of the two genes by PCR. The objectives were to

- 1 Examine the co-existence of two genes expressing alternative metabolic enzymes for the degradation of 3-Cba. and 4-Cba (the *chlA* and *chaA* genes) in a continuous culture enrichment

- 2 Compare the frequency of these genes with culturable colony plate counts and total genomic DNA isolated from the culture

6.2 MATERIALS AND METHODS

6.2.1 Continuous culture conditions

A Versatec 1.2 L continuous culture fermentor was used to culture the mixed groundwater community from Well A at Hyde Park. The dilution medium (FG2) was designed to mimic the amended feed for the sequencing batch reactors at Hyde Park, including the major organic and inorganic contaminants present (Wyndham *et al.* 1994b). FG2 was prepared as follows: 1.4 strength medium A+N pH 8.0, 0.005% yeast extract, 0.016 mM copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 0.006 mM lead (Lead Acetate $\cdot 3\text{H}_2\text{O}$), 0.48 mM zinc ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 0.74 mM 2-chlorobenzoate, 0.22 mM 3-Cba, 0.25 mM 4-Cba, 2.38 mM benzoic acid. Following autoclaving, 0.025 mM toluene, 1.0 mM phenol and 0.006 mM 2,4-dichlorophenol were added. The final pH was 7.8. The dilution rate of the continuous culture was 0.05h^{-1} , giving a retention time equal to that of the sequencing batch reactors at Hyde Park (Ying *et al.* 1992). This continuous culture enrichment was maintained at room temperature (22 °C) for 84 days.

6.2.2 Sampling and total DNA isolation

Samples of 50 ml were collected aseptically from the continuous culture after 0, 9, 15, 22, 31, 38, 45, 53, 61, 69, 76 and 84 days of incubation. 50 μl of each sample was serially diluted in 1:10th strength medium A+N. Total counts were obtained from the 10^{-2} to 10^{-5} dilutions using the spread plate technique on Casitone-Peptone-Starch (CPS) agar. These plates were incubated at room temperature and counted after 18 days. The remaining samples were centrifuged, the supernatants removed, and the cell pellets resuspended in 1 ml of 20% glycerol and frozen at -70°C . At the end of the enrichment period (84 days), a biofilm sample was collected by scraping the sides of the vessel with a sterile spatula. This sample was also resuspended in 20% glycerol and frozen at -70°C . At the end of the continuous culture experiment, these glycerol stocks were thawed and

centrifuged to remove the glycerol. The pellets were resuspended in 100 μ l TE and the previously described method for isolating total genomic DNA was performed (Ausubel *et al.* 1989). The resulting DNA pellets were dissolved in 60 μ l sterile distilled water. DNA quantitation was determined spectrophotometrically (A_{260}).

6.2.3 Detection of *clcA* and *chaA* genes

The *clcA* and *chaA* genes were detected by polymerase chain reaction (PCR) and subsequent dot blot hybridization. The primers and amplification conditions for the *clcA* (chlorocatechol-1,2-dioxygenase) gene and *chaA* (chlorobenzoate-3,4-(4,5)-dioxygenase) gene were described in Chapter 4. Template genomic DNA (10 ng), calculated from the A_{260} value, was amplified for all samples. Following the first amplification, 10 μ l of each product was re-amplified under the same conditions.

PCR products were chloroform extracted to remove the mineral oil from the final amplification products. DNA was ethanol precipitated and resuspended in 60 μ l sterile distilled water. 12 μ l of this DNA was 5-fold serially diluted in sterile distilled water in volumes of 100 μ l. Dot blots were performed as described in Chapter 5. *Alcaligenes* sp. BR60 (pBRC60) and *Pseudomonas putida* AC866 (pAC27) genomic DNA were 10-fold serially diluted beginning with 90 μ g of DNA. Negative control samples, lacking DNA, were also 10-fold serially diluted. Dot blot hybridizations were conducted as described in Chapter 5. The sizes of the *clcA* and *chaA* amplification products were confirmed by Southern hybridization as described in Chapter 5.

6.2.4 Minimal level of detection of the *clcA* and *cbaA* genes

This was performed as described in Chapter 5. A total of 10 ng template DNA was amplified with different concentrations of target *clcA* or *cbaA* DNA (10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg *Pseudomonas putida* AC866 (pAC27) or *Alcaligenes* sp. BR60 (pBRC60)) in order to determine the minimal amount of target DNA that could be detected using the PCR amplification and dot blot techniques described above. Non-target template DNA was isolated from non-*cbaAB* and non-*clcABD* probe positive isolates described in Chapter 3.

6.3 RESULTS

6.3.1 Culturable colony counts and DNA quantitation

The initial culturable colony count after inoculation was 1.13×10^8 CFU/ml. With the exception of days 22, 69 and 76, the culturable colony counts were approximately in this range (Table 6.1). The concentration of total DNA recovered from the continuous culture varied throughout the 84 day experimental period (Table 1). The DNA concentration on day 0 was the highest observed during the entire experimental period (1750 ng/ml).

6.3.2 The effect of amplification on target DNA

Using variable amounts of *clcA* target template DNA, and following the PCR amplification, dilution, dot blot and hybridization conditions in the Methods section, the minimal level of detection of the *clcA* gene was 100 pg of *Pseudomonas putida* AC 800 (pAC27) template DNA in the presence of 10 ng non-target template DNA (Figure 6.1). Assuming an approximate genome size of 5×10^6 bp (Holloway *et al.* 1990), that there is one copy of this gene per cell and a size of 362 bp for the amplified *clcA* product, this amounts to 7.2 fg of target *clcA* DNA.

The same *chaA* probe was used for dot blot hybridization in Chapters 5 and 6. In Chapter 5 (Figure 5.5A) it was shown that the minimal level of detection of the *chaA* gene was 10 pg of *Alcaligenes* sp. BR60 (pBRC60) template DNA in the presence of 10 ng non-target template DNA. This corresponds to 2.1 fg of target *chaA* DNA.

Table 6.1 Chemostat Total Culturable Bacteria Counts and DNA Concentrations

Date	Culturable Colony Counts CFU ml ⁻¹	Total DNA (ng/ ml)
10/03/92 (T = 0)	1.13×10^8	1750
19/03/92 (T = 9 days)	1.66×10^8	912
25/03/92 (T = 15 days)	1.34×10^8	756
01/04/92 (T = 22 days)	3.58×10^8	1045
10/04/92 (T = 31 days)	1.02×10^8	469
17/04/92 (T = 38 days)	1.88×10^8	448
24/04/92 (T = 45 days)	2.02×10^8	448
02/05/92 (T = 53 days)	1.68×10^8	1091
10/05/92 (T = 61 days)	8.80×10^7	46.0
18/05/92 (T = 69 days)	4.20×10^7	437
25/05/92 (T = 76 days)	4.20×10^7	0.45
02/06/92 (T = 84 days)	8.40×10^7	147
Biofilm (T = 84 days)	-	3678

Figure 6.1: Minimal Level of Detection of *clcA* Target Template DNA

Using variable amounts of *clcA* target template DNA, and following the PCR amplification, dilution, dot blot and hybridization conditions outlined in the Methods section and in Chapter 5, the minimal level of detection of the *clcA* gene was 100 pg of *Pseudomonas putida* sp. AC866 (pAC27) DNA (two dots). Assuming an approximate genome size of 5×10^6 bp, one copy per genome and an amplified fragment of 362 bp for the *clcA* gene this amounts to 7.2 fg of target *clcA* DNA sequence.

A maximum of 4 dots was detected with 10 ng *Pseudomonas putida* sp. AC866 (pAC27) DNA

<i>clcA</i> Target DNA	AC866 Genomic DNA	5-fold dilutions			
720 fg	10 ng	●	●	●	◊
72 fg	1 ng	●	●	●	
7.2 fg	100 pg	●	●		
720 ag	10 pg				

6.3.3 *clcA* and *cbaA* gene detection

The limit of detection for non-amplified genomic *Pseudomonas putida* AC866 (pAC27) and *Alcaligenes* sp. BR60 (pBRC60) DNA was 90 ng (6.5 pg target sequence) and 9 ng (1.9 pg target sequence), respectively (Figures 6.2 and 6.3). All continuous culture DNA samples had at least 7.2 fg of *clcA* DNA assuming a fixed genome size of 5×10^6 bp, one copy of target sequence per cell and the length of the PCR amplification product (362 bp). Following this same rationale, all samples showed a minimum of 2.1 fg *cbaA* target DNA.

The *clcA* gene was consistently present throughout the experimental period (Figure 6.2). The *clcA* gene was present at a higher copy number than the *cbaA* gene during the first 31 days of continuous culture enrichment. There was approximately 7.2 fg of target *clcA* sequence in 10 ng template DNA from day 0, at the time of inoculation. The *clcA* target copies in 10 ng template DNA remained between 7.2 and 72 fg for the duration of the experiment. The biofilm sample contained approximately 72 fg of target *clcA* sequence in 10 ng template DNA. This was 10-fold more target sequence than was present in the liquid culture medium at the same sampling time (day 84). Thus over the course of continuous culture enrichment, the amount of *clcA* target DNA in 10 ng template DNA ranged from 7.2 fg to 72 fg.

The *cbaA* gene was barely detectable prior to day 38 (Figure 6.3). These samples contained approximately 2.1 fg target *cbaA* sequence in 10 ng template DNA. From day 38-84 the *cbaA* gene copy number increased to 21 fg. After 84 days of continuous culture enrichment, the biofilm on the sides of the culture vessel contained approximately a 10-fold greater copy number of the *cbaA* gene (210 fg) compared to the liquid culture medium (21 fg) for an equivalent amount of total genomic template DNA. The amount of *cbaAB* target DNA in 10 ng template DNA ranged from 2.1 fg to 210 fg during the experimental period.

Figure 6.2 *clcA* Gene Detection in Chemostat Enrichments

10 ng of total genomic DNA from chemostat enrichment samples was amplified with *clcA* primers. Five-fold serial dilutions of the amplification products were dot-blotted onto nylon membranes and subjected to hybridization and chemiluminescence detection with a Digoxigenin-labelled *clcA* probe. The positive (+) control was a ten-fold serial dilution of non-amplified 90 µg *Pseudomonas putida* AC866 (pAC27) genomic DNA. The negative (-) control was a ten-fold serial dilution of distilled water amplified under the same conditions as the total genomic DNA from the continuous culture enrichment.

Non-amplified *Pseudomonas putida* AC866 DNA was detected down to 90 ng or 6.5 pg non-amplified target *clcA* DNA sequence, assuming one target copy/genome.

Figure 6.3 *chaA* Gene Detection in Chemostat Enrichments

10 ng of total genomic DNA was amplified with *chaAB* primers from chemostat enrichment samples. Five-fold serial dilutions of the amplification products were dot-blotted and subjected to hybridization and chemiluminescence detection with a Digoxigenin-labelled *chaA* probe. The positive (+) control was a ten-fold serial dilution of 90 µg of *Alcaligenes* sp. BR60 (pBRC60) genomic DNA. The negative (-) control was a ten-fold serial dilution of distilled water amplified under the same conditions as the total genomic DNA from the continuous culture enrichment. Non-amplified *Alcaligenes* sp. BR60 (pBRC60) DNA was detected down to 9 ng or 1.9 pg non-amplified target *chaA* DNA sequence, assuming 1 target copy/genome.

6.4 DISCUSSION

A continuous culture inoculated with a groundwater sample from Hyde Park was sampled over an 84-day period in order to monitor the incidence of the *clcA* and *cbaA* genes. The culture feed, which closely mimicked the amended groundwater feed used in the industrial bioremediation facility operating at Hyde Park, contained mixed organic and inorganic pollutants including several chlorobenzoate congeners. Culturable counts were determined, and total cellular bacterial DNA was extracted in order to establish if there was a relationship between the total count and the proportion of the two genes, both of which are involved in chlorobenzoate degradation.

The variations in total genomic DNA recovered are likely a reflection of methodology limitations rather than a reflection of total genomic DNA variations in the continuous culture (Table 6.1). The fact that the culturable colony counts did not decrease significantly during day 76 and the total DNA recovered did strongly suggests a technical glitch in DNA isolation. The culturable colony count peaked at day 22. The fluctuations observed in total culturable colony counts suggests that the cell population in the chemostat was changing either due to culture instability or due to normal fluctuations between culturable and non-culturable cells. There was a consistently lower number of culturable cells in the continuous culture between days 61 and 84. This may be a reflection of cells entering viable but non-culturable state. Nutrient stress may render cells non-culturable. These viable but non-culturable cells are usually physiologically different than the same strains in a "normal" state. Microscopic examination has revealed that viable but non-culturable cells are smaller and rounder than the same strain of cells in nutrient rich medium (Roszak and Colwell 1987). It was concluded that synthetic and metabolic growth processes continue in the absence of cell division under stressful conditions. DNA synthesis is temporarily blocked, preventing cell division whereas other growth processes, including RNA and protein synthesis, continue.

The fluctuating total culturable colony counts between days 61 and 84 in the continuous culture liquid medium may also be due to the migration of cells from the liquid medium to the biofilm on the sides of the vessel. Continuous culture enrichments

operating at low dilution rates select for slow-growing populations with high affinity nutrient uptake systems and against specialist microorganisms that grow actively only during periods of high nutrient influxes. The latter may be washed out of the culture vessel at low dilution rates

There was no clear correlation between the total DNA concentrations and the qualitative assessment of the copy number of the *clcA* and *cbaA* genes. This supports the batch culture enrichment results in Chapter 3, which showed that less than 5% of the chlorobenzoate catabolic isolate collection hybridized to either the *clcABD* or *cbaAB* genes.

Initially, there were approximately 7.2 fg and 2.1 fg of target *clcA* and *cbaA* gene sequences in 10 ng template DNA, respectively. During the first 31 days of continuous culture enrichment, there were between 7.2 and 72 fg of *clcA* target sequence. The *cbaA* gene remained steady at 2.1 fg target sequence (Figures 6.2 and 6.3). For the second half of the enrichment period (days 38 to 84), the *cbaA* gene was present in a higher copy number (21 to 210 fg target sequence) than the *clcA* gene (7.2 fg target sequence) with the exception of day 69. The decrease in the *cbaA* gene but not the *clcA* gene during this sampling suggests that the *cbaA* gene may not have been stable in the continuous culture enrichment. The fact that these two genes were present in approximately the same copy number at the end of the enrichment period (72 fg and 21 fg of *clcA* and *cbaA* target sequences, respectively) indicates that there was no tendency for one gene to replace the other in the continuous culture.

During the second half of the enrichment period the *cbaA* gene was selected following adaptation of the inoculum to the FG2 medium. At the end of the experimental period this gene had increased in copy number by at least 100-fold compared to the initial quantity (2.1 versus 210 fg target DNA sequence). In contrast the *clcA* gene was present in the same copy number at the end of the experiment as on day 0 (7.2 fg target DNA sequence).

These results suggest that the two alternative pathways for chlorobenzoate degradation can co-exist. A mixed culture such as the Well A-derived community growing on a complex contaminant mixture will have numerous spatial and chemical

niches available for colonization, so that co-existence of strains competing for the same chlorinated compounds is possible. Additionally, it has been suggested that under environmental conditions heterotrophic microbes are not specializing in the metabolism of one particular carbon substrate for growth but are simultaneously utilizing many. In a model system for three distantly related bacterial strains in a carbon-limited continuous culture, there was evidence that pollutant-degrading microorganisms were usually mineralizing the pollutant together with alternative carbon sources of a natural origin (Egli *et al.* 1995). Furthermore, it appeared that the level of expression of the pollutant-catabolizing enzymes was significantly affected by the ratio of pollutant to utilizable carbon of natural origin. In my chemostat enrichment the hosts of the *clcA* and *cbaA* genes may have been utilizing carbon sources other than chlorobenzoates including benzoate, and sugars and organic acids in the yeast extract.

The predominant host strains of the *clcA* gene confirmed in this Thesis and in other studies are members of the Γ -Proteobacteria. This subclass of the Proteobacteria has the characteristics of r-strategists and are considered to be opportunistic. Rapid growth rates allow them to dominate when resources (nutrients) are plentiful (Atlas and Bartha 1987). By contrast, members of the β -subclass of the Proteobacteria are the primary hosts of the *cbaA* gene. These organisms tend to be K-strategists. They reproduce more slowly and tend to be successful in nutrient limited situations, primarily because they are better scavengers. Wagner *et al.* (1993) found that the β -Proteobacteria dominated in low nutrient conditions in sewage but were replaced with members of the Γ -Proteobacteria when nutrient concentrations increased. Krooneman *et al.* (1996) noted that isolates that degrade 3-Cba via a pathway not involving catechols (such as the *cbaAB* pathway) seem to have significantly lower maximum specific growth rates than isolates that metabolize 3-Cba via catechol. These observations also suggest that the hosts of the non-catechol routes of degradation of 3-Cba are K-strategists.

In a related competition study between two microbial isolates originally isolated from the Hyde Park site: a *cbaAB* harbouring isolate, NR-4, and a *clcABD* probe positive isolate, 6-9, in a flow-through biofilm reactor, showed similar results (Kosolen 1995). NR-4, a β -Proteobacterium, grew slowly and was able to survive well in nutrient poor

environments. Conversely the isolate 6-9, a γ -Proteobacterium, thrived under nutrient rich conditions which is characteristic of r-strategists. These observations suggested that it was the host bacterium that predicted the ability to compete under different environmental conditions, and not the specific catabolic genotype (*cbaAB* or *clcABD*) that it harboured.

The relative success of the *clcA* gene during the first half of the experiment can be explained using r-strategy principles (Andrews and Harris 1986). Hosts of this genotype could initially outcompete the hosts of the *cbaA* genotype due to their high initial numbers and rapid growth rates. The observed increase in the total number of *cbaA* target genes during the second half of the enrichment period is a typical response of K-strategist hosts to nutrient poor conditions. The slow growth rate and low initial copy number prevented this genotype from dominating until the latter half of the enrichment period. Hosts of the *cbaAB* genotype were observed to be more efficient at 3-Cba uptake and degradation under low (μM) concentrations (Bott and Kaplan 1996, Nakatsu *et al.* 1995a, Rosolen 1995).

There were 10-fold more copies of the *clcA* gene in the biofilm sample compared to the continuous culture broth at day 84. In the same sample, there were at least 10-fold more copies of the *cbaA* gene in the biofilm compared to the culture broth. Thus, both host groups preferentially established themselves in biofilms. These microorganisms were provided with stability from being washed out of the chemostat and could more effectively scavenge nutrients in a biofilm environment. In a study comparing conjugation frequencies on solid and in liquid environments between aquatic bacteria, a solid environment (filter or plate mating) was consistently superior to a liquid (broth) environment (Genthner *et al.* 1988). The biofilm on the chemostat in this study may have provided a solid surface which encouraged conjugation of the *cbaAB* and *clcABD* genotypes encoded on high molecular weight plasmids. There is also a higher cell density in the biofilm compared to the liquid medium. The increased proximity of cells to one another would also encourage more frequent conjugation.

CHAPTER 7

Conclusions and Future Research Considerations

The major goal of this Thesis was to thoroughly investigate the distribution of chlorobenzoate and biphenyl catabolic genotypes in an industrially contaminated region. The long history of chloroaromatic contamination in the Niagara watershed, including the Hyde Park landfill site, made it an ideal location for this study. This research was carried out on two levels. First, batch culture enrichments of the culturable portion of the microbial population in this region enabled me to evaluate the chlorobenzoate degradation potential and conduct more detailed genetic and phenotypic analysis of the culturable bacteria harbouring the four catabolic genotypes chosen for this study. Second, a total DNA extraction approach was included in order to obtain a more complete view of the selected genotypes in this region. At the time of this PhD investigation, there had been no long-term or comprehensive analyses of a contaminated environment investigating all of these parameters. Thus the results of this investigation were deemed novel. This concluding chapter will re-iterate the objectives listed in Chapter 1 and briefly discuss the results. Finally, future research considerations based on these investigations will be proposed.

Objective 1: To perform a long-term investigation of the effects of Hyde Park contamination, the sample season, enrichment temperature and sample year on the degradation potential of 3-Cba, 4-Cba and 3,4-dCba in water/sediment batch culture enrichments.

In Chapter 2 GC-MS studies of water samples from BRC, DHC and FC confirmed that BRC and DHC received contaminants from the Hyde Park site. Sediment/water enrichments from BRC and DHC consistently showed a higher frequency of complete microbial degradation of 3-Cba, 4-Cba and 3,4-dCba when compared to enrichments of

control samples from FC. The chlorobenzoate isomer showing the most frequent degradation in all batch culture enrichments was 4-Cba. Seasonal variations were observed for 3-Cba degradation only. Year to year variations in microbial chlorobenzoate degradation were noted for 4-Cba and 3,4-dCba enrichments. Although 4°C batch culture enrichments consistently showed less frequent degradation of chlorobenzoates during all seasons sampled, some sites showed complete degradation at this temperature. This suggested that the culturable microbial consortia responsible for chlorobenzoate degradation were facultative psychrophiles. The Hyde Park enrichment cultures showed similar degradation potential results as the creeks contaminated by this site. SBR1 showed better degradation of 3-Cba and 4-Cba. It is possible that these chlorobenzoate isomers are degraded primarily in the first SBR in the bioremediation facility.

7.2 Objective 2: To study the incidence and distribution of four previously characterized catabolic genotypes (*clcABD*, *cbaAB*, *fcB* and *bphA*) in the culturable populations of the Niagara watershed over an 18-month sampling period.

In Chapter 3 a collection of 818 microorganisms from the Niagara watershed batch culture enrichments was screened for four chlorobenzoate and biphenyl catabolic genotypes. Although less than 5% of the isolates hybridized to any one of the 4 genotypes screened, all catabolic genotypes were stable in this environment over the 18-month sampling period.

The chlorocatechol *ortho*-ring-fission pathway (*clcABD*) characterized in *Pseudomonas putida* AC866 (pAC27) was detected in 7% and 1% of all 3-Cba and 4-Cba catabolic isolates, respectively. The prevalence of this genotype was higher in isolates collected from Hyde Park enrichments. The 3-chlorobenzoate 3,4-(4,5)-dioxygenase pathway (*cbaAB*) from *Alcaligenes* sp. BR60 (pBRC60) was the only chlorobenzoate catabolic pathway previously detected in this environment. It has continually been detected in the Niagara watershed since 1985. A total of 3% of the combined 3-Cba and

3-Cba(3CBP) catabolic isolates, 7% of the 3,4-dCba catabolic isolates and 1% of the putative 3CBP catabolic isolates were found to harbour this genotype. The enzyme that determines congener specificity in the 4-Cba hydrolytic dehalogenase pathway (*fcB*) from *Pseudomonas* sp. CBS3 was also detected in this region in 2% of the microbial isolates capable of 4-Cba mineralization. Many of the *fcB* and *clcABD* probe positive isolates were collected from 4°C enrichment cultures implicating them as psychrophilic microorganisms. Finally, 2% of the putative biphenyl degrading microorganisms harboured the biphenyl dioxygenase (*bphA*) genotype from the archetypal bacterium *Burkholderia* sp. LB400. These results showed that microorganisms can naturally evolve or recruit catabolic genotypes and that genotypes with the same chlorobenzoate range can co-exist. As well, these results indicated that there exists a vast range of uncharacterized genotypes that comprised greater than 95% of the isolate collection. These isolates may express catabolic pathways related to the known pathways but with divergent gene sequences, or they may express alternative pathways not yet elucidated.

7.3 Objective 3: To perform detailed phenotypic and genetic analyses of all probe positive isolates from the batch culture enrichments including a comparison with the archetypal microorganisms harbouring each genotype.

The results of this investigation were detailed in Chapter 4. An examination of ERIC PCR DNA patterns from all probe positive isolates revealed that only 4 pairs of *clcABD* probe positive isolates were clonal bacteria. An analysis based on carbon source substrate range revealed that all unique probe isolates could be divided into 5 phenotypically distinct groups. Microorganisms in groups I and II primarily mineralized sugars in the BIOLOG GN plates. The reference strains in these groups were members of the β -Proteobacteria. While the Γ -Proteobacteria were represented in groups III and IV, group V had member belonging to the α -Proteobacteria. Microorganisms in these three groups metabolized sugars and organic acids in the BIOLOG GN plates. The fluorescent

Pseudomonads, which were previously believed to be the dominant hosts of chloroaromatic catabolic genotypes, actually represented the smallest phenotypic grouping (group III). The hosts of each of the four catabolic genotypes had representatives in at least two different phenotypic groups. This was surprising considering the metabolic organization required in regulating converging catabolic pathways in hosts that may have acquired catabolic genes by horizontal transfer. Nevertheless, the *clcABD* genotype was usually harboured in Γ -Proteobacteria hosts and the *cbaAB* genotype was most often detected in representatives of the β -Proteobacteria.

The chlorobenzoate substrate range of all probe positive isolates was also investigated. With the exception of the *fcbb* probe positive isolates which had a chlorobenzoate substrate range restricted to 4-Cba, the chlorobenzoate mineralization pattern varied within isolates harbouring the same genotype.

There were three RFLP patterns for the *clcABD* genes which all differed from the archetypal bacterium *Pseudomonas putida* AC866 (pAC27). The genetic diversity of these genes suggests horizontal transfer from a source other than *Pseudomonas putida* AC866 (pAC27). One isolate had the same RFLP pattern as the *clcABD* genes in *Burkholderia* sp. LB400, suggesting a common source for these two isolates. *Burkholderia* sp. LB400 was the only probe positive strain that harboured more than one of the four genotypes (*bphA* and *clcABD* genotypes). This is the first report of this well-studied archetypal isolate harbouring the *clcABD* genotype.

The chlorobenzoate substrate range of the *cbaAB* probe positive isolates was divided into 4 groups. Some isolates were capable of mineralizing all three chlorobenzoate isomers. Despite these variations in chlorobenzoate substrate range the RFLP pattern for the *cbaAB* genes in all probe positive isolates was identical to the RFLP pattern from the archetypal isolate, *Alcaligenes* sp. BR60 (pBRC60). More detailed RFLP mapping of Tn5271 showed that the *cbaAB* probe positive isolates had two variations of Tn5271, *Alcaligenes* sp. BR60-like and *Alcaligenes* sp. OCC43-like. The latter group was missing approximately 800 bp of the right side of Tn5271. It is possible

that these two versions of Tn5271 evolved independently through sequential intermolecular and intramolecular transposition events.

All *fcB* probe positive isolates had unique RFLP patterns for the dehalogenase gene that differed from *Pseudomonas* sp. CBS3, the archetypal microorganism suggesting that this gene has a high genetic diversity. Finally, 75% of the *bphA* probe positive isolates had the same RFLP pattern as *Burkholderia* sp. LB400 suggesting a common source of evolution for the *bphA* catabolic gene in these isolates.

The insertion element IS1071 which flanks Tn5271 was detected in a large proportion of probe positive isolates containing one of the four catabolic genotypes as well as many of the archetypal catabolic strains. The presence of IS1071 in numerous nonhomologous catabolic genotypes may be a valuable indicator of microbial adaptation to environmental contamination.

7.4 Objective 4: To detect the prevalence of the *cbaA* and *bphA* genes in total DNA isolated from Niagara watershed samples.

In Chapter 5 total DNA extraction, PCR amplification and dot blot hybridization methods were optimized for the Niagara watershed water/sediment samples in order to better assess the natural distribution of the *cbaA* and *bphA* genes in this environment and in Hamilton Harbour samples. The results showed that although the *cbaA* gene was detected in all environmental samples, it was generally more prominent in chloroaromatic- or PCB-contaminated sites. By contrast, the *bphA* gene was present in more-or-less equal copy numbers at almost all sites. The level of contamination did not increase the target copy number of this gene which was high at all sites. As well, no seasonal or yearly variation was noted for either of these genes.

7.5 Objective 5: To study the co-existence of the *cbaA* and *clcA* genes in a continuous culture enrichment of chlorobenzoate contaminated groundwater from Hyde Park.

Over an 84-day enrichment period, in a continuous culture that closely mimicked the Hyde Park bioremediation facility, the *cbaA* and *clcA* genes co-existed. There was no tendency for one gene to replace the other. Although the *cbaA* gene was present in a lower copy number in the inoculum, it increased in copy number approximately 100-fold over the course of the enrichment period. The *clcA* gene was present at virtually the same copy number throughout the experimental period. It was concluded that hosts harbouring the *cbaA* gene were typical of K-strategists while the hosts harbouring the *clcA* gene were likely r-strategists. The biofilm DNA contained more copies of target DNA for both genes.

7.6 Future Research Consideration

The results of this research have raised many additional research possibilities.

The degradation potential of other chlorobenzoate isomers in the Niagara watershed, including 2-chlorobenzoate and trichlorobenzoates could be assessed. Microbial anaerobic PCB dehalogenation preferentially removes *meta*- and *para*-substituted chlorines, resulting in the accumulation of *ortho*-substituted PCB congeners and subsequently *ortho*-substituted chlorobenzoates. This would give a more complete understanding of the microbial chlorobenzoate degradation potential in this region.

Only a small proportion of the isolate collection was probe positive (5%) and 1 screened for the major genetically characterized catabolic genotypes that mineralized chlorobenzoates via different routes. Further genetic characterization of the remaining isolate collection would reveal if there was a dominant chlorobenzoate catabolic genotype or a multitude of different co-existing genotypes in this region. In order to quickly screen

the microbial collection for alternative degradative pathways, ERIC PCR could be employed to establish genetically unique isolates.

I cannot comment definitively on the role or the importance of these four catabolic genotypes in nature, other than to note that they were all present. The question always arises as to how significant the detection of these catabolic genotypes is. Despite the fact that the catabolic genotypes have been detected, there is no evidence that the catabolic genes were actually expressed. The results of Chapter 2 showing enhanced biodegradation potentials in creeks receiving Hyde Park contamination are only an indirect indication that selection (and therefore presumably gene expression) has occurred. There is evidence that in the environment heterotrophic microbes are not specializing in the metabolism of one particular carbon substrate for growth (ie. a specific pollutant). Rather, they are simultaneously utilizing multiple carbon sources. Thus although the genes are present, they may not be expressed at all times. The fact that the genotypes used in this study are often encoded on high molecular weight plasmids and/or transposons suggests the necessity of some kind of genetic selection in order to ensure that they are maintained in the host organism. By detecting mRNA instead of DNA target sequences, the degradative activity of a microbial community may be assessed directly.

It would be of interest to establish how the different catabolic genotypes evolved in order to better understand the natural gene flow of these genotypes in this environment. This could be achieved by sequencing the PCR amplified gene products. DNA nucleotide differences in catabolic genes may also reveal sequence domains that could account for the broader substrate range for chlorobenzoates that was observed for some of the isolates. Phylogenetic studies using genetic techniques such as 16SrRNA sequencing to show the evolutionary relationships between the hosts that carry the different genotypes would give a more complete understanding of the effective natural host range of the catabolic genotypes.

Finally, it had previously been noted and was confirmed in this Thesis that IS1071 is present in many phenotypically diverse hosts harbouring nonhomologous catabolic genotypes. Almost half of all probe positive isolates in this study (and all *cbaAB* probe

positive isolates) harboured copies of *IS1071*. Due to its global distribution and association with a wide range of pollutant-degrading microorganisms, screening environments for this IS element may provide a general indicator of environmental contamination and pollutant degradation potential. *IS1071* could be detected using the basic methods described in this Thesis, batch culture enrichment or PCR amplification of environmental DNA.

Appendix I

3-Chlorobenzoate Batch Culture Enrichment Results Used in the Study of the Microbial Degradation Potential in the Niagara Watershed

Sample HPLC peak areas, fractional degradation results, contingency tables and Chi-square analyses . See Chapter 2 for experimental details.

Summary of Areas Under the 3-Cba Peaks Following HPLC Analysis
(254 nm, 50% methanol, 0.5% acetic acid)

	May-93			Nov-92			May 93		
	First Sampling			Second Sampling			Third Sampling		
Site	4°C	22°C	32°C	4°C	22°C	32°C	4°C	22°C	32°C
MR1	877344	0	0	2149824	2579172	1988745	1058584	1462378	1477636
MR2	1603840	450428	0	2058734	5093463	0	1375396	418642	837032
BRC1	1624846	472707	331999	3578915	2224034	0	1733792	1274639	973589
BRC2	1649846	0	3708679	2358715	4026303	0	932309	0	0
BRC3	1904279	0	0	2948725	1839289	7135362	797743	0	0
BRC4	1677426	3528646	0	2045914	2062284	1227264	1451	0	0
BRC5	1934941	0	0	3815751	4167749	0	1491	0	0
BRC6									
DHC1	1632356	943167	0	4058735	5558089	4061872	1435367	1224034	0
DHC2	1449768	0	0	3587164	4356933	3957897	573540	1193182	1445777
DHC3	1657616	1904189	0	2971546	2278140	2632207	538293	1388407	1429758
DHC4	1554500	2247400	0	3157814	5259271	3171107	718488	1402904	763738
DHC5	1636374	1590936	0	3617815	405674	2217505	702387	1328437	1489217
DHC6	1689766	3264673	3183083	2048755	5331369	1438853	658960	1178740	1545269
FC 5									
FC1	1950017	1474545	1481805	3348751	3804639	2095002	1398688	1410696	1454109
FC2	1615914	1780313	2635590	2841768	2300108	3951262	1406120	1469366	1452400
FC3	1606490	1802276	420766	3518767	3747673	2246375	1453500	1385359	1444726
FC4	1651939	2792593	2334635	3154795	3963860	1887818	1432373	1330660	1427243
FC5	1746686	1531190	1529996	3948175	3639198	1469160	1388388	1462434	1471959
	Aug-93			Nov 93					
	Fourth Sampling			Fifth Sampling					
Site	4°C	22°C	32°C	4°C	22°C	32°C			
MR1	1531826	929435	1087440	1389901	1065847	805291			
MR2	1564979	998969	0	1277097	1166	1421196			
BRC1	1537036	1151193	0	1275496	1393380	1216971			
BRC2	1402867	1319039	876849	1275431	1485443	1412109			
BRC3	1469223	1288293	356956	1106149	0	1436152			
BRC4	1442914	0	0	1134068	1496801	1419265			
BRC5	1583598	1177481	0	1192921	1436064	1341546			
BRC6	1626896	1322132	993840	1288530	1418986	1263810			
DHC1	1456332	1405896	1229223	1315141	1500016	1402933			
DHC2	1615878	1276333	1250243	1394838	1245187	1385881			
DHC3	1590960	1452037	411523	1468341	1267874	1317779			
DHC4	1501978	1249521	238393	1437384	1317733	1420780			
DHC5	1847319	1382772	1131281	1383996	1089120	1241987			
DHC6	1597303	1385795	1383614	1298920	1428581	1349907			
FC 5	1589733	1361245	1370935						
FC1	1569983	1493332	1418703	1000703	1417084	1480168			
FC2	1541906	1463065	1434863	1481928	1305305	1456897			
FC3	1412927	1489175	1405920	1324565	1496533	1427070			
FC4	1643785	1470652	1446936	1467362	1530821	1415772			
FC5	1629120	1434684	1440074	1458234	1325171	1339793			

Fractional Degradation Results for 3 Cba Batch Culture Enrichments

Site	May 93 First Sampling			Nov-92 Second Sampling			May-93 Third Sampling		
	4°C	22°C	32°C	4°C	22°C	32°C	4°C	22°C	32°C
MR1	2	5	5	1	1	1	1	1	1
MR2	1	4	5	1	1	5	1	4	2
BRC1	1	4	4	1	1	5	1	1	2
BRC2	1	5	1	1	1	5	2	5	5
BRC3	1	5	5	1	1	1	2	5	5
BRC4	1	1	5	1	1	1	5	5	5
BRC5	1	5	5	1	1	5	5	5	5
BRC6									
DHC1	1	2	5	1	1	1	1	1	5
DHC2	1	5	5	1	1	1	3	1	1
DHC3	1	1	5	1	1	1	3	1	1
DHC4	1	1	5	1	1	1	3	1	2
DHC5	1	1	1	1	4	1	3	1	1
DHC6	1	1	1	1	1	1	3	1	1
FC 5									
FC1	1	1	1	1	1	1	1	1	1
FC2	1	1	1	1	1	1	1	1	1
FC3	1	1	4	1	1	1	1	1	1
FC4	1	1	1	1	1	1	1	1	1
FC5	1	1	1	1	1	1	1	1	1

Site	Aug 93 Fourth Sampling			Nov 93 Fifth Sampling		
	4°C	22°C	32°C	4°C	22°C	32°C
MR1	1	2	1	1	1	2
MR2	1	2	5	1	5	1
BRC1	1	1	5	1	1	1
BRC2	1	1	2	1	1	1
BRC3	1	1	4	1	5	1
BRC4	1	5	5	1	1	1
BRC5	1	1	5	1	1	1
BRC6	1	1	2	1	1	1
DHC1	1	1	1	1	1	1
DHC2	1	1	1	1	1	1
DHC3	1	1	4	1	1	1
DHC4	1	1	5	1	1	1
DHC5	1	1	1	1	1	1
DHC6	1	1	1	1	1	1
FC 5	1	1	1			
FC1	1	1	1	1	1	1
FC2	1	1	1	1	1	1
FC3	1	1	1	1	1	1
FC4	1	1	1	1	1	1
FC5	1	1	1	1	1	1

	Rank	HPLC Peak Area	For the Contingency Table
No Degradation	1	$1 > 1.0 \times 10^7$	Row 1: Complete Degradation (Rank 5)
Partial Degradation	2	$1.0 - 0.75 \times 10^7$	Row 2: Partial Degradation (Rank 2-4)
	3	$7.5 - 5.0 \times 10^6$	Row 3: No Degradation (Rank 1)
	4	$5.0 - 2.5 \times 10^6$	
Complete Degradation	5	$< 2.5 \times 10^6$	

H₀ = Degradation (All Temps, All Seasons) is Independent of HP Contamination.

Data:

Col:	1	2
1>	38	0
2>	24	1
3>	139	77

Chi-square = 28.214 with 2 degrees of freedom; P = 0.000

H₀ = Degradation (All Temps, All Seasons) is Independent of Sampling Sites.

Data:

Col:	1	2	3
1>	30	8	0
2>	15	9	1
3>	66	73	17

Chi-square = 43.914 with 4 degrees of freedom; P = 0.000

H₀ = Degradation (All Temps, MR, BRC, DHC) is Independent of Sampling Year.

Data:

Col:	1	2
1>	19	19
2>	6	18
3>	53	86

Chi-square = 3.958 with 2 degrees of freedom; P = 0.136

Appendix II

A Summary of the Confirmed Chlorobenzoate and "Putative" Biphenyl and Chlorobiphenyl Catabolic Isolates from Niagara Watershed Batch Culture Enrichments

Isolates were collected from different sampling trips and enrichment temperature.

These results are also summarized in Table 3.3

3-Chlorobenzoate Catabolic Isolates

4-Chlorobenzoate Catabolic Isolate Distribution

3 4-Dichlorobenzoate Catabolic Isolates

Biphenyl "Putative" Catabolic Isolates

3-Chlorobiphenyl "Putative" Catabolic Isolates

Appendix III

**Sample Chart used to Quantitate the High Molecular Weight DNA Plugs from
Environmental DNA Extractions.**

SUMMARY OF AREA UNDER THE CURVE FROM ENVIRONMENTAL TOTAL DNA EXTRACTIONS
USING THE BIO RAD IMAGING DENSITOMETER MODEL GS-670 (MOLECULAR ANALYST VER. 1.1.1)

Gel MP1 Lane	1 Sample	Area	DNA Conc				Weight of			
			Calc. DNA (ng)	Vol. of Plug (uL)	in Plug (ng/uL)	Dil Conc. (ng/uL)	Vol. (uL) Loaded	HMW DNA Extracted (ug)	HMW DNA Extracted (ng/mL)	Average HMW DNA (ng/mL)
	2 MR N-5a	0.18	658.4	230	2.86	1.43	60	2.19	219	
	3 MRN-5b	0.16	569.6	230	2.48	1.24	60	1.9	190	204
	4 MRN-5c	0.17	611.3	190	3.22	1.61	60	2.04	204	
	5 DHC N-5a	0.19	694.6	190	3.66	1.83	60	2.32	232	
	6 DHCN-5b	0.17	611.3	220	2.77	1.39	60	2.04	204	245
	7 DHCN-5c	0.24	902.9	190	4.75	2.38	60	3	300	
Lambda	wt 23.1	0.11								
Marker	M9.64	0.07								
800 ng	M2.26	0.02								

r2: 0.37, Y-int 2.33E-2, slope of line: 2.40E-04

HMW: High Molecular Weight (> 23 kbp)

Appendix IV

Carbon Sources in BIOLOG GN Microtiter Plates

A1 water	A2 α-cyclodextrin	A3 dextrin	A4 glycogen	A5 Tween 40	A6 Tween 80	A7 N-aryl D- galactosamine	A8 N-acyl D- galactosamine	A9 ascorol	A10 L-arabinose	A11 D-arabitol	A12 α-biose
B1 erythritol	B2 D-fructose	B3 L-fucose	B4 D-galactose	B5 gentobiose	B6 α-D-glucose	B7 mannitol	B8 α-lactose	B9 α-D-lactose lactulose	B10 maltose	B11 D-mannitol	B12 D-mannose
C1 D-melibiose	C2 β-methyl D-glucoside	C3 psiose	C4 D-raffinose	C5 L-rhamnose	C6 D-sorbitol	C7 sucrose	C8 D-trehalose	C9 turandose	C10 xytol	C11 methyl pyruvate	C12 methyl methyl succinate
D1 acetic acid	D2 cis-aconitic acid	D3 ortho acid	D4 formic acid	D5 D-galactonic acid lactone	D6 D-galacturonic acid	D7 D-gluconic acid	D8 D-glucosaminic acid	D9 D-glucuronic acid	D10 α-hydroxy butyric acid	D11 β-hydroxy butyric acid	D12 γ-hydroxy butyric acid
E1 p-hydroxy phenylacetic acid	E2 itaconic acid	E3 α-keto butyric acid	E4 α-keto glutamic acid	E5 α-keto valeric acid	E6 D-L-lactic acid	E7 malonic acid	E8 propionic acid	E9 succinic acid	E10 D-saccharic acid	E11 succinic acid	E12 succinic acid
F1 bromo succinic acid	F2 succinamic acid	F3 glutaronamide	F4 alaninamide	F5 D-alanine	F6 L-alanine	F7 L-α-amino glycine	F8 L-asparagine	F9 L-aspartic acid	F10 L-glutamic acid	F11 glycyl L-aspartic acid	F12 glycyl L-glutamic acid
G1 L-histidine	G2 hydroxy L-proline	G3 L-leucine	G4 L-ornithine	G5 L-phenyl alanine	G6 L-proline	G7 α-amino glutamic acid	G8 D-serine	G9 L-serine	G10 L-threonine	G11 DL-carnitine	G12 γ-aminobutyric acid
H1 crotonic acid	H2 inosine	H3 inosine	H4 thymine	H5 phenyl ethylamine	H6 putrescine	H7 2-amino ethanol	H8 α-D-glucose butyrol	H9 glycerol	H10 D-glucose glycerol phosphate	H11 glucose-1- phosphate	H12 glucose-6- phosphate

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