Maximizing Biodegradation of Chlorinated Paraffins in a Cyclic Batch Bioreactor

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Abstract

Chlorinated paraffins are toxic, persistent, and bioaccumulative. Commercial products are mixtures of congeners and are used in industry as plasticizers, high temperature and pressure lubricants, in coatings and as flame retardants. They are the largest group of chlorinated substances still produced, and are mobilized to the environment *via* sediments. They have been found in marine life in Canada, milk products in the U.K. and in the breast milk of Northern residents of Québec. The aerobic biodegradation of commercial mixtures of chlorinated paraffins is not well studied. The goal of this work was to maximize the biodegradation of the commercial chlorinated paraffin Cereclor S52 using an axenic culture of *Pseudomonas* sp. 273. To this end, an automated, cyclical bioreactor with a control strategy based on carbon dioxide evolution was developed. Attempts at automating the bioreactor using a chloride specific probe as the control parameter were unsuccessful. Tests of the adherence of growing cultures to hydrocarbon substrates showed differences between Cereclor S52 and a readily degradable chlorinated alkane, 1,10-dichlorodecane (DCD). In the bioreactor, Cereclor S52 was degraded by up to 73% by means of multiple additions of DCD. Cereclor was emulsified by mechanical means to maximize bioavailability. The highest reported degradation of chlorinated paraffins reported elsewhere is 57% (Omori, 1987). The degree of degradation achieved using twelve sequential additions of DCD was 63%. This is indicative of an upper limit for the degradation of Cereclor S52 in this system. The degradative enzymes in *Pseudomonas* sp. 273 did not appear to be induced by Cereclor itself, by yeast extract, or by hexadecane. Under abiotic conditions, the equilibrium partitioning of the added Cereclor emulsion favoured the reactor walls (90%) over the mixed liquor (10%). When DCD was added to this system, partitioning increased to 50%. The work suggests that DCD acted to induce degradative enzymes, as a cosubstrate, and as a solvent to improve the bioavailability of Cereclor S52 in the bioreactor. Based on a comparison of gas chromatographs of Cereclor in the mixed liquor of the bioreactor through the course of the experiments, there appears selective degradation of certain fractions of the Cereclor mixture.

Résumé

Les paraffines chlorées peuvent etre toxiques, persistentes, et bioaccumulatives. Les produits commerciaux sont des mélanges de congénères et sont utilisés en industrie comme plastifiants, lubrifiants pour températures et pressions élevées, et ignifuges. Ils font partie du plus grand groupe de substances chlorées encore produites et s'accumulent dans l'environnement via les sédiments. Ils ont été retracés dans la vie aquatique au Canada, les produits laitiers au Royaume-Uni et dans le lait maternel des habitants du Grand Nord québécois. La biodégradation aérobique des mélanges commerciaux de paraffines chlorées n'a pas été étudiée en profondeur, avec seulement deux rapports antérieurs. L'objectif de ce projet était de maximiser la biodégradation de la paraffine chlorée commune Cereclor S52 en utlisant une culture aérobique et axénique de *Pseudomonas* sp. 273, connue pour dégrader les chloroalcanes. Pour y parvenir, un bioréacteur cyclique et automatisé utilisant une stratégie de contrôle basée sur l'évolution du dioxide de carbone a été développé. Des essais non-fructueux ont été effectués dans le but d'automatiser le bioréacteur en utilisant une sonde spécifique au chlore comme paramètre de contrôle. Des tests d'adhérence des cultures en croissance aux substrats d'hydrures de carbone ont démontré des différences entre Cereclor S52 et un alcane chloré facilement dégradable, le 1,10-dichlorodécane (DCD) . Cercelor S52 a été dégradé jusqu'à 73% dans le bioréacteur, conséquamment à trois additions séquentielles de DCD. Le Cereclor a été émulsifié de façcon mécanique afin de maximiser la biodisponibilité. La dégradation maximale de paraffines chlorées connue ailleurs est de 57% (Omori, 1987). La dégradation obtenue après douze additions séquentielles de DCD est de 63%. Ceci est une indication de la présence d'une limite supérieure pour la dégradation de Cereclor S52 dans ce système. Les enzymes de *Pseudomonas* sp. 273 responsables de la degradation n'ont pas semblées être induites par le Cereclor lui-même, par l'extrait de levure ou par l'hexadécane. En conditions abiotiques, la repartition à l'équilibre de l'addition de l'émulsion de Cereclor favorisait les murs du bioéracteur (90%) plutôt que la liqueur mixte (10%). Lorsque du DCD a été ajouté au système, le Cereclor qui adhérait aux murs s'est retrouvé dans la liqueur mixte. Ces résultats suggèrent que le DCD agit comme inducteur des enzymes dégradatives en tant que co-substrat, et comme solvant pour augmenter la biodisponibilité du Cereclor S52 dans le bioréacteur. En se basant sur la comparaison de chromatographes en phase gazeuse du Cereclor dans la liqueur mixte du bioréacteur au cours de l'expérimentation, il y aurait dégradation sélective de certaines fractions du mélange de Cereclor.

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1. LITERATURE REVIEW

1.1 CHLORINATED PARAFFINS

Chlorinated alkanes are classified primarily according to halogen content and carbon chain length. Some researchers use the term "chloroparaffins" to refer only to compounds having carbon chain lengths longer than 10 atoms long (C_{10}) (Allpress and Gowland 1999, Omori, Kimura et al. 1987) while the term chloroalkane is used in the more general sense. Others use the term chloroalkane to refer to all chain lengths. Often, the term "chloroparaffin" refers to the industrial products that are mixtures of compounds of varying chlorine content and carbon chain length. It is this definition that is adopted in this thesis. The Canadian toxic substances management policy classifies short chain chlorinated paraffins as having carbon chain lengths between C_{10} and C_{13}, medium chain as being C_{14}-C_{17} and long chain as greater than or equal to C_{18} (Environment Canada 1997). The industrial mixtures are very low in aromatics, olefins and cyclo alkane content (Exxon Mobil 2001).

Chloroparaffins are marketed in Canada under the trade name Cereclor by Pioneer Chemicals (formerly a division of ICI Canada). These compounds are produced in Canada solely in Cornwall, Ontario. Elsewhere, chlorinated paraffins are marketed under the trade names Toyoparax (Japan), Chlorowax (USA), Paroil (USA), Cloparin (Italy), and Witaclor (Germany) among many others. A more extensive list of other trade names used worldwide is provided in Appendix 3.

Due to their stability under extreme conditions, chloroparaffins are used mainly in high temperature and pressure applications. In 1999 an estimated 3500 - 5000 tonnes of chloroparaffins were used in Canada while annual world use is estimated to be 300,000 T/yr (Omori, Kimura et al. 1987). Figure 1 illustrates the breakdown by application in the USA, according to a chemical industry marketing group (Chemexpo, 1999). The compounds are also used in flame retardants foams and in other materials.

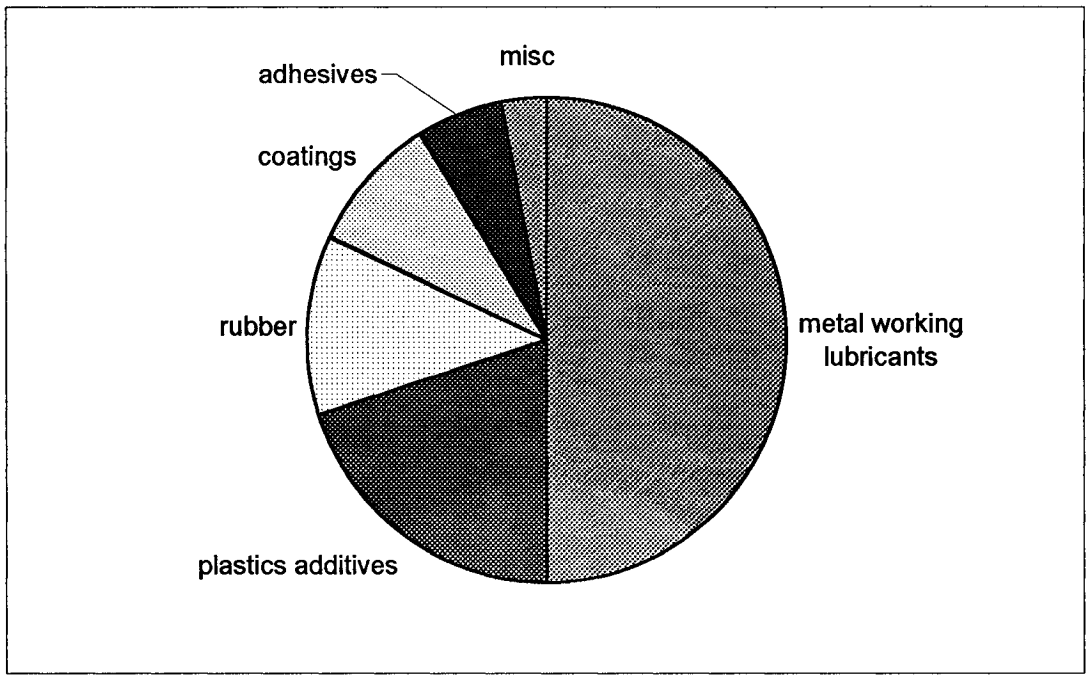

Figure 1. Industrial use of Cereclor compounds by sector in the United States (source: OPD, 1999)

1.2 ENVIRONMENTAL SIGNIFICANCE

Chlorinated alkanes are the largest group by mass of chlorinated hydrocarbons still produced. A significant proportion of the production of chloroparaffins is discharged as waste and carry off on product from metal working operations (United Nations 1996). While the compounds have low volatility and low aqueous solubility, they have a high affinity for particulate matter and may be mobilized to the environment *via* sediments in storm water. While vapour pressures are low at ambient temperatures, the Henry's law constants are similar to those associated with certain pesticides like toxaphene and chlordane which are transported in the atmosphere and accumulate in the Arctic (Canada 1997). Measurable levels of these compounds have been found in marine life in Quebec and Ontario. Chlorinated paraffins have also found their way into dairy products and edible oils in the UK (United Nations 1996) and into the breast milk of Northern Canadian aboriginal people (Tomy, Fisk et al. 1998).

The halogen substitutions that impart high temperature stability also make them persistent in the environment. The compounds may be incorporated into cellular lipids (Kulakova 1995, Wischnak, 1998) and be thereby bioaccumulative. The Canadian government has judged short chain chlorinated paraffins to be predominantly

anthropogenic, persistent, bioaccumulative and toxic, and has therefore initiated steps to phase out their use under the Toxic Substance Management Policy (Canada 1997).

1.3 TREATMENT

A biological treatment system may be envisaged to treat the waste from the largest world user of industrial chlorinated alkanes, the metal working industry. Waste from this industry is mixed with rinse or runoff water and has intermittent flows. Biological treatment is feasible mainly for aqueous effluents and therefore could be applied here. Also, a batch or fed-batch system is suited for the treatment of intermittent flows. Therefore a batch biological treatment system might be proposed, which could degrade industrial chlorinated alkanes and produce a non-toxic effluent.

The key issues in understanding the mechanisms involved in the biodegradation of chloralkanes are as follows (Maier, Pepper et al. 2000):

- bioavailability (defined as the access of the culture to the substrate, including the case of growth on hydrophobic substrates.)
- activity of cultures: (defined as the kinetics of growth, the possibility of cometabolism and diauxic growth, as well as issues of oxygen transfer.)
- enzymology : (defined as the effect on growth of the chain length of the substrate, the number and position of substitutions, the biochemical mechanisms leading to dehalogenation, the expression of catabolic genes, and substrate specificity; this may also be called genetic potential and contaminant structure)

These issues are discussed in more detail in subsequent sections.

1.4 SELF CYCLING FERMENTER

The Self Cycling Fermenter (SCF) is a reactor system developed for the culture of micro-organisms. It is a cycling batch treatment process where the key elements are that the substrate of interest is the limiting nutrient, and the end of a cycle is determined by the exhaustion of the limiting nutrient. Thus, the cycle time is determined by the state of the culture itself, and not by a timer or other means. In this manner, the system is self-regulating. At the end of the cycle, exactly half of the reactor contents are drained and refilled with feed. Thus, under stable repetitive cycling, the cycle time and the doubling time of the culture are equivalent (Brown 1991). At the time that the reactor is drained and re-filled, the reactor contents must be homogeneous.

In a traditional cyclical batch treatment system the cycle time is predetermined. In the SCF, the cycle time is determined by the state of the culture, in particular the cessation of catabolic activity due to the disappearance of the limiting nutrient. Therefore, the selection of a control parameter from which the state of the culture can be inferred is critical for the operation of a SCF. Examples of control parameters that may be used are dissolved oxygen, pH, carbon dioxide evolution, product evolution, and the disappearance of the limiting nutrient. The control parameter is selected based on its' correlation with the disappearance of the limiting nutrient, the ease of interpreting the cycle end point, and the robustness of the measuring element.

Since the cycle time is equal to the doubling time of the culture, and exactly half of the reactor contents are removed each cycle, cell synchrony is induced (Brown 1991). Cell synchrony refers to the temporal alignment of the cell processes in the culture. In the idealized case, each cell in the culture divides once, exactly at the end of the cycle. Irrespective of the time that cell division occurs, it will take place once each cycle. Cell synchrony allows us to use the bulk parameters of the culture to measure metabolic events in the cell. This is an important benefit of the use of the SCF for the study of biological processes.

The batch system envisaged for the treatment of metal shop effluent is not necessarily a SCF, but it may contain elements of the SCF design. Such a batch treatment system has certain advantages. For example, using a control parameter to determine the cycle end time has the advantage of optimizing the removal efficiency and the cycle time, and thus the throughput. In the case of an intermittently fed culture the selection of an appropriate control parameter would be important to ensure adequate removal efficiency. Further, there may be metabolic advantages if cell synchrony is achieved (Bailey 1986).

On the other hand, a batch system may not be a good choice where the feed is very toxic to the culture. The advantage of a continuously fed system in this case is that the feed is diluted by the reactor contents and the culture is not exposed to high levels of the toxic substances. Alternatively, a fed batch system may be used for the same reasons.

1.5 BIOLOGICAL REMOVAL OF IMMISCIBLE SUBSTRATES

The biodegradation of sparingly soluble liquids has been well studied (Maier, Pepper et al. 2000). Commonly, three mechanisms are reported to be important in these cases (Velankar, Barnett et al. 1975, Nakahara, Erickson et al. 1977, Gutierrez and Erickson 1977):

1. Dissolution of the substrate and subsequent uptake by organism. Although the compounds under study are "insoluble" there is a finite amount that will dissolve.

2. "Solubilization" of the substrate through emulsification. The emulsifying agents may be either excreted by the cell or added to the system. Although the substrate is not dissolved, the cell interacts with the emulsified material as if it were soluble.

3. Direct contact of substrate with cells, including both random contact and cell adherence. In particular, this mechanism is critical for systems where the substrate cannot pass into the cell, and extracellular enzymes are important for biodegradation. Cellular floc formation can encourage direct contact through the occlusion of substrate droplets.

Emulsifiers may affect the surface properties of the organisms, and thus influence uptake through both mechanism "2" and "3"

Thus, both abiotic and biotic mechanisms are important. Mass transfer in a particular system may determine the rate of degradation. In some cases, the rates of transfer can be so slow that the compound may be considered refractory in spite of the genetic potential of the organism (Maier, Pepper et al. 2000).

Researchers have sought to enhance biodegradation of these compounds through the use of additives (Belkin; Efroymson and Alexander 1991; Jimenez and Bartha 1996; Churchill and Churchill 1997; Bouchez-Naitali, Blanchet et al. 2001). Two groups of additives are of particular interest: surfactants and solvents. Some research has been published which seeks to link the effects of each. When the substrate is degraded only from the soluble fraction, the degradation rate is limited by the rate of dissolution of the compound. In the case of the degradation of chlorinated paraffins, the solubility of many of the compounds is so low that this mechanism is not important (Maier, Pepper et al. 2000). When dissolution is important, the growth rate will be greater for substrates that have greater solubilities, unless the solubility approaches the half-saturation constant (K_s) defined by Monod kinetics.

Considerable research has been dedicated to the effect of emulsifiers on biodegradation of hydrocarbons. Emulsifiers are even produced by certain micro-organisms as a mechanism to take advantage of insoluble substrates (Zuckerberg, Diver et al. 1979). However, conflicting results have been obtained. The rate of biodegradation of a refractory hydrocarbon, pyrene, was enhanced by the addition of detergent below the critical micelle concentration, but above that level it was severely limited (Jimenez and Bartha 1996). In a study of bioremediation of crude oil fractions following the Exxon Valdez spill, (Button, Robertson et al. 1992) the effects of widely applied surfactants were 'unremarkable' after 1.5 months. However Churchill *et al,* 1997, reported on the importance of surfactants in biodegradation following the same spill in a different study.

Other work has attempted to elucidate the effect of surfactant on the cell wall/ oil interface. This work measures cell surface hydrophobicity using the "bacterial adherence to hydrocarbon" (BATH) test (Rosenberg 1984). Further, the hydrophobicity of the substrate has been measured by means of the octanol-water partition test (ASTM 1997). One group (Al-Tahhan, Sandrin et al. 2000) reported that low levels of biosurfactants enhance the cell surface hydrophobicity. This in turn affects the direct adherence of hydrophobic substrates to the cell surface. Octadecane degradation was enhanced (Churchill and Churchill 1997) by the addition of surfactants in two bacterial strains (*Pseudomonas* sp. and *Rhodococcus* sp.), while there was no effect was observed in two other strains, both *Acinetobacter* species. The rate of degradation in the *Acinetobacter* strains was much higher than in the other two strains and it was hypothesised that the difference was related to the fact that the *Acinetobacter* cultures were hydrophobic and the others relatively hydrophillic. Again, according to Zhang et al., (Zhang and Miller 1994), fast degraders of octadecane had high cell surface hydrophobicities while slow degraders had low hydrophobicities, based on the BATH assay results.

Other work has focused on the enhancement of the adherence of the substrate to the cell through the use of hydrophobic solvents. The work on surfactants referenced above (Jimenez and Bartha 1996) included comparative work on the use of solvents for the degradation of pyrene using a culture of *Mycobacterium* with hydrophobic properties. Certain solvents, including heptamethylnonane were inhibitory, while others more than doubled the mineralization rates of pyrene without being metabolised themselves. It was hypothesised that upon attachment of the cell to the hydrophobic solvent, access to the substrate of interest was enhanced. Another study using an *Arthrobacter* strain found heptamethlynonane to enhance degradation of naphthalene (Efroymson and Alexander 1991). Also, it has been reported (Kanaly, Bartha et al. 2000) that the addition of diesel fuel augmented benzo-pyrene degradation.

In a study of a strain *Rhodococcus equi* that did not produce biosurfactants, flocculation of cells was observed to affect the biodegradation rate of hydrophobic substrates (Bouchez-Naitali, Blanchet et al. 2001). In this case it is reported that cellular hydrophobicity tended to cause cells to agglomerate. The cells exhibited an initial period of exponential growth that was independent of the interfacial area between the substrate and the media. This was followed by a long period of linear growth that was also independent of interfacial area. The linear growth, which can be indicative of mass transfer limitation, was correlated to the onset of flocculation that was assumed to reduce cells' exposure to the immiscible substrate. In the linear growth phase the biodegradation rate was observed to increase with increasing stirring speed, which was attributed to breaking up of flocs.

According to Maier *et al.* (Maier, Pepper et al. 2000), the uptake of short and long chain organic solvents occurs by separate mechanisms, and may explain why cells have evolved separate modes of metabolism for these compounds. Short chain organics, usually more soluble, infiltrate the cell wall and accumulate in the membrane, effectively imparting toxicity through disruption of the membrane. Long chain compounds, on the other hand are generally much less soluble and do not penetrate and thus damage the cell membrane. The biodegradability of the longer chain substances is found to be correlated to their hydrophobicity, as measured by the octanol-water partition coefficient (K_{ow}). This observation also points to the importance of the direct contact of the substrate with the organism.

Finally, the importance of direct contact is highlighted by the work of Moo-Young *et al* (Moo-Young and Shimizu 1971) that correlated the size of the droplets of immiscible substrates with the growth rate. This work found that for well agitated systems the droplet size affected the growth rate but the substrate concentration, between 0.1 and 1% (v/v) did not.

1.6 THERMODYNAMICS

1.6.1 Energetics

Alkanes are highly reduced compounds, and as such, are good substrates to act as electron donors in biosystems. The catabolic pathway for alkanes typically consists of three oxidation steps, to yield a fatty acid. Chloralkanes are similarly oxidized, yielding a fatty acid, a proton and a chloride ion. The fatty acid is broken down by β-oxidation which then feeds the tricarboxylic acid (TCA) cycle (Lehninger 1982).

β-oxidation of a fatty acid utilizes the cofactor, Coenzyme A (CoA), which is used in the catabolism of carbohydrates *via* the TCA cycle. Coenzyme A forms a thioester bond with the fatty acid at the expense of the energy from the conversion of one molecule of ATP to AMP. The fatty acyl-CoA thus formed then yields an acetyl-CoA, thereby removing two carbon atoms from the fatty acid. This step results in the reduction of the electron carriers FAD^+ and NAD^+ to $FADH_2$ and NADH respectively. The cell uses these reduced compounds to regenerate ATP from ADP. Acetyl-CoA is then oxidized to CO_2 and H_2O *via* the TCA cycle reducing further FAD^+ and NAD^+. Continuing in this way, the fatty acid is broken down two carbon atoms at a time with an overall yield of 5 ATP molecules resulting from the cleavage of each fatty acid chain, and 11 ATP molecules per Acetyl-CoA produced (Lehninger 1982).

Many chlorinated compounds are not easily biodegraded, although the biochemical or thermodynamic reasons are not obvious (Pries, Ploeg et al. 1994). Chlorinated alkanes are more oxidized than their non-chlorinated analogues, so the energy available *via* mineralization to HCl, H_2O and CO_2 is reduced. Therefore, the degree of chlorination and the length of the carbon chain have important effects on the thermodynamics (Janssen, Pries et al. 1994). Table 1 provides estimates of the Gibbs free energy of reaction, ΔG_r, for the mineralization of various chlorinated and non-chlorinated alkanes. Estimates of the Gibbs free energy of formation, ΔG_F, for the alkanes were taken from literature (Dolfing and Janssen 1994).

Table 1 - Gibbs Energies of Formation and Mineralization for Selected Alkanes and Chloralkanes (Dolfing and Janssen, 1994)

Compound	ΔG°_f (kcal/mol)	ΔG°_r (mineralization) (kcal/mol)
ethane	-41.7	-351
ethanol	-43.3	-311
chlorethanol	-48.8	-290
1,2 Dichloroethane	-17.4	-306
1,1,1,2 tetrachloroethane	-18.6	-273

The following statements may be made for the compounds listed:

- The energy of formation decreases with degree of chlorination. A lower energy of formation for a reactant results in a less favourable mineralization reaction.
- The energy of mineralization is negative (favourable reaction) even for heavily chlorinated compounds like tetrachloroethane.
- The energy released by mineralization decreases with increasing chlorination (less negative a ΔG°_r, and therefore less favourable)
- Partially oxidized reactants such as ethanol yield less energy upon mineralization

This analysis is supported by observation in the context of the capabilities of dehalogenating organisms. In the case of *Rhodococcus erythtopolis* Y2, the biomass yield was 3-6 fold greater when growing on unchlorinated alkanes than on the corresponding 1-chloroalkanes.(Armfield, Sallis et al. 1995).

In the special case where an oxygenolytic enzyme is used, the dehalogenation reaction consumes $NADH_2$. This enzyme system is discussed in detail below. One mole of $NADH_2$ is required for each mole of chlorine removed. $NADH_2$ is produced through the oxidation of the alkane. In the case of tetradecane, 34 moles of $NADH_2$ are produced from β-oxidation of the alkane. So for hexachlorotetradecane, 6 moles of $NADH_2$ are required, which is 18% of the available amount, thus impacting the energy balance.

1.6.2 Cometabolism

The definition of cometabolism is the transformation of an organic compound of interest by a microorganism with no contribution to energy or biomass yield (Chang, Voice et al. 1993; Criddle 1993). That is, the compound is not an electron donor in the energy sense and there is no significant uptake by cells from that compound of carbon, nitrogen, phosphorous, sulphur, etc.. The organism may be proliferating on a separate substrate, called a cosubstrate, or using energy reserves. Cometabolism may be termed fortuitous catabolism, and arises when broad substrate specificity is associated with the action of enzymes and cofactors (Maier, Pepper et al. 2000). The more general term cosubstrate may be both applied in the case of cometabolism or where two growth substrates are present.

A growth substrate is an electron or energy donor that is a carbon source. An energy substrate is an electron donor but it does not provide a carbon source for growth, as in, for example, in the case of the use of H_2 as an electron donor in members of methane generating consortia (Criddle 1993).

Generally, the definitions are applied to the original target compounds and not to metabolic intermediates. For example, when a metabolite is a growth substrate the original compound from which it was derived is considered to be a growth substrate (Maier, Pepper et al. 2000). Diauxic growth is defined as the distinct separation of growth on one substrate from growth on a second substrate.

A relevant example of cometabolism involved the degradation of short chain chloralkanes in the presence of active methanotrophs or phenol degraders (Fetzner and Lingens). In this case, the enzymes produced while growing on toluene are capable of degrading TCE.

1.7 ENZYMOLOGY

1.7.1 Biodegradation of Haloalkanes

A general view of aerobic cell metabolism is illustrated in Figure 2, for the case of hydroxylation of hydrocarbons mediated by an oxygenase. The pathway may be

summarized as follows: hydroxylation to form an alcohol, dehydrogenation of the alcohol to yield an aldehyde, further dehydrogenation of the aldehyde to yield a carboxylic acid, β-oxidation, and finally mineralization to CO_2 and H_2O *via* the tricarboxylic acid cycle. Many variations on this pathway have been reported, although all the pathways are typically *via* a carboxylic acid (Yokota, Fuse et al. 1986).

The biodegradation of haloalkanes follows a similar pathway with initial hydroxylation being associated with dehalogenation. Chlorine is released as chloride ion together with a proton, maintaining electroneutrality. Therefore, the dehalogenation process will drop the pH of the solution.

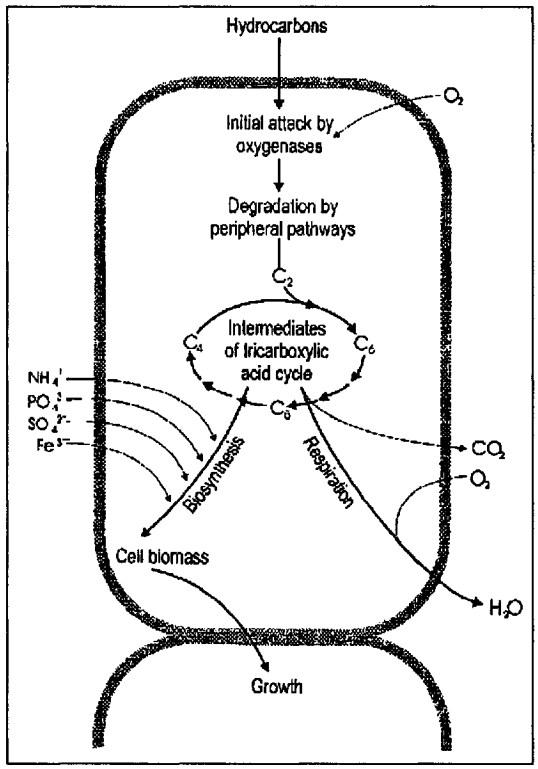

Figure 2 Overview of Metabolism of Alkanes,
from (Maier, Pepper et al. 2000)

Most of the research on the biodegradation of haloalkanes has been concerned the degradation of short chain haloalkanes, halo fatty acids, and alkenes (Janssen, Pries et al. 1994),(Belkin; Copley; Fetzner and Lingens; Hardman 1991; Janssen, van der Ploeg et al. 1994; Janssen, van der Ploeg et al. 1995). In particular compounds with carbon chain lengths less than five (C_5) have been examined extensively. This research has been driven by the acute toxicity of these compounds and their proliferation in the environment. In contrast, very little research has been conducted on the degradation of paraffins ($>C_{10}$) with multiple halogen substitutions.

In general, examples in nature of organisms capable of degrading many chloralkanes are rare. One group of researchers (Omori and Alexander 1978) isolated 500 species from a contaminated soil sample but found only three that exhibited dehalogenation activity on dichlorononane. In many cases, the organisms possessing these enzymes were isolated by enrichment on the substrate of interest from contaminated soil found at industrial sites (Hardman 1991; Armfield, Sallis et al. 1995). In other cases dehalogenating organisms were isolated from surface water samples (Allpress and Gowland 1999) and sewage samples (Omori and Alexander 1978).

The process of dehalogenation refers to the cleavage of the carbon-halogen bond and is often the rate limiting step in the biodegradation of chlorinated alkanes (Slater, Bull et al. 1997), (Hardman 1991). In more general terms, microorganisms that can grow on halogenated alkanes are typically capable of producing these enzymes (Janssen, Pries et al. 1994). However, in some cases organisms growing on other substrates can carry out dehalogenation through cometabolic processes (Omori, Kimura et al. 1987).

The enzymes responsible for aerobic dehalogenation are classified according to the six known mechanisms for dehalogenation (Fetzner and Lingens): hydrolytic, oxygenolytic, thiolytic, substitution, dehydrohalogenation and hydration. Dehydrohalogenation, the term referring to the simultaneous removal of adjacent hydrogen and halogen to leave a double bond, has not been reported in microbial systems (Hardman 1991). The hydrolytic and oxygenolytic mechanisms are often associated with the biodegradation of chloralkanes by bacteria under aerobic conditions (Hardman 1991).

Both the hydrolytic and oxygenolytic mechanisms involve the hydroxylation of the substrate and simultaneous release of the halide. However, the mechanisms for hydroxylation are different. These reactions are described in detail below. Product inhibition has been observed in organisms dehalogenating short chain substrates (Janssen, Jager et al. 1987) where an alcohol dehydrogenase is active in the absence of an aldehyde dehydrogenase. In this case dehydrogenation and not dehalogenation is the rate limiting step in the degradation of these compounds.

In some cases dehalogenation will occur spontaneously from unstable intermediates formed during catabolism (Fetzner and Lingens). In this case the enzyme responsible for the initial catabolic step might not have affinity for only halogenated substrates, but non-halogenated analogues as well.

1.7.2 Dehalogenase Enzymes

As discussed above, the most prominent enzymes for aerobic degradation of haloalkanes are the hydrolytic and oxygenolytic dehalogenases. The hydrolytic enzymes are active

for certain short chain substrates (those up to C_9) and the oxygenolytic dehalogenases, including monooxygenases, have demonstrated activity for both short and medium chain haloalkanes. For example, *Pseudomonas sp.* Strain 273, a gram negative motile rod, released stoichiometric quantities of chloride from α,ω-dichloroinated alkanes containing 5-12 carbon atoms using a monooxygenase (Wischnak, Loffler et al. 1998). An interesting case is *Rhodococcus erythropolis* Y2, which is thought to produce both an oxygenase dehalogenase and a hydrolytic dehalogenase. The expression of the hydrolytic enzyme was induced by short chain chloralkanes and the expression of the oxygenase was induced by medium chain chloralkanes (Armfield, Sallis et al. 1995).

1.7.2.1 Oxygenolytic Dehalogenases

Oxygenolytic dehalogenases are characterized by the requirement for elemental oxygen and NAD(P)H in order for dehalogenation to occur in cell free extracts (Tomy, Fisk et al. 1998). Some reports suggest that Fe^{2+} is also required (Bock and Muller 1996). Oxygenolytic dehalogenases can be further classified as either monooxygenase dehalogenases or dioxygenase dehalogenases. The reaction catalyzed by a monooxygenase incorporates one oxygen atom from O_2 into the organic substrate. The other oxygen atom is reduced to H_2O. Fortuitous, or cometabolic, dehalogenation of aromatic substrates has been reported in methanotrophic cultures that employ these enzyme types (Han, S et al. 1999). Medium and long chain chloralkanes are reported to be degraded by oxygenolytic enzymes (Allpress and Gowland 1999) (Curragh, Flynn et al. 1994),(Yokota, Fuse et al. 1986) but cometabolism was not always implicated. Monooxygenase dehalogenases are sometimes called hydroxyases.

The general reaction, where A is the substrate and B is the co-substrate, is (Lehninger 1982):

(1) $AH + BH_2 + O_2 \Rightarrow AOH + B + H_2O$

In the case of the dehalogenation of haloalkanes, the following reaction is proposed:

(2) $R\text{-}CH_2\text{-}Cl + BH_2 + \frac{1}{2} O_2 \Rightarrow R\text{-}CH_2\text{-}OH + HCl + B$

where B is $NAD(P)^+$. Alternatively, a different compound (or a series of compounds) may take the place of $NAD(P)^+$ in the dehalogenation reaction, but this compound will ultimately transfer electron(s) to $NAD(P)^+$.

An example of an alternative electron carrier is the rubredoxin protein, which has been implicated with ω-hydroxyases involved in alkane degradation (Fritsche 1999).

However, this protein is more commonly associated with anaerobic pathways (Fritsche 1999).

In the case of a dioxygenase both oxygen atoms in O_2 are incorporated into the substrate. An example of a reaction catalyzed by a dioxygenase is the oxidation of benzene to catechol or certain cases of the dehalogenation of polychlorinated phenol (Scholtz, Messi et al.). The dioxygenase group of proteins is not thought to be important for the degradation of chlorinated paraffins.

The organism *Pseudomonas* sp. 273 is reported to use a monoxygenase dehalogenase based on the observed requirement for elemental oxygen and NADH (Wischnak, Loffler et al. 1998).

1.7.2.2 Hydrolytic Dehalogenases

Reactions catalyzed by hydrolytic dehalogenases are characterized by a displacement reaction, without involvement of cofactors. Water is a substrate (thus the term hydrolytic), as follows (Janssen, Pries et al. 1994):

(3) $R_1\text{-}CHCl\text{-}R_2 + H_2O \Rightarrow R_1\text{-}CHOH\text{-}R_2 + HCl$

Note that this initial catabolic step can take place in the absence of oxygen but the overall mineralization process involves oxygen as the terminal electron acceptor. The importance of hydrolytic dehalogenases may therefore be demonstrated experimentally by subjecting chloroalkanes to cell free extracts in the absence of oxygen.

Similarly, the mineralization of a short chain chloralkanes has been demonstrated by a number of gram positive bacteria through the action of the hydrolytic dehalogenase produced by the gene dhaA. (Poelarends, Zandstra et al. 2000). The reaction may be described as follows:

(4) $R\text{-}CH_2Cl + H_2O \Rightarrow HCl + R\text{-}CH_2OH$

(5) $R\text{-}CH_2OH + X \Rightarrow XH_2 + R\text{-}CHO$

(6) $R\text{-}CHO + Y + H_2O \Rightarrow YH_2 + R\text{-}COOH$

Equation 4), which occurs in the presence of the enzyme coded by DhaA, illustrates the action of the hydrolytic dehalogenase (Janssen, Pries et al. 1994). The carboxylated end product is then mineralized, as discussed above, through β-oxidation. Equation 5) occurs in the presence of the enzyme coded by AdhA and equation 6) occurs in the presence of the enzyme coded by AldA.

Study of the degradation of 2-monochloro and 2,2-dichloro carboxylic acids (Slater, Lovatt et al. 1979) demonstrated that subterminal hydroxylation is possible, as illustrated in Figure 3. The hypothesis that the subterminal hydroxylation pathway may be associated with dehalogenation has not been tested to date. Another example is the case of *Rhodococcus erythropolis* Y2 (Armfield, Sallis et al. 1995) where the activity of resting cell suspensions to a series of internally substituted alkanes was measurable when the culture was induced with hexadecane. Where an internally substituted haloalkane is first degraded through β-oxidation, the substrate for the dehalogenase is a 2-chloro carboxylic acid. Figure 3 provides a comparison of the terminal and subterminal pathways for *n*-alkane degradation. Evidence for subterminal hydroxylation-dehalogenation has been established in work with hexachlorocyclohexane (Nagata, Miyauchi et al. 1997). However others report that resting cell systems had greater activity towards terminally substituted substrates than toward mid chain substituents (Armfield, Sallis et al. 1995).

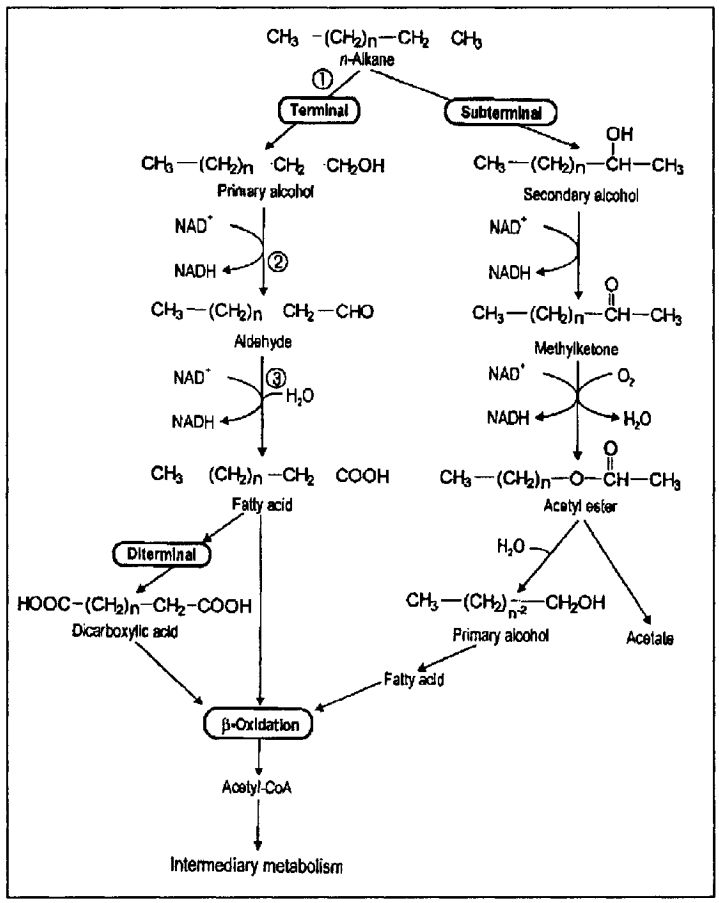

Figure 3 Overview of Terminal and Subterminal Hydroxylation of Alkanes (White 2001)

A large number of hydrolytic dehalogenases have been characterized in the literature (Hardman 1991; Janssen, Pries et al. 1994). These have been classified according to, for example, substrate specificity, physical characteristics and DNA sequence. The enzyme produced by *Xanthobacter autotrophicus* GJ10, originally identified by Janssen *et al.* is the best characterized dehalogenase with over 125 references on the subject published to date (Janssen, Pries et al. 1994).

Activity of hydrolytic dehalogenases has not been observed for chain lengths longer than C_9. Indeed many hydrolytic enzymes demonstrate narrow substrate specificity, as is the case of the gram positive coccus *Xanthobacter autotrophicus* GJ10, which is active only for C_2 and C_3 compounds (Janssen, Scheper et al. 1985). Therefore, these enzymes are of no importance for the initial dehalogenation of chlorinate paraffins, since these consist of chlorinated paraffins that have lengths $>C_{10}$. However, they play a possible role where organisms with hydrolytic dehalogenase are capable of degrading long chain mono chlorinated compounds by first conducting β-oxidation on an unchloriniated end and then by dehalogenating the ω-chloro carboxylic acid. (Kulakova, 1997).

1.7.2.3 Location of the Catabolic Genes

Six strains of *Rhodococcus* sp., utilizing short chain haloalkanes, isolated from different parts of the world are reported to display closely matching dehalogenation genes (Poelarends, Zandstra et al. 2000). These gram positive organisms (Y2, TB2, m15-3, HA1, NCIMB13064 and GJ70) contained catabolic genes on 70-100 kb plasmids. Each plasmid contained dhaA, adhA and aldA genes reported to be responsible for, respectively, dehalogenation, alcohol dehydrogenation and aldehyde dehydrogenation. All the plasmids also contained the genes invA and dhaR, genes which were purported to be involved in the regulation of the dehalogenation process. In a study of NCIMB 13064 (Kulakova, Larkin et al. 1997) the incorporation of the catabolic gene from the plasmid into the chromosomes resulted in loss of dehalogenation activity. GJ10 produces a hydrolytic dehalogenase that is coded by the gene dhlA (Janssen, Scheper et al. 1985). In the case of *Pseudomonas sp.* Strain 273 (Wischnak, Loffler et al. 1998), the location of the catabolic genes has not been established.

1.7.3 Biodegradation of Chloro-Paraffins

Only two studies have been published related to the biodegradation of the commercial mixtures of chloralkanes with medium chain lengths (Omori, Kimura et al. 1987, Allpress and Gowland 1999).

An early study (Omori, Kimura et al. 1987) reported release of up to 57% of the stoichiometric amount of chloride from Toyoparax CP-150 in a jar fermenter with a

mixed culture of a series of known chlorinated alkane degraders using hexadecane as a co-substrate. CP-150 is an industrial chloroparaffin with mean chain length of 15.4 and a chlorine content of 50% (w/w). The initial substrate concentration was 360 mg/L. In shake flasks, the axenic cultures achieved only up to 13% chlorine release on this substrate. These researchers demonstrated that certain organisms in their mixed culture were able to dehalogenate 2-chloro carboxylic acids, and others not. All the cultures could degrade 3-chloro carboxylic acids as well as 1-chloro and α-ω chloroalkanes but they never observed 100% release, possibly due to a drop in pH that is associated with the dechlorination process (equations 2,3). The authors hypothesised that the organisms first removed the terminal chlorine of the substrate, and then produced either a 2 or a 3-chloro fatty acid *via* β-oxidation.

In other work on short chain compounds, the same group of researchers found that (Yokota, Fuse et al. 1986):

- terminal chlorinations were dehalogenated by cultures possessing either oxygenases or hydrolases,
- 2-chloro aliphatic acids were dehalogenated by cultures possessing hydrolases,
- 3-chloro aliphatic acids were dehalogenated by enzymes in the *β*-oxidation system.

They proposed that multiple substitutions and adjacent substitutions contributed to incomplete degradation (Omori, Kimura et al. 1987).

Interestingly, other researchers have found that certain isolates could dehalogenate one but not both optical isomers of 2-halogenated alkanoic acids while certain isolates could dehalogenate both (Motosugi, Esaki et al. 1982). The enzyme from both isolates had the same response to gel electrophoresis with activity staining.

In work with a strain of *Rhodococcus* (Allpress and Gowland 1999) a series of 1-chloro medium chain alkanes were degraded releasing 80-100% of chlorine as chloride. Also, 49% of available chloride was released after 100 days of incubation in shake flasks of Cereclor S49L without a cosubstrate. The substrate was added at a concentration of 1% v/v. S49L has chain lengths of 10-13 carbon atoms and is 49% chlorine by weight. A similar experiment was conducted using Cereclor S45, which has chain lengths of 14-17 atoms and is 58.5% chlorine by weight. In this case 20% of available chlorine was released. The organism did not grow on the following compounds: chloroacetic acid, dichloroacetic acid, 2-chloropropionic acid, chloroacetaldehyde, 2-chloroethanol, pentachlorophenol, 3-chlorobenzoic acid, 1,2-dichloroethane, 1,1,2,2-tetrachloroethane, 1,1,2,2-tetrafloro 3-chloropropane, 1-chlorbutane, 1-chlorooctane, 1-chlorononane, 1,9-dichlorononane, 1-chlorodecane, Cereclor S58 (C_{14}-C_{17}) or Cereclor 63L (C_{10}-C_{13}).

Neither of these researchers analysed the residual chloroparaffin to determine whether selective degradation had taken place.

Work with *Rhodococcus erythropolis* Y2 (Armfield, Sallis et al. 1995) indicated a wider substrate specificity in the case of the oxygenase than in the case of the hydrolase. For example, 3-monochloropropionic acid was effectively dehalogenated, as were medium chain haloalkanes, when the oxygenase was induced with the medium chain substrates but not when the hydrolase was induced using short chain substrates. This research investigated a series of α and β substituted haloalkanes but not mid-chain substituted substrates. Therefore, the mode of dehalogenation is known to be different between substrates with chain lengths less than C_9 compared to those longer than C_9 (Poelarends, Zandstra et al. 2000).

It is important to note that there is evidence of a competing abiotic dehalogenation mechanism. A release of 35 mg/L chloride was observed in an un-inoculated control after 5 days from 1% v/v Cereclor S45 (Allpress and Gowland 1999). Also, 48 mg/L bromide was released from 500 mg/L of bromomethane after ten days of incubation (Janssen, Scheper et al. 1985) while other flasks exhibited less than 3.5 mg/L abiotic release. Similarly, from <3.5 to 30 mg/L halide were observed in sterile controls of shake flask experiments on a range of haloalkanes (Janssen, Jager et al. 1987). The researchers did not speculate on the mechanism of release or explain the variability in results of abiotic experiments. In particular, they did not speculate on the possibility of contamination of abiotic experiments.

1.7.4 Enzyme Expression

While there are many reports of constitutive monooxygenases involved in catabolism, such as the degradation of trichloroethylene and dichloromethane by methanotrophs (see section 1.6.2), the importance of enzyme induction is highlighted in most reports of oxygenolytic dehalogenation of medium and long chain haloalkanes and of hydrolytic dehalogenation of short chain haloalkanes. For example, a report of 1,2 dichloroethane dehalogenation by a monooxygenases in a non-methanotroph concluded the enzyme is induced (Hage and Hartmans 1999).

In the case of *Pseudomonas* sp. 273, induction is brought about by the chloroalkane and by the unchlorinated alkane with the same chain length (Wischnak, Loffler et al. 1998), (Bock and Muller 1996). However, in these same studies it was reported that cells previously grown on decane dehalogenated 1,10 dichlorodecane (DCD) with reduced activity compared to cells previously grown on DCD. In most cases no activity for DCD

was immediately observed when the starter culture was grown on nutrient broth or glucose, but activity returned after a delay.

Early research (Omori, Kimura et al. 1987) reported that a *Pseudomonas aeruginosa* strain dechlorinated DCD when grown on *n*-hexadecane. However the organism did not grow on DCD alone. Thus, the *n*-alkane was necessary for the degradation of the corresponding chloroalkane. In contrast, work with *Rhodococcus erythropolis* Y2 indicated that the oxygenase dehalogenase activity was induced by growth in the presence of C_7 to C_{18} 1-chloralkanes and by the corresponding *n*-alkanes. These results were similar to those using *Pseudomonas* sp. 273 (Wischnak, Loffler et al. 1998), (Bock and Muller 1996) where DCD degradation was induced by both DCD and *n*-decane.

2. OBJECTIVES

The objective of this study was to maximize the degradation of chlorinated alkanes, in particular the Cereclor class of compounds through:

- developing a suitable process control strategy for a semi-continuous system
- identifying cultures, isolated by others, that are known chloroalkane degraders
- identifying potential co-substrates that act to induce the degradative enzymes
- maximizing bioavailability using known techniques such as solvents, and emulsification
- develop tools for the analysis of Cereclor compounds by gas chromatography

3. MATERIALS AND METHODS

3.1 CULTURES

All the haloalkane degraders used in the study were isolated by enrichment by other researchers. Table 2 provides a list of the haloalkanes degraders that were considered in this work.

The cultures were obtained either on agar plates or stabs, or freeze dried in vials. The cultures were first grown up in liquid mineral salts media (described below) in shake flasks at 30°C with a variety of α-ω dichlorinated alkanes as the carbon source. They were then plated on nutrient agar. Single colonies were selected from agar plates and grown a second time under the selection pressure of the α-ω dichlorinated alkane media. These cultures were examined under the microscope and the gram stain was performed to

help confirm the identities of the organisms. Cultures were taken from these flasks in late log growth phase or early stationary phase and frozen in 1.5 mL centrifuge tubes at -70°C in a mixture of nutrient broth and 10% glycerol. The frozen cultures were used to inoculate shake flasks in all experiments. This starter culture was then used to inoculate test flasks and/or the reactor.

Table 2 Candidates for Chloroparaffin Degradation
Cultures considered in this study. "A" was originally designated *Actinomycetes* "B" was originally designated *Rhodococcus Rhodochrous*

Organism	Demonstrated Substrate	Gram Stain	Isolated By
Rhodococcus Erythropolis Y2	short and medium chain	+ve	(Sallis, Armfield et al. 1990)
Pseudomonas sp. 273	1,10, dichlorodecane	-ve	(Wischnak, Loffler et al. 1998)
Rhodococcus Erythropolis GJ70 (A)	pesticides, long chain pca	+ve	(Janssen, Jager et al. 1987)
Rhodococcus Erythropolis NCIMB 13064 (B)	various, mainly short chain	+ve	(Curragh, Flynn et al. 1994)
Rhodococcus sp. S45-1	medium chain chloralkanes	+ve	(Allpress and Gowland 1999)
Xanthobacter Autotrophicus GJ10	short chain haloalkanes	+ve	(Janssen, Scheper et al. 1985)
Corynebacterium sp. m15-3	chloroparaffins	+ve	(Yokota, Omori et al. 1987)

3.2 CULTURE CONDITIONS

The carbon source was the limiting nutrient for all fermentations. Nitrogen and other nutrients were added to the mineral salts media in excess of growth requirements. The defined carbon sources used for fermentation work were: 1,10-dichlorodecane (DCD), 1, 2-dichlorododecane (DCDD), and hexadecane (C16) (99%, 98%, 99% purity respectively, Sigma Aldrich). Cereclor S52 (S52), (Pioneer Chemical Co., lot no. 12-12-01) and yeast extract (Difco Laboratories) were also used. DCDD is solid at room temperature and was therefore warmed in a water bath to 30°C prior to use.

The mineral salts medium (MSM) for all fermentations was prepared according to Table 3. Trace minerals were supplied from the concentrate solution described in Table 4. This media had an ionic strength of approximately 50 mM. NaOH was added as required to

adjust the pH to 7.0 prior to autoclaving. For reactor work, media was autoclaved in 10L volumes for 3h at 121°C and 1.03 bar (gauge). Carbon sources were added to the media following autoclaving, to avoid thermal decomposition, using aseptic techniques.

Table 3 Mineral Salts Media

Component	g/L
$Na_2HPO_4 \cdot 2H_2O$	2.67
KH_2PO_4	1.36
$(NH_4)_2SO_4$	0.5
$MgSO_4 * 7H_2O$	0.2
Yeast extract	0.5
Trace Minerals Solution	1 mL/L

Table 4 Trace Minerals Solution

Component	g/L
NaOH	20
$MgSO_4 \cdot 7H_2O$	10
$ZnSO_4 \cdot 7H_2O$	4
$CuSO_4 \cdot 5H_2O$	1
$MnSO_4 \cdot H_2O$	3.2
$Fe_2SO_4 \cdot 7H_2O$	20
Na_2SO_4	100
$NaMoO_4 \cdot 2H_2O$	1
H_2SO_4 (mL)	1
EDTA	30

3.3 PHYSICAL CHARACTERISTICS OF SUBSTRATES

The three chlorinated substrates used in the study are DCD, DCDD and Cereclor S52. The properties DCD and DCDD are summarized in the table below. The vapour pressure of DCD is 5 orders of magnitude greater than that of Cereclor S52.

Table 5 Characteristics of DCD and DCDD

	units	1,10 DCD	1,12 DCDD
Water solubility*	µg/L	257	22.4
Sub Cooled Vapour Pressure*	Pa (25°C)	0.5	0.068
Viscosity**	MPA (cP) (20°C)	4.72	n/a
Melting Point	C	15.6	29
SG	g/cm3	0.9960	0.9530

*(Drouillard Ken, Hiebert et al.)

** Sigma-Aldrich

3.4 REACTOR

A process and instrument diagram illustrating fermenter system used is pictured in Figure 4.

The system consisted of a 3 L stainless steel cylindrical reactor vessel with a working volume of 2 L. The system was fully automated, including sampling systems, by means of a data aquisition and control system, described in section 3.5. The reactor was equipped with instrumentation to quantify offgas CO_2, dissolved oxygen and chloride ion. A four bladed radial turbine impeller 7.5 cm in diameter was driven by a variable speed mixer (Caframo model RZR1) set to 920 rpm. Air was introduced to the reactor directly below the turbine at a rate of 1.5 L/min. The overall oxygen transfer coefficient, k_La, was estimated in water to be 155 min^{-1} according to an empirical relationship based on mixer speed and air flow rate developed for the equipment (Brown 1998). Mineral media was introduced to the vessel by weight (MLP 25 pressure transducer, Transducer Techniques Inc.) through a stainless steel solenoid valve. The filling process was calibrated to deliver a 1.00L volume. Medium was drained from the vessel through an overflow complete with height adjustment. The height of the overflow determined the residual volume, that is, the volume left in the reactor after draining, with the mixer shut off. The residual volume was set to 1.00 L. Spent broth was drained through an automated sampling system to a waste container.

The samples were kept in a refrigerator at 4°C. The reactor vessel was fitted with a temperature control loop incorporating a thermocouple, an electrical heater, and a temperature controller (Omega Instrument Co.) with proportional-integral control using factory default tuning parameters to maintain the reactor temperature at 30 °C ±0.1°C. Air was regulated to the reactor by means of a gas flow indicator/controller coupled to the CO_2 analyser. The gas was dispersed through a rotometer, a glass wool air filter, a solenoid valve and a diffuser, consisting of four orifices each 0.1 mm in diameter. Offgas

passed through a water cooled condenser fitted over the reactor and through another glass wool air filter. Condensate was returned to the reactor. Offgas was sampled by the CO_2 analyser through an air dryer.

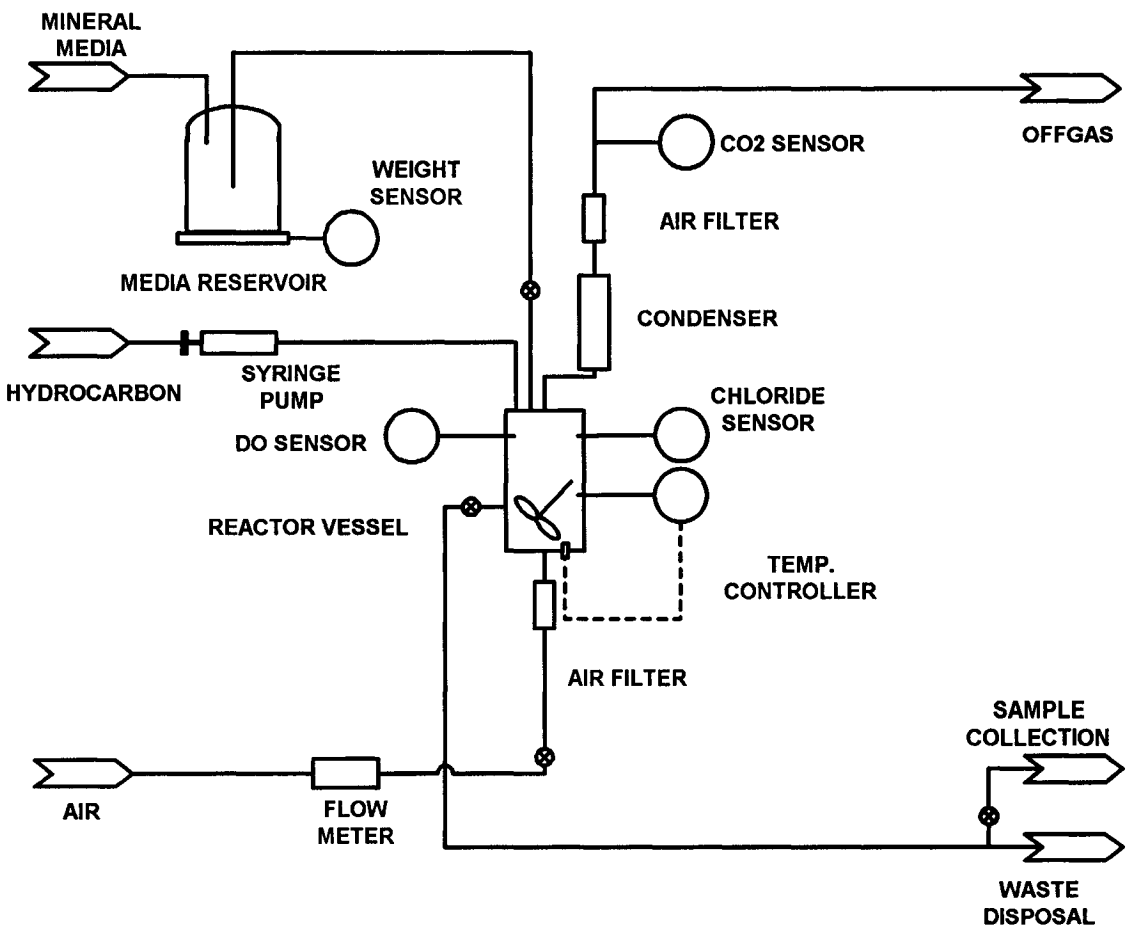

Figure 4 Self Cycling Fermenter Process and Instrument Diagram

The following terminology is used in describing the reactor experiments:

- A **cycle** consists of filling the reactor, following another cycle, from the 50% level to 100% level with mineral medium, adding the carbon source, carrying out the desired biological reactions, and draining half the reactor contents.

- A **run** consists of a series of cycles starting from the sterile, un-inoculated reactor. At the end of a run the interior of the reactor was rinsed with solvent to recover residual hydrocarbon.

- A **stage** consists of a portion of a **cycle** with a distinct operating regime, such as a fed batch period.

3.5 PROCESS CONTROL

The data acquisition and control system was as described by Brown (Brown 1998) and was based on an Intel Pentium compatible PC with a data acquisition board (Data Translation Inc. DT2801), a screw terminal board (DT707) and a relay board (Opto22 Inc. PB16A with OAC5 optically isolated relays). The relays turned on and off all the solenoid valves, the mixer motor, and the syringe pump. Larger electrical loads had separate relays. The load cell, chloride transmitter and dissolved oxygen transmitter provided 0-5 VDC signals to the data aquisition board through signal conditioning circuits that employed AD624 integrated amplifier chips (Analog Devices Inc.). Where a transmitter supplied a 4-20 mA signal, the leads were shorted using resistors. The amplifier circuit board provided a conditioned power supply for all amplifier chips and the pressure transducer.

The data acquisition system was interfaced through LABTECH software (LABTECH Inc.) that linked the data acquisition board with buffers in the RAM of the control PC. These buffers were in turn linked using a macro, written in MS Visual Basic for Applications, to a workbook in MS Excel. This macro was open coded, that is, the code was available for editing, so it was possible to modify the algorithm to provide the control functions for the fermenter. A listing of the control macro that linked the buffers to the workbook and provided data acquisition and control is provided in Appendix 1.

The following subroutines were necessary to provide data acquisition and control of the SCF.

1. A series of subroutines established a two way link between the workbook and the buffers. These subroutines provided the status and contents of the buffers available to the spreadsheet for data acquisition, and read certain cells from the spreadsheet back to the buffers for control. These subroutines also set up the spreadsheet to accept the data and initialized certain macro variables. These subroutines were provided with the LABTECH software and were modified to suit the purposes of the SCF.

2. A single subroutine served as the host for all control subroutines. This subroutine, called "GetData()" ran at a time interval specified by the user and contained calls to all other subroutines. It copied the current value of each of the

buffers to a worksheet together with a time stamp. This subroutine called itself, so it was an endless loop which ran until a "stop" routine was activated by the user. This vendor supplied routine was rewritten to control the SCF.

3. Three parameters were accessed by the "GetData" host routine: the chloride electrode output, dissolved oxygen and the medium bottle weight. The CO_2 system was controlled by its own PC and therefore was not connected to the data acquisition board. Instead the CO_2 system was networked to the control PC using a network hub and MS DOS networking software. A subroutine, named "get_co2_data" was written which accessed the text file produced by the CO_2 system and copied the latest value to the control workbook, permitting for automated control of the reactor based on this parameter. The call frequency was set to correspond to the frequency that the CO_2 system read the CO_2 value. The CO_2 system would crash if both the CO_2 system and the reactor control system accessed the CO_2 data file simultaneously, so a subroutine was written to prevent this event from occurring.

4. A subroutine was written to calculate the average value of a parameter and the change in a parameter over time. When implemented, the end of a cycle could be identified when the rate of change reached a threshold. The average value of a parameter was in other cases compared directly with a threshold to activate reactor cycling. A manual cycling mode was also available, with which the end of a cycle could be initiated by the operator.

5. A series of subroutines were written to perform the sequence of actions required to cycle the fermenter. This sequence was as follows:
   - turn off the agitator
   - open the drain valve until the contents have drained to the overflow level into the temporary sample vessel (not shown in Figure 4)
   - open the appropriate sample valve to drain the temporary sample vessel to the sample jar and write the sample date and time to the spreadsheet.
   - close the drain valve and the sample valve
   - activate the syringe pump to add substrate
   - turn on the agitator
   - open the fill valve and add 1.00L of medium based on medium bottle weight
   - close the medium fill valve
   - set a timer to prevent the possibility of cycling for 30 minutes

6. A subroutine was written to allow the macro to be run on simulated data to check the operation of the cycle detection subroutines.
7. A subroutine was written to allow for fed batch addition of substrate from the syringe pump based on the signal from the carbon dioxide analyzer.
8. A subroutine was written to randomize the quantity of substrate addition at each cycle.
9. A subroutine was written to add, on demand, a measured quantity of medium to the reactor according to the weight of the bottle.
10. A subroutine was written to measure the quantity of medium added to the reactor when the medium valve was activated manually.

3.6 CULTURE CONDITIONS

DCD was added to the reactor using a syringe pump (Model 200, KDScientific) equipped with a 30 mL glass syringe, 2mm I.D. teflon tubing and a stainless steel hypodermic needle. DCDD was added by pipette aseptically through an open port after heating to 30°C in a water bath. Similarly, C16 was added by pipette using aseptic techniques. Cereclor S52 was added using the following procedures. First, the hydrocarbon was taken up in a 10 mL syringe and warmed to 30°C. A 100 mL volume of sterile distilled water, was added to a 400 mL glass beaker, was placed in an ice bath on a homogenizer (Model 125-D, Willem-Polytron, Brinkman Instruments, with 3.5 cm dia PT45 generator, rated 20000 rpm). The homogenizer was operated at an estimated 10000 rpm (PCU-2-110 speed control, Kinematica GmbH). The diameter of the beaker was only slightly greater than that of the generator head. A 5.0 mL volume of hydrocarbon was added through a wide bore hypodermic needle to the base of the impeller. The procedure was carried out over a period of 3 minutes creating a milky homogenate. The homogenate was taken up in a sterile 10 mL pipette and added aseptically to the reactor. In parallel, a sample of the homogenate was added to a flask containing MSM and this flask was extracted to determine the precise concentration of hydrocarbon in the homogenate. An abiotic experiment was conducted to confirm that this procedure did not result in measurable chloride release. Microscopic examination of the homogenate indicated that most droplets were less than 5 μm in diameter. However the complete particle size distribution was not measured.

A 100 mL volume of medium was added to each 500 mL shake flask. The flasks were stoppered with foam or glass wool plugs. Shake flasks were incubated at 250 rpm and 30 °C (model G25 Incubator Shaker, New Brunswick Scientific Co.). Where Cereclor was fermented in flasks, C16 and DCD were added as cosubstrates. Flasks were adjusted for

evaporative losses by the addition of sterile distilled water, by weight, prior to the collection of each sample.

3.7 ANALYTICAL PROCEDURES

3.7.1 Biomass

Biomass was estimated using one of three methods: gravimetrically, by absorbance, or using a colourimetric protein assay.

In the gravimetric method, a 10 mL sample was centrifuged at 10,000 x g for 5 minutes. The supernatant was decanted and the pellet was rinsed to a dessicated, tared weighing dish using deionized water. The dishes were dried to constant weight in an 80°C oven (Isotemp, Fisher Scientific Ltd.) and then cooled in a dessicator for at least 2 hrs. Samples cooled in the laboratory environment outside the dessicant jar were observed to gain weight due to the absorption of ambient humidity. Gravimetric analyses were conducted using an analytical balance with a weighing accuracy +/- 0.0001 g, (Denver Instrument Co. Model TR 204) using 10 mL of sample volume. For dilute samples, 20 mL was used resulting in a resolution of 10 mg/L dry weight.

In the absorbance assay, biomass was inferred from optical density measured at 560 nm using a Bio 100 spectrophotometer (Varian Inc.) with distilled water as the reference. Samples were less than 15 minutes removed from the fermentation vessel, but more than 5 minutes old to allow suspended gases to escape. Biomass samples were diluted to an absorbance of less than 1 AU with distilled water and the dilution factor was then applied to the absorbance to determine the "adjusted absorbance". A calibration curve for adjusted absorbance *vs.* measured dry weight is provided in Figure 5. This curve was used subsequently to determine dry weight. This curve was applied only to samples at the cycle endpoint, to avoid interference in the absorbance measurement by residual, suspended hydrophobic substrate.

Due to the interference effect of suspended hydrophobic substrate on the absorbance test, an alternate test was developed which utilized a colourimetric protein assay (D_c Protein Assay, Biorad Laboratories Ltd.). Samples were centrifuged and the pellet was re-supended in deionized water. In order to improve the resolution of the assay, the samples were concentrated either 2x or 4x by reducing the amount of deionized water. The protein assay test was conducted on samples that had not been frozen. A sample of the supernatant was used as the reference in the spectrophotometer. The calibration curve was prepared against dry weight results from gravimetry (Figure 6).

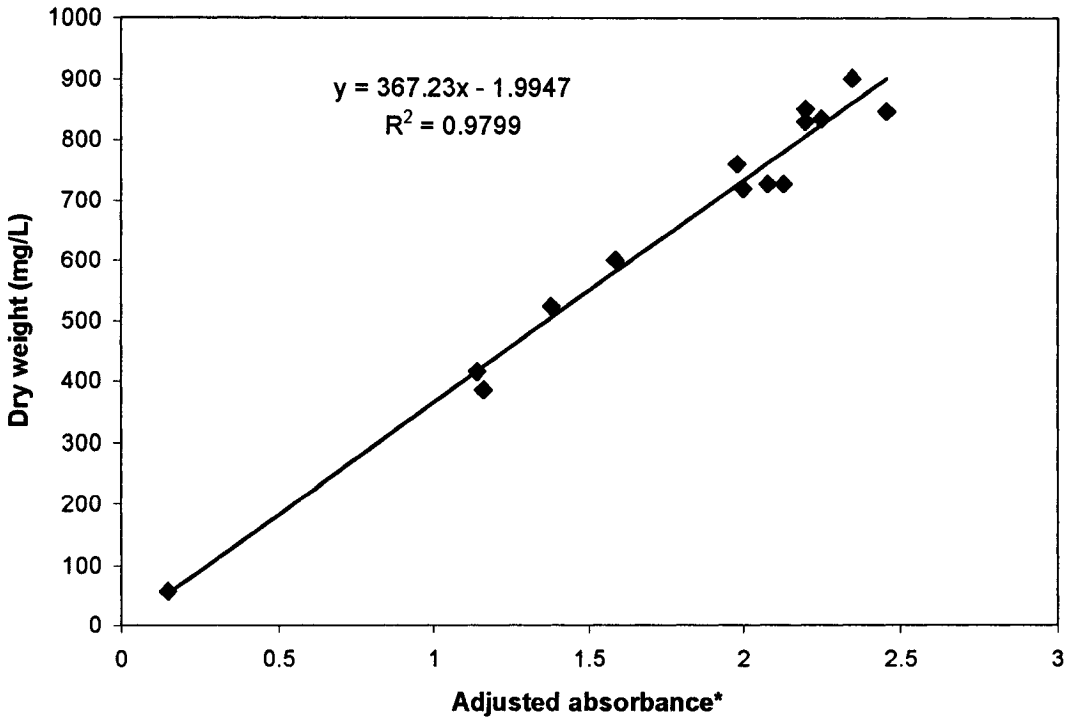

Figure 5 Calibration Curve for Biomass by Absorbance (560nm)
*Adjusted for dilution of sample to below 1.0 AU

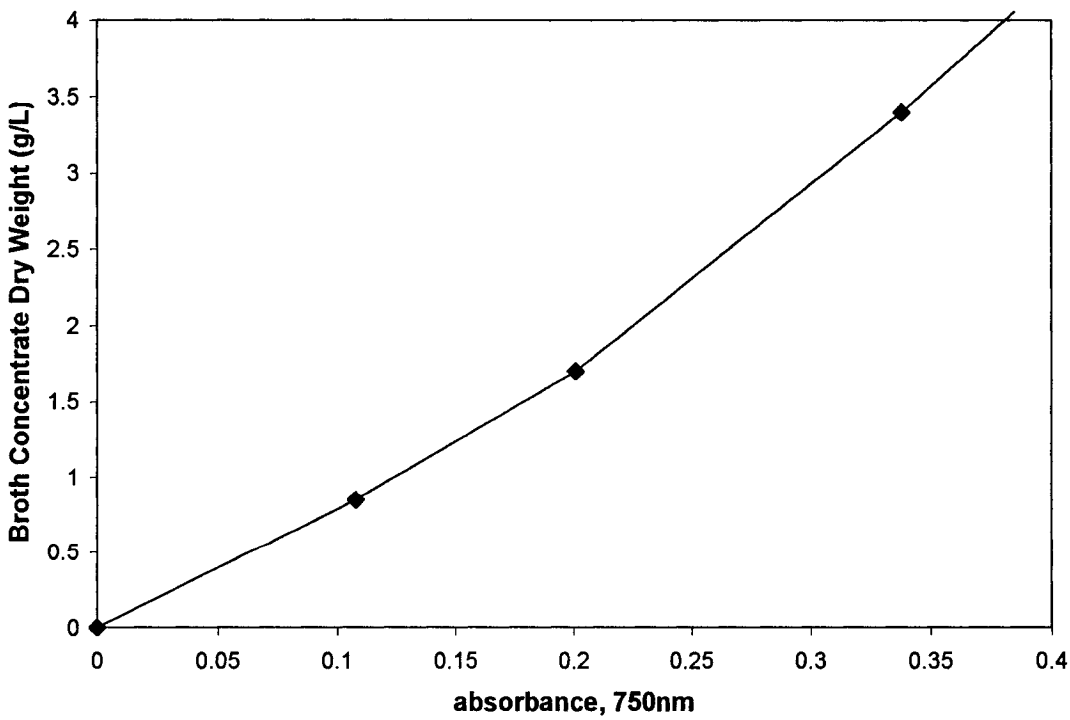

Figure 6 Protein Assay Calibration Curve

3.7.2 Chloride

Quantification of chloride ion was conducted on a DX100 ion chromatograph (Dionex Corp.) with a Dionex IonPac AS12A anion exchange column, an AG12A guard column and a conductivity detector. Eluent was composed of 0.3 mM $NaHCO_3$ and 2.9 mM $NaCO_3$ solution in deionized water (<19uS/cm). An anion suppressor was utilized to increase the sensitivity of the detector to the analyte by lowering the ionic strength of the eluent. The suppressor wass located downstream of the chromatography column but upstream of the detector.

A 2 mL sample was centrifuged, and the supernatant frozen in new glass test tubes. For analysis, the sample was thawed, and a sub sample of 0.50 mL was transferred to a cleaned 15 mL test tube. 10.0 mL of internal standard solution was then added to provide a 20:1 dilution, and the test tube was mixed for at least 30 seconds on a Fisher Vortex Genie Model 2. The internal standard solution consisted of 89 ppm NH_4Br in distilled water. Blanks and spikes were performed routinely as controls. Three point calibration curves were prepared prior to each set of analyses. Standards were prepared using NaCl in mineral salts media and diluted with internal standard, following the same procedures as for sample preparation. All calibrations, showing the variability between calibrations, are illustrated in Figure 7. The effect of the initial pH of the samples on the response of the IC, illustrated in Figure 8, was insignificant.

Attempts were made to measure the chloride concentration in the reactor continuously using a chloride ion specific probe. Due to a lack of sensitivity of the chloride probe, these measurements were used only to plot relative levels within cycles. The chloride specific probe (Pheonix Electrodes model 27502-13, supplied by Cole Parmer Insturment Co.) was fitted to the reactor in a teflon housing and connected to a Horizon Ecology Model 5997 Eh transmitter (Cole Parmer Instrument Co.). The chloride specific probe was routinely calibrated using a 1000 ppm Cl^- solution of sodium chloride according to the manufacturer's instructions. The electrode was routinely polished prior to calibration using mylar polishing strips supplied with the probe.

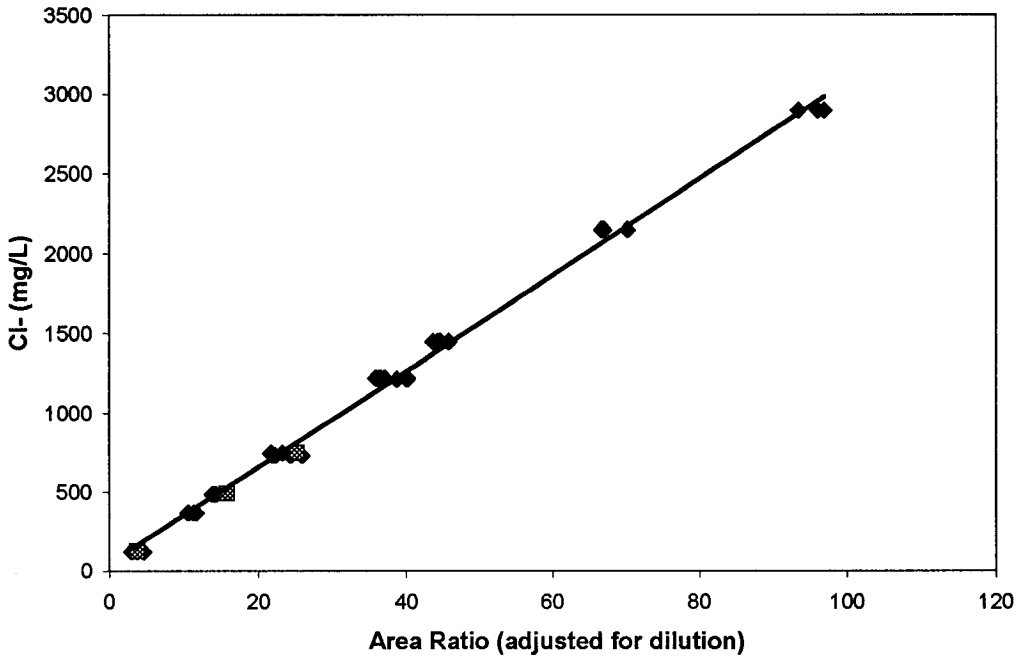

Figure 7 Calibration Curve for Ion Chromatograph
◆-untreated standards ■- treated standards

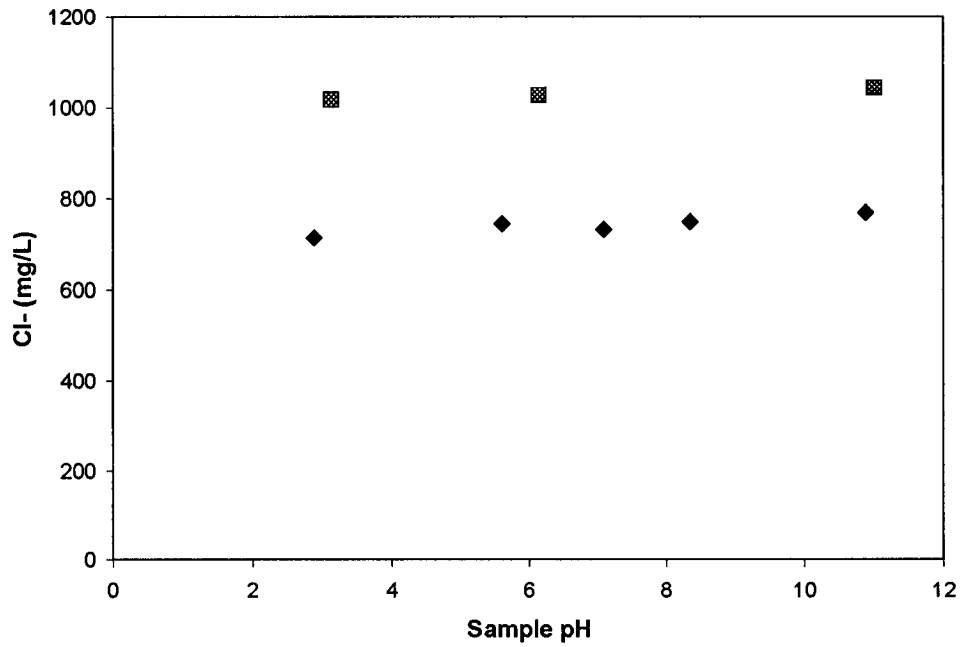

Figure 8 Dependence of IC measurement on sample pH.
◆747 ppm ■1021 ppm Cl^- Samples were prepared in MSM and diluted 20:1 in internal standard solution

The probe is an electrochemical cell. Therefore, this probe should be stored dry to prevent unnecessary dissolution of the cathode. The probe response, that is the slope of the log of the concentration vs. to the probe electrical potential, was -54 mV per decade for a new probe. Over time the probe response decreased, possibly due to the loss of the silver surface area. In addition, the probe offset varied with pH. A typical calibration curve, at two pH values, is provided in Figure 9.

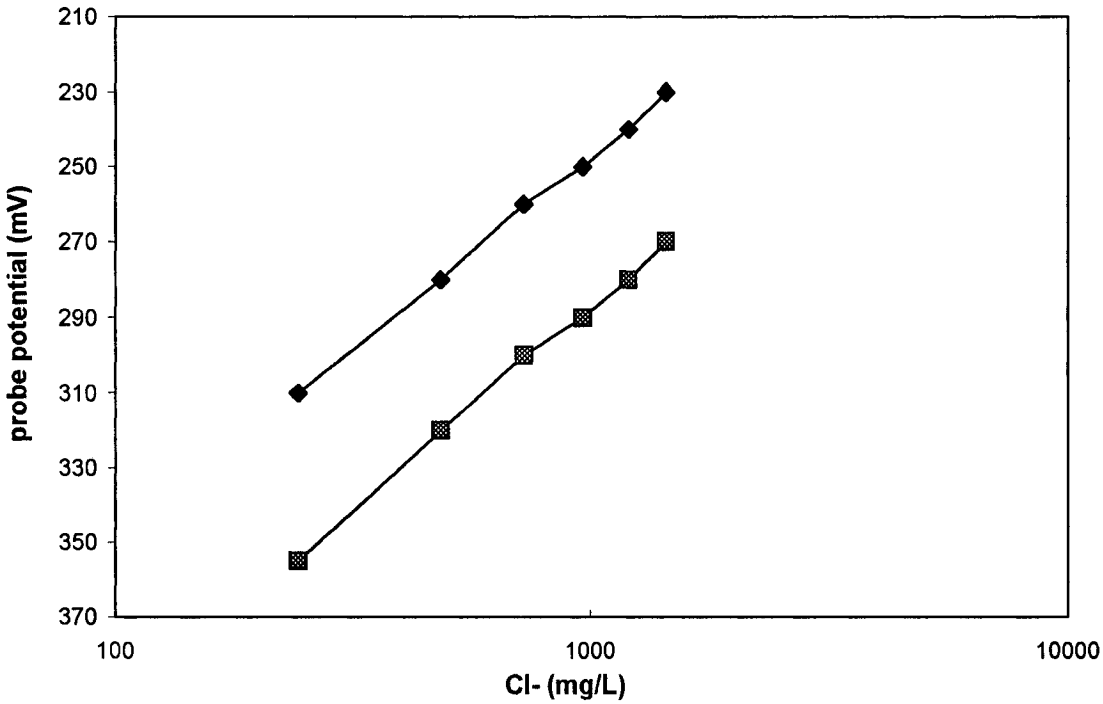

Figure 9 Chloride Probe Calibration Curve with respect to pH
■ pH 4 and ◆ pH 7, showing a probe response of between 44 and 46 mV/decade respectively

3.7.3 Treatment Procedure for Chloride Samples

Selected samples were pre-treated prior to analysis. Pre-treatment was discovered to be necessary to recover all chloride from the reactor liquor under certain conditions. The procedure consisted of freezing a 10 mL sample volume, thawing, and then adjusting the pH to 10.0 using 5N NaOH solution. The basic sample was mixed for between fifteen minutes and two hours, then centrifuged in 2 mL micro-centrifuge tubes and analysed by ion chromatography. The results were adjusted to account for dilution by the NaOH solution.

3.7.4 Reactor Liquor Dissolved Oxygen

Dissolved oxygen was measured in the reactor using an Ingold autoclavable polarographic probe (Cole Parmer Model E05644-02) connected to a Cole Parmer transmitter (Model 01971-00). The probe body was grounded to the instrument ground of the data acquisition system.

The probe was polarized overnight prior to calibration. A two point calibration method was employed at the operating temperature of 30 °C. The instrument response was zeroed with the probe disconnected as directed in the instrument's operating manual. This value was compared to the response in tap water stripped of oxygen with a stream of nitrogen gas and found to be identical. The saturation point was then defined by the response of the probe in tap water saturated with air. Subsequently, dissolved oxygen values were reported as a percentage of the saturated value.

The probe was autoclaved in the reactor body following calibration.

3.7.5 Carbon Dioxide in Reactor Offgas

Reactor offgas carbon dioxide was measured by means of an Oxymax 451 series infrared photometer coupled with a gas flow control valve (Columbia Instrument Co.) and an Oxymax MS-DOS based data acquisition and control system. The system was calibrated using a two point method according to the manufacturer's instructions using nitrogen gas as a zero point and dry compressed air as the second point.

3.7.6 Hydrophobic Substrate

Assays for dichlorodecane (DCD) and dichlorododecane (DCDD) were conducted on a Varian CP 3800 gas chromatograph with a Flame Ionization Detector (FID). Separation was achived using a Supelco SPB-5 column which was 30m long and had a bore of 0.32mm. Alternatively, a 0.50 mm i.d. column was used for certain analyses involving DCD, S52 and C16. The immobilized phase of the SPB-5 column is poly(5% diphenyl/95% dimethylsiloxane). The film thickness was 0.25um. The column oven program was as follows: start at 100°C and hold for 0.5 min, ramp at a rate of 30°C/min to 300°C, and hold for 5 min. The total run length was 12.2 min. The injector temperature was 280°C. The best results were obtained using splitless injection, changing to a split of 100:1 after 20 seconds. The detector temperature was 300°C.

The calibration curve was generated by spiking known quantities of hydrocarbons into fermentation broth, including biomass at approximately 0.8 g/L dry weight. Frozen samples of fermentation broth, including cells, were thawed, and the pH of the aqueous phase was lowered to 2.0 using concentrated hydrochloric acid. Samples were then

contacted with an equal volume of hexanes containing either 137 g/L DCDD or 120 g/L 1-chlorohexadecane (CHD) as an internal standard, depending on the substrates being analysed. DCDD was used as the internal standard for all analyses except for fermentations of DCDD, in which case CHD was used as an internal standard. Extractions were conducted using hexanes in 20 mL sample vials with foil tops, in 250 mL separatory funnels and in 2 L separatory funnels, depending on the volume of the sample.

The mixture was agitated at high power on a vortex shaker (Vortex Genie Model 2, Fisher Scientific) for 3.0 minutes. Samples formed a stable emulsion, that was broken through centrifugation in the test vial at 4xg for 3 minutes. The centrifugation speed was set at the maximum allowable without breaking the test vials. Using test vials avoided the errors associated with transferring the samples to centrifugation tubes, and the given speed was more than adequate to break the emulsions. A second extraction was conducted on several test samples. The calibration curves for DCDD, DCD, C16 and S52 for both chromatographs utilized in the study are provided in Figure 10 and Figure 11.

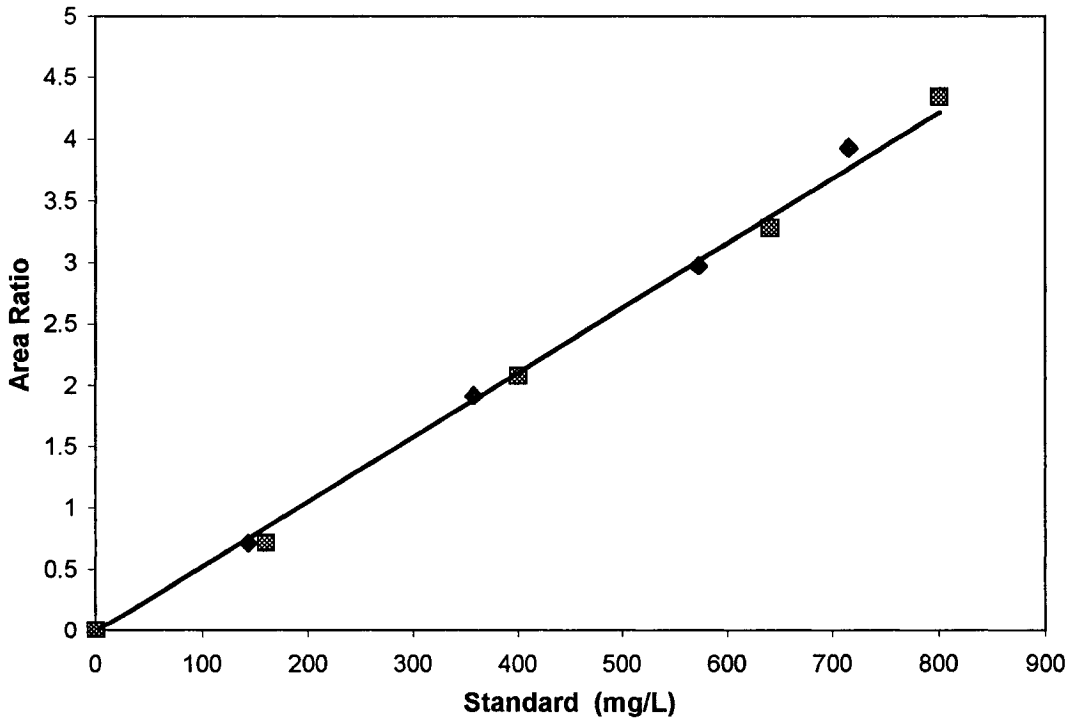

Figure 10 Calibration Curve for DCD and DCDD with CHD as Internal Standard
■ DCD/CHD ◆ DCDD/ CHD - 0.32 bore column

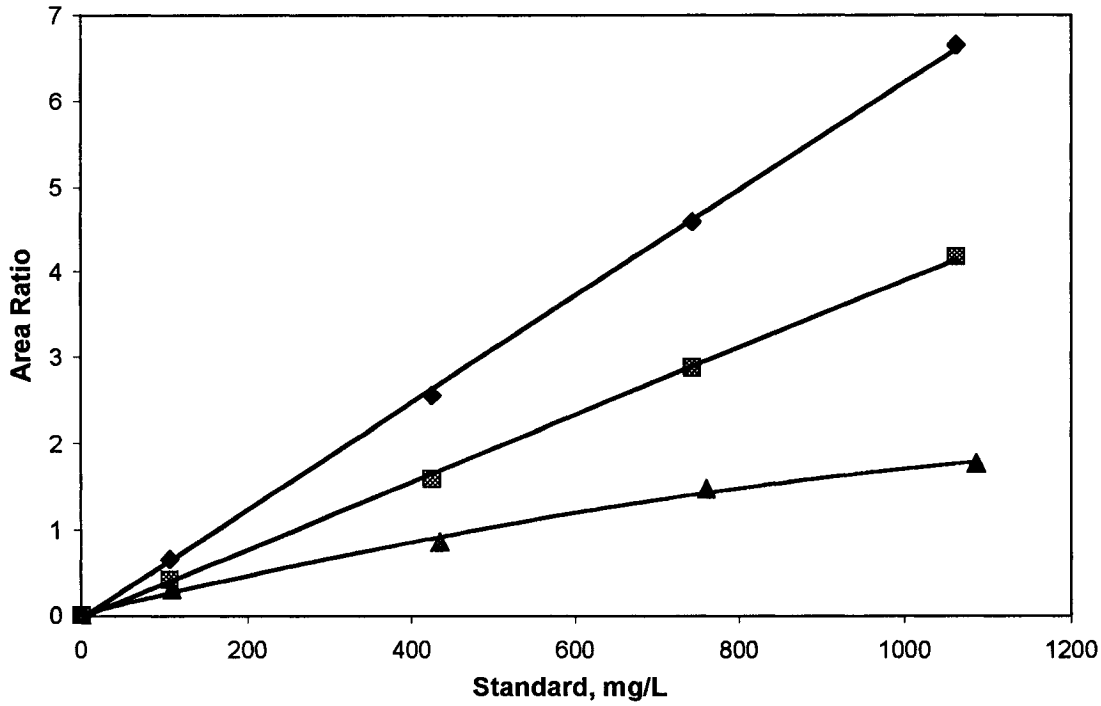

Figure 11 Calibration Curve for DCD, C16 and Cereclor S52 with DCDD as Internal Standard
■ DCD ◆ C16 ▲ Cereclor S52 - 0.5 bore column.

A linear calibration curve was utilized for the compounds: DCD, DCDD, and C16. A non linear calibration curve was utilized for S52.

Certain chromatograms were converted from the Varian export file format and analysed in MS Excel using custom software written in MS Visual Basic for Applications. The code for this software is provided in Appendix 2. This software automated the manipulation of the raw data from the chromatograph allowing for more a complete evaluation, as discussed in Section 4.8, than provided by the commercial instrument control software.

The reactor was rinsed following shutdown to recover biomass and hydrocarbons that accumulated on the walls of the vessel. The first 20 mL of media was flushed from the bottom drain to a 2L tared separatory funnel to recover oils which had collected in the drain orifice. The remainder of the culture was removed and reported separately. Then the reactor was scrubbed and rinsed with distilled water in the fume hood to the same separatory funnel. The volume of the rinse was determined by the differential weight of

the funnel. A sample of the rinse water was taken for biomass determination and the remainder was acidified. Finally, the reactor was flushed with 100 mL of hexane, containing the internal standard, to the separatory funnel to rinse the remaining hydrocarbon from the reactor. Additional hexane / internal standard solution was added to the funnel until the volumes of hexane and rinse water were equal. The extraction procedure described above was repeated to determine the hydrocarbon content on the reactor rinse, because depending on the experiment the solvent was sometimes saturated with hydrocarbon. The mass of hydrocarbon remaining on the walls was calculated knowing the volume of the solvent used.

3.7.7 Bacteria Adherence to Hydrocarbon (BATH) Assays

Hydrocarbon / biomass surface interactions were measured using a procedure modified from Rosenberg *et al.* ((Rosenberg 1984). The procedure measures adherence of the biomass to hydrocarbon. This is achieved by measuring the drop in absorbance of the aqueous phase containing biomass following contact with the hydrocarbon phase. Four hydrocarbons were tested using the experimental design illustrated in Table 6. Biomass was grown under a series of conditions as described. Biomass was harvested and then immediately washed in mineral salts media (pH 7.0) and diluted to an absorbance of 0.5 AU. 10 mL samples were agitated in 20 mL vials for between 0.5 min and 2 min at maximum power (Fisher Vortex Genie 2). All tests were conducted in a water bath at 30°C. The absorbance change was in most cases measured after the agitated sample had settled for 15 min.

3.7.8 pH

Samples of fermentation broths were analysed for pH in 3 mL volumes using a Cole Parmer pH meter and an Acumet combination pH probe with Ag/AgCl reference cell. Probes were calibrated using the two point method using pH 4.00 and pH 7.00 standards.

Table 6 BATH Assay Experimental Design Matrix

	Hydrocarbon Tested					Biomass Washing Procedure			Agitation Procedure				Growth Phase and Culture System				HC Conc.		
Test Name	YE	C16	S52	DCD	DCDD	washed 3x in MSM	washed 5x in MSM	washed 1x in MSM	1/2 min strong agitation	1min strong agitation	1 min weak agitation	2 min violent agitation	Reactor stationary phase	Reactor early log phase	Flask stationary phase	Flask early log phase	10% Hydrocarbon	1% Hydrocarbon	0.1% Hydrocarbon
B1.1				X				X					X					X	X
B1.2					X			X					X					X	X
B1.3		X						X					X					X	X
B1.4			X					X					X					X	X
B2.1				X		X					X		X				X		
B2.2					X	X					X		X				X		
B2.3		X				X					X		X				X		
B2.4			X			X					X		X				X		
B3.1				X			X			X						X	X		
B3.2					X		X			X						X	X		
B3.3		X					X			X						X	X		
B3.4			X				X			X						X	X		
B4.1				X			X		X							X	X		
B4.2					X		X		X							X	X		
B4.3		X					X		X							X	X		
B4.4			X				X		X							X	X		
B4.5			X				X		X							X	X		
B4.6	X			X			X		X							X	X		
B4.7	X			X			X		X							X	X		
B4.8			X				X					X				X	X		
B4.9			X	X			X		X							X	X		
B5.1		X					X		X				X				X		
B5.2	X		X				X		X				X				X		
B5.3	X	X					X		X				X				X		
B5.4		X					X					X	X				X		
B6.1		X					X		X						X		X		
B7.1		X					X		X					X			X		
B7.1				X			X		X					X			X		

4. RESULTS

4.1 MINERALIZATION ENERGETICS

The energetics of the mineralization of chlorinated alkanes was investigated to identify possible thermodynamic barriers to the biodegradation of these compounds. Chlorinated

alkanes are more oxidized than their non-chlorinated analogues, so it was expected that the Gibbs free energy of the mineralization reaction would be higher (less negative), meaning the mineralization reaction would be thermodynamically less favourable.

Published data for the Gibbs free energy of reaction, ΔG_r, for the mineralization of various chloroalkanes, and the Gibbs free energy of formation, ΔG_f for these substrates were not available for all the chlorinated alkanes that were studied. Instead, these parameters were estimated using the group contribution method (Dolfing and Janssen 1994). In Table 7, estimates ΔG_f based on the group contribution method reported in the literature are compared to our estimates using the same method.

Table 7 - Group Contribution Method Estimates of Gibbs Energies of Formation and Mineralization for Selected Alkanes and Chloralkanes.
a - Dolfing and Janssen, 1994 b - calculated using the method reported in "a"

Compound	Carbon Atoms, C	ΔG_f° (kcal/mol)	ΔG_r° (kcal/mol)	ΔG_r°/ C
ethane	2	-41.7[a]	-351	-175
1,2 Dichloroethane	2	-17.4[a]	-306	-153
1,1,1,2 tetrachloroethane	2	-18.6[a]	-273	-137
1,10 dichlorodecane	10	-3[b]	-1520	-152
1,14 dichlorotetradecane	14	3.8[b]	-2181	-156
Hexachlorotetradecane	14	10.5[b]	-2070	-148
Tetradecachlorotetradecane	14	-70[b]	-1863	-133

4.2 SHAKE FLASK EXPERIMENTS

4.2.1 Culture Screening

Preliminary experiments were conducted in shake flasks using *Rhodococcus Erythropolis* Y2, *Pseudomonas* sp.273, *Rhodococcus Erythropolis* GJ70, and *Rhodococcus Erythropolis* NCIMB 13064 with 10mM DCD as the carbon source. *Pseudomonas* sp.273 grew well on DCD, normally achieving an absorbance of over 1 AU, while the other cultures grew only slightly, reaching an absorbance of less than 0.1 AU.

Rhodococcus Erythropolis Y2, *Rhodococcus Erythropolis* NCIMB 13064 and *Rhodococcus Erythropolis* GJ70 are gram positive cultures isolated on short chain chloroalkanes. Therefore, these cultures were grown with 1,6 dichlorohexane at 100 µL in 100 mL added in two successive additions of 50 µL. The addition of greater quantities of 1,6 dichlorohexane resulted in the death of these cultures. The gram negative culture, *Pseudomonas* sp. 273, was enriched using 1, 10 dichlorodecane at 100 uL/100 mL, and

grew under all concentrations tested, up to 10 mM. The other cultures grew well on 1,6 dichlorohexane (DCH). Based on these results, *Pseudomonas* sp.273 was selected for further study as the long chain alkanes were of interest.

4.2.2 Refinements to Culture Medium

Initially, the fermentation medium used was as described in the literature (Wischnak, Loffler et al. 1998). This medium included vitamins B12, pyridoxal, riboflavin, and thiamine. A trial was conducted which measured the biodegradation of DCD by *Pseudomonas* sp.273 in medium with and without vitamins. The results demonstrated that there was no significant difference in the growth, rate of growth or final hydrocarbon concentration (results not shown). In subsequent fermentations that included yeast extract, vitamins were omitted from the media to minimize the chance of contamination. The trace mineral solution was made up with only the minimum necessary EDTA to dissolve metals because of literature reports that EDTA inhibited dehalogenation in cell-free enzyme extracts (Wischnak, Loffler et al. 1998). The concentration of EDTA in the trace minerals solution was 30 g/L.

4.2.3 DCD Degradation by *Pseudomonas* sp. 273 in Shake Flask

Initial fermentations were conducted on DCD in shake flasks, to demonstrate growth and degradation and to develop analytical techniques. DCD was selected because *Pseudomonas* sp.273 had been isolated on this substrate (Wischnak, Loffler et al. 1998).

Flasks inoculated with active cultures of *Pseudomonas* sp. 273 grown on DCD typically reached an absorbance of over 1.0 AU after 1-3 days and exhibited a concomitant release of chloride. The release of chloride and the production of biomass indicated growth of *Pseudomonas* sp. 273 on DCD as the "sole" carbon source. Yeast extract was present in the mineral media as a growth factor and the quantity of carbon added as yeast extract was small compared to the amount added as DCD. Experimental controls indicated that the biomass production in the presence of mineral media and DCD far exceeded that observed in the presence of mineral media alone (results not shown). The final pH of these flasks was typically 3.8 - 4.5, and although residual substrate was observed, further incubation resulted in no additional chloride release or biomass increase (results not shown).

4.2.4 Medium Titration Curve

In order to estimate the buffering capacity of the media during dehalogenation, a titration curve was prepared using HCl as the titrant. This curve is presented in Figure 12. The figure illustrates that to reach a pH of 4.2, a typical endpoint in the flask fermentations,

requires the addition of 38 mM chloride as HCl. This quantity is equivalent to 19 mM or about 4g/L of DCD, since one proton is released with each chloride ion during dehalogenation (equation 2).

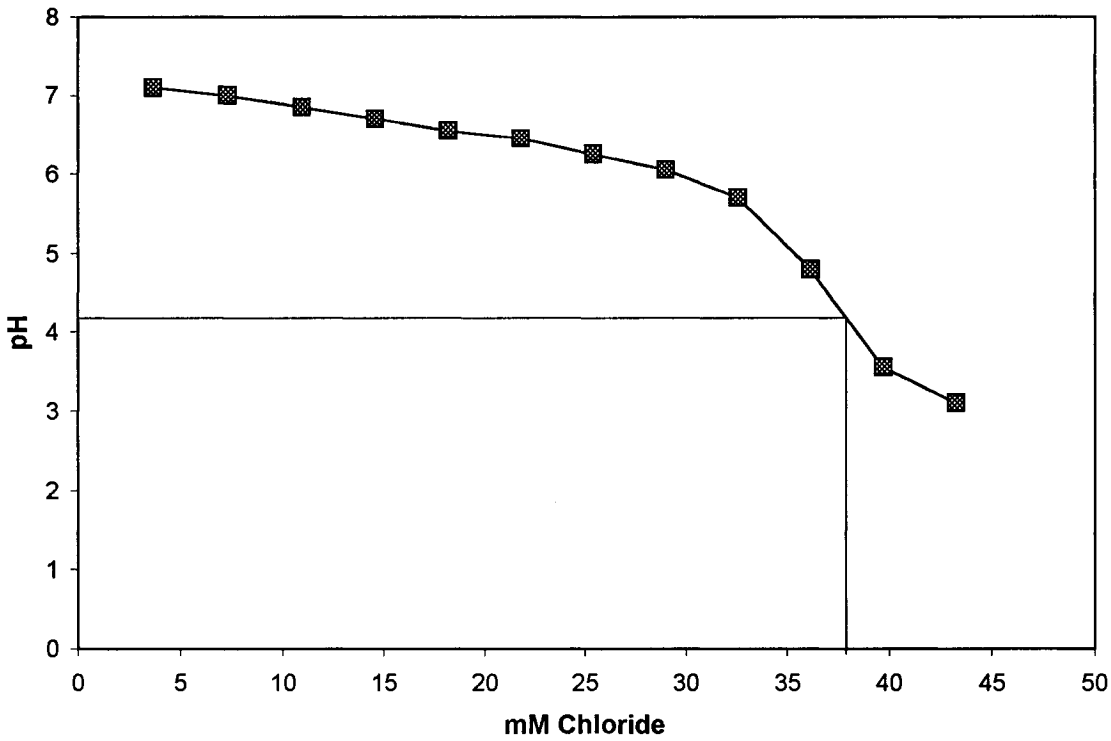

Figure 12 Medium Titration Curve
38 mM of acid (equivalent to 19 mM DCD) required to reach the typical pH endpoint of 4.2 for flask fermentations

4.3 CELL/HYDROCARBON ADHERENCE TESTS

The bacteria adherence to hydrocarbon (BATH) assay was used to help elucidate the relationship between the biodegradation results on various substrates with the adherence of the substrates to the biomass.

The cultures were grown either on DCD or on yeast extract prior to the tests. The results, X, are expressed as the difference in absorbance of the aqueous phase after agitation compared to before agitation as in equation 7.

$$X = \frac{A_f - A_i}{A_i} x100 \qquad (7)$$

All the tests were conducted with equal volumes of substrate and medium. A negative number in the results means biomass was removed from the aqueous phase to the hydrocarbon layer. Results are expected to be more negative for hydrocarbons and biomass which show greater adherence.

Selected results are shown in Table 8. The results of BATH assays were strongly correlated to the phase of growth of the culture, the degree of agitation and to the hydrocarbon used for the adherence test. DCD proved to reduce the absorbance to a lesser extent compared to DCDD and hexadecane. However in the case of DCD the aqueous phase following the test contained tiny droplets of hydrocarbon and this increased the light scattering in these samples. This effect was not visible in the other cases. The absorbance of the DCD samples decreased if a longer settling time was allowed, whereas with other hydrocarbons the effect was less pronounced. The results were generally zero or had positive values for the case where biomass was harvested from the reactor from the stationary phase and stored in the refrigerator overnight before testing. Thus it appears that, for aged biomass the absorbance increased due to the contribution of residual oil suspended in the aqueous phase. In these cases, the absorbance of the DCD samples rose more than that of the other hydrocarbons (results not shown). The addition of yeast extract to the medium in the test had no appreciable effect on the absorbance change.

Table 8 Summary of BATH Assay Results
In tests marked with *, early log flask grown cells were used because reactor grown cells were not available. Reported values are quoted as per equation 7.

	CHANGE IN ABSORBANCE, X (560nm)		
Hydrocarbon	Agitation Time		
	1/2 min	1 min	2 min
C16	-61%	-62%	
S52	-7%	-4%	-17%
DCD	-38%	-55%	
DCDD	-49%	-83%	
S52 + DCD	-23%		

	Reactor Grown Cells, ½ min agitation	
Hydrocarbon	Stationary Phase	Early Log Phase
C16	-15%	-55%
S52	+43%	* -4%
DCD	-1%	-31%
DCDD	+4%	* -49%

4.4 PROCESS CONTROL

4.4.1 Selection of Control Parameter

The output of the chloride electrode was chosen initially as the parameter to determine the time of cycling of the reactor. Chloride release is directly linked to the degradation of the substrate. A series of fermentations was run to monitor the intra cycle chloride probe responses on two carbon sources: DCD and DCDD. The chloride profiles for such experiments are provided in Figure 13 and Figure 14, respectively. Fermentations of DCD showed a distinct change in the slope of the chlorine probe output which coincided with the exhaustion of the substrate in the mixed liquor (see also Figure 18). The DCDD fermentations did not exhibit this feature.

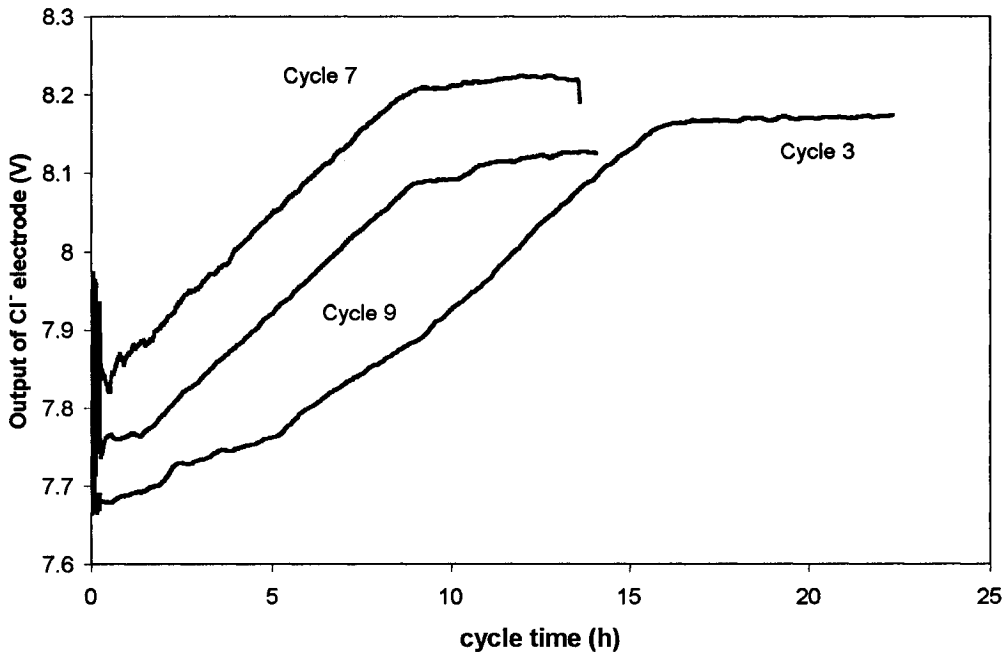

Figure 13 Typical chloride electrode response for reactor fermentations of DCD
The electrode output was transposed and amplified so the units are arbitrary

Dissolved oxygen (DO) was also considered for use as a control parameter. A typical DO profile for a fermentation on DCD is shown in Figure 15. However, at a low cell density and a high oxygen transfer rate, the dissolved oxygen concentration did not vary through the course of a cycle (results not shown).

The offgas CO_2 was also screened as a control parameter. This necessitated the establishment of a real time connection between the CO_2 system PC and the control PC. Profiles for chloride, DO and offgas CO_2 for a fermentation of DCD are provided in Figure 15.

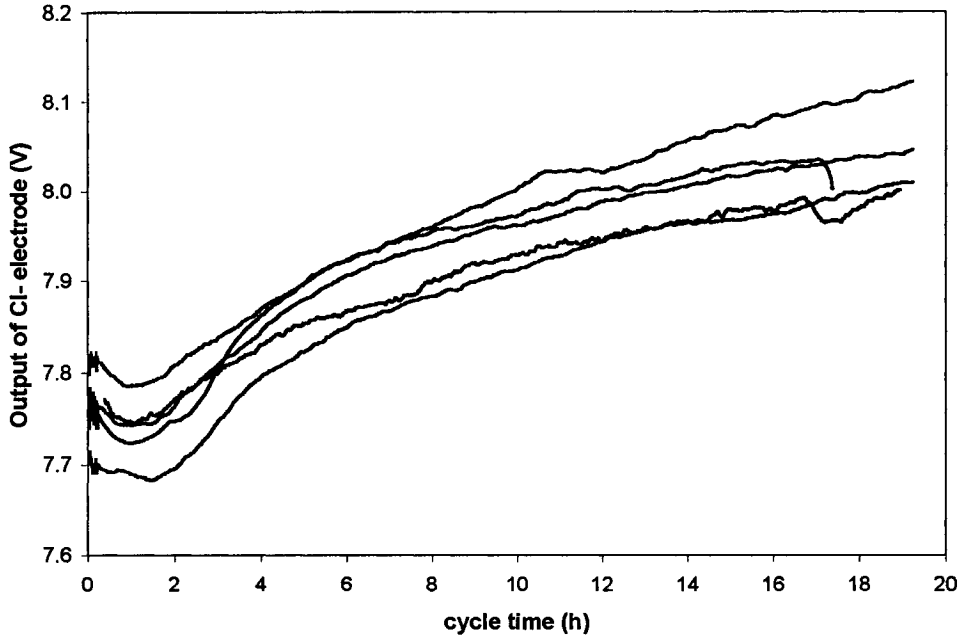

Figure 14 Typical chloride electrode responses for reactor fermentations of DCDD The electrode output units are arbitrary.

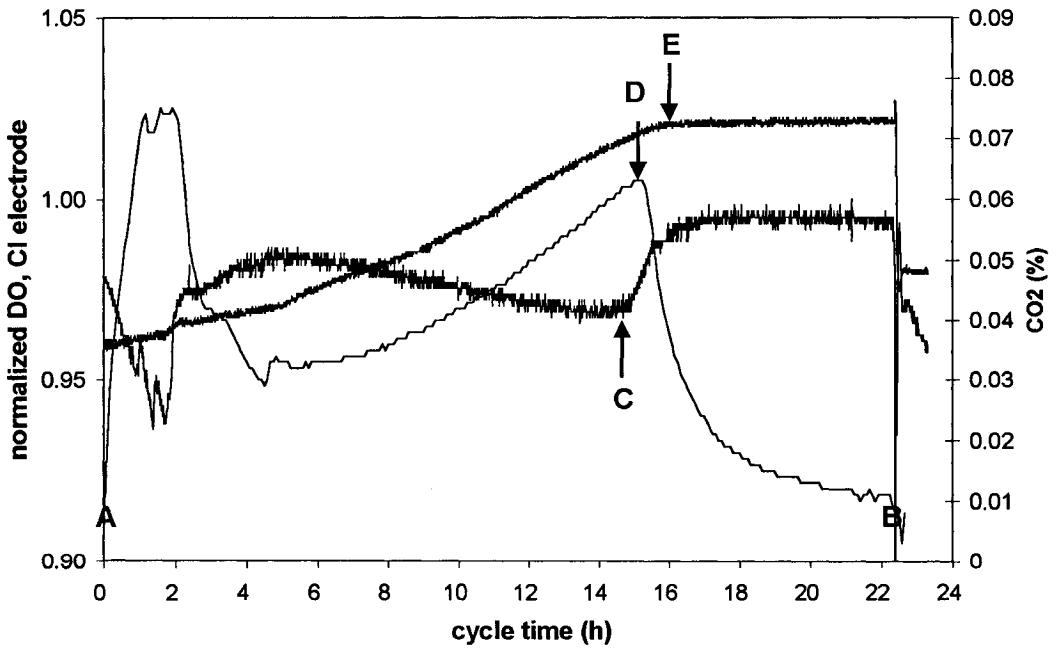

Figure 15 Characteristic CO_2, DO and Cl^- probe profiles for DCD degradation A - start of cycle (addition of medium and hydrocarbon), B - end of cycle (full degradation of added hydrocarbon), C - local minimum for DO at 14.4h, D - local maximum for CO_2 at 15.2 h, E - change of slope of chloride probe output at 15.9h

4.5 ABIOTIC CERECLOR CONTROLS

An abiotic experiment was conducted to assess the rate of chloride release under normal conditions in the reactor in the absence of bacteria. The issue of bioavailability was also explored. The experiment used homogenized Cereclor S52 in sterile mineral salts media with 84 mg/L added sodium chloride. The sodium chloride was added to simulate the chloride levels of other experiments, where chloride had accumulated in the medium due to the degradation of chloroalkanes in previous cycles. After fourteen days, DCD was added to the reactor in a single spike to determine the effect of DCD on the bioavailability of the Cereclor S52.

The results of the abiotic control are provided in Figure 16. The measured level of chloride at the start of the experiment was slightly lower than added amount (84 mg/L *vs.* 100 mg/L) which was attributed to errors in weighing due to the weight of water absorbed by the NaCl from the atmosphere during storage. The chloride level was constant throughout the run within the error of the assay. The S52 concentration dropped by 50% after 3 hours and 95% had been removed from the mixed liquor after 5 days (Figure 16). Then, with the addition of DCD, the S52 levels rose to about 50% of the amount originally added. The measured suspended DCD levels fluctuated between 43 and 60 % of the amount added. When the reactor was shut down and disassembled, accumulated hydrocarbons were observed on the reactor walls but were not quantified.

4.6 FERMENTATIONS OF DICHLORODECANE AND DICHLORODODECANE

Fermentations of DCD with *Pseudomonas* sp.273 were conducted to test the reactor systems and associated controls, as discussed above. *Pseudomonas* sp.273 grew well on DCD in the reactor and 5mM of substrate (1 g/L) was degraded after 18-24 h (results not shown). A series of runs were conducted with DCD alone, DCDD alone, and the two substrates combined to determine the effect on biodegradation results of slight changes in the length of the carbon backbone.

The CO_2 profiles for separate fermentations of yeast extract and of DCD are illustrated in Figure 17. The sum of these two curves is characteristic of a typical fermentation starting with these combined substrates as shown in Figure 18 and Figure 19.

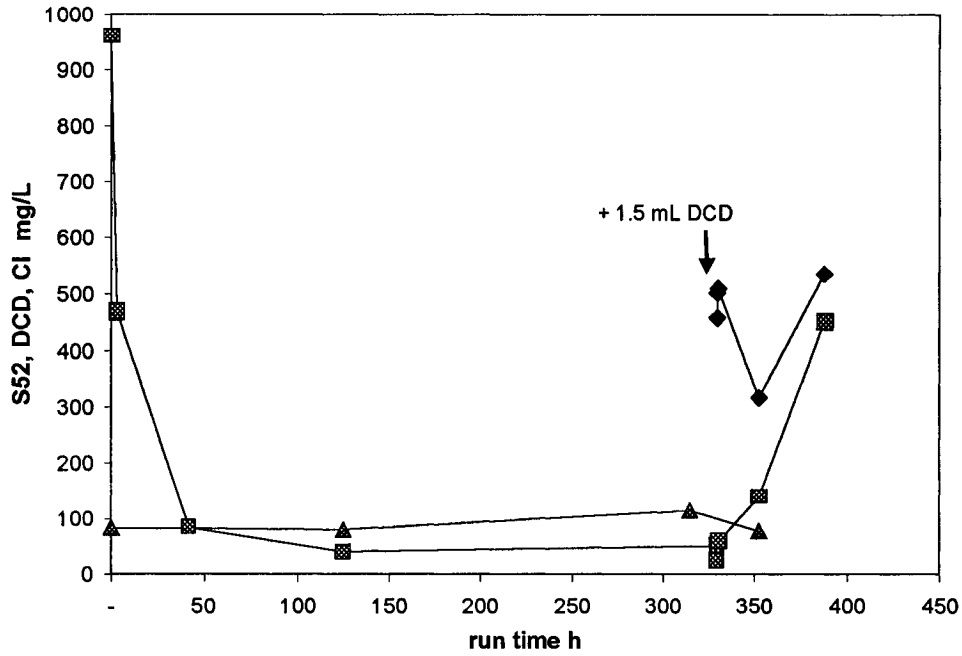

Figure 16 Abiotic Experiment with Cereclor S52 and DCD
■- Cereclor S52; ◆- DCD; ▲- chloride; 1000 ppm S52 added at t=0, 740 ppm DCD added at t=330 h; Initial chloride = 80 ppm added as NaCl

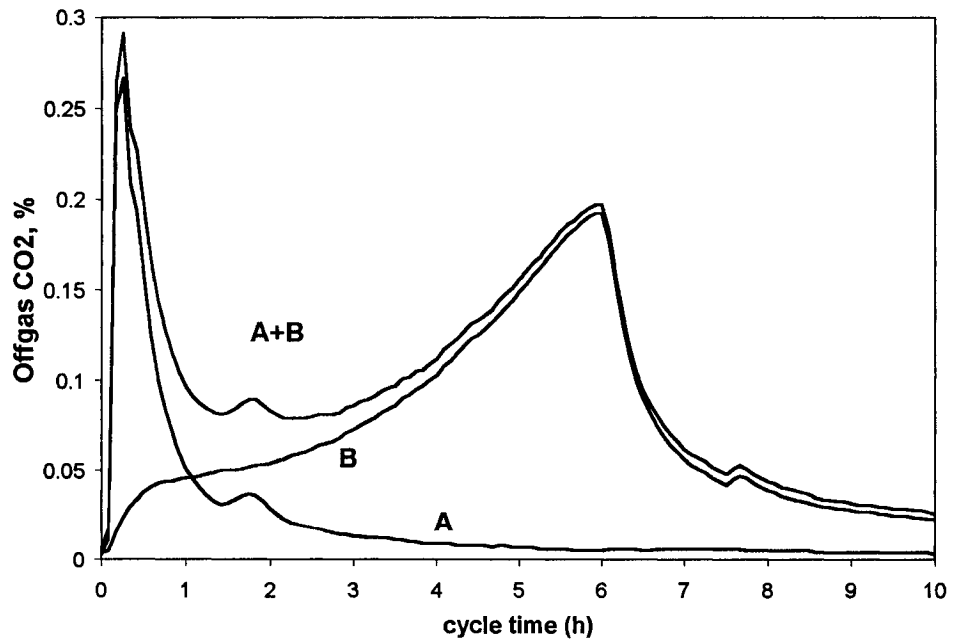

Figure 17 Superimposed CO_2 Profiles for Fermentations on YE and DCD
A - Fermentation of yeast extract alone, B - DCD alone, A+B - composite curve showing similarity to the profile where the two substrates are fermented together

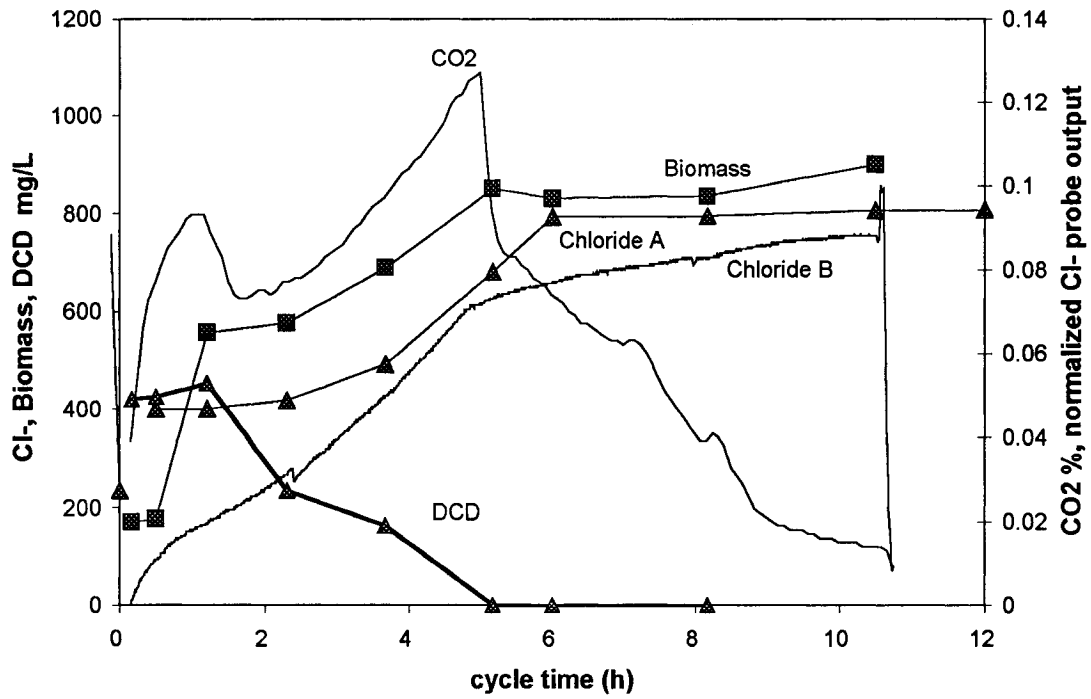

Figure 18 Intra Cycle Sampling Results for a Fermentation of DCD "Chloride A" - by ion chromatograph and "Chloride B" - by online chloride probe, suspended DCD by GC, Biomass by dry weight and CO_2 by online analyser. Chloride measurement by IC peaks after the disappearance of the suspended substrate.

Chloride, DO, and the suspended concentration of DCD are superimposed on the real time data for a cycle in which DCD was added as the growth substrate in Figure 18. Results of a repeat experiment are provided in Figure 19. This figure also illustrates the effect of sodium hydroxide addition on the output of the chloride probe. NaOH was added to adjust the pH in the experiment.

The biomass yield on DCD for the experiment illustrated in Figure 19 was 0.35 g biomass/g DCD. When biomass was measured gravimetrically, interference was apparent during the early part of the cycle due to loss of solids that adhered to the oily substrate and did not form part of the pellet during centrifugation. The oily substrate also interfered with biomass determinations by absorbance because during the early stages of fermentations oil droplets contributed to light scattering. Both these techniques for the determination of biomass produced repeatable results at the end of the fermentation when the oily substrate was exhausted.

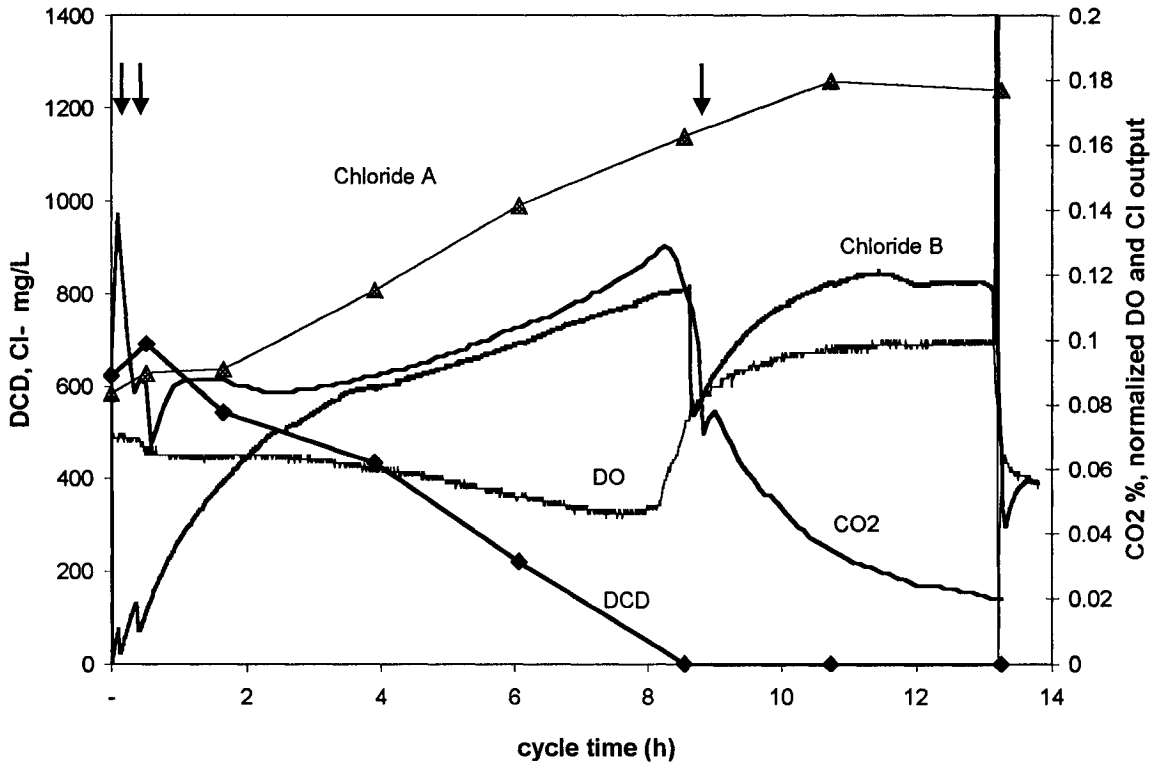

Figure 19 Fermentation of DCD
Repeat of experiment described by Figure 18, but with NaOH addition. "Chloride A" - by IC and "Chloride B" - by online chloride probe, suspended DCD by GC, CO_2 by online analyser. Arrows indicate times of NaOH addition to neutralize medium, showing and the effect on the chloride probe.

A test was conducted under alternating pH and substrate limiting conditions. The rate of metabolism was gauged by the concentration of CO_2 in the offgas. The results are illustrated in Figure 20. A similar test was conducted whereby a spike of ammonium sulphate solution was added to the reactor following the drop in CO_2 output, which demonstrated the drop was not due to nitrogen limitation (results not shown).

During typical operation of the reactor, the CO_2 profile was repeatable from cycle to cycle. However, during the startup period, the profile changed from cycle to cycle, as illustrated in Figure 21. These changes are reflected in the differences in the transient evolution rates and in the cycle to cycle variations in total biomass production (Figure 22).

Results from fermentations using both DCD and DCDD as growth substrates are illustrated in Figure 23 and Figure 24.

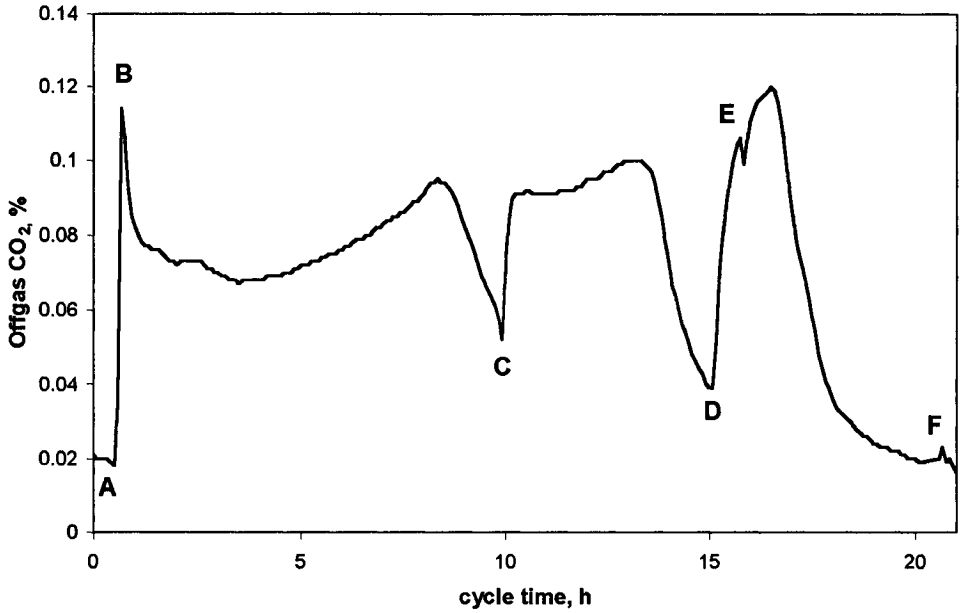

Figure 20 Effect of Substrate and pH limitation on offgas CO_2 concentration
A - end of cycle [DCD]=2.6, pH 5.8, B - cycle start, add 2mL DCD, C - pH 3.9, [DCD]=150 mg/L, add 0.62g NaOH, D - pH 5.9, add 0.12 mL DCD, E - add 0.25 g NaOH, F - cycle end pH=4.6, [DCD] = 12.2 mg/L

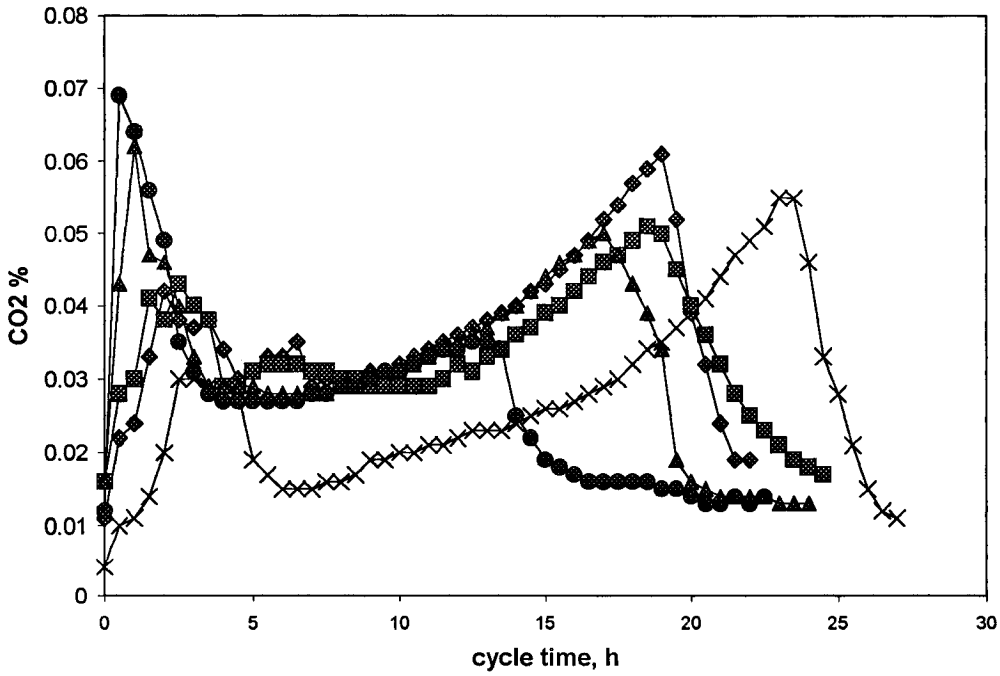

Figure 21 Changes to the CO_2 Evolution Profiles during Early Cycles
X cycle 4, ◆ cycle 5, ■ cycle 6, ▲ cycle 7, ● cycle 8 demonstrating a trend to shorter fermentation times with the development of biomass and with acclimatization

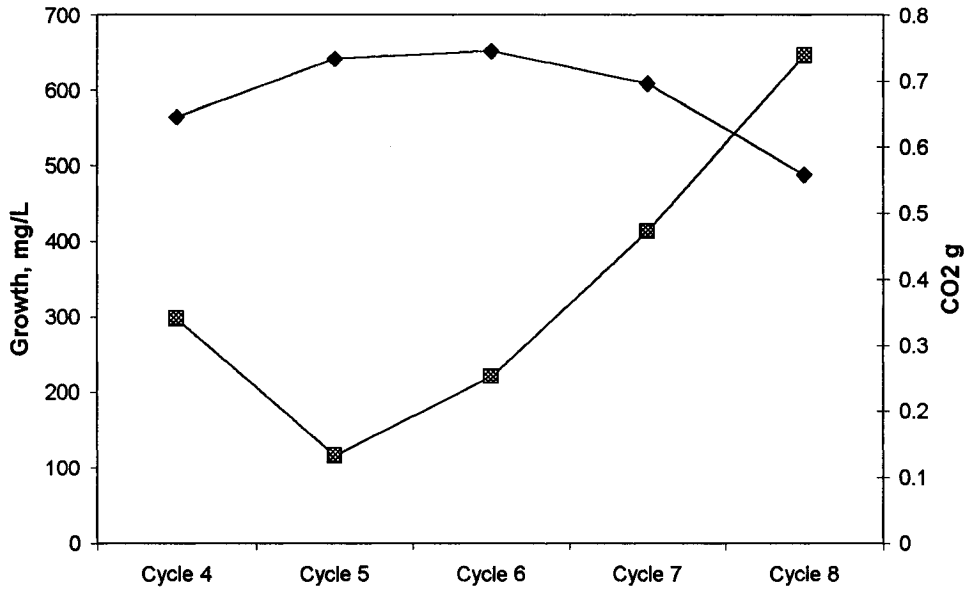

Figure 22 CO_2 and Biomass Produced over a Complete Cycle
■ growth in biomass over the cycle, dry weight ▲ CO_2 production per cycle. These data are from the same experiment illustrated in Figure 22

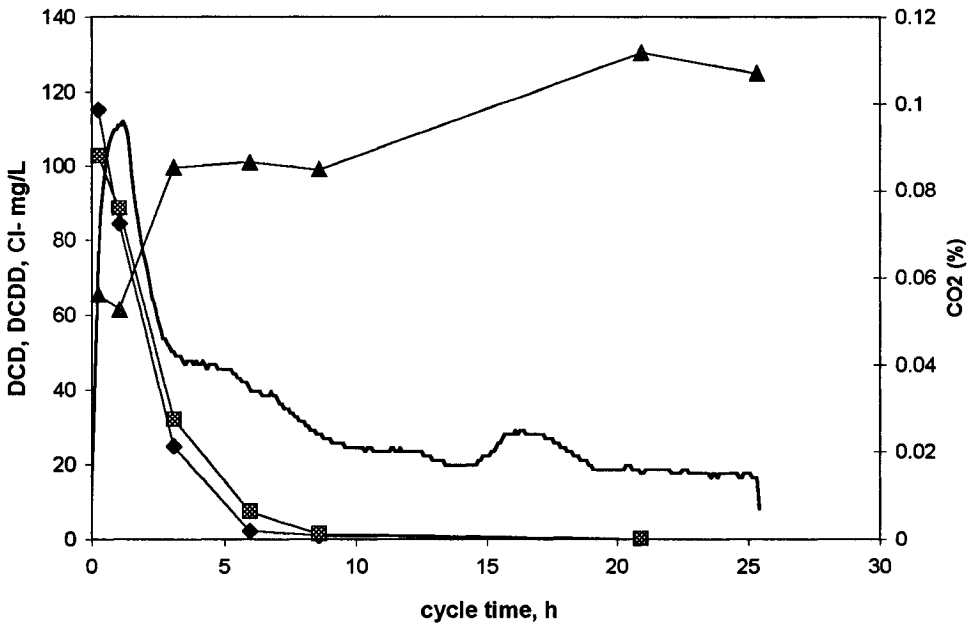

Figure 23 Combined Fermentation of DCD and DCDD
◆-DCD, ■-DCDD, ▲-chloride, - Offgas CO_2, starting concentration DCD=246 mg/L, DCDD = 238 mg/L

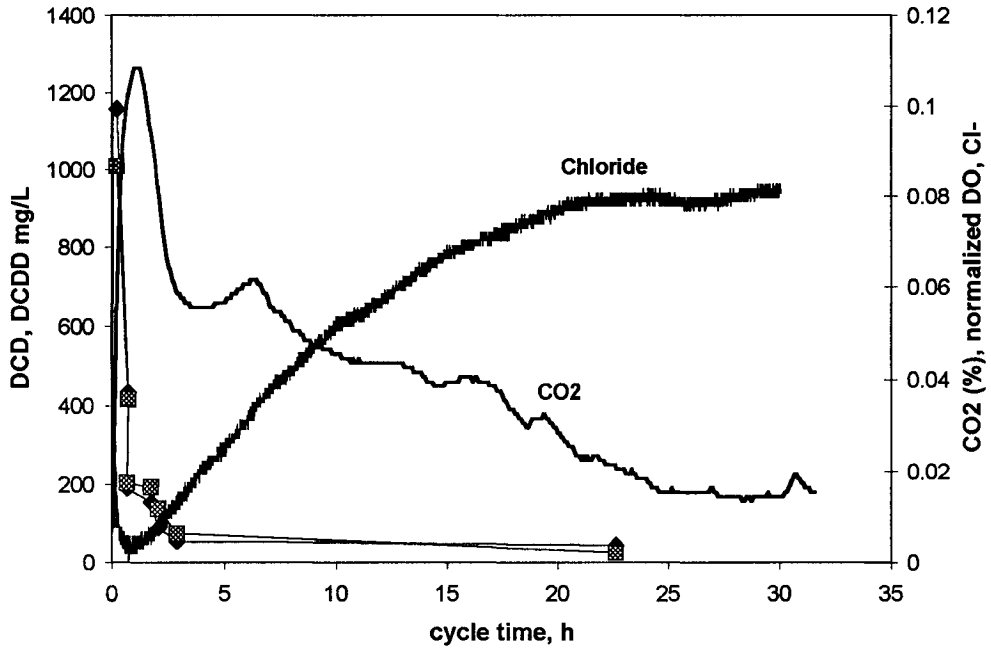

Figure 24 Combined Fermentation of DCD and DCDD (repeat)
◆-DCD, ■-DCDD, starting concentration DCD=948 mg/L, DCDD = 954 mg/L

4.7 FERMENTATIONS OF CERECLOR S52

4.7.1 Shake Flask Fermentations using Cereclor

Shake flask fermentations were conducted with 1.4 g/L Cereclor S52 homogenate in the presence of the cosubstrates DCD and hexadecane. DCD was chosen as a cosubtrate because it is known to induce the oxygenolytic dehalogense of *Pseudomonas.* sp. 273. Hexadecane was chosen because it is non-chlorinated substrate with low volatility which is known to be degradable by a host of bacteria.

Flasks incubated with *Pseudomonas.* sp. 273 and DCD resulted in full degradation of DCD, as indicated by extraction, as well as 12-21% degradation of S52 based on chloride released after 30 d. Flasks incubated with C_{16} yielded only 28 mg/L Cl^- corresponding to about 5% of the added S52. An abiotic flask containing DCD, S52 and C_{16} showed 28 mg/L Cl^- after 30 days but this figure rose to 106 mg/L after 54 days. The abiotic flask showed signs of growth of biomass, therefore contamination was suspected to be the cause of the chloride release later in the experiment (data not shown).

An un-explained mechanism resulted in the loss or sequestration of chloride in flasks incubated for long duration. Between 30 and 54 days of incubation, chloride dropped by almost 50% in the two flasks that initially contained DCD and S52. The chlorine

balance based on measured chloride and extractions of flasks at the end of this fermentation could not be reconciled; only about half of the chlorine added as DCD or S52 could be accounted for. The reason for this discrepancy was never elucidated and the sample treatement procedure (see section 3.7.3) did not affect the chloride results for these samples.

4.7.2 Reactor Fermentations of Cereclor with DCD

The biodegradation tests which were started in shake flasks were also carried out in the reactor. The reactor instrumentation allowed more detailed analysis than the shake flask.

The culture was grown on DCD through multiple cycles to build biomass and to induce the degradative enzymes. In five separate experiments, Cereclor was then added as a homogenate to the induced culture, then a day later subsequent additions of DCD were made spaced 1 day apart with intervening pH adjustment using NaOH. The experiments differed in the amount, and timing of the DCD additions. In the first experiment, Cereclor was added as a homogenate followed by two additions of DCD. In this experiment the reactor containing induced culture was cycled following the depletion of the added DCD, then Cereclor S52 was added and fermented without co-substrate. The reactor was then monitored for 2 days without addition of DCD.

In the second and third experiments, two additions of DCD were made to a Cereclor fermentation, and then the reactor was shut down to recover undegraded Cereclor from the reactor walls in order to close the chlorine balance. In the fourth experiment, a smaller concentration of Cereclor was added to the reactor. In the fifth experiment, twelve consecutive additions of DCD were made, spaced one day apart, with no cycling. In this experiment, ammonium sulphate was added to ensure that sufficient nitrogen was available to degrade all the added carbon source. The pH was adjusted as required using NaOH.

The results of the first experiment are illustrated in Figure 25. The initial biomass was 1.1 g/L, obtained from a previous fermentation on DCD. Biomass rose to 1.4 g/L following the addition of S52 and two spikes of DCD over 96 hours. Biomass then dropped to 1.26 g/L over about 50 hours following cycling and the addition of further S52, without DCD addition. A further drop to 0.96 g/L was observed following another cycle and the addition of S52 alone. Intracycle biomass measurements were not taken as they were influenced by the presence of the homogenized S52 and DCD.

Up to the point labelled "B" (Figure 25), 30 mg/L of chloride, corresponding to 4% of the added hydrocarbon, was released prior to DCD addition. After the first addition of DCD, 269 mg/L of chloride was released, of which 166 mg/L could be attributed to the

DCD and the balance was attributed to Cereclor degradation. After the second addition of DCD at C, 134 mg/L of chloride was released while again the potential release from DCD alone was 166 mg/L, indicating incomplete degradation of the added DCD. Meanwhile, extractions showed a decrease in S52 in the reactor mixed liquor over time after each addition.

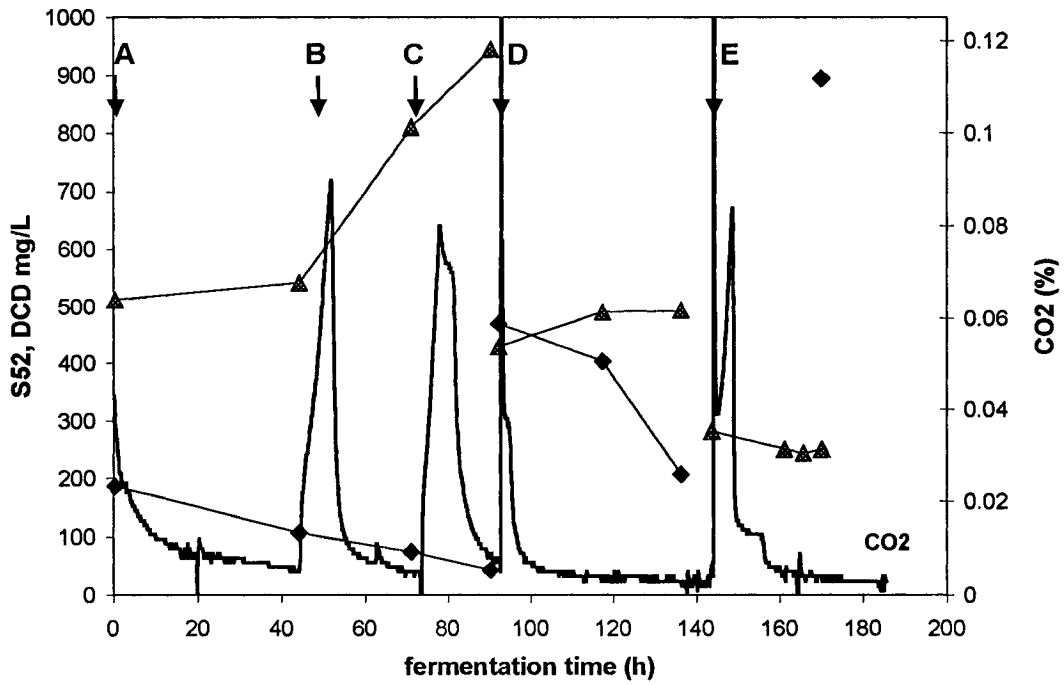

Figure 25 Two sequential DCD additions in a fermentation of Cereclor S52
◆-S52; ▲-chloride; A - added 2.9g homogenized S52 to end of the previous cycle; B,C - added 1g DCD at each point; D - cycled and added 3.8g S52; E-cycled and added 4.4g S52

The chloride concentration in the reactor dropped by half at each cycle point due to the dilution of the reactor contents with new chloride-free media. The overall S52 degradation was 14% from A to D, while no degradation of the added S52 was measured in the subsequent cycles (Figure 25).

This experiment was repeated with minor variations and the results are provided in Figure 26 and Table 9. The procedure for this experiment differed from the previous one in that the S52 homogenate was added to the beginning of a cycle along with the yeast extract, instead of at the end of a previous cycle on DCD. The experiment had a duration of 34 days. The chloride removed through sampling of the mixed liquor was 90 mg/L or about 10% of the total produced through biodegradation. At the end of the run, 0.42 g of S52, 48 mg of DCD and 14 g of biomass solids were recovered from the walls of the

reactor. The chlorine recovered as hydrocarbon from the reactor walls was 40% of amount added as S52 and DCD. 43% of the added S52 was degraded over the first 70 hours through two additions of the DCD cosubstrate.

A drop in the measured chloride occurred after the first DCD spike at the point labelled "D" in Figure 26. Frozen aliquots had been saved and these were assayed using the caustic treatment procedure described in section 3.7.3. An increase in chloride was noted in those samples following treatment. These samples are labelled as treated samples in Figure 26.

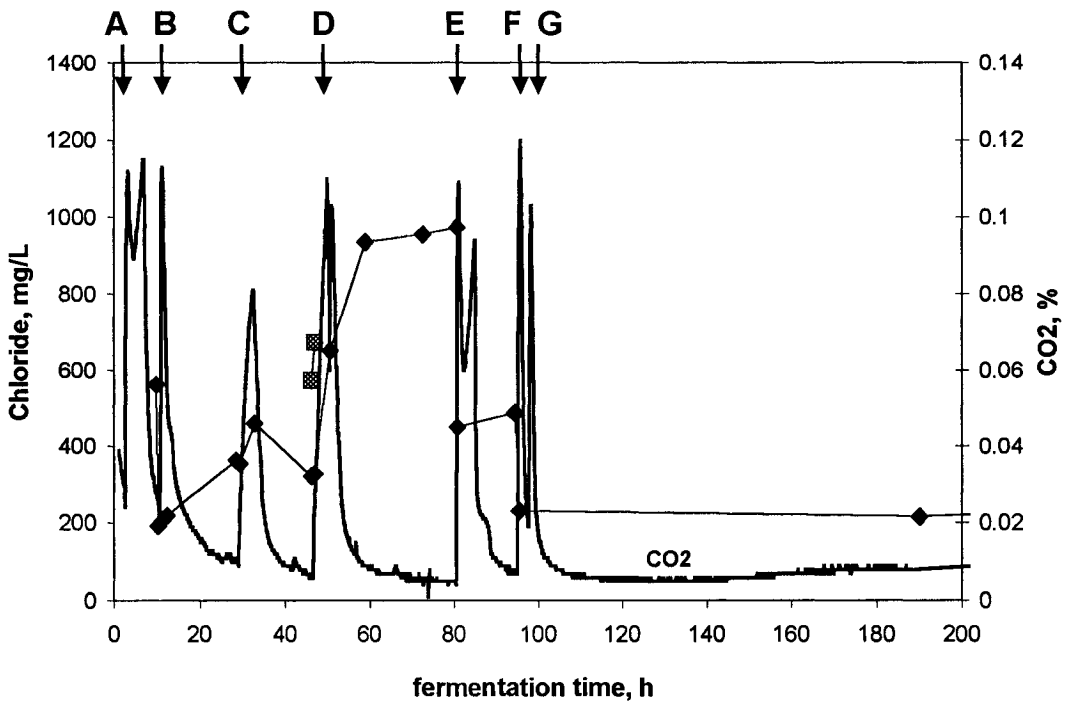

Figure 26 Sequential Fermentations of Cereclor S52 with DCD Additions
◆-chloride; ■- chloride (treated samples); A - cycle on DCD; B- cycle on 3.8 g S52; C,D - add 1g DCD with pH adjustment; E- cycle with 2.6g S52; F- cycle with 2.6g S52; G- cycle without hydrocarbon addition

Table 9 Summary of Biodegradation of Cereclor S52 with DCD Addition
(see figure 25) * based on treated samples; ** losses due to withdrawal of samples

	Mass Cl Added as DCD	Mass Cl Added as S52	Measured Chloride Initial	Measured Chloride Final*	Change	Sampling Losses**	Degradation %S52
	mg	mg	mg	mg	mg Cl-	mg Cl-	
29 h, prior to 1st DCD addition		1960	383	710	327	15	17%
46 h, prior to 2nd DCD addition	329		710	1268	558	41	14%
75 h, after 2nd DCD addition	329		1268	1752	484	70	12%
Total A-D (see figure 26)	658	1960			1369	127	43%

A third repeat experiment achieved only 32% degradation of added S52. However this experiment was complicated by a mixer failure and the reactor contents went unmixed for a period of 10 hours (data not shown).

The fourth experiment consisting of sequential additions of DCD to a fermentation of Cereclor. The initial S52 concentration was 1.6 g/L. The results of this experiment are summarized in Table 10 and Figure 28. S52 was degraded by 53%. This experiment served as a precursor to fed batch experiments which are discussed below (see section 4.7.3)

Table 10 Summary of Biodegradation of Cereclor S52 with DCD

	Mass Cl Added as DCD	Mass Cl Added as S52	Measured Chloride Initial	Measured Chloride Final	Change	Sampling Losses	Degradation
	mg	mg	mg	mg	mg Cl-	mg Cl-	%S52
Before DCD Spike		1642	735	955	221	32	15%
DCD Addition 1	329		955	1366	411	43	8%
DCD Addition 2	329		1366	2157	791	23	30%
Total	658	1642			1423	98	53%

A fifth experiment extrapolated to twelve stages the sequential addition of DCD to a Cereclor fermentation. The results of this trial are summarized in Table 11, Table 12, and Figure 27. Cumulative chloride results throughout the experiment were considered, taking into account sampling losses. Chloride release showed variations from cycle to cycle, including some cycles where full degradation of DCD was not achieved. The reason for this was not unexplained. Measured chloride levels dropped in stage 2 but increased when the sample was treated with NaOH. In stage 2 (that is, following the 2nd addition of DCD) an unexplained peak in CO_2 production was observed at the 30 hour point.

At the end of the fifth experiment, the chloride concentration was 133 mg/L above the chlorine added as DCD. This represents 63% degradation of the added S52. The figure for S52 degradation is obscured by the fact that the vast majority of the chlorine added was as DCD (95%), making the proportion which was added as S52 close to the sensitivity of the measurement. Upon shutdown after this experiment, a solvent rinse of the reactor walls recovered 25% of the S52 added while 2.8% remained in the reactor liquor. The overall chlorine balance considering added hydrocarbons, recovered

hydrocarbons, and recovered chloride, for this run closed to within 0.1%. Assuming the balance for chlorine attributed to DCD closed to 100%, then a balance on S52 of 91% was realized (Table 12). Abiotic release was assumed negligible since no chloride release was observed in an abiotic control experiment (section 4.5). The S52 concentration dispersed in the liquid phase varied with time as illustrated in Figure 27.

Table 11 Summary of Biodegration of Cereclor S52 with 12 Sequential Additions of DCD Cosubstrate.
The estimated net biodegradation of Cereclor S52 was 63%

	Mass Cl Added as DCD mg	Mass Cl Added as S52 mg	Measured Chloride Initial mg	Final mg	Increase mg Cl-	Sampling Losses mg Cl-	Net Increase less DCD
Added S52		211	497	546	49	17	66
DCD Addition 1	329		546	673	127	18	-184
DCD Addition 2	329		673	1015	343	35	49
DCD Addition 3	329		1015	1434	418	37	126
DCD Addition 4	329		1434	1774	340	41	52
DCD Addition 5	329		1774	2018	244	55	-30
DCD Addition 6	329		2018	2409	392	37	100
DCD Addition 7	329		2409	2772	363	44	77
DCD Addition 8	329		2772	3066	295	136	101
DCD Addition 9	329		3066	3040	-26	52	-303
DCD Addition 1	329		3040	3491	451	49	170
DCD Addition 1	329		3491	3677	186	52	-91
DCD Addition 1	329		3677	3831	154	176	1
Total	3948	211			3334	747	133

Table 12 Chlorine Balance (see fig 27)

Chlorine Balance	g Cl
Added	
chloride at start	0.497
as S52	0.211
as DCD	3.949
	4.657
Removed	
Sampling	0.747
Remaining	
chloride at end	3.831
as S52	0.059
as DCD	0.013
	4.650
	99.9%

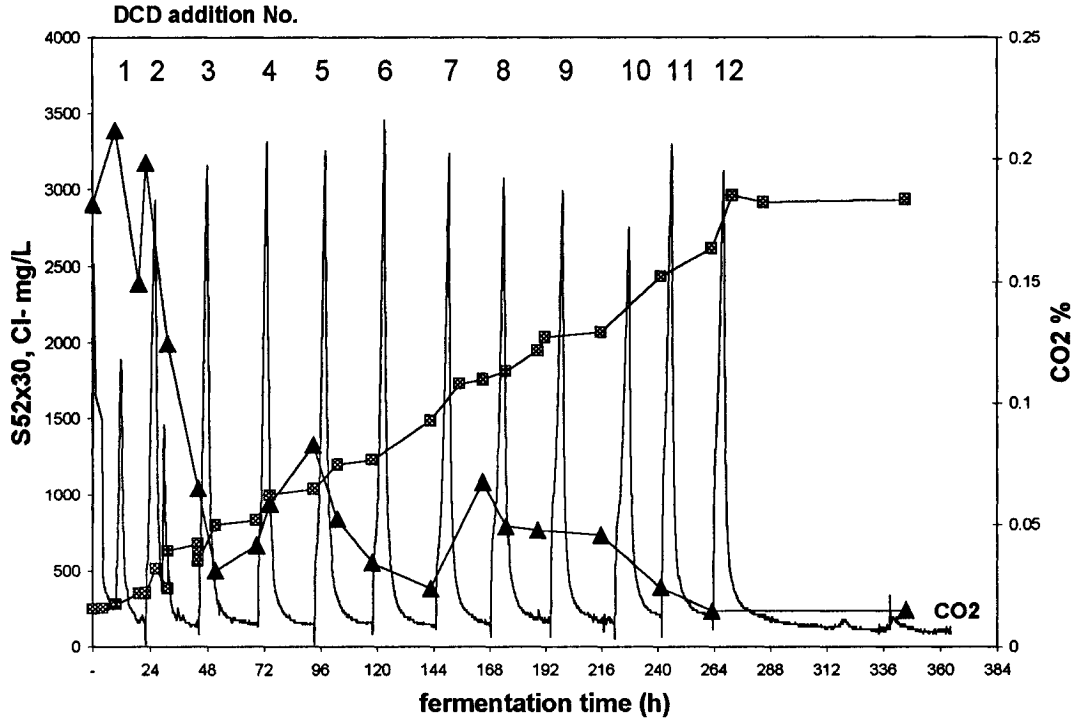

Figure 27 Biodegration of Cereclor S52 with Twelve Sequential Additions of DCD ■ - chloride; ▲- Cereclor S52 (suspended in mixed liquor) The S52 concentration is adjusted to suit the Y scale of the plot.

4.7.3 Fed Batch Experiments using Yeast Extract

A fed batch experiment was conducted to test whether Cereclor degradation could be sustained in the absence of DCD but with sustained growth of the culture. The fed batch trail was started with both Cereclor and DCD present to repeat the initial conditions of previous experiments. The reactor was topped up with mineral media to compensate for evaporation and sampling losses at the 127 h point prior to the start of the fed batch experiment (Figure 28). With residual Cereclor present, a concentrated yeast extract solution was added in a fed batch manner. The feed rate of yeast extract solution was controlled by the offgas CO_2 level to achieve a mean CO_2 level of 0.02%. This level of metabolic activity was chosen because chloride release had been observed at this level in prior experiments. The control system maintained the level between 0.01 and 0.03% CO_2 for four days. No pH adjustment was necessary. Then further Cereclor was added as a homogenate, and a further fed batch trial was conducted using yeast extract. During the initial fed batch portion of the experiment, after the 125 h point, when the culture was in the induced state, 10% of the added S52 was released as chloride. Later, no release of chloride was observed.

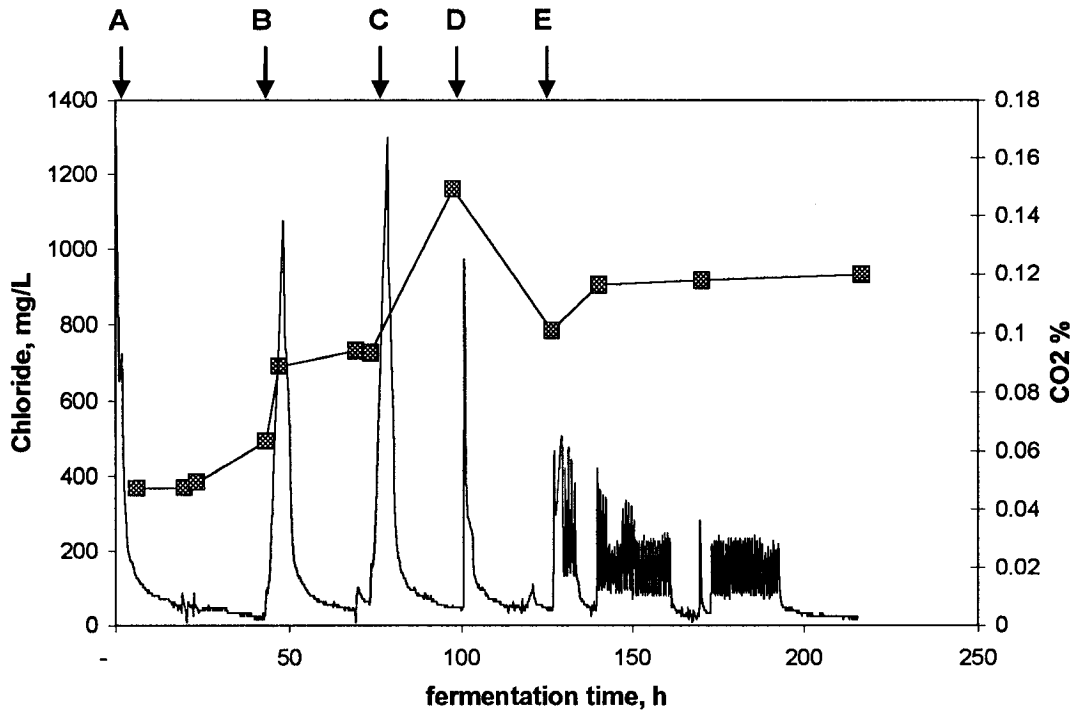

Figure 28 Fermentation of Cereclor S52 with DCD addition and Fed Batch addition of Yeast Extract
■- chloride; A- cycle with 3.2 g Cereclor S52; B,C- add 1g DCD; D-replace lost volume; E- start of fed batch with yeast extract.

Following the first fed batch experiment, the reactor was flushed with 1.5 L of sterile media but not shut down and cleaned out. A second fed batch experiment using yeast extract in the presence of S52 resulted in negligible release of chloride, only 1% of added S52 was degraded. The results of this experiment are provided in Table 13 and Figure 29 (<75h). No biodegradation was observed.

4.7.4 Fermentation of S52 with Hexadecane

After the completion of the second fed batch trial, at the 75 hr point in Figure 29, a spike addition of hexadecane was made. Hexadecane was used because it is generally a readily degradable substrate and it contains no chlorine. Following the addition, 2% of added S52 was released as chloride after 5 days. The results are corrected for the losses of reactor volume due to evaporation over long periods. No degradation of the hexadecane was observed.

Subsequent additions of DCD were made to demonstrate that the culture remained viable through the period of yeast extract and hexadecane addition, but the first DCD addition

resulted in the release of only the chlorine added as DCD. The results are summarized in Table 13 and Figure 29.

Table 13 Summary of S52 Biodegradation in 2nd Fed Batch and Hexadecane Experiment

	Mass Cl Added as DCD mg	Mass Cl Added as S52 mg	Measured Chloride Initial mg	Final mg	Increase mg Cl-	Sampling Losses mg Cl-	Degradation %S52
Added S52		1160					
YE Fed Batch			177	177	0	12	1%
C16 Trial			177	196	19	4	2%
DCD Addition 1	329		196	458	262	39	-2%
DCD Addition 2	329		458	1471	1013	25	61%
DCD Addition 3	329		1471	1838	366	94	11%
Total	987	1160			1660	174	73%

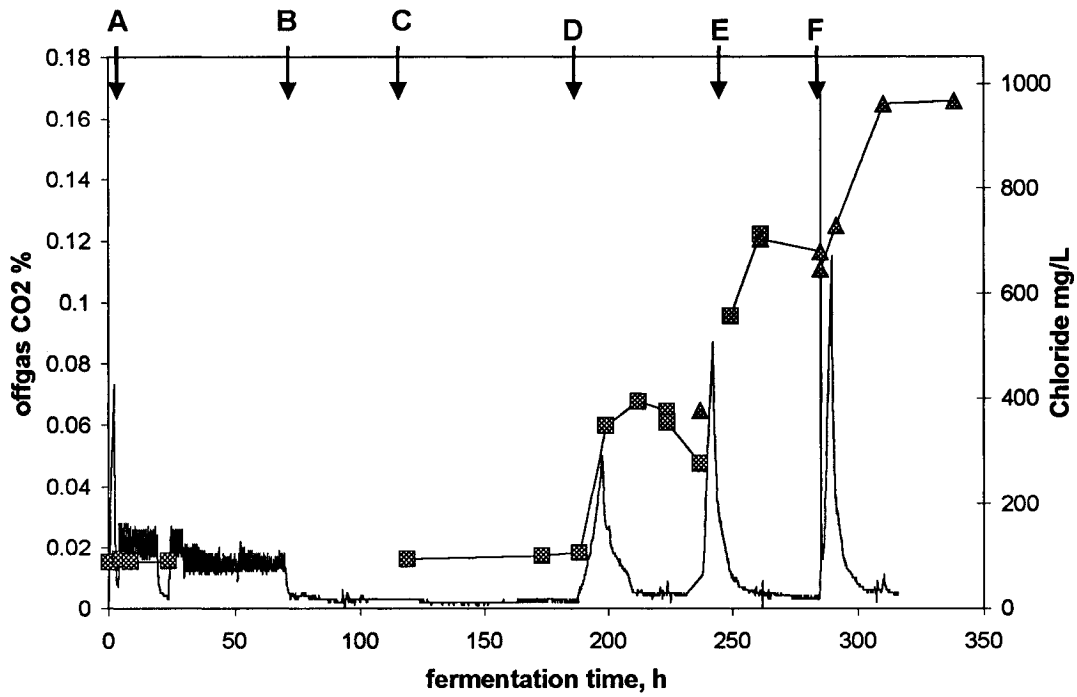

Figure 29 Fermentation of S52 with Fed Batch Yeast Extract, Hexadecane, DCD ■- chloride; ▲- chloride (treated samples); A-B- fed batch with yeast extract; C-addition of hexadecane; D,E,F additions of DCD to demonstrate the viability of the culture

4.8 SELECTIVITY OF CERECLOR DEGRADATION

A typical gas chromatogram for a solution of S52 in hexane is provided in Figure 30. This figure displays only the portion of the trace in the region that S52 is eluted. The

instantaneous response is adjusted by the magnitude of the internal standard peak and corrected using a solvent blank.

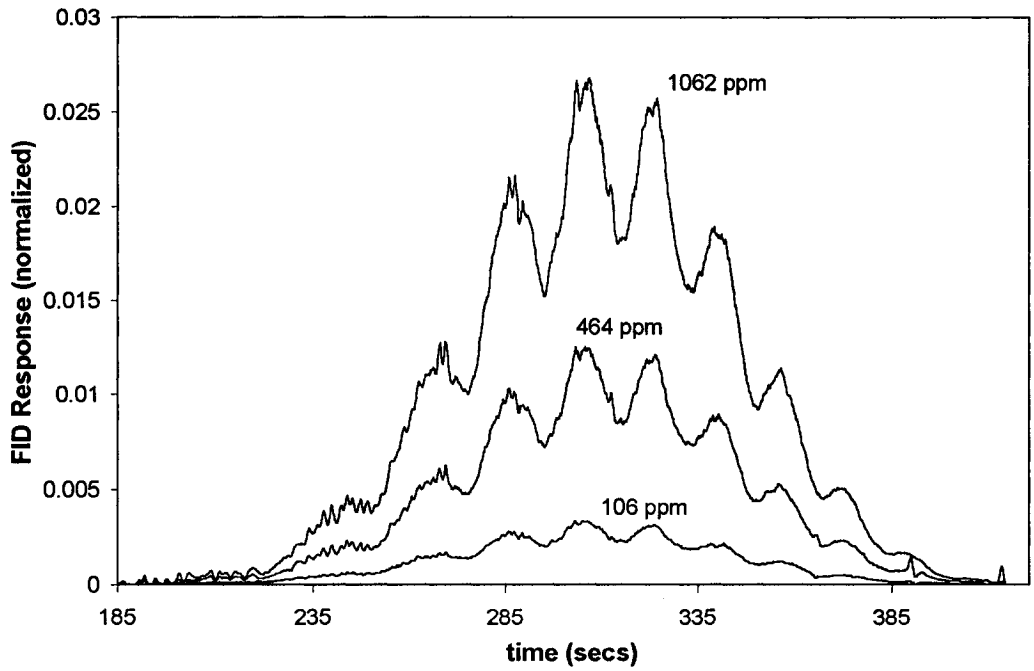

Figure 30 Gas chromatographs of S52 standards, normalized with respect to the area of the internal standard peak (area ratio)

Biodegradation of S52 was monitored using chloride release and the results were validated using measurements of Cereclor concentration. However, changes in the gas chromatograms of extractions through the course of a fermentation were noted. (Figure 31). Three techniques were employed to interpret the results of Cereclor gas chromatography:

1. The instantaneous relative response was plotted, which is the response of the FID detector divided by the peak area of the internal standard for each time increment The relative response for a fermentation of Cereclor in the presence of DCD is provided in Figure 31. The chromatogram labelled "feed" is the result of an extraction of the homogenate that was fed to the reactor. The rinse water from the walls of the reactor upon shutdown was also analysed and is shown.

2. The areas under the curves of the instantaneous response factor represent the total abundance. The calibration curve is provided in section 3.7.6.

3. The "standard response" was defined as the ratio of the relative response to the relative response of the feed sample. This ratio is intended to measure differences in the relative response through the course of a fermentation using the feed chromatogram as the basis. This measures changes to the profile of the chromatogram from sample to sample. The standard responses of these curves are provided in Figure 32.

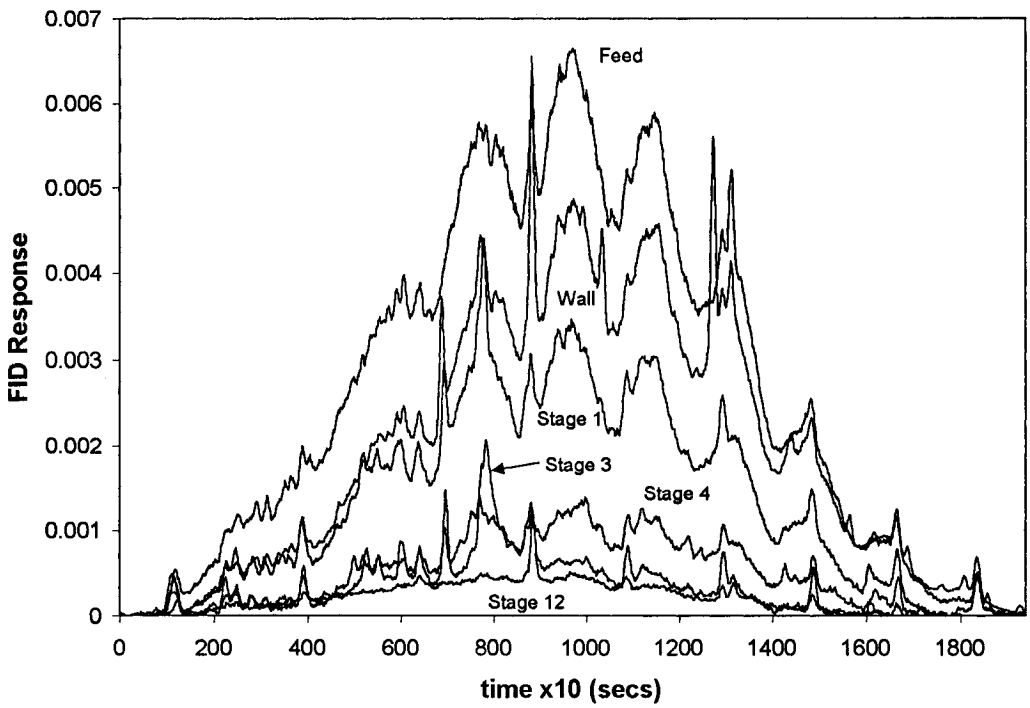

Figure 31 Cereclor Chromatographs through 12 Sequential Additions of DCD
DCD was added after each stage. "Wall" sample was recovered from reactor walls upon shutdown. The time datum was based on the appearance of the internal standard peak.

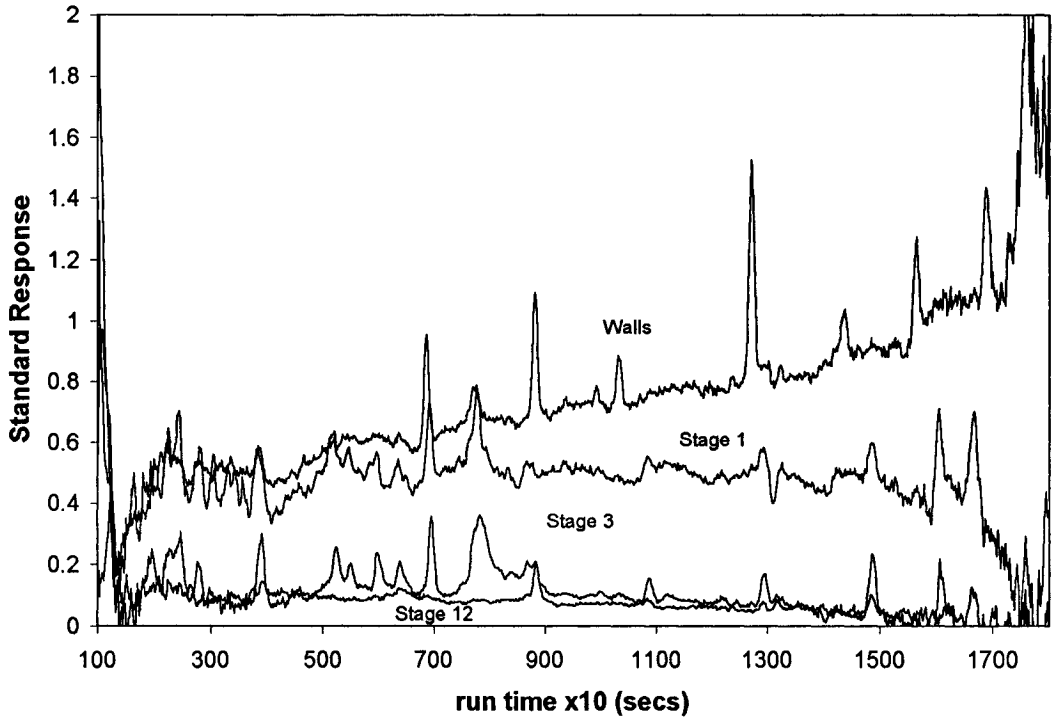

Figure 32 Standard Response in Biodegradation of Cereclor S52 with DCD (see text) "walls" sample was recovered from reactor walls upon shutdown

5. DISCUSSION

5.1 CULTURE SCREENING

The cultures *Rhodococcus Erythropolis* Y2, *Rhodococcus Erythropolis* GJ70, and *Rhodococcus Erythropolis* NCIMB 13064 grew very little or not at all on DCD. The small amount of growth which was noted in preliminary shake flask experiments may be attributed either to growth on the yeast extract contained in the mineral media, or to a very slow rate of growth on DCD. This question was not investigated, and *Pseudomonas* sp.273 was selected for further work because it clearly grew better on the medium chain chlorinated alkane.

Pseudomonas sp.273 is reported to posses a mono-oxygenase which as active for chlorinated substrates with carbon chain lengths longer than nine atoms (Wischnak, Loffler et al. 1998). *Pseudomonas* sp.273 was isolated on DCD, while the other organisms were isolated on short chain chloroalkanes. The choice of enrichment substrate is known to influence the type of dehalogenation system selected (Armfield, Sallis et al. 1995). The use of short chain chloroalkanes selects for the hydrolase system,

and the use of medium chain chloroalkanes selects for the oxygenase system. *Pseudomonas* sp.273, utilizing the oxygenase system, is considered the best candidate for the degradation of commercial mixtures of medium chain chloralkanes.

Rhodococcus Erythropolis Y2, *Rhodococcus Erythropolis* GJ70, and *Rhodococcus Erythropolis* NCIMB 13064 grew well on 1,6 dichlorohexane at 0.5 mL/L, supporting the hypothesis that they posses the hydrolas enzyme system. These cultures showed inhibition at a concentration of 1 mL/L, while *Pseudomonas* sp.273 did not exhibit any negative impacts when it was grown in the presence of up to 2 mL/L. This supports the hypothesis of Maier (Maier, Pepper et al. 2000), that the short chain chloroalkanes are more toxic to bacteria than medium and long chain chloroalkanes because the short chain substrates infiltrate and damage the cell wall.

5.2 SUBSTRATES

5.3 FERMENTATION OF α,ω-DICHLOROALKANES

5.3.1 Process Control

The remediation process was carried out in an unsteady state bioreactor. Therefore, it was desireable to have an on-line estimate of when the dechlorination process was complete. This estimation was used to determine the cycling time of the reactor.

The process control strategy was developed through a series of fermentations on 1,10-dichlrorodecane (DCD) and 1,12 dichlorododecane (DCDD) as the carbon sources. The traces using DCD as substrate show a plateau in the probe response curve that occurred when the substrate was exhausted (Figure 13). This was expected, as the chloride electrode is selective for the product of DCD degradation. Based on these curves, the chloride probe showed promise as a control parameter because the reactor could be cycled shortly after the plateau was reached. A control subroutine was written which calculated a running average slope for a given number of data points, and triggered the cycle sequence when the average slope dropped below a threshold. Figure 33 shows data from a typical cycle. In order to implement this control strategy, a timer was required which prevented the triggering of a cycle for the first hours of a cycle since the variability of the slope was large during this period. A 15 minute moving average was used to calculate the slope. A shorter period resulted in greater variability in the slope and greater chance of the triggering of a cycle prematurely, that is, prior to the plateau (data not shown)

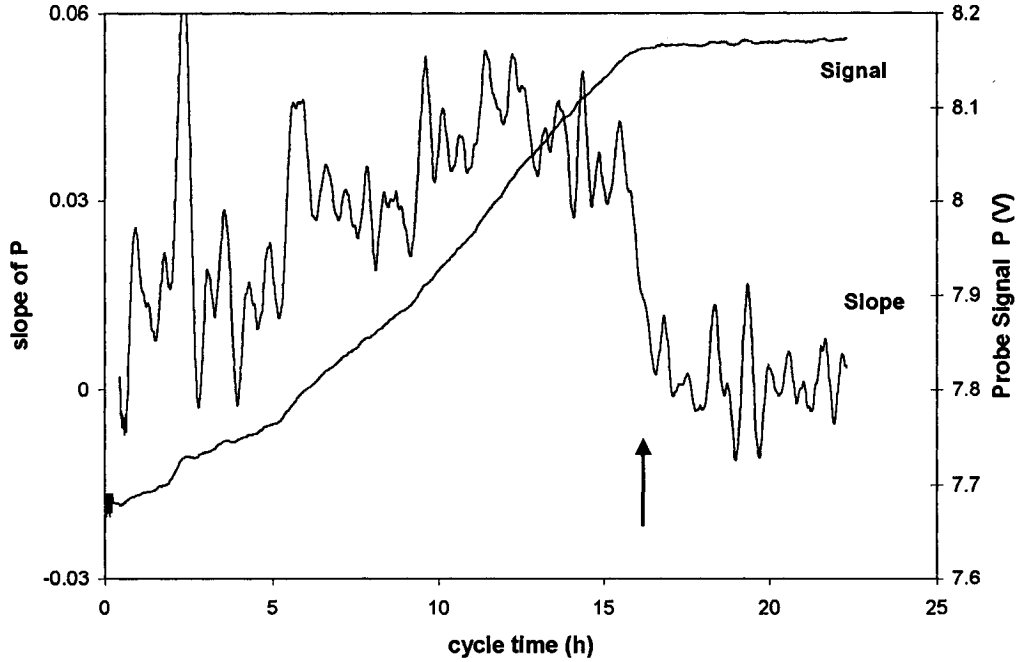

Figure 33 Typical Analysis of Chloride Probe Response for a Fermentation of DCD. The slope was based on a running average of 25 consecutive probe signal data points (15 min). The cycle time is detected at the arrow.

Unfortunately, the probe response profiles with DCDD as the substrate did not have a plateau (Figure 14) making the algorithm inappropriate. The reason for this difference was not elucidated. Furthermore, the slope of these profiles exhibited greater variability throughout the cycle, as opposed to just at the start of the cycle as for DCD (data not shown). This could potentially trigger a cycle before the exhaustion of substrate, which would lead to incomplete degradation. The use of filtering was investigated in order to address the issue of signal variability, and the wavelet transform method was identified as the best candidate. However, due to the complexity of implementing this method it was not attempted.

The use of the chloride probe as a control parameter was further complicated by the response of the probe to pH. As chloride is released due to degradation, the pH drops, subject to the buffering capacity of the media. Theoretically, a complete fermentation of 10 mM DCD results in a pH drop from 7 to 4, and releases up to 646 mg/L of chloride. However, as the probe was sensitive to pH, a response of only 10 mV (300 to 290 mV) is expected. This scenario was validated experimentally, as illustrated in Figure 34.

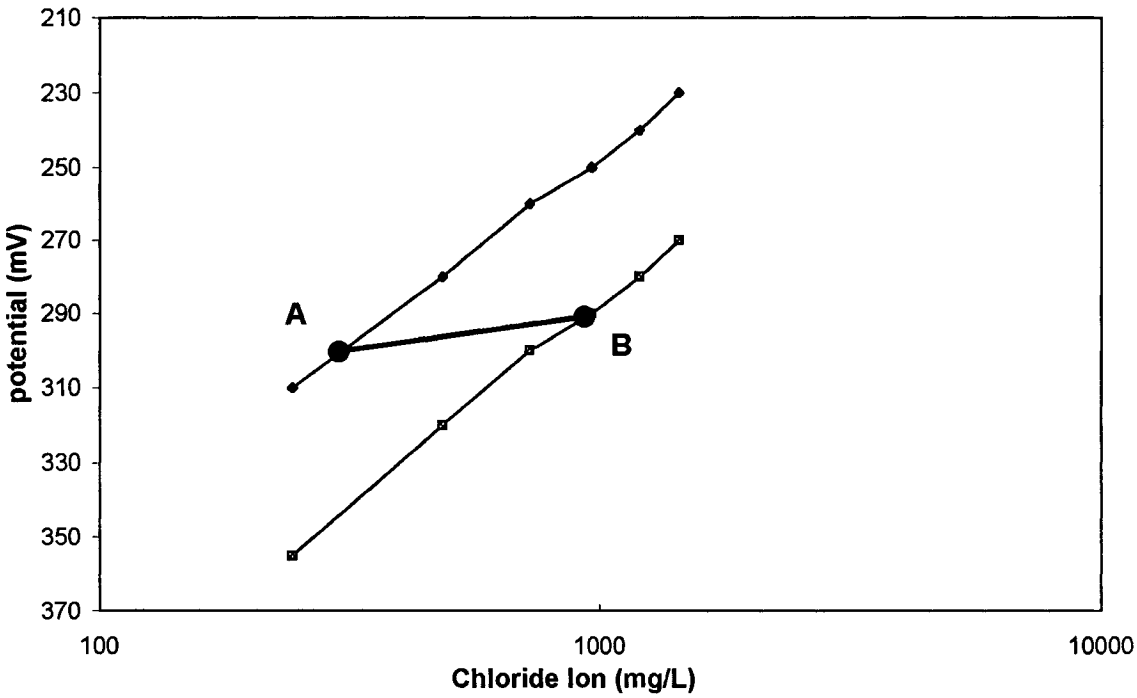

Figure 34 Loss of Probe Sensitivity due to pH Change during Fermentation
For a 10 mM DCD fermentation from A: pH 7, 300 mg/L Cl^-, 300 mV to B: pH4, 946 mg/L, 290 mV resulting in a probe response of only -8.7 mV/decade

Other effects were also found to interfere with the electrode response. For instance, the output from the chloride probe indicated rising chloride levels throughout the initial stages of the fermentation, that were contradicted by ion chromatography. The discrepancy was not resolved. Based on this discussion, the chloride probe was abandoned as a possible control parameter.

Dissolved oxygen was then considered as a possible control parameter. The characteristic profiles (Figure 15) exhibit sharp rises in the DO level following exhaustion of each of the chlorinated substrates. This response could easily be incorporated into a control scheme based on a threshold for the rate of change of the moving average of the dissolved oxygen probe response, as is discussed above. However, the sensitivity of this control strategy, defined in this context as the difference between the maximum and minimum values of the parameter through the course of the fermentation, is dependant on the balance between the oxygen supply and the demand. If the system is not mass transfer limited, no change in the dissolved oxygen levels through the course of the fermentation will occur. This was the case for fermentations at low cell densities. The drawback of process control using DO is therefore that the maximum

sensitivity of the parameter to detect the cycle end is achieved when the system is closest to oxygen starvation. Nonetheless, dissolved oxygen was monitored throughout each fermentation and was considered an acceptable control parameter under many circumstances.

The sensitivity of offgas CO_2 as a parameter for process control also varies with oxygen transfer rate (OTR) but for other reasons. With increasing sparging rate, the concentration of CO_2 in the offgas decreases for a constant metabolic rate due to dilution with the sparging gas. However, since the OTR is more strongly influenced by mixing rate than by sparging rate, and the mixing rate has no effect on the sensitivity of the CO_2 measurement, this parameter may be considered a more robust control parameter for the system than DO. The end of cycle was inferred if the CO_2 evolution rate fell below a user-defined threshold value.

CO_2 was used as the control parameter for all subsequent work.

5.3.2 Intracycle Profiles for Growth on Dichloroalkanes

The transient chloride output profiles discussed above (Figure 13, Figure 14), reveal that there exist significant differences in the metabolism of the two chlorinated alkanes α,ω-dichlorodecane and α,ω-dichlorododecane. The rate of CO_2 production for fermentations with DCD increased with time, in a manner that suggested that the degradation reaction was roughly first order in biomass. Measurements of biomass with respect to time indicated that the biomass increased in a similarly shaped curve. These observations are consistent with the condition of substrate mass transfer not being limiting.

In contrast, fermentations with DCDD showed a declining reaction rate with time. This is consistent with a model where substrate mass transfer is limiting, because the reaction rate decreased as the substrate concentration decreased. This hypothesis was not tested.

While estimates in the literature of the solubilities of these compounds vary, DCDD is reported to have less than 10% the solubility of DCD, so the hypothesis that the latter compound appears to have a higher rate of mass transfer to the cell could be related to the importance of substrate utilization from the dissolved fraction. However, the absolute solubilities of both of the compounds are low, and therefore direct metabolism from the oil phase is assumed to be important (Jimenez and Bartha 1996). The BATH assay results for DCD and DCDD are similar, indicating these compounds adhere to the cell wall of *Pseudomonas* sp. 273 to a similar degree (Table 8). Cell-free controls that were conducted during BATH testing revealed, however, that DCD was more prone to forming fine droplets after vigorous shaking and that these droplets persisted in suspension (data

not shown). The degree of emulsification under the conditions tested was not, however, sufficient to invalidate the results of the BATH assay. Similarly, when BATH testing was conducted with a sample of biomass that did not adhere to hydrocarbon, the absorbance of the supernatant increased in the case of DCD but not in the case of DCDD, and this increase was attributed to the oil droplets in suspension, based on visual inspections with a magnifying glass. These results indicate that the differences in the CO_2 profiles may be attributed to differences in the degree to which the two substrates dispersed as fine droplets in the media. The differences might be evident in the surface properties of the two compounds, however comparative data for surface tension and viscosity were not gathered. This observation warrants further investigation.

When yeast extract and DCD were fermented separately and the CO_2 profiles were superimposed, the composite curve resembles the CO_2 profile for when the two substrates were fermented together (Figure 17). This is an indication of diauxic growth, that is, the organism exhausted the readily degradable substrate before attacking the chlorinated alkane. Additional evidence of diauxy is provided by the chloride measurements obtained from ion chromatography, as shown in Figure 18, and Figure 19. The measured chloride levels were unchanged through the spike in CO_2 production from yeast extract, and then rose while DCD was being degraded.

Un-degraded hydrophobic substrate was recovered from the reactor walls following completion of the experiment by means of a solvent rinse. Using this technique significant quantities of hydrophobic substrate were recovered. The quantity recovered from the reactor walls was greatest following upset conditions, such as cases when runs were abandoned due to equipment failure. Abiotic tests demonstrated that the DCD partitioned to the reactor walls upon addition (Figure 16). However in some fermentations of DCD almost no substrate was recovered upon shut down, as was the case for run 9 where only 0.3% of added DCD remained on the reactor walls (Table 12). These results demonstrated that the substrate could be utilized by the organism from the walls of the reactor, or the turbulence of the reactor re-entrained DCD from the walls.

The final drop in CO_2 production coincided with the exhaustion of the chlorinated substrate in suspension. The peak in chloride levels, measured by ion chromatography, did not appear until well after the drop in metabolic activity. This phenomenon may be explained either by continued metabolism (although at a lower rate) of substrate adsorbed to the walls of the reactor, or by a delay in the release of chloride from the biomass after degradation was complete, or both. These hypotheses were not investigated further.

The shape of the CO_2 profile was consistent for most cycles. However the magnitude of the peaks and the duration of the runs varied. In general, the initial cycles following

startup showed decreasing cycle length. The quantity of biomass remaining in the reactor at the end of the cycle grew concomitantly. This, and acclimatization, were assumed to be responsible for the shortening cycle length. Figure 21 and Figure 22 illustrate the change in the cycle length for selected cycles during startup. Figure 22 demonstrates the inverse relationship between CO_2 production and growth and demonstrates the observed acclimatization.

Attempts were made to close the chlorine mass balances for a series of fermentations on DCD. The results were within the accuracy of the chloride measurement when full degradation occured, that is, stoichiometric quantities of chloride ion were observed to be released to the media. When incomplete degradation was observed, the mass balance was frequently not closed, except when the reactor was shut down and the interior walls analysed for hydrocarbon.

Figure 23 and Figure 24 illustrate the results of fermentations with DCD and DCDD combined. The substrates were mixed in a 1:1 volume ratio prior to addition. Chloride release for both compounds followed the same pattern observed in fermentations of DCD alone, namely, chloride release started upon exhaustion of the yeast extract.

The data shown for substrate represent the suspended concentrations, not the quantity adsorbed to the reactor walls. Therefore, the decrease early in the fermentation, prior to the start of the release of chloride, is a measure of adsorption not degradation. Abiotic controls demonstrated that a significant quantity of DCD was not adsorbed. Thus, after the start of chloride release the observed substrate decreases are assumed to be at least partly related to biodegradation. Therefore, the DCD/DCDD system is not diauxic as was the DCD/yeast extract system. DCD and DCDD are utilized simultaneously, though GC data illustrated in both Figure 23 and Figure 24 show that DCD dropped more quickly than DCDD. This observation was not surprising as these compounds are thought to be degraded by the same enzyme system (Wischnak, Loffler et al. 1998). Figure 24 illustrates a plateau in the suspended substrate concentrations that may be the result of an adsorption isotherm prior to the start of degradation. However, this observation was not noted in subsequent experiments.

5.4 DEGRADATION OF CERECLOR S52

A number of factors are recognised determinants in the biodegradation of refractory, immiscible carbon sources in stirred tank reactors. The work with S52 addressed the issues of genetic potential, substrate characteristics, co-substrates, and bioavailability. The results are discussed below.

5.4.1 Catabolic Enzymes

5.4.1.1 Types of dehalogenases

It has been reported that *Pseudomonas* sp.273 possesses a monooxygenase (Wischnak, Loffler et al. 1998), which in aerobic metabolism is responsible for the degradation of a wide range of chloralkanes, including those with ten or more carbons. In contrast, *Rhodococcus Erythropolis* GJ70 and *Rhodococcus Erythropolis* NCIMB 13064 are known to posses hydrolytic dehalogenases. This type of enzyme is recognized to be active for alkanes, aldehydes and carboxylic acids with chain lengths less than C_9. Indeed, these two organisms grew well with 1,6 dichlorohexane (data not shown). No attempt was made to grow *Pseudomonas* sp.273 on 1,6 dichlorohexane in the current study. *Rhodococcus Erythropolis* Y2 has been shown to posses both a hydrolytic dehalogenase and another enzyme, possibly a monooxygenase (Armfield, Sallis et al. 1995) but this culture did not grow as well on DCD as *Pseudomonas* sp.273 during screening tests. Since the goal of the work was to degrade chlorinated paraffins, that consist of mixtures of alkanes C_{10} and longer, *Pseudomonas* sp.273 was considered the best candidate.

When long chain polychlorinated substrates are broken down biochemically, shorter chain chlorinated compounds such as chlorinated alcohols or carboxylic acids are likely to be the metabolites. It follows that if an organism is capable of mineralizing a chlorinated paraffin, or at least breaking it down to an appreciable degree, then the enzymes responsible for further degradation of the resulting shorter chain compounds should be present. However, researchers (Allpress and Gowland 1999), (Hardman 1991), (Armfield, Sallis et al. 1995) have observed that some organisms which are capable of degrading long chain compounds can not degrade the short chain analogues. They speculated that either the enzymes involved are not active for the shorter chain substrates, or the observed recalcitrance is due to differences in membrane transport. Noting that their experiment considered whole cells and not cell free extracts, a number of other explanations are possible, such as toxicity effects of the shorter chain alkanes. Also, others have observed the activity of monoxygenases towards short chain chloroalkanes (Hage and Hartmans 1999) demonstrating that at least some monooxygenase dehalogenases are active for certain short chain chloralkanes.

Alternatively, one could speculate that a second unrecognized dehalogenating enzyme is expressed in *Pseudomonas* sp.273 such as the case of *Rhodococcus Erythropolis* Y2. That is, the oxygenase might break the long chain chloroparaffins down to shorter chain length chloro-carboxylic acids which are accessible to another dehalogenase type. However for *Pseudomonas* sp.273 this has not been proven. Previous work on

chlorinated paraffins found a higher rate of degradation with a defined mixture of organisms possessing different enzymatic capabilities than with axenic cultures. Axenic cultures of these organisms demonstrated peak activity on different sybstrates (Omori 1987). Others state that dechlorination may be carried out by the enzymes responsible for β-oxidation (Yokota, 1986). In this case, the dehalogenase would be necessary only to convert the chlorinated paraffin to a chloroalcohol, whereupon enzymes responsible for the mineralization process, including β oxidation, could complete the dehalogenation.

Another hypothesis is that enzymatic removal of several chlorine atoms from the carbon backbone is required prior to chain shortening through β oxidation. The consensus is that dehalogenation, not β oxidation, is rate limiting (Hardman 1991). This hypothesis is based on work with short chain alkanes, since the body of literature on the biodegradation of chlorinated paraffins is limited. So if multiple dehalogenations were favoured to occur simultaneously (which seems unlikely), or if the presence of adjacent, or even distal, chlorine substitutions were shown to inhibit β oxidation, then this theory could have relevance because chain shortening could not occur prior to dehalogenation.

5.4.1.2 Induction of dehalogenases

The available literature indicates that induction is important in the action of dehalogenases in general, and in the degradation of α,ω-dichloroalkanes by *Pseudomonas* sp.273 in particular. This suggests that these enzymes are not constitutive. Interestingly, other researchers have identified non-chlorinated hydrocarbons such as decane and hexadecane as inducing compounds. In the current work, however, hexadecane did not induce the degradation of S52 and was undegraded itself in the reactor after 3 days (Figure 29).

Cereclor S52 itself did not induce the production of the degradative enzymes since biodegradation occurred only in the presence of DCD or biomass that was already induced. The reduced bioavailability of S52 does not explain the total lack of activity on S52.

5.4.2 Bioavailability

5.4.2.1 Mixing

Cereclor S52 was mechanically emulsified prior to all of the fermentations. S52 is a viscous oil which does not emulsify easily under agitation alone. Other researchers have noted the importance of beads in shake flasks to improve the degradation of chlorinated paraffins (Allpress and Gowland 1999). They attributed this importance to mass transfer

effects. Note that the homogenization process did not contribute to abiotic degradation, based on the abiotic reactor experiment (Figure 16).

Degradation of S52 in shake flasks resulted in lower rates of degradation and lower final percent degradation compared to experiments in the well mixed reactor. The maximum rate of degradation in flasks was 21% (see section 4.7.1) while in the reactor the maximum was 73% (see Figure 27), while the latter result considered multiple additions of DCD. Flask fermentations of mixtures of S52 and DCD showed maximum degradation after up to 30 days, while chloride levels reached a plateau after 24 hours in the reactor. Differences in the rate of degradation between flask and reactor culture of DCD was similarly observed.

These differences may be due to substrate mass transfer. The reactor system generates greater surface area for cell adherence (see section 5.4.2.3). Estimates of k_La were similar for the shake flasks and the reactor under the given conditions (data not shown), so oxygen mass transfer in particular is not likely to be responsible for the difference. These data indicate that mass transfer was the limiting factor for the degradation of S52 in the presence of an induced culture.

5.4.2.2 Sorption and Solvent Effects

Cereclor S52 was observed to accumulate on the reactor walls, as was the case in reactor experiments with DCD, and it was recovered at the end of experiments by a solvent rinse of the reactor interior. The results of an abiotic reactor experiment, illustrated in Figure 16, demonstrated that the levels of S52 suspended in the reactor dropped rapidly from about 1 g/L to less than 100 mg/L. With the addition of 750 mg/L DCD, which acted as a solvent, the levels of suspended Cereclor increased from 50 mg/L to 500 mg/L. This demonstrates the importance of sorption of S52 to the walls of the reactor on the bioavailability of the S52, and the benefits of DCD as a solvent for Cereclor.

Further evidence of the importance of sorption and solvent effects is provided by the variablility of S52 concentration suspended in the reactor during multiple additions of DCD (Figure 27). S52 apparently was repeatedly immobilized and re-suspended throughout the course of the experiment, however these results could also be explained by sampling variability.

5.4.2.3 BATH Assays

The BATH assays were used to help elucidate the relationship between the biodegradation results and the adherence of the substrate to the biomass. The BATH assay results are important because it is thought that utilization *via* direct contact is

important for the degradation of substrates with limited solubility. The procedure measures the affinity of the biomass for the hydrophobic substrate by measuring the drop in absorbance in the mixed liquor before and after the addition of a large quantity of the substrate. In these experiments the agitation time could be set, but the intensity of agitation was subjective. Therefore, there was difficulty comparing the results with those of other researchers.

Table 8 shows a large difference between the results of S52 and DCD, but less of a difference between those of DCD and DCDD. Early log phase cells adhered well to DCDD, resulting in a greater than 80% drop in absorbance of the medium, but not as well to S52, resulting in a less than 10% drop. Meanwhile the same test resulted in an over 50% drop for the case of DCD. In the case of S52, a longer agitation time enabled greater adherence; the absorbance dropped by up to 17% in tests with S52 with longer agitation times. Longer agitation times did not improve the adherence in the case of other substrates (data not shown). This may indicate Cereclor takes longer than the less viscous oils to reach a stable droplet size under given agitation strength, or that Cereclor has less affinity for cells, or both. The viscosity of the substrate will affect the oil droplet size in the agitated system and the BATH results indicate this affects adherence. So, these data indicate that emulsification of S52 prior to fermentation was a good approach. S52 was not emulsified prior to conducting the BATH assays.

The difference in the results with DCD compared to DCDD may be partly explained by the observation of greater quantities of residual DCD in the aqueous phase following settling compared to DCDD. The residual oil also contributed to the absorbance increasing light scattering. This it is possible that a greater proportion of the cells adhered to the oil than previously concluded. However, this observation suggests that dispersion plays a role in bioavailability of DCD. This effect indicates that while DCD has a low solubility, the substrate surface area of the un-dissolved oil in the mixed system is greater than for the other substrates tested. This may contribute to the rapid degradability of DCD.

5.4.2.4 Effect of pH.

A culture pH of 4.2 has been shown by others to render *Pseudomonas* sp.273 inactive (Wischnak, Loffler et al. 1998). Fermentations in shake flasks demonstrated that low pH endpoints correlated with partial consumption of DCD. These results indicated that the cultures had reached a pH endpoint. Insufficient buffering capacity was present in the media to counter the release of protons associated with the degradation of the carbon substrate.

The shake flask fermentations with DCD that demonstrated pH endpoints had initial substrate concentrations of 10 mM (1056 mg/L). Based on the titration curve of the medium (Figure 12), the pH endpoint observed by Wischnak of 4.2 requires the release of 19mM chloride, or about 4 g/L DCD. Therefore, the pH change was in excess of what was anticipated based on DCD addition. The difference can be attributed to the release of protons from growth on yeast extract.

5.4.3 Cosubstrates

The work with various cosubstrates involved aspects of both the physical and genetic factors discussed above.

Figure 25, Figure 26, and Figure 28 illustrate three experiments consisting of two successive additions of DCD to a fermentation of S52. DCD was easily degraded in most cases and S52 was degraded to between 14% and 73% of the quantity added. DCD had been previously demonstrated to be an easily degraded substrate and as an inducing agent, and it improved the bioavailability of the S52 through the solvent effect discussed above (see section 1).

A readily degradable soluble substrate, yeast extract, was tested in fed batch experiments for ability to enhance the degradation of Cereclor. The test was conducted over 75 hours (Figure 28). Approximately 10% of the S52 added to the fed batch experiment was degraded rapidly following its addition, but subsequently no further degradation of S52 was observed. A subsequent cycle and the further addition of S52 resulted in no further release of chloride in the presence of yeast extract (Figure 29). These results suggest that a small portion of the S52 is degradable with an induced culture but that further degradation was not enhanced by the presence of yeast extract. Thus, Cereclor is not an inducing agent for the enzyme.

The addition of hexadecane to a culture in the presence of S52 is illustrated in figure 15. Although C16 is reported to be an inducer of dehalogenating enzymes, a three day incubation period was insufficient to allow the culture to consume even the C16 itself.

Based on the success of the experiments with DCD (see section 4.7.2), this methodology was extended to 12 successive additions of DCD. In this case NaOH and $(NH_4)_2 SO_4$ were added to compensate for pH decreases and nitrogen consumption respectively. This experiment is illustrated in Figure 27. In spite of 12 successive additions of DCD, only 63% of the added S52 was degraded. The remainder was accounted for upon shutdown in a solvent rinse of the reactor walls. Since twelve successive additions of DCD did not achieve a higher degree of degradation of S52 than three additions did, then it is suspected that the un-degraded fraction represents the congeners of S52 that are

refractory. In order to validate this hypothesis, a sample of chloralkane collected from the reactor walls from this experiment could be re-injected to a fermentation. This, however, was not performed.

The mass balance on chlorine from S52 was typically closed to within less than 10%. That is, the chlorine added as S52 was recovered either as chloride or as residual S52 in the liquor or attached to the reactor walls. This is an indication that S52 biodegradation does not result in the formation of large quantities of a polar chlorinated metabolite, that is, a metabolite that is detected by neither the ion chromatograph nor the gas chromatograph.

5.4.4 Gas Chromatography of Cereclor S52

Extractions in hexane of S52 from standard solutions resulted in characteristic gas chromatographs, as illustrated in Figure 30. These results are not unexpected for samples containing a mixture of congeners. Other researchers have reported that the successive secondary "humps" represent groups of compounds with the same numbers of chlorine atoms (Tomy, Billeck et al. 2000). The number of chlorine atoms on the carbon backbone has a greater impact on the GC retention time than does the length of the carbon chain, since the groups of congeners with equal chlorine content elute together. This result is confirmed by the results of research on the volatility of chlorinated alkanes. The liquid vapour pressure at 25°C of decane is 350 times that of DCD, while only eleven times that dodecane. A dichlorodecane was reported to be 17 times that of a tetrachlorodecane (Drouillard Ken, Tomy Gregg et al.).

The gas chromatograph results were standardized using the peak area of the internal standard, DCDD. The relationship between the area ratio and the concentration for chromatography with Cereclor S52 could be adequately described using a two parameters model in the form:

$$y = \frac{Ax}{x + B} \tag{8}$$

Samples from all S52 fermentations were analysed using the gas chromatograph. Note however, that the degradation was estimated from the chloride data, not from the gas chromatography data, as discussed in section 3. The calibration curve illustrated in Figure 11 must be used with caution in relating the area ratio to the S52 concentration, as the shape of the GC profile changed through the course of the fermentations. The decrease in peak area is not necessarily directly related to the increase in mixed liquor chloride because all congeners did not have identical response factors. Further, the

decrease could have been due either to degradation or to sequestration of S52 to the walls of the reactor. We nevertheless do observe an overall decreasing trend in the concentration of S52 with time as measured by chromatography throughout the course of a fermentation (Figure 25). For the experiment where DCD was added to the fermentation through twelve successive stages, significant variability in the measured S52 level was observed (Figure 27). This variability was attributed to the solvent effect as discussed above which served to remove some Cereclor from the reactor walls.

The changes in the GC profile for this experiment are illustrated in Figure 31. Comparing stage 3 with stage 4, we conclude that S52 was resuspended from the walls to the bulk liquid, and that a component that eluted at 80 seconds was present at the start of the experiment in stage 3 but was removed in stage 4. Further, the chromatograph after 12 stages did not exhibit the secondary structure that was characteristic of S52. The profile for a sample taken from the reactor rinse after shutdown labelled "walls" in Figure 31, is presented as well in order to compare the secondary structure. The area under this curve is not of interest, because the concentration of S52 being attached to the walls is not analogous to that of the suspended substrate and it depends on the rinse solvent volume. Differences are evident, however, in the profile of the rinse sample (walls) and the profile of the S52 that remained suspended in the reactor. These differences are highlighted in Figure 32, in which the ratios of the profiles to the feed sample are plotted. Recalling that the retention time is correlated to the degree of chlorination first, and then to the chain length, the rising trend in this plot suggests that the sample collected from the reactor walls contained a higher proportion of compounds with higher degree of chlorination. If chain length alone determined the composition of the hydrocarbon which was sequestered to the walls, then the standard response plot for this sample would have contained the humps that characterize the secondary structure of the chromatographs. The effect could be either due to a greater propensity for such compounds to adsorb to the walls, to lesser propensity to be degraded, or both.

Using the empirical model adopted to represent the GC response to Cereclor compounds, (equation 8) and assuming that the constants A and B for each of the individual components of the compound are not identical, then a mixture such as Cereclor, represented by a single curve could be resolved into a family of curves. Each curve in the family would represent a single congener found in Cereclor, and the combined response to the mixture would be a weighted average of each curve in the family. It is unlikely that each of the congeners in Cereclor would have the same relationship between concentration and GC response.

The family of curves may be transformed to the domain of elution time and the ratio of abundances may be calculated. This process is the same as the way the standard response

ratio was calculated to measure changes in the secondary structure of the chromatograph in Figure 32. This ratio of abundances then provides information on the anticipated effect on the standard response ratio of changes to the parameters of the model. Interestingly, the constant A, (equation 8) which is the maximum response, has no effect on the relative response ratio (data not shown). The constant B, however, is directly related to the standard response ratio in the time domain. Therefore, if B drops with elution time then the standard response ratio will drop with elution time, and *vice versa*. Also, this indicates that some of the congeners might approach a linear calibration curve, as do DCD and DCDD, and the constant B (equation 8) for the GC response could be directly related to chlorine content. Further work is required to test this hypothesis.

6. CONCLUSIONS

- The chloride specific probe was not a suitable process control parameter for the experimental reactor in fermentations with chlorinated substrates. The probe did not provide reliable real time quantitative measurements for chloride. The response of the probe to a drop in the pH is the opposite to that of an increase in chloride. As a result, the probe lacks sensitivity when the two phenomena occur simultaneously, as is the case of the biodegradation of chlorinated paraffins.

- The rate of production of CO_2 and the mixed liquor dissolved oxygen level were both suitable parameters for control of the reactor under most conditions

- Gibbs free energies of formation and Gibbs free energies of reaction for the mineralization of various chlorinated substrates were estimated using the group contribution method (Dolfing, 1994). For all the chloroalkanes considered, the thermodynamics of the mineralization reactions were favourable. However, an increasing degree of chlorination resulted in a less favourable free energy of reaction.

- The CO_2 production profiles were different for 1,10-dichlorodecane (DCD) than for 1,12-dichlorododecane (DCDD). While DCD and DCDD have similar chemical structures, their physical properties, including melting point and behaviour during a bacterial adherence test, were different. Cereclor S52 was the least adherent hydrocarbon tested, possibly due to its higher viscosity.

- DCD was fully degraded in the bioreactor, releasing stoichiometric quantities of chloride. This substrate is a known inducer of the dehalogenases possessed by *Pseudomonas* sp. 273. Cereclor S52, emulsified by mechanical means, was degraded by up to 73% by means of sequential additions of DCD to the bioreactor. The highest reported degradation of chlorinated paraffins reported elsewhere is 57% (Omori, 1987). The percent degradation was not higher (63%) for twelve successive additions than for three (up to 73%). This work is indicative of an upper limit for the degradation of Cereclor S52 in this system. The degradative enzymes in *Pseudomonas* sp. 273 did not appear to be induced by Cereclor itself, by yeast extract, or by hexadecane.

- Cereclor was deposited on the reactor walls, and recovered upon shutdown by means of a solvent rinse. Using this technique the mass balance on chlorine was closed to within the error of the analytical procedures used to quantify chloride and hydrocarbon. Under abiotic conditions, the equilibrium partitioning of the

added Cereclor emulsion favoured the reactor walls (approx. 90%) over the mixed liquor (approx. 10%). When DCD was added to this system, Cereclor was stripped from the walls to the mixed liquor. This suggests that DCD acted not only as a cosubstrate and to induce degradative enzymes, but also as a solvent to improve the bioavailability of Cereclor S52 in the bioreactor.

- Based on a comparison of gas chromatographs of Cereclor in the mixed liquor of the bioreactor through the course of the experiments, there appears selective degradation of certain fractions of the Cereclor mixture. Also, certain components of the Cereclor mixture were selectively deposited on the reactor walls. It is not known whether these components were deposited to the walls because they were non degradable, or *vice versa*.

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APPENDIX 1 - PROCESS CONTROL CODE

```
Option Explicit

Declare Function LTsAttachOrCreateScaledBuffer Lib "SWAY32.DLL" _
   (ByVal tag_type As Integer, ByVal tag_scale As Double, ByVal tag_offset As Double, ByVal
tag_application As String, ByVal tag_topic As String, _
   ByVal tag_item As String, ByVal tag_buffersize As Long, _
   ByVal tag_launch_flag As Integer) As Long
Declare Function LTsGetNextPoint Lib "SWAY32.DLL" _
   (ByVal tag_handle As Long, ByVal tag_datatype As Integer, _
   tag_voidpointer As Any, tag_timestamp As Double) As Integer
Declare Sub LTsDetachFromBuffer Lib "SWAY32.DLL" _
   (ByVal tag_handle As Long)
Declare Function LTsSenderActive Lib "SWAY32.DLL" _
   (ByVal tag_ReceiverID As Long) As Integer
'added
Dim new_average As Double
Dim duration As Date
Dim current_average As Double
Dim control_start As Date
Dim current_time As Date
Dim start_time As Date
Dim count1 As Long
Dim count2 As Integer
Dim cycle_count As Integer
Dim threshold As Single
Dim electrode(10) As Double
Dim load_cell(6) As Double
Dim electrode_average(4) As Double
Dim time_array(4) As Double
Dim local_slope(4) As Double
Dim max_slope As Double
Dim load_cell_average As Double
Dim sample_freq As Date
Dim sample_duration As Date
Dim increase_flag As Integer
Dim average_flag As Integer
Dim valve_flag As Integer
Dim auto_drain_fill As Integer
Dim sample_time As Date
Dim target_valve As Integer
Dim target_cell As Integer
Dim filling As Integer
Dim lc_decrease As Double
Dim current_lc As Double
Dim elec_av As Double
Dim elec_sd As Double
Dim sim_data_flag As Integer
Dim handle(1 To 20) As Long          ' handle to a receiver object
Dim row(1 To 20) As Long             ' current spreadsheet row
Dim D_DOUBLE As Integer     ' constant for buffer attach
Dim D_INT As Integer        ' constant for buffer creation
Dim firsttime As Double     ' the initial time for the run
Dim delta_time As Date      ' The time interval for auto cycling.
Dim num_tags As Integer     ' the number of tags to get.
Dim auto_run As Integer     ' Auto-run flag.
```

```
Dim ts_each As Integer      ' flag to time stamp each column
Dim stop_after_N_rows As Single    ' Stop after this many rows.
Dim start_row As Integer           ' Start putting data in this row.
Dim b_scale As Double       ' buffer scale factor
Dim b_offset As Double         ' buffer offset
'Dim samp As Integer

Dim automation_flag As Integer
Dim co2_next_time As Date
Dim co2_call_interval As Date
Dim last_co2_call As Date
Dim co2_data_value As Double
Dim co2_time As Single
Dim co2_topic As String
Dim co2_sample As Long
Dim co2_row As Long
Dim co2_date As Single
Dim co2_write As Date
Dim co2_get_time As Date
Dim co2_adj As Single

Dim syringe_rnd As Single
Dim syringe_auto As Integer

Dim fed_batch_flag As Integer
Dim fed_batch_cycles As Integer
Dim fed_batch_threshold As Double
Dim load_row As Integer



Sub Auto_Open()
   If Worksheets("Adv. Settings").Range("auto_time") = 1 Then
      auto_collect  ' Start auto-collecting data.
   Else
      ' Give the user a start button.
      Worksheets("LT Data").Buttons.Add 250, 5, 60, 17
      Worksheets("LT Data").Buttons.Caption = "Start"
      Worksheets("LT Data").Buttons.OnAction = "start_button"
   End If
End Sub

Sub start_button()  ' The user just pushed the start button.
   Dim i As Integer
   Worksheets("LT Data").Buttons.Delete  ' Delete all buttons.

   load_row = 2
   count1 = 0
   cycle_count = 1
   valve_flag = 0
   filling = 0
   current_time = Date + Time
   control_start = Date + Time + Sheet3.Cells(16, 2).Value / 60 / 24
   auto_drain_fill = Sheet3.Cells(15, 2).Value
   co2_call_interval = Worksheets("Adv. Settings").Cells(25, 2) / 3600 / 24
```

```
    co2_next_time = current_time + co2_call_interval
    co2_data_value = 1
    co2_row = 2
    automation_flag = Worksheets("Adv. Settings").Cells(24, 2)
    threshold = Sheet3.Cells(18, 2).Value
    Sheet1.Cells(2, 7) = 0 'sets cumulated pulses to zero

    syringe_auto = Sheet3.Cells(22, 2)
    If syringe_auto = 1 Then
       syringe_rnd = Int(randomnum(1, 15, 2) + 0.5)
    Else
       syringe_rnd = Sheet3.Cells(20, 2)
    End If

    Sheet3.Cells(20, 2) = syringe_rnd
    Sheet4.Cells(31, 7) = syringe_rnd
    Sheet4.Cells(18, 7).Value = cycle_count
    Sheet4.Cells(17, 7).Value = valve_flag
    Sheet4.Cells(16, 7).Value = filling
    Sheet4.Cells(19, 7).Value = (control_start - current_time) * 24 * 60
    Sheet4.Cells(21, 7).Value = auto_drain_fill
    Sheet4.Cells(22, 7).Value = co2_call_interval
    Sheet4.Cells(23, 7).Value = automation_flag
    Sheet4.Cells(24, 7).Value = threshold
    Sheet4.Cells(25, 7).Value = co2_data_value

    'Sheet3.Cells(10, 2).Value = 1          'turn on mixer
    'Sheet3.Cells(9, 7).Value = 1           'turn on air
    'increase_flag = 0
    'Sheet4.Cells(15, 7).Value = increase_flag

    For i = 0 To 9
       electrode(i) = 0
    Next i
    For i = 0 To 3
       electrode_average(i) = 0
    Next i

    MsgBox "Turn off syringe pump while relay is initialized" & vbCr _
       & "Set the injection volume to 0.2 mL and Synchronize Clocks" _
       , vbOKOnly + vbInformation, "Startup Check"
    Sheet1.Cells(2, 2).Value = Date + Time
    auto_collect  ' Start auto-collecting data.
End Sub

Sub auto_collect()                    ' Start collecting data.
    start_row = 4  ' Row to start our data.
    Attach  ' Attach to LT-SPEEDWAY buffer.
    auto_run = 1   ' Run continuously.
    delta_time = Worksheets("Adv. Settings").Range("time_interval")
    stop_after_N_rows = Worksheets("Adv. Settings").Range("stop_after_N") + start_row - 1
    Application.OnTime Now + delta_time, "GetData"
    Worksheets("LT Data").Buttons.Add 250, 5, 60, 17
    Worksheets("LT Data").Buttons.Caption = "Stop"
    Worksheets("LT Data").Buttons.OnAction = "Halt"
```

```
End Sub

Sub Attach()
   Dim app As String          ' string to identify sender app
   Dim topic As String        ' string to identify sender document
   Dim item As String         ' string to identify data item
   Dim buffersize As Long     ' size of the buffer
   Dim launch_flag As Integer ' 0 for no launch, 1 for launch
   Dim i_tag As Integer       ' tag loop counter
   Dim any_error              ' nonzero if any buffer is not found

   D_DOUBLE = 5               ' Create a buffer to store doubles.
   buffersize = 1000          ' Set the size of the buffer.
   any_error = 0              ' Initialize.
   app = "RUNTIME"            ' Set the application string.
   ' Get the topic string.
   topic = Worksheets("Settings").Range("setup_name") + ".LTC"
   launch_flag = 0            ' Initialization.
   'clear_datasheet           ' Clear data in the "LT Data" worksheet.
   firsttime = -777           ' flag for initialization
   num_tags = Worksheets("Settings").Range("Number_of_Tags")
   ts_each = Worksheets("Adv. Settings").Range("time_stamp_each")
   D_INT = 1
   b_scale = 1
   b_offset = 0


   For i_tag = 1 To num_tags

      row(i_tag) = start_row - 1    ' Start putting data at this row.
      ' Get the item string.
      item = Worksheets("Settings").Range("tag_name").Offset(i_tag - 1, 0)
      ' Only launch if all buffers are there.
      If i_tag = num_tags Then launch_flag = 1
      ' Attach to the buffer, and set its size.
      handle(i_tag) = LTsAttachOrCreateScaledBuffer(D_DOUBLE, b_scale, b_offset, app, topic, _
            item, buffersize, launch_flag)
      If handle(i_tag) <> 0 Then
         ' Put time label on top of column.
         If i_tag = 1 Or ts_each = 1 Then
            Worksheets("LT Data").Cells(3, 1).Offset(0, (i_tag - 1) * 2).Value _
               = "Sample Time"  ' Label for time stamp column.
         End If
         'Put item name on top of column.
         If ts_each = 0 Then
            Worksheets("LT Data").Cells(3, 2).Offset(0, i_tag - 1).Value = item
         Else
            Worksheets("LT Data").Cells(3, i_tag * 2).Value = item
         End If
         ' Put the LT-SPEEDWAY buffer status on the Settings sheet.
         Worksheets("Settings").Range("tag_name").Offset(i_tag - 1, 1).Value = _
            "Created"
      Else
         ' Unsuccessful.  No buffer extant.
         Worksheets("Settings").Range("tag_name").Offset(i_tag - 1, 1).Value = _
            "Error - LT-SPEEDWAY buffer not created or found."
```

```
        any_east one buffer not found.
      End If
    Next i_tag

    ' Put status information on the spreadsheet.
    Worksheets("LT Data").Cells(1, 1) = "Status:"
    If any_error = 0 Then
      ' Successful!
      Worksheets("LT Data").Cells(1, 2) = "LT-SPEEDWAY buffer(s) active"
    Else
      Worksheets("LT Data").Cells(1, 2) = _
          "Error - LT-SPEEDWAY buffer(s) not created or found."
    End If
End Sub

Sub GetData() 'Get data now

    Dim data_value As Double        ' the data point fetched from the buffer
    Dim new_data_flag As Integer    ' flag to indicate if there's new data
    Dim timestamp As Double     ' a timestamp
    Dim i_tag As Integer        ' tag loop counter
    Dim any_data As Integer     ' flag if there is any data for this tag
    Dim max_row As Integer      ' The maximum row number we are on.
    Dim i As Integer
    Dim j As Integer
    Dim p As Integer

    max_row = 0    ' Initialize.
' only write data to LT Data sheet if flag is set
    For i_tag = 1 To num_tags       ' Do for each tag or block.
      any_data = 0              ' We have no data yet.

      If handle(i_tag) <> 0 Then  'Get data only if there's a receiver object.

          Do
           ' Get the next data point from the buffer.
           new_data_flag = LTsGetNextPoint(handle(i_tag), D_DOUBLE, _
                           data_value, timestamp)

           If new_data_flag = 1 Then          ' If there's new data...
             any_data = 1                 ' We have some data.
             If (i_tag <> num_tags) Then    'not for load cell
               row(i_tag) = row(i_tag) + 1    ' Move down one row in that column
               Worksheets("Control Data").Cells(14, 7).Value = row(i_tag)
             End If

             If row(i_tag) > max_row Then   ' Track the max. row. it is used to stop after the maximum
               max_row = row(i_tag)
             End If

             If firsttime <> -777 Then  'set to -777 at attach once only
               ' Adjust time origin to start of run.
               timestamp = timestamp - firsttime
             Else
               firsttime = timestamp ' Save time for time origin.
               timestamp = 0#
```

```
            End If

            If i_tag = 1 Or ts_each = 1 Then
               ' Put the timestamp in the spreadsheet.
               Worksheets("LT Data").Cells(row(i_tag), 1) _
                     .Offset(0, (i_tag - 1) * 2).Value = timestamp / 3600
            End If
            ' Put the data in the spreadsheet.
            If ts_each = 0 Then
               If i_tag <> num_tags Then       'not for load cell
                  Worksheets("LT Data").Cells(row(i_tag), i_tag + 1). _
                     Value = data_value
               End If
            Else
               Worksheets("LT Data").Cells(row(i_tag), i_tag * 2). _
                  Value = data_value
            End If
         End If
      Loop Until new_data_flag = 0   ' Stop looping if no more new data.
      If (any_data = 1) Then
         ' Put the status on the Settings sheet.
         With Worksheets("Settings").Range("tag_name")
            .Offset(i_tag - 1, 2).Value = row(i_tag)
            .Offset(i_tag - 1, 3).Value = data_value
            .Offset(i_tag - 1, 4).Value = timestamp
         End With
      End If
   End If
Next i_tag
'num_tags = Worksheets("Settings").Range("Number_of_Tags")
'electrode(row(i_tag) Mod 10) = Sheet2.Cells(3, 5).Value

get_co2_data

Calculate              ' Recalculate the worksheet.

count1 = count1 + 1   'counter for getdata loop

load_cell(count1 Mod 6) = Sheet2.Cells(5, 5).Value

'close the target sample valve after a delay if valve_flag = 1
close_samp_valve

'check to see if a manual fill is requested
manual_fill_check

'check to close medium valve based on load cell
close_medium_valve

'check the cycling criteria and the cycling automation flag
CheckSelfCycle

'override control_start (cycling criteria delay) if the program was restarted part way through the cycle
current_time = Date + Time
If Sheet3.Cells(30, 2) = 1 Then
   control_start = Date + Time + 10 / 3600 / 24  '10sec delay expressed in days
```

```
    Sheet3.Cells(30, 2) = 0
  End If

  'copy load cell monitor
  If Sheet3.Cells(33, 2) = 1 Then
    Sheet7.Cells(load_row, 2) = Sheet2.Cells(5, 5)
    Sheet7.Cells(load_row, 1) = Sheet2.Cells(5, 6)
    load_row = load_row + 1
  End If
  If Sheet3.Cells(34, 2) = 1 Then 'reset load sheet
    Sheet7.Range("A1:b5000").ClearContents
    load_row = 2
    Sheet3.Cells(34, 2) = 0
  End If


  'implement stopping criteria
  If auto_run = 1 Then
    ' There are two stopping criteria:
    '  (1) We ave gotten the number of rows of data requested.
    '  (2) There are no more active senders.
    If (max_row >= stop_after_N_rows) Or (AnySenders = 0) Then
      Halt  ' Stop the automatically-timed operation.
    Else
      Application.OnTime Now + delta_time, "GetData"
    End If
  End If

  'update diagnostics
  Sheet4.Cells(19, 7).Value = (control_start - current_time) * 24 * 60 'displays in minutes
  Sheet4.Cells(18, 7).Value = cycle_count
  Sheet4.Cells(17, 7).Value = valve_flag
  Sheet4.Cells(16, 7).Value = filling
  Sheet4.Cells(21, 7).Value = auto_drain_fill
  Sheet4.Cells(22, 7).Value = co2_call_interval
  Sheet4.Cells(23, 7).Value = automation_flag
  Sheet4.Cells(24, 7).Value = threshold
  Sheet4.Cells(25, 7).Value = co2_data_value
  Sheet4.Cells(26, 7) = co2_next_time
  Sheet4.Cells(28, 7) = Date + Time
  Sheet4.Cells(27, 7) = co2_get_time - co2_write
End Sub
Public Sub open_reactor_valve()

  'this procedure opens reactor valve for specified time
  duration = Sheet3.Cells(12, 2).Value / 3600 / 24 'draining time expressed in days

  Sheet3.Cells(10, 2).Value = 0          'turn off mixer

  'setting timer
  start_time = Date + Time
  current_time = Date + Time

  Sheet3.Cells(9, 5).Value = 1           'open drain valve

  'loop to keep valve open for specified duration
```

```
    While currentration)
      current_time = Date + Time
    Wend

    Sheet3.Cells(9, 5).Value = 0              'close valve

    'write syringe rnd to controls sheet
    Sheet4.Cells(cycle_count + 4, 3) = syringe_rnd

    'activate syringe for pca injection
    syringe_rnd = Sheet3.Cells(20, 2)
    Sheet4.Cells(29, 7) = syringe_rnd
    syringe_activate
    syringe_rnd_generator

    Sheet3.Cells(10, 2).Value = 1             'turn mixer on


End Sub

Public Sub open_medium_valve()
    Dim i As Integer

    load_cell_average = 0

      For i = 0 To 5
        load_cell_average = load_cell_average + load_cell(i)
      Next i

    load_cell_average = load_cell_average / 6

    Sheet3.Cells(9, 8).Value = 1        'open valve

    lc_decrease = Sheet3.Cells(14, 2)

    filling = 1
    Sheet4.Cells(16, 7).Value = filling

End Sub

Public Sub open_sampling_valve()

    valve_flag = 1
    Sheet4.Cells(17, 7).Value = valve_flag

    target_valve = cycle_count Mod 4  'mod returns the remainder of count/4 - hence when cycle_count is 1
or 5 target valve is 1
    If target_valve = 0 Then target_valve = 4  'mod returns 0 for 4/4 in cycle 4, 8 etc.
    target_cell = target_valve + 1

    If target_cell = 5 Then
      target_cell = 6
    End If

    Sheet3.Cells(9, target_cell).Value = 1
    sample_time = Date + Time
```

```
Public Sub get_co2_data() 'gets co2 dataq value, adjusts get time and checks fed batch flag


Dim col_index As Integer
Dim row_index As Integer
Dim check As String
Dim stop_flag As Integer
   automation_flag = Worksheets("Adv. Settings").Cells(24, 2)
   co2_call_interval = Sheet3.Cells(25, 2).Value / 3600 / 24
   co2_topic = Sheet3.Cells(23, 2)
   current_time = Date + Time
   If co2_next_time <= current_time And automation_flag = 1 Then
      co2_get_time = current_time
      co2_next_time = current_time + co2_call_interval
      Application.DisplayAlerts = False 'this statement avoids causing a hang if the file is not available
      Workbooks.OpenText Filename:=co2_topic, Origin:=xlWindows, _
         StartRow:=1, DataType:=xlDelimited, TextQualifier:=xlDoubleQuote, _
         ConsecutiveDelimiter:=False, Tab:=True, Semicolon:=False, Comma:=True, _
         Space:=False, Other:=False, FieldInfo:=Array(1, 1)

      Application.Goto Reference:="R36C1"

     stop_flag = 0
     row_index = 0

     Do While stop_flag = 0
        row_index = row_index + 1
        check = Selection.Offset(row_index, 0).Value
        If check = "" Then
           co2_data_value = Selection.Offset(row_index - 1, 11).Value
           co2_time = Selection.Offset(row_index - 1, 2).Value
           co2_sample = Selection.Offset(row_index - 1, 0).Value
           co2_date = Selection.Offset(row_index - 1, 1).Value
           stop_flag = 1
        End If
     Loop

     ActiveWorkbook.Close
     Application.DisplayAlerts = True

     'adjusts co2_next_time to ensure get and write don't coincide
     co2_next_adjust

     'put data on settings sheet and co2 data sheet
     Worksheets("Settings").Cells(9, 5).Value = co2_data_value
     Worksheets("Settings").Cells(9, 4).Value = co2_row
     Worksheets("Settings").Cells(9, 6).Value = co2_time
     'Worksheets("CO2").Cells(co2_row, 2).Value = co2_date
     'Worksheets("CO2").Cells(co2_row, 3).Value = co2_time
     'calculate co2 data time in terms of common origin and in hours
     Worksheets("CO2").Cells(co2_row, 3).Value = ((co2_date + co2_time) - Sheet1.Cells(2, 2).Value) *
24
     Worksheets("CO2").Cells(co2_row, 1).Value = co2_sample
     Worksheets("CO2").Cells(co2_row, 5).Value = co2_data_value
     Worksheets("CO2").Cells(co2_row, 4).Value = co2_date + co2_time
```

```
      Worksheets("CO2").Cells(co2_row, 6).Value = co2_write
      Worksheets("CO2").Cells(co2_row, 7).Value = co2_get_time
      Worksheets("CO2").Cells(co2_row, 8).Value = co2_adj
      co2_row = co2_row + 1

      fed_batch

   End If
End Sub
' runs once each time new co2 value is available
Public Sub fed_batch()
   fed_batch_flag = Sheet3.Cells(26, 2).Value
   fed_batch_threshold = Sheet3.Cells(29, 2).Value

   If fed_batch_flag = 1 And co2_data_value < fed_batch_threshold Then
      Sheet1.Cells(2, 7).Value = Sheet1.Cells(2, 7).Value + Sheet3.Cells(28, 2).Value ' increments the fed
batch pulse cumulator
      syringe_rnd = Sheet3.Cells(28, 2).Value ' temporarily take over syringe_rnd variable for fed batch
operation
      syringe_activate
   End If
syringe_rnd = Sheet3.Cells(20, 2) ' sets value back ready for automated pca injection  both automation and
fed batch use the same syringe relay
'stops automation if syringe is empty
If Sheet1.Cells(2, 7) > Sheet3.Cells(31, 2) Then Sheet3.Cells(26, 2) = 0

End Sub

Sub copy_simulation()
  sim_data_flag = Worksheets("Adv. Settings").Cells(21, 2).Value
  ' copy electrode simulation data to LT Data and Settings _
          if simulation flag is set
        If i_tag = 2 And sim_data_flag = 1 Then
          Worksheets("Settings").Cells(3, 5).Value = _
            Worksheets("Simulation Data").Cells(row(i_tag), 2).Value
          Worksheets("LT Data").Cells(row(i_tag), 2).Value = _
            Worksheets("Settings").Cells(3, 5).Value
          'Worksheets("Settings").Cells(3, 6).Value = _
          '   Worksheets("Simulation Data").Cells(row(i_tag), 1).Value
          'Worksheets("LT Data").Cells(row(i_tag), 1).Value = _
          '   Worksheets("Simulation Data").Cells(row(i_tag), 1).Value

          'halt the process if the sim data is finished
          If row(i_tag) = 65000 Then
            'put halt here
          End If
        End If
End Sub
Public Sub close_samp_valve()
'if cycle has occurred and sample valve is open
   If valve_flag = 1 Then
      current_time = Date + Time
      sample_duration = Sheet3.Cells(13, 2).Value / 3600 / 24
      If current_time >= (sample_time + sample_duration) Then
        Sheet3.Cells(9, target_cell).Value = 0     'close sample valve
        valve_flag = 0
```

```
        Sheet4.Cells(17, 7).Value = valve_flag
      End If
    End If
End Sub
Public Sub manual_fill_check()
' check to see if a manual fill is requested
    If Sheet3.Cells(19, 2) = 1 Then
      Sheet3.Cells(19, 2) = 0
      open_medium_valve
    End If
End Sub
Public Sub close_medium_valve()
'if medium valve is open check to see if it's time to close it
    If filling = 1 Then
    'initial load cell level is an average but the stop point is not
      current_lc = Sheet2.Cells(5, 5).Value
      If current_lc < (load_cell_average - lc_decrease) Then
        Sheet3.Cells(9, 8).Value = 0
        filling = 0
        Sheet4.Cells(16, 7).Value = filling
      End If

    End If
End Sub
Public Sub CheckSelfCycle()
    Dim i As Integer
    Dim j As Integer

    threshold = Sheet3.Cells(18, 2).Value
    current_time = Date + Time
    auto_drain_fill = Sheet3.Cells(15, 2).Value

    If auto_drain_fill = 1 And current_time >= control_start _
        And co2_data_value <= threshold Then
          open_reactor_valve   'drain Rx, wait for drain and inject pca
          open_medium_valve  'open fill valve and set filing flag to 1
          open_sampling_valve  'set target valve open it and set sample time

          Sheet4.Cells(cycle_count + 4, 2) = Now()
          cycle_count = cycle_count + 1
          count1 = 0
          Sheet4.Cells(18, 7).Value = cycle_count
          control_start = Date + Time + Sheet3.Cells(16, 2).Value / 60 / 24
    End If
End Sub

Function AnySenders() As Integer
    ' Check to see if there are any active senders.
    Dim i_tag As Integer        ' tag loop counter

    AnySenders = 0              ' Initialize
    If row(1) >= start_row Then     ' Do only if we have some data.
      For i_tag = 1 To num_tags   ' Do for each tag or block.
        ' Check only if there is a receiver object.
        If handle(i_tag) <> 0 Then
          ' Check for an active sender.
```

```
            Ii_tag)) = 1 Then
               AnySenders = 1
            End If
         End If
      Next i_tag
   Else
      AnySenders = -1  ' We don't have data. Wait for senders to arrive.
   End If
End Function

Sub Detach()
   Dim i_tag As Integer        ' tag loop counter

   For i_tag = 1 To num_tags
      ' Detach only if there is a receiver object.
      If handle(i_tag) <> 0 Then
         ' Detach from the buffer.
         LTsDetachFromBuffer (handle(i_tag))
         handle(i_tag) = 0       ' We no longer have a valid handle.
         ' Tell the user we're detached.
         Worksheets("LT Data").Cells(1, 2) = _
               "Detached from LT-SPEEDWAY buffer(s)"
         ' Put the LT-SPEEDWAY buffer status on the Settings sheet.
         Worksheets("Settings").Range("tag_name").Offset(i_tag - 1, 1) _
            .Value = "Detached"
      End If
   Next i_tag
End Sub

Sub Halt()  ' Stop the aoutmatically-timed operation.
   auto_run = 0 ' Stop running continuously.
   Application.OnTime Now + delta_time + delta_time, "Detach"
   Worksheets("LT Data").Buttons.Delete
   Worksheets("LT Data").Buttons.Add 250, 5, 60, 17
   Worksheets("LT Data").Buttons.Caption = "Clear"
   Worksheets("LT Data").Buttons.OnAction = "clear"
End Sub

Sub clear()
   Worksheets("LT Data").Buttons.Delete
   clear_datasheet               ' Clear the "LT Data" worksheet.
   Worksheets("Settings").Range("C3:F50").clear  ' Clear status info.
   Calculate                     ' Recalculate the worksheet.
End Sub

Sub clear_datasheet()
   ' Clear data values and titles.
   Worksheets("LT Data").Range("A1:H5000").ClearContents
End Sub

Sub co2_next_adjust()

   co2_topic = Worksheets("Adv. Settings").Cells(23, 2)
   co2_write = FileDateTime(co2_topic)

   If co2_get_time - co2_write > 160 / 3600 / 24 Then
```

```
    co2_next_ / 3600 / 24
    co2_adj = -10
  ElseIf co2_get_time - co2_write < 140 / 3600 / 24 Then
    co2_next_time = co2_next_time + 10 / 3600 / 24
    co2_adj = 10
  Else
    co2_next_time = co2_next_time
    co2_adj = 0
  End If

End Sub
Public Function randomnum(seed, upper, lower)
'generates random numbers between upper and lower bounds
'if seed is negative then the function is repeatable
  randomnum = Rnd(seed) * (upper - lower) + lower

End Function

Sub syringe_activate()
' turns the syringe relay bit on and off  number of times dictated by syringe_rnd

  While syringe_rnd <> 0
    current_time = Date + Time
    start_time = Date + Time
    Sheet4.Cells(30, 7).Value = 1   'bit status
    Sheet3.Cells(9, 9).Value = 1   'set relay on
    While current_time <= (start_time + 2 / 3600 / 24)  'wait 2 secs
      current_time = Date + Time
    Wend
    Sheet4.Cells(30, 7).Value = 0
    Sheet3.Cells(9, 9).Value = 0   'set relay off
    current_time = Date + Time
    start_time = Date + Time
    While current_time <= (start_time + 5 / 3600 / 24)  'wait 5 secs to allow pump to finish
      current_time = Date + Time
    Wend
    syringe_rnd = syringe_rnd - 1
    Sheet4.Cells(29, 7) = syringe_rnd
  Wend


End Sub
Sub syringe_rnd_generator()
'random integer generator between 2 and 15 syringe cycles (0.4 and 3 mL @0.2 mL/cycle)
  syringe_auto = Sheet3.Cells(22, 2)
  If syringe_auto = 1 Then
    syringe_rnd = Int(randomnum(1, 15, 2) + 0.5)
    Sheet3.Cells(20, 2) = syringe_rnd
  Else
    syringe_rnd = Sheet3.Cells(20, 2)
  End If

  Sheet4.Cells(31, 7) = syringe_rnd

End Sub
Sub Test()
```

```
Sheet1.Cells(2, 2).Value = Date + Time
co2_next_time = current_time
get_co2_data

End Sub
```

APPENDIX 2 GAS CHROMATOGRAPH PROCESSING CODE

```
Option Explicit
'use an even number for interval
Const interval = 24
'these variable are from the interval macro
Dim col As Integer
Dim f As Double
Dim count As Double
Dim first As Integer
Dim last As Integer
Dim target As Double
Dim time As Double
Dim average As Double
Dim avg(1 To interval) As Double '0 to interval

'these variable are from the baseline and single column macros
Dim source_col As Integer
Dim target_row As Integer
Dim source_row As Integer
Dim start_col As Integer
Dim start_row As Integer
Dim target_col As Integer
Dim n As Integer
Dim actual_run_time_length As Double
Dim est_count As Integer
Dim peak1 As Single
Dim p1minus As Single
Dim p1plus As Single
Dim i As Integer
Dim j As Integer
Dim p1(2) As Single  ' 0 is hte index(from the start row), 1 the time(varian base) and 2 the value
Dim p2(2) As Single
Dim slope As Double
Dim yint As Double
Dim put_col As Integer

Sub baseline()

Select_From_Active ("start_row")
start_row = ActiveCell.Row + 4
peak1 = Cells(start_row + 9, 10)
p1minus = Cells(start_row + 9, 11)
p1plus = Cells(start_row + 9, 12)
put_col = Cells(start_row + 9, 13)
'n = Cells(start_row + 1, 19)

i = 0
'find the start point of hte baseline
Do While Cells(start_row + i, 18) < peak1 - p1minus
   i = i + 1
   'add an error check here for i>n
Loop
p1(0) = i
p1(1) = Cells(start_row + i, 18)
p1(2) = Cells(start_row + i, 19) ' replace this with avg routine if necessary
'remove this code which averages a series of readings
'For j = 0 To 5
'   p1(2) = p1(2) + Cells(start_row + i - 3 + j, 19)
'Next j
'p1(2) = p1(2) / 6
'find the endpoint of the baseline
Do While Cells(start_row + i, 18) < peak1 + p1plus
   i = i + 1
```

```
Loop
p2(0) = i
p2(1) = Cells(start_row + i, 18)
p2(2) = Cells(start_row + i, 19) ' replace this with avg routine if necessary
' remove code as above
'For j = 0 To 5
'    p2(2) = p2(2) + Cells(start_row + i - 3 + j, 19)
'Next j
'p2(2) = p2(2) / 6

' convert this to a for loop is desired
Cells(29, 9) = p1(0)
Cells(29, 10) = p1(1)
Cells(29, 11) = p1(2)
Cells(30, 9) = p2(0)
Cells(30, 10) = p2(1)
Cells(30, 11) = p2(2)

slope = (p2(2) - p1(2)) / (p2(1) - p1(1))
yint = p2(2) - slope * p2(1)

'DESCRIBE THE line
i = p1(0)
Do While i <= p2(0)
   Cells(start_row + i - p1(0), put_col) = Cells(start_row + i, 18) 'time index
   Cells(start_row + i - p1(0), put_col + 1) = slope * Cells(start_row + i, 18) + yint ' calculated baseline
   i = i + 1
Loop

End Sub
'move to a cell containing a given string
Sub Select_From_Active(stringtofind As String)
'cells.find method uses a variant or object type
Dim firstcell

Set firstcell = Cells.Find(what:=stringtofind, After:=ActiveCell, LookIn:=xlFormulas, lookat:= _
      xlPart, SearchOrder:=xlByRows, searchdirection:=xlNext, MatchCase:=False, matchbyte:=True)


If firstcell Is Nothing Then MsgBox "Not Found" Else firstcell.Select

End Sub
'organises data from a set sheet format to a set sheet format

Sub single_column()
'
Select_From_Active ("ordinate_values") ' searches active sheet for a string and leaves pointer on the cell
source_row = ActiveCell.Row
source_col = ActiveCell.Column
start_col = source_col
start_row = source_row

target_row = source_row
target_col = source_col + 18
n = 0

actual_run_time_length = InputBox("enter the run time length (secs)")
'actual_run_time_length = Cells(10, 2)
est_count = InputBox("enter the estimated count - use the raw data not the varian file")
'est_count = Cells(4, 2)
```

```
Cells(start_row - 2, target_col) = est_count

Cells(start_row - 4, target_col - 1) = "start_row =" & start_row
Cells(start_row - 3, target_col - 1) = "actual count"
Cells(start_row - 2, target_col - 1) = "est count"
Cells(start_row - 1, target_col) = "FID Out"
Cells(start_row - 1, target_col - 1) = "real time secs"
Cells(start_row - 1, target_col - 2) = "varian time secs"

Do While Cells(source_row, source_col).Value <> " " And _
      Cells(source_row, source_col).Value <> ""
   Do While Cells(source_row, source_col).Value <> " " And _
         Cells(source_row, source_col).Value <> ""
      n = n + 1
      Cells(target_row, target_col).Value = Cells(source_row, source_col).Value
      Cells(target_row, target_col - 1) = n * (actual_run_time_length / est_count)
      Cells(target_row, target_col - 2) = n * 0.1
      Cells(start_row - 3, target_col) = n
      source_col = source_col + 1
      target_row = target_row + 1
      Cells(start_row - 2, start_col) = n
   Loop
   source_col = start_col
   source_row = source_row + 1
Loop

If est_count <> n Then MsgBox "warning, rerun with a new estimated count"
Cells(start_row, 9) = "peak table copied here"
Cells(start_row + 7, 9) = "values in secs use varian time"
'Cells(start_row + 5, 13) = "values in count"
Cells(start_row + 8, 10) = "peak secs"
Cells(start_row + 8, 11) = "minus"
Cells(start_row + 8, 12) = "plus"
Cells(start_row + 8, 13) = "col for baseline"
'Cells(start_row + 6, 14) = "avg plus"
Cells(start_row + 9, 9) = "peak 1"
Cells(start_row + 10, 9) = "peak 2"


Select_From_Active ("peak_retention_time")
Range(Selection.Offset(0, 0), Selection.Offset(2, 7)).Copy
Cells(start_row + 1, 9).Select
ActiveSheet.Paste
Select_From_Active ("peak_area")
Range(Selection.Offset(0, 0), Selection.Offset(2, 7)).Copy
Cells(start_row + 4, 9).Select
ActiveSheet.Paste



End Sub


Sub integrate()
'place cursor in col to be processed
'check the first, last, target col(normaly 7) and make space 6 col from current
'values before running macro

first = 4
last = 2086
col = ActiveCell.Column
```

```
f = 0 'actual counter is the time-200*10
count = 1 'actual counter
target = first 'target row number
time = 207.9 'actual time
'count f = 0 to interval mod interval


For i = first To last   ' i is the source row number

 If count = interval Then
   Cells(target, 7) = time - (interval / 2)
   average = 0
   For count = 1 To interval
   average = average + avg(count)
   Next count
   average = average / interval
   Cells(target, col + 6) = average
   target = target + 1
 End If

 time = time + 0.1
 count = f Mod interval + 1
 avg(count) = Cells(i, col)
f = f + 1
Next i


End Sub
```

APPENDIX 3 - LIST OF TRADE NAMES OF CHLORINATED PARAFFINS

Manufacturer	Trade Name
Oxychem, USA	Chlorowax *
Keil Chemical Div., USA	CW-*
Dover Chemical Corp.	Paroil-*, Chlorez -*
Plastifax, Inc., USA	Plastichlor *
ICI, Australia; Canada; UK; France	Cereclor *
Neville Chemical Co.,	Unichlor *
Pearsall Chemical Co.,	FLX-* CPF-*
Hüls AG, Germany	Chlorparaffin *
Dynamit Nobel, Germany	Witaclor *
Caffaro, Italy	Cloparin *
Hoechst AG, Germany	Chlorparaffin-* Hordaflex-* Hoechst -*
Rhône-Poulenc, France	Alaiflex-* Ribeclor -*

Others Manufacturers:
Bann Quimica (Brazil),
Excel Industry (India),
Ajinomoto (Japan),
Tosoh (Japan),
Asahi Denka (Japan),
Plasticlor (Mexico),
NCP (South Africa)