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Biodegradation of chlorinated alkanes and their commercial mixtures by *Pseudomonas* sp. strain 273

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Abstract The biodegradation of chlorinated alkanes was studied under oxic conditions with the objective of identifying favorable and unfavorable intramolecular chlorination sequences with respect to the enzymes studied. Several dehalogenating bacterial strains were screened for their ability to degrade middle-chain polychlorinated alkanes as well as a commercial mixture. Of the organisms tested, the most promising was *Pseudomonas* sp. strain 273, which possesses an oxygenolytic dehalogenase. The effects of carbon chain length (C_6–C_{16}), halogen position, and overall chlorine content (14–61% w/w) were examined using both commercially available compounds and molecules synthesized in our laboratory. The effects of co-substrates, solvents, and inducing agents were also studied. The results with pure chlorinated alkanes showed that the relative positions of the chlorine atoms strongly influenced the total amount of dehalogenation achieved. The greatest dehalogenation yields were associated with terminally chlorinated alkanes. The α- and α,ω-chlorinated compounds yielded similar results. Vicinal chlorination had the most dramatic impact on degradation. When present on both ends or at the center of the molecule, no dehalogenation was detected. Although partial dehalogenation of 1,2-dichlorodecane was observed, it was likely due to a combination of β-oxidation and an abiotic mechanism. Cereclor S52 was appreciably dehalogenated in shake flasks only when 1,10-dichlorodecane was present as a co-substrate and after increasing the oil surface area through mechanical emulsification, demonstrating the importance of abiotic factors in degrading commercial polychlorinated alkane mixtures.

Keywords Bacterial degradation · Chlorinated paraffins · Degradation · Dehalogenase · Paraffins · Polychlorinated alkanes

Abbreviations 1,2-DCD: 1,2-Dichlorodecane · 1,10-DCD: 1,10-Dichlorodecane · 5,6-DCD: 5,6-Dichlorodecane · 1,6-DCH: 1,6-Dichlorodecane · HCD: 1,2,5,6,9,10-Hexachlorodecane · PCAs: Polychlorinated alkanes · TCD: 1,2,9,10-Tetrachlorodecane · UABL: Upper aerobic biodegradation limit

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Introduction

Polychlorinated alkanes (PCAs) are the largest group of chlorinated hydrocarbons still produced in Western Europe and North America. Their large-scale production started in 1930 and had reached an estimated 300 kt in 1993 [31]. Due to their chemical stability, PCAs are often used as softeners in plastics, as flame retardants, and as additives in gear and cutting oils [6, 29]. Commercially, PCAs are produced by the free-radical chlorination of *n*-alkanes with chain lengths of C_{10}–C_{30} [28]. They are classified according to their carbon chain length, as short (C_{10}–C_{13}), medium (C_{14}–C_{17}), or long ($>C_{17}$), and by their chlorine content, typically 30–70% (w/w). The chlorination process has low positional selectivity, resulting in PCA mixtures composed of thousands of isomers and congeners [24, 28].

A large percentage of PCAs is not recycled and thus finds their way into the environment. Once introduced, their long-range transport is possible via sediments [6]. This is of concern, as commercial mixtures of PCAs have

been shown to bioaccumulate and have been implicated as carcinogens, potential mutagens, teratogens, and endocrine disrupters [6, 28, 35]. Thus, the widespread use of PCAs has raised concern and interest in their environmental fate.

A number of microorganisms have demonstrated abilities to dehalogenate chlorinated alkanes [13, 25, 26, 34, 36]. Most known dehalogenases are either classified as hydrolytic or oxygenolytic. The extent of the degradation is related not only to the specific enzymes present, but also to other biotic and abiotic factors, including bioavailability. In general, PCAs are relatively volatile, have low solubilities in water, and tend to adsorb to solid surfaces [9, 10]. These characteristics tend to limit the bioavailability of PCAs and make their biological degradation difficult. Other factors affecting the degradation of PCAs include nutrient conditions, mass transfer, enzyme expression levels, and toxicity, to name a few [4, 17]. Some of these factors, such as the transport issues, can be addressed through the application of sound engineering principles.

The ultimate degree to which a mixture of PCAs can be degraded once all transport, bioavailability, and expression constraints have been removed is referred to in this paper as the "upper aerobic biodegradation limit" (UABL). This quantity is solely a function of the specificity of the enzymes for the many isomers in the PCA mixture. Quantifying the UABL of a PCA mixture requires both a complete chemical characterization of the PCA mixture and an assessment of the affinity of the various enzymes for the individual components identified. With these two inputs, it is possible to quantify the UABL and, hence, the feasibility of treating a particular PCA mixture biologically.

Unfortunately, no analytical method has been proposed that can adequately resolve the PCA mixture into its constituent components [12, 20, 27, 29]. Our research group has recently addressed this shortcoming using a modeling approach. The composition of the PCA mixture was estimated using a Monte Carlo simulation, based on a set of governing rules related to the relative reactivities of the various intermediates generated during the chlorination process. The distribution predicted by the model compared remarkably well with the available analytical data pertaining to the gross characterization of the mixture. The results from this work will be disseminated separately.

The current study is focused on the second issue required to quantify the UABL of a dehalogenase. Namely, it was desired to expand upon the limited set of available data related to the biological dehalogenation of long-chain chloroalkanes. To date, most studies have focused on chlorinated hydrocarbons with chain lengths less than C_4 [13, 26]. Results on microbial dehalogenation of chlorinated hydrocarbons with carbon chain lengths greater than C_9 are sparse. A notable exception is the work performed with α- and α,ω-chlorinated alkanes [2, 14, 33]. With regards to mixtures, there have been several attempts to biodegrade industrial mixtures of polychlorinated alkane [1, 21], but only the total amount of chloride released from the alkane mixture was reported.

To fill this gap in the literature, the biodegradation of chlorinated alkanes with carbon chain lengths between C_6 and C_{16} was examined. The aim was to elucidate the effect of chlorine position and carbon chain length on biodegradation. The work focused on pure compounds, as current analytical techniques cannot resolve the complex industrial PCA mixtures into its substituents. As mainly only α-chlorinated and α,ω-dichloro alkanes are commercially available, internally chlorinated alkanes were synthesized in our laboratory. The gross degradation of a commercial mixture of chlorinated alkanes was also examined.

Materials and methods

Chemicals

Olefins (1-decene, 5-decene, 1,9-decadiene, and 1,5,9-decatriene), chlorinated alkanes (1-chlorodecane, 1,6-dichlorohexane, 1,10-DCD, 1-chlorohexadecane) and chlorine gas were purchased from Sigma-Aldrich Canada (Mississauga, Ont., Canada). All chemicals had purities ranging from 95 to 99.5%. Approximately 96% of the 1,5,9-decatriene was present as the *cis* isomer. A commercial mixture of chlorinated paraffins was supplied by PCI Chemicals (Cornwall, Ont., Canada), and contained 52% (Cereclor S52) chlorine, by weight. Sodium chloride and bromide salts, and dichloromethane (DCM, HPLC grade) were purchased from Fisher Scientific (Nepean, Ont., Canada). Tetrachloromethane (CCl_4, spectrophotometric grade) was purchased from J.T. Baker (Phillipsburg, NJ., USA).

Hydrocarbon syntheses

Alkenes can undergo substitution or addition reactions when in the presence of halogens [19]. The halogen is spontaneously added across the double bond when the reaction is carried out in DCM at ambient temperature (19–22°C) and pressure (1 atm) [19]. A competing reaction is the chlorine free-radical substitution of hydrogen atoms on the alkane chain. This undesirable side reaction can be minimized by carrying out the reaction in the absence of light and freshly distilled, peroxide-free solvent. Upon completion of the reaction, 1-decene, 5-decene, 1,9-decadiene, and 1,5,9-decatriene were expected to be transformed to 1,2-dichlorodecane (1,2-DCD), 5,6-dichlorodecane (5,6-DCD), 1,2,9,10-tetrachlorodecane (TCD), and 1,2,5,6,9,10-hexachlorodecane (HCD), respectively. The reaction apparatus consisted of a 250-ml round-bottom flask charged with 2 ml alkenes and 10 ml solvent. The reaction mixture was gently mixed with a magnetic stir bar. Chlorine gas was connected to the reaction vessel with Teflon tubing and was bubbled

through the solution at a rate of two to six bubbles per second. The extent of reaction was tracked by gas chromatography. Upon completion of the reaction, the clear reaction mixture turned yellow due to the presence of dissolved chlorine gas, which was subsequently stripped with nitrogen gas to avoid the undesirable free-radical chlorination reactions. The solvent was removed from the chlorinated alkane product by roto-evaporation. The structure of the synthesized chlorinated alkanes was determined by mass spectrophotometry (27:2:2 Atlanta, Calif., USA) in conjunction with gas chromatography (GC/MS, Thermo Finnigan, Austin, Tx., USA), high-resolution mass spectrometer (HR/MS, Kratos Analytical MS25RFA, UK) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR, 300 MHz Varian Mercury). Mass spectra were generated with a PolarisQ mass spectrometer quadrupole ion trap unit. The separation was carried out with a capillary column RTX-5MS (Restek, Bellefonte, Penn., USA, 30 m×0.25 mm ID, 0.25-μm film thickness). Both electron ionization (EI) and chemical ionization (CI, positive and negative) were used. In the latter technique, methane was the reagent gas. Other operating conditions were as follows: injector temperature (150 or 290°C), oven temperature (1 min at 60°C, ramped at 20°C min^{-1}, final temperature of either to 250 or 310°C), transfer line temperature (10°C above the final column oven temperature), and ion source temperature (250°C). When the high mass ions were desired, the ion source temperature was lowered to 120°C, and the trap offset was increased to between 3 and 10 V, as suggested in the literature [30].

The HR/MS was operated at 70°C, with direct sample insertion. The ion source temperature was 200°C. Two modes of ionization were applied in the HR/MS analysis: EI with electron energy of 70 eV and positive chemical ionization with NH_3 as the reagent gas. The mass range used was 35–1000 amu and the scan rate was 3 scan/decade. ^{1}H and ^{13}C NMR spectroscopy (300 MHz) were carried out at 25.0°C with samples dissolved in $CDCl_3$.

Culture and culture conditions

Three bacterial strains (*Pseudomonas* sp. strain 273, *Rhodococcus* sp. strain NCIMB 13064 and *Rhodococcus erythropolis* Y2) that are known to dehalogenate chlorinated alkanes were studied [2, 16, 33]. The bacterium *R. erythropolis* Y2 has been shown to posses two types of dehalogenases: a hydrolytic dehalogenase (halidohydrolase), induced by C_3–C_6 1-haloalkane substrates, and at least one oxygenase, induced by C_7–C_{16} 1-haloalkanes and *n*-alkanes [2]. Less is known about the dehalogenases associated with *Pseudomonas* sp. strain 273, although there are several reports on the ability of this bacterium to dehalogenate chlorinated alkanes [4, 33].

The three bacterial strains were screened for dehalogenating ability on solid media, with the most active retained for further study in liquid culture. Agar plates were prepared by adding agar at a concentration of 18 g l^{-1} to the base mineral medium. Screening tests were performed with and without the addition of yeast extract as a co-substrate, at a concentration of 0.5 g l^{-1}. After inoculation, the plates were sprayed uniformly with a solution of hydrocarbon source (1,10-dichlorodecane (1,10-DCD) or Cereclor S52) in ether (1:1, vol:vol). The solvent was evaporated from agar plates prior to incubation at 30°C. Positive growth was indicated by the formation of zones of clearing.

Biodegradation tests were carried out in 500-ml shake flasks containing 100 ml of mineral salt media. Per liter, the medium contained 2.12 g Na_2HPO_4, 1.36 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$ and 0.2 g $MgSO_4{\cdot}7H_2O$, 500 mg yeast extract and 1 ml of microelement solution [5]. In some cases, shake flasks were prepared without yeast extract in order to determine microbial growth on chlorinated alkane as sole carbon source. The microelements solution consisted of, per liter, 20 g NaOH, 10 g $MgSO_4{\cdot}7H_2O$, 4 g $ZnSO_4{\cdot}7H_2O$, 1 g $CuSO_4{\cdot}5H_2O$, 3.2 g $MnSO_4{\cdot}H_2O$, 20 g $Fe_2(SO_4)_3{\cdot}7H_2O$, 100 g Na_2SO_4, 1 g $NaMoO_4{\cdot}2H_2O$, 1 ml 96% H_2SO_4 and 120 g EDTA. Shake flasks were incubated at 30°C and agitated at 200 rpm. A volume of 50 or 100 μl of chlorinated hydrocarbons was added to the mineral medium. Previous studies have demonstrated the ability of *Pseudomonas* sp. strain 273 to dehalogenate 1,10-DCD [4, 33]. Therefore, all cells used for inoculation were taken from a starter culture, grown on 1,10-DCD and were considered to be in an induced state with respect to the dehalogenating enzymes [7]. Bacteria for inoculation were harvested in the mid- to late-exponential phase.

Cereclor S52, at concentrations of 0.5 and 1% v/v, was tested as the sole carbon source and in combination with dodecane or 1,10-DCD. The chloroalkane 1,10-DCD served as both a co-substrate for energy production and as an enzyme inducing agent. In media containing chlorinated alkane, 10 g of glass beads 3 mm in diameter (Fisher Scientific) were added to facilitate dispersion of the substrate [1]. In order to further increase the surface area of Cereclor S52, 0.5 g of the oil was mechanically emulsified with 10 ml of water using a homogenizer (Brinkman Instruments, Model 125-D, with a 3.5-cm PT45 generator) prior to addition to the mineral medium. All shake flasks were covered either with sterilized foam stoppers or rubber stoppers wrapped in aluminum foil. Abiotic controls were prepared and incubated under the same conditions as the inoculated flasks. Chloride release in the abiotic controls was subtracted from the measurements obtained from biological experiments. Biotic controls were also performed, in which a preferred substrate of *Pseudomonas* sp. strain 273, 1,10-DCD, was used to confirm the presence and activation of dehalogenating enzymes in the bacteria.

Hydrocarbon analysis

Chlorinated alkanes were analyzed on a Varian CP-3800 gas chromatograph equipped with a Supelco SPB-5 column (30 m×0.32 mm ID, 0.25 μm film thickness) and a

flame ionization detector. The carrier gas was UHP grade helium (Megs Specialty Gases, Montreal, Que., Canada) at a column flow of 3 ml min^{-1} and split ratio of 40:1. The temperature program for all hydrocarbon analyses was as follows: initial 100°C, hold for 1 min, ramp to 300°C at 15°C min^{-1}, hold for 5 min. The injector temperature was 300°C and the detector temperature was 320°C. Samples were prepared for injection by contacting the aqueous phase of a shake flask with a 50 ml of chloroform or dichloromethane containing 750 ppm 1,12-dichlorododecane (1,12-DCDD) as the internal standard. Where indicated, extraction efficiencies were determined using triplicate samples, which typically contained biomass at concentrations on the order of 0.4 g l^{-1}.

Analysis of chloride release

Dehalogenation of chlorinated compounds was quantified by measuring the chloride ion concentration in the growth medium using ion chromatography (IC). A Dionex DX-100 ion chromatograph (Dionex Canada, Oakville, Ont., Canada) equipped with an ion pack anion exchange AS 12A guard column (4 mm×50 mm), AS 12A column (4 mm×200 mm) and self-regenerating suppressor was used. Anion detection was performed by electrical conductivity. A solution of 2.7 mmol l^{-1} Na_2CO_3 and 0.3 mmol l^{-1} $NaHCO_3$ was used as the eluent, at a flow rate of 1.5 ml min^{-1}.

Biomass

Biomass was estimated optically, using a standard curve of absorbance at 560 nm versus dry weight of biomass. Absorbance measurements were done using a Bio 100 spectrophotometer (Varian). For the dry weight determination, a 30 ml sample of growth medium was centrifuged at 10,000× *g*. The pellet was then re-suspended in distilled water, transferred to an aluminum weigh tray, and dried at 105°C to constant weight. The standard curve was linear for absorbance values less than unity, with an adjusted r^2 value of 0.95. The biomass formed due to growth on yeast extract, when present, was determined independently, and was subtracted from all values in which hydrocarbons were present. Therefore, the biomass reported corresponds to that of the hydrocarbon only.

Results

Synthesis of hydrocarbons

The reaction time for complete chlorination of 1-decene, 5-decene, 1,9-decadiene, and 1,5,9-decatriene was 30–60 min, as indicated by the complete disappearance of the parent compound and the gradual formation of a new peak on the chromatogram (data not shown). The product of the chlorination reaction was identified using ^{1}H and ^{13}C NMR and HR/MS with direct sample insertion using both EI and positive CI. The purity of the chlorinated alkanes was estimated by gas chromatography.

The products of the chlorination of 1-decene, 5-decene, 1,9-decadiene were 1,2-dichlorodecane, 5,6-dichlorodecane, and 1,2,9,10-tetrachlorodecane, respectively, as verified by ^{13}C and ^{1}H NMR. The NMR spectral data are as follows (^{13}C at 75.4 MHz in $CDCl_3$, ^{1}H at 300 MHz in $CDCl_3$). 1,2-dichlorodecane: ^{13}C NMR δ 61, 48, 35, 31.5, 29.9, 29.8, 29.7, 26, 23, 14; ^{1}H NMR δ 4.05 (m, 1 H), 3.78 (dd, 1 H), 3.65 (dd, 1 H), 1.95 (m, 2 H), 1.70 (m, 2 H), 1.35 (m, 10 H), 0.90 (t, 3 H). 5,6-dichlorodecane: ^{13}C NMR δ 66, 35, 28.5, 21.5, 14.2; ^{1}H NMR δ 3.95 (m, 2 H), 2.0 (m, 2 H), 1.8 (m, 2 H), 1.6 (m, 2 H), 1.39 (m, 6 H), 0.90 (t, 6 H). 1,2,9,10-tetrachlorodecane: ^{13}C NMR δ 61, 48, 35, 29, 26; ^{1}H NMR δ 4.05 (m, 2 H), 3.78 (dd, 2 H), 3.65 (dd, 2 H), 1.95 (m, 2 H), 1.70 (m, 2 H), 1.4 (m, 8 H).

Chlorination of 1,5,9-decatriene gradually led to the formation of chromatographic peaks suspected to be associated with HCD. In order to verify the identity of the synthesized molecules, the reaction product mixture was analyzed on HR/MS. The CI (positive) and EI mass spectra of the unknown gave similar fragmentation patterns. In the CI mass spectra, a peak at *m*/*z* of 364 was observed, which corresponds to $[M+NH_4]^+$. The presence of the molecular peak at an *m*/*z* of 346, in conjunction with fragments associated with the loss of the six chlorine atoms (*m*/*z* of 311, 275, 239: base ion, 203, 167, 131), confirmed its identity. The two chlorine isotopes were also present in all high-molecular-weight fragments. The number and relative areas of the peaks in the gas chromatograms also supported the above analyses. Since the structure of the major product was confirmed by HR/MS, as described above, further investigation was not carried out.

Screening of biodegradation capacity

The bacterial strains selected for study have all been previously shown to posses the ability to dehalogenate haloalkanes [2, 22, 34]. Relative dehalogenating ability was assessed by screening on solid medium. After 3 days of incubation, only *Pseudomonas* sp. strain 273 demonstrated dehalogenating ability on all chlorinated substrates, both with and without yeast extract. *Rhodococcus* sp. strain NCIMB 13064 and *R. erythropolis* Y2 formed zones of clearing only when yeast extract was included as a co-substrate. The sole exception was *Rhodococcus* sp. NCIMB 13064, which demonstrated some ability to dehalogenate Cereclor S52 in the absence of yeast extract during the first 3 days.

After 10 days of incubation, growth was observed on all agar plates (with and without yeast extract) by all bacteria. However, as *Pseudomonas* sp. strain 273 showed the quickest adaptation and the largest zones of clearing on agar plates, this organism was chosen for further work. Negative and positive controls all behaved as expected, removing the physiological state of the organism and abiotic processes as factors. The possible

effect of the solvent (ether) on growth was eliminated using the appropriate control.

Biodegradation of chlorinated alkanes

Pseudomonas sp. strain 273 was tested with a variety of chlorinated alkanes (1-chlorodecane, 1-chlorohexadecane, 1,6-DCH, 1,10-DCD, 1,2-DCD, 5,6-DCD, TCD, HCD), differing in carbon backbone length, and position and degree of chlorination.

Monochloroalkanes

Initial attempts to degrade 1-chlorodecane resulted in the loss of 65% of the initial 1-chlorodecane added to the abiotic control flasks by volatilization. This problem was successfully addressed by sealing the flasks with rubber stoppers wrapped in aluminum foil, resulting in negligible abiotic loss of chlorinated hydrocarbons in subsequent experiments (up to 90% dechlorination in an 8-day incubation) and no substrate left in solution at the end of incubation period. Based on chloride release, complete dehalogenation of 100 µl of 1-chlorohexadecane was achieved in duplicate shake flasks after 22 days of incubation (103 and 100% dehalogenation). This was confirmed by the amount of residual substrate recovered in the spent broth at the end of the incubation period (0 and 2%). The abiotic control confirmed that none of the compound had been lost due to volatilization.

The dehalogenation rates of 1-chlorodecane and 1-chlorohexadecane were compared in experiments in which both monochlorinated alkanes were added to separate shake flasks on an equimolar chlorine basis (196 µl of 98% 1-chlorodecane, 302 µl of 95% 1-chlorohexadecane and 101 µl of 99% 1,10-DCD). Here, 1,10-DCD was included as a control. Duplicates were not performed in this series. Flasks were inoculated from the same source and were covered with impermeable stoppers. For all three compounds, over 97% of the chlorine contained in each substrate was recovered as chloride within 17 days of incubation (Fig. 1). In descending order, the average rates of dehalogenation over the first 4 days were: 1-chlorodecane (65 mg $Cl^- l^{-1}$ day^{-1}) > 1,10-DCD (27 mg $Cl^- l^{-1}$ day^{-1}) > 1-chlorohexadecane (25 mg $Cl^- l^{-1}$ day^{-1}). However, it is not clear whether the difference between the dehalogenation rates of the latter two compounds is significant.

Dichloroalkanes

There is some debate in the literature regarding the ability of the oxygenolytic dehalogenases to dehalogenate short-chain chlorinated hydrocarbons. Wischnak and coworkers [33] reported that the growth of *Pseudomonas* sp. strain 273 on short-chain α,ω-chlorinated hydrocarbons (e.g., C_5 and C_6) was inhibited when these compounds were present at concentrations above

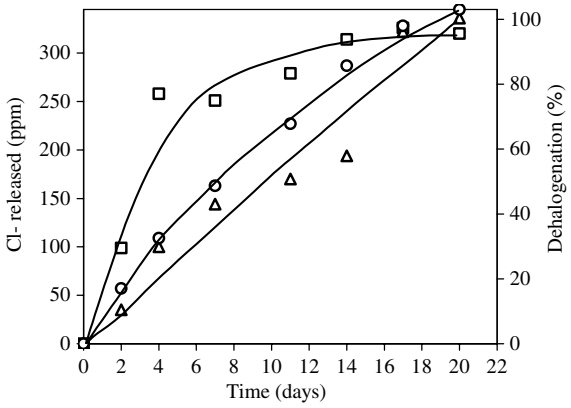

Fig. 1 Chloride release during biodegradation of the monochloroalkanes 1-chlorodecane (*open squares*), 1-chlorohexadecane (*open triangles*). The dichloroalkane 1,10-DCD (*open circles*) was included as a control

0.31 g l^{-1}. The dehalogenating ability of *Pseudomonas* sp. strain 273 on shorter chloroalkanes was tested by adding 1,6-DCH to a series of shake flasks in concentrations of 0.75 and 0.27 g l^{-1}. Less than 5% dehalogenation was achieved for both concentrations. In comparison, complete dehalogenation of 100 µl of 1,10-DCD was achieved in 5–17 days of incubation, depending upon the amount and physiological state of the innoculum used. No 1,10-DCD was detected in any shake flask at the end of the incubation period.

In order to evaluate the impact of intramolecular chlorine concentration, five shake flasks, containing 50 µl of 1,2-DCD as the sole carbon and energy source (no yeast extract) were inoculated and incubated. Two shake flasks were sampled every few days to monitor the chloride release and biomass formation. The remaining three flasks were analyzed only at the end of the incubation period. Possible co-metabolic degradation of 1,2-DCD was investigated by incubating duplicate flasks with 50 µl 1,2-DCD and 50 µl 1,10-DCD. Both abiotic (50 µl 1,2-DCD) and biotic (50 µl 1,10-DCD) controls were included in the series.

Over a 50-day incubation period, 51–56% of the chlorine associated with 1,2-DCD was released into the growth medium as chloride ions. Biomass levels tracked the increase in chloride levels until day 24, when the biomass reached stationary phase while chloride release continued (Fig. 2). Chloroform extraction and GC analysis of the flasks' contents showed that an estimated 2–10% of the chloroalkane was still present in the growth medium after 50 days. When the 1,2-DCD extract was analyzed by GC, a peak was observed on the chromatogram at a retention time (R_t) of 11.7 min. This peak was not present in samples of the starting oil. Upon subsequent injections of the same extraction solvent, the size of this peak decreased until it disappeared approximately 2 h after the chloroform extraction. Surprisingly, GC/MS analysis of fresh chloroform extract did not yield a peak at the expected retention time.

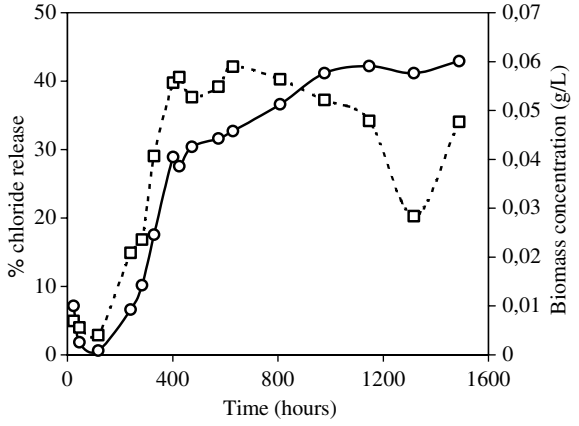

Fig. 2 Biomass formation and chloride release during the biodegradation of 1,2-DCD with *Pseudomonas* sp. strain 273. Chloride release (*open circles*) and biomass (*open squares*) are shown

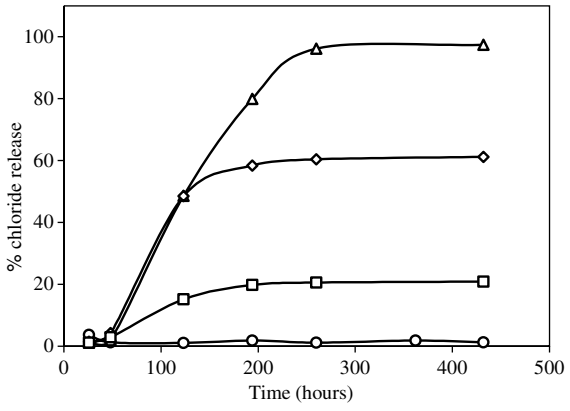

Fig. 3 Biological dehalogenation of 50 µl of pure 5.6-dichlorodecane (*open circles*), 50 µl pure 1,10-DCD (*open triangles*), a mixture containing 25 µl each of 5,6-dichlorodecane and 1,10-DCD (*open diamonds*), and a mixture containing 25 µl each of 1,2,9,10-TCD and 1,10-DCD (*open squares*)

Flasks incubated with a mixture of 1,10-DCD and 1,2-DCD showed 70 and 81% release of chloride over the same period. Chloroform extraction of the growth medium showed that negligible amounts (< 1%) of 1,10-DCD or 1,2-DCD remained. When 1,10-DCD was added as the sole substrate, it was completely dehalogenated in 10 days. In abiotic controls, 85–100% of 1,2-DCD was recovered upon extraction after 50 days.

A similar series of shake-flask experiments were performed with 5,6-DCD (90%) as substrate. Five flasks were incubated with 50 µl 5,6-DCD as sole source of carbon and energy, duplicate abiotic flasks were incubated with 50 µl 5,6-DCD, another set were incubated with a mixture of 25 µl 5,6-DCD and 25 µl 1,10-DCD, and two flasks were incubated with 50 µl 1,10-DCD, serving as positive control. Of the five flasks incubated with pure 5,6-DCD, no chloride release above that measured for the abiotic control (~1%) was measured after a period of 35 days (Fig. 3). Chloroform extraction and GC analysis of the growth medium resulted in the recovery of 5,6-DCD for two of the shake flasks in quantities similar to those extracted from the abiotic controls. However, the remaining three flasks showed no trace of 5,6-DCD although heterogeneous droplets and an oily substance on the surface of the growth medium was clearly visible. The extracts of one of the flasks with no 5,6-DCD remaining showed the presence of an unknown peak with an R_t of 11.7 min similar to that observed for the flask extracts of the 1,2-DCD degradation experiment. Again, subsequent injections of this chloroform extract showed the decrease of the peak size until it had disappeared after 2 h of extraction, indicating that the compound giving rise to the peak mentioned is unstable in chloroform.

For all five flasks, only a slight increase in biomass was observed (data not shown). Flasks with mixtures of 5,6-DCD and 1,10-DCD showed 57 and 61% total chloride release after 9 days with no further increase after 35 days (Fig. 3). This corresponds to complete dehalogenation of 1,10-DCD added with no or very little dehalogenation of 5,6-DCD. GC analysis of chloroform extracts of these flasks showed no trace of either 1,10-DCD or 5,6-DCD.

Polychlorinated alkanes

TCD is a viscous oil at room temperature and did not disperse well in the mineral salts growth medium. The addition of 50 µl of TCD to a shake flask resulted in the formation of three or four large droplets that would not break up into smaller droplets when shaken at 200 rpm. No chloride was released into the media after 35 days of incubation, while complete dehalogenation of 50 µl of 1,10-DCD was achieved after 11 days in the positive control. Since the lack of chloride release could be due to mass-transfer limitations rather than lack of enzymatic specificity, additional flasks with mixtures of 25 µl TCD and 25 µl 1,10-DCD were prepared. There are several reports that the bioavailability of a substrate can be increased by adding a solvent [11, 15, 23]. In these cases, the solvent increased the affinity of the cell for the organic/aqueous interface. This decreases the free cell density in suspension, while increasing bioavailability. Thus, in the current work, 1,10-DCD not only served as a solvent, but also as an inducer of the dehalogenating enzymes, and as a co-substrate. The addition of 1,10-DCD resulted in much better dispersion of TCD. However, after 9 days of incubation only 21% chloride release was measured with no additional chloride released after 35 days (Fig. 3). Chloroform extraction of the flask content resulted in the recovery of the undegraded TCD (~100%) and 1,10-DCD (60–85%). During the first 9 days of incubation, the size of the suspended oil droplets increased slightly. Biomass levels correlated strongly with the chloride release (data not shown).

HCD is a solid at room temperature, with a melting point above 40°C. Since the optimal incubation temperature for *Pseudomonas* sp. strain 273 is 30°C, dehalogenation experiments could not be performed using

pure HCD in the liquid state. Therefore, solid HCD was incubated at 30°C. No chloride was released into the media after 35 days. This was not surprising as it has been reported in the literature that hydrocarbons in the solid state do not support growth of *Pseudomonas* sp. strain 273 [33]. In order to increase its bioavailability, HCD was dissolved in 1,10-DCD and added as a solution at a ratio of 1:10 (vol:vol). This ratio was confirmed by GC. However, in spite of this, all of the chloride released into the media could be accounted for by the degradation of 1,10-DCD, suggesting that no appreciable dehalogenation of HCD was achieved after 40 days. Extraction of the contents of shake flasks confirmed the above hypothesis, as no 1,10-DCD was present at the end of incubation. The extraction efficiency was not determined in the case of HCD due to the limited amount of the material synthesized. Assuming 100% extraction efficiency, 83% of HCD was recovered. The chloride release and extent of biodegradation of the chlorinated alkanes are summarized in Table 1.

Attempts were made to degrade Cereclor S52 at concentrations of 0.5 and 1% (v/v), with and without co-substrates and enzyme inducing agents (dodecane and 1,10-DCD, added in separate experiments). All flasks contained glass beads. Chloride release was followed during 4 months of incubation on a weekly basis. The detected chloride release accounted for the dehalogenation of 1,10-DCD only. The final pH also suggested no Cereclor S52 dehalogenation. A pH of 6.7 was measured in flasks with only yeast extract as co-substrate. A pH of 6.2–6.3 was determined in flasks with 1,10-DCD and Cereclor and a pH of 6.5 where dodecane was added as co-substrate. When Cereclor was added in emulsified form (10 ml of water/oil emulsion containing 0.5 g of Cereclor S52), 27% of the total chlorine content was released after 30 days of incubation.

Discussion

The catabolism of PCAs requires that terminal chlorine atoms be removed so that the enzymes responsible for further breakdown can access the carbon backbone. Breakdown of the carbon chain through β-oxidation is very energetically favorable, while it is plausible that dechlorination is not. Therefore, it is possible that a critical degree of intramolecular chlorination exists beyond which mineralization is no longer energetically feasible. This hypothesis is plausible in a general sense, regardless of the specificity of the dehalogenating enzymes.

In order to explore this issue, it was necessary to calculate the Gibbs free energy of reaction associated with the relevant processes. Unfortunately, thermodynamic data on longer-chain chlorinated alkanes are sparse. Therefore, a group contribution method was used to estimate the free energy of formation for the substrates of interest [8]. For biological systems, the framework for this method was provided by Mavrovouniotis et al. [18], and later expanded upon to include chlorinated alkanes by Dolfing and Janssen [8]. The basic premise of the method is that any compound can be decomposed into functional groups, and each group assigned a free energy. The accuracy of the method is reported to be better than 20 kJ mol^{-1}, which is more than adequate for evaluating these compounds for their potential to serve as energy-yielding substrates.

In their work, Dolfing and Janssen [8] concluded that it was not possible to assign a single free energy to a chlorine atom in a molecule. Rather, the value depended upon the relative position of the halogen on the carbon backbone, and on the presence and location of other chlorine atoms. The estimates of free energies of formation associated with the compounds used in the current study are shown in Table 2. The values were extracted from the data of Dolfing and Janssen [8] using a regression of the available data, relating the group contribution to a number of molecular attributes. The identifying features used in the regression were the total number of chlorine atoms, the carbon chain length, the number of chlorine atoms on primary and secondary carbons, the number of di- and tri-chlorinated carbons, and the number of vicinal chlorine atoms. The resulting regression equation had an adjusted correlation coefficient (r^2) of 0.84. The estimates provided in Table 2 should be used with caution, as the compounds used to

Table 1 Summary of chloride release from chlorinated hydrocarbons during their biodegradation by *Pseudomonas* sp. strain 273

Substrate	Chloride released (%)	Incubation time (days)	Recovery of substrate after incubation
1-Chlorodecane	90	8	0%
1-Chloro hexadecane	102	22	1%
1,6-DCH	< 5	25	9–13%
1,10-DCD	100	5–17	0%
1,2-DCD	54	50	2–10%
1,2-DCD + 1,10-DCD (1:1)	76	50	< 1%
5,6-DCD	1	35	2 flasks ~100%, 3 flasks 0%
5,6-DCD + 1,10-DCD (1:1)	59	35	0%
1,2,9,10-TCD	0	35	100%
1,2,9,10-TCD and 1,10-DCD	21	35	100% TCD, 60–85% 1,10-DCD
HCD	0	35	> 89%
HCD + 1,10-DCD (1:10)	85	40	0% 1,10-DCD, > 83% HCD

Table 2 Gibbs free energies of formation for selected chlorinated alkanes. Free energies of reaction corresponding to Eq. 1 are also tabulated. All calculations considered the components in the aqueous phase, as described by Dolfing et al. [8] Free energies of formation for the chlorinated compounds were estimated as described in the text

Substrate	$\Delta G_f^{0\prime}$ (kJ/mol)	$\Delta G_R^{0\prime}$ (kJ/mol)
1-Chlorodecane	4	−6,445
1-Chlorohexadecane	45	−10,248
1,2-Dichlorodecane	−24	−4,421[a]
5,6-Dichlorodecane	−28	−6,347
1,10-Dichlorodecane	−31	−6,344
1,6-Dichlorohexane	−46	−3,822
1,2,5,6,9,10-Hexachlorodecane	−311	−5,801
1,2,9,10-Tetrachlorodecane	−114	−6,129

[a]Free energy of reaction associated with the conversion of 1,2-dichlorodecane to 3,4-dichlorobutyric acid based on Eq. 2

assign a group contribution to the chlorine atoms differ significantly in carbon chain length from those used by Dolfing and Janssen [8]. However, this error is expected to be small, as the primary effect in adding a chlorine atom to the molecule is the gross oxidation of the carbon. The intramolecular chemical environment of the chlorine atom has only a secondary role in defining the energy of formation.

As the system is aerobic, the maximum possible energy release is that associated with the reaction:

$$C_nH_{2n+2-m}Cl_m + \left(\frac{3n-m+1}{2}\right)O_2 + (m-1)H_2O \rightarrow nHCO_3^- + (m+n)H^+ + mCl^- \quad (1)$$

From the free energy estimates associated with this reaction, it is apparent that all compounds studied can potentially support growth (Table 2). While the average carbon in the molecule does become more oxidized as more chlorine is added, this effect is offset by the energy released in completely oxidizing the carbon to CO_2. This result does not change, even in the limiting case in which all hydrogen atoms have been replaced by chlorine [8]. Thus, the hypothesis of existence of a critical degree of chlorination beyond which the compound can no longer support growth is not substantiated. Based on the free energy estimates, the single largest determining factor seems to be the carbon chain length.

While Gibbs free energy provides the maximum amount of energy released, the actual amount available to the organism depends upon the biochemical pathways by which catabolism are carried out. Furthermore, rates of reaction cannot be inferred from free energy data. For instance, no degradation of HCD was observed, although this process is energetically favorable (Table 2). Thus, it appears as if the enzymes do not have an appreciable affinity for this compound, limiting the rate of reaction. For those compounds in which degradation was observed, assuming similar overall catabolic pathways, it is reasonable to expect similar metabolic efficiencies for the various chlorinated alkanes. Furthermore, if it is assumed that negative effects of these substrates on cell

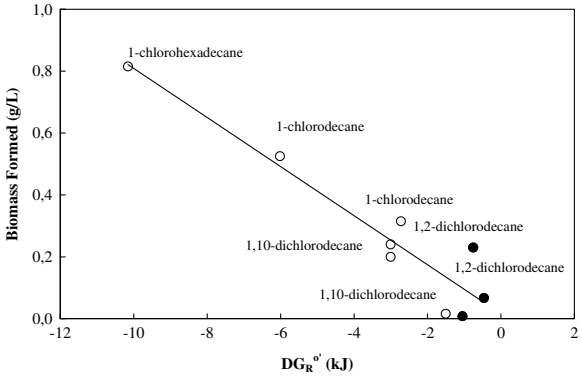

Fig. 4 Correlation between biomass formed and Gibbs free energy of release corresponding to Eq. 1. The free energy of reaction associated with the conversion of 1,2-dichlorodecane to 3,4-dichlorobutyric acid is also shown (*filled circles*). Gibbs free energy data were taken from Table 2

growth, if any, are similar, then it is expected that the Gibbs free energy of reaction should correlate with the amount of biomass produced. This approach has been demonstrated by others [3]. For the current study, the hypothesis was confirmed, as all data in which reliable biomass measurements were attained (1-chlorodecane, 1,2-DCD, 1,10-DCD, 1-chlorohexadecane) showed strong linearity when biomass was plotted against the free energy of reaction (Fig. 4).

It has been well established for insoluble substrates that direct contact between the cell and the organic phase is often required for growth [17]. In these cases, cell affinity and surface area of the organic phase are critical factors. Thus, when significant growth was not observed, the possibility that bioavailability or lack of enzyme induction was the limiting factor was addressed by emulsifying the chlorinated alkanes and by adding 1,10-DCD as a co-substrate. When applied to 1,2-DCD, 5,6-DCD, TCD and HCD, these actions had no effect on the observed outcome, with chloride release attributable to the degradation of the co-substrate. The rates of degradation were the same in the presence or absence of 1,10-DCD (Fig. 3). These results suggest that the limiting step in the degradation of the pure haloalkanes is the enzymatic dehalogenation. In the case of the commercial PCA mixture, however, increasing the surface area of oil through emulsification was required in order to achieve any degree of dehalogenation. This result has also been confirmed in studies in a batch bioreactor, establishing the influence of abiotic factors on growth (data not shown).

The focus of the current work was to elucidate some details pertaining to the specificity of the oxygenases for various chlorinated isomers. In order to assess the gross ability of the enzymes to act on the various substrates, overall yield data are easiest to interpret, as a time scale does not appear in this quantity. The data show that *Pseudomonas* sp. strain 273 can readily degrade monochlorinated alkanes in which the chlorine atom resides on the primary carbon. The amount of biomass

produced decreased with carbon chain length, in proportion to the estimated free energies of reaction (Fig. 4). Rates of conversion of the monochlorinated species seemed independent of chain length, suggesting that the active site interacts with a subunit less than ten carbon atoms in length.

Not all dichlorinated substrates were degraded to the same extent. The ease with which the organism handled 1,10-DCD, a preferred substrate, was not surprising. Due to its chain length, the two chlorine atoms are adequately separated so each one did not influence the other. Thus, the enzyme likely interacts with the compound in the same way as with its monochlorinated analogue. Still, it is possible that the location of the second carbon could change the affinity of the substrate for the active site. In fact, there is some weak evidence that the presence of the second chlorine actually enhances the interaction with the enzyme, as 1,10-DCD seemed to be degraded more quickly.

The molecule 1,6-dichlorohexane has the same intramolecular distribution as 1,10-DCD. However, only ~5% of the compound was degraded, suggesting that the degradation of shorter-chain chloroalkanes are problematic for *Pseudomonas* sp. strain 273 either as a result of inhibitory effects or lack of substrate affinity of the dehalogenase enzymes. It was not possible to obtain accurate biomass readings for these experiments, and thus the data could not be reconciled against the Gibbs free energy estimates. Lowering the amount of substrates added did not improve the yield, as it had in other studies [33].

When present in a vicinal arrangement, chloride release occurred to a much lesser extent or not at all. TCD and HCD with two and three pairs of vicinal chloride atoms were not degraded, even when expression, energy, and bioavailability issues were addressed through addition of 1,10-DCD, which acted as an inducing agent, co-substrate, and organic solvent. This result suggests that the vicinal carbons present on the ends of the molecule are not dehalogenated and therefore is not a suitable substrate for oxygenolytic dehalogenase enzymes. This hypothesis was supported by the fact that no chloride release was measured during the attempt to biodegrade 5,6-DCD. However, in three out of five flasks initially incubated with 5,6-DCD only, there was no trace of 5,6-DCD while an oily substance was clearly visible. This indicates that partial degradation of 5,6-DCD had occurred without the participation of the dehalogenase enzymes. The fact that no traces of 1,10-DCD or 5,6-DCD were detected upon chloroform extraction of flasks that were incubated with mixtures of those chlorinated alkanes supports this result. β-oxidation of 5,6-DCD was most likely responsible for this finding and proceeded until the vicinal chlorine atoms were encountered. Possible metabolic byproducts of this partial degradation process include 3,4-dichlorooctanoic acid, 3,4-dichlorooctanedioic acid, and 3,4-dichlorohexanedioic acid. Future studies involving HPLC are planned to identify the compounds.

The small amounts of biomass observed in all flasks with 5,6-DCD as sole carbon and energy source is likely the reason that two out of five flasks showed no degradation of 5,6-DCD. The fact that the chloride release results from mixtures of 5,6-DCD and 1,10-DCD is 60% rather than the expected 50% is like an artifact of the difficulty in accurately pipetting the viscous oil into the shake flasks. Also, it is likely that the estimated 10% impurities in the 5,6-DCD contain chlorinated isomers that can be dehalogenated by *Pseudomonas* sp. strain 273.

Growth on 1,2-DCD as a sole carbon and energy source was obtained in all cases. Chloride release increased asymptotically to approximately half (51–56% measured) of that available in the original oil. In flasks with mixtures of 1,2-DCD and 1,10-DCD, approximately three quarters (70–81% measured) chloride release was measured, which correspond to complete dehalogenation of 1,10-DCD and approximately 50% chloride release from 1,2-DCD. The fact that the actual results for chloride release are slightly higher is due to factors including pipetting error and chloride release due to impurities as mentioned above.

Based on the interpretation of the tetrachlorodecane and hexachlorodecane results, it is postulated that vicinal dehalogenation is problematic in the case of 1,2-DCD as well. Therefore, as suggested by the results of the 5,6-DCD degradation, β-oxidation likely commences at the unchlorinated end of the molecule. In this case, β-oxidation would continue until a 5,6-dichlorhexanoic acid or, more likely, 3,4-dichlorobutyric acid is produced. This compound could then spontaneously form a lactone, releasing chloride ions in the process. Analogous compounds, such as 4-chlorobutanoic acid and 4-hydroxybutanoic acid, are known to be unstable and spontaneously form lactones [7, 32]. Thus, the release of chloride resulting from the degradation of 1,2-DCD is due to an abiotic mechanism rather than the action of the dehalogenase enzyme. Also, the possibility of 3,4-dichlorobutyric acid as an end product of metabolism is not refuted by the Gibbs free energy analysis based on the reaction in Eq. (2), as shown in Fig. 4:

$$C_nH_{2n}Cl_2 + \left(\frac{3n-9}{2}\right)O_2 \rightarrow (n-4)HCO_3^- + (n-3)H^+ + Cl_2C_4H_5O_2^- + H_2O \quad (2)$$

It was postulated that the unknown peak seen in the chromatogram of the extracted content of the 1,2-DCD flask (R_t of 11 min) was associated with either the lactone or acid form of 4-hydroxy-3-chlorbutyric acid. However, due to the instability of this compound associated with this peak, it was not possible to identify it and this postulate remains speculative. Another possibility is that *Pseudomonas* sp. strain 273 has the ability to incorporate the chlorinate substrates or metabolic byproducts directly into the cell architecture, as has been shown with other organisms [15]. Again, future studies

involving HPLC are planned to identify the metabolic intermediate of this degradation process.

In order to draw a more comprehensive picture of the enzyme specificity associated with the dehalogenase of *Pseudomonas* sp. strain 273, degradation experiments with multiple monochlorinated secondary carbons are needed. The required substrates can be synthesized in a manner similar to that described in this study. For instance, as noted here, partial chlorination of 1,5,9-decatriene will lead mainly to addition across the internal double bond. Thus, through treatment with chlorine or hydrogen chloride, internally chlorinated substrates of interest can be synthesized. These activities are currently underway in our laboratory. Dehalogenation of gem chlorination of primary and secondary carbons is not of great interest for the case of PCA degradation. Although the free radical chlorination has low positional selectivity, a second chlorine does not readily substitute for hydrogen at a carbon already bound to a chlorine atom [28].

The work presented provides important information about the aerobic biodegradation and dehalogenation of PCAs by oxygenolytic enzymes. Firstly, enzymatic dehalogenation of PCAs with terminally substituted chlorine atoms must occur before β-oxidation can proceed. However, dehalogenation of PCAs is not a requirement for the initiation of β-oxidation of the hydrocarbon chain, provided the chlorine on the carbon chain is located some distance from the primary carbon atom. Thus, PCAs can be partially degraded, resulting in complex mixtures of chlorinated carboxylic acids as metabolic byproducts. Some of these can spontaneously release chloride. Future studies will show whether such acids are recalcitrant to further aerobic degradation and whether they have toxic effects on biological systems.

Secondly, PCAs with chlorine atoms substituted in a vicinal arrangement are not acted upon by the oxygenolytic dehalogenase enzymes produced by *Pseudomonas* sp. strain 273. Future studies will show whether this substrate specificity is general for all oxygenolytic enzymes or specific for the enzymes studied. Our group is currently working on a model that will allow for complete characterization of PCA mixtures. Once completed, the results regarding the substrate specificity of the dehalogenase enzymes will be incorporated into that model. This will provide insight into the extent to which the oxygenolytic enzymes can be expected to dehalogenate PCA mixtures and into the possible metabolic byproducts that are the likely results of the biodegradation process.

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