Biodegradation (2006) 17:535–544
DOI 10.1007/s10532-005-9024-8

Biodegradation kinetics of 2,4,6-Trichlorophenol by an acclimated mixed microbial culture under aerobic conditions

Christopher J.P. Snyder[1], M. Asghar[2], Jeno M. Scharer[3] & Raymond L. Legge[3,*]
[1]*Golder Associates Ltd., 2 180 Meadovale Boulevard, L5N 5S3, Mississauga, ON, Canada;* [2]*Department of Chemistry, University of Agriculture, Faisalabad, Pakistan;* [3]*Biochemical Engineering Group, Department of Chemical Engineering, University of Waterloo, N2L 3G1, Waterloo, ON, Canada (*author for correspondence: e-mail: rllegge@engmail.uwaterloo.ca)*

Accepted 27 September 2005

Key words: biodegradation, biomass, bioremediation, kinetics, mixed microbial culture, 2,4,6-trichlorophenol

Abstract

The objective of this study was to achieve a better quantitative understanding of the kinetics of 2,4,6-trichlorophenol (TCP) biodegradation by an acclimated mixed microbial culture. An aerobic mixed microbial culture, obtained from the aeration basin of the wastewater treatment plant, was acclimated in shake flasks utilizing various combinations of 2,4,6-TCP (25–100 mg l^{-1}), phenol (300 mg l^{-1}) and glycerol (2.5 mg l^{-1}) as substrates. Complete primary TCP degradation and a corresponding stoichiometric release of chloride ion were observed by HPLC and IEC analytical techniques, respectively. The acclimated cultures were then used as an inoculum for bench scale experiments in a 4 l stirred-tank reactor (STR) with 2,4,6-TCP as the sole carbon/energy (C/E) source. The phenol acclimated mixed microbial culture consisted of primarily Gram positive and negative rods and was capable of degrading 2,4,6-TCP completely. None of the predicted intermediate compounds were detected by gas chromatography in the cell cytoplasm or supernatant. Based on the disappearance of 2,4,6-TCP, degradation was well modelled by zero-order kinetics which was also consistent with the observed oxygen consumption. Biodegradation rates were compared for four operating conditions including two different initial 2,4,6-TCP concentrations and two different initial biomass concentrations. While the specific rate constant was not dependent on the initial 2,4,6-TCP concentration, it did depend on the initial biomass concentration (X_{init}). A lower biomass concentration gave a much higher zero-order specific degradation rate. This behaviour was attributed to a lower average biomass age or cell retention time (θ_x) for these cultures. The implications of this investigation are important for determining and predicting the potential risks associated with TCP, its degradation in the natural environment or the engineering implications for *ex situ* treatment of contaminated ground water or soil.

Introduction

The widespread production and industrial use of trichlorophenols (TCPs) as biocides in wood treatment, flame retardants (Atuanya et al. 2000), preservatives for leather and textile goods (Kharoune et al. 2002), solvents and as reagents in synthetic chemistry (Aranda et al. 2003) and their toxicity and persistence (Armenante et al. 1995) has led to their detection in soils, sediments, and natural water (Boyd et al. 1989; Chang et al. 1999; Gardin et al. 2001) as well as in food and in human urine (Crosby 1981). Aquatic environments may contain chlorophenols resulting from the Kraft bleaching process (Alexander & Scow 1989; Andreoni et al. 1998; Martinez et al. 2000) or

chlorination of water supplies for the purpose of disinfection (Ahlborg & Thunberg 1980).

The environmental fate of chlorophenols may include degradation by naturally occurring microorganisms (Aranda et al. 1999; Martinez et al. 2000). Anaerobic microorganisms are able to degrade chlorophenolic compounds but in anaerobic conditions, multichlorophenols are degraded by reductive dehalogenation into mono- or dichlorophenols which remain in the environment because of low degradation rates (Mohn & Kennedy 1992; Fahmy et al. 1994; Chang et al. 1999). These mono- and dichlorophenols can be degraded by aerobic catabolism through aerobic oxidation of the aromatic ring (Kafkewitz et al. 1992; Clément et al. 1995).

Most of the information available on biodegradation pathways is for pure cultures, even though mixed indigenous cultures are predominantly used for large-scale treatment (Boyd et al. 1989; Bae et al. 1997; Andreoni et al. 1998). Biodegradation by mixed microbial cultures has been treated as a "black box" with little information known or concern about the details of the actual processes. Some primary degradation rates have been reported for mixed cultures (Maltseva & Oriel 1997; Langwaldt et al. 1998; Dominguez et al. 2002; Aranda et al. 2003) while rates for the degradation of biodegradation intermediates (and potential rate-limiting steps in biodegradation) have not been well studied. Once the initial contaminant disappears from solution (primary degradation), the compound is assumed to be completely degraded and/or detoxified. This reasoning is typically followed in determining the fate of organics in waste treatment plants (Boyd et al. 1989) and in laboratory studies (Brock & Madigan 1991; Chakrabarty 1992). However, the possibility exists that only partial degradation occurs resulting in intermediates with higher toxicity than the original compound (Exon & Koller 1986). Slow biodegradation of intermediate products may also occur (Fahmy et al. 1994; Gardin et al. 2001). For the case of contaminated soil, the potential implications of this include producing a soil with a greater toxicity than the original or the reuse of only partially decontaminated soil that may take a significant additional time period to become decontaminated to an acceptable level, if at all.

In this study, 2,4,6-trichlorophenol (2,4,6-TCP) was chosen as a model contaminant. 2,4,6-TCP is a common constituent of wood preservative formulations (Chudoba et al. 1989; Aranda et al. 1999; Martinez et al. 2000) and is often found as a contaminant in soil (Dominguez et al. 2002). 2,4,6-TCP has a low Henry's law constant and as such undergoes minimal air stripping in forced aeration systems. 2,4,6-TCP is less recalcitrant than pentachlorophenol but more recalcitrant than phenol (as a model, this allows biodegradation studies of manageable time periods). Additional information through chloride ion analysis and appropriate material balances, can aid in understanding the biodegradation process.

Several different kinetic models have been proposed for the biodegradation of different chlorophenolics (Crosby 1981; Armenante et al. 1995; Dominguez et al. 2002; Chen et al. 2003). This information is essential for determining and predicting the potential risk that a compound carries during its exposure to, and degradation in the natural environment or the time and size of equipment required for *ex situ* treatment of contaminated ground water or soil. Primary degradation of the compound may not correlate well with the ultimate removal of the compound's environmental toxicity. Chloride ion release, coupled with other factors, may also be utilized to follow chlorophenolic degradation (Aranda et al. 2003).

In this study, the aim was to achieve a better quantitative understanding of the biodegradation of 2,4,6-TCP. This involved the study of 2,4,6-TCP's breakdown products, its related biodegradation pathway, and degradation rates for an acclimated (ability to completely degrade 2,4,6-TCP) mixed microbial culture. This information is used for the development of a mathematical model to describe the kinetics of 2,4,6-TCP biodegradation.

Materials and methods

Acclimation of mixed microbial culture

An aerobic mixed microbial culture was obtained from the aeration basin of the Waterloo Sewage Treatment Plant (Waterloo, ON). The mixed culture was first acclimated to phenol (300 mg l^{-1}) and glycerol (2 g l^{-1}) in a 20 l STR with a 12-l working volume for approximately a

four and a half month period. The phenol and glycerol acclimated culture was then used as an inoculum for shake flask experiments. Shake flask experiments utilized various combinations of 2,4,6-TCP, phenol, and glycerol as substrates. A series of five shake flask experiments were performed over approximately a 6 month period during which time complete primary TCP degradation and stoichiometric chloride ion release were observed. The shake flask cultures that had been acclimated to 2,4,6-TCP as the sole carbon/energy (C/E) source were then used as an inoculum for bench scale experiments in a 4 L stirred-tank reactor (STR) with a 2 l working volume and 2,4,6-TCP as the sole C/E source. Aseptic conditions were maintained only for preparation and sampling of control experiments during the shake flask studies.

Reactors

Two bioreactors were employed in this study. A 20 l STR (Marubishi type MSJ-20 L-1, Marubishi Lab Equipment, Tokyo, Japan) with a 12 l working volume was operated in a fed-batch mode using automatic pH control (New Brunswick Scientific Model pH-40, New Brunswick, NJ) with base addition (NaOH 0.5 mol l^{-1}) as required. Agitation was achieved using two 6-bladed disk turbine impellers (12 cm diameter, 230 rpm) and 4 internal baffles on the perimeter of the vessel. Air was sparged at a rate of 0.33 VVM.

The 4 l bioreactor was a Chemap® CMF 100 (Chemap AG, Volketswil, Switzerland) with a 2 l working volume. The reactor pH and dissolved oxygen concentration were continuously monitored by Ingold oxygen and pH electrodes. Agitation was achieved using one 6-bladed disk turbine impeller (48 mm diameter, 400 rpm) and two diametrically opposed baffles. The air sparging rate (~0.015 VVM) was low enough to observe a decrease in DO concentration during the biodegradation of 2,4,6-TCP. Temperature and agitation speed were continuously monitored with all probes and sensors connected to a CBC 10 data logger (Chemap AG, Volketswil, Switzerland). Data was also routed to a printer (Epson LX-850) and printed every 1, 4, or 12 min. No external pH control was used. The pH ranged from 7.40 to7.7 at the start and 6.9 to 7.6 at the end of the experiment. Antifoam control was not required.

Culture conditions

Shake flask experiments (in triplicates) were performed using either 0.075 l or 0.125 l working volumes in 0.25 l or 0.5 l Erlenmeyer flasks, respectively. Foam bungs were used as caps. The flasks were agitated at 150 rpm on a rotary orbital shaker (Eberbach Corp., Ann Arbour, MI) at room temperature. The composition of media was K_2HPO_4, 0.5 and $(NH_4)_2SO_4$, 0.667 g l^{-1}. Added to this was: (i) 25, 50, or 100 mg l^{-1} TCP (Sigma Chemical Co., St. Louis, MO) for TCP as sole C/E source or (ii) 50 mg l^{-1} TCP and 300 mg l^{-1} phenol or (iii) 50 mg l^{-1} TCP, 300 mg l^{-1} phenol, and 2.5 g l^{-1} glycerol.

A microbial inoculum from the 20 l STR was used for the first shake flask experiments and several subsequent shake flask experiments. Control flasks contained autoclaved media and TCP or autoclaved biomass. For the later shake flask experiments, the microbial inoculum was prepared by amalgamating all TCP degrading cultures from earlier experiments and centrifuging it in 2–250 ml centrifuge bottles for 10 min at 2000 × *g* (IEC Central-HN bench top centrifuge, rotor #268). The supernatant was discarded and the biomass pellet was re-suspended with 75 ml tap water and used as an inoculum (8.33 ml/flask).

The 20 l STR was operated in fed-batch mode at 23 °C. The mixed aerobic culture was recultured twice per week. During each reculture, one fourth of the culture was withdrawn and replaced with 3 l fresh medium containing 8.33 g l^{-1} glycerol (BDH Chemical, Toronto, ON), 2 g K_2HPO_4 (Mallinckrodt, Paris, KY), 2.67 g l^{-1} $(NH_4)_2SO_4$ and 1.33 g l^{-1} phenol (Mallinckrodt, Paris, KY) to bring the volume back to 12 l. The 4 l STR was operated in batch mode at 23 °C. During re-culture, the contents of the reactor were allowed to gravity settle and 1.5 l of supernatant was withdrawn and replaced with 1.4 l medium containing 0.71 g l^{-1} K_2HPO_4, 0.95 g l^{-1} $(NH_4)_2SO_4$ and 200 mg or 50 mg 2,4,6-TCP for a 100 mg l^{-1} or a 25 mg l^{-1} 2,4,6-TCP initial concentration (from a 600 mg l^{-1} 2,4,6-TCP solution in tap water). Tap water was added to bring to a final working volume to 2 l.

Analytical

Biomass determinations

Dry weight determinations were carried out by filtering 10 ml samples through a 0.2 μm pore size cellulose nitrate membrane filter (Sartorius, Canlab, Toronto, ON). Residues were then washed with 10 ml of deionized water and biomass transferred to tarred aluminium foil dishes and dried at 103 °C for 24 h or until constant weight. Mixed liquor volatile suspended solids (MLVSS) and mixed liquor suspended solids (MLSS) were determined by Standard Methods (Beltrame et al. 1982) using borosilicate microfibre glass filters (No. AP40 047 05 filters, Millipore, Bedford, MA).

Chemical analysis

Analyses of the supernatants and cell cytoplasm were performed using high pressure liquid chromatography (HPLC; column, reversible ODS II (Regis, Morton Grove, IL), 15 cm × 4.6 mm; gradient pump, LKB 2249 (Bromma, Sweden); integrator, Spectra Physics SP 4270 (San Jose, CA), attenuation, 8, chart recorder speed, 0.5 cm min^{-1}; UV detector, Gilson 116 (Middleton, WI), UV detector wavelength, 254 nm at 0.1 AUFS; dilutor, Gilson 401 (Villiers Le Bel, France); sample injector, Gilson 231 (Villiers Le Bel, France); eluent, (v/v) 50:50:0.1 acetonitrile: deionized distilled water: glacial acetic acid (Analytical Reagent, BDH, Toronto, ON) flowrate, 0.85 ml min^{-1}) and gas chromatography (GC; Hewlett Packard 5880A; column, J&W DB-5 fused silica capillary (Chromatographic Specialties, Brockville, ON), 30 m × 0.25 mm ID, 0.25 μm thick film (polymethyl (5% phenyl) siloxane); detector, flame ionization (300 °C); injection (275 °C), manual (1–2 μl), splitless (30 s), initial oven temperature (80 °C) for 1 min followed by ramping (rate of 10 °C min^{-1}) to final oven temperature (300 °C); integrator, Hewlett Packard 5880A Series, Level Three) for determination of phenol, 2,4,6-TCP, and potential intermediate biodegradation products.

Chloride ion analysis was performed by ion chromatography (IC; Dionex DX-300 series gradient pump; column, IonPac™ AS 11 (4 mm ID column); conductivity detector linked to a microcomputer equipped with Dionex AI-450 chromatography software). The eluent composition was 0.17 mM $NaHCO_3$ and 0.18 mM Na_2CO_3 in high purity water (>18 MΩ cm Resistivity) at a flow rate, 2.0 ml min^{-1}; conductivity detector temperature compensation, 1.7; regenerant solution, 0.025 N H_2SO_4.

Correction for TCP adsorption to biomass

The majority of the 4 l STR experiments and some of the later shake flask experiments utilized a procedure to correct for TCP adsorption to the biomass as follows. 1000 μl of the fermentation broth was transferred to a clean 1.5 ml polypropylene microcentrifuge tube using a micropipettor (Eppendorf Model 4810). 500 μl of acetonitrile (Omni-Solv® spectrophotometry grade, BDH, Toronto, ON) was added using a micropipettor to the microcentrifuge tube. Contents of the microcentrifuge tube were mixed using a Vortex Genie™ (Scientific Industries, Bohemia, NY) on maximum speed for 30 s. The tubes were centrifuged as above and stored at −4 °C until analysis. Acetonitrile was used as a solvent because all components of interest were soluble in it and it was a major component of the HPLC eluent.

Cell morphology

The morphology of representative samples from the fermentation broth of the 20 l STR, the shake flask TCP fed culture, and the 4 l bench scale STR were determined using scanning electron microscopy (SEM). The cells were prepared for SEM following the method described by Hayat (1989). A Hitachi model S-570 SEM was used at an accelerating voltage of 15 kV. Periodic examination of all three cultures by light microscopy was performed to observe possible changes in the microbial composition of the mixed culture.

Results

Primary 2,4,6-TCP degradation and chloride ion mass balance

Scanning electron microscopy performed on the acclimated 2,4,6-TCP degrading mixed culture showed the culture to consist almost exclusively of floc particles containing mostly rod shaped bacteria. The rod-shaped microflora comprised almost equal distribution of Gram positive and Gram negative bacteria. Representative results obtained in the 4 l batch STR experiments are shown in

Figure 1 indicating 2,4,6-TCP concentrations and actual and theoretical chloride (Cl^-) ion concentrations versus time at a MLVSS concentration of 120 mg l^{-1}. The actual Cl^- ion concentration was the concentration measured in the fermentation broth. The theoretical Cl^- ion concentration was the stoichiometric concentration calculated assuming complete dechlorination occurring with primary degradation of 2,4,6-TCP. Volatilization and abiotic loss of 2,4,6-TCP was ruled out based on control shake flask experiments. All data presented has been corrected for adsorption of 2,4,6-TCP to the biomass. Dechlorination of 2,4,6-TCP was observed to occur within 30 h. The pattern of actual chloride accumulation was comparable to the theoretical value although the chloride concentration on a theoretical basis was slightly higher.

Oxygen Uptake and pH

Air was supplied to the STR at a low rate allowing changes in the dissolved oxygen concentration (DO) to be followed during the biodegradation of 2,4,6-TCP. In most batch scale experiments, the start of the decrease in DO and the minimum value of DO corresponded with the start and the end of the primary degradation, respectively. An example of this trend is shown in Figure 2 where the decrease in DO (as % of saturation) is plotted against time (time scale arbitrary to correspond to the start of 2,4,6-TCP primary degradation) during 2,4,6-TCP biodegradation. This data is for the experiment shown in Figure 1. Based on linear regression ($R^2 = 0.9866$) of the DO data shown in Figure 2, oxygen uptake rates follow zero-order kinetics during primary 2,4,6-TCP degradation. Similar patterns were observed for pH; the pH started to decrease with the onset and the minimum value roughly corresponded to the end of primary 2,4,6-TCP degradation (data not shown).

Zero-order modelling of 2,4,6-TCP degradation kinetics

The linear portions of the 2,4,6-TCP degradation curves were modelled as zero-order kinetics. In Table 1 the effects of high and low initial 2,4,6-TCP concentrations and high and low MLVSS concentrations on zero-order specific TCP degradation rate constants are compared. Based on zero-order kinetics, 2,4,6-TCP concentrations as a function of time during two typical experiments are compared with the predicted 2,4,6-TCP concentrations in Figure 3.

Effect of biomass and 2,4,6-TCP concentration on kinetics

The zero-order specific biodegradation rates obtained for the four different operating conditions (Table 1) were compared to determine if the initial biomass or initial 2,4,6-TCP concentration had a significant effect on the observed specific biodegradation rates. For true zero-order biodegradation kinetics, the specific biodegradation rate should be

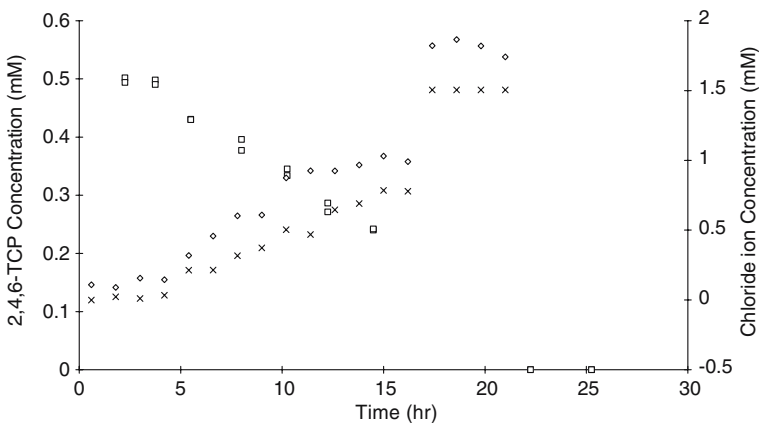

Figure 1. Degradation of 2,4,6-TCP by acclimated mixed microbial culture with an initial 2,4,6-TCP concentration of 100 mg l^{-1}. The mixed liquor volatile suspended solids concentration was 120 mg l^{-1}. □, 2,4,6-TCP; ◇, chloride ion (actual); ×, chloride ion (theoretical – assuming complete dechlorination occurred with primary degradation).

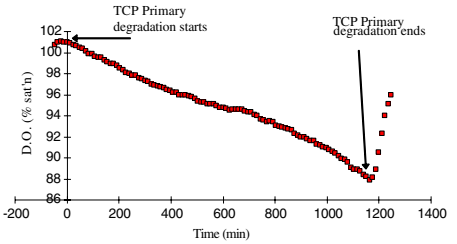

Figure 2. Dissolved oxygen concentration during degradation of 2,4,6-TCP by acclimated mixed microbial culture. The 2,4,6-TCP concentration was 100 mg l^{-1}. The mixed liquor volatile suspended solids concentration was 230 mg l^{-1}.

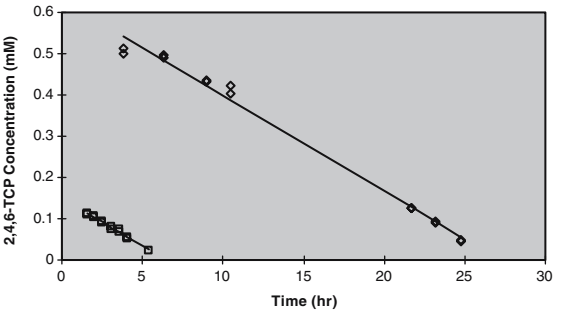

Figure 3. 2,4,6-TCP concentration during two typical experiments with predicted concentrations based on zero-order kinetics. □, mixed liquor volatile suspended solids concentration was 120 mg l^{-1}; ◇, mixed liquor volatile suspended solids concentration was 128 mg l^{-1}.

independent of both the initial limiting substrate concentration and the initial biomass concentration (X_{init}) with all else constant. Analysis of variance (ANOVA), at a 95% confidence level, showed that the zero-order specific degradation rate was not a function of initial 2,4,6-TCP concentration, further supporting zero-order kinetics. However, the observed zero-order specific degradation rate was found to be a function of X_{init}. The lower X_{init} gave higher (1.5–2 fold increase) zero-order specific degradation rates.

Search for intermediate compounds

There are several potential biodegradation intermediates that are dependent on the biodegradation pathway utilized by the mixed microbial culture (Fahmy et al. 1994; Gardin et al. 2001). These include products of dehalogenation before ring cleavage (2,4- and 2,6-dichlorophenol, 2- and 4-chlorophenol, phenol, and catechol) and dehalogenation after ring cleavage (3,5-dichlorocatechol). None of the above potential intermediate compounds were detected in the cell cytoplasm or supernatant based on HPLC analysis (data not shown). Concentrations of 3,5-dichlorocatechol (approximately 3 μg l^{-1}) were just above the detection limit in lysed cell pellets. Dichlorocatechol was not detected in the supernatant.

Discussion

The critical step in the degradation of chlorinated compounds is the cleavage of the carbon-chlorine

Table 1. Zero-order modelling of 2,4,6-TCP degradation by a mixed microbial culture from bench scale stirred-tank reactor experiments

Initial TCP Concentration (mg l^{-1})	Initial Biomass Concentration (mg l^{-1})	Zero-order Specific TCP Degradation Rate (mg TCP/(g MLVSS*H)[a]	Linear Regression (R^2) for Zero-order Fit
25	120	38.2	0.9887
25	120	40.5	0.9698
25	120	39.9	0.9854
100	120	37.4	0.9761
100	120	35.8	0.9905
100	120	28.7	0.9883
25	240	19.6	0.9847
25	240	20.9	0.9913
25	240	23.6	0.9438
100	240	21.4	0.9863
100	240	18.7	0.9672
100	240	24.3	0.9923

[a]MLVSS – mixed liquor volatile suspended solids.

bond (Dasappa & Loehr 1991; Mohn & Kennedy 1992; Fahmy et al. 1994). Two different catabolic pathways appear to have evolved for chlorophenol degradation. The first pathway involves dehalogenation after ring cleavage, common for mono-, di-, and some trichlorophenolics. The *ortho* ring cleavage of 3,5-dichlorocatechol (DCC) may be the rate-limiting step in the dehalogenation of 2,4,6-TCP after ring cleavage. It has been postulated (Ditzelmuller et al. 1989) that the ring cleavage of halocatechols is the crucial reaction step in haloaromatic degradation because of the inefficient *ortho* transformation of halocatechols by ordinary (non-chloroaromatic) ring-cleavage enzymes (Dorn & Knackmuss 1978; Kafkewitz et al. 1992; Clément et al. 1995) coupled with the formation of suicide metabolites from the *meta* pathway. 2,4,6-TCP may be biodegraded through a similar pathway (Ettala et al. 1992; Greenberg et al. 1992) as other chlorophenols.

The second pathway involves dehalogenation before ring cleavage, early in the degradation mechanism. This second approach is common for polychlorinated phenolics – penta-, tetra-, and some trichlorophenols (Mohn & Kennedy 1992; Fahmy et al. 1994; Gardin et al. 2001). As with most classification systems, there are exceptions, as with partial dechlorination of chlorophenols before ring cleavage.

After 2 months of acclimation experiments, the 2,4,6-TCP acclimated culture consistently degraded 0.5 mM 2,4,6-TCP in batch experiments. Chloride ion analysis performed on reactor broth samples during the primary degradation of 2,4,6-TCP allowed a mass balance on chlorine during the primary degradation of 2,4,6-TCP providing some insight into the mechanistic aspects of 2,4,6-TCP biodegradation. In most previous studies, chloride ion analysis was performed infrequently or only at the beginning and at the end of the biodegradation study period (Karns et al. 1983; Haggblom et al. 1989; Kelly et al. 1989; Liu & Pacepivicius 1990). The mass balance developed from chlorinated substrate analysis coupled with chloride ion analysis may show the degree of dechlorination as a function of time during primary chloro-organic biodegradation, giving some insight into the biodegradation mechanism (Lora et al. 2000). One example of such a system has been reported by Kelly et al. (1989) in which 2,4-D analysis was coupled with chloride ion analysis during mixed culture biodegradation studies. A stoichiometric quantity of chloride ion was released during 2,4-D biodegradation, unequivocally showing complete dechlorination of the compound without the accumulation of chlorinated intermediates. Togna et al. (1995) and Gardin et al. (2001) showed rapid dehalogenation by enriched anaerobic cultures with an optimum at a pH between 8 & 9 and 7.5 respectively.

Biodegradation of 2,4,6-TCP in this study followed zero-order kinetics. No significant accumulation of intracellular or extracellular chlorinated intermediates was observed. If significant accumulation of chlorinated intermediates had occurred, one would expect that the actual Cl^- ion concentration would have been lower than the theoretical Cl^- ion concentration at some time during 2,4,6-TCP biodegradation, reflecting incomplete dechlorination of intermediates. The results (see Figure 1) showed that the actual Cl^- ion concentration was usually higher than the theoretical Cl^- ion concentration and that this pattern was observed regardless of the initial starting biomass concentration. This would suggest that 2,4,6-TCP was immediately dechlorinated as soon as primary degradation or uptake had occurred without significant accumulation of chlorinated intermediates. Similar results have been reported for 2,4-DCP, 2,4-D and 2,4,6-TCP by Beltrame et al. (1982), Kharoune et al. (2002) and Aranda et al. (2003), respectively. This disappearance of 2,4,6-TCP via adsorption to the biomass, volatilization, and abiotic loss of 2,4,6-TCP could be ruled ruled-out on the basis of control shake flask experiments. Therefore, it appears that 2,4,6-TCP is rapidly dechlorinated upon uptake by the microbes. Based on linear regression of the DO data shown in Figure 2, oxygen concentration decreases linearly during primary 2,4,6-TCP degradation. Since complete dechlorination and the start and finish of increased oxygen uptake correspond directly to 2,4,6-TCP primary degradation, complete dechlorination and complete biodegradation appear to occur nearly simultaneously. The rate-limiting step in the kinetics of biodegradation of 2,4,6-TCP by a 2,4,6-TCP acclimated mixed culture may be the initial uptake of 2,4,6-TCP by the microbes which coincide with the initiation of primary degradation of 2,4,6-TCP.

The values of the correlation coefficient (R^2) shown in Table 1 confirm that the biodegradation

2,4,6-TCP by an acclimated mixed culture was adequately modelled by zero-order kinetics. No significant bias was observed in the residuals plots for the zero-order modelling. The data presented in Figure 3 indicate a good zero-order kinetics fit of 2,4,6-TCP degradation during batch experiments using a 2,4,6-TCP acclimated mixed microbial culture. The concentration of the 2,4,6-TCP utilizable by the microbes was low relative to the initial cell concentration so 2,4,6-TCP was probably utilized at near constant cell mass. The initial 2,4,6-TCP concentrations of 100 mg l^{-1} (0.5 mM) and 25 mg l^{-1} (0.125 mM) were probably much greater than the half saturation constant (K_s) for 2,4,6-TCP for this mixed culture. Chudoba et al. (1989) found $K_S = 0.6$ mg COD l^{-1} for the biodegradation of a feed concentration of 88.6 mg l^{-1} 2,4-DCP (2,4-DCP as 20% of total COD) in a completely mixed system using an acclimated mixed culture at constant volumetric loading with variable solids retention times. Similar operating conditions were used for the bench STR experiments. Due to the chemical similarity of 2,4-DCP and 2,4,6-TCP coupled with the possibility of similar biodegradation pathways similar values of K_s may be expected. Under these conditions (initial substrate concentration much greater than K_s and non-growing microbes) the biodegradation rate is essentially constant, zero-order (Crosby 1981; Dominguez et al. 2002). Kelly et al. (1989) also reported near zero order kinetics for the primary degradation of 1000 mg l^{-1} 2,4-DCP as the sole carbon and energy source in mixed culture experiments. Kharoune et al. (2002) observed zero order kinetics for degradation of 200–400mg l^{-1} 2,4,6-TCP by a mixed aerobic consortium. In contrast, Blades-Fillmore (1980) observed neither first nor zero-order biodegradation kinetics but kinetics related to 2,4,6-TCP concentration for 2,4,6-TCP degradation by river water/sediment microbes at an initial concentration of 50 μg l^{-1}. Nyholm et al. (1992) reported first order degradation kinetics of 20–1000 μg^{-1} 2,4,6-TCP as a secondary carbon source. Dasappa and Loehr (1991) and Chang et al. (1999) also reported first order kinetics, although the reaction order in first case seems closer to zero order, for 300–600 mg 2,4,6-TCP kg^{-1} in soil and its water soluble fraction.

Zero-order kinetics have also been observed when the nutrient that limits the growth of the active bacterial population is made available to the cells at a constant rate, but the rate does not fully meet the demand of the organisms. Uptake of chloroaromatics as the first step in the catabolic pathway is often ignored. The actual mechanism of uptake into the cell and its regulation can have a major effect on the observed biodegradation kinetics. Energy dependent transport systems for haloaromatic uptake by the bacterial cell have been suggested, but not until the 1990s has a carrier-mediated transport system for uptake of a haloaromatic been elucidated (Crosby 1981; Neilson 1990). If the limiting substrate is linked to an active transport system for transport across the cell membrane into the cell and this rate is slower (and constant) than the rate of substrate utilization within the cell, then zero-order kinetics may be observed (Crosby 1981; Martinez et al. 2000; Dominguez et al. 2002). This may have some merit for 2,4,6-TCP at the high concentration used in these experiments. As discussed earlier, the rate limiting step in 2,4,6-TCP degradation appears to be its uptake by the microbes. Chlorophenolics are inherently toxic molecules acting as inhibitors of oxidative phosphorylation. Oxidative phosphorylation in procaryotes occurs via electron transport components embedded in the plasma membrane of the cell (Brock & Madigan 1991). Microbes may have evolved or adapted to minimize the inhibitory effect of the high 2,4,6-TCP concentration by somehow controlling the transport of 2,4,6-TCP through the plasma membrane. If this controlled 2,4,6-TCP transport mechanism occurs at a near constant rate and is the rate limiting step in 2,4,6-TCP biodegradation, then zero-order kinetics would be observed.

The zero-order specific biodegradation rates obtained for the four different operating conditions given in Table 1 were compared to determine if the initial biomass or initial 2,4,6-TCP concentration had a significant effect on the observed specific biodegradation rates. For true zero-order biodegradation kinetics, the specific biodegradation rate is independent of both the initial limiting substrate and the initial biomass concentration (X_{init}) with all else constant. It was shown by statistical analysis (ANOVA) that the zero-order specific degradation rate was not a function of the initial 2,4,6-TCP concentration. However, the observed zero-order specific degradation rate was found to be a function of X_{init}. The lower X_{init} resulted in higher

zero-order specific degradation rate constants. This can be attributed to a lower average biomass age, also referred as the cell retention time (θ_x), in experiments where lower X_{init} occurred. This likely occurred because of the order in which the experiments were executed. All experiments using the higher X_{init} the viable biomass fraction was depleted by normal biological decay and by less frequent replacement of medium. The overall effect of these ''biomass growth'' experiments was to increase the θ_x during the experiments using the higher X_{init}. All experiments utilizing the lower X_{init}, the mass of cells withdrawn made up a greater fraction of the total biomass in the STR. These factors together were responsible for a lower θ_x and higher fraction of active cells.

Acknowledgements

This work was supported by a Strategic Grant from the Natural Science and Engineering Research Council of Canada (NSERC).

References

Ahlborg UG & Thunberg TM (1980) Chlorinated phenols: Occurrence, toxicity, metabolism, and environmental impact. In: CRC Critical Reviews in Toxicology, Vol. 7, Boca Raton, FL

Alexander M & Scow KM (1989) Kinetics of biodegradation in soil. In: Reactions and Movement of Organic Chemicals in Soils. Soil Science Society of America and American Society of Agronomy, Madison, WI, pp. 243–269

Andreoni V, Baggi M, Cavalca M, Zangrossi M & Bernasconi S (1998) Degradation of 2,4,6-trichlorophenol by a specialized organism and by indigenous soil microflora:bioaugmentation and self-remediability for soil restoration. Lett. Appl. Microbiol. 27: 86–92

Aranda C, Godoy F, Becerra J, Barra R & Martinez M (2003) Aerobic secondary utilization of a non-growth and inhibitory substrate 2,4,6-trichlorophenol by *Sphingopyxis chilensis* S37 and *Sphingopyxis*-like strain S32. Biodegradation 14: 265–274

Aranda C, Godoy F, González B, Homo J & Martínez M (1999) Effects of glucose and phenylalanine upon 2,4,6-trichlorophenol degradation by *Pseudomonas paucimobilis* S37 cells in a nogrowth state. Microbios 100: 73–82

Armenante PM, Shu H, Huang CR, Kung C & Kafkewitz D (1995) Kinetics of the sequential degradation of 2,4,6-trichlorophenol by an anaerobic microbial population. Biotechnol. Lett. 17: 663–668

Atuanya EI, Purohit HJ & Chakrabarti T (2000) Aerobic and anaerobic biodegradation of chlorophenols using UASB and ASG bioreactors. World J. Microbiol. Biotechnol. 16: 95–98

Bae HS, Lee JM & Lee ST (1997) Biodegradation of a mixture of 2,4,6-trichlorophenol and 4-chlorophenol and phenol by a defined mixed culture. J. Gen. Appl. Microbiol. 43: 97–103

Beltrame P, Beltrame PL, Carniti P & Pitea D (1982) Kinetics of biodegradation of mixtures containing 2,4-dichlorophenol in a continuous stirred reactor. Water Res. 16: 429–433

Blades-Fillmore LA (1980) The Biodegradation of 2,4,6-Trichlorophenol in the Delaware River Water and Sediments and in Urban Runoff. MASc Thesis, Rutgers University, New Brunswick, NJ

Boyd SA, Mikesell MD & Lee J (1989) Chlorophenols in soils. In: Reactions and Movement of Organic Chemicals in Soils. Soil Science Society of America and American Society of Agronomy, Madison, WI. pp. 209–229

Brock TD & Madigan MT (1991) The Biology of Microorganisms. 6th edition, Prentice Hall, Englewood Cliffs, NJ p. 110

Chakrabarty AM (1992) Bioremediation: How does the environment modulate microbial gene evolution?. In: Ladisch MR & Bose A (Eds)Harnessing Biotechnology for the 21st Century: Proceedings of the Ninth International Biotechnology Symposium and Exposition, August 19–21. Crystal City, VA, (pp. 422–426). American Chemical Society, Washington, DC

Chang BV, Chiang CW & Yuan SY (1999) Microbial dechlorination of 2,4,6-trichlorophenol in anaerobic sewage sludge. J. Environ. Sci. Health B34: 491–507

Chen Y, Zhang H, Chen H, Fu S & Zhang X (2003) Coupled anaerobic/aerobic biodegradation of 2,4,6-trichlorophenol. J. Environ. Sci. 15: 469–474

Chudoba J, Albokova J, Lentge B & Kummel R (1989) Biodegradation of 2,4-dichlorophenol by activated sludge microorganisms. Water Res. 23: 1439–1442

Clément P, Matus V, Càrdenas L & Gonzàlez B (1995) Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. FEMS Microbiol. Lett. 127: 51–55

Crosby DG (1981) Environmental chemistry of pentachlorophenol. Pure Appl. Chem. 53: 1051–1080

Dasappa SM & Loehr RC (1991) Toxicity reduction in contaminated soil bioremediation processes. Water Res. 25: 1121–1130

Ditzelmuller G, Loidl M & Streichsbier F (1989) Isolation and characterization of a 2,4-dichlorophenoxyacetic acid-degrading soil bacterium. Appl. Microbiol. Biotechnol. 31: 93–96

Dominguez VM, Correa J, Vidal G, Lopez A & Marttinez M (2002) 2,4,6-Trichlorophenol degradation by river sediments exposed to bleached kraft mill discharge. Bull. Environ. Contam. Toxicol. 69: 463–470

Dorn E & Knackmuss H-J (1978) Chemical structure and biodegradability of halogenated aromatic compounds-substituent effects on 1,2-dioxygenation of catechol. Biochem. J. 174: 85–94

Ettala M, Koskela J & Kiesila A (1992) Removal of chlorophenols in a municipal sewage treatment plant using activated sludge. Water Res. 26: 797–804

Exon JH & Koller LD (1986) Toxicity of 2-chlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol. Water Chlorination: Environ. Impact Health Effects 5: 307–330

Fahmy M, Heinzle E & Kut OM (1994) Treatment of bleaching effluents in aerobic/anaerobic fluidized biofilm systems. Water Sci. Technol. 24: 179–187

Gardin H, Lebeault JM & Pauss A (2001) Degradation of 2,4,6-trichlorophenol (2,4,6-TCP) by co-immobilization of anaerobic and aerobic microbial communities in an upflow reactor under air-limited conditions. Appl. Microbiol. Biotechnol. 56: 524–530

Greenberg AE, Clesceri LS & Eaton AD (1992) Standard Methods for the Examination of Water and Wastewater. In: Greenberg AE, Clesceri LS & Eaton AD (Eds), (pp. 2–57). 18th edition, American Public Health Association, Washington, DC

Haggblom MM, Janke D, Middeldorp PJM & Salkinoja-Salonen MS (1989) Hydroxylation and dechlorination of tetrachlorohydroquinone by *Rhodococcus sp*. strain CP-2 cell extracts. Appl. Environ. Microbiol. 55: 516–519

Hayat MA (1989) Principles and Techniques of Electron Microscopy. 3rd edition, MacMillan Press, Houndsmills, U.K p. 79

Kafkewitz D, Armenante PM, Lewandowski G & Kung CM (1992) Dehalogenation and mineralization of 2,4,6-trichlorophenol by the sequential activity of anaerobic and aerobic microbial communities. Biotechnol. Lett. 14: 143–148

Karns JS, Kilbane JJ, Duttagupta S & Chakrabarty AM (1983) Metabolism of halophenols by 2,4,5-trichlorophenoxyacetic acid-degrading *Pseudomonas cepacia*. Appl. Environ. Microbiol. 46: 1176–1181

Kelly MP, Hallberg KB & Tuovinen OH (1989) Biological degradation of 2,4-dichlorophenoxyacetic acid: chloride mass balance in stirred tank reactors. Appl. Environ. Microbiol. 55: 2717–2719

Kharoune L, Kharouni M & Lebeault JM (2002) Aerobic degradation of 2,4,6-trichlorophenol by a microbial consortium - selection and characterization of microbial consortium. Appl. Microbiol. Biotechnol. 59: 112–117

Langwaldt JH, Männistö MK, Wichmann R & Puhakka JA (1998) Simulation of *in situ* subsurface biodegradation of polychlorophenols in air-lift percolators. Appl. Microbiol. Biotechnol. 49: 663–668

Liu D & Pacepivicius G (1990) A systematic study of the aerobic and anaerobic biodegradation of 18 chlorophenols and 3 cresols. Toxicol. Assessment 5: 367–387

Lora PO, Sjölund M, Tracol C & Morvan C (2000) Adaptation of an inoculum to 2,4,6-trichlorophenol biodegradation in an activated-sludge bioreactor. Water Sci. Technol. 42: 179–183

Maltseva O & Oriel P (1997) Monitoring of alkaline 2,4,6-trichlorophenol degrading enrichment culture by DNA fingerprinting methods and isolation of the responsible organism, haloalkophilic *Nocardioides sp*. strain M6. Appl. Environ. Microbiol. 63: 4145–4149

Martinez M, Campos A, Garcia A & Gonzalez CL (2000) Marine bacteria tolerant to chlorophenol degradative bacteria isolated from river receiving pulp mill discharges. Toxicol. Environ. Chem. 77: 159–170

Mohn WW & Kennedy KJ (1992) Limited degradation of chlorophenols by anaerobic sludge granules. Appl. Environ. Microbiol. 58: 2131–2136

Neilson AH (1990) The biodegradation of halogenated organic compounds. J. Appl. Bacteriol. 69: 445–470

Nyholm N, Jacobsen BN, Pedersen BM, Poulsen O, Damborg A & Schultz B (1992) Removal of organic micropollutants at PPB levels in laboratory activated sludge reactors under various operating conditions: biodegradation. Water Res. 26: 339–353

Togna MT, Kafkewitz D & Armenante PM (1995) Rapid dehalogenation of 2,4,6-trichlorophenol at alkaline pH by an anaerobic enrichment culture. Lett. Appl. Microbiol. 20: 113–116