

## AN ABSTRACT OF THE THESIS OF

Sheryl L. Stuart for the degree of Doctor of Philosophy in Civil Engineering presented on October 28, 1996. Title: The Effect of Environmental Conditions on the Reductive Dechlorination of Pentachlorophenol by a Mixed, Methanogenic Culture.

Abstract approved: **Redacted for privacy**

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Reductive dechlorination is an energy-yielding process that some bacteria use to transform chlorinated aromatic compounds. Understanding the conditions under which microbially mediated reductive dechlorination occurs, and evaluating reaction rates are important for the design of some bioremediation strategies. In this research, pentachlorophenol-(PCP)-dependent, reductive dechlorination rate increases within a mixed, methanogenic culture were evaluated. Further, the related effects of H<sub>2</sub> concentration and redox potential on the reductive dechlorination of PCP were investigated.

In 6- to 12-day experiments, a computer-monitored/feed-back-controlled bioreactor was used to hold temperature, pH, acetate concentration, redox potential and H<sub>2</sub> concentration constant at desired levels while transformation of multiple PCP additions was monitored. Transformation of PCP yielded 3,4,5-trichlorophenol (3,4,5-TCP) via 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP). Below an initial PCP concentration of 0.5 μM, PCP was transformed to 3,4,5-TCP within three to six hours. Biomass concentration changes were small, and PCP and 2,3,4,5-TeCP transformations were modeled as pseudo-first order reactions. Growth of a bacterial subpopulation that used PCP and 2,3,4,5-TeCP as terminal electron acceptors was suggested by increases in pseudo-first order rate coefficients that were directly related to the amount of PCP transformed to 3,4,5-TCP, and were independent of the acetate consumed, overall biomass concentration changes, and experimental duration. An average rate coefficient

doubling time, assumed to be equivalent to a subpopulation doubling time, of 1.7 days (1.4 to 2.3 days) was estimated.

The addition of  $H_2$  sustained reductive dechlorination activity for a prolonged period without methanogenesis, and purging  $H_2$  from the reactor decreased rates of reductive dechlorination.  $H_2$  was identified as the primary electron donor for the reductive dechlorination reaction, and a theoretical 2:1,  $H_2$ :PCP stoichiometry was observed. However, increases in pseudo-first order rate coefficients were smaller during continuous addition of  $H_2$  than when  $H_2$  was endogenously supplied during acetoclastic methanogenesis. During periods of elevated apparent  $E_H$  to -0.1 V, methanogenesis stopped, the  $H_2$  concentration decreased, and transformation of PCP and 2,3,4,5-TeCP continued at progressively slower rates.  $H_2$  added while the apparent  $E_H$  was maintained at -0.1 V, caused reductive dechlorination rates to increase, suggesting that decreased transformation rates at elevated apparent  $E_H$  is caused by a deficiency of  $H_2$ .

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The Effect of Environmental Conditions on the Reductive Dechlorination of  
Pentachlorophenol by a Mixed, Methanogenic Culture

by

Sheryl L. Stuart

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 Sheryl L. Stuart, author

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# **The Effect of Environmental Conditions on the Reductive Dechlorination of Pentachlorophenol by a Mixed, Methanogenic Culture**

## **Introduction and Background**

This dissertation contains three manuscripts describing research conducted with a methanogenic enrichment culture to investigate factors affecting reductive dechlorination of pentachlorophenol (PCP). The culture originally was obtained from a municipal anaerobic digester and has been maintained with continuous acetate and PCP feed for approximately six years (74). In their first experiments, Nicholson et al. observed a PCP dechlorination pathway characterized by removal of chlorine atoms in the *ortho* positions, yielding 3,4,5-trichlorophenol (3,4,5-TCP) via 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) (74). After a six month acclimation period, they observed a variety of dechlorination pathways, including removal of chlorine atoms in the *ortho*, *meta*, and *para* positions, and PCP was dechlorinated to the level of di- and monochlorophenols (74). However, after approximately two years of continuous laboratory culture, only the initial pathway was observed; chlorine atoms were removed at the *ortho* positions, no dichlorophenols were detected, and 3,4,5-TCP accumulated (29). The reason for this apparent pathway shift was unknown, but was thought potentially to result from a change in environmental conditions. (Similar pathway shifts or loss of activity have been observed in other long-term incubations of cultures able to transform haloaromatic compounds (1, 4, 19, 25, 30, 43, 116).) In initial work with this culture, batch experiments were performed, with single additions of acetate and a chlorophenol. The experiments generally lasted less than two days, and no significant growth of the culture was observed, based on volatile suspended solids (VSS) measurements. However, fluctuations in environmental conditions, such as pH and redox potential, were unavoidable as acetate and chlorophenol concentrations changed (73). Such fluctuating conditions made changes in rates of chlorophenol transformation difficult to interpret.

The goal of my research was to assess the effect of environmental conditions, such as hydrogen concentration and redox potential, on the reductive dechlorination of PCP by the culture. This was done using a computer-monitored and feed-back controlled bioreactor system to control key environmental parameters, including temperature, pH, acetate concentration, redox potential, and head-space gas composition. With this system, changes in the reductive dechlorination pathway or rate of chlorophenol transformation could be evaluated under specific, controlled environmental conditions. I hypothesized that changes in environmental conditions could affect either the pathway through which PCP was transformed, or the rate of reductive dechlorination of PCP. Specifically, since reductive dechlorination of aromatic compounds is most often observed in highly reduced, methanogenic environments, I hypothesized that more reduced conditions, as indicated by a low apparent redox potential, or  $E_H$ , would enhance reductive dechlorination, and conversely conditions resulting in elevated apparent redox potentials would hinder reductive dechlorination. I also hypothesized that sulfide concentration could serve as a surrogate parameter for apparent  $E_H$  measurements. This hypothesis was disproven early in the research, and was not pursued further. However, testing the remaining hypotheses proved to be challenging and complex.

Due to the toxicity of PCP and its metabolites (2, 16, 36, 73, 87, 113), initial PCP concentrations greater than approximately 8  $\mu\text{M}$  resulted in total loss of culture activity. Therefore, to compare rates of PCP transformation at different environmental conditions, initial PCP concentrations generally were kept below 0.5  $\mu\text{M}$ . This enabled multiple PCP additions to be transformed under a variety of conditions in experiments lasting up to two weeks. The experimental approach was first to establish dechlorination pathways and rates at one set of conditions, then at a second set of conditions, and finally at the original set of conditions. In this way, transformation pathways and rates at two different sets of conditions could be compared.

Before the effect of environmental conditions on PCP transformation could be evaluated, the baseline behavior of the culture to repeated addition of PCP had to be established. Experiments were conducted in which multiple PCP additions were made

while the acetate concentration, pH, and apparent  $E_H$  were held constant, and acetate consumption, gas production, and VSS and chlorophenol concentrations were monitored. From these experiments it became apparent that the rate of PCP and 2,3,4,5-TeCP transformation tended to increase with the amount of PCP transformed to 3,4,5-TCP. This relationship was independent of the amount of acetate consumed, changes in the VSS concentration, and experimental duration, and suggested growth of a bacterial subpopulation capable of using PCP and 2,3,4,5-TeCP as electron acceptors in a growth-supporting process. Therefore, in evaluating effects of environmental conditions, the additional effect of growth of a reductively dechlorinating subpopulation on reductive dechlorination rates had to be considered. The first paper presents results from these experiments, including the first-order model used to represent chlorophenol progress curves, the relationship between rate increases and the amount of PCP transformed, and a method for determining reductive dechlorination activity doubling times.

After the baseline behavior of the culture had been established, investigation of the influence of  $H_2$  concentration and apparent  $E_H$  on both methanogenesis and reductive dechlorination of PCP could proceed. However, since  $H_2$  is a redox-active species which can affect the apparent  $E_H$ , differentiating between  $H_2$  effects and redox potential effects can be difficult. The second and third papers present results from experiments designed to isolate these related parameters. The second paper primarily establishes the importance of  $H_2$  in the reductive dechlorination of PCP and 2,3,4,5-TeCP by the culture, and the third paper concentrates on the effect of elevated apparent  $E_H$  on methanogenesis,  $H_2$  concentration, and rates of reductive dechlorination.

## Kinetic Evidence for Pentachlorophenol-dependent Growth of a Subpopulation in a Pentachlorophenol- and Acetate-fed, Methanogenic Culture

### SUMMARY

Reductive dechlorination of pentachlorophenol (PCP) by a PCP- and acetate-fed, methanogenic culture was evaluated in a computer-monitored/feed-back-controlled bioreactor. In 6- to 12-day experiments, environmental conditions such as pH, acetate concentration, temperature and redox potential were held constant while transformation of multiple PCP additions was monitored. PCP was reductively dechlorinated at the *ortho* position, yielding 3,4,5-trichlorophenol (3,4,5-TCP) via 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP). Below an initial PCP concentration of 0.5  $\mu\text{M}$ , PCP was completely transformed to 3,4,5-TCP within three to six h. Biomass concentration changes were small during this period, and PCP and 2,3,4,5-TeCP transformations were modeled as pseudo-first-order reactions. Increases in pseudo-first-order rate coefficients for PCP and 2,3,4,5-TeCP were directly related to the amount of PCP transformed to 3,4,5-TCP, suggesting enrichment of a PCP-catabolizing population. Moreover, rate coefficient increases were independent of the amount of acetate consumed, changes in the overall volatile suspended solids (VSS) concentration, and the experimental duration. When PCP was added to the reactor at increasingly shorter time intervals, an exponential addition pattern was achieved, and increases in pseudo-first-order rate coefficients were exponential. An average rate coefficient doubling time of 1.7 days (1.4 to 2.3 days) was estimated. While the VSS concentration of the culture increased 60% in an 8-day period, pseudo-first-order rate coefficients increased by a factor of approximately six. This large increase in transformation rate coefficients suggests growth of a bacterial subpopulation capable of using PCP and 2,3,4,5-TeCP as terminal electron acceptors.

## INTRODUCTION

Assessing growth of a bacterial subpopulation in a mixed, enrichment culture is a difficult problem for scientists and engineers interested in assessing bioremediation technologies. While individual species capable of carrying out a given transformation may be isolated, few tools are available for assessing growth of a particular subpopulation in mixed cultures. Bulk parameters such as volatile suspended solids (VSS), protein concentration, ATP concentration, optical density measurements and plate counts have been employed to assess overall growth of enrichment cultures. However, changes in a small fraction of the overall population are not detected by these gross measurements. In addition, growth characteristics of an isolated species in pure culture may differ substantially from growth in mixed culture or natural systems. For example, growth may be highly dependent on environmental conditions, the presence or absence of inhibitors, electron donors or electron acceptors, and complex relationships between syntrophic species.

Most probable number (MPN) techniques frequently have been employed to measure growth. These methods range from actual bacterial counts to implied population increases based on the the ability of serially diluted cultures to degrade a target compound (40, 86, 96). Success of these methods depends on providing suitable conditions for bacterial growth or compound transformation within the dilution vials and on having a sufficient number of replicates at the proper dilution to achieve a statistically significant response. In addition, if the target bacterial species is involved in a syntrophic relationship with other members of a consortium, then the “supporting bacteria” must be present in larger numbers than the target species to insure that the growth requirements of the target species are met after dilution. Bacteria with particularly fastidious growth requirements conceivably could be missed by an MPN method.

Numerous mathematical models, of varying complexity, have been proposed to evaluate growth based on the disappearance of a substrate (83, 84, 88-90, 93, 94, 106). The appropriateness and predictive capacity of these models appear to depend on the particular system being modeled. Relevant factors include substrate concentration, the

presence of more than one growth substrate, and the source of the inoculum. Many model formulations are based on Michaelis-Menten or Monod kinetics, and include a relatively large number of parameters. As demonstrated by Robinson (82, 83) and Suflita et al. (106), parameter estimates from models based on Monod kinetics may be highly correlated, and therefore unique parameter estimates may be difficult to obtain. Also, sizeable errors may be introduced if model assumptions regarding growth or substrate concentration are not satisfied.

In this study, an alternate method employing first-order kinetics, was used to assess changes in activity of an anaerobic mixed culture resulting from repeated exposure to a target compound. Pentachlorophenol (PCP), is toxic to the culture at concentrations above approximately 9  $\mu\text{M}$  (2.5 mg/L), but was chosen as a model target compound because of its presence as a contaminant in many soil and groundwater environments and for its potential to serve as an electron acceptor in anaerobic, growth yielding processes (18, 20, 22, 57, 61, 67). PCP was transformed by the culture via an *ortho* dechlorination pathway: 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) was formed transiently, and 3,4,5-trichlorophenol (3,4,5-TCP) accumulated. A computer-monitored and feed-back controlled, quasi-batch bioreactor was used to maintain constant environmental conditions, such as pH, acetate concentration and redox potential, while PCP was added incrementally to the culture. Initial PCP concentrations were kept within a first-order transformation range, and pseudo-first-order rate coefficients for both PCP and 2,3,4,5-TeCP transformation were determined. While the overall biomass concentration, as measured by volatile suspended solids (VSS), increased approximately 60% during experiments, pseudo-first-order rate coefficients for PCP transformation increased as much as an order of magnitude. Increases in pseudo-first-order rate coefficients were directly related to the amount of PCP transformed, suggesting growth of a reductively dechlorinating subpopulation. Changes in rate coefficients were used to estimate an “activity yield,” or the change in rate coefficient per  $\mu\text{M}$  of PCP transformed. In addition, in experiments in which pseudo-first-order rate coefficients increased exponentially, an “activity doubling time” was determined. This doubling time was

within the range of growth doubling times determined for pure cultures of bacteria using chloroaromatic compounds as electron acceptors.

## **METHODS AND MATERIALS**

### **Reactor system.**

A two-reactor system was used: a continuous-flow reactor for maintenance of the enrichment culture at steady-state conditions, and a second, smaller reactor for batch experiments in which conditions were manipulated. Both reactors were maintained at  $30 \pm 2$  °C. This two reactor approach helped ensure that all long-term batch experiments were begun with inocula grown under similar conditions. The continuous-flow reactor (10 L liquid volume) was constructed of Kimax beaded process pipe (6 in I.D.) with stainless steel, Teflon-lined flange fittings (Ace Glass Co.; Vineland, N.J.) which sealed aluminum top and bottom plates. The reactor lid contained ports for nutrient addition, liquid and headspace sampling, and gas venting. Feed solution was pumped with an FMI RP-G6 piston pump (Fluid Metering Inc.; Oyster Bay, N.Y.) from a refrigerated feed reservoir, through Teflon and glass tubing, to a point approximately 15 cm from the reactor bottom. Effluent was removed by gravity flow from 5 cm below the liquid surface. To provide mixing, an electric motor (Bodine Electric Co.; Chicago, IL) continuously rotated a glass shaft (60 rpm) with two, 45 cm<sup>2</sup> Teflon paddles, located at the end of the shaft and at the shaft's midpoint. Transfer from the continuous-flow reactor to the batch reactor was accomplished by siphon through the glass feed tube at the top of the reactor.

The 2.5 L batch reactor included the following features: 1) continuous monitoring of pH (Orion Ross; 81-01), sulfide (Orion; 94-16 BN), and redox potential (platinum) (Analytical Sensors, Inc.; OR100031 BN) electrodes at a controllable time interval, and 2) feedback-controlled pH maintenance coupled to acetate concentration maintenance. Although the redox potential and headspace composition of the culture could also be

controlled by the reactor system, these controls were not used in the experiments described here. All electrodes were referred to a single reference electrode (Orion double junction; 90-02 BN) to avoid problems caused by multiple reference electrodes. In the batch reactor, parameters such as acetate concentration, pH, and temperature could be held constant while multiple PCP additions were made over experimental periods lasting up to two weeks. Aliquots of PCP were sequentially added to the reactor in a number of patterns. A linear addition pattern was obtained by making PCP additions of approximately equal amounts equally spaced in time. An exponential pattern was achieved by adding PCP to the reactor soon after the prior PCP addition had been transformed to 2,3,4,5-TeCP. Thus, if PCP and 2,3,4,5-TeCP transformation rates increased, the rate of PCP addition also was increased.

### **Culture conditions.**

The culture used in this study was originally obtained from anaerobic digester sludge from the Corvallis, OR municipal wastewater treatment plant as described previously (74). The culture was maintained in a continuous-flow reactor with a 10 day hydraulic detention time for approximately six years. The reactor feed contained 91 mM acetate and  $3.5 (\pm 0.6) \mu\text{M}$  PCP along with a trace mineral and vitamin formulation based on a modification of medium developed by Owen et al. (77). The steady state biomass concentration was  $290 (\pm 60) \text{ mg/L}$  volatile suspended solids (VSS) over a period of three years (Appendix A).

Nutrients were provided in sufficient excess to ensure that they would not become limiting during long-term, semi-batch experiments. The trace mineral and vitamin composition of the medium, in mg/L, was as follows:  $\text{NaHCO}_3$ , 1050;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 225.5;  $\text{NH}_4\text{Cl}$ , 61.7;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1620.0;  $\text{KCl}$ , 170.5;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 18.0;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 27.0;  $\text{H}_3\text{BO}_3$ , 5.1;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 2.4;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 2.3;  $\text{ZnCl}_2$ , 1.9;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 18.4;  $(\text{NH}_4)_2\text{HPO}_4$ , 146.8; biotin, 0.036; folic acid, 0.036; pyridoxine hydrochloride, 0.180; riboflavin, 0.090; thiamin, 0.090; nicotinic acid, 0.090; pantothenic acid, 0.090;  $\text{B}_{12}$ ,

0.002; p-aminobenzoic acid, 0.090; and thiocetic acid, 0.090. In addition,  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  (500 mg) and  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$  (370 mg) were added manually to the continuous-flow reactor every other day. A 0.75 mM aqueous PCP solution with a pH of approximately 10 was manually injected into the batch reactor.

### **Analytical techniques.**

For chlorophenol and acetate analysis, liquid samples were withdrawn from the batch reactor in 3.5 to 5 mL quantities using a 5-mL ground glass syringe with a six inch, 18-gauge needle. (For solids determinations, 26 mL were withdrawn in a 30 mL syringe.) Reactor samples were filtered through Gelman type A/E glass fiber filters with a nominal 1  $\mu\text{m}$  pore size, and approximately the first 3 mL of filtrate were discarded. Total suspended solids (TSS) and volatile suspended solids were measured as described in Standard Methods for the Examination of Water and Wastewater (35).

Chlorophenol samples were prepared for gas chromatographic analysis using a modification of the acetylation and extraction method developed by Voss et al. and Perkins et al. (79, 111). Culture tubes with Teflon-lined screw caps were used to mix 100  $\mu\text{L}$  of sample with 0.5 mL of a solution containing 30.4 g/L  $\text{K}_2\text{CO}_3$  (Mallinckrodt; Paris, KY) and 250  $\mu\text{g/L}$  2,4,6-tribromophenol as internal standard (Aldrich Chemical Co.; Milwaukee, WI). Acetic anhydride (Mallinckrodt) (75  $\mu\text{L}$ ) was added and the tubes were capped and shaken on a wrist action shaker for 20 minutes. The acetylated products were then extracted into 1 mL of hexane during another 10 minutes of shaking. The hexane fraction was transferred to a 2 mL autosampler vial and sealed with a Viton septum and crimp-seal cap. Chlorophenols in the hexane fraction were analysed using a Hewlett-Packard model 5890 gas chromatograph with an electron capture detector. Splitless injection (1  $\mu\text{L}$ ) was performed by a Hewlett-Packard model 7673 autosampler. A 30-m DB5 column (0.32 mm I.D. with 0.25  $\mu\text{m}$  film thickness; J & W Scientific # 123-3032) was used for compound separation. The injector temperature was 250  $^\circ\text{C}$ , and the detector temperature was 320  $^\circ\text{C}$ . The initial oven temperature, 45 $^\circ\text{C}$ , was held for 2 min

followed by a 15 °C/min ramp to 100 °C. Following a second 5 °C/min ramp, the temperature was held at 215 °C for 3 min prior to a 30°C/min temperature increase to 245°C. This final temperature was held for 10 min. A column head pressure of 5 psi was maintained by the carrier gas, helium, at a total flow rate of 20 mL/min. The make-up gas was 95% argon/5% methane at a flow rate of 60 mL/min. Compounds were identified by comparison with retention times of authentic standards. The chlorophenol detection limit was approximately 0.01 µM for the more highly chlorinated compounds and 0.1 µM for compounds with 2 chlorines or less.

Acetate was analyzed using a Dionex 4000I ion chromatograph with a conductivity detector. An Ionpac<sup>®</sup> AS4A anion analysis column and AG4A guard column with anion suppression were used for separation. Samples were eluted with a 1.8 mM carbonate/1.7 mM bicarbonate eluant at a flow rate of 2 mL/min. Dilute sulfuric acid (13.6 mM) was used as regenerant. Samples were diluted in eluant (1:25) to avoid water interference with the acetate peak.

Headspace samples were obtained with 250 µL, 500 µL, or 1 mL Pressure-lok gas tight syringes (Dynatech Precision Sampling Corp.; Baton Rouge, LA). A Fisher model 25V gas partitioner was used to measure CO<sub>2</sub>, O<sub>2</sub>, N<sub>2</sub> and CH<sub>4</sub> in 100 µL headspace samples. Hydrogen was detected in headspace samples using a Hewlett Packard Model 5890 Series II gas partitioner with a thermal conductivity detector and argon carrier gas. A 4 ft. x 1/8 in. stainless column, packed with molecular sieve 13X 45/60 (Supelco, MR 58723) was used for separation. The hydrogen detection limit was approximately 16 ppm (vol./vol.) in a 1 mL sample.

## **Chemicals.**

Pentachlorophenol (99+%) was obtained from Aldrich Chemical Co.; 2,3,4,5-tetrachlorophenol (TeCP) and 3,4,5-trichlorophenol (TCP) (95+%) were obtained from Ultra Scientific (North Kingstown, RI).

## THEORY

### Model derivation.

When appropriate, (i.e. at low initial PCP concentrations) PCP transformation and 2,3,4,5-TeCP formation and transformation were modeled using a first-order model. Given two assumptions, 1) changes in active biomass or intracellular enzyme concentration are relatively small over the course of the reaction, and 2) the initial substrate concentration is well below the half-saturation constant, the model may be derived from a traditional Monod growth model.

$$(1) \quad \frac{dS}{dt} = -\frac{\mu_m X S}{Y(K_s + S)}$$

If  $X \approx X_o$  and  $S \ll K_s$ , then

$$(2) \quad \frac{dS}{dt} = -\frac{\mu_m X_o}{YK_s} S = -kS$$

Where

$S$  = substrate concentration,  $\mu\text{M}$

$X$  = biomass concentration,  $\text{mg/L}$

$X_o$  = the initial biomass concentration,  $\text{mg/L}$

$K_s$  = the half-saturation constant,  $\mu\text{M}$

$\mu_m$  = the maximum specific growth rate,  $\text{h}^{-1}$

$Y$  = growth yield,  $\text{mg VSS}/\mu\text{mol substrate}$

$\mu_m X_o / YK_s = k = \text{a pseudo-first-order rate constant, } \text{h}^{-1}$

Pseudo-first-order biotransformation rate constants for PCP and 2,3,4,5-TeCP ( $k_P$  and  $k_T$ , respectively) and initial PCP and 2,3,4,5-TeCP concentrations ( $P_o$  and  $T_o$ ) were estimated by simultaneous, nonlinear,  $\chi^2$  optimization of the integrated forms of two pseudo-first-order rate equations.

$$(3) \quad P = P_o \left( e^{-k_P t} \right)$$

$$(4) \quad T = \frac{k_P P_o}{k_T - k_P} [e^{-k_P t} - e^{-k_T t}] + T_o (e^{-k_T t})$$

Where

$P, T$  = PCP and 2,3,4,5-TeCP concentrations, respectively,  $\mu\text{M}$

$P_o, T_o$  = initial PCP and 2,3,4,5-TeCP concentrations,  $\mu\text{M}$

$k_P, k_T$  = pseudo-first-order rate coefficients for PCP and 2,3,4,5-TeCP,  $\text{h}^{-1}$

$t$  = time on the individual progress curve time scale, h

Equation 3 accounts for the first-order transformation of PCP to 2,3,4,5-TeCP and Equation 4 includes first-order production and consumption of the intermediate, 2,3,4,5-TeCP. A non-zero initial 2,3,4,5-TeCP concentration,  $T_o$ , was encountered when successive PCP additions were performed before all of the 2,3,4,5-TeCP was converted to 3,4,5-TCP.

The optimization program (Microsoft Excel 5.0; Solver) uses the generalized reduced gradient algorithm as described by Lasdon et al. (55). Confidence intervals (95%) for the individual rate parameters ( $k_P$ ,  $k_T$ , and  $P_o$ ) were obtained iteratively. A parameter value above or below the best fit value was arbitrarily selected, and the remaining parameter values were optimized by minimizing  $\chi^2$ , as above. The parameter of interest was then adjusted and the  $\chi^2$  minimization was repeated until a change in the optimum  $\chi^2$ , representing 95% confidence with one degree of freedom ( $\Delta\chi^2 = 3.814$ ), was obtained (81).

A first-order model also was used to describe the exponential pattern of cumulative PCP ( $\Sigma P$ ) addition and corresponding exponential increases in pseudo-first-order rate coefficients with time.

$$(5) \quad C = C_o (e^{a\tau})$$

Where

$C$  = value of parameter of interest ( $k_P$ ,  $k_T$ ,  $\Sigma P$ ) at time,  $\tau$ .

$C_o$  = value of parameter at time zero.

$a$  = exponential rate coefficient,  $\text{h}^{-1}$

$\tau$  = time on the overall experimental time scale, h

For exponentially increasing pseudo-first-order rate coefficients, Equation 5 becomes,

$$(6) \quad (k_n)_\tau = (k_n)_{\tau=0} e^{a\tau}$$

$$(7) \quad \left( \frac{\mu_m}{YK_s} \right)_n (X_{on})_\tau = \left( \frac{\mu_m}{YK_s} \right)_n (X_{on})_{\tau=0} e^{a\tau}, \text{ or}$$

$$(8) \quad (X_{on})_\tau = (X_{on})_{\tau=0} e^{a\tau}$$

Equation 8 is an equation for exponential growth. If the same population of bacteria is responsible for transforming both PCP and 2,3,4,5-TeCP, then the subscript,  $n$ , in Equation 8 may be dropped, since  $X_P = X_T = X$ . Increases in the concentration of enzyme(s) responsible for the chlorophenol transformations also may contribute to increases in pseudo-first-order rate coefficients. In this case, rather than being constant, the quotient  $\mu_m/YK_s$  would be a function of time.

A parameter doubling time,  $\tau_{double}$ , may be determined from Equations 5-8 as follows:

$$(9) \quad \frac{\ln 2}{a} = \tau_{double}$$

Since pseudo-first-order rate coefficients provided an indication of the culture's dechlorinating activity,  $\tau_{double}$  for  $k_P$  and  $k_T$  were defined as "activity" doubling times. If increases in activity resulted from growth of a bacterial subpopulation, then activity doubling times are equivalent to subpopulation doubling times.

### **Initial subpopulation concentration estimate.**

A number of bacterial species can obtain energy and grow by reductive dechlorination of chloroaromatic compounds. If growth at the expense of PCP transformation to 3,4,5-TCP is the process which sustains a reductively dechlorinating bacterial subpopulation within the continuous flow reactor, then a bacterial subpopulation concentration may be estimated based on an electron acceptor growth yield,  $Y_{EA}$ , and the hydraulic properties of the reactor. Table 1 presents the theoretical electron acceptor growth yield estimates for each two electron transfer (single chlorine removal), PCP to

2,3,4,5-TeCP, and 2,3,4,5-TeCP to 3,4,5-TCP, with hydrogen as the electron donor. The theoretical yields were estimated using the thermodynamic data of Dolfig and Harrison (23), and the yield estimation method of Heijnen et al. (41, 42) with hydrogen as the electron donor. Actual electron acceptor growth yields of several bacterial species capable of reductive dechlorination of haloaromatic compounds also are presented for comparison. The overall electron acceptor growth yield estimate for the conversion of PCP to 3,4,5-TCP is 11 g VSS/mol PCP converted, which appears to compare well with electron acceptor yields determined experimentally.

Using an electron acceptor yield estimate of 11 g VSS/mol PCP converted, and based on the feed and hydraulic conditions of the continuous flow reactor, the population of the dechlorinating organisms in the continuous flow reactor (and thus the initial population in the semi-batch experiments) was estimated at 0.02 mg VSS/L ( $\approx 7 \times 10^4$  cells/mL), or 0.008% of the total biomass.

Table 1. Theoretical and actual electron acceptor yields for some haloaromatic compounds.

Reaction	Organism	Yield (gVSS/mol electron acceptor)	Reference
PCP → 2,3,4,5 TeCP	theoretical	6 <sup>a</sup>	
2,3,4,5 TeCP → 3,4,5 TCP	theoretical	5 <sup>a</sup>	
3-chlorobenzoate → benzoate	<i>Desulfomonile tiedjei</i>	3-6 <sup>b</sup>	(67)
3-chlorobenzoate → benzoate	<i>D. tiedjei</i>	11 <sup>b</sup>	(22)
2-chlorophenol → phenol	2CP-1	5 <sup>b</sup>	(14)
3-Cl-4-OHPA <sup>d</sup> → 4-OHPA	<i>Desulfitobacterium hafniense</i>	≈17 <sup>b,c,e</sup>	(13)
3-Cl-4-OHPA <sup>d</sup> → 4-OHPA	<i>Desulfitobacterium dehalogenans</i>	23 <sup>e</sup> 4 <sup>f</sup>	(61)

<sup>a</sup> Estimated as stated in text.

<sup>b</sup> An estimated protein content of 60% was used to convert from protein yields to dry weight (VSS) yields.

<sup>c</sup> Estimated from graph of yield data.

<sup>d</sup> 3-chloro-4-hydroxyphenylacetic acid

<sup>e</sup> Measured with pyruvate as the electron donor.

<sup>f</sup> Measured with formate as the electron donor.

## RESULTS

### Transformation pathway, mass balance, and determination of pseudo-first-order rate coefficients.

PCP reductive dechlorination was evaluated in several experimental series during which temperature ( $30 \pm 2$  °C), acetate concentration (12-20 mM) and pH ( $7.0 \pm 0.1$ ) were externally controlled while the apparent  $E_H$  ( $-0.25 \pm 0.002$  V) and headspace gas composition ( $\text{CH}_4:\text{CO}_2:\text{H}_2 \approx 1.2:1:10^{-3}-10^{-4}$ ) remained at natural, steady values. Experiments typically were conducted over a 100-200 h time period, during which multiple PCP additions were made as described above. In this way, the changes in activity of a small bacterial subpopulation responsible for the reductive dechlorination of PCP could be assessed within a mixed culture environment. During periods of active acetate consumption/methanogenesis, changes in activity, as reflected by changes in pseudo-first-order rate coefficients, were directly related to the amount of PCP converted to 3,4,5-TCP. An “activity yield,” or the change in pseudo-first-order rate coefficient per  $\mu\text{M}$  of PCP converted to 3,4,5-TCP was determined. Also, for experiments in which the pseudo-first-order rate coefficients increased exponentially with time, “activity doubling times” were calculated.

Typical PCP transformation progress curves, shown in Figure 1, indicate that removal of PCP's *ortho* chlorines yielded 2,3,4,5-TeCP transiently and an accumulation of 3,4,5-TCP. Pseudo-first-order model curves for PCP and 2,3,4,5-TeCP also are shown. The initial PCP concentration for the addition shown in Figure 1 was approximately  $0.36 \mu\text{M}$ , while the 3,4,5-TCP concentration was nearly an order of magnitude higher as a result of previous PCP additions and 3,4,5-TCP transferred from the continuous flow reactor. The 3,4,5-TCP concentration in the effluent of the continuous flow reactor was approximately 74% (Standard deviation = 17%) of the influent PCP concentration, suggesting possible partial transformation of this compound. However, no other metabolites were detected in either the continuous flow reactor or during long-term batch experiments.

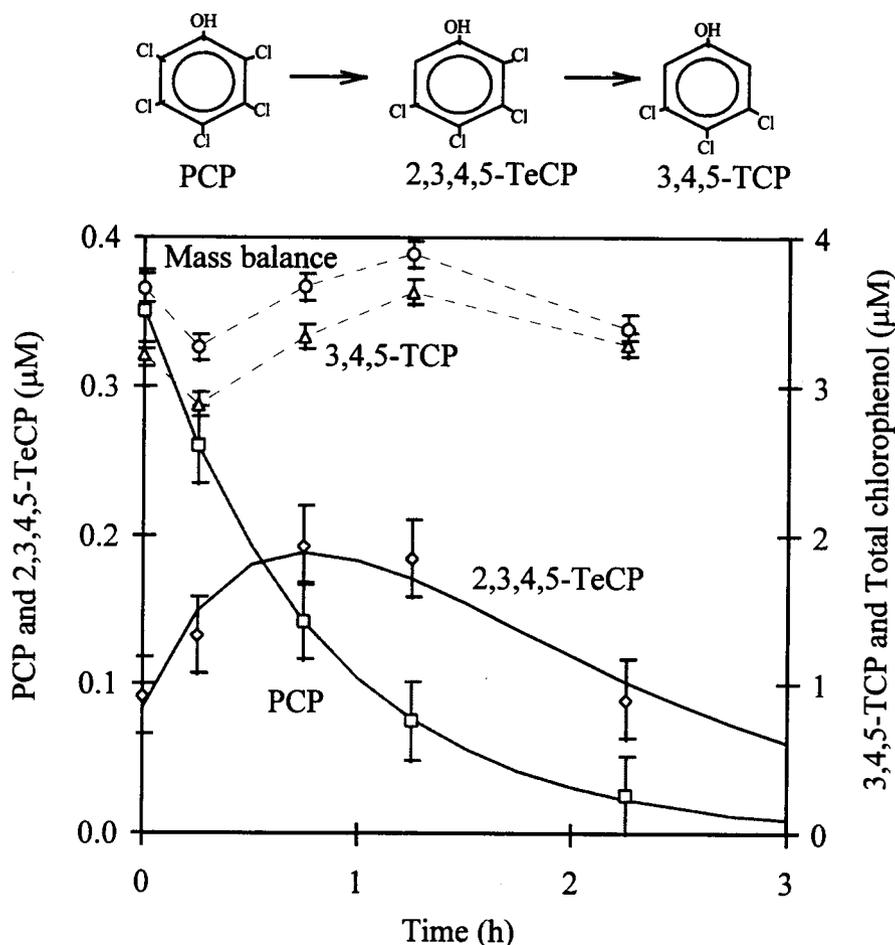


Figure 1. Observed pathway for the reductive dechlorination of PCP, and a typical PCP ( $\square$ ) transformation progress curve to 2,3,4,5-TeCP ( $\diamond$ ) and 3,4,5-TCP ( $\Delta$ ). Solid lines indicate pseudo-first-order model optimizations. The 3,4,5-TCP and chlorophenol mass balance ( $\circ$ ) is an order of magnitude higher due to accumulation from prior PCP additions. Error bars indicate 1 standard error.

Data from two, 150-h experiments in which PCP was added to the bioreactor multiple times, are shown in Figure 2. PCP additions were evenly spaced in time to form a linear pattern of cumulative addition in the experiment shown in Figure 2A, while cumulative PCP additions formed an exponential pattern in the experiment shown in Figure 2B. In both experiments, the pseudo-first-order rate coefficients for PCP transformation increased with sequential PCP additions, and the pattern of increase matched the pattern of PCP addition/transformation. In both experiments, acetate

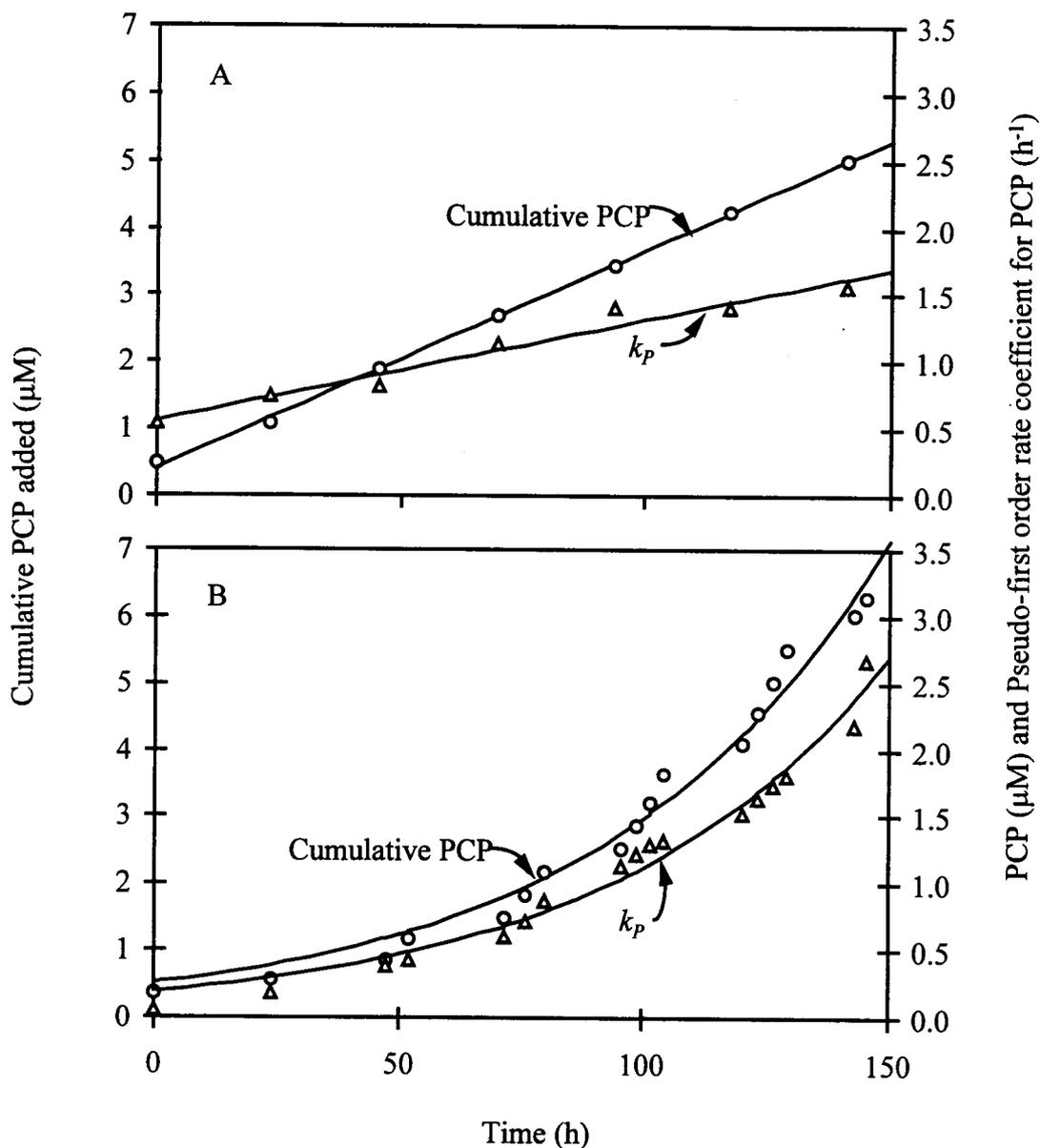


Figure 2. Two experiments in which aliquots of PCP were added to the reactor multiple times in both a linear pattern (A) and an exponential pattern (B) while environmental conditions were held constant. Pseudo-first-order rate coefficients ( $\Delta$ ), and the cumulative amount of PCP ( $\circ$ ) added to the reactor and transformed to 3,4,5-TCP are shown. Although some initial PCP concentrations approached  $0.8 \mu\text{M}$  in the experiment shown in Figure 2A, only data below about  $0.5 \mu\text{M}$  was used for rate coefficient determinations.

consumption was steady at an average rate of 2.7 mmol acetate/mgVSS-h, and the VSS concentration increased approximately 60% from the beginning to the end of the experiment. During the same period, the pseudo-first-order rate coefficient for PCP transformation increased by about 2.5 times in the experiment shown in Figure 2A, and by about 5.5 times in the experiment shown in Figure 2B. Clearly, neither the amount of acetate consumed nor the overall increase in biomass, as measured by VSS, could account for the increase in PCP transformation rate in either experiment.

### **Rate coefficients versus cumulative PCP added; "activity yield" calculation.**

The increase in pseudo-first-order transformation rate coefficient with PCP addition during periods of active acetate consumption/methanogenesis, shown in Figure 2, was a generally observed phenomenon. Figure 3 shows pseudo-first-order transformation rate constants for PCP (Figure 3A) and 2,3,4,5-TeCP (Figure 3B) regressed against cumulative PCP added to the bioreactor (in a variety of patterns) in ten different experiments. The data have not been adjusted for fluctuations in biomass concentration, as measured by VSS, or for minor changes in the influent PCP concentration to the continuous flow reactor, which may have influenced the initial population of dechlorinating bacteria transferred to the batch reactor. The best fit regression equations, shown as solid lines in Figure 3A and B, are presented in Table 2 along with the associated standard errors of the slope and intercept parameter estimates.

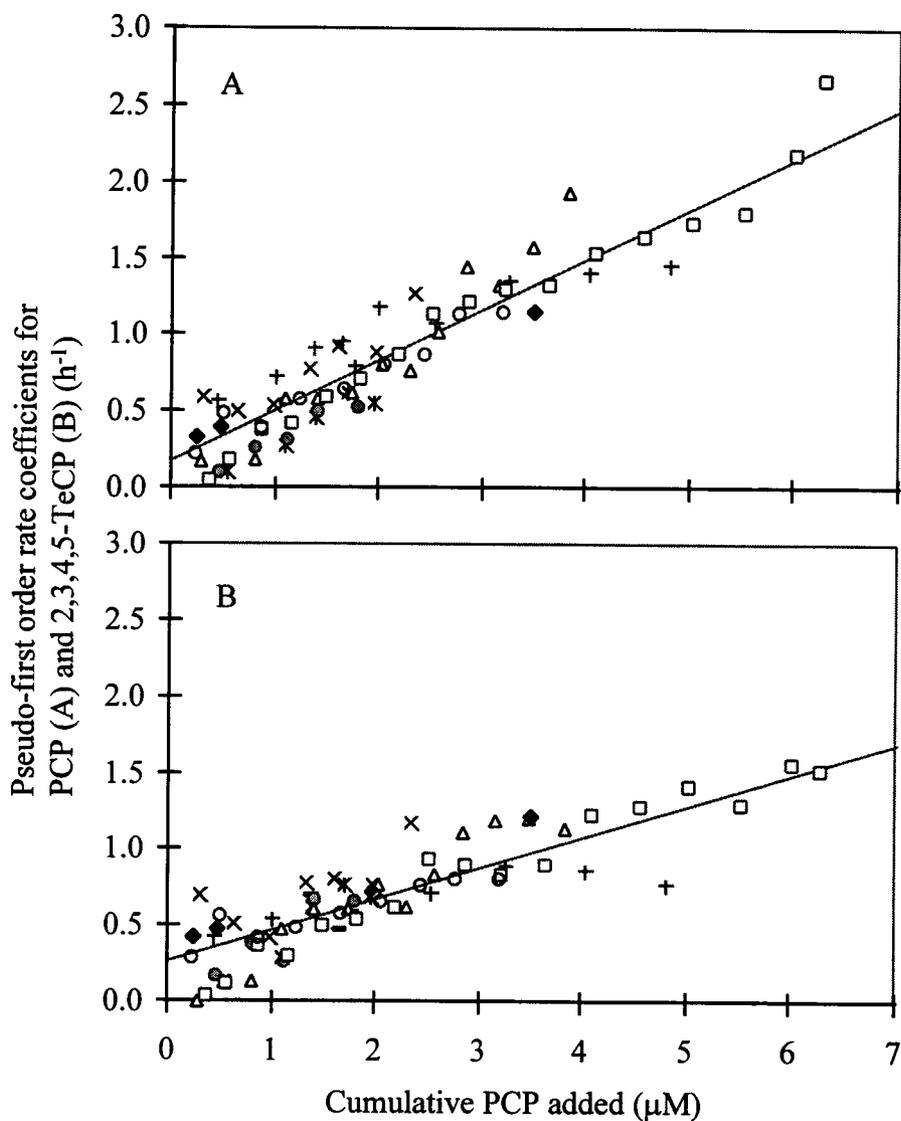


Figure 3. Pseudo-first-order rate coefficients for PCP (A) and 2,3,4,5-TeCP (B) versus the cumulative concentration of PCP added to the reactor. The slopes of the regression lines indicate “activity yields.” The different symbols correspond to ten individual experiments. Solid diamonds indicate pseudo-first-order rate coefficients of the first, second and fourth PCP additions from the experiment shown in Figure 4.

Table 2. Regression equations for PCP and 2,3,4,5-TeCP versus Cumulative PCP. Standard error for parameter estimates are given in parentheses.

PCP <sup>a</sup> :	Rate Coefficient, h <sup>-1</sup>	= 0.33(ΣPCP added, μM)	+ 0.17 h <sup>-1</sup>
		(0.013)	(0.036)
2,3,4,5-TeCP <sup>a</sup> :	Rate Coefficient, h <sup>-1</sup>	= 0.20(ΣPCP added, μM)	+ 0.26 h <sup>-1</sup>
		(0.012)	(0.034)

<sup>a</sup>The degrees of freedom for each regression was 69.

The slopes of these regressions represent the changes in activity resulting from conversion of PCP to 3,4,5-TCP or, “activity” yields. The change in pseudo-first-order rate coefficient for PCP per μM of PCP converted to 3,4,5-TCP was 0.33 (h)<sup>-1</sup>(μM PCP)<sup>-1</sup>, with a 95% confidence interval of 0.31 to 0.35 (h)<sup>-1</sup>(μM PCP)<sup>-1</sup>. The change in pseudo-first-order rate coefficient for 2,3,4,5-TeCP per μM of PCP converted was 0.20 (h)<sup>-1</sup>(μM PCP)<sup>-1</sup>, with a 95% confidence interval of 0.18 to 0.22 (h)<sup>-1</sup>(μM PCP)<sup>-1</sup>.

The activity yield relationship shown in Figure 3 was determined using data from two types of experiments. In the first, initial PCP concentrations were kept relatively low (Figure 2), and in the second, a single high concentration PCP addition was substituted for a number of lower concentration additions. By adding a large concentration of PCP rather than smaller concentrations spaced in time, a higher cumulative PCP concentration was transformed over a shorter time interval in the second type of experiment than in the first type. Figure 4 shows chlorophenol progress curves from an experiment with four PCP additions. The initial PCP concentrations of the first, second, and fourth additions were within the first-order concentration range, while the initial PCP concentration of the third addition was nearly ten times higher, thus precluding determination of pseudo-first-order constants for addition three. The pseudo-first-order rate coefficients determined for

the first, second and fourth PCP additions, shown as solid diamonds in Figure 3, are in good agreement with the linear regression relationships.

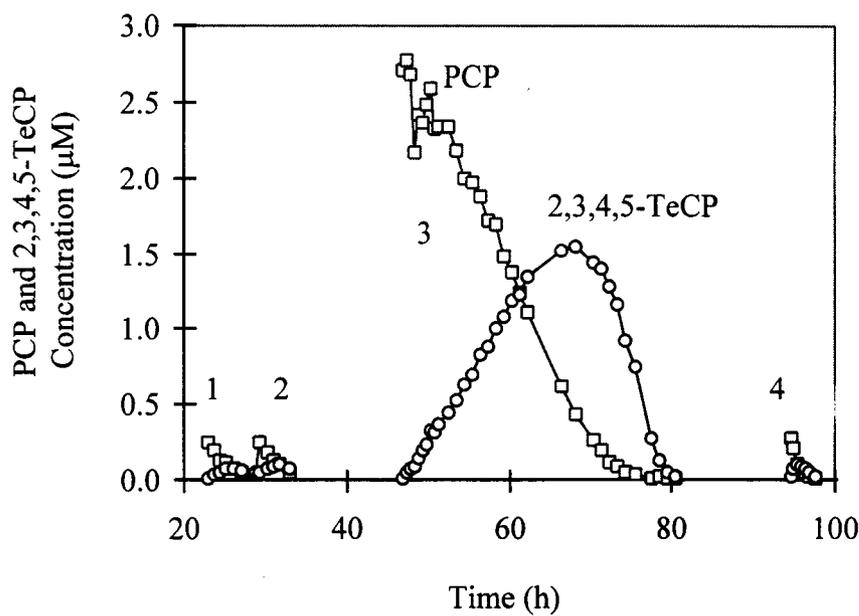


Figure 4. PCP (□) and 2,3,4,5-TeCP (○) progress curves from an experiment in which four PCP additions were made: the initial PCP concentrations of the first, second, and fourth additions were within the first-order concentration range, while the initial PCP concentration of the third addition was nearly ten times higher.

By 95 h, 3.2  $\mu\text{M}$  of PCP had been transformed, and the pseudo-first-order rate coefficient for PCP was  $1.2 \text{ h}^{-1}$  (95% CI =  $0.9\text{-}1.4 \text{ h}^{-1}$ ). In contrast, by 95 h in the experiment shown in Figure 2B, 1.2  $\mu\text{M}$  of PCP had been transformed, and the pseudo-first-order rate coefficient for PCP was only  $0.6 \text{ h}^{-1}$  (95% CI =  $0.5\text{-}0.8 \text{ h}^{-1}$ ). Therefore, changes in pseudo-first-order rate coefficient were not merely a function of experimental duration, but depended on the cumulative amount of PCP transformed to 3,4,5-TCP.

### Exponential additions yield exponential increase in $k_p$ and $k_T$ .

An “activity doubling time,” or the time required for the pseudo-first-order rate coefficients to increase by a factor of two, was estimated from four experiments in which PCP was added incrementally to the reactor in an exponential pattern. PCP was added at concentrations which were completely transformed to 3,4,5-TCP within a period of three to six h. Therefore, over the larger 100+ h time scale of the experiments, the pattern of PCP addition corresponded to the pattern of PCP transformation to 3,4,5-TCP.

As shown in Equations 6-8 above, growth of a reductively dechlorinating subpopulation may be responsible for increases in pseudo-first-order rate coefficients with time. Each dechlorination reaction may or may not be carried out by the same subpopulation. If a common subpopulation ( $X_p = X_T = X$ ) is responsible for the transformation of both PCP and 2,3,4,5-TeCP, then optimization of the individual exponential curves for the pseudo-first-order rate coefficients ( $k_p$  and  $k_T$ ) should yield a common exponential coefficient,  $a$ . Further, since increases in pseudo-first-order rate constant appeared to be directly linked to PCP conversion (as shown in Figure 3 above), then optimization of the cumulative PCP curve should also provide the same exponential rate coefficient.

Cumulative PCP, and pseudo-first-order rate coefficient data, and exponential model curves from one experiment are shown in Figure 5. Error bars indicate 95% confidence intervals for the individual parameters obtained from progress curves such as shown in Figure 1. The time axes are offset to avoid overlap of data. The model curves presented in Figure 5 share a common exponential rate coefficient,  $a$ , but initial values of each parameter ( $k_p$ ,  $k_T$ ,  $\Sigma P$  at  $\tau = 0$ ) vary.

Estimates of the exponential rate coefficient,  $a$ , obtained from optimizing Equation 5 for each of the  $k_p$ ,  $k_T$ , and  $\Sigma P$  data sets from four experiments are presented in Table 3. Analysis of variance was used to evaluate whether differences in the means of the rate coefficients obtained from the separate data sets were statistically significant.

Evidence for the existence of different means was suggestive but inconclusive (F-statistic = 4.2 with 2 and 9 degrees of freedom; p-value = 0.052.).

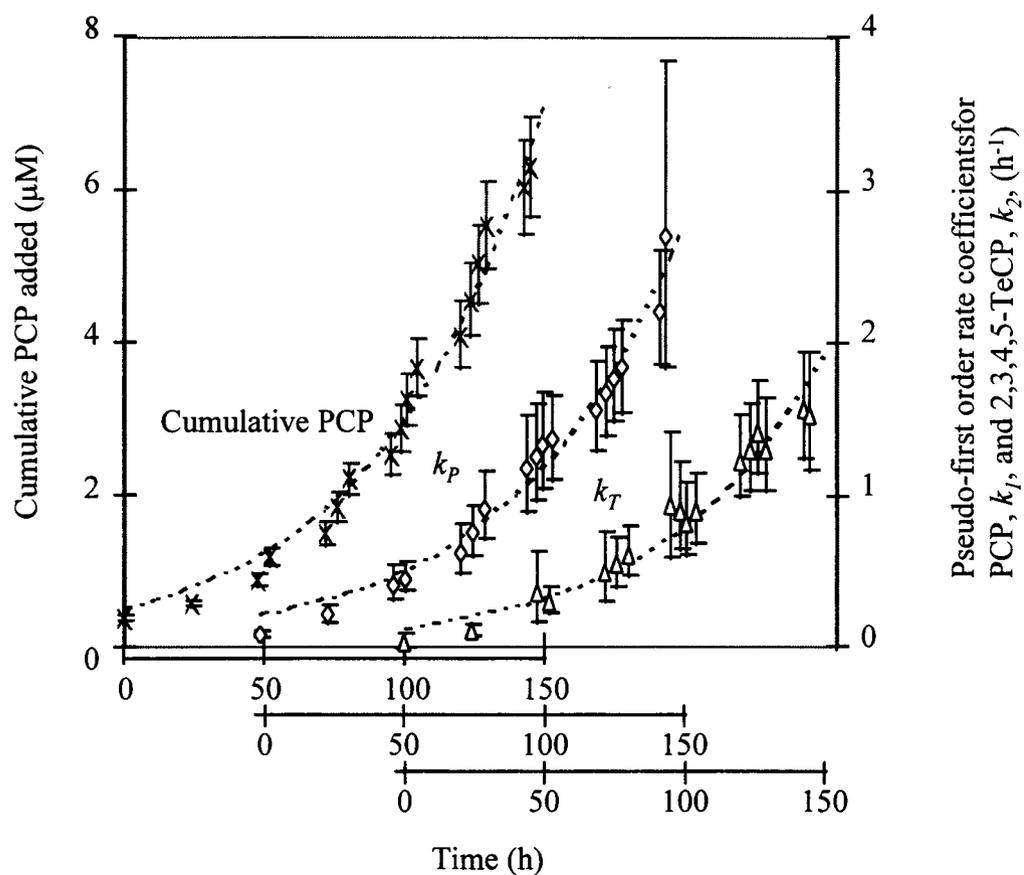


Figure 5. Cumulative PCP added ( $\times$ ), and corresponding pseudo-first-order rate coefficients for PCP ( $\diamond$ ) and 2,3,4,5-TeCP ( $\Delta$ ) obtained during a period of active acetate consumption and methanogenesis. Dashed lines are from exponential model optimizations. All three model curves share a common rate coefficient,  $a$ , but initial values vary.

Table 3. Exponential rate coefficients

Experiment	Number of data points per curve	Optimized Exponential Rate Coefficients, $a$ , ( $\text{h}^{-1}$ )			Residual sum of squares for individual curve optimization		
		$k_P$	$k_T$	$\Sigma P$	$k_P$	$k_T$	$\Sigma P$
10/94	11	0.016	0.015	0.014	0.20	0.21	0.32
11/94	17	0.017	0.015	0.018	0.21	0.15	1.17
1/95	9	0.018	0.010	0.024	0.048	0.048	0.35
10/95	7	0.016	0.014	0.025	0.051	0.12	0.19

In general, the first two PCP transformations were slower than predicted by the exponential model. Acclimation of the culture to the acetate- and PCP-rich environment of the quasi-batch reactor and recovery from the shock of transfer from the continuous flow reactor may have been responsible for initially sluggish rates. Since the first two data points had a proportionately large influence on the rate coefficient estimates from the experiments with fewer data points (experiment 1/95 and 10/95), the exponential rate coefficients determined from these experiments tended to be more disparate than the exponential rate coefficients determined from experiments with more data points.

When the first two data points were excluded from all data sets in each of the experiments, there was virtually no evidence for a difference in the means of the exponential coefficients determined from the individual data sets (F-statistic = 1.1 with 2 and 9 degrees of freedom, p-value, 0.36). The overall mean exponential rate coefficient determined by averaging all twelve rate coefficients was  $0.017 \text{ h}^{-1}$  with a standard deviation of  $0.004 \text{ h}^{-1}$  regardless of whether or not the first two data points were excluded from each data set. From Equation 9, a rate coefficient of  $0.017 \text{ h}^{-1}$  corresponds to an activity doubling time of 1.7 days (range of 1.4 to 2.3 days).

Electron acceptor yield and initial dechlorinating biomass concentration estimates were used to determine whether or not growth could reasonably account for the changes observed in pseudo-first-order rate coefficients. Given an estimated electron acceptor yield of 11 gVSS/mol PCP converted to 3,4,5-TCP, and an initial biomass concentration of 0.02 mgVSS/L, the maximum final biomass concentration achievable from a cumulative PCP addition of 6.4  $\mu\text{M}$  (shown in Figure 5) would be approximately 0.09 mgVSS/L, which represents a fivefold increase in biomass. This increase is reasonably close to the approximately sixfold increase in pseudo-first-order rate coefficients shown in Figure 5.

An estimate of the maximum substrate utilization rate ( $\mu\text{m}/Y$ ) may be obtained from a pseudo-first-order rate coefficient,  $k$ , a biomass concentration,  $X$ , and a half-saturation constant,  $K_S$  (Equation 2). Although a half-saturation constant was not determined, it was estimated to be greater than approximately 0.5  $\mu\text{M}$  because of the goodness-of-fit of the pseudo-first-order model below an initial PCP concentration of 0.5  $\mu\text{M}$ . The pseudo-first order rate coefficient for PCP at the end of the experiment shown in Figure 5 was 2.6  $\text{h}^{-1}$ . Using a biomass estimate of 0.09 mgVSS/L, and  $K_S$  equal to 0.5  $\mu\text{M}$  (as a minimum estimate), a maximum specific substrate utilization rate would be approximately 14 mmol PCP/gVSS-h, or assuming VSS is composed of 60% protein, 24 mmol PCP/g protein-h. Higher estimates of  $K_S$  would yield higher maximum substrate utilization rates.

### **Validity of first-order model.**

An inherent assumption of the first-order model for chemical transformations, presented in equations 3 and 4 above, is that the rate coefficients,  $k_p$  and  $k_T$ , are “constants” and therefore do not change appreciably during the course of the reaction. The fact that  $k_p$  and  $k_T$  have been shown to change with time, as illustrated in Figures 2, 4 and 5, appears to contradict this assumption. However, in these experiments most PCP transformations to 3,4,5-TCP were essentially complete within three to six h. (See Figure

1 above.) With a 1.7 day activity doubling time, the maximum increase in rate coefficient within a six h period would be approximately 11%--a change considered sufficiently small to justify use of the first-order model approximation.

## DISCUSSION

Several lines of evidence suggest that growth of a bacterial subpopulation resulted from the reductive dechlorination of PCP and 2,3,4,5-TeCP: 1) Overall changes in culture biomass (VSS) could not account for the increases in transformation rate coefficients, and the increases could not be directly correlated to the amount of acetate consumed or the time that the culture was in the quasi-batch reactor; 2) Energy conserved from reductive dechlorination of haloaromatic compounds is theoretically sufficient to support growth (22, 26) and a reasonable estimate for the change in pseudo-first-order rate coefficients based on growth of a subpopulation could be predicted, for the conditions of this culture, using a theoretical electron acceptor growth yield; 3) Several bacterial species capable of obtaining energy and growing at the expense of reductive dechlorination of chlorinated aromatic compounds have been isolated (13, 14, 18, 22, 26, 61, 64, 67); 4) Growth rates or doubling times reported for these pure cultures are within the range of the 1.7 day activity doubling time reported here. Growth rates of  $0.026 \text{ h}^{-1}$  to  $0.009 \text{ h}^{-1}$  (doubling times = 1.1 to 3.2 days) were reported for *D. teidjei* cultures grown with varying concentrations and combinations of pyruvate, 3-chlorobenzoate, lactate, and various inhibitors (18). A 2,4,6-TCP dechlorinator (DCB-2, later named *Desulfitobacterium hafniense* (13)) had a doubling time of approximately 2 days when grown in a medium containing pyruvate and yeast extract (64), and a species capable of growth from the reductive dechlorination of 2-chlorophenol had a 3.7 day doubling time (14). However, because no independent assessments of bacterial growth, such as MPN determinations, were performed during these experiments, increases in PCP and 2,3,4,5-TeCP transformation rates with time and PCP addition cannot be attributed conclusively to growth of a particular bacterial subpopulation.

The estimated maximum specific PCP utilization rate of 24 mmol Cl/g protein-h for the culture used in this study is higher than rates observed for two pure cultures of reductively dechlorinating bacteria. DCB-2 had a maximum specific 2,4,6-TCP utilization rate of 1.8 mmol Cl/g protein-h and a maximum specific PCP utilization rate 0.029 mmol Cl/g protein-h (64). Similarly, *D. tiedjei* had a maximum specific PCP utilization rate of 0.054 mmol Cl/g-protein-h (69). Since the culture used in this study was specifically enriched to use PCP, the higher PCP utilization rate may reflect selection for a highly efficient PCP-utilizing subpopulation. The higher rate may also result from the particular environmental conditions present in our mixed culture.

A common bacterial population responsible for transformation of both PCP and 2,3,4,5-TeCP was suggested since there was little evidence for a difference in doubling times determined for the pseudo-first-order rate coefficient data for both compounds. Several pure cultures of bacteria capable of carrying out both of these *ortho* dechlorinations have been isolated (7, 13, 108).

Enzymes required for reductive dechlorination reactions are induced in a number of bacterial isolates and enrichment cultures (14, 64, 69, 108). The culture used in the quasi-batch experiments described here was grown in a reactor with a continuous supply of PCP in the feed. However, the PCP and 2,3,4,5-TeCP concentrations within the continuous flow reactor were generally below the detection limit of approximately 0.01  $\mu\text{M}$ . The slower than expected initial transformation rates of the first two PCP additions within the batch experiments may reflect metabolic adjustment to the higher PCP and/or acetate concentrations within the quasi-batch reactor. In addition, inadvertent exposure to oxygen during the transfer between the continuous flow reactor and the quasi-batch reactor also may have contributed to initially lower rates.

The method of determining an activity doubling time from exponential changes in pseudo-first-order rate coefficients is yet to be assessed fully. This approach may only be valid for slow-growing bacterial species capable of processing a substrate on a time scale much faster than their rate of growth. Suflita et al. acknowledge that growth may occur during apparently first-order degradation processes and presented a model, based on pseudo-first-order kinetics but including an additional parameter to account for growth

(106). Although the method presented by Suflita et al. was attempted in modeling the data from the current study, meaningful and consistent parameter values could not be obtained. This failure to obtain the additional “growth” parameter from the first-order progress curves may have been a function of the relatively short time interval of individual progress curves relative to the overall trend in activity, i.e., activity changes during a given progress curve were too subtle to warrant an additional parameter.

A positive feature of the approach presented here is that incremental additions of low concentrations of a substrate reduces the potential for substrate toxicity that may be encountered in methods that require large initial substrate doses, such as the “substrate-induced growth-response” model proposed by Schmidt (88). Toxicity was a concern in early work with *D. tiedjeii*. Although 3-chlorobenzoate was used as an electron acceptor and was later shown to support growth of *D. tiedjeii*, concentrations of 3-chlorobenzoate had to be controlled to avoid inhibition of growth and activity (24, 67, 91). A similar situation was encountered in our work with a PCP-degrading, acetate-fed methanogenic consortium.

Use of the approach presented here may be limited to specific culture conditions. Zhang and Wiegel observed increases in the rate of reductive dechlorination of 2,4-DCP to 4-CP for the first three or four out of approximately forty repeated additions in serum bottle experiments with sediment enrichments, but the rate stabilized, and remained constant for the remaining additions (116). The observed stabilization in rate may have resulted from nutrient limitations or the accumulation of 4-CP. Scow et al. concluded that models derived from the Monod equation, including the first-order model, did not adequately describe the mineralization kinetics of low concentrations of compounds added to soil (90). Nevertheless, the use of changes in pseudo-first-order rate coefficients to determine activity yields and activity doubling times provides an alternate approach to characterizing the activity of a small subpopulation of bacteria within a complex, mixed culture.

The results presented here underscore the need for thorough kinetic studies, which include repeated exposures to target compounds, when designing and implementing bioremediation technologies. Rate coefficient data from single progress curves are not

adequate to fully evaluate a xenobiotic compound's resistance to degradation. Culture growth may affect transformation rates and should be factored into system design.

## CONCLUSIONS

- Increases in PCP and 2,3,4,5-TeCP transformation rate coefficients were directly related to the amount of PCP transformed via 2,3,4,5-TeCP to 3,4,5-TCP up to a cumulative PCP concentration of approximately 7  $\mu\text{M}$ , at which point accumulated 3,4,5-TCP began to affect the culture.
- Increases in PCP and 2,3,4,5-TeCP transformation rates could not be accounted for by the overall increase in biomass as measured by VSS, by the amount of acetate consumed, or by the duration of experiments.
- PCP and 2,3,4,5-TeCP may serve as electron acceptors in growth yielding reactions.
- A reductive dechlorination activity doubling time of 1.7 days was estimated.
- A unique method was presented for estimating activity increases in a mixed bacterial culture, in response to a potentially toxic xenobiotic compound.

## **The Effect of Hydrogen on the Reductive Dechlorination of Pentachlorophenol by an Acetate-fed, Methanogenic Enrichment Culture**

### **SUMMARY**

The role of  $H_2$  in the reductive dechlorination of pentachlorophenol (PCP) in an acetate-fed, methanogenic enrichment culture was evaluated. PCP was reductively dechlorinated at the ortho position, yielding 3,4,5-trichlorophenol (3,4,5-TCP) via 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP). Below an initial PCP concentration of  $0.5 \mu\text{M}$ , PCP was completely transformed to 3,4,5-TCP within three to six hours. Biomass concentration changes were small during this period, and PCP and 2,3,4,5-TeCP transformations were modeled as pseudo-first order reactions. Pseudo-first order transformation rate coefficients increased with the amount of PCP transformed to 3,4,5-TCP during active methanogenesis from acetate, but decreased when methanogenesis was inhibited by the accumulation of 3,4,5-TCP. The addition of  $H_2$  sustained reductive dechlorination activity for a prolonged period in the absence of methanogenesis, and removal of  $H_2$  from the reactor by purging with  $H_2$ -free gas mixtures decreased the rates of reductive dechlorination. A theoretical 2:1,  $H_2$ :PCP stoichiometry was observed. However, increases in pseudo-first order rate coefficients with the amount of PCP transformed to 3,4,5-TCP were not as large during continuous addition of  $H_2$  as when  $H_2$  was endogenously supplied during methanogenesis from acetate. Some nutritional requirement supplied to the reductively dechlorinating bacteria during methanogenesis from acetate may not be supplied during methanogenesis from  $H_2$  and  $CO_2$ .

### **INTRODUCTION**

Reductive dehalogenation, the reduction of a carbon atom with the replacement of a bonded halogen atom by a hydrogen atom, is one of several mechanisms for the

biotransformation of halogenated alkyl solvents, and is the primary biotransformation mechanism for highly halogenated aromatic compounds (71, 110). To assess the potential of reductive dehalogenation to transform aryl halide pollutants in natural environments and in treatment systems, much research has focused on understanding the microbial, physical and chemical conditions required for reductive dehalogenation. Experiments have been conducted with mixed cultures obtained from several sources, including freshwater and estuarine environments, aquifer material, anaerobic digesters, and lab-scale bioreactors (12, 34, 36, 43, 54, 71, 74, 79, 95, 114). In addition to these studies, research using enrichment and pure cultures has provided valuable information about the influence of specific environmental conditions such as temperature, redox potential, salinity, and the availability of alternative electron donors and acceptors (14, 22, 24, 26, 31, 38-40, 44, 50, 62-64, 116).

Interspecies hydrogen transfer is a well-established phenomenon (10). In coculture studies with several bacterial species capable of using a variety of electron acceptors ( $\text{CO}_2$ ,  $\text{S}^0$ ,  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$ ), Cord-Ruwisch et al. demonstrated that reducing equivalents in the form of  $\text{H}_2$  were preferentially transferred to and used by the species capable of reducing the most energetically favorable electron acceptor (15). Halogenated aromatic compounds also may serve as electron acceptors and reductive dehalogenation is an energy yielding reaction that can support bacterial growth. (13, 14, 22, 26, 61, 64, 67, 70).

In natural anoxic environments,  $\text{H}_2$  is produced during fermentation of complex carbon sources to acetate and other short-chain fatty acids and alcohols (10). Although some methanogens consume  $\text{H}_2$  in the reduction of  $\text{CO}_2$  to  $\text{CH}_4$ , acetoclastic methanogens also have been shown to produce  $\text{H}_2$  during methanogenesis from acetate (3, 8, 9, 52, 59, 80, 117). Several researchers have grown cocultures in which an acetate-consuming,  $\text{H}_2$ -producing bacterium supplied sufficient  $\text{H}_2$  for the growth of a bacterium incapable of using acetate (9, 80, 117).

The role of  $\text{H}_2$  as an electron donor for the reductive dechlorination of 3-chlorobenzoate (3-CB) by a three-membered bacterial consortium was reported by Dolfing and Tiedje (24). In the triculture, two members of the consortium were unable to

use 3-CB for growth. The third organism, the dechlorinating bacterium DCB-1, now *Desulfomonile tiedjeii*, was unable to use benzoate for growth. The bacterial species cooperated in a syntrophic relationship: *D. tiedjeii* reductively dechlorinated 3-CB to benzoate using  $H_2$  produced during the acetogenic oxidation of benzoate by another member of the consortium.

Although the role of  $H_2$  as a source of reducing equivalents for reductive dehalogenation has been investigated in a number of studies (17, 20, 24, 57, 63, 68, 79, 101, 116), this work is not exhaustive. In many studies,  $H_2$  has been supplied exogenously at only one or two concentrations for the reductive dehalogenation of a limited number of compounds. Also, the role of acetogens and methanogens in supplying  $H_2$  to dehalogenating species, or in maintaining low  $H_2$  concentrations in mixed cultures, has not been thoroughly evaluated.

In previous work we have demonstrated that during active methanogenesis, increases in the rates of pentachlorophenol (PCP) and 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) transformation were directly related to the amount of PCP transformed to 3,4,5-trichlorophenol (3,4,5-TCP). Growth of a bacterial subpopulation capable of using PCP and 2,3,4,5-TeCP as electron acceptors was suggested (103). In this paper, we present evidence suggesting that  $H_2$ , produced during methanogenesis, served as the electron donor for the reductive dechlorination reactions.

In this study we investigated the role of  $H_2$  in the reductive dechlorination of PCP by a mixed, continuously grown, acetate- and PCP-fed enrichment culture. Our objectives were (1) to demonstrate a relationship between conditions of active methanogenesis and the reductive dechlorination of PCP and 2,3,4,5-TeCP, (2) to determine if exogenously supplied  $H_2$  could support reductive dechlorination in the absence of methanogenesis from acetate, and (3) to investigate the effect of added  $H_2$  on the rates of PCP and 2,3,4,5-TeCP transformation. In addition, the stoichiometry of  $H_2$  consumption and PCP transformation was examined.

## METHODS AND MATERIALS

### Culture and Reactors.

The culture used in this study was obtained from a municipal anaerobic digester (Corvallis, OR), and was maintained in a 10 L continuous-flow reactor with a 10 d hydraulic detention time (74, 103). The feed solution contained 91 mM acetate and approximately 3.4  $\mu$ M PCP, along with a trace mineral and vitamin formulation based on a modification of medium developed by Owen et al. (77), and described previously (103). A second, 2.5 L reactor was used for experiments in which environmental conditions were controlled at a variety of operating conditions while multiple PCP additions were made. Both reactors were maintained at  $30 \pm 2$  °C. This two-reactor approach helped ensure that all long-term batch experiments were begun with inocula grown under essentially identical conditions.

In the 2.5 L batch reactor (liquid volume = 2.2-2.4 L), parameters such as acetate concentration, pH, and apparent  $E_H$  (redox potential) could be held constant during experimental periods lasting up to two weeks. The batch reactor had the following features: 1) continuous monitoring, at a controllable time interval, of pH (Orion Ross; 81-01), sulfide (Orion; 94-16 BN), and apparent  $E_H$ , or redox potential (platinum) (Analytical Sensors, Inc.; OR100031 BN) electrodes, 2) the ability to change and maintain the apparent  $E_H$  at a pre-determined value using a variety of oxidants and reductants, 3) the ability to purge with a defined gas mixture of up to three gasses (Tylan General mass flow controllers), and 4) feedback-controlled pH maintenance coupled to acetate concentration maintenance using small volumes of a concentrated acetic acid/acetate buffer. All electrodes were referred to a single reference electrode (Orion double junction; 90-02 BN) to avoid problems caused by multiple reference electrodes. A 0.75 mM aqueous PCP solution with a pH of approximately 10 was used for PCP addition to the batch reactor.

Two experiments were performed in which the mass flow controllers were used with H<sub>2</sub>, CO<sub>2</sub>, and CH<sub>4</sub> or N<sub>2</sub> to continuously purge the reactor at total flow rates of 100-170 ml/min. Flows of the individual gasses were adjusted to control the H<sub>2</sub> concentration of the headspace at values ranging from 0.008% to 1.3%. The pH was controlled at 7.0 ± 0.1 using approximately 30% CO<sub>2</sub>, and acetate concentrations were adjusted manually as necessary to maintain a constant concentration. When H<sub>2</sub> was found as a contaminant in the CH<sub>4</sub>, N<sub>2</sub> was substituted as the balance of the gas mixture. O<sub>2</sub> was removed from the CH<sub>4</sub>, N<sub>2</sub> and H<sub>2</sub> using OMI-2 oxygen traps (Supelco; 2-3906). A 1% H<sub>2</sub> in N<sub>2</sub> mixture was used to obtain H<sub>2</sub> concentrations less than 0.02 %.

### **Analytical Techniques.**

For chlorophenol and acetate analysis, liquid samples were withdrawn from the batch reactor in 3.5 to 5 mL quantities using a 5 mL ground glass syringe with a six inch, 18-gauge needle. (For solids determinations, 26 mL were withdrawn in a 30 mL syringe.) Reactor samples were filtered through Gelman type A/E glass fiber filters with a nominal 1 µm pore size, and the first 3 mL of filtrate was discarded. Total suspended solids and volatile suspended solids were measured as described in Standard Methods for the Examination of Water and Wastewater (35).

Chlorophenol samples were prepared for gas chromatographic analysis using a modification of the acetylation and extraction method developed by Voss et al. and Perkins et al. as described previously (79, 103, 111). A Hewlett-Packard model 5890 gas chromatograph with an electron capture detector was used for chlorophenol analyses (103).

Acetate was analyzed using a Dionex 4000I ion chromatograph with a conductivity detector. An Ionpac<sup>®</sup> AS4A anion analysis column and AG4A guard column with anion suppression were used for separation. Samples were eluted with a 1.8 mM carbonate/1.7 mM bicarbonate eluant at a flow rate of 2 mL/min. Dilute sulfuric acid (13.6 mM) was used as regenerant. Samples were diluted in eluant (1:25 or 1:10) to

avoid interference of water with the acetate peak. Because sample dilution was required, the detection limit for acetate was approximately 0.2 mM.

Headspace samples were obtained with 250  $\mu$ L, 500  $\mu$ L, or 1 mL Pressure-lok gas tight syringes (Dynatech Precision Sampling Corp.; Baton Rouge, LA).  $H_2$  was detected in headspace samples using a Hewlett Packard Model 5890 Series II gas partitioner with a thermal conductivity detector and argon carrier gas. A 4 ft x 1/8 in stainless steel column, packed with molecular sieve 13X 45/60 (Supelco, MR 58723) was used for separation. The  $H_2$  detection limit was approximately 16 ppm (vol./vol.) in a 1 mL sample. Dissolved  $H_2$  concentrations were calculated from headspace measurements using a Henry's Law constant at 30 °C, of  $1.31 \times 10^3$  L-atm/mol (107).

### **Chemicals.**

Pentachlorophenol (99%) was obtained from Aldrich Chemical Co.; 2,3,4,5-tetrachlorophenol and 3,4,5-trichlorophenol (95+%) were obtained from Ultra Scientific (North Kingstown, RI).

### **RESULTS**

The possible role of methanogens and other members of the mixed, methanogenic culture in supplying  $H_2$  for the reductive dechlorination of PCP, and the effect of exogenously supplied  $H_2$  on PCP and 2,3,4,5-TeCP transformation rates were investigated in an acetate- and PCP-fed, methanogenic culture. In the mixed culture, reductive dechlorination of PCP and 2,3,4,5-TeCP was evaluated in the absence of acetoclastic methanogenesis.

### PCP transformation pathway, progress curves, and mass balance.

Data from a typical PCP degradation experiment, shown in Figure 6, indicate that PCP transformation yielded 2,3,4,5-TeCP transiently while 3,4,5-TCP accumulated. The initial PCP concentration for the addition in Figure 6 was approximately 0.38  $\mu\text{M}$ , while

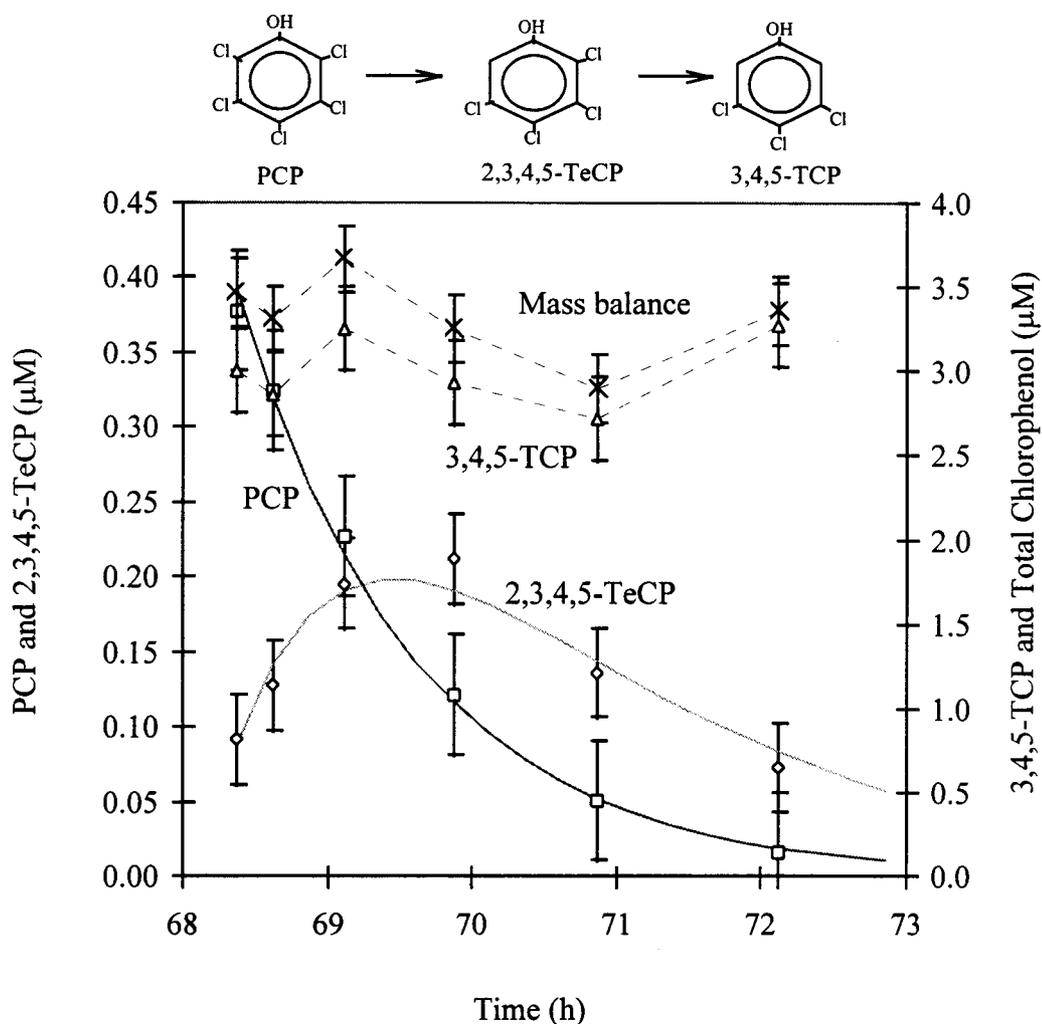


Figure 6. Observed pathway for the reductive dechlorination of PCP, and a typical PCP ( $\square$ ) transformation progress curve to 2,3,4,5-TeCP ( $\diamond$ ) and 3,4,5-TCP ( $\Delta$ ). Solid lines indicate pseudo-first order model optimizations. The 3,4,5-TCP and the chlorophenol mass balance ( $\times$ ) is an order of magnitude higher due to accumulation from prior PCP additions. Error bars indicate one standard error.

the 3,4,5-TCP concentration was nearly an order of magnitude higher as a result of PCP additions prior to 68 h. Methanogenesis was greatly reduced at 3,4,5-TCP concentrations above about 6  $\mu\text{M}$ , and reductive dechlorination ceased at 3,4,5-TCP concentrations between 9 and 12  $\mu\text{M}$ . Therefore, to avoid rapid accumulation of 3,4,5-TCP, initial PCP concentrations were kept low, usually less than 0.5  $\mu\text{M}$ . Low initial PCP concentrations also allowed use of a first-order kinetic model. However, in some experiments relatively large initial PCP concentrations intentionally were used to inhibit methanogenesis by the accumulation of the resulting 3,4,5-TCP. A first-order model was not used for initial PCP concentrations above approximately 0.6  $\mu\text{M}$ . When appropriate, first-order PCP and 2,3,4,5-TeCP biotransformation rate constants and initial concentrations were estimated by nonlinear,  $\chi^2$  optimization as described previously (55, 81, 103).

#### **Inhibition of methanogenesis and reductive dechlorination by 3,4,5-TCP.**

In experiments during which multiple PCP additions were made, first-order degradation rates generally increased with the amount of PCP transformed to 3,4,5-TCP, suggesting growth of a PCP-dechlorinating subpopulation (103). This trend was sustained during active methanogenesis from acetate, without addition of exogenous  $\text{H}_2$ , but was disrupted when methanogenesis was inhibited by accumulated 3,4,5-TCP. The effect of accumulated 3,4,5-TCP on acetate degradation and PCP transformation is shown in Figure 7. By 170 h, the 3,4,5-TCP concentration accumulated to approximately 6  $\mu\text{M}$  (Figure 7A). Because the acetate concentration within the reactor was held relatively constant, the slope of the cumulative acetate curve in Figure 7B represents the rate of acetate degradation by the consortium. At 170 h, the specific acetate degradation rate began to slow from an average of 2.7 mmol/mgVSS-h, until by 195 h, acetate degradation essentially stopped (Figure 7B). Gas production also was reduced considerably (data not shown).

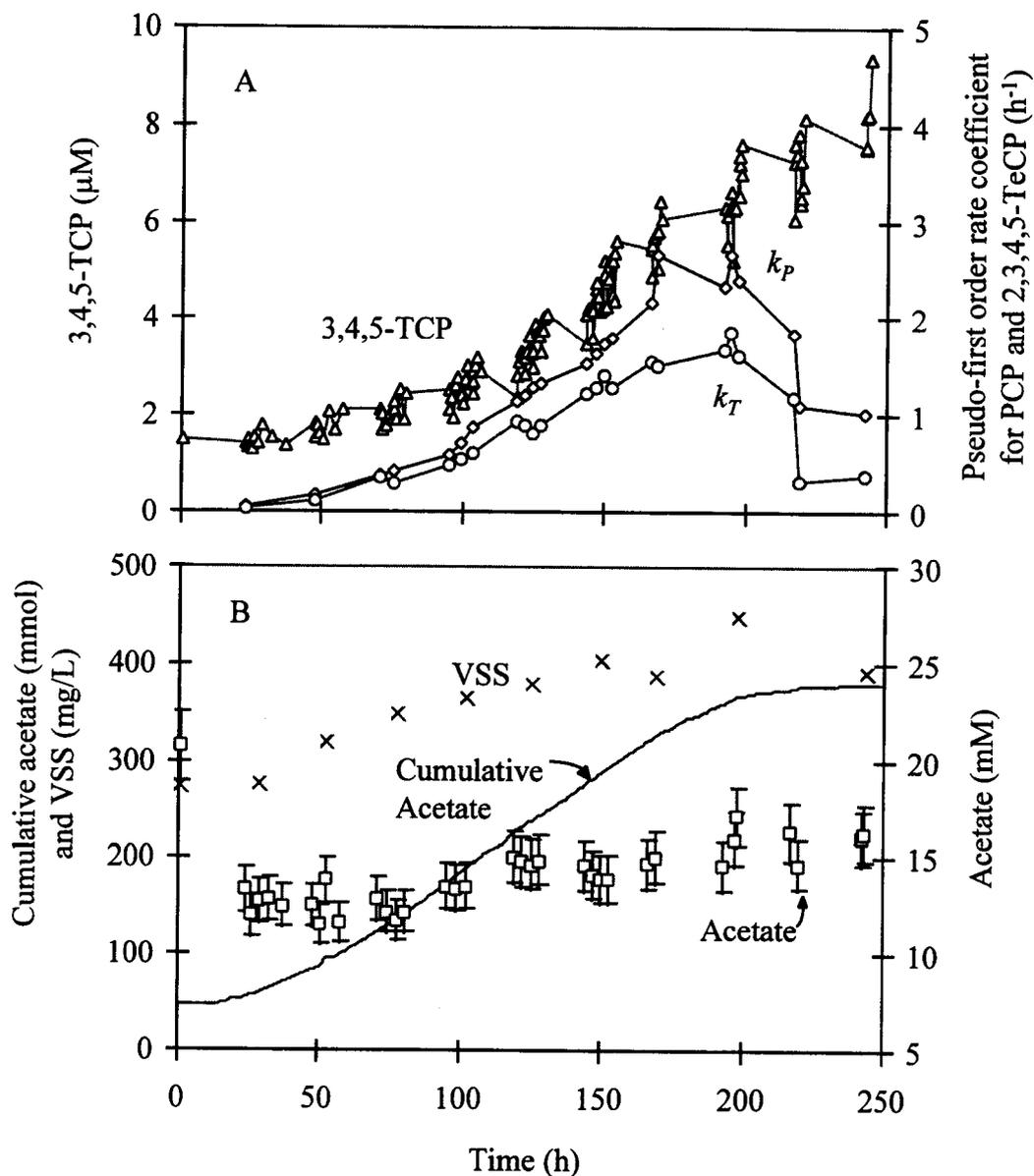


Figure 7. Chlorophenol transformation rate data and acetate degradation data from an experiment in which multiple PCP additions were made. (A) 3,4,5-TCP concentration ( $\Delta$ ), and pseudo-first order rate coefficients for PCP ( $\diamond$ ) and 2,3,4,5-TeCP ( $\circ$ ) with time. (B) Cumulative acetate added (—), VSS ( $\times$ ) and acetate concentration ( $\square$ ) data versus time. VSS data represent single samples. Error bars represent one standard error. The apparent  $E_H$  and pH were stable at  $-0.25\text{V} \pm 0.002\text{V}$  and  $7.0 \pm 0.02$ , respectively, after a 23 hour equilibration period.

Coincident with the slowing of acetate degradation, PCP and 2,3,4,5-TeCP transformation rate coefficients also were reduced (Figure 7A). Prior to 170 h, the rates of PCP and 2,3,4,5-TeCP transformation, as represented by pseudo-first order rate coefficients, continually increased with each PCP addition. After acetate degradation and corresponding methanogenesis began to slow, the rates of chlorophenol transformation failed to increase appreciably during the next few PCP additions, and then decreased. Chlorophenol transformation did not stop entirely, however. At the end of the experiment, the pseudo-first order rate coefficients remained higher than initial transformation rate coefficients.

In this experiment, pH was controlled;  $E_H$  was monitored but not controlled. After a 24 hour equilibration period, the pH and apparent  $E_H$  were constant at  $7.0 \pm 0.02$  and  $-0.25 \pm 0.002$  V (905 data points), respectively, for the remainder of the experiment, and the acetate concentration remained constant (Figure 7B). Therefore, changes in PCP transformation rate coefficients could not be attributed to changes in pH, apparent  $E_H$ , or acetate concentration, but were concurrent with changes in the rate of acetate degradation and methanogenesis.

### **Effects of $H_2$ addition on transformation rates without methanogenesis.**

The effect of  $H_2$  addition on PCP transformation rates in the absence of methanogenesis was investigated in an experiment in which methanogenesis was stopped by the accumulation of 3,4,5-TCP and the addition of  $1.5 \mu\text{M}$  of PCP. While reductive dechlorination of PCP and 2,3,4,5-TeCP continued after methanogenesis had stopped, the rates of transformation decreased.  $H_2$  was added to determine if exogenously supplied  $H_2$  could increase the rate of PCP transformation in the absence of methanogenesis.

The 3,4,5-TCP concentration reached  $6 \mu\text{M}$  after about 100 h (Figure 8A), and as in the experiment shown in Figure 7, the rate of acetate consumption began to slow (Figure 8B). At 110 h, PCP was added to a concentration of  $1.5 \mu\text{M}$ , and acetate consumption and gas production stopped. Between 110 and 126 h, three PCP

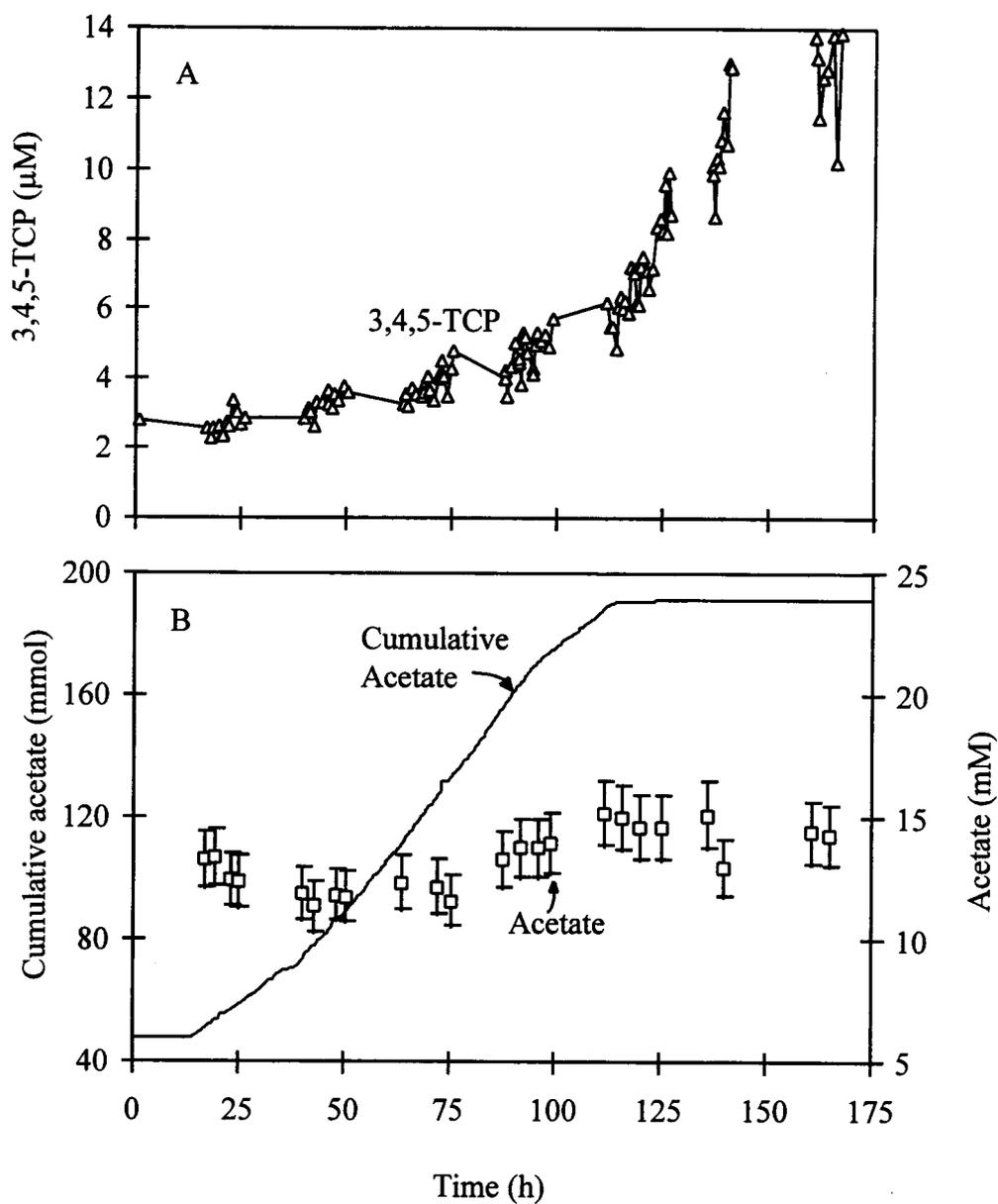


Figure 8. The 3,4,5-TCP concentration ( $\Delta$ )(A), and acetate consumption history (B) in an experiment in which  $\text{H}_2$  addition allowed reductive dechlorination to continue without methanogenesis. The rate of cumulative acetate addition (—) approached zero at 112 h while the acetate concentration ( $\square$ ) remained constant indicating cessation of acetate consumption.

additions totaling 4.6  $\mu\text{M}$ , were reductively dechlorinated to 3,4,5-TCP in the absence of methanogenesis (Figure 8). Since the initial PCP concentrations of these additions were above the first-order transformation range, first-order rate coefficients were not determined. The three PCP progress curves are shown on the same time axis in Figure 9.

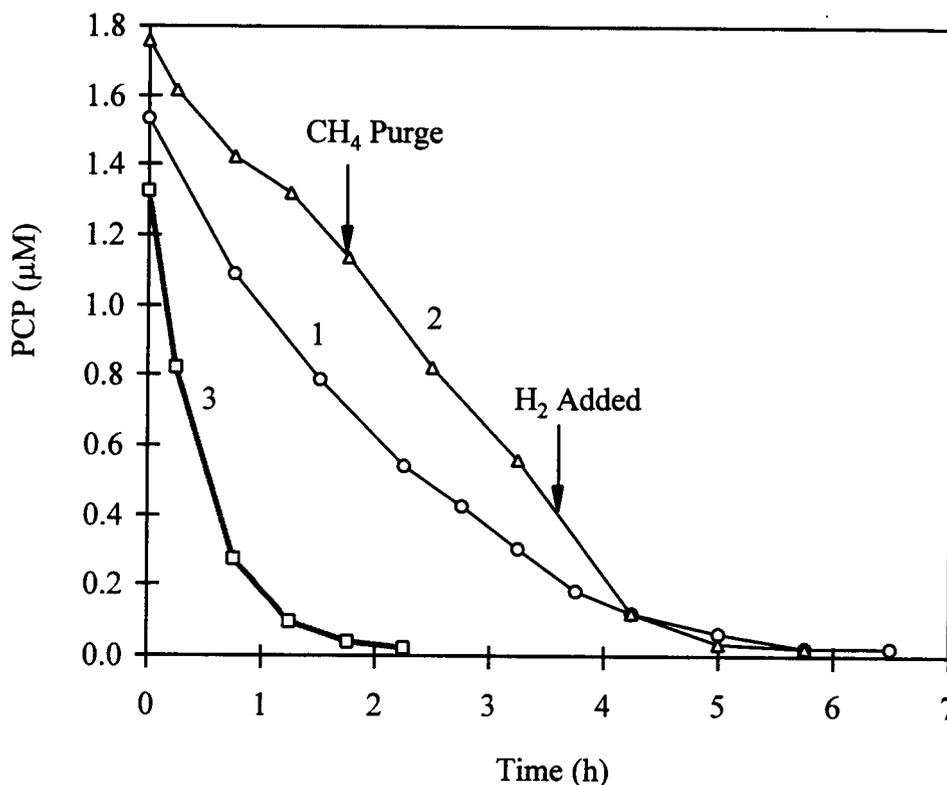


Figure 9. PCP transformation progress curves, shown on the same time scale, for three sequential additions after acetate consumption and methanogenesis were stopped due to 3,4,5-TCP. The first PCP addition (○) was transformed readily. The second PCP addition (Δ) was transformed at a slower rate until  $\text{H}_2$  was added to the reactor after approximately 3 h. A third PCP addition (□) was transformed rapidly without methanogenesis.

In the first addition (labeled 1), PCP was readily biotransformed. The PCP transformation rate was lower initially during transformation of the second PCP addition (labeled 2), though the rate appeared to rebound slightly when the reactor was purged briefly with methane. (This was done to relieve a vacuum created by repeated sampling

without gas production. Hydrogen was later found to be a low-level contaminant in the methane.) About 1.5 h later, H<sub>2</sub> was purged through the reactor at a flow rate of 6.1 mL/min for ten minutes, causing a dramatic increase in the rate of reductive dechlorination. A third PCP injection was transformed rapidly in the absence of methanogenesis, but in the presence of exogenously supplied H<sub>2</sub>. The time required for the reductive dechlorination of 1.3 μM of PCP fell from approximately 6 h before H<sub>2</sub> addition (curve 1) to 2.5 h after H<sub>2</sub> addition (curve 3). While fermentation of cellular materials may also have supplied H<sub>2</sub> for the reductive dechlorination of PCP, when methanogenesis was halted, these sources of H<sub>2</sub> apparently were not able to maintain H<sub>2</sub> concentrations comparable to those during active methanogenesis.

To determine if reductive dechlorination activity was still viable after 26 h with no methanogenesis, H<sub>2</sub> was again purged for 10 minutes prior to addition of 4.4 μM of PCP at 136.6 h (Figure 10). Although this PCP concentration was approximately double that of the previous three additions, and ten times the concentration shown in Figure 6, it was readily transformed to 3,4,5-TCP without methanogenesis. Since the initial PCP concentrations were well above the first-order concentration range, zero-order biotransformation rate constants were determined by evaluating the linear portion of each curve. The maximum PCP reductive dechlorination rate was estimated to be 1.3 μM/h (SE = 0.1 μM/h) for the PCP addition at 124.9 h (third addition in Figure 9) and 1.6 μM/h (SE = 0.05 μM/h) for the PCP addition at 136.6 h (first addition shown in Figure 10).

Even though H<sub>2</sub> was again added to the reactor at 161 h, a subsequent 5.5 μM PCP addition was only slightly transformed in approximately seven h (Figure 10). A combined PCP and 3,4,5-TCP concentration approaching 20 μM was probably toxic to the reductively dechlorinating organisms. The cessation of PCP transformation at elevated total chlorophenol concentrations suggests that the PCP transformations following H<sub>2</sub> addition were biologically mediated, and were not the result of an abiotic reaction associated with elevated H<sub>2</sub> concentrations.

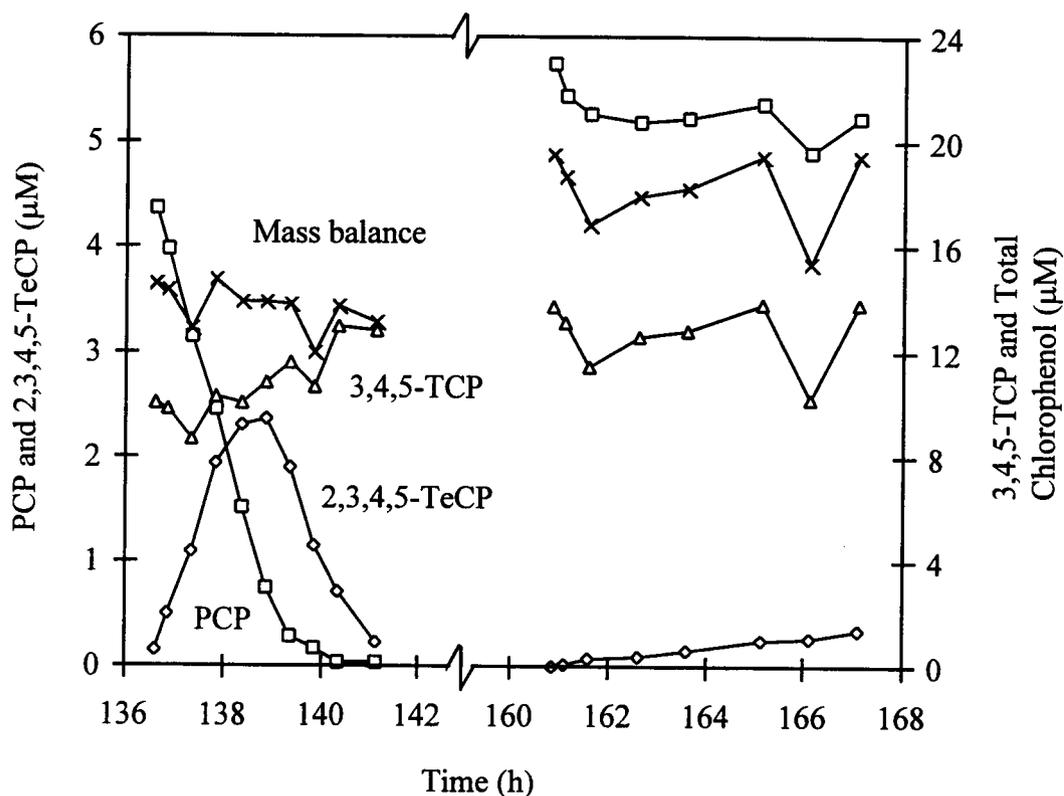
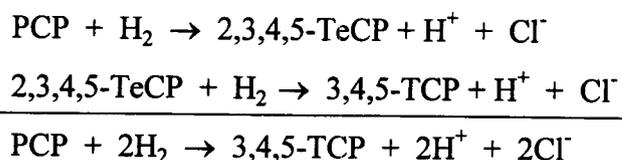


Figure 10. PCP transformation progress curves from PCP additions 26 and 51 h after methanogenesis had stopped.  $H_2$  was added prior to each PCP addition. Toxicity of the accumulated chlorophenols eventually stopped PCP transformation. Symbols: PCP (□); 2,3,4,5-TeCP (◇); 3,4,5-TCP (Δ); Mass balance (×).

### Determination of $H_2$ :PCP stoichiometry.

The stoichiometry of  $H_2$  consumption and PCP transformation was evaluated by comparing rates of  $H_2$  removal and 3,4,5-TCP formation. To curtail production of endogenous  $H_2$  and reduce the background  $H_2$  concentration, sufficient PCP was added to the reactor to stop methanogenesis by the accumulation of 3,4,5-TCP. Following cessation of methanogenesis, additional PCP was added to consume residual  $H_2$ . The theoretical stoichiometry for reduction of PCP to 3,4,5-TCP via 2,3,4,5-TeCP, with  $H_2$  as the electron donor is as follows:



Once the endogenous H<sub>2</sub> concentration was reduced to below detection, H<sub>2</sub> and PCP were added simultaneously, and progress curves were obtained (Figure 11).

Prior to the period shown in Figure 11, 3,4,5-TCP accumulated to approximately 6.5 μM, no acetate consumption or gas production were observed for approximately 24 h, and H<sub>2</sub> was not detected in the headspace. (Data not shown.) Pulses of H<sub>2</sub> were added to the reactor at 121 h (8 mL) and at 128 h (5 mL), along with pulses of PCP (Figure 11). Relatively high initial concentrations of both H<sub>2</sub> and PCP were used to ensure that concentration changes could be detected easily. Progress curves for the conversion of two PCP additions to 3,4,5-TCP via 2,3,4,5-TeCP are presented in Figure 11A. The maximum zero-order PCP reductive dechlorination rate was estimated to be 1.0 μM/h for the first addition, and 1.3 μM/h for the second addition (Figure 11A).

The cumulative concentration of PCP transformed to 3,4,5-TCP, and corresponding H<sub>2</sub> concentration data are shown in Figure 11B. Although the rate of conversion of PCP to 3,4,5-TCP was not strictly linear since 2,3,4,5-TeCP transiently accumulated, Figure 11B shows that for both PCP additions, the slope of the H<sub>2</sub> consumption data was twice the slope of the 3,4,5-TCP formed, suggesting that two H<sub>2</sub> molecules were consumed for every PCP molecule converted to 3,4,5-TCP. H<sub>2</sub> consumption by bacterial species other than the reductive dechlorinators cannot be ruled out, since the effect of accumulated 3,4,5-TCP on all members of the mixed culture was not known. Nevertheless, the theoretical 2:1 H<sub>2</sub> to PCP stoichiometry was observed for both PCP additions even though the zero order rate of PCP transformation increased.

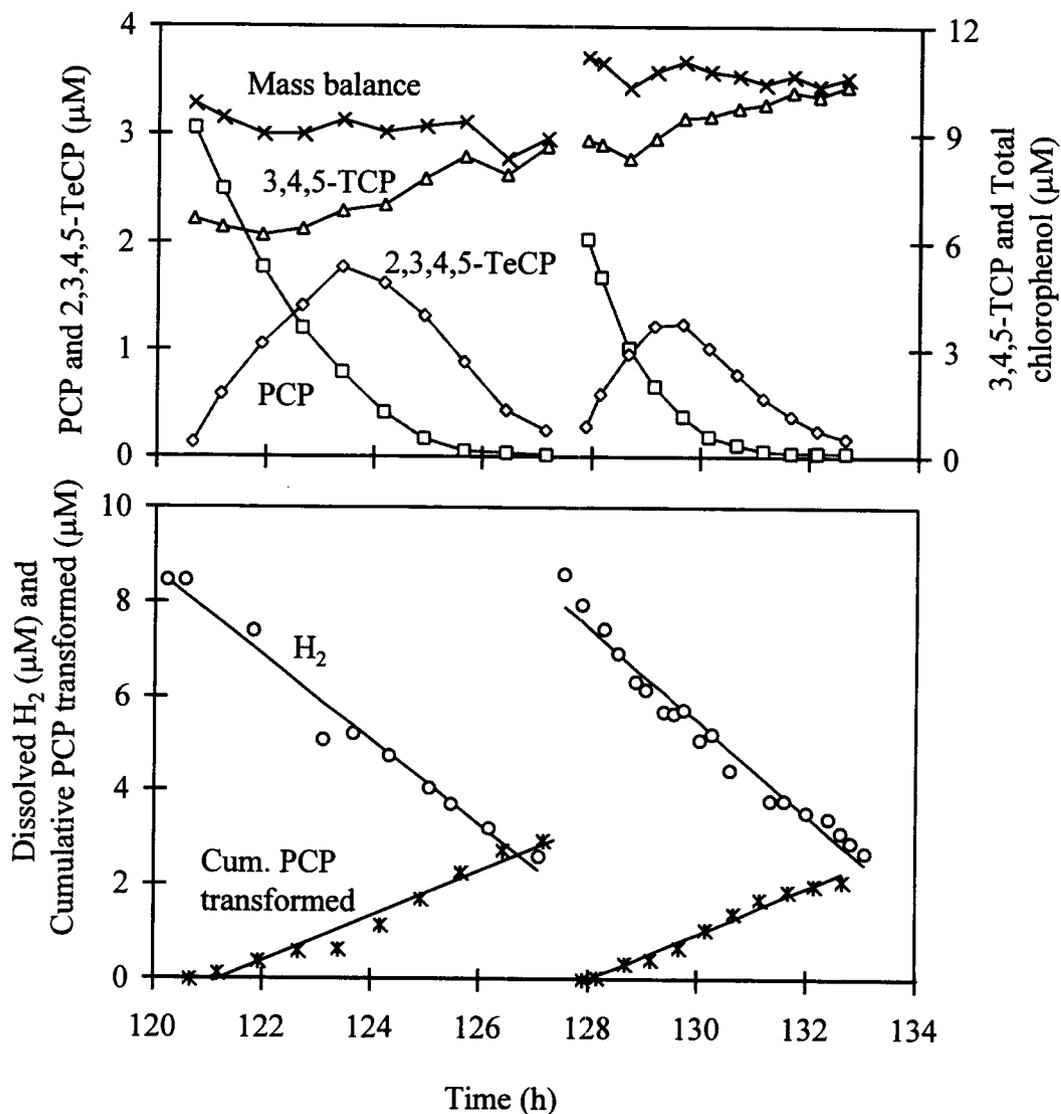


Figure 11. Chlorophenol and H<sub>2</sub> concentration data used to determine stoichiometry. (A) Progress curves for the transformation of PCP (□) to 3,4,5-TCP (Δ) via 2,3,4,5-TeCP (◇) during a period without acetate consumption, but with added H<sub>2</sub>. The chlorophenol mass balance (x) is also shown. (B) H<sub>2</sub> data (○) and cumulative PCP transformed to 3,4,5-TCP (✱) for corresponding progress curves in Figure 11A. Linear regressions, shown as solid lines, indicate that two H<sub>2</sub> molecules were consumed for every PCP molecule transformed to 3,4,5-TCP.

### Reactor sparging with 1+% H<sub>2</sub>.

To establish further a relationship between H<sub>2</sub> consumption and PCP transformation, experiments were conducted in which gas mixtures containing different proportions of H<sub>2</sub> were sparged through the reactor. The objective of the first experiment was to compare rates of PCP transformation in the presence of H<sub>2</sub>, to rates of PCP transformation when H<sub>2</sub> was removed from the reactor by sparging with H<sub>2</sub>-free gas mixtures. In addition, to determine if H<sub>2</sub> alone could support the reductive dechlorination of PCP, acetate was not added to the reactor initially.

The H<sub>2</sub> content of the gas mixture and pseudo-first order rate coefficients for PCP transformation are presented in Figure 12. The gas mixture contained CO<sub>2</sub>, CH<sub>4</sub> or N<sub>2</sub>, and 1.1-1.3% H<sub>2</sub> intermittently, and was supplied at a total gas flow rate of approximately 120 mL/min. H<sub>2</sub> was removed from the gas stream from 60 to 80 h and from 148 to 167 h.

Ten PCP additions were transformed to 3,4,5-TCP prior to acetate addition. The H<sub>2</sub> concentration was maintained above 1% for additions 1 through 7 and 10, but was removed from the purge gas for additions 8 and 9. (Although the H<sub>2</sub> gas flow was turned off, the remaining gas mixture contained CH<sub>4</sub> which, as noted above, contained H<sub>2</sub> as a contaminant. Therefore, some H<sub>2</sub> continued to enter the reactor.) Reducing the H<sub>2</sub> concentration strongly influenced the rate of PCP transformation. The pseudo-first order rate coefficients for PCP additions 8 and 9 were approximately one half and one third, respectively, of the pseudo-first order rate coefficients for additions 7 and 10 in the presence of 1.2 % H<sub>2</sub>.

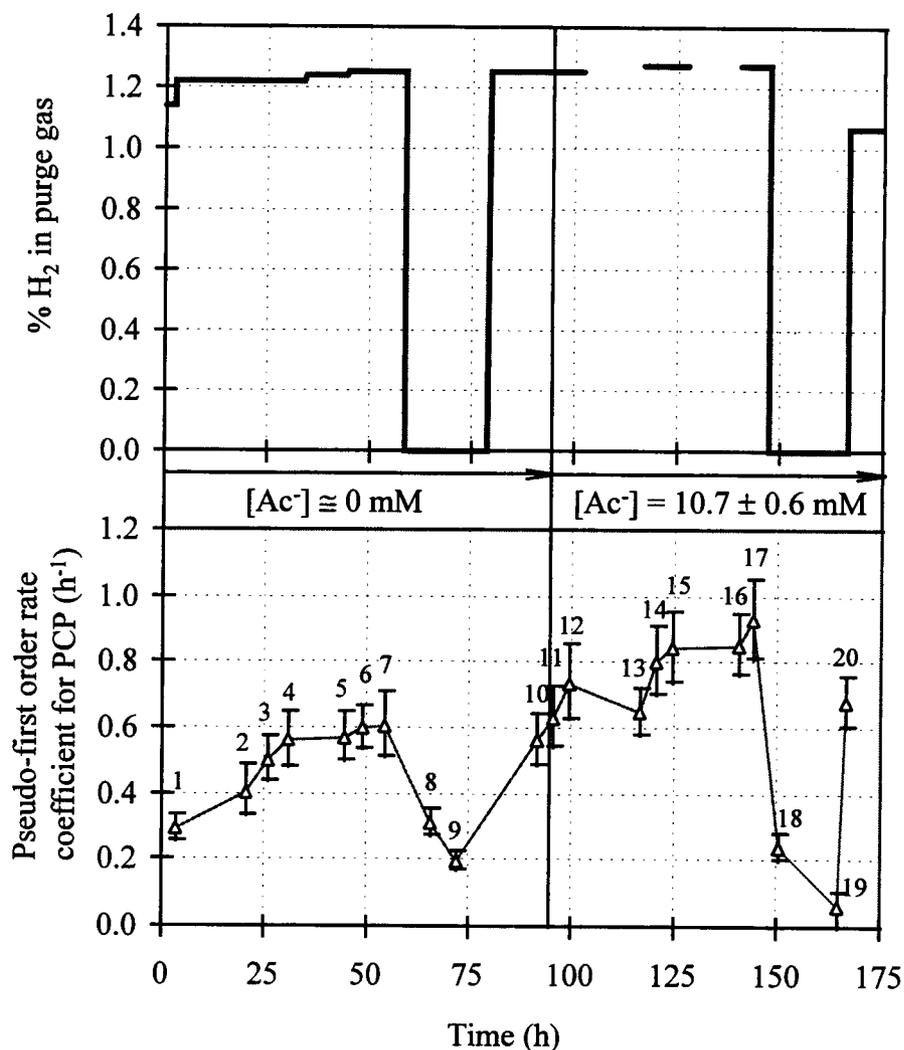


Figure 12. Concentration of H<sub>2</sub> in the purge gas, and PCP transformation rate coefficients from an experiment in which H<sub>2</sub> concentrations were externally controlled between 0% and 1.3%. A) The percent of H<sub>2</sub> in the gas mixture, and B) pseudo-first order rate coefficients for PCP. H<sub>2</sub> was removed from the purge gas at approximately 60 and 148 h. Gaps in the % H<sub>2</sub> data indicate periods in which purging was stopped. Acetate was added at 95 h, and concentrations are indicated. Error bars indicate the 95% confidence intervals for the rate coefficient estimates.

Pseudo-first order rate coefficients appeared to stabilize at approximately  $0.6 \text{ h}^{-1}$  for additions 4-7 and 10 (Figure 12 B), rather than increasing as previously observed (Figure 7) (103). To try to stimulate reductive dechlorination and achieve further rate coefficient increases, acetate was added at 95 h to a concentration of  $10.7 \pm 0.6 \text{ mM}$ . The acetate concentration remained constant without further acetate addition for the duration of the experiment, indicating no acetate consumption. However, small changes in acetate concentration, on the micromolar scale, would not have been detected by the analysis used. Following acetate addition, ten more PCP additions were transformed to 3,4,5-TCP. By addition 17, the pseudo-first order rate coefficient had increased to approximately  $0.9 \text{ h}^{-1}$ , suggesting that acetate may play a role in pseudo-first order rate coefficient increases.

To verify the effect of lowered  $\text{H}_2$  concentration on the rate of PCP transformation,  $\text{H}_2$  was again removed from the purge gas at 148 h. In addition,  $\text{CH}_4$  was replaced with  $\text{N}_2$  to eliminate the  $\text{H}_2$  contaminant, and the gas flow rate was increased from 120 mL/min to 170 ml/min for three hours and then maintained at 140 ml/min, to more effectively flush  $\text{H}_2$  from the reactor. Once again, removing  $\text{H}_2$  from the purge gas dramatically lowered the rate of PCP transformation from  $0.9 \text{ h}^{-1}$  for addition 17 to  $0.2 \text{ h}^{-1}$  and  $0.06 \text{ h}^{-1}$  for additions 18 and 19, respectively. During both periods when  $\text{H}_2$  was essentially absent from the gas stream, PCP was transformed at sequentially lower rates: i.e. the pseudo-first order rate coefficient for addition 8 was greater than for addition 9, and likewise the pseudo-first order rate coefficient for addition 18 was greater than for addition 19. Residual  $\text{H}_2$ , not yet fully purged from the reactor, may have caused the higher initial transformation rates. When  $\text{H}_2$  was returned to the gas stream, increases in the rates of PCP and 2,3,4,5-TeCP transformation were nearly instantaneous. PCP and 2,3,4,5-TeCP data and first order model curves for representative pre- and post-  $\text{H}_2$  periods are shown in Figure 13.

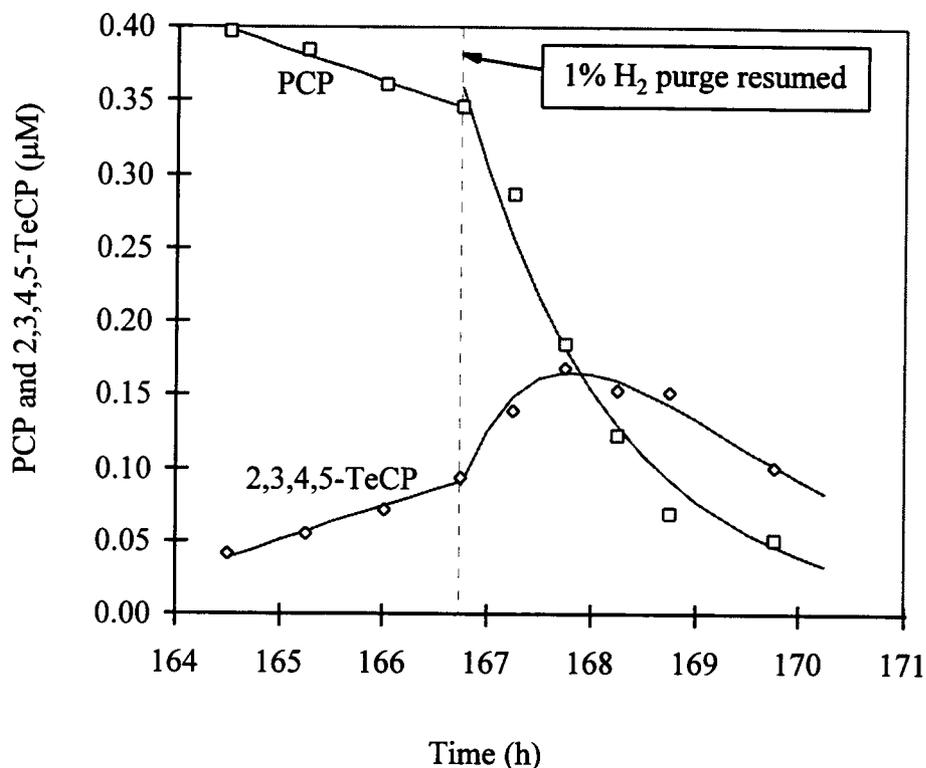


Figure 13. Progress curves showing PCP (□) and 2,3,4,5-TeCP (◇) transformation before and after H<sub>2</sub> was returned to the gas stream. Transformation rates rapidly increased when 1% H<sub>2</sub> flow was resumed. Solid lines indicate pseudo-first order model fits.

While pseudo-first order rate coefficients increased with the concentration of PCP transformed to 3,4,5-TCP when acetate was added to the reactor, the maximum pseudo-first order rate coefficient for PCP, achieved after transformation of 6 μM of PCP, was 0.9 h<sup>-1</sup> (95% CI = 0.8-1.1 h<sup>-1</sup>) as compared to 2.2 h<sup>-1</sup> (95% CI = 1.8-2.6 h<sup>-1</sup>) from experiments without gas sparging (103). To determine if the relatively high (1+ %) H<sub>2</sub> concentration may have negatively influenced increases in PCP transformation rates, the experiment was repeated using lower H<sub>2</sub> concentrations.

#### Reactor sparging with 0.008-0.019% H<sub>2</sub>.

Endogenous dissolved H<sub>2</sub> concentrations measured during reductive dechlorination of PCP ranged from 0.2 to below 0.01 μM (data not shown). To more

closely simulate these H<sub>2</sub> concentrations, measured during methanogenesis from acetate, the H<sub>2</sub> composition of the gas stream was maintained between 0.008% and 0.019% (Dissolved concentration at equilibrium = 0.06-0.1 μM). As in the experiment shown in Figure 12, acetate was not added to the reactor initially. The H<sub>2</sub> composition of the purge gas and pseudo-first order rate coefficients for PCP are shown in Figure 14. The total gas flow rate was approximately 100 mL/min.

In contrast to the experiment in which a 1.2% H<sub>2</sub> concentration was maintained in the headspace (Figure 12), at a H<sub>2</sub> concentration of 0.008% without acetate, the pseudo-first order rate coefficients of the first four PCP additions decreased rather than increased. This low concentration of H<sub>2</sub> apparently was not able to support transformation rate increases without acetate. In addition, the apparent E<sub>H</sub> was reduced to only -0.17 V as compared to -0.25 V typically observed (data not shown). When the H<sub>2</sub> concentration in the gas stream was doubled to 0.016% at 47 hours the PCP transformation rate appeared to increase immediately, although the 95% confidence intervals of the transformation coefficients overlapped. At 50 h, acetate was added to a concentration of 9 mM and pseudo-first order rate coefficients began to increase in a fashion comparable to the experiment with a 1.2% H<sub>2</sub> purge. Also, the apparent E<sub>H</sub> fell to below -0.25V. Since the H<sub>2</sub> concentration was increased, acetate was added, and the apparent E<sub>H</sub> decreased, increases in pseudo-first order rate coefficients can not be attributed solely to any of these parameters. Never-the-less, the maximum pseudo-first order rate coefficient achieved with 0.016% H<sub>2</sub> and 9 mM acetate was approximately 1.0 h<sup>-1</sup>.

While acetate consumption was not detected when the gas stream contained 1.2% H<sub>2</sub>, at the lower H<sub>2</sub> concentrations of the experiment shown in Figure 14, an average specific acetate consumption rate of 0.9 mmol acetate/g VSS-h was determined. This rate was one-third the 2.7 mmol acetate /gVSS-h observed in experiments without gas purging (Figure 7B).

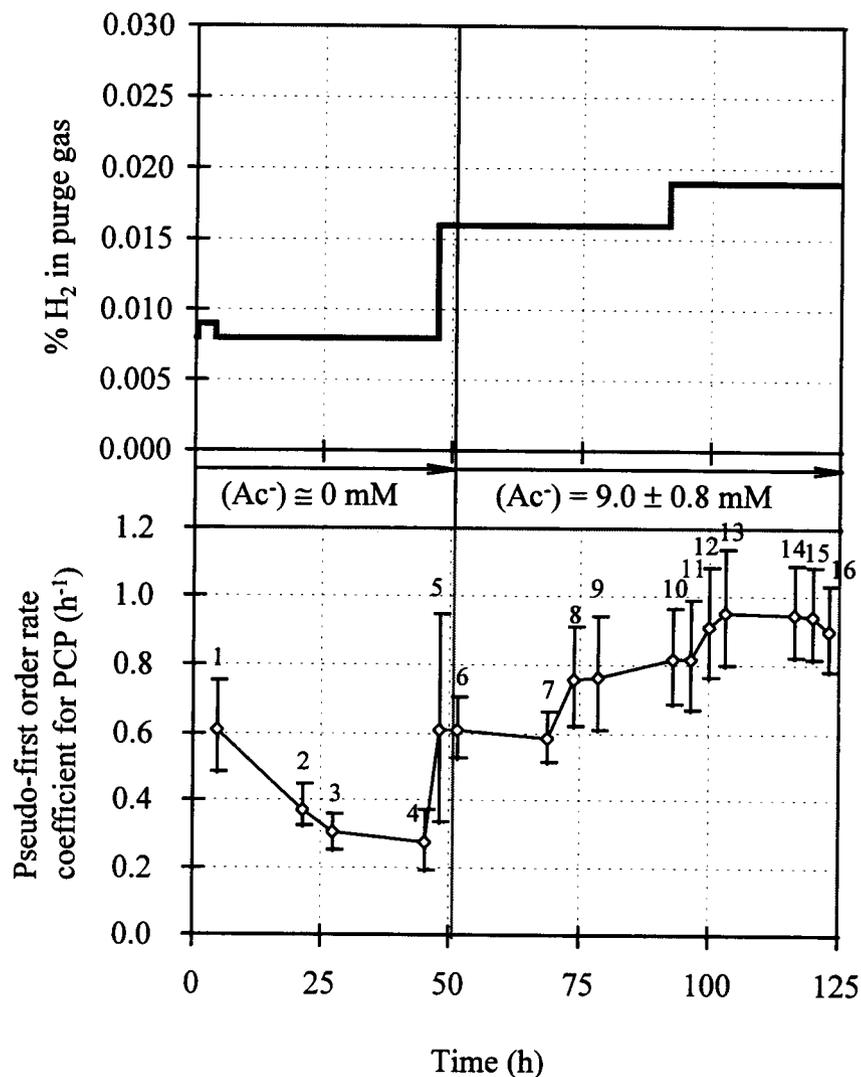


Figure 14. Concentration of H<sub>2</sub> in the purge gas, and PCP transformation rate coefficients from an experiment in which H<sub>2</sub> concentrations were externally controlled at 0.008%, 0.016%, and 0.019%. (A) Percent of H<sub>2</sub> in the gas mixture, and (B) pseudo-first order rate coefficients for PCP. Error bars indicate 95% confidence intervals for the rate coefficient estimates.

### Comparison of rate increases with and without added H<sub>2</sub>.

In previous work, without added H<sub>2</sub>, linear relationships between increases in pseudo-first order rate coefficients for PCP and 2,3,4,5-TeCP and the cumulative concentration of PCP transformed to 3,4,5-TCP were described (103). Similar linear relationships were observed for the two experiments in which different concentrations of H<sub>2</sub> were continuously sparged through the reactor. Rate coefficient data and linear regression results from experiments with and without exogenous H<sub>2</sub> are presented in Figure 15, and the linear regression equations are presented in Table 4. In experiments

Table 4. Slope and intercept parameters for linear regressions from experiments with and without exogenous H<sub>2</sub>.

Compound	H <sub>2</sub>	Slope	SE of Slope	Intercept	SE of Intercept	df <sup>a</sup>
PCP :	No	0.33	0.013	0.17	0.036	69
	Yes	0.10	0.025	0.35	0.11	14
2,3,4,5-TeCP:	No	0.20	0.012	0.26	0.034	69
	Yes	0.069	0.016	0.40	0.068	14

<sup>a</sup> df = degrees of freedom

with exogenous H<sub>2</sub>, only rate coefficient data obtained during periods with acetate and with a H<sub>2</sub> concentration of 0.016% or greater were included in the regressions. The slopes of the regressions represent the amount that the pseudo-first order rate coefficient increased per  $\mu\text{M}$  of PCP transformed to 3,4,5-TCP, and the intercept was an estimate of initial rate coefficients. The slopes of the regressions from experiments with added H<sub>2</sub> were roughly one third of the slopes from experiments without H<sub>2</sub>, and the intercepts were roughly double. Clearly, the increases in pseudo-first order rate coefficients per  $\mu\text{M}$  of PCP converted to 3,4,5-TCP were less when the culture was sparged with gas mixtures

containing 0.016% to 1.3%  $H_2$ , than when  $H_2$  was endogenously supplied during methanogenesis.

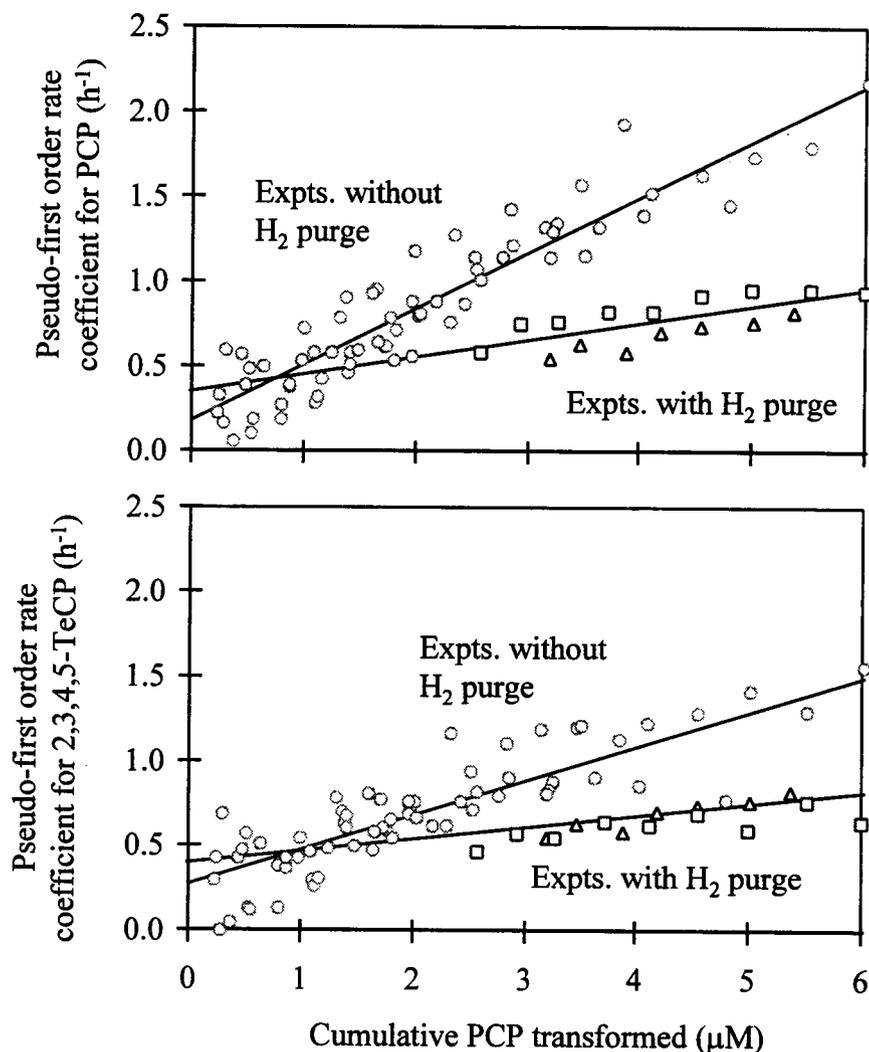


Figure 15. Pseudo-first order rate coefficients for PCP (A) and 2,3,4,5-TeCP (B) versus the cumulative concentration of PCP added to the reactor. Pseudo-first order rate coefficients from experiments in which  $H_2$  concentrations were not externally controlled (O) are shown, along with rate coefficients from two experiments in which  $H_2$  concentrations were controlled ( $\square, \Delta$ ).

## DISCUSSION

Our results suggest that a syntrophic relationship, similar to those studied by Zinder and Koch, Phelps et al., Boone et al., and Cord-Ruwisch et al., exists between acetoclastic methanogens and a reductively dechlorinating subpopulation in our enrichment culture (9, 15, 80, 117). H<sub>2</sub> from other sources such as acetogenic degradation of cellular byproducts also may have contributed to the H<sub>2</sub> pool. However, even without this source of H<sub>2</sub>, evidence from pure culture studies suggests that H<sub>2</sub> production from acetate may have been sufficient to supply the dechlorinating population. *Methanosarcina thermophila* TM-1 maintained a dissolved H<sub>2</sub> concentration of 0.3 μM during growth from acetate (3). In addition, H<sub>2</sub> was produced at 0.5-2% of the rate of CH<sub>4</sub> formation in both resting and growing cultures of *Methanosarcina barkeri* strain MS during methanogenesis from acetate (52). At the acetate consumption rate observed in this study, this H<sub>2</sub> production rate would have been more than adequate to supply H<sub>2</sub> for the reductive dechlorination of the small concentrations of PCP added to the reactor. The theoretical energy yield from reductive dehalogenation is greater than that obtained from methanogenesis from H<sub>2</sub> and CO<sub>2</sub>, and may drive H<sub>2</sub> transfer from the methanogens to the reductively dechlorinating species (15, 23).

Previous studies evaluating the role of H<sub>2</sub> in reductive dehalogenation have yielded differing results depending on the H<sub>2</sub> concentration employed, the halogenated compound tested, and the microbial species or consortium used. Some researchers have reported that added H<sub>2</sub> has had either no effect, or has decreased the rate of reductive dehalogenation or growth of the dehalogenating culture (18, 20, 57, 68, 101, 116). Others have reported stimulation of reductive dehalogenation (57, 63, 79, 116). Results of the above studies suggest that the impact of H<sub>2</sub> is concentration and species dependent. Either too much or too little H<sub>2</sub> may decrease growth, the rate of reductive dehalogenation, or both. Furthermore, reductive dehalogenation of more highly halogenated compounds may tolerate (or require) higher concentrations of H<sub>2</sub> than compounds with fewer halogen atoms (116).

Our results also reflected this apparent paradox in the effect of H<sub>2</sub> on bacterial cultures. Added H<sub>2</sub> allowed PCP transformation to continue at increased rates after transformation rates were reduced following cessation of methanogenesis (Figures 4, 5, and 6). In addition, removing H<sub>2</sub> from the reactor by sparging greatly reduced the rate of PCP transformation, and returning H<sub>2</sub> to the gas stream increased the rate of transformation (Figure 12). However, when H<sub>2</sub> was supplied exogenously to the culture through sparging, transformation rate coefficients failed to increase as much as during experiments without exogenous H<sub>2</sub> (Figure 15), perhaps indicating repression of growth of the dechlorinating subpopulation. In contrast, initial transformation rates from the experiments with added H<sub>2</sub> apparently were higher (larger intercepts) than the initial transformation rates from experiments without added H<sub>2</sub> (Figure 15, Table 4). Exogenously supplied H<sub>2</sub> may have reduced the effects of inadvertent exposure to oxygen during transfer from the continuous flow reactor to the batch reactor, and thereby increased initial PCP transformation rates over those from experiments in which H<sub>2</sub> wasn't added. Alternately, higher initial H<sub>2</sub> concentrations in experiments with added H<sub>2</sub> may have allowed greater initial transformation rates.

A dechlorinating subpopulation has been estimated to make up only about 0.008% of the total dry weight of the culture (103). Even though the energy yield from reductive dechlorination is relatively high, the sheer numbers of methanogens may have out-competed the dechlorinating population for the limited H<sub>2</sub> when a H<sub>2</sub> composition of 0.008% was supplied without acetate, thus contributing to decreases in reductive dechlorination rates (Figure 14). Metabolic stresses caused by the higher than usual apparent E<sub>H</sub> of -0.17 V may also have contributed to decreases in reductive dechlorination rates.

Metabolic changes during methanogenesis from H<sub>2</sub> and CO<sub>2</sub> as opposed to acetate may have contributed to lower increases in transformation rate coefficients when H<sub>2</sub> was supplied. Perhaps a nutritional requirement, supplied to the dechlorinating bacteria during methanogenesis from acetate, may have been missing when H<sub>2</sub> and CO<sub>2</sub> were preferentially used. Extracellular corrinoid production increased when acetate was added

to methanol grown cells of *M. barkeri* strain Fusaro, suggesting differences in potentially useful cellular products under different metabolic conditions (65, 92).

Initial work with *D. tiedjeii* in a defined consortium indicated that H<sub>2</sub> is the preferred source of reducing equivalents for the reductive dechlorination of 3-chlorobenzoate by this organism (24). However, in pure culture studies, Stevens et al. reported reduced cell yield when H<sub>2</sub> was supplied during growth of *D. tiedjeii* on pyruvate (101), and DeWeerd et al. reported decreased growth when *D. tiedjeii* was cultured under a H<sub>2</sub>:CO<sub>2</sub> atmosphere (18). A similar reduction in growth may have occurred in our mixed culture when H<sub>2</sub> was exogenously supplied. When grown in a complex medium with pyruvate and rumen fluid, the reductive dechlorination of 3-chlorobenzoate by *D. tiedjeii* was inhibited at a H<sub>2</sub> concentration of 80 kPa (0.8 atm; 618 μM dissolved concentration), but resumed when the H<sub>2</sub> concentration was reduced to below 2.7 kPa (0.03 atm; < 20 μM dissolved) (57). Using resting cells of *D. tiedjeii*, DeWeerd and coworkers showed that in the absence of growth, *D. tiedjeii* uses H<sub>2</sub> as an electron donor for the reductive dehalogenation of 3-chloro-, 3-bromo-, and 3-iodobenzoate (17).

In freshwater sediments, a H<sub>2</sub> partial pressure up to 1 atm stimulated the rate of 2,4-dichlorophenol reductive dechlorination and no decreases in reductive dechlorination were observed up to 3 atm. However, at H<sub>2</sub> partial pressures above 0.64 atm, the reductive dechlorination of 4-chlorophenol, and phenol and benzoate degradation were totally interrupted (116). In a 2-chlorophenol-degrading enrichment culture, H<sub>2</sub> and CO<sub>2</sub> could not serve as sole co-substrates for reductive dechlorination, but the culture was able to use H<sub>2</sub> slowly from the gas phase, and the addition of H<sub>2</sub> to crude cell extracts allowed complete dechlorination (20). PCP transformation rates were greatly reduced when sulfate was added to a PCP-degrading, methanogenic enrichment culture. Reduction in transformation rates was attributed to lower endogenous H<sub>2</sub> concentrations under sulfate reducing conditions than under conditions of methanogenesis from yeast extract. When sulfate reduction was inhibited by the addition of molybdate, H<sub>2</sub> concentrations and PCP

transformation rates were comparable to those observed under methanogenic conditions (63).

Perkins et al. investigated the relative importance of methanogenesis versus eubacterial activity on the reductive dechlorination of 2,4,6-TCP by an anaerobic digester sludge enrichment culture (79). In a series of serum bottle experiments, bromoethanesulfonic acid (BESA) was used to inhibit methanogenesis, and vancomycin was used to inhibit the activity of eubacteria.  $H_2$ , acetate, or fructose plus titanium citrate were supplied as electron donors. The reductive dechlorination of 2,4,6-TCP was inhibited under all treatments with vancomycin, indicating that eubacteria rather than methanogens were responsible for carrying out reductive dechlorination. This result is consistent with our observation of reductive dechlorination in the absence of methanogenesis. Of the BESA treatments, reductive dechlorination was only inhibited in the acetate-fed bottles. Perkins et al. suggested that the dechlorinating population had a “syntrophic relationship” with the acetoclastic methanogens, but they did not speculate that  $H_2$  was the missing component in the acetate-fed bottles.  $H_2$  was provided in one set of bottles and was presumably produced by acetogens (not susceptible to BESA) during the degradation of fructose in the other set of bottles. Only  $H_2$  production by the acetoclastic methanogens would have been halted by BESA (21).

Addition of supplemental, short-chain carbon sources to complex, bacterial cultures stimulated reductive dechlorination of chloroaromatic compounds in several studies (32, 44, 53).  $H_2$  production has been suggested as one possible cause of enhanced reductive dechlorination activity (17, 32, 44, 53). Our research suggests that while  $H_2$  may serve as an electron donor for reductive dechlorination of PCP, maximum transformation rates may not be achieved by addition of  $H_2$  alone. Although not shown conclusively, acetate may also contribute to increased transformation rates. Furthermore, other metabolic interactions appear to influence the rate of reductive dechlorination and perhaps growth of a dechlorinating subpopulation.

## CONCLUSIONS

The importance of interspecies  $H_2$  transfer in the reductive dechlorination of PCP in an acetate-fed, methanogenic enrichment culture was evaluated. We have determined that 1) reductive dechlorination rates increased with the amount of PCP transformed to 3,4,5-TCP during active methanogenesis, but 2) they decreased with the amount of PCP transformed when methanogenesis was inhibited by the accumulation of 3,4,5-TCP; 3) the addition of  $H_2$  sustained reductive dechlorination activity for a prolonged period in the absence of methanogenesis; 4) removal of  $H_2$  from the reactor by purging with  $H_2$ -free gas mixtures decreased the rates of reductive dechlorination; 5) a theoretical 2:1  $H_2$ :PCP stoichiometry was observed; 6) increases in pseudo-first order rate coefficients with the amount of PCP transformed were not as large during continuous addition of  $H_2$  at concentrations above 0.016% as when  $H_2$  was endogenously supplied during methanogenesis from acetate.

## **The Effect of Redox Potential Changes on Reductive Dechlorination of Pentachlorophenol and the Degradation of Acetate by a Methanogenic Culture**

### **SUMMARY**

The effect of redox potential changes on methanogenesis from acetate, and on the reductive dechlorination of pentachlorophenol (PCP) was evaluated using a computer-monitored and feed-back-controlled bioreactor. PCP was transformed via 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) to 3,4,5-trichlorophenol (3,4,5-TCP). In 6- to 12-day experiments, pH, acetate concentration, and temperature were held constant, and the redox potential was maintained at different set points, while transformation of multiple PCP additions was monitored. Without redox potential control, the apparent  $E_H$  of the culture was approximately -0.26 V. The apparent  $E_H$  was elevated from -0.26 V for periods up to ten h, by computer-controlled, repeated addition of  $H_2O_2$  or  $K_3Fe(CN)_6$ . Methanogenesis continued during a relatively mild apparent  $E_H$  shift to -0.2 V with  $H_2O_2$ , but was halted when the apparent  $E_H$  was raised to -0.1 V with either  $H_2O_2$  or  $K_3Fe(CN)_6$ . Methanogenesis resumed when the apparent  $E_H$  returned to -0.26 V. During periods of large apparent  $E_H$  shifts in which methanogenesis stopped, transformation of PCP and 2,3,4,5-TeCP continued at progressively slower rates. The rate of 2,3,4,5-TeCP transformation was decreased more by elevated redox potentials than was the rate of PCP transformation.  $H_2$  added while the apparent  $E_H$  was maintained at -0.1 V, caused reductive dechlorination rates to increase, suggesting that decreased  $H_2$  concentrations during periods of elevated apparent  $E_H$  may contribute to decreased reductive dechlorination rates.

## INTRODUCTION

Reductive dehalogenation of haloaromatic compounds, or the microbially mediated removal of a halogen atom and replacement with a hydrogen atom, is most commonly observed in highly reduced anaerobic environments characteristic of methanogenesis (30, 33, 71, 105). In some enrichment cultures, particularly those from estuarine environments, reductive dehalogenation has been shown to occur under sulfate reducing conditions (39, 49). However, in other cultures, the presence of electron acceptors typical of less reduced environments such as sulfate, ferric iron, nitrate, or oxygen has failed to enhance or has decreased reductive dehalogenation activity (30, 33, 40, 51, 53, 63, 68, 85). Whether or not reductive dehalogenation activity is impacted by alternative electron acceptors may depend on the presence of microbial consortia or abiotic processes capable of competing with reductively dehalogenating organisms for reducing equivalents (5, 33, 38, 63). The availability of reducing equivalents may be reflected in the concentration of dissolved  $H_2$  present (58, 60, 63) or in the redox potential of the culture. Hale et al. (40) found that variations in pH, redox potential, and the concentration of sulfate and nitrate present in five different sediment samples accounted for 83% of the variation in the rates of reductive dechlorination of two dichlorophenols. Aside from this work, relatively little is known about the effect of redox potential on the rate of reductive dechlorination reactions. The aim of our research was to examine the response of a methanogenic, pentachlorophenol-degrading consortium, to elevated apparent redox potentials in a quasi-batch bioreactor in which environmental conditions, such as acetate concentration, pH, temperature, and apparent  $E_H$  were controlled. (The adjective “apparent” is used to emphasize that redox potential values are measured using a platinum electrode and may not fully represent the equilibrium redox potential of the solution.)

Investigations of the effect of altered redox potential (apparent  $E_H$ ) on growth of methanogenic (27, 47, 48) and nonmethanogenic (75, 109, 112) anaerobic bacteria have found species-specific responses to redox potential manipulations. For example,

Vainshtein and Gogotova reported different redox potential ranges for optimal rates of H<sub>2</sub>S production by four different sulfate reducing bacterial species (109). Although methanogenesis is normally expected to occur at redox potentials below -0.2 V (27, 60, 76, 115), Fetzer and Conrad reported that *Methanosarcina barkeri* was able to reduce the apparent E<sub>H</sub> of a redox buffer solution containing ferric and ferrous cyanide from an initial value of +0.43 V to +0.05V. Methane production began at +0.05 V. The ability of *M. barkeri* to reduce ferricyanide was dependent on methanol concentration and the density of the bacterial suspension (27). Another strict anaerobe, *Clostridium acetobutylicum*, grew at an apparent E<sub>H</sub> of + 0.37 V artificially maintained with K<sub>3</sub>Fe(CN)<sub>6</sub>, but not at an apparent E<sub>H</sub> of +0.10 V maintained by aeration. In addition, growth of the ferricyanide poised culture was halted by aeration even though the apparent E<sub>H</sub> was not changed (72, 75), indicating that the presence or absence of O<sub>2</sub> was more critical to the survival of this particular species than redox potential.

In previous work, we have shown that at an apparent E<sub>H</sub> of -0.25 V the rates of pentachlorophenol (PCP) and 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) transformation increase with the amount of PCP converted to 3,4,5-trichlorophenol (3,4,5-TCP), suggesting growth of a bacterial subpopulation responsible for reductive dechlorination (103). While reductive dechlorination continues in the absence of methanogenesis, the rate of PCP transformation remains constant or decreases rather than increases (102). However, exogenously supplied hydrogen can support reductive dechlorination without methanogenesis (102).

In this study, two oxidants, H<sub>2</sub>O<sub>2</sub> and K<sub>3</sub>Fe(CN)<sub>6</sub>, were used for apparent redox potential control. H<sub>2</sub>O<sub>2</sub> was selected because of its common use as an oxygen substitute in in situ bioremediation processes (11, 28, 45, 56). K<sub>3</sub>Fe(CN)<sub>6</sub> has been used for redox control in a variety of studies (27, 48, 72, 112). It is non-toxic to three intestinal bacterial species at concentrations as high as 2% (112), and causes no inhibition of methanogenesis from methanol by *M. barkeri* at concentrations less than 0.5 mM (27).

Using these oxidants, we evaluated acetate degradation, methanogenesis and the rates of reductive dechlorination of PCP and 2,3,4,5-TeCP during apparent redox potential perturbations. During periods of large apparent E<sub>H</sub> shifts, methanogenesis

ceased while transformation of PCP and 2,3,4,5-TeCP continued at progressively slower rates. The rate of 2,3,4,5-TeCP transformation appeared to be reduced more by elevated redox potentials than the rate of PCP transformation. Evidence suggesting that rates of reductive dechlorination are decreased at elevated redox potentials due to a decrease in the concentration of H<sub>2</sub> available to the reductively dechlorinating organisms is presented.

## METHODS AND MATERIALS

### Reactors.

A two reactor system was used: one continuous-flow reactor (10 L) for maintenance of the enrichment culture at steady-state conditions, and a second, smaller reactor (2.5 L) for batch experiments in which reductive dechlorination was evaluated under controlled conditions. Both reactors were maintained at  $30 \pm 2$  °C. In the batch reactor, parameters such as acetate concentration, pH, and redox potential were held constant at different values, while multiple PCP additions were made over experimental periods lasting up to two weeks. This two reactor approach helped ensure that all long-term batch experiments were begun with inocula grown under nearly identical conditions. Details of the reactors are described elsewhere (103).

The 2.5 L batch reactor included the following features: 1) continuous monitoring, at a controllable time interval, of pH (Orion Ross; 81-01), sulfide (Orion; 94-16 BN), and redox potential (platinum) (Analytical Sensors, Inc.; OR100031 BN) electrodes, 2) feedback-controlled pH maintenance coupled to acetate concentration maintenance, 3) the ability to change and maintain the redox potential at a pre-determined value using a variety of oxidants and reductants, 4) tracking of reactor volume changes due to sample removal, or addition of reagents, and 5) the ability to purge the reactor with a defined gas mixture of up to three gasses (Tylan General mass flow controllers). All electrodes were referred to a single reference electrode (Orion double junction; 90-02 BN) to avoid problems caused by multiple reference electrodes. Potentials measured with the platinum

electrode were adjusted to refer to a standard hydrogen electrode by subtracting the reference electrode potential of -0.242 V.

### **Apparent $E_H$ control.**

A computer-controlled system was used to maintain the apparent redox potential at desired set points. Just as pH buffer capacities are calculated based on the change in solution pH after addition of an acid or base, “redox buffer capacities” were calculated based on the change in response of a platinum electrode after the addition of an oxidant or reductant. The apparent  $E_H$  was measured immediately prior to oxidant addition and thirty to sixty seconds after addition to allow for mixing. Calculated redox buffer capacities were stored in a six-membered array that was continually updated (i.e. the most recent value replaced the oldest value in the array). For each apparent  $E_H$  adjustment, the average of the redox buffer capacity array was multiplied by the initial apparent  $E_H$  measurement to determine the amount of oxidant (or reductant) necessary to maintain a given redox potential. This volume was then added, a new apparent  $E_H$  was measured, and a new redox buffer capacity was calculated. In this way solution changes were automatically followed and appropriate adjustments in oxidant/reductant addition were made.

A redox shift was accomplished in two stages. Since the redox buffer capacity of the solution at different redox potentials was generally unknown, the first stage of a redox shift was designed to gain redox buffer capacity information with which to proceed. Small arbitrarily chosen volumes of oxidant/reductant solution were added to the reactor, and redox buffer capacities were calculated and stored every three to five minutes until the six-membered array was filled. In the second stage, the average redox capacity was used to determine the amount of oxidant/reductant necessary for a 30 mV shift toward the desired set point. This stage proceeded until the redox potential was within 20 mV of the desired set point. The  $E_H$  maintenance stage followed. Redox buffer capacities were used to calculate the volume of oxidant or reductant solution necessary to maintain the

apparent  $E_H$  at the desired set point. Deaerated solutions of two oxidants, hydrogen peroxide ( $H_2O_2$ ) and potassium ferricyanide ( $K_3Fe(CN)_6$ ) were used for apparent  $E_H$  control.

### **Pt electrode care and response.**

Platinum electrodes were routinely cleaned using either a warm ( $70\text{ }^\circ\text{C}$ ) aqua regia solution (1:3 solution of concentrated  $HNO_3$  and  $HCl$ ) (35) or detergent and 1 M  $HCl$  as recommended by the electrode manufacturer (Analytical Sensors, Inc.). Calibration was performed using ZoBell's solution ( $3 \times 10^{-3}$  M potassium ferrocyanide and  $2 \times 10^{-2}$  M potassium ferricyanide in 0.1 M  $KCl$  (+0.43 V vs. SHE)) (35), and saturated quinhydrone solutions in pH 4 and pH 7 buffers. Electrode performance in standard solutions did not always reflect performance at the lower redox potentials and less highly poised redox environment of the reactor solution. Although electrode performance in the standard solutions was not changed, loss of electrode sensitivity in the reactor was observed over time. This loss of sensitivity was identified by unusually high redox buffer capacities, large  $E_H$  swings due to increased electrode response time, and changes in the relative response between the platinum and sulfide electrodes. To help identify reduced platinum electrode sensitivity, a second platinum electrode was added to the reactor system to serve as a check for the apparent  $E_H$ -controlling electrode. Electrodes were periodically replaced with new electrodes before signs of lost sensitivity appeared.

### **Culture conditions.**

The culture used in this study was originally obtained from municipal anaerobic digesters as described previously (74). The culture was maintained in a continuous-flow reactor with a 10 day hydraulic detention time for approximately six years. The volatile suspended solids concentration of the culture was maintained at 290 mg/L (SD = 60 mg/L) for three years. The reactor feed contained 91 mM acetate and approximately 3.4

$\mu\text{M}$  PCP, along with a trace mineral and vitamin formulation based on a modification of medium developed by Owen et al. (77) and described elsewhere (103). Nutrients were provided in sufficient excess to ensure that they would not become limiting during long-term, semi-batch experiments. A 0.75 mM aqueous PCP solution with a pH of approximately 10 was manually injected into the batch reactor.

### **Analytical techniques.**

For chlorophenol and acetate analysis, liquid samples were withdrawn from the batch reactor in 3.5 to 5.0 mL quantities using a 5 mL ground glass syringe with a six inch, 18-gauge needle. (For solids determinations, 26 mL were withdrawn in a 30 mL syringe.) Reactor samples were filtered through Gelman type A/E glass fiber filters with a nominal 1  $\mu\text{m}$  pore size, and approximately the first 3 mL of filtrate were discarded. Total suspended solids and volatile suspended solids were measured as described in Standard Methods for the Examination of Water and Wastewater (35).

Chlorophenol samples were prepared for gas chromatographic analysis using a modification of the acetylation and extraction method developed by Voss et al. and Perkins et al. (79, 111) and described elsewhere (103). Acetylated chlorophenols extracted in hexane were analyzed using a Hewlett-Packard model 5890 gas chromatograph with an electron capture detector as described previously (103). Compounds were identified by comparison with retention times of authentic standards.

Acetate was analyzed using a Dionex 4000I ion chromatograph with a conductivity detector. An Ionpac<sup>®</sup> AS4A anion analysis column and AG4A guard column with anion suppression were used for separation. Samples were eluted with a 1.8 mM carbonate/1.7 mM bicarbonate eluant at a flow rate of 2 mL/min. Dilute sulfuric acid (13.6 mM) was used as regenerant. Samples were diluted in eluant (1:25) to avoid water interference with the acetate peak.

A Fisher model 25V gas partitioner was used to measure  $\text{CO}_2$ ,  $\text{O}_2$ ,  $\text{N}_2$  and  $\text{CH}_4$  in 100  $\mu\text{L}$  headspace samples obtained with a 250  $\mu\text{L}$  Pressure-lok gas tight syringe

(Dynatech Precision Sampling Corp.; Baton Rouge, LA). Hydrogen was detected in headspace samples using a Hewlett Packard Model 5890 Series II gas partitioner with a thermal conductivity detector and argon carrier gas (reference flow = 7 mL/min; carrier flow = 17 mL/min). A 4 ft x 1/8 in stainless steel column, packed with molecular sieve 13X 45/60 (Supelco, MR 58723) was used for separation. Dissolved hydrogen concentrations in equilibrium with the headspace were calculated from headspace measurements using a Henry's Law constant at 30 °C, of  $1.31 \times 10^3$  L-atm/mol (107). The hydrogen detection limit was approximately 16 ppm (vol./vol.) in a 1 mL sample. This is equivalent to a detection limit in the aqueous phase of 0.01  $\mu$ M.

Gas production was periodically measured using either a bubble flow meter for instantaneous measurement, or by displacement of a saturated sodium sulfate/sulfuric acid solution in a closed gas trap.

### **Chemicals.**

Pentachlorophenol (99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). 2,3,4,5-Tetrachlorophenol (TeCP) and 3,4,5-trichlorophenol (TCP) (95+%) were obtained from Ultra Scientific (North Kingstown, RI).

### **RESULTS**

The effect of elevated redox potential on the rates of methanogenesis from acetate and the reductive dechlorination of PCP and 2,3,4,5-TeCP were evaluated in a bioreactor in which temperature, pH, and acetate concentration were held constant while the apparent  $E_H$  was controlled at different values using  $H_2O_2$  or  $K_3Fe(CN)_6$ .

### **First-order transformation of PCP and 2,3,4,5-TeCP.**

PCP was transformed by the culture via 2,3,4,5-TeCP to 3,4,5-TCP which accumulated. Below an initial PCP concentration of about 0.5  $\mu\text{M}$ , PCP transformation and 2,3,4,5-TeCP formation and transformation were modeled using a first-order kinetic model. Pseudo-first order PCP and 2,3,4,5-TeCP biotransformation rate constants and initial PCP and 2,3,4,5-TeCP concentrations were estimated by nonlinear,  $\chi^2$  optimization as described previously (55, 81, 103). Chlorophenol data and model curves for representative chlorophenol progress curves are shown in Figure 16. The concentration of 3,4,5-TCP is an order of magnitude higher than the other chlorophenols as a result of previous PCP additions. Changes in pseudo-first order transformation coefficients were used to evaluate the effect of elevated redox potential on reductive dechlorination. We have shown previously that transformation rate coefficients tend to increase with the amount of PCP converted to 3,4,5-TCP (103). Therefore, failure of pseudo-first order rate coefficients to increase with the amount of PCP transformed, or a tendency for coefficients to decrease was defined as an adverse effect.

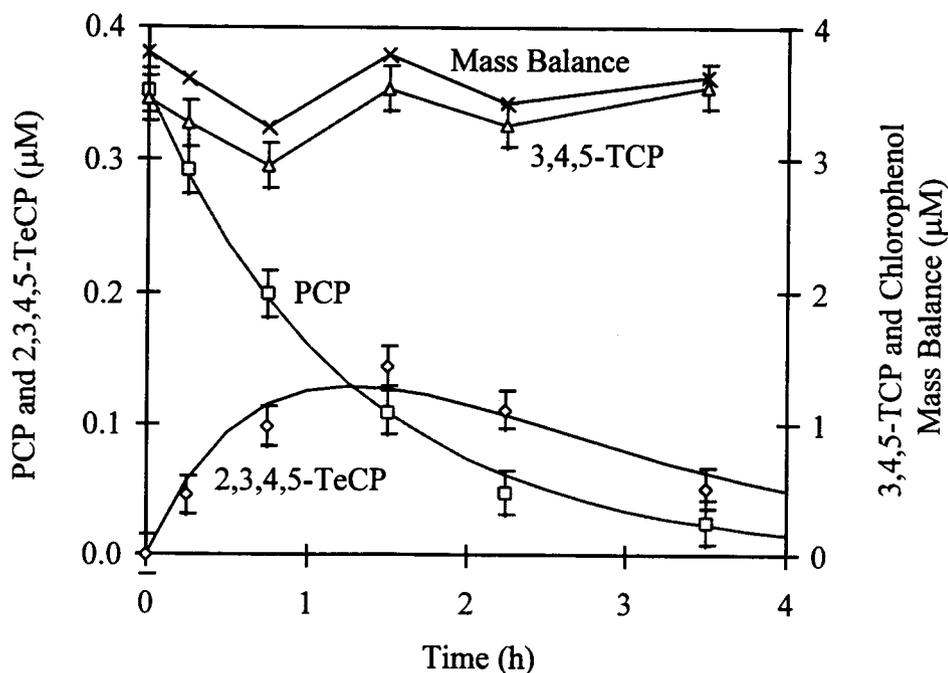


Figure 16. Representative progress curves and mass balance for PCP transformation to 3,4,5-TCP via 2,3,4,5-TeCP. First order model fits are indicated by solid lines. Error bars represent 1 standard error. 3,4,5-TCP concentrations are high due to accumulation from previous PCP transformations.

#### Apparent $E_H$ elevation with $H_2O_2$ .

Results from an experiment in which the apparent  $E_H$  was raised with  $H_2O_2$  on three occasions to three different potentials are shown in Figure 17. Prior to 75 h, the apparent  $E_H$  was allowed to stabilize at -0.26 V, acetate degradation was established at a specific degradation rate of 2.8 mmol acetate/g VSS-h, and 5 PCP additions totaling 1.7  $\mu$ M were reductively dechlorinated to 3,4,5-TCP (data not shown). The apparent  $E_H$  and the cumulative  $H_2O_2$  necessary to maintain each  $E_H$  are shown in Figure 17A. Since the apparent  $E_H$  returned to the steady, “natural” value of  $-0.26 \pm 0.01$  V between  $H_2O_2$  additions, cumulative  $H_2O_2$  added was reset to zero at the beginning of each redox maintenance period. As expected, the rate of  $H_2O_2$  addition (indicated by the slope of the cumulative  $H_2O_2$  curve) required to maintain a given apparent  $E_H$  increased as the  $E_H$  set

point was raised farther from the system's well poised apparent  $E_H$  of -0.25 to -0.26 V. The required rate of  $H_2O_2$  addition increased from approximately 0.033 mmol/h (SE = 0.0004 mmol/h) at an  $E_H$  set point of -0.20 V to 0.19 mmol/h at an  $E_H$  set point of -0.10 V (SE = 0.004 mmol/h) and approximately 0.36 mmol/h (SE = 0.001 mmol/h) at an  $E_H$  set point of 0 V. Increased difficulty in maintaining the reactor apparent  $E_H$  away from the natural system  $E_H$  was reflected in the wider scatter in the apparent  $E_H$  data as the  $E_H$  set point was increased (Figure 17). (For  $E_H$  set point = -0.2 V, Average  $E_H$  = -0.21 V; SD = -0.006 V. For  $E_H$  set point = -0.1 V, Average  $E_H$  = -0.099 V; SD = -0.006 V. For  $E_H$  set point = 0 V, Average  $E_H$  = -0.008 V; SD = 0.023 V).

Acetate consumption and methanogenesis were more rapidly and dramatically influenced by changes in the apparent  $E_H$  than were PCP and 2,3,4,5-TeCP transformation. Figure 17B shows the cumulative amount of acetate added to the reactor over the time period, and the measured acetate concentration. Since the acetate concentration remained relatively constant, the slope of the acetate addition curve equals the rate of acetate consumption by the consortium. During the first, relatively minor,  $E_H$  shift to -0.20 V, the rate of acetate degradation was slowed from approximately 2.7 mmol/gVSS-h to 2.4 mmol/gVSS-h. During the second, more pronounced  $E_H$  shift to -0.10 V, acetate consumption slowed and stopped approximately 4.5 h after redox control was initiated. Acetate consumption did not resume until the apparent  $E_H$  had returned to -0.26 V, 3.5 h after redox control was terminated. Acetate consumption and gas production continued until the apparent  $E_H$  was raised to 0 V, and did not resume at former levels even though the apparent  $E_H$  returned to -0.26 V.

Failure of acetate consumption to resume may have resulted from the combined effects of  $H_2O_2$  toxicity and inhibition due to the accumulation of 3,4,5-TCP to approximately 6  $\mu$ M. In three other experiments in which  $H_2O_2$  was added at comparable rates (0.30 to 0.46 mmol/h), acetate consumption resumed within 10% of the rate prior to apparent  $E_H$  elevation in all cases (Appendix C). Therefore,  $H_2O_2$  addition alone did not appear to be toxic to the culture at the doses used. Under normal redox conditions (-0.26

V), acetate consumption was reduced after the 3,4,5-TCP concentration exceeded  $6 \mu\text{M}$  (103).

In contrast to acetate consumption, reductive dechlorination of PCP to 2,3,4,5-TeCP and 3,4,5-TCP continued throughout periods of apparent  $E_{\text{H}}$  control. As shown in Figure 17C, the rate of PCP transformation increased during the first two periods of elevated apparent  $E_{\text{H}}$ . These rate increases were comparable to rate increases during experiments without  $\text{H}_2\text{O}_2$  addition, and may be due to growth of a subpopulation of reductively dechlorinating bacteria (103). Only when the apparent  $E_{\text{H}}$  was raised to 0 V, with a  $\text{H}_2\text{O}_2$  addition rate of 0.36 mmol/h, was the rate of PCP transformation slowed (Figure 17C). This decrease in the reductive dechlorination rate was exposure dependent: the pseudo-first order rate coefficient for PCP fell from  $1.9 \text{ h}^{-1}$ , two h after the initiation of redox control at 0 V, to  $0.2 \text{ h}^{-1}$  after approximately five h of redox potential control at 0 V. Although the rate of PCP transformation after the apparent  $E_{\text{H}}$  returned to -0.26 V from 0 V was higher than the lowest rate observed at an apparent  $E_{\text{H}}$  of 0 V, it was only 30% of the rate observed prior to the  $E_{\text{H}}$ -shift at 148 h. This reduction in dechlorinating activity may suggest that the dechlorinating organisms were sensitive to the relatively large amount of  $\text{H}_2\text{O}_2$  used to maintain the apparent  $E_{\text{H}}$  at 0 V. Alternatively, the reduced chlorophenol transformation rates may be related to the cessation of acetate consumption and methanogenesis (102).

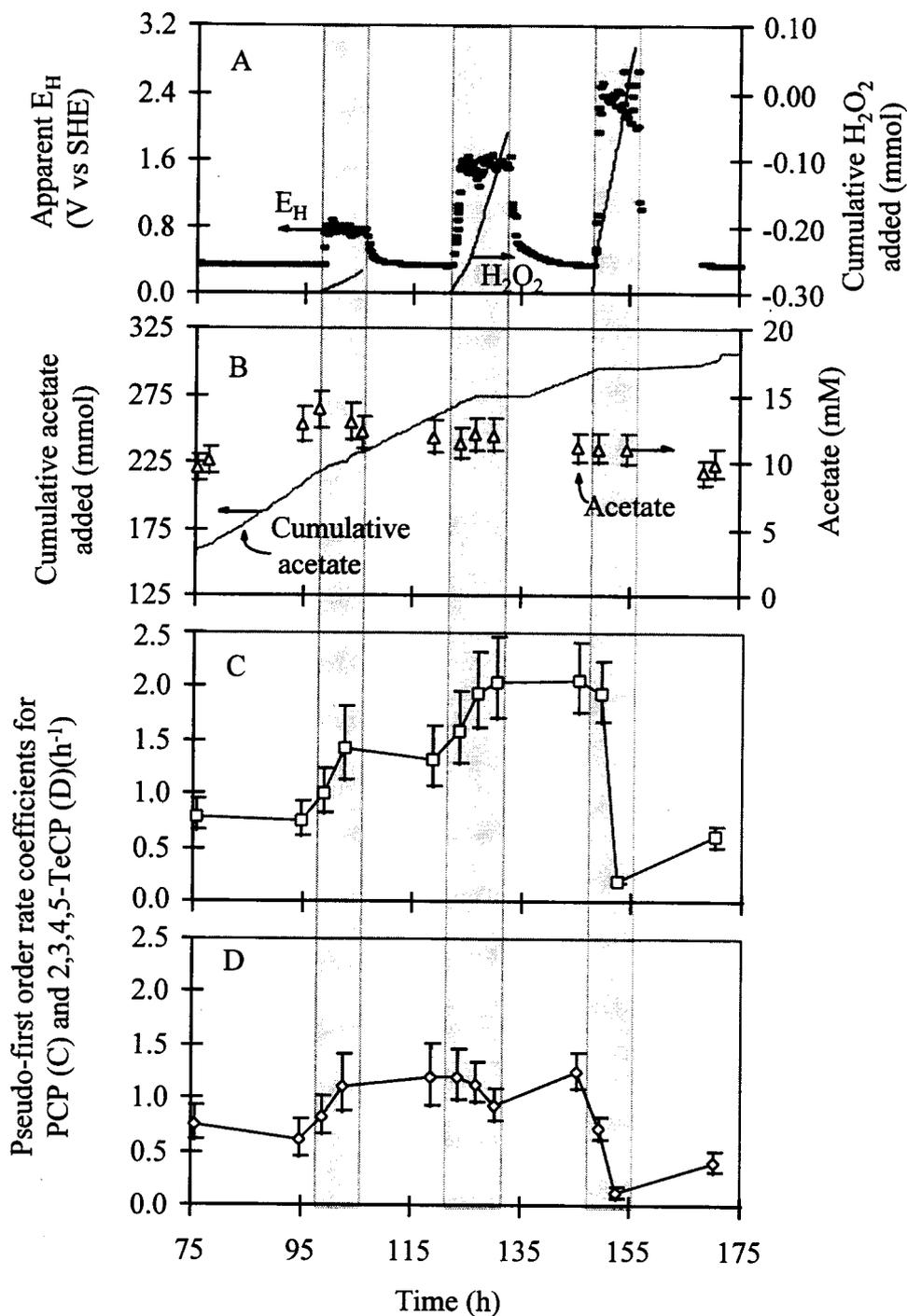


Figure 17. Amount of  $H_2O_2$  added, apparent  $E_H$ , acetate, and chlorophenol transformation rate data from an experiment in which the apparent  $E_H$  was elevated three times with  $H_2O_2$ . (A) Cumulative  $H_2O_2$  and apparent  $E_H$  measurements; (B) cumulative acetate added and the amount of acetate present within the reactor; and (C) best fit and 95% confidence interval for pseudo-first order rate coefficients and for PCP and (D) 2,3,4,5-TeCP. Shaded areas are periods of apparent  $E_H$  control. (A gap in the  $E_H$  data between h 156 and 167 was the result of computer difficulties.)

The rate of 2,3,4,5-TeCP transformation also increased during the first period of redox control (-0.2 V), but remained constant or decreased during the second period (-0.1 V), and was greatly reduced during the third period (0 V)(Figure 17D). Slowing of the 2,3,4,5-TeCP transformation rate was also exposure dependent. When the apparent  $E_H$  was raised to 0 V, the 2,3,4,5-TeCP transformation rate was reduced sequentially from  $0.7 \text{ h}^{-1}$ , 2 h after the initiation of redox control, to  $0.1 \text{ h}^{-1}$  after approximately 5 h of redox potential control.

In other experiments without  $\text{H}_2\text{O}_2$  addition, pseudo-first order rate coefficients for 2,3,4,5-TeCP and PCP either increased or decreased simultaneously (103). However, the rate of 2,3,4,5-TeCP transformation was affected more than the rate of PCP transformation by increases in the apparent  $E_H$  (e.g. the second period of apparent  $E_H$  control to -0.1 V in Figure 17). In addition, in separate experiments, the apparent  $E_H$  was elevated fourteen times, and a total of twenty-four PCP additions were transformed to 3,4,5-TCP. In 19 of these 24 PCP additions at elevated redox potentials, the pseudo-first order rate coefficient for 2,3,4,5-TeCP either increased by a smaller percentage or decreased by a greater percentage (compared to the immediately preceding rate coefficient) than the pseudo-first order rate coefficient for PCP (Appendix 4).

### **$\text{H}_2$ addition during periods of elevated apparent $E_H$ .**

Theoretical, or “true,” redox potential is inversely related to  $\text{H}_2$  concentration, i.e. lower redox potentials occur at higher  $\text{H}_2$  concentrations (104). *Apparent*  $E_H$  measurements may or may not reflect  $\text{H}_2$  concentrations, depending on the chemical matrix (78). In previous work we observed sequential reductions in PCP and 2,3,4,5-TeCP transformation rates by this culture when  $\text{H}_2$  was depleted (102). The exposure-dependent decrease in PCP and 2,3,4,5-TeCP transformation rates at elevated redox potentials was similar to rate reductions following  $\text{H}_2$  depletion (102). To determine if lowered  $\text{H}_2$  concentrations during periods of elevated redox potential contributed to

reduced chlorophenol transformation rates,  $H_2$  was added to the reactor while an elevated apparent  $E_H$  was maintained.

Results from an experiment in which the apparent  $E_H$  twice was raised to  $-0.10$  V with  $K_3Fe(CN)_6$  added at a rate of approximately  $0.28$  mmol/h are shown in Figure 18. During the first 90 h of this experiment the apparent  $E_H$  stabilized at  $-0.26$  V, the specific acetate degradation rate stabilized at  $2.4$  mmol/gVSS-h, and six PCP additions totaling  $2$   $\mu$ M were transformed to 3,4,5-TCP (data not shown). Apparent  $E_H$ , and dissolved  $H_2$  data after 90 h are presented in Figure 18A.  $H_2$  concentrations fell to below detection approximately 2.5 h after each redox shift was begun.  $H_2$  was added to the reactor headspace at 104 and 126 h, during transformation of PCP additions 3 and 5, and while the apparent  $E_H$  was maintained at  $-0.10$  V (Figure 18).

The apparent  $E_H$  was not particularly sensitive to changes in  $H_2$  concentration. The measured  $H_2$  concentration fell nearly an order of magnitude between 140 and 150 h (Figure 18A), while the measured apparent  $E_H$  remained constant at  $-0.26$  V. (No external apparent  $E_H$  control was used during this period.) In addition, actual  $H_2$  concentrations tended to be one to two orders of magnitude higher than would be calculated by applying the Nernst equation (at pH 7 and  $30$   $^{\circ}$ C) to apparent  $E_H$  measurements. For example, the expected  $H_2$  concentration at equilibrium with an  $E_H$  of  $-0.26$  V is  $4.5 \times 10^{-6}$  atm ( $3.4 \times 10^{-3}$   $\mu$ M dissolved), not the  $1.3 \times 10^{-5}$  to  $1.3 \times 10^{-4}$  atm partial pressure ( $0.01$  to  $0.1$   $\mu$ M dissolved) observed between 140 and 150 h (Figure 18A).

As in the experiment in which  $H_2O_2$  was used as the oxidant (Figure 17B), when  $K_3Fe(CN)_6$  was used, acetate consumption and gas production stopped shortly after the apparent  $E_H$  was raised to  $-0.1$  V (Figure 18B). When the apparent  $E_H$  returned to  $-0.26$  V, acetate consumption resumed at a reduced rate of approximately  $1$  mmol/gVSS-h, compared to the initial acetate consumption rate of  $2.4$  mmol/gVSS-h. The rate of acetate consumption was further reduced to approximately  $0.6$  mmol/gVSS-h after the second period of redox potential elevation (Figure 18B). These decreases in the rate of acetate consumption may indicate that  $K_3Fe(CN)_6$  was more toxic to the methanogens than

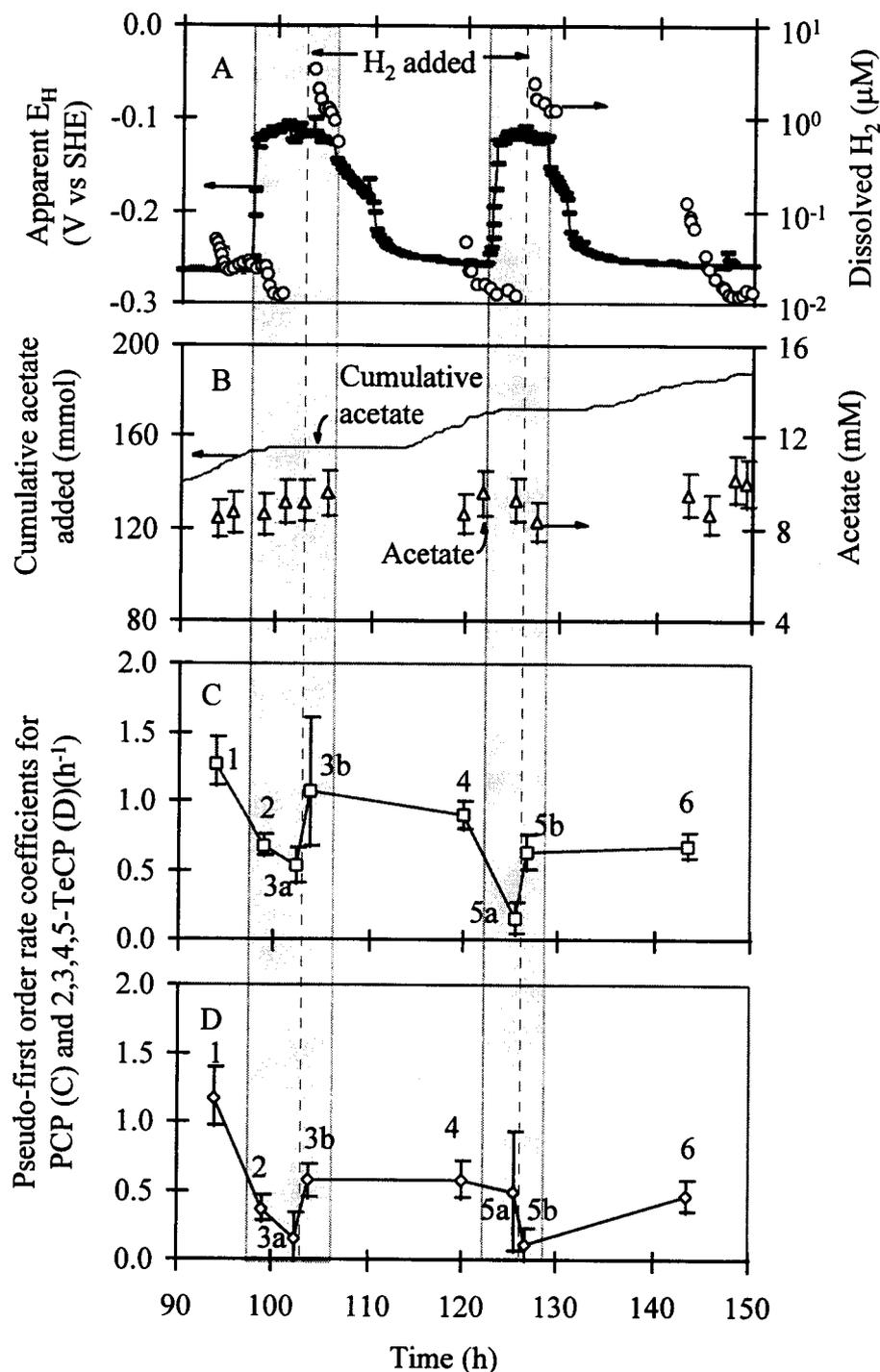


Figure 18.  $H_2$  concentration, apparent  $E_H$ , acetate, and chlorophenol transformation rate data from an experiment in which the apparent  $E_H$  was elevated twice with  $K_3Fe(CN)_6$ . (A) Dissolved  $H_2$  and apparent  $E_H$ ; (B) cumulative acetate added and acetate concentration; and (C) best fit and 95% confidence interval for pseudo-first order rate coefficients for PCP and (D) 2,3,4,5-TeCP.  $H_2$  (2 mL) was added to the reactor headspace during transformation of additions 3 and 5. Rate coefficients before  $H_2$  addition are labeled "a," and after  $H_2$  addition are labeled "b."

$\text{H}_2\text{O}_2$  at the concentrations necessary for apparent  $E_{\text{H}}$  control. (Recall that when  $\text{H}_2\text{O}_2$  was used as the oxidant, acetate consumption generally resumed within 10% of initial rates.) However, since  $\text{H}_2$  was added to the reactor, a shift in methanogenic metabolism from acetate consumption to  $\text{H}_2$  and  $\text{CO}_2$  consumption also may account for reductions in the rate of acetate degradation.

Two PCP additions were made after the apparent  $E_{\text{H}}$  was elevated to  $-0.1$  V at 97 h (labeled 2, 3a and 3b in Figure 18). The pseudo-first order rate coefficient for PCP addition 2, was roughly half of the rate coefficient for PCP addition 1 (prior to the apparent  $E_{\text{H}}$  elevation). The initial rate coefficient of addition 3 (3a, prior to  $\text{H}_2$  addition) was decreased further. Similarly, the initial pseudo-first order rate coefficient for addition 5 (5a), at  $-0.1$  V was greatly decreased compared to the pseudo-first order rate coefficient of addition 4, at  $-0.26$  V. However, during both periods of apparent  $E_{\text{H}}$  control at  $-0.1$  V, when  $\text{H}_2$  was added to the reactor, the pseudo-first order rate coefficient for PCP transformation increased two to four times (Figures 18A and 18C).

Data and first-order model fits for additions 1, 2, and 3 are presented in Figure 19. PCP addition 1, made at an apparent  $E_{\text{H}}$  of  $-0.26$  V, was substantially transformed to 3,4,5-TCP within three h (Figure 19). PCP addition 2, made at  $-0.1$  V, was less rapidly transformed, and about half of the initial PCP concentration remained as PCP or 2,3,4,5-TeCP after three h. When  $\text{H}_2$  was added to the reactor during transformation of PCP addition 3, PCP and 2,3,4,5-TeCP progress curves were rapidly influenced. Dashed lines indicate the chlorophenol progress curves predicted from data before  $\text{H}_2$  addition and solid lines indicate the first-order model fit of actual concentration data (Figure 19). Progress curves following  $\text{H}_2$  addition showed more rapid transformation of both PCP and 2,3,4,5-TeCP than was predicted by first-order model fits of concentration data prior to  $\text{H}_2$  addition (Figure 19).

When the apparent  $E_{\text{H}}$  was raised to  $-0.1$  V with  $\text{H}_2\text{O}_2$ , pseudo-first order rate coefficients for PCP continued to increase with the amount of PCP transformed to 3,4,5-TCP (Figure 17). In contrast, when  $\text{K}_3\text{Fe}(\text{CN})_6$  was used to maintain the apparent  $E_{\text{H}}$  at  $-0.1$  V, pseudo-first order rate coefficients for both PCP and 2,3,4,5-TeCP decreased (Figure 18). This difference in response of reductive dechlorination rates to the two

oxidants may indicate greater toxicity of  $K_3FeCN_6$  relative to  $H_2O_2$ . An alternative explanation is variation in measurement of the apparent  $E_H$  between the two experiments.

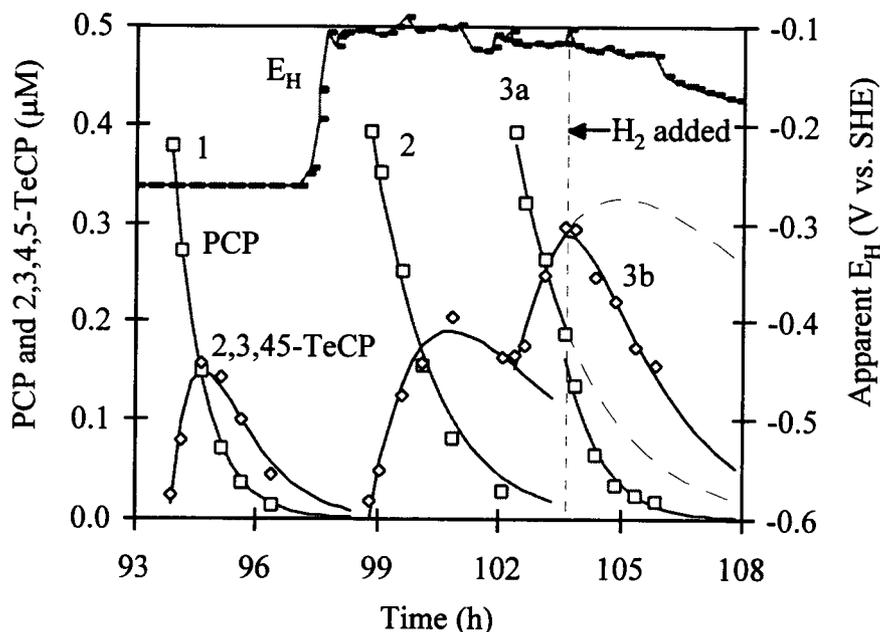


Figure 19. PCP and 2,3,4,5-TeCP progress curves for PCP additions 1, 2, and 3. Pseudo-first order model fits are indicated by solid lines. The time of addition of 2 mL of  $H_2$  is indicated. Dashed lines indicate the predicted PCP and 2,3,4,5-TeCP concentrations if  $H_2$  hadn't been added.

Pseudo-first order rate coefficients for 2,3,4,5-TeCP were also reduced by elevations in apparent  $E_H$  (Figure 18D). The 2,3,4,5-TeCP transformation rate coefficient for PCP addition 2, at -0.1 V, was one third of the rate coefficient of addition 1, at -0.26 V, and the transformation coefficient for 3a was half of the rate coefficient for addition 2 (Figure 18D). Just as  $H_2$  addition increased the rate of PCP transformation,  $H_2$  added during transformation of addition 3 caused the rate coefficient for 2,3,4,5-TeCP to increase by a factor of 3 (Figures 18D, and 19). Rate coefficient data for 2,3,4,5-TeCP during the second period of apparent  $E_H$  control (addition 5) were anomalous. Wide error bars on transformation coefficient 5a made interpretation of the results difficult.  $H_2$  addition, appeared to have little effect on the rate of 2,3,4,5-TeCP transformation during

the second period of apparent  $E_H$  maintenance. Data and first-order model fits for addition 5 are presented in Figure 20. As in PCP addition 3, addition of  $H_2$  altered the progress curves from what was predicted prior to  $H_2$  addition. The large 95% confidence interval for 2,3,4,5-TeCP rate coefficient 5a, and unexpectedly low 2,3,4,5-TeCP rate coefficient 5b (Figure 18D), following  $H_2$  addition, may have resulted from a lack of sufficient data points to adequately define first-order curves during this period (Figure 20). Nevertheless, the addition of  $H_2$  rapidly increased the rate of PCP transformation even though the apparent  $E_H$  was maintained at -0.1 V.

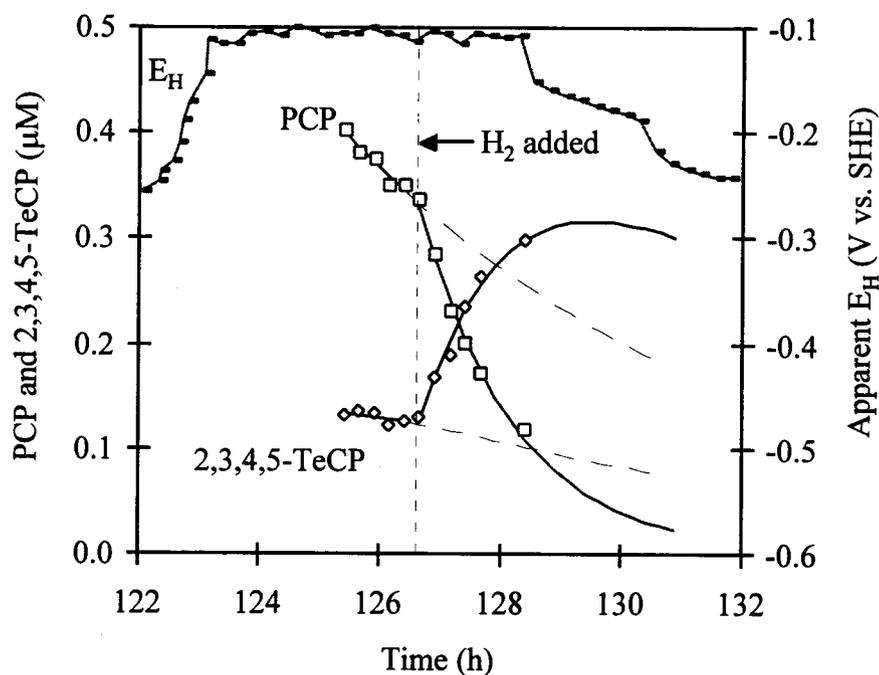


Figure 20. The effect of  $H_2$  addition on the reductive dechlorination of PCP addition 5. The apparent  $E_H$  was maintained at -0.1 V with  $K_3Fe(CN)_6$ . Pseudo-first order model fits are indicated by solid lines. The time of addition of 2 mL of  $H_2$  is indicated. Dashed lines indicate first-order model predictions if  $H_2$  hadn't been added.

## DISCUSSION

Prior studies of pure cultures of anaerobic bacteria have shown that some species exhibit a range of tolerance to elevated apparent  $E_H$  (27, 75, 109, 112). In the current study, PCP and 2,3,4,5-TeCP reductive dechlorination was shown to persist under conditions of elevated redox potential that inhibited methanogenesis. Acetate degradation and methane production were slowed or stopped at lower apparent redox potentials and after shorter exposure times than were reductive dechlorination of PCP and 2,3,4,5-TeCP. However, the highest dechlorination rates were obtained during periods of low redox potential (-0.25 V) and active methanogenesis from acetate.

Fetzer and Conrad reported methanogenesis from methanol by *M. barkeri* at redox potentials as high as +0.05 V in the absence of oxygen (27). The ability to produce methane at this elevated apparent  $E_H$  was dependent on cell density, methanol concentration and the total amount of oxidant (mixtures of  $K_4Fe(CN)_6$  and  $K_3Fe(CN)_6$ ) used to maintain the apparent  $E_H$ . In contrast, in our study, methanogenesis was halted at an apparent redox potential of -0.1 V maintained with either  $H_2O_2$  or  $K_3Fe(CN)_6$  (Figures 17 and 18). The observed difference in tolerance to elevated apparent  $E_H$  may be explained in part by lower cell densities in the current study (approximately 20 to 30% of those reported by Fetzer and Conrad), and the longer duration of exposure. In addition, in experiments in which  $K_3Fe(CN)_6$  was added, the cumulative amount of oxidant necessary to maintain a given apparent  $E_H$  yielded a final concentration greater than 0.5 mM.  $K_3Fe(CN)_6$  concentrations above 0.5 mM were found to inhibit methanogenesis from methanol by *M. barkeri* (27).

The return to an apparent  $E_H$  of -0.26 V between redox potential perturbations was a function of both abiotic (reaction with components in the chemical matrix such as reduced iron and sulfide) and biotic activity, such as the effect observed by Fetzer and Conrad (27). Interestingly, the apparent redox potential did not reflect all changes in  $H_2$  concentration such as the order of magnitude change between h 140 and 150 in the experiment in which the apparent  $E_H$  was elevated with  $K_3Fe(CN)_6$  (Figure 18). In

addition, as mentioned previously, actual  $H_2$  concentrations tended to be one to two orders of magnitude higher than theoretical  $H_2$  concentrations based on the apparent  $E_H$ . Such discrepancies often arise from the “non-equilibrium” behavior of complex redox systems.

The nearly instantaneous increase in reductive dechlorination rate when  $H_2$  was added during periods of elevated apparent  $E_H$  (Figures 19 and 20) suggests that the apparent  $E_H$  may be less important than the availability of hydrogen to the rate of PCP transformation by this culture. Madsen and Aamand found a similar dependence on hydrogen by a PCP-dechlorinating, methanogenic enrichment culture also derived from municipal digester sludge (63). Yeast extract served as the source of endogenous hydrogen in serum bottle cultures with and without added sulfate. PCP and 2,3,4,5-TeCP transformation rates were reduced significantly when hydrogen concentrations were lowered due to the activity of sulfate reducing bacteria (63).

In previous work, we have shown that rates of PCP and 2,3,4,5-TeCP transformation depend on the availability of  $H_2$  (102). In addition, it has been suggested that acetoclastic methanogens may contribute a significant portion of the endogenous  $H_2$  produced by the culture (52, 59, 80, 102). Since  $H_2$  production by methanogens is linked to methane production (52, 59, 80), conditions of elevated apparent  $E_H$  sufficient to curtail methane production probably stopped  $H_2$  production as well. As demonstrated in Figure 18, when an oxidant was added to maintain an elevated apparent  $E_H$ , endogenous  $H_2$  production was not sufficient to maintain  $H_2$  concentrations above the detection limit of approximately  $0.01 \mu\text{M}$ . Reductions in PCP transformation rates with time during periods of elevated apparent  $E_H$  may reflect this depletion of the  $H_2$  pool.

Pseudo-first order rate coefficients for 2,3,4,5-TeCP tended to be lower than those for PCP (Figures 17 and 18), and were more greatly decreased during periods of elevated apparent  $E_H$ . The first order model shown in Figure 16 and presented elsewhere (103) is based on the assumption that both compounds are metabolized concurrently rather than sequentially. This indicates that under normal, low redox conditions ( $-0.25 \text{ V}$  to  $-0.26 \text{ V}$ ), PCP is not significantly preferred over 2,3,4,5-TeCP as an electron acceptor. Under these

conditions, the ratio of pseudo-first order rate coefficients for 2,3,4,5-TeCP transformation to PCP transformation ranges from approximately 0.8 to 1.0. However, under conditions of elevated apparent  $E_H$  and corresponding lowered reductant ( $H_2$ ) supply, the ratio of pseudo-first order rate coefficients for 2,3,4,5-TeCP transformation to rate coefficients for PCP transformation falls as low as 0.2 to 0.4. PCP may be preferentially dechlorinated, since the theoretical energy yield from the conversion of PCP to 2,3,4,5-TeCP is somewhat higher than for the conversion of 2,3,4,5-TeCP to 3,4,5-TCP (23).

Since both  $H_2$  and  $O_2$  strongly influence the redox chemistry of aqueous environments, separating concentration effects from redox potential effects is difficult. Several researchers have focused on the relationship between  $O_2$ , apparent redox potential and the viability of several species of anaerobic bacteria (27, 47, 48, 72, 109, 112). In general,  $O_2$  concentration appears to be more important in determining the growth of a variety of anaerobes than the apparent  $E_H$  of the media in which they are cultured (27, 72). Similarly, in this study, apparent  $E_H$  measurements did not appear to be as critical as  $H_2$  concentration in influencing the rates of reductive dechlorination of PCP and 2,3,4,5-TeCP.

## CONCLUSIONS

- Methanogenesis continued during a relatively mild apparent  $E_H$  shift to -0.2 V with  $H_2O_2$ , but was halted when the apparent  $E_H$  was raised to -0.1 V with either  $H_2O_2$  or  $K_3Fe(CN)_6$ . Methanogenesis resumed when the apparent  $E_H$  returned to -0.26 V.
- During periods of large apparent  $E_H$  shifts in which methanogenesis stopped, transformation of PCP and 2,3,4,5-TeCP continued at progressively slower rates.
- The rate of 2,3,4,5-TeCP transformation was reduced more by elevated redox potentials than was the rate of PCP transformation.

- $H_2$  concentrations fell to below detection when the apparent  $E_H$  was raised to -0.1 V with  $K_3Fe(CN)_6$ .
- $H_2$  added during periods of elevated apparent  $E_H$  caused reductive dechlorination rates to increase.

## Conclusions and Engineering Significance

This chapter summarizes the main findings of this research, briefly discusses their implications for design of biological systems, and presents suggestions for future research.

### **Growth of a dechlorinating subpopulation, as suggested by increases in reductive dechlorination rates.**

**Conclusions:** Increases in pseudo-first-order rate coefficients for PCP and 2,3,4,5-TeCP were related to the amount of PCP transformed to 3,4,5-TCP. Rate coefficient increases occurred up to cumulative PCP additions of  $6 \pm 1 \mu\text{M}$ , at which point enough 3,4,5-TCP had accumulated to adversely affect the culture i.e. acetate consumption and methanogenesis began to slow. Rate coefficient increases were greater than could be accounted for by the overall increase in biomass (as measured by VSS), the amount of acetate consumed, or the duration of experiments. However, bacterial growth, supported by reductive dechlorination of PCP and 2,3,4,5-TeCP, could account for the observed rate increases. The time for reductive dechlorination activity to double, which may be assumed to approximate a population doubling time, was estimated from exponential curves of pseudo-first order rate coefficients versus time using non-linear optimization techniques. The estimated activity doubling time of 1.7 d was comparable to population doubling times of pure cultures of reductively dechlorinating bacteria (14, 18, 64).

**Engineering Significance:** Determining the growth and nutritional requirements of a small subpopulation of bacteria in a mixed culture is difficult. Complex interactions between bacterial species vary depending on the species present and on environmental conditions. Considerable effort and time may be spent isolating and characterizing pure bacterial cultures, but not all species are culturable using methods currently available. Furthermore, behavior of a species in pure culture may not be indicative of its behavior or

survivability in a mixed culture or in the environment. Therefore, methods are needed for evaluating how subpopulations in mixed cultures or natural systems respond (e.g., growth, death, metabolic activity) to environmental changes. Such methods would facilitate the design and optimization of bioremediation and treatment approaches that employ complex communities of microorganisms.

In this research, a method was developed for estimating a PCP-dependent, reductive dechlorination activity yield and activity doubling time for a subpopulation in an anaerobic mixed culture. A similar methodology may be applicable to other anaerobic systems and halogenated aromatic compounds. Results of this research indicate that the potential growth of a bacterial subpopulation in response to low concentrations of a halogenated aromatic contaminant should not be discounted when estimating degradation rates and compound persistence. Furthermore, response of the culture to accumulation of a toxic metabolite (3,4,5-TCP), and observed increases in PCP and 2,3,4,5-TeCP transformation rates in response to repeated, small additions of PCP, indicate an advantage of obtaining rate parameters from repeated additions of a test compound rather than relying on single progress curves.

**Research Needs:** MPN techniques, or investigations with defined bacterial consortia could be used to confirm the relationship between activity increases and bacterial growth. The general applicability of this method to other anaerobic, bacterial consortia and other halogenated compounds should be investigated further.

### **Role of H<sub>2</sub> supply in the reductive dechlorination of PCP.**

**Conclusions:** During active methanogenesis, PCP and 2,3,4,5-TeCP reductive dechlorination rates increased with time. Conversely, transformation rates decreased with time when methanogenesis was inhibited by 3,4,5-TCP accumulation, even though acetate concentrations remained constant. This indicates that the availability of acetate, alone, did not support continued transformation rate increases. However, when H<sub>2</sub> was added manually to the reactor in the absence of methanogenesis, reductive dechlorination activity continued for a prolonged period. In combination, these observations suggest

that  $H_2$  produced during methanogenesis may serve as the electron donor for reductive dechlorination of PCP to 3,4,5-TCP by this culture. Further, a 2:1,  $H_2$ :PCP, stoichiometry was observed, as would be predicted from reaction equations. During continuous addition of  $H_2$  at concentrations above 0.016%, observed increases in pseudo-first order rate coefficients (described above) were smaller than when  $H_2$  was endogenously supplied during methanogenesis, suggesting that excessive  $H_2$  concentrations may adversely affect reductive dechlorination.

**Engineering Significance:** Establishing conditions favorable for growth of bacteria capable of reductive dechlorination is key to successful bioremediation of some pollutants. Results of this research suggest that optimum dechlorination rates may be achieved when  $H_2$  concentration is controlled between certain levels. When  $H_2$  was not sufficiently available, the culture used in this research was not able to use acetate as an electron donor to sustain rapid rates of reductive dechlorination of PCP. The same may be true of other cultures as well. When  $H_2$  was exogenously supplied at a concentration above 0.016 %, optimum rates of PCP transformation did not occur, perhaps indicating that  $H_2$  can interfere with growth of key species. Alternatively, an unknown nutritional requirement may be supplied during acetoclastic methanogenesis that is not supplied during methanogenesis from  $H_2$  and  $CO_2$ . Complex substrates, such as pyruvate or fatty acids other than acetate, that are anaerobically converted to both  $H_2$  and acetate, may be more effective than acetate alone in supporting reductive dechlorination reactions.

**Research Needs:** The role of  $H_2$  as electron donor in reductive dechlorination reactions, and its possible interference with growth of reductively dechlorinating bacteria should be investigated further. Pure cultures of reductively dechlorinating bacteria could be grown at several different concentrations of  $H_2$  to determine  $H_2$  dependent differences in growth rates or yields. The role of syntrophic bacteria in supplying  $H_2$  or some other nutritional requirement to bacteria performing reductive dechlorination should also be investigated. Nutritional requirements can be identified through experiments in which different compounds are systematically tested to see if growth of a target bacterial species is enhanced. Experiments with defined co-cultures or highly enriched mixed cultures (i.e.

with only a few species of bacteria) may also be performed to identify syntrophic relationships.

### **Influence of elevated apparent $E_H$ on methanogenesis and reductive dechlorination.**

**Conclusions:** Methanogenesis continued during relatively minor apparent  $E_H$  shifts from -0.26 V to -0.2 V with  $H_2O_2$ , but stopped when the apparent  $E_H$  was raised to -0.1 V with either  $H_2O_2$  or  $K_3Fe(CN)_6$ . Methanogenesis resumed when the apparent  $E_H$  returned to -0.26 V. When methanogenesis stopped during periods of large apparent  $E_H$  shifts, PCP and 2,3,4,5-TeCP transformation rates declined, with 2,3,4,5-TeCP transformation rates being more adversely affected than PCP transformation rates. Transformation rate declines at elevated apparent  $E_H$  resembled rate reductions observed when  $H_2$  was removed from the reactor by purging with  $H_2$ -free gas. When the apparent  $E_H$  was elevated to -0.1 V with  $K_3Fe(CN)_6$ , the  $H_2$  concentration dropped to below detection (approximately  $1.3 \times 10^{-5}$  atm; 0.01  $\mu$ M aqueous). When  $H_2$  was added to the reactor, while maintaining the apparent  $E_H$  at -0.1 V, an immediate increase in the PCP transformation rate occurred. These observations suggest that apparent  $E_H$ , alone, is not a good indicator of the rate of reductive dechlorination processes.

**Engineering Significance:** Because apparent  $E_H$  and  $H_2$  concentration are closely related, it is difficult to change one without affecting the other. Although results of this research suggest that the apparent  $E_H$  is not as direct an indicator of PCP and 2,3,4,5-TeCP reductive dechlorination rates as  $H_2$  concentration is, apparent  $E_H$  measurement is generally less difficult to perform than measurement of very low concentrations of  $H_2$ . Therefore, a monitoring protocol for a bioremediation or treatment process might include continuous apparent  $E_H$  measurement with periodic  $H_2$  concentration measurement.

The observation that reductive dechlorination continued during periods when methanogenesis was inhibited indicates that methanogenesis, in itself, is not critical to the reductive dechlorination process (except, perhaps, to provide a nutrient, as mentioned above). However, in natural systems, high ambient  $H_2$  concentrations typically are

associated with regions of decreased redox potential and active methanogenesis (58, 60). Therefore, reductive dechlorination in natural systems is more likely to be supported in zones of active methanogenesis where  $H_2$  is available. However, because both methanogens and bacteria conducting reductive dechlorination could survive for up to ten hours at elevated apparent  $E_H$ , it appears that short-term exposure to elevated redox conditions need not severely impact a treatment process.

**Research Needs:** To eliminate questions regarding potential oxidant toxicity, other oxidants, besides the two used in these experiments, should be investigated for their effect on reductive dechlorination rates. Also, the effect of decreasing the apparent  $E_H$  (below the “natural” level of -0.26 V) on reductive dechlorination rates should be investigated to determine if reductive dechlorination rates could be enhanced by more reduced conditions. Because 2,3,4,5-TeCP transformation rates were more severely affected by elevated apparent  $E_H$  than PCP transformation rates were, investigations into the relationship between the number of chlorine substituents and the effect of elevated apparent  $E_H$  could be illuminating.

### **Reductive dechlorination pathway.**

**Conclusion:** Reductive dechlorination of PCP by this culture occurred only at the *ortho* positions; 2,3,4,5-TeCP was produced transiently and 3,4,5-TCP accumulated. No additional metabolites were observed under any environmental conditions investigated.

**Engineering Significance:** Pentachlorophenol's *ortho* dechlorination products tend to be more toxic than *meta* and *para* dechlorination products (2, 16, 36, 73, 87, 100, 113). In addition, less highly chlorinated chlorophenols are more soluble, making them more mobile in aquifers, (6) and more easily degraded aerobically (37, 98, 99),. Therefore, accumulation of 2,3,4,5-TeCP and 3,4,5-TCP would be an undesirable outcome in a bioremediation or treatment process. Although complete mineralization of PCP by an anaerobic enrichment culture has been reported (66), most studies with

anaerobic consortia have shown only partial dechlorination of PCP (12, 43, 44, 46, 54, 74, 114). Sequential anaerobic/aerobic treatment of PCP would be a desirable approach.

**Research Needs:** A greater understanding of the nutritional and environmental requirements of bacterial communities capable of carrying out all three (*ortho*, *meta*, and *para*) reductive dechlorination steps is needed. The feasibility of PCP bioremediation and treatment strategies employing sequential anaerobic/aerobic processes should be evaluated.

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## **Appendices**

## Appendix A

### Culture Conditions and Feed Preparation

**Culture description.** The culture used in this study was originally obtained from anaerobic digester sludge from the Corvallis, OR municipal wastewater treatment plant. The culture was maintained in a continuous-flow reactor with a 10 day hydraulic detention time for approximately six years. The reactor feed contained 91 mM acetate and  $3.5 (\pm 0.6) \mu\text{M}$  PCP along with a trace mineral and vitamin formulation based on a modification of medium developed by Owen et al. (77). The steady state biomass concentration was 290 (SD =  $\pm 60$ ) mg/L volatile suspended solids (VSS) over a period of approximately three years.

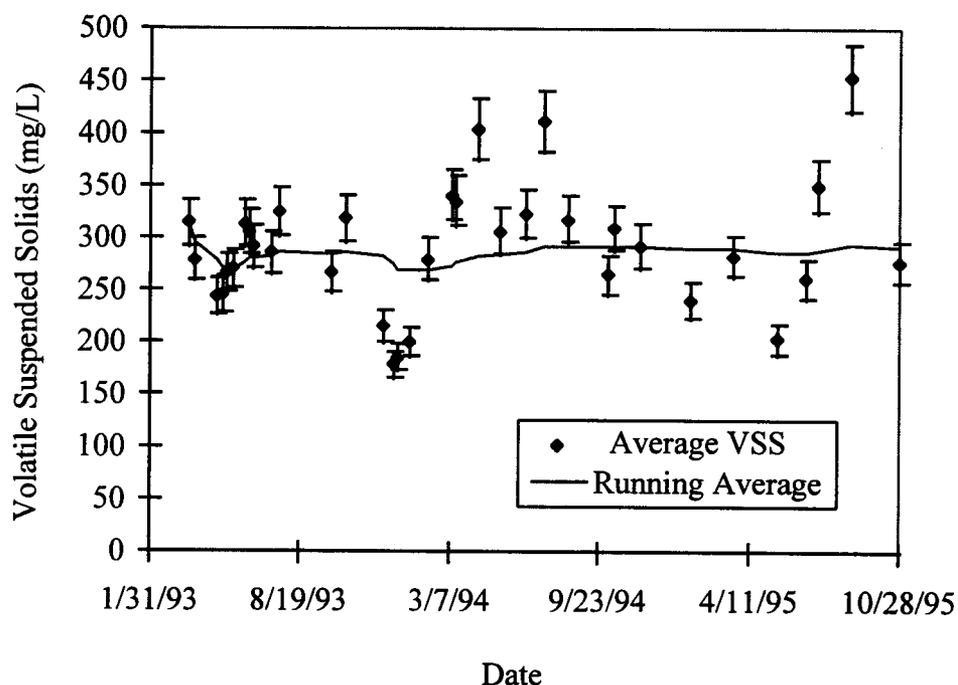


Figure A.1 Volatile suspended solids concentration within the continuous-flow reactor.

**Continuous-flow reactor design.** A schematic of the continuous-flow reactor in which the methanogenic culture was maintained is presented in figure A.2. The reactor (10 L liquid volume) was constructed of Kimax beaded process pipe (6 in I.D.) with stainless steel, Teflon-lined flange fittings (Ace Glass Co.; Vineland, N.J.) which sealed aluminum top and bottom plates. The reactor lid contained ports for nutrient addition, liquid and headspace sampling, and gas venting. Feed solution was pumped with an FMI

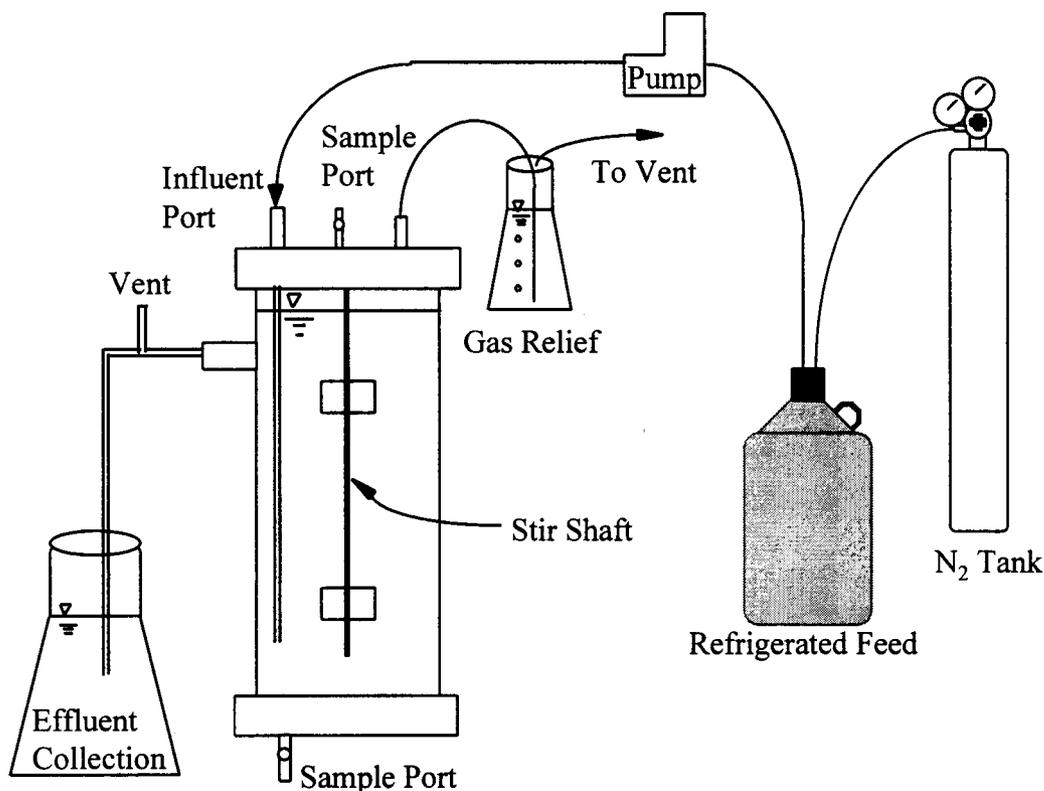


Figure A.2. Schematic of continuous flow reactor

RP-G6 piston pump (Fluid Metering Inc.; Oyster Bay, N.Y.) from a refrigerated feed reservoir, through Teflon and glass tubing, to a point approximately 15 cm from the reactor bottom. Effluent was removed by gravity flow from 5 cm below the liquid surface. To provide mixing, an electric motor (Bodine Electric Co.; Chicago, IL) continuously rotated a glass shaft (60 rpm) with two, 45 cm<sup>2</sup> Teflon paddles, located at the

end of the shaft and at the shaft's midpoint. Transfer from the continuous-flow reactor to the batch reactor was accomplished by siphon through the glass feed tube at the top of the reactor.

**Feed storage and preparation.** Feed was prepared in 12 L batches in a 24 L glass carboy and was stored in 4 L amber glass reagent bottles with screw caps in a refrigerator maintained at approximately 4 °C. Feed was pumped directly from each bottle, in turn, through teflon tubing inserted through a rubber stopper which replaced the cap of the feed bottle. The rubber stopper also contained a port through which N<sub>2</sub> was continuously supplied at a low rate. N<sub>2</sub> purging was done to reduce the O<sub>2</sub> concentration within the feed and to lessen microbial growth within the feed container.

The trace mineral and vitamin formulation was based on a modification of medium developed by Owen et al. (77). Stock solutions labeled S3 through S8, after Owen et al., are presented in Table A.1. Concentrations of stock solutions and volumes added to the feed were adjusted to provide excess nutrients during prolonged quasi-batch experiments in which acetate was added, but nutrients were not. Concentrations were also modified so that standard volumetric pipet sizes such as 50- and 100-mL volumes could be used in feed preparation. In addition, NiCl<sub>2</sub>·6H<sub>2</sub>O was added to the mineral stock solution (S4) to satisfy the Ni requirement of some anaerobes (97). Stock solutions S3 through S7 were refrigerated.

The saturated PCP solution (S8) was prepared by adding excess PCP crystals to deionized water (treated by reverse osmosis) in a 2 L reagent bottle. The PCP solution was continuously stirred on a magnetic stirrer for a minimum of 12 d. Crystals were visible in the mixture. The PCP solution was filtered through a Gelman A/E glass fiber filter to remove residual crystals. A brown paper bag was placed over the PCP solution to limit light exposure.

Table A.1 Stock solutions for feed preparation

Solution	Volume prepared (L)	Compound	Mass Added (g)
S3	1	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	35.24
S4	2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	27.06
		NH <sub>4</sub> Cl	43.10
		MgCl <sub>2</sub> ·6H <sub>2</sub> O	194.40
		KCl	140.46
		MnCl <sub>2</sub> ·6H <sub>2</sub> O	2.16
		CoCl <sub>2</sub> ·6H <sub>2</sub> O	3.24
		H <sub>3</sub> BO <sub>3</sub>	0.616
		CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.292
		Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.276
		ZnCl <sub>2</sub>	0.227
		NiCl <sub>2</sub> ·6H <sub>2</sub> O	2.20
		S5	0.1
S6	0.1	Na <sub>2</sub> S·9H <sub>2</sub> O	50
S7	0.5	biotin	0.10
		folic acid	0.10
		pyridoxine hydrochloride	0.50
		riboflavin	0.25
		thiamin	0.25
		nicotinic acid	0.25
		pantothenic acid	0.25
		B <sub>12</sub>	0.0053
		p-aminobenzoic acid	0.25
		thioctic acid	0.25
S8	---	Saturated PCP solution	

Feed was prepared as follows:

1. A 20 L glass carboy was filled with approximately 8 L of deionized water and 1.2 L of tap water. (Tap water (10%) was added after December, 1993 as a potential source of trace nutrients not provided in the original feed recipe.)
2. Glacial acetic acid (30 mL) was measured in a 50-mL graduated cylinder and added.
3. Anhydrous sodium acetate (46.7 g) gradually was added through a plastic funnel, and rinsed into the carboy with deionized water.
4. The  $(\text{NH}_4)_2\text{HPO}_4$  stock solution (S3) (50 mL) was measured in a volumetric pipet and added.
5. Vitamin stock solution (S7) (100 mL) was added. Since the stock solution was not completely dissolved, it was vigorously mixed on a magnetic stirrer while 100 mL of solution was measured with a volumetric pipet.
6. Mineral stock solution (S4) (200 mL) was added. This solution was also mixed on a magnetic stirrer while 2 x 100 mL was measured with a 100-mL volumetric pipet.
7. Filtered, saturated PCP solution (1.2 L) was added.
8.  $\text{NaHCO}_3$  (12.6 g) was added.
9. The fluid volume was brought up to 12 L, and the feed was shaken, with periodic venting of  $\text{CO}_2$ , until all of the  $\text{NaHCO}_3$  was dissolved.
10. Feed was transferred to amber glass storage bottles and placed in the refrigerator.
11. To avoid formation of iron sulfide precipitates in the feed reservoirs, 1-mL aliquots of stock solutions containing  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  (S5) and  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  (S6) were added to the reactor directly, every other day.

The measured PCP concentration of the feed for the continuous flow reactor is shown in Figure A.3.

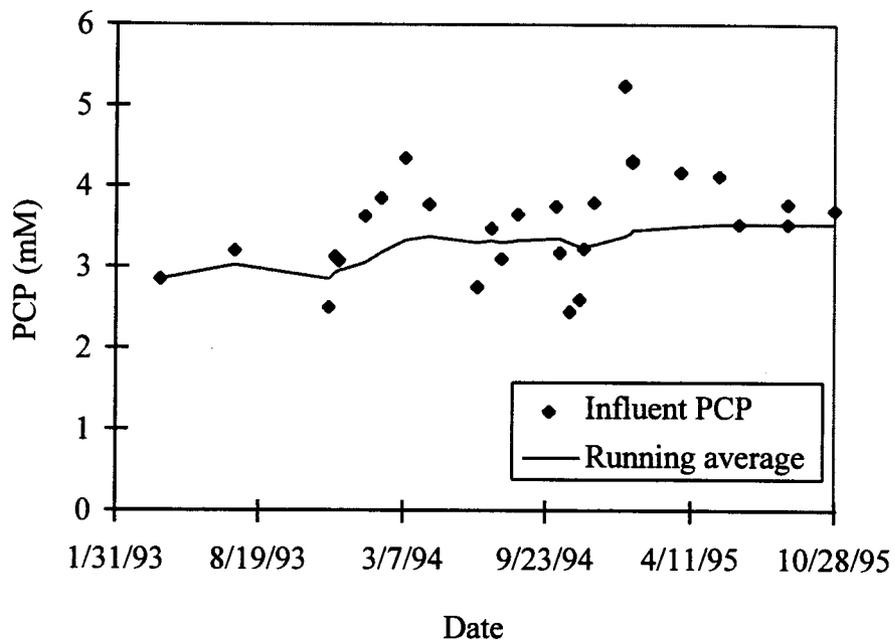


Figure A.3 PCP concentration in the influent to the continuous-flow reactor.

## Appendix B

### Experimental Protocol

Quasi-batch experiments were performed in a computer-monitored and feed back controlled 2.5 L bioreactor. A schematic of the reactor system is shown in Figure A.4.

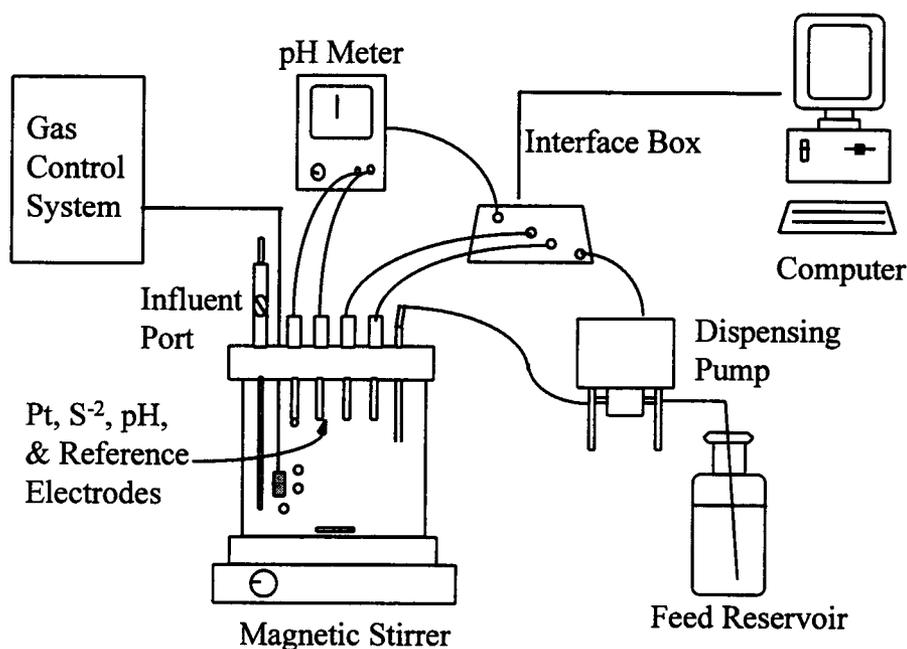


Figure A.4. Schematic of the quasi-batch bioreactor.

The 2.5 L batch reactor included the following features: 1) continuous monitoring of pH (Orion Ross; 81-01), sulfide (Orion; 94-16 BN), and redox potential (platinum) (Analytical Sensors, Inc.; OR100031 BN) electrodes at a controllable time interval, and 2) feedback-controlled pH maintenance coupled to acetate concentration maintenance, 3) the ability to change and maintain the redox potential at a pre-determined value using a variety of oxidants and reductants, 4) tracking of reactor volume changes due to sample removal, or addition of reagents, and 5) the ability to purge the reactor with a defined gas mixture of up to three gases (Tylan General mass flow controllers). To avoid large

changes in liquid volume during acetate concentration, pH and redox potential maintenance, low-volume piston pumps, (Model RHSY, Fluid Metering, Inc., Oyster Bay, N.Y.) modified to accept digital input signals, were used to deliver microliter quantities of concentrated reagents to the reactor. All electrodes were referred to a single reference electrode (Orion double junction; 90-02 BN) to avoid problems caused by multiple reference electrodes. In the batch reactor, parameters such as acetate concentration, pH, and temperature could be held constant while multiple PCP additions were made over experimental periods lasting up to two weeks.

A schematic of a typical experiment is shown in Figure A.5, and a step by step protocol follows.

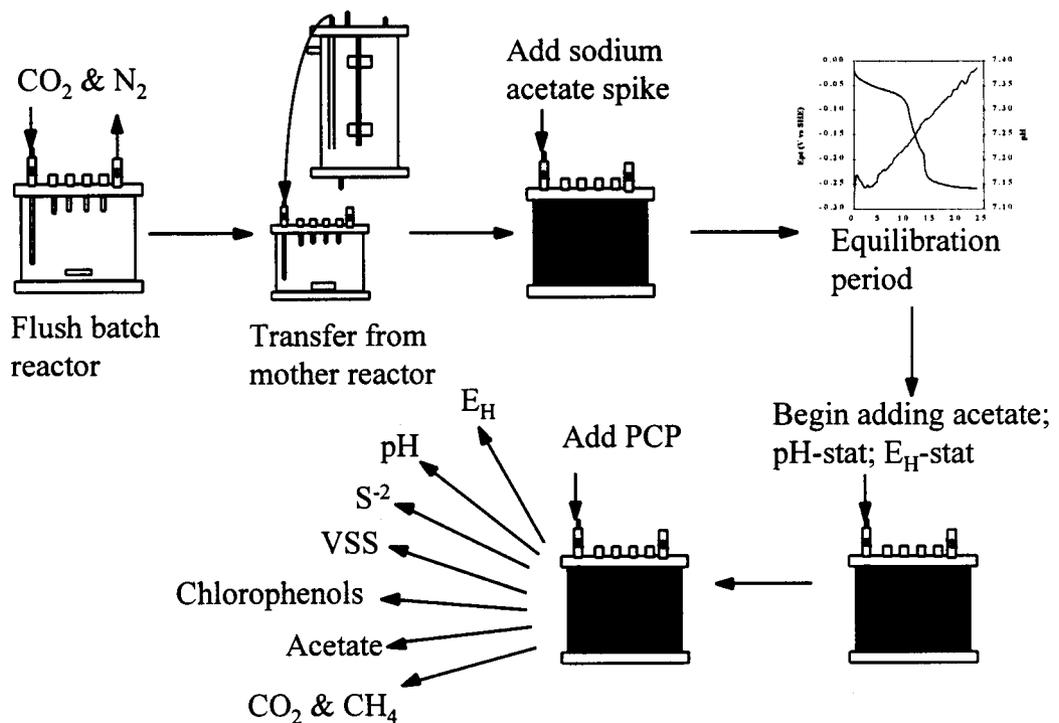


Figure A.5. Schematic of experimental protocol.

## Experimental protocol

### Phase I--Transfer

1. Rinse, dry and tare glass fiber filters for solids analyses.
2. Turn on gas flow controller and allow it to warm up for thirty minutes.
3. Prepare acetate and sulfate primary standard by adding 0.6950 g NaAc and 0.200 g  $\text{Na}_2\text{SO}_4$  to 500 mL of deionized water in a volumetric flask. (Concentrations:  $[\text{Ac}^-] = 1000 \text{ mg/L} = 16.95 \text{ mM}$ ;  $[\text{SO}_4^{2-}] = 270.5 \text{ mg/L} = 2.82 \text{ mM}$ .) This solution may be kept up to one month if refrigerated, but bacterial growth may occur.
4. If refrigerated, remove standard solution from the refrigerator and allow it to come to room temperature before preparing standards.
5. Allow Fisher gas partitioner and/or HP 5890 GC with a thermal conductivity detector to warm up for 20-30 minutes.
6. Mix 4 L of IC eluant (Add 0.382 g dried  $\text{Na}_2\text{CO}_3$  and 0.286 g  $\text{NaHCO}_3$  to 2 L volumetric flask fill to volume with deionized water.) and 4 L of IC regenerant (Fill 2 L volumetric flask half full with deionized water. Add 1.45 mL of concentrated  $\text{H}_2\text{SO}_4$ , and fill to volume.). (Save 2 L of eluant for standard and sample dilution.)
7. Replace IC solutions, and purge for 15 minutes.
8. Replace reference electrode's internal (saturated Ag/AgCl) and external (10%  $\text{KNO}_3$ ) filling solutions.
9. Insert teflon rods in electrode holders and connect reactor to gas line.
10. Set gas flow controllers to desired settings: e.g.  $\text{CO}_2 = 110\%$  of full scale;  $\text{CH}_4 = 85\%$  of full scale. (Total flow =  $166.5 \text{ std. cm}^3/\text{min}$ , and the  $\text{CO}_2:\text{CH}_4$  ratio is 24.4:75.6.) Open gas valves, and begin purging reactor. (Purge 2-3 hours.) Place gas relief tubing in a water reservoir to observe pressure release.
11. Prepare acetate and sulfate standard dilutions by adding 1, 2, 3, 4, and 5 mL of primary standard to IC eluant in 100-mL volumetric flasks. (Concentrations:  $[\text{Ac}^-] = 0.17, 0.34, 0.51, 0.68, 0.85 \text{ mM}$ ;  $[\text{SO}_4^{2-}] = 0.028, 0.056, 0.085, 0.11, 0.14 \text{ mM}$ .)
12. Run an acetate and sulfate standard curve on the IC.

13. Dissolve 4.92 g of sodium acetate anhydrous in 100 mL of distilled deionized water in a 125-mL ehrlenmeyer flask. Cap with a rubber septum.
14. Calibrate pH electrodes.
15. Prepare 1:10 dilution of glacial acetic acid and 0.3 M acetate solution. Add 25 mL of glacial acetic acid and 6.15 g anhydrous sodium acetate to deionized water in a 250-mL volumetric flask. Flush dispenser. ( $[Ac^-] = 2.04 \text{ M}$ .) Glacial acetic acid is always prepared at the same concentration, but different concentrations of sodium acetate may be used.
16. When  $O_2$  is no longer measurable in the headspace, exchange rods in the electrode holders with electrodes. Continue purging 30-60 minutes.
17. Purge sodium acetate solution (prepared in step 13) with nitrogen.
18. Check reactor atmosphere with gas partitioner or GC.
19. Change reactor septum.
20. Transfer culture from the continuous-flow reactor to the batch reactor. Siphon through the reactor feed tube (suspended to mid-depth). Start siphon with a pipet bulb. Place gas relief tubing in water reservoir to observe pressure release.
21. Add acetate solution.
22. Connect electrodes and begin the program to monitor electrodes.
23. Measure gas composition of reactor head space.
24. Briefly bubble  $CO_2$  into the reactor to relieve a vacuum created by  $CO_2$  in the reactor headspace dissolving into the liquid. Stop bubbling when a positive pressure develops within the reactor, and bubbles are apparent at the vent.

#### **Phase II--Acetate Maintenance**

25. Establish the pH set point. (Acetate won't be added if the pH is below this set point. When the pH rises due to acetate consumption, the program automatically begins adding acetate/acetic acid solution.)
26. Wait for the apparent  $E_H$  to stabilize (generally below  $-0.25 \text{ V}$ ).
27. Measure acetate periodically, and adjust the ratio of acetate to acetic acid in the feed as necessary.
28. Take 30 mL VSS sample.

**Phase III--PCP addition and monitoring**

29. Place 25 mL of 200 mg/L (0.75 mM) PCP solution in a small ehrlenmeyer flask capped with a septum. Purge with nitrogen.
30. After a period of steady acetate concentration is established, add the PCP solution to the reactor with a syringe to achieve the desired concentration.
31. Periodically take samples for acetate and PCP analysis. Perform head space analyses.
32. Repeat PCP addition and sampling, as desired.
33. Continue monitoring acetate, and VSS. Adjust acetate concentration of the feed as necessary to maintain constant acetate concentration within the reactor.

**Phase IV--Apparent  $E_H$  control**

34. Purge 300 mL of deionized water with  $N_2$  gas.
35. Prepare oxidant solution: 0.05 M  $K_3FeCN_6$  (Add 1.65 g to 60 mL deaerated water in a 100-mL volumetric flask. Fill to volume.) or 0.05 M  $H_2O_2$  (Add 1.5 mL of 30%  $H_2O_2$  to 200 mL deaerated water in a 250-mL volumetric flask. Fill to volume.)
36. Calibrate dispense pump for oxidant addition.
37. Flush pump with oxidant, and place tubing in reactor lid. (Purge reactor while placing tubing in the lid so that  $O_2$  is not introduced.)
38. Begin  $E_H$ -stat.

## **Appendix C**

### **Data**

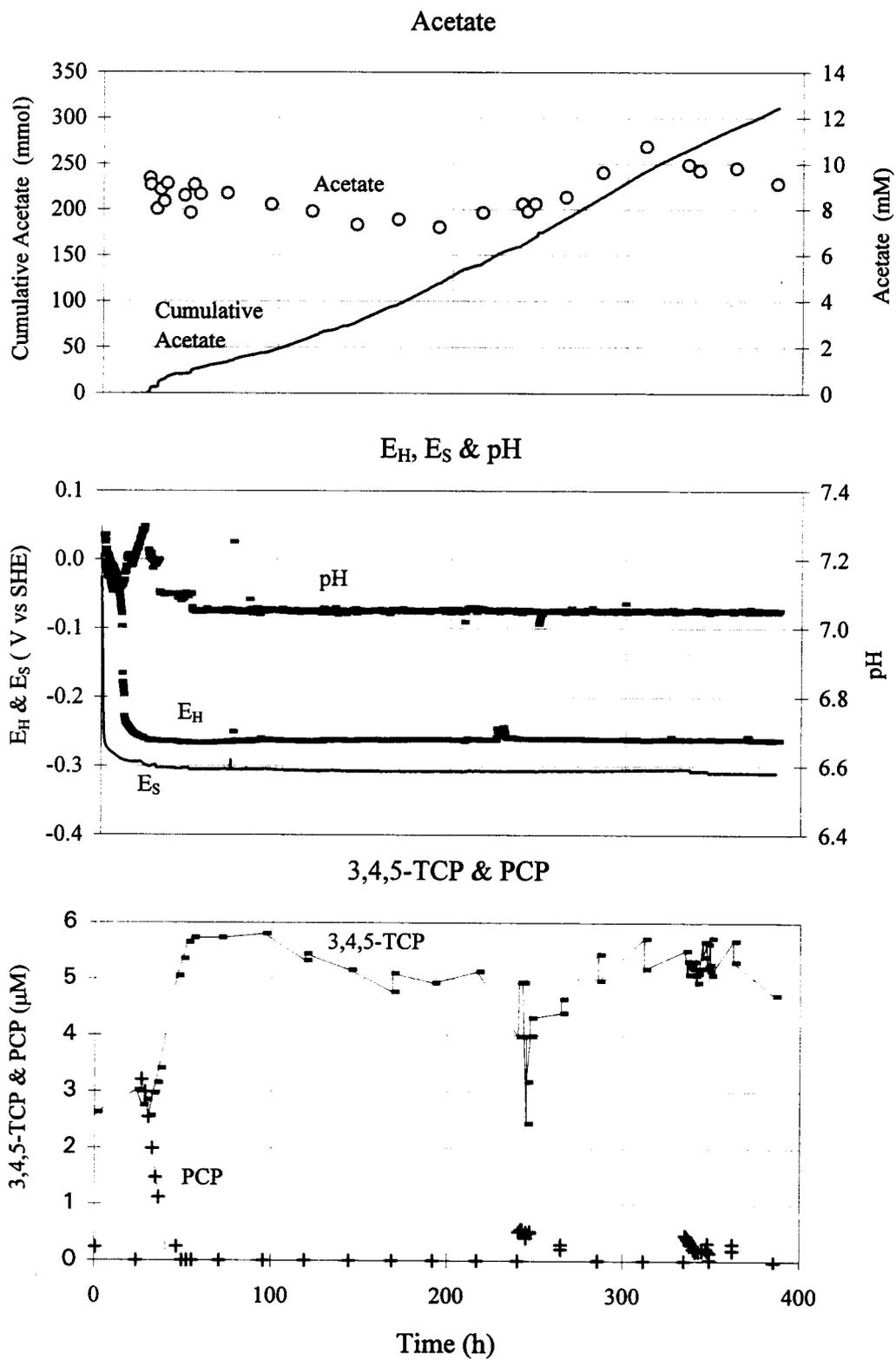


Figure A.6. Summary data for February '94 experiment.

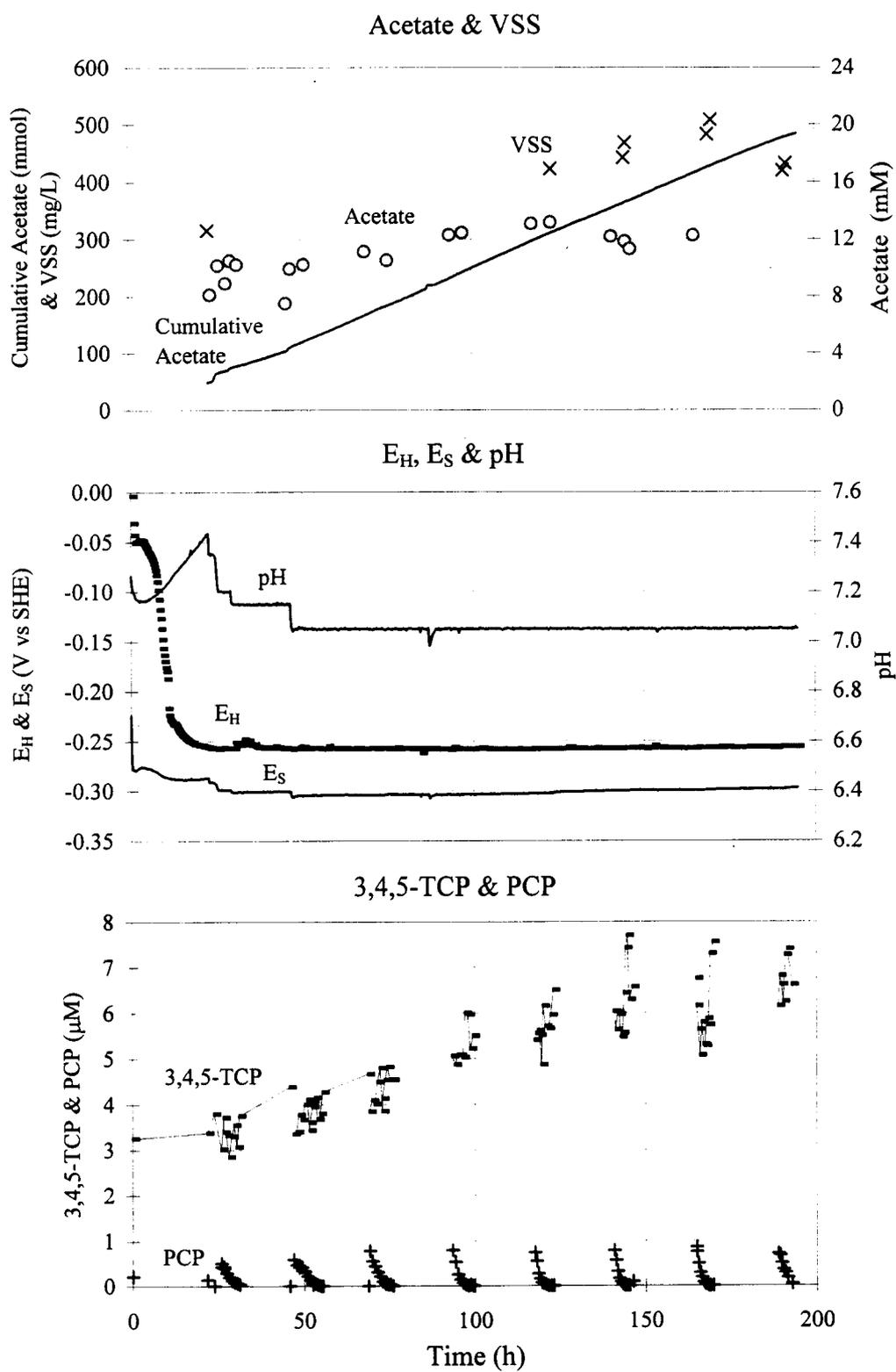


Figure A.7. Summary data for March '94 experiment.

Table A.3. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
March '94

Date	Add Time	Addition	$E_H$ (V)	$k_P$ ( $h^{-1}$ )	Upper $k_P$ ( $h^{-1}$ )	Lower $k_P$ ( $h^{-1}$ )	PCP(0)	LSS PCP	$\chi^2$ PCP
17-Mar	47.1	1	-0.26	0.54	0.68	0.41	0.61	1.2E-03	1.79
18-Mar	74.2	2*	-0.26	0.75	0.86	0.65	0.34	1.8E-03	2.66
19-Mar	96.0	3	-0.26	0.82	0.97	0.69	0.31	2.1E-03	3.18
20-Mar	99.9	4	-0.26	1.13	1.43	0.91	0.27	1.7E-03	2.53
21-Mar	118.6	5	-0.26	1.40	1.74	1.15	0.34	1.1E-03	1.62
22-Mar	124.8	6	-0.26	1.41	1.69	1.18	0.31	2.9E-04	0.44
23-Mar	149.0	7	-0.26	1.57	1.94	1.29	0.39	3.6E-03	5.45
24-Mar	172.9	8	-0.26	1.08	1.27	0.29	0.53	1.4E-02	22.25

\*Stirring was interrupted prior to this period.

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_P$
1	0.41903	nd	nd	0.18	5.0E-04	3.11	4.90	1.29
2	0.553074	nd	nd	0.26	2.9E-03	18.05	20.70	1.35
3	0.606129	nd	nd	0.39	3.2E-03	20.53	23.72	1.35
4	0.717852	nd	nd	0.37	2.5E-03	15.88	18.41	1.58
5	0.888539	nd	nd	0.29	3.0E-03	18.97	20.58	1.58
6	0.864378	nd	nd	0.35	9.4E-03	59.34	59.78	1.63
7	0.777753	nd	nd	0.25	1.4E-03	8.51	13.97	2.01
8	0.45308	nd	nd	0.31	1.6E-03	10.61	32.87	2.39

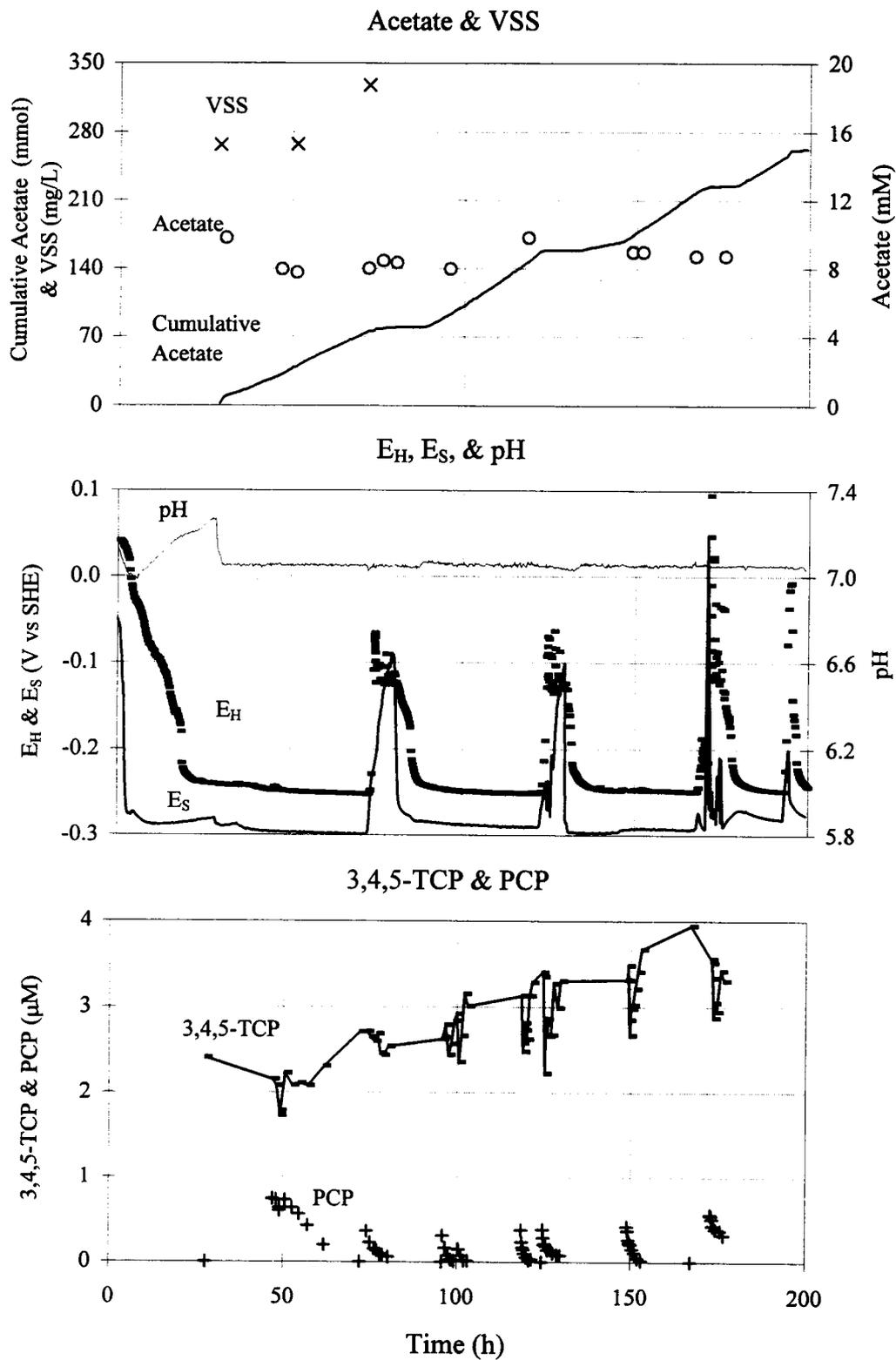


Figure A.8. Summary data for June '94 experiment.

Table A.3. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
June '94

Date	Add Time	Addition	$E_H$ (V)	$k_P$ ( $h^{-1}$ )	Upper $k_P$ ( $h^{-1}$ )	Lower $k_P$ ( $h^{-1}$ )	PCP(0)	LSS PCP	$\chi^2$ PCP
21-Jun	47.1	1	-0.25	0.18	nd	nd	0.61	4.8E-03	3.64
22-Jun	74.2	2	-0.10	0.32	0.42	0.23	0.34	1.4E-03	2.37
23-Jun	96.0	3	-0.25	0.90	1.34	0.60	0.31	1.9E-04	3.30
23-Jun	99.9	4	-0.25	0.94	1.48	0.49	0.27	3.4E-04	1.42
24-Jun	118.6	5	-0.25	1.17	1.55	0.86	0.34	2.3E-03	5.77
24-Jun	124.8	6	-0.10	0.40	0.56	0.28	0.31	1.1E-02	9.51
25-Jun	149.0	7	-0.25	0.61	0.78	0.47	0.39	3.7E-03	8.24
26-Jun	172.9	8	-0.10	0.15	0.20	0.10	0.53	7.5E-03	7.21

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_P$
1	0.12	nd	nd	0.08	2.9E-02	27.86	31.50	0.69
2	0.14	0.24	0.04	0.04	7.4E-04	0.36	2.731	0.43
3	0.70	1.11	0.41	0.05	1.9E-04	0.85	4.145	0.77
4	0.47	0.78	0.21	0.12	2.3E-04	2.87	4.283	0.50
5	0.73	1.08	0.49	0.04	2.8E-03	2.26	8.034	0.63
6	0.10	0.18	0.02	0.07	8.8E-04	3.30	12.813	0.24
7	0.44	0.62	0.29	0.05	2.8E-03	0.80	9.046	0.72
8	0.17	0.47	0.00	0.00	1.7E-03	1.70	8.916	1.12

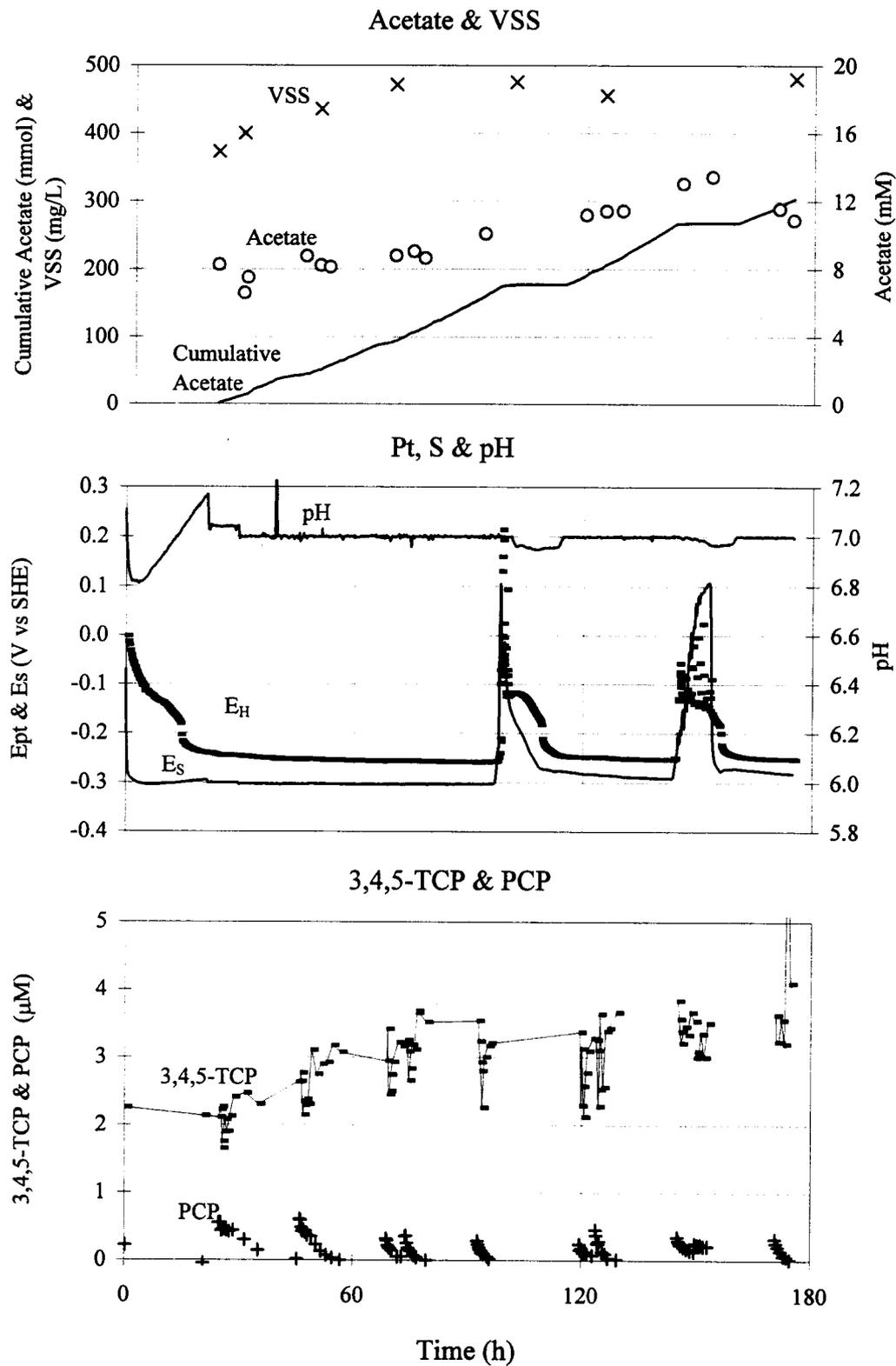


Figure A.9. Summary data for July '94 experiment.

Table A.4. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
July '94

Date	Add Time	Addition	$E_H$ (V)	$k_P$ ( $h^{-1}$ )	Upper $k_P$ ( $h^{-1}$ )	Lower $k_P$ ( $h^{-1}$ )	PCP(0)	LSS PCP	$\chi^2$ PCP
12-Jul	24.83	1	-0.25	0.1	0.13	0.10	0.55	2.3E-02	12.26
13-Jul	46.08	2	-0.25	0.28	0.32	0.25	0.61	1.9E-02	9.83
14-Jul	68.83	3	-0.25	0.47	0.63	0.32	0.30	2.4E-03	1.19
14-Jul	73.83	4	-0.25	0.62	0.77	0.48	0.35	7.0E-03	3.38
15-Jul	92.83	5	-0.25	0.56	0.78	0.36	0.25	3.7E-03	1.77
16-Jul	119.58	6	-0.25	0.25	0.46	0.08	0.18	4.3E-03	2.08
16-Jul	123.83	7	-0.25	0.60	0.72	0.49	0.42	8.6E-03	4.22
17-Jul	145.33	8	-0.1	0.23	0.33	0.14	0.31	3.1E-03	1.52
17-Jul	149.83	9	-0.1	0.06	0.21	0.00	0.24	2.8E-04	0.14
18-Jul	170.85	10	-0.25	0.51	0.71	0.36	0.29	2.3E-03	1.12

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_P$
1	0.13	0.16	0.11	0.01	5.6E-03	17.09	29.349	0.8
2	0.30	0.33	0.26	0.03	1.6E-02	48.82	58.642	1.0
3	0.63	0.90	0.42	0.03	7.4E-04	2.30	3.487	0.7
4	0.77	0.98	0.60	0.05	1.0E-03	3.07	6.452	0.8
5	0.69	1.05	0.40	0.02	5.6E-04	1.74	3.517	0.8
6	0.18	0.55	0.00	0.03	2.6E-04	0.80	2.884	1.4
7	0.41	0.49	0.34	0.09	1.0E-02	31.54	35.762	1.5
8	0.11	0.25	0.00	0.03	4.2E-04	1.31	2.827	2.1
9	0.02	0.22	0.00	0.15	2.9E-04	0.89	1.033	3.6
10	0.48	0.69	0.30	0.05	3.1E-04	0.95	2.066	1.1

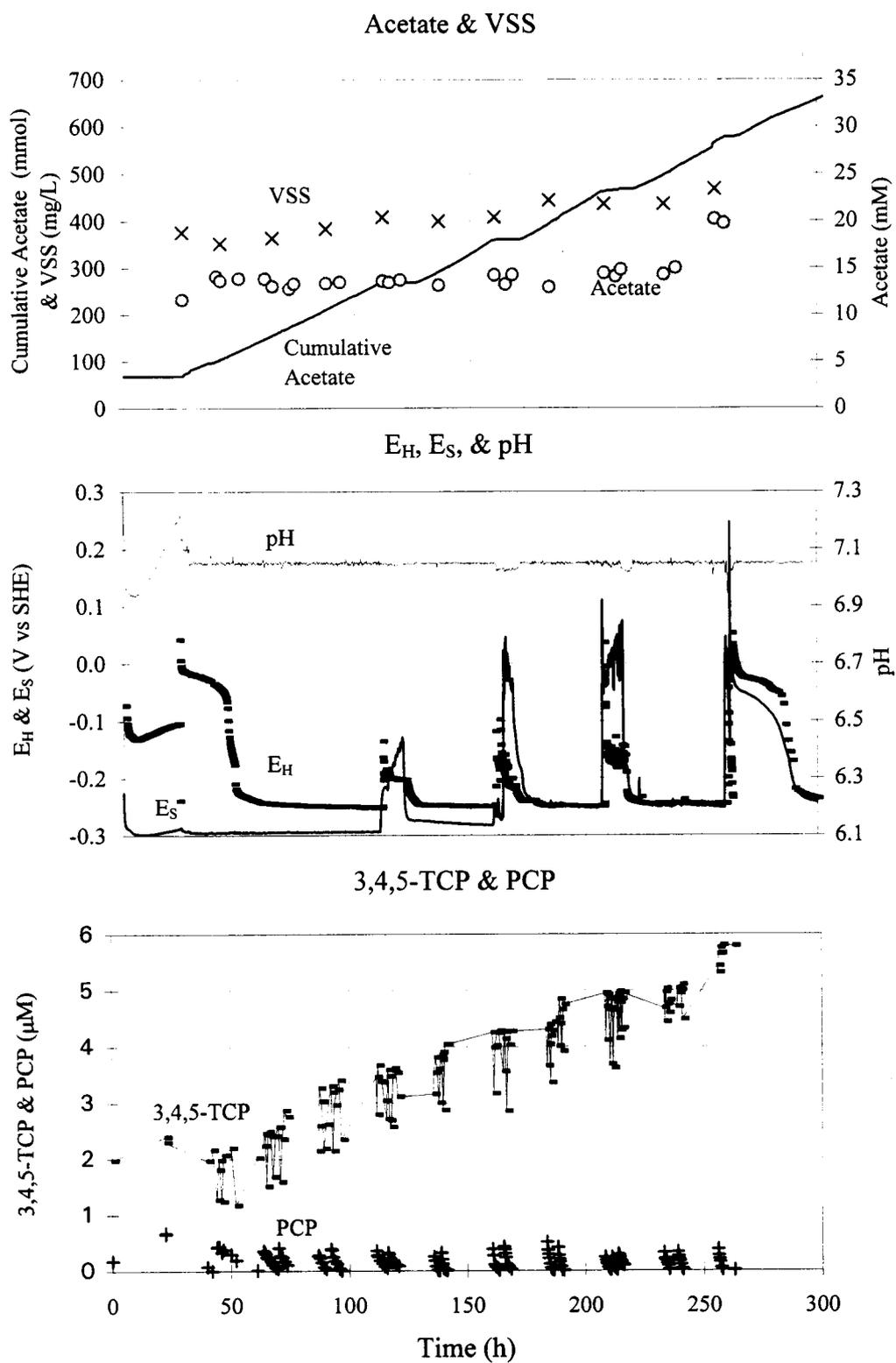


Figure A.10. Summary data for August '94 experiment.

Table A.5. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
August '94

Date	Add Time	Addition	$E_H$ (V)	$k_p$ ( $h^{-1}$ )	Upper $k_p$ ( $h^{-1}$ )	Lower $k_p$ ( $h^{-1}$ )	PCP(0)	LSS PCP	$\chi^2$ PCP
15-Aug	22.4	1	-0.25	nd	nd	nd	0.7	8.3E-01	2234.40
16-Aug	44.0	2	-0.25	0.10	0.12	0.09	0	1.2E-02	15.42
17-Aug	63.7	3	-0.25	0.27	0.32	0.22	0.35	2.6E-03	3.19
17-Aug	70.0	4	-0.25	0.32	0.40	0.24	0.38	2.1E-03	2.62
18-Aug	86.8	5	-0.25	0.51	0.69	0.36	0.30	2.6E-03	3.13
18-Aug	92.3	6	-0.25	0.53	0.63	0.44	0.40	3.0E-03	3.58
19-Aug	111.5	7	-0.20	0.39	0.49	0.31	0.34	1.4E-03	1.65
19-Aug	116.3	8	-0.20	0.26	0.34	0.19	0.30	2.6E-03	3.12
20-Aug	135.8	9	-0.25	1.45	1.93	1.08	0.27	5.3E-04	0.62
20-Aug	138.9	10	-0.25	1.78	2.27	1.39	0.32	2.7E-04	0.31
21-Aug	160.6	11	-0.20	1.14	1.40	0.93	0.36	3.3E-03	3.93
21-Aug	165.4	12	-0.20	0.81	0.94	0.69	0.43	4.0E-03	4.91
22-Aug	183.7	13	-0.25	1.22	1.38	1.09	0.51	1.1E-03	1.31
22-Aug	188.3	14	-0.25	1.49	1.72	1.29	0.43	1.3E-03	1.52
23-Aug	208.3	15	-0.15	1.17	1.49	0.90	0.25	3.8E-04	0.45
23-Aug	211.0	16	-0.15	0.98	1.29	0.72	0.23	9.6E-04	1.12
23-Aug	213.8	17	-0.15	0.57	0.74	0.41	0.31	8.1E-04	0.99
24-Aug	233.2	18	-0.25	0.79	0.98	0.62	0.31	4.1E-03	4.90
24-Aug	238.9	19	-0.25	0.91	1.09	0.74	0.33	1.6E-03	1.97
25-Aug	256.0	20	-0.25	1.21	1.37	0.94	0.39	1.5E-03	1.84

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_p$
1	0.2	nd	nd	0.000	0.0E+00	0.0	nd	nd
2	0.17	0.22	0.13	0.00	1.5E-03	5.9	21.28	1.7
3	0.38	0.48	0.29	0.03	1.6E-03	6.1	9.26	1.4
4	0.26	0.38	0.15	0.08	1.1E-03	4.5	7.16	0.8
5	0.68	0.93	0.48	0.05	2.7E-04	1.1	4.19	1.3
6	0.65	0.80	0.54	0.03	2.2E-03	8.6	12.19	1.2
7	0.37	0.49	0.26	0.01	1.9E-04	0.7	2.37	0.9
8	0.14	0.20	0.07	0.13	4.1E-03	16.6	19.74	0.5
9	0.83	1.06	0.63	0.07	1.7E-04	0.7	1.28	0.6
10	1.23	1.49	1.02	0.14	7.3E-04	2.9	3.22	0.7
11	0.49	0.60	0.39	0.08	5.8E-04	2.3	6.27	0.4
12	0.35	0.42	0.28	0.11	5.3E-03	21.9	26.76	0.4
13	0.56	0.63	0.49	0.08	6.0E-03	24.8	26.11	0.5
14	0.62	0.71	0.53	0.13	4.4E-03	18.3	19.81	0.4
15	0.50	0.66	0.35	0.05	1.3E-03	5.3	5.72	0.4
16	0.29	0.40	0.19	0.15	4.5E-03	18.4	19.55	0.3
17	0.17	0.25	0.08	0.22	7.0E-03	29.6	30.58	0.3
18	0.34	0.50	0.19	0.03	3.1E-04	1.2	6.11	0.4
19	0.46	0.59	0.34	0.08	2.4E-03	9.5	11.44	0.5
20	0.74	0.74	0.40	0.05	2.5E-03	9.9	11.74	0.6

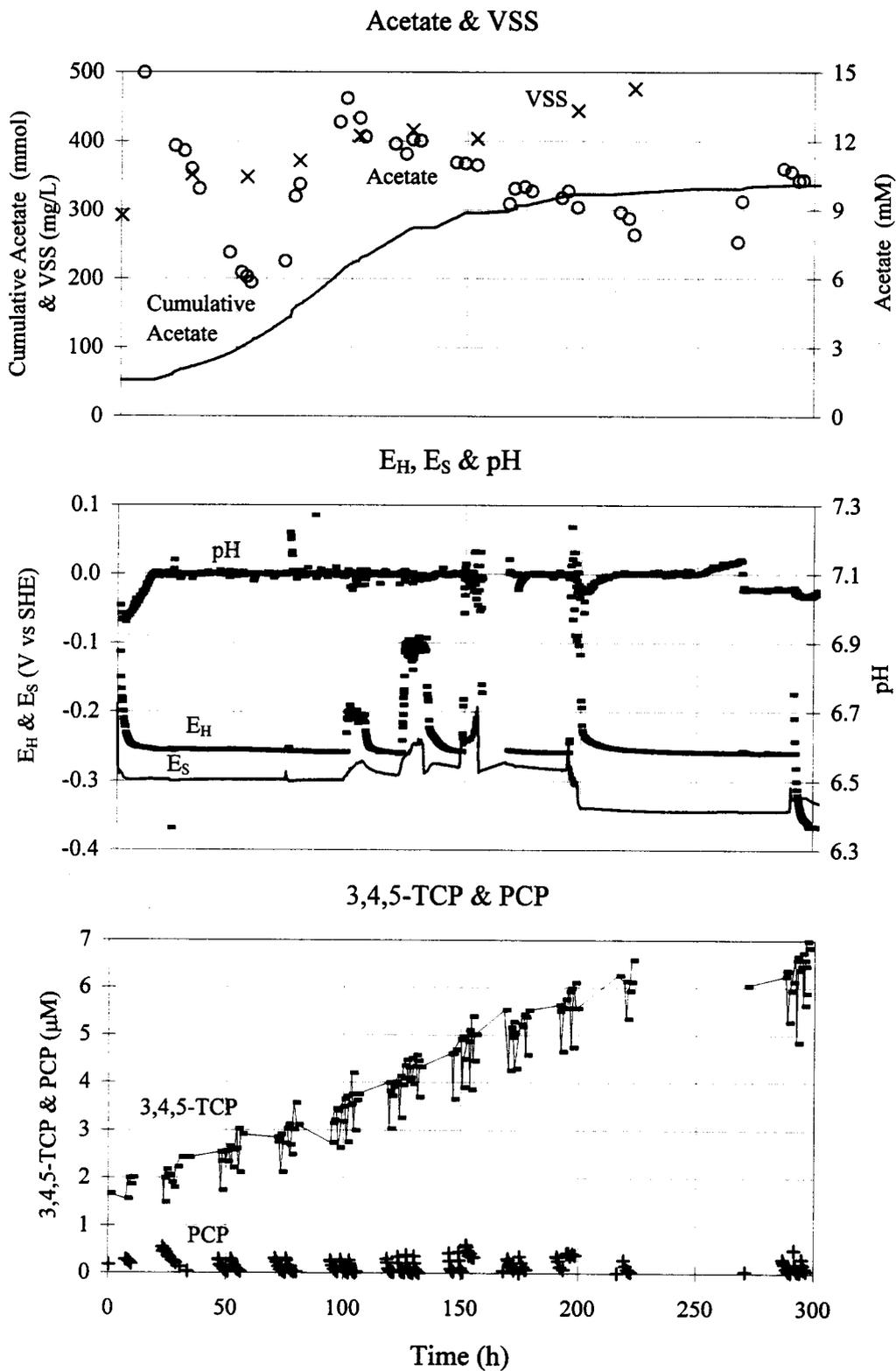


Figure A.11. Summary data for October '94 experiment.

Table A.6. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
October '94

Date	Add Time	Addition	E <sub>H</sub> (V)	kp (h <sup>-1</sup> )	Upper kp (h <sup>-1</sup> )	Lower kp (h <sup>-1</sup> )	PCP(0)	LSS PCP	χ <sup>2</sup> PCP
13-Oct	7.48	1	-0.25	0.2	0.250	0.1	0.29	4.9E-04	1.6
14-Oct	23.23	2	-0.25	0.18	0.20	0.17	0.54	9.1E-03	29.8
15-Oct	46.98	3	-0.25	0.59	0.69	0.49	0.29	6.7E-04	2.0
15-Oct	52.23	4	-0.25	0.58	0.68	0.49	0.32	1.2E-03	3.8
16-Oct	70.98	5	-0.25	0.62	0.73	0.53	0.33	1.5E-03	4.7
16-Oct	75.73	6	-0.25	0.80	0.96	0.67	0.31	3.0E-04	0.9
17-Oct	94.73	7	-0.25	0.76	0.93	0.62	0.28	2.4E-04	0.7
17-Oct	98.98	8	-0.20	1.01	1.24	0.82	0.29	2.4E-04	0.7
17-Oct	102.73	9	-0.20	1.43	1.82	1.13	0.27	7.7E-04	2.3
18-Oct	118.73	10	-0.25	1.33	1.64	1.07	0.30	5.6E-04	1.7
18-Oct	123.48	11	-0.10	1.58	1.96	1.28	0.33	7.0E-04	2.1
18-Oct	126.98	12	-0.10	1.93	2.32	1.62	0.37	7.6E-04	2.3
18-Oct	130.23	13	-0.10	2.05	2.47	1.70	0.36	1.1E-04	0.3
19-Oct	145.23	14	-0.25	2.06	2.41	1.76	0.43	8.2E-04	2.5
19-Oct	149.23	15	0.00	1.93	2.23	1.67	0.44	1.9E-04	0.6
19-Oct	152.48	16	0.00	0.20	0.23	0.17	0.57	7.7E-03	26.1
20-Oct	170.23	17	-0.25	0.61	0.71	0.52	0.32	9.4E-04	2.9
20-Oct	175.23	18	-0.25	0.69	0.81	0.58	0.36	5.4E-04	1.7
21-Oct	190.98	19	-0.25	0.56	0.66	0.46	0.36	1.3E-03	4.3
21-Oct	195.23	20	0.00	0.02	0.05	0	0.42	2.1E-03	6.9
22-Oct	216.57	21	-0.25	0.80	0.98	0.65	0.28	1.0E-04	0.3
25-Oct	287.07	23	-0.25	0.70	0.84	0.58	0.31	6.6E-04	2.0
25-Oct	291.73	24	-0.30	1.18	1.52	0.87	0.26	4.8E-04	1.5
25-Oct	294.98	25	-0.35	1.09	1.33	0.87	0.29	3.2E-03	9.7

Table A.6. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
October '94, Continued.

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_P$
1	0.00	nd	nd	0.047	4.1E-05	0.13	1.70	0.00
2	0.13	nd	nd	0.013	6.6E-03	22.45	52.23	0.71
3	0.47	nd	nd	0.000	5.5E-04	1.80	3.83	0.80
4	0.61	nd	nd	0.072	8.2E-03	27.14	30.98	1.05
5	0.60	nd	nd	0.024	4.7E-04	1.54	6.25	0.97
6	0.76	0.93	0.62	0.071	2.5E-03	8.15	9.08	0.95
7	0.62	0.81	0.45	0.029	4.3E-04	1.39	2.13	0.81
8	0.83	1.02	0.67	0.088	4.7E-04	1.54	2.27	0.82
9	1.11	1.40	0.89	0.086	2.7E-04	0.88	3.20	0.78
10	1.19	1.52	0.94	0.062	2.2E-04	0.73	2.42	0.90
11	1.20	1.45	0.99	0.067	3.2E-04	1.06	3.17	0.76
12	1.13	1.33	0.96	0.114	3.5E-04	1.17	3.46	0.59
13	0.93	1.10	0.79	0.142	4.1E-04	1.41	1.75	0.46
14	1.25	1.43	1.08	0.136	1.6E-03	5.36	7.87	0.61
15	0.72	0.83	0.62	0.167	1.6E-03	5.65	6.25	0.38
16	0.12	0.17	0.07	0.222	4.7E-03	16.75	42.85	0.60
17	0.41	0.51	0.31	0.043	7.7E-04	2.56	5.46	0.67
18	0.41	nd	nd	0.130	6.8E-04	2.33	4.01	0.60
19	0.35	nd	nd	0.033	6.9E-05	0.23	4.48	0.64
20	0.04	nd	nd	0.145	5.2E-04	1.74	8.69	1.61
21	0.40	nd	nd	0.046	5.6E-05	0.19	0.50	0.49
23	0.41	nd	nd	0.063	9.1E-04	3.04	5.08	0.58
24	0.78	nd	nd	0.162	1.4E-03	4.80	6.27	0.67
25	0.61	nd	nd	0.127	3.9E-04	1.34	11.08	0.56

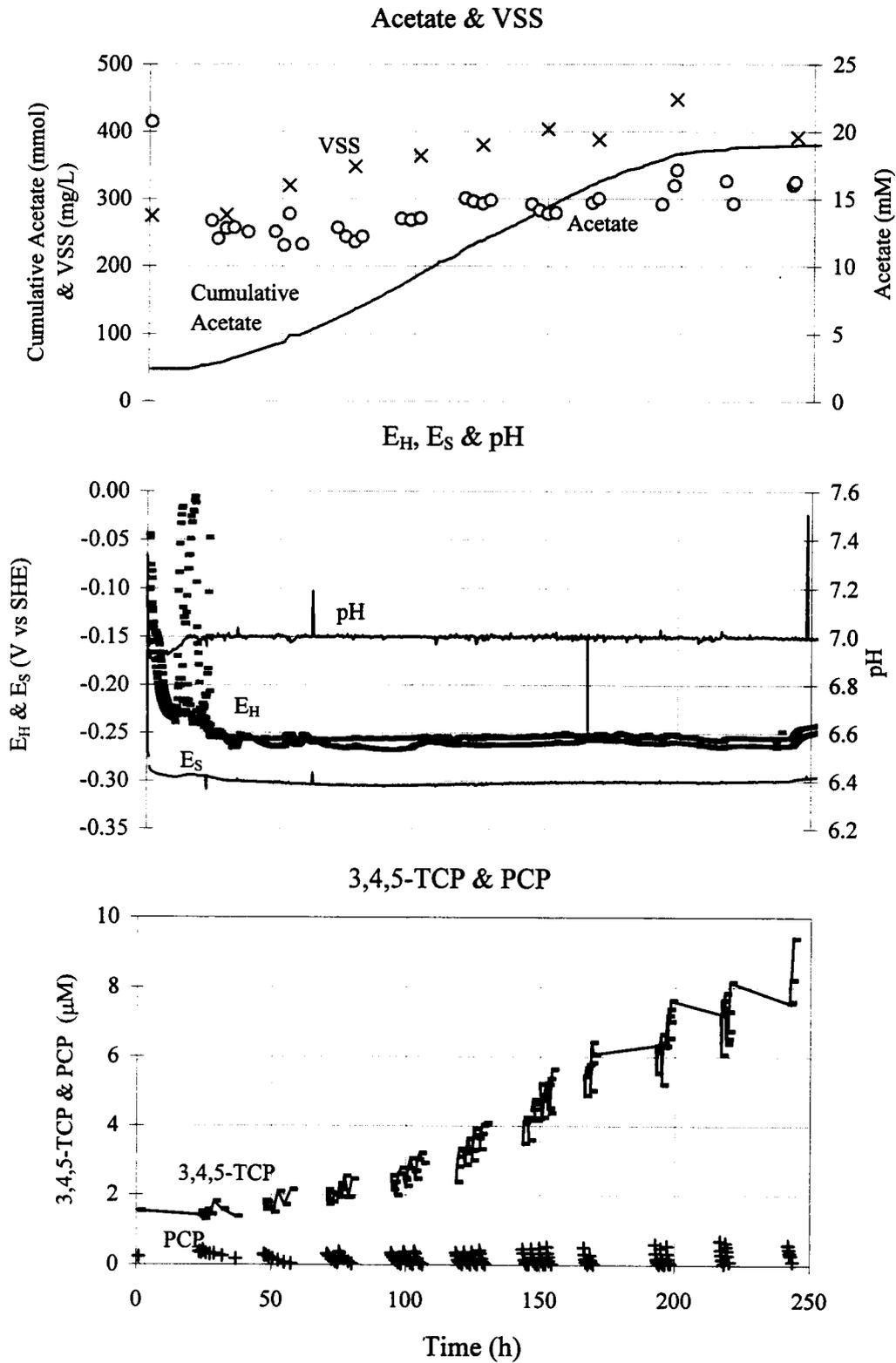


Figure A.12. Summary data for November '94 experiment.

Table A.7. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
November '94

Date	Add Time	Addition	E <sub>H</sub> (V)	k <sub>p</sub> (h <sup>-1</sup> )	Upper k <sub>p</sub> (h <sup>-1</sup> )	Lower k <sub>p</sub> (h <sup>-1</sup> )	PCP(0)	LSS PCP	χ <sup>2</sup> PCP
18-Nov	1	23.39	-0.25	0.05	0.07	0.04	0.38	7.6E-03	10.8
19-Nov	2	47.39	-0.25	0.19	0.24	0.14	0.29	1.1E-03	1.6
20-Nov	3	70.89	-0.25	0.39	0.51	0.29	0.31	1.8E-04	0.3
20-Nov	4	75.39	-0.25	0.43	0.54	0.33	0.36	6.4E-04	0.9
21-Nov	5	94.89	-0.25	0.60	0.77	0.46	0.32	3.4E-04	0.5
21-Nov	6	99.39	-0.25	0.72	0.90	0.57	0.36	6.7E-04	1.0
21-Nov	7	103.39	-0.25	0.88	1.12	0.69	0.38	7.2E-04	1.0
22-Nov	8	118.89	-0.25	1.14	1.48	0.86	0.33	8.9E-05	0.1
22-Nov	9	121.89	-0.25	1.21	1.56	0.93	0.35	1.5E-05	0.0
22-Nov	10	124.64	-0.25	1.30	1.64	1.01	0.37	6.5E-04	0.9
22-Nov	11	127.64	-0.25	1.33	1.63	1.07	0.43	9.0E-04	1.3
23-Nov	12	143.64	-0.25	1.53	1.85	1.26	0.45	7.6E-04	1.1
23-Nov	13	146.89	-0.25	1.63	1.95	1.36	0.46	8.6E-04	1.2
23-Nov	14	149.89	-0.25	1.73	2.06	1.45	0.47	6.4E-04	0.9
23-Nov	15	152.64	-0.25	1.80	2.12	1.51	0.51	8.9E-04	1.3
24-Nov	16	166.39	-0.25	2.18	2.58	1.82	0.49	3.8E-04	0.5
24-Nov	17	168.64	-0.25	2.67	3.81	1.81	0.28	7.5E-04	1.1
25-Nov	18	192.89	-0.25	2.33	2.71	2.00	0.57	6.3E-04	0.9
25-Nov	19	194.89	-0.25	2.67	3.26	2.18	0.44	1.3E-03	1.9
25-Nov	20	197.14	-0.25	2.39	2.80	2.03	0.53	8.5E-04	1.2
26-Nov	21	216.89	-0.25	1.84	2.08	1.62	0.67	7.8E-04	1.1
26-Nov	22	218.89	-0.25	1.10	1.26	0.95	0.64	3.7E-03	5.1
27-Nov	23	242.14	-0.25	1.02	1.19	0.85	0.56	5.8E-04	0.8

Table A.7. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
November '94, Continued.

Addition	$k_T$ ( $h^{-1}$ )	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	2,3,4,5(0)	LSStet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_p$
1	0.04	0.09	0.00	0.06	7E-04	1.0	11.7	0.8
2	0.12	0.16	0.08	0.12	3E-03	4.8	6.4	0.6
3	0.37	0.62	0.18	0.01	4E-04	0.6	0.9	1.0
4	0.31	0.40	0.22	0.12	2E-03	2.7	3.6	0.7
5	0.50	0.77	0.30	0.01	3E-04	0.4	0.9	0.8
6	0.55	0.72	0.41	0.09	1E-03	1.7	2.6	0.8
7	0.62	0.80	0.47	0.11	2E-03	2.4	3.4	0.7
8	0.94	1.41	0.60	0.03	2E-04	0.3	0.5	0.8
9	0.90	1.23	0.64	0.08	7E-04	1.0	1.0	0.7
10	0.83	1.08	0.61	0.11	2E-03	2.8	3.8	0.6
11	0.90	1.14	0.69	0.11	2E-03	2.5	3.8	0.7
12	1.23	1.52	0.99	0.08	5E-04	0.7	1.8	0.8
13	1.29	1.61	1.03	0.10	8E-04	1.2	2.5	0.8
14	1.41	1.76	1.13	0.13	3E-04	0.4	1.3	0.8
15	1.30	1.63	1.03	0.13	2E-03	3.5	4.8	0.7
16	1.56	1.95	1.24	0.13	5E-04	0.7	1.3	0.7
17	1.51	1.95	1.15	0.27	8E-04	1.2	2.3	0.6
18	1.67	nd	nd	0.14	3E-04	0.4	1.3	0.7
19	1.86	nd	nd	0.20	2E-03	2.8	4.7	0.7
20	1.62	nd	nd	0.19	9E-04	1.4	2.6	0.7
21	1.19	nd	nd	0.14	4E-03	5.8	6.9	0.6
22	0.33	nd	nd	0.20	2E-03	2.6	7.7	0.3
23	0.39	nd	nd	0.08	5E-04	0.8	1.6	0.4

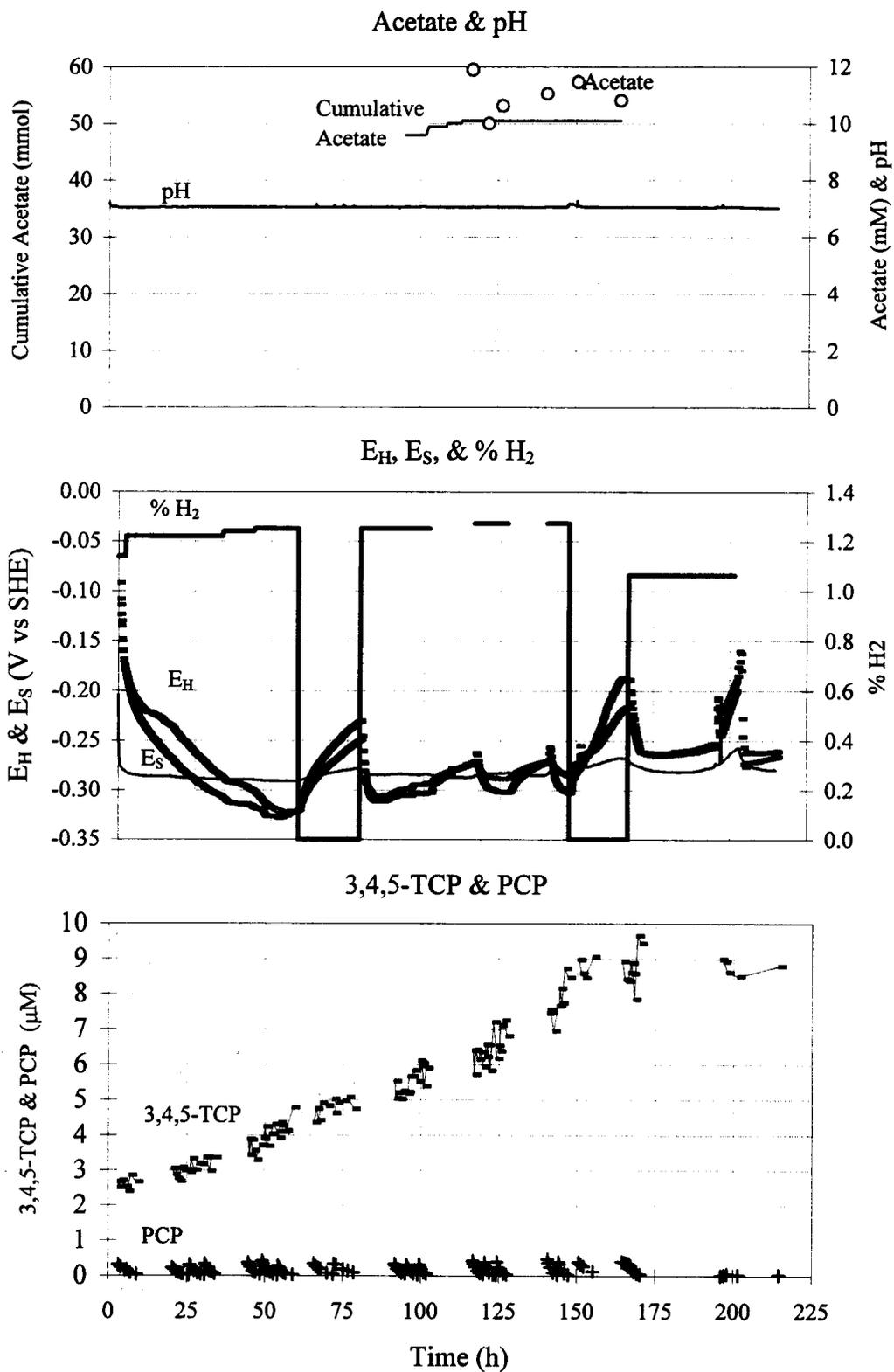


Figure A.13. Summary of data from March '95 experiment.

Table A.8. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
March '95

Date	Add Time	Addition	% H <sub>2</sub>	k <sub>p</sub> (h <sup>-1</sup> )	Upper k <sub>p</sub> (h <sup>-1</sup> )	Lower k <sub>p</sub> (h <sup>-1</sup> )	PCP(0)	LSS PCP	χ <sup>2</sup> PCP
21-Mar	3.3	1	1.2	0.29	0.3	0.3	0.31	2.0E-03	10.67
22-Mar	20.5	2	1.2	0.41	0.49	0.34	0.22	1.7E-04	0.89
22-Mar	26.0	3	1.2	0.50	0.58	0.44	0.31	2.7E-04	1.44
22-Mar	31.0	4	1.2	0.56	0.65	0.48	0.33	2.3E-04	1.24
23-Mar	44.8	5	1.2	0.57	0.65	0.50	0.37	3.3E-04	1.81
23-Mar	49.3	6	1.2	0.60	0.67	0.54	0.41	4.8E-04	2.57
23-Mar	54.3	7	1.2	0.61	0.71	0.52	0.29	5.0E-04	2.69
24-Mar	65.8	8	0	0.31	0.35	0.28	0.35	6.7E-04	3.62
24-Mar	72.3	9	0	0.20	0.23	0.17	0.34	3.1E-04	1.71
25-Mar	91.8	10	1.3	0.56	0.64	0.49	0.33	3.7E-04	2.00
25-Mar	95.5	11	1.3	0.63	0.73	0.54	0.31	2.5E-04	1.33
25-Mar	99.5	12	1.3	0.74	0.86	0.63	0.32	1.1E-04	0.61
26-Mar	116.8	13	1.3	0.65	0.72	0.58	0.41	5.0E-04	2.70
26-Mar	120.5	14	1.3	0.80	0.91	0.70	0.37	6.8E-04	3.63
26-Mar	124.5	15	1.3	0.84	0.95	0.74	0.39	2.6E-04	1.41
27-Mar	140.8	16	1.3	0.85	0.95	0.76	0.47	2.0E-04	1.06
27-Mar	144.3	17	1.3	0.93	1.1	0.82	0.40	3.0E-04	1.60
27-Mar	150.7	18	0	0.24	0.28	0.20	0.39	1.5E-04	0.81
28-Mar	164.5	19a	0	0.06	0.10	0	0.40	2.1E-05	0.12
28-Mar	166.8	19b	1.1	0.68	0.76	0.60	0.36	1.8E-03	9.50

Addition	k <sub>T</sub> (h <sup>-1</sup> )	Upper k <sub>T</sub> (h <sup>-1</sup> )	Lower k <sub>T</sub> (h <sup>-1</sup> )	2,3,4,5(0)	LSStet	χ <sup>2</sup> Tet	χ <sup>2</sup> Total	k <sub>T</sub> /k <sub>p</sub>
1	0.43	nd	nd	0.07	4.6E-04	2.5	13.2	1.5
2	0.57	nd	nd	0.01	2.4E-04	1.3	2.2	1.4
3	0.55	nd	nd	0.04	2.3E-04	1.2	2.7	1.1
4	0.63	nd	nd	0.08	6.2E-04	3.4	4.6	1.1
5	0.66	nd	nd	0.02	2.0E-04	1.1	2.9	1.2
6	0.61	nd	nd	0.10	1.1E-03	5.6	8.2	1.0
7	0.58	nd	nd	0.09	2.0E-04	1.1	3.8	1.0
8	0.25	nd	nd	0.01	4.8E-04	2.6	6.2	0.8
9	0.10	nd	nd	0.11	1.9E-04	1.0	2.7	0.5
10	0.70	nd	nd	0.03	1.3E-04	0.7	2.7	1.2
11	0.57	nd	nd	0.10	1.9E-04	1.0	2.3	0.9
12	0.69	nd	nd	0.11	2.3E-04	1.2	1.8	0.9
13	0.65	nd	nd	0.03	3.3E-04	1.8	4.5	1.0
14	0.75	nd	nd	0.13	6.0E-04	3.2	6.8	0.9
15	0.78	nd	nd	0.11	1.1E-04	0.6	2.0	0.9
16	0.79	nd	nd	0.06	2.4E-04	1.3	2.4	0.9
17	0.80	nd	nd	0.13	1.3E-04	0.7	2.3	0.9
18	0.08	nd	nd	0.05	8.6E-05	0.5	1.3	0.3
19a	0.00	nd	nd	0.04	2.4E-05	0.1	0.3	0.0
19b	0.71	nd	nd	0.09	2.3E-04	1.2	10.7	1.0

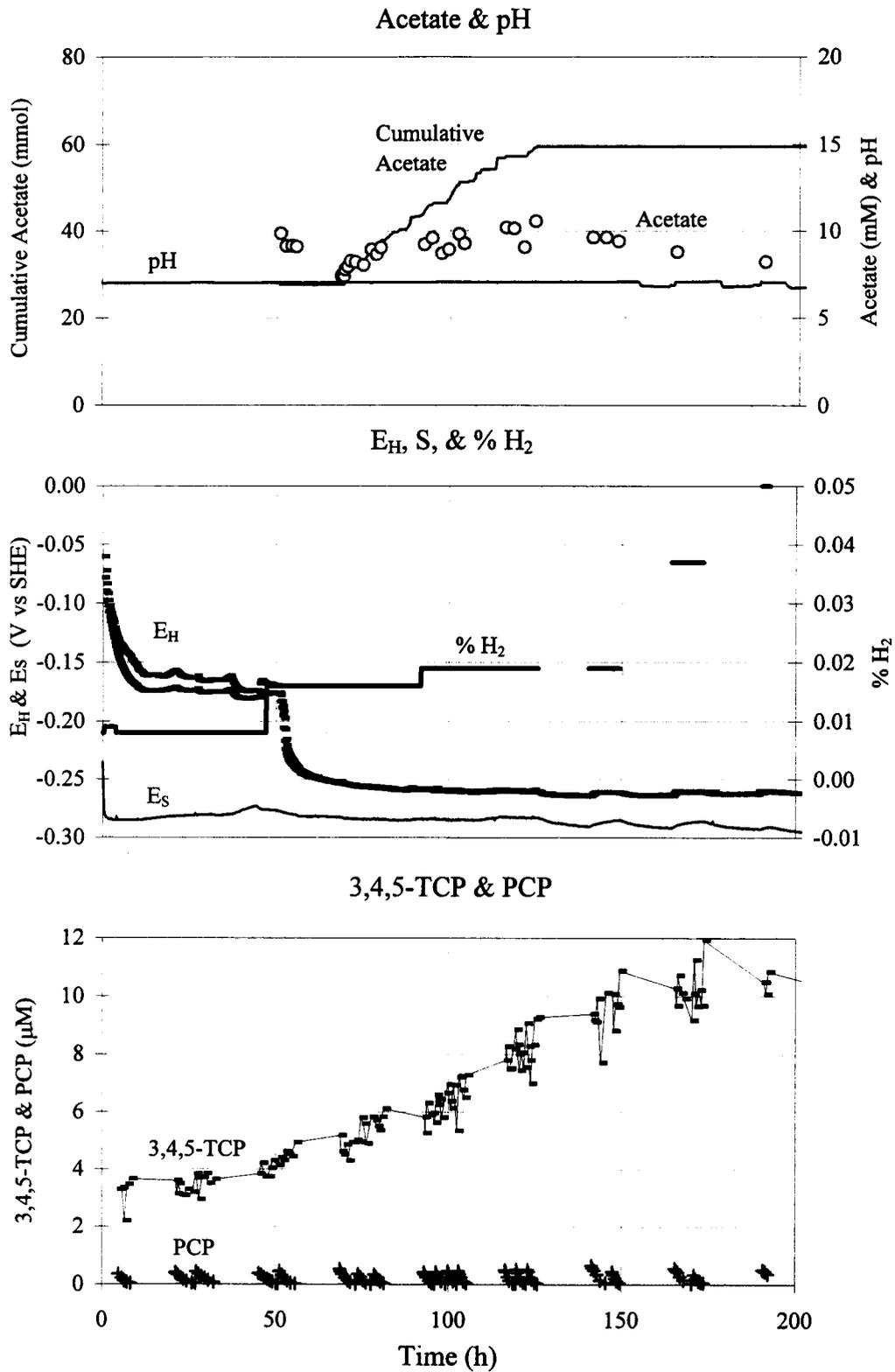


Figure A.14. Summary of data from May '95 experiment

Table A.9. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
May '95-- H<sub>2</sub> purge between 0.008 % and 0.016%.

Date	Add Time	Addition	E <sub>H</sub> (V)	k <sub>p</sub> (h <sup>-1</sup> )	Upper k <sub>p</sub> (h <sup>-1</sup> )	Lower k <sub>p</sub> (h <sup>-1</sup> )	PCP(0)	LSS PCP	χ <sup>2</sup> PCP
21-May	4.75	1	-0.17	0.61	0.75	0.5	0.36	5.0E-04	0.64
22-May	21.25	2	-0.17	0.37	0.45	0.32	0.41	8.3E-04	1.08
22-May	27.25	3	-0.17	0.30	0.36	0.25	0.44	5.4E-04	0.71
23-May	45.25	4 all	-0.17	0.34	0.40	0.29	0.40	2.6E-03	3.32
23-May	45.25	4 a	-0.17	0.27	0.37	0.19	0.39	4.4E-04	0.56
23-May	47.75	4 b	-0.17	0.61	0.95	0.34	0.20	2.1E-04	0.27
23-May	51.25	5	-0.26	0.61	0.71	0.53	0.47	1.9E-03	2.47
24-May	68.75	6	-0.26	0.58	0.66	0.51	0.53	8.6E-04	1.11
24-May	74.00	7	-0.26	0.76	0.92	0.62	0.38	3.7E-03	4.65
24-May	78.75	8	-0.26	0.77	0.95	0.61	0.35	2.6E-04	0.33
25-May	93.00	9	-0.26	0.82	0.97	0.69	0.44	9.4E-04	1.21
25-May	96.50	10	-0.26	0.82	0.99	0.67	0.42	4.9E-04	0.64
25-May	100.00	11	-0.26	0.92	1.09	0.76	0.46	2.8E-04	0.36
25-May	103.00	12	-0.26	0.96	1.14	0.80	0.47	9.6E-04	1.24
26-May	116.50	13	-0.26	0.95	1.10	0.82	0.52	2.1E-03	2.67
26-May	119.75	14	-0.26	0.95	1.09	0.82	0.52	2.8E-04	0.36
26-May	123.00	15	-0.26	0.90	1.03	0.78	0.52	1.4E-03	1.86
27-May	141.50	16	-0.26	0.49	0.55	0.44	0.66	6.0E-03	7.95
27-May	147.50	17	-0.26	0.90	1.09	0.74	0.43	2.9E-04	0.38
28-May	165.50	18	-0.26	0.45	0.52	0.40	0.56	3.4E-03	4.44
28-May	170.50	19	-0.26	0.69	0.82	0.56	0.44	1.3E-03	1.65
29-May	190.75	20	-0.26	0.21	0.28	0.15	0.51	3.6E-04	0.49

Table A.9. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
May '95 Continued.

Addition	$k_T$ ( $h^{-1}$ )	2,3,4,5(0)	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	LSS Tet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_p$
1	0.53	0.09	nd	nd	3.9E-04	1.30	1.95	0.87
2	0.29	0.00	nd	nd	9.4E-04	3.07	4.14	0.77
3	0.18	0.11	nd	nd	6.6E-04	2.22	2.93	0.61
4	0.27	0.00	nd	nd	1.6E-03	5.25	8.57	0.80
4	0.13	0.00	nd	nd	1.5E-05	0.05	0.61	0.46
4	0.43	0.17	nd	nd	1.4E-04	0.48	0.74	0.70
5	0.52	0.12	nd	nd	1.8E-03	6.19	8.66	0.84
6	0.46	0.01	nd	nd	3.1E-03	10.24	11.35	0.80
7	0.57	0.10	nd	nd	8.3E-04	2.78	7.42	0.75
8	0.55	0.08	nd	nd	2.4E-04	0.80	1.13	0.72
9	0.64	0.03	nd	nd	7.2E-04	2.38	3.59	0.79
10	0.62	0.12	nd	nd	4.9E-04	1.64	2.27	0.76
11	0.69	0.13	nd	nd	2.0E-03	6.86	7.21	0.76
12	0.60	0.18	nd	nd	5.0E-04	1.71	2.95	0.63
13	0.76	0.05	nd	nd	1.4E-04	0.47	3.13	0.80
14	0.65	0.14	nd	nd	1.1E-03	3.69	4.05	0.68
15	0.57	0.17	nd	nd	4.1E-03	13.88	15.74	0.64
16	0.38	0.03	nd	nd	1.5E-03	4.90	12.85	0.78
17	0.60	0.13	nd	nd	1.9E-04	0.63	1.01	0.67
18	0.43	0.03	nd	nd	5.8E-04	1.93	6.37	0.94
19	0.48	0.19	nd	nd	6.9E-04	2.35	4.01	0.70
20	0.33	0.04	nd	nd	2.4E-04	0.78	1.26	1.56

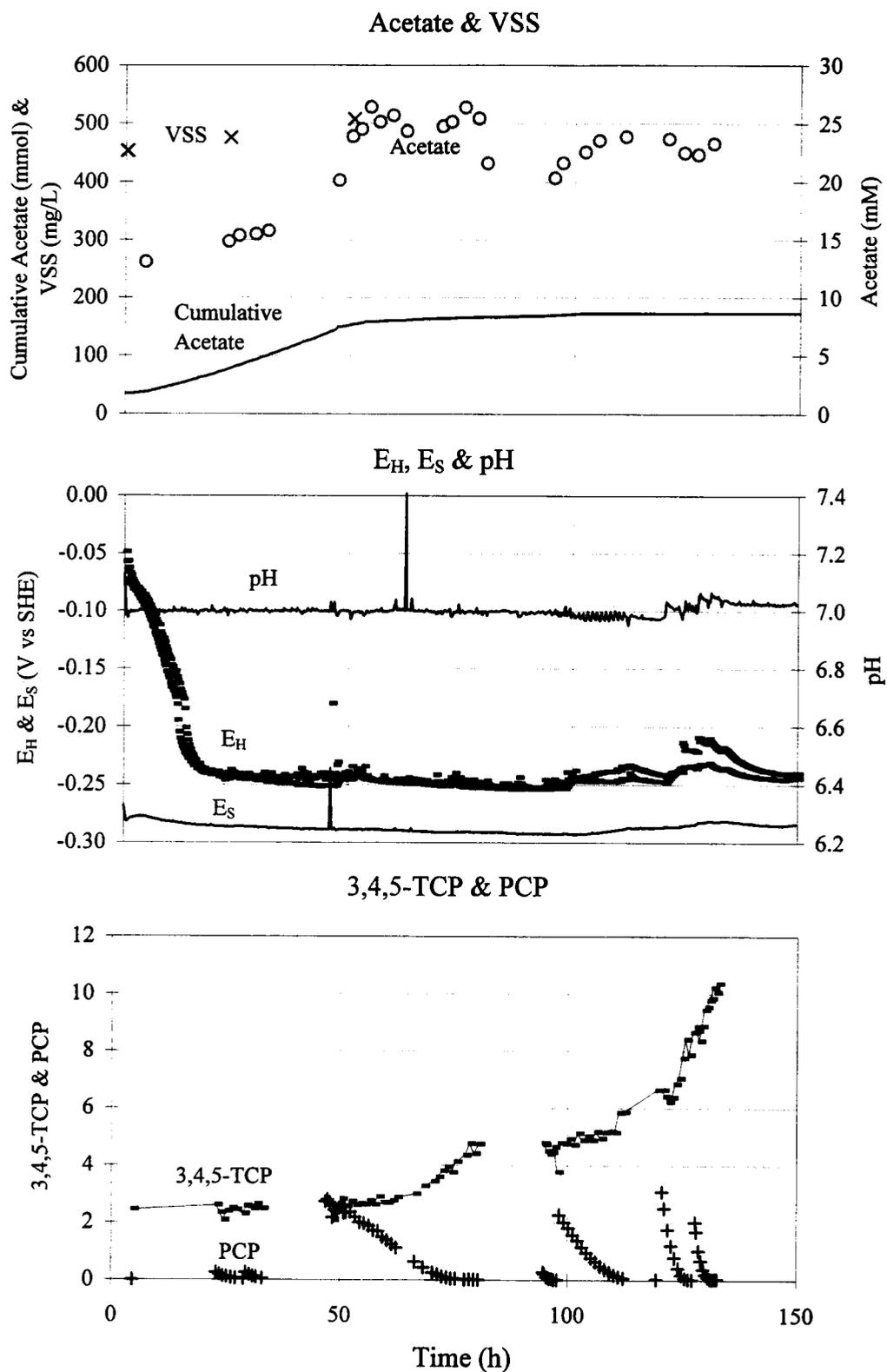


Figure A.15. Summary of data from August '95 experiment.

Table A.10. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
August '95

Date	Add Time	Addition	$E_H$ (V)	$k_P$ ( $h^{-1}$ )	Upper $k_P$ ( $h^{-1}$ )	Lower $k_P$ ( $h^{-1}$ )	PCP(0)	LSS PCP	$\chi^2$ PCP
25-Aug	22.9	1	-0.25	0.33	nd	nd	0.244	3.2E-04	0.7
25-Aug	29.4	2	-0.25	0.39	nd	nd	0.244	2.1E-04	0.4
28-Aug	94.7	4	-0.25	1.15	1.41	0.93	0.276	4.3E-04	0.9

Addition	$k_T$ ( $h^{-1}$ )	2,3,4,5(0)	Upper $k_T$ ( $h^{-1}$ )	Lower $k_T$ ( $h^{-1}$ )	LSS Tet	$\chi^2$ Tet	$\chi^2$ Total	$k_T/k_P$
1	0.43	0.00	nd	nd	3.0E-04	2.0	2.7	1.3
2	0.48	0.05	nd	nd	1.6E-04	1.1	1.5	1.2
4	1.21	0.03	nd	nd	6.1E-05	0.4	1.3	1.1

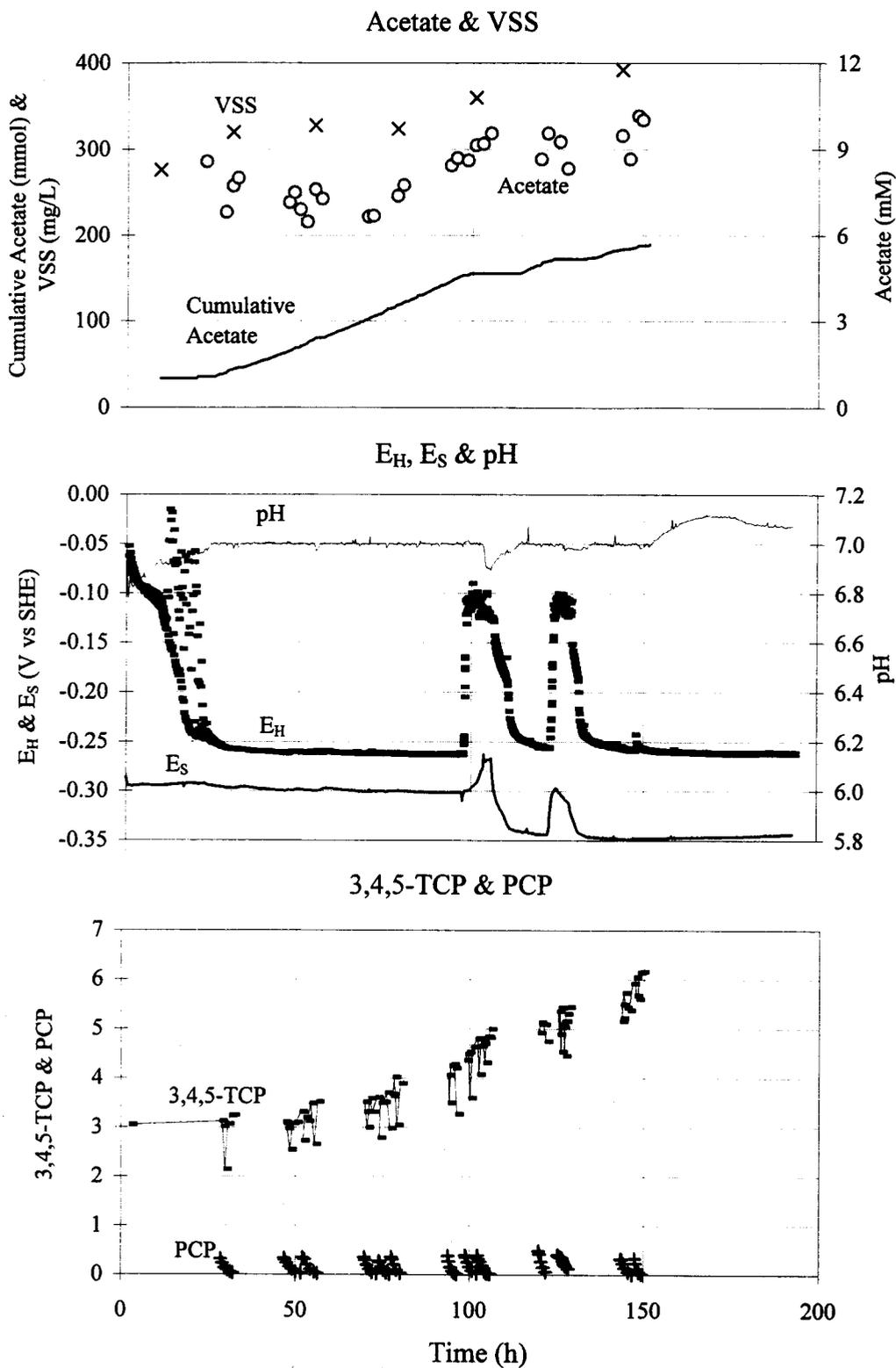


Figure A.16. Summary of data from October '95 experiment.

Table A.11. First-order kinetic constants for PCP and 2,3,4,5-TeCP transformation.  
October '95

Date	Add Time	Addition	E <sub>H</sub> (V)	k <sub>P</sub> (h <sup>-1</sup> )	Upper k <sub>P</sub> (h <sup>-1</sup> )	Lower k <sub>P</sub> (h <sup>-1</sup> )	PCP(0)	LSS PCP	χ <sup>2</sup> PCP
29-Oct	29	1	-0.25	0.59	0.69	0.51	0.30	4.3E-04	1.49
30-Oct	46.85	2	-0.25	0.50	0.57	0.44	0.33	1.8E-04	0.62
30-Oct	52.13	3	-0.25	0.54	0.61	0.47	0.37	1.8E-03	6.13
31-Oct	69.88	4	-0.25	0.78	0.90	0.68	0.35	1.9E-04	0.66
31-Oct	74.13	5	-0.25	0.92	1.09	0.78	0.29	1.1E-03	3.95
31-Oct	77.63	6	-0.25	0.88	1.03	0.76	0.37	4.6E-04	1.63
1-Nov	93.88	7	-0.25	1.27	1.46	1.11	0.38	6.3E-05	0.22
1-Nov	98.83	8-- -0.1V	-0.1	0.68	0.77	0.60	0.40	1.3E-03	4.66
1-Nov	102.37	9(-0.1V all)	-0.1	0.78	0.86	0.71	0.42	4.9E-03	16.91
1-Nov	102.37	9(-0.1V 1st)	-0.1	0.53	0.66	0.41	0.38	3.9E-04	1.35
1-Nov	103.87	9(-0.1V H <sub>2</sub> )	-0.1	1.07	1.61	0.67	0.13	2.5E-04	0.86
2-Nov	119.93	10	-0.25	0.90	0.99	0.80	0.51	2.1E-03	7.39
2-Nov	125.42	1(-0.1V All)	-0.1	0.28	0.32	0.25	0.42	1.2E-02	43.52
2-Nov	125.42	11(1st)	-0.1	0.15	0.26	0.04	0.40	1.4E-04	0.50
2-Nov	126.67	11(H <sub>2</sub> )	-0.1	0.63	0.75	0.51	0.33	2.7E-04	0.99
3-Nov	143.43	12	-0.25	0.68	0.77	0.59	0.34	3.2E-03	11.30
3-Nov	147.42	13--CH <sub>3</sub> O	-0.25	1.37	1.61	1.16	0.34	2.9E-04	0.99

Addition	k <sub>T</sub> (h <sup>-1</sup> )	Upper k <sub>T</sub> (h <sup>-1</sup> )	Lower k <sub>T</sub> (h <sup>-1</sup> )	2,3,4,5(0)	LSStet	χ <sup>2</sup> Tet	χ <sup>2</sup> Total	k <sub>T</sub> /k <sub>P</sub>
1	0.70	0.90	0.53	0.00	7.9E-04	3.56	5.049	1.172
2	0.51	0.64	0.40	0.00	1.1E-03	4.82	5.443	1.021
3	0.43	0.51	0.35	0.04	5.4E-04	2.46	8.599	0.794
4	0.78	0.97	0.63	0.00	8.3E-04	3.73	4.389	0.999
5	0.81	1.00	0.64	0.04	5.9E-04	2.68	6.637	0.872
6	0.76	0.94	0.61	0.04	2.8E-04	1.26	2.891	0.860
7	1.17	1.40	0.98	0.01	7.2E-04	3.25	3.472	0.921
8-- -0.1V	0.37	0.47	0.28	0.00	1.1E-03	5.18	9.839	0.548
9(-0.1V all)	0.47	0.51	0.42	0.16	3.2E-03	14.82	31.727	0.595
9(-0.1V 1st)	0.16	0.34	0.00	0.15	5.1E-04	2.40	3.746	0.292
9(-0.1V H <sub>2</sub> )	0.57	0.70	0.46	0.29	4.5E-04	2.12	2.983	0.535
10	0.58	0.71	0.45	0.01	9.2E-04	4.21	11.602	0.643
1(-0.1V All)	0.07	0.14	0.00	0.08	1.4E-02	64.80	108.322	0.237
11(1st)	0.49	0.93	0.07	0.13	5.9E-05	0.27	0.771	3.318
11(H <sub>2</sub> )	0.11	0.23	0.00	0.12	3.2E-04	1.48	2.472	0.174
12	0.46	0.58	0.35	0.00	8.5E-04	3.85	15.156	0.673
13--CH <sub>3</sub> O	0.74	0.89	0.60	0.09	6.2E-04	2.86	3.854	0.540