Biodegradation **4**: 3–21, 1993.

Biodegradation kinetics of a mixture containing a primary substrate (phenol) and an inhibitory co-metabolite (4-chlorophenol)

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Received 10 March 1992; accepted in revised form 3 November 1992

Key words: cometabolism, cosubstrate, 4-chlorophenol, inhibition, kinetics, modeling, monooxygenase, phenol, substrate interactions

Abstract

Batch experiments on the simultaneous utilization of phenol (primary substrate) and 4-chlorophenol (cometabolic secondary substrate) demonstrated two critical substrate interactions. First, the cometabolic degradation of 4-chlorophenol was proportional to the rate of phenol oxidation, which provided the electrons for the initial monooxygenase reaction. Second, 4-chlorophenol inhibited the oxidation of the primary substrate, phenol. Modeling analyses of the degradation of phenol alone and of phenol and 4-chlorophenol together showed that the proportionality between phenol and 4-chlorophenol degradation rates averaged 0.1 mg 4-CP/mg phenol, which corresponds to 0.5% of the electrons generated by phenol oxidation being used as a cosubstrate for the monooxygenase reaction of 4-chlorophenol. In addition, modeling analyses suggest that 4-chlorophenol was a noncompetitive inhibitor of phenol oxidation for high phenol concentrations, but a competitive inhibitor for low phenol concentrations.

Abbreviations: GC – gas chromatography; FID – flame-ionization detector; DO – dissolved oxygen, 4-CP – 4-chlorophenol; Ph – phenol; RLS – relative least squares criterion; NAD – nicotinamide adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate

Introduction

Biodegradation of a cometabolite is a situation for which the explicit consideration of substrate interactions is essential if the kinetics are to be applied accurately for process design or fate prediction. For all cometabolites, a primary electron-donor substrate is required to grow and sustain the biomass capable of degrading the cometabolite, which is an obligate secondary substrate (Stratton et al. 1983). However, additional sophisticated interactions can occur: e.g., (1) enzyme induction by a structurally similar primary substrate; (2) a direct cosubstrate requirement, such as for O_2 in monooxygenase reactions; (3) a requirement for an internal, electron-donating cosubstrate, such as NADH or NADPH in monooxygenase or reductive-dehalogenation reactions; and (4) inhibition of primary-substrate utilization by the cometabolite.

4-Chlorophenol (4-CP) provides an excellent example of an environmentally significant compound that exhibits these substrate interactions. Saéz & Rittmann (1991) demonstrated that 4-chlorophenol is a cometabolite that is degraded by phenol-in-

duced cells and is inhibitory to its own degradation. Furthermore, their results and previous work of Gottschalk (1986) and Spain & Gibson(1988) supported that the initial step of 4-chlorophenol degradation, a monooxygenase reaction to 4-chlorocatechol, requires O_2 and NADPH as cosubstrates. Finally, the results presented herein demonstrate that phenol oxidation supplies the electrons to produce the NADPH cosubstrate and that 4-chlorophenol inhibits the oxidation of the cells' primary substrate, phenol.

The focus of this work is on quantification of two of the substrate interactions: the cosubstrate effect of phenol oxidation and 4-chlorophenol's inhibition of phenol oxidation. In order to evaluate the interactions, two series of experiments were necessary. The first series evaluated the kinetics of phenol utilization with no 4-chlorophenol present. These experiments demonstrated that phenol alone behaves as a self-inhibitory primary substrate and provided the data needed to estimate the three parameters describing self-inhibition kinetics by the Haldane equation (Andrews 1968). The second series of experiments defined the kinetics for the simultaneous utilization of phenol and 4-chlorophenol. They demonstrated that 4-chlorophenol inhibited phenol oxidation and that the rate of 4-chlorophenol degradation was proportional to the rate of phenol oxidation, which provided the electron flow for the monooxygenase reaction.

The unique contribution of this work is the complete quantification of each interaction. Data interpretation through modeling analyses allowed us to conclude that less than 1% of the electron flow from phenol is used in 4-chlorophenol degradation, that 4-chlorophenol acts as a noncompetitive inhibitor at high phenol concentrations, and that 4-chlorophenol acts as a competitive inhibitor when phenol is nearly exhausted. In addition, these quantitatively based conclusions allowed us to infer mechanistic explanations of how 4-chlorophenol and phenol interact internally.

Experimental design and methods

The experimental data used for the kinetic studies were obtained using dispersed-growth batch reactors that were seeded with the effluent from a chemostat operated at room temperature and actively biodegrading phenol, 2-chlorophenol, and 4-chlorophenol (4-CP) as the only organic compounds present in the influent. The use of a steady-state chemostat as the source of cells for the batch tests assured a cell supply with constant characteristics for the different batch experiments. The bacteria used were *Pseudomonas putida* PpG4 (ATCC 17453), which cometabolically oxidizes chlorophenols (Saéz & Rittmann 1991).

The chemostat, having a liquid volume of 10 L and operated at an hydraulic retention time of 40 days, received filter-sterilized air and a sterile mixture of organic compounds [phenol (1000 mg/L), 2-chlorophenol (50 mg/L), and 4-chlorophenol (50 mg/L)] in a mineral-medium solution (Saéz & Rittmann 1991). The high influent phenol concentration assured a relatively high biomass concentration inside the chemostat, while the long hydraulic retention time ensured active and stable biodegradation without inhibition and instabilities. The chemostat provided very high removals: the effluent phenol concentration ranged from 3 to 6 μg/L, and the effluent 2- and 4-chlorophenol concentrations were less than 10 μg/L (Saéz & Rittmann 1991).

The batch tests were performed using 3-L glass flasks filled with a liquid volume of 2 L. The dissolved oxygen (DO) concentration within the batch reactor was held near saturation by supplying sterile air through a stainless-steel perforated pipe. The temperature and pH during the tests were 23–26 °C and 6.6–6.8, respectively. Completely mixed conditions were provided by aeration and a magnetic stirring bar.

The batch reactors were first supplied with 1,880 mL of autoclaved mineral medium, which provided buffer capacity, nutrients, and trace elements (Saéz & Rittmann 1991). After addition of the mineral medium, the batch reactors were seeded with 100 mL of fresh chemostat effluent, which resulted in an initial suspended solids (SS) concentration of 5 mg/L (coefficient of variation = 27%). Then, stirring and aeration proceeded for 5 min to allow for homogenization of the reactor contents and for reaching DO concentrations near saturation. Final-

ly, small volumes (5–30 mL) of the concentrated stock solutions of phenol and 4-chlorophenol were added to give the desired initial concentrations.

Air flowrate, DO, pH, and temperature were continuously monitored. Ten-mL samples were removed by syringe at appropriate times, immediately acidified to a pH of 2 with 6N sulfuric acid, and divided into two portions. Absorbance at 420 nm was measured in one portion, which was converted to SS concentration using a calibration curve of absorbance versus dry weight of SS. The dispersed-growth biomass, measured indirectly by absorbance, corresponded to the total biomass, because wall growth was absent.

The second portion was pressure filtered through a 0.45-μm nylon membrane and extracted with methylene chloride. The extract was analyzed for phenol and 4-chlorophenol using a Hewlett-Packard gas chromatograph (GC) model 5890A equipped with FID. The GC output was analyzed by a Hewlett-Packard integrator model 3396A. The difference in the retention times and the almost constant peak width, independently of the retention time, allowed very good compound separation and consistent data integration. The details of the analytical techniques are described in Saéz & Rittmann (1991).

Biodegradation tests were carried out to assess the biodegradation kinetics of phenol/4-chlorophenol mixtures. Biodegradation tests were performed in systems in which the substrates were phenol alone or phenol and 4-chlorophenol together. The experiments with phenol alone determined its intrinsic kinetic behavior, while substrate interactions were then evaluated via experimentation in the bi-substrate system. Saéz & Rittmann (1991) already established that phenol and 4-chlorophenol were not lost by abiotic mechanisms in the batch systems.

Biotic tests with phenol alone were carried out at four different initial nominal concentrations: 50, 100, 175, and 250 mg/L. To assay reproducibility, three replicates for the initial concentrations of 50 and 175 mg/L were run. Also, to investigate if the biomass adapted after being grown in a batch mode, two experiments were performed in which a second spike of phenol, yielding approximately the same initial concentration than the first spike, was given after the phenol from the first spike was consumed. These second-spike tests had initial concentrations of 50 and 100 mg/L.

Six biodegradation experiments with phenol and 4-chlorophenol together were performed. The initial nominal concentrations of phenol/4-chlorophenol in mg/L were: 50/25, 50/50, 50/75, 175/25, 175/50, and 175/100.

Table 1 summarizes the 16 biodegradation experiments performed and provides a key for identifying each test.

Experimental results

Phenol-alone tests

The batch tests with phenol as the only substrate showed two important characteristics. First, the environmental conditions, as measured by the pH, temperature, and DO, remained nearly constant during the tests. The pH and temperature ranges were 6.64–6.81 and 23–26 °C, respectively. Although the DO concentration decreased with time during most of the biodegradation phases, the lowest value measured was 5.6 mg/L, which indicates that fully aerobic conditions prevailed during all

Table 1. Summary of the biodegradation batch experiments.

Test number	Initial nominal concentration, mg/L	
	Phenol	4-Chlorophenol
Phenol-alone tests (10 tests)		
PA1-PA2-PA3	50	0
PA4	100	0
PA5-PA6-PA7	175	0
PA8	250	0
PA9 (second spike)	50	0
PA10 (second spike)	100	0
Phenol/4-chlorophenol (6 tests)		
PC1	50	25
PC2	50	50
PC3	50	75
PC4	175	25
PC5	175	50
PC6	175	100

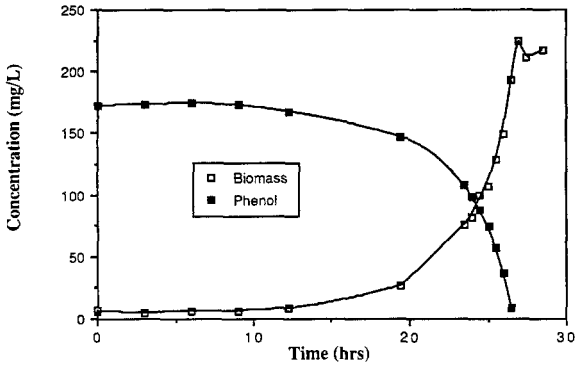

Fig. 1. Time-dependent phenol and biomass concentrations for batch test PA5.

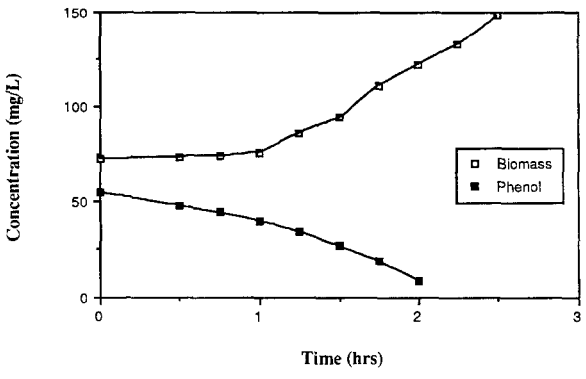

Fig. 2. Time-dependent biomass and phenol concentrations for batch test PA9.

tests. The constancy of the temperature and of the pH and redox conditions during the experiments assured that the changes in the phenol biodegradation kinetics were caused solely by changes in the phenol and biomass concentrations. The second important characteristic was the good reproducibility of the phenol assay, indicated by low values of the coefficients of variation for triplicate samples: the coefficient of variation was consistently lower than 1%, and its highest value was 3.35%.

First-spike experiments

Time-dependent phenol and biomass concentrations for first-spike experiments, exemplified in Fig. 1 for test PA5, illustrate the typical pattern of all the batch tests with phenol. A lag period had no detectable biomass growth or substrate utilization. Then, the rates of cell growth and phenol utilization increased steadily with time during most of the biodegradation phase. Finally, a short stationary period occurred after the substrate was depleted. The lag period varied from 9 to 28 hours, with longer lag periods associated with high initial phenol concentrations.

This characteristic behavior is explained by increases in biomass and by self-inhibition. Once the lag phase was completed, significant increases in cell mass occurred. Because the substrate-utilization rate is proportional to the biomass concentration, the rate of substrate utilization accelerated during the test in response to increasing biomass. The biomass effect was enhanced by a self-inhibition effect. As is quantified later in this paper, phenol behaved as a self-inhibitory substrate. Thus, as the initially high phenol concentrations decreased, self inhibition was relieved, thereby further accelerating substrate utilization. This accelerating substrate utilization occurred until almost all the substrate was consumed.

Reproducibility of the experiments was studied by performing two sets of three identical tests. Although the lengths of the acclimation periods varied among replicates, the results were nearly identical for the biodegradation phases (Saéz and Rittmann 1991).

Second-spike experiments

The second-spike experiments were performed to study the cell's adaptation. A comprehensive comparison between first- and second-spike experiments cannot be achieved by a purely qualitative analysis of the time-dependent curves, because the starting cell concentrations were different for both types of experiments. However, qualitative analysis of the trends from the batch curves still provides valuable information. A quantitative comparison is performed later.

Figure 2 presents the results from test PA9, a good representative. The first clear observation is that the second-spike experiments had no lag phase: thus, the cells needed no further acclimation. Second, although phenol was consumed immediately, the biomass concentration, as measured by absorbance measurements, did not change appre-

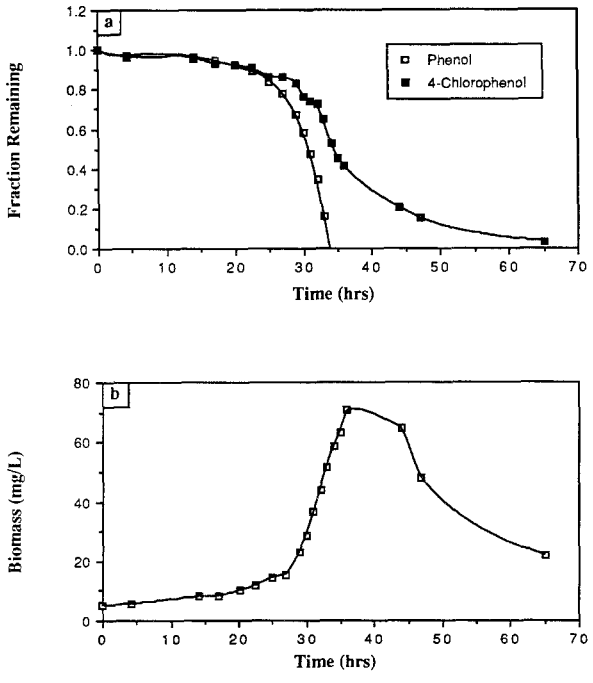

Fig. 3. Time-dependent curves for the concentrations of: a) phenol and 4-chlorophenol, and b) biomass for a complete-removal experiment (test PC1). The initial concentrations of phenol and 4-chlorophenol were 50.2 and 23.0 mg/L, respectively.

ciably at the beginning of the experiment. Third, the biomass concentration, as measured again by absorbance measurements, continued increasing for about 30 min after all the phenol in the bulk solution was depleted. The second and third observations are explained by the concept that absorbance measurements were correlated more to cell number than to cell mass. At the beginning and end of an experiment, the change in cell mass did not correlate perfectly with cell divisions, because cell size increased or decreased. Consequently, information about the biomass concentrations, determined through optical absorbances, at the beginning and end of the biodegradation phase is not totally reliable. This consideration becomes especially important in the procedure used to estimate the cell-growth parameters discussed later in this paper.

Phenol/4-chlorophenol tests

The results for the bisubstrate experiments can be classified into two groups. In the first group (tests PC1, PC4, and PC5), complete removal of 4-chlorophenol was observed; these tests are termed complete 4-CP removal experiments. On the other hand, the removal of 4-chlorophenol was only partial in the second group of tests (tests PC2, PC3, and PC6), which are termed partial 4-CP removal experiments.

Figure 3 shows typical time-dependent phenol, 4-chlorophenol, and biomass concentrations for the complete 4-CP removal experiments; the data shown correspond to test PC1, but the results are similar for the other complete-removal experiments. After a lag period, in which no appreciable changes in the concentrations of both substrates and biomass were observed, the biodegradation phase started. During the biodegradation period, the growth of biomass and *simultaneous* transformation of both substrates occurred. Phenol was transformed more rapidly than 4-chlorophenol, which resulted in phenol being depleted before 4-chlorophenol. Exhaustion of phenol coincided with the beginning of the negative cell-growth period and with a sharp decrease in the 4-chlorophenol transformation rate; the implications of these coincidences, discussed in the next paragraph, are critical. Finally, the removal of the remaining 4-chlorophenol was completed during the negative cell-growth period.

Analysis of the batch curves yields two conclusions that have paramount significance for the kinetic modeling, which is presented later. First, the coincidence of the beginning of the negative cell-growth period with phenol exhaustion strongly reinforces that 4-chlorophenol cannot support growth, even in the presence of phenol. That 4-chlorophenol is a co-metabolite was previously demonstrated for situations in which 4-chlorophenol was present alone (Saéz & Rittmann 1991). Second, since the 4-chlorophenol curve 'followed' the phenol curve before the phenol was depleted and since the 4-chlorophenol transformation rate slowed sharply at the point of phenol exhaustion, the transformation rates of phenol and 4-chlorophenol were linked to each other. The explanation is that phenol was supplying the electrons required for the initial

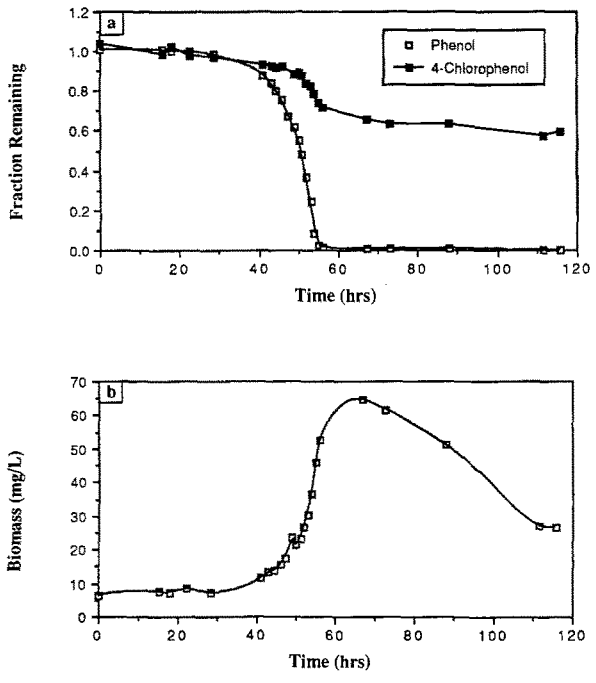

Fig. 4. Time-dependent curves for the concentrations of: a) phenol and 4-chlorophenol, and b) biomass for a partial-removal experiment (test PC2). The initial concentrations of phenol and 4-chlorophenol were 56.4 and 54.1 mg/L, respectively.

monooxygenase step of 4-chlorophenol degradation. This link is quantified later.

Figure 4 shows typical batch curves for a partial 4-CP removal experiment (test PC2). Most of the characteristics of the batch curves for partial 4-CP removal experiments are identical to those for complete 4-CP removal experiments: the presence of a lag phase, the simultaneous transformation of both substrates, the more rapid transformation of phenol, the link between the phenol and 4-chlorophenol removal rates, and the coincidence of the beginning of the negative cell-growth period with the halting of the phenol degradation.

Two characteristics of the batch curves for partial 4-CP removal experiments are markedly different from those for the complete-removal experiments. First, the 4-chlorophenol remaining when the biodegradation of phenol stopped was not appreciably transformed during the negative cell-growth period. Second, the phenol biodegradation rate sharply dropped before phenol became undetectable (test PC6) or levelled off at a nonzero (i.e., detectable)

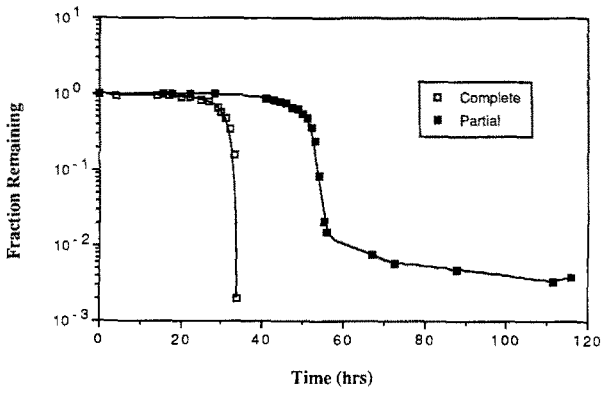

Fig. 5. Typical time-dependent phenol concentration curves (logarithmic scale) for tests in which a complete or a partial 4-chlorophenol removal was observed. The curves shown for the complete and partial removal correspond to tests PC1 and PC2, respectively. The initial conditions for those tests are given in Figures 3 (test PC1) and 4 (test PC2).

concentration (tests PC2 and PC3). This sharp decrease, observed in partial removal-experiments, but not in complete-removal experiments, is illustrated in Fig. 5, which shows typical time-dependent phenol concentration curves on an expanded logarithmic scale. The sharp decrease in the phenol degradation rate for partial 4-CP removal experiments was due to inhibition caused by 4-chlorophenol, which is quantified later.

Table 2 summarizes the duration of the lag and the character of the 4-chlorophenol removal (complete or partial) for the bisubstrate tests. The results for the biodegradation of phenol alone also are included to facilitate comparison. Analysis of Table 2 yields two conclusions. First, the duration of the lag period increased as the initial 4-chlorophenol concentration (I_i) increased (for a fixed initial phenol concentration, S_i). Second, the complete or partial character of the experiment was totally determined by the 4-chlorophenol/biomass (I/X) ratio evaluated at the I and X experimental values corresponding to the first measurement when phenol degradation stopped. Complete 4-CP removal experiments had $I/X \leq 0.21$ mg 4-CP/mg SS, while partial-removal experiments had $I/X \geq 0.38$ mg 4-CP/mg SS. These results are completely concordant with those obtained for the 4-chlorophenol-alone tests (Saéz & Rittmann 1991), where complete-removal tests

Table 2. Duration of the lag and character of the 4-chlorophenol removal for the phenol/4-chlorophenol tests.[1]

Test	S_i[2] (mg/L)	I_i[3] (mg/L)	Lag length (hours	4-CP removal character	First I/X measured when S = 0
PA1	50.84	0.0	<15.0	not applicable	not applicable
PA2	50.66	0.0	<14.7	not applicable	not applicable
PA3	51.15	0.0	<15.5	not applicable	not applicable
PC1	50.18	22.96	<14.1	complete	0.21
PC2	56.44	54.09	28.5–41.0	partial	0.55[3]
PC3	50.32	68.17	20.0–38.0	partial	1.10[3]
PA5	173.24	0.0	9.0–12.25	not applicable	not applicable
PA6	168.19	0.0	21.5–27.0	not applicable	not applicable
PA7	182.54	0.0	25.0–28.0	not applicable	not applicable
PC4	174.16	25.06	38.0–44.1	complete	0.065
PC5	170.92	48.28	47.8–61.9	complete	0.17
PC6	172.50	94.59	69.0–78.5	partial	0.38

Notes: [1] The data for the phenol-alone tests are included for comparison. [2] For those tests (experiments PA5-PA7, and PC2-PC6) where a lag phase was clearly observed, the initial concentrations S_i and I_i were taken as the mean of all observations performed during the lag. [3] Phenol degradation stopped when its concentration was zero in all tests except tests PC2 and PC3; the I/X ratio in those cases was taken when phenol degradation stopped.

had I/X ≤ 0.25 mg 4-CP/mg SS and partial-removal tests had I/X ≥ 0.59 mg 4-CP/mg SS.

Kinetic modeling and interpretation

Criterion for goodness of fit of a model and its parameters

The first step to correctly find a model and its best parameters that represent a data set is to analyze the error structure of the variables, if that information is available. Since the phenol assay involved performing three independent determinations for each experimental point, the experimental error structure for the phenol determinations can be estimated. The standard deviation (STD) for triplicate samples for the phenol determinations increased as the phenol concentration increased, as illustrated in Fig. 6 (test PA5). Hence, the variance of the absolute error for the phenol assay was nonconstant. On the other hand, the coefficient of variation (COV) for the triplicate samples did not have a dependence on the phenol concentration. The data in Fig. 6 indicate that the relative error for the phenol assay had an approximately constant variance. Similar results were obtained when analyzing the standard deviations and coefficients of variation for the other phenol-alone biodegradation tests. In conclusion, the error structure of the phenol assay is such

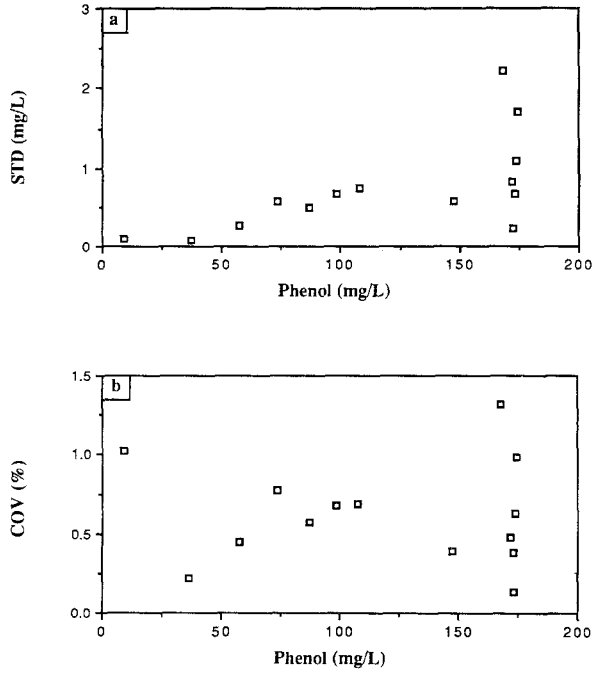

Fig. 6. a) Standard Deviation (STD), and b) coefficient of variation (COV) of three replicate samples as a function of the mean phenol concentration for batch test PA5.

that the *relative error* has mean zero and constant variance.

The constant-variance characteristic of the relative error requires that estimation of the model parameters employ a relative least-squares (RLS) criterion if the concentration ranges are large (Saéz & Rittmann 1992). Inspection of Fig. 1 demonstrates that phenol and biomass concentrations varied over 2 to 3 orders of magnitude. Thus, the RLS criterion was essential to estimate the model parameters in this research. The RLS criterion used is F_2, which is defined as the sum of the squares of the differences between the normalized model-predicted value and 1.0, where the normalization is achieved by division by the experimental value (Saéz & Rittmann 1992).

Phenol-alone tests

Model equations

The basic mass-balance equations for a single-species, single-substrate batch reactor are

$$\frac{dS}{dt} = r_s \tag{1}$$

and

$$\frac{dX}{dt} = r_x \tag{2}$$

in which

S = concentration of phenol (M_sL^{-3}, where M_s is mass of phenol and L is length),
X = concentration of active biomass (M_xL^{-3}, where M_x is mass of active bacteria),
t = time from the beginning of the experiment (T, where T is time),
$-r_s$ = phenol utilization rate ($M_sL^{-3}T^{-1}$), and
r_x = biomass growth rate ($M_xL^{-3}T^{-1}$).

Because phenol exhibited self-inhibition, Haldane kinetics (Andrews 1968) were assumed for the phenol utilization rate. Combining Haldane kinetics with a biomass growth rate controlled by synthesis and maintenance (Roels 1983) yields

$$-r_s = \frac{q_s X S}{K_s + S + \frac{S^2}{K_h}} \tag{3}$$

and

$$r_x = Y_s (-r_s) - b X \tag{4}$$

in which

q_s = maximum specific phenol utilization rate in absence of self inhibition and when phenol is present alone ($M_sM_x^{-1}T^{-1}$),
K_s = half-maximum-rate phenol concentration in absence of self inhibition and when phenol is present alone (M_sL^{-3}),
K_h = self-inhibition constant for phenol (M_sL^{-3}),
Y_s = true yield coefficient of biomass per unit of phenol consumed ($M_xM_s^{-1}$), and
b = decay coefficient (T^{-1}).

Combining Eqs. (1)–(4) yields

$$\frac{dS}{dt} = -\frac{q_s X S}{K_s + S + \frac{S^2}{K_h}} \tag{5}$$

and

$$\frac{dX}{dt} = Y_s \left(-\frac{dS}{dt}\right) - b X \tag{6}$$

For the special case in which Y_s (–dS/dt) is much greater than bX, a typical situation during the biodegradation phase in batch reactors, Eq. (6) can be reduced to

$$\frac{dX}{dt} = Y_s \left(-\frac{dS}{dt}\right) \tag{7}$$

which can be integrated to give

$$X = X_o + Y_s (S_o - S) = -Y_s S + constant \tag{8}$$

where S_0 and X_0 are the initial phenol and biomass concentrations for the biodegradation phase, respectively. Equation (8) indicates that, if decay is negligible, a linear relationship exists between X and S, and the slope of a graph of X versus S is $(-Y_s)$.

The remainder of this section describes how estimates for the cell-growth parameters (Y_s and b) and the phenol-utilization parameters (q_s, K_s, and K_h)

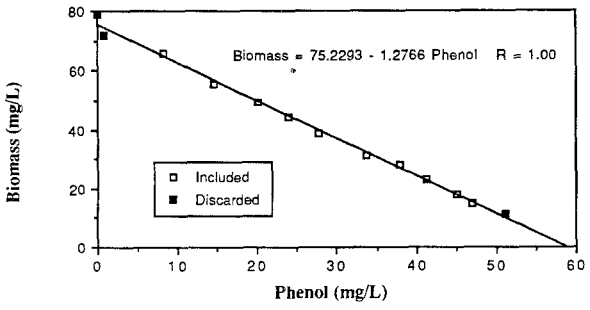

Fig. 7. Biomass concentration as a function of the phenol concentration for test PA3. Points included in and discarded from the regression are identified.

were obtained when phenol was present as a single substrate.

Estimation of the cell-growth parameters

As suggested by Eq. (8), a linear relationship exists between biomass and phenol concentrations, if the decay rate (bX) is negligible compared to the rate of new growth [Y_s (–dS/dt)]. Figure 7 shows the biomass concentration as a function of the phenol concentration for test PA3; the results for the other tests are similar. Clearly, the biomass and phenol concentrations are linearly related. An absolute least-squares method was used to estimate the parameters of the linear model: it gave the same estimates as the RLS for this situation, because of the almost perfect linearity of the data. Some experimental points were not included in the regressions. The points discarded, which did not fit on the straight lines, were always located in the lag phase, at the very beginning of the biodegradation phase, or at the very end of the biodegradation phase. These points were discarded because they were taken from regions in which cell-mass concentrations were not well correlated to light absorbance, as discussed previously. Thus, the discarded X values were not reliable.

The results for the linear regressions, summarized in Table 3, yield three conclusions. First, the coefficients of correlation were almost identical to 1.00, indicating an almost perfect linear relationships between biomass and phenol concentrations in all cases. This linearity demonstrates, in turn, that the decay coefficient was negligible during the biodegradation phase of the batch tests. Second, the yield coefficient for phenol was not correlated to the initial phenol concentration or to the number of phenol spikes given. Third, the mean value for the yield coefficient for phenol, computed as the average for all 10 tests, is 1.24 mg SS/mg phenol (0.74 mg COD_{cells}/mg COD_{phenol}) and has a standard deviation of 0.14 mg SS/mg phenol and a coefficient of variation of 11.6%. In summary, good estimates for Y_s and b are 1.24 mg SS/mg phenol and negligible, respectively, for the biodegradation period.

Estimation of the phenol-utilization parameters

Since decay is negligible during the biodegradation phase, Eqs. (5) and (8) can be combined to give the model equation governing the phenol concentration

$$\frac{dS}{dt} = -\frac{q_s [X_0 + Y_s (S_0 - S)] S}{K_s + S + \frac{S^2}{K_h}} \qquad (9)$$

The main practical problem for using Eq. (9) to determine the parameters q_s, K_s, and K_h is that Eq. (9) requires a perfect knowledge of the initial concen-

Table 3. Yield coefficients for the phenol-alone biodegradation tests.

Test	Initial phenol concentration (mg/L)	Yield coefficient (mg SS/mg Phenol)	Coefficient of correlation
First-spike tests			
PA1	50.84	1.14	1.00
PA2	50.66	1.38	1.00
PA3	51.15	1.28	1.00
PA4	101.82	1.39	1.00
PA5	173.24	1.16	1.00
PA6	168.19	1.33	1.00
PA7	182.54	1.17	1.00
PA8	251.13	1.08	1.00
Second-spike tests			
PA9	54.89	1.47	0.99
PA10	108.95	1.05	0.99
Mean (mg SS/mg phenol)		1.24	
Standard deviation (mg SS/mg phenol)		0.14	
Coefficient of variation (%)		11.6	

trations for the biodegradation phase, X_0 and S_0. Slight variations in X_0 and S_0 may yield very different model-parameter estimates, because the variations are amplified over time. The concentrations at the start of the test, X_i and S_i, cannot be used to approximate X_0 and S_0, since a lag phase existed and the concentrations were measured at discrete times. Furthermore, since the biomass concentration at the start of the test was very low (≈ 5 mg/L), it could not be measured accurately by absorbance measurements.

To overcome the limitations in the exact determination for X_0 and S_0, the following three-step procedure was employed. First, several determinations of the biomass concentration at the start of the different tests were performed by dry weight measurements. The average value (5 mg/L; coefficient of variation = 27%) was considered as X_i for all the tests.

The second step consisted of estimating X_0 and S_0 for the biodegradation phase of each test. The different initial conditions were given by

$$S_0 = S_{measured} \tag{10}$$

First-spike experiments

$$X_0 = X_i + Y_s\,(S_i + S_0) = 5.0 + 1.24\,(S_i - S_o) \tag{11}$$

Second-spike experiments

$$X_0 = 5.0 + 1.24\,\Delta S + 1.24\,(S_i - S_0) \tag{12}$$

where

$S_{measured}$ = a measured S value after the start of the biodegradation phase, and

ΔS = the initial concentration of phenol given during the first spike.

Equations (11) and (12) are consistent with the observation that decay is negligible during the biodegradation phase. Different phenol concentrations, all measured during the biodegradation phase of each test, were tried as $S_{measured}$ for that test. The data set for a given run (for each test) consisted of all the experimental observations taken after the condition selected as $S_{measured}$, so long as at least 4 experimental points were available.

Although Eq. (9) can be analytically integrated to give an implicit solution for S, the differential form of the equation was solved numerically. The previously estimated value for Y_s of 1.24 mg SS/mg phenol was always employed.

The third step was to compute the best-fit values of q_s, K_s, and K_h for each run and S_0 value. The relative least-squares (RLS) criterion was employed for estimating the model parameters (Saéz & Rittmann 1992).

Simultaneous estimation of all three Haldane parameters – q_s, K_s, and K_h – for each individual test was attempted first. The RLS parameter estimates varied widely among the different tests, and several combinations of 'best' parameter estimates were infeasible. These problems occurred because of the high correlation between the parameters, which resulted in very different sets of parameters values providing almost identical fits. Hence, simultaneous estimation of all three parameters failed.

The following two-stage methodology overcame the correlation problems. First, the parameters q_s and K_s were estimated by considering together the data from the 3 replicate experiments (tests PA1-PA3) having the lowest initial phenol concentrations (50 mg/L). This step is logical and efficient because the parameter K_s becomes a dominant determinant of the kinetics as the phenol concentration decreases; on the other hand, the effects of K_s are overwhelmed by the term S^2/K_h (substrate-inhibition term) for high values of S [see Eq. (9)]. Also, considering together the data for tests PA1-PA3 provided more reliability, compared to an individual-test estimation procedure, because the fits were based on more experimental points. The second stage was to estimate for each test the self-inhibition parameter, K_h, using the values of q_s and K_s determined in the first stage. The results for both stages are discussed below.

The best fit value for K_s and q_s were determined for runs having an initial nominal phenol concentration equal to 50 mg/l. The best-fit parameters were K_s = 1.22 mg/L and q_s = 0.356 mg/mg-h. Figure 8 shows how the RLS function F_2 (sum of the squares of the relative residuals) evaluated at the given K_s and with the q_s and K_h RLS best estimates corresponding to that K_s, depends on K_s for these

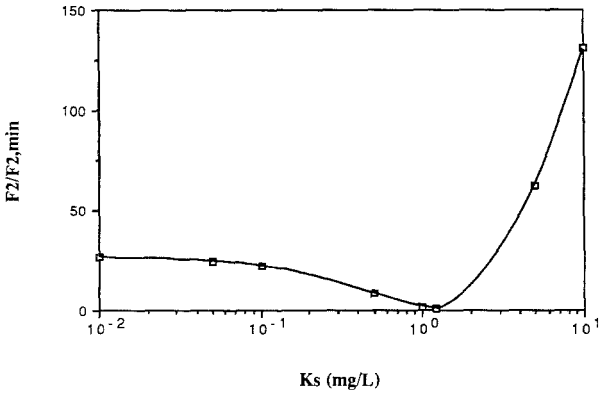

Fig. 8. Ratio between the RLS function (F_2, evaluated at the given K_s value and at the q_s and K_h RLS best estimates for the given K_s) and the minimum RLS function ($F_{2\text{-min}}$, obtained at $K_s = 1.22$ mg/L, $q_s = 0.356$ mg/mg-hr, and $K_h = 36.8$ mg/L) considering only the data for the tests having initial nominal phenol concentrations equal to 50 mg/L (i.e., tests PA1, PA2, and PA3).

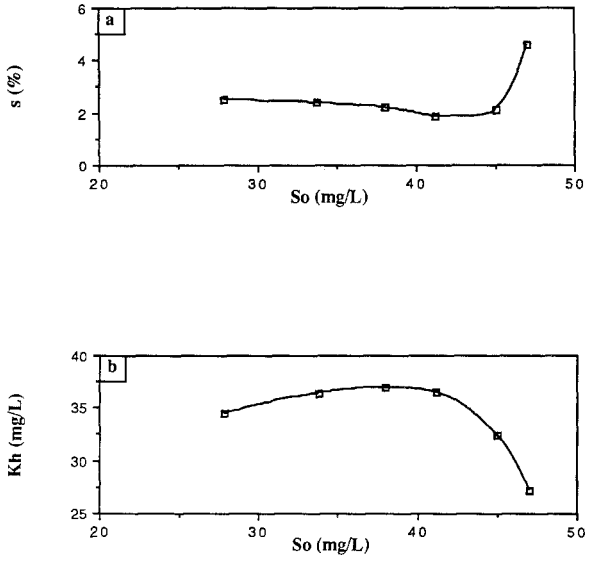

Fig. 9. Effect of S_o on the: a) standard deviation (s) and b) K_h estimate for the RLS method for test PA3.

tests. The figure gives F_2 normalized to the minimum F_2 obtained, $F_{2\text{-min}}$. The function $F_2/F_{2\text{-min}}$has a sharp minimum at $K_s = 1.22$ mg/L, indicating that K_s is well determined and unique. The corresponding RLS best estimate for K_h is 36.8 mg/L.

Next, the parameter-estimation procedure found, for each individual test, the RLS estimate for K_h by using the values for q_s (= 0.356 mg/mg-h) and K_s (= 1.22 mg/L) obtained when analyzing the results for tests with low initial phenol. Typical results are shown in Figs. 9 and 10 for batch tests PA3 and PA8, respectively. Each figure presents the standard deviation, s, of the nonlinear regression, and the RLS estimate for the self-inhibition parameter, K_h. The results are presented as functions of the $S_0 = S_{measured}$for the biodegradation phase [see Eqs. (10)-(12)].

The experiments can be divided into two groups. In the first group – tests PA1, PA2, PA3 (Fig. 9), PA5, PA6, and PA7 – the estimates for K_h stabilize when S_0 decreases. In other words, K_h does not depend on the chosen initial condition when S_0 is lower than a certain value, an indication that the parameter is well determined. This good behavior is corroborated by the low values for the standard deviation of the regressions, which are consistently less than 2% in the stabilization range.

Statistically stabilized behavior occurs for two reasons. First, as the S_0 decreases, it is more likely that points in the transition zone between the acclimation and biodegradation phases, which probably is not well represented by the Haldane model, are not considered in the regression analysis. Second, the X_i part of Eq. (11) becomes small compared to

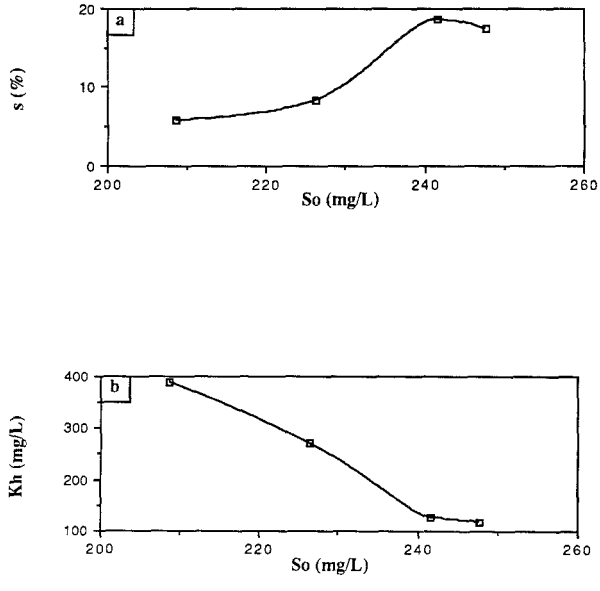

Fig. 10. Effect of S_o on the: a) standard deviation (s) and b) K_h estimate for the RLS method for test PA8.

$Y_s (S_i - S_0)$. Since the phenol concentrations were accurately measured and the estimate of Y_s was based on many points, it is very likely that the term $Y_s (S_i - S_0)$ was estimated more accurately than the term X_i. If this is the case, the error in X_0, which affects the parameter estimates, decreases as S_0 decreases, because the term $Y_s (S_i - S_0)$ becomes more dominant.

In the second group of experiments – tests PA4, PA8 (Fig. 10), PA9, and PA10 – the values for K_h continuously increase as the chosen S_0 decreases, and a stabilized region is not observed. This observation indicates that K_h is not as well estimated, which is corroborated by higher values for the standard deviations of the regressions, compared to the first group of experiments.

The data for all the tests are summarized in Table 4, which shows the best results for each test as determined by the lowest values obtained for the standard deviations of the regressions. Analysis of Table 4 yields four conclusions:

- First, the standard deviations for the regressions for the first group of tests – experiments with stabilized statistical behavior – are very low, ranging from 0.4 to 1.9%. This demonstrates that the Haldane model represents extremely well the biodegradation of phenol as a single substrate; on the other hand, the standard deviations are considerably higher for the second group of tests (4.4–8.8%).
- Second, the results for the triplicate tests PA1-PA3 and for the triplicate tests PA5-PA7 are almost identical, which demonstrates that the biodegradation-phase experimental results were totally reproducible.
- Third, the estimate for K_h increases with increasing initial phenol concentrations, suggesting that some kind of cell adaptation occurred during the lag phase.
- Finally, while some kind of adaptation apparently occurred after a second-spike for an initial concentration of 50 mg/L (compare tests PA1-PA3 with test PA9), the results at 100 mg/L for first- and second-spike experiments were almost identical (compare tests PA4 and PA10).

Table 5 summarizes the best Haldane-parameter estimates for the biodegradation of phenol as a single substrate. Only the results with stabilized statistical behavior are included, which excluded the results from the runs with nominal initial phenol concentrations of 175 mg/L (PA7) and 250 mg/L (PA8). The estimates for K_h shown in this table were obtained by considering simultaneously all the data for the three replicates at each initial concentration and by using the previously estimated values for q_s

Table 4. RLS estimates for K_h and the standard deviations of the regressions for the phenol-alone biodegradation tests.[a]

Test	S_i (mg/L)[b]	S_0 (mg/L)[c]	K_h (mg/L)	s (%)
First-spike tests				
PA1	50.84	32.64	36.6	0.61
PA2	50.66	43.54	38.7	1.18
PA3	51.15	41.22	36.5	1.88
PA4[d]	101.82	79.94	261.8	5.39
PA5	173.24	107.91	140.7	0.48
PA6	168.19	99.11	164.3	0.44
PA7	182.54	139.99	140.3	1.12
PA8[d]	251.13	208.68	398.3	5.74
Second-spike tests				
PA9[d]	54.89	39.73	155.3	4.38
PA10[d]	108.95	87.62	276.4	8.80

[a] These results consider the following parameter values: $Y_s = 1.24$ mg SS/mg phenol, $q_s = 0.356$ mg/mg-h, and $K_s = 1.22$ mg/L.
[b] Phenol concentration measured at the beginning of the test.
[c] Phenol concentration that, when used as the initial condition for the model, gives the best fit, as measured by the standard deviation of the regression (see text).
[d] The model results for these tests may be less reliable (see text).

Table 5. Summary of the best parameters for the batch biodegradation of phenol alone and for initial phenol concentrations of 50 and 175 mg/L.

Inital nominal phenol concentration (mg/L)	Y_s	b	q_s	K_s	K_h
50	1.24	negligible	0.356	1.22	36.8
175	1.24	negligible	0.356	1.22	138.0

The units for the model parameters are:
Y_s = mg SS/mg phenol
b = 1/hr
q_s = mg phenol/(mg SS-hr)
K_s = mg phenol/L
K_h = mg phenol/L

(= 0.356 mg/mg-h) and K_s (= 1.22 mg/L). The tests at 50 and 175 mg/L could be modeled well using the same values for q_s and K_s. However, the estimates for K_h increased as the initial phenol concentration increased, an indication that the cells adapted during the lag phase. The physiological basis of the adaption was not investigated.

Phenol/4-chlorophenol tests

Model equations

The governing mass-balance equations for a single-species, batch reactor in the presence of one primary substrate (S) and one secondary substrate (I) are Eqs. (1), (2), and (13).

$$\frac{dI}{dt} = r_i \tag{13}$$

in which

I = concentration of 4-chlorophenol (M_iL^{-3}, where M_i is mass of 4-chlorophenol), and

$-r_i$ = 4-chlorophenol transformation rate ($M_iL^{-3}T^{-1}$).

Considering Haldane kinetics for the biodegradation of the primary substrate when it is present alone (this was demonstrated to be true for the phenol-alone biodegradation), and postulating that the presence of the secondary substrate affects the Haldane coefficients q_s and K_s for phenol via interaction terms of the form

$\left[1+\frac{z_i^{nl}}{K_i}\right]$, where the function z_i = I or I/S (z_1 is for q_s and z_2 is for K_s), transform Eq. (1) to

$$\frac{dS}{dt} = -\frac{\dfrac{q_s}{\left[1+\dfrac{z_2^{nl}}{K_1}\right]} X S}{K_s\left[1+\dfrac{z_2^{nl}}{K_2}\right] + S + \dfrac{S^2}{K_h}} \tag{14}$$

in which

K_1 = inhibition constant associated with the maximum specific phenol utilization rate ($M_i^{n1}L^{-3nl}$ when z_1 = I, or $M_i^{n1}M_s^{-n1}$ when z_1 = [I/S]),

K_2 = inhibition constant associated with the half-maximum-rate phenol concentration ($M_i^{n2}L^{-3n2}$ when z_2 = I, or $M_i^{n2}M_s^{-n2}$ when z_2 = [I/S]),

n_1, n_2 = empirical positive exponents (dimensionless).

When the secondary substrate is transformed via the electrons generated by the primary-substrate oxidation and/or through electrons generated by biomass oxidation (Saéz & Rittmann 1991), the rate of secondary-substrate transformation (–dI/dt) should be proportional to the rate of primary-substrate oxidation (–dS/dt) and to the rate of biomass decay (bX), both of which can supply electrons. Then, Eq. (13) takes the form

$$\frac{dI}{dt} = \alpha\frac{dS}{dt} - \beta bX \tag{15}$$

in which

α = amount of 4-chlorophenol transformed per unit of phenol consumed ($M_iM_s^{-1}$), and

β = amount of 4-chlorophenol transformed per unit of biomass oxidized ($M_iM_x^{-1}$).

Saéz & Rittmann (1991) found that β was 0.92 mg 4-CP/mgSS when 4-CP degradation was uninhibited.

The rate of net biomass growth remains the difference between the growth associated with the primary-substrate utilization and the biomass decay (Eq. (6)).

Equations (15) and (6) can be simplified when decay is negligible, the typical situation for batch tests. The simplified forms are

$$\frac{dI}{dt} = \alpha\frac{dS}{dt} \tag{16}$$

and Eq. (7). They can be integrated analytically to give

$$I = I_0 - \alpha\,(S_0 - S) \tag{17}$$

and Eq. (8).

The procedure used to analyze the kinetic behavior of the bisubstrate system was the following. First, the yield coefficient for phenol in the presence of 4-chlorophenol was estimated by using Eq. (8) and the experimental results in which cell decay was negligible. Second, the parameter α was eval-

uated from Eq. (17) with 4-chlorophenol data also obtained when decay was negligible. Third, the parameters n_1, n_2, K_1, and K_2 were estimated using the RLS criterion for the governing Eq. (14). Different combinations for the functions z_1 and z_2 were evaluated. Recall that the q_s, K_s, and K_h values already were obtained by analyzing the phenol-alone biodegradation tests. Each of the steps is discussed in detail in the remainder of this section.

Cell-growth rate and yield

The growth yield coefficient for phenol in the presence of 4-chlorophenol (Y_s) was computed using Eq. (8), which can be rearranged to give

$$Y_s = \frac{X - X_0}{S_0 - S} \tag{18}$$

Equation (18), when applied between the start of the test (with concentrations S_i and X_i) and the experimental time at which the measured biomass was maximum (with concentrations S = 0 and X = X_{max}), takes the form

$$Y_s = \frac{X_{max} - X_i}{S_i} = \frac{X_{max} - 5.0}{S_i} \tag{19}$$

because the initial concentration at the start of the test was taken as 5.0 mg/L for all tests.

Table 6, which summarizes the yield coefficients for the phenol/4-chlorophenol tests, leads to two conclusions. First, Y_s did not correlate to the initial 4-chlorophenol concentration; consequently, it can be taken as a constant for all the tests. Second, the mean for the yield coefficient for phenol in the presence of 4-chlorophenol – 1.16 mg SS/mg phenol, with a standard deviation and a coefficient of variation of 0.10 mg SS/mg phenol and 8.7%, respectively – was only 6.5% less than the mean for Y_s in the absence of 4-chlorophenol (1.24 mg SS/mg phenol, with a coefficient of variation of approximately 10%). Thus, the two means were not different at a significance level of 5%, based on the t-statistic for comparing the differences between means. The two conclusions can be combined to finally conclude that the yield coefficient for phenol was not affected by the presence of 4-chlorophenol; consequently, a value for Y_s = 1.24 mg SS/mg phenol (the value obtained for the biodegradation of phenol alone) is used for modeling the phenol/4-chlorophenol system.

Table 6. Estimation of the yield coefficient for phenol/4-chlorophenol tests.

Test	S_i (mg/L)	I_i (mg/L)	X_{max} (mg/L)	Y_s (mg SS/mg phenol)
PC1	50.18	22.96	70.72	1.31
PC2	56.44	54.09	64.69	1.06
PC3	50.32	68.17	62.12	1.14
PC4	174.16	25.06	199.68	1.12
PC5	170.92	48.28	219.36	1.25
PC6	172.50	94.59	188.72	1.07
	Mean (mg SS/mg phenol)			1.16
	Standard deviation (mg SS/mg phenol)			0.10
	Coefficient of variation (%)			8.7

4-Chlorophenol transformation rate

Since Eq. (17) can be written as

$$I = \alpha S + constant \tag{20}$$

the slope of a linear regression between I and S provides the least-squares estimate for α. Figure 11 shows the experimental results and the linear regression for test PC1; the other tests behaved similarly. The points included in the regressions are those that belong to the biodegradation phase before phenol was depleted. The linear relationship observed between the 4-chlorophenol and phenol concentrations clearly demonstrates that the 4-chlorophenol transformation rate was dependent on the phenol biodegradation rate. This dependence occurred because the NADPH consumed during the transformation of 4-chlorophenol to 4-chlorocatechol (Saéz & Rittmann 1991) was regenerated by the electrons produced from phenol oxidation. In the absence of a primary substrate, NADPH regeneration was via biomass oxidation (Saéz & Rittmann 1991), but NADPH regeneration occurred much more rapidly through phenol oxidation when phenol was present.

The results of the linear regressions between I and S are summarized in Table 7, which shows the least-squares estimates for α and I_i^* (the predicted

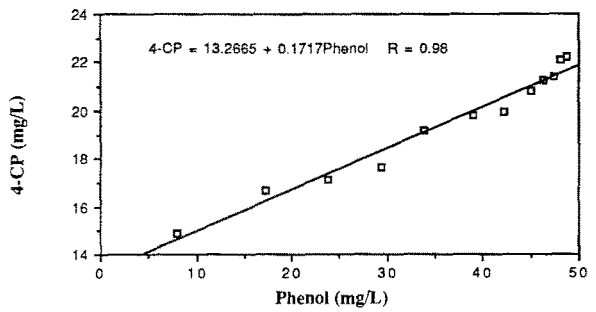

Fig. 11. Linear regression used to estimate the parameter α for test PC1.

initial 4-chlorophenol concentration), along with the coefficients of correlation (R). The I_i* values were computed by substituting the value for the measured initial phenol concentration (S_i) into the regressions. The analysis of Table 7 yields two conclusions. First, α was not related to S_i or I_i in any clear way. Second, the mean for α was 0.101 mg 4-CP/mg phenol, having a standard deviation and a coefficient of variation of 0.059 mg 4-CP/mg phenol and 58.4%, respectively. The large value for the coefficient of variation indicates that the parameter α was not well determined or that it was not truly constant.

Similarly to the parameter β for the transformation of 4-chlorophenol when present alone (Saéz & Rittmann 1991), the parameter α represents the electrons employed for NADPH regeneration (used to transform 4-chlorophenol to 4-chlorocatechol) per unit of electron generated by phenol oxidation. Using the mean value for α(= 0.10 mg 4-CP/mg phenol), the electron ratio can be computed as (0.10 mg 4-CP/mg phenol) × (94 mg phenol/28 e^- meq from phenol oxidation) × (2 e^- meq to NADPH regeneration/128.5 mg 4-CP). The resultant ratio of 0.005 e^- to NADPH regeneration/e^- from phenol oxidation. In other words, the biomass was shunting about 0.5% of the electrons gained by phenol oxidation to regeneration of NADPH for 4-chlorophenol transformation. This very low diversion of electrons, by itself, should not produce any significant change in the yield coefficient in the presence of 4-chlorophenol, compared to the corresponding value in the absence of 4-chlorophenol. This analysis is consistent with the conclusion that the yield coefficient was not affected by the presence of 4-chlorophenol. When phenol was absent, the bacteria shunted about 9% of the electrons generated by biomass oxidation to 4-CP degradation (Saéz & Rittmann 1991).

Phenol biodegradation rate

Substitution of Eqs. (17) and (8) into Eq. (14) yields the governing equation for the concentration of phenol. For the case in which $z_1 = I$ and $z_2 = I/S$ (remember that z_1 is for q_s and z_2 for K_s), the governing equation becomes

$$-\frac{dS}{dt} = \frac{\dfrac{q_s}{\left[1+\dfrac{\{I_0-\alpha(S_0-S)\}^{n1}}{K_1}\right]}[X_0+Y_s(S_0-S)]\,S}{K_s\left[1+\dfrac{\{[I_0-\alpha(S_0-S)]/S\}^{n2}}{K_2}\right]+S+\dfrac{S^2}{K_h}} \qquad (21)$$

Other choices for z_1 and z_2 yield slightly modified forms.

Because Eq. (21) has a complicated form and contains 9 parameters (q_s, K_s, K_h, Y_s, α, K_1, n_1, K_2, and n_2), simultaneous estimation of all nine parameters is an impossible task. But, the step-by-step procedure followed in this research allowed the previous estimation of 5 of the 9 parameters (q_s, K_s, K_h, Y_s, and α), which greatly simplified the mathematical analysis for the remaining four (K_1, n_1, K_2, and n_2).

The following four-step procedure was used to

Table 7. Estimation of the parameter α for phenol/4-chlorophenol tests.

Test	S_i [mg phenol / L]	I_i [mg 4-CP / L]	α [mg 4-CP / mg phenol]	I_i* [mg 4-CP / L]	R
PC1	50.18	22.96	0.172	21.88	0.98
PC2	56.44	54.09	0.176	51.96	0.99
PC3	50.32	68.17	0.079	71.22	0.94
PC4	174.16	25.06	0.041	24.71	1.00
PC5	170.92	48.28	0.053	46.04	0.98
PC6	172.50	94.59	0.081	94.60	0.97

Mean (mg 4-CP/mg phenol)	= 0.101
Standard Deviation (mg 4-CP/mg phenol)	= 0.059
Coefficient of Variation (%)	= 58.4

determine the unknown functions z_1 and z_2 and to estimate the unknown parameters K_1, n_1, K_2, and n_2.

- First, functional relationships for z_1 and z_2 were assumed. The functions evaluated were given by $z_i = I$ or I/S.
- Second, the parameters q_s, K_s, K_h, Y_s, and α were assumed to be constant and known for a given test. The values for q_s, K_s, and K_h used corresponded to the RLS estimates obtained for the biodegradation of phenol alone (these values are given in Table 5). Y_s was taken as 1.24 mg SS/mg phenol, as previously determined. Since the parameter α varied significantly for the different tests, individual α values were considered for each test. The values taken for α correspond to those given in Table 7.
- Third, the model parameters K_1, n_1, K_2 and n_2 were estimated (via the RLS criterion) by using different initial conditions for the biodegradation phase of each test, in a similar way to that explained in detail for phenol alone. The initial conditions used were given by Eqs. (10), (11), and (22).

$$I_0 = I_i^* - \alpha\,(S_i - S_0) \qquad (22)$$

where I_0 is the 4/chlorophenol concentration taken as initial for the biodegradation phase, I_i^* is the predicted 4-chlorophenol concentration at the start of the test (taken from Table 7), X_i was 5 mg/L, the average value determined by dry weight measurements, and S_i was the phenol concentration at the start of the test. The best initial condition for a given test was the one that minimized the standard deviation of the regression, as was described previously for phenol alone.

- Fourth, new functional relationships for z_1 and z_2 were assumed, and the first three steps were repeated. Comparison between the different z_i functions was carried out by comparing the standard deviations of the regressions.

Table 8 summarizes the RLS estimates for K_1, K_2, and n_2 for tests PC1-PC6 when $z_1 = I$, $n_1 = 1$, and $z_2 = I/S$. For $z_1 = I$ and $z_2 = I/S$, two derived parameters are defined. They are $K_1' = K_1^{1/n1}$ and $K_2' = K_2^{1/n2}$, which represent, respectively, the I concentration at which the specific phenol utilization is one-half of the value in the absence of I (for the same S) and the I/S ratio at which the half-maximum-rate phenol concentration is double the value in the absence of I.

Analysis of Table 8 yields three conclusions. First, the effect of 4-chlorophenol on the phenol degradation was inhibitory for five experiments (tests PC1-PC3, and PC5-PC6), because K_1' and/or K_2' were positive and much lower than infinity; in other words, the presence of 4-chlorophenol made q_s smaller and/or K_s larger. On the other hand, 4-chlorophenol was stimulatory in test PC4, since K_1' was negative. The explanation for the counter-intuitive result in test PC4 is not yet clear. Second, the standard deviations of the regressions, s, were very low for tests PC1-PC5, which indicates that the model represented the data extremely well. A value

Table 8. Parameter estimates for phenol utilization during the phenol/4-chlorophenol tests, when $z_1 = I$, $n_1 = 1$, and $z_2 = I/S$.[1]

Test	S_i[2] $[\frac{mg\ Ph}{L}]$	S_o[3] $[\frac{mg\ Ph}{L}]$	K_1'[4] $[\frac{mg\ Ph}{L}]$	n_2 (-)	K_2 $[\frac{mg\ 4\text{-}CP}{mg\ Ph}]^{2.5}$	K_2'[4] $[\frac{mg\ 4\text{-}CP}{mg\ Ph}]$	s (%)
PC1	50.18	38.99	52.20	2.5	∞	∞	2.73
PC2	56.44	49.70	61.50	2.5	371.8	10.67	1.46
PC3	50.32	45.27	137.8	2.5	55.07	4.97	4.61
PC4	174.16	166.98	− 83.69	2.5	∞	∞	1.86
PC5	170.92	104.23	37.30	2.5	73598	88.46	1.89
PC6	172.50	137.26	∞	0.1	0.020	1.0×10^{-17}	22.32

Notes: [1] Parameters used: $q_s = 0.356$ mg Ph/(mg SS-hr); $K_s = 1.22$ mg Ph/L; $K_h = 36.8$ mg Ph/L (for tests PC1-PC3) or 138.0 mg Ph/L (for tests PC4-PC6); $Y_s = 1.24$ mg SS/mg Ph; α = indivual value for each test, taken from Table 7. [2] Phenol concentration at the start of the test. [3] Selected phenol concentration for the beginning of the biodegradation phase (see text). [4] $K_1' = K_1^{(1/n1)}$ and $K_2' = K_2^{(1/n2)}$.

of n_2 = 2.5 provided good fits for all those tests. However, the fit for test PC6 was poorer, as indicated by the high s value. Third, tests PC1, PC4, and PC5 – the complete 4-CP removal experiments – demonstrated that the main inhibition effect was on q_s, since K_2' was very large. One the other hand, the inhibitory effect of 4-chlorophenol was manifested mainly on the parameter K_s for tests PC2, PC3, and PC6 – the partial 4-CP removal experiments – since the K_1' values were large. In summary, the inhibition by 4-chlorophenol on phenol degradation was expressed mainly on the parameter q_s for complete 4-CP removal experiments and mainly on K_s for partial 4-CP removal experiments.

A possible explanation for this inhibition pattern is the following. At the beginning of the experiments, the I/X ratios were high (on the order of 5–20 mg 4-CP/mg SS), a value much larger than that which can be tolerated in the absence of phenol (Saéz & Rittmann 1991). Once the oxidation of phenol started, it clearly compensated the inhibition caused by 4-chlorophenol, at least to some degree. However, not all the inhibition could be suppressed by the oxidation of phenol, and the residual inhibition was manifested through a lowering of q_s. As the reaction proceeded, phenol utilization caused a decrease in the 4-chlorophenol concentration and an increase in the biomass concentration, which resulted in a decrease in the I/X ratio. When phenol was no longer available at high enough concentrations to partially compensate the inhibition by 4-chlorophenol, the inhibition degree was controlled by the I/X ratio. A sharp decrease in phenol degradation occurred if the I/X ratio at that point was higher than about 0.25–0.30 mg 4-CP/mg SS, similar to the 4-chlorophenol-alone studies (Saéz & Rittmann 1991). This sharp decrease was expressed through an increase in K_s, an increase that could be adequately modeled using the I/S ratio. On the other hand, no decrease in the phenol degradation rate was observed when the I/X ratio at the time when phenol was exhausted was lower than 0.25–0.30 mg 4-CP/mg SS, which meant that K_s was not affected.

The effect of I on q_s when phenol was present suggests that phenol and 4-chlorophenol compete for the monooxygenase (Saéz & Rittmann 1991) in a noncompetitive way. A reasonable hypothesis is that an oxidized intermediate of phenol competes with 4-chlorophenol for a site of regulatory binding. When phenol is being oxidized at a high enough rate, the product is present and binds to the site; then, 4-chlorophenol cannot bind, and enzyme deactivation is overcome. When phenol is not available at high enough concentrations, 4-chlorophenol binds, and noncompetitive deactivation of the enzyme occurs when the I/X ratio is high.

The competitive inhibition response of 4-chlorophenol when phenol was nearly exhausted can be explained by the substrate function of 4-chlorophenol. At low I/X ratios – i.e., representing low ratios between 4-chlorophenol and the monooxygenase – the substrate function and the relatively large amounts of monooxygenase keep the internal concentration of 4-chlorophenol too low for any deactivation function to be expressed. Under these circumstances, K_s is not affected, because 4-chlorophenol is at too low of an internal concentration to be an effective competitive inhibitor. On the other hand, the competition function was dominant at high I/X ratios, because the internal 4-chlorophenol concentration was high, due to its self-inhibition (Saéz & Rittmann 1991). The end result is manifested by an increase in K_s, a sign of competitive inhibition.

The I/X ratio evaluated at the time when phenol was totally depleted determined if 4-CP removal was complete or partial, which in turn, affected the main way in which 4-chlorophenol inhibited phenol degradation. This ratio can be predicted by dividing Eq. (17) by Eq. (8) and setting S = 0,

$$\frac{I}{X}\Big|_{s=0} = \frac{I_i - \alpha S_i}{X_i + Y_s S_i} \tag{23}$$

which can be simplified by noting that $Y_s S_i \gg X_i$ for the batch tests performed in this research, yielding

$$\frac{I}{X}\Big|_{s=0} = \frac{1}{Y_s}\left(\frac{I_i}{S_i} - \alpha\right) \tag{24}$$

Since the ratio (I/X at S = 0) should be lower than approximately 0.25–0.30 mg 4-CP/mg SS for a complete 4-CP removal, a condition for the initial ratio I_i/S_i can be developed from Eq. (24), the result being

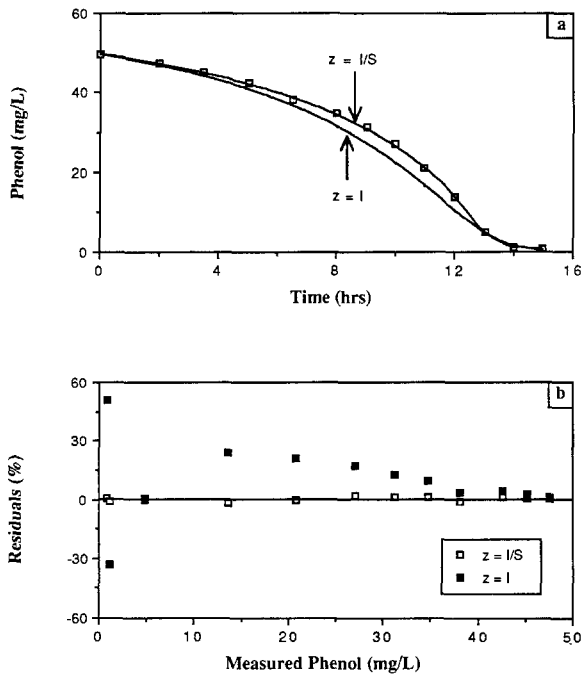

Fig. 12. a) Predicted concentrations and b) model residuals for a partial 4-chlorophenol removal experiment (test PC2). For both models shown, $z_1 = I$ and $n_1 = 1$. The model for $z_2 = I/S$ considers $n_2 = 2.5$, $K_1' = 61.50$ mg 4-CP/L, and $K_2' = 10.67$ mg 4-CP/mg phenol (RLS estimates taken from Table 8). The model for $z_2 = I$ considers $n_2 = 1.0$, $K_1' = 119.18$ mg 4-CP/L, and $K_2' = 4.22$ mg 4-CP/mg phenol (RLS estimates for $n_2 = 1.0$). The start of the biodegradation phase (t = 0) was taken for $S_o = 49.70$ mg Ph/L.

$$\frac{I_i}{S_i} < (0.25 - 0.30)\, Y_s + \alpha \qquad (25)$$

Taking the average values for Y_s (= 1.24 mg SS/mg phenol) and α (= 0.10 mg 4-CP/mg phenol), the condition for complete 4-CP removal is $I_i/S_i < 0.41$–0.47 mg 4-CP/mg phenol. The I_i/S_i ratios for the complete 4-CP removal tests PC1, PC4, and PC5 were 0.46, 0.14, and 0.28 mg 4-CP/mg phenol, respectively, and the I_i/S_i ratios for the partial 4-CP removal tests PC2, PC3, and PC6 were 0.96, 1.35, and 0.55 mg 4-CP/mg phenol, respectively. The experimental results are consistent with the condition given by Eq. (25).

In summary, the following rules, obtained from analysis of Table 8 and from the development presented in the last paragraph, can be applied to model the effect of 4-chlorophenol on the biodegradation of phenol. For $I_i/S_i < 0.41$–0.47 mg 4-CP/mg phenol, the effect of 4-chlorophenol can be modeled by a noncompetitive decrease in q_s, using $z_1 = I$, $n_1 = 1$, $K_1' = 40$–50 mg 4-CP/L, and $K_2' = \infty$. These conditions represent well the complete removal tests PC1 and PC5, but not the complete removal test PC4 (because it was stimulatory). For $I_i/S_i > 0.41$–0.47 mg 4-CP/mg phenol, successful modeling was with a competitive increase in K_s, given by $K_1' = \infty$, $z_2 = I/S$, $n_2 = 2.5$, and $K_2' = 5$–11 mg 4-CP/mg phenol. Partial 4-CP removal tests PC2 and PC3 are well modeled by these conditions; on the other hand, the partial 4-CP removal test PC6 is not as well represented by the given conditions. [Note that $K_1' = \infty$ means that q_s is not affected and that $K_2' = \infty$ means that K_s is not affected.]

Finally, K_s-affected experiments could not be modeled well using $z_2 = I$; the functional relationship $z_2 = I/S$ gave much better fits, as illustrated in Fig. 12 for the partial 4-CP removal test PC2. The function $z_2 = I$ could not predict the sharp decrease in the phenol degradation rate that occurred in the period between 12 and 15 h. The residuals plot shows that the function $z_2 = I$ gave systematic errors for phenol concentrations from around 10 to 35 mg/l. On the other hand, the function $z_2 = I/S$ yielded a very good fit, having low residuals that were well behaved with mean near zero and a constant variance.

A likely reason why the effect on K_s for partial 4-CP removal tests was better modeled by the ratio I/S, instead of by the value I, is the following. When phenol was present at concentrations too low to partially compensate the inhibition caused by 4-chlorophenol binding, the controlling variables of the inhibition degree were the I/S ratio and I, both of which controlled the internal concentration of 4-chlorophenol. I/S probably gave better fits than I, because the I/S ratio provided a better mathematical representation of the on/off switch for monooxygenase deactivation when phenol and its product were depleted. The regulatory binding of 4-chlorophenol caused a rapid increase in the internal concentration of 4-chlorophenol, which made it a much more potent competitive substrate to phenol. This very sudden deactivation of the monooxygenase, like an on/off switch, cannot be represented very well by continuous functions, but the $(I/S)^{2.5}$ func-

tion simulates the effect reasonably well, because it quickly goes to infinity as S becomes very small.

Conclusions

Batch experiments coupled with modeling analyses lead to the following key conclusions about the substrate interactions occurring when phenol and 4-chlorophenol were utilized simultaneously.

- The cometabolic degradation of 4-chlorophenol was greatly accelerated by the presence of phenol, whose oxidation overcame self-inhibition by 4-chlorophenol.
- The 4-chlorophenol degradation rate was proportional to the rate of phenol oxidation. The average proportionality coefficient, α = 0.1 mg 4-CP/mg Ph, corresponds to shunting 0.5% of the electrons generated by phenol oxidation to the monooxygenase reaction with 4-chlorophenol.
- 4-chlorophenol inhibited phenol oxidation in a noncompetitive manner (i.e., $q_s' = q_s/(1 + I/K_1)$, and K_1 = 40–50 mg 4-CP/L) for high phenol concentrations.
- 4-chlorophenol inhibited phenol oxidation in a competitive manner (i.e., $K_s' = K_s\ [1 + [(I/S)/K_2')^{2.5}]$, and K_2' = 5–11 mg 4-CP/mg Ph) when the phenol concentration was very low.
- For batch tests, complete removal of 4-chlorophenol occurred only when the initial ratio of 4-chlorophenol to phenol was less than 0.41–0.47 mg 4-CP/mg Ph. When the initial ratio was larger, the 4-chlorophenol concentration present when phenol was exhausted was inhibitory to further 4-chlorophenol degradation.

Acknowledgement

The economical support of the government of Chile, the Catholic University of Chile, and the U.S. National Science Foundation (Grant No. CES 8351844) is greatly appreciated.

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