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Biodegradation of phenolic wastes*

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Abstract

Phenolic biodegradation kinetics were determined in bioreactors with large solids retention times (SRT). Long term kinetic experiments were conducted in pulse-fed batch reactors for single substrate (phenol) and multiple substrates (combinations of glucose, phenol and pentachlorophenol). Short term initial rate experiments were also conducted on the single and multiple substrate reactors. Results indicate that phenol is metabolized at a maximum rate of 0.55 h^{-1} with a half saturation coefficient of 10 mg/l. Phenol concentrations in excess of 50 mg/l inhibit the biodegradation rate. Our results also indicate that pentachlorophenol is cometabolized in the presence of phenol. It can be concluded that biodegradation of phenolic waste is a viable treatment option because the organisms, through their metabolic processes, reduced the waste concentrations below our detection limits.

Introduction

Many xenobiotic compounds thought to be too toxic for microorganisms to degrade are being degraded under aerobic, anaerobic, or anoxic conditions. Biodegradation for these compounds is being studied in soils, groundwater, traditional activated sludge units, fixed film reactors, and combinations of processes. The merits of single organism cultures are argued against natural mixed microbial populations. There are commercially available freeze dried microorganisms claimed to be capable of degrading ranges of recalcitrant compounds in a multitude of environments. Clearly, the use of microorganisms to reme-

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diate contaminated sites or break down toxic effluent wastes is gaining wide acceptance and practice. Biodegradation is versatile, inexpensive, and can potentially turn a toxic material into harmless end-products.

The versatility and adaptability that microbial degradation of hazardous wastes has demonstrated makes it tempting to promise potentially too much in the current atmosphere to find immediate solutions to our in-place pollutant problems and to meet tighter effluent discharge permits. Biodegradation truly has great potential in meeting our needs to clean up contaminated soils and water, but it has its limitations. Time is a key factor as the biodegradation of recalcitrant compounds can range from hours to years. Anaerobic degradation may be slower, but it typically generates fewer residuals. Any sludge generated from hazardous compounds is automatically considered hazardous until proven otherwise (40 CFR 261.3; 40 CFR 260.22). Growth can be inhibited not just by the presence of toxic compounds, but by the availability of macro- and micronutrients. Depending on the process chosen, such as aerobic versus anaerobic, generation of more dangerous compounds is possible as in the formation of vinyl chloride during the anaerobic degradation of trichloroethylene (TCE) [1]. These are a few of the considerations necessary when evaluating the appropriateness of biodegradation for the elimination of hazardous wastes.

Reported research on the biodegradation of hazardous compounds ranges from bench scale studies with single cultures to *in situ* or field level investigations. Bench scale reactor studies include investigations on a single type of organism's ability to degrade a compound through to acclimated mixed microbial populations consuming mixed wastes. These organisms are cultured most commonly in batch, batch-fed, continuous flow, or continuous flow with recycle reactors. Numerous studies have been conducted on single compounds acting as the sole substrate, yet in many applications it is more likely to encounter multiple substrates. These substrates may interact to enhance (synergism) or reduce (antagonism) degradation. Pilot scale treatability studies are commonly performed to determine the feasibility of a process to scale up to full unit operation. Several field investigations have been reported for the remediation of contaminated soils and groundwater by *in situ* efforts or pump-and-treat methods. In the present climate to repair the damaged environment, we have jumped from the laboratory to the field and in some cases it has been premature.

Fundamental studies on the kinetics of biodegradation under controlled conditions cannot be stressed enough. The mechanisms responsible for the successful degradation of hazardous compounds are not understood completely and few of the degradation pathways have been unraveled. The mathematical models available rely on Monod based kinetics with inhibition included. There is a need for more studies at the bench scale examining the effects of multiple substrates on biodegradation to determine those kinetic parameters necessary for the design of treatment facilities. Fortunately, the microbiology community

has become keenly interested in the degradation of these compounds and have been prolific in their research efforts. They can provide the resolution necessary to elucidate the mechanisms by which microorganisms are capable of degrading these compounds. Combining the talents of these scientists with those of the engineering community is a necessary step in the development of processes which will efficiently and effectively eliminate target compounds.

The objectives of our research were to determine the biodegradation kinetics of single substrate and multiple substrate phenolic compounds. Kinetic experiments were conducted using bioreactors with a relatively large mean solids retention time (SRT $\approx$ 65 days). These reactors were operated in a pulsed fed mode using single substrates of phenol and multiple substrates made with combinations of glucose, phenol and pentachlorophenol. Experiments were conducted to determine the rate and extent of biodegradation and to determine if there are synergisms in multiple substrates.

Background

The large scale synthesis of industrially important hydrocarbons and chlorinated organics has occurred only during the past few decades. Synthetic hydrocarbons, chlorinated organics and many of their end-products represent one class of xenobiotic and persistent compounds. Many of these organic chemicals exhibit not only acute toxicity but also chronic toxic effects including mutagenic, carcinogenic, and teratogenic manifestations. Synthetic organic compound are commonly found in industrial wastewater streams and can produce toxic upsets in wastewater treatment units. The resulting decrease in treatment efficiency may allow substantial amounts of these compounds to be discharged in effluents or concentrated in generated sludge.

Biodegradation represents an important process for the elimination of unwanted organic compounds. The activated sludge process is one the most common engineering solutions for the biodegradation of organic pollutants in industrial and municipal wastewaters. It has been shown by several investigators that a priority pollutant can be successfully degraded in an activated sludge laboratory reactor [2,3]. However, most industrial treatment plants contain complex influent wastewaters comprised of multiple combinations of toxic organic compounds. The mathematical relationship between cell growth and substrate depletion, necessary to design treatment facilities, are determined by performing kinetic studies on mixed cultures. It is important that kinetic studies be conducted in multicomponent media due to the multicomponent aspects of wastewaters, the role of auxiliary substrates, and mixed microbial populations in the degradation process [4]. Kinetic studies should be performed by using a feed that contains several biogenic compounds in order to mimic the characteristics of industrial and municipal wastewaters.

Induction of degradative enzymes

It has been well documented that prior exposure to some compounds can enhance or inhibit the degradation of compounds to which the organisms are subsequently exposed. Templeton and Grady [5] discuss the importance of culture history on the performance of microorganisms and consequently on the values of the determined kinetic parameters. They contend that cells physiologically adapt to their previous environment. These authors found that as the dilution rate at which the cells are grown in a chemostat is increased, the organisms contain higher levels of RNA and enzymes. If these cells were placed into batch or fed-batch reactors without substrate limitations, they could be expected to generate high growth rates relative to what the opposite culture conditions would generate. Consequently, evaluating a set of kinetic parameters determined during batch cultivation may not be representative of activated sludge systems.

The presence of enzymes and their concentrations could be expected to influence the rate and extent of degradation. Biodegradation of a xenobiotic compound can occur if the organisms present are capable of synthesizing the proper enzymes under the given set of environmental conditions. Gratuitous metabolism and cometabolism are often used to explain the biodegradation of xenobiotic compounds [6].

Enzymes are usually specific to their catalytic functions but they are much less specific to substrate binding. As a result, enzymes can be induced to cleave a certain chemical bond on a xenobiotic compound that resembles the functional groups of a natural substrate. According to Grady [4], if the functional groups do not greatly alter the charge makeup of the enzyme active site, then it is possible for the enzymes to catalyze its specific reaction on the xenobiotic compound. This induction of an existing enzyme which has the appropriate catalytic activity toward a new substrate is called gratuitous metabolism. Gratuitous metabolism is thought to be the major mechanism for biodegradation of aromatic hydrocarbons [4].

Dalton and Stirling [7] define cometabolism as the "transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound". Non-growth substrates will not support cell growth and maintenance. The non-growth substrate cannot act as the sole source of carbon and energy for a pure culture of bacteria. Transformation can only occur when there is another growth substrate present. This requirement is the distinction between gratuitous metabolism and cometabolism. Cells will eventually die if there is more energy required to transform the xenobiotic compound or to transport it into the cell than there is energy derived from the reaction. A growth substrate must be present in order to yield energy to drive the initial transformation reaction.

Assessing biodegradability of phenolic compounds

The extent to which phenolic compounds will be degraded can be determined by extensive individual testing or estimated by models which predict compound behavior based on similar, well characterized compounds. These models are loosely defined as Quantitative Structure–Activity Relationships (QSARs) which can be used to predict toxicity and fate in the environment. Using a compound's structure, molar refractivity, connectivity, among other parameters, behavior of the compound under a given set of conditions can be approximated. QSARs have been successfully applied in predicting carcinogenic activity using a structural analogy to identify structural components associated with carcinogenic activity and analyzing a new compound with respect to a standard set of descriptors for potential carcinogenicity [8]. Known chemical carcinogens such as aromatic amines, azo compounds, polynuclear hydrocarbons, alkylating agents, halogenated aliphatic and aromatic hydrocarbons, and carcinogenic metals such as cadmium and chromium are used as standards. However, structural criteria do not allow prediction of carcinogenic activity for totally new structural types.

QSARs have been used to predict certain physicochemical parameters necessary to assess environmental risks of chemicals, but which are difficult to measure. The main reasons for the use of QSARs are due to the difficulty in measuring the parameters and the varying inter-laboratory results. QSARs have been successful in predicting solubility, boiling points, density, heats of formation, and Henry's constants. Other studies have used QSARs to analyze toxicity and inhibition of bioactivity, biosorption, bioconcentration, and biodegradation [9]. An analysis of six classes consisting of seventy-nine aromatic and aliphatic compounds revealed that degradation of a series of congeners proceeds by a common mechanism which involves bonds common to all compounds in the series [10]. It was also concluded that electronic factors dominate the degradation process in the initial microbial attack. While these models represent a potentially powerful tool which can save time and money, they are not yet perfected to the point where specific experimental verification is no longer necessary.

For the family of phenolic compounds their basic structure, and that of most aromatic hydrocarbons, is the benzene ring. Benzene is the simplest six-membered aromatic carbon. The benzene molecule and benzene derivatives are less reactive and more stable than single-chain (aliphatic) compounds because of the larger amount of energy required to break apart a cyclic structure [11]. Positional locations of substituents are significant to the overall reactivity of an aromatic molecule since substituents greatly influence the mode of reaction for a chemical. These structural characteristics certainly influence the biodegradability of the compounds.

The pervasive use of petroleum based products has led largely to the prevalence of phenolic compounds in effluents and waste materials. Phenolics are

generated by coking plants, oil refineries, and several chemical industries during the processing of resins, plastics, dyes, pharmaceuticals and pesticides among other products. Another related compound is pentachlorophenol, the primary constituent of formulations used for the preservation of wood and as a fungicide, bactericide, insecticide and herbicide. Pentachlorophenol (PCP) has been widely used as a representative test compound to study the kinetic behavior of chlorinated hydrocarbons [12]. For these reasons our research team elected to study the biodegradation of phenol and pentachlorophenol in batch- fed reactors with single and multiple substrates. We evaluated biodegradation kinetics and have compared our findings to those already reported in the literature.

Phenolic compound degradative pathways

Several investigators have focused attention on elucidating metabolic pathways for phenolic compound biodegradation. In reviewing the literature a common metabolic step emerges for aerobic organisms. This step consists of the consumption of oxygen [13] and addition of a hyroxyl group to the phenol structure. The catechol type compound generated is a common metabolic step for biodegradation of phenolic compounds and has also been shown for biodegradation of benzene rings [14] as well as biphenyls [15–17]. Substitution of two hydroxyl groups, ortho to each other, allow enzymatic ring fission to occur, yielding organic acids that can undergo further reactions to common metabolites that can enter the TCA cycle (Fig. 1).

Halogenated phenolic rings present an additional problem to organisms attempting to biodegrade them. Fortunately, organisms have been exposed to well over 200 naturally occurring halogenated organic compounds [18]. Consequently, they have had a significant period of time within which to develop methods to degrade these naturally occurring compounds which may be an asset in degrading xenobiotic halogenated compounds [4]. In aerobic environments Wood [13] shows that the degradation of haloaromatics with low halogen counts (e.g. 2,4-dichlorophenol or 2,4-dichlorophenoxyacetic acid) will generally leave the carbon–halogen bonds intact until the ring structure has been split by oxygenases (Fig. 2, [19]) with subsequent halogen removal as hydrogen halides [4]. In cases where the haloaromatics have a high halogen count (e.g. pentachlorophenol) a halide must be substituted by a hydroxyl group on the intact ring before the ring fission reaction can begin (Fig. 3). It should be emphasized that all of the above oxygenase catalyzed reactions require an aerobic growth environment. It should be stated that haloaromatics have been dehalogenated in anaerobic environments [20] but problem end products have also been reported [1].

Biodegradation kinetics

The inhibition of microbial growth is often due to the inhibition of enzyme

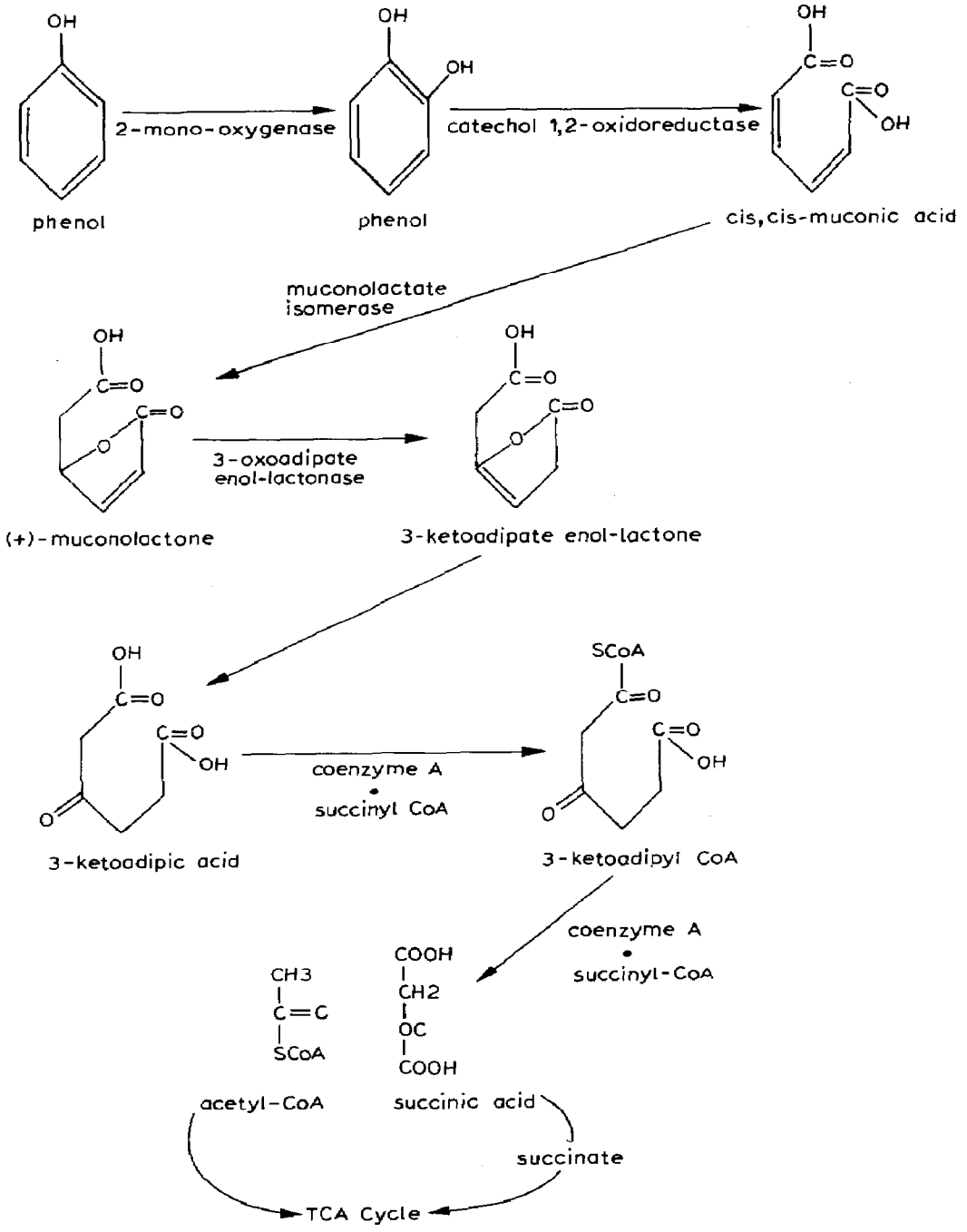


Fig. 1. Ortho-cleavage pathway of phenol metabolism by bacteria (adapted from Doelle [34]).

systems. An enzyme inhibitor reduces the rate of an enzymatically catalyzed reaction by binding with either the free enzyme and/or with the enzyme–substrate complex [21]. The Briggs and Haldane models for enzyme inhibition

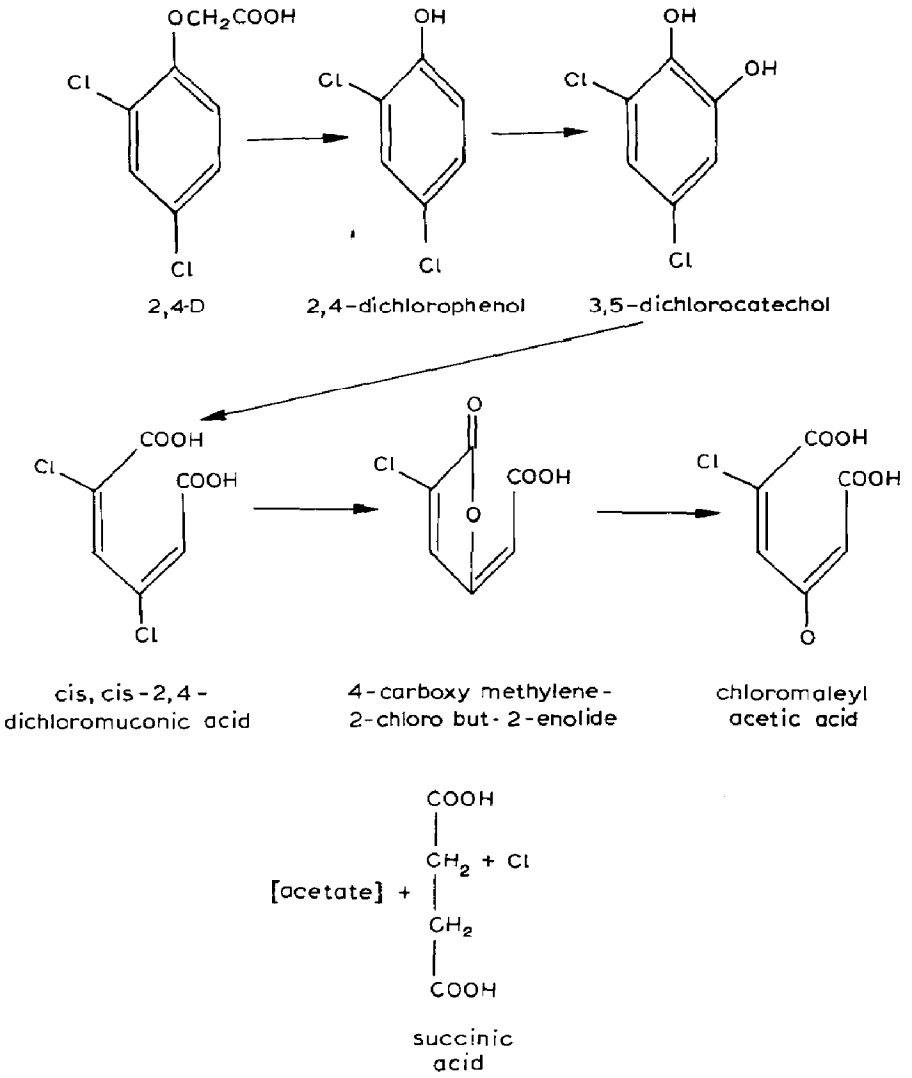


Fig. 2. Proposed degradative pathway of 2,4-D and 2,4-dichlorophenol (adapted from Cripps and Roberts [19]).

are often used to explain the behavior of cell growth inhibition. The growth rate of a cell population may be restricted by the presence of an inhibitor, high substrate concentration, or inhibitory by-product concentration. There are three types of models used to explain cell growth inhibition: competitive, non-competitive, and uncompetitive. Competitive inhibition occurs when a substrate competes with another substrate for a site on either the cell or the enzyme [22]. Non-competitive inhibition occurs when the inhibitor can combine with both the free cell or enzyme and the cell/enzyme–substrate complex. An uncompetitive inhibitor binds with the cell/enzyme–substrate complex which cannot undergo further reaction to yield product. Empirical correlations show

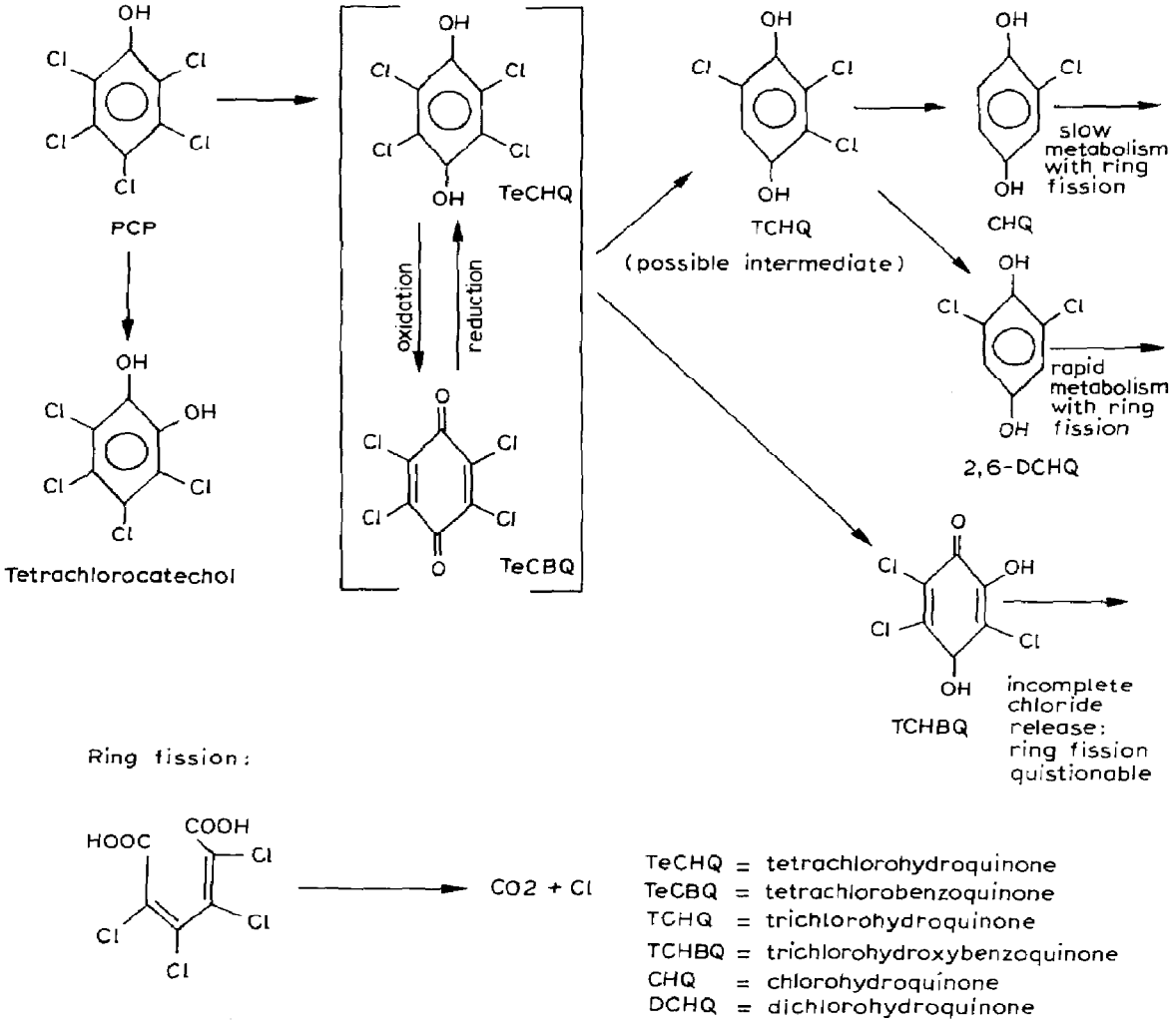


Fig. 3. Speculated route for PCP decomposition (adapted from McGinnis et al. [41] and Rochkind et al. [12]).

that the uncompetitive inhibition model is the most frequently used to describe cell growth and substrate utilization in mixed cultures of bacteria [21]. Andrews [23] applied a kinetic expression of the Monod form that allowed the substrate to inhibit the reaction at high concentrations. The variables and coefficients in this equation are defined the same as in the classical Monod equation, that is:

$$q = \frac{\mu_{max}}{Y} \frac{S}{K_s + S + S^2/K_i} \quad (1)$$

q is the specific substrate removal rate (t^{-1}); μ is the specific growth rate of microorganisms, (t^{-1}); μ_{max} is the maximum specific growth of microorga-

TABLE 1

Kinetic parameters[a] for phenol and pentachlorophenol (PCP)

Compound	μ_{max} (h^{-1})	K_s (mg/l)	K_i (mg/l)	Reference
Phenol	0.57 -1.18	0.634–245	7.5 -700	Rozich et al. [26] Beltrame et al. [40]
PCP	0.029–0.09	0.020– 0.09	0.80– 1.5	Klecka and Maier [39] Moos et al. [6]

[a] μ_{max} = maximum specific growth rate; K_s = half-saturation constant; and K_i = inhibition constant.

nisms, (t^{-1}); Y is a dimensionless yield coefficient ($\mu_{max}/y = q_m$); S is the concentration of the material being degraded with concentration units; and, K_s is the half-saturation coefficient, also with units of concentration. The inhibition term:

$$S^2/K_i \qquad (2)$$

is what differentiates this kinetic expression from the classical Monod expression. It can be seen that as S increases, the magnitude of this term increases and q is attenuated unless K_i is also very large.

The inhibitory effects of phenol biodegradation have been extensively investigated in both pure and mixed cultures [25–27]. Sokol and Howell [28] used a continuous flow reactor scheme with a pure culture of *Pseudomonas putida* to determine the kinetic parameters of phenol degradation. Using both the Andrews model (uncompetitive inhibition) and a two parameter kinetic model to describe cell growth and phenol depletion, these investigators report that the latter model successfully approximated the inhibitory behavior of phenol. Other investigators used the Andrews equation to evaluate their kinetic parameters from chemostat systems which are compiled in Table 1.

These kinetic models have shortcomings and are limited. Many times more complex models fail to give significantly better fits to biodegradation data [29]. The Andrews equation does not predict critical substrate concentration above which growth ceases [30]. The research we are reporting represents a preliminary assessment of these kinds of problems with emphasis on multiple substrate utilization kinetics.

Approach

Glucose, phenol, and pentachlorophenol were chosen as carbon sources because these substrates are common wastewater constituents; the latter two particularly in petrochemical wastes. Glucose is the most commonly used compound for the study of non-inhibitory organic kinetics [4]. Phenol is a primary

constituent in many industrial wastewaters because it can be a by-product in the production of fossil fuel energy [26]. Pentachlorophenol (PCP) is widely used in a variety of agricultural and industrial applications as a fungicide, bactericide, insecticide, and herbicide. It is most widely used in the United States for wood preservation in newly cut timber and slime control in pulp and paper production. PCP is most frequently used to study the kinetic behavior of chlorinated hydrocarbons [12]. A mixture of these three constituents represents a non-inhibitory substrate (glucose), a somewhat inhibitory substrate (phenol), and a very inhibitory substrate (PCP). Additionally, phenol and PCP are structurally similar with the substitution of chlorine groups around the aromatic ring for PCP.

Long-term studies and short-term kinetic experiments were performed on activated sludge bacteria grown in pulse-fed (high cell recycle) batch reactors. The experiments were designed to study the biodegradation of a multicomponent substrate feed in an aqueous suspension of acclimated microorganisms. Samples were drawn from each reactor to determine (1) long term biodegradation rates of the priority pollutants, and (2) cell growth rates and substrate utilization rates by means of short-term kinetic experiments. Nine reactors consisting of different combinations and concentrations of three carbon sources were studied. Speitel and DiGiano [31] show that the initial rate method is faster and less time-consuming than chemostat studies. This technique was used to obtain kinetic coefficients for the multiple substrate reactors. The initial rate method also eliminates the lag time that results when batch studies are performed.

The pulse-fed reactor system was designed for complete mass balance closure. Simple round-bottom flasks served as the bioreactors which held an aqueous suspension of microorganisms taken from an activated sludge basin and fed synthetic media of trace nutrients, buffers, and the target compounds [32]. An organic trap composed of XAD-3 resin was placed on the effluent air stream to trap any volatilized components. Microorganisms were taken from the aeration basin of an industrial wastewater treatment facility and the Texas A&M University Wastewater Treatment Plant.

Culturing techniques

Templeton and Grady [5] note that the biodegradation potential of microorganisms is directly related to culture history. Microorganisms in the wastewaters of the industrial wastewater treatment facility were initially acclimated to glucose. Seven long-term experiments were conducted on these reactors. However, these organisms were not vigorous and the cultures were difficult to maintain for longer than six months. Therefore, University wastewater microorganisms were initially acclimated to the designated substrate feed. All initial rate experiments and four long-term experiments were conducted on these reactors.

The bioreactors were aerated and cultivated by adding additional substrate when the previous feed was depleted. Operation of the reactors was such that cell wastage was minimized thus approaching a plug flow regime with cell recycle rates ranging between 90 to 98% and an average sludge age of 55.6 days. Reactors reached steady-state when the suspended solids concentration became constant. For mixtures of (1) PCP and glucose and (2) PCP, phenol, and glucose steady state was attained after 50 and 75 days, respectively. Substrate concentrations were based on reported phenolic concentrations in industrial wastewater which range from 25–500 mg/l phenol and 0.1–20 mg/l PCP [3,6,33]. The nutrient feed consisted of a carbon:nitrogen:phosphorus molar ratio of 22.2:5.1:1. This ratio was based on the amount of carbon, nitrogen, and phosphorus needed by bacterial cells to sustain growth and maintenance [3, 34]. The remaining salts and trace metals in the nutrient media were thermodynamically stable and buffered to a pH of 6.8–7.2 [35].

Analytical methods

The reactors were monitored on a daily basis to determine the changes in substrate concentrations in the dissolved and solid phases. Substrate concentrations were measured via HPLC, GC, and in the case of glucose, an enzymatic assay (Sigma Chemical Company, St. Louis, MO, USA).

Mixed liquor suspended solids (MLSS) were analyzed by standard procedures [36] substituting a microwave drying procedure (Model AVC80, CEM Industries). Using five triplicate analyses, the mean MLSS for activated sludge was 1095 mg/l ($\pm$6.7 mg/l) and 159 mg/l ($\pm$3.1 mg/l) for the starting samples in the initial rate experiments. An electronic particle counting system (Coulter Electronics, Louton, England) was used to determine the number and size of particles, i.e. the microbial flocs. These analyses were used to quantify biomass changes for the initial rate experiments. It was found to be the most sensitive method for detecting and quantifying small changes in biomass for the determination of growth rates. Total organic carbon (TOC) was also monitored.

Experimental approach

Long-term kinetic experiments were run to determine a particular substrate's total residence time in the reactor and elucidate the sequence of multiple substrate degradation. These experiments were conducted by adding the substrate feed to the reactors immediately after complete degradation had occurred from the previous substrate addition. Bioreactor samples were taken every two hours. The bioreactor samples were then centrifuged and the supernatant injected into the HPLC to detect dissolved phase phenol and PCP concentrations. Dissolved phase organic carbon was also determined. The remaining portion of the dissolved phase was analyzed for glucose. In each experiment, suspended solids were monitored every four hours.

The method of initial rates is frequently used to obtain kinetic parameters for enzymatic reactions in which there are several substrates present in the medium [21]. It is difficult to obtain kinetic values when there are several sequential or simultaneous degradations occurring. The initial rate method enables the determination of the cell growth rate and substrate utilization values separately for each component. Further, biodegradation rates and growth rates can be determined for pulse-fed reactors over a relatively short time period.

The experimental protocol for the initial rate method was adapted as described for our application. A constant mass of substrate free microorganisms was added to each of a series of beakers. The appropriate test substrate was added to initiate the experiments. The initial rate experiments were conducted on four sets of reactors acclimated to the following substrate combinations: phenol only, phenol and glucose, PCP and glucose, and the triple substrate reactor (PCP + phenol + glucose). For a single substrate reactor, only one set of beakers with the same biomass in each at five phenol concentrations was prepared. For a dual substrate reactor, one set of beakers were prepared for each substrate singly and another set for the combination of substrates all in increasing concentrations within a set. And for the triple substrate reactor, there were three sets of single substrate feed, two sets for dual substrates, and a third set for all three substrates.

To begin the initial rate experiments, microorganisms in the pulse-fed reactor had to be actively degrading the substrate of concern. For example, the initial rate experiment for glucose in a glucose–phenol reactor proceeded when the cells were actively degrading glucose. A phenol initial rate experiment was begun when the cells were actively degrading phenol. However, an initial rate experiment for both glucose and phenol required that the cells had reduced the glucose concentration to a minimum and were beginning to degrade phenol. PCP experiments were conducted in the same manner. Samples were taken from the initial rate batch reactors when 3 to 20% biodegradation had occurred which translates into 10 to 25 minute incubation periods.

Results and discussion

Phenol represents a commonly occurring toxic and inhibitory substrate which can be degraded under a variety of conditions. The inhibitory concentration is generally reported to range between 50 mg/l [25] and 200 mg/l [24], but cultures have recovered readily from shock loadings of 500–1000 mg/l [26]. Microorganisms indigenous to wastewater treatment facilities were found to quickly adapt to phenol as their sole source of carbon, but true equilibration was not considered to be fully established before forty days. We initially introduced the activated sludge microorganisms to glucose and then added increasing amounts of phenol. We also tried adapting cultures directly to phenol as the sole source of carbon. These latter cultures were found to be more vigorous,

degrading the substrate four times faster than cultures initially acclimated to only glucose. This response may indicate the selection of hardier microorganisms during the acclimation process. Templeton and Grady [5] note that cells physiologically adapt to their previous growth environment. Further, they contend that values of the Monod kinetic parameters obtained by short-term batch procedures are strongly influenced by biomass culture history.

Microorganisms from a set of batch-fed reactors fed between 100–700 mg phenol/l every other day, were used to determine kinetic experiments on starving as well as well fed microorganisms using various levels of initial substrate to microorganism ratios. When bacteria, who had been consistently fed 600 mg/l, were starved for two days and then fed a low dose feed, zero order degradation was observed (Fig. 4a). But a high dose feed resulted in Monod kinetics (Fig. 4b). Well fed microorganisms showed biodegradation rates obeying Haldane kinetics (Fig. 4c). These three responses from the same microorganisms represent a concentration independent growth, substrate saturated growth, and inhibition growth, respectively. Such responses indicate that the yield coefficient cannot be constant and must be very dependent on the physiological state of the microorganisms prior to the initiation of the experiment.

The use of radiolabeled compounds in parallel with traditional monitoring parameters can help elucidate the degradation kinetics of substrates. Using ^{14}C labelled phenol in the substrate feed, a distinction between the distribution of phenol and its derivatives could be made for the suspension. Combining gas chromatography, total suspended solids, and liquid scintillation analyses, the concentration of phenol remaining in the solution, associated or incorporated into the biomass, and formation of exogenous products was determined. Phenol was completely consumed. The ^{14}C found in solution represents a secondary product, probably an organic acid as indicated by a decrease in solution pH. As phenol in solution was consumed, the biomass concentration leveled off when the phenol concentration became limiting. However, the amount of ^{14}C on the microorganisms was significantly higher (Fig. 5a). On initial inspection, this difference might be attributed to adsorbed substrate except that the microorganisms were washed to prevent carry-over. It was assumed that the yield factor, Y, was constant. Yet, if the yield factor is determined from a plot of the amount of biomass (X) versus substrate (S), the slope changes indicating a change in yield (Fig. 5b). This difference was likely due to storage of the substrate which is not consumed until the solution substrate concentration is exhausted. Figure 6(a) shows the plot of X versus S for three different cases and how the slopes (yield) change is presented in Figs. 6(b) and 6(c). When these experiments were repeated under identical conditions, the same rate parameters are generated within acceptable accuracy limits.

To determine what synergistic or antagonistic relationships might exist for a multiple substrate feed solution, cultures fed combinations of glucose, phenol,

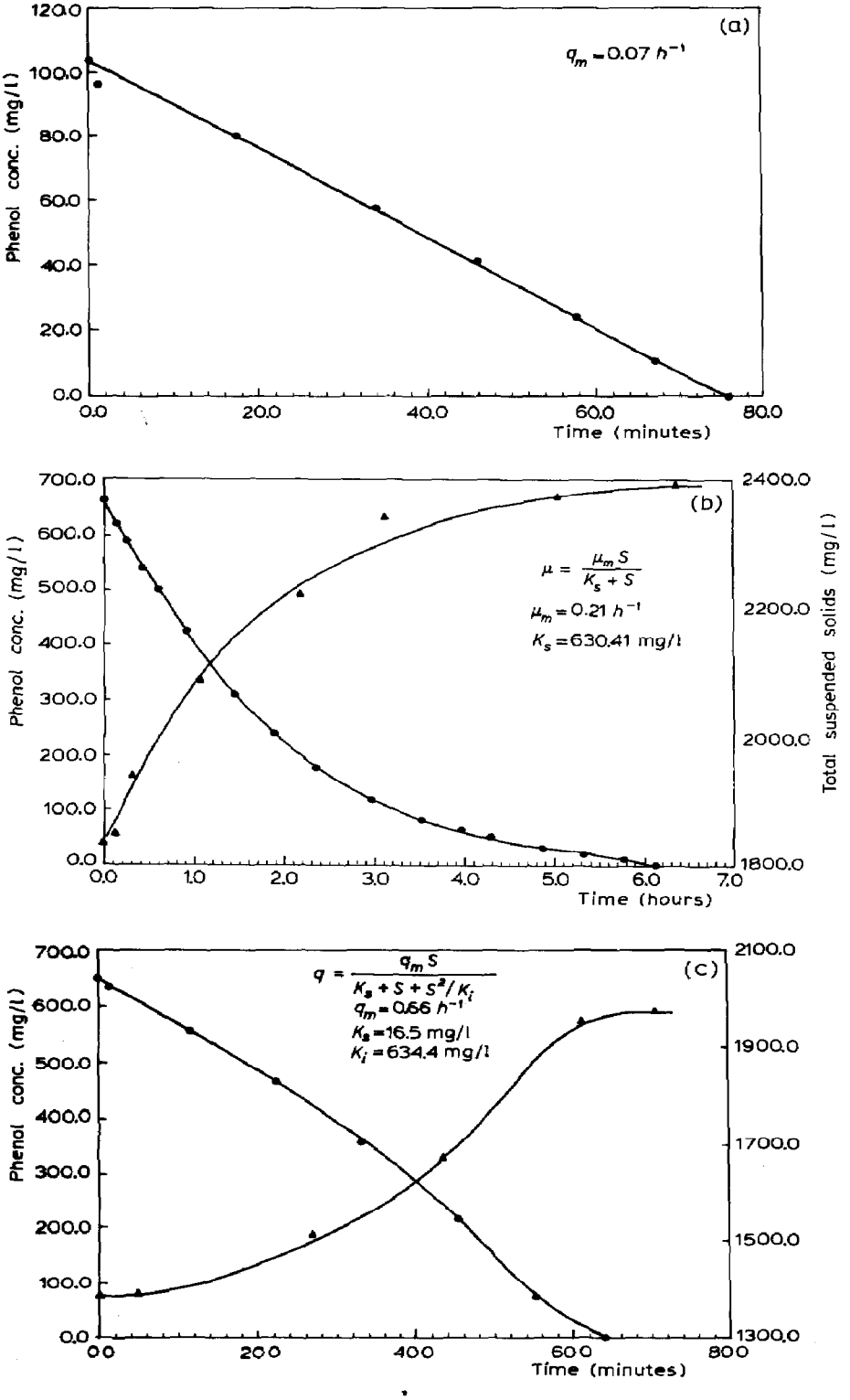


Fig. 4. (a) Phenol degradation by starved bacteria at low initial substance concentration. (b) Phenol degradation by starved bacteria at high substrate concentration: (●) phenol removal, and (▲) biomass growth. (c) Phenol degradation by non-starving bacteria at high initial substrate concentration (symbols as in b).

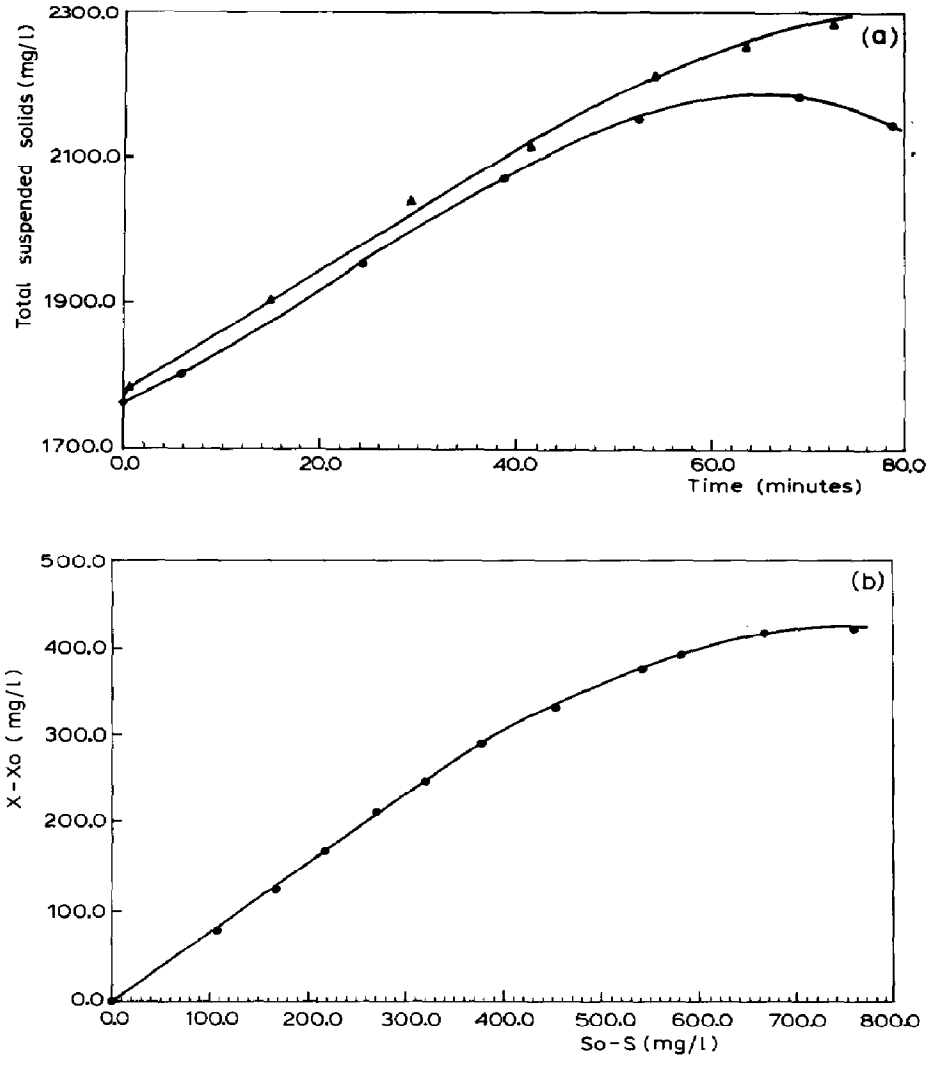
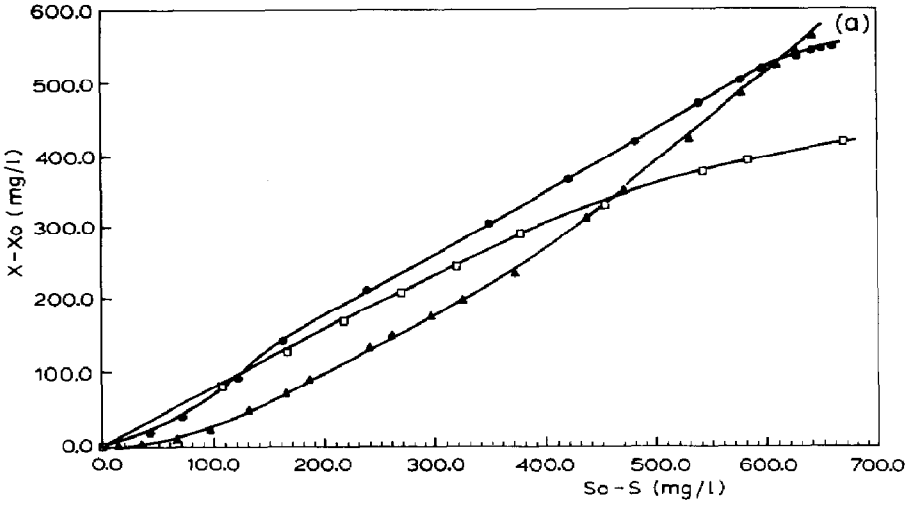
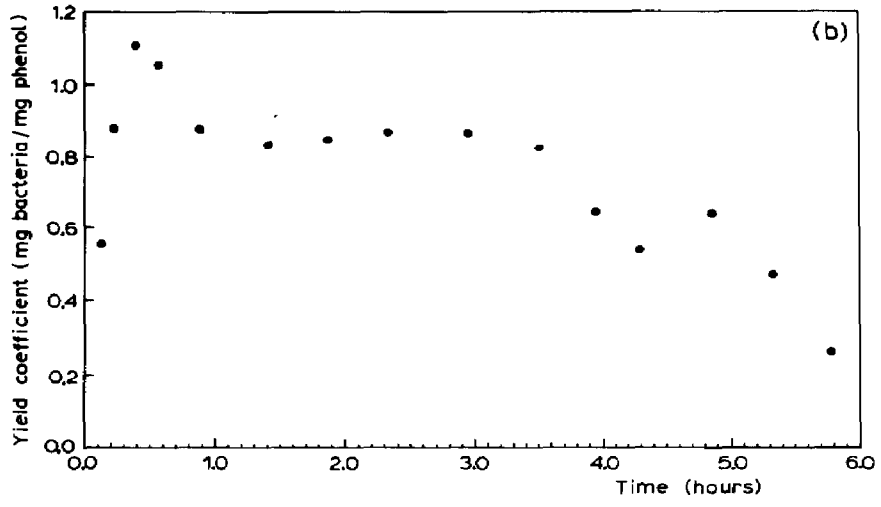


Fig. 5. (a) Change in biomass concentration measured in terms of total suspended solids (●) and by liquid scintillation for ^{14}C building blocks (▲) in biomass. (b) Yield factor curve.

and pentachlorophenol were acclimated. The microbial responses were examined by monitoring the change in the concentration of each substrate through the degradation period. These tests were initiated when the previous pulse addition of substrates had been completely degraded resulting in a somewhat starved condition for the microorganisms. The same mixtures to which the cultures had been acclimated were added and the change in concentrations measured in time. By monitoring the disappearance or degradation of each substrate individually, we were able to evaluate microbial substrate preferences. Glucose is commonly reported to be the preferred substrate [37] as was the case in our experiments. For all mixtures glucose was always consumed





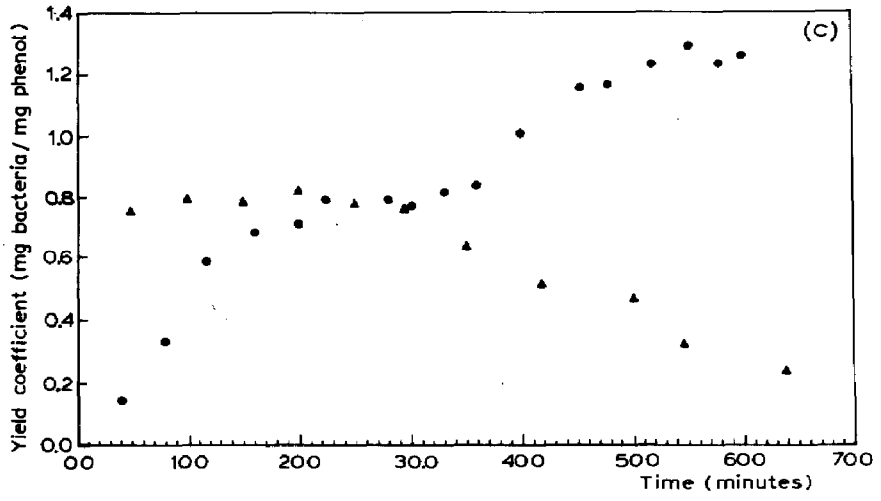


Fig. 6. Yield factor curves, (a). (●) Case I: Monod kinetics, starved bacteria; (▲) Case II: Haldane kinetics, non-starved bacteria; (□) Case III: Monod kinetics, starved bacteria. (b) Yield factors for Case II, (●), (a). (c) Yield factors for Cases II (●) and III (▲), (a).

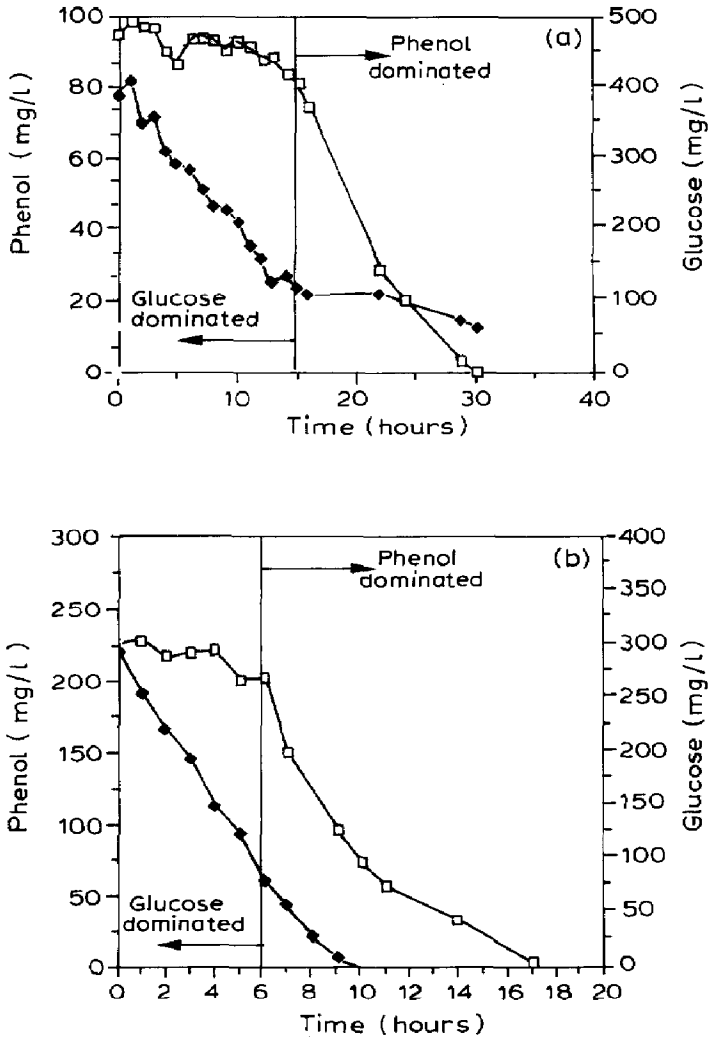


Fig. 7. Preferential degradation of glucose (♦) over phenol (□) in two reactors: (a) acclimated to 400 mg/l glucose and 100 mg/l phenol; and (b) acclimated to 300 mg/l glucose and 200 mg/l phenol.

first. The rate of degradation depended on the concentration of the competing substrate. For substrate ratios of 400:100 (mg/l glucose:mg/l phenol), microorganisms consumed glucose almost exclusively until it reached 20 ppm after which phenol degradation began (Fig. 7a). This occurred in approximately fifteen hours. However, for substrate ratios of 300:200, a much closer ratio, phenol degradation began at a higher residual glucose concentration (80 ppm) and in half the time (Fig. 7b). For cultures fed glucose and PCP (499 ppm:1 ppm), glucose was consumed to negligible concentrations after which PCP degradation was initiated. In a triple substrate reactor containing glucose, phenol and PCP, glucose was the preferred substrate. No distinction was made

by the microorganisms between phenol and PCP which were consumed simultaneously. However, once the phenol was degraded, PCP degradation slowed considerably. It is likely that PCP degradation is a result of cometabolism due to the presence of phenol.

Whether multiple substrates are consumed simultaneously or sequentially depends on the manner in which the microorganisms have been cultured. Grady [38] discusses the conditions under which simultaneous and sequential degradation occur and their relevance to activated sludge systems. Sequential degradation is expected when microorganisms are undergoing high growth rates. Simultaneous degradation occurs when microbial growth is slow such as in systems with large solids retention times. An example of such a system would be an activated sludge basin. For microorganisms cultured in batch or batch-fed conditions where new substrate additions are made subsequent to complete substrate degradation, the microorganisms undergo periods of starvation. When new substrate is added, the microorganisms quickly assimilate the available substrate resulting in fast growth rates as seen in our experiments. However, when microorganisms are continuously exposed to constant concentrations of the multiple substrates as in a continuous stirred tank reactor (CSTR), they are expected to degrade those substrates simultaneously.

The initial rate experiments were conducted to evaluate kinetic parameters from the batch-fed reactors. For multiple substrate reactors, each individual substrate was tested and then each possible combination of substrates. By monitoring the change in substrate and biomass concentrations as a function of time, estimates of the growth rates could be determined. Initial rate experiments were conducted on two single substrate reactors containing only phenol (50 and 200 mg/l), two phenol plus glucose reactors (450 mg/l glucose to 50 mg/l phenol; 300 mg/l glucose to 200 mg/l phenol), a glucose plus PCP reactor (499 mg/l glucose to 1.0 mg/l PCP), and one triple substrate reactor (400 mg/l glucose to 100 mg/l phenol to 1 mg/l PCP). In addition to the determination of the kinetic parameters, an understanding of the interactions between substrates was evaluated.

Once again the culture history of the microbial population was found to significantly influence the response to substrate degradation efficiency. A culture acclimated to 50 mg/l phenol exhibited typical Monod or saturation growth. Whereas, at 200 mg/l the response in terms of growth rate versus substrate concentration appears to be that of inhibition kinetics such as Haldane growth (Fig. 8). The maximum growth rates for the two cultures were comparable at 0.55–0.53 h^{-1} for the 50 and 200 mg/l, respectively with inhibition from the higher phenol reactor microorganisms at phenol concentrations in excess of 50 mg/l.

In all of the dual substrate reactors glucose was present with either phenol or PCP. PCP as the sole carbon source could not support a high enough biomass concentration for testing purposes. In the phenol and glucose reactors,

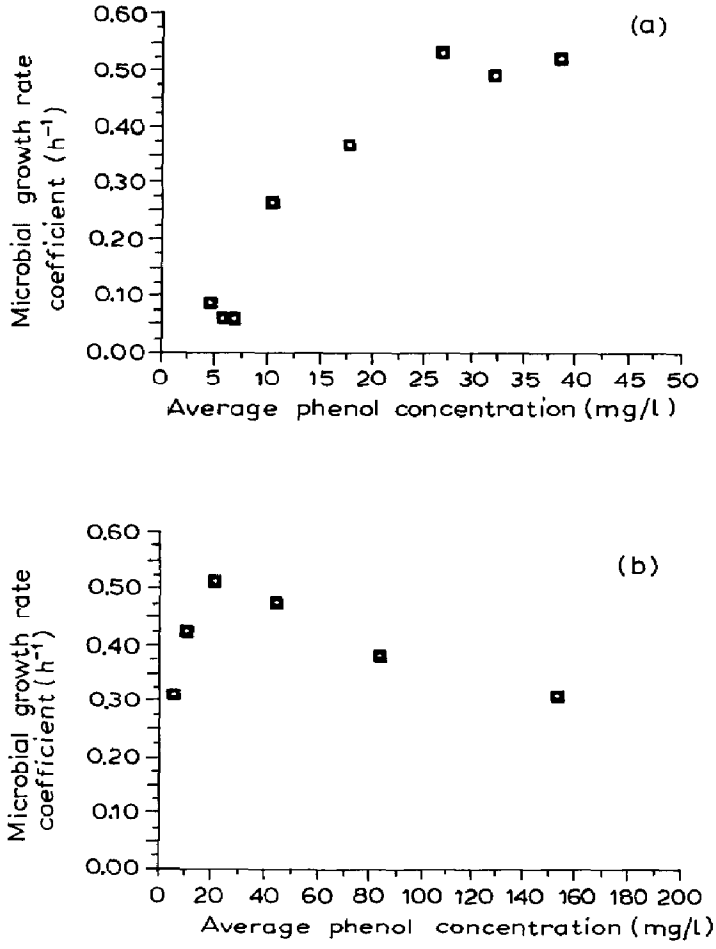


Fig. 8. Microbial growth rates for (a) acclimated single substrate 50 mg/l phenol reactors at non-inhibitory concentrations, and (b) for acclimated single substrate 200 mg/l phenol reactors at inhibitory concentrations.

the overall microbial growth rate in the presence of both substrates represented the cumulative effect of degradation of both carbon sources. The 450:50 reactor generated a maximum growth rate (μ_{max}) of 0.84 h^{-1} which was attributable to degradation of both phenol and glucose Fig. 9). However, at the higher phenol concentration (the 200:300 reactor), the lower overall growth rate of 0.3 h^{-1} was largely due to the degradation of glucose due to the inhibitory effect of the higher phenol concentration. Comparing these results to those of the single substrate reactors (50 and 200 mg/l phenol), phenol inhibition was more pronounced when glucose was present (Table 2). At the lower, non-inhibitory phenol concentration the cumulative growth is enhanced with both substrates. However, at the higher, inhibitory phenol concentration, growth is reduced. Phenol was not efficiently degraded in this reactor without glucose being present indicating a cometabolic process.

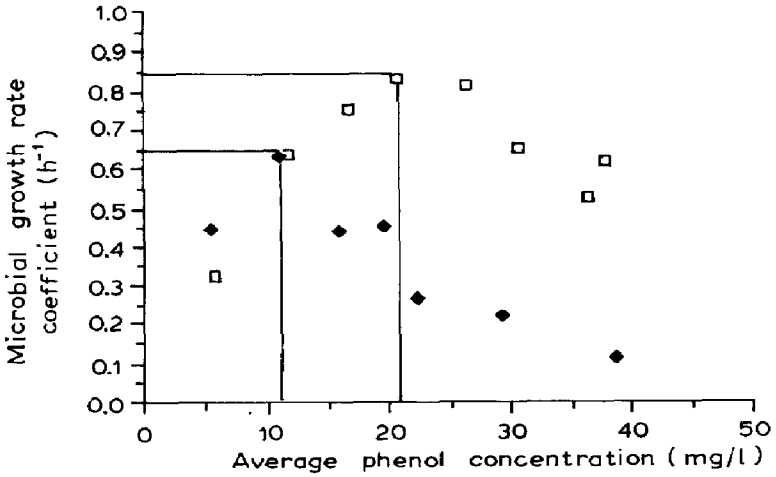


Fig. 9. Contrast in microbial response to single (◆) and dual (□) substrate feed. Growth rates for an acclimated dual substrate reactor fed 450 mg/l glucose and 50 mg/l phenol.

TABLE 2

Maximum specific growth rate coefficients and the associated substrate values

Reactor description	Substrate[a]	Cumulative μ_{max} (h^{-1})	Substrate @ μ_{max} (mg/l)		
			Glucose	Phenol	PCP
Phenol: 50 ppm	Ph	0.55	—	27	—
Phenol: 200 ppm	Ph	0.53	—	40	—
Glucose: 400 ppm	Glu	0.40	175	—	—
+	Ph	0.65	—	10	—
Phenol: 50 ppm	Glu+Ph	0.84	160	20	—
Glucose: 300 ppm	Glu	0.35	200	—	—
+	Ph	0.06	—	30	—
Phenol: 200 ppm	Glu+Ph	0.30	50	20	—
Glucose: 499 ppm	Glu	1.10	50	—	—
+	PCP	0.53	—	—	0.04
PCP: 1 ppm	Glu+PCP	0.50	25	—	0.40
Glucose: 400 ppm	Glu	0.66	150	—	—
+	Ph	0.45	—	10	—
Phenol: 100 ppm	Glu+Ph	0.32	180	20	—
+	Glu+PCP	0.44	100	—	0.2
PCP: 1 ppm	Ph+PCP	0.55	—	22.5	0.43
	Glu+Ph +PCP	0.46	180	22	0.25

[a]Abbreviations used: Ph = phenol; Glu = glucose; and PCP = pentachlorophenol.

The proposed metabolic pathways for glucose and PCP (Figure 3) do not show any overlapping steps for glucose and PCP degradation. Our investigation on the PCP/glucose reactor found that at PCP concentrations in excess of 1.0 mg/l, microbial growth was severely inhibited. The very high growth rate generated when glucose alone was fed to the microorganisms reflects the extent of inhibition that low PCP concentrations can exert (Table 2). The microorganisms were able to use PCP as a sole carbon source. Glucose, however, was not considered to aid in the metabolism of PCP.

From the initial rate experiments on the triple substrate reactor, both growth enhancement and inhibition were observed due to multiple substrate culturing. Each of the growth rates from the different substrate combinations were compared, so that the effects of a multicomponent substrate media on cell growth could be evaluated. Glucose growth rates followed Monod-type saturation kinetics generating a maximum growth rate coefficient of 0.65 h^{-1}. Both the glucose plus phenol and the glucose plus PCP growth rates exhibited substrate inhibition. However, in the glucose plus phenol test, inhibition was less pronounced and did not occur until a substrate ratio of 200:60 was reached (Table 2). PCP inhibited the microbial growth rate more in the dual substrate media with glucose and PCP than in the phenol plus PCP. PCP was more inhibitory in the presence of glucose than with phenol indicating that phenol aided in the metabolism of PCP thereby mitigating the inhibition of PCP (Fig. 10). A residual inhibition effect may account for some of the suppressed growth response in the absence of any PCP. Also, at 100 mg/l phenol is considered to be inhibitory and growth rates would be expected to be less than at a 50 mg/l feed (Table 2). Having been previously cultivated with multiple substrates does

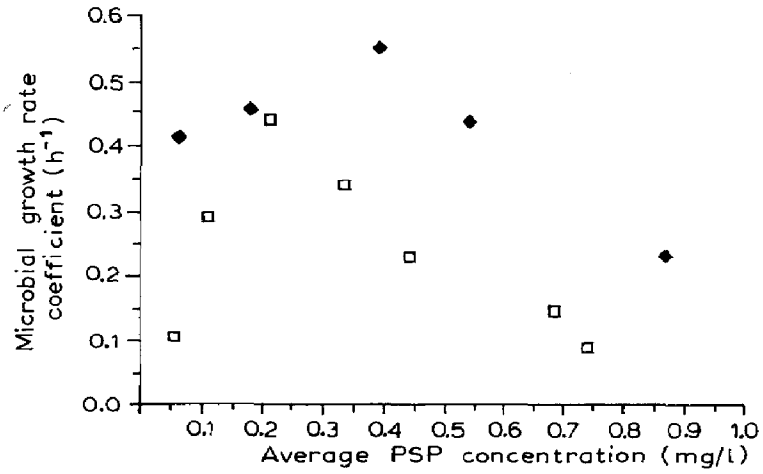


Fig. 10. Effect of PCP on growth rates in dual substrate feed. Microbial growth rates for an acclimated triple substrate reactor fed glucose plus phenol (□), and phenol plus PCP (♦).

affect the microbial growth rates until the microorganisms reacclimated to their new environment.

The response of the triple substrate reactor with glucose, phenol, and PCP indicates potential experimental artifacts in the use of the initial rate method for multiple substrate reactors. The high PCP removal rates for the triple substrate reactor (Table 2) may be due to the "starvation" of the microorganisms. In the dual substrate experiments (glucose plus PCP and phenol plus PCP), the cells were removed from the reactor when they were actively degrading PCP. In the triple substrate experiments, the bacteria for the glucose only experiment were removed from the acclimated reactor when they were actively degrading both phenol and PCP and the glucose was already degraded. The microorganisms in the triple substrate reactor did not actively degrade PCP until the glucose was consumed. When the cells were exposed to the glucose and PCP during the initial rate experiments, they may have removed some PCP in addition to the glucose because they had not been fed either glucose or phenol for several hours. The bacteria may have responded to this by removing a large amount of glucose since it is a preferred substrate. The growth rates may be strongly influenced by the "condition" of the organisms at the start of the experiment.

Conclusions

The batch biodegradation kinetics of phenol are strongly affected by the physiology of the bacteria prior to the initiation of the batch experiments. When the bacteria were starved, the biodegradation kinetics followed the Monod model. Inhibition of the bacterial growth by phenol occurred when the ratio of the initial amount of phenol fed to the initial amount of bacteria present exceeded 0.47, even though the bacteria were starved. The biodegradation of phenol was modelled by the Haldane equation when the bacteria were well fed before the batch experiments were performed. The observed cell yield coefficient also changed during a single batch study. The observed cell yield coefficient was determined to be strongly dependent on the feeding frequency of the bacteria and on the presence of any inhibition. The observed cell yield coefficient was constant in the phenol batch studies when the bacteria were not starved for more than one day and when there was no inhibition by phenol. The yield coefficient increased when the microorganisms were starved for more than one day and when growth was inhibited either due to high initial phenol concentration or when the microorganisms were well fed prior to an experiment.

Three mechanisms were significant in the biodegradation of multicomponent substrates: culture history, preferential degradation of substrates, and gratuitous metabolism or cometabolism of inhibitory substrates. Culture history dictated the biodegradation rate and efficiency of substrate removal. Reactors that were acclimated to glucose first degraded phenol and PCP at a

much slower rate than reactor that were initially acclimated to both the glucose and the inhibitory substrate(s).

Glucose was observed to be the preferred substrate in the multiple substrate reactors studied and under our test conditions. Phenol degradation occurred only after the glucose had been degraded to less than 75 mg/l in both of the dual substrate glucose and phenol reactors studied (450:50 and 300:200). PCP degradation commenced only after the glucose was fully degraded in the acclimated glucose and PCP reactor studied. In the acclimated triple substrate reactor, glucose degradation occurred first, then phenol and PCP were degraded simultaneously.

The initial rate experiments demonstrated that cometabolism is a significant removal mechanism for both phenol and PCP. In acclimated dual substrate glucose and phenol reactors, higher growth rates and greater phenol removal occurred when the substrate feed contained both glucose and phenol. The most efficient PCP removal occurs when phenol is a primary substrate. Phenol cometabolized PCP, thus indicating either an induced enzyme system or joint pathways for phenol and PCP metabolism exists.

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