Bull. Environ. Contam. Toxicol. (1996) 57:69-76

Bulletin of Environmental Contamination and Toxicology

Toxicity of Phenol and Monochlorophenols to Growth and Metabolic Activities of Pseudomonas

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Received: 8 July 1995/Accepted: 8 February 1996

Phenolic compounds are toxic to many organisms (Babich and Stotzky 1985) and are often present in the effluents from oil refineries and the petrochemical, pesticide, and color and textile industries. Several authors have demonstrated a characteristic pattern of behavioral responses in fishes during phenol exposure (Hansen and Kawatski 1976; Swift 1981). Cenci et al. (1987) have also evaluated the toxicity of halogenated phenolic compounds by screening for effects on the specific growth rates (SGR) and the dehydrogenase activity (DHA) of *Escherichia coli.* However, little work has been done to determine the effects on biota from short exposures at relatively high concentrations of phenol or monochlorophenols that might occur following a deliberate or accidental discharge to a receiving water.

Microorganisms with phenol-degrading capacity have been studied intensively, including cyanobacteria such as *Nostoc linckia* (Megharaj et al. 1991), yeast such as *Trichosporon cutaneum* (Neujahr and Gaal 1973), bacteria such as *Pseudomonas putida,* and other unidentified species (Solomon et al. 1994; Molin and Nilsson 1985). Among these *Pseudomonas* has received the most attention and several mutants have been prepared to degrade substituted phenols (Bayly and Wigmore 1973; Evans et al. 1971; Gaunt and Evans 1971).

In general microorganisms degrade aromatic compounds to catechols via the benzoate dioxygenase or phenol hydroxylase pathways. The ultimate fate of catechols is either cis,cis-muconic acid (ortho fission pathway) or 2-hydroxymuconic semialdehyde (meta fission pathway). Since the effective toxicity of phenol and monochlorophenols toward microorganisms might be highly dependent on their degradation pathways, two different strains of *Pseudomonas* have been employed: *Pseudomonas* I (ATCC 23973) which can utilize benzoate via the meta fission pathway and *Pseudomonas* II (isolated from soil) with pyrocatechase

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activity, which is able to grow on phenol as the sole carbon source.

In our previous studies of phenol biodegradation by *Pseudomonas,* it was observed that upon incubation of cells with a phenol-free mineral medium no significant oxygen uptake rate can be observed. Because oxygen uptake by the cells is only initiated in the presence of proper substrates, it is implied that the cells' activity can be determined by measuring the oxygen uptake rates.

The aim of this study is to investigate the initial response of *Pseudomonas* I and II upon exposure to high concentrations of phenol and chlorophenols by measuring the oxygen uptake rates using an oxygen electrode. In order to compare these kinetic results with standard microbial tests, a series growth experiments were also conducted in the current studies.

MATERIALS AND METHODS

Cell growth was normally monitored by optical density (OD) measurement, using a spectrophotometer (Shimadzu UV-120-02) at a wavelength of 595 nm. Oxygen concentrations were measured with a Yellow Springs Instruments Model 53 biological oxygen monitor.

Pseudomonas I was grown at 30 °C in medium A (Huang 1987) containing 3.0 g (20.8 m*M)* of sodium benzoate and 0.5 g of yeast extract per liter. Typically the exponential growth phase was complete in approximately 20 hr (OD 2.1). 50-mL aliquots of medium A in 125-mL Erlenmeyer flasks were autoclaved and supplemented with aqueous solutions of phenol, o-chlorophenol (OCP), m-chlorophenol (MCP) or p-chlorophenol (PCP) at the desired concentrations. A suitable amount of sodium benzoate was added (without adding yeast extract) so as to keep the total amount of carbon equal to that of 20.8 m*M* sodium benzoate. A 0.2 mL aliquot of a primary culture (OD 1.6) was used as inoculum for all sets of growth experiments. In the case of phenol molar fractions of 0, 10, 20, 30 and 40% corresponding to sodium benzoate were studied. However, owing to their limited solubilities in water, the studies of monochlorophenols were only performed at concentrations of 0.101, 0.169, 0.506, 1.010 and 1.690 m*M.*

The initial oxygen-uptake studies with *Pseudomonas* I were carried out at 30 °C in an oxygen electrode chamber. Phenol, OCP, MCP, and PCP were first dissolved in ethanol and then diluted with medium A to the desired concentrations. Each set of solutions at a fixed concentration of phenols was assayed in the presence of sodium benzoate (in medium A) at concentrations of 4.8, 2.4, 1.2, 0.8 and 0.6 m*M.* Finally, a 2.9 mL reaction mixture containing 5 µL of ethanol, sodium benzoate, and

phenols was preequilibrated at 30 °C in an oxygen electrode chamber, and 0.1 mL of washed whole cells (harvested from 20 mL of an OD 1.3 culture, washed and resuspended in 4 mL of medium A) was added to initiate the oxidation reaction, and the oxygen uptake rate was recorded.

Pseudomonas II was isolated by Dr. C. M. Huang (Taiwan Sugar Research Institute) and determined to be a species of *Pseudomonas* which was capable of growing on phenol as the sole carbon source. Typically, the exponential growth phase was concluded at OD 1.4, giving an inoculum of approximately 1.7 g/L of cells (wet weight). Inoculations of *Pseudomonas* II at various phenol and monochlorophenol concentrations were studied. Each inoculum contained of OCP, MCP, or PCP at various molar fractions from 0.5 to 50% corresponding to phenol, keeping the total carbon equal to 10.6 m*M* (1000 ppm) phenol.

The initial oxygen uptake of *Pseudomonas* II in the presence of monochlorophenols was also determined at 30 °C. A 100 mL (OD 0.86) *Pseudomonas* II cell culture was harvested by centrifugation at 15,000 rpm for 10 min. The cells were resuspended in 10 mL of mineral medium after having been washed twice with 100 mL of the same medium; this cell suspension was defined as solution A. Various concentrations (4.50, 3.00, 1.80, 0.90, 0.60, 0.45, 0.39, 0.345, 0.30, 0.255 m*M)* of phenol solutions were prepared. A 3 mL aliquot of reaction mixture containing 1 mL of the phenol solution and 1.9 mL of mineral medium was preequilibrated at 30 °C in the oxygen electrode chamber. Solution A (0.1 mL) was added to initiate the phenol oxidation reaction, and the oxygen uptake rate was recorded. Inhibition studies with *Pseudomonas* II were carried out at three fixed monochlorophenol concentrations (0.37, 1.1 and 3.3 m*M)* and various phenol concentrations (0.60, 0.30, 0.15, 0.10, 0.075 and 0.067 m*M).*

RESULTS AND DISCUSSION

Various molar fractions of phenol-benzoate have been applied to exponentially growing cells of *Pseudomonas* I. The lag times of these cultures were similar; however, their maximum absorbance (595 nm) at the end of the exponential phase decreased significantly as the molar fraction of phenol increased. A plot of the maximum absorbance obtained at the end of the exponential growth phase of each culture vs the molar fraction of phenol present in the corresponding growth medium gave a linear relationship (Figure 1). Obviously, the decrease in the maximum absorbance is due to the limited benzoate substrate present in the culture media, while the molar fraction of phenol is increased. These results suggest that phenol is neither a substrate nor a good inhibitor of *Pseudomonas* I.

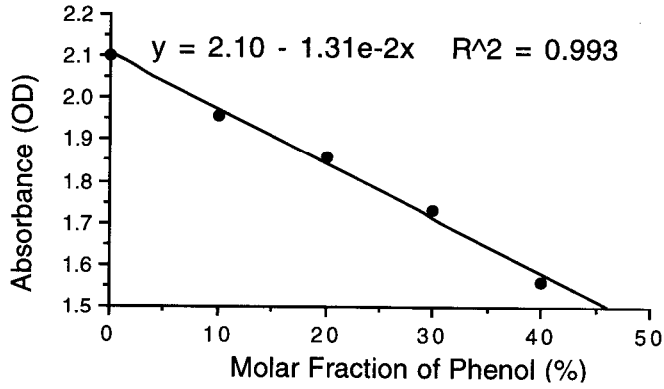

Figure 1. Linear correlation of the maximum absorbance obtained at the end of the exponential growth phase of each culture vs the molar-fraction of phenol present in the corresponding growth medium.

Upon incubation of monochlorophenols with *Pseudomonas* I, a significant changed in the growth rate is observed when the concentrations of OCP, MCP, and PCP are higher than 1.686, 0.506 and 1.010 m*M,* respectively. These results suggest that the toxic effect of phenols towards *Pseudomonas* I decreases in the order of MCP, PCP, OCP, and phenol.

The initial effects of phenol and monochlorophenols on the oxidation of benzoate by *Pseudomonas* I were analyzed and expressed as oxygen uptake rates. Double reciprocal plots for the inhibition of *Pseudomonas* I by phenols were prepared, as shown in Figure 2. When sodium benzoate was varied in the presence of a set of concentrations of phenols, a family of straight lines intersecting at the left of the vertical axis and having a common intercept on the 1/[benzoate] axis was obtained. This result implied that phenol and monochlorophenols are noncompetitive inhibitors of *Pseudomonas* I. From linear replots of slope vs phenol concentration, the apparent Ki's of phenol, OCP, MCP, PCP were calculated to be 67.5, 14.5, 3.9, and 10.0 m*M,* respectively. When the concentrations of phenol, OCP, MCP, and PCP were 3.3 m*M,* the remaining maximum velocities of oxygen uptake (Vmaxi) were 95, 81, 54 and 69%, respectively, of the velocity of the control reaction (Vmax; without adding inhibitors; data not shown). This result is similar to the result from growth experiments, indicating that the toxicity to *Psedomonas* I decreases in the order MCP, PCP, OCP, and phenol.

Pseudomonas II is able to grow on phenol, an inhibitory substrate, as the sole carbon source. It appears that both MCP and PCP can retard the rate of cell growth completely if their molar fraction (corresponding to phenol) exceeds 6 and 8%, respectively. In the case of OCP, in spite of lengthening lag-times of up to 40 hr, the inoculum survived even when the molar fraction of OCP was increased to 50%. Such results show that

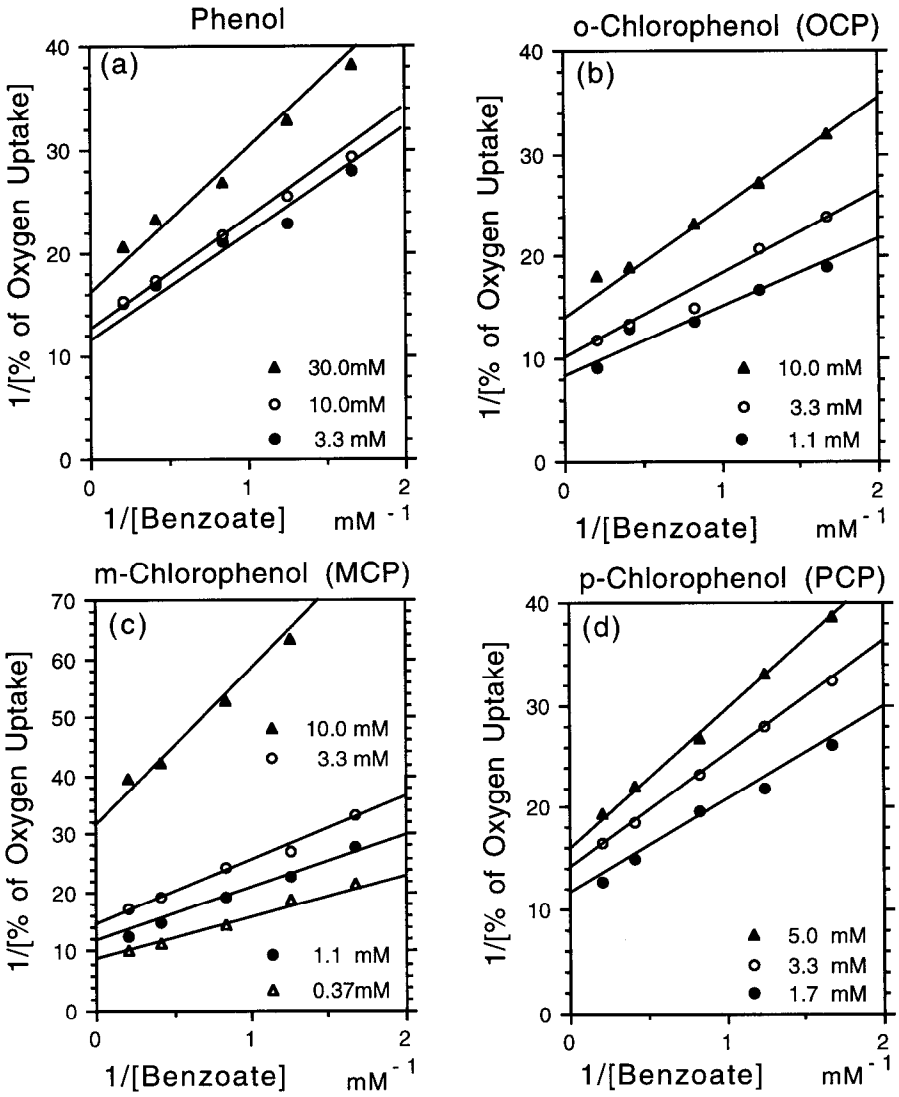

Figure 2. Double reciprocal plot for the inhibition of oxygen uptake by *Pseudomonas* I by (a) phenol (b) OCP (c) MCP(d) PCP. The oxygen electrode method was used for these measurements, and the velocities were expressed as the percentage of oxygen uptake per min per unit amount of cells.

the toxic effect of chlorinated phenols decreases in the order MCP, PCP, and OCP.

In spite of the inhibitory effect of phenol, initial velocity studies with *Pseudomonas* II were also carried out in essentially the same manner except that a series of curves were obtained (Figure 3). From linear

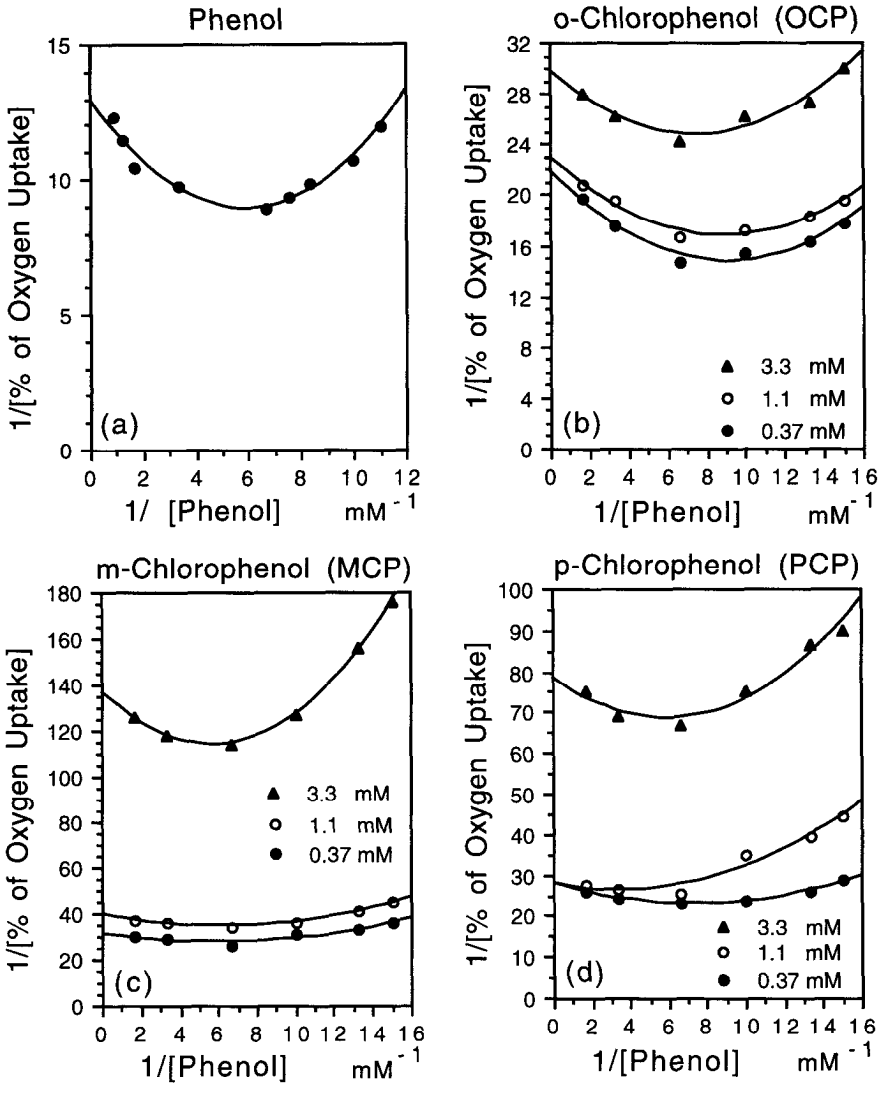

Figure 3. Double reciprocal plot for the inhibition of oxygen uptake by *Pseudomonas* II by (a) phenol (b) OCP (c) MCP (d) PCP. The method is as described in Figure 2.

replots of limiting slopes at low concentrations of phenol taken from the reciprocal plots, the apparent Ki's of OCP, MCP, and PCP were calculated to be 3.6, 0.14, and 0.90 m*M,* respectively. The Vmaxi was calculated directly from the oxygen uptake rate at the curve minimum. Thus, in the presence each of OCP, MCP and PCP, fixed at 3.3 m*M,* the corresponding Vmaxi are 36.7, 7.8, and 13.7 % of Vmax, respectively. These kinetic results are also consistent with a toxicity descending in the order MCP, PCP and OCP observed in growth experiments.

Despite the differences in the metabolic pathways of Pseudomonas I and II, the order of toxicity of phenol and monochlorophenols towards these two strains are similar. This result could be due to the fact that MCP, PCP and OCP are noncompetitive inhibitors of both benzoate-growth *Pseudomonas* I and phenol-growth *Pseudomonas* II. Obviously, the Vmaxi reflects the toxicity or inhibitory effect directly, but unfortunately, the Vmaxi is highly dependent on the concentration of inhibitors, so that it is difficult to evaluate the relative toxicity of compounds unless they are assayed at the same concentration. Inasmuch as most compounds inhibit noncompetively, the Vmaxi can be expressed as follows:

$$\text{Vmaxi} = \text{Vmax}\ \frac{\text{Ki}}{\text{Ki} + [\text{I}]} \qquad \text{where } [\text{I}] = \text{inhibitor concentration}$$

Thus, 1/Ki might be a good parameter to express the relative toxicity of different compounds. Accordingly, the relative toxicity (RT) of phenols may also be evaluated from the relative 1/Ki values (Table 1). These results are in agreement with the relative phenol toxicity estimates from Cenci's SGR and DHA methods.

In this study we have demonstrated that the toxicity of phenolic compounds may be evaluated by kinetic studies. The Vmaxi's can reflect the toxicity of inhibitors directly, whereas the apparent Ki is a good parameter to evaluate the relative toxicity of compounds.

Acknowledgments. This work was generously supported by the National Science Council of the Republic of China (Grant NSC 79-0211-B-006-03 and NSC 79-0211-B-006-05)

Table 1. Relative toxicity (RT) of phenol and monochlorphenols towards *Pseudomonas* and *E. coli.*

Compounds	*Pseudomonas* I			*Pseudomonas* II			*E. coli*	
	Ki	Vmaxi[a])	RT[b])	Ki	Vmaxi	RT	SGR[c])	DHA[d])
Phenol	67.5	95	1.0	-	-	-	1.0	1.0
OCP	14.5	81	4.6	3.6	37	1	8.3	1.7
MCP	3.9	54	17	0.14	8	26	9.5	4.2
PCP	10.0	69	6.7	0.90	14	4	-	-

a) Vmaxi at inhibitor concentrations of 3.3 m*M.* b) Relative toxicity from relative 1/Ki c) Relative toxicity from specific growth rate of *E. coli.* d) Relative toxicity from dehydrogenase activity (Cenci et al. 1987)

REFERENCES

Babich H, Stotzky G (1985) A microbial assay for determining the influence of physicochemical environmental factors on the toxicity of organisms: Phenol. Arch Environ Contam Toxicol 14:409-415.

Bayly RC, Wigmore GJ (1973) Metabolism of phenol and cresols by mutants of *Pseudomonas putida.* J Bacterial 113:1112-1120.

Cenci G, Caldini G, Morozzi G (1987) Chlorinate phenol toxicity by bacterial and biochemical tests. Bull Environ Contam Toxicol 38: 868-875.

Evans WC, Smith BSW, Fernley HN, Davies JI (1971) Bacterial metabolism of 4-chlorophenoxyacetate. Biochem J 122:509-518.

Gaunt JK, Evans WC (1971) Metabolism of 4-chloro-2-methyphenoxyacetate by a soil *Pseudomonas.* Biochem J 122:519-526.

Hansen CR, Kawatski JA (1976) Application of 24-hour post exposure observation to acute toxicity studies with invertebrates. J Fish Res Board Can, 33: 1198-1201.

Huang DS (1987) Alternate substrates and inhibitors of catechol dioxygenases. Ph.D. thesis, Princeton University.

Megharaj M, Pearson HW, Venkateswarlu K (1991) Toxicity of phenol and three nitrophenols towards growth and metabolic activities of *Nostoc linckia,* isolated from soil. Arch Environ Contam Toxicol 21:578-584.

Molin G, Nilsson I (1985) Degradation of phenols by *Pseudomonas putida* ATCC 11172 in continuous culture at different ratios of biofilm surface to culture volume. Appl Environ Microbiol, 50:946-950.

Neujahr HY, Gaal A (1973) Phenol hydroxylase from yeast. Purification and properties of the enzyme from *Trichlosporon cutaneum.* Eur J Biochem 35:386-400.

Solomon BO, Posten C, Harder MPF, Hecht V, Deckwer WD (1994) Energetics of *Pseudomonas cepacia* G4 growth in a chemiostat with phenol limitation. J Chem Tech Biotechnol, 60:275-282.

Swift DJ (1981) Changes in selected blood components of rainbow trout, exposed to hypoxia or sub-lethal concentrations of phenol or ammonia. J Fish Biol 19:45-61.