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Quantitative Structure-Toxicity Relationship of Halogenated Phenols on Bacteria

D. Liu, K. Thomson, and K. L. E. Kaiser

Environmental Contaminants Division, National Water Research Institute, Burlington, Ontario, Canada L7R 4A6

Toxicants are generally present in effluents and natural ecosystems as a mixture. As a result, the toxic impact on biota from such mixture can not adequately be estimated from a series of chemical analyses alone. Consequently, biological monitoring is an important early warning system for safeguarding the total ecosystem (CAIRNS et al. 1980).

Microorganisms have several attributes which make them attractive for use in the screening of chemical toxicity. In particular, they are easily handled and require relatively little space (MOWAT 1976), representing a substantial saving in the operation cost. Moreover, their rapid growth rate is desirable because of the possibiltity to observe toxicity effects over several generations in a very short time period.

Bacteria are now widely used in the assessment of chemical toxicity and have undergone sufficient evaluation and refinement to be considered reliable and reproducible (PATTERSON et al. 1970, RYSSOV-NIELSEN 1975, BROUZES et al. 1978, NARKIS & ZUR 1979, ANDERSON & ABDELGHANI 1980). The objectives of this study were to report the modification of the resazurin reduction procedure (LIU 1981) for measuring chemical toxicity and to demonstrate the application of this improved test in the study of the toxicity/structure evaluation.

MATERIALS AND METHODS

Medium: The growth medium (GM) contained the following ingredients: KH_2PO_4, 1.64 g; K_2HPO_4, 2.64 g; glucose, 0.20 g; sodium acetate, 0.20 g; nutrient broth, 0.80 g, distilled water 1 L. Sterilization of the medium was accomplished by autoclaving at 121°C for 15 min. The medium was so developed after extensive testings that too high a level of organic nutrients in the GM was found to decrease the bacterial response to the test toxicant. The use of a strong buffering system in the GM has also improved the reproducibility of the resazurin reduction procedure.

Bacterial culture: The test bacterium, identified as a *Bacillus* sp., (TL81), was isolated from activated sludge using the following procedure. To a 125-mL Erlenmyer flask containing 50 mL of GM, 0.1 mL of fresh activated sludge was added and the mixture

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was incubated overnight on a shaker at room temperature (21°C). After appropriate dilutions, the mixed culture was plated onto the GM agar plates. Further purification and identification of the isolates were based on the conventional microbiological techniques. One of the isolates, TL81, was chosen as the test bacterium because of its rapid response to the toxicants. Culture TL81 was maintained on the GM broth and was transferred three times before using in the test procedure. This was to ensure that the cell's dehydrogenase was at the optimal level. The cells were transferred as late as possible in the afternoon and used at the same time every morning so that the age of the cells was always the same (18-20 h) for each day's testing. The growth achieved was consistently 0.49 ± 0.01 O.D.(625 nm) and the culture was, therefore, directly used without adjustment.

Chemicals: Phenol, monochlorophenol (MCP), dichlorophenols (DCP), trichlorophenols (TCP), tetrachlorophenols (TTP), pentachlorophenol (PCP), monobromophenols (MBP) and dibromophenols (DBP) were obtained from Supelco Inc, Bellefonte, PA. 16823. They were first dissolved in dilute NaOH, followed by neutralization to pH 7.0 with HCl and then made to volume in a 10-mL volumetric flask. Stock solutions were normally prepared at the concentration of 50 mg mL^{-1}.

Reagents: Resazurin solution was prepared by dissolving one reszurin tablet (5 mg per tablet from BDH) in 50 mL of distilled water using a volumetric flask. The resazurin solution was stored in a brown bottle and was stable for approximately one week at 4°C. Phthalate-HCl buffer (0.05 M) was made up by dissolving 1.02 g of potassium biphthalate in 90 mL of distilled water and the pH was adjusted to 3.0 with 6N HCl (final volume = 100 mL). Sodium bicarbonate and n-amyl alcohol (solvent) were laboratory reagent grade.

Procedures: The toxicity effect on the bacterial culture (TL81) was determined by the following scheme:

A. Reagent control: 4 mL GM + 1 mL resazurin solution
B. Cell control: 3 mL GM + 1 mL resazurin solution + 1 mL cells (0.49 O.D. at 625 nm)
C. Reaction mixture: 3000 - Y μL GM + 1 mL resazurin solution + 1 mL cells (Y = μL test chemical's stock solution)

The experiments were conducted in standard 2 x 15 cm test tubes at room temperature (21°C). The resazurin solution was added last and the contents were quickly mixed and placed in the dark. After an exact incubation of 30 min, 10 mL of n-amyl alcohol and 1 mL of phthalate-HCl buffer solution were rapidly added to each tube to stop the reaction.

The contents were vigorously mixed for 10 sec on a vortex mixer followed by centrifugation in the same test tube at 1,000 rpm for 5 min. The upper solvent layer was transferred into a clean test

tube containing approximately 2 g of sodium bicarbonate. The contents were gently mixed and the absorbance of the supernatant was read on a spectophotometer at 610 nm (the maximum absorbance of unreduced resazurin). If a colorimeter is used such as Spectronic 20, the absorbance is preferably read at 625 nm to avoid the interference from the absorbance of resorufin (reduced resazurin with absorbance maximum at 585 nm). Toxicity to the culture TL81, expressed as % inhibition, of the test chemicals was calculated using the following:

$$I = \frac{(A-B) - (A\ C)}{(A-B)} \times 100$$

where A = final O.D. of reagent control
B = final O.D. of cell control
C = final O.D. of reaction mixture

The term IC_{50} used in this study refers to the effective concentration of the toxicant causing 50% inhibition of the bacterial dehydrogenase activity. The value of IC_{50} was graphically determined by plotting log % inhibitions vs log concentrations of toxicant.

RESULTS AND DISCUSSIONS

The modified resazurin reduction procedure was designed to be a simple and effective screening technique for rapid assessment of chemical toxicity to microorganisms. Consequently, some commercial pipetting apparatuses were tried and incorporated into the routine laboratory testing procedure, resulting in substantial time saving. The Eppendorf Repeater 4780 with a Combitip (12.5 mL size) was used to deliver resazurin solution (1 mL) or phthalate--HCl buffer (1 mL), because of its ability to allow for 11 possible repetitive pipettings. However, the cell suspension (TL81) was added using a regular 1 mL-size Eppendorf micropipette due to the tendency of the cells to settle. The solvent (n-amyl alcohol) used to extract the resazurin from the aqueous phase was added via an Oxford pipetter.

The reproducibility of the modified resazurin test was examined using pentachlorophenol (10 ppm) and mercuric chloride (5 ppm) as the test toxicants at three different days with ten replicates each for the cells control, cells plus PCP, and cells plus $HgCl_2$. The results (Table 1) show that within a batch, the error of the calculated % inhibition is less than 12%, which is considered acceptable. In addition, a comparison of the results from different batches revealed a very good overlapping within the calculated error. In view of the complexity involved with the response of a biological system to any foreign toxicant, the modified resazurin procedure can be considered having very good reproducibility (average r.s.d. 3%). Since the standard deviation does show

Table 1. % inhibition of microbial dehydrogenase by PCP and $HgCl_2$

	Batch one			Batch two			Batch three		
	Mean*	r.s.d. (%)	% I#	Mean*	r.s.d. (%)	% I#	Mean*	r.s.d. (%)	% I#
Control	0.115	7		0.094	3		0.088	8	
PCP	0.360	1	53±5	0.341	1	53±3	0.320	1	52±5
$HgCl_2$	0.540	1	91±8	0.543	1	96±5	0.550	2	98±12

*Absorbance at 610 nm. #Absolute error.

some tolerable variation from day to day experiment, it would be advisable to use replicate tests if absolute accuracy is of the primary concern. Thus triplicate tests were used in the study of halogenated phenols toxicity/structure evaluation (Table 2). While the generalization may still hold that the toxicity of

Table 2. IC_{50} of various chlorophenols and bromophenols to the culture TL81 expressed in mg L^{-1} and as logarithms of the inverse molar concentrations C $[mol\ L^{-1}]$

Chemicals	IC_{50}(ppm)	$\log \frac{1}{C}$	Chemicals	IC_{50}(ppm)	$\log \frac{1}{C}$
Phenol	2300	-1.39	2,3,4-TCP	13	1.18
2-MCP	700	-0.74	2,3,5-TCP	10	1.30
3-MCP	450	-0.54	2,3,6-TCP	190	0.02
4-MCP	400	-0.49	2,4,5-TCP	12	1.22
2,3-DCP	130	0.10	2,4,6-TCP	240	-0.08
2,4-DCP	75	0.34	3,4,5-TCP	5	1.60
2,5-DCP	85	0.28	2,3,4,5-TTP	4	1.76
2,6-DCP	550	-0.53	2,3,5,6-TTP	54	0.63
3,4-DCP	52	0.50	PCP	9	1.47
3,5-DCP	25	0.81	2-MBP	550	-0.50
			3-MBP	380	-0.34
			4-MBP	400	-0.36
			2,4-DBP	60	0.62
			2,6-DBP	500	-0.30

chlorophenols to microorganisms increases with the degree of chlorination in the phenol nucleus (LIU 1981), a precaution, however, must be exercised when such a generalization is used to predict the toxicity of new chemicals based on the study of only a few bench-marker compounds. Theroretically, all isomers should exhibit somewhat similar reactions and behaviours including the toxicity to the biota, but this is not exactly so with chloro-

phenols (Table 2). For instance, 3,5-DCP (IC_{50} = 25 ppm) was approximately 22 times more toxic than 2,6-DCP (IC_{50} = 550 ppm), while 2,4,6-TCP was 48 times less toxic than 3,4,5-TCP, implying a significant variation of chemical toxicity among the isomers of chlorophenols.

The data in Table 2 suggest that the positions of the chlorine substitutents in the phenol profoundly affect the chlorophenols' toxicity to the culture TL81. The para-substituted phenols are more toxic than the ortho-ismoers and this observation is in agreement with the results of ZITKO (1975). When both ortho positions (2,6) of phenol are substituted by chlorines, the molar toxicity is significantly less than that of other dichlorophenols, conversely, chlorine substitution of both meta (3,5) postions causes a strong increase of toxicity. Thus 2,6-DCP (IC_{50} = 550 ppm) was found to be the least toxic of all dichlrophenols and 3,5-DCP (IC_{50} = 25 ppm) the most toxic. A similar observation applies to the TCPs; 2,3,5-TCP (IC_{50} = 10 ppm) was found to be more toxic than either 2,4,6-TCP (IC_{50} = 240 ppm) or 2,3,6-TCP (IC_{50} = 190 ppm).

It appears that the ameliorating effect of 2,6-chlorine substitution on phenol dominates over the enhancing effect of 3,5-substitution on the chlorophenols' toxicity. Thus 2,3,5,6-TTP (IC_{50} = 54 ppm) was approximately 14 times less toxic than 2,3,4,5-TTP (IC_{50} = 4 ppm). This observation may explain why pentachlorophenol (2,3,4,5,6-PCP) was less toxic than the lower chlorinated 2,3,4,5-TTP. In general, the bromophenols studied exhibited a similar pattern of toxicity to the culture TL81 as that of chlorophenols. As expected, 2,6-DBP (IC_{50} = 500 ppm) was found to be less toxic than 2,4-DBP (IC_{50} = 60 ppm).

As reported repeatedly and summarised by ZITKO (1975) and HANSCH and LEO (1979), the toxicities of phenol derivatives frequently correlate with their octanol/water partition coefficients (P). This observation is confirmed by structure-activity analysis of our data. Using the available experimental partition coefficients (HANSCH & LEO 1979), augmented by those calculated from the hydrophobic fragment constants, the observed toxicities can be predicted from the linear regression equation (1):

$$\log \frac{1}{C} = 0.84 \log P - 2.54 \quad (1)$$
$$(N = 24, R^2 = 0.81)$$

With an additional variable $\sum\delta$, δ being the Hammett's constant for ortho substitution, the quality of the regression can be improved to that shown in equation (2):

$$\log \frac{1}{C} = 0.98 \log P - 0.94 \sum o - 2.68 \quad (2)$$
$$(N=24, R^2 = 0.90, \text{s.d. } 0.28)$$

As seen from equation (2), the toxicity of chlorophenols correlates well with the lipophility (log P) of the compound and decreases with the sum of Hammett's ortho constants.

To further illustrate the usefulness and reliability of the modified resazurin reduction procedure in the study of toxicity/structure, the toxicity of some chlorophenols to various biota is summarized in Table 3. The results demonstrate that the

Table 3. IC_{50} of some chlorophenols to the biota

Chemicals	TL81 IC_{50} (ppm)	Trout * IC_{50} (ppm)	Fathead minnow # 96-h IC_{50} (ppm)
2,4-DCP	75	1.7	8.3
2,6-DCP	550	4.0	9.7
2,3,5-TCP	10	0.8	
2,4,5-TCP	12	0.9	
2,4,6-TCP	240	1.1	

*from HATTULA et al. (1981) #from PHIPPS et al. (1981)

toxicity data derived from the modified resazurin procedure agreed qualitatively with the trout and fathead minnow bioassay tests (HATTULA et al. 1981, PHIPPS et al. 1981). It may be of interest to note here that 2,4,6-TCP exhibited less toxicity than other TCP isomers to trout (HATTULA et al. 1981), which would substantiate our finding that 2,6-chlorine substitution on phenol nucleus decreased the chlorophenols' toxicity.

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