Bull. Environ. Contam. Toxicol. (1987) 38:868-875

Environmental Contamination and Toxicology

Chlorinated Phenol Toxicity by Bacterial and Biochemical Tests

G. Cenci, G. Caldini, and G. Morozzi

Institute of Hygiene, University of Perugia, Via del Giochetto, I-06100 Perugia, Italy

The presence of micropollutants in the environment can interact with natural biological systems and some direct or indirect consequences are possible on both human or animal health and in the ecological field (Becking 1979; Bitton 1983).
Microbial tests, together with some biochemical tests, seem just as useful as rapid in vitro assay (Liu 1981; Ribo 1984; Adams et al. 1985) to study chemical toxicity in the environment.
Therefore it might be important to verify the concordance rate of bacterial and enzymatic effect indicators against various inorganic and organic toxic agents and state if a complementary or alternative use of these is possible.
In previous works (Morozzi et al. 1982; Cenci et al. 1985) the above mentioned aspect was considered emphasizing for some heavy metals a slight concordance between growth, or respiration inhibition, and both the dehydrogenase and beta-galactosidase activity of Escherichia coli.
The aim of the present investigation, carried out using E. coli as the test organism and poly-chlorophenols as the toxic organic compounds, was to define: (i) the activity ranges of different chlorinated phenols in the series from monochlorophenol to pentachlorophenol; (ii) the effect of the above mentioned compounds on growth and viability parameters, correlating experimental results with those obtained by enzymatic activities (dehydrogenase and beta-galactosidase); (iii) the relationships between toxicity and some physico-chemical properties of the considered organic compounds. The choice of chlorophenols depends on their implication in important industrial cycles (i.e. tannery, textile, feed (rise), paper and wood) and on their high toxicity for biological systems, so that their probable presence in the final effluents can be regarded also in terms of environmental toxicity (Douglas and Dougherty 1980; Leuenberger et al. 1983; Boyd and Shelton 1984). The phenol was selected as representative organic pollutant (Babich and Stozsky 1985) and the toxicity of other compounds was also expressed as relative phenol toxicity.

Send reprint requests to Prof. G. Cenci at the above address.

MATERIALS AND METHODS

The toxicity of different compounds was evaluated using as test organism Escherichia coli (NCTC 9001) and a salt glucose medium (SGM), pH 7, with glucose as the carbon source (Cenci et al. 1985).

Phenol, ortho-Monochlorophenol (o-MCP), para-Monochlorophenol (p-MCP) and 2,4,6-Trichlorophenol (TCP) were purchased from Carlo Erba (Italy); 3,5-Dichlorophenol (DCP) from Koch-Light Laboratories (England); Pentachlorophenol (PCP) from K & K Laboratories (U.S.A). Except for phenol (which was dissolved in water) the chemicals were dissolved in ethyl alcohol and then diluted with water to suitable concentrations. The solutions were filtered throught 0.45 µm membrane (Millipore) and stored in sterile glass screw-capped tubes at 4°C.

The toxicity of various compounds was determined by both growt experiments in SGM and enzymatic tests. The cultures were incubated with shaking at 37°C and the growth was monitored by an automatic Bonet-Maury biophotometer. Cell concentrations were obtained by a calibration curve. The following growth and enzymatic parameters were used:

- maximum growth yield (MGY), i.e. absolute growth yield (cells/ml) and relative growth yield (RGY), when the ratio MGY to control was considered;
- specific growth rates (SGR), calculated during exponential phase according to the equation:

$$SGR = \log N(2) - \log N(1) \; / \; t(2) - t(1)$$

  where N(1) and N(2) represent the cell concentrations at t(1) and t(2), in hours, respectively;
- lag-times (LAG), defined as the time (in hrs) required to attain mid-point of growth curve;
- dehydrogenase activity (DHA) and beta-galactosidase activity (GAL) was determined by colorimetric tests as previously described (Cenci et al. 1985).

RESULTS AND DISCUSSION

The lowest experimental concentrations with significant effect (LCSE) were found using either the growth or enzymatic parameters described in Methods, by testing differences using Student's "t" for independent samples (Table 1). In the same table the highest no observed effect concentrations (NOEC) was also shown.
After a previous check of homoscedasticity (Bartlett's test chi-square = 1.776, df = 3, p = 0.62), a multiple comparison of the LCSE data by two-way ANOVA using logarithmic trasformation, showed that the differences in results obtained by the four considered parameters were not significant. Instead, different degree of toxicity was observed for different phenol compounds ($F(5;15)$; $p < 0.01$).

Comparing the percentages between cell numbers of exposed and control cultures after different times, the toxic effect of chlorinated phenols was evaluated as follows.

Table 1. Millimolar values of LCSE and NOEC (in parentheses) from indicated parameters for E. coli cultures treated with phenol and chlorinated phenols.

Compounds	MGY	SGR	LAG	DHA	GAL
Phenol	15.0 (7.5)	7.5 (3.75)	3.75 (3.0)	3.0 nd	nr (30.0 mt)
o-MCP	1.5 (0.37)	0.75 (0.37)	0.75 (0.37)	0.75 (0.37)	nr (6.0 mt)
p-MCP	1.5 (0.75)	0.37 nd	1.5 (0.75)	1.5 (0.75)	nr (3.0 mt)
DCP	0.075 (0.037)	0.075 (0.037)	0.075 (0.037)	0.187 (0.075)	nr (0.75 mt)
TCP	0.075 (0.037)	0.15 (0.075)	0.075 (0.018)	0.15 (0.075)	nr (0.30 mt)
PCP	0.094 (0.046)	0.094 (0.046)	0.094 (0.046)	0.018 (0.046)	nr (0.075 mt)

LCSE and NOEC was determined by Student's "t" for independent samples, testing at 0.01 level; nr, not reached; nd, not determined; mt, maximum tested.

Under the experimental conditions used (SGM at 37°C, inoculum 10^7/ml) the time necessary to obtain starting of acclimation of growing cultures to phenol was 3 hours, while for chlorinated compounds it was about 2 hours longer (Figure 1). For the tested ranges of concentration (LCSE and their scalar dilutions) in no case was the start of acclimation related either to the concentration or the number of Cl substituents.
It was observed that generally in the presence of sublethal concentrations the effect was transitory and the acclimation of E. coli to tested doses of phenols was relatively high at the control MGY, i.e. after 8 hours incubation. This indicates that the principal effect was on the lag-phase.
However, while for phenol the 8 hours MGY was reached up to 7.5 mM, for all chlorinated phenols the same result was obtained at longer times compared to control cultures, even when the experimental concentrations was low. The MGY mean cell/ml, together with respective times and the relative percentages in respect to controls, which are reported in Table 2, showed that for each compound the highest experimental concentrations (LCSE) increase the MGY considerably (up to 10 - 16 hrs), and that correspondingly the RGY for phenol and monochlorophenols only diminishes. This was not so in the case of poly-chlorinated phenols.

Important deductions about phenol toxicity emerge by plotting the specific growth rates, normalized to controls, against the different phenol concentrations (log mM).

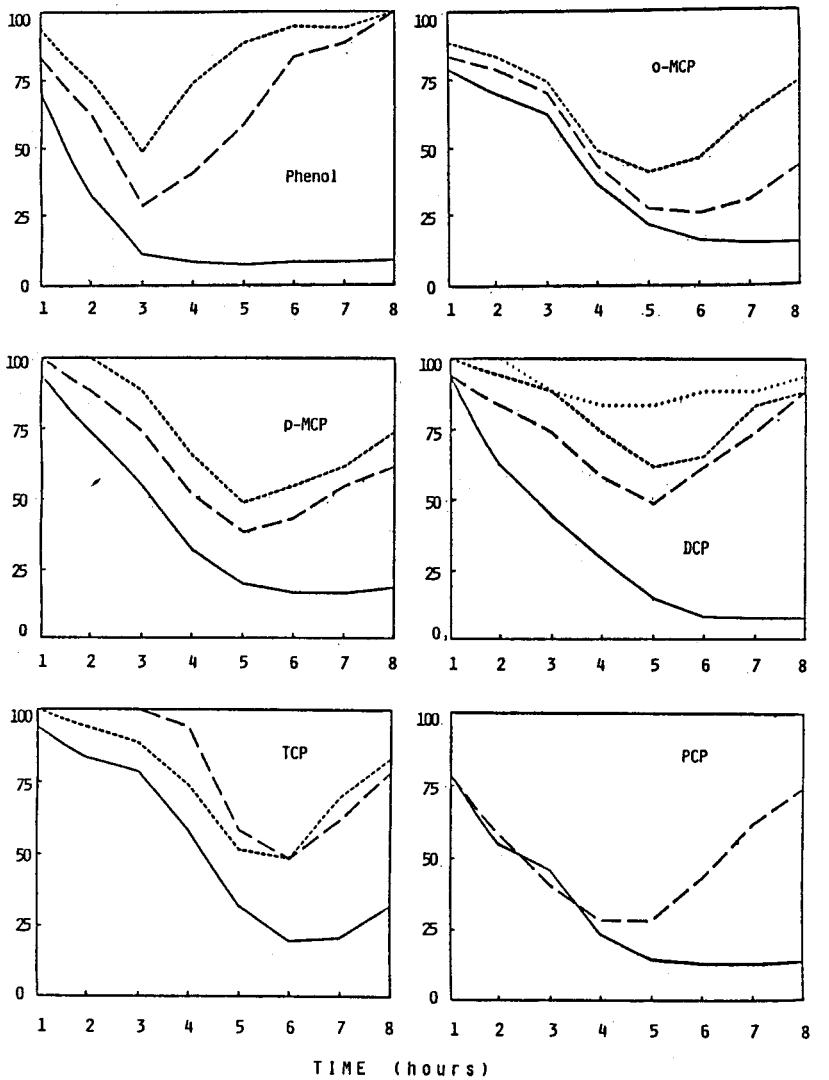

Figure 1. Percentage of control cell number against the time of E. coli cultures treated with different concentrations of indicated compounds. Symbols (———) LCSE, (— — —) 1/2 LCSE, (------) 1/4 LCSE, (·····) 1/8 LCSE.

Figure 2a indicates in fact that the slopes of the linear tract of regression lines for six compounds differ little and that by IC(50) values a good discrimination among different compounds can be estimated. Moreover three different groups can be noted. These include compounds with analogous toxicity: (i) phenol, (ii) o- and p-MCP, (iii) DCP, TCP and PCP. The results obtained with lag-time study, not reported here, were in line with those described for SGR values as regards the toxicity order.

The validity of dehydrogenase activity in determining the toxicity

Table 2. Maximum growth yield (MGY) at the indicated times and relative percentage respect to control (RGY). Mean values from two concord tests.

Compounds	Concentration (mM)	M G Y cells/ml ($\times 10^8$)	M G Y after hours	R G Y
Phenol	0	2.81	8	100
	3.75	2.81	8	100
	7.50	2.81	8	100
	15.00	0.58	18	20.64
o-MCP	0	2.57	8	100
	0.375	2.13	10	82.87
	0.750	1.86	12	72.37
	1.500	1.07	24	41.63
p-MCP	0	2.63	8	100
	0.375	1.94	9	73.76
	0.750	1.62	10	61.59
	1.500	0.50	12	19.01
DCP	0	2.63	8	100
	0.009	2.20	10	83.65
	0.018	2.20	10	83.65
	0.037	2.51	10	95.43
	0.075	1.99	24	75.66
TCP	0	2.51	8	100
	0.018	2.51	8	100
	0.037	2.29	10	91.23
	0.075	2.29	24	91.23
PCP	0	2.50	8	100
	0.046	1.99	10	79.60
	0.094	2.34	18	93.60

of various phenol compounds by discriminating among different concentrations was also proved. A synthetic picture of experiments carried out (Figure 2b) shows similar DHA trend behaviours for phenol treated cultures with respect to those described by SGR values. A good profile separation was also confirmed by DHA assay, even if IC(50) values were different compared to those by SGR. Instead it was observed that all phenol compounds tested do not inhibit the beta-galactosidase activity of E. coli even if the tested doses were high.

In this study since SGR and DHA appear the more responsive parameters for quantitative toxicity evaluation we compared the results obtained from the two mentioned tests for each phenol compound (Table 3). The relative toxicity values show that for both SGR and DHA the values obtained were lower than 10 for mono-

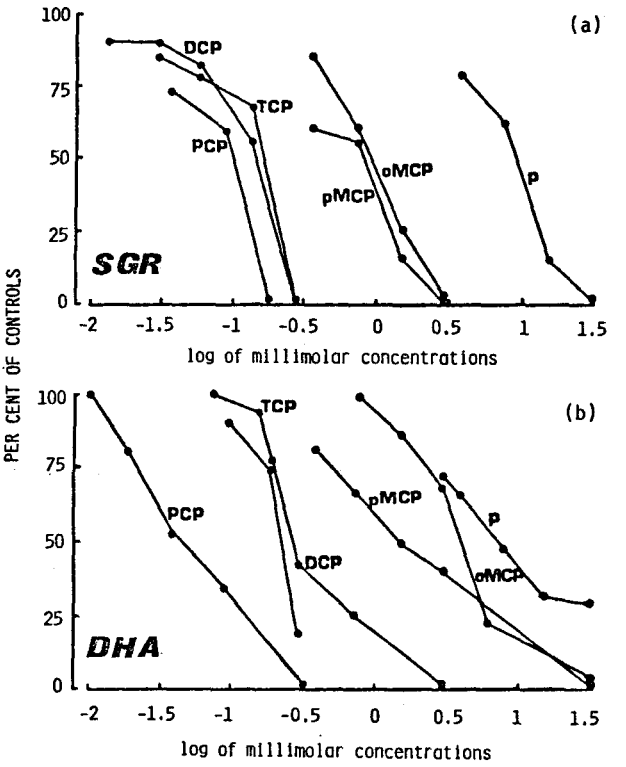

Figure 2. The effect of chlorinated phenols on specific growth rate (a) and on dehydrogenase activity (b) of E. coli.

Table 3. Relative phenol toxicity (RPT) from specific growth rate (SGR), dehydrogenase activity (DHA), microtox test and agar plate method. The mM IC(50) are reported.

Compounds	S G R		D H A		Microtox[a]		Agar plate[b]
	IC(50)	RPT	IC(50)	RPT	IC(50)	RPT	RPT
Phenol	7.90	1.0	6.76	1.0	0.26	1.0	1
o-MCP	0.95	8.3	3.98	1.7	0.17	1.5	1
p-MCP	0.83	9.5	1.60	4.2	-	-	-
DCP	0.15	52	0.26	26	-	-	2
TCP	0.16	49	0.25	27	0.03	8.6	4
PCP	0.10	79	0.04	169	0.0003	866	40

[a] from Beckman (1983) - [b] from minimal inhibitory concentrations (Liu and Kwasniewska 1981)

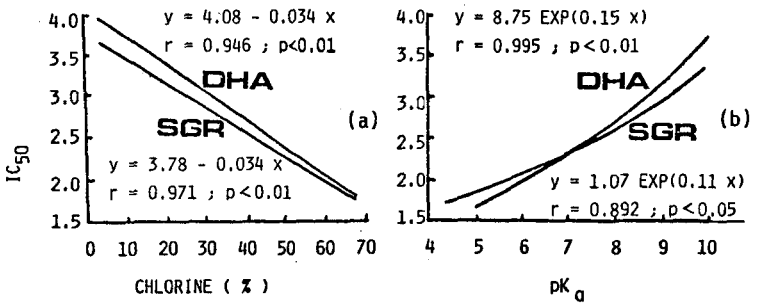

Figure 3. Regression and correlation of percent of chlorine (a) and ionization constants (b) of phenol compounds and their IC(50) values, from SGR and DHA respectively.

chlorophenols and higher than 25 for polychlorinated phenols; the high relative phenol toxicity (RPT) of PCP must be emphasized. SGR and DHA RPTs were related by exponential regression (DHA = 1.28 EXP(0.06 SGR); r=0.958; p 0.01). The above mentioned data confirm the same toxicity order observed by Liu and Kwasniewska (1981), using the agar plate method for rapid assessment of chemical inhibition to microbial populations, and also that by microtox assay (Beckman 1983). This last test however was more sensitive than SGR and DHA in evidentiating the toxic properties of polychlorinated compounds (TCP and PCP). Both experimental SGR and DHA values are always in the range defined by agar plate and microtox test.

An attempt to estabilish relations between some physicochemical properties of phenol compounds and toxicity was made. It was found that both SGR and DHA inhibition were directly related to percent of chlorination in tested phenols (Figure 3a), i.e. IC(50) values progressively decrease with increasing substituent number. This result is in agreement with Liu's (1980) data relative to evaluation of toxic effect of the same phenolic compounds using the system of mixed culture-resazurin test. Dependance of toxicity on phenol ionization constants was also found (Figure 3b), and in this case the regression was defined by exponential equation (DHA $p < 0.01$; SGR $p < 0.05$) and the IC(50) increased by pKa.
No substantial differences were noted between DHA and SGR IC(50) profiles when the pKa ranged from 7 to 8.

On the basis of the experimental results, this work underlines the concordance of growth parameters in revealing sublethal toxicity for E. coli. The data also confirmed the reliability of DHA assay, while a lack of sensitivity to phenol compounds was shown for beta galactosidase activity.
We can therefore conclude by emphasizing the validity of the laboratory model: E. coli-SGR-DHA in screening to evaluate the chemical toxicity of halogenated phenol compounds.

REFERENCES

Adams N, Goulding KH, Dobbs AJ (1985) Toxicity of eight water-soluble organic chemicals to Selenastrum capricornutum: a study of methods for calculating toxic values using different growth parameters. Arch Environ Contam Toxicol 14: 409-415

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

Becking CA (1979) Report of the workshop on biological screening tests, EPA-600/79-000, USEPA, Las Vegas, Nevada

Beckman (1983) Microtox Application Notes

Bitton G (1983) Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: a review. CRC - Critical Reviews Environmental Control 13: 51-67

Boyd SA, Shelton DR (1984) Anaerobic biodegradation of chlorophenols in fresh and acclimate sludge. Appl Environ Microbiol 47: 272-277

Cenci G, Morozzi G, Caldini G (1985) Injury by heavy metals in Escherichia coli. Bull Environ Contam Toxicol 34: 188-195

Douglas WK, Dougherty RC (1980) Pentachlorophenol in the environment. Evidence for its origin from commercial pentachlorophenol by negative chemical ionization mass spectrofotometry. Environ Sci Technol 14: 447-449

Leubenberger C, Coney R, Graydon JW, Molnar-Kubica E, Giger W (1983) Schwer abbaure organische Stoffe in Abwässern der Zellstoffherstellung: Auftreten und Verhalten in einer biologischen Kläranlange. Chemia 37: 345-354

Liu D (1981) A rapid biochemical test for measuring chemical toxicity. Bull Environ Contam Toxicol 26: 145-149

Liu D, Kwasniewska K (1981) An improved agar plate method for rapid assessment of chemical inhibition to microbial populations. Bull Environ Contam Toxicol 27: 289-294

Morozzi G, Cenci G, Caldini G (1982) The tolerance of an environmental strain of Escherichia coli to some heavy metals. Zbl Bakt Hyg I Abt Orig B 176: 55-62

Ribo JM (1984) Chlorinated aromatic compounds: microtox test results and structure-toxicity relationships. National Water Research Institute, Report no. 84-3

Received September 19, 1986, accepted November 24, 1986