Bull. Environ. Contam. Toxicol. (1985) 34:331–339

Bulletin of Environmental Contamination and Toxicology

Effect of Bacterial Cultures on Microbial Toxicity Assessment

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For a long period of time, soil and aquatic environments have been taken for granted as a depository for industrial and domestic wastes. In the United States alone, more than 280 million metric tons of wastes are estimated to be annually disposed of into ponds, lagoons and landfills (Bauer et al. 1981). Due to inadequate planning and poor management, many of the toxic chemicals such as mirex and chlorobenzenes from these wastes have found their ways into rivers, lakes and groundwater (Kaiser 1978; Oliver and Nicol 1982). To safeguard the public health, there is a need to establish criteria for identifying toxic substances in the total ecosystem and in determining their levels of toxicity. Due to the vast number of existing and newly introduced chemicals, the number of toxicity assessments required is increasing at a faster rate than are the corresponding experimental screening works (Bobra et al. 1983). Thus a wide range of short-term bioassays using algae, fish, invertebrates and bacteria has been developed for use in the rapid screening of chemical toxicity (Wong et al. 1979; Sastry and Agrawal 1979; van Dijk et al. 1977; Trevors et al. 1981).

Bacteria are particularly suitable for use in the rapid bioassay of chemical toxicity, because they are inexpensive to cultivate, grow rapidly and contain physiological and enzymatic processes also found in higher organisms. Since approximately 90% of hazardous wastes are placed in soil and water for permanent disposal (Bauer et al. 1981), it is logical to expect that micro-organisms (bacteria) contained in these environments would be the first biota exposed to these toxic insults, and thus they could be used to establish initial toxicity levels. However, the field of microbial toxicity screening is still in its infancy and discrepancies in test results between various assaying procedures have been reported (Dutka and Kwan 1982). Various factors such as bacterial types, pH, temperature, cell age and organic nutrients are suspected to have an influence on the outcome of microbial toxicity tests (Bitton 1983). Thus the objective of this paper was to investigate the effect of various bacterial species on the microbial toxicity test using 6 different bacterial cultures, 12 selected chemicals and a standard set of conditions.

MATERIALS AND METHODS

The six strains of bacteria used in the present study were isolated from various environmental sources. Cultures A, B, and C were isolated from a laboratory activated sludge (Liu et al. 1981), while cultures E and F were obtained from the activated sludge of Elmira (Ontario) sewage treatment plant which receives some wastewater from local industries (Liu et al. 1983). Culture G was isolated from a Lake Erie sediment sample. The purity of these cultures was constantly monitored by phase microscopic examination and nutrient agar plating. The pure bacterial strains were grown separately on a liquid medium containing the following ingredients (g/L): K_2HPO_4, 1.32; KH_2PO_4, 0.82; glucose, 0.2; sodium acetate, 0.2; nutrient broth, 1.0; and yeast extract, 1.0. The medium was sterilized by autoclaving at 121°C for 15 min and the cultures were grown separately in 125-mL Erlenmyer flasks containing 50 mL of growth medium each on a shaker (200 rpm) at room temperature (21°C). The culture broth was used in the toxicity assay after 20 hr of growth without any adjustment of the cells density.

The twelve chemicals tested for their toxicity to bacteria were obtained from commercial sources with the following minimum purity : $HgCl_2$ (99.5%), KCN (97%), phenol (99.5%), o-cresol (99.5%), m-cresol (99.5%), o-MCP (o-monochlorophenol 98%), m-MCP (m-monochlorophenol 98%), DCP (2,3-dichlorophenol 98), PCP (pentachlorophenol 98%), o-DCB (o-dichlorobenzene 98%), parathion (98.5%), toxaphene (95%). KCN and $HgCl_2$ were prepared as aqueous solutions and all phenols as sodium salt. Parathion, toxaphene and o-DCB were made up in DMSO (dimethyl sulfoxide). In the case of the last three chemicals, a modified resazurin reduction procedure (Liu and Thomson 1983) was employed for the toxicity assessment.

The toxicity of the chemicals to bacterial cultures was determined using the resazurin reduction procedure (Liu 1981). The inhibition test mixture contained the following components: *X* µL of test chemical solution, 3000 - *X* µL fresh growth medium, 1 mL of cells and 1 mL of resazurin solution. Total volume of the test reaction mixture was 5 mL. Since some of the bacterial cultures was found to be much more active than others in reducing the dye resazurin, the reaction mixture were incubated for different length of time (30-60 min). The 12 chemicals were tested over a range of concentrations in order to reveal any chronic (sublethal) and acute toxicity effect that may arise from the exposure of bacterial cultures to these toxicants. The results were plotted as " inhibitions " *vs* log concentrations to generate the IC_{50} value, which was defined as the toxicant's concentration that cause 50% inhibition of the resazurin reduction. The % inhibition was calculated using the following equation.

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

where
A = absorbance of reagent control (610 nm)

B = absorbance of cell control
C = absorbance of testing mixture.

The resazurin test for toxicity measures the microbial dehydrogenase activity by accepting electrons flowing from the cell's metabolic processes. In the presence of toxicant, the dehydrogenase activity is suppressed, resulting in less electrons flowing available for resazurin reduction. Thus toxicity assay as assessed by the resazurin reduction method is actually a measure of rate inhibition (rate of resazurin reduction in a control *vs* its reduction rate in a test mixture), ie, an incubation time is not very critical as long as no more than 90-95% of the dye in the cell control is reduced. The variation in incubation time (30-60 min) would ensure the weaker resazurin-reducing cultures to be accurately assessed with a spectrophotometer.

RESULTS AND DISCUSSION

Table 1 presents summary results of all the 12 test chemicals for their acute toxicity effects (IC_{50}) to 6 different bacterial strains as measured by the resazurin reduction method. The simultaneous use of several bacterial cultures from various environmental sources in the determination of a chemical's toxicity is beneficial in that some insight information regarding the interaction between microorganism and chemical can be observed. For instance,

Table 1. IC_{50} values (ppm) of 12 chemicals to 6 bacterial cultures.

Chemicals	IC_{50}/Bacterial cultures					
	A	B	C	E	F	G
$HgCl_2$	1.6	< 0.05	0.05	0.9	0.8	0.4
KCN	260	9	12	7	44	9
phenol	>1000	280	280	>500	>500	>500
o-cresol	>500	260	330	380	>500	>500
m-cresol	>500	225	410	360	>500	>500
o-MCP	>500	130	125	170	>500	>500
m-MCP	>500	54	50	66	320	>500
2,3-DCP	>500	22	21	35	94	>500
PCP	13	21	23	9	14	240
o-DCB	>500	80	48	>500	>500	190
toxaphene	>500	>500	>500	>500	>500	>500
parathion	>500	>500	>500	>500	>500	>500

the results in Table 1 clearly demonstrate the difficulty of selecting a representative bacterial culture for use in a short-term bioassay of an environmental toxicant. The 6 cultures tested did not always respond in the same way to the presence of a toxicant in terms of inhibition sensitivity. Culture A exhibited a profound resistence to $HgCl_2$ toxicity (IC_{50}= 1.6 ppm) when compared with the culture B (IC_{50} < 0.05 ppm). However, at the same time the

culture A was also found to be more susceptible to PCP's toxicity than was the latter one with the IC_{50} values of 13 and 21 ppm respectively.

Perhaps the most important public health aspect of this study is the finding that a chemical's toxicity seems to vary dramatically from one species to another, even assayed by the same testing procedure. Thus the results in Table 1 suggest that there is little possibility of finding an ideal bacterial culture that is highly sensitive to all toxic compounds. A considerable diversity in sensitivity among algae to organochlorine insecticides was also observed recently (Lal 1983). Therefore, the interaction between biota and a toxicant is indeed very complicated without a general pattern to follow. In a natural environment microorganisms tend to exist in a community for muntal benefit and protection, because such a community offers the organisms a greater potential to cope with environmental stress caused by contaminants due to its genetic diversity. For this reason, a single pure culture study in the laboratory should not be liberally used or freely extrapolated to predict the ultimate consequence of new chemicals in the environment.

Table 2. % Inhibition and stimulation (-) of 6 bacterial cultures by $HgCl_2$ as measured by the resazurin method.*

Cultures	% inhibition								
	$HgCl_2$ concentrations (ppm)								
	0.05	0.10	0.20	0.50	1.00	2.00	4.00	5.00	10.00
A				0	6	70	87	90	97
B	70	98	92	99	88	91	93	100	100
C	48	91	91	90	83	92	92	100	100
E	0	8	6	33	54	90	94	87	100
F		0	-1	7	72	95	95	100	100
G		0	9	63	60	86	94	83	80

* average of 8 experiments from different dates.

From the ecotoxicological view point, determination of a chemical's IC_{50} values has little scientific merit, because such values do not provide any subtle or sublethal signal of toxicity other than the biological end-point, such as cell death (Dagani 1983). Therefore, the various concentrations of the 12 test chemicals producing any inhibition or stimulation are presented in Tables 2 and 3. When comparing the data in Tables 1 and 2, the limitation of using IC_{50} value in the ecotoxicological study is evident. For instance, $HgCl_2$ showed an almost identical toxicity to both cultures E and F in terms of IC_{50} (Table 1). However, when the concentration of $HgCl_2$ in the reaction mixture increased from 0.5 to 1.0 ppm level (Table 2), there was a greater increase in $HgCl_2$ toxicity to the culture F (65%) than was to culture E (21%), implying the former being more susceptible to $HgCl_2$ at lower concentrations. Thus, the interaction between a toxicant

Table 3. % Inhibition and stimulation (-) of 6 bacterial cultures by 11 chemicals as measured by the resazurin method.*

Chemicals	Culture	% Inhibition and Stimulation Chemical concentrations (ppm)											
		5	10	20	25	50	100	150	200	250	400	500	1000
KCN	A	31			28				42			86	
	B	31	57				89					97	
	C	35	49				86					95	
	E	36	69				76					96	
	F	-14	11				72					97	
	G	17	53				64					92	
phenol	A		-5				0						36
	B		4				6					74	
	C		5				2					76	
	E		0				14					20	
	F		3				-9					-16	
	G		0				-2					-2	
o-cresol	A		-6				-6	-31				-6	
	B		6				-9	35				85	
	C		-9				6	17				76	
	E		0				8	19				64	
	F		7				-21	-19				-36	
	G		-12				-15	0				-15	
m-cresol	A		0				25					25	
	B		6				10			55		71	
	C		0				6			13		64	
	E		2				0			18		79	
	F		5				-8					-20	
	G		7				1					-6	
o-MCP	A					0	0		12			22	
	B					17	36		73			83	
	C					21	38		75			79	
	E					20	27		57			85	
	F					-5	-5		2			23	
	G					-1	-2		-3			-2	
m-MCP	A					14	9		18			-2	
	B					48	72		80				
	C					51	70		79				
	E					32	73		83				
	F					-4	-4		16			80	
	G					-1	-1		-1			9	

Table 3. (continued)

Chemicals	Culture	% Inhibition and Stimulation											
		Chemical concentrations (ppm)											
		5	10	20	25	50	100	150	200	250	400	500	1000
2,3-DCP	A		0		5	17	18		3			-6	
	B		35		52	79	73					40	
	C		39		53	79	76					33	
	E		11		30	70	72					56	
	F		-2		-5	10	54					73	
	G		2		-1	6	31				32	46	
PCP	A	23	39		82	63	60					82	
	B	12	30		54	69	75					88	
	C	22	34		52	58	73					75	
	E	29	53		78	75	78					81	
	F	18	33		79	82	79					78	
	G		13			39	29		35		91	91	
o-DCB	A	-9	-18		-27	-38	-86			35		32	
	B			-9		11	68		29		3	-18	
	C			-5		53	73		26		8	-12	
	E			-2		-20	4		18		13	13	
	F			-8		-20	-36		-1		3	-5	
	G	-3	-8		-13	-30	-20			78		80	
toxaphene	A		0				0	4		11	14	24	
	B		0				0	16		32	31	33	
	C		0				0	16		14	27	28	
	E		0				0	22		29	31	37	
	F		0				0	-24		-13	1	30	
	G		0				0	2		6	6	0	
parathion	A		0				0	-10		-17	-20	-12	
	B		0				0	-15		10	23	33	
	C		0				0	-24		0	35	37	
	E		0				0	-1		4	3	8	
	F		0				0	-41		-36	-29	-9	
	G		0				0	0		-11	-21	-4	

* average of 8 experiments from different dates.

and microorganisms determined at the IC_{50} concentration level sometimes could be substantially deviated from those assayed at the lower toxicant concentrations. It is believed that comparing the effect of a toxicant on two or more different bacterial species at various concentration levels may provide valuable data that can not be obtained from the comparison of IC_{50} values alone.

Of the 11 chemicals listed in Table 3, only from KCN and PCP could a dose concentration-response relationship for the 6 bacterial cultures be obtained. The other 9 compounds caused the cultures to yield a variety of toxicity responses including the stimulation of dehydrogenase activity at low toxicant concentrations and the subsequent enzyme inhibition at higher levels of concentration. The lack of a general pattern between toxicants and microorganisms interaction clearly demonstrates the fallacy of those attempting to predict the environmental behaviour of new chemicals based on a few sets of physical and chemical parameters. It is of interest to note that the toxicity of 2,3-DCP to the 4 cultures (A, B, C, and E) exhibited a peculiar pattern which initially followed a dose-response relationship at lower concentrations (10-100 ppm), then subsequently accompanied by a decrease of the chemical's toxicity at higher levels of concentration (100-500 ppm). At this time, we have no explanation for the behaviour of 2,3-DCP to the four cultures, but a recent study showed that phenol at low concentrations was more toxic to the rotifer, *Brachionus rubens*, than at higher ones (Halbach et al. 1983).

Table 4. Effect of mixing two bacterial cultures on the assessment of chemical toxicity.*

Chemicals	% inhibition and stimulation (-) cultures		
	A	F	A + F (50/50)
10 ppm KCN	30 ± 3	2 ± 17	16 ± 10
1 ppm $HgCl_2$	0 ± 4	63 ± 21	32 ± 9
100 ppm o-MCP	-3 ± 7	-1 ± 9	-2 ± 8
500 ppm o-MCP	16 ± 7	41 ± 15	29 ± 11

* average of 6 experiments from different dates.

The response of the 6 cultures to o-DCB showed more variations (Table 3) with four strains (A, E, F, and G) exhibiting an intense stimulation of the dehydrogenase activity at the lower chemical concentrations, and then followed by inhibition of the enzyme activity at the higher o-DCB concentrations. From the acute toxicity view point, only one strain (G) reached an IC_{50} value at the upper limit of concentration, while two other cultures (B and C) after reaching an IC_{50} in the mid-concentration range, again displayed a stimulation of the dehydrogenase activity at the upper concentration limit of 500 ppm. A literature search on chlorobenzenes yielded little information on the mechanism of these chemicals' biotoxicity. Thus, no obvious explanation for the dramatic differences in o-DCB's toxicity to various bacterial cultures is available. However, disturbance of the cellular function by a toxicant, as evidenced by the inhibition or stimulation of the dehydrogenase activity, is harmful to an organism, because of the possible interruption of the cell's normal metabolic process (Gupta and Sastry 1981). Thus it is suggested that the determination of a chemical's toxicity should include both the

acute (IC_{50}) and subtle effects.

The limitation of using a single biota species in the toxicity assessment of environmental contaminants was recently recognized (Babich and Stotzky 1977; Hedtka and Puglis 1982; Basha et al. 1983; Borgmann and Ralph 1983). Therefore, another approach involving the use of a two-species algal bioassay procedure for detecting the toxicity of chemicals was developed (Lundy et al. 1984). A two-species system is not only likely to be more sensitive to chemical stress, but is also one step closer to the multi-species, competitive natural environment. Thus, two cultures (A and F) were grown separately and were immediately mixed prior to the toxicity testing to produce a two-species mixed bacterial culture and to see if the results obtained would be the average of those from each pure strain's response (Table 4). In these experiments, cultures A and F were tested separately and as a 50/50 (v/v) mixture with three chemicals (KCN, $HgCl_2$, and o-MCP). A comparison of the results obtained using the mixed culture and the average calculated from the response of the individual strains (A and F) shows a good agreement between the two values within the limits of their absolute errors (standard deviation). This is expected since the resazurin reduction test is short term (< 1 hr) and there is insufficient time for a resistant strain to increase numerically, thus clouding the results for the bacterial mixture.

The present study demonstrates the extreme complexity and unpredictability of the biota-toxicant interaction. The diverse responses of bacterial cultures to different concentrations of the same chemical would also suggest that the linear " dose-response " models often used to explain toxicological responses are inadequate in ecotoxicological research on toxic substances in the natural environment. Apparently, it is not appropriate to assess the toxicity of a chemical by a single bacterial species. Thus, the battery approach involving the use of at least two or more bacterial species with the test chemical assayed at a wide range of concentrations to yield both acute and subtle effects is recommended.

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Received September 4, 1984; Accepted October 26, 1984.