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A RAPID AGAR PLATE METHOD FOR TOXICITY SCREENING OF WATER-SOLUBLE AND -INSOLUBLE CHEMICALS

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MANAGEMENT PERSPECTIVE

Public concern about environmental and health effects from the toxic substances led to the establishment of the Environmental Contaminants Act. One method of defining the potential toxicity of these chemical compounds is the use of bioassays. Because of the vast numbers of existing and newly introduced chemicals in the environment, it is virtually impossible to rely on the use of the conventional animal tests to assess their environmental impact. During the past few years considerable efforts were directed towards the establishment of short-term bioassays that are inexpensive, rapid, sensitive and reproducible for such impact assessment.

Microbial toxicity tests have been rapidly gaining international recognition as an efficient short-term bioassay for environmental toxicants. A major advantage of microbial toxicity tests over chemical analysis is their direct assessment of potential biotic impact without the uncertainty of extrapolation from chemical analysis. The agar plate diffusion method reported here will enable the private industries and governmental agancies to conduct a rapid toxicity assessment on both water-soluble and -insoluble compounds. The technique can also be used by basic researchers in, for example, quantitative structure-toxicity relationship studies.

PERSPECTIVES DE GESTION

L'inquiétude du public au sujet des effets des substances toxiques sur l'environnement et la santé a conduit à l'adoption de la Loi sur les contaminants de l'environnement. Une des méthodes pour évaluer la toxicité potentielle de ces composés chimiques consiste à utiliser les essais biologiques. En raison du très grand nombre de produits chimique déjà présents ou nouvellement introduits dans 'environnement, il est pratiquement impossible de se fier aux seuls essais classiques sur les animaux pour évaluer leurs effets au niveau de l'environnement. Ces dernières années, on s'est orienté énergiquement vers la mise au point d'essais biologiques à court terme, qui sont peu coûteux, rapides, sensibles et reproductibles, pour l'évaluation de ces effets.

Les mesures de toxicité par essais microbiens ont rapidement été acceptées à l'échelle internationale comme essais biologiques à court terme d'une grande efficacité pour les toxiques de l'environnement. Le principal avantage des essais de toxicité microbienne, par rapport aux analyses chimiques: l'évaluation directe de l'effet biotique potentiel, sans l'incertitude de l'extrapolation à partir de l'analyse chimique. La méthode de diffusion sur plaque de gélose, décrite ici, permettra à l'industrie privée et aux organismes gouvernementaux d'effectuer rapidement des évaluations de toxicité, aussi bien de composés solubles qu'insolubles dans l'eau. La même technique peut être employée pour la recherche fondamentale, comme, par exemple, dans les études de la relation quantitative structure-toxicité.

ABSTRACT

This paper presents a simple, rapid, sensitive and reproducible technique for determining the chemical toxicity of both water-soluble and -insoluble compounds by using a direct agar diffusion assay. The procedure involves the use of a dimethyl sulfoxide-glycerol carrier solvent system to dissolve the test chemicals and the incubation of the seeded agar plate at 30°C to speed up the bacterial growth rate. Optimal conditions for performing the test have been established and test results are obtainable in about 4 hours. The technique has been applied to evaluate the toxicity of organic, inorganic as well as organometallic compounds. The method is simple, inexpensive and is suitable for the toxicity screening purpose as well as for use in structure-toxicity studies.

RÉSUMÉ

La présente communication décrit une technique simple, rapide, sensible et reproductible pour mesurer la toxicité chimique de composés aussi bien solubles qu'insolubles dans l'eau, grâce à un essai de diffusion directe en gélose. La méthode emploie un système de solvant avec véhicule de diméthylsulfoxyde-glycérol pour dissoudre les produits chimiques analysés et l'incubation de la plaque de gélose ensemencée à 30°C afin d'activer le taux de croissance batérienne. Des conditions optimales ont été prévues pour l'essai, dont les résultats peuvent être obtenus en l'espace de 4 heures. La technique a été utilisée pour évaluer la toxicité de composés organiques, minéraux et organométalliques. La méthode est simple, peu coûteuse, et elle convient aussi bien pour le dépistage de la toxicité, que pour des études de toxicité par rapport à la structure.

A Rapid Agar Plate Method for Toxicity Screening of Water-soluble and -insoluble Chemicals

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INTRODUCTION

Toxicity assessment is one of the important parameters used in the characterization of a chemical's effects on the environment. This assessment is now required as an integral part of the hazard evaluation of new chemicals in the toxic substances legislation testing schemes of many countries. For example, the U.S. Toxic Substances Control Act calls for the testing of new chemicals prior to their manufacture (17).

There are approximately 63,000 chemicals in common use (10) not to mention the numerous new ones continuously introduced. Detailed toxicity assessment of all the existing chemicals by conventional animal tests is time-consuming and impractical, in terms of operational cost and space. Thus there is a genuine need for simple and inexpensive procedures for the rapid screening for their toxic effect. Such need has led to the development of a wide range of short-term bioassays, using bacteria, yeast, fungi protozoa and algae (3,7).

Microorganisms have several attributes which make them ideal test biota in the short-term bioassays for rapid screening of chemical toxicity. They are easily handled and require

relatively little space (11). The toxicity of a chemical to a microorganism is normally measured in terms of growth inhibition (12), oxygen consumption (15), ATP level (13), enzyme activity (4), or colony formation on agar plate (1). The present study describes the development of a convenient agar plate method for rapid screening of toxicant activity for both water-soluble and -insoluble compounds, and its applications to a variety of organic, inorganic and organometallic compounds.

MATERIALS AND METHODS

Media: The liquid broth medium used in culturing the test bacteria contained 2 g each of glucose, sodium acetate and yeast extract, 1.64 g of KH_2PO_4 and 2.64 g of K_2HPO_4 per litre of distilled water. Initially the agar plate medium was also prepared from the same medium with an agar supplement of 15 g per litre. The buffer components (KH_2PO_4 and K_2HPO_4) in the agar medium caused cloudiness in the pour plate and were subsequently omitted from the medium. The omission of phosphate buffer components from the medium had no observable detrimental effect on colony formation on the agar plate. Sterilization of the medium was accomplished by autoclaving at 121° C for 15 min. The agar plates have a shelf life of approximately 6 weeks at 4° C in a sealed plastic bag. The plates should be pre-dried before bacterial seeding to facilitate the adsorption of the liquid. In our practice the agar plates (with lids on) are dried over night in a drying oven at 30°C.

Culture: The test bacterial culture was Bacillus cereus,

originally isolated from local municipal activated sludges (16). The organism was grown in 50 ml of liquid broth medium contained in a 125 ml Erlenmeyer flask. After 16-18 hrs growth on a rotary shaker (220 rpm) at room temperature (21°C), 0.1 ml of the culture was transferred into another flask containing fresh medium. The transfer was repeated three times to establish actively growing culture. The cell concentration was adjusted to 0.50 o.d. (620 nm) and used immediately to seed the agar plates. The mixed bacterial culture was developed by adding 0.1 ml of fresh domestic activated sludge to a 125 ml Erlenmeyer flask containing 50 ml of the liquid broth medium. The other steps in manipulating the mixed culture were the same as those observed with the pure culture.

Chemicals: Twenty-four chemical compounds (14 organics, 10 inorganics, 4 organometallics) representing a wide range of environmental contaminants were employed to evaluate the suitability and practicability of using the agar plate method for rapid toxicity screening as well as for ranking the test chemicals in terms of hazard. The 14 organics studied included (a) 5 phenols: 2,4-DCP (2,4-dichlorophenol), 3,4-DCP (3,4-dichlorophenol), 2,3,5-TCP (2,3,5-trichlorophenol), and PCP (pentachlorophenol); (b) 5 pesticides: CBL (carbaryl), FNT (fenitrothion), TXP (toxaphene), 2,4 -D (2,4-dichlorophenoxy acetic acid), and 2,4,5-T (2,4,5-trichlorophenoxy acetic acid); (c) 2 chlorobenzenes, i.e. m-DCB (m-dichlorobenzene) and 1,3,5-TCB (1,2,5-trichlorobenzene), (d) 2 organic contaminants, Aroclor 1221 (a commercial PCB formulation), and 2,4-DNT (2,4 -

dinitrotoluene). The 9 inorganic compounds encompassed lead acetate, copper nitrate, chromium chloride, silver nitrate, copper sulfate, cadmium chloride, cobalt chloride, nickel chloride, potassium dichromate. The 4 organometallics used in the study were all alkyllead compounds, i.e. trimethyllead acetate (Me_3PbOAc), triethyllead acetate (Et_3PbOAc), triethyllead chloride (Et_3PbCl) and tripropyllead acetate (Pr_3PbOAc). All test chemicals were dissolved in a mixture of 80% DMSO and 20% glycerol (w/w) at a concentration of 20 mg ml^{-1}, unless otherwise stated in the text. All dilutions on test chemicals were made using the DMSO-glycerol mixture. A 10 ul standard spotting volume for the test chemical was used on the seeded agar plate.

Test Procedure: Adjust the young (16-18 hrs) bacterial culture with fresh liquid broth medium to a final concentration of 0.50 o.d. (620 nm). An aliquot of 0.5 ml of the cell suspension is evenly spread onto the surface of the pre-dried agar plate with the aid of a rotary auto-plater. Alternatively, a bent glass rod can be used to spread the culture manually across the agar plate. After approximately 5-10 min or when the liquid is completely absorbed, the seeded agar plates are ready for use in chemical toxicity assessment. Ten ul of solution containing 0.01-200 ug of the test chemical plus one control (DMSO-glycerol) are placed onto the surface of the seeded agar plate by using a 10 ul Eppendorf micropipette. Usually 3-4 different concentrations of the test chemical and one control can be accommodated on a standard 100 mm petri dish. The control is always placed at the

10-11 o'clock position on the seeded agar plate, followed clockwise by the test chemical arranged in a concentration gradient pattern. The test agar plates are incubated in a 30°C incubator for approximately 3-4 hrs, and the zone of inhibition can be clearly, although at times faintly observed, when the plates are held at an angle of 30-45 degrees. The diameter of the inhibition zone is measured in mm with a micrometer and the zone size is used in assessing a chemicals's toxicity. The control produces no zone of inhibition on the seeded agar plate. Alternatively the agar plates may be kept at room temperature (21°C) overnight for measurement in the following morning if desired.

RESULTS AND DISCUSSIONS

The agar plate method is one of a few short-term bioassays which do not require the use of sophisticated laboratory equipment or expensive reagents. It is ideal for the preliminary screening for chemical toxicity. Our attempts were first concentrated on the standardization of the procedure for optimal conditions to perform the test, followed by the development of a low toxicity carrier solvent system for solubilizing both the water-soluble and -insoluble chemicals.

Experiments were conducted to determine the optimal concentration of cell suspension required for seeding the agar plate in order to have visible bacterial growth within the time frame of a working day, i.e. 8 hrs. Fresh (16 hr) cell suspensions of Bacillus cereus was adjusted to 0.50 and 1.50 o.d. (620 nm)

respectively, with fresh liquid broth medium. Then 0.5 or 1 ml of these adjusted cell suspensions were used to seed the pre-dried agar plates. The 1 ml seeding application was later rejected because of the long drying time, while the 0.5 ml seeding solution especially for the 0.50 o.d. cell suspension, dried much faster in only 5-10 min. Agar plates seeded with high cell concentrations have a mottled appearance which interfered with zone measurements. An aliquot of 0.5 ml of a 0.5 o.d. (620nm) cell suspension was judged to be the optimal amount of bacteria for seeding. Agar plates seeded in this manner allow the detection of the growth inhibition zone by toxicant after a 5-6 hr incubation at room temperature (21° C).

The possibility of reducing the agar plate incubation time was also investigated. Seeded agar plates in triplicates were spotted with 10 ul volume of water (control), $HgCl_2$ (100 ug), o-MCP (sodium salt, 50 ug), and 3,4-DCP (sodium salt, 50 ug). Each set of these plates were then incubated at three different temperatures at 21°C, 30°C, and 35°C. The higher incubation temperature (30 and 35°C) speeded up the bacterial growth which could be easily observed and measured after only a 3 hr incubation (Fig. 1). There was no appreciable difference between the plates incubated at 30°C or at 35°C. The 30°C was arbitrarily chosen as the standard incubation temperature for agar plate in all subsequent experiments. This temperature (30°C) is considered to be the optimal growth temperature for Bacillus cereus(5).

To assess whether the agar plate method has the potential for use

in the field, where difficulties may be experienced in agar plate preparation, culture growing and plate seeding, experiments were conducted to determine the storageability of the pre-dried agar plate for field use. Five agar plates were seeded with fresh Bacillus cereus suspension and four of these were immediately stored in a refrigerator at 4°C for subsequent tests with toxicants on day 1, 2, 8 and 12. The fifth plate was immediately (day 0) spotted with the test toxicant (100 ug $HgCl_2$, 50 ug o-MCP, and 50 ug 3,4-DCP), and incubated at 30°C for the earliest possible observation of toxicity in time (hr) required to produce the zone of growth inhibition on the agar plate. The results in Table 1 indicate that the pre-seeded agar plate could be stored at 4°C for a period of 8 days without apparent change in response to the test chemicals. Although the seeded cells on the agar plate showed a slower growth after storage, nevertheless, experimental results were still obtainable within a working day (8 hr). The present technique is therefore suitable in screening toxicants in the field.

In order to determine whether water-insoluble chemicals could be tested by the present method, several common solvents were spotted onto the seeded agar plates to check their toxicity level to the seeding cells. Methanol, ethanol and acetone (10 ul) were found to spread out rapidly in large and irregular diameters ranging from 20 to 30 mm, only DMSO stayed in a compact and even drop with a diameter of 5-6 mm. After drying the solvent spots, the agar plates were incubated at room temperature (21°C) for 4-6 hrs to produce a visible bacterial growth developed on the

plates. The spots of methanol, ethanol, and acetone were totally clear, indicating the complete kill of the seeded cells by these solvents. DMSO, however, showed a zone of less dense growth, but not totally clear, indicating some survivals of the seeded bacteria. It appears that DMSO is the most suitable carrier solvent with relatively low toxicity carrier solvent for use, and it was investigated further at different dilution to see if toxicity could be reduced. Aliquots of 10 ul of various diluted DMSO solutions (100, 80, 60 and 40%) were spotted onto the seeded plates, followed by room temperature incubation of these plates for 4-6 hrs. The results indicated that all diluted DMSO solutions, except with the full strength DMSO, did not exhibit any toxicity to the seeded bacteria culture Bacillus cereus. A 80% DMSO was used to prepare stock solutions of several water-insoluble chemicals (m-MCB, 1,3,5-TCB, PCP, TXP and 2,4-DNT) at the level of 20 mg ml^{-1}.

For chemicals not soluble in the DMSO-water mixture, it was found that the use of glycerol to replace water constituted a solvent mixture (DMSO/glycerol, 80:20, w/w), which is non-toxic to the seeding bacteria, and capable of dissolving all the above water-insoluble compounds at a level of 20 mg ml^{-1}(20,000 ppm) or higher. The solvating power of the DMSO/glycerol (80:20) carrier system in solubilizing test chemicals for toxicity assessment by the agar plate method was further evaluated using 12 test compounds with a wide range of water solubility, ranging from the water insoluble chlorobenzene to the very soluble inorganic salt $HgCl_2$. The compounds were tested for their toxicities at 3

concentration levels of 2, 20 and 200 ug per spot with a control spot of 10 ul of DMSO/glycerol (80:20) on each seeded agar plate to ensure that the observed toxicity was promarily due to the test chemical per se. The results in Table 2 clearly indicate that this DMSO/glycerol mixture is indeed a powerful and versatile carrier solvent, capable of solubilizing both water-soluble and -insoluble compounds. The reliability and reproducibility of the DMSO-glycerol solvent system in conjunction with the use of agar plate method, for toxicity assessment of chemicals is apparent, as the bacterial responses, from batch to batch at different dates to the test chemicals is reproducible.

Other compositions of DMSO/glycerol as to their suitability as carrier solvent were also evaluated because one batch of the mixed bacterial culture showed some sensitivity to the DMSO/glycerol (80:20) solvent system. It was found that the DMSO/glycerol at 70:30 composition has completely eliminated the toxic effect of DMSO/glycerol to the mixed culture, although its solvating power was not as good as the 80/20 composition. For this reason, a control spot of the DMSO/glycerol carrier solvent is recommended to be included in each experiment. For most uses, DMSO/glycerol compositions of 80:20 and 70:30 would be sufficient for handling most water-soluble and -insoluble chemicals by the present method.

The rapid solubilization of the inorganic salt $HgCl_2$ in the DMSO glycerol mixture prompted the feasibility study of determining the toxicity of other heavy metals on agar plate using this

solvent mixture. Heavy metals are ubiquitous contaminants and some of these such as mercury and cadmium have caused a great deal of environmental concern (2,14). The development of a rapid, simple and reliable screening method would be useful in monitoring and impact assessment. Inorganic salts of various heavy metals encompassing lead,. chromium, silver, cadmium, cobalt, mercury and nickel were dissolved separately in the DMSO/glycerol (80:20) mixture, as welll as in distilled water, at three concentration levels of 2, 20, 200 ug per 10 ul (spot). This was done to assess whether there was any mediation of the heavy metals' toxicity to Bacillus cereus by the carrier solvent. The results in Table 3 indicate that the DMSO/ glycerol (80:20) mixture had no apparent mediating influence on the activity of the test toxicants. The 10 inorganic salts of heavy metals tested, with the exception of $CrCl_3$, all showed exactly the same pattern of response (size of the growth inhibition zone), whether these salts were dissolved in water or in the DMSO/glycerol solvent.

Organometallics have found a fairly wide range of applications in industries, agriculture and forestry as plastic stabilizers, herbicides, and biocides. The frequent usage of these chemicals have contributed to their distribution in the aquatic environment (9). Alkyllead compounds are one of the few groups of organometallics, which have been extensively studied, mainly because of problem and concern regarding lead pollution in the environment (8). Four alkyllead compounds (Me_3PbOAc, Et_3PbOAc, Et_3PbCl, Pr_3PbOAc) and one inorganic lead ($PbOAc_2$) were dissolved

in DMSO/glycerol (80:20) mixture and the toxicity test was carried out on five different days, covering a three week span in order to check its reliability and reprodicibility (Table 4). Bacterial growth on the agar plates was inhibited by the tripropyl and trimethyl lead at the level of 0.2 ug/10 ul test spot. However, the cells response to the trimethyl lead at this concentration level was actually at borderline, i.e. decreased growth but the zone being not totally clear. A dosage of 0.02 ug Pb/10 ul solution of the tripropyllead acetate showed no inhibition. The minimum amount required for inhibition for the two triethyllead compounds was at the level of 2 ug/10 ul spot. Apparently the identity of the anion did not influence the toxicity of the triethyllead compounds, in agreement with the literature data (6). Lead acetate required a dose of 200 ug/spot before the cell growth inhibition was seen. It may be of interest to note that the clear zones for the organolead compounds tested increased with increasing amounts of chemicals, whereas the inorganic lead acetate showed only a fixed diameter of 10 mm, regardless of the concentration. At present we do not have an explanation for this observation. Based on the results in Table 4, the toxicity ranking of the lead compounds as determined by the agar plate method is : Pr_3PbOAc, Me_3PbOAc, Et_3PbOAc, Et_3PbCl, $Pb(OAc)_2$ in decreasing order.

The toxicity of the five lead compounds was also assessed by the respiration inhibition test for comparison. Results tabulated in Table 5 clearly indicated that the tripropyl lead acetate has the highest toxic potency (IC_{50} = 32 ppm), while the inorganic lead

acetate was the least toxic with IC_{50} value being unable to reach. When compared Tables 4 and 5, it is obvious that the order of toxicity of lead compounds to the test bacterium B. cereus followed the same pattern, as evaluated by the agar plate method and the respiration test.

The high sensitivity of the agar plate method in detecting the toxicity of alkyllead compounds prompted us to explore the application of this method in measuring the toxicity of other contaminants. Aroclor 1221 (a PCB formulation) and toxaphene (insecticide) were chosen as test toxicants, maimnly because of their water-insolubility and their environmental significance. The two chemicals were dissolved into DMSO/glycerol (80:20) mixture before being applied onto the seeded agar plates. To enhance the photographic contrast, the agar plates were allowed to incubate at 30°C for 10 hrs (Fig. 2). The toxicity of toxaphene could be demonstrated at the 0.1 ug level, while the PCB Aroclor 1221 being less toxic at 1 ug level.

While the agar plate method is valuable for toxicity screening of both water-soluble and -insoluble chemicals, it may also be applied in other areas of toxicological studies. Quantitative results could be obtained from the experimental data. To illustrate this point, the toxicity data of lead compounds in Table 4 were used to generate the dose-response curves for the alkyllead compounds by plotting the toxicant's concentration (ug/spot) vs. the diameters (mm) of the clear inhibition zone on the agar plates (Fig.3). The linearity of these curves suggests that the agar plate method has the potential to be used for

quantitative estimation or for prediction of the toxic potency of other analogous series or new chemicals, particularly under the situation when such chemicals are available in only very small quantities. The present agar plate method requires only a 10 ul solution for each test spot, making it economical to perform toxicity assessment on expensive or new chemicals. Moreover, the test chemicals may be easily recovered after toxicity testing, simply by cutting off the area of clear zone on the agar plate with a clean spatula and extracting it with an appropriate solvent. Such a practice may be necessary in studying the interaction between microorganism and the test chemicals such as biotransformation reactions during the test period.

The present method is unique that test results are readily available within 3-4 hrs of plate incubation. Most water-soluble and -insoluble compounds including organic, inorganic, organometallic can be easily assessed for their toxicity. Wide applicability and simplicity make the present method ideal for use in the preliminary screening for toxicity.

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REFERENCES

1. Anderson, A.C., and A.A. Abdelghani. 1980. Toxicity of selected arsenical compounds in short term bacterial bioassays. Bull. Environ. Contam. Toxicol. 24: 124-127.

2. Attar, E.N., and E.J. Maly. 1982. Acute toxicity of cadmium, zinc and cadmium-zinc mixtures to Daphnia magna. Arch. Environ. Contam. Toxicol. 11: 291-296.

3. Bitton, G. 1983. Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: A review. CRC Crit. Rev. Environ. Control. 13 (1): 51-67.

4. Bitton, G., T. Khafif, N. Chataigner, J. Bastide, and C.M. Coste. 1986. A direct INT-dehydrogenase assay (DIDHA) for assessing chemical toxicity. Toxicity Assess. 1: 1-12.

5. Breed, R.S., E.G.D. Murray, and N.R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed. The Williams & Wilkins Co., Baltimore.

6. Chau, Y.K., and P.T.S. Wong. 1984. Organic lead in the aquatic environment, p.21-31. In P. Grandjean and E.C. Granjean (ed), Biological effects of organolead compounds. CRC Press Inc., Boca Raton.

7. Dutka, B.J., and G. Bitton. 1986. Toxicity testing using microorganisms. vol.2, CRC Press Inc., Boca Raton.

8. Grandjean, P., and E.C. Grandjean. 1984. Biological Effects of Organolead Compounds. CRC Press Inc., Roca Raton.

9. Maguire, R.J., Y.K. Chau, G.A. Bengert, and E.J. Hale. 1982. Occurrance of organotin compounds in Ontario lakes and rivers. Environ. Sci. Technol. 16: 698-702.

10. Maugh, T.H. 1978. Chemicals: How many are there? Sci. 199: 162.

11. Mowat, A. 1976. Measurement of metal toxicity by biological oxygen demand. J. Water Poll. Control. Fed. 48: 853-866.

12. Narkis, N., and C. Zur. 1979. Toxicity test accompanying biodegradation test of anionic surfactants. Bull. Environ. Contam. Toxicol. 22: 449-456.

13. Parker, C.E., and E.J. Pribyl. 1984. Assessment of bacterial ATP response as a measurement of aquatic toxicity, p. 283-293. In D. Liu and B.J. Dutka (ed.), Toxicity screening procedures using bacterial systems. Marcel Dekker, Inc., New York.

14. Peterson, R.L., D. Arnold, D.G. Lynch, and S.A. Price. 1980. A heavy metal bioassay based on percent spore germination of the sensitive fern, Onoclea sensibilis. Bull. Environ. Contam. Toxicol. 24: 489-495.

15. Slabbert, J.L., and W.O.K. Grabow. 1986. A rapid water toxicity screening test based on oxygen uptake of Pseudomonas putida. Toxicity Assess. 1: 13-26.

16. Thomson, K., D. Liu and K.L.E. Kaiser. 1986. A direct resazurin test for measuring chemical toxicity. Toxicity Assess. 1: 407-418.

17. U.S. Environmental Protection Agency. 1979. Toxic substances control act premanufacture testing of new chemical substances. Fed. Reg. 16240-16292.

TABLE 1. Effect of the storage time on the performance of the seeded agar plate

Day	Diameter of the clear zone (mm)				Time required to view results (hr)
	H_2O	$HgCl_2$	3,4-DCP	o-MCP	
0	-	20	12	8	3-4
1	-	20	12	8	4-6
2	-	20	12	8	4-6
8	-	20	12	8	6-7
12	-	22	14	8	overnight

TABLE 2. DMSO 80%/glycerol 20% as a carrier solvent for various test chemicals in the agar plate method

Chemicals	ug required to produce clear zone						
	Batch 1	Batch 2		Batch 3			
	Day 0	Day 0	Day 1	Day 0	Day 1	Day 2	Day 3
m-DCB	20	20	20	20	20	20	20
1,3,5-TCB	20	20	20	20	20	20	20
PCP	2	2	2	2	2	2	2
2,3,5-TCP	2	2	2	2	2	2	2
2.4-DCP	20	20	20	20	20	20	20
2,4-D	20	20	20	20	20	20	20
FNT	20	20	20	20	20	20	20
CBL	200	200	200	200	20	20	20
2,4,5-T	20	20	20	20	20	20	20
2,4-DNT	200	200	200	200	200	20	20
Phenol	200	200	200	200	200	200	200
$HgCl_2$	2	2	2	2	2	2	2

TABLE 3. Comparison of heavy metals toxicity as assessed in water and in 80% DMSO/20% glycerol by the agar plate method

Chemicals	ug required to produce clear zone			
	in DMSO/glycerol		in water	
	trial 1	trial 2	trial 1	trial 2
$HgCl_2$	2	2	2	2
$K_2Cr_2O_7$	2	2	2	2
$CdCl_2.2\frac{1}{2}H_2O$	2	2	2	2
$AgNO_3$	2	2	2	2
$CrCl_3.6H_2O$	20	20	2	2
$NiCl_2.6H_2O$	20	20	20	20
$CoCl_2.6H_2O$	20	20	20	20
$CuSO_4.5H_2O$	20	20	20	20
$Cu(NO_3)_2.3H_2O$	20	20	20	20
$Pb(CH_3COO)_2.3H_2O$	200	200	200	200

TABLE 4. Toxicity of alkylleads and lead acetate as assessed by the agar plate method*

Chemicals	Diameter of clear zone (mm)				
	100 ug	20 ug	2 ug	1 ug	0.2 ug
Me_3PbOAC	25.6±1.4	21.2±2.8	14.4±1.6	12.5±0.5	7.5±1.5
Et_3PbOAC	22.4±1.6	14.0±3.0	7.3±0.8		
Et_3PbCl	21.1±0.8	14.4±0.6	7.8±1.3		
Pr_3PbOAC	29.8±0.2	25.2±0.8	18.4±2.6	13.0±2.0	7.4±0.6
$Pb(OAC)_2$	10±0	10±0			

* average of 5 experiments at different dates

Table 5. IC_{50} (ppm) of lead compounds as determined by the respiration inhibition test.*

Test compounds	IC_{50} (ppm)
Me_3PbOAC	93
Et_3PbOAC	104
Et_3PbCl	103
Pr_3PbOAC	32
$Pb(OAC)_2$	not reached

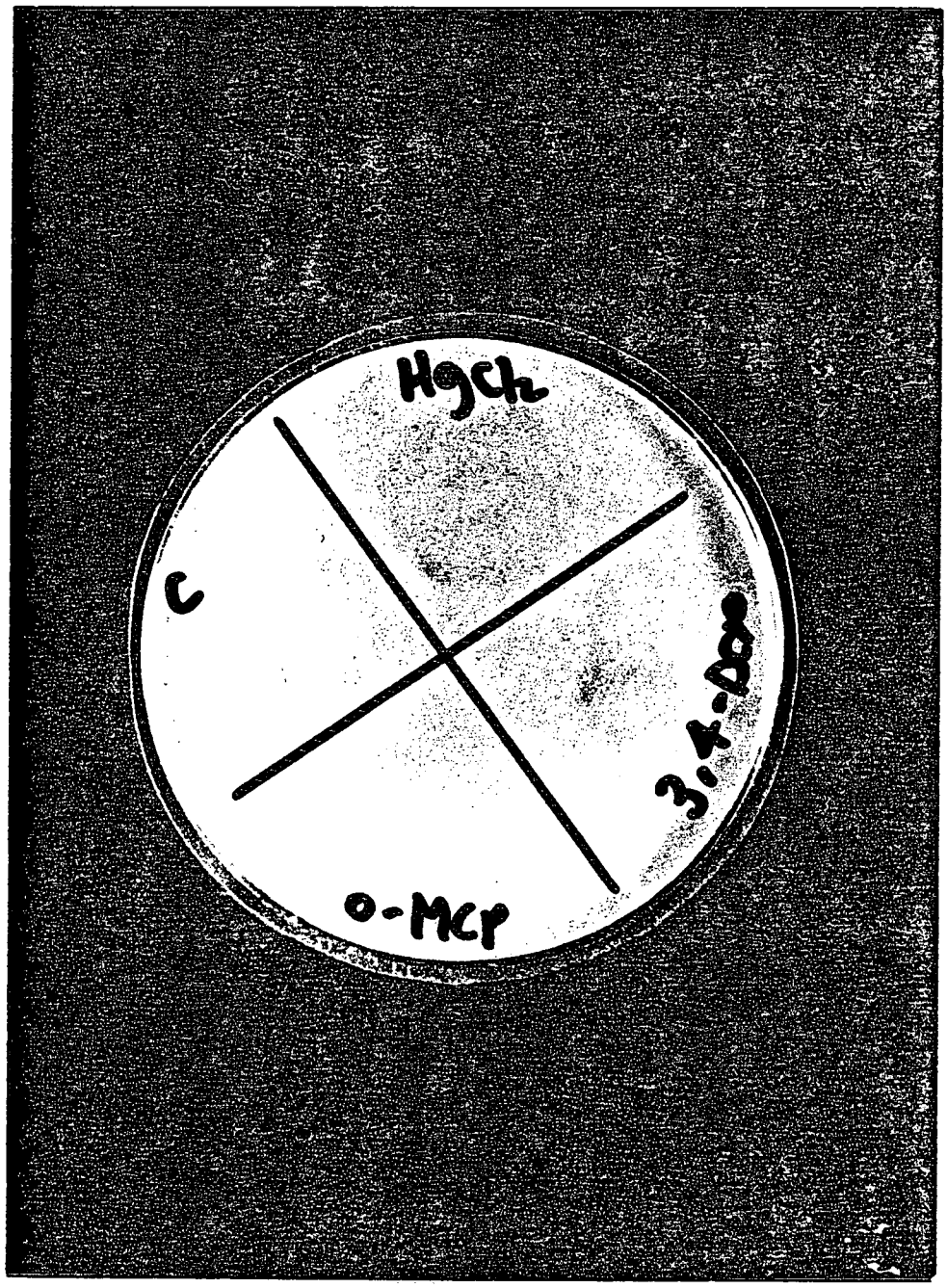
* Experiments were conducted using a gilson differential respirometer operated at 23°C. A typical reaction mixture comprised 3.75 ml fresh liquid medium, 1 ml cell suspension (2 O.D. at 620 nm), 0.25 ml DMSO solution containing the test compound (in the side arm of the warburg flask), and 0.2 ml of 20% KOH in the center well for CO_2 absorption.

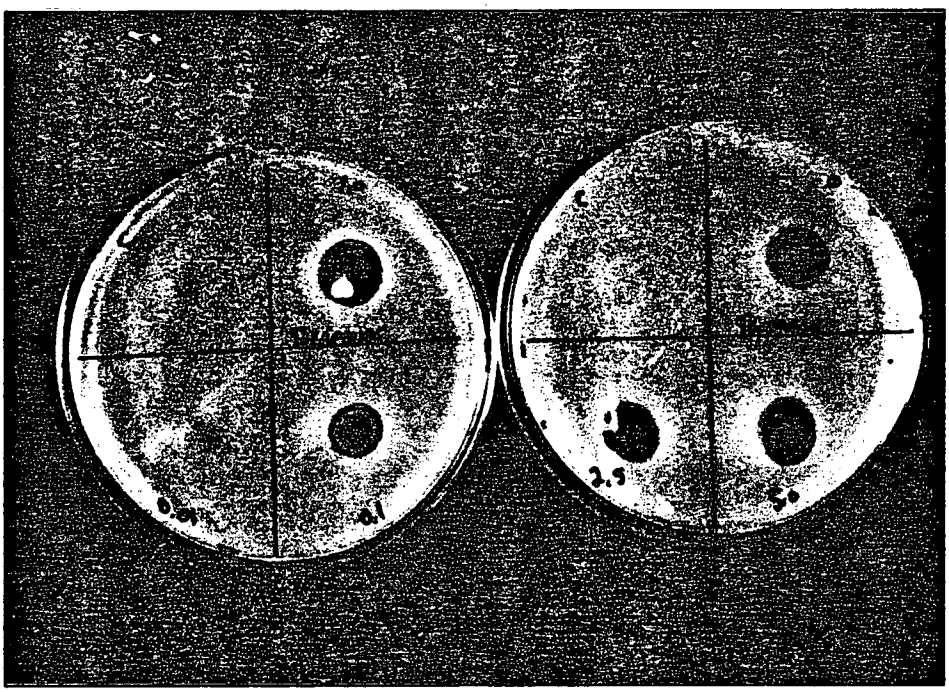
FIGURE LEGENDS

Fig. 1 Formation of the growth inhibition zone by toxicants on the seeded agar plate after 3 hr incubation at 30°C.

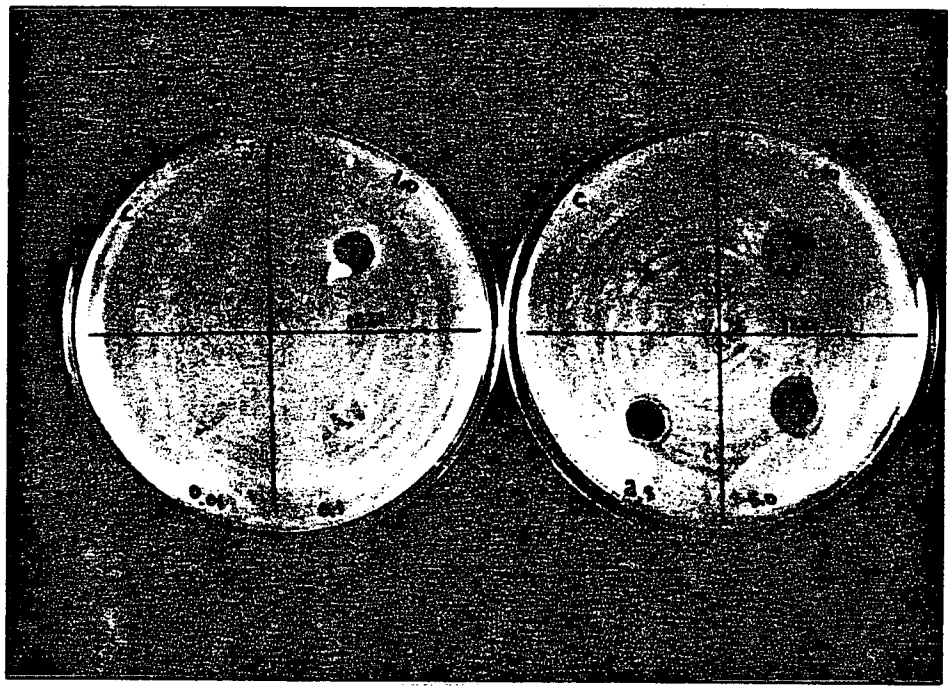
Fig. 2 Toxicity screening for toxaphene (A) and Aroclor 1221 (B) using the agar plate method.

Fig. 3 Dose response of clear zone size to concentrations of lead compounds on assay plates.


HgCl2
C
3,4-DCP
O-MCP



A



B

Fig-2

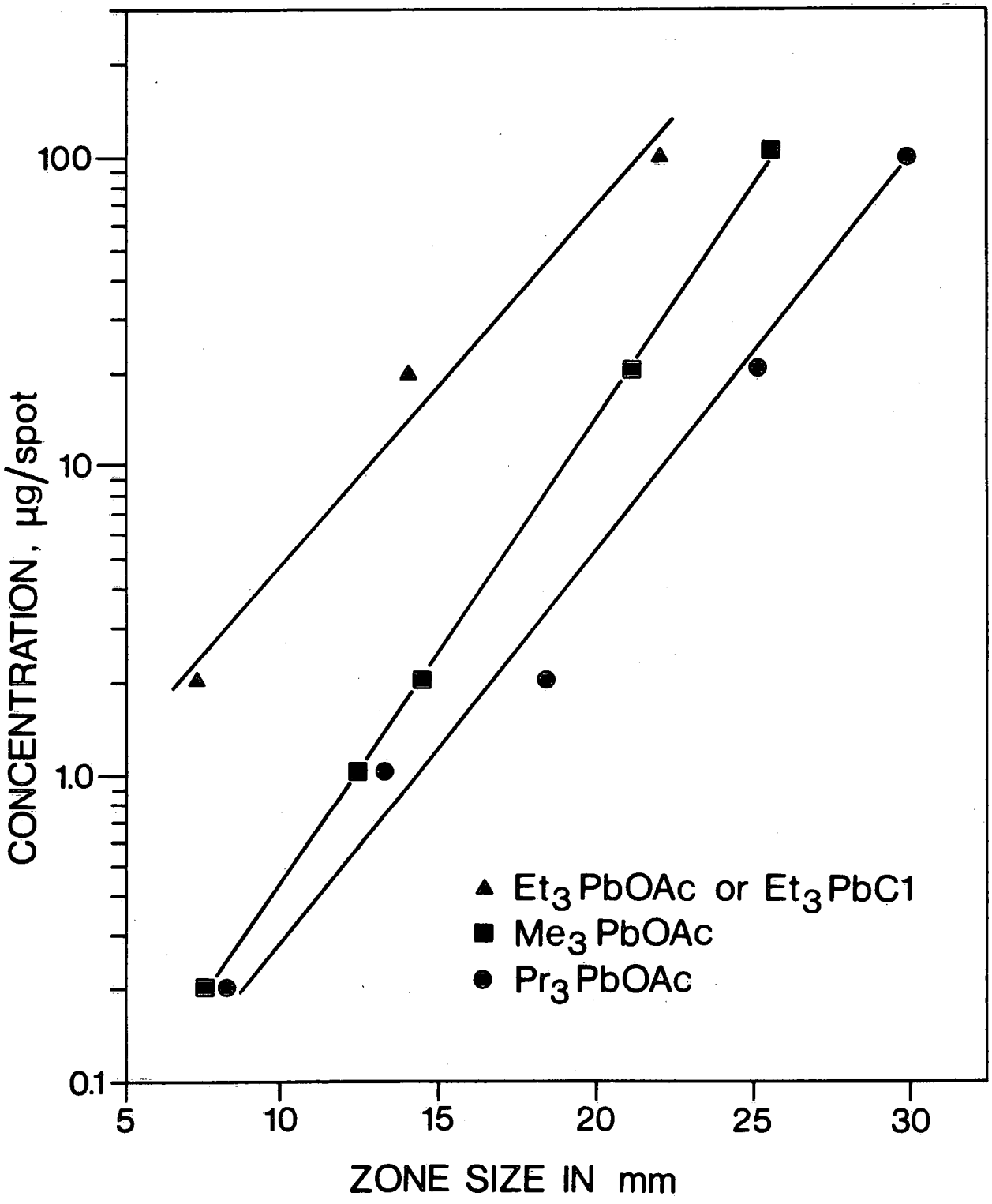

100
10
1.0
0.1
CONCENTRATION, µg/spot
5
10
15
20
25
30
ZONE SIZE IN mm
▲ Et3PbOAc or Et3PbCl
■ Me3PbOAc
● Pr3PbOAc

Fig - 3