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THE USE OF CYTOTOXICITY ASSAYS FOR THE ASSESSMENT OF TOXICITY (EHT 9329)

Final Report to the Department of the Environment

DoE 1823-M/3

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Report No: DoE 1823-M/3

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PREFACE

On 1 April 1985, the Department of the Environment placed a contract (Ref PECD 7/7/168) with the Water Research Centre (WRc) to evaluate the use of cytotoxicity assays for the assessment of toxicity. The contract ended on 30 April 1988.

This final report summarises the work undertaken between April 1985 and April 1988 both at WRc and in the laboratories of other organisations working under contract to WRc.

SUMMARY

I OBJECTIVES

a) To evaluate the performance of *in vitro* cytotoxicity assays in relation to toxicological screening of chemicals, materials and water samples.

b) To adapt suitable test systems for use with extracts of water samples and leachates from materials.

c) To optimise at least one test system, as appropriate, for use with chemicals, materials and water samples.

II REASONS

Tissue culture has been used in biology since the beginning of this century and its application to toxicity testing has been of increasing interest over recent years. Cytotoxicity assays using cells in tissue culture may provide one answer to the problem of assessing the biological activity of complex mixtures in environmental pollution.

III CONCLUSIONS

1. *In vitro* cytotoxicity assays are a potentially useful tool in the study of toxic compounds of complex mixtures. This usefulness is restricted by the relative lack of development of such assays at the present time and the unpredictability of problems in their use. They can only be used as screening tests since data cannot be easily extrapolated directly to man.

2. The lack of xenobiotic metabolism, which is lost in most cell lines and which is necessary for determining the toxicity of indirect acting toxicants can at present only be overcome by the use of primary cell cultures. This is a serious drawback to the use of cell lines in cytotoxicity testing. New cell lines which maintain a high degree of metabolic integrity are just being developed.

3. The main characteristics necessary in a cytotoxicity assay to be used routinely as a screen are sensitivity, reproducibility, robustness and ease of use.

4. Cloning efficiency appeared to be the most sensitive assay of the ones examined and visual assessment of morphology the least sensitive.

5. The period of exposure is extremely important since some chemicals are not cytotoxic unless the cells are exposed for a sufficiently long period. Very short assays must be considered with some suspicion.

6. Cytotoxicity assays appear to be of little value in examining drinking water but may have some uses in screening effluents.

7. The use of cytotoxicity assays to screen leachates from materials is severely hampered by the variation in sensitivity to toxic components of materials.

8. Where such assays are used to examine leachates from materials the purpose and limitations of the assay must be clearly defined in order to prevent over-interpretation of the results by non-specialists.

9. Toxin producing blooms of the blue-green alga Microcystis aeruginosa can be detected by means of a cytotoxicity assay which provides an alternative to the use of laboratory animals.

IV RECOMMENDATIONS

The cytotoxicity assays presently used for screening leachates from materials should be considered more critically to determine precisely what they will measure, whether they can be improved or whether they should be specified for use only with certain materials.

The development of cytotoxicity assays based on cell culture is very fragmented and suffers from too much duplication of simple experimental work. Their role in toxicology and the development of alternatives to laboratory animals is being hindered by this fragmentation. It is recommended that this be better co-ordinated and further fundamental research into cells in culture, particularly in toxicology, be carried out.

IV RESUME OF CONTENTS

Practical studies on the performance of *in vitro* cytotoxicity assays and their use for screening water and effluent samples, materials used in water supply and the hepatotoxin of *Microcystis aeruginosa* are described.

Of the assays examined, cloning efficiency appeared to be the most sensitive while assessment of cell morphology was the least sensitive. The period of exposure to toxins was found to be extremely important, the cells requiring extended exposure to some toxins before evidence of cytotoxicity was observed.

The cytotoxicity assays examined appeared to have little value in examining drinking water but may have some uses in screening effluents where the concentration of toxins is higher. The assays showed substantial variation in sensitivity to the toxic components which leach from materials in contact with water. It is therefore shown to be necessary to define the purpose and limitations of such assays to prevent over interpretation of results by non-specialists.

Toxin producing blooms of the blue-green alga *Microcystis aeruginosa* were shown to be detectable by means of a cytotoxicity assay rather than by the current methods using laboratory animals.

The value of cytotoxicity assays is restricted by the relative lack of development of such assays at the present time, the unpredictability of problems in their use and the lack of xenobiotic metabolism in cell lines.

CONTENTS

APPENDICES

SECTION 1 - INTRODUCTION

One problem increasingly facing those studying pollution in the environment is that of assessing the biological significance of mixtures of chemicals for which analytical techniques are either unavailable or inappropriate. In many instances the answer to this problem has been to use biological activity in one form or another as a means of measuring or assessing such mixtures. Such an approach has been used with great success to study the biological activity of complex mixtures in chlorinated drinking water by means of bacterial mutagenicity tests.

Mutagenicity tests use a specific kind of toxicity, toxicity to genetic material, as an endpoint. However, there are many compounds which are of interest and which do not affect DNA. In these circumstances a more general biological/toxicological assay system would be more appropriate. In this context cytotoxicity assays using cells in tissue culture are of considerable interest.

1.1 TISSUE CULTURE TECHNIQUES

Tissue culture has been used in biology since the beginning of this century but its application in toxicity testing has only been seriously considered in the last decade. There are a number of reasons for this increase in interest including increasing cost of animal studies, moral considerations on the use of animals, and greater recognition of the importance of mechanisms of toxicity at the cellular level. Research into in vitro techniques as alternatives to animal testing is now progressing rapidly and considerable effort is being made in validation and standardisation of methods.

There are three principal types of tissue culture which have been used in toxicity studies.

i) Organ culture

Whole organs or sections of tissue can be cultured in vitro for limited periods.

ii) Primary culture

Cells are isolated from tissues and grown for up to a month usually as monolayers.

iii) Continuous cell lines

These cells can be grown and subcultured in vitro almost indefinitely.

There are advantages and disadvantages with each of the different types of tissue culture. Unlike organ culture and primary culture, continuous cell lines do not require regular sacrifice of animals as a source of tissue. However, organ and primary culture more closely mimic cells in vivo. Continuous cell lines tend to be dedifferentiated, that is they have lost the ability to perform organ-specific biochemical functions. Cell lines generally have a poor drug-metabolising capability and are therefore unsuitable for studying chemicals which require metabolism to express their toxicity. However, despite their present limitations, continuous cell lines are useful as biological models. They can be used to predict the intrinsic acute toxic potential of chemicals and the range of possible end-points can provide some information on mechanisms of action.

1.2 APPLICATION IN THE WATER INDUSTRY

There are a number of applications for which tissue culture methods could be of value to scientists in the water industry.

i) Cytotoxicity assays could be used to study a wide range of chemicals, natural products or materials involving complex mixtures for which a bio-assay may be more appropriate than analytical methods.

ii) Cytotoxicity testing could be applied to water samples to study or monitor specific effluents or the effect of a combination of effluents on a receiving water.

iii) An *in vitro* cytotoxicity assay already forms part of the testing of non-metallic fittings for use in contact with potable water (United Kingdom Water Fittings Byelaws Scheme 1985). However, such techniques could be of value in the testing of water industry materials on a wider basis.

iv) Compounds identified in water that have not been tested for toxicity may be screened using *in vitro* test systems particularly when comparison can be made with related compounds of known toxicity.

1.3 THE CONTRACT

In April 1985, the Water Research Centre began a three year contract for the Department of the Environment on the use of cytotoxicity assays for the assessment of toxicity. The objectives of the contract were as follows:

a) To evaluate the performance of *in vitro* cytotoxicity assays in relation to toxicological screening of chemicals, materials and water samples.

b) To adapt suitable test systems for use with extracts of water samples and leachates from materials.

c) To optimise at least one test system as appropriate for use with chemicals, materials and water samples.

The programme was designed so that a range of cytotoxicity assays would be examined using pure chemicals and the most suitable assays applied to water samples and materials. The basis of the project was therefore comparison of *in vitro* assays with one another rather than an attempt to specifically develop and validate methods for replacing animals used in any particular *in vivo* test. Nonetheless the work would contribute to progress in alternatives to using whole animals.

SECTION 2 - LITERATURE REVIEW

As part of the early stages of the contract a comprehensive review of the literature relating to cytotoxicity testing using cell-lines was prepared. This was issued to the Department as an interim report DoE 1297-M. The report reviewed the use of continuous cell lines for in vitro cytotoxicity testing with particular emphasis on the suitability of different end-points for broad screening of chemicals, water samples and materials. Published studies on the cytotoxicity testing of water samples and materials were also considered.

The conclusions were that different end-points tended to rank chemicals in the same order and therefore the simpler, robust, test systems were preferable to more sensitive but elaborate methods. Different cell lines appeared to give similar results with respect to sensitivity and so ease of culture and applicability to the chosen end-point were the most appropriate primary selection criteria.

SECTION 3 - SELECTION OF TEST SYSTEMS

We initially considered 4 test systems, which were the MIT-24 test, FRAME test, inhibition of cell growth (vital dye method) and cloning efficiency assay. A comparison of these test systems was then made using a single cell line.

3.1 SELECTION OF CELL LINE

Two readily obtainable cell lines were in use in our laboratory for assessing chromosome damage by concentrated drinking water extracts in vitro. These were CHO-KI, a Chinese hamster ovary cell line, and the V79/4 Chinese hamster lung fibroblast cell line. On the basis of slightly greater ease of handling under our laboratory conditions the V79/4 cell line was selected for experimental work on the test programme. Details of maintenance and culture procedures are given in Appendix A.

3.2 MIT-24 TEST

Following a visit to Dr B Ekwall in Sweden, the MIT-24 test used in his laboratory was chosen for a combination of three reasons:

a) it is simple to perform;
b) it can be used in microtitre systems;
c) it is well documented for use on a wide range of chemicals.

Dr Ekwall uses HeLa cells but the system can be used with any cell line. The test, called the MIT-24 test, uses two end-points to assess cytotoxicity, cell morphology after incubation for 24 hours and inhibition of metabolism after incubation for 7 days. The use of microtitre plates enables a large number of samples to be tested in a short time at low cost.

In Dr Ekwall's laboratory the MIT-24 test has been used to study the 'basal' cytotoxicity of several types of substances including drugs, plasticisers, alcohols and foodstuffs. Basal cytotoxicity refers to toxicity to cell structure and function common to all cell types. One drawback with the MIT-24 test as used by Dr Ekwall is that it is a qualitative, not a quantitative assay. Both the morphological (observed microscopically) and metabolic inhibition (measured by change in pH of medium as indicated by the colour of the medium) end-points are somewhat subjective.

Details of the test are given in Appendix A.

3.3 FRAME TEST

In 1982, The Fund for the Replacement of Animals in Medical Experiments (FRAME) established a research programme in collaboration with four laboratories for the development and validation of an in vitro cytotoxicity test protocol (Balls and Horner 1985). The test selected was based on assessing the inhibition of cell growth by means of measuring protein production using Kenacid Blue, a protein dye.

In this test the cells were grown in plates with 24 wells for 24 hours after which the test solutions were added. Following incubation for a further 72 hours protein production was measured. The test is less simple to use than the MIT-24 test but has a more objective end-point. Details are given in Appendix A.

3.4 INHIBITION OF CELL GROWTH (VITAL DYE METHOD)

This method of measuring inhibition of cell growth utilises the uptake of certain dyes, known as vital dyes, by living cells. The method was developed primarily by Borenfreund and Puerner (1985) and is based on the uptake of neutral red which is thought to enter living cells by non-ionic diffusion and accumulates in lysosomes. This dye is excluded from dead cells.

The cells are grown in tissue culture flasks until nearly confluent, harvested and aliquots grown in 24 well or microtitre tissue culture plates for 24 hours after which the test solutions are added. After incubation for a further 24 hours, the test solutions are removed and inhibition of cell growth measured.

This is a simple and fairly rapid test with an objective end-point which can be used in microtitre systems. Microtitre systems are valuable because they use less material and space, they increase the number of replicates and are easily automated resulting in a substantial saving in time and man-power. This test was later modified, in the light of our findings, to give the cells an increased period of exposure to the test solutions. Details of both the basic method and our modification to it are given in Appendix A.

3.5 CLONING EFFICIENCY

This technique is based on the ability of a cell to form a colony. In principle it is also measuring inhibition of cell growth except that the ability of individual cells to grow is considered rather than an effect on the growth of a culture. It has been used extensively in studies on

the potency of anti-cancer drugs but has also been used in a wide variety of other cytotoxicity studies. It is less simple to perform than some other test systems but is reputed to be very sensitive.

The cells are grown in tissue culture flasks until near confluency, harvested, diluted and aliquots grown in petri-dishes. Following 24 hours incubation, the medium is removed and replaced with test solutions, and incubated for a further 24 hours. The test solutions are then replaced by fresh medium, and the cells incubated for a further 7-9 days, after which the colonies are fixed, stained and counted. This test was later modified when applied to water samples and leachates from materials to give an increased period of exposure to the test solutions. Full details of the method are given in Appendix A.

SECTION 4 - VALIDATION OF TEST SYSTEMS

The term 'validation' can have a variety of meanings but in the context of this project it relates to the comparison of the test systems using a small group of pure compounds. In order to assess the comparative sensitivity and reproducibility of cytotoxicity assays it is important to make comparisons on the basis of well- defined substances. This is not possible with complex mixtures of largely unknown composition.

Thirteen chemicals were selected primarily for their suitability for in vitro cytotoxicity testing (Table 1). However, a number of the compounds had also been tested in studies by other groups and therefore provided a useful basis for inter-laboratory comparison.

The majority of the chosen compounds were direct acting but one (cyclophosphamide) was chosen because it requires activation to reactive intermediates by the metabolic enzymes of the cell.

Table 1 - Compounds chosen for validation of test systems

Catechol
p-Chloro-mercuribenzoic acid
Cycloheximide
Cyclophosphamide (requires metabolic activation)
Dichloroacetic acid
Dimethyl sulphoxide
2,4-Dinitrophenol
Methanol
Phenol
Sodium dodecyl sulphate
Trichloroacetic acid
Triton X-100
Vincristine sulphate

4.1 METHOD

The thirteen compounds were dissolved in MEM medium or dimethyl sulphoxide (DMSO). In cases where the latter solvent was used to initially dissolve the compound, the solution was diluted with MEM medium to ensure that the final concentration of DMSO in the test solution did not exceed 1%. The assays were compared by testing the compounds over a range of doses. In the MIT-24 assay, each of the 13 compounds was tested at 6 dose levels, ranging from 50 000 to 0.5 µg/ml. In the other three assays, doses were selected following dose ranging studies.

4.2 RESULTS

The results of the validation studies using the MIT-24 test are shown in Table 2.

The MIT-24 test was found to be simple to perform and reproducibility of results was good. However, we found no evidence that the 7-day end-point (inhibition of metabolism) could provide any more information than that obtained after 24 hours (effect on cell morphology) by visual assessment of the cultures.

The results of validation studies using the other tests are given in Table 3. The 50% inhibitory dose (ID50) for each compound was determined from a graph of dose versus percentage absorbance of control.

The data obtained from these studies indicate that in general all four assays tended to rank the 13 compounds tested in the same order for toxicity. However, some differences in the sensitivity of the assays were apparent, and the inhibition of cell growth (vital dye method) was evidently the least sensitive assay, with some compounds (notably cycloheximide, vincristine sulphate and dinitrophenol) showing much lower cytotoxicity in this test system. It seems probable that the major factor in the lower sensitivity of inhibition of cell growth (vital dye method) was the short exposure period. Whereas a 72-hour exposure period was employed for inhibition of cell growth (protein method) it was only 24 hours for inhibition of cell growth (vital dye method). The exposure period in the cloning assay was also 24 hours but in this case there was a further incubation period of up to 9 days in which toxic effects could become apparent. Moreover, although cloning efficiency has many similarities to inhibition of cell growth it remains a slightly different end-point and may be inherently more sensitive to certain types of toxic insult.

The importance of exposure period has also been noted by other workers. Riddell et al (1986), using very similar methods but a different cell line (3T3-L1), found the same effect with cycloheximide, vincristine sulphate and dinitrophenol. These compounds were less cytotoxic when a 24-hour exposure period was employed rather than a 72-hour exposure period. In fact, they reported that differences in exposure period were important for about half of the 50 compounds that they tested.

In order to determine if an extension of exposure time would improve the sensitivity of the cell growth assay (vital dye method) the exposure time was increased from 24 hours to 72 hours. The results of repeat testing of six of the selected compounds compared with the original assay are given in Table 3.

Table 2 - Results of MIT-24 tests

	Methanol*	Dimethyl sulphoxide *	DCA	TCA	Phenol	SDS|	DNP|	Triton| X-100	p-CMBA|	Catechol	Cyclo-heximide|	Vincristine sulphate	Cyclo-phosphamide|
50 000 ug/ml	+	+											
5 000 ug/ml	-	-	+	+									
500 ug/ml	-	-	-	-	+	+	+	+	+	+			-
50 ug/ml	-	-	-	-	-	-	+/-	+/-	+	+			-
5 ug/ml			-	-	-	-	-	-	-	+/-			-
0.5 ug/ml					-	-	-	-	-	-	+	+	-

TCA = Trichloroacetic acid
DCA = Dichloroacetic acid
SDS = Sodium dodecyl sulphate
DNP = 2,4-Dinitrophenol
p-CMBA = p-chloro-mercuribenzoic acid

Results are based on at least three experiments carried out on different days

\+ cytotoxic
+/- some evidence of toxicity
\- no evidence of toxicity

| made up in DMSO though the solvent concentration did not exceed 1% in the test solutions

* Dimethyl sulphoxide and methanol were tested at 5%, 0.5% (v/v) etc which approximately correspond to the dose levels given in the table

Table 3 - Results of inhibition of cell growth (protein and vital dye methods) and cloning efficiency tests using V79/4 cells

Compound	Inhibition of cell growth (protein method)	Inhibition of cell growth (vital dye method)	Cloning efficiency	Inhibition of cell growth (72 hours exposure)
Catechol	21 1 1	20 1 1	7 1 2	3.9 1 0.3
p-Chloromercuribenzoic acid	9.4 1 3.0	8.0 1 0.8	7.7 1 0.8	
Cycloheximide	0.19 1 0.05	6.1 1 1.3	0.31 1 0.07	0.028 1 0.004
Cyclophosphamide	>2000	>2000	>2000	
Dichloroacetic acid	1820 1 220	2770 1 200	1390 1 70	
Dimethyl sulphoxide	2.4% 1 0.3%	3.4% 1 0.2%	2.7% 1 0.4%	2.1% 1 0.2
2,4-Dinitrophenol	53 1 3	586 1 215	268 1 92	33.5 1 4.0
Methanol	6.7% 1 0.2%	6.2% 1 0.5%	5.8% 1 0.1%	
Phenol	362 1 48	737 1 56	520 1 130	
Sodium dodecyl sulphate	83 1 2	69 1 7	89 1 2	78.9 1 7.4
Trichloroacetic acid	1550 1 60	2020 1 180	1570 1 160	
Triton X-100	16 1 2	51 1 6	34 1 8	
Vincristine sulphate	0.056 1 0.019	0.48 1 0.08	0.016 1 0.004	0.065 1 0.019

Data are expressed in 5g/ml except dimethyl sulphoxide and methanol, which are expressed as percentage volume:volume.

Results are based on at least three experiments carried out on different days and are expressed as the mean 1 standard error of the mean.

All compounds were dissolved in medium except sodium dodecyl sulphate, 2,4-dinitrophenol, Triton X-100, p-chloromercuribenzoic acid, cycloheximide and cyclophosphamide which were originally made up in dimethyl sulphoxide, though the solvent concentration did not exceed 1% in the test solutions.

NB: The data given for the inhibition of cell growth (protein method) are slightly different from those given in a previous progress report (Water Research Centre 1986). This is due to minor changes in the way in which the results were calculated

The data show that an extension of exposure time resulted in an increase in the sensitivity of the assay to the compounds tested. Sodium dodecyl sulphate was found to be an exception, and a decrease in cytotoxicity was demonstrated with increasing time of exposure from 24 to 72 hours.

4.3 DISCUSSION

When examining a range of cytotoxicity assays in order to choose one for a particular application, it is important to consider comparability, reproducibility, ease of use and sensitivity. In addition, the success of any cytotoxicity assay may be dependent on its operation by experienced or well-trained staff.

Each of the assays investigated in this study were relatively easy to perform and reproducible but the cloning efficiency assay proved to be more intensive in terms of time needed to carry out the test. All four assays tended to rank compounds in the same order for toxicity. Inhibition of cell growth (vital dye method), using an exposure period of 24 hours, was the least sensitive assay, particularly to the effects of cycloheximide, dinitrophenol and vincristine sulphate. However, an increase in the period of exposure to 72 hours resulted in an increased sensitivity of the assay to these compounds.

Of the four assays, results from the MIT-24 test are the most difficult to compare as the principal end-point, visual assessment of cells, is subjective in nature and hence the test is not directly quantifiable. It was therefore decided to use relatively clear-cut assessments (only three grades of effect) and to test the compounds at wider dose ranges. However, the data from this test system still compare quite well with the directly quantifiable test methods. Moreover, in vitro cytotoxicity tests of the type currently being investigated are only likely to be used as crude indicators of cytotoxic potency and hence very accurate quantification of results may not be particularly useful.

Reproducibility is a very important requirement for an in vitro cytotoxicity assay. The reproducibility of results in this study was good and, of equal importance, the data also compare favourably with

those published by other workers (Knox et al 1986, Stark et al 1986). It appears that groups of compounds tested in similar assays but in different laboratories, tend to give similar results without rigorous attention to exact duplication of methods. In terms of ease of operation, visual assessment of cultures is probably the simplest method although its subjectivity is a drawback. However, microscopic examination of cells can easily be included as an additional end-point when measuring inhibition of cell growth. Of the other assays that we studied, the cloning method was more costly in terms of time and materials compared to the inhibition of cell growth methods. There is little to choose between the two methods for inhibition of cell growth, but fewer washing steps render the vital dye method more applicable for use in 96-well plates than the protein method. Testing in 96-well plates has perhaps slight advantages compared to testing in 24-well plates as more data points are generated and less reagents are required. When testing pure compounds, the method which we would favour for future use is measurement of inhibition of cell growth by the vital dye method, using a 72-hour rather than 24-hour exposure period, and also including visual assessment of cultures after 24 hours.

The sensitivity of a test system does not appear to be particularly important when testing groups of pure compounds as they tend to be ranked in a similar order even by different assays. However, since sensitivity may be a more significant factor when testing complex mixtures it was decided to use the more sensitive assays (namely cell cloning and 72-hour vital dye method) in evaluating the application of cytotoxicity assays to water samples and leachates from materials.

SECTION 5 - APPLICATION OF CYTOTOXICITY ASSAYS

5.1 A COMPARISON OF THE CYTOTOXICITY OF N-HALOALKANES

One use of in vitro cytotoxicity assays is to assess the toxicity of homologous series of chemicals in order to test theoretical structure activity relationships. The benefits of such an approach are twofold.

First there are many groups of compounds such as some of those observed as micropollutants in drinking water for which toxicity data exist for only a few members. In this case it would be of great value to make an assessment of toxicity of all the compounds using structure activity. However, structure activity relationships breakdown at some point and confidence would be greatly increased by knowing when this breakdown occurs. Secondly, such an approach would enable decisions to be made as to what animal testing might be required and to reduce the amount of testing carried out. However, to be of wide practical value cytotoxicity assays need to be used which can give results with compounds which are not obvious candidates for testing in these assays. Such compounds may be of high volatility or of low aqueous solubility or miscibility.

The halo-alkanes are one such group of compounds which are of interest in the context of water contaminants. There is little toxicological data available on them and they would not be obvious candidates for testing in cytotoxicity assays because they are not a family of new chemicals being examined by industry.

The chloroalkanes from C4-C10 and bromoalkanes from C4-C6 were chosen for testing. The C1-C3 homologues were too volatile to test, ie, they were gases at room temperature.

Initial range-finding was carried out in the MIT test using 96-well plates. Each compound was tested at 8 different dose levels using five wells for each dose level, which allowed two compounds to be tested per plate.

The dilutions were made up at double strength and then serially diluted to give the final dose range. The test dilutions (100 µl) were then pipetted into appropriate wells. The control wells received medium only. Each well was then sealed with 100 µl of sterile mineral oil and the plates sealed with self-adhesive plastic sheets before being incubated for 24 hours at 37 °C. After 24 hours the plates were examined for morphological abnormalities and cell death using light

microscopy. Comparative cytotoxicity testing was then carried out in the FRAME test. Each compound was tested at 6 dose levels on at least two separate occasions. Details of the assay methods are given in Appendix A.

The bromoalkanes proved to be extremely immiscible with the medium. The C4-C10 members of this group all formed an emulsion on mixing with the medium which separated into two layers on standing. Higher members of the series were unable to be tested even when a carrier solvent was used to aid solution. The cytotoxicity of those bromoalkanes tested was extremely variable probably due to variations in solubility.

The chloroalkanes also presented some difficulties with solubility and chlorobutane gave wide variations in dose response at or around the ID50. There was no smooth change in toxicity with increasing number of carbon atoms (Table 4).

The results of this study showed the haloalkanes to be of low in vitro cytotoxicity but it increased slightly with increasing number of carbon atoms. This dose response trend peaked at chloroheptane. There was considerable variation between experimental results. However, this trend was consistent in all experiments which suggests that this was a true characteristic of the group and not the result of experimental error.

Table 4 - Calculated ID50 values for the chloroalkanes investigated using the FRAME assay

Chloroalkane	ID50 (mg/ml)	1 SEM
Chloropentane	6.1	0.2
Chlorohexane	6.7	0.2
Chloroheptane	0.9	0.1
Chlorooctane	2.8	0.2
Chlorodecane	2.7	0.2

It is possible that this phenomenon was due to a detergent effect. Therefore the compounds could be inserting into the plasma membrane and disrupting the membrane at the critical concentration for micelle formation.

This cytotoxicity test was of limited value in assessing *in vitro* cytotoxicity of the haloalkanes. Particular problems were encountered with the volatility of the compounds and their immiscibility with aqueous medium. These difficulties would be applicable to all *in vitro* cytotoxicity tests currently being investigated.

The current state of the art limits the use of cytotoxicity assays to compounds which are of low volatility, miscible with or soluble in water and which do not require metabolic activation to reactive intermediates.

5.2 WATER SAMPLES

5.2.1 Treated water

Previous experience in our laboratory has demonstrated that bacterial mutagenicity assays used with water samples require pre-concentration of the samples in order to obtain unequivocal results (Forster *et al* 1983). Studies by Fauris *et al* (1985), using an assay which did not require sample pre-concentration, investigated the cytotoxicity of water samples taken from a treatment plant, with respect to the type of treatment, dosage used and treatment stage. The assay, which used inhibition of RNA synthesis as an endpoint, demonstrated good sensitivity, and was reported to detect cytotoxic compounds both present in raw water, and those produced as a result of chlorination. In the light of these encouraging results, we decided to investigate the use of our cytotoxicity assays for their application to water samples. Our preliminary studies using drinking water samples gave no evidence of cytotoxicity, however, in order to confirm these results, we investigated the effects of treated and raw water samples in more detail.

A group of five samples was taken from a pilot scale water treatment plant which treats lowland river water by alum coagulation followed by rapid gravity sand filtration and finally granular activated carbon (GAC) filtration. Samples iii) and v) were chlorinated to give a free residual of 0.5 mg/l chlorine after a contact time of 30 minutes. The samples taken were:

i) Raw water

ii) After coagulation/rapid gravity sand filtration

iii) After coagulation/rapid gravity sand filtration and hand chlorinated

iv) After coagulation/rapid gravity sand/GAC filtration

v) After coagulation/rapid gravity sand/GAC filtration and hand chlorinated

The samples were collected from the pilot plant in glass bottles and returned to the laboratory, where they were filtered through Whatman GF/C filters and sterilised using 0.22 µm Millipore filters.

Tissue culture medium was prepared by diluting a ten-times liquid concentrate of MEM medium with the water sample to be tested. MEM concentrate diluted with double distilled water was used as a control. The media were then supplemented with 10% foetal calf serum, 20 mM HEPES buffer, 0.22% sodium bicarbonate solution, 100 units/ml of penicillin/streptomycin and 2 mM L-glutamine and the pH was maintained between 7.2 and 7.4.

The samples were tested for inhibition of cell growth by the vital dye method (72 hour incubation), but a visual assessment of cell density and cell morphology was made after 24 hours of incubation. The samples were also evaluated for effects on cloning efficiency, using an 8 to 10 day exposure period. Each sample was tested on two separate occasions

(denoted a and b). The results obtained are given in Table 5, in which values for inhibition of cell growth and cloning efficiency are given as percentage of control.

These results indicate that the samples of water from various stages in water treatment were not cytotoxic in any of the assays. It may be the case that the cytotoxicity test used by Fauris et al (1985) is more sensitive but equally the method is more complex to perform and requires expensive, specialist equipment including a scintillation counter. Moreover, since this gives little information with regard to possible hazards to consumers in comparison with that obtained from others such as genotoxicity assays, it may be argued that benefits of such a test do not merit the effort involved.

Table 5 - Results with treated water samples

Sample	24-hour morphology		Inhibition of cell growth		Cloning efficiency	
	a	b	a	b	a	b
(i)	-	-	96	98	95	118
(ii)	-	-	96	94	96	135
(iii)	-	-	82	95	93	110
(iv)	-	-	88	99	109	118
(v)	-	-	100	97	111	111

- No evidence of cytotoxicity

5.2.2 Effluents

Since no cytotoxicity was detected by our cytotoxicity assays using samples of treated water, in contrast to the positive cytotoxicity results obtained by Fauris et al (1985), studies using effluents were initiated, and the potential application of the assays for monitoring changes in any observed activity investigated.

Following preliminary investigations, six sampling sites designated Q, R, S, T, U and V were chosen for the study. These comprised two river water samples (Q and T), three samples of domestic sewage effluent (R, U and V) and one effluent from a paper mill (S).

On the day of sampling, samples were passed through a Whatman GF/C filter in order to remove particulate matter, then sterilised by passing through a 0.22 µm Millipore filter. Each sample was then tested after both one week and six weeks of laboratory storage at 4 °C. Tissue culture medium was prepared by dilution of ten-times liquid concentrate MEM medium with the water sample to be tested. The control was MEM concentrate diluted with double distilled water. The media were supplemented with 10% foetal calf serum, HEPES and bicarbonate buffer, L-glutamine and penicillin- streptomycin.

The three types of water sample (river water, domestic sewage effluent and industrial effluent) were tested for cytotoxicity in the inhibition of cell growth (24 hour vital dye method) and cloning efficiency assays. In order to ascertain whether laboratory storage of samples altered their cytotoxic activity, we also investigated the effect of sample storage on cytotoxicity in these assays. Full experimental details of these assays are given in Appendix A, with the following modifications:

i) In the vital dye method, volumes of test sample in each well were 0.2 ml rather than 0.1 ml.

ii) For cloning efficiency, cells were exposed to water samples for both 24 hours and 8-10 days. The 24 hour exposure period allowed direct comparison with the vital dye method.

No evidence of cytotoxicity was observed for either of the river water samples (Q and T) when tested for inhibition of cell growth by the vital dye method (Figure 1). Similarly, these two samples gave little or no evidence of cytotoxicity in the cloning efficiency test (Figure 2). However, some evidence of cytotoxicity was observed in the cloning efficiency test for the other samples following 24 hours exposure, with

exposure of cells for 8-10 days resulting in increased cytotoxicity. Under these conditions of prolonged exposure, the samples of domestic sewage effluents gave good evidence of cytotoxic activity, although sample R was more toxic than samples U and V, and potent cytotoxicity was demonstrated by the industrial effluent, sample S (Figure 3). Therefore, a direct comparison of these two methods would indicate that cloning efficiency shows greater sensitivity in its application to the testing of such effluent samples.

Laboratory storage of water samples for six weeks was shown to have no effect on cytotoxicity using the vital dye method. However, in the cloning efficiency test using 24 hours exposure time, one water sample (S) was highly cytotoxic after storage for both one week and six weeks (Figure 2). Samples R and U also appeared to become more cytotoxic after storage for six weeks. Prolonged storage appeared to have little effect on cytotoxicity in the cloning efficiency assay using an exposure period of 8-10 days (Figure 3).

Following discussions with a water utility, an industrial effluent was identified that could be of interest to the project. This particular site discharges relatively large quantities of material, although the complex nature of the effluent renders it difficult to evaluate by analytical methods. Bioassay techniques might, however, provide some useful information.

Two test samples, designated X and Y, were tested. Sample X was the final effluent from the sewage treatment works that receives the discharge. Sample Y was the effluent from the industrial site.

Each sample was passed through a Whatman GF/C filter to remove particulate matter, and filter-sterilised using a 0.22 µm Millipore filter. The samples were tested for inhibition of cell growth by the vital dye method (with 72 hour incubation period and evaluation of morphology after 24 hours) and for effects on cloning efficiency. Full details of the methods are given in Appendix A. Two sets of controls were used, normal MEM medium and concentrated medium diluted with double distilled water.

One set of samples was taken but these were tested on up to four occasions (denoted a to d). Results for the three end-points are presented in Tables 6-8. Unless stated the test samples were compared to the distilled water control.

Table 6 - Effects on morphology after 24 hours

SAMPLE	a	b	c	d
X	-	-	-	-
Y	ND	ND	+	+

- No cytotoxicity observed
+/- Some evidence of cytotoxicity
+ Cytotoxic
ND Not done

Table 7 - Results of inhibition of cell growth (percentage of control)

SAMPLE	a	b	c	d
X	104	88~	105	106~
Y	ND	ND	0	0~

~ Compared to ordinary MEM control due to variation with the distilled water control.
ND Not done

There was no evidence for <u>in vitro</u> cytotoxicity of Sample X in any of the assays. However, Sample Y was markedly cytotoxic in all tests. The cytotoxic components in the effluent may therefore have been modified, degraded or diluted by the sewage treatment process.

Table 8 - Results of cloning efficiency (percentage of control)

SAMPLE	a	b	c	d
X	93	102	96	100
Y	ND	ND	0	0

ND Not done

Further experiments were therefore performed to assess the effect of dilution on the cytotoxicity of the effluent, using inhibition of cell growth (vital dye method) and cloning efficiency methods.

Samples were again collected, designated X′ and Y′ as before and tested on two occasions, a and b. Sample and media preparation, and assay methodology followed the protocol previously outlined. The percentage concentration of water sample used in the final media was tested at 6 doses for both samples.

Table 9 - Results of inhibition of cell growth (percentage of control)

Dose (proportion of effluent in test sample)	Total percentage of control			
	Sample Y′		Sample X′	
	a	b	a	b
100%	5	42	72	89
80%	8	51	82	93
60%	21	62	101	93
40%	45	71	106	94
20%	77	90	105	91
10%	94	83	102	80

Table 10 - Results of cloning efficiency (percentage of control)

Dose (proportion of effluent in test sample)	Total percentage of control			
	Sample Y'		Sample X'	
	a	b	a	b
100%	0	0	94	98
80%	0	0	101	96
60%	0	0	106	98
40%	0	0	93	106
20%	22	76	108	99
10%	87	85	115	101

These results indicate again that sample X', the sewage effluent, was not cytotoxic before or after dilution whilst sample Y' showed cytotoxicity before dilution in both assays. However, it appears that the cytotoxic activity observed with sample Y' may be reduced or eliminated by the effect of dilution. In both assays, a 1:10 dilution of sample Y' (ie 10% sample concentration) demonstrated a marked reduction in cytotoxicity. It is possible, therefore, that the observed cytotoxicity of the industrial effluent would be removed by dilution in the receiving works.

Although studies using an industrial effluent have shown positive cytotoxicity results in our assays these results do not indicate a hazard to man but merely demonstrate the presence of a biologically active compound or compounds. In addition, negative cytotoxicity results, such as those given by the effluent from the treatment works, do not indicate that no hazard exists, but may simply indicate that the compound or compounds are present at concentrations below the detection limits of the assays, the experiments with dilution providing some support for this hypothesis.

5.3 MATERIALS

There is a requirement to assess the potential of leachates from materials used in contact with potable water to pose a hazard to the health of consumers. However, the chemicals which leach are often poorly characterised, and toxicological information may be defective in one or more aspects. In addition, reaction products from the manufacturing process may be present. Those authorities making an assessment of the suitability of such materials are faced with a dilemma since adequate analytical characterisation of the leachate may be extremely difficult and expensive. Under these circumstances the use of in vitro test systems to screen for the presence of biologically active compounds which would require further investigation is an alternative proposition.

At present in the UK, a voluntary scheme for the testing of non-metallic materials for use in contact with potable water (United Kingdom Water Fittings Byelaws Scheme 1985) includes a cytotoxicity test in African Green Monkey kidney (VERO) cells. It is based on evaluation of cell morphology after 24 hours of exposure. A failure in this test does not mean that a toxic hazard to consumers exists but it does indicate the need for further investigations if the material is to be used in contact with potable water.

In our studies, we originally intended to include the Water Byelaws Test but we experienced difficulty in obtaining a commercial supply of VERO cells, over a period of just under a year. Consequently the experiments were carried out with the V79 cell line, that had been used for all previous work on this project.

5.3.1 Rubbers

Synthetic rubber materials are widely used in various types of water fittings and many are employed in preference to natural rubber. The change to synthetic materials was based on such factors as relative costs, availability of raw materials and mechanical properties, and the

increasing awareness that natural materials underwent biodeterioration and encouraged the growth of coliform organisms. During the seventies the widespread recognition that materials could impair water quality in a number of ways (Burman and Colbourne 1976, 1977 and 1979; Taylor 1973) led to a systematic assessment of the effects of materials and to the development of processes for the testing of materials.

Elastomeric materials are used in the manufacture of numerous components of water fittings, eg, tap washers, gaskets, "O" rings etc, and form the main type of material used to make effective joints in water distribution mains. There is documented evidence of microbial degradation of some of these (Hutchinson and Ridgway 1977) and elastomeric materials are the most common materials to give positive results in the in vitro cytotoxicity test used by the UK Water Fittings Byelaws Scheme. Rubbers were therefore chosen as suitable materials for evaluation of the cytotoxicity assays in this project. The following six samples of different rubbers were obtained from WRc Swindon:

Sample 1 Natural rubber
Sample 2 Natural/Styrene-butadiene blend
Sample 3 Styrene-butadiene rubber
Sample 4 Ethylene propylene diene methyl rubber/Sulphur cured
Sample 5 Ethylene propylene diene methyl rubber/Peroxide cured
Sample 6 Nitrile rubber

From experience gained in the testing of chemicals and water samples, three endpoints were chosen for study:

i) Cell morphology after 24 hours

ii) Measurement of inhibition of cell growth by the vital dye (neutral red) method at 72 hours and

iii) Cloning efficiency using an 8-10 day exposure period.

Preparation of the samples and testing was carried out in compliance with recommendations of the UK Water Fittings Byelaws Scheme (BS6920 1988).

Each sample of rubber was cut to size (15 600 mm^2 ± 10%, with a thickness of 2 mm, length 130 mm and width 60 mm). The rubber sample was immersed in 1 litre of tapwater in a sealed jar, which was stored in the dark for 24 hours. The water collected from this procedure was designated Leachate I. The rubber was then reimmersed in a fresh litre of water, and stored for another 24 hours, providing Leachate II. A further 24 hour leachate sample (III) and a 72 hour leachate sample (IV) were also collected. A blank sample consisted of one litre of tapwater that was stored in a sealed jar, in the dark but in the absence of any material, throughout the duration of the experiment (144 hours). Each leachate was sterilised by passage through a 0.22 µm Millipore filter, then stored for short periods at 4 °C until required for preparation of medium.

Tissue culture medium was prepared by diluting a ten-times liquid concentrate of MEM medium with the leachate sample to be tested. MEM concentrate diluted with double distilled water was used as a negative control. The media were supplemented with 10% foetal calf serum, 20 mM HEPES buffer, 0.22% sodium bicarbonate solution, 100 units/ml of penicillin/streptomycin and 2 mM L-glutamine and the pH was adjusted to between 7.2 and 7.4 before testing.

The samples were tested for inhibition of cell growth by the vital dye method (72 hour incubation). Included as part of this assay was visual assessment of the cultures for effects on cell density and cell morphology after 24 hours of incubation. The samples were also evaluated for effects on cloning efficiency. Details of the cytotoxicity assays are given in Appendix A.

Results are based on up to three experiments carried out on different days. Experiments A, B and C represent three separate collections of four leachate samples from each rubber material. Medium prepared from

each leachate was then tested on two occasions, denoted a and b. The results of these experiments are given in Appendix B, Tables 11-28. Results for inhibition of cell growth and cloning efficiency are presented as a percentage of the tapwater control (blank).

(a) Discussion

The results of the assays used in these studies give evidence that cytotoxic compounds leach from rubbers 3, 4, and 6, although the 24 hour morphology assay was shown to be less sensitive to this activity than the inhibition of cell growth and cloning efficiency assays. For example, using the cell morphology assay, cytotoxicity was only observed with leachates I and IV of rubber 3, leachates I and IV of rubber 4 and leachate IV of rubber 6. However, the other two assays were less discriminating, and indicated all leachates of rubbers 3, 4 and 6 to be cytotoxic.

Some evidence of cytotoxicity was observed for leachates I and IV of rubber 1, using the cell morphology assay. However, this result was only demonstrated on one occasion, and no evidence of cytotoxicity was given for any leachates of this rubber using the inhibition of cell growth (vital dye method) or cloning efficiency assays. There was no evidence of cytotoxicity with leachates from rubbers 2 and 5 in any of the test systems used.

The 72 hour leachate (Leachate IV) samples were found to be the samples giving the most evidence of cytotoxicity for rubbers 3, 4 and 6 in the cell morphology assay. This is particularly interesting since the Water Byelaws Testing Scheme, which also employs cell morphology as an endpoint, only tests sequential 24 hour leachate samples, and does not include a 72 hour leachate sample.

The main conclusions from this work concerning the application of our cytotoxicity assays for testing rubber materials are:

1. Under the conditions of the tests, the cloning assay would appear to be more sensitive than inhibition of cell growth which is in turn more sensitive than microscopic assessment of the cells after 24 hours of exposure. This supports our earlier findings on the importance of exposure period with respect to sensitivity.

2. The variation of results with the quantitative tests is such that figures of between 80% and 120% of control are typical negative values. Only when values are much lower, for example 50% of control or less, can there be confidence in the conclusion of a cytotoxic effect. However, if dose-response experiments were performed then more confidence might be attained in interpretation of results.

3. The inclusion of a distilled water control is important to monitor the response of the blank and the overall performance of the test. The results given for the distilled water show the variability between that and the blank. With one or two exceptions the blank and distilled water values were similar.

4. Cytotoxicity assays which are more sophisticated than that used in the Water Byelaws scheme can still only be used as a very crude screen for the toxicity of leachates from rubbers.

5. These results confirmed that cytotoxic activity was observed in the leachates from styrene-butadiene rubber, ethylene propylene diene methyl rubber/sulphur cured and nitrile rubber.

5.3.2 Other materials

Cytotoxicity tests have been used for some time to screen the toxicity of plastics for medical use. Since the same problems are encountered in evaluating the possible hazards from water pipes and pipe lining materials as outlined in Section 5.3, in vitro cytotoxicity assays may provide an indication of whether biologically active compounds are present in leachate from plastics used in this way. In addition

previous studies of leachates from uPVC plastics gave positive results in cytotoxicity assays, although the active principles were not identified (Wilsnack 1973, 1976).

In the present studies leachates from glass reinforced plastic (GRP), cPVC and blue polyethylene MDPE pipes (manufactured by Upnor, BP, Stewart and Lloyds, and Polypin, which were designated blue polyethylene I, II, III and IV respectively) were examined in two cytotoxicity assays. In addition, the sensitivity of the assay systems to compounds known to leach from various types of plastic pipe and relining materials was assessed using pure compounds.

(a) Methods

Each sample of pipe was cut to give a standard surface area (15 600 mm^2 ± 10%). The sample was immersed in 1 litre of tapwater, and stored in a sealed jar in the dark for 24 hours. The water collected from this procedure was designated Leachate I. Further leachate samples were collected according to the method previously outlined for rubbers (Section 5.3.1), and designated II, III and IV as before. Each leachate was sterilised using a 0.22 µm Millipore filter before using to dilute a ten-times liquid concentrate of MEM medium. The media were supplemented with 10% foetal calf serum, 20 mM HEPES buffer, 0.22% sodium bicarbonate solution, 100 units/ml of penicillin/streptomycin and 2 mM L-glutamine and the pH was adjusted to between 7.2 and 7.4. MEM concentrate diluted with double distilled water was also included as a negative control.

The samples were tested in the inhibition of cell growth (72 hour vital dye method), which included visual assessment after 24 hours incubation, and in the cloning efficiency assay using an 8-10 day exposure period. Details of the assays are given in Appendix A.

In addition to the testing of leachates, the cytotoxicity of the following compounds, which are known to leach from plastic pipes and epoxy resins, was also tested: Bisphenol A diglycidyl ether and Bisphenol F diglycidyl ether, which are components of epoxy resins;

dimethyltin dichloride, dibutyltin dichloride and lead(II) chloride, used as stabilisers in uPVC and cPVC pipes; Santanox (6,6'-ditertiarybutyl-4,4/-thiodi-m-cresol) and Irganox (pentaerythrityl-tetrakis[3-(3,5-ditertiarybutyl-4-hydrophenyl)propionate]), which are antioxidants in polyethylene pipes; and trimethylhexamethylene diamine, used as a hardener in epoxy resins.

Following initial dose range-finding studies, the compounds were tested at 6 dose levels using the inhibition of cell growth (72 hours vital dye method) and cloning efficiency assays. Details of the method are given in Appendix A. A contract was also placed with Thames Water Services to test these compounds in the Water Byelaws scheme, which uses VERO cells.

(b) Results

The results of these studies are given in Appendix B, Tables 29-46. There was no evidence of cytotoxicity for the leachates of cPVC, Blue polyethylene II and blue polyethylene III. GRP showed some evidence of cytotoxicity in the cell morphology assay, but these results were not reproducible, and no cytotoxicity was observed for leachates of this pipe sample in the other assays.

Blue polyethylene I leachates showed no cytotoxicity in the cell morphology and cell cloning assays. However, this compound did appear to be cytotoxic on one occasion in the vital dye assay, but since the distilled water sample also gave cytotoxic results on this occasion, these results may be of little significance.

Cytotoxicity was observed in the 72 hour leachates of blue polyethylene IV, with each assay. However, it appeared that this toxicity occurred only following a leaching time of greater than 24 hours. In addition, the medium in the assays was observed to be particularly alkaline following 8 days incubation in the cloning assay. Therefore it is unclear whether the cytotoxic effects observed are due to the toxic nature of the leachate or to interference with the buffering system of the medium.

Compounds known to exist in the leachates of plastic pipes and epoxy resins were tested, and the 50% inhibitory dose (ID50) for each compound was determined from a graph of dose versus percentage control. Results are given in Appendix B, Table 47.

However, a number of problems were experienced in obtaining these results. For example, although inhalation or skin contact with most of the compounds tested should be avoided, it is difficult to perform the assays (in particular the cell cloning assay) in a confined space such as in a glove box. Secondly, some compounds (for example lead(II) chloride and trimethylhexamethylene diamine) may alter the pH of the medium to a level beyond the physiological range for cell growth. Therefore it is unclear whether a true ID 50 can be calculated from the results obtained, or whether this merely represents the concentration leading to a pH value intolerable for cell growth. In addition, the results of these compounds had a low level of reproducibility, similar to those of the plastic pipe leachates.

During our studies, it became evident that the application of our cytotoxicity assays for the testing of materials was subject to difficulties resulting from the unpredictable behaviour of the samples during testing. For example, problems were experienced in attaining and maintaining pH values amenable to cell growth with both the leachates and pure compounds tested. Therefore, although it is possible to obtain an indication of the cytotoxicity of plastic pipe leachates and their components using these assays, the potential problems experienced in producing these results may outweigh their ultimate value.

The results of the Vero cell studies carried out by Thames Water Services are given in Appendix C. The ID 50 is a calculation of the dose of test compound which inhibits cell growth by 50%. Since the results obtained in the WBAS test are subjective, and are not quantifiable, an ID 50 value cannot be obtained. Instead, the results are expressed using the terms of non-toxic (the highest dose at which no toxicity is observed), toxic (the lowest dose resulting in an observable toxic response) and the dose which results in complete cell death. The

determination of the values which fall into these categories are therefore dependent on the dose levels chosen for testing. In addition, if a large margin between dose levels is used in the WBAS test, an estimation of the approximate ID 50 value is even more difficult. We have compared the concentration determined as toxic in the WBAS test with the ID 50 values obtained in the inhibition of cell growth assay (72 hour vital dye method) (Appendix B, Table 48).

The two tests generally demonstrated similar sensitivity to the toxicity of the compounds tested, with particularly good comparison of the values obtained for bisphenol A diglycidyl ether, bisphenol F diglycidyl ether, dibutyltin dichloride, lead (II) chloride and Santanox. In each of these cases, the toxic values of the WBAS test are seen to be slightly lower than the ID 50 value. This might be expected, since the toxic classification represents the lowest concentration at which toxicity is observed, and is therefore likely to be of lower comparative value than the ID 50.

The ID 50 values obtained for dimethyltin dichloride and Irganox 1010 are shown to be significantly lower than the values stated as non-toxic in the WBAS test, indicating the inhibition of cell growth assay to be of greater sensitivity to these compounds. The WBAS test demonstrated greater sensitivity to only one compound, namely trimethylhexamethylene diamine. This was the compound with which we experienced problems in maintaining pH values amenable to cell growth in our assay.

In addition to testing after 24 hours exposure, the compounds were also tested in the WBAS test using an extended exposure period of 48-72 hours. However, unlike those results obtained in our studies, the WBAS test did not detect any resulting increase in toxicity. This might have been due to large differences between the dose levels used or possibly differences in sensitivity between the two test systems.

These results demonstrate the ability of both tests to detect the toxicity of materials known to leach into water from plastic pipes and epoxy resins to the cells used. However, the ID 50's and the

concentrations determined as toxic for most compounds in the tests far exceed those likely to exist in leachates in practice. Therefore, in order to test the toxicity of such compounds in leachate samples, considerable concentration of the leachate would first be necessary. However, the procedures available for concentrating mixtures of compounds to the extent necessary for testing may result in some loss of the compound of interest, or its interaction with other compounds present. The toxicity of the compound of interest may also be masked by simultaneous concentration of other compounds of greater toxicity. In addition to such technical problems, the expense incurred from concentration procedures may outweigh the benefit of the results obtained. Therefore, the value of these tests for the screening of leachates of materials needs to be assessed in order to determine whether they merit the necessary expenditure.

5.4 ALGAL TOXINS

5.4.1 Introduction

Algal blooms appear to have become increasingly prevalent in lakes and reservoirs throughout the UK. Many blooms in which blue-green algae are dominant may produce toxic compounds, many of which are not well characterised nor amenable to chemical analysis. This is so for the toxins produced by the most common species, Microcystis aeruginosa. The method used to assess whether Microcystis blooms are toxin-producing is to inject extracts of freeze dried material into mice. In vitro cytotoxicity assays would be useful in studying the toxicity of such blooms since tests could be carried out in the laboratories of water utilities. In addition in vitro assays would provide a replacement for a technique which requires the use of laboratory animals.

This part of the project was linked to a programme of research funded by WRc at the University of Dundee on the topic of Microcystis aeruginosa toxicity.

5.4.2 Extraction of toxin

Freeze dried samples of pure strains of toxin and non-toxin producing Microcystis aeruginosa were supplied by Dr G Codd of Dundee University. Freeze dried material (0.5 g) was extracted into 100 ml 5% n-butanol/20% methanol in water. This was stirred for 2 hours on ice and spun at 20 000 g for 1 hour. The supernatant was poured off and retained. The procedure was repeated once using the pellet, and the two supernatants pooled.

The extract was filtered through a Whatman GF/C filter, rotary evaporated to about 10 ml and centrifuged at 1500 rpm for 20 minutes. The supernatant was filter sterilised with a 0.2 micron filter.

5.4.3 Cytotoxicity assays

These toxins are hepatotoxic in vivo causing massive disruption of hepatocytes. The mechanism of, and the reason for this organ specific action are not clear but when we started this work there was nothing to suggest that the toxin would be specific for hepatocytes, and so we decided to use our cell lines based on CHO cells. There was, however, a suggestion that the toxins may have an effect on cell membranes, and this was investigated at the Robens Institute using fluorescence polarography (see Section 5.5) but the technique used to measure changes in membrane fluidity was unsuccessful.

The toxin and non-toxin producing strains were tested for comparative cytotoxicity in the MIT test and FRAME test. The ID50 of the two strains was similar in the MIT test with the non-toxin producing strain being considerably more toxic than might have been expected. This could be due to a number of factors including the presence of other substances in the extract, unrelated to toxin production, which were able to inhibit metabolism of the cells. However, in the FRAME test there was a clear differentiation between the two strains with ID50s of 12 mg/ml for the non-toxin producing strain and 2 mg/ml to 5 mg/ml for the toxin producing strain.

There was some concern that the extraction procedure was extracting substances which would have an inhibitory effect on cell growth, particularly in the MIT test, although not associated with toxin production. In addition it was possible that the presence of serum in the medium may have been reducing the toxicity of the algal toxin. Therefore additional studies were undertaken.

The measurement of lactate dehydrogenase leakage into the medium is considered a sensitive means of measuring toxicity where membrane damage has occurred. As it was suspected that algal toxins do affect cell membranes the technique was applied to this investigation. This was carried out in our laboratory as part of a CASE award studentship with the University of Dundee by Mr I Priestley which was funded by WRc not the Department. The results of this work are presented here since they are an extension of the work carried out on behalf of the Department in this contract.

Preparation of extracts of toxic and non-toxin producing strains was carried out using the methods of Brooks and Codd (1986) and Poon et al (1987). These methods gave an improved extraction of toxin.

Cytotoxicity was measured in the FRAME test, inhibition of cell growth (vital dye method) and by measuring LDH leakage from the cells. The assays were, however, modified so that some cells were exposed to toxin in the absence of serum for the first 3 hours.

The results of all three assays showed little effect of toxin when incubated in the presence of serum throughout. However, there was a clear toxic response observed when a serum-free incubation step was included (Figures 4-6). In addition, the dose of extract giving 50% inhibition of cell growth (ID50) was comparable to the LD50 in mice except for inhibition of cell growth (vital dye method) which was less sensitive than the other two assays.

These data indicate that in the absence of a specific analytical technique for Microcystis toxin an in vitro cytotoxicity assay could be used to screen blooms for toxicity and so avoid the use of intact animals.

5.5 INVESTIGATION OF A RAPID CYTOTOXICITY ASSAY

There are a number of possible applications of cytotoxicity assays in the water industry for which an answer in a few hours would be either desirable, or in some cases, essential. Examples of such applications are in the monitoring of effluents or the use of such assays as part of a battery of monitors for protection of surface water intakes from chemical spills.

A contract was placed with the Robens Institute of the University of Surrey who were one of the original collaborating bodies on the development of the FRAME assay. The aim of this contract was to investigate the possibility of developing an assay which would give an answer within 24 hours in the first instance. The details of these studies are given in the final report (Appendix D).

Three main lines of approach were chosen. These were adaptions of the inhibition of cell growth (vital dye method) to increase sensitivity and obtain results within 24 hours, measurement of ATP and ATP/ADP ratio, and evaluation of a mitochondrial function test. Some investigation of the use of fluorescence polarography to measure charges in membrane fluidity were also carried out (see also Section 5.4).

A range of compounds were tested in each assay. All the tests gave essentially similar results though a variant of the vital dye method, in which the cells and test compound were plated concomitantly and using a 24 hour exposure period, was marginally the most sensitive. The mitochondrial function test also showed promise as a sensitive assay.

However, both this work and the validation studies carried out at WRc (Section 4) and elsewhere (Riddell et al 1986) have now shown that a short exposure period can be a drawback when testing some compounds. It

would appear that some compounds require exposure periods of at least 48 or 72 hours before cytotoxic effects become apparent. Short exposure periods may therefore markedly underestimate in vitro cytotoxicity in these cases.

The conclusion of this work is that the development of cytotoxicity assays for monitoring water is not likely to be a practical possibility where a short time of response is required.

SECTION 6 - DISCUSSION

Interest in the use of cytotoxicity assays has increased substantially in recent years for two reasons. First because they are of low cost compared to conventional toxicity tests using laboratory animals and are short-term in nature, an answer being available in a few days. Second, there is a real desire to replace experiments using laboratory animals by alternatives wherever possible.

The purpose of this contract was to investigate the potential for use of cytotoxicity assays by the water industry and to suggest an assay or assays which could be further developed if this was appropriate. A secondary purpose was to critically review current work on the development of cytotoxicity assays to be used as alternatives to animals in toxicity studies. There are a number of potential uses for cytotoxicity assays in the water industry. Since one of the problems frequently encountered is that of screening complex mixtures of chemicals in water, a problem which does not readily lend itself to solution by means of conventional chemical analysis, bio-assay may provide a suitable alternative.

A considerable amount of research into disinfection by-products in drinking water has been carried out using bio-assays with genotoxicity as an end-point. These include assays using mammalian cells in tissue culture to determine the activity of concentrated extracts of drinking

water. Cytotoxicity assays would be less appropriate for use with concentrated extracts but they could provide a different end-point for comparison of the biological activity of treated waters.

Monitoring of effluents is also a problem of studying changes of complex mixtures with time. In this case the mixture may or may not be well characterised but it is likely to be more concentrated than a drinking water sample.

Materials used in contact with potable water present a somewhat different problem. Under certain conditions of use such as high surface to volume ratio and/or where materials may be inefficiently applied or manufactured it is conceivable that ingredients may leach into the water in sufficient quantities to represent some risk to public health. There is therefore a requirement for some means of detecting such substances. In some circumstances it will be obvious from the materials which substances may be leached and in these cases standard analytical techniques may be applied. However, when the nature of the leachate is unknown it is necessary to use some form of 'blanket' detecting method which, in the absence of suitable analytical procedures would indicate the use of a biological screen.

Another possible role for cytotoxicity assays is the detection of high concentrations of toxic chemicals which may threaten public water supplies. The problem of intake protection is one which is not amenable to broad spectrum analysis and one which bio-assays such as fish monitors have been used to solve. In this case high sensitivity combined with a rapid response would be required.

Finally there are many groups of chemicals found in water for which there is little or no data on toxicity. The use of structure activity relationships (SAR) is an attractive means of assessing whether they pose a hazard to health. Unfortunately SARs are notoriously unreliable and some means of knowing at which point in a homologous series of chemicals the relationship breaks down is essential. It is possible that such a method could be provided by in vitro cytotoxicity assays.

The present study has, however, exposed some of the reasons why the potential applications of cytotoxicity assays are not being exploited. First, it is essential that the assays are carried out by experienced operators, in order to obtain reproducible results. Second, problems may arise resulting from factors intrinsic to the assay itself. These may either be related to the chemical or sample being tested. For example changes in pH which the cells are unable to tolerate as experienced in our work with leachates from materials may cause problems, or they may result from cellular factors, including ageing or contamination.

One of the main limitations of cytotoxicity assays is that the results cannot be extrapolated directly to man. There are several reasons why this is so including no measure of absorption and distribution (pharmacokinetics), differences in sensitivity compared with target cells in vivo and lack of cell metabolism. It does appear, however, that cells in tissue culture could be used to achieve considerably more than at present but there is a need for more basic research into the function of cells in culture and the development of cell lines before this potential is realised. The newly developed HepG2 cell line, which is reputed to retain a high degree of metabolic integrity, is an example of the way in which the technique could develop.

When selecting a cytotoxicity assay for a particular application, it is important to consider a number of characteristics of the assay, including robustness, reproducibility, ease of use and sensitivity. The reproducibility of the assays investigated was found to be good in studies using pure compounds and water samples. However, the studies using leachates of materials were less reproducible, giving unpredictable results on some occasions.

In terms of ease of operation, visual assessment of cell morphology is probably simplest. However, this was shown to be the least sensitive assay in each of our studies, and can also be easily included as an additional endpoint when measuring inhibition of cell growth. Cloning was more costly in terms of time and materials than the other assays,

and is probably the least simple assay. There is little to choose between the two methods for inhibition of cell growth, but fewer washing steps render the vital dye more applicable for use in 96-well plates than the protein method. Testing in 96-well plates has perhaps slight advantages compared to testing in 24-well plates, as more data points are generated and less materials per plate are required.

Sensitivity was not demonstrated to be a particularly important factor in the testing of groups of pure compounds, since each assay ranked the chemicals in a similar order of toxicity. However, as demonstrated by the studies using water samples and leachates from materials, sensitivity is a vital component of the assay. This is not surprising since this is analogous with the detection limit in analytical chemistry. The results of these studies indicated that cloning efficiency was more sensitive than inhibition of cell growth (vital dye method), which was not entirely due to differences in exposure period. Some of the water samples showed evidence of cytotoxicity in the cloning assay following 24 hours exposure, but a longer exposure period (8-10 days) did increase the sensitivity of this assay. With respect to the assay of different water samples by cloning efficiency, little or no evidence of cytotoxicity was given by the river water samples, although the industrial effluent was markedly cytotoxic. In some cases, laboratory storage of water samples was found to increase the cytotoxic activity. Therefore the timing of sampling and testing is an important consideration of such tests with the possibility that re-testing may be complicated by changes in activity.

The assays, unlike those of Fauris et al (1985), failed to detect cytotoxic activity in samples of treated and raw water, perhaps as a result of insufficient sample concentration. However, the results of studies comparing the cytotoxicity of effluents indicated the potential use of the assays for screening industrial discharges and for measuring the dilution required to eliminate any observed cytotoxic activity. This application might then be of greater value if used in combination with other bioassays, perhaps using bacteria or aquatic organisms.

This would increase the range of chemicals which could be detected and would also increase confidence in any marginal results by providing confirmatory data.

The results of testing the leachates from rubber and plastic materials showed that cytotoxicity assays were able to detect leaching of cytotoxic compounds. However, this is dependent on the extraction period and surface-to-volume ratio of the material and extracting water. The test used in the Water Byelaws Scheme includes 24-hour extractions but the use of increased extraction periods would certainly find more toxic samples. It is therefore necessary to determine the purpose of the test and what it is intended to achieve. If it is intended that the assay act as a screen then the sensitivity will be important in determining a pass or fail. Unfortunately without specific studies with support from analytical chemistry the selection of extraction period, and so sensitivity, is a purely arbitrary procedure.

Consideration should be given to the value of such a screening test in the absence of the requirement for further investigation of the leachate analytically. The cytotoxicity testing of pure compounds known to leach from materials revealed a wide variation in toxicity which would compromise the value of cytotoxicity assays as a first detection screen prior to more detailed chemical analysis. In addition problems were encountered with changes in pH which can be a serious difficulty. However, it is possible that cytotoxicity assays could be used as bio-assays for the detection of specific substances for which methods of analysis may be particularly difficult. Therefore, if a particular cytotoxic component was known to be present in a formulation, its capability to leach may be screened using a cytotoxicity assay. There would also appear to be a role for cytotoxicity assays in the quality control of materials for use in contact with potable water.

Cytotoxicity assays have been shown to be potentially useful in the detection of toxin- producing blooms of Microcystis aeruginosa. To this end they could be used to largely replace the mouse assay. However, it is considered that even in this case cytotoxicity assays would ideally be secondary to a good analytical technique.

There are some circumstances, such as the potential use in intake protection, in which a rapid answer would be required of a cytotoxicity assay. In such cases the assay needs to be of very high sensitivity to be of practical value. However, our studies have shown the importance of exposure period in relation to sensitivity. Therefore, rapidity and sensitivity must at present be considered to be mutually exclusive and we consider such applications as not feasible, at least at this time.

At present there is a considerable research effort directed at developing and applying cytotoxicity assays in order to reduce the use of animals in toxicology. This effort is rather fragmented with many small groups using a variety of assays to screen for toxicity or irritancy, mostly as a way of selecting compounds of least toxicity from a number of candidate chemicals. There have been some successes, notably the use of in vitro cytotoxicity in screening for anti-cancer drugs and the quality control of some medical plastics.

However, there is a need for better co-ordination of the research effort in this field and funding of more basic research into the function of cells in culture and the development of new cell lines such as the newly developed HepG2, mentioned above.

There is also a need for a more critical approach which will aim for the development of cytotoxicity assays to determine how far they can become more important and sophisticated components of toxicity testing. At present there is too ready an acceptance of the simplicity and limitations of cell systems. To this end it is important that further fundamental research be carried out.

SECTION 7 - CONCLUSIONS

1. In vitro cytotoxicity assays are a potentially useful tool in the study of toxic compounds of complex mixtures. This usefulness is restricted by the relative lack of development of such assays at the

present time and the unpredictability of problems in their use. They can only be used as screening tests since data cannot be easily extrapolated directly to man.

2. The lack of xenobiotic metabolism, which is lost in most cell lines and which is necessary for determining the toxicity of indirect acting toxicants can at present only be overcome by the use of primary cell cultures. This is a serious drawback to the use of cell line in cytotoxicity testing. New cell lines which maintain a high degree of metabolic integrity are just being developed.

3. The main characteristics necessary in a cytotoxicity assay to be used routinely as a screen are sensitivity, reproducibility, robustness and ease of use.

4. Cloning efficiency appeared to be the most sensitive assay of the ones examined and visual assessment of morphology the least sensitive.

5. The period of exposure is extremely important since some chemicals are not cytotoxic unless the cells are exposed for a sufficiently long period. Very short assays must be considered with some suspicion.

6. Cytotoxicity assays appear to be of little value in examining drinking water but may have some uses in screening effluents.

7. The use of cytotoxicity assays to screen leachates from materials is severely hampered by the variation in sensitivity to toxic components of materials.

8. Where such assays are used to examine leachates from materials the purpose and limitations of the assay must be clearly defined in order to prevent over-interpretation of the results by non-specialists.

9. Toxin producing blooms of the blue-green alga Microcystis aeruginosa can be detected by means of a cytotoxicity assay which provides an alternative to the use of laboratory animals.

SECTION 8 - RECOMMENDATIONS FOR FURTHER WORK

The cytotoxicity assays presently used for screening leachates from materials should be considered more critically to determine precisely what they will measure, whether they can be improved or whether they should be specified for use only with certain materials.

The development of cytotoxicity assays based in cell culture is very fragmented and suffers from too much duplication of simple experimental work. Their role in toxicology and the development of alternatives to laboratory animals is being hindered by this fragmentation. It is recommended that this be better co-ordinated and further fundamental research into cells in culture, particularly in toxicology, be carried out.

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APPENDIX A - IN VITRO CYTOTOXICITY TEST METHODS

SECTION A1 - THE V79/4 CELL LINE

All experiments were performed using the V79/4 Chinese hamster lung fibroblast cell line (Ford and Yerganian 1958). The cell line was kindly supplied by Mr M O'Donovan (The Boots Company Limited).

V79/4 cells were grown as monolayer cultures in 75 cm^2 plastic tissue culture flasks using Eagle's MEM medium with Earle's salts supplemented with 10% foetal calf serum, 2mM L-glutamine, and 20mM HEPES buffer maintaining a pH of 7.2-7.4 at 37 °C. Cells were grown in a 5% carbon dioxide atmosphere and subcultured every 2-3 days. For subculture, a mixture of trypsin (0.0125% w/v) and EDTA (0.02% w/v) in saline was used. No antibiotics were present during routine culture but penicillin-streptomycin was employed during test procedures. The culture was checked regularly for mycoplasma contamination by the DNA fluorescent staining technique (Hoechst kit).

SECTION A2 - MIT-24 TEST

This test is based upon two end-points, visual assessment of cultures after 24 hours and evaluation of metabolic inhibition after 7 days (the medium contains phenol red pH indicator and therefore changes colour as the cells produce acidic metabolites).

The MIT-24 test was carried out according to the method of Ekwall (1980). Near-confluent monolayer cultures of V79/4 cells were harvested and diluted to a cell density of 5×10^4 cells/ml in MEM medium. The suspension culture was then mixed for about 30 minutes using a magnetic stirrer before 0.1 ml aliquots were dispensed into each of 96 wells of a tissue culture plate. Test chemicals were made up at four different concentrations in MEM medium in glass bijou bottles. For each dilution, 0.1 ml aliquots were added to each of 5 wells. Hence for one plate,

four chemicals were tested and 16 wells received 0.1 ml of medium only to act as controls. Wells were sealed with 0.1 ml of paraffin oil and the whole plate covered with a polythene film. Plates were incubated at 37 °C for seven days.

After 24 hours of incubation, cultures in each well were evaluated by light microscopy for changes in cell numbers and differences in cell morphology. At the end of seven days, colour of the medium in each well was assessed. Typically, at the beginning of the experiment all the wells were pink (about pH 7.4) but after seven days, cell metabolism in control wells had turned the medium to an orange colour (about pH 7.1). Toxicity was indicated where wells remained a similar colour to the original pink.

SECTION A3 - FRAME TEST

V79/4 cells were harvested and diluted to a final concentration of 5 x 10^3 cells/ml in MEM medium. The cell suspension was shaken for about 30 minutes and then 1 ml aliquots were added to each well of a 24-well tissue culture plate. Plates were incubated for 24 hours to allow the cells to attach. The medium covering the cells was then removed and 1 ml aliquots of the solutions of test chemicals added. Six dose levels were made up for each compound and three wells were used for each dose level. The remaining six wells were controls and received 1 ml of medium only. Plates were then incubated for 72 hours. At the end of this period, the medium covering the cells was removed and the cells washed twice with phosphate buffered salts without magnesium or calcium (PBS'A') at physiological pH. The final wash was removed and 1 ml of fixative (3% glutaraldehyde in PBS'A') added to each well and left for 2 hours. On removal of the fixative, cells were stained for 30 minutes with 1 ml/well of the dye solution*. The dye was then removed and 1 ml

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* 0.4 g of Kenacid Blue R was dissolved in 250 ml of ethanol and 630 ml of water. Immediately before use 12 ml of glacial acetic acid was added to each 88 ml of stain and the solution filtered.

of destain (ethanol:glacial acetic acid:water in the ratio 2:1:17) added to each well. The plate was agitated for 5 minutes and the destain removed. The destain procedure was repeated, agitating for 15 minutes, until the destain no longer took up the blue colouration of the dye. The final destain was removed and 1 ml/well of desorbing solution (1M potassium acetate in 70% ethanol) added. The absorbance of the solution in each well was measured at 590 nm using a Cecil CE292 ultraviolet spectrophotometer. The average absorbance at each dose level was calculated and plotted as percentage absorbance of control against dose. The dose which inhibited cell growth by 50% (ID50) was then determined from the graph.

SECTION A4 - INHIBITION OF CELL GROWTH (24 HOUR VITAL DYE METHOD)

V79/4 cells were harvested and diluted to a concentration of 5×10^4 cells/ml in MEM medium. Aliquots (0.1 ml) of the cell suspension were dispensed into each of 84 wells of 96-well tissue culture plates. A row of 12 wells were left empty to act as spectrophotometric blanks. The plates were incubated for 24 hours to allow the cells to attach. Medium covering the cells was then removed and 0.1 ml aliquots of solutions of the test compound were added. Six dose levels were made up for each test compound and one row containing 12 wells was used for each dose level. The remaining 12 wells that contained cells received medium only and acted as controls. The plates were then incubated for a further 24 hours. After this time, the test solutions were removed and the cells washed by addition of 0.1 ml PBS'A' to each well. Subsequently, 0.2 ml aliquots of freshly prepared neutral red solution (50 µg/ml in medium) were added to each well and the plates incubated for 3 hours. At the end of this period, the neutral red solution was removed and the cells washed again with PBS'A'. Each well then received 0.1 ml of a solution of 50% ethanol/1% glacial acetic acid and the plates were carefully agitated on an IKA shaker for about 15 minutes. The absorbance of each well was read at 540 nm using a Dynatech MR700 microplate reader. The

average absorbance at each dose level was calculated in terms of percentage of control and the ID50 determined from a graph of dose versus percentage absorbance of control.

SECTION A5 - INHIBITION OF CELL GROWTH (72 HOUR VITAL DYE METHOD)

V79/4 cells were grown as monolayers in 75 cm^2 plastic tissue culture flasks until they were nearing confluency. The cells were then harvested and diluted to a concentration of 1 x 10^4 cells/ml in Eagles Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum, glutamine, HEPES buffer and penicillin/streptomycin. Aliquots (0.1 ml) of the cell suspensions were dispensed into each of 84 wells of 96-well tissue culture plates. A row of 12 wells was left empty to act as spectrophotometric blanks. The plates were incubated for 24 hours to allow the cells to attach. Medium covering the cells was then removed and 0.2 ml aliquots of medium containing the test sample were added (normally one row containing 12 wells was used for each sample or dose level). After 24 hours of incubation the cultures were examined microscopically*. The plates were then incubated for a further 48 hours giving a total incubation period of 72 hours. After this time, the test solutions were removed and the cells washed by addition of 0.1 ml of PBS'A' to each well. Subsequently, 0.2 ml aliquots of freshly prepared neutral red solution (50 µg/ml in MEM medium), prewarmed to 37 °C to prevent precipitation, were added to each well and the plates incubated for 3 hours. At the end of this period, the neutral red solution was removed and the cells washed again with PBS'A'. Each well then received 0.1 ml of a solution of 50% ethanol/1% glacial acetic acid and the

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* The cell cultures were examined microscopically for effects on cell density and cell morphology. Classification was divided into three simple categories, no observable effect, some evidence of cytotoxicity, and clear cytotoxic effect. This visual assessment is easily incorporated as part of the test for inhibition of cell growth and can provide useful information as well as ensuring that the performance of the growth test is monitored effectively.

plates carefully agitated on an IKA shaker for about 15 minutes. The absorbance of each well was then read at 540 nm using a Dynatech MR700 microplate reader. The average absorbance of each sample or dose level was calculated and converted into percentage of control.

SECTION A6 - CLONING EFFICIENCY

V79/4 cells were harvested and counted accurately using a ZM Coulter counter. The cell suspension was diluted to 30 cells/ml in MEM medium and 5 ml aliquots dispensed into 60 mm petri-dishes. The dishes were incubated for 24 hours after which the medium was removed and replaced with 5 ml of fresh medium containing the test compound. Compounds were tested at six dose levels, three dishes per dose. Cells were incubated in the presence of the test compound for 24 hours. At the end of this period, the medium in each dish was again removed and replaced with fresh medium. The dishes were then incubated for a further 7-9 days. At the end of the experiment, the colonies were fixed using 3:1 methanol:glacial acetic acid, stained with 10% giemsa, washed and dried, and then counted on an Artek 880 automatic colony counter. The average cloning efficiency of each sample was calculated in terms of percentage of control.

$$\text{Cloning efficiency} = \frac{\text{Number of colonies formed}}{\text{Number of cells seeded}} \times 100$$

SECTION A7 - ENZYME LEAKAGE ASSAY

V79/4 cells were harvested and diluted to a concentration of 1×10^4 cells/ml in MEM medium. Aliquots of 1 ml were then added to each well of a 24-well tissue culture plate, and incubated for 24 hours in order to allow the cells to attach. The medium covering the cells was then removed and 1 ml aliquots of the test solutions added. The toxic extract of the cyanobacterium Microcystis aeruginosa was diluted with

MEM medium to give a range of 6 doses, and 3 wells were used for each dose level. The remaining six wells were controls and received 1 ml MEM medium. The plates were then incubated for 24 hours, after which the medium in the wells was removed and assayed for LDH activity. The cell layer was simultaneously treated with the solubilising agent, and then assayed for LDH activity. Each sample to be assayed (100 µl) was incubated with the enzyme substrate and cofactor for 20 minutes at 37 °C, after which colour reagent (1 ml) was added and the mixture left for a further 20 minutes at room temperature. Colour was then developed by the addition of 10 ml of 0.4M NaOH. Each assay mixture was read at 460 nm, and enzyme activity expressed in Berger-Broida (B-B) units read from a pre-plotted calibration graph.

One B-B unit = the amount of LDH that will reduce 4.8×10^{-4} Mol of pyruvate per minute at 25 °C.

A commercial diagnostic kit (Sigma, procedure No 500) was used for the assay of LDH activity (Cabaud and Wroblewski 1958), and contained the following reagents:

Solubilising agent: Triton X-100 (0.1g) + bovine serum albumin in 0.9% saline (0.1g)

Enzyme substrate: Sodium pyruvate 0.75 Mol/l

Cofactor: NADH 1.28 Mol per 100 µl of sample

Colour reagent: 0.2 mg/ml 2,4-dinitrophenyl- hydrazine in 1M HCl
0.4M NaOH

SECTION A8 - VERO CELLS (WATER BYELAWS SCHEME)

Vero cells were harvested and 10^3 to 10^5 cells added to 0.3 mls of growth medium and 2.7 mls of a sterile solution of the test compound. Portions (1 ml) of the resulting suspension were transferred into each

of three sterile tissue-culture containers with airtight closures. The containers were capped tightly and incubated at 37 ± 1 °C for 24 hours and 72 hours.

After incubation the condition of the cells in each container were examined microscopically. The presence or absence of a confluent cell layer was recorded and the presence of any irregularly shaped cell or cells showing signs of rounding off were noted. If confluent growth was not observed, the appearance of any cells floating in the growth medium was recorded. If more than one of the three containers showed effects on morphology the compound was considered to be cytotoxic.

Full details of all solutions and methods used in this test are given in BS 6920, Section 2.5, 1988.

APPENDIX B

RESULTS OF CYTOTOXICITY ASSAYS WITH LEACHATES FROM MATERIALS

Table 11 - Results of cell morphology after 24 hours for Rubber 1

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	ND	-	-	-	-	-
1 I	ND	-	+/-	-	-	-
1 II	ND	-	-	-	-	-
1 III	ND	-	-	-	-	-
1 IV	ND	-	+/-	-	-	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 12 - Results of inhibition of cell growth (vital dye method) for Rubber 1

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	102	106	120	95	93	ND
1 I	93	94	127	92	99	ND
1 II	83	88	127	85	99	ND
1 III	95	99	127	93	106	ND
1 IV	100	96	126	89	101	ND

ND Not done

Table 13 - Results of cloning efficiency for Rubber 1

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	101	109	102	114	103	ND
1 I	107	108	96	124	94	ND
1 II	100	111	123	126	88	ND
1 III	105	108	115	92	88	ND
1 IV	105	113	ND	96	93	ND

ND Not done

Table 14 - Results of cell morphology after 24 hours for Rubber 2

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	ND	-	-	-	-	-
2 I	ND	-	-	-	-	-
2 II	ND	-	-	-	-	-
2 III	ND	-	-	-	-	-
2 IV	ND	-	-	-	-	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 15 - Results of inhibition of cell growth (vital dye method) for Rubber 2

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	107	110	118	84	93	ND
2 I	97	ND	104	101	100	ND
2 II	93	98	107	107	99	ND
2 III	91	99	119	106	102	ND
2 IV	99	103	117	102	89	ND

ND Not done

Table 16 - Results of cloning efficiency for Rubber 2

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	101	109	102	114	103	ND
2 I	102	98	142	110	95	ND
2 II	105	109	117	59	101	ND
2 III	105	106	114	112	94	ND
2 IV	102	99	ND	102	99	ND

ND Not done

Table 17 - Results of cell morphology after 24 hours for Rubber 3

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	-	-	-	-	-	-
3 I	+/-	+/-	+/-	-	-	-
3 II	-	-	-	-	-	-
3 III	-	-	-	-	-	-
3 IV	+	+	+	-	+	+

\- No cytotoxicity observed
\+ Cytotoxic
+/- Some evidence of cytotoxicity

Table 18 - Results of inhibition of cell growth (vital dye method) for Rubber 3

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	257	86	89	158	97	97
3 I	4	15	5	65	46	33
3 II	59	43	4	82	51	27
3 III	55	53	3	80	44	34
3 IV	51	16	11	63	4	0

Table 19 - Results of cloning efficiency for Rubber 3

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	99	105	94	98	100	100
3 I	0	0	0	0	0	0
3 II	0	0	0	0	0	0
3 III	0	0	0	0	0	0
3 IV	0	0	0	0	0	0

Table 20 - Results of cell morphology after 24 hours for Rubber 4

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	-	-	-	-	-	-
4 I	+	+	+	-	-	-
4 II	+/-	+/-	+/-	-	-	-
4 III	+/-	+/-	+/-	-	-	-
4 IV	+	+	+	-	+	+

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic

Table 21 - Results of inhibition of cell growth (vital dye method) for Rubber 4

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	257	122	100	155	97	85
4 I	15	4	4	49	29	9
4 II	2	7	4	50	45	27
4 III	29	26	6	56	47	14
4 IV	11	4	3	34	18	0

Table 22 - Results of cloning efficiency for Rubber 4

SAMPLE	EXPERIMENT A		EXPERIMENT B		EXPERIMENT C	
	a	b	a	b	a	b
Distilled	99	105	94	98	100	100
4 I	0	0	0	0	0	0
4 II	0	0	0	0	0	0
4 III	0	0	0	0	0	0
4 IV	0	0	0	0	0	0

Table 23 - Results of cell morphology after 24 hours for Rubber 5

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	ND	-	ND	-
5 I	ND	-	ND	-
5 II	ND	-	ND	-
5 III	ND	-	ND	-
5 IV	ND	-	ND	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 24 - Results of inhibition of cell growth (vital dye method) for Rubber 5

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	110	97	112	125
5 I	94	95	97	93
5 II	95	93	96	101
5 III	101	96	99	107
5 IV	98	92	87	101

Table 25 - Results of cloning efficiency for Rubber 5

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	88	103	101	105
5 I	83	78	94	89
5 II	91	85	103	92
5 III	97	102	103	110
5 IV	93	100	97	106

Table 26 - Results of cell morphology after 24 hours for Rubber 6

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	ND	-	ND	-
6 I	ND	-	ND	+/-
6 II	ND	-	ND	-
6 III	ND	-	ND	-
6 IV	ND	+	ND	+

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 27 - Results of inhibition of cell growth (vital dye method) for Rubber 6

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	104	100	113	111
6 I	23	8	14	13
6 II	23	5	22	22
6 III	26	11	24	22
6 IV	0	1	0	0

Table 28 - Results of cloning efficiency for Rubber 6

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	88	103	101	105
6 I	0	0	0	0
6 II	0	0	0	0
6 III	0	0	0	0
6 IV	0	0	0	0

Table 29 - Results of cell morphology after 24 hours for cPVC

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	-	-	-
cPVC I	-	-	-	-
cPVC II	-	-	-	-
cPVC III	-	-	-	-
cPVC IV	-	-	-	-

-	No cytotoxicity observed	+	Cytotoxic
+/-	Some evidence of cytotoxicity	ND	Not done

Table 30 - Results of inhibition of cell growth (vital dye method) for cPVC

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	107	110	93	89
cPVC I	99	111	99	91
cPVC II	105	121	82	80
cPVC III	91	125	94	95
cPVC IV	71	107	90	75

ND Not done

Table 31 - Results of cloning efficiency for cPVC

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	94	ND	86	99
cPVC I	99	ND	92	84
cPVC II	97	ND	94	97
cPVC III	96	ND	91	92
cPVC IV	94	ND	97	99

ND Not done

Table 32 - Results of cell morphology after 24 hours for GRP

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	-	-	-
GRP I	+/-	-	-	-
GRP II	+/-	-	-	-
GRP III	+/-	-	-	-
GRP IV	+/-	+/-	-	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 33 - Results of inhibition of cell growth (vital dye method) for GRP

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	106	112	89	77
GRP I	99	105	97	87
GRP II	103	114	84	77
GRP III	94	108	82	68
GRP IV	66	105	74	73

ND Not done

Table 34 - Results of cloning efficiency for GRP

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	94	ND	86	99
GRP I	101	ND	84	96
GRP II	94	ND	87	99
GRP III	93	ND	93	99
GRP IV	88	ND	89	99

ND Not done

Table 35 - Results of cell morphology after 24 hours for Blue Polyethylene 1 (Upnor)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	-	-	-
BP1 I	-	-	-	-
BP1 II	-	-	-	-
BP1 III	-	-	-	-
BP1 IV	-	-	-	-

-	No cytotoxicity observed	+	Cytotoxic
+/-	Some evidence of cytotoxicity	ND	Not done

Table 36 - Results of inhibition of cell growth (vital dye method) for Blue Polyethylene 1 (Upnor)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	20	ND	95	77
BP1 I	36	ND	101	102
BP1 II	40	ND	103	99
BP1 III	45	ND	98	98
BP1 IV	40	ND	96	89

ND Not done

Table 37 - Results of cloning efficiency for Blue Polyethylene 1 (Uponor)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	ND	106	83	101
BP1 I	ND	85	84	92
BP1 II	ND	85	86	91
BP1 III	ND	84	83	83
BP1 IV	ND	91	89	90

ND Not done

Table 38 - Results of cell morphology after 24 hours for Blue Polyethylene 2 (BP)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	-	-	-
BP2 I	-	-	-	-
BP2 II	-	-	-	-
BP2 III	-	-	-	-
BP2 IV	-	-	-	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 39 - Results of inhibition of cell growth (vital dye method) for Blue Polyethylene 2 (BP)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	27	108	91	74
BP2 I	44	94	93	90
BP2 II	102	93	102	103
BP2 III	103	108	100	93
BP2 IV	25	83	86	89

ND Not done

Table 40 - Results of cloning efficiency for Blue Polyethylene 2 (BP)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	ND	106	83	101
BP2 I	ND	74	94	97
BP2 II	ND	74	90	97
BP2 III	ND	79	88	100
BP2 IV	ND	82	95	93

ND Not done

Table 41 - Results of cell morphology after 24 hours for Blue Polyethylene 3 (Stewart and Lloyd)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	ND	-	-
BP3 I	-	ND	-	-
BP3 II	-	ND	-	-
BP3 III	-	ND	-	-
BP3 IV	-	ND	-	-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 42 - Results of inhibition of cell growth (vital dye method) for Blue Polyethylene 3 (Stewart and Lloyd)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	110	ND	88	81
BP3 I	72	ND	106	106
BP3 II	80	ND	98	100
BP3 III	77	ND	105	110
BP3 IV	75	ND	78	105

ND Not done

Table 43 - Results of cloning efficiency for Blue Polyethylene 3 (Stewart and Lloyd)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	106	ND	100	106
BP3 I	79	ND	95	104
BP3 II	92	ND	99	103
BP3 III	83	ND	100	94
BP3 IV	ND	ND	95	101

ND Not done

Table 44 - Results of cell morphology after 24 hours for Blue Polyethylene 4 (Polypin)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	-	ND	-	-
BP4 I	-	ND	-	-
BP4 II	-	ND	-	-
BP4 III	-	ND	-	-
BP4 IV	-	ND	-	+/-

\- No cytotoxicity observed
+/- Some evidence of cytotoxicity
\+ Cytotoxic
ND Not done

Table 45 - Results of inhibition of cell growth (vital dye method) for Blue Polyethylene 4 (Polypin)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	113	ND	95	87
BP4 I	94	ND	87	92
BP4 II	56	ND	106	112
BP4 III	24	ND	105	105
BP4 IV	0	ND	24	25

ND Not done

Table 46 - Results of cloning efficiency for Blue Polyethylene 4 (Polypin)

SAMPLE	EXPERIMENT A		EXPERIMENT B	
	a	b	a	b
Distilled	106	ND	100	106
BP4 I	73	ND	91	105
BP4 II	82	ND	93	106
BP4 III	0	ND	95	90
BP4 IV	0	ND	6	0

ND Not done

Table 47 - Results of inhibition of cell growth (72 hour vital dye and cell cloning methods) for compounds known to leach from plastic pipes or epoxy resins

Compound	Inhibition of cell growth (ID_{50}) vital dye method	cell cloning method
Bisphenol A diglycidyl ether	18.75 +/- 2.5	28.48 +/- 2.1
Bisphenol B diglycidyl ether	21.0 +/- 1.75	30.91 +/- 1.0
Dimethyltin dichloride	3.96 +/- 0.71	6.605 +/- 0.09
Dibutyltin dichloride	0.055 +/- 0.015	ND
Santanox	1.099 +/- 0.24	ND
Irganox 1010	213.4 +/- 31.0	ND
Trimethylhexamethylene diamine	0.244 +/- 0.07	ND
Lead(II) chloride	125.0 +/- 15.0	ND

Data are expressed in µg/ml except trimethylhexamethylene diamine and lead (II) chloride, which are expressed as µl/ml and mg/ml, respectively.

Results are based on at least two experiments carried out on different days and are expressed as the mean +/- standard error of the mean.

Table 48 - Results of testing 8 compounds in WBAS scheme (carried out by Thames Water) and by inhibition of cell growth (vital dye) method (carried out by WRc)

	Thames Water results						WRc results
	24 hours exposure			48 hours exposure			Vital dye method (72 hours exposure)
Compound	Non-toxic	Toxic	Complete cell death	Non-toxic	Toxic	Complete cell death	ID 50
Bisphenol A diglycidyl ether	5.0	15.0	25.0	5.0	15.0	25.0	18.75 +/- 2.5
Bisphenol F diglycidal ether	5.0	15.0	25.0	<5.0	5.0	25.0	21.0 +/- 1.75
Dimethyltin dichloride	10	20	20	10	20	20	3.96 +/- 0.71
Dibutyltin dichloride	0.01	0.05	0.5	0.01	0.05	0.5	0.055 +/- 0.01
Sanatox	0.5	1.0	5.0	0.5	1.0	5.0	1.099 +/- 0.24
Irganox 1010	600	>600	>600	600	>600	>600	213.4 +/- 31.0
Trimethyl-hexamethylene diamine	0.01	0.075	0.1	0.01	0.075	0.1	0.244 +/- 0.07
Lead (II) chloride	<50	50	300	<50	50	300	125.0 +/- 15.0

Data are expressed in µg/ml, except trimethylhexamethylene diamine and lead (II) chloride, which are expressed as µl/ml and mg/ml respectively

APPENDIX C

TOXICOLOGICAL SCREENING OF SUBMITTED COMPOUNDS BY THAMES WATER SERVICES

Thames Water Services

16 Jun 1988

New River Head Laboratories
177 Rosebery Avenue, London EC1R 4TP
Telephone Direct Line: 01-833 6105
Please reply to :01-833 6104
Telex: 267216 TWMTLB G
Fax: 01-833 6123

Report Ref:
Our Ref: WQS/MT/2176
Enquiry: P 9,670

WRC MEDMENHAM LABS
HENLEY ROAD, MEDMENHAM,
MARLOW,
BUCKS SL7 2HD

Attn.MS PAULA TILLING

TEST REPORT: TOXICOLOGICAL SCEENING OF SUBMITTED COMPOUNDS

Ten compounds were submitted for toxicological screening against monkey kidney cell line ATCC No.CCL81 to assess their effect on potable water.

Six dose levels where requested to be assesed for each compound; these are shown on the result sheet for each compound.

Each result sheet gives 1) Toxicity dose level 24h
2) Toxicity dose level 48/72h
3) Toxic response
4) Cell death dose level

All controls flasks gave a satisfactory response.

yours sincererly

David J Pratt

David J Pratt; Materials Testing Officer
Thames Water Services (Materials)

Thames Water

WQS/MT/2176/P9,670

Results
COMPOUND No.1

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 0.5/1.0/2.5/5.0/7.5/10.0

Toxicity dose level 24h

Non toxic : 2.5 ug/l

Toxic : 5.0 ug/l

Toxic response : Granular Inclusions

Complete cell death / 100% rounding :)10 ug/l

Toxicity dose level 48h

Non toxic : 1.0 ug/l

Toxic : 2.5 ug/l

Toxic response : Granular Inclusions

Complete cell death / 100% rounding :)10 ug/l

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.2

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 0.5/1.0/3.5/7.0/10.0/20.0

Toxicity dose level 24h

Non toxic : 10.0 ug/l

Toxic : 20.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding :)20 ug/l

Toxicity dose level 48h

Non toxic : 10.0 ug/l

Toxic : 20.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding :)20 ug/l

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.3

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 0.005/0.01/0.05/0.1/0.5/1.0

Toxicity dose level 24h

Non toxic : 0.01 ug/l

Toxic : 0.05 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 0.5 ug/l

Toxicity dose level 72h

Non toxic : 0.01 ug/l

Toxic : 0.05 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 0.5 ug/l

WQS/MT/2176/P9,670

Results

COMPOUND No.4

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (mg/ml): 0.05/0.1/0.3/0.7/1.0/1.5

Toxicity dose level 24h

Non toxic :(0.05 mg/l

Toxic : 0.05 mg/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 0.3 mg/l

Toxicity dose level 72h

Non toxic :(0.05 mg/l

Toxic : 0.05 mg/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 0.3 mg/l

Thames Water

THAMES WATER SERVICES

Page of

WQS/MT/2176/P9,670

Results
COMPOUND No.5

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 0.05/0.1/0.5/1.0/5.0/10.0

Toxicity dose level 24h

Non toxic : 0.5 ug/l

Toxic : 1.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 5.0 ug/l

Toxicity dose level 72h

Non toxic : 0.5 ug/l

Toxic : 1.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 5.0 ug/l

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.6

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 50/100/200/300/450/600

Toxicity dose level 24h

Non toxic : 600.0 ug/l

Toxic : Not within range tested.

Toxic response : Nil

Complete cell death / 100% rounding : Nil.

Toxicity dose level 72h

Non toxic : 600.0 ug/l

Toxic : Not within range tested.

Toxic response : Nil

Complete cell death / 100% rounding : Nil.

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.7

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 5/15/25/35/45/55

Toxicity dose level 24h

Non toxic : 5.0 ug/l

Toxic : 15.0 ug/l

Toxic response : Rounding

Complete cell death / 100% rounding : 25.0 ug/l

Toxicity dose level 72h

Non toxic : 5.0 ug/l

Toxic : 15.0 ug/l

Toxic response : Rounding

Complete cell death / 100% rounding : 25.0 ug/l

Thames Water

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.8

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ug/ml): 5/15/25/35/45/60

Toxicity dose level 24h

Non toxic : 5.0 ug/l

Toxic : 15.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 25 ug/l

Toxicity dose level 72h

Non toxic : (5.0 ug/l

Toxic :=)5.0 ug/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 25 ug/l

WQS/MT/2176/P9,670

Results
COMPOUND No.9

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ul/ml): 0.01/0.075/0.1/1.0/5.0/15.0

Toxicity dose level 24h

Non toxic : 0.01 ul/l

Toxic : 0.075 ul/l

Toxic response : Granular Inclusions

Complete cell death / 100% rounding : 0.1 ul/l

Toxicity dose level 48h

Non toxic : 0.01 ul/l

Toxic : 0.075 ul/l

Toxic response : Granular Inclusions

Complete cell death / 100% rounding : 0.1 ul/l

THAMES WATER SERVICES

WQS/MT/2176/P9,670

Results
COMPOUND No.10

This compound gave the following response when tested against monkey kidney cell line ATCC No.CCL81. Six dose levels were tested, using three 25cm2 tissue culture flasks for each dose level.

Dose levels (ul/ml): 5/15/25/35/45/55

Toxicity dose level 24h

Non toxic : 15.0 ul/l

Toxic : 25.0 ul/l

Toxic response : Stressed Growth

Complete cell death / 100% rounding : 55 ug/l

Toxicity dose level 48h

Non toxic : 15.0 ul/l

Toxic : 25.0 ul/l

Toxic response : Rounding

Complete cell death / 100% rounding : 25 ug/l

THAMES WATER SERVICES

APPENDIX D

THE USE OF CYTOTOXICITY ASSAYS FOR THE ASSESSMENT OF WATER TOXICITY

Toxicology Unit

THE ROBENS INSTITUTE

The Robens Institute of Industrial & Environmental Health & Safety
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Director & Professor of Toxicology
Dr J.W. Bridges

THE USE OF CYTOTOXICITY ASSAYS FOR THE ASSESSMENT OF WATER TOXICITY

FINAL REPORT

Principle Scientist : Miss S. Good

Study Director : Dr. D.J. Benford

Date Issued : 25.9.87

Susan Good

University of Surrey

INTRODUCTION

This Report is a summation of the studies performed at the Robens Institute for the Water Research Centre on the use of cytotoxicity assays for the assessment of water toxicity (EHT 9329). In a preliminary Project Assessment Report (dated 4.9.86) it was stated that the following studies would be performed :-

i. Further development of the Neutral Red Test (using microtitre plates) to increase the sensitivity and minimise the time taken to obtain results.

ii. Develop a test for ATP and ATP/ADP using the luciferin/luciferase assay.

iii. Adapt methods to incorporate a metabolising system (when significant advances have been made with the FRAME study).

iv. Determine whether fluorescence polarography would be useful to investigate the effects of Microcystis aeruginosa toxin on membrane fluidity.

A group of test compounds, selected by the WRc, have been subjected to a series of possible cytotoxicity test protocols.

An Interim Report (dated 18.2.87) concluded that the Neutral Red Test in microtitre plates could be increased in sensitivity and performed more rapidly by adding the test compound at the same time as plating the cells. Omission of serum made little difference to the sensitivity. We have now completed our analysis of variations on the Neutral Red Test. Preliminary

studies with the ATP assay using the luciferin assay suggested this would be more sensitive than the Neutral Red Test. The complete series of test compounds have now been applied to the ATP assay and, for comparison, also to a mitochondrial function test (Mosmann, 1983).

The fluorescence polarography technique was reported in the Interim Report. Results were highly variable and treatment of V79 cells with selected toxins did not produce a dose response. In view of the fact that Microcystis aeruginosa toxin in the absence of serum was found by the WRc to be highly toxic in cytotoxicity tests, work on the fluoresence polarography was discontinued. Suggestions were made for studies on a metabolising cytotoxicity test using the human hepatoma cell line Hep G2. However there was insufficient time within the current contract for any progress to be made on this.

This Final Report presents an overall analysis of the cytotoxicity studies performed for this project.

MATERIALS AND METHODS

Cell Line

V79/4 cells were provided by the WRc and stocks maintained frozen in liquid nitrogen.

Culture Media and Plastic Ware

All media constituents were purchased from Gibco Biocult (Uxbridge, Middlesex). Tissue culture plasticware was purchased either from Becton Dickinson, Cowley, Oxford (Falcon microtitre plates and 25cm^2 flasks) or from Gibco Biocult, Uxbridge, Middlesex (24-well plates).

Test Compounds

Vincristine sulphate (approx 99%) : Sigma Chemical Co, Poole, Dorset.

Cycloheximide (purity not stated) : Sigma

pCMB : p-Chloromercuribenzoic acid (purity not stated) : Sigma

Catechol (99+%) : Aldrich, Gillingham, Dorset

Triton X-100 (purity not stated) : Hopkins & Williams, Chadwell Heath, Essex.

SDS : Sodium dodecylsulphate (95%) : Sigma

DNP : 2,4-Dinitrophenol (90-95%) : Sigma

Phenol (99.5%) : BDH Ltd, Poole, Dorset

DCA : Dichloroacetic acid (purity not stated) : Sigma

TCA : Trichloroacetic acid (99.5%) : BDH Ltd

Methanol (AR) : BDH Ltd

Dimethylsulphoxide (AR) : BDH Ltd

Cyclophosphamide (purity not stated) : Sigma

Reagents

Neutral Red (CI 50040), 3-(4,5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemical Co (Poole, Dorset). ATP monitoring reagents were purchased from LKB Wallac (Croydon, Surrey). All other reagents were of highest available purity from either BDH Ltd (Poole, Dorset) or Sigma Chemical Co (Poole, Dorset).

METHODS

Test Compounds

Test compounds were dissolved in DMSO at stock concentrations 100x those required. These were then added to EMEM at a dilution of 1/100 to achieve the desired concentration. Thus the final solvent concentration was 10 µl/ml and this was also added to controls. Seven doses of test compound in quadruplicate were used for calculation of ID_{50} values.

Culture Conditions

V79/4 cells were cultured in Eagles Minimum Essential Medium supplemented with 10% fetal calf serum and 100 µg/ml kanamycin (EMEM) stocks of cells were grown up in Falcon 25cm^2 flasks. Falcon microtitre or Nunc 24-week plates were used for testing. The cells were maintained at 37^{o} in 5% CO_2/95% air at all times.

Neutral Red Test

a. Incubation Conditions

Basic Neutral Red Test.

i. V79/4 cells were seeded at 800 cells in 0.2ml EMEM in microtitre plates and incubated for 24 hours.

ii. Test compounds were added in fresh EMEM and the cells were incubated for 24 hours before assessment of Neutral Red uptake as below.

Variation 1.

Fetal calf serum was omitted from the EMEM/test compound mixture.

Variation 2.

Test compound and 800 cells in 0.2ml EMEM were added to the wells at the same time followed by a 24 hour exposure period.

Variation 3

Test compound and 800 cells in 0.2ml EMEM were added to the wells at the same time followed by a 48 hour exposure period.

Variation 4

Test compound and 5000 cells in 0.2ml EMEM were added to the wells at the same time followed by a 5 hour exposure period.

b. **Determination of Neutral Red Uptake** (Borenfreund & Puerner, 1985).

1. Neutral Red was prepared as an aqueous 4% (w/v) stock solution and filter sterilised prior to addition to EMEM at a final concentration of 50 µg/ml.

2. The medium in the wells was removed, the cells were washed with PBS'A' then 0.2ml of Neutral Red in EMEM was added to each well and incubated for 3 hours.

3. The cells were washed and fixed briefly with 4% formaldehyde/1% calcium chloride (no more than 5 sec) then

destained in 1% acetic acid/50% ethanol for 20 minutes on a Luckam suspension mixer (1 rotation/second).

4. The absorbance at 540nm was measured on a Dynatech MR590 Plate Reader.

Luciferin/Luciferase Assay for determination of ATP content

Cells were seeded in 24 well multiwells and treated as for the basic Neutral Red Test. After a 24 hour treatment period the ATP was extracted as recommended in LKB Wallac Application Note 507, i.e.

1. Trichloracetic acid (TCA) extractant was prepared just before use by mixing 1 volume of 10% w/v TCA with 1 volume 4mM disodium EDTA in distilled water.

2. The cells were washed with PBS'A' then 1ml of TCA extractant was added to each well and left for 10 minutes.

3. Before assay the ATP extract was diluted 1:50 in Tris-Acetate Buffer (0.1m Tris-acetate buffer, 2mM EDTA. pH 7.75±0.05 at 25°C) to reduce the TCA content to a non-inhibitory level.

4. The assay was then performed as described in LKB Wallac Application Note 507, i.e.

   100 µl ATP Monitoring Reagent

   300 µl Tris-Acetate Buffer

   10 µl 2M Potassium Acetate

added to cuvette - luminescence reading taken to use as a blank (B).

5. 100 µl sample added and mixed (30 secs) - luminescence reading taken -(S).

6. 10 µl 10^{-6} M ATP standard added and mixed (30 secs) - luminescence reading taken (I).

7. If an ADP determination is required, 10µl 20mM phosphophenol pyruvate (PEP), 10 µl pyruvate kinase (35-50 units/mg protein) added to cuvette and mixed for 1 minute - luminescence reading taken (S_2).

8. The results are calculated as follows :-

$$\text{ATP (mol)} = \frac{S1 - B}{I} \times \text{Amount of ATP standard added (mol).}$$

$$\text{ADP (mol)} = \frac{S1 - S1}{I} \times \text{Amount of ATP standard added (mol).}$$

c. MTT Test

This test is based on the reduction of a tetrazolium salt to its blue formazan product by succinate dehydrogenase in the mitochondrial membrane. The reaction occurs only in active mitochondria, i.e. only in living cells, and so can be incorporated into a quantitative cytotoxicity test.

The procedure described here is based on the method of Mosmann (1983) as modified by Denizot and Lang (1986) [See Results Section].

1. MTT stock solution was prepared at 5mg/ml in PBS and filtered to remove small amounts of insoluble residue. This was then kept at 4°C.

2. MTT stock solution was added to EMEM at 1ml per 10mls medium.

3. Test compound was dissolved in DMSO and added to a suspension of V79/4 cells (2×10^5 cells/ml) to give a final concentration of DMSO of 10 µl/ml medium. Seven concentrations of each test compound were used in microtitre plates (0.2ml/well).

4. After 5 hours the plates were washed 3 times with PBS, and 100µl MTT in EMEM was added to each well.

5. Plates were incubated for a further 2 hours by which time blue formazan crystals had formed in the bottom of each well.

6. The medium was removed, wells were washed once with PBSA and 100 µl isopropanol was added to each well to dissolve the crystals. Plates were mixed for 1 minute on a Luckham Rocker to ensure even dispersal of colour.

7. Absorbance was read on a Dynatech Minireader MR590 at 570nm.

Evaluation of Data

For Neutral Red, ATP and MTT tests the values for quadruplicate wells were averaged and expressed as percentage of solvent control for calculation of ID_{50} values (that dose

resulting in a test value of 50% of control). All results are expressed as mean ± standard error of the mean. Comparison of test data was made by linear regression analysis on a log scale.

RESULTS

a. Neutral Red Uptake

Table 1 shows a comparison of the ID_{50} values determined for 10 of the 13 test compounds. Cyclophosphamide (3mg/ml), Methanol (50mg/ml) and DMSO (10mg/ml) had little or no effect in the basic Neutral Red Test and were consequently not included for subsequent tests. The data demonstrates that some compounds gave lower ID_{50}s when added to cells in suspension (variations 2 and 3) rather than to attached cells in monolayer (basic method and variation 1).

b. ATP/ADP

The possibility of using ATP measurements and ADP/ATP ratios as indicators of sublethal effects on cells was investigated. Readings on dilutions of ADP standard indicated that the minimal limit of detection for ADP was 10^{-8} M; below this concentration interference from residual amounts of ATP in the enzyme mixture masked the reaction. At the cell density used, untreated V79 cells contain approximately 10^{-8} M ATP, so only if almost all of this is dephosphorylated would ADP be detectable.

Exploratory experiments were performed using DNP which is a mitochondrial uncoupler and therefore would be expected to have the greatest effect on ATP/ADP ratios, but as expected no ADP signal was obtained above the background.

Consequently only ATP determinations were made on the test compounds. The LD50's obtained did not differ from those

determined by the basic Neutral Red Test, except in the case of DNP, as was expected (Table 2).

In general the Assay was found to be expensive in time and money and inter- and intra- assay variations were much greater than with Neutral Red. An attempt was made to cut down the cost of the Assay by reducing the constituents of the cuvette to 25% of the original, but it was not possible to reproduce the results obtained using the normal method in this manner.

c. MTT Test

A standard curve was obtained using the same method as Mosmann (1983) with doubling dilutions of cell number. Good results were obtained with less than 10% error, but the linear portion of the curve only extended to 25,000 cells/well. At cell densities above this the solubility of the formazan product became limiting. It was also found that in a few of the wells the serum protein precipitated, thus interfering with the assay.

These faults were also described recently by Denizot and Lang (1986). One of the ways which they suggested of overcoming the problem was to eliminate the medium and MTT before dissolving the crystals in pure solvent. This made three improvements.

1. If all the medium is removed no serum precipitation will occur.

2. The presence of HCl is no longer necessary to acidify the phenol red which interfered with the absorbance reading. The presence of HCl previously altered the spectral qualities of the formazan reducing its absorption peak and thus reducing sensitivity.

3. The use of pure solvent meant that the formazan dissolved completely and almost instantaneously.

Using this revised method the linearity extended to 50,000 cells/well which agrees with the results obtained by Mosmann. As it was intended to use a starting concentration of 40,000 cells/well for the cytotoxicity test this result seemed adequate. The Dynatech MR590 is not sensitive enough to resolve a reading below 1,000 cells/well, but this range is acceptable for obtaining ID50 estimations. An additional step of washing once with PBSA before adding isopropanol to the wells was performed to eliminate the presence of the residual medium.

ID50 values obtained with the MTT test are shown in Table 2. Several of these were significantly lower than obtained with the basic Neutral Red Test.

DISCUSSION

Cytotoxicity Test Methods

Different methods of assessing cytotoxicity have been compared in order to determine the most sensitive and quickest method with a view to adopting its use for assessing water quality. All the tests gave essentially similar results. Table 3 presents the linear regression analysis of the ID50 values obtained (expressed in log form), each method being compared with the basic Neutral Red Test. The plots of these data are presented in Appendix 1. The correlation coefficients show that the values are very closely related. The slopes demonstrate that the most sensitive test was Variation 2 of the Neutral Red Test, ie. cells and test compound plated concommitantly with a 24 hour exposure period. However the differences in slopes are extremely small and do not represent significant differences for all test compounds (see Tables 1 and 2). Thus it appears that all the methods tested here have essentially similar sensitivities and preferences should be made on the basis of convenience and rapidity alone. The Neutral Red uptake and MTT tests can both be performed within one working day (approximately 8 hours). The ATP assay as performed here took 3 working days to complete. This could be decreased but it is unlikely to fit into one working day due to the time taken to measure the ATP content. This is a much more complex and lengthy procedure than with Neutral Red or MTT and as it requires expensive reagents it is the least preferred method. Neutral Red and MTT are equally convenient to measure with the MTT procedure being marginally

faster. The preference between these two tests is dependent upon familarity and availability of the appropriate minireader filter (540nm for Neutral Red, 570nm for MTT).

The Relevance of Cytotoxicity Data

Cytotoxicity tests should detect all substances which are acutely toxic by mechanisms not dependent upon specialised cellular functions. Linear regression analysis of the *in vitro* data with rat oral LD50s (Table 4) produces a straight line with correlation coefficient of 0.773 ($P<0.05$) however there is really insufficient data and the distribution is too uneven to state which values are the outliers (Figure 1). It is therefore more useful to compare the cytotoxicity data with the *in vivo* potency classification (Figure 2). If corresponding *in vitro* classifications are drawn at <10, 10-100, 100-1000 and >1000 µg/ml it can be shown that the Neutral Red Test produced no false negatives and was oversensitive to Triton X-100, catechol, dichloracetic acid and possibly SDS. These are all irritants and it is therefore probably desirable that they should be positive in a cytotoxicity test. However it should be noted that these *in vitro* categories were selected to produce the most favourable comparison.

FURTHER WORK

Methods should be adapted to permit detection of substances requiring metabolic activation. Probably the most convenient method of achieving this would be by using a cell line which possesses some metabolising capacity (e.g. the human hepatoma line Hep G2).

If this proved unsuccessful exogenous metabolising systems could be employed. Ideally these should be designed to support the full range of possible metabolic activation pathways. A single system such as rat liver 9000g supernatant (S9) plus an NADPH regenerating system is unlikely to be sufficient.

Finally the value of cytotoxicity testing must be assessed by screening water samples containing different types of contaminant. For this it is likely that methods should be adapted to use concentrated culture medium diluted with the test sample. Analytical methods of detecting water contaminants, where available, will probably always be more sensitive and provide more information than biological tests. In order for biological tests to become widely accepted it must be shown that they are advantageous in terms of time taken to obtain results and ability to detect the presence of all possible toxic contaminants.

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Borenfreund, E. and Puerner, J. (1985). Toxicol Lett. 24. 119.

Denizot, F. and Lang, R. J. Immunological Methods. 89. 271-277.

Mosmann (1983). J. Immunological Methods. 64. 55-63.

TABLE 1. Comparison of ID50 values obtained by variations of the Neutral Red Test.

TEST COMPOUND	BASIC METHOD	VARIATION 1	VARIATION 2	VARIATION 3	VARIATION 4
Vincristine	0.075	-	0.05±0.026	0.05±0.018	-
Cyclohex-mide	0.033±0.009	1.40±0.31 *	0.10±0.03	0.06±0.01	-
pCMB	3.7±1.3	0.77±0.37	1.67±0.68	2.33±0.93 *	0.68±0.29
Catechol	47.0±6.2	63.8±12.3	41.5±4.7	28.3±2.0	-
Triton X-100	49.3±4.9	45.0±13.9	32.0±2.0 *	18.7±2.5 *	-
SDS	113±3	57.3±23.5	97.7±9.4	102±4.7	-
DNP	180±0	200±15	66.7±3.3 *	40.0±5.8 *	282±29 *
Phenol	3430±1070	3500±1080	660±160	590±80	-
DCA	1300±0	1870±570	800±240	1200±320	-
TCA	13300±1700	10200±2400	5200±2400 *	6200±2200	10000±800

ID_{50} values are expressed as mean ± S.E.M (n=3) in µg/ml.

For abbreviations see Materials Section.

Basic Method - 24h preincubation (attachment period), 24h exposure

Variation 1 - 24h preincubation, 24h exposure without serum

Variation 2 - No preincubation, 24h exposure

Variation 3 - No preincubation, 48h exposure

Variation 4 - No preincubation, 5h exposure

* Significantly different from ID50 value obtained with the basic method determined by Students't test, $P<0.05$.

TABLE 2. ID_{50} values obtained using MTT and ATP determinations.

TEST COMPOUND	MTT	ATP
Vincristine	-	0.05±0.02
Cycloheximide	0.03±0.02	0.07±0.04
pCMB	0.73±0.19 *	2.33±0.42
Catechol	119±32	33.3±0.9
Triton X-100	19.3±4.8 *	34.7±6.2
SDS	48.7±0.9 *	96.3±65.4
DNP	245±27.5	58.3±10.9 *
Phenol	2383±73	1300±626
DCA	1917±192 *	817±369
TCA	6700±3800	19500±8501

ID_{50} values are expressed as means ± SEM (n = 3) in µg/ml. For abbreviations see Materials Section.

* Significantly different from ID_{50} value obtained with the basic Neutral Red method (Table 1) determined by Students' t test, $P < 0.05$.

TABLE 3. Linear regression analyses of ID50 values obtained by different cytotoxicity tests.

	CORRELATION COEFFICIENT	SLOPE
NR Variation 1	0.9302 (P<0.001)	0.9120
NR Variation 2	0.9904 (P<0.001)	0.8849
NR Variation 3	0.9890 (P<0.001)	0.9032
NR Variation 4	0.9848 (P<0.1)	1.1689
MTT	0.9850 (P<0.001)	1.0016
ATP	0.9917 (P<0.001)	0.9564

TABLE 4. Oral rat LD50 values for the test compounds.

		LD50[a] (mg/kg)	POTENCY
1.	Vincristine	NA	
2.	Cycloheximide	2.0	VP
3.	pCMB	NA	
4.	Catechol	3890	NSE
5.	Triton X100	1800	M
6.	SDS	1288	M
7.	DNP	30	P
8.	Phenol	414	M
9.	DCA	2820	NSE
10.	TCA	5000	NSE

a LD50 values obtained from the Register for Toxic Effects of Chemical Substances.

b Classified according to the guidelines in Annex VId to the VIth Amendment of the EEC Directive on the classification of Dangerous Substances (67/548/EEC), i.e.

VP	-	very potent	< 20mg/mg
P	-	potent	⩾ 20 - <200mg/kg
M	-	marginal	⩾ 200- <2000mg/kg
NSE	-	no significant effect	⩾ 2000mg/kg

NA not available.

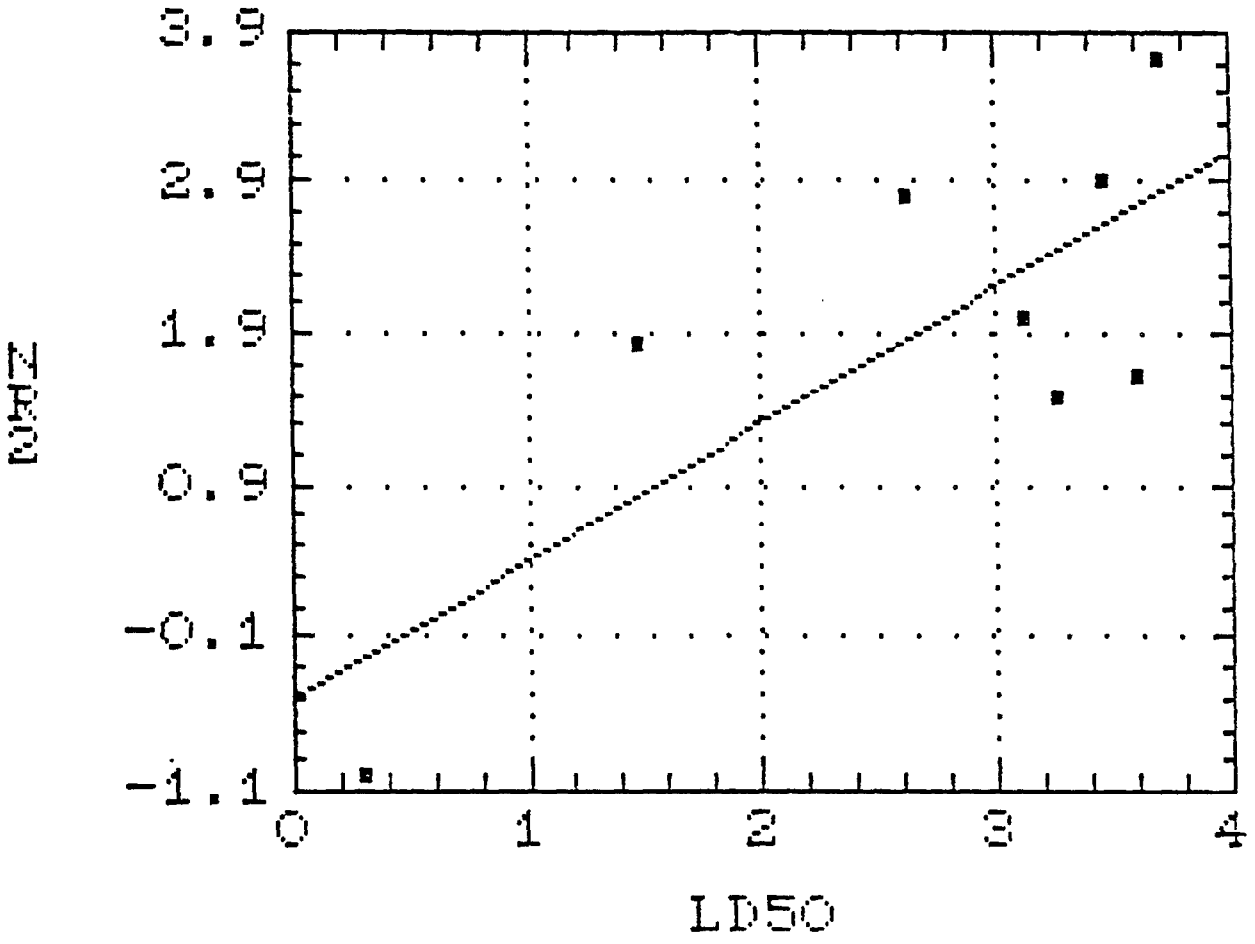

FIGURE 1. Linear regression analysis of ID_{50} values obtained in the Neutral Red Test variation 2 compared with rat oral LD_{50} values (see Table 4).

Values are $\log_{10} ID_{50}$ and $\log_{10} LD_{50}$.

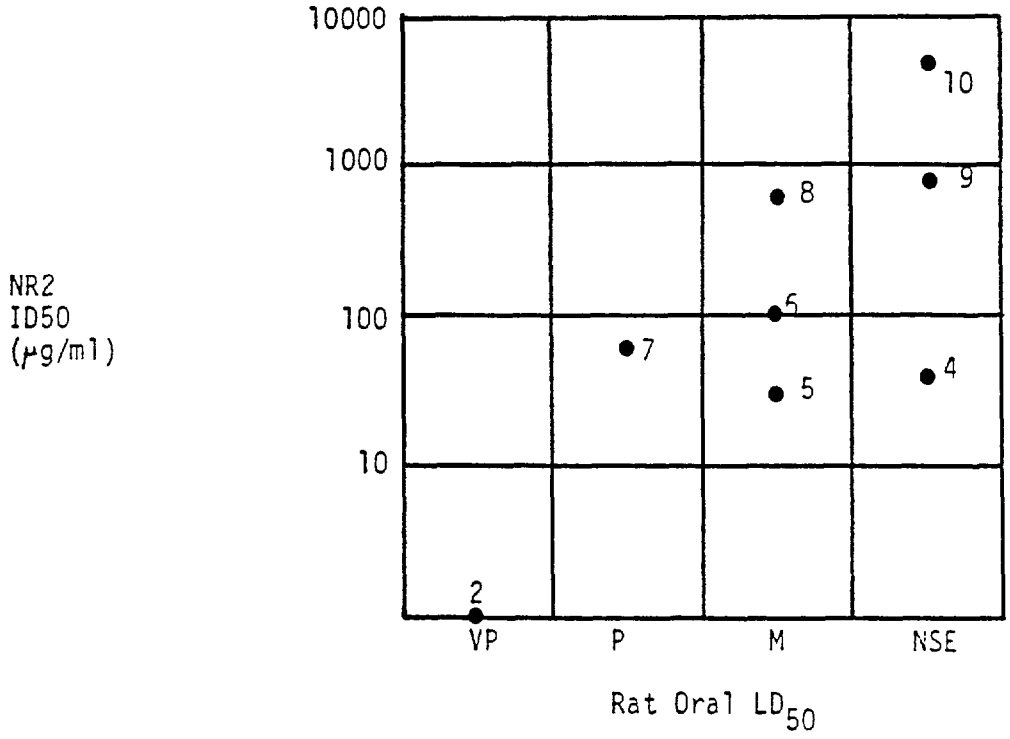

FIGURE 2. Comparison of Neutral Red ID_{50} and Rat Oral LD_{50} data.

See Table 4 for classifications of rat oral data.

APPENDIX 1 - LINEAR REGRESSION ANALYSIS

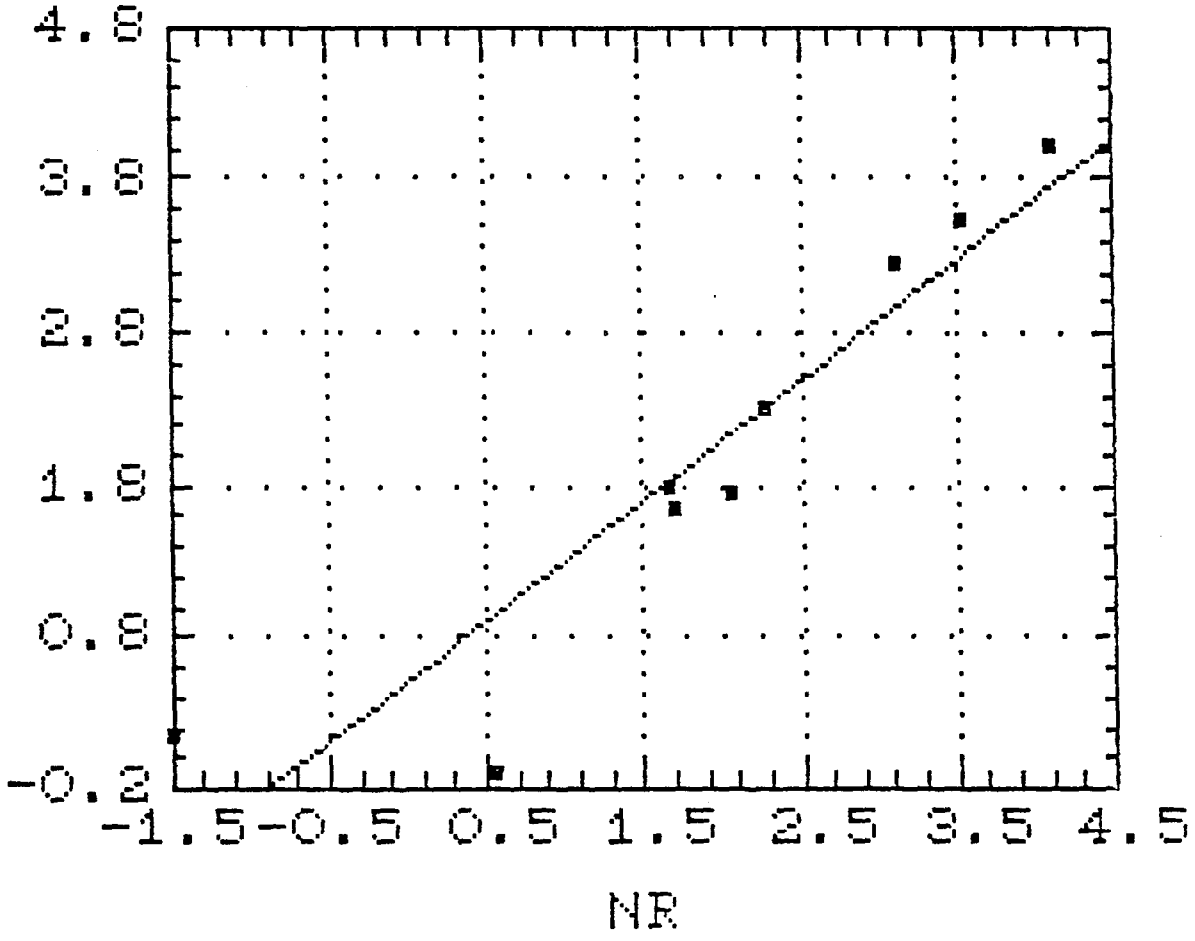

FIGURE 3. Linear Regression Analysis of ID_{50} values obtained in the Neutral Red Test variation 1 compared with the basic test.

Values are $\log_{10} ID_{50}$.

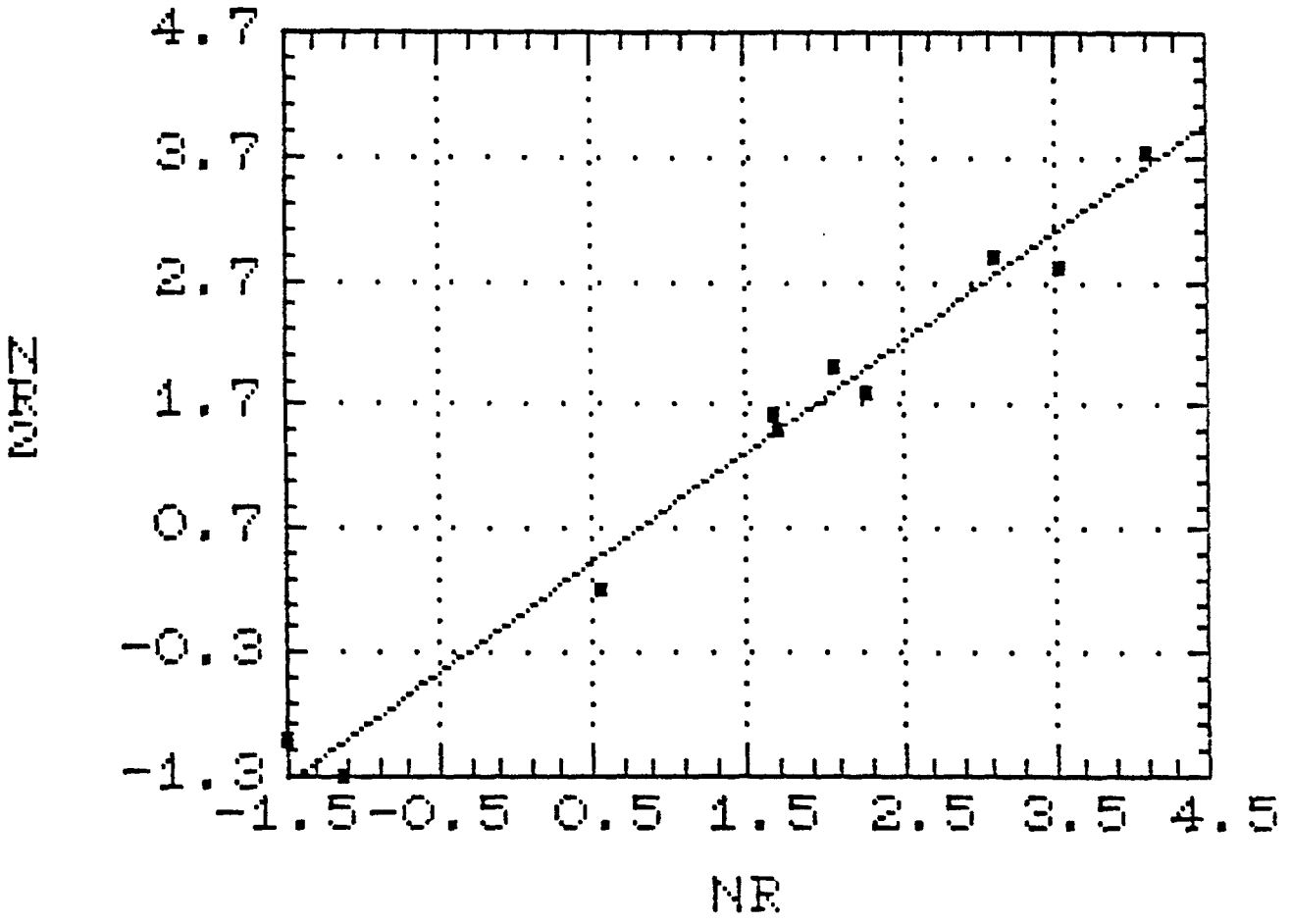

FIGURE 4. Linear Regression Analysis of ID_{50} values obtained by the Neutral Red Test variation 2 compared with the basic test.

Values are $\log_{10}$ ID_{50}

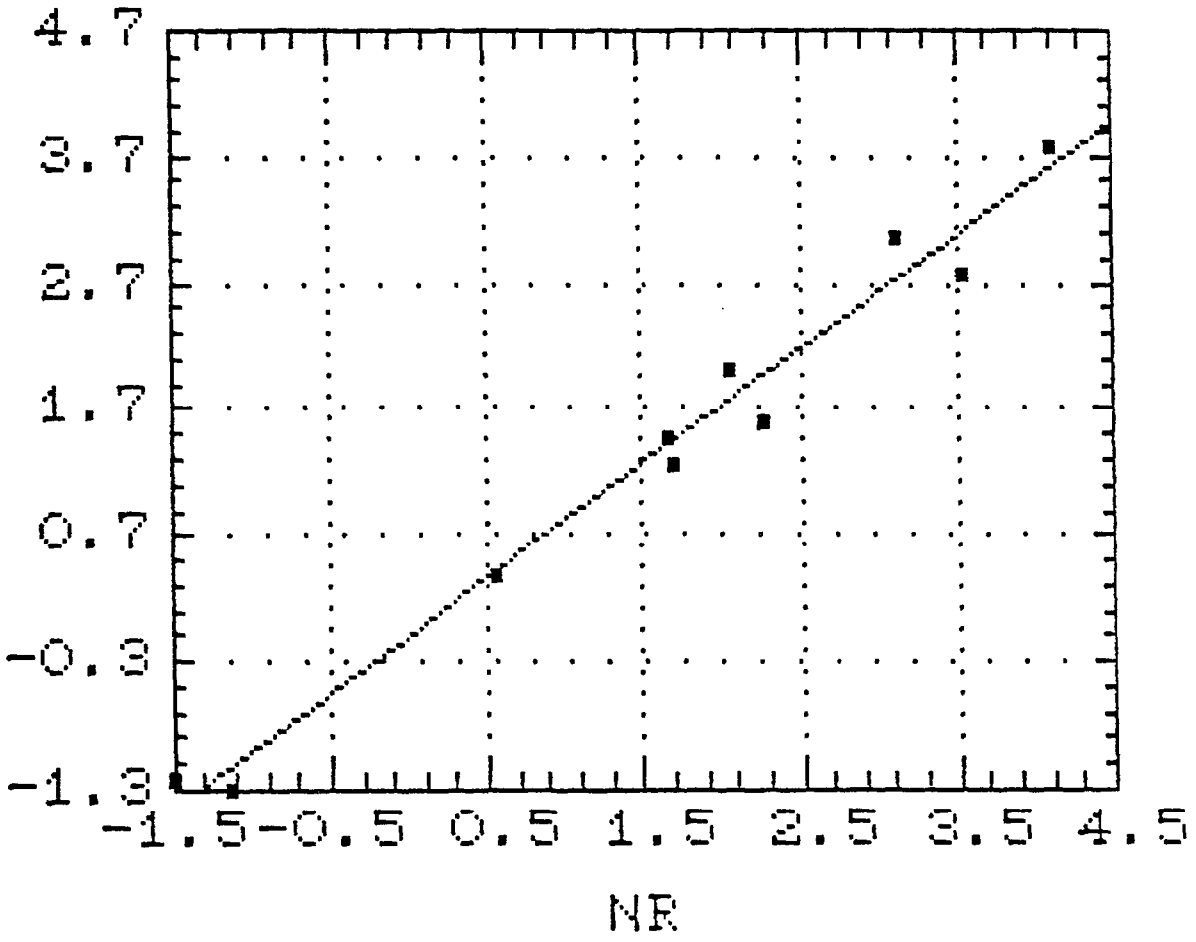

FIGURE 5. Linear Regression Analysis of ID_{50} values obtained by the Neutral Red Test variation 3 compared with the basic test.

Values are $\log_{10}$ ID_{50}.

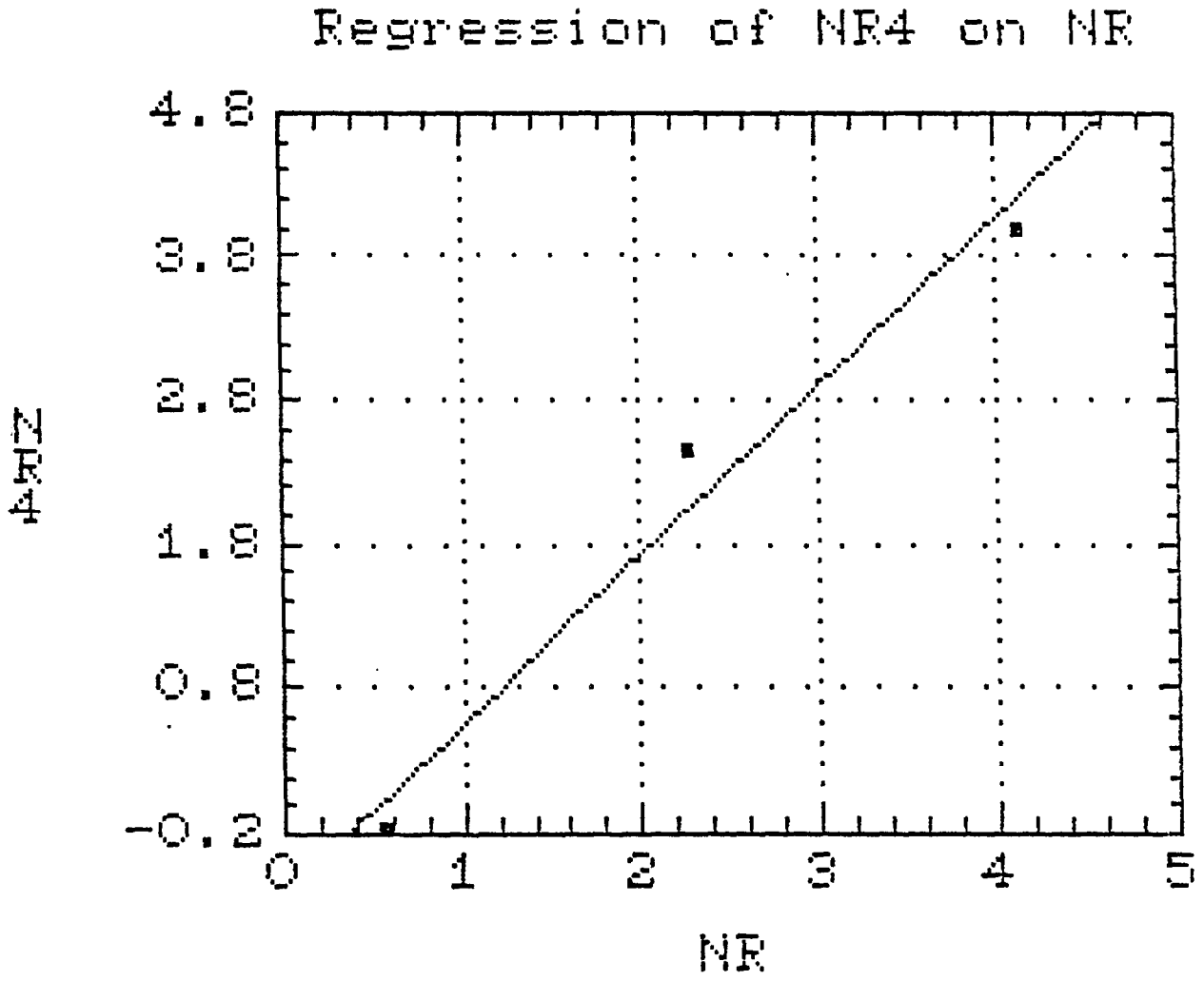

FIGURE 6. Linear Regression Analysis of ID_{50} values obtained by the Neutral Red Test variation 4 compared with the basic test.

Values are $\log_{10}$ ID_{50}.

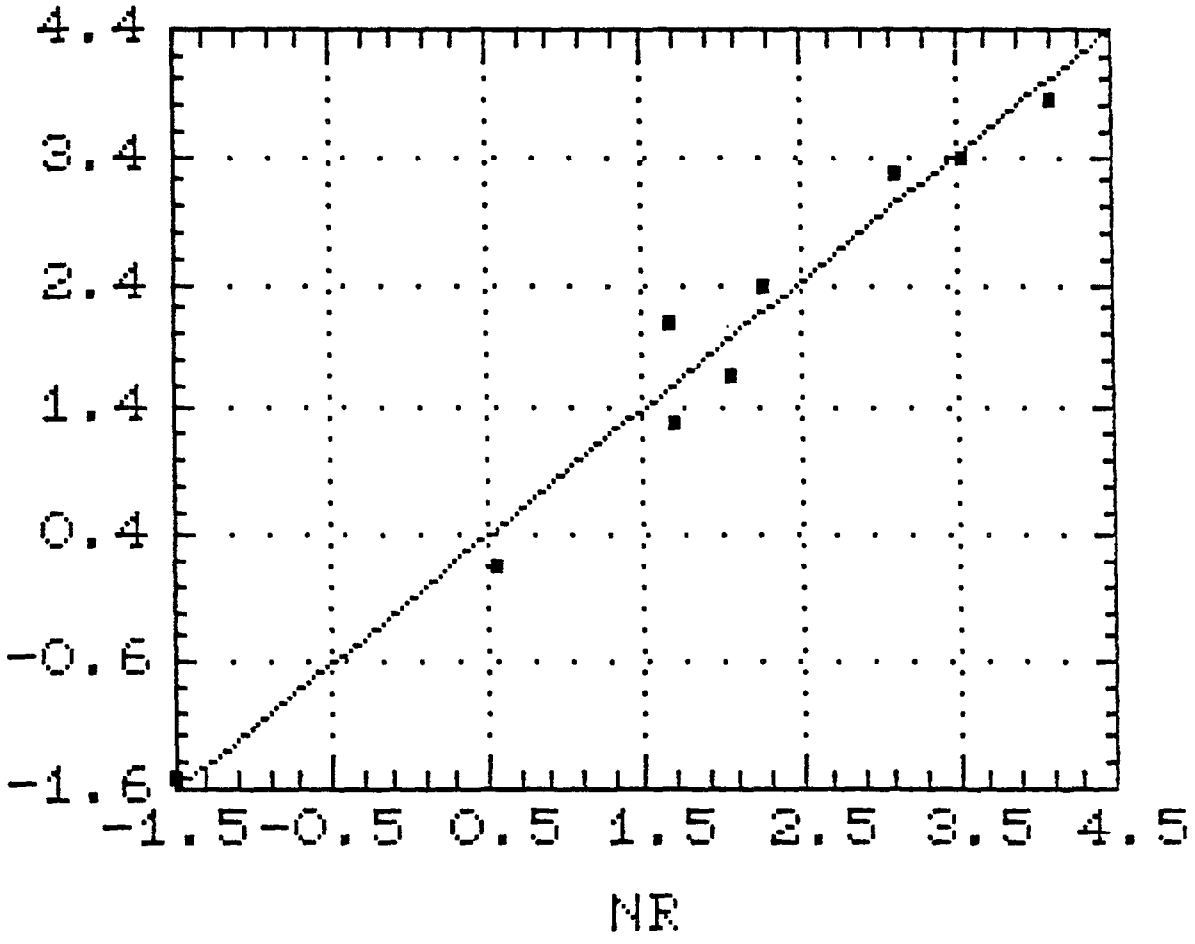

FIGURE 7. Linear Regression Analysis of ID_{50} values obtained by the MTT test compared with the basic Neutral Red Test.

Values are $\log_{10}$ ID_{50}.

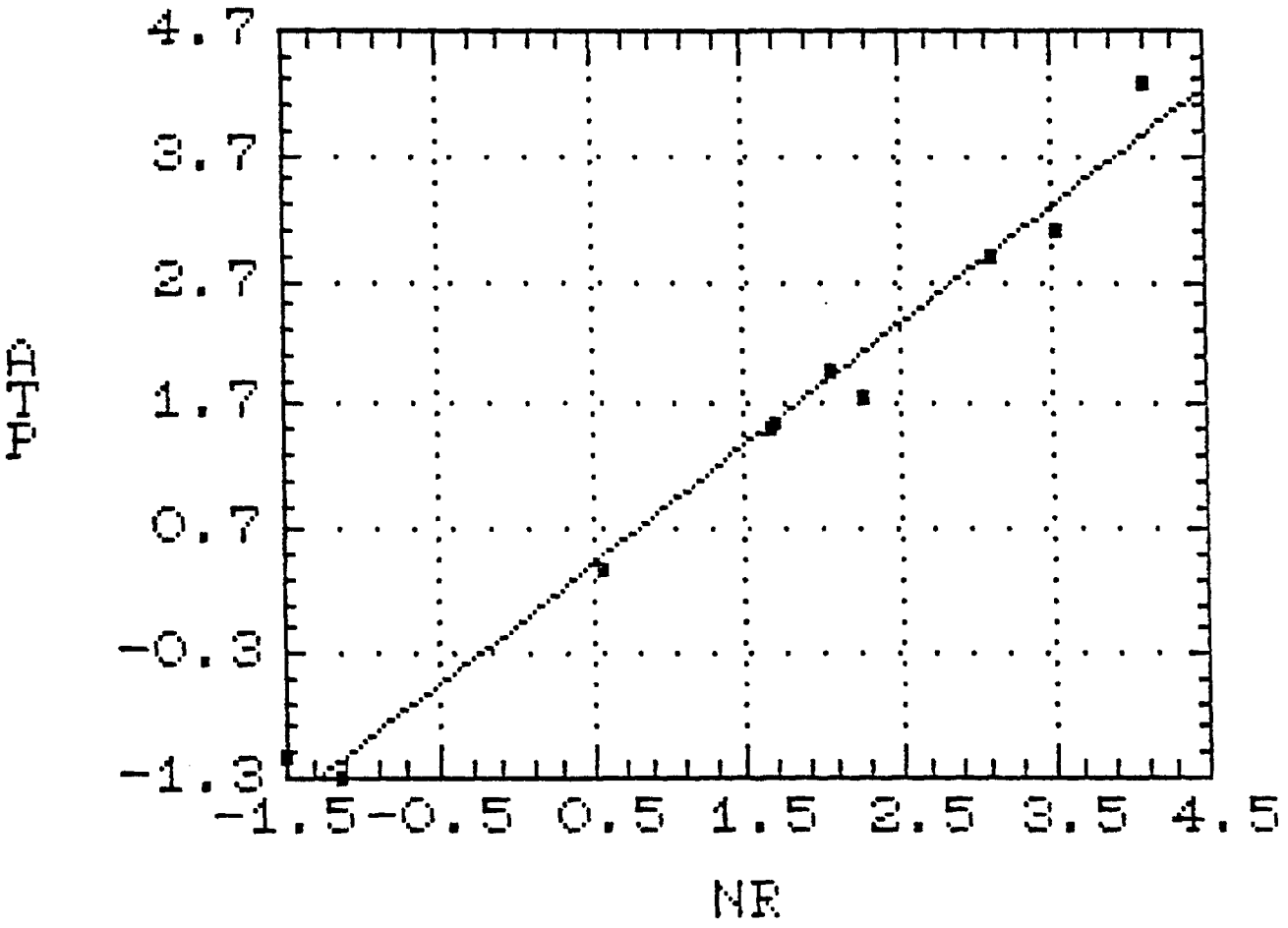

Figure 8 Linear Regression Analysis of ID_{50} values obtained by ATP determination compared with the Basic Neutral Red Test.

Values are $\log_{10}$ ID_{50}.