BIOMONITORING OF WASTE WATER

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Incorporating as an Addendum:

COMPARATIVE EVALUATION STUDY WITH THE WEAVER HUMAN CELL TEST: THE WEAVER HUMAN CELL TEST IN COMPARISON WITH OTHER BIO-SENSOR TESTS USED TO ASSESS THE TOXIC EFFECTS OF POLLUTED WATER

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EXECUTIVE SUMMARY

Motivation

Access to an adequate supply of usable natural water is a basic requirement for human life. At the present time, when the right to such access is being written into law in some countries, there is a diminishing likelihood of its being met for billions of people living in Asia, Africa and Latin America.

Many different factors are involved, the most obvious being the increased demand for water due to population growth and urbanisation, without any matching increase in the supply of fresh water. In the past, wastewater contained much biodegradable material, able to be processed and removed by living organisms. Today's effluents also carry other substances, some of them incompatible with any form of life.

An obvious solution is to deal with the problem at source, where substance-based end-of-pipe methods of analysis are most cost-effective, and regulatory controls can be enforced without difficulty.

The current approach is to use effect-based monitoring systems, including ecological ones, to detect the existence of a problem, and then trace it back to its end-of-pipe source by means of whole organism survival tests. These have their own limitations, and even the simplified kit-type tests now available are not universally applicable.

The alternative to one-off testing is to use integrated batteries of tests designed to meet the requirements of specific practical situations, and for this purpose, human cell culture assays have both advantages and shortcomings, which are discussed in detail in the following pages.

Objectives

The original aim of the project was to develop a rapid, low-cost human cell toxicity test that could be used for the universal monitoring of complex effluents. This would be based upon earlier work listed in the **References**, and the aims listed below were formulated as necessary stages in reaching this final objective:

- Determining the range and sensitivity of the assay
- Simplifying the assay
- Controlling 'drift' in cellular responses to toxic agents.

Results and discussion

The problem of drift (discussed in detail later in this report) is essentially the changing responsiveness of the same cells to the same amounts of the same toxic agents. Cells of higher organisms function by means of biochemical pathways each of which consists of a train of molecular reactions governed by mass action. The pathways are linked together through source molecules and end products, and it is by regulating the availability of these that cells are able to express their different functions. Unused biochemical functions can become attenuated during successive cell divisions and may eventually be lost altogether.

One solution to this problem is to use primary cell cultures at a fixed passage number. This approach requires extensive back-up facilities in the way of animal (or fish) colonies and holding cultures. Another solution is to use established cell lines and select for their ability to maintain alternative metabolic pathways by ‘bouncing’ them between different cell culture environments. We find the following media:

- serum-free with glutamine,

- glutamine-free with serum,
- with glutamine and serum, and
- serum-free, glutamine-free (for the assay).

go a long way towards solving this problem, apparently by stabilizing glutamate metabolism prior to and during the assay.

A major factor impacting on the cost and convenience of cell culture assays is the need to maintain sterile working conditions. We have introduced three simplifying modifications. The first is to work under clean, non-sterile conditions up to the final stages of setting up an assay. The entire plate is then sterilised by brief irradiation with UV light before adding the cells and starting the incubation. The second is to re-sterilise disposable equipment with 70% ethanol while it is still in use and then flush it immediately afterwards with sterile water. This saves time and reduces the consumption of disposable plastic-ware. A third factor adding significantly to costs is the level of staff training and time needed for interpreting results. We have tackled this problem by putting appropriate controls into the test and by using computer-controlled procedures for reading the assays and calculating results.

Under our experimental conditions, each assay generates a number between -30 and 100, which is indicative of the level of cytotoxicity of the sample being tested. It is evident from our results that the values we obtained for known toxic agents all occur at concentrations well above their permissible limits in Class 2 drinking water.

The window between the lower limits of sensitivity of the assay and the upper limits of safety for human consumption can often be closed by concentrating individual samples 20-fold, a feasible approach when working with the small volumes required for the assay. However, because it adds to the costs in consumables and time, it would not be realistic to treat large numbers of non-toxic samples in this way.

In practice, the opposite situation in which a significant percentage of samples are off-scale with respect to toxicity is common in South African mining and industrial effluents. Such samples are easily assayed by sequential dilution.

A series of replicate assays carried out on unknown samples of supposedly usable water from several environmental sources showed characteristic cytotoxicity profiles for some of them.

During the course of the project, several minor changes were made in the methodology with the aim of reducing the risk of variability due to operator bias. These are noted in the report.

Conclusions and recommendations

Cell culture-based toxicity assays, and specifically the human cell assays described here have an obvious place in any battery of tests for evaluating water quality. Their level of sensitivity covers the range of toxicity found in many complex effluents and can be correlated with clinical databases. In addition they integrate easily with currently used methods of chemical analysis.

We conclude that effluents showing cytotoxicity in this assay should be regarded as hazardous for human health until such time as the agents causing the effects have been identified.

ACKNOWLEDGEMENTS

The project was initiated and completed with financial support from the Water Research Commission and from Highveld Biological (Pty) Ltd. Both are gratefully acknowledged.

We thank Dr Steve Mitchell and Dr Elke Bey for their very considerable personal input in helping to focus the direction and aims of this project.

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CHAPTER 1: INTRODUCTION

Access to an adequate supply of usable natural water is a basic requirement for human life, and has been throughout history. It is perhaps ironic that at a time when the right to such access is being written into law in some countries, the likelihood of its being met is fast receding for billions of people living in Asia, Africa and Latin America (GLEICK, 2001).

Apart from the uncertainties linked to global warming, many different factors are involved, perhaps the most obvious being the increased demand for water due to population growth and urbanisation, without any matching increase in existing supplies of fresh water. Despite greater efficiencies in utilisation, growing human interaction with usable source water is converting it into unusable wastewater at an ever-increasing rate.

Part of the problem is obviously due to bigger loads of 'natural' waste (sewage, salts and suspended solids), which could still be dealt with by conventional procedures. However, nowadays this waste often carries with it a cocktail of synthetic chemicals including plasticisers, pesticides, detergents, endocrine disrupters, drug metabolites and even pseudo-biologicals (genetically-modified organisms and viruses). Such effluents cannot be relied upon to clean themselves up under natural conditions, even in large wetlands, and remain a long-term threat both to the environment and to human health.

Consensus opinion is that the problems of pollution (including the cost of cleaning up) remain those of the polluter, and this applies particularly in the case of cross-boundary pollution. The obvious solution is to deal with such problems at source, where substance-based end-of-pipe methods of analysis are most cost-effective and regulatory controls can be enforced with little difficulty.

The problem assumes a different order of magnitude once these single source contaminants have joined the mainstream complex effluent. Synergistic toxic enhancement is increasingly likely, analytical identification of the component elements becomes prohibitively expensive, and tracking down the origins of individual pollutants elusive and time-consuming.

The current approach is to use effect-based monitoring systems, including ecological ones, to detect the existence of a problem and then trace it back to its end-of pipe source. Whole effluent toxicity testing methods (WET tests) using fish, *daphnia* and algae have been successfully applied in several first-world countries (BLAISE, 1991; KNOPS et al., 2001) as well as in South Africa (SLABBERT et al., 1998A, 1998B). Although subject to limitations, they are currently the best hope for regulatory enforcement.

What are the limitations of these first generation whole organism survival tests? To start with, they are difficult to scale up without an adequate infrastructure to provide, for example, an ongoing supply of test organisms of uniform age, size and sensitivity. They require a fair amount of subjective input by the person carrying out the assay, particularly when quantitative assessments are required, and the methodologies are generally inapplicable to problems of chronic toxicity, mutagenicity, endocrine disruption and oncogenesis.

Even the simplified kit-type tests now available are not fully inclusive and need to be read against a background of other information. The current reality is that a universal test has yet to be developed that gives a sufficiently sensitive numerical assessment of water quality, at low cost, and without additional interpretative input from highly trained staff. It is doubtful if any one test will ever answer the ultimate question of whether a sustainable ecological balance can be maintained, short of actually doing such a survey.

In the meantime, the demand for water is increasing, more water-quality testing is needed, and the alternative to one-off testing is to use integrated batteries of tests that best meet the requirements of specific situations.

Several research groups have approached this problem by looking first at lower organisms for evidence of an immediate toxic impact from complex effluents, and only subsequently at higher life forms for information on delayed responses such as endocrine disruption.

The project being reported on here was initiated on the assumption that test systems based on the biological properties of higher organisms could provide at least as much general information as those based on lower organisms, and possibly more in situations with a direct bearing on human health. It represents a practical implementation of procedures and findings previously reported on to the WRC (DAMELIN and ALEXANDER, 2000), and many of the methods used in the present case are based upon similar methods outlined in that report. These in turn incorporated the improvements of CARMICHAEL et al. (1987) and SCUDIERO et al. (1988) on the original procedure of MOSMANN (1983).

Toxicity assays using human cell cultures have certain advantages (MEYER et al., 2001; SHOJI et al., 2000A; VALENTIN et al., 2001). Apart from the obvious fact that they work by detecting interference with cellular biochemical processes identical to those responsible for maintaining human life, there is also a very large background literature available, including much that correlates directly with clinical situations. The cultures tend to be longer lived than cell cultures from the corresponding tissues of small mammals, and are:

- readily available from many sources,
- able to be stored indefinitely,
- ideal for fast micro-assays,
- compatible with numerous existing analytical methods,
- suitable for automated reading, recording and computation, and
- able to be scaled up or down at short notice.

Perhaps the most important advantage of cell cultures is their ability to respond to very low levels of cytotoxic agents after natural or induced genetic modification, and through phenomena such as hormesis (STEBBING, 1982; CALABRESE and BALDWIN, 2000; DAMELIN et al., 2000).

There are also disadvantages. Cell culture systems require:

- an infrastructure able to support sterile work,
- which increases running costs,
- necessitates expensive capital equipment, and
- requires highly trained staff.

Another serious problem is the tendency of cell cultures to 'drift' if they are not constantly re-sourced. This can be due to genetic de-repression, to the introduction of new genetic material by adventitious infections, or to chromosomal shuffling in aneuploid cultures. DAMELIN and ALEXANDER (2000) used this property of adaptation to develop a cell line resistant to cadmium cytotoxicity. Whatever the cause may be, the end result is a lack of reproducibility in the experimental data over time, as levels of toxicant that were once easy to detect cease to be detectable.

The original aim of this project was to develop a rapid, low-cost assay that could be used for universal monitoring of complex effluents. Direct contact with the practicalities of testing complex effluents for toxicity caused this single aim to be re-formulated as the following three objectives:

1. To determine the range and sensitivity of cell culture-based toxicity assays so that they can be included in the integrated batteries of tests needed to monitor toxicity in specific practical situations.

2. To simplify the standard cell culture assay procedures so that they can be carried out rapidly, at low cost, with minimal laboratory facilities.

3. To control the 'drift' in response of cell cultures so that assays would remain consistently reproducible over an extended period of time and from one laboratory to another.

The question of the inter-laboratory reproducibility of the Weaver Human Cell Test is dealt with at the end of this Report in the Addendum by Elizabeth Wittekindt, at that time senior scientist on the staff of the Federal Institute of Hydrology, in Germany.

It is also possible to compare the results obtained in this test with those of various other laboratories that have used and are using *in vivo* and *vitro* test systems. This can be done by correlating the EC_{50} data given in Table 2 with the corresponding data for the same compounds listed in the Guidance Document published by the National Institute of Environmental Health Sciences of the U.S. Public Health Service. (See Guidance Document on Using In Vitro Data to Estimate In Vivo Starting Doses for Acute Toxicity, 2001).

CHAPTER 2: MATERIALS AND METHODS

Materials

- 37° C incubator (set at 5.9% CO_2, Jouan type EG 115 IR)
- 96-well plate (U-type, Nunc or similar, see comment in **Discussion**)
- Activation Medium (Highveld Biological)
- Cabinet for sterile work (Paul Lundie and Associates)
- Colour Development Kit (Highveld Biological)
- Control water (double RO, 60 μS/m)
- Digital pipette (20 - 200 μl, Finnpipette)
- Dispensing pipette (10 μl - 1 ml, Eppendorf)
- Dispensing sheet (see Figure 1)
- Filter funnel and paper (150 mm)
- Hanks BSS (Ca and Mg free, Highveld Biological)
- Incubation Medium (10x conc., Highveld Biological)
- Incubation Buffer (20x conc., Highveld Biological) (see **Discussion**)
- K-562 cells (ATCC or Highveld Biological)
- McCartney bottles (20 ml - sterile)
- Maintenance Media A and B (Highveld Biological)
- Medical specimen bottles (Plastpro Scientific)
- Microplate reader and computer (Labsystems Multiskan RC)
- MTT Reagent (0.25% in water, Sigma)
- Multichannel pipette (Finnpipette 4 - 8-channel)
- Parafilm (3M)
- Self-sealing plastic bags (120 x 180 mm)
- Standard control solution (10 μg/L $CdCl_2$, Rand Water)
- Standard control sample (above solution diluted 10x with control water)
- Sterilizing filters (25 mm disposable, Sartorius or Millipore)
- Suspension Medium (Highveld Biological)
- Trypsin/versene solution (Highveld Biological)
- UV plate steriliser (UV Technology SA)

Collection of samples

Water samples (20 ml) should be collected in medical specimen bottles (40 ml) and stored at 4°C until needed. Clear samples can be used directly or by allowing suspended solids to sediment and then decanting the supernatant water. A clarifying centrifugation or filtration may sometimes be necessary.

Preparation of cell suspension

The cells used for the cytotoxicity assay are derived from the K-562 human leukaemia cell line (ATCC CCL 243) after adaptation to serum-free growth in Maintenance Medium A (supplemented with alanyl glutamate). A confluent culture of these cells is centrifuged down in the presence of trypsin/versene, is re-suspended in Activation Medium (glutamate-free) supplemented with 5% FBS, and is incubated at 37°C for 7 days, after which it is again centrifuged down prior to re-suspension in Maintenance Medium B (containing 5% FBS). Cells may be harvested 1 to 2 days after the second or subsequent changes of Medium B. Cells still remaining in the flask after 3 weeks in culture should be returned to Maintenance Medium A.

Harvested cells ($5x10^6$) are washed with Hanks BSS and are then re-suspended in 2.5 ml of Suspension Medium prior to storage at 4°C for 1 to 4 hours (see comments on cell suspension in **Discussion**).

Acute cytotoxicity assay

- Identify the intended positions of test samples by noting their code numbers in the appropriate squares on the dispensing sheet (Figure 1). Give the dispensing sheet a unique code number and mark this number on a U-type 96-well plate.

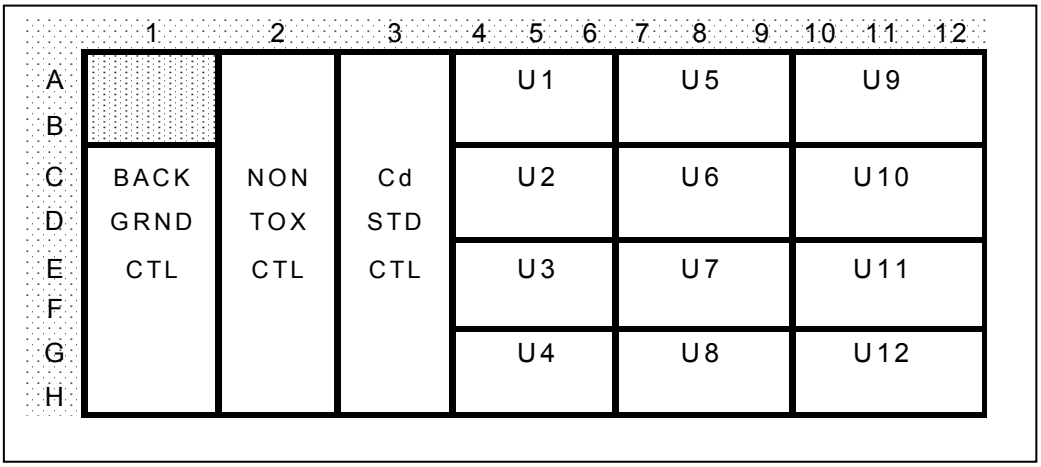

Figure 1. Dispensing sheet used to identify samples and record information during the assay

- Dispense 7 x 170 μl control water into wells 1C, E, G and 2A, C, E, G of the 96-well plate.
- Dispense 4 x 170 μl of the standard control sample into wells 3A, C, E, G.
- Dispense 3 x 170 μl from each of 12 test specimens into adjacent wells in the range A4-12, C4-12, E4-12, and G4-12.
- Sterilise the samples by standing the plate (uncovered) for 15 minutes in the UV plate steriliser in the sterile cabinet.
- Add 20 μl of Incubation Medium to all wells except 1A and B, followed by 10 μl of Incubation Buffer to the same wells; repeat this step once if the phenol red indicator remains yellow.
- Mix and split each of the samples with the multi-channel pipette by withdrawing (5 times) and replacing (4 times) 100 μl aliquots and depositing the final 100 μl in the row of empty wells immediately below (rows B, D, F, H).
- Insert the plate into a plastic bag (unsealed). Gas to an orange colour with CO_2 if all wells are not already this colour.
- Finally, dispense 10 μl of the cold K-562 human cell suspension ($\pm$ $2x10^6$ cells/ml) into wells 2A to 12H, replace the plate cover, and incubate for 18 hours at 37°C in air containing 5.9% CO_2. Confirmatory assays should be incubated for 24 hours under the same conditions.
- At the end of this time, examine columns 5, 8 and 11 of the plate on an inverted microscope and record features of interest on the plate's dispensing sheet. In particular note whether precipitated material is present.

MTT assay

- Dispense 10 μl aliquots of MTT reagent into all wells except 1A and B and incubate the plate for 3 hour at 37°C.

- Terminate the assay by adding 10 μl of Detergent Solution (from the Colour Development Kit) to each of the above wells, followed after 5 minutes by 10 μl Acid. Lightly tap the plate to promote mixing before dispensing 50 μl aliquots of Heavy Oil into the same wells. Use parafilm slivers to break any of the larger bubbles that may have formed.

- Stand for 1 hour at 37°C and 1 hour at 4°C to allow the purple formazan dye to become uniformly distributed in the drop of oil, then bring the plate to room temperature before reading it.

- Measure the O.D. of each well on a microplate reader at 570 μm, blanking against air in each case (Figure 2). Control readings are provided by wells 1C-H (background), 2A-H (non-toxic) and 3A-H (standard). The colour is stable for several weeks at 4°C.

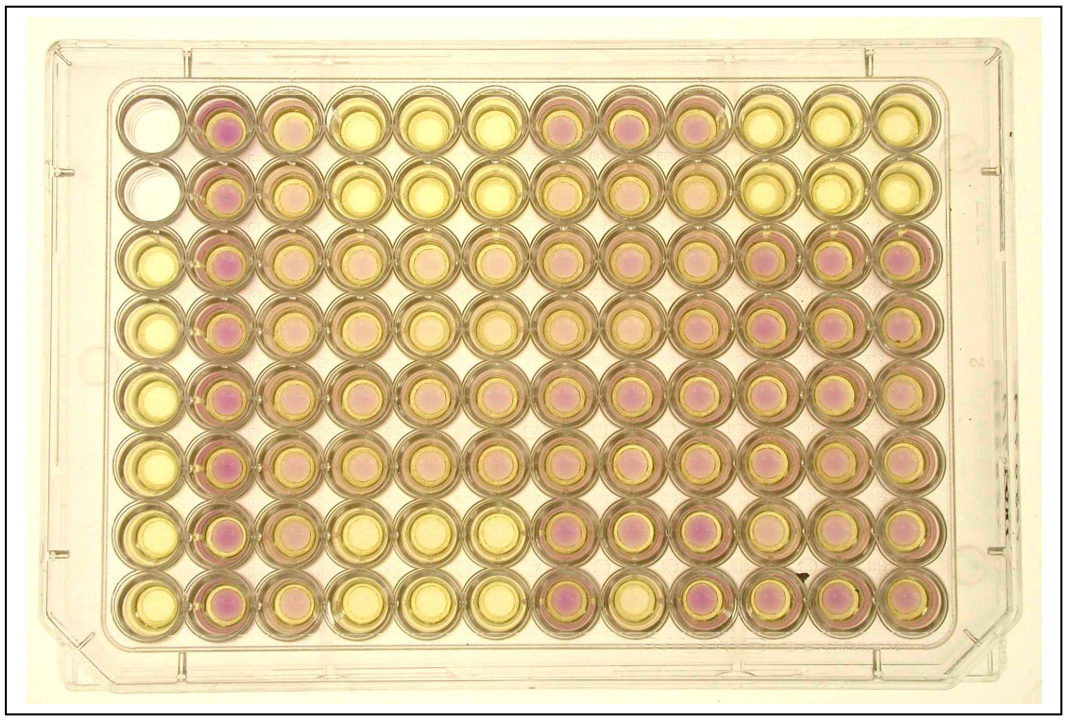

Figure 2. Photograph of 96-well plate before reading
Note: Columns 1, 2 and 3 are the toxic control, the non-toxic control and the Cd standard control respectively. The 12 assay samples are arranged in blocks of six wells each (3 samples across and 4 down). Well G8 is clearly an outlier and its reading would be excluded before calculating the mean cytotoxicity of this sample.

Calculation and interpretation of results

It is first necessary to determine the mean and standard deviation values of the control groups (wells 1C-H, 2A-H and 3A-H). If necessary, exclude up to two readings falling outside the range of mean ±10%.

Sample group readings falling outside the range covered by sample mean ±10% may be excluded up to a maximum of two.

Sample groups with readings having too high a standard deviation should be re-tested, although this decision will obviously depend on the type of sample being tested. In general, standard deviations of 5% or less are acceptable.

The program will invalidate plates with:

- mean background control reading >0.090 optical density units,
- mean non-toxic control reading <4x mean background control reading or <0.300 optical density units,
- cytotoxicity of mean standard control outside of preset range (see comment in **Discussion**)

Mean sample readings can be correlated to one another and to readings obtained on other occasions by calculating their percentage cytotoxicity according to the following formula:

$$\% \text{ cytotoxicity} = 100 - \frac{100 \times (\text{sample mean} - \text{background mean})}{\text{non-toxic control mean} - \text{background mean}}$$

Samples giving values >50% should be retested after twofold dilution with control water. Extremely cytotoxic samples may require serial 2.7-fold dilution. This gives a 20-fold dilution down the plate from wells A to H.

Cytotoxicity values >20% indicate an overt response by the cell culture to agents present in the water sample. Values in this range require confirmation by repeat assay or, if >50%, by dilution assay.

Values between 11% and 20% indicate either marginal cytotoxicity or metabolic imbalance. The former would be due to the presence of low levels of cytotoxic agents, and the latter to much larger amounts of substances not usually regarded as toxicants. Extending the incubation time to 24 hours may help to distinguish between the two. For more certainty, fractionation or concentration assays would have to be done.

Samples giving values from -10% to 10% must be regarded as not detectably cytotoxic.

Values below -10% indicate a stimulatory response by the cells. This could be due to nutritional stimulation or hormetic (non-toxic) effects, or it could be a precursor indication of subsequent cytotoxic effects (chronic toxicity). In all cases, they represent a response by the cells to substances that should not be there.

It is convenient to work on known compounds as mg/l of the actual chemical in the test specimen or as micromoles of the toxic component, e.g. 1 mg/l of $CdCl_2$ or 5.46 μM Cd. In the process of carrying out the assay, the specimen is diluted to 1.3x its original volume; this factor can be ignored if all specimens are handled in the same way.

Unknown cytotoxic solutions may be compared with one another and with known standards by determining the relative dilutions (or concentrations) of each that are able to produce EC_{10} or EC_{50} responses in the cell cultures.

CHAPTER 3: RESULTS

Control assays

Altogether more than 6000 individual assays were carried out during the investigation; this report is based only on the final 3000, which were captured in a database. By this stage, the basic procedures had been largely standardised and the project aims were focused on controlling 'drift' and identifying problems that might require special handling.

Analysis of 212 background control assays (with no cells present) gave a mean O.D. reading of 0.069 units.

Analysis of 57 duplicate (on the same plate) non-toxic control assays gave a standard deviation of 6.44%. This translates into a 99.7% probability that test samples reading >20% are in fact cytotoxic, and a fair probability that many of those reading >10% are also.

The cells used in the assays are responding to multiple biochemical equilibria (see Figure 11), and it is necessary to evaluate some of the factors likely to affect the reproducibility of their response. Two such factors are the time needed by the cells to equilibrate with the normal test medium, and the rate of sequential interference with their functioning by cytotoxic agents. Table 1 shows the results obtained in a modular plate experiment carried out over 30 hours.

Table 1. Effect of incubation time upon the non-toxic control, the 1mg/l $CdCl_2$ standard control solution, and an environmental test sample

Time in hours	Non-toxic control in O.D. units	1 mg/l $CdCl_2$ % cytotoxicity	Environmental sample % cytotoxicity
15	0.564 (78%)	57	27
18	0.648 (90%)	80	24
21	0.686 (95%)	93	31
24	0.700 (97%)	95	32
27	0.725 (101%)	96	32
30	0.717 (100%)	96	29

Figure 3 gives the distribution of mean cytotoxicity (%) for 178 individual 8-fold assays that were carried out on the standard control solution (1 mg/l $CdCl_2$) in the course of assaying a series of environmental samples.

There are indications of three peaks, or distinct metabolic states, in which the control standard shows 36-40%, 46-55% and 90-95% cytotoxicity.

In the earlier experiments, we attempted to bring the Cd standard reading as close to 50% as possible. This proved to be difficult. In later experiments, we found that replacing 30% to 50% of Maintenance Medium B with fresh medium every day (i.e. allowing the cells to condition the medium) put the cells into a stable metabolic state with a reproducible 75% to 100% sensitivity to the 1mg/l $CdCl_2$ control standard. This is shown in Figure 4. Recent work suggests that it may also be possible to put the cells into an alternative metabolic state with a reproducible 10% to 35% sensitivity to the $CdCl_2$ control standard.

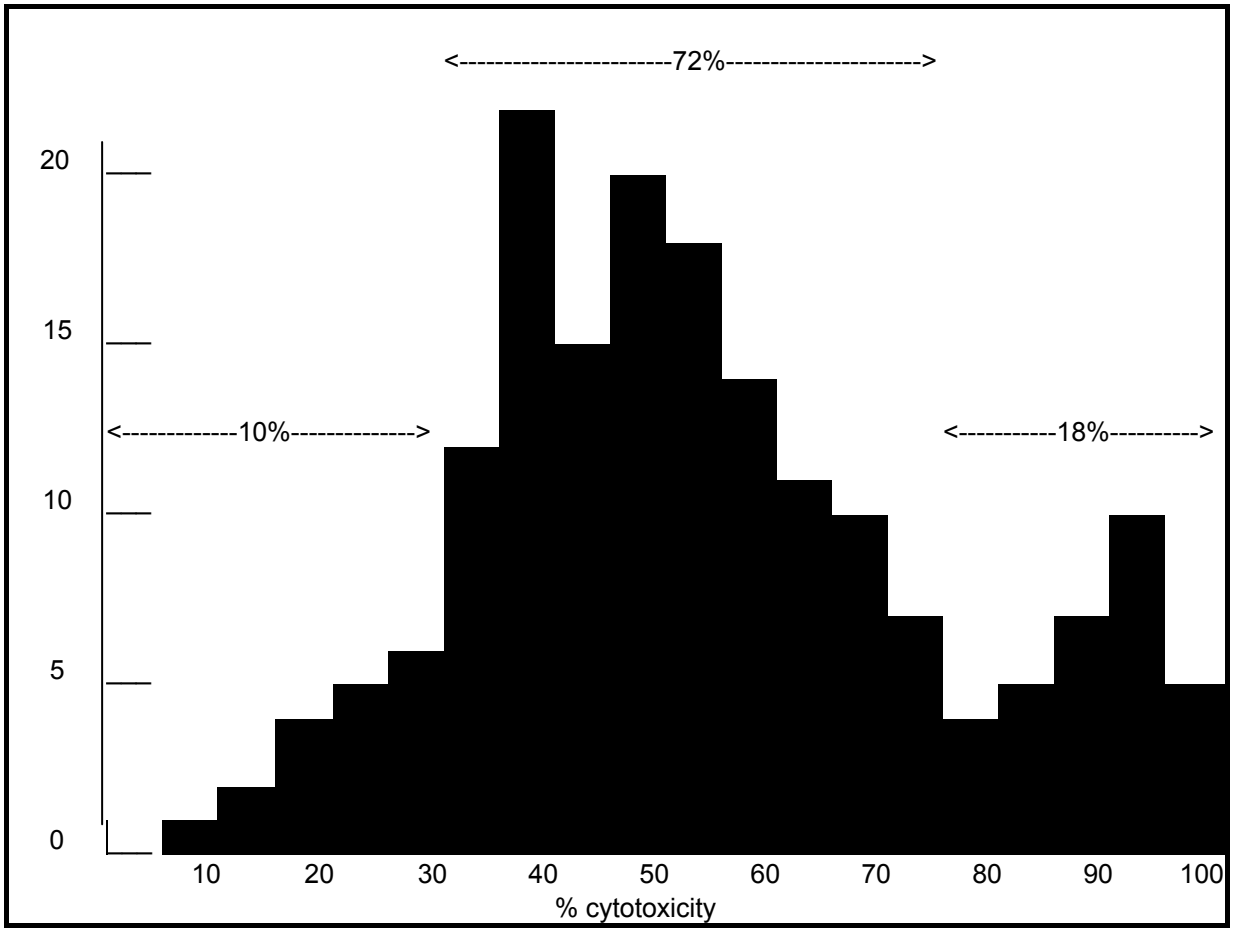

Figure 3. Distribution of cytotoxicity after 178 assays on a control solution of $CdCl_2$ (1 mg/l)

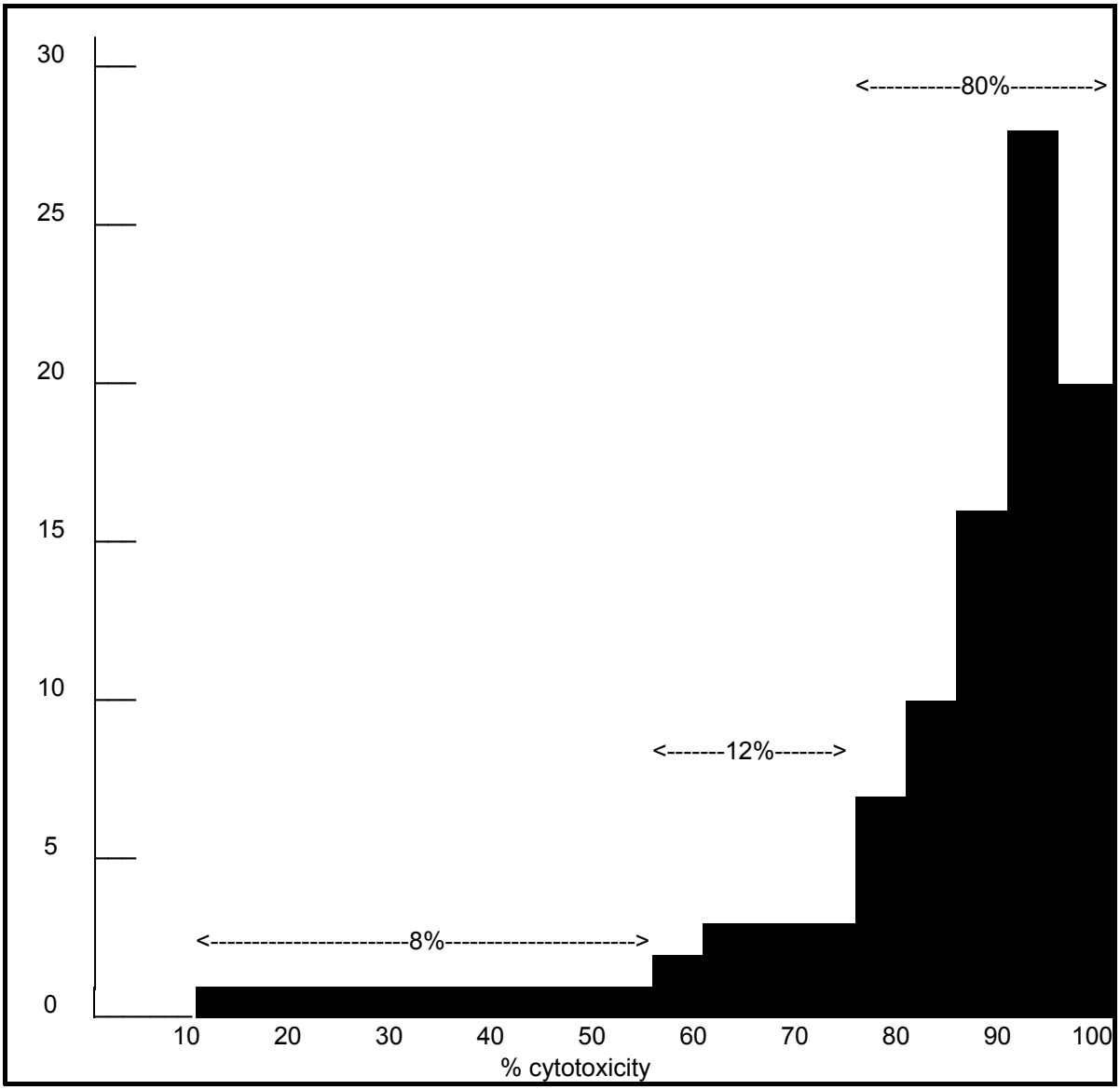

Figure 4. Distribution of cytotoxicity after 114 assays on a control solution of $CdCl_2$ (1 mg/l)

Assays carried out on known toxic chemicals

Table 2 shows the results obtained with various chemical compounds known to show toxicity in specific situations. Comments on the effects of individual chemicals are given as footnotes.

Table 2. Toxicity of various chemicals on K-562 cells *in vitro*

Name	**Toxicity mg/kg = ppm LD_{50}	Toxicity ug/ml = ppm	
		EC_{50}	EC_{10}
acetone	10700	6600	1050
acetonitrile	3800	-	>10000
acridine orange	-	3.8	0.6
9-aminoacridine	78	9.5	1.5
atropine	622	33.1	5.3
cadmium (ionic)	54.0.61	0.097	
cadmium chloride	88	1.0	0.16
cadmium chloride/EDTA *(a)	-	>12	>2
caffeine	127	20.9	3.3
calcium chloride *(b)	42	315	250
chromium ($K_2Cr_2O_7$)	-	1.9	0.31
copper sulphate	960	19.1	3.0
dimethyl formamide	6800	-	4800
dimethyl sulphoxide	17900	-	6050
2:4-dinitrophenol	30	20.9	3.3
EDTA	-	47.9	7.6
ethanol 1% *(c)	10600	-	>10000
fluoro-acetic acid	5	>63	>10
glyphosate adsorbed *(d)	1568	<2.5	<0.4
glyphosate free	1568	6.3	1.0
lead nitrate	-	50.1	7.9
lead nitrate/2-mercaptoethanol *(e)	-	14.4	2.3
magnesium chloride *(f)	8100	-	4300
2-mercaptoethanol *(g)	345	>12	>2
mercuric chloride	19	0.50	0.078
mercuric chloride/2-mercaptoethanol *(h)	-	0.10	0.016
methanol 1% *(i)	1500	-	>10000
4-nitrophenol	467	36.3	5.8
pentachlorophenol *(j)	146	25.0	4.0
phenol	530	43.7	6.9
potassium chloride	-	3980	632
potassium cyanide	10	16.6	2.6
proflavine	140	11.5	1.8
sodium arsenate	6	>63	>10
sodium chloride *(k)	-	-	5000
sodium dodecyl sulphate	-	4.6	0.73
sodium fluoride	180	44	6.9
sodium nitrite	180	1.0	0.16
thymol	980	>63	>10
zinc sulphate	-	5.8	0.91

*Footnotes

a) Cadmium toxicity is neutralized by EDTA (antagonism).

b) Several biological functions are dependent upon having a correct calcium balance. Calcium overloads sufficient to disturb this balance are distinguishable from toxic effects by their 'dog-leg' dilution curves (see Fig.6).
c) Ethanol at low concentrations is virtually non-toxic to human blood cells.
d) Glyphosate preparations usually contain surfactants to promote toxic adherence.
e) Lead toxicity is enhanced 5-fold in the presence of 2-mercapto-ethanol (synergism).
f) K-562 cells tolerate variable concentrations of magnesium (unlike calcium).
g) 2-Mercaptoethanol is non-toxic at synergistic concentrations.
h) Synergism (see lead).
i) Methanol (like ethanol) is non-toxic to blood cells.
j) Pentachlorophenol causes severe hormetic effects at 1 mg/l.
k) This is in addition to the 6800 mg/l of sodium chloride present in the culture medium.

**LD_{50} values are taken from *The Merck Index*, twelfth edition, published by Merck Research Laboratories, Whitehouse Station, NJ. 1996.

In order to obtain this information, it was necessary to carry out dilution assays on these chemicals. In most cases, there was a linear relationship between cytotoxicity readings in the 20% to 80% range and the logarithm of the concentration of the toxicant. The graphs tended to become asymptotic (sigmoid) as they approached 0% and 100% cytotoxicity, but the linear section could be extrapolated to intersect the X-axis at a characteristic threshold point (EC_0). Two such graphs, apparently corresponding to the first peak of Figure 3 and the peak of Figure 4, are shown for $CdCl_2$ in Figure 5. These translate into $CdCl_2$ detection limits (21% cytotoxicity) of 155 μg/l and 489 μg/l or 0.846 μM and 2.67 μM respectively.

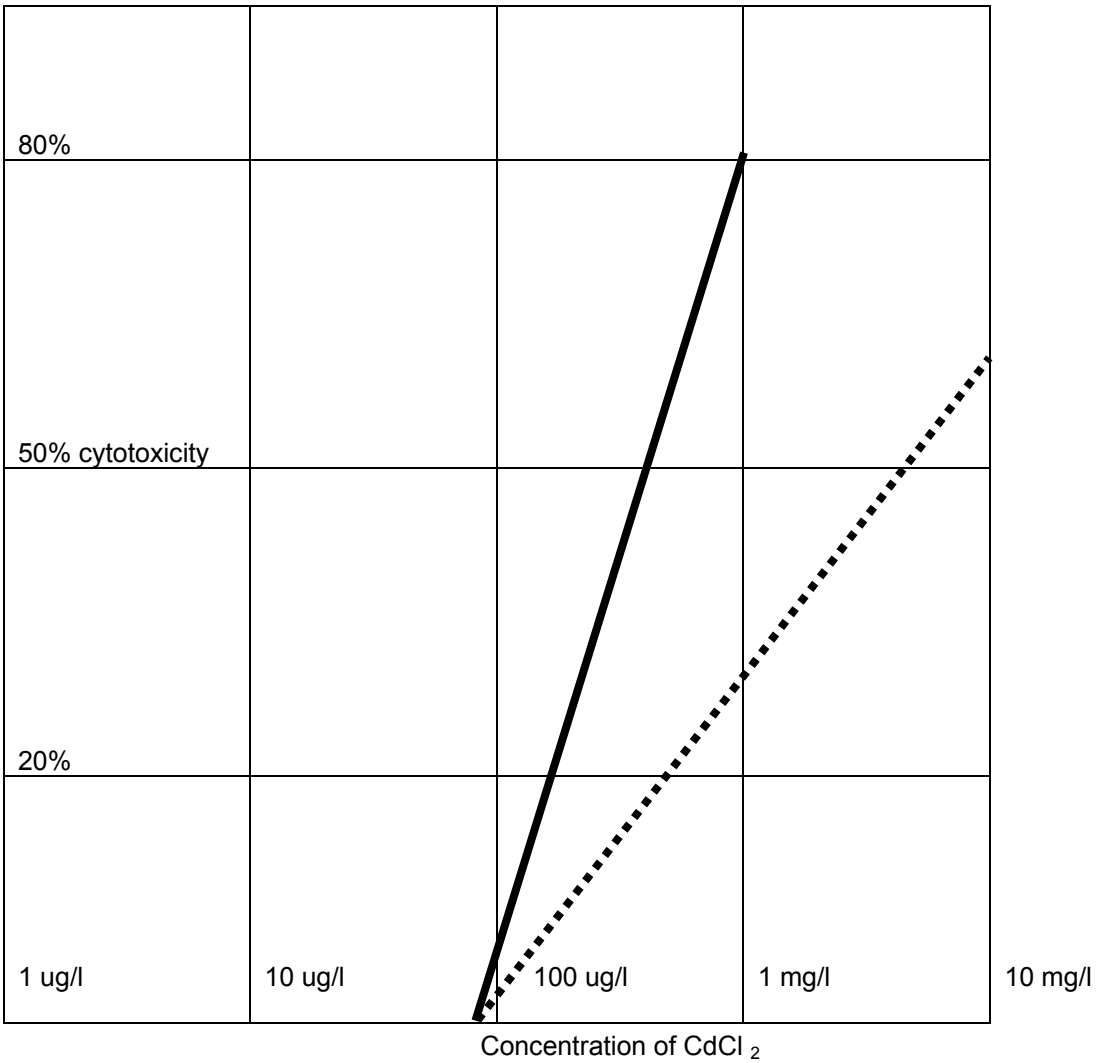

Figure 5. Cytotoxicity of $CdCl_2$ when tested against K-562 cells of higher and lower sensitivity to cadmium

With some chemicals, a reproducible ‘dog-leg’ effect was obtained (Figure 6).

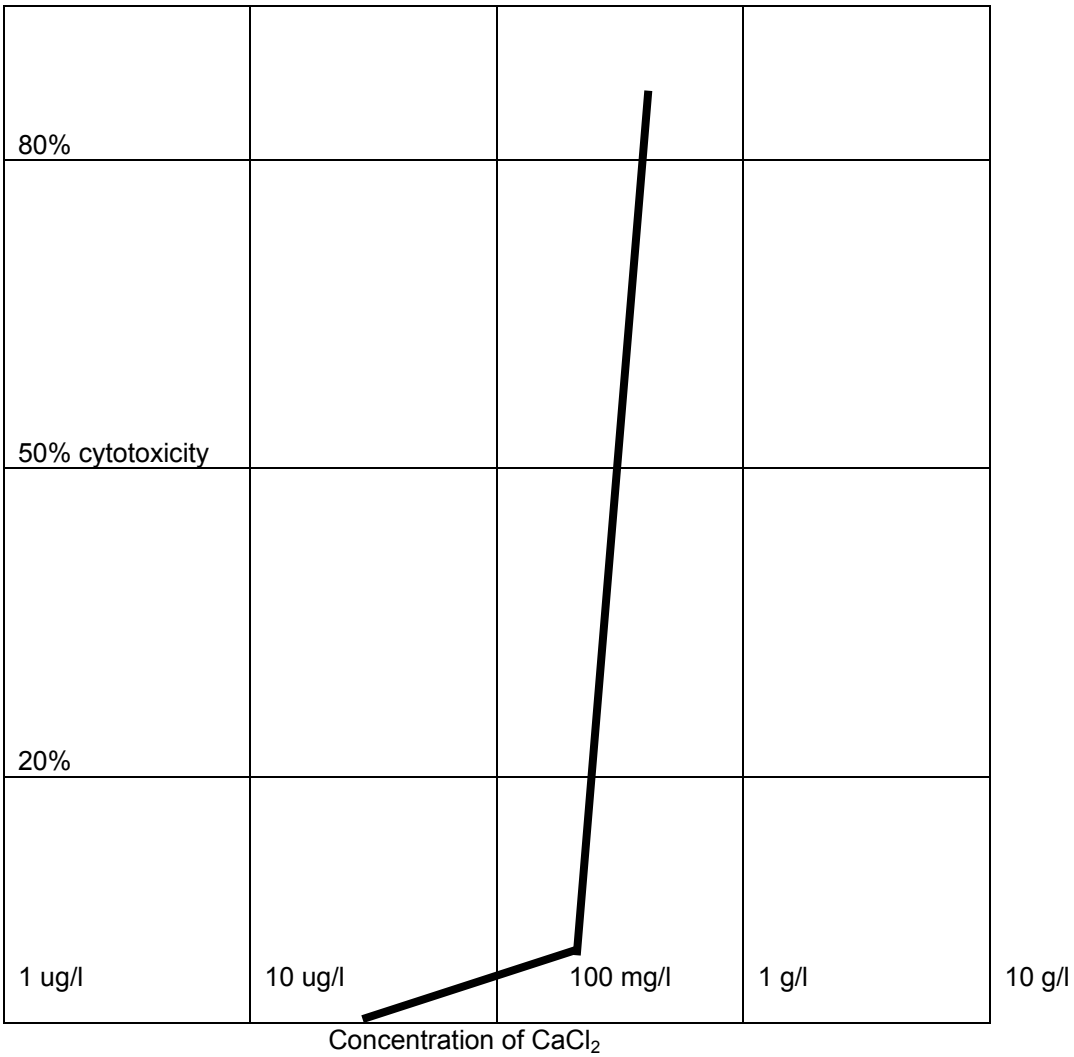

Figure 6. Cytotoxicity of calcium chloride when tested against K-562 cells

Table 3 and Figures 7 to 10 summarise the cumulative results of assays carried out on groundwater samples from defined environmental sources. The data have been edited to include only those assays for which the corresponding 1mg/l cadmium standard control falls within the 30% to 70% toxicity range.

Assay results for samples #92 and #101 (Table 3) were all clustered within the non-cytotoxic range with S.D. values of 9% and 8% respectively. These results do not differ significantly from those of the non-toxic control. In contrast, the other three samples (#98a, #98b and #79) showed a different pattern of response, with most of the results in the cytotoxic range. For sample #79, this pattern persists even when the edited assay results are retained (Figure 10).

Table 3. Multiple cytotoxicity assays carried out on groundwater samples from defined environmental sources

Sample identification	#79	#92	#98a	#98b	#101
Number of assays	13	12	40	29	15
Assays within Cd_{30-70} range	6	10	22	21	12
Assays within cytotoxic range	5	0	19	19	0
Mean cytotoxicity (%)	51	4	52	53	5
Conductivity (mS/m)	50	43	162	129	40

Note: Samples #98a and #98b were taken from the same source nine weeks apart. Calculations are based upon those assays where the 1mg/l Cd standard control fell within the 30-70% range.

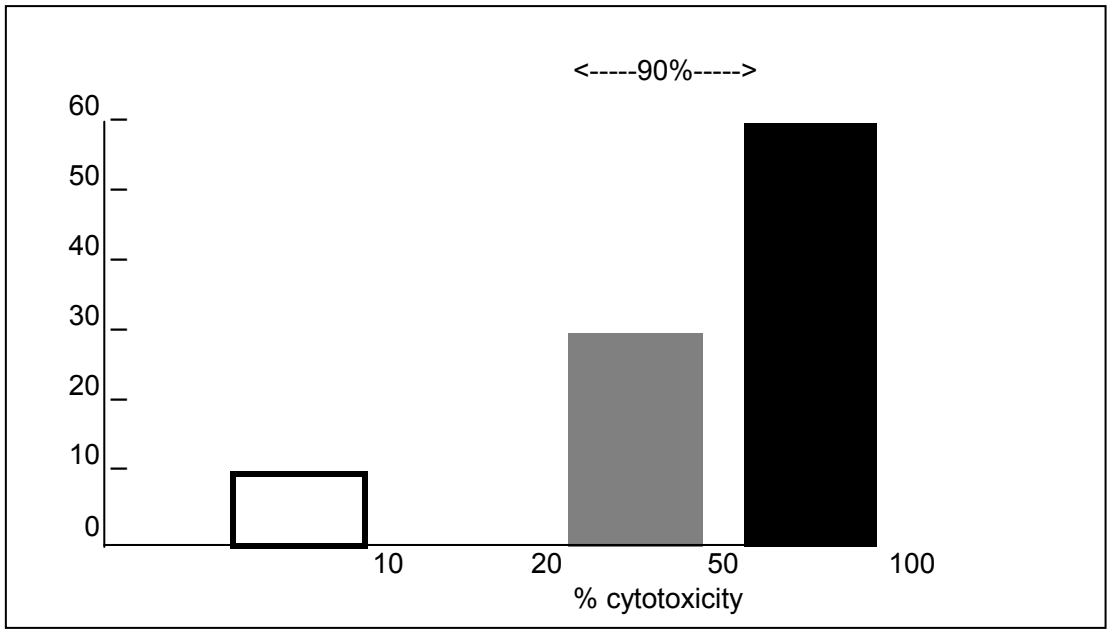

Figure 7. Distribution of cytotoxicity after 22 assays on a sample of surface water from source # 98a

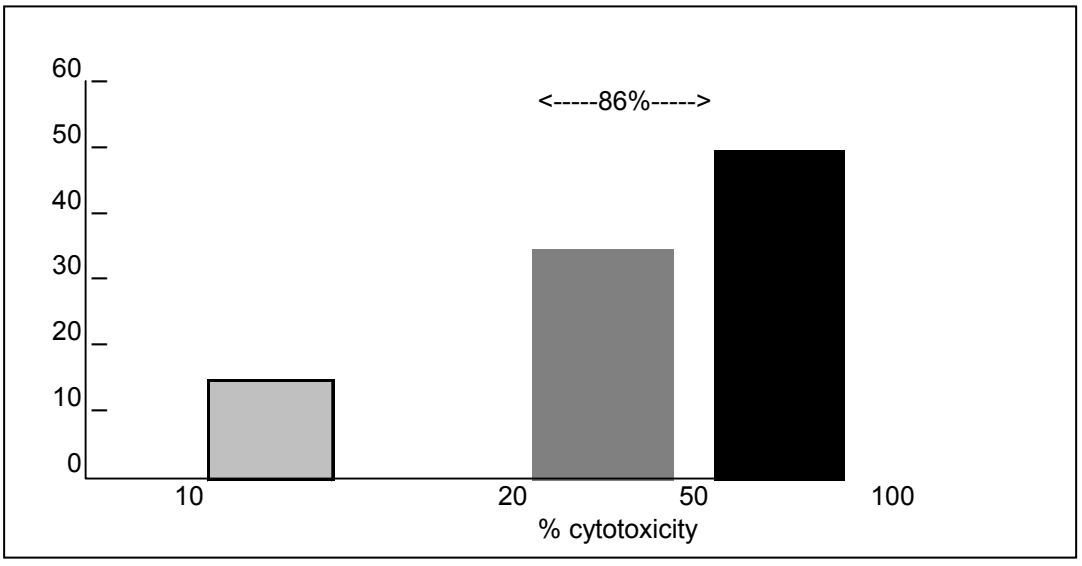

Figure 8. Distribution of cytotoxicity after 21 assays on a sample of surface water from source # 98b

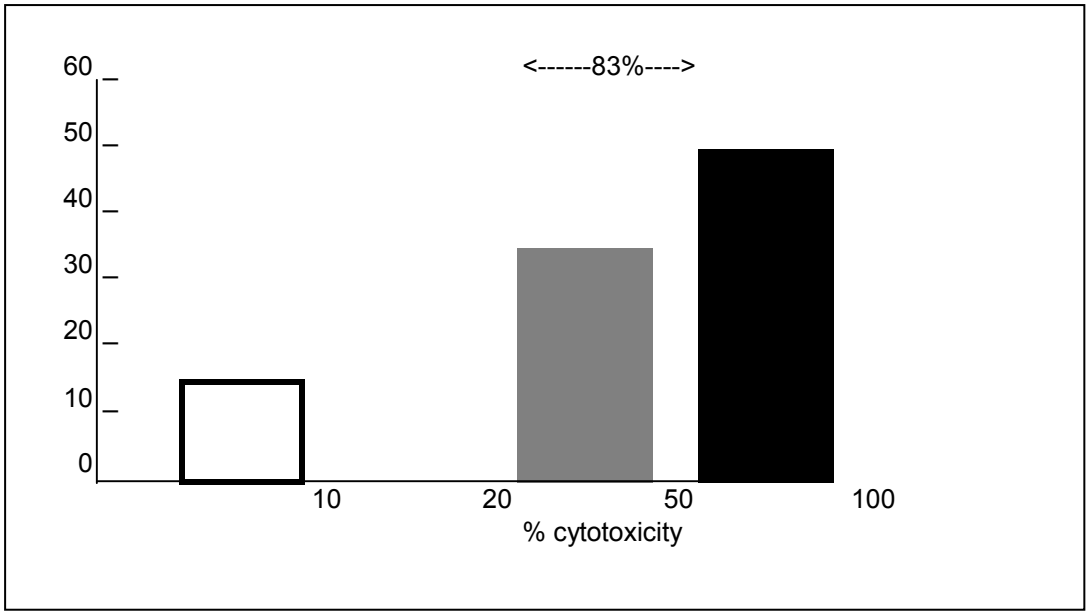

Figure 9. Distribution of cytotoxicity after 6 assays on a sample of groundwater from source # 79

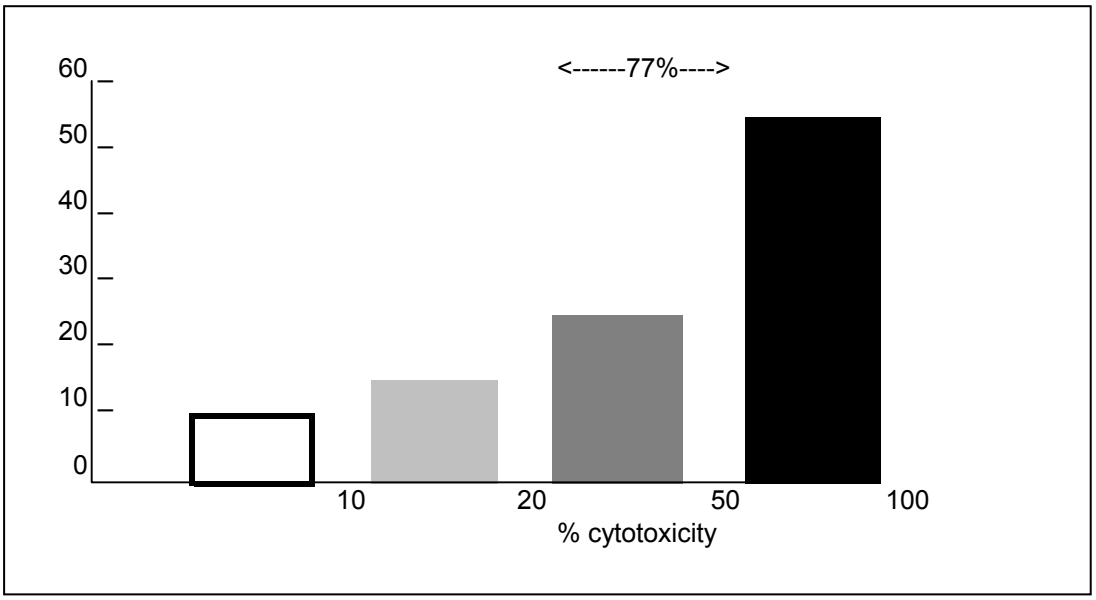

Figure 10. Distribution of cytotoxicity after 13 assays on a sample of groundwater from source # 79

CHAPTER 4: DISCUSSION

The **Introduction** defines the purpose of this project as being the development of a universal monitoring system for wastewater. Various experimental difficulties had to be overcome during the course of developing the project, and the aims listed below were formulated as necessary interim stages that had to be reached before the final objective could be attained.

- Determining the range and sensitivity of the assay
- Simplifying the assay
- Controlling the 'drift' in cellular response to cytotoxic agents.

For practical reasons, it proved easier to deal with these aims in reverse order.

Controlling drift

Biomonitoring presupposes a high degree of stability in the biological component of the test system. However, it also presupposes a high degree of sensitivity, and sensitive biological systems are inherently unstable. The problem is therefore to retain stability without losing sensitivity. Comparative variations in the cytotoxicity of $CdCl_2$ under mildly different experimental conditions are shown in Figure 3 and illustrate this point.

Changing the experimental conditions under which an assay is carried out can be expected to affect sensitivity. Drift may be defined as a reproducible shift in sensitivity when the experimental conditions have not been changed, and is a recurring feature of experimental systems involving long-term mammalian cell cultures. There are several reasons for this.

All mammalian cells contain some genetic material that is in continuous use, other genetic material that is periodically used, and an excess of genetic material that is only metabolically active when it is replicated. Cells growing in long-term culture tend to adjust their metabolism towards one that is economical for their current environment, and if this environment remains stable for a prolonged period, the tendency is to lose those functions associated with unused genes by progressive natural selection. In particular, cells will tend to lose functions that enhance their vulnerability to environmental cytotoxins in situations where there are alternative metabolic pathways available. This need not even be a total loss; a significant shift in statistical balance can be enough to change the response of an entire cell population provided that enough cytotoxic material can be removed by resistant cells to protect the more vulnerable ones.

DAMELIN and ALEXANDER (2000) were able to isolate a population of cadmium-resistant cells by following the above procedure.

The MTT reagent used to measure cytotoxicity responds to enzyme systems active in the central energy pathways of the cell (MOSMANN, 1983; FRESHNEY, 1994; BERRIDGE et al., 1996). These pathways are linked to all the metabolic processes likely to be affected by cytotoxic agents (Figure 11), and their levels of enzyme activity are constantly re-adjusted during the normal course of events. In addition to metabolic balance, the rate of cell metabolism and the possibility of cell division must also be taken into account. All these factors will affect the cellular response both to cytotoxic substances present in the assay samples and to the MTT reagent.

Glutamate metabolism is one of the processes linked to the central metabolic pathways, and we found that regulating glutamate metabolism by adjusting the levels of glutamate available to the cells during and preceding the assay helps to stabilise the cellular metabolic balance and thus the assay. However, it is also necessary for the glutamate pathways to be active so

that the cells can function at an adequate metabolic rate, and this is ensured by staged changes in the maintenance media prior to the assay.

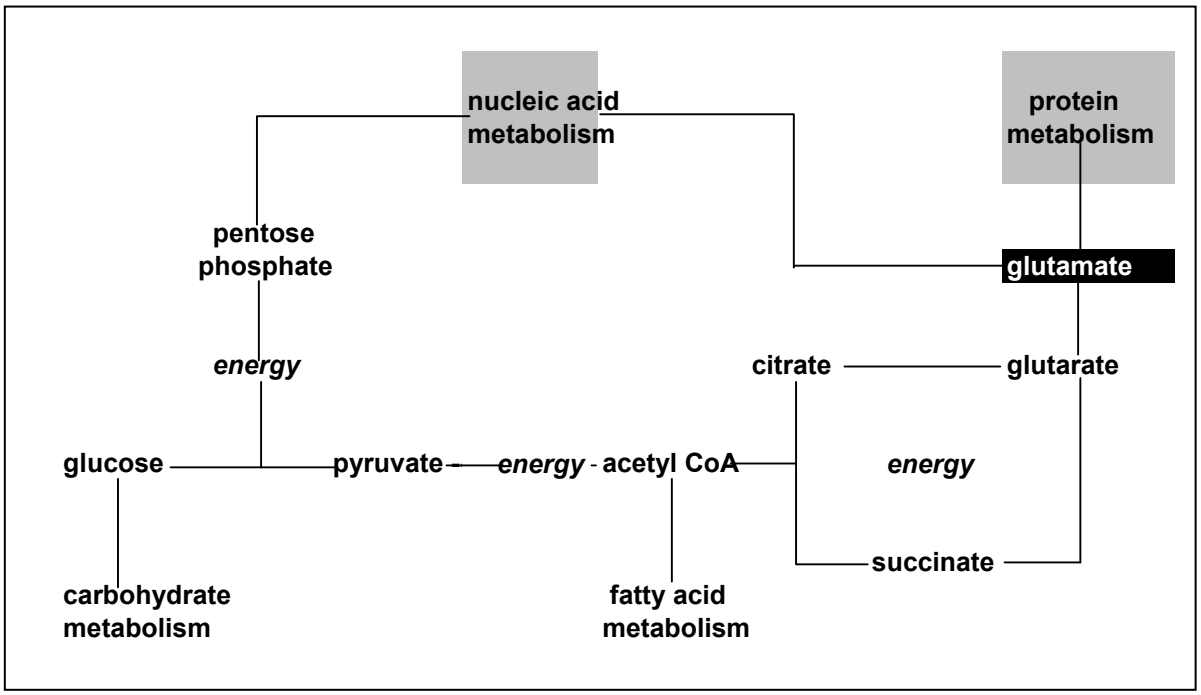

Figure 11. A simplified diagram of the central metabolic pathways active in mammalian cells

The following culture media were used:

- serum-free with glutamine,
- glutamine-free with serum,
- with glutamine and serum, and
- serum-free, glutamine-free (for the assay).

Relevant information about the responses of cell cultures to their nutrient media may be found in BARNES and SATO (1980) and EAGLE (1955, 1959).

Glutamine is a non-essential (cell-synthesised) amino acid involved in several key metabolic functions (Figure 11), including some that determine cytotoxic response, and serum components can apparently substitute for it in some respects, although not in others. We found that stabilising glutamate metabolism by the above procedure, which is described in detail in the **Methods** section, goes a long way towards solving the problem of 'drift'.

An alternative solution to the problem of drift lies in using early passage diploid cultures at a constant stage of outgrowth. Such cultures are resource-intensive and of limited metabolic flexibility, although the reproducibility of results is generally high in the earlier stages of culture (see for example DOWLING and MOTHERSILL, 2001). In previous work with cells of this type, we have found them to be rather insensitive to cytotoxins in the short term (12 to 24 hours) when compared with some of the more sensitive permanent cell lines. The latter are in any case far easier to handle on a routine basis, which is why they were chosen (see also SAUVANT et al., 1997; SHOJI et al., 2000B; EDITORIAL, 2001).

Metabolic variability

'Drift' should not be confused with the reversible changes in cell sensitivity associated with changing metabolic patterns. For example, sub-confluent cell cultures in growth phase (after a change of medium) may respond differently to the same cytotoxic agents as they do when they become confluent cultures in stationary phase (in conditioned medium).

Cells that have conditioned their growth medium give stronger cytotoxic responses to Cd and Hg compounds. This is apparently the reason for the high 90% to 95% Cd cytotoxicity peak in Figure 3. There is also a clearly defined low Cd cytotoxicity peak at 35% to 40%, and a broader peak at 50% to 55% Cd cytotoxicity. The 35% to 40% peak seems to be associated with cellular metabolic patterns required in early exponential cell growth. For cells in this particular state, Cd cytotoxicity is reduced, while the toxicity of detergents and acridines seems in contrast to be enhanced (unpublished results). The shifts appear to be discrete rather than gradual, hence the peaks and valleys in Cd toxicity shown in Figure 3.

Since different cytotoxins do not all work in the same way, we prefer to use $CdCl_2$ as our standard control, because it identifies the prevailing metabolic pattern of the target cells. This makes it possible to limit the assay to stable cell cultures giving Cd responses either in the 30% to 70% or in the 75% to 100% range.

It is only possible to make comparisons between assays carried out on cells that are functioning in the same way, and the methodologies described in this report have all been developed with this end in view. At the same time, it should be recognised that the complex effluents being tested may include substances that alter the normal functioning of cells, and in particular their response to cytotoxic components. From Table 1 it can be seen that this response is not instantaneous, which is possibly the reason for the characteristic, spread out profiles shown by the unidentified environmental cytotoxins in Figures 7 to 10.

Simplifying the assay

'Bouncing' cell cultures from one nutrient medium to another is not a simplification. However, the procedure does not add significantly to the cost of the assay in terms of time and materials used because in the long run the savings balance the losses.

The major factor impacting on the cost and convenience of cell culture assays is the need to maintain sterile working conditions. This could become unacceptably burdensome if translated into a need to sterilise all incoming specimens under conditions that do not affect their toxicity, as can happen during heat sterilisation, for example.

We have introduced two modifications. The first is to do all work under clean, non-sterile conditions right up to the final stages of setting up an assay. The entire plate is then sterilised by brief irradiation with UV light, which is able to penetrate the microliter volumes of test material present and destroy contaminating micro-organisms. The addition of sterile media and cells is done immediately afterwards, just before the start of incubation.

The second modification is to re-sterilise equipment such as dispensers with 70% ethanol while still in use, then flush the item immediately with sterile 2x RO water. This allows for rapid processing of large numbers of specimens without an equivalent depletion of sterile laboratory consumables.

Cell culture assay systems are sufficiently complex to require interpretive input by highly trained staff, and this is yet another factor adding significantly to cost structures.

We have tackled the problem by including various internal controls within the test and by using computer-controlled procedures for reading the assays and calculating the results. This eliminates the need for the staff carrying out the assays to have more than a basic grounding in toxicology. All that is required is normal manual dexterity, the ability to follow written instructions, and a conscientious and accurate approach to record-keeping. The computer program identifies specimens and assay plates that give anomalous results, and these should be evaluated by someone with a wider background of training and experience before the assays are repeated.

A number of reasons for rejecting assay results are listed below:

- *Background control readings too high*. 96-well plates made of plastics that soften in Heavy Oil could be the cause. Another reason is that the incubation medium and buffer, neither of which contains antibiotics, have become contaminated by micro-organisms.

- *Non-toxic control readings too low*. This could be due to insufficient numbers of cells or to an unacceptably low rate of metabolic activity in the stock cell culture. Deficiencies in the nutrient media or other cell-culture problems could be the reason.

- *Non-toxic control readings too high*. Too many cells or contamination of the cell culture with micro-organisms. Such problems tend not to occur with experienced staff.

- *Duplicate assay results too scattered*. This is usually due to an uneven distribution of cells in the suspension medium, or to dispensing equipment that requires servicing.

- *Mean standard (Cd) control outside the preset range*. This signals a different metabolic pattern in the test cells. The results are valid but not necessarily comparable with results obtained in other assays.

Range and sensitivity of the assay

The MTT assay is based on the observation that over a wide range of cell concentrations the amount of coloured formazan produced in each well is directly proportional to the viable cell count (MOSMANN, 1983; WAN et al., 1994). Provided that equal numbers of cells are delivered to each well, the assay can thus be used to assess cell viability, and the procedures outlined in **Materials and methods** are designed to ensure this. Under our experimental conditions, each assay generates a number between -30 and 100, which is indicative of the level of toxicity of the sample being tested. This can be compared with the corresponding figure for the standard control specimen present on the same plate. Negative readings below -10 indicate toxicant concentrations capable of inducing a stress response in the test cells, a phenomenon known as hormesis (STEBBING, 1982; CALABRESE and BALDWIN, 2000; DAMELIN et al., 2000) and may be regarded as highlighting a risk of long-term adverse effects.

Table 2 shows the assay results obtained with a range of chemical standards. Of particular interest are the high toxic thresholds of sodium chloride, ethyl alcohol and dimethyl sulphoxide. This relative insensitivity allows test specimens to be fractionated by chromatography and then eluted, for example, with HCl, which can subsequently be neutralised. Following such a procedure, which can be carried out in minutes, it is possible to classify cytotoxic specimens into broad categories of potential toxicants, as well as to establish whether synergistic effects are operative. Similarly, the lack of response of K-562 cells to small amounts of ethyl alcohol enables it to be used as an interim sterilising agent for the dispensing equipment needed to set up the assays (see above).

It can be deduced from Table 2 that the assay system is not sensitive to variable sub-threshold concentrations of non-toxic salts. This allows the pHs of unknown acid and alkaline test samples to be brought within the optimal range of the assay by adjusting the amount of Incubation Buffer used in the assay (see under **Materials and methods**).

It is also evident from Table 2 that the EC_{10} values for known toxic chemicals all occur at concentrations well above the permissible limits for drinking water (SABS, 1999). A value of 11% is used as the threshold for marginal cytotoxic activity, and 21% upwards as evidence of an acute cytotoxic response. These figures are based upon the reproducibility of the assay in the hands of different operators under variable conditions of cell culture, and take into account the probability that most operator errors will be picked up by the computer at the time the assays are read.

In evaluating environmental samples, it was found to be necessary to distinguish between acute cytotoxicity and overload cytotoxicity. The former is characterised by progressive damage over time and shows up as a ±20% increase in cytotoxicity during the 18 to 30 hour incubation period (Table 1). It also displays normal toxicant kinetics on dilution (Figure 5). Overload cytotoxicity shows itself initially as a reversible metabolic imbalance (11% to 20% apparent cytotoxicity not changing significantly with incubation time) and then jumps suddenly to 50% cytotoxicity or more. Dilution curves follow typical dog-leg kinetics (Figure 6); twofold dilution either makes little difference to the level of cytotoxicity or returns it to the 11% to 20% marginal range. These comments pre-suppose the absence of other toxicants in the test sample.

Anomalies and interfering factors

We have already referred to the problem of 'drift' (i.e. the accumulation of insensitive cells in the indicator cell population). Micro-organisms are in general less sensitive to toxic chemicals than mammalian cells, and if present in very large numbers can detoxify specimens before they are put up for assay (unpublished results). Killing them by UV irradiation immediately prior to carrying out the assay does not necessarily release the toxic material they have already taken up, and the best solution is to keep the samples cool after collection and remove the micro-organisms by syringe filtration as soon as possible thereafter.

Various chemical and biochemical agents are also able to change the cellular response by blocking surface receptors or by providing competing molecular sites; EDTA is a case in point for Cd (Table 2). This is also a reason why cytotoxicity assays should not be carried out in the presence of scavenging proteins such as serum albumen. Such effects may be regarded as the reverse of transport synergism (see below).

Prior adaptation of the cultures to serum-free growth in media similar to that in which they are assayed (see above) reduces their response to the small amounts of nutrients sometimes present in test specimens, which would otherwise show up as negative cytotoxicity.

Declining cytotoxic effects are likely to be found when re-testing specimens previously disinfected with short-lived cytocidal agents such as chlorine and peroxide.

Under appropriate conditions, some reducing agents (ascorbic acid, sulphydryl compounds, bisulphites etc.) are able to reduce MTT directly. It may be necessary to show that no such agents are present in samples by doing parallel repeat assays to which cells have and have not been added.

Single cell-type assays are usually unable to detect 'knock-on' toxicity, where for example fluoroacetate is metabolised to fluorocitrate by one cell type and then blocks the citric acid cycle in cells of another type. In line with this, FAA is non-toxic to K-562 cells (Table 2). Toxins that target specific cell types (methanol, KCN) also exhibit lower than expected cytotoxicity to K-562 cells.

Synergistic effects can easily be studied in this assay system by adding the synergist to the Incubation Buffer at a concentration that keeps it below its toxic threshold in the final test solution. From our preliminary investigations, it appears that synergists cause a shift in the background cellular metabolic balance or rate, resulting in an amplified response to the test agent. Thus far, two chemicals have been identified that appear to be associated with this kind of synergism, pentachlorophenol (and possibly other chlorinated aromatic compounds) and the fluoride ion, both active at concentrations less than 1 mg/l. Fluoride is of particular concern because it appears to be synergistic with nitrite ions at levels that are permissible in Class II drinking water (see SABS 241, 2001).

We suggest that both synergistic and antagonistic effects are caused by potentially toxic substances at sub-threshold concentrations that are none-the-less high enough to disturb the metabolic balance shown in Figure 11 or the metabolic rate apparent in Table 1. The presence of a second toxic agent should then generate a response in accordance with the altered sensitivity pattern as shown in Figure 3 (i.e. more cytotoxic or less cytotoxic depending upon whether the metabolic pathways being targeted are attenuated or enhanced by the presence of the synergist). If this view is correct, agents causing hormesis should also be potential synergists or antagonists.

A second type of synergism is transport synergism. In this case, the two components interact to give a complex that is more readily transported to the target site than are the components separately. Mercuric chloride and mercaptoethanol illustrate this particular situation (Table 2). Lead compounds behave in the same way; cadmium does not; also, unlike lead and mercury it shows a considerable drop in toxicity in the presence of EDTA (Table 2).

Evaluation of unknown water samples

Table 3 and Figures 7 to 9 show the pooled assay results obtained by using the above procedures on environmental water samples of varying origin, though not under ideal conditions because much of the work was done while the procedures were still being developed. Different workers carried out the assays using K-562 cell cultures in various stages of growth. Results were later edited to exclude all assays in which the Cd standard control readings fell outside the 30-70% cytotoxicity range.

Assay results for two of the samples (#92 and #101 of Table 3) are tightly clustered in the non-cytotoxic range (<20%) with standard deviations comparable to that of the non-toxic controls (9%, 8% and 6.44%). Electrical conductivity readings are uncommonly high for natural water but despite this there is no evidence from the assay results of any interference with the normal functioning of K-562 cells, and therefore of the presence of cytotoxic agents.

Results from the other three samples are loosely distributed in the cytotoxic range (>20%). Samples #98a and #98b, taken from the same flowing source nine weeks apart show virtually identical mean cytotoxicities, and their conductivity measurements, though differing, place them in the questionable range.

From Figure 6, we can see that for $CaCl_2$ overload cytotoxicity comes into effect at concentrations in excess of 300 mg/l, i.e. when the conductivity exceeds 70 mS/m. This is well below the conductivities shown by environmental samples #98a and #98b (Table 3). One is obliged to ask whether the apparent toxicity of these samples is not simply due to an overload of Ca salts. Fractionation and dilution assays have since shown that overload cytotoxicity is a component of the problem but not the only one.

Sample #79 from a different source some distance away shows a very similar pattern despite its lower conductivity. Interestingly, its cytotoxicity pattern is not much changed by including the results of tests falling outside the preset standard control range.

Water from sources #79 and #98 obviously contains substances able to interfere with the normal functioning of K-562 cells and must therefore be regarded as being potentially harmful to human health.

General comments

For practical purposes, we find it helpful to group cytotoxic samples in two classes depending on whether their assay results are greater than or less than 50%. Toxicity is a process rather than a product, and for analytical purposes it is more informative to make comparisons between samples by running them on the same plate for a time insufficient for the cytotoxic process to go to completion in the standard control sample. With the system described here, the control response is 80% complete at 18 hours and virtually 100%

complete after 24 hours (Table 1). This also makes it possible to apply simple fractionation procedures to cytotoxic samples in order to determine whether the agent responsible is likely to be cationic, anionic or organic, and whether synergistic or antagonistic factors are involved.

Statistical analysis of replicate and dilution assays is also helpful and is obviously required for assigning EC_{50} values, although in our opinion, such figures are only meaningful as approximate guidelines in respect of pre-defined environmental and metabolic states. For example, the EC_{50} values for Cd on K-562 cells derived from the two graphs shown in Figure 5 differ by a factor of 15. Under such circumstances, we find it difficult to make comparisons between the many different kinds of toxicity tests currently available, and it is increasingly apparent that a suite approach, adaptable to individual situations, is the only sensible answer.

From time to time, suggestions are made that an ideal toxicity test could be developed along the lines of an instant dipstick test, sensitive to picogram amounts of toxicants. This point of view overlooks several relevant factors:

- Detecting sub-threshold levels of toxicants defeats the purpose of the test, which is to find out whether or not there is a problem.
- Threshold levels differ for different organisms.
- Instant tests detect products rather than processes. In this respect, they are little different from the chemical assays, many of which are already available in dipstick form.
- Different organisms have different biochemical mechanisms for responding to toxicants. Those of mammalian species can take as long as 14 hours to become fully functional (DAMELIN, 2001).

In our opinion, both ultra-rapid and conventional toxicity tests need to be interpreted in the light of all the information available. They are in fact management tools, the purpose of which is only achieved when the sources and natures of the toxic pollutants they detect have been identified, generally by other procedures.

What is clear from this investigation is that a significant percentage of wastewater samples are still able to generate cytotoxic responses after finding their way back into the natural environment.

The last result also illustrates an important feature of this assay – the cellular response is very uniform when negligible amounts of toxicants are present. As the load increases, the cellular response will vary, depending upon which metabolic pathways are predominantly active at the time of the assay, as well as how active they are. There is, of course, no reason why every pathway should be equally sensitive to all cytotoxins, and we consider the variability in itself to be an indication of interference with metabolic pathways.

This is a monitoring assay. Its purpose is to detect the presence of biologically active agents in water and to give an estimate of how serious an impact they are likely to have on living systems, in particular human beings. In order to carry out this function effectively, the cells need to be metabolically both resilient and versatile – resilient, so as not to respond to variables such as passage number, age, and to some extent population size, all of which are outside the scope of the assay; versatile, so as to have on tap a full range of metabolic resources, any of which can be interfered with by the appropriate toxicants. Given a particular type of interference, the cellular response should be in proportion to the concentration of the causative agent, which is not possible with exceptionally fragile cells.

The project outlined above was aimed at designing an *in vitro* assay system that satisfies all these requirements, and to some extent this objective has been achieved. Nevertheless, there is still scope for further development. Application of the assay to unknown environmental samples, even before a final protocol was decided on, has generated a spectrum of responses, some of which deserve more thorough investigation.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

Cell culture-based toxicity assays, and specifically human ones such as that described here, have an obvious place in any battery of tests for evaluating water quality. Their level of sensitivity conveniently covers the range of toxicity found in complex urban and industrial effluents. Like other toxicity assays, they are not stand-alone tests. However, they can easily be integrated with other tests, in particular with chemical analyses.

The methodology described in this report is complex, despite the simplifications introduced. For this reason, it cannot be cost-effective at low levels of monitoring. Its correct application is as a management tool in project and surveillance monitoring, where large numbers of samples have to be evaluated over short periods of time. In such situations, it can provide quantitative information, in electronic format, within 24 hours, at considerably less cost than many other assay methods.

Despite its sensitivity to mildly toxic effluents we have yet to detect persistent false positives, unless residual chlorine qualifies as such. In contrast, false negatives are not unknown and include such highly toxic compounds as fluoroacetates. Events invalidating the assay, such as chemical reduction of MTT and accidental precipitation, have to be guarded against, and can usually be bypassed by repeating the assay at twofold dilution.

The main advantage of this assay is its flexibility; it can be done with different cells in different media over periods extending up to several days. The small volumes required permit quick, easy processing as well as simple pre-treatment of specimens. The possibility of using faster and less general sensors than MTT opens the door to other options.

From the evidence assembled during this investigation, we can only conclude that specimens giving confirmed cytotoxic readings must, without exception, be regarded as hazardous to human health until the causative agents have been identified.

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ADDENDUM 1: COMPARATIVE EVALUATION STUDY WITH THE WEAVER HUMAN CELL TEST

THE WEAVER HUMAN CELL TEST IN COMPARISON WITH OTHER BIO-SENSOR TESTS USED TO ASSESS THE TOXIC EFFECTS OF POLLUTED WATER
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Name of the sensing test: Weaver Human Cell Test

Type of assay: Effect on the energy pathway of K-562 cells *in vitro*

EXECUTIVE SUMMARY

In South Africa, the quality of source water and wastewater is presently coming under tough scrutiny as new legislation is in the process of being prepared by Government to protect a valuable and scarce natural resource from the toxic impact of pollution.

A meaningful assessment of the impact on the environment of polluted, toxic industrial effluents, rivers and catchment areas, as well as groundwater and sediments, requires the development of eukaryotic bioassays, using either multicellular organisms, such as fish and frogs, or single cell populations (sub-organism test systems). We can only begin to understand the extent of present and potential future damage if we have relevant biological response systems.

There are a number of biological test systems that can detect the toxic effects of substances that appear in contaminated water. Each kind of test with its specific bio-sensor indicates a different focus of interest and concern: the fish test is significant for the assessment of the aquatic environment such as rivers and streams, as are the *daphnia* and algae tests; the bacterial tests give information on alterations in the genetic make-up and energy pathways; other specific tests focus at the impact of toxic water on plant life. Human and animal cells *in vitro* are used to understand pathological responses to hormones and other important modulating factors, which are sometimes present in water at alarming levels.

The assessment of water quality has three aspects: (1) microbiological testing, (2) chemical analysis, and (3) toxic environmental impact. The Weaver Human Cell Test is concerned only with the third aspect, the possible negative effect of toxic water on human or other mammalian cells *in vitro*. Any conclusion on how the results of these *in vitro* tests give information about human and animal health are at this stage tentative until parallel studies have been carried out in toxicological laboratories.

The present investigation was initiated in order to obtain the following information:

- How sensitive the Weaver Human Cell Test is compared to similar tests on other biological systems
- How reproducible is the test in terms of intra-laboratory and inter-laboratory studies.

The present study is confined to making a statement about these questions under the given conditions described below. The recent analyses are not assigned for German DIN and ISO standardisation indications. In this regard, more comprehensive investigations and expert knowledge should be requested.

Standard solutions of single substance and complex toxic contents were applied in double blind tests. These solutions have been extensively investigated and tested by the Federal Institute of Hydrology in Berlin, Germany, using the major ecotoxicological test systems. The results indicate congruent results between the response of the Weaver Human Cell Test and other tests that are based on very different bio-sensors. This indicates that the test is suitable to be included in a battery of tests, particularly if it is found to be more convenient and cheaper to carry out and to provide new information with respect to human health.

The reproducibility of the Weaver Human Cell Test in-house and in a different laboratory, carried out by the author following written instructions, was within acceptable limits as discussed below. A stringent statistical analysis of the results of this comparative study showed that the effective concentration limit (EC) has to be raised to between 15% and 20%, which means that response levels up to 20% are considered still to be within normal limits. The variations are within detectable limits and correspond to other biological test systems.

This preliminary study indicates that the Weaver Human Cell Test would be a suitable tool for monitoring the quality of drinking water and would also be applicable for remediation processes for water that is considered to be of drinking water standard. Furthermore, it has been shown that the Weaver Human Cell Test is a sufficiently robust test that could be used as a guiding tool during clean-up processes, particularly as it is a 24-hour test and not expensive compared with other tests.

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INTRODUCTION

The *in vitro* assay Weaver Human Cell Test is appropriate as a toxicity and vitality test using human cell lines (chronic myelogenous leukemia cells) to record responses of these cells to contaminations in test samples (different source) over 24 hours. To assess acute cytotoxic influence with an *in vitro* test system, human cells were used as biosensors. It must be pointed out that the test system is possibly not able to detect toxic agents that are non-toxic to human beings.

Description of the system

The Weaver Human Cell Test allows for the assessment of adverse effects. In the test's existing format, MTT is used for assessing the activity of cellular oxidative pathways. In previous studies, the endpoint was adapted to adherent human cells. The biochemical targets for the induction of toxic effects can be described as the 'central biochemical pathways' including the respiratory chain oxido-reductases, so mitochondria and cytoplasmic organelles are most probably affected (BERRIDGE et al., 1996).

The test system is based on the fact that the respiratory energy chain of exposed cells shows a reduced capacity of enzymatic activity in comparison with the control population (BERRIDGE and TAN, 1993; BERRIDGE et al., 1996).

The overall level of activity of these pathways has been described as a convenient marker of viability because it declines when cells respond to stress and it completely disappears when they die (WHITCUTT, 2000; MEYER et al., 2001).

As a biotest for measuring ecotoxicological effects, the Weaver Human Cell Test has been developed with non-adherent human leucocytes by Highveld Biological Association (HBA) (DAMELIN et al., 2000; WHITCUTT, 2000; MEYER et al., 2001).

The endpoint measured in the Weaver test is the reduction of MTT, [3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide], which leads to a conversion of the substrate into a coloured formazan end-product. A linear relationship of colour release and cell viability was demonstrated in prior investigations.

The applied substrate MTT is used to measure the activity of the oxidative pathways (biophysiological target structure). In general, this procedure enables toxic inhibition of biochemical energy pathways to be detected (WHITCUTT, 2000).

The adaptation of the testing procedure for rapid mass testing of water samples lowers the costs of monitoring studies and enhances their applicability for regulatory approaches.

Performance of the comparative evaluation (inter-laboratory study) with the Weaver Human Cell Test

Six blind sample probes (certified mono substances, sediment extracts and non-toxic water) were tested in three independent test sets.

Date 1: 16-07-2002 intra-laboratory assessment in Highveld Biological Association
Date 2: 17-07-2002 intra laboratory assessment in Highveld Biological Association
Date 3: 17-07-2002 inter-laboratory assessment in University of Pretoria, (Department of Virology; Prof. Dr. W Grabow)

The samples were tested according to the Highveld Biological Association procedure described in the final report of WRC project K5/1121/1/04 entitled 'An integrated approach to biomonitoring wastewater for the presence of biologically active agents' by JM Whitcutt, RA Emmett, R Tseki, Z Mbatha and P Humphries.

In Table 1, the standard solutions of single substance and complex toxic agents are listed. The comparative study was employed in double blind tests. These solutions have been previously investigated and tested by the Federal Institute of Hydrology in Berlin, Germany, using the major ecotoxicology test systems, DIN EN ISO 11348 (luminescence test), DIN 38 412-33, (algae test with *Scenedesmus suspicatus*) and ISO/DIS 13829 (umu-test, bacterial genotoxicity test).

Table 1. Comparative evaluation study with the Weaver Human Cell Test. Blind probe test performance (only code numbers have been expressed for intra- and inter laboratory study)

Sample code number	Source	Solvent control	Test concentration starting point
1	Industrial station [F3 Aug01] original sample contains 30% DMSO	DMSO 3%	[1:10]
2	4-NQO 4-Nitroquinoline-N-Oxide [1 g/l]	DMSO 0.1%	[1:1000]
3	Cr(VI) Potassium-di-chromate [50 mg/l]	A. dest.	direct testing (test 1) [1:10; test 2, 3]
4	Industrial station [F5 Aug01]	DMSO 3%	[1:10]
5	Phthalate ester-mixture No. 3 [1 g/l each]	MeOH 0.1%	see certificate [1:1000]
6	Drinking water	A. dest.	Direct testing

For each sample, the following dilution scheme was applied:

Dilution series:			
Dilution no.	Dilution factor	Sample content (%)	
1	0	100	original sample
2	2,7	37	dil2
3	7,3	13,7	dil3
4	20	5	dil4

In summary, for the comparative evaluation study (three independent tests), 58 single tests, normally including 6 parallels per sample dilution, were performed by the author and Dr Whitcutt.

For the inter-laboratory comparison, the third test was performed entirely at the University of Pretoria, using equipment from the department (double distilled water, sterile working materials, pipettes, sterile working benches and incubators). The final spectrophotometric measurements were performed at Highveld Biological Association.

The exposure preparation of blind samples with their appropriate solvent controls was performed exclusively by the author (step 1; test set up, exposure). Substrate MTT addition, stopping the enzyme reaction and plate reading (step 2 and 3) were performed by Dr. Whitcutt and the author.

Johannesburg tap water was tested in two independent tests, instead of German tap water (no. 6) – as previously planned. During the first test run, the German tap water induced precipitation effects – possibly due to chemical reactions of the waterborne calcium content with the carbonate buffer system (medium constituents). Single tests were performed with 6 parallels for each dilution step (with the exception that the test with tap water was performed without further dilutions but with N = 18 parallels for each test).

For each plate, a positive control with 1 mg/l cadmium solution (cadmium chloride) was introduced, as well as 8 standard negative controls, in addition to 8 solvent controls, if necessary).

Variance analysis

The variance analyses of raw data and toxicity calculations were performed in accordance with DIN 38 402 T 41/42 (preparation, performance and evaluation of ring tests; German Standardisation Procedure). For the specific recommendations of the Weaver Human Cell Test, adapted Excel working sheets for the calculation of test results and the variance analysis had been prepared.

Definition and treatment of outliers

The raw data (N >550; controls and treatment pools) were scored for outliers through the documentation of errors during performance for single test wells as well as the outsourcing of ‘optical outliers’, which showed deviations from the means that were higher than the limit of 3SD. For each treatment group, the total number of outliers for the raw data was assessed to be <5%.

For laboratory variance calculations and for defining the precision of the method (coefficient of variance for repeatability), the following data were excluded from calculations:

- Tests with enhanced enzyme activity measures as negative toxicity data
- Data close to 0% toxicity limit (+/- 5%).

RESULTS AND DISCUSSION

Variance analysis

Because several test samples contain a solvent, the data were calculated using the corresponding solvent control. For DMSO, a backward shift of controls was measured, resulting in an MTT release potential of 20% to 25% lower.

The common quality criteria for each single test run were fulfilled in congruence with the test protocol, defined as the mean blank reading <0.095 optical density units:

- mean non-toxic control reading,
- >4 x mean blank reading and 0.300 optical density units,
- % cytotoxicity of 1 mg/L cadmium chloride standard between 31% and 70%.

The detailed results of the inter-laboratory study are shown in Table 2.1 (laboratory variance) and Table 2.2 (precision, repeatability, intra- and inter-laboratory comparison). For the toxicity data in graphical format, see Appendix B.

Samples 1 to 4 have effectively caused adverse effects, with high toxicity levels in a close dose response manner. The data from individual test performances showed a closed effect range with low variation coefficients (Table 2.1). For heavy metals such as cadmium and the test substance Cr(VI); sample no. 3), the lowest variance could be assessed.

For low toxicity values and enzyme activity measures in the range of +5% to -5%, higher variances will normally be detected for mathematical reasons. These data were excluded from further variance analysis.

For single tests, a low mean CV (laboratory coefficient of variance) of 12% was calculated. In independent test performances, the first test tends to lead to slightly higher coefficients of variance (20%) compared with test 2 (12%), and test 3, which was performed in a different laboratory (20%). Nevertheless, these results show the high practicability of the Weaver Human Cell Test for trained persons, given the high reproducibility in independent tests.

The results obtained from experiments performed at Highveld Biological and the University of Pretoria (Institute of Virology) showed no significant differences. The variance of independent tests did not differ between the two groups (Table 2.2 – see intra-laboratory and inter-laboratory VR).

False positive results were not found. For sample no. 5 (phthalate ester mixture) as well as for the tap water control, no inhibition of MTT reduction could be detected in 3 independent tests.

A high degree of reproducibility between replicate assays was shown for contaminated probes as well as for negative controls. The tap water control was performed with 18 instead of 6 parallels per tests. For non-toxic waters, low laboratory standard deviations were assessed (Table 2). In the case of sample no. 5, higher coefficients of variation were obtained, due to the chemical interaction of several compounds mixed in the probe and possibly because of problems of diffuse chemical interactions of the complex mixture of test compounds during exposure. Table 3 gives an overview considering the precision of the Weaver Human Cell Test. The variance coefficient of repeatability (VR) could be shown to be in comparable ranges for biological variances. In analogue studies, VR values about 15 up to 30% can be obtained, whereas the inter laboratory variance can be shown to be mostly in higher ranges. No differences between the intra- and inter laboratory precision have been evaluated. For the application of the test in different laboratories / and different trained personal, comparable variance data for the intra- and inter laboratory precision data can be assumed.

Table 2.1. Comparative evaluation study with the Weaver Human Cell Test. Analysis of laboratory variance

Weaver Human Cell Test						**Results Validation experiments**								
Results		Test 1	Test 2	Test 3	Test1-3	Test 1	Test 2	Test 3	Test 1-3	**Variance for parallels in single tests**		
		Toxicity	Toxicity	Toxicity	Toxicity	SD	SD	SD	SD	**CV1 (%)**	**CV2 (%)**	**CV3 (%)**
Sample	source	mean, N = 6			mean	for parallels (single tests; N = 6)			mean	**test 1**	**test 2**	**test 3**
Sample 1	Sediment	92.63	88.34	85.59	**88.85**	1.66	1.33	5.22	**2.74**	1.79	1.51	6.10
1/dil2	fraction	87.48	60.49	74.21	**74.06**	3.02	1.6	0.34	**1.65**	3.45	2.65	0.46
1/dil3	F3 EtAc/hex	52.2	18.43	34.8	**35.14**	1.97	3.25	2.04	**2.42**	3.77	17.63	5.86
1/dil4		-7.5	2.03	18.26	**4.26**		0.2	1.89	**1.05**		9.85	10.35
EC-20 / LID		EC-20 = 4 mg sed./ml extract			**LID = 20**							
Sample 2	4-NQO	45.78	68.41	71.87	**62.02**	5.88	2.13	1.42	**3.14**	12.84	3.11	1.98
2/dil2	1 mg/L	43.39	47.78	55.71	**48.96**	6.4	1	6.73	**4.71**	14.75	2.09	12.08
2/dil3		21.09	22.55	11.29	**18.31**	6.96	2.95	5.27	**5.06**	33.00	13.08	46.68
2/dil4		-8	11.4	1.53	**1.64**		1.02	0.49	**0.76**		8.95	32.03
EC-20 / LID		EC-20 = 0.05 mg/L										
Sample 3	CrVI	different	95.29	79.81	**87.55**		0.28	11.37	**5.83**		0.29	14.25
3/dil2	5 mg/L	dosage	91.34	79.41	**85.38**		1.74	16.75	**9.25**		1.90	21.09
3/dil3		tested	82.94	81.82	**82.38**		1.58	0.55	**1.07**		1.90	0.67
3/dil4			61.67	64.05	**62.86**		1.66	0.87	**1.27**		2.69	1.36
EC-20 / LID		EC-20 < 0.25 mg/L										
Sample 4	Sediment	75.1	71.37	78.45	**74.97**	13.42	3.94	4.91	**7.42**	17.87	5.52	6.26
4/dil2	fraction	56.15	27.23	36.51	**39.96**	4.71	6.1	2.8	**4.54**	8.39	22.40	7.67
4/dil3	F5 methanol	28.78	7.18	16.85	**17.60**	9.57	3.6	3.58	**5.58**	33.25	50.14	21.25
4/dil4		9.38	1.41	10.3	**7.03**	0.11	0.34	1.87	**0.77**	1.17	24.11	18.16
EC-20 / LID		EC-20 = 11 mg sed./ml extract			**LID = 7,3**							
Sample 5	Phthalate	-21.05	-22.5	-2.48	**-15.34**							
5/dil2	Esters	-14.04	-8	-10.65	**-10.90**							
5/dil3	Mix3	-25.3	-1.19	-9.2	**-11.90**							
5/dil4	1mg/L 17x	-26.57	-1.4	-3.1	**-10.36**					**mean CV1**	**mean CV2**	**mean CV3**
EC-20 / LID		enhanced enzyme activity								**13.03**	**10.49**	**12.89**
Sample 6	tap water	not calcul.	-3.8	-10.6	**-7.200**	N = (2x 18 parallels) for tap water						
EC-20 / LID		no effect observed			**LID = 1**							
									3.58	**12.01**		
									mean SD	**mean CV**		
									for parallels			
									N = 43	**N = 43**		

Table 2.2. Comparative evaluation study with the Weaver Human Cell Test. Analysis of variance

Weaver Human Cell Test	**Results validation experiments**				
Variance between independent tests				**Repeatability**	
Test 1-3				**(Precision)**	
mean	**VR1**	**VR2**	**VR3**	**mean VR**	**meanVR**
SI				**intra-lab**	**inter-lab**
2.90	3.13	3.28	3.38	**3.20**	**3.26**
11.02	12.60	18.22	14.85	**15.41**	**15.22**
13.79	26.42		39.62	**26.42**	**33.02**
10.63					
11.57	25.27	16.91	16.10	**21.09**	**19.43**
5.10	11.75	10.67	9.15	**11.21**	**10.52**
5.00	23.71	22.17	44.28	**22.94**	**30.05**
7.92					
7.74		8.12	9.70	**8.12**	**8.91**
5.96		6.53	7.51	**6.53**	**7.02**
0.56		0.68	0.68	**0.68**	**0.68**
1.19		1.93	1.86	**1.93**	**1.89**
2.89	3.85	4.05	3.69	**3.95**	**3.86**
12.06	21.47	44.28	33.02	**32.87**	**32.92**
8.83	30.70		52.43	**30.70**	**41.56**
3.99	42.55		38.75	**42.55**	**40.65**
9.11					
2.47					
10.03					
11.49	**mean VR1**	**mean VR2**	**mean VR3**		
	20.14	**12.44**	**19.65**		
				Repeatability/precision	
				VR (%)	**VR (%)**
				Intra-lab	**Inter-lab**
7.21				**16.26**	**17.02**
SI (N = 56)				**N = 21**	**N = 35**

Table 2 legend

Comparative evaluation study with the Weaver Human Cell Test. Analysis of variance

CV laboratory coefficient of variance CV (%)

VR (%) coefficient of variance for repeatability = precision

SD laboratory standard deviation

SI standard deviation of repeatability

LID Lowest ineffective dose (corresponding EC_{20} value)

*) outliers for variance analysis were defined as CV values, obtained from data close to the no effect level
(here positive and negative values were obtained close to sero point; for sample no. 5 and 6)

Dilution series:

Dilution no.	dil.factor	sample content (%)	
1	0	100	sample
2	2.7	37	dil2
3	7.3	13.7	dil3
4	20	5	dil4

In summary, the comparative evaluation study has shown that for routine testings, the EC_{10} level cannot be defined as a significant level for the detection of adverse environmental effects in the Weaver Human Cell Test, but EC_{20} levels should be calculated.

Table 3. Comparative evaluation study with the Weaver Human Cell Test. Analysis of variance

Weaver Human Cell Test	SD	SI	CV	VR (%) intra-lab.	VR (%) inter-lab.
Blind samples (mean values after exclusion of outliers)	3,58	7,21	11,64	16,26	17,02
N	43	56	43	21	35

SD = laboratory standard deviation
SI = standard deviation of repeatability
CV = laboratory coefficient of variance
VR = comparative variation coefficient (as a measure of repeatability and precision)

Comparability with alternative *in vitro* and *in vivo* assays

An overview of the toxicity approach is shown in Table 4. Sample no. 1 (sediment extract fraction probe; F3, in ethyl acetate/hexane), originated from an industrial station in Germany, induced comparable inhibitory effects with different test systems. The Weaver Human Cell Test showed toxic effects in congruence with tests that are based on different trophic levels (bacteria, algae). The sample also showed toxicity to *Daphnia*, if pore water was used (data not shown). In contrast to sample no. 1, an approximately 3-fold higher sensitivity was detected with the Weaver Human Cell Test with sample no. 4, which was from the same origin as no. 1, but was sub-fractionated in the more polar solvent methanol.

It can be considered from the industrial sample tested that the Weaver Human Cell Test should be more responsible and possibly better suited for water-soluble environmental contaminants than comparable *in vitro* tests. This tendency should be investigated and confirmed by further comparative studies.

The mono substance in sample no. 2 is a well-known genotoxine (BAUN et al., 1999). For this substance, genotoxic effects can be shown in lower test concentrations of between 10- and 100-fold, compared with cytotoxic influences (DUIS et al., 1996; UNRUH, 2001). In addition, the nitroaromate 4-NQO is known to be toxic for fish-embryos and mussels (Table 4).

The Weaver Human Cell Test showed vitality effects at relatively low test concentrations, for which adverse effects on the ecosystem level (e.g. lethality in mussels, fish embryos, fish larvae and adult fish) were described. The nitroaromate 4-NQO (>0,07 mg/L) is able to induce embryotoxic effects in the early life stages *of Danio rerio* (WITTEKINDT et al., 2001).

Table 4. Comparative evaluation study with the Weaver Human Cell Test: comparison of results with respect to sensitivity

Sample characterisation	**Bioassay comparison**			
Sample no. (nature of sample, original concentration)	Weaver Human Cell Test	Lumi-nescence+	Algae-toxic++	Other specific effects
	Toxicity LID values	Toxicity LID / EC_{20}	Toxicity LID / EC_{20}	Genotoxicity+++ embryotoxic for fish esterase inhibition
Industrial station [F3Aug0167601] 80 mg Sediment per ml EtAc-extracts	**20**	**16**	**32**	Genotoxicity +++ **16** cytotoxic to MCF-7 cell line
4-NQO 4-Nitroquinoline-N-Oxide [1 mg/L]	0.05 mg/L Embryotoxicity (*Danio rerio* fish larvae), Wittekindt et al., 2001 0.125 mg/L	n.t.	n.t. Mutagenic effects, Rao et al., 1995	Lethality (*Dreissena polymorpha)* Wittekindt et al., 2000a,b, 0.250 mg/L genotoxicity +++ 0,05 mg/L Baun et al., 1999
Cr(VI) Potassium dichromate [5 mg/l]	< 0.25 mg/L	20-80% range 18.7 mg/L	n.t.	Genotoxic +++ (0,2 mg/L)
Industrial station [F5Aug0167601] 80 mg Sediment per ml MeOH-extract	**7.3**	**2**	**1**	Esterase inhibition **2** (*Acanthamoeba castellanii)*
Phthalate Ester-mix No. 3 17 compounds [1 mg/L each]	Enhancement of MTT reduction 1 mg/L	n.t.	n.t.	Growth inhibition with 0.1 mg/L with MCF-7 cell line
Drinking water	1	1	1	

Note: *In vitro*-bioassays: +) DIN EN ISO 11348, ++): Algae test: DIN 38 412-33, +++): ISO/DIS 13829 (umu-test, bacterial genotoxicity test)
LID = Lowest ineffective dose; EC_{20}.

The samples were tested according to the Highveld Biological Association procedure described in the final report of WRC project K5/1121/1/04 entitled 'An integrated approach to biomonitoring wastewater for the presence of biologically active agents' by JM Whitcutt, RA Emmett, R Tseki, Z Mbatha and P Humphries.

For sample no. 4 (potassium dichromate), analogue sensitivity ranges (EC_{50} values) were obtained during the evaluation study as described in the final report of WRC project K5/1121/1/04.

False positive results have not been detected under the given conditions. For sample no. 5 (phthalate ester mixture) as well as for the tap water control, no inhibition of MTT reduction could be detected in 3 independent tests. The phthalate ester mixture is a relatively new substance mixture known to show hormone-like activity with no or low accompanying toxicity. Enhanced mitotic activity was not found in MCF-7 cell clones, indicating that a different pathway from oestrogen receptor induction should be responsible for hormone-like activity. The positive induction of MTT breakdown in the lymphoid K-562 cell line (chronic myelogenous leukaemia cells) is an interesting result, which indicates the need for future research studies, because it shows the ability of phthalates to enhance the activity of the respiratory system.

In summary, the preliminary study showed a high congruence of toxicity induction in the Weaver Human Cell Test with representative samples, with respect to different *in vitro* and *in vivo* test parameters using different aquatic organisms.

The highest comparability with respect to toxicity and sensitivity for representative samples investigated can be shown between the Weaver Human Cell Test and the luminescence test according to DIN EN ISO 11348. In both cases, respiratory pathway parameters are selected as target parameters that express high conservation of bio-structures during evolution.

CONCLUDING REMARKS

The present investigation was initiated in order to obtain the following information:

- How sensitive the Weaver Human Cell Test is compared to similar tests on other biological systems
- How reproducible the test is in terms of intra-laboratory and inter-laboratory studies.

Standard solutions of single substance and complex toxic contents were employed in double blind tests.

From the technical side of test performance, the Weaver Human Cell Test showed a high degree of optimisation with respect to preventing shifts in sensitivity during cell passages.. This problem is solved by establishing cell lines and selecting them for their ability to maintain alternative metabolic pathways by 'bouncing' them between different cell culture environments with the following media: (1) serum-free with glutamine, (2) glutamine-free with serum, (3) with glutamine with serum and (4):serum-free, glutamine-free for the assay itself. This maintenance cycle seems likely to be responsible for the stable sensitivity ranges in replicate tests, irrespective of the cell cycle number. Concerning the cell quality, the different steps during the performance of the Weaver Human Cell Test seem to represent an optimum. No further changes can be recommended.

A stringent statistical analysis of the results of this comparative study have shown that the effective concentration limit (EC) has to be raised to between 15% and 20%. The intra- and inter-laboratory VR values can be shown to be in equal ranges (16% and 17%). No differences between the intra- and inter-laboratory precision were found.

The variations within detectable limits correspond with other biological test systems. The data from individual test performances demonstrate a close effect range with low variation coefficients. The variance coefficient of repeatability (VR) could be shown to be in comparable ranges for biological variances. For the application of the test in different laboratories with different trained personnel, comparable variance data for the intra- and inter-laboratory precision can be assumed.

The comparative evaluation study has therefore shown that the EC_{10} effect level cannot be defined as a significant level for the detection of adverse environmental effects in the Weaver Human Cell Test. The calculated EC_{20} toxicity data have been shown to be of high significance for the detection of adverse environmental effects.

For future routine tests, basic measures and calculated toxicity data should be given with SD and the corresponding coefficients of variance (CV), adding an appropriate significance test. Dunnett's Test is recommended (a parametric test for statistical significance calculation; a multivariate procedure which considers different N for controls and samples).

The results indicate congruent results between the response of the Weaver Human Cell Test and other tests that are based on very different bio-sensors. For water contaminants of higher polarity, the test showed higher sensitivity than common used tests (e.g. luminescence test). This observation should be confirmed by further comparative studies.

Recent results indicate that the test is suitable to be included in a battery of tests, particularly if it is found to be more convenient and cheaper to carry out and to provide new information with regard to human health aspects. An appropriate test battery should also include effect parameters on different ecologically relevant trophic levels, to detect genotoxic, endocrine and possibly immunotoxic effects.

Further applications and recommendations

This preliminary study indicates that the Weaver Human Cell Test would be a suitable tool for monitoring the quality of drinking water and that it seems to be generally applicable for the technical treatment process of remediation for water that is considered to be of drinking water standard. Apart from these Public Health applications, the test results suggest further ecological applications, namely, analyses of surface water, groundwater and sewage in risk assessment studies. It has been shown that the Weaver Human Cell Test is a sufficiently robust test for use as a guiding tool during clean-up processes, particularly as it is a 24-hour test that provides economical features compared to other tests.

The present study makes possible a statement about the Weaver Human Cell Test with regard to its sensitivity and comparability with other ecotoxicological standard tests using a set of reference substances and samples. This evaluation is confined to giving a statement on the basis of the analyses performed under the conditions described above. The recent analyses are not assigned for German DIN and ISO standardisation indications. In this regard, more comprehensive investigations and expert knowledge should be requested.

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APPENDIX A: BACKGROUND INFORMATION

The Federal Institute of Hydrology (*BfG*) is a higher authority of the Federal Republic of Germany under the jurisdiction of the Federal Ministry of Transport, Building and Housing (*BMVBW*) with its Headquarters at Koblenz and a Branch Office at Berlin.

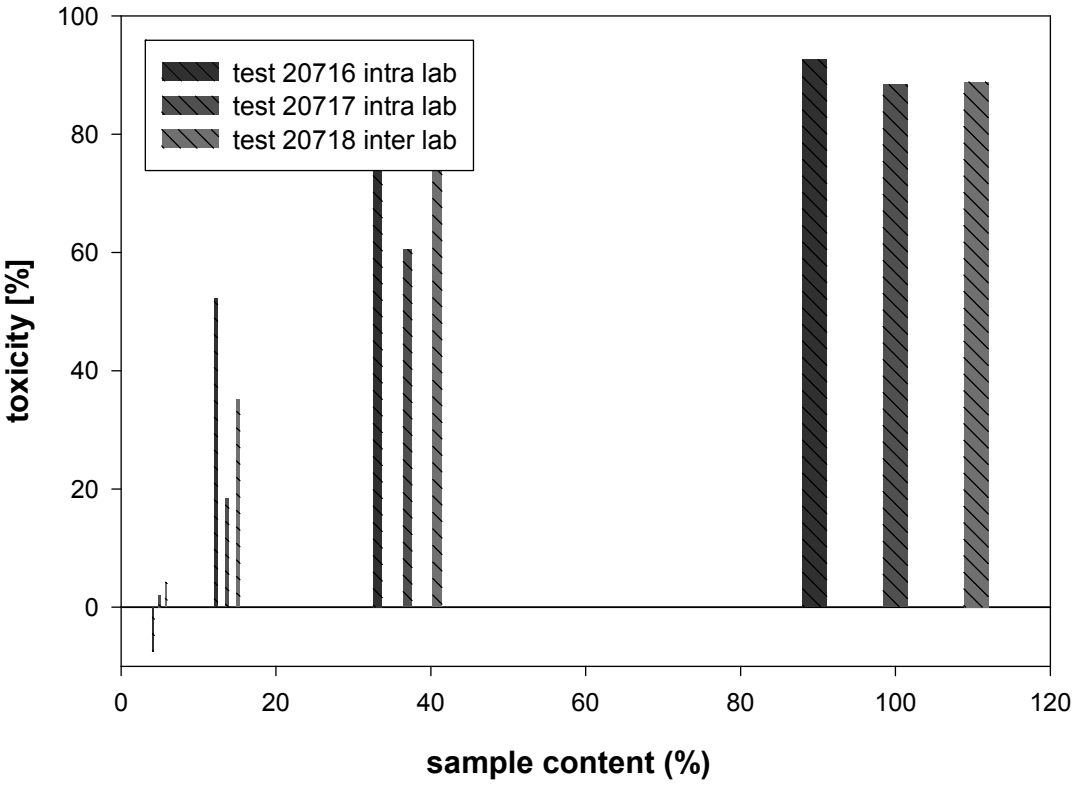
It is the scientific institute of the Federal Government for research and consulting in the fields of hydrology, water-resources management, ecology, and conservation of water.

The *BfG* also renders services to the Ministry of the Environment, Nature Conservation and Nuclear Safety (*BMU*). Above all this refers to issues of transboundary waters.

The *BfG* has at present around 350 staff members, of which about two thirds work at the Headquarters at Koblenz.

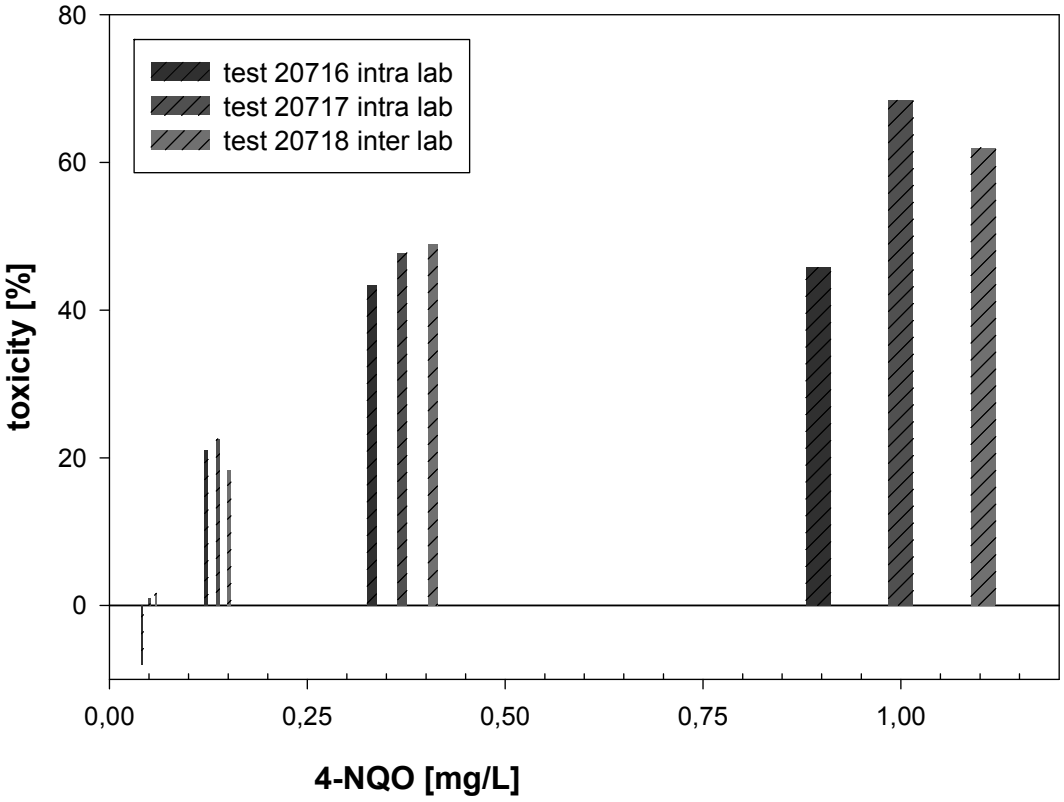
APPENDIX B: TOXICITY DATA

Weaver Human Cell Test evaluation experiment sample 1 / Industrial Station / sediment fraction F3



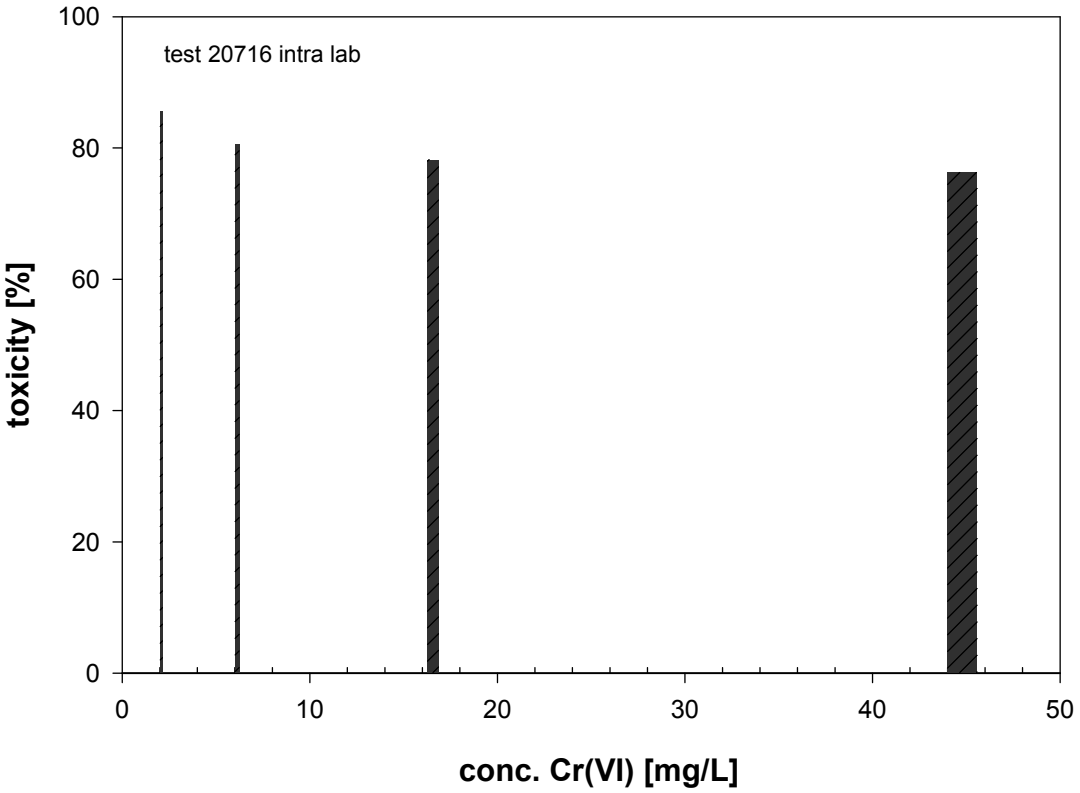
100% = 80 mg/ml sediment equivalent sample no. 1: August01 extraction in Acetone/hexane, subfraction ethylene acetate-hexane - subsequent resolved: 3% DMSO were used for test.

Weaver Human Cell Test evaluation experiment sample 2 / 4-Nitroquinoline-N-oxide

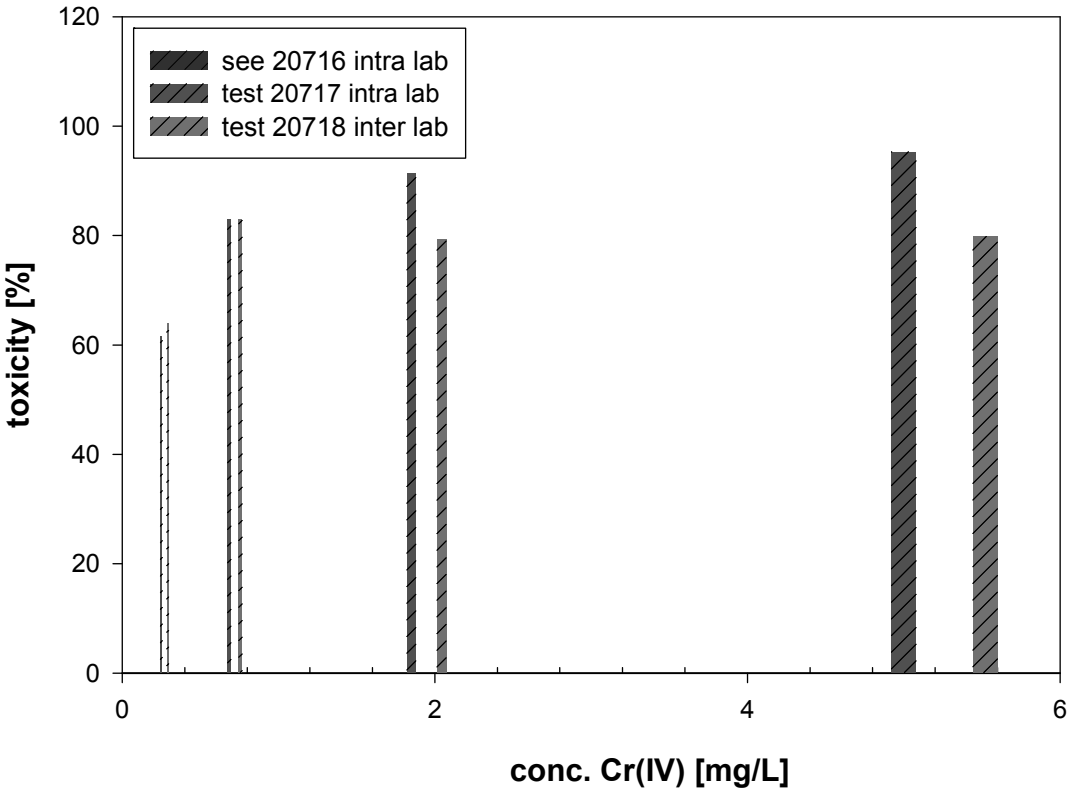


4-Nitroquinoline-N-oxide in A. bidest.

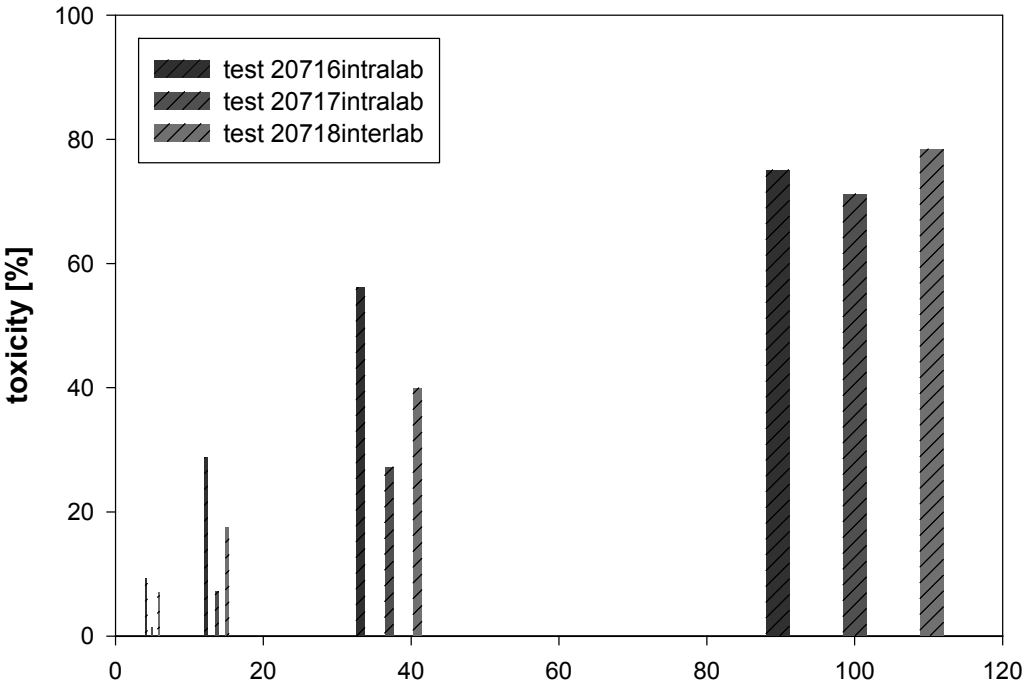
Weaver Human Cell Test validation experiment sample 3: potassium-dichromate CrVI



Weaver Human Cell Test validation experiment sample 3: potassium-dichromate CrVI



Weaver Human Cell Test validation experiment sample 4 / Industrial Station / sediment fraction F5



100% = 80 mg/ml sediment equivalent sample no. 4: 67601 August01 extraction in Acetone/hexane, subfraction methanol - subsequent resolved: 3% DMSO were used for test.

Weaver Human Cell Test validation experiment sample 5: Phthalate Ester Mix No. 3 (Ehrenstorfer)