# AN INVESTIGATION OF THE USE OF CELL CULTURE TECHNIQUES IN ECOTOXICITY TESTING

**John J. Bartlett B.Sc. (Hons)**

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From research work done at,
School of Science
Regional Technical College
Ballinode
Sligo

Supervisors: **Mr. John W. Gault** B.A.(Mod) MIBI. MICI.
**Mr. Eugene M. Brady** B.Sc. MIBI.

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## Dedication

To Angela.

Forsan et haec olim meminisse iuvabit...
....Durate, et vosmet rebus servate secundis.

Virgil, Aeneid.
Bk. 1, 203...207.

## Declaration

This thesis has not previously been submitted to this, or any other college. With acknowledged exception, it is entirely my own work.

John J. Bartlett. B.Sc. (Hons)

## Acknowledgements

I would like to thank Angela .....for everything (we'll always have Paris).

I would like to thank John Gault and Eugene Brady for their continued faith in me, and in cell culture.

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A special thanks to my family (Bartlett and McCarthy) and friends, who have wondered, perhaps as often as I, will it ever end?

## Abstract

The usefulness of cell culture methods in environmental analysis was assessed by direct comparison of a battery of *in vitro/in vivo* test methods. Criteria for comparison were the ease of handling, sensitivity of the test species, reproducibility, rate of generation of data etc. The experimental work was divided into two main areas, the first related to predictive hazard assessment, the second to environmental monitoring, as follows;

1:- Predictive Hazard Assessment.

The ecotoxicity of three chemicals of interest to the fish farming industry (Nuvan [dichlorvos], ivermectin and malachite green) was assessed. *In vitro* methods used were the cytotoxicity of the chemicals to two cell lines (L929 and RTG-2), each quantified by two dye end-points (crystal violet and neutral red). *In vivo* methods used were standard toxicity testing (fish acute toxicity, algal growth inhibition) and proposed screening methods (*Artemia*, *Brachionus* and *Streptocephalus* immobilisation).

The cell culture methods were found to be rapid, reproducible and versatile. There was no significant difference between the sensitivity of the two end-points. There was no significant difference in the sensitivity of the two cell lines to ivermectin. Sensitivity to Nuvan was largely similar. RTG-2 cells were significantly more sensitive to malachite green. The sensitivity of the cell lines relative to the whole organism trials varied from low (ivermectin), to median (Nuvan), to high (malachite green). Interpretation of the cell culture data in conjunction with Structure Activity Relationship (SAR) data greatly increased this sensitivity.

The most sensitive *in vivo* system was the fish acute toxicity test, the least sensitive was the algal growth inhibition test. Invertebrate screening trials were rapid and reproducible. Invertebrate sensitivity varied between species and toxin.

Overall, cell culture methods exhibited a number of advantages over *in vivo* methods. These included greater statistical strength, versatility, and a wide range of theoretical applicability. Limitations include a lack of systemic factors. However, the use of battery *in vitro* tests and the interpretation of cytotoxicity data in conjunction with SAR data would decrease these limitations.

2:- Environmental Monitoring.

(a) Metalliferous effluent study.

The toxicity of three fractions of a metalliferous effluent, one low pH, and two neutral, were determined using the L929 cell line (crystal violet end point) and *Artemia* Experiments on the effects of serum, exposure time and seeding density were carried out on the cell culture system. Effluent fractions were also analysed by AAS.

The sensitivity of the cell line to the metalliferous effluents was high. There were no major technical problems with the operation of the cell culture tests. Comparison with AAS data showed that metal concentrations were poor indicators of effluent toxicity. Increasing media serum concentration decreased the indication of toxicity given, at approximately double the rate seen with the sheep dip. All of the effluent toxicity was expressed within 24 hours. Increased seeding density reduced the toxicity indicated.

*Artemia* sensitivity to metalliferous effluents was low. A high sensitivity to the low pH effluent was shown to be related to pH.

( b ) Sheep dip study.

The toxicity of an organophosphate based sheep dip was assessed using L929 cells (including the effect of serum concentration, seeding density and incubation time on toxicity indicated) and *Artemia*, as above.

A column study to assess the efficacy of a peat based treatment system for the removal of sheep dip from solution was carried out using GC analysis, and toxicity analysis using L929 and *Artemia* , as above.

The sensitivity of the cell line to the sheep dip was found to be relatively low, which limited the amount of data that could be generated on the monitoring of column effluents. Increased media serum concentration reduced the toxicity indicated. Most of the toxic effect occurred within 48 hrs. Higher seeding density reduced toxicity indicated.

The sensitivity of *Artemia* was classed as medium, and was useful for the monitoring of column effluents.

Overall, results from the study demonstrated that there are a number of areas of environmental analysis to which cell culture methodologies could make a useful contribution. Analysis of materials in parallel with other ecotoxicity methods demonstrated a number of problems which are a feature of existing methods. Cell culture results improved on a significant proportion of these.

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# CHAPTER 1
# INTRODUCTION

## 1:1 The Impetus For The Development Of New Bioassay Systems.

### 1:1.1 The use and deficiencies of current bioassay systems.

There are two main approaches which are taken in the scientific assessment of environmental pollutants (Persoone, 1988; Maltby and Calow, 1989). These are;

(a) Predictive Hazard Assessment (which includes toxicity testing and other laboratory scale studies); and

(b) Retrospective Hazard Assessment (i.e. environmental monitoring).

Within each of these categories, there are a range of physical, chemical and biological methods which can be used to generate useful data. However, biological methods are considered the most directly relevant because physical and chemical (P/C) methods alone cannot determine the ecological effects of pollutants on complex communities. Also, environmentally significant levels of many toxins cannot be measured by current P/C methods (APHA- AWWA, 1980; Murphy, 1983; OECD, 1984; Wall and Hanmer, 1987).

Environmental management involves the use of data, initially from predictive studies to determine standards and controls (i.e. policy formulation), and subsequently from monitoring studies, to adjust any control mechanisms used (Depauw, 1981; Wall and Hanmer, 1987).

However, despite the importance of biological data, physical and chemical analyses contribute the bulk of data used in environmental management. This is because there are a number of significant restrictions on the use of biological tests (bioassays).

Because of the complexity of ecological communities, the ideal biological assessment level would be the community level, and because of the unique characteristics of many individual sites, the ideal study environment would be the intended site of release. However, these ideals are not realistic. As a result, the classical laboratory-scale $LD_{50}$ test has developed as the most sophisticated bioassay currently in use in environmental analysis.

Any departure from ideal automatically involves restrictions. The major scientific limitations inherent in these bioassays include the use

of a small number of easily handled species as assay material (which are often tolerant and, by definition, ecologically unrepresentative), the use of optimum test conditions for each species (also, by definition, environmentally unrepresentative), and the use of a small number of individuals (i.e. statistically weak) in each test (Persoone, 1988).

There are also physical limitations. Current bioassay systems are time consuming, require large amounts of space and materials, and, as a result, are more expensive than other systems. There are a large number of new chemicals synthesised each year (up to 1000), and the time requirement alone for current bioassays precludes the full predictive environmental hazard assessment of each of these by biological methods. As a result, many materials go into commercial production with little or no environmental toxicity data available. In fact, of the approx. 70,000 chemicals currently in commercial use, extensive ecotoxicological data is available for less than 1000 (Murphy, 1983; LaFlamme, 1984; Richards and Shieh, 1986; Babich and Borenfreund, 1987a, 1988a, 1990; Wall and Hanmer, 1987; Persoone, 1988).

In particular, there are few biological assays which are flexible enough to be used in continuous environmental monitoring studies. This has meant that monitoring studies have been restricted to point estimates of selected parameters (i.e. limited P/C analysis). As monitoring studies are the mechanism by which the release of effluents is most effectively controlled, this leaves a large gap in the environmental toxicity database.

Thus, the use of bioassays is limited, generally to tests in the predictive hazard assessment category, and to materials thought to represent specific, major hazards, as a result of intended use.

### 1:1.2 The requirements of new bioassay systems.

Because of the limitations on the usefulness of physical/chemical and classical $LD_{50}$ systems in predictive hazard assessment (both for new materials, and a backlog of untested existing materials) and the lack of an effective biological system for environmental monitoring, a need has been identified to develop new bioassay systems to increase the size and extent of the ecotoxicological database. To be a viable development, these would have to be rapid, precise, sensitive, reproducible, flexible, statistically strong and low-cost (Marion and Denizeau, 1983a,b; Wall and Hanmer, 1987).

The test would need to be applicable to the assessment of pure compounds (including formulated products), complex effluents, and environmental samples.

## 1:2 Study Design And Objectives.

### 1:2.1 A proposal for the use of cell culture in ecotoxicity testing, and an approach to examining this.

Cell culture methodologies have been proposed as a suitable test system which would satisfy the above requirements, and a number of studies have examined specific aspects of environmental applications (section 3:2.3, p. 49). A number of authors have stated that studies on the development of *in vitro* trials should compare results obtained to those obtained from *in vivo* trials carried out in the same laboratory (Christian *et al.*, 1973; Waters *et al.*, 1978; Marion and Denizeau, 1983a,b; Fry *et al.*, 1988; Babich and Borenfreund, 1990; Brandao *et al.*, 1992). No study to date has carried out such direct comparisons. The study described herein was designed in line with this principle, including the comparison of a number of different cell culture methodologies for relative usefulness.

An application to authentic environmental situations was regarded as most useful. For the study of the usefulness of cell culture tests in predictive toxicity testing, three chemicals of interest to the fish farming industry were examined. These were Nuvan (dichlorvos), ivermectin (a proposed alternative to dichlorvos) and malachite green.

To determine usefulness in environmental monitoring, two actual environmental cases were examined. The first was the ability of specific cell culture tests to contribute data to the assessment of the toxicity of a metalliferous effluent. The second was the ability of specific cell culture tests to contribute data to the assessment of the efficacy of a peat based treatment system for the removal of sheep-dip from solution. Each was compared to results from a specific invertebrate assay.

Figures 1.1 and 1.2 show the matrix of trials that resulted, in the two broad experimental areas. A precursor to cytotoxicity trials is the maintenance in culture of the chosen cell lines. This was common to both areas.

Figure 1.1

Predictive Hazard Assessment Trials

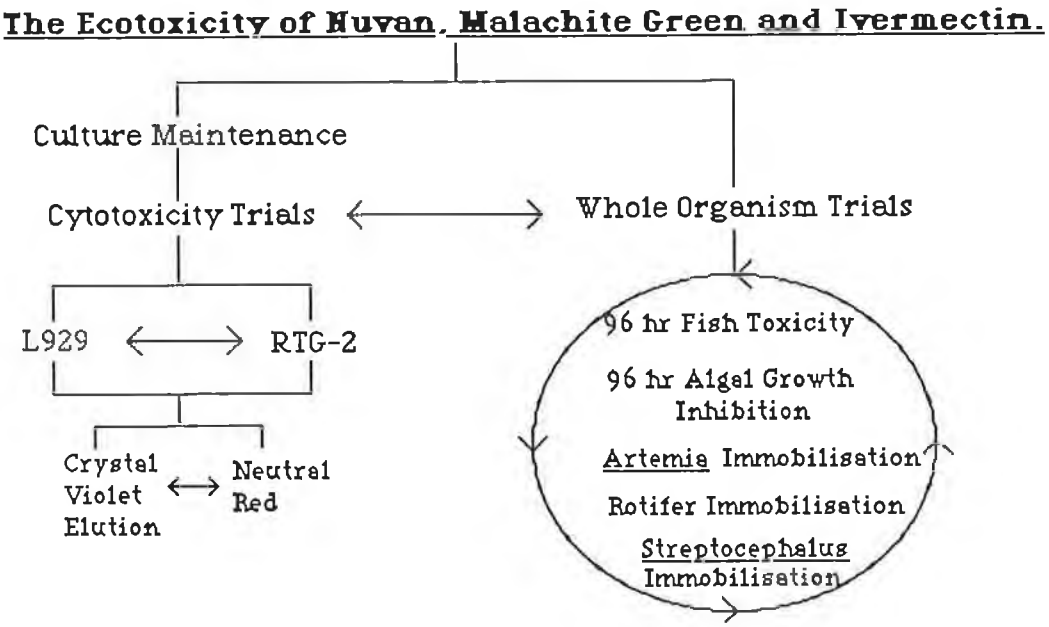
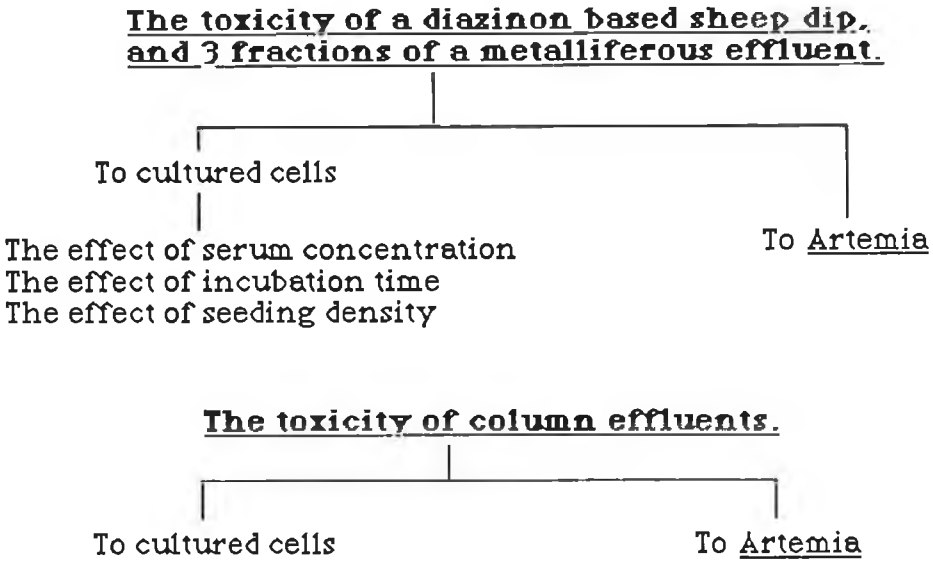



Figure 1.2

Environmental Monitoring Trials



1:2.2 Objectives.

The specific objectives of the study are as follows;

1. To examine the use of two cell lines (one mammalian, one fish), and two dye end-points (one vital, one non-vital), in predictive ecotoxicity trials by establishing the cytotoxicity of three chemicals associated with the fish-farming industry.

2. To compare the results from the cell systems to those from a group of standard whole-organism trials (three invertebrate, algal and fish toxicity) also carried out by the author .

3. To investigate the toxicity of three metalliferous effluents using chemical and biological analysis (i.e. a representative cell culture trial, and whole-organism trial), and to compare the usefulness of these systems in defining them.

4. To carry out an investigation into the efficacy of a peat-based treatment system for the treatment of sheep-dip, using physical/chemical and biological analysis (as above), and to compare the usefulness of the these systems in monitoring its activity.

5. To determine, using the results from the above, whether cell culture can be a useful addition to the discipline of environmental analysis.

## 1:3 Layout of Thesis.

As noted, there are two distinct areas of investigation in this study. These are predictive hazard assessment, and environmental monitoring trials. Each generates data which would be used in different ways to achieve the overall objective of environmental management. The layout of the thesis reflects these categories also.

The cell culture, whole organism and other analytical methodologies used were common to all areas of the study, and a description of these is contained in a single chapter, Materials and Methods.

There is a great deal of literature on the topic of environmental analysis generally, and within each of the specific areas investigated. A large amount of data was also generated in each set of experiments. In order to maintain clarity and to allow the focus to be retained on the literature relevant to each area, the two sets of experiments have been presented as separate chapters, each with their own Literature Review, Results and Discussion.

In order to place the specific findings in the context of overall environmental analysis, a further short chapter is presented on the findings re: the overall usefulness of cell culture in environmental analysis.

The resulting layout of the thesis is as follows;

**Chapter 1:** Introduction.

**Chapter 2:** Materials and methods.

**Chapter 3:** Predictive hazard assessment trials.

**Chapter 4:** Environmental monitoring trials.

**Chapter 5:** Selection of bioassays for environmental analysis

**Chapter 6:** Conclusions/Recommendations

**Chapter 7:** References

**Chapter 8:** Appendices

# CHAPTER 2
# MATERIALS AND METHODS

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## Chapter 2: Materials and Methods

## 2:1 Introduction.

This study involved two distinct areas of experimentation, as described in chapter 1, i.e. predictive hazard trials and monitoring trials. However, as similar methodologies were used in both, presentation of materials and methods in this way would not be appropriate. The methods themselves fall into three broad categories, cell culture maintenance, cell culture trials, and ecotoxicity trials. A fourth major category is a description of the column experiments, which are a significant portion of the monitoring trials.

The following chapter on materials and methods is divided into these four broad sections, along with sections referring to the toxins studied, data handling and waste management.

## 2:2 Cell Culture Maintenance.

### 2:2.1 Chosen cell lines.

Two different cell lines were used in these trials, one mammalian, and one fish cell line as follows.

#### 2:2.1.1 L929.

Mouse areolar and adipose tissue, fibroblast morphology. Supplied by Medical Supply Company (MSC). Flow catalogue 03-439. This cell line, being of mammalian origin, has a temperature optimum of 37 $^{o}$C, and was cultured in 25 cm$^2$ culture flasks (MSC. Cell cult. 3205P), in a standard 37 $^{o}$C incubator (WT Binder, D-100).

#### 2:2.1.2 RTG-2.

Pooled male and female gonadal tissues from rainbow trout, fibroblast morphology. (MSC. Flow. 02-734). This cell line has a temperature optimum of 20-22 $^{o}$C. As no such incubator was available, the $CO_2$ incubator for mammalian trials was adapted by inserting a cooled water coil which succeeded in maintaining the temperature at 19-22 $^{o}$C during all RTG-2 trials.

### 2:2.2 Growth media.

The same growth media was used for both cell lines during similar procedures. However, different media were used for maintenance and toxicity trials, as follows.

2:2.2.1 Maintenance media.

Both cell lines were maintained in Eagles Modified Essential Media (EMEM 1X, with 20 mM hepes buffer) (MSC. Flow. 12-104-54). Before use, the following were added to 500 ml; 11 ml penicillin (5,000 IU/ml)/streptomycin (5,000 µg/ml) solution (MSC. Flow. 16-700-49); 5.5 ml L-glutamine (200 mM) solution (MSC. Flow. 16-801-49), 5.5 ml Non Essential Amino Acids (100X solution. MSC. Flow. 16-810-49); 27.5 ml foetal bovine serum (to 5%. MSC. Flow. 29-191-54).

2:2.2.2 Trial media.

Cytotoxicity trials are carried out in 96 well plates (MSC. Cell cult. 33F96LC). As these multiwell plates are not sealed, pH balance is maintained by incubation in a 5% $CO_2$ atmosphere (MSC. Flow. IR 1500 incubator), using a media which does not contain hepes buffer, but which does contain 2.0 g/l sodium bicarbonate (MSC. Flow. 12-102-54). One other significant difference is that no antibiotics are used in trial media, as these are known to interfere, in some cases (Horner and Shah, 1984, Clothier *et al.*, 1985). All other additives were the same. All of the above products were obtained sterile.

2:2.3 Media replenishment and passaging.

Extensive cell growth is accompanied by a reduction in available nutrients, and a reduction in media pH (caused by the release of acidic metabolites), indicated by a yellowing of the media additive, phenol red indicator. It is important to change culture media regularly, so as to maintain optimum growth and condition. This media replenishment was carried out, as necessary, throughout the study.

Cultured cells can grow, either in a layer, attached to the bottom of a culture flask, or in suspension. Both cell lines used in this study grow attached. These cells replicate according to individual population dynamics, but all will eventually reach a point of 'confluence', when all available space in the culture flask is taken up.

Overgrowth causes stress on the cells in culture (indicated by increased vacuolisation, ragged cell membranes etc.). Thus, when confluence is reached, it is necessary to divide up the cells. This is done according to a preselected split ratio, and is called passaging. The technique involves the use of an enzyme solution which causes the cells to round up and detach from the flask (trypsin/EDTA. Flow. 16-891-49).

Passaging not only maintains optimum conditions, but also

provides material for assays. It is important for the even distribution of the cells in the multiwell plates, that disaggregation of the monolayer produces a single cell suspension. This was the case with the L929 cell line. As no protocol was available in the literature for the RTG-2 cell line, the same protocol was used in initial trials for both lines. However, it was extremely difficult to achieve a single cell suspension using this protocol with the RTG-2 cells, and a second protocol was developed, as outlined below.

2:2.3.1 L929 passaging.

The culture media was poured off, and the cell layer washed with phosphate buffered saline (PBSA tabs. MSC. Flow. 28-103-49). This was poured off, and 1ml of the trypsin/EDTA solution was added. This was swirled around for 60 seconds, and then poured off. The cell layer was then incubated for 2-3 minutes, until detachment occurred.

5 ml of media was added, and the cell suspension was drawn gently up and down a 1 ml pipette to achieve a single cell suspension. An aliquot was withdrawn aseptically, and counted on a haemocytometer. The split ratio was determined from this, and the requisite volume of suspension was removed to the appropriate number of flasks. Media was then added to the new flasks, to bring the final volume in each to 15 ml. All flasks were marked with the date and passage number, as well as the name of the cell line.

2:2.3.2 RTG-2 passaging.

The basic technique for passaging RTG-2 cells was the same as in section 2:2.3.1, except that the above trypsin contact regime did not give good disaggregation, and cell clumps caused a number of problems during a number of early trials.

A series of tests were carried out to determine the optimum trypsinisation conditions for RTG-2 cells, and the following modifications were adopted;

(1) The trypsin/EDTA solution was not poured off, but was allowed to contact the cells until after incubation, which was extended to 10 minutes. This is significant because trypsin is regarded as toxic to cells, and extended contact is not generally recommended.

(2) The incubation temperature was changed from the RTG-2 cell temperature optimum of 20 $^{o}$C, used in initial attempts, to the mammalian temperature optimum of 37 $^{o}$C. This is significant because no previous reference could be found to the use of 37 $^{o}$C with a non-mammalian cell line. However, the above protocol works well, particularly when the cell/media suspension is drawn gently up and down a sterile 10 ml pipette.

2:2.4 Freezing and recovery.

It is desirable to maintain stocks of cultured cells in liquid nitrogen, both as a reference material, and as a replacement in case of stock loss. It is recommended that a permanent laboratory should build up permanent stocks at regular intervals. In this study, both L929 and RTG-2 cells were frozen at approximately 5-passage intervals, according to the following protocol.

2-3 confluent flasks were trypsinised, and resuspended in media to achieve a final concentration of approximately $1x10^{6}$ cells/ml in EMEM. 10% v/v sterile glycerol was added as a cryostatic agent. This was then dispensed into 3 sterile cryotubes (MSC. Cell Cult. 35002). These were left on ice for 30 minutes, transferred to a container of dry ice for 2 hours, and then transferred on cryocanes (Nalgene. 5015-001) to the gas phase of a liquid nitrogen storage unit (MSC. Taylor Wharton 10 HCL).

Recovery of cells was achieved by immersing in a 37 $^{o}$C water bath, directly after removal from the liquid nitrogen. The contents of the vial were transferred to a culture flask, and 10 ml fresh media was added very slowly (over 3-5 minutes), to avoid osmotic shock.

2:2.5 Sterile technique.

All of the above methods apply advanced sterile techniques. A number of points of handling technique, related to the use of multiwell plates were developed during this study to achieve this aseptic requirement, but are best noted during the section on cytotoxicity trials (section 2:4, p. 17). The following is a list of sterile materials and methods used during general culturing.

A laminar flow hood (Gelaire, BSB4) was used throughout the study. No materials were stored within this cabinet. The UV sterilising lamp was operated for half an hour before any operation, and at regular intervals

during extended sessions. Before each session, the cabinet was swabbed down with 95% EtOH. Sterile 1 ml and 10 ml pipettes were used at all times (Costar. 4012. Sterilin. 47110), as well as sterile petri-dishes for waste etc. (Sterilin. 109132). All materials were swabbed with EtOH on introduction to the cabinet. The necks of containers were flamed, where appropriate.

Individually wrapped sterile multiwell plates were used for trials, as well as sterile culture flasks. Also for trials, the multichannel pipettes (Flow. 77-827-00, Eppendorf. 4788, 200.00. 4780,000.010) were sterilised by swabbing, and the pipette tips were autoclaved (MSC. Flow. 61-210-00. 61-228-C2, Eppendorf. 102-122-110).

## 2:3 **Toxins.**

Three toxins used in the fish farming industry, were chosen for the predictive hazard trials. For the monitoring trials, a group of metalliferous effluents, and a diazinon based sheep dip were chosen.

### 2:3.1 Nuvan.

Dichlorvos (2,2-dichlorovinyl dimethylphosphate [IUPAC], $C_4H_7Cl_2O_4P$) is an organophosphate insecticide, whose action is based on direct cholinesterase inhibition. It has a very broad spectrum of uses in a number of areas of agriculture and horticulture. In the fish farming industry it is used to control adult stages of the sea louse. 1 litre of 'Aquaguard Sea Lice Treatment' (Nuvan), a 500 g/l solution of dichlorvos, manufactured by Ciba-Geigy Agrichemicals, was obtained as a gift from Ciba-Geigy Ireland ltd, Waterford.

### 2:3.2 Malachite green.

Malachite green is a triarylmethane dye ($C_{23}H_5N_2$), which has been used widely since 1936 as a fungicide and ectoparaciticide in the fish farming industry. 200 ml of '50% Malachite Green (Zinc Free)', an aqueous solution of 535 g/l malachite green (containing some acetic acid and some sulphuric acid), manufactured by Hygeia chemicals ltd, Oranmore, Co. Galway, was obtained as a gift from Bayer ltd, Dun Laoighre, Co. Dublin.

### 2:3.3 Ivermectin.

Ivermectin is an 80/20 mixture of 22,23-dihydroavermectin $B_{1a}$ ($C_{48}H_{74}O_{14}$) and 22,23-dihydroavermectin $B_{1b}$ ($C_{47}H_{72}O_{14}$), introduced as an agricultural pesticide and an antiparasitic drug in 1985. It is being proposed

for use in the fish farm industry as an alternative to dichlorvos, against sea lice. 50 ml of 'Ivomec', a 1% w/v solution of ivermectin was purchased from a local pharmacy.

2:3.4 Metalliferous effluents.

The metalliferous effluents used in the monitoring trials were obtained from an Irish electroplating company.

The waste disposal system on site involves collection of alkaline waste streams and acidic waste streams in separate tanks. These are then mixed to a pH between 6-8, and sodium metabisulphite added to coagulate precipitated metals. These are removed by filter press, and the liquor directly discharged.

A sample from each tank was taken, and three fractions of metalliferous waste prepared in the laboratory for these trials, as follows;

(1) A sample of the low pH effluent was filtered through a O.45µm filter, which had the dual effect of removing suspended solids, and sterilising the aliquot for cell culture analysis.

(2) A sample of simulated 'discharge' effluent was prepared by mixing the low and high pH fractions of the effluent, to pH 7.4, and adding the prescribed amount of sodium metabisulphite (25 kg/2500 gallons). This was then filtered, as above.

(3) A third fraction was prepared by neutralising an aliquot of the low pH sample with 6N NaOH, and filtering as above.

Each of these three fractions were measured for metal content (Cu, Cr, Ni, Zn), by atomic absorption spectrophotometry, using methods as described by the APHA (1981), and standard conditions, as described by the instrument manufacturers, Perkin Elmer.

It must be stressed that the quantities of metals found in the discharge fraction are not indicative of those discharged by the company. The fraction analysed was a simple laboratory simulation designed to provide a sample significantly different from the low pH effluent, using the broad treatment system used in the plant.

2:3.5 Sheep dip.

The sheep dip used in the monitoring trials was Osmonds Scab Approved Gold Fleece (Fly and Winter Dip). This is a solvent based diazinon product containing 60% w/w diazinon (an organophosphate insecticide), 32% solvent, and 8% emulsifiers. Recommended bath preparation is by adding 600 ml of product to 900 L (200 gal.) of water (conc. 400 mg/l a.i.). Recommended treatment is a contact time of at least one minute.

The batch used in this study was obtained as a gift from Osmonds (Cross Vetpharm group).

## 2:4 Cytotoxicity Tests.

The matrix of cell culture tests, as described in chapter 1, were chosen to address a number of questions raised by the proposed use of cell culture as an environmental analysis tool. These include the effect of the different sensitivities of cell lines, and of end-points, and the range of materials and situations to which the techniques could be applied.

Thus, two cell lines were chosen, one mammalian, and one fish. Two end-points were chosen, a non-vital dye (which stains all cells remaining, regardless of viability), and a vital dye (which stains only actively metabolising cells). The range of chemicals tested included organophosphates, avermectins, dyes and complex effluents, including metals. The range of situations examined included pure products, factory effluents, and a long term waste treatment experiment.

In both the predictive hazard trials and monitoring trials (excepting column effluents), and with all cell lines, end-points and chemicals, range-finding trials were carried out, to determine a set of concentrations for initial toxicity trials. The range was then refined, according to results generated, in subsequent trials.

In the predictive hazard trials, a complete set of product and end-point trials were carried out, firstly on one cell line, and then on the other. This was to keep the number of passages within each population set to a minimum.

Also in the predictive hazard trials, with each cell line, and each product, both cell lines were assessed simultaneously, using cell material from a single passage.

In the monitoring trials, the L929 cell line, and the crystal violet elution assay were selected for use. Each of the two sets of experiments were carried out over a small number of passages.

2:4.1 Plate seeding.

The first step in a cytotoxicity trial is the seeding of a multiwell plate with the required cell line. As previously noted, the production of a single cell suspension at this point is particularly important. There were some differences in seeding between the cell lines.

2:4.1.1 L929 seeding.

The optimum seeding density (i.e. that which allowed the population to be in log-phase after 24 hours incubation, and to reach sub-confluence over the trial period), was determined by growth curve experiments to be $3 \times 10^4$ cells/ml. Two to three flasks of near confluent, unstressed cells were passaged as in 2:2.3.1 (p. 13), and pooled into 5-10 ml of trial media. The cell number was determined by haemocytometer. The volume of cell suspension required for dilution was drawn off to a sterile culture flask, and the requisite volume of trial media added. This was mixed thoroughly by inverting the flask gently (excessive mixing causes frothing of serum proteins, and increases the risk of contamination).

An aliquot of seeding suspension was poured into the bottom half of a sterile petri-dish, and 0.1 ml per well of cell suspension was dispensed by multichannel micropipette to eleven columns of the multiwell plate (beginning at column two, as column one was retained as a reagent blank). Between the seeding of separate plates, extra mixing was carried out. The plates were covered and incubated for 24 hours at 37 $^{o}$C in a 5% $CO_2$ atmosphere.

2:4.1.2 RTG-2 seeding

Some difficulty was experienced with disaggregation of RTG-2 cells, using the initial protocol. In fact, cell clumps caused the failure of a number of early trials, through the retention of excessive amounts of dye, and the subsequent distortion of results. The protocol of passaging described in section 2:2.3.2 (p. 13) was developed to counter these problems.

With RTG-2 cells, a seeding density of $1 \times 10^5$ cells/ml was determined to be the optimum.

2:4.2 Toxin dilutions.

The next step was the dilution of test chemicals to trial concentrations. Identical dilution technique was applied for each cell line.

As Nuvan, malachite green and sheep dip were all reasonably water soluble, a similar dilution technique was used for all three. There were some differences in the dilution of ivermectin, column effluents and metalliferous effluents.

2:4.2.1 Nuvan, malachite green, sheep dip.

An appropriate volume of product was diluted to 25 ml in sterile, double distilled water, to provide a 10,000 mg/l stock solution. This was serially diluted in increments of 1/10 ml to give the required working solution. From this, a series of standard concentrations were made up as 10x the required media concentrations. From these, 1 ml was pipetted to 9 ml aliquots of trial media.

In this way, the same volume of media was used at all concentrations (i.e. the same concentrations of nutrients/salts etc. were applied). 1 ml of sterile double distilled water was added to a blank (zero concentration) universal.

2:4.2.2 Ivermectin.

This is only slightly water soluble, and so had to be dissolved in an organic solvent. 95% EtOH (0.2 µm filter sterilised) was used as the solvent for ivermectin in these trials.

Dilution was as in 2:4.2.1, except that 1 ml was removed by sterile syringe to 9 ml EtOH to give a solution of 1,000 mg/l, and intermediate standard solutions were prepared as 100x required media concentrations. These were then diluted in media as 0.1 ml in 9.9 ml media. This was to keep the media solvent concentrations as < 1%. A preliminary trial showed that this concentration of EtOH did not inhibit cell growth in either cell line.

2:4.2.3 Metalliferous effluents.

Because the constituents of the effluents were not known, the expression of concentration used was percentage (v/v) of effluent in solution. The highest concentration that could be applied was 10%, as there is a constant 1/10 dilution in media used as the final step in the protocol. Lower concentrations were achieved by preparing intermediate dilutions in sterile double distilled water, as before.

During the range finding trials, an additional plate was prepared using PBSA as the diluent, to determine whether this provided any

buffer against the toxic effects of pH. However, no significant buffering was seen.

2:4.2.4 Column effluent.

In the case of column effluents, the sample for analysis contained that amount of pesticide components which were not held in the peat, as well as any materials leached from the peat.

The pesticide concentration was known, as it was measured by GC. However, as there were components other than the active ingredient present, an expression of concentration as mg/l diazinon would not be appropriate. The expression used was the percentage of effluent applied.

As with the metalliferous effluents, the highest concentration of column effluent that could be applied in cell culture trials was 10% (i.e. 1 ml of effluent added directly to the media). Other dilutions were prepared in double distilled sterile water.

2:4.3 Toxin application.

The same technique for toxin application was used in all cases. The protocol described here was developed in order to apply the toxins in the most reproducible and consistent fashion possible.

Twenty four hours after seeding, the plate was taken from the $CO_2$ incubator to the laminar flow air hood, and the media was removed by inverting the plate over a waste tray. Each end of the plate was 'flicked' over the tray to remove the maximum amount of liquid from each half. This technique was found to be the most effective. Individual column removal, using the multichannel pipette, was found to be extremely time consuming, and often caused cells to be scraped from the bottom of the well. The technique was used in all relevant stages of toxin application, and all relevant stages of each end point procedure.

The cells were washed twice with sterile PBSA (from a sterile petri-dish) prior to toxin application. The blank media aliquot was mixed thoroughly by inversion, and poured into the bottom half of a sterile petri-dish. 0.1 ml of the solution was dispensed to the blank wells of the plate (columns 1-3). This procedure was then repeated for each subsequent concentration of toxin, using the same micropippette tips, but flushing with each concentration.

The plate was then covered, marked with an identification code, and returned to the $CO_2$ incubator for the trial period (96 hrs).

2:4.4 End points.

There are a number of end points that can be used to assess the effect of a chemical on the cultured cells, after the incubation period. These include dyes, enzyme leakage, DNA damage etc. For a routine environmental assay, the requirement would be that it would be quick, low-cost, and would not require sophisticated technology. With this in mind, two dye methods were chosen, as follows.

2:4.4.1 Crystal violet elution assay.

The crystal violet elution assay described here was adapted from Dykes *et al.* (1984). The multiwell plate was removed from the $CO_2$ incubator. The toxin media was removed from the wells by inversion. The wells were washed twice with 0.1 ml of non-sterile PBSA. The cells were fixed by addition of 0.1 ml of 10% formaldehyde (non-sterile), and left for 15 minutes. This was removed, and the cells were washed twice as before. The cells were dyed with 0.1 ml of 0.25% crystal violet (non-sterile), and left for 30 minutes.

This was removed, and the cells were washed at least five times with PBSA to remove excess crystal violet (this washing is particularly important). Retained dye was eluted with 0.1 ml of 33% glacial acetic acid (non-sterile). Elution during the predictive hazard trials was for a minimum of one hour. During the monitoring trials, a plate-shaker was used (Heidolf, DSG-304/M4), reducing elution time to 10 minutes. Absorbances were read at 540 nm on a plate scanning spectrophotometer (Titertek, Multiscan MCC) (ref. $\lambda$ 620 nm).

2:4.4.2 Neutral red assay.

The protocol used in this study was as described by Borenfreund and Puerner (1985). It is broadly similar to the crystal violet elution assay except that, after toxin removal, the cells were re-incubated for 3 hours in 0.1 ml of a sterile solution of 50 µg/ml neutral red, prepared in media.

The fixing solution was 0.1 ml of a formaldehyde/calcium chloride mixture for 1 minute (10 ml 40% formaldehyde + 10 ml 10% calcium chloride + 80 ml water [non-sterile]). The elution solution was 0.1 ml of an acetic acid/ethanol mixture (1 ml glacial acetic acid in 50% EtOH [non-sterile]).

The reading and reference wavelengths were the same as for the crystal violet elution assay.

2:4.5 Serum experiments.

Media serum concentration is known to have an effect on the toxicity of some pollutants. A series of trials were carried out to examine this, using the low pH fraction of the metalliferous effluents, and the sheep dip. Three media serum concentrations were examined, these were 1, 5 and 10%.

The cell line used was L929; plate seeding was as in 2:4.1; toxin dilution was as in 2:4.2.3 and 2:4.2.4; toxin application was as in 2:4.3, and assay was by crystal violet assay, as in 2:4.4.1.

2:4.6 Incubation time experiments.

Length of incubation is known to have an effect on the toxicity of some pollutants. To examine this, a series of trials were carried out using the low pH fraction of the metalliferous effluents, and the sheep dip.

The cell line used was L929, and the media serum concentration was 5%. Seeding, dilution, toxin application and assay were as in 2:4.5. With the metalliferous effluent, the seeding density was higher than standard, at $6x10^4$/ml. With the sheep dip, there were three seeding densities, as described in 2:4.7.

2:4.7 Seeding density experiment.

Seeding density can also effect the outcome of a toxicity trial, though to a more controllable degree than serum concentration or time, as the optimum seeding density for a particular cell line, serum concentration, media, and culture environment are determined in advance, and applied in subsequent toxicity tests.

To examine the effect in this particular study system, a seeding density trial was carried out in tandem with the sheep dip time trial (i.e. not with the metalliferous effluent). A series of plates were seeded at three densities, $3x10^4$/ml, $6x10^4$/ml, and $1x10^5$/ml. Cell line, serum concentration, dilution, toxin application and assay were as in 2:4.6.

## 2:5 Ecotoxicity Tests.

There are a number of standard whole animal ecotoxicity tests in regulatory use, such as those recommended by the OECD (1984). There are also a number of new systems being developed, or in tentative use, such as those promoted by the University of Ghent, Belgium.

Because of the need to provide directly comparable results for the cytotoxicity results; wishing also to demonstrate the complex ecological systems which any toxicity test would need to model; and bearing in mind the characteristics of the toxins under trial, a number of tests were chosen, as follows;

(a) The 96 hr. Fish Toxicity Test
This was chosen as it is the standard reference test for aquatic animal toxicity. Rainbow trout were chosen as the test species to provide direct comparison with the RTG-2 results.

(b) The 96 hr. Algal Growth Inhibition Test
This was chosen as it is the standard reference test for toxicity to aquatic flora, and would increase the available information on the effects of the test chemicals on the aquatic food chain.

(c) *Artemia* Immobilisation
Though not a standard test, this seawater crustacean test is used by a number of industries and regulatory bodies as an additional test. *Artemia* belong to a taxonomic group which may be affected by the use of a number of the toxins examined here.

(d) *Brachionus* Immobilisation
Among the group of trials developed by the Ghent University team, rotifer toxicity refers to the effect of the test chemicals on one of the lowest links in the freshwater food chain.

(e) *Streptocephalus* Immobilisation
Also among the tests developed at Ghent, *Streptocephalus* is a freshwater crustacean, whose toxicity would further extend the matrix of investigations being carried out.

Range finding trials were carried out for each chemical, and with each test system (except the fish trials) to give initial trial

concentrations. These were then refined, according to results generated, in subsequent trials.

2:5.1 The 96 hour fish toxicity trials.

These trials were carried out at the Eolas Environmental Toxicity Laboratory, Shannon, Co. Clare. 2,500 rainbow trout fingerlings were purchased from Finure farms on the 6th of march 1991. These were kept in a stock tank, using Shannon municipal supply water (Plate 2.1). They were initially fed three times a day, but this was down to once a day at the beginning of these trials. Daily selection was carried out for mortalities, disease and cannibalism.

The test protocol used was as described by the OECD (1984). The trials were 96 hr. static batch tests, using non-replenishment, and with constant aeration. 10 fish per test volume were used. As the fish were sub-two gramme in weight, twenty litre trial volumes were used to keep loadings below the maximum recommended 1 g/l (Plate 2.2). The fish were starved for 24 hours before the trial. Mortalities and oxygen levels and temperature were monitored throughout each day, and pH was monitored daily. Dead fish were removed daily.

Initially one Nuvan trial and one ivermectin trial were carried out in plastic containers, because of lack of conclusive information on their attachment to glass. However, these two failed within 24 hours, due to extensive escapement.

Because there was published information on the toxicity to rainbow trout of ivermectin; taking note of restrictions on time and facilities; and noting that previous fish toxicity and chemical trials on Nuvan were carried out in glass, it was decided to dispense with the ivermectin trial, and to re-start the Nuvan trial in glass containers.

The malachite green trial was also carried out in glass containers. Reducing the number of trials to two allowed extension of the Nuvan concentration range, while retaining a direct comparison between experimental results and previously published results for at least one chemical (i.e. malachite green).

**Plate 2.1**
**Rainbow trout stock tank**

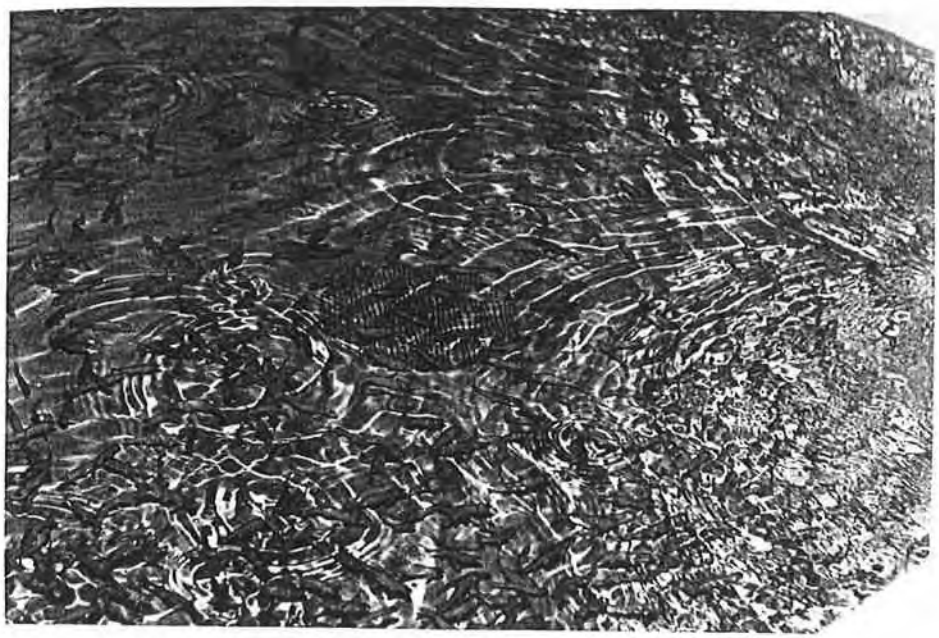
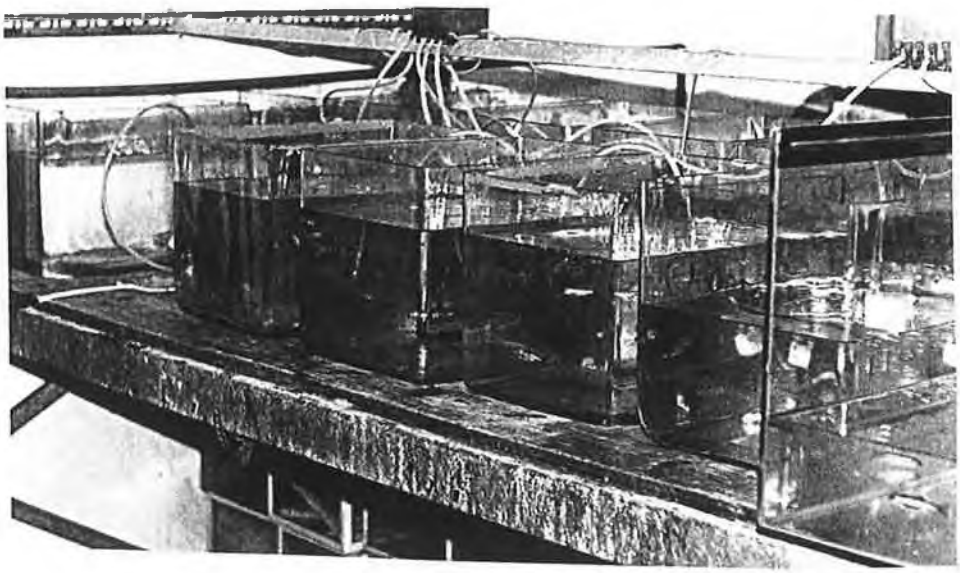


**Plate 2.2**
**Rainbow trout toxicity trial apparatus**



2:5.1.1 Nuvan.

No information was available on the toxicity of Nuvan to Rainbow trout. From the information on toxicity to other fish, and from other results generated during this study, it was decided to apply a range of 0.01 mg/l to 1.0 mg/l in the initial trial. With the results generated from this, the range applied in the second test was 0.0056 mg/l, 0.01 mg/l, 0.056 mg/l, 0.1 mg/l, 0.32 mg/l, 0.56 mg/l, 1.0 mg/l.

Dilutions were made up in 10 ml volumes, in double distilled water. Concentrations were made up as 2,000x the desired strength, which meant that the volume of toxin added to the 20 litre tanks was 10 ml.

2:5.1.2 Malachite green.

As there was published data available on the toxicity of malachite green to Rainbow trout (96 hr. $LC_{50}$ = 0.248 mg/l, Bills *et al.*, 1977), the applied range was designed around this, and to demonstrate 100% mortality and the No Observable Effect Concentration (NOEC). Dilutions were prepared as in 2:5.1.1. The chosen range was 0.056 mg/l, 0.10 mg/l, 0.32 mg/l, 0.56 mg/l and 1.0 mg/l. After 24 hours, the concentrations 0.018 mg/l and 0.01 mg/l were added.

2:5.2 96 hour algal growth inhibition trials.

The protocol used for these trials was that described by the OECD (1984). An initial series of experiments were carried out on each of the recommended species (*Selenastrum capricornutum* [CCAP 278/4], *Scenedesmus subspicatus* [CCAP 276/20], and *Chlorella vulgaris* [CCAP 211-11S]), to determine the most suitable species for the type of cultivation being used, and for analysis by optical density. From these *Chlorella vulgaris* was chosen as the test species. A calibration curve relating cell number (determined by haemocytometer) to absorbance (689.6 nm) was established, and used to measure growth in the trials. The information required under the OECD protocol is given below.

2:5.2.1 Test chemicals.

Information on test chemicals is given in chapter 3.

2:5.2.2 Test organism.

*Chlorella vulgaris* (CCAP 211-11S) was obtained from The Freshwater Biological Association, Cumbria. Laboratory cultivation was by

shaken culture in 250 ml closed Erlenmeyer flasks (sterile), under > 4,000 lux illumination, at 20 $^{o}$C. 100 ml volumes were cultured in OECD recommended media. The concentrated stock media ingredient solutions were prepared and autoclaved in double distilled water, the final media was prepared in sterile double distilled water.

50 ml of the culture volume was transferred to fresh media every week. Pre-culture was as described by the OECD.

2:5.2.3 Toxin dilutions.

Sufficient volumes of the test organism, in lots of 1 litre sterile culture media were prepared at 2 x $10^5$ cells/ml (2x). Toxin dilutions were prepared in sterile media, in sterile 150 ml containers, in lots of 150 ml (3 replicates of 50 ml), at 2x the required concentration. The dilution technique was similar to that described in 2:4.2 (p. 18).

The 1 litre volumes of *Chlorella vulgaris* were constantly stirred by (sterile) stirring bar. 50 ml aliquots of algal culture were dispensed aseptically to the required number of sterile 250 ml Erlenmeyer flasks, stoppered with cotton wool and aluminium foil, which were placed at random on a bench.

50 ml aliquots of the toxins were aseptically dispensed to randomly selected flasks. These were then placed at random on a grill above a constant light source, in a controlled temperature chamber (20 $^{o}$C). The cultures were shaken every day, and absorbance readings were taken at 48 hrs. and 96 hrs.

2:5.2.4 Ivermectin.

The only exception to the above protocol was ivermectin, which had to be dissolved in EtOH. Non-aqueous solvents must not be present at a concentration > 0.1 ml/l in the test, and so 10 µl volumes of 10,000x concentrations were aseptically dispensed by microsyringe to 100 ml volumes of 1 x $10^5$ cell/ml algal cultures.

2:5.3 *Artemia* immobilisation.

Dormant cysts of the brine shrimp, *Artemia salina*, are readily available in pet shops, as fish food. An ecotoxicity screening trial using these has been in use for some time by researchers, industries, and some regulatory authorities. The advantages of the test are speed, sensitivity

(though the test is less sensitive than some others) and cost effectiveness. These are the reasons that the test was chosen for this study.

The protocol used in this study is an adaptation of that developed by the research team of Professor G. Persoone, at the State University of Ghent, Belgium. The cysts were obtained in a local aquarian shop, manufactured by King British Aquarium Accessories ltd., Bradford and Yorkshire.

2:5.3.1 *Artemia* hatching.

Standard sea water (unsterilised), as described by the APHA (1981) was used for cyst hatching, and for all trials. 48 hours before the toxicity test, a quantity of *Artemia salina* cysts were places in a petri dish. 9 ml of standard seawater was added and swirled. The petri dish was covered and exposed to a light source of >4000 lux for one hour at 25 $^{o}$C. This was then incubated at 25 $^{o}$C in darkness for 24 hours.

After 24 hours, the hatched larvae (Instar I) were transferred to a further 9 ml fresh media, and re-incubated for a further 24 hrs., to moult into Instar II-III. To maximise the collection of the Instar I larvae, a light was shone on one side of the dish, the other covered with aluminium foil. As the larvae are positively phototactic, they congregated on the illuminated side, and were more easily captured, using a Pasteur pipette. As the larvae feed endogenously for 3-4 days (Nikonenko, 1987), they are not fed during the trial.

2:5.3.2 Toxin dilutions.

The toxin dilution protocol was similar to that used in the cell culture trials (section 2:4.2, p. 18). A set of dilutions were prepared in sea-water at 10x, then each was diluted 1/10 in seawater, to test concentration. This was in order to provide a direct comparison between the invertebrate and cell tests, and meant that the highest dilution of the media with toxin was 1/10 for the invertebrate, predictive hazard trials also. This allowed maintenance of salinities at a consistently high level.

However, this is not a strict requirement of the test, as *Artemia* are very tolerant to changes in salinity, and reductions by up to 80% have been shown to cause no adverse effects (Persoone and Wells, 1987).

In the monitoring trials, with the metalliferous and column effluents, where 10% effluent was found to produce no toxicity, the concentration was raised to a maximum of 75%, in order to establish an $IC_{50}$.

2:5.3.3 Toxicity trials.

24 well plates were used (Corning. 25820). Five concentrations and one blank were tested on each plate. A solvent blank was included in the ivermectin trial.

2 ml of toxin solution was added to each of 4 wells in one column. Three of these were test wells, the fourth was used as a rinsing trough to minimise dilution of the toxins during transfer of the larvae. A quantity greater than 30 larvae was delivered to each rinsing trough via an apparatus made up of a micropippette tip attached to a disposable 10 ml pipette, and controlled by a 10 ml pipette pump. A fresh tip was used for each concentration to avoid carrying any toxin back to the larval stock. Delivery was monitored using a 10x microscope, with illumination from a separate light source.

10 larvae were then dispensed to each of the 3 associated toxin wells from each of the rinsing wells, beginning with the blank. As the sequence was from the blank up to high concentrations, it was not necessary to use separate pipette tips for this part of the procedure.

The plate was covered and incubated in the dark at 25 $^{o}$C. After 24 hours, the number of dead and living larvae in each well were recorded. The larvae were considered dead if they did not show any movement during 10 seconds of observation.

2:5.4 *Brachionus* immobilisation.

This test, using juveniles of the rotifer *Brachionus calyciflorus* hatched from cysts, is another adapted from the group of tests developed by the State University of Ghent. It was chosen for this study because of the important position that rotifers occupy in the freshwater food chain.

The test protocol was broadly similar to the *Artemia* protocol, with the following differences;

2:5.4.1 Test media.

The hatching and trial media was Standard Freshwater (Hard), as described by the APHA (1981).

2:5.4.2 *Brachionus* hatching.

Cysts were obtained in vials from the State University of Ghent. The contents of one vial were emptied into one well of a 24 well plate. 2 ml of

media was added. The cysts were exposed to a light source of >4000 lux for 15 minutes. They were then incubated at 25 $^{o}$C for 16 hours. Trials were initiated within 2 hours of hatching.

2:5.4.3 *Brachionus* handling.

Rotifers are too small to dispense effectively using the apparatus described in the *Artemia* protocol. Instead, the tip of a glass Pasteur pipette was stretched as thinly as practicable, using heat, and this was used for the transfers. The flow was controlled with a 2 ml pipette pump.

2:5.4.4 Toxicity trials.

In range finding tests, 10 rotifers per well were used. In trials, 8 per well were used. A toxin volume of 1 ml was used.

2:5.5 *Streptocephalus* immobilisation.

The third screening test used the larvae of the fairy shrimp *Streptocephalus proboscideus*. It was chosen as a direct freshwater comparison to the *Artemia* immobilisation test. The protocol used was identical to that described in 2:5.3, with the following exceptions;

2:5.5.1 Test media.

The hatching and trial media was Standard Freshwater (Hard), as described by the APHA (1981).

2:5.5.2 *Streptocephalus* hatching.

Cyst hatching was initiated 24 hours prior to the trials. the contents of one phial (obtained from the State University of Ghent) was emptied into a petri dish. 9 ml of media was added and swirled. This was covered and incubated at 25 $^{o}$C under >4000 lux illumination for 18 hours. After 18 hours, the larvae were transferred to fresh media. These were re-incubated under illumination at 25 $^{o}$C for a further 6 hours before the trials.

2:6 **Column Experiments.**

This portion of the study had a dual purpose, to provide samples for analysis by cell culture and by *Artemia*, and to assess the efficacy of a peat based treatment system for the removal of sheep dip from solution.

This required the building of a series of peat columns, the development of a GC method for analysis of diazinon in sheep dip and

effluents, and the implementation of a column loading experiment over an extended period. These are described in the following section.

2:6.1 The Peat.

The peat used in this study was Bord na Mona Peat Fibre, provided by Bord na Mona Peat Research Centre, Newbridge, Co. Kildare. A second material, peat nodules, was also provided.

2:6.2 Column design.

The columns were designed as in Figure 2.1. They were constructed from PVC piping, 10.5 cm internal diameter, 75 cm tall. The base of each column was closed with a bung of the same material, with holes drilled to allow effluent to leave. A layer of acid-washed pea gravel, 7.5 cm deep, was placed at the bottom of each one, to prevent clogging.

Five columns were constructed. Three were filled with peat fibre, and two were filled with a 20/80 v/v mix of peat fibre and peat nodules (37/63 w/w). A 60 cm peat depth was used. A compaction rate of 25% was required (i.e. 60 cm used was equivalent to 80 cm uncompacted peat). This took 750 g of peat, wet weight (423 g.d.w.).

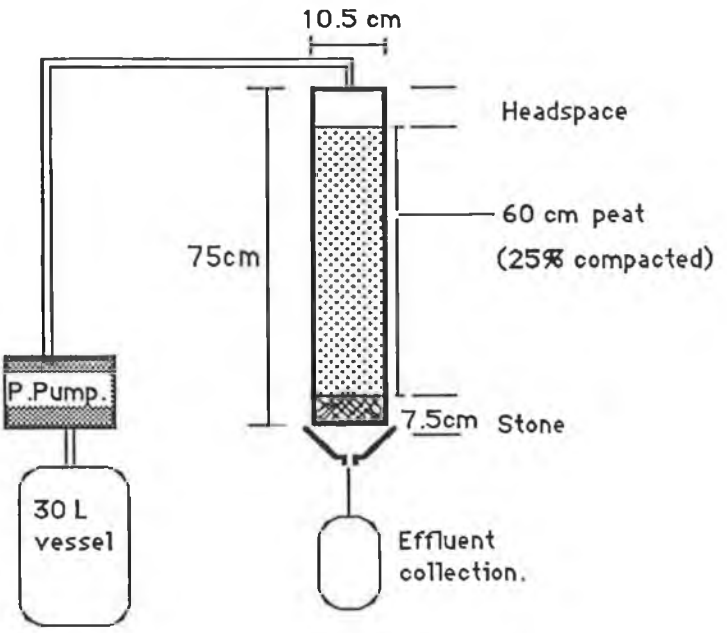
The columns were secured to a laboratory wall. In the initial stages of operation, a funnel was attached to the base of each, sealed with parafilm, and connected by tube to a 5 litre collection vessel. This facilitated continuous collection of the column effluents, and prevented evaporation from the base (Plate 2.3).

Evaporation from the top of the column was also prevented using parafilm. The development of channel flow in the peat was controlled by use of a splash grid, which was a small portion of steel grid, suspended by elastic band from the sides of each column, under the drop from the feed tube, but slightly off centre (Plate 2.4).

This had the effect of distributing the drop from the feed tube by splashing a large portion of the drop away from the centre at constantly changing rates, determined by the irregular movement of the grid, itself caused by the drop hitting the grid obliquely.

**Figure 2.1**

**Peat column construction**




2:6.3 Column operation.

Initially, it was intended that the columns would be continuously fed by peristaltic pump throughout the period of the study. The flow rate was controlled by the internal diameter of the peristaltic tubing. Two 30 litre reservoirs were installed under the peristaltic pump, one for distilled water, another for the sheep dip preparation.

The mixture was constantly stirred, using a paddle wheel(Plate 2.5). The reservoirs were at ground level, and tubing brought the sheep dip from the common sump to each column (Plate 2.6). Sheep dip batches were prepared daily. Samples were taken every 4-8 hours for the first four days, and then every 24 hours to the end of the first two weeks of operation. Samples were frozen for subsequent analysis.

After 2 weeks it was necessary to modify the column operation. The number of columns were reduced to 2 (one each of the two media types). Column feed was changed to gravity feed (Figure 2.2), with each column fed from a separate 30 litre reservoir. The dip was prepared every 3-4 days, and was not constantly stirred. Flow from the reservoirs was controlled by Teflon taps, and the effluent was collected intermittently (every 3-4 days), and frozen, as before.

**Plate 2.3**
**Peat column construction**

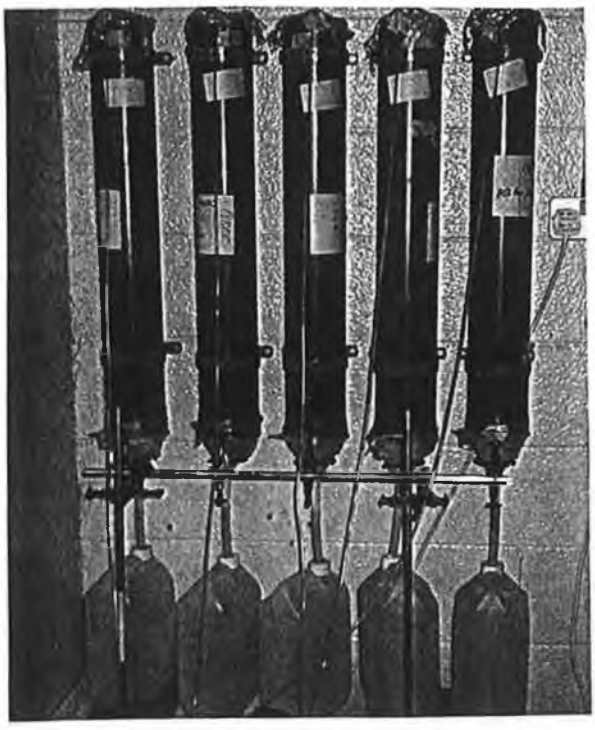


**Plate 2.4**
**Splash grid**

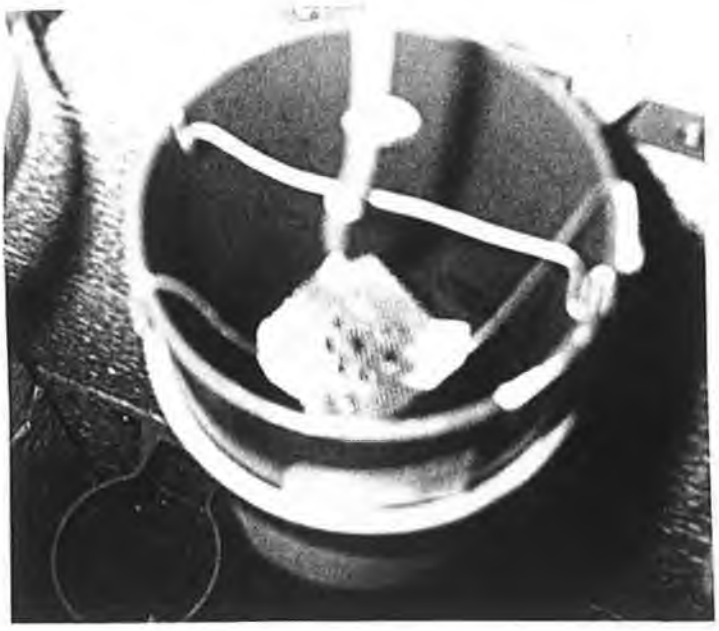


**Plate 2.5**
**Sheep dip reservoirs**

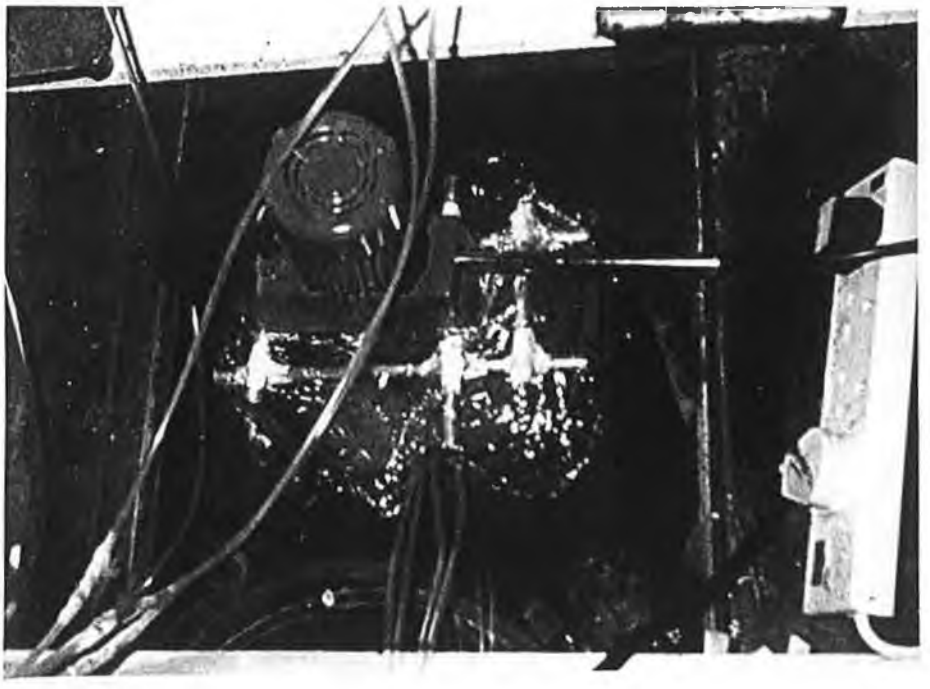


**Plate 2.6**
**Peristaltic delivery of sheep dip**

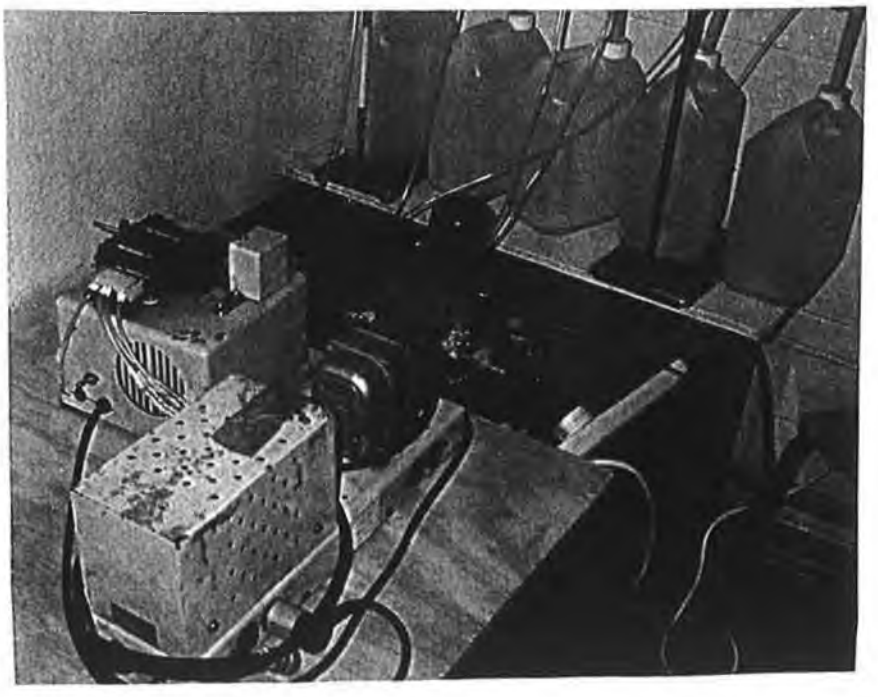
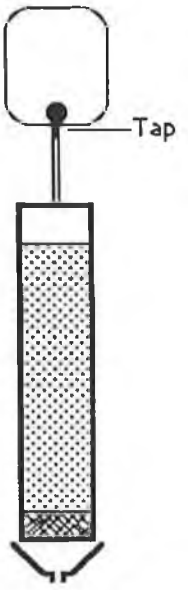


**Figure 2.2**

**Gravity column feed**




Intermittent Collection.

2:6.4 GC Analysis of Sheep Dip/Effluent.

2:6.4.1 Development of GC program.

The GC column chosen for analysis of sheep dip/diazinon was that described by Suett and Padbury (1982), i.e. a 1.5 m packed glass column, with WHP support, 80-100 mesh, and 4% OV-101 stationary phase. The column was obtained from Phase Separations Limited, and column conditioning was as described by the manufacturer. The instrument used for analysis was a Perkin Elmer, model Sigma 3, gas chromatograph, with FID detector.

As the sheep dip contains a dozen or more unknown materials, it was necessary to establish a program which would isolate diazinon from these. It would also have to separate the diazinon from any unknowns in the leached peat fibre column effluent. A short telephone survey of the laboratories carrying out pesticide analysis in Ireland determined that no-one is currently analysing for diazinon on a routine (i.e. continuous) basis.

Many of those who analyse intermittently by GC also require extraction procedures from food/soil/tissue or water, before determination. Given the high concentrations used in this study, and the relatively pure nature of effluents from the test material, it was envisaged that extraction, clean-up or concentration steps may not be necessary. Nevertheless, some

initial solvent extractions were carried out, to examine the logistics of sample preparation, and to identify a suitable solvent.

A range of operating conditions used by previous workers were examined, and used to guide initial investigations into diazinon separation. An acetone solution of pure diazinon (Reidel-de-Haen, cat. 45428) was used to identify the peak initially, then aqueous solutions of the pure sheep dip were ran to determine whether unknowns would interfere in this region. Finally, a portion leached through peat was used to determine if peat leachates would interfere with the diazinon peak. None were found, therefore it was decided that direct aqueous injection of the dip/effluent would be appropriate.

A suitable range of operating conditions for this study were then determined, these are set out in Table 2.1.

**Table 2.1**
**GC operating conditions**

| Parameter | Operating Condition |
|---|---|
| Oven Temp ($^{o}$C) | 195 |
| Injector/Detector temp ($^{o}$C) | 250 |
| Nitrogen (ml/min) | 40 |
| Air (ml/min) | 30 |
| Hydrogen (ml/min) | 20 |
| Retention time (min) | 5.0 |

2:6.4.2 Selection of internal standard.

An internal standard was required for injections, to minimise the effect of injection errors. Other organophosphate pesticides are commonly used as internal standards for selected OPs, however, this would not be practical in this situation, as the quantities required would not be appropriate for routine effluent analysis. A suitable i.s. would have to be readily available, structurally similar to the test material (i.e. have a useful retention time), stable etc.

A range of materials were tested, including Thiamine, Biotin, Glucose-6-Phosphoric acid, Glucose-1-Phosphate, and others. The material chosen was Di-N-Butyl Phthalate (DNBP). This has a retention time of 7.5 min under the conditions described in the previous section. This material was suitable for the conditions in this study, where fresh solutions were prepared

each day, but would not be suitable for long-term routine analysis, as aqueous solutions are not stable over long periods.

Peak height ratio (of diazinon to DNBP) curves were prepared for three ranges; 2-8 mg/l (active ingredient) diazinon (using 6 mg/l DNBP, prepared as 1 ml of 30 mg/l aqueous DNBP standard plus 4 ml aq. diazinon standard), 10-80 mg/l diazinon (using 30 mg/l DNBP, prepared as 1/4 from a 150 mg/l aq. DNBP std.), and 120-360 mg/l diazinon (using 150 mg/l DNBP, prepared as 2/2 from a 300 mg/l DNBP std.). In subsequent trials, and effluent analysis, samples within these ranges were prepared in a similar fashion.

2:6.4.3 Storage experiment.

Aliquots of 60 mg/l a.i. sheep dip were prepared weekly, over a four week period. The aliquots were split into two portions, half of which were frozen in glass universals, and half of which were maintained, also in glass, at room temperature. At the end of this period, the samples were analysed for diazinon, and the results compared to determine the extent of any breakdown.

2:6.4.4 Routine effluent analysis.

Column effluent samples were taken in two portions, a 100 ml portion for toxicity analysis, and a 10 ml sample for GC analysis. Both were frozen immediately. The GC samples were analysed on a daily basis, for up to two weeks, when analysis frequency was changed to every 7-14 days. Samples were prepared with internal standard as per 2:6.4.2.

## 2:7 Waste Management.

Throughout the study, hazardous wastes of two main types were generated. The first was all materials which came in contact with cultured cells (flasks, media, pipettes etc.). While no definite biohazard is known to exist, the precautionary principle was applied, and the material was regarded as a biohazard. The second was toxin residues, which represented a definite hazard. These materials were disposed of as follows;

2:7.1 Biohazard materials.

All materials which came in contact with cultured cells were retained, and later disposed of by incineration.

2:7.2 Toxin residues.

Dichlorvos, ivermectin and malachite green are known to be detoxified at alkaline pH. All toxin residues were retained in spare media bottles. Concentrated NaOH was added, and the residues were stored for more than 6 months. They were then disposed of by dilution to sewer. A similar protocol was applied to diazinon, except that acidic conditions promote breakdown, and a pH of less than 2 was used for storage.

## 2:8 Data Handling.

2:8.1 Processing of primary data.

All of the cell culture and invertebrate trials were carried out in multiwell plates (96 for cell assays, 24 for invertebrate assays). The results from the replicate wells of each of the individual columns (or the total number of wells in duplicate columns, where used) in the multiwell plates were pooled and averaged, and standard deviations were calculated. For algal trials, the results from the replicate flasks were pooled also. Percentage inhibitions were calculated, in relation to the blank wells (or flasks), according to;

$$(1 - \text{abs. tox.}/\text{abs cont.}) \times 100$$

where; abs tox. was the absorbance (or survival) in the toxin well.

abs cont. was the absorbance (or survival) in the control well.

(Isenberg, 1993).

2:8.2 Estimation of median toxicity data.

Three standard measures of median toxic effects were calculated during the study, depending on the system being used. These were; the $EC_{50}$ (median effective concentration), used for cell culture, algal, and fish trials; the $IC_{50}$ (concentration causing 50% immobilisation), used for invertebrate trials; and the $LC_{50}$ (median lethal concentration), used for fish toxicity trials.

The term effective is defined for individual assay systems, such as inhibition of neutral red uptake for the cell culture-neutral red system, or induction of specific symptoms (such as convulsions) in Rainbow trout in the fish acute toxicity system.

There are a number of mathematical methods available for calculating these measures, each requiring different types of data sets, and most producing different types of information. Only one of these methods was applicable to all of the various data sets generated during this study, this was the log-conc. versus percent-mortality method (also known as the graphical interpolation method) as described by Peltier and Weber (1985).

This linear regression method (using 6 data points for the cell culture, and 5 data points for the algal and invertebrate trials) is used by a number of researchers (Babich *et al.*, 1989; Babich and Borenfreund, 1990; Ortego and Benson, 1992; Persoone *et al.*, 1993; Isenberg, 1993).

2:8.3 Statistical assessment of association.

The primary topic of interest in the study was the performance of the cell culture assays. Thus, a statistical measure of the difference in the response of the various treatments was of interest.

Each pair of cell culture treatments (L929 crystal violet vs. L929 neutral red etc.) were examined for association by single factor analysis of variance (F-test), as described by Campbell (1989).

This is the standard procedure to determine whether the differences between any of the observed sets of concentrations from paired populations are statistically significant (Gelber *et al.*, 1985).

The test was carried out using the Minitab programme.

# CHAPTER 3
# PREDICTIVE HAZARD ASSESSMENT

Chapter Index Page

## 3:1 Introduction.

The following chapter outlines a set of experiments on the toxicity of a number of chemicals, as assessed by cell culture methods, and by a range of whole organism tests. As described in chapter 1, these tests are in the category of Predictive Hazard Assessment, as their objective is to indicate the toxicity of single chemicals or products intended for direct discharge to the environment.

The products investigated are used in the fish farming industry. Thus, the whole organism tests used are aquatic, both freshwater and haline. Using results generated, the tests are compared for sensitivity, speed, reproducibility, applicability, and other parameters which determine whether a particular toxicity trial has advantages over any other.

## 3:2 Literature Review.

### 3:2.1 The need for new cell culture bioassays.

The objective of environmental analysis is to provide data that can be used to determine the effects of materials discharged to the environment (either directly, through use, or as effluents). This data is then used to limit or prevent any discharges from exerting potential effects. The greatest difficulty faced in this regard is that target ecologies consist of complex communities, with widely variant sensitivities.

It is generally accepted that physical and chemical analyses alone cannot assess the ecological effects of chemicals or mixtures on a diverse community of flora and fauna. However, there are problems also with the types of biological test systems currently available (Burdick, 1967: OECD, 1984).

Ecotoxicologists use *in vivo* laboratory bioassays with selected representatives of ecological communities to generate useful specific toxicity data, under controlled conditions. There are a large number of such bioassays (section 3:2.5, p. 59), and a wide variety of applications for which they are useful (OECD, 1984; APHA- AWWA, 1980). However, the potential of most *in vivo* bioassays is limited by the restrictive culture conditions necessary, and the statistical limitations imposed by the use of a small number of test organisms (Richardson *et al.*, 1977).

These factors, combined with cost and other logistics, reduce the flexibility of *in vivo* bioassays in environmental analysis, particularly the amount and type of data which can be generated on materials of interest.

Also, few, if any of these bioassays, have any great potential for the routine monitoring of *in vivo* environments, including effluent processing and discharge, receiving waters, and accident/emergency situations.

This situation is of particular concern, when considering the fact that there are an estimated 70,000 commercially available synthetic chemicals. For up to 80% of these, no ecotoxicity information at all is available (LaFlamme, 1984). Up to 1000 new chemicals are synthesised per year, and the restrictions mentioned prohibit exhaustive ecotoxicological assessment of all of these before commercial production. As a result, the priority for testing has necessarily been the assessment of materials related in some way to direct human exposure (NRC, 1984; Babich and Borenfreund, 1987a, 1988a; Persoone, 1988).

New ecotoxicity bioassays are necessary therefore, which could generate large amounts of varied and relevant data. To be an improvement on existing methods, any proposed assay would need to be easy to perform, sensitive, quick, reproducible, statistically strong, flexible and cost effective.

The use of cultured cells in ecotoxicity testing has been proposed as such a test system. The scientific basis for their use is the biological principle that toxic mechanisms are expressed first at molecular level, and then, through successive levels of biological organisation, to the intact animal. The cell, as the primary unit of this organisation, is the ultimate target of toxic mechanisms, and, as cells in culture retain most of their functions in the intact animal, they can be used to assess the toxic effect of chemical agents (Waters *et al.*, 1978; Murphy, 1983).

### 3:2.2 Aquaculture: problems with parasites.

Atlantic salmon are farmed in more than twelve countries world-wide. Commercially, the value of these fisheries is considerable (for example, a harvest of 31,000t, in 1989, was worth £116m in Scotland), particularly as they are often concentrated in remote communities. There are many diseases, parasites and predators that the fish farmer has to contend with. Amongst the most damaging of these are the sea-lice, which first appeared in Norwegian fish farms in the 1960s, and in Scottish fish farms in the 1970s (Pike, 1989).

Sea-lice are a natural part of the ecology of salmonids and other fish. In fact, in wild salmon they are an indication of 'freshness', as they can't survive in freshwater, and drop off soon after the fish returns from the sea. However, in intensive culture, which is carried out in estuarine pens,

sea-lice numbers can multiply to the extent where heavy commercial losses are caused. The damage is caused by lesions (following attachment), which are sites for the development of infection, leading to osmotic shock (Rae, 1979; Pike, 1989).

There are two main species of lice which attach to salmonids, *Lepeoptheirus salmonis* and *Caligus elongatus*. The former causes most damage. There are 10 stages in the life cycle of *L. salmonis*, the first four are free-living, the next four attach to the fish by filament, the last two (adult) attach by sucker. Reproduction in the lice goes on all year round (generation time at 10 $^{o}$C is six weeks), but numbers peak in Autumn and Spring (Pike, 1989). Heaviest losses of farmed fish tend to occur in Winter.

In 1976, Marine Harvest Limited identified the critical life stages of the sea-lice, and studied a number of chemicals as anti-parasitic agents. The organophosphate insecticide dichlorvos was chosen. This was found to be effective only against the adult, or sucking stages, and so repeated treatments were necessary. A method of application was devised which uses pens around the sea cages, constant oxygenation, and an exposure of 1 mg/l for 1 hour. A series of environmental trials, including decay-rates, fish residues and effects on plankton and other animals were carried out. The results of these were described as 'favourable', and limited authorisation was granted to Marine Harvest in 1979 by the UK MAFF for the use of 'Nuvan 500 EC' (Rae, 1979).

Fish farms in general have long been the focus of critical debate in relation to the actual or perceived pollution potential that they represent. The build-up of organic sediments underneath cages, containing waste food (20% food loss is an average estimate), faeces and therapeutic agents, has been shown to cause enrichment, deoxygenation, and evolution of toxic gases. They can also act as a sink for toxins (Gowen and McClusky, 1988). The prophylactic use of antimicrobial agents (including malachite green) has also been criticised. Some consider that this could produce antibiotic resistant microbes (Brown, 1989).

Concern grew in the 1980s about the use of dichlorvos in aquaculture, initially because of perceived gaps in the toxicological information, and subsequently because of a number of negative reports on its toxicity to non-target organisms (Ross and Horsman, 1988). With the planned expansion of the salmon farming industry, a heated debate developed over the use of the chemical. Official sanction for dichlorvos was maintained, however, on the basis that no viable alternative had been

developed, and the projected commercial losses did not justify complete withdrawal (Pike, 1989).

The development of an alternative to dichlorvos has become a priority for the industry, and several approaches have been taken. These have ranged from a series of alternative chemicals to biological methods such as the use of wrasse (including Corkwing, Goldsinny and Rockbrook wrasse), and the use of plants, including onions and garlic (Costello, 1991).

One of the most promising, and most controversial of these, has been the proposed use of ivermectin. The efficacy of this chemical against sea-lice was first demonstrated by Palmer *et al.* (1987). There are a number of studies in progress, particularly in Ireland, on the logistics of its use, incorporated in the food of the fish and dosed at 0.2 mg/kg. Details of these studies have not yet been published.

The controversy surrounding its use stems from its extreme toxicity to aquatic animals. Media reports have referred to statements from the manufacturer, that they are not involved with, and do not sanction, the use of this chemical in aquaculture. The Scottish Salmon Growers Association have said that they will expel any of their members using ivermectin.

The generation of toxicity data on both of these chemicals is a priority for ecotoxicologists. The use of cell culture methods, along with whole organism trials, to produce some of this data would serve the dual purpose of allowing an assessment of the relative merits of the various systems. This is the approach taken in this study.

### 3:2.3 The use of cell culture in environmental studies.

#### 3:2.3.1 General.

Because of the number and diversity of species which could be affected by discharge of materials to the environment, an equally diverse range of cultured cells have been used to assess possible ecotoxicity. Also, because of the complexity of ecological relationships, and the ways in which environmental exposures can occur, a number of different approaches have been taken to generating relevant data. These can be divided into three main categories; the assessment of discrete toxicity; the assessment of the toxicity of mixtures; and investigations into the mechanisms of toxicity.

3:2.3.2 Discrete cytotoxicity.

The most frequent application has been the determination of the cytotoxicity of single chemicals (or formulated products) intended for release to the environment. Predictive hazard assessment is the use of this data to assign a specific environmental risk to such chemicals. Both mammalian and fish cells have been used for this purpose, but the majority of studies have used non-mammalian cells.

Rachlin and Perlmutter (1967) first used Fathead minnow cells (FHM) to assess the toxicity of zinc, and found the cells to be more sensitive than the intact animal (end-point: mitotic index). Marion and Denizeau (1983a,b) used Rainbow trout gonad (RTG-2) and human skin epithelial cells (NCTC 2544) to assess the toxicity of lead and cadmium. They found the human cells to be more sensitive than the fish cells to cadmium, but found the opposite with lead (end-points: protein/nucleic acid extraction).

Babich *et al.* (1986) used the Bluegill sunfish (BF-2) cells to assess the toxicity of eighteen metal salts (end-point: neutral red). A good correlation was found between the cytotoxicity of divalent metals and their chemical softness (the chemical softness {$\sigma$, Hansch} parameter describes the specific ligand to which metals may bind, and thus exert a biological effect), and between *in vitro* and *in vivo* data for these metals. The same was not the case however for the anionic metals. This was attributed to the established tolerance of fish to these metals.

Differences were found in the sensitivity of two cell lines derived from Bluegill sunfish (BF-2 and an unnamed line derived from the fin) in response to several organochlorine pesticides by Babich and Borenfreund in another study (end-points: neutral red and mono-oxygenase activity)(1987b). An attempt to compare this set of data to that from other fish cell-lines highlighted the problem of the effect of incubation temperature on toxicity.

Babich and Borenfreund (1987c) also assessed the toxicity of organotins, organochlorine pesticides, PCBs, PAHs and phenolic compounds, in order to determine the usefulness of FHM cells in comparison to BF-2 cells. The FHM cells were more sensitive, but the BF-2 cells correlated better with the corresponding *in vivo* data (end-points: neutral red, coomassie blue protein assay, cell detachment).

Kfir and Prozesky (1981) assessed the use of three mammalian cell lines (HeLa [human carcinoma], ML [mouse lymphoma] and BGM [Buffalo Green monkey]) to determine the toxicity of six pure toxicants (five

metal salts and phenol) and three effluents (settled sewage, biofilter humus tank and activated sludge effluents). The three cell lines showed different sensitivities, with HeLa cells least sensitive, and BGM cells most sensitive to the six pure toxins. The BGM line was also the most sensitive to the effluent samples, all of which were found to be toxic. Raw sewage was found to be the most toxic of the three. In comparison to the electronic fish biomonitoring system, cell culture assays were twice as sensitive (end-point: cloning efficiency).

3:2.3.3 The cytotoxicity of mixtures.

A second application has been the assessment of the toxicity of complex environmental mixtures, from waters to effluents. This is an application where cell culture can make a particularly valuable contribution to increasing the understanding of environmental toxicity mechanisms, and can generate data directly useful in environmental systems management. It is a topic that is examined further in the monitoring trials section of this study, but is reviewed here also, in order to give a complete review of the use of cell culture in environmental analysis to date.

One of the first such studies was that of Christian *et al.* (1973), who proposed a cell culture assay, using L929 cells, for the toxicity screening of drinking water, and, in particular, reused water. The protocol involved culturing cells on disposable test tubes, using the test water to reconstitute powdered media, and assay by the Lowrey protein method. A military water re-use system, producing 100% recycled water, was studied. Cytotoxicity was used both to determine the toxicity of drinking water, and to determine the efficiency of a number of treatment steps. The recommendations for future study included correlation of the cell system with whole animal trials.

In later studies, another team from the University of Cincinnati showed that concentration by distillation of non-toxic tapwater increased (or produced) toxicity. A simulated treated wastewater (ultrafiltration and reverse-osmosis) was not toxic, but its concentrate after distillation was. This indicated the need to concentrate aqueous environmental samples of low toxicity. Their method was vacuum fractional distillation, and recombination of the pre-water (low-boiling) fractions with the water concentrate (Cody *et al.*, 1975, 1979).

The same cell culture test was applied to coal extracts from two coal mines. Extracts from a mine where Coal Workers Pnuemonoconiosis was prevalent were far more toxic than those from a mine where it was less

common (Christian *et al.*, 1975). This is significant, as no direct relationship would be expected between aqueous cytotoxicity and a disease which was understood to be caused by inhalation of coal dust. A later cell culture assay, however, showed that it was leaching of dust particles by lung biofluids that released a toxic product which caused the pnuemonoconiosis. A qualitative correlation was established between the cytotoxicity of these leachates and their *in vivo* ability to cause the disease (Christian and Nelson, 1978).

In a study aimed at developing a mammalian cell-culture assay for assessing oil-refinery effluents, Richardson *et al.* (1977) refer to age, sex, reproductive condition, previous environment, life cycle and the low numbers used, as sources of variability in whole animal ecotoxicity trials. They assessed the use of L-M cells (clone 939, a.k.a LM/ML, L929) and a cell counting end point in the determination of the aquatic toxicity of these effluents. The results were compared to those obtained with *Daphnia*. They conclude that an aquatic bioassay should be sensitive, rapid, based on reproduction (not survival), and statistically significant. The study showed the cell culture assay to be suitable, according to each of these requirements (particularly so in comparison to *Daphnia*).

In an early, comprehensive review of the general topic of mixture toxicity assessment, Waters *et al.* (1978) list a number of studies. Examples of mixtures assessed include motor exhaust emissions (Chang conjunctival cells: cell growth and division assays), aqueous coal extracts and drinking water (L929 cells: protein/DNA assays), and industrial particulate samples (organ-specific alveolar macrophage cells: dye exclusion assay).

In a later review, Hunt *et al.* (1986), of the UK Water Research Centre, detail the toxicological assessment of a wide range of environmental samples, including raw waters (Vero cells: morphology assay; HeLa cells: morphology assay; KB cells: cell growth/survival assays; L [mouse lymphoma] cells: cell growth [using crystal violet] assay; HeLa cells: DNA inhibition assay; HeLa cells: RNA inhibition assay), treated waters (Vero cells: morphology assay; HeLa cells: morphology assay; L929 cells: protein/ DNA/counting assays; Balb/c 3T3 [mouse embryo fibroblast] cells: cloning efficiency assay), effluents (L-M cells: cell growth assay; BGM/ML cell lines: cloning efficiency assay; BGM cells: oxygen uptake assay) and materials used in the water industry (Vero cells: morphology assay).

They conclude that cytotoxicity assays are especially useful in four main areas of aquatic toxicity testing. These are; the study of chemicals/ products/mixtures, the study of waters and effluents, the study of materials

for use in the water industry; and the screening of unknown compounds isolated from water.

In a study following from this review, another team from the WRC, (Hunt *et al.*, 1987), compared four such cytotoxicity assays (MIT-24 , KBr, neutral red and cloning efficiency) used to assess the toxicity of thirteen chemicals, and a range of water/effluent samples (neutral red and cloning efficiency assays only), to V79/4 (Chinese hamster lung) cells.

All four assays were found to be easy to perform and reproducible, but cloning efficiency had a higher time requirement. All four gave the same toxicity ranking, but the neutral red method was found to be the least sensitive overall, and, in particular, did not indicate any toxicity in the water/effluent samples. This lack of sensitivity was attributed to a short exposure time used (24 hrs.).

Results from the MIT-24 assay were considered the most difficult to interpret. The KBr assay was found to be reproducible and sensitive, but was less useful in terms of ease of applicability, particularly in 96 well assays, because of extra washing steps. Overall, the neutral red method (with a recommended 72 hr. incubation) was selected as the most useful of the four methods.

3:2.3.4 Investigation of toxic mechanisms.

A third application of cell culture in environmental analysis has been the investigation of the mechanisms of toxicity of materials of interest. Information from this type of study can also be used to assess the usefulness of particular cell culture methodologies.

Babich and Borenfreund (1988a) used BF-2 cells to establish Structure Activity Relationships (SARs) for several groups of toxins (end-point: neutral red). An interesting feature of this study was that 34 $^{o}$C was used as the test temperature, as opposed to 25 $^{o}$C, which is the temperature optimum of the fish species. This was because the cell culture had been found in previous studies to adopt a new temperature optimum, and to exhibit higher sensitivity at this new temperature. In the study, no correlation was found between toxicity and lipophilicity for a number of butylated tins, but was found for a number of diorganotin salts. A similarly linear relationship between toxicity and lipophilicity (and degree of chlorination) was found for chlorinated benzenes and analines.

Garza-Ocanas *et al.* (1990) assessed the toxicity of eighteen diverse chemicals to six cell lines, of different tissue origin, derived from the

same species. No significant differences were found between the cell lines, which were derived from rat kidney (NRK-52E), lung (L-2), small intestine (IEC-6), liver (C-9), heart (H9c2) and skin (FR) (end-point: cell attachment).

Hunt *et al.* (1986) also refer to differences in sensitivity in their review of the use of cytotoxicity assays in the water industry. They conclude that, although there are differences in sensitivity between cell lines, the sensitivity of a cell line is not the most important characteristic. They state that "the ease with which a cell line is cultured, and its applicability to the chosen end-point are the major criteria when assessing suitability".

The significance of tissue of origin was also assessed by Linseman *et al.* (1990). Three cell lines, Chang (human liver), SIRC (rabbit cornea) and LLC-PK1 (pig kidney) were compared for sensitivity to four aminoglycoside antibiotics. The kidney proximal tubule origin of the LLC-PK1 cells might be expected to confer a greater sensitivity to these materials than the cells of different origin, as this is the site where they are concentrated *in vivo*. However, this was not found. In fact the LLC-PK1 cell-line was the least sensitive of the three, and the least effective at ranking the toxicity, relative to *in vivo* toxicity. A possible explanation suggested was that neither of the two end-points used reflected specific kidney function, and that such an end-point may yield different results (end-points: LDH leakage and cell proliferation).

Benford and Good (1987), in a study comparing the neutral red, MTT and ATP assays (V79/4 cells) note that the neutral red and MTT assays examine toxicity to lysosomes and mitochondria respectively. They demonstrate how the method of application determines the actual toxic mechanism under study (e.g. concomitant application of toxin and cells gives the assessment of the effect of the toxin on the success of attachment; more than 24 hour incubation with the toxin gives the assessment of the effect on cell proliferation, etc.). However, in an examination of the toxicity of 10 chemicals, these variations of the neutral red assay, and also the MTT and ATP assays, all correlated very well with the basic neutral red method.

An investigation into the environmental significance of toxin antagonism, in particular the previously documented relationship between arsenic and selenium, has been investigated by Babich *et al.* (1989). BG/G (Bluegill gill) and BG/F (Bluegill fin) cells were assessed for arsenic-selenium toxicity by the neutral red method. The toxicities of arsenite and arsenate indicated by these two cell lines were consistant with those from BF-2 cells, and also the BALB/c 3T3 and HFF (human foreskin fibroblast) cells.

However, all three Bluegill cell lines were more resistant to selenite and selenate (seven and twenty times respectively) than the two mammalian cell lines. No explanation is proffered for this result. The antagonistic relationship between the two compounds was demonstrated across a range of ionic states and concentrations.

A number of studies have investigated the environmental significance of mutagenic materials, as well as the mutagenic potential of polluted waters and effluents, using cell culture methodologies. An early investigation by Kocan *et al.* (1979) examined the cytotoxicity of eight mutagens to three fish cell lines (RTG-2, BF-2 and STE [Steelhead embryo] cells: growth inhibition [cell counting] assay). The sensitivities of all three cell lines were shown to be similar to that of a positive control, the HFF cell line, with BF-2 cells the most sensitive. This was despite the differences in incubation temperatures. Both BF-2 and RTG-2 cells were shown to be capable of metabolising some promutagens to mutagens.

Mutagenic activation has also been demonstrated by Coulombe *et al.* (1982). Initial studies used the Rainbow trout S20 hepatic fraction to demonstrate the activation of a series of aflatoxins, which correlated well with Rainbow trout *in vivo* data. In a further study, this principle was extended to the use of Rainbow trout and Coho salmon hepatocytes, co-cultured *in vitro* with *Salmonella* TA98. The Rainbow trout cells were shown to have a much higher metabolic activity. However, overall cell activity was significantly less, in some cases, than that of the isolated mitochondrial fraction (Coulombe *et al.*, 1984).

Wilcox and Williamson (1986) investigated chromosomal aberrations caused in CHO (Chinese hamster ovary) and human lymphocyte cells *in vitro* by concentrated chlorinated drinking water samples. This was then correlated with clastogenic effects *in vivo*.

A series of chlorinated waters were shown to have a dose-related cytogenic activity with the CHO cells, but the lymphocyte assay was less effective. Unchlorinated raw waters also showed weak cytogenic activity, but the major source of the treated water activity was shown to be associated with the chlorination process. This activity was also shown to be reduced by media serum in an inverse concentration relationship. The *in vivo* assay was negative, which correlates with previously documented hepatic detoxification noted both *in vivo* and *in vitro*, preventing the mutagen from reaching the bone marrow. However, it is noted that other target cells, for example in the intestinal tract, may be at risk.

Ahmed *et al.* (1977) demonstrated the unscheduled DNA synthesis (UDS) caused in human cells (VA-4) by pesticides. Six out of thirteen pesticides induced UDS with or without metabolic activation. Three more showed no UDS with or without metabolic activation. Another three showed UDS activity only after activation, and the initial UDS activity of one pesticide (chlordane) was removed by activation. The repair profiles for four of the pesticides from the above group of six were examined (using photolysis of BUdR), there was a difference in the profiles between three of the four, which were insecticides, and the fourth, which was a herbicide.

Walton *et al.* (1983) extended these principles to a comparison of the processes involved in several fish and mammalian cell lines (HF [human fibroblast], CHO, RTG, RTO [Rainbow trout ovary], FHM and CH [Chum salmon heart] cells). A dose dependant UDS activity was found with the four mutagens tested in all cell lines. The sensitivities of all the fish cell lines were essentially the same, both mammalian lines were more sensitive than the fish lines, and the HF line was more sensitive than the CHO line. This pattern was carried on through DNA repair following UV exposure, assessed using the HF, CHO and RTG lines.

3:2.3.5 Summary.

The above review shows that cell culture methodologies have been used in a number of environmental investigations. The objectives of most of these have been to examine specific chemicals, or specific aspects of the cell culture methods. Some have also examined a wide range of environmental samples, including effluents and receiving waters. While the studies reviewed have been divided into three broad categories, some studies have examined aspects of two, or all of these, at the same time.

None, however, have made a comprehensive examination of a number of cell culture methods, with reference to all aspects of the methods (including sensitivity, ease of operation, statistical strength, applicability etc.), and in direct comparison to whole organism methods, carried out in parallel. In particular, no study has examined the operation of cell culture methods in monitoring studies, or in comparison to existing physical and chemical analysis.

These are the objectives of the study described herein.

3:2.4 Cell culture methodologies.

3:2.4.1 The L929 cell line.

The L929 cell line is a mouse areolar/adipose fibroblast cell line. It is relatively easy to culture, and has a number of handling characteristics which would favour its use in routine assays (section 3:3.2.1, p. 85). It is also useful as an *in vitro* comparison to established *in vivo* mouse $LD_{50}$ data. Mouse acute toxicity is used to produce a significant amount of the available *in vivo* toxicity data.

It was chosen for this study for these reasons, but also as a direct mammalian comparison to the data to be generated by the RTG-2 cells. Apart from the comparison of two species, use of the two cell lines also allows a comparison of the effect of toxins on cells of different tissue origin, which would help to extend the environmental significance of any hazard determined. Epithelial cells throughout the body are those in direct contact with the environment, and thus are the primary target of pollutants (Marion and Denizeau, 1983)

As seen in section 3:2.3 (p. 47) there has been no extensive previous use of this cell line in ecotoxicological studies, although a number of other mammalian cell lines have been used in a variety of such applications.

3:2.4.2 The RTG-2 cell line.

The Rainbow trout gonad (RTG-2) fish cell line (the first fish cell line ever developed) was first described by Wolf and Quimby in 1962. It was derived from the primary cultivation of pooled normal gonads (mostly ovaries) of fingerling yearling fish. Cold trypsinisation was used for disaggregation, and a mixed monolayer of epithelial-like and fibroblast-like cells developed.

Subsequent subcultures were at one to two week intervals in 10-15% serum EMEM. The morphology was seen to be influenced by media, age of culture and location within the cell sheet, but was mostly fibroblast-like. Optimum growth pH was found to be 7.3, with 7.4 and over found to be inhibitory. Cultures were grown successfully at a range of temperatures between 4 and 26 $^{o}$C, however all cells died after 24 hours at 30 $^{o}$C. Metabolism decreased significantly with reduced temperature, but long periods of no feeding or attention were tolerated (up to 5 months at 19 $^{o}$C, even longer at lower temperatures), making the handling of the cells more flexible than

many other vertebrate lines (Wolf and Quimby, 1962). In fact, RTG-2 cells have been stored for up to two years at 4 $^{o}$C without a medium change, while still retaining viability. The cell line has been stored and recovered at -80 $^{o}$C for up to 10 years (Wolf and Mann, 1980).

By 1980, there were 61 fish cell lines, representing 17 families and 36 species. Fish cell lines are considered particularly useful for environmental work because of their wide temperature ranges, most having optima around 20-25 $^{o}$C. A number of studies have referred to the temperature optimum of RTG-2 as 20 $^{o}$C (Babich and Borenfreund, 1987; Wolf and Mann, 1980; Yoshimizu *et al.*, 1988).

RTG-2 cells have been used in particular for clinical toxicology work, to investigate biochemical mechanisms in fish, and to study viral diseases in fish (where it has been shown to be susceptible to a number of fish viruses), amongst other applications (Lee and Bols, 1980; Yoshimizu *et al.*, 1988). However, some workers regard the replication rate of RTG-2 to be too slow to be of use in routine environmental studies (Babich and Borenfreund, 1988).

Previous ecotoxicological studies using RTG-2 cells include assessment of the ecotoxicity of metals, such as cadmium and lead. Marion and Denizeau cultured RTG-2 cells at 15 $^{o}$C, and used protein/nucleic acid extraction and radiolabelled isotope incorporation as endpoints. They found that serum in the media reduced the cytotoxicity of cadmium, but not lead. The exposure time in these studies was determined by the time taken for a predetermined seeding density to reach confluence in a petri-dish (i.e. 18-20 days for a seeding density of $10^5$ cells in a 60mm dish). The concentrations applied were related to *in vivo* $LD_{50}$s, and according to this comparison, the RTG-2 cells were more sensitive to lead than the fish *in vivo* (Marion and Denizeau, 1983a,b).

Tarazona *et al.* (1992) used RTG-2 cells to screen for the toxicity of water samples (Algerciras Bay, Cadiz, Spain) that had been concentrated by passage through SEP-PAC C-18 cartridges, followed by elution with acetonitrile. These were then evaporated to dryness, and resuspended in DMSO for analysis. The neutral red end-point was used, with a 24 hour incubation time. All water samples were toxic to RTG-2, at concentration factors of between 1 and 10.

3:2.4.3 The crystal violet end point.

Crystal violet is a common biological dye. It is most often used in cytotoxicity assays to stain cell colonies in the Colony Forming Efficiency (CFE) method (Martin, 1989). It has not been used often in general cytotoxicity (cell proliferation) assays (section 3:2.3, p. 7). It was chosen for this study because of its ease of use, and as a comparison to the vital dye staining process involved in the neutral red method.

The crystal violet method records all remaining cells in a well, and does not differentiate between healthy and unhealthy cells. However, it is quick and easy to apply, and uses materials widely available in biological laboratories. The method used in this study was as described by Dykes *et al.* (1984).

3:2.4.4 The neutral red end point.

The neutral red method for the measurement of the cytotoxicity of environmental materials was first proposed by Borenfreund and Puerner (1985), as a supplementary procedure to a morphological end-point, the Highest Tolerated Dose (HTD) method. It was initially developed as an alternative to the Draize test, but was soon applied to a wider field (Babich and Borenfreund, 1990). It is based on the uptake of the weakly cationic neutral red dye by non-ionic diffusion. The dye then accumulates in the lysosomes of actively metabolising cells (Nemes *et al.*, 1979). It is not clear whether the uptake of the neutral red through the cell membrane is an active or passive mechanism.

Borenfreund and Puerner (1985) demonstrated that three hours was the optimum incubation time for the uptake of the dye, and that it has an absorbance peak in the region of 540nm. They also demonstrated that this uptake was linear over cell concentrations from $1 \times 10^3$ to $4 \times 10^4$ cells per well. In subsequent studies, they showed that the method produces about double the optical density of comparable methods, such as the MTT method, and so less cells per well are required (Babich and Borenfreund, 1988).

In subsequent years, the method was used by a number of laboratories to examine a wide range of environmental materials. They found that the ranking of the toxicity of different materials was the same, despite intercellular differences in sensitivity. Their recommendations included an incubation time of 24 hours for direct acting toxins, and the use of longer times (3 to 6 days), or the incorporation of S9 for those materials requiring metabolism. A good correlation between the neutral red method

and the Draize test was demonstrated. A good correlation between aquatic $LC_{50}$ data and cytotoxicity data from specific cell lines was said to be due to the similarity between physicochemical parameters affecting the bioavailability and rate of uptake of test agents from the water column of cells of the whole animal and those affecting uptake from aqueous growth medium into cultured cells (Babich and Borenfreund, 1988).

Riddel *et al.* (1986) found a very close correlation between the NR method and the Kenacid Blue r (KBr) protein method, and also a good correlation with the HTD method. They recognised the possibility that chemicals could cause an increase in the size and/or number of lysosomes in a cell, without an associated increase in the number of cells, but did not find this in thirty general chemicals tested. One chemical, chloroquine sulphate is known to specifically inhibit lysosomes, and was significantly more toxic by the NR method than by the KBr or HTD methods.

Babich and Borenfreund (1988) found this lysosomal swelling to be caused by sub-lethal concentrations of several pesticides, and attributed it to the lipophilicity of the materials, and subsequent membrane destabilisation.

Hunt *et al.* (1986) compared the NR method to the MIT-24, the KBr and the cloning efficiency (or CFE) methods in their review of environmental applications of cytotoxicity testing methods. The same rank order of toxicity was found for the four methods, but the NR method was said to be less sensitive than others. In the testing of selected environmental water samples, the NR method detected no toxicity, while the cloning efficiency method detected toxicity on two out of six. This lack of sensitivity was attributed to the short exposure time used (24 hours). However, Benford and Good compared four variations of the NR method, all with incubation times of 24 hours or less, and found the method to be more sensitive than either the MTT or the ATP methods (Benford and Good, 1987).

In a later study, Hunt *et al.* used comparability, reproducibility, ease of use and sensitivity as parameters, and according to these the NR method was found to be more useful than the KBr method. Similar parameters led to a similar conclusion by Benford and Good (Hunt *et al.*, 1987; Benford and Good, 1987).

### 3:2.5 Whole organism methodologies.

#### 3:2.5.1 General.

In determining the effects of a toxin on an ecosystem, the ideal test would use the entire ecosystem. However, this is not a practical option. Some such methods have in fact been designed, including the testing of materials *in situ* (Dejoux, 1975), but these are technically involved, and are not useful for generating significant amounts of data on a large number of new materials.

By definition therefore, any test which can be used in this way will suffer from a lack of ecological factors, such as the ability to incorporate system repair mechanisms, the ability of populations to adapt to adverse conditions, bioaccumulation and biomagnification (Burdick, 1967; OECD,1984).

The increasing tendency to search for, or apply, a single test, or small number of tests, fails to appreciate these factors (Dagani, 1980), as discussed by the OECD Expert Group on Ecotoxicology: "the imperative prescription of one standard test species to be used in environmental testing is irrational. To do so would suggest an accuracy which does not exist, and, moreover would be contrary to the philosophy of model systems in ecotoxicological testing" (OECD, 1984)

One systems approach which has been used to bridge the gap between single species trials and whole ecosystem studies, is the use of a number of species, chosen from defined taxonomic or functional groups within a particular community. 'Representative', or 'sensitive' examples from groups present are selected, and a battery of whole organism trials are carried out (OECD, 1984). This approach is most useful when coupled with information on the biological activity and environmental fate of any material, and when data is interpreted within the constraints of the individual test systems.

In keeping with this functional group approach, the whole organism trials carried out in this study (to generate data which could be compared to the cell culture results), include vertebrate, invertebrate and algal species from both freshwater and haline environments.

#### 3:2.5.2 The 96 hr. fish acute toxicity test.

Fish were one of the first bioassay organisms used in environmental toxicity tests. Standard conditions have been established for the handling of test fish, including acclimatisation periods, feeding etc.

(Hunn *et al.*, 1968). Standard methods for conducting fish trials have also been the subject of a great deal of research, and have been described by a number of authorities, including the ASTM (1980) and the OECD (1984).

The choice of test type (e.g. static, flow-through etc.) is governed by data needs and resources, but the length of static trials is set at 96 hours, because of fluctuations in toxin concentration, oxygen levels, pH, and the build up of metabolites and degradation products after this period (ASTM, 1980). Other time scales have, however, been suggested, such as the *incipient* $LC_{50}$, which is the $LC_{50}$ determined over a period long enough to demonstrate cessation of acute lethality (Sprague, 1970). The trials carried out in this study followed the protocols as described by the OECD and the ASTM (OECD, 1984; ASTM, 1980).

It is recognised that the acute lethal data generated by these assays is limited by the statistical constraints of the small number of organisms used in the test, and by factors such as varying water quality characteristics and the motility of affected organisms in their natural environments (Sprague, 1970). It is also recognised that sub-lethal effects, such as growth and reproduction impairment are not measured in standard acute tests. However, other sub-lethal effects, such as motility and behaviour can be incorporated to produce $EC_{50}$ data which can be very useful (Sprague, 1971). The acute fish toxicity test then, is designed to generate data that may indicate the need for more extensive trials in a comprehensive risk assessment process (ASTM, 1980; Dagani, 1980).

#### 3:2.5.2.1 *Salmo gairdneri.*

The acute fish toxicity test organism used in this study was the Rainbow trout. This was designated as *Salmo gairdneri* (Richardson), until 1989, when Smith and Stearly (1989) demonstrated that the Rainbow trout was the same species as the Kamchatka trout, and that it was more closely related to the Pacific salmon (*Oncorhynchus*) than to the Atlantic salmon (*Salmo*). Their conclusion was that the Rainbow trout should now become *Oncorhynchus mykiss*. However, since this has not been universally accepted as yet, I have referred to the Rainbow trout as *Salmo gairdneri* in this study, except where it is specifically referred to in other studies as *Oncorhynchus mykiss* (e.g. Cusack and Johnson, 1990).

Rainbow trout have been used extensively in aquatic toxicity trials, including acute tests (Davies *et al.*, 1976), embryo-larval tests, where the embryo has often been shown to be more sensitive than the adult fish,

including a high susceptibility to the bioaccumulation of xenobiotics (Meyer and Jorgenson, 1983; Metcalfe, 1989; DeGraeve *et al.*, 1980; Davies *et al.*, 1976), and carcinogenicity tests (Metcalfe, 1989; Kobayashi *et al.*, 1990). They have also been used in multi-species trials on environmental contaminants (Phipps and Holcombe, 1985).

Along with other salmonids, Rainbow trout were considered for some time to be a very sensitive species. This is often the case, however, it has been shown that they, and other salmonids, have a range of sensitivities which are often in line with non-salmonid species (Sprague, 1970; DeGraeve *et al.*, 1980).

The hepatic biotransformation of environmental chemicals by Rainbow trout has been demonstrated *in vivo* (Pedersen *et al.*, 1976). This can also be measured using fish hepatic cell culture systems, but should be borne in mind when comparing data from non-hepatic cells.

Biotransformation is closely linked to bioaccumulation and biomagnification, two other vital ecological processes which cannot be detected either by cell systems, or single species test systems, and which have been shown to be important in the toxicity of some materials to Rainbow trout (Lech and Bend, 1980).

Rainbow trout have been used previously in studies examining the toxicity of pesticides and antibiotics (as were examined in this study). They have been shown to be very sensitive to organophosphate insecticides, and to have a range of sensitivities to a number of other pesticides, from sensitive to relatively tolerant (Matthieson *et al*, 1988; Livingston, 1977; Phipps and Holcombe, 1985). Several commonly used antibiotics have been shown to be toxic to Rainbow trout, with the relevant safety margins dependant on the pharmaco-kinetics of the materials in the fish (Lauren *et al.*, 1989; Girer, 1990).

Rainbow trout toxicity data has been used in a number of studies to establish correlations with other test systems. Khangorot and Ray (1987) compared published $LC_{50}$ data to their results for the 48 hr. *Daphnia magna* test. A strong correlation was found between the two tests when analysed by regression analysis ($r^2 = 0.814$), and by rank order of toxicity ($r_S = 0.929$).

3:2.5.3 The 96 hr. algal growth inhibition test.

The algal growth inhibition test is a standard OECD methodology, a legal requirement by the EC for chemicals produced in quantities exceeding 100 t/yr. It was initially a development of existing eutrophication trials, the

shorter test time being chosen to maximise data generation, to allow standardisation of test parameters, and to avoid increases in cell volume which would distort results (Bolier and Donze, 1989). The test species used in this study was *Chlorella vulgaris*, one of the three species recommended by the OECD (along with *Scenedesmus*, and *Selenastrum*). It was used in environmental toxicity trials as early as 1957 (Butler, 1977).

Algae are used because they are important primary producers, and an effect on an algal species could affect the entire ecosystem. The test, as applied, requires significant glassware and instrument resources. However, at the same time, it is the only standard ecotoxicity (whole organism) trial to incorporate some of the advantages of cell culture systems, such as the use of large homogenous single cell populations and the ability to determine the effect of a chemical over several generations.

Significant variation has been found in a number of trials, but this is often due to failure to control physico/chemical parameters. Examples of such critical parameters include constant light saturation, adsorption of materials by high biomass and pH control (particularly in static tests). Wide interspecies variations have also been found, and battery tests have been recommended to counter these (Nyholm and Kallqvist, 1989).

The statistic used to calculate $EC_{50}$ data is the specific growth rate. This is because figures drawn from growth rate data, as opposed to biomass data, are less affected by test system parameter variation (Nyholm, 1985). Further statistical analysis is normally curvilinear, as the data is quantitative (being drawn from a large number of identical organisms) rather than the quantal data generated by animal trials. Walsh *et al.* (1987), in a study on 21 pesticides, 18 organotin compounds and 4 algal species (187 trials) applied graphical interpolation, moving average, probit and binomial analysis to the results and found that they were essentially the same for all four. In the absence of confidence intervals, graphical interpolation was preferred to the more complex methods.

Stimulatory growth responses have sometimes been noted at low concentrations, prior to inhibition. This is possibly due to increased reproduction, as a response to environmental stress, before the log-phase of the toxic response. The procedure commonly used in these cases is to ignore the initial curve in data treatment, and to note it in the report (Nyholm and Kallqvist, 1989).

The toxicity of chemicals to algae has been shown to be related to their octanol-water partition coefficients, with organotins and

chlorobenzenes, which are more soluble in octanol, shown to be more toxic. This increased toxicity is contributed to higher bioavailability after penetration of lipoprotein membranes (Wong *et al.*, 1982; Wong *et al.*, 1984).

An ability to adapt to toxins has also been demonstrated. Butler *et al.* (1980) showed a tolerance to copper in *Chlorella vulgaris* isolated from environments with elevated levels of the metal. This adaptation had previously been seen with other toxins, and was as a result of exclusion of toxin, or the formation of intracellular compartments.

3:2.5.4 The *Artemia* immobilisation test.

The brine shrimp *Artemia salina* has been used in toxicological studies for over 30 years. Like *Daphnia* spp., it was widely used at that time as a food source for fish used in acute and chronic toxicity work. Its wide availability and the familiarity with its culture methods made it a suitable candidate for toxicity investigations, but the development of a standard toxicity trial methodology was not formalised until 1975. At that time the University of Ghent developed the *Artemia* Reference Centre (ARC) test. This was assessed by an 80 laboratory calibration trial in 1981, and found to have an acceptable level of variability.

The ARC test was described as "an acute screening-testing protocol of intermediate sensitivity, satisfactory repeatability and reproducibility, low cost, minimum maintenance of animals, and universal, year-round applicability." It is a regulatory requirement under the EEC Directive on dumping of titanium wastes, but is not required under the Oslo convention. Some criticisms of the test include the high haline tolerance of *Artemia* (and accompanying assumption of resistance to chemicals), and the fact that *Artemia* are not present in the sea, and so are not a representative species (Persoone and Wells, 1987).

*Artemia* have been used in acute toxicity trials (Kissa *et al.* {1984}, found *Artemia* very tolerant to Cd, Co and Ni, and sensitive to Cr), chronic toxicity trials (Nikonenko {1987}, demonstrated adaptation to toxins), and reproductive toxicity trials (Kissa *et al.* {1984}, found the eggs of *Artemia* to be more sensitive than the nauplii in general, as did Rafiee *et al.* {1986}, Rao and Latheef {1989} and Pandey and MacRae {1991}), amongst others.

The toxicity of naphthalene derivatives to *Artemia* has been positively correlated with the Octanol-Water Partition Coefficient, reinforcing the view that toxicity has more to do with the organism/water availability of the material than it does with its molecular structure, up to a

point where the correlation is limited by extreme low water solubility (Foster and Tullis, 1984).

Abernathy *et al.* (1986) also examined the role of organism-water partitioning using *Artemia*, and concluded that, amongst the hydrocarbons, toxicity is non-selective, and no particular group of hydrocarbons is dominantly toxic. They stress the use of the hypothetical subcooled liquid solubility ($C_L$) for solids, and not the actual solid solubility ($C_S$), as the proper parameter for predictive comparisons. In general, *Artemia* was found to be less sensitive than *Daphnia*, but this did not take account of the shorter exposure time in the *Artemia* assay.

**3:2.5.4.1** *Artemia salina.*

Members of the genus *Artemia* are found in hypersaline environments, including estuaries, lakes and lagoons. These environments are characterised by large fluctuations in critical parameters, such as salinity, oxygen and temperature, and *Artemia* survive in these environments by their extremely high adaptability (Lenz, 1984).

The species name *Artemia salina* has, in the past, been applied to a number of different strains from the genus *Artemia*. In fact, there are many different species, often delineated by geographical origin. However, most of these species have been shown to have very similar tolerances. Vanhaeke *et al.* (1984) found that thirteen different strains survived over a wide range of temperatures and salinities, with differences in tolerance only at high temperatures and low salinities. The common temperature optimum for the thirteen was 20 to 25 $^{o}$C.

Exposure to light has been shown to be a trigger in the hatching of cysts, which is strongest in the region of 575nm. It has been suggested that this is due to the presence of a photoreceptive pigment, augmented by the presence of haematin (Linden *et al.*, 1986). Positive phototaxis in the nauplii is also used as an important handling parameter.

3:2.5.5 The *Brachionus* immobilisation test.

At the time that *Artemia* trials were being developed, another genus of aquatic invertebrate was commercially available as a fish food. This was the rotifer genus *Brachionus*. A number of species were examined by Ghent University in the late 1980's, including *Brachionus plicatilis* and *Brachionus rubens*. The species used in this study was the freshwater

rotifer, *Brachionus calyciflorus*. This was the species found to have the closest correlation with *Daphnia* sensitivity (Persoone, 1989a).

*Brachionus plicatilis* was the first to be assessed, by Snell and Persoone (1989a). They found hatching to be temperature and salinity dependant. Their test used 24 well plates, a 1 ml test volume and 10 test organisms per well. They found mortality from starvation to begin at 48 hrs. Differences in sensitivity were noted if the test was not started within 3 hours of hatching, but apart from this, it compared very favourably with other trials, such as *Daphnia* and *Artemia* . General sensitivity to chemicals was found to range from more to less sensitive than other species.

Serrano *et al.* (1986) found that the organochlorine and organophosphate (including trichlorfon) pesticide resistances of three strains of this species were about 1000 times greater than other organisms, and that sub-lethal effects on growth and reproduction were minimal. This supported field observations of large increases in rotifer numbers, and concomitant decreases in other groups, in ponds treated with pesticides. They correlated this with the species osmoregulatory capability. However, there were large differences in sensitivity between strains, which was correlated with geographical origin.

A second study by Snell and Persoone (1989b), used the freshwater species *Brachionus rubens*, which was found to have similar hatching characteristics to *Brachionus plicatilis* (except for the salinity profile). However, significant and unexplained differences in hatching times were noted in different batches and different populations.

Their test medium was moderately hard freshwater. The rank order of sensitivity of *Brachionus rubens* was the same as *Brachionus plicatilis*, except for cadmium, but *Brachionus rubens* was more sensitive than *Brachionus plicatilis* .

The *Brachionus calyciflorus* test was chosen for this study because of the important position they occupy in aquatic ecosystems as grazers of phytoplankton, their role in nutrient cycling, and as a food source for fish. The design of the test offers the same advantages as the *Artemia* test (the use of cryptobiotic eggs, standardisation etc.), and the added advantage of comparison of results from a freshwater invertebrate system.

3:2.5.6 The *Streptocephalus* immobilisation test.

This is a third test being developed by the Ghent University laboratories, using the freshwater shrimp species *Streptocephalus proboscideus*. It was chosen as a direct freshwater comparison to the haline shrimp *Artemia salina*.

All of the advantages and characteristics of the other tests apply to this test also. As this is amongst the newest of the tests being developed, there has been very little work done previously using this species. No reports on previous pesticide or antibiotic studies could be found.

3:2.6 Nuvan/dichlorvos.

3:2.6.1 Chemistry.

Dichlorvos is an organophosphate insecticide, a direct acting cholinesterase (ChE) inhibitor. Its chemical formula is $C_4H_7Cl_2O_4P$ (mol. mass 221); chemical structure is $Cl_2C{=}CHOP^{=O}(OCH_3)_2$; chemical names are 2,2-dichloroethenyl dimethylphosphate (CAS [62-73-7]) and 2,2-dichlorovinyl dimethylphosphate [IUPAC]); common trade names include Dedevap, Nogos, Nuvan, Aquaguard, Phosvit and Vapona; its solubility is about 10 g/litre in water at 20 °C and it is miscible with most organic solvents and aerosol propellants; it is stable to heat, but is hydrolysed by water, a saturated aqueous solution at room temperature is converted to dimethylphosphate at a rate of about 3% per day, more rapidly in alkali (WHO, 1989).

It is manufactured either by the dechlorination of trichlorphon (chlorophos), through the action of caustic alkalis in aqueous solution at 40-50 °C (yield 60%), or the reaction of chloral with trimethyl phosphite (yield 93%) (Hofer, 1981; WHO, 1989)

It breaks down rapidly by both abiotic (e.g. photodegredation and hydrolysis) and biotic factors in water and humid air to form acidic products (including dichloro-ethanol, dichloroacetaldehyde [DCA], dichloroacetic acid, dimethylphosphate and dimethylphosphoric acid) and other water soluble products, which are eventually mineralised (WHO, 1989). Tests on the half-life of dichlorvos in sea-water at two temperatures, two pH values and two aeration regimes gave the results seen in Table 3.1.

Dichlorvos was found to be more stable than trichlorphon in sea water (Samuelson, 1987). The half-life of dichlorvos hydrolysis in deionised water was 240 hours at pH 7 and 120 hours at pH 8 (Hofer, 1981). However, it

has been suggested that dichlorvos formulated as Nuvan breaks down at a much slower rate, because of shielding by the emulsifier (Fraser *et al.*, 1989).

**Table 3.1**
**The effect of pH, temperature and aeration on the breakdown of dichlorvos in sea-water**

| Temperature ($^0$C) | pH 7.7* | pH 8.0* | pH 8.0+ |
|---|---|---|---|
| | $T_{1/2}$ (hrs.) | $T_{1/2}$ (hrs.) | $T_{1/2}$ (hrs.) |
| 4.5 | 195 | 178 | 122 |
| 13.5 | 166 | 153 | 93 |

From Samuelson (1987). * = low aeration rate. + = high aeration rate.

Also, in tests where the toxicity of Nuvan to a number of algal species was shown to be due to the non-dichlorvos fraction, storage at room temperature, under illumination, for 23 days did not affect toxicity (Raine *et al.*, 1990).

3:2.6.2 Pesticide use.

Dichlorvos has been used widely as a broad spectrum insecticide since 1961. Global production in 1989 was approximately 4 million Kg/year, with 60% of this used in plant protection, 30% for public hygiene and vector control, and 10% to protect stored products. Various formulations include emulsifiable and oil soluble concentrates, ready-for-use liquids, aerosols, granules and impregnated strips (WHO, 1989).

Nuvan 500 EC is a 50% w/v solution of dichlorvos in di-n-butyl phthalate (with other unidentified constituents, including an emulsifier), used to control infestations of the sea-lice *Lepeoptheirus salmonis* (Kroyer), and *Caligus elongatus* (von Nordmann). The recommended treatment is to surround the fish net with a tarpaulin, and to dose with a concentration of 1 mg/l dichlorvos for 1 hour, at temperatures between 5 and 16 $^0$C. Then the tarpaulin is removed, and the solution is released. Because dichlorvos kills only the pre-adult and adult sea-lice, repeated treatments are necessary at intervals of a few weeks, particularly during the summer (Pike, 1989; Ross, 1989; Raine *et al.*, 1990).

Trichlorphon, the dichlorvos precursor, is also used (as Neguvon) against sea-lice (300 mg/l for 15-16 min), its toxic action is through degradation to dichlorvos over time. Variations in exposure which

result from this, have led to mortalities (Salte *et al.*, 1987), and so a preference has developed for the use of dichlorvos directly.

3:2.6.3 Absorption, mechanism of toxicity, metabolism, excretion.

Dichlorvos is readily absorbed via all routes of exposure. In the rat, after oral administration, it is metabolised in the liver before it reaches the circulatory system. One hour after oral administration of $^{32}$P-dichlorvos, maximum concentrations of radioactivity are found in the kidneys, liver, stomach and intestines. In bone, the increase is slower, due to inorganic phosphate entering the phosphate pool of the organism. It is distributed eventually to all tissues (WHO, 1989).

The major toxic mechanism of dichlorvos is the inhibition of acetylcholinesterase (AChE) activity in the nervous system, and in other tissues. Brain ChE inhibition in a number of fish (Largemouth bass [*Micropterus salmoides*], Bluegill [*Lepomis macrochirus*], Golden shiner [*Notemigonus crysoleucas*], goldfish [*Carasissus auratus*], and Fathead minnow [*Pimephales promelas*]) was found at doses of 0.1 mg/l and lower, but recovery of ChE activity took place when they were returned to clean water (over 30 days) (Weiss, 1961). An investigation of a fatal dose of Neguvon to salmonids in pens showed that dead fish had brain-AChE activity reduced to 4% of unexposed fish, those that survived had brain-AChE levels 30% of the unexposed fish (Salte *et al.*, 1987).

There are three mechanisms of AChE antagonism; (1) reversible binding of dichlorvos to the enzyme, which is rapid; (2) reaction with the enzyme to form a dimethylphospho-enzyme derivative, with the loss of DCA, which is also rapid but can only be reversed by hydrolysis, or by agents such as 2-PAM; and (3) "ageing" of the phospho-enzyme compound to a more stable methylphospho-enzyme derivative, which is relatively slow and gives a stable product (WHO, 1989).

Maximum inhibition generally occurs within one hour, and is followed by rapid recovery, if removed from the source. Potentiation is slight when administered with a number of other organophosphates, but is marked in combination with malathion. Immune suppression has been reported in tests with rabbits (WHO, 1989).

Dichlorvos is metabolised mainly in the liver. The metabolism of dichlorvos in different species, including man, is rapid and uses similar pathways. Differences between species relate to the rate of metabolism rather than a difference in metabolites. No evidence of the accumulation of

dichlorvos or potentially toxic metabolites has been found, using a range of laboratory mammals. The major route for the elimination of the phosphorus portion was via the urine, with expired air being a less important route. However, the vinyl portion is mainly eliminated in the expired air, and less so in the urine (WHO, 1989).

3:2.6.4 Toxicity to algae and bacteria.

Raine *et al.* (1990) examined the toxicity of Nuvan and dichlorvos to photosynthetic $CO_2$ fixation by five unialgal cultures (*Chaetoceros calcitrans* [Paulsen], *Isochrysis galbana* [Parke], *Pavlova lutheri* [Droop], *Pseudoisochrysis paradoxa* [Ott nom. nud.], and *Skeletonema costatum* [Greville]), and natural assemblages of phytoplankton (collected at Black Rock, Salthill, Co. Galway). They found Nuvan at a concentration of greater than 1.0 mg/l dichlorvos to be toxic to all organisms, but dichlorvos itself was not toxic. The carrier in which dichlorvos is prepared is di-n-butyl phthalate. Tests were not carried out on this, and information on the emulsifier was not available.

The conclusion was that the Nuvan toxicity was due either to the carrier, to a degradation product of dichlorvos, to another chemical present in the formulation such as an emulsifier, or to a combination of such factors (Raine *et al.*, 1990). The topic of toxicity in formulated products of constituents other than the active ingredient is one that is discussed further in chapter 4, section 4:3.6 (p. 184).

A concentration of 3.5 mg/l diazinon produced a 50% reduction in the growth of *Euglena gracilis* (Butler, 1977). In eutrophic carp ponds, 0.325 mg/l killed *Cladocera* (predominantly *Bosmina* and *Daphnia* species), decreased *Copepoda* (mainly *Cyclops* species), and increased Rotatoria (mainly *Polyarthra* and *Brachionus* species) as well as phytoplankton (mainly *Scenedesmus* and *Pediastrum* species), giving only a slight change in total biomass (WHO, 1989).

The effect of dichlorvos on micro-organisms is variable and species dependent. Certain micro-organisms have the ability to metabolise dichlorvos, but the pesticide may interfere with the endogenous oxidative metabolism of the organism. In some micro-organisms, it inhibits growth, in others it either has no effect, or it causes stimulation. It has little or no effect on sewage micro-organisms, up to 100 mg/l (WHO, 1989).

3:2.6.5 Toxicity to aquatic animals.

Dichlorvos has been measured as having a high acute toxicity to a number of species of freshwater and estuarine fish (Table 3.2).

**Table 3.2**

**The toxicity of dichlorvos to fish**

| Test Organism | T($^0$C) | Wt(g) | $LC_{50}$ (mg/l) 24/48/96 hrs | Source | Other |
|---|---|---|---|---|---|
| *Salmo clarki* Cutthroat trout | 12 | 2.5 | ---/---/0.170 | Johnson & Finley | --- |
| *Salvelinus namaycush* Lake trout | 12 | 0.3 | ---/---/0.187 | Johnson & Finley | --- |
| *Mugil cephalus* Striped mullet | 20<br>20 | 6.4<br>1.0 | 0.74/0.66/0.20<br>1.75/1.25/0.25 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Lepomis macrochirus* Bluegill sunfish | 18 | 1.5 | ---/---/0.869 | Johnson & Finley | --- |
| *Menidia menidia* Atlantic silverside | 20 | 0.8 | 5.70/3.40/1.25 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Thallasoma bifasciatum* Bluehead | 20 | 5.4 | 1.75/1.75/1.44 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Anguilla rostrata* American eel | 20 | 0.14 | 2.30/2.30/1.80 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Sphaeroides macullatus* Northern puffer | 20 | 100 | 2.25/2.25/2.25 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Fundulus majalis* Striped killifish | 20 | 0.92 | 2.40/2.40/2.30 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Fundulus heteroditus* Mummichog | 20 | 1.7 | 3.41/2.68/2.68 | Eisler (1970a) | pH 8.0 Sal. 24 |
| *Gambusia affinis* Mosquito fish | 17 | 0.2 | ---/---/5.27 | Johnson & Finley | --- |
| *Cyprinus carpio* Carp | -- | 8 mm | ---/---/0.34 | Verma *et al.* | $TL_{50}$ |
| *Pimephales promelas* Fathead minnow | 17 | 0.7 | ---/---/11.6 | Johnson & Finley | |

In tests with seven species of estuarine fish, Eisler (1970a) concludes that, in sensitivity to organophosphate insecticides, teleosts are intermediate between the relatively resistant molluscs and the highly susceptible crustaceans. He recommends that future marine toxicity assays should use species from a minimum of three widely divergent groups, including teleosts, crustaceans, and molluscs.

A strong correlation has also been demonstrated between the incidence of cataracts in wild fish, including salmon and Sea-trout, and the use of Nuvan in fish farms. *In vitro* trials with Rainbow trout corneas showed that incubation with a concentration of 1.34 x $10^{-4}$M (c. 30 mg/l) Nuvan caused rapid depolarisation, and, overnight, led to a significant increase in opacity. While such concentrations in sea-water associated with the use of Nuvan are unlikely to ever occur, it is the formation of droplets containing dichlorvos in sea-water which is the basis of concern.

Because of the high lipophilicity of dichlorvos (chloroform/water partition co-efficient 100/1), it is unlikely that dichlorvos will leave the emulsifier until it contacts lipid. The emulsifier forms droplets of 3 µm to 70 µm (95% > 20 µm) diameter. It has been calculated that one droplet of 175 µm entering the cornea of a fish (which would not be a significant barrier to lipid soluble materials) would be sufficient to raise the corneal concentration to greater than 1.34 x $10^{-4}$M (Fraser *et al.*, 1989).

A number of invertebrates have been measured as having an extreme sensitivity to dichlorvos (96hr. $IC_{50}$ to *Daphnia pulex* 0.07 µg/l, Table 3.3). However, other invertebrates have shown a lower sensitivity. A study to determine the influence of a number of pesticides on the "hatchability" of *Artemia salina* dry eggs found no effect at 10 mg/l (Kuwabara *et al.*, 1980).

In the autumn of 1985, fishermen holding lobster (*Homarus americanus*) in sea crates, in the vicinity of an Atlantic salmon farm, reported total lobster mortalities which they associated with Neguvon treatment in the salmon farm. Following this, Egidius and Moster (1987) carried out trials on Neguvon and Nuvan with a small number of lobster (*Homarus gammarus*), crabs (*Cancer pagurus, Cancer maenas*) and Blue mussels (*Mytilus edulis*). 100% lobster mortality was caused by 0.1 mg/l Nuvan after 7.5 hours. Both species of crab survived 1.0 mg/l Nuvan for 10 hours, but one *Cancer pagurus* died after 24 hours at 1.0 mg/l. The mussels survived 1.0 mg/l Nuvan for 24 hours.

Although the trial results suffered from the lack of controls, and a small number of test organisms, it was these tests which first raised

concern over the use of dichlorvos. They concluded that the lobster kill probably was caused by the Neguvon. They considered that long term effects of the two chemicals were probably slight, but that more attention needed to be paid to the short time interval, immediately after treatment, when high levels existed.

**Table 3.3**

**The toxicity of dichlorvos to aquatic invertebrates**

| Test Organism | T(°C) | $IC_{50}$ (µg/l) 24/48/96 hrs. | Source | Other |
|---|---|---|---|---|
| *Daphnia pulex* Water flea | 15 | ---/0.07/--- | Sanders & Cope | Fresh-water |
| *Pteronarcys californica* Stonefly | 15 | ---/---/0.10 | Johnson & Finley | --- |
| *Simocephalus serrulatus* Water flea | 15<br>21 | ---/0.26/---<br>---/0.28/--- | Sanders & Cope | Fresh-water |
| *Gammarus lacustris* Amphipod | 21 | ---/---/0.50 | Johnson & Finley | Fresh-water |
| *Crangon septemspirosa* Sand-shrimp | 20 | 18/ 12/ 4 | Eisler (1969) | 24 ppt. salinity. |
| *Palaemontes vulgaris* Grass-shrimp | 20 | 390/300/15 | Eisler (1969) | 24 ppt salinity. |
| *Pagarus longicarpus* Hermit crab | 20 | 150/52/45 | Eisler (1969) | 24 ppt salinity. |
| Calonid Copepoda | -- | 90/---/--- | Saward *et al.* | --- |
| *Scylla serrata* Marine edible crab | -- | 4160*/3380/ 1860/1220 | Rao *et al.* | 33 ppt salinity |

* = 12 hours

Cusack and Johnson (1990) performed laboratory and field trials on the toxicity of dichlorvos to larval, juvenile and adult lobsters (*Homarus americanus*), mussels (*Mytilus edulis*), periwinkles (*Littorina littoralis*), zooplankton (natural populations), and phytoplankton (including *Platymonas* spp., *Chaetoceros calcitrans*, *Chaetoceros gracilis* and *Isochrysis galbana*). Dichlorvos was not toxic to the mussels or the periwinkles at 1

mg/l for 1 hr. (although behavioural changes were noted with the periwinkles), it was toxic to the larval and adult lobsters, the zooplankton and the phytoplankton at this level in laboratory trials (although not at 0.1 mg/l). In field trials it was not toxic to larval and juvenile lobsters housed adjacent to treated sea-cages. The phytoplankton growth was inversely proportional to the concentration of dichlorvos.

3:2.6.6 Factors affecting toxicity.

Variations in water hardness from 44 to 162 mg/l and in pH from 6 to 9, did not alter the toxicity of dichlorvos for Cutthroat or Lake trout (Johnson and Finley, 1980).

Experiments on the Mummichog concluded that the toxicity of organophosphate insecticides to this fish was directly proportional to temperature between 10 and 30 $^{o}$C, and to salinity between 12 and 36 ppt, and inversely proportional to pH in the range 5.5 to 10.0. Thus, at least three regimen of each should be tested (Eisler, 1970b).

The toxicity of dichlorvos to grass shrimp increased with salinity from 12 to 30 ppt, possibly due to lowered resistance of the organism. It was considered that inter-species and intra-species variation in sensitivity to organophosphorus insecticides is related to the number and types of esterases present (Eisler, 1970a)

3:2.6.7 Mutagenicity, reproductive toxicity.

Dichlorvos is mutagenic in a number of microbial systems, but there is no evidence of mutagenicity in mammals, where it is rapidly degraded by esterases in blood and other tissues. From acute and chronic studies, it is clear that the metabolites of dichlorvos are all less toxic than the parent compound. Only dichloroacetaldehyde (DCA) was positive in a few mutagenicity tests (WHO, 1989).

Dichlorvos is an alkylating agent, its metabolites are not. It binds *in vitro* to bacterial and mammalian nucleic acids, but the genotoxic effect of this alkylating activity is regarded as slight (WHO, 1989). Genotoxic trials by Aquilina *et al.* (1984) on dichlorvos, trichlorfon and DCA showed unscheduled DNA synthesis in the human epithelial-like cell (EUE) caused by the two pesticides, but not by DCA. None of the three were mutagenic in tests with Chinese hamster cells (V79).

In several reproduction studies on rats and domestic animals, no effects were seen on reproduction, and there was no embryotoxicity at dose

levels that did not cause maternal toxicity. At toxic doses, dichlorvos may cause reversible disturbances of spermatogenesis in mice and rats. It was not teratogenic in several studies carried out on rats and rabbits. Carcinogenicity trials for mice (600 mg/kg) and rats (234 mg/kg) have proved negative, although there are some equivocal trial results (WHO, 1989).

### 3:2.7 Ivermectin.

#### 3:2.7.1 Chemistry.

Avermectins are 16 membered macrocyclic lactones which have neither antibacterial nor antifungal properties, and do not inhibit protein or chitin synthesis (Fisher and Mrozic, 1989; Burg and Stapley, 1989).

Ivermectin is a semisynthetic derivative of the avermectin group, containing at least 80% of 22,23-dihydroavermectin $B_{1a}$ ($C_{48}H_{74}O_{14}$, mol. mass 874, R=$C_2H_5$, MK-932) and less than 20% of 22,23-dihydro-avermectin $B_{1b}$ ($C_{47}H_{72}O_{14}$, mol. mass 860, R=$CH_3$); synonyms are 22,23-dihydroavermectin $B_1$, 22,23-dihydro C-076$B_1$ and MK-0933; trade names include Heartgard 30, Cardomec, Equalan, Ivomec, Zimectrin, Mectizan; production is through fermentation and solvent extraction, giving an off-white powder (Campbell, 1989). The commercial formulation of Ivomec is a 1% (w/v) solution of ivermectin in 60% (v/v) propylene glycol and 40% (v/v) glycerol formal (Fink and Porras, 1989).

Avermectins are highly lipophilic, but are only slightly soluble in water (0.006-0.009 mg/l). They are acid-sensitive, and strong alkalis can be used to destroy avermectin residues for safe disposal. They are also rapidly degraded by UV light (Fisher and Mrozic, 1989).

Studies on environmental fate show that it binds strongly to soil and organic matter (retaining 99.5% of the parent material, leaching less toxic metabolites). Half-lives in soil/faeces mixtures were temperature dependant (inversely related) ranging from 7 to 240 days. Degradation by sunlight on glass had a half-life of only 3 hours (Halley *et al.*, 1989a).

#### 3:2.7.2 Pesticide use.

Fermentation of *Streptomyces avermitilis*, an actinomycete, produces four anthelminth-active compounds (Burg and Stapley, 1989; Campbell *et al.*, 1983). All of these, and many of their derivatives were shown to be toxic to gastrointestinal parasites in farm animals. One derivative, 22,23-dihydro avermectin $B_1$ (ivermectin) was shown to be sufficiently

potent, and safe, to warrant commercial production under the name ivermectin (Fisher and Mrozic, 1989). It was first released for this purpose in 1981 (Campbell, 1985).

Ivermectin is extremely potent against two major phyla, the nematodes and the arthropods (including sea-lice), and is used in the treatment of these in most farm animals, as well as pets and humans (Campbell, 1985).

General agricultural usage is at doses of 0.2-0.3 mg/kg body weight (normally single doses), with environmental loadings resulting from excretion, primarily in faeces, of up to 60-80% of the dose, with up to 50% of this being the parent drug (Halley *et al.*, 1989b).

Ivermectin was first suggested as a possible controller of sea-lice (*L. salmonis* and *C. elongatus*) in 1987 by Palmer *et al.* Commercial salmon pellets were coated with Ivomec and fed at a 1% body weight ration. Drug doses were multiples of the recommended mammalian dose of 0.2 mg/kg. Ivomec was found to be effective against the adult sea-lice, and remained so for up to 20 days. The therapeutic doses were not toxic to either seawater salmon or freshwater Rainbow trout (24 hours), but double this concentration (0.4 mg/kg) was. At 48 hours, 0.2 mg/kg caused mortality in freshwater salmon. This leaves a very narrow safety margin.

3:2.7.3 Absorption, mechanism of toxicity, metabolism, excretion.

The acute dermal $LD_{50}$ of ivermectin to rats and mice is significantly higher than the oral $LD_{50}$, suggesting that ivermectin demonstrates poor dermal absorption (Lankas and Gordon, 1989).

A definitive mode of action for avermectins has yet to be established. It is known that they have a number of sites of action, and that a number of target species have very different sensitivities. This may be due to differential ability to reach the site of action in a particular animal. The generalised mode of action is the increase in membrane chloride ion permeability, but there are certainly a number of others.

In the nervous system, effects on chloride reactions with Gamma-Amino-Butyric-Acid (GABA, causing increased release from nerve endings and binding of GABA to its receptors), lead to neural failure (Campbell, 1985).

The specific mode of action in invertebrates is different to that in vertebrates (Turner and Schaeffer, 1989). Also, invertebrates have GABA

distributed throughout their systems, while mammals have GABA only in their central nervous systems (Campbell *et al.*, 1983).

Radioactive labelling studies have shown that ivermectin deposits mostly in the fatty tissues and the liver of animals (Campbell *et al.*, 1983), and least so in the brain, independent of the route of exposure. The liver metabolism of a range of animals was similarly efficient at breaking down the chemical. The majority of ivermectin has been found to be excreted in faeces, with, as noted, excretion rates of 60%-80% of the dosage found. Only a very small proportion is excreted in the urine (Campbell, 1985; Chiu and Lu, 1989).

Because of the extremely low tissue levels of metabolites from therapeutic doses, *in vitro* systems have been used in metabolism studies. These offered the dual advantages of concentration of the metabolites and higher purity. Studies with rat and cattle hepatocytes produced 24-hydroxymethyl-$H_2B_{1a}$ and 24-Hydroxymethyl-$H_2B_{1b}$ as the major metabolites, with monosaccharides of these two as minor metabolites. Swine hepatocytes produced 3"-0-desmethyl-$H_2B_{1a}$ and 3"-0-desmethyl-$H_2B_{1b}$. *In vivo* studies have produced similar results (Chiu and Lu, 1989).

It has also been shown that the polar products (i.e. not the swine hepatocyte metabolites) go on to be esterified into fatty-acid esters, and to be stored in the fatty tissues as non-polar products of the liver metabolites. This leads to higher fatty tissue levels than liver tissue levels in all animals studied. It is unusual because classic metabolism pathways produce polar products ready for excretion (Campbell *et al.*, 1983; Chiu and Lu, 1989).

3:2.7.4 Toxicity to aquatic animals and plants.

Because there is no manufacturer-promoted use of ivermectin in aquatic environments, there has been very little research into the toxicity of the chemical to aquatic organisms. The bulk of this work to date was carried out in a wide-ranging series of trials by Halley *et al.* (1989a).

Results from these trials include the 48 hr. $IC_{50}$ to *Daphnia magna*, which was 0.025 µg/l, with an NOEC of 0.01 µg/l. Ivermectin metabolites were found to be 16 to 640 times less toxic. The 96 hr. $LC_{50}$s to two species of fish, Rainbow trout (*Salmo gairdneri*) and Bluegill sunfish (*Leponis macrochinis*) were 3.0 and 4.8 µg/l respectively.

Ivermectin at 10 mg/l increased the growth of the algae *Chlorella pyrenoidosa* over the first week of a 14 day trial, but this rate of growth slowed in the second week. An overall drop in standing crop was

attributed to the solvent, and no toxicity was attributable to ivermectin (Halley *et al.*, 1989a).

The 24 hr. $IC_{50}$ of ivermectin to the snail *Biomphalaria glabrata* was 30 µg/l (Matha and Weiser, 1988).

3:2.7.5 Mutagenicity, reproductive toxicity.

In tests for genotoxic activity, including the Ames test (*S. typhimurium*, with and without S9), the Mouse Lymphoma Assay (Fischer L5178Y cells), and Unscheduled DNA Synthesis trials (IMR-90 cells), no genotoxic activity was attributed to ivermectin. In reproductive toxicity trials, developmental toxicity was only found at concentrations which were toxic to the mother, indicating that it is not a selective developmental toxin (Campbell *et al.*, 1983; Lankas and Gordon, 1989).

3:2.8 Malachite green.

3:2.8.1 Chemistry.

Malachite green, a green or yellow crystalline powder with a metallic sheen, is an arylmethane dye with antifungal, antibacterial and antiparasitic action. Its chemical formula is $C_{27}H_{34}N_2O_4S$ (mol. mass 364.9); chemical name is 4-[P-(dimethylamino)-a-phenyl-benzylidene]-2,5-cyclohexa-dien-1-xylidene dimethyl ammonium chloride; common trade names include C.I. Basic Green 4, C.I. 42,000, Aniline Green, China Green, Victoria Green B or WB, New Victoria Green Extra O, I, or II, Diamond Green B, BX or Pextra, Solid Green O, Light Green N, Benzol Green, Benzaldehyde Green, Fast Green, Astramalachite green and Astrazon Green, amongst others; it is soluble in water, less so in alcohol; its $pK_a$ is 6.90 (at pH 7.4 it is only 25% ionised); an aqueous solution is blue-green, or yellow at pH < 2; it is stable in a slightly acid aqueous solution (Nelson, 1974a; Alderman, 1985).

It is produced by condensing benzaldehyde with N,N-dimethylalanine, oxidising the resultant bis(P-dimethylaminophenyl) phenylmethane, and treating with HCL (Nelson, 1974a). Commercial production commonly achieves a yield of 65%, which is then diluted with bulking agents to the desired strength.

Confusion as to the correct malachite green content can stem from its use as a dye, where dye products are expressed as a proportion of the dyeing ability of the dry dye. However, a saturated aqueous dye contains

only 7.6% malachite green. Excess acetic and hydrochloric acids are used to achieve contents of over 30% (Alderman, 1985).

Malachite green is commonly produced as oxalate or hydrochloride salts. The Astrazon Green product used in fish farming is a 50% solution in a mixture of acetic and either sulphuric or hydrochloric acids. However, there are many other products used in the industry, and the sources and quality can vary greatly (Hygeia Chemicals ltd., Product Data Sheet; Alderman, 1985).

The ionic equilibrium of malachite green is between the dye salt cation and the carbinol or pseudobase. The combination of the cation with hydroxyl ions to produce the non-ionized pseudobase is slow (speed of equilibrium from 100% to half the $pK_a = 2.1$ hours, as opposed to normal instantaneous reactions). It results in the deposition of the carbinol as a green-white sludge (Alderman, 1985). The non-ionic pseudobase is lipophilic and can enter cells easily, but it is the dye which is regarded as bioactive, and so degradation to the less water soluble carbinol could be considered a detoxification mechanism (Nelson, 1974a). However, studies on the effect of pH on malachite green toxicity indicated that there is no difference in toxicity between the two. This may be due to re-ionization of the dye within the flesh of the organism (Lanzing, 1965; Bills *et al.*, 1977)

Malachite Green has been shown to bind strongly to organic material, including the nuclei of animal cells. Reduction of antibiotic activity has also been shown to be caused by binding to serum proteins (Alderman, 1985). Activated carbon has been shown to remove malachite green at a rate of 23.4 mg/g carbon (Bills *et al.*, 1977). This feature is under investigation as the basis for a possible effluent treatment system, using peat (Henry, 1990).

Malachite green is subject to slow photodegradation (over weeks), to p-dimethylbenzophenone (aerobic), or malachite green leucobase (anaerobic). This is not likely to be significant in terms of ecotoxicology (Alderman, 1985). In bioassays, storage over 1, 2 and 3 weeks did not reduce its toxicity (Bills *et al.*, 1977).

3:2.8.2 Pesticide use.

Malachite green has many applications as a dye, but was first used as a fungicide dip for fish and fish eggs in 1936 by Foster and Woodbury (Nelson, 1974a; Bills *et al.*, 1977). It is the activity of malachite green against oomycetes and other fungal infections in fish and crustaceans which is of

most use to the fish farming industry. It is used in salmonid fish culture (amongst others) as a flush treatment, concentration 1-5 mg/l, for 1 hour. Current use in the UK fish farming industry has been quoted as around 1000 kg/yr (Alderman, 1988).

Its antifungal activity was underlined in trials on a new fungicide screening method, where malachite green was significantly more active than three alternative fungicides, and was proposed as a reference chemical for the new method (Bailey, 1983).

In tests to find a replacement for malachite green, in the light of fears of possible carcinogenicity, only 2 out of over 200 candidates showed any promise, and these were still significantly less effective than malachite green (Schnick, 1988). This prompted the US fish and Wildlife Service to apply for a licence for its continued use at state hatcheries. This was allowed, and a hatchery maximum effluent level of 50 µg/l applied (Anon, 1989).

A synergistic effect between malachite green and formalin has been demonstrated against fish pathogens, reducing the concentrations necessary for each material. Thus, the two are often used in combination as a fish farming antifungal agent (Nelson, 1974b; Gilbert *et al.*, 1979).

The chemical used in this study was '50% Malachite Green, zinc free (Astrazon Green)', as marketed by Hygeia Chemicals ltd., Galway. It contains a 50% solution of malachite green (i.e. 535 g/l at 1.07 g/ml density).

3:2.8.3 Mechanism of toxicity, metabolism.

The mode of action of arylmethane dyes is respiratory enzyme antagonism (Fizhenko and Braun, 1967). Specifically, malachite green acts as an irreversible poison against thiol-containing enzymes. In hepatocyte culture it has been shown to cause destruction of mitochondria, which can be prevented using cytochrome C. This cytochrome C mediation of toxicity has also been found *in vivo* in rats (Werth, 1958; Alderman, 1985).

An immunosuppressive action in the treated fish was associated with malachite green (indicated by a reduction in blood lymphocyte numbers), and was considered for some time to negate the fungicidal benefits, but this was shown in studies by Hlavek and Bulkley (Rainbow trout), and Pickering and Pottinger (Brown trout), to be a transient stress-induced phenomenon, associated with irregular, or single treatments. They demonstrated that Brown trout acclimatised to daily dosages without any ill-effects, and recommended prophylactic application (Hlavek and Bulkley, 1980; Pickering and Pottinger, 1985).

3:2.8.4 Toxicity to bacteria.

The simple triphenylmethane dyes of the arylmethane group have significant antimicrobial activity. They are most active against G+ bacteria, less so against G- bacteria and least against acid-fast bacteria and bacterial spores. As noted, this antibacterial activity has been shown to be inhibited by serum proteins (Fischer *et al.*, 1952; Alderman, 1985).

3:2.8.5 Toxicity to aquatic animals.

In a study with a large number of test organisms (Table 3.4), Bills *et al.* found that salmonids were less sensitive than ictalurids (catfish etc.), who in turn were less sensitive than centrarchids (Bluegills, bass etc.). In all cases, toxicity increased with exposure time (Bills *et al.*, 1977).

**Table 3.4**
**The toxicity of malachite green to fingerling* fish in soft water at 12°C**

| Test Species | 3hr $LC_{50}$ (mg/l) | 6hr $LC_{50}$ (mg/l) | 24hr $LC_{50}$ (mg/l) | 96hr $LC_{50}$ (mg/l) |
|---|---|---|---|---|
| *Oncorhynchus tshawytscha* Chinook salmon | 1.72 | 1.38 | 0.292 | 0.224 |
| *Oncorhynchus kisutch* Coho salmon | -- | >3.00 | 0.569 | 0.383 |
| *Salmo salar* Atlantic salmon | 3.56 | 1.09 | 0.497 | 0.283 |
| *Salmo trutta* Brown trout | 1.73 | 1.27 | 0.352 | 0.248 |
| *Salmo gairdneri* Rainbow trout | 1.41 | 0.760 | 0.360 | 0.248 |
| *Salvelinus fontinalis* Brook trout | 3.00 | 1.44 | 0.300 | 0.220 |
| *Ictalurus punctatus* Channel catfish | >3.00 | 1.10 | 0.181 | 0.112 |
| *Micropterus salmoides* Largemouth bass | -- | -- | 0.282 | 0.0728 |
| *Micropterus dolomieui* Smallmouth bass | 1.36 | -- | 0.154 | 0.0453 |
| *Lepomis macrochirus* Bluegill sunfish | 6.00 | 2.19 | 0.231 | 0.0305 |

Adapted from Bills *et al.* (1977). * = 0.5-1.5g.

However, it should be noted that there are a large number of studies which have reported widely ranging toxic doses. Interpretation of these is complicated by lack of attention to the grade of malachite green used,

and thus confusion as to the true concentrations applied. Also, test temperatures have often been omitted (Alderman, 1985).

For example, in a comprehensive review of the literature on the use of formal-malachite green, Nelson (1974b) quotes a number of $LC_{50}$ values which are significantly different to those found by Bills *et al.* in their work. These include (result from Bills *et al.* in parentheses); Channel catfish (*Ictalurus punctatus*), 0.299 mg/l (0.112 mg/l); Largemouth bass (*Micropterus salmoides*), 0.0453 mg/l (0.0728 mg/l); Smallmouth bass (*Micropterus dolomieni*), 0.0200 mg/l (0.0453 mg/l); Bluegill (*Lepomis machrochirus*), 0.261 mg/l (0.0305 mg/l) (Nelson, 1974b; Bills *et al.*, 1977). It is not possible to reconcile these differences, as no description of test conditions, or of the type of malachite green used is given by Nelson. For this reason, the values used as reference in this study are from Bills *et al.*

Aquatic invertebrates and molluscs were found in general to be less sensitive than fish (Table 3.5), with the Asiatic clam resistant at over 100 mg/l The most sensitive invertebrate tested was a mayfly nymph(*Callibaetis* sp.), whose 96 hr. $IC_{50}$ was 0.079 mg/l (Bills *et al.*, 1977).

**Table 3.5**
**The toxicity of malachite green to selected invertebrates in limed water at 16°C**

| Test Species | 6hr. $IC_{50}$ (mg/l) | 24hr. $IC_{50}$ (mg/l) | 96hr. $IC_{50}$ (mg/l) |
|---|---|---|---|
| *Corbicula leana* Asiatic clam | -- | -- | 122 |
| *Cypridopsis* spp. Ostracod | 5.85 | 5.85 | 3.45 |
| *Palaemonetes kadiakensis* Freshwater prawn | -- | 9.10 | 1.90 |
| *Tanytarsus dissimilis* Midge larvae | 5.00 | 1.00 | 0.510 |
| *Callibaetis* spp. Mayfly naiads | 5.75 | 2.75 | 0.0790 |

Adapted from Bills *et al.*, (1977).

Survival of the nauplii of the shrimp *Penaeus stylirostris* was zero after 12 hours at a concentration of 2.0 mg/l malachite green. At 0.4 mg/l, survival was greater than 80% after 12 hours, but less than 5% after 24 hours. A concentration of 0.08 mg/l caused no mortalities after 24 hours. Metamorphosis was found to be a more sensitive parameter, being reduced at a concentration of 0.08 mg/l, but not at 0.016 mg/l. This may, however, be an

expression of the time-dependant toxicity of malachite green. Overall, malachite green was more toxic than erythromycin, minocycline and formalin to *Penaeus stylirostris* (Castille and Lawrence, 1986).

3:2.8.6 Factors affecting toxicity.

The toxicity of malachite green to Rainbow trout fingerlings was found by Lanzing (1965) and Alderman (1985) to be directly related (positively) to temperature.

However, Bills *et al.* (1977) conclude that this relationship is not significant, despite such a trend in their data. They did conclude that the opposite relationship in Channel catfish was significant. No significant temperature difference was found with Bluegill. Changes in water pH did not affect the toxicity of malachite green to Rainbow trout or Channel catfish, but lowered pH reduced its toxicity to Bluegills. Water hardness had no significant effect on its toxicity to any of these three species.

3:2.8.7 Reproductive toxicity, mutagenicity, carcinogenicity.

Malachite green has never been registered as a therapeutic agent for the fish farming industry because of fears of teratogenicity and carcinogenicity raised by Werth and others. Werth (1958) found a series of hereditary tumours in the progeny of animals treated with malachite green. These tumours did not appear in the progeny of a group which had been administered cytochrome C simultaneously, or in the control group. The conclusion was that the teratogenic activity was caused by the reduced performance or production of respiratory enzymes.

However, Kimura *et al.* (1990) point to the increased longevity of the progeny in that study, who were given malachite green alone, and attributes the increased incidence of tumours found to spontaneous tumours in long-lived rats.

In a study by Clemmensen *et al.* (1984), malachite green was found to be non-mutagenic by the Ames assay, with or without metabolic activation, to the strains TA 1535, TA 1537, and TA 100. The microsomal fraction also reduced the cytotoxic effects of malachite green, as seen by Werth on intravenous administration of cytochrome C.

However, metabolic activation with strain TA 98 produced a dose dependant toxic effect in the range 20-70 µg/plate (there was no effect without activation). This dose is significantly higher than is used in fish culture. There were no clastogenic effects noted in the micronucleus test,

carried out with the CHO cell line. The conclusion was that more extensive *in vivo* tests were necessary before recommendation for use leading to intentional consumption or contact.

Meyer and Jorgenson (1983) carried out such tests with Rainbow trout eggs, and with pregnant rabbits. Malachite green at 1-5 mg/l (1 hour treatments, 5, 10 and 15 applications over 34 days) reduced the percentage of Rainbow trout ova that developed to the eyed stage, increased the hatching time, reduced the size of larvae and increased deformities. However, the proportion of eyed eggs that hatched increased, probably due to control of fungal infections.

It also caused significant teratogenic effects in rabbits, with both skeletal and visceral anomalies found, though with no dose relationship. There was also significant weight loss in the parental animals. Although the doses used were higher than would be used in fish culture, they were sub-lethal and gave significant cause for concern. Another cause for concern was also noted in the study, and that was the acute pulmonary toxicity of malachite green when accidentally aspirated (coughed) into the lungs of test animals. This raised significant questions as to the occupational safety of malachite green.

In their own tests for carcinogenicity, Kimura *et al.* (1990) applied concentrations which did not cause weight loss in mice, and found no evidence of carcinogenicity. The same team, under Kobayashi (1990), examined the carcinogenicity of malachite green to Rainbow trout, because of their reported susceptibility to carcinogenic materials. Treatments were 0.3 and 2.0 mg/l for 1 hour, twice weekly for 52 weeks. No tumours were found, and neither was there any weight loss. An $LC_{50}$ for this treatment method was said to lie between 4 and 8 mg/l., with no effect at 2 mg/l.

These results indicate that the eggs of Rainbow trout are much more sensitive to malachite green than the hatched fish, indicating again that any carcinogenicity is likely to be developmental.

## 3:3 Results and Discussion.

### 3:3.1 Introduction.

For cell culture methodologies to be a useful addition to environmental analytical techniques they must perform well in a number of different areas. These fall into two broad categories, qualitative and quantitative, as shown in Table 3.6.

**Table 3.6**
**Parameters for assessment of toxicity test methods**

| Qualitative | Quantitative |
|---|---|
| Ease of use | Sensitivity |
| Relevance | Reproducibility |
| Flexibility | Statistical strength |
| Cost | Volume of data |
| Resource requirements | Duration |

In the following Results and Discussion section, all of the methods will be compared to each other, using all of these parameters. Because the two categories provide very different kinds of data, the section will be divided into qualitative/quantitative results. The quantitative category is further divided into results from individual systems, and comparison of multiple systems.

### 3:3.2 Qualitative comparison of methods.

For a technique to be useful in routine monitoring, it must be practical. Any particular method can provide useful environmental data, but if it is technically involved, and not realistically applicable in a range of laboratories, use will not be made of it. An example of this is *in situ* toxicity testing, which provides the most directly relevant data of all, but is almost completely impractical, particularly in relation to the volume of ecotoxicity data that is required.

Qualitative performance, therefore, is very important when assessing any particular method, or group of methods. The following section is an assessment of the cell culture and whole organisms tests used in this study according to parameters in the qualitative category.

3:3.2.1 The L929 cell line.

The L929 cell line has not been used extensively in previous ecotoxicity trials. No previous reference is available, therefore, to culturing characteristics, or use in environmental assays. For example, in a series of papers by Christian *et al.* (section 3:2.3.3, p. 49), no reference was made to the handling of the cell line, or the methodology of the assay.

In this study, there were no significant problems with routine culture, or in cytotoxicity assays. The generation time (c. 16 hours) was shorter than that of RTG-2 cells (c. 24-36 hours), and seeding a 25 $cm^2$ flask at 1 x $10^4$ cells/ml produced confluence in 3-4 days. Once confluence was reached, media nutrients were used up quickly, and contact inhibition led to death and detachment within 48-72 hours (Plate 3.1). Regular sub-culture therefore, was essential.

Disaggregation in Trypsin/EDTA routinely produced a single-cell suspension. Occasionally, this failed, requiring a second, or a third trypsinisation. There was no set of circumstances common to these failures (e.g. trypsin batch, temperature, confluence level, length of exposure time to trypsin etc.), therefore no comment can be made on any possible cause.

A growth assay in 24-well plates established the correct seeding density (to give a lag-phase of less than 24 hours, and sub-confluence in 96-well plates after 96 hours), as 3 x $10^4$ cells/ml (5% serum media).

It was noted that L929 cells cultured in Hepes media sometimes exhibited a degree of vacuolisation (Plate 3.2). When the same cells were incubated in bicarbonate media (i.e. under a 5% $CO_2$ atmosphere), this vacuolisation was effectively removed, and the mitotic fraction increased (Plate 3.3). This indicates that the Hepes buffer may cause a degree of stress to L929 cells in culture. This was not a factor in the ecotoxicity trials, where the bicarbonate media was used. It may, however, be a consideration for long-term maintenance.

Overall, the handling characteristics of the L929 cell line in this study indicate that it would be an effective ecotoxicity assay material. Its culture conditions are easily standardised, and it forms a single-cell suspension readily, which is important for even plate seeding. It is a mammalian epithelial cell line, and so represents the primary target cell group of environmental toxins.

Plate 3.1 Contact inhibition, L929 Cells

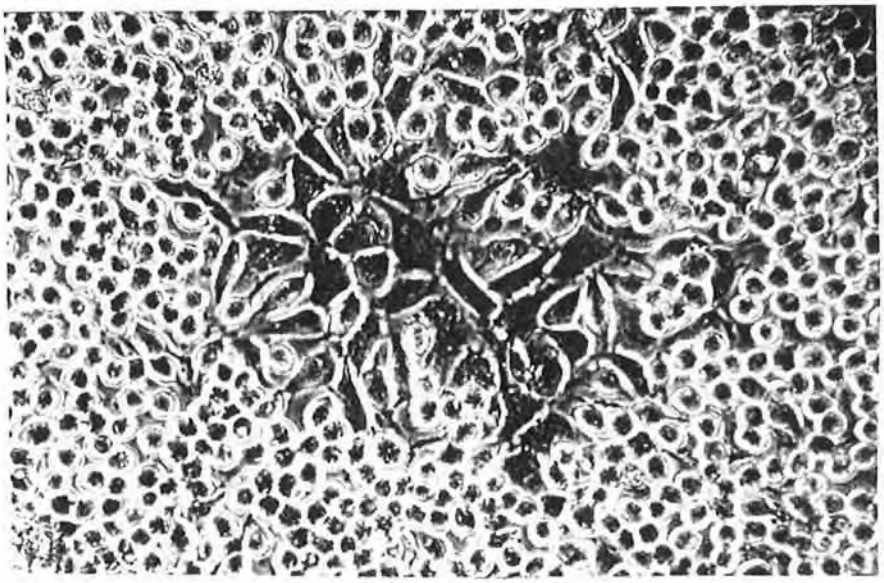


Plate 3.2
The morphology of L929 cells grown in hepes buffer media

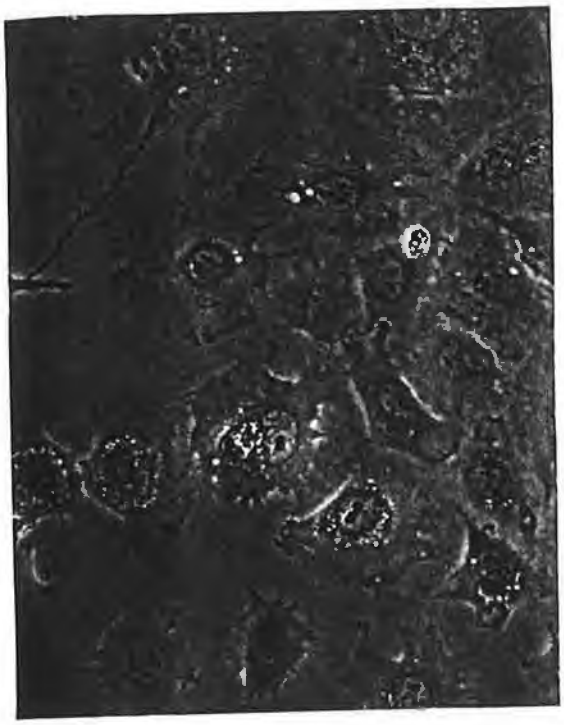
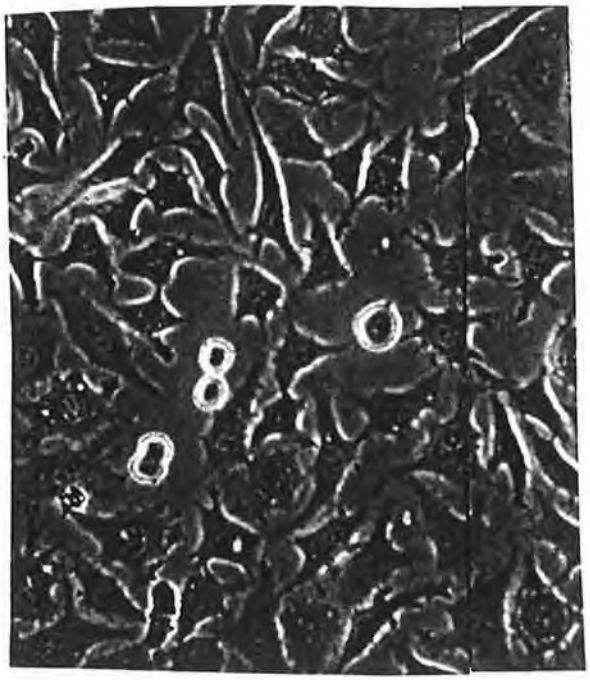


Plate 3.3
The morphology of L929 cells grown in bicarbonate media



Equipment and other resources required for cell culture are common to all cell lines. These include standard incubators, $CO_2$ incubators, laminar flow air hoods, and general microbiological facilities. Expertise required are a high standard of aseptic technique, and training in specific cell culture techniques.

These are significantly less resources than are required for, say, fish acute toxicity testing, or sophisticated chemical instrumental analysis. It is considered here that these resource requirements do not represent any particular bar to the use of cell culture in environmental analysis.

### 3:3.2.2 The RTG-2 cell-line.

In terms of systems, Babich and Borenfreund (1990) considered that a comparison between aquatic whole animal and cell culture systems would be best, because of the similar route of administration. Comparison between Rainbow trout *in vivo/in vitro* data is an example of such a comparison.

The RTG-2 cell-line, as used in this study, was, in some ways, more difficult to culture, and more difficult to apply in assays than the L929 line, but it also had some distinct advantages, as follows.

#### 3:3.2.2.1 Temperature.

The temperature optimum of the RTG-2 cell line is 20 $^{o}$C. However, the cells can grow over a wide range of temperatures, which is considered an advantage in routine maintenance. Metabolism decreases along with temperature from the optimum. Viable cells were obtained in this study after incubation at 4 $^{o}$C for two months, without replenishment. Thus, the routine maintenance requirements of RTG-2 are somewhat less stringent than those of L929.

In some cases, flexibility in cultivation temperature can be an advantage in cytotoxicity tests also. One of the problems of expanding the ecotoxicity database is the range of test conditions used, in particular temperatures, which makes comparison of results very difficult. In situations where direct comparisons with data at a range of temperatures are necessary, it would be possible to carry out RTG-2 trials at any temperature, up to 25 $^{o}$C. This is a particular advantage of fish cells.

For example, Kocan *et al.* (1979) used an incubation temperature of 18 $^{o}$C with RTG-2 (and an exposure time of 72 hours). Walton *et al.* (1983) also used 18 $^{o}$C in a study on DNA repair synthesis. Marion and Denizeau

(1983, a-b) used a temperature of 15 °C with RTG-2 in studies on cadmium and lead. At a 10% serum concentration, they found the RTG-2 generation time at this temperature to be 96 hours. Wolf and Quimby (1962) report that, in comparison to growth at 24 °C, the growth rate at 18 °C was 1.3 times slower, at 12.5 °C it was 2.2 times slower, and at 4 °C it was 3.5 times slower.

Some do not consider this an advantage however, Babich and Borenfreund (1987b) considered the longer replication times at these low temperatures severely limit the use of RTG-2 cells in cytotoxicity studies.

In this study, a temperature of 20 °C was used for the RTG-2 trials (the optimum growth temperature), while a temperature of 12 °C was used for the Rainbow trout trial.

**3:3.2.2.2** Trypsinisation.

The first trypsinisation method tried (L929 standard) did not detach RTG-2 cells effectively. Some detached after extended trypsin contact, but in large clumps, which were not useful for cytotoxicity assays, as clumping caused extra dye to be retained in the wells, relative to others in the same column. This resulted in high intra-column and inter-column variation. A number of alternative protocols were tried. Further extended contact gave a greater proportional yield, but did not significantly decrease the degree of clumping.

The regime which eventually gave useful recovery and disaggregation was application of 1 ml Trypsin/EDTA to the cell layer, incubation (without pouring off) for 10 minutes at 37 °C, followed by drawing up and down, very gently, into a pipette. The flask should not be tapped to dislodge the cells, this should be done by tilting and gentle washing with the Trypsin/EDTA solution.

This is significant, as the temperature used is 17 °C above the optimum, and 7 °C above that considered lethal (albeit over a number of hours). There is no precedent in the literature for this protocol, but it was used in the study as one that was effective, and necessary.

Wolf and Quimby (1962), in the original paper, described the initial passaging of the cells as utilising extended cold trypsinisation (0.25% trypsin for 10 min.). However, they subsequently stated a preference for the use of disodium versenate (20 mg/100 ml). Kocan *et al.* (1979) used 0.5% trypsin in 0.02% EDTA (no reported contact time), and reported no difficulties in passaging. Similarly, Marion and Denizeau (1982) used 3 mM EDTA in 50 mM sodium bicarbonate, and reported no difficulties.

As noted, extended cold trypsinisation was not effective in this study, but the extended warm trypsinisation was (disodium versenate was not tried). Once the trypsinisation difficulty was resolved, the amount of clumping was reduced to a manageable level.

**3:3.2.2.3** Replication rate.

RTG-2 cells had a longer generation time than L929, at 24-36 hours. This meant that a higher seeding density was required, to give 90% confluence over 96 hours. A growth assay established the correct seeding density as 1 x $10^5$ cells/ml (5% serum).

RTG-2 cells were also much larger than L929 cells (Plate 3.4), and they did not round up on confluence. Instead, they overgrew into a number of layers (Plate 3.5). The metabolism of the media by the RTG-2 cells became much slower at this point than that of L929, and overgrowth was maintained for several weeks without a significant reduction in viability (this, again, would be an advantage in culture maintenance). However, subsequent trypsinisation was much more difficult.

The larger cell size, longer generation time, and resultant smaller increase in cell number over the incubation period (possibly leading to lower relative absorbances), is a factor which may make the RTG-2 cell line less useful than others in routine assays. To achieve the five or six generations which occur over 96 hours in the L929 cell line, the RTG-2 assay would require 120 - 216 hours.

It also means that shorter-term assays, such as 4 - 24 hour assays, which might be used in effluent monitoring, would be limited in interpretation to simple cytotoxicity rather than growth inhibition or other reproductive effects.

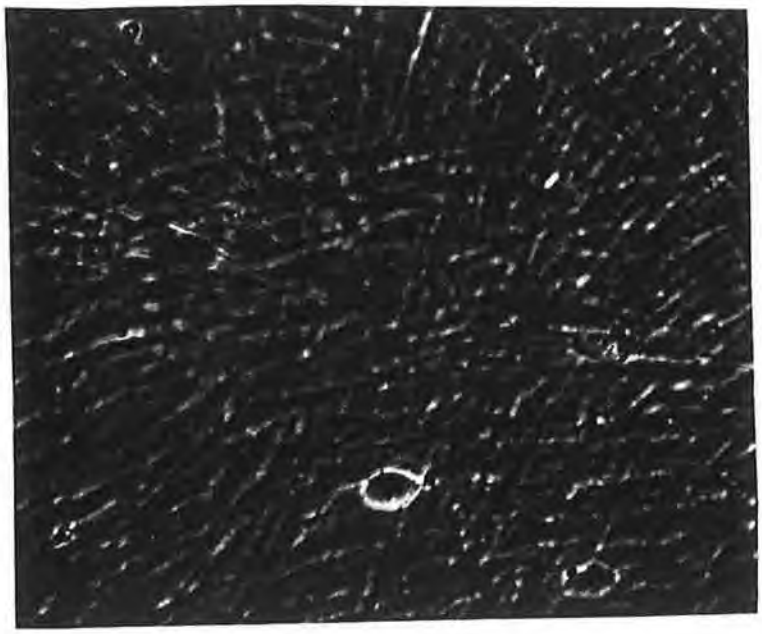
A minor point, also related to cell number, is discussed by Wolf and Quimby (1962). They report that a large cell number is not required by RTG-2 cells for growth (i.e. that several hundred cells will give a successful sub-culture). With the L929 line, a critical number is required for seeding. This would be an advantage in the routine culture of the line

**Plate 3.4**

**The morphology of RTG-2 cells**

**Plate 3.5**

**Layered growth, RTG-2 cells**



**3:3.2.2.4** Morphological observations.

From microscopic examination of RTG-2 cells in contact with Nuvan and malachite green, two different mechanisms of cytotoxicity were noted. Cells in contact with Nuvan tended to develop a heavily granulated cytoplasm initially. This was followed by contraction of the cell, particularly those in colonies, where the entire colony would take on the appearance of a single mass, covered in cellular debris. This would be followed shortly afterwards by detachment.

Cells in contact with malachite green took on a very different appearance. The first alteration was a darkening of the wall of the nucleus. This was followed by elongation and narrowing of the cells, where colonies tended to separate out. The central portion of the cell, including the nucleus would then swell considerably. This was followed by detachment.

While no reference was available to previous observations on morphological changes in L929 or RTG-2 cells, such changes have been observed in other mammalian cell lines. Rodrigues and Mattei (1987) describe the morphological changes of two cell lines (CHO and IB-RS-2) on exposure to ivermectin. Granulisation was followed by intense vacuolisation, contraction and lysis of cells. Riddel *et al.* (1986) noted significant morphological change at < 1% of the $EC_{50}$ for 5 chemicals, including paracetamol and aspirin, to 3T3 L1 cells.

The ability to make these observations routinely in cell culture assays, and the possibility of quantifying these sub-lethal effects, is another useful feature of cell culture in general, which could add further meaningful data to the ecotoxicity database.

Observed effects on specific organelles, or cellular processes could help to interpret the toxicity of a substance in relation to extrapolation to organ or system level effects. Waters *et al.* (1978) state that "the correlation of structural, biochemical and cytochemical alterations *in vivo* with similar changes *in vitro* has led to a better understanding of the sequence of morphological events occurring in cell injury and death." In this study, sub-lethal effects are also noted, and discussed, for Rainbow trout (section 3:3.3.5.2, p. 113).

**3:3.2.2.5** Summary.

While the RTG-2 cell line has particular advantages in environmental analysis, in particular the direct comparison with Rainbow trout data, there are a number of significant points of handling which

reduce its effectiveness. The most important of these, as seen in this study, is the long generation time, which reduces the volume of data that can be generated. A second is the tendency to clump, possibly increasing variation in the 96-well plates.

On balance, taking handling characteristics alone, it is considered here that the L929 cell line is a better choice, overall, for routine environmental analysis. The two are not, of course, exclusive, a strength of cell culture being that many different cell lines, from different species, and different tissues, can be cultured and tested concurrently. The conclusion reached here is simply in relation to the two cell lines under study.

3:3.2.3 The crystal violet end point.

There were five steps in the crystal violet end-point. Some workers using this protocol do not include the formaldehyde fixing step, but it was found to be essential in this study. None of the steps presented any significant difficulty, except washing off the dye, which required a minimum of five applications of fresh PBSA. If this was not done, there was significant retention of the dye on the walls of the well. Desorption with glacial acetic acid was thorough, though complete mixing was important prior to reading. The L929 cell layer absorbed crystal violet strongly (c. 0.2 - 0.5 abs. units), the RTG-2 line less so (0.05 - 0.2 abs. units).

The difficulties seen here resulted from manual methods applied, and were resolved by development and application of standard protocols. However, both washing and mixing (along with other steps) can be automated. In the monitoring trials section of the study an automatic plate shaker was used. Thus, no part of the method restricts its use in routine environmental analysis.

Previous use of the crystal violet method has been limited, but has included assays where crystal violet was used to stain cells prior to coulter counting (Hunt *et al.*, 1986). There has been little discussion on its merits relative to other methods. In this study, it was found to be quick, simple to use, and reliable.

3:3.2.4 The neutral red end point.

This end-point has been used in a number of cytotoxicity studies since its initial use by Borenfreund and Puerner (Borenfreund and Puerner, 1985; Riddel *et al.*, 1986; Babich and Borenfreund, 1986; Hunt *et al.*, 1987; Babich and Borenfreund, 1987a; Benford and Good, 1987; Babich *et al.*, 1989).

There were eight steps in the neutral red end-point, including overnight incubation of the dye, and a further 3 hr. incubation of the cell layer, after toxin incubation. There were some particular handling and operational characteristics of note, as outlined below.

**3:3.2.4.1** Formal-calcium rinsing.

The formal-calcium rinsing step was somewhat problematic, in that extended contact caused elution of some of the dye from the cells. As this is also the fixing step, it is important that the contact is long enough to achieve fixation, and short enough to avoid elution of the dye (recommended time is a 'rapid rinse'). In a number of trials where this contact time was varied, results ranged from failure due to non-fixation of the cell layer, through successful trials, to failure due to elution of some dye (causing absorbances to be too low for useful calculation), and failure due to complete elution of the dye. Standardisation of this contact time is essential to successful completion of the assay.

Using the results from these trials, a standard application of 30 seconds was decided, and applied throughout the rest of the study. In this way, it did not cause any significant problem overall.

**3:3.2.4.2** Reduction of formaldehyde concentration.

From an industrial health and safety point of view, reduction of the formaldehyde concentration to the minimum necessary for fixation would also be a positive development.

Although it has not been discussed in isolation in previous papers, there has been a progressive move in this direction by those developing the method. In the original paper, Borenfreund and Puerner (1985) used 40% formaldehyde and 10% calcium chloride. Babich *et al.* (1986) used a 10% solution of these (i.e. 4%/1%, as used in this study), and the following year Babich and Borenfreund (1987c) used a 1%/1% solution. By 1990, Babich and Borenfreund had again reduced the formaldehyde concentration to a 0.5%/1% solution with calcium chloride.

There have also been some studies where a different approach has been taken. Riddel *et al.* (1986) omitted the formal-calcium step altogether, with 3T3-L1 cells, saying that the glacial acetic acid step was both a fixant and an eluant. This was in a study where the cells, after elution of the neutral red dye, were then subject to a protein determination (KBr).

Hunt *et al.* (1987), with V79/4 cells, also omitted the fixation step, using PBSA as a washing step.

These approaches are inconsistent with the results seen in this study, for the two cell lines used (in particular, the L929 cell line). For both, fixation by formaldehyde was essential, and failure of the formal-calcium step led to failure of the assay.

**3:3.2.4.3** Mixing.

Elution with acetic acid-ethanol was much slower than that with glacial acetic acid (used in the crystal violet elution method), and mixing was extremely important. Significant differences were found in readings obtained from a range of mixing regimes. The regime finally applied was a minimum desorption period of two hours, followed by manual shaking for two minutes.

**3:3.2.4.4** Absorbances.

Neutral red gave higher absorbances for both cell-lines, with the RTG-2 cell-line again giving lower absorbances than the L929 line. This is an advantage of the method, as it meant that the numeric differences between toxin absorbances and blank absorbances were larger, giving more confidence in calculations of growth inhibition. These high absorbances have been seen previously. Babich and Borenfreund (1990) found that the neutral red method gave twice the absorbance of the MTT method, but described the advantage as being an ability to use less cells.

**3:3.2.4.5** Negative inhibitions.

For malachite green and Nuvan, with both cell lines (and for ivermectin with RTG-2), there were a number of low concentration responses that were negative to some degree. In the original paper, Borenfreund and Puerner (1985) found < 0% inhibition with two out of five surfactants tested on 3T3 cells (Triton X1-555 and sodium lauryl sulphate).

While negative inhibitions can result from promotion of growth by low levels of toxins, and are seen with a number of different methods, there is a particular aspect of the neutral red method that should be noted as a possible cause. This is referred to as lysosomal swelling.

Riddel *et al.* (1986) state that, in theory, a chemical could cause an increase in the size or number of lysosomes without an increase in cell number. They relate this to the response to chloraquine sulphate (an anti-

malarial drug that increases the pH in lysosomes and is a known inhibitor), which they cite as an example of lysosomal swelling. In their trial, there was a higher toxicity indication for the drug from the neutral red assay than from the KBr assay.

However, this is not the effect swollen lysosomes would have, higher neutral red concentrations relative to the control (than would be seen with an equitoxic chemical that doesn't cause lysosomal swelling) would depress the $EC_{50}$. Therefore chloraquine sulphate, as described, is not an example of a toxin causing lysosomal swelling.

Babich and Borenfreund (1989) noted these negative inhibitions with a number of organochlorine pesticides, assessed using the BF-2 line and a Bluegill fin line. They also considered that xenobiotics can induce alterations in lysosomal membranes, causing an increase in lysosomal volume, creating giant lysosomes, and causing subsequent membrane destabilisation. They correlated this with the high lipophilicity of the organochlorine pesticides.

Negative inhibitions have also been found with other end-points, and have often been attributed to metabolism by the cells of low levels of toxin. Christian *et al.* (1973) found them with the L929 cell line using a protein determination for barium (-10 to -25%) and other chemicals (< -10%) and Kocan *et al.* (1979) found them with a cell counting assay for 2-aminofluorene in a study which demonstrated that RTG-2 cells in culture were capable of metabolising a number of mutagens and promutagens. Shopsis and Sathe (1984) found two compounds (allyl alcohol and sodium hypochlorite) caused a stimulation of uridine uptake (10-40%) at concentrations well below the $UI_{50}$ (concentration causing a 50% reduction in the uptake of uridine), but cause inhibition at higher concentrations (leading to a $UI_{50}$ determination). The $UI_{50}$s themselves were well below the $EC_{50}$ values (cytotoxicity).

### 3:3.2.4.6 Summary.

The main reason that the neutral red method is promoted as a better method than others, is that neutral red is a vital dye, taken up in the lysosomes of actively metabolising cells only. Thus, it is said to give a more subtle distinction between toxins than methods which dye all remaining cells in the well, irrespective of viability, such as the crystal violet method. This has nothing to do with its handling characteristics.

In comparing the two methods used here, solely on the handling of the protocols, the crystal violet method is easier to use, is shorter term, and is more reliable and reproducible. For these reasons, it is considered here (on the basis of a qualitative comparison only) to be more suitable for routine environmental analysis.

3:3.2.5 The 96 hr. fish acute toxicity test.

The fish acute toxicity test system is the longest established ecotoxicity test, and is the reference system for the production of aquatic animal $LC_{50}$ data. The importance of fish as representative ecotoxicity assay material should not be underestimated. Wolf and Mann (1980) point out that fish make up 50% of all vertebrates. Fish toxicity is, however, one of the systems that require the greatest degree of expertise and management, such as stock maintenance (Hunn *et al.*, 1968) and standardisation of test variables, like test water, oxygen, organism etc. (Burdick, 1967).

As with the algal growth system, there are a number of test variations, including the method of toxin application, and the standard species that can be applied. The system used here was a static batch system with non-replenishment, constant aeration, and using Rainbow trout (*Salmo gairdneri*). Rainbow trout were chosen as a direct comparison to the established RTG-2 fish cell line. Redesignation of the scientific name of the Rainbow trout has been described in section 3:2.5.2.1 (p. 60).

**3:3.2.5.1** Trial procedures.

In this study, an initial trial with Nuvan and ivermectin failed, as the (plastic) containers were too shallow, and there was high escapement overnight. Because of the space limitations, and as a published $LC_{50}$ was available for ivermectin, it was decided to end the ivermectin trial. The Nuvan trial was initially started in plastic, as there was no conclusive data on its non-attachment to glass. However, it was noted that a number of previous studies were carried out in glass. As glass containers would preclude escapement, and allow more concentrations to be used, the Nuvan trial was restarted in these. The malachite green trial had been initiated in glass, and was completed as same.

There were no problems with the operation of the test methodology in glass, although it should be noted that this was helped significantly by the opportunity to use dedicated fish toxicity facilities, where routine husbandry and test conditions have been long established.

The main limitations of the test are the small number of individuals, concentrations and replicates that can be applied. Also more monitoring (pH, oxygen and temperature) is required for the fish toxicity test than for any other ecotoxicity test. Thus, the time requirement is higher for the this test than for any other.

Also, large amounts of space and glassware were required, as well as a cold room and fish husbandry facilities. One advantage is that sub-lethal effects can be easily measured, to generate $EC_{50}$ data (effective concentration, specified symptoms).

**3:3.2.5.2** $EC_{50}$ determination.

From visual monitoring of Rainbow trout in contact with Nuvan and malachite green, two distinct mechanisms of toxicity were noted. The symptoms resulting from these mechanisms were used as the basis of an $EC_{50}$ index. The $EC_{50}$ index for Nuvan was convulsions, and for malachite green was lethargy. A quantitative measure of the difference between the $EC_{50}$ and the $LC_{50}$ is contained in section 3:3.3.5.2 (p. 113).

The complete sub-lethal effects of Nuvan were lethargy, convulsions and discolouration (darkening of the skin), and the sub-lethal effects of malachite green were agitation (at very low levels) followed by lethargy and senescence, and also darkening of the skin and 'coughing'.

Fish affected by Nuvan swam on their sides, in convulsions, often for a number of days, at the end of the trial, almost all of the fish remaining in tanks where there had been mortality were affected in at least one of these ways. Affected fish would gather at the bottom corners of the tanks. Dead fish showed bleeding from the gills. The number of fish showing these symptoms increased over the four days, it is unlikely that the fish remaining in the two highest concentrations (0.56 and 1.00 mg/l) would have survived for long.

Fish affected by malachite green initially became agitated, for a short while, and then became very lethargic. They would go to the bottom of the tanks, occasionally rising to the surface 'gasping'. All of the fish affected by malachite green showed these symptoms after 24 hours. Some became progressively worse and died, but most were in the same condition after 96 hours as they were after 24 hours (with the exception of those in the middle concentration, 0.32 mg/l).

**3:3.2.5.3** Summary.

The fish acute toxicity trial produces data that is directly relevant, through using a species that is likely to be affected by the discharge of a chemical or effluent. It is also capable of producing quantifiable sub-lethal data.

However, considering the test from a handling point of view, extensive equipment and expertise resources are required, which limit quite severely the amount of data that can be generated. Thus, only a small number of institutions are capable of carrying out extensive fish toxicity studies. In relation to environmental monitoring in particular, individual manufacturers, or local agencies do not have these facilities, and the testing of effluents etc. is limited to central agencies.

For these reasons, it is not considered to be useful for routine environmental analysis.

3:3.2.6 The 96 hr. algal growth inhibition test.

A freshwater algal growth inhibition test is a base-set test recommended by the OECD and required by the EC for Level 1 chemicals (i.e. > 100t/yr. produced {Nyholm and Kallqvist, 1989}).

It is the only standard ecotoxicity system which incorporates aspects of cell culture systems, such as large, homogenous test populations. The methodology is long established, and there are several types of test system, including static or shaken systems, a number of standard species, and a number of growth monitoring methods. The test system used here was a static system, using *Chlorella vulgaris* and growth monitoring by absorption.

In this study, apart from the fish toxicity trial, the algal growth inhibition test required the greatest amount of resources in terms of glassware and other equipment. However most of this glassware and equipment would be found in any general laboratory.

An initial growth-cycle trial was carried out on the three OECD standard species, *Scenedesmus subspicatus*, *Selenastrum capricornutum* and *Chlorella vulgaris*. *Scenedesmus* was the most highly coloured of the three. However, under the culture conditions in use, the cells clumped, and so were limited in their application in measurement of cell number by absorbance. *Selenastrum* grew well, but did not absorb significantly at any point in the visible spectrum. *Chlorella vulgaris* grew well, and, though small, could be easily counted by haemocytometer.

An absorption peak was found at 689.6nm (the λ max. of chlorophyll). Following absorption experiments (section 3:3.3.6, p. 115), which established a correlation between algal cell number and concentration, and which established that the toxins do not absorb at the chosen wavelength, absorbance was chosen as the preferred growth measurement method. From the correlation curve between absorbance and cell number, a conversion equation was established, and this was used to calculate cell number, specific growth rate, and thus percentage growth inhibition (calculated by graphical interpolation).

Nyholm (1985) concluded that specific growth rate was a better response variable than biomass, because $EC_{50}$ figures estimated from growth rates are less dependant on particular test parameters. The OECD recommend that, when growth inhibition is calculated in this way, the times used should be incorporated into the designation of the $EC_{50}$. In the case of this study, the recommended designation would be $EC_{50}(48\text{-}96)$.

Walsh *et al.* (1982), in analysing the toxicity of 39 chemicals over 187 tests, and using 4 calculation methods, doubt whether traditional statistical methods (e.g. moving average and probit analysis) would be applicable to data from algal growth inhibition tests. This is because statistical methods for quantal data (as produced in whole animal trials) are based on analysis of tolerance distribution for individual test organisms. Algal data, on the other hand, is quantitative, and based on a large number of identical individuals (Nyholm and Kallqvist, 1989). The results obtained by Walsh *et al.* were essentially the same for the four methods examined, and so they recommended the use of graphical interpolation in algal tests. This maintains consistency, as graphical interpolation was the method of median toxicity determination used for all other systems in this study.

The test methodology was straightforward, except the requirement for < 0.1 ml/l of organic solvents in the media. As this was 0.01% v/v, it meant that the concentration of ivermectin (as Ivomec) that could be applied was severely limited. In other test systems, solvent concentrations of up to 1% v/v are allowed.

Another limiting factor, though not so in this study, would be the concentration at which a substance would cloud the media, and reduce light penetration. This could decrease growth without causing direct toxicity, and could reduce the applicability of absorbance as a growth assay.

The main limitations of the test were the amount of space required per number of toxins and per number of replicates, and the time requirement was higher than for most other tests.

These would be considered severe limitations, in relation to the volume of data that could be generated. For these reasons, it is considered here that the usefulness of algal growth inhibition tests in routine environmental analysis, using the OECD protocol, would be limited.

3:3.2.7 The *Artemia* immobilisation test.

Although *Artemia* have been used for some time in toxicity testing, there is no regulatory standard method, and the toxicological principles of the test are applied in a number of varied protocols. Examples include; Foster and Tullis (1984) where 25 nauplii per 125 ml volume in Erlenmeyer flasks were used, and the flasks were illuminated during the trial; Kissa *et al.* (1984), where 50/100 ml glass tubes were used; Abernathy *et al.* (1986), where 33 ml phials were used. In this study, the method used was that of the University of Ghent, which is being promoted as a standard methodology.

*Artemia salina* cysts are readily available, and so a sufficient harvest can be guaranteed. Hatching success was high, and the nauplii were highly phototactic, which made collection and handling efficient. In place of the recommended Pasteur pipette, a handling device was made up from a multichannel pipette tip attached to a 10 ml pipette, with the pressure controlled by a 10 ml pipette pump. This gave better control over the collection and dispensing of the nauplii.

The nauplii were robust and very active, making the interpretation of toxic effects very clear. If more concentrations were routinely applied, it would be possible to use the sub-lethal symptoms as the basis for the calculation of $EC_{50}$ data. In this study, the surviving nauplii in the lowest concentrations of Nuvan and ivermectin applied were sluggish, and swimming less randomly. This would have made them less ecologically effective, and more susceptible to predation. For malachite green, the surviving nauplii at the lowest concentration applied were unaffected. As the current protocol uses only five concentrations, the point at which sub-lethal effects disappeared was not seen for Nuvan or ivermectin.

Overall, there were no logistic problems in the operation of the Ghent University protocol. The use of transfer wells was of particularly obvious benefit, dispensing the required number from these to the toxin

wells was a very practical and effective option. The apparatus described above worked well, increasing efficiency of nauplii transfer.

In summary, and from the point of view of handling characteristics, the *Artemia salina* protocol worked well. It uses a species which could be affected by the discharge of materials to the environment. It could be used to generate large amounts of data quickly, and it is low cost. For these reasons, it is considered to be useful in routine environmental analysis.

3:3.2.8 The *Brachionus* immobilisation test.

There are also a number of different methods also currently being developed for the use of rotifers in ecotoxicity trials. Examples include Serrano *et al.* (1986) who used 0.5 ml in glass phials (5 individuals) under light for *Brachionus plicatilis*, (marine), and fed them *Tretaselmis* spp. during the trials; Snell and Persoone (1989) used 24 well plates, and a 1 ml volume for *Brachionus plicatilis* and *Brachionus rubens* (freshwater). There was no feeding, and the trials were conducted in the dark. Wide variations in hatching success were reported for *Brachionus rubens*.

The cysts used in this study were obtained in kit form, from the University of Ghent (*Brachionus calyciflorus*). They did not hatch as well as the *Artemia*, and therefore, on some occasions, sufficient nauplii were not available for the number of trials that one phial is purported to cater for. There were, however, sufficient numbers of individuals in the phials for the overall number of trials required, as ten individuals per well used in the range-finding, and eight per well in the toxin trials.

They are not positively phototactic, and cannot be concentrated in the same way as *Artemia*. They are, however, very slow, and are collected by concentrating them in a smaller volume of water. This inherent lack of activity made interpretation of toxic effects somewhat more difficult, particularly any calculation of $EC_{50}$ data from sub-lethal symptoms.

The rotifers were less than one quarter the size of *Artemia*, and therefore required more delicate handling. The bore of the multichannel pipette tip, as used in the *Artemia* trials, was too big for effective handling. As an alternative, the tip of a glass Pasteur pipette was stretched as thinly as possible, and a 2 ml pipette pump was used for pressure control.

The purpose-made plates recommended in the Ghent protocol were not available, and so 24-well plates were used. These have been used in other rotifer trials, and require a higher well volume (1.0 ml) than used in the Ghent protocol (0.5 ml) to give sufficient liquid depth in the well. This

was, nonetheless, half the volume used in the shrimp trials, and, coupled with the greater difficulty in collecting and dispensing the nauplii, resulted in greater dilution of the toxins, relative to the *Artemia* trials.

Overall, the handling of the protocol was somewhat more difficult than with the *Artemia salina* test, but it was still relatively straightforward. Rotifer cysts are not as widely available as those of the brine shrimp, and are being sold as dedicated toxkits, which are relatively expensive. That notwithstanding, it offers many of the same advantages, and has many of the same limitations, as the *Artemia* test. For these reasons, it is considered here, from the point of view of handling characteristics, to be useful in routine environmental analysis.

3:3.2.9 The *Streptocephalus* immobilisation test.

No further information was available on the use of *Streptocephalus* in ecotoxicity trials, and so no reference can be made to previous experience with the test methodology.

A small quantity of *Streptocephalus proboscideus* cysts were obtained, also in kit form, from Ghent University. The amount contained should have been enough to carry out three replicates of each trial, with sufficient material to repeat any failed trials. However, *Streptocephalus* hatching was significantly less successful than that of *Artemia*. Also, they were far less phototactic, but just as active as *Artemia*, which made their handling the most difficult of the three invertebrate methods.

As a result, it was only possible to carry out duplicate trials for Nuvan and malachite green, and no trial was completed for ivermectin. The range finding tests for Nuvan and malachite green indicated a useful toxic range, and the trials were based on these. The operation of the test method was similar to that of the *Artemia* test, but there was significantly more dilution of the toxin wells because of the difficulties in handling the nauplii.

The concentrations used for the ivermectin range finding trial were based on the reported lower sensitivity of *Artemia* than *Daphnia* (i.e. suggesting that shrimp were a less sensitive group). However, all the specimens died at the lowest concentration applied (0.1 mg/l), with no blank mortalities. In the next, extended trial, 100% were also dead at the lowest concentration applied (1.0 µg/l), however there was 50% mortality in the blank. This meant that the result could not be used. At that point, no more nauplii were available.

As a result of these difficulties, the data presented here is regarded as tentative, and therefore is not considered sufficient for the designation of $IC_{50}$ concentrations. It is presented here in order to provide a comparison of the methodology, and an assessment of the usefulness of the test system, in comparison to the other systems used.

3:3.3 Quantitative results - individual systems.

The quantitative results from the predictive hazard assessment trials are presented in two sections. The first, below, examines the results from each system in isolation, from the points of view of absolute sensitivity and reproducibility. The second, section 3:3.4 (p. 122) compares the relative sensitivity of the various systems statistically.

3:3.3.1 Overall toxicity data.

The median toxicity data for each of the species and test methods used are presented in Table 3.7. The data files for these results are contained in appendix 8.2, as follows; L929 cell line, Tables 8.1-8.18, appendix pages iv-vi; RTG-2 cell line, Tables 8.19-8.36, appendix pages vi-viii; *Artemia salina*, Tables 8.37-8.45, appendix pages ix-x; *Brachionus calyciflorus*, Tables 8.46-8.54, appendix pages x-xi; *Streptocephalus proboscideus*, Tables 8.55-8.58, appendix page xi; algal growth inhibition, Tables 8.59-8.65, appendix page xii; fish acute toxicity, Tables 8.66-8.69, appendix pages xiii-xiv.

The data presented are from a minimum of three replicate trials, with some exceptions, as noted (i.e. only one fish toxicity and two *Streptocephalus* trials were possible). These are presented accordingly.

It can be seen from Table 3.7 that, to Nuvan, the most sensitive species was Rainbow trout, and the least sensitive was *Chlorella*. The RTG-2 cell line was slightly less sensitive than the L929 line. *Artemia* was slightly less sensitive than the RTG-2 cells. The sensitivity of *Streptocephalus* was close to that of Rainbow trout. *Brachionus* sensitivity was between those of Rainbow trout and cultured cells. The nine test systems gave median toxicity concentrations within a range of two orders of magnitude, and, when excluding algae, the eight animal systems gave indices largely within one order of magnitude.

To ivermectin, the most sensitive species was *Artemia*, with an almost equal index from Rainbow trout. *Streptocephalus* is likely to be highly sensitive also. The least sensitive species was again *Chlorella*, to which ivermectin would be classed as non-toxic. There was no significant

difference between the cell lines. *Brachionus* sensitivity was slightly less than that of the cultured cells. The eight test systems to which ivermectin was toxic differed in sensitivity by three orders of magnitude, the widest range found in the study. This is in keeping with the wide range of sensitivities of target organisms, attributed to the number of sites of action that ivermectin has by Campbell (1985).

**Table 3.7**
**Overall median toxicity data for all ecotoxicity test methods and all toxins**

| Test System | Toxin | | | | | |
|---|---|---|---|---|---|---|
| | Nuvan (mg/l) | S.D. | Ivermectin (mg/l) | S.D. | Malachite green (mg/l) | S.D. |
| L929 (c. violet) | 5.31 | ± 2.11 | 1.73 | ± 0.50 | 0.195 | ± 0.053 |
| L929 (n. red) | 5.90 | ± 1.94 | 2.75 | ± 0.46 | 0.242 | ± 0.085 |
| RTG-2 (c. violet) | 9.56 | ± 1.32 | 2.25 | ± 0.28 | 0.023 | ± 0.006 |
| RTG-2 (n. red) | 9.45 | ± 2.27 | 3.30 | ± 1.38 | 0.020 | ± 0.004 |
| Algae | 79.9 | ± 10.2 | <0+ | -- | 5.97 | ± 3.25 |
| *Artemia* | 12.92 | ± 0.64 | 0.0027 | ±.0002 | 0.710 | ± 0.032 |
| *Brachionus* | 3.69 | ± 0.51 | 2.50 | ± 0.00 | 0.524 | ± 0.049 |
| *Streptocephalus* | 0.743 | ± .001 | <0.10* | -- | 0.557 | ± 0.12 |
| Rainbow trout | 0.512 | -- | 0.003" | -- | 0.297 | -- |

+ = Net promotion (to 1.0 mg/l). * = No $IC_{50}$ obtained (100% death at 0.10 mg/l).
" = Published data (Halley *et al.*, 1989). *Streptocephalus* results tentative.

The system most sensitive to the toxic effects of malachite green was RTG-2 cells in culture. This was the only occasion that RTG-2 cells were significantly more sensitive than L929. L929 cells gave a toxicity index similar to that of Rainbow trout. *Chlorella* was again the least sensitive species. The most sensitive whole organism species was Rainbow trout, with the invertebrate species all giving indices in the same range, which lay between that of L929 cells and *Chlorella*. The nine test systems gave toxicity indices within two orders of magnitude. As was the case with Nuvan, if *Chlorella* is excluded, this range was reduced to one order of magnitude.

The lack of sensitivity of the algal system is the only significant trend which can be seen in this data. No animal system, either *in vivo* or *in*

*vitro* is consistently the most sensitive, or occupies a consistent position in relation to the sensitivity of the other methods. However, the majority of systems (with the exception of the ivermectin batch) gave toxicity indications largely within one order of magnitude. The same trend is to be found in sub-groups, such as the cell culture data, or the invertebrate data.

Rainbow trout *in vivo* can be seen to be particularly sensitive, although it is the most sensitive on only one occasion. Significantly, the largest gap between the Rainbow trout and the most sensitive species for any chemical is with malachite green, where the RTG-2 cells are one order of magnitude more sensitive.

3:3.3.2 The L929 cell line.

**3:3.3.2.1** Sensitivity.

Although the L929 cell line is of mouse adipose/areolar tissue origin, and the extrapolation of data to non-mammalian species is a cited as a weakness of the system, the sensitivity of the cultured cells was, in the majority of cases, within one order of magnitude of the most sensitive system used (Table 3.7). In all cases, the L929 cell line was more sensitive than the algal growth inhibition test.

In four out of nine cases, it was more sensitive than the invertebrate systems, (to two of the others, *Brachionus*-Nuvan and ivermectin, it was only slightly less sensitive).

It was least effective in indicating toxicity to Rainbow trout, being one order of magnitude less sensitive to Nuvan, three orders of magnitude less sensitive to ivermectin, but slightly more sensitive to malachite green.

The relatively good sensitivity to the organophosphate is of particular note, as the major mode of action of the pesticide is AChE inhibition, which, theoretically, should not cause toxicity in cultured cells. These results indicate that there is a secondary, but significant toxic mechanism of dichlorvos, which affects the cells in culture.

Other non AChE effects have been seen previously, but have not been extensively discussed. These include teratogenic effects, unrelated to AChE activity, which have been seen with a number of organophosphates. Also, cell specific effects have been noted in *in vivo* histopathological studies, including vacuolisation of cytoplasm, enlargement of nuclei, and rupturing of cell membranes (Hassal, 1990).

Rodrigues and Mattei (1987) showed the toxicity of ivermectin to two other mammalian cells, CHO and IB-RS-2 (pig kidney) to be reduced by serum (as shown by substitution with Ultroser G). A number of other studies have shown the toxicity of materials known to bind to protein (e.g. metals) to be reduced by serum in media (sections 3:2.3, 4:2.1, pp. 47, 132). However, Shopsis and Sathe (1984) make the point that such binding could, in theory, also increase the toxicity of insoluble materials by acting as a transport mechanism across cell membranes during metabolism of the serum.

Halley *et al.* (1989a) also found that ivermectin binds strongly to organic matter. Thus, serum binding could be a factor in the low sensitivity of the L929 cell line, relative to Rainbow trout and two of the invertebrates. In a full toxicity testing programme, $EC_{50}$ data would be interpreted in the light of SAR data, and wider safety margins applied, where necessary.

Few other studies have used the L929 cell line. Those that have, found a range of sensitivities. Christian *et al.* (1973) found significant inhibition to L929 cells at 1 order of magnitude above the Maximum Allowable Concentration (MAC) for arsenic, lead, silver and cyanide. They also demonstrated antagonism between cadmium and zinc. Kfir and Prozesky (1981) found LM cells to be twice as sensitive as the fish avoidance test to activated sludge effluents.

### 3:3.3.2.2 Intra-trial reproducibility

One of the main limitations of whole animal trials is the small number of individuals used, and the poor statistical strength that results. With cell culture tests, there are c. $10^6$ cells in each of 96 wells, and a number of replicates at each concentration (8 in this study). This, immediately, gives a higher significance to any result obtained.

There are, however, a number of possible sources of variation. The most prominent is inherent biological variation, which is a feature of any biological experiment, and which cannot be completely eliminated. There are also a number of steps in the protocol, such as seeding, toxin application, eluant application and instrument variation which could lead to operator error as a source of variation. The following is a discussion of the observed variation, in relation to the performance of the L929 cell line in predictive hazard ecotoxicity trials.

The individual results (intra-trial) for the various trials are presented in appendix 8.2 (appendix page iv), as percentage inhibition per toxin concentration, and the replicate standard deviation (% inhibition) for

each point. In the case of cell culture, there were eight replicates per concentration, in the case of the invertebrate trials, and the algal trials, there were three replicates per concentration. In the case of the fish trials, due to limitations on space and other resources, there were no replicates.

Overall, as can be seen in the appendix tables, intra-trial variation in cell culture trials was low. There are two points of note from these results. The first is that, throughout the trials, toxin concentrations which registered above c. 20% inhibition had very low column standard deviations (average 3.34%, as % inhibition, n=71). At concentrations where inhibition was less than c. 20%, variation was, on average, higher (average 6.10%, n= 39). The second is that there was no regular 0% inhibition response, and, on occasion, low toxin concentrations registered negative inhibitions (i.e. promotion of growth).

An example of these trends is given in Figure 3.1. The data for the Figure is from appendix Tables 8.12 (malachite green, neutral red-3) and 8.16 (Nuvan, neutral red-1). The tables are reproduced below, for direct comparison with the Figure.

**Table 3.8**
**The toxicity of malachite green and Nuvan to L929 cells (neutral red assay)**

M. Green *

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.050 | 1.54 | 6.55 |
| 0.075 | 8.52 | 5.15 |
| 0.100 | 13.71 | 3.74 |
| 0.250 | 39.63 | 2.62 |
| 0.500 | 95.68 | 0.97 |
| 1.000 | 99.65 | 0.21 |

* = Appendix Table 8.12.

Nuvan +

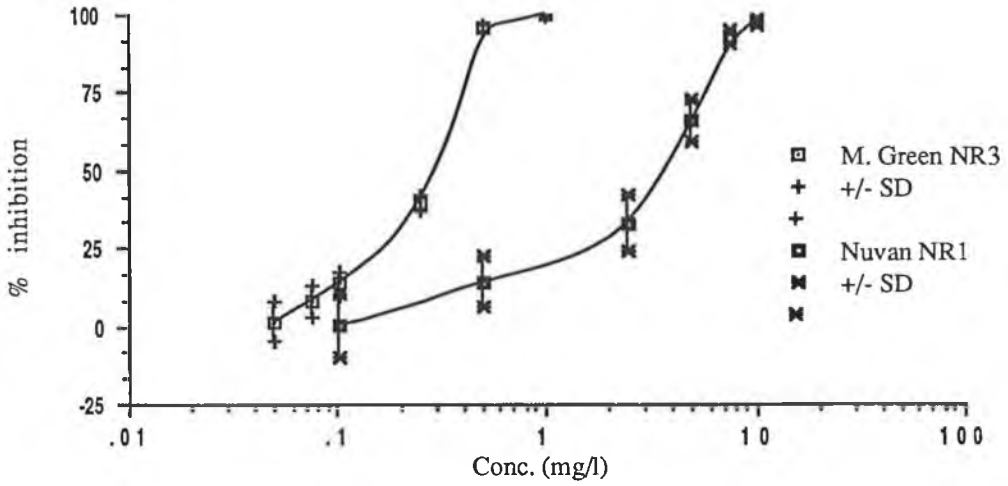
| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.42 | 10.31 |
| 0.50 | 14.35 | 8.05 |
| 2.50 | 33.17 | 8.75 |
| 5.00 | 66.25 | 6.82 |
| 7.50 | 92.61 | 2.24 |
| 10.00 | 97.31 | 1.05 |

+ = Appendix Table 8.16.

The malachite green data is from a typical data set, with standard deviations of up to 6.5% (inhibition), the Nuvan data is from a data set with some of the highest standard deviations seen in the study, of 8-10%.

**Figure 3.1**

**The toxicity of malachite green and Nuvan to L929 cells**




As can be seen from the Figure, the highest standard deviations are seen at the lowest concentration of toxin. Given that the Nuvan data set contains some of the highest standard deviations seen in the study, it can be seen that these are not excessive. Negative inhibitions also occur at low concentrations of toxin.

It is unlikely that this variation is caused by uneven seeding of cells, as it would have to be consistently in the low concentration set of wells alone. As the cell sheets in these wells are likely to be confluent, or near confluent, at the end of the incubation period, it is possible that it was due to uneven retention of dye by confluent sheets of cells (i.e. contact inhibition causing reduction of cell permeability).

This level of intra-column variation, and higher in a number of cases, has been seen in a number of other studies, and has been regarded as acceptable. Examples include Richardson *et al.* (1977), Cody *et al.* (1979), Marion and Denizeau (1983a,b), Babich *et al.* (1986), Knox *et al.* (1986), Benford and Good (1987), Hunt *et al.* (1987), Babich and Borenfreund (1987, 1990), Brandao *et al.* (1990).

It should be noted that the variation seen with the cell culture trials, are either comparable, or, more often, significantly better than those seen in whole animal trials (Kuwabara *et al.*, 1980; 10-20% for *Artemia.*, Blust *et al.*, 1986 and Pandey and MacRae, 1991).

Negative inhibitions have also been found by a number of workers, but have not previously been discussed in isolation. Christian *et al.*

(1973) regarded < 5% inhibition as no effect, and Richardson *et al.* (1977) regarded < 15% as being no effect. Cody *et al.* (1979 regarded a range of -5% to +5% inhibition as no effect. Others include Hunt *et al.* (1987), Babich and Borenfreund (1987, 1990).

Overall then, the level of intra-trial variation is considered to be acceptable. The protocols applied in this portion of the study included application of cells and chemicals with a 250µl capacity pipette (requiring a fill for each column of the plate), and manual mixing of the plates. It is probable that automation, which is available for almost all steps of a cell culture cytotoxicity test, would reduce variation further.

**3:3.3.2.3** Inter-trial reproducibility.

Inter-trial variation is also of importance, and these figures are presented in Table 3.7, section 3:3.3.1 (p. 104), as the standard deviation of triplicate (except where otherwise stated) experiments.

The variation seen, compares favourably (and is better in many cases) with that seen in a number of other studies (including many of those cited in 3:3.3.2.2), such as Sanders and Cope (1966), Butler *et al.* (1980), Kissa *et al.* (1984), Khangorot and Ray (1987), Nikonenko (1987), Ortego and Benson (1992), Persoone *et al.* (1993).

Knox *et al.* (1986), when discussing a large multicentre trial aimed at validating *in vitro* methods, state that a twofold difference in values around the mean is acceptable. The reproducibility achieved here was significantly better than that.

Low inter-trial reproducibility is a feature of whole animal toxicity tests, and is one of the main problems that needs to be addressed by the introduction of new test methods. For example, an EPA listing of $LC_{50}$ results for the toxicity of diazinon was examined. In 18 measures of the acute oral toxicity to Sprague Dawley rats, the range of values cited was from 100 mg/kg to 8,200 mg/kg. The average was 3,448 mg/kg, and the standard deviation was $\pm 2614$.

As the measure of the usefulness of cell culture methods is whether it represents an improvement on the existing systems, or not, it is considered here that the level of inter-trial variation seen with the L929 cell line in this study is acceptable.

**3:3.3.2.4** Summary.

From the results, it can be seen that the L929 cell line can be classed as sensitive to the toxins tested, which is particularly significant when considering that two of the toxins act, primarily, on the nervous system. The high number of individuals applied in each well, and the high number of replicates per toxin concentration are particular advantages of cell culture system. This is reflected in the low intra- and inter-trial variation. An improvement in the statistical strength of environmental bioassays is one of the major objectives in research into new test systems.

For these reasons, and recalling the quantitative results, it is considered here that the L929 cell line would be useful in routine environmental monitoring.

3:3.3.3 The RTG-2 cell line.

The RTG-2 cell assay is directly comparable to the Rainbow trout test, and offers an indication of the effect of extrapolation between a cellular system and a whole organism system. Its use also offers a comparison between a fish cell line (i.e. from a species likely to be affected by environmental discharges), and a mammalian cell line, as well as a comparison between cells of different organ origin.

**3:3.3.3.1** Sensitivity.

As can be seen in Table 3.7 (p. 104) the RTG-2 cells were slightly less sensitive than L929 to Nuvan and ivermectin, and one order of magnitude more so to malachite green.

The most significant result is its sensitivity to malachite green, which was over one order of magnitude higher than any other system. This is particularly interesting when considering the use of malachite green in fish farming. It may be related to the gonadal origin of the cells, Meyer and Jorgenson (1983) found the toxicity of malachite green to Rainbow trout ova to be high, and to lead to deformities in surviving hatchlings.

The range of toxicity of malachite green to a number of fish species, as seen in Table 3.4 (p. 80) extends from c. 0.03 mg/l to 0.3 mg/l. It is used therapeutically at 1 mg/l for 1 hour, but studies have shown that variation in environmental conditions from the ideal causes significant toxic effects at this concentration (section 3:2.8.3, p. 79). The sensitivity of RTG-2 cells reflects this high *in vivo* sensitivity well.

Overall, however, the measured sensitivity of the RTG-2 cell line in this study exhibited many of the same trends as the L929 cells. As with the L929 line, RTG-2 cells were always more sensitive than the algal cells. Also, in the majority of cases, the RTG-2 sensitivity was within one order of magnitude of the whole animal systems, and it was more sensitive in 5 out of 9 cases than the invertebrate systems.

Also similar to the L929 cell line, the least effective indication given by RTG-2 cells was its relationship to the Rainbow trout *in vivo* toxicity. It was more than one order of magnitude less sensitive to Nuvan, and three orders of magnitude less sensitive to ivermectin. As noted, the low toxicity of ivermectin to cultured cells, relative to Rainbow trout toxicity, may be related to serum binding, and would be interpreted as such in a full toxicity testing programme.

A number of other studies have found varying sensitivities for the RTG-2 cell line. Kocan *et al.* (1979) found RTG-2 to be just as sensitive to mutagens as a human cell line, despite the temperature difference. Marion and Denizeau (1982a) found RTG-2 cells to be more sensitive to lead than the human skin epithelial cell line, NCTCC-2544. This sensitivity was unaffected by serum. However, the opposite was the case for cadmium (1982b). Walton *et al.* (1983) found RTG-2 response to four mutagens to be similar to that of RTO, CHO and FHM cells.

Other fish cell lines have also shown variable sensitivities. Rachlin and Perlmutter (1968) found FH/M cells to be more sensitive than intact Fathead minnow. One tenth of the TLm had no effect, and this was considered to be a better safety margin. Babich *et al.* (1989) found BF/2 and BF/G cells to be 1-2 orders of magnitude less sensitive than two mammalian cell lines to selenium compounds.

**3:3.3.3.2** Reproducibility.

The intra- and inter-trial variation profiles of the RTG-2 cell line were very similar to those of the L929 cell line. With intra-trial variation in particular, inhibitions above 20% had lower standard deviations than those below 20%, as before. However, on average, the degree of variation in both categories was higher than with L929 (above 20% average = 5.16%, n = 82; below 20% average = 7.53%, n = 23). That notwithstanding, it is still considered to be not excessive.

**3:3.3.3.3** Summary.

Overall, the RTG-2 cell line performed, in many respects, in a very similar manner to the L929 cell line. While there was an overall higher level of variation, there was also a marked higher sensitivity to malachite green, relative to all other test system used.

On balance, and taking into account the handling characteristics of the RTG-2 cell line (section 3:3.2.2, p. 87), it is considered here that the assay can be used effectively in routine environmental analysis. However, it is also considered that it doesn't offer any significant advantages over the L929 cell line. As noted previously, the two are not exclusive, this conclusion is in relation to the systems used in the study.

3:3.3.4 The cell culture end points.

An analysis of the operation of the end points is inherent in the analysis of the cell lines above, and in the qualitative results. A discussion of the qualitative results for the end points in isolation is not, therefore, appropriate. They are further discussed in section 3:3.4.1 (p. 122), when comparing the results from cell culture systems statistically.

3:3.3.5 The 96 hr. fish acute toxicity test.

The high sensitivity of Rainbow trout to many aquatic toxins has been well documented (Davies *et al.*, 1976; DeGraeve *et al.*, 1980; Phipps and Holcombe; Khangorot and Ray, 1987). Of particular note is the importance of hepatic biotransformation to toxicity of xenobiotics (Pedersen *et al.*, 1976; Lech and Bend, 1980).

Interpretation of data from fish acute toxicity trials is dependant, in many cases, on the particular operating conditions of the trials. In this study, the average fish weight was 1.75g (n = 60), therefore 20 litre test volumes were used to keep the fish loading under the 1 g/l limit permissible under the OECD protocol (OECD, 1984). The average temperature in the fish tanks was 12 $^{o}$C. While this is one degree below the 13 $^{o}$C minimum recommended for the species in the OECD protocol, fluctuations did not exceed the +/- 1 $^{o}$C deviation from the average, as required by the OECD.

Also, other investigators have recommended a temperature range for rainbow trout which has 12 $^{o}$C as its minimum (ASTM, 1980), and other studies have used test temperatures as low as 11 $^{o}$C, and below (Davies *et al.*, 1976; Pedersen *et al.*, 1979). The importance of the effect of test temperature on fish toxicity is well documented, and the metabolism of

xenobiotics is known to be greatly enhanced at elevated temperatures (Girer, 1990). This can be either a toxifying or a detoxifying mechanism (Buhler and Williams, 1988).

The average pH in the tanks was 7.7 for both chemicals. The permissible range of 6.0 - 8.5, as required by the OECD, was not exceeded at any time. The oxygen regimes for the Nuvan and malachite green were slightly different throughout the trial, but on no occasion was the 60% saturation minimum breached (also as required by the OECD).

**3:3.3.5.1** Sensitivity.

Rainbow trout acute toxicity, as measured in this study, was consistently among the most sensitive of the systems used. To Nuvan, it was the most sensitive system of all. The measured $LC_{50}$ was similar to that found for a range of other fish (0.17 - 11.6 mg/l dichlorvos, Table 3.2, p. 70). *Artemia* was slightly more sensitive to ivermectin, but Rainbow trout were more sensitive than all remaining systems.

The concentrations that had been used for ivermectin were from 0.56 µg/l to 10 µg/l. It was noted on day 0, four hours after the initiation of the trial, that all of the fish in the ivermectin containers were grouped around the air stones. Oxygen levels at the time were over 90% saturation.

The most notable result was with malachite green, where both cell lines were more sensitive than Rainbow trout. L929 was slightly more sensitive, but RTG-2 was one order of magnitude more sensitive. The measured Rainbow trout $LC_{50}$ for malachite green in this study (0.297 mg/l) was very close to the published $LC_{50}$ (0.248 mg/l), which is in the upper section of a range of 1 order of magnitude found by Bills *et al.* (1977) for 10 fish species (Table 3.4, p. 80).

**3:3.3.5.2** Calculation of $EC_{50}$.

While the historic toxicity index is death (an easily measurable parameter), there are a number of conditions which would reduce the ecological effectiveness of an organism, and lead to death by predation etc. (Dagani, 1980). Calculation of an $EC_{50}$, or Effective Concentration (effective being defined by the particular index used), allows more subtle interpretation of sub lethal effects, which can often be expressed at concentrations far below those causing death.

In this study, it was possible to calculate $EC_{50}$ data from such a parameter for Nuvan and malachite green. This was the observed symptoms

of toxicity, which were convulsions with Nuvan, and lethargy with malachite green. These symptoms were monitored at a number of time intervals, in order to establish the extent of differences between sub-lethal toxicity and lethal toxicity, and the rate at which toxicity occurred. The data for these two measures are contained in appendix Tables 8.66/67 (malachite green), appendix page xiii, and appendix Tables 8.68/69 (Nuvan), appendix page xiv. The resulting $LC_{50}/EC_{50}$ data are presented in Table 3.9.

**Table 3.9**
**Rainbow trout toxicity: $LC_{50}$ vs. $EC_{50}$**

| Time (hrs.) | Nuvan $LC_{50}$ (mg/l) | $EC_{50}$ (mg/l) | Malachite green $LC_{50}$ (mg/l) | $EC_{50}$ (mg/l) |
|---|---|---|---|---|
| 1 | > 1.00 | > 1.00 | > 1.00 | > 1.00 |
| 3 | > 1.00 | > 1.00 | > 1.00 | > 1.00 |
| 24 | > 1.00 | 0.56 | 0.380 | 0.320 |
| 48 | 0.89 | 0.283 | 0.360 | 0.292 |
| 72 | 0.76 | 0.283 | 0.320 | 0.292 |
| 96 | 0.512 | 0.283 | 0.297 | 0.292 |

> 1.00 = no mortality at the highest concentration applied, 1 mg/l.

Nuvan, as assessed by both indices, exerted progressive toxicity over the four days. There was significant sub-lethal toxicity expressed within 24 hours, at which time there was no mortality. The expression of sub-lethal toxicity peaked at 48 hours, and remained at that for the rest of the trial. The $LC_{50}$ indication increased at a more regular rate.

The four $EC_{50}$ indications of toxicity for Nuvan are significantly lower than those from the $LC_{50}$. A point of note is that the 96 hr. $EC_{50}$ for Nuvan is lower than either indication for malachite green, which would classify it as more toxic, according to this end point. This indicates that Nuvan has a significant sub-lethal toxicity, which would have implications for the setting of environmental limits. The $LC_{50}$ data would have to be used with extreme caution, and a more sensitive index would be better.

Malachite green exerted a progressive toxicity also (both indices), but the rate of increase is lower than with Nuvan. The majority of the $LC_{50}$ indication was expressed within 24 hours. The majority of the $EC_{50}$ indication was also expressed in the first 24 hours. However, unlike Nuvan, both indices for malachite green gave broadly similar indications of toxicity

over the four days, with almost identical values after 96 hours. This indicates that there is not a high sub-lethal toxicity with malachite green.

These sub-lethal effects are particularly interesting, when considering the morphological differences in the responses of the RTG-2 cell line to Nuvan and malachite green, as discussed in section 3:3.2.2.4 (p. 91).

### 3:3.3.5.3 Summary

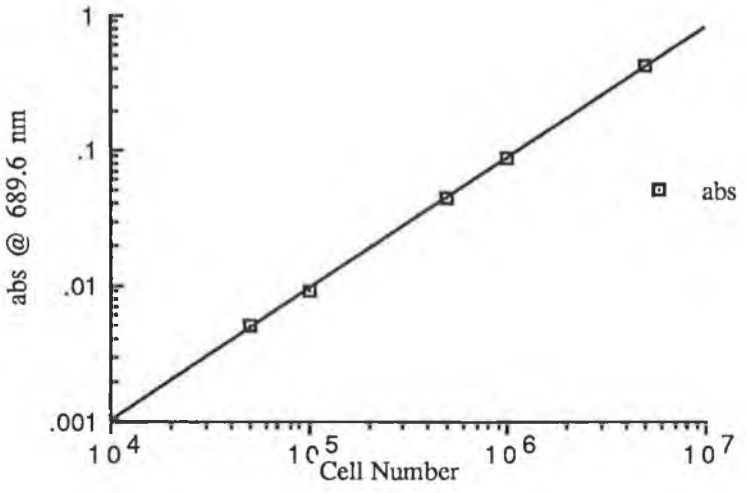
The 96 hr. fish acute toxicity test is one of the most widely used, and accepted, ecotoxicity test methods. It has proven sensitivity, and can generate sub-lethal data easily. However, its capacity to generate large volumes of data is severely restricted by the extensive resources required to carry out the tests effectively.

Despite its high sensitivity, as measured in this study, it is considered here to be of limited value in routine environmental analysis.

### 3:3.3.6 The 96 hr. algal growth inhibition test.

A growth-curve experiment was carried out at a number of seeding densities, using both shaken and static systems. This also facilitated a correlation between haemocytometer counts and absorbance readings. From this, a static system was chosen, with a seeding density of 1 x $10^5$ cells/ml. A correlation of 0.9998 was obtained between cell number and optical density at 689.6 nm (Figure 3.2), therefore absorbance was chosen as the growth measurement method.

**Figure 3.2**
**Correlation of algal cell number with absorbance at 689.6 nm**



The absorbance spectra of the toxins were scanned. Nuvan does not absorb significantly at any point in the visible range, however, it begins to precipitate in algal medium at concentrations over 100 mg/l. Malachite green has two absorbance peaks in the visible range, one minor peak at 424.4 nm, and a major peak at 617.6 nm. It does not absorb at 689.6 nm. The colour of the dye is first visible to the naked eye in algal media at 10.0 mg/l, and at 100 mg/l the media is dark blue. The highest concentration possible for ivermectin was 1.0 mg/l, as no more than 0.1 ml/l of an organic solvent is allowed (OECD, 1984), and the stock concentration was 10,000 mg/l. It did not precipitate in the algal media at this level, and it does not absorb at 689.6 nm.

**3:3.3.6.1** Sensitivity.

The sensitivity of the algal growth inhibition test was the lowest of all the systems used, in all cases. To Nuvan and malachite green it was more than one order of magnitude less sensitive than the next system. Ivermectin was found to be non-toxic to *Chlorella vulgaris* up to the highest concentration used (1.0 mg/l). In fact, the results show promotion of growth, with two different profiles, relating to three concentrations where ethanol was used as a solvent, and the two highest concentrations, where undiluted Ivomec was used.

An ivermectin solvent blank (propylene glycol/glycerol-formal) was not applied in this or any test in this study because, according to the ASTM test protocol, ASTM (1980), no ingredient of a commercial formulation is considered a solvent unless extra amounts are used to prepare stock solutions.

While no work had been done previously on these chemicals for this species of algae, some results are available on various permutations with other species. Butler *et al.* (1977) found the $EC_{50}$ of dichlorvos to *Euglena gracilis* to be 3.5 mg/l, and Grahl *et al.* (cited in WHO, 1989) found 0.325 mg/l dichlorvos increased the growth of *Scenedesmus* and *Pediastrum* species. Halley *et al.* (1989b) found 10 mg/l ivermectin to increase the growth of *Chlorella pyrenoidosa.*

Butler *et al.* (1977) describe how *Chlorella vulgaris* was seen to metabolise lindane, and a number of other algae have been found to metabolise other insecticides. Wide discrepancies between species sensitivities and insecticide toxicities were noted. Wong *et al.* (1984), however, found that another freshwater algae, *Ankistrodesmus falcatus*, was

sensitive to 12 chlorobenzenes. This sensitivity correlated almost perfectly with the Octanol/Water Partition Coefficient (O/W coefficient). Reported toxicity of 4 of these to *Chlorella vulgaris* was lower, but also had a high correlation with the O/W coefficient (0.98). This would appear to be the opposite of the situation found in this study, where ivermectin, the most lipophilic of the three toxins, was the least toxic to *Chlorella vulgaris*, and malachite green, the least lipophilic, was the most toxic.

Despite this wide variation in species sensitivity, Nyholm and Kallqvist (1989) point out that algae are frequently more sensitive than fish or *Daphnia*, and say that some of the species variation may be due to the non-standardisation of physico-chemical parameters.

Also, it is the only representative of the primary producers examined in the study.

**3:3.3.6.2** Reproducibility.

It is recognised that there are a number of algal test factors which can lead to a great deal of inter-trial variation. Nyholm and Kallqvist (1989) in a discussion on the effect of physico-chemical variations on test variability, note growth medium, pH, $CO_2$ supply, biomass concentration, duration, light and temperature, end-point and response variable as important factors.

Intra-trial reproducibility was good in the majority of cases (appendix Tables 8.59-8.65, appendix page xii), and reading by absorbance was a practical and effective option. Inter-trial variation was higher than with the other systems (Table 3.7, p. 104), but, given that orders of magnitude are often seen between trials (Nyholm and Kallqvist, 1989), it is considered to be relatively good.

Bolier and Donze (1989) found that artificial medium gave the best reproducibility. They also noted that, in a number of studies, a second growth phase was observed, which was an increase in cell volume, not in cell number (and so not an increase in specific growth rate). The absorption method would register this as increased cell number, and give a false indication of toxic response. However, the time this occurred was outside the time-scale used for this study.

**3:3.3.6.3** Summary.

Overall, the lack of sensitivity, and the significant space resources required for the algal growth inhibition test lead to the conclusion

that it is not likely to be of significant value, using current protocols, in routine environmental monitoring.

However, it should be noted that, despite restrictions, the test uses an organism representative of an ecology that is not represented by any other in the study. The results are of particular value for this reason alone.

While the documented high inter-trial variation was not seen in this study, this would further add to the problems with its application.

### 3:3.3.7 The *Artemia salina* test.

As noted in section 3:2.5.4 (p. 63), *Artemia* have been used for a number of years in toxicity testing. Their tolerance to variations in environmental conditions is a very useful handling characteristics, but is considered by some to be the basis of their observed low sensitivity to some chemicals, and groups of chemicals (such as metals).

#### **3:3.3.7.1** Sensitivity.

The sensitivity of *Artemia salina* to the three toxins used in this study varied from low (Nuvan) to median (malachite green) to high (ivermectin), relative to the other animal systems used. Overall, its sensitivity was largely within one order of magnitude of the most sensitive species used.

The most significant result is its extreme sensitivity to ivermectin, which was the only other system to correlate with the *in vivo* Rainbow trout toxicity.

Another result of note is the observation of sub-lethal effects in the trials. At the lowest concentrations tested there were definite sub-lethal effects on surviving nauplii, which would have made the affected individuals ecologically non-viable. With Nuvan, the effect seen was that the survivors were swimming less randomly. With ivermectin, the surviving nauplii were somewhat sluggish. This was not the case with malachite green, where the surviving nauplii appeared unaffected.

Measuring sub-lethal effects has been discussed previously in section 3:3.3.5 (p. 112), but has not been discussed previously in the literature, in reference to *Artemia*. The test end point, no movement for 15 seconds on observation, does not allow the interpretation of these effects, and it is considered here that this is a significant underuse of the potential sensitivity of the test. In this study, significant sub-lethal effects were noted at one order of magnitude below the $IC_{50}$.

Foster and Tullis (1984) found the toxicity of naphthalene derivatives to *Artemia* to be directly proportional to their Octanol/Water coefficients, which could explain this extreme toxicity of ivermectin to *Artemia* and to *Streptocephalus* (as ivermectin is highly lipophilic), but not its low toxicity to *Brachionus*. They note a cut-off point in this relationship, when a chemical becomes too lipophilic, and passage between the polar and non-polar phases is inhibited by extreme hydrophobicity.

The NRC (1984) also refer to this cut-off point, and note that some carcinogens (aromatic hydrocarbons) become inactive above log P = 8. Abernathy *et al.* (1986) also concluded that the toxicity of hydrocarbons and chlorinated hydrocarbons is largely determined by organism-water partitioning. Relating toxicity to the O/W coefficient suggests that the chemical is a non-specific physical toxicant (i.e. mode of action by disrupting membranes etc.). There are some cases of true molecular toxicity, and deviations from the Octanol/Water model indicate the extent of these. In general, Abernathy *et al.* found *Artemia* to be less sensitive than *Daphnia* to the 38 chemicals tested.

Kissa *et al.* (1984) found *Artemia* to be resistant to cadmium, nickel and cobalt, but sensitive to chromium. In all cases, toxicity increased over time, and hatching rate was a more sensitive end-point than toxicity to the nauplii. *Artemia* are noted to have a low sensitivity to metals, this is discussed further in section 4:2.1.3 (p. 135).

**3:3.3.7.2** Reproducibility.

The intra-trial reproducibility of the *Artemia* results was poorer than that seen with the cultured cells, but good in comparison to that seen on other *Artemia* studies (10-20%, Blust *et al.*, 1986 and Pandey and MacRae, 1991). The protocol, with some experience, can be standardised well, and variability minimised. However, it should be noted that the number of individuals used is small (10 per well), which would automatically magnify the effect of any variation, in comparison to cell culture tests.

The statistical strength of these tests could be improved by using a number of 24 well plates in parallel, or by the manufacture of specialised plates, with more, or larger wells.

Inter-trial variation also compared well with variation found in round-robin tests of the *Artemia* protocol by Snell and Persoone (1989).

**3:3.3.7.2** Summary.

The range of sensitivities found in these trials reflects the range of sensitivities noted in the literature for *Artemia*. Overall, however, it would be classed from these results as sensitive, particularly in relation to the index given for the toxicity of ivermectin.

Also in the light of data from the literature, the intra- and inter-trial variation seen was low. However, the test protocol itself is restricted in the number of individuals applied. Suggestions are made for improving this.

Overall, the test performed well, and it is considered here, from the results obtained, that the *Artemia salina* immobilisation test is useful in routine environmental monitoring.

3:3.3.8 The *Brachionus* immobilisation test.

**3:3.3.8.1** Sensitivity.

The sensitivity of the rotifer, *Brachionus calyciflorus*, as measured in this study, was median to Nuvan and malachite green, and low to ivermectin (3 orders of magnitude), relative to the other systems used. The low sensitivity to ivermectin is particularly significant in light of the extreme sensitivity of the other two invertebrates used, and of Rainbow trout.

Initial range finding concentrations for ivermectin were based on a reported link between rotifer sensitivity and *Daphnia* sensitivity (Snell and Persoone, 1989a). However, it was found that *Brachionus calyciflorus* was significantly less sensitive than *Daphnia*. Sub-acute toxic responses were not seen for any of the toxins.

Its sensitivity to Nuvan and malachite green was within one order of magnitude of the most sensitive system used, and its sensitivity to ivermectin was similar to that of the cultured cells.

Serrano *et al.* (1986) reported the sensitivity of another *Brachionus* species (*Brachionus plicatilis*, marine) to be low to one organochlorine and five organophosphates. There was also significant variation (3 orders of magnitude) between three strains of the same species. Sub-lethal doses, however, had little effect on fertility, which shows why *Brachionus* often becomes dominant in pesticide polluted situations. Grahl *et al.* (cited in WHO, 1989), found 0.325 mg/l dichlorvos increased species of *Brachionus* (unspecified) and other rotifers, while killing cladocerans (*Bosmina* and *Daphnia*) and reducing copepods (*Cyclops*).

Snell and Persoone (1989a) found a similar pattern for *Brachionus plicatilis*, with a range of sensitivities from high, to copper, through low, to malathion. They link this resistance to pesticides to the species' noted osmoregulatory ability, which is central to its ecological success. This osmoregulatory ability is essentially a reversal of the Octanol/Water phenomenon. A second trial, with *Brachionus rubens* (freshwater, 1989b) showed the same rank order of toxicity, but *B. rubens* was generally more sensitive.

**3:3.3.8.2** Reproducibility.

The intra-trial reproducibility of the *Brachionus* trials, was, on average, similar to that seen with *Artemia*. Snell and Persoone (1987a,b) report a similar variability for *B. plicatilis*, but a higher variation for *B. rubens*. There was a particularly sharp log response between 1.8 and 3.2 mg/l leading to zero deviation with ivermectin. This is an unusual result.

Inter-trial reproducibility was also similar.

**3:3.3.8.3** Summary.

A number of the qualitative and quantitative characteristics of the *Brachionus* test are similar to those seen with the *Artemia* test. The handling of the test is good, sensitivity is varied, but is generally good, and reproducibility is good, despite the low number of individuals applied.

For these reasons, and noting that it is a freshwater comparison to saline species, it is considered here that, from the results obtained, the *Brachionus calyciflorus* immobilisation test would be useful in routine environmental monitoring.

3:3.3.9 The *Streptocephalus* immobilisation test

No further information was available in the literature on the sensitivity of *Streptocephalus proboscideus*. Although the results from the trials in this study should be regarded as tentative, the measured sensitivity of the species places it amongst the most sensitive of the invertebrate species used. This is particularly significant when viewed in the context the poor handling characteristics of the nauplii, and the higher degree of toxin dilution that results (section 3:3.2.9, p. 102).

For Nuvan and malachite green, the toxicity indicated by *Streptocephalus* is median to high, the results being within one order of magnitude of the most sensitive system used. For ivermectin, the only

quantifiable indication given is two orders of magnitude less sensitive than Rainbow trout or *Artemia*. However, a second test, which could not be used because of blank mortalities, suggests that its true sensitivity is very high.

A discussion of the intra- and inter-trial reproducibility of the results is not appropriate, as the handling characteristics, and low numbers of replicates requires that the results be regarded as tentative.

In summary, there were a number of handling problems with this test, leading to a small number of successful trials. However, the results which were obtained suggest that the species has a high sensitivity. For these reasons, it is considered here that, with further refinement of the test protocol, this test system could be the useful in routine environmental monitoring.

### 3:3.4 Quantitative results - comparison of systems.

The results from all the ecotoxicity trials have been presented and discussed in the previous sections as individual systems. A further discussion, comparing the relationship between the test systems, would also be of interest.

Statistical comparison of the result sets would give a quantifiable expression of the relationships that can be seen in Table 3.7 (p. 104), where the result sets are paired (i.e. originate from the same treatment, e.g. cell culture etc.). Where data sets are not paired (e.g. comparing cell culture results with *Artemia* results), ranking of the data can give a more graphical representation of the relationship between the different tests.

In the following sections, the cell culture results are analysed statistically, and all of the results are ranked, according to sensitivity, and according to toxicity.

#### 3:3.4.1 F- test of association between cell culture systems.

It can be seen from Table 3.7 (p. 104) that there is very little difference between the results of the two end-points, for either cell line, or any toxin. However, the conclusion is a subjective one, as there is no measure of this relationship.

In order to provide a statistical measure of the association between the sets of cell culture results, each pair of results is analysed in the following section for association by the Single Factor Anova (F-test). The methodology has been described in section 2:8.3 (p. 39).

The anova test is the standard test to determine whether there is a statistical difference between the means of particular pairs of populations (Gelber *et al.*, 1985). The null hypothesis examined is whether the two sample means are equal. If the null hypothesis is accepted (+), this means that the two sets cannot be said to be statistically distinct. For example, if the L929 crystal violet and neutral red results for Nuvan lead to acceptance of the null hypothesis, the two sets of results are judged to be equal (i.e. from the same sample population), despite the crystal violet average value being lower than the neutral red.

If the test leads to rejection of the null hypothesis, the two sets of results are proved to be statistically distinct, regardless of how close the average values are. In this case, the relationship was tested at both the 5% and the 1% levels.

Table 3.10 presents the results of the Single Factor Anova (F-test) assessment of the association between the various cell culture systems examined.

**Table 3.10**

**Comparison of $EC_{50}$ data for all toxins from cell culture ecotoxicity test methods, using the single-factor anova (F-test) at 5% and 1% (in parenthesis) probability levels**

| | | L929 (c. violet) | RTG-2 (c.violet) | L929 (pooled) |
|---|---|---|---|---|
| Nuvan | L929 (n. red) | + (+) | | |
| | RTG-2 (n. red) | | + (+) | |
| | RTG-2 (pooled) | | | - (-) |
| Ivermectin | L929 (n. red) | + (+) | | |
| | RTG-2 (n. red) | | + (+) | |
| | RTG-2 (pooled) | | | + (+) |
| M. Green | L929 (n. red) | + (+) | | |
| | RTG-2 (n. red) | | + (+) | |
| | RTG-2 (pooled) | | | - (-) |

Null Hypothesis = median toxicity measures from the different tests are equal.
Accept = +, Reject = -.

These results show that there is no statistical difference between the crystal violet end point and the neutral red end point for any toxin, with

the L929 cell line (column 1 of the table). Similarly, there is no difference between the two end points for any toxin with RTG-2 (column 2).

When this was established, the results for each cell line were pooled, and the sensitivity of the overall data from each cell line tested. This was possible because the data sets for each batch of crystal violet/neutral red results (for each cell line) are paired. That is, each pair originate from the same flask of seed cells, were subject to the same plate seeding event, were subject to the same toxin dilution event, and were incubated at the same time, under identical conditions.

There is a statistically significant difference between the sensitivity of the two cell lines for Nuvan and malachite green, but not with ivermectin (column 3 of the table).

The crystal violet elution assay and the neutral red assay are based on two different principles. The former measures the uptake of dye in cellular cytoplasm, which is said to be irrespective of the condition of the cells, and the latter measures active metabolism of a vital dye in the lysosomes of the cell, which occurs only when the cell is in good condition (i.e. actively metabolising). It might be expected, therefore, that the neutral red method would be more sensitive to the sub-lethal effects of a toxin, and thus a more sensitive method overall.

As can be seen in the Table, this was not the case. One possibility is that the early response of a cell to toxic injury, i.e. reduced cell permeability (Rodrigues and Mattei, 1987), excludes the crystal violet dye, effectively measuring only metabolising cells also, making it substantially equivalent to the neutral red method.

Other studies have found a difference in sensitivity between the neutral red method, and a range of others. Hunt *et al.* (1987) found neutral red to be less sensitive than the MIT-24 assay, the KBr assay, and the colony counting assay. However, they had used a 24 hour incubation time, and considered this to be the cause of a relative lack of sensitivity. Benford and Good (1987) compared 4 variations of the neutral red assay (i.e. standard incubation, 24 hour incubation, 5 hour incubation, and substitution of serum). Excellent correlation was found between all four, with the greatest degree of variation being with the serum-free variation. All four correlated well with the MTT and the ATP assays ($r^2 > 0.95$).

3:3.4.2 Rank order of sensitivity.

Another method of demonstrating the relative sensitivity of a particular system is to rank its sensitivity over a number of investigations, in this case, the three chemicals whose toxicity is being measured. The rank order of sensitivity of the seven systems used in this study (cell line data composited) is presented in Table 3.11.

**Table 3.11**

**The rank order of sensitivity of seven ecotoxicity test systems to Nuvan, ivermectin and malachite green**

| Test System | Nuvan | Ivermectin | Malachite Green |
|---|---|---|---|
| L929 | 4 | 4 | 2 |
| RTG-2 | 5 | 5 | 1 |
| Algae | 7 | 7 | 7 |
| *Artemia* | 6 | 1 | 6 |
| *Brachionus* | 3 | 6 | 4 |
| *Streptocephalus* | 2 | 3 | 5 |
| Rainbow Trout | 1 | 2 | 3 |

In all cases, *Chlorella* was the least sensitive species. For the two neuro-toxins, cultured cells were amongst the least sensitive of the seven (with L929 more sensitive than RTG-2). For the thiol antagonist cultured cells were the most sensitive (with RTG-2 more sensitive than L929). Rainbow trout was always amongst the most sensitive species, but was only ranked 1 for Nuvan. Of the invertebrate species, *Streptocephalus* was generally the most sensitive, and *Artemia* the least, except for ivermectin, where *Artemia* was particularly sensitive. The sensitivity of *Brachionus* was generally median, relative to other whole animal systems, except for ivermectin, to which it showed some resistance.

Results from other studies have showed varying success in sensitivity ranking by other systems. Riddel *et al.* (1986) found that the neutral red method and the KBr method gave the same ranking, and largely the same $EC_{50}$s, for 30 chemicals. The HTD method gave a somewhat similar ranking, but for five chemicals, including paracetamol and aspirin, significant morphological change was seen at less than one tenth of the $EC_{50}$, as calculated by the neutral red or the KBr methods.

Garza-Ocanas *et al.* (1990) investigated the toxicity of 18 compounds to 6 cell lines from various rat organs. For the majority, the sensitivity of the lines was almost exactly the same, meaning also that, for the majority, the rank order of toxicity was the same.

In summary, these results are a different way of expressing the findings outlined in 3:3.3 (p. 103), and in the F-tests. It is a method which is useful for the quick evaluation of a large amount of test results, and which can highlight particular sensitivities, or tolerances, of particular methods.

3:3.4.3 Rank order of toxicity.

A corollary of the rank order of sensitivity shown in Table 3.11 is the rank order of toxicity of a chemical, and this is presented in Table 3.12 (cell line data composited).

**Table 3.12**

**The rank order of toxicity of Nuvan, ivermectin and malachite green to seven ecotoxicity test systems**

| Test System | Nuvan | Ivermectin | Malachite Green |
|---|---|---|---|
| L929 | 3 | 2 | 1 |
| RTG-2 | 3 | 2 | 1 |
| Algae | 2 | 3 | 1 |
| *Artemia* | 3 | 1 | 2 |
| *Brachionus* | 3 | 2 | 1 |
| *Streptocephalus* | 3 | 1 | 2 |
| Rainbow Trout | 3 | 1 | 2 |

Differences in the mode of action of a chemical, and in the sensitivity of a particular species are essentially expressions of the same thing. However, it is useful to highlight differences in the mode of action of a chemical also, in order to further the assessment of the ecotoxicological effectiveness of a particular test system, and to generate more data that can be used in the management of the chemicals.

According to the batch of seven tests used in this study, Nuvan was the least toxic of the three chemicals under investigation, except for the single case where ivermectin was found to be non-toxic to *Chlorella*. The cell culture systems assessed malachite green as the most toxic of the three, while the whole animal systems, with the exception of the *Brachionus* test, assessed

ivermectin as the most toxic of the three. This split is essentially due to the failure of the cell culture systems, and the *Brachionus* system to respond to the toxicity of ivermectin, which is likely to be related to its lipophilicity. It is clear that, given this, ivermectin is by far the most toxic of the three.

This is a very significant result, given that ivermectin is being proposed as a replacement for dichlorvos in fish farming. The proposed dose is lower than the current dose of dichlorvos, but the method of application, coated on feed, could lead to uncontrolled discharge of significant quantities of the chemical.

Results from other studies have showed varying success in toxicity ranking by other systems. Linseman *et al.* (1990) found that three cell lines, Chang (liver), SIRC (corneal) and LLC-PK1 (kidney), did not rank four aminoglycoside antibiotics in the same way. These compounds are known to accumulate in the kidney, and to be nephrotoxic. However, the kidney cell line was less sensitive than the liver cell line.

In summary, the expression of rank order of toxicity allows the relative toxicities of the chemicals to be highlighted. It also highlights a distinction between the cellular and the whole animal systems.

### 3:3.5 Summary.

The results from the predictive hazard assessment trials show that cell culture methods offer a number of advantages to environmental analysis, and can operate favourably alongside most others.

There was no single method which was better than all others in either their handling, and their data generating characteristics. Neither was there one single method which was significantly more sensitive than the others. All of the methods contributed some data to the overall assessment of the toxicity of the three materials.

The criteria for determining whether or not cell culture methods were a useful addition to the battery of standard whole organism tests applied included ease of use, statistical strength, relevance, and volume of data generated. The cell systems used excelled in these categories.

The conclusion, therefore, is that cell culture techniques have a great deal to offer in routine environmental analysis.

# CHAPTER 4
# ENVIRONMENTAL MONITORING

Chapter Index Page

## 4:1 Introduction.

As described in chapter 1, environmental analysis can be divided into two main categories, predictive hazard assessment, and environmental monitoring (retrospective hazard assessment). Both of these categories are currently restricted by the lack of a practical biological test system which could generate large amounts of relevant data, quickly, reproducibly, with high statistical strength, at low cost, and having a significant degree of flexibility.

Environmental monitoring in particular lacks such a system, as priority decrees that products set for commercial production (and generally only those intended for human contact) receive the major share of attention.

It is considered here that cultured cells possess many of the characteristics required of a good environmental monitoring system. However, few studies have examined aspects of this, and none have examined the topic in detail, applying a direct *in vivo/in vitro* comparative approach. There is a need, therefore, for a study to examine the technical aspects of such a system, to establish whether it can satisfy the rigorous requirements of routine monitoring.

The following chapter outlines two sets of effluent monitoring experiments, designed to test the usefulness of a cell culture system (L929 cells, crystal violet end point), through comparison with an established *in vivo* test (*Artemia* immobilisation), and with chemical analysis (AAS and GC).

The first was analysis of a complex metalliferous effluent. Metal effluents offer particular technical problems for toxicity analysis, including low pH, the effect of organics on toxicity, and chemical speciation.

In general, routine analysis of such effluents would be for specific metals, and for other parameters involved in classical oxygen and nutrient management (e.g. BOD, COD, N, P, etc.). Management of effluent discharge would then be through the setting of specific limits for a certain number of these, with no account taken of the toxicity of the effluent, or the likelihood of changing effluent characteristics over time.

By analysing the effluent using cell culture and *Artemia* immobilisation, it is intended to show whether toxicity tests, and cell culture tests in particular, can help to characterise the effluent to a higher degree.

The second was an experiment designed to assess the efficacy of a peat based treatment system for the removal of an organophosphate insecticide sheep dip from solution. In general, such experiments would be monitored by chemical analysis of the active ingredient alone. By analysing

the product, and the column effluents using cell culture and *Artemia* immobilisation, it is intended to show whether toxicity tests, and cell culture in particular, have a contribution to make to such experiments, and to assess the degree of any contribution.

In carrying out this experiment significant time and other resources were required for the design and construction of the peat column apparatus, and for the development of a GC programme for the analysis of diazinon. While the emphasis of the overall study is on the operation of the cell culture system, the technical requirements of chemical analyses should also be considered, as these are often rigorous, and severely restricting. An assessment of the limitations of chemical analysis is important for the proper comparison of other test systems, such as cell culture.

## 4:2 Literature Review.

### 4:2.1 Metal toxicity.

#### 4:2.1.1 General.

Metals, as a group of elements, represent one of the most complex groups of xenobiotics in environmental pollution and its management. They are present in the environment, in significant quantities, in a variety of rock types, and are central to the metabolism of almost all parts of the food chain. However, there are also a number of anthropogenic sources, principally factory effluents, landfill leachates and municipal wastewaters. A particular case for concern in the 1980s was the use of tributyltins (TBTs) as antifouling paints on ships. In 1985, 30% of the worlds fleet were using these (Clark *et al.*, 1988).

As elements, metals do not undergo further breakdown, and can concentrate in various environmental sinks. When metals increase above background levels, they can represent some of the most potent toxins in existence. As such, they are of extreme importance.

A further consideration is that metals present particular problems in the measurement of environmental toxicity, because of their strong affinity for organic material, and because of the complexity of equilibria equations (in particular, the disparity between the toxicity of dissolved, and particulate metals).

All of these features make a metal effluent of interest in a study such as this, particularly since metal effluents can often contain many more

toxins than the metals alone, such as cyanides, etc. Also, they are often at extremes of pH. Cultured cells are very pH sensitive, and reduction of toxicity by media serum is a topic that is receiving a great deal of attention.

4:2.1.2 To cultured cells.

A number of previous studies have involved cultured cells and metals, though not metal effluents. These have had many objectives, from assessing the response of the cell systems, to assessing the toxicity of the materials, and to establishing toxic mechanisms.

Marion and Denizeau, in a study with lead (1983a), found that 2.4 and 10 mg/l lead, in either 1% or 10% serum, had no effect on cultured human cells. However, 2.4 mg/l did inhibit metabolic processes in RTG-2 cells. This compares with 96 hour $LC_{50}$ data from Rainbow trout of 1.40 mg/l (hard water), and 1.17 mg/l (soft water) (Davies *et al.*, 1976).

In that study with Rainbow trout, Davies *et al.* (1976), noted that lead is complexed in natural waters into non-toxic complexes. It is the dissolved (or 'available') fraction that is toxic. This highlights the difficulties and dangers in interpreting environmental levels of xenobiotics on the basis of chemical information alone. In their study, the equivalent total lead concentration was c. 500 mg/l. They suggest filtration through a 0.45µm filter to distinguish between dissolved and particulate metal fraction.

In a subsequent study with cadmium, Marion and Denizeau (1983b) found that 2.4 mg/l cadmium was highly toxic to RTG-2 cells, but only when the serum concentration was dropped from 10% to 1%. In that study also, the cultured human cells were affected. A similar serum effect was noted by Babich and Borenfreund (1990), where there was a four fold difference between the toxicity of cadmium at 1% and 10% serum.

Stark *et al.* (1986) previously noted the reduction of metal toxicity by chelators. The toxicity of $Cd^{2+}$, $Co^{2+}$, and $Ni^{2+}$ to BALB-c 3T3 cells was reduced by EDTA, and to a lesser extent by nitrilotriacetic acid. The reduction of $Cu^{2+}$ toxicity was somewhat lower.

Christian *et al.* (1973), in a study with L929 cells, found $Cd^{2+}$ and $Zn^{2+}$ antagonistic at equimolar and 10x molar mixes. Antagonism between metals *in vivo* has previously been noted. Babich *et al.*, (1989) found that *in vivo* antagonism between arsenic and selenium was demonstrable *in vitro* also, using BG/G and BG/F cells.

Cherian (1985) states that the use of cell culture systems has enhanced the knowledge of the cellular and molecular toxicity of metals. As

many of these effects are seen in the renal proximal tubules, their studies were carried out on rat kidney cells. They recognise the problems in the analysis of metal toxicity by cell culture as including quantitation, extrapolation and solubility, but note that these are in common with other systems. They suggest the use of buffers to reduce solubility problems.

Biological response to toxic insult by metals, is often the production of proteins, such as metallothionein (MT), which bind metals, and reduce toxicity (Rafiee *et al.*, 1986). In this way, MT binds lead, cadmium, zinc, mercury and other metals. Copperchelatin binds copper (Marion and Denizeau, 1983a). However, paradoxically, metal-metallothionein complexes have been shown in many cases to be highly toxic themselves.

Another response is the formation of intranuclear inclusion bodies, which isolate the toxins, and prevent expression of toxicity. Butler *et al.* (1990) attribute the tolerance to copper seen with *Chlorella vulgaris*, to exclusion of the metal, or to the formation of intracellular compartments.

Cherian found that CdMT was far more toxic than $CdCl_2$, despite the uptake of $Cd^{2+}$ from $CdCl_2$ being three times that from CdMT. This was postulated to be due to the mechanisms of transport of the two, with CdMT entering by endocytosis (as opposed to diffusion for $CdCl_2$) causing membrane leakage. Significantly, pre-treatment of the cells with low levels of metals reduced their toxicity, by inducing the formation of inclusion bodies, demonstrating possible adaptation to metals.

Quantitative Structure Activity Relationships (QSARs), used to predict the toxicity of other groups of toxins, have been examined by cell culture for metals also, but have produced anomalous results.

The chemical softness parameter ($\sigma$, Hansch parameter) classifies metals, according to softness and hardness. It describes the specific ligand to which they may bind, and thus exert a biological effect. Babich and Borenfreund (1988) found the sequence of toxicity for divalent organotins (cationic) to BF-2 and BALB/c 3T3 cells to correlate strongly with the Hansch parameter (0.91/0.93). In the same study, they found the toxicity of diorganotins (but not butyltins) correlated strongly with lipophilicity also (0.96, BF-2 cells).

Hulme *et al.* (1989) also considered the Hansch parameter as a possible QSAR for metals, and compared 52 metal toxicities *in vivo* (rat oral and mouse intraperitoneal) with toxicity to 3T3-LI cells, and with $\sigma$. They found the softness parameter to be of questionable value, and considered the

salt of the metal to be of more importance. They found $SO_4$ salts to be more toxic than $Cl_2$, $B_2$ and $I_2$.

This is an important point, when assessing the comparability of test results, as many studies (including cell culture studies) have prepared metal solutions from salts, without prior consideration of the form of the salt, or the availability of the metal in solution.

Interestingly, Hulme *et al.* (1989) found the toxicity of chloride salts in cell culture to correlate best with mouse intraperitoneal data. Chloride is the dominant anion in cell culture media, and intraperitoneal administration of toxins is a better approximation to the situation in cell culture, as the digestive system is bypassed.

Another unusual result from the assessment of metal toxicity by cell culture was a shift in the temperature optimum of BF-2 cells, from 26 $^{o}$C to 34 $^{o}$C, a phenomenon previously not seen. The shift further demonstrated a temperature dependant effect on the toxicity of diethyltin dichloride, and other classes of toxins (Babich and Borenfreund, 1987).

4:2.1.3 To *Artemia*.

*Artemia* have been used in a number of studies on metal toxicity. These studies have included $IC_{50}$ determinations, effects of metals on various life stages, and examination of toxic mechanisms. A point of note is that *Artemia* is a regulatory requirement for testing under the 1978 EEC Directive on the dumping of titanium (Persoone and Wells, 1987).

*Artemia* have been shown to have a very high resistance to the toxicity of a number of metals. Kissa *et al.* (1984) found the (48hr.) $IC_{50}$ of the following metals to be; $Cd^{2+}$, 159.6 mg/l; $Cr^{6+}$, 7.911 mg/l; $Ni^{2+}$, 63.0 mg/l; $Co^{3+}$, 171.7 mg/l. The concentrations causing 50% inhibition of hatching were, however, significantly lower, at; $Cd^{2+}$, 4.898 mg/l; $Cr^{6+}$, 4.66 mg/l; $Ni^{2+}$, 10.233 mg/l; $Co^{3+}$, 10.256 mg/l.

These toxicity indications were low, in comparison to the (48hr.) $IC_{50}$ figures obtained by Khangorot and Ray (1987) for *Daphnia magna*; $Cd^{2+}$, 1.88 mg/l; $Cr^{6+}$, 1.79 mg/l; $Ni^{2+}$, 7.59 mg/l; $Cu^{2+}$, 0.093 mg/l, and for *Salmo gairdneri* (96 hr.); $Cd^{2+}$, 2.5 mg/l; $Cr^{6+}$, 11.2 mg/l (fish are the exception to the established high environmental toxicity of $Cr^{6+}$, Babich *et al.* {1986}), $Ni^{2+}$, 3.55 mg/l; $Cu^{2+}$, 0.253 mg/l.

Nikonenko (1987) considered that a study on possible adaptation to low levels of toxins would be appropriate, as actual environmental levels are often low. He studied the effect of low levels of nickel, phenol and DDT on

*Artemia salina*. After exposure to sub-threshold concentrations of the three, the concentrations required to cause 100% immobilisation were; nickel control = 18 mg/l, test = 20 mg/l (this is significantly lower than the figure obtained by Kissa *et al.*, but the data are not directly comparable, as the method of application of concentrations in this study was cumulative, over 20 days); phenol control = 100 mg/l, test = 200 mg/l; DDT control = 0.012 mg/l, test = 0.02 mg/l. Thus *Artemia* show ability to adapt to low levels of a range of pollutants, including metals.

A similar study was that by Rafiee *et al.* (1986), who showed that 0.1-10 µM cadmium progressively delayed the hatching of *Artemia*, but that washing reversed this effect. The emerged nauplii, however, showed marked differences in colour and shape. The measured $IC_{50}$ to *Artemia* was between 1-10 mM, in comparison to that of *Daphnia magna*, 0.27 µM (30 µg/l). Metal toxicity at low concentrations to the rate of development of *Artemia* has also been noted by Pandey and MacRae (1991), in a study on organic mercury compounds. The effect was shown to be relative to the weight of the compound, and not the ionisation state.

Rao and Latheef, (1989) assessed the effect of copper on the hatching of *Artemia*, and on growth, either from the media, or when introduced in feed. The concentration of copper required to reduce hatching by 50% was 40 mg/l, and copper in the media had more of an effect on growth than that from feed.

Antagonism between metals has also been noted with *Artemia*. Verriopoulos *et al.* (1987), in toxicity tests with 25 day old adults, found that mixtures of $IC_{50}$ quantities of copper and chromium exerted a less than additive effect.

4:2.2 Sheep dip - diazinon.

The use of sheep dip in Ireland is a topic which is causing increased concern in recent times, as the volumes of dip used increase, and knowledge of the environmental effects grows. There are few useful environmental bioassays that could generate data which would be useful in addressing the topic. The use of cell culture as a bioassay for the monitoring of a sheep dip treatment system is examined in this study.

The following section reviews the current situation with regard to the use of sheep dip in Ireland, and the use of diazinon in particular.

4:2.2.1 The use of sheep dip in Ireland.

The dipping of sheep in Winter for scab, using organophosphate insecticides, is compulsory in Ireland. Summer dip, against fly-strike is also common. Dipping must be at least one minute to allow the acaricide to reach the lanolin in the wool (O'Brien, 1992). Current disposal methods are to store the dip in slurry, and to spray this mixture onto fallow land, away from watercourses, or to dilute the dip 1/20 before spreading (O'Brien, 1992).

The Irish national sheep flock varies from 5.5 million to 9 million ewes before the June cull (O'Brien, 1992), which makes sheep husbandry an important agricultural activity. Until recently at least, there was a perception amongst controlling bodies that sheep dipping in Ireland did not represent a significant environmental hazard, despite up to 400,000 gallons of sheep dip being used here every year, with up to 30% of these currently containing phenol (Henry, 1993).

That perception is, however, changing rapidly. Pesticide review groups have recently been set up in a number of countries, including Ireland, where a committee has been set up between the Dept. of Agriculture and the Dept. of the Environment (O'Brien, 1992).

4:2.2.2 The use of diazinon in sheep dip.

Diazinon was first registered as an organophosphate insecticide in 1956. It has a wide variety of applications, including aerosols, sprays, pet collars, ear tags, dips, aerial spray and soil incorporation. In America, over 10 million pounds per annum are used (EPA, 1988).

Diazinon is not compatible with phenol. Thus, as a sheep dip, it is most commonly prepared as solvent based. It is the most widely used organophosphate in Ireland for this purpose (followed by propetamphos, phoxin, and pyrethrites). Phenol based sheep dips are set to be phased out, so it follows that, after the phase-out is complete, its use is likely to increase (O'Brien, 1992; Morgan, 1992).

The product used in this study was Osmonds Gold Fleece sheep dip, containing 60% diazinon, and 32% of an unnamed solvent. There are known to be up to 16 other compounds present, including 3% liquid paraffin, and a stabiliser, epoxidised castor oil (Morgan, 1992).

4:2.2.3 The chemistry of diazinon.

Diazinon is an organophosphate insecticide which acts by cholinesterase inhibition. Chemical names include, phosphorothioic acid (O,O,-diethyl-O-{6-methyl-2-[1-methylethyl]-4-pyrimidynyl} ester); thiophosphoric acid (2-isopropyl-4-methyl-6-pyrimidyl diethyl ester); O,O,-diethyl O-2-isopropyl-4-methyl-6-pyrimidyl thiophosphate; and diethyl 2-isopropyl-4-methyl-6-pyrimidyl thiophosphate (Windholz *et al.*, 1976). Trade names are numerous, according to the area of application.

It is an amber/brown liquid, with a mild, sweet aromatic smell (EPA, 1988). Its molecular formula is $C_{12}H_2N_2O_3PS$; mol. wt. 304.36; solubility in water at 20 $^{o}C$ is 40 mg/l; it is miscible with alcohol, ether, petroleum ether, cyclohexane, benzene and other similar hydrocarbons (Windholz *et al.*, 1976).

4:2.2.4 The toxicity of diazinon.

The EPA fact sheet on the toxicity of diazinon is characterised by two trends, the variability of the toxicity of diazinon in various systems, and the number of data gaps which still exist.

It has a low acute oral toxicity (618 mg/kg), a low acute dermal toxicity (> 2000 mg/kg), it causes no dermal irritation, no ocular irritation, and low dermal sensitisation. It is not oncogenic, and not teratogenic up to 100 mg/kg/day. However, it is classed as very highly toxic in avian acute tests (3-10 mg/kg), highly toxic in avian dietary tests (47-245 mg/kg), very highly toxic to Rainbow trout (0.09 mg/l) (Cope {1965} also reported a 24 hr $LC_{50}$ of 0.380 mg/l), highly toxic to Bluegill sunfish (0.136 mg/l), and very highly toxic to *Gammarus fasciatus* (0.2 mg/l). In the marine environment, it is classed as moderately toxic to Sheepshead minnow (1.4 mg/l). Its toxicity to non-target organisms (honey bees) is classed as high (0.2 µg/bee).

There are data gaps for acute delayed neurotoxicity, subchronic toxicity, chronic feeding, metabolism, reproduction, mutagenicity, and metabolism and persistence in plants and animals. Immediate data requirements identified include acute toxicity (both technical grade and product), and all ecological effects. Significantly, the EPA state that the potential of diazinon to contaminate groundwater is unknown (EPA, 1988).

Other invertebrate systems classify its toxicity as high/very high, including *Daphnia magna* (96 hr. $IC_{50}$ = 0.002 mg/l; Meier *et al.*, 1976), *Simocephalus serratus* (48 hr. $IC_{50}$ = 1.4 µg/l), and *Daphnia pulex* (48 hr. $IC_{50}$ = 0.9 µg/l) (Sanders and Cope, 1966), or moderate, including *Gillia aliatis* (4 hr.

$IC_{50}$ 93 mg/l, 96 hr $IC_{50}$ = 11 mg/l; Robertson and Mazella, 1989), and *Brachionus calyciflorus* (24 hr. $IC_{50}$ = 29 mg/l; Fernandez-Casalderrey *et al.*, 1992). Kuwabara *et al.* (1980) found that 10 mg/l diazinon had no effect on the hatching of *Artemia*.

Toxicity to the freshwater fish, *Channa punctatus* was found to be 3.1 mg/l (96 hr. $LC_{50}$) by Sastry and Malik (1981). Ferrando *et al.* (1991) found the $LC_{50}$ of diazinon for the European eel, *Anguilla anguilla*, ranged from 0.16 mg/l (24 hr.) to 0.08 mg/l (96 hr.), the second most toxic of eight insecticides tested. Weiss (1961) found that 0.5 mg/l diazinon killed 50% of Largemouth bass in 1 hour, and that brain AChE activity in the remainder was 17.5%. The same concentration killed 50% of Fathead minnow in 80 minutes, the remaining individuals had an AChE activity of 29.1% of normal.

This range of toxicities, and the disparity between the mammalian and 'environmental' toxicity indices demonstrates the complexity of the activity of toxins, and the hazards involved in making environmental management decisions based on limited data. The environmental implications of the use and disposal of diazinon are obviously far more significant than might be suggested from a set of data that did not include ecotoxicity indices. Also, it is clear that a great deal more data is required in this area. The EPA are restricting new and current uses of diazinon, until these data gaps can be filled (EPA, 1988).

4:2.2.5 The environmental fate of diazinon.

As many of the uses of diazinon are crop related (it is used on over 75 food crops [Robertson and Mazzella, 1989], e.g. against wireworms, corn rootworms, root maggots, etc.), there is a great deal of literature on the fate of diazinon in soils.

Early studies showed that the concentration of the pesticide, organic content, moisture content and microbial populations all play an important part in determining its half-life. Initial figures for half-life in target soils varied from 2-4 weeks in lab and field trials, with around 10% remaining after 20 weeks (Getzin and Rosefield, 1966; Bro-Rasmussen *et al.*, 1968). A later study confirmed these rates (the fastest of eight OPs studied), and showed that, while field breakdown is initially very fast, later breakdown rates approach horizontal, indicating that intense adsorption of trace amounts may occur (Bro-Rasmussen *et al.*, 1970).

A number of studies focused on the microbial aspects of diazinon degradation. Gunner *et. al.* (1966) showed that water carried diazinon was a

poor source of carbon, but that solvent carried diazinon led to rapid and profuse growth. This indicates that, initially at least, microbial attack is limited by water solubility. The lack of a lag phase with subsequent additions showed that the microbial population had adapted to the pesticide. It was also shown to be suitable as a sole source of sulphur (the initial site of attack), phosphate and nitrogen, though the latter was least useful because of shielding of the N by the ring portion of the molecule (also Alexander, 1981).

The microbial population was increased by diazinon additions, though not generally, indicating that specific groups (primarily a coccoidal bacterium) were able to perform specific portions of the degradation. This finally led to a rapid increase in *Streptomyces* species, 180 days after application.

This *Streptomyces* breakdown was again seen by Sethunathan and MacRae (1969) in a study on submerged soils. They found that a concentration of 9.57 mg/l fell to 6.7 mg/l over 10 days in a sterile media, but that *Streptomyces* increased this rate by reducing 11.75 mg/l to 3.23 mg/l over the same period (a higher rate than in aerobic soils). However, to achieve this in such a short space of time, an additional carbon source (glucose) was necessary. Persistence was found to be lower in the submerged soils also, at 2-6% after 50-70 days.

In contrast to these results, Gunner *et al.* (1968) refer to the isolation of an organism from soil capable of using diazinon as a sole carbon source, as well as S, P and N. However, no further characterisation of the organism was carried out.

Konrad *et al.* (1967), in a batch study, showed that an initial large reduction in diazinon concentration was due to adsorption by the soil, and that a subsequent slow reduction was due to breakdown. They concluded that breakdown in batch systems was by chemical reaction at the adsorption sites, catalysed by the adsorption, as microbial acclimatisation was slow. They measured the breakdown of the pesticide at the adsorption sites as 6-11% per day, with associated release of breakdown products into solution (they note also the possible high toxicity of these products, specifically diethyl thiophosphoric acid).

This breakdown was strongly catalysed by pH, with rapid removal at pH 2, intermediate removal at pH 4, and stable diazinon at pH 6. This was supported by others, such as Getzin (1968), who showed that degradation was rapid at pH 4.3, and persistence longest at pH 8.1. Specific catalysis of diazinon hydrolysis by copper has been demonstrated in mineral

soils, and a number of ion-exchange materials, by Mortland and Raman (1967), though not so in organic soils.

Later studies concentrated on the chemical hydrolysis of diazinon, and the soil factors that affect this. Schoen and Winterlin (1987) showed again that the factors affecting breakdown were pH (the major effect), soil type (organic content), moisture and pesticide concentration. Moisture was shown to have less of an effect in organically amended soils. Increasing concentration had a significant effect, with microbial activity ceased at 1000 mg/l.

In summary, these results show that diazinon applied to soils is adsorbed, and that breakdown of diazinon occurs both by chemical hydrolysis (catalysed by pH, some metals and other factors), and by microbial breakdown, both of the parent molecule and hydrolysis products. The importance of microbial breakdown depends on a number of factors also, such as other carbon sources, oxygen profile, etc.

### 4:2.3 The nature of peat.

The waste treatment system examined in the study was a biofibrous peat product for the removal of sheep dip from solution. The operation of the column was followed by cell culture and *Artemia* toxicity testing, as well as the standard GC analysis.

Peat has significant pollutant abatement properties, mainly attributable to its high ion exchange capacity, and its cellular structure. These properties have been used to develop a number of environmental products in recent years. The following is a review of the literature on the characteristics of peat.

#### 4:2.3.1 The formation of peat.

Peat is a product of the organic detritus cycle which occurs in a specific ecosphere, that where the degradation of vegetation is slow due to high moisture content, and associated anaerobic conditions. It is defined as soil with greater than 65% organic matter.

Within the ecosphere of peat formation, there are many specific niches, and many different types of peat, with significantly variant properties. The peat used in this study was 'peat fibre', which consists largely of the root system of the common cottongrass, *Eriophorum angustifolium*. It is a fraction of the peat that can make up as little as 1%

d.w., and is extracted from air-dried milled peat by gravity separation screening (Coffey *et al.*, 1989).

4:2.3.2 The constituents of peat.

Because of the very slow rate of breakdown of plant material in bogs, the subsequent concentration of stable breakdown products, and along with the changing communities of bog plants during its evolution, peat consists of a hugely complex mixture of organic compounds (Fuchsman, 1980). These constituents are characterised largely according to chemical fractionation, and the range of materials involved are significant because of their implications in the use of peat materials in pollution control (both useful and limiting properties).

There are four major fractions identifiable in peat (Fuchsman, 1980). These are; solvent soluble materials, or bitumens; acid soluble carbohydrates, which include pectins, hemicelluloses, etc.; strong alkaline soluble lignins; aqueous alkaline soluble materials, principally humic acids.

These are also a number of other biologically significant materials, such as vitamins (principally B vitamins), and toxin antagonists. This antagonism has been demonstrated with a number of peat products. One cottongrass-sedge peat product, Torfot, has been shown to inhibit the toxicity of strophantin and strychnine (Fuchsman, 1980), and Christera *et al.* (1974) reported that humates counteracted the phytotoxic effects of various thiophosphates and chlorinated fungicides.

The most important of these groups is the humic portion, which can be further divided into humic acids, fulvic acids and humins. Humic acids can make up as much as 40% of the dry weight of peat. Fulvic acids leach easily from peat, giving a yellow-brown colour (Fuchsman, 1980).

Humic acids are particularly important because they account for most of the ion-exchange capacity of peat, the basis of the majority of its pollution abatement properties (Dissanayake *et al.* {1981} found that removal of humic acids significantly reduced the adsorptive capacity of peat for metals). This feature is well characterised, to the extent that humic acids are used to manufacture ion-exchange resins.

Despite the complex nature of peat, and therefore any soluble extracts, water extracts from peat have not been well studied, with existing analysis mostly in the area of carbohydrates (Fuchsman, 1980).

4:2.3.3 The use of peat in pollution studies.

The use of peat in pollution reduction has been the focus of a large number of studies. The emphasis in these studies has been the removal of specific materials from waste streams etc., and consideration has not generally been given to the effect of materials that might be leached from the peat, or to ultimate disposal of the peat. These are topics that are discussed in later sections, in reference to interpretation of toxicity results.

**4:2.3.3.1** General.

The first reported use of peat in pollution studies was for municipal wastewater treatment in Finland, where a peat-ditch system has been in use since 1957 (Surakka and Kamppi, 1971). Since then numerous studies have examined this application in great detail. These have included Nichols and Boelter (1982), Rock *et al.* (1982, 1984a) and Rana and Viraraghavan (1987).

Peat has also been found to be very useful for the removal of oil from oil-in-water emulsions by the dual processes of absorption, and physical removal through coalescence of large droplets. It has been shown to absorb up to 8 times its weight in oil, being selectively wetted by the oil, and releasing the water (McKay, 1980).

Another effective application of peat in pollution studies is the proven ability of peat to remove heavy metals from solution. This capacity was noted as early as 1930 by Nikol'skii and Parmanova (1939), who demonstrated the removal of Cu, Zn, Pb, and Hg, within the pH range of 3 (below which metals leached) and 8.5 (above which the peat decomposed).

**4:2.3.3.2** Removal of pesticides.

Very little work has been carried out to date on the removal of pesticides by peat, as examined in this study.

Brown *et al.* (1979) used peat to remove dieldrin from river water, sewage effluents and dyehouse effluents. A removal rate of 88% from water and sewage effluents compared very favourably with removal by activated carbon (99%). However, removal from dyehouse effluent was less successful, at 57%, even after coagulation with ferric chloride. They attribute this to competition with other organics in the effluent. They also note that passage through the peat filter cleaned the chromatogram of chlorinated organics, which were present in large numbers in the untreated effluent fractions.

McCarthy (1990a) used an acid treated peat to remove organic pesticides, paraquat, diquat and amitrole. Removal efficiencies of close to 100% were achieved, except for amitrole at pH 7 (it becomes cationic at pH 5.5). Under the conditions used, a range of flow rates, pH values and ionic strengths did not markedly reduce removal efficiency.

4:2.3.4 Column studies.

A number of investigators have used both batch and column systems in the study of the pollution abatement properties of peat. Batch measures of capacity have been consistently lower than column measures.

Ayyaswami and Viraraghavan (1985) found that peat removal of organics in batch study was just 40-50%. In a further study (Viraraghavan and Ayyaswami, 1989), they increased these efficiencies to 60% for BOD, but were still well below those seen in column studies. Higher concentrations of peat showed reduced efficiencies, due to the leaching of organics from the peat (2.5g/100ml of peat leached 3.1 mg/l BOD, 120.5 mg/l COD, 51.8 mg/l TOC, 1.28 mg/l PO4-P, and 4.68 mg/l TKN).

Because peat in solution leaches undefined organic materials, effluents that arise from the application of chemicals, such as sheep dip, to peat columns cannot be defined. In this way, they resemble the effluents from standard waste treatment plants far more than those from, say, resin adsorption systems (which have a similar mode of action).

As a result of the complex nature of peat leachates and effluents, it was considered here that the suitability of the peat would be best assessed by the inclusion of a toxicity test in the study, rather than by chemical analysis in isolation, which is normally used. Thus, the effluents were analysed by cell culture and *Artemia* also.

Among the many column studies that have examined the use of peat for the removal of specific pollutants, there have been many different types of column design, and variations of operating conditions. Examples of these are given in Table 4.1.

The primary conditions of interest for effective operation are the peat bed-depth, the bulk density, and the flow rate. Bed-depth and flow rate determine the contact time of the liquid phase with the peat. The bulk density also affects this, but is more important in relation to ponding, as a result of clogging.

Table 4.1

Operating conditions of some previous peat column studies

| Col. diam. (cm) | Bed depth (cm) | Bulk density ($kg/m^3$) | Loading rate (cm/day) | Pollutant | Reference |
|---|---|---|---|---|---|
| 5 | 25 | 137 | 2934-6885 | 2,4-D | Cloutier *et al.* (1985) |
| 10 | 30 | --- | 200/800/ 5000 | Oil | Mathavan and Viraraghavan (1989) |
| 10 | 20/25/30 /35/50 | 82-100 | 6.37/8.91/ 11.46/14.0 | Septic tank effluent | Rana and Viraraghavan (1987) |
| 10 | 10/20/30 /60/90 | 100-120 | 2.5-61.1 | Septic tank effluent | Rock *et al.* (1984) |
| 10 | 25/30 | --- | 200 | Oil | Viraraghavan and Mathavan (1988) |
| 10 | 30 | --- | 355 | Food industry wastes | Viraraghavan and Kikkeri (1988) |

4:2.2.7 The use of peat 'fibre'.

There are few documented studies on the use of peat fibre as a specific portion of peat. Kavanagh *et al.* (1990) demonstrated its IEC to be lower (72 meq/g) than those of other Bord na Mona peats (110-118 meq/g). However, its hydraulic properties are better than those of raw peat, and for this reason its use in pollution treatment applications is being investigated (Henry, 1993).

Coffey and Kavanagh (1989) describe the use of peat fibre in a biofilter. The airflow is cooled to bacterial optimum temperature before passing over the bed, humidity is carefully controlled, and vaporised fats and other solids are scrubbed out. Greater than 90% efficiency is predicted, with the possibility of specific seeding being required. Filter life is expected to be very high, at 5-10 years, if no fouling occurs.

## 4:3 Results and Discussion.

This section is divided into the results and discussion of the toxicity of the metalliferous effluents, and the sheep dip study. Common to both sets of experiments are the protocols used for pre-treatment of samples. As these are an important considerations in *in vitro* toxicity testing, their implications are discussed in an initial section.

### 4:3.1 Pre-treatment of effluent samples for cell culture analysis.

There have not been many previous studies on the use of cultured cells for the analysis of effluents, or other environmental samples. Those that have been carried out to date have tended to be directed towards assessment of the sensitivity of specific cell assays, rather than the analysis of a large volume of environmental pollutants, or the comparison of cell assays with *in vivo* systems (as was the objective of this study). For this reason, there has not been a great deal of discussion in the literature on the technical aspects of analysing undefined effluents etc. *in vitro*.

There are a number of technical points which must be considered. The most fundamental of these is whether the sample should be pre-treated in any way. In chemical analysis, physical or chemical pre-treatment of the sample is common, and is designed to isolate and preserve the chemical species of interest for analysis.

For example, samples for analysis of metals are amended with acid, to solubilise all of the entrained metal. However, consideration of this type of amendment and fractionation is not always given when interpreting the levels of particular parameters analysed. This can lead to incorrect conclusions on the speciation of constituents in a sample. The effect of quoting 'available', or 'unavailable' metals have been discussed previously in section 4:2.1. (p. 132)

In effluent toxicity testing, the material is almost completely undefined. With *in vivo* testing, effluents are often applied untreated (in particular, samples of low toxicity are rarely concentrated). However, with *in vitro* testing, there are two pre-treatments which are often necessary. The first is sterilisation of the sample, by filtration or heat, the second is concentration of low toxicity samples.

Both could have implications for interpretation of cell culture results. Filtration could fractionate a waste, possibly removing some toxins, while failure to sterilise samples could introduce uncontrolled variables, such as inhibition of the cells by microbes, or metabolism of the sample by

microbes during the test. Heat sterilisation could destroy heat labile toxins. Concentration of samples, by distillation, resins etc., could create chemical species that did not exist in the sample, or break down toxins that did exist.

The studies that have been carried out to date have used a variety of protocols, emphasising the options that are available, and the lack of discussion on the topic.

Christian *et al.* (1973) assessed the use of an L929 assay for the analysis of drinking water. Samples were untreated, treated by reverse osmosis/activated carbon or concentrated by fractional distillation. There is no discussion on sterilisation of the samples, or the implications of pre-treatment. Richardson *et al.* (1977) also used L929 for the determination of the toxicity of oil-refinery effluents. They prepared samples by reconstituting double strength media with the effluents. The effluents had been previously sterilised by 0.45µm filtration, but this was not discussed.

Christian and Nelson (1978) used almost exactly the same protocol for assessing the toxicity of coal extracts (0.22µm filtration), but also applied sterilised coal particles directly. It was concluded that it was not possible to separate the effects of particulates, and that of materials possibly leached from them over the test period. It was decided that all future samples should be filtered. Again, there was no discussion of the possible effects of pre-treatment.

Kfir and Prozesky (1981) used sewage effluents to reconstitute powdered media, followed by 0.22µm filtration. Sewage effluents in particular are likely to have large amounts of materials associated with particulates. There is no discussion of this.

Wilcox and Williamson (1986) carried out extensive pre-treatment of drinking water and raw water samples, by concentrating on resin, eluting with acetone, evaporating, reconstituting in water, 0.45µm filtering, and storage by freezing. There is no discussion on the possible effects of pre-treatment, but the treated water samples were shown to have clastogenic effects, which were attributed to the chlorination process.

Terazona *et al.* (1991) used an almost identical system for the concentration of estuarine samples for analysis by RTG-2, but did not discuss the assay in any detail.

Hunt *et al.* (1986), in a review of *in vitro* assays applied to the water industry, do discuss the topics of sterilisation and concentration. They recognise the possibility of the production of artefacts during concentration, or removal of toxins during filtration. They recommend assessing each

situation individually, but recognise that concentration and sterilisation may often be essential. In a later study (1987), they assessed the toxicity of a number of water samples. Pre-treatment was 0.22µm filtration, and the samples were used to prepare media from 10x concentrate. There is no further discussion of the topic of pre-treatment.

While the implications of pre-treatment for cell culture appear similar to those for chemical analysis, they assume a greater importance in the toxicity test, as the toxic reaction is a cumulative effect of the interactions between all of the chemical species present.

The ability to assay concentrated samples could be looked on as an advantage, when the objective is to determine the effectiveness of, for example, a treatment system, as with Wilcox and Williamson. However, with effluents for discharge, or environmental samples (natural waters etc.), there is a distinct possibility that concentration could give an inaccurate picture of toxicity. Where the objective is to compare several samples of different origin, it may be feasible, but to determine absolute toxicity, it must be regarded as a suspect strategy.

The conclusion reached from review of these studies, the implications of pre-treatment, and the situation with chemical testing is that pre-treatment of samples is a topic that needs to be addressed at the outset of each study. Results generated must be interpreted within the confines of the parameters chosen. The requirement for sterilisation is considered here to be essential, and unlikely to be an issue where there are many options.

In this study, the metal samples were 0.45µm filtered, as part of the replica treatment process, and so would not be regarded as additional pre-treatment. The column effluents were autoclaved at 110 $^{o}$C, a less harsh regime than is used in other cases.

### 4:3.2 Metalliferous effluents.

#### 4:3.2.1 Source/composition of effluents.

The metalliferous effluent used in this study was obtained from an Irish electroplating company. The production system uses a series of 33 dip tanks which contain various solutions used in the dipping processes for a number of possible plating operations. These solutions include hot alkali (pH 12), electrolytic alkali, $H_2SO_4$ (various strengths), CuCN, sodium hypochlorite, phosphoric acid, boric acid, hydrazine, sodium metabisulphite, lacquer (including unspecified solvents), water, and various nickel and chromium

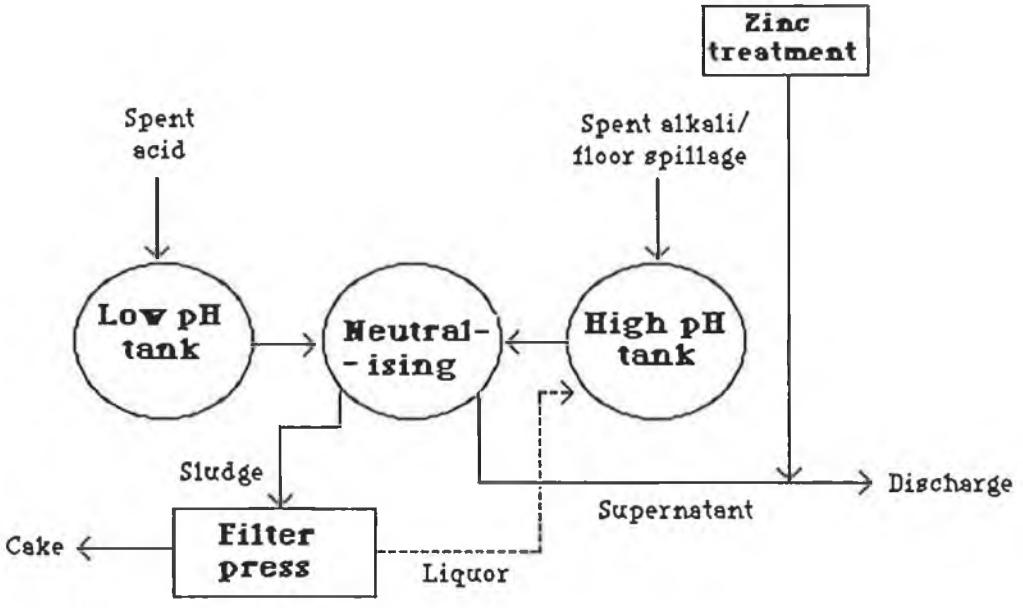
solutions. Zinc polishing (prior to plating) is carried out in a separate system, creating a separate waste.

The waste treatment system, as shown in Figure 4.1, consists of channelling of the wastes from the separate tanks through a series of underground pipes, according to pH, into either a spent acid tank (referred to in later sections as the low pH tank), or a spent alkali tank (referred to in later sections as the high pH tank). Floor spillages are channelled to the high pH tank, as are liquors from the main filter press.

Effluents from these two tanks are fed into a third (neutralising) tank, at a controlled rate, to achieve a pH of 6-9. This causes metals to precipitate. Precipitation is aided by a set (w/v) addition of sodium metabisulphide, and a settlement time of 72 hours. This produces a sludge, which is fed to a filter press, and a liquor, which is the final effluent.

The final effluent from the neutralising tank, along with the effluent from a separate zinc filter bed, are discharged directly to a waterway. The sludge from both filter presses is drummed, and exported for disposal.

**Figure 4.1**
**Electroplating waste treatment process**




As described in section 2:3.4 (p. 16), samples were taken from the low pH tank and from the high pH tank. From these, three fractions of

effluent were prepared in the laboratory. These were a low pH sample (hereafter referred to as the Low pH fraction), which was the low pH effluent, amended only by 0.45µm filtration, and two neutral fractions (pH 7.4). The first neutral fraction (hereafter referred to as the Discharge fraction) was prepared by mimicking the nominal procedure at the waste treatment plant (substituting 0.45µm filtration for the filter press), the second was by neutralisation with 6N NaOH, again 0.45µm filtered (hereafter referred to as the NaOH neutralised fraction).

These waste fractions were analysed for metal content by atomic absorption spectrophotometry, and the concentrations obtained are presented in Table 4.2. It must be stressed that the quantities of metal in the simulated discharge effluent are not those found in a normal effluent discharged, as evidenced by routine analysis results.

The normal process would be continually monitored for metal content, and discharge controlled in this way. The fraction was prepared in order to provide an effluent that would have gone through at least the basic waste treatment process, and thus would be substantially different from the Low pH fraction.

When the laboratory simulation was being carried out, a volume of 2.75 litres of high pH was required to neutralise 500 ml of low pH effluent. It was considered at the time that this may possibly cause unacceptable dilution of the Discharge fraction, and create a sample that may be of very low toxicity. In order to maximise the contrast between the Low pH and neutral fractions, it was decided to create a third fraction. This was prepared by neutralising the low pH effluent with 6N NaOH. In this case, just 15 ml of 6N NaOH was required to neutralise 300 ml of the low pH waste.

**Table 4.2**
**The concentrations of four selected metals in three metalliferous effluents (mg/l)**

| Metal | Low pH | Discharge | NaOH neutralised |
|---|---|---|---|
| Zinc | 540 | 38.1 | 45.8 |
| Copper | 77.5 | 8.1 | 9.0 |
| Nickel | 120.2 | 16.2 | 59.8 |
| Chromium | 24.1 | 2.03 | 2.33 |

As can be seen from the table, the Discharge fraction does contain significantly less metals than the NaOH neutralised fraction (1:1.81, total metal content), though not as low as the dilution might have suggested (c. 1:5). This indicates that the high pH waste also contains significant amounts of metals.

The waste treatment system described above is based on the premise that the major pollutant in the waste is the metals, and that the effluent for discharge can be controlled by reducing the metal content, and by monitoring metal concentrations in the final effluent. The treatment system itself is little more than straight pH precipitation of the metals.

This approach is echoed also in the licence for discharge, prepared by the local authority. The parameters specified are those which describe classical oxygen and nutrient management, along with cyanide, and the major metals used in the process. Thus, pH, oxygen, solids, BOD, ammonia, phosphorus, temperature and cyanide are given specific limits. The metal concentration for discharge is controlled by a 'total metals' limit of 1 mg/l.

The frequency of analysis is daily for the metals, cyanide, oxygen, pH and temperature. Nutrient parameters are measured weekly. Occasional samples must also be taken for lead and cadmium.

This would be considered a typical licence for such a plant, and stems from the prevailing view that effluents can be managed by controlling the discharge of specific constituents, without regard for the effect of the effluent as a whole.

4:3.2.2 Toxicity to cultured cells.

The toxicity of the metal effluents, as well as a number of test variables thought to influence the indication of toxicity given, were examined by cell culture using the L929 cell line, and the crystal violet elution assay.

**4:3.2.2.1** $EC_{50}$ determination.

The data files for the determination of the toxicity of the metalliferous effluent fractions by cell culture are presented in appendix 8:3.1, as follows; Discharge fraction, Tables 8.70-8.72, appendix page xv; Low pH fraction, Tables 8.73-8.75, appendix page xv; NaOH neutralised fraction, Tables 8.76-8.78, appendix page xv. The resulting $EC_{50}$ data is presented in Table 4.3 (p. 152).

**Table 4.3**
**The toxicity of three metalliferous effluents to cultured L929 cells**

| Effluent | $EC_{50}$ (% effluent) | SD (% inhibition) |
|---|---|---|
| Low pH | 0.554 | 0.055 |
| Discharge | 2.07 | 0.59 |
| NaOH neutralised | 1.49 | 0.059 |

The most toxic of the three effluents was the Low pH fraction, which also had the highest concentration of total metal (Table 4.2, p.150). The least toxic was the Discharge fraction, which had the lowest concentration of total metal.

With the Low pH fraction, there was no expression of acidity (yellowing of the media) at the highest concentration applied. Therefore, it is concluded that pH did not interfere with the $EC_{50}$ determination of toxins in the effluent.

Inter-trial and intra-trial variation was similar to that seen in the predictive hazard assessment trials, and discussed in section 3:3.3 (p. 103).

**4:3.2.2.2** Technical considerations.

Metal (and many other) effluents are often at low pH. Cultured cells are extremely pH sensitive, which makes pH itself a significant toxic mechanism. The Low pH fraction of effluent was chosen to examine the effect of pH on the cell culture system. This fraction (pH 1.28) contained the highest amount of metal (all in solution).

While pH itself is toxic, a technical problem envisaged was that this effect (as indicated by yellowing of the media) would mask any toxicity of the other components of the waste. On discharge of an acidic effluent, low pH would be buffered out, but other toxins could still be present.

This would be a significant limitation to the use of cell culture for this waste. In the range finding trials, yellowing of the media was seen down to 1% of this effluent. As an additional trial, PBSA was used as diluent, to establish whether this would provide any further buffering of the effluent. As noted in 2:4.2.3 (p. 19), no significant buffering was found.

However, in the toxicity trials, with careful mixing, none of the trial concentrations were affected (highest conc. used, 1.8%), thus none of the measured toxicity can be attributed to low pH. Also, there was no

precipitation seen, either in the dilution phials, or in the wells of the test plates over the incubation period. This suggests that all of the metals present in the test concentrations were in solution. Thus, pH was not an obstacle to the analysis of this effluent.

A second important consideration, which is a corollary of the above, is the fraction of the total metal content that actually exerts toxicity. At the pH of most ecotoxicity tests (7.0), and cell culture tests in particular (7.4), an equilibrium exists, between metals in solution and particulate metals. Davies *et al.* (1976) have shown that it is the available (or soluble) portion of the total metal content of a sample that is toxic, and therefore that only this portion should be measured/considered.

They suggest filtration through a 0.45μm filter to distinguish between 'total' and 'dissolved' metal portions. This protocol was used here, and had the dual function of sterilising the sample for analysis. Thus all of the metals present in the Discharge and NaOH neutralised fractions can also be said to be in solution.

A third technical consideration is the origin of the metal in solution. In testing the toxicity of metals, there are a number of ways in which metal solutions can be prepared. These include numerous salts, and acidified standards (either neutralised before a trial, or retained at low pH). The choice of preparation is very important, but is a consideration that has not received a great deal of attention from researchers comparing the toxicity of various metals in various systems.

The majority of these prepare metals from salts, but without describing a rationale for the choice of salt (Rachlin and Perlmutter, 1968; Cherian, 1985; Knox *et al.*, 1986; Babich and Borenfreund, 1986, 1987, 1988, 1990). A small number have neutralised acidified standards (Marion and Denizeau, 1983a,b). None previously have used the acidified forms directly (as was done with the Low pH fraction here).

Hulme *et al.* (1989) have considered the question of the salt of metal used, and conclude that this is more important than QSARs such as the Hansch parameter. They found the sulphate salts to be the most toxic, and the chloride salts to give the best correlation between *in vivo/in vitro* data.

In this study, the origin of the metal in solution is not a variable, as the samples are complex effluents. Thus, no information is available on the speciation of the metals. This, along with the divergence of approach to toxin preparation in the literature makes the comparison of the results with published data very difficult.

**4:3.2.2.3** Interpreting and applying toxicity data.

As can be seen from Table 4.3, the most toxic of the three was the Low pH fraction, the second most toxic was the NaOH neutralised fraction, and the least toxic was the Discharge fraction. This was roughly in keeping with the total selected metal content of the three (i.e. copper, zinc, nickel and chromium). The Low pH fraction contained 761.8 mg/l total metal, NaOH neutralised fraction contained 116.93 mg/l total metal, and Discharge fraction contained 64.43 mg/l total metal (Table 4.2, p. 150).

If all of the toxicity was due to the metal content of the effluents, then a reduction in metal content should produce a concomitant reduction on toxicity (as indicated by the metal/metal or toxicity/toxicity ratios for any pair of effluents). Thus, the metal/toxicity ratio sets should be largely equivalent. However, this is not the case.

The ratio of metal content in the Low pH fraction to the NaOH neutralised fraction is 1:6.51, whereas the ratio of toxicity is 1:3.74. The ratios for the Low pH fraction to the Discharge fraction are 1:11.82 (metal) and 1:2.69 (toxicity). The ratios for the NaOH neutralised fraction to the Discharge fraction are 1:1.81 (metal) and 1:1.39 (toxicity).

The conclusion, therefore, is that there are other toxins present in the effluent fractions than the specified metals, as shown by cell culture results, and that the relative importance of these is higher in the neutralised fractions of the waste. Another example of this is the comparison of the toxicity results with some from the literature.

Specific examples of metal toxicity to cultured cells (as opposed to studies on mechanisms of toxicity etc.) from the literature are few, and not directly comparable. However, they do, in general, indicate that metal toxicity is generally relatively low in these systems. Two examples are given in Table 4.4, along with the results from this study, given as the measured concentration of each metal present at the $EC_{50}$ of the effluents (e.g. 540 mg/l zinc in Low pH fraction x $EC_{50}$ {0.554%} = 2.99 mg/l zinc).

What this table shows is that metals in the three effluent fractions at the point of $EC_{50}$ are at much lower concentrations than were required to cause median toxicity ($EC_{50}$s) with other cultured cells. This suggests that the concentrations of metals present are unlikely to be responsible for all of the toxicity at this point. This is before consideration of the proven antagonism shown by a number of metal matrices (4:2.1.2, 4:2.1.3, pp. 133, 135).

Table 4.4

**Examples of selected metal toxicities to cultured cells, compared to their concentrations in each effluent, at the point of $EC_{50}$**

| Metal | Cell line | | Effluent fraction | | |
|---|---|---|---|---|---|
| | BF/2* | 3T3-L1+ | Low pH | NaOH neut. | Discharge |
| | Metal $EC_{50}$ (mg/l) | Metal $EC_{50}$ (mg/l) | (mg/l at effluent $EC_{50}$) | (mg/l at effluent $EC_{50}$) | (mg/l at effluent $EC_{50}$) |
| Zinc | 12.42 | 4.56 | 2.99 | 0.682 | 0.789 |
| Copper | 34.95 | 1.27 | 0.429 | 0.134 | 0.168 |
| Nickel | 117.5 | 18.79 | 0.666 | 0.891 | 0.335 |
| Chromium | 1445.5 | 291.2 | 0.134 | 0.035 | 0.042 |

* = Babich *et al.* (1986). + = Hulme *et al.* (1989).

While there are some factors restricting the comparison of these data, such as uncertainty of the 'available' metal concentrations in the literature studies (as metals were prepared from salts, and no measurements were made), it is likely that the general trend holds true.

Examination of the production process also strongly supports the view that other toxins are likely to be present in the effluents in significant quantities. Many toxic materials other than the metals specified in the licence are used in the process. Among these are cyanide, NaOH, $H_2SO_4$, sodium hypochlorite, boric acid, phosphoric acid, hydrazine, sodium metabisulphite, water based lacquer, unspecified solvents, and other materials (including other metals, albeit low toxicity metals such as potassium, sodium, calcium etc.). Control of discharge by selected parameters, such as total metal content, while being the most common approach to the management of such effluents, demonstrates a lack of awareness of the importance of toxicity, and of the complexity of the topic.

Individual effluents can be thought of as unique entities, particularly when considering the constantly changing nature of production systems, and waste treatment systems. In designing waste discharge licences, account should be taken of the unique characteristics of each system, and blanket limits for specific parameters (such as 1 mg/l total

metal content) should not be used unless they can be shown to be directly linked to the environmental hazards posed by the changing effluent characteristics. In reality, no single chemical parameter is capable of being used in this way. A biological toxicity index is the only parameter capable of mapping the effects of variable effluents.

In this particular case, the effluent licence specifies a number of parameters for analysis. As is generally the case, the majority of these refer to classical effluent oxygen and nutrient management. The parameters specified which could be said to be directly intended to control toxicity are limited to cyanide (0.05 mg/l), and total metals (copper, nickel, zinc and chromium) at less than 1 mg/l.

A practical example of the limitations of blanket limits, such as 1 mg/l total (selected) metal contents is as follows. The (total metal) $EC_{50}$ of the Low pH fraction was 4.22 mg/l (total metal content x 0.554%), that of the NaOH neutralised effluent was 1.74 mg/l, and that of the Discharge effluent was 1.33 mg/l. As a factor of 1/100 to 1/1000 of the $EC_{50}$ should be applied as a safety margin for such an effluent (giving an effective metal limit of 1-4 µg/l total metal), a limit of 1 mg/l is wholly insufficient, as it would allow discharge of these quantifiably toxic fractions.

This is quite apart from the fact that some metals, such as copper are, in isolation, toxic to aquatic life at levels well below 1 mg/l ($Cu^{2+}$ 48 hr. $IC_{50}$ to *Daphnia magna* = 0.093 mg/l, 96 hr. $LC_{50}$ to *Salmo gairdneri* = 0.253 mg/l {Khangorot and Ray, 1987}). A fact that should preclude such limits in the first instance.

Only a biological system, such as cell culture, could demonstrate these concepts and effects, and the application used here shows that complex effluents can be analysed without major technical problems. Most importantly, the results show that the cell culture system could be used effectively to determine a safe discharge regime for these effluents, and is capable of providing monitoring information, to constantly adjust any such management system.

**4:3.2.2.4** The effect of serum.

The data files for the cell culture media serum experiments on metalliferous effluent (Low pH fraction), as described in section 2:4.5 (p. 22), are presented in appendix 8:3.1.1, Tables 8.79-8.87, appendix page xvi. The resulting $EC_{50}$ data are presented in Table 4.5, and also in Figure 4.2.

**Table 4.5**

**The effect of media serum on the toxicity of a metalliferous effluent (Low pH) to L929 cells**

| Serum conc. (%) | $EC_{50}$ (% inhibition) | SD (% inhibition) |
|---|---|---|
| 1 | 0.418 | 0.012 |
| 5 | 0.601 | 0.018 |
| 10 | 1.483 | 0.371 |

Metal complexation and equilibrium reactions in the environment are well documented. Also, the affinity of proteins for metals is the basis of an important biological response to metal toxicity, i.e. the production of metallothioneins. This has led to extensive discussion in the literature on the effect of media serum on the toxicity of metals to cultured cells. The effects have been studied for a range of individual metals (Marion and Denizeau, 1983a,b; Cherian, 1985; Stark *et al.*, 1986; Blust *et al.*, 1986), though not for complex metal effluents.

Reduction in toxicity indication is due to the dense protein nature of serum, which binds to materials, making many xenobiotics (most notably metals) 'unavailable', in terms of expressing their toxicity. The observed effect is the basis of criticism levelled at the use of cell culture tests.

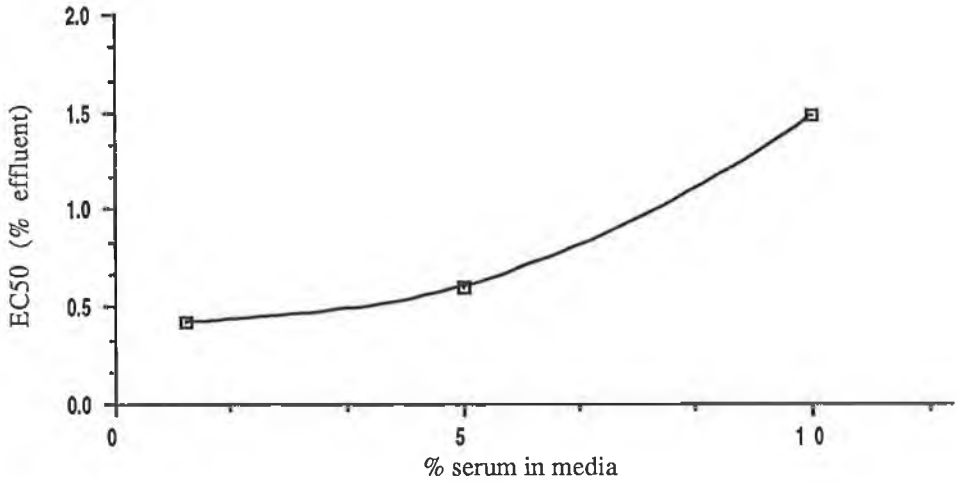
The results in this study show that the serum content in the media does have a quantifiable effect on the toxicity of metalliferous effluents to L929 cells in culture. However, the degree of effect does not reflect the difference in media serum concentration, with a tenfold increase in serum concentration producing a 3.5 fold reduction in toxicity indication (this compares with results from Babich and Borenfreund {1990} who found that an increase in media serum from 1-10% caused a four fold reduction in the toxicity indicated for cadmium).

While the result is significant, it is not considered to be a major effect, over the range of serum concentration tested, and not one that would preclude the use of cell culture systems for this type of analysis. Also, these effects are in common with almost all other test systems (Davies *et al.*, 1976; Cherian, 1985; Blust *et al.*, 1976).

Any analytical system is carried out according to an established protocol, and the results can only be precisely interpreted within the particular parameters of the test. However, much wider effective use can often be made of the data.

**Figure 4.2**

**The effect of media serum on the toxicity of a metalliferous effluent (Low pH) to L929 cells**




For example, a 5 day BOD test describes only the oxygen demand of a material at 20 $^{o}$C, over 5 days, in the dark. Despite these restrictions, it is a very valuable tool in predicting the oxygen demand of a range of materials, in a variety of environmental conditions.

Similarly, media serum is necessary for the culture of cells, and cannot currently be dispensed with. However, if a standard amount of serum is used for the culture of any one cell line, then the results should be expressed as being obtained in the presence of that amount of serum. The data can then be used freely, for comparative purposes.

While the effect measured is significant, it is considered here to be one that could be managed by standardisation of serum content, and interpretation of results within the limitations of standard protocols.

In fact, it is considered here that cell culture has particular advantages in this area, in that the effects of serum can be quantitatively measured, and other important reactions can also be studied. These include reactions varying from metal metabolism (Cherian, 1985; Babich and Borenfreund, 1988), to metal antagonism (Christian *et al.*, 1973; Babich *et al.*, 1989), to acclimation to metal toxicity (Cherian, 1985).

**4:3.2.2.5** The effect of exposure time.

The data files for the cell culture exposure time/seeding density experiments on metalliferous effluent (Low pH fraction, $6x10^4$ cells/ml), as described in section 2:4.6 (p. 22), are presented in appendix 8:3.1.2, Tables 8.88-8.92, appendix page xvii. The resulting $EC_{50}$ data are presented in Table 4.6, and also in Figure 4.14.

A single seeding density was used in this experiment. This was chosen as $6x10^4$ cells/ml, as earlier indicated that growth of a lower seeding density would not give sufficient absorbance over the first 24 hours to calculate $EC_{50}$s accurately.

**Table 4.6**

**The effect of exposure time on the toxicity of a metalliferous effluent (Low pH) to L929 cells**

| Exposure time (hours) | $EC_{50}$ (% effluent) |
|---|---|
| 9 | 0.924 |
| 26 | 0.713 |
| 47 | 0.715 |
| 73 | 0.741 |
| 95 | 0.735 |

Both exposure time and seeding density can affect the toxicity of a chemical indicated for a cell line. However, the effect of seeding density on response in routine studies would be minimal, as a precursor to any such study would be the establishment of the optimum seeding density for the particular cell line and conditions used, i.e. that which would give log phase growth after 24 hours (for toxin exposure), and which would give sub-confluent growth (80-95%) over the course of the trial.

Thus, the choice of seeding density would be objective, and, to a large degree, a non-variable. As with serum concentration in the preceding section, the results would be interpreted as being within these parameters.

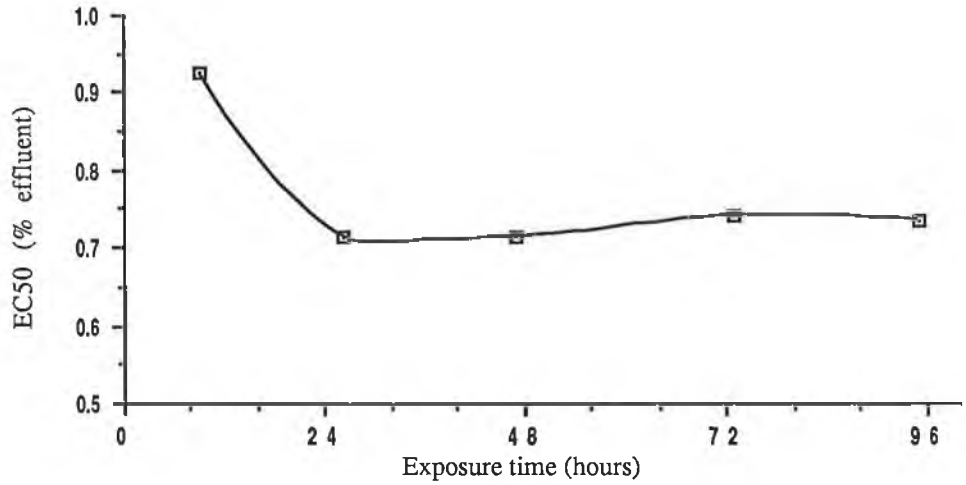
The effect of incubation time has to do with whether the toxin is direct acting, or whether it requires metabolism before exerting its effect. This is a more subtle point, and one which should always be considered when assessing the toxicity of a material.

Benford and Good (1987) examined a number of end points and time regimes. They make the point that an incubation time of over 24 hours

introduces the effect of the toxin on the replication of the cell line. Hunt *et al.* (1987) used a 24 hour incubation time for a comparison of neutral red with other methods. They found it to be relatively insensitive, and attributed this to the short incubation time. They suggest a 72 hour incubation. On the other hand, Babich and Borenfreund (1988) recommend a 24 hour exposure time, with the neutral red assay, for direct acting toxins, and 3-6 days, or the inclusion of S9, for those requiring metabolic activation.

In the case of this study, a 96 hour exposure was chosen, in order to give a direct comparison to the standard whole organism trials also under investigation in the predictive hazard trials (i.e. algal growth inhibition and fish acute toxicity).

**Figure 4.3**
**The effect of exposure time on the toxicity of a metalliferous effluent (Low pH) to L929 cells**




The results show that the effluent was a direct acting toxin, with all of its effect exerted over the first 24 hours. In fact, as the exposure time increases, there seems to be a slight drop in toxicity indicated. This may be due to the biological responses to metal toxicity (i.e. complexation of the metals by metallothioneins, or the isolation of the metals by the production of nuclear inclusion bodies), becoming more effective over time.

It may also be due to contact inhibition, resulting in closed cell membranes, and a degree of protection from the effects of the toxin as cell numbers approach confluence. This would happen sooner than normal in

this assay, because of the higher seeding density. In any case, the effect was small, and did not significantly alter the toxicity indicated.

These results indicate that a shorter exposure time can be successfully applied in this cell culture system, increasing the amount of data that can be generated, and thus the usefulness of the system.

This trial was carried out at a single seeding density, 6 x $10^4$ cells/ml (higher than normal, for reasons described above). Because of this, no direct measurement can be made of the effect of seeding density. However, comparing the 96 hr. result (0.735), with the earlier 3 x $10^4$ cell/ml 96 hr. result (0.554), suggests that a higher seeding density reduces the toxicity indicated. Again, it is a variable that would be defined at the beginning of any study. As such, it is not considered to be problematic.

4:3.2.3 Toxicity to *Artemia*.

The data files for the determination of the toxicity of the metalliferous effluent fractions by *Artemia* immobilisation are presented in appendix 8:3.1.4, as follows; Discharge fraction, Tables 8.93-8.95, appendix page xviii; Low pH fraction, Tables 8.96-8.98, appendix page xviii; NaOH neutralised fraction, Tables 8.99-8.101, appendix page xviii. The resulting $EC_{50}$ data are presented in Table 4.7.

**Table 4.7**
**The toxicity of three metalliferous effluents to *Artemia salina***

| Effluent | $EC_{50}$ (% effluent) | SD (% inhibition) |
|---|---|---|
| Low pH | 0.866 | 0.008 |
| Discharge | 46.63 | 0.48 |
| NaOH neutralised | 75.40 | 2.08 |

The results show that the indication of toxicity given by *Artemia salina* for the three metalliferous effluents was significantly lower than that given by cultured cells. There are two particular points of note.

The first is that the apparent high toxicity of the Low pH fraction was due to the pH itself. This was measured in the wells, and found to be 1.0% = pH 4.02, 0.75% = pH 6.38, 0.56% = pH 6.80, 0.32% = pH 7.29, 0.10% = pH 7.81. This reduction is at a lower % effluent than seen with the cell culture

trials, as there is a much lower buffering capacity in the artificial seawater media than in cell culture media.

While pH in itself is a toxic mechanism, and would lead to toxicity if discharged directly to the environment, the point of note here is that the *Artemia* system was not able to distinguish between toxicity due to pH, and toxicity due to other components, as was the cell culture system.

The second point of note is that the Discharge fraction of the waste was 1.6 times more toxic than the NaOH neutralised fraction of waste, despite containing 1.8 times less metals.

These results are in keeping with the established low sensitivity of *Artemia salina* adults to metals (other life stages have been shown to be more sensitive), as noted in section 4:2.1.3 (p. 135).

*Artemia* have not been used previously in many studies of effluents, and in particular, of metal effluents. The only major reference available on *Artemia* and effluents was a study where agricultural effluents were used to promote the growth of *Artemia* populations. Only one type (poultry litter and biogas slurry) inhibited growth. All of the others (various combinations of groundnut oil cake, decayed cabbage leaves, super phosphate and bakers yeast) increased growth (Basil and Pandian, 1991).

One other was the examination of the toxicity of a number of combined toxins, including copper, chromium, oil, and an oil dispersant. Verriopoulos *et al.* (1987) used 25 day old adults to examine these relationships. They found that the toxicities of individual metals + oil or oil dispersant were additive, copper and chromium together were slightly antagonistic, but that any mixture of metals + oil and/or oil dispersant was strongly antagonistic.

The test system did have some technical advantages, in that the tolerance of wide ranges of salinity meant that dilutions of up to 75% effluent (6 ppt salinity) could be tested directly (Persoone and Wells, 1987, say that *Artemia* can tolerate 5-150 ppt salinity; Rao and Latheef, 1989, hatched them at 5-75 ppt). However, it is this tolerance of widely variant environmental conditions that is said also to be the basis of the high tolerance to some toxins, such as metals (Persoone and Wells, 1987).

While there is an established low sensitivity to metals, it is also of note that *Artemia* were not sensitive to the other toxic components in the media either, as identified in the cell culture analysis of the effluents. This is of interest, as *Artemia* have been shown to be sensitive to a number of other groups of chemicals (for example in the predictive hazard trials section of

this study), and might have been expected to reflect this. As this was not the case, the conclusion must be that *Artemia* are not useful for the monitoring of metalliferous effluents.

Once again, this reinforces the point that a single organism approach to toxicity testing can be as misleading as a chemical-only approach. The integration of physical/chemical and biological methods is the only approach that will provide all of the information necessary to manage the discharge of effluents, and other materials, to the environment successfully.

4:3.3 Sheep dip.

The diazinon based sheep dip, used in the assessment of the efficacy of a peat based treatment system for the removal of sheep dip from solution, was assessed for toxicity by cell culture and *Artemia* assays, as in 4:3.2. The effect of serum, incubation time and seeding density were also examined in the cell culture assay.

4:3.3.1 Toxicity to cultured cells.

**4:3.3.1.1** $EC_{50}$ determination.

The 96 hour $EC_{50}$ of the diazinon sheep dip to cultured L929 cells, assayed by the crystal violet elution end point, was 26.29 +/- 1.22 mg/l (a.i). The data files for these trials are presented in appendix 8:3.2, Tables 8.102-8.107, appendix page xix.

This classifies the product as moderately toxic, according to this assay, and is less toxic than of any previous index, bar one (when considered as conc. of active ingredient), as noted in section 4:2.2.4 (p. 138). The only assay to give a lower $EC_{50}$ was the rotifer, *Brachionus calyciflorus*, at 29 mg/l. However, it is within one order of magnitude of the indication given by a number of commonly used organisms, such as the invertebrates *Artemia salina* (9.41 mg/l, in this study), and *Gillia aliatis* (11mg/l), and the catfish, *Channa punctatus* (3.1 mg/l). Thus, it would not be seen as a particularly insensitive system.

This is significant when considering the fact that cultured L929 cells are not targeted by the main mode of action of the organophosphate insecticide group (acetylcholinesterase inhibition), and is in keeping with the relative sensitivity of the cell line to dichlorvos in the predictive hazard trials. This again indicates that organophosphates may have a second,

significant, toxic mechanism which is generally eclipsed by the acute toxicity of AChE inhibition.

There is evidence to support this in the literature. Teratogenic effects, unrelated to AChE activity have been seen with a number of organophosphates, but especially diazinon. Histopathological effects have been noted *in vivo*, including vacuolisation of cytoplasm, enlargement of nuclei, and rupturing of cell membranes (Hassal, 1990).

**4:3.1.1.2** The effect of serum.

The data files for the cell culture media serum experiments on sheep dip, as described in section 2:4.5 (p. 22), are presented in appendix 8:3.2.2, Tables 8.108-8.113, appendix page xx. The resulting $EC_{50}$ data are presented in Table 4.8, and also in Figure 4.4.

**Table 4.8**
**The effect of media serum on the toxicity of a diazinon-based sheep dip to L929 cells**

| Serum conc. (%) | $EC_{50}$ mg/l (a.i.) | SD (mg/l) |
|---|---|---|
| 1 | 21.30 | 1.35 |
| 5 | 26.29 | 1.22 |
| 10 | 37.77 | 2.30 |

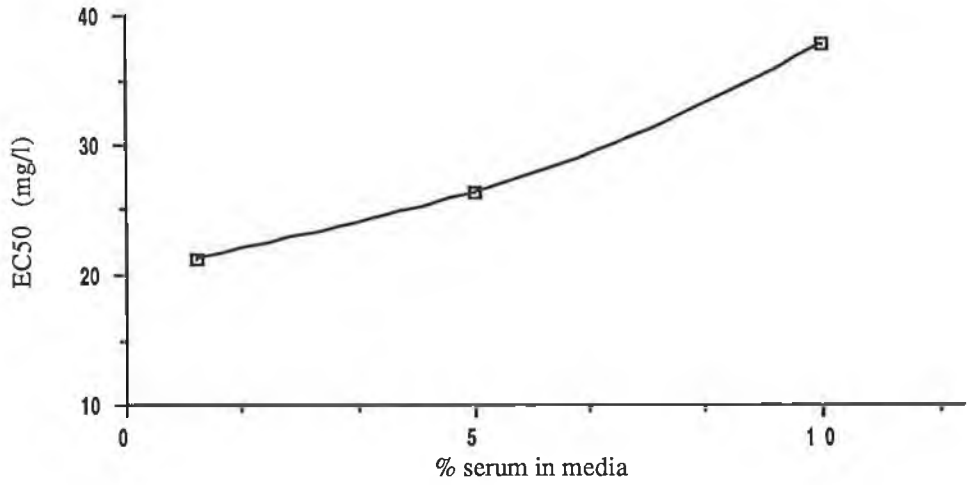
As can be seen, increasing amounts of media serum reduced the indication of toxicity for the dip, with a ratio of 1:1.77 for a tenfold difference in serum concentration (1/10%). This is half the reduction seen with the metalliferous effluents.

This reduction in toxicity is in keeping with the results seen in a number of previous studies. Marion and Denizeau (1983a,b) found that media serum reduced the index of toxicity for cadmium, but not (at the conc. used) of lead. Alderman (1985) found that the antibiotic activity of malachite green was reduced by serum proteins.

Wilcox and Williamson (1986) noted an inverse concentration relationship between the concentration of media serum, and the clastogenic effect of chlorinated water samples. In that study, a concentration difference of 0-15% serum caused a reduction in chromosomal aberrations from 84% to 24%. Rodrigues and Mattei (1987) showed that serum in media reduced the index of toxicity for ivermectin. Babich and Borenfreund (1989),

in their studies, have found DDT, toxaphene, heptachlor and chlordane were more toxic at 2% serum than at 10% serum.

**Figure 4.4**
**The effect of media serum on the toxicity of a diazinon-based sheep dip to L929 cells**




The results here show that, as with the metalliferous effluent, an effect on toxicity is exerted by media serum. However, with the sheep dip, the extent of the effect is about half, with less than a twofold effect over a tenfold decrease in serum concentration. As with the metal effluent, it is unlikely to be considered a major effect, and is not one that would preclude the use of cell culture systems for this type of analysis.

**4:3.3.1.3** The effect of exposure time/seeding density.

The data files for the cell culture exposure time/seeding density experiments on sheep dip, as described in section 2:4.6/2:4.7 (p. 22), are presented in appendix 8:3.2.3, Tables 8.117-8.124, appendix page xxi. The resulting $EC_{50}$ data are presented in Table 4.9, and also in Figure 4.5.

No result was calculable for the $3x10^4$ seeding density at 14 or 24 hours, as the absorbances were too low, and too variable to give a reading. It was a feature of all seeding densities that absorbances in the first 24 hours after toxin application were low, and replicates were highly variable. These figures would not be used to designate $EC_{50}$ data, and are presented in the context of the exposure time/seeding density experiments only.

**Table 4.9**

**The effect of exposure time and seeding density on the toxicity of a diazinon-based sheep dip to L929 cells**

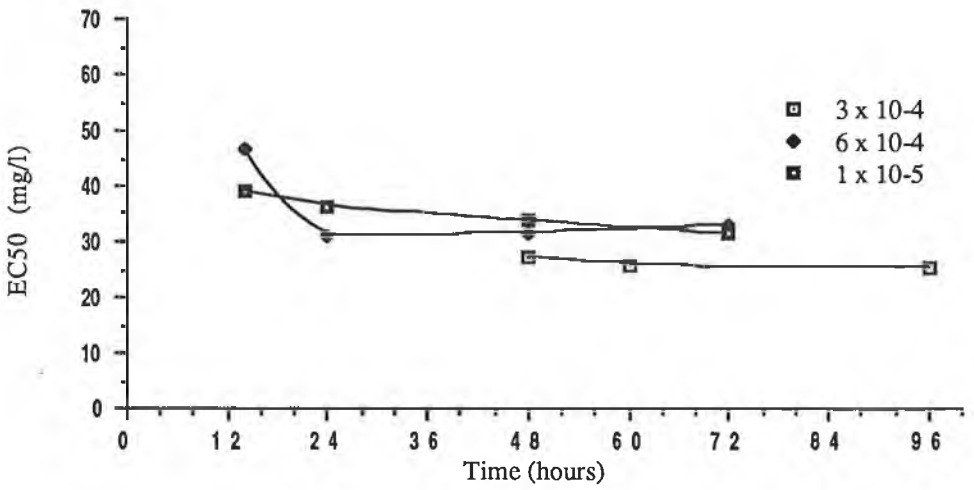
| Time (hrs) | $EC_{50}$ (mg/l) | | |
|---|---|---|---|
| | Seeding density (cells/ml) | | |
| | $3 \times 10^4$ | $6 \times 10^4$ | $1 \times 10^5$ |
| 14 | n.r. | 46.62 | 39.18 |
| 24 | n.r. | 31.13 | 36.27 |
| 48 | 27.06 | 31.49 | 33.65 |
| 72 | 25.89* | 32.92 | 31.20 |
| 96 | 25.08 | n.t. | n.t. |

n.r. = no result. n.t. = no trial. * = 60 hours.

However, in general, results show that higher seeding densities are give a slightly lower indication of toxicity for the sheep dip. This may be due to higher seeding densities reaching confluence before the end of the trial, resulting in contact inhibition. Cells affected by contact inhibition close off cell membranes, and this may give a degree of protection from the effects of the toxin.

**Figure 4.5**

**The effect of exposure time and seeding density on the toxicity of a diazinon-based sheep dip to L929 cells**



In all cases, the most of the toxic effect was exerted after 24/48 hours, indicating that significant metabolism (or metabolic activation) of the sheep dip was not required to express toxicity.

As with the metalliferous effluent, analysis of the sheep dip could be carried out over a shorter period, without any significant loss of sensitivity. This would further extend the applicability of the cell assay in the analysis of this material in the environment, as a shorter assay period would mean that more samples could be analysed, and that results could be obtained, interpreted and applied in a shorter period.

4:3.3.2 Toxicity to *Artemia*.

The $IC_{50}$ of diazinon sheep dip to *Artemia salina* was 9.41 +/- 0.60 mg/l (a.i.). The data files for these trials are presented in appendix 8:3.2.4, Tables 8.125-8.127, appendix page xxiii.

This would classify the sheep dip as moderately toxic, according to this assay. This is less toxic than the majority of invertebrate and fish toxicity indices noted for pure diazinon in section 4:2.2.4 (p. 138), including *Daphnia magna* (0.002 mg/l), *Daphnia pulex* (0.8 mg/l), *Gammarus fasciatus* (0.2 mg/l), Bluegill sunfish (0.136 mg/l), *Channa punctatus* (3.1 mg/l) and rainbow trout (0.09 mg/l). However, it is more toxic than indicated by a small number of species of invertebrates studied, i.e. *Gillia aliatis* (11 mg/l), and *Brachionus calyciflorus* (29 mg/l).

This range of toxicities again underlines the limitations of basing environmental management decisions on single or few parameters, whether they be physical/chemical or biological (Meier *et al.*, 1976; Sastry and Malik, 1981; EPA, 1988; Johnson and Finley; Robertson and Mazzella, 1989; Fernandez-Casalderrey *et al.*, 1992).

4:3.4 Peat experiment.

The final part of the study was the assessment of the efficacy of a peat based treatment system for the removal of sheep dip from solution. Generally, experiments like this would be carried out using chemical analysis of specific parameters only, in this case analysis of diazinon, the active ingredient in the sheep dip.

On this occasion, an additional objective was to determine whether toxicity assay methods could be used to monitor the quality of the effluents from the columns, and to increase the database on the system

generally. This would give a direct comparison of the usefulness of chemical and biological methods, over an extended experimental period.

Cell culture (L929 cell line, crystal violet elution assay) and invertebrate (*Artemia*) tests were used. The significant difference between these experiments, and the metalliferous effluent experiments is that the construction and operation of the peat columns was a long-term exercise, where the toxicity information would be used to assess the operation of the column, as well as describe individual samples.

The following section presents the results of the chemical analysis of the column effluents.

4:3.4.1 GC analysis of column effluents.

The results of the GC analysis of column effluents are presented in Table 4.10. The use of peat fibre in pollution studies is based largely on its adsorptive capacity 9in particular, its low cost relative to other adsorbents, such as activated charcoal, or resins). The physico-chemical nature of adsorption has been well characterised (Freundlich, 1907; Langmuir, 1916; Weber and Gould, 1966, Allen *et al.*, 1988), to the extent that mathematical models can be prepared for particular systems from batch and column studies. However, these models can only describe adsorption in isolation.

If adsorption was the only removal mechanism operating on these peat columns, column life would be expected to be in the order of 3-7 days, at the weight of fibre/concentration of dip/flow rate used.

This was not the case. Removal of sheep dip continued long beyond the projected time, indicating that there was a second, significant removal mechanism operating on the column. Experiments were carried out to test and characterise the second mechanism. These are further discussed in section 4:3.4.1.3 (p.173).

After 14 days of operation, it was necessary to decommission three of the columns. The blank column had given consistent results, and so these could be reasonably extrapolated for the duration of the study. One of each of the duplicate columns was retained (i.e. 100% fibre and fibre/peat mix). The study was ended when the columns ponded. This happened earlier for the fibre/peat mix (45 days) than for the 100% fibre column (66 days). This result was in keeping with the reported superior hydraulic properties of the peat fibre.

Table 4.10:Concentration of diazinon in column effluents (mg/l)

| Time (days) | Blank 100% fibre | Column 1 100% fibre | Column 2 100% fibre | Column 3 Fib./nod. mix | Column 4 Fib./nod. mix |
|---|---|---|---|---|---|
| 0.31 | 0 | 124.7 | 153.0 | 57.7 | 151.6 |
| 0.48 | 0 | 118.7 | 114.2 | 44.5 | 141.2 |
| 0.65 | 0 | 109.6 | 118.7 | 37.7 | 150.1 |
| 1.00 | 0 | 115.7 | 94.2 | 39.4 | 98.8 |
| 1.15 | 0 | 118.7 | 77.0 | 36.0 | 101.9 |
| 1.31 | 0 | 114.2 | 81.7 | 34.3 | 84.8 |
| 1.46 | 0 | 97.5 | 75.4 | 34.3 | 73.8 |
| 1.58 | 0 | 91.1 | 86.4 | 34.3 | 80.1 |
| 1.98 | 0 | 92.7 | 81.7 | 34.3 | 75.4 |
| 2.14 | 0 | 108.1 | 91.1 | 34.3 | 94.4 |
| 2.31 | 0 | 89.5 | 75.4 | 41.1 | 69.0 |
| 2.59 | 0 | 81.7 | 78.6 | 34.3 | 70.6 |
| 2.98 | 0 | 78.6 | 75.4 | 34.3 | 73.8 |
| 3.52 | 0 | 65.8 | 84.9 | 46.1 | 70.6 |
| 4.00 | 0 | 70.6 | 67.4 | 42.8 | 67.4 |
| 4.52 | 0 | 39.1 | 44.9 | 34.9 | 50.1 |
| 4.98 | 0 | 38.6 | 42.0 | 33.0 | 42.7 |
| 5.48 | 0 | 38.3 | 46.4 | 41.6 | 55.2 |
| 6.00 | 0 | 37.2 | 44.9 | n.r. | 51.2 |
| 6.52 | 0 | 41.4 | 46.8 | 49.7 | 59.9 |
| 6.98 | 0 | 43.5 | 50.4 | 52.6 | 61.4 |
| 7.46 | 0 | 54.5 | 56.3 | 56.3 | 62.0 |
| 7.98 | 0 | 52.1 | 56.7 | 53.5 | 71.1 |
| 8.50 | 0 | 78.6 | 75.4 | 73.8 | 89.5 |
| 8.98 | 0 | 69.0 | 62.6 | 65.8 | 64.2 |
| 9.50 | 0 | 62.6 | 61.0 | 75.4 | 59.3 |
| 9.98 | 0 | 59.3 | 72.2 | 56.1 | 80.7 |
| 10.42 | 0 | 67.4 | 69.0 | 54.4 | 70.6 |
| 11.00 | 0 | 64.2 | 61.0 | 73.8 | 83.3 |
| 11.38 | 0 | 64.2 | 57.7 | 65.8 | 49.5 |
| 12.00 | 0 | 59.3 | 59.3 | 61.0 | 70.4 |
| 12.33 | 0 | 61.0 | 69.0 | 81.7 | 69.0 |
| 13.00 | Decommissioned | 64.2 | 66.7 | 78.6 | 80.1 |
| 13.42 | --- | 64.2 | 69.0 | 81.7 | 91.1 |
| 14.00 | --- | 75.4 | 70.6 | 89.5 | 77.0 |
| 17.00 | --- | 56.1 | Decommissioned | 73.8 | Decommissioned |
| 21.00 | --- | 57.7 | --- | 88.0 | --- |
| 25.00 | --- | 91.1 | --- | 80.1 | --- |
| 28.00 | --- | 109.6 | --- | 115.7 | --- |
| 31.00 | --- | 139.7 | --- | 176.5 | --- |
| 34.00 | --- | 202.4 | --- | 211.0 | --- |
| 38.00 | --- | 193.8 | --- | 225.2 | --- |
| 42.00 | --- | 216.7 | --- | 206.7 | --- |
| 45.00 | --- | 188.0 | --- | 237.9 | --- |
| 49.00 | --- | 202.4 | --- | Ponded | --- |
| 56.00 | --- | 236.5 | --- | --- | --- |
| 59.00 | --- | 211.0 | --- | --- | --- |
| 63.00 | --- | 216.7 | --- | --- | --- |
| 66.00 | --- | 216.7 | <---Ponded | --- | --- |

The specific operation of each type of column is discussed in the following sections. No long term studies have been done previously on the use of peat columns for the removal of emulsions from solution. No data was available, therefore, for direct comparison.

**4:3.4.1.1** The 100% fibre columns.

The two duplicate 100 % biofibrous peat columns operated similarly over the 14 days of concurrent operation (Figure 4.6). There was an initial removal of approximately 70%, which improved over the first 5 days to approx. 90%. This dropped gradually over 10 days to approx. 82%. The duplicate column was then decommissioned (Column 2).

The remaining 100% fibre column (Column 1) operated steadily over the next 10 days (Figure 4.7), improving slightly to 85%, when performance began to disimprove sharply to approx. 50%, over the following 10 days. From this point, to the end of the test period, when the column ponded (c. 66 days), performance was reasonably steady, ending at 46% diazinon removal.

**4:3.4.1.2** The fibre/peat mix columns

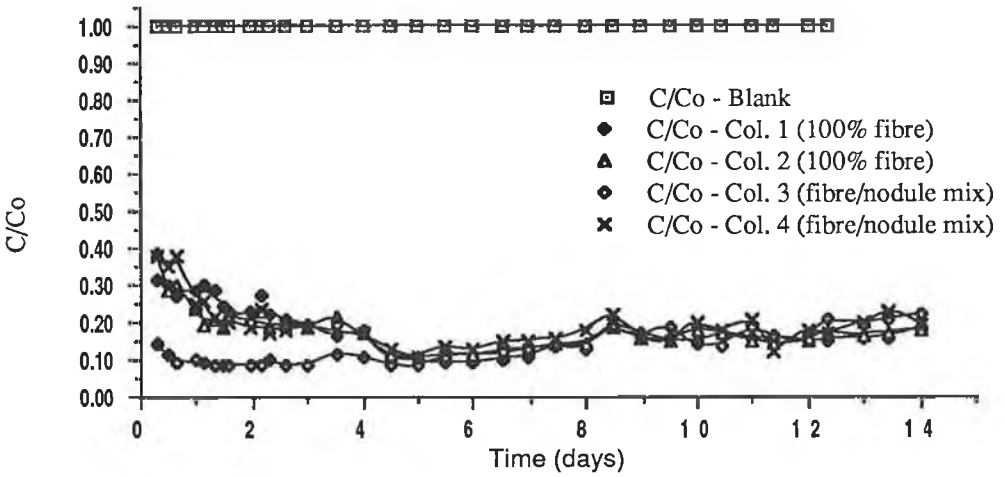
Results from the two fibre/peat mix columns were somewhat different. The first of these (Column 3) had a much better initial performance than any other, at 90% removal over the first 4 days (Figure 4.6). However, over the subsequent 10 days, when the pesticide removal by the other three columns improved, Column 3 performance agreed closely with the others.

It was this column that was retained for the longer-term operation Its performance in the long-term period of operation was very similar to that of the retained 100% fibre column (Column 1), up until the time it ponded (Figure 4.7). However, as noted, this was significantly earlier than the 100% fibre (at 45 days).

The results from the second fibre/peat mix column (Column 4) were different from its duplicate in that they followed the trend seen in the 100% fibre columns closely (Figure 4.8). However, it should be noted that it also generally performed slightly worse than any of these (Figures 4.6, 4.8). It was decommissioned after 14 days.

**Figure 4.6**

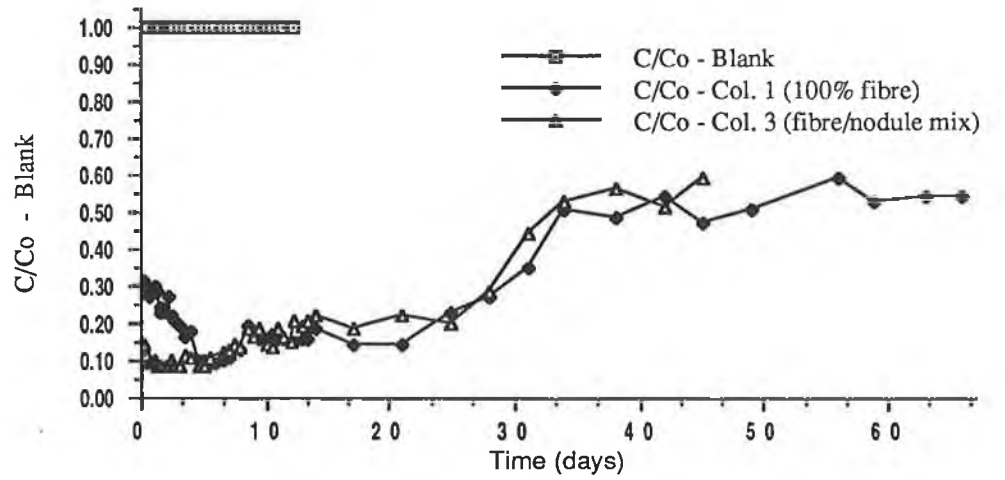
**The concentration of diazinon in column effluents over the first 14 days of operation (all columns)**




C/Co = effluent concentration/influent concentration, giving a ratio which rises to 1.00 when adsorbent is completely exhausted.

**Figure 4.7**

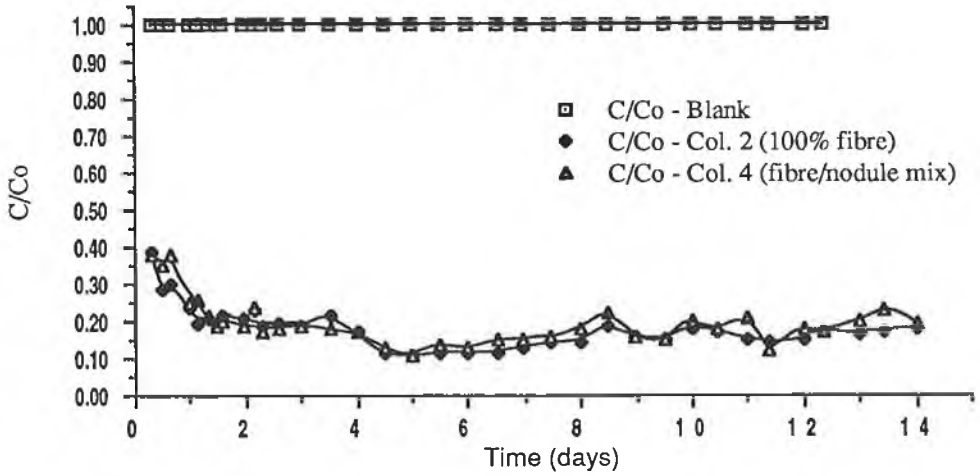
**The concentration of diazinon in column effluents over their entire period of operation: Columns 1 and 3**




C/Co = effluent concentration/influent concentration, giving a ratio which rises to 1.00 when adsorbent is completely exhausted.

**Figure 4.8**

**The concentration of diazinon in column effluents over their entire period of operation: Columns 2 and 4**




C/Co = effluent concentration/influent concentration, giving a ratio which rises to 1.00 when adsorbent is completely exhausted.

There is no explanation for the difference in performance between the two fibre/peat mix columns. If they had both operated at a higher level than the 100% fibre columns, then it could be attributed to the higher adsorptive capacity of the peat portion of the mix, particularly in the light of the fact that this occurred over the first four days of operation (a lifespan for the column that could reasonably be expected from adsorption alone at this concentration and flow rate).

However, Column 4 not only showed a poorer performance, it also was in close agreement with the 100% fibre columns, which might indicate that the errant column is Column 3. If, on the other hand, it was found that there was a difference in compaction, with Column 4 being less compact, it could be surmised that this was allowing a higher rate of flow through the column, and subsequent poorer performance.

As a result of the disparity between the duplicate columns, neither of these can be concluded. It should also be noted again that they operated similarly for the final 10 days of concurrent operation.

4:3.4.1.3 Long-term column trends.

The major pollutant removal mechanism associated with peat is its high ion exchange capacity. This adsorptive capacity is finite, and can be mathematically modelled for families of dose rates, flow rates, peat concentrations, etc., using data from column studies such as this one. From previous studies, the adsorptive capacity of this particular under the particular conditions applied in the study, might have been expected to last approximately 3-7 days.

As can be seen from the results, this was not the case, and the columns continued to remove pesticide until they ponded at 45-65 days. This indicated that there was a second, significant removal mechanism for the sheep dip associated with the peat.

To test this hypothesis, and to determine the nature of the second removal mechanism, batch and leaching experiments were carried out on the peat/dip. The results showed that the second mechanism was the physical removal of large droplets of dip, due to the emulsion status of the aqueous preparation.

A description of these experiments, their results and implications are presented in appendices 8.4 and 8.5.

The ultimate significance of the results, is the implications for quantity of removal and final disposal of the peat fibre. The results show that, over the entire period of operation, the column removed approximately 150 grammes of pesticide, from 230 grammes added. The results from appendix 8.5 show that large amounts of the entrained pesticide could be washed directly from the peat (aside from leaching of adsorbed pesticide).

Thus, the peat can be said to effectively remove large amounts of sheep dip from solution, and product design could maximise this removal. However, careful consideration should be given to the ultimate disposal of the spent peat, as significant quantities of pesticide are likely to be loosely bound, and would be washed out easily.

4:3.4.4 Analysis and storage of sheep dip.

In comparing the efficiency of chemical vs. biological methods in this experiment, consideration must be given to the design and operation of the chemical method also. The following section describes the use of the method, and the results of a storage experiment, which have implications for both chemical and biological assays.

The method developed for the analysis of diazinon in aqueous solution and in aqueous peat column effluents worked well, aided by the high concentrations of pesticide in use in the study, and by the relatively pure nature of the effluents (from a chromatographic point of view).

The relationship between analyte and internal standard peak heights are presented in Table 4.11, and Figure 4.9. The ranges chosen were in anticipation of an initial high rate of removal, followed at some point by a breakthrough, and eventual failure of the column.

**Table 4.11**
**Ratios of DNBP To diazinon peak height for selected diazinon concentration ranges**

| Diazinon Concentration (mg/l) | Ratio - DNBP/Diazinon P.H. |
|---|---|
| DNBP conc. = 6 mg/l | Addition 1/4 |
| 1.6 | 2.6312 |
| 3.2 | 1.3661 |
| 6.4 | 0.6815 |
| n = 3 | Av % SD = 5.13 |
| DNBP conc. = 30 mg/l | Addition 1/4 |
| 8 | 2.4598 |
| 16 | 1.2195 |
| 32 | 0.6064 |
| 64 | 0.2749 |
| n = 3 | Av % SD = 2.57 |
| DNBP conc. = 150 mg/l | Addition 2/2 |
| 60 | 1.6514 |
| 120 | 0.7536 |
| 180 | 0.5282 |
| n = 3 | Av % SD = 2.05 |

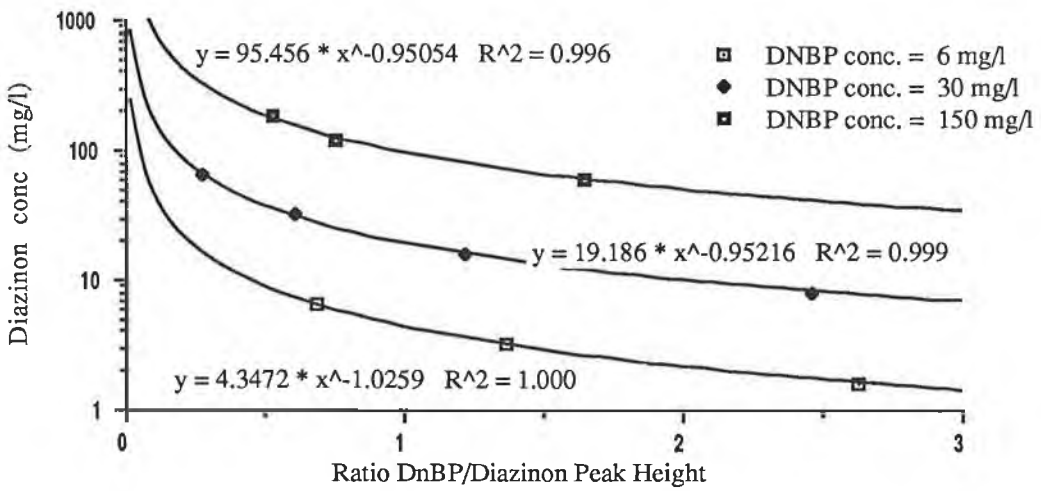
The linearity of the relationships is very good, at virtually 1 for each range. The internal standard was prepared fresh daily, and worked well for the during the study.

One of the possible problems initially envisaged was that materials leached from the peat could cause significant interference in the chromatogram. There are a large number of possible leachates, as outlined

in section 4:2.3 (p. 141). There have been previous examples of humic acids interfering with the recovery of pesticides by solid-phase extraction (Johnson *et al.*, 1991).

In this study, humic acids did not interfere with either the rate of recovery, or the chromatographic resolution. In fact, in the region of diazinon elution, no interferences at all were present. A similar situation was noted by Brown and Bellinger (1979). They found that hexane extractions of a dyehouse effluent contained numerous peaks, due to chlorinated organics, which would have appeared in the dieldrin region. Passage of the effluent through peat columns virtually eliminated these peaks, and also (just as significantly) did not show any donated peaks.

**Figure 4.9**
**Diazinon analysis:**
**Internal Standard curves**




Another factor of interest in the logistics of the study was the need to store effluent samples for a period of time, for toxicity and chemical analysis, and the effect that this might have on concentration.

While GC analysis was carried out immediately over the first 14 days, and weekly over the remainder of the trial, it was necessary to store samples (frozen) for longer periods to allow for toxicity testing. The results of the storage experiment, as described in section 2:6.4.3 (p. 37), are presented in Table 4.12.

The rapid breakdown of diazinon in solution has already been noted (section 4:2.2.5, p. 139). While the solutions used in this study were of

pure sheep dip (as opposed to soil or other seeded solutions), the results show that breakdown at room temperature was nonetheless rapid, with a half life of c. 16 days (60 mg/l conc.). This compares with half-lives of 9, 10 and 17 days obtained by Sethunathan and MacRae (1969) in three types of submerged soils, applied at 50 mg/l. In uninnoculated culture medium, they found 9.57 mg/l to be reduced to 6.7 mg/l over 10 days.

**Table 4.12**
**The effect of storage on the concentration of diazinon**

| Time (Days) | Measured Conc. in Frozen Samples (mg/l)* | % of Measured Conc. in Room Temperature Samples |
|---|---|---|
| 0 | 60.0 (100.0) | 100.00 |
| 7 | 64.0 (106.7) | 68.84 |
| 13 | 65.2 (108.7) | 70.85 |
| 22 | 65.6 (109.3) | 9.19 |
| 27 | 61.0 (101.7) | 8.45 |

* = % of nominal concentration (60 mg/l) in frozen samples in parenthesis.

In contrast, no breakdown was seen in the frozen samples over the 4 week storage period. This is in keeping with the results seen in a number of other studies, where reduced temperature was seen to significantly retard breakdown (Getzin, 1968; Bro-Rasmussen *et al.*, 1970; Suett and Padbury, 1982). This facilitated the storage of effluent samples by freezing without further chemical preservation, for subsequent toxicity analysis.

4:3.4.3 Summary of chemical analysis.

In this study, significant time, and material resources, were required in establishing a chemical diazinon analysis method. In recent years, the number of laboratories analysing for organophosphate insecticides are relatively few, as many researchers consider that their generic short half-lives deem organophosphates to be of low environmental hazard (despite their high acute toxicity, and the fact that a number of organophosphates have quite long half-lives). Thus, no current method for analysis of the sheep dip by GC-FID was available.

Devising the new GC programme was time-consuming and technically difficult. However, once established, it worked well, and supplied direct monitoring data on the operation of the column. Daily analysis was very time consuming, due to the set-up and shut-off times required for the instruments, but, on the other hand, when samples were stored, a large number could be analysed at one time.

The main limitation of the chemical analysis was that it was for one ingredient of the formulation only, the active ingredient. Thus, chemical analysis was not able to demonstrate, in itself, the existence of other important removal mechanisms, as became evident in other parts of the study (although it was used to confirm results, in the batch and leaching experiments). Also, it was not able to define the effluents in terms of toxicity, or identify materials other than the active ingredient.

In conclusion then, chemical analysis was a most useful monitoring tool, from the point of view of following the day-to-day operation of the columns. However, it was of limited use in understanding the removal processes at work, or in defining the precise hazard posed by the dip, or the column effluents.

### 4:3.5 Column effluent toxicity.

The following sections present the results of toxicity analysis of the column effluents. There are two aspects to the interpretation of the results. The first is the absolute toxicity of each column effluent, and whether the toxicity assay could be used to define the effluent for discharge. The second is if the results over the entire period of the experiment could be used to monitor the operation of the columns (as did the chemical method.

#### 4:3.5.1 Toxicity to cultured cells.

The cell culture assay has been demonstrated in the predictive hazard experiments and the metalliferous effluent experiments to be sensitive, and easy to use. In the sheep dip experiments, its sensitivity was lower than that of other systems. While this was not a limitation in the analysis if the product, analysis of the effluents was confined by two experimental constants. These were the field dip application concentration used for the columns, of 400 mg/l active ingredient sheep dip. The second was the final 1/10 dilution of toxins in media used in the particular cell protocol for this study.

The effect that these constants had on the analysis of column effluents is described in the following sections.

**4:3.5.1.1** Cytotoxicity results.

The data files for the analysis of column effluents by cell culture analysis (L929 cell line, crystal violet elution assay) are presented in appendix 8:3.3, as follows; Blank column, Tables 8.128-8.134, appendix page xvxiv; Column 1, Tables 8.135-8.157, appendix page xxv; Column 2, Tables 8.158-8.165, appendix page xxvii; Column 3, Tables 8.166-8.183, appendix page xxix; Column 4, Tables 8.184-8.191, appendix page xxxi.

As previously noted in 4:3.3.1.1 (p. 163), the $EC_{50}$ of diazinon sheep dip to cultured L929 cells was 26.29 mg/l (a.i). Because of the inherent 1/10 dilution in the test protocol, the concentration of diazinon would therefore need to rise above 262.9 mg/l before an $EC_{50}$ could be established from column effluents (assuming that all of the toxicity of the dip was due to the active ingredient, and that no toxins were eluted from the peat fibre).

In an adsorption study where the analyte was in true solution, the concentration of analyte would rise from low levels (when adsorption was removing all, or virtually all, of the material from solution), past a pre-set breakthrough point (used to calculate effective adsorbent life, generally 5% or less of influent concentration), to influent levels, when the adsorptive capacity of the fibre was used up (i.e. absolute column life, or adsorbent exhaustion).

The combination of low dose rate and relatively low sensitivity of the cell line meant that cell culture analysis (using the study protocol) would not give information on the breakthrough of analyte in the effluent (20 mg/l a.i). However, the cell culture trials could be reasonably expected to give information on the 'exhaustion' of the peat fibre (400 mg/l), and on any unknown toxins in the effluent.

However, as discussed in section 4:3.4.1.3 (p. 173), the sheep dip is not in true solution, and is removed by processes other than adsorption, which led to extended column life, and maximum effluent diazinon levels, as noted, of 240 mg/l. This meant that subsequent cell culture analysis should not have been able to assign $EC_{50}$ values to any of the effluents (i.e. the effluents could only be classified as non-toxic, up to 10% effluent concentration).

This was the case with effluents from the Blank column, Column 1 and Column 2. However, it should be noted that the highest inhibitions

recorded in Columns 1 and 2 were over the first four days, when relatively high inhibitions were also seen in the Blank column. This would indicate the elution of loosely bound materials, as described in sections 4:2.3 (p. 141), and that these materials were somewhat toxic to cultured cells.

Early results from Column 3 and Column 4, however, gave a somewhat different picture. These columns were a mixture of peat fibre and peat nodules. Results from Columns 3 and 4 also showed significant inhibition over the first four days, but to a greater extent, so much so that $EC_{50}$ values were calculable for four of the samples. Three of these were from Column 3 (0.979 days, = 10.20%, 1.979 days = 6.03%, and 3.792 days, = 10.70%), and one from column 4 (1.979 days = 9.20%).

These results indicate that there are toxic materials leached from the peat in the first few days of operation, which cannot be identified by analysis of the sheep dip active ingredient.

The results are particularly significant when considering the fact that the diazinon removal performance of these duplicate columns were significantly different from each other over this time period. In fact, the concentration of diazinon in Column 3 effluents was 2-3 times less than those from Column.

This further points to the measured toxicity as being from unknown leachates from the peat itself, with more of such materials being leached from the peat nodules than from the peat fibre.

Another point of note is that, when the concentration of diazinon went above 150 mg/l (c. 31 days), inhibition in the column samples could be expected to begin to rise, and to reach circa 30% at 240 mg/l (effective conc. in media 24 mg/l). This was not the case in either Column 1, or Column 4, which operated to the end of the column experiment. This indicates that all of the toxicity of the formulated product cannot be attributed to the active ingredient, a point that is discussed extensively, as noted, in section 4:3.6.

**4:3.5.1.2** The usefulness of cell culture data.

As can be seen from the above, cell culture was not useful on this occasion for following precisely the operation of the columns, in the way that GC analysis was. This was due to the requirements of the particular protocol used, and to the mechanisms of removal of the sheep dip by the peat.

However, even under these difficult circumstances, cell culture was able to supply information on the toxicity of the product, and on the

performance of the adsorbent, that standard physical or chemical analysis alone could not. Thus, the cell culture results indicated that there is leaching of undefined toxic materials from the peat in the early stages of operation, and that there may be other toxic components in the product than the active ingredient. This would make monitoring of the environmental effects of the product by active ingredient alone insufficient.

For these reasons, it can be said that cell culture analysis was useful in extending the study database, and increasing the understanding of the operation of this adsorbent.

While this particular cell culture protocol could not follow the operation of the columns, in this particular case, there are a number of other protocols, as described in the following section, which would increase the effective concentration of toxin, and sensitivity of the cell line.

Also, as in other areas of the study, the trials produced rapid, reproducible, low-cost data, which are useful characteristics.

**4:3.5.1.3** Extending the application of cell culture tests.

As noted, there were two reasons for the limitation of the cell culture protocol, as applied in the environmental monitoring portion of this study. The first was the fixed dose rate required for the column experiment. The second was the constant 1/10 dilution of the toxicant in media.

The cell culture protocol was chosen initially for the predictive hazard trials, to maintain nutrient and osmotic levels both at a high, and a consistent level in the media, and was retained in the monitoring trials unamended, as a direct comparison to these.

A direct dilution of toxin in 1X media is used by a number of workers in the field. Rachlin and Perlmutter (1968), in their study of fish cells for the analysis of aquatic pollutants, used a 1/10 final dilution in media. Christian *et al.* (1973), in their study of the toxicity of metals, used a 1/100 final dilution in media.

This is not a limitation in the analysis of pure toxins, where stock solutions can be prepared at 10x (as was the case in the predictive trials), but would be for the analysis of effluents, if the highest concentration that could be analysed were 10%, and those levels were not toxic (as was the case in the monitoring trials).

However, there are a number of strategies that can be used to solve this problem. The most commonly used is the reconstitution of powdered media with the effluent (Christian *et al.*, 1973), or of concentrated

media (Hunt *et al.*, 1987). Another is the concentration of effluents, by distillation (Cody *et al.*, 1975, 1979), or by adsorption, elution and solvent extraction (Wilcox and Williamson, 1986; Tarazona *et al.*, 1991).

Thus the limitations seen in this particular study are not major impediments to the use of cell culture assays in general. Another approach would be to use a more sensitive assay than cell death, a point that is discussed in section 3:3.2.2.4 (p. 91).

4:3.5.2 Toxicity *Artemia.*

The data files for the analysis of column effluents by *Artemia salina* immobilisation are presented in appendix 8:3.4, as follows; Blank column, Tables 8.192-8.198, appendix page xxxiii; Column 1, Tables 8.199-8.201, appendix page xxxiv; Column 3, Tables 8.222-8.239, appendix page xxxvii.

The *Artemia* test, as applied in earlier 'pure' toxin studies, used a protocol whereby the maximum dilution of the seawater media in the test wells would be 10%. This guaranteed high salinity, as well as consistent salinity across the wells. This is not, in fact, a requirement of the test, as the *Artemia* are very tolerant to low salinity, and to changes in salinity (Vanhaeke *et al.*, 1984). In salinity tests carried out by the author, up to 80% reduction in salinity caused no mortalities, or sub-lethal symptoms.

In this study, as the measured toxicity of the sheep dip to *Artemia* was 9.41 mg/l (a.i), a concentration of greater than 94.1 mg/l diazinon would be required to generate $IC_{50}$ data, if the original protocol was to be maintained (as with the cell culture protocol). This would limit calculation of toxicity data. Instead, higher concentrations of effluent were used (up to 75% with Blank column effluents).

The ranges of concentrations used in the trials were designed around the 'expected' $IC_{50}$, calculated from the published (i.e. measured in section 4:3.3.1.1, p. 163) sheep dip $IC_{50}$ and the GC measured diazinon concentration, as follows.

$$\text{Expected effluent } IC_{50}(\%) = \frac{9.41}{(\text{GC conc.}/100)}$$

This is the way in which chemical data is normally extrapolated to give a measure of expected toxicity for effluents etc., in the absence of a routine toxicity monitoring programme.

Column effluent toxicity was measured in the Blank, Column 1 and Column 3 (the long-term columns) for selected samples from the first fourteen days of operation, and for all samples thereafter, until ponding occurred. These results are presented in Tables 4.13 and 4.14. There was no measured toxicity to *Artemia* with any of the blank samples.

As can be seen from the tables, the toxicity of the column samples followed the measured diazinon concentration (inversely) in both columns.

**Table 4.13**

**The toxicity of effluent from Column 1 to *Artemia* ($IC_{50}$ expressed as % of effluent applied)**

| Time (Days) | Diazinon concentration (GC-mg/l) | Effluent $IC_{50}$ (%) |
|---|---|---|
| 0.63 | 109.57 | 12.04 |
| 0.98 | 115.67 | 14.18 |
| 1.98 | 92.65 | 16.43 |
| 3.79 | 70.61 | 25.32 |
| 6.00 | 37.15 | 35.00 |
| 8.50 | 78.55 | 20.03 |
| 11.00 | 64.18 | 22.72 |
| 13.00 | 64.18 | 22.53 |
| 14.00 | 75.38 | 21.57 |
| 17.00 | 56.06 | 38.17 |
| 21.00 | 57.69 | 19.05 |
| 25.00 | 91.10 | 18.83 |
| 28.00 | 109.59 | 9.45 |
| 31.00 | 139.71 | 8.61 |
| 34.00 | 202.40 | 4.98 |
| 38.00 | 193.79 | 9.03 |
| 42.00 | 216.67 | 9.53 |
| 45.00 | 188.03 | 8.38 |
| 49.00 | 202.40 | 8.83 |
| 56.00 | 236.49 | 8.62 |
| 59.00 | 210.97 | 6.80 |
| 63.00 | 216.70 | 6.96 |
| 66.00 | 216.70 | 7.25 |

These results were as expected, and demonstrates how a toxicity test can be used to trace the performance of a column, in the same way that a chemical method can. This is an important point, because, as previously stated, toxicity data in general gives a more complete picture of the effect of the discharge of a material than limited chemical analysis can. It

automatically includes measurement of unknown chemical species, as well as measurement of any synergism or antagonism.

However, a trend that was not expected is that the measured toxicity was lower than the calculated toxicity, in all cases. This topic is discussed further in section 4:3.6.

This led to the range of concentrations being somewhat low for the initial three samples in Column 3, precluding calculation of some $IC_{50}$s.

**Table 4.14**

**The toxicity of effluent from Column 3 to *Artemia* ($IC_{50}$ expressed as % of effluent applied)**

| Time (Days) | Diazinon concentration (GC-mg/l) | Effluent $IC_{50}$ (%) |
|---|---|---|
| 0.63 | 37.70 | n.r.[1] |
| 0.98 | 39.40 | n.r.[2] |
| 1.98 | 34.27 | n.r.[3] |
| 3.79 | 42.78 | 32.12 |
| 6.00 | n.r.[4] | 32.00 |
| 8.50 | 73.80 | 20.67 |
| 11.00 | 73.80 | 23.59 |
| 13.00 | 78.55 | 23.11 |
| 14.00 | 89.54 | 20.67 |
| 17.00 | 73.80 | 34.69 |
| 21.00 | 87.98 | 33.44 |
| 25.00 | 80.13 | 11.67 |
| 28.00 | 115.67 | 35.68 |
| 31.00 | 176.45 | 7.79 |
| 34.00 | 210.97 | 8.58 |
| 38.00 | 225.18 | 9.15 |
| 42.00 | 206.69 | 7.78 |
| 45.00 | 237.88 | 8.42 |

n.r. = no result. [1]56% eff = 27% inh. [2]56% eff = 38% inh. [3]75% eff = 16% inh. [4]Loss of sample.

In summary, the analysis of column effluents by the *Artemia salina* assay generated data whose trends generally followed the chemical analysis of the active ingredient. However, the measured toxicity was lower in all cases than that extrapolated from the sheep dip toxicity, and the effluent diazinon concentration. This indicates that there are other toxic materials in the dip than the active ingredient.

Only a toxicity assay could demonstrate this, which again highlights the inaccuracies that are inherent in a chemical definition of toxicity, a biological phenomenon.

For these reasons, the *Artemia* assay is considered to have been very useful in the analysis of column effluents, and in interpreting column operation.

4:3.6 Extrapolation of data between analytical systems.

Because of the complexity of ecological communities, environmental analytical data is almost always extrapolated to some degree, and often on a number of different levels. For example the concentration of phosphate in grab samples at a one site on a stream may be taken as a measure of phosphate concentration in the whole stream, and furthermore, several such stream estimates may be used to establish a eutrophication management plan for a lake.

Similarly, with toxicity analysis, the concentration of a chemical causing 50% lethality under laboratory conditions may be used to define safe levels of that chemical for that species in its natural environment (where temperatures etc. vary considerably), and furthermore, may be used to define safe levels of that chemical for entire communities in particular ecospheres.

There are many obvious flaws with such an approach, but it is often the only strategy available for control of effluents etc., particularly where toxicity analysis is not carried out.

The most common extrapolation is the analysis of a formulated product, or complex effluent, for 'active ingredients', or single parameters which are perceived to be the most hazardous constituents (e.g. metal content in the metal effluents, diazinon concentration in the sheep dip). The actual hazard is then taken to be a function of that single parameter. Over time, the relationship is assumed to be constant.

In the following sections the data from the GC analysis Artemia analysis of column effluents are manipulated in this way, to demonstrate the implications that it can have on the proper management of such materials.

The results also demonstrate aspects of the operation of the peat columns that cannot be determined using chemical analysis alone.

4:3.6.1 Calculation of expected effluent toxicity.

In this study, the toxicity of the sheep dip is defined in terms of the diazinon content. Thus, $IC_{50}$s are expressed as mg/l active ingredient (a.i.). In a situation where toxicity analysis were not carried out on the effluents (as would generally be the case), toxicity would normally be calculated (extrapolated) as a function of effluent diazinon concentration.

Also, the formulated product is applied to an adsorbent which is a highly heterogenous, and largely undefined material. Effluents arising from the columns cannot be assumed to be free of materials leached from the adsorbent. In fact, it is an accepted fact that significant amounts of undefined materials are eluted. This raises two important questions.

1) Are there any toxic materials leached from the adsorbent itself?

2) Are there any other contributors to the toxicity of the product than the 'active ingredient'?

There is ample precedent for both of these. The possible leachates from the peat are described in section 4:2.3 (p. 141). The toxicity of non a.i. components in formulated products has been described by other researchers, such as Raine *et al.* (1990), who found the toxicity of Nuvan (as mg/l dichlorvos) significantly higher than that of technical grade dichlorvos. However, the recommended treatment of salmon with Nuvan is based on the concentration of dichlorvos (1 mg/l a.i.).

**4:3.6.1.1** Leaching from the columns.

In section 4:2.3.4 (p. 144), it is can be seen that a large number of materials are easily leached from peat. This was clear in this study from visual observation, where effluents were highly coloured over the first 6 days, and did not completely lose the colour for up to 10 days.

Leached materials would include loosely bound water soluble substances, as well as those displaced by ion exchange with the sheep dip. It was possible that a number of these could affect the toxicity of the effluents, either from the point of view of adding significant toxicity, or through toxin antagonism (as described in section 4:2.3.2, p. 142).

Still further, there is the possibility of toxic by-products from the pesticide itself (Bell and Tzesos, 1988). Up to 20 water soluble products

have been detected from diazinon applied to soils (Getzin and Rosefield,1966). Weber and Gould (1966) note that, with organophosphates, the products of oxidation can often be more toxic than the parent compound. Konrad *et al.* (1967) showed that a number of water soluble products resulted from chemical hydrolysis, and concluded that these could have a significant contribution to the observed toxicity of the pesticide.

According to the *Artemia* results, there were no significantly toxic components in any of the Blank effluents (though inhibitions of up to 26% were seen over the first four days to cell culture). There was no evidence of additional toxicity in the column effluents (as assessed by comparison of expected effluent $IC_{50}$s with measured effluent $IC_{50}$s in the following sections).

Only a toxicity assay could generate this type of data.

**4:3.6.1.2** Other contributors to toxicity.

The second question is a more subtle one, and is addressed in this, and the following section. If the only contributor to the toxicity of the sheep dip was diazinon, then the 'expected' toxicity of the column effluent could be calculated (extrapolated) directly. The formula used, as described in 4:3.5.2 (p. 181), is as follows;

$$\text{Expected effluent } IC_{50}(\%) = \frac{9.41}{(\text{GC conc.}/100)}$$

Thus, if the GC measured diazinon concentration was actually 9.41 mg/l, then the $IC_{50}$ of the effluent (expressed as % of effluent) would be 100%. If the concentration was 94.1 mg/l, the $IC_{50}$ would be 10%, etc.

Expected column effluent toxicity was calculated, using the above calculation, from the $IC_{50}$ of the sheep dip to *Artemia*, and GC measurements of diazinon, for all samples which were assessed by *Artemia*. The results are presented in Table 4.12, and Figure 4.10.

As can be seen, the expected toxicity is always higher (lower numerical value) than the measured toxicity. Thus calculating the toxicity by the active ingredient in this particular system overestimates the toxicity of the effluents. This indicates that there are more contributors, in the formulated product, to the measured toxicity, than the 'active ingredient'.

These results also rule out the possibility of toxicity of the leachates, synergism from components in the leachates, or production of

more toxic by products of diazinon from breakdown during passage through the column.

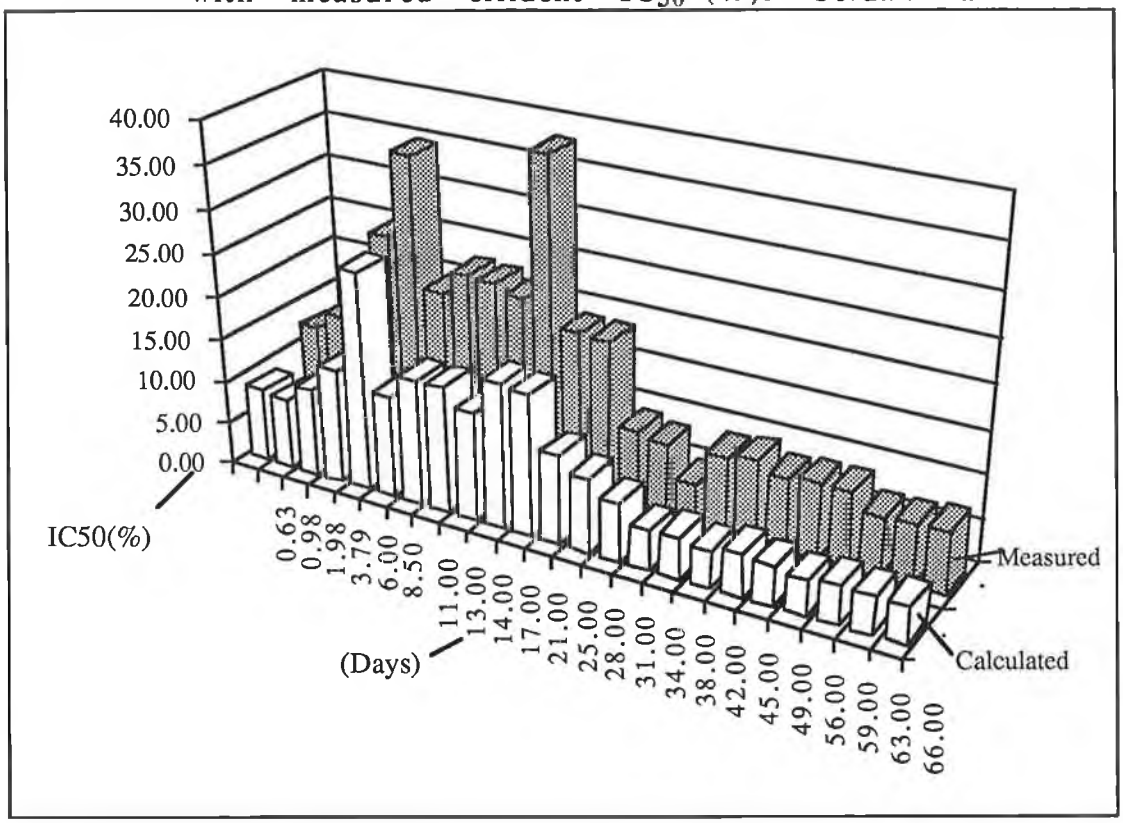
**Table 4.12**
**Calculation of expected effluent $IC_{50}$ from measured diazinon concentration: Column 1**

| Time (Days) | Diazinon concentration (GC-mg/l) | Measured Effluent $IC_{50}$ (%) | Calculated Effluent $IC_{50}$ (%) |
|---|---|---|---|
| 0.63 | 109.57 | 12.04 | 8.59 |
| 0.98 | 115.67 | 14.18 | 8.14 |
| 1.98 | 92.65 | 16.43 | 10.16 |
| 3.79 | 70.61 | 25.32 | 13.33 |
| 6.00 | 37.15 | 35.00 | 25.33 |
| 8.50 | 78.55 | 20.03 | 11.98 |
| 11.00 | 64.18 | 22.72 | 14.66 |
| 13.00 | 64.18 | 22.53 | 14.66 |
| 14.00 | 75.38 | 21.57 | 12.48 |
| 17.00 | 56.06 | 38.17 | 16.79 |
| 21.00 | 57.69 | 19.05 | 16.31 |
| 25.00 | 91.10 | 18.83 | 10.33 |
| 28.00 | 109.59 | 9.45 | 8.59 |
| 31.00 | 139.71 | 8.61 | 6.74 |
| 34.00 | 202.40 | 4.98 | 4.65 |
| 38.00 | 193.79 | 9.03 | 4.86 |
| 42.00 | 216.67 | 9.53 | 4.34 |
| 45.00 | 188.03 | 8.38 | 5.00 |
| 49.00 | 202.40 | 8.83 | 4.65 |
| 56.00 | 236.49 | 8.62 | 3.98 |
| 59.00 | 210.97 | 6.80 | 4.46 |
| 63.00 | 216.70 | 6.96 | 4.34 |
| 66.00 | 216.70 | 7.25 | 4.34 |
| 67.00 | 199.50 | 7.42 | 4.72 |
| 68.00 | 185.10 | 5.60 | 5.08 |

It follows from this, that the difference in toxicity would have to be due to preferential removal by the peat of the non-diazinon toxins (whether by adsorption, or by facilitating breakdown/conversion of these components).

Figure 4.10 shows the relationship graphically. The closeness of the trend over time can be seen clearly, as well as the extent of the overestimation of toxicity by calculation from the measured diazinon concentration.

**Figure 4.10**
**Comparison of expected effluent $IC_{50}$ (%) with measured effluent $IC_{50}$ (%): Column 1**




There is also a second theoretical explanation for the reduction in toxicity. That is the amelioration of the diazinon toxicity by other substances in the peat leachate (i.e. toxin antagonism, as seen in section 4:2.3, p. 141). Between them, they give rise to the following permutations;

1) That all the toxicity is actually due to diazinon, and that the reduction in toxicity shown is completely due to antagonism.

2) That all the toxicity is not due to diazinon, and the reduction is due to some degree (not calculable) of preferential removal of the non-diazinon toxins over diazinon, with no antagonism from the leachate.

3) That a combination of differential removal and antagonism is extending the gap beyond that resulting from differential removal alone.

On balance, as the pattern is continued throughout the life of the columns, and toxin antagonists could be expected to be leached out before this, it is more likely that option 2 pertains.

The implications of the results, are significant. In short, an expression of toxicity by active ingredient alone does not take into consideration other toxins in formulated products, or complex interactions between toxins and environmental processes (in this case, a waste treatment process). This could lead to a potentially hazardous situation.

There are three possible combinations of situations that can result, one of which carries an additional hazard.

1) If the additional (non-a.i.) toxins were not preferentially removed, and the toxicity of the formulated product was determined from the product itself (as opposed to technical grade a.i., as is often done), then an expression of toxicity as a.i. would encompass all contributors to toxicity, and no additional hazard would result.

2) If toxicity is determined from the product, as above, and the additional toxins are preferentially removed (as is postulated in this case), overestimation of the toxicity results, and again, no additional hazard results.

3) If, however, the toxicity of the product (effluent etc.) was taken to be the toxicity of the active ingredient, and the toxicities of subsequent unknowns (e.g. effluents, waters etc.) were extrapolated from measurements of the concentration of that ingredient alone, then an underestimation of true toxicity would result. This would be an additional hazard.

This is a fundamental limitation of decision making based on physical/chemical data alone, and again highlights the need for the introduction of readily produced and incorporated toxicity data.

4:3.6.2 Summary.

Overall, the *Artemia* toxicity results show that the performance of the columns can be followed using a toxicity index. More importantly, it is

possible to make distinctions between toxic fractions based on this data, and to arrive at a more subtle appreciation of the processes at work on the columns than would be possible from chemical means alone.

While, in this study, expression of the toxicity of the formulated product as mg/l a.i. led to an overestimation of toxicity, in others it could lead to a significant underestimation of the same. Actual toxicity analysis, as opposed to extrapolations based on chemical analysis, would then be clearly invaluable.

4:3.7 Summary of environmental monitoring trials.

Environmental monitoring is the area of environmental analysis that has received the least amount of attention with respect to toxicity testing. Paradoxically, it is the area that could gain most from it, as it is the management of the ultimate discharge of waste materials that translates toxic hazard into toxic effect. A practical toxicity test would provide a quantum expansion of the quality as well as the extent of the database available for such management decisions.

To date, toxicity tests used for environmental monitoring have varied from ecological analysis of *in situ* communities, to on-line fish avoidance tests. Where toxicity analysis is carried out on effluents for discharge, it tends to be on an intermittent basis, generally to satisfy licence requirements. There is little or no routine toxicity analysis of effluents.

The main reason for this is that existing tests are cumbersome, expensive, time consuming, inflexible, statistically weak, and provide data which is not always easily interpreted. Thus, a new test would have to improve on some, and ideally all, of these areas.

It is considered here that cultured cells offer advantages in all of these areas. A series of experiments were designed to test the usefulness of a selected cell culture system. No testing system is without limitations, and criticisms of the use of cultures cells include the lack of organisational systems, and the effect of media serum on the toxicity of materials. The experiments were designed with these limitations in mind, as well as the intention of assessing the flexibility, and the statistical strength of the assay.

4:3.7.1 Metalliferous effluent experiments.

The set of effluents examined were a group of metalliferous effluents. The analysis of metals is known to have a number of technical difficulties attached, regardless of the test system. These include the effect of

pH, availability of metals in solution, affinity for organic material, and others. Three metal fractions were prepared, to examine a number of these factors in relation to cultured cells. Analysis included chemical, *in vitro* (cell culture) and *in vivo* (*Artemia*).

Cell culture results showed that the effect of the toxicity of effluent components was not eclipsed by the effect of pH. Media serum reduced the indication of toxicity given, to a degree approximately double that seen in the analysis of sheep dip. However, this is considered manageable, with standardisation and proper interpretation of results. The toxic effects of the effluent were seen to be entirely expressed within 24 hours, which means that data can be obtained for this type waste in a significantly shorter time than in the protocol applied (96 hrs.).

Given the range and extent of the known difficulties with the toxicological analysis of metals, these cell culture results show a high degree of applicability, and flexibility.

It was possible to demonstrate, through comparison of the cell culture results with chemical analysis of the effluents, that there are other contributors to the toxicity of the effluent than the metal content.

Two significant points stand out from this series of experiments. The first is the extreme weakness of chemical analysis in the proper characterisation of the toxic hazard posed by the metal effluents. This is particularly significant when considering the fact that the normal basis for monitoring and control of the discharge of these wastes relies heavily on metal analysis.

The second is the very low sensitivity of the *in vivo* system, *Artemia*, to the metal effluents. This was not simply a relative insensitivity, as it reversed the indication of the toxicity of the two neutral effluent fractions. This again underlines the principle of integrated assessment, and the dangers of relying on any one system.

It is concluded then, that cell culture analysis is very useful for the analysis of this type of effluent, and offers some distinct advantages over existing systems.

4:3.7.2 Sheep dip experiments.

The second set of experiments was the assessment of the efficacy of a peat based treatment system for the removal of an organophosphate based sheep dip from solution. Such experiments would normally be carried out by chemical analysis of the active ingredient.

The toxicity of the sheep dip was assessed, along with the effect of serum concentration, incubation time and seeding density on this toxicity .

The challenge for the cell culture system in these experiments was to establish whether the system could be used to monitor the operation of the columns, and/or could provide data which would increase the understanding of the processes at work.

The results showed that L929 cells in culture, as assayed by crystal violet elution, had a relatively low sensitivity to the sheep dip (though significance is increased when considering the major mode of action). The concentration of serum in the media reduced the indication of toxicity. However, the effect is not considered to be excessive, and could be managed by standardisation. Most of the toxic effect of the sheep dip was expressed within 48 hours, which meant that the incubation time for the test, as applied, could be halved without loss of sensitivity.

A significant result, however, was that the sensitivity of the cell line was not sufficient to allow continuous monitoring of the operation of the column, using the chosen protocol. The feed dip concentration was dictated by field application rates to be 400 mg/l, which was approximately 16 times the $EC_{50}$ of the dip. Because there is an inherent 1/10 final media dilution of an effluent in the protocol used in this study, the true feed concentration was effectively 1.6 times the $EC_{50}$. Thus, levels of dip in the column effluents would have to reach 63% of the feed before an $EC_{50}$ could be calculated.

This is well above the breakthrough point, which restricted the amount of data that could be generated from the cell culture test. Eventually, the experiment ended without breakthrough occurring, which meant that cell culture $EC_{50}$s could not be calculated for many effluent samples.

While data on the monitoring of the column was not generated by cell culture assay in this particular study, there are a number of strategies which could be used to increase the maximum amount of effluent that could be tested in routine use. These include concentration of effluents (by distillation, resins etc.), and reconstitution of powdered media, or of concentrated liquid media with effluent. Thus, the problem encountered is not one that should preclude the use of cell culture systems generally.

Also, cell culture results were able to provide useful information on a number of other aspects of column operation. Firstly, measurable toxicity in the peat/fibre columns in the early days of operation indicated that toxic materials were being leached from the peat itself.

Secondly, following on from analysis of the sheep dip, some

level of toxicity was expected when column diazinon reached 150 mg/l. This was not seen, which indicated that all of the toxicity of the dip is not due to the active ingredient alone. This point is extensively discussed in reference to *Artemia* results. Again, chemical analysis could not demonstrate this.

There is general agreement that no single organism or system is a panacea for the problems and requirements of environmental analysis, and none should be pursued as such. All experiments in this study were carried out in parallel with a minimum of one *in vivo* system (several in the predictive hazard trials), and/or chemical analysis.

For the monitoring trials, the *in vivo* comparison was the *Artemia salina* immobilisation test. *Artemia* were seen to be sensitive to the sheep dip, though not significantly more so than the cultured cells (a factor of 2.5). However, it was sensitive enough to allow continuous monitoring of the column operation.

Extensive comparison of effluent $IC_{50}$ data, along with chemical analysis, allowed full examination of the trend seen in the cell culture tests, i.e. that toxicity in the effluents did not tally with that extrapolated from the concentration of diazinon, indicating that there are other toxic materials in the formulated product than diazinon. This again proves that, monitoring of active ingredient alone is not sufficient to control the toxic hazard of products or complex effluents.

Chemical analysis of the sheep dip required a time consuming development of a GC system for the analysis of diazinon. Once developed, it worked well, and provided the best day-to day monitoring data on the operation of the columns. However, it was not able to define the hazard posed by the dip, or the column effluents.

In summary, the cell culture system applied in this study did not facilitate continuous monitoring of the operation of a series of column trials. However, it did provide valuable information on other aspects of the column study. As there are a number of strategies available to remove the limitation encountered in this case, it is not considered significant. It is concluded that cell culture can make a valuable contribution to this type of study.

The *in vivo* system used also gave valuable results, and again underlined the importance of toxicological information when assessing the hazard posed by effluents, and when managing their discharge.

This too was the conclusion from the chemical analysis results, which gave valuable monitoring data, but which could not address the subtleties of other parts of the experiment.

# CHAPTER 5
# THE SELECTION OF BIOASSAYS FOR ENVIRONMENTAL ANALYSIS

Chapter Index Page

**Chapter 5 The selection of bioassays for environmental analysis**

## 5:1 Introduction.

The preceding chapters outline a number of ecotoxicity experiments, designed to compare the effectiveness of cell culture methodologies with a number of standard whole organism trials. The experiments were divided into two broad categories, predictive hazard assessment trials, and environmental monitoring trials. The results are discussed relevant to the overall results from each section.

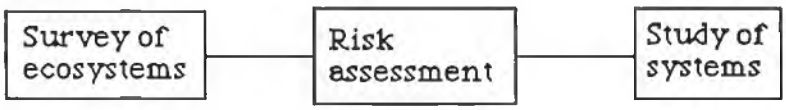
The following chapter aims to set the entire set of results in the context of environmental analysis, and to demonstrate where cell culture methods may be of potential use.

## 5:2 The Need for Bioassays in Environmental Analysis.

### 5:2.1 Environmental analysis.

There are three main areas in the discipline of environmental analysis. These, as shown in Figure 5.1, are; survey of ecosystems, their structures, processes and functions (in order to increase knowledge of the workings of ecological communities); risk assessment of materials intended for discharge to the environment (in order to manage these discharges, so as to minimise impacts); and experimental study of specific systems (e.g. receiving waters and waste treatment) (in order increase the efficiency of environmental controls).

**Figure 5.1**
**Environmental analysis**




Each of these three areas of operation use chemical, physical and biological methods of analysis, to different degrees. Surveys of ecosystems have tended to use mostly biological methods, with some physical and chemical analysis. Studies of systems, on the other hand, use physical and chemical methods, almost exclusively. Environmental risk assessment is the area that most involves the principles of ecotoxicology.

Given that toxicity is a biological effect, it might be assumed that biological methods have a major role to play in risk assessment. However, this has not been the case. The vast majority of data used in environmental

risk assessments to date has tended to be physical and chemical. The reasons for this trend, and the difficulties it creates, are outlined below.

5:2.2 Ecotoxicology and risk assessment.

Ecotoxicology is a combination of chemistry, ecology and toxicology, which seeks to combine information on the patterns of production and use of chemicals, with information on their fate in the environment, and information on their interactions with the environment (toxicity).

This is to provide a measure, firstly, of the environmental risk that they represent, and, secondly, of the actual effects that they have (Calow, 1993; Richardson, 1993). Despite the labelling of materials as toxic, or non-toxic, all chemicals are potential toxins (Maki and Bishop, 1985). It is the way in which they are used that determines whether a hazard becomes an effect or not. Risk assessment is the term used for the process of quantification of the likelihood that a hazard will be translated into a toxic effect (Paasivirta, 1991).

There are a wide variety of environmental analytical techniques involved in risk assessment. As noted, current environmental risk analysis relies on chemical and physical methodologies. This is because there are a number of restrictions and limitations associated with biological methods. However, also as noted, toxicity is a biological effect, and, as such, is best determined by a biological assay.

The ultimate determination of ecotoxicity would be to measure the effect of chemicals *in situ*, using the affected communities. While such tests exist, they are long-term, expensive, and often do not produce data which is easily used (Macek, 1985; Hopkin, 1993). The limitations associated with existing short-term, single species bioassays include resource requirements, statistical weakness, lack of reproducibility, variations in sensitivity, and problems with extrapolation to ecological situations (Cairns and Pratt, 1989).

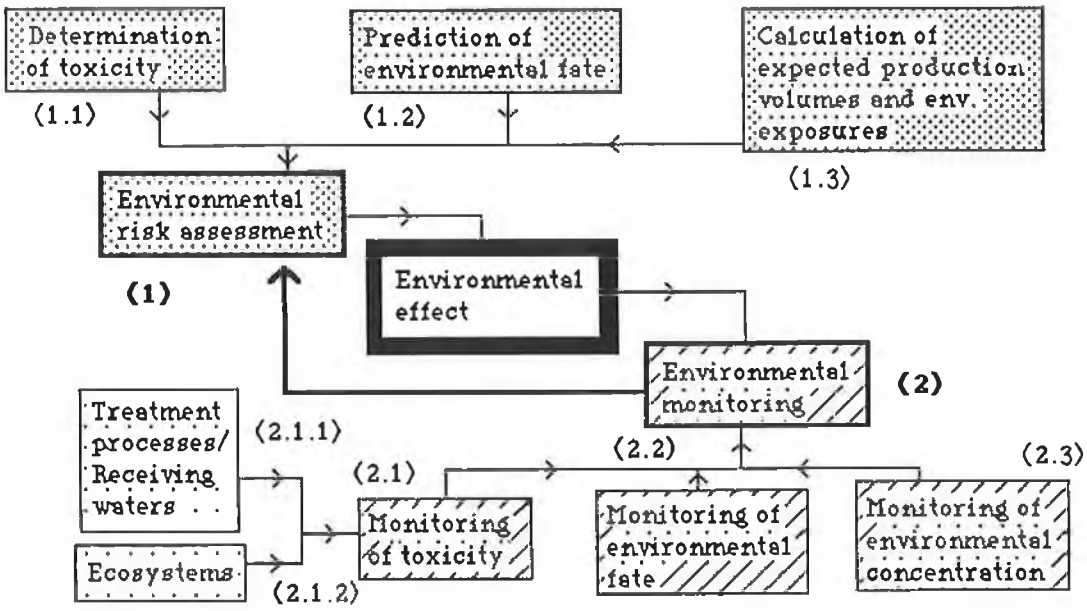
With over 70,000+ synthetic chemicals in use today, with approximately 1,000 added each year (Maki and Bishop, 1985), and with 80% of these lacking a significant amount of ecotoxicity information (section 1:1.1, p. 2), the importance of developing new methodologies is amplified, and a great deal of research is directed towards this objective.

The process of risk assessment has two main parts, assessment of the projected risk, prior to release, and the assessment of actual effects on

discharge. The two form a loop, which allows the process to be constantly updated, to minimise the effects of the discharge of materials. As can be seen in Figure 5.2, each part has a number of distinct categories, with separate objectives, all of which contribute to the overall management of discharges.

**Figure 5.2**

**Environmental risk assessment**




Environmental toxicity is a result of a potential hazard being translated by use patterns. As can be seen from the figure, each category of projected risk assessment requires separate data gathering. Projected effect cannot accepted as the true environmental effect because of the extremely complex environmental mediation processes that can occur. Thus, monitoring is an integral part of risk assessment. The overall process of environmental risk assessment is designed to constantly provide data which can increase the understanding of environmental interactions.

This study has examined the contribution that cell culture techniques may be able to make to the overall area of environmental analysis, and environmental risk assessment in particular.

Within the category of projected environmental risk assessment (1), it can be seen that there is no analytical data used in the calculation of expected production volumes etc. (1.3). Within the category of environmental monitoring (2), physical and chemical data is best suited to the analysis of the environmental concentrations of specific chemicals (2.3),

and to the analysis of the environmental fate of specific chemicals (2.2). In the monitoring of toxicity, ecological studies are best suited to monitoring the effects of discharges on ecosystems (2.1.2) (Cairns and Pratt, 1989).

From the results obtained in the study, it is considered that cell culture methodologies can be used effectively in all other portions of risk assessment. Within the wider field of environmental analysis (Figure 5.1), ecological studies are best suited to the survey of ecosystems, but it is considered that cell culture methods can contribute to the study of systems also (e.g. studies on process control etc.).

Of all of these, the area of the monitoring of toxicity (treatment systems and receiving waters {2.1.1}) is the area that can benefit the most from bioassay analysis, and thus from cell culture methods. This is because of the almost entire lack of biological information in this critical portion of the risk assessment process.

Despite the biological nature of toxicity, bioassay data is rarely included in licence requirements, and even less commonly in the assessment of the efficiency of waste treatment systems. An example of the use of toxicity data in licensing which demonstrates the benefits of its inclusion is that of the Clyde River Purification Board (Mackay, 1989).

In a survey, it was determined that only two of the ten UK water authorities had ever required toxicity information in licences. One of these was the Clyde River Purification Board (CRPB). The use of chemical data alone in licences for discharges assumes that the toxicity of every listed constituent of a waste is known, and that the waste consists entirely of the listed constituents (a very unlikely situation).

The CRPB have used toxicity testing in the screening of effluents for potential toxicity, testing compliance with licence specifications, screening receiving waters for toxicity and determining the toxicity of specific substances.

With one particular waste, toxicity analysis agreed with the expected toxicity from the listed constituents, and monitoring of the waste was set at routine chemical analysis, and occasional toxicity analysis. For some time, this was acceptable. However, there was then a sharp increase in effluent toxicity, which could not be explained by chemical analysis. Further toxicity analysis identified the offending chemical, which was not being routinely measured, as it was believed to be non-toxic. The licence was then modified to require routine toxicity testing.

Thus, it can be seen that toxicity testing can provide information on materials for discharge that cannot be supplied by any other type of testing, and can generate data that can be used to achieve effective environmental management.

Macek (1985) also describes these principles, and describes a protocol for setting target effluent toxicities, based on discharge volumes, and receiving water flows.

In this study, cell culture methodologies were used to determine the predicted toxicity of a number of materials, to assess the efficacy of a new waste treatment system, and to analyse the toxicity of a group of metalliferous wastes.

Determination of the toxicity of the products presented few technical problems, and gave indices of toxicity which ere comparable with several other bioassays, carried out in parallel. While few results were obtained from the cell assay with the new treatment system, this was attributable to the specific protocol used, and a number of other protocols are described which would help to increase the database.

Also, there were no problems with the handling of the assay. With the metalliferous effluents, cell assays were able to provide data on relative toxicities of effluents which could not be predicted from chemical analysis. In this case also, there were no problems with the handling of the assay.

Based on these results, it is considered here that cell culture methods would be very useful in environmental analysis.

### 5:3 Selecting Suitable Bioassays.

Almost any organism, or cell line, that can be cultured in the laboratory, can be used in a bioassay procedure. The potential candidates can be divided into a number of classes, such as algae, macrophytes, invertebrates, fish, mammals, various classes of cultured cells, and many more. Increasingly, research is being directed to the use of non-animal systems. Under legislation, such as the UK Animals (Scientific Procedures) Act of 1986, and EC Directive 86/609/EEC, alternative non-animal methods must be used whenever possible (Fentem and Balls, 1993).

Selecting suitable bioassay procedures involves the setting of criteria for measuring effectiveness, and for comparison with existing methods. One approach that is to be studiously avoided is the attempt to select

a single system, or species, to produce data in all areas of environmental analysis, or in all areas of risk assessment.

This is a view which is held by many researchers, including Isenberg (1993), who states that no single test species is uniformly sensitive to all classes of xenobiotics. A battery of tests with different sensitivity profiles would give a better measure of the toxicities of the wide classes of chemicals that exist. He believes that no species is better or worse than another, just different, and that each organism contributes its own involuntary judgement on the toxicity of a chemical. Thus, each test has its own merits.

Persoone and Wells (1987) also hold this view. They state, "No single organism or testing protocol fulfils all criteria to determine the toxicity of materials, and there are inherent dangers in single species approach". Fentem and Balls (1993) believe that, currently, fish are used as representative species, and, as such, the toxicity data obtained provides only approximate guidance for environmental risk assessment purposes. They consider that there is absolutely no need for determination of precise $LC_{50}$ data, as safety factors of at least one order of magnitude will subsequently be applied to the data in any risk assessment procedure.

They envisage a battery of *in vitro* tests to replace fish lethality tests, including Microtox, cytotoxicity and Toxkits, and say that further studies need to be undertaken, to assess the suitability of some of these tests for inclusion in a battery approach. In particular, they point out that, when screening for basal cytotoxic effects of chemicals, toxicity tests with commonly mammalian cells may give comparable results to fish cell lines, and that few studies have examined this.

The criteria for selecting particular bioassay for use, are related to the specific handling and data characteristics of particular tests. These include ease of use, speed, sensitivity, precision, data interpretation, reproducibility and cost-effectiveness (Macek, 1985; Cairns and Pratt, 1989; Isenberg, 1993; Calow, 1993).

### 5:4 The Performance of Cell Culture Assays.

In this study, two cell lines, and two end points were uses as cell culture assays. They were compared, according to the above criteria, to a range of invertebrate and other single species bioassays carried out by the author. Performance in these categories is discussed here.

5:4.1 Ease of use.

For a new bioassay to be of any significant, wide-spread use, it must be possible to carry out the assay with relatively few resources, including sophisticated training. While some current bioassays, such as fish acute toxicity, have high resource requirements (i.e. facilities and expertise), there are several others, notably new invertebrate assays, which require few resources (Persoone *et al.* (1993).

While cell culture systems have some high initial set-up costs associated, these are not excessive, when compared to instrumentation such as GC, HPLC etc. Routine test requirements are very low, and are comparable to the operation of a small microbiological laboratory. To carry out sophisticated cell culture studies would require special training. However, it is considered here that executing standard protocols, which have specific objectives for routine environmental analysis, would require very little extra training above that of a microbiology technician, skills which are widely available. Further skills, or resources, for advanced cell culture investigations, could be provided on a regional, or a central basis, as is commonly the case with chemical methodologies.

5:4.2 Statistical strength.

Cell culture is the only animal bioassay which uses $10^6$-$10^7$ individuals, can apply multiple replicates in one test, and can feasibly allow multiple tests (with the same, or other cell lines) in parallel.

New invertebrate assays have increased the number of individuals that can be used, and further modifications would allow multiple replicates/trials to be used, but nothing like the numbers used in cell culture could ever be achieved.

Inter-trial and intra-trial variability is a significant feature of all current bioassay methodologies. Dose-response curves are typically sigmoid, and each mean (concentration) has an associated variation, due to different responses of individual organisms. The least variation seen in the curve is normally at the 50% level of response, which is why the $LC_{50}$ measure is used. Essentially, its use is arbitrary, as the ultimate goal of toxicity tests is to designate a concentration where no effect is caused (Gelber *et al.*, 1985; Rand and Petrocelli, 1985; Cairns and Pratt, 1989).

This, in itself, is complicated by the promotion of growth at low levels of toxins, which Ramade (1987) refers to as *Hormetic* action. He estimates that 50% of all potentially toxic substances can have a beneficial

effect on living things in low doses. He cites the particular case of the well known ability of various insecticides to increase the fertility of female insects in low doses, and states, "it is no longer possible to talk about....a dose without effect".

Intra-trial variation in current toxicity testing is commonly 25-40% (Macek, 1985). This is because of the heterogenicity of the sample population, and the small number of individuals used, which amplifies the statistical significance of random variations.

Inter-trial variations are often considerable. Common algal multiple test variations are >1 order of magnitude (Lewis, 1993). Sprague (1985) describes how sets of copper toxicity to Rainbow trout experiments in his laboratory ranged by factors from 1.39 to 5.5, and sets of organo-phosphate insecticide experiments over 2 years varied by a factor of 5, or more. He states that common inter-laboratory variations are a factor of 10 or more (also quoted by Lewis {1993} for inter-laboratory variation with algae).

In this study, the intra-trial and inter-trial variations were relatively low for all systems, including the cell culture assays. Because of the larger numbers of individuals and replicates in use in the cell assays, cell culture results would have a higher statistical significance.

An increase in the statistical strength of the data generated is one of the main contributions to be made by cell culture methodologies to environmental analysis.

5:4.3 Sensitivity.

This is one of the most important considerations when choosing a suitable bioassay, but one that should also be interpreted within the context of the overall objective of environmental analysis, i.e. increasing the environmental database. As noted earlier, the choice of species, with respect to sensitivity is, to a degree, arbitrary, if the objective is to rank chemicals in order of importance, for management purposes.

The question of sensitivity is one which has been addressed by a number of researchers. Isenberg (1993), when assessing the sensitivity of the Microtox assay relative to fish sensitivity, shows how 65% of results are within 0.5 orders of magnitude, and 95% within 1.5 orders of magnitude. This is regarded as excellent. Algal sensitivity, on the other hand, is noted for wide variations. Lewis (1993) states that variation in sensitivity in algal species is commonly 1-3 orders of magnitude.

In this study, two of the toxins studied are classed as neurotoxins. The lack of systemic factors is one of the main criticisms levelled at cell culture. While this certainly presents some particular difficulties, the results from this study show that, relative to the other systems examined in the study, and for all toxins examined, the cell culture assays would be classed as having medium to high sensitivity.

5:4.4 Data generation.

An important point of comparison between bioassays is the type of data that can be generated (i.e. the amount of data that can be generated in a short time, and the use that can be made of the data). These considerations include the length of the tests, whether the tests can be used in studies on the mechanisms of toxicity, and whether the data can be easily incorporated into management systems (including being easily understood by non-scientists).

It is particularly important, in the light of the backlog of chemicals without ecotoxicity information, the rate of production of new chemicals, and the compartments of environmental analysis which do not yet have a useful bioassay, that new bioassays should produce large amounts of meaningful and easily understood data.

Current bioassay systems are rigid, with respect to time, and thus the amount of data that can be generated. Also, they do not lend themselves well to studies on the mechanisms of toxicity, without significant expertise resources. However, they are legally accepted, and understood by policy makers and legislators. The death of an animal is an easily understood concept.

In this study, the cell assays were found to be very flexible, in relation to the length of time required for data generation. There are many types of cell culture assays, and these can be carried out in time spans that range from 1 hour, to chronic studies. Results from the study also give information on the mechanisms of toxicity of some of the toxins examined.

Conceptual problems related to the extrapolation of data from cellular systems to animal systems are the main reason why cell culture assays have not gained wide-spread legislative and policy acceptance. This is despite the fact that the extrapolation of data from cellular systems to whole animals has no less scientific basis than the extrapolation from one species to another, as is the practice in classical mammalian toxicity testing.

While the many projected technical and other problems expected with the use of cell culture assays are addressed, and solved, the greatest barrier to their wide-spread use may yet be a conceptual one.

## 5:5 Specific Integration Strategies.

While the design of specific ecotoxicity testing programmes is beyond the ambit of the study, the following are some suggestions for specific approaches that could be taken in using cell culture methods in environmental analysis.

### 5:5.1 Determination of toxicity.

For determination of the toxicity of specific materials, where the objective is to build on the dossier of environmental information, a battery of cell types could be used, to maximise the identification of any organ specific toxicity. Three representative tissue types would include hepatocytes (which provide biotransformation), epithelial cells (which represent the barrier tissue for almost all systemic effects), and gonadal cells (which are a representative reproductive cell type). For any given species, these would provide a basal dossier which could be applied to general toxins.

This could be expanded further by selecting cell lines from a number of species, representative of major groups within an ecosystem. At an even finer level, agencies working on a river catchment basis, with a much smaller universe of chemicals to deal with, could develop primary cultures direct from specific species present in each, unique ecosystem.

Cell culture can also contribute to data to categories other than lethal toxicity. This has already been acknowledged in the application of cytogenetic assays to assay for mutagenicity, carcinogenicity and teratogenicity (Walton *et al.*, 1983).

### 5:5.2 Waste management.

A study by Wall and Hanmer (1987) shows how the use of cell culture assays can be developed and integrated into waste management programmes. They refer to a cell culture study by local authorities in Florida, which found 60% of all municipal effluents to violate state toxicity limits for discharge. A similar situation was found with industrial wastes where chemically 'safe' wastes were shown to be highly toxic.

They describe a cell culture battery screening system used by a number of American states to define wastes as part of regional management plans. This has led to self-monitoring (using cell culture) by industrialists.

Models such as this could easily be developed for use in any area.

5:5.3 Research.

*In vitro* tests in research do not need to satisfy the requirements of a model system, and do not necessarily need to be correlated with other methods. Often, they are designed to answer a specific question only, and thus to further scientific knowledge.

The use of cell culture in environmental research includes studies on the biological action of toxins, on synergism and antagonism (e.g. Babich *et al*, 1988 on arsenic and selenium), on toxifying and detoxifying mechanisms (e.g. Kocan *et al.*, {1979} on the activation of mutagens by 3 cell lines) etc.

There have also been studies on the effectiveness of various treatment systems, using cell culture (e.g. Cody *et al.*, 1979 on water treatment and re-use options; Wilcox and Williamson, 1986 on the effect of water treatment on the mutagenicity of drinking water).

The possible applications of cell culture in research are myriad, and represent the area of environmental analysis in which cell culture methods are currently most often used.

5:6 **Summary.**

There is no panacea for the problems of environmental analysis. These problems are considerable, and there is an urgent need for useful bioassay methods which could be used to increase the environmental database, for the more effective management of materials discharged to the environment.

No existing or proposed bioassay method meets all of the requirements of the perfect bioassay. Of the range examined in this study, the cell culture tests used came the closest. It is considered from the results that cell culture methodologies could be of use in a number of areas of environmental analysis.

# CHAPTER 6
# CONCLUSIONS/
# RECOMMENDATIONS

## 6:1 Conclusions.

From the results of the study, as described, the following conclusions are drawn;

(1) The cell culture assays examined in the study were useful in a number of environmental applications.

(2) The cell culture methods presented fewer technical difficulties than many of the whole organism trials. They generated more data, and results had greater statistical strength than those from whole organism trials.

(3) No single cell culture or whole organism trial performed consistently better, in terms of sensitivity or handling, than all of the other methods. Each had a particular advantage in some area of assessment. Overall, cell culture methods performed better than any single whole organism trial.

(4) There was no significant difference in the indication of toxicity given by the two cell culture dye end-points, crystal violet elution, and neutral red for the toxins tested.

(5) The sensitivity of the two cell lines, L929 and RTG-2 was largely similar to Nuvan and ivermectin. RTG-2 cells were significantly more sensitive to malachite green.

(6) Algal growth inhibition, was the least sensitive, and one of the most technically problematic systems, but was the only system representative of the primary producer trophic level.

(7) Nuvan was the least toxic of the three fish farming chemicals tested. Ivermectin and malachite green were each assessed as being the most toxic by different systems.

(8) L929 cells were highly sensitive to three fractions of a metalliferous waste. The order of toxicity followed the metal concentration, but not in a 1:1 fashion.

(9) The most toxic of the three was the Low pH effluent, which contained the highest amount of metals. The toxicity was not associated with pH. Increasing serum concentration reduced the indication of toxicity, at a rate twice that seen with the sheep dip. Increasing seeding density reduced the indication of toxicity. Most of the toxic effect was exerted within 24 hours.

(10) *Artemia* sensitivity to the metalliferous effluents was low.

(11) L929 cells had a relatively low sensitivity to a diazinon based sheep dip. Indication of toxicity decreased with increasing serum concentration, and with increasing seeding density. Most of the toxic effect was exerted within 48 hours.

(12) Overall, the effect of serum concentration on the toxicity of the sheep dip and the metalliferous effluent was not considered to be a bar to the use of cell culture methods.

(13) Because of its relatively low sensitivity, cell culture toxicity analysis was not able to follow the operation of the columns, using the particular protocol applied in the study. A number of other protocols were identified which would enable the system to be used.

(14) Cell culture results were able to identify the leaching of toxic materials from the peat, not identifiable by chemical analysis, or *Artemia*.

(15) Both cell culture and *Artemia* results were useful in determining that there were more toxins in the sheep dip than the active ingredient, diazinon.

(16) *Artemia salina* sensitivity to the sheep dip was classed as medium, and the test was used to follow the operation of the peat columns.

## 6:2 Recommendations.

From the results of the study, as described, the following recommendations are made;

(1) Further studies which compare the sensitivities of various cell culture methods to whole organism methods, carried out in parallel, would be useful.

(2) The designation of a standard cytotoxicity assay, and its use in a systematic study for the determination of the toxicity of existing xenobiotics is required. The use of a mammalian cell line, and the crystal violet dye end point is recommended.

(3) A study establishing a number of representative cell lines from a particular ecosystem, aimed at establishing a toxicity management programme for that ecosystem would be of interest.

(4) A study aimed at using a cytotoxicity assay to monitor process control at a wastewater treatment plant, or chemical production plant would be of interest.

# CHAPTER 7
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# CHAPTER 8
# APPENDICES

## 8:1 Introduction.

The following appendix chapter presents the data files for all toxicity trials, as well as two experiments from the sheep dip study. The first data section (8:2) contains all of the files from the predictive hazard assessment trials. The second section (8:3) contains all of the files from the monitoring trials.

In section 8:2, the files are sub-divided into the assay systems used. Within each assay system, the files are presented according to toxin, with the replicate files for ivermectin presented, then the replicate files for malachite green, then the replicate files for Nuvan. Within the cell line sections, the files for the crystal violet elution trials are presented, then the neutral red files. In this section, the concentrations of toxins are given as mg/l, or µg/l active ingredient (e.g. Nuvan = mg/l as dichlorvos).

With the cell culture trials, the index measured was the inhibition of the uptake of crystal violet or neutral red dye. Thus the toxic effect is referred to as % inhibition (% Inh. in table headings). With the algal trials, the toxic effect is growth inhibition, and is also referred to as % inhibition. With the invertebrate trials, the toxic effect is immobilisation of the nauplii (as opposed to death), and is referred to as % immobilisation (% Imm.). With the fish trials, there were two toxic effects, mortality (% Mort.), and specific symptoms, referred to as effects (% Eff.).

In section 8:3, the files are sub-divided into those from the metalliferous effluent trials, and those from the sheep dip trials, those from the column experiment - cell culture trials, and those from the column experiment -*Artemia* trials. The conventions for headings etc. in 8.3 follow those from 8.2. In this section, the concentrations of the toxins are given as % effluent (% eff. in table headings).

The metalliferous and cell culture sections are each further sub-divided into cell culture (and then to standard conditions, serum experiment and time experiment) and *Artemia* files. The column experiment sections are further sub-divided into files from the various columns.

In section 8:4, a batch experiment on the removal of sheep dip by peat is presented.

In section 8:5, an experiment investigating the leaching of pesticide from peat is presented.

8:2 **Predictive Hazard Trials**

8:2.1 L929 cell line

Table 8.1: Ivermectin - Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 3.02 | 7.59 |
| 0.50 | 18.77 | 5.29 |
| 0.75 | 15.78 | 3.14 |
| 1.00 | 29.63 | 5.65 |
| 5.00 | 93.89 | 4.53 |
| 10.00 | 98.14 | 1.37 |

Table 8.2: Ivermectin -Crystal Violet-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 23.11 | 7.86 |
| 0.50 | 29.59 | 7.13 |
| 1.00 | 45.81 | 5.20 |
| 2.50 | 69.21 | 3.30 |
| 5.00 | 96.56 | 6.38 |
| 10.00 | 96.53 | 1.10 |

Table 8.3: Ivermectin - Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 10.19 | 4.43 |
| 0.50 | 30.65 | 4.43 |
| 1.00 | 37.08 | 3.47 |
| 2.50 | 66.48 | 4.10 |
| 5.00 | 98.10 | 1.19 |
| 10.00 | 98.80 | 0.95 |

Table 8.4: Ivermectin - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 15.63 | 5.73 |
| 0.50 | 14.63 | 7.39 |
| 1.00 | 22.28 | 5.77 |
| 2.50 | 30.38 | 5.89 |
| 5.00 | 97.42 | 0.80 |
| 10.00 | 99.58 | 0.44 |

Table 8.5: Ivermectin - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 12.35 | 9.75 |
| 0.50 | 14.28 | 4.06 |
| 1.00 | 17.03 | 8.92 |
| 2.50 | 54.90 | 7.22 |
| 5.00 | 96.07 | 0.98 |
| 10.00 | 99.81 | 0.25 |

Table 8.6: Ivermectin - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 10.39 | 4.97 |
| 0.50 | 16.53 | 5.98 |
| 1.00 | 22.69 | 2.43 |
| 2.50 | 45.69 | 4.55 |
| 5.00 | 96.97 | 0.71 |
| 10.00 | 99.89 | 0.13 |

Table 8.7: M. Green - Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.005 | 2.52 | 2.92 |
| 0.010 | 18.50 | 4.90 |
| 0.100 | 22.97 | 4.33 |
| 0.500 | 94.10 | 4.62 |
| 1.000 | 96.31 | 3.74 |
| 5.000 | 95.95 | 3.80 |

Table 8.8: M. Green - Crystal Violet-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.01 | 18.45 | 1.76 |
| 0.05 | 26.68 | 1.57 |
| 0.10 | 33.36 | 4.45 |
| 0.20 | 68.28 | 3.23 |
| 0.30 | 97.76 | 1.91 |
| 0.50 | 98.99 | 0.78 |

Continuing: Predictive hazard trials, L929 cell line.

Table 8.9: M. Green - Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.050 | 8.67 | 3.29 |
| 0.075 | 11.09 | 4.57 |
| 0.100 | 22.00 | 4.07 |
| 0.250 | 70.69 | 5.57 |
| 0.500 | 98.30 | 1.04 |
| 1.000 | 98.66 | 1.24 |

Table 8.10: M. Green - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | 4.12 | 9.64 |
| 0.050 | 7.43 | 9.66 |
| 0.100 | 9.38 | 12.23 |
| 0.500 | 96.77 | 0.82 |
| 1.000 | 98.70 | 1.26 |
| 5.000 | 99.68 | 0.29 |

Table 8.11: M. Green - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.01 | 6.21 | 7.73 |
| 0.05 | 4.45 | 3.43 |
| 0.10 | 20.23 | 9.47 |
| 0.20 | 87.85 | 3.82 |
| 0.30 | 99.45 | 0.41 |
| 0.50 | 99.73 | 0.30 |

Table 8.12: M. Green - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.050 | 1.54 | 6.55 |
| 0.075 | 8.52 | 5.15 |
| 0.100 | 13.71 | 3.74 |
| 0.250 | 39.63 | 2.62 |
| 0.500 | 95.68 | 0.97 |
| 1.000 | 99.65 | 0.21 |

Table 8.13: Nuvan - Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 15.08 | 5.01 |
| 0.50 | 19.54 | 4.20 |
| 2.50 | 46.65 | 2.17 |
| 5.00 | 63.52 | 3.18 |
| 7.50 | 81.76 | 2.44 |
| 10.00 | 91.48 | 2.44 |

Table 8.14: Nuvan - Crystal Violet-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.50 | 14.44 | 5.72 |
| 1.00 | 21.43 | 2.54 |
| 2.50 | 24.53 | 6.43 |
| 5.00 | 34.81 | 6.99 |
| 7.50 | 52.58 | 5.18 |
| 10.00 | 70.53 | 3.44 |

Table 8.15: Nuvan - Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 3.32 | 5.89 |
| 1.00 | 12.06 | 3.97 |
| 2.50 | 28.82 | 4.66 |
| 5.00 | 43.94 | 5.30 |
| 10.00 | 81.96 | 4.00 |
| 50.00 | 99.63 | 0.42 |

Table 8.16: Nuvan - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.42 | 10.31 |
| 0.50 | 14.35 | 8.05 |
| 2.50 | 33.17 | 8.75 |
| 5.00 | 66.25 | 6.82 |
| 7.50 | 92.61 | 2.24 |
| 10.00 | 97.31 | 1.05 |

Continuing: Predictive hazard trials, L929 cell line.

Table 8.17: Nuvan - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.50 | -3.40 | 7.16 |
| 1.00 | 12.22 | 8.74 |
| 2.50 | 12.21 | 8.70 |
| 5.00 | 34.76 | 8.28 |
| 7.50 | 62.62 | 5.05 |
| 10.00 | 86.71 | 4.19 |

Table 8.18: Nuvan - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 16.21 | 3.10 |
| 2.50 | 25.03 | 5.51 |
| 5.00 | 31.93 | 3.11 |
| 7.50 | 49.24 | 6.78 |
| 10.00 | 74.51 | 4.43 |
| 50.00 | 99.84 | 0.09 |

8:2.2 RTG-2 cell line

Table 8.19: Ivermectin-Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -3.63 | 7.98 |
| 0.18 | 8.46 | 7.78 |
| 0.32 | 18.45 | 6.00 |
| 1.00 | 20.39 | 5.59 |
| 1.80 | 39.56 | 9.76 |
| 3.20 | 74.21 | 3.51 |

Table 8.20: Ivermectin-Crystal Violet 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.56 | 24.77 | 7.51 |
| 1.00 | 27.76 | 8.70 |
| 1.80 | 38.73 | 9.63 |
| 3.20 | 59.83 | 9.46 |
| 5.60 | 82.95 | 3.95 |
| 10.00 | 97.30 | 2.06 |

Table 8.21: Ivermectin-Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 10.96 | 4.58 |
| 0.56 | 25.15 | 7.70 |
| 1.00 | 28.30 | 6.63 |
| 1.80 | 48.88 | 3.62 |
| 3.20 | 57.44 | 7.43 |
| 5.60 | 90.15 | 1.77 |

Table 8.22: Ivermectin - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 1.69 | 8.87 |
| 0.18 | 3.95 | 9.43 |
| 0.56 | 16.25 | 5.91 |
| 1.00 | 13.42 | 9.45 |
| 1.80 | 28.58 | 8.08 |
| 3.20 | 80.07 | 7.84 |

Table 8.23: Ivermectin - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.56 | -10.19 | 9.56 |
| 1.00 | -1.31 | 5.84 |
| 1.80 | 20.65 | 7.48 |
| 3.20 | 40.31 | 4.69 |
| 5.60 | 54.15 | 6.26 |
| 10.00 | 97.45 | 3.04 |

Table 8.24: Ivermectin - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.18 | 12.12 | 4.58 |
| 0.32 | 12.65 | 4.71 |
| 1.00 | 21.72 | 3.13 |
| 1.80 | 38.90 | 5.38 |
| 3.20 | 57.52 | 5.87 |
| 5.60 | 97.19 | 2.05 |

Continuing: Predictive hazard trials, RTG-2 cell line.

Table 8.25: M. Green - Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.001 | 0.53 | 9.01 |
| 0.010 | 20.88 | 6.73 |
| 0.018 | 52.28 | 5.51 |
| 0.032 | 84.76 | 7.84 |
| 0.100 | 85.68 | 7.21 |
| 0.320 | 93.63 | 3.38 |

Table 8.26: M. Green - Crystal Violet-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | -6.38 | 3.83 |
| 0.018 | 22.29 | 9.51 |
| 0.032 | 55.76 | 6.93 |
| 0.056 | 90.54 | 3.32 |
| 0.100 | 83.44 | 7.18 |
| 0.180 | 89.19 | 7.87 |

Table 8.27: M. Green - Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | -4.17 | 7.79 |
| 0.018 | 37.17 | 8.46 |
| 0.032 | 75.46 | 6.50 |
| 0.056 | 92.39 | 2.83 |
| 0.100 | 98.05 | 1.30 |
| 0.180 | 98.76 | 1.59 |

Table 8.28: M. Green - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | 17.32 | 9.70 |
| 0.018 | 40.71 | 6.16 |
| 0.032 | 86.21 | 3.93 |
| 0.180 | 84.15 | 3.29 |
| 0.320 | 88.47 | 3.05 |
| 0.560 | 98.56 | 0.58 |

Table 8.29: M. Green - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | 11.95 | 8.45 |
| 0.018 | 47.65 | 7.80 |
| 0.032 | 54.07 | 8.75 |
| 0.056 | 85.01 | 5.54 |
| 0.180 | 94.59 | 3.05 |
| 0.560 | 94.90 | 3.52 |

Table 8.30: M. Green - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.010 | 39.85 | 7.72 |
| 0.018 | 56.12 | 7.50 |
| 0.032 | 82.10 | 8.45 |
| 0.056 | 91.83 | 3.63 |
| 0.100 | 94.55 | 2.82 |
| 0.180 | 95.33 | 3.16 |

Table 8.31: Nuvan - Crystal Violet-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 5.52 | 11.40 |
| 3.20 | 20.78 | 10.12 |
| 5.60 | 23.71 | 8.67 |
| 10.00 | 44.03 | 4.22 |
| 18.00 | 96.15 | 4.31 |
| 32.00 | 93.58 | 4.31 |

Table 8.32: Nuvan - Crystal Violet-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | 9.88 | 5.03 |
| 1.00 | 21.38 | 3.65 |
| 5.60 | 37.27 | 4.40 |
| 10.00 | 58.21 | 2.00 |
| 18.00 | 71.61 | 6.20 |
| 32.00 | 97.46 | 2.14 |

Continuing: Predictive hazard trials, RTG-2 cell line.

Table 8.33: Nuvan - Crystal Violet-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 19.26 | 8.35 |
| 3.20 | 17.40 | 9.72 |
| 5.60 | 11.04 | 7.64 |
| 10.00 | 55.05 | 4.44 |
| 18.00 | 71.76 | 6.38 |
| 32.00 | 98.52 | 1.55 |

Table 8.34: Nuvan - Neutral Red-1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 29.77 | 8.97 |
| 3.20 | 32.86 | 10.51 |
| 10.00 | 38.05 | 8.30 |
| 18.00 | 86.01 | 5.89 |
| 32.00 | 97.56 | 1.37 |
| 56.00 | 97.00 | 2.66 |

Table 8.35: Nuvan - Neutral Red-2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 9.00 | 7.68 |
| 1.00 | 27.70 | 3.44 |
| 5.60 | 39.09 | 3.47 |
| 10.00 | 54.51 | 6.02 |
| 18.00 | 63.19 | 3.17 |
| 32.00 | 90.28 | 3.17 |

Table 8.36: Nuvan - Neutral Red-3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 23.11 | 3.15 |
| 3.20 | 36.63 | 3.65 |
| 5.60 | 45.57 | 4.10 |
| 10.00 | 55.17 | 2.66 |
| 18.00 | 79.59 | 3.88 |
| 32.00 | 93.48 | 3.71 |

Continuing: Predictive hazard trials

8:2.3 *Artemia salina*

Table 8.37: Ivermectin - 1

| Conc. (µg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 12.73 | 11.06 |
| 1.00 | 25.45 | 13.76 |
| 3.20 | 56.06 | 6.94 |
| 5.60 | 82.81 | 8.99 |
| 10.00 | 95.00 | 4.41 |

Table 8.38: Ivermectin - 2

| Conc. (µg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 14.95 | 4.35 |
| 1.00 | 31.67 | 7.64 |
| 3.20 | 52.78 | 4.81 |
| 5.60 | 75.91 | 3.72 |
| 10.00 | 94.84 | 4.51 |

Table 8.39: Ivermectin - 3

| Conc. (µg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 14.14 | 5.50 |
| 1.00 | 30.20 | 3.04 |
| 3.20 | 59.44 | 0.96 |
| 5.60 | 78.50 | 1.54 |
| 10.00 | 95.40 | 3.99 |

Table 8.40: Malachite Green - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.18 | 13.03 | 6.05 |
| 0.32 | 13.33 | 5.77 |
| 0.56 | 38.79 | 2.10 |
| 1.00 | 65.56 | 5.09 |
| 1.80 | 86.67 | 5.77 |

Table 8.41: Malachite Green - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.18 | 5.56 | 9.62 |
| 0.32 | 9.39 | 0.52 |
| 0.56 | 40.39 | 4.88 |
| 1.00 | 69.26 | 8.91 |
| 1.80 | 87.78 | 3.85 |

Table 8.42: Malachite green - 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.18 | 16.06 | 5.33 |
| 0.32 | 12.22 | 10.72 |
| 0.56 | 43.33 | 15.28 |
| 1.00 | 67.66 | 5.35 |
| 1.80 | 87.27 | 11.06 |

Table 8.43: Nuvan - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 6.67 | 5.77 |
| 5.60 | 5.81 | 5.04 |
| 10.00 | 34.81 | 8.34 |
| 18.00 | 70.91 | 10.12 |
| 32.00 | 81.11 | 10.18 |

Table 8.44: Nuvan - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.00 | 10.00 | 0.00 |
| 5.60 | 13.10 | 4.44 |
| 10.00 | 34.94 | 4.41 |
| 18.00 | 90.00 | 0.00 |
| 32.00 | 93.33 | 11.55 |

Continuing: Predictive hazard trials, *Artemia salina*

Table 8.45: Nuvan - 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 3.70 | 6.42 |
| 5.60 | 10.00 | 0.00 |
| 10.00 | 31.00 | 6.46 |
| 18.00 | 78.33 | 2.89 |
| 32.00 | 90.56 | 0.96 |

8:2.4 *Brachionus calyciflorus*

Table 8.46: Ivermectin - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 0.00 | 0.00 |
| 1.80 | 0.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |
| 4.20 | 100.00 | 0.00 |
| 5.60 | 100.00 | 0.00 |

Table 8.47: Ivermectin - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 0.00 | 0.00 |
| 1.80 | 0.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |
| 4.20 | 100.00 | 0.00 |
| 5.60 | 100.00 | 0.00 |

Table 8.48: Ivermectin - 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 0.00 | 0.00 |
| 1.80 | 0.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |
| 4.20 | 100.00 | 0.00 |
| 5.60 | 100.00 | 0.00 |

Table 8.49: Malachite Green - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 3.70 | 6.42 |
| 0.18 | 11.57 | 0.80 |
| 0.32 | 12.50 | 0.00 |
| 0.56 | 48.15 | 3.21 |
| 1.00 | 88.89 | 11.11 |

Table 8.50: Malachite Green - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.00 | 0.00 |
| 0.18 | 13.56 | 4.19 |
| 0.32 | 20.83 | 7.22 |
| 0.56 | 61.85 | 15.96 |
| 1.00 | 92.13 | 6.85 |

Table 8.51: Malachite Green - 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 3.70 | 6.42 |
| 0.18 | 10.53 | 1.77 |
| 0.32 | 18.70 | 7.03 |
| 0.56 | 60.19 | 4.01 |
| 1.00 | 93.94 | 10.50 |

Continuing: Predictive hazard trials, *Brachionus calyciflorus*

Table 8.52: Nuvan - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 7.87 | 6.85 |
| 1.00 | 25.00 | 12.50 |
| 3.20 | 40.48 | 10.91 |
| 5.60 | 68.52 | 11.23 |
| 10.00 | 97.22 | 4.81 |

Table 8.53: Nuvan - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 4.17 | 7.22 |
| 1.00 | 7.50 | 6.61 |
| 3.20 | 41.67 | 14.43 |
| 5.60 | 68.06 | 6.36 |
| 10.00 | 85.00 | 6.01 |

Table 8.54: Nuvan - 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 8.33 | 7.22 |
| 1.00 | 23.15 | 11.23 |
| 3.20 | 51.39 | 14.63 |
| 5.60 | 75.00 | 0.00 |
| 10.00 | 90.28 | 8.67 |

8:2.5 *Streptocephalus proboscideus*

Table 8.55: Malachite Green - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.32 | 10.37 | 0.64 |
| 0.56 | 40.00 | 0.00 |
| 1.00 | 93.33 | 11.55 |
| 1.80 | 100.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |

Table 8.56: Malachite Green - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.32 | 10.00 | 10.00 |
| 0.56 | 73.33 | 23.09 |
| 1.00 | 100.00 | 0.00 |
| 1.80 | 100.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |

Table 8.57: Nuvan - 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 22.22 | 6.94 |
| 1.00 | 89.26 | 11.13 |
| 1.80 | 100.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |
| 5.60 | 100.00 | 0.00 |

Table 8.58: Nuvan - 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.56 | 33.33 | 15.28 |
| 1.00 | 73.33 | 11.55 |
| 1.80 | 100.00 | 0.00 |
| 3.20 | 100.00 | 0.00 |
| 5.60 | 100.00 | 0.00 |

Continuing: Predictive hazard trials

8:2.6 Algal growth inhibition test

Table 8.59: Nuvan - 1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 3.20 | 8.82 | 3.58 |
| 10.00 | 7.80 | 1.94 |
| 32.00 | 22.19 | 4.69 |
| 100.00 | 59.07 | 1.73 |
| 320.00 | 89.01 | 1.72 |

Table 8.60: Nuvan - 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 10.00 | -9.61 | 5.08 |
| 32.00 | 10.68 | 12.63 |
| 100.00 | 58.46 | 0.78 |
| 320.00 | 94.19 | 1.91 |
| 560.00 | 96.16 | 0.87 |

Table 8.61: Nuvan - 3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 10.00 | -2.10 | 2.94 |
| 32.00 | 24.56 | 8.02 |
| 100.00 | 72.13 | 5.72 |
| 320.00 | 97.12 | 0.20 |
| | | |

Table 8.62: Malachite Green - 1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -3.59 | 4.76 |
| 1.00 | 9.81 | 1.50 |
| 10.00 | 88.92 | 0.00 |
| 100.00 | 100.00 | 3.70 |
| 1000.00 | 100.00 | 0.00 |

Table 8.63: Malachite Green - 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | -9.19 | 3.57 |
| 1.00 | 1.41 | 19.45 |
| 3.20 | 13.55 | 5.17 |
| 10.00 | 53.59 | 7.10 |
| 32.00 | 98.17 | 1.91 |

Table 8.64: Malachite Green - 3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | -7.75 | 8.04 |
| 1.00 | 12.51 | 6.35 |
| 3.20 | 55.18 | 7.01 |
| 10.00 | 87.62 | 4.78 |
| 32.00 | 100.00 | 0.66 |

Table 8.65: Ivermectin 1 (Range Finding)

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.056 | 8.00 | - - |
| 0.100 | 15.90 | - - |
| 1.000 | -3.81 | - - |
| 5.600 | -18.66 | - - |
| 10.000 | -13.95 | - - |

Continuing: Predictive hazard trials

8:2.7 Fish acute toxicity test (Rainbow trout, 96 hrs.)

Table 8.66: Malachite green ($LC_{50}$)

| Conc(mg/l) | % Mort. 1hr. | % Mort. 3hr. | % Mort. 24hr. | % Mort. 48hr. | % Mort. 72hr. | % Mort. 96hr. |
|---|---|---|---|---|---|---|
| 0.000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.010 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.056 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.100 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.180 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.320 | 0 | 0 | 40 | 40 | 50 | 60 |
| 0.560 | 0 | 0 | 80 | 100 | 100 | 100 |
| 1.000 | 0 | 0 | 100 | 100 | 100 | 100 |

Table 8.67: Malachite green ($EC_{50}$)

| Conc(mg/l) | % Eff. 1hr. | % Eff. 3hr. | % Eff. 24hr. | % Eff. 48hr. | % Eff. 72hr. | % Eff. 96hr. |
|---|---|---|---|---|---|---|
| 0.000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.010 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.056 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.100 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.180 | 0 | 0 | 0 | 10 | 10 | 10 |
| 0.320 | 0 | 0 | 50 | 60 | 60 | 60 |
| 0.560 | 0 | 0 | 90 | 100 | 100 | 100 |
| 1.000 | 0 | 0 | 100 | 100 | 100 | 100 |

Continuing: Predictive hazard trials, fish toxicity.

Table 8.68: Nuvan ($LC_{50}$)

| Conc(mg/l) | % Mort. 1hr. | % Mort. 3hr. | % Mort. 24hr. | % Mort. 48hr. | % Mort. 72hr. | % Mort. 96hr. |
|---|---|---|---|---|---|---|
| 0.0000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0056 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0100 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0560 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.3200 | 0 | 0 | 0 | 0 | 10 | 10 |
| 0.5600 | 0 | 0 | 0 | 20 | 40 | 60 |
| 1.0000 | 0 | 0 | 30 | 60 | 70 | 80 |

Table 8.69: Nuvan ($EC_{50}$)

| Conc(mg/l) | % Eff. 1hr. | % Eff. 3hr. | % Eff. 24hr. | % Eff. 48hr. | % Eff. 72hr. | % Eff. 96hr. |
|---|---|---|---|---|---|---|
| 0.0000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0056 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0100 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.0560 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.3200 | 0 | 0 | 0 | 60 | 60 | 60 |
| 0.5600 | 0 | 0 | 50 | 80 | 90 | 90 |
| 1.0000 | 0 | 0 | 100 | 100 | 100 | 100 |

8:3 **Monitoring Trials**

8:3.1 Metalliferous effluents

8:3.1.1 Cell culture - standard conditions

Table 8.70: Discharge fraction-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.56 | 1.99 | 2.23 |
| 0.75 | 20.80 | 1.38 |
| 1.00 | 15.98 | 2.80 |
| 1.80 | 67.75 | 6.04 |
| 3.20 | 97.96 | 3.47 |
| 10.00 | 99.56 | 0.39 |

Table 8.71: Discharge fraction-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.75 | 11.84 | 4.95 |
| 1.00 | 14.97 | 6.54 |
| 1.80 | 43.30 | 3.10 |
| 3.20 | 98.93 | 0.89 |
| 5.60 | 99.05 | 0.82 |
| 10.00 | 99.05 | 1.00 |

Table 8.72: Discharge fraction-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.75 | 14.12 | 3.67 |
| 1.00 | 15.72 | 6.47 |
| 1.80 | 25.70 | 6.62 |
| 3.20 | 62.27 | 5.24 |
| 5.60 | 98.77 | 0.40 |
| 10.00 | 99.31 | 0.32 |

Table 8.73: Low pH fraction-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | 4.88 | 2.77 |
| 0.42 | 19.35 | 3.57 |
| 0.56 | 38.59 | 4.29 |
| 0.75 | 78.17 | 1.67 |
| 0.87 | 99.54 | 0.24 |
| 1.00 | 99.79 | 0.19 |

Table 8.74: Low pH fraction-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | 20.51 | 2.36 |
| 0.42 | 29.59 | 3.08 |
| 0.56 | 53.23 | 1.84 |
| 0.75 | 99.29 | 0.69 |
| 0.87 | 99.27 | 0.59 |
| 1.00 | 98.87 | 1.09 |

Table 8.75: Low pH fraction-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | 27.22 | 2.56 |
| 0.42 | 32.28 | 2.14 |
| 0.56 | 60.54 | 1.91 |
| 0.75 | 98.01 | 3.06 |
| 0.87 | 98.78 | 0.80 |
| 1.00 | 99.29 | 0.59 |

Table 8.76: NaOH neutral. fraction-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.32 | 15.95 | 5.99 |
| 0.56 | 23.23 | 4.91 |
| 1.00 | 34.30 | 5.16 |
| 1.80 | 60.45 | 2.22 |
| 3.20 | 81.87 | 1.90 |
| 7.50 | 99.26 | 0.39 |

Table 8.77: NaOH neutral. fraction-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.56 | 26.84 | 6.09 |
| 1.00 | 40.65 | 5.72 |
| 1.80 | 57.76 | 2.77 |
| 2.40 | 69.25 | 2.72 |
| 3.20 | 80.14 | 2.90 |
| 5.60 | 98.72 | 0.61 |

Continuing: Monitoring trials, metalliferous effluent, cell culture

8:2.1.2 Cell culture - serum experiment

Table 8.78: NaOH neutral. fraction-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.56 | 22.54 | 3.47 |
| 1.00 | 41.39 | 2.92 |
| 3.20 | 75.60 | 2.07 |
| 5.60 | 96.71 | 0.69 |
| 7.50 | 99.48 | 0.62 |
| 10.00 | 99.23 | 0.62 |

Table 8.79: 1% serum-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 8.33 | 4.14 |
| 0.32 | 22.11 | 2.68 |
| 0.56 | 95.11 | 2.22 |
| 0.75 | 99.22 | 0.52 |
| 1.00 | 99.11 | 0.53 |
| 1.80 | 99.28 | 0.43 |

Table 8.80: 1% serum-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -4.03 | 3.39 |
| 0.32 | 21.98 | 3.17 |
| 0.56 | 97.01 | 2.20 |
| 0.75 | 98.81 | 0.44 |
| 1.00 | 98.59 | 0.69 |
| 1.80 | 98.86 | 0.73 |

Table 8.81: 1% serum-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 5.60 | 2.18 |
| 0.32 | 18.40 | 1.17 |
| 0.56 | 86.34 | 2.99 |
| 0.75 | 99.18 | 0.59 |
| 1.00 | 99.36 | 0.54 |
| 1.80 | 99.39 | 0.34 |

Table 8.82: 5% serum-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -0.53 | 3.60 |
| 0.32 | 3.15 | 4.24 |
| 0.56 | 37.33 | 1.84 |
| 0.75 | 77.82 | 2.31 |
| 1.00 | 99.11 | 0.41 |
| 1.80 | 98.98 | 0.84 |

Table 8.83: 5% serum-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.95 | 4.20 |
| 0.32 | 11.03 | 2.79 |
| 0.56 | 44.19 | 1.86 |
| 0.75 | 89.37 | 3.21 |
| 1.00 | 99.64 | 0.24 |
| 1.80 | 99.16 | 0.62 |

Table 8.84: 5% serum-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -0.31 | 5.83 |
| 0.32 | 8.35 | 5.30 |
| 0.56 | 40.75 | 4.77 |
| 0.75 | 84.09 | 2.52 |
| 1.00 | 95.43 | 3.00 |
| 1.80 | 95.63 | 3.06 |

Table 8.85: 10% serum-1

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -2.56 | 3.66 |
| 0.32 | 3.05 | 4.20 |
| 0.56 | 12.89 | 3.00 |
| 0.75 | 24.00 | 2.23 |
| 1.00 | 43.62 | 2.33 |
| 1.80 | 51.52 | 3.12 |

Continuing: Monitoring trials, metalliferous effluent, cell culture

Table 8.86: 10% serum-2

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -4.63 | 5.02 |
| 0.32 | 3.23 | 3.20 |
| 0.56 | 10.12 | 2.95 |
| 0.75 | 24.48 | 3.07 |
| 1.00 | 42.98 | 2.11 |
| 1.80 | 50.53 | 1.92 |

Table 8.87: 10% serum-3

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.07 | 2.48 |
| 0.32 | 6.11 | 3.01 |
| 0.56 | 16.60 | 1.83 |
| 0.75 | 31.86 | 2.17 |
| 1.00 | 49.44 | 2.65 |
| 1.80 | 57.22 | 2.14 |

8:2.1.3 Cell culture - metalliferous effluent - time experiment (Low pH fraction, 6x10-4 cells/ml)

Table 8.88: 09 hrs incubation.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -7.21 | 3.69 |
| 0.32 | -6.23 | 4.81 |
| 0.56 | -1.31 | 4.74 |
| 0.75 | 14.18 | 4.49 |
| 0.87 | 39.18 | 5.98 |
| 1.00 | 65.00 | 10.96 |

Table 8.89: 26 hrs incubation.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.89 | 3.45 |
| 0.32 | 4.27 | 2.28 |
| 0.56 | 18.12 | 3.29 |
| 0.75 | 57.69 | 1.67 |
| 0.87 | 84.77 | 2.33 |
| 1.00 | 98.71 | 0.56 |

Table 8.90: 47 hrs incubation.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -1.86 | 4.70 |
| 0.32 | 5.86 | 4.21 |
| 0.56 | 19.47 | 3.57 |
| 0.75 | 56.98 | 2.60 |
| 0.87 | 81.93 | 1.12 |
| 1.00 | 98.77 | 0.68 |

Table 8.91: 73 hrs incubation.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.85 | 2.13 |
| 0.32 | 5.36 | 2.18 |
| 0.56 | 18.86 | 2.72 |
| 0.75 | 51.53 | 1.67 |
| 0.87 | 71.66 | 1.89 |
| 1.00 | 97.86 | 3.46 |

Table 8.92: 95 hrs incubation.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 0.10 | -2.38 | 8.12 |
| 0.32 | 20.83 | 6.16 |
| 0.56 | 20.27 | 6.23 |
| 0.75 | 53.01 | 2.50 |
| 0.87 | 68.65 | 1.48 |
| 1.00 | 98.17 | 0.79 |

Continuing: Monitoring trials, metalliferous effluent

8:2.1.4 *Artemia salina* - metalliferous effluent

Table 8.93: Discharge fraction-1

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 6.06 | 5.25 |
| 56.00 | 78.84 | 2.61 |
| 65.00 | 100.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Table 8.94: Discharge fraction-2

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 22.73 | 6.36 |
| 32.00 | 30.76 | 8.08 |
| 56.00 | 61.21 | 10.22 |
| 65.00 | 100.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Table 8.95: Discharge fraction-3

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 15.15 | 13.89 |
| 32.00 | 21.11 | 18.36 |
| 56.00 | 69.80 | 3.04 |
| 65.00 | 100.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Table 8.96: Low pH fraction-1

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.00 | 0.00 |
| 0.32 | 0.00 | 0.00 |
| 0.56 | 0.00 | 0.00 |
| 0.75 | 0.00 | 0.00 |
| 1.00 | 100.00 | 0.00 |

Table 8.97: Low pH fraction-2

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.00 | 0.00 |
| 0.32 | 0.00 | 0.00 |
| 0.56 | 6.67 | 5.77 |
| 0.75 | 10.00 | 10.00 |
| 1.00 | 100.00 | 0.00 |

Table 8.98: Low pH fraction-3

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 0.10 | 0.00 | 0.00 |
| 0.32 | 0.00 | 0.00 |
| 0.56 | 16.67 | 11.55 |
| 0.75 | 10.00 | 10.00 |
| 1.00 | 100.00 | 0.00 |

Continuing: Monitoring trials, metalliferous effluent, *Artemia salina*

Table 8.99: NaOH neutral. fraction-1

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 8.59 | 8.34 |
| 56.00 | 22.73 | 6.36 |
| 65.00 | 26.87 | 6.68 |
| 75.00 | 45.15 | 5.01 |

Table 8.100: NaOH neutral. fraction-2

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 6.67 | 5.77 |
| 32.00 | 18.03 | 8.14 |
| 56.00 | 33.33 | 5.77 |
| 65.00 | 30.20 | 3.04 |
| 75.00 | 53.33 | 5.77 |

Table 8.101: NaOH neutralised fraction-3

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 12.42 | 5.01 |
| 56.00 | 26.77 | 9.86 |
| 65.00 | 26.67 | 5.77 |
| 75.00 | 50.00 | 10.00 |

8:3.2 Sheep dip

8:3.2.1 Cell culture - standard conditions

Table 8.102: Sheep dip - 1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -3.26 | 4.66 |
| 10.00 | 4.74 | 6.35 |
| 18.00 | 36.78 | 4.09 |
| 32.00 | 65.67 | 2.37 |
| 56.00 | 95.53 | 2.21 |
| 100.00 | 99.02 | 0.86 |

Table 8.103: Sheep dip - 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -18.06 | 10.24 |
| 10.00 | -11.05 | 6.97 |
| 18.00 | 28.63 | 4.42 |
| 32.00 | 63.77 | 3.72 |
| 56.00 | 95.15 | 2.26 |
| 100.00 | 98.60 | 1.12 |

Table 8.104: Sheep dip - 3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -25.07 | 5.82 |
| 10.00 | -3.25 | 6.68 |
| 18.00 | 37.16 | 3.59 |
| 32.00 | 65.10 | 4.38 |
| 56.00 | 96.16 | 2.41 |
| 100.00 | 97.96 | 1.66 |

Table 8.105: Sheep dip - 4

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -11.64 | 7.87 |
| 10.00 | -6.49 | 10.16 |
| 18.00 | 22.76 | 13.91 |
| 32.00 | 69.52 | 7.37 |
| 56.00 | 93.61 | 4.78 |
| 100.00 | 90.35 | 5.66 |

Table 8.106: Sheep dip - 5

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -10.29 | 8.54 |
| 10.00 | -2.52 | 8.69 |
| 18.00 | 22.49 | 8.09 |
| 32.00 | 68.23 | 5.43 |
| 56.00 | 98.96 | 1.52 |
| 100.00 | 98.73 | 1.51 |

Table 8.107: Sheep dip - 6

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -26.48 | 4.09 |
| 10.00 | -12.02 | 13.22 |
| 18.00 | 8.56 | 8.75 |
| 32.00 | 65.27 | 9.47 |
| 56.00 | 93.79 | 4.06 |
| 100.00 | 92.35 | 5.41 |

8:3.2.2 Cell culture - serum experiment

Table 8.108: 1% serum - 1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 3.20 | 1.64 | 7.15 |
| 5.60 | 10.58 | 6.18 |
| 10.00 | 18.61 | 3.38 |
| 18.00 | 35.58 | 8.09 |
| 32.00 | 91.97 | 4.94 |
| 56.00 | 95.26 | 5.72 |

Table 8.109: 1% serum - 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 3.20 | 4.23 | 4.46 |
| 5.60 | 15.85 | 8.00 |
| 10.00 | 24.48 | 5.52 |
| 18.00 | 43.90 | 3.15 |
| 32.00 | 90.54 | 5.38 |
| 56.00 | 97.18 | 1.50 |

Continuing: Monitoring trials, sheep dip, cell culture, serum experiment

Table 8.110: 1% serum - 3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 3.20 | -19.51 | 9.77 |
| 5.60 | 4.69 | 5.60 |
| 10.00 | 8.33 | 6.16 |
| 18.00 | 32.04 | 9.11 |
| 32.00 | 87.78 | 5.47 |
| 56.00 | 93.52 | 4.48 |

Table 8.111: 10% serum - 1

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | 6.02 | 6.78 |
| 10.00 | 9.33 | 5.56 |
| 18.00 | 19.22 | 4.71 |
| 32.00 | 44.73 | 2.95 |
| 56.00 | 79.17 | 7.44 |
| 100.00 | 91.20 | 1.45 |

Table 8.112: 10% serum - 2

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -10.05 | 1.83 |
| 10.00 | -4.69 | 3.63 |
| 18.00 | 6.82 | 3.81 |
| 32.00 | 37.61 | 6.35 |
| 56.00 | 73.75 | 7.57 |
| 100.00 | 90.87 | 2.01 |

Table 8.113: 10% serum - 3

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | 1.37 | 4.28 |
| 10.00 | 5.23 | 6.60 |
| 18.00 | 14.25 | 3.65 |
| 32.00 | 43.09 | 5.71 |
| 56.00 | 73.78 | 6.02 |
| 100.00 | 89.78 | 3.53 |

8:3.2.3 Cell culture - sheep dip - time/seeding density experiment (3x10-4 12 and 24 hr not calculable, due to insufficient growth)

Table 8.114: 3x10-4, 48 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -1.73 | 13.13 |
| 10.00 | 11.53 | 4.08 |
| 18.00 | 25.94 | 8.49 |
| 32.00 | 63.11 | 7.39 |
| 56.00 | 96.83 | 2.11 |
| 100.00 | 97.41 | 2.60 |

Table 8.115: 3x10-4, 60 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -2.95 | 8.88 |
| 10.00 | 15.19 | 5.32 |
| 18.00 | 21.94 | 4.11 |
| 32.00 | 71.73 | 6.03 |
| 56.00 | 97.26 | 2.20 |
| 100.00 | 93.46 | 3.31 |

Table 8.116: 3x10-4, 96 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.70 | 10.49 |
| 10.00 | 13.47 | 6.85 |
| 18.00 | 19.97 | 4.33 |
| 32.00 | 79.32 | 4.69 |
| 56.00 | 97.32 | 2.38 |
| 100.00 | 96.04 | 2.53 |

Continuing: Monitoring trials, sheep dip, cell culture, time/seeding density experiment

Table 8.117: 6x10-4, 14 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -10.86 | 18.56 |
| 10.00 | -4.21 | 15.51 |
| 18.00 | -4.21 | 17.88 |
| 32.00 | 18.40 | 14.44 |
| 56.00 | 70.29 | 13.40 |
| 100.00 | 90.24 | 11.96 |

Table 8.118: 6x10-4, 24 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -14.40 | 16.38 |
| 10.00 | -6.80 | 12.68 |
| 18.00 | 20.00 | 11.73 |
| 32.00 | 52.00 | 14.40 |
| 56.00 | 90.80 | 9.74 |
| 100.00 | 89.60 | 9.49 |

Table 8.119: 6x10-4, 48 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -9.99 | 7.68 |
| 10.00 | 3.74 | 6.11 |
| 18.00 | 20.80 | 4.26 |
| 32.00 | 51.10 | 3.22 |
| 56.00 | 85.17 | 2.83 |
| 100.00 | 96.98 | 2.89 |

Table 8.120: 6x10-4, 72 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -5.65 | 5.27 |
| 10.00 | 2.07 | 4.01 |
| 18.00 | 13.98 | 2.66 |
| 32.00 | 48.59 | 2.11 |
| 56.00 | 85.29 | 2.32 |
| 100.00 | 98.91 | 0.72 |

Table 8.121: 1x10-5, 14 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -14.77 | 17.19 |
| 10.00 | -15.25 | 7.10 |
| 18.00 | -8.47 | 13.74 |
| 32.00 | 38.98 | 14.46 |
| 56.00 | 75.79 | 11.10 |
| 100.00 | 89.35 | 7.68 |

Table 8.122: 1x10-5, 24 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -13.39 | 9.98 |
| 10.00 | 5.47 | 12.60 |
| 18.00 | 18.75 | 19.26 |
| 32.00 | 42.45 | 11.52 |
| 56.00 | 84.90 | 9.50 |
| 100.00 | 85.94 | 9.82 |

Table 8.123: 1x10-5, 48 hr.

| Conc. (mg/l) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -7.63 | 8.92 |
| 10.00 | 3.30 | 1.77 |
| 18.00 | 17.76 | 4.78 |
| 32.00 | 48.45 | 4.93 |
| 56.00 | 83.66 | 4.20 |
| 100.00 | 96.58 | 3.93 |

Table 8.124: 1x10-5, 72 hr.

| Conc. (mg/l ) | % Inh. | SD (% Inh) |
|---|---|---|
| 5.60 | -0.53 | 2.97 |
| 10.00 | 5.17 | 5.43 |
| 18.00 | 14.17 | 6.47 |
| 32.00 | 51.21 | 3.68 |
| 56.00 | 87.48 | 2.25 |
| 100.00 | 98.77 | 1.06 |

Continuing: Monitoring trials, sheep dip

8:3.2.4 *Artemia salina*

Table 8.125: Sheep Dip 1

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 18.89 | 1.92 |
| 5.60 | 20.74 | 1.28 |
| 10.00 | 51.26 | 6.53 |
| 13.30 | 78.18 | 4.81 |
| 18.00 | 92.99 | 6.69 |

Table 8.126: Sheep Dip 2

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 23.33 | 5.77 |
| 7.50 | 26.87 | 6.68 |
| 10.00 | 53.89 | 14.56 |
| 13.30 | 78.18 | 4.81 |
| 32.00 | 100.00 | 0.00 |

Table 8.127: Sheep dip 3

| Conc. (mg/l) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 30.00 | 0.00 |
| 7.50 | 40.00 | 0.00 |
| 10.00 | 60.91 | 11.39 |
| 13.30 | 71.11 | 7.70 |
| 32.00 | 100.00 | 0.00 |

Continuing: Monitoring trials

8:3.3 Column experiment - Cell culture

8:3.3.1 Blank column

Table 8.128: Blank. 0.625 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.59 | 6.43 |
| 3.20 | -4.19 | 5.88 |
| 5.60 | -11.69 | 3.76 |
| 7.50 | -17.79 | 8.07 |
| 8.70 | -3.82 | 6.53 |
| 10.00 | 9.28 | 6.46 |

Table 8.129: Blank, 0.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -0.58 | 4.94 |
| 3.20 | -9.38 | 4.61 |
| 5.60 | -10.94 | 5.83 |
| 7.50 | -3.79 | 4.88 |
| 8.70 | 5.82 | 2.70 |
| 10.00 | 16.53 | 3.24 |

Table 8.130: Blank, 1.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.71 | 12.16 |
| 5.60 | -9.59 | 6.95 |
| 8.70 | -7.31 | 11.55 |
| 10.00 | 6.30 | 3.58 |
| | | |
| | | |

Table 8.131: Blank, 3.792 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 7.00 | 1.89 |
| 3.20 | 8.39 | 5.62 |
| 5.60 | 1.87 | 4.65 |
| 7.50 | 18.83 | 5.33 |
| 8.70 | 25.23 | 6.50 |
| 10.00 | 26.97 | 5.52 |

Table 8.132: Blank, 6.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -3.39 | 5.58 |
| 3.20 | -11.03 | 7.58 |
| 5.60 | -10.22 | 2.16 |
| 7.50 | -17.79 | 5.28 |
| 8.70 | -13.73 | 2.92 |
| 10.00 | -7.66 | 3.34 |

Table 8.133: Blank, 8.5 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.42 | 3.93 |
| 3.20 | -4.00 | 4.42 |
| 5.60 | -8.72 | 5.59 |
| 7.50 | -14.81 | 4.46 |
| 8.70 | -5.91 | 4.56 |
| 10.00 | -1.05 | 4.14 |

Table 8.134: Blank, 11.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.96 | 3.56 |
| 3.20 | -5.79 | 7.08 |
| 5.60 | -17.86 | 6.34 |
| 7.50 | -7.40 | 3.37 |
| 8.70 | 8.30 | 4.01 |
| 10.00 | 3.16 | 4.86 |

Continuing: Monitoring trials, column experiment, cell culture

8:3.3.2 Column 1

Table 8.135: Column 1, 0.625 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.22 | 5.60 |
| 3.20 | -5.82 | 7.19 |
| 5.60 | -14.17 | 11.57 |
| 7.50 | -8.71 | 5.30 |
| 8.70 | 7.07 | 7.33 |
| 10.00 | 22.37 | 6.75 |

Table 8.136: Column 1, 0.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.71 | 8.13 |
| 3.20 | 1.16 | 5.64 |
| 5.60 | -10.63 | 6.44 |
| 7.50 | -9.95 | 4.06 |
| 8.70 | 0.26 | 4.85 |
| 10.00 | 22.74 | 3.32 |

Table 8.137: Column 1, 1.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.98 | 7.24 |
| 3.20 | -3.24 | 3.18 |
| 5.60 | -11.63 | 7.43 |
| 7.50 | -6.89 | 9.93 |
| 8.70 | 2.53 | 12.10 |
| 10.00 | 1.54 | 9.19 |

Table 8.138: Column 1, 3.792 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -7.06 | 11.21 |
| 5.60 | -3.72 | 5.03 |
| 7.50 | 0.80 | 9.26 |
| 8.70 | 7.36 | 5.78 |
| 10.00 | 15.77 | 4.16 |
| | | |

Table 8.139: Column 1, 6.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -13.13 | 5.82 |
| 3.20 | -25.84 | 10.54 |
| 5.60 | -20.17 | 15.05 |
| 7.50 | -23.80 | 8.03 |
| 8.70 | -11.77 | 5.07 |
| 10.00 | -2.72 | 3.84 |

Table 8.140: Column 1, 8.5 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.03 | 4.16 |
| 3.20 | -0.57 | 4.38 |
| 5.60 | -6.46 | 5.35 |
| 7.50 | -6.28 | 3.53 |
| 8.70 | 2.30 | 4.00 |
| 10.00 | 8.89 | 3.91 |

Table 8.141: Column 1, 11.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -10.18 | 7.22 |
| 3.20 | -13.71 | 2.81 |
| 5.60 | -16.03 | 4.07 |
| 7.50 | -17.61 | 10.81 |
| 8.70 | -5.24 | 5.86 |
| 10.00 | 6.24 | 6.00 |

Table 8.142: Column 1, 13.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -5.69 | 5.31 |
| 3.20 | -12.81 | 7.97 |
| 5.60 | -9.76 | 4.20 |
| 7.50 | -11.32 | 3.37 |
| 8.70 | -2.24 | 3.15 |
| 10.00 | 2.93 | 3.72 |

Table 8.143: Column 1, 14.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -0.64 | 3.61 |
| 3.20 | -4.80 | 2.99 |
| 5.60 | -12.93 | 3.28 |
| 7.50 | -20.67 | 5.61 |
| 8.70 | -6.27 | 8.56 |
| 10.00 | 2.51 | 11.99 |

Table 8.144: Column 1, 17.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.61 | 3.29 |
| 3.20 | -1.58 | 3.83 |
| 5.60 | -1.44 | 2.45 |
| 7.50 | -6.73 | 3.70 |
| 8.70 | -3.39 | 5.57 |
| 10.00 | 3.39 | 4.66 |

Table 8.145: Column 1, 21.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 2.09 | 2.47 |
| 3.20 | -0.23 | 3.11 |
| 5.60 | -3.18 | 3.45 |
| 7.50 | -5.15 | 2.47 |
| 8.70 | -3.58 | 3.03 |
| 10.00 | 2.45 | 4.64 |

Table 8.146: Column 1, 25.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 0.43 | 2.97 |
| 3.20 | 6.95 | 1.50 |
| 5.60 | -6.91 | 2.92 |
| 7.50 | -4.33 | 2.11 |
| 8.70 | -0.34 | 1.94 |
| 10.00 | -1.20 | 2.47 |

Table 8.147: Column 1, 28.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -0.09 | 5.17 |
| 3.20 | -5.20 | 3.50 |
| 5.60 | -5.12 | 3.55 |
| 7.50 | -7.36 | 4.88 |
| 8.70 | -2.56 | 5.58 |
| 10.00 | 0.36 | 5.68 |

Table 8.148: Column 1, 31.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.52 | 4.04 |
| 3.20 | -5.22 | 4.69 |
| 5.60 | -2.12 | 4.65 |
| 7.50 | -4.89 | 4.82 |
| 8.70 | -1.98 | 7.42 |
| 10.00 | 2.94 | 4.80 |

Table 8.149: Column 1, 34.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -3.42 | 1.99 |
| 3.20 | -5.00 | 2.07 |
| 5.60 | -4.77 | 2.08 |
| 7.50 | -5.64 | 2.36 |
| 8.70 | -2.85 | 3.03 |
| 10.00 | -2.00 | 2.01 |

Table 8.150: Column 1, 38.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.86 | 8.86 |
| 3.20 | -2.57 | 6.64 |
| 5.60 | -5.86 | 5.60 |
| 7.50 | -7.01 | 7.22 |
| 8.70 | 1.92 | 3.57 |
| 10.00 | 12.32 | 4.46 |

Table 8.151: Column 1, 42.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -9.05 | 4.51 |
| 3.20 | -8.89 | 5.55 |
| 5.60 | -13.67 | 4.74 |
| 7.50 | -12.71 | 4.65 |
| 8.70 | -2.48 | 3.94 |
| 10.00 | 3.22 | 3.77 |

Table 8.152: Column 1, 45.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -6.12 | 2.65 |
| 3.20 | -11.05 | 2.76 |
| 5.60 | -12.09 | 4.49 |
| 7.50 | -11.52 | 2.87 |
| 8.70 | -7.21 | 8.93 |
| 10.00 | -0.61 | 11.78 |

Table 8.153: Column 1, 49.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.78 | 4.30 |
| 3.20 | -5.04 | 2.93 |
| 5.60 | -7.21 | 2.37 |
| 7.50 | -10.95 | 2.85 |
| 8.70 | -4.97 | 2.12 |
| 10.00 | -2.90 | 3.43 |

Table 8.154: Column 1, 56.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 1.80 | 4.00 |
| 3.20 | 3.31 | 4.14 |
| 5.60 | 1.26 | 2.49 |
| 7.50 | -1.11 | 3.36 |
| 8.70 | -2.77 | 5.59 |
| 10.00 | -2.31 | 2.31 |

Table 8.155: Column 1, 59.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.49 | 3.87 |
| 3.20 | 0.95 | 3.68 |
| 5.60 | -1.98 | 3.46 |
| 7.50 | -1.66 | 2.82 |
| 8.70 | 1.34 | 2.34 |
| 10.00 | 1.11 | 2.84 |

Table 8.156: Column 1, 63.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.07 | 2.24 |
| 3.20 | 2.83 | 5.56 |
| 5.60 | 2.15 | 7.37 |
| 7.50 | 2.30 | 7.12 |
| 8.70 | 4.45 | 7.14 |
| 10.00 | 9.26 | 8.12 |

Table 8.157: Column 1, 66.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.52 | 3.67 |
| 3.20 | 6.35 | 9.72 |
| 5.60 | 3.20 | 8.59 |
| 7.50 | 1.99 | 11.28 |
| 8.70 | 0.66 | 10.57 |
| 10.00 | 6.15 | 8.92 |

Continuing: Monitoring trials, column experiment, cell culture

8:3.3.3 Column 2

Table 8.158: Column 2, 0.625 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -14.40 | 7.24 |
| 3.20 | -13.21 | 9.83 |
| 5.60 | -15.41 | 11.54 |
| 7.50 | -10.05 | 5.05 |
| 8.70 | 5.71 | 5.36 |
| 10.00 | 16.98 | 7.54 |

Table 8.159: Column 2, 0.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -4.72 | 7.60 |
| 3.20 | -3.31 | 4.72 |
| 5.60 | -7.43 | 12.56 |
| 7.50 | -7.03 | 5.20 |
| 8.70 | 2.33 | 7.04 |
| 10.00 | 20.02 | 3.65 |

Table 8.160: Column 2, 1.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -3.63 | 7.82 |
| 3.20 | 2.52 | 11.36 |
| 5.60 | 6.73 | 7.68 |
| 7.50 | -1.48 | 7.63 |
| 8.70 | 19.14 | 4.85 |
| 10.00 | 30.90 | 4.87 |

Table 8.161: Column 2, 6.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -11.06 | 5.73 |
| 3.20 | -9.42 | 3.04 |
| 5.60 | -12.37 | 10.82 |
| 7.50 | -6.75 | 8.76 |
| 8.70 | 3.41 | 4.30 |
| 10.00 | 10.53 | 3.58 |

Table 8.162: Column 2, 8.5 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.03 | 3.06 |
| 3.20 | -2.41 | 3.74 |
| 5.60 | -5.44 | 7.53 |
| 7.50 | -7.42 | 6.08 |
| 8.70 | -0.67 | 5.07 |
| 10.00 | 7.11 | 3.75 |

Table 8.163: Column 2, 11.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -0.60 | 5.09 |
| 3.20 | -6.89 | 4.14 |
| 5.60 | -10.64 | 5.78 |
| 7.50 | -13.62 | 4.50 |
| 8.70 | 3.72 | 6.07 |
| 10.00 | 10.84 | 5.92 |

Table 8.164: Column 2, 13.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -4.80 | 6.55 |
| 3.20 | -5.53 | 6.81 |
| 5.60 | -13.39 | 7.23 |
| 7.50 | -7.10 | 6.31 |
| 8.70 | -3.47 | 4.45 |
| 10.00 | -2.30 | 7.01 |

Table 8.165: Column 2, 14.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 0.42 | 4.29 |
| 3.20 | -1.59 | 2.79 |
| 5.60 | -7.82 | 2.68 |
| 7.50 | -12.12 | 2.91 |
| 8.70 | -3.97 | 3.30 |
| 10.00 | -0.93 | 3.72 |

Continuing: Monitoring trials, column experiment, cell culture

8:3.3.4 Column 3

Table 8.166: Column 3, 0.625 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.12 | 7.09 |
| 3.20 | 0.33 | 6.90 |
| 7.50 | 2.26 | 7.74 |
| 8.70 | 28.52 | 9.44 |
| 10.00 | 39.58 | 7.83 |
| | | |

Table 8.167: Column 3. 0.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.37 | 4.70 |
| 3.20 | -10.08 | 5.04 |
| 5.60 | -13.37 | 3.78 |
| 7.50 | -7.47 | 9.69 |
| 8.70 | 20.85 | 8.12 |
| 10.00 | 46.09 | 6.14 |

Table 8.168: Column 3, 1.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 1.39 | 4.21 |
| 3.20 | 7.15 | 4.71 |
| 5.60 | 24.69 | 9.16 |
| 7.50 | 65.52 | 9.31 |
| 8.70 | 78.21 | 6.45 |
| 10.00 | 92.81 | 3.21 |

Table 8.169: Column 3, 3.792 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 11.91 | 7.07 |
| 3.20 | 13.54 | 5.09 |
| 5.60 | 17.88 | 7.14 |
| 7.50 | 20.30 | 5.55 |
| 8.70 | 31.43 | 6.51 |
| 10.00 | 43.48 | 5.31 |

Table 8.170: Column 3, 6.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -11.30 | 5.63 |
| 3.20 | -28.81 | 17.64 |
| 5.60 | -25.33 | 5.68 |
| 7.50 | -35.90 | 9.41 |
| 8.70 | -24.41 | 8.21 |
| 10.00 | -18.47 | 9.64 |

Table 8.171: Column 3, 8.5 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 3.20 | -9.28 | 5.70 |
| 5.60 | -16.73 | 4.45 |
| 7.50 | -13.08 | 5.46 |
| 8.70 | 1.97 | 4.24 |
| 10.00 | 8.76 | 2.45 |
| | | |

Table 8.172: Column 3, 11.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.18 | 4.38 |
| 3.20 | 3.96 | 6.40 |
| 5.60 | 11.68 | 6.05 |
| 7.50 | 8.69 | 11.05 |
| 8.70 | -4.66 | 9.34 |
| 10.00 | -12.57 | 7.12 |

Table 8.173: Column 3, 13.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -0.51 | 3.05 |
| 3.20 | -4.35 | 5.72 |
| 5.60 | -11.04 | 8.07 |
| 7.50 | -9.89 | 4.99 |
| 8.70 | -1.43 | 6.05 |
| 10.00 | 9.84 | 5.99 |

Table 8.174: Column 3, 14.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -4.99 | 4.96 |
| 3.20 | -5.75 | 2.83 |
| 5.60 | -10.54 | 2.75 |
| 7.50 | -10.54 | 2.27 |
| 8.70 | -5.42 | 2.03 |
| 10.00 | 4.46 | 2.91 |

Table 8.175: Column 3, 17.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.86 | 1.71 |
| 3.20 | 1.56 | 2.02 |
| 5.60 | 0.64 | 4.04 |
| 7.50 | -1.36 | 3.13 |
| 8.70 | 0.41 | 3.50 |
| 10.00 | 0.53 | 3.43 |

Table 8.176: Column 3, 21.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.15 | 2.43 |
| 3.20 | -1.35 | 1.94 |
| 5.60 | -3.81 | 1.55 |
| 7.50 | -5.65 | 5.36 |
| 8.70 | -1.95 | 4.99 |
| 10.00 | 2.00 | 4.18 |

Table 8.177: Column 3, 25.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -8.10 | 7.52 |
| 3.20 | -14.37 | 6.04 |
| 5.60 | -15.68 | 4.45 |
| 7.50 | -12.89 | 4.38 |
| 8.70 | -4.84 | 4.87 |
| 10.00 | 7.22 | 6.07 |

Table 8.178: Column 3, 28.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -3.40 | 2.00 |
| 3.20 | -3.17 | 2.12 |
| 5.60 | -6.04 | 2.08 |
| 7.50 | -4.78 | 2.50 |
| 8.70 | -0.55 | 3.98 |
| 10.00 | 3.71 | 4.12 |

Table 8.179: Column 3, 31.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.56 | 3.79 |
| 3.20 | -3.08 | 3.11 |
| 5.60 | -5.33 | 2.52 |
| 7.50 | -4.68 | 4.12 |
| 8.70 | 2.60 | 7.02 |
| 10.00 | 8.77 | 6.73 |

Table 8.180: Column 3, 34.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -2.10 | 3.36 |
| 3.20 | -3.14 | 2.16 |
| 5.60 | -5.96 | 3.63 |
| 7.50 | -3.89 | 2.14 |
| 8.70 | -1.74 | 4.96 |
| 10.00 | -1.34 | 3.09 |

Table 8.181: Column 3, 38.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 1.73 | 3.44 |
| 3.20 | -1.12 | 3.84 |
| 5.60 | -3.95 | 4.64 |
| 7.50 | -5.21 | 5.14 |
| 8.70 | 0.28 | 5.91 |
| 10.00 | 10.90 | 3.13 |

Table 8.182: Column 3, 42.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -4.61 | 5.08 |
| 3.20 | -7.68 | 5.10 |
| 5.60 | -9.92 | 5.49 |
| 7.50 | -8.31 | 4.08 |
| 8.70 | 0.10 | 6.68 |
| 10.00 | 9.59 | 4.87 |

Table 8.183: Column 3, 45.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 2.59 | 5.90 |
| 3.20 | -1.71 | 3.40 |
| 5.60 | 0.58 | 6.94 |
| 7.50 | 2.26 | 10.69 |
| 8.70 | 4.42 | 10.32 |
| 10.00 | 10.58 | 13.37 |

8:3.3.5 Column 4

Table 8.184: Column 4, 0.625 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -8.58 | 3.34 |
| 3.20 | -15.66 | 5.64 |
| 5.60 | -16.79 | 11.02 |
| 7.50 | -10.09 | 8.25 |
| 8.70 | 9.74 | 7.36 |
| 10.00 | 29.14 | 8.93 |

Table 8.185: Column 4, 0.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -8.09 | 9.72 |
| 3.20 | 1.26 | 6.54 |
| 5.60 | -7.98 | 13.81 |
| 7.50 | -6.26 | 5.08 |
| 8.70 | 7.35 | 5.17 |
| 10.00 | 24.57 | 7.20 |

Table 8.186: Column 4, 1.979 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.98 | 7.72 |
| 3.20 | 9.77 | 7.71 |
| 5.60 | 13.83 | 10.49 |
| 7.50 | 21.91 | 9.65 |
| 8.70 | 43.20 | 7.24 |
| 10.00 | 60.86 | 9.96 |

Table 8.187: Column 4, 3.792 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 5.23 | 5.55 |
| 3.20 | 4.09 | 5.59 |
| 5.60 | -5.81 | 11.07 |
| 7.50 | 15.23 | 6.91 |
| 8.70 | 23.30 | 5.77 |
| 10.00 | 29.86 | 7.28 |

Table 8.188: Column 4, 8.5 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -10.37 | 7.04 |
| 3.20 | -16.67 | 8.11 |
| 5.60 | -17.75 | 11.98 |
| 7.50 | -21.37 | 8.16 |
| 8.70 | -13.89 | 4.27 |
| 10.00 | -7.64 | 7.80 |

Table 8.189: Column 4, 11.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -1.78 | 5.20 |
| 3.20 | -6.70 | 6.24 |
| 5.60 | -10.33 | 10.24 |
| 7.50 | 4.30 | 4.43 |
| 8.70 | 15.51 | 5.26 |
| 10.00 | 23.08 | 6.81 |

Table 8.190: Column 4, 13.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | -5.17 | 5.64 |
| 3.20 | -9.09 | 5.99 |
| 5.60 | -10.07 | 10.89 |
| 7.50 | -7.89 | 5.60 |
| 8.70 | 10.07 | 9.89 |
| 10.00 | 14.75 | 9.53 |

Table 8.191: Column 4, 14.0 days.

| Conc. (% eff) | % Inh. | SD (% Inh) |
|---|---|---|
| 1.00 | 5.27 | 2.99 |
| 3.20 | 2.00 | 2.94 |
| 5.60 | 1.00 | 2.11 |
| 7.50 | 0.45 | 1.66 |
| 8.70 | -1.59 | 4.74 |
| 10.00 | 0.52 | 3.72 |

Continuing: Monitoring trials

8:3.4 Column experiment - *Artemia salina*

8:3.4.1 Blank column

Table 8.192: Blank, 0.625 days

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 0.00 | 0.00 |
| 5.60 | 0.00 | 0.00 |
| 10.00 | 6.67 | 5.77 |
| 18.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |

Table 8.193: Blank, 0.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 3.33 | 5.77 |
| 5.60 | 3.33 | 5.77 |
| 10.00 | 0.00 | 0.00 |
| 18.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |

Table 8.194: Blank, 1.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 42.00 | 0.00 | 0.00 |
| 56.00 | 0.00 | 0.00 |
| 75.00 | 0.00 | 0.00 |

Table 8.195: Blank, 3.791 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 42.00 | 0.00 | 0.00 |
| 56.00 | 0.00 | 0.00 |
| 75.00 | 0.00 | 0.00 |

Table 8.196: Blank, 6.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 42.00 | 0.00 | 0.00 |
| 56.00 | 0.00 | 0.00 |
| 75.00 | 0.00 | 0.00 |

Table 8.197: Blank, 8.5 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 42.00 | 0.00 | 0.00 |
| 56.00 | 0.00 | 0.00 |
| 75.00 | 0.00 | 0.00 |

Table 8.198: Blank, 11.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.6.00 | 0.00 | 0.00 |
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 56.00 | 0.00 | 0.00 |
| 75.00 | 0.00 | 0.00 |

Continuing: Monitoring trials, column experiment, *Artemia*

8:3.4.2 Column 1

Table 8.199: Column 1, 0.625 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 17.68 | 18.20 |
| 5.60 | 9.70 | 10.01 |
| 10.00 | 46.26 | 13.35 |
| 18.00 | 60.91 | 11.39 |
| 32.00 | 93.27 | 5.92 |

Table 8.200: Column 1, 0.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 12.73 | 11.06 |
| 5.60 | 20.00 | 17.32 |
| 10.00 | 32.42 | 6.70 |
| 18.00 | 66.06 | 18.21 |
| 32.00 | 83.94 | 5.33 |

Table 8.201: Column 1, 1.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 29.35 | 1.84 |
| 32.00 | 100.00 | 0.00 |
| 42.00 | 100.00 | 0.00 |
| 56.00 | 100.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Table 8.202: Column 1, 3.791 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 71.82 | 1.57 |
| 42.00 | 77.58 | 4.20 |
| 56.00 | 77.58 | 4.20 |
| 75.00 | 84.87 | 5.00 |

Table 8.203: Column 1, 6.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 45.45 | 12.73 |
| 42.00 | 60.61 | 13.89 |
| 56.00 | 64.24 | 12.41 |
| 75.00 | 66.11 | 6.74 |

Table 8.204: Column 1, 8.5 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 24.85 | 18.66 |
| 32.00 | 80.00 | 10.00 |
| 42.00 | 80.00 | 10.00 |
| 56.00 | 100.00 | 0.00 |
| 75.00 | 93.33 | 5.77 |

Table 8.205: Column 1, 11.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.00 | 0.00 |
| 10.00 | 3.03 | 5.25 |
| 32.00 | 84.24 | 9.97 |
| 56.00 | 93.89 | 5.36 |
| 75.00 | 100.00 | 0.00 |

Table 8.206: Column 1, 13.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.00 | 0.00 |
| 10.00 | 8.84 | 0.44 |
| 32.00 | 81.11 | 1.92 |
| 56.00 | 86.67 | 5.77 |
| 75.00 | 90.30 | 0.52 |

Continuing: Monitoring trials, column experiment, *Artemia*

Table 8.207: Column 1, 14.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.00 | 0.00 |
| 10.00 | 7.41 | 6.42 |
| 32.00 | 88.33 | 12.58 |
| 56.00 | 96.67 | 5.77 |
| 75.00 | 100.00 | 0.00 |

Table 8.208: Column 1, 17.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 15.76 | 14.12 |
| 18.00 | 34.85 | 15.96 |
| 32.00 | 40.00 | 10.00 |
| 56.00 | 81.21 | 1.05 |
| 75.00 | 100.00 | 0.00 |

Table 8.209: Column 1, 21.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 28.33 | 2.89 |
| 18.00 | 46.97 | 9.46 |
| 32.00 | 87.58 | 13.80 |
| 56.00 | 90.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Table 8.210: Column 1, 25.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 9.39 | 0.52 |
| 5.60 | 9.44 | 10.05 |
| 10.00 | 22.78 | 12.51 |
| 32.00 | 90.61 | 9.11 |
| 75.00 | 100.00 | 0.00 |

Table 8.211: Column 1, 28.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 6.67 | 5.77 |
| 5.60 | 13.33 | 11.55 |
| 10.00 | 55.15 | 21.69 |
| 32.00 | 90.30 | 0.52 |
| 56.00 | 100.00 | 0.00 |

Table 8.212: Column 1, 31.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 8.68 | 1.19 |
| 3.20 | 12.42 | 5.01 |
| 5.60 | 26.06 | 21.13 |
| 10.00 | 61.11 | 9.62 |
| 32.00 | 96.67 | 5.77 |

Table 8.213: Column 1, 34.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 10.37 | 10.02 |
| 3.20 | 23.33 | 15.28 |
| 5.60 | 59.39 | 4.58 |
| 10.00 | 93.33 | 5.77 |
| 32.00 | 100.00 | 0.00 |

Table 8.214: Column 1, 38.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.33 | 5.77 |
| 3.20 | 15.76 | 5.84 |
| 5.60 | 21.06 | 3.53 |
| 10.00 | 58.18 | 3.15 |
| 32.00 | 100.00 | 0.00 |

Continuing: Monitoring trials, column experiment, *Artemia*

Table 8.215: Column 1, 42.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 16.06 | 5.33 |
| 3.20 | 12.78 | 6.31 |
| 5.60 | 22.12 | 11.37 |
| 10.00 | 53.33 | 5.77 |
| 32.00 | 100.00 | 0.00 |

Table 8.216: Column 1, 45.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 13.03 | 15.38 |
| 3.20 | 22.73 | 6.36 |
| 5.60 | 23.33 | 5.77 |
| 10.00 | 65.50 | 9.65 |
| 32.00 | 100.00 | 0.00 |

Table 8.217: Column 1, 49.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 16.36 | 11.82 |
| 3.20 | 21.06 | 3.53 |
| 5.60 | 18.15 | 13.16 |
| 10.00 | 61.52 | 7.84 |
| 32.00 | 100.00 | 0.00 |

Table 8.218: Column 1, 56.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.33 | 5.77 |
| 3.20 | 12.73 | 6.30 |
| 5.60 | 17.98 | 8.71 |
| 10.00 | 64.65 | 9.26 |
| 32.00 | 100.00 | 0.00 |

Table 8.219: Column 1, 59.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 9.14 | 0.83 |
| 3.20 | 26.52 | 9.19 |
| 5.60 | 39.60 | 6.07 |
| 10.00 | 77.78 | 15.75 |
| 32.00 | 100.00 | 0.00 |

Table 8.220: Column 1, 63.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 6.73 | 5.92 |
| 3.20 | 24.36 | 5.12 |
| 5.60 | 27.56 | 21.27 |
| 10.00 | 100.00 | 0.00 |
| 32.00 | 100.00 | 0.00 |

Table 8.221: Column 1, 66.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 17.78 | 16.78 |
| 3.20 | 5.13 | 8.88 |
| 5.60 | 20.13 | 4.81 |
| 10.00 | 100.00 | 0.00 |
| 32.00 | 100.00 | 0.00 |

Continuing: Monitoring trials, column experiment, *Artemia*

8:3.4.3 Column 3

Table 8.222: Column 3, 0.625 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.00 | 0.00 |
| 10.00 | 0.00 | 0.00 |
| 32.00 | 18.18 | 15.75 |
| 42.00 | 10.44 | 1.17 |
| 56.00 | 26.67 | 25.17 |

Table 8.223: Column 3, 0.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 3.03 | 5.25 |
| 10.00 | 7.04 | 6.12 |
| 32.00 | 6.67 | 5.77 |
| 42.00 | 6.67 | 5.77 |
| 56.00 | 38.15 | 15.96 |

Table 8.224: Column 3, 1.979 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 0.00 | 0.00 |
| 42.00 | 6.67 | 11.55 |
| 56.00 | 10.00 | 17.32 |
| 75.00 | 16.11 | 21.10 |

Table 8.225: Column 3, 3.791 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 0.00 | 0.00 |
| 32.00 | 49.70 | 8.40 |
| 42.00 | 74.44 | 13.47 |
| 56.00 | 76.97 | 15.68 |
| 75.00 | 86.97 | 6.05 |

Table 8.226: Column 3, 6.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 3.03 | 5.25 |
| 32.00 | 50.00 | 0.00 |
| 42.00 | 65.45 | 6.56 |
| 56.00 | 84.44 | 5.09 |
| 75.00 | 87.22 | 6.31 |

Table 8.227: Column 3, 8.5 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 10.00 | 23.33 | 15.28 |
| 32.00 | 78.33 | 10.41 |
| 42.00 | 90.61 | 0.52 |
| 56.00 | 93.33 | 5.77 |
| 75.00 | 100.00 | 0.00 |

Table 8.228: Column 3, 11.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 0.00 | 0.00 |
| 10.00 | 0.00 | 0.00 |
| 32.00 | 80.91 | 8.67 |
| 56.00 | 90.61 | 0.52 |
| 75.00 | 97.22 | 4.81 |

Table 8.229: Column 3, 13.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 3.33 | 5.77 |
| 10.00 | 3.03 | 5.25 |
| 32.00 | 81.82 | 0.00 |
| 56.00 | 100.00 | 0.00 |
| 75.00 | 100.00 | 0.00 |

Continuing: Monitoring trials, column experiment, *Artemia*

Table 8.230: Column 3, 14.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 5.60 | 3.33 | 5.77 |
| 10.00 | 15.45 | 4.72 |
| 32.00 | 86.67 | 5.77 |
| 56.00 | 93.64 | 5.53 |
| 75.00 | 100.00 | 0.00 |

Table 8.231: Column 3, 17.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 0.00 | 0.00 |
| 5.60 | 23.03 | 12.07 |
| 10.00 | 28.79 | 8.25 |
| 32.00 | 46.67 | 7.35 |
| 75.00 | 100.00 | 0.00 |

Table 8.232: Column 3, 21.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 9.09 | 0.00 |
| 5.60 | 17.04 | 11.24 |
| 10.00 | 20.00 | 10.00 |
| 32.00 | 48.48 | 10.34 |
| 75.00 | 93.94 | 10.50 |

Table 8.233: Column 3, 25.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 0.00 | 0.00 |
| 5.60 | 12.12 | 13.89 |
| 10.00 | 46.67 | 11.55 |
| 32.00 | 90.61 | 9.11 |
| 75.00 | 100.00 | 0.00 |

Table 8.234: Column 3, 28.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 3.20 | 10.00 | 0.00 |
| 5.60 | 10.37 | 10.02 |
| 10.00 | 15.56 | 5.09 |
| 32.00 | 41.54 | 4.07 |
| 56.00 | 96.67 | 5.77 |

Table 8.235: Column 3, 31.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 23.77 | 15.51 |
| 3.20 | 28.15 | 14.11 |
| 5.60 | 18.89 | 1.92 |
| 10.00 | 81.41 | 10.59 |
| 32.00 | 96.67 | 5.77 |

Table 8.36: Column 3, 34.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.33 | 5.77 |
| 3.20 | 9.39 | 9.11 |
| 5.60 | 36.67 | 23.54 |
| 10.00 | 56.36 | 3.15 |
| 32.00 | 100.00 | 0.00 |

Table 8.37: Column 3, 38.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 12.73 | 11.06 |
| 3.20 | 18.59 | 10.59 |
| 5.60 | 22.12 | 6.82 |
| 10.00 | 56.67 | 15.28 |
| 32.00 | 100.00 | 0.00 |

Continuing: Monitoring trials, column experiment, *Artemia*

Table 8.238: Column 3, 42.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 3.03 | 5.25 |
| 3.20 | 23.25 | 6.67 |
| 5.60 | 32.14 | 8.52 |
| 10.00 | 68.18 | 12.82 |
| 32.00 | 100.00 | 0.00 |

Table 8.239: Column 3, 45.0 days.

| Conc. (% eff) | % Imm. | SD (% Inh) |
|---|---|---|
| 1.00 | 6.67 | 5.77 |
| 3.20 | 6.67 | 11.55 |
| 5.60 | 20.37 | 11.56 |
| 10.00 | 66.67 | 11.55 |
| 32.00 | 100.00 | 0.00 |

8:4 **Peat Batch Experiment**

Six 100 ml aliquots of 400 ppm a.i. sheep dip were prepared in Erlenmeyer flasks (ground glass stoppered). To these were added 0, 1.0, 2.5, 5.0, 7.5 and 10 grammes (wet weight) of peat fibre. These were shaken for 4 hours, a time greater than required for equilibrium in similar systems, with pesticides (Cloutier *et al.*, 1985). Then, the ready eluant was removed (i.e. no strong vacuum removal). An aliquot was removed for analysis, the remainder was GFC filtered, and an aliquot removed from this portion also. These were analysed to the effect of sample filtration.

The results are presented in Table 8.4.1. The results show that filtration removes significant amounts of diazinon from solution.

**Table 8.4.1**

**The uptake of diazinon by peat fibre in a batch experiment: the effect of sample filtration**

| Sample | Treatment | Mls of eluant | Conc. (mg/l) | Uptake / g.d.w. | Redctn (%) on Filtering |
|---|---|---|---|---|---|
| Seed | | | 389.11* | | |
| Seed | GFC filtered | | 144.13 | | 63 |
| Seed | 0.45 µ filtered | | 157.11 | | 60 |
| 1.0 g peat | | 98 | 256.83 | **24.39** | |
| 1.0 g peat | GFC filtered | 91 | 173.70 | 41.00 | 32 |
| 2.5 g peat | | 92 | 195.28 | **14.88** | |
| 2.5 g peat | GFC filtered | 86 | 134.57 | 19.42 | 31 |
| 5.0 g peat | | 86 | 128.80 | **9.89** | |
| 5.0 g peat | GFC filtered | 81 | 75.40 | 11.63 | 42 |
| 7.5 g peat | | 80 | 88.62 | **7.53** | |
| 7.5 g peat | GFC filtered | 73 | 54.76 | 8.27 | 38 |
| 10.0 g peat | | 78 | 62.35 | **6.04** | |
| 10.0 g peat | GFC filtered | 70 | 33.48 | 6.50 | 46 |

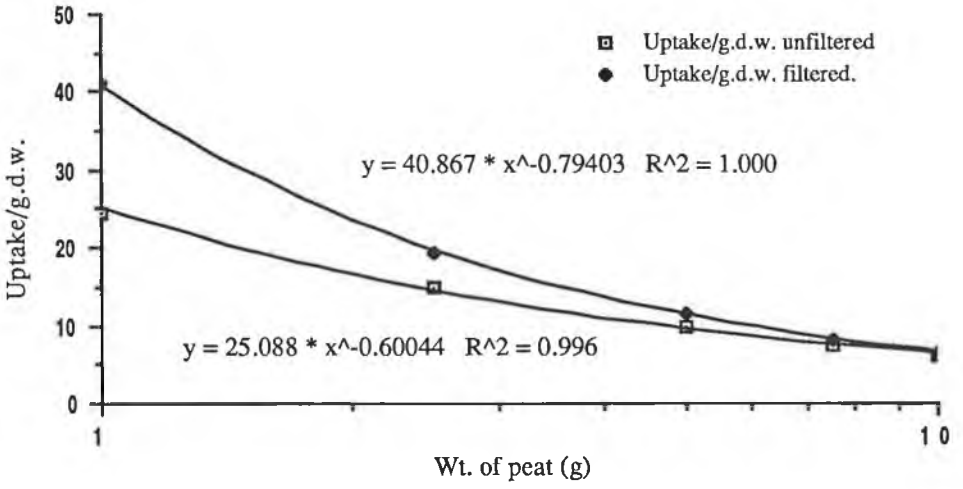
* = 100 ml solution, i.e. total 38.911 mg in solution.

As can be seen from the table, glass fibre paper filtration of the field concentration dip 'solution' removed 63% of the diazinon from the solution. Millipore (0.45 µm) filtration, while using a different material and pore size, did not remove a significantly different quantity (60%). Glass fibre

paper filtration of the eluant from the batch flasks removed amounts varying from c. 30-50%.

Figure 8.4.1 shows the relationship between peat concentration and mass adsorbed, along with the effect of filtration on this.

**Figure 8.4.1**
**Batch removal of diazinon: the effect of filtration**




It can be seen that failure to take into account physical filtration would lead to a gross overestimation of the adsorptive capacity of the fibre for the pesticide. An assessment of the fibre performance based on such figures would assume that the pesticide was tightly bound, which would have serious implications for disposal.

The explanation proposed for removal by filtration is that the sheep dip is not in true solution, but rather is present as an emulsion, and that a significant quantity of the emulsion droplets are large enough to be removed by physical filtration. This is a principle that has been previously described, and applied in environmental protection situations (Coupal and Lalancette, 1976; Chaney and Hundeman, 1979; McKay, 1980; Viraraghavan and Mathavan, 1988,89; McCarthy, 1990a).

The removal of low-water solublity pesticides from water samples by filtration has been reported previously by others. Brown and Bellinger (1979), in a study of dieldrin in river catchments, and potential methods of removal, present results which indicate that passage of untreated dyehouse effluent, containing 31.4 µg/l of dieldrin, through a GF/F filter

(0.7μm) removed 30% of the dieldrin. However, no further results are shown, and no discussion is made of this result.

Viraraghavan and Mathavan, in a number of studies on the use of peat for the removal of oil-in water emulsions, reported the need for glass fibre filtration of oil samples, as conventional filtration would remove large emulsion droplets from solution (Viraraghavan and Mathavan, 1988, 1989).

A number of other workers have examined the removal oil-in-water emulsions by peat in waste treatment systems (McKay, 1980; McCarthy, 1990a). However, in these cases, there was no requirement for the pollutant to be tightly bound, to facilitate subsequent land disposal. Also, breakthrough rates were not of great concern, as the process was being used as an improving, or damage limitation option, rather than a complete waste treatment option.

These results have significant implications for the operation of the columns, and for the ultimate use of the product. A column of the size and compaction rate used in this study could reasonably be expected to act as a physical filter to any material which can be removed in this way.

The most immediate concern was that no realistic prediction could be made for expected mass adsorbed/gramme, and so for breakthrough, or column life. If the peat bed was to physically filter the emulsion, then the capacity for a 60 cm, 25% compacted bed to do so could be very large indeed.

Also, if pesticide was to be removed in this way, it would not be tightly bound, and would have significant potential for leaching, which would severely restrict the end disposal of the used peat. The leaching experiment, as described in appendix 8:5 was designed to determine if this was the case.

## 8:5 Peat Leaching Experiment

This experiment was designed to give qualitative data on whether or not diazinon would be easily leached (or more correctly washed out) from the peat. At the end of the period of operation of the duplicate 100% fibre column (i.e. 14 days, Column 2), the column was allowed to drain for 12 hours, then the fibre was removed as a single mass.

A series of 10 g (w.w.) samples were taken from five equidistant points along the length of the compacted fibre, beginning 2-3 cm from the top, and added to 100 ml of water in 250 ml glass stoppered Erlenmeyer flasks. These were shaken gently for four hours, and the water was analysed by GC for diazinon content.

The results are expressed as wet weight, as oven drying of peat with large quantities of pesticide entrained would not be appropriate. Thus the result is not quantified in terms of the amount of pesticide applied/entrained/discharged.

**Table 8.5.1**

**The number of mg of diazinon leached by 10g (w/w) samples of peat fibre to 100 ml distilled water**

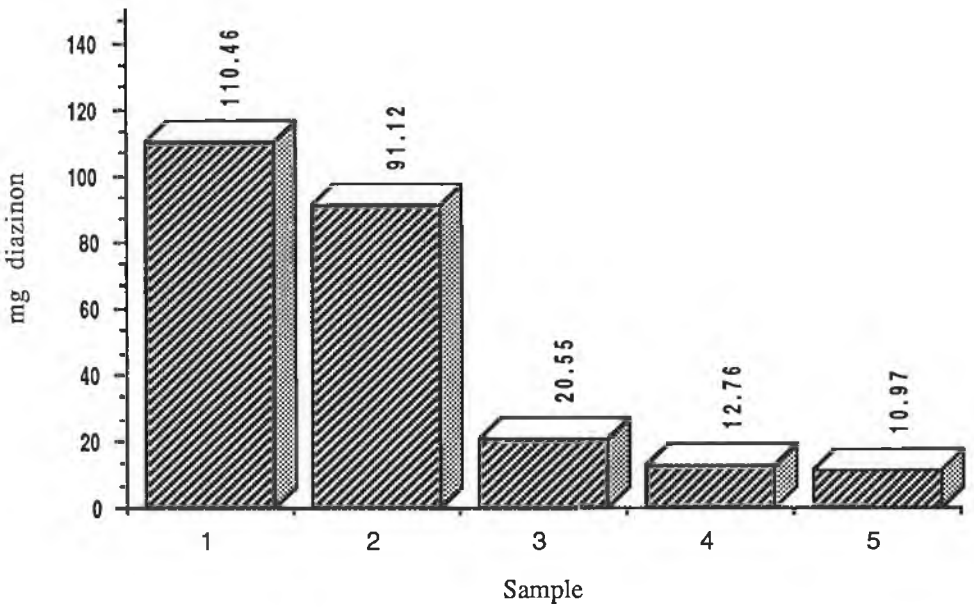
| Sample Number | No. of mg Diazinon Leached |
|---|---|
| 1 | 110.5* |
| 2 | 91.1 |
| 3 | 20.6 |
| 4 | 12.8 |
| 5 | 11.0 |

* = 1,105 mg/l, in 100 ml.

As can be seen from Table 8.5.1, and Figure 8.5.1, large amounts of pesticide were readily washed out by gentle shaking. While some of this may have been elution of lightly bound pesticide (which would still be of concern), the huge quantities released could only be a result of physically entrained emulsion being redistributed to the solvent by the shaking action. This can be readily seen in Plate 8.5.1, which shows the solutions from the upper portions of the column as completely white (flask one measured 1,100 mg/l, flask two measured 911 mg/l).

These results confirm the hypothesis of physical filtration of emulsion droplets, and raises important questions about the use and disposal of the material in the removal of sheep dip from solution.

**Figure 8.5.1**
**The number of mg of diazinon leached by 10g (w/w) samples of peat fibre to 100 ml distilled water**




These results indicate that the material is not suitable for landfill after use, which would often be the disposal method used for spent adsorbents.

If adsorption were the only mechanism at work, there would be a range of disposal options available, including using the fibre to produce peat blocks which could be used in horticulture, as a source of diazinon in pest control. As stated, this is not the case here.

Incineration of the fibre would seem to be an obvious option, with complete destruction of the pesticide, along with recovery of the calorific value of the fibre. However, care should be taken to ensure that toxic by-products would not arise from such treatment. For example, while there is no chlorine in the pesticide, there may be in the unknown portions of the dip, most significantly the solvent, which could, in theory, give rise to chlorinated by-products, which would be of some concern.

Plate 8.5.1

Wash out of sheep dip from fibre

There are conflicting opinions in the literature about the ultimate disposal of peat used for pollutant removal. While Chaney and Hundeman (1979) used peat to precipitate suspended Cadmium, and other metals (clearly an effluent improvement stage), they then state that disposal of the peat by burial gives insignificant leaching (below background levels). It is difficult to imagine that this would remain the case in the long term.

Also McCarthy (1990a), using acid treated peat to remove paraquat, diquat and amitrole from solution, showed that high salts solutions removed 80-100% of the adsorbed pesticide (3M NaCl removed 100% amitrole, 5.5M NaCl removed 92% diquat and 5.5M $NH_4Cl$ removed 83% paraquat). While these salts levels would not occur in normal soil systems, they could, in theory, occur in landfill leachates.

While peat has not been previously used to remove diazinon, reports from soil studies have regarded soil applied diazinon to be adsorbed and not available for leaching. However, Littlejohn and Melvin (1991) have shown diazinon in surface waters to peak after dipping, and also after high rainfall. A specific discharge to a soakaway (which complied with Department of Agriculture regulations) appeared in a stream, 400m away, within 3 hours.