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Determining water quality: Development of a cell culture cytotoxicity assay

By

Petro Humphries

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Determining water quality: Development of a cell culture cytotoxicity assay

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Abstract

Not only in South Africa, but also all over the world, pollution is threatening the quality of water resources. More than 100,000 chemicals are produced worldwide and some of these chemicals enter into the aquatic environment, posing a potential threat to humans, animals as well as other organisms. Therefore, determining the quality of water is of great importance. The testing of the toxicity of water samples are either based on a biological model or chemical model. In the biological model, organisms are utilized as indicators for toxicity of a particular sample, this sample may then be cytotoxic to the cells of the organism. In the chemical model the amount of a specific chemical present in water samples are predicted. Although, within the biological model there are many cytotoxicity screening assays (also called bioassays) that assess quality of water, there is a need for a fast, efficient and cost-effective cell culture system that may act as first screening procedure in a range of tests.

Of particular importance in the context of this thesis, are bioassays that measure specific biochemical parameters, e.g. the Uridine uptake bioassay, MTT (3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) assay and the bioluminescent assay. An important advantage of these assays are their sensitivity and the fact that the tests are principally based on cell mortality as a consequence of membrane damage or influence on cell metabolism. Researchers at the Highveld Biological Laboratory in South Africa have the past few years paid particular attention to the

MTT assay, originally developed by Mosmann, and have modified this assay. In this thesis the adjusted method is referred to as the Modified Highveld Biological assay. There were, however, still a few adjustments to be made in order to use this modified method to its full potential. The Bioluminescent assay, also were thought to have potential to be modified as water quality screening test. Therefore, the hypotheses investigated in the current thesis were:

- The original Mosmann MTT method can be modified successfully to provide a sensitive, reproducible mass screening method for determining chemical cytotoxicity and water quality, by using the K-562 cell line.
- Sensitivity of the assay can be increased by utilizing a 3 phase medium cycle (Medium type 1-3) instead of the previously used single culture medium.
- Furthermore, this cytotoxicity assay can be successfully utilized to determine whether any chemical solution or water sample is cytotoxic.

Thus information was needed to determine whether cytotoxicity correlates with real life toxicity. This requires time-consuming epidemiological surveys unless results can be correlated with previous surveys.

The research in this thesis attempted to indicate that the obtained cytotoxicity can be used to predict toxicity of a water sample, and that the cytotoxicity findings of this dissertation may indeed be meaningful. The culturing conditions of the Modified Highveld Biological MTT assay were modified successfully and could thus be utilized as the first assay in a battery of tests to determine overall cytotoxicity by utilising K-652 cells in culture. Due to successful modification of the assay, this MTT assay now reduces assay duration, thereby saving important resources.

An attempt was also made to optimise the Bioluminescent assay. This method is based on the fact that adenosine triphosphate (ATP) is present in all living cells. The method utilizes an enzyme, firefly luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly dependent on the ATP concentration and is measured using a luminometer. The following problems were, however, identified that renders this method unsatisfactory as cytotoxicity indicator for water samples.

- The luciferase enzyme is too sensitive to too many different substances used in the cell culture medium and in the preparation procedures of the cells (ions, salts etc.) which makes it less suited for usage for fast, effective testing of water toxicity.

- This method will probably function better when luciferase can be carried into the cells or form part of the cells' genetic material.
- We therefore suggest that, the Bioluminescent assay might possibly be a method to determine cytotoxicity, if the sensitivity of the luciferase enzyme could be further investigated.

Thus it could be concluded that luminescence is not suitable for assaying complex mixtures because it is possible for unknown non-toxic agents, present in test samples, to interfere with the process of light emission (quenching).

Lastly, known chemical solutions as well as unknown water samples were screened using the adjustments to the Modified Highveld Biological Method proposed in this thesis. The cytotoxicity of unknown water samples and chemical solutions were successfully determined and different cytotoxic effects were obtained, e.g. synergism, antagonism, additive effects and neutral effects. It was also possible to reduce or remove the cytotoxicity of certain water samples by applying pre-treatment with either Na_2CO_3 (removing possible toxic divalent and polyvalent metals) or SepPak cartridge clean up (removing organic toxicants). Results indicated that the method is very sensitive and can detect even low traces of toxicants.

Thus, it can be concluded that the method was successfully adjusted to be useful as a first screening assay for toxicity analysis of a series of environmental water samples.

Opsomming

Besoedeling is besig om in Suid-Afrika, sowel as die res van die wêreld, watervoorrade te bedreig. Meer as 100,000 chemikalieë word wêreldwyd vervaardig en sommige daarvan beland in die akwatiese omgewing. Dit is 'n potensiële bedreiging vir mense, diere sowel as ander organismes. Om hierdie redes is dit uiters belangrik om die kwaliteit van water te bepaal. Die bepaling van die toksisiteit van water monsters word tans gebaseer op 'n biologiese of chemiese model. In die biologiese model word organismes gebruik as indikators vir toksisiteit van 'n spesifieke monster, hierdie monster mag dan sitotoksies wees vir die selle van die organisme. In die chemiese model word die hoeveelheid van 'n spesifieke chemiese middel teenwoordig in die watermonster bepaal. Alhoewel daar baie sitotoksiese siftingstoetse, ook biosiftingstoetse genoem ("screening assay" of "bioassay") bestaan, wat deel uitmaak van die biologiese model en gebruik word om die kwaliteit van water te bepaal, is daar 'n behoefte vir die ontwikkeling van 'n vinnige, koste-effektiewe selkultuursisteem wat as eerste sitotoksisiteitsindikator in 'n reeks siftingsprosedures gebruik kan word.

Van besondere belang binne die konteks van hierdie tesis, is biosiftingstoetse wat spesifieke biologiese parameters meet, bv. die "Uridine uptake bioassay", "MTT assay" en die "Bioluminescent assay". 'n Belangrike voordeel van hierdie toetse is hulle sensitiwiteit en omdat die toetse hoofsaaklik gebasseer is op seldood as gevolg van membraanbeskadiging of invloed op sel metabolisme. Navorsers by die Highveld Biological Laboratorium in Suid-Afrika het die laaste paar jaar spesifiek aandag gegee aan die MTT (3-(4,5-dimethielthiazol-2-iel-2,5-diphenieltetrazolium bromied) toets, oorspronklik ontwikkel deur Mosmann, en het hierdie toets aangepas. In hierdie tesis word na die aangepaste metode verwys as die "Modified Highveld Biological Method". Dit was egter nodig om nog 'n paar veranderinge te maak voordat hierdie metode se volle potensiaal benut kon word. Daar is ook geglo dat die "Bioluminescent assay" potensïeel aangepas kan word vir die gebruik as waterkwaliteit siftingstoets. Die volgende hipoteses is dus ondersoek in hierdie tesis:

- Die oorspronklike Mosmann MTT toets metode kan suksesvol aangepas word om 'n sensitiewe, herhaalbare grootskaalse siftingsmetode te ontwikkel vir die bepaling van chemiese sitotoksisiteit en water kwaliteit, deur gebruik te maak van die K-562 sellyn.

- Sensitiwitiet van die toets kan verhoog word deur gebruik te maak van 'n 3-fase mediumsiklus (Medium tipe 1-3) in plaas van die oorspronklike enkel kultuurmedium.
- Verder kan hierdie sitotoksisiteitstoets suksesvol gebruik word om te bepaal of enige chemiese oplossing of watermonster sitotoksies is.

Dus word daar gepoog om uit te vind of sitotoksisiteit gekorreleer kan word met regte toksisiteit. Om bogenoemde te vermag, moet tydrowende epidemologiese opnames onderneem word, tensy verkrygde resultate met bestaande opnames gekorreleer kan word.

Die navorsing in die tesis het gepoog om aan the dui dat die sitotoksisiteit wat verkry word, toegepas kan word om toksisiteit te voorspel, wat dan tot die volgende resultate ly wat in die tesis verkry is. Die kultuurkondisies van die "Modified Highveld Biological MTT assay" is suksesvol aangepas om gebruik te word as eerste toets in 'n battery toetse om algehele sitotoksisiteit te bepaal deur gebruik te maak van die K-562 sellyn. Nadat die toets susksesvol aangepas is, word die tydsduur van die toetse nou verminder, en bespaar so belangrike hulpmiddels.

Daar was ook gepoog om die "Bioluminescent assay" te optimaliseer. Die metode is gebaseer op die feit dat Adenosien Trifosfaat (ATP) teenwordig is in alle lewende selle. Die metode gebruik 'n ensiem, die vuurvlieg-lusiferase-ensiem wat die omskakeling van lig vanaf ATP en lusiferien kataliseer. Die vrygestelde lig intensiteit is liniêr afhanklik van die ATP konsentrasie en word gemeet d.m.v. 'n luminometer. Die volgende tekortkominge is egter geïdentifiseer wat die metode ongeskik maak vir die gebruik as sitotosisiteitsindikator:

- Die lusiferase-ensiem is te sensitief vir te veel verskillende substanse (ione, soute ens.) wat in die selkultuurmedium en voorbereiding van die selle gebruik word; dit maak die gebruik daarvan minder geskik vir vinnige, effektiewe bepaling van watertoksisiteit.
- Die metode sal waarskynlik beter funksioneer as lusiferase in die sel self geïnkorporeer kan word of deel vorm van die sel se genetiese material.
- Ons stel dus voor dat die "Bioluminescent assay" moontlik as sitotoksisiteitstoets gebruik kan word, indien die sensitiwiteit van die luciferase-ensiem verder nagevors is.

Dus kan die gevolgtrekking gemaak word dat "Luminescence" nie geskik is om komplekse mengsels te toets nie, aangesien dit moontlik is dat nie-toksiese agente ook teenwoordig mag wees in die toets monster, wat dan inmeng met die proses van lig uitstraling.

Laastens was bekende chemise oplossings sowel as onbekende watermonsters gesif deur gebruik te maak van die aanpassings tot die "Modified Highveld Biological Metode" soos voorgestel in hierdie tesis. Die sitotoksisiteit van onbekende water monsters en chemiese oplossings was suksesvol bepaal en verskillende sitotoksiese effekte was verkry, naamlik sinergisme, antagonisme, optelling effekte en neutrale effekte. Dit was ook moontlik om die sitotoksisiteit van sekere van die water monsters te verminder of te verwyder. Dit was moontlik deur die monsters vooraf te behandel met of Na_2CO_3 (verwyder moontlike toksiese divalente en polivalente metale) of 'n SepPak kolom (verwyder organiese toksienes). Resultate het aangedui dat die metode baie sensitief is en dat dit selfs baie lae konsentrasies van toksiene kan bepaal.

Dus kan daar tot die gevolgtrekking gekom word dat die metode suksesvol aangepas is om gebruik te kan word as die eerste siftingstoets vir toksisiteitsanalise van 'n reeks omgewinswater monsters.

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Chapter 1: Introduction

Background

Due to an ever-changing global environment, the availability and quality of water is increasingly becoming important as an economic and life sustaining commodity. Pollution of water is threatening water supplies throughout the world; pollutants can be regarded as anything that makes water unfit for its intended domestic, industrial, agricultural and recreational use or destroys the availability to aquatic ecosystems. Natural river water contains minute concentrations of trace metals, such as mercury, cadmium, chromium, zinc, copper and nickel. These metals, however, occur in relatively high concentrations in wastewater products of various industries such as metal finishing and plating, battery manufacturing, mineral processing, and steel and alloy works. Trace metals in high concentrations can impact negatively on water supplies; e.g. too much manganese and copper can stain clothing when oxidised by detergents, too much calcium can lead to kidney stones and fluoride, over optimum levels, lead to brittle bones (1). Some trace metals lead to chronic and acute toxicity at elevated concentrations for aquatic life and can accumulate in the tissues of the organisms, which are hazardous when eaten by birds, animals or humans (1).

Currently, determining the quality of water is of great importance, and the testing of the cytotoxicity of the water is either based on a biological model or chemical model. In the biological model, organisms are utilized as indicators for toxicity, the product then being cytotoxic to the cells of the organism. This type of **toxicity** can be defined as **cytotoxicity**, and is a more descriptive definition for the type of cytotoxicity where living cells or organisms are involved. In the chemical model the amount of a specific chemical present in water samples are tested (e.g. analysing the sample by means of High Pressure Liquid Chromatography (HPLC)).

Ecosystems contain a variety of life forms including bacteria, fungi, protozoa's, insects, larvae, snails, worms, plankton, large plants, fish, amphibians, reptiles, birds and mammals. The aquatic organisms living in the ecosystems play a key role in the regeneration of the rivers and water supplies. These organisms break down organic material such as animal and plant waste into carbon dioxide, water and nutrients like nitrogen and phosphorous. These organisms also play a vital role as biological indicators of cytotoxicity in an ecosystem. As mentioned previously, they can be

applied in biological cytotoxicity testing where they (the cellular organisms themselves) act as indicators of water quality and the degree of cytotoxicity that is present. In biological cytotoxicity testing, the response of living organisms or biological material to the combined effect of all constituents of complex effluents is evaluated. It has the advantage over chemical-specific analysis, which cannot identify unknown pollutants in an effluent and cannot detect toxicity due to chemical interaction, e.g. synergism, antagonism and addition (2). Biological cytotoxicity testing in general considers surrogate life forms to model the cytotoxicity of the chemicals to the organisms in the ecosphere. Algae are used in aquatic systems; fish are used to represent the effect on the food chain; activated sludge is used to represent the effect on treatment systems and rats are used to represent the effect on terrestrial warm-blooded animals (2).

Chemical toxicity and biological cytotoxicity test models can be utilised on their own or together. Biological cytotoxicity tests are important additions to chemical-specific measurements because they:

- respond to compounds which are not readily identifiable or measurable by analytical techniques,
- respond to unknown compounds,
- detect effects due to chemical interactions, e.g. synergism, antagonism and addition,
- provide information on the type of hazardous chemical activity in an effluent i.e. toxicity, mutagenicity, potential carcinogenicity and teratogenicity,
- provide information on the impact on particular groups of target organisms (2).

The bases of biological cytotoxicity models

Biological cytotoxicity models utilise *in vitro* cytotoxicity assays (also known as bioassays) that make use of sensitive cells in cultures exposed to particular samples. The process of cytotoxicity is a complex *in vitro* cellular process, and its expression in the cell may manifest itself in a wide spectrum of cellular events. These events range from simple cell death (apoptosis or necrosis), caused by the harmful effects of toxic chemicals to functional changes, causing alterations in normal cellular activity necessary for survival. These causes are triggered by the presence of foreign substances e.g. water pollutants.

If any biological test system is employed as cytotoxic indicator, it must be sensitive enough to detect small concentrations of cytotoxic substances and must be as easy as possible and also performed in as short period of time as possible. Thus, the aim of all cytotoxicity tests is to determine the effect on a test organism, exposed to a toxicant for differing periods of time (2). Acute cytotoxicity can be observed in less than 96 hours. In contrast, chronic cytotoxicity leads to sub-lethal effects, for instance reduced and retarded growth, reduced reproduction, etc. (2).

All the cytotoxicity tests currently employed (discussed in detail in the literature review) are of great importance for the determination of water quality. These assays deliver valuable information regarding the cytotoxicity of water samples and the possible threat it contain for the specific test organism utilised for the tests. Despite the aforementioned, the need for testing methods to determine overall cytotoxicity, not only specific toxicity, are still eminent. It is this specific need that inspired the research for this thesis.

In this thesis the following toxicity definitions and terminology will be used:

- **Toxicity**: Total toxic effect of pollutant on an entire organism.
- **Cytotoxicity**: Toxic effect of a pollutant on cells in culture.

The research methods followed in this thesis included a critical analysis of all relevant literature in order to provide a thorough background and information basis for the second part, a laboratory investigation. A laboratory investigation was undertaken to develop a reliable cellular screening assay system, using human leukaemic cells as indicator of overall cytotoxicity.

Purpose of the study

The purpose of this study was to develop a refined, cost effective cytotoxicity assay, with high accuracy, objectivity and reproducibility, to be applied as the first screening assay of a battery of tests, to determine the overall toxicity of a specific water sample. This assay utilised the human leukaemic cell line, known as the K-562 cell line as cellular model to act as indicator to assess the cytotoxicity of several given chemical solutions or water samples (Table 1,Materials and Methods). The original Mosmann MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay, an assay used to determine cell viability, was modified by the Highveld Biological Laboratory, to act as sensitive indicator for cytotoxicity. Furthermore, this study aimed to determine the toxicity of different chemical solutions, in order to create a database of standard cytotoxicity values obtained for a range of chemicals (Table 1). This will enable researchers to make informed decisions regarding the impact that a given chemical, at a specific concentration, will have on the environment.

Hypotheses

- The original Mosmann MTT assay can be modified successfully to provide a sensitive, reproducible mass screening method for determining chemical cytotoxicity and water quality, by using the K-562 cell line.
- Sensitivity of the assay can be increased by utilizing a 3 phase medium cycle instead of the previously used single culture medium.
- Furthermore, this cytotoxicity assay can be successfully utilized to determine whether any chemical solution or water sample is cytotoxic. Thus, cytotoxicity are used to predict toxicity

Chapter 2: Literature Study

Introduction

Cytotoxicity testing has become an integral part of modern life due to an increase in the number of chemicals that is produced and introduced on the market each day. More than 100,000 chemicals are produced world-wide and some of these chemicals enter into the aquatic environment, posing a potential threat to the aquatic organisms. Humans who come into contact with these new chemicals, either directly through consumption of the contaminated water or by contact with the aquatic organisms, are also confronted with a potential threat of toxicity poisoning. As soon as test systems become refined and efficient for specific chemicals, more chemicals are produced by industry that also has to be tested for toxicity. Especially, in urban areas, a variety of chemicals are used in economic activities. For the hazard assessment of these chemicals, simple, rapid, reproducible and inexpensive cytotoxicity tests are needed (3).

As mentioned in the introduction, two toxicity test models exist, namely a chemical and a biological model. Chemical-based analysis of toxicants determines the constituents of a specific sample, and not the cytotoxic effects it could have on organisms. In the biological model, cytotoxicity tests determine the effects of the sample on living material (2). These types of tests are important additions to chemical-specific measurements. When using a biological model, the test organism should adhere to the following requirements:

- have a known origin and history,
- be disease free,
- acclimatised to test conditions,
- be handled and treated carefully to limit variation in their response (2).

More specialised and effective biological cytotoxicity testing is urgently needed for the evaluation of the response of living organisms or biological material to the combined effects of all constituents of complex effluents (2) The effects of individual toxic chemicals on the aquatic environment have, however, been studied extensively, using various test organisms (4). When pollution occurs several chemicals are usually present simultaneously, leading to interactions between them (4). These may

interact additively, synergistically or antagonistically, or the observed effect may be due to the toxicity of the dominant constituent (2).

Chemical interactions that may play an important role in cytotoxicity

Narcosis

Organic industrial chemicals can be classified by modes of toxic action or manifestation of cytotoxicity at the organismal or physiological level of interaction. Over 70% of organic chemicals cause cytotoxicity by non-covalent narcosis (reversible physical alterations in cells that are non-binding to macromolecules). There are multiple mechanisms (such as interactions at the biochemical or molecular level) of narcosis including non-polar or neutral narcosis, polar narcosis, amine narcosis, and di-ester narcosis. The remainder of industrial organic chemicals (approximately 30%) elicits cytotoxicity that is non-reversible. These reactions are typically covalent, resulting in chemical changes in biological systems, most commonly alkylation or arylation to soft nucleophiles. Electro(nucleo)philic chemicals may be either direct-acting electrophiles, or require transformation (biotic or a-biotic) to the electrophilic metabolite from the benign parent structure (5).

Addition, Neutral, Partial additive and Antagonistic interactions

Much of the research on toxic interaction of aquatic pollutants has utilised fish models for assessing and predicting combined cytotoxicity. Because of the variety of toxicant concentrations that biological species react to, the battery approach using various testing systems is recommended (4). In such tests, combined effects are described as **additive** when the observed effect is equal to the sum of the individual toxicities, and **neutral** if due entirely to the cytotoxicity of the dominant constituent. **Partial additive** interaction occurs when the observed effect is intermediate between neutral and additive cytotoxicity. Interaction is **antagonistic** if the combined effect is less than the neutral effect, and **synergistic** if the effect is larger than the additive effect (4).

Results obtained by Slabbert and Maree in 1986 (4) show that the concentration of individual chemicals in a mixture can play an important role in the type of combined effect exhibited. A combined cytotoxic effect is not simply the result of exogenous interaction between chemicals, but can be interpreted in terms of the differing

responses of an organism (4). Individually, some chemicals (As^{3+}, Cr^{3+}, Zn^{2+} and CN) have serious inhibiting effects; others have none (C_6H_5OH and NH_3) or only a slight effect (BO_3^{3-}, F^-, Cu^{2+} and Pb^{2+}). A mixture of these ten chemicals causes an additive effect of toxicities and a 100% inhibition (4). Slabbert and Maree also determined that a combination of certain chemicals at lower concentrations could have cytotoxic effects much greater than that specific chemical at a higher individual concentration. In wastewater, the occurrence of **synergistic**, **additive**, **partially additive** (more cytotoxic) and **neutral effects** can usually be expected to outweigh obtained **antagonistic** effects (less cytotoxic) (4).

All the water toxicity tests that are currently available on the market can be applied for testing both single chemicals and mixtures of different chemicals (utilised in the chemical model previously mentioned). There is, however, a need for bioassays to detect combined effects of chemicals. (4).

The process of toxicity testing

The following paragraphs will discuss the definition of cytotoxicity range, cytotoxic effects to human health, measurement of cytotoxicity and method of applying toxicity test assays.

Definition of cytotoxicity range

Potency information used for regulatory, modelling or risk assessment purposes are mainly collected in a laboratory. A chemical's toxicity is determined by defining the range of chemical concentrations that produce a readily observable cytotoxic effect in groups of the same test species under controlled conditions (5). The degree of cytotoxicity can be defined where 1 specific chemical is more toxic than another, if the first chemical causes the same adverse effects as the second chemical, only at a lower concentration (2).

Cytotoxic effects to human health

From results obtained with cytotoxicity measurements, it is apparent that human health may be affected if cellular cytotoxicity is present. These cytotoxic effects can be divided into 2 groups:

Non-threshold effects: Carcinogenicity

A small number of molecular events can lead to changes in a single cell that can result in uncontrolled cellular proliferation. With these effects there is no level of exposure that does not pose a small, finite probability of causing uncontrolled growth (2).

Threshold effects: Acute, sub-acute or chronic toxicity

Humans can have an identifiable exposure level below which no effects are observable and which are presumed to be safe. These effects range from effects other than cancer, affecting function of organ systems, e.g., an altered cellular metabolic rate (2).

Measurement of cytotoxicity

Present day contamination of aquatic ecosystems is characterised by long-term exposure of organisms to low doses of complex chemical mixtures. Emphasis should be placed on the development of ecotoxicological effects methodology, and assessment of sub-lethal and chronic cytotoxicity, together with measurement of acute, lethal effects of chemicals (6). Chemicals exhibit different reaction periods. Acute cytotoxicity tests give an indication of the cytotoxic effects present in the organisms after a relatively short exposure period. No knowledge will be gained regarding long-term exposure periods. Thus, it would be profitable to utilise both acute and chronic test methods simultaneously (6). The molecular and cellular mechanisms utilised for particular classes of chemicals should be known in order to understand the cytotoxic effects of specific toxins in organisms (6). Diagnostic markers could then be derived for the classification of chemicals with respect to their sub-lethal or chronic cytotoxicity and for hazard detection in environmental surveillance and monitoring (6).

Method of applying toxicity test assays

To apply a water cytotoxicity test assay, the toxicologist has to test a wide spectrum of chemicals to ensure that a range of reactions are observed and recorded. For instance, representatives of the following groups of toxins could be tested:

- toxic metals,
- pesticides,
- non-ionic surfactants present in the aquatic environment (6).

The cytotoxic responses in the test organism should be quantitative and measured, not in terms of incidence, but in a unit of response. This response is used to

compare unexposed controls with test organisms and determine whether the differences between these values are statistically significant. A basic principle of aquatic cytotoxicity is that the response of exposed organisms is dependent on the ambient concentration of the toxicant (5). Toxicogenetics (chemicals presenting cytotoxicity causing genetic mutations in a cell) and toxicodynamics (toxic substances may affect the normal functioning of the cell, thus biochemistry may change due to presence of toxicant) govern this response (5). Unicellular organisms are useful test subjects because simplified toxicokinetics (e.g. uptake and distribution of the site of action) is proportional to water-lipid partitioning. Among the more frequently used unicellular organisms in ecotoxicological assessments are members of the *Ciliophora*, especially species of *Tetrahymena* (5) (discussed later).

In the previous paragraphs the two different models available to determine cytotoxicity were mentioned as well as the process of cytotoxicity tests. The following paragraphs will focus on the specific bioassays currently used.

Bioassays currently used

The development and application of microbiotests otherwise known as bioassays began in the 1960's, but the biological tests grew both in quantity and quality in the 1970-1980's. From the beginning of the 1990's, bioassays collectively represented an important ecotoxicological tool with which anthropogenic stresses on the environment could be diagnosed (7). The development of biotesting has been driven to increased use of bioassays, due to the following:

- a persistent need for multi-trophic level ecotoxicity assessment,
- requirements for simplicity and cost efficiency during biotesting,
- availability for large-scale screening of xenobiotics (foreign substances that enter the living organism, causing alterations in the morphology, function and biochemistry of the cell), singularly or in mixtures (7).

Thus, bioassays have become an essential part of ecotoxicological assessment, and they inspire new and varied activities in research and development (7).

The use of short-term cytotoxicity assays for the initial screening of chemicals not only aids in establishing priorities for the selection of chemicals that should be tested

in vivo, but also decreases the time in which potential toxicants can be evaluated. As many xenobiotics enter into the aquatic environment, there is a need for rapid, cost-effective bioassays to screen the vast number of chemicals for their potential ecotoxicology (8). A few examples of bioassays include the Algaltoxkit, Protoxkit, Daphtoxkit etc. (7) A large number of different bioassays are currently in use for determining water quality and aquatic ecotoxicology. Each of the different tests utilizes different test organisms to assess the cytotoxicity present in the environment. The bioassays can be divided as follow (7):

Table 2.1: Different bioassays currently available

Test organism	Number of species utilised for tests	Test name
Bacterial	8	Ames test / Mutagenicity test Dehydrogenase activity test Motility inhibition test Microtox test TOXI-Chromotest SOS-Chromotest ECHA biocide monitor
Protozoan	3	Growth inhibition test Respiratory inhibition test Chemo-attraction inhibition test
Invertebrate	7	Marine rotifer lethality test Freshwater rotifer lethality test Cladoceran lethality test Cladoceran 7-day life cycle test Mysid shrimp lethality test Nematode lethality/ mutagenicity test Teratogenicity test
Cell cultures	Non-mammalian (e.g. Fish cells) Mammalian (e.g. Hamster cells)	Cytotoxicity tests

Bioassays are most effective when they accurately determine environmental impacts of pollutants in a cost- and time-efficient manner, but in some cases, several types of bioassays may be required to accurately describe a system (9). Table 2.2 classifies the different categories of bioassays and were compiled form Shoji *et al.*, 2000 (10)

Table 2.2: Bioassay categories

Category	Characteristics	Advantages	Disadvantages
Type 1	Developed and utilised for Pharmacology. Employs cultured mammalian cells		Special techniques and facilities are necessary. Long exposure periods, thus cannot be applied for monitoring the quality of environmental waters.
Type 2	Direct monitoring of toxicity in environmental samples.	Evaluate toxicity very quickly and easily.	Majority cannot detect toxicity in environmental waters due to lower sensitivity.

Groups of bioassays available

The different bioassays can be divided into 3 groups, according to the endpoint that is being measured:

- Assays that allow for overall evaluation of the cytotoxic effects, the rate of division, and the amount of the cellular biomass. These assays allow for the detection of overall and late disturbances of cells.
- Assays that explore the integrity of specific cellular functions only in living cells, especially the cell membrane integrity and also the mitochondrial and lysosomal functions. Thus, these assays allow for the underlining of sublethal effects.
- Assays e.g. the RNA synthesis assay, which is a very sensitive, but also very specific test (11). This test measures the effectiveness of RNA synthesis after exposure to pollutants.

<u>Cell proliferation assays (Table 2.2: Category 2) can be subdivided into 2 groups:</u>

- Cloning methods – slower assays with more difficulty to perform (12, 13).
- Methods that rely on cell metabolism – these assays measure increase in cell population (12, 13).

A bioassay used for screening studies must be sensitive and it must detect all major cellular disturbances. Initially, toxicologists utilised only a single assay, to explore the

cellular growth rate and viability because it allowed cell functions to be explored as a whole (11). More recently, toxicologists have given preference to a battery of bioassays, which specifically explore the various cellular functions (11). Additionally, toxicological experiments are orientated to determine sub-lethal effects of the toxicant to cellular function, as this information can indicate future increase in cytotoxicity – they may therefore act as early warning systems. These effects are obtained with *in vitro* cultures of fish or mammalians cells and have been proven to correlate well with *in vivo* test models. Thus, they can be utilised to predict the undesirable effects of various pollutants. Among the various *in vitro* assays, the neutral red incorporation, the MTT reduction assay, and the Coomassie blue assay, are among the most frequently used bioassays. (11). These assays utilise mammalian cell lines and have been developed to evaluate the toxic effect of chemicals on human health (3).

The above paragraphs discussed toxicity and toxicity testing in general (definition, interactions etc.) and the characteristics needed for a "good" cytotoxicity test. The following paragraphs discuss the different types of bioassays that are available.

Bacteria utilised in bioassays

The Ames test is named after Bruce Ames (14). This test utilises mutated bacteria that have lost the ability to form colonies *in vitro*. Exposure to mutagenic compounds can lead to reverse mutations (wrong base replaced with right one). After exposure, the bacteria are capable of forming colonies again, although histidine is not present. The number of colonies formed acts as a measure for mutagenicity of the test compound; the more colonies, the more mutagenic the toxin (14).

The Mutatox™ test works a little differently than the Ames test (14). The principle of the Mutatox™ test is based on the use of a dark variant of the luminescent saltwater bacteria *Vibrio fisheri*. Genotoxic damage induces the return of luminescence, which is used as a measure for the genotoxicity of the tested sample (14).

Daphnia magna utilised in bioassays

As an epifuanal zooplankter, *Daphnia magna* is often observed to skim the sediment surface, and therefore is exposed to both water-soluble contaminants and particle-bound contaminants on the sediment surface through ingestion. Low pH, high

conductivity and high water column metal concentrations correlates with low survival of daphnia's in both water column and sediment cytotoxicity tests (9).

Another method utilising *Daphnia magna* for determining metabolism is the analysis of the rate of oxygen consumption of the organism. This assay method measures the effects that toxicants could have on the metabolism of the whole organism. In a study where D*aphnia magna* were utilised with the above-mentioned assay, it was found that the chemicals tested did not cause measurable additional metabolic costs at the organismal level (15). Thus, the metabolic rate of the organism was altered, although acclimation is known to occur with respect to different heavy metals tested (CTAB, copper and cadmium). Further investigations are thus required to decide whether energetic demands due to stress responses are small or whether possible additional costs are masked by other effects (15).

Daphnia magna was also utilised in a test for the determination of iron cytotoxicity. Most metals are more soluble at lower pH levels, which result in leaching of metals from sediments and/or prevention of absorption of water column metals to sediments. Iron in sediments may or may not have a casual role in sediment toxicity. Ferric precipitates from acid mine drainage sites may have a smothering effect on biota and they may even cause physical aberration. To evaluate these claims in the laboratory, a toxicity test was conducted with ferric hydroxide as bottom substrate. Though all test organisms died within 48 hours, no dissolved iron was detected in the overlying water. Waterborne metals have a greater influence on aquatic biota than sediment bound metals in an area influenced by mining activities (9).

Daphnia pulex utilised in bioassays

Using the cladoceran *Daphnia pulex* in a Daphnidtox-based assay can also monitor chronic cytotoxicity of water samples. This assay utilises *Daphnia pulex* organisms as biosensors, as an indicator of cytotoxicity. Acute cytotoxicity tests will not allow for the determination of the concentration necessary to protect aquatic organisms over their entire life cycles. Thus, it is necessary to be able to conduct a chronic test to measure the long-term effect of pollutants on aquatic organisms. The parameters observed in this test are the survival and reproduction, and the results of the test are expressed in terms of the highest concentration that has no statistically significant observed effect when compared to the controls (16, 17).

Tetrahymena pyriformis utilised in bioassays

During recent years a number of rapid microbiological screening tests have been developed, one of which was the *Tetrahymena pyriformis* oxygen uptake bioassay, which provides results within 10 minutes. This method is advantageous because of its short generation time, and a doubling rate of less than 3 hours in defined medium. Based on physiological functions, these microbiological tests detect low levels of toxicants. *Tetrahymena pyriformis* has been applied to test mixtures of chemicals commonly found in water (4). Results form L-929 murine fibroblasts and *Tetrahymena pyriformis* were compared with each other to study their acute cytotoxicity response to different chemicals. Viability of the L-929 fibroblasts was evaluated by Trypan Blue dye exclusion followed by counting of cells with a hematocytometer. *Tetrahymena pyriformis* viability and mobility were monitored by examination with a photonic microscope. Most of the substances tested on the *Tetrahymena pyriformis* system indicated dose-dependant and time-dependant inhibitory effects on the cell proliferation rate, which were more prevalent for inorganic than organic substances (18).

In a study by Sauvant and co-workers in 2000, *Tetrahymena pyriformis* was utilised for the determination of the potential toxicity of aluminium (Al) to this aquatic model. Al is among the most abundant elements in the environment (19) and it represents approximately 8.1% of the Earth's crust weight. The chemistry of Al is complex and closely connected with the pH of the Al surrounding environment (20). It is naturally present in water or may result from anthropogenic activities, such as the industrial process or the use of Al salts (alum) as coagulant for the purification of water. Epidemiological studies have indicated that exposure to Al present in drinking water might result in a number of health threatening diseases. Al is described as a 'hard' trivalent metal ion and consequently, it can bind strongly to the oxygen-donor ligands (20). Moreover, the presence of other compounds, (i.e. essentially fluoride, silicon, phosphate or organic matter) and the hardness of water may greatly interact on the formation and the balance between the soluble and indissoluble forms of Al (20).

Cell cultures utilised in bioassays

Isolated cells that maintain essential traits of the *in vivo* state during *in vitro* culture offer a number of advantages for investigative as well as predictive toxicology. *In vitro* cell cultures allow more rapid and less expensive testing than *in vivo*

experiments. Since basal cellular structures and functions may be considered highly conserved biological entities, cellular studies provide an approach to detect general mechanisms of cytotoxicity. Cell cultures satisfy a societal desire to reduce the use of animals in toxicological research and testing. The advantage of using animal cells in culture for toxicological research and screening has been recognised for many years in the field of biomedical sciences. The absence of systemic influences, which is an advantage of *in vitro* systems, can be at the same time a disadvantage because the relevance of systemic effects in cytotoxicity and toxicant metabolism will not be reflected from the *in vitro* results. Isolating cells from various potential target tissues utilised in the detoxification process of toxicants, and exposing them *in vitro* can address this problem. Comparing toxicant responses in primary cultures of cells from organs which are involved in toxicant uptake, metabolism and effect can lead to the better understanding of the relative importance and interaction of these tissues in chemical toxicity (6).

There are many different bioassays available that utilise both non-mammalian and mammalian cell lines for cytotoxicity determination. The following paragraphs will discuss these different testing methods.

Non-mammalian cell cultures

Fish and fish cell line cultures

Fish are part of the natural diet of both aquatic mammals and birds. Humans living in developing countries are dependent on fish as a protein source, both directly and indirectly as a feedstock for their domestic animals. The approach of placing fish of hatchery-reared origin in cages at selected points in an aquatic ecosystem offers several advantages compared with investigations based only on feral fish. With *in situ* exposure tests, a homogenous group of individuals are exposed at a specific location for a specific period of time. To determine the biological effects of trace elements on fish, the gills (which has a large surface area and come into direct contact with the water; utilised for respiration, osmoregulation, excretion of nitrogenous waste products, acid-base balance) and liver (which is utilised for metabolization and detoxification process) are of primary interest. Thus, the usage of cell lines originating from these organs are of great importance in predicting possible cytotoxicity (21).

According to Fauconneau and Paboeuf in 2001 (22), fish muscle growth could be altered by pollutants by means of hyperplasia (myosatelite cells fuse together to give new fibres) and hypertrophy (myosatelite cells proliferate and fuse with existing fibres). These muscle stem cells are named satellite cells. Satellite cells studied both *in situ* and *in vitro* provide a valuable tool to analyse the specific effect of pollutants on muscle hyperplasia and hypertrophic growth (22). For each function, (ion, water and gas exchange, detoxification, and reproduction) and corresponding tissues (skin, gill, kidney, liver, and gonad) affected by pollutants, the response of specific cells could also be analysed. The adhesion capacity of satellite cells could be one of the specific targets of xenobiotics. The use of muscle satellite cells system to assess the effect of different pollutants on muscle growth is complementary to the use of other cells models (22).

The neutral red inhibition assay was used for the determination of cytotoxicity of four different metal salts (cadmium chloride; copper chloride; zinc sulphate; nickel chloride) on five monolayer-cultured fish cell lines, with one suspension cell line for comparison (3). It was found that the strength of the cytotoxicity was observed in order of Cadmium chloride > Zink sulphate > Copper chloride > Nickel chloride (3). Rainbow trout primary epithelial cell cultures contain two cell types, keratinocytes and goblet mucus cells. After treatment, these cells were classified as 1.) normal, 2.) goblet, 3.) necrotic and 4.) apoptotic. Table 2.3 is a summary of the characteristics of different cells exposed to four different chemicals as determined by Dowling and Mothersill in 2001 (8).

Table 2.3: Characteristics of different cells after treatment with four chemicals

Type of cell	Characteristics
Normal	Normal epithelium
Goblet	Basophilic cytoplasm, basally located nuclei, accumulation of mucus secretary granules, giving rise to goblet features.
Necrotic	Swelling of cytoplasm and organelles with only slight changes in nucleus Organelle dissolution and rupture of plasma membranes, thus leakage of contents into the extracellular space.
Apoptotic	Must show two or more of the following characteristics: • cell volume shrinkage and picnotic nucleus (chromatin condensation) • cytoplasmic blebbing • nuclear fragmentation • development into apoptotic bodies

This study indicated that as the concentration of the chemical increased, the number of cells decreased, even the goblet cells. The number of cells covered with mucus increased as the amount of chemical treatment increased. Fish cells have to be cultured at room temperature (~ 20°C). This slows down their biochemical responses, thus the assay takes much longer to complete than the corresponding mammalian cell assays done at 37°C Personal communication, Dr Whitcutt).

Another study by Risso-de Faverney and co-workers, tested on the same cell-line, obtained results that suggested that the composition of the medium utilised, plays a very important role in cytotoxicity (23).

Mammalian cell cultures

Many cytotoxic parameters have been used to screen the cytotoxic potential of xenobiotics available for determining toxicity, and a few of them are:

- trypan blue dye exclusion assay (determining the breakdown of the cellular permeability barrier), (24)
- neutral red assay (25, 26)
- lactate dehydrogenase assay determine the glutamic oxaloacetic transaminase leakage, (27, 28, 29)

- assay to determine the protein content (30) or highest tolerated dose, (31)

Table 2.4 gives a summary of the associated characteristics of trypan blue dye exclusion assay, neutral red assay and lactate dehydrogenase assay.

Table 2.4: Different cell culture test methods and their associated characteristics

Test method	Characteristics	Determining Toxicity
Trypan blue exclusion test (24)	Only demonstrate either viable or dead cells without detecting cells with sublethal injury.	Only badly damaged and dead cells will be coloured by the trypan blue dye.
Neutral red assay (25, 26)	*In vitro* incorporation of a supravital dye, neutral red, into lysosomes of living cultured mammalian cells.	Damage to the cell surface or lysosomal membranes = decreased dye uptake. Neutral red is extracted for quantitative measurement of cell viability and cytotoxicity of toxicants.
Lactate dehydrogenase leakage assay (27, 28, 29)	Measurement of lactate dehydrogenase (LDH) in culture supernatant. Based on 2 measurements: LDH amount of the culture medium and the intracellular LDH amount	A result of leakage is an indicator of cytotoxicity referring to disorders of membrane functions.

There are many different types of mammalian cell culture bioassays and the techniques focus on the detection of potential carcinogens and toxicants in water. Cytotoxicity can be detected by applying for e.g. the colony formation assay, the cloning efficiency assay, the transformation assay, the oxygen uptake rate assay, low density lipoprotein uptake assay, the nutrient uptake assay, the comet assay, the MTT assay and the bioluminescent assay. Particularly the colony formation assay, cloning efficiency assay, the transformation assay and oxygen uptake rate assay are

bioassays often used as water quality indicators in cytotoxicity testing. These bioassays will now be discussed.

Colony formation assays

One of the most widespread and accepted *in vitro* cytotoxicity methods is the measurement of the proliverative capacity of single cells to form colonies. Among these methods, the colony formation and the clonogenicity measurement in soft agar using a double layer agar system are the most widely used. This assay, however, has problems with low plating efficiencies, clumping artefacts and length of assay period. In an attempt to overcome the clonogenic assay problems, many short-term assays have been developed, including dye exclusion techniques, tritiated thymidine uptake, radio-labelled glucose utilisation and automated image analysis of Coomassie blue stained cells. The MTT assay was also applied with the clonogenic assay, due to the formation of formazan crystals, which gives spindle-shaped colonies that could be scored and detected by means of an automated apparatus. It was also profitable to see whether this assay method could shorten the technical time applied to score colonies, which were grown either in a T-flask or in soft agar (32).

Results from a study by Lemieux and co-workers in 1993 indicated that it is indeed possible to stain microcolonies with a tetrazolium salt and that it can also shorten the assay period, since no eye-visible colonies are needed to start counting the newly formed colony. The MTT assay, used in this way, is suitable to stain microcolonies and to monitor the kinetics of a cell population. This test could be of great importance for many types of clonogenic assays (32).

Cloning efficiency assay

A sensitive mammalian cell culture is utilised to determine cloning efficiency. HeLa (human Carcinoma), mouse lymphoma (ML) and BGM (Buffalo green monkey kidney cells) cell cultures is utilised in this technique. These cultures were found to be twice as sensitive as the electronic fish biomonitoring system (33). Kfir in 1981 suggested that the cloning efficiency method was able to detect half the minimum concentration of toxicants present to effect fish negatively. This assay is based on the ability of a single cell to metabolise and divide to form a cell colony. The assay is also widely applied for studies in radiology and pharmacology (33).

Kfir also found that the BGM cell line was more sensitive than the HeLa or ML cell lines. HeLa cells appeared to be the least sensitive. This phenomenon can be explained when taking into account that HeLa cells are from a tumour cell line, with tumour cells having a higher resistance and growth rate (33).

Toxicants tested by Kfir were Copper sulphate, Mercury chloride, Phenol, Cadmium chloride, Ammonium sulphate, and Lead chloride. Toxicity was determined by the % survival after treatment:

$$\frac{\text{Average number of colonies in treated plates}}{\text{Average number of colonies in control plates}} \times 100$$

Each result reported was the average of 16 tests. Each plate contained 200 cells. The higher concentrations of toxicants were the minimum concentrations detected by an electronic fish biomonitoring system. Although this is a very sensitive method, it is labour intensive because the number of colonies have to be scored by microscope, and this can only be done by one person to prevent deviation in the counting method (33).

The transformation assay

The transformation assay can be applied for detection of potential carcinogens, while it can simultaneously indicate toxicants present in water. Utilising mammalian cell cultures provide a system for the detection of changes in cell growth rate and pattern, induced by chemical carcinogens, thus leading to transformation. The transformation assay is based on evidence that transformed cells start to grow on each other with loss of contact inhibition. This method is proven to be more sensitive than the *Ames Salmonella*/microsome mutagenicity assays (mentioned earlier). The transformation assay is sensitive and as a mammalian cell system is used, it is considered to approximate the effect of a chemical on humans. Primary golden hamster embryonic cells were tested with a known carcinogen (33). This carcinogen also serves as a positive control and the negative control is fresh medium made up with distilled water (33).

The total number of transformed colonies in each set of treated plates is counted and

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the % transformation is calculated as follows:

$$\frac{\text{Number of transformed colonies on treated plates}}{\text{Total number of colonies on treated plates}} \times 100$$

Oxygen uptake rate as an indicator of cytotoxicity

This assay utilised the BGM cell line. Slabbert and co-workers in 1984 (34) found that results could be obtained within 10 minutes. Parameters such as bioluminescence, nitrification or enzyme activity serves as indicators for cytotoxicity measurements. This specific assay is based on the oxygen uptake rate of *Tetrahymena pyriformis* (previously described), now substituted with BGM cells. Oxygen uptake is recorded continuously before (noted as the reference), during (mixing) and after (test) sample addition. Test uptake rates compared to reference uptake rates served as a measure for the effect of different toxicants on the oxygen uptake rate of the BGM cells. All results were expressed in relation to a standard reference uptake rate of 5%/min. Results are obtained 1-5 min after mixing. Slabbert and coworkers in 1984 performed this assay testing copper, cadmium, mercury, lead, cyanide, phenol and ammonia. Water samples were also tested. Lower concentrations of some of the individual toxicants could be detected. When the water samples were also tested, the sensitivity of the cell culture technique to the total toxicant concentration did not appear to be any better than that of the *Tetrahymena pyriformis* bioassay system (34).

Low density- lipoprotein uptake assay

Shoji *et al.* in 2000 developed a quick and easy bioassay, known as the fluorescein isothiocyanate (FITC)-labelled low-density lipoprotein (LDL)-uptake activity assay utilising 96-well plates as a rapid and sensitive bioassay. LDL is one of the most important nutrients for mammalian cells, and the LDL-uptake rate is essentially very high. Therefore, the LDL-uptake rate when cells are exposed to toxicants can be used as a sensitive index for the cytotoxicity of environmental samples. Although this assay is sensitive enough to evaluate cytotoxicity of environmental water within 4 hours, it was found not to be applicable for the on-site evaluation of environmental water cytotoxicity, because cultured cells, that must be manipulated aseptically, cannot be used repeatedly (10). Shoji and co-workers (35) then modified the above-mentioned assay. This is more rapid and a disposable bioassay device using human

cells immobilised at a high density in micro-carriers that can be used for daily water quality management. This disposable device employed a filter tip as a container for the human cells immobilised in porous collagen micro-carriers. The only requirement is to introduce sample water into the tip by using a micropipette. By enhancing the cell density in the tip, the device was able to detect the cytotoxicity of river water and chemicals within only 2 hours. By using a hydrophobic membranous filter tip to pack immobilised cells in micro-carriers, one can detect cytotoxicity of environmental water easily on-site merely by pipetting sample water using a micropipette. Only a portable fluorometer is required for on-site evaluation. Comparative tests were run with cells in culture dishes and in the filter tips (10, 35).

Comet assay

Techniques that permit the sensitive detection of DNA damage and repair are critically important to fields of toxicology ranging from ageing and clinical investigations to genetic toxicology and molecular epidemiology (36, 37, 38). The three methods most commonly used for asserting DNA damage or repair involve the scoring of chromosomal aberrations, micronuclei, and/or sister chromatid exchanges in proliferating cell populations, the detection of DNA repair synthesis (so-called unscheduled DNA synthesis, UDS) in individual cells, and the detection of single-strand DNA breaks and alkali-labile sites in pooled cell populations (36). Table 2.5 name representative tests for various biochemical-cellular lesions, compiled from de Maagd and Tonkes in 2000 (14).

Table 2.5: Representative tests for various biochemical-cellular lesions

Genotoxic Lesion	Test
Gene mutations	Ames
Primary DNA damage	Mutachromoplate SOS chromo UmuC Mutatox™
Clastogenesis	Sister chromatid exchange
Aneuploidy	Comet assay Allium

The comet test is named after the comet-like structure that emerges when DNA is

separated by gel electrophoresis. Strand breaks lead to the formation of small DNA fragments that lead to an increasing length of the comet tail, the longer the tail, the more DNA damage (14, 39).

The potential utility of the SCG/Comet assay to human studies, especially when combined with an ability to identify selected cell populations and to recognise different classes of DNA damage using specific repair enzymes, is enormous. The potential applications of the SCG/Comet assay in such areas as radiobiology, genetic toxicology, environmental biomonitoring, and human studies are almost unlimited (36).

In addition to its value as in laboratory studies, the SCG/Comet assay is becoming a major tool for environmental biomonitoring. Adverse exposure situations resulting from the improper disposal of hazardous wastes are generally identified by analytical techniques characterising the levels of known pollutants. However, these techniques do not provide insight into the biological hazards associated with complex mixtures as they interact or are acted upon by various environmental pathways (36). Rydberg and Johanson in 1978 were the first researchers to directly quantitate DNA damage in individual cells by applying the mild alkaline version of the SCG assay. The degree of DNA damage was quantitated by measuring the ratio green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer (40).

To improve the sensitivity of detecting DNA damage in individual cells, Östling and Johanson (1984) (41) developed an improved microgel electrophoresis technique. In this bioassay, DNA fragmentation in cells undergoing apoptosis (programmed cell death) is measured by flow cytometry, after staining with DNA binding dyes like propidium iodide, ethidium bromide, DAPI, etc., or by agarose gel electrophoresis of oligoneuclosomal sub-units produced by specific endonucleases after chromatin cleavage (36). It may, however, not be possible to observe oligonucleosomal ladders obtained after DNA extraction and agarose gel electrophoresis, where a mixture of unaffected and apoptotic cells are extracted. These observations suggest that the microelectrophoresis assay of individual cells is a rapid, simple and inexpensive method for estimation of DNA fragmentation in cells undergoing apoptosis (41, 42).

Bioassays that measure specific biochemical parameters

These bioassays include the Uridine uptake bioassay, MTT assay and the Bioluminescent assay. These bioassays measure specific biochemical parameters. They are sensitive and the tests are principally based on cell mortality state as a consequence of membrane damage and alteration to metabolic pathways (43). However, before the cells die, they exhibit biochemical perturbations (morbidity state) induced by the toxic compound under study. Thus, agents that alter the integrity of plasma membranes, either directly or as a secondary result of damage to some other cell component, will alter the net rates of nutrient uptake in the cells. Furthermore, the rate of uptake processes is well-regulated aspects of the metabolism of cultured cells changing in reproducible ways in response to alterations in cellular growth state. Thus, agents that stimulate or modify cell growth may manifest their actions by perturbations in nutrient uptake rates (43).

Uptake studies allow for the temporal separation of cytotoxic treatment and assessment of damage, thus enabling a quantitative characterisation of cytotoxic effects at any time during or after treatment. In contrast, assays that rely on the release of previously accumulated radioactive substrates are limited in duration because of spontaneous leakage of labelled material and are not readily adaptable to measurements of recovery (43).

Uridine uptake bioassay

Uridine uptake is a more sensitive indicator of cytotoxic action than the loss of protein from culture dishes. Changes in uridine uptake rates can be induced by several mechanisms, which include alterations in influx, in the rate of phosphorylative trapping, and in rates of efflux (43). Kjeldgaard in 1963 (44) has shown that DNA and protein synthesis are directly proportional to cellular growth rate, while RNA synthesis is proportional to the square of cellular growth rate. The loss of membrane activity and alterations in phosphorylation caused by altered growth status, or changes in concentrations of intracellular high-energy metabolites should be revealed by changes in uridine uptake. This uridine uptake inhibition cytotoxicity test is based on the quantitative inhibition of the RNA synthesis rate of cells in the presence of toxic compounds. Uridine uptake rates in cultured cells can be measured rapidly and reproducibly, and may provide a quantitative index of the degree of damage induced by xenobiotics (45, 46, 47).

The simplicity and low cost of cell culture systems relative to intact animal tests has led to an increase in their use in cytotoxicity studies. The most frequently used endpoints in cellular cytotoxicity detect the breakdown of the cellular permeability barrier, measured by vital dye penetration, the release of intracellular enzymes, or the leakage of previously accumulated radioactive material (46).

The original Mosmann MTT assay

A great interest in colorimetric and fluorometric assays that can be miniaturised in 96-well micro titre plates have developed in recent years (48). Ideally, a colorimetric assay for living cells should utilise a colourless substrate that's modified to a coloured product by any living cell, but not by any dead cells or tissue culture medium (49). Tetrazolium salts are attractive candidates, since they measure the activity of various dehydrogenase enzymes (49, 50). The tetrazolium ring is cleaved in active cells, and so the reaction only occurs in living cells (49). The MTT assay is an example of such a colometric assay. When using the MTT assay, the amount of formazan crystals that form, serve as an estimate of the number of active mitochondria and thus the amount of ATP present in the cells. MTT, a hydrogen acceptor, is a water-soluble tetrazolium salt that is reduced to violet water-insoluble formazan crystals by the respiratory chain and other electron transport systems in mitotically active cells only (51). Cytotoxicity is then assessed by using an automatic ELISA spectrophotometric microtitre plate reader that offers great advantages in speed, simplicity, cost and safety (52). Wan and colleagues in 1994 suggested that the MTT assay is a sensitive, precise, convenient, rapid and economical test method for determining relative cytotoxicity (52). Since the introduction and development of the MTT assay by Mosmann in 1983 (49), numerous changes have been made to serve different purposes, especially to overcome certain limitations of the method (53, 54, 55). The Denizot and Lang method will now be discussed (53).

Modified MTT assay by Denizot and Lang

Denizot and Lang modified the Mosmann method in 1986. They found that the sensitivity of the [3H] Thymidine uptake assay was greater than that obtained for the MTT assay as described by Mosmann in literature (49). This Mosmann method uses serum in the incubation medium that could cause this lowered sensitivity. Thus, Denizot and Lang suggested that the serum should be removed during the extraction of the formazan-dye complex (53). The washing steps involved, made their MTT

technique less attractive, since there exists a risk of losing cells and formazan during the washing procedures. Even the loss of a few thousand cells readily results in a substantial drop in optical density (51, 53).

Modified MTT assay for evaluation of cytotoxicity in water: The Highveld Biological Method

Damelin and co-workers (including Dr Whitcutt from the Highveld Biological Laboratory in South Africa where the research for this thesis was completed) developed a modified MTT test, utilising McCoy cells in culture (55, 56, 57).

The experimental procedure of Damelin and co-workers **involved culturing of cells in one specific medium** before trypsinisation of the McCoy cells, seeding of the cells into micro titre plates, and exposure of the cells to varying concentrations of heavy metal salts (cupric chloride, mercury chloride, copper chloride, zinc chloride and cadmium chloride), with or without chelating agents, and incubated for periods up to 24 hours. The tetrazolium salt MTT was added to the wells for 4 hours. Formosan precipitates were solubilized and concentrated and absorbency measured at 570nm. All necessary controls were included (55, 56, 57).

The study indicated that a rapid colorimetric assay might be used to quantify the degree of cytotoxicity for cells exposed to heavy metals. When metals and added ligands/chelators were tested, the assay demonstrated that changes in cytotoxicity were due to the changes in metal speciation. At sub-inhibitory metal concentrations, increased or hormetic activity occurred. Thus, hormesis is an indication of cytotoxicity at very low concentrations. The increase in activity (MTT reduction) is due to overcompensation for a change in the cells' biochemistry or genetics. The onset of hormetic activity and metal concentration was determined by the MTT assay with the following chemicals: cupric, mercuric and cadmium chloride at concentrations of 0.4, 0.8, 1.6 and 3.2mg/ml. Hormetic toxicity was obtained at 0.2mg/ml, plateau at 1.3mg/ml and LC_{50}-value at 6.2mg/ml (55).

Whitcutt at Highveld Biological Laboratory further modified the methods used in the study of Damelin and co-workers by replacing the McCoy cells with the K-562 cell-line (a human myelogenous leukaemia cell-line). The cell population has been characterised as highly undifferentiated and of the granulocytic series. K-562 blasts

are multi-potential, haematopoietic malignant cells that spontaneously differentiate into recognisable progenitors of the erythrocytic, granulocytic and monocytic series. These cells have a doubling time of 26-30 hours over a 5-7 day period.

Table 2.6 compares the three different methods discussed in the previous paragraphs with each other.

Table 2.6: Comparison between the original Mosmann MTT assay, the Denizot and Lang modified method and the Modified Highveld Biological method

Original Mosmann MTT assay	**MTT assay as Modified by Denizot and Lang**	**Modified Highveld Biological assay as utilised by Damelin *et al.***
Incubation medium contains both serum and phenol red. Isopropanol added to solubilise formazan crystals. Tested on a range of mouse lymphoma cell-lines	Incubation medium can contain serum that should be removed during extraction of the formazan crystals. Two cell-lines were tested, namely EL4 (T—lymphoma) and LB3 (an IL-2-dependant T – lymphocyte cell-line)	Serum containing incubation medium. McCoy mouse cells were used as cytotoxicity cell line.

The MTT assay has been employed in various medical, microbiological and toxicological approaches (54, 58 - 68). Also, the bio-reduction of tetrazolium salts is used in a wide range of biological assays, including measurements of activity of oxidoreductases, sub-cellular localisation of oxidoreductases, detection of superoxide radicals, testing of cell viability and growth, and mycoplasma screening (63).

Bioluminescent assay

The nucleotide adenosine triphosphate (ATP) plays a central role in energy exchanges in biological systems. The firefly luciferase enzyme *Phontinus pyralis* has had a long history of use in biology, especially for the detection of ATP. In principle, the enzyme can be used to measure total ATP in a sample, or it can be coupled to ATP producing or consuming reactions. This assay is very sensitive and as little as

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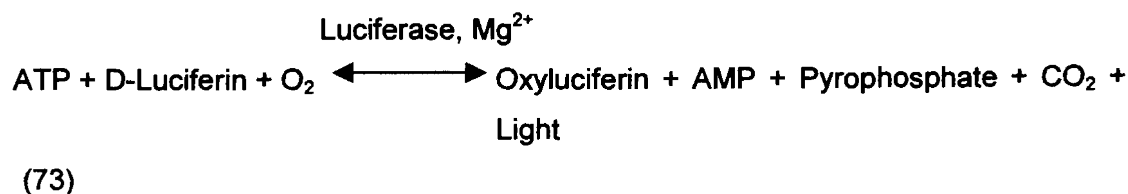
10^{-14}mol of ATP can be accurately measured. ATP has been used as a tool for the functional integrity of living cells since all cells require ATP to remain alive and carry out their specialised functions. Most ATP is found within living cells and links catabolic and anabolic processes. Cell injury or oxygen substrate depletion results in a rapid decrease in cytoplasmic ATP. Measurement of ATP is therefore fundamental to the study of living processes (64, 69).

Many methods have been used for ATP determination, but now the most widely used, because of its sensitivity, is the luciferin-luciferase bioluminescent assay. The amount of ATP being monitored relates directly to the number of cells present in culture. Luciferases have been used in soluble and immobilised/co-immobilised forms in assays for a variety of enzymes, substrates and cofactors (69 - 76). ATP bioluminescence has been used for determining levels of ATP in a number of different cell types, including muscle cells (77), bovine brain microvessels, platelets (78), erythrocytes, bacterial/microbial cells (79 - 81) and epithelia cells (82).



$MgATP_2$- converts the luciferin into a form, which is capable of being catalitically, oxidised by the luciferase in a high quantum yield chemiluminescent reaction. Cellular ATP can be measured by direct lysis of the cells with a suitable detergent, the released ATP is then free to react with the luciferin-luciferase and leading to light emission. Firefly luciferase from *Photinus pyralis* has been extensively studied by many investigators since it was first crystallised by Green and McElroy in 1956 (83 - 89)

For high throughput screening of compounds inducing cytotoxicity or cell proliferation, untreated cells can be used as a control. Accordingly, the output in Relative Light Units or RLUs of untreated cells (RLU control) is set at 100% and the effect of cytotoxic compounds is calculated by comparing to this value:

100% x (RLU sample/RLU control)

Unlike the measurement of tritiated thymidine uptake, which requires a 4-hour pulse with radioisotope after the initial incubation time, ATP bioluminescence will give results within 15 min of the termination of cell incubation (88, 89, 90).

The MTT assay relies on intact mitochondrial function and therefore only measures living cells. Alley and co-workers in 1988 modified the original MTT assay to use a microtitre plate format, and a modification known as the microculture tetrazolium assay has been used in this study (91). The ATP assay is based on the measurement of ATP using firefly luciferase-luciferin system (92, 93). Since ATP degrades rapidly after cell death and its concentration is related to cell number, its measurement provides an assessment of biomass (93). The ATP assay is the more sensitive assay, easily detecting 1500 cells/well with a luminescence count of approximately 28,000, which is more than 100 x background luminescence. The relationship between cell number and luminescence is linear. In contrast, the original MTT assay has difficulty in detecting less than 25,000 cells/well although it also shows good linearity. The reproducibility of the ATP assay was found to be slightly greater than the original MTT assay (59).

As mentioned in the introduction, the hypothesis of this thesis was that, the original Mosmann MTT method can be modified successfully to provide a sensitive, reproducible mass screening method for determining chemical cytotoxicity and water quality with a high degree of reproducibility and sensitivity, by using the K-562 cell line. Furthermore, that the sensitivity of the assay can be increased by utilising a 3 phase medium cycle instead of the previously used single culture medium. I also hypothesised that this cytotoxicity assay can be successfully utilised to determine whether any chemical solution or water sample, may pose a possible hazardous effect to human health. Leading from this hypothesis, the following aims were set for this thesis.

Objectives of the study

Although the researchers from the Highveld Biological Laboratory modified the MTT assay protocol as mentioned in Table 2.6, the need existed for optimising the cell culturing procedures to improve sensitivity of the assay in order to determine the cytotoxicity of chemical solutions and water samples.

- The first aim of this study was therefore to optimise the culturing of cells in

different culture media, so that these cells could be used in the Modified Highveld Biological MTT assay as the first assay in a battery of tests to determine overall cytotoxicity by utilising human cells in culture. This assay may reduce cytotoxicity assay duration, thereby saving important resources.

- The second aim of the study focused on bioluminescence as an indication of the ATP level in the cells after treatment. This method is based on the fact that ATP is present in all living cells. The bioluminescent method utilises an enzyme, firefly luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly dependent on the ATP concentration and is measured using a luminometer.
- Thirdly, to determine the cytotoxicity of various water samples and chemical solutions with the MTT assay as modified in this thesis as an indication of toxicity.
- Lastly, the results form the two above-mentioned bioassays were examined and compared.

The following research questions thus directs this thesis:

Research questions

1. What are the correct order of cell culture media to be used in the Modified Highveld Biological MTT assay and the bioluminescent assay to obtain an optimal functioning cytotoxicity screening assay? (Referred to as the Modified Highveld Biological MTT assay in this thesis)
2. Can this Modified Highveld Biological MTT assay be utilised as a screening assay for cytotoxicity testing of chemical solutions, but more importantly for water samples?
3. What are the advantages associated with this Modified Highveld Biological MTT assay, if applied in a water toxicity testing system?
4. Which of the two test systems (the Modified Highveld Biological MTT assay or the bioluminescent assay utilised for determining metabolic ATP) was most suitable for regular usage?

Chapter 3: Materials and Methods

Research question 1

The following procedures were performed in order to answer the following research question 1: *What are the correct order of cell culture media to be used in the Modified Highveld Biological MTT assay and the bioluminescent assay to obtain an optimal functioning cytotoxicity screening assay?*

K-562 cells were used in the assays. **Cell culturing conditions** of these K-562 cells **needed to be optimised** before the two assays could be utilised to perform water sample cytotoxicity testing (Research Question 2). This included optimising the medium cycle in which the cells were cultured, as the culturing of cells in only one medium did not provide the required sensitivity to indicate cytotoxicity of water samples (personal communication, Dr Whitcutt). The cells were cultured according to the procedures noted in the next paragraphs and then exposed to the Modified Highveld Biological MTT assay that utilises a cadmium chloride standard (cytotoxicity set between 30 - 70%) as a cytotoxicity control (measuring assay sensitivity). After optimising the cell culture conditions for the Modified Highveld Biological MTT assay, the bioluminescent assay was optimised.

<u>The following culturing media were used to answer this research question:</u>

(a) (KSLMS with glutamate) medium with alanyl glutamate (serum free medium)

(b) (KSLMS without glutamate) medium, supplemented with bovine serum (serum medium 1)

(c) (KSLMS with glutamate) medium, supplemented with both alanyl glutamate and bovine serum (serum medium 2)

(d) (MEM without glutamate) medium – Cells suspended in this medium are used in the Modified Highveld Biological MTT assay.

<u>The following culturing schedule was followed to answer this research question:</u>

Cells were cultured in (a) → (b) → (c) → (a) → (b) → (c) → (a) etc.

Where (a), (b) and (c) were different medium formulations described below.
Cells from the 2nd serum-containing medium were used in the Modified Highveld Biological assay to determine sensitivity via cadmium chloride standard control. Refer to Highveld Biological for the complete media formulations.

Culturing of the continuous K-562 cells

K-562 cells were seeded into appropriate culture flasks. According to the volume of the flask, a corresponding 5-20ml-culture medium was added to the flask. The medium was routinely replaced with fresh medium, as soon as the pH of the medium became to acidic indicated by a colour change from salmon pink to yellow. It is very important to utilise an indicator in the medium, to ensure that the medium could be replaced when all the nutrients were depleted. To remove the exhausted medium, the attached cells were removed from the inner surface of the flask, but care was taken to prevent damage to the cells. In order to accomplish this, the flask was moderately shaken to loosen the attached cells. Half of the shaken medium suspension was discarded into sterile waste bottles and was replaced with an equal volume of fresh medium. This method was only applied when the cells were used in a test that same day or the following day. To add fresh medium for further culturing and sub-culturing, the entire content of the flask was collected in a sterile McCartney bottle, centrifuged and replaced with the entire volume of fresh medium. The cells were then returned to the same flask, only if severe sterility measures were followed, or else the cells were seeded in a new flask.

Determining culturing conditions for cells in different medium solutions

The cells were cultured in three different medium solutions as part of a medium cycle to determine whether optimal sensitivity for the cytotoxicity-screening assay could be

obtained.

- **Medium Type 1:** (KSLMS with glutamate) medium with proteins, supplemented with alanyl glutamate (2.5mg/ml) and antibiotics (Gentamicin, 1%). Cells were cultured in this medium for ± 4 weeks or long enough to enable them to grow. Cells were then transferred to medium 2.
- **Medium Type 2:** (KSLMS without glutamate) medium without proteins, supplemented with bovine serum (5%) and antibiotics (Gentamicin, 1%) (Serum medium 1). Cells taken from medium 1 are cultured in this medium for at least 1 week. If cell growth is satisfactory, they are seeded into medium 3.
- **Medium Type 3**: (KSLMS with glutamate) medium without proteins but supplemented with bovine serum (5%), alanyl glutamate and antibiotics (Gentamicin, 1%) (Serum medium 2). Cells are taken from medium 2 and cultured in this medium for 3 – 4 days.

Medium 1 is used for cells that have just been thawed. This medium is thus supplemented with proteins, seeing that it is an important constituent to improve the quality of the cells. Proteins are necessary for building and repair of cells and also for maintaining a healthy state. After the cells displayed healthy growth, they could be used in the following medium, medium 2.

Cells are kept on this medium cycle of 3 media for continuos culturing. Cells are taken from the 3rd medium when a cytotoxicity-screening test is to be performed. The cells that were not used in an experiment were returned to serum-free medium (Medium type 1) to complete the medium cycle. The cytotoxicity assay was performed in MEM medium without glutamine. To determine whether this cycle were successful, the sensitivity of the cells were measured by applying the Modified Highveld Biological MTT Assay.

Preparation of the wells for adding of MTT solution

Preparation of a configuration sheet

The Modified Highveld Biological MTT test makes use of a configuration sheet (Table 3.1) that notes the designation of a specific sample/chemical on the 96-well plate and any other relevant information about that specific sample/chemical, e.g.:

- Sample being tested, e.g. any chemical or unknown sample (NaCl, KCN, $NaNO_3$, unknown 1).
- The concentration of the sample, e.g. 1, 2.5, 5, 10 µl/ml etc. The tested chemical standard had a certain chosen concentration, after which the relevant volume was determined to maintain this concentration during the test. For example, for $Pb(NO_3)_2$, you have a stock solution of 1mg/ml, but the chosen concentration for a test is 10µg/ml. 400µl of the stock solution is diluted in 20 ml sterile water to give a solution with a concentration of 20µg/ml (solution 2). The total volume in each well is equal to 170µl, thus 135µl of solution 2 is added to 135 µl sterile water, resulting in an end concentration of 10µg/ml.
- Any additional treatment performed on a sample, for example filtration, treatment with sodium carbonate etc. (this section of the configuration sheet was completed when water samples and chemical solutions were tested after optimising of the test – Research Question 2).

Table 3.1: Configuration sheet

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	P	Cd	S1			S5			S9		
B	B	P	Cd									
C	N	P	Cd	S2			S6			S10		
D	N	P	Cd									
E	N	P	Cd	S3			S7			S11		
F	N	P	Cd									
G	N	P	Cd	S4			S8			S12		
H	N	P	Cd									

A – H = Vertical rows of wells on the plate

1 – 12 = Horizontal rows of plates

N = negative control

P = positive control

Cd = cadmium chloride standard

S = specimen

Allocation of the 96 wells

Each plate has its own full set of controls: Negative control (N, 6 wells), Positive

control (P, 8 wells) and Cadmium chloride standard control (Cd - 1µg/ml or 1ppm, 8 wells). It also allows for another 12 samples to be tested (S1 - S12, six wells per sample) per plate (this part of the procedures will be discussed under the Materials and Methods of Research Question 2).

For each set of wells (as allocated above), only half the number of wells allocated for that specific control was filled, with double the amount necessary, by utilising a digital pipette with a range between 10µl and 200µl. For example, for the negative control, C1, E1 and G1 were filled with 170µl of sterile water in stead of 85µl. After the plate was sterilised (by means of an enclosed UV microtitre plate steriliser) the following was added with an Eppendorf type pipette:

- 20µl of incubation medium (10 x MEM, without antibiotics (d))
- 10µl of incubation buffer (10 x $NaHCO_3$).

Thus, the volume in the 3 wells was equal to 200µl. Half the amount of sterile water and incubation medium (100µl) was transferred to the empty wells, D1, F1 and H1 by means of a 6-channel Titertek pipetter, to lessen pipetting time and prevent evaporation from wells.

This is an example of how the wells were filled:

- Negative control : C1, E1, G1
- Positive control : A2, C2, E2, G2
- Cadmium control: A3, C3, E3, G2

Preparation and utilising the K-562 cells for the Modified Highveld Biological MTT test

The cells that were prepared and cultured in the three different medium solutions are now ready to be utilised in the Modified Highveld Biological MTT assay. The entire contents of a small flask of cells in serum-containing medium (Medium type 3) were sufficient for setting up two 96-well microtitre plates. One flask with approximately $4x10^6$ cells, and between 15 000 - 25 000 cells/well was needed for each test.

A confluent flask of K-562 cells was moderately shaken (caution not to damage cells) and the entire contents were emptied into a sterile McCartney bottle. Hanks balanced salt solution (HBSS) was added to the flask and placed in the fridge while the cell

suspension was being spun down. After centrifugation of the cell suspension, the culture medium was emptied into a sterile waste bottle. The flask with the HBSS was then shaken to loosen the cells that stayed behind after emptying the flask. This HBSS suspension was added to the spun down cells and centrifuged again. After centrifugation, HBSS was discarded into the sterile waste bottle and the cells were washed once again with HBSS, followed with centrifugation.

The supernatant HBSS solution was emptied into the sterile waste bottle and 2 - 2.5ml incubation medium (MEM, without alanyl glutamate and serum, with antibiotics (d)) was added. According to the number of cells present and the amount of cells wanted, the extra amount of medium needed, was calculated as follows:

$$\frac{\textbf{Amount of cells counted}}{\textbf{Amount of cells wanted per well}} \times 1$$

For example = $\frac{\textbf{40 652} \text{ (counted amount of cells)}}{\textbf{15 000} \text{ (amount of cells wanted)}}$

= **2.7ml medium to be added**

Thus, an additional amount of 2.7ml of incubation medium was added to the cell suspension to ensure that 15 000 cells would be present in the 10µl of cell suspension seeded into each well. The prepared cell suspension was placed in the fridge at 4°C for one hour, till needed for the test.

After mixture and dispensing of the medium-solution to the empty wells, the cells were added. The cells prepared as described above (Utilising K-562 cells in the modified MTT test), was taken directly from the fridge, placed in a container with cold water. The Eppendorf type pipette was used to dispense 10µl of cells into each well, except for the 6 negative control wells. It is very important to dispense the cells at a constant speed and also to hold the pipette at a 45-degree angle. The plate was placed in a zip-seal bag, which was left open for gas exchange and placed in a CO_2 incubator. The plate was incubated for 18 hours at 37°C, 5.9% CO_2 and 100% humidity.

Protocol for addition of the MTT solution to the different wells

After an 18-hour incubation period, the plates were removed from the CO_2 incubator and 10µl of MTT solution (Sigma M 5655, 2.5mg/ml) added into each well. The plates were then incubated in the CO_2 incubator for another 3 hours. After completion of incubation period, the plates were taken from the incubator for termination. Firstly, 10µl detergent solution (10% NP 40 in water) was added to each well. The plates were left to stand for 5 minutes. 10µl of 1N HCl was then added to each well. The plates were lightly tapped to promote mixing. Care was taken not to spill the contents of the wells. 50µl of colour developer (Heavy oil ©; specific constituents under copyright) was added into the centre of each well. Each well was checked for bubbles. Surface bubbles were removed by touching them with a sharp pointed piece of parafilm. The plates were covered with their lids, placed back in the zip-seal bags. The plates were placed at 37°C for 1 hour. After the hour, the plates were transferred to the fridge at 4°C for at least an hour.

After completion of the test, the plate was read on an ELISA reader (Biorad or Labsystems) at 570nm using the software program provided. The results were transferred to an Excel program sheet to determine the percentage cytotoxicity present in each group. The test utilises a cadmium standard to specifically monitor the response of the cells to the toxic cadmium standard. A lethal dose response of between 30-70% to 1ppm (10µl) of cadmium chloride hemipentahydrate (490ppb Cd^{2+}) was taken as being acceptable. Values falling outside this range were presumed as unacceptable and the test was considered invalid.

Protocol for the bioluminescent assay

The CytoPro HTS Kit (6410000) and the ATP Biomass Kit (BO 1243 - 118) bioluminescent assay kits were used in the experimental procedures. These kits were originally developed for detecting contaminating ATP in water samples (e.g. the presence of bacteria in water). The ATP Biomass Kit protocol (provided by the distributors) was modified, as the original protocol was not suited for testing total water cytotoxicity.

The cell culturing procedures for the K-562 cells were the same as described for the MTT assay (from paragraph with title: Culturing of the continuous K-562 cells to paragraph with title: Preparation and utilising the K-562 cells for the Modified

Highveld Biological MTT assay). The microtitre plates were prepared as described above except where MTT assay protocol was followed; the bioluminescent kit protocol was followed.

The CytoPro HTS Kit

This rapid assay kit is intended for use in determining cytotoxicity and cell proliferation using bioluminescent ATP technology. The kit is formulated to work for high throughput screening in 96 and 384 well microplates. The kit has an advantage in that it allows for a single step homogenous assay suitable for utilising mammalian cell lines in culture.

Assay method: original protocol

The Lyophilised ATP monitoring reagent was reconstituted with Somalyse. The recommended total sample volume for each well in the 384 well plates are 25µl and for the 96 well plates are 100µl of the sample. The ATP monitoring reagent/Somalyse solution was stabilised at room temperature before use. The optimal temperature for the reaction was 22°C. After completion of the 18-hour incubation time, the microplate was loaded into the luminometer. Table 3.2 is an example of the protocol programmed into the luminometer.

Table 3.2: The protocol programmed into the luminometer

Step	Protocol for the Instrument	96-well plate
1	Dispense: ATP Monitoring Reagent/Somalyse	100µl
2	Shake; Diameter - 1; Speed – 600	5 sec
3	Incubate	2 min 40 sec
4	Measure, integration time	1000 ms

The direct luminometer output is in relative light units (RLU, that amount of light given off as indication of amount of ATP present in cells). The output in RLUs of unexposed cells ($RLU_{control}$) was set as 100% and the effect of cytotoxic compounds is calculated by comparing to this value:

$$100\% \times (RLU_{sample}/RLU_{control})$$

Thus, this method gives an indication of the number of living cells after exposure.

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The ATP Biomass Kit

This assay kit is intended for use in environmental, industrial and research applications for the measurement of total ATP using the bioluminescence technique. The kit measures the total biomass and does not make a distinction between different ATP sources, like bacteria, yeast, mold, animal or plant cells.

In contrast with the CytoPro HTS kit assay, this assay kit contains separate ATP monitoring reagent and ATP releasing reagent. Thus, the amount of both can be varied according to preference, but the method is not a single step assay as the CytoPro HTS kit.

Assay method: original protocol

100µl of sample were pipetted into each well of the 96 well plate. 100µl of ATP releasing reagent was added into each well, followed by 500µl of diluted ATP monitoring reagent.

The plate was lightly shaken and then put into the luminometer to read the light output. The concentration of the ATP could be calculated as follows:

$$\text{ATP } (\mu\text{mol}) = \frac{S - B}{I - (S - B)} \times \text{ATP standard } (\mu\text{mol})$$

Where,

- S = light emission of the sample
- B = background light emission
- I = light emission internal standard

The following experiments were performed to optimise the ATP Biomass Kit

Determining the optimal volume of ATP monitoring reagent

A small modification was made to the previously described protocol for use of the kit. Instead of using 500µl of diluted ATP monitoring reagent, 100µl of original reconstituted ATP monitoring reagent was used, because the wells were too small to

add the volume suggested in the original protocol.

For use in the original protocol:

ATP monitoring reagent: Tris Buffer

1 : 5 dilution

Thus, rather than diluting the reagent and utilising 500µl, the reagent remains undiluted and was used as a volume of 100µl.

The ATP standard concentration was used to range between 1.1ng/ml, 37,5ng/ml, 93.75ng/ml, 0.25µg/ml and 0.5µg/ml. The amount of ATP monitoring reagent was used to range between 70 to 120 µl.

Determining the optimal time for measuring the light output obtained

Three ATP standard concentrations were used, namely, 0.5µg/ml, 62.5ng/ml and o.3125ng/ml. Three time intervals were chosen, namely 2,3, and 4 minutes and three controls, namely Tris, water and a blank control were used to ensure absence of ATP contamination.

Determining the influence of different experimental solutions used during normal culturing procedures of the K-562 cells on the RLU's produced

Different solutions used in normal culturing of the K-562 cells might influence the RLU's obtained (Table 3.3). To determine whether these solutions have an influence, their RLU's were determine, without cells, but with an ATP standard control using the luminometer.

Table 3.3: Different cell culture solutions tested

Positive control (ATP standard, 0.5µg/ml))
Blank
Tris Buffer
Water
Hanks Balanced salt solution (Without Ca; Without Mg)
ATP with 10µl Hanks Balanced salt solution
ATP standard with MEM (with Phenol red)
ATP standard With MEM (without Phenol red)
ATP with HBSS – without Ca, without Mg
ATP with HBSS – with Ca; with Mg
ATP with Dulbellco's Phosphate Buffered Saline – Without Ca; and without Mg
ATP with another ATP releasing reagent – NP 40
ATP with MEM (with Phenol Red; with Vitamins)
ATP with MEM (with Phenol Red; with non-essential amino acids)

Materials and Methods for Research Question 2

The following material and methods were used to answer Research Question 2: *Can this Modified Highveld Biological MTT assay be utilised as a screening assay for cytotoxicity testing of chemical solutions, but more importantly for water samples?*

Different known chemical solutions were tested to determine their cytotoxicity (Table 3.4). In addition to this, a number of water samples of unknown origin were tested to determine their cytotoxic potential.

Known chemical solutions exposed to cells in culture

The chemical solutions that were tested are summarised in the Table 3.4.

Table 3.4 Known chemical solutions to be tested

Chemical	Concentration (µg/ml)
$HgCl_2$*	0.25; 0.5; 1; 1.4; 2; 2.5; 3.7; 5; 10
2-Mercaptoethanol*	1; 0.5
KCN	20
Phenol*	0.5; 1.4; 3.7; 10
SDS	10
$CuSO_4$	10
Methanol*	1%
Pentachlorophenol*	1; 10
2:4-Dinitrophenol	10
DMF*	0.05%, 0.14%, 0.4%,1%
$Pb(NO_3)_2$*	10
$CdCl_2$*	0.5, 1, 1.4, 3.7, 10
$NaNO_2$*	10
Acridine Orange	10; 5; 3.7; 1.4
$K_2Cr_2O_7$	1; 5; 1.7; 0.7; 0.2; 2.5; 10; 3.7; 1.4; 0.5
$CaCl_2$*	5000; 1900; 686; 254
Ethanol	Serial dilution of 2.7
DMSO	1%

All the chemicals given in Table 3.4 were dissolved in water, unless indicated

otherwise. Pentachlorophenol needs NaOH to dissolve in water, to prevent precipitation, but this could alter the pH of the assay. Thus, 1mg/ml pentachlorophenol was dissolved in DMSO (no cytotoxicity) and then diluted 100 x in water.

Additional cytotoxicity tests were performed using chemicals with asterisk (*), as the toxicity of these chemicals are know in the literature (3, 8).

The chemicals marked with * were also subjected to a number of additional treatments (see Table 3.5) to see whether any change in toxicity occur.

Table 3.5: Additional treatments for chemicals marked * in Table 3.4.

Additional Treatment	**Concentration**	**Solvent**
$NaHCO_3$	7.5 and 10 x concentrate	Sterile water
Pentachlorophenol	1µg/ml	Sterile water
Pentachlorophenol	1µg/ml	DMSO
Pentachlorophenol together with DMSO	1µg/ml	Sterile water
Fluoride	10µg/ml	Sterile water
Methanol	20%	Sterile water
Ethanol	20%	Sterile water
$CaCl_2$	5mg/ml	Sterile water
$MgCl_2$	5mg/ml	Sterile water

Unknown water samples exposed to cells in culture

The unknown water samples that were tested are noted in Table 3.6. The experiments were conducted as double blind tests. Because the content of the water sample was not known at time of testing, and therefore the cytotoxicity was unknown, no dilutions or concentrations of the sample was made. Two pre-treatments were with the samples. Na_2CO_3 were used to precipitate toxicants in the sample. The sample was filtered through a SepPak cartridge to remove organic toxicants that might be present.

Table 3.6: Unknown water samples tested

Unknown water sample
SW – Unknown 1
TW – Unknown 2
Rain – Unknown 3
Ch – Unknown 4
SP 707.3 – Unknown 5
BE – Unknown 6
LH 57 – Unknown 7
LH 56 – Unknown 8
LH 73 – Unknown 9

Research Questions 3 and 4

- *What are the advantages associated with this Modified Highveld Biological MTT assay, if applied in a water toxicity testing system?*
- *Which of the two test systems (the Modified Highveld Biological MTT assay or the bioluminescent assay) utilised for determining metabolic ATP was most suitable for regular usage?*

These two research questions will be answered as part of the discussion, as it does not include empirical research.

Chapter 4: Results

Results of Research Question 1

Question 1: *What are the correct order of cell culture media to be used in the Modified Highveld Biological MTT assay and the bioluminescent assay to obtain an optimal functioning cytotoxicity screening assay?*

Cell sensitivity is the optimal parameter regulating the optimal functioning of the cytotoxicity screening conditions.

Optimising cell culture conditions

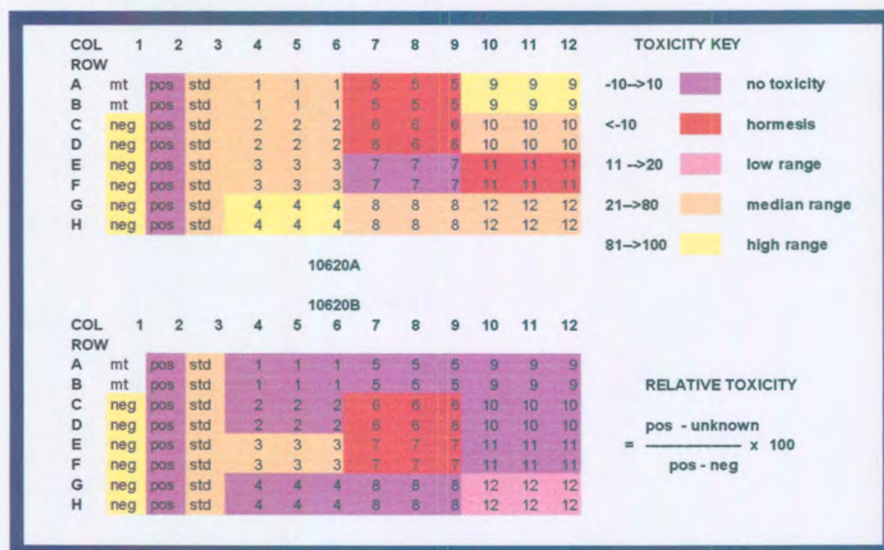
Introducing the cells to a medium cycle

As mentioned in the Materials and Methods, the cells was cultured in three different medium solutions (Medium type 1 -3) to determine whether sensitivity of the cells in culture could be increased. To determine whether this cycle were successful, the sensitivity of the cells were measured by applying the Modified Highveld Biological MTT Assay.

Results determining the sensitivity of the cells by applying the Modified Highveld Biological MTT Assay

Cells were taken from 3rd medium type (d) and exposed to the Modified Highveld Biological MTT assay. To determine whether this cycle as prescribed by the Highveld Biological Association gives satisfactory results, the sensitivity of the cells was measured by applying the Modified Highveld Biological MTT assay to different toxic chemicals, validating each assay with the cadmium chloride control. See Figure 4.1 to indicate how the 30-70% cytotoxicity was calculated.

Figure 4.1a: Determining the 30-70% cytotoxicity values (Compiled by the Highveld Biological Laboratory)



10620A

COL / ROW	1	2	3	4	5	6	7	8	9	10	11	12
A	mt	pos	std	1	1	1	5	5	5	9	9	9
B	mt	pos	std	1	1	1	5	5	5	9	9	9
C	neg	pos	std	2	2	2	6	6	6	10	10	10
D	neg	pos	std	2	2	2	6	6	6	10	10	10
E	neg	pos	std	3	3	3	7	7	7	11	11	11
F	neg	pos	std	3	3	3	7	7	7	11	11	11
G	neg	pos	std	4	4	4	8	8	8	12	12	12
H	neg	pos	std	4	4	4	8	8	8	12	12	12

10620B

COL / ROW	1	2	3	4	5	6	7	8	9	10	11	12
A	mt	pos	std	1	1	1	5	5	5	9	9	9
B	mt	pos	std	1	1	1	5	5	5	9	9	9
C	neg	pos	std	2	2	2	6	6	6	10	10	10
D	neg	pos	std	2	2	2	6	6	6	10	10	10
E	neg	pos	std	3	3	3	7	7	7	11	11	11
F	neg	pos	std	3	3	3	7	7	7	11	11	11
G	neg	pos	std	4	4	4	8	8	8	12	12	12
H	neg	pos	std	4	4	4	8	8	8	12	12	12

TOXICITY KEY

Range	Key
-10–>10	no toxicity
<-10	hormesis
11 –>20	low range
21–>80	median range
81–>100	high range

RELATIVE TOXICITY

$$= \frac{\text{pos - unknown}}{\text{pos - neg}} \times 100$$

Figure 4.1b: Example of completed MTT test with indicated controls

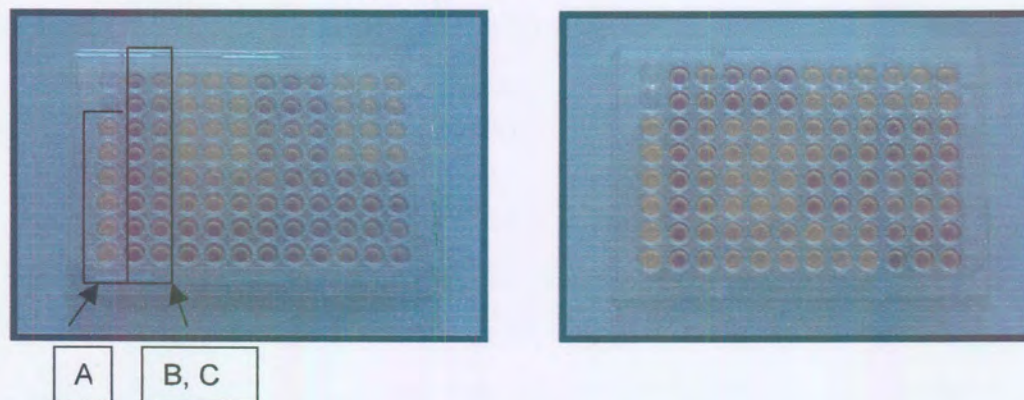
A – Negative control

B – Positive control

C – Cadmium chloride standard control

Positive control cytotoxicity are set as 100% living cells

Cadmium control cytotoxicity: $\frac{\text{Pos} - \text{CdCl}_2 \text{ control}}{\text{Pos - Neg}} \times 100$



Optimising the Bioluminescent assay

As mentioned in the Materials and Methods, the two kits, the CytoPro HTS Kit and the ATP Biomass Kit were tested for use as cytotoxicity indicators for water samples.

No results was obtained when the CytoPro HTS Kit were used as indicated in the

original kit protocol, because it is a one step protocol that cannot be adjusted for use as a water cytotoxicity screening assay.

The ATP Biomass Kit was modified as mentioned in the Material and Methods.

Determining the optimal volume of ATP monitoring reagent to produce the highest RLU possible

A high RLU for the standard is important to be able to establish cytotoxicity when later testing water samples for their cytotoxicity. Table 4.1 is a summary of the ATP standard concentrations tested, the amount of ATP monitoring reagent added and the relative RLU's obtained.

Table 4.1: Initial results indicating volumes of ATP Monitoring Reagent tested and subsequent relative light units obtained (Figure 4.2a – c; Graphic representation of results contained in table below)

ATP standard Concentration	Amount of ATP Monitoring Reagent (µl)	Relative light units Obtained
1.1ng/ml	70 80 90	0.0027 0.0039 0.0064
37.5ng/ml	70 80 90	0.0860 0.1062 0.1230
93.75ng/ml	70 80 90	0.1938 0.2324 0.2609
0.25µg/ml	70 80 90	0.7889 0.9458 1.060
0.5µg/ml	70 80 90	1.284 1.518 1.728

Measurements were taken 30 seconds after addition of the ATP releasing reagent. The original ATP biomass kit protocol suggests using 50µl of ATP monitoring reagent, but in the current experiments when 50µl of ATP monitoring reagent was used, the RLU-readings were less than 0.01. Therefore, as indicated in Table 4.1, volumes of 70 to 90µl of ATP monitoring reagent were used. As indicated in Table 4.1, 90µl of ATP monitoring reagent delivered the best RLU reading. As the RLU obtained with 90µl of ATP monitoring reagent for all 5 ATP standard concentrations (1.1ng/ml, 37.5ng/ml, 93.75ng/ml, 0.25µg/ml and 0.5µg/ml) was still too low for

measuring toxicity, the experiment was repeated, only with one ATP standard concentration of 0.5µg/ml (highest RLU obtained), but with volumes ranging from 90 to 120µl of ATP monitoring reagent (Table 4.2).

Table 4.2: ATP concentration of 0.5µg/ml with volumes ranging from 90 – 120µl ATP Monitoring Reagent (Figure 4.3; Graphic representation of results obtained in table below)

Volume of ATP Monitoring Reagent (µl)	**Relative light units obtained**
90	1.869
100	2.008
110	**2.217**
120	1.964

Results indicated that 110µl of ATP monitoring reagent delivers the best RLU's for the enzyme reaction (Table 4.2). ATP monitoring reagent also has the ability to quench the light output reaction if it is present in too high quantities. Here it can be seen that 120µl of ATP monitoring reagent is too high because the number of RLU's obtained are starting to decrease.

Figure 4.2 a – c indicated the RLU obtained for the three volumes of ATP monitoring reagent (70 to 90µl). From the graphs, it is clear that the 90µl of ATP monitoring reagent produced the highest RLU for the initial experiments with the concentration of ATP standard ranging from 1.1ng/ml to 0.5 µg/ml.

Figure 4.2a: RLU obtained for 70µl ATP monitoring reagent

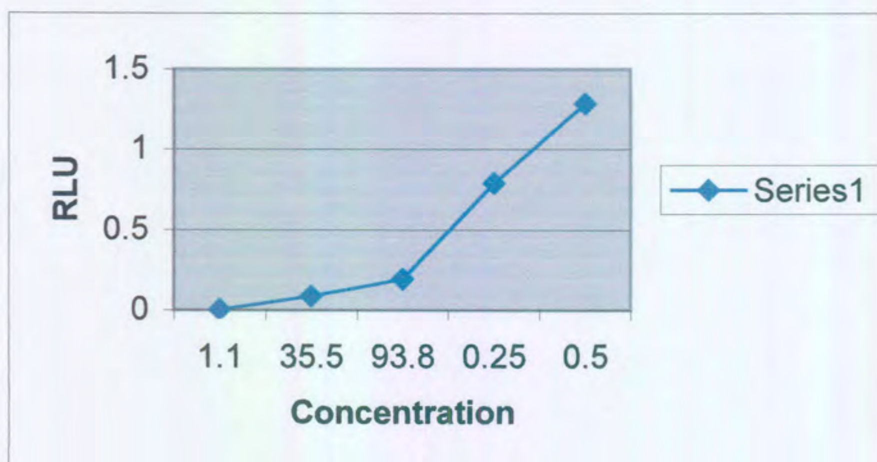


Figure 4.2b: RLU obtained for 80µl ATP monitoring reagent

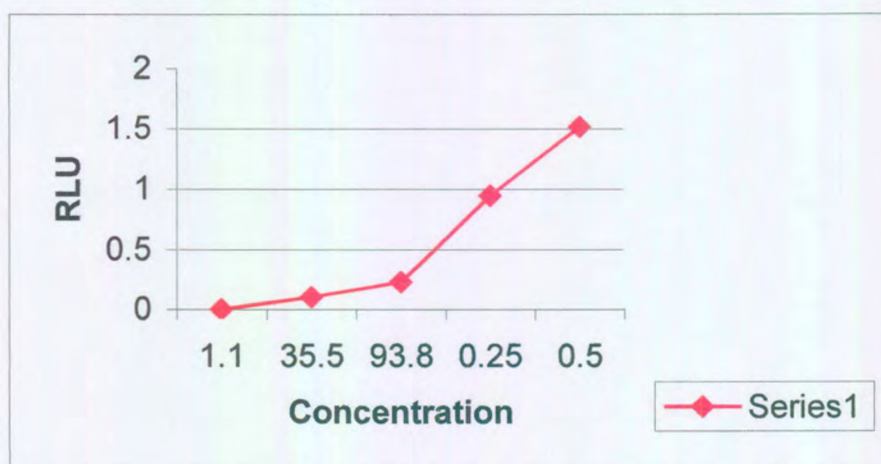


Figure 4.2c: RLU obtained for 90µl ATP monitoring reagent

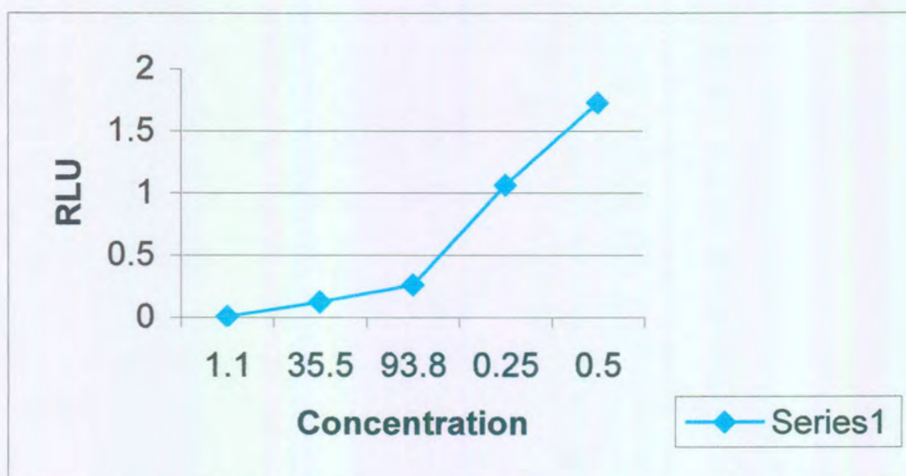
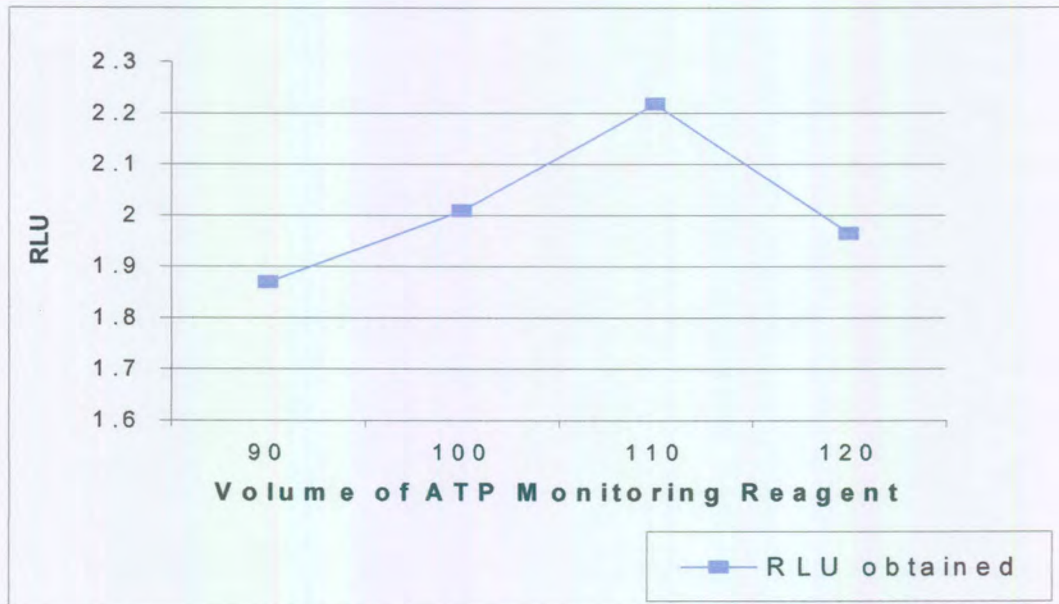


Figure 4.2a – c represents the results as displayed in Table 4.1. The X-axis represented the concentrations tested, and the Y-axis represent the relative light units that was obtained with different volumes of ATP monitoring reagent.

Figure 4.3 is a graph of the results of Table 4.2 where only one concentration (0.5 µg/ml) of ATP standard was used to test volumes ranging from 90 to 120µl of ATP monitoring reagent.

Figure 4.3: 0.5 µg/ml ATP standard used to test volumes ranging from 90 to 120µl of ATP monitoring reagent



This figure represents the results obtained in Table 4.2. The X-axis indicates the different volumes of ATP monitoring reagent tested, with the obtained relative light units, indicating that 110 µl is the best suited volume.

Experiment to determine the optimal time for measuring the light output obtained

Three time intervals, namely 2,3 and 4 minutes were used to determine the optimal time period for measuring the RLU's. Table 4.4 indicates the different ATP standard concentrations ranging from 0.5µg/ml to 0.3125ng/ml, time period exposed and RLU's obtained for particular ATP standard concentration at a specific time interval. Three different controls were used, Tris, water and a blank control was used to indicate the absence of contaminating ATP.

Table 4.4: Different standard ATP concentrations and controls versus time period

ATP standard Concentration	Time Period (min)	Relative light units obtained	Relative light units obtained for Controls
0.5µg/ml 62.5ng/ml 0.3125ng/ml	2	0.7255 0.0989 0.001	Tris – 0.00015 H_2O – 0.00025 Blank – 0.00065
0.5µg/ml 62.5ng/ml 0.3125ng/ml	3	0.6890 0.0928 0.001	Tris – 0.00015 H_2O – 0.00025 Blank – 0.00035
0.5µg/ml 62.5ng/ml 0.3125ng/ml	4	0.6575 0.0914 0.001	Tris – 0.00035 H_2O – 0 Blank – 0.00005

Three different ATP concentrations were tested and the relative light units documented for the readings obtained at 3 different time intervals, 2, 3 and 4min respectively.

The test was repeated, at the same concentrations and time intervals, but with newly constituted ATP monitoring reagent to determine whether the duration after constitution of the ATP monitoring reagent influenced the obtained RLU's (Table 4.5).

Table 4.5: Relative light units obtained with newly constituted newly ATP monitoring reagent

ATP standard Concentration	Time Period (min)	Relative light units obtained	Relative light unit obtained for Controls
0.5µg/ml 62.5ng/ml 0.3125ng/ml	2	0.8876 0.1193 0.0018	Tris – 0.00015 H_2O – 0.00025 Blank – 0.00065
0.5µg/ml 62.5ng/ml 0.3125ng/ml	3	0.8432 0.1147 0.00165	Tris – 0.00015 H_2O – 0.00025 Blank – 0.00035
0.5µg/ml 62.5ng/ml 0.3125ng/ml	4	0.8061 0.1086 0.0013	Tris – 0.00035 H_2O – 0 Blank – 0.00005

Results indicated in Table 4.4 and 4.5, suggested that there were no significant difference between the RLU values. I, however, suggest that it is probably better to use newly constituted ATP monitoring reagent for each test performed. Also, there is no significant difference between the RLU readings obtained for the different time intervals (Table 4.4 and 4.5). Thus, the reaction seem to stay constant for 2 to 4 minutes and the measurement can be taken any time from 2 to 4 minutes after addition of the ATP releasing reagent.

Determining the influence of different experimental solutions used during normal culturing procedures of the K-562 cells on the RLU's produced

RLU results for the different cell culture solutions are presented in Table 4.6 and Figure 4.4. The relevant number indicating a specific solution in Figure 4.4, is given below Figure 4.4.

Table 4.6: Experiment to determine the influence of different cell culture solutions on the Relative light units

Solution Tested	RLUs obtained
Experiment 1	
Positive control (ATP standard, 0.5µg/ml)	0.1040
Blank	0.00085
Tris Buffer	0.0004
Water	0.0003
Hanks Balanced salt solution (Without Ca; Without Mg)	0.0003
ATP with 10µl Hanks Balanced salt solution	0.0856
ATP standard with MEM (with Phenol Red)	0.01075
ATP standard With MEM (without Phenol Red)	0.03025
Experiment 2 (Repeat of Experiment 1)	
ATP with HBSS – without Ca, without Mg	1.447
ATP with HBSS – with Ca; with Mg	1.474
ATP with Dulbellco's Phosphate Buffered Saline – Without Ca; without Mg	1.448
ATP with another ATP releasing reagent – 10% NP 40	1.103
ATP with MEM (with Phenol Red; with Vitamins)	1.470
ATP with MEM (with Phenol Red; with non-essential amino acids)	1.773

Figure 4.4: RLU reading of different cell culture solutions

Table 4.6 and Figure 4.4 represents the results obtained when different cell culture solutions were tested. The X-axis of the graph represents the different solutions tested, as described below, and the Y-axis the relative light units obtained. Number 1 and 9 indicate the positive controls for the 2 repeated tests.

Positive control (ATP standard, 0.5µg/ml) - **1**

Blank - **2**

Tris Buffer - **3**

Water - **4**

Hanks Balanced salt solution(Without Ca; Without Mg) - **5**

ATP with 10µl Hanks Balanced salt solution - **6**

ATP standard with MEM (with Phenol red) - **7**

ATP standard With MEM (without Phenol red) - **8**

ATP Standard – **9**

ATP with HBSS – without Ca, without Mg – **10**

ATP with HBSS – with Ca; with Mg – **11**

ATP with Dulbellco's Phosphate Buffered Saline – Without Ca; without Mg - **12**

ATP with another ATP releasing reagent – NP 40 - **13**

ATP with MEM (with Vitamins) - **14**

ATP with MEM (with non-essential amino acids) - **15**

Although the RLU measurements obtained were very low, it could still be utilized to determine whether the different solutions would inhibit the reaction of the Luciferase-enzyme. The RLU value of the Positive control was taken as 100% light output. The inhibiting effect of each different cell culture solution could then be determined. For the blank, Tris buffer and water controls (negative controls), the RLU value must be lower than 0.0001 RLU or else contamination with ATP has occurred and all the results would then be invalid. The clean HBSS had the same readings as the negative controls, thus, there were no ATP present in the solution and no contamination had taken place.

Table 4.7 indicates the % of RLU obtained for each of the cell culture solutions as measured against the positive control, where the positive control is 100% RLU.

Table 4.7: Different cell culture solutions and their influence on RLU's

Solution Tested	**Percentage Light out put Obtained**
Experiment 1	
Positive control	100%
ATP with 10µl Hanks Balanced Salt solution	82%
ATP with MEM (with Phenol Red)	10%
ATP with MEM (without Phenol Red)	29%
Experiment 2 (Repeat of experiment 2)	
Positive control	100%
ATP with HBSS – without Ca, without Mg	65.3%
ATP with HBSS – with Ca; with Mg	66.5%
ATP with Dulbellco's Phosphate Buffered Saline – Without Ca; without Mg	65.3%
ATP with another ATP releasing reagent – NP 40	49.75%
ATP with MEM (with Phenol Red; with Vitamins)	66.3%
ATP with MEM (with Phenol Red; with non-essential amino acids)	79.97%

<u>The following RLU-inhibiting results were obtained:</u>

- ATP with 10µl of HBSS – 82%. Thus, the luciferase-enzyme reaction was inhibited by 18%.
- ATP with MEM (with Phenol Red) – 10%. Thus, the light reaction was inhibited by 90%. Phenol red tends to reduce the light out put by half due to the fact that it absorbs at 570nm. Here we see it can even be more.
- ATP with MEM (without Phenol Red) – 29%. Thus the light reaction was inhibited by 71%. Thus, without Phenol Red being present, the light reaction was still quenched. Thus, some constituent of the MEM-medium was also inhibiting the Luciferase-enzyme reaction.

The above-mentioned test was repeated, and the RLU reading obtained for the positive control (0.5µg/ml) was equal to 2.217 RLU. Thus, this would be 100% light output production.

<u>The following RLU-inhibiting results were obtained:</u>

- ATP with HBSS – without Ca, without Mg - 65.3%. Thus, the Luciferase-enzyme reaction was inhibited by 34.7%
- ATP with HBSS – with Ca; with Mg - 66.5%. Thus, the Luciferase-enzyme reaction was inhibited by 33.5%
- ATP with Dulbellco's Phosphate Buffered Saline – Without Ca; without Mg - 65.3%. Thus, the Luciferase-enzyme reaction was inhibited by 34.7%.
- ATP with another ATP releasing reagent – NP 40 - 49.75%. Thus, the Luciferase-enzyme reaction was inhibited by 50.25%
- ATP with MEM (with Phenol Red; with Vitamins) - 66.3%. Thus, the Luciferase-enzyme reaction was inhibited by 33.7%.
- ATP with MEM (with Phenol Red; with non-essential amino acids) - 79.97%. Thus, the Luciferase-enzyme reaction was inhibited by 20%. It appears as though non-essential amino acids partly override the light inhibition.

Thus, some of the constituents of the different solutions were definite inhibitors of the Luciferase-enzyme reaction.

Results of Research Question 2

Chemical Solutions

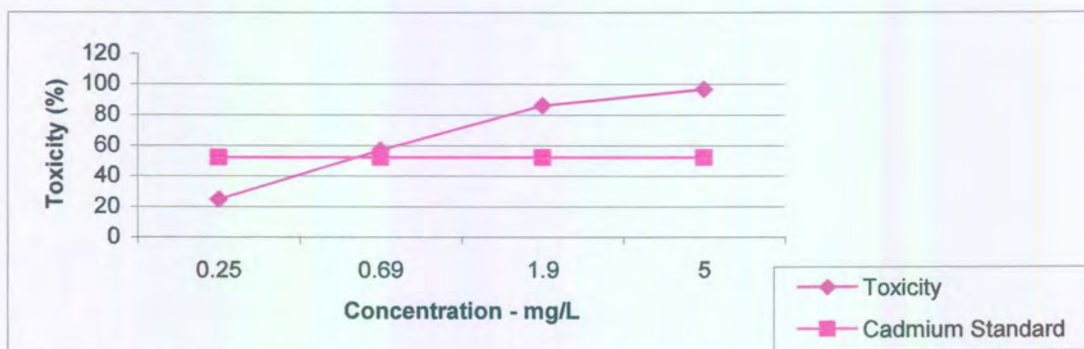
The following results for Research Question 2 were obtained: *Can this Modified Highveld Biological MTT assay be utilised as a screening assay for cytotoxicity testing of chemical solutions, but more importantly for water samples?*

The chemical solutions and unknown water samples (noted in Table 3.4 and 3.5 - Materials and Methods) were subjected to the Modified Highveld Biological MTT assay. Results are presented below. Because of the unreliability of the Bioluminescent assay, and the fact that more refining is needed to obtain the same sensitivity as obtained with the Modified Highveld Biological assay, it was decided not to subject the chemicals and water samples to this assay.

$HgCl_2$

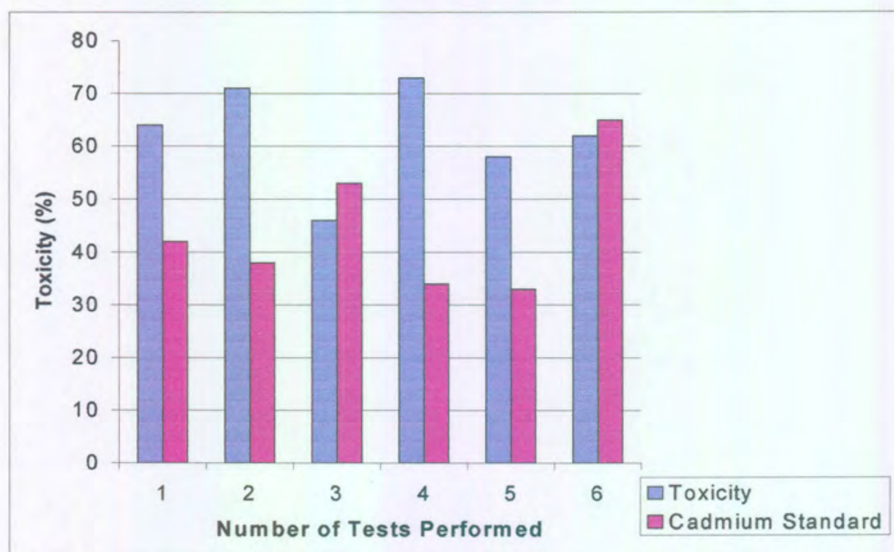
$HgCl_2$ was subjected to a serial 2.7 dilution. Cytotoxicity decreased from 97% (5µg/ml) to 25% (0.25µg/ml) (Figure 4.5a and Table 4.8). When cytotoxicity was determined at a concentration of 2µg/ml, the cytotoxicity varied between 50 and 70% cytotoxicity with a mean cytotoxicity value of 76.3% (Figure 4.5b, Table 4.9). For the serial dilution, at a concentration of 1.9µg/ml, the cytotoxicity value obtained was equal to 86% (Table 4.8). Thus, it is clear that $HgCl_2$ is a very toxic substance, even at very low concentrations.

Figure 4.5a: Toxicity of $HgCl_2$ after a serial dilution



This graph indicates the cytotoxicity obtained (Y-axis) for different concentrations (X-axis).

Figure 4.5b:Toxicity of $HgCl_2$ - concentration 2mg/L



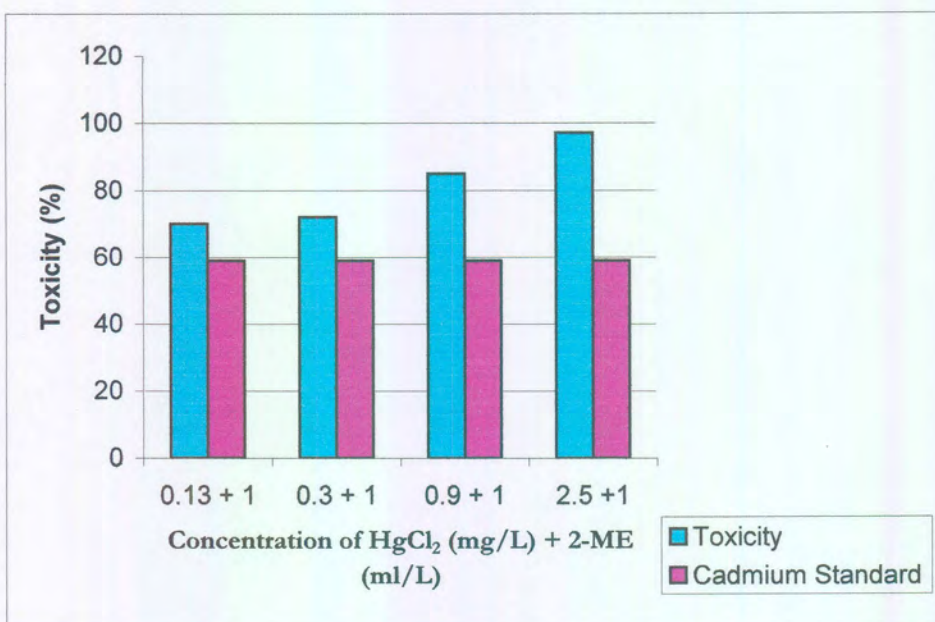
2-Mercaptoethanol (2-ME)

Cytotoxicity of 2-Mercaptoethanol was determined for 2 different concentrations. 5% cytotoxicity was obtained for a concentration of 0.5ml/L and 9% at a concentration of 1ml/L. Thus, it is evident that 2-Mercaptoethanol is a non-cytotoxic chemical, when present individually (Table 4.9).

Interaction between $HgCl_2$ and 2-ME

The mean cytotoxicity value for $HgCl_2$ alone is equal to 78.7% for a concentration of 2.5µg/ml. The mean cytotoxicity value for 2-ME at 1ml/L was equal to 9% (Table 4.9). For the test where $HgCl_2$ (2.5µg/ml) and 2-ME (1ml/L) was utilised, the cytotoxicity obtained was equal to 97% (Table 4.10, Figure 4.6). When the cytotoxicities of the individual chemicals were calculated, after addition, it was 88%. Thus, $HgCl_2$ and 2-ME together produces a synergistic effect.

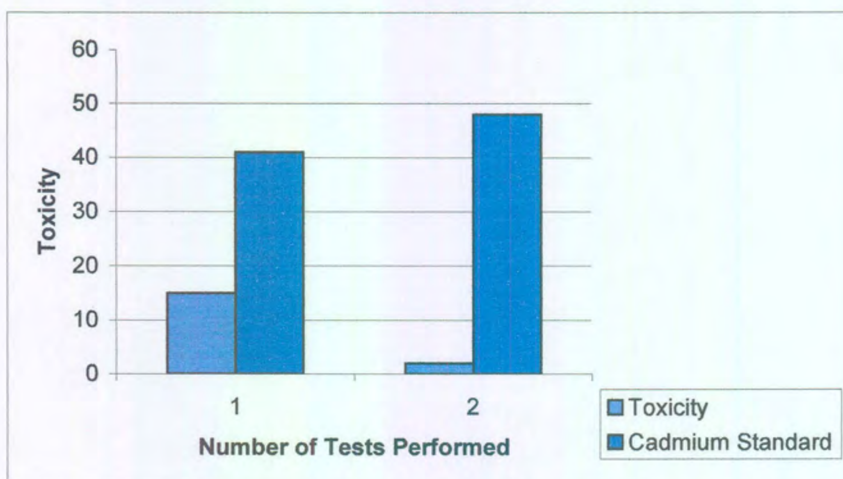
Figure 4.6: Interactions Between $HgCl_2$ and 2-ME



$Pb(NO_3)_2$

The cytotoxicity obtained for this chemical substance was between 2 and 15% cytotoxicity at a concentration of 10µg/ml, with a mean cytotoxicity value of 8.5%. As an individual chemical, this substance is non-cytotoxic (Figure 4.7).

Figure 4.7: Toxicity of $Pb(NO_3)_2$ at 10mg/L

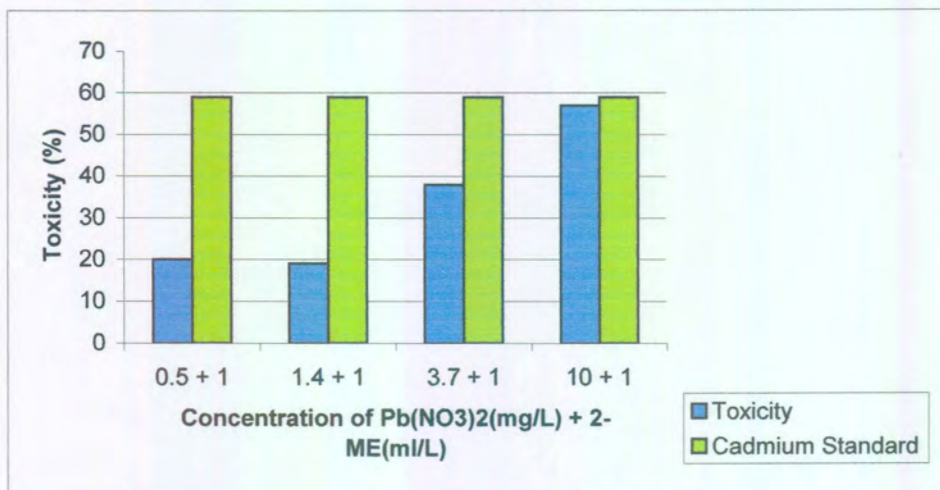


Interaction between $Pb(NO_3)_2$ and 2-ME

The toxicity for $Pb(NO_3)_2$ as an individual chemical at a concentration of 10µg/ml, was equal to 15% (Figure 4.7). The cytotoxicity of 2-ME was 9%. If added, the

cytotoxicity was 24%. The obtained cytotoxicity was 57%, indicating a synergistic effect (Table 4.10 and Figure 4.8).

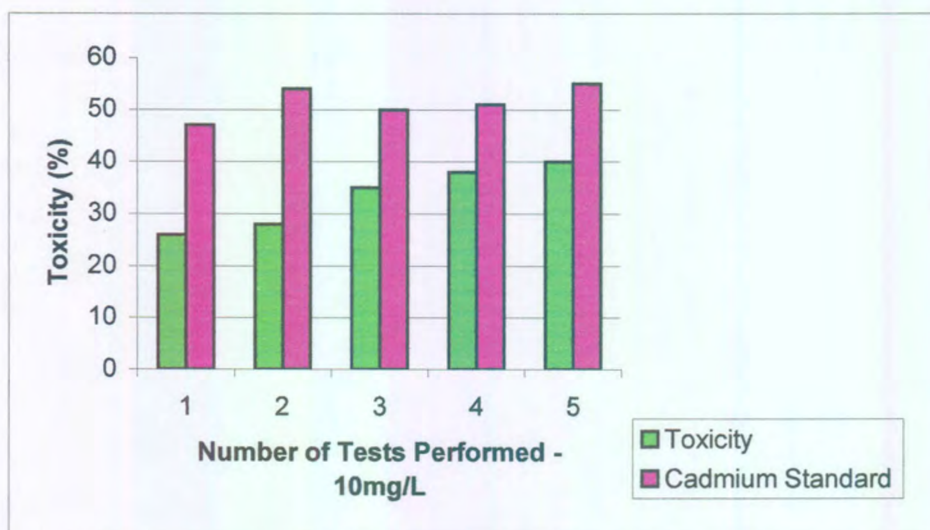
Figure 4.8: Interactions between $Pb(NO_3)_2$ and 2-ME



<u>Pentachlorophenol</u>

Cytotoxicity obtained for the different tests were between 25 to 40%. The mean cytotoxicity value obtained was 33.4% at a concentration of 10µg/ml (Table 4.9 and Figure 4.9).

Figure 4.9: Toxicity of Pentachlorophenol

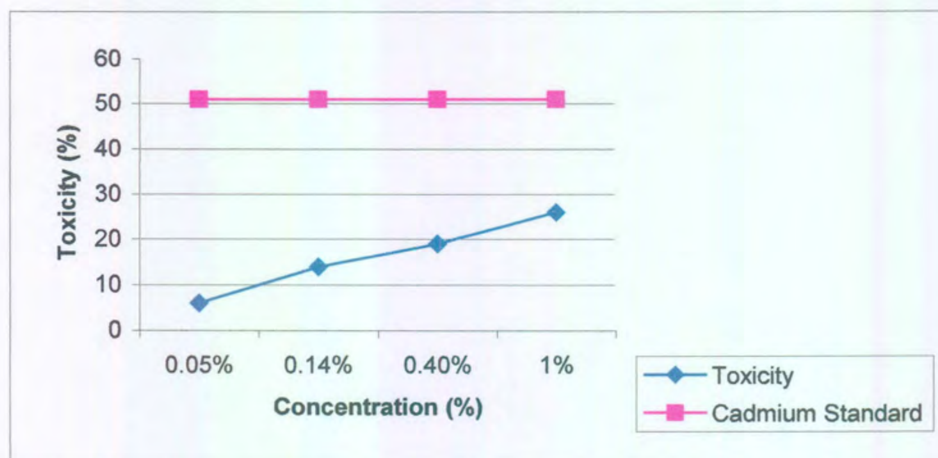


<u>DMF</u>

The cytotoxicity of DMF was determined after a serial dilution of 2.7 (Table 4.8).

Cytotoxicity decreased from 26% (1% concentration) to 6% (0.05% concentration) (Figure 4.10).

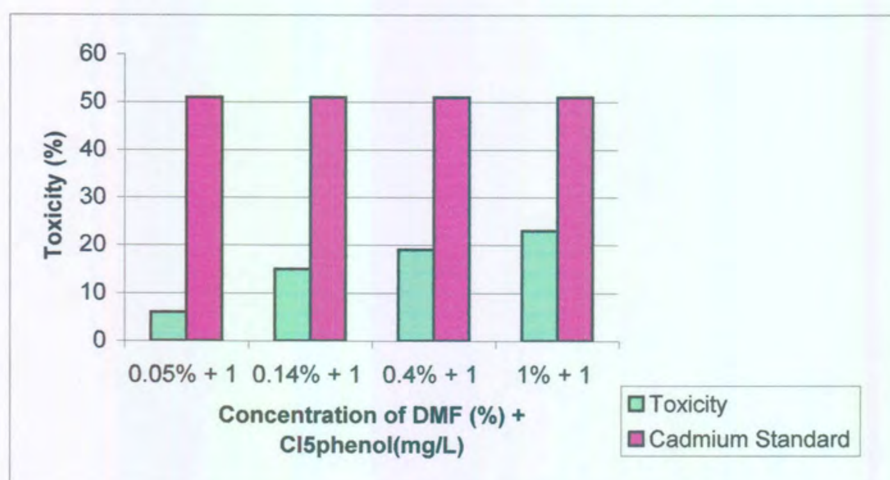
Figure 4.10: Toxicity of DMF at different concentrations



<u>Interaction between DMF and Pentachlorophenol</u>

The cytotoxicity of pentachlorophenol at a concentration of 10µg/ml was equal to 33.4%. An estimated value for pentachlorophenol at a concentration of 1µg/ml, would be 5%. Figure 4.11 clearly indicates that DMF together with pentachlorophenol result in almost exactly the same cytotoxicity reading. The mean cytotoxicity value obtained for the chemical mixture was 22%. Thus, a neutral effect can be observed (Table 4.8 and 4.10).

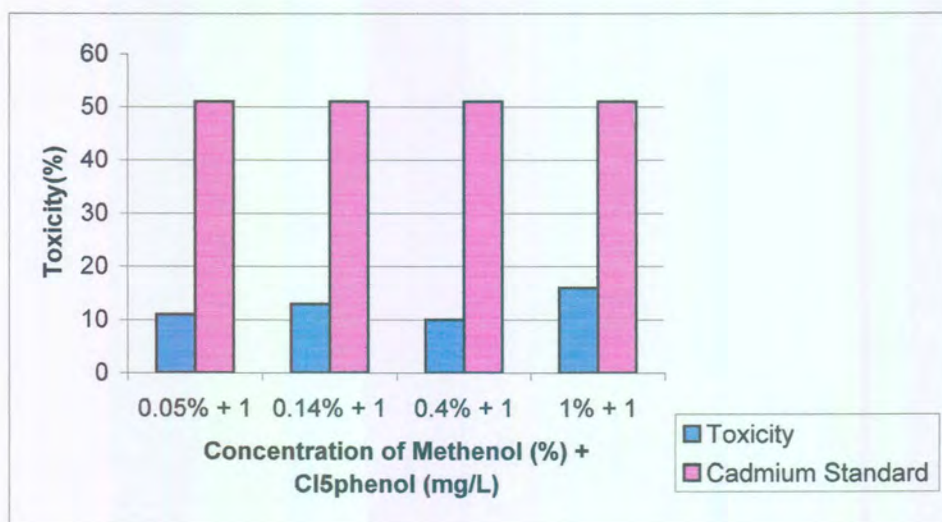
Figure 4.11: Interactions between DMF and Pentachlorophenol



Interaction between Methanol and Pentachlorophenol

The mean cytotoxicity value for methanol at a concentration of 1% was 26% (Table 4.9). When the interaction between methanol and pentachlorophenol was determined, methanol was tested at four different concentrations, while pentachlorophenol was used at a concentration of 1µg/ml. As mentioned above, the estimated cytotoxicity value for pentachlorophenol at 1µg/ml was 5%. Results given in Figure 4.12 and Table 4.10 indicate that the cytotoxicity of methanol is slightly reduced to below the 20% toxicity cut-off line.

Figure 4.12: Interactions between methanol and Pentachlorophenol

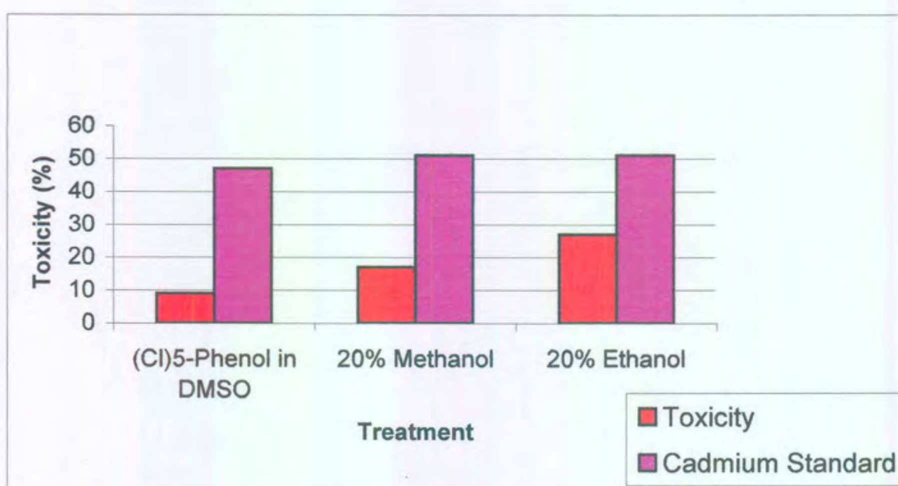


Cytotoxicity of chemical solutions due to certain chemical treatments

Phenol

Phenol alone has a mean cytotoxicity value of 33% at a concentration of 10µg/ml (Table 4.9). Phenol was co-cultured with Pentachlorophenol dissolved in DMSO. The cytotoxicity reading obtained was 9%. Further, phenol was also co-cultured with 20% Methanol and 20% Ethanol. The cytotoxicities obtained were 17% and 27% respectively. Thus, all three these chemical treatments decreases the cytotoxicity of phenol (Table 4.11, Figure 4.13).

Figure 4.13: Toxicity reading of Phenol after certain treatments



$NaNO_2$

The cytotoxicity for $NaNO_2$ at 10µg/ml was 46.6% (Table 4.9). $NaNO_2$ was tested with different chemicals, and for each test, the concentration was set at 10µg/ml. Treatments that lowered $NaNO_2$ cytotoxicity were:

- $NaHCO_3$ (7.5 % concentration, 7.5g/100ml) – cytotoxicity value: 24%
- $NaHCO_3$ (10 % concentration, 10 g/ml) – cytotoxicity value: 26%
- Fluoride – 2 cytotoxicity values: 26% and 41%
- Pentachlorophenol – cytotoxicity value: 30%
- Pentachlorophenol dissolved in DMSO – cytotoxicity value: 19% (Table 4.11).

Other chemicals tested did not lead to a marked change in cytotoxicity:

- $MgCl_2$ (5mg/ml) – cytotoxicity value: 40%
- $CaCl_2$ (1mg/ml) – cytotoxicity value: 41%
- Pentachlorophenol together with DMSO – cytotoxicity value 44% (Table 4.11).

Thus, the first group of chemicals will lead to a decrease in cytotoxicity, whilst the second group will cause no change in the cytotoxicity (Figure 4.14, 4.15).

Figure 4.14: Toxicity of $NaNO_2$ at concentration 10mg/L

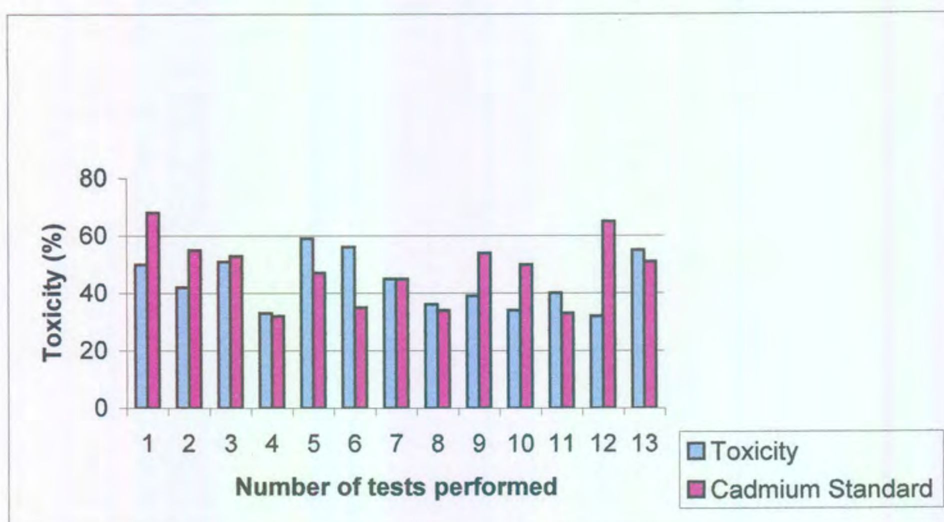
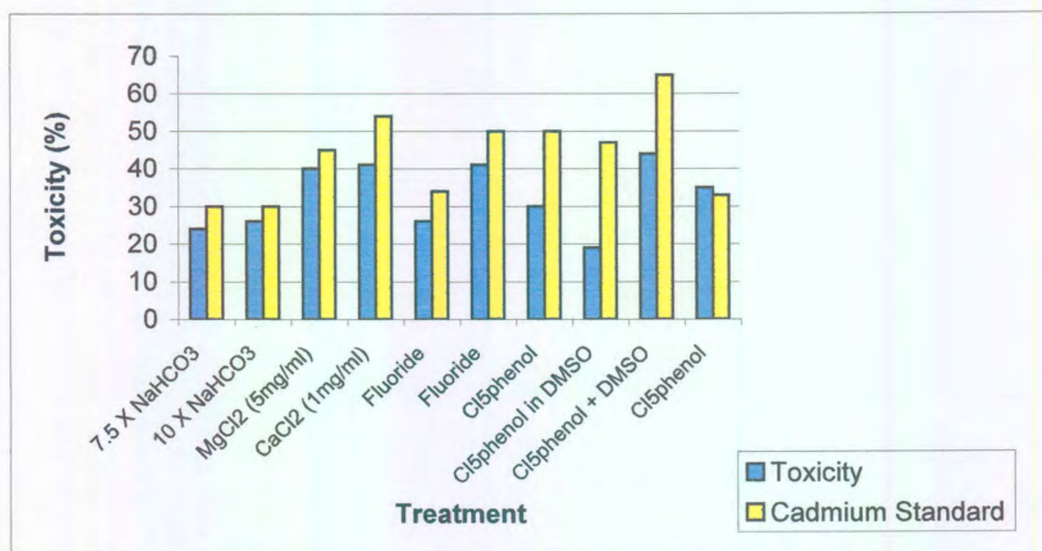


Figure 4.15: Toxicity of $NaNO_2$ with different treatments

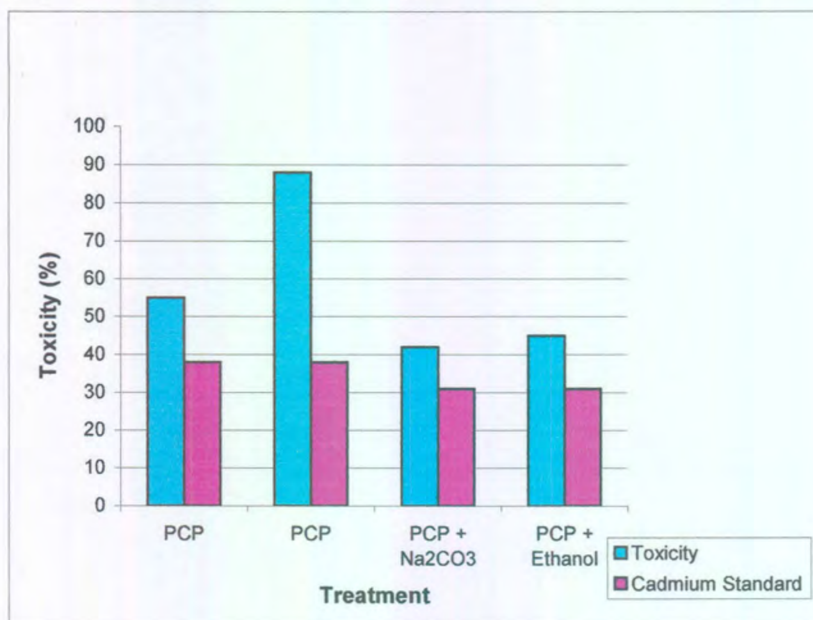


$CdCl_2$

The cytotoxicity obtained for $CdCl_2$ at 3.7µg/ml was 87.7%, (a high cytotoxicity reading for such a low concentration) (Table 4.9). At the same concentration, but in the presence of pentachlorophenol, the cytotoxicity reading was 55% (Table 4.11). $CdCl_2$ at a concentration of 10µg/ml delivered toxicity reading equal to 95% (Table 4.9). At the same concentration, but also in the presence of pentachlorophenol, delivered a cytotoxicity reading of 88% (Table 4.11). Thus, in both cases the cytotoxicity of $CdCl_2$ decreased in the presence of pentachlorophenol. When the

cytotoxicity of $CdCl_2$ in the presence of pentachlorophenol and Na_2CO_3 was determined, the cytotoxicity was 42% (Table 4.11). $CdCl_2$ cytotoxicity, in the presence of pentachlorophenol and ethanol, was 45% (Table 4.11 and Figure 4.16). The cytotoxicity value for $CdCl_2$ at 0.5µg/ml was 41.4% (Table 4.9). Thus, these 2 mixtures of chemicals have no effect on the cytotoxicity.

Figure 4.16: Toxicity of $CdCl_2$ after different treatments



Toxicity of CaCl2 at different concentrations

$CaCl_2$ was non-cytotoxic up to a concentration of 370µg/ml. A sudden rise in cytotoxicity occurred after the above-mentioned concentration from 10% to 70% cytotoxicity (370 µg/ml to 686µg/ml). This was followed by a gradual increase in cytotoxicity from 70 to 80% to 97 and to 99% (Figure 4.17). Thus, $CaCl_2$ are non-cytotoxic at small concentrations, but an extreme increase in cytotoxicity occurred at 500µg/ml.

Figure: 4.17: Toxicity of $CaCl_2$ at different concentrations

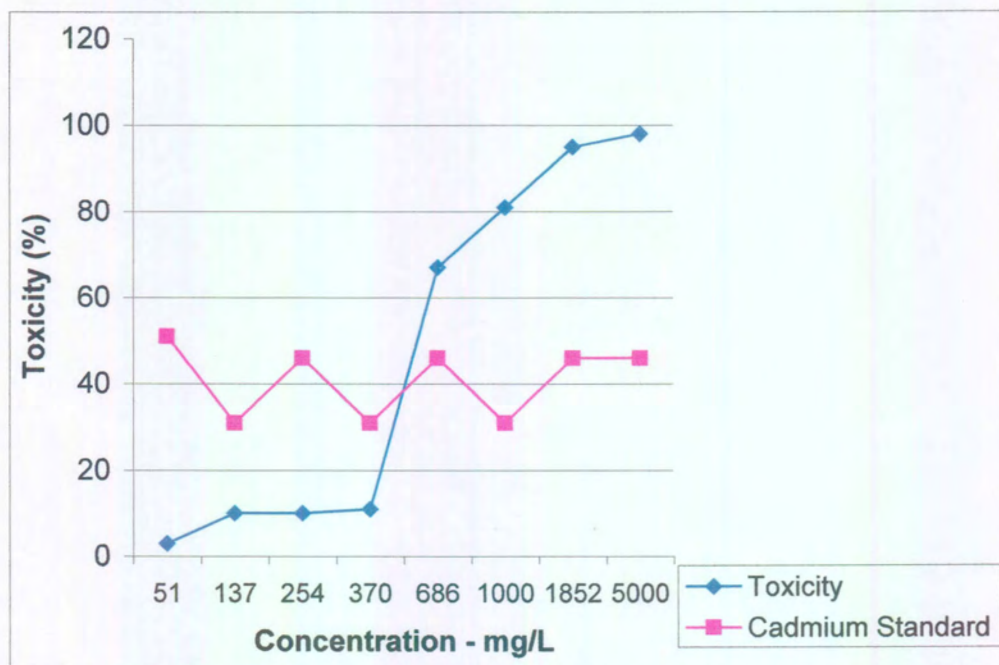


Table 4.8: Cytotoxicity (%) of chemicals after dilutions

Chemical	Cytotoxicity	Concentration – µg/ml	Cadmium
$HgCl_2$	25	0.25	52
	57	0.69	
	86	1.9	
	97	5	
DMF	6	0.05%	51
	14	0.14%	
	19	0.4%	
	26	1%	
SDS	-17	0.5	42
	-21	1.4	
	-2	3.7	
$CaCl_2$	3	51	51
	10	137	31
	10	254	46
	11	370	31
	67	686	46
	81	1000	31
	95	1852	46
	98	5000	46

Table 4.9: Cytotoxicity (%) of different chemicals at different concentrations

Chemical Tested	Concen-tration μg/ml	Times Test Repeated	Cyto-toxicity	SD	Cadmium Standard	SD
$HgCl_2$	0.25	4	13.56	7.8	43.5	11.2
	0.5	6	25.3	7.8	56	13
	1	4	38.5	3.3	46	11.4
	1.4	6	50.3	10.8	56	13
	1.9	2	47	2.8	43.5	10.6
	2	9	76.3	19.6	49.3	13
	2.5	6	78.7	4.0	45.6	9.3
	3.7	5	77	12.1	56	13
	5	2	72	2.8	43.5	10.6
	10	6	85.9	7.1	54	11.1
2-Mercaptoethanol	0.5	1	5	-	67	-
	1	1	9	-	67	-
KCN	20	7	25.9	5.64	49.75	11.17
Phenol	0.5	1	28	-	54	-
	1.4	2	29	1.4	62	11.3
	3.7	1	28	-	54	-
	10	3	33.3	14.6	55.3	14.05
SDS	10	5	30.8	13.2	50.2	9.5
$CuSO_4$	10	3	24	10.4	42.7	9.6
Methanol	1%	5	26	6.1	49	9.14
Pentachlorophenol	10	6	29.7	8.1	49.5	5.7
$CdCl_2$	0.5	7	41.1	9.4	47.5	11.0
	1.4	8	45.7	18.7	53.9	12.2
	3.7	5	87.7	11.2	59.5	9.95
	10	6	94.9	3.98	56.4	12.2
2:4-DNP	10	5	30.8	10.4	52.7	8.82
NH_4Cl	20	2	2.5	4.95	54.5	3.5
Thymol	10	1	0	-	48	-
NaF	10	2	18	-	46	-

Chemical Tested	Concen - tration µg/ml	Times Test Repeated	Cyto-toxicity	SD	Cadmium Standard	SD
Ethanol	0.1	2	6	5.7	62	11.3
	0.3	2	15.5	4.9	62	11.3
	0.7	2	29.5	23.3	62	11.3
	0.9	1	11	-	58	-
	1	2	20.5	17.7	60	13.4
	2	2	9.5	2.1	55	1.4
	2.5	1	14	-	58	-
$NaNo_2$	10	15	46.6	11.05	46.7	10.9
$Pb(NO_3)_2$	10	2	8.5	9.2	44.5	4.95
Acridine Orange	1.4	1	21	-	66	-
	3.7	5	30.4	13.6	44.4	12.6
	5	4	25	4.7	44	9.42
	10	10	48.4	23.5	51.2	15
$K_2Cr_2O_7$	0.2	1	25	-	32	-
	0.5	6	27.7	5.0	57.7	13.7
	0.7	1	28	-	51	-
	1	4	38.5	3.3	46	11.4
	1.4	8	51	10.9	54	12.8
	1.7	1	28	-	36	-
	1.9	2	47	2.8	43.5	10.6
	2	2	60	2.8	49	22.6
	3.7	8	78.9	11.2	55.5	12.8
	2.5	8	80.3	8.9	47.5	12.5
	5	2	72	2.8	43.5	10.6
	10	9	89.1	8.6	55.9	10.3

For comparing these results, calculated EC_{50} (effective concentration) values would have been preferable to the raw data. More determinations would be necessary to calculate the EC_{50} values. This was not possible for this thesis, it could be possible to calculate these values for future use or for when it will be utilised more regularly.

Table 4.10: Interactions between different chemical solutions

Chemicals Tested	Cytotoxicity	Concentration	Cadmium	Type of Interaction
$HgCl_2$ + 2-ME	70 72 85 97	0.13 + 1 0.3 + 1 0.9 + 1 2.5 + 1	59 59 59 59	Additive - Synergism
$Pb(NO_3)_2$+ 2-ME	20 19 38 57	0.5 + 1 1.4 + 1 3.7 + 1 10 +1	59 59 59 59	Synergism
DMF + Pentachlorophenol	6 15 19 23	0.05% + 1 0.14% + 1 0.4% +1 1% + 1	51 51 51 51	Neutral
Methanol + Pentachlorophenol	11 13 10 16	0.05% +1 0.14% +1 0.4% +1 1% + 1	51 51 51 51	Antagonism
$CdCl_2$ + Pentachlorophenol	36 90	0.5 0.5	69 67	Neutral - Synergism

Table 4.11: Cytotoxicity (%) of chemicals after different chemical treatments

Chemical Tested	Toxicity	Cadmium	Concentration of Chemical Tested (µg/ml)	Treatment
$HgCl_2$	63	33	2	PCP
	63	65	2	DMSO+PCP
	63	34	2.5	Fluoride
	84	47	5	PCP in DMSO
KCN	10	33	10	PCP
	15	34	10	Fluoride
	2	47	10	PCP in DMSO
Phenol	9	47	10	PCP in DMSO
	17	51	10	20% MeOH
	27	51	10	20% EtOH
SDS	63	33	10	PCP
	55	47	10	PCP in DMSO
	57	65	10	DMSO+PCP
PCP	17	51	10	20% MeOH
	36	51	10	20% EtOH
$CdCl_2$	55	38	3.7	PCP
	88	38	10	PCP
	42	31	0.5	PCP+Na_2CO_3
	45	31	0.5	PCP+EtOH
Thymol	8	47	10	PCP in DMSO
NaF	5	33	10	PCP
$CaCl_2$	71	51	588	Fluoride+PCP
	1	51	588	Fluoride
$NaNO_2$	24	30	10	7.5 x $NaHCO_3$
	26	30	10	10 x $NaHCO_3$
	40	45	10	$MgCl_2$ 5mg/ml
	41	54	10	$CaCl_2$ 1mg/ml
	26	34	10	Fluoride
	41	50	10	Fluoride
	30	50	10	PCP
	19	47	10	PCP in DMSO
	44	65	10	PCP+DMSO
	35	33	10	PCP
Acridine Orange	79	41.5	10	PCP
	47.5	42	10	Fluoride
	78	65	10	DMSO+PCP
	57	47	10	PCP in DMSO
$K_2Cr_2O_7$	84	47	5	PCP in DMSO
	68	34	2.5	Fluoride
	63	33	2	PCP
	72	65	2	DMSO+PCP

Results for unknown water samples

Nine different unknown water samples (Table 3.6 of the Material and Methods) were tested for their cytotoxicity by means of the Modified Highveld Biological MTT assay. Different tests were performed to determine the extent of the toxicity present in each sample. According to tests done by Dr. E Wittekindt from the Federal Institute of Hydrology, Koblenz, Berlin (**Appendix A**) for verification of the method, any sample that exhibited cytotoxicity below 20% (EC_{20}), could not be seen as being cytotoxic.

As mentioned in the paragraph above, cytotoxicity can only be determined from 20% and upwards. From the 9 unknown water samples tested, 5 samples exhibited definite cytotoxicity (above 20%). Only the results from these 5 water samples (samples 1, 4,6,7 and 9) will now be discussed. Unknown samples 2 and 8 exhibited low cytotoxicity that may lead to problems in future. Samples with cytotoxicity values above 20% were randomly chosen to be exposed to different tests (Na_2CO_3 and SepPak cartridge clean-up) to see whether toxicity could be lowered or removed (Table 4.12). Na_2CO_3 was used to precipitate toxicants in the sample. The sample was filtered through a SepPak cartridge remove any organic toxicants that could be present.

Unknown 4: (Figure 14.18 a and b)

Unknown water sample 4 had a mean cytotoxicity value of 58.14%. The Modified Highveld Biological MTT assay was repeated 21 times, and the cytotoxicity for 13 of the repeats were above 40% (Figure 4.18a). Thus, this sample may be very hazardous to potential consumers. When the sample was pre-treated with Na_2CO_3 (Figure 4.18b), cytotoxicity fell to 10%.

Figure 4.18a: Toxicity Of unknown sample 4, (untreated)

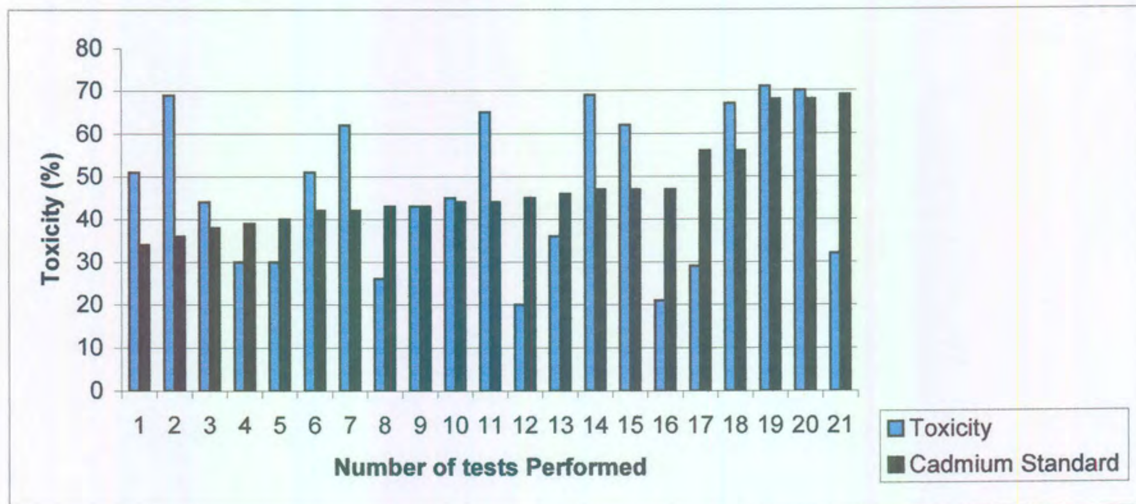
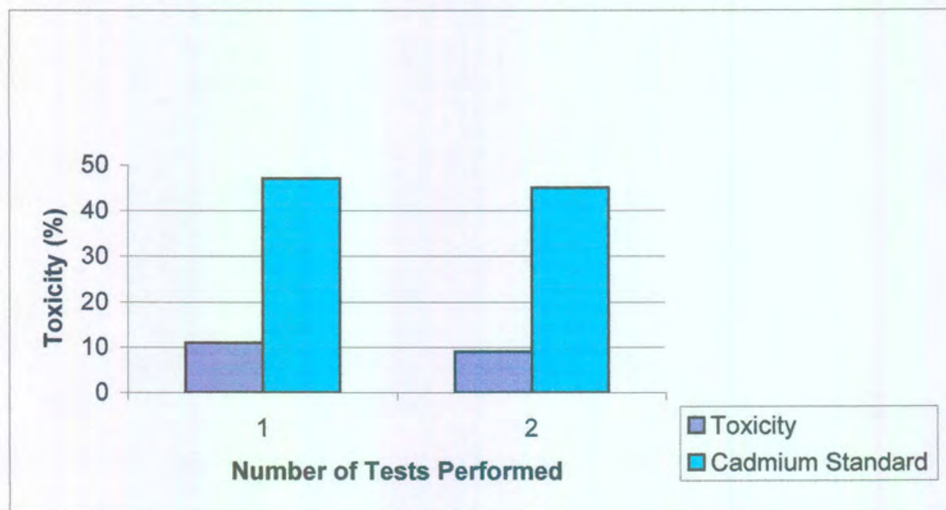


Figure 4.18b: Toxicity of unknown sample 4 after pre-treatment with Na_2CO_3



Unknown 6: (Figure 4.19)

Cytotoxicity was between 40 and 45% for all the tests performed. The mean cytotoxicity was 42%. Thus, this sample also exhibits a high cytotoxicity (Figure 4.19).

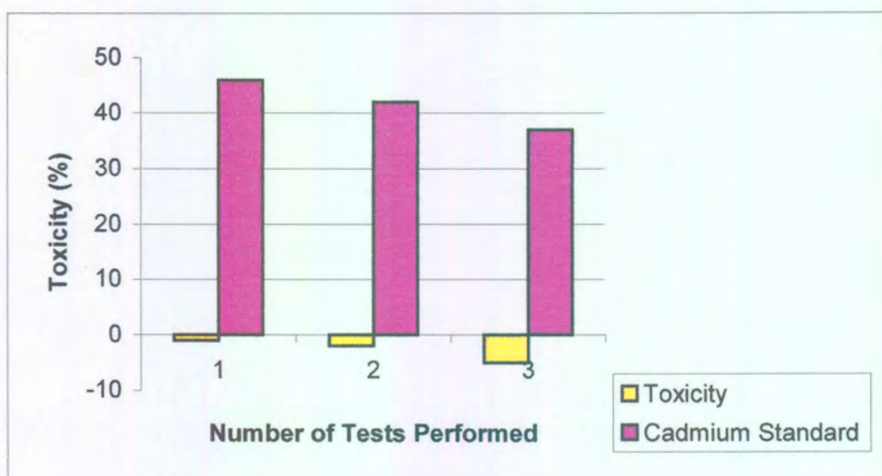
Figure 4.19: Toxicity of unknown sample 6

Unknown 7: (Figure 4.20 a and b)

The mean cytotoxicity of the untreated water sample was 75.5%. Cytotoxicity was more than 50% for all the repeated tests performed (Figure 4.20a), thus this sample exhibited very high cytotoxicity. When the sample was pre-treated with Na_2CO_3 (Figure 4.20b) cytotoxicity was less than 0%, indicating a hormetic effect. This sample was then filtered through a SepPak cartridge, causing cytotoxicity to decrease from 75.5 to 51.5%. Thus, some of the organic components leading to cytotoxicity were removed, but not all.

Figure 4.20a: Toxicity of unknown sample 7 (untreated)

Figure 4.20b: Toxicity of unknown sample 7, (pre-treated with Na_2CO_3)



Unknown 8: (Figure 4.21 a and b)

This water sample exhibited a mean cytotoxicity of 25.5% (Figure 4.21a). This sample exhibits a borderline cytotoxicity, but it may cause health problems in future. When the sample was pre-treated with Na_2CO_3, cytotoxicity decreased to 2% (Figure 4.21b)

Figure 4.21a: Toxicity of unknown sample 8, (untreated)

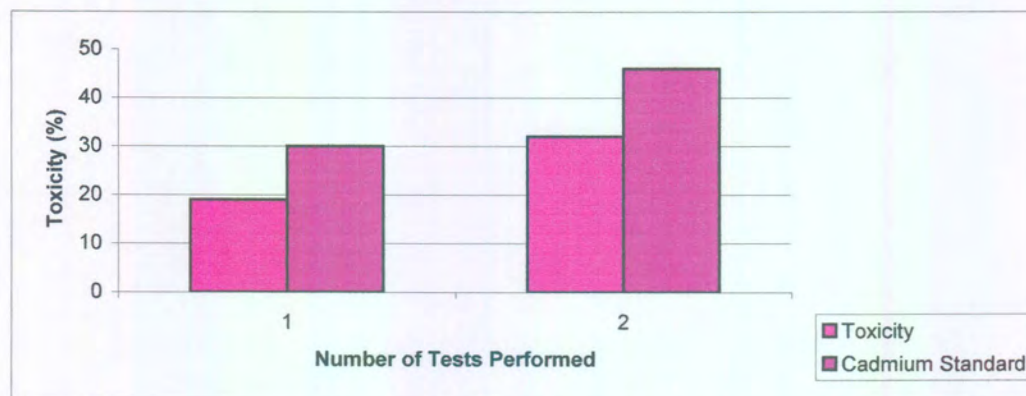
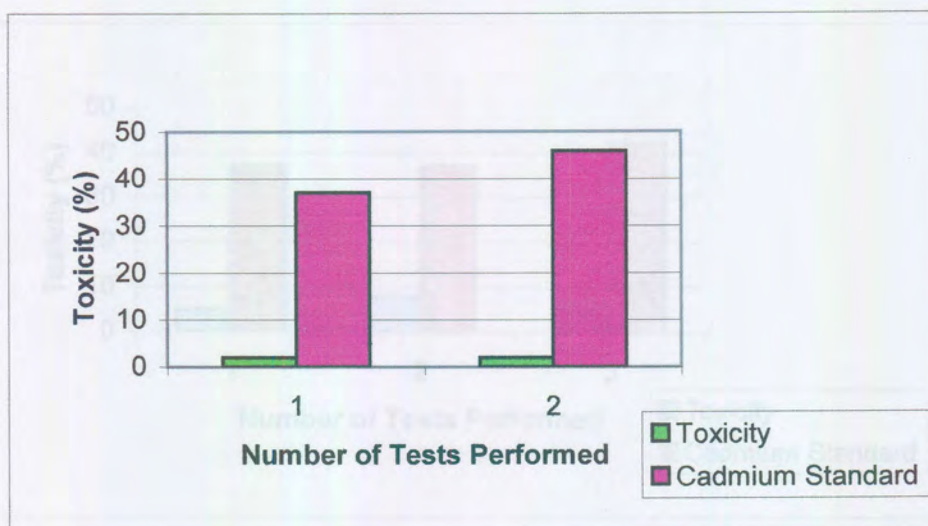


Figure 4.21b: Toxicity of unknown sample 8 (pre-treated with Na_2CO_3)



Unknown 9: (Figure 4. 22 a and b)

The individual cytotoxicity values varied between 50-75% cytotoxicity. The mean cytotoxicity value obtained was equal to 59% (Figure 4.22a). This water sample was also pre-treated with Na_2CO_3 and cytotoxicity was lowered to 7.75% (Figure 4.22b). The sample was also filtered through a SepPak cartridge, but cytotoxicity increased to 72.5%.

Figure 4.22a: Toxicity of unknown sample 9 (untreated)

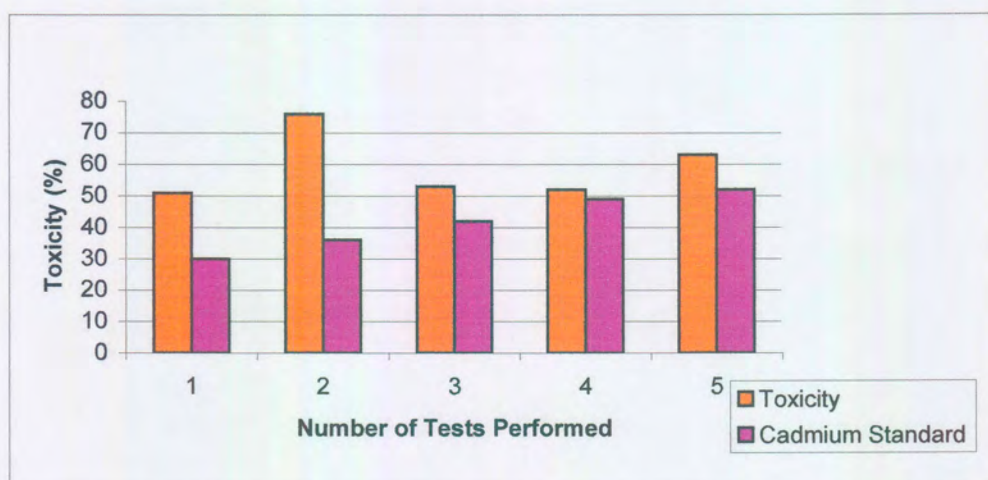


Figure 4.22b: Toxicity of unknown sample 9 (pre-treated with Na_2CO_3)

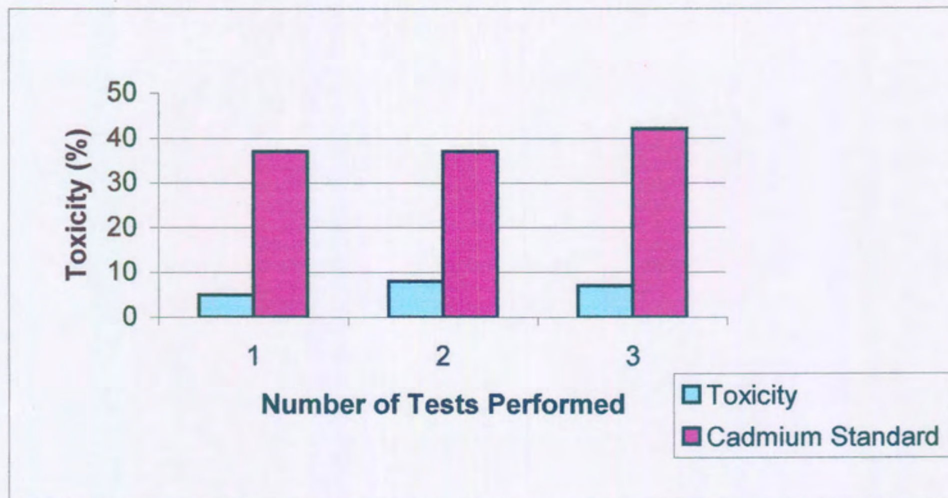


Table 4.12: Toxicity (%) of unknown water samples

Unknown Tested	Toxicity	SD (Times test repeated)	Cadmium	SD
Unknown 1	57	-	68	-
Unknown 2	25	-	68	-
Unknown 3	5.5	3.54 (2)	67	-
Unknown 4 -				
Untreated Sample	58.14	24.7 (28)	47.4	9.5
Pre-treated with Na_2CO_3	13	5.3 (3)	48.7	4.7
Unknown 5	13	5.9 (7)	46.9	11.4
Unknown 6	42.5	23.1 (4)	54.3	15.14
Unknown 7 -				
Untreated Sample	75.5	13.98 (8)	49.4	12.8
Pre-treated with Na_2CO_3	-5.25	5.4 (4)	44.25	6.3
SepPak cartridge Clean-up	51.5	8.5 (2)	33	4.2
Unknown 8 –				
Untreated Sample	25.5	22.4 (6)	42.5	7.6
Pre-treated with Na_2CO_3	2.75	6.99 (4)	44.3	6.3
Unknown 9 –				
Untreated Sample	59	10.65 (5)	41.8	8.1
Pre-treated with Na_2CO_3	7.75	2.5 (4)	40.5	4.4
SepPak cartridge Clean-up	72.5	4.9 (2)	33	4.2

Chapter 5: Discussion

Discussion of Research Question 1

Question 1: *What are the correct order of cell culture media to be used in the Modified Highveld Biological MTT assay and the bioluminescent assay to obtain an optimal functioning cytotoxicity screening assay?*

The aim of this research question was to develop a fully functional screening assay system for use in water quality assessment. The past few years, researchers at the Highveld Biological Laboratory made a number of changes to the original Mosmann MTT assay (called in this thesis the Modified Highveld Biological assay). One of the main objectives of the research in this thesis, was to refine the Highveld Biological MTT assay by determining optimal cell culture conditions (maximum sensitivity when determining cytotoxicity) for this assay. Furthermore, two existing Bioluminescent assay kits were tested (CytoPro HTS kit and the ATP Biomass kit). The first kit was found not to be suitable for cytotototoxicity testing of water samples as it is a single step method and cannot be manipulated for higher sensitivity. Although the ATP Biomass kit could be manipulated for higher sensitivity, too many variables were present that interfered with cytotoxicity readings. We therefore decided that, although this bioluminescent method has potential to be further developed as a cytotoxicity indicator for water samples, this part of the process will not be included in the present research for the thesis.

The following will now be discussed:

- Why were K-562 cells used?
- Why was there a need to implement a 3-phase medium cycle?
- Why did the Highveld Biological Research team modify the original Mosmann MTT Method?
- How did the Highveld Biological Laboratory modify the original Mosmann MTT method?
- What were the limitations of the Bioluminescent assay?

Use of the K-562 cell-line

K-562 cells are a continuous cell-line that was originally obtained from a patient with chronic myelogenous leukaemia. This cell-line has a number of advantages associated with its use in a cell culture cytotoxicity screening assay:

- It is a permanent cell-line, with a rapid growth cycle, dividing every 26-30 hours.
- It is a human cell-line, thus it can give an indication of possible negative effects to the humans.

Apart from the reasons given above, the fact that the K-562 cells normally grow in suspension simplifies their use in a routine assay. This is not the case when an attached cell line would be used. Previous work in the Highveld Biological Laboratory showed that these K-562 cells are more sensitive to toxins when glutamine and serum are absent from the assay medium. Thus the rotating culture system was set up to ensure that the cells remain responsive to these more sensitive conditions.

Instead of culturing the cells in a single medium, the cells were cultured in a medium cycle that contained 3 medium types, excluding the incubation medium used during the modified Highveld Biological MTT assay (as mentioned in Materials and Methods). This was done to improve the reproducibility and the sensitivity of the cells. The cells that were utilised in an assay had to exhibit a healthy metabolic state. By transferring the cells from one phase of the cycle to the next, the different metabolic pathways remained open and fully functional (**Appendix B**). The cells had to grow in both serum-free (Medium type 1) and serum-containing medium (Medium type 2) respectively. Cells had to grow maximally in the serum-free medium, with alanyl glutamate and serum containing medium, without alanyl glutamate, to be utilised in a toxicity test. This procedure was carried out with success (Figure 4.1). Cells were taken from the 3rd medium (c) of the cycle and exposed to the Modified Highveld Biological MTT assay. If the cytotoxicity of the Cadmium control were between the set parameters of 30-70% cytotoxicity, this was an indication of appropriate sensitivity of the cells and taken as an indication that the medium cycle does increase the sensitivity of the cells, thus increasing the sensitivity of the assay. Cytotoxicity of between 30-70% was obtained (Figure 4.1).

Reasons for culturing the K-562 cells in the different media of the medium cycle

The cells in the three-phase medium cycle are continuously cultured; when cytotoxicity screening assays need to be performed, the cells are taken from phase 3 (Medium type 3, (c)) and placed in the incubation medium (d).

Cells were cultured in serum-free medium in order for these cells to adapt to growing in medium that contains no serum proteins, only alanyl glutamate. Cells were then seeded to serum-containing medium in order for them to adapt to growing in medium with bovine serum, but that contains no alanyl glutamate. The incubation medium that was utilised during the cytotoxicity-screening assay (when cells were in microtitre plates) did not contain any bovine serum or alanyl glutamate. Thus, due to the fact that the cells were exposed to similar growth conditions during the first three phases (Medium type 1-3), they do not enter a shock stage when now placed in the incubation medium in the microtitre plates. If the cells did not adhere or grow during the continuous cell-culturing period, they were placed back into serum-free medium.

Reasons for applying the Modified Highveld Biological MTT Assay

The procedure originally described by Mosmann involved incubating cells in culture medium (containing serum and phenol red), with the yellow MTT solution, to produce water-insoluble formazan crystals. An equal volume of isopropanol was added to solubilize the formazan crystals. Because of the presence of phenol red indicator in the medium, which also absorbs at 570nm, hydrochloric acid was added to convert the indicator to a yellow, non-interfering counterpart. Denizot and Lang in 1986 (53), who also modified Mosmanns' method, suggested that the serum should be removed during the extraction of the formazan-dye complex. The washing steps involved, made their MTT technique less attractive, since there exists a risk of losing cells and formazan during the washing procedures. Even the loss of a few thousand cells readily results in a substantial drop in optical density (62).

Limitations of the original Mosmann MTT assay

In addition to the overall satisfactory results obtained with the original Mosmann MTT assay, possible difficulties and recommendations for optimisation of the assay have been described. Difficulties associated with the their assay are summarised as follows:

- High and variable background.
- The presence of serum proteins.
- The acid pH of the solute.
- The wavelength chosen for absorbency measurement.
- All of the above mentioned contribute to reduced sensitivity.

The colorimetric assay has a high and variable background, due to the precipitation of serum proteins from the medium when the organic solvent is added (53). Isopropanol, as organic solvent, is known to precipitate serum proteins and this effect may be even more pronounced at low pH levels (below 4) at which most of the serum proteins will carry a basic charge, increasing the possibility of precipitation even further. This can give rise to a light scattering phenomenon (62). Thus, it is necessary to remove all the serum proteins that may be present on the cell surface or in the medium. A further complication is the slow solubility of the formazan product in the final isopropanol mix (53). Denizot and Lang also indicated that hydrochloric acid or an acid pH may cause changes in the spectral properties of formazan salts (53), although it is necessary for changing phenol red to its yellow counterpart which do not absorb at the same absorbency as MTT.

Changes made by the Highveld Biological Laboratory

Due to the limitations associated both the Mosmann and Denizot and Lang methods, modifications were made by the Highveld Biological Laboratory to eliminate all the above mentioned problems.

Firstly, care had to be taken to remove all serum proteins that could have been attached to the plasma membrane receptors before being used for a cytotoxicity screening test. Also, after completion of the test, during termination, detergent solution was added to each well to solubilise the cell membrane (phospholipid structure) to release the formed formazan crystals. This step was followed by addition of hydrochloric acid to the wells to change the phenol red indicator to its yellow counterpart. The addition of hydrochloric acid could cause a dual negative response. Firstly, if any serum proteins were present, precipitation of serum proteins would occur, interfering with determining the absorbency. Also, the spectral characteristics of the formazan crystals may change. This phenomenon was prevented by adding an amount of heavy oil to the wells, and so concentrates the

dissolved formazan crystals in the oil. The highly coloured "oil bead" which resulted was then measured spectrophotometrically at the wavelength of 570nm.

As already mentioned, the serum proteins have to be removed from the plasma membrane receptors. For this purpose, HBSS (without Ca^{2+}; with Mg^{2+}; with HEPES) were utilised for washing the cell to remove all interfering substances that could be attached to the cellular surface. The plasma membrane receptors are utilised as "message molecules" that can alter the cellular metabolism. Therefore, any toxic effect that any solution may have on the bio-indicator could be altered or removed if serum proteins were present, thus causing reduced and modified cellular effects. As seen from literature, an organic chemical is more toxic in serum-free media.

Furthermore, the cells were placed into the assay culture medium and then placed into the fridge at 4°C for at least 1 hour. This was necessary for synchronising the cells to the same stage of the cell growth cycle. The media that were utilised did not contain any Ca due to the fact that the cells form a better suspension and the cells tend to adhere to a lesser extend to the flask and well surface.

Discussion of the Bioluminescent assay

This Bioluminescent ATP monitoring assay has previously been successfully used with genetically engineered cells that contain the luciferase genes (*luc* and *lux*). Solutions have also been utilised to promote the permeability of cell membranes, thus allowing quick entrance into the cells (88).

The firefly luciferase that is utilised in this method has a number of factors that should be taken into account before being able to utilise this system to its full entirety. Substances that interfered with the luciferase enzyme (88) are high concentrations of ions, high concentrations of salts, high levels of oxyluciferin, also the light output is reduced by the presence of phenol red. As seen from the obtained results, phenol red quenched the light reaction by 90%. Also, the solutions that were tested (Table 3.3; Materials and Methods) interfered with the RLU's as seen in Table 4.6 (Results).

A further consideration when using the assay kit is that it requires white microtitre plates to be utilised during the test to prevent loss of light. Light escapes through the sides and bottom of a transparent tissue culture plate, thus leading to false results.

Seeing that this assay is based on utilising a cell-line for determining changes in ATP production due to cytotoxicity, it is very important to be able to study the cells during the test seeing that contamination might occur when culturing cells. When using white microtitre plates, it is impossible to study the cells during the tests, thus it is impossible to determine whether any contamination has occurred or the cells are still viable. Precipitation may also occur, leading to false results.

Although we did not use this method further in the research for the present thesis, because it could produce false cytotoxicity screening results, with further development and testing this method could become a very valuable tool for determining water cytotoxicity. Furthermore, this method can be very easily automated, if the above mentioned shortcomings can be overcome, rendering it as a good cytotoxicity screening method.

Discussion of Research Question 2

Question 2: *Can this Modified Highveld Biological MTT assay be utilised as a screening assay for cytotoxicity testing of chemical solutions, but more importantly for water samples?*

As indicated in the above paragraphs discussing the results of Research Question 1, only the Modified Highveld Biological MTT assay, with modifications suggested by results from the present thesis, were used. The cytotoxicity of different chemical solutions (Table 3.4 - Materials and Methods) and unknown water samples (Table 3.6 – Materials and Methods) were successfully determined by only using the Modified Highveld Biological MTT assay.

The following results will now be discussed:

- Using Cadmium (Cd) as an internal standard.
- Discussion of the results of the chemical solutions used.
- Discussion of the results of the unknown water samples.

Using Cadmium (Cd) as an internal standard

An internal standard system (negative control, positive control and Cd cytotoxicity control) is important in any cytotoxicity screening system. In this thesis, each microtitre plate contained 6 negative control wells (sterile water, culture medium with

no cells) were allocated. The absorbency readings for the negative controls were never higher than 100 (0.1) and the test was repeated if a higher absorbency was obtained. There were 8 positive control wells allocated on each plate (sterile water, culture medium, 10µl cells). Absorbency readings between 400 (0.4) and a 1000 (1.0) were acceptable. If the reading was below 400, it was too difficult to distinguish differences between the positive control and the chemical or water sample. If the reading was more than a 1000, to many cells were present to obtain the true cytotoxicity of the given sample. A Cd standard, known for its cytotoxicity, was also utilised on each plate to determine the sensitivity of the cells. The reading obtained for the Cd standard had to fall between 30-70% cytotoxicity. This reading gave an indication of the sensitivity of the cells and indicated their metabolic status.

Cytotoxicity is dependent on the time of exposure and the concentration of the given sample that were tested. An exposure period of less than 14 hours is too short for significant changes to take place. By increasing the exposure period to 18 hours, the time period is long enough for the toxin to alter any important cellular functions. Also a long enough period is allowed to gather information on possible cytotoxic effects that may be present. If 18 hours exposure time is allowed, it is also convenient because it allows the plate to be cultured overnight, and the test can be terminated the following day. Thus, test results are available within 24 hours.

Chemical solutions

In total, 14 chemical solutions was analysed (Table 3.3 – Materials and Methods). The results for these chemicals will now be discussed according to the figures form the Results section.

Discussion of results: $HgCl_2$ (Figure 4.5a and b)

The results indicate that $HgCl_2$ is a cytotoxic chemical. When a serial dilution was performed, cytotoxicity increased linearly (Figure 4.5a). Even at very low concentrations, $HgCl_2$ remains very cytotoxic (Figure 4.5b). Thus, if $HgCl_2$ was present in any water sample, it would induce serious cellular functional changes, leading to cellular death via either apoptoses or necrosis.

Discussion of results: $HgCl_2$ in the presence of 2-ME (Figure 4.6)

This test was performed to observe the possible reaction of $HgCl_2$ if 2-ME was

present in the same solution at a concentration of 1µl/ml. $HgCl_2$, as an individual chemical, has a mean toxicity value of 76.3%; if present at a concentration of 2µg/ml (Figure 4.6). When $HgCl_2$ was present in solution at a concentration of 2.5µg/ml, in the presence of 2-ME, cytotoxicity increased to almost 100%. 2-ME, at a concentration of 1µl/ml, exhibited a cytotoxicity of 9%. Thus, if these two chemicals were present in the same test solution, the cytotoxicity increased, leading to a synergistic effect. $HgCl_2$ on its own will induce cellular death, while 2-ME will not.

Discussion of results: $Pb(NO_3)_2$ (Figure 4.7 and 4.8)

$Pb(NO_3)_2$ is a non-cytotoxic chemical if present individually and at a concentration of 10µg/ml. The reaction of $Pb(NO_3)_2$ in the presence of 2-ME was also determined. The cytotoxicity obtained for a mixture of these two chemicals, resulted in a value higher than 50% cytotoxicity (Figure 4.7 and 4.8). The two chemicals, as individual chemicals, are entirely non-toxic, but when in mixture, they lead to synergistic cytotoxicity, not just a additive cytotoxic effect. The additive effect is noted when the two original cytotoxicities add up to the resultant cytotoxicity. Synergistic effect is noted when the resultant cytotoxicity is greater then the sum of the two individual toxicities (mentioned in the Literature).

Discussion of results: Pentachlorophenol and DMF (Figure 4.9 – 4.11)

Pentachlorophenol has a cytotoxic nature at a concentration of 10µg/ml (Figure 4.9). When a serial dilution was performed on DMF, its cytotoxicity increased linearly to 25%. Thus, DMF is slightly cytotoxic at a concentration of 1%, but if the concentration were to increase, cytotoxicity may also increase (Figure 4.10). These two above mentioned chemicals were tested in mixture to note the reaction of DMF in the presence of pentachlorophenol, present at a concentration of 1µg/ml. The cytotoxicity obtained was exactly the same as that obtained for DMF alone, thus these two chemicals has a neutral effect on each other (Figure 4.11).

Discussion of results: Methanol and Pentachlorophenol (Figure 4.12)

Methanol is sightly cytotoxic if present at a concentration of 1%. If methanol would to be present at a higher concentration, its cytotoxicity could possibly increase. Just like methanol, pentachlorophenol is also slightly cytotoxic if present at a concentration of 10µg/ml. At 10µg/ml, pentachlorophenol has a mean cytotoxicity of 29.7% (Figure 4.12). Thus, if pentachlorophenol were to be present at a

concentration of 1µg/ml, the estimated cytotoxicity would be 2.9%. Methanol were also tested at different concentrations in the presence of pentachlorophenol. The obtained cytotoxicity remained almost the same. Cytotoxicity actually decreased to below 20%. Thus, it is hypothesized that pentachloroophenol can lower the cytotoxicity of methanol to below dangerous levels. Pentachlorophenol has an antagonistic cytotoxicity effect, if found together with methanol.

Pentachlorophenol and sodium fluoride are two chemicals which showed both synergistic and antagonisitc effects when other chemicals were present. The interactions are complex and the results reported here still have to be confirmed.

Discussion of results from Phenol (Figure 4.13)

Phenol was subjected to 2.7 x serial dilution. The concentrations used, varried from 0.5µg/ml to 10µg/ml and the cytotoxicity obtained, increased only dismally from the lowest to highest tested concentration. Phenol, even at a concentration of 10µg/ml, is not that cytotoxic. Phenol was further subjected to a number of treatments (Figure 4.13):

- Pentachlorophenol that was dissolved in DMSO – Penols toxicity was lowered to below 10%. Thus pentachlorophenol induces a antagonistic toxicity effect if in the presence of phenol. Thus, this chemical mixture can serve as a great tool for removing toxicity induced by phenol.
- Methanol (20%) – Phenol's toxicity was lowered to below 20%. Thus, even methanol had a antagonistic toxicity effect in the presence of phenol. Toxicity was lowered, thus methanol could also serve as a good tool for lowering phenols' toxicity.
- Ethanol (20%) – Toxicity of phenol in the presence of ethanol was equal to almost 30%. Phenol as an individual chemical can reach a toxicity of 33%, thus ethanol has a neutral toxicity effect on phenol.

Discussion of results: $NaNO_2$ (Figure 4.14 and 4.15)

$NaNO_2$, at a concentration of 10µg/ml, produced a cytotoxic effect of 46.6%. Thus, this chemical alone is very cytotoxic. $NaNO_2$ was also subjected to a number of chemicals as a pre-treatment. Firstly, the routinely applied method utilises 10 x concentrated $NaHCO_3$ as a buffer. $NaNO_2$ was tested with $NaHCO_3$ at a 7.5 x concentration. The obtained cytotoxicity was exactly the same as the previously

obtained cytotoxicity, thus the concentrations did not make any difference in cytotoxicity obtained. All the treaments utilised to test in the presence of $NaNO_2$ lowered the cytotoxicity to below 50%. Pentachlorophenol dissolved in DMSO lowered the cytotoxicity significantly to below 20%. Fluoride and pentachlorophenol alone lowered cytotoxicity to below 30%. $MgCl_2$ and pentachlorophenol alone lowered the cytotoxicity to below 40% and $CaCl_2$ and pentachlorophenol together with DMSO, lowered cytotoxicity to 50% and below. Thus, all of these chemical solutions are antagonists, thus, they could be applied as tools for lowering the cytotoxicity of $NaNO_2$.

Discussion of results: $CdCl_2$ (Figure 4.16)

Cytotoxicity of $CdCl_2$ was determined in the presence of a number of chemical solutions. $CdCl_2$ was tested for a concentration range of 0.5 – 10µg/ml. The cytotoxicity ranged from 41% to 95%. Thus, $CdCl_2$ is a very cytotoxic chemical, this is also the reason why this chemical was utilised as an internal standard. $CdCl_2$ was also tested with the following:

- Pentachlorophenol and $CdCl_2$ - cytotoxicity was increased (Figure 4.16). The obtained interaction was less than a synergistic or addative effect. When the test was repeated, the obtained toxicity was equal to a additive to synergistic effect.

Discussion of results: $CaCl_2$ (Figure 4.17)

The cytotoxicity of $CaCl_2$ was also determined at different concentrations. This chemical was non-cytotoxic up to a concentration of 370µg/ml. While at a concentration of 686 µg/ml, it became highly cytotoxic. Thus, this chemical can be taken up by the cells, up to a certain concentration, where after it suddenly becomes cytotoxic (Figure 4.17). Thus, this chemical is non-cytotoxic at low concentrations, but extremely cytotoxic at higher concentrations.

Water samples

Discussion of results: Unknown 4 (Figure 4.18a and b)

Unknown water sample 4 was tested for its cytotoxicity and from Figure 4.18a it is clear that this water sample was very cytotoxic. More than half the readings obtained, after repeating the test a number of times, indicated definite cytotoxicity. The cytotoxicity obtained for the water sample was cytotoxic enough for 40-70% of

the cells to die. When this sample was pre-treated with Na_2CO_3, the cytotoxicity of the sample fell to below 10%, thus this suggests that the toxic agent is likely to be a divalent or polyvalent metal. This could be a good way of pre-treatment of the water sample before releasing it to the surrounding community, seeing that cytotoxicity is completely removed.

Discussion of unknown water sample 6 (Figure 4.19)

Unknown water sample 6 was tested for its potential cytotoxicity. As seen from the results, the sample exhibited definite cytotoxicity (Figure 4.19). The sample itself is very cytotoxic, seeing that it induced death of 40% of the incubated cells. It would therefore be suggested that this sample should be subjected to further testing to determine the origin of the high degree of cytotoxicity. Possible Gas Chromatography or High Pressure Liquid Chromatography is suggested to be used for further investigation.

Discussion of unknown water sample 7 (Figure 4. 20 a and b)

The potential cytotoxicity of unknown water sample 7 was also tested (Figure 4.20a). The tests were repeated 8 times, and for each of the results obtained, the sample exhibited a cytotoxicity level of between 55-90%. Thus, this would mean that half to more than three-quarters of the cells died on exposure to this water sample. Definite steps to detoxify this sample would be necessary. The same sample was exposed to pre-treatment with Na_2CO_3. The cytotoxicity of the sample was totally removed and became hormetic. Hormesis is the phenomenon of increased biological activity due to the presence of trace amounts of toxins or inhibitors (as mentioned in the Literature Review). It can also be due to the response to low levels of ionising radiation. Low levels of toxic metals induced this phenomenon, that is, an increase in the formazan reduction in cells exposed to "chronic" levels of metals (55). Hormesis is possibly a form of energy compensation initiated by cells exposed to extreme environmental conditions, which require production of high levels of stress proteins over a short time period. That is, steady-state energy levels may be temporarily distributed and a burst of energy production (hormesis) serves to restore energy homeostasis to the cell (55).

Discussion of unknown water sample 8 (Figure 4.21a and b)

The cytotoxicity obtained from the unknown water sample 8 is indicated in Fig 4.21a.

This sample was also subjected to further testing by pre-treatment with Na_2CO_3. Once again, the pre-treatment removed the cytotoxicity completely, via precipitation.

Discussion of unknown sample 9 (Figure 4.22a and b)

The cytotoxicity of unknown sample 9 is presented in Figure 4.22a. More than half of the cultured cells died due to exposure to this water sample. This sample was therefore subjected to further testing. The sample was pre-treated with Na_2CO_3, and the cytotoxicity was totally removed. Thus, once again by subjecting this sample to treatment with Na_2CO_3, the cytotoxic constituent/s (divalent and polyvalent metals) was removed via induced precipitation.

Discussion of Research Question 3

Research Question 3: *What are the advantages associated with this Modified Highveld Biological MTT assay, if applied in a water toxicity testing system?*

The method as modified in this thesis exhibited a number of advantages.

- Due to the fact that 12 samples can be tested per plate and only a small amount of sample is required, this method has a high throughput of samples within a short time period (less that 24 hours) thus giving reproducible results within a short time period.
- Also, in each plate the sample testing is repeated 6 times, thus a more accurate mean cytotoxicity value can be obtained. Also the tests can be repeated with very little variation on the cytotoxicity obtained (**Appendix A**).
- This method is very flexible; various constituents of the method can be changed to observe effect, some subject to further testing and research in future.
- The results that were obtained were highly accurate and objective, due to the fact that damage to the cells (colour production via formazan crystals) are measured by computer-linked spectrophotometric determination.

Discussion of Research Question 4

Research Question 4: *Which of the two test systems (the Modified Highveld Biological MTT assay or the bioluminescent assay utilised for determining metabolic ATP) was most suitable for regular usage?*

As mentioned previously, the Bioluminescent assay still has to be modified considerably. We therefore suggest that at the present time, only the Modified Highveld Biological method be used for cytotoxicity testing of water samples. The Modified Highveld Biological MTT assay can be applied to determine cytotoxicity of either water samples or chemical solutions, enabling the scientist to set up a database of information regarding the inherent cytotoxicity of any chemical solution, also the cytotoxicity of different water samples taken from different water sources. This is an easy method to use, which delivers reproducible results (**Appendix A**) within a short time period.

The main problem associated with the Bioluminescent ATP monitoring assay, is the fact that the luciferase enzyme is too sensitive to too many different substances (ions, salts etc.) which makes it less suited for usage for fast, effective testing of water toxicity. This method will probably function better when luciferase can be carried into the cells or form part of the cells' genetic material. Thus, it would be better suited for bioengineering (as was originally suggested). We therefore suggest that, the bioluminescent assay will definitely be a method to determine cytotoxicity, if the sensitivity of the luciferase enzyme could be better controlled.

Conclusion

The aims of this study was as follows:

- to optimise the culturing of cells in different culture media, so that these cells could be used in the Modified Highveld Biological MTT assay as the first assay in a battery of tests to determine overall cytotoxicity by utilising human cells in culture. This assay may reduce cytotoxicity assay duration, thereby saving important resources.
- to optimise the bioluminescent assay as an indication of the ATP level in the cells after treatment. This method is based on the fact that ATP is present in all living cells. The bioluminescent method utilises an enzyme, firefly luciferase, which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly dependent on the ATP concentration and is measured using a luminometer.
- to determine the cytotoxicity of various water samples and chemical solutions with the MTT assay as modified in this thesis.
- to compare the two above-mentioned bioassays.

We believe that all above mentioned aims were successfully achieved and that the method developed and discussed in this thesis will provide not only valuable information for South African water resource management, but for the protection of the world's most valuable resource: Water.

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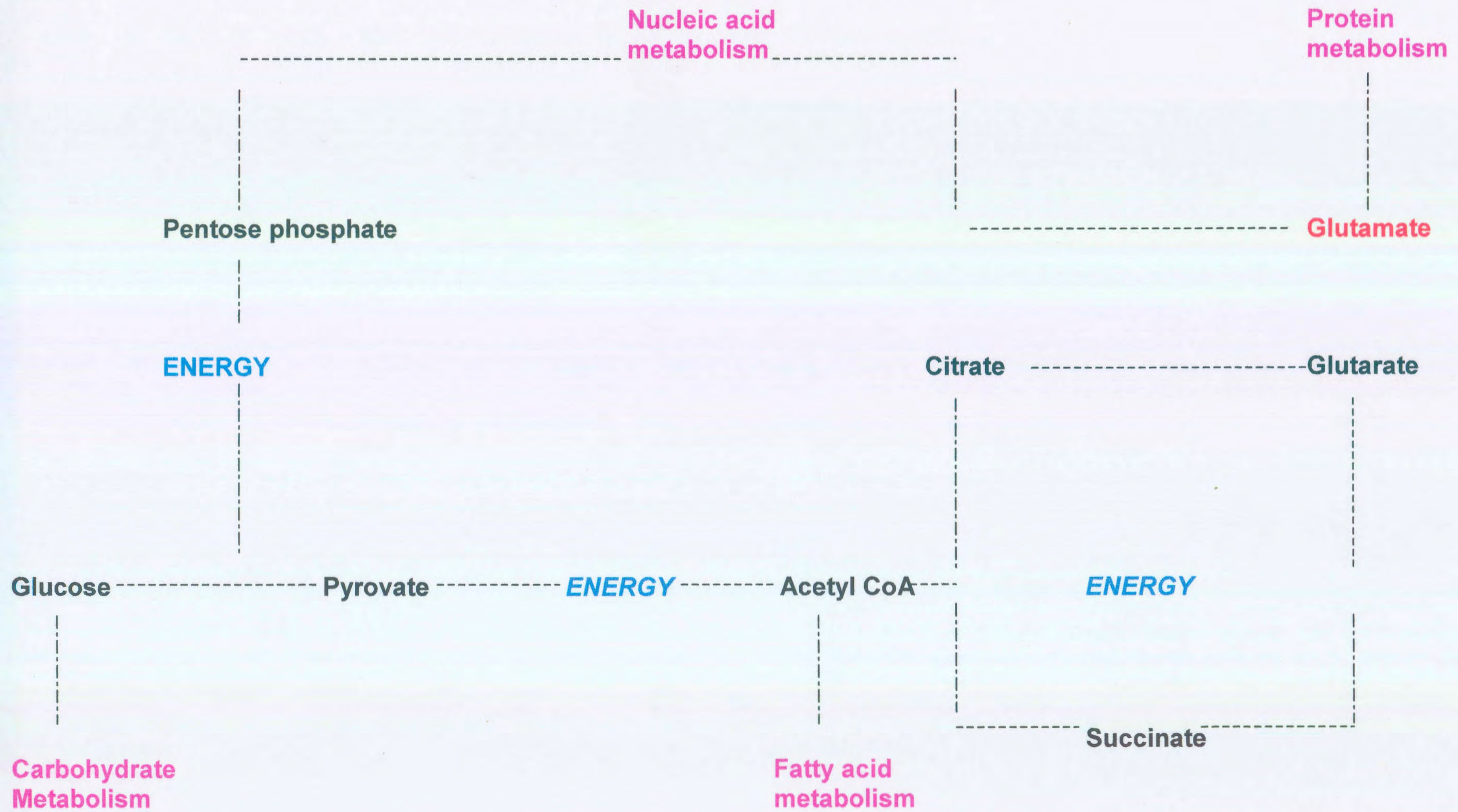
Appendix A

A comparative evaluation study with the Weaver human cell test performed by Elisabeth Wittekindt from the Federal Institute of Hydrology Koblenz, Berlin.

Appendix B

K-562 cells that were utilised in the Modified Highveld Biological MTT assay had to exhibit a healthy metabolic state. This was achieved by transferring the K-562 cells from one phase of the medium cycle to the next, thus the different metabolic pathways remained open and fully functional. These pathways are indicated in this appendix, with glutamate being the main cellular metabolic component.


Nucleic acid metabolism
Protein metabolism
Pentose phosphate
Glutamate
ENERGY
Citrate
Glutarate
Glucose
Pyrovate
ENERGY
Acetyl CoA
ENERGY
Succinate
Carbohydrate Metabolism
Fatty acid metabolism

COMPARATIVE EVALUATION STUDY WITH THE WEAVER HUMAN CELL TEST

THE WEAVER HUMAN CELL TEST IN COMPARISON TO OTHER BIO-SENSOR TESTS USED TO ASSESS THE TOXIC EFFECTS OF POLLUTED WATER JOHANNESBURG 14 – 19 JULY 2002

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NAME OF THE SENSING TEST: WEAVER HUMAN CELL TEST
TYPE OF ASSAY: EFFECT ON THE ENERGY PATHWAY OF K-562 CELLS *IN VITRO*

EXECUTIVE SUMMARY

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

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

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

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

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

a) How sensitive is the Weaver Human Cell Test compared to similar tests on other biological systems?

b) How reproducible is the test in terms of intra laboratory and inter laboratory studies?

The present study is confined to make a statement about these questions under the given conditions described below. The recent analyses were not assigned for German DIN- and ISO standardisation. If this is required, more comprehensive investigations and expert knowledge should be requested.

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

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

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

Introduction

The *in vitro assay* "Weaver Human Cell Test" is appropriate as a toxicity and vitality test using a human cell line (K-562 chronic myelogenous leukemia cells) to record the responses of these cells to contaminations in test samples (from different sources) over 24 hours. It must be pointed out that the test system is possibly not able to detect agents toxic for other organisms, which are not toxic for human beings.

Description of the System

The Weaver Human Cell Test allows the adverse effects of environmental agents to be assessed, using MTT to measure the activity of cellular oxidative pathways. In previous studies, the endpoint was adapted to adherent human cells. The biochemical targets for the induction of toxic effects can be described as "general

biochemical pathways" such as the respiratory chain and others involving oxido-reductases. Mitochondria and cytoplasmic organelles are most probably affected (Berridge et al., 1996).

The test system is based on the fact that the energy chain of cells exposed to toxic agents shows reduced enzymatic activity in comparison with that of the control population (Berridge and Tan, 1993: Berridge et al., 1996).

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

As a biotest for measuring ecotoxicological effects, the Weaver Human Cell Test have been developed with non-adherent human leucocytes by Highveld Biological Association (HBA) (Damelin et al., 2000: Whitcutt, 2000: Meyer et al., 2001).

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

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

The adaptation of the testing procedure for rapid mass testing of water samples should lower the cost of monitoring studies and enhance its applicability for regulatory approaches.

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

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

Date 1: 02-07-16 intra laboratory assessment in Highveld Biological Association
Date 2: 02-07-17 intra laboratory assessment in Highveld Biological Association
Date 3: 02-07-17 inter laboratory assessment in University of Pretoria (Department of Virology; Prof. Dr. W. Grabow)

The samples were tested according to the procedure of Highveld Biological Association along the DRAFT FINAL REPORT - PROJECT K5/1121/0/1 (promoted by the South African Water Research Commission):Title of the report: "An integrated approach to biomonitoring waste water for the presence of biologically active agents" (Authors: J.M. Whitcutt R.A. Emmett R. Tseki Z. Mbatha P. van Heerden).

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

Sample code number	Source	Solvent control	Test concentration starting point
1	Industrial station [F3 Aug01] original sample contains 30% DMSO	DMSO 3%	[1:10]
2	4-NQO 4-Nitroquinoline-N-Oxide [1 g/l]	DMSO 0,1%	[1:1000]
3	Cr(VI) Potassium-di-chromate [50 mg/l]	A. dist.	direct testing (test 1) [1:10; test 2, 3]
4	Industrial station [F5 Aug01]	DMSO 3%	[1:10]
5	Phthalate ester-mixture No. 3 [1 g/l each]	Methanol 0,1%	see certificate [1:1000]
6	Drinking water	A. dist.	Direct testing

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

For each sample, the following dilution scheme was applied:

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

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

For the inter laboratory comparison, the third test was performed completely at University of Pretoria. Additionally, the equipment was used from the department (aqua 2x dist, sterile working materials, water, pipettes, sterile working benches, incubators). The final measurements were made at Highveld Biological Association.

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

Johannesburg tap water was tested in two independent tests instead of German tap water (no. 6) – as previously planned. During the first test run, the German tap

water induced precipitation effects – possibly due to chemical reactions of the water-born calcium content with the carbonate buffer system (medium constituents). Single tests were performed with 6 parallels for each dilution step (exception: tap water was performed without further dilutions, but with N = 18 parallels for each test).

For each plate, a standard control with 1 mg/l cadmium solution (cadmium chloride) was introduced as well as maximum toxicity (no cells) and zero toxicity (laboratory water) controls and a solvent control, if necessary.

Variance analysis

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

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

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

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

Results and discussion

Variance analysis

Due to the fact that several test samples contain a solvent, the data were calculated using the corresponding solvent control. For DMSO a backward shift of controls was measured and about 20 - 25% lower MTT release potential resulted.

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

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

The detailed results of the inter laboratory study are shown in Table 2.1 (laboratory variance) and Table 2.2 (precision, repeatability, intra- and inter laboratory comparison). The toxicity data, demonstrated in graphs - see appendix.

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

For low toxicity values and enzyme activity measures in the range of = (5) - (-5) %, higher variances have been detected for mathematical reasons. These data were excluded from further variance analysis.

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

The obtained results from experiments performed in Highveld Biological and the University of Pretoria (Institute of Virology) showed no significantly differences. The variance of independent tests were not different from both groups (Table 2.2 / see intra laboratory and inter laboratory VR).

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

An high degree of reproducibility between replicate assays was shown for contaminated probes as well as for negative controls. The tap water control was performed with 18 instead of 6 parallels per tests. For non-toxic waters, low laboratory standard deviations have been assessed (Tab. 2). In case of sample no. 5, higher coefficients of variations were obtained, due to the chemical interaction of

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Weaver Human Cell Test					Results Validation experiments							
Results		Test 1	Test 2	Test 3	Test1-3	Test 1	Test 2	Test 3	Test 1-3	Variance for parallels in single tests		
		Toxicity	Toxicity	Toxicity	Toxicity	SD	SD	SD	SD	CV1 (%)	CV2 (%)	CV3 (%)
sample	source	mean, N = 6			mean	for parallels (single tests; N = 6)			mean	test 1	test 2	test 3
sample 1	Sediment	92.63	88.34	85.59	**88.853**	1.66	1.33	5.22	**2.737**	1.79	1.50	6.10
1/dil2	fraction	87.48	60.49	74.21	**74.060**	3.02	1.6	0.34	**1.653**	3.45	5.50	0.45
1/dil3	F3 EtAc/hex	52.2	18.43	34.8	**35.143**	1.97	3.25	2.04	**2.420**	3.77	17.64	5.85
1/dil4		-7.5	2.03	18.26	**4.263**		0.2	1.89	**1.045**	36.00	10.01	10.30
EC20 / LID		EC20 = 4 mg sed./ml extract			**LID = 20**							
sample 2	4-NQO	45.78	68.41	71.87	**62.020**	5.88	2.13	1.42	**3.143**	12.85	3.11	1.97
2/dil2	1 mg/L	43.39	47.78	55.71	**48.960**	6.4	1	6.73	**4.710**	14.89	2.09	11.41
2/dil3		21.09	22.55	11.29	**18.310**	6.96	2.95	5.27	**5.060**	32.98	13.80	46.70
2/dil4		-8	11.4	1.53	**1.643**	4.9	1.02	0.49	**2.137**	6.50	8.98	2.80
EC20 / LID		EC20 = 0.05 mg/L										
sample 3	CrVI	different	95.29	79.81	**87.550**		0.28	11.37	**5.825**		0.29	14.20
3/dil2	5 mg/L	dosage	91.34	79.41	**85.375**		1.74	16.75	**9.245**		1.90	21.10
3/dil3		tested	82.94	81.82	**82.380**		1.58	0.55	**1.065**		1.90	0.66
3/dil4			61.67	64.05	**62.860**		1.66	0.87	**1.265**		2.68	1.36
EC20 / LID		EC20 < 0.25 mg/L										
sample 4	Sediment	75.1	71.37	78.45	**74.973**	13.42	3.94	4.91	**7.423**	17.88	5.52	6.30
4/dil2	fraction	56.15	27.23	36.51	**39.963**	4.71	6.1	2.8	**4.537**	8.40	22.40	7.67
4/dil3	F5 methanol	28.78	7.18	16.85	**17.603**	9.57	3.6	3.58	**5.583**	33.23	12.40	21.21
4/dil4		9.38	1.41	10.3	**7.030**	4.09	3.4	1.87	**3.120**	43.64	10.73	18.07
EC20 / LID		EC20 = 11 mg sed./ml extract			**LID = 7,3**					mean CV1	mean CV2	mean CV3
sample 5	Phthalat	-21.05	-22.5	-2.48	**-15.343**	outliners for variance*				17.95	7.53	11.01
5/dil2	Esters	-14.04	-8	-10.65	**-10.897**	laboratory variance not calculated						
5/dil3	Mix3	-25.3	-1.19	-9.2	**-11.897**	for sample no. 5						
5/dil4	1mg/L 17x	-26.57	-1.4	-3.1	**-10.357**							
EC20 / LID		enhanced enzyme activity			**LID = 1**						CV from rough data	
sample 6	tap water	not calcul.	-3.8	-10.6	**-7.200**	N = (2x 18 parallels) for tap water					2.89	3.56
EC20 / LID		no effect observed			1							
									3.811	**11.64**		
									mean SD	**mean CV**		
									for parallels			
									N = 43	N = 43		

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

Weaver Human Cell Test		**Results Validation experiments**			
Variance between independend tests				**Repeatibility (Precision)**	
Test 1-3 mean SI	**VR1**	**VR2**	**VR3**	**mean VR intra lab**	**meanVR inter lab**
2.90	3.127	3.279	3.385	**3.20**	**3.26**
11.02	12.596	18.216	14.849	**15.41**	**15.22**
13.79	26.415		39.623	**26.42**	**33.02**
10.63					
11.57	25.273	16.913	16.098	**21.09**	**19.43**
5.10	11.750	10.670	9.152	**11.21**	**10.52**
5.00	23.706	22.171	44.283	**22.94**	**30.05**
7.92					
7.74	different	8.123	9.698	**8.12**	**8.91**
5.96	dosage	6.531	7.512	**6.53**	**7.02**
0.56	tested	0.675	0.684	**0.68**	**0.68**
1.19		1.930	1.858	**1.93**	**1.89**
2.89	3.851	4.052	3.686	**3.95**	**3.86**
12.06	21.472	44.276	33.022	**32.87**	**32.92**
8.83	30.696		52.429	**30.70**	**41.56**
3.99	42.555		38.754	**42.55**	**40.65**
	mean VR1	mean VR2	mean VR3		
9.11	20.14	12.44	19.65		
2.47					
10.03					
11.49					
3.40					
11.78 mean SI for 3 independent tests SI (N = 56)				**Repeatibility/precision**	
				VR (%) intra lab	**VR (%) inter lab**
				16.26	**17.02**
				N = 21	**N = 35**

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

Tab. 2 Comparative evaluation study with the Weaver Human Cell Test. Analysis of variance

CV laboratory coefficient of variance CV (%)
VR (%) coefficient of variance for repeatibility = precision
SD laboratory standard deviation
SI standard deviation of repeatibility
LID Lowest ineffective dose (correspondending EC20 value)
*) outliners for variance analysis were defined as CV values, obtained from data close to the no effect level (here positive and negative values vere obtained close to sero point; for sample no. 5 and 6)

Dilution series:

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

several compounds mixed in the probe and possibly because of problems of diffuse chemical interactions of the complex mixture of test compounds during exposure. Table 3 gives an overview considering the precision of the Weaver Human Cell Test. The variance coefficient of repeatability (VR) could be shown to be in comparable ranges for biological variances. In analogue studies, VR values about 15 up to 30% can be obtained, whereas the inter laboratory variance can be shown to be mostly in higher ranges. No differences between the intra- and inter laboratory precision have been evaluated. For the application of the test in different laboratories / and different trained personal, comparable variance data for the intra- and inter laboratory precision data can be assumed.

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

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

Table 3 Comparative evaluation study with the Weaver Human Cell Test.
Analysis of variance
SD = laboratory standard deviation
SI = standard deviation of repeatability
CV = laboratory coefficient of variance
VR = comparative variation coefficient
(as a measure of repeatability and precision).

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

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

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

Sample characterisation	Bioassay comparison			
Sample no. (nature of sample, original concentration)	Weaver Human Cell Test	Lumi-nescence+	Algae-toxic++	Other specific effects
	Toxicity LID values	Toxicity LID / EC20	Toxicity LID / EC20	Genotoxicity+++ embryotoxic for fish esterase inhibition
Industrial station [F3Aug0167601] 80 mg Sediment per ml EtAc-extracts	**20**	**16**	**32**	Genotoxicity +++ **16** cytotoxic to MCF-7 cell line
4-NQO 4-Nitroquinolin-N-Oxide [1 mg/L]	0.05 mg/L Embryotoxicity (*Danio rerio* fish larvae), Wittekindt et al., 2001 0.125 mg/L	n.t.	n.t. Mutagenic effects, Rao et al., 1995	Lethality (*Dreissena polymorpha*) Wittekindt et al., 2000a,b, 0.250 mg/L genotoxicity +++ 0,05 mg/L Baun et al., 1999
Cr(VI) Potassium dichromate [5 mg/l]	< 0.25 mg/L	20-80% range 18.7 mg/L	n.t.	Genotoxic +++ (0,2 mg/L)
Industrial station [F5Aug0167601] 80 mg Sediment per ml MetOH-extract	**7.3**	**2**	**1**	Esterase inhibition **2** (*Acanthamoeba castellanii)*
Phthalat Ester-mix No. 3 17 compounds [1 mg/L each]	Enhancement of MTT reduction 1 mg/L	n.t.	n.t.	Growth inhibition with 0.1 mg/L with MCF-7 cell line
Drinking water	1	1	1	

Tab. 4 Comparative evaluation study with the Weaver Human Cell Test.
Comparison of results with respect to sensitivity.
In vitro-bioassays:+): DIN EN ISO 11348, ++): Algae test: DIN 38 412-33,
+++): ISO/DIS 13829 (umu-test - bacterial genotoxicity test).
LID = Lowest ineffective dose; EC20.

The mono substance in sample no. 2 is a well known genotoxine (Baun et al., 1999). For this substance, genotoxic effects can be shown in 10 up to 100-fold lower test concentrations, compared with cytotoxic influences (Duis et al., 1995; Unruh, 2001). In addition, the nitroaromate 4-NQO is known to be toxic for fish-embryos and mussels (Table 4).

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

For sample no. 4 (potassium dichromate), analogue sensitivity ranges (EC_{50} values) were obtained during the evaluation study as described in former reports (FINAL REPORT - PROJECT K5/1121/0/1 - investigating the Weaver Human Cell Test).

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

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

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

Concluding remarks

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

1. How reproducible is the test in terms of intra laboratory and inter laboratory studies?

2. How sensitive is the Human Cell Test compared to similar tests on other biological systems?

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

From the technical side of test performance, the Weaver Human Cell Test showed an high degree of optimisation – with respect to preventing shifts in sensitivity during cell passages. This problem is solved by establishing cell lines and select them for

their ability to maintain alternative metabolic pathways by 'bouncing' them between different cell culture environments with the following media: (1): serum-free with glutamine, (2): glutamine-free with serum, (3): with glutamine with serum and (4): serum-free, glutamine-free for the assay itself. This maintenance cycle seems likely to be responsible for the stable sensitivity ranges in replicate tests, irrespective of the cell cycle number. Concerning the cell quality, the different steps during the performance of the Weaver Human Cell test seem to represent an optimum, no further changes can be recommended.

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

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

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

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

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

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

<u>Further applications and recommendations</u>

These preliminary study indicate that the Weaver Human Health Test would be a suitable tool to monitor the quality of drinking water and seems to be in general applicable for technical treatment process of remediation processes for water that is considered to be of drinking water standard. Beside this applications with respect to Public Health aspects, the test results suggest further ecological applications:

analyses of surface water, ground water and sewage in risk assessment studies. It has been shown that the Weaver Human Cell Test is a sufficiently robust test to be used as a guiding tool during clean-up processes, particularly as it is a 24 hour test which provide economic features compared to other tests.

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

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Appendix - Information

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

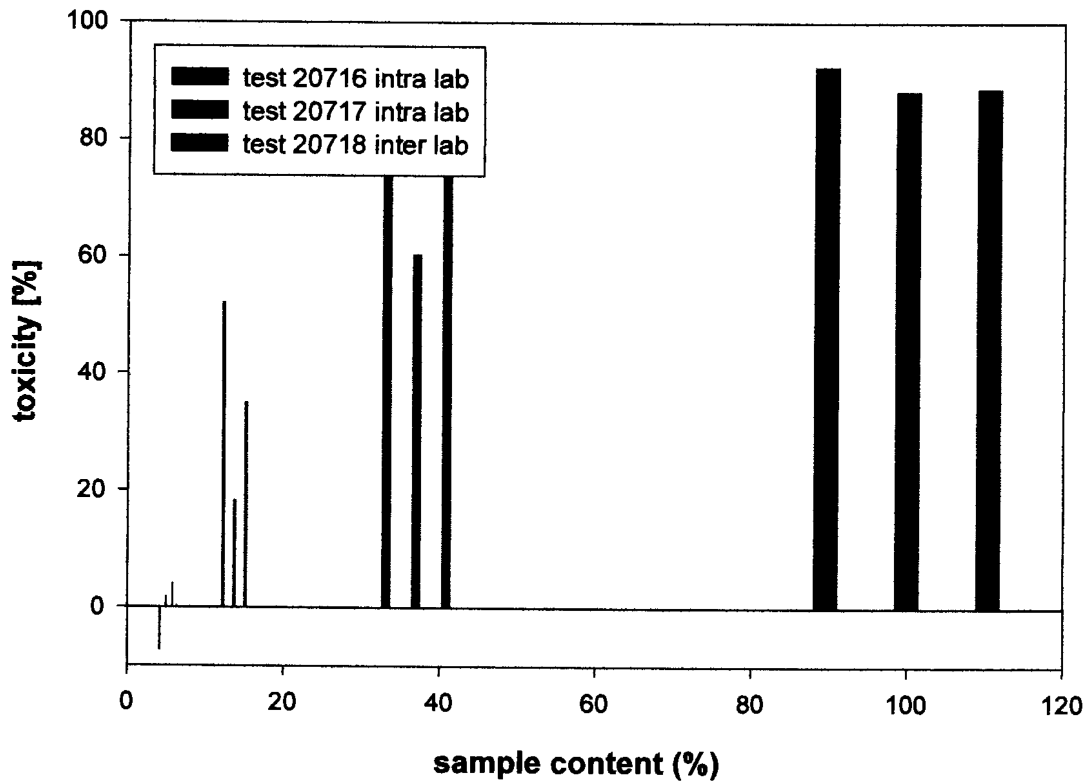
It is the scientific institute of the Federal Government for research and consulting in the fields of hydrology, water-resources management, ecology, and conservation of waters.

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

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

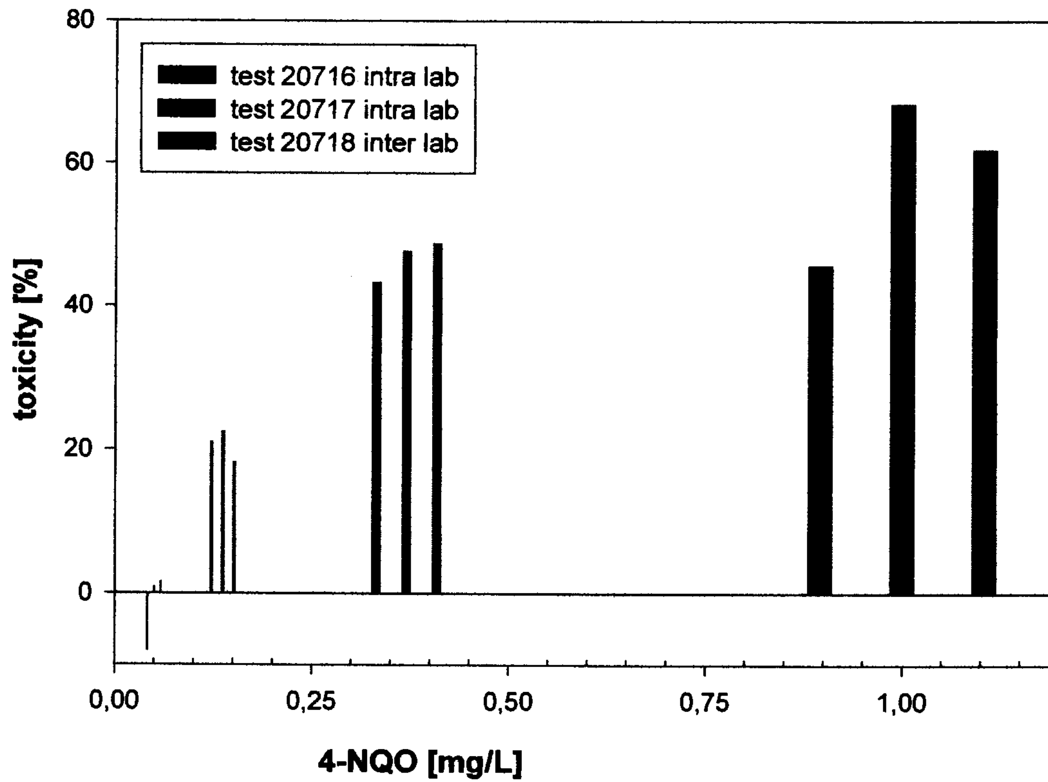
Appendix – Toxicity data

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



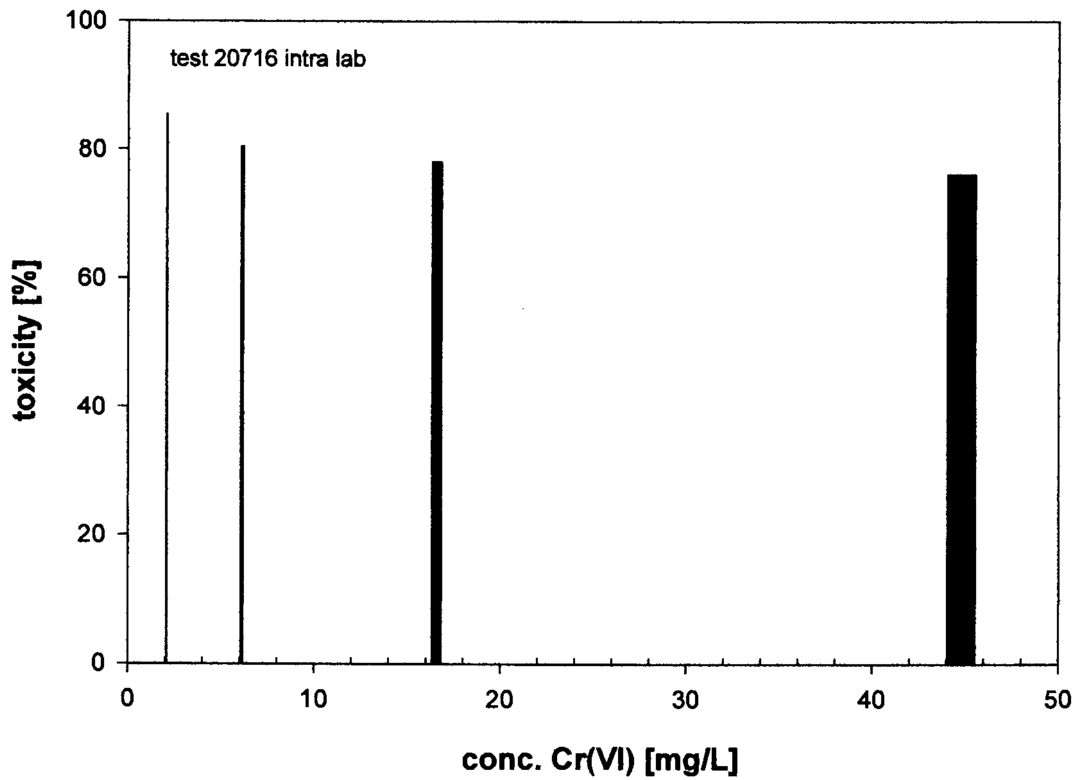
100% = 80 mg/ml sediment equivalent sample no. 1: August01 extraction in Acetone/hexane, subfraction ethylene acetate-hexane - subsequent resolved: 3% DMSO were used for test.

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

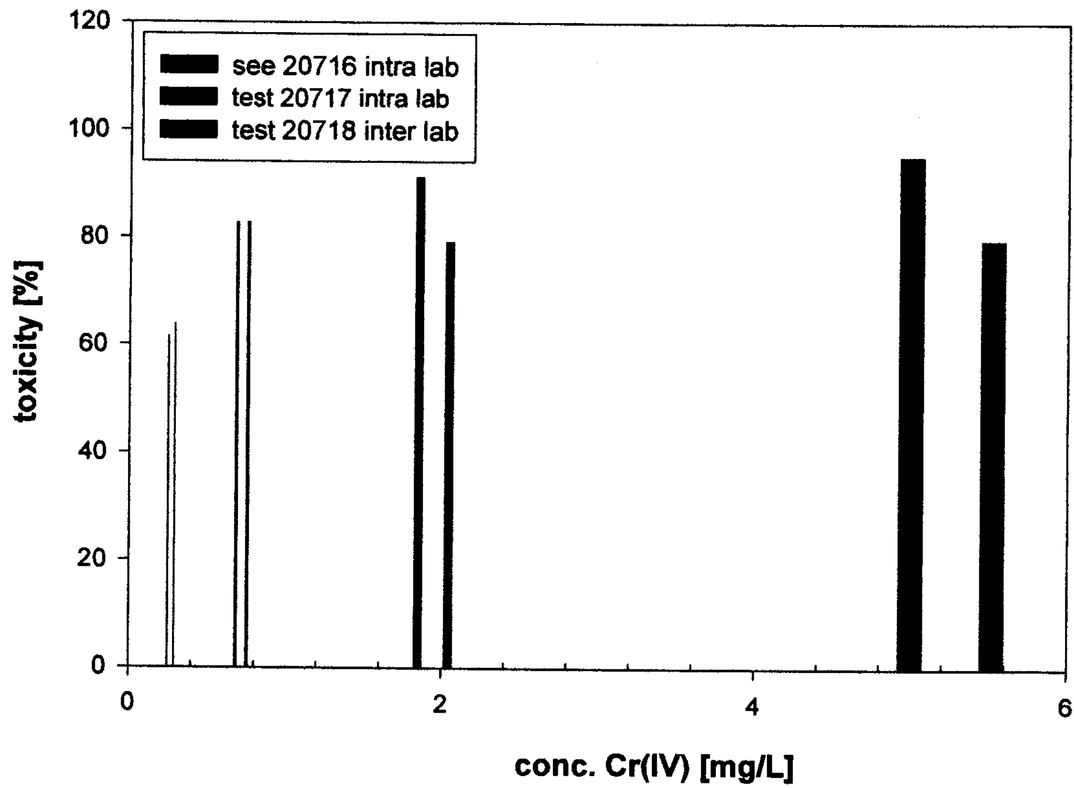


4-Nitroquinoline-N-oxide in A. bidest.

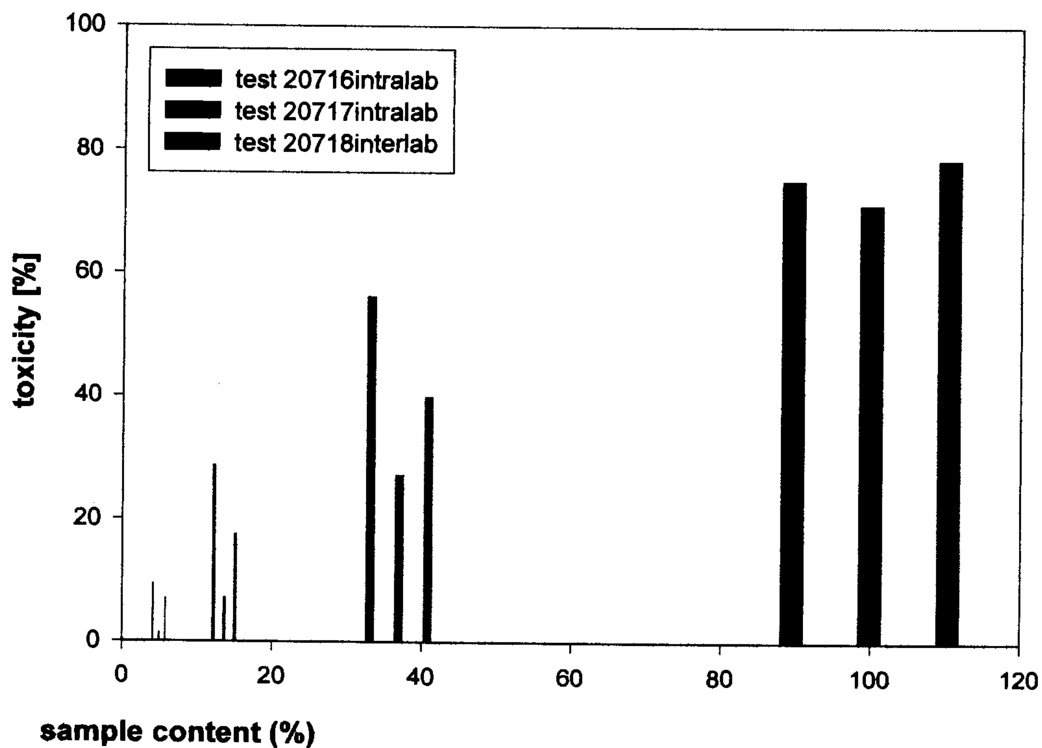
Weaver Human Cell Test validation experiment sample 3: potassium-dichromate CrVI



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

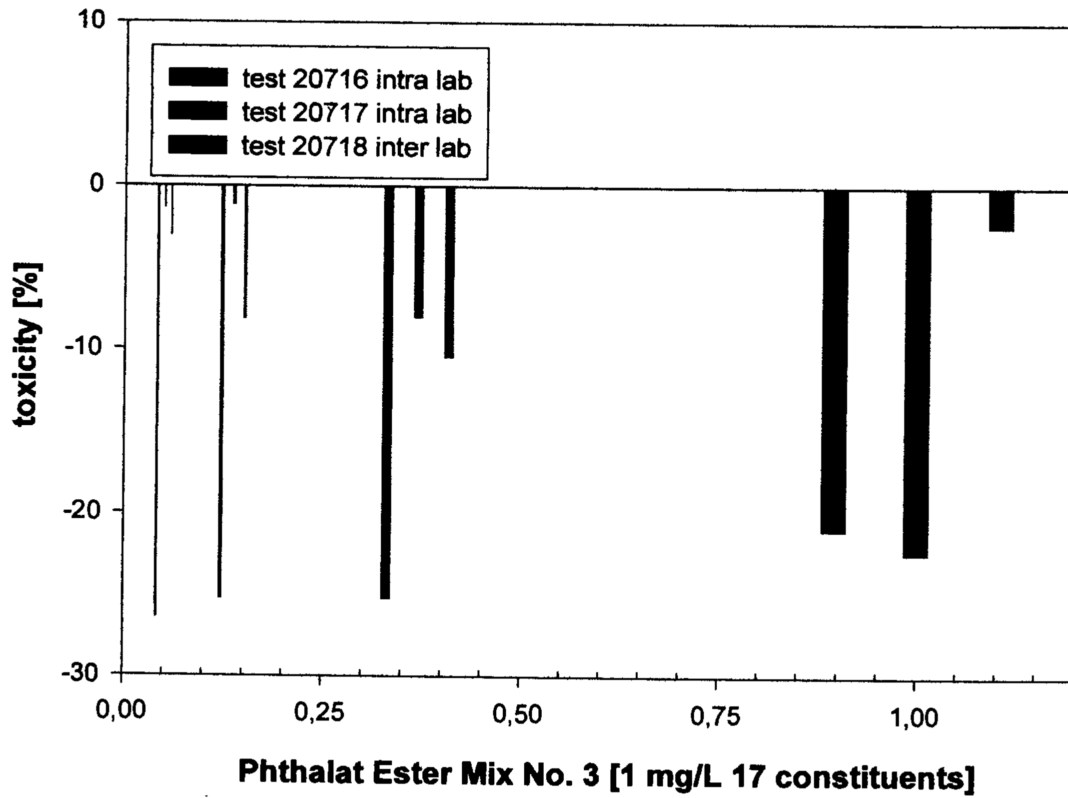


Weaver Human Cell Test validation experiment sample 4 / Industrial Station / sediment fraction F5



sample content (%)

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


Weaver Human Cell Test validation experiment
sample 5: Phthalate Ester Mix No. 3 (Ehrenstorfer)
test 20716 intra lab
test 20717 intra lab
test 20718 inter lab
toxicity [%]
10
0
-10
-20
-30
0,00
0,25
0,50
0,75
1,00
Phthalat Ester Mix No. 3 [1 mg/L 17 constituents]