Surface water toxicity assessment by ecotoxicological and *in vitro* toxicologocical assays

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Abstract

Environmental pollution is an ever-growing problem in today's consumer world. Disposal of chemicals and their unwanted by-products into the natural environment poses significant risks to ecosystems and human health. The receiving natural environment is a sink to chemical mixtures and this triggers a range of potential environmental and health problems. It is therefore imperative to develop rapid reliable techniques for initial screening and establishment of toxic potential and risks. The selection of the catchment study locations in Tasmania (creeks stream and rivers) was the result of recorded observations following rainfall of anomalous oyster population mortality and adverse human health in the geographical catchment area. A biological impact was identified in the study area but the casual agents and environmental triggers were unclear hence the need for *in vitro* toxicology assessment techniques for identifying the triggers and risks associated with exposure to environmental contaminants. The aim of this paper is to present findings of *in vitro* cytotoxicity assays and their potential in assessing contaminated river water toxicity using human cell cultures. The key to the approach adopted was to investigate exposure to the environmental pollutants and generate human toxicity profiles with a high degree of confidence using cell viability and damage as end points. Furthermore, correlation of the toxicity results using human cell cultures as ecological receptors in the study area clearly indicated the usefulness of and sensitivity of human-derived cells in detecting the toxicity of unknown chemical mixtures.

Keywords: water samples, human skin cells, human skin fibroblasts, HepG2, A549, toxicity, cytotoxicity, ecotoxicity, MTS, sea urchin development test, bivalve larval development test.

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1 Introduction

Chemicals represent an integral part of our life and play an important role in promoting our lifestyle. However, the impact of pollutants on human health and the environment is of major concern to individuals and regulators especially with increasing awareness as a result of pesticide use from the 1960s [1].

This paper investigates the impacts of unknown chemical mixtures in a natural environment by using eco-toxicological tests combined with modified *in vitro* assays to measure the toxic potential of environmental pollutants. The selection of biological tests rather than chemical identification as a starting point is based on the Australian and New Zealand Environment and Conservation Council (ANZECC) environmental guidelines for conservation and sustainable development, which recognises a hierarchy of evidence, based assessment. This assessment ranges from most powerful (biological effects) to least powerful (chemical identification and measurement) [2]. Furthermore, toxicity assessment of chemical mixtures is a challenging task and requires an understanding of interactions between chemicals and characteristics of the chemicals present [3]. Unfortunately in ecosystems, it is not always possible to identify all individual chemicals in a mixture and their interactions [4], hence the need for a rapid and accurate screening technique to assess samples toxicity. Natural systems are complex systems and it is virtually impossible to understand the full mechanism of migration, accumulation and biotransformation of chemicals introduced in such open systems, but it is hoped that the results presented here could elucidate the cumulative toxic potential of mixtures [4].

This study investigated runoffs downstream from a timber plantation in Tasmania into an otherwise pristine natural environment and their toxic potential. Collected surface samples (using the skimmer box method) toxicity assessment was undertaken using ecological indicators such as the bivalve larval development test, daphnid immobilization tests and sea urchin development tests. The early development tests are recognized as a potent model for cell cycle analysis [5, 6] and toxicological studies [7]. Furthermore the samples were tested looking at cell death as an end point using human-derived cell cultures that represents potential target organs (skin, lung and liver) in the human body. These cells by virtue of their location, numbers and ease of growth in culture could be used as possible indicators of cellular damage caused by contaminant exposure [8].

The non-invasive *in vitro* study showed the sensitivity and usefulness of using non-animal models for chemical mixtures toxicity assessment. Human-derived cell use assisted in eliminating uncertainties in data extrapolation (animals to humans) and the use of animals in testing [8]. Furthermore, the techniques used are rapid and reproducible as they generate accurate toxicity profiles within hours of conducting the assays.

2 Material and methods

2.1 Bivalve larval development toxicity test

In this test embryos of the rock oyster *Saccostrea commercialis* were used for toxicity assessment of water samples derived from the catchment area. The larval development tests are commonly used in North America using APHA and ASTM protocols [9]. The development test was undertaken using a serial dilution of the sample provided (100, 50, 25, 12.5 and 6.3%). The eggs were fertilised and introduced into a test vessel (kept at 24°C). The established end point was D-veliger larval development 48 hour post exposure to the test materials. The normal and abnormal developed larvae were counted and subjected to statistical analysis to determine the EC_{50} (concentration causing 50% inhibition in larval development). Artificial and filtered sea waters (ASW, FSW) were used as controls.

2.2 Sea urchin larval development toxicity test

This test involved exposure of fertilised eggs from wild caught *Heliocidaris tuberculata* (Maroubra, NSW, Australia) to water samples from the contaminated area and assessment of normal development to Pluteus stage 72 hours post exposure. The same dilution protocol as the bivalve larval development test was followed. The end points were larval development 72 hour post exposure. EC_{50} values were also established.

2.3 Cladoceran immobilisation toxicity test

This test involved exposing laboratory reared freshwater daphnids *Ceriodaphnia cf dubia* to water samples from the contaminated area. This test is similar to the widely used tests using northern hemisphere daphnids and is based on USEPA methods adopted in 1993. It is a 48 hour acute test with immobilisation as its end point. This test was used to calibrate between human cell lines and animal models

2.4 Human-derived cell cultures

Primary fibroblast cell cultures were derived from human skin biopsies [Children's Hospital Westmead (Australia)] and maintained in short term cell culture. Cells were subcultured as adherent cells in 75 cm^2 tissue culture flasks with 0.2 μm vented seals (Falcon). The culture media consisted of colour free Dulbeco's modified eagle medium (DMEM): RPMI 1640 (1:1) purchased from Sigma Chemicals, supplemented with 5% foetal calf serum (Trace Bioscience), 3% Sigma antibiotics [penicillin (100 U/ml), streptomycin (0.1 mg/ml) and L-glutamine (2 mM)]. The cell lines (HepG2 and lung cells (A549)) were cultured at 37°C at sub-confluence in a humidified incubator set to a mixture of 5% CO_2/95% air. Cell viability was over 95% as measured by tryptan blue dye exclusion.

Confluent cells in log phase of growth were released from the bottom of the culture flask using Trypsin EDTA (Gibco, USA), and then washed three times with cell culture medium.

The seeding density of cells to 96 well plates was previously determined and cells were seeded at a density of 500,000 cells/ml based on the linearity range studies (cell concentration versus absorbance) previously conducted [10].

2.5 Sample preparation

The sample used in the bivalve, daphnid, sea urchin tests and human cells testing consisted of reconstituted fresh samples serially diluted and used directly in the tests. This approach was necessary as previous test established a complete loss of toxicity in 10 days old samples, thus multiple fresh samples were required. Individual samples were calibrated to each other using the daphnid test. The skimmer collected samples were subjected to methanol fractionation, by passing the fresh sample through a C18 column. The C18 columns were later subjected in the lab to methanol extraction. The total volume of each methanol extract was 2 ml. This final volume of 2 ml was reconstituted with water for the bivalve, daphnid and sea urchin tests, and filtered water for the *in vitro* tests prior to conducting testing.

2.6 Cytotoxicity assays

The MTS assay (Promega, USA) was selected for measuring the number of active cells in the culture (based on the lactate dehydrogenase activity in the mitochondria). The MTS assay measuring the conversion of a soluble tetrazolium salt to a formazan product by viable cells [11]. The assay consisted of an MTS solution prepared by mixing a solution of MTS (42 mg MTS powder in 21 ml of DPBS pH 6.0-6.5) with a PMS solution (0.92 mg/ml PMS in DPBS) to the cells to be tested in a ratio of 1:5. The MTS was then incubated with the cells for a period of 2 h at 37°C in the dark. After 2 h, the cellular supernatant absorbance was measured. The amount of reduced Formazan was assessed by measuring the optical density at 492 nm using a Labsystem Multiskan MS plate reader. Data was plotted as a dose response curve exposure versus absorbance reading.

2.7 Statistical analysis

The dose response curves reported were plotted from the experimental data and the background absorbance subtracted from the presented graphical pots. All the data reported was expressed as the mean ± SD of 4 replicated wells. Statistical procedures and graphical analysis were performed using Microsoft Excel software.

3 Results

3.1 Water sample toxicity assessments using sea urchin and oyster development tests (South George)

The toxicity assessment with sea urchin, daphnid and oyster tests was adopted to screen samples obtained from 5 different areas and tributaries along the river

system. The samples from all areas were analysed for toxicity and it was established that South George tributary samples were the most toxic as they contributed to 100% death of the test species used. The other samples obtained from different tributaries significantly reduced ($P<0.05$) number of normal larval but did not match the 100% kill rate observed with the South Georges area samples. The results of the oyster and sea urchin development tests are presented in Figure 1.

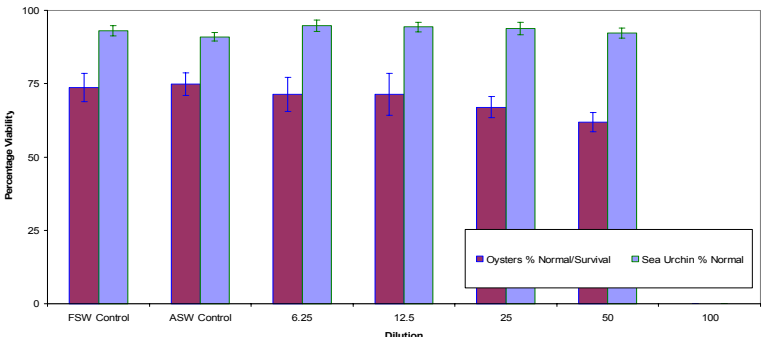

Figure 1: Oyster and sea urchin development test (South George).

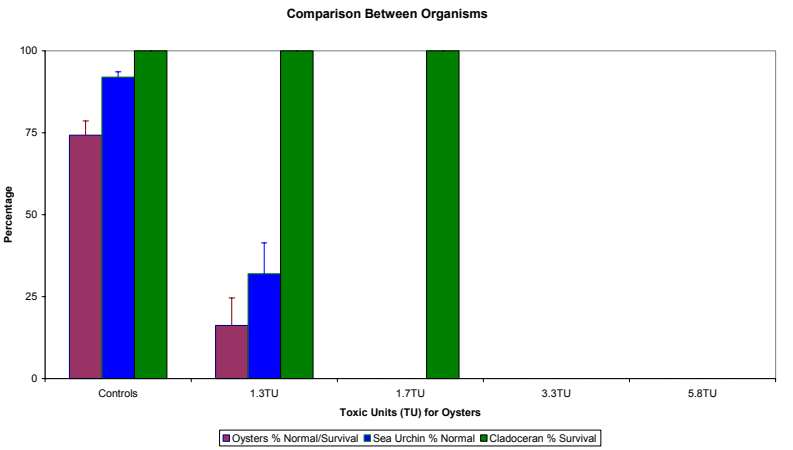

Figure 2: Comparison between animal models against toxic units (TU) for oysters (TU=1/EC50).

Comparison between organisms indicated that daphnids are slightly less sensitive than oysters and sea urchins (Figure 2), however this organism does not require sea salt manipulation and is readily available as it is easily cultured in the laboratory. For these reasons it was decided that daphnids would be used to calibrate the human cell lines and the animal models.

3.2 Water sample cytotoxicity assessments using human skin fibroblasts and MTS assay (South George)

The experimental investigations used cells (skin fibroblasts/HepG2 and lung cells) cultures at a density of 50,000 cells/100 µl exposed to dilution of a concentrated river sample (methanol extraction from C18 column concentrates) with damage levels assessed by MTS assay 24 h post exposure (Figure 3).

The results are summarized in Table 1.

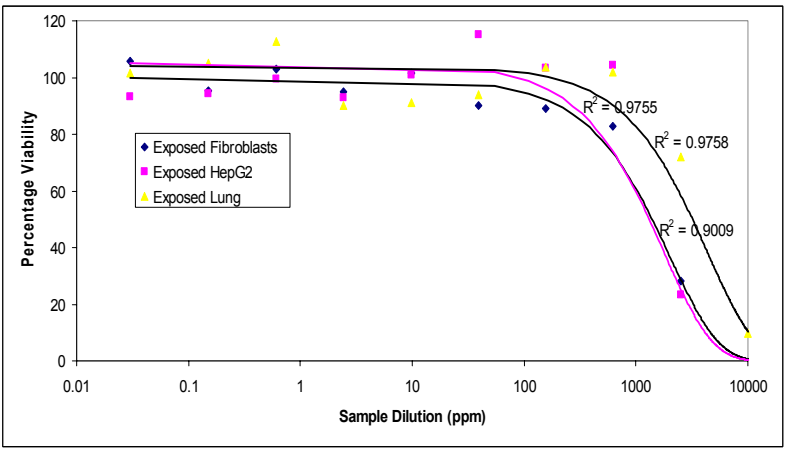

Figure 3: Cytotoxic effects of exposure in human cells as reported using the MTS assay.

Table 1: Calculated toxicity parameters.

Toxicity tests	*in vitro* toxicity data IC_{50} (ppm)	EC_{50} (ppm) from Dapnid EC_{50} of 3030 ppm
Fibroblasts	1809.88±466	-
HepG2	1100.23± 95	-
Lung (A549)	3465.7± 0.017	-
Oyster Development	-	1515 ± 350
Sea Urchin Development	-	1614± 50

4 Discussion

Testing of environmental samples can be undertaken at any biological level, from the molecular to the whole organism [12]. The majority of pollutants will initiate the toxic response at the molecular level and this is later translated at the physiological level though development and presence of diseases [13]. This phenomenon is further compounded by accumulation, transformation and

biomagnifications and transport of pollutants in any ecosystem [14]. The responses can be measured at any of the biological levels and we selected to measure the development/cellular response to damage so as to understand the mechanisms of toxicity triggered by the pollutants. The ecological versus *in vitro* testing was adopted to assess surface water toxicity of a receiving ecological environment in Tasmania contaminated with runoff from a timber plantation. The tests conducted clearly indicated a potential toxic threat to the development and survival of ecological communities as revealed by the sea urchin and oyster tests data (Figure 1). The two tests generated similar toxicity profiles. The data showed the harmful effects of exposure to undiluted samples from the river system contributed to 100% death of the test organisms. Furthermore careful examination of Figure 1 indicated that the observed lethal effects recorded by the development tests are lost upon dilution of the sample. Diluting sample to 50% will lead to non significant variation in viability as established by the student t-test ($P<0.05$). The observed development disruption supports the theory of chemical response and agrees with published literature on herbicide products disrupting cell division in sea urchin embryos [15].

The establishment of a biological impact as determined by the tests conducted does not establish human toxicity, as the mechanisms of toxicity could be different. Therefore the need to conduct human toxicity testing to establish whether the same toxic profile are observed in humans and whether any association could be established between the influence of the pollutant on proliferation of ecological community and human risk exposure. The human toxicity data was generated by in *vitro methods* investigating a number of potential exposures pathways via skin lung and liver. Cell cultures using fibroblasts (skin), lung cells and HepG2 (liver) were used and cellular activity/ death was established as endpoint as measured by the MTS assay. The data reported in Figure 3 clearly indicated reduction of cell viability in a dose dependent manner as a response to high concentrations of contaminated water samples. Cell viability in the three cell types used was reduced to 50% at concentrations of 1100–3465 ppm as indicated in Table 1. The data also indicates a higher sensitivity by the test assay (by comparison to the ecotoxicological tests performed previously) in recording toxic exposure with detection of toxicity at doses as low as 1000 ppm dilution. The three cell types used were equally affected and displayed a similar dose response curve. HepG2 cells were most sensitive to damage, followed by fibroblasts, with lung cells the least sensitive (Table 1). The observed toxicity profile are similar to those observed using the ecological test, with undiluted test sample being very toxic and killing 100% of the cell population, but the *in vitro* tests displayed higher sensitivity in detecting the early toxic effects with lower dilutions affecting cell viability.

The combination of ecological and human cell testing appeared to be appropriate in detecting environmental pollutants toxicity from an ecological and human perspective (comparable IC_{50} and EC_{50} as shown in Table 1). A better correlation could perhaps be established by plotting the sea urchin and human fibroblasts per cent viability data together (Figure 4). The data indicated similar

toxicity detection with higher sensitivity of human cell cultures in detecting toxic potential of lower dilutions than the oyster development test (Figure 4a). Furthermore if the recorded viabilities are correlated (R^2=0.75) there is a good agreement between the two techniques in detecting toxic damage (Figure 4b).

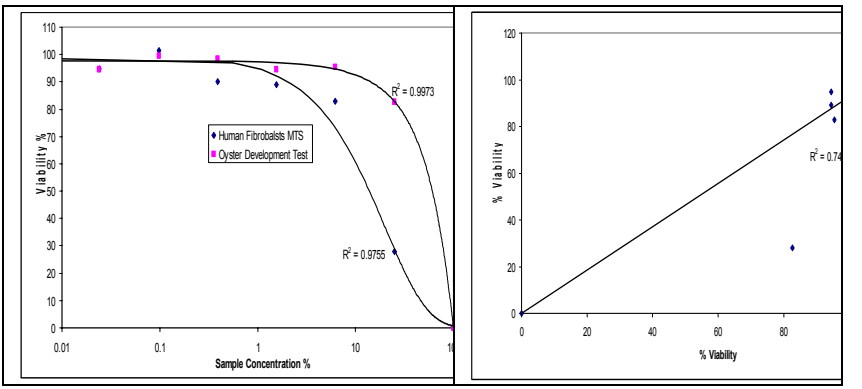

Figure 4: Viability versus exposure using human skin fibroblasts and oyster development test.

It is concluded from the data presented that there is a good correlation between ecological and *in vitro* toxicological tests with respect to the contaminated area we investigated (Table 1). This agrees with published literature where it is stated that there is a good agreement between animal toxicity tests (LD_{50}) and *in vitro* cytotoxicity assays (IC_{50}) for a wide range of chemicals [16–19]. The observed toxicity could be resulting from a toxin (with a half life of a few days) and/or a mixture of chemicals and further studies including chemical analysis will be required to identify and quantify the compounds triggering the observed toxic response. It would also be beneficial to investigate the effects on other key ecological receptors in the area. The link to human impacts was also explored and the results reported by the human cell tests performed clearly indicate acute toxic effect. Hence there is an urgent need to further investigate human and animal diseases especially where a distinctive link could be established between the symptoms and exposure in the contaminated geographical area with a focus on chronic exposure and its impacts on the exposed populations. Future studies are being conducted looking at immunotoxic/genotoxic effect of exposure to the contaminants.

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