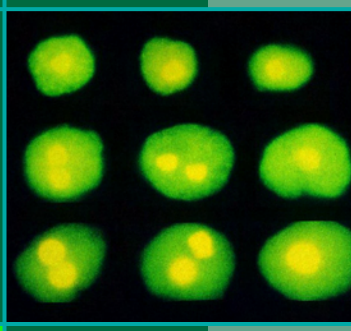
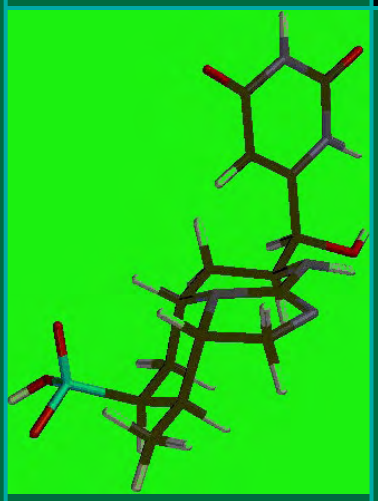
The Cooperative Research Centre for
Water Quality and Treatment

Screening Assays for Water-borne Toxicants





Research Report 60

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Screening Assays for Water-borne Toxicants

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Research Report No 60

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FOREWORD

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EXECUTIVE SUMMARY

The aim of this project was to develop a panel of screening assays that have potential to replace the standard mouse bioassay for detecting toxicants in water. The focus was placed on developing assays to detect the cyanotoxins relevant to Australian drinking water sources; cylindrospermopsins (CYN), microcystins (MCYST), and saxitoxins (STX).

Cylindrospermopsin

Initial cytotoxicity studies showed that CYN produced toxic effects in a range of cell lines including those derived from the liver (HepG2, C3A), gastrointestinal tract (HCT-8, LLC-MK2, HCT-8) and kidneys (Vero). Cells were incubated for 7 days following exposure to 0.25 µM, 2.5 µM or 25 µM toxin. Progressive loss of viability to death of nearly all cells was seen over 1-3 day incubation in cells treated with the two highest concentrations of toxin. The liver derived cell lines were most sensitive to CYN, which was apparent at the lowest concentration tested (0.25µM). The protracted nature of CYN's toxic effects was also evident. Although cytotoxicity was not detected immediately following 1-6 hr exposure to CYN, toxic effects were induced at 24 hr even when the cell had only been exposed for these short time periods. The results were most prominent in the C3A liver cell line. The sensitivity of the liver cells to CYN in all cytotoxicity experiments conducted supports the idea that metabolites produced by liver cytochrome P450 (CYP450) enzymes are involved in the toxicity process. The protracted cytotoxicity induced by CYN means detection in less than 24 hr is not possible when cytotoxicity is used as the assay endpoint.

To further investigate rapid assays for CYN with detectable endpoints within a few hours (same day), two assay formats were evaluated based on CYN's mechanism of action; protein synthesis inhibition. These included (1) use of reticulocyte lysates as a model for cell-free protein synthesis; and (2) generation of cell-lines expressing green fluorescent protein (GFP) as a cellular assay for protein synthesis.

Using commercially available reticulocyte lysate, the cell-free protein synthesis inhibition assay for CYN was established. The IC_{50} for CYN was 110 nM, and unknown samples could be quantified over a 5X concentration range, completing the assay within ~4 hrs. The assay set-up was then modified to lower the number of ribosomes and associated translation components present. Reticulocyte lysates with 7- and 10-fold reduced ribosome content were prepared and found to improve the assay sensitivity to CYN ≥10-fold. Low nM (<10 nM) concentrations of CYN could be detected in this assay system. This is close to 1 µg/L (2.4 nM) CYN that has been suggested as a drinking water guideline level. It was also noted that the slope of the concentration-response curve was altered in the diluted lysates, potentially broadening the quantifiable concentration range. Further assay optimisation should be able to lower the detection limit further, but it was not possible to investigate this within the limits of the current project.

C3A and Vero cell lines were stably transfected with GFP to establish a cellular assay for protein synthesis. Importantly, the GFP protein used had a short half-life (1.5- 2 hrs), to allow rapid monitoring for toxin-induced changes in protein synthesis. Most work on the GFP assay was carried out using Vero-GFP (kidney) cells, as they were more amenable to flow cytometric analysis compared with the C3A-GFP (liver) cells. Treatment of Vero-GFP cells with CYN (10µM) or other protein synthesis inhibitors rapidly reduced GFP levels, demonstrating that detection of toxin was possible within 4 hrs. Preliminary results indicated that the assay was more sensitive to the inhibitor emetine compared with CYN, despite knowledge that CYN was a more potent inhibitor in a cell-free assay. This may reflect differences in cellular uptake of the toxins. An additional CRC project (2.0.1.2.2.3) was established to further optimise this GFP assay for CYN.

Microcystin

For MCYST two analogues, MCYST-LR and MCYST-LA, were tested for cytotoxicity. Of all cell lines tested, Vero cells were the only ones that showed sensitivity to MCYST-LR with 50 µM toxin reducing cell viability to 10% in 6-7 days. In contrast all cell lines (HepG2, C3A, LLC-MK2, HCT-8 and Vero), were susceptible to 50 µM MCYST-LA treatment, causing near complete cell death in 6-7 days. Cells were not sensitive to lower concentrations (0.5 µM and 5 µM) of the toxins. The difference in cytotoxicity results with the two MCYST analogues is likely to be due to differences in uptake. The

more hydrophobic analogue MCYST-LA may be able to transfer across the lipid membrane to a greater extent that MCYST-LR. However, overall the results showed that these cells have limited sensitivity to the toxins. This is thought to be due to the requirement of OATP transport proteins, not expressed by these cell lines, for cellular uptake of MCYST. Due to low sensitivity and extended incubation times required for detection it was considered that further development of a cell based assay for MCYST was not warranted.

Saxitoxin

A cell-based assay for STX known as the Neuroblastoma assay has previously been extensively validated for use with shellfish extracts, and was also recently validated for use with cyanobacterial extracts. The standard assay set up is not rapid - the endpoint is measured 24 hr after treatment. Neuro-2A (nerve) cells are treated with veratridine and ouabain to open the sodium channels and allow a lethal influx of sodium into the cells. The addition of STX blocks the sodium influx and allows the cells to survive at the 24 hr time point. In this study we investigated more rapid endpoints based on detecting changes in cell membrane potential that occur following treatment with toxins acting on sodium channels. Results showed that the fluorescent dye $DiBAC_4(3)$ could be used with Neuro-2A cells to rapidly detect toxin induced changes in membrane potential. Cells were analysed by flow cytometry within ~4 hrs of treatment. The assay worked well for detecting changes in membrane potential induced by veratridine alone or in combination with ouabain. Initial results showed that STX could reduce the veratridine-induced effects on membrane potential, although the extent of protection afforded by STX was variable over the experiments carried out. Given the rapid nature of the assay further work may be warranted to optimise the assay set-up.

Finally, a range of invertebrate bioassays were also evaluated as rapid toxicity screening assays. The results from these studies are reported separately as part of a CRC PhD student thesis being written by David Ruebhart.

Summary and Conclusions

The aim of this project was to develop a range of screening assays that can be used to detect toxicants in water. The focus was on detection of the cyanotoxins, CYN, MCYST and saxitoxin. The research outcomes identified a number of assays that may have potential use in a screening capacity.

For CYN, cell-lines expressing GFP were created to measure cellular protein synthesis and these were demonstrated to respond to the inhibitory effects of the toxin within 4 hrs of exposure. This greatly improves the turn around time over the use of cytotoxic endpoints for CYN that require at least 24 hr incubation. Optimisation of this assay is planned in CRC project 2.0.1.2.2.3 and will concentrate on improving sensitivity to CYN.

Also for CYN, modification of the cell-free (reticulocyte lysate) protein synthesis assay enabled detection of much lower concentrations of the toxin than had previously been achieved. Detection of concentrations <10 nM (equiv to < 4 µg/L) was possible, close to the suggested 1 µg/L drinking water guideline level for CYN. The improved sensitivity achieved in this assay has potential practical application for detection of CYN in source water samples without sample concentration. Validation with different water types will be required.

Given the limited effects of MYCST in cell lines tested, rapid cell based assays for detection of this toxin type is not possible. As demonstrated by other research groups, modification of the cells to express organic anion transport proteins is required to facilitate uptake of the toxin.

For saxitoxin, a rapid cell-based assay for saxitoxin utilizing changes in membrane potential has been previously established in other laboratories, and so we attempted to develop a similar assay. However, due to lack of time we were not able to reproduce the published results and so further research work would be required to fully evaluate the potential of our own assay system.

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ABBREVIATIONS

ANOVA	Analysis of Variance
ADWG	Australian Drinking Water Guidelines
ATCC	American Type Culture Collection
CYN	Cylindrospermopsin
CHEX	Cycloheximide
DMSO	Dimethylsulfoxide
FL1	Fluorescence Channel 1
FSC	Forward Scatter
GFP	Green Fluorescent Protein
MCYST	Microcystin
MEM	Minimal Essential Medium
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
NHMRC	National Health and Medical Research Council (Australia)
OATP	Organic Anion Transporter Protein
PBS	Phosphate Buffered Saline
PSP	Paralytic Shellfish Poison
STX	Saxitoxin
SSC	Side Scatter
WHO	World Health Organisation

1 INTRODUCTION

1.1 Aim

The aim of this project was to develop a panel of screening assays that have potential to replace the standard mouse bioassay for detecting toxicants in water. While emphasis was placed on developing assays to detect the cyanotoxins, the assays were designed to have the potential to pick up other possible toxic contaminants in potable water.

1.2 Background

The cyanobacterial toxins are just one group of water toxicants that are recognised at the regulatory level, other classes of toxins include pesticides, heavy metals, and disinfection by-products (Refer to ADWG, 2004). Following evaluation of health related data the World Health Organisation recommended a guideline level of 1 µg/L for the cyanotoxin microcystin-LR in drinking water (WHO, 2003). The National Health and Medical Research Council (NHMRC) has also reviewed the data as part of the Australian Drinking Water Guidelines (ADWG, 2004) and recommend that the concentration of total microcystins does not exceed of 1.3 µg/L (expressed as microcystin-LR equivalents). Although there is insufficient data available for the NHMRC to set guideline values for other cyanobacterial toxins, cylindrospermopsin, nodularin and saxitoxin are also recognised to pose a health risk and need to be monitored. Some water authorities use the health alert levels as a guide, 10 µg/L nodularin and 3 µg/L saxitoxin (STX equivalents) (Fitzgerald *et al.*, 1999) or 1µg/L as proposed for cylindrospermopsin (Humpage and Falconer, 2002; Humpage and Falconer, 2003).

The *in vivo* mouse bioassay has been used for many years in the water industry to determine toxicity of a cyanobacterial bloom. Although the method can provide an indication of toxicity in an uncharacterised bloom sample, it has limited sensitivity and specificity and is subject to ethical concerns. It cannot be used with low concentrations of toxins. Furthermore, when cylindrospermopsin is suspected to be present, the test is not rapid - observations must be carried out for 7 days due to the protracted nature of its toxicity. From an ethical viewpoint it is now widely recognised in the literature that the mouse bioassay is unacceptable (Negri and Llewellyn, 1998; Flanagan *et al.*, 2001; Usleber *et al.*, 2001). It has been banned for routine toxicity testing in Europe and there is strong pressure from the NHMRC Animal Welfare Committee to follow. The water industry therefore needs to act proactively to validate alternative assay systems to detect the toxins.

Over the last decade a number of alternative methods have been developed for the detection of cyanobacterial toxins in water samples. These include a range of chemical analytical methods, enzyme inhibition assay, immunological detection, invertebrate bioassays, cell culture assays and PCR based detection of toxin genes. This project assessed both cell based assays and invertebrate bioassays as toxicity screening tools as alternatives to the mouse bioassay. Only the cell based assays are reported here.

The cyanobacterial toxins that form the basis of the project are the microcystins (hepatotoxins), cylindrospermopsins (cytotoxins) and the saxitoxins (neurotoxins). The nature of the toxins, their cellular uptake and mechanism of action require consideration when designing the assays. The toxicity assays are designed to detect unknown cytotoxic compounds as well as the specific biochemical events diagnostic for known toxins / toxin classes. This approach allows unknowns to be detected in addition to those toxins suspected to be present based on identification of cyanobacterial species in the water body. This is an important consideration, since new cyanobacterial toxins continue to be found (Baker *et al.*, 2001). Assays are designed for rapid detection of toxicity in water samples. Analytical chemical tests can then be used more efficiently by focussing on samples which give a positive result in the toxicity screen.

Microcystins

The hepatotoxic microcystins (MCYSTs) are cyclic peptides, molecular weight 800-1,100 Daltons. These toxins are produced in Australia mainly by *Microcystis aeruginosa* (Baker and Humpage, 1994). Over 60 different structural variants have been identified, with MCYST-LR the most common. The LD_{50} for MCYST-LR is 50 µg/kg bodyweight in mice by intraperitoneal injection. See Figure 1 for the toxin structure. MCYST-LR was found to be a potent inhibitor of protein phosphatases 1 and 2A (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990). The MCYSTs are not directly cell permeant due to their relatively large size and hydrophilic nature, thus their specificity for the liver is due to active uptake by specific transporter proteins expressed by the liver. The organic anion transporting polypeptides (OATP) have recently been shown to mediate the uptake of microcystin (Fischer *et al.*, 2005) Once inside the cell, inhibition of protein phosphatases causes unregulated phosphorylation of the cell cytoskeleton, leading to its collapse. Liver cells lose contact with each other and blood pools in the spaces which are created, leading to death from hypovolaemic shock. At lower concentrations, more subtle effects on regulated phosphorylation are believed to underlie the tumour promoting effects of microcystin (IARC, 2006).

Cell-free assays based on inhibition of these phosphatases have been proposed (An and Carmichael, 1994; Heresztyn and Nicholson, 2001a; Heresztyn and Nicholson, 2001b) although concerns have been raised about the sensitivity to other co-occurring phosphatases that may interfere with this assay (Sim and Mudge, 1993). Most immortalised cell-lines are relatively insensitive to microcystin because they lack the OATP (Eriksson *et al.*, 1987). Recently microcystin sensitive cell lines have been developed by stable transfection of the OATP transporters that allow cellular uptake (Fischer *et al.*, 2007; Monks *et al.*, 2007) although these cells are not commercially available.

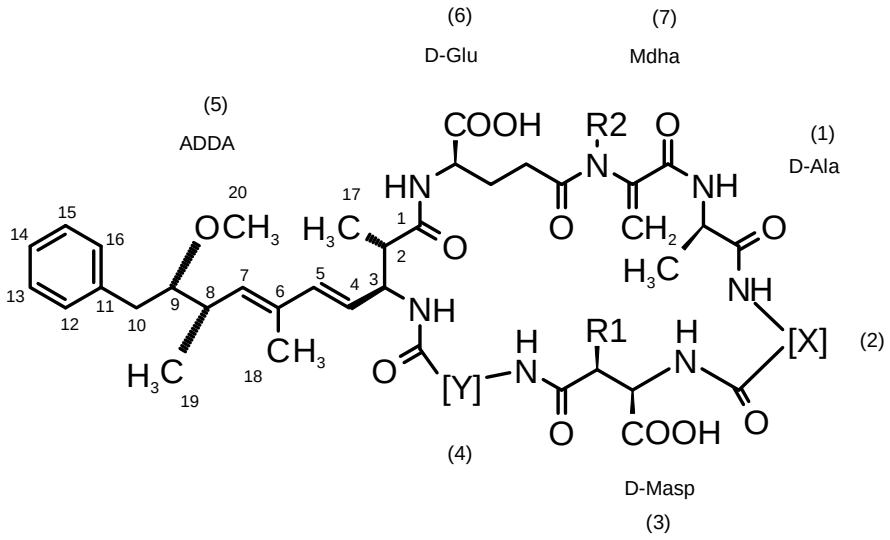


Figure 1. General structure for the microcystins. The variable amino acids are represented by X and Y. In Microcystin-LR, X = L-Leucine (L) and Y=L-Arginine (R), while for microcystin-LA Y = Alanine.

Cylindrospermopsin

Cylindrospermopsin (CYN) is an alkaloid toxin with molecular weight 415 Daltons (Figure 2). It is produced in Australia mainly by *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* (Hawkins et al., 1997; Shaw et al., 1999). Following poisoning, CYN is primarily hepatotoxic, and also causes extrahepatic lesions of variable location and severity including in the kidneys, gastrointestinal tract, lungs and thymus (Terao *et al.*, 1994; Hawkins *et al.*, 1997; Falconer *et al.*, 1999). The LD_{50} in mice is 2 mg/kg at 24 hr and 0.2 mg/kg at 5-6 days by intraperitoneal injection (Ohtani *et al.*, 1992). CYN is a potent inhibitor of protein synthesis (Terao *et al.*, 1994). In primary hepatocytes the effects on protein synthesis are an early indicator of exposure to CYN and occur at subtoxic concentrations and well before the onset of toxicity at higher concentrations (Froscio *et al.*, 2003). The mechanism of

CYN uptake is not clear, both passive diffusion and involvement of the bile acid transport system have been suggested (Chong *et al.*, 2002).

The cell-free protein synthesis inhibition assay has been used to quantify the amount of CYN present in cyanobacterial samples (Froscio *et al.*, 2001), although the sensitivity is limited to concentrated samples (>200 µg/L CYN). Cell based cytotoxicity endpoints can also be used to determine the presence of CYN. Primary hepatocytes have been shown to be a sensitive model for exploring cytotoxic events induced following CYN exposure (Runnegar *et al.*, 1994; Runnegar *et al.*, 1995; Froscio *et al.*, 2001; Humpage *et al.*, 2005). CYN also shows cytotoxic effects in a range of immortalised cell lines (Bain *et al.*, 2007; Neumann *et al.*, 2007), which are more suitable for development of a screening assay.

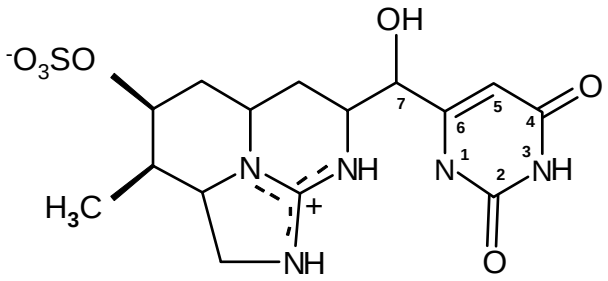


Figure 2. Structure of cylindrospermopsin.

Saxitoxins

The saxitoxins are a group of alkaloid neurotoxins which include a range of hydroxylated and sulphated analogues (Figure 3). They were first described in cyanobacteria as aphantoxins and subsequently shown to be identical to the paralytic shellfish poisons (PSPs) produced by marine dinoflagellate species (Alam *et al.*, 1978; Ikawa *et al.*, 1982). In Australia they are produced by *Anabaena circinalis* (Humpage *et al.*, 1994). They are fast acting neurotoxins that inhibit nerve conduction by blocking sodium channels. The LD_{50} of saxitoxin is 10 µg kg-1 (administered intraperitoneally to mice). The toxicity of related analogues varies approximately 100-fold (Humpage *et al.*, 1994). A cell-based assay for these toxins has been extensively validated for use with shellfish extracts, and was recently also validated for use with extracts of *A. circinalis* (Humpage *et al.*, 2007).

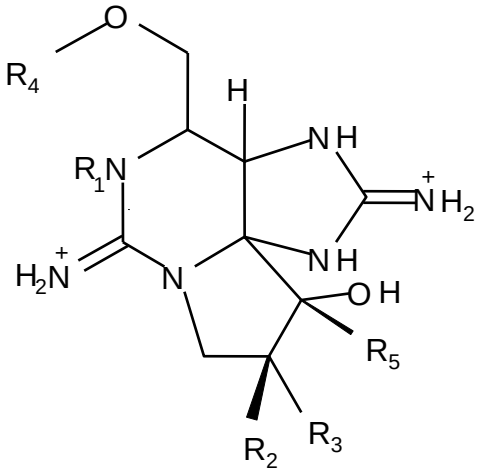


Figure 3. General structure of the saxitoxins. The variable chemical groups are indicated. R_1 = H or OH, R_2 = H or SO_3^-, R4 = H, $CONH_2$ or $CONHSO_3^-$ and R5 = H or OH.

Cyanotoxin assays based on lower trophic level organisms.

A number of lower trophic level organisms have been investigated as bioassays for the detection of cyanotoxins. Most analyses have been carried out in Europe where the focus has been directed towards detecting the MCYSTs. These tests have used fruit flies (Swoboda *et al.*, 1994), house flies (Ross *et al.*, 1996), locusts (McElhiney *et al.*, 1988), invertebrates such as *Daphnia* or *Artemia* (Lawton *et al.*, 1994), bacteria such as *Photobacterium phosphereum* (Microtox test) or *Serratia marcescens* (Lawton *et al.*, 1994), or the larvae of a freshwater crustacean *Thamnocephalus platyurus* (Thamnotox kit; (Torokne, 1999). The *Daphnia*, *Artemia* and *Thamnocephalus* based assays have been shown to produce results that correlate with the known cyanotoxin contents of the test samples. Of these the *Artemia* and *Thamnocephalus* based tests appear to be the most easily implemented. Both of these microbiotests have also been used for analysis of cylindrospermopsin (Falconer, 2005)

1.3 Methods used in this project

The assay methods used in this study included bioassays using whole organisms of lower trophic levels such as daphnia, screening assays using mammalian cell lines, or tests using mammalian cell extracts. The use of these types of assay format was designed to allow detection of a wide range of compounds. The bioassays were included to allow detection of compounds with complex modes of action that become apparent at the level of the organism. Use of cell culture and cell derived extracts allowed the development of more sensitive and specific assays in mammalian, including human, derived systems.

1.3.1 Alternative bioassays using lower trophic level organisms

A range of invertebrate and microbial bioassays that may present alternatives to the mouse bioassay for the detection of cyanotoxins in water supplies were evaluated in this project. This evaluation formed a CRC PhD project by David Ruebhart, Griffith University, Queensland. The three main components of this PhD work included: 1) *Artemia salina* (brine shrimp) nauplii bioassay, 2) insect bioassay e.g. *Nauphoeta cinera* and 3) a range of assays using microbial species e.g. *Photobacterium leiognathi*. Assays were tested for sensitivity to purified toxin and cyanobacterial extracts. Results are reported in CRC PhD Student Thesis, David Ruebhart - *Alternative Bioassays for the Detection of Cyanotoxins*, Griffith University, Queensland, Australia.

1.3.2 *In vitro* assays using mammalian cell lines or cell extracts

A number of cell lines were chosen for assay development. These were selected to be representative of different tissue types and cover the major organs targeted by the cyanobacterial toxins. Cell lines derived from liver (HepG2, C3A), gastrointestinal tract (HCT-8), kidneys (Vero) and nervous system (Neuro-2A) were included. The first aim was to screen the range of cell lines for sensitivity to the cyanobacterial toxins, using a cytotoxicity endpoint. Cell lines responsive to toxin(s) were then chosen for development of assays with biochemical endpoints diagnostic for the presence of specific toxins or toxin classes. In this project rapid assays for CYN and saxitoxin were developed.

Cytotoxicity endpoint

Cell lines were screened for sensitivity to CYN, MCYST-LR and MCYST-LA. The MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess cytotoxicity following toxin exposure. In this assay, the mitochondrial dehydrogenase activity of viable cells converts the MTT salt to a coloured formazan product. The extent of formazan production relies on the amount and activity of viable cells present. Therefore a decrease in amount formed indicates the degree of cytotoxicity caused by the test material.

Rapid assays: cylindrospermopsin

Cylindrospermopsin (CYN) is a potent inhibitor of protein synthesis in cell lysates (Terao *et al.*, 1994) and this activity can be used to readily quantify the amount of toxin present in a sample (Froscio *et al.*, 2001). Reticulocyte lysates were used to establish a protein synthesis inhibition assay for CYN at the Australian Water Quality Centre (AWQC). This was established for use as a comparative CYN detection method for new cell culture-based assays under development. A validated cell-based assay for CYN does not exist, and was a major focus of this project.

In cell culture, CYN's effects on protein synthesis can be used as an early indicator of exposure to the toxin, occurring at subtoxic concentrations and well prior to the onset of toxicity at higher concentrations (Froscio *et al.*, 2003). Based on this mechanism of action a diagnostic cell-based assay for CYN can be established, although standard assays for protein synthesis generally require extensive processing of the cell samples and the use of radio-labelled precursors.

An alternative method that was investigated in this project was to introduce a highly-expressed reporter protein, such as green fluorescent protein (GFP) into the cell and monitor its expression to obtain a measure of protein production. The use of a destabilised GFP protein with high turnover rate is ideal as changes in protein production can be rapidly detected.

A number of researchers have used the expression of luminescent or fluorescent reporter proteins in bacteria as a rapid method for screening samples for inhibitors of prokaryotic protein synthesis or general antibacterial properties (Lampinen *et al.*, 1995; Collins *et al.*, 1998). Mammalian cell cultures stably expressing GFP have been used in a variety of screening assays generally for cytotoxic endpoints (Chen *et al.*, 2005). Here we developed transfected cell-lines that express a destabilised GFP reporter protein to rapidly monitor for changes in protein synthesis, as induced by CYN.

Rapid assays: saxitoxin

The neuroblastoma assay has been previously validated and used for the detection of saxitoxins (Jellett *et al.*, 1992; Jellett *et al.*, 1995; Humpage *et al.*, 2007). In this assay, neuroblastoma cells were challenged with veratridine and ouabain which enhance the sodium ion influx, reduce sodium exit and cause the cell to swell and lyse. The presence of saxitoxin (STX) blocks the sodium channels, protecting the cells from the effects of veratridine and ouabain and allowing the cells to survive. Cell viability is determined by MTT assay after 24 hr treatment. For more rapid detection, recent studies have focussed on the detection of toxin-induced membrane depolarisation using voltage sensitive fluorescent dyes (Louzao *et al.*, 2000; Louzao *et al.*, 2003; Manger *et al.*, 2007). This rapid endpoint was evaluated in this study for detection of saxitoxins.

2 MATERIALS AND METHODS

2.1 Cyanobacterial toxins

Purified CYN was obtained from AWQC prepared as detailed in Humpage *et al.* (2005). Microcystin analogues MCYST-LR and -LA were obtained from Alexis Biochemicals (AXXORA LLC, CA USA) or Sapphire Biosciences (Australia). Saxitoxin and other PSP certified reference material standards were obtained from the Institute of Marine Biosciences (National Research Council, Halifax, Canada).

2.2 Reagents

Minimal Essential Medium (MEM) with Earle's salts, foetal bovine serum, new born calf serum and non-essential amino acids were obtained from Gibco BRL (MD, USA), 10X Medium 199 and RPMI-1640 were obtained from Sigma-Aldrich (MO, USA). Trypan Blue, DMSO and MTT and the media supplements, L-glutamine, sodium bicarbonate, sodium pyruvate, phenol red, gentamycin, penicillin and streptomycin were also obtained from Sigma-Aldrich (MO, USA).

2.3 Cell culture

Cell lines used in this project included C3A, HepG2, Vero, LLC-MK2, HCT-8, and Neuro-2a. All cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Details of cell type are provided in Table 1. Growth media for each of the cell lines is defined below. Cell lines were grown at 37°C, 5% CO_2 and passaged as required using 0.25% trypsin-EDTA solution (Sigma-Aldrich St Louis, MO, USA). Tissue culture flasks (25 cm^2 or 75 cm^2) were obtained from Greiner Bio-one (CELLSTAR, Germany).

C3A Growth Medium

C3A cells were maintained in minimal essential medium (MEM) with Earle's salts supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.06mg/ml streptomycin, 0.1mg/ml penicillin and 10% foetal bovine serum .

HepG2 Growth Medium

HepG2 cells were maintained in MEM with Earle's salts supplemented with 2 mM L-Glutamine, 2.2 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 0.06 mg/ml streptomycin, 0.1mg/ml penicillin, 10% foetal bovine serum.

Table 1. Cell lines used in cytotoxicity assays

Cell line	Origin	Cell type
C3A	ATCC CRL-10741	Human hepatocellular carcinoma
Hep-G2	ATCC HB-8065	Human hepatocellular carcinoma
VERO	ATCC CCL-81	African Green Monkey kidney
LLC-MK2	ATCC CCL-7	Rhesus monkey kidney
HCT-8	ATCC HRT-18	Human ileocecal adenocarcinoma
Neuro-2a	ATCC CCL-131	Mouse neuroblastoma

Vero Growth Medium

Vero cells were maintained in 1X Medium 199 with Earle's salts supplemented with 0.6 mM L-Glutamine, 0.04 mg/ml gentamycin, 20 mM sodium bicarbonate, 0.0005% (w/v) phenol red and 6% new-born calf serum.

LLC-MK2 Growth Medium

LLC-MK2 cells were maintained in MEM, supplemented with 1.85 mM L-Glutamine, 0.1 mM non-essential amino acids and 6% non heat inactivated foetal bovine serum.

HCT-8 Growth Medium

HCT-8 cells were maintained in RPMI-1640 (Sigma-Aldrich, MO, USA) supplemented with 3.9 g/L HEPES, 2 g/L sodium bicarbonate, 0.06 mg/ml streptomycin, 0.1 mg/ml penicillin and 10% heat inactivated foetal bovine serum.

Neuro-2a Growth Medium

Neuro-2a cells were maintained in RPMI-1640 (Sigma-Aldrich, MO, USA) supplemented with 2.0 g/L sodium bicarbonate, 100 mM sodium pyruvate, 0.06 mg/ml streptomycin, 0.1 mg/ml penicillin, 10% new born calf serum.

2.4 Cytotoxicity assays

2.4.1 MTT assay

The MTT assay was based on the procedure described by Mosmann (1983). Immediately prior to use, MTT (5 mg/ml) was dissolved in phosphate buffered saline (PBS), pH 7.4 ± 0.05, and further diluted in cell culture medium to give a 0.7 mg/ml solution. For assay, medium was removed from microplate wells and 60 µl MTT solution (0.7 mg/ml) added. Plates were returned to the incubator (37ºC, 5% CO_2) for 30 mins. MTT solution was then removed and 100 µl of DMSO added to solubilise the reduced formazan product. Plates were place on a shaker for 20 minutes prior to determining the absorbance at 570 nm /reference at 650 nm using a VICTOR^3 plate reader (Perkin Elmer, Wellesley, MA, USA).

2.5 Screening cell lines for sensitivity to cyanotoxins – 7 day studies

The response of C3A, HepG2, Vero, HCT-8, LLC-MK2 cell lines was compared following exposure to CYN, MCYST-LR or MCYST-LA over 7 days. Cells were seeded into 96-well flat bottom plates (Sarstedt, Australia) at a density of 1 x10^4 cells/well for the 1 or 2 day treatments and 5 x 10^3 cells/well for the 3, 6 and 7 day treatments. Following incubation at 37ºC, 5% CO_2 for 24 hrs, cells were treated with CYN (0.25 µM, 2.5 µM, 5 µM), MCYST-LA (0.5 µM, 5 µM, 50 µM) or MCYST-LR (0.5 µM, 5 µM, 50 µM). For each treatment, 6 replicate wells were used. Untreated cells were included as a control. The MTT assay was carried out at the required time points as described above.

2.6 Short term exposure to CYN

Time- and concentration-response to CYN was carried out in C3A and Vero cell lines. A seeding density of 1 x 10^4 cells/well was used. Cells were exposed to CYN (0.01-22 µM) for 1, 2, 4, 6 or 24hr and then the MTT assay carried out as detailed above. Alternatively, to determine the length of exposure time required to induce CYN toxicity, cell were exposed to the toxin for 1, 2, 4, or 6 hr. The medium containing toxin was then removed and cells were washed twice with PBS before adding fresh growth medium. Incubation was continued at 37ºC, 5% CO_2 for 24 hrs MTT assay was carried out as detailed above. A further plate was incubated for 72 hrs for MTT assay.

2.7 Rapid assays: Protein synthesis inhibition assay for CYN in cell extracts

2.7.1 *In vitro* translation

In vitro protein synthesis (translation) was performed using a commercially prepared rabbit reticulocyte lysate obtained from Promega (Madison, WI, USA). Translation mixes (25µl) were prepared on ice comprising 70% rabbit reticulocyte lysate, 20 µM unlabeled amino acids and 1 unit/µl RNasin® ribonuclease inhibitor. Reactions were made up to volume using sterile MilliQ water. Translation was initiated by the addition of 20 µg/ml luciferase mRNA (0.5X recommended) and reaction mixes were incubated at 30ºC, 90 mins. A negative control (no mRNA) was processed with each experiment

2.7.2 Post-translational analysis: Luciferase assay

The extent of protein synthesis was determined by assaying for the luciferase protein using the Luciferase assay system (Promega, Madison, WI, USA). Translation mixes were diluted 10^4 fold in lysis buffer (25 mM tris-phosphate, 2 mM DTT, 2 mM 1,2-diaminocyclohexane N,N,N'N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). Samples (20 µl) were added to 80 µl of luciferase assay reagent (Promega, Madison, WI, USA) in white 96 well microplates (Optiplates, Packard). Samples were read (3 second time delay, 10 second read time) on a luminescence VICTOR^3 microplate reader (Perkin Elmer, Wellesley, MA, USA). Each translation reaction was assayed in duplicate.

2.7.3 Inhibition of protein synthesis by CYN – concentration response

Dilution series of CYN were prepared in MilliQ water on each day of use. For each sample tested, 2.5 µl of toxin were added to a 25 µl translation reaction. The reaction mix was pre-incubated for 5 min at 30ºC in the presence of the toxin prior to initiation of the translation reaction with mRNA. Assay results were reported as percent protein synthesis inhibition (PSI) in toxin treated samples with respect to the control (MilliQ water). The concentration-response curve was modelled to a sigmoidal dose-response (variable slope). Using the modelled data, the linear portion excluded by the 99% CI of the top and bottom plateaus was defined (9.2–92% PSI). The CYN concentration range producing a percent of PSI response of 9.2-92% was calculated (50-240 nM CYN).

2.7.4 Translation mixes with reduced ribosome content

In vitro translation mixes were prepared to contain a reduced number of ribosomes and associated translation components. The standard reaction mix described in Section 2.7.1 above contains a final concentration of 70% reticulocyte lysate. 10% lysate and 6% lysate reaction mixes were prepared in 3.3 mM Hepes-KOH buffer to maintain pH and supplemented as follows: Translation mixes (25 µl) were prepared on ice comprising 3.3 mM Hepes-KOH, pH 7.5, 79 mM potassium acetate, 0.5 mM $MgCl_2$, 2 mM DTT, 250 µM GTP, 20 µM unlabeled amino acids, 1 unit/µl RNasin® ribonuclease inhibitor and 10 or 6% rabbit reticulocyte lysate (Promega, Madison, WI, USA). Reactions were made up to volume using sterile nuclease free water. Translation was initiated by the addition of 10 ng/µl luciferase mRNA and reaction mixes were incubated at 30ºC, 90 mins. A negative control (no mRNA) was processed with each experiment. Samples were diluted 10^2 in lysis buffer before carrying out the luciferase assay as described in Section 2.7.2.

2.8 Rapid assays: GFP reporter protein assay for CYN

2.8.1 Chemicals and reagents

The selection agent G-418 sulphate was obtained from Promega (Madison, WI, USA). Kanamycin, ampicillin and cycloheximide (CHEX) were obtained from Sigma-Aldrich (MO, USA). The pTurboGFP-dest1 vector was obtained from Evrogen (Moscow).

2.8.2 Plasmid propagation

The plasmid was propagated in *E.coli* DH5-α competent cells (Invitrogen) and plasmid DNA isolated from transformants using UltraClean™ Endotoxin-Free Plasmid extraction kit (MOBIO Laboratories, CA, USA). The plasmid was linearised with ApaI prior to transfection experiments.

2.8.3 Stable transfection of cell lines

C3A (1.5 x10^6 cells/plate) and Vero (1 x 10^6 cells/plate) cell lines were seeded onto 100 mm plates and incubated overnight at 37°C, 5% CO_2. The cell monolayer (~40% confluent) was then transfected with 0, 10 or 20 µg linearised vector in a calcium phosphate precipitate (Profection Mammalian Systems, Promega, MO, USA). Growth media was replaced at 24 hr. At 48 hrs cells were passaged by trypsin digest, and cells were dispensed into growth media supplemented with G-418 for selection of resistant colonies. The selection media contained 400µg/ml G-418 for C3A cells and 600 µg/ml G-418 for Vero cells. After 3 weeks colonies were picked and expanded in 24-well plates. GFP expression in clones was screened by fluorescence microscopy. PCR analysis of cell extracts confirmed the presence of the destabilised GFP sequence.

2.8.4 Measurement of GFP

Microplate Reader

Monolayers of Vero-GFP cells or C3A-GFP cells were analysed for GFP fluorescence using a VICTOR^3 microplate reader (Perkin Elmer, Wellesley, MA, USA). Black View-Plates were used and fluorescence was detected a 485 nm (15 nm bandwidth) excitation filter and 535 nm (25 nm bandwidth) emission filter. Cell seeding densities of 1x10^4 – 1x10^5 cells/well were trialled.

Flow Cytometry

Vero-GFP cells (5x10^4 cells /well) were seeded into wells of 48-well plate (500 µl) and grown overnight to produce a monolayer. Cells were treated with CYN, CHEX or emetine and incubation continued for up to 6 hrs. Cells were then trypsinised to produce a single cell suspension, and levels of GFP expression analysed by flow cytometry. Samples were run on a FACSCalibur flow cytometer (BD Biosciences, CA, USA). Excitation was provided by the argon ion laser (488 nm) and data was recorded using CellQuest™ software (BD Bioscience for MacIntosh). The cell population was analysed by forward scatter (FSC), side scatter (SSC), and GFP fluorescence detected with a 530/30 nm bandpass filter (FL1). For each sample 10,000 events were evaluated.

2.9 Rapid assays: Saxitoxin assay

2.9.1 Chemicals and reagents

Veratridine and ouabain were obtained from Sigma-Aldrich (MO, USA) and made up to stock concentrations in DMSO. The bis-oxonal membrane potential-sensitive dye $DiBAC_4$ (3) was obtained from Invitrogen-Molecular Probes Inc, (USA).

2.9.2 $DiBAC_4$(3) membrane potential assay

$DiBAC_4$(3) is a lipophilic and anionic bis-oxonal membrane potential-sensitive dye. Uptake of the dye is restricted to depolarised cells or cells with disrupted cytoplasmic membranes. The fluorescent dye accumulates inside the cells by binding to intracellular membranes and proteins, increasing green fluorescent intensity. To assess the suitability of $DiBAC_4$(3) for detecting membrane depolarisation in Neuro-2A cells, a heat shock treatment was used as a stressor to disrupt the membrane potential. Cells were heat treated at 37°C, 50°C and 60°C for 20 mins, cells were then trypsinised to produce a single cell suspension, and stained with the membrane potential sensitive dye $DiBAC_4$(3) (4µM) before flow cytometric analysis. Samples were run on the FASCalibur flow (BD Biosciences, CA, USA). Excitation was provided by the argon ion laser (488nm) and data was recorded using CellQuest^{Tm} software (BD Bioscience for MacIntosh). The cell population was analysed by forward scatter (FSC) and side scatter (SSC), and the green fluorescence detected with a 530nm/30nm bandpass filter (FL1). For each sample a minimum of 10,000 events were evaluated.

2.9.3 Saxitoxin treatments

Neuro-2a cells (2×10^5 cells/ml) were seeded into a 48-well plate (500µl) and grown overnight to produce a monolayer. Cells were pre-incubated with saxitoxin for 1 hr in fresh media, before incubation for 3 hrs with various concentrations of veratridine and ouabain. The cells were then treated with $DiBAC_4$(3) (4µM) for flow cytometric analysis as described in section 2.9.2.

2.10 Statistical analysis

GraphPad Prism Version 4.02 for Windows (GraphPad Software, SanDiego, CA, USA) was used for all statistical analyses and graphing

3 RESULTS

3.1 Screening cell lines for sensitivity to cyanotoxins – 7 day studies

The response of the cell lines C3A, HepG2, Vero, HCT-8, and LLC-MK2 to CYN, MCYST-LR or MCYST-LA exposure over 7 days was compared.

3.1.1 CYN

For all cell lines, treatment with 2.5 µM or 25 µM CYN showed a reduction in cell viability compared to the control (Figure 4). CYN effects on cell viability were time-dependent, with progressive loss of viability occurring over 1- 3 day incubation resulting in near complete cell death for most cell lines. For HCT-8 cells treated with 2.5 µM CYN, 6-7 day incubation was required to cause maximal effects. Sensitivity to lowest concentration tested, 0.25 µM CYN, varied between the different cell lines. The liver-derived cell lines C3A and HepG2 were most susceptible. At day 7 cell viability was reduced to 30% in C3A cells and 71% in HepG2 cells following treatment with 0.25 µM CYN. In contrast, the viability of Vero, HCT-8 and LLC-MK2 cells was not reduced over 7 days following exposure to 0.25 µM CYN.

3.1.2 MCYST-LR

Of all the cell lines tested, Vero was the only cell line to show sensitivity to MCYST-LR exposure (Figure 5). Incubation with 50 µM MCYST-LR reduced Vero cell viability to 10% after 6-7 days. Lower concentrations of MCYST-LR (0.5 µM and 5 µM) showed no effect. The viability of C3A, HepG2, HCT-8 and LLC-MK2 cell lines was not affected over the 7 day incubation period following exposure to the toxin (Figure 5).

3.1.3 MCYST-LA

All cell lines were susceptible to 50µM MCYST-LA treatment. Reductions in cell viability were evident within 2-3 days resulted in near complete cell death in 6-7 days. Results are shown in Figure 6. Following 5µM MCYST-LA treatment, only Vero cells showed some susceptibility, reducing viability to 75% in 6-7 days. Treatment with 0.5 µM MCYST-LA did not show effects over the 7 day incubation period.

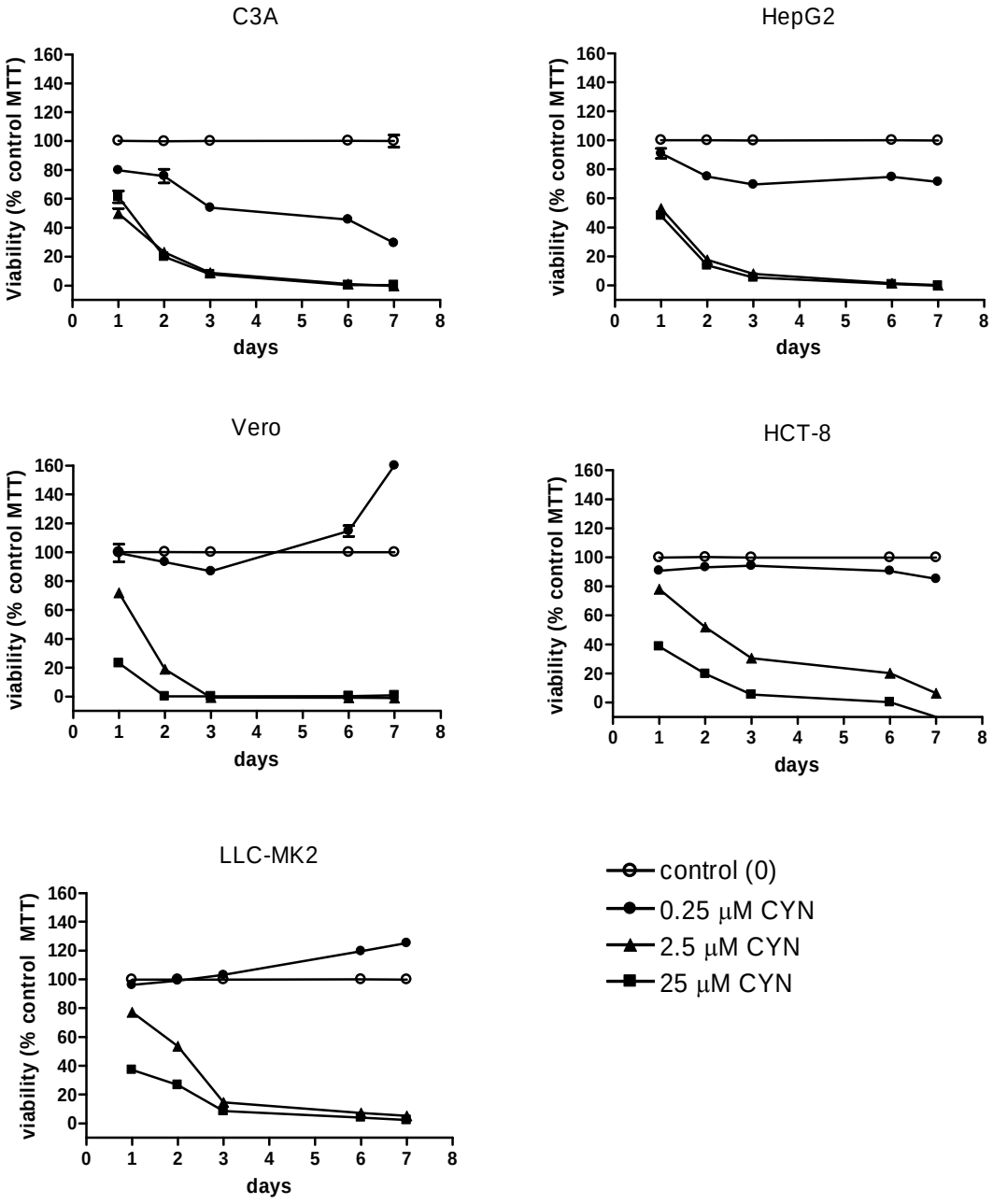


Figure 4. Screening cell lines for sensitivity to CYN. C3A, HepG2, Vero, HCT-8 and LLC-MK2 cell lines were exposed to CYN (0, 0.25 µM, 2.5 µM and 25 µM) for 7 days**.** Data are Mean ± SE of 6 replicates.

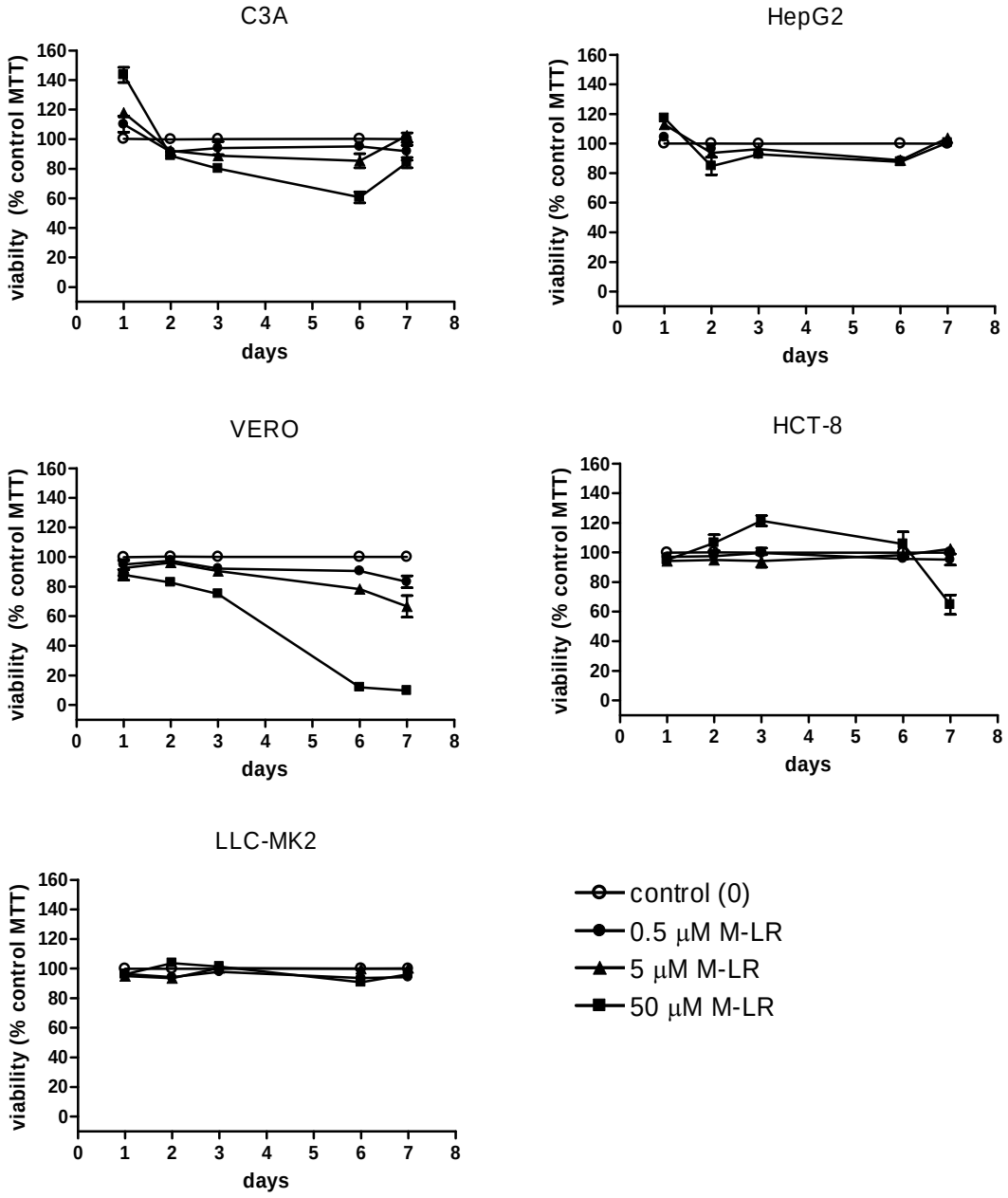


Figure 5. Screening cell lines for sensitivity to MCYST-LR. C3A, HepG2, Vero, HCT-8 and LLC-MK2 cell lines were exposed to MCYST-LR (0, 0.5μM. 5.0 μM and 50μM) for 7 days. Data are Mean ± SE of 6 replicates.

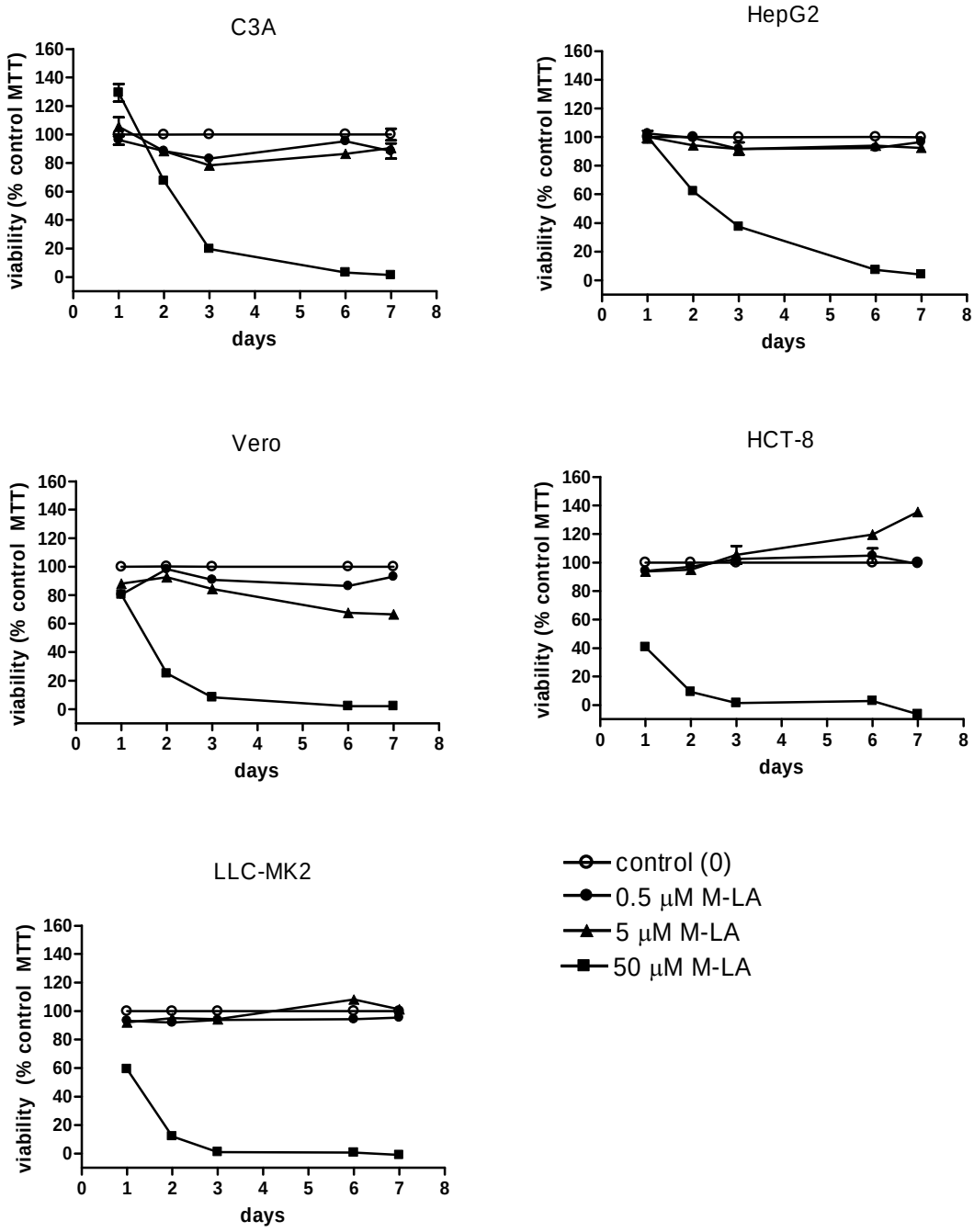


Figure 6. Screening cell lines for sensitivity to MCYST-LA. C3A, HepG2, Vero, HCT-8 and LLC-MK2 cell lines were exposed to MCYST-LA (0, 0.5 μM, 5.0 μM and 50 μM) for 7 days. Data are Mean ± SE of 6 replicates.

3.2 Short-term exposure to CYN

Results from the 7 day cell culture studies showed CYN's adverse effects were evident within 24 hr, with further progression over extended time frames. In the next set of experiments, work was carried out to determine whether short term exposure to CYN could induce toxicity at later time points. Experiments were run in parallel using C3A and Vero cell lines.

It was first established that incubation with CYN (0.01-22 µM) for only 1 hr, 2 hr, 4 hr, or 6 hr produced limited effects in both C3A and Vero cell lines (Figure 7). No loss of cell viability was observed following 1-4 hr exposure. Exposure to high concentrations of CYN for 6 hr did reduce cell viability to a limited extent. For the C3A cell line, 6 hr exposure to 22 µM CYN reduced the cell viability to 84 ± 4.3% ($p < 0.05$, one-way ANOVA). Similarly in Vero cells, exposure to 22µM CYN for 6 hr reduced cell viability to 82 ± 2.0% ($p < 0.05$, one-way ANOVA). A concentration-response curve was obtained at 24 hr with concentrations ≥ 0.83 µM CYN in C3A cells and ≥ 2.5 µM CYN in Vero cells resulting in a significant reduction in cell viability from the control ($p < 0.05$, one-way ANOVA). Statistical analyses are not shown on the graphs for clarity.

3.2.1 Short-term exposure with recovery

It was found that short-term exposure (1-6 hr) of C3A or Vero cells to CYN followed by a recovery period still induced concentration-dependent cytotoxicity when assayed at 24 hr (Figure 8). Following 1 or 2 hr exposure with concentrations of ≥ 7 µM CYN, or 4-6 hr with concentrations ≥ 2.5 µM the viability of C3A cells was significantly reduced at 24 hr from the control. Analyses were carried out by one-way ANOVA, although not shown on graph for clarity. In Vero cells, short term exposure to CYN also reduced cell viability at 24 hr, although the sensitivity of these cells was lower. The highest concentration tested, 22 µM CYN, produced a significant decrease in viability at 24 hr following 2 hr, 4 hr or 6 hr exposure followed by further incubation in the absence of toxin (Figure 8). Further progression of toxic effects with short-term exposure was also observed at 72 hrs in both cell lines (data not shown).

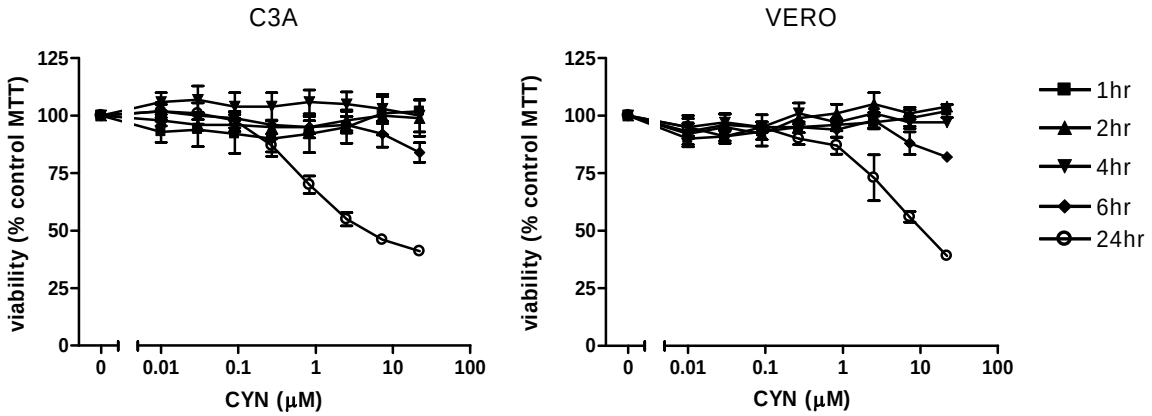


Figure 7. Continuous treatment with CYN. Viability of C3A or Vero cells was determined following 1, 2, 4 , 6 or 24 hr continuous exposure to CYN. Data are Mean ± SE of 3 independent experiments.

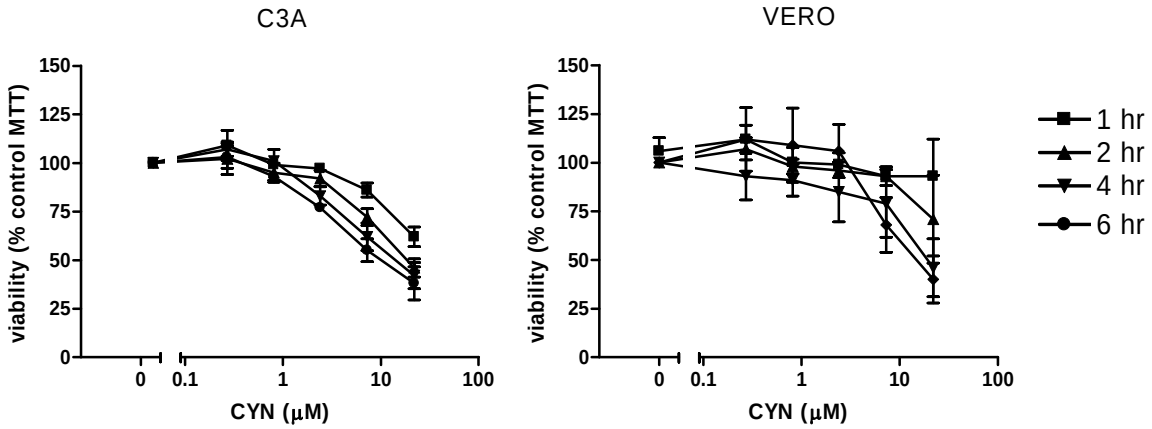


Figure 8. Short-term exposure to CYN with recovery. Viability of C3A or Vero cells following 1, 2 , 4 ,or 6 hr exposure to CYN with further incubation in the absence of toxin up to 24 hr. Data are mean ±SE of 3 independent experiments.

3.3 Rapid assays: Protein synthesis inhibition assay for CYN in reticulocyte lysate

Cell free protein synthesis (translation) was inhibited by CYN in a concentration-dependent manner (Figure 9). The IC_{50} for CYN was 110 nM; 95% CI = 95 -126 nM. The quantifiable concentration-range as defined by the linear portion of the curve was determined to be 50-275 nM CYN (Table 2). Samples were run within 4 hrs.

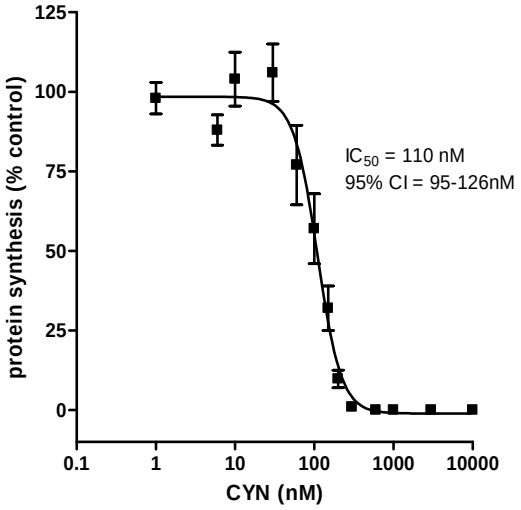


Figure 9. Sigmoidal concentration-response curve illustrating the effect of CYN on cell free protein synthesis. Data are mean ± SE of 4 independent experiments. $R^2 = 0.93$.

Table 2. CYN concentration-response curve fit.

Best-fit values	
BOTTOM	-1.07
TOP	98
IC_{50}	110 nM
Hillslope	-2.9
95% Confidence Interval	
IC_{50}	95-128
99% Confidence Intervals	
BOTTOM	-7.4-5.2
TOP	92-105
X data excluded by 99% CI	50-275 nM CYN

3.3.1 Improved sensitivity to CYN in modified reticulocyte lysate

To determine if a reduction in the number of ribosomes and associated translation components in the cell lysate would reduce the amount of CYN required to inhibit protein synthesis and improve assay sensitivity, diluted lysates were prepared. *In vitro* translation mixes were prepared to contain final concentrations of 70% (standard mix as supplied), 10% and 6% reticulocyte lysate, theoretically reducing the ribosome content up to 10-fold. Results shown in Figure 10 illustrate that assay sensitivity was improved in lysates with reduced ribosome content. The IC_{50} for CYN was reduced from 108 nM in the 70% lysate to 11 nM and 4 nM in the 10% and 6% lysate preparations respectively. A change in the slope of the modelled sigmoid concentration response curve was also noted in the diluted lysates (Table 3). Hillslopes were reported as -2.9 (70%), -1.7 (10%) and -1.1 (6%).

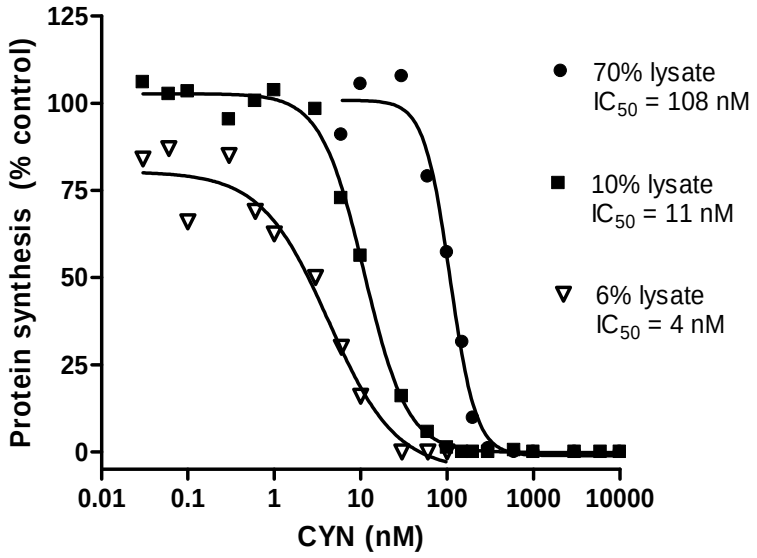


Figure 10. Effect of CYN on protein synthesis in cell lysates with reduced ribosome contents. Data are mean of 2-4 independent experiments and modelled to a sigmoid concentration response curve. The r^2 = 0.93 (70%), 0.96 (10%) and 0.90 (6% lysate).

Table 3. CYN concentration-response curve fit for reticulocyte lysates with either standard ribosome (70%) or reduced ribosome content (10% and 6%).

Best-fit values	**70% lysate**	**10% lysate**	**6% lysate**
BOTTOM	-1.0	-0.16	-5.4
TOP	101	103	80
IC_{50}	108nM	11nM	4.2 nM
Hillslope	-2.9	-1.7	-1.1
95% Confidence Interval			
IC_{50}	94-125	9.2-13	2.0-9.1
99% Confidence Intervals			
BOTTOM	-7.0-4.9	-3.9-3.6	-28-18
TOP	93-108	98-107	65-95
X data excluded by 99% CI	47-275 nM CYN	2.1-66 nM CYN	1.1-8.6 nM CYN

3.4 Rapid assays: GFP reporter protein assay for CYN

In this set of experiments, C3A and Vero cell lines were transfected with a destabilised GFP reporter protein to rapidly monitor for CYN-induced changes in protein synthesis.

3.4.1 Generation of cell lines stably expressing GFP

Both C3A and Vero cell lines were stably transfected with the pTurboGFP-dest1 vector (Figure 11). Cell samples were analysed by both fluorescence microscopy and flow cytometry and shown to express GFP over many generations. As shown in Figure 11, the C3A-GFP cell line was seen to express the GFP protein to a higher extent than the Vero-GFP cells and in a greater overall percentage of the cell population.

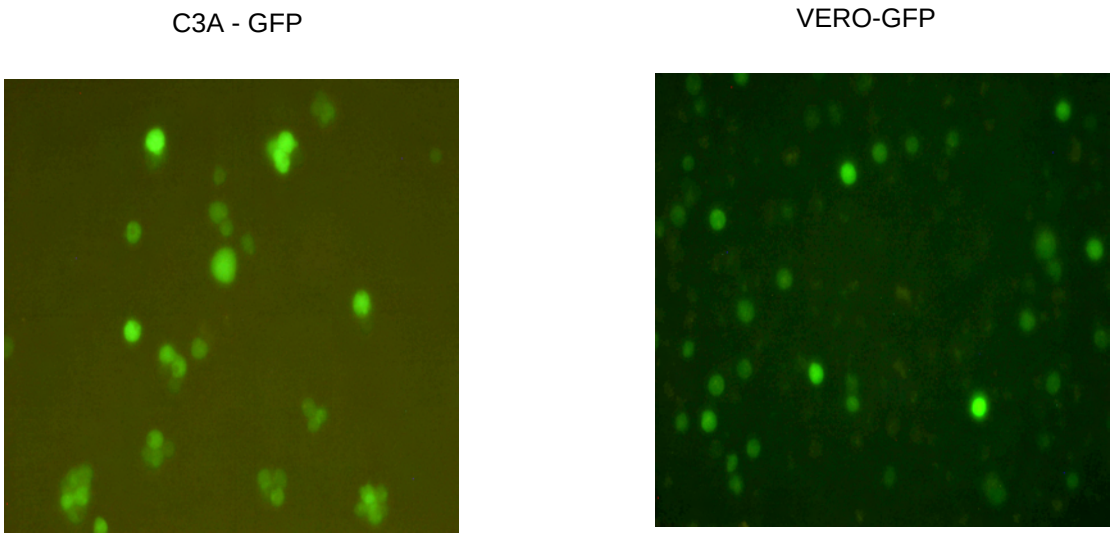


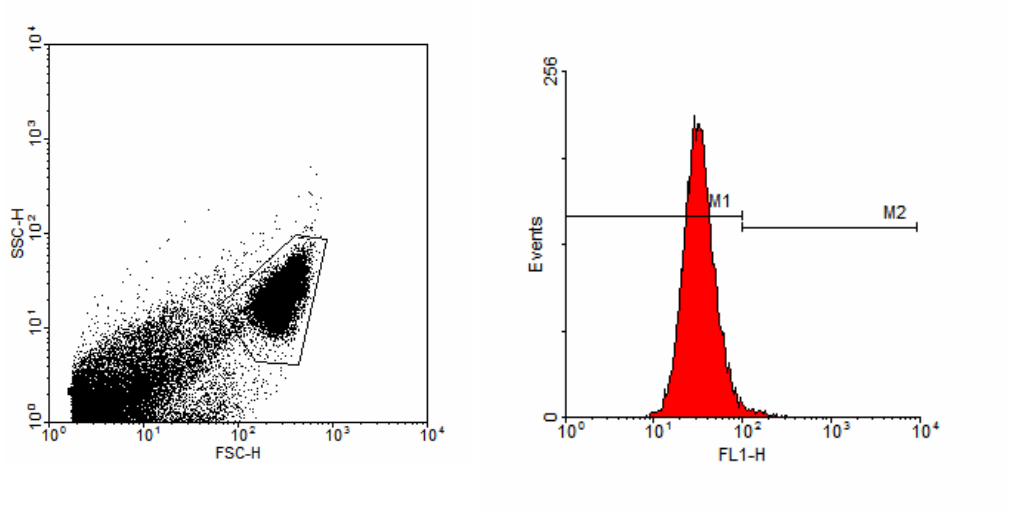
Figure 11. Fluorescence of C3A and Vero cell lines expressing pTurboGFP-dest1. Cells were visualised by fluorescence microscopy with WB excitation

3.4.2 Quantifying the GFP signal.

Quantification of the GFP signal was trialled both on the microplate reader and by flow cytometry. While the GFP signal could be quantified on the microplate reader with the signal proportional to cell number, the sensitivity was limited. The signal could not be detected at the normal cell seeding density, requiring high cell numbers to be plated per microplate well. High autofluorescence of the non-transfected cell lines was also noted.

Flow cytometry of cell suspensions provided the most sensitive method for quantifying the GFP signal and further work continued using this method. Vero-GFP cells were used as they easily formed a single cell suspension following trypsin treatment. C3A-GFP cells were prone to clumping even after trypsin digestion (as seen in Figure 11) making the flow cytometric analysis difficult. Analysis of Vero-GFP cells by flow cytometry showed two cell populations with approximately 60% of cells fluorescent and 40% non-fluorescent (Figure 12 B). Comparisons were made with non-transfected Vero cells (Figure 12 A). The GFP fluorescence expressed by the Vero-GFP had approximately 10 fold higher signal intensity than the non-fluorescent cells. A small percentage of the population were highly fluorescent, with approximately 100-fold higher intensity than the non-fluorescent population (Figure 12 B).

A. VERO



B. VERO-GFP

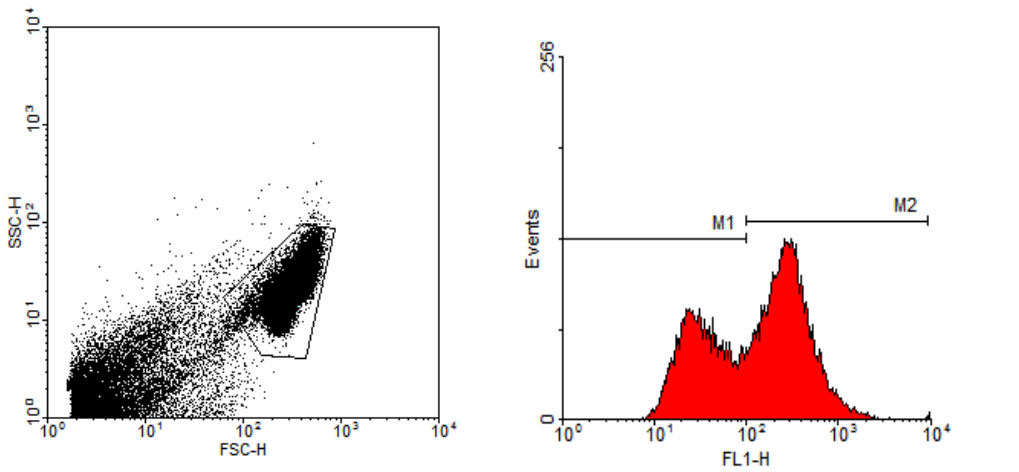


Figure 12. Flow cytometric analysis of (a) Vero and (b) Vero-GFP cell lines. To analyse GFP levels, the cell population was first gated on the FSC vs SSC scatter plots. The fluorescence intensity of the gated cells was then analysed on the histograms (FL1 vs event frequency) and the number of events falling into the GFP fluorescent (M2) and non-fluorescent (M1) regions determined.

3.4.3 Exposure of Vero-GFP cells to protein synthesis inhibitors

Treatment of Vero-GFP cells with the protein synthesis inhibitors CYN, CHEX or emetine was shown to decrease the GFP signal. Time and concentration responses following inhibitor treatments were subsequently determined.

Vero-GFP cells were exposed to 10 µM CYN or 10 µM CHEX for 2, 4, or 6 hrs, and decrease in GFP signal monitored at each time point. The data were fit to a one phase exponential decay curve (r^2 = 0.99) and the half life of the GFP protein was determined to be 1.2-1.5 hr (Figure 13).

Pilot studies were carried out to determine the sensitivity of the assay following 4 hr treatment of Vero-GFP cells with protein synthesis inhibitors. A range of inhibitor concentrations for CYN and emetine (0.1-10 µM) were used. Preliminary results indicate that the assay was sensitive to low concentrations of emetine (≤0.1 µM), while higher concentrations of CYN were required (≥3 µM) (Figure 14). Further work on optimising the sensitivity of this assay to CYN is being carried out in a CRC project 2.01. 2.2.3 'Optimisation of Cylindrospermopsin Toxicity Assay'.

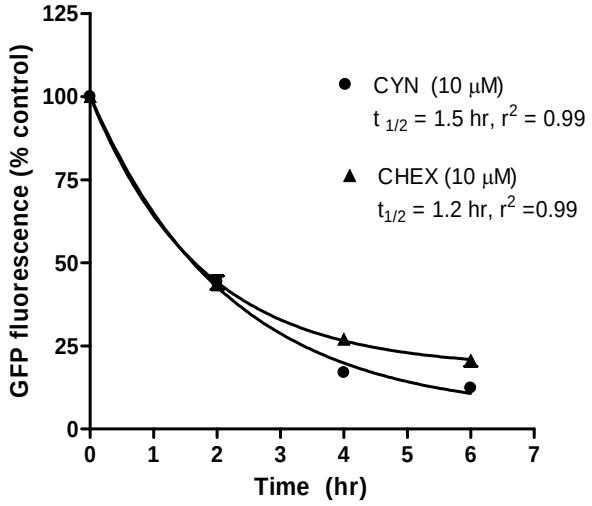


Figure 13. TurboGFP decay curve. Decrease in GFP-signal over time in Vero-GFP cells following treatment with 10 µM cylindrospermopsin (CYN) or 10 µM cycloheximide (CHEX).

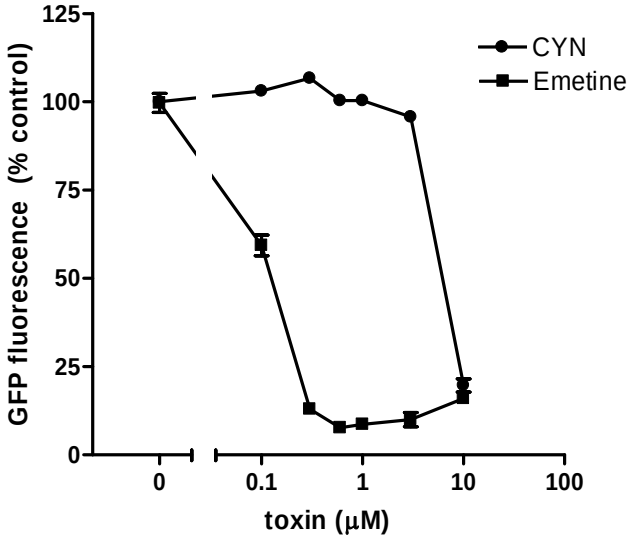


Figure 14. Sensitivity of the GFP assay. Cells were exposed to 0.1-10 µM CYN or Emetine for 4hrs and the percentage of GFP cells in the population determined. Data are mean ± SE of 3 replicates.

3.5 Rapid assays: Saxitoxin assay

Saxitoxin is a fast acting neurotoxin that inhibits nerve conduction by blocking sodium channels. In the neuroblastoma assay the cells are challenged with veratridine and ouabain to produce cytotoxicity, an effect that can be reversed in the presence of saxitoxin (Jellett *et al.*, 1992; Jellett *et al.*, 1995; Humpage *et al.*, 2007). The theory behind this assay is that the treatment with veratridine opens the sodium channel. The open state allows a potentially lethal influx of sodium ions into the cells. Ouabain blocks the Na^+/K^+ ATPase pump, inhibiting the transport of sodium ions back out of the cell, and so potentiating the effects of veratridine. Saxitoxin, being a sodium channel blocker, counters the effect of veratridine and ouabain. In the standard neuroblastoma assay, the viability of the cells is determined by MTT assay 24 hr after treatment, with an increase in survival of the cells indicating the presence of saxitoxin. More recently, detection of the membrane depolarisation that occurs with the influx of sodium has been investigated using voltage sensitive fluorescent dyes (Manger *et al.*, 2007). In theory, this should produce a much more rapid assay because the sodium influx is detected rather than cell death which occurs at a later time.

3.5.1 $DiBAC_4(3)$ membrane potential assay

Heat shock treatment

To assess the suitability of $DiBAC_4(3)$ for detecting membrane depolarisation in Neuro-2A cells, a heat shock treatment was used as a stressor to disrupt the membrane potential. Cells were heat treated at 37°C, 50°C and 60°C for 20 mins, before staining with 4 µM $DiBAC_4(3)$. As shown in Figure 15, a strong increase in fluorescence was shown following heat treatment of neuroblastoma cells, indicating cell depolarisation. Effects were seen following the 50°C treatment, and were more pronounced following the 60°C treatment. These results suggested that this dye may be suitable for measuring changes in membrane potential in the presence of veratridine, and interference of saxitoxin.

Treatment with Veratridine and Ouabain

Different concentrations of veratridine (50 µM-150 µM) and ouabain (250 µM) were applied to Neuro-2a cells for 3 hrs to determine if the cell membrane could be rapidly depolarised as measured by $DiBAC_4(3)$. Veratridine alone was shown to depolarise the cell membrane with 30% and 40 % of cells depolarised following treatment with 100 µM or 150 µM veratridine respectively (Figure 16). This was potentiated when used in combination with 250 µM ouabain. This concentration of ouabain (250 µM) did not depolarise the cell membrane alone, although higher concentrations ≥ 500 µM did so (data not shown).

Treatment with Saxitoxin

To determine if saxitoxin could protect the cells from veratridine and ouabain induced membrane depolarisation, cells were pre-incubated with 0.01-100 ng/ml saxitoxin before the addition of 70 µM veratridine and 250 µM ouabain. Cells were stained with $DiBAC_4(3)$ and analyses were carried out by flow cytometry.

As shown in Figure 17, pretreatment of cells for 1 hr with saxitoxin had a protective effect against membrane depolarisation induced by treatment with veratridine (75 µM) and ouabain (250 µM). Although saxitoxin was tested over a range of concentrations, the protective effect was not concentration dependent. The results in Figure 17 are representative of one experiment. Similar results were obtained in > 3 independent experiments.

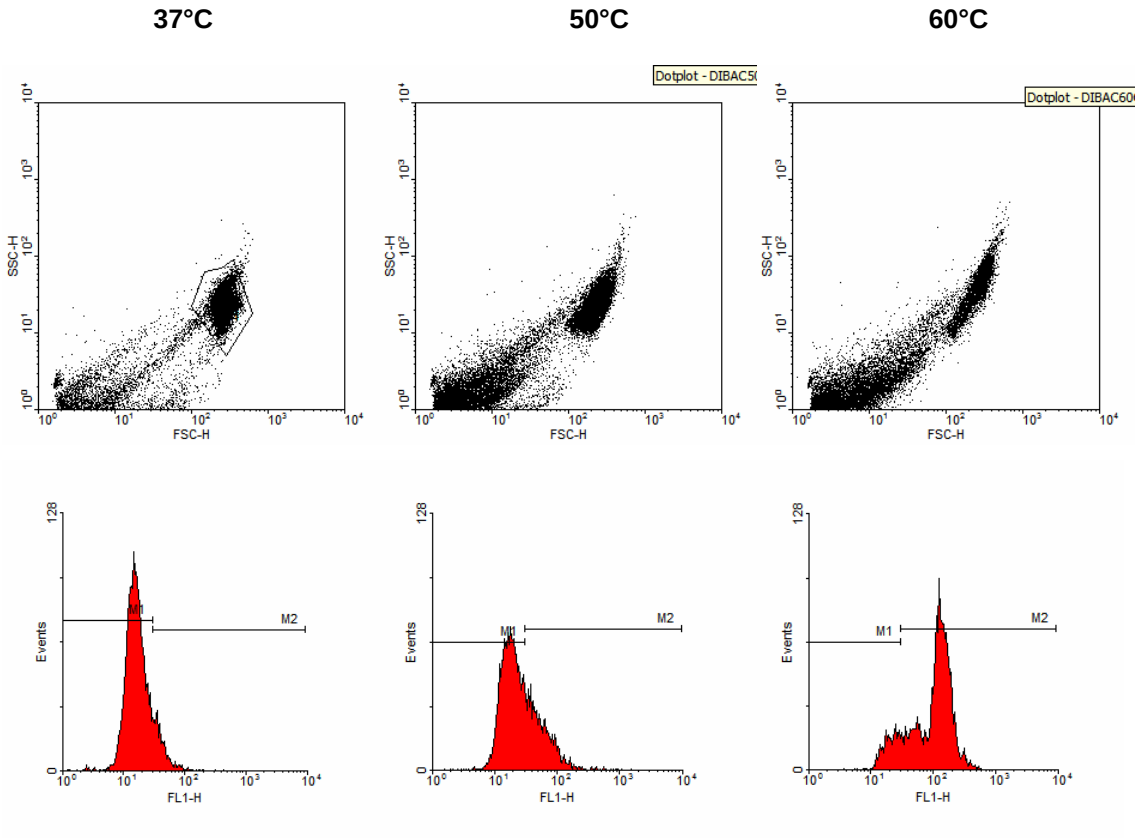


Figure 15. Heat shock increases $DiBAC_4(3)$ membrane potential dye staining in neuroblastoma cells. Control cells (37°C) and heat treated cells at 50°C or 60°C. Scatter plots show the FSC and SSC of the cell population with the first plot indicating the "gate" used to select cells for display in the histograms. Histograms illustrate the level of $DiBAC_4(3)$ staining measured in the FL1 channel

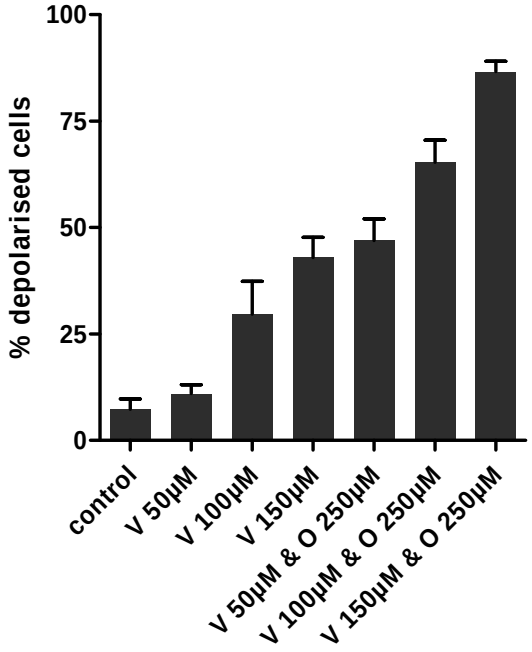


Figure 16. Depolarisation of Neruo-2a cell membrane following 3hr treatment with 50-100 µM veratridine (V) or a combination of veratridine and 250 µM ouabain (O). Data are mean ± SE of triplicates from a representative experiment.

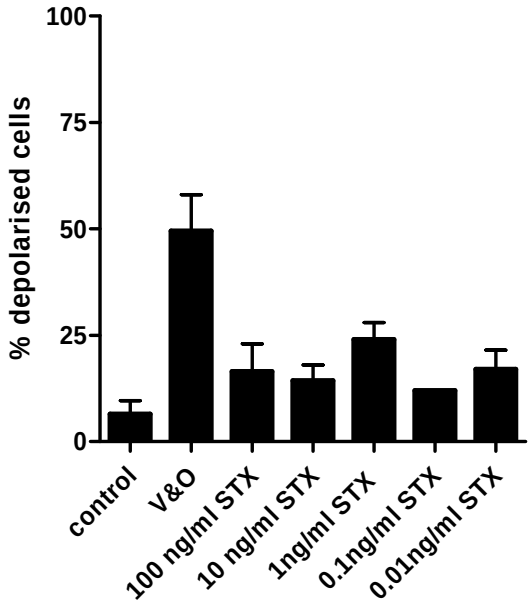


Figure 17. Saxitoxin treatment (1hr) of Neuro-2a cells provides some protection from veratridine and ouabain induced membrane depolarisation (3 hr treatment). Data are mean ± SE of triplicates from a representative experiment.

4 DISCUSSION

The need for alternatives to the mouse bioassay for cyanotoxin testing has led to the assessment of a number of new techniques for this purpose. In this project lower trophic level bioassays and cell-based screening methods were evaluated for suitability. The invertebrate bioassays are discussed in the CRC PhD project, results of the cell-based screening methods and cell extract assays are discussed below.

4.1 Screening cell lines for sensitivity to cyanotoxins – 7 day studies

The cell lines C3A, HepG2, Vero, HCT-8, and LLC-MK2 were screened for sensitivity to the cyanobacterial toxins CYN, MCYST-LR and MCYST-LA. Saxitoxin was not included in the screening experiments due to the very cell-specific nature of its activity in blocking voltage sensitive sodium channels such as those expressed by neuronal cell lines. For example, relatively high (5 µg/ml) concentrations of saxitoxin induced no cytotoxic effects in HepG2 cells following 24 hr incubation (Flanagan *et al.*, 2001).

The observation that all cell lines were sensitive to CYN exposure was consistent with CYN being a general cytotoxin. The liver-derived cell lines C3A and HepG2 were most susceptible to CYN's effects, which is consistent with both *in vivo* observations that CYN is primarily hepatotoxic (Terao *et al.*, 1994; Hawkins *et al.*, 1997; Falconer *et al.*, 1999) and the suggestion that liver derived toxic metabolites may be involved in the toxicity process (Runnegar *et al.*, 1995; Froscio *et al.*, 2003). C3A and Vero cells were chosen for further development of the cell based assays. The kidney derived Vero cells were the next most sensitive to CYN after the liver cell lines, and have also been observed to be easy to work with in the laboratory.

While high concentrations (50 µM) of MCYST-LR only induced cytotoxic effects in the Vero cell line over extended incubation periods, 50 µM MCYST-LA induced cytotoxicity in all cell lines tested after 2-3 days incubation. The difference in cytotoxicity results with the two MCYST analogues is likely to be due to differences in uptake. MCYST-LA is less polar than MCYST-LR suggesting some MCYST-LA may be able to transfer across the lipid membrane. A positive relationship between the toxicity and hydrophobicity of MCYST analogues has been shown previously (Ward and Codd, 1999). Uptake of MCYST's has recently been shown to be facilitated by organic anion transporter proteins (OATPs) known to be expressed by liver cells (Fischer *et al.*, 2005). The fact that the liver cell lines C3A and HepG2 were no more sensitive to MCYST than other tissue derived cell lines shows that these cell lines do not express the organic anion transporter proteins involved in its uptake. A MCYST assay based on these cell-lines would therefore be (a) of low sensitivity, and (b) give highly variable results with different analogues of similar *in vivo* toxicity. Recently, transfected cell lines expressing recombinant OATP's have been shown to be sensitive to MCYST analogues in the nM concentration range (Fischer *et al.*, 2007; Monks *et al.*, 2007). These cell lines are not currently commercially available. Due to lack of sensitivity of the cell lines tested to MCYST, no further cell based work was carried out with this toxin.

4.2 Short term exposure to CYN

CYN's effects were further investigated using the C3A and Vero cell lines to provide an understanding of treatment protocol required when developing assays for this toxin. Experiments were carried out to determine whether short term exposure to CYN could induce detectable toxicity immediately or at later time points. Results in this study clearly illustrate the protracted nature of CYN toxicity in these cell-lines. Although cytotoxicity was not detected immediately following a 1-6 hr exposure to CYN, the toxin was able to induce toxicity at 24 hr, even when the cells were only exposed for these short time periods. The results were most prominent in the liver C3A cell line. Similar results, albeit with reduced sensitivity were obtained using Vero (kidney) cells. This again may indicate the potential involvement of liver derived metabolites in CYN's toxicity process as discussed above.

The observation that increased cytotoxicity was seen with incubation times of 4-6 hr compared to 1 hr with the same concentration of toxin may suggest slow uptake of the toxin. Once the toxin has entered the cells, another slow intra-cellular process, probably inhibition of protein synthesis and/or metabolism of the toxin to an active form, occurs which eventually causes the observed cell death.

In terms of assay development, these results show that a cytotoxicity endpoint following short-term CYN exposure is not suitable for detection of this toxin. This is consistent with previous research showing CYN induced cytotoxicity in both primary hepatocytes and cell lines develops over extended incubation periods (Runnegar *et al.*, 1995; Chong *et al.*, 2002; Froscio *et al.*, 2003). This *in vitro* data is also supportive of *in vivo* observations where long lasting effects of CYN have also been observed (Falconer and Humpage, 2001; Rogers *et al.*, 2007). However the results also show that enough CYN entered the cells by 4-6 hr for cytotoxicity to occur by 24 hr or other later time point. It has previously been shown in primary hepatocytes that inhibition of protein synthesis occurred within 4 hrs although cytotoxicity was not seen until 18 hrs (Froscio *et al.*, 2003). Furthermore, CYN inhibits protein synthesis in a cell-free system and so does not require metabolic activation for this effect. Therefore, a cell-based assay based on detection of protein synthesis inhibition would be expected have the advantages of rapid, specific, detection of CYN and sensitivity to unknown toxins.

4.3 Rapid assays: Protein synthesis inhibition assay for CYN in cell extracts

CYN has been shown to be potent inhibitor of protein synthesis in reticulocyte lysate (Terao *et al.*, 1994). In previous work these inhibitory effects were used to quantify the amount of CYN present in cyanobacterial samples (Froscio *et al.*, 2001), with the rate of protein synthesis quantified as [^{3}H]-Leucine incorporation in a protein precipitate. In this study the rate of synthesis of luciferase (directed by luciferase-coding mRNA) was determined by a secondary luciferase assay. Both methods show similar sensitivity for CYN detection, with $IC_{50's}$ for CYN of ~110 nM reported. As expected, due to the nature of CYN's action and steep slope of the concentration-response curve, there was only a narrow (5X) concentration range for quantification of CYN of unknown samples. The samples were assayed in the laboratory within a few ~ 4 hrs.

Based on receptor-ligand binding theory, it was hypothesised that a reduced number of ribosomes and/or associated translation components in the reticulocyte lysate would mean that less CYN was required to inhibit protein synthesis, hence improving assay sensitivity. Note that recent research indicates that CYN's target may not be the ribosome itself, but rather one of the soluble protein associated with protein synthesis (Froscio *et al.*, 2008). Reticulocyte lysate with 7- and 10-fold reduced ribosome / associated protein content were prepared. This improved assay sensitivity to CYN approximately 10-fold. Low nM (<10 nM) concentrations of CYN could be detected in this assay system which is close to the 1 µg/L (2.4 nM) CYN in drinking water that has been suggested as a suitable drinking water level based on available data (Humpage and Falconer, 2003). It was also noted that the slope of CYN's concentration response curve was modified in diluted lysates, potentially broadening the range over which the toxin can be quantified. Further assay optimisation should be able to lower the detection limit further and assess quantifiable concentration range, but it was not possible to investigate these within the limits of the current project.

4.4 Rapid assays: GFP reporter protein assay for CYN

C3A and Vero cell lines were transfected with pTurboGFP-dest1 vector and shown to stably express the GFP protein over many generations. Using flow cytometric analysis, Vero-GFP cell populations were shown to consist of both fluorescent and non-fluorescent cells. It is not clear if the lack of GFP expression in some of the Vero-GFP cells was due to: depletion of amino acids, a cell-cycle dependent expression of GFP or if there is always a population of non-fluorescent cells present. Interestingly, the ratio of fluorescent to non-fluorescent cells was routinely observed to be 60:40 for a log growth phase culture. The percentage of non-fluorescent cells in the population increased in older cultures, which may suggest cell-cycle dependent expression. It should be noted that the growth medium was not replaced, also allowing depletion of substrate. The cells were responsive to treatment with CYN and the protein synthesis inhibitors CHEX and emetine, shifting the fluorescent cell population into the non-fluorescent region. Following a complete block of protein synthesis, the half life of the GFP protein was shown to be 1.2-1.5 hrs, consistent with that described for the pTurboGFP-dest1 expression vector (Evrogen Product data sheet: EVN-FP19, www.everogen.com). This shows that rapid detection of toxins inducing protein synthesis inhibition is possible. Preliminary results indicating that the assay is more sensitive to emetine than CYN may be due to differences in uptake of

the toxins. It is known that using the cell-free reticulocyte assay to determine protein synthesis inhibition, CYN is a more potent inhibitor than emetine (personal communication, Stella Fanok, AWQC). The contrast in results from the cell-free to the cell system would suggest that that CYN is not efficiently transported across the cell membrane. Chong *et al.* (2002) suggested both passive diffusion and the bile acid transport system are involved CYN uptake. The short-term exposure work with CYN in this project suggests that this toxin is slowly taken up by C3A and Vero cells (discussed in Section 3.2) consistent with a diffusion or facilitated diffusion process. As a result, a further CRC project has been developed to optimise the GFP reporter protein assay and its sensitivity for CYN detection (CRC 2.0.1.2.2.3). The new project's aims are to (1) enhance GFP expression in the Vero-GFP cell line and (2) facilitate entry of CYN to the cells to enhance sensitivity in the assay. To determine if GFP expression in all cells can be enhanced, it is proposed to supplement the Vero-GFP growth medium with additional amino acids as substrate for the highly expressed GFP protein. To facilitate delivery of CYN into the Vero-GFP cells a number of potential methods are proposed for trial including: DMSO treatment, use of cationic mediated lipid transfection agents and electroporation.

4.5 Rapid assays: Saxitoxin assay

A number of researchers have recently investigated the use of fluorometric dyes for detecting changes in cell membrane potential induced by toxins that interfere with sodium channels, and their application for rapid saxitoxin detection (Louzao *et al.*, 2000; Louzao *et al.*, 2003; Manger *et al.*, 2007). Results in this study using the membrane potential-sensitive dye $DiBAC_4(3)$ with the neuroblastoma cell line showed that changes in membrane potential can be rapidly detected by flow cytometry. While the assay worked well for detecting changes induced by veratridine alone or in combination with ouabain, the results were not as expected following pre-treatment with saxitoxin. While saxitoxin clearly could reduce veratridine's effects on membrane potential, concentration-dependency was not observed. The extent of protection afforded by saxitoxin was also noted to be quite variable over a number of different experiments with the conditions employed. At this stage it is not clear why variable results were obtained. Veratridine and saxitoxin interact with different sites on the sodium channel and it is not thought that they compete directly. However, the channel is known to change its configuration depending upon the membrane potential, and so it is possible that this changes its interaction with one or other of these toxins. Given the rapid nature of the assay further optimisation may be warranted.

5 SUMMARY AND CONCLUSIONS

The aim of this project was to develop a range of screening assays that can be used to detect toxicants in water. The focus was on detection of the cyanotoxins, CYN, MCYST and saxitoxin. The research outcomes identified a number of assays that may have potential use in a screening capacity.

For CYN, cell-lines expressing GFP were created to measure cellular protein synthesis and these were demonstrated to respond to the inhibitory effects of the toxin within 4 hrs of exposure. This greatly improves the turn around time over the use of cytotoxic endpoints for CYN that require at least 24 hr incubation. Optimisation of this assay is planned in CRC project 2.0.1.2.2.3 and will concentrate on improving sensitivity to CYN.

Also for CYN, modification of the cell-free (reticulocyte lysate) protein synthesis assay enabled detection of much lower concentrations of the toxin than had previously been achieved. Detection of concentrations <10 nM (equiv to < 4 µg/L) was possible, close to the suggested 1 µg/L drinking water guideline level for CYN. The improved sensitivity achieved in this assay has potential practical application for detection of CYN in source water samples without sample concentration. Validation with different water types will be required.

Given the limited effects of MYCST in cell lines tested, rapid cell based assays for detection of this toxin type is not possible. As demonstrated by other research groups, modification of the cells to express organic anion transport proteins is required to facilitate uptake of the toxin.

For saxitoxin, a rapid cell-based assay for saxitoxin utilizing changes in membrane potential has been previously established in other laboratories, and so we attempted to develop a similar assay. However, due to lack of time we were not able to reproduce the published results and so further research work would be required to fully evaluate the potential of our own assay system.

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- Curtin University of Technology
- Department of Human Services Victoria
- Griffith University
- Melbourne Water Corporation
- Monash University
- Orica Australia Pty Ltd
- Power and Water Corporation
- Queensland Health Pathology & Scientific Services
- RMIT University
- South Australian Water Corporation
- South East Water Ltd
- Sydney Catchment Authority
- Sydney Water Corporation
- The University of Adelaide
- The University of New South Wales
- The University of Queensland
- United Water International Pty Ltd
- University of South Australia
- University of Technology, Sydney
- Water Corporation
- Water Services Association of Australia
- Yarra Valley Water Ltd