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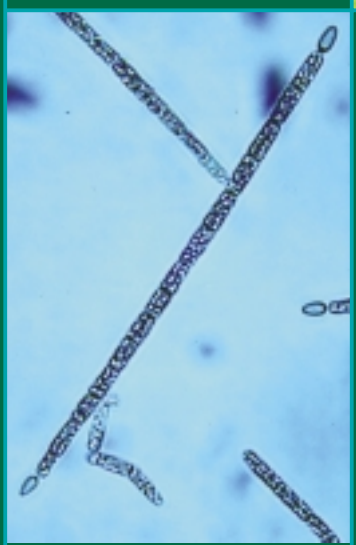
Water Quality and Treatment

Oral Toxicity of Cylindrospermopsin:
No Observed Adverse Effect Level Determination in Male Swiss Albino Mice



Research Report 13

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COOPERATIVE RESEARCH CENTRE FOR WATER QUALITY AND TREATMENT

Oral Toxicity of Cylindrospermopsin: No Observed Adverse Effect Level Determination in Male Swiss Albino Mice

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Foreword

Title: Oral Toxicity to Swiss Albino Mice of the Cyanobacterial Toxin Cylindrospermopsin Administered Daily over Eleven Weeks

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CRC for Water Quality and Treatment Project No. 1.3.1.1 – Tumour Promotion by Cyanobacterial Toxins

Executive Summary

To provide safe Guideline Values for chemicals in drinking water, the World Health Organisation requires toxicological data. The protocols for experimental provision of this data are formulated in an international context by the OECD and US National Toxicology Program, and the results of toxicology trials are published and available to all Governments and their agencies. The WHO Guideline Values are used as a basis for legislation where appropriate, or national recommendations as in the Australian Drinking Water Guidelines. National agencies re-evaluate the toxicology data and the WHO Guidelines, as appropriate for the circumstances in their country.

The cyanobacterial toxins have recently undergone evaluation by WHO as toxic chemicals in drinking water, and a Guideline Value for one toxin - microcystin-LR, has been determined. The WHO expert group developing these guidelines considered that the data available at the time of discussion were inadequate for assessment of the cyanobacterial toxin cylindrospermopsin (CYN). As a result they encouraged research into the toxicology of this alkaloid, as it occurs widely in drinking water sources in the sub-tropics and tropics of the world.

The cyanobacteria (blue-green algae) producing cylindrospermopsin are commonly found in drinking water sources in Northern Australia. Hence the majority of published research worldwide into the toxic species *Cylindrospermopsis raciborskii* and the toxin cylindrospermopsin has been undertaken in Australia. Following investigations of this toxin by research groups in Adelaide and Brisbane, the Cooperative Research Centre for Water Quality and Treatment approved funding for this study.

Research design

The research undertaken comprised a preliminary range-finding trial and a final subchronic oral toxicity trial in mice using purified cylindrospermopsin. This was aimed at determining the No Observed Adverse Effect Level for the toxin, and followed the protocol set out by the OECD. The research was carried out by Dr Andrew Humpage and Professor Ian Falconer, in the Department of Clinical and Experimental Pharmacology, University of Adelaide Medical School. Approval was given by the University of Adelaide Animal Ethics Committee for the experimental animal usage. The experimental design required extraction of toxin from cultures of *C. raciborskii* strain AWT 205 grown by Dr. Peter Hawkins at AWT EnSight, West Ryde, NSW, Australia, followed by the isolation and purification of cylindrospermopsin. Purification was carried out by Dr. Andrew Humpage with the assistance of Mrs Lidia Sledz at the Australian Water Quality Centre, Salisbury, South Australia.

Two oral exposure trials were carried out. The preliminary trial (1) used an extract of lysed *C. raciborskii* cells supplied in the drinking water of mice over 10 weeks, at doses reaching levels likely to cause measurable injury. On the basis of the results of this trial, a lower dose trial (2) was undertaken to find the No Observed Adverse Effect Level. The design of toxicology trial (2) employed administration of purified toxin by mouth (gavage) daily to Swiss Albino mice over a period of eleven weeks. The dose rates employed were aimed at rising from a dose with no likely effects, to the lower end of the dose rate of the initial trial, at which adverse changes had been observed.

Towards the end of the experiments urine was collected to assess kidney function. Blood samples were collected under anaesthesia for clinical biochemistry and haematology, which were carried out by a NATA accredited laboratory. Euthanasia at the end of the dosing period was done by Nembutal (phenobarbitone) injection. Detailed post-mortem examination of each mouse was carried out, and all organs specified by the OECD protocol together with a range of neural tissues were prepared for histopathological examination. Tissue sections were examined by two independent observers, with critical liver sections re-examined by a further histopathologist.

Results

Clinical observations of the animals were carried out continuously, and intensively prior to the end of the dosing period. No clinical changes in appearance or behaviour were seen at any dose, though the upper dose rates in the preliminary trial resulted in reduced growth rate. No changes in response to sensory stimuli, grip strength or motor activity were observed. In both trials a reduction in water consumption was seen at all dose levels of toxin. There were no observed changes in food consumption.

Gross pathology. No externally visible evidence of organ or tissue damage could be observed at post mortem examination at any dose employed in either trial.

Body and organ weights. The body, liver, spleen, kidneys, adrenal glands, heart, testis, epididymis and brain were weighed. Bodyweight was significantly higher in mice at the 30 and 60 μg/kg/d doses of CYN by gavage, but the trend over the dose range of 0 to 240 μg/kg/d was not significantly affected. In the preliminary drinking water trial mice receiving 423 and 657 μg/kg/d had significantly reduced bodyweight. Kidney weight as a percentage of body weight was significantly raised with all doses of toxin at or above 60 μg/kg/d in both trials. Liver weight was increased as a percentage of body weight at or above 216 μg/kg/d in both trials. Testis weight showed a statistical increase at 60 and 120 μg/kg/d, prior to correction for body weight, after which the increase became non-significant. Other organs showed no significant weight changes with dose of toxin.

Urine analysis. Measurements were made of 14 clinical indicators of renal function during weeks 9/10 of dosing in the gavage experiment. Urine specific gravity, protein, glucose, ketones, creatinine, sodium, potassium, chloride, calcium, bicarbonate, phosphate, pH, volume and the presence of blood were determined. Expressed per unit creatinine in the urine, a statistically significant reduction in urine protein was seen at 60, 120 and 240 μg/kg/d of CYN, and a reduction in sodium and specific gravity only at 240 μg/kg/d. A reduction in potassium (suspected to be caused by analytical problems) was seen at all doses in the gavage trial, but only at 432 μg/kg/d in the preliminary trial.

Serum analysis. Measurements were made of serum total protein, albumin, globulin, glucose, creatinine, urea, total bilirubin, total bile acids, cholesterol, triglycerides, sodium, potassium, calcium, chloride, bicarbonate, creatine kinase, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. The only serum parameter showing a statistically significant change over the dose range tested in the gavage trial was cholesterol, which increased at 30 and 60 μg/kg/d but not at higher doses. Examination of trends in serum parameters showed that serum albumin and total bilirubin increased over the range of 60 to 240 μg/kg/d, whereas bile acids and phosphate decreased. Each of these parameters showed statistically significant changes (in the same direction as the gavage trial) in the preliminary trial at 216 and 423 μg/kg/d of CYN. Serum enzymes indicative of liver function showed no significant changes in the gavage trial, but alkaline phosphatase showed a significant rise at 423 μg/kg/d in the preliminary trial.

Haematology. Red cell counts, haemoglobin, packed cell volume, white cell total and differential counts were done on blood samples collected at euthanasia. Bone marrow smears were also examined and scored for cell types. The only significant difference observed was an increase in blood lymphocytes at the dose of 30 μg/kg/d, which did not recur at higher doses.

Histopathology. Histological examination of organ sections was carried out on liver, kidney, heart, lungs, thymus, thyroid, trachea, salivary glands, adrenal glands, epididymis, testis, prostate, gall bladder, oesophagus, stomach, duodenum/small intestine, large intestine, pancreas, spleen, urinary bladder, eyes, lymph nodes, aorta, cerebrum, cerebellum, cervical, thoracic and lumbar spinal cord, and peripheral nerve. Only in the liver were dose-related changes in tissue pathology observed. An increase in mitotic figures, inflammatory foci and necrotic cells was seen with increasing dose in liver sections. Approximately double the extent of tissue damage compared to controls was seen at 240 μg/kg/d, with evidence of increased injury at 60 and 120 μg/kg/d in the gavage trial. In the preliminary trial mice receiving a dose of 432 μg/kg/d showed clearly increased tissue injury. At a dose of 240 μg/kg/day in the gavage trial, proximal tubule damage was seen in the kidneys, similar to that seen at all doses in the preliminary trial.

Conclusions

At very low dose rates of toxicants different parameters of organ and tissue damage show different sensitivities in response. The decision over the extent of an 'Observed Adverse Effect' therefore becomes a balance of evidence from several sources. In general, a conservative outcome is preferable to looking for more severe injury to resolve the lowest dose rate that has caused injury in a toxicity trial. At the lowest dose rate used in these experiments, 30 μg/kg/d, the change in bodyweight was positive, and the main indicators of tissue injury did not show any significant alteration. At the next dose of 60 μg/kg/d, there was evidence of changes in organ weight, urine and serum parameters, and histopathology of the liver, which together can be regarded as adverse. Above this dose rate the changes increasingly appear adverse.

Guideline derivation

It is therefore concluded that the oral No Observed Adverse Effect Level is 30 μg/kg/d, and the Lowest Observed Adverse Effect Level is 60 μg/kg/d for cylindrospermopsin over 11 weeks in male Swiss Albino mice.

Applying this data to the determination of a Tolerable Daily Intake (TDI) and Guideline Value (GV) for cylindrospermopsin in drinking water using the accepted uncertainty factors and WHO standard values for bodyweight and water consumption:

TDI = 30 μg cylindrospermopsin/kg/d (NOAEL) ÷ uncertainty factors

Uncertainty factors:	Intraspecific human variability	10x
	Interspecific variability	10x
	Limitations in data	10x

Therefore, TDI = 0.03μg/kg/day

GV = 0.03 (TDI) x 60 (kg bodyweight) x 0.9 (proportion of toxin derived from drinking water)
÷ 2 (litres water /d)

= 0.81 μg/litre

or to round off

GV = 1.0 μg cylindrospermopsin/litre drinking water.

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Abbreviations

ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
APTT	Activated partial thrombin time
AST	Aspartate aminotransferase
AWT	Australian Water Technologies
Bwt.	Body weight
Chol.	Cholesterol
Creat.	Creatinine
CYN	Cylindrospermopsin
EPA	Environmental Protection Agency
FD	Freeze-dried (lyophilised)
GFR	Glomerular filtration index
GIT	Gastro-intestinal tract
GV	Guideline Value
Hb	Haemoglobin
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatography - tandem mass-spectrometry
LOAEL	Lowest Observed Adverse Effect Level
Lymph.	Lymphocytes
MCH	Mean cell haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCT	Multiple comparison test
MCV	Mean cell volume
MS/TOF	Time of flight mass-spectrometry
NATA	National Association of Testing Agencies
Neuts.	Neutrophils
NHMRC	National Health and Medical Research Council (Australia)
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program (US)
OECD	Organisation for Economic Cooperation and Development
PCV	Packed cell volume
PT	Prothrombin time
RBC	Red blood cell
RFI	Renal failure index
TBA	Total bile acids
TBilli	Total bilirubin
TDI	Tolerable Daily Intake
Trig.	Triglycerides
USG	Urine specific gravity
VPS	Veterinary pathology services
WCC	White cell count
WHO	World Health Organisation

1. INTRODUCTION

1.1 Aim

To provide safe Guideline Values for chemicals in drinking water, the World Health Organisation require toxicological data. The WHO expert group developing these guidelines considered that the data available were inadequate for assessment of the cyanobacterial toxin cylindrospermopsin (CYN). The research undertaken aimed at determining the No Observed Adverse Effect Level for the toxin in male Swiss Albino mice, and was based on the protocol set out by the OECD.

1.2 Background

The cyanobacterial toxin cylindrospermopsin is an important potential contaminant of Australian water supplies. It is produced by a cyanobacterium (blue-green alga) named *Cylindrospermopsis raciborskii* which occurs in the rivers, lakes and drinking water reservoirs of Australia. Originally considered a mainly tropical and sub-tropical species, over recent years it appears to have spread into the Murray-Darling system in Australia (Baker and Humpage, 1994), and into Central Europe and North and South America (Padisak, 1997). The apparent proliferation of this species may be a consequence of the general warming of the environment, with the emergence of neophytes better adapted to temperate conditions, or a combination of these processes. The cyanobacterium first came into prominence as a result of the poisoning of 138 aboriginal children and 10 adults on Palm Island, a tropical island off Townsville in central Queensland, Australia. The origin of this poisoning was unclear at the time, though the clinical picture was well described (Byth, 1980). The affected individuals had fever, headache, vomiting, swollen livers, initial constipation, kidney damage with loss of water, electrolytes and protein, and considerable pain. Later they suffered bloody diarrhoea, further adding to their dehydration. The more severe cases were transferred to intensive care. Overall 69% of individuals required intravenous therapy for electrolyte imbalance, and in the most severe cases for hypovolemic and acidotic shock. The illness lasted up to 26 days and fortunately there were no fatalities.

The onset of the poisoning followed complaints from the population of unpleasant taste and odour in the drinking water, and the subsequent treatment of a water bloom of cyanobacteria in Solomon Dam, the water supply reservoir, with copper sulphate. The Palm Island water supply had a conventional filtration and chlorination system and there was no evidence of bacterial or viral pathogens in the drinking water (Bourke et al., 1983; Bourke et al., 1986). Solomon Dam was and is the major water supply for the population on Palm Island and is still the site of frequent water-blooms of cyanobacteria. As a result of the problems arising from these blooms extensive limnological studies have been carried out, and amelioration strategies undertaken (Griffiths et al., 1998).

While several toxic cyanobacterial species form dense floating scums on lakes and rivers during periods of light winds, *C. raciborskii* forms bands well below the surface. These bands may be at 5 or more meters depth in stratified lakes and rivers, close to the metalimniun, and up to cell densities of hundreds of thousands per millilitre (McGregor and Fabbro, 2000). This exacerbates the problem for water supply authorities, as the cyanobacteria may not be visible from the surface, and occur at depths where water intakes are located.

1.2.1 Cylindrospermopsis raciborskii toxicity

Following the poisoning incident in 1979, the predominant cyanobacterial species in the reservoir was isolated and put into culture, the species identified and toxicological studies undertaken (Hawkins et al., 1985). The species was found to be *C. raciborskii*, first described in Indonesia. Cell extracts were found to be highly poisonous in mouse toxicity trials, with damage seen in the liver (showing centrilobular to massive necrosis), kidneys, adrenal glands, lungs and intestine. The lethal dose for 50% of mice (LD_{50}) within 24 h was 64±5 mg of cell culture /kg bodyweight.

Since this early research, toxic *C. raciborskii* has been identified in numerous rivers, reservoirs, farm water supply dams and ornamental lakes in Australia (Baker and Humpage, 1994; Fabbro et al., 2001; Hawkins et al., 1997) and implicated in livestock deaths (Saker et al., 1999; Thomas et al., 1998). In Florida, USA, *C. raciborskii* was found to dominate one eutrophic lake all the year round and appear in others seasonally (Chapman and Schelske, 1997). There have been suggestions that pelican and alligator deaths in Florida may be linked to *C. raciborskii* dominance in some lakes. Mouse testing indicated that these organisms in Florida were toxic (Burns et al., 2000). In Lake Balaton, Hungary, which is not a highly eutrophic lake, *C. raciborskii* was the cause of a massive water-bloom in 1994 which was toxic to fish and insects (Hiripi et al., 1997).

1.2.2 Other cyanobacterial species producing cylindrospermopsin

A single species of cyanobacteria may produce more than one toxin, and the same toxin may be produced by quite different species (Chorus and Bartram, 1999). The toxin cylindrospermopsin and the species *C. raciborskii* are examples of both characteristics. In Japan a new species of filamentous cyanobacterium, *Umezakia natans*, has been shown to contain cylindrospermopsin (Harada et al., 1994; Terao et al., 1994). This organism was isolated from a temperate region lake, Lake Mikata, Fukui, Japan (Watanabe, 1987). In Lake Kinneret in Israel (Sea of Galilee), which has a Mediterranean climate, the cyanobacterial species *Aphanizomenon ovalisporum* produces cylindrospermopsin (Banker et al., 1997). Lake Kinneret is the main freshwater reservoir in Israel, and in 1994 a large water-bloom of *A. ovalisporum* reached 4,000 trichomes/ml, evenly distributed in the epilimnium. Waterblooms of this density cause concern in drinking water supplies due to turbidity, taste and odour problems, as well as potential for toxicity. Cylindrospermopsin-producing *A. ovalisporum* blooms have also occurred in Australia (Shaw et al, 1999; P. Baker, Pers. Com.).

There is evidence that some strains of *C. raciborskii* in Brazil are neurotoxic, containing paralytic shellfish poisons (Lagos et al., 1999). Other Brazilian waterbodies, however, contain cyanobacteria producing the hepatotoxin cylindrospermopsin, though the species concerned are not yet identified (Carmichael et al., 2001).

1.2.3 Cylindrospermopsin

The original isolate of *C. raciborskii* from Palm Island was grown for the extraction, purification and structural identification of the toxin cylindrospermopsin. The toxin is an alkaloid consisting of a tricyclic guanidinium moiety linked to hydroxymethyl uracil (Fig. 1). The tricyclic rings carry a sulphate group, so the compound is a highly water soluble zwitterion (Ohtani et al., 1992). The molecular weight is 415.43. The toxin is chemically stable to sunlight, high temperature and a wide range of pH (Chiswell et al., 1999). The toxin has now been completely synthesised, and there is considerable interest in alternative synthetic pathways (McAlpine and Armstrong, 2000; Murphy and Thomas, 2001; Xie et al., 2000).

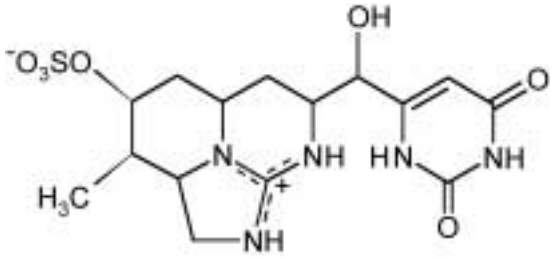

Figure 1. Cylindrospermopsin

The biosynthetic pathway remains to be clarified, but the genetic analysis of toxic cyanobacteria has shown a polyketide synthetase gene which is characteristic of several strains and species of organisms which produce cylindrospermposin (Schembri et al., 2001; Wilson et al., 2000). The biosynthetic precursors may include guanidoacetic acid (Burgoyne et al., 2000).

1.2.4 Analytical methods

The basic approach to quantification of cylindrospermopsin in water-bloom concentrates or cell culture has been cell lysis followed by high performance liquid chromatography (HPLC) (Harada et al., 1994; Hawkins et al., 1997). As a result of the characteristic uracil spectrum, diode array detection with HPLC provides a preliminary quantitation of the toxin. Superimposed nucleoside or amino acid peaks may however result in overestimation. A more precise method for measurement of the toxin in biological samples or drinking water is HPLC followed by tandem mass spectrometry (MS-MS). This will not provide data on impurities that are present in the HPLC peak when it is operated as an analytical tool. However, mass spectrometry separates and quantitates the toxin's characteristic mass fragments, thus providing positive identification of the molecule as well as accurate measurement (Eaglesham et al., 1999). Several groups are developing Enzyme-Linked Immunosorbent Assays (ELISA) for measurement of cylindrospermopsin at the very low concentrations likely to be found in drinking water. These are not yet published. Nuclear magnetic resonance was used in the study reported here in order to identify any impurities, as well as HPLC-MS/MS for identification and quantitation of the purified toxin.

1.2.5 Cylindrospermopsin Toxicology

Ohtani and colleagues first purified and identified cylindrospermopsin from *C. raciborskii* (Ohtani et al., 1992). They published a 24 hour LD_{50} for intraperitoneal injection of 2,100 μg/kg and a 5-6 day LD_{50} of 200 μg/kg bodyweight in mice. From the earlier laboratory studies

on *C. raciborskii* toxicity it was apparent that the poisoning substantially affected the liver, though damage was not restricted to this organ. Intraperitoneal administration of cell extracts to mice caused death within 6-9 hours, while lower doses caused deaths up to 5 days later. The pathological examination of these animals showed mottled swollen livers, with congestion in lungs, kidneys, adrenal glands and small intestine. The liver damage depended on dose and length of time of survival. Animals receiving a high dose showed centrilobular to massive hepatocyte coagulative necrosis with haemorrhage and necrotic material in the central veins. The kidneys showed epithelial cell necrosis in the proximal tubules, the adrenal cortex showed epithelial cell degeneration and necrosis, the lungs embolic material in the small arteries and capillaries. In animals at lower doses surviving up to 5 days, accumulation of lipid in hepatocytes was an obvious feature (Hawkins et al., 1985). The LD_{50} at 24 hours was 64 mg freeze-dried culture/kg bodyweight and it was noted that some mice dosed at the LD_{50} survived up to 5 days.

Subsequent studies have supported and extended these finding. As a result of identification and quantitation of the alkaloid toxin, it was possible to express extract toxicity in terms of toxin content. The intraperitoneal toxicity of cell extracts was shown to be in the range of LD_{50} 100- 200 μg cylindrospermopsin/kg over 7 days. Electron microscopic examination of kidneys of mice administered intraperitoneal *C. raciborskii* extracts showed the extent of proximal tubule damage, illustrated by the disintegration of the cells and leakage of cell contents into the dilated luminal space. Blood flow through the glomeruli was also reduced as indicated by the red cell numbers (Falconer et al., 1999b). Comparison of toxicity between batches of *C. raciborskii* showed different relative toxicities to liver and kidney, which may imply the presence of further toxins (Falconer et al., 1999b). Other evidence of toxicity variation not consistent with cylindrospermopsin content has been reported (Hawkins et al., 1997).

Oral administration of *C. raciborskii* extracts required higher doses of toxic material for lethality. The lethal dose was between 4.4 - 6.9 mg cylindrospermopsin equivalent per kg of mouse, with death occurring at 2 - 6 days after treatment. The animals showed a fatty liver with periacinar necrosis, acute renal tubular necrosis, atrophy of the lymphoid tissue of the spleen and thymus, haemorrhage in the heart and ulceration of the upper part of the gastric mucosa. The authors noted that substantial amounts of the freeze-dried culture were present in the ulcerated stomach at autopsy, indicating that the lethal dose estimate may have over-estimated the toxic dose (Falconer et al 1999b; Seawright et al 1999).

Oral dosing studies with purified cylindrospermopsin indicated an approximate LD_{50} for a single dose of 6.0 mg/kg for mice. Repeated oral dosing over 14 days provided a No Observed Adverse Effect Level of 150 μg/kg/d (Shaw et al. 2001). In an experiment with a cell-free extract of *C. raciborskii* supplied in drinking water to mice over 90 days, no pathological changes were recorded up to the maximum dose of 150 μg/kg/d (Shaw et al 2001).

1.2.6 Sub-chronic toxicity studies

At present there are no sub-chronic studies on the oral toxicity of purified cylindrospermopsin published in scientific journals. Without this data it is not possible to obtain the required No Observed Adverse Effect Level or Lowest Observed Adverse Effect Level doses of toxin, which are the prerequisites for determination of Tolerable Daily Intake for cylindrospermopsin. Extrapolations of short-term oral exposure to purified toxin, or from oral exposure to toxic cell extracts, or from intraperitoneal injection of toxin, are not accepted by WHO for drinking water Guideline Value determination. Several protocols exist for subchronic toxicity evaluation, from the US EPA and from the OECD (OECD, 1998; EPA, 1998). A draft of harmonised guidance notes has been prepared by the Chemicals Unit, Department of Health and Aged Care, Canberra, Australia, for the OECD (Chemicals Unit, 2001). The methodology followed by this study used the OECD protocol as the basis, taking into consideration the evaluation procedures likely to be applied, and local limitations.

1.2.7 Mechanism of toxicity and toxin metabolism

Studies of the body distribution of ^{14}C-labelled cylindrospermopsin have shown that the main excretory route is through the kidneys, with nearly 50% of an intraperitoneally administered dose appearing in the urine in 6 hours. At this time 20% of the dose was present in the liver. At 48 hours, 13% of the dose was in the liver, with a small amount retained in the kidney. Faecal excretion was very variable, accounting for about 10% of the dose (Norris et al., 2001). Extraction of the liver showed a hydrophilic metabolite and bound radioactivity. Comparison of distribution of the radioactive label between blood and tissue indicated some concentration of label in liver, kidney and lungs, but not at levels indicating an active transport into cells (Norris et al., 2001). Studies of cylindrospermopsin uptake into isolated hepatocytes provided evidence of both bile acid transporter involvement and another, as yet undefined, means of uptake (Chong et al, 2002).

Cylindrospermopsin purified from *Umezakia natans* in Japan was used for an electron-microscope based study of toxicity to mice. The 5-6 day LD_{50} dose of 200 μg/kg was administered intraperitoneally and a time series of

electron microscopic tissue examinations carried out. Injury was seen in liver, thymus, kidneys and heart. They observed in the liver a cessation of protein synthesis as exhibited by dissociation of ribosomes from the endoplasmic reticulum, followed by membrane proliferation, fat droplet accumulation and finally cell death (Terao et al., 1994).

This has been further elucidated by Froscio and colleagues who used the cell-free assay for protein synthesis based on reticulocyte lysates to measure cylindrospermopsin toxicity (Froscio et al 2001). This assay system does not contain the xenobiotic metabolising enzymes present in hepatocytes, implying that unmodified toxin is the active agent in this system.

Runnegar and colleagues observed the depletion of glutathione preceding the loss of cell viability in isolated hepatocytes incubated with cylindrospermopsin. This may indicate conjugation between glutathione and the toxin or a metabolite of the toxin (Runnegar et al., 1994). Further studies showed that inhibition of glutathione synthesis was a component of the depletion. However some protection against cell toxicity was shown by ∝-napthoflavone, an inhibitor of xenobiotic oxidative metabolism through cytochrome P-450. This implies that a more toxic metabolite of cylindrospermopsin is involved in cell death in culture, generated by cytochrome P-450 oxidation (Runnegar et al., 1995).

The interaction between cylindrospermopsin toxicity through protein synthesis inhibition and through generation of more toxic metabolites in the liver remains to be explored.

1.2.8 Mutagenicity and carcinogenicity

The mutagenic activity of cylindrospermopsin has been explored in cell culture. A human lymphoblastoid cell line was exposed to purified toxin in the cytokinesis-blocked micronucleus assay (Humpage et al., 2000). A significant increase in micronuclei in binucleate cells was seen, demonstrating DNA strand breaks and loss of portions of chromosomes at cell division due to cylindrospermopsin. Fluorescent labelling of the cells with nucleotide sequences complementary to centromeric DNA showed that many but not all of the micronuclei contained centromeres. This indicates whole chromosome loss from the treated cells at cell division. Both of these effects of cylindrospermopsin result in genetic damage, which may increase the risk of carcinogenicity.

In vivo carcinogenicity has been explored in mice. In preliminary studies mice were orally dosed with 1 to 3 damaging but sub-lethal amounts of *C. raciborskii* extract, or saline solution. Thereafter the dosed and control animals were divided into two groups, one receiving in their diet tetradecanoylphorbol acetate - a promoter of tumour growth - and the other not. After 30 weeks the animals were euthanased and the major organs and tissues examined visually and histologically. Three well developed tumours and two precancerous lesions were found in cylindrospermopsin-dosed mice while control animals remained cancer-free. No effect of the tumour promoter was seen (Falconer and Humpage, 2001). On the basis of this evidence for mutagenicity and possible carcinogenicity of toxic *C. raciborskii,* it is apparent that further research is needed to verify the origin and nature of the genetic damage and to substantiate the *in vivo* carcinogenicity of cylindrospermopsin.

1.2.9 Determination of Guideline Values for safe drinking water supply.

The procedure for determining the Guideline Value for a chemical toxicant in drinking water is set out in the WHO publication Toxic Cyanobacteria in Water (Chorus and Bartram, 1999). The relevant chapters on Human Health Aspects (Kuiper-Goodman et al., 1999) and Safe Levels and Safe Practices (Falconer et al., 1999a) set out the methodology. The basic data from which the Guideline Level is determined is the experimental identification of the maximum dose to which an animal can be continuously exposed orally over 10-13 weeks, without any significant adverse effects. This is the No Observed Adverse Effect Level (NOAEL). At very low doses of toxicants non-adverse effects may be seen, such as a raised growth rate or improved food conversion efficiency. Small non-adverse changes may be seen in blood or urine components or in histology, which do not affect the health of the animal. Adverse effects are those likely to result in reduced bodyweight, increased liability to infection, or negative physiological changes. For hepatotoxins, critical findings are damage to liver tissue as observed by histology, raised liver-derived enzymes in serum, and increases in metabolic products normally excreted via the liver such as bile pigments (Chemicals Unit, 2001).

Having evaluated the data from a sub-chronic toxicity study and resolved the value for the NOAEL, this is then converted to a Tolerable Daily Intake (TDI) by application of safety or uncertainty factors. The standard factors for rodent toxicity trials are 10 for intraspecific differences between individual people and 10 for interspecific differences between rodents and people. A variable additional factor is included for extrapolating from sub-chronic exposure to lifetime exposure, use of Lowest Observed Adverse Effect Level when the NOAEL is not clear, or when there are gaps in the data, for example no teratology information or suspected carcinogenicity.

In the case of cylindrospermopsin there is limited toxicological data, no information on teratogenicity or reproductive effects, and no lifetime carcinogenicity data.

An additional factor of 10 is suggested.

This provides an overall safety factor of 10x10x10=1,000 to be applied to the NOAEL, hence

Tolerable Daily Intake = NOAEL ÷ 1,000

Therefore, for example,

say NOAEL = 50 µg/kg/d,
then TDI = 50 ÷ 1,000
= 50 ng/kg/day.

This is the lifetime daily dose considered not to cause harm due to chemical toxicity.

To obtain the Guideline Value (GV) for drinking water the bodyweight of a person (60 kg or 70 kg), the volume of drinking water consumed (2 litres), and the proportion of toxin coming through the drinking water (normally taken as 0.8 or 0.9), are applied:

$$GV = \frac{TDI \times wt \times proportion}{volume} = \frac{50 \times 70 \times 0.9}{2.0} = 1575 \text{ ng/litre}$$

or 1.6 µg/litre

2. MATERIALS AND METHODS

2.1 Animal Husbandry

Male Swiss Albino mice (20 - 25 g) were used for both trials. They were obtained from University of Adelaide Animal Services and housed, up to 10 per cage, at the University of Adelaide Medical School Animal House. They were provided with food and water *ad libitum*, unless otherwise stated, and maintained on a 12hr/12hr light/dark cycle at 19 - 23°C. Water and food consumption were measured throughout. All procedures were approved by the University of Adelaide Animal Ethics Committee.

2.2 Toxic *C. raciborskii* Material

Cultured *C. raciborskii* strain AWT205 was kindly supplied by Dr Peter Hawkins, AWT Ensight, Sydney, Australia. (Hawkins et al. 1997). The cells plus the medium from two cultures (approx. 20 L total volume) were freeze-dried and pooled. The toxicity of the cultured material was determined by both intraperitoneal and oral mouse bioassay, and by LC-MS/MS (Eaglesham et al. 1999).

2.3 Preparation of an Aqueous Extract

An aqueous extract of the *C. raciborskii* material was prepared as follows: Fourteen grams of the pooled freeze-dried material was suspended in 100 ml MilliQ.H_2O and sonicated (10 min, 50 W, Labsonic 1510 probe sonicator), and then frozen (-20°C) overnight. After thawing, the volume was increased to 500 ml, mixed and then refrozen. The freeze-thaw cycle was repeated twice more, giving 4 cycles in all, then the volume was increased to 900 ml before centrifugation (15,000 x g, 20 min, Sorval centrifuge). The supernatant was made up to 1 L with MilliQ.H_2O, aliquoted into 100 ml lots and frozen until use. This material was used (diluted as required) for the first chronic dosing experiment, and was also the starting material for the purification of cylindrospermopsin (CYN) for the second experiment.

2.4 Cylindrospermopsin Purification

The purification protocol was based on that of Banker et al. 2000, with some modification. Three hundred and twenty ml of the aqueous extract containing approx. 18 mg CYN was loaded onto 6 conditioned Biotage KP-C18-HS cartridges (approx. 55 ml/cartridge) and the toxin washed through with 150 ml MilliQ.H_2O per cartridge. The pooled eluate was concentrated by rotary evaporation to 5 ml (later diluted 1/4 in MilliQ.H_2O for the next stage). Sephadex G-10 size exclusion chromatography was performed on a 4 cm x 35 cm (400 ml) column with MilliQ.H_2O at a flow rate of 1.8 ml MilliQ.H_2O/min. Ten ml fractions were collected and pooled over the 10 runs needed to process all of the material. Fractions were assessed for CYN content by analytical HPLC (see below) and the fractions containing most toxin (23 - 35) were pooled in 4 groups based on toxin content. These were concentrated 40 - 80 fold by rotary evaporation for the final stage. Preparative HPLC purification was performed on a Waters Bondapak C18 125Å 10mm 7.8 x 300 mm column with isocratic 5% MeOH in MilliQ.H_2O at a flow rate of 2 ml/min. Fifty-nine injections of between 600 ml and 1000 ml were made. One minute fractions were collected and same-time fractions pooled. Fractions were analysed by analytical HPLC as before, and those spanning the toxic peak (23 - 26) were pooled to provide the final product.

Analytical HPLC used a Waters 600 controller, a Waters 717plus autosampler, and a Waters 996 photodiode array detector. The column was an Alltech Alphabond C18 125Å 10U, 3.9 x 300 mm, and the mobile phase was isocratic 5% aqueous methanol, 0.6 ml/min. Injections were 50 ml.

2.5 Chronic Oral Dosing Studies

Two chronic dosing trials were performed for the determination of a No Observable Adverse Effect Level (NOAEL) for CYN. The dose ranges and number of mice used in each experiment are given in Table 1.

2.5.1 Experiment 1: *C. raciborskii* extract in drinking water

In the first experiment, the crude aqueous extract of cultured *C. raciborskii* was supplied for 10 weeks at appropriate dilutions in the animals' drinking water. Animal weight and extract consumption were recorded throughout the experiment, with the actual CYN dose rate being calculated at the end. The dose ranges and number of mice used are given in Table 1.

Using metabolic cages, urine was collected from each mouse over a 20 hour period before treatment began, at 5 weeks, and again at 10 weeks. Nutritionally complete liquid food (Nutrison Multi-fibre, Nutricia (Asia-Pacific) Ltd) was supplied *ad libitum* during these collection periods to avoid contamination of urine samples with

food dust and to stimulate urine production. Volume of liquid diet consumed was recorded.

At the end of the experiment, mice were anaesthetised with pentobarbital before blood was collected by cardiac puncture. Major organs were weighed, and post mortem and histological examinations of organs were performed (see Table 2). Urine and serum chemistries were analysed as listed in Tables 3 and 4 by a National Association of Testing Authorities (NATA)-accredited veterinary laboratory (IDEXX Veterinary Pathology Services, Adelaide, Australia).

2.5.2 Experiment 2: Purified CYN daily by gavage

In the second trial, animals were dosed with the purified CYN daily by gavage for 11 weeks. The dose ranges and number of mice used are given in Table 1. In this trial urine was only collected for a single 20 hour period during the last week of the trial during which these mice were also put onto the liquid diet. Animal weights and consumption of food and water were again monitored, and at the end of the experiment the animals and their tissues were processed essentially as previously, following the OECD recommendations for tissues to be examined by histology (OECD,1998; see Table 2). In this experiment, a clinical examination was conducted after 9 weeks of treatment, paying attention to the signs listed in Section 3.3.1. Due to serum volume requirements, serum chemistry and haematology could not both be done for the same mouse. Therefore, serum chemistry was analysed in 5 of each of the control, 30, 60, and 120 μg/kg/d groups, and all 6 of the 240 μg/kg/d group. Haematology was done for 5 of each of the control, 30, 60 and 120 μg/kg/d groups only (see Tables 3 - 5). Urine and serum chemistries, and haematology were again performed under NATA-accredited procedures (IDEXX Veterinary Pathology Services, Adelaide, Australia).

Table 1. Treatment Regimes

Drinking Water Experiment - 10 weeks			Gavage Experiment - 11 weeks	
Dose[a] (mg FD/kg/d)	Dose equiv. (μg CYN/kg/d)	Number of mice	Dose (μg CYN/kg/d)	Number of mice
0.0	0.0	12	0.0	10
43	216	10	30	10
85	432	10	60	10
130	657	10	120	10
135[b]	687	5	240	6

Notes: **a** This is the actual dose of freeze – dried extract, calculated from consumption data. The equivalent CYN dose is used in the text and graphs for ease of comparison with Experiment 2.

b In this dose, the mice reduced their liquid intake and became dehydrated. Therefore, the data were excluded from the analysis.

Table 2. Organs examined at post mortem, weighed, and examined histologically

Organ/Tissue	Organs weighed	Histopathology
Brain	2	2: cerebrum, cerebellum, medulla
Spinal chord	–	2: cervical, thoracic, lumbar
Thyroid	–	2
Thymus	2	2
Oesophagus	–	2
Salivary glands	–	2
Stomach	–	2
Intestine	–	2: duodenum, ileum, colon
Liver	1,2	1,2
Gall bladder	–	2
Pancreas	–	2
Kidneys	1,2	1,2
Adrenals	2	2
Spleen	1,2	1,2
Heart	2	2
Trachea	–	2
Lungs	–	2
Aorta	–	2
Testes	2	2
Epididymes	2	2
Prostate	–	2
Urinary bladder	–	2
Lymph nodes (axial)	–	2
Peripheral nerve (femoral)	–	2
Bone marrow	–	2
Eyes	–	2

Notes: 1 = Experiment 1; 2 = Experiment 2; - = Not examined.

Table 3. Serum analysis parameters

Parameter	Units	Experiment
Cholesterol	mmol/L	2
Triglycerides	mmol/L	2
Total Bile Acids	µmol/L	1,2
Glucose	mmol/L	2
Phosphate	mmol/L	2
Sodium	mmol/L	1,2
Potassium	mmol/L	1,2
Bicarbonate	mmol/L	1,2
Chloride	mmol/L	1,2
Calcium	mmol/L	2
Urea	mmol/L	1,2
Creatinine	mmol/L	1,2
Protein	g/L	1,2
Albumin	g/L	1,2
Globulins	g/L	1,2
Total Bilirubin	mmol/L	1,2
Alkaline Phosphatase	U/L	1,2
Alanine Amino Transferase	U/L	1,2
Aspartate Amino Transferase	U/L	1,2
Creatinine Kinase	U/L	2
Lipase	U/L	1

Table 4. Urine analysis parameters

Parameter	Units	Experiment
Specific Gravity (USG)		1,2
Protein	g/L	1,2
Glucose	mmol/L	1,2
Ketones	dipstick	1
Creatinine	mmol/L	1,2
Sodium	mmol/L	1,2
Potassium	mmol/L	1,2
Chloride	mmol/L	1
Calcium	mmol/L	1
Bicarbonate	mmol/L	1
Phosphate	mmol/L	1,2
pH		1,2
Blood cells	+/-	2

Table 5. Haematology analysis parameters (Experiment 2 only)

Category	Parameter	Units
Haematology	Red blood cell count	x 10^{12}/L
	Haemoglobin	g/L
	Packed cell volume	L/L
	Mean cell volume	fl
	Mean cell haemoglobin	pg/L
	Mean corpuscular haemoglobin concentration	L/L
	White cell count	x 10^{9}/L
Differential white cell count	Bands	%, x 10^{9}/L
	Neutrophils	%, x 10^{9}/L
	Lymphocytes	%, x 10^{9}/L
	Monocytes	%, x 10^{9}/L
	Eosinophils	%, x 10^{9}/L
	Basophils	%, x 10^{9}/L
	Others	%, x 10^{9}/L
Cell appearance	Red blood cells	
	Platelets	
Coagulation	Prothrombin time	seconds
	Activated partial thrombin time	seconds

2.6 Statistics

Data were generally analysed by One-way ANOVA followed, if $p < 0.05$, by Dunnet's Multiple Comparison Test (MCT). However, if Barlett's Test for Homogeneity of Variance indicated that variances were significantly unequal, then the Kruskal-Wallis Test was used instead followed, if $p < 0.05$, by Dunn's MCT. Two-way ANOVA with repeated measures was used to analyse the chemistry data from three urine collections taken over the duration of the first experiment. Sample matching was not significant in the USG data, so this was analysed by two-way ANOVA without the assumption of repeated measures. All statistical analyses and the preparation of graphs were performed using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. RESULTS

3.1 Characterisation of the Freeze-dried *Cylindrospermopsis raciborskii* Culture Material

3.1.1 Cylindrospermopsin content

Cultured *C. raciborskii* cells plus the medium from two cultures (approx. 20 L total volume) were freeze-dried and pooled. Cylindrospermopsin (CYN) content of the freeze-dried material was 5.5 mg/g, as determined by LC-MS/MS (Eaglesham et al 1999) of a 5% acetic acid extract.

3.1.2 Acute toxicity via the intraperitoneal route

Intraperitoneal toxicity of a saline suspension of the freeze-dried culture (FD) was tested by mouse bioassay. Three mice dosed with 50 mg/kg died in less than 24 hrs, as did 2/3 mice dosed with 40 mg/kg, whilst all 3 mice tested at 30 mg/kg survived 24 hrs. However, the 30 mg/kg dosed mice were exhibiting clear signs of toxicity (piloerection, immobility) when euthanased at 29 hrs. Post mortem examination of these mice showed changes in the kidney (pale, mottled), liver (pale brick red, size normal, gall bladder enlarged and dark green/yellow), and spleen (enlarged, normal colour), while the stomach was grossly distended but the intestine empty. Three mice dosed at 15 mg/kg were alert and normal until killed at 48 hrs. Their organs showed only relatively slight effects. This indicates an approximate i.p. lethal dose (24 hrs) of 40 mg FD/kg or 220 μg CYN/kg.

3.1.3 Acute toxicity via the oral route

Oral toxicity of a saline suspension was determined in single mice by gavage with 1200, 1600, 2000, 2500, 3000, and 3500 mg/kg. The 1200, 1600, and 2000 mg/kg treated mice (including a second mouse dosed later with 2000 mg/kg) survived for 7 days. The most obvious early effect in these mice was a palpable swelling of the abdomen, evident 48 hrs post-dosing but generally subsiding later. At post mortem, the liver was slightly enlarged, and the contents of the duodenum were red/brown. The rest of the GIT, and other organs, appeared normal. The mouse dosed at 2500 mg/kg survived 5 1/2 days, whilst those given 3000 and 3500 mg/kg survived 4 days. Again the stomach was palpable, by 24 hrs in the top-dose mouse and by 48 hrs in the 3000 mg/kg mouse. All 3 mice were affected by 24 hrs (ruffled fur, with top dose also showing hunched posture, deep and slow breathing, eyes shut), although the 2500 mg/kg mouse appeared to recover later. They became increasingly moribund during the last 24 hrs before death. At post mortem, all 3 mice had very full stomachs containing dry food and bedding material, pale and mottled livers of reduced size, enlarged straw – yellow gall bladders, pale kidneys and spleens, and minimal intestinal contents (grey/green liquid, or gas). Histology of the organs from the 3500 mg/kg treated mouse showed: coagulative necrosis with focal haemorrhage, hepatocyte vacuolation, and thickening/fibrosis of the bile ducts in the liver; tubular dilation and tissue repair/remodelling in the kidney; and follicular atrophy in the spleen. The livers from 2500 mg/kg and 3000 mg/kg treated mice showed less necrosis and haemorrhage, and more vacuolation. Other organs were similar to those of the 3500 mg/kg treated mouse.

The oral lethal dose was therefore approximately 2,500 mg FD/kg equal to 13.75 mg CYN/kg over 7 days.

3.1.4 Toxin content of the aqueous *C. raciborskii* extract

The theoretical maximum CYN content of the aqueous extract was expected to be 77 mg CYN/L, based on 5.5 mg CYN/g and 14 g/L (this assumes that the 5% acetic acid extraction used for the LC-MS/MS analysis of the freeze-dried material was 100% efficient). In fact, analysis of the aqueous extract indicated 51.6 mg CYN/L by HPLC and 61.6 mg CYN/L by protein synthesis inhibition assay (Froscio et al 2001), hence the relative extraction efficiency was about 70%.

3.1.5 Toxin content of the purified cylindrospermopsin

The final product was not pure. LC-MS/MS analysis showed that the CYN content was 10.4 mg. However, the dry weight was 22 mg. Analysis by NMR and MS/TOF showed that the contaminant was phenylalanine (Dr Simon Pyke, Chemistry Department, Adelaide University). No other contaminant was evident in this analysis nor by analytical HPLC. This is similar to the finding of Harada et al. 1994 who also noted co-elution of CYN with phenylalanine, as well as tyrosine, some nucleotides, and uracil.

3.2 Trial 1: *C. raciborskii* Extract in Drinking Water

3.2.1 Consumption of toxic material

Mice in all extract-treated groups reduced their water consumption due to the presence of the extract. Therefore, the water available to the control group was reduced to 5 ml/mouse/day to match the consumption of the lowest treatment group. The mice in the highest dose group reduced their consumption to the extent that they became dehydrated and so this treatment was excluded from the analysis.

3.2.2 Effects on mouse body weight, and the weights of major organs

The final average body weight in the 432 and 657 μg/kg/d dose groups was significantly reduced in comparison with control mice. The weight of mice in the 216 μg/kg/d group was only slightly and non-significantly reduced in comparison with controls (Fig 2). Nevertheless, the weights of livers and kidneys were significantly increased in this and all other dose groups, and this is the case whether the data were analysed as absolute weights or as a percentage of body weight (only the latter are shown, Figs 3 and 4). Spleen weight was not significantly changed (not shown).

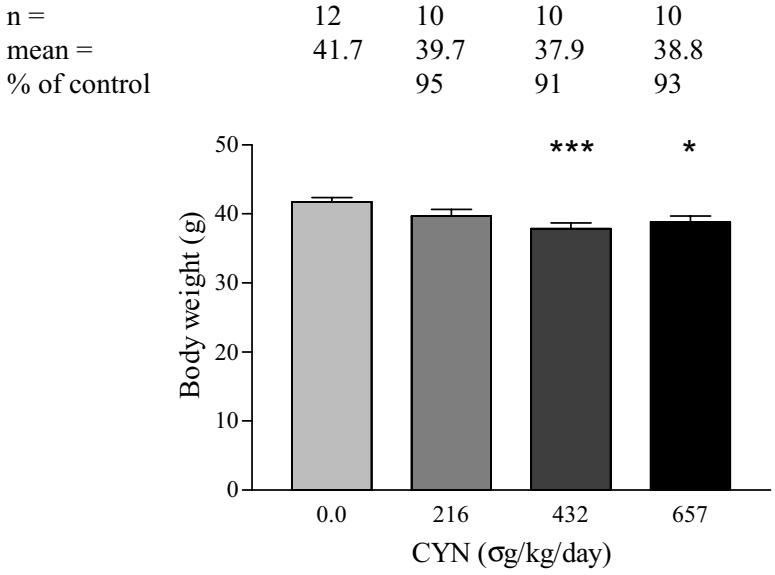

Figure 2. Experiment 1: Average final mouse bodyweight (g). One way ANOVA, Dunnett's MCT * p<0.05, *** p<0.001

n =	12	10	10	10
mean =	5.5	7.0	7.5	8.1
% of control		127	136	147

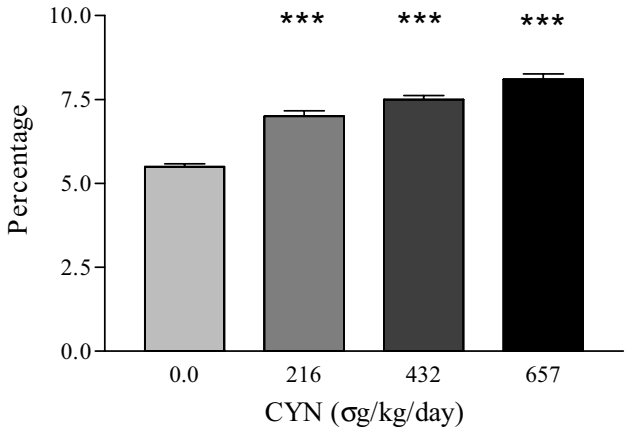

Figure 3. Experiment 1: Liver weight as a percentage of body weight. One way ANOVA, Dunnett's MCT *** p<0.001

n =	12	10	10	10
mean =	1.45	1.88	2.06	2.07
% of control		130	142	143

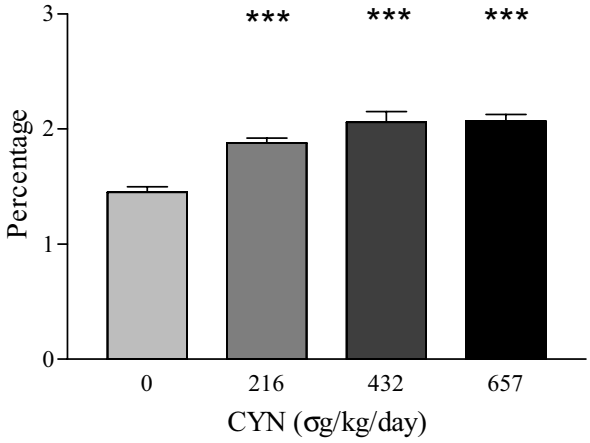

Figure 4. Experiment 1: Kidney weight as a percentage of body weight. One way ANOVA, Dunnett's MCT *** p<0.001

3.2.3 Effects on serum parameters

Serum analyses were carried out on blood from mice dosed at 0, 216 and 432 µg CYN/kg/day. Both of these toxin dose rates showed significant effects, with elevated albumin and total bilirubin levels, reduced total bile acids, and reduced serum phosphate (Figs 5 - 8). Serum alkaline phosphatase was increased at the 432 µg/kg/d dose only, and there was no significant effect on alanine aminotransferase (Figs 9 and 10). The graph for serum urea, although no significant effect occurred, is included for comparison with that from trial 2 (Fig 11).

n =	11	10	10
mean =	25.8	28.7	29.5
% of control		111	114

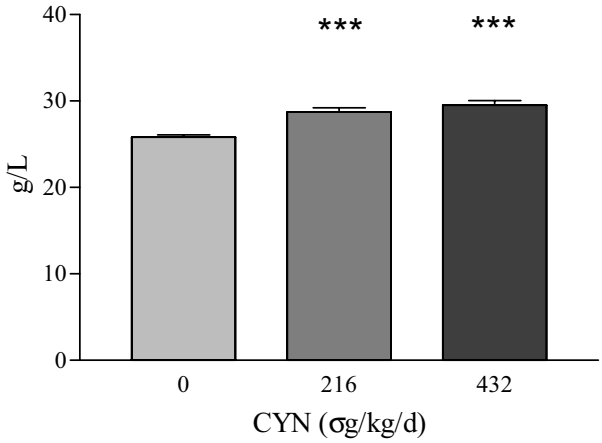

Figure 5. Experiment 1: Serum albumin concentration (g/L). One way ANOVA, Dunnett's MCT *** p<0.001

n =	12	12	12
mean =	2.06	2.65	3.59
% of control		129	174

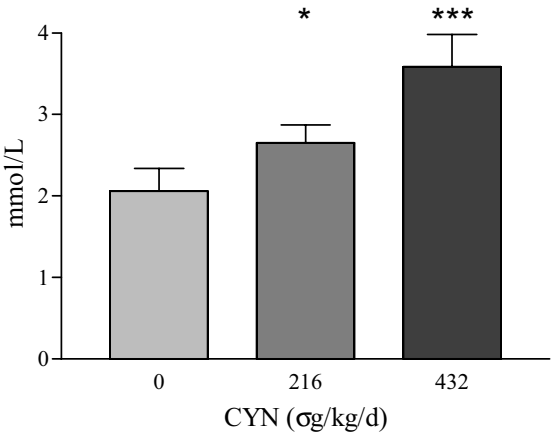

Figure 6. Experiment 1: Serum total bilirubin concentration (mmol/L). One way ANOVA, Dunnett's MCT * p<0.05, *** p<0.001

n =	9	8	10
mean =	8.32	4.46	3.93
% of control		54	47

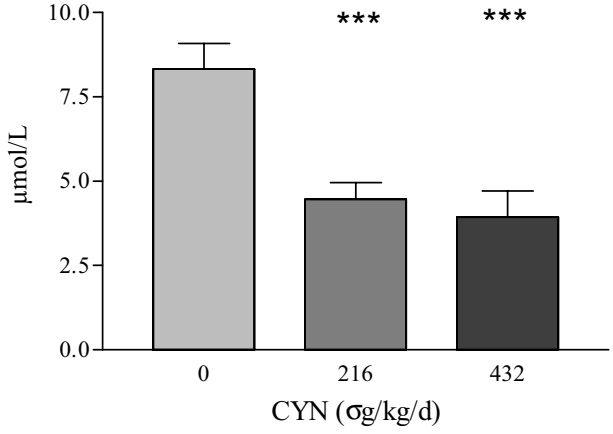

Figure 7. Experiment 1: Serum total bile acids concentration (µmol/L). One way ANOVA, Dunnett's MCT *** p<0.001

n =	11	10	10
mean =	3.77	2.73	2.71
% of control		72	72

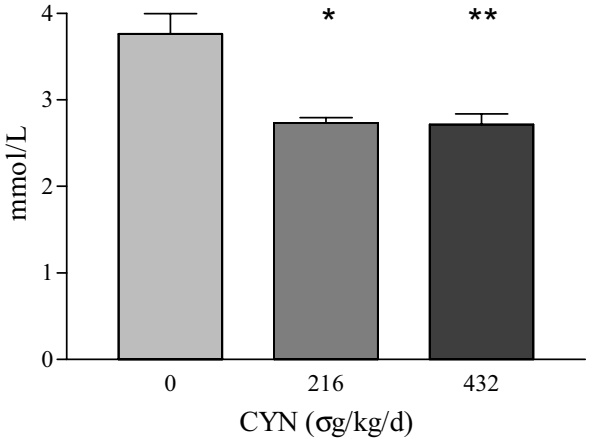

Figure 8. Experiment 1: Serum phosphate concentration (mmol/L). Kruskal-Wallis, Dunn's MCT * p<0.05, ** p<0.01

n =	12	10	10
mean =	54.8	59.3	161.8
% of control		108	295

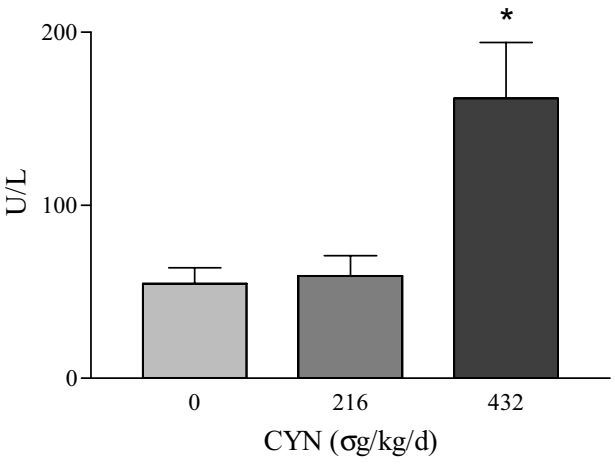

Figure 9. Experiment 1: Serum alkaline phosphatase (U/L). Kruskal-Wallis, Dunn's MCT * p<0.05

n =	5	9	10
mean =	167.8	188.1	303.8
% of control		112	181

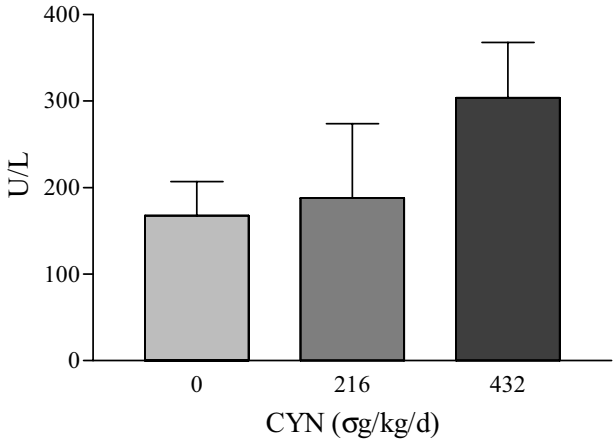

Figure 10. Experiment 1: Serum alanine amino-transferase (U/L).
Kruskal-Wallis p=0.1048

n =	12	10	10
mean =	268.2	159.3	234.6
% of control		59.4	87.5

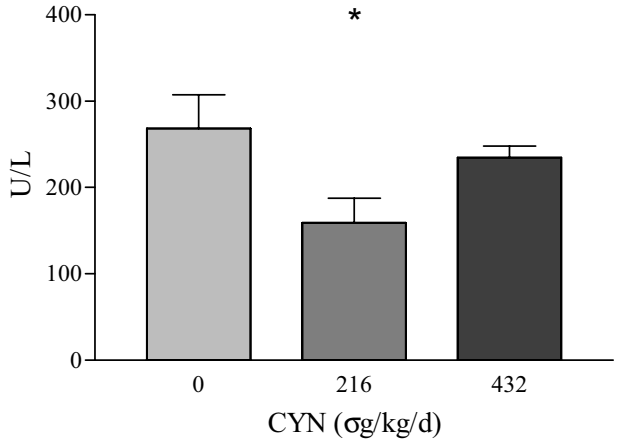

Figure 11. Experiment 1: Serum aspartate amino-transferase (U/L).
Kruskal-Wallis, Dunn's MCT * p<0.05

n =	11	10	10
mean =	10.8	11.1	10.7
% of control		103	100

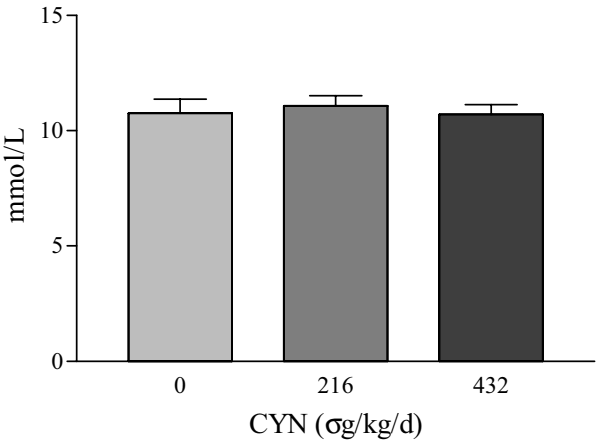

Figure 12. Experiment 1: Serum urea (mmol/L).
One-way ANOVA, p=0.8561

3.2.4 Effects on urine parameters

All data were normalised to creatinine concentration to account for variation in urine production (Figs 13 and 14). No significant effects were observed in the urines of the 216 μg/kg/day group. Urinary protein was reduced in both the 432 and 657 μg/kg/day groups (Fig 15). The 432 μg/kg/day group had reduced urinary potassium, phosphate, and renal failure index (RFI; sodium loss as a function of creatinine loss) (Figs 16, 17, and 20). Urine sodium, specific gravity and glomerular filtration rate were not significantly affected but the graphs are included for comparison with Experiment 2 (Figs 18, 19 and 21). There were no significant treatment effects on the volume of liquid food consumed during urine collection, nor on the volume of urine produced.

Urine samples were also collected before the experiment began and after 5 weeks of exposure to the *C. raciborskii* extract. The data for protein/creatinine, Na/creatinine, K/creatinine, and USG are presented in Figs 22-25. USG, and particularly protein/creatinine, showed clear dose and time dependency, with extract treatment apparently reducing the magnitude of underlying increases occurring in the controls. The were no significant dose-dependent changes in the Na/creatinine or K/creatinine concentrations.

n =	12	10	10	10
mean =	8.9	7.9	9.2	9.6
% of control		89	103	108

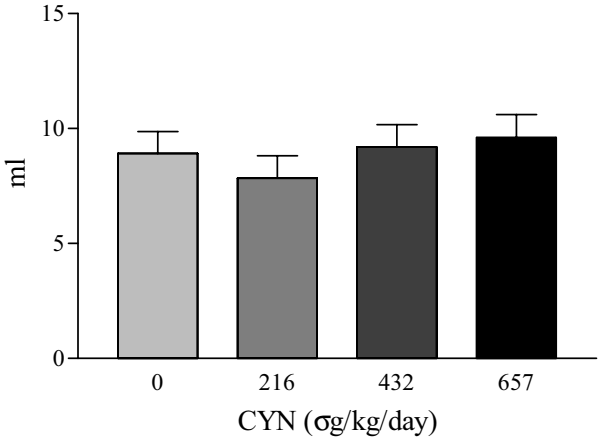

Figure 13. Experiment 1: Urine volume (ml).
One-way ANOVA, p=0.6445

n =	12	10	10	10
mean =	0.71	0.75	0.74	0.68
% of control		106	104	96

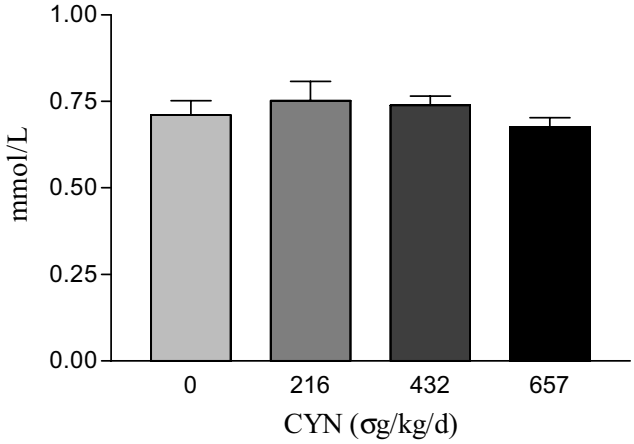

Figure 14. Experiment 1: Urine creatinine concentration (mmol/L).
One-way ANOVA, p=0.5694

n =	12	10	10	10
mean =	3.72	2.24	0.62	0.48
% of control		60	17	13

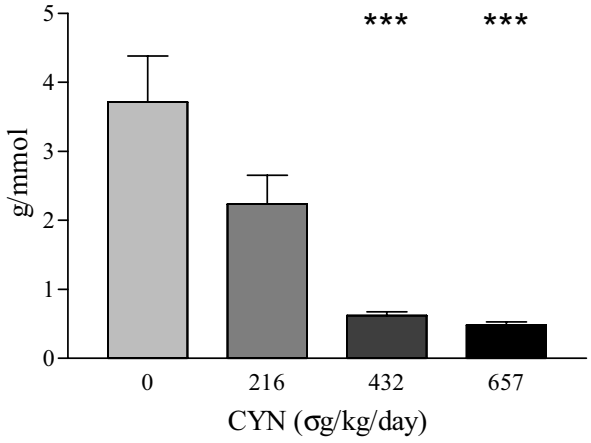

Figure 15. Experiment 1: Urine protein/creatinine (g/mmol).
Kruskal-Wallis, Dunn's MCT *** p<0.001

n =	12	10	10	10
mean =	75.9	66.4	58.5	65.9
% of control		87	77	87

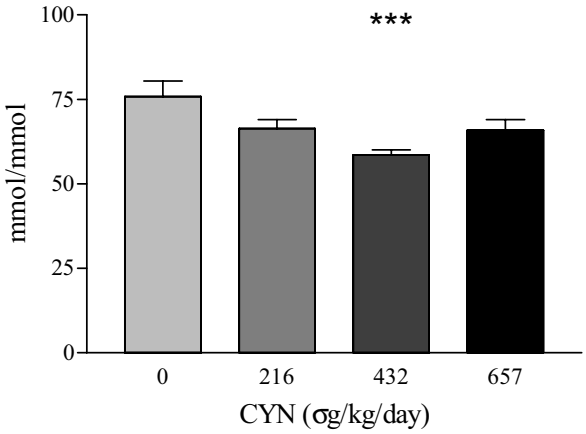

Figure 16. Experiment 1: Urine potassium/creatinine (mmol/mmol).
Kruskal-Wallis, Dunn's MCT *** p<0.001

n =	12	10	10	10
mean =	9.02	9.23	5.08	9.78
% of control		102	56	108

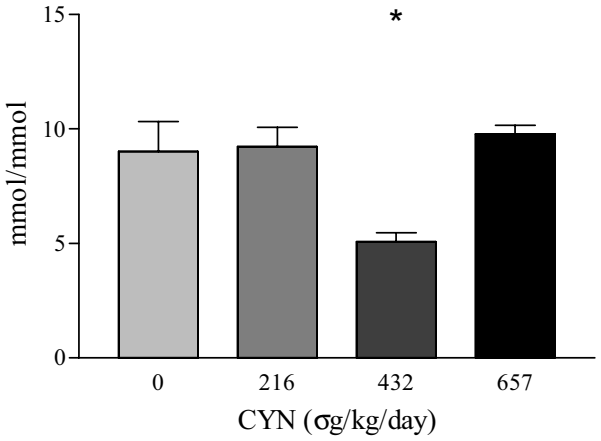

Figure 17. Experiment 1: Urine phosphate/creatinine (mmol/mmol). Kruskal-Wallis, Dunn's MCT * p<0.05

n =	12	10	10	9
mean =	89.6	86.2	84.4	92.1
% of control		96	94	103

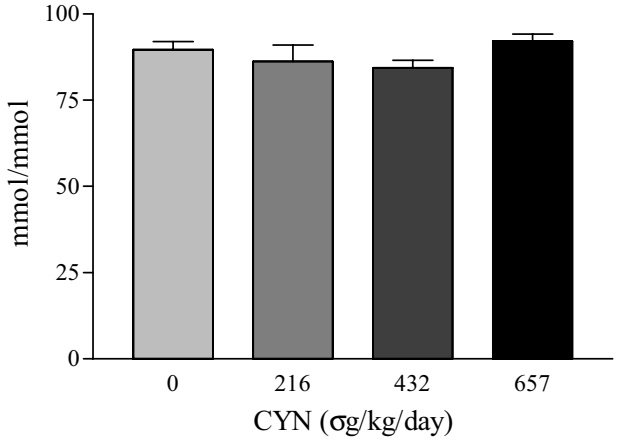

Figure 18. Experiment 1: Urine sodium/creatinine (mmol/mmol). One-way ANOVA, p=0.1241

n =	12	10	10	10
mean =	1.47	1.41	1.38	1.51
% of control		96	94	103

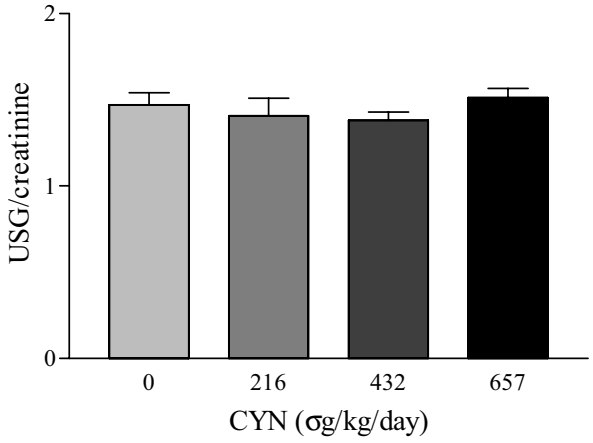

Figure 19. Experiment 1: Urine specific gravity/creatinine.
One-way ANOVA, p=0.5768

n =	12	10	10
mean =	3.52	3.34	2.71
% of control		95	77

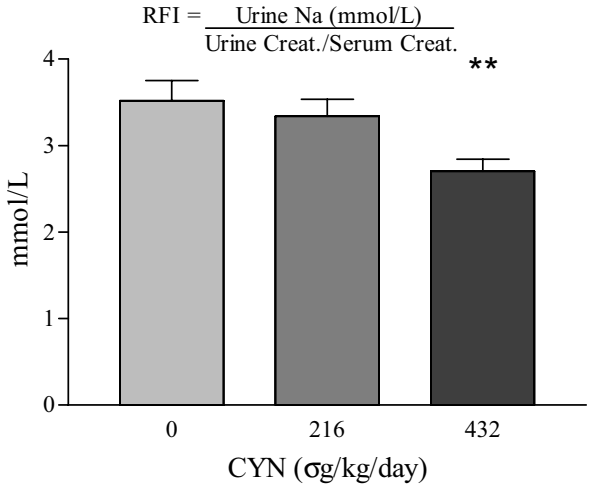

Figure 20. Experiment 1: Renal failure index (RFI, mmol/L).
Kruskal-Wallis, Dunn's MCT ** p<0.01

n =	12	10	10
mean =	4.02	3.58	5.7
% of control		89	142

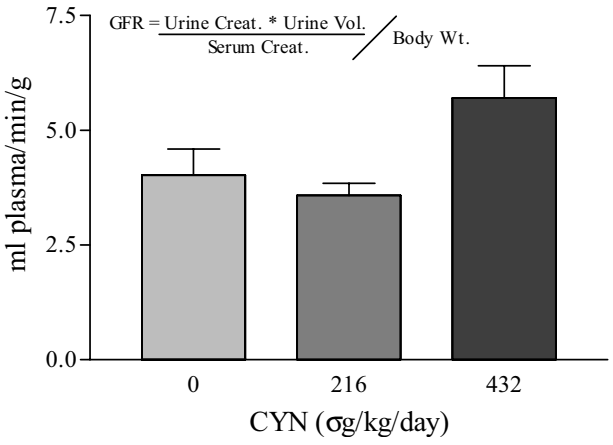

Figure 21. Experiment 1: Glomerular filtration rate (GFR, ml/min/g). Kruskal-Wallis p=0.0754

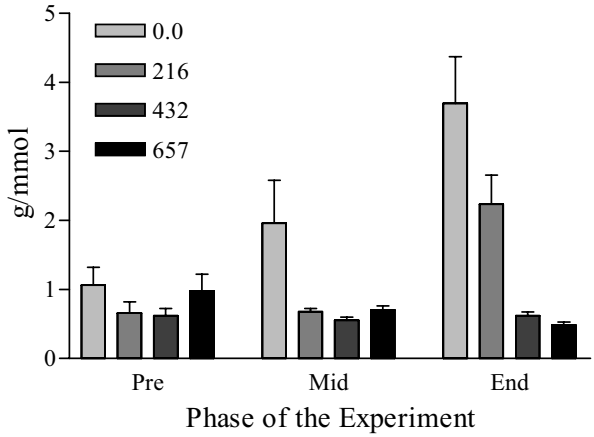

Figure 22. Experiment 1: Change in total protein/creatinine (g/mmol) over the course of the experiment.

Repeated measures 2-way ANOVA:

Source of Variation	% of total variation	P value
Interaction	15.85	P<0.0001
Time	9.42	P<0.0001
Dose	23.72	0.0001
Subjects (matching)	20.7760	0.0068

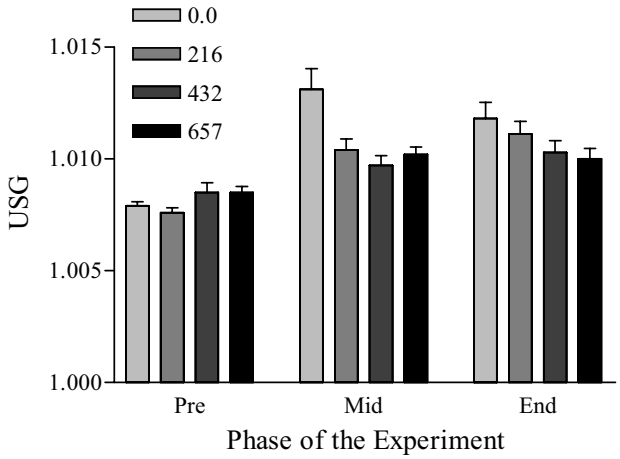

Figure 23. Experiment 1: Change in urine specific gravity over the course of the experiment.

Two-way ANOVA
(not Repeated Measures as Matching not significant):

Source of Variation	% of total variation	P value
Interaction	9.55	0.0034
Dose	7.21	0.0020
Time	33.98	P<0.0001

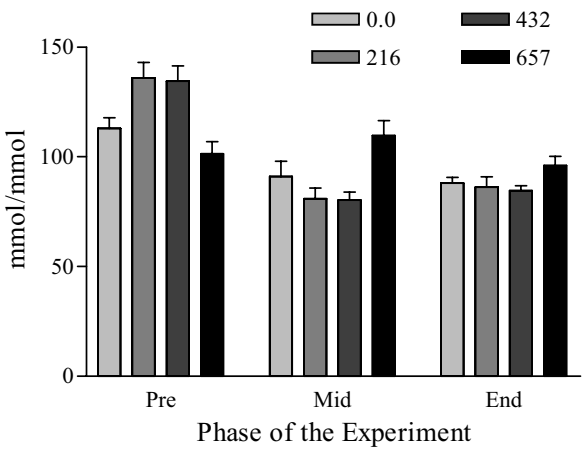

Figure 24. Experiment 1: Change in sodium/creatinine (mmol/mmol) over the course of the experiment.

Repeated measures 2-way ANOVA:

Source of Variation	% of total variation	P value
Interaction	20.26	P<0.0001
Time	37.06	P<0.0001
Dose	0.56	0.8944
Subjects (matching)	25.1058	P<0.0001

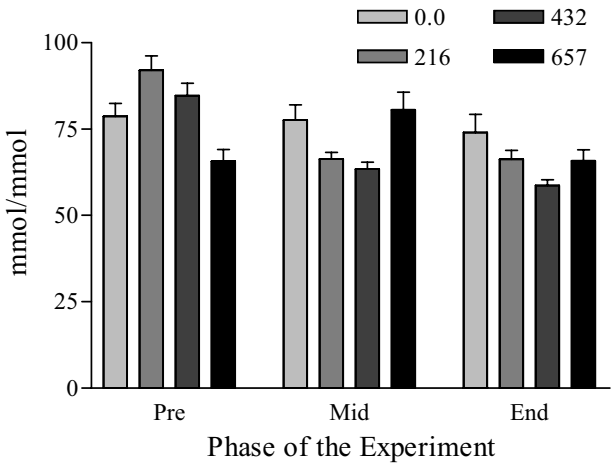

Figure 25. Experiment 1: Change in potassium/creatinine (mmol/mmol) over the course of the experiment.

Repeated measures 2-way ANOVA:

Source of Variation	% of total variation	P value
Interaction	23.22	P<0.0001
Time	15.83	P<0.0001
Dose	4.74	0.2409
Subjects (matching)	28.6959	P<0.0001

3.2.5 Tissue histology

Liver, spleen, lung, stomach, duodenum, pancreas and kidney sections were examined from two mice at each dose level in the experiment. In both livers examined from mice receiving 432 µg/kg/day of cylindrospermopsin in the cyanobacterial extract there was evidence of hepatocyte damage not seen in mice at a lower dose of extract or in the controls. Kidney tissue from a range of mice examined, including controls, showed white cell invasion and evidence of tubular degeneration. The most severe degeneration was visible in kidneys of mice receiving the highest concentration of extract, which had been noted to have a much reduced water consumption and consequent dehydration. A mouse in this group had a polyp in the duodenum. There was no dose-related injury seen in the other tissues examined.

3.3 Trial 2: Purified CYN Daily By Gavage

3.3.1 Clinical examination

In addition to daily inspection during gavage, the mice were formally examined for clinical signs of toxicity after 9 weeks of treatment. The examination included: Skin, fur, eyes, mucous membranes, respiration, pupil size, gait, posture, grooming, behaviour pattern, noise response, visual response, touch response, grip strength, motor activity, and evidence of lachrymation or abnormal excretions. No adverse effects were seen in any of the mice.

3.3.2 Post-mortem examination

No treatment-related pathology was observed in any organ or tissue of any mouse at post-mortem examination. Tissues were excised and fixed for histological examination as listed in Table 2.

3.3.3 Effects on mouse body weight and the weights of major organs

Individual body weights and water consumption per cage were monitored throughout the trial. The results, averaged for the last 4 weeks of the trial, are shown in Figs 26 and 27. Average mouse weight was significantly increased with respect to controls in the 30 and 60 µg/kg/d groups. Water consumption was significantly reduced in all treatment groups by this stage of the trial.

n =	10	10	9	9	6
mean =	32	35.6	36.8	35.4	34.3
% of control		119	115	111	107

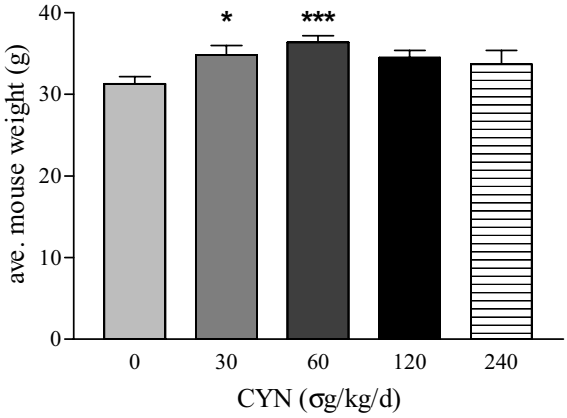

Figure 26. Experiment 2: Average mouse weight over the last 4 weeks of the experiment. One way ANOVA, Dunnett's MCT * p<0.05, *** p<0.001

n =	8	8	8	8	4
mean =	184.2	97.1	125.3	131.9	124.7
% of control		53	68	72	68

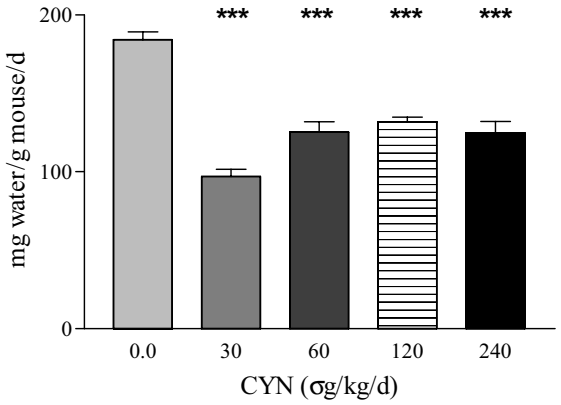

Figure 27. Experiment 2: Average mouse water consumption over the last 4 weeks of the experiment. One way ANOVA, Dunnett's MCT *** p<0.001

Weights of the liver, spleen, adrenals, kidneys, epididymes, testes, heart, thymus, and brain were recorded at post mortem (see Table 2). Liver weight was significantly increased in the 60 and 240 μg/kg/d groups (Fig 28), as were the adrenals in the 120 μg/kg/d group (Fig 29), and the kidneys and testes in the 60, 120, and 240 μg/kg/d groups (Figs 30 and 31). However, when these data were normalised to bodyweight, only percent liver weight in the 240 μg/kg/d group, and the percent kidney weight in the 60, 120, and 240 μg/kg/d groups remained significantly different from control (Figs 32 and 33).

n =	10	10	9	9	6
mean =	1.69	1.92	2.13	2.02	2.08
% of control		114	126	119	123

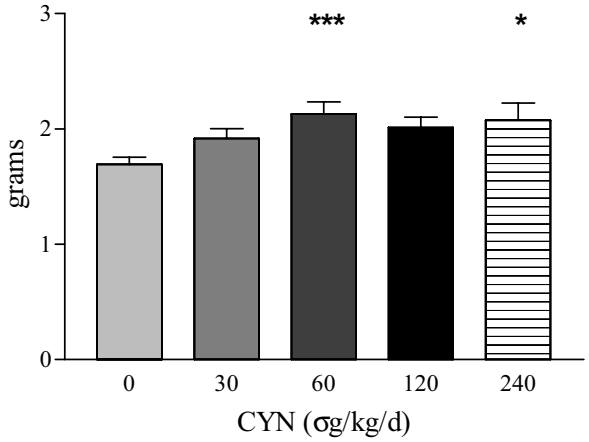

Figure 28. Experiment 2: Liver weight.
One way ANOVA, Dunnett's MCT* p<0.05, *** p<0.001

n =	10	10	9	9	6
mean =	0.0054	0.0063	0.0067	0.0078	0.0074
% of control		117	124	144	137

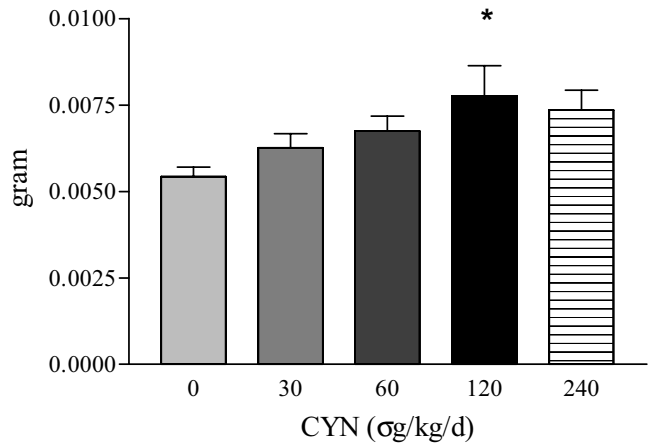

Figure 29. Experiment 2: Adrenals weight.
One way ANOVA, Dunnett's MCT* $p<0.05$

n =	10	10	9	9	6
mean =	0.47	0.56	0.61	0.64	0.61
% of control		119	130	136	130

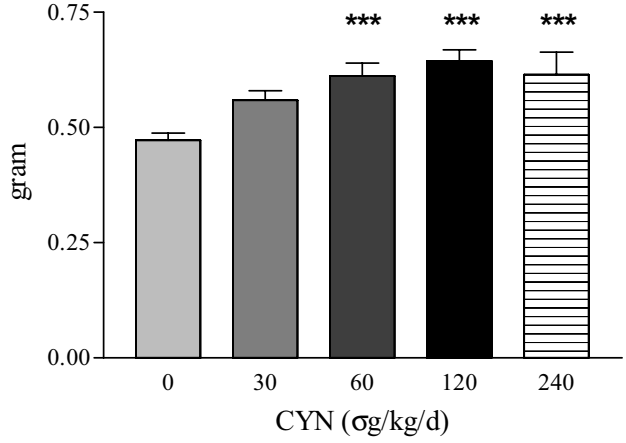

Figure 30. Experiment 2: Kidneys weight.
One way ANOVA, Dunnett's MCT*** $p<0.001$

n =	10	10	9	9	6
mean =	0.19	0.22	0.23	0.23	0.23
% of control		116	121	121	121

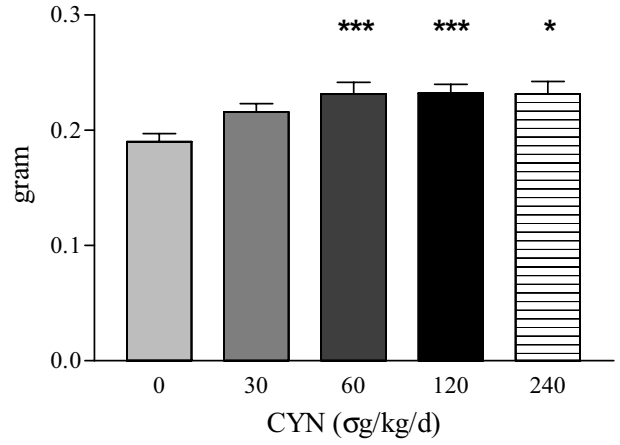

Figure 31. Experiment 2: Testes weight.
One way ANOVA, Dunnett's MCT * p<0.05, *** p<0.001

n =	10	10	9	9	6
mean =	5.3	5.4	5.8	5.7	6.0
% of control		102	109	108	113

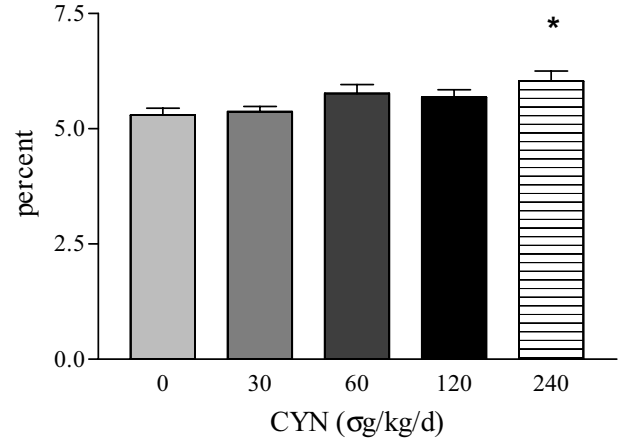

Figure 32. Experiment 2: Liver weight as a percentage of bodyweight.
One way ANOVA, Dunnett's MCT * p<0.05

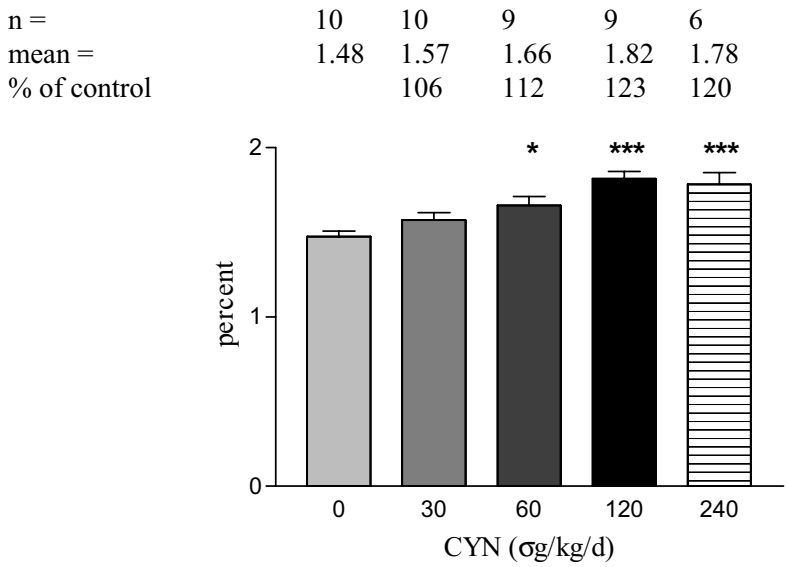

Figure 33. Experiment 2: Kidney weight as a percentage of bodyweight. One way ANOVA, Dunnett's MCT * $p<0.05$, *** $p<0.001$

3.3.4 Effects on serum parameters

A full range of serum parameters were measured (see Table 3), however only cholesterol and glucose showed significant treatment effects by one way ANOVA. It is relevant that the group size was smaller for the serum analyses in this experiment due to the requirement for blood for haematology, so a similar degree of effect to that seen in experiment 1 will be less likely to be found statistically significant in experiment 2. For cholesterol (Fig 34), the 30 and 60 μg/kg/d groups, but not the 120 and 240 μg/kg/d groups, had significantly higher levels than control by Dunnett's post-hoc test. There was a statistically significant reduction in glucose concentration with increasing dose assessed by one-way ANOVA, but due to low sample numbers no individual treatment was significantly different from control (Fig 35). Serum urea, albumin, and total bile acids appeared to have biologically interesting trends but statistical significance was not demonstrated (Figs 36 - 39). Similarly serum enzyme activities did not show significant changes with dose, nor did serum anions or cations, triglycerides, creatinine, total protein or globulin (Figs 40 and 41).

n =	5	5	4	5	6
mean =	3.26	4.60	4.65	3.68	4.08
% of control		141	143	113	125

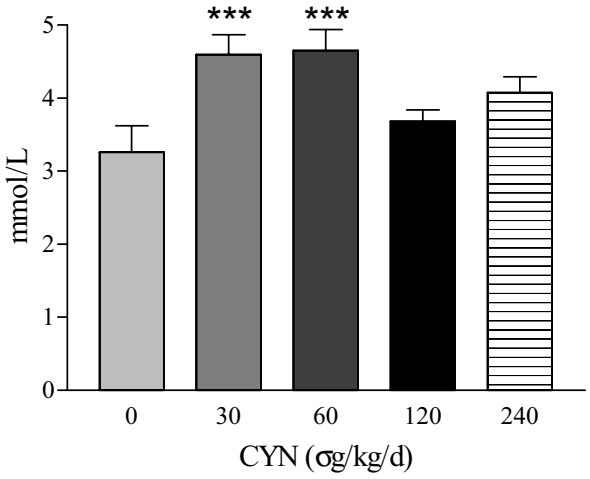

Figure 34. Experiment 2: Serum cholesterol concentration (mmol/L). One way ANOVA, Dunnett's MCT *** p<0.001

n =	5	5	4	5	6
mean =	9.52	9.80	10.8	8.08	7.22
% of control		103	114	85	76

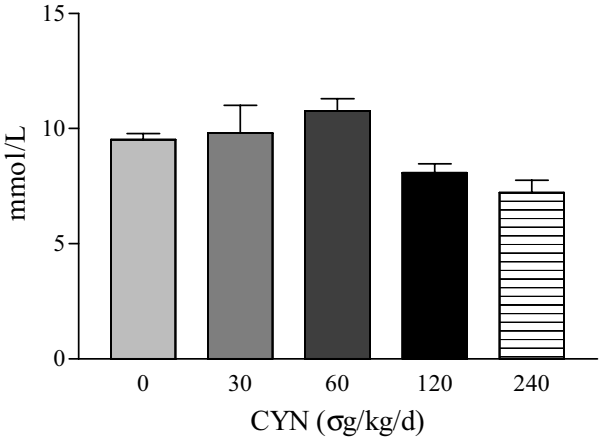

Figure 35. Experiment 2: Serum glucose concentration (mmol/L). One way ANOVA p=0.0104, Dunnett's MCT none sig.

n =	5	5	4	5	6
mean =	9.24	9.22	8.55	7.54	7.92
% of control		100	93	82	86

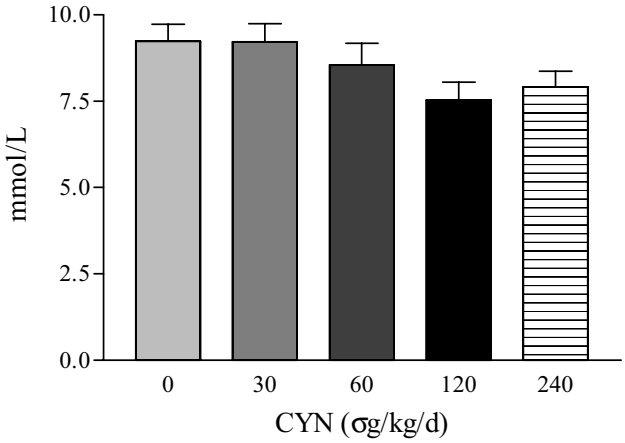

Figure 36. Experiment 2: Serum urea concentration (mmol/L).
One way ANOVA p=0.0986

n =	5	5	4	5	6
mean =	23.8	26.6	26.0	26.0	25.8
% of control		112	109	109	108

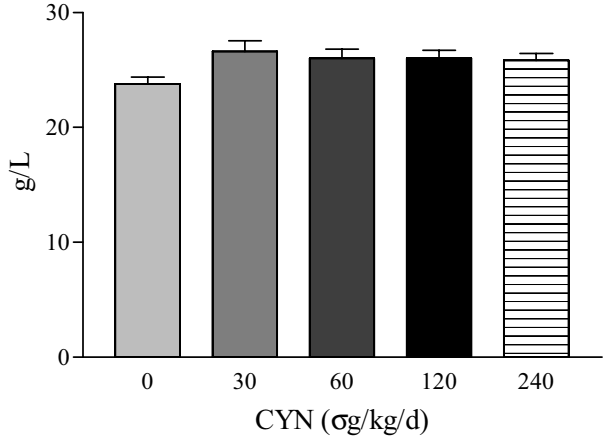

Figure 37. Experiment 2: Serum albumin concentration (mmol/L).
One way ANOVA p=0.1095

n =	4	4	3	5	4
mean =	8.5	7.8	5.5	6.4	6.6
% of control		92	65	75	78

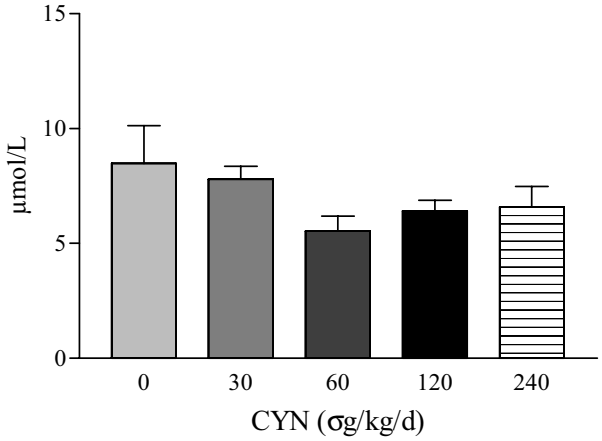

Figure 38. Experiment 2: Serum total bile acids concentration (µmol/L). One way ANOVA p=0.2742

n =	5	5	4	5	6
mean =	2.62	2.72	2.88	3.06	3.07
% of control		104	110	117	117

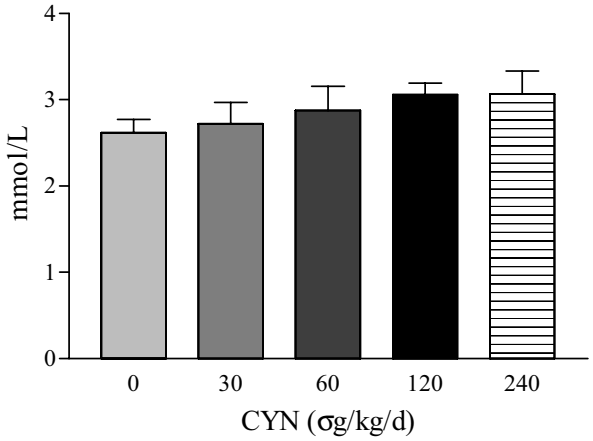

Figure 39. Experiment 2: Serum total bilirubin concentration (mmol/L). One way ANOVA p=0.4874

n =	5	5	4	5	6
mean =	157.0	156.2	154.0	155.4	155.2
% of control		99.5	98.1	99.0	98.9

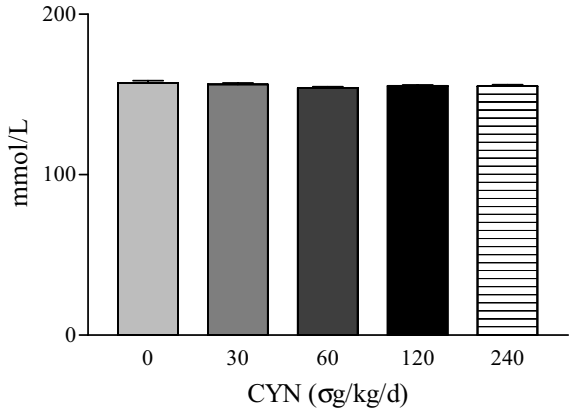

Figure 40. Experiment 2: Serum sodium ion concentration (mmol/L).
One way ANOVA p=0.3626

n =	5	5	4	5	6
mean =	112.4	112.2	111.5	110.4	109.8
% of control		99.8	99.2	98.2	97.7

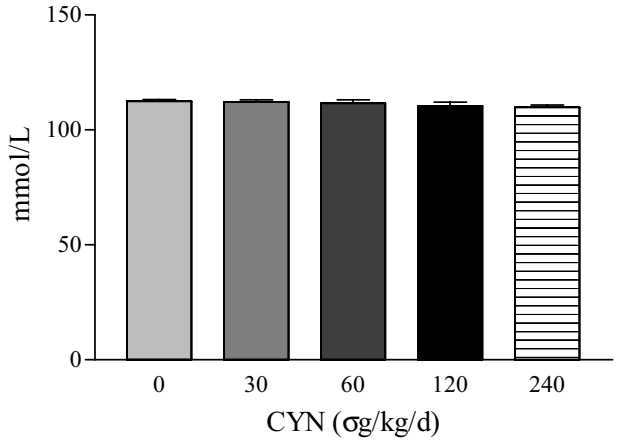

Figure 41. Experiment 2: Serum chloride ion concentration (mmol/L).
One way ANOVA p=0.4529

3.3.5 Effects on urine parameters

Urine parameters were sensitive to the effects of the toxin, more parameters reaching significant differences with dose rate than were seen with serum parameters. The highest dose rate of CYN suppressed water consumption to 68% of control, whereas lower doses did not (Fig 42). The decreased volume at this dose is reflected in increased urine creatinine concentration (Fig 43). Expressing the urine concentration data as parameter per unit creatinine removes variations due to urine volume changes. Urine specific gravity per unit creatinine was significantly reduced at 240 μg/kg/d, urine protein was reduced at 120, and 240 μg/kg/d, urine sodium was reduced in the 30 and 240 μg/kg/d groups, and urine potassium was reduced in all groups (Figs 44 - 47). However the potassium results showed some instability in testing, suggesting possible interfering substances present in the mouse urines. Hence the low potassium results may not be valid and should be interpreted with caution. Urine phosphate showed a statistically significant treatment effect, but no individual dose was different from control (Fig 48). Renal failure index and glomerular filtration rate were not significantly altered (Figs 49 and 50).

n =	10	10	9	9	6
mean =	9.85	11.18	10.38	11.74	6.74
% of control		114	105	119	68

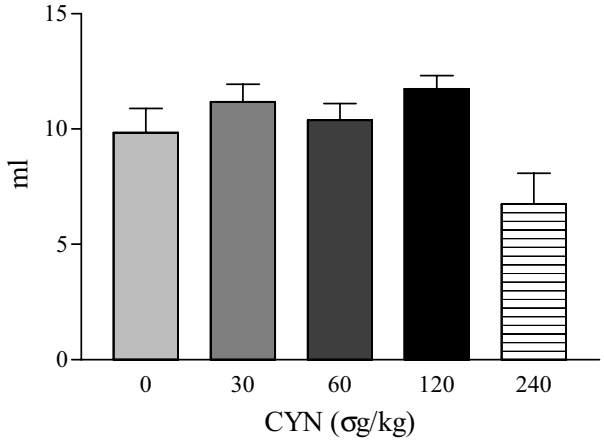

Figure 42. Experiment 2: Urine volume (ml).
One way ANOVA p=0.0107, Dunnett's MCT none sig.

n =	10	10	9	9	6
mean =	0.57	0.49	0.54	0.51	0.72
% of control		86	95	90	126

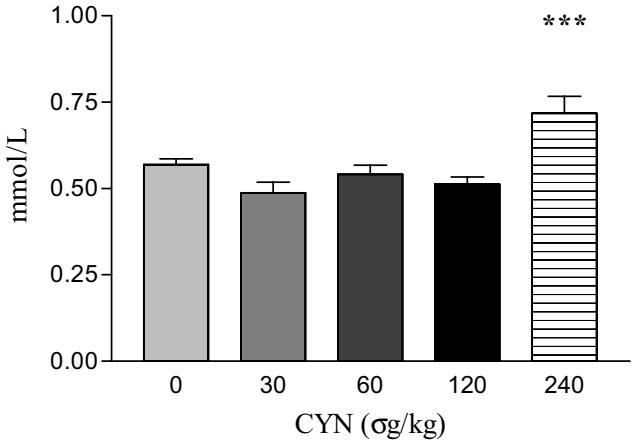

Figure 43. Experiment 2: Urine creatinine concentration (mmol/L). One way ANOVA, Dunnett's MCT *** p<0.001

n =	10	9	9	9	6
mean =	1.79	2.04	1.91	1.99	1.44
% of control		114	107	111	80

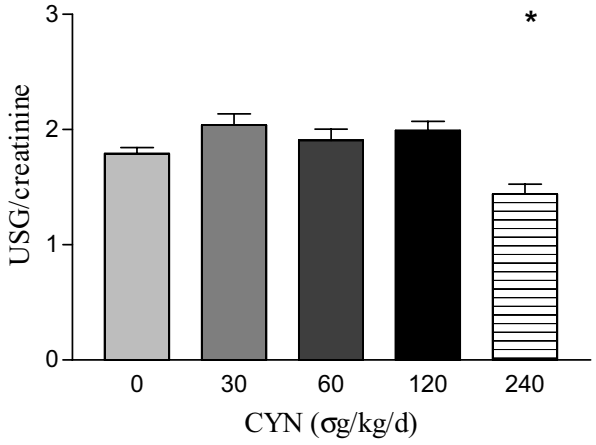

Figure 44. Experiment 2: Urine specific gravity/creatinine. One way ANOVA, Dunnett's MCT * p<0.05

n =	10	10	8	9	6
mean =	4.3	3.6	3.3	2.2	1.6
% of control		70	77	51	37

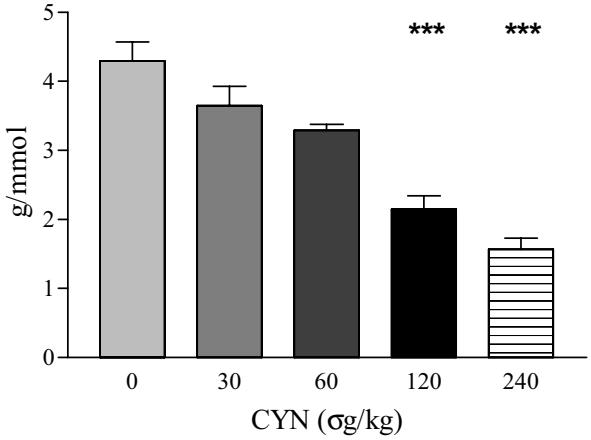

Figure 45. Experiment 2: Urine protein/creatinine (g/mmol). Kruskal-Wallis, Dunn's MCT *** p<0.001

n =	10	10	9	9	6
mean =	132.3	121.2	127.5	127.1	107.2
% of control		92	96	96	81

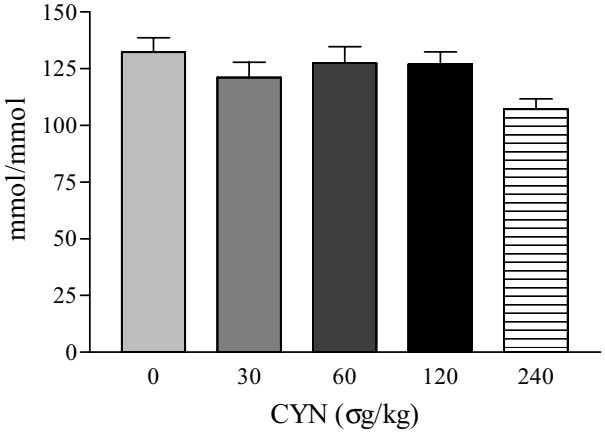

Figure 46. Experiment 2: Urine sodium/creatinine (mmol/mmol). One-way ANOVA p=0.1445

n =	10	10	9	9	6
mean =	68.3	51.1	51.8	49.5	51.5
% of control		75	76	73	75

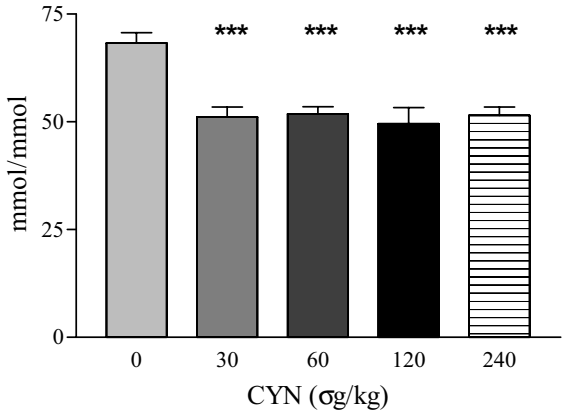

Figure 47. Experiment 2: Urine potassium/creatinine (mmol/mmol). One-way ANOVA, Dunnett's MCT *** p<0.001

n =	10	10	9	9	6
mean =	10.9	8.9	11.2	8.8	7.9
% of control		82	103	81	73

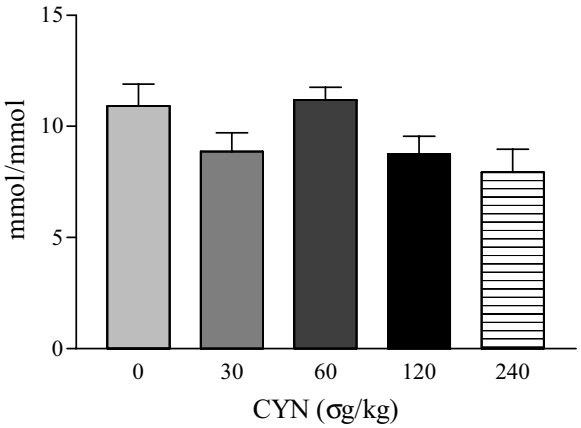

Figure 48. Experiment 2: Urine phosphate/creatinine (mmol/mmol). One-way ANOVA p=0.0483, Dunnett's MCT none sig.

n =	5	5	4	5	6
mean =	4.3	4.3	4.5	3.6	3.6
% of control		100	105	84	84

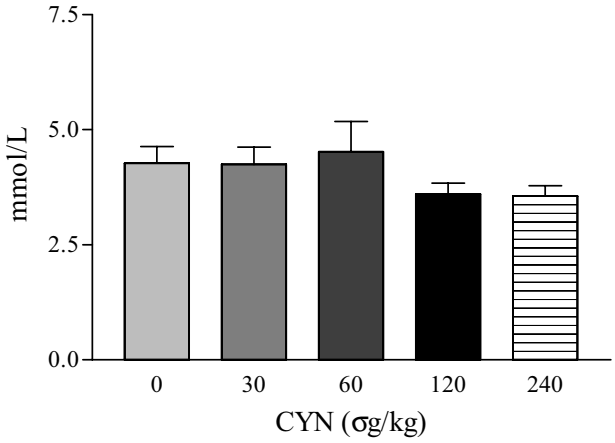

Figure 49. Experiment 2:Renal failure index.
One-way ANOVA p=0.2697

n =	5	5	4	5	6
mean =	6.24	4.54	4.53	5.80	4.00
% of control		73	73	93	64

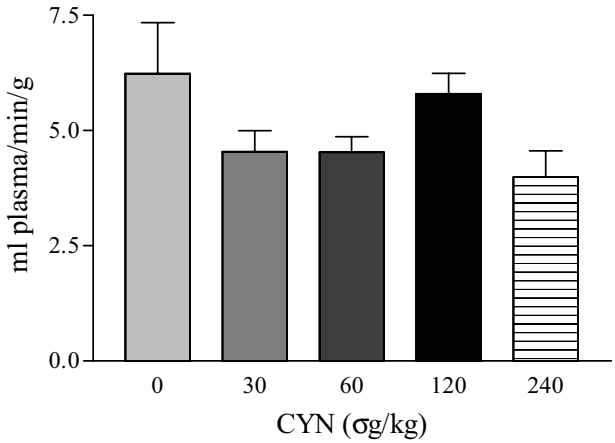

Figure 50. Experiment 2:Glomerular filtration rate.
One-way ANOVA p=0.1112

3.3.6 Effects on haematological parameters

There was no difference between treatments in red cell counts or in any of the other erythrocyte parameters (Hb, PCV, MCV, MCH, MCHC, Table 5). However, there was a non-significant rise in white cell numbers in the 30 μg/kg/d group, and this was found to be due to a significant increase in lymphocyte rather than neutrophil numbers in this group (see Figs 51 - 53). Clotting time was analysed using Prothrombin Time and Activated Partial Thrombin Time. No significant effects were observed.

n =	5	5	5	4
mean =	3.44	5.48	3.72	3.73
% of control		159	108	108

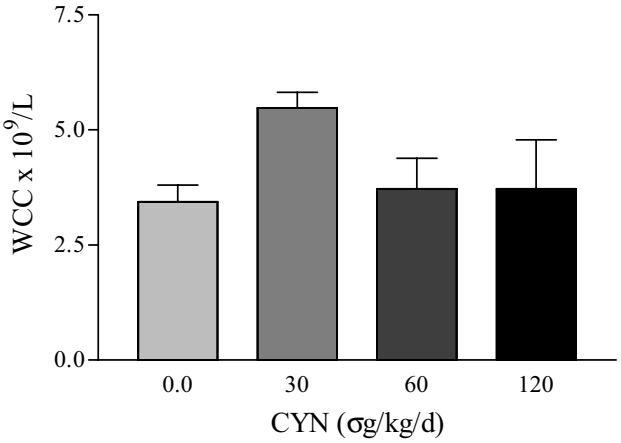

Figure 51. Experiment 2:White cell count.
One-way ANOVA p=0.1091

n =	5	5	5	4
mean =	2.02	1.88	1.90	1.98
% of control		93	94	98

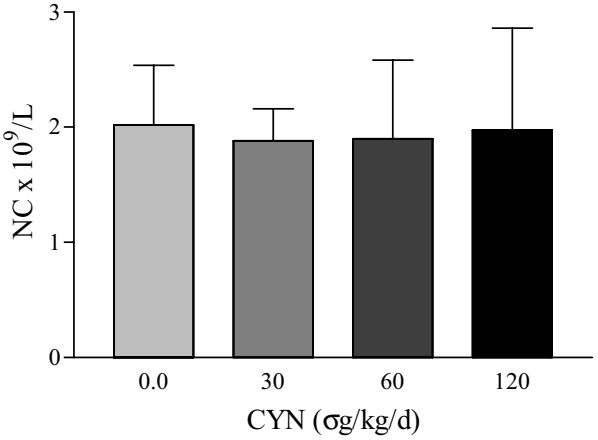

Figure 52. Experiment 2:Neutrophil count.
One-way ANOVA p=0.9981

n =	5	5	5	4
mean =	1.40	3.42	1.76	1.75
% of control		244	126	125

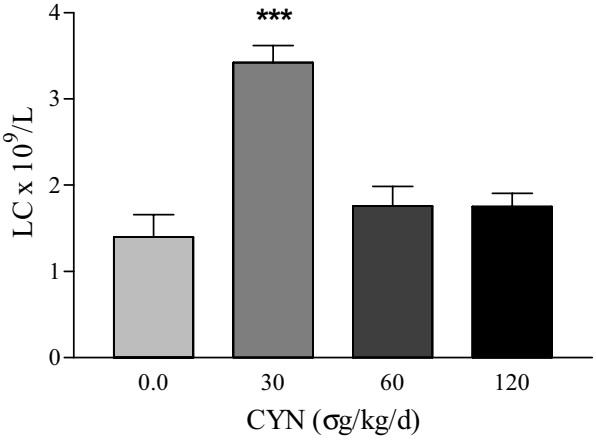

Figure 53. Experiment 2: Lymphocyte count.
One-way ANOVA, Dunnett's MCT *** p<0.001

3.3.7 Organ and Tissue Histopathology

The organs and tissues listed in Table 2 were examined for histopathological evidence of injury or adverse treatment effects. Initially the sections from all organs and tissues from control mice and from the 240 μg CYN/kg/d group were examined as blind specimens. Where any pathological evidence was observed the sections were scored for extent of injury. Only in liver sections were any clearly dose-related changes seen. None of the other organs which showed injury in previous higher dose or acute experiments were observed to have dose–related injury in the 240 μg CYN/kg/d group.

Following identification of liver injury, all liver sections from mice at all dose rates were examined as blind samples for histopathological evidence of injury or adverse histological changes. Sections were scored for extent of injury, examining in particular cellular necrosis or past evidence of necrosis, inflammatory foci and bile duct changes. Dose-related injury was observed, with an increase in histological damage over controls at 120 and 240 μg/kg/d (Table 6)

Table 6. Percentage of mice in group with necrotic or inflammatory foci in liver section

Treatment (CYN μg/kg/d)	Mice affected (%)
0.0	10
30	10
60	20
120	60
240	90

In the 240 μg CYN/kg/d group of mice, one kidney section showed substantial proximal tubule damage similar to that seen in the previous drinking water CYN trial and another section showed small groups of proximal tubule cells degenerating with leucocyte infiltration. None of the lower dose rates or the controls showed any histopathological evidence of kidney injury. None of the other organs which showed injury in previous higher dose or acute experiments were observed to have dose–related injury in the 240 μg CYN/kg/d group.

Table 7. Summary of data from both trials (Key at bottom of table)

	Dose as σg CYN/kg/d						
	Trial 2				Trial 1		
Parameter	**30**	**60**	**120**	**240**	**216**	**432**	**657**
Body weight	ŷŷŷ	ŷŷŷ	ŷ	ŷ	Ź	ŹŹŹ	ŹŹŹ
% Liver weight	ź	ŷ	ŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ
% Kidney weight	ŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ	ŷŷŷ
% Spleen weight	ź	ŷ	ŷ	ŷ	Ź	Ź	ŷ
% Brain weight	Ź	Ź	Ź	ź	nd	nd	nd
% Thymus weight	ź	ź	ŷ	ź	nd	nd	nd
% Adrenals weight	ź	ŷ	ŷ	ŷ	nd	nd	nd
% Heart weight	Ź	ź	ź	ź	nd	nd	nd
% Testes weight	ź	ŷ	ŷ	ŷ	nd	nd	nd
% Epididymes weight	ź	ź	ŷ	ź	nd	nd	nd

Continued over…

Key

Significantly reduced	ŹŹŹ	Non-significant increase [a]	ŷ
Non-significant reduction [a]	Ź	Significantly increased	ŷŷŷ
No change from control	ź	Not determined	nd

Notes: [a] Mean of treatment group falls outside SEM of control group mean.

Table 7. Summary of data from both trials (Key at bottom of table) – continued

	Dose as σg CYN/kg/d						
	Trial 2				Trial 1		
Parameter	**30**	**60**	**120**	**240**	**216**	**432**	**657**
Serum							
Total protein	ŷ	ŷ	ŷ	ŷ	ŷ	Ź	nd
Albumin	ŷ	ŷ	ŷ	ŷ	ŷŷŷ	ŷŷŷ	nd
Globulins	ź	ŷ	ź	ŷ	Ź	ŹŹŹ	nd
Total bilirubin	ź	ŷ	ŷ	ŷ	ŷŷŷ	ŷŷŷ	nd
Total bile acids	ź	Ź	Ź	Ź	ŹŹŹ	ŹŹŹ	nd
Cholesterol	ŷŷŷ	ŷŷŷ	ŷ	ŷ	nd	nd	nd
Triglycerides	Ź	ŷ	ŷ	Ź	nd	nd	nd
Alkaline phosphatase	ŷ	ź	ź	ź	ź	ŷŷŷ	nd
Alanine aminotransferase	ŷ	ź	ź	ŷ	Ź	ŷ	nd
Aspartate aminotransferase	ź	Ź	Ź	ź	ŹŹŹ	Ź	nd
Creatine kinase	ŷ	ź	ź	ŷ	nd	nd	nd
Lipase	nd	nd	nd	nd	ź	ŷ	nd
Glucose	ź	ŷ	Ź	Ź	nd	nd	nd
Creatinine	ź	ź	ź	ź	ź	ŹŹŹ	nd
Urea	ź	ź	Ź	Ź	ź	ź	nd
Sodium	ź	Ź	Ź	Ź	nd	ź	nd
Potassium	Ź	ź	ź	ź	nd	ź	nd
Chloride	ź	ź	Ź	Ź	nd	ŷŷŷ	nd
Bicarbonate	Ź	ź	ŷ	ź	Ź	ź	nd
Anion gap	ź	Ź	ź	ź	ź	Ź	nd
Phosphate	ź	Ź	Ź	Ź	ŹŹŹ	ŹŹŹ	nd
Calcium	ź	ź	ź	ź	ź	ź	nd
pH	nd	nd	nd	nd	ŷŷŷ	ŷŷŷ	ź

Continued over…

Key

Significantly reduced	ŹŹŹ	Non-significant increase [a]	ŷ
Non-significant reduction [a]	Ź	Significantly increased	ŷŷŷ
No change from control	ź	Not determined	nd

Notes: [a] Mean of treatment group falls outside SEM of control group mean.

Table 7. Summary of data from both trials (Key at bottom of table) – continued

	Dose as σg CYN/kg/d						
	Trial 2				Trial 1		
Parameter	**30**	**60**	**120**	**240**	**216**	**432**	**657**
Urine							
USG/creatinine	ź	ź	ź	ŹŹŹ	ź	Ź	ź
Protein/creatinine	Ź	Ź	ŹŹŹ	ŹŹŹ	Ź	ŹŹŹ	ŹŹŹ
Glucose/creatinine	ŷ	ź	ź	ź	Ź	Ź	Ź
Ketones	nd	nd	nd	nd	ź	ź	ź
Sodium/creatinine	ź	ź	ź	Ź	ź	Ź	ź
Potassium/creatinine	ŹŹŹ	ŹŹŹ	ŹŹŹ	ŹŹŹ	Ź	ŹŹŹ	Ź
Chloride/creatinine	nd	nd	nd	nd	ź	Ź	ź
Bicarbonate/creatinine	nd	nd	nd	nd	Ź	ŷ	ź
Phosphate/creatinine	Ź	ź	Ź	Ź	ź	ŹŹŹ	ź
Calcium/creatinine	nd	nd	nd	nd	ŷ	ź	ŷ
pH	ŷŷŷ	ź	ź	ź	Ź	ź	ŹŹŹ
Blood cells (positive/total)	0/10	0/9	0/9	1/6	nd	nd	nd
Renal failure index	ź	ź	Ź	Ź	ź	ŹŹŹ	nd
Glomerular filtration rate	Ź	Ź	ź	Ź	ź	ŷ	nd
Haematology							
White cell count	ŷ	ź	ź	nd	nd	nd	nd
Lymphocytes	ŷŷŷ	ź	ź	nd	nd	nd	nd
Neutrophils	ź	ź	ź	nd	nd	nd	nd
Other white cells	ź	ź	ź	nd	nd	nd	nd
Red cell count	ź	ź	ŷ	nd	nd	nd	nd
Haemoglobin	Ź	ź	ź	nd	nd	nd	nd
Packed cell volume	ŷ	ŷ	ŷ	nd	nd	nd	nd
Mean cell volume	ź	ź	ź	nd	nd	nd	nd
Mean cell haemoglobin	Ź	Ź	Ź	nd	nd	nd	nd

Continued over…

Key

Significantly reduced	ŹŹŹ	Non-significant increase [a]	ŷ
Non-significant reduction [a]	Ź	Significantly increased	ŷŷŷ
No change from control	ź	Not determined	nd

Notes: [a] Mean of treatment group falls outside SEM of control group mean.

Table 7. Summary of data from both trials (Key at bottom of table) – continued

	Dose as σg CYN/kg/d						
	Trial 2					Trial 1	
Parameter	**30**	**60**	**120**	**240**	**216**	**432**	**657**
Mean corpuscular Hb conc.	Ź	Ź	Ź	nd	nd	nd	nd
Red cell polychromasia	ŷ	ŷ	ŷ	nd	nd	nd	nd
Red cell anisocytosis	ź	ź	ź	nd	nd	nd	nd
Appearance: Platelets	ź	ź	ź	nd	nd	nd	nd
Prothombin time	ŷ	ź	ź	nd	nd	nd	nd
Activated partial thrombin time	ŷ	ŷ	ŷ	nd	nd	nd	nd

Key

Significantly reduced	ŹŹŹ	Non-significant increase [a]	ŷ
Non-significant reduction [a]	Ź	Significantly increased	ŷŷŷ
No change from control	ź	Not determined	nd

Notes: [a] Mean of treatment group falls outside SEM of control group mean.

3.4 Comparison of the two experiments

Overall the type and direction of effects seen as a result of exposure to oral CYN were reasonably consistent between the experiments, although the magnitude of effect differed between parameters. Organ weights as a % of body weight illustrate this as progressive increases in liver and kidney weights occur with dose across both experiments (Fig 3, 4: 32, 33)

Changes in serum parameters are harder to interpret because in experiment 2 group sizes are only half those of experiment 1 due to half the blood samples being used for haematology. Therefore, changes in parameters of similar size are more likely to reach statistical significance in experiment 1 than in experiment 2. There are also differences between the control levels of some serum parameters in the two experiments For example, serum albumin was raised 108% at a dose of 240 μg/kg/d from a control value of 25.8 g/L in experiment 2 (non-significant) and 111% at 216 μg/kg/d from a control value of 23.8 g/L in experiment 1 (significant). Similarly, serum bilirubin was raised 112% at a dose of 240 μg/kg/d from a control value of 2.74 mmol/L in experiment 1 (non-significant), and 129% at 216 μg/kg/d from a lower control value of 2.06 mmol/L in experiment 2 (significant).

The urine parameter group sizes were comparable between the two experiments Control values were somewhat different, but these differences were not as large as the treatment effects. In experiment 2, urine protein/creatinine was reduced to 37% of control (4.3 g/mmol) at 240 μg/kg/d and to 51% at 120 μg/kg/d, whereas in experiment 1 it was reduced (non-significant) to 60% of control (3.72 g/mmol) at 216 μg/kg/d

Urine sodium/creatinine was not markedly affected in either experiment. Urine volume was slightly increased (~10%) in the 3 lower experiment 2 treatment groups, but any slight dilution effect this had should have been controlled by normalising the parameters to creatinine (see reciprocity of creatinine and urine volume graphs).

Histopathological damage was seen in livers of higher dose groups in both trials, though accurate quantitation was not possible. At or above 120 μg/kg/d hepatocyte injury was increased. In the gavage trial at 240 μg/kg/d proximal tubule degeneration was observed.

4. DISCUSSION

4.1 Resumé of results

In determining the Lowest Observed Adverse Effect Level (LOAEL) and the No Observed Adverse Effect Level (NOAEL) for toxin exposure, dose rates are employed that cause minimal injury. At very low toxin doses, the metabolic processes which underlie homeostasis will cause compensating changes to maintain key physiological parameters. The most apparent of these is organ growth, where the toxin affects organ function. A parallel which is well understood is the overgrowth of the thyroid gland resulting from iodine deficiency in the diet or a genetic defect in hormone biosynthesis (Falconer, 1965). It can therefore be anticipated that a toxin with demonstrated function as a blocking agent in cell metabolism, such as cylindrospermopsin which inhibits protein synthesis (Terao et al., 1994; Froscio et al., 2001), will cause growth of organs which synthesise proteins required for homeostasis. These increases may be regarded as compensatory hypertrophy, not necessarily pathological in nature.

Bodyweight itself may be affected by small changes in metabolic efficiency, as well as changes in food or water intake. In a situation where protein synthesis is restricted by a toxin, without a change in food intake, the additional available energy is likely to be stored as fat, hence increasing bodyweight. In this study body weight increased at low toxin doses (30 and 60 μg/kg/d; with non significant increases at 120 and 240 μg/kg/d) and decreased at high doses (432 and 657 μg/kg/d). Decreased bodyweight following toxin exposure was clearly an adverse effect, indicating non-compensated metabolic injury. Thus the dose range employed in this study ranged from toxin exposure capable of homeostatic compensation to that causing adverse effects. Expressing organ weights as a percentage of body weight, increased liver and kidney weights were observed as dose increased (liver, significant increases at: 216, 240, 432 and 657 μg/kg/d, non-significant increases at 60 and 120 μg/kg/d; kidney, significant increases at 60-657 μg/kg/d, non-significant increase at 30 μg/kg/d).

In the serum, albumin and bilirubin levels were increased with increasing toxin exposure, whereas bile acids and phosphate concentrations were reduced. Serum glucose was reduced at increasing toxin doses. In the urine, protein and possibly potassium excretion was reduced, while phosphate and sodium may also have been reduced but the variable effects in different dose groups make this uncertain. In experiment 1, urine analyses at three different stages of the experiment showed that this relative decrease in loss of protein to the urine occurred progressively against a background of increasing urine protein in the controls. A decrease in renal protein loss could be a response to reduced protein production in the kidney. The increased serum albumin may reflect the increased size of the liver, which is responsible for the synthesis of this serum protein. It is, however, difficult to reconcile the action of a protein synthesis inhibitor with an increase in a serum protein. This increase was also observed when serum albumin was expressed as a percentage of total serum proteins, possibly indicating a shift in the mix of proteins produced.

Serum bilirubin was non-significantly increased at 60 - 240 μg/kg/d in parallel with the similar trend in albumin in this dose range, and may be due to the increased binding capacity that the albumin represents. However, at 216 μg/kg/d and especially at 432 μg/kg/d, the increase in serum bilirubin is greater than the albumin increase, indicating a more seriously reduced hepatic function at these doses. Serum bile acids follow a similar but inverse pattern to bilirubin, also indicating changes in hepatic function

The clinical indicator enzymes for liver injury in diseased states, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) do not appear to be sensitive to this toxin in the mouse. Serum ALP is only raised significantly in the 432 μg/kg/d dose group, and the other enzymes show no significant changes. The elevation of ALP is low in comparison with changes in hepatobiliary disease (Loeb and Quimby,1989), and may relate to the long-term low dose exposures to a protein synthesis inhibiting toxin in these experiments. The rise in serum cholesterol at the lower doses of 30 and 60 μg/kg/d (non-significant increases at 120 and 240 μg/kg/d) is difficult to interpret, but may indicate changes in lipoprotein production in the liver due to the toxin.

An increase in lymphocyte numbers was seen in the 30 μg/kg/d group, possibly indicating a low grade infection. However, this finding was consistent across most of the mice housed in two separate cages in this group, but did not recur in other toxin doses, and thus remains an anomaly.

4.2 Conclusions

The preliminary conclusion from trial 1 in which *C. raciborskii* extract was provided in the drinking water experiment was that all doses employed caused statistically significant adverse changes compared to

control animals. The lowest dose of cylindrospermopsin of 216 μg/kg/d raised %liver weight, %kidney weight, serum albumin and total bilirubin concentrations and depressed total serum bile acid and phosphate concentrations and urine potassium/creatinine and protein/creatinine ratios.

Therefore, the NOAEL must be less than 216 μg/kg/d.

In trial 2, employing oral gavage of purified cylindrospermopsin, mouse body weight was elevated at the 30 and 60 μg/kg/d doses, with liver, kidney and testis weights increased at 60 μg/kg/d and above. As a percentage of body weight, kidney weight increased at 60 μg/kg/d and above, whereas liver weight was only significantly increased at 240 μg/kg/d. Similar organ weight changes were also observed in trial 1.

At 120 and 240 μg/kg/d doses there was increased histopathological damage to the liver, and at 240 μg/kg/d damage to the proximal tubules of the kidney. Haematology showed an increase of lymphocytes at the 30 μg/kg/d dose only, however no significant changes were seen in bone marrow smears or the spleen.

Serum parameters showed little change with CYN dose, however some indication of a change was observed between the controls, 120 and 240 μg/kg/d doses, similar to those seen at a higher dose in experiment 1. Serum glucose and phosphate appeared reduced at 120 and 240 μg/kg/d doses, however only an increase in serum cholesterol at 30 and 60 μg/kg/d was statistically significant. The indicators of liver injury, serum enzymes alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total bilirubin did not show statistically significant changes between 30 and 240 μg/kg/d doses, however mean serum ALT and AST did (non significantly) increase between 120 and 240 μg/kg/d doses.

Urine parameters showed greater sensitivity to toxin dose. There was significant depression of urine protein/creatinine concentration at 120 and 240 μg/kg/d doses and urine specific gravity was depressed at 240 μg/kg/d dose.

While the lack of significant changes in tissue injury markers in serum indicates little cell destruction over the lower dose range in trial 2, the trends that are seen relate directly to the injury demonstrated in trial 1 at a higher dose. That metabolic changes were occurring at the lower doses is indicated by the organ weight and urine parameter changes. The two experiments clearly bracket the dose range within which the No Observed Adverse Effect Level occurs, however the point considered to be the one in which an adverse effect is seen is subjective, as different parameters provide different dose rates having no effect. Adverse effects (liver histopathology, increased serum bilirubin, decreased serum bile acids) are seen at 240 μg/kg/d in trial 2 and at 216 μg/kg/d in trial 1, hence the NOAEL is below this dose rate.

Organ weight changes may be compensatory hypertrophy for suppressed protein synthesis, and occurred in kidneys at 60 μg/kg/d and liver at 240 μg/kg/d doses, and urine protein/creatinine was reduced at the 120 and 240 μg/kg/d doses. Urine potassium reduction is difficult to relate to an adverse metabolic change, particularly when no significant changes in urine sodium are occurring concurrently. There appears to be an increase in serum potassium and decrease in serum chloride with dose, though they are not statistically significant.

Thus, if organ weight is regarded as the most reliable indicator of adverse change, the NOAEL is 30 μg CYN/kg/d. If urine protein is considered a better indicator of suppression of protein synthesis and therefore an adverse change, then 60 μg/kg/d is the NOAEL.

Examination of changes in enzymic indicators of liver damage show low sensitivity, with only a non-significant increase in ALT at 240 μg/kg/d in trial 1 and a significant rise in ALP at 432 μg/kg/d in trial 2. It is possible that inhibition of enzyme synthesis may reduce enzyme release in injured cells, hence suppressing serum changes.. The increases in serum cholesterol and blood lymphocytes at the lowest doses were not matched at higher doses, and so their relevance is questionable.

Therefore, the strongest and most consistent effect seen was on organ weights, the lowest doses showing statistically significant effects being 240 μg/kg/d for liver weight and 60 μg/kg/d for kidney weight. If this increased size is a response to reduced metabolic capacity rather than increased cell death, then one may well only see the subtle effects on serum levels of hepatic metabolites (albumin, bile acids, bilirubin) that were observed. The reduced urinary excretion of protein, significant at 120 μg/kg/d, fits with this picture. If increased organ weight is a response to reduced metabolic capacity, then it is a marker of that adverse effect. The renal system seems to be a more sensitive marker of this effect than the hepatic, and so a NOAEL of 30 μg/kg/d seems appropriate.

4.3 Application to the calculation of a safe level of CYN in drinking water for human consumption.

The standard safety or uncertainty factors apply to this *in vivo* mouse oral dosing study.

Tolerable Daily Intake (TDI) determination

Safety factors	
Intraspecific human variability	10
Interspecific variation	10
Limitations in data - particularly sub-chronic to lifetime exposure, possibility of mutagenicity or carcinogenicity, lack of data for teratogenicity or reproductive effects	10
Overall uncertainty factor	1000
NOAEL dose rate (conservative option)	30 μg/kg/d

Tolerable Daily Intake is NOAEL/Uncertainty factor, hence

30/1,000 = 0.03 μg/kg/d

The Guideline Value for safe drinking water is calculated as

TDI x Body Wt (kg) x Proportion of toxin consumed in drinking water/Volume consumed daily(litres).

Applying the WHO standard body weight of 60 kg, an assumed proportion of 0.9 of total toxin intake coming in drinking water, and a water consumption of 2.0 L/d.

GV = 0.03 x 60 x 0.9/2 = 0.81 μg/L

For practical purposes 1.0 μg/L of drinking water is an effective Guideline Value.

5. RECOMMENDATIONS

- That this report be provided to the WHO Chemical Safety Committee in time for consideration for their next meeting in 2003 for the rolling revision of the Drinking Water Guidelines.
- That this report be provided to the Australian NH&MRC expert committee on cyanobacterial toxins in drinking water for consideration for revision of the Australian Drinking Water Guidelines
- That this report be provided to the New Zealand Department of Health for consideration for their current drinking water guidelines revision.
- That this report be provided to the Brazilian Federal Department of Health for consideration in revision of their drinking water guidelines.
- That this report be provided to the Florida Department of Health, the US EPA and the National Institute of Environmental Health Sciences.

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APPENDIX I - EXPERIMENT 1 RAW DATA

This data is also available over the internet at http://www.waterquality.crc.org.au/cyano.htm
For abbreviations, see p 15.

Table 8. Experiment 1. Body and organ weights

Cage	Mouse Code	Dose (mg Cyl Extract /kg Mouse /day)	Kidneys	Liver	Spleen	Body weight	Kidney% Bwt	Liver% Bwt	Spleen% Bwt
1	Blue 1	0	0.4929	2.3412	0.1172	41.07	1.20%	5.70%	0.29%
1	Blue 2	0	0.6045	2.5240	0.1192	43.96	1.47%	6.15%	0.29%
1	Blue 3	0	0.5382	2.3074	0.1400	42.79	1.31%	5.62%	0.34%
1	Blue 4	0	0.5998	2.2447	0.0972	45.19	1.46%	5.47%	0.24%
1	Red 1	0	0.6853	2.2888	0.1275	41.41	1.67%	5.57%	0.31%
1	Red 2	0	0.6660	2.2869	0.0990	44.49	1.62%	5.57%	0.24%
1	Red 3	0	0.6049	2.2440	0.1187	39.38	1.47%	5.46%	0.29%
1	Red 4	0	0.5371	2.4286	0.1143	41.10	1.31%	5.91%	0.28%
1	Black 1	0	0.5824	2.0519	0.1054	37.30	1.42%	5.00%	0.26%
1	Black 2	0	0.5515	2.2450	0.0795	42.06	1.34%	5.47%	0.19%
1	Purple 1	0	0.6957	2.3004	0.1348	41.52	1.69%	5.60%	0.33%
1	Purple 2	0	0.6795	2.0646	0.1671	40.57	1.65%	5.03%	0.41%
2	Purple 1	43	0.6444	2.6910	0.1254	37.82	1.57%	6.55%	0.31%
2	Purple 2	43	0.7310	3.1173	0.0975	40.52	1.78%	7.59%	0.24%
2	Purple 3	43	0.8270	3.0145	0.1253	45.80	2.01%	7.34%	0.31%
2	Purple 4	43	0.8312	3.0003	0.1390	40.82	2.02%	7.31%	0.34%
2	Green 1	43	0.7620	2.2955	0.0685	38.92	1.86%	5.59%	0.17%
2	Green 2	43	0.7506	2.8523	0.1260	39.37	1.83%	6.94%	0.31%
2	Green 3	43	0.7085	2.6334	0.0901	37.62	1.73%	6.41%	0.22%
2	Green 4	43	0.7262	2.7531	0.0863	37.90	1.77%	6.70%	0.21%
2	Blue 1	43	0.7260	3.0553	0.0951	43.06	1.77%	7.44%	0.23%
2	Blue 2	43	0.7263	2.3031	0.0810	34.95	1.77%	5.61%	0.20%
3	Red 1	85	0.7010	2.4540	0.1230	33.15	1.71%	5.98%	0.30%
3	Red 2	85	0.6085	3.0180	0.0822	40.82	1.48%	7.35%	0.20%
3	Red 3	85	0.9104	2.9751	0.1091	37.34	2.22%	7.24%	0.27%
3	Red 4	85	0.8833	2.8706	0.1006	39.43	2.15%	6.99%	0.24%
3	Black 1	85	0.9256	2.8515	0.0966	39.91	2.25%	6.94%	0.24%
3	Black 2	85	0.7269	3.2993	0.0903	39.65	1.77%	8.03%	0.22%
3	Black 3	85	0.7962	2.7260	0.1190	38.88	1.94%	6.64%	0.29%
3	Black 4	85	0.8556	2.7151	0.0880	36.75	2.08%	6.61%	0.21%
3	Purple 1	85	0.6600	2.5942	0.0822	33.85	1.61%	6.32%	0.20%
3	Purple 2	85	0.6961	2.8566	0.1023	38.71	1.69%	6.96%	0.25%
4	Green 1	130	0.8587	3.3170	0.1320	39.63	2.09%	8.08%	0.32%
4	Green 2	130	0.8306	3.1251	0.0966	38.18	2.02%	7.61%	0.24%
4	Green 3	130	0.8598	3.6030	0.1253	40.45	2.09%	8.77%	0.31%
4	Green 4	130	0.7442	2.9950	0.1065	40.44	1.81%	7.29%	0.26%
4	Blue 1	130	0.9713	3.2594	0.1101	43.04	2.36%	7.94%	0.27%
4	Blue 2	130	0.6854	2.9331	0.1126	36.66	1.67%	7.14%	0.27%
4	Blue 3	130	0.8411	2.9401	0.1169	39.90	2.05%	7.16%	0.28%
4	Blue 4	130	0.6208	2.7843	0.1137	35.17	1.51%	6.78%	0.28%
4	Red 1	130	0.9143	3.3361	0.1060	40.57	2.23%	8.12%	0.26%
4	Red 2	130	0.7422	2.9476	0.1280	34.14	1.81%	7.18%	0.31%
5	Purple 3	135	0.6350	2.7400	0.1151	35.70	1.55%	6.67%	0.28%
5	Purple 4	135	0.7405	2.5103	0.0945	33.57	1.80%	6.11%	0.23%
5	Red 1	135	0.7860	2.9122	0.0846	39.13	1.91%	7.09%	0.21%
5	Red 2	135	0.6611	2.6353	0.0801	33.74	1.61%	6.42%	0.20%
5	Red 3	135	0.6085	2.3564	0.0767	34.28	1.48%	5.74%	0.19%

Table 9. Experiment 1. Serum chemistry

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	pH	Na (mmol/L)	K (mmol/L)	Bicarb (mmol/L)	Cl (mmol/L)	Urea (mmol/L)	Creatinine (mmol/L)	Protein (g/L)
Cage 1	Black 1	0	7.27	–	–	14.2	–	10	0.04	53
Cage 1	Black 2	0	7.22	147	6.5	15.6	102	11.2	0.04	51
Cage 1	Blue 1	0	7.04	148	6.9	14.5	103	13.8	0.04	56
Cage 1	Blue 2	0	7.17	164	3.2	16	77	14.1	0.05	50
Cage 1	Blue 3	0	–	–	–	–	–	–	0.02	50
Cage 1	Blue 4	0	7.09	149	7.4	17.9	105	11.6	0.04	49
Cage 1	Purple 1	0	7.34	147	5.4	22.3	104	11	0.03	51
Cage 1	Purple 2	0	7.25	147	4.0	15.7	103	9.1	0.04	56
Cage 1	Red 1	0	7.13	152	7.1	16.6	106	11.7	0.05	55
Cage 1	Red 2	0	7.27	–	–	9.8	–	7.8	0.04	54
Cage 1	Red 3	0	7.24	146	5.5	15.5	101	8.7	0.04	51
Cage 1	Red 4	0	7.24	153	6.1	15.5	105	9.3	0.04	52
Cage 2	Blue 1	43	7.34	–	–	8.8	–	10.9	0.04	57
Cage 2	Blue 2	43	7.37	–	–	16.5	–	9.6	0.04	58
Cage 2	Green 1	43	7.31	–	–	12.2	–	11.6	0.04	57
Cage 2	Green 2	43	7.25	–	–	16.2	–	10.5	0.03	49
Cage 2	Green 3	43	7.39	–	–	–	–	10.6	0.05	54
Cage 2	Green 4	43	7.38	–	–	16.2	–	11.0	0.03	48
Cage 2	Purple 1	43	7.33	–	–	14.5	–	14.4	0.04	57
Cage 2	Purple 2	43	7.45	–	–	–	–	11.0	0.04	56
Cage 2	Purple 3	43	7.39	–	–	15.8	–	11.8	0.04	55
Cage 2	Purple 4	43	7.21	–	–	13	–	9.4	0.04	51
Cage 3	Black 1	85	7.3	150	5.7	19.2	107	11.3	0.03	50
Cage 3	Black 2	85	7.28	152	4.9	16.4	108	9.8	0.03	51
Cage 3	Black 3	85	7.23	148	6.2	16.1	108	11.2	0.03	47
Cage 3	Black 4	85	7.24	151	5.3	14.0	110	10.0	0.03	50
Cage 3	Purple 1	85	7.32	151	5.6	15.8	108	9.2	0.03	53
Cage 3	Purple 2	85	7.25	139	5.4	13.0	107	9.4	0.03	55
Cage 3	Red 1	85	7.44	152	5.6	17.7	107	11.0	0.04	50
Cage 3	Red 2	85	7.31	152	5.4	15.9	106	13.4	0.04	56
Cage 3	Red 3	85	7.39	154	6.0	17.7	110	12.0	0.03	51
Cage 3	Red 4	85	7.33	146	5.9	17.6	107	9.8	0.03	51
Cage 4	Blue 1	130	7.21	Sample lost						
Cage 4	Blue 2	130	7.26	Sample lost						
Cage 4	Blue 3	130	7.27	Sample lost						
Cage 4	Blue 4	130	7.22	Sample lost						
Cage 4	Green 1	130	7.18	Sample lost						
Cage 4	Green 2	130	7.22	Sample lost						
Cage 4	Green 3	130	7.22	Sample lost						
Cage 4	Green 4	130	7.26	Sample lost						
Cage 4	Red 1	130	7.24	Sample lost						
Cage 4	Red 2	130	7.24	Sample lost						
Cage 5	Purple 3	135	7.09	147	4.5	14.7	102	8.1	0.06	52
Cage 5	Purple 4	135	7.07	147	5.9	11.2	104	10.3	0.04	42
Cage 5	Red 1	135	7.18	147	5.5	15.8	105	8.9	0.04	50
Cage 5	Red 2	135	7.04	150	5.6	13.3	106	9.4	0.05	48
Cage 5	Red 3	135	7.23	147	5.3	16.9	103	12.5	0.04	51

Table 9 continued

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	ALB g/L	T bili mmol/L	Ca mmol/L	Phosphate mmol/L	ALP U/L	ALT U/L	AST U/L	Lipase U/L
Cage 1	Black 1	0	26	2.8	2.53	4.65	50	–	614	350
Cage 1	Black 2	0	25	1.2	2.54	3.89	66	–	257	135
Cage 1	Blue 1	0	27	2.4	2.57	4.65	144	242	265	506
Cage 1	Blue 2	0	25	2.7	2.52	4.45	62	–	377	425
Cage 1	Blue 3	0	–	3.1	–	–	44	–	368	390
Cage 1	Blue 4	0	25	2.7	2.46	3.78	40	221	261	419
Cage 1	Purple 1	0	25	2.7	2.51	2.79	32	79	209	253
Cage 1	Purple 2	0	26	0.0	2.18	2.30	17	64	145	254
Cage 1	Red 1	0	27	2.4	2.60	4.50	45	233	271	272
Cage 1	Red 2	0	27	1.1	2.26	3.29	47	–	120	392
Cage 1	Red 3	0	25	1.0	2.26	3.48	63	–	134	216
Cage 1	Red 4	0	26	2.6	2.45	3.64	48	–	197	403
Cage 2	Blue 1	43	30	2.3	2.54	2.82	75	202	119	304
Cage 2	Blue 2	43	31	4.0	2.67	2.80	49	96	191	302
Cage 2	Green 1	43	29	3.3	2.54	2.93	67	75	108	229
Cage 2	Green 2	43	27	2.9	2.60	2.93	154	955	378	360
Cage 2	Green 3	43	29	1.9	2.49	2.77	32	78	101	286
Cage 2	Green 4	43	26	2.9	2.43	2.59	29	117	215	288
Cage 2	Purple 1	43	31	2.3	2.61	2.88	49	70	85	310
Cage 2	Purple 2	43	28	1.7	2.40	2.49	31	70	97	305
Cage 2	Purple 3	43	28	2.2	2.33	2.35	48	81	113	278
Cage 2	Purple 4	43	28	3.0	2.33	2.76	59	137	186	**1920**
Cage 3	Black 1	85	27	1.0	2.36	2.41	61	305	235	507
Cage 3	Black 2	85	28	3.1	2.43	2.61	43	181	256	262
Cage 3	Black 3	85	28	3.7	2.42	2.24	157	441	282	289
Cage 3	Black 4	85	30	3.9	2.53	2.59	240	550	261	529
Cage 3	Purple 1	85	32	5.3	2.54	2.85	280	683	273	361
Cage 3	Purple 2	85	32	4.8	2.52	3.13	206	107	228	295
Cage 3	Red 1	85	28	4.4	2.54	3.57	287	315	200	394
Cage 3	Red 2	85	31	2.7	2.54	2.69	64	146	244	262
Cage 3	Red 3	85	30	4.2	2.48	2.64	242	262	231	248
Cage 3	Red 4	85	29	2.8	2.36	2.41	38	48	136	385
Cage 4	Blue 1	130								
Cage 4	Blue 2	130								
Cage 4	Blue 3	130								
Cage 4	Blue 4	130								
Cage 4	Green 1	130								
Cage 4	Green 2	130								
Cage 4	Green 3	130								
Cage 4	Green 4	130								
Cage 4	Red 1	130								
Cage 4	Red 2	130								
Cage 5	Purple 3	135	28	3.9	2.62	3.71	33	74	84	587
Cage 5	Purple 4	135	25	3.0	2.34	4.37	141	128	137	389
Cage 5	Red 1	135	28	2.6	2.55	3.15	30	73	98	320
Cage 5	Red 2	135	28	3.6	2.60	3.58	316	490	175	349
Cage 5	Red 3	135	28	2.7	2.41	2.80	102	69	117	189

Table 9 continued

Cage No	Mouse No Dose (mg Cyl Extract /kg	Mouse /day)	TBA umol/L	Globulins (Prot - Alb)
Cage 1	Black 1	0	–	27
Cage 1	Black 2	0	7.5	26
Cage 1	Blue 1	0	8.7	29
Cage 1	Blue 2	0	8.1	25
Cage 1	Blue 3	0	–	–
Cage 1	Blue 4	0	9.6	24
Cage 1	Purple 1	0	3.4	26
Cage 1	Purple 2	0	11.8	30
Cage 1	Red 1	0	7.5	28
Cage 1	Red 2	0	–	27
Cage 1	Red 3	0	10	26
Cage 1	Red 4	0	8.3	26
Cage 2	Blue 1	43	5.2	27
Cage 2	Blue 2	43	5	27
Cage 2	Green 1	43	4.9	28
Cage 2	Green 2	43	3.3	22
Cage 2	Green 3	43	–	25
Cage 2	Green 4	43	5.6	22
Cage 2	Purple 1	43	5.8	26
Cage 2	Purple 2	43	–	28
Cage 2	Purple 3	43	1.6	27
Cage 2	Purple 4	43	4.3	23
Cage 3	Black 1	85	2.5	23
Cage 3	Black 2	85	1.3	23
Cage 3	Black 3	85	5.8	19
Cage 3	Black 4	85	5.3	20
Cage 3	Purple 1	85	4.6	21
Cage 3	Purple 2	85	2.4	23
Cage 3	Red 1	85	9.6	22
Cage 3	Red 2	85	2.7	25
Cage 3	Red 3	85	2.2	21
Cage 3	Red 4	85	2.9	22
Cage 4	Blue 1	130		
Cage 4	Blue 2	130		
Cage 4	Blue 3	130		
Cage 4	Blue 4	130		
Cage 4	Green 1	130		
Cage 4	Green 2	130		
Cage 4	Green 3	130		
Cage 4	Green 4	130		
Cage 4	Red 1	130		
Cage 4	Red 2	130		
Cage 5	Purple 3	135	5.7	
Cage 5	Purple 4	135	10.0	
Cage 5	Red 1	135	4.7	
Cage 5	Red 2	135	10.6	
Cage 5	Red 3	135	10.3	

Table 10. Experiment 1. Urine analysis

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	Urine volume (ml)	Wt. Food Drunk (g)	Vol. Food Drunk (ml)	Urine % of Food	Our pH	VPS pH
Cage 1	Black 1	0	12.5	29.51	26.88	46.51%	7.70	8.0
Cage 1	Black 2	0	7.5	16.77	15.27	49.11%	7.66	8.0
Cage 1	Blue 1	0	11.0	21.81	19.86	55.38%	7.97	8.0
Cage 1	Blue 2	0	9.0	19.95	18.17	49.53%	7.49	7.5
Cage 1	Blue 3	0	9.5	32.04	29.18	32.56%	7.88	8.0
Cage 1	Blue 4	0	2.0	25.41	23.14	8.64%	8.86	8.5
Cage 1	Purple 1	0	14.0	36.96	33.66	41.59%	7.34	7.5
Cage 1	Purple 2	0	9.0	24.52	22.33	40.30%	7.25	7.0
Cage 1	Red 1	0	9.5	26.18	23.84	39.84%	7.69	8.0
Cage 1	Red 2	0	10.5	24.75	22.54	46.58%	7.69	8.0
Cage 1	Red 3	0	8.5	18.94	17.25	49.28%	7.45	7.5
Cage 1	Red 4	0	4.0	28.37	25.84	15.48%	8.23	8.0
Cage 2	Blue 1	43	11.0	25.41	23.14	47.53%	7.58	8.0
Cage 2	Blue 2	43	6.0	26.45	24.09	24.91%	7.13	7.0
Cage 2	Green 1	43	5.5	25.76	23.46	23.44%	7.67	8.0
Cage 2	Green 2	43	13.5	31.66	28.83	46.82%	7.12	7.5
Cage 2	Green 3	43	9.0	29.09	26.49	33.97%	7.30	7.5
Cage 2	Green 4	43	4.5	27.13	24.71	18.21%	7.37	8.0
Cage 2	Purple 1	43	8.0	25.27	23.01	34.76%	7.69	8.0
Cage 2	Purple 2	43	9.0	23.63	21.52	41.82%	7.06	7.0
Cage 2	Purple 3	43	8.5	26.44	24.08	35.30%	7.30	7.5
Cage 2	Purple 4	43	3.5	25.26	23.01	15.21%	8.22	8.5
Cage 3	Black 1	85	4.0	27.68	25.21	15.87%	7.68	8.0
Cage 3	Black 2	85	9.5	24.83	22.61	42.01%	7.43	7.5
Cage 3	Black 3	85	12.5	30.31	27.60	45.28%	7.26	7.5
Cage 3	Black 4	85	13.0	29.31	26.69	48.70%	7.42	7.5
Cage 3	Purple 1	85	11.5	26.76	24.37	47.19%	7.82	8.0
Cage 3	Purple 2	85	11.5	26.79	24.40	47.13%	7.65	8.0
Cage 3	Red 1	85	6.5	27.45	25.00	26.00%	7.77	8.0
Cage 3	Red 2	85	7.5	22.44	20.44	36.70%	8.11	8.0
Cage 3	Red 3	85	10.0	28.03	25.53	39.17%	7.36	8.0
Cage 3	Red 4	85	6.0	27.53	25.07	23.93%	8.44	8.5
Cage 4	Blue 1	130	15.0	36.75	33.47	44.82%	6.25	6.0
Cage 4	Blue 2	130	6.5	32.63	29.72	21.87%	7.02	7.0
Cage 4	Blue 3	130	9.5	24.56	22.37	42.47%	7.39	8.0
Cage 4	Blue 4	130	8.5	24.02	21.88	38.86%	7.17	7.5
Cage 4	Green 1	130	4.0	33.84	30.82	12.98%	7.87	8.5
Cage 4	Green 2	130	12.5	28.44	25.90	48.26%	7.29	7.5
Cage 4	Green 3	130	12.5	31.78	28.94	43.19%	6.97	7.0
Cage 4	Green 4	130	10.5	27.69	25.22	41.64%	7.67	8.5
Cage 4	Red 1	130	8.0	27.55	25.09	31.88%	7.39	7.5
Cage 4	Red 2	130	9.0	28.93	26.35	34.16%	7.27	7.5
Cage 5	Purple 3	135	9.0	28.85	26.28	34.25%	7.30	7.5
Cage 5	Purple 4	135	8.0	29.35	26.73	29.93%	6.76	7.0
Cage 5	Red 1	135	10.5	28.79	26.22	40.05%	7.01	7.5
Cage 5	Red 2	135	10.0	28.67	26.11	38.30%	8.00	8.5
Cage 5	Red 3	135	5.5	19.63	17.88	30.76%	7.05	7.0

Table 10 continued

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	Ketones	USG	Na mmol/L	K mmol/L	Cl mmol/L	Bicarb mmol/L	Creatinine mmol/L	Glucose mmol/L
Cage 1	Black 1	0	Neg	1.011	56	53	69	9.1	0.58	0.0
Cage 1	Black 2	0	Neg	1.011	58	46	67	7.0	0.64	0.2
Cage 1	Blue 1	0	Neg	1.010	57	51	68	14.0	0.67	0.0
Cage 1	Blue 2	0	Neg	1.011	64	48	74	4.9	0.76	0.0
Cage 1	Blue 3	0	Neg	1.012	64	59	74	11.5	0.76	0.0
Cage 1	Blue 4	0	Neg	1.018	85	89	106	31.6	1.10	0.0
Cage 1	Purple 1	0	Neg	1.010	51	30	65	15.3	0.55	0
Cage 1	Purple 2	0	Neg	1.011	57	31	68	7.3	0.75	0
Cage 1	Red 1	0	Neg	1.012	60	55	73	8.2	0.62	0.1
Cage 1	Red 2	0	Neg	1.012	60	58	73	7.7	0.62	0.3
Cage 1	Red 3	0	Neg	1.013	64	57	76	3.5	0.67	0.1
Cage 1	Red 4	0	Neg	1.018	80	69	90	11.6	0.80	1.8
Cage 2	Blue 1	43	Neg	1.009	56	39	67	10.8	0.60	0.2
Cage 2	Blue 2	43	Neg	1.012	62	56	80	5.0	0.81	0.0
Cage 2	Green 1	43	Neg	1.013	74	63	87	8.9	0.85	0.0
Cage 2	Green 2	43	Neg	1.008	55	38	66	9.2	0.48	0.0
Cage 2	Green 3	43	Neg	1.010	61	44	73	7.2	0.69	0.0
Cage 2	Green 4	43	Neg	1.012	65	54	81	6.4	0.77	0.0
Cage 2	Purple 1	43	Neg	1.010	64	43	73	10.0	0.69	0.0
Cage 2	Purple 2	43	Neg	1.011	57	51	71	3.7	0.77	0.0
Cage 2	Purple 3	43	Neg	1.012	66	47	78	7.1	0.71	0.0
Cage 2	Purple 4	43	Neg	1.014	67	55	82	13.6	1.15	0.1
Cage 3	Black 1	85	Neg	1.014	77	56	80	12.8	0.85	0.0
Cage 3	Black 2	85	Neg	1.009	57	40	66	12.0	0.68	0.0
Cage 3	Black 3	85	Neg	1.009	54	39	64	8.4	0.69	0.0
Cage 3	Black 4	85	Neg	1.010	60	42	70	8.0	0.85	0.0
Cage 3	Purple 1	85	Neg	1.009	55	37	68	19.1	0.62	0.0
Cage 3	Purple 2	85	Neg	1.009	55	36	65	10.0	0.69	0.0
Cage 3	Red 1	85	Neg	1.011	71	52	82	15.0	0.79	0.0
Cage 3	Red 2	85	Neg	1.010	57	40	70	16.1	0.69	0.0
Cage 3	Red 3	85	Neg	1.010	61	42	69	10.7	0.70	0.0
Cage 3	Red 4	85	Neg	1.012	77	49	85	22.2	0.83	0.0
Cage 4	Blue 1	130	Neg	1.009	59	41	69	1.50	0.58	10.9*
Cage 4	Blue 2	130	Neg	1.013	72	58	80	3.80	0.88	0
Cage 4	Blue 3	130	Neg	1.009	56	39	68	12.10	0.66	0
Cage 4	Blue 4	130	Neg	1.010	62	40	69	6.20	0.69	0
Cage 4	Green 1	130	Neg	1.012	86	59	92	15.50	0.66	0
Cage 4	Green 2	130	Neg	1.009	58	44	70	11.20	0.60	0
Cage 4	Green 3	130	Neg	1.008	58	37	67	8.00	0.60	0
Cage 4	Green 4	130	Neg	1.010	61	43	71	11.70	0.68	0
Cage 4	Red 1	130	Neg	1.010	65	46	77	9.20	0.72	0
Cage 4	Red 2	130	Neg	1.010	67	37	75	5.40	0.69	0
Cage 5	Purple 3	135	Neg	1.010	55	28	68	9.5	0.85	0
Cage 5	Purple 4	135	Neg	1.011	58	29	73	4.6	0.95	0
Cage 5	Red 1	135	Neg	1.009	52	27	66	6.9	0.71	0
Cage 5	Red 2	135	Neg	1.009	51	26	67	15.7	0.75	0
Cage 5	Red 3	135	Neg	1.011	60	31	72	4.3	0.92	0

* Glucose result was checked by VPS

Table 10 continued

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	Calcium mmol/L	Phosphate mmol/L	Protein g/L	USG/Creatinine	Na/Creatinine	K/Creatinine	Cl/Creatinine
Cage 1	Black 1	0	0.90	6.90	0.75	1.74	96.55	91.38	118.97
Cage 1	Black 2	0	0.30	8.00	3.70	1.58	90.63	71.88	104.69
Cage 1	Blue 1	0	0.40	4.85	0.67	1.51	85.07	76.12	101.49
Cage 1	Blue 2	0	0.70	8.05	3.20	1.33	84.21	63.16	97.37
Cage 1	Blue 3	0	0.90	5.75	0.74	1.33	84.21	77.63	97.37
Cage 1	Blue 4	0	0.60	6.30	4.20	0.93	77.27	80.91	96.36
Cage 1	Purple 1	0	1.30	4.64	2.80	1.84	92.73	54.55	118.18
Cage 1	Purple 2	0	1.68	3.87	2.00	1.35	76.00	41.33	90.67
Cage 1	Red 1	0	0.70	7.05	4.40	1.63	96.77	88.71	117.74
Cage 1	Red 2	0	0.11	1.78	3.10	1.63	96.77	93.55	117.74
Cage 1	Red 3	0	0.10	13.25	4.50	1.51	95.52	85.07	113.43
Cage 1	Red 4	0	1.00	4.05	0.75	1.27	100.00	86.25	112.50
Cage 2	Blue 1	43	0.6	4.0	0.63	1.68	93.33	65.00	111.67
Cage 2	Blue 2	43	0.7	11.5	1.60	1.25	76.54	69.14	98.77
Cage 2	Green 1	43	0.7	7.6	2.90	1.19	87.06	74.12	102.35
Cage 2	Green 2	43	0.4	4.4	0.41	2.10	114.58	79.17	137.50
Cage 2	Green 3	43	0.7	6.5	2.20	1.46	88.41	63.77	105.80
Cage 2	Green 4	43	0.6	6.8	1.80	1.31	84.42	70.13	105.19
Cage 2	Purple 1	43	0.5	7.2	0.72	1.46	92.75	62.32	105.80
Cage 2	Purple 2	43	3.5	9.1	2.70	1.31	74.03	66.23	92.21
Cage 2	Purple 3	43	1.4	6.0	3.10	1.43	92.96	66.20	109.86
Cage 2	Purple 4	43	0.6	5.0	0.71	0.88	58.26	47.83	71.30
Cage 3	Black 1	85	0.80	5.10	0.65	1.19	90.59	65.88	94.12
Cage 3	Black 2	85	1.80	4.60	0.37	1.48	83.82	58.82	97.06
Cage 3	Black 3	85	0.85	3.80	0.33	1.46	78.26	56.52	92.75
Cage 3	Black 4	85	0.75	5.00	0.65	1.19	70.59	49.41	82.35
Cage 3	Purple 1	85	0.45	2.65	0.30	1.63	88.71	59.68	109.68
Cage 3	Purple 2	85	1.25	3.10	0.35	1.46	79.71	52.17	94.20
Cage 3	Red 1	85	0.75	4.65	0.35	1.28	89.87	65.82	103.80
Cage 3	Red 2	85	0.50	3.00	0.65	1.46	82.61	57.97	101.45
Cage 3	Red 3	85	0.25	3.65	0.45	1.44	87.14	60.00	98.57
Cage 3	Red 4	85	1.25	2.05	0.57	1.22	92.77	59.04	102.41
Cage 4	Blue 1	130	0.90	6.05	0.16	1.74	101.72	70.69	118.97
Cage 4	Blue 2	130	2.00	8.30	0.45	1.15	81.82	65.91	90.91
Cage 4	Blue 3	130	0.55	5.25	0.23	1.53	84.85	59.09	103.03
Cage 4	Blue 4	130	0.80	6.85	0.34	1.46	89.86	57.97	100.00
Cage 4	Green 1	130	1.35	7.15	0.24	1.53	130.30	89.39	139.39
Cage 4	Green 2	130	0.65	7.05	0.29	1.68	96.67	73.33	116.67
Cage 4	Green 3	130	0.60	6.10	0.39	1.68	96.67	61.67	111.67
Cage 4	Green 4	130	0.70	7.05	0.49	1.49	89.71	63.24	104.41
Cage 4	Red 1	130	1.20	5.95	0.48	1.40	90.28	63.89	106.94
Cage 4	Red 2	130	1.90	6.00	0.21	1.46	97.10	53.62	108.70
Cage 5	Purple 3	135	1.73	3.35	0.53	1.19	64.71	32.94	80.00
Cage 5	Purple 4	135	1.85	5.72	0.30	1.06	61.05	30.53	76.84
Cage 5	Red 1	135	1.62	6.26	0.46	1.42	73.24	38.03	92.96
Cage 5	Red 2	135	2.07	3.40	0.20	1.35	68.00	34.67	89.33
Cage 5	Red 3	135	1.46	3.12	0.69	1.10	65.22	33.70	78.26

Table 10 continued

Cage No	Mouse No	Dose (mg Cyl Extract /kg Mouse /day)	Bicarb/ Creatinine	Creatinine/ Creatinine	Glucose/ Creatinine	Calcium/ Creatinine	Phosphate/ Creatinine	Protein/ Creatinine
Cage 1	Black 1	0	15.69	1.00	0.00	1.55	11.90	1.29
Cage 1	Black 2	0	10.94	1.00	0.31	0.47	12.50	5.78
Cage 1	Blue 1	0	20.90	1.00	0.00	0.60	7.24	1.00
Cage 1	Blue 2	0	6.45	1.00	0.00	0.92	10.59	4.21
Cage 1	Blue 3	0	15.13	1.00	0.00	1.18	7.57	0.97
Cage 1	Blue 4	0	28.73	1.00	0.00	0.55	5.73	3.82
Cage 1	Purple 1	0	27.82	1.00	0.00	2.36	8.44	5.09
Cage 1	Purple 2	0	9.73	1.00	0.00	2.24	5.16	2.67
Cage 1	Red 1	0	13.23	1.00	0.16	1.13	11.37	7.10
Cage 1	Red 2	0	12.42	1.00	0.48	0.18	2.87	5.00
Cage 1	Red 3	0	5.22	1.00	0.15	0.15	19.78	6.72
Cage 1	Red 4	0	14.50	1.00	2.25	1.25	5.06	0.94
Cage 2	Blue 1	43	18.00	1.00	0.33	1.00	6.67	1.05
Cage 2	Blue 2	43	6.17	1.00	0.00	0.86	14.20	1.98
Cage 2	Green 1	43	10.47	1.00	0.00	0.82	8.94	3.41
Cage 2	Green 2	43	19.17	1.00	0.00	0.83	9.17	0.85
Cage 2	Green 3	43	10.43	1.00	0.00	1.01	9.42	3.19
Cage 2	Green 4	43	8.31	1.00	0.00	0.78	8.83	2.34
Cage 2	Purple 1	43	14.49	1.00	0.00	0.72	10.43	1.04
Cage 2	Purple 2	43	4.81	1.00	0.00	4.55	11.82	3.51
Cage 2	Purple 3	43	10.00	1.00	0.00	1.97	8.45	4.37
Cage 2	Purple 4	43	11.83	1.00	0.09	0.52	4.35	0.62
Cage 3	Black 1	85	15.06	1.00	0.00	0.94	6.00	0.76
Cage 3	Black 2	85	17.65	1.00	0.00	2.65	6.76	0.54
Cage 3	Black 3	85	12.17	1.00	0.00	1.23	5.51	0.48
Cage 3	Black 4	85	9.41	1.00	0.00	0.88	5.88	0.76
Cage 3	Purple 1	85	30.81	1.00	0.00	0.73	4.27	0.48
Cage 3	Purple 2	85	14.49	1.00	0.00	1.81	4.49	0.51
Cage 3	Red 1	85	18.99	1.00	0.00	0.95	5.89	0.44
Cage 3	Red 2	85	23.33	1.00	0.00	0.72	4.35	0.94
Cage 3	Red 3	85	15.29	1.00	0.00	0.36	5.21	0.64
Cage 3	Red 4	85	26.75	1.00	0.00	1.51	2.47	0.69
Cage 4	Blue 1	130	2.59	1.00	–	1.55	10.43	0.28
Cage 4	Blue 2	130	4.32	1.00	0.00	2.27	9.43	0.51
Cage 4	Blue 3	130	18.33	1.00	0.00	0.83	7.95	0.35
Cage 4	Blue 4	130	8.99	1.00	0.00	1.16	9.93	0.49
Cage 4	Green 1	130	23.48	1.00	0.00	2.05	10.83	0.36
Cage 4	Green 2	130	18.67	1.00	0.00	1.08	11.75	0.48
Cage 4	Green 3	130	13.33	1.00	0.00	1.00	10.17	0.65
Cage 4	Green 4	130	17.21	1.00	0.00	1.03	10.37	0.72
Cage 4	Red 1	130	12.78	1.00	0.00	1.67	8.26	0.67
Cage 4	Red 2	130	7.83	1.00	0.00	2.75	8.70	0.30
Cage 5	Purple 3	135	11.18	1.00	0.00	2.04	3.94	0.62
Cage 5	Purple 4	135	4.84	1.00	0.00	1.95	6.02	0.32
Cage 5	Red 1	135	9.72	1.00	0.00	2.28	8.82	0.65
Cage 5	Red 2	135	20.93	1.00	0.00	2.76	4.53	0.27
Cage 5	Red 3	135	4.67	1.00	0.00	1.59	3.39	0.75

APPENDIX II - EXPERIMENT 2 RAW DATA

This data is also available over the internet at http://www.waterquality.crc.org.au/cyano.htm
For abbreviations, see p 15.

Table 11. Experiment 2. Body and organ weights

Mouse	Dose (µg CYN/kg/d)	Body wt	Liver	spleen	adrenals	kidneys	epididymes	testes
C5M1	0	33.9636	1.5503	0.1086	0.0052	0.502	0.1147	0.2153
C5M2	0	32.6408	1.573	0.0871	0.0051	0.5142	0.1081	0.1819
C5M3	0	30.8476	1.5085	0.074	0.0053	0.4508	0.0713	0.169
C5M4	0	31.8404	1.9018	0.0906	0.0066	0.4639	0.0735	0.1864
C5M5	0	31.108	1.8781	0.0623	0.0056	0.5186	0.0726	0.1903
C9M1	0	28.0736	1.5784	0.0902	0.0067	0.3771	0.0738	0.1487
C9M2	0	27.6963	1.4169	0.074	0.0039	0.3983	0.0899	0.1746
C9M3	0	36.5795	1.9797	0.1127	0.0046	0.4885	0.0956	0.2105
C9M4	0	31.7518	1.6783	0.0721	0.0051	0.4918	0.1047	0.2032
C9M5	0	35.5978	1.8676	0.0925	0.0062	0.514	0.0924	0.2185
C4M1	30	31.2034	1.563	0.0785	0.0057	0.4964	0.0967	0.1973
C4M2	30	38.644	2.1985	0.1302	0.0091	0.6354	0.1007	0.2464
C4M3	30	39.9144	2.2497	0.1025	0.0065	0.583	0.1071	0.2438
C4M4	30	36.4854	1.809	0.0924	0.0053	0.6523	0.1063	0.2386
C4M5	30	31.9224	1.603	0.0787	0.0048	0.5605	0.0942	0.2048
C8M1	30	35.7632	2.0844	0.0914	0.0079	0.5302	0.0613	0.2038
C8M2	30	29.7748	1.5465	0.0756	0.0056	0.4532	0.084	0.1764
C8M3	30	34.4438	2.0198	0.1191	0.0057	0.566	0.1008	0.2145
C8M4	30	36.6673	1.9831	0.1391	0.0057	0.5004	0.0949	0.232
C8M5	30	41.5969	2.1311	0.0975	0.0063	0.614	0.097	0.1979
C3M1	60	32.7101	1.8835	0.12	0.0079	0.535	0.0974	0.197
C3M2	60	38.9312	2.6103	0.118	0.0048	0.6675	0.1001	0.2359
C3M3	60	38.9117	2.2631	0.1551	0.0054	0.7655	0.1143	0.2503
C3M4	60	38.2001	2.2804	0.1272	0.0056	0.7048	0.1122	0.2564
C3M5	60	40.188	2.5558	0.119	0.0067	0.6262	0.1153	0.2738
C7M1	60	35.9262	1.7382	0.0659	0.0087	0.5421	0.0998	0.2073
C7M2	60	36.8661	1.9277	0.0984	0.0073	0.5673	0.0904	0.2328
C7M3	60	34.398	1.8212	0.0812	0.0079	0.5437	0.0817	0.1787
C7M4	60	–	–	–	–	–	–	–
C7M5	60	34.8648	2.0705	0.0844	0.0064	0.5523	0.1047	0.2485
C2M1	120	38.7706	2.1568	0.1213	0.0129	0.6625	0.1195	0.2595
C2M2	120	35.8325	2.41	0.154	0.0114	0.6079	0.0998	0.2392
C2M3	120	36.4268	2.0423	0.1249	0.0064	0.6907	0.1134	0.2637
C2M4	120	30.7117	1.6737	0.081	0.0069	0.5411	0.0918	0.2044
C2M5	120	31.6371	1.5332	0.1254	0.0078	0.5461	0.1176	0.242
C6M1	120	–	–	–	–	–	–	–
C6M2	120	35.864	2.1105	0.0687	0.0061	0.6321	0.0948	0.2276
C6M3	120	35.6103	1.9886	0.0864	0.007	0.6824	0.1061	0.1947
C6M4	120	37.6308	2.1405	0.1086	0.0053	0.7791	0.1124	0.2276
C6M5	120	35.7624	2.0752	0.1007	0.0061	0.653	0.1005	0.2295
C1M1	240	34.1408	1.8682	0.1298	0.0083	0.6233	0.0956	0.2724
C1M2	240	26.7174	1.6098	0.0723	0.0055	0.4698	0.0853	0.2221
C1M3	240	39.4359	2.7036	0.1322	0.0096	0.8274	0.1002	0.2023
C1M4	240	34.4627	2.0012	0.116	0.0069	0.5966	0.1111	0.2462
C1M5	240	33.0047	2.1271	0.1054	0.0069	0.5389	0.1073	0.2386
C1M6	240	38.2454	2.1444	0.131	0.0069	0.6303	0.0913	0.2067

Table 11 continued

Mouse	Dose (µg CYN/kg/d)	heart	thymus	brain	Liver%Bwt	Spleen%Bwt	Adrenals%Bw	Kidneys%Bwt
C5M1	0	0.172	0.0273	0.4619	4.565%	0.320%	0.015%	1.478%
C5M2	0	0.1506	0.0519	0.4133	4.819%	0.267%	0.016%	1.575%
C5M3	0	0.1717	0.0191	0.4592	4.890%	0.240%	0.017%	1.461%
C5M4	0	0.1682	0.0452	0.4466	5.973%	0.285%	0.021%	1.457%
C5M5	0	0.1525	0.0454	0.4474	6.037%	0.200%	0.018%	1.667%
C9M1	0	0.1403	0.0172	0.4208	5.622%	0.321%	0.024%	1.343%
C9M2	0	0.1328	0.0392	0.4242	5.116%	0.267%	0.014%	1.438%
C9M3	0	0.1743	0.0569	0.4936	5.412%	0.308%	0.013%	1.335%
C9M4	0	0.1638	0.0397	0.4619	5.286%	0.227%	0.016%	1.549%
C9M5	0	0.151	0.0655	0.4681	5.246%	0.260%	0.017%	1.444%
C4M1	30	0.1487	0.0526	0.4619	5.009%	0.252%	0.018%	1.591%
C4M2	30	0.2089	0.0757	0.514	5.689%	0.337%	0.024%	1.644%
C4M3	30	0.1911	0.0458	0.4847	5.636%	0.257%	0.016%	1.461%
C4M4	30	0.1781	0.0378	0.4713	4.958%	0.253%	0.015%	1.788%
C4M5	30	0.1243	0.0218	0.4446	5.022%	0.247%	0.015%	1.756%
C8M1	30	0.1738	0.0472	0.4835	5.828%	0.256%	0.022%	1.483%
C8M2	30	0.1555	0.0455	0.4448	5.194%	0.254%	0.019%	1.522%
C8M3	30	0.1526	0.0624	0.4987	5.864%	0.346%	0.017%	1.643%
C8M4	30	0.1622	0.04	0.4719	5.408%	0.379%	0.016%	1.365%
C8M5	30	0.165	0.0688	0.5014	5.123%	0.234%	0.015%	1.476%
C3M1	60	0.1572	0.0423	0.4444	5.758%	0.367%	0.024%	1.636%
C3M2	60	0.177	0.0539	0.4873	6.705%	0.303%	0.012%	1.715%
C3M3	60	0.1811	0.0509	0.4955	5.816%	0.399%	0.014%	1.967%
C3M4	60	0.1774	0.0456	0.4797	5.970%	0.333%	0.015%	1.845%
C3M5	60	0.2129	0.0411	0.4653	6.360%	0.296%	0.017%	1.558%
C7M1	60	0.1622	0.0622	0.4563	4.838%	0.183%	0.024%	1.509%
C7M2	60	0.178	0.0483	0.4941	5.229%	0.267%	0.020%	1.539%
C7M3	60	0.1735	0.0567	0.463	5.294%	0.236%	0.023%	1.581%
C7M4	60	–	–	–	–	–	–	–
C7M5	60	0.1732	0.0344	0.4951	5.939%	0.242%	0.018%	1.584%
C2M1	120	0.1843	0.0572	0.4544	5.563%	0.313%	0.033%	1.709%
C2M2	120	0.2022	0.0575	0.4711	6.726%	0.430%	0.032%	1.697%
C2M3	120	0.1785	0.0688	0.5275	5.607%	0.343%	0.018%	1.896%
C2M4	120	0.16	0.051	0.4276	5.450%	0.264%	0.022%	1.762%
C2M5	120	0.1714	0.0443	0.4394	4.846%	0.396%	0.025%	1.726%
C6M1	120	–	–	–	–	–	–	–
C6M2	120	0.1517	0.053	0.5086	5.885%	0.192%	0.017%	1.762%
C6M3	120	0.1547	0.0395	0.4868	5.584%	0.243%	0.020%	1.916%
C6M4	120	0.1521	0.0587	0.4955	5.688%	0.289%	0.014%	2.070%
C6M5	120	0.1548	0.0473	0.4797	5.803%	0.282%	0.017%	1.826%
C1M1	240	0.1682	0.0399	0.5181	5.472%	0.380%	0.024%	1.826%
C1M2	240	0.1161	0.0194	0.4083	6.025%	0.271%	0.021%	1.758%
C1M3	240	0.208	0.0532	0.4863	6.856%	0.335%	0.024%	2.098%
C1M4	240	0.1844	0.056	0.4923	5.807%	0.337%	0.020%	1.731%
C1M5	240	0.1601	0.0523	0.4858	6.445%	0.319%	0.021%	1.633%
C1M6	240	0.1946	0.0543	0.4727	5.607%	0.343%	0.018%	1.648%

Table 11 continued

Mouse	Dose (µg CYN/kg/d)	Epididymes %Bwt	Testes %Bwt	Heart%Bwt	Thymus%Bwt	Brain%Bwt
C5M1	0	0.338%	0.634%	0.506%	0.080%	1.360%
C5M2	0	0.331%	0.557%	0.461%	0.159%	1.266%
C5M3	0	0.231%	0.548%	0.557%	0.062%	1.489%
C5M4	0	0.231%	0.585%	0.528%	0.142%	1.403%
C5M5	0	0.233%	0.612%	0.490%	0.146%	1.438%
C9M1	0	0.263%	0.530%	0.500%	0.061%	1.499%
C9M2	0	0.325%	0.630%	0.479%	0.142%	1.532%
C9M3	0	0.261%	0.575%	0.476%	0.156%	1.349%
C9M4	0	0.330%	0.640%	0.516%	0.125%	1.455%
C9M5	0	0.260%	0.614%	0.424%	0.184%	1.315%
C4M1	30	0.310%	0.632%	0.477%	0.169%	1.480%
C4M2	30	0.261%	0.638%	0.541%	0.196%	1.330%
C4M3	30	0.268%	0.611%	0.479%	0.115%	1.214%
C4M4	30	0.291%	0.654%	0.488%	0.104%	1.292%
C4M5	30	0.295%	0.642%	0.389%	0.068%	1.393%
C8M1	30	0.171%	0.570%	0.486%	0.132%	1.352%
C8M2	30	0.282%	0.592%	0.522%	0.153%	1.494%
C8M3	30	0.293%	0.623%	0.443%	0.181%	1.448%
C8M4	30	0.259%	0.633%	0.442%	0.109%	1.287%
C8M5	30	0.233%	0.476%	0.397%	0.165%	1.205%
C3M1	60	0.298%	0.602%	0.481%	0.129%	1.359%
C3M2	60	0.257%	0.606%	0.455%	0.138%	1.252%
C3M3	60	0.294%	0.643%	0.465%	0.131%	1.273%
C3M4	60	0.294%	0.671%	0.464%	0.119%	1.256%
C3M5	60	0.287%	0.681%	0.530%	0.102%	1.158%
C7M1	60	0.278%	0.577%	0.451%	0.173%	1.270%
C7M2	60	0.245%	0.631%	0.483%	0.131%	1.340%
C7M3	60	0.238%	0.520%	0.504%	0.165%	1.346%
C7M4	60	–	–	–	–	–
C7M5	60	0.300%	0.713%	0.497%	0.099%	1.420%
C2M1	120	0.308%	0.669%	0.475%	0.148%	1.172%
C2M2	120	0.279%	0.668%	0.564%	0.160%	1.315%
C2M3	120	0.311%	0.724%	0.490%	0.189%	1.448%
C2M4	120	0.299%	0.666%	0.521%	0.166%	1.392%
C2M5	120	0.372%	0.765%	0.542%	0.140%	1.389%
C6M1	120	–	–	–	–	–
C6M2	120	0.264%	0.635%	0.423%	0.148%	1.418%
C6M3	120	0.298%	0.547%	0.434%	0.111%	1.367%
C6M4	120	0.299%	0.605%	0.404%	0.156%	1.317%
C6M5	120	0.281%	0.642%	0.433%	0.132%	1.341%
C1M1	240	0.280%	0.798%	0.493%	0.117%	1.518%
C1M2	240	0.319%	0.831%	0.435%	0.073%	1.528%
C1M3	240	0.254%	0.513%	0.527%	0.135%	1.233%
C1M4	240	0.322%	0.714%	0.535%	0.162%	1.429%
C1M5	240	0.325%	0.723%	0.485%	0.158%	1.472%
C1M6	240	0.239%	0.540%	0.509%	0.142%	1.236%

Table 12. Experiment 2. Serum chemistry

AnimalID	Dose (µg CYN/kg/d)	Chol mmol/L	Trig mmol/L	Phos mmol/L	CK U/L	Na mmol/L	K mmol/L	Bicarb mmol/L	CL mmol/L	Urea mmol/L	Creat mmol/L	Protein g/L	ALB g/L
C5M1	0	2.7	1	3.6	182	157	6.6	21	110	7.3	0.03	44	23
C5M2	0	2.4	X	3.1	258	155	6.3	19	115	9.9	0.03	44	23
C5M3	0	3.9	1.2	2.9	382	154	6.2	20.7	113	9.5	0.03	44	23
C9M1	0	4.3	0.61	2.8	138	163	5.3	17.1	112	9.9	0.04	49	26
C9M2	0	3	0.78	2.7	195	156	6.5	16.7	112	9.6	0.04	46	24
C4M1	30	3.7	0.7	3.4	616	154	6.8	16	113	9.8	0.04	46	24
C4M2	30	4.9	0.5	2.9	891	155	5.7	17	112	10.2	0.03	47	25
C4M3	30	4.9	0.6	3.1	391	158	5.1	19	114	8	0.03	51	28
C8M1	30	4.3	X	2.7	115	156	5	18.2	109	10.2	0.03	50	27
C8M2	30	5.2	0.72	2.9	81	158	6.12	18.4	113	7.9	0.04	50	29
C3M4	60	3.9	0.9	3	241	155	6.6	26	108	7.5	0.03	47	24
C3M5	60	4.8	1.3	2.4	296	152	6.3	19	110	10	0.04	50	26
C7M1	60	5.3	X	2.8	378	155	6	14.5	113	9.2	0.04	52	28
C7M2	60	4.6	0.95	2.7	188	154	5.7	17.3	115	7.5	0.03	49	26
C2M3	120	4.2	1.1	3.1	207	155	6.7	22	106	7.1	0.03	48	26
C2M4	120	3.7	1.4	2.8	168	157	6.5	22	113	6.5	0.03	47	25
C2M5	120	3.2	0.5	2.8	139	156	6.5	23	114	6.6	0.03	44	24
C6M2	120	3.6	2.6	2.7	270	155	6.1	19.8	107	8.5	0.03	49	27
C6M3	120	3.7	0.6	2.5	600	154	6.4	20	112	9	0.03	50	28
C1M1	240	4.36	0.5	2.5	298	153	6.82	22	106	9.1	0.04	52	27
C1M2	240	3.5	0.58	3.1	237	159	5.7	16.8	109	7.3	0.03	48	26
C1M3	240	4.8	X	2.5	198	156	5.4	16.2	110	8.4	0.03	51	26
C1M4	240	3.5	0.33	3	1140	156	6.4	16.4	113	8.1	0.03	49	27
C1M5	240	4.4	1.6	3.1	229	154	6.2	22	109	8.6	0.04	49	26
C1M6	240	3.9	0.39	2.9	378	153	7.4	25	112	6	0.03	43	23

Table 12 continued

AnimalID	Dose (µg CYN/kg/d)	Glob g/L	T Bili mmol/L	Ca mmol/L	ALP U/L	ALT U/L	AST U/L	Glucose mmol/L	TBA umol/L
C5M1	0	21	2.9	2.5	31	27	59	8.8	8
C5M2	0	21	3.1	2.2	X	36	161	9.5	0
C5M3	0	21	2.6	2.3	25	33	77	9.7	4.1
C9M1	0	23	2.5	2.6	16.9	43	187	9.2	11.8
C9M2	0	22	2.6	2.4	44	48	114	10.4	10
C4M1	30	22	2	2.3	46	72	218	8.4	9.3
C4M2	30	22	2.4	2.2	42	92	141	6.3	6.6
C4M3	30	23	3.1	2.3	54	151	189	9.6	7.7
C8M1	30	23	2.7	2.4	X	52	72	11.3	X
C8M2	30	21	3.4	2.6	37	24	49	13.4	7.6
C3M4	60	23	3.5	2.4	35	49	70	9.3	6.8
C3M5	60	24	2.4	2.4	29	27	44	11	5.2
C7M1	60	24	2.4	2.3	X	31	120	11	X
C7M2	60	23	3.2	2.3	33	22	58	11.8	4.6
C2M3	120	22	3.2	2.6	26	39	60	8	7.6
C2M4	120	22	3.4	2.3	20	22	80	9	7.2
C2M5	120	20	2.8	2.3	23	23	56	7	5.1
C6M2	120	22	3.2	2.4	36	47	58	7.5	6.5
C6M3	120	22	2.7	2.4	38	38	91	8.9	5.7
C1M1	240	25	2.5	2.4	35	45	82	7.3	7.6
C1M2	240	22	4.2	2.3	28	53	103	5.3	0
C1M3	240	25	3.2	2.3	X	61	110	8.2	0
C1M4	240	22	2.5	2.3	X	122	136	8.4	4.4
C1M5	240	23	2.7	2.6	48	57	106	5.9	8.4
C1M6	240	20	3.3	2.3	21	42	77	8.2	6

Table 13. Experiment 2. Urine chemistry

Mouse	Dose (µg CYN/kg/d)	Urine volume (ml)	Wt. food drunk (g)	Urine % of Food	USG	pH	Blood/Blood Cells	Protein (g/L)	Glucose (mmol/L)	Creatinine (mmol/L)	Na (mmol/L)	K (mmol/L)
C5M1	0	11.72	27.6	42.5%	1.014	8.0	NEG	2.1	0.4	0.81	77	36.0
C5M2	0	16.97	29.28	58.0%	1.011	8.0	NEG	1.74	25.4	0.46	70	27.0
C5M3	0	8.23	28.93	28.4%	1.012	8.0	NEG	1.95	0.7	0.51	77	21.0
C5M4	0	11.03	31.89	34.6%	1.012	8.0	NEG	3	0.9	0.59	69	30.5
C5M5	0	6.57	25.16	26.1%	1.013	8.0	NEG	2.2	0.2	0.56	88	35.0
C9M1	0	5.09	22.34	22.8%	1.016	8.0	NEG	3.25	0.4	0.71	86	42.5
C9M2	0	10.78	24.05	44.8%	1.012	8.0	NEG	2.7	2.2	0.63	72	35.0
C9M3	0	8.18	29.85	27.4%	1.012	8.0	NEG	2.4	0.2	0.59	79	37.0
C9M4	0	11.29	25.00	45.2%	1.010	8.0	NEG	2.65	0.1	0.47	63	28.5
C9M5	0	8.59	26.77	32.1%	1.011	8.0	NEG	2.75	0.2	0.53	78	31.0
C4M1	30	12.37	31.52	39.2%	1.009	8.0	NEG	1.83	0.4	0.42	55	23.5
C4M2	30	14.00	31.89	43.9%	1.009	8.5	NEG	1.26	0.5	0.48	60	25.5
C4M3	30	13.35	26.57	50.2%	1.010	8.5	NEG	1.98	0.4	0.49	55	24.0
C4M4	30	11.4	26.55	42.9%	1.010	8.0	NEG	1.68	0.3	0.50	55	23.0
C4M5	30	8.92	19.88	44.9%	1.011	8.0	NEG	2.16	0.4	0.67	59	31.0
C8M1	30	8.11	25.51	31.8%	1.010	8.5	NEG	1.53	0.4	0.47	60	26.5
C8M2	30	9.64	20.51	47.0%	1.009	8.0	NEG	1.62	0.3	0.46	58	24.5
C8M3	30	12.01	21.65	55.5%	1.008	8.0	NEG	1.44	0.3	0.44	56	21.0
C8M4	30	7.69	17.27	44.5%	1.010	8.5	NEG	1.92	0.4	0.62	61	23.5
C8M5	30	14.28	29.75	48.0%	1.008	8.5	NEG	1.89	0.2	0.33	55	21.5
C3M1	60	10.02	35.05	28.6%	1.011	8.0	NEG	14.7	0.5	0.51	69	27.5
C3M2	60	5.36	17.15	31.3%	1.013	8.0	NEG	2.00	0.3	0.68	61	33.0
C3M3	60	12.4	23.54	52.7%	1.012	8.0	NEG	1.92	0.4	0.55	67	23.0
C3M4	60	10.69	29.05	36.8%	1.011	8.0	NEG	1.86	0.1	0.58	68	31.0
C3M5	60	11.06	34.68	31.9%	1.012	8.0	NEG	2.13	0.2	0.62	70	35.5
C7M1	60	13.06	23.3	56.1%	1.009	8.0	NEG	1.38	0.3	0.42	67	22.5
C7M2	60	9.48	20.4	46.5%	1.010	8.0	NEG	1.74	0.3	0.54	66	27.5
C7M3	60	10.27	21.58	47.6%	1.010	7.5	NEG	1.62	0.0	0.53	70	26.0
C7M4	60	11.04	25.83	42.7%	1.009	8.0	NEG	1.62	0.1	0.44	69	25.5
C7M5	60	–	–	–	–	–	–	–	–	–	–	–
C2M1	120	13.97	28.78	48.5%	1.011	8.0	NEG	0.93	0.4	0.52	67	29.0
C2M2	120	13.83	30.8	44.9%	1.011	8.0	NEG	0.90	22.4	0.52	68	12.5
C2M3	120	13.51	25.33	53.3%	1.010	8.0	NEG	1.14	0.4	0.52	60	29.5
C2M4	120	9.28	26.18	35.4%	1.010	8.0	NEG	0.75	0.4	0.58	63	26.0
C2M5	120	10.81	24.34	44.4%	1.010	8.0	NEG	1.35	0.5	0.62	65	27.5
C6M1	120	–	–	–	–	–	–	–	–	–	–	–
C6M2	120	11.31	19.94	56.7%	1.019	8.0	NEG	0.81	0.2	0.45	67	23.0
C6M3	120	10.11	21.46	47.1%	1.009	8.0	NEG	1.32	0.4	0.52	64	27.0
C6M4	120	10.63	24.21	43.9%	1.009	8.0	NEG	1.41	0.1	0.43	64	27.0
C6M5	120	12.24	23.4	52.3%	1.009	8.0	NEG	1.17	0.0	0.46	62	25.0
C1M1	240	4.36	23.65	18.4%	1.015	8.0	NEG	1.68	0.5	0.90	82	46.5
C1M2	240	2.48	15.7	15.8%	1.014	8.0	MOD	1.02	0.2	0.84	81	45.0
C1M3	240	7.72	21.64	35.7%	1.011	8.0	NEG	0.87	0.2	0.65	78	33.5
C1M4	240	6.56	19.73	33.2%	1.011	8.0	NEG	0.87	0.3	0.64	73	30.5
C1M5	240	7.28	22.82	31.9%	1.012	8.5	NEG	0.93	0.4	0.66	75	39.0
C1M6	240	12.06	22.69	53.2%	1.010	8.0	NEG	1.38	0.4	0.62	67	28.0

Table 13 continued

Mouse	Dose (µg CYN/kg/d)	P (mmol/L)	USG/Creat	Prot/Creat	Gluc/Creat	Na/Creat	K/Creat	P/Creat
C5M1	0	5.28	1.25	2.59	0.49	95.06	44.44	6.52
C5M2	0	4.60	2.20	3.78	55.22	152.17	58.70	10.00
C5M3	0	6.00	1.98	3.82	1.37	150.98	41.18	11.76
C5M4	0	5.35	1.72	5.08	1.53	116.95	51.69	9.07
C5M5	0	6.21	1.81	3.93	0.36	157.14	62.50	11.09
C9M1	0	10.77	1.43	4.58	0.56	121.13	59.86	15.17
C9M2	0	4.48	1.61	4.29	3.49	114.29	55.56	7.11
C9M3	0	6.62	1.72	4.07	0.34	133.90	62.71	11.22
C9M4	0	6.99	2.15	5.64	0.21	134.04	60.64	14.87
C9M5	0	6.12	1.91	5.19	0.38	147.17	58.49	11.55
C4M1	30	5.11	2.40	4.36	0.95	130.95	55.95	12.17
C4M2	30	3.18	2.10	2.63	1.04	125.00	53.13	6.63
C4M3	30	3.81	2.06	4.04	0.82	112.24	48.98	7.78
C4M4	30	3.97	2.02	3.36	0.60	110.00	46.00	7.94
C4M5	30	3.04	1.51	3.22	0.60	88.06	46.27	4.54
C8M1	30	5.20	2.15	3.26	0.85	127.66	56.38	11.06
C8M2	30	4.95	2.19	3.52	0.65	126.09	53.26	10.76
C8M3	30	2.77	2.29	3.27	0.68	127.27	47.73	6.30
C8M4	30	5.59	1.63	3.10	0.65	98.39	37.90	9.02
C8M5	30	4.12	3.05	5.73	0.61	166.67	65.15	12.48
C3M1	60	5.70	1.98	28.82	0.98	135.29	53.92	11.18
C3M2	60	6.64	1.49	2.94	0.44	89.71	48.53	9.76
C3M3	60	6.74	1.84	3.49	0.73	121.82	41.82	12.25
C3M4	60	5.90	1.74	3.21	0.17	117.24	53.45	10.17
C3M5	60	9.36	1.63	3.44	0.32	112.90	57.26	15.10
C7M1	60	4.37	2.40	3.29	0.71	159.52	53.57	10.40
C7M2	60	6.23	1.87	3.22	0.56	122.22	50.93	11.54
C7M3	60	5.39	1.91	3.06	0.00	132.08	49.06	10.17
C7M4	60	4.48	2.29	3.68	0.23	156.82	57.95	10.18
C7M5	60	–	–	–	–	–	–	–
C2M1	120	5.95	1.94	1.79	0.77	128.85	55.77	11.44
C2M2	120	3.69	1.94	1.73	43.08	130.77	24.04	7.10
C2M3	120	2.93	1.94	2.19	0.77	115.38	56.73	5.63
C2M4	120	4.94	1.74	1.29	0.69	108.62	44.83	8.52
C2M5	120	4.78	1.63	2.18	0.81	104.84	44.35	7.71
C6M1	120	–	–	–	–	–	–	–
C6M2	120	5.77	2.26	1.80	0.44	148.89	51.11	12.82
C6M3	120	5.50	1.94	2.54	0.77	123.08	51.92	10.58
C6M4	120	2.87	2.35	3.28	0.23	148.84	62.79	6.67
C6M5	120	3.86	2.19	2.54	0.00	134.78	54.35	8.39
C1M1	240	5.78	1.13	1.87	0.56	91.11	51.67	6.42
C1M2	240	4.34	1.21	1.21	0.24	96.43	53.57	5.17
C1M3	240	4.41	1.56	1.34	0.31	120.00	51.54	6.78
C1M4	240	6.12	1.58	1.36	0.47	114.06	47.66	9.56
C1M5	240	4.99	1.53	1.41	0.61	113.64	59.09	7.56
C1M6	240	7.54	1.63	2.23	0.65	108.06	45.16	12.16

Table 14. Experiment 2. Blood coagulation rates

Mouse	Dose (µg CYN/kg/d)	PT Seconds	APTT Seconds
C5M4	0	10.3	18.0
C5M5	0	11.7	20.0
C9M3	0	11.0	18.3
C9M4	0	11.0	17.3
C9M5	0	10.7	17.4
C4M4	30	11.3	21.4
C4M5	30	11.6	22.0
C8M3	30	13.0	18.0
C8M4	30	11.0	22.0
C8M5	30	11.4	18.0
C3M1	60	11.1	21.0
C3M3	60	11.4	21.0
C7M3	60	11.4	18.0
C7M5	60	11.0	18.0
C2M1	120	10.6	18.0
C2M2	120	11.4	19.3
C6M3	120	11.0	21.5
C6M4	120	11.1	22.0
C6M5	120	11.1	22.0

Table 15. Experiment 2. Haematology

Mouse	Dose (µg CYN/kg/d	RBC ($x10^{12}$/L)	Hb (g/L)	PCV (L/L)	MCV (fl)	MCH (pg/L)	MCHC (L/L)	WCC ($x10^9$/L)	Neuts ($x10^9$/L)	Lymph ($x10^9$/L)a
C5M4	0	7.9	134	0.39	49	16.8	341	4.3	3.6	0.6
C5M5	0	7.6	128	0.39	52	16.8	327	4.3	2.9	1.4
C9M3	0	7.4	123	0.36	49	16.7	344	3.1	1.2	1.9
C9M4	0	7.7	129	0.39	51	16.7	330	2.5	1.4	1.1
C9M5	0	7.3	124	0.36	49	17.0	349	3.0	1.0	2.0
C4M4	30	7.0	100	0.35	50	14.3	286	5.0	1.1	3.8
C4M5	30	7.5	106	0.39	52	14.2	275	6.8	2.7	3.9
C8M3	30	7.9	135	0.39	49	17.1	349	5.3	2.3	2.8
C8M4	30	8.0	134	0.40	50	16.8	338	5.0	1.6	3.3
C8M5	30	7.9	130	0.40	50	16.3	327	5.3	1.7	3.3
C3M1	60	7.3	115	0.37	50	15.7	314	5.0	3.4	1.5
C3M2	60	7.1	119	0.37	51	16.8	327	3.6	1.1	2.5
C3M3	60	7.0	117	0.36	51	16.8	328	5.3	3.7	1.6
C7M3	60	8.6	141	0.43	50	16.3	325	3.0	0.9	2.0
C7M5	60	8.2	137	0.41	50	16.6	333	1.7	0.4	1.2
C2M1	120	8.5	135	0.42	49	15.8	324	6.6	4.2	2.0
C2M2	120	7.5	118	0.37	49	15.7	323	4.0	2.6	1.3
C6M4	120	7.9	130	0.39	49	16.4	337	2.4	0.6	1.8
C6M5	120	7.2	128	0.37	52	17.7	343	1.9	0.5	1.3

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