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Toxicity and recovery in the pregnant mouse after gestational exposure to the cyanobacterial toxin, cylindrospermopsin

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ABSTRACT: Cylindrospermopsin (CYN) is a tricyclic alkaloid toxin produced by fresh water cyanobacterial species worldwide. CYN has been responsible for both livestock and human poisoning after oral exposure. This study investigated the toxicity of CYN to pregnant mice exposed during different segments of gestation. The course of recovery and individual responses to the toxin were evaluated. Adverse effects of CYN were monitored up to 7 weeks post-dosing by clinical examination, histopathology, biochemistry and gene expression. Exposure on gestational days (GD) 8–12 induced significantly more lethality than GD13–17 exposure. Periorbital, gastrointestinal and distal tail hemorrhages were seen in both groups. Serum markers indicative of hepatic injury (alanine amino transferase, aspartate amino transferase and sorbitol dehydrogenase) were increased in both groups; markers of renal dysfunction (blood urea nitrogen and creatinine) were elevated in the GD8–12 animals. Histopathology was observed in the liver (centrilobular necrosis) and kidney (interstitial inflammation) in groups exhibiting abnormal serum markers. The expression profiles of genes involved in ribosomal biogenesis, xenobiotic and lipid metabolism, inflammatory response and oxidative stress were altered 24 h after the final dose. One week after dosing, gross, histological and serum parameters had returned to normal, although increased liver/body weight ratio and one instance of gastrointestinal bleeding was found in the GD13–17 group. Gene expression changes persisted up to 2 weeks post-dosing and returned to normal by 4 weeks. Responses of individual animals to CYN exposure indicated highly significant inter-animal variability within the treated groups. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: cylindrospermopsin; cyanobacteria; toxicity; mouse; serum chemistries; gene expression

INTRODUCTION

Cylindrospermopsin (CYN) is a potent tricyclic alkaloid toxin produced by fresh water cyanobacteria that has been associated with toxicity and illness in people after environmental exposures. CYN and several structurally related chemicals are known to be produced by a number of cyanobacterial species that have been identified worldwide. These organisms include *Cylindrospermopsis raciborskii* in Australia (Ohtani *et al.*, 1992), New Zealand (Stirling and Quilliam, 2001), Thailand (Li *et al.*, 2001a) and the USA (Burns, 2008); *Umezakia natans* in Japan (Harada *et al.*, 1994); *Aphanizomenon ovalisporum* in Israel (Banker *et al.*, 1997); *Raphidiopsis curvata* in China (Li *et al.*, 2001b); *Anabaena bergii* in Australia (Fergusson and Saint, 2003); and *Aphanizomenon flos-aquae* in Germany (Preussel *et al.*, 2006). The widespread occurrence and increasing range of *C. raciborskii* (Briand *et al.*, 2004), coupled with its ability to bioaccumulate (White *et al.*, 2006, 2007), indicate that it possesses the potential to cause adverse health effects in cattle (Thomas *et al.*, 1998) and human populations.

The health hazard of CYN to human populations was first noted in 1979 following an illness on Palm Island, Australia, that affected 148 people, a majority of whom required hospitalization. The affected individuals exhibited anorexia, hepatomegaly, abnormal levels of protein, glucose and ketones and, in later stages, blood

in the urine, acidotic shock, bloody diarrhea and bleeding mucous membranes (Byth, 1980). Five days prior to the onset of the symptoms, the reservoir serving as the town's drinking water source experienced an algal bloom that was treated with the algicide, copper sulfate. The water that was distributed to the town of Palm Island was subsequently chlorinated as part of the standard water treatment protocol. It was noted that people living in homes not supplied with water from the reservoir did not exhibit the symptoms and the possibility of algal toxicity was suggested (Bourke *et al.* 1983). Hawkins *et al.* (1985) identified *C. raciborskii* as one of the most prevalent species of bloom-forming cyanobacteria in the reservoir at that time and found that administration of this lyophilized algae to mice by intraperitoneal (i.p.)

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injection produced hepatic toxicity as well as toxic effects in other organs. Ohtani *et al.* (1992) identified the hepatotoxic chemical produced by *C. raciborskii* as CYN and the toxin has now been isolated from different populations of this species. Saker *et al.* (1999) identified CYN as the probable cause for cattle mortality on a farm in Queensland, and the numerous observations of bioaccumulation, death and developmental toxicity in a variety of organisms have led some workers to suggest that the toxin may be an ecological hazard (see Kinnear *et al.*, 2009).

Mice treated with purified CYN or with CYN-containing cyanobacterial lyophilates exhibit dose-related hepatic and renal damage, as well as adverse effects in other organ systems. The distribution and elimination of CYN has been determined at doses within the lethal range (Norris *et al.*, 2001a) and the half-life was found to be in the range of 12 h. At 12 h post-dosing, less than 5% of the dose remained in the liver with much less present in the kidneys or blood. Liver damage, characterized by centrilobular necrosis, is a constant finding in all studies involving the effects of CYN on mammals (Seawright *et al.*, 1999; Bernard *et al.*, 2003; Griffiths and Saker, 2003; Humpage and Falconer, 2003). Kidney damage involving proximal tubule necrosis is often noted, as well as other changes in the basic architecture of the organ (Falconer *et al.*, 1999). Hemorrhages in the lungs (Hawkins *et al.*, 1985; Bernard *et al.*, 2003) and heart (Hawkins *et al.*, 1985; Seawright *et al.*, 1999) have been reported. Comparison of exposure to cellular extracts of *C. raciborskii* and pure CYN revealed differences in the type and severity of toxicity, leading a number of workers to suggest the possibility of other toxins being produced by the cyanobacteria (Falconer *et al.*, 1999; Metcalf *et al.*, 2002; Humpage and Falconer, 2003). It has been hypothesized that CYN may be metabolized to an active compound based on data showing that cytochrome P450 inhibition protects hepatic cell cultures from CYN effects (Runnegar *et al.*, 1995; Humpage *et al.*, 2005); and treatment with the cytochrome P450 inhibitor piperonyl butoxide protected mice from CYN toxicity (Norris *et al.*, 2002), although the active metabolite(s) have not been identified. It should be noted that CYN itself did induce significant gene expression changes in three types of cell cultures not thought to be capable of the metabolic activation of CYN (Bain *et al.*, 2007). Different strains of *C. raciborskii* can produce different toxins, or produce none, and chemical variants of CYN are present in different strains (see Bernard *et al.*, 2003). Two investigators have reported on hepatotoxic effects of cell-free extracts from *C. raciborskii* strains that did not contain measurable levels of CYN (Fastner *et al.*, 2003; Saker *et al.*, 2003).

Our laboratory has been engaged in a series of studies designed to evaluate the potential developmental toxicity of cyanobacterial toxins in mammals (microcystin-LR, Chernoff *et al.*, 2002; anatoxin-a, Rogers *et al.*, 2005; and CYN, Rogers *et al.*, 2007). In Rogers *et al.* (2007), the data indicated that maternal exposure to CYN during pregnancy reduced litter size but was not otherwise developmentally toxic when administered on gestational days (GD) 8–12, the major period of organogenesis. When fetuses were exposed later in pregnancy (GD13–17), however, CYN produced a variety of other adverse effects in addition to reduced litter size. At birth, pups were noted with reduced weight and bleeding in the gastrointestinal tract and tail similar to that noted in the pregnant females. All CYN-exposed pups were cross-fostered to control dams but significantly decreased survival was observed. After weaning, a persistent reduced body weight was seen up to 1½ years after birth. The offspring toxicity data are summarized in Rogers *et al.* (2007). The data reported

here summarize an effort to characterize the adult post-exposure toxicity and subsequent course of recovery after CYN exposure in the post-partum adult females from the 2007 study using a variety of endpoints: gross observations, clinical chemistry markers of hepatic and renal function, histopathology and analysis of gene expression in liver tissue.

MATERIALS AND METHODS

Animals

The CD-1 (Swiss–Webster) mice used in the current study were post-parturitional adult females whose litters were used to evaluate the postnatal developmental toxicity of CYN (Study 1 in Rogers *et al.*, 2007). The dams were obtained from Charles River Laboratories (Raleigh, NC, USA) and were singly housed in polycarbonate cages on heat-treated pine shaving bedding in animal rooms with a controlled temperature range (22–26 °C). They were fed commercial rodent chow (Purina Prolab®) and water *ad libitum*. Maternal animals treated during two different dosing periods were evaluated: early in gestation (GD8–12) or the end of gestation (GD13–17). GD0 was defined by the presence of a vaginal sperm plug. All studies were conducted after approval by the National Health and Ecological Effects Research Laboratory Institutional Animal Care and Use Committee using the guidelines of the National Research Council (1996).

Compound

CYN used in these studies was supplied by the Australian Water Quality Center (AWQC), a business unit of the South Australian Water Corporation, Adelaide, Australia. It was purified from bulk cultures of *Cylindrospermopsis raciborskii* Seenayya Subba Raju using a proprietary method based on that published by Norris *et al.* (2001b). The purified toxin (C₁₅H₂₁N₅O₇S; CAS no. 143545-90-8, molecular weight 415.42) was characterized using nuclear magnetic resonance spectrometry, direct infusion electrospray mass spectrometry and analysis by liquid chromatography using photodiode array and full-scan positive-ion mode mass spectrometric detection. The toxin was shown to be free from organic impurities and present as the sodium salt. The estimated purity was >98%.

Experimental Design

Pregnant mice were administered daily i.p. injections of 50 µg kg⁻¹ CYN in 0.1 ml distilled water for five days on GD8–12 (42 animals) or GD13–17 (42 animals). The dose was calculated based on the GD7 weight and the quantity of CYN administered was not altered during the course of dosing. Concurrent controls received 0.1 ml distilled water alone on either GD8–12 (26 animals) or GD13–17 (26 animals). Toxicity induced by CYN after i.p. injection indicates that the hepatic and renal effects are qualitatively similar to those obtained after oral dosing, although the oral route is less toxic by a ~25-fold factor (Falconer *et al.*, 1999). The similarity of the observed toxicity to the effects noted in the human population, coupled with the difficulty in obtaining an adequate supply of CYN, was the basis of our choice of the i.p. route for these studies. Dose–response data from the study (data not shown, see Rogers *et al.*, 2007) indicated that 50 µg kg⁻¹ per

day induced effects that were similar to those seen in humans (i.e. hepatic and renal toxicity and gastrointestinal bleeding). Levels of 32 µg did not induce these effects and doses higher than 50 µg kg⁻¹ per day induced death in the great majority of test animals. Animals were weighed prior to treatment, during the dosing period and on the day they were euthanized. Subsets of animals (two or three controls and three to five CYN-exposed) were euthanized the day following the last dose and on post-treatment days 7 and 14 for both exposures; days 28 and 42 for the GD8–12 exposure, and on days 35 and 49 for the GD13–17 exposures. Blood, liver and kidney samples were obtained at each time point for further analyses. Endpoints measured included maternal weight, clinical signs of toxicity, during and after the dosing period; serum chemistries indicative of hepatic and/or renal function and general homeostasis, histopathology of liver and kidney tissues, and hepatic gene expression after the dosing period.

Blood Collection and Serum Preparation

Animals were euthanized by CO₂, and removed when breathing ceased. Access to the descending aorta was obtained by making a 'V'-shaped cut in the abdominal wall and displacing the organs to visualize the vessel just below the diaphragm. Blood was collected from the descending aorta immediately by inserting a 26G 3/8 inch needle attached to a 1 cm³ syringe into the vessel. The collected blood was transferred to a 4 ml Vacutainer serum separator tube, allowed to clot for approximately 1 h, and spun at 3000 rpm for 10 min to separate the serum. Serum was transferred to an autoanalyzer cup and stored at -80 °C.

Serum Chemistries

Serum clinical chemistry markers of hepatic and renal function and injury were measured using the Konelab Arena 30 Clinical Analyzer (Thermo Clinical Labsystems, Espoo, Finland). Hepatic function was assessed with AAT (alpha-1 antitrypsin), ALP (alkaline phosphatase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), LDH (lactate dehydrogenase) and SDH (sorbitol dehydrogenase). Renal function endpoints were BUN (blood urea nitrogen) and creatinine. Glucose and albumin were measured as markers of general toxicity. All assays were performed using commercially available clinical chemistry kits. LDH, BUN, glucose, ALT, AST and ALP kits were obtained from Thermo Electron Corp., Melbourne, Australia. Creatinine and SDH kits are from Diagnostic Chemicals Ltd, Oxford, CT. Albumin and AAT kits were obtained from DiaSorin Inc. (Stillwater, MN, USA).

Tissue Preparation for Histological Evaluation

The liver and kidneys were excised, weighed, and examined for visible lesions. Liver tissue samples approximately 3 mm thick were obtained from both the median and left lobes of 56 animals and fixed in >10× volume 10% neutral buffered formalin (NBF). Both kidneys were collected from the same animals, the right kidney cut longitudinally and the left cross-sectionally, and also fixed in >10× volume 10% NBF. Tissues were placed on a horizontal shaker for at least 18 h and then transferred to 70% ethanol for not more than 72 h. Sections of liver and kidney from each animal were evaluated. Tissues were routinely processed, embedded in

paraffin, sectioned at approximately 5 µm, stained with hematoxylin and eosin, and examined histologically. Tissues were examined using an Olympus BX51, with a 7-ocular nosepiece ranging from 100× to 600×.

Gene Array and Real-time RT-PCR Analysis of Hepatic Tissue

A tissue sample, adjacent to that collected for histopathology, was obtained from the liver for gene array analysis. The tissue was immediately homogenized in TRI reagent (Sigma-Aldrich, St Louis, MO, USA) and processed through alcohol precipitation on the same day. RNA pellets were then stored at -80 °C until further use. Following resuspension in RNase free water, RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington DE), purified through RNeasy spin columns (Qiagen, Valencia, CA, USA), evaluated using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and subsequently stored at -80 °C. Only samples with an RNA Integrity number of at least 8.5 (2100 Expert software, version B.01.03) were included in the study. RNA labeling, microarray hybridization and scanning, and data reduction were done at the EDGE² Program, University of Wisconsin, using a two-channel custom-spotted microarray developed for evaluation of liver toxicity (see Hayes *et al.*, 2005 for details on generation of arrays and techniques used to obtain the gene array data).

Real-time RT-PCR analysis was performed using genes selected to verify the effects noted by microarray analysis. These analyses were conducted using the same RNA samples utilized for microarray analysis with the addition of either two or three samples per group from the GD13–17 dose group that were included to evaluate treatment recovery at 7, 16, and 28 days post-dosing. Two micrograms total RNA was digested using 2 units DNaseI (no. M6101, Promega Corporation, Madison, WI, USA) for 30 min at 37 °C followed by 10 min at 65 °C in a buffer containing 40 mM Tris (pH 8.0), 10 mM MgSO₄ and 1 mM CaCl₂. The RNA was then quantified using a Quant-iT RiboGreen RNA assay kit according to the manufacturer's protocol (no. R11490, Invitrogen Corporation, Carlsbad, CA, USA) and approximately 1.5 µg RNA reverse transcribed using a High Capacity cDNA Archive Kit according to the provided protocol (no. 4322171, Applied Biosystems, Foster City, CA, USA). Amplification was performed on an Applied Biosystems model 7900HT Fast Real-Time PCR System in duplicate using 25 ng cDNA and TaqMan Universal PCR Master Mix (no. 4304437, Applied Biosystems) in a total volume of 12 µl. Beta-2-microglobulin (B2m, Entrez no. 12010) was used as an endogenous reference gene. The following TaqMan assays (Applied Biosystems) were included in the study: B2m (no. Mm00437762_m1), Ehhadh (no. Mm00470091_s1), Cyp2b10 (no. Mm00456591_m1), Cyp2E1 (no. Mm00491127_m1), Fabp1 (Mm00444340), Gclc (Mm00802655_m1), Nfe2l2 (Mm00477784_m1), Rpl6 (Mm01198491_g1), Rps12 (Mm03030276_g1), F10 (Mm00484177_m1), C4a (no. Mn00550309_m1), C9 (Mm00442739_m1) and Hpx (Mm00457510_m1). Fold change was estimated using the 2^{-ΔΔCT} method of Livak and Schmittgen (2001).

Data Evaluation

The weight and clinical chemistry data were analyzed by analysis of variance (ANOVA) using SASversion 9.2 software (SAS Institute Inc., 2008). Means, standard errors and coefficients of variation

were calculated in Proc Means. In many cases the variances of outcome measures were not homogeneous across the treatment groups; therefore a Box Cox transformation was calculated (SAS Proc Transreg) to stabilize the variance (Box and Cox, 1964). Proc GLM was used to perform one-way ANOVAs on the transformed data across the 10 treatment by day groups. Pairwise *t*-tests were calculated within the ANOVAs to test for differences between the treated and control groups at each time point; a Bonferroni correction was used to adjust for multiple comparisons. Where a direction of change was predicted *a priori*, a one-sided *t*-test was used; otherwise a two-sided test was used. Levene's test (Levene, 1960) was used to evaluate the statistical significance of the difference in variance between the treated group one day after the last dose and the pool of all other treatment groups. In the array data, gene transcripts that deviated more than 2-fold from control were further evaluated for relevance to canonical pathways and biological functions using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Heat maps were generated using Eisen Lab Cluster and Treeview software (<http://rana.lbl.gov/EisenSoftware.htm>).

RESULTS

Clinical Findings

Clinical signs of toxicity are summarized in Table 1. GD8–12 animals were affected earlier and to a greater degree than the GD13–17 group. Maternal weight gain was significantly reduced after the initial dose of CYN in the GD8–12 animals and this effect continued throughout the treatment period (Fig. 1a). After the fifth dose, maternal weights were significantly reduced, but this effect did not persist (Table 2). Vaginal bleeding was observed on GD11 following the third dose of CYN along with reduced activity, blood in the distal area of the tail, and mild to moderate hemorrhage in and around one or both eyes following the fourth dose. Vaginal and/or periorbital hemorrhages were often associated with death before GD17. Mortality and morbidity-required euthanasia initially occurred after the fourth dose on GD12 (8/42 animals). Eight died on GD13 after the fifth dose with an additional five moribund animals euthanized at that time. Single instances of death occurred on GD16 and GD18. Gestational length in treated animals did not differ from controls.

The body weights of the GD13–17 dosed animals were not as severely affected; the only significant weight differences noted were after the second dose (Fig. 1b) and 49 days after the last dose (Table 3). There were no deaths in this group. Intraluminal blood in the gastrointestinal tract of GD13–17 animals was found

in three of four euthanized one day after the last dose and 1 of 5 on the seventh day after the last dose. A single instance of bleeding around the eyes was observed. Treated animals gave birth earlier than controls with 58% of the CYN animals giving birth by 1:00 p.m. on GD18/PND0 as compared with 31% of the controls.

No other clinical signs of toxicity were observed in either group after 7 days post-dosing.

Serum Biochemistries and Organ/body Weight Ratios

The data for the GD8–12 and GD13–17 dose groups are summarized in Tables 2 and 3. The mean levels of three markers of hepatic function (AAT, ALT and AST) were significantly elevated ($P \leq 0.01$) on the day following the last dose in both the GD8–12

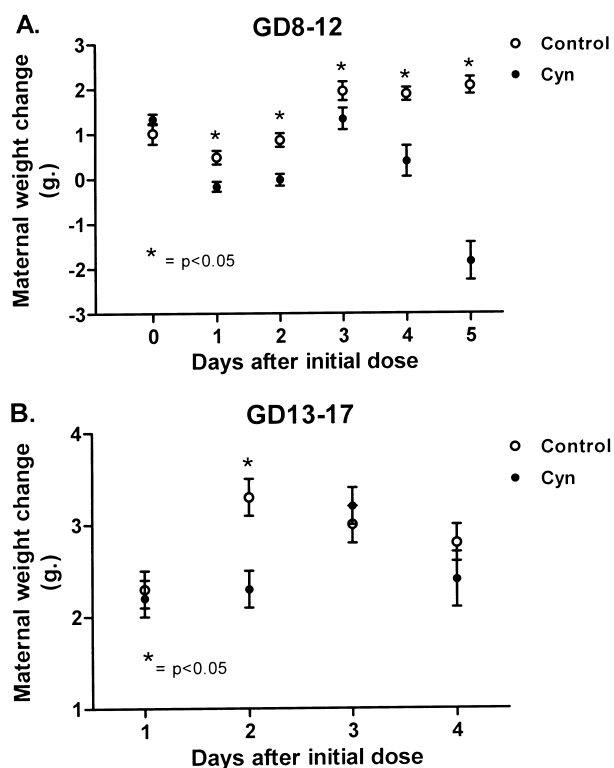


Figure 1. Maternal weight change during cylindrospermopsin administration: (A) GD8–12; (B) GD13–17. Note that animals in the GD13–17 group had given birth after the fifth dose and therefore these data are not shown.

Table 1. Maternal effects after exposure to 50 $\mu\text{g}/\text{kg}$ cylindrospermopsin

Exposure	Treatment	No. mice	No. died (%)	Vaginal	Bleeding (no.) ^a			Gastrointestinal
					Orbital	Tail		
GD 8–12	Control	26	0	0	0	0	0	
	CYN	42	23(55) ^b	13	2	2	0	
GD 13–17	Control	26	0	0	0	0	0	
	CYN	42	0	0	1	0	3 ^c	

^aMultiple effects occurred in single animals.

^b $p < 0.01$.

^cTwo instances of intestinal bleeding were noted in the four animals killed on GD 13 and one instance was seen 7 days post-dosing.

Table 2. Clinical chemistries and organ/body weight ratios for GD8–12 animals

Days after last dose	No.	Kill/Wt	L/BW	K/BW	AAT (mg dl ⁻¹)	ALP (IU l ⁻¹)	ALT (IU l ⁻¹)	AST (IU l ⁻¹)	LDH (IU l ⁻¹)	SDH (IU l ⁻¹)	BUN (mg dl ⁻¹)	Creatinine (mg dl ⁻¹)	Glucose (mg dl ⁻¹)	Albumin (g dl ⁻¹)
1	Con	3	40.2 ± 1.5	6.2 ± 0.2	1.1 ± 0.1	10.3 ± 0.6	30.6 ± 2.3	82.1 ± 4.8	378.5 ± 22.4	39.9 ± 3.4	12.1 ± 1.2	0.35 ± 0.01	150.9 ± 15.4	2.2 ± 0.0
	%cv		6.5	6.6	9.6	6.8	12.8	10.2	10.3	14.6	16.7	6.61	17.7	0.6
	Cyn	5***	32.2* ± 2.1	6.5 ± 0.5	1.2 ± 0.1	34.1* ± 16.9	112.1* ± 37.0*	219.3* ± 35.9*	692.2 ± 361.8*	50.5 ± 7.7*	129.3* ± 30.9*	0.60** ± 0.13*	130.2 ± 11.4	0.9* ± 0.2
	%cv		14.4	15.8	22.0	70.1	65.4	40.6	98.7	40.9	50.6	64.60	21.5	46.1
7	Con	3	36.0 ± 2.4	6.0 ± 0.6	1.3 ± 0.1	9.8 ± 0.1	37.8 ± 6.0	65.7 ± 1.8	255.2 ± 43.7	32.1 ± 3.8	17.8 ± 0.7	0.45 ± 0.04	218.2 ± 31.7	3.4 ± 0.3
	%cv		11.6	17.1	3.3	1.9	27.3	4.8	29.6	20.7	6.5	14.64	25.1	14.3
	Cyn	5	34.9 ± 1.7	6.3 ± 0.6	1.2 ± 0.1	10.0 ± 0.5	38.2 ± 0.4	65.2 ± 3.2	227.1 ± 16.8	33.7 ± 3.5	15.4 ± 2.5	0.39 ± 0.02	204.1 ± 22.8	3.1 ± 0.1
	%cv		10.7	21.1	8.9	10.7	22.3	11.1	16.5	23.3	36.6	8.87	25.0	7.7
14	Con	3	34.4 ± 1.7	5.6 ± 0.1	1.2 ± 0.1	9.9 ± 0.2	47.3 ± 6.3	73.7 ± 8.8	309.9 ± 27.6	41.8 ± 1.7	17.8 ± 1.4	0.54 ± 0.05	259.5 ± 11.3	3.4 ± 0.1
	%cv		8.7	3.5	5.2	3.3	23.0	20.7	15.4	7.2	13.3	17.13	7.5	7.5
	Cyn	3	34.6 ± 0.2	6.1 ± 0.5	1.3 ± 0.1	9.1 ± 1.1	48.3 ± 3.0	125.5 ± 13.1	451.9 ± 51.3	57.1 ± 7.2	20.9 ± 2.0	0.43 ± 0.05	207.9 ± 13.3	3.4 ± 0.1
	%cv		1.2	13.8	7.8	21.3	6.6	18.0	19.7	21.9	16.7	19.55	11.1	5.6
28	Con	3	36.8 ± 2.0	5.2 ± 0.4	1.0 ± 0.1	10.5 ± 0.9	46.3 ± 5.4	65.9 ± 4.6	290.0 ± 91.9	35.7 ± 8.9	20.6 ± 5.3	0.39 ± 0.02	281.3 ± 1.8	3.0 ± 0.1
	%cv		9.4	13.8	1.3	13.5	20.3	12.0	54.9	43.1	44.2	7.71	1.1	7.0
	Cyn	3	37.6 ± 0.6	5.4 ± 0.4	1.2 ± 0.1	9.8 ± 0.6	38.8 ± 9.2	32.2 ± 18.6	247.6 ± 65.4	35.2 ± 8.0	16.6 ± 4.4	0.37 ± 0.01	244.9 ± 13.5	3.1 ± 0.2
	%cv		2.1	11.4	4.7	10.1	41.2	68.4	45.7	39.4	45.9	6.80	9.6	10.6
42	Con	3	38.2 ± 2.3	4.7 ± 0.1	1.2 ± 0.1	10.4 ± 0.3	45.1 ± 7.4	81.3 ± 7.4	296.2 ± 5.5	36.8 ± 0.6	22.1 ± 0.1	0.40 ± 0.00	278.3 ± 24.6	3.1 ± 0.1
	%cv		10.5	2.1	13.3	4.6	28.4	15.8	3.2	2.7	0.4	1.82	15.3	4.7
	Cyn	3	36.0 ± 0.8	5.0 ± 0.1	1.1 ± 0.1	10.2 ± 0.2	42.9 ± 5.7	65.6 ± 6.4	207.1 ± 6.3	30.8 ± 3.8	19.6 ± 0.6	0.40 ± 0.00	247.7 ± 3.3	3.1 ± 0.1
	%cv		3.9	4.9	15.1	3.8	23.1	16.9	5.3	21.5	4.9	11.38	2.3	4.7

%cv, % coefficient of variation.

**P < 0.01.

***P < 0.05.

Note that significances are given for both means and variance.

AAT, antitrypsin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; SDH, sorbitol dehydrogenase; BUN, blood urea nitrogen; K/BW, kidney/body weight ratio.

***AAT, two samples; LDH, BUN, four samples.

Table 3. Clinical chemistries and organ/body weight ratios for GD13–17 animals

Days after last dose	No.	Kill/Wt	L/BW	K/BW	AAT (mg dl ⁻¹)	ALP (IU l ⁻¹)	ALT (IU l ⁻¹)	AST (IU l ⁻¹)	LDH (IU l ⁻¹)	SDH (IU l ⁻¹)	BUN (mg dl ⁻¹)	Creatinine (mg dl ⁻¹)	Glucose (mg dl ⁻¹)	Albumin (g dl ⁻¹)
1	Con	2	30.1 ± 3.0	6.6 ± 0.6	1.3 ± 0.1	9.2 ± 0.1	42.3 ± 4.9	57.4 ± 3.9	277.1 ± 49.3	33.3 ± 8.2	26.2 ± 0.1	0.45 ± 0.02	210.7 ± 9.7	3.6 ± 0.7
	%cv		14.1	13.5	15.9	2.1	16.2	9.7	25.1	35.1	0.8	5.03	6.5	26.8
	Cyn	4	31.5 ± 2.7	7.5 ± 0.9	1.2 ± 0.2	28.5* ± 5.5*	117.6	314.1* ± 90.6*	992.1* ± 232.9*	119.0* ± 41.9*	33.9 ± 6.2*	0.46 ± 0.04	97.2* ± 11.9	1.7* ± 0.4*
	%cv		17.2	24.6	26.3	38.3	34.5	57.7	46.9	70.3	36.3	17.68	24.5	52.3
7	Con	3	30.3 ± 1.3	5.6 ± 0.3	1.3 ± 0.1	10.7 ± 0.1	46.8 ± 2.0	80.1 ± 17.0	243.6 ± 75.1	30.9 ± 8.2	17.4 ± 1.0	0.42 ± 0.03	240.3 ± 41.4	3.5 ± 0.2
	%cv		7.6	9.3	15.8	0.5	7.4	36.8	53.4	46.0	10.2	9.97	22.6	6.4
	Cyn	5	32.1 ± 1.5	8.5 ± 0.6*	1.4 ± 0.1	12.2 ± 1.3	74.7 ± 31.6	70.2 ± 8.3	340.9 ± 74.4	44.5 ± 8.7	19.1 ± 3.2	0.41 ± 0.02	214.1 ± 5.0	3.2 ± 0.2
	%cv		10.4	17.0	6.4	24.6	94.8	26.6	48.8	44.1	37.4	11.06	5.2	6.6
14	Con	3	33.1 ± 1.0	4.9 ± 0.2	1.4 ± 0.1	9.7	41.6 ± 2.1	60.4 ± 1.1	233.7 ± 31.7	36.5 ± 8.4	12.7 ± 1.7	0.45 ± 0.05	324.6 ± 36.5	2.9 ± 0.2
	%cv		5.5	8.5	5.1	nd	8.8	3.0	23.5	40.0	23.2	17.32	19.5	9.5
	Cyn	5	31.9 ± 0.6	5.7 ± 0.3	1.3 ± 0.1	9.6 ± 0.2	40.6 ± 2.2	64.1 ± 5.0	294.2 ± 38.8	34.2 ± 4.9	14.8 ± 1.4	0.45 ± 0.03	277.2 ± 16.5	3.5 ± 0.1
	%cv		4.0	12.5	8.0	5.0	11.9	17.6	29.5	31.9	21.1	15.73	13.3	2.9
35	Con	2	34.2 ± 1.3	4.5 ± 0.3	1.2 ± 0.1	9.9 ± 0.2	49.4 ± 9.2	73.1 ± 9.5	271.8 ± 38.8	36.1 ± 3.4	21.1 ± 4.5	0.42 ± 0.01	327.6 ± 79.9	3.1 ± 0.1
	%cv		5.4	9.5	9.7	3.4	26.3	18.3	20.2	13.4	29.9	4.04	34.5	3.9
	Cyn	4	33.1 ± 1.6	5.0 ± 0.2	1.2 ± 0.1	10.0 ± 0.3	46.0 ± 6.0	63.7 ± 2.2	247.5 ± 39.3	32.6 ± 2.5	12.7 ± 1.1	0.41 ± 0.02	312.5 ± 17.6	3.2 ± 0.1
	%cv		9.9	9.2	3.7	5.6	26.3	7.0	31.7	15.1	17.1	7.93	11.3	2.9
49	Con	3	38.2 ± 1.0	3.9 ± 0.1	1.0 ± 0.1	8.9 ± 0.7	42.0 ± 5.2	61.7 ± 2.9	243.0 ± 28.5	35.1 ± 3.9	21.6 ± 2.2	0.40 ± 0.05	235.9 ± 12.6	3.1 ± 0.1
	%cv		4.3	1.7	5.4	14.5	21.6	8.2	20.3	19.4	17.5	23.62	9.2	4.6
	Cyn	5	33.1 ± 0.9**	4.6 ± 0.2	1.3 ± 0.1	10.0 ± 0.2	35.3 ± 2.5	57.2 ± 3.9	183.7 ± 17.7	27.6 ± 1.7	15.4 ± 0.7	0.42 ± 0.02	263.2 ± 21.6	3.1 ± 0.1
	%cv		5.9	12.0	66.0	5.4	16.1	15.3	21.5	13.7	10.7	8.37	18.3	5.5

%cv, % coefficient of variation.

**P < 0.01.

***P < 0.05.

Note that significances are given for both means and variance.

AAT, antitrypsin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; SDH, sorbitol dehydrogenase; BUN, blood urea nitrogen; K/BW, kidney/body weight ratio.

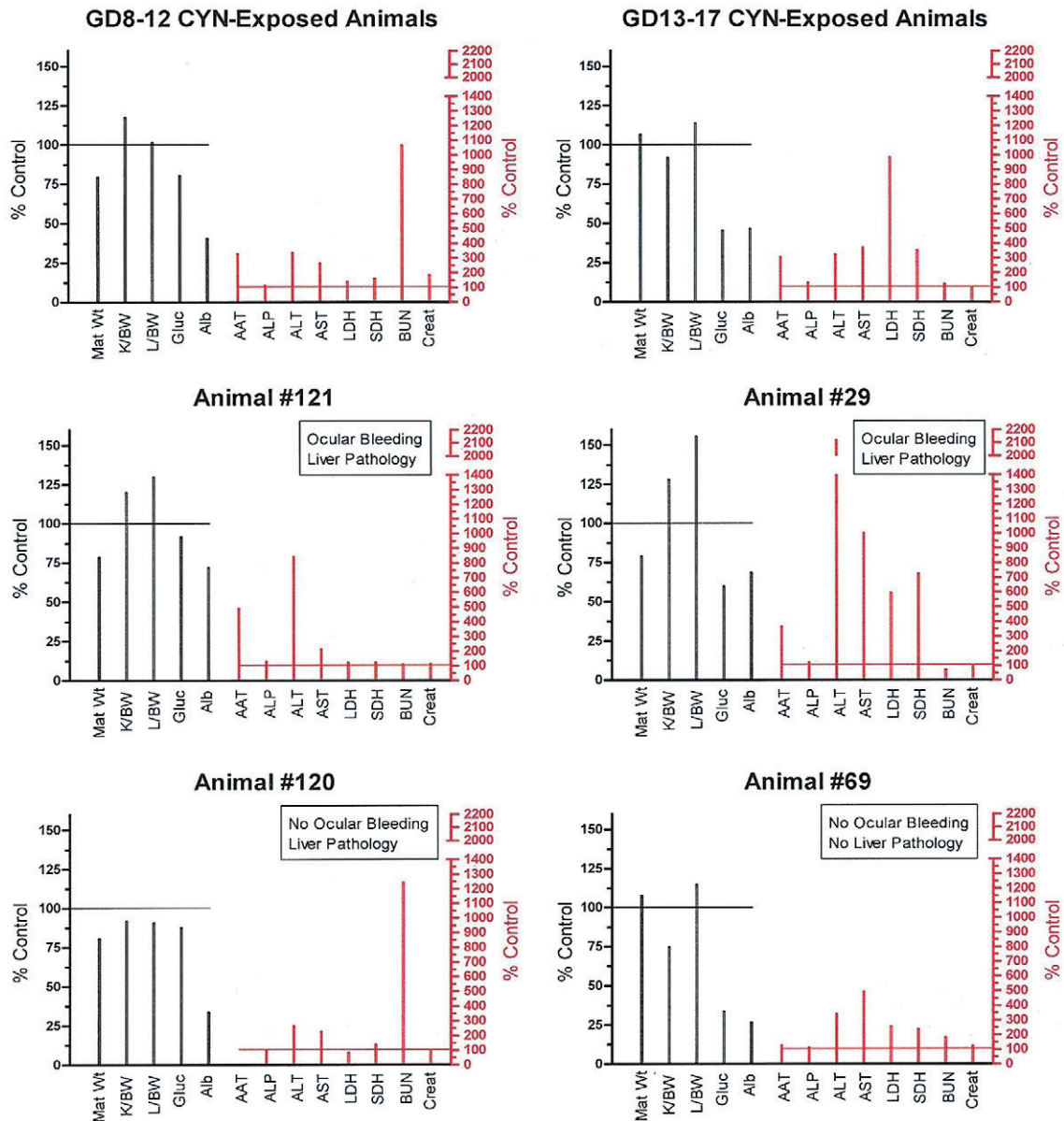


Figure 2. Toxicity profiles of averaged GD8–12 and GD13–17 animals' responses to CYN and individual animals within each of these dosing regimen groups. The left y-axis represents data on body and organ weights and general markers of metabolic homeostasis compared with control values (controls = 100%). The right y-axis shows the degree of change in the hepatic and renal markers of toxicity as a percentage of the control values (controls = 100%).

and GD13–17 groups. LDH and SDH were significantly elevated ($P < 0.01$) in the GD13–17 group while showing a trend in the same direction in the GD8–12 group. At this time, serum albumin levels were significantly reduced ($P \leq 0.01$) in both groups. One day after the last dose, both BUN ($P \leq 0.01$) and creatinine ($P \leq 0.05$) levels were significantly elevated in the GD8–12 group. Serum glucose levels were significantly lower ($P \leq 0.01$) in the GD13–17 group while the serum glucose levels showed a trend in the same direction in the GD8–12 group. Values for clinical chemistries returned to normal 7 days after the last dose in both groups. No significant differences in kidney/body weight ratio were noted in either group. The liver/body weight ratio was not different in the GD8–12 group but were significantly elevated in the GD13–17 animals on day 7 post-dosing ($P < 0.01$) and remained higher than the control values for the remainder of the study.

Measures of variance (standard error) and variance relative to the mean (percentage coefficient of variation or %cv) are listed in Tables 2 and 3. The variability in response across GD8–12 CYN-exposed individuals was significantly greater one day after the last dose for serum levels of ALT, AST, LDH, SDH, BUN, creatinine and the kidney/body weight ratio ($P \leq 0.01$). The response of the GD13–17 animals was generally similar with the following exceptions: the variance of AAT was significantly increased ($P \leq 0.01$), as were the serum levels of albumin ($P \leq 0.01$), but no significant effects were noted for either creatinine or kidney/body weight ratios.

An illustration of individual differences in response to CYN is shown in Fig. 2. This figure summarizes the GD8–12 and GD13–17 group 'toxicity profiles' of weight changes and clinical chemistries as well as individual profiles of pairs of animals selected because

Table 4. Incidence of histologic lesions in female mice treated with CYN either during GD8–12 or GD13–17

Dose in $\mu\text{g}/\text{kg}$	GD8–12		GD13–17	
	0	50	0	50
Hepatocyte necrosis ^a	1/9 (11%)	7/19 (37%)	1/9 (11%)	4/19 (21%)
Nephrosis and/or renal inflammation	0/9	5/19 (26%)	0/9	0/19

^aPredominantly individual cell necrosis in centrilobular and/or subcapsular regions and accompanied by subacute to chronic inflammation.

of the range of responses they displayed. Although the type and the direction of changes induced by CYN were consistent, the degree of response varied considerably among animals. For example, in the GD13–17 animals illustrated, animals 29 and 69 differed from the group average. Animal 29 exhibited ocular bleeding, liver pathology, reduced body weight, increased liver/body weight ratio and extremely high levels of hepatic toxicity markers, effects that were either absent or present to a much lesser degree in animal 69.

Histopathology

The incidence of histologic lesions is given in Table 4. Minimal to moderate centrilobular hepatocyte necrosis and apoptosis, primarily of scattered individual cells, were observed in 1/9 (11%) of both GD8–12 and GD13–17 controls compared with 7/19 (37%) GD8–12 and 4/19 (21%) of GD13–17 CYN-exposed animals one day after the last dose. Scattered hepatocytes were either individually dying (via necrosis and/or apoptosis) or occasionally undergoing coagulative necrosis in groups, sometimes accompanied by varying degrees of inflammatory cell infiltrates (lymphocytes with occasional macrophages and neutrophils; Fig. 3). Lesions generally affected <10% of the parenchyma. Minimal to moderate kidney lesions were noted only in 5/19 (26%) treated GD8–12 dams. Affected animals had multifocal, minimal to moderate, chronic interstitial nephritis or minimal nephrosis seen as acute tubular epithelial necrosis (data not shown).

Gene Array and Real-time PCR Analyses

Microarray analysis

The transcriptional response to CYN was similar among individual animals and did not differ markedly across the two exposure groups. Canonical pathways or functional groups found to be altered by CYN one day following the last dose included ribosome biogenesis (Fig. 4), lipid metabolism (Fig. 5a), xenobiotic metabolism (Fig. 5b), the inflammatory response (Fig. 6a) and the nuclear factor E2-related factor 2 (Nrf2) response to oxidative stress (Fig. 6b).

Real-time qRT-PCR analysis

The results for real-time quantitative RT-PCR are shown in Fig. 7. The genes chosen for PCR analysis represent a subset of genes shown by microarray analysis to be altered by CYN in the livers of the adult mice. A comparison of the microarray and RT-PCR data indicates that the direction of the gene expression changes was

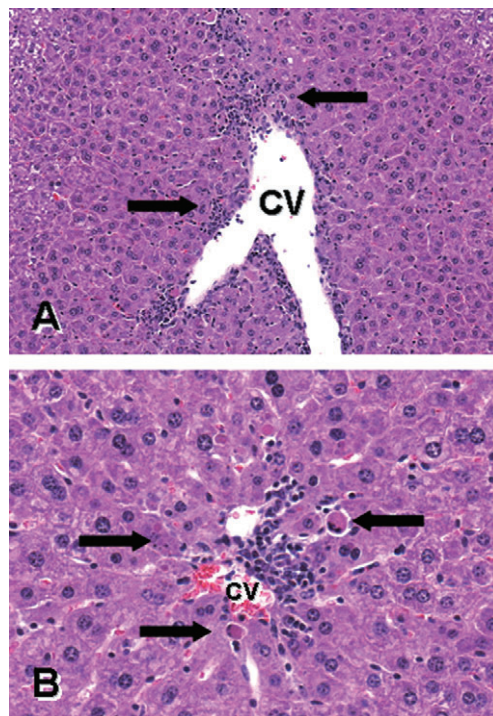


Figure 3. Photomicrographs of H&E sections of liver from a mouse treated with $50 \mu\text{g kg}^{-1}$ of cylindrospermopsin during GD13–17 and killed on GD18, demonstrating centrilobular necrosis and apoptosis. The figures show different areas of the central vein (CV). Magnification is (A) 180 \times and (B) 380 \times . The arrows in (A) point to cell death and inflammation; arrows in B point to apoptotic hepatocytes.

similar for most genes, although scattered differences in the degree of changes were observed in individual animals. Discrepancies were observed with regard to expression of Nrf2, especially among animals dosed on GD8–12. Increased expression of glutamate cysteine ligase (Gclc), a Nrf2 regulated gene (Wild *et al.*, 1999; Han *et al.*, 2008), was confirmed in all but one animal. Hepatic recovery from the changes induced by CYN was apparent as early as one week after dosing with expression levels close to control levels by post-dosing day 28.

DISCUSSION

Cylindrospermopsin is a toxin that is produced by a variety of cyanobacterial species, especially *C. raciborskii*, which has been rapidly spreading globally and is now the source of many blooms worldwide. It has already been shown to have the potential to induce human toxicity via chlorinated drinking water. Surveys

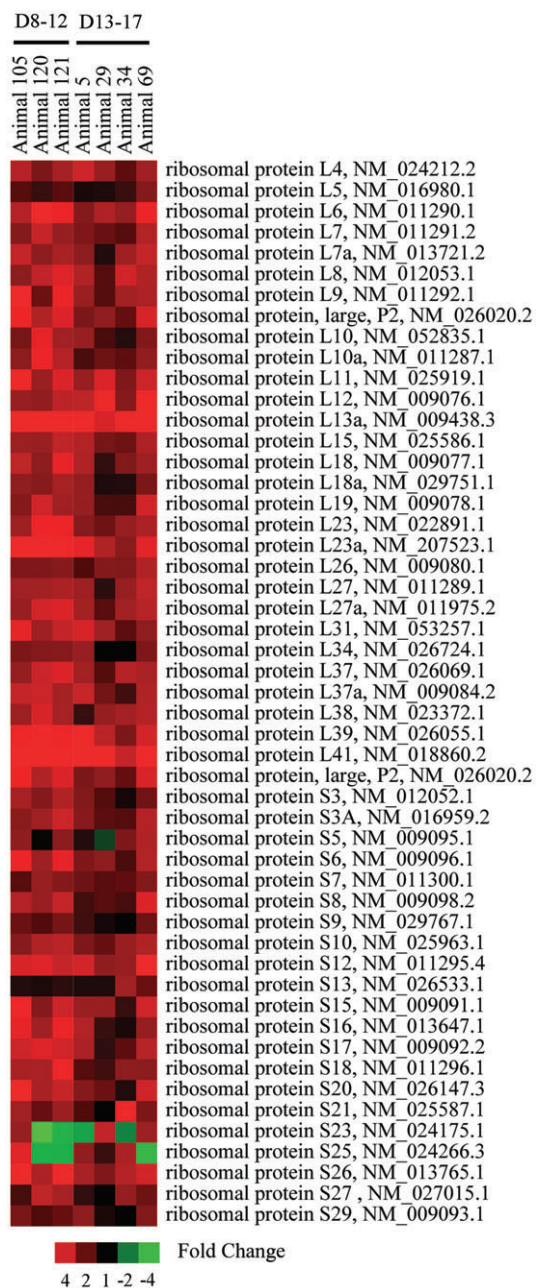


Figure 4. Effect of CYN on genes associated with ribosome. Many of the genes in this functional group were up-regulated by CYN in both dose groups. Red and green correspond to up- and down-regulation, respectively.

have identified blooms of *C. raciborskii* in Indiana (Jones and Sauter, 2005), Illinois (Holland *et al.*, 2006) and Florida (Burns, 2008) in the USA. This organism also tends to decrease the biodiversity of phytoplankton species when it becomes established in a water system (Dobberfuhl, 2005). Increased dependence on surface waters for drinking coupled with climatic conditions that promote cyanobacterial growth make *C. raciborskii* an increasing threat to both the environment and human populations (Briand *et al.*, 2004; Jones and Sauter, 2005; Burns, 2008).

Exposure of pregnant mice to CYN on GD8–12 induced greater toxicity than on GD13–17. Toxicity in GD8–12 animals was first noted as a significant reduction in weight gain after the initial

dose that persisted throughout the dosing period. In contrast, there was no clear pattern of weight change in the GD13–17 dams. Mortality and vaginal bleeding occurred in 55 and 31% of the GD8–12 animals, respectively, vs 0% in the GD13–17 group. The reason for these differences is unknown. If CYN is distributed across the fetal–maternal compartments, differences in maternal toxicity noted between treatment groups could be related to the changing volume of the embryo/fetal compartment during gestation. Although placental transfer studies of CYN have not been done, toxicity of CYN to pups exposed late in gestation (GD13–17) indicates an absence of a placental barrier. The GD13–17 animals have a significantly larger fetal compartment and therefore the maternal exposure and associated toxicity would be much less at this time. In contrast, early in gestation the dose to the maternal organism exclusive of the embryo/fetus would be significantly higher and the resultant maternal toxicity greater.

The bleeding that occurred in treated animals is a relatively uncommon xenobiotic-induced effect. Hemorrhage in multiple sites in both the dam and fetus suggests a CYN-induced coagulopathy and/or vascular injury consistent with an alteration in coagulation factors and the coagulation cascade. Gene expression analysis indicated a down regulation of Coagulation Factor X. Deficiencies in the expression of this gene have been associated with bleeding in both humans (Peyvandi *et al.*, 1998) and mice (Dewerchin *et al.*, 2000), but a relationship to CYN-induced bleeding remains to be determined. Similar responses to CYN intoxication, i.e. bloody diarrhea and bleeding in the mucous membranes, were noted in affected humans (Byth, 1980). Protein synthesis inhibition is generally considered to be a key component of CYN-induced toxicity (Terao *et al.*, 1994; Froschio *et al.*, 2001, 2008; Metcalf *et al.*, 2002; Neumann *et al.*, 2007; Falconer, 2008). The degree to which such inhibition is responsible for CYN toxicity remains unknown. Froschio *et al.* (2003) failed to show a relation between the degree of protein synthesis inhibition in mouse hepatocytes and the leakage of LDH into the medium. They concluded that protein synthesis inhibition may not be fully responsible for CYN hepatocyte toxicity. Considerable cell proliferation in dermal and mouth epithelial layers of *Bufo marinus* tadpoles exhibiting CYN-induced toxicity was not consistent with general protein synthesis inhibition (Kinnear *et al.*, 2007). The protein synthesis inhibitor, cycloheximide, does not induce liver necrosis (Farber, 1971) while others such as carbon tetrachloride (Smuckler *et al.*, 1961) and galactosamine (Decker and Keppler, 1974) do result in hepatic necrosis. The bleeding that is a prominent characteristic of CYN toxicity has been reported after exposure to the protein synthesis inhibitor, T-2, a fungal metabolite. Also, placental bleeding has been described in both rats (Doi *et al.*, 2008) and mice (Schiefer, 1984; Rousseaux *et al.*, 1986) exposed to T-2. This bleeding did not occur after the exposure to the protein synthesis inhibitor, emetine, but adverse clinical signs included vomiting and diarrhea (National Cancer Institute, 1978).

Hepatic toxicity was indicated by serum markers of liver function and histopathology. Elevations in AAT, ALP, ALT, AST, LDH and SDH indicated that the liver was adversely affected in both GD8–12 and GD13–17 dams one day after the last dose. Biochemical indicators of hepatic damage returned to normal after one week in the GD13–17 group and by 4 weeks in the GD8–12 animals. Reduction of serum albumin was also noted in both groups, indicating possible renal and/or hepatic injury. The hepatic histopathology consisted of centrilobular hepatocyte necrosis and was similar in both the GD8–12 and GD13–17 groups. These effects were not observed one week after dosing.

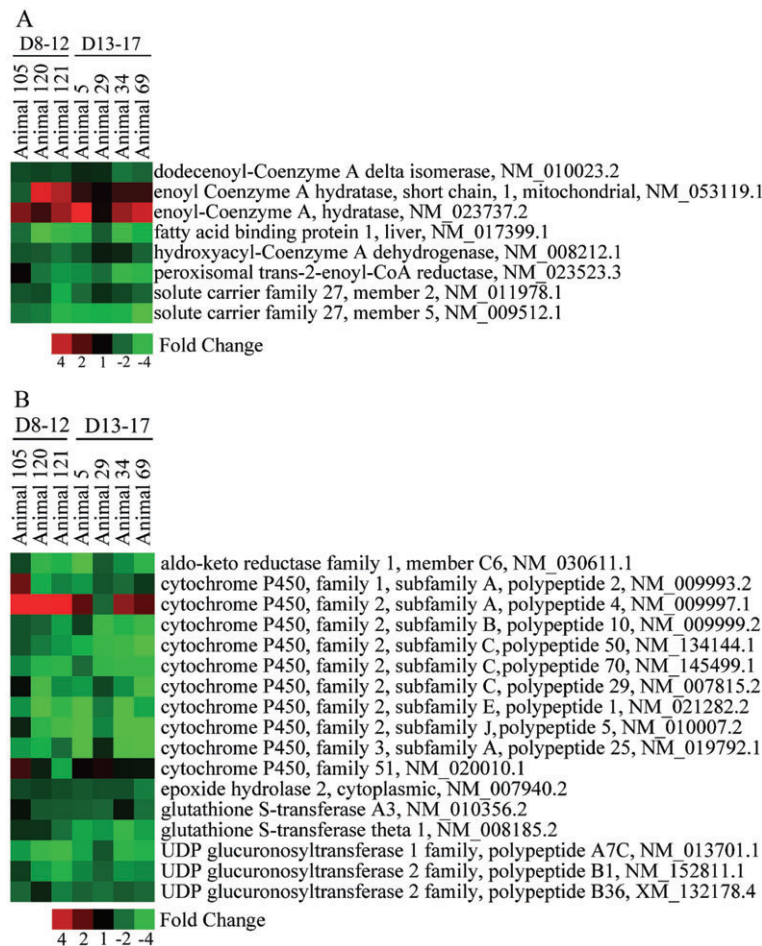


Figure 5. Effect of CYN on genes related to lipid metabolism (A) and xenobiotic metabolism (B). Genes in these functional categories showed uniform changes across animals in each treatment group, an effect consistent with hepatic injury. Red and green correspond to up- and down-regulation, respectively.

The liver has been described as a primary site of CYN-induced toxicity (Hawkins *et al.*, 1985; Harada *et al.*, 1994; Bernard *et al.*, 2003; Griffiths and Saker, 2003; Terao *et al.*, 1994). Our data do not necessarily support this hypothesis. Serum chemistries indicated similar hepatic toxicity in both treatment groups. However, five out of six serum markers indicative of hepatic toxicity showed greater changes from control values in the GD13–17 group, although significantly less lethality was seen in these animals. The incidence of hepatic histopathology was also similar in both groups but the severity of the changes was inconsistent with liver damage as a cause of death.

Markers for renal injury, BUN and creatinine, were increased in the GD8–12 but not in GD13–17 animals. Although these data are consistent with histopathological findings of renal tissue damage (nephrosis) in the GD8–12 group, it should be noted that the observed increase in BUN and disproportional slight increase in creatinine suggest that other possible sources, such as intraluminal hemorrhage, may have contributed to elevated BUN.

Similar changes in gene expression were observed in both dose groups when evaluated by microarray analysis 24 h after the final dose of CYN. These changes included altered expression of genes associated with ribosome biogenesis, lipid metabolism, xenobiotic metabolism, the inflammatory response and the Nrf2 response to oxidative stress. Transcriptional changes involving

genes related to fatty acid metabolism and ribosome biogenesis were also reported by Chung *et al.* (2005) in the rat liver following a single toxic exposure to carbon tetrachloride, indicating that these changes may be common to hepatic injury. In addition, reduced cytochrome P450 enzyme activity and/or decreased expression of cytochrome P450 genes by specific inducers of liver inflammation has been described elsewhere (for reviews, see Gerrish and Malarkey, 2007; Renton, 2004; Morgan, 2001) and has previously been reported for CYN-exposed mice (Terao *et al.*, 1994). Indeed, CYN is a known hepatotoxin (Seawright *et al.*, 1999; Fastner *et al.*, 2003; Terao *et al.*, 1994) and while liver injury was apparent in the current study, the inability to draw a distinction between the two dose groups based on the gene expression data provides additional support to the argument that liver injury may not be the principal mode of action associated with the toxicity observed in CYN-exposed mice. Additional studies using broad gene expression arrays at different time points following CYN exposure are currently underway and may provide a more complete understanding of the course of CYN-induced toxicity.

In recent years, there has been increased interest in identifying factors that are responsible for the range of responses observed within a population following exposure to a toxicant (see Fraser, 2004; Varki *et al.* 2008; Liao *et al.*, 2008). Several investigators have noted the tendency of CYN to induce an unusually wide breadth

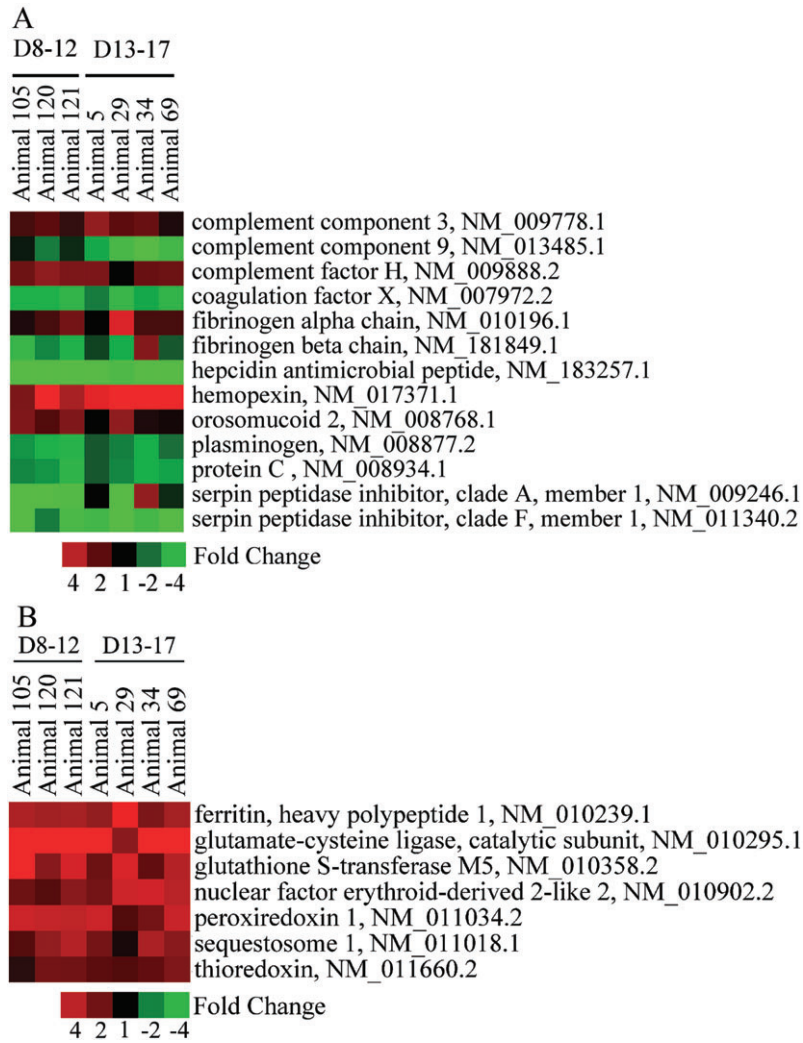


Figure 6. Effect of CYN on genes associated with the inflammatory response (A) and the Nrf2 response to oxidative stress (B). Changes probably related to liver injury were observed across animals in each dose group. Red and green correspond to up- and down-regulation, respectively.

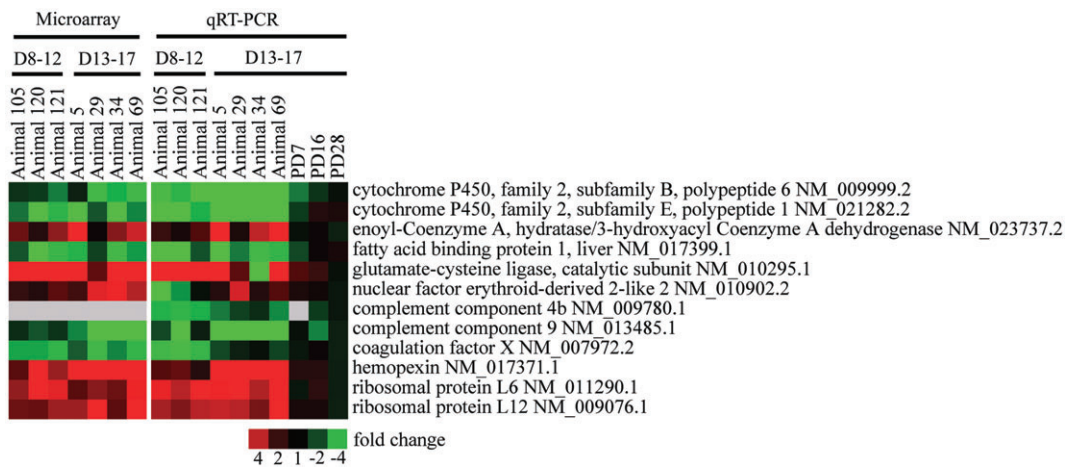


Figure 7. Real-time PCR evaluation of selected genes. Good agreement was found between the results from either microarray or RT-PCR analysis. Differences were observed with respect to expression of Nrf2, especially among animals dosed on GD8–12, although increased expression of glutamate cysteine ligase (Gclc), an Nrf2 regulated gene, was confirmed in all but one animal. Red and green correspond to up- and down-regulation, respectively.

of individual responses to similar exposures in both mammals (Seawright *et al.*, 1999; Norris *et al.*, 2001a) and amphibians (White *et al.*, 2007). The current study appears to support these observations. The degree of individual variability in response to specific toxicants is expressed by the standard error and the percentage coefficient of variation (%cv) of the treatment groups. The significant increase in variance seen in the treated groups was evidence of the tendency of CYN to elicit a wide range of individual responses. The %cv supports these observations in that the increase in variance relative to the mean is also highly noteworthy. The variance was significantly increased in both GD8–12 and GD13–17 groups for most of the hepatic toxicity measures. The variability in measures of renal function appeared to be greater in the GD8–12 group although serum BUN variability was also significantly elevated in the GD13–17 group.

The increasing global range of *C. raciborskii*, the identification of other cyanobacterial species that have been shown to be capable of producing CYN and the known adverse health impacts of exposure to CYN in humans and other mammalian species may be a cause for concern. Burns (2008) noted levels of CYN ranging from 8.1 to 97.1 $\mu\text{g l}^{-1}$ in treated drinking water from Florida, an indication that that CYN may constitute a potential health risk in the USA. Therefore, there is a need for an increased research in numerous areas. The first, and arguably the most important, of these is a concerted effort to produce large quantities of well-characterized CYN or CYN-containing algal lyophilates so that the needed research can be undertaken in multiple laboratories. As long as CYN remains difficult to obtain, research efforts will be impeded. Research priorities should include: epidemiology studies to determine if there is an association between CYN in drinking water and adverse health effects similar to those noted in the 'Barcoo' disease of the Australia outback for which an association with CYN ingestion has been suggested (Cleland, 1911; Hayman, 1992); the identification of additional potential toxicants in *C. raciborskii* and active metabolites of CYN; the long-term effects of CYN exposure including potential carcinogenicity, immunotoxicity, adverse effects of prenatal exposure, CNS toxicity, reproductive impairment and organ toxicity; the course of CYN toxicity in the individual and the identification of the basal causes of the toxicity; the environmental effects of the spread of *C. raciborskii*; and the environmental conditions that determine the amounts of CYN produced in blooms and the retention of the CYN in bodies of water.

The study reported here was designed to make a thorough assessment of the impact of CYN administered to adult mice by i.p. injection during either early (GD8–12) or late (GD13–17) gestation and the subsequent recovery from the induced toxicity. A range of endpoints including clinical observations, gross and microscopic pathology, serum biochemistry and hepatic gene expression were evaluated. The exposed animals exhibited toxic effects similar to those described in a human population (Palm Island, Australia) exposed to CYN via chlorinated drinking water, that is, gastrointestinal bleeding and hepatic and renal toxicity. The similarity in response demonstrates that i.p. administration of CYN in mice is a valid model for CYN-induced toxicity in humans. The data indicate a significant variability in individual responses to CYN intoxication. Evidence of a return to normalcy was noted by PD7 with a small number of exceptions. One week after dosing, gastrointestinal bleeding was observed in a single GD13–17 CYN-exposed animal and elevated liver/body weight ratios were seen in the same dose group. Recovery appeared to be complete at time points examined after 2 weeks post-dosing.

A comparable rate of recovery was also noted for most genes evaluated by RT-PCR although changes in the expression of specific genes (e.g. Gclc and hemopexin) persisted for up to 2 weeks post-dosing. All gene expression levels had returned to normal values by 4 weeks post-dosing.

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