NCEA-C-1765
November 2006

Toxicological Reviews of Cyanobacterial Toxins: Microcystins LR, RR, YR and LA

National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, OH 45268

DISCLAIMER

This report is an external draft for review purposes only and does not constitute Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

TABLE OF CONTENTS

TABLE OF CONTENTS cont.

TABLE OF CONTENTS cont.

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

AFB1	Aflatoxin B1
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMD	Benchmark dose
BMDL	Benchmark dose, lower confidence limit
BMDS	Benchmark dose software
BMR	Benchmark response
BUN	Blood urea nitrogen
CI	Confidence interval
DEN	Diethylnitrosamine
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ESR	Electron spin resonance
FETAX	Frog Embryo Teratogenicity Assay-Xenopus
GD	Gestation day
GFR	Glomerular filtration rate
GGT	γ-Glutamyltransferase
GST-P	Glutathione S-transferase (placental form)
HPLC	High pressure liquid chromatography
i.p.	Intraperitoneal
i.v.	Intravenous
LC_{50}	Concentration lethal to 50% of population
LD_{50}	Dose lethal to 50% of population
LDH	Lactate dehydrogenase
LOAEL	Lowest-observed-adverse-effect level
MAPK	Mitogen-activated protein kinase
MCAR	Microcystin-AR

MCLA	Microcystin-LA
MCLR	Microcystin-LR
MCRR	Microcystin-RR
MCYM	Microcystin-YM
MCYR	Microcystin-YR
MPT	Mitochondrial permeability transition
MW	Molecular weight
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect level
OATP	Organic acid transport protein
PAS	Periodic acid-Schiff
POD	Point of departure
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
RfC	Reference concentration
RfD	Reference dose
ROS	Reactive oxygen species
RVR	Renal vascular rate
SDH	Sorbitol dehydrogenase
SRR	Standardized rate ratio
TDI	Tolerable Daily Intake
TEF	Toxicity equivalency factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling
UF	Uncertainty factor
VAF	Virus antibody free
WHO	World Health Organization

PREFACE

The Safe Drinking Water Act (SDWA), as amended in 1996, requires the Environmental Protection Agency (EPA) to publish a list of contaminants which, at the time of publication, are not subject to any proposed or promulgated national primary drinking water regulations, that are known or anticipated to occur in public water systems, and which may require regulations under SDWA. This list, known as the Contaminant Candidate List (CCL), was first published in 1998 and then again in 2005. The 1998 and 2005 CCLs include "cyanobacteria (blue-green algae), other freshwater algae, and their toxins" as microbial contaminants.

In 2001, a meeting was held among EPA, researchers from the drinking water industry, academia and government agencies with expertise in the area of fresh water algae and their toxins. The goal of this meeting was to convene a panel of scientists to assist in identifying a target list of algal toxins that are likely to pose a health risk in source and finished waters of the drinking water utilities in the U.S. Toxin selection was based on four criteria: health effects, occurrence in the United States, susceptibility to drinking water treatment and toxin stability. Microcystins were identified at this meeting as being toxins of high priority based on those criteria.

The National Center for Environmental Assessment has prepared this Toxicological Review of Cyanobacterial Toxins: Microcystins (LR, RR, YR and LA) as one in a series of dose-response assessments to support the health assessment of unregulated contaminants on the CCL. The purpose of this document is to compile and evaluate the available data regarding microcystin toxicity to aid the Office of Water in regulatory decision making. It is not intended to be a comprehensive treatise on the chemical or toxicological nature of microcystins.

In Section 6, Major Conclusions in the Characterization of Hazard and Dose Response, EPA has characterized its overall confidence in the quantitative and qualitative aspects of the hazard and dose response by addressing knowledge gaps, uncertainties, quality of data and scientific controversies. The discussion is intended to convey the limitations of the assessment and to aid and guide the Office of Water in the ensuing steps of the human health risk assessment of microcystins.

AUTHORS, CONTRIBUTORS AND REVIEWERS

AUTHORS

Belinda Hawkins, Ph.D. (Chemical Manager)
National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, OH

Heather Carlson-Lynch
Syracuse Research Corporation
Syracuse, NY

Marc Odin
Syracuse Research Corporation
Syracuse, NY

Julie Stickney
Syracuse Research Corporation
Syracuse, NY

REVIEWERS

INTERNAL EPA REVIEWERS

Joyce Donohue, Ph.D.
Office of Water
Washington, DC

Elizabeth Hilborn, D.V.M., M.P.H.
National Health and Environmental Effects Research Laboratory
Office of Research and Development
Research Triangle Park, NC

James Sinclair, Ph.D.
Office of Water
Cincinnati, OH

Jeff Swartout
National Center for Environmental Assessment
Office of Research and Development
Cincinnati, OH

1. INTRODUCTION

This toxicological review presents background and justification for hazard and dose-response assessments of microcystins LR, RR, YR and LA. U.S. Environmental Protection Agency (EPA) toxicological reviews may include oral reference dose (RfD) and inhalation reference concentration (RfC) values for chronic and less-than-lifetime exposure durations and a carcinogenicity assessment.

The RfD and RfC provide quantitative information for use in risk assessments for health effects known or assumed to be produced through a nonlinear (possibly threshold) mode of action. These reference values are defined as an estimate of an exposure, designated by duration and route, to the human population (including susceptible subgroups), that is likely to be without an appreciable risk of adverse effects. Reference values may be derived for acute ($\leq$24 hours), short-term (up to 30 days), subchronic (up to 10% of average lifespan) and chronic (up to lifetime) exposures, all considered to be continuous exposures throughout the duration specified. A reference value is derived from a BMDL (a statistical lower confidence limit on the benchmark dose), a no-observed-adverse effect level (NOAEL), lowest-observed-adverse-effect level (LOAEL) or other suitable point of departure with uncertainty/variability factors applied to reflect limitations of the data used. The RfD is expressed in units of mg/kg-day, and the RfC in units of mg/m^3.

The carcinogenicity assessment provides information on the carcinogenic hazard potential of the substance in question and quantitative estimates of risk from oral exposure and inhalation exposure. The information includes a weight-of-evidence judgment of the likelihood that the agent is a human carcinogen and the conditions under which the carcinogenic effects may be expressed. Quantitative risk estimates are presented in three ways. The *slope factor* is the result of application of a low-dose extrapolation procedure and is presented as the risk per mg/kg-day. The *unit risk* is the quantitative estimate in terms of either risk per μg/L drinking water or risk per $\mu g/m^3$ air breathed. Another form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000; 1 in 100,000; or 1 in 1,000,000.

Development of these hazard identification and dose-response assessments for microcystins has followed the general guidelines for risk assessment as set forth by the National Research Council (1983). EPA guidelines that were used in the development of this assessment include the following: *Guidelines for the Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 1986a), *Guidelines for Mutagenicity Risk Assessment* (U.S. EPA, 1986b), *Guidelines for Developmental Toxicity Risk Assessment* (U.S. EPA, 1991), *Guidelines for Reproductive Toxicity Risk Assessment* (U.S. EPA, 1996), *Guidelines for Neurotoxicity Risk Assessment* (U.S. EPA, 1998a), *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (U.S. EPA, 2005b), *Recommendations for and Documentation of Biological Values for Use in Risk Assessment* (U.S. EPA, 1988), (proposed) *Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity* (U.S. EPA, 1994a), *Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry* (U.S. EPA, 1994b), *Use of the Benchmark Dose Approach in Health Risk Assessment* (U.S. EPA, 1995), *Science Policy Council*

Handbook: Peer Review (U.S. EPA, 1998b, 2000a, 2005c), *Science Policy Council Handbook*: *Risk Characterization* (U.S. EPA, 2000b), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000c), *Supplemental Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (U.S. EPA, 2000d) and *A Review of the Reference Dose and Reference Concentration Processes* (U.S. EPA, 2002).

Microcystins are a group of at least 80 naturally occurring hepatotoxins produced by freshwater cyanobacteria (blue-green algae) including *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc* and *Oscillatoria* (Duy et al., 2000). Microcystins were first isolated from cyanobacterial extracts in the 1980s (WHO, 1999).

Much of the toxicological research on microcystins has focused on a single congener, microcystin-LR (MCLR). In addition to MCLR, this report focuses on three other major microcystin congeners: microcystin-YR, microcystin-RR and microcystin-LA (abbreviated as MCYR, MCRR and MCLA throughout this document). Literature searches were conducted for studies relevant to the derivation of toxicity and carcinogenicity values for these four microcystin congeners. The following databases were searched: MEDLINE (PubMed), TOXLINE, BIOSIS, CANCERLIT, TSCATS, CCRIS, DART/ETIC, EMIC, GENETOX, HSDB and RTECS. The relevant literature was reviewed through May 2006.

2. CHEMICAL AND PHYSICAL INFORMATION

Microcystins are monocyclic heptapeptide toxins produced by a number of cyanobacterial species, including members of *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc* and *Oscillatoria* (Duy et al., 2000). At least 80 microcystin congeners have been identified. A general structure for microcystins is shown in Figure 2-1.

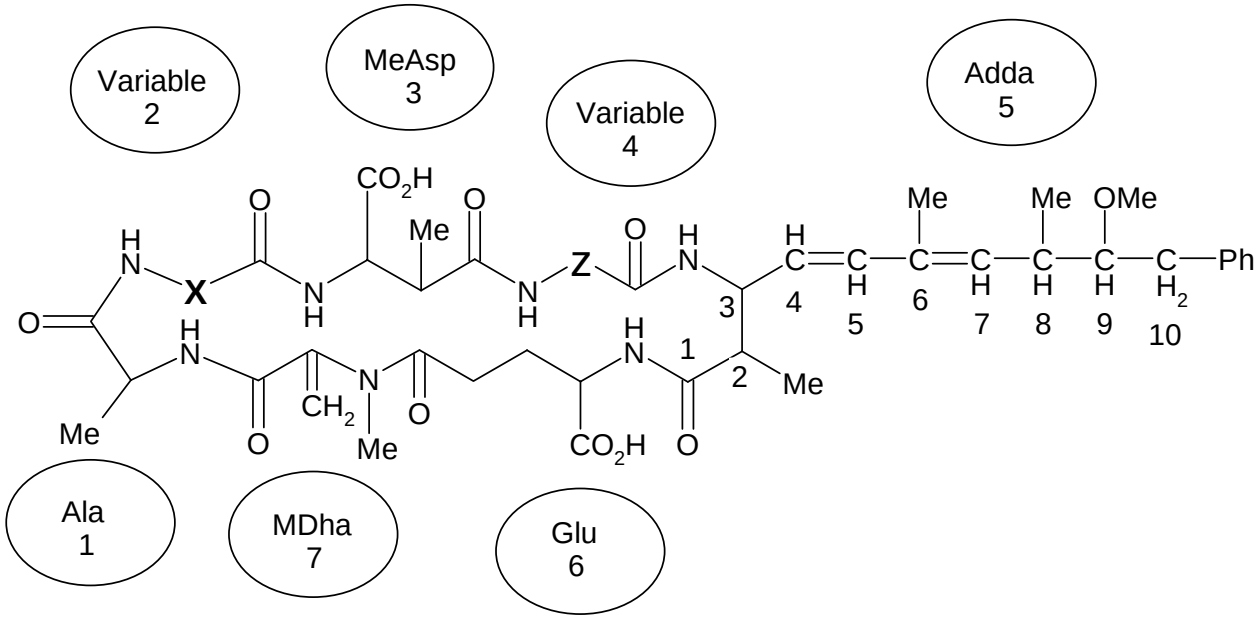


Figure 2-1. General Structure of Microcystins

Microcystins are monocyclic heptapeptides consisting of D-alanine (Ala); two variable amino acids (at positions X and Z in Figure 2-1); D-β-methylaspartic acid (MeAsp); (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda); iso-linked D-glutamic acid (Glu) and *N*-methyl dehydroalanine (MDha). Structural variations occur in all seven of the amino acid peptides, but most commonly in the L-amino acids at positions X and Z in Figure 2-1 (shown as "variable" amino acids in the figure). The most common L-amino acids at position X are leucine (L), arginine (R) and tyrosine (Y), while those at position Z are arginine (R) and alanine (A). The congeners take their names from the L-amino acids in these positions. For example, the microcystin congener with leucine in the X position and arginine in the Z position is microcystin-LR.

Little information on the chemical and physical properties of microcystins was located. Duy et al. (2000) provided the most thorough (albeit general) review of the properties of microcystins. The microcystins identified to date have molecular weights (MWs) ranging from 900 to 1200. Microcystins are nonvolatile and generally quite hydrophilic, although a few have lipophilic properties. Microcystins are soluble in water, ethanol and methanol, and insoluble in

acetone, ether, chloroform and benzene. Laboratory studies show microcystins to be stable under changes in temperature and pH. Microcystins are stable in sunlight; however, in the presence of pigments (type unspecified) and sunlight, microcystins can be decomposed or isomerized (Duy et al., 2000). Limited information suggests that microcystins can bioaccumulate in aquatic organisms; these toxins have been measured in a number of fish and aquatic invertebrates.

3. TOXICOKINETICS

The available information on toxicokinetics of microcystins is primarily focused on exposure via injection routes, either intravenous or intraperitoneal. Few data are available on the oral and inhalation exposure routes. No data on the absorption, metabolism or elimination of microcystins after *in vivo* oral or inhalation exposure in humans or animals were located in the literature. Toxicokinetic data available from parenteral routes of exposure are of uncertain relevance to oral and inhalation exposure routes. The database on microcystins does not contain any toxicokinetic models for microcystins.

A number of studies on the toxicokinetics of microcystins have used ^{3}H-dihydro-MCLR (usually produced by reduction of the MDha moiety in MCLR with tritiated sodium borohydride) as a test material. While there are similarities between the organ distribution, hepatocellular uptake and clinical syndrome after exposure to dihydroMCLR and MCLR (Meriluoto et al., 1990), there are differences in the binding of these compounds to molecular targets. Craig et al. (1996) showed that, while dihydroMCLA was capable of inactivating protein phosphatase 2Ac through a rapid binding mechanism, it did not subsequently form a covalent bond with PP2Ac, while MCLA did. Further information on the structural requirements for microcystin toxicity is provided in Section 4.4.9. Potential differences in the binding of dihydro-microcystin analogs dictates that caution be exercised in generalizing toxicokinetic information derived using these compounds to microcystins as a group. In particular, information on the subcellular localization of dihydromicrocystins may not be applicable to microcystins containing an intact MDha residue.

3.1. ABSORPTION

Pulmonary absorption of MCLR (purified from a bloom sample) was rapid following intratracheal instillation in mice (Ito et al., 2001). Immunostaining of the lung occurred within 5 minutes, followed by a lag period of 60 minutes before staining was observed in the liver. Based on the positive immunostaining of alveoli, it was concluded that absorption occurred at the alveoli. The lethal dose level for intratracheal injection was similar to a lethal dose for intraperitoneal (i.p.) injection (i.e., both about 100 μg/kg). The authors reported that the lungs were not affected by microcystin administration, but it is unclear whether a detailed histopathological evaluation of the lungs was conducted in addition to the immunostaining.

The occurrence of hepatotoxicity and lethality following oral exposure to microcystins (see Section 4.2) is evidence of oral absorption of the toxin. However, quantitative assessments of oral absorption were not located. Ito et al. (1997a) qualitatively studied the oral absorption and distribution of MCLR (purified from a bloom sample) following gavage dosing in mice (500 μg/kg). Immunostaining techniques indicated that MCLR was absorbed primarily in the small intestine, although some absorption did occur in the stomach. Erosion was observed in the surface epithelial cells and in the lamina propria of the small intestine villi. Erosion may facilitate uptake of the toxin into the bloodstream.

The oral bioavailability of MCLR was indirectly studied in *in situ* experiments using isolated intestinal loops of rats (Dahlem et al., 1989). Rats given an infusion of MCLR (>95% pure by high pressure liquid chromatography (HPLC) with UV detection) into the ileum showed clinical signs (i.e., labored breathing and circulatory shock) and evidence of liver toxicity within 6 hours of a single 5 mg/kg dose. Liver toxicity was assessed as an increase in the liver-to-body weight ratio and the presence of gross and histopathological liver lesions characteristic of microcystin toxicity (i.e., enlarged livers, hepatocyte rounding and disassociation, hemorrhage). Infusion of a similar dose into a jejunal loop produced a lower degree of liver toxicity, as compared to the ileal loop infusion. These results suggest that there could be site-specificity in intestinal absorption of MCLR; however, differences in absorptive surface area were not taken into account in the experiment. *In vitro* experiments reported in this publication indicated that cholestyramine, a bile acid sequestrant, bound MCLR, and the presence of cholestyramine in the ileal loop infusion significantly reduced MCLR liver toxicity (Dahlem et al., 1989).

Oral absorption of ^{3}H-dihydromicrocystin was also demonstrated using ileal loop exposure in swine (Stotts et al., 1997a,b). In the exposed swine, the maximum blood concentration of the toxin occurred 90 minutes after dosing.

3.2. DISTRIBUTION

The distribution of microcystins is limited due to the poor ability of these toxins to cross cell membranes. Microcystins are primarily taken up into the liver by the multispecific active transport system for bile acids. Once inside the cell, these toxins bind covalently to cytosolic proteins, resulting in retention in the liver. The cytosolic proteins bound by microcystins have been identified as the protein phosphatase enzymes (PP1 and PP2A). It should be noted that dihydromicrocystin analogs do not appear to form covalent bonds with PP1 and PP2A, although they are able to rapidly bind and inactivate the enzymes (Craig et al., 1996). Binding to and inhibition of these phosphatase enzymes are directly related to the mechanism of toxicity for microcystins and are further discussed in Section 4.4.7.3. This section will describe the overall organ distribution, cellular uptake, subcellular localization and protein binding of microcystins.

3.2.1. Organ Distribution

The organ distribution of a ^{125}I-labelled heptapeptide toxin (MW 1019) isolated from *Microcystis aeruginosa* (while not identified by the study authors as such, probably because the toxin had not yet been named, this is assumed to be a microcystin) was investigated in female rats following intravenous (i.v.) administration (Falconer et al., 1986; Runnegar et al., 1986). The heptapeptide toxin was purified by HPLC prior to reaction with ^{125}I in the presence of NaI and lactoperoxidase. Labelling of the toxin was confirmed by HPLC and mouse bioassay. The highest tissue concentrations of microcystins were detected in the liver and kidney. After 30 minutes, 21.7% of the administered dose was present in the liver, 5.6% was present in the kidneys, 7% remained in the gut contents, and 0.9% was cleared in the urine (Falconer et al., 1986). The balance of the administered dose was not reported; however, the authors reported that no significant accumulation was observed in other organs or tissues.

Brooks and Codd (1987) reported extensive liver uptake following i.p. injection of 125 μg/kg of a ^{14}C-labelled toxin extracted from *M. aeruginosa* strain 7820 (assumed to be a microcystin) in mice. Seventy percent of the radiolabel was found in the liver after 1 minute, increasing to almost 90% after 3 hours. Radiolabel was also found in the lungs, kidneys, heart, large intestine, ileum and spleen.

The distribution of ^{3}H-MCLR (>95% pure) was evaluated following i.p. injection of a sublethal (45 μg/kg) or lethal (101 μg/kg) dose in mice (Robinson et al., 1989). The tissue distribution of radiolabel was similar after injection of either a lethal or a sublethal dose. Liver accumulation reached a maximal value of 60% by 60 minutes. For the 101 μg/kg dose, the liver, intestine and kidney contained 56, 7 and 0.9% of the radiolabel, respectively. Heart, spleen, lung and skeletal muscle each contained less than 1% of the radiolabel.

Immunostaining methods were used to evaluate the organ distribution following intratracheal instillation of MCLR purified from a bloom sample (Ito et al., 2001). Following instillation of a lethal dose (100 μg/kg), the lung, liver, small intestine and kidney were positively stained for MCLR. Intense staining was observed in the lung by 5 minutes post-instillation, followed by the kidney (10 minutes), the small intestine (45 minutes) and the liver (60 minutes). After approximately 90 minutes, bleeding began around the hepatic central vein. The authors described the pathological changes in the liver as essentially the same as those seen following oral or i.p. injection exposure routes. Intratracheal instillation of a sublethal dose (50 μg/kg) resulted in immunostaining of the lung, liver, kidney, cecum and large intestine (Ito et al., 2001). No discernable pathological changes were observed at this dose level. Ito et al. (2002) synthesized glutathione and cysteine conjugates of microsystin-LR and administered them by intratracheal instillation in mice. These conjugates are, according to the authors, known metabolites of microcystins. The metabolites were demonstrated to be less toxic than the parent compound (lethal doses were about 12-fold higher than the MCLR lethal dose) and were distributed primarily to the kidney and intestine, as opposed to the liver.

The distribution of MCLR (purified from a bloom sample) following oral gavage administration to mice (500 μg/kg) was investigated using immunostaining methods (Ito et al., 1997a). MCLR was detected in large amounts in the villi of the small intestine. Erosion of the villi was observed, which may have enhanced absorption of the toxin into the bloodstream. MCLR was also present in the blood plasma, liver, lungs, kidneys and heart.

The distribution of ^{3}H-dihydroMCLR in mice was shown to differ for the oral and i.p. injection routes of exposure (Nishiwaki et al., 1994). Intraperitoneal injection of ^{3}H-dihydroMCLR resulted in rapid and continuous uptake in the liver, with approximately 72% of the administered dose present in the liver after 1 hour. The ^{3}H-dihydroMCLR was synthesized by reduction of N-methyldehydroalanine from microcystin-LR. Small amounts of radiolabel were found in the small intestine (1.4%), kidney and gallbladder (0.5%), lungs (0.4%) and stomach (0.3%) following i.p. injection. Oral administration of ^{3}H-dihydroMCLR resulted in much lower concentrations in the liver, with less than 1% of the administered dose found in the liver at either 6 hours or 6 days post administration. ^{3}H-DihydroMCLR is rapidly distributed to the liver of swine following i.v. injection or ileal loop infusion (Stotts et al., 1997a,b). Smaller amounts were distributed to the kidneys, lungs, heart, ileum and spleen.

MCLR was not found in the milk of dairy cattle that were exposed to *M. aeruginosa* cells via drinking water (Orr et al., 2001) or ingestion of gelatin capsule containing the cells (Feitz et al., 2002).

3.2.2. Cellular Uptake

The cellular uptake of ^{3}H-dihydroMCLR was evaluated using primary rat hepatocytes in suspension and in isolated perfused rat liver (Eriksson et al., 1990a; Hooser et al., 1991a). The uptake (as measured by scintillation counting of washed cells) of a mixture of unlabelled MCLR and ^{3}H-dihydroMCLR was shown to be specific for freshly isolated rat hepatocytes (Eriksson et al., 1990a). Uptake was negligible in human hepatocarcinoma cells (Hep G2), mouse fibroblast (NIH-3T3) and human neuroblastoma cells (SH-SY5Y). The uptake of ^{3}H-dihydroMCLR was also shown to be inhibited by bile acid transport inhibitors such as antamanide, sulfobromophthalein and rifampicin, and by the bile salts cholate and taurocholate (by competing for the bile acid transporter).

The uptake of ^{3}H-dihydroMCLR was demonstrated to be rapid for the first 5-10 minutes, followed by a plateau, in both rat hepatocyte suspensions and the isolated perfused rat liver (Hooser et al., 1991a). Uptake was measured as radioactivity in fractionated cells versus radioactivity in medium. The uptake of ^{3}H-dihydroMCLR was inhibited by incubation of suspended rat hepatocytes at 0°C, suggesting the involvement of an energy-dependent process (Hooser et al., 1991a). Uptake was also inhibited by preincubation of hepatocytes with rifampicin, presumably via competitive inhibition of the bile acid transporter (Hooser et al., 1991a).

Many studies have demonstrated that inhibition of microcystin uptake at the bile acid transporter reduces or eliminates the liver toxicity observed following *in vitro* or *in vivo* exposures (Runnegar et al., 1981, 1995a; Runnegar and Falconer, 1982; Hermansky et al., 1990a,b; Thompson and Pace, 1992). The human organic acid transport protein (OATP) was shown to mediate the transport of ^{3}H-microcystin (type not specified) in *Xenopus laevis* oocytes, and this uptake was inhibited by sulfobromophthalein and taurocholate. This transport protein is found in the human brain and may be related to the acute neurotoxicity seen in hemodialysis patients exposed to microcystins (see Section 4.4.5.1).

Runnegar et al. (1991) studied the influence of dose level and exposure time on the uptake of ^{125}I-microcystin-YM in isolated rat hepatocytes (measured as radioactivity in centrifuged cell pellet). Hepatocyte uptake was initially rapid with a plateau in the uptake rate observed after 10 minutes. The initial uptake rate (in the first minute of exposure) increased with increasing concentration, but cumulative uptake ceased at a dose that resulted in plasma membrane blebbing.

Microcystin-YM uptake by isolated rat hepatocytes was temperature-dependent and was inhibited 70-80% by the addition of sodium deoxycholate or sulfobromophthalein (Runnegar et al., 1995b). This provides evidence to indicate that microcystin uptake occurs by carrier mediated transport, most likely via the bile acid transporter. Pretreatment of mice with bile acid

transporter inhibitors (cyclosporine A, rifamycin, trypan blue and trypan red) abolished microcystin toxicity, suggesting limited or no uptake of microcystins (Runnegar et al., 1995b). Further, *in vitro* preincubation of hepatocytes with bile acids or bile acid transport inhibitors (taurocholate, trypan blue, cholate, sulfobromophthalein, cyclosporine A, trypan red and rifamycin) each decreased the uptake of microcystin-YM, as measured by assays for protein phosphatase inhibition in cell lysates (Runnegar et al., 1995b). Pretreatment with protein phosphatase inhibitors (i.e., microcystin-YM and calyculin A) also resulted in the inhibition of both microcystin-YM uptake and protein phosphatase inhibition, suggesting that the bile acid transporter is itself regulated by serine/threonine phosphorylation.

Many cell types and established cell lines, including both rodent and some human cells, have been evaluated for potential susceptibility to microcystin uptake and toxicity. Primary isolated hepatocytes have been shown to be the most sensitive to cytotoxicity, due to the presence of the organic ion/bile acid transport system (Eriksson et al., 1990b). In addition, primary cultures of liver cells cease to express these bile acid transport proteins after 2-3 days of being maintained in culture. Therefore, established liver cell lines are generally not useful for evaluating microcystin toxicity (Eriksson and Golman, 1993; Heinze et al., 2001). Chong et al. (2000) evaluated microcystin toxicity in eight permanent cell lines (including rodent, primate and human cell lines), only two of which (human oral epidermoid carcinoma KB cells and rat Reuber H35 hepatoma H-4-II-E cells) showed cytotoxicity following MCLR exposure. The toxic response in these cells was most evident if MCLR was added when the cells were seeded. Established monolayers were more resistant to microcystin toxicity. Mechanistic studies that evaluate organ and cell type specificity for microcystins are further discussed in Section 4.4.7.1.

3.2.3. Subcellular Localization and Cytosolic Protein Binding

Tissue distribution was evaluated in mice given i.v. injection of a sublethal dose of ^{3}H-MCLR (Robinson et al., 1991a). The liver contained approximately 67% of the radiolabel by 60 minutes, and the amount of hepatic radioactivity did not change throughout the 6-day study period, despite urinary and fecal elimination of 24% of the administered dose. The subcellular distribution of radioactivity in the liver demonstrated that approximately 70% of the hepatic radiolabel was present in the cytosol. *In vitro* experiments showed that radiolabeled microcystin in the liver was bound to high molecular weight cytosolic proteins (Robinson et al., 1991b). The nature of the binding was demonstrated to be covalent, saturable and specific for a protein with a molecular weight of approximately 40,000. Binding was inhibited by okadaic acid (a potent inhibitor of serine/threonine phosphatases [1 and 2A]), suggesting that the target protein is protein phosphatase 1 or 2A. A discussion of protein phosphatase binding and inhibition by microcystins is provided under mechanistic studies in Section 4.4.7.3, below. Binding proteins for MCLR were found in cytosol derived from several different organs, suggesting that liver specificity is not due to limited distribution of target proteins. Covalent binding to hepatic proteins may be responsible for the long retention of microcystins in the liver. Lin and Chu (1994) evaluated the kinetics of MCLR distribution in serum and liver cytosol derived from mice. Uptake of pure MCLR, as analyzed by direct competitive enzyme-linked immunosorbent assay (ELISA), into the serum was shown to be rapid following an i.p. injection of 35 μg/kg (sublethal dose). The toxin reached a maximum concentration in the serum by 2 hours and in liver cytosol by 12 hours post-injection. MCLR was shown to be bound to liver cytosolic

proteins and the kinetics of binding were correlated with inhibition of protein phosphatase 2A activity. The maximum decrease in enzyme activity was observed 6-12 hours following injection.

Pace et al. (1991) demonstrated significant accumulation of ^{3}H-MCLR in isolated perfused liver despite a low overall extraction ratio (16% in liver, 79% in perfusate). In the liver, radiolabel corresponding to MCLR (15%) and a more polar metabolite (85%) was primarily found in the cytosolic fraction.

The subcellular distribution of ^{3}H-dihydroMCLR was evaluated using primary rat hepatocytes in suspension and the isolated perfused rat liver (Hooser et al., 1991a). ^{3}H-dihydroMCLR was primarily localized in the cytosolic fraction in both the hepatocytes and liver. In the hepatocytes, precipitation with trichloroacetic acid indicated that approximately 50% of the ^{3}H-dihydroMCLR was found as free toxin, while the remaining 50% was bound to cytosolic proteins. Since little of the radiolabel was in the insoluble pellet containing insoluble actin and other elements, the authors suggested that ^{3}H-dihydroMCLR did not bind significantly to actin or other cytoskeletal proteins (Hooser et al., 1991a).

The subcellular protein binding of ^{3}H-dihydroMCLR was evaluated in rat liver homogenates (Toivola et al., 1994). Most of the radiolabeled toxin (80%) was bound to cytosolic proteins. ^{3}H-dihydroMCLR was shown to bind both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A); however, PP2A was detected primarily in the cytosol, while PP1 was found in the mitochondrial and post-mitochondrial particulate fraction (membrane proteins). The binding of microcystins to PP1 and PP2A and the inhibition of protein phosphatase activity is further discussed in Section 4.4.7.3 (Mechanistic Studies).

Limited information in humans exposed to microcystins intravenously via dialysate indicates that a large proportion of microcystins in the blood are bound to proteins. Hilborn et al. (2005) compared two techniques for measuring microcystin in the serum of six patients. Use of ELISA, which detects free microcystins, resulted in serum microcystin concentrations ranging from 8 to 51% of the concentrations obtained using gas chromatography/mass spectrometry (GC/MS) detection of 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB, which is derived from both free and protein-bound microcystins by chemical oxidation). These results indicate that microcystins are bound to proteins in human blood, and that analysis for microcystins using ELISA techniques may underestimate total blood concentrations.

3.3. METABOLISM

Urinary and fecal metabolites of MCLR were analyzed in samples collected 6 and 12 hours following i.v. injection of a sublethal dose of ^{3}H-MCLR in mice (Robinson et al., 1991a). Approximately 60% of the radiolabel in both the urine and the feces was associated with the parent compound. MCLR was metabolized in liver cytosol preparations to a product that binds to a high molecular weight cytosolic protein (Robinson et al., 1991b). The parent compound also binds to this protein, which has been suggested to be the catalytic subunit of protein phosphatase 2A. In isolated perfused rat liver, binding of both the parent toxin (^{3}H-MCLR) and a more polar metabolite to cytosolic proteins was also demonstrated (Pace et al., 1991). Polar

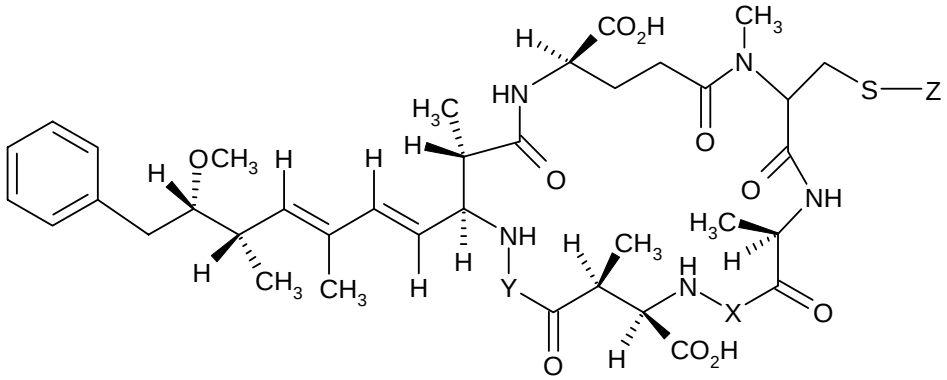
metabolites accounted for 65-85% of the hepatic cytosol radiolabel. Metabolites of MCLR were not further characterized in these studies.

^{3}H-Dihydromicrocystin is not extensively metabolized in swine liver after i.v. injection or ileal loop exposure, and is primarily present in hepatic tissues as the parent compound (Stotts et al., 1997a,b).

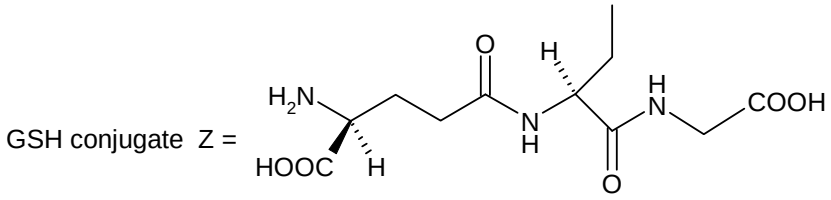
Administration of 125 μg/kg of *Microcystis* toxin 7820 to mice resulted in decreased levels of cytochrome b5 and cytochrome P450 (Brooks and Codd, 1987). Pretreatment of mice with microsomal enzyme (mixed function oxidase) inducers (β-naphthoflavone, 3-methylcholanthrene and phenobarbital) was shown to eliminate this effect on hepatic enzymes and to extend survival and reduce liver toxicity (i.e., changes in liver weight). In an *in vitro* study using mouse liver microsomes, cytochrome P450 associated enzyme activity (i.e., metabolism of aminopyrene and p-nitrophenol) was not altered by an unidentified toxin isolated from *M. aeruginosa* (assumed to be a microcystin; Cote et al., 1986).

The hepatic metabolism of MCRR and MCLR (purified from blooms) was studied following i.p. injection in mice and rats (Kondo et al., 1996). Glutathione and cysteine conjugates were identified at 3 and 24 hours in both mouse and rat livers. Structural modification of the 3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) and methyldehydroalanine (MDha) moieties of the toxins was indicated. Figure 3-1 shows the glutathione and cysteine conjugates of microcystins.

Kondo et al. (1992) demonstrated that glutathione and cysteine conjugates of MCLR and MCYR were less toxic than the parent compounds based on LD_{50} estimates, but were still significantly toxic (LD_{50} values ranged from 217 to 630 μg/kg in mice). Glutathione and cysteine conjugates of MCLR were shown to inhibit protein phosphatases 1 and 2A *in vitro* to the same degree as MCLR; however, these metabolites were primarily distributed to the kidney and intestine following intratracheal instillation in mice (Ito et al., 2002). This result suggests that the lower toxicity of glutathione and cysteine conjugates may be related to distribution to excretory organs and elimination of metabolites *in vivo*. Metcalf et al. (2000) also demonstrated that microcystin conjugates with glutathione, cysteine-glycine and cysteine were less toxic in the mouse bioassay; however, these conjugates were also shown to be weaker inhibitors of protein phosphatases 1 and 2A *in vitro*. Takenaka (2001) illustrated that glutathione conjugates of MCLR are formed by glutathione S-transferase enzymes found in both rat liver cytosol and microsomes.



	X	Y
Microcystin LR	Leu	Arg
Microcystin YR	Tyr	Arg
Microcystin RR	Arg	Arg



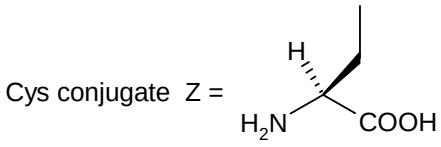


Figure 3-1. Structures of GSH and Cys Conjugates of Microcystins LR, YR and RR (Kondo et al., 1992)

Several studies have demonstrated an increase in hepatic glutathione levels following exposure to microcystins (Ding et al., 2000a; Bouaïcha and Maatouk, 2004; Gehringer et al., 2004). MCLR was shown to induce the *de novo* synthesis of glutathione in mice exposed to a toxic sublethal dose (75% of the LD_{50}) (Gehringer et al., 2004). Increased transcription of glutathione-S-transferase was also demonstrated in this study.

3.4. ELIMINATION

Limited information on the elimination of microcystins from the human body is available from follow-up of dialysis patients exposed to microcystins intravenously (see Section 4.4.5.1 for further detail). In two separate incidents in Brazil (one in Caruaru, one in Rio de Janeiro), microcystins were detected in patients' serum more than 50 days after documented exposure (Hilborn et al., 2005; Soares et al., 2006).

The excretion of microcystins was investigated in female rats (Falconer et al., 1986). The blood half-life was measured following i.v. administration of a ^{125}I-labelled heptapeptide toxin extracted from *M. aeruginosa* (MW 1019, assumed to be a microcystin). A biphasic blood elimination curve was demonstrated, with the first component having a half-life of 2.1 minutes and the second component having a half-life of 42 minutes. After 120 minutes, 9.4% of the administered dose was present in the intestinal contents and 2.9% was present in the urine, suggesting that biliary excretion plays a significant role in elimination of microcystins. Biliary excretion was also demonstrated in isolated perfused rat liver (Pace et al., 1991). In the bile collected 10-20 minutes after toxin exposure, 78% of the radiolabel was associated with the parent toxin, while the remaining radiolabel was associated with more polar metabolites.

MCLR excretion was also evaluated in mice (Robinson et al., 1991a). A biexponential plasma elimination curve was observed following i.v. injection of a sublethal dose of ^{3}H-MCLR. Plasma half-lives of 0.8 and 6.9 minutes were reported for the first and second phase of elimination, respectively. Approximately 24% of the administered dose was eliminated in the urine (9%) and feces (15%) throughout the 6-day study period. Approximately 60% of the excreted microcystin, measured at 6 and 12 hours following injection, was present as the parent compound.

Ito et al. (1997a) demonstrated that MCLR is secreted in the mucous of goblet cells from both the small and large intestine of mice following administration by oral gavage (500 μg/kg). MCLR was not detected in urine in this study.

Stotts et al. (1997a,b) evaluated the toxicokinetics of ^{3}H-dihydroMCLR in swine following i.v. injection and ileal loop exposure. Elimination of ^{3}H-dihydromicrocystin was rapid and followed a biphasic pattern, suggesting that the liver rapidly removes the toxin from the blood. Clearance from the blood is slower at higher dose levels, presumably due to the liver toxicity and circulatory shock observed at high doses. ^{3}H-Dihydromicrocystin was detected in the bile as early as 30 minutes after i.v. injection. Following ileal loop exposure, the concentration of toxin was consistently higher in the portal venous blood as compared to peripheral blood. This suggests that first pass metabolism may play a role in the clearance of dihydroMCLR.

3.5. PHYSIOLOGICALLY-BASED TOXICOKINETIC MODELS

No physiologically based toxicokinetic models have been developed for microcystins.

4. HAZARD IDENTIFICATION

4.1. STUDIES IN HUMANS – EPIDEMIOLOGY, CASE REPORTS, CLINICAL CONTROLS

A number of case reports and epidemiological investigations have examined the relationship between human exposure to cyanobacteria and various health endpoints. In all of these studies, humans were exposed to blooms of cyanobacteria in environmental settings. As a result, the potential for co-exposure to multiple cyanobacterial toxins and/or other microorganisms or compounds to contribute to observed toxicity cannot be ruled out.

4.1.1. Oral Exposure

4.1.1.1. Short-Term Studies and Case Reports

Dillenberg and Dehnel (1960) reported on a series of animal poisonings and human exposures to cyanobacterial blooms in various lakes of Saskatchewan, Canada during the previous year. Several cases of individual or group human exposures during recreational activities were reported. In general, the symptoms were gastrointestinal in nature, including nausea, stomach pain and diarrhea; headache and muscle weakness were also reported. Stool samples from three of the victims showed evidence of cyanobacteria (*Microcystis* and *Anabaena*). In addition, water samples from the lakes in which the victims had been swimming showed cyanobacteria. At the time of this report, cyanobacterial toxins had not been fully characterized. Thus, no data on the nature or quantity of toxins in the affected waters were provided.

Billings (1981) reported a series of outbreaks of human illness potentially associated with exposure to cyanobacteria in two Pennsylvania lakes. Swimmers in both lakes reported symptoms, including headache, abdominal cramping, nausea, vomiting, diarrhea, hay fever-like symptoms, ear aches, eye irritation, sore throat, sneezing, runny nose and swollen lips within a few hours of swimming in the waters. Investigation by the state Departments of Environmental Resources and Health served to rule out common bacterial, protozoal and viral agents in the outbreaks. In the second lake, a bloom of *Anabaena* was identified. Indirect evidence (rapid onset of symptoms, absence of other potential causative agents and consistency with previous reports of health effects after exposure to cyanobacteria) led the investigators to postulate a role for exposure to *Anabaena* in the health outcomes.

Turner et al. (1990) reported a similar type of outbreak among army recruits who had consumed reservoir water during canoe exercises. Detailed case reports were presented for two recruits. Both 16-year-old recruits presented with several days' history of malaise, sore throat, blistering around the mouth, dry cough, pleuritic pain and abdominal pain. One also had experienced vomiting and diarrhea. Physical examination revealed fever, left basal pulmonary consolidation (pneumonia) and abdominal tenderness in both patients. Blood tests revealed low platelet counts in both patients. Both were tested for a variety of pathogens, including *Leptospira*, *Legionella*, *Chlamydia*, *Coxiella*, *Mycoplasma* and influenza and adenovirus, all

with negative result. The authors reported similar symptoms (sore throat, headache, abdominal pain, dry cough, diarrhea, vomiting and blistered mouth) in 16 additional soldiers who had taken part in the canoe exercises. The reservoir contained a bloom of cyanobacteria, primarily *M. aeruginosa*. Further, a sample of the bloom taken the day after the patients were admitted into the hospital showed microcystins to be present, including MCLR. High levels of *Escherichia coli* were also found in reservoir water sampled 2 weeks later. The authors suggested that microcystin exposure may have had a role in the pulmonary consolidation and low platelet count of the two patients, citing evidence from studies in mice (the authors cited Falconer et al., 1981 and Slatkin et al., 1983).

Teixeira et al. (1993) characterized an epidemic of gastroenteritis, primarily among children, in the vicinity of the Itaparica Dam in Bahia, Brazil. The authors collected data on the incidence of treatments for diarrhea between February and May of 1988. Timing of 1118 cases of diarrhea in local health units was recorded, showing a spike in the incidence of gastroenteritis coinciding with the flooding of the Itaparica Dam reservoir. Most (about 70%) of the cases involved children under the age of 5 years. Additional data were collected on the age, residence, symptoms, foods consumed, source of drinking water and travel for 76 patients given outpatient treatment for diarrhea. Fecal, blood and urine samples were collected from these patients and analyzed for chemical and biological contaminants (i.e., bacteriologic, virologic, cholinesterase, heavy metals). In addition, water samples were analyzed for chemical and biological contaminants (i.e., organophosphates, carbamates, heavy metals, fecal coliform). Analysis of biological samples showed no contaminants. Untreated water samples showed high levels of fecal coliform, but samples of treated water did not contain significant levels. Untreated water samples also revealed high counts (1104-9755 units per mL) of *Anabaena* and *Microcystis* cells, 4-32 times the World Health Organization (WHO) maximum acceptable cell count for untreated water (300 units/mL at the time). No data were provided on levels of cyanobacteria in the treated water. It is not clear from the publication whether affected persons were exposed to treated or untreated water. This study does not provide information on health effects of microcystin exposure, but provides some circumstantial evidence for gastrointestinal effects from exposure to cyanobacteria.

A case control study investigated the incidence of gastrointestinal and dermatological symptoms among persons exposed to Murray River water (Australia) (el Saadi and Cameron, 1993; el Saadi et al., 1995). Physicians in 8 of 11 towns along the Murray River participated in the study, recruiting 102 gastrointestinal and 86 dermatological cases between January and March, 1992. Gastrointestinal cases were patients with abdominal pain, vomiting or diarrhea; dermatological cases had rash, itching or blistering of the mouth. Control patients (132) were selected as the next patient entering the office after each case, when possible. For each study participant, age, sex, primary source of drinking and domestic water (rain/spring, untreated river water or chlorinated river water from a town supply) and recreational water contact (none, river or lake contact, or other, such as pool contact) during the previous week were recorded. River water samples were collected and cyanobacteria identified and quantified. *Anabaena*, *Aphanizomenon* and *Planktothrix* were the most common cyanobacteria identified in the samples; small numbers of *M. aeruginosa* were infrequently identified. Both univariate and multivariate analysis of the data showed the odds of having gastrointestinal symptoms to be raised in persons drinking chlorinated river water or using untreated river water for domestic

purposes. Likewise, both types of statistical analyses showed increased odds of having dermatological symptoms for persons less than 20 years of age and for persons using untreated river water for domestic purposes. The proportion of patients with gastrointestinal symptoms and the proportion with dermatological symptoms both correlated with mean weekly log cyanobacterial cell count in the river, although the correlation was statistically significant only for gastrointestinal symptoms. However, when upper and lower reaches of the river were analyzed separately, nonsignificant correlations were observed.

No data on cyanobacterial toxins in the river water were provided. The symptoms reported in this study cannot be readily attributed to any particular toxin (if they are indeed associated with toxin exposure rather than exposure to the living cyanobacterial cells) due to the absence of toxin analyses, as well as the identification of genera with potential to produce multiple toxins. In addition, the potential for other microbial or chemical contaminants in the untreated river water was not evaluated in this study.

Pilotto et al. (1997) conducted a prospective study of gastrointestinal and dermatological symptoms among people exposed to cyanobacteria at water recreation sites in Australia. Study participants were individuals 6 years of age and older who were present at one of several water bodies that were both used for recreational purposes and expected to have algal blooms during the summer. Interviewers visited these sites on several Sundays and invited all individuals to participate. Participants completed a questionnaire to evaluate health status and the nature and duration of water-contact activities. In addition, subjects were asked whether they had symptoms or recreational water contact in the 5 days prior to study initiation, in order to control for the effects of prior health conditions and prior exposure on study findings. Five hundred and fourteen persons had either pre-existing symptoms or water contact in the days prior to initial interview. Participants were contacted by telephone 2 and 7 days later, at which time the occurrence of diarrhea, flu-like symptoms, skin rashes, mouth ulcers, fevers or eye or ear infections in the intervening time was recorded. Water samples for cyanobacterial cell count and toxin analysis were collected at 10 a.m. and 2 p.m. on the day of initial interview. Toxin presence was assessed by mouse bioassay (i.p. injection of 500 mg freeze-dried cells/kg body weight).

Of 1029 persons invited to participate, 921 persons participated in the study (Pilotto et al., 1997). Interviewers were able to contact 845 of these persons by telephone 2 days after initial interview, and 852 persons 7 days later. No differences in the reporting of gastrointestinal and dermatological symptoms were found between those who had water contact and those without water contact (on the day of the initial interview) when contacted 2 days later (Pilotto et al., 1997). However, when subjects with water contact or symptoms prior to initial interview were excluded, a significant trend to increasing symptom occurrence with duration of exposure was observed among persons contacted 7 days after initial interview. Cyanobacterial cell count showed some correlation with symptom occurrence, but presence/absence of hepatotoxins did not. The authors postulated that any association between symptoms and exposure resulted from the allergenic nature of the cells rather than exposure to toxins.

Pilotto et al. (1999) evaluated the relationship between cyanobacterial exposure and perinatal outcomes in an ecological study conducted in Australia. Cyanobacterial monitoring

data (cell counts) were collected from raw drinking water supplies in 156 towns. Perinatal outcome data were obtained from several registries (for calendar years 1992-1994) and the following variables assessed: premature birth (≤36 weeks), low birth weight (<2.5 kg), very low birth weight (<1.5 kg) and congenital defects (at least one). Maternal residence at birth was used to assess exposure based on cyanobacterial cell counts. Exposure was assessed at various gestational periods, either as the proportion of time with cyanobacterial exposure (proportion of weeks with non-zero levels) or average alert level (alert level 1 = <2000 cells/mL; alert level 2 = 2000-15,000 cells/mL; alert level 3 = <15,000 cells/mL). Data on 32,700 births were collected, although the numbers with exposure data in different gestational periods varied. A significant difference in the incidence of low birth weight and very low birth weight babies was observed between mothers with and without cyanobacterial exposure during the first trimester. Very low birth weight incidences increased with increasing exposure to cyanobacteria (as measured by the proportion of first trimester with non-zero cyanobacterial cell counts). At the highest exposure category (exposure to cyanobacteria during 100% of first trimester), the odds ratio (OR) was 1.42 (95% confidence interval [CI] = 1.00-2.02). When exposure was assessed as the average alert level (cell concentration interval), there was a significant increase in congenital defects at the highest average alert level of 2.5-3.0 (OR = 2.03, 95% CI = 1.37-3.01), but the trend was not significant. This study had a number of limitations, including a lack of individual exposure data and lack of data on cyanobacteria or toxins in the finished water (after various treatment processes). Further, because the measure of exposure was cyanobacteria rather than toxin, it is difficult to interpret this study in the context of microcystin effects.

Falconer et al. (1983) compared the hepatic enzyme levels in patients served by a public water supply contaminated with a bloom of *M. aeruginosa* with levels in patients living in areas served by other water supplies. Enzymes assessed in the study were γ-glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). The study population consisted of all patients served by a single hospital laboratory and referred for liver function tests before, during and after a bloom of *M. aeruginosa* in the Malpas Dam reservoir of Australia. Patients were classified either as residents of the city of Armidale, which uses the reservoir for drinking water supply, or residents of neighboring towns with independent water supplies. Liver function test results within each comparison group were further sorted by date into three categories: testing during the 5 weeks before the first signs of the bloom appeared, testing during the 3-week bloom or the 2 weeks following copper sulfate treatment of the bloom (identified as the high-risk time interval due to the cell lysis and subsequent toxin release) or testing during the 5 weeks that followed.

Results of plasma enzyme analyses were compared before, during and after the bloom among residents of Armidale and surrounding areas (Falconer et al., 1983). Analysis of variance was used to assess differences in enzyme levels between comparison groups and between times within comparison groups. Results of the statistical analysis indicated a significant rise in GGT levels in residents of Armidale during the bloom period. ALT levels in Armidale residents increased during the bloom period, but the change was not statistically significant.

The authors noted substantial variability in enzyme levels, attributing this finding to the imprecise method of selecting study participants (Falconer et al., 1983). It should be noted that several of the enzyme measurements for the referent population were associated with one

individual requiring repeat analysis for chronic kidney disease. Alcoholism, which can increase GGT levels, was reported to occur at about the same proportion (7-10%) in the groups assessed before and during the bloom, although it was substantially lower in the post-bloom group of Armidale residents. The authors concluded that the change in GGT among Armidale residents before and during the bloom period might potentially be associated with exposure to *M. aeruginosa.*

4.1.1.2. Long-Term Studies and Epidemiological Studies

Zhou et al. (2002) conducted a retrospective cohort analysis of colorectal cancer and exposure to microcystins in drinking water in a Chinese province in which an association had been reported previously (Jiao et al., 1985; Chen et al., 1994). Cases of primary colorectal adenocarcinoma between 1977 and 1996 from eight randomly selected towns within Haining City of Zhejiang Province were used as the study population. Cases were identified using the local cancer registry and independently verified by two pathologists. Drinking water source used longest during the lifetime was used as a surrogate measure of exposure to microcystins. Information on drinking water source was obtained by interview of patients or family members of deceased cases. In each of the eight towns, 10 water sources (3 rivers, 3 ponds, 2 wells and 2 taps) were randomly selected and sampled for microcystins twice in each of the months of June through September (total of eight samples from each source). Water samples were analyzed for microcystins by ELISA; the authors did not specify the targeted congeners. The authors do not specify the nature of the "tap" water sources, but the text implies that the tap water derives from one or more treatment plants.

The average incidence rate of colorectal cancer across all of the study areas was 8.37/100,000 per year. The incidence rate was compared among the four different water sources, with well water users serving as the referent population. Compared with the incidence among well water users, the colorectal cancer incidence rates among users of the other water sources were significantly increased. Tap water use was associated with a relative risk of 1.88, while river and pond water use were both associated with a relative risk over 7.0. There was no difference in colorectal cancer incidence between river and pond water users. The authors suggested that exposure to trihalomethane compounds might account for the increase in incidence among tap water users. Table 4-1 shows the incidence rate, relative risk and 95% CIs for these exposure comparisons.

Microcystins were detected at concentrations exceeding 50 pg/mL (considered by the authors to be the limit for positive detection) only in river and pond water, and the average concentrations in these sources were substantially higher (30- to 50-fold) than well or tap water. A similar proportion (about 25%) of the residents in each of the eight towns used river and pond water for drinking water, allowing an analysis comparing the average microcystin concentration in river and pond water in each town with the incidence rate by town. This analysis showed a strong correlation between incidence rate and concentration of microcystin (Spearman correlation

1

Table 4-1. Relative Risk of Colorectal Cancer and Microcystin Concentration by Drinking Water Source (Zhou et al., 2002)

Water Source	Colorectal Cancer Incidence Rate per 100,000	Relative Risk of Colorectal Cancer	95% CI	Number of Microcystin Samples >50 pg/mL	Mean Microcystin Concentration (pg/mL)	Maximum Microcystin Concentration (pg/mL)
Well water	3.61	-	-	0/ 12	0.73	9.13
Tap water	6.77	1.88	1.39-2.54	0/ 17	4.85	11.34
River water	28.5	7.94	6.11-10.31	25/ 69	141.08	1083.43
Pond water	27.76	7.7	5.75-10.30	6/ 35	106.19	1937.94

coefficient = 0.88, $p<0.01$). Figure 4-1 shows the relationship between colorectal cancer incidence and average microcystin concentration.

This study provides suggestive evidence for an association between microcystin exposure and colorectal cancer. It is also consistent with earlier reports of an association between drinking river or pond water and incidence of colorectal cancer in the Zhejiang Province of China (Jiao et al., 1985; Chen et al., 1994; studies published in Chinese and not translated for this review). However, because demographic information was not provided in the report, it is not clear whether dietary, genetic and lifestyle factors associated with colorectal cancer have been adequately controlled in the analysis. Further, other potential biological and chemical contaminants in the river and pond water were not considered.

Several epidemiological studies have examined the relationship between drinking water source (well, river, pond or ditch) and liver cancer in Haimen City, Jian-Su Province, China, an area with an elevated hepatocarcinoma incidence (Yu, 1989; Yu et al., 1989). These studies, published in Chinese and not translated for this review, showed an increased risk of primary liver cancer associated with consumption of pond or ditch water (Ueno et al., 1996). According to Health Canada (2002), Yu (1989) showed that consumption of pond or ditch water was associated with an 8-fold increase in liver cancer incidence when compared with well water consumption. Health Canada (2002) reported that a larger study of 65 counties in China, also published in Chinese (Chen et al., 1991), showed the opposite; that consumption of deep well water was associated with an increased risk of liver cancer.

Ueno et al. (1996) conducted a survey of microcystin content in drinking water supplies in Haimen City to test the hypothesis that microcystins in surface drinking water supplies could contribute to the higher incidence of liver cancer. Microcystins were measured by ELISA in shallow and deep wells, as well as in ponds/ditches and river waters. The authors did not indicate which congeners were targeted by the ELISA. Occurrence of microcystins was higher in pond/ditch water (17% reported as positive with concentration >50 pg/mL) and river water (32% positive) samples than in shallow wells (4% positive) or deep wells (no detections >50 pg/mL). Further, microcystin concentrations averaged across the drinking water types were different, averaging 101, 160 and 68 pg/mL in pond/ditch, river and shallow well samples, respectively. These data, while suggestive, do not directly associate exposure to microcystins and liver cancer, since individual exposures were not measured or estimated, and other biological or chemical contaminants in the surface waters have not been ruled out.

In a case-control study of liver cancer in Haimen City, conducted by Yu et al. (2002), a variety of liver cancer risk factors were evaluated, including hepatitis B and C virus infection, aflatoxin B1 or microcystin exposure, smoking, drinking, diet and genetic polymorphisms. From a pool of 248 patients with hepatocellular carcinoma and 248 age-, sex- and residence-matched controls, 134 paired cases and controls assented to blood samples for virus infection and ALDH2 and CYP2E1 gene polymorphism analyses. Data from these analyses were combined with questionnaire information on possible lifestyle and dietary risk factors for liver cancer. Microcystin exposure was assessed categorically based on drinking water supply (tap, deep or

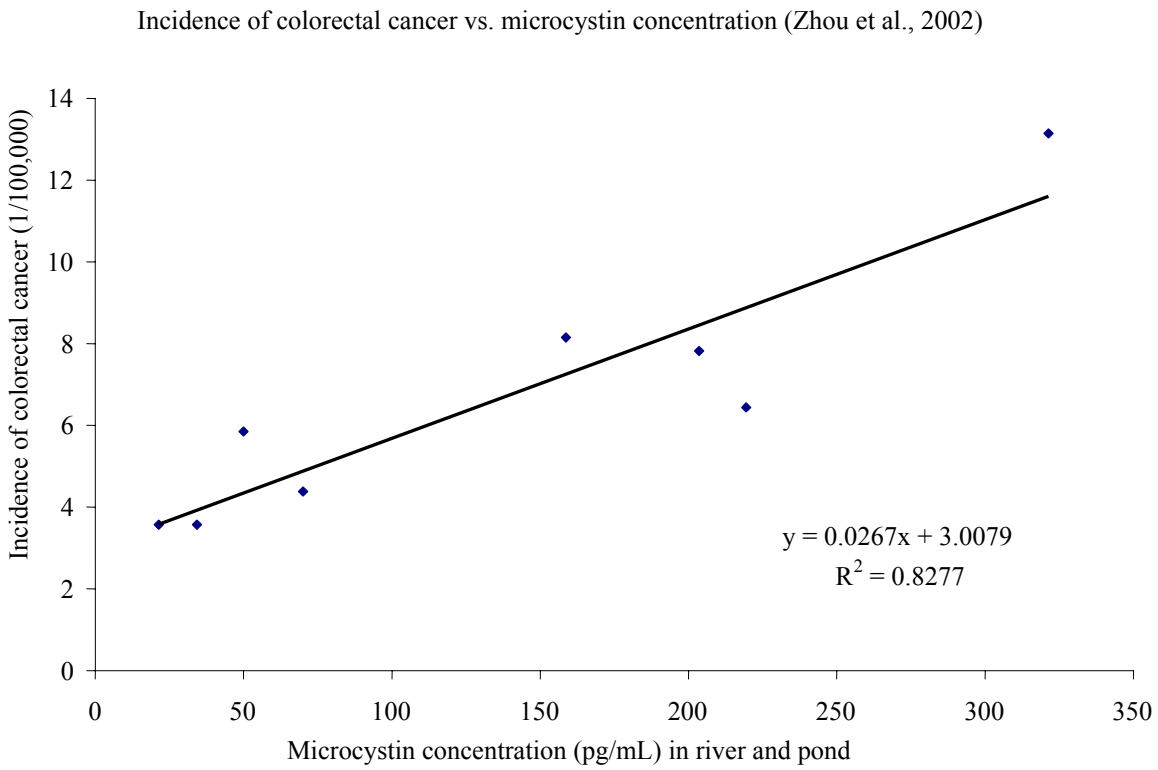


Figure 4-1. Relationship between Colorectal Cancer and Microcystin Concentration in River and Pond Water in Haining City, China (Zhou et al., 2002)

shallow well, river, ditch or pond), as in earlier studies (Yu et al., 1989). Neither univariate nor multivariate analysis of the data indicated an association between consumption of river, pond or ditch water and hepatocellular carcinoma. Hepatitis B virus infection was strongly associated with primary liver cancer, and history of i.v. injection was also identified as a risk factor (Yu et al., 2002).

Fleming et al. (2002) conducted an ecological epidemiological investigation of the relationship between drinking water source and incidence of primary liver cancer in Florida. The study was prompted by data showing cyanobacteria and toxins, especially microcystins, in surface drinking water sources in Florida. The study population consisted of all cases of primary hepatocellular carcinoma reported to the Florida state cancer registry between 1981 and 1988. The study population was divided into comparison groups consisting of those served by surface drinking water supply and those using other sources. Residence at the time of diagnosis was used to place cases into the various comparison groups. Surface water treatment plants and their service areas were geocoded, as were deep groundwater treatment plants. Several comparisons were made. First, incident cases residing in the service area of a surface water treatment plant were compared with those residing in the service area of a deep groundwater treatment plant. Within this comparison, there were several referent groups; one randomly sampled from the available groundwater service areas, one matched on median income and rent, one matched on ethnic makeup and one matched on income, rent and ethnicity. Second, incident cases in the surface water service area were compared with equally-sized buffer areas surrounding the surface water service area, but not served by the treatment plant. Finally, incident cases were compared with the incidence in the general Florida population.

Evaluation of the individual incidence rates in the 18 surface water service areas with the groundwater service areas did not reveal any statistically significant differences among the individual incidence rates. When the service areas were pooled, residence in a surface water service area was associated with a statistically significant reduced risk of hepatocellular carcinoma compared with either groundwater service areas (standardized rate ratios [SRR] ranged from 0.8 to 0.98 for the four groundwater comparison groups) or the general Florida population (SRR of 0.8). It should be noted that the measure of exposure, residence within a surface water service area, was estimated as the average size plus two standard deviations of the service area for this comparison.

When comparisons were made between residence in the actual (i.e., not estimated as above) surface water service areas and residence in the buffer areas surrounding the service areas, a statistically significant increase in the incidence of hepatocellular carcinoma was observed for those residing within the surface water service area (SRR = 1.39, CI = 1.38-1.4). Analyses of 1990 census data suggested that the ethnic and socioeconomic backgrounds of the service areas and buffer areas were similar, although the authors did not report these data. Interestingly, the incidence of hepatocellular carcinoma in the buffer areas was significantly lower than that in the general Florida population (SRR = 0.59).

An ecological study such as this is useful for generating hypotheses, but not for establishing an exposure-response relationship due to the lack of exposure data on individuals. In this case in particular, there is strong potential for misclassification of exposure. Residence in

a surface water service area at the time of diagnosis of hepatocellular carcinoma is a poor measure of potential exposure to cyanobacterial toxins, especially given residential mobility and likely latency time for cancer development. Further, the initial comparisons with groundwater service areas used GIS-generated estimates of surface water service areas rather than actual service areas, leading to greater potential for misclassification.

Fleming et al. (2004) also conducted an ecological study assessing the relationship between incident colorectal cancer and proximity to a surface drinking water treatment plant, with the latter representing a surrogate for exposure to cyanobacteria. Methods for this study were identical to those described above for Fleming et al. (2002) except that colorectal cancer data were abstracted from the Florida Cancer Data System from 1981-1999. As with Fleming et al. (2002), comparisons were made between the colorectal cancer incidence rates in the 18 surface water treatment service areas with several referent groups (a random group of groundwater treatment service areas, a group of groundwater treatment service areas matched on median income and rent, a group of groundwater treatment service areas matched on ethnic makeup, a group of groundwater treatment service areas matched on both median income and ethnicity, groups residing in an equally-sized buffer areas surrounding the surface water service area and, finally, the general Florida population). Mann Whitney rank sum tests of all comparisons did not suggest an association between colorectal cancer and residence at time of diagnosis in a surface water treatment area (details not provided). This ecological study is subject to the same limitations as described above for Fleming et al. (2002).

4.1.2. Inhalation Exposure

No studies of human exposure to microcystins via inhalation were identified in the materials reviewed for this document.

4.2. ACUTE, SHORT-TERM, SUBCHRONIC AND CHRONIC STUDIES AND CANCER BIOASSAYS IN ANIMALS – ORAL AND INHALATION

Early research on cyanobacterial toxins examined the effects of exposure to cyanobacterial organisms rather than on the toxins now known to produce many of the toxicological effects. In the case of microcystins, the isolation and characterization of important toxin(s) did not occur until the 1980s (WHO, 1999). As a result, many studies have used various extracts of cyanobacterial blooms as test substances in toxicological experiments. These studies contribute to the hazard identification for cyanobacterial toxins, but, as discussed below, are not useful for dose-response assessment.

The quantity of an individual cyanobacterial toxin in different bloom samples and extracts varies widely, being influenced by a number of different factors. Some toxins are produced by more than one genus of cyanobacteria. For example, microcystins have been shown to be produced by *Microcystis*, *Anabaena*, *Planktothrix*, *Nostoc* and others (WHO, 1999). Some species (e.g., *Anabaena*) can produce more than one cyanotoxin (WHO, 1999). Even within a species, different strains produce varying levels of toxin; some produce little or no toxin at all. Growth conditions can also contribute to the level of toxin produced by a given species and strain (WHO, 1999). Finally, any given sample may contain multiple genera, species or strains

of cyanobacteria, as well as other contaminating organisms. Some of these variables will also apply to cyanobacterial cells cultured in a laboratory, although clonal cultures may be characterized as to toxin content. In general, there is no clear means of predicting the toxin content in a given bloom sample or cell extract.

In addition to the variations in toxin production within bloom and/or culture samples, there are variations in toxin concentration depending on the method used for producing a material for toxicological administration. Among the studies reviewed, the administered material included lyophilized bloom samples in solution, cell-free extracts, cell-free lysates, partially purified toxins, purified toxins and others. As endotoxins, microcystins exist primarily within the cyanobacterial cell, and are released when cells are lysed. As a result, studies of extracts obtained by removing intact cells may not contain much, if any microcystin. Purified or partially-purified toxins were used in a number of studies; however, the specific microcystin congener or congeners may not have been identified. As a result, data using bloom samples, cell extracts or partially purified toxins cannot be considered reliable information in relating exposure to a given toxin with toxicological effect.

Giving due consideration to the limitations of algal extract studies, a distinction is made between toxicological data obtained using purified microcystins and toxicological data obtained using a bloom sample or extract. These data are discussed separately in this report, with the latter data being considered supplemental due to the confounding factors outlined above. As a result, studies of cyanobacterial extracts are not reviewed in depth.

4.2.1. Oral Exposure

4.2.1.1. Acute Studies

4.2.1.1.1. *Purified Microcystins*

Fawell et al. (1999) conducted acute, subchronic and developmental toxicity studies of MCLR in mice and/or rats. In the acute portion of the study, single oral gavage doses of MCLR (purity not specified) in aqueous solution were given to male and female CR1:CD-1(ICR)BR(VAF plus) mice and CR1:CD(SD)BR(VAF plus) rats (five per sex per species). Doses of 500, 1580 and 5000 μg/kg body weight were administered. Untreated control groups were not included. The animals were observed for up to 14 days prior to sacrifice and necropsy. Microscopic examinations of the lung and liver were conducted. LD_{50} values were calculated.

Oral LD_{50} values were estimated to be about 5000 μg/kg for mice and over 5000 μg/kg for rats. Animals that died showed clinical signs, including hypoactivity and piloerection; however, clinical signs were absent in survivors. Body weights among surviving animals were not affected during the 14-day follow-up. Necropsy of the animals that died showed darkly discolored and distended livers, as well as pallid kidneys, spleen and adrenals. Livers of all animals that died had moderate or marked centrilobular hemorrhage. The incidence and severity of liver lesions increased in a dose-dependent fashion, as shown in Table 4-2.

Table 4-2. Incidence of Liver Lesions in Mice and Rats Treated with a Single Dose of MCLR (Fawell et al., 1999)

Dose (μg/kg)	Number Animals Treated	Mortality	Diffuse Hemorrhage	Moderate Centrilobular Hemorrhage	Marked Centrilobular Hemorrhage	Centrilobular Necrosis	Cytoplasmic Vacuolation
Mice							
500	10	0	2	0	0	0	0
1580	10	1	2	2	1	0	0
5000	10	5	1	7	0	2	0
Rats							
500	10	0	8	0	0	0	0
1580	10	0	7	0	0	0	0
5000	10	1	8	1	1	1	1

Diffuse hemorrhage in the liver was seen in rats and mice of all dose groups, but the incidence was not clearly related to dose. Because an untreated control group was not included in this study, it is not possible to say whether the liver effects were treatment-related; thus, neither a NOAEL nor a LOAEL could be determined. The single mouse death at 1580 μg/kg indicates that this is a frank effect level (FEL) in this species; the FEL in rats was 5000 μg/kg with a single rat death at this dose.

Yoshida et al. (1997) assessed the acute oral toxicity of purified MCLR (>95% pure by HPLC) in female BALB/c mice. Preliminary experiments using doses of 16.8 and 20 mg/kg resulted in death within 160 minutes in two mice; therefore, doses of 8.0, 10.0 and 12.5 were chosen for LD_{50} determination. MCLR in saline solution was administered via gavage to a total of five 6-week-old mice. Two control mice received saline via gavage. Mortality was observed over a 24-hour period, and dead animals, including those in the preliminary study, were immediately necropsied. One surviving mouse was sacrificed and necropsied 24 hours after treatment; the remainder were sacrificed and necropsied after a week. The liver, kidneys and lung were sectioned and examined by light microscopy. Electron microscopy was used to identify apoptotic cells in the livers of treated mice. The remaining tissues were subjected to histopathological analysis.

Mortality within 24 hours was 0/1 at 8 mg/kg, 0/2 at 10 mg/kg and 2/2 at 12.5 mg/kg. The oral LD_{50} was calculated to be 10.9 mg/kg. No effects on the stomach, intestine, skin or

organs other than the liver and kidneys were observed. Liver effects were pronounced in animals that died, including centrilobular hemorrhage and hepatocyte degeneration, as well as free hepatocytes in the veins of mice receiving doses in excess of 12.5 mg/kg (in the preliminary experiments). Effects on the kidneys included proteinaceous eosinophilic materials in the Bowman's spaces of mice receiving high doses (>12.5 mg/kg). In a single mouse treated with 10.0 mg/kg and sacrificed after 24 hours, evidence of hepatocellular necrosis was observed in the centrilobular and midzonal regions, and single cell death (possibly apoptotic) was reported in the centrilobular region, as well as surrounding necrotic areas. In the other mouse treated with 10 mg/kg and the two mice treated with 8.0 mg/kg (all sacrificed 1 week after treatment), the livers contained hypertrophic hepatocytes in the centrilobular region and fibrosis in the centrilobular and midzonal regions. A few apoptotic cells were observed in these animals. No kidney effects were reported in animals that survived treatment for at least 24 hours. No control group was included, so neither a NOAEL nor a LOAEL could be determined from this study. The deaths of both mice given 12.5 mg/kg MCLR indicate that this is an FEL.

Ito et al. (1997a) compared the acute effects of MCLR on the livers and gastrointestinal tracts of young and aged mice. Single doses of 500 μg/kg MCLR (purity not specified) dissolved in ethanol and diluted in saline were administered via oral gavage to aged (29 mice aged 32 weeks) and young (12 mice aged 5 weeks) male ICR mice. Three aged and three young untreated mice served as controls. Twenty-two aged mice were sacrificed at 2 hours, five mice at 5 hours, and two mice at 19 hours after treatment; four young mice were sacrificed at each time point. Liver damage and gastrointestinal erosion were evaluated.

The results showed marked differences between young and aged mice in both liver damage and gastrointestinal effects. In young mice, no liver pathology or gastrointestinal changes were reported. In contrast, 18 of 29 aged mice treated with the same dose developed pathological changes of the liver. Among the aged mice, 8 of 29 had liver injury of the highest severity, characterized as bleeding, disappearance of many hepatocytes in the whole liver and friable tissue (severity rating of +4). Five of 29 mice had liver changes characterized by bleeding and disappearance of hepatocytes in centrilobular region (severity rating of +3). Necrosis in the centrilobular region was observed in 4 of 29 mice (severity rating of +2) and eosinophilic changes in the centrilobular region were indicated for one mouse (severity rating of +1).

Gastrointestinal effects observed in the treated aged mice included necrosis to one-third depth of the mucosa and severe duodenal damage (including decreased villi density, separation of epithelial cells from lamina propria and edema of both the submucosa and villi). Details of the incidence of these effects were not reported; however, the authors indicated that the degree of liver injury was related to the severity of gastrointestinal effects. Regeneration of intestinal tissues was evident in some of the mice sacrificed at later time points (5 and 19 hours after treatment). Among untreated aged mice, serum enzyme levels (AST and ALT) were not different, but gastrointestinal condition was somewhat compromised in the aged mice. Aged mice had thinning of surface epithelial cells with consequent exposure of lamina propria and glands in some areas. The authors postulated that the oral uptake of MCLR was dependent on gastrointestinal tract erosion and the loss of permeability in capillaries of the villi. This study

identified a freestanding LOAEL of 500 μg/kg (in aged mice only) for liver and gastrointestinal effects.

In a poorly described study, Fitzgeorge et al. (1994) administered MCLR via gavage to newly weaned CBA/BALBc mice. The commercially-obtained compound was described only as "suitably purified." The LD_{50} was estimated to be 3000 μg/kg, and increases in liver (43%) and kidney (5.9%) weights were reported. The authors reported that there was no change in lung or spleen weight; other endpoints were either not examined or not reported.

Rogers et al. (2005) evaluated the potential synergism between MCLR and anatoxin-a administered by gavage to CD-1 mice (sex not specified). A total of 60 fasted mice were given gavage doses of 0, 500 or 1000 μg/kg MCLR (purity ≥98%) followed 50 minutes later with gavage doses of 0, 500, 1000 or 2500 μg/kg anatoxin-a (purity ≥95%). Controls were given distilled water by gavage. While not specified, group sizes are presumed to have been five animals per treatment. The animals were observed for clinical signs of toxicity, loss of appetite and mortality; body weight was measured before treatment and 3 hours later. The duration of observation was not reported. No deaths, clinical signs of toxicity or differences in body weight were observed. Effect levels cannot be identified from this study due to inadequate reporting and because few toxicological endpoints were evaluated.

4.2.1.1.2. *Cyanobacterial Extracts*

Rao et al. (2005) compared the acute oral effects of microcystin extract in aged (36 weeks old) and young (6 weeks old) Swiss albino mice. A single LD_{50} dose of extract was administered to male mice; mortality occurred after 4-5 hours. Both groups of mice had increased relative liver weight and DNA fragmentation compared to control, but there was no difference between the age groups. In contrast, glutathione depletion and lipid peroxidation were significantly greater in the aged mice when compared with young mice. Further, while most serum enzymes were increased over controls in both groups, GGT was increased to a greater extent in aged mice than in young mice.

4.2.1.2. Short-Term Studies

4.2.1.2.1. *Purified Microcystins*

Heinze (1999) evaluated the effects of MCLR in drinking water on 11-week-old male hybrid rats (F1 generation of female WELS/Fohm x male BDIX). Groups of 10 rats were given doses of 0, 50 or 150 μg/kg body weight for 28 days in drinking water. Water consumption was measured daily and rats were weighed at weekly intervals. Dose estimates provided by the authors were not adjusted to account for incomplete drinking water consumption (3-7% of supplied water was not consumed over the 28-day period). The test material was obtained commercially, but the authors did not report a measure of purity. After 28 days of exposure, rats were sacrificed by exsanguination under ether anesthesia. Organ weights (liver, kidneys, adrenals, thymus and spleen) were recorded and hematology, serum biochemistry and histopathology of liver and kidneys were evaluated.

Hematological evaluation demonstrated an increase in the number of leukocytes in rats in the highest dose group (38% increase). Serum biochemistry showed significantly increased mean levels of ALP and lactate dehydrogenase (LDH) in both treatment groups (84 and 100% increase in LDH, 34 and 33% increase in ALP in low and high doses, respectively) and no changes in mean levels of ALT or AST. A dose-dependent increase in relative liver weights was observed (17 and 26% at the low and high doses, respectively). Table 4-3 shows the mean enzyme levels and relative liver weights.

Table 4-3. Serum Enzyme Levels and Relative Liver Weights (Mean ± Standard Deviation) in Rats Ingesting MCLR in Drinking Water (Heinze, 1999)

Parameter	Control n=10	50 μg/kg n=10	150 μg/kg n=10
Relative liver weight (g/100 g body weight)	2.75 ± 0.29	3.22 ± 0.34*	3.47 ± 0.49*
Lactate dehydrogenase (microkatals/L)	16.64 ± 4.48	30.64 ± 5.05*	33.58 ± 1.16*
Alkaline phosphatase (microkatals/L)	9.67 ± 2.20	13.00 ± 3.81*	12.86 ± 1.85*

* $p \leq 0.05$ when compared with control

The authors also reported a dose-dependent increase in absolute liver weights, although the data were not provided. No statistically significant changes in other organ weights or body weights were observed. In treated animals, histopathological alterations in the liver were classified as toxic hepatosis. The incidence of liver lesions is summarized in Table 4-4. Lesions were spread diffusely throughout the parenchyma and included increased cell volume, increased mitochondria, cell necrosis, activation of Kupffer cells and increased amounts of periodic acid-Schiff (PAS)-positive substances, indicating cell damage. Liver lesions were observed in both treatment groups, but the severity of the damage was increased in the 150 μg/kg dose group. No effects on the kidneys were observed. A NOAEL could not be determined from this study. The lowest dose tested (50 μg/kg) represents a LOAEL based on liver lesions, increased relative liver weights and changes in liver enzymes (ALP and LDH).

4.2.1.2.2. *Cyanobacterial Extracts*

Davidson (1959) treated groups of three mice with drinking water or feed mixed with different extracts (crude, aqueous and filtered) from a *Nostoc rivulare* water bloom for 21 days. The only effects reported were ruffled hair and nervousness in the mice treated with crude extract. Kalbe (1984) observed no change in body weight among juvenile mice and rats given filtrates from two different water blooms of *M. aeruginosa* for 2-8 weeks.

1

Table 4-4. Incidence of Liver Lesions in Rats Ingesting MCLR in Drinking Water for 28 Days (Heinze, 1999)			
Liver Histopathology	Control n=10	50 μg/kg n=10	150 μg/kg n=10
Degenerative and Necrotic Hepatocytes with Hemorrhage			
Slight	0	4	0
Moderate	0	6	6
Intensive damage	0	0	3
Degenerative and Necrotic Hepatocytes without Hemorrhage			
Slight	0	0	0
Moderate	0	0	1
Intensive damage	0	0	0
PAS-positive Material			
Slight	1	5	0
Moderate	0	5	8
Intensive damage	0	0	2
Activation of Kupffer Cells			
Slight	0	0	0
Moderate	0	10	10
Intensive damage	0	0	0
Lipid Granules and Droplets			
Slight	0	4	0
Moderate	1	2	1
Intensive damage	0	0	0

Orr et al. (2003) exposed yearling beef cattle to live cultures of *M. aeruginosa* in drinking water in an effort to evaluate whether microcystins accumulated in the liver or blood of the animals. Four steers were treated for 28 days, and four untreated steers served as controls. No effects on body weight, weight gain, food or water consumption or plasma enzymes (GGT, glyceraldehydes dehydrogenase, AST or bilirubin) were observed. The authors reported no detectable microcystins (by HPLC and gas chromatography-mass spectrometry) in either plasma samples collected throughout treatment or in samples of the liver collected upon sacrifice at the termination of exposure. Analysis of the liver samples by ELISA showed measurable microcystins; however, the authors indicated that these results likely represented cross-reaction with something besides microcystins, given the failure of the more sensitive HPLC analysis to detect microcystins.

Schaeffer et al. (1999) reported the results of an unpublished 1984 study in which *Aphanizomenon flos-aquae*, a cyanobacterium consumed as a food supplement, was fed to mice in the diet. The authors used recent analysis of the *A. flos-aquae*, which often coexists with *Microcystis* species, to estimate the microcystin content in the material consumed by the mice. Analysis of the *A. flos-aquae* samples used in the feeding study showed an average concentration of 20±5 μg MCLR per gram of *A. flos-aquae*. The authors estimated the daily exposure of MCLR in the exposed mice to range from 43.3 μg/kg body weight per day to 333.3 μg/kg-day. No clinical signs of toxicity were reported, and no effects on mortality, body weight, organ weights or histology were observed in the treated mice. In addition, no effects on reproductive parameters were reported in five treated mice (highest dose group) allowed to breed.

4.2.1.3. Subchronic Studies

4.2.1.3.1. *Purified Microcystins*

Fawell et al. (1999) conducted acute, subchronic and developmental toxicity studies of MCLR given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice (age not specified). MCLR was obtained commercially and administered in distilled water. The concentration in the dosing solution was verified by HPLC with UV detection. Daily oral gavage doses of 0, 40, 200 or 1000 μg/kg body weight were given to groups of 15 male and 15 female mice for 13 weeks. Daily clinical observations were made, body weight and food consumption were recorded weekly, and eye examinations were conducted prior to and at the conclusion of treatment. Hematology and serum biochemistry were evaluated for seven mice of each treatment group during the final week of treatment. Upon sacrifice after 13 weeks, gross examination of organs and microscopic evaluation of tissues were performed. All tissues were examined in the control and high dose animals, while only lungs, liver and kidney were examined in the other treated animals.

Mean body weight gain was decreased approximately 15% in all treated male groups. Mean terminal body weights differed from controls by about 7% in these groups. No dose-related trends were evident for body weight gain or body weight in males. The only body weight change observed in females was an increase in body weight gain in the 200 μg/kg-day group. Hematological evaluation showed slight (10-12%) decreases in mean hemoglobin concentration, red blood cell count and packed cell volume among females receiving 1000 μg/kg body weight.

ALP, ALT and AST levels were significantly elevated (2- to 6-fold higher) in the high-dose males, and ALP and ALT were likewise elevated (2- and 6-fold higher, respectively) in high dose females. ALT and AST were also elevated (2-fold) in the mid-dose males. GGT was slightly decreased in some treatment groups. Serum albumin and protein were reduced (13%) in males of the mid- and high-dose groups. Table 4-5 shows the blood chemistry results.

Table 4-5. Blood Chemistry Results (Mean ± Standard Deviation) for Mice Treated with MCLR for 13 Weeks (Fawell et al., 1999)

MCLR Dose (μg/kg-day)	Alkaline Phosphatase (ALP) (U/l)	Alanine Aminotransferase (ALT) (U/l)	Aspartate Aminotransferase (AST) (U/l)	Gamma Glutamyl Transaminase (GGT) (U/l)	Total Protein (g%)	Albumin (g%)
Male						
Control	91 ± 22.2	27 ± 8.0	68 ± 27.7	6 ± 1.0	5.5 ± 0.32	3.2 ± 0.19
40	95 ± 29.2	37 ± 17.2	64 ± 12.2	4 ± 0.7	5.1 ± 0.26	3.0 ± 0.13
200	94 ± 32.3	59[a] ± 28.0	121[b] ± 43.7	3[c] ± 0.4	4.8[b] ± 0.29	2.8[c] ± 0.13
1000	232[b] ± 103.2	159[c] ± 75	121[b] ± 26.3	4 ± 0.4	4.8[c] ± 0.21	2.8[c] ± 0.11
Female						
Control	167 ± 24.6	32 ± 11.3	101 ± 38.3	4 ± 1.0	5.1 ± 0.30	3.1 ± 0.14
40	187 ± 76.2	25 ± 7.8	74 ± 13.2	3 ± 0.5	5.2 ± 0.28	3.2 ± 0.16
200	156 ± 33.4	27 ± 9.4	74 ± 22.1	3 ± 0.0	5.3 ± 0.31	3.4[a] ± 0.14
1000	339[b] ± 123.7	220[b] ± 149.1	144 ± 71.7	3 ± 0.4	5.1 ± 0.22	3.1 ± 0.18

[a] Significantly different from controls at $p<0.05$
[b] Significantly different from controls at $p<0.01$
[c] Significantly different from controls at $p<0.001$

Histopathological changes in the liver were reported in the males and females of the mid- and high-dose groups, with a dose-related increase in incidence and intensity. The liver lesions were multifocal and observed throughout the liver lobule. Table 4-6 summarizes the incidence of liver histopathological changes. Sex-related differences in liver pathology were not apparent. No lesions were found in other tissues.

The authors characterized the 40 μg/kg body weight dose as a clear NOAEL and indicated that histopathological changes observed in the 200 μg/kg dose group were not severe. The mid dose (200 μg/kg-day) represents a LOAEL based on the liver histopathological changes and statistically significant blood chemistry changes. The WHO (1999) used the NOAEL value of 40 μg/kg-day from this study as the basis for its provisional Tolerable Daily Intake for MCLR.

1

Table 4-6. Incidence of Liver Histopathology in Mice Treated with MCLR for 13 Weeks (Fawell et al., 1999)

Liver Histopathology	Control	40 μg/kg-day	200 μg/kg-day	1000 μg/kg-day
Male	n=15	n=15	n=15	n=15
Acute inflammation	0	1	0	0
Chronic inflammation	1	2	4	15
Congestion	3	0	0	1
Hepatocyte vacuolation	5	5	6	3
Hemosiderin deposits	0	0	0	15
Hepatocyte degeneration	0	0	1	14
Female	n=15	n=15	n=15	n=15
Autolysis	0	0	0	1
Chronic inflammation	5	8	8	14
Congestion	0	0	0	1
Hepatocyte vacuolation	5	5	11	8
Hemosiderin deposits	0	0	1	14
Hepatocyte degeneration	0	0	1	9

4.2.1.3.2. *Cyanobacterial Extracts*

Falconer et al. (1994) administered dried bloom materials in the drinking water of pigs for 44 days. Plasma samples collected over 56 days showed dose- and time-dependent increases in GGT, ALP and total bilirubin, as well as a decrease in plasma albumin. Dose-related changes in the incidence and severity of histopathological changes of the liver were also observed, including cytoplasmic degeneration, hepatic cord disruption, single cell necrosis, periacinar degeneration, congestion and Kupffer cell proliferation.

4.2.1.4. Chronic Studies

4.2.1.4.1. *Purified Microcystins*

Ueno et al. (1999) evaluated the toxicity of MCLR in mice chronically exposed via drinking water. Two hundred 6-week-old female BALB/c mice were randomly assigned to receive either no treatment or drinking water (*ad libitum*) containing 20 μg/L MCLR for 7 days/week. The MCLR had been isolated from lyophilized algal bloom materials from Lake Suwa in Nagano, Japan and had been characterized as 95% pure by HPLC. Twenty animals from each group were sacrificed at 3, 6 and 12 months, while the remaining 40 animals were retained for chronic toxicity evaluation and sacrificed at 18 months.

Weekly estimates of food and water consumption and daily observations for clinical signs of toxicity, morbidity and mortality were recorded. Body weights were recorded weekly for the first 2 months, biweekly up until the first year and monthly until sacrifice. At 3, 6, 12 and 18 months, blood was obtained from 20 animals from each group. Samples from 10 animals per group were used for hematological evaluation, and samples from 10 additional animals were used for serum biochemistry evaluation. At each scheduled sacrifice time, complete necropsy of 10 animals per group was conducted. Animals from the chronic toxicity group were necropsied when moribund or dead (prior to scheduled sacrifice) or upon sacrifice at 18 months. Relative and absolute organ weights (liver, kidneys, spleen, thymus, adrenal, ovaries, brain, heart and uterus) were recorded for 9-10 animals per group at each scheduled sacrifice, and histopathological evaluation of these and numerous other organs was conducted. Finally, three to five animals per group were subjected to immunohistochemistry of the liver upon sacrifice to determine the distribution of MCLR in the liver.

Based on weekly estimates of water consumption, the authors calculated the average total intake of MCLR over 18 months to be 35.5 μg/mouse. No clinical signs of toxicity were observed in either of the groups, and survival in the control and chronic treatment groups was similar. No statistically significant differences in body weight, food consumption, water consumption or hematology were observed; however, hematology data from the 3-month sacrifice were lost due to sampling errors. Treated mice were reported to have a statistically significant decrease in ALP at month 12 (13%) and a significant increase in cholesterol at month 18 (22%). Neither effect was considered by the authors to be toxicologically significant in the absence of other treatment-related effects; however, the increase in cholesterol could be related to the interaction of MCLR with bile acid transport in the liver.

A decrease in heart weight among treated mice sacrificed at month 12 was not considered treatment-related in the absence of histopathological changes. Sporadic changes in absolute and relative thymus weight in treated mice were observed, but histological and morphometric evaluation of the thymus revealed no abnormalities attributable to exposure. In contrast to other studies, the authors observed no difference in the incidence of liver histopathology between treated and control mice. Immunohistochemistry of the liver revealed no accumulation of MCLR. This study identifies a free-standing NOAEL of 2.7 μg/kg-day in female mice (calculated assuming a 24.5 g body weight and an exposure duration of 548 days).

Ito et al. (1997b) evaluated the carcinogenicity and liver toxicity of chronic gavage doses of MCLR. A water bloom from Lake Suwa, Japan served as the source of the MCLR, which was isolated and dissolved in ethanol and saline for dosing. The purity of the isolated MCLR was not specified. Twenty-two ICR mice (13 weeks old) were given either 80 or 100 gavage doses of 80 μg/kg MCLR over the course of 28 weeks. Ten mice were sacrificed after 80 treatments, five were sacrificed after 100 treatments and seven were withdrawn from treatment after 100 doses and sacrificed 2 months later. Three mice served as untreated control. Although the authors did not specify the nature of the postmortem examinations, it appears that the liver was the only organ examined. No change in mean liver weight was observed in the MCLR-treated animals compared with controls. The authors reported "light" injuries to hepatocytes in the vicinity of the central vein in 8 of 15 mice sacrificed immediately after treatment, and in 5 of 7 mice that were withdrawn from treatment for 2 months after exposure. No fibrous changes or neoplastic nodules were observed. Analysis for MCLR and its metabolites by immunohistochemistry failed to detect either the parent compound or any metabolites in the livers of mice sacrificed immediately after treatment.

Thiel (1994) briefly reported the results of a chronic toxicity study of MCLA in vervet monkeys. The report is a brief summary published in the proceedings of an international workshop; a published version of this study was not located. According to the summary, three monkeys were given increasing intragastric doses of MCLA for 47 weeks, while three other monkeys served as controls. Doses increased from 20 μg/kg at the commencement of the study to 80 μg/kg at study termination. The rate of dosage increase was not reported. Monthly measures of body weight and clinical signs (respiration, pulse, temperature) showed no effect of treatment. Blood was withdrawn monthly; hematological parameters examined were hematocrit, bilirubin, hemoglobin, erythrocyte and leukocyte count and platelet count. No statistically significant changes in hematological parameters were observed. No changes were observed in serum biochemistry analyses (albumin, globulins and electrolytes, as well as AST, LDH, ALP, ALT and GGT). Histopathological examination of the liver and other organs (not specified) did not show any differences in treated monkeys when compared with controls.

4.2.1.4.2. *Cyanobacterial Extracts*

Falconer et al. (1988) conducted a chronic exposure experiment using an extract of a *M. aeruginosa* water bloom in Swiss Albino mice. A concentration-dependent increase in mortality, reduced body weight and a concentration-dependent increase in ALT levels were observed among groups of mice receiving serial dilutions of the extract as their drinking water for a year. There was some evidence that bronchopneumonia incidence was related to concentration of

extract. No significant differences in liver histopathology were observed, although the observed liver changes were slightly more prevalent in treated animals. The data showed some indication of sex differences in susceptibility; male mice showed effects (including mortality and enzyme level increases) at lower concentrations than females.

4.2.1.5. Initiation/Promotion Studies – Cyanobacterial Extracts

Falconer (1991) and Falconer and Buckley (1989) reported evidence of skin tumor promotion by extracts of *Microcystis*. *Microcystis* extract was administered via drinking water to mice pretreated topically with an initiating dose of dimethylbenzanthracene (DMBA). The total skin tumor weight in mice drinking *Microcystis* extract was significantly higher than that of mice receiving only water after initiation. The number of tumors per mouse was only slightly increased in mice receiving extract; the weight difference was largely due to the weight of individual tumors (Falconer and Buckley, 1989). The total weight of tumors in this group also exceeded that of mice pretreated with DMBA and subsequently treated with topical croton oil, with or without concurrent consumption of *Microcystis* extract. Details of the tumor incidence in the mice were not provided by the authors. When *Microcystis* extract was provided in the drinking water of mice pretreated with two oral doses of N-methyl-N-nitroso-urea, no evidence of promotion of lymphoid or duodenal adenomas and adenocarcinomas was observed. No primary liver tumors were observed (Falconer and Humpage, 1996).

Humpage et al. (2000) administered *M. aeruginosa* extract in drinking water to mice pretreated with azoxymethane. Mice were sacrificed at intervals up to 31 weeks after commencement of extract exposure. Enzyme analysis showed a concentration-dependent increase in ALP and decrease in albumin in mice treated with extract. The authors observed a concentration-dependent increase in the mean area of aberrant crypt foci of the colon, although the number of foci per colon and the number of crypts per focus were not different among the groups. The authors proposed that increased cell proliferation caused the increase in size of foci. Histological examination of the livers of mice treated with extract showed more leukocyte infiltration in animals treated with the highest concentration of extract compared to those receiving a low concentration.

4.2.2. Inhalation Exposure

All available studies of inhalation exposure used MCLR, and there were no inhalation studies using cyanobacterial extracts.

4.2.2.1. Acute Studies

Fitzgeorge et al. (1994) conducted experiments in CBA/BALBc mice with MCLR administered via intranasal instillation and inhalation. This study is poorly described, giving few details of study design and findings. A single experiment with mice (number unspecified) inhaling a fine aerosol (particle size 3-5 μm) with 50 μg/L MCLR for an unspecified duration of time did not result in any deaths, clinical signs of toxicity or histopathological changes. The nature of the examinations was not reported. The authors estimated the delivered dose of MCLR to be very small (about 0.0005 μg/kg).

A brief abstract describes a study of acute MCLR exposure via inhalation (Creasia, 1990). Details of study design and results were not reported. The LC_{50} for mice exposed to a MCLR aerosol (nose only) for 10 minutes was reported to be 18 μg/L (mg/m^3) air with a 95% confidence interval of 15.0-22.0 μg/L (mg/m^3). Based on studies of lung deposition after exposure of mice to the LC_{50} concentration, an LD_{50} of 43 μg/kg body weight was estimated. The authors reported that histological lesions in mice killed by aerosol exposure were similar to those in mice dosed intravenously with MCLR.

4.2.2.2. Short-Term Studies

Benson et al. (2005) exposed groups of six male BALB/c mice to monodisperse submicron aerosols of MCLR via nose-only inhalation for 30, 60 or 120 minutes each day for 7 consecutive days. The concentration of MCLR was 260-265 μg/m^3 and doses deposited in the respiratory tract were estimated to be 3, 6 and 12.5 μg/kg body weight. Control mice were exposed to the aerosolized vehicle (20% ethanol in water). Clinical signs were recorded daily. Sacrifice by injection of Euthasol occurred the day after the last exposure. Blood was collected by cardiac puncture and serum was subjected to clinical chemistry analysis (blood urea nitrogen [BUN], creatinine, total bilirubin, ALP, AST, ALT, total protein, albumin and globulin). Organ weights (adrenals, lung, liver, kidney, spleen and thymus) were recorded and histopathological examinations of the liver, respiratory tract tissues, adrenals, kidney, spleen, thymus, gastrointestinal tract and testes were conducted.

No clinical signs or effects on body weight or organ weights were observed following exposure to MCLR aerosol. Histopathological examination revealed treatment-related lesions in the nasal cavity only. Lesions were not observed in the liver, other organs or in other parts of the respiratory tract. As indicated in Table 4-7, the incidence and severity of nasal lesions increased with length of the daily exposure period. The lesions consisted primarily of necrosis or inflammation of respiratory epithelial cells and degeneration, necrosis and atrophy of olfactory epithelial cells. Necrotic lesions of olfactory epithelial cells were generally larger patches, while few cells were involved in respiratory epithelial cell necrosis.

4.2.2.3. Subchronic and Chronic Studies

No subchronic or chronic animal studies evaluating the inhalation route of exposure were identified in the materials reviewed for this document.

Table 4-7. Incidence and Severity of Nasal Cavity Lesions in Mice Inhaling Microcystin Aerosol for 7 Days (Benson et al., 2005)

Lesion	Severity	Control	Daily Exposure Period (minutes)		
			30	60	120
Respiratory Epithelial Necrosis					
Turbinate 1	Minimal	0/6	1/6	0/6	0/6
	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	2/6
Turbinate 2	Mild	0/6	0/6	6/6	3/6
	Moderate	0/6	0/6	0/6	3/6
Respiratory Epithelial Inflammation					
Turbinate 1	Mild	0/6	1/6	0/6	1/6
Turbinate 2	Mild	0/6	1/6	0/6	0/6
Olfactory Epithelial Degeneration, Necrosis and Atrophy					
Turbinate 1	Mild	0/6	0/6	0/6	4/6
	Moderate	0/6	0/6	0/6	1/6
Turbinate 2	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	6/6
Turbinate 3	Mild	0/6	0/6	6/6	0/6
	Moderate	0/6	0/6	0/6	4/6
	Marked	0/6	0/6	0/6	2/6

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES – ORAL AND INHALATION

4.3.1. Oral Exposure

4.3.1.1. Purified Microcystins

Fawell et al. (1999) conducted acute, subchronic and developmental toxicity studies of MCLR given via gavage to Cr1:CD-1(ICR)BR (VAF plus) mice (age not specified). MCLR (0, 200, 600 or 2000 μg/kg) was administered to groups of 26 mice on days 6-15 of pregnancy. The mice were sacrificed on day 18 and necropsied. Weight and sex of the fetuses were recorded, and external, visceral and skeletal examinations performed. Seven of 26 dams receiving 2000 μg/kg died and 2 others were sacrificed prematurely due to morbidity. Altered liver appearance was noted during gross examination of these animals. Surviving dams in this group did not display any clinical signs of toxicity or differences in body weight or food consumption. The authors reported that fetal body weight was significantly lower than controls and there was delayed skeletal ossification at the highest dose; however, the data were not presented in the publication. These effects may be associated with maternal toxicity. According to the authors, no effects on resorption or litter size were observed, nor were there increases in external, visceral or skeletal abnormalities in fetuses of any treatment group. Data on reproductive and developmental parameters were not provided. Based on the authors' description of the findings, it seems evident that the 600 μg/kg-day dose represents a NOAEL for both maternal and developmental toxicity in mice. Further, the deaths of seven dams receiving 2000 μg/kg-day clearly identify this dose as a maternal FEL. However, in the absence of the data showing incidences of reproductive and developmental parameters, it is difficult to determine conclusively whether the high dose also represents a LOAEL for developmental toxicity.

4.3.1.2. Cyanobacterial Extracts

Falconer et al. (1988) conducted a limited study of reproductive effects using an extract from an *M. aeruginosa* bloom sample. Eight female mice that had been given $1/4^{th}$ dilution of the extract as drinking water (estimated to contain 14 μg/mL of unspecified microcystin toxin) since weaning were mated with similarly treated males. No difference in number of litters, pups per litter, sex ratio or litter weight were observed. Reduced brain size was reported to occur in 7 of 73 pups from treated parents and in none of 67 pups from controls. The litter distribution of the affected pups was not reported by the authors. One of the small brains was examined histologically, revealing extensive damage to the hippocampus.

4.3.2. Inhalation Exposure

No reports of developmental or reproductive toxicity by the inhalation route of exposure were identified in the materials reviewed for this document.

4.4. OTHER STUDIES

4.4.1. Neurological Effects

Neurological effects have been reported to occur with acute lethal doses of MCLR. Clinical signs such as hypoactivity and piloerection have been observed in mice and rats exposed to lethal doses of MCLR by oral or i.p. administration (Hooser et al., 1989a; Fawell et al., 1999). These signs were observed in animals that subsequently succumbed to the lethal exposures. No reports of neurotoxicity by the inhalation route of exposure were identified in the materials reviewed for this document.

Maidana et al. (2006) reported that long-term memory retrieval (as assessed by step-down inhibitory avoidance task) was impaired in rats receiving intrahippocampal injection of 0.01 or 20 μg/L of a microcystin extract from *Microcystis* strain RST 9501. Exposure at 0.01 μg/L also impaired spatial learning (as assessed by performance on the radial arm maze), but exposure at the higher concentration did not. The authors indicated that the primary microcystin produced by this strain is [D-Leu^1] MCLR, a variant of MCLR. Oxidative damage, as measured by lipid peroxides and DNA damage, was increased in tissue homogenates of the hippocampus from treated animals.

Foxall and Sasner (1981) conducted limited *in vitro* studies on the neurological effects of a crude extract from a bloom of *M. aeruginosa*. Little detail on experiment design was reported; frog and mouse heart, frog sartorius muscle, frog sciatic nerve and mouse ileum were used in the experiments. The authors reported that the extract had no effect on electrical or mechanical events.

4.4.2. Immunological Effects

Evaluation of the immunotoxicity of microcystins *in vivo* was reported in only two studies, both of which used a cyanobacterial cell extract rather than purified microcystins. Shirai et al. (1986) reported that mice, immunized i.p. with either live or sonicated cells from a *Microcystis* water bloom, developed delayed-type hypersensitivity when challenged 2 weeks later with a subcutaneous injection of sonicated cells. Delayed hypersensitivity was assessed by footpad swelling, which was increased approximately 2-fold over controls at the highest doses of cells. It is not clear whether an endotoxin in the bloom sample was responsible for the development of hypersensitivity, or whether the antigenic epitope existed on other components of the sample.

Shen et al. (2003) assessed the effect of cyanobacterial cell extract on immune function. Mice received 14 daily i.p. injections containing a cell-free extract from a water bloom dominated by *M. aeruginosa*. Doses were reported as 16, 32 and 64 mg lyophilized cells/kg body weight or as 4.97, 9.94 and 19.88 μg/kg microcystin equivalents. HPLC analysis indicated that the microcystin content of the extract was 79.53%, although specific congeners in the extract were not reported. The following immunotoxicity endpoints were examined: phagocytosis, lymphocyte proliferation and antibody production in response to sheep red blood cells. Phagocytic capacity was reduced at the two highest doses, but percentage phagocytosis was not

affected. B-lymphocyte proliferation was significantly reduced (33% compared to controls at 32 mg/kg), while changes in T-lymphocyte proliferation were mild, and deemed biologically insignificant. Finally, humoral immune response, as measured by antibody-forming plaques, was reduced in a dose-dependent manner in treated mice.

Chen et al. (2004, 2005) evaluated the role of nitric oxide generation and macrophage related cytokines on the reduced phagocytic capacity induced by pure MCLR. A dose-dependent inhibition of nitric oxide production was observed in activated macrophages, and a repressive effect was seen in cytokine formation at the mRNA level (e.g., IL-1β, TNF-α, GM-CSF, IFN-γ) after either 24 hour (Chen et al., 2004) or 6 hour treatment (Chen et al., 2005). Hernandez et al. (2000) indicated that MCLR enhanced the early spontaneous polymorphonuclear leukocyte (PMN) adherence (not late or PMN stimulated early or late) at low concentrations, suggesting that microcystins may affect the immune system.

4.4.3. Hematological Effects

Several studies have noted thrombocytopenia in laboratory animals treated with microcystins or bloom extracts purportedly containing microcystins (Slatkin et al., 1983; Adams et al., 1985, 1988; Takahashi et al., 1995). Early investigations explored whether microcystins had a direct effect on platelets, and whether platelets might be responsible for pulmonary thrombi (Slatkin et al., 1983; Jones, 1984). However, *in vitro* studies have shown that MCLR neither induces nor impedes the aggregation of platelets (Adams et al., 1985). Pulmonary thrombi apparently consist of necrotic hepatocytes circulating in the blood (see Section 4.4.5.2.1). More recent information supports the hypothesis that hematological effects observed in animals acutely exposed to microcystins are secondary effects of liver hemorrhage (Takahashi et al., 1995).

Takahashi et al. (1995) reported dose-dependent reductions in erythrocyte count, leukocyte count, hemoglobin concentration, hematocrit and coagulation parameters 1 hour after rats were exposed to MCLR (100 and 200 μg/kg i.p). None of these parameters changed until after massive liver hemorrhage commenced. Further, hematological changes such as increased prothrombin time and fibrin deposition in the renal glomeruli were not observed. The authors concluded that the depletion of blood components occurred as a result of liver hemorrhage.

Interestingly, mild thrombocytopenia was reported in 1-week-old mice treated with a large i.p. dose of MCLR, even though none of these mice died (Adams et al., 1985).

4.4.4. Effects by Dermal Exposure

No animal studies evaluating the effects in animals of dermal exposure to purified microcystins were identified in the materials reviewed for this document; only one study using a cyanobacterial extract was located. Davidson (1959) applied a crude extract from a bloom of *N. rivulare* to the shaved backs of three mice every 2 hours for a total of 12 hours. Heavy scales were observed on the treated areas; the scales were gone within 4 days and hair regrowth occurred in the following weeks. Dermal application of an aqueous extract or aqueous filtrate did not result in any effects.

4.4.5. Effects by Parenteral Exposure

4.4.5.1. Effects in Humans after Parenteral Exposure

In February of 1996, unfinished water from a reservoir with a cyanobacterial bloom was used at a hemodialysis center in Caruaru Brazil, leading to numerous deaths among the patients treated with the water (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). The first report of this incident was published by Jochimsen et al. (1998), and Pouria et al. (1998) published follow-up information on the status of the patients. Azevedo et al. (2002) provided the most up-to-date information on patient status after the incident. Using water samples from the reservoir, serum and tissue samples from patients, and a variety of methods, each publication identified microcystins as the primary causative factor in the deaths and disease among patients. Carmichael et al. (2001) used analytical results from tissue samples with dialysate volumes to estimate the concentration of microcystins in the water to which patients were exposed, as a direct measure of exposure concentration was not available.

Of the 131 patients undergoing dialysis during the 4 days when unfinished water was supplied to the center, 116 experienced symptoms, including visual disturbances, nausea, vomiting and muscle weakness; 100 developed acute liver failure; and 52 had died as of December, 1996 (Azevedo et al., 2002). The acute presentation of the disease included malaise, weakness, dizziness, vertigo, visual disturbances and blindness, nausea, vomiting and abdominal pain. Clinical signs included hepatomegaly and jaundice. Biochemistry showed high concentrations of bilirubin and ALP, moderate increases in AST and ALT, hypoglycemia, hypoalbuminemia and severe hypertrigliceridemia. Major hematology findings were slightly low platelet count (within normal range) and reduced platelet aggregation, as well as red blood cell abnormalities (anisocytosis, acanthocytosis and schistocytosis) in some patients. Liver biopsy and autopsy samples showed severe, diffuse individual hepatocyte necrosis throughout the liver lobule, with cell-plate disruption and apoptosis; however, no intrahepatic hemorrhage was observed. Leukocyte infiltration and canalicular cholestasis were also observed.

Microcystins were implicated as the major contributing factor to patient death and morbidity (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). Evidence for the role of microcystins was derived from a variety of sources discussed by Azevedo et al. (2002) and Carmichael et al. (2001). Quantitative analysis of the phytoplankton in the reservoir from which the water was supplied to the dialysis center showed that cyanobacteria represented about 99% of the phytoplankton in the reservoir, although the species present during the outbreak were not identified. Analyses of filter systems in the dialysis center showed microcystins; the carbon filter also had cylindrospermopsin. Analyses of patient sera and liver samples provided additional evidence. Patient sera were analyzed for other potential toxins (chlorines, chloramines, trace elements, heavy metals, agricultural compounds and pesticides), but none were found (Pouria et al., 1998). Both serum and liver analyses for microcystins revealed MCYR, MCLR and MCAR. None of the biological samples contained cylindrospermopsin. Finally, physiological effects observed in the patients closely mirrored effects observed in laboratory animals exposed to microcystins. Specifically, the liver damage observed in patients was similar to that observed in mice (Jochimsen et al., 1998). Using

microcystin concentrations measured in patients' livers and typical dialysis volume, Carmichael et al. (2001) estimated the average concentration of microcystins in the dialysate to be 19.5 μg microcystins/L.

Soares et al. (2006) reported another incident involving human exposure to microcystins via dialysate. In November, 2001, 44 patients of a renal dialysis center in Rio de Janeiro, Brazil, were assumed to be exposed to microcystins after a bloom of *Microcystis and Anabaena* occurred in the reservoir supplying water to the center. The concentration of microcystins in the drinking water was 0.4 μg/L by ELISA. In the water used at the center (after treatment by activated carbon column), the concentration was 0.32 μg/L. Of the 44 patients exposed, 90% had serum microcystin concentrations above the limit of detection (0.16 ng/mL). Twelve of the patients were selected for 2-month follow-up monitoring of serum levels. Over the follow-up period, serum concentrations ranged from <0.16 to 0.96 ng/mL. The highest serum concentrations occurred 1 month after initial exposure. The authors did not provide any information on health effects from the exposure.

Pilotto et al. (2004) reported that about 20% of 114 volunteers subjected to skin-patch testing using cell suspensions and extracts of various cyanobacterial cultures (including two strains of *M. aeruginosa*) experienced a mild dermal skin reaction (erythema). The reaction did not vary with cyanobacterial species.

4.4.5.2. Effects in Animals after Parenteral Exposure

4.4.5.2.1. *Acute Studies with Parenteral Exposure*

The acute toxicity of microcystins administered i.p. has been extensively studied. The primary target organ for acute microcystin toxicity is the liver; effects have also been observed in the kidney, lungs and gastrointestinal tract. Some of the effects observed in organs other than the liver are believed to be secondary to liver effects.

A number of references report LD_{50} estimates for injected MCLR (Slatkin et al., 1983; LeClaire et al., 1988; Lovell et al., 1989a; Hermansky et al., 1990c; Miura et al., 1991; Stotts et al., 1993; Gupta et al., 2003); two report LD_{50} values for MCRR and MCYR (Stotts et al., 1993; Gupta et al., 2003). Table 4-8 summarizes the available estimates of microcystin LD_{50} values after injection exposure to purified microcystins.

As the table shows, the LD_{50} for MCLR in mice ranges between 30 and 60 μg/kg. The acute lethality of MCYR is slightly lower than MCLR; LD_{50} estimates for MCYR were 111 and 171 μg/kg (Gupta et al., 2003 and Stotts et al., 1993, respectively). The LD_{50} for MCRR is higher still, with LD_{50} values estimated as 235 μg/kg (Gupta et al., 2003) and 650 μg/kg (Stotts et al., 1993). In rats, the LD_{50} for MCLR was similar to that in mice. There is some evidence that

Table 4-8. LD_{50} Values of Purified Microcystin Congeners by Intraperitoneal Administration						
Sex/Strain	Purity	Vehicle	LD_{50} (95% CI)	Duration	Comments	Reference
MCLR-Mice						
Male/Balb/C	75%	NS*	32.6 ($\pm$1.2) μg/kg	24 hours	Impurities tested for toxicity at high doses with negative results	Lovell et al., 1989a
Female/Swiss albino Hale-Stoner	NS	NS	60 μg/kg	NS		Slatkin et al., 1983
Female/NIH non-Swiss	>95%	Distilled water	61 μg/kg	NS		Hermansky et al., 1990c
Male/Swiss Webster	NA	0.09% saline	<100 μg/kg	NS	LD_{50} by up and down method	Stotts et al., 1993
Not specified/ CBA/Balbc	NS	NS	250 μg/kg	NS		Fitzgeorge et al., 1994
Female/Swiss albino	NS	NS	43.0 μg/kg (37.5-49.4)	24 hours	LD_{50} by up and down method	Gupta et al., 2003
Male/Swiss albino	NS	Methanol and PBS	43 μg/kg (37.5-49.4)	24 hours	LD_{50} by up and down method	Rao et al., 2005

Table 4-8 cont.

Sex/Strain	Purity	Vehicle	LD_{50} (95% CI)	Duration	Comments	Reference
MCLR-Rats						
Male/Fischer	NS	NS	50 μg/kg (36-68)	72 hours	Intraarterial injection. Abstract only	LeClaire et al., 1988
Male/Fischer 344	>95%	saline	Fed rats: 122 μg/kg (106-141) Fasted rats: 72 μg/kg (60-83)	25 hours		Miura et al., 1991
MCRR-Mice						
Male/Swiss Webster	NS	0.09% saline	~650 μg/kg	NS	LD_{50} by up and down method	Stotts et al., 1993
Female/Swiss albino	NS	NS	235.40 μg/kg (202.3-272.8)	24 hours	LD_{50} by up and down method	Gupta et al., 2003
MCYR-Mice						
Male/Swiss Webster	NS	0.09% saline	~171 μg/kg	NS	LD_{50} by up and down method	Stotts et al., 1993
Female/Swiss albino	NS	NS	110.6 μg/kg (81.7-149.6)	24 hours	LD_{50} by up and down method	Gupta et al., 2003

* Not specified.

the LD_{50} for MCLR was higher in fed rats (122 μg/kg) than in fasted rats (72 μg/kg) (Miura et al., 1991).

In general, death occurs quickly in animals receiving a lethal injected dose of MCLR. Mice typically die within 1-2 hours of a lethal i.p. dose of MCLR (Adams et al., 1988; Gupta et al., 2003). Mean time to death for mice exposed to a lethal dose of MCRR or MCYR is also within 2 hours (Gupta et al., 2003). In mice, an i.p. dose of 100 μg/kg MCLR typically results in 100% mortality (Adams et al., 1988; Hooser et al., 1989a; Hermansky et al., 1990c). Hooser et al. (1989a) compared the effects of MCLR in mice and rats and observed significant differences in survival time; both male and female rats given less than 240 μg/kg survived between 20 and 32 hours; rats receiving higher doses died within 8 hours. In contrast, female mice receiving 100 μg/kg died within 1.5 hours (Hooser et al., 1989a). Miura et al. (1991) administered MCLR i.p. to fed and fasted rats and reported a protective effect of feeding. Median time to death for rats given 100 μg/kg MCLR was 32 hours in fed rats and less than 2 hours in fasted rats.

The sequence of events leading to death in laboratory rodents has been extensively studied (Slatkin et al., 1983; Adams et al., 1988; Hooser et al., 1989a,b, 1990; Takahashi et al., 1995). In general, similar effects have been reported in both rats and mice, but effects occur later in rats than in mice (Hooser et al., 1989a). Gross and microscopic changes in swine treated intravenously are similar to those observed in rodents (Lovell et al., 1989b).

Ten minutes after mice received a lethal i.p. dose, clinical signs, enzyme changes and liver weight changes were generally absent (Slatkin et al., 1983; Adams et al., 1988). Beginning approximately 20 minutes after dosing in mice, liver weights increased as the livers became suffused with blood (Slatkin et al., 1983; Adams et al., 1988; Hermansky et al., 1990c). Adams et al. (1988) estimated that as much as 44% of the total blood volume was located in the liver 30 minutes after a lethal dose of MCLR in mice. Similarly, in swine treated intravenously, blood volume lost to the liver was estimated to be about 40% (Beasley et al., 2000). At 30 minutes, there were isolated areas of hepatic necrosis, and at 45 minutes, there was marked liver congestion and widespread hepatic necrosis (Hermansky et al., 1990c). Pulmonary thrombi observed at the time of death were generally believed to result from necrotic hepatocytes (Adams et al., 1988). Other pulmonary effects observed at 30-60 minutes after exposure included congestion, bronchial epithelial hyperplasia and necrosis, edema and hemorrhage (Gupta et al., 2003). In general, hepatic enzyme levels show little or no change until 20-30 minutes after dosing, when hepatic hemorrhage is beginning (Adams et al., 1988; Hooser et al., 1989a; Hermansky et al., 1990c; Takahashi et al., 1995).

At 60 minutes, there was severe disassociation of hepatocytes, hepatocyte loss and hemorrhage, with disintegration of liver architecture (Hooser et al., 1991b; Guzman et al., 2003). Thrombocytopenia coincided with hepatic hemorrhage as blood accumulated in the liver (Slatkin et al., 1983; Adams et al., 1988; Takahashi et al., 1995).

The available studies demonstrate a very steep dose-response curve for MCLR acute toxicity. In female NIH non-Swiss outbred mice, the only change observed after i.p. administration of 50 μg MCLR/kg was Kupffer-cell hyperplasia, while all mice receiving 100

μg/kg died (Hermansky et al., 1990c). Hooser et al. (1989a) reported that male rats dosed i.p. with 20, 40 or 80 μg/kg and females dosed with 40 μg/kg MCLR showed no clinical signs of toxicity nor gross or microscopic lesions in the liver or other organs examined, while 120 μg/kg was lethal in some rats and 160 μg/kg was consistently lethal. Lovell et al. (1989a) administered a sublethal i.p. dose (about 25 μg/kg) of MCLR to male mice and reported a significant increase in liver weight (8.7%), but no clinical signs or hepatic lesions.

Induction of apoptosis is believed to be one mechanism for liver damage in acutely exposed animals (Hooser, 2000). Yoshida et al. (1998) treated mice with single sublethal doses of MCLR (20, 30 and 45 μg/kg i.p.) and observed them for 7 days. Multiple apoptotic cells were noted in the centrilobular regions of the livers of these mice. Hooser (2000) reported widespread apoptosis in most hepatocytes after rats were treated with a single i.p. dose of 500 μg/kg MCLR. Yoshida et al. (1998) reported the occurrence of two types of apoptotic hepatocytes in mice given sublethal doses of MCLR; one that revealed MCLR by immunohistochemistry and one that did not. The authors suggested that the latter type may contain MCLR that had lost the antigenic epitope, or may have become apoptotic via other means, including ischemia or hypoxia. Apoptosis induction is not restricted to the liver; Botha et al. (2004) reported significantly increased apoptotic indices in the gastrointestinal tract of BALB/c mice as early as 8 hours after a single 75% LD_{50} dose (specific dose not reported) of MCLR i.p. The authors observed immunohistochemical evidence of MCLR in the lamina propria and postulated that MCLR was involved in the induction of apoptosis. The role of apoptosis in microcystin-induced liver toxicity is further discussed in Section 4.4.7.5 (Mechanistic Studies).

Effects outside the liver have been reported after acute injection exposure to MCLR. It remains unclear whether such effects may be indirectly related to hepatotoxicity. Some studies have shown increases in kidney weight (Hooser et al., 1989a; Lovell et al., 1989a) or other signs of kidney damage (LeClaire et al., 1988; Zhang et al., 2002) in rats and mice after injection of sublethal doses of MCLR. Lovell et al. (1989a) observed dilation of cortical tubules and eosinophilic granular or fibrillar material in the cortical tubules after MCLR administration. In addition to reports of kidney effects, there are scattered reports of cardiac effects, including degeneration and necrosis of myocardial cells after i.p. or i.v. exposure to MCLR (LeClaire et al., 1988; Zhang et al., 2002). LeClaire et al. (1995) reported that mechanisms such as reflex tachycardia and increased cardiac output, which typically would allow the heart to compensate for the acute hypotension caused by blood pooling in the liver, were impaired in rats given a lethal dose of MCLR. The authors suggested a cardiogenic component to the toxicity of MCLR. Oishi and Watanabe (1986) observed tachycardia in mice 20 minutes after i.p. injection of lyophilized cells from *M. aeruginosa*. Finally, a few studies suggest that acute i.p. exposure to MCLR can affect lipid peroxidation levels in both the intestinal mucosa (Moreno et al., 2003) and liver (Towner et al., 2002) of rats.

Two injection studies support the finding by Ito et al. (1997a) by oral exposure that young animals are not as susceptible to the acute toxic effects of microcystins as older animals. Adams et al. (1985) administered lethal doses of MCLR via i.p. injection to 1-, 2- and 3-week-old mice. None of the 1- or 2-week-old mice died, whereas 23 of 31 mice aged 3 weeks died within 2 hours. The 3-week-old mice that survived were rechallenged with MCLR a week later,

and all died. Rao et al. (2005) reported that time to death decreased with age in mice treated i.p. with MCLR.

Guzman and Solter (2002) evaluated the acute effects of repeated injection of MCLR. Male BALB/c mice were injected with 45 μg/kg MCLR daily for 2, 4 or 7 days. Livers of mice receiving four or seven daily doses were pale and moderately enlarged, with an accentuated reticular pattern; absolute and relative liver weights were statistically increased over controls. Histopathology revealed apoptotic hepatocytes in the centrilobular region of mice receiving two doses, and marked hepatocytomegaly, disorganized hepatocytes, multinucleated hepatocytes and cytoplasmic vacuolation in mice receiving four or seven doses. Guzman et al. (2003) reported immunostaining of some centrilobular hepatocytes following two doses of 45 μg/kg; however, protein phosphatase activity was not affected.

Acute toxicity of bloom extracts is highly variable, likely reflecting the variable toxin content of algal blooms. LD_{50} values estimated for various bloom extracts range from 14 mg dry weight cells/kg body weight to 1924 mg dry weight cells/kg (see Table 4-9). These studies used a variety of test materials, including lyophilized cyanobacterial cells, cell-free lysates, etc.

Early studies of purified but unidentified toxins from *M. aeruginosa* show essentially the same pattern of acute hepatotoxicity and time to death after i.p. injection of lethal doses in rats and mice that is seen with purified MCLR (Elleman et al., 1978; Foxall and Sasner, 1981; Falconer et al., 1981; Theiss et al., 1984, 1985, 1988; Jones and Carmichael, 1984; Siegelman et al., 1984; Dabholkar and Carmichael, 1987). In addition, many studies of cyanobacterial bloom extracts (primarily *M. aeruginosa*) administered via i.p. injection to laboratory rodents show similar effects (Ashworth and Mason, 1945; Ohtake et al., 1989; Rao et al., 1994; Porfino et al., 1999; Sabour et al., 2002).

Jackson et al. (1984) also observed liver pathology in sheep exposed intraruminally to a bloom sample identified as *M. aeruginosa*. Time to death ranged from 18 to 48 hours post exposure. In animals that succumbed, the livers were hemorrhagic and necrotic.

Effects on organs other than the liver have been reported in some studies of bloom extracts. Bhattacharya et al. (1997) observed changes suggesting distal tubular dysfunction (proteinurea, as well as decreases in kidney LDH and AST levels) in rats injected with LD_{50} doses of *M. aeruginosa* extract, but no histopathological changes. Dose-dependent increases in urea and creatinine and decreases in total protein and albumin were also observed. Picanco et al. (2004) reported that i.p. injection of an extract from a culture of *M. aeruginosa* (strain NPJB-1) into either young or mature mice resulted in increased alveolar collapse and increased number of polymorphonuclear and mononuclear cell infiltrations when compared with saline-treated controls. There was a very low concentration of contaminating bacteria in the culture (i.e., the culture was not axenic) used in this study, and the authors acknowledged that materials in the extract other than microcystins may have contributed to the pulmonary effects.

1

Table 4-9. Intraperitoneal LD_{50} Values for Bloom Extracts

Microcystin Source	Species	Sex/Strain	Vehicle	LD_{50} (95% CI)	Duration	Comments	Reference
Solution of lyophilized cells of *M. aeruginosa*	Rats	Male/Jcl: Wistar	Saline	67.4 mg dry weight cells/kg, 2 hours	2 hours		Oishi and Watanabe, 1986
Solution of lyophilized cells of *M. aeruginosa*	Mice	Male/Jcl:ICR	Saline	14.4 mg dry weight cells/kg, 1 hour	1 hour	None of mice surviving past 1 hour died within 1 week	Oishi and Watanabe, 1986
Solution of lyophilized cells of *M. ichthyoblabe*	Mice	Male/Swiss	0.9% saline	502-1924 mg cell dry weight/ kg body weight	Not specified	Microcystin content ranged from 0.73-0.78 μg/g	Sabour et al., 2002
Cell-free lysate of *M. aeruginosa*	Mice	Male/Swiss	0.9% saline	431 mg/kg dry weight, 24 hours	24 hours	LD_{50} by up and down method	Rao et al., 1994
Solution of lyophilized bloom sample	Mice	Not specified/ Balb/C	Saline	25-250 mg dry weight phytoplankton sample/kg	Not specified	Bloom dominated by *M. aeruginosa*. MCLR content of samples ranged from 53-952 μg/g DW biomass	Tarczynska et al., 2000
Solution of lyophilized bloom sample	Mice	Male/Swiss albino	0.9% saline	154.28 mg algae/kg, 48 hours	48 hours	*M. aeruginosa* was 95% of bloom biomass	Porfino et al., 1999
Cell-free lysate of *M. aeruginosa*	Mice	Male/Swiss albino	Not specified	3.5 g extract/kg, 24 hours	24 hours	MCLR dose approximately 9.625 mg/kg	Rao et al., 2005
Purified toxin from *M. aeruginosa*	Mice	Not specified/ white, strain unspecified	Ethanol and water	466 $\pm$ 13 μg/kg	Not specified	Congener not identified/specified	Bishop et al., 1959
purified toxin of *M. aeruginosa*	Mice	Male/white, strain unspecified	Not specified	56 μg/kg (43-60)	Not specified	Congener not identified/specified	Elleman et al., 1978

In further support for the findings of age-dependent liver effects from purified microcystins, Foxall and Sasner (1981) reported that neonatal and juvenile (age not specified) mice had no clinical symptoms or liver pathology after i.p. injections of a crude lysate from a bloom of M. *aeruginosa*. Details of the experiment and results were not provided. The authors reported that the mice did not die until they were 20 days of age, but it is not clear from the publication whether the mice treated as neonates died later or only mice that were at least 20 days old when treated died. Mature mice were also treated for comparison, but the results were not reported. The authors concluded that "young animals were not sensitive to the toxin but developed sensitivity as they matured".

4.4.5.2.2. *Short-Term Studies with Parenteral Exposure*

Guzman and Solter (1999) and Solter et al. (1998, 2000) evaluated the effects on rats of short-term administration of MCLR via continuous i.p. infusion. After 28 days of exposure at 16, 32 or 48 μg/kg-day, there were dose-dependent increases in serum levels of sorbitol dehydrogenase (SDH), AST, GGT, ALP and bile acids, while a dose-dependent decrease in serum albumin and a decrease in ALT synthesis were also observed (Solter et al., 1998, 2000).

Immunohistochemistry on the liver showed evidence of bioaccumulation of MCLR in liver cytosol, with measured liver concentrations increasing at a greater rate than the administered dose (Solter et al., 1998). Apoptotic cells and cytoplasmic vacuolation were observed in the livers of rats receiving 32 and 48 μg/kg-day. A later study exposing rats at the same doses showed evidence for oxidative damage in the liver, as measured by dose-dependent increases in malondialdehyde, a lipid peroxidation byproduct (Guzman and Solter, 1999). This observation is consistent with evidence for oxidative stress after short-term exposure to MCLR.

4.4.5.2.3. *Subchronic Studies with Parenteral Exposure*

Shi et al. (2002; Chinese publication, only abstract reviewed) reported oxidative stress in rats injected with i.p. doses of 4, 8 or 12 μg/kg-day MCLR for 35 days. Serum GGT and whole blood glutathione were decreased, while LDH and AST increased after exposure, with no change in ALT levels. Hepatocyte proliferation and apoptosis were also observed. Oxidative stress and apoptosis are discussed further in Section 4.4.7 (Mechanistic Studies).

Elleman et al. (1978) administered daily i.p. injections of a purified toxin from a bloom of *M. aeruginosa* to white male rats (strain unspecified) for 6 weeks. Doses were reported as fractions of the LD_{100} (0.75, 0.5, 0.25 and 0). These doses correspond to 52.5, 35 and 17.5 μg/kg based on the reported LD_{100} (70 μg/kg). Two mice from each group were sacrificed weekly for necropsy and histopathological examination of the liver, kidney, heart, lung, spleen and brain. Eleven of 16 high-dose and 5 of 14 mid-dose mice died prior to scheduled sacrifice; none of the mice receiving the low dose died early. Of the 11 high dose animals that died prematurely, nine died during the first week with symptoms of acute toxicity and liver hemorrhage. In the remaining groups, progressive liver changes were seen with each week, and dose-dependent pathology was observed. The authors noted numerous mitotic figures in hepatocytes of the low-dose mice early on. Other histopathological findings in the liver were hepatocyte degeneration,

scattered necrosis, fibrosis and mononuclear cellular infiltration; details of the severity of these findings in each group were not reported.

4.4.5.2.4. *Chronic Studies with Parenteral Exposure*

Milutinovic et al. (2002, 2003) evaluated the kidney effects of chronic i.p. administration of MCLR and MCYR in rats. Doses of 10 μg/kg were administered to groups of five male Wistar rats every other day for 8 months. After sacrifice under CO_2 anesthesia, the kidneys were removed, fixed, sectioned and stained. During exposure, the treated rats exhibited clinical signs of toxicity and reduced body weight. Microscopic examination of the kidneys of treated animals showed collapsed glomeruli and dilated tubules with eosinophilic casts and some cytoplasmic vacuolation. The interstitial space was infiltrated with lymphocytes. More renal corpuscles were significantly damaged in the MCLR-treated group than the MCYR-treated group. Cytoskeletal abnormalities and DNA damage typical of apoptosis or necrosis were also observed in tubular epithelial cells (Milutinovic et al., 2002, 2003). Although they did not report details of the liver pathology in this study, the authors noted that the kidneys were more damaged than the livers, suggesting that adaptation to exposure may have occurred in the livers.

4.4.5.2.5. *Initiation/Promotion Studies with Parenteral Exposure*

Nishiwaki-Matsushima et al. (1992) demonstrated that i.p administration of MCLR could enhance the number and area of glutathione S-transferase (placental form; GST-P) positive foci in a medium-term rat liver bioassay. In male F344 rats pretreated with 200 mg/kg diethylnitrosamine (DEN) and partially hepatectomized, the number of GST-P positive foci was significantly increased when the rats were subsequently treated with 10 μg/kg MCLR i.p. twice a week. In a follow-up experiment, rats were pretreated with DEN and then given twice weekly i.p. doses of 10, 25 or 50 μg/kg MCLR. A dose-dependent increase in the number and area of GST-P positive foci was observed in the animals treated with MCLR (Nishiwaki-Matsushima et al., 1992).

Ohta et al. (1994) also used the two-stage rat liver bioassay model to evaluate the promotion capability of MCLR in rats pretreated with DEN, but not subjected to partial hepatectomy. After treatment with 200 mg/kg DEN, rats given twice weekly i.p. injections of 25 μg/kg MCLR had significantly more GST-P positive foci and significant increases in the area of such foci when compared with DEN pretreatment alone. MCLR alone had negligible initiating capability.

Hu et al. (2002) reported significant enhancement of gamma-GT foci in a two-stage medium-term rat bioassay. Microcystin treatment (congener not specified) in DEN-pretreated rats resulted in 100% incidence of gamma-GT foci, while DEN treatment alone resulted in foci in only 22% of rats. Immunohistochemistry showed that microcystin exposure reduced expression of the bax gene and increased expression of the bcl-2 gene. The Bax protein induces apoptosis, while the Bcl-2 protein inhibits apoptosis (Klassen, 2001). This finding suggests that apoptosis is inhibited by low doses of microcystin; in contrast, higher doses appear to induce apoptosis (see Section 4.4.7.5 below).

Sekijima et al. (1999) conducted a similar experiment using either DEN or aflatoxin B1 (AFB1) as an initiator (with partial hepatectomy) and MCLR or a combination of AFB1 and MCLR for promotion. In rats pretreated with 200 mg/kg DEN and subsequently given 10 μg/kg MCLR i.p., there were increases in both the number and area of GST-P positive foci, but this difference did not reach statistical significance. In rats pretreated with DEN and subsequently given either AFB1 alone or a combination of MCLR and AFB1, a statistically significant increase in number and area of foci was observed. The effect on GST-P positive foci of combined treatment with MCLR and AFB1 was not synergistic, however; the number and area of foci in animals treated with both was not larger than the sum of the foci induced by each compound individually. When altered hepatic foci of all types (including basophilic and eosinophilic/clear, rather than only GST-P positive foci) were analyzed, the number of foci was significantly greater in rats treated with both MCLR and AFB1 than in those treated with either toxin alone. To assess the effect of MCLR on initiation by AFB1, Sekijima et al. (1999) pretreated rats with AFB1 followed by twice weekly i.p. injections of 1 or 10 μg/kg MCLR for 6 weeks. The number and area of GST-P positive foci were significantly increased in animals given MCLR compared with controls. There was no difference in number or area of foci between the two doses of MCLR. The authors suggested that the higher dose may have had cytotoxic effects on hepatocytes.

Ito et al. (1997b) treated 13 ICR mice with 100 i.p. injections (5 times/week) of 20 μg/kg MCLR over 28 weeks. Five mice were sacrificed immediately after the last injection, while eight mice were withdrawn from treatment and sacrificed 2 months later. Three untreated mice served as controls. Relative liver weights were 4.7% of body weight in the control mice, 9% in mice sacrificed immediately after the last treatment, and 6.8% in those withdrawn from treatment and sacrificed later. Statistical comparisons among the relative liver weights were not provided, nor were the data with which to perform these comparisons. Neoplastic nodules were observed in the livers of all mice of both treatment groups. The nodules ranged in size up to 5 mm in diameter. The mean numbers of nodules in the treated animals (7.7 and 9.9 nodules per cm^2 area in the groups sacrificed immediately and 2 months later, respectively) were not significantly different from the controls (control data not provided). The incidence of nodule development in the few control mice was not reported. The types of nodules were characterized as A) weakly staining with hematoxylin and eosin and with small nuclei; B) intensely staining with eosin but not with PAS or C) mainly occupied with fat droplets. The small number of animals in the treatment and control groups limits the usefulness of these data.

4.4.5.2.6. *Developmental/Reproductive Studies with Parenteral Exposure*

Chernoff et al. (2002) investigated the developmental toxicity of MCLR in CD-1 mice. Pregnant mice were treated with i.p. or subcutaneous doses of MCLR (95% pure) on gestation days (GDs) 7-8, 9-10 or 11-12. Doses of 0, 32, 64 and 128 μg/kg were administered i.p., while only the 128 μg/kg dose level was administered subcutaneously. Mice were sacrificed by CO_2 inhalation on GD 17. After litter and gravid uterus weights were recorded, fetuses were examined for gross malformations and preserved for skeletal examination. Livers of the dams were examined grossly and subjected to histopathology. No effects on maternal weight gain, litter size, average fetal weight or incidence of gross or skeletal abnormalities were observed. Histological examination of the maternal livers showed no effects of treatment.

In a separate experiment by the same researchers (Chernoff et al., 2002), pregnant mice treated with 32-128 μg/kg MCLR (via i.p. injection on GD 7-8, 9-10 or 11-12) were allowed to give birth, and the growth and viability of the offspring were followed for 5 days. A different, apparently more potent, lot of MCLR was used in this experiment. Mortality exceeded 50% (19 of 35 dams) in the 64 μg/kg dose group and only 1 of 34 animals in each of the 96 and 128 μg/kg dose groups survived treatment. Among the surviving animals, there were no effects on viability, birth weight or growth of litters during the brief follow-up period.

Experiments with rabbit whole embryo cultures *in vitro* suggest that low concentrations of MCLR (10-20 μM) can alter the organization of actin filaments and microtubules, although cell morphology is not significantly affected (Frangez et al., 2003; Zuzek et al., 2003). At high concentrations (100 μM), MCLR causes cell rounding and loss of adhesion properties, with consequent cell detachment and dispersion. Frangez et al. (2003) showed that the zona pellucida (a glycoprotein envelope surrounding the ovum) forms an effective barrier against the effects of MCLR, as rabbit whole embryos embedded in zona pellucida were not affected even at high concentrations of MCLR.

Development of mouse embryos in culture was inhibited by a purified toxin from a bloom dominated by *Microcystis* (Sepulveda et al., 1992). At a concentration of 120 μg/mL of toxin in the culture medium, development of two-cell embryos was halted and cytolysis occurred in some embryos. Disruption of the actin cortex was also observed in these embryos. At 60 μg/mL, two-thirds of the embryos divided once more, and the remainder did not develop further. In eight-cell embryos treated with 120 μg/mL toxin, compaction was prevented or reversed. Embryos at this stage treated with 240 μg/mL did not develop further; cells were rounded and lysed. The specific toxin used in this experiment was not identified.

Conflicting results have been observed in teratogenicity testing of purified microcystins in the Frog Embryo Teratogenicity Assay-Xenopus (FETAX) assay with *X. laevis* embryos. In one study, MCLR at concentrations of 25-250 μg/L was shown to induce both skeletal and soft tissue malformations in *X. laevis* embryos (Dvoráková et al., 2002). In contrast, Fischer and Dietrich (2000) reported no effects on mortality, malformation or growth in these embryos after exposure to either MCLR or MCRR at concentrations up to 2000 μg/L. Dvoráková et al. (2002) attributed the differing results to interlaboratory variability or variability in sensitivity of the embryos. O'Brien et al. (2003) reported no effects of MCYR on *X. laevis* embryo mortality, malformation or growth.

Interestingly, Dvoráková et al. (2002) demonstrated that biomass from a *Microcystis* species (*wesenbergii*) that does not produce microcystins could induce malformations in *Xenopus* embryos. O'Brien et al. (2003) also tested two extracts each from *Plantothrix rubescens* and *M. aeruginosa*, reporting that all four extracts resulted in facial narrowing and growth retardation in 96-hour *Xenopus* embryos, while purified MCYR had no effects.

4.4.6. Effects by Intratracheal or Intranasal Instillation

Ito et al. (2001) evaluated the distribution of MCLR after intratracheal instillation of lethal doses in male ICR mice and included a limited description of toxic effects. MCLR in saline solution was instilled at various doses (50, 75, 100, 150 and 200 μg/kg) into 34 mice; 3 mice were sham-exposed as controls. Mortality was 100% in 12 mice receiving doses of 100 μg/kg and greater. At 75 μg/kg, two of four mice died, while no deaths occurred in 18 mice given 50 μg/kg intratracheally. The time course of hepatotoxicity was further evaluated in eight mice given intratracheal doses of 100 μg/kg. One mouse was sacrificed at each of 5, 10, 20, 30, 45, 60, 90 and 120 minutes. Immunostaining for MCLR showed the toxin in the lungs within 5 minutes and in the liver after 60 minutes. Hemorrhage in the liver was observed after 90 minutes and became severe by 120 minutes.

Fitzgeorge et al. (1994) conducted experiments in CBA/BALBc mice with MCLR administered via intranasal instillation and inhalation. This study is poorly described, giving few details of study design and findings. The LD_{50} for intranasal instillation of MCLR was equal to the i.p. LD_{50} (250 μg/kg). Liver and kidney weights were increased in the animals receiving MCLR intranasally (41.6 and 7.5% respectively). The authors further evaluated the relationship between dose and liver weight increase after intranasal instillation of MCLR. At single intranasal doses of 31.3, 62.5, 125, 250 and 500 μg/kg, liver weight increased proportionally (0, 1.5, 24.4, 37.4 and 87%). Seven daily intranasal doses of 31.3 μg/kg, a dose that had produced no liver weight change after a single dose, resulted in a liver weight increase of 75%. Fitzgeorge et al. (1994) reported histopathological findings, but failed to specify which findings resulted from single doses and which resulted from the multiple-dose experiment reported in the same publication. Findings included necrosis of respiratory and olfactory epithelium in the nasal mucosa and centrilobular necrosis with hemorrhage in the liver. Early changes in the liver included vacuolar degeneration and necrosis of hepatocytes near the central vein. The adrenal glands showed effects as well, with vacuolation and necrosis of the inner cortex, as well as congestion of medullary blood vessels. No histopathological changes were observed in the trachea, lungs, esophagus, pancreas, spleen, lymph nodes, kidneys or brain.

4.4.7. Mechanistic Studies

Many mechanistic studies have been conducted to characterize the toxicology of microcystins. These studies include *in vivo* investigations in laboratory animals, *in situ* studies in isolated perfused organ systems and *in vitro* assays in isolated cell preparations. Mechanistic studies have evaluated many aspects of microcystin toxicity, including: 1) the reason for target organ and cell type specificity of microcystins, 2) description of the subcellular effects that occur in susceptible cells, 3) interaction with serine and threonine protein phosphatases (i.e., PP1 and PP2A) as the molecular target for microcystins, 4) the role of cytoskeletal effects, 5) the importance of oxidative stress and apoptosis as a mode of toxic action and 6) the use of chemoprotectants to reduce toxicity. Each of these topics is discussed in further detail below. Mechanistic data related to the genotoxicity of microcystins is presented below in Section 4.4.8 (Genotoxicity and Cell Proliferation).

4.4.7.1. Target Organ/Cell Type Specificity

Oral and injection studies in laboratory animals have demonstrated that the liver is the primary target organ for microcystin toxicity (see Section 4.2). Mechanistic studies suggest that the target organ specificity is directly related to the limited ability of microcystins to cross cell membranes in the absence of an active transport system, such as the bile acid transporter in hepatocytes. Evidence of the importance of the bile acid transporter to liver toxicity is provided by studies that used bile acids and bile acid transport inhibitors (Runnegar et al., 1981, 1993, 1995b; Runnegar and Falconer, 1982; Eriksson et al., 1990a; Hermansky et al., 1990a,b; Hermansky et al., 1991). These studies demonstrated that the liver toxicity produced by *in vitro* or *in vivo* exposures to microcystins was reduced or eliminated by inhibition of hepatocellular uptake using bile acid transport inhibitors (e.g., antamanide, sulfobromophthalein and rifampicin) and bile salts (i.e., cholate and taurocholate). Additional discussion of the cellular uptake of microcystins is provided in Section 3.2 (Distribution).

Runnegar et al. (1993) demonstrated that i.p. injection of mice with MCYM or MCLR caused inhibition of liver protein phosphatase activity followed by evidence of liver toxicity (i.e., increased liver weight). Kidney protein phosphatase activity was unchanged following the *in vivo* exposure even at lethal doses. *In vitro* exposure of kidney extracts to microcystins did result in a decrease in kidney phosphatase activity, and no difference in sensitivity was observed between liver and kidney phosphatase inhibition. This result suggests that target organ specificity is most likely due to slower intercellular uptake of microcystins in the kidney.

The cell type specificity of microcystins was investigated using isolated rat hepatocytes, rat renal epithelial cells (ATCC 1571) and rat skin fibroblasts (ATCC 1213) (Khan et al., 1995; Wickstrom et al., 1995). The time course of light microscopic and ultrastructural effects was examined following *in vitro* exposure to MCLR (Khan et al., 1995). Effects were noted after 4 minutes in hepatocytes, 1 hour in renal cells and 8 hours in fibroblasts. Similar lesions observed in all cell types included blebbing, loss of cell-cell contact, clumping and rounding, cytoplasmic vacuolization and redistribution of cellular organelles. Effects that were seen only in hepatocytes include loss of microvilli, whirling of rough ER, dense staining and dilated cristae of mitochondria and pinching off of membrane blebs. The nuclear changes typical of apoptosis were seen in renal cells and fibroblasts. Cell type differences may be related to the specific proteins that were overphosphorylated within each cell type. The authors postulated that the lack of apoptotic changes in hepatocytes in this study might be related to the short exposure duration or the failure of their transmission electron microscopic method to examine severely damaged cells that had detached from the coverslips. Wickstrom et al. (1995) evaluated the changes in cytoskeletal morphology after MCLR exposure in these cell types. High concentrations and long incubation times were required for cytoskeletal changes in kidney and skin cells; however, the nature of the changes was similar in all cell types (e.g., actin aggregation). Wickstrom et al. (1995) suggested that microcystins may enter kidney cells and fibroblasts via pinocytosis.

McDermott et al. (1998) treated several cell types with MCLR (primary rat hepatocytes, human fibroblasts, human endothelial cells, human epithelial cells and rat promyelocytes). Hepatocytes underwent membrane blebbing, cell shrinkage, organelle redistribution, chromatin

condensation and, in some cells, DNA fragmentation. Similar changes were observed in the other cell types, but a longer duration of exposure was required.

Matsushima et al. (1990) demonstrated that injection of MCYR into mouse skin epithelial cells and human fibroblasts resulted in morphological changes in cell shape (i.e., spindle shape to round forms). These cells were thought to be resistant to microcystins; however, microcystins YR, LR and RR produced a dose-dependent inhibition of protein phosphatase activity using the partially purified enzyme derived from mouse skin cell cytosol. The authors suggest that absence of a direct effect in these cells is due to lack of penetration through the cell membrane.

Many cell types and established cell lines have been evaluated for potential susceptibility to microcystin uptake and toxicity. Primary isolated hepatocytes have been shown to be the most sensitive to cytoxicity, due to the presence of the organic ion/bile acid transport system (Eriksson et al., 1987, 1990b). Uptake was negligible in human hepatocarcinoma cells (Hep G2), mouse fibroblasts (NIH-3T3), erythrocytes and human neuroblastoma cells (SH-SY5Y). Hepatic endothelial cells have also been shown to be resistant to microcystin toxicity (Solow et al., 1989; Runnegar et al., 1994). Primary cultures of liver cells cease to express these bile acid transport proteins after 2-3 days of being maintained in culture. Therefore, established liver cell lines are not generally useful for evaluating microcystin toxicity (Eriksson et al., 1994; Battle et al., 1997; Heinze et al., 2001).

4.4.7.2. Characterization of Subcellular Effects in the Liver

The liver effects that occur following *in vivo* exposures to microcystins are generally discussed in Section 4.2 (Animal Studies). Many additional mechanistic studies describe liver histopathology, ultrastructural changes and biomarkers of cytotoxicity in either isolated perfused rat liver (Berg et al., 1988; Pace et al., 1991; Runnegar et al., 1995b) or primary isolated hepatocytes (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Runnegar and Falconer, 1986; Berg and Aune, 1987; Runnegar et al., 1987; Falconer and Runnegar, 1987a,b; Thompson et al., 1988; Solow et al., 1989; Mereish et al., 1989; Mereish and Solow, 1990; Eriksson et al., 1990a; Boe et al., 1991; Khan et al., 1995; Runnegar et al., 1995b; Yea et al., 2001; Batista et al., 2003).

Toxicological effects of microcystins in the isolated perfused rat liver were similar to those demonstrated following *in vivo* exposure (Pace et al., 1991). During a 60-minute exposure, MCLR caused liver engorgement and cessation of bile flow. Electron microscopy revealed loss of sinusoidal architecture, dilation of bile canaliculi and the space of Disse and decreased intracellular contact. Mitochondrial swelling, disruption of endoplasmic reticulum and formation of whorls and loss of desmosomal intermediate filaments were also observed. Mitochondrial function was impaired, with inhibition of state 3 respiration and a decrease in the respiratory control index.

Runnegar et al. (1995b) demonstrated a decrease in protein phosphatase activity in perfused rat liver exposed to MCYM. Cessation of bile flow, increased perfusion pressure, decreased protein secretion and decreased glucose secretion were also observed. Histological

changes included hepatocytes swelling, loss of sinusoidal architecture, pyknotic nuclei and extensive necrosis. Exposure to high concentrations of toxin extracts in the isolated perfused liver produced loss of cord architecture due to hepatocyte disassociation, membrane damage and cytolysis and nuclear effects (pyknosis, karyokinesis, karyolysis) (Berg et al., 1988). Ultrastructural effects included swollen mitochondria, vacuoles, necrosis, abnormal nuclei, bile canaliculi lacking microvilli and whorls of rough endoplasmic reticulum.

Studies in primary isolated hepatocytes have described the morphological and histopathological changes induced by microcystins that relate to loss of sinusoidal architecture and cytotoxicity (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Runnegar and Falconer, 1986; Berg and Aune, 1987; Runnegar et al., 1987; Falconer and Runnegar, 1987a,b; Thompson et al., 1988; Solow et al., 1989; Mereish et al., 1989; Mereish and Solow, 1990; Eriksson et al., 1990a; Boe et al., 1991; Runnegar et al., 1995b; Khan et al., 1995; Yea et al., 2001; Batista et al., 2003). Microcystin exposure to hepatocytes in suspension or cultured in a monolayer results in membrane blebbing that becomes more pronounced and localized in one region of the cell surface. Cells are observed to be rounded in appearance and become dissociated from one another. Microfilaments are reorganized as a compact spherical body in the vicinity of the blebbing, while the rest of cell is depleted of filamentous actin. MCLR disrupts hepatocellular morphology within minutes, leading to loss of sinusoidal architecture and hemorrhage. Morphological changes in hepatocytes (i.e., blebbing, rounding) have been shown to occur prior to any effect on cell membrane integrity (measured as LDH leakage or release of radiolabeled adenine nucleotides) or cell viability (generally measured as decreased trypan blue exclusion) (Runnegar et al., 1981; Runnegar and Falconer, 1982; Aune and Berg, 1986; Ding et al., 2000a).

Thompson et al. (1988) described the time course of cellular effects of microcystins (type not specified) on cultured rat hepatocytes. Disintegration of attachment matrix occurred by 15 minutes, followed by cells clustered in groups with no extracellular material at 1 hour, and release of cells from plates between 2 and 4 hours. LDH release did not occur until after these visual effects, but was dose-related.

Similar toxicological effects were observed in isolated human hepatocytes (Yea et al., 2001; Batista et al., 2003). MCLR produced blebbing, fragmentation and hepatocyte disassociation. Cytotoxicity, as measured by LDH leakage, occurred after morphological changes were evident. Yea et al. (2001) indicated that cytotoxicity in human hepatocytes was observed at a concentration (1 μM) that did not affect rat hepatocytes. Batista et al. (2003) also reported a slightly higher susceptibility to microcystin-induced morphological change in human hepatocytes as compared to rat hepatocytes.

The ultrastructural effects observed following microcystin exposure in isolated rat hepatocytes (i.e., condensation of chromatin, segregation of organelles, separated by apoptotic microbodies, decrease in cell volume and increase in cell density) suggest that hepatocyte cell death is apoptosis and not necrosis (Boe et al., 1991). Microcystin exposure in hepatocytes causes cell shrinkage, externalization of membrane phosphatidylserine, DNA fragmentation and chromatin condensation, indicating a rapid apoptosis (Ding et al., 2000b). Apoptosis is discussed in further detail below as a possible mode of action for microcystin liver toxicity.

Several studies have suggested that microcystins may increase the release and decrease the reincorporation of arachadonic acid into cellular membranes (Adams et al., 1985; Naseem et al., 1990, 1991; Nobre et al., 2001).

4.4.7.3. Molecular Target: Inhibition of Type 1 and 2A Protein Phosphatases

The primary molecular target of microcystins has been identified as serine and threonine protein phosphatases PP1/PP2A. Protein phosphatases dephosphorylate proteins while protein kinases phosphorylate them. Together, protein kinases and phosphatases maintain the balance of phosphorylation and dephosphorylation of key proteins involved in cell cycle regulation. Because more than 97% of protein phosphates occur at serine and threonine residues (Gehringer, 2004), the PP1 and PP2A protein phosphatases are particularly important. Inhibition of these enzymes results in the increased phosphorylation of a number of regulatory proteins. Importantly, PP1 is believed to be the major phosphorylase *a* phosphatase in the liver (Runnegar et al., 1993). PP2A, the major soluble serine/threonine phosphatase, regulates several mitogen-activated protein kinases (Gehringer, 2004).

Microcystins bind to these enzymes under both *in vivo* and *in vitro* study conditions, resulting in an inhibition of enzyme activity leading to an increase in protein phosphorylation. Microcystins have been shown to directly inhibit the activity of PP1 and PP2A derived from several different species (i.e., fish, mammals, plants) and cell types (Honkanen et al., 1990; MacKintosh et al., 1990; Matshushima et al., 1990; Yoshizawa et al., 1990; Sim and Mudge, 1993; Xu et al., 2000; Leiers et al., 2000; Becchetti et al., 2002). Microcystins do not alter protein kinase activity, suggesting the balance of phosphorylation and dephosphorylation is related to protein phosphatase inhibition alone. Microcystins have been used as a tool to investigate the importance of serine and threonine phosphorylation to specific cellular functions. The regulatory effects of phosphorylation on sodium channel opening in renal cells (Becchetti et al., 2002), smooth and skeletal muscle contraction (Hayakawa and Kohama, 1995; Knapp et al., 2002) and insulin secretion (Leiers et al., 2000) have been studied.

Runnegar et al. (1993) demonstrated the inhibition of PP1 and PP2A activity in the liver following i.p. injection of MCYM and MCLR. Increased protein phosphorylation preceded the observed increase in liver weight and was correlated with hepatotoxicity. Decreased phosphatase activity was also demonstrated in the isolated perfused liver (Runnegar et al., 1995b).

The relationship between phosphatase inhibition by microcystins and changes in cytoskeletal structure and cell morphology has been reviewed (Eriksson and Golman, 1993). Inhibition of protein phosphatase activity by MCLH and 7-dmMCRR was associated with an increased phosphorylation of cytoskeletal and cytosolic proteins (Eriksson et al., 1990b). Concentrations that produce a marked increase in protein phosphorylation were accompanied by a complete reorganization of microfilament network. The cytoskeletal effects of microcystins are discussed in further detail below. Microcystin LH and 7-dmMCRR were equipotent inhibitors of purified PP1 and PP2A; however, higher concentrations of 7-dmMCRR were required to increase protein phosphorylation. Table 4-10 shows studies with comparative data on inhibition of protein phosphatases (IC_{50}s) by MCLR, MCYR, MCRR and MCLA.

Table 4-10. Studies Comparing Protein Phosphatase Inhibition Activity of Microcystin Congeners

Reference	IC_{50} (nM)			
	MCLR	MCLA	MCYR	MCRR
PP2A Inhibition				
Craig et al., 1996	0.15	0.16		
Nishiwaki-Matsushima et al., 1991	0.28			0.78
Matsushima et al., 1990	7.6		4.5	5.8
PP1 Inhibition				
MacKintosh et al., 1995	0.2		0.2	
Mixture of PPs				
Yoshizawa et al., 1990	1.6		1.4	3.4

The molecular interaction between microcystins and protein phosphatases has been evaluated using immunoprecipitation, autoradiography, reverse phase liquid chromatography, X-ray crystallography, nuclear magnetic resonance (NMR) solution structures, and molecular dynamics simulation (Runnegar et al., 1995b; MacKintosh et al., 1995; Goldberg et al., 1995; Craig et al., 1996; Bagu et al., 1997; Mattila et al., 2000; Mikhailov et al., 2003; Maynes et al., 2004, 2006). Molecular modeling and molecular dynamics simulations have indicated that microcystins bind in a Y-shaped groove containing the catalytic site on the surface of PP1 (Mattila et al., 2000). Studies with PP1 suggest that the C-terminal β12-β13 loop of PP1 (containing residues 268-281) is important for microcystin-protein phosphatase interactions as well as for substrate recognition (Maynes et al., 2004, 2006). Information available to date indicates that the binding process primarily involves the amino acids Glu, Adda, Leu and MDha of microcystins. Figure 4-2 shows a schematic representation of the interactions between microcystin-LR and protein phosphatase 1; these interactions are discussed further below.

Microcystins LR, LA and LL interact with the catalytic subunits of PP1 and PP2A in two phases. The first phase occurs within minutes and consists of rapid inactivation of the phosphatase. The second, slower phase of interaction represents a covalent interaction that takes place within several hours (Craig et al., 1996). The initial binding and inactivation of protein phosphatases appears to result from several non-covalent interactions that are still being elucidated. Mattila et al. (2000) demonstrated an interaction of the Glu amino acid (reported as

IGlu in the publication) carboxyl group of MCLR with a metal ion (Fe, Mn) in the PP1 catalytic site. Glu appears to be an important component because esterification eliminates toxicity (Namikoshi et al., 1993; Rinehart et al., 1994). Herfindal and Selheim (2006), in a review of the mechanisms of microcystin toxicity, indicated that the Adda side chain is involved in a hydrophobic interaction between the Trp 206 and Ile130 residues in the hydrophobic groove of PP1. Mattila et al. (2000) suggested that the long side chain of the Adda residue may contribute to orienting the toxin into the catalytic site. The Adda amino acid residue of microcystins plays an important role in the inhibition of protein phosphatase activity (Nishiwaki-Matsushima et al., 1991; Gulledge et al., 2002, 2003a,b). Isomerization of the diene from 4E,6E to 4E,6Z on the Adda chain (see Figure 2-1) eliminates the toxic activity of microcystins (Harada et al., 1990; Nishiwaki-Matsushima et al., 1991; Stotts et al., 1993). Microcystin analogues containing only Adda and one additional amino acid are capable of substantial inhibition of PP1 and PP2A, while modifications to the Adda structure abolished the inhibition (Gulledge et al., 2003b). Finally, Herfindal and Selheim (2006) indicated that the L-Leu of MCLR participates in a hydrophobic interaction with Tyr 272 of PP1 (on the β12-β13 loop).

The second phase of interaction between microcystins and protein phosphatase consists of covalent bonding (Craig et al., 1996). Immunoprecipitation and autoradiography methods indicate that covalent bonds result from the interaction between the methylene of the MDha residue of microcystins and the thiol of Cys273 located at the C-terminal of PP1. NMR solution structures and X-ray crystallography data on the MCLR/PP1 complex illustrate the covalent linkage at Cys-273 (Goldberg et al., 1995; Bagu et al., 1997). Site-directed mutagenesis replacing Cys273 in PP1 results in a loss of microcystin binding (MacKintosh et al., 1995; Maynes et al., 2004). Based on sequence similarity between PP1 and PP2A, it has been suggested that Cys-266 is the site of equivalent covalent linkage between PP2A and microcystins (Craig et al., 1996).

Microcystin analogues containing a reduced MDha residue are not capable of covalent binding to protein phosphatases. MacKintosh et al. (1995) reported that a reduction of the MDha residue of MCYR by ethanethiol abolished covalent binding to PP1. Likewise, Craig et al. (1996) showed that reduction of the MDha residue of MCLA abolished the covalent binding phase with PP2A. Maynes et al. (2006) confirmed the lack of covalent interaction by determining the crystal structure of dihydroMCLA bound to PP1. Their work showed that the β12-β13 loop of PP1 takes on a different conformation when the covalent bond is absent, and that other interactions (including hydrogen bonding) are responsible for the bond between dihydroMCLA and PP1.

The importance of covalent bonding between microcystins and protein phosphatases to toxicity resulting from the enzyme inhibition is uncertain, as other interactions are apparently responsible for the rapid inactivation of the enzymes (Herfindal and Selheim, 2006). Modifications to either molecule (microcystin or protein phosphatase) to prevent covalent bonding generally decrease, but do not eliminate, the toxic action (Meriluoto et al., 1990; MacKintosh et al., 1995; Hastie et al., 2005).

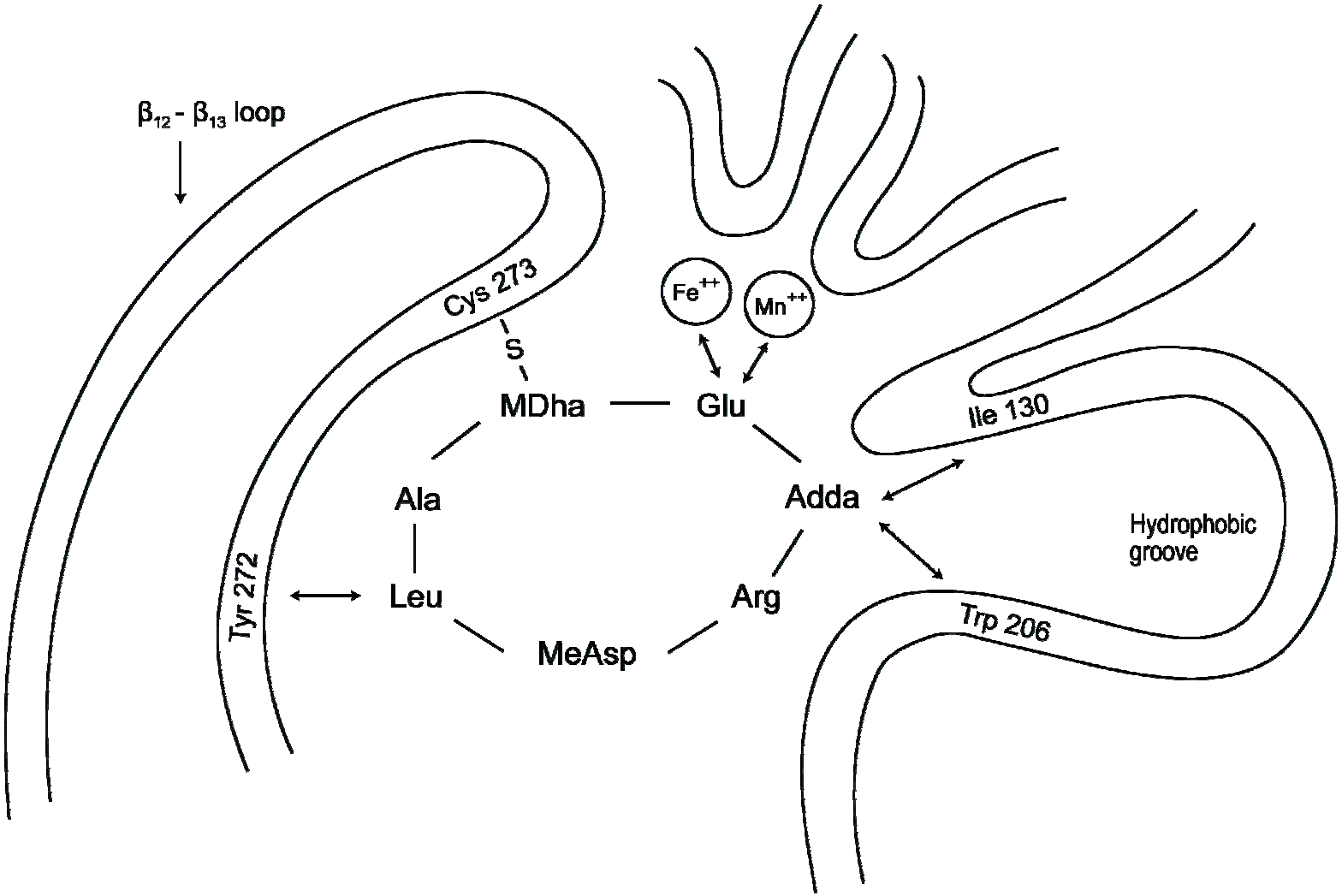


Figure 4-2. Schematic Representation of Interactions between Microcystin-LR and the Catalytic Site of Protein Phosphatase 1

Microcystins may also bind to other molecular targets in addition to protein phosphatases. Chen et al. (2006) used bioinformatic approaches to identify human liver aldehyde dehydrogenase 2 (ALDH2) as a potential molecular target of MCLR. After screening a phage display library to identify potential ligands specific for MCLR, Chen et al. (2006) used molecular docking studies to show that MCLR could bind to ALDH2. The authors postulated that this interaction could lead to aldehyde-induced reactive oxygen species (ROS) and apoptosis.

4.4.7.4. Cytoskeletal Effects

The cytoskeletal effects of microcystins in the liver have been visually demonstrated in several studies using light, electron and fluorescent microscopy (Runnegar and Falconer, 1986; Eriksson et al., 1989; Hooser et al., 1989a,b, 1991b; Falconer and Yeung, 1992). Ultrastructural changes in rats given a lethal dose of microcystin A[1] include the following: a widening of intracellular spaces; progressive disassociation followed by rounding, blebbing and invagination of hepatocytes; loss of microvilli in the space of Disse; breakdown of the endothelium; hemorrhage; and loss of lobular architecture (Hooser et al., 1989b). No effects were noted in endothelial cells or Kupffer cells. In isolated hepatocytes, actin aggregates were seen at the base of the membrane blebs. As membrane blebs grew larger and were drawn toward one pole of the cell, the microfilaments were organized toward the same pole, resulting in rosette formation with a condensed band of microfilaments at the center (Runnegar and Falconer, 1986; Eriksson et al., 1989; Hooser et al.,1991b; Falconer and Yeung, 1992; Wickstrom et al., 1995; Ding et al., 2000a). Frangez et al. (2003) also demonstrated cytoskeletal changes in rabbit primary whole embryo cultured cells. Actin and microtubule disorganization was demonstrated to lead to detachment and cellular toxicity.

The observed reorganization of microfilaments that leads to alteration of hepatocyte morphology was not shown to be due to effects on actin polymerization (Runnegar and Falconer, 1986; Eriksson et al., 1989; Falconer and Yeung, 1992). Instead, microcystins cause an increase in the phosphorylation of cytokeratin intermediate filament proteins (Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995; Blankson et al., 2000). Toivola et al. (1997) evaluated the effects of MCLR on hepatic keratin intermediate filaments in primary hepatocytes cultures. A disruption of the desmoplakin[2] organization at the cell surface (disorganization of desmosomes) is followed by a dramatic reorganization of the intermediate filament and microfilament networks, resulting in intermediate filaments being organized around a condensed actin core. The major target proteins for microcystin-induced hyperphosphorylation include keratins 8 and 18 and desmoplakin (DP) I/II. Keratins 8 and 18 are the major proteins of intermediate filaments in hepatocytes; DP I and II attach keratin filaments in epithelial cells to desmosomes. Hyperphosphorylation of DPI/II leads to loosening of cell junction and loss of interactions with cytoplasmic intermediate filaments. The hyperphosphorylation of keratin proteins leads to increased solubility (caused by disassembly or prevention of subunit polymerization), leading to the observed morphological effects. Phosphopeptide mapping shows

[1] The authors refer to the test compound as microcystin-A, which may reflect an old nomenclature no longer in use. Available information is insufficient to identify the congener with current nomenclature.

[2] Desmoplakin is the principal plaque protein in a desmosome, which is a localized thickening of membrane that serves as an adhesion junction connecting contiguous cells.

four specific tryptic peptides in soluble keratin 18 that are highly phosphorylated; however, no specific phosphorylation sites have been identified for keratin 8. A Ca^{2+}/calmodulin-dependent kinase may be involved in regulating the serine-specific phosphorylation of keratin proteins 8 and 18.

Some investigators have suggested that generation of reactive oxygen species may play a role in the cytoskeletal changes induced by microcystins. Ding et al. (2001) illustrated generation of superoxide and hydrogen peroxide radicals preceding microfilament disorganization and cytotoxicity. Hepatocellular glutathione levels were affected by microcystins, and administration of N-acetylcysteine was shown to protect against cytoskeletal alterations (Ding et al., 2000a).

4.4.7.5. Apoptosis

The ultrastructural changes observed in hepatocytes after microcystin exposure suggest that cell death is related to apoptosis and not necrosis. These changes include cell shrinkage (decreased volume and increased density), condensation of chromatin and segregation of organelles separated by apoptotic microbodies (Boe et al., 1991; Fladmark et al., 1998; McDermott et al., 1998; Ding et al., 2000b; Mankiewicz et al., 2001). The effects of microcystins on the signaling pathways involved in rapid apoptosis have been investigated in several studies (Ding et al., 1998a,b, 2000b, 2001, 2002; Ding and Ong, 2003). Mitochondrial permeability transition (MPT) is considered to be a critical rate-limiting event in apoptosis. Oxidative stress may play a role in the induction of MPT and the onset of apoptosis. In cultured hepatocytes exposed to microcystins, an increase in the generation of ROS preceded the onset of MPT, mitochondrial depolarization and apoptosis. A dose- and time-dependent increase in ROS and lipid peroxidation, measured as malondialdehyde formation, was shown to precede morphological changes in hepatocytes and release of LDH. The addition of deferoxamine or cyclosporine A inhibited the formation of ROS and delayed the onset of MPT and cell death. The addition of superoxide dismutase prevented collapse of cytoskeleton and release of LDH from isolated hepatocytes. An early surge of mitochondrial Ca^{2+} was shown to occur prior to MPT and cell death. Prevention of this Ca^{2+} surge by one of several methods (i.e., chelation of intracellular Ca^{2+}, blockage of the mitochondrial Ca^{2+} uniporter or use of mitochondrial uncoupler) prevented MPT and cell death. Electron transport chain inhibitors (e.g., rotenone, actinomycin A, oligomycin or carbonyl cyanide m-chlorophenylhydrazone) also inhibited the onset of MPT. MCLR caused the release of cytochrome c through MPT, which is considered universal in mitochondrial apoptosis; however, caspases -9 and -3 were not activated. The increase in intracellular Ca^{2+} may instead facilitate the activation of calpain, which occurred following exposure to microcystins (Ding and Ong, 2003). Botha et al. (2004) demonstrated that apoptosis and oxidative stress can be induced in nonhepatic cells by microcystins. LDH leakage and increased apoptotic indices were observed in the human colon carcinoma cell line (CaCo2) and MCF-7 cells (deficient in pro-caspase-3). These changes were accompanied by increased H_2O_2 formation and increased calpain activity.

Western blot analysis has been used to show that MCLR increases the expression of p53 and the pro-apoptotic Bax protein in both cultured rat hepatocytes treated with MCLR *in vitro* and rat liver after *in vivo* exposure (Fu et al., 2005). Expression of the anti-apoptotic protein

Bcl-2 was decreased *in vitro*, but *in vivo* MCLR treatment did not lead to a difference in the expression of this protein. This finding suggests that MCLR may induce apoptosis through other mechanisms in addition to the inhibition of protein phosphatases.

4.4.7.6. Lipid Peroxidation

Several studies have investigated the role of glutathione homeostasis and lipid peroxidation in microcystin-induced liver toxicity (Runnegar et al., 1987; Eriksson et al., 1989; Bhattacharya et al., 1996; Ding et al., 2000a; Towner et al., 2002; Gehringer et al., 2003a,b, 2004; Bouaïcha and Maatouk, 2004). Ding et al. (2000a) indicated that microcystin exposure in isolated hepatocytes resulted in an initial increase in glutathione synthesis followed by a later depletion of glutathione. MCLR was shown to induce the *de novo* synthesis of glutathione in mice exposed to a toxic sublethal dose (75% of the LD_{50}) (Gehringer et al., 2004). Increased transcription of glutathione-S-transferase was also demonstrated in his study. Gehringer et al. (2004) suggest that increased lipid peroxidation induced by microcystins is accompanied by an increase in glutathione peroxidase, transcriptional regulation of glutathione-S-transferase and glutathione peroxidase and *de novo* synthesis of glutathione. Bouaïcha and Maatouk (2004) also reported that a low noncytotoxic concentration (2 ng/mL) of MCLR in primary rat hepatocytes caused an initial increase in ROS formation and an increase in glutathione; however, a decrease in lipid peroxidation was observed in this study. Electron spin resonance (ESR) spin trapping techniques have demonstrated the formation of two possible lipid-derived free radical metabolites in rat liver following *in vivo* exposure to MCLR (Towner et al., 2002). Vitamin E and selenium supplementation in mice provided some protection against liver toxicity and lethality by MCLR (Gehringer et al., 2003a,b). Measures of liver toxicity included serum enzyme determination, lipid peroxidation, glutathione levels and histopathology. Hermansky et al. (1991) reported that membrane active antioxidants (i.e., vitamin E, silymarin and glutathione) provided some protection from microcystin toxicity (i.e., LDH leakage) and lethality; however, free radical scavengers and water soluble antioxidants were ineffective (see below).

Several studies have reported MCLR-induced increases in lipid peroxidation as well as decreases in antioxidant enzymes (Moreno et al., 2005; Jayaraj et al., 2006). Jayaraj et al. (2006) measured oxidative stress in mice treated i.p. with an LD_{50} dose of MCLR. Significant increases in heat shock protein-70 and hepatic lipid peroxidation were observed. Further, GSH was depleted, and there were decreases in the activity of glutathione peroxidase, superoxide dismutase, catalase, glutathione reductase and glutathione-S-transferase in the animals treated at the LD_{50}. Similarly, Moreno et al. (2005) reported significant reductions in glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase, along with increases in lipid peroxidation, in both the liver and kidney of rats treated intraperitoneally with single doses of MCLR.

Some studies report the absence of lipid peroxidation during microcystin-induced hepatotoxicity. A time-dependent leakage of LDH, ALT and AST was observed in liver slices with no change observed for glutathione content or lipid peroxidation (Bhattacharya et al., 1996). In addition, Runnegar et al. (1987) indicated that glutathione depletion did not occur until after morphological changes (i.e., blebbing) were observed. Eriksson et al. (1989) indicated that rapid

deformation of isolated rat hepatocytes by MCLR was not associated with alterations in glutathione homeostasis.

4.4.7.7. Prevention of Liver Toxicity and Lethality

Several types of agents have been evaluated as potential chemoprotectants against microcystin toxicity, including inhibitors of bile acid transport, microsomal enzyme inducers, calcium channel blockers, free radical scavengers, water-soluble antioxidants and membrane active antioxidants. It was initially reported that preincubation of hepatocytes with chemicals that interfere with uptake of bile acids (sulfobromophthalein, rifampicin, sodium cholate and sodium deoxycholate) also prevents hepatocyte deformation (Runnegar et al., 1981; Runnegar and Falconer, 1982).

Hermansky et al. (1991) evaluated several possible chemoprotectants by measuring LDH leakage and lethality following i.p. injection of MCLR. No protective effect was observed using calcium channel blockers, free-radical scavengers or water-soluble antioxidants administered prior to MCLR. Membrane active antioxidants, such as vitamin E, silymarin and glutathione, provided some protection from microcystin toxicity and lethality. Phenobarbital provided partial protection; however, tetrachlorodibenzo-*p*-dioxin did not afford protection, suggesting that the protective effect may not be related to microsomal enzyme induction. Rifampicin and cyclosporine A provided complete protection by blocking uptake of microcystins at the bile acid transporter. Hermansky et al. (1990a,b) reported that rifampicin can be given 15 minutes after MCLR injection and still prevent lethality, while cyclosporine A prevents lethality only if given 0.5-3 hours before MCLR injection.

Silymarin and dithioerythritol, both antioxidants, were shown to reduce MCLR toxicity, as measured by LDH and adenine nucleotide release and light microscopy in primary cultures of adult rat hepatocytes (Mereish and Solow, 1990). Dithioerythritol and silymarin have both been shown to increase the content of reduced thiols (i.e., glutathione). Silymarin has additionally been shown to stabilize membranes, inhibit lipoxygenase, reduce leukotrienes, scavenge free radicals and increase protein synthesis.

Mereish et al. (1991) indicated that silymarin pretreatment inhibited microcystin liver toxicity in mice (evidenced by histopathology and serum enzyme levels) following i.p. injection, but not oral administration. As discussed above, vitamin E and selenium supplementation in mice provided some protection against liver toxicity and lethality by MCLR (Gehringer et al., 2003a,b).

Thompson and Pace (1992) evaluated several types of agents for their ability to protect against MCLR toxicity in cultured hepatocytes. Toxicity was measured as morphology under light microscopy, LDH release and protein synthesis inhibition. The uptake of MCLR into hepatocytes was also measured. Cytochalasins D&E, fungal metabolites that interfere with actin polymerization into microfilaments, were shown to protect against LDH release and provided moderate protection from rounding and clustering of cells; however, these compounds produced cytotoxicity themselves at concentrations that were required for protection against microcystin toxicity. Cholate and deoxycholate are competitive inhibitors for the bile acid transporter.

These compounds provided some protection against LDH leakage, but were also cytotoxic. Trypan blue and trypan red also provided some protection related to blocking microcystin uptake into hepatocytes. The antibiotic rifampicin was shown to prevent microcystin uptake and toxicity at low non-cytotoxic concentrations, suggesting a possible therapeutic use in microcystin poisoning.

Rao et al. (2004) confirmed that pretreatment with cyclosporine-A, rifampin and silymarin each provided 100% protection against a lethal dose of MCLR (although the route was not specified, it is assumed to be i.p., based on the lethal dose). Protected animals had significantly reduced glutathione and increased hepatic lipid peroxidation up to 7 days after treatment, but levels were returned to normal by 14 days.

Adams et al. (1985) demonstrated that MCLR lethality following i.p. injection in mice was reduced by pretreatment with a single subcutaneous injection of carbon tetrachloride. Lethality was also reduced in young mice in this study (no deaths at 1 and 2 weeks of age, 23/31 deaths at 3 weeks of age), suggesting that normal hepatic function is necessary for the uptake and hepatotoxicity of MCLR. The microsomal enzyme inhibitors SKF525A and cobalt chloride produced no effect on MCLR lethality, indicating that microsomal metabolism is not critical for MCLR toxicity. The administration of hydrocortisone was also shown to protect against MCLR lethality in mice, possibly due to a decrease in the release of arachadonic acid from membrane phospholipids. Naseem et al. (1990) demonstrated that pretreatment of cultured rat hepatocytes with glucocorticoids (flucinolone, dexamethasone and hydrocortisone) reduced the release of arachadonic acid and metabolites caused by MCLR.

β-Carotene and lutein inhibited the effect of MCLR on hepatocyte morphology in mouse primary hepatocyte cultures (Matsushima-Nishiwaki et al., 1995). β-Carotene protected the cytokeratin network from disassembly and suppressed the hyperphosphorylation of cytokeratins 8 and 18. Several carotenoid analogs were evaluated, and the protective effect appeared to be related to the number of *trans* configured double-bonds in the carotenoid.

4.4.7.8. Extra-Hepatic Effects of Microcystins

An isolated perfused kidney model was used to evaluate the kidney toxicity of MCLR (Nobre et al., 1999, 2001). MCLR produced vascular, glomerular and tubular effects in the exposed kidney. An increase in perfusion pressure was followed by an increase in the glomerular filtration rate (GFR), increased urinary flow rate and a reduction in tubular transport at the proximal tubules. Histopathological changes included protein in the urinary spaces, but were not further described. Dexamethazone and indomethacin were shown to antagonize the effects of MCLR on perfusion pressure, renal vascular resistance (RVR), GFR and urinary flow. These results suggest a role for phospholipase A2 and cyclooxygenase in the kidney toxicity of microcystins. Nobre et al. (2003) utilized rat peritoneal macrophages exposed to MCLR to further investigate the role of inflammatory mediators in the isolated perfused kidney model. Macrophage supernatants from exposed rats caused an increase in RVR, GFR and urinary flow and reduced Na^+ transport. These effects were reduced by cyclohexamide, dexamethasone and quinacrine, further suggesting the involvement of PLA_2 and other inflammatory mediators in microcystin-induced kidney toxicity.

Moreno et al. (2003) investigated the effects of MCLR on intestinal physiology following an i.p. injection of 100 μg/kg in rats. Lipid peroxidation was increased in both the serum and the intestinal mucosa of treated rats. With the exception of sucrase, intestinal brush border enzymes were unaffected by MCLR exposure. An increase in the specific activity of acid phosphatase and succinate dehydrogenase in intestinal homogenates suggests an effect of MCLR on lysosomal and mitochondrial membranes, respectively. Nobre et al. (2004) used perfused rat ileal segments and ligated intestinal loops to evaluate the effect of MCLR on electrolyte and water secretion. MCLR caused significant secretion of water, sodium, potassium and chloride. Aziz (1974) observed that a dialyzable component of whole cell lysate from *M. aeruginosa* caused fluid accumulation in the ligated small intestine of guinea pigs.

Sicińska et al. (2006) evaluated the effects of MCLR on human erythrocytes *in vitro.* MCLR exposure resulted in the formation of echinocytes, hemolysis, conversion of oxyhemoglobin to methemoglobin, and a decrease in membrane fluidity. In addition, measures of oxidative stress were affected in treated erythrocytes; glutathione reductase and superoxide dismutase activity were decreased, while ROS and lipid peroxidation were increased.

Several studies have evaluated the effects of MCLR on immune system components *in vitro* (Lankoff et al., 2004; Teneva et al., 2005; Chen et al., 2005; Kujbida et al., 2006). Lankoff et al. (2004) reported that MCLR inhibited B-cell proliferation in human and chicken peripheral blood lymphocytes at all concentrations tested, and decreased T-cell proliferation only at the highest concentration. Apoptosis was enhanced in both human and chicken lymphocytes (Lankoff et al., 2004). Similarly, MCLR was cytotoxic to mouse splenocytes, and caused apoptosis in B-cells but not in T-cells (Teneva et al., 2005).

Kujbida et al. (2006) assessed the effects of MCLR and [Asp3]-MCLR on human polymorphonuclear lymphocytes (PMNs) *in vitro*. Both compounds caused migration of neutrophils in a chemotaxis chamber, suggesting that PMNs may migrate from the blood stream to organs that concentrate microcystins, such as the liver. In addition, both caused a dose-related increase in ROS production as measured by chemiluminescence of PMN degranulation products that accompany ROS production. The phagocytosis of *Candida albicans* by PMNs was increased after exposure to either compound, but only MCLR increased the intracellular killing of *C. albicans*. These findings suggest the possibility that PMNs may mediate some of the toxic effects of microcystins.

4.4.8. Genotoxicity and Cell Proliferation

Available data give conflicting results when purified MCLR has been tested for mutagenicity. Pure MCLR did not induce mutations in the Ames assay either with or without metabolic activation, although microcystin-containing extracts did induce mutations (Ding et al., 1999). A crude toxin extracted from *M. aeruginosa* did not induce mutations in the Ames assay (Grabow et al., 1982). In contrast, Suzuki et al. (1998) observed increased ouabain resistance mutation frequency in human embryo fibroblast cells treated with MCLR (purity not specified). Similarly, Zhan et al. (2004) reported a 5-fold increase over control in the frequency of thymidine kinase mutations when human lymphoblastoid TK6 cells were treated with

commercially-obtained MCLR. More slow-growing mutants were observed than fast-growing mutants, suggesting that the mutation damage was larger than the TK locus, and that MCLR induced large deletions, recombinations or rearrangements. Repavich et al. (1990) reported that Ames assays (using strains TA98, TA100 and TA102) of a purified hepatotoxin (supplied by Wright State University and presumed to be microcystin) were negative, as were *Bacillus subtilis* multigene sporulation assays.

The conflicting information on mutagenicity may be related to differences in the cell uptake of MCLR. For example, the failure of MCLR to induce mutations in bacterial cells may be related to poor uptake. Zhan et al. (2004) reported that MCLR is not taken up by many cell types, including bacteria; however, the authors did not provide references to support this assertion. While hepatocytes take up MCLR at a significant rate, other cell types show limited or no uptake unless measures are taken to enhance the penetration of the cells by MCLR. The cellular uptake of microcystins is discussed in detail in Sections 3.2 (Distribution) and 4.4.7 (Mechanistic Studies).

A number of studies have reported DNA damage after MCLR treatment *in vivo* (Rao and Bhattacharya, 1996), and in primary rat hepatocytes (Ding et al., 1999) and human hepatoma cells (Zegura et al., 2003, 2004). Recent studies suggest that apoptosis may be intimately linked to observations of DNA damage in cells treated with MCLR. Lankoff et al. (2004) showed a strong correlation between DNA damage, as measured by the comet assay, and the induction of apoptosis, as measured by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, in human lymphocytes. Other evidence has suggested that the comet assay can give a false positive measure of DNA damage when apoptosis is induced, as DNA fragmentation is one consequence of apoptosis induction (Lankoff et al., 2004). The authors postulated that earlier reports of DNA damage measured by the comet assay may have been related to early stages of apoptosis due to cytotoxicity rather than a direct effect on DNA. The induction of apoptosis appears to be dose-related. Humpage and Falconer (1999) showed that low (picomolar) concentrations of commercially-obtained MCLR induced cytokinesis and inhibited apoptosis in primary mouse hepatocytes, while higher (nanomolar) concentrations resulted in the inverse effects.

Bouaïcha et al. (2005) reported that noncytotoxic concentrations of MCLR did not cause the formation of hydrophobic DNA adducts in primary cultured rat hepatocytes treated *in vitro*, but did decrease the amount of endogenous hydrophobic adducts. MCLR was also shown to cause a dose- and time-dependent increase in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (a measure of oxidative DNA damage) both in cultured hepatocytes and in rat liver cells after *in vivo* treatment via i.p. injection (Maatouk et al., 2004; Bouaïcha et al., 2005).

Conflicting results have been reported in studies of MCLR-induced clastogenicity. MCLR (commercially-obtained) has induced micronuclei in human lymphoblastoid cells and mouse bone marrow erythrocytes (Ding et al., 1999; Zhan et al., 2004). Lankoff et al. (2004) observed no effect of MCLR on the incidence of chromosomal aberrations in human peripheral blood lymphocytes. Observations of polyploidy in MCLR-treated cells (Humpage and Falconer, 1999; Lankoff et al., 2003) may be related to its effects on cytokinesis. Lankoff et al. (2003) showed that MCLR, through its effect on microtubules, damages the mitotic spindle, leading to

the formation of polyploid cells. Repavich et al. (1990) reported a dose-related increase in chromosome breakage in human lymphocytes exposed to a purified hepatotoxin (presumed to be a microcystin).

Mechanistic evidence provides support for the hypothesis that MCLR can act as a promoter at low doses. Zhu et al. (2005) reported that MCLR can transform immortalized colorectal crypt cells, resulting in anchorage-independent growth and enhanced proliferation. MCLR has been shown to increase the expression of the bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002). Further, MCLR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003). Gehringer (2004) reviewed the molecular mechanisms leading to promotion by MCLR and the related tumor promoter, okadaic acid. Gehringer (2004) reported that MCLR inhibits protein phosphatase PP2A, which regulates several mitogen-activated protein kinases (MAPK). The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. In addition, activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. Finally, Gehringer (2004) noted that MCLR has been reported to increase phosphorylation of p53, which is involved in the regulation of the cell cycle and apoptosis as well as the carcinogenic process.

4.4.9. Structure-Activity Relationships

With a few exceptions, microcystin congeners exhibit i.p. LD_{50} values between 50 and 300 μg/kg in mice (Rinehart et al., 1994; WHO, 1999). MCLR is one of the most potent congeners (i.p. LD_{50} approximately 50 μg/kg). Limited comparative testing of *in vitro* protein phosphatase inhibition (IC_{50}) of MCLR, -RR and -YR resulted in IC_{50} values of 1.6, 3.4 and 1.4 nM, respectively (Yoshizawa et al., 1990), indicating that microcystin congeners may be relatively similar in protein inhibition potency. Pharmacokinetic differences among the various microcystin congeners may be at least partially responsible for observed variations in lethal potency (Ito et al., 2002). Microcystin congeners of varying hydrophobicity were shown to interact differently with lipid monolayers (Vesterkvist and Meriluoto, 2003). Effects on membrane fluidity could alter the cellular uptake of these toxins.

Wolf and Frank (2002) proposed toxicity equivalency factors (TEFs) for the four major microcystin congeners based on LD_{50} values obtained after i.p. administration. The proposed TEFs, using MCLR as the index compound (TEF=1.0) were 1.0 for MCLA and MCYR and 0.1 for MCRR. The application of TEFs based on i.p. LD_{50} values to assessment of risk from oral or dermal exposure is questionable given that differences in liphophilicity and polarity of the congeners may lead to variable absorption by non-injection routes of exposure.

The molecular interaction between microcystins and the catalytic subunits of protein phosphatases has been extensively studied (see Section 4.4.7.3 for more detail). The interaction was shown to occur in two phases. The first phase occurs within minutes and results in rapid inactivation of the phosphatase (Craig et al., 1996). The amino acids Glu and Adda appear to be important for the rapid inactivation of the protein phosphatases and for subsequent toxicity (Harada et al., 1990; Nishiwaki-Matsushima et al., 1991; Namikoshi et al., 1993; Rinehart et al., 1994; Gulledge et al., 2002, 2003a,b). The carboxyl group of the Glu residue in MCLR

apparently interacts with a metal (Fe, Mn) ion in the PP1 catalytic site (Mattila et al., 2000). The Adda side chain is involved in a hydrophobic interaction between the Trp 206 and Ile130 residues in the hydrophobic groove of PP1 (Herfindal and Selheim, 2006). The few apparently non-toxic (i.p. LD_{50} values >1000 μg/kg) microcystin congeners exhibit structural alterations in the Adda or Glu regions (Harada et al., 1990; Stotts et al., 1993; Rinehart et al., 1994).

The second, slower phase of interaction represents a covalent interaction between the methylene of the MDha residue of microcystins and the thiol of Cys273 located at the C-terminal of PP1 that takes place over several hours (Craig et al., 1996). Microcystin analogues containing a reduced MDha residue are not capable of covalent binding to protein phosphatases (MacKintosh et al., 1995; Craig et al., 1996; Maynes et al., 2006). The importance of covalent bonding to the toxic effect of microcystins is uncertain, as other interactions are apparently responsible for the rapid inactivation of the enzymes (Herfindal and Selheim, 2006). Modifications to either molecule (microcystin or protein phosphatase) to prevent covalent bonding generally decrease, but do not eliminate the toxic action (Meriluoto et al., 1990; MacKintosh et al., 1995; Hastie et al., 2005; Herfindal and Selheim, 2006).

4.5. MODE OF ACTION – NONCANCER AND CANCER

Microcystins appear to result in different cellular effects depending on dose. In a review of the mechanistic data on microcystins, Gehringer (2004) postulated a dualistic response for microcystins and okadaic acid (another potent inhibitor of PP1 and PP2A). Gehringer (2004) outlined evidence suggesting that at high doses, microcystins cause alterations in cellular structure and function that may lead to cell death via apoptosis or necrosis, while at low doses, microcystins inhibit apoptosis and cause cell proliferation. The high-dose effects are likely to be responsible for the acute toxicity and lethality of microcystins and are discussed below.

4.5.1. Target Organ Specificity

The liver is the primary site of toxicological action for microcystins after oral, i.v., i.p. and intranasal instillation exposure. Acute and short-term exposures to microcystins have resulted in intrahepatic hemorrhage in both rats and mice (Ito et al., 1997a; Fawell et al., 1999; Heinze, 1999). One subchronic study in mice also showed liver effects (Fawell et al., 1999), including hepatocyte degeneration, chronic inflammation and hemosiderin deposits, but no hemorrhage. Liver hemorrhage, resulting from apoptosis and necrosis of hepatocytes leading to disintegration of hepatic architecture, appears to be the most prominent effect observed in available toxicological studies,. The main reason for this target organ specificity is the greater cellular uptake of microcystins by hepatocytes compared with other cells. Microcystins are actively transported into hepatocytes by the bile acid transporter system, while uptake by other cell types is limited by the lack of an active transport system. *In vitro* studies demonstrate that preincubation of hepatocytes with compounds that block the uptake of bile acids prevent damage to the hepatocyte from microcystin exposure (Runnegar et al., 1981, 1993, 1995a; Runnegar and Falconer, 1982; Eriksson et al., 1990a; Hermansky et al., 1990a,b, 1991). Treatment of various cell types with microcystins results in rapid damage to hepatocytes, while changes in other cell types occur after much longer exposure (Khan et al., 1995; Wickstrom et al., 1995; McDermott et al., 1998).

4.5.2. Key Events in the Mode of Action for Liver Toxicity and Hemorrhage

Available mechanistic data indicate that the hepatotoxic effects of microcystins begin with one or two molecular effects: inhibition of protein phosphatases and induction of oxidative stress. At present, it is not clear whether the two effects are linked, whether they lead independently to similar cellular effects (e.g., cytoskeletal damage and apoptosis), or whether one effect is the predominant cause of hepatocellular damage. In both cases, the initial cellular effects are associated with cytoskeletal changes and the induction of apoptosis. These alterations in the structural integrity and function of hepatocytes lead to profound hepatotoxicity and hemorrhage. In summary, the key events in the hepatotoxicity of microcystins appear to be:

1. Molecular events (inhibition of protein phosphatase and/or induction of oxidative stress);
2. Cellular effects (cytoskeletal damage and/or apoptosis); and
3. Tissue damage (altered liver structure and function, and intrahepatic hemorrhage).

The molecular events and cellular effects leading to tissue damage are discussed further below.

4.5.2.1. Molecular Events

Protein Phosphatase Inhibition. As discussed above in Section 4.4.7.3, microcystins are potent inhibitors of serine and threonine PP1 and PP2A. Inhibition of these protein phosphatases results in aberrant phosphorylation of a number of cellular proteins, with the potential for multiple effects on the cell. Current data suggest that the inhibition of PP1/PP2A by microcystins can trigger cytoskeletal damage and apoptosis.

One outcome of microcystin-induced inhibition of PP1 and PP2A is the hyperphosphorylation of cytokeratin intermediate filament proteins (Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995; Blankson et al., 2000). Specifically, microcystin exposure results in hyperphosphorylation of keratins 8 and 18 and desmoplakin I/II (Toivola et al., 1997). It has been suggested that protein kinases PKC, PKA or the calcium/calmodulin-dependent kinase may play a role in the hyperphosphorylation of these proteins (Gehringer, 2004). The hyperphosphorylation of desmoplakin I/II results in the loosening of cell junctions and loss of interactions with cytoplasmic intermediate filaments, while the hyperphosphorylation of keratins 8 and 18 leads to increased solubility. Some of the morphological changes observed in hepatocytes (e.g., blebbing, rounding) may result from the hyperphosphorylation of cytokeratin intermediate filament proteins.

Guzman et al. (2003) reported an increase in the phosphorylation of p53 in rat livers after i.p. exposure to MCLR. PP1 and PP2A help to regulate the activity of p53 through dephosphorylation. Thus, inhibition of these enzymes can result in hyperphosphorylation of p53. Increases in the phosphorylation of p53 can cause an increase in the transcription of $p21^{WAF1}$, which in turn inhibits cyclin D-, E- and A-dependent kinases. The result of the latter inhibition is to stall the cell cycle in G1 phase (Gehringer, 2004). This disruption of cell cycling can allow for repair of DNA damage or for apoptosis to occur. For MCLR, there is evidence of

hyperphosphorylation of p53, but other steps in this cascade of events have not yet been investigated.

Oxidative Stress. A number of studies indicate that oxidative stress may play a role in microcystin-induced hepatotoxicity. As noted above in Section 4.4.7.5, studies of chemoprotectants show that several antioxidants can provide protection against the toxicity of microcystins. Among the antioxidants shown to protect against the effects of microcystin, either *in vitro* or *in vivo*, are vitamin E, silymarin, dithioerythritol, desferoxamine, N-acetylcysteine, superoxide dismutase and glutathione. Further, dose and time-dependent increases in reactive oxygen species have been shown to precede morphological changes in hepatocytes, and the addition of superoxide dismutase prevents the cytoskeletal collapse caused by microcystins. Ding and Ong (2003) have proposed two primary pathways by which microcystins increase oxidative stress leading to cell death. First, microcystins may deplete glutathione, leading to oxidative damage and cell death. Second, microcystins may increase the production of ROS by disrupting the mitochondrial electron transport chain, leading to mitochondrial permeability transition and apoptosis.

Microcystins may enhance oxidative stress by altering glutathione homeostasis; however, the importance of glutathione homeostasis in MCLR-induced hepatotoxicity is not clear. Glutathione serves as an intracellular antioxidant, by scavenging free radicals, by serving as a substrate for the reduction of hydrogen peroxide by glutathione peroxidase and by detoxifying xenobiotics. In addition, depletion of glutathione can disrupt microfilament structures in some cell types (Ding and Ong, 2003). MCLR lethality has been prevented in mice by pretreatment with glutathione (Hermansky et al., 1991), and hepatocytes pretreated with a glutathione precursor were likewise protected from MCLR toxicity. Some studies have shown glutathione depletion after microcystin exposure (Runnegar et al., 1987); however, depletion did not occur until after membrane blebbing had been observed. Other studies have reported an increase in glutathione after MCLR exposure (Ding et al., 2000a; Bouïacha and Maatouk, 2004). Finally, Eriksson et al. (1989) indicated that the rapid deformation of rat hepatocytes after MCLR exposure was not associated with changes in glutathione levels. Thus, the role of glutathione homeostasis in MCLR-induced hepatotoxicity has not yet been determined.

A variety of studies have demonstrated the importance of mitochondrial permeability transition in the apoptotic cascade induced by MCLR (see Ding and Ong, 2003; Gehringer, 2004). In particular, studies have shown that pretreating hepatocytes with cyclosporin A, a specific inhibitor of MPT, prevented cell death from microcystin exposure (Ding and Ong, 2003; Gehringer, 2004). Ding and Ong (2003) outlined the following pathways for MCLR-induced apoptosis via MPT. First, microcystin disrupts the mitochondrial electron transport chain, leading to the release of reactive oxygen species from mitochondria and mitochondrial permeability transition. MPT triggers a release of cytochrome c and mitochondrial calcium. Cytochrome c may activate one or more caspases that trigger apoptosis, although neither caspase-9 nor caspase-3 appear to be involved based on current information (Ding and Ong, 2003; Gehringer, 2004). The release of mitochondrial calcium activates calpain and calcium/calmodulin-dependent protein kinase II, both of which also lead to apoptosis.

4.5.2.2. Cellular Effects

Cytoskeletal Changes. Morphological changes observed in hepatocytes treated *in vitro* with MCLR include membrane blebbing, cell rounding and dissociation. Membrane blebs become localized in one region of the cell, and microfilaments are reorganized as a compact spherical body near the blebbing (Runnegar and Falconer, 1986; Eriksson et al., 1989; Hooser et al., 1991b; Falconer and Yeung, 1992; Wickstrom et al., 1995; Ding et al., 2000a). These morphological changes occur before cell viability or cell membrane integrity is affected. Electron microscopy of isolated perfused rat liver showed that these cellular effects led to loss of sinusoidal architecture, dilation of bile canaliculi and the space of Disse and decreased intercellular contact (Pace et al., 1991). Intrahepatic hemorrhage results from the breakdown of liver structure, and the liver is rapidly engorged with blood.

Apoptosis. Membrane blebbing is also a characteristic of the apoptotic process (Gehringer, 2004). A growing body of evidence indicates that microcystin exposure can trigger apoptosis. Hooser (2000) used several visualization (light and electron microscopy) and analytical techniques (TUNEL and electrophoresis to evaluate DNA laddering) to demonstrate widespread apoptosis in the livers of rats 3 hours after an i.p. dose of MCLR. Characteristic apoptotic changes including cell rounding, shrinkage, disassociation, loss of microvilli and chromatin margination and condensation were observed in a majority of hepatocytes. The author postulated that the rapidity with which the apoptotic process occurred overwhelmed the phagocytic capacity of the liver, such that apoptotic hepatocytes depleted their energy stores and later underwent necrosis. In mice, apoptotic hepatocytes have also been observed, but not to the degree reported in rats. Hooser (2000) postulated that intrahepatic hemorrhage and death occurred so quickly in mice that cellular changes characteristic of apoptosis did not have time to develop.

4.5.3. Conclusion

The mechanisms by which microcystins induce hepatic damage have not been fully elucidated**.** Available evidence suggests roles for both protein phosphatase inhibition and oxidative stress as important molecular events, since chemoprotectant studies show that pretreatment with compounds that inhibit either of these effects can protect against hepatotoxicity in MCLR-treated animals. It is possible, even likely, that microcystin exposure triggers a series of independent or linked events that cause cytoskeletal damage and/or apoptosis, given the numerous cellular functions controlled by PP1 and PP2A, as well as the number of effects triggered by oxidative stress. These cytoskeletal and apoptotic changes apparently lead to the altered hepatic structure/function and intrahepatic hemorrhage observed in animal studies.

4.6. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS

The preponderance of toxicological data on the effects of microcystins is restricted to the MCLR congener. A single, poorly-described study, reported only in a secondary source is available for the LA congener. Data on the YR and RR congeners are limited to i.p. LD_{50} values and measures of relative inhibition of protein phosphatases. As a result, this section largely describes the available information on the toxic effects of MCLR, with limited reference to other congeners.

Anecdotal reports indicate that, in humans, exposure to cyanobacterial blooms (including microcystin-producing genera) can result in neurological, gastrointestinal and dermatological symptoms, such as headache; muscle weakness; eye, ear and throat irritation; nausea; stomach pain; diarrhea; blistering around the mouth; and hay-fever like symptoms (Dillenberg and Dehnel, 1960; Billings, 1981; Turner et al., 1990; Teixeira et al., 1993; el Saadi and Cameron, 1993). Effects were reported in persons exposed via recreational contact (swimming, boating) and drinking water. Turner et al. (1990) also reported pneumonia in army recruits exposed to a cyanobacterial bloom. Symptoms occurring after exposure to cyanobacteria cannot be directly attributed to microcystin toxins (or other endotoxins); some effects may result from exposure to the cyanobacterial cells themselves, or from exposure to multiple toxins in the bloom.

The primary noncancer health effect of exposure to MCLR is liver damage. The liver is targeted largely because hepatocytes are among only a few cell types that actively take up microcystins, which do not readily cross the cell membrane. Severe liver damage (diffuse individual hepatocyte necrosis, cell-plate disruption and apoptosis) occurred in dialysis patients exposed to microcystins[3] in dialysate (Jochimsen et al., 1998; Pouria et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002). At high acute doses in laboratory animals, MCLR caused potentially fatal hemorrhaging within the liver. While the liver is the usual target of microcystin toxicity, there have been some reports of effects in other systems, including hematological, kidney, cardiac, neurological and gastrointestinal effects. It has been suggested that some effects in other organs observed after high doses of MCLR may result from ischemia or hypoxia caused by hepatic hemorrhage. However, some effects outside the liver have been observed in the absence of hemorrhage.

Much of the toxicological data on microcystins are limited to reports of liver effects after single lethal or sublethal doses administered via i.p. injection. These studies indicate that injected doses of 50-200 μg/kg MCLR or MCYR are usually lethal in mice and rats within a few hours (Adams et al., 1988; Hooser et al., 1989a; Hermansky et al., 1990c; Stotts et al., 1993; Gupta et al., 2003; Rao et al., 2005). Miura et al. (1991) showed that the median time to death is greatly increased in fed rats (32 hours) when compared with fasted rats (less than 2 hours). The authors suggest that fasting may increase the sensitivity of animals to the mitochondrial toxicity of microcystins, although this could not be conclusively demonstrated. In the liver, MCLR destroys the cytoskeleton of hepatocytes, leading to hepatocyte disassociation, degeneration,

[3] Exposure was to untreated water containing cyanobacteria. The presence of microcystins was confirmed in patient biopsy samples; however, it is possible that the patients may also have been exposed to other microbial or chemical contaminants.

apoptosis and necrosis (Hermansky et al., 1990c; Hooser et al., 1991b). Hepatic hemorrhage and disintegration of the liver architecture follow quickly (Hooser et al., 1991b). Effects reported to occur outside the liver include pulmonary thrombi derived from necrotic hepatocytes, kidney effects such as dilation of cortical tubules and eosinophilic material in the cortical tubules, and degeneration and necrosis of myocardial cells (Adams et al., 1988; LeClaire et al., 1988; Zhang et al., 2002). As previously stated, some of these effects may occur secondary to hepatic hemorrhage.

Injection studies suggest a very steep dose-response curve for acute liver effects from microcystin exposure. In several studies, mice and rats receiving single i.p. doses of 20-40 μg/kg MCLR showed no clinical toxicity and few or no gross or microscopic effects in the liver or other organs (Hooser et al., 1989a; Lovell et al., 1989a; Hermansky et al., 1990c), while i.p. doses of 50-200 μg/kg are usually lethal within a few hours (Hooser et al., 1989a; Hermansky et al., 1990c; Stotts et al., 1993; Gupta et al., 2003).

4.6.1. Oral

Table 4-11 provides a summary of the noncancer effects from repeated-dose oral studies of MCLR toxicity in laboratory animals. The table includes all of the studies that used purified microcystins as the test substance. As the table indicates, the toxicological database for effects of microcystins after oral exposure is limited.

Liver Effects. One study of human exposure to drinking water before, during and after a bloom of *M. aeruginosa* reported a significant increase in GGT levels during the bloom compared with levels before the bloom (Falconer et al., 1983). The study population consisted of all persons subjected to liver function tests in the area served by the affected drinking water supply; as such, it is not representative of the general population. The liver is the primary target organ when laboratory animals are exposed to high doses of MCLR. Oral exposure to single 500 μg/kg doses of MCLR caused diffuse hemorrhage in the liver of mice and rats; more pronounced liver damage occurred at higher doses (Ito et al., 1997a; Fawell et al., 1999). Young mice (5 weeks old) did not develop signs of hepatotoxicity at 500 μg/kg MCLR, while aged mice (32 weeks old) developed clear signs (Ito et al., 1997a). This difference may result in part from differences in gastrointestinal absorption of microcystins, but cannot be entirely explained by absorption differences, since similar age-dependent effects were reported after i.p. exposure (Adams et al., 1985; Rao et al., 2005).

A single 28-day study of oral exposure to 50 or 150 μg/kg MCLR in drinking water showed increased liver weight, slight to moderate liver lesions with hemorrhages and increased ALP and LDH in rats exposed at 50 μg/kg-day (Heinze, 1999). A subchronic study in mice using a similar dose range identified a LOAEL of 200 μg/kg (Fawell et al., 1999). At this dose, mild liver lesions including chronic inflammation, hemosiderin deposits and single hepatocyte degeneration were observed, as well as increased ALT and AST in male animals. The 40 μg/kg dose was identified as a NOAEL. Mild hepatocyte injury was reported in mice given 80 or 100 gavage doses of 80 μg/kg each over 28 weeks, corresponding to time-weighted average doses of 33-41 μg/kg-day (Ito et al., 1997b). Based on the report, it appears that a limited postmortem

Table 4-11. Summary Noncancer Results in All Animal Studies of Oral Exposure to Purified Microcystin-LR								
Species	Sex	Average Daily Dose (μg/kg-day)	Exposure	NOAEL (μg/kg-day)	LOAEL (μg/kg-day)	Responses	Comments	Reference
Acute Exposure								
Rat	M/F	500, 1580, 5000	Single gavage	ND	5000*	Mortality; diffuse hepatic hemorrhage at lower doses	No untreated controls. Dose-dependent increase in hepatotoxicity	Fawell et al., 1999
Mouse	M/F	500, 1580, 5000	Single gavage	ND	1580*	Mortality; diffuse hepatic hemorrhage at lower dose	No untreated controls. Dose-dependent increase in hepatotoxicity	Fawell et al., 1999
Mouse	F	8000, 10000, 12500	Single gavage	ND	12500*	Mortality (2/2); hypertrophic hepatocytes, fibrosis in centrilobular and midzonal regions at lower doses	No untreated controls. 1-2 animals/dose group.	Yoshida et al., 1997
Mouse	M	0, 500	Single gavage	ND	500 (aged mice only)	Centrilobular hepatic hemorrhage and necrosis; necrosis of intestinal mucosa and duodenal damage	Effects observed in aged (32 week-old) mice; no effects on liver or gastrointestinal tract in young (5 week-old) mice	Ito et al., 1997a
Short-Term Exposure								
Rat	M	0, 50, 150	Drinking water, 28 day	ND	50	Slight to moderate degenerative and necrotic hepatocytes with hemorrhages; increased serum enzymes (ALP and LDH)		Heinze, 1999

1

Table 4-11. cont.

Species	Sex	Average Daily Dose (μg/kg-day)	Exposure	NOAEL (μg/kg-day)	LOAEL (μg/kg-day)	Responses	Comments	Reference
Subchronic Exposure								
Mouse	M/F	0, 40, 200, 1000	Daily gavage, 13 weeks	40	200	Minimal/slight chronic inflammation with hemosiderin deposits and single hepatocyte degeneration; increased serum enzymes (ALT and AST)		Fawell et al., 1999
Chronic Exposure								
Mouse	F	0, 3	Drinking water, 18 months	3	ND	No effects on survival, body weight, hematology, serum biochemistry, organs or histopathology	Minor changes in ALP and cholesterol not considered toxicologically significant by researchers	Ueno et al., 1999
Mouse	Not given	Not available	Gavage, 80 μg/kg, 80-100 times over 28 weeks	ND	ND	Light injuries to hepatocytes in the vicinity of the central vein	Only liver examined; only three control animals; dosing frequency unclear	Ito et al., 1997b
Developmental Toxicity								
Mouse	F	0, 200, 600, 2000	Gavage, GD 6-15	600	2000*	Maternal mortality (7/26) and morbidity (2/26 humanely sacrificed); reduced fetal body weight, delayed skeletal ossification	Authors defined 600 μg/kg-day as NOAEL but did not present data on reproductive or developmental parameters to support identification of LOAEL	Fawell et al., 1999

2 * Frank Effect Level (FEL)

examination was conducted in this study, which was primarily aimed at evaluating carcinogenicity. No liver or other toxicity was reported in female mice given approximately 3 μg/kg-day MCLR in drinking water for 18 months (Ueno et al., 1999).

Neurological Effects. The database contains scattered reports of neurological symptoms after exposure to high doses of MCLR. Dialysis patients exposed to microcystins in dialysate reported symptoms such as visual disturbance, blindness, vertigo, headache and muscle weakness (Jochimsen et al., 1998). Clinical signs in mice and rats orally exposed to lethal doses (about 5000 μg/kg) include hypoactivity and piloerection (Fawell et al., 1999).

Other Organs. Gastrointestinal effects (necrosis, duodenal damage) were observed in aged mice exposed orally to single 500 μg/kg doses of MCLR (Ito et al., 1997a). Kidney effects including eosinophilic materials in the Bowman's spaces were observed in two mice exposed to a lethal dose of 12.5 mg/kg (Yoshida et al., 1997). Female mice exposed subchronically to 1000 μg/kg had slight increases in hemoglobin concentration, erythrocyte count and packed cell volume (Fawell et al., 1999). Milutinovic et al. (2002, 2003) briefly reported that kidney effects are more pronounced than liver effects in rats chronically exposed to i.p. doses of MCLR and MCYR (time weighted average dose, 5 μg/kg for 8 months). Details of the liver examinations were not reported in this study, limiting the usefulness of these data.

Developmental Effects. A single oral study of developmental toxicity in mice reported maternal toxicity, liver effects and deaths in some dams treated at the highest dose of MCLR (2000 μg/kg during GD 6-15), along with reduced fetal body weight and delayed skeletal ossification. No effects on reproductive or developmental parameters were observed in other treatment groups, and 600 μg/kg was identified as a NOAEL for developmental toxicity (Fawell et al., 1999). One study of developmental toxicity after i.p. injection of 32-238 μg/kg MCLR in mice confirmed the lack of developmental or reproductive effects in the absence of maternal toxicity (Chernoff et al., 2002). A study in which an extract of *M. aeruginosa* (estimated to contain about 14 μg/L unspecified toxin) was administered in the drinking water to mice before and during pregnancy revealed small brains in 7 of 73 pups from treated parents and none in untreated controls (Falconer et al., 1988). The litter distribution of the affected pups was not reported by the authors. It is not possible to attribute this effect to microcystin exposure, as the extract may have contained other compounds.

In vitro studies suggest that MCLR can disrupt the cytoskeleton of embryonic cells, causing cell detachment, retarding division or causing cytolysis (Sepulveda et al., 1992; Frangez et al., 2003; Zuzek et al., 2003). MCLR effects on these and other cell types may be limited by the degree of uptake. Frangez et al. (2003) showed that an intact zona pellucida prevented effects in rabbit whole embryo cultures.

4.6.2. Inhalation

Very limited information is available on the toxicity of MCLR via inhalation exposure. The available data indicate that short-term inhalation of a low concentration of MCLR can cause local damage to the epithelial cells of the nasal cavity. A single study of inhalation exposure in

mice revealed dose-dependent damage to the respiratory and olfactory epithelial cells of the nasal cavity (Benson et al., 2005). Exposure occurred over 7 days at 260 μg/m^3 for 30, 60 and 120 minutes per day (authors estimated the deposited doses as 3, 6 and 12 μg/kg). No effects on the liver or other organs were observed.

Several limited lines of evidence suggest that high doses of MCLR via respiratory exposure routes can lead to systemic uptake with subsequent liver effects. Systemic uptake of MCLR by respiratory routes of exposure has been demonstrated in studies of acute, high-dose exposure (Creasia, 1990; Fitzgeorge et al., 1994; Ito et al., 2001). Importantly, the LD_{50} for MCLR given via either intranasal or intratracheal instillation is similar to that of MCLR given via i.p. injection (Fitzgeorge et al., 1994; Ito et al., 2001). As with i.p. and oral exposure, liver hemorrhage is the proximate cause of death in animals lethally dosed via intranasal or intratracheal instillation (Fitzgeorge et al., 1994; Ito et al., 2001). Further evidence of systemic effects comes from a brief abstract describing lethality in mice exposed via inhalation (nose only) to MCLR aerosols. Creasia (1990) reported an LC_{50} for MCLR of 18 mg/m^3 air for 10 minutes (authors estimated the deposited dose as 45 μg/kg), and indicated that histopathological findings in deceased mice were similar to those reported after i.v. dosing. Ito et al. (2001) suggested that MCLR could enter the bloodstream either via local damage to the nasal mucosa leading to exposure of the nasal blood vessels, or through transport to the lung and absorption into alveolar capillaries.

4.7. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION

4.7.1. Summary of Overall Weight of Evidence

Applying the Guidelines for Carcinogen Risk Assessment (U.S. EPA, 2005a), there is *inadequate information to assess carcinogenic potential* of microcystins by the oral, dermal or inhalation routes of exposure. One poorly-described long-term carcinogenicity bioassay found no increase in neoplastic liver nodules after gavage administration in mice (Ito et al., 1997b). The few available epidemiological studies that suggest a positive association between liver or colorectal cancers and microcystins are limited by ecological study design, poor measures of exposure, potential coexposure to other microbial or chemical contaminants and, in some cases, failure to control for known liver and colorectal risk factors. MCLR has been shown to have a promotional effect in two-stage rat liver bioassays using i.p. administration; however, the relevance of this effect to environmental exposures is uncertain. Mechanistic information provides some support for a possible promotional effect of MCLR.

4.7.2. Synthesis of Human, Animal and Other Supporting Evidence

Several human epidemiological studies have reported an association between consumption of drinking water containing cyanobacteria and microcystins and liver or colon cancer in certain areas of China (Yu et al., 1989 and Yu, 1989 as cited in Ueno et al., 1996; Zhou et al., 2002). In all of these studies, the use of a surface drinking water supply was used as a surrogate for exposure to microcystins. Individual exposure to microcystins was not estimated.

Further, it is not clear whether these studies adequately controlled for confounding factors, such as hepatitis infection or aflatoxin exposure.

Ito et al. (1997b) conducted the only study of oral carcinogenicity of a purified microcystin. In this study, chronic gavage doses of MCLR over 28 weeks failed to induce neoplastic nodules of the liver in mice. Limited information from two-stage, medium-term rat liver bioassays where MCLR was administered i.p. suggest that MCLR can act as a promoter, increasing the number and/or size of GST (placental form) positive foci in livers of rats pretreated with an initiating agent (Nishiwaki-Matsushima et al., 1992; Ohta et al., 1994; Sekijima et al., 1999; Hu et al., 2002). In one such study, MCLR alone showed no initiating activity (Ohta et al., 1994). Ito et al. (1997b) observed an increase in the size of neoplastic liver nodules in mice given 100 i.p. injections of MCLR without an initiating agent; however, the numbers of treated and control animals were small.

Studies of cyanobacterial extract also suggest a possible promotional effect. In mice given an extract of *M. aeruginosa* in drinking water, the mean area of aberrant crypt foci of the colon was significantly increased, although the number of foci was not affected (Humpage et al., 2000). Similarly, the total weight of skin tumors was increased in mice given an extract of *Microcystis* in drinking water after topical DMBA pretreatment (Falconer and Buckley, 1989; Falconer, 1991). It is not possible to determine whether the observed effects resulted from exposure to microcystins or to other contaminants in the extracts.

Mechanistic data indicate that at low doses, MCLR may increase cell proliferation. MCLR has been shown to increase the expression of the bcl-2 protein (that inhibits apoptosis) and decrease the expression of the bax protein (that induces apoptosis) (Hu et al., 2002). Further, MCLR upregulates the transcription factors c-fos and c-jun, leading to abnormal proliferation (Zhao and Zhu, 2003). Gehringer (2004) reviewed the molecular mechanisms leading to promotion by MCLR and the related tumor promoter, okadaic acid. Gehringer (2004) reported that MCLR inhibits protein phosphatase PP2A, which regulates several MAPKs. The MAPK cascade regulates transcription of genes required for cell proliferation, including c-jun and c-fos. In addition, activation of the MAPK cascade has been postulated to inhibit apoptosis and thus increase cell proliferation. Finally, Gehringer (2004) noted that MCLR has been reported to increase phosphorylation of p53, which is involved in the regulation of the cell cycle and apoptosis.

Genotoxicity studies of MCLR have given conflicting results, with negative findings in Ames assays (Grabow et al., 1982; Ding et al., 1999) while positive results were observed with human cell lines (Suzuki et al., 1998; Zhan et al., 2004). Evidence for MCLR-induced DNA damage as measured by the comet assay has been called into question by the finding that apoptosis can lead to false positive findings in this assay (Lankoff et al., 2004). There is some evidence for a clastogenic effect of MCLR (Ding et al., 1999; Zhan et al., 2004).

4.8. SUSCEPTIBLE POPULATIONS AND LIFE STAGES

Little information is available on potentially susceptible populations. Studies in laboratory rodents suggest that the acute effects of MCLR may be more pronounced in adult or aged animals than in juvenile animals (Adams et al., 1985; Ito et al., 1997a; Rao et al., 2005). In these studies, young animals showed little or no effect at MCLR doses lethal to adult animals. Age-dependent differences in toxicity were observed after both oral and i.p. exposure, suggesting that differences in gastrointestinal uptake were not entirely responsible for the effect of age. The relevance of these age-related differences to acute toxicity in humans is unclear.

Available information does not suggest any pronounced gender differences in response to microcystins. Studies with algal extracts suggest the possibility that male mice may be more sensitive than female mice to oral exposure to algal extracts (Falconer et al., 1988). However, the relevance of this finding to human microcystin exposure is uncertain given the potential for coexposure to other contaminants in algal extracts.

Because microcystins inhibit the action of protein phosphatases (PP1 and PP2A), coexposure to other compounds that inhibit these enzymes (for example, okadaic acid) may enhance the toxicological effects of microcystins.

5. DOSE RESPONSE ASSESSMENTS

5.1. NARRATIVE DESCRIPTION OF THE EXTENT OF THE DATABASE

The available information on the toxicokinetic behavior of microcystins in humans or animals after oral or inhalation exposure is limited to a single study of the organ distribution of dihydro-MCLR. No other data are available on the absorption, distribution, metabolism or elimination of microcystins via environmentally relevant exposure routes. Acute lethality data show a significant difference in lethal doses via injected and oral routes of exposure, suggesting that the toxicokinetic behavior of microcystins is an important determinant of health effects after oral exposure; thus, the deficiencies in this category of data are significant.

The mode by which microcystins affects its primary target organ, the liver, is remarkably well-studied. There are abundant mechanistic data ranging from target organ specificity down to molecular targets. *In vitro* studies using human hepatocytes show effects similar to those in animal hepatocytes, indicating that the mode of toxicological action is similar. Human hepatocytes appear to be more susceptible to the action of MCLR than rat hepatocytes (Yea et al., 2001; Batista et al., 2003). Further evidence for the relevance of this mode of toxicological action to humans comes from reports of human exposure. Liver histopathology on humans exposed to MCLR via dialysate showed effects similar to those seen in animals, although intrahepatic hemorrhage was not observed (Azevedo et al., 2002).

The toxicological database for microcystins is almost exclusively limited to data on a single congener, MCLR. Data on the other congeners is restricted to *in vitro* studies of protein phosphatase inhibition, i.p. LD_{50} measures and a single, poorly-described toxicological evaluation of MCLA in primates. The database on the oral toxicity of MCLR is adequate to support the derivation of RfD values. Human data on the oral toxicity of MCLR are limited by potential co-exposure to other cyanobacterial toxins and microorganisms. There are three studies of acute oral exposure to MCLR in two laboratory animal species; however, none of these identified a NOAEL, and the minimum dose tested was the same for all three. Further, neither of the two experiments published in Fawell et al. (1999) nor the experiment by Yoshida et al. (1997) used an untreated control group. One animal study evaluated the oral toxicity of MCLR after short-term (<30 days) exposure and and one after subchronic (30-90 days) exposure. The short-term study (Heinze, 1999) used a small number of animals (10/dose) and did not identify a NOAEL, but was otherwise of good study quality. The subchronic study (Fawell et al., 1999) used an adequate number of animals (30/dose) and identified both a NOAEL and LOAEL. Two chronic exposure studies are available, but one of these (Ito et al., 1997b) apparently conducted only a limited examination of the liver for toxicity, and the other (Ueno et al., 1999) used a single dose and did not identify a LOAEL. A single, well-conducted developmental toxicity study in the mouse is available; however, the results are presented only briefly and without any supporting data in the publication (Fawell et al., 1999). The noncancer database is missing a chronic toxicity study in a second species, as well as a multigeneration reproductive toxicity study and neurotoxicity study.

The database on the inhalation toxicity of MCLR is inadequate for the derivation of any RfC. There are no human data on the inhalation of MCLR. There is a single well-reported animal study addressing inhalation exposure to MCLR for 7 days (Benson et al., 2005). This study used only one exposure concentration with daily exposure for 30, 60 or 120 minutes and, as such, is not adequate for short-term RfC derivation.

The available data on carcinogenicity are inadequate for carcinogenicity assessment. There is no well-conducted long-term carcinogenicity bioassay for microcystin. Several studies using an initiation-promotion protocol are available, as are limited mechanistic data suggesting a potential promoting effect of microcystins.

5.2. ORAL REFERENCE DOSE

Data considered in deriving oral reference dose for each exposure duration are summarized in the following exposure-response array (Figure 5-1) as well as in Table 5-1 below. Due to the limited toxicological database for microcystins, both the table and the figure include all studies in the published literature that examined the oral toxicity of purified MCLR in laboratory animals, with one exception. The publication by Ito et al. (1997b) did not provide the dosing frequency or information to estimate an average daily dose; thus, this study is not included in the table or figure.

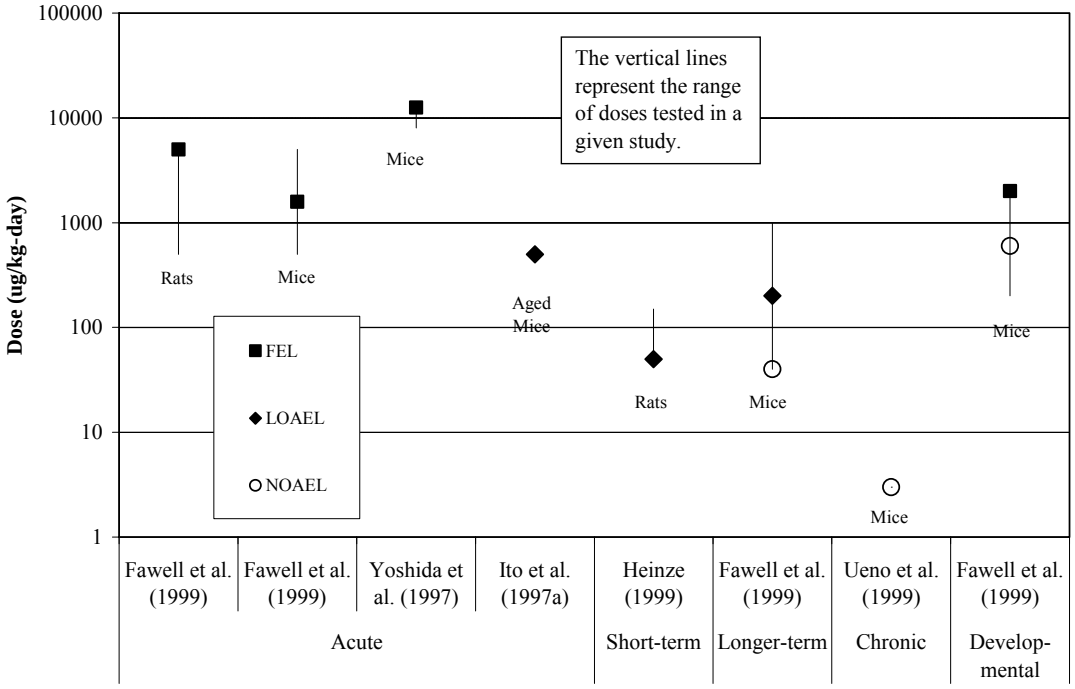


Figure 5-1. Exposure Response Array for Oral Exposure: All Studies of Purified Microcystin-LR

Table 5-1. Available Dose-Response Information for Oral Exposure to Purified MCLR

Species	Sex	Average Daily Dose (μg/kg-day)	Exposure	NOAEL (μg/kg-day)	LOAEL or FEL (μg/kg-day)	Reference
Acute Exposure						
Rat	M/F	500, 1580, 5000	Single gavage	ND	5000*	Fawell et al., 1999
Mouse	M/F	500, 1580, 5000	Single gavage	ND	1580*	Fawell et al., 1999
Mouse	F	8000, 10000, 12500	Single gavage	ND	12500*	Yoshida et al., 1997
Mouse	M	0, 500	Single gavage	ND	500 (aged mice only)	Ito et al., 1997a
Short-Term Exposure						
Rat	M	0, 50, 150	Drinking water, 28 d	ND	50	Heinze, 1999
Subchronic Exposure						
Mouse	M/F	0, 40, 200, 1000	Daily gavage, 13 weeks	40	200	Fawell et al., 1999
Chronic Exposure						
Mouse	F	0, 3	Drinking water, 18 months	3	ND	Ueno et al., 1999
Developmental Toxicity						
Mouse	F	0, 200, 600, 2000	Gavage, GD 6-15	600	2000*	Fawell et al., 1999

* Frank Effect Level (FEL)

5.2.1. Acute Oral RfD

The acute oral data for MCLR are inadequate for the derivation of an acute RfD. There are four studies of acute exposure to MCLR (Yoshida et al., 1997; Ito et al., 1997a; Fawell et al., 1999); however, none of the available studies identified a NOAEL. Yoshida et al. (1997) conducted a LD_{50} determination using a small number of mice (5) treated with a single gavage dose, and no untreated controls. Histopathology of surviving mice (one at 8 mg/kg and two at 10 mg/kg) showed liver lesions (hypertrophic hepatocytes with centrilobular and midzonal fibrosis). The high dose in this study was an FEL based on deaths of the 2 treated animals. Fawell et al.

(1999) administered single gavage doses of 500, 1580 or 5000 μg/kg MCLR to groups of rats and mice (10/dose/species). There were no untreated control groups in this study. In both species, diffuse hepatic hemorrhage was observed at the low dose (500 μg/kg); however, in the absence of untreated controls for comparison, this dose cannot be identified as a LOAEL. Ito et al. (1997a) administered single gavage doses of 500 μg/kg to young (5 weeks old) and aged (32 weeks old) mice. Centrilobular hemorrhage and hepatocyte necrosis, as well as gastrointestinal lesions, were observed in the aged mice, while no effect was observed in the young mice. This study identifies a freestanding LOAEL. The absence of an acute study of adequate quality precludes derivation of an acute oral RfD.

5.2.2. Short-Term Oral RfD

5.2.2.1. Choice of Principal Study and Critical Effect

A single short-term study and a single developmental toxicity study of orally-administered MCLR are available. Heinze (1999) evaluated the effects of MCLR in drinking water in 11-week-old male hybrid rats. Groups of 10 rats (5 of each sex) were given approximate doses of 0, 50 or 150 μg/kg body weight for 28 days. Serum biochemistry showed significantly increased mean levels of ALP and LDH in both treatment groups (84 and 100% increase in LDH, 34 and 33% increase in ALP in low and high doses, respectively). A dose-dependent increase in relative liver weights was observed at both dose levels (17 and 26% at the low and high doses, respectively). Liver lesions were observed in both treatment groups, but the severity of the damage was increased in the 150 μg/kg dose group. Moderate to severe degenerative and necrotic hepatocytes with hemorrhage was observed in 0 of 10 controls, 6 of 10 low-dose and 9 of 10 high-dose rats.

Fawell et al. (1999) evaluated the developmental toxicity of MCLR administered via gavage to mice at doses of 0, 200, 600 and 2000 μg/kg on GDs 6-15. Seven of 26 dams receiving 2000 μg/kg died and two others were sacrificed prematurely due to morbidity. At this dose, fetal body weight was significantly lower than controls and there was delayed skeletal ossification; these effects may have been associated with maternal toxicity. Data on reproductive and developmental parameters were not provided in the reference; thus, a LOAEL for developmental toxicity could not be determined. This study identified a NOAEL of 600 μg/kg-day for both developmental and maternal effects. The high dose of 2000 μg/kg was an FEL based on maternal mortality. The study by Heinze (1999) identified a lower LOAEL and more sensitive effect (hepatotoxicity) than the developmental toxicity study did; thus, this study was chosen as the basis for the short-term RfD.

5.2.2.2. Methods of Analysis

Liver toxicity observed by Heinze (1999) included liver lesions, serum enzyme changes, and changes in relative liver weight. All three of these endpoints were considered for determining the point of departure for RfD derivation.

All quantal models in U.S. EPA's Benchmark Dose (BMD) software (version 1.3.2) were fit to the incidence data for liver lesions in rats (males and females combined) exposed to MCLR in the drinking water for 28 days (Heinze, 1999). The incidence data for liver lesions are reported in Table 4-4 (Section 4.2.1.2.1). Of the liver lesions observed, the category of degenerative and necrotic hepatocytes with hemorrhage showed a strong dose-related trend with greater incidence and severity with higher dose, and no control animals were affected. As the table shows, at 50 μg/kg, 4/10 rats had slight lesions and 6/10 had lesions of moderate severity. At 150 μg/kg, 6/10 rats had moderate lesions and 3/10 had intensive damage. For BMD modeling, the moderate and severe lesion categories were collapsed into one. The data modeled are shown in Table 5-2.

Table 5-2. Incidence of Liver Lesions Used for BMD Modeling (Heinze, 1999)

	0 μg/kg-day	50 μg/kg-day	150 μg/kg-day
Lesion incidence	0/10	6/10	9/10

In accordance with the U.S. EPA (2000c) BMD methodology, the default benchmark response (BMR) of 10% increase in extra risk was used. The high response rate (60%) at the lowest dose with a positive response precludes the use of a lower BMR for this analysis. Models were run using the default restrictions on parameters built into the BMDS. The modeling results are shown in Table 5-3. Adequate fits were achieved with all models, except the quantal quadratic. While the gamma, multistage, quantal linear and Weibull all converged on the same model, the log probit model gave the best fit, as assessed by AIC. Figure 5-2 shows the fit of the log probit model to the data. Appendix A contains the full model outputs. The BMD and BMDL estimated by the log probit model for the liver lesion data are 11.0 and 6.4 μg/kg-day, respectively.

The linear model for continuous data was fit to the increased LDH and ALP levels reported in Heinze (1999). These data are shown in Table 4-3 (Section 4.2.1.2.1). With only three observations in each of these datasets, there were not enough data points to use the remaining models (polynomial, power, or Hill), which each have more than three parameters.[4]

The linear model did not provide adequate fit to either dataset as measured by goodness-of-fit criteria (see Appendix A for model outputs). The linear model was also fit to the relative liver weight changes (also three observations) reported in Heinze (1999). These data are shown in Table 4-3 (Section 4.2.1.2.1). In accordance with the U.S. EPA methodology, the default BMR of one standard deviation change from the control mean was used, and the polynomial coefficients were restricted to be positive. The linear model provided an adequate fit to the data (see Appendix A for model output). The BMD and BMDL estimated by the linear model for the relative liver weight data are 85 and 58 μg/kg-day, respectively.

[4] The number of parameters describing the shape of the dose-response curve cannot exceed the number of dose groups (U.S. EPA, 2000c).

Table 5-3. BMD Modeling Results for Heinze (1999) Liver Lesion Data

Model	Degrees of Freedom	χ^2	χ^2 Goodness of Fit *p*-Value	AIC	BMD (μg/kg-day)	BMDL (μg/kg-day)
Log-probit (slope ≥1)	2	0.01	0.99	21.97	11.04	6.38
Gamma (power ≥1)	2	0.09	0.96	22.05	6.31	3.92
Multistage (degree=1)*	2	0.09	0.96	22.05	6.31	3.92
Quantal Linear	2	0.09	0.96	22.05	6.31	3.92
Weibull (power ≥1)	2	0.09	0.96	22.05	6.31	3.92
Log-logistic (slope ≥1)	1	0.00	1	23.96	10.14	1.24
Quantal Quadratic	2	5.77	0.06	26.17	24.81	19.01
Logistic	1	3.43	0.06	28.31	19.43	11.40
Probit	1	3.50	0.06	28.43	19.69	12.31

* Degree of polynomial initially set to (n-1) where n= number of dose groups including control; model selected is lowest degree model providing adequate fit. Betas restricted to ≥0.

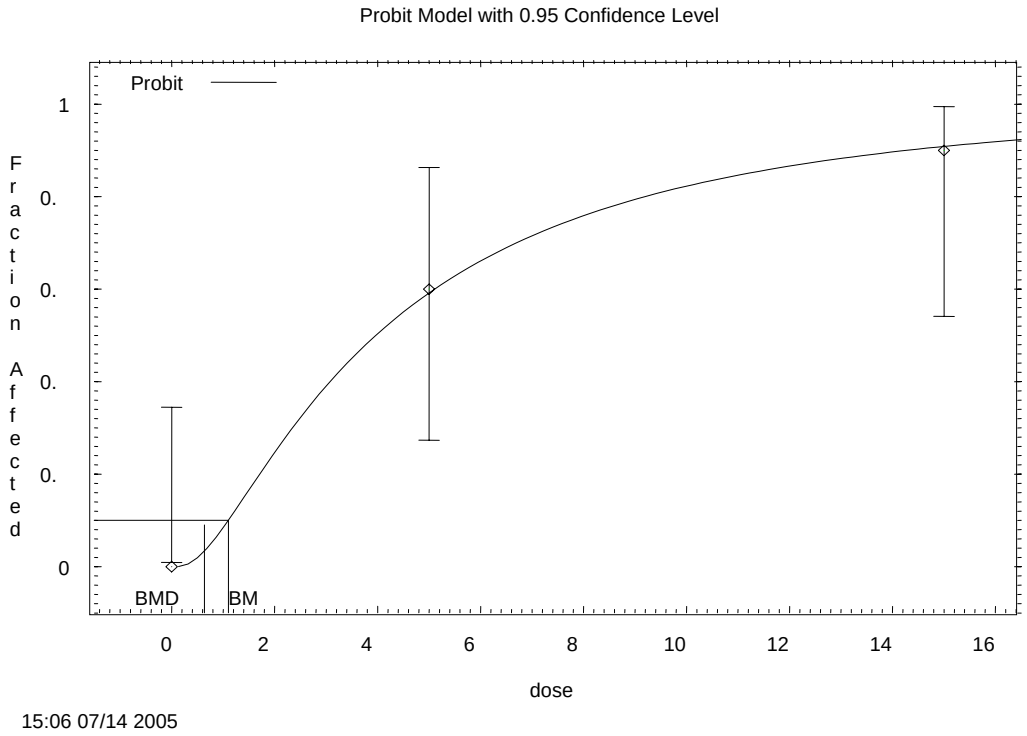


Figure 5-2. Probit Model Fit to Liver Lesion Incidence Data from Heinze (1999)

5.2.2.3. RfD Derivation

The BMDL of 6 μg/kg-day from the Heinze (1999) data is the lowest BMDL among the modeled datasets and was used as the point of departure (POD) for the short-term RfD. Dividing the BMDL of 6 μg/kg-day by a composite uncertainty factor (UF) of 1000 results in a short-term RfD for MCLR of $6x10^{-6}$ mg/kg-day.

Short-term RfD	=	BMDL ÷ UF
	=	6 μg/kg-day ÷ 1000
	=	**0.000006 mg/kg-day or $6x10^{-6}$ mg/kg-day**

The composite UF of 1000 includes a factor of 10 for interspecies extrapolation, a factor of 10 to account for interindividual variability in the human population and a factor of 10 for database limitations, as follows.

- A default 10-fold UF for intraspecies differences was used to account for potentially susceptible individuals in the human population. There is insufficient information on the toxicity of microcystins in exposed humans. Cases of human poisoning have been attributed to ingestion of water containing microcystin-producing cyanobacteria, but no dose-response information is available. There is no information on the degree to which humans of varying gender, age, health status or genetic makeup might vary in the disposition of, or response to, ingested microcystins. There are some data to suggest that adult or aged rodents may be more susceptible than young rodents to the acute toxicity of MCLR (Ito et al., 1997a). Further, studies with algal extracts suggest the possibility that male mice may be more sensitive than female mice to oral exposure to algal extracts (Falconer et al., 1988); however, the relevance of this finding to human microcystin exposure is unclear.

- An interspecies UF of 10 was used to account for differences in response between laboratory rodents and humans. No information is available on the toxicity of purified microcystins in humans, and data on toxicokinetic differences between animals and humans in the disposition of ingested microcystins are not available. Limited data from *in vitro* studies suggests that human hepatocytes may be more susceptible to the effects of MCLR than rat hepatocytes (Yea et al., 2001; Batista et al., 2003), supporting the use of a full 10-fold UF.

- A 10-fold UF is used to account for deficiencies in the database. Database deficiencies include the lack of a detailed developmental toxicity study, a neurotoxicity study, a multi-generation reproductive toxicity study and supporting information on systemic toxicity in a second species.

5.2.3. Subchronic Oral RfD

5.2.3.1. Choice of Principal Study and Critical Effect

A single subchronic oral toxicity study is available for MCLR. Fawell et al. (1999) identified both a NOAEL (40 μg/kg-day) and a LOAEL (200 μg/kg-day for slight liver injury) after subchronic exposure of male and female mice. Fawell et al. (1999) administered daily oral gavage doses of 0, 40, 200 or 1000 μg MCLR per kg body weight to groups of 15 male and 15 female mice for 13 weeks. Histopathological changes in the liver and serum enzyme changes were reported in the mid- and high-dose groups. Both the histopathology and the serum enzymes showed dose-dependent changes. The authors considered the liver changes in the 200 μg/kg-day dose group to represent a minimal effect.

The NOAEL from this 90-day study (40 μg/kg-day) is only slightly lower than the LOAEL identified in the 28-day study above (50 μg/kg-day). Further, Heinze (1999) observed more severe effects in rats exposed to 50 μg/kg-day for 28 days in drinking water than Fawell et al. (1999) observed in mice exposed via gavage doses of 200 μg/kg-day for 90 days. The reason for this difference in response is not clear. Both studies used a commercially-produced test material from the same manufacturer. In the drinking water study, MCLR was dissolved in ethanol and diluted to a stock solution that was subsequently used to prepare drinking water. It is possible that the small intake of ethanol may have potentiated the hepatic effects of MCLR; however, there are no data to determine whether this is likely or not. In the gavage study, test solutions were prepared with distilled water and the concentration was confirmed by HPLC with UV detection (Fawell et al., 1999). The accuracy of dosing in the gavage study was likely to be greater than in the drinking water study. The authors of the drinking water study indicated that the MCLR solution was prepared daily, and water consumption was measured daily. Between 3 and 7% of the water solution administered over the 28 days was not consumed, and the dose estimates were not corrected for this loss (Heinze, 1999). This loss of administered dose would lead to a small overestimate of the LOAEL in the drinking water study, leading to a further discrepancy in the results of the two studies.

The drinking water study used smaller group sizes (10 males/dose) than the gavage study (15/sex/dose or 30/dose). However, the incidence of liver lesions (with necrosis and hemorrhage) in the drinking water study increased from 0% to 100% (including slight, moderate, and intensive lesions) between the control and low-dose group, and there was a dose-related change in the severity of the lesions, leaving little question that the effect was treatment-related.

These studies appear to contradict evidence from acute parenteral studies indicating that mice are more sensitive to the acute effects of MCLR. Typically, mice die within a few hours of a lethal injected dose, while rats may survive 24-48 hours. Species-specific differences in oral absorption of MCLR do not appear to account for the discrepancy between these studies; in an acute study of orally-administered MCLR using both mice and rats, mortality occurred at a lower dose in mice (1580 μg/kg) than in rats (5000 μg/kg; Fawell et al., 1999). It is possible that the more mild effects in the mice in the subchronic study resulted from an adaptive response to MCLR exposure. The longer exposure duration may have allowed for liver regeneration and

repair that was not possible in the shorter-term study; however, there is no information to support this hypothesis. In fact, Ito et al. (1997) reported that light injuries to hepatocytes were still evident in five of seven mice 2 months after treatment with MCLR had ceased.

5.2.3.2 Methods of Analysis

The data from both Heinze (1999) and Fawell et al. (1999) were considered for identifying the point of departure for the subchronic RfD derivation. Results of the modeling for Heinze (1999) are reported in Section 5.2.2.2. Among the liver lesions reported by Fawell et al. (1999), only chronic inflammation showed evidence of a dose-response relationship. Consequently, all quantal models in U.S. EPA's Benchmark Dose Software (BMDS) were fit to the incidence data for chronic liver inflammation in male and female mice reported by Fawell et al. (1999). The incidence data are reported in Table 4-6. In accordance with the U.S. EPA methodology, the default BMR of 10% increase in extra risk was used. Models were run using the default restrictions on parameters built into the BMDS. Adequate fits were achieved with all models. For both male and female mice, the probit model provided the best fit, as assessed by AIC. Table 5-4 gives the results for the best fit models; Appendix A contains the full outputs for all models.

Table 5-4. BMD Modeling Results for Fawell et al. (1999) Chronic Liver Inflammation Data

	Degrees of Freedom	X^2	χ^2 Goodness of Fit *p*-Value	AIC	BMD (μg/kg-day)	BMDL (μg/kg-day)
Male: Probit Model	2	0.21	0.90	40.75	107.59	66.45
Female: Probit Model	2	0.94	0.63	72.84	86.34	56.92

Fawell et al. (1999) also reported significant increases in ALP, AST, and ALT in high-dose animals. These data are shown in Table 4-5 (Section 4.2.1.3.1). Of these, only the increase in ALT in male mice showed a dose-response trend amenable to modeling. All continuous models but the Hill model[5] were fit to the ALT data for male mice reported in Fawell et al. (1999). In accordance with the U.S. EPA methodology, the default BMR of one standard deviation change from the control mean was used, and the polynomial coefficients were restricted to be positive. Only the linear model with a nonhomogenous variance provided an adequate fit to the data. Table 5-5 gives the results from the linear model (see Appendix A for model output). The BMD and BMDL estimated by the linear model (nonhomogenous variance) for the ALT increases in male mice are 82 and 58 μg/kg-day, respectively.

5 There were too few dose groups to apply the Hill model.

Table 5-5. BMD Modeling Results for Fawell et al. (1999) ALT Data in Male Mice					
	Degrees of Freedom	Goodness of Fit *p*-Value	AIC	BMD (μg/kg-day)	BMDL (μg/kg-day)
Linear model, non-homogenous variance	2	0.10	447.68	81.84	58.37

The BMDL from the 28-day drinking water study (6 μg/kg-day) is approximately an order of magnitude lower than any of the BMDL values from the 90-day gavage study (57-66 μg/kg-day). Details of the BMD modeling and analysis of the data from Heinze (1999) are provided above in Section 5.2.2.2, Method of Analysis, under Short-Term Oral RfD.

5.2.3.3. RfD Derivation

The BMDL of 6 μg/kg-day from the Heinze (1999) data is used as the POD for the subchronic RfD. A composite UF of 1000 is used to derive the subchronic RfD, including a factor of 10 for interspecies extrapolation, a factor of 10 for interindividual variability, and a factor of 10 for database limitations (see Section 5.2.2.3 above for details). Although the BMDL comes from a 28-day study, a UF for exposure duration is not proposed, based on the lower toxicity observed in the 90-day gavage study conducted by Fawell et al. (1999). The subchronic RfD is, therefore, set equal to the short-term RfD of 0.006 μg/kg-day or $6x10^{-6}$ mg/kg-day.

Subchronic RfD = BMDL ÷ UF
= 6 μg/kg-day ÷ 1000
= **0.006 μg/kg-day or $6x10^{-6}$ mg/kg-day**

5.2.4. Chronic Oral RfD

5.2.4.1. Choice of Principal Study and Critical Effect

Two chronic studies of the oral toxicity of MCLR were identified. Ito et al. (1997b) conducted a chronic gavage study in mice with 80 to 200 doses (80 μg/kg-day) given over 28 weeks; however, the control group was very small (3 animals) and the postmortem examination was apparently limited to the liver. This study was not given further consideration for RfD development given these study quality concerns.

Ueno et al. (1999) evaluated the toxicity of MCLR in female mice chronically exposed via drinking water 7 days/week. The authors conducted a comprehensive postmortem examination. No treatment-related effects were identified, and the authors observed no

difference in the incidence of liver histopathology between treated and control mice. It is important to note that immunohistochemistry of the liver revealed no accumulation of MCLR. This study identified a free-standing NOAEL of approximately 3 μg/kg-day in female mice.

Although Ueno et al. (1999) used only a single dose level and identified a freestanding NOAEL, it was chosen for RfD derivation because it was a well-conducted chronic study using a relevant exposure route (drinking water). The BMDL of 6 μg/kg-day from modeling (see Section 5.2.2.2) of Heinze (1999) compares favorably with the free-standing NOAEL of 3 μg/kg-day reported by Ueno et al. (1996), providing support for the use of the NOAEL from Ueno et al. (1999).

5.2.4.2. RfD Derivation

As noted above, the NOAEL of 3 μg/kg-day from the study by Ueno et al. (1999) is used as the POD for the chronic RfD. Because this study used a single dose, it was not possible to use BMD modeling to identify the POD. A composite UF of 1000 is used to derive the chronic RfD, including a factor of 10 for interspecies extrapolation, a factor of 10 for interindividual variability and a factor of 10 for database limitations (see Section 5.2.2.3 above for details). Dividing the NOAEL of 3 μg/kg-day by a composite UF of 1000 results in a chronic RfD for MCLR of $3x10^{-6}$ mg/kg-day.

Chronic RfD	=	NOAEL ÷ UF
	=	3 μg/kg-day ÷ 1000
	=	**0.003 μg/kg-day or $3x10^{-6}$ mg/kg-day**

In 1999, the WHO published a provisional Tolerable Daily Intake (TDI) for MCLR based on the subchronic gavage study later published by Fawell et al. (1999). The WHO used the NOAEL of 40 μg/kg-day with a composite UF of 1000 to derive a TDI of 0.04 μg/kg-day or $4x10^{-5}$ mg/kg-day. The composite uncertainty factor included UFs of 10-fold each for interindividual variability, interspecies extrapolation, and database deficiencies (WHO specifically cited the lack of chronic toxicity and carcinogenicity studies). WHO (1999) did not evaluate either Ueno et al. (1999) or Heinze (1999), which may not have been published at the time.

Table 5-6 provides a summary of the RfD values derived for MCLR in this report.

5.3. INHALATION REFERENCE CONCENTRATION

The available data do not provide adequate information for the derivation of inhalation RfCs for MCLR. Two acute inhalation studies were identified in the literature. In a poorly described study, Fitzgeorge et al. (1994) conducted a single experiment with mice (number unspecified) inhaling a fine aerosol (particle size 3-5 μm) with 50 μg/L MCLR for an unspecified duration of time. There were apparently no deaths, clinical signs of toxicity or histopathological changes; however, the authors gave few details of study design and findings. A brief abstract describes a study of acute microcystin exposure via inhalation (Creasia, 1990). The LC_{50} for

1

Table 5-6. Summary of Reference Dose Values			
	RfD (mg/kg-day)	Critical Effect	Principal Study
Acute	NA		
Short-term	6×10^{-6}	Hepatotoxicity	Heinze, 1999
Subchronic	6×10^{-6}	Hepatotoxicity	Heinze, 1999
Chronic	3×10^{-6}	No effects observed	Ueno et al., 1999

mice exposed to a MCLR aerosol (nose only) for 10 minutes was reported to be 18 μg/L (mg/m^3) air with a 95% confidence interval of 15.0-22.0 μg/L (mg/m^3). The authors reported that histological lesions in mice killed by aerosol exposure were similar to those in mice dosed intravenously with MCLR. Neither of these studies provides adequate basis for an acute RfC.

Only one well-conducted study of inhalation exposure to MCLR was identified. Benson et al. (2005) exposed groups of six male BALB/c mice to monodisperse submicron aerosols of MCLR via nose-only inhalation for 30, 60 or 120 minutes each day for 7 consecutive days. The concentration of MCLR was 260-265 μg/m^3. Histopathological examination revealed treatment-related lesions in the nasal cavity only. The incidence and severity of nasal lesions increased with daily exposure duration. This study used only one exposure concentration, and as such, the data are of limited utility for RfC derivation. Further, extrapolation of the effects from this study for the purpose of deriving a short-term RfC would be associated with substantial uncertainty given the brief exposure time (30-120 minutes/day) and duration (7 days). There are no subchronic or chronic animal studies evaluating the inhalation route of exposure.

Route-to-route extrapolation is not considered appropriate for microcystins based on current data. Limited available information indicates that inhalation exposure to microcystins may cause point-of-entry effects (Benson et al., 2005), while oral exposure leads to hepatotoxicity. Data from intratracheal and intranasal instillation studies show hepatic effects after exposure via these routes; however, the relevance of this information to inhalation exposures is uncertain.

5.4. CANCER ASSESSMENT

No dose-response or other information is available regarding the carcinogenicity of pure microcystins.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATIONS OF HAZARD AND DOSE-RESPONSE

6.1. HUMAN HAZARD POTENTIAL

Microcystins are a group of naturally occurring hepatotoxins produced by freshwater cyanobacteria. No studies of the absorption, distribution, metabolism or elimination of microcystins LR, YR, RR or LA *in vivo* have been conducted. Acute lethality data suggest a significant difference in the lethal dose after oral exposure to MCLR when compared with injection routes of exposure, suggesting low absorption of orally-administered MCLR. The cellular uptake and distribution of MCLR has been extensively studied, showing preferential uptake of MCLR by hepatocytes due to the presence of a bile acid transporter.

Data on human exposures to microcystin-producing cyanobacteria have shown gastrointestinal and dermal effects; however, it is not clear whether these are effects of microcystins, other endotoxins, or the microorganisms themselves. The preponderance of the available toxicological studies in animals employed the MCLR congener. Both oral and parenteral exposure studies in laboratory animals point to the liver as the primary target organ of MCLR, and parenteral exposure studies suggest a steep dose-response curve for the hepatotoxic effects. The toxicological database is limited to only a few studies using oral exposure to purified microcystins; all of these used the MCLR congener, and most were conducted in mice. The database includes four studies of acute exposure, one 28-day and one 90-day study, and two chronic studies. In all of the studies where a toxicological effect was observed, the primary target organ was the liver (one study also identified gastrointestinal lesions). The liver effects included hepatocyte degeneration and necrosis, inflammation, fibrosis, hypertrophy and hemorrhage. Humans exposed to microcystins via dialysate suffered acute liver failure and, in many cases, death. Liver biopsies conducted on the decedents showed hepatocyte necrosis and apoptosis, but no intrahepatic hemorrhage.

A single well-described study of short-term (7 days) inhalation exposure identified the upper respiratory tract as a target organ. Damage to the respiratory and olfactory epithelial cells of the nasal cavity was observed in mice in this study. Studies using intratracheal or intranasal instillation have shown systemic effects, including liver toxicity, after these exposures.

A single oral developmental toxicity study with inadequate data reporting indicated that developmental effects in the absence of maternal toxicity are not likely, and that developmental effects, if any, would occur at much higher doses than liver effects.

Mechanistic studies indicate that the inhibition of protein phosphatases 1 and 2A (a well-established molecular effect of microcystin exposure) and/or oxidative stress play a role in the hepatotoxicity of MCLR. Cellular effects in hepatocytes include membrane blebbing, cell rounding and dissociation, and apoptosis. These cellular effects lead to alterations in the liver structure and function as well as intrahepatic hemorrhage.

6.2. DOSE RESPONSE

The available oral data were sufficient for derivation of short-term, subchronic and chronic oral RfDs for MCLR. Based on a BMDL of 6 μg/kg-day for hepatotoxicity in rats exposed to MCLR in drinking water for 28 days (Heinze, 1999), an RfD of 0.006 μg/kg-day ($6x10^{-6}$ mg/kg-day) was derived for short-term and subchronic exposure durations. A UF of 1000 was used to derive the RfD. The UF comprises component factors of 10 for interspecies extrapolation, 10 for interindividual variability and 10 for database deficiencies. The subchronic RfD did not include an additional UF for extrapolating from a 28-day study because a subchronic (90-day) gavage study identified a higher NOAEL. A chronic RfD of 0.003 μg/kg-day ($3x10^{-6}$ mg/kg-day) was derived from a free-standing NOAEL of 3 μg/kg-day in female mice chronically exposed via drinking water. A composite UF of 1000 was used, with factors of 10 each for interindividual variability, interspecies extrapolation and database deficiencies. Inhalation RfCs were not derived since there were no studies of adequate quality for this purpose. There is inadequate evidence to evaluate the carcinogenicity of microcystins LR, RR, YR and LA.

7. REFERENCES

Adams, W.H., R.D. Stoner, D.G. Adams et al. 1985. Pathophysiologic effects of a toxic peptide from *Microcystis aeruginosa*. Toxicon. 23(3):441-447.

Adams, W.H., J.P. Stone, B. Sylvester et al. 1988. Pathophysiology of cyanoginosin-LR, *in vivo* and *in vitro* studies. Toxicol. Appl. Pharmacol. 96(2):248-257.

Ashworth, C.T. and M.F. Mason. 1945. Observations on the pathological changes produced by a toxic substance present in blue-green algae (*Microcystis aeruginosa*). Am. J. Pathol. 22:369-383.

Aune, T. and K. Berg. 1986. Use of freshly prepared rat hepatocytes to study toxicity of blooms of the blue-green algae *Microcystis aeruginosa* and cyanotoxin *Oscillatoria agardhii*. J. Toxicol. Environ. Health. 19(3):325-336.

Azevedo, S.M.F.O., W.W. Carmichael, E.M. Jochimsen et al. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru, Brazil. Toxicology. 181-182:441-446.

Aziz, K.M.S. 1974. Diarrhea toxin obtained from a waterbloom-producing species, *Microcystis aeruginosa* Kützing. Science. 183:1206-1207.

Bagu, J.R., B.D. Sykes, M.M. Craig and C.F.B. Holmes. 1997. A molecular basis for different interactions of marine toxins with protein phosphatase-1. Molecular models for bound motuporin, microcystins, okadaic acid, and calyculin A. J. Biol. Chem. 272(8):5087-5097.

Batista, T., G. de Sousa, J.S. Suput et al. 2003. Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes. Aquat. Toxicol. 65(1):85-91.

Battle, T., C. Touchard, H.J. Moulsdale et al. 1997. New cell substrates for *in vitro* evaluation of microcystin hepatocytoxicity. Toxicol. In Vitro. 11(5):557-567.

Beasley, V.R., R.A. Lovell, K.R. Holmes et al. 2000. Microcystin-LR decreases hepatic and renal perfusion and causes circulatory shock, severe hyperglycemia, and terminal hyperkalemia in intravascularly dosed swine. J. Toxicol. Environ. Health-Part A. 61(4):281-303.

Becchetti, A., B. Malik, G. Yue et al. 2002. Phosphatase inhibitors increase the open probability of ENaC in A6 cells. Am. J. Physiol. Renal Physiol. 283(5):F1030-F1045.

Benson, J.M., J.A. Hutt, K. Rein et al. 2005. The toxicity of microcystin LR in mice following 7 days of inhalation exposure. Toxicon. 45(6):691-698.

Berg, K. and T. Aune. 1987. Freshly prepared rat hepatocytes used in screening the toxicity of blue-green algal blooms. J. Toxicol. Environ. Health. 20(1/2):187-197.

Berg, K., J. Wyman, W.W. Carmichael and A.S. Dabholkar. 1988. Isolated rat liver perfusion studies with cyclic heptapeptide toxins of *Microcystis* and *Oscillatoria* (freshwater cyanobacteria). Toxicon. 26(9):827-837.

Bhattacharya, R., P.V.L. Rao, A.S.B. Bhaskar et al. 1996. Liver slice culture for assessing hepatoxicity of freshwater cyanobacteria. Human Exp. Toxicol. 15(2):105-110.

Bhattacharya, R., K. Sugendran, R.S. Dangi and P.V.L. Rao. 1997. Toxicity evaluation of freshwater cyanobacterium *Microcystis aeruginosa* PCC-7806. 2. Nephrotoxicity in rats. Biomed. Environ. Sci. 10(1):93-101.

Billings, W.H. 1981. Water-associated human illness in northeast Pennsylvania and its suspected association with blue-green algae blooms. In: The Water Environment: Algal Toxins and Health, W.W. Carmichael, Ed. Plenum Press, New York, NY. p. 243-255.

Bishop, C.T., E.F.L.J. Anet and P.R. Gorham. 1959. Isolation and identification of the fast-death factor in *Microcystis aeruginosa* NRC-1. Can. J. Biochem. Physiol. 37(3):453-471.

Blankson, H., E.M. Grotterod and P.O. Seglen. 2000. Prevention of toxin-induced cytoskeletal disruption and apoptotic liver cell death by the grapefruit flavonoid, narigin. Cell Death Diff. 7(8):739-746.

Boe, R., B.T. Gjersten, O.K. Vintermyr et al. 1991. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 195(1):237-246.

Botha, N, M. van de Venter, T.G. Downing et al. 2004. The effect of intraperitoneally administered microcystin-LR on the gastrointestinal tract of Balb/c mice. Toxicon. 43(3):251-254.

Bouaïcha, N. and I. Maatouk. 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured rat hepatocytes. Toxicol. Lett. 148(1-2):53-63.

Bouaïcha, N., I. Maatouk, M.J. Plessis and F. Perin. 2005. Genotoxic potential of microcystin-LR and nodularin *in vitro* in primary cultured rat hepatocytes and *in vivo* in rat liver. Environ. Toxicol. 20(3):341-347.

Brooks, W.P. and G.A. Codd. 1987. Immunological and toxicological studies on *Microcystis aeruginosa* peptide toxin. Br. Phycol. J. 22(3):301.

Carmichael, W.W., S.M.F.O. Azevedo, J.S. An et al. 2001. Human fatalities from cyanobacteria: Chemical and biological evidence for cyanotoxins. Environ. Health Perspect. 109(7):663-668.

Chen, J., T.C. Campbell, J. Li and R. Peto. 1991. Diet, Life-style and Mortality in China. A Study of the Characteristics of 65 Chinese Counties, Cornell University Press, Ithaca, NY. (As cited in Health Canada, 2002).

Chen, K., Y.Z. Shen and G.F. Shen. 1994. Study on incidence rate of some cancer in areas with difference in drinking water sources. Chin. J. Public Health. 12(3):146-148 (As cited in Zhou et al., 2002). (Chinese)

Chen, T., X. Zhao, Y Liu et al. 2004. Analysis of immunomodulating nitric oxide, iNOS and cytokines MRNA in mouse macrophages induced by microcystin-LR. Toxicology. 197(1):67-77.

Chen, T., P. Shen, J. Zhang et al. 2005. Effects of microcystin-LR on patterns of iNOS and cytokine mRNA expression in macrophages *in vitro*. Environ. Toxicol. 20(1):85-91.

Chen, T., J. Cui, Y. Liang et al. 2006. Identification of human liver mitochondrial aldehyde dehydrogenase as a potential target for microcystin-LR. Toxicology. 220(1):71-80.

Chernoff, N, E.S. Hunter III, L.L. Hall et al. 2002. Lack of teratogenicity of microcystin-LR in the mouse and toad. J. Appl. Toxicol. 22(1):13-17.

Chong, M.W.K., K.D. Gu, P.K.S. Lam et al. 2000. Study of the cytotoxicity of microcystin-LR on cultured cells. Chemosphere. 41(1-2):143-147.

Cote, L-M., R.A. Lovell, E.H. Jeffrey et al. 1986. Failure of blue-green algae (*Microcystis aeruginosa*) hepatotoxin to alter *in vitro* mouse liver enzymatic activity. J. Toxicol.- Toxin Rev. 52(2):256.

Craig, M., H.A. Luu, T.L. McCready et al. 1996. Molecular mechanisms underlying the interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. Biochem. Cell Biol. 74(4):569-578.

Creasia, D.A. 1990. Acute inhalation toxicity of microcystin-LR with mice. Toxicon. 28(6):605.

Dabholkar, A.S. and W.W. Carmichael. 1987. Ultrastructural changes in the mouse liver induced by hepatoxin from the freshwater cyanobacterium *Microcystis aeruginosa* strain 7820. Toxicon. 25(3):285-292.

Dahlem, A.M., A.S. Hassan, S.P. Swanson et al. 1989. A model system for studying the bioavailability of intestinally administered microcystin-LR, a hepatotoxic peptide from the cyanobacterium *Microcystis aeruginosa.* Pharmacol. Toxicol. 64(2):177-181.

Davidson, F.F. 1959. Poisoning of wild and domestic animals by a toxic waterbloom of *Nostoc rivulare* Küetz. J. Am. Water Works Assoc. 51:1277-1287.

Dillenberg, H.O. and M.K. Dehnel. 1960. Toxic water bloom in Saskatchewan, 1959. Can. Med. Assoc. J. 83:1151-1154.

Ding, W.X. and C.N. Ong. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol. Lett. 220(1):1-7.

Ding, W.X., H.M. Shen, Y. Shen et al. 1998a. Microcystic cyanobacteria causes mitochondrial-membrane potential alteration and reactive oxygen species formation in primary cultured rat hepatocytes. Environ. Health Perspect. 106(7):409-413.

Ding, W.X., H.M. Shen, H.G. Zhu and C.N. Ong. 1998b. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. Environ. Res. 78(1):12-18.

Ding, W.X., H.M. Shen, H.G. Zhu, B.L. Lee and C.N. Ong. 1999. Genotoxicity of microcystic cyanobacterial extract of a water source in China. Mutat Res. 442(2):69-77.

Ding, W.X., H.M. Shen and C.N. Ong. 2000a. Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. Environ. Health Perspect. 108(7):605-609.

Ding, W.X., H.M. Shen and C.N. Ong. 2000b. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. Hepatology. 32(3):547-555.

Ding, W.X., H.M. Shen and C.N. Ong. 2001. Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. J. Toxicol. Environ. Health Part A. 64(6):507-519.

Ding, W.X., H.M. Shen and C.N. Ong. 2002. Calpain activation after mitochondrial permeability transition in microcystin-induced cell death in rat hepatocytes. Biochem. Biophys. Res. Commun. 291(2):321-331.

Duy, T.N., P.K.S. Lam, G.R. Shaw et al. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev. Environ. Contam. Toxicol. 163:113-185.

Dvoráková, D., K. Dvoráková, L. Bláha et al. 2002. Effects of cyanobacterial biomass and purified microcystins on malformations in *Xenopus laevis*: Teratogenesis assay (FETAX). Environ. Toxicol. 17(6):547-555.

Elleman, T.C., I.R. Falconer, J.R. Jackson and M.T.C. Runnegar. 1978. Isolation, characterization and pathology of the toxin from a *Microcystis aeruginosa (=Anacystis cyanea)* bloom. Aust. J. Biol. Sci. 31(3):209-218.

el Saadi, O. and A.S. Cameron. 1993. Illness associated with blue-green algae. Med. J. Aust. 158(11):792-793.

el Saadi, O., A.J. Esterman, S. Cameron and D.M. Roder. 1995. Murray River water raised cyanobacterial cell counts, and gastrointestinal and dermatological symptoms. Med. J. Aust. 162(3):122-125.

Eriksson, J.E. and R.D. Golman. 1993. Protein phosphatase inhibitors alter cytoskeletal structure and cellular morphology. Adv. Prot. Phosphatases. 7:335-357.

Eriksson, J.E., J.A.O. Meriluoto and T. Lindholm. 1987. Cyanobacterial toxins – physiological and ecological effects. In: Rapport till Finlans Akademi. p. 1-12.

Eriksson, J.E., G.I.L. Paatero, J.A.O. Meriluoto et al. 1989. Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. Exp. Cell Res. 185(1):86-100.

Eriksson, J.E., L. Gronberg, S. Nygard et al. 1990a. Hepatocellular uptake of 3H-dihydromicrocystin-LR, a cyclic peptide toxin. Biochim. Biophys. Acta. 1025(1):60-66.

Eriksson, J.E., D. Toivola, J.A.O. Meriluoto et al. 1990b. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. Biochem. Biophys. Res. Commun. 173(3):1347-1353.

Eriksson, J.E., D.M. Toivola, M. Reinikainen et al. 1994. Testing of toxicity in cyanobacteria by cellular assays. In: Detection Methods for Cyanobacterial Toxins, G.A. Codd, T.M. Jefferies, C.W. Keevil and E. Potter, Eds. Royal Society of Chemistry, Cambridge. p. 75-84.

Falconer, I.R. 1991. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. Environ. Toxicol. Water Qual. 6(2):177-184.

Falconer, I.R. and T.H. Buckley. 1989. Tumour promotion by *Microcystis sp*., a blue-green alga occurring in water supplies. Med. J. Aust. 150(6):351.

Falconer, I.R. and A.R. Humpage. 1996. Tumour promotion by cyanobacterial toxins. Phycologia. 35:74-79.

Falconer, L.R. and M.T.C. Runnegar. 1987a. Effects of the peptide toxin from *Microcystis aeruginosa* on intracellular calcium, pH and membrane integrity in mammalian cells. Chem-Biol. Interact. 63(3):215-225.

Falconer, L.R. and M.T.C. Runnegar. 1987b. Toxic peptide from the blue-green alga *Microcystis aeruginosa*- effects on hepatocytes and thymocytes. Biochem. Soc. Trans. 15(3):468-469.

Falconer, I.R. and S.K. Yeung. 1992. Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorylation of cell proteins. Chem-Biol. Interact. 81(1-2):181-196.

Falconer, I.R., A.R.B. Jackson, J. Langley and M.T.C. Runnegar. 1981. Liver pathology in mice in poisoning by the blue-green alga *Microcystis aeruginosa*. Aust. J. Biol. Sci. 34(2):174-187.

Falconer, I.R., A.M. Beresford and M.T.C. Runnegar. 1983. Evidence of liver damage by toxin from a bloom of the blue-green alga, *Microcystis aeruginosa*. Med. J. Aust. 1(11):511-514.

Falconer, I.R., T. Buckley and M.T.C. Runnegar. 1986. Biological half-life organ distribution and excretion of ^{125}I-labeled toxic peptide from the blue-green alga *Microcystis aeruginosa*. Aust. J. Biol. Sci. 39(1):17-21.

Falconer, L.R., J.V. Smith, A.R.B. Jackson et al. 1988. Oral toxicity of a bloom of the cyanobacterium *Microcystis aeruginosa* administered to mice over periods of up to one year. J. Toxicol. Environ. Health. 24(3):291-305.

Falconer, I.R., M.D. Burch, D.A. Steffensen, M. Choice and O.R. Coverdale. 1994. Toxicity of the blue-green alga (Cyanobacterium) *Microcystis aeruginosa* in drinking water to growing pigs, as an animal model for human injury and risk assessment. Environ. Toxicol. Water Qual. 9:131-139.

Fawell, J.K., R.E. Mitchell, D.J. Everett and R.E. Hill. 1999. The toxicity of cyanobacterial toxins in the mouse. 1. Microcystin-LR. Human Exp. Toxicol. 18(3):162-167.

Feitz, A.J., T. Lukondeh, M.C. Moffitt et al. 2002. Absence of detectable levels of the cyanobacterial toxin (microcystin-LR) carry-over into milk. Toxicon. 40(8):1173-1180.

Fischer, W.J. and D.R. Dietrich. 2000. Toxicity of the cyanobacterial cyclic heptapeptide toxins microcystin-LR and –RR in early lifestages of the African clawed frog. Aquat. Toxicol. 49(3):189-198.

Fitzgeorge, N.L.M., S.A. Clark and C.W. Kelvin. 1994. Routes of intoxication. In: Detection Methods for Cyanobacterial (Blue-Green Algal) Toxins. G.A. Codd, T.M. Jeffreies, C.W. Kelvin and E. Potter, Eds. Royal Society of Chemistry, Cambridge, U.K. p. 69-74.

Fladmark, K.E., M.H. Serres, N.L. Larsen et al. 1998. Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. Toxicon. 36(8):1101-1114.

Fleming, L.E., C. Rivero, J. Burns et al. 2002. Blue green algal (cyanobacterial) toxins, surface drinking water and liver cancer in Florida. Harmful Algae. 1(2):157-168.

Fleming, L.E., C. Rivero, J. Burns, C. Williams, J.A. Bean and W.B. Stephan. 2004. Cyanobacteria exposure, drinking water and colorectal cancer. In: Harmful Algae 2002. Proceedings of the Xth International Conference on Harmful Algae. K.A. Steidinger, J.H. Landsberg, C.R. Tomas and G.A. Vargo, Eds. Florida Fish and Wildlife Conservation Commission and Intergovernmental Oceanographic Commission of UNESCO, Tallahassee, FL. p. 470-472.

Foxall, T.L. and J.J. Sasner, Jr. 1981. Effects of a hepatic toxin from the cyanophyte *Microcystis aeruginosa.* In: The Water Environment: Algal Toxins and Health, W.W. Carmichael, Ed. Plenum Press, New York, NY. p. 365-387.

Frangez, R., M.C. Zuzek, J. Mrkun et al. 2003. Microcystin-LR affects cytoskeleton and morphology of rabbit primary whole embryo cultured cells *in vitro*. Toxicon. 41(8):999-1005.

Fu, W.Y., J.P. Chen, X.M. Wang and L.H. Xu. 2005. Altered expression of p53, Bcl-2 and Bax induced by microcystin-LR *in vivo* and *in vitro*. Toxicon. 46(2):171-177.

Gehringer, M.M. 2004. Microcystin-LR and okadaic acid-induced cellular effects: A dualistic response. FEBS Lett. 557(1-3):1-8.

Gehringer, M.M., S. Govender, M. Shaw and T.G. Downing. 2003a. An investigation of the role of vitamin E in the protection of mice against microcystin toxicity. Environ. Toxicol. 18(2):142-148.

Gehringer, M.M., K.S. Downs, T.G. Downing et al. 2003b. An investigation into the effects of selenium supplementation on microcystin hepatotoxicity. Toxicon. 41(4):451-458.

Gehringer, M.M., E.G. Shephard, T.G. Downing et al. 2004. An investigation into the detoxification of microcystin-LR by the glutathione pathway in Balb/c mice. Int. J. Biochem. Cell Biol. 36(5):931-941.

Goldberg, J., H.B. Huang, Y.G. Kwon, et al. 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376(6543)745-753.

Grabow, W.O.K., W.C. Du Randt, O.W. Prozesky and W.E. Scott. 1982. *Microcystis aeruginosa* toxin: Cell culture toxicity, hemolysis, and mutagenicity assays. Appl. Environ. Microbiol. 43(6):1425-1433.

Gulledge, B.M., J.B. Aggen, H.B. Huang et al. 2002. The microcystins and nodularins: cyclic polypeptide inhibitors of PP1 and PP2A. Curr. Med. Chem. 9(22):1991-2003.

Gulledge, B.M., J.B. Aggen and A.R. Chamberlin. 2003a. Linearized and truncated microcystin analogues as inhibitors of protein phosphatases 1 and 2A. Bioorg. Medicinal Chem.. Lett. 13(17):2903-2906.

Gulledge, B.M., J.B. Aggen, H. Eng et al. 2003b. Microcystin analogues comprised only of Adda and a single additional amino acid retain moderate activity as PP1/PP2A inhibitors. Bioorg. Medicinal Chem. Lett. 13(17):2907-2911.

Gupta, N., S.C. Pant, R. Vijayaraghavan and P.V. Rao. 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology. 188(2-3):285-296.

Guzman, R.E. and P.F. Solter. 1999. Hepatic oxidative stress following prolonged sublethal microcystin-LR exposure. Toxicologic Pathol. 27(5):582-588.

Guzman, R.E. and P.F. Solter. 2002. Characterization of sublethal microcystin-LR exposure in mice. Vet. Pathol. 39(1):17-26.

Guzman, R.E., P.F. Solter and M.T. Runnegar. 2003. Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. Toxicon. 41(7):773-781.

Harada, K., K. Ogawa, K. Matsuura et al. 1990. Structural determination of geometrical isomers of microcystins LR and RR from cyanobacteria by two-dimensional NMR spectroscopic techniques. Chem. Res. Toxicol. 3(5):473-481.

Hastie, C.J., E.B. Borthwick, L.F. Morrison et al. 2005. Inhibition of several protein phosphatases by a non-covalently interacting microcystin and a novel cyanobacterial peptide, nostocyclin. Biochim. Biophys. Acta. 1726:187-193.

Hayakawa, K. and K. Kohama. 1995. Reversible effects of okadaic acid and microcystin-LR on the ATP-dependent interaction between actin and myosin. J. Biochem. 117(3):509-514.

Health Canada. 2002. Guidelines for Canadian Drinking Water Quality: Supporting Documentation — Cyanobacterial Toxins–Microcystin-LR. Water Quality and Health Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. Available at http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/doc_sup-appui/index_e.html.

Heinze, R. 1999. Toxicity of the cyanobacterial toxin microcystin-LR to rats after 28 days intake with the drinking water. Environ. Toxicol. 14(1):57-60.

Heinze, R., J. Fastner, U. Neumann and I. Chorus. 2001. Testing cyanobacterial toxicity with primary rat hepatocyte and cell-line assays. In: Cyanotoxins: Occurrence, Causes, Consequences, I. Chorus, Ed. Springer-Verlag, New York, NY. p. 317-324.

Herfindal, L. and F. Selheim. 2006. Microcystin produces disparate effects on liver cells in a dose dependent manner. Mini. Rev. Med. Chem. 6(3):279-285.

Hermansky, S.J., P.J. Casey and S.J. Stohs. 1990a. Cyclosporin A--a chemoprotectant against microcystin-LR toxicity. Toxicol. Lett. 54(2-3):279-285.

Hermansky S.J., S.N. Wolff and S.J. Stohs. 1990b. Use of rifampin as an effective chemoprotectant and antidote against microcystin-LR toxicity. Pharmacology. 41(4):231-236.

Hermansky, S.J., S.J. Stohs, R.S. Markin and W.J. Murray. 1990c. Hepatic lipid peroxidation, sulfhydryl status, and toxicity of the blue-green algal toxin microcystin-LR in mice. J. Toxicol. Environ. Health. 31(1):71-91.

Hermansky, S.J., S.J. Stohs, Z.M. Eldeen et al. 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. J. Appl. Toxicol. 11(1):65-73.

Hernandez, M., M. Macia, C. Padilla and F.F. Del Campo. 2000. Modulation of human polymorphonuclear leukocyte adherence by cyanopeptide toxins. Environ. Res. 84(1):64-68.

Hilborn, E.D., W.W. Carmichael, M. Yuan et al. 2005. Serologic evaluation of human microcystin exposure. From: Interagency International Symposium on Cyanobacterial Harmful Algal Blooms, Research Triangle Park, NC.

Honkanen, R.E., J. Zwiller, R.E. Moore et al. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J. Biol. Chem. 265(32):19401-19404.

Hooser, S.B. 2000. Fulminant hepatocyte apoptosis *in vivo* following microcystin-LR administration to rats. Toxicol. Pathol. 28(5):726-733.

Hooser, S.B., V.R. Beasley, R.A. Lovell et al. 1989a. Toxicity of microcystin LR, a cyclic heptapeptide hepatoxin from *Microcystis aeruginosa* to rats and mice. Vet. Pathol. 26(3):246-252.

Hooser, S.B., L.L. Waite, V.R. Beasley et al. 1989b. Microcystin-A induces morphologic and cytoskeletal hepatocyte changes *in vitro*. Toxicon 27(1):50-51.

Hooser, S.B., V.R. Beasley, E.J. Basgall, W.W. Carmichael, and W.M. Haschek. 1990. Microcystin-LR–induced ultrastructural changes in rats. Vet. Pathol. 27(1):9-15.

Hooser, S.B., M.S. Kuhlenschmidt, A.M. Dahlem et al. 1991a. Uptake and subcellular localization of tritiated dihydro-microcystin-LR in rat liver. Toxicon. 29(6):589-601.

Hooser, S.B., V.R. Beasley, L.L. Waite et al. 1991b. Actin filament alterations in rat hepatocytes induced *in vivo* and *in vitro* by microcystin-LR, a hepatoxin from the blue-green alga, *Microcystis aeruginosa*. Vet. Pathol. 28(4):259-266.

Hu, Z., H. Chen, Y. Li et al. 2002. [The expression of bcl-2 and bax genes during microcystin induced liver tumorigenesis.] Zhonghua Yu Fang Yi Xue Za Zhi. 36(4):239-242. (Chinese)

Humpage, A.R. and I.R. Falconer. 1999. Microcystin-LR and liver tumor promotion: Effects on cytokinesis, ploidy, and apoptosis in cultured hepatocytes. Environ. Toxicol. 14(1):61-75.

Humpage, A.R., S.J. Hardy, E.J. Moore et al. 2000. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J. Toxicol. Environ. Health Part A. 61(3):155-165.

Ito, E., F. Kondo and K.-I. Harada. 1997a. Hepatic necrosis in aged mice by oral administration of microcystin-LR. Toxicon. 35(2):231-239.

Ito, E., F. Kondo, K. Terao and K.-I. Harada. 1997b. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. Toxicon. 35(9):1453-1457.

Ito, E., F. Kondo and K. Harada. 2001. Intratracheal administration of microcystin-LR, and its distribution. Toxicon. 39(2-3):265-271.

Ito, E., A. Takai, F. Kondo et al. 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. Toxicon. 40(7):1017-1025.

Jackson, A.R.B., A. McInnes, I.R. Falconer and M.T.C. Runnegar. 1984. Clinical and pathological changes in sheep experimentally poisoned by the blue-green alga *Microcysstis aeruginosa*. Vet. Pathol. 21(1):102-113.

Jayaraj, R., T. Anand and P.V. Rao. 2006. Activity and gene expression profile of certain antioxidant enzymes to microcystin-LR induced oxidative stress in mice. Toxicology. 220(2-3):136-146.

Jiao, D.A., G.F. Shen, Y.Z. Shen and G.M. Zheng. 1985. The case-control study of colorectal cancer. Chin. J. Epidemio. 6:285-288 (As cited in Zhou et al., 2002). (Chinese)

Jochimsen, E.M., W.W. Carmichael, J.S. An et al. 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. New Engl. J. Med. 338(13):873-878.

Jones, C.L.A. 1984. Biochemical, hematological, and hepatotoxicological studies of hepatotoxins from *Microcystis aeruginosa* strain 7820, and *Anabaena flos-aquae* strain S-23-G. M.S. Thesis, Wright State Univ., Dayton, OH.

Jones, C.L.A. and W.W. Carmichael. 1984. Comparison of hepatotoxins from the cyanobacteria *Anabaena flos-aquae* and *Microcystis aeruginosa.* Fed. Proc. 43(3):A1716.

Kalbe, L. 1984. Animal experiments on the oral toxicity of blue-green algae waterblooms. Limnol. (Berlin) 15(2):559-562.

Khan, S.A., S. Ghosh, M.L. Wickstrom et al. 1995. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts and renal epithelial cells. Natural Toxins. 3(3):119-128.

Klassen, C.D., Ed. 2001. Casarett and Doull's Toxicology: The Basic Science of Poisons, 6th ed. McGraw-Hill, New York, NY.

Knapp J., S. Aleth, F. Balzer et al. 2002. Calcium-independent activation of the contractile apparatus in smooth muscle of mouse aorta by protein phosphatase inhibition. Naunyn Schmiedebergs Arch. Pharmacol. 366(6):562-569.

Kondo, F., Y. Ikai, H. Oka et al. 1992. Formation, characterization, and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. Chem. Res. Toxicol. 5(5):591-596.

Kondo, F., H. Matsumoto, S. Yamada et al. 1996. Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. Chem. Res. Toxicol. 9(8):1355-1359.

Kujbida, P., E. Hatanaka, A. Campa, P. Colepicolo and E. Pinto. 2006. Effects of microcystins on human polymorphonuclear leukocytes. Biochem. Biophys. Res. Commun. 341(1):273-277.

Lankoff, A., A. Banasik, G. Obe et al. 2003. Effect of microcystin-LR and cyanobacterial extract from Polish reservoir drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. Toxicol. Appl. Pharmacol. 189(3):204-213.

Lankoff, A., L. Krzowski, J. Glab et al. 2004. DNA damage and repair in human peripheral blood lymphocytes following treatment with microcystin-LR. Mutat. Res. 559(1-2):131-142.

LeClaire, R.D., W.B. Lawrence, K.A. Bostian and K.A. Mereish. 1988. Acute toxicity of microcystin-LR in the rat: A comparative dose-response study using serum chemistries and mortality as indices. Toxicologist. 8(1):221.

LeClaire, R.D., G.W. Parker and D.R. Franz. 1995. Hemodynamic and calorimetric changes induced by microcystin-LR in the rat. J. Appl. Toxicol. 15(4):303-311.

Leiers, T., A. Bihlmayer, H.P.T. Ammon and M.A. Wahl. 2000. $[Ca^{2+}](i)$- and insulin-stimulating effect of the non-membranepermeable phosphatase-inhibitor microcystin-LR in intact insulin-secreting cells (RINm5F). Br. J. Pharmacol. 130(6):1406-1410.

Lin, J.R. and F.S. Chu. 1994. Kinetics of distribution of microcystin-LR in serum and liver cytosol of mice: an immunochemical analysis. J. Agric. Food Chem. 42(4):1035-1040.

Lovell, R.A., D.J. Schaeffer, S.B. Hooser et al. 1989a. Toxicity of intraperitoneal doses of microcystin-LR in two strains of male mice. J. Environ. Pathol. Toxicol. Oncol. 9(3):221-237.

Lovell, R.A., K.R. Holmes, W.W. Carmichael and V.R. Beasley. 1989b. Hemodynamic and pathologic effects of microcystin-LR administered intravenously to swine. Toxicon. 27(1):60. (Abstract)

Maatouk, I., N. Bouaicha, M.J. Plessis and F. Perin. 2004. Detection by 32P-postlabelling of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA as biomarker of microcystin-LR- and nodularin-induced DNA damage *in vitro* in primary cultured rat hepatocytes and *in vivo* in rat liver. Mutat. Res. 564(1):9-20.

MacKintosh, C., K.A. Beattie, S. Klumpp et al. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264(2):187-192.

MacKintosh, R.W., K.N. Dalby, D.G. Campbell, P.T. Cohen, P. Cohen and C. MacKintosh. 1995. The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. FEBS Lett. 371(3):236-240.

Maidana, M., V. Carlis, F.G. Galhardi et al. 2006. Effects of microcystins over short- and long-term memory and oxidative stress generation in hippocampus of rats. Chem. Biol. Interact. 159(3):223–234.

Mankiewicz, J., M. Tarczynska, K.E. Fladmark et al. 2001. Apoptotic effect of cyanobacterial extract on rat hepatocytes and human lymphocytes. Environ. Toxicol. 16(3):225-233.

Matsushima, R., S. Yoshizawa, M.F. Watanabe et al. 1990. *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin on mouse skin and fibroblasts. Biochem. Biophys. Res. Commun. 171(2):867-874.

Matsushima-Nishiwaki, R., Y. Shidoji, S. Nishiwaki et al. 1995. Suppression by carotenoids of microcystin-induced morphological changes in mouse hepatocytes. Lipids. 30(11):1029-1034.

Mattila K., A. Annila and T.T. Rantala. 2000. Metal ions mediate the binding of cyanobacterial toxins to human protein phosphatase I: A computational study. Oulu University Library, Oulun Yliopisto, Oulu.

Maynes JT, K.R. Perreault, M.M. Cherney, H.A. Luu, M.N. James and C.F. Holmes. 2004. Crystal structure and mutagenesis of a protein phosphatase-1:calcineurin hybrid elucidate the role of the β12-β13 loop in inhibitor binding. J. Biol. Chem. 279(41):43198-43206.

Maynes, J.T., H.A. Luu, M.M. Cherney et al. 2006. Crystal structures of protein phosphatase-1 bound to motuporin and dihydromicrocystin-LA: Elucidation of the mechanism of enzyme inhibition by cyanobacterial toxins. J. Mol. Biol. 356(1):111-120.

McDermott, C.M., C.W. Nho, W. Howard and B. Holton. 1998. The cyanobacterial toxin, microcystin-LR can induce apoptosis in a variety of cell types. Toxicon. 36(12):1981-1996.

Mereish, K.A. and R. Solow. 1990. Effect of antihepatotoxic agents against microcystin-LR toxicity in cultured rat hepatocytes. Pharmacol. Res. 7(3):256-259.

Mereish, K.A., R. Solow, Y. Singh et al. 1989. Comparative toxicity of cyclic polypeptides and depsipeptides on cultured rat hepatocytes. Toxicologist. 9(1):68.

Mereish, K.A., D.L. Bunner, D.R. Ragland and D.A. Creasia. 1991. Protection against microcystin-LR induced hepatoxicity by Silymarin: Biochemistry, histopathology and lethality. Pharm. Res. 8(2):273-277.

Meriluoto, J.A., S.E. Nygard, A.M. Dahlem and J.E. Eriksson. 1990. Synthesis, organotropism and hepatocellular uptake of two tritium-labeled epimers of dihydromicrocystin-LR, a cyanobacterial peptide toxin analog. Toxicon. 28(12):1439-1446.

Metcalf J.S., K.A. Beattie, S. Pflugmacher and G.A. Codd. 2000. Immuno-crossreactivity and toxicity assessment of conjugation products of the cyanobacterial toxin, microcystin-LR. FEMS Microbiol. Lett. 189(2):155-158.

Mikhailov, A., A.S. Härmälä-Braskén, J. Hellman et al. 2003. Identification of ATP-synthase as a novel intracellular target for microcystin-LR. Chem-Biol. Inter. 142(3):223-237.

Milutinovic, A., B. Sedmak, I. Horvat-Znidarsic and D. Suput. 2002. Renal injuries induced by chronic intoxication with microcystins. Cell Mol. Biol. Lett. 7(1):139-141.

Milutinovic A, M. Zivin, R. Zore-Pleskovic, B. Sedmak and D. Suput. 2003. Nephrotoxic effects of chronic administration of microcystins-LR and –YR. Toxicon. 42(3):281-288.

Miura, G.A., N.A. Robinson, W.B. Lawrence and J.G. Page. 1991. Hepatoxicity of microcystin-LR in fed and fasted rats. Toxicon. 29(3):337-346.

Moreno, I.M., A. Mate, G. Repetto et al. 2003. Influence of microcystin-LR on the activity of membrane enzymes in rat intestinal mucosa. J. Physiol. Biochem. 59(4):293-299.

Moreno, I., S. Pichardo, A. Jos et al. 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon. 45(4):395-402.

Namikoshi M., BW Choi, F Sun, KL Rinehart, WR Evans and W.W. Carmichael. 1993. Chemical characterization and toxicity of dihydro derivatives of nodularin and microcystin-LR, potent cyanobacterial cyclic peptide hepatotoxins. Chem. Res. Toxicol. 6(2):151-158.

Naseem, S.M., Hines, H.B. and D.A. Creasia. 1990. Inhibition of microcystin-induced release of cyclooxygenase products from rat hepatocytes by anti-inflammatory steroids. Proc. Soc. Exp. Biol. Med. 195(3):346-349.

Naseem, S.M., K.A. Mereish, R. Solow and H.B. Hines. 1991. Microcystin-induced activation of prostaglandin synthesis and phospholipids metabolism in rat hepatocytes. Toxicol. In Vitro. 5(4):341-345.

National Research Council. 1983. Risk Assessment in the Federal Government: Managing the Process. National Academy Press, Washington, DC.

Nishiwaki R., T. Ohta, E. Sueoka et al. 1994. Two significant aspects of microcystin-LR: specific binding and liver specificity. Cancer Lett. 83(1-2):283-289.

Nishiwaki-Matsushima, R., S. Nishiwaki, T. Ohta et al. 1991. Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatase. Jpn. J. Cancer Res. 82(9):993-996.

Nishiwaki-Matsushima, R., T. Ohta, S. Nishiwaki et al. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118(6):420-424.

Nobre, A.C.L., M.C.M. Jorge, D.B. Menezes et al. 1999. Effects of microcystin-LR in isolated perfused rat kidney. Brazilian J. Med. Biol. Res. 32(8):985-988.

Nobre, A.C.L., G.R. Coelho, M.C.M. Coutinho et al. 2001. The role of phospholipase A(2) and cyclooxygenase in renal toxicity induced by microcystin-LR. Toxicon. 39(5):721-724.

Nobre, A.C.L., A.M.C. Martins, A. Havt et al. 2003. Renal effects of supernatant from rat peritoneal macrophages activated by microcystin-LR: Role protein mediators. Toxicon. 41(3):377-381.

Nobre, A.C.L., S.M. Nunes-Monteiro, M.C.S.A. Monteiro et al. 2004. Microcystin-LR promote intestinal secretion of water and electrolytes in rats. Toxicon. 44:555–559.

O'Brien, E., S. Altheimer, N. Kreke and D.R. Dietrich. 2003. Teratogenicity of cyanobacterial extracts to FETAX embryos. Toxicol. Sci. 72(Suppl. 1):246.

Ohta, T., R. Nishiwaki, J. Yatsunami et al. 1992. Hypersphosphorylation of cytokeratins 8 and 18 by microcystin-LR, a new liver tumor promoter, in primary cultured rat hepatocytes. Carcinogenesis. 13(12):2443-2447.

Ohta, T., E. Sueoka, N. Iida et al. 1994. Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. Cancer Res. 54(24):6402-6406.

Ohtake, A., M. Shirai, T. Aida et al. 1989. Toxicity of *Microcystis* species isolated from natural blooms and purification of the toxin. Appl. Environ. Microbiol. 55(12):3202-3207.

Oishi, S. and M.F. Watanabe. 1986. Acute toxicity of *Microcystis aeruginosa* and its cardiovascular effects. Environ. Res. 40(2):518-524.

Orr, P.T., G.J. Jones, R.A. Hunter et al. 2001. Ingestion of toxic *Microcystis aeuruginosa* by dairy cattle and implications for microcystin contamination of milk. Toxicon. 39(12):1847-1854.

Orr, P.T., G.J. Jones, R.A. Hunter and K. Berger. 2003. Exposure of beef cattle to sub-clinical doses of *Microcystis aeruginosa*: Toxin bioaccumulation, physiological effects and human health risk assessment. Toxicon. 41(5):613-620.

Pace, J.G., N.A. Robinson, G.A. Miura et al. 1991. Toxicity and kinetics of [^{3}H]microcystin-LR in isolated perfused rat livers. Toxicol. Appl. Pharmacol. 107(3):391-401.

Picanço M.R., R.M. Soares, V.R. Cagido, S.M.F.O. Azevedo, P.R.M. Rocco and W.A. Zin. 2004. Toxicity of cyanobacterial extract containing microcystins to mouse lungs. Braz. J. Med. Biol. Res. 37(8):1225-1229.

Pilotto, L.S., R.M. Douglas, M.D. Burch et al. 1997. Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. Aust. NZ J. Pub. Health. 21(6):562-566.

Pilotto, L.S., E.V. Klewer, R.D. Davies, M.D. Burch and R.G. Attewell. 1999. Cyanobacterial (blue-green algae) contamination in drinking water and perinatal outcomes. Aust. NZ J. Pub. Health. 23(2):154-158.

Pilotto L., P. Hobson, M.D. Burch, G. Ranmuthugala, R. Attewell and W. Weightman. 2004. Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers. Aust. NZ J. Pub. Health. 28(3):220-224.

Porfino, Z., M.P. Ribeiro, C.S. Esstevam et al. 1999. Hepatospenomegaly caused by an extract of cyanobacterium *Microcystis aeurignosa* bloom collected in the Manguaba lagoon, Alagoas-Brazil. Rev. Microbiol. 30(3):278-285.

Pouria, S, A. de Andrade, J. Barbosa et al. 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaro, Brazil. Lancet. 352:21-26.

Rao, P.V.L. and R. Bhattacharya. 1996. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver *in vivo*. Toxicology. 114(1):29-36.

Rao, P.V.L., R. Bhattacharya and S.D. Gupta. 1994. Isolation, culture, and toxicity of the cyanobacterium (blue-green alga) *Microcystis aeruginosa* from a freshwater source in India. Bull. Environ. Contam. Toxicol. 52(6):878-885.

Rao, P.V., R. Jayaraj and A.S. Bhaskar. 2004. Protective efficacy and the recovery profile of certain chemoprotectants against lethal poisoning by microcystin-LR in mice. Toxicon. 44(7):723-730.

Rao P.V.L., N. Gupta, R. Jayaraj et al. 2005. Age-dependent effects on biochemical variables and toxicity induced by cyclic peptide toxin microcystin-LR in mice. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 140(1):11-19.

Repavich, W.M., W.C. Sonzogni, J.H. Standridge, R.E. Wedepohl and L.F. Meisner. 1990. Cyanobacteria (blue-green algae) in Wisconsin waters: Acute and chronic toxicity. Water Res. 24(2):225-231.

Rinehart K.L., M. Namikoshi and B.W. Choi. 1994. Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). J. Appl. Phycol. 6:159-176.

Robinson, N.A., G.A. Miura, C.F. Matson et al. 1989. Characterization of chemically tritiated microcystin-LR and its distribution in mice. Toxicon. 27(9):1035-1042.

Robinson, N.A., J.G. Pace, C.F. Matson et al. 1991a. Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. J. Pharmacol. Exp. Therapeut. 256(1):176-182.

Robinson, N.A., C.F. Matson and J.G. Pace. 1991b. Association of microcystin-LR and its biotransformation product with a hepatic-cystolic protein. J. Biochem. Toxicol. 6(3):171-180.

Rogers, E.H., E.S. Hunter, V.C. Moser et al. 2005. Potential developmental toxicity of anatoxin-a, a cyanobacterial toxin. J. Appl. Toxicol. 25(6):527-534.

Runnegar, M.T.C. and I.R. Falconer. 1982. The *in vivo* and *in vitro* biological effects of the peptide hepatotoxin from the blue-green alga *Microcystis aeruginosa*. S. Afr. J. Sci. 78:363-366.

Runnegar, M.T.C. and I.R. Falconer. 1986. Effect of toxin from the cyanobacterium *Microcystis aeruginosa* on ultrastructural morphology and actin polymerization in isolated hepatocytes. Toxicon. 24(2):109-115.

Runnegar, M.T.C., I.R. Falconer and J. Silver. 1981. Deformastion of isolated rat hepatocytes by a peptide hepatoxin from the blue-green alga *Microcystis aeruginosa*. Nayn-Schmied Arch. Pharmacol. 317(3):268-272.

Runnegar, M.T.C., I.R. Falconer, T. Buckley and A.R.B. Jackson. 1986. Lethal potency and tissue distribution of ^{125}I-labelled toxic peptides from the blue-green alga *Microcystis aeruginosa*. Toxicon. 24(5):506-509.

Runnegar, M.T.C., J. Andrews, R.G. Gerdes and I.R. Falconer. 1987. Injury to hepatocytes induced by a peptide toxin from the cyanobacterium *Microcystis aeruginosa*. Toxicon. 25(11):1235-1239.

Runnegar, M.T.C., N. Berndt and N. Kaplowitz. 1991. Identification of hepatic protein phosphatases as novel critical targets for the hepatotoxicity of microcystin *in vivo*. Hepatology. 14(4):A159.

Runnegar, M.T.C., S. Kong and N. Berndt. 1993. Protein phosphatase inhibition and *in vivo* hepatotoxicity of microcystins. Am. J. Physiol. 265(2):G224-G230.

Runnegar, M.T.C., L.D. Deleve and N. Berndt. 1994. The effects of the protein phosphatase inhibitors and microcystin and calyculin A differ in hepatocytes and hepatic endothelial cells. FASEB J. 8(7):1231.

Runnegar, M., N. Berndt, S.M. Kong, E.Y. Lee and L. Zhang. 1995a. *In vivo* and *in vitro* binding of microcystin to protein phosphatases 1 and 2A. Biochem. Biophys. Res. Commun. 216(1):162-169.

Runnegar, M.T.C., N. Berndt and N. Kaplowitz. 1995b. Microcystin uptake and inhibition of protein phosphatases: effects of chemoprotectants and self-inhibition in relation to known hepatic transporters. Toxicol. Appl. Pharmacol. 134(2):264-272.

Sabour, B., M. Loudiki, B. Oudra et al. 2002. Toxicology of a *Microcystis ichthyoblabe* waterbloom from Lake Oued Mellah (Morocco). Environ. Toxicol. 17(1):24-31.

Schaeffer, D.J., P.B. Malpas and L.L. Barton. 1999. Risk assessment of microcystin in dietary *Aphanizomenon flos-aquae*. Ecotoxicol. Environ. Safety. 44(1):73-80.

Sekijima, M., T. Tsutsumi, T. Yoshida et al. 1999. Enhancement of glutathione S-transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B_1-initiated rats. Carcinogenesis. 20(1):161-165.

Sepulveda, M.S., M. Rojas and F. Zambrano. 1992. Inhibitory effect of a *Microcystis* sp. (cyanobacteria) toxin on development of preimplantation mouse embryos. Comp. Biochem. Physiol.-C Pharmacol. Toxicol. Endocrinol. 102(3):549-553.

Shen, X., G.R. Shaw, G.A. Codd et al. 2003. DNA microarray analysis of gene expression in mice treated with the cyanobacterial toxin, cylindrospermopsin. Can. Tech. Rep. Fish Aquat. Sci. 2498:49-51.

Shi, W., H. Zhu, X. Yan and Z. Zhou. 2002. [Sub-acute hepatotoxicity of low doses of microcystins.] Huan Jing Ke Xue. 23(5):47-51. (Chinese)

Shirai, M., Y. Takamura, H. Sakuma et al. 1986. Toxicity and delayed type hypersensitivity caused by *Microcystis* blooms from Lake Kasumigaura. Microbiol. Immunol. 30(7):731-735.

Sicińska, P., B. Bukowska, J. Michalowicz and W. Duda. 2006. Damage of cell membrane and antioxidative system in human erythrocytes incubated with microcystin-LR *in vitro*. Toxicon. 47(4):387-397.

Siegelman, H.W., W.H. Adams, R.D. Stoner and D.N. Slatkin. 1984. Toxins of *Microcystis aeruginosa* and their hematological and histopathological effects. In: Seafood Toxins, E.P. Ragelis, Ed. American Chemical Society, Washington, DC. p. 407-413.

Sim, A.T.R. and L.M. Mudge. 1993. Protein phosphatase activity in cyanobacteria: consequences for microcystin toxicity analysis. Toxicon. 31(9):1179-1186.

Slatkin, D.N., R.D. Stoner, W.H. Adams et al. 1983. Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. Science. 220:1383-1385.

Soares, R.M., M. Yuan, J.C. Servaites et al. 2006. Sublethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro, Brazil. Environ. Toxicol. 21(2):95-103.

Solow, R., K. Mereish, G.W. Anderson, Jr. et al. 1989. Effect of microcystin-LR on cultured rat endothelial cells. Toxicologist. 9(1):160.

Solter, P.F., G.K. Wollenberg, X. Huang et al. 1998. Prolonged sublethal exposure to the protein phosphatase inhibitor microcystin-LR results in multiple dose-dependent hepatotoxic effects. Toxicol. Sci. 44(1):87-96.

Solter, P., Z.L. Lui and R. Guzman. 2000. Decreased hepatic ALT synthesis is an outcome of subchronic microcystin-LR toxicity. Toxicol. Appl. Pharmacol. 164(2):216-220.

Stotts, R.R., M. Namikoshi, W.M. Haschek et al. 1993. Structural modifications imparting reduced toxicity in microcystins from *Microcystis* spp. Toxicon. 31(6):783-789.

Stotts, R.R., A.R. Twardock, W.M. Haschek et al. 1997a. Distribution of tritiated dihydromicrocystin in swine. Toxicon. 35(6):937-953.

Stotts, R.R., A.R. Twardock, G.D. Koritz et al. 1997b. Toxicokinetics of tritiated dihydromicrocystin-LR in swine. Toxicon. 35(3):455-465.

Suzuki, H., M.F. Watanabe, Y.P. Yu et al. 1998. Mutagenicity of microcystin-LR in human RSA cells. Int. J. Mol. Med. 2(1):109-112.

Takahashi, O., S. Oishi and M.F. Watanabe. 1995. Defective blood coagulation is not causative of hepatic haemorrhage induced by microcystin-LR. Pharmacol. Toxicol. 76(4):250-254.

Takenaka, S. 2001. Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin-LR by F344 rat cytosolic and microsomal glutathione S-transferases. Environ. Toxicol. Pharmacol. 9(4):135-139.

Tarczynska M., G. Nalecz-Jawecki, M. Brzychcy et al. 2000. The toxicity of cyanobacterial blooms as determined by microbiotests and mouse assays. In: New Microbiotests for Routine Toxicity Screening and Biomonitoring, Persoone et al., Eds. Kluwer Academic/Plenum Publishers, New York, NY. pp. 527-532.

Teixeira, M.G.L.C., M.C.N. Costa, V.L.P. Carvalho et al. 1993. Gastroenteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil. Bulletin of PAHO. 27(3):244-253.

Teneva, I., R. Mladenov, N. Popov and B. Dzhambazov. 2005. Cytotoxicity and apoptotic effects of microcystin-LR and anatoxin-a in mouse lymphocytes. Folia. Biol. (Praha). 51(3):62-67.

Theiss, W.C., W.W. Carmichael, M.M. Mullins and R.H. Bruner. 1984. Hepatotoxicity of the blue-green algae (cyanobacteria) *Microcystis aeruginosa*. Toxicologist. 4(1):12.

Theiss, W.C., W.W. Carmichael, W.W. Mullins et al. 1985. Direct rapid hepatotoxicity of the cyanobacteria *Microcystis aeruginosa*. Toxicon. 23(1):40-41.

Theiss, W.C., W.W. Carmichael, J. Wyman and R. Bruner. 1988. Blood pressure and hepatocellular effects of the cyclic heptapeptide toxin produced by *Microsystis aeruginosa* strain PCC-7820. Toxicon. 26(7):603-613.

Thiel, P. 1994. The South African contribution to studies on the toxic cyanobacteria and their toxins. In: Toxic Cyanobacteria: Current Status of Research and Management. Proceedings of an International Workshop. Adelaide, Australia, March 22-26. D.A. Steffensen and B.C. Nicholson, Ed. Australian Centre for Water Quality Research, Salisbury, Australia. pp. 23-27.

Thomspon, W.L. and J.G. Pace. 1992. Substances that protect cultured hepatocytes from the toxic effects of microcystin-LR. Toxicol. In Vitro. 6(6):579-587.

Thompson, W.L., M.B. Allen and K.A. Bostian. 1988. The effects of microcystin on monolayers of primary rat hepatocytes. Toxicon. 26(1):44.

Toivola, D., J.E. Eriksson and D.L. Brautigan. 1994. Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates. FEBS Lett. 344(2-3):175-180.

Toivola, D.M., R.D. Goldman, D.R. Garrod and J.E. Eriksson. 1997. Protein phosphatases maintain the organization and structural interactions of hepatic keratin intermediate filaments. J. Cell Sci. 110(Pt. 1):23-33.

Towner, R.A., S.A. Sturgeon and K.E. Hore. 2002. Assessment of *in vivo* oxidative lipid metabolism following acute microcystin-LR-induced hepatotoxicity in rats. Free Radical Res. 36(1):63-71.

Turner, P.C., A.J. Gammie, K. Hollinrake and G.A. Codd. 1990. Pneumonia associated with contact with cyanobacteria. Br. Med. J. 300(6737):1440-1441.

Ueno, Y., S. Nagata, T. Tsutsumi et al. 1996. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis. 17(6):1317-1321.

Ueno, Y., Y. Makita, S. Nagata et al. 1999. No chronic oral toxicity of a low-dose of microcystin-LR, a cyanobacterial hepatoxin, in female Balb/C mice. Environ. Toxicol. 14(1):45-55.

U.S. EPA. 1986a. Guidelines for the Health Risk Assessment of Chemical Mixtures. Fed. Reg. 51(185):34014-34025.

U.S. EPA. 1986b. Guidelines for Mutagenicity Risk Assessment. Fed. Reg. 51(185):34006-34012.

U.S. EPA. 1988. Recommendations for and Documentation of Biological Values for Use in Risk Assessment. U.S. Environmental Protection Agency, Office of Research and Development, Washington, DC. EPA 600/6-87/008. NTIS PB88-179874/AS.

U.S. EPA. 1991. Guidelines for Developmental Toxicity RiskAssessment. Fed. Reg. 56(234):63798-63826.

U.S. EPA. 1994a. Interim Policy for Particle Size and Limit Concentration Issues in Inhalation Toxicity Studies. Fed. Reg. 59(206):53799.

U.S. EPA. 1994b. Methods for Derivation of Inhalation Reference Concentrations and Application of Inhalation Dosimetry. U.S. Environmental Protection Agency, Office of Research and Development, Washington, DC. EPA/600/8-90/066F. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 1995. Use of the Benchmark Dose Approach in Health Risk Assessment. U.S. Environmental Protection Agency. U.S. Environmental Protection Agency, Office of Research and Development, Washington, DC. EPA/630/R-94/007. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 1996. Guidelines for Reproductive Toxicity Risk Assessment. Fed. Reg. 61(212):56274-56322.

U.S. EPA. 1998a. Guidelines for Neurotoxicity Risk Assessment. Fed. Reg. 63(93):26926-26954.

U.S. EPA. 1998b. Science Policy Council Handbook: Peer Review. Prepared by the Office of Science Policy, Office of Research and Development, Washington, DC. EPA/100/B-98/001. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2000a. Science Policy Council Handbook: Peer Review, 2nd ed. U.S. Environmental Protection Agency, Office of Research and Development, Office of Science Policy, Washington, DC. EPA/100/B-00/001. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2000b. Science Policy Council Handbook: Risk Characterization. U.S. Environmental Protection Agency, Office of Research and Development, Office of Science Policy, Washington, DC. EPA/100/B-00/002. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2000c. Benchmark Dose Technical Guidance Document: External Review Draft. U.S. Environmental Protection Agency, Office of Research and Development, Office of Science Policy, Washington, DC. EPA/630/R-00/001. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2000d. Supplemental Guidance for Conducting for Health Risk Assessment of Chemical Mixtures. U.S. Environmental Protection Agency, Office of Research and Development, Office of Science Policy, Washington, DC. EPA/630/R-00/002. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2002. A Review of the Reference Dose and Reference Concentration Processes. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC. EPA/630/P-02/0002F. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2005a. Guidelines for Carcinogen Risk Assessment. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC. EPA/630/P-03/001B. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2005b. Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. U.S. Environmental Protection Agency, Risk Assessment Forum, Washington, DC. EPA/630/R-03/003F. Available at http://www.epa.gov/iris/backgr-d.htm.

U.S. EPA. 2005c. Peer Review Handbook. 3rd edition. Review draft. Science Policy Council, Washington, DC. Available at: http://intranet.epa.gov/ospintra/scipol/prhndbk05.doc.

Vesterkvist, P.S. and J.A. Meriluoto. 2003. Interaction between microcystins of different hydrophobicities and lipid monolayers. Toxicon. 41(3): 349-355.

WHO (World Health Organization). 1999. Toxic Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring, and Management, I. Chorus and J. Bartram, Eds. E&FN Spon, London, UK

Wickstrom, M.L., S.A. Khan, W.M. Haschek et al. 1995. Alterations in microtubules, intermediate filaments and microfilaments induced by microcystin-LR in cultured cells. Toxicologic. Pathol. 23(3):326-337.

Wolf, H.-U. and C. Frank. 2002. Toxicity assessment of cyanobacerial toxin mixtures. Environ. Toxicol. 17(4): 395-399.

Xu, L., P.K.S. Lam, J. Chen et al. 2000. Comparative study on *in vitro* inhibition of grass carp (*Ctenopharyngodon idellus*) and mouse protein phosphatases by microcystins. Environ. Toxicol. 15(2):71-75.

Yea, S.S., Y.I. Yang, W.H. Jang and K.H. Paik. 2001. Microcystin-induced proinflammatory cytokines expression and cell death in human hepatocytes. Hepatology. 34(4 Pt. 2 Suppl.):516A

Yoshida, T., Y. Makita, S. Nagata et al. 1997. Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin in mice. Nat. Toxins. 5:91-95.

Yoshida, T., Y. Makita, T. Tsutsumi et al. 1998. Immunohistochemical localization of microcystin-LR in the liver of mice: A study on the pathogenesis of microcystin-LR-induced hepatotoxicity. Toxicologic. Pathol. 26(3):411-418.

Yoshizawa, S., R. Matsushima, M.F. Watanabe et al. 1990. Inhibition of protein phosphatases by *Microcystis* and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. 116(6):609-614.

Yu, S.-Z. 1989. Drinking water and primary liver cancer. In: Primary Liver Cancer, Z.Y. Tang, M.C. Wu and S.S. Xia, Ed. China Academic Publishers, New York, NY. p. 30-37 (as cited in Ueno et al., 1996 and Health Canada, 2002).

Yu, S.-Z., Z.-Q. Chen, Y.-K. Liu, Z.-Y. Huang and Y.-F. Zhao. 1989. The aflatoxins and contaminated water in the etiological study of primary liver cancer. In: Mycotoxins and Phycotoxins '88, S. Natori, K. Hashimoto and Y. Ueno, Eds. Elsevier, Amsterdam. p. 37-44 (as cited in Ueno et al., 1996 and Health Canada, 2002).

Yu, S.Z., X.E. Huang, T. Koide et al. 2002. Hepatitis B and C viruses infection, lifestyle and genetic polymorphisms as risk factors for hepatocellular carcinoma in Haimen, China. Jpn. J. Cancer Res. 93(12):1287-1292.

Zegura, B., B. Sedmak and M. Filipic. 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon. 41(1):41-48.

Zegura, B., T.T. Lah and M. Filipic. 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology. 200(1):59-68.

Zhan, L., M. Sakamoto, M. Sakuraba et al. 2004. Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells. Mutat. Res. 557(1):1-6.

Zhang, Z., S. Kang, C. Chen et al. 2002. [The acute toxic effects of microcystin-LR in SD rats.] Zhonghua Yu Fang Yi Xue Za Zhi. 36(5):295-297. (Chinese)

Zhao, J.M. and H.G. Zhu. 2003. [Effects of microcystins on cell cycle and expressions of c-fos and c-jun.] Zhonghua Yu Fang Yi Xue Za Zhi. 37(1):23-25. (Chinese)

Zhou, L., H. Yu and K. Chen. 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed. Environ. Sci. 15(2):166-171.

Zhu, Y., X. Zhong, S. Zheng, Z. Ge, Q. Du and S. Zhang. 2005. Transformation of immortalized colorectal crypt cells by microcystin involving constitutive activation of Akt and MAPK cascade. Carcinogenesis. 26(7):1207-1214.

Zuzek, M.C., M. Kosec, J. Mrkum et al. 2003. Microcystin-LR causes reorganization of actin filaments and microtubules in rabbit whole embryo cultures. Toxicol. Lett. 144(Suppl 1):568.

APPENDIX A

Contents: Benchmark Dose Modeling Output Files for:

1) Heinze, 1999 Liver Lesions
2) Fawell et al., 1999 Male Chronic Liver Inflammation
3) Fawell et al., 1999 Female Chronic Liver Inflammation
4) Fawell et al., 1999 Male and Female (combined) Chronic Liver Inflammation
5) Heinze, 1999 Relative Liver Weight Changes
6) Heinze, 1999 Lactate Dehydrogenase Changes
7) Heinze, 1999 Alkaline Phosphatase Changes
8) Fawell et al., 1999 Male Alanine Aminotransferase Changes

Heinze,1999. Liver Lesions

```
================================================================
         $Revision: 2.2 $ $Date: 2001/03/14 01:17:00 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                        Thu Jul 14 15:03:08 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cummulative Gamma distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope =    0.0153804
                          Power =      1.02976


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background    -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

                  Slope

     Slope            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                   NA
          Slope          0.0166997           0.00500499
          Power                  1                   NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
```

Full model	-9.98095				
Fitted model	-10.0255	0.089063	2	0.9564	
Reduced model	-20.7944	21.6269	2	<.0001	

AIC: 22.051

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0
50.0000	0.5661	5.661	6	10	0.2162
150.0000	0.9183	9.183	9	10	-0.2115

Chi-square = 0.09 DF = 2 P-value = 0.9553

Benchmark Dose Computation

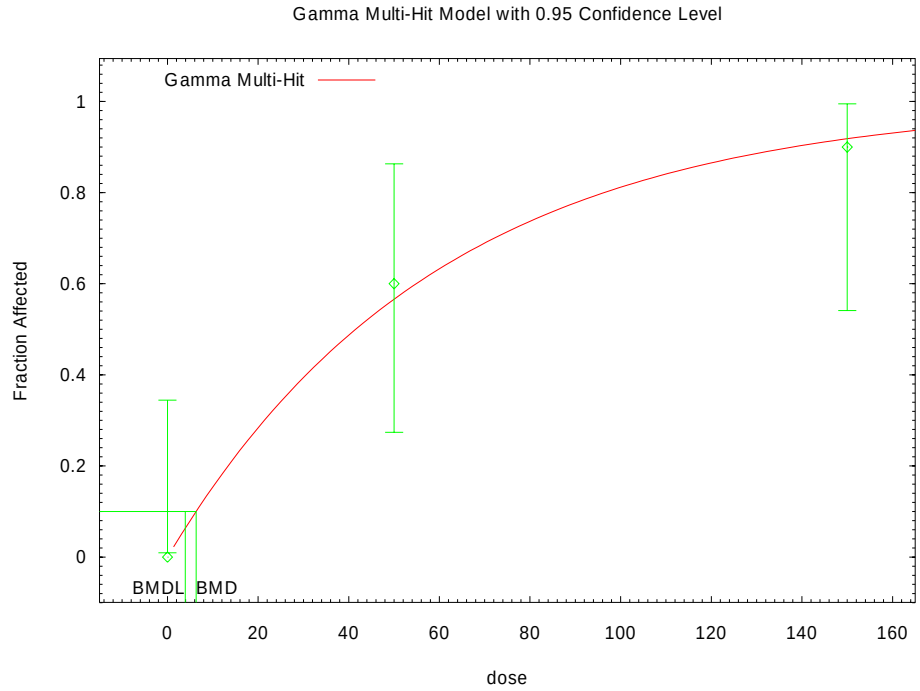
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 6.30914

BMDL = 3.92229



```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\HEINZE_LIVER_LESIONS.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\HEINZE_LIVER_LESIONS.plt
                                          Thu Mar 09 11:41:20 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = 1/[1+EXP(-intercept-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     background =            0   Specified
                      intercept =     -2.28075
                          slope =    0.0300564


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.75

     slope        -0.75            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
      intercept            -2.02314            0.772069
          slope           0.0344016           0.0123743


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -9.98095
     Fitted model        -12.1529       4.34392      1         0.03714
```

Reduced model	-20.7944	21.6269	2	<.0001

AIC: 28.3058

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1168	1.168	0	10	-1.15
50.0000	0.4248	4.248	6	10	1.121
150.0000	0.9584	9.584	9	10	-0.9248

Chi-square = 3.43 DF = 1 P-value = 0.0639

Benchmark Dose Computation

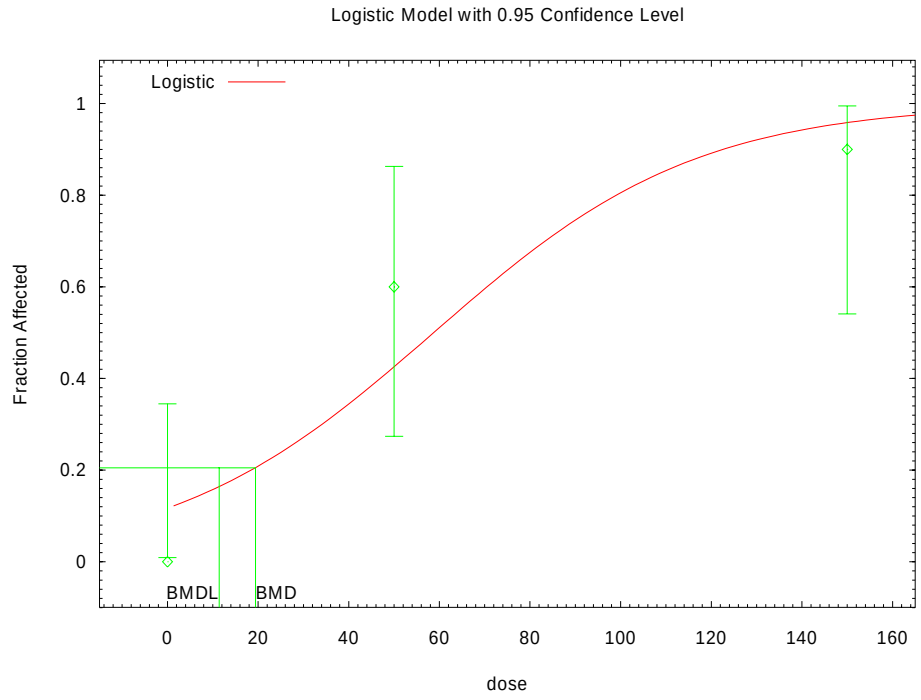
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 19.4327

BMDL = 11.4032



```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                        Thu Jul 14 15:04:46 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial Parameter Values
                    background =            0
                     intercept =     -5.97477
                         slope =      1.63093


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.99

     slope        -0.99            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
     background                  0                   NA
      intercept            -5.97477             4.77026
          slope             1.63093             1.12507

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.

 Warning: Likelihood for the fitted model larger than the Likelihood for the
full model.
```

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value	
Full model	-9.98095				
Fitted model	-9.98095	-3.55271e-015	1		-1
Reduced model	-20.7944	21.6269	2	<.0001	

AIC: 23.9619

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0
50.0000	0.6000	6.000	6	10	-3.44e-015
150.0000	0.9000	9.000	9	10	-1.123e-014

Chi-square = 0.00 DF = 1 P-value = 1.0000

Benchmark Dose Computation

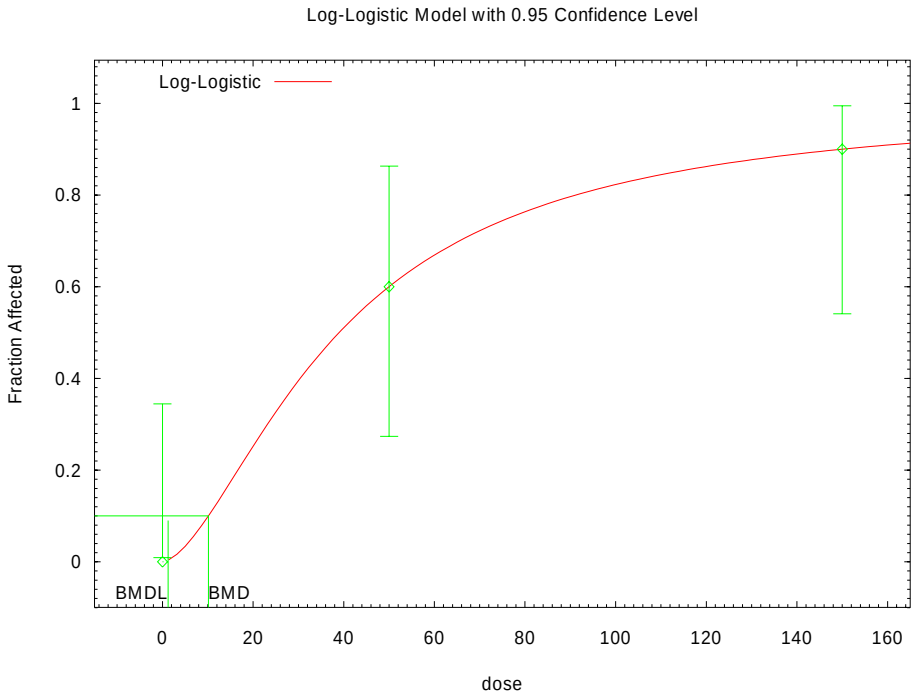
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 10.137

BMDL = 1.23783



1 15:04 07/14 2005

```
=================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\HEINZE_LIVER_LESIONS.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\HEINZE_LIVER_LESIONS.plt
                                          Thu Mar 09 11:37:17 2006
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 3
 Total number of specified parameters = 0
 Degree of polynomial = 2


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =    0.0617654
                        Beta(1) =     0.015138
                        Beta(2) =            0


            Asymptotic Correlation Matrix of Parameter Estimates

 ( *** The model parameter(s)  -Background    -Beta(2)
 have been estimated at a boundary point, or have been specified by the
user, and do not appear in the correlation matrix )

                Beta(1)

   Beta(1)            1



                           Parameter Estimates

        Variable           Estimate             Std. Err.
      Background                  0                   NA
         Beta(1)          0.0166997           0.00582148
         Beta(2)                  0                   NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.
```

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-9.98095			
Fitted model	-10.0255	0.089063	2	0.9564
Reduced model	-20.7944	21.6269	2	<.0001

AIC: 22.051

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	10	-1.000
i: 2					
50.0000	0.5661	5.661	6	10	0.138
i: 3					
150.0000	0.9183	9.183	9	10	-0.244

Chi-square = 0.09 DF = 1 P-value = 0.7623

Benchmark Dose Computation

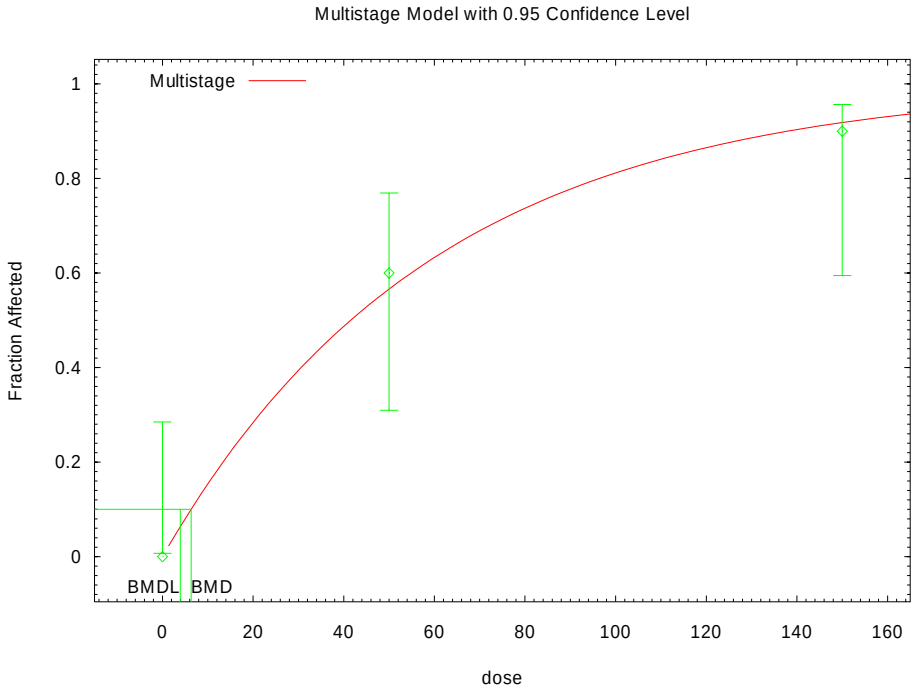
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 6.30914

BMDL = 3.92229


Multistage Model with 0.95 Confidence Level
Multistage
Fraction Affected
1
0.8
0.6
0.4
0.2
0
BMDL
BMD
0
20
40
60
80
100
120
140
160
dose
11:37 03/09 2006

1
2
3

```
 ======================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                          Thu Jul 14 15:05:31 2005
 ======================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 3
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =    0.0617654
                        Beta(1) =     0.015138


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

                Beta(1)

   Beta(1)            1



                           Parameter Estimates

        Variable           Estimate             Std. Err.
      Background                  0                   NA
         Beta(1)          0.0166997           0.00582148

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.
```

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-9.98095			
Fitted model	-10.0255	0.089063	2	0.9564
Reduced model	-20.7944	21.6269	2	<.0001

AIC: 22.051

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0000	0.000	0	10	0.000
i: 2					
50.0000	0.5661	5.661	6	10	0.138
i: 3					
150.0000	0.9183	9.183	9	10	-0.244

Chi-square = 0.09 DF = 2 P-value = 0.9553

Benchmark Dose Computation

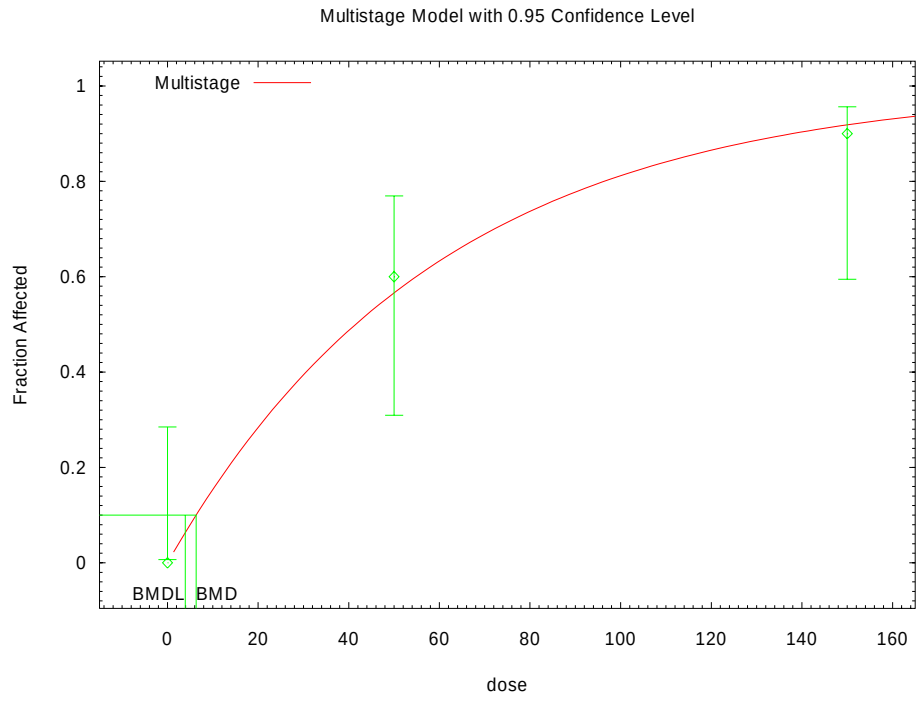
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 6.30914

BMDL = 3.92229



15:05 07/14 2005

1

```
1
2   ========================================================================
3           Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
4           Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
5   TOX REV\HEINZE_LIVER_LESIONS.(d)
6           Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
7   DOCUMENTS\_CYANO TOX REV\HEINZE_LIVER_LESIONS.plt
8                                             Thu Mar 09 11:49:47 2006
9    ========================================================================
10
11   BMDS MODEL RUN
12  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
13
14     The form of the probability function is:
15
16     P[response] = CumNorm(Intercept+Slope*Dose),
17
18     where CumNorm(.) is the cumulative normal distribution function
19
20
21     Dependent variable = COLUMN2
22     Independent variable = COLUMN1
23     Slope parameter is not restricted
24
25     Total number of observations = 3
26     Total number of records with missing values = 0
27     Maximum number of iterations = 250
28     Relative Function Convergence has been set to: 1e-008
29     Parameter Convergence has been set to: 1e-008
30
31
32
33                    Default Initial (and Specified) Parameter Values
34                       background =            0   Specified
35                        intercept =     -1.57069
36                            slope =    0.0201403
37
38
39              Asymptotic Correlation Matrix of Parameter Estimates
40
41              ( *** The model parameter(s)  -background
42                    have been estimated at a boundary point, or have been
43   specified by the user,
44                    and do not appear in the correlation matrix )
45
46                 intercept        slope
47
48    intercept            1        -0.75
49
50        slope        -0.75            1
51
52
53
54                              Parameter Estimates
55
56           Variable           Estimate             Std. Err.
57          intercept              -1.209             0.433188
58              slope           0.0190194          0.00553748
59
60
61
62                           Analysis of Deviance Table
63
64           Model      Log(likelihood)  Deviance  Test DF     P-value
```

Full model	-9.98095				
Fitted model	-12.2154	4.46887	1	0.03452	
Reduced model	-20.7944	21.6269	2	<.0001	

AIC: 28.4308

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1133	1.133	0	10	-1.131
50.0000	0.3982	3.982	6	10	1.304
150.0000	0.9499	9.499	9	10	-0.7234

Chi-square = 3.50 DF = 1 P-value = 0.0613

Benchmark Dose Computation

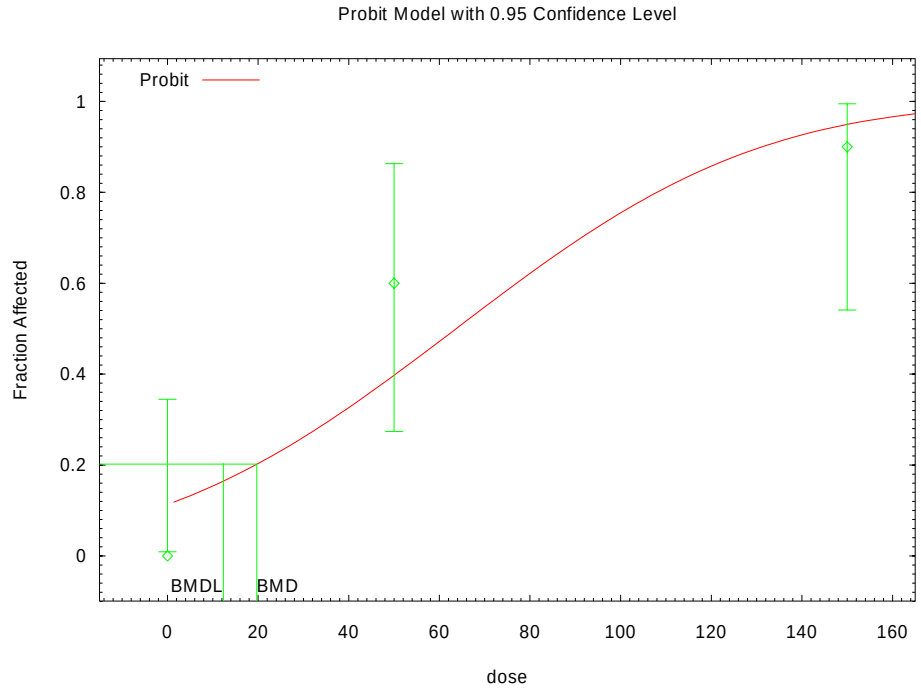
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 19.6901

BMDL = 12.3138



```
==========================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                          Thu Jul 14 15:06:15 2005
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial (and Specified) Parameter Values
                    background =            0
                     intercept =     -3.68466
                         slope =            1


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background    -slope
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept

 intercept            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
     background                  0                   NA
      intercept            -3.68338             0.323658
          slope                  1                   NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus has no standard error.
```

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-9.98095			
Fitted model	-9.98638	0.0108688	2	0.9946
Reduced model	-20.7944	21.6269	2	<.0001

AIC: 21.9728

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0
50.0000	0.5904	5.904	6	10	0.06155
150.0000	0.9078	9.078	9	10	-0.08513

Chi-square = 0.01 DF = 2 P-value = 0.9945

Benchmark Dose Computation

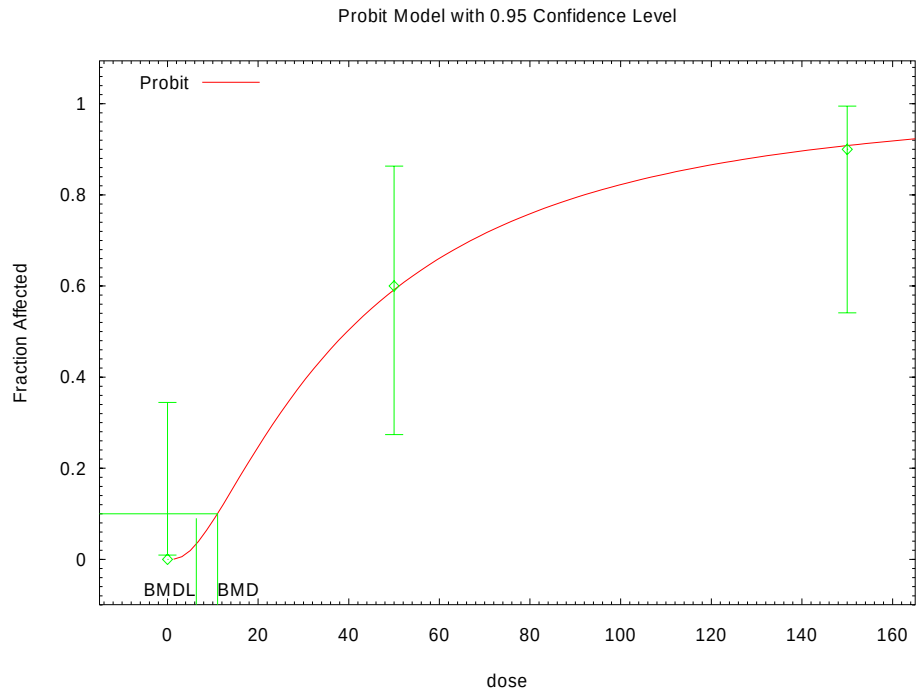
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 11.0433

BMDL = 6.37572



```
 ================================================================
        Quantal Linear Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                      Thu Jul 14 15:07:01 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope =    0.0129727
                          Power =            1   Specified


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background    -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

                  Slope

     Slope            1



                          Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                   NA
          Slope          0.0166997           0.00500498

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                        Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -9.98095
    Fitted model         -10.0255     0.089063      2          0.9564
   Reduced model         -20.7944      21.6269      2         <.0001

           AIC:           22.051
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0
50.0000	0.5661	5.661	6	10	0.2162
150.0000	0.9183	9.183	9	10	-0.2115

Chi-square = 0.09 DF = 2 P-value = 0.9553

Benchmark Dose Computation

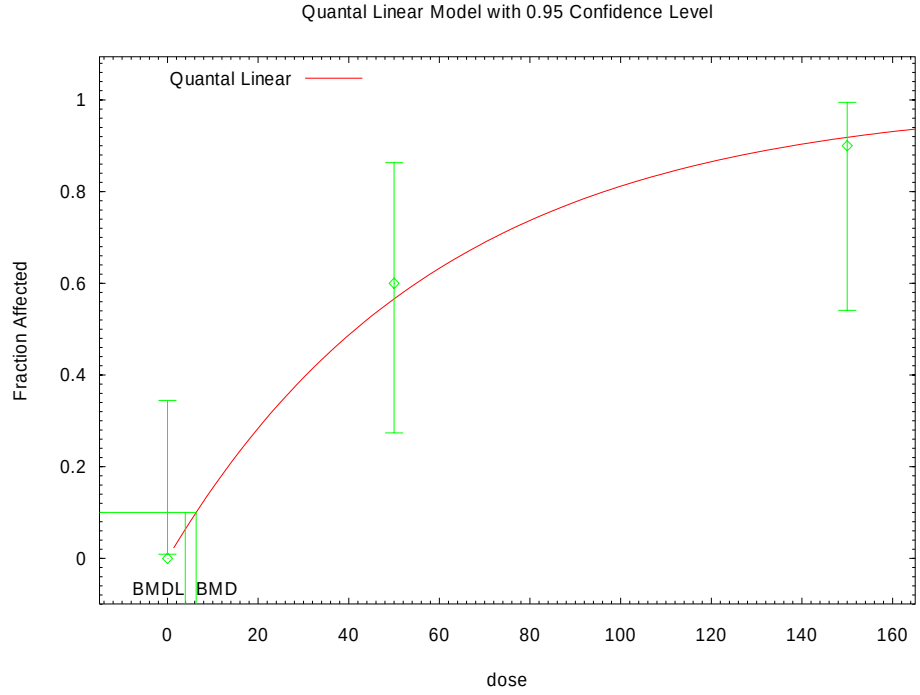
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 6.30914

BMDL = 3.92229



```
 ================================================================
        Quantal Quadratic Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                        Thu Jul 14 15:07:40 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^2)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope = 8.64849e-005
                          Power =            2   Specified


            Asymptotic Correlation Matrix of Parameter Estimates

            ( *** The model parameter(s)  -Background    -Power
                  have been estimated at a boundary point, or have been
specified by the user,
                  and do not appear in the correlation matrix )

                   Slope

     Slope            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                    NA
          Slope        0.000171114          5.74454e-005

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -9.98095
    Fitted model         -12.0873       4.21262      2          0.1217
   Reduced model         -20.7944       21.6269      2         <.0001

            AIC:         26.1745
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0000	0.000	0	10	0
50.0000	0.3480	3.480	6	10	1.673
150.0000	0.9787	9.787	9	10	-1.725

Chi-square = 5.77 DF = 2 P-value = 0.0558

Benchmark Dose Computation

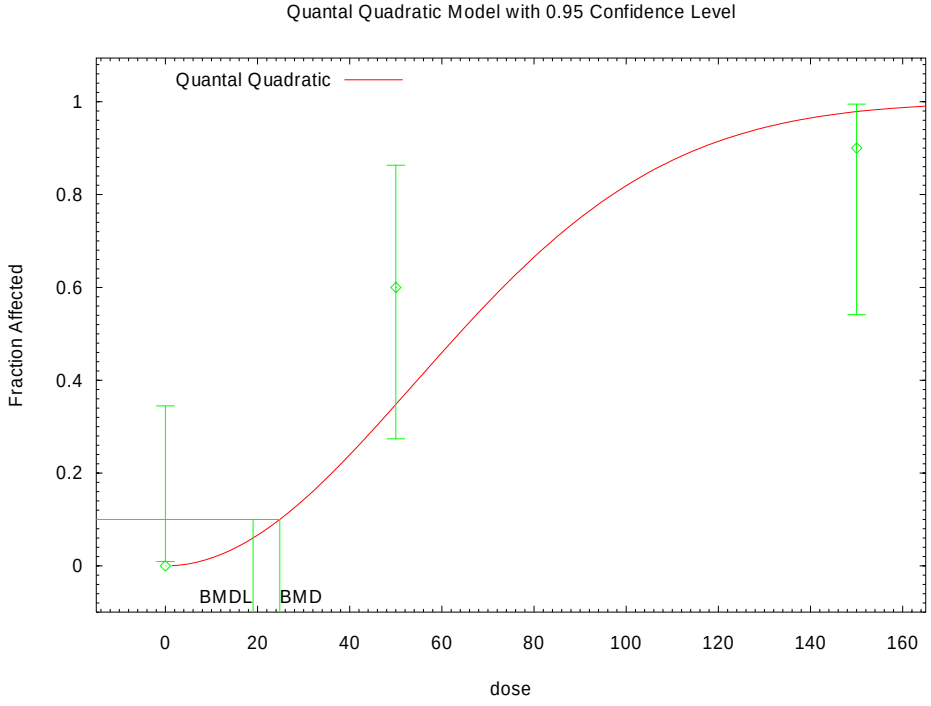
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

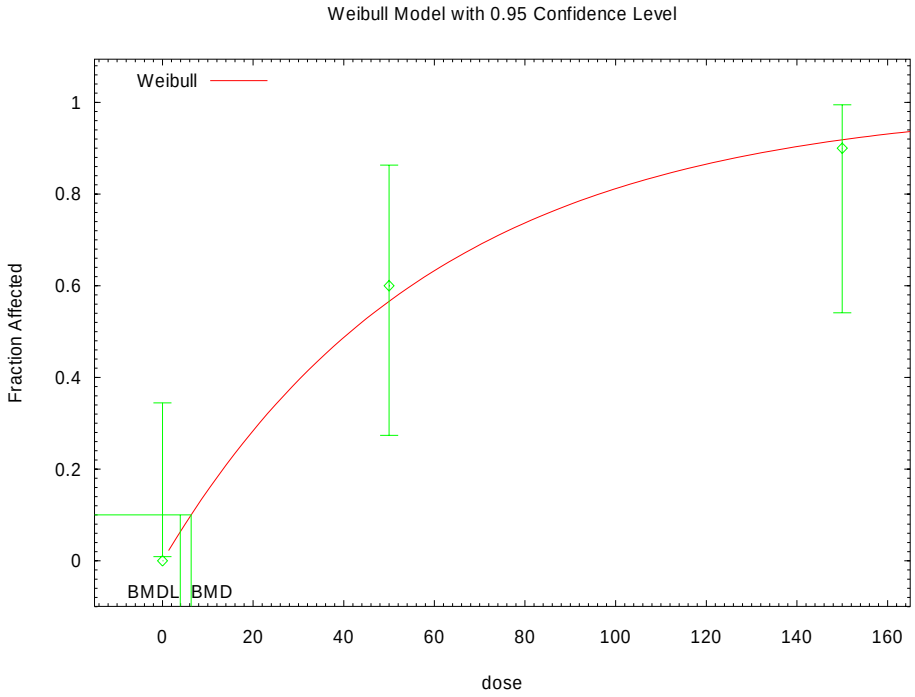
BMD = 24.814

BMDL = 19.01



```
 ================================================================
        Weibull Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.(d)
        Gnuplot Plotting File:  C:\BMDS\HEINZE_MOD_AND_INT_WITH_HEMORRHAGE.plt
                                          Thu Jul 14 15:08:20 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial (and Specified) Parameter Values
                     Background =    0.0454545
                          Slope =    0.0129727
                          Power =            1


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Background    -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

                  Slope

     Slope            1


                          Parameter Estimates

       Variable           Estimate             Std. Err.
     Background                  0                   NA
          Slope          0.0166997           0.00500498
          Power                  1                   NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                        Analysis of Deviance Table

       Model      Log(likelihood)  Deviance  Test DF     P-value
     Full model        -9.98095
    Fitted model       -10.0255     0.089063      2          0.9564
```

```
Reduced model        -20.7944       21.6269      2         <.0001

          AIC:          22.051


                    Goodness  of  Fit

                                                                          Scaled
    Dose      Est._Prob.    Expected    Observed      Size        Residual
  ------------------------------------------------------------------------------
   0.0000      0.0000          0.000          0          10               0
  50.0000      0.5661          5.661          6          10          0.2162
 150.0000      0.9183          9.183          9          10         -0.2115

 Chi-square =       0.09     DF = 2        P-value = 0.9553


   Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =      Extra risk

Confidence level =           0.95

             BMD =        6.30914

            BMDL =       3.92229
```



Fawell et al.,1999 Male Chronic Inflammation

```
=================================================================
         $Revision: 2.2 $ $Date: 2001/03/14 01:17:00 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:45:24 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cummulative Gamma distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.09375
                          Slope =   0.00193589
                          Power =          1.3


            Asymptotic Correlation Matrix of Parameter Estimates

              Background        Slope        Power

Background            1        0.037        0.041

     Slope        0.037            1            1

     Power        0.041            1            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background          0.0999826            0.054818
          Slope          0.0276638            0.488219
          Power            8.09035             116.456


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
    Fitted model         -18.4512     0.376765      1          0.5393
   Reduced model         -39.4295      42.3333      3         <.0001
```

AIC: 42.9024

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1000	1.500	1	15	-0.4301
40.0000	0.1000	1.500	2	15	0.4304
200.0000	0.2667	4.001	4	15	-0.0002949
1000.0000	1.0000	15.000	15	15	0.00692

Chi-square = 0.37 DF = 1 P-value = 0.5429

Benchmark Dose Computation

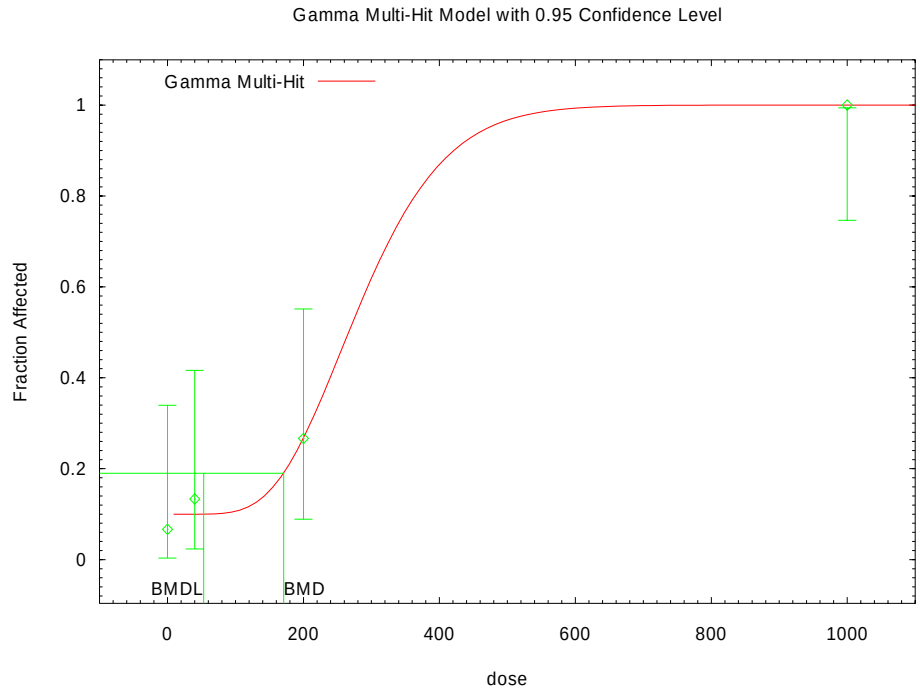
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 170.825

BMDL = 53.1183



```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 11:56:58 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = 1/[1+EXP(-intercept-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                     background =            0   Specified
                      intercept =     -2.07771
                          slope =   0.00552538


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.72

     slope        -0.72            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
      intercept            -2.49527            0.612166
          slope          0.00805129          0.00337645


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
     Fitted model        -18.4278      0.329996      2          0.8479
    Reduced model        -39.4295       42.3333      3         <.0001

           AIC:          40.8556
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0762	1.143	1	15	-0.139
40.0000	0.1022	1.533	2	15	0.3983
200.0000	0.2921	4.382	4	15	-0.217
1000.0000	0.9962	14.942	15	15	0.2408

Chi-square = 0.28 DF = 2 P-value = 0.8680

Benchmark Dose Computation

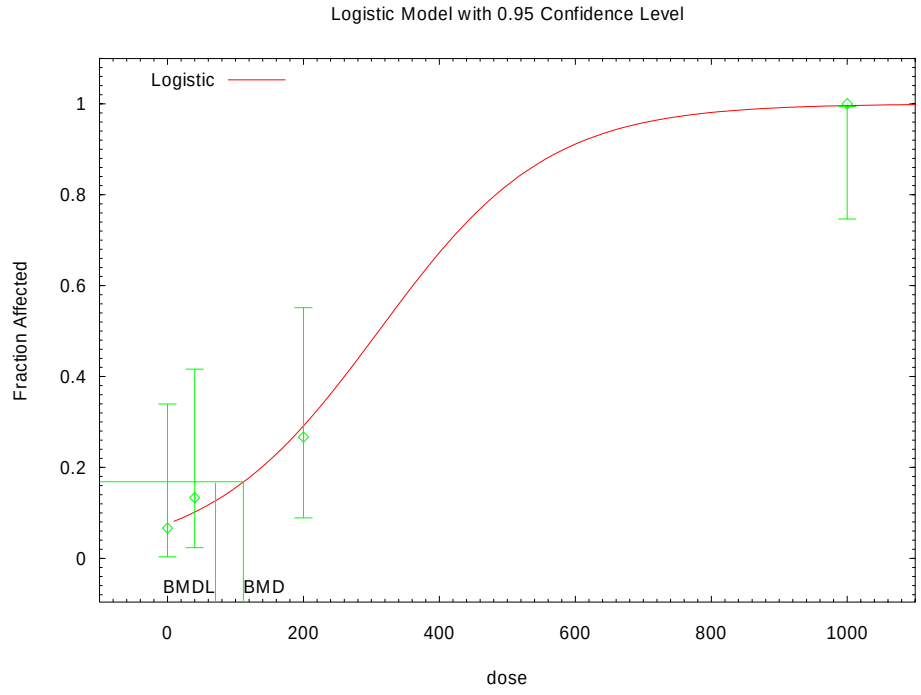
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 111.719

BMDL = 70.686



```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:47:11 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial Parameter Values
                    background =    0.0666667
                     intercept =     -10.0178
                         slope =      1.86367


           Asymptotic Correlation Matrix of Parameter Estimates

             background    intercept        slope

background            1       0.0022      -0.0023

 intercept       0.0022            1           -1

     slope      -0.0023           -1            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     background          0.0999997           0.0547725
      intercept           -65.9875             5466.64
          slope            12.1748             1031.77


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
     Fitted model        -18.4512     0.376844      1          0.5393
    Reduced model        -39.4295      42.3333      3         <.0001

            AIC:         42.9024
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1000	1.500	1	15	-0.4303
40.0000	0.1000	1.500	2	15	0.4303
200.0000	0.2667	4.000	4	15	-4.206e-005
1000.0000	1.0000	15.000	15	15	0.0004285

Chi-square = 0.37 DF = 1 P-value = 0.5428

Benchmark Dose Computation

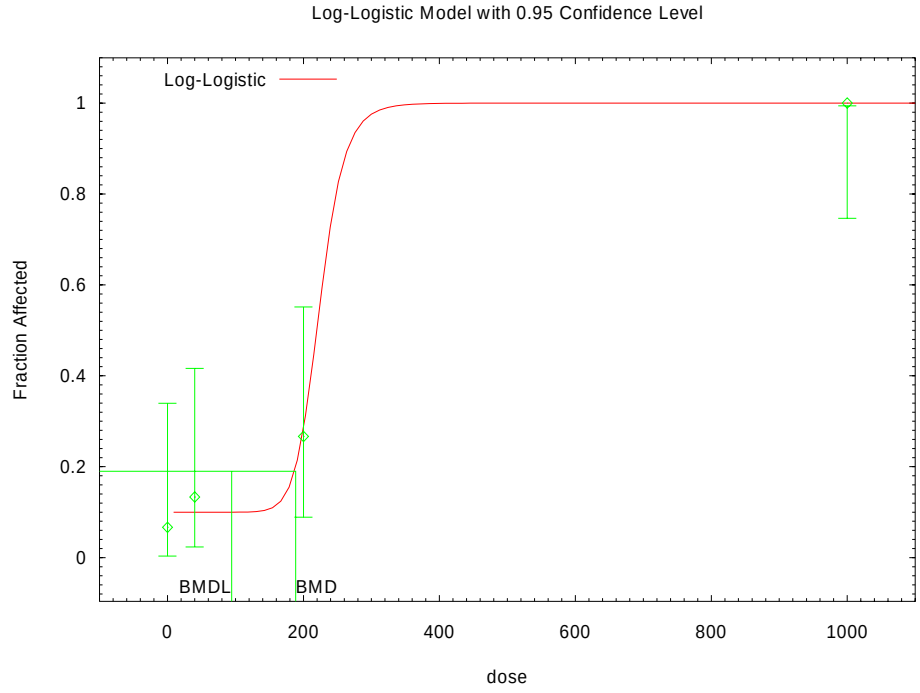
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

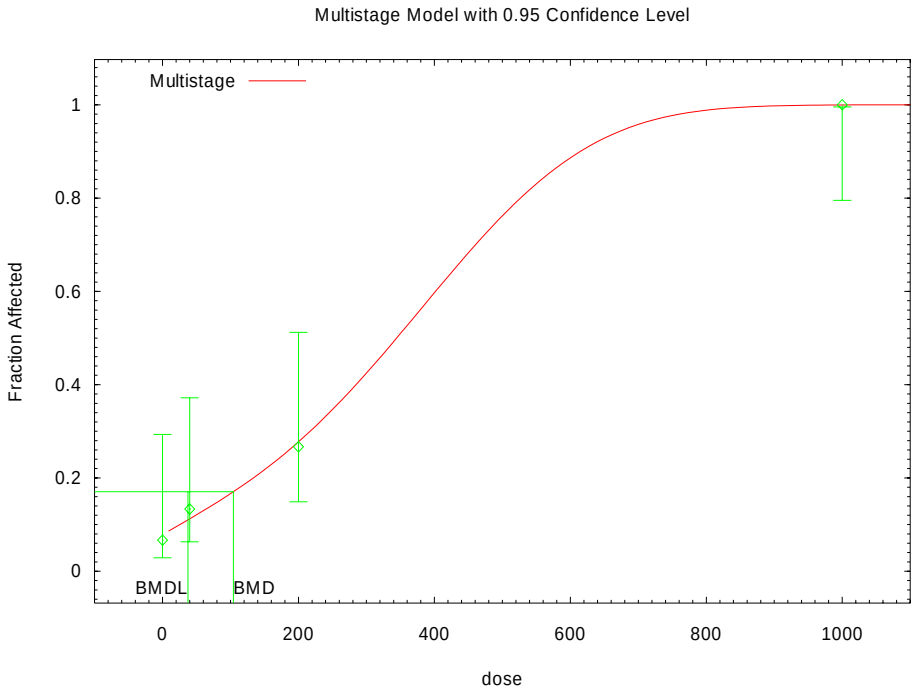
BMD = 188.582

BMDL = 94.487



```
================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                          Thu Mar 09 11:59:44 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                     Background =            0
                        Beta(1) =            0
                        Beta(2) =            0
                        Beta(3) = 1.00264e+011


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Beta(2)
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

             Background      Beta(1)      Beta(3)

Background            1        -0.65         0.14

   Beta(1)        -0.65            1        -0.42

   Beta(3)         0.14        -0.42            1


                          Parameter Estimates

        Variable           Estimate             Std. Err.
      Background          0.0782274            0.200883
         Beta(1)         0.000933123          0.00219636
         Beta(2)                   0                  NA
         Beta(3)        7.10432e-009        1.57018e-008
```

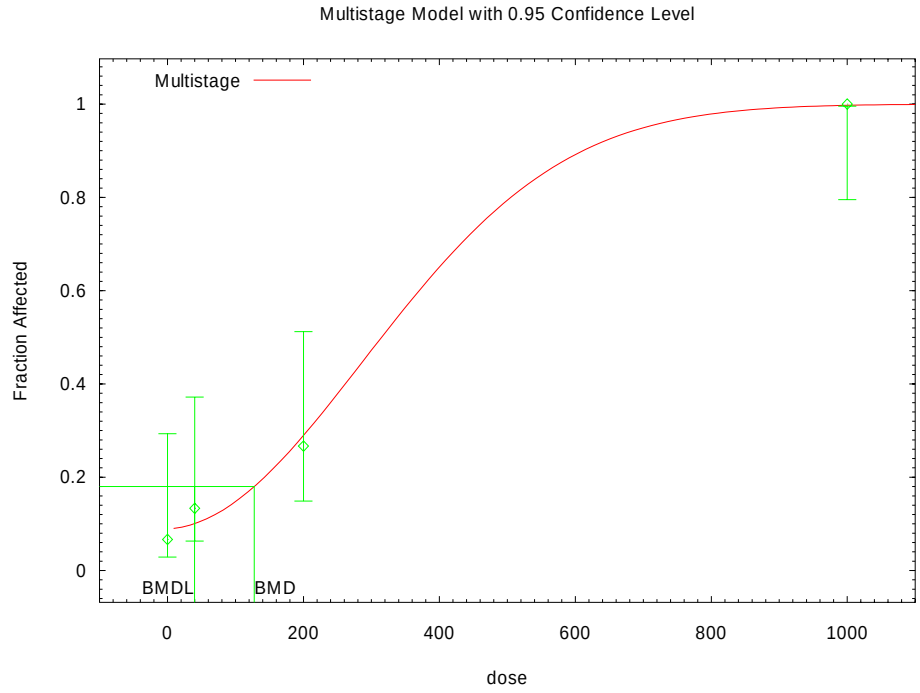
```
NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.

                        Analysis of Deviance Table

       Model      Log(likelihood)  Deviance  Test DF     P-value
     Full model         -18.2628
    Fitted model        -18.3175       0.10941      1          0.7408
   Reduced model        -39.4295       42.3333      3         <.0001

            AIC:          42.635


                     Goodness  of  Fit

      Dose     Est._Prob.    Expected    Observed     Size     Chi^2 Res.
  ------------------------------------------------------------------------
i: 1
    0.0000     0.0782         1.173         1          15      -0.160
i: 2
   40.0000     0.1124         1.686         2          15       0.210
i: 3
  200.0000     0.2774         4.161         4          15      -0.054
i: 4
 1000.0000     0.9997        14.996        15          15       1.000

 Chi-square =       0.11     DF = 1        P-value = 0.7438


   Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =      Extra risk

Confidence level =           0.95

             BMD =        104.279

            BMDL =        37.4815
```


Multistage Model with 0.95 Confidence Level
Multistage
Fraction Affected
1
0.8
0.6
0.4
0.2
0
BMDL
BMD
0
200
400
600
800
1000
dose
11:59 03/09 2006

1
2

```
=================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 12:00:48 2006
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 3
 Total number of specified parameters = 0
 Degree of polynomial = 2


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =            0
                        Beta(1) =            0
                        Beta(2) = 1.01264e+014


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)      Beta(2)

Background            1        -0.65         0.33

   Beta(1)        -0.65            1        -0.75

   Beta(2)         0.33        -0.75            1


                          Parameter Estimates

        Variable           Estimate             Std. Err.
      Background          0.0889744             0.205309
         Beta(1)        8.80277e-005           0.00292558
         Beta(2)        5.76916e-006          6.80122e-006


                        Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
```

```
       Full model         -18.2628
     Fitted model         -18.4529       0.380206       1          0.5375
    Reduced model         -39.4295        42.3333       3         <.0001

              AIC:          42.9058


                       Goodness  of  Fit

       Dose      Est._Prob.    Expected    Observed      Size      Chi^2 Res.
  ---------------------------------------------------------------------------
i: 1
     0.0000      0.0890         1.335         1           15       -0.275
i: 2
    40.0000      0.1005         1.508         2           15        0.363
i: 3
   200.0000      0.2893         4.340         4           15       -0.110
i: 4
  1000.0000      0.9974        14.961        15           15        1.003

 Chi-square =        0.35     DF = 1        P-value = 0.5556


    Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =       Extra risk

Confidence level =             0.95

              BMD =         127.726

             BMDL =         39.8667
```



```
=================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:47:43 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =            0
                        Beta(1) = 1.04991e+017


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)

Background            1        -0.37

   Beta(1)        -0.37            1



                           Parameter Estimates

        Variable           Estimate             Std. Err.
      Background            0.048937             0.16314
         Beta(1)           0.0025684         0.000904753


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
     Fitted model        -20.3189       4.11219      2           0.128
   Reduced model         -39.4295       42.3333      3         <.0001

            AIC:          44.6378
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Chi^2 Res.
i: 1					
0.0000	0.0489	0.734	1	15	0.381
i: 2					
40.0000	0.1418	2.127	2	15	-0.070
i: 3					
200.0000	0.4310	6.465	4	15	-0.670
i: 4					
1000.0000	0.9271	13.906	15	15	1.079

Chi-square = 2.94 DF = 2 P-value = 0.2298

Benchmark Dose Computation

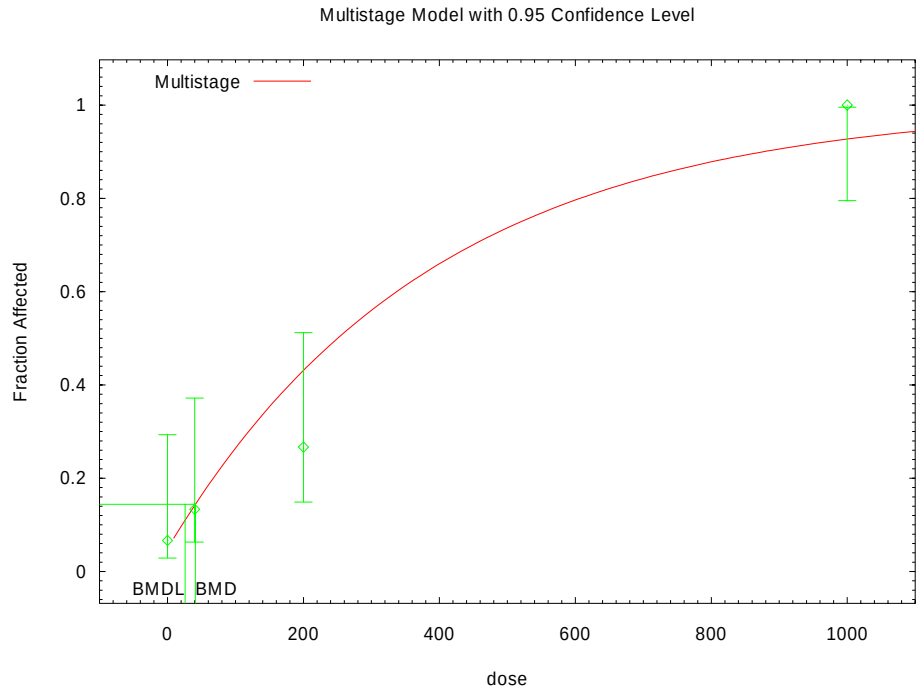
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 41.0219

BMDL = 25.943



```
==========================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 12:02:26 2006
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = CumNorm(Intercept+Slope*Dose),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     background =            0   Specified
                      intercept =     -1.32259
                          slope =   0.00345221


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                  have been estimated at a boundary point, or have been
specified by the user,
                  and do not appear in the correlation matrix )

               intercept        slope

 intercept            1        -0.65

     slope        -0.65            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
      intercept            -1.42508            0.306506
          slope          0.00435347          0.00171942


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
     Fitted model        -18.3773      0.229031      2          0.8918
    Reduced model        -39.4295       42.3333      3         <.0001
```

AIC: 40.7546

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0771	1.156	1	15	-0.151
40.0000	0.1055	1.582	2	15	0.3512
200.0000	0.2897	4.345	4	15	-0.1963
1000.0000	0.9983	14.974	15	15	0.16

Chi-square = 0.21 DF = 2 P-value = 0.9002

Benchmark Dose Computation

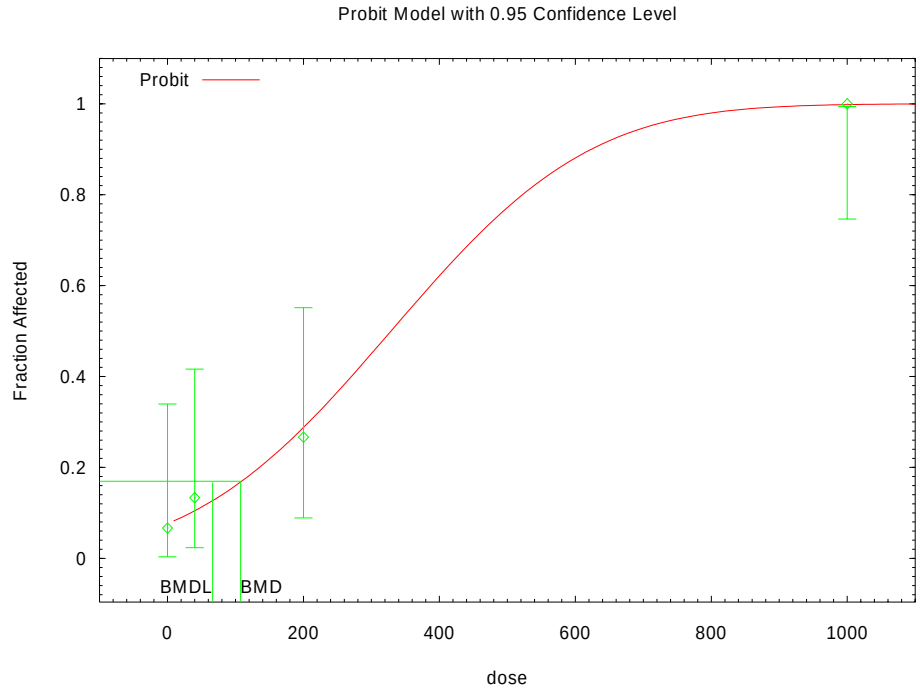
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 107.589

BMDL = 66.4468



```
=============================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:48:13 2005
 =============================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                  Default Initial (and Specified) Parameter Values
                     background =    0.0666667
                      intercept =     -5.60926
                          slope =      1.03389


           Asymptotic Correlation Matrix of Parameter Estimates

             background    intercept        slope

background            1       0.0018       -0.002

 intercept       0.0018            1           -1

     slope       -0.002           -1            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
     background                 0.1            0.0547723
      intercept            -22.1534              1087.37
          slope             4.01215              205.229


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
```

Fitted model	-18.4512	0.376844	1	0.5393
Reduced model	-39.4295	42.3333	3	<.0001

AIC: 42.9024

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.1000	1.500	1	15	-0.4303
40.0000	0.1000	1.500	2	15	0.4303
200.0000	0.2667	4.000	4	15	-1.174e-005
1000.0000	1.0000	15.000	15	15	0.0004249

Chi-square = 0.37 DF = 1 P-value = 0.5428

Benchmark Dose Computation

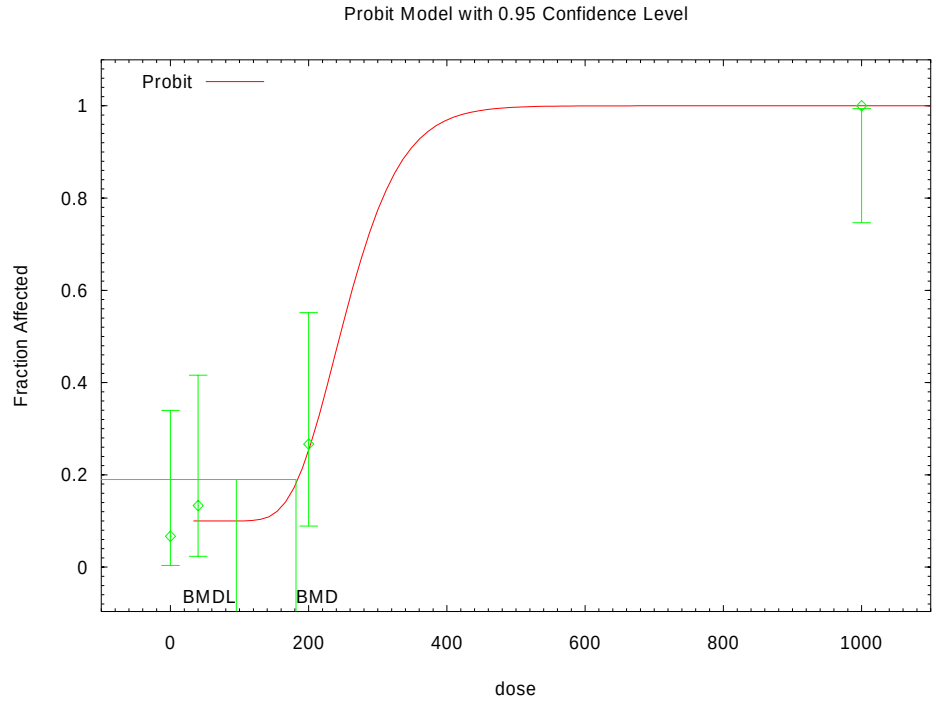
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 181.665

BMDL = 95.2612



```
 ================================================================
        Quantal Linear Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:48:41 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.09375
                          Slope =    0.0033673
                          Power =             1   Specified


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Power
                  have been estimated at a boundary point, or have been
specified by the user,
                  and do not appear in the correlation matrix )

             Background        Slope

Background            1        -0.23

     Slope        -0.23            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background           0.048937            0.0439067
          Slope           0.0025684          0.000746108



                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
    Fitted model         -20.3189       4.11219      2           0.128
   Reduced model         -39.4295       42.3333      3         <.0001

           AIC:          44.6378
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0489	0.734	1	15	0.3183
40.0000	0.1418	2.127	2	15	-0.09393
200.0000	0.4310	6.465	4	15	-1.285
1000.0000	0.9271	13.906	15	15	1.086

Chi-square = 2.94 DF = 2 P-value = 0.2298

Benchmark Dose Computation

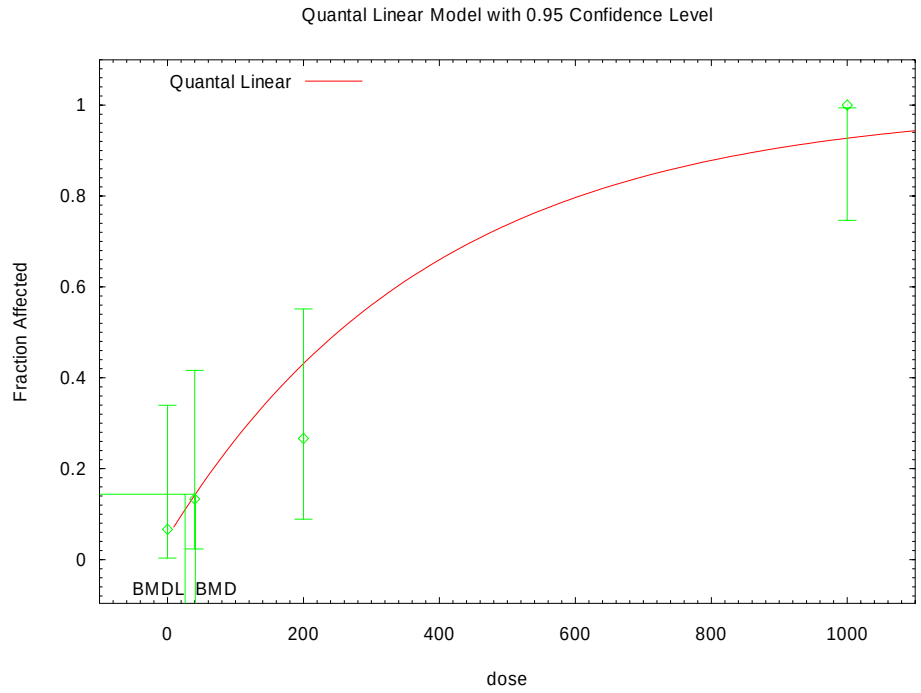
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 41.0219

BMDL = 25.943



```
=================================================================
        Quantal Quadratic Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:49:14 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^2)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.09375
                          Slope =  3.3673e-006
                          Power =            2   Specified


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

             Background        Slope

Background            1        -0.32

     Slope        -0.32            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background            0.091177           0.0532315
          Slope        6.03482e-006        3.53595e-006


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
    Fitted model         -18.4542     0.382862      2          0.8258
   Reduced model         -39.4295      42.3333      3         <.0001

           AIC:          40.9085
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0912	1.368	1	15	-0.3298
40.0000	0.0999	1.499	2	15	0.4317
200.0000	0.2861	4.291	4	15	-0.1665
1000.0000	0.9978	14.967	15	15	0.1808

Chi-square = 0.36 DF = 2 P-value = 0.8372

Benchmark Dose Computation

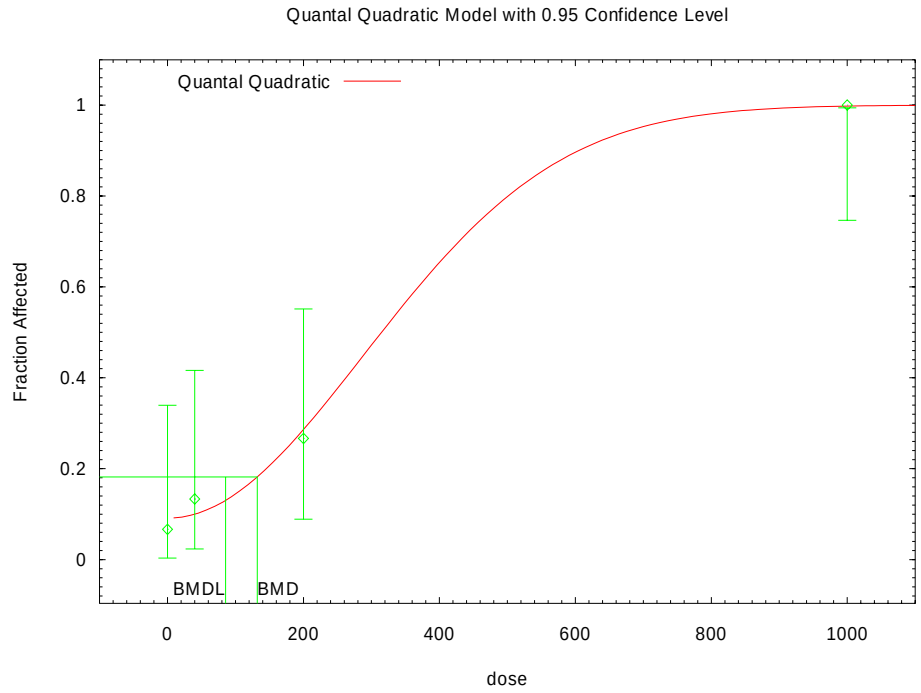
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 132.132

BMDL = 85.4708



15:49 12/28 2005

```
==========================================================================
        Weibull Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_MALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:49:46 2005
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^power)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial (and Specified) Parameter Values
                     Background =      0.09375
                          Slope = 4.78181e-007
                          Power =      2.28256


            Asymptotic Correlation Matrix of Parameter Estimates

              Background        Slope        Power

Background            1        -0.33         0.31

     Slope        -0.33            1           -1

     Power         0.31           -1            1


                           Parameter Estimates

        Variable           Estimate             Std. Err.
      Background          0.0959027           0.0577365
           Slope       1.27341e-006        1.17833e-005
           Power            2.27222             1.67024


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -18.2628
    Fitted model         -18.4284      0.331283      1          0.5649
   Reduced model         -39.4295       42.3333      3         <.0001

            AIC:         42.8569
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.0959	1.439	1	15	-0.3845
40.0000	0.1009	1.514	2	15	0.4168
200.0000	0.2712	4.067	4	15	-0.03917
1000.0000	0.9998	14.997	15	15	0.05665

Chi-square = 0.33 DF = 1 P-value = 0.5678

Benchmark Dose Computation

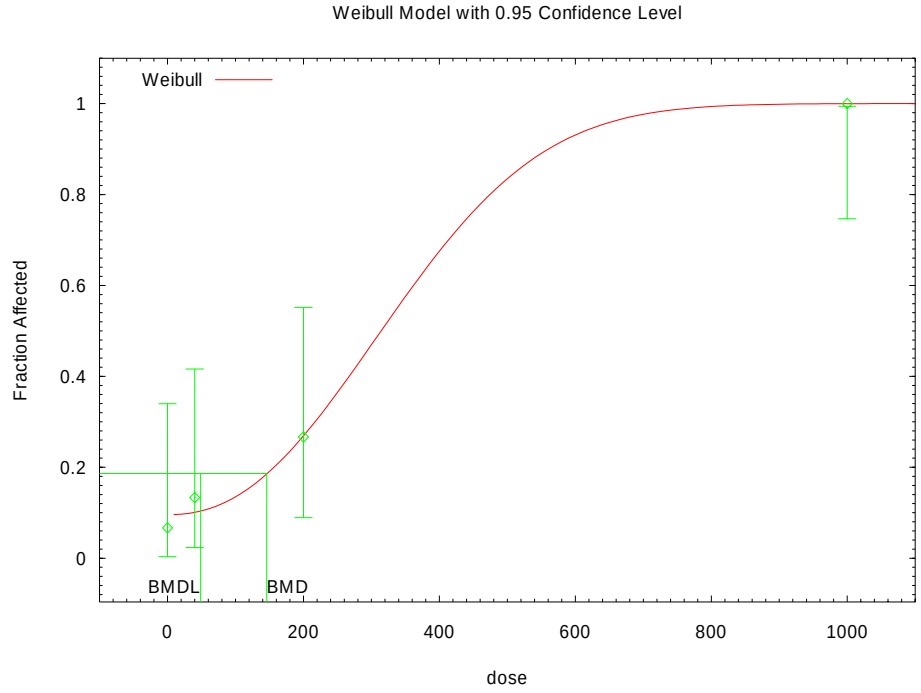
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 145.973

BMDL = 48.796



Fawell et al., 1999 Female Chronic Inflammation

```
=================================================================
         $Revision: 2.2 $ $Date: 2001/03/14 01:17:00 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:50:58 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response]= background+(1-background)*CumGamma[slope*dose,power],
   where CumGamma(.) is the cummulative Gamma distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Power parameter is restricted as power >=1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.34375
                          Slope =   0.00408479
                          Power =      2.14573


            Asymptotic Correlation Matrix of Parameter Estimates

            ( *** The model parameter(s)  -Power
                  have been estimated at a boundary point, or have been
specified by the user,
                  and do not appear in the correlation matrix )

              Background        Slope

Background             1        -0.42

     Slope        -0.42            1



                           Parameter Estimates

        Variable           Estimate             Std. Err.
     Background            0.385764           0.0913888
          Slope           0.0020737         0.000852726
          Power                   1                  NA

NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.
```

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-33.9494			
Fitted model	-34.4584	1.01801	2	0.6011
Reduced model	-40.7516	13.6045	3	0.003496

AIC: 72.9167

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.3858	5.786	5	15	-0.4172
40.0000	0.4347	6.520	8	15	0.7709
200.0000	0.5943	8.914	8	15	-0.4808
1000.0000	0.9228	13.842	14	15	0.1531

Chi-square = 1.02 DF = 2 P-value = 0.5996

Benchmark Dose Computation

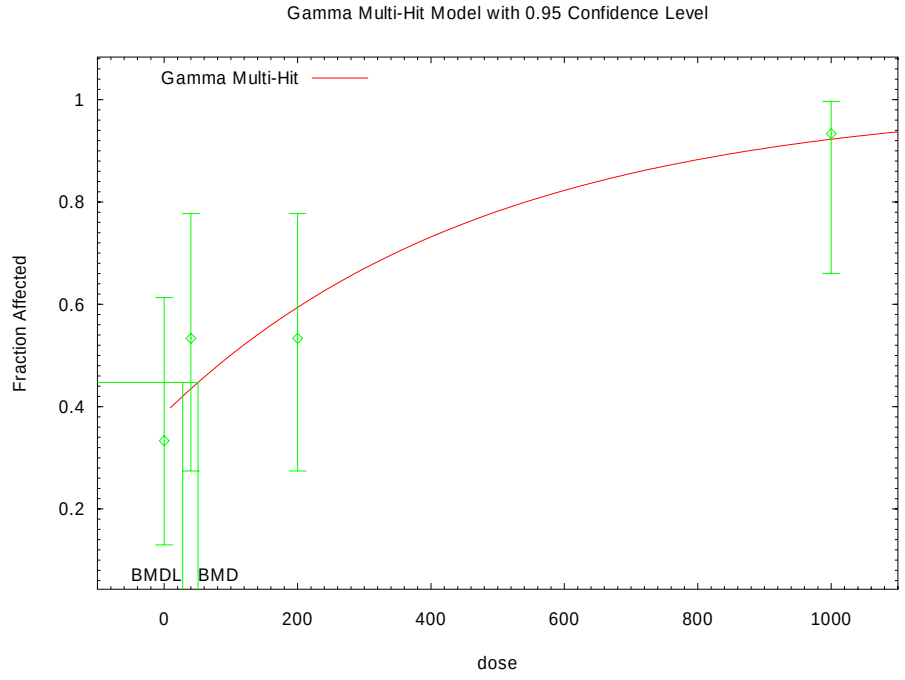
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 50.8081

BMDL = 27.7368



```
================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 12:16:37 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = 1/[1+EXP(-intercept-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                     background =            0   Specified
                      intercept =    -0.342416
                          slope =   0.00261455


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.51

     slope        -0.51            1


                           Parameter Estimates

       Variable           Estimate             Std. Err.
      intercept          -0.374017            0.335179
          slope         0.00300128          0.00110665


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
     Full model         -33.9494
    Fitted model        -34.4216     0.944503      2          0.6236
   Reduced model        -40.7516      13.6045      3        0.003496

           AIC:          72.8432
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4076	6.114	5	15	-0.5851
40.0000	0.4368	6.553	8	15	0.7534
200.0000	0.5563	8.345	8	15	-0.1792
1000.0000	0.9326	13.989	14	15	0.0114

Chi-square = 0.94 DF = 2 P-value = 0.6243

Benchmark Dose Computation

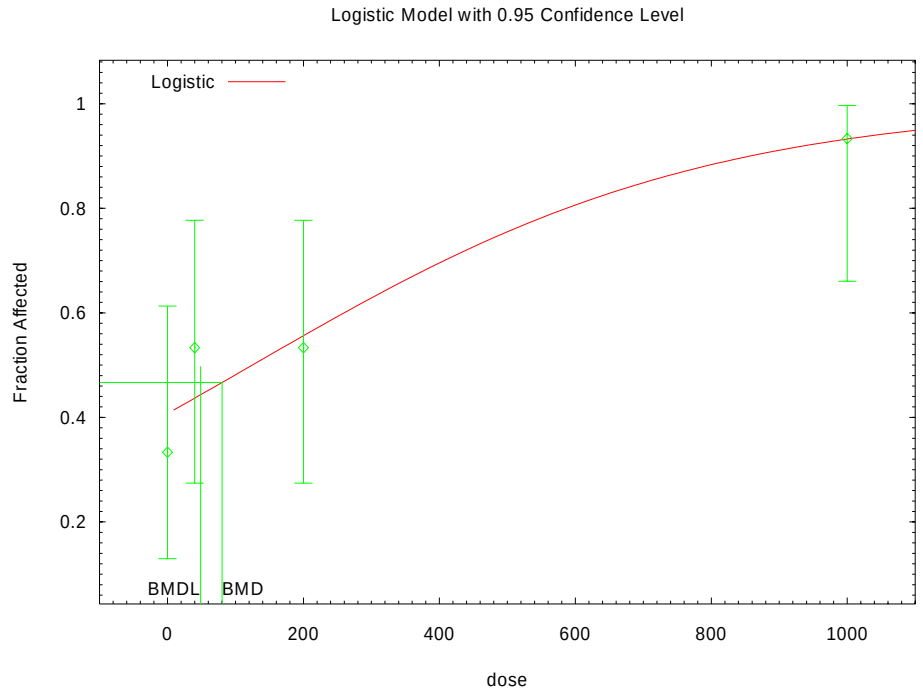
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 80.3245

BMDL = 48.7137



```
 1
 2  ================================================================
 3          Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
 4          Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
 5          Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
 6                                          Wed Dec 28 15:51:45 2005
 7   ================================================================
 8
 9   BMDS MODEL RUN
10  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
11
12     The form of the probability function is:
13
14     P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]
15
16
17     Dependent variable = COLUMN2
18     Independent variable = COLUMN1
19     Slope parameter is restricted as slope >= 1
20
21     Total number of observations = 4
22     Total number of records with missing values = 0
23     Maximum number of iterations = 250
24     Relative Function Convergence has been set to: 1e-008
25     Parameter Convergence has been set to: 1e-008
26
27
28
29     User has chosen the log transformed model
30
31
32                    Default Initial Parameter Values
33                       background =     0.333333
34                        intercept =     -5.18041
35                            slope =            1
36
37
38              Asymptotic Correlation Matrix of Parameter Estimates
39
40                background    intercept        slope
41
42  background            1        -0.51         0.47
43
44   intercept        -0.51            1        -0.99
45
46       slope         0.47        -0.99            1
47
48
49
50                             Parameter Estimates
51
52          Variable           Estimate             Std. Err.
53        background            0.426119           0.0985863
54         intercept            -12.3243             8.90473
55             slope             2.07233             1.36766
56
57
58                           Analysis of Deviance Table
59
60           Model      Log(likelihood)  Deviance  Test DF     P-value
61         Full model         -33.9494
62        Fitted model        -34.5387       1.17872      1          0.2776
63       Reduced model        -40.7516       13.6045      3        0.003496
64
```

AIC: 75.0774

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4261	6.392	5	15	-0.7267
40.0000	0.4314	6.471	8	15	0.7971
200.0000	0.5448	8.172	8	15	-0.08904
1000.0000	0.9310	13.966	14	15	0.03513

Chi-square = 1.17 DF = 1 P-value = 0.2789

Benchmark Dose Computation

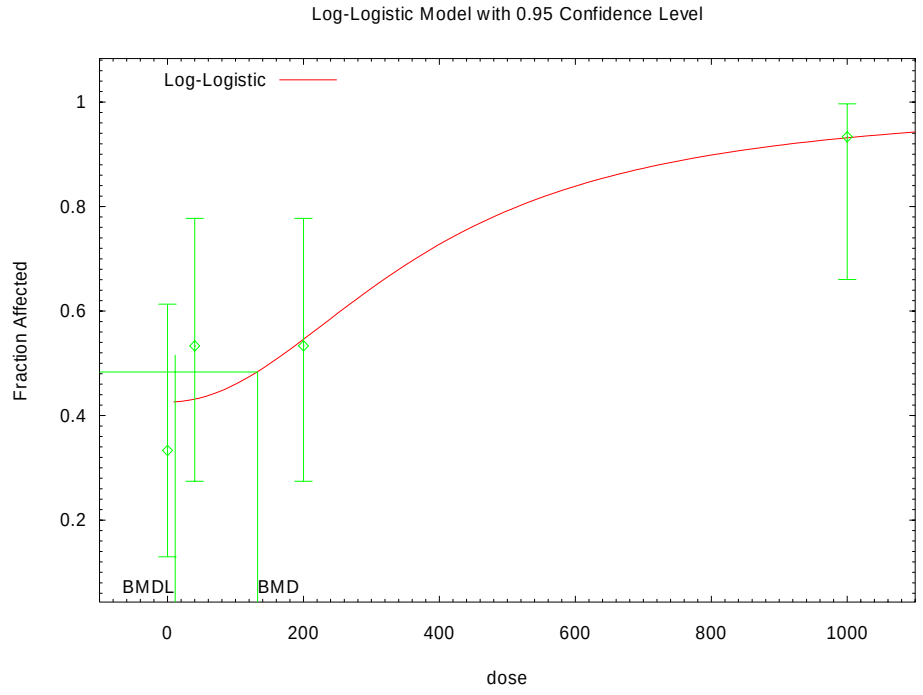
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

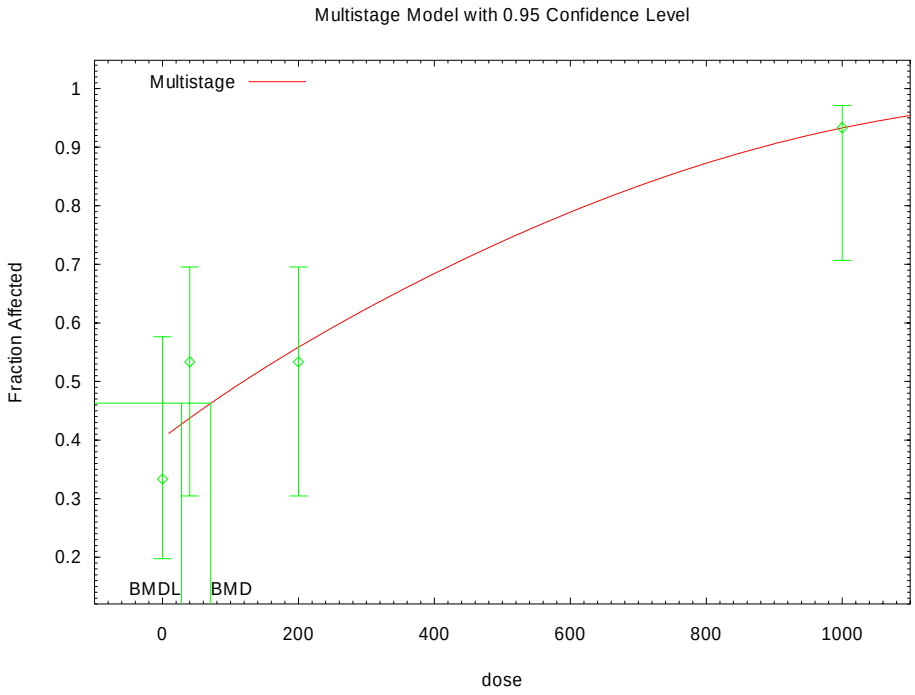
BMD = 132.532

BMDL = 11.3311



```
================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 12:17:59 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2-beta3*dose^3)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 4
 Total number of specified parameters = 0
 Degree of polynomial = 3


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008


                  Default Initial Parameter Values
                     Background =     0.417955
                        Beta(1) =   0.00124572
                        Beta(2) =            0
                        Beta(3) =  9.2085e-010


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Beta(2)
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

             Background      Beta(1)      Beta(3)

Background            1        -0.66         0.55

   Beta(1)        -0.66            1        -0.93

   Beta(3)         0.55        -0.93            1


                          Parameter Estimates

        Variable           Estimate             Std. Err.
      Background           0.403311             0.162784
         Beta(1)          0.00148059           0.00267062
         Beta(2)                   0                   NA
         Beta(3)        7.06214e-010        2.70652e-009

```

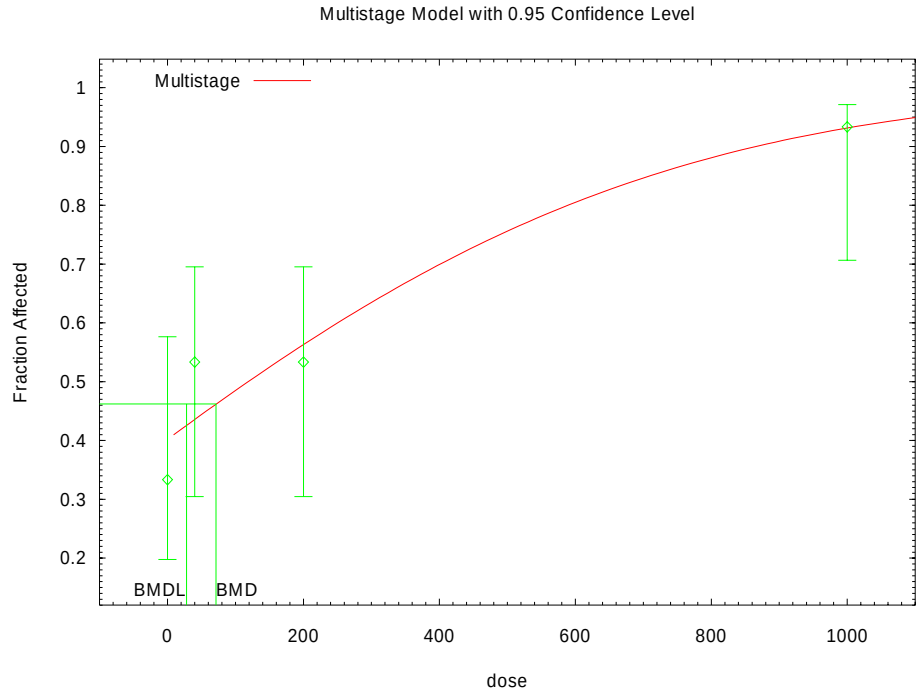
```
NA - Indicates that this parameter has hit a bound
     implied by some inequality constraint and thus
     has no standard error.


                        Analysis of Deviance Table

       Model      Log(likelihood)  Deviance  Test DF     P-value
     Full model         -33.9494
    Fitted model        -34.4013      0.903921      1          0.3417
   Reduced model        -40.7516       13.6045      3         0.003496

           AIC:          74.8026


                     Goodness  of  Fit

      Dose     Est._Prob.    Expected    Observed     Size     Chi^2 Res.
  ------------------------------------------------------------------------
i: 1
    0.0000     0.4033         6.050         5          15      -0.291
i: 2
   40.0000     0.4376         6.565         8          15       0.389
i: 3
  200.0000     0.5587         8.381         8          15      -0.103
i: 4
 1000.0000     0.9330        13.995        14          15       0.005

 Chi-square =       0.90     DF = 1        P-value = 0.3421


   Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =      Extra risk

Confidence level =           0.95

             BMD =        70.9904

            BMDL =        28.0638
```



12:17 03/09 2006

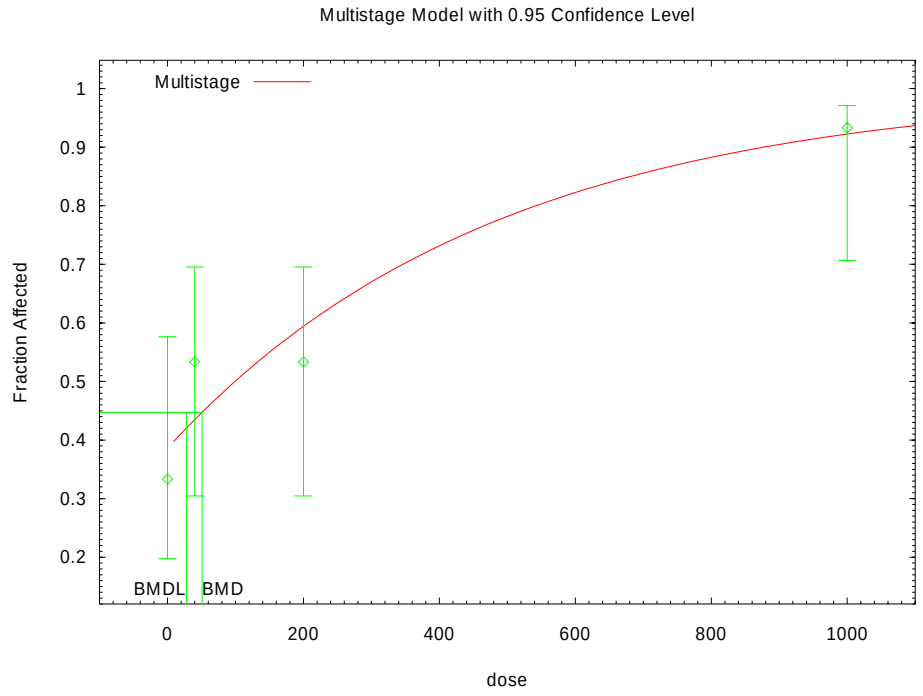
```
=================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:52:16 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1-beta2*dose^2)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 3
 Total number of specified parameters = 0
 Degree of polynomial = 2


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =     0.417495
                        Beta(1) =   0.00110779
                        Beta(2) = 1.05844e-006


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)      Beta(2)

Background            1        -0.67         0.58

   Beta(1)        -0.67            1        -0.95

   Beta(2)         0.58        -0.95            1


                          Parameter Estimates

        Variable           Estimate             Std. Err.
      Background            0.402286            0.165802
         Beta(1)          0.00142259          0.00323206
         Beta(2)        7.46169e-007        3.28803e-006


                        Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
```

```
        Full model         -33.9494
      Fitted model         -34.4149        0.93098       1          0.3346
     Reduced model         -40.7516        13.6045       3        0.003496

              AIC:          74.8297


                       Goodness  of  Fit

       Dose      Est._Prob.    Expected    Observed      Size      Chi^2 Res.
   ------------------------------------------------------------------------
i: 1
     0.0000      0.4023          6.034          5          15       -0.287
i: 2
    40.0000      0.4360          6.540          8          15        0.396
i: 3
   200.0000      0.5635          8.453          8          15       -0.123
i: 4
  1000.0000      0.9317         13.975         14          15        0.026

  Chi-square =        0.93     DF = 1         P-value = 0.3347


    Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =       Extra risk

Confidence level =             0.95

              BMD =         71.3892

             BMDL =         27.9852
```



```
================================================================
        Multistage Model. $Revision: 2.1 $ $Date: 2000/08/21 03:38:21 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                          Thu Mar 09 12:20:10 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(
-beta1*dose^1)]

   The parameter betas are restricted to be positive


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

 Total number of observations = 4
 Total number of records with missing values = 0
 Total number of parameters in model = 2
 Total number of specified parameters = 0
 Degree of polynomial = 1


 Maximum number of iterations = 250
 Relative Function Convergence has been set to: 1e-008
 Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                     Background =     0.377465
                        Beta(1) =   0.00221127


           Asymptotic Correlation Matrix of Parameter Estimates

             Background      Beta(1)

Background            1        -0.45

   Beta(1)        -0.45            1



                           Parameter Estimates

        Variable           Estimate             Std. Err.
      Background            0.385756               0.1375
         Beta(1)           0.00207378          0.000944904


                         Analysis of Deviance Table

         Model      Log(likelihood)  Deviance  Test DF     P-value
       Full model         -33.9494
      Fitted model        -34.4584       1.01801      2          0.6011
     Reduced model        -40.7516       13.6045      3        0.003496
```

```
            AIC:         72.9167


                      Goodness  of  Fit

      Dose      Est._Prob.     Expected     Observed      Size      Chi^2 Res.
   ------------------------------------------------------------------------
i: 1
     0.0000      0.3858         5.786          5           15       -0.221
i: 2
    40.0000      0.4347         6.520          8           15        0.402
i: 3
   200.0000      0.5943         8.914          8           15       -0.253
i: 4
  1000.0000      0.9228        13.842         14           15        0.148

  Chi-square =       1.02     DF = 2        P-value = 0.5996


    Benchmark Dose Computation

Specified effect =            0.1

Risk Type        =      Extra risk

Confidence level =           0.95

             BMD =        50.8061

            BMDL =        27.7368
```



```
==========================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                        Thu Mar 09 12:21:25 2006
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = CumNorm(Intercept+Slope*Dose),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is not restricted

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                 Default Initial (and Specified) Parameter Values
                    background =            0   Specified
                     intercept =    -0.216871
                         slope =   0.00162326


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -background
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

              intercept        slope

 intercept            1        -0.53

     slope        -0.53            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
      intercept           -0.223386             0.20693
          slope          0.00172875         0.000560405


                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -33.9494
     Fitted model         -34.421      0.943277      2           0.624
    Reduced model        -40.7516       13.6045      3         0.003496
```

AIC: 72.842

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4116	6.174	5	15	-0.6161
40.0000	0.4387	6.581	8	15	0.7385
200.0000	0.5487	8.230	8	15	-0.1196
1000.0000	0.9339	14.008	14	15	-0.00859

Chi-square = 0.94 DF = 2 P-value = 0.6252

Benchmark Dose Computation

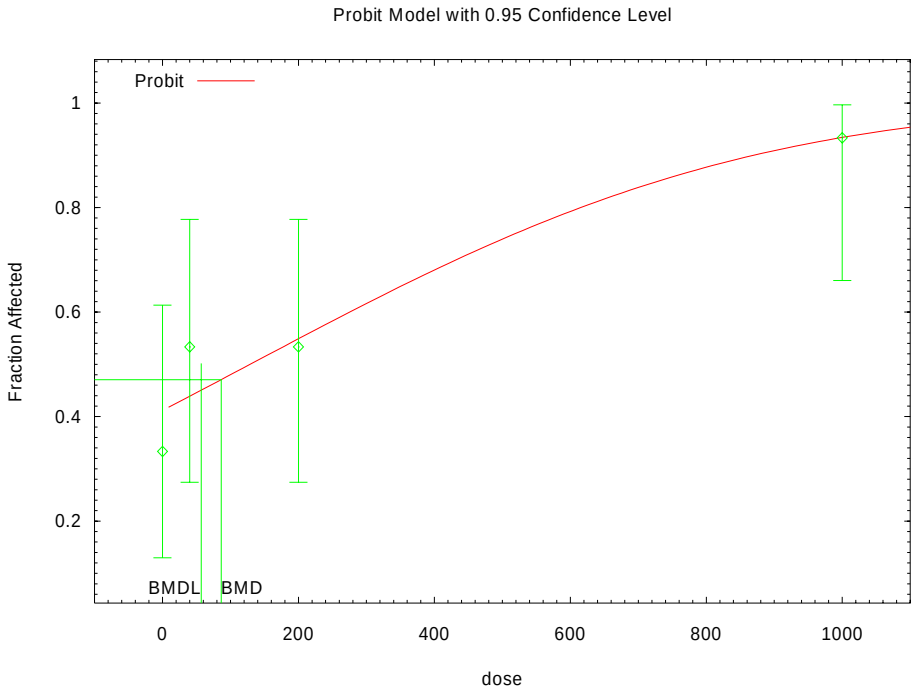
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 86.341

BMDL = 56.9234



1 12:21 03/09 2006

```
================================================================
        Probit Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:53 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:52:51 2005
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = Background
               + (1-Background) * CumNorm(Intercept+Slope*Log(Dose)),

   where CumNorm(.) is the cumulative normal distribution function


   Dependent variable = COLUMN2
   Independent variable = COLUMN1
   Slope parameter is restricted as slope >= 1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



   User has chosen the log transformed model


                 Default Initial (and Specified) Parameter Values
                    background =     0.333333
                     intercept =     -5.62294
                         slope =            1


           Asymptotic Correlation Matrix of Parameter Estimates

             background    intercept        slope

background            1        -0.46         0.43

 intercept        -0.46            1        -0.99

     slope         0.43        -0.99            1


                          Parameter Estimates

       Variable           Estimate             Std. Err.
     background            0.431473           0.0937984
      intercept              -7.732             4.97551
          slope              1.2906             0.75516


                        Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -33.9494
```

Fitted model	-34.5602	1.22166	1	0.269
Reduced model	-40.7516	13.6045	3	0.003496

AIC: 75.1204

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4315	6.472	5	15	-0.7674
40.0000	0.4323	6.485	8	15	0.7897
200.0000	0.5370	8.055	8	15	-0.02871
1000.0000	0.9327	13.991	14	15	0.009746

Chi-square = 1.21 DF = 1 P-value = 0.2706

Benchmark Dose Computation

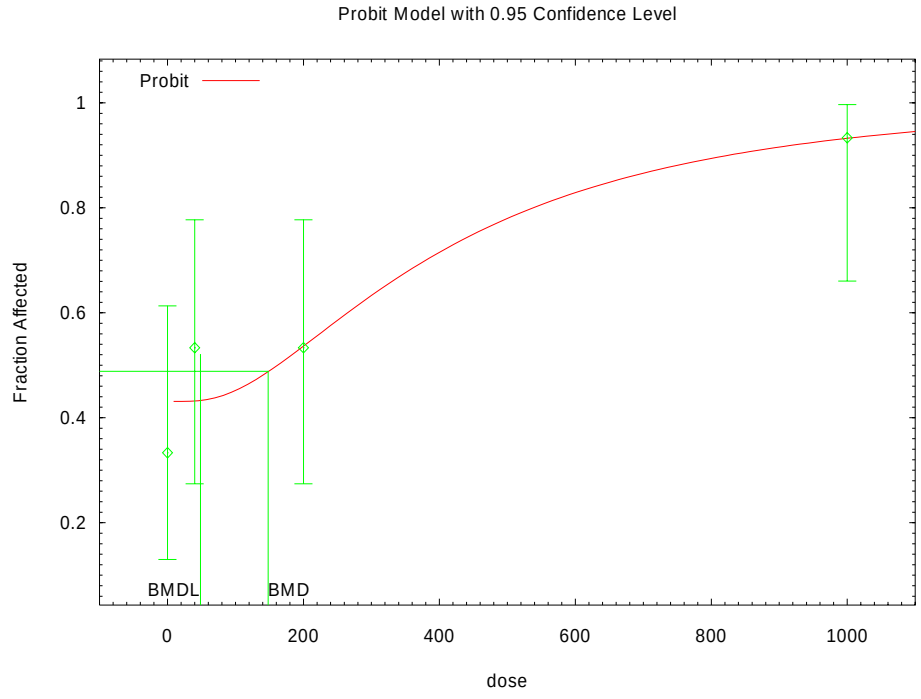
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 148.118

BMDL = 48.5214



```
=================================================================
        Quantal Linear Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                          Wed Dec 28 15:54:53 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.34375
                          Slope =   0.00194591
                          Power =            1   Specified


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

             Background        Slope

Background            1        -0.42

     Slope        -0.42            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background           0.385756            0.0913888
          Slope          0.00207382          0.000852767



                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -33.9494
    Fitted model         -34.4584       1.01801      2          0.6011
   Reduced model         -40.7516       13.6045      3        0.003496

           AIC:          72.9167

```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.3858	5.786	5	15	-0.4171
40.0000	0.4347	6.520	8	15	0.771
200.0000	0.5943	8.914	8	15	-0.4808
1000.0000	0.9228	13.842	14	15	0.153

Chi-square = 1.02 DF = 2 P-value = 0.5996

Benchmark Dose Computation

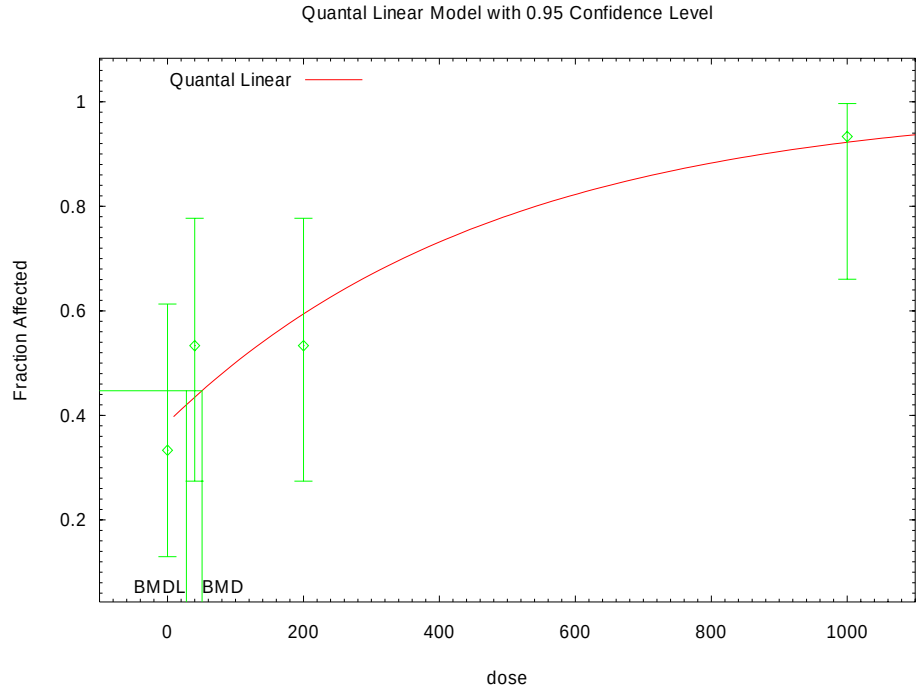
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 50.8051

BMDL = 27.7368



```
=================================================================
        Quantal Quadratic Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
        Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
        Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
                                        Wed Dec 28 15:55:22 2005
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the probability function is:

   P[response] = background + (1-background)*[1-EXP(-slope*dose^2)]


   Dependent variable = COLUMN2
   Independent variable = COLUMN1

   Total number of observations = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial (and Specified) Parameter Values
                     Background =      0.34375
                          Slope = 1.94591e-006
                          Power =            2   Specified


           Asymptotic Correlation Matrix of Parameter Estimates

           ( *** The model parameter(s)  -Power
                 have been estimated at a boundary point, or have been
specified by the user,
                 and do not appear in the correlation matrix )

             Background        Slope

Background            1        -0.23

     Slope        -0.23            1



                           Parameter Estimates

       Variable           Estimate             Std. Err.
     Background            0.449003           0.0779228
          Slope        2.16129e-006        1.00801e-006



                         Analysis of Deviance Table

        Model      Log(likelihood)  Deviance  Test DF     P-value
      Full model         -33.9494
    Fitted model         -34.6163       1.33385      2          0.5133
   Reduced model         -40.7516       13.6045      3        0.003496

           AIC:          73.2326

```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4490	6.735	5	15	-0.9007
40.0000	0.4509	6.764	8	15	0.6416
200.0000	0.4946	7.420	8	15	0.2998
1000.0000	0.9365	14.048	14	15	-0.05091

Chi-square = 1.32 DF = 2 P-value = 0.5181

Benchmark Dose Computation

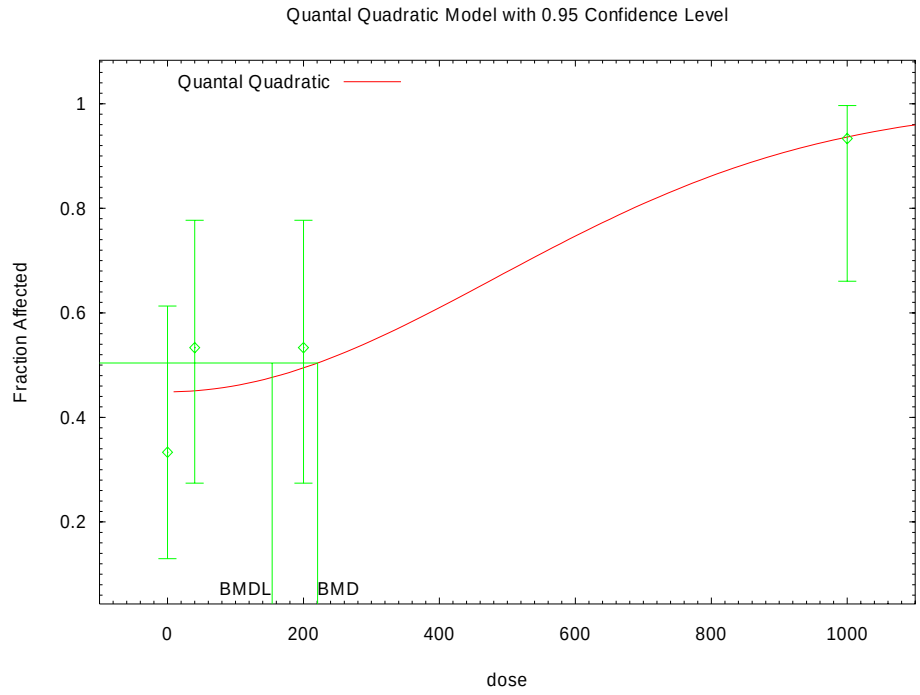
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 220.792

BMDL = 153.94



15:55 12/28 2005

```
1
2   ================================================================
3           Weibull Model $Revision: 2.2 $ $Date: 2000/03/17 22:27:16 $
4           Input Data File: C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.(d)
5           Gnuplot Plotting File:  C:\BMDS\FAWELL_FEMALE_CHRONIC_INFLAMMATION.plt
6                                             Wed Dec 28 15:55:56 2005
7    ================================================================
8
9    BMDS MODEL RUN
10  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
11
12     The form of the probability function is:
13
14     P[response] = background + (1-background)*[1-EXP(-slope*doseˆpower)]
15
16
17     Dependent variable = COLUMN2
18     Independent variable = COLUMN1
19     Power parameter is restricted as power >=1
20
21     Total number of observations = 4
22     Total number of records with missing values = 0
23     Maximum number of iterations = 250
24     Relative Function Convergence has been set to: 1e-008
25     Parameter Convergence has been set to: 1e-008
26
27
28
29                     Default Initial (and Specified) Parameter Values
30                        Background =      0.34375
31                             Slope =  0.000217656
32                             Power =      1.31712
33
34
35              Asymptotic Correlation Matrix of Parameter Estimates
36
37                Background        Slope        Power
38
39  Background             1        -0.77         0.76
40
41       Slope         -0.77            1           -1
42
43       Power          0.76           -1            1
44
45
46
47                              Parameter Estimates
48
49          Variable           Estimate             Std. Err.
50        Background           0.396812             0.136488
51             Slope          0.00106435           0.00719029
52             Power            1.09925             0.999439
53
54
55
56                            Analysis of Deviance Table
57
58           Model      Log(likelihood)  Deviance  Test DF     P-value
59         Full model         -33.9494
60       Fitted model         -34.4529       1.00699      1          0.3156
61      Reduced model         -40.7516       13.6045      3        0.003496
62
63              AIC:          74.9057
64
```

Goodness of Fit

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.3968	5.952	5	15	-0.5025
40.0000	0.4327	6.491	8	15	0.7864
200.0000	0.5792	8.689	8	15	-0.3601
1000.0000	0.9271	13.906	14	15	0.09327

Chi-square = 1.01 DF = 1 P-value = 0.3151

Benchmark Dose Computation

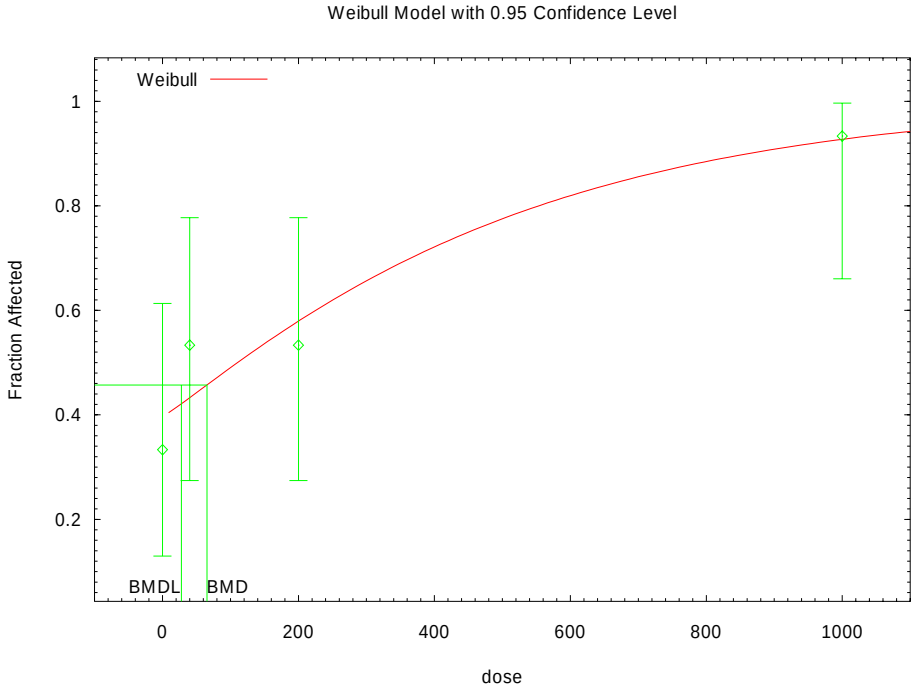
Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 65.3741

BMDL = 27.7679



Heinze, 1999 Relative Liver Weight Changes

```
=============================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.plt
                                            Thu May 18 09:37:38 2006
 =============================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =       0.1466
                            rho =            0   Specified
                         beta_0 =      2.84857
                         beta_1 =   0.00447143


                                  Parameter Estimates

                                                          95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
          alpha         0.143276        0.0369936           0.0707695
0.215782
         beta_0          2.84857         0.101163              2.6503
3.04685
         beta_1       0.00447143       0.00110819          0.00229942
0.00664343


           Asymptotic Correlation Matrix of Parameter Estimates

                    alpha       beta_0       beta_1
     alpha              1     5.6e-009     1.9e-009
    beta_0     5.6e-009            1        -0.73
    beta_1     1.9e-009        -0.73            1


      Table of Data and Estimated Values of Interest
```

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	2.75	0.29	2.85	0.379	-0.824
50	10	3.22	0.34	3.07	0.379	1.24
150	10	3.47	0.49	3.52	0.379	-0.412

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	15.381120	4	-22.762240
A2	16.880747	6	-21.761494
fitted	14.144766	2	-24.289533
R	7.133405	2	-10.266809

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	19.4947	4	<.0001
Test 2	2.99925	2	0.2232
Test 3	2.47271	1	0.1158

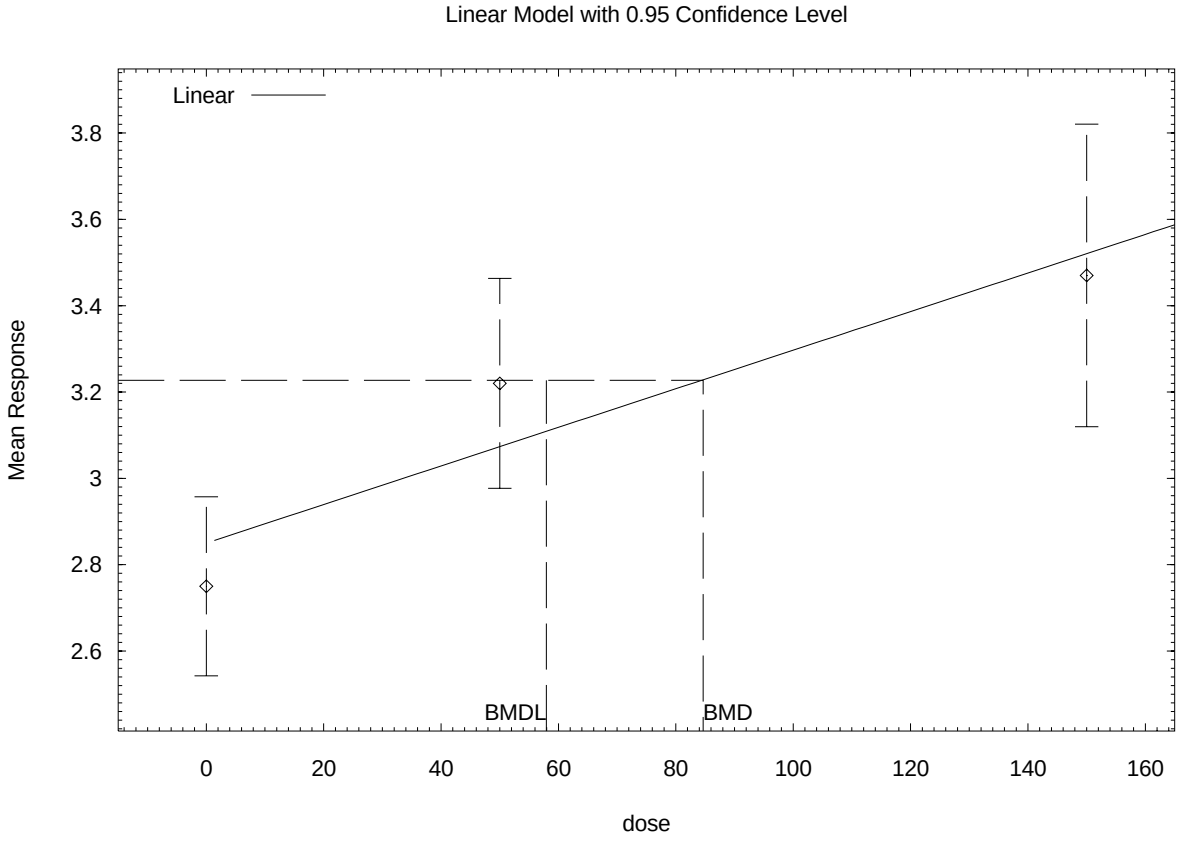
The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A homogeneous variance
model appears to be appropriate here

The p-value for Test 3 is greater than .05. The model chosen appears
to adequately describe the data

Benchmark Dose Computation

```
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

              BMD =       84.6525


             BMDL =       57.9321
```



09:37 05/18 2006

Heinze, 1999 Lactate Dehydrogenase Changes

```
================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.plt
                                          Thu May 18 09:40:36 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =      15.6395
                            rho =            0   Specified
                         beta_0 =        20.22
                         beta_1 =        0.101


                                Parameter Estimates

                                                        95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
          alpha           29.028          7.49498             14.3381
43.7179
         beta_0            20.22          1.43994             17.3978
23.0422
         beta_1            0.101        0.0157738            0.070084
0.131916


           Asymptotic Correlation Matrix of Parameter Estimates

                    alpha       beta_0       beta_1
     alpha              1     6.2e-015     1.5e-015
    beta_0     6.2e-015            1        -0.73
    beta_1     1.5e-015        -0.73            1


      Table of Data and Estimated Values of Interest
```

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	16.6	4.48	20.2	5.39	-2.1
50	10	30.6	5.05	25.3	5.39	3.15
150	10	33.6	1.16	35.4	5.39	-1.05

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	-54.666589	4	117.333177
A2	-46.093905	6	104.187810
fitted	-65.523922	2	135.047844
R	-78.954450	2	161.908899

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	65.7211	4	<.0001
Test 2	17.1454	2	0.0001892
Test 3	21.7147	1	<.0001

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05. Consider running a
non-homogeneous variance model

The p-value for Test 3 is less than .05. You may want to try a
different model

Benchmark Dose Computation
Specified effect = 1

```
1   Risk Type        =     Estimated standard deviations from the control mean
2
3
4   Confidence level =          0.95
5
6                BMD =       53.3442
7
8
9               BMDL =       39.9199
```

```
==========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.plt
                                          Thu May 18 09:42:43 2006
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   The polynomial coefficients are restricted to be positive
   The variance is to be modeled as Var(i) = alpha*mean(i)^rho

   Total number of dose groups = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =      15.6395
                            rho =            0
                         beta_0 =        20.22
                         beta_1 =        0.101


                                  Parameter Estimates

                                                          95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
          alpha     2.44639e+006     4.30425e+006       -5.98979e+006
1.08826e+007
            rho         -3.55294         0.556161              -4.643
-2.46289
         beta_0          21.3722          1.64609              18.146
24.5985
         beta_1        0.0863674        0.0134905           0.0599266
0.112808


           Asymptotic Correlation Matrix of Parameter Estimates

                    alpha          rho       beta_0       beta_1
     alpha              1        -0.99         0.19        -0.23
       rho          -0.99            1        -0.21         0.25
    beta_0           0.19        -0.21            1        -0.89
    beta_1          -0.23         0.25        -0.89            1


      Table of Data and Estimated Values of Interest
```

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	16.6	4.48	21.4	6.79	-2.2
50	10	30.6	5.05	25.7	4.9	3.2
150	10	33.6	1.16	34.3	2.93	-0.808

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = alpha*(Mu(i))^rho

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	-54.666589	4	117.333177
A2	-46.093905	6	104.187810
A3	-54.022155	5	118.044309
fitted	-61.092641	4	130.185281
R	-78.954450	2	161.908899

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels?
(A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	65.7211	4	<.0001
Test 2	17.1454	2	0.0001892
Test 3	15.8565	1	<.0001
Test 4	14.141	1	0.0001696

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05. A non-homogeneous variance
model appears to be appropriate

```
The p-value for Test 3 is less than .05.  You may want
to consider a
different variance model

The p-value for Test 4 is less than .05.  You may want
to try a different
model


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

              BMD =       78.6091


             BMDL =       63.5158
```

Heinze, 1999 Alkaline Phosphatase Changes

```
=================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY DOCUMENTS\_CYANO
TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\HEINZE_ENZYMES_AND_LIVER_WT.plt
                                        Thu May 18 09:46:03 2006
 =================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 3
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                          alpha =      7.59287
                            rho =            0   Specified
                         beta_0 =      10.6414
                         beta_1 =            0


                                Parameter Estimates

                                                        95.0% Wald Confidence
Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
           alpha          7.93453          2.04869             3.91918
11.9499
         beta_0          10.6414          0.75283             9.16591
12.117
         beta_1        0.0180286       0.00824683          0.00186506
0.0341921


           Asymptotic Correlation Matrix of Parameter Estimates

                    alpha       beta_0       beta_1
     alpha              1     1.1e-006    -4.7e-007
    beta_0     1.1e-006            1        -0.73
    beta_1    -4.7e-007        -0.73            1
```

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	10	9.67	2.2	10.6	2.82	-1.09
50	10	13	3.81	11.5	2.82	1.64
150	10	12.9	1.85	13.3	2.82	-0.545

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	-43.827730	4	95.655461
A2	-40.832314	6	93.664628
fitted	-46.068366	2	96.136733
R	-48.794188	2	101.588375

Test 1: Does response and/or variances differ among dose levels
(A2 vs. R)
Test 2: Are Variances Homogeneous (A1 vs A2)
Test 3: Does the Model for the Mean Fit (A1 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	15.9237	4	0.0003485
Test 2	5.99083	2	0.05002
Test 3	4.48127	1	0.03427

The p-value for Test 1 is less than .05. There appears to be a
difference between response and/or variances among the dose levels.
It seems appropriate to model the data

The p-value for Test 2 is greater than .05. A homogeneous variance
model appears to be appropriate here

The p-value for Test 3 is less than .05. You may want to try a
different model

Benchmark Dose Computation

```
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

              BMD =       156.243


             BMDL =       87.6609
```

Fawell et al., 1999 Male Alanine Aminotransferase Changes

```
==============================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.plt
                                          Tue May 09 12:59:55 2006
 ==============================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   rho is set to 0
   The polynomial coefficients are restricted to be positive
   A constant variance model is fit

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                  Default Initial Parameter Values
                         alpha =            1
                           rho =            0   Specified
                        beta_0 =      30.4717
                        beta_1 =     0.129124


                                  Parameter Estimates

                                                          95.0% Wald
Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
          alpha          1584.79           289.34             1017.69
2151.88
         beta_0          30.4717          6.47011             17.7905
43.1529
         beta_1         0.129124        0.0126792            0.104273
0.153974


            Asymptotic Correlation Matrix of Parameter Estimates

```

```
                    alpha       beta_0       beta_1
      alpha             1    -6.1e-008     9.2e-007
     beta_0    -6.1e-008            1        -0.61
     beta_1     9.2e-007        -0.61            1


      Table of Data and Estimated Values of Interest

 Dose       N    Obs Mean    Obs Std Dev   Est Mean   Est Std Dev   Chi^2
Res.
------     ---   --------    -----------   --------   -----------   ---------
-

    0    15         27            8         30.5         39.8         -0.338
   40    15         37         17.2         35.6         39.8          0.133
  200    15         59           28         56.3         39.8          0.263
 1000    15        159           75          160         39.8         -0.0579


   Model Descriptions for likelihoods calculated


 Model A1:        Yij = Mu(i) + e(ij)
           Var{e(ij)} = Sigma^2

 Model A2:        Yij = Mu(i) + e(ij)
           Var{e(ij)} = Sigma(i)^2

 Model  R:         Yi = Mu + e(i)
            Var{e(i)} = Sigma^2


                       Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1         -250.943933       5     511.887866
              A2         -216.540867       8     449.081734
           fitted        -251.046212       2     506.092423
              R          -281.663312       2     567.326624

 Test 1:  Does response and/or variances differ among dose
levels
           (A2 vs. R)
 Test 2:  Are Variances Homogeneous (A1 vs A2)
 Test 3:  Does the Model for the Mean Fit (A1 vs. fitted)

                     Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df     p-value

   Test 1              130.245          6          <.0001
   Test 2              68.8061          3          <.0001
   Test 3             0.204557          2          0.9028

The p-value for Test 1 is less than .05.  There appears
to be a
```

```
difference between response and/or variances among the
dose levels.
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  Consider
running a
non-homogeneous variance model

The p-value for Test 3 is greater than .05.  The model
chosen appears
to adequately describe the data


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

              BMD =       308.304


             BMDL =       252.245

```

```
================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.plt
                                          Tue May 09 13:00:39 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   The polynomial coefficients are restricted to be positive
   The variance is to be modeled as Var(i) = alpha*mean(i)^rho

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                 Default Initial Parameter Values
                        alpha =            1
                          rho =            0
                       beta_0 =      30.4717
                       beta_1 =     0.129124


                                Parameter Estimates

                                                        95.0% Wald
Confidence Interval
       Variable         Estimate        Std. Err.     Lower Conf. Limit
Upper Conf. Limit
          alpha         0.098324         0.116485           -0.129982
0.32663
            rho          2.15231         0.291636             1.58072
2.72391
         beta_0          28.6439          2.33865             24.0602
33.2276
         beta_1         0.141692        0.0184799            0.105472
0.177912


           Asymptotic Correlation Matrix of Parameter Estimates
```

	alpha	rho	beta_0	beta_1
alpha	1	-0.99	0.06	-0.17
rho	-0.99	1	-0.065	0.18
beta_0	0.06	-0.065	1	-0.39
beta_1	-0.17	0.18	-0.39	1

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	15	27	8	28.6	11.6	-0.549
40	15	37	17.2	34.3	14.1	0.739
200	15	59	28	57	24.3	0.321
1000	15	159	75	170	79	-0.556

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = alpha*(Mu(i))^rho

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	-250.943933	5	511.887866
A2	-216.540867	8	449.081734
A3	-217.537818	6	447.075636
fitted	-219.838125	4	447.676251
R	-281.663312	2	567.326624

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels?
(A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

Tests of Interest

Test	-2*log(Likelihood Ratio)	Test df	p-value
Test 1	130.245	6	<.0001
Test 2	68.8061	3	<.0001
Test 3	1.9939	2	0.369
Test 4	4.60062	2	0.1002

The p-value for Test 1 is less than .05. There appears to be a difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05. A non-homogeneous variance model appears to be appropriate

The p-value for Test 3 is greater than .05. The modeled variance appears to be appropriate here

The p-value for Test 4 is greater than .05. The model chosen seems to adequately describe the data

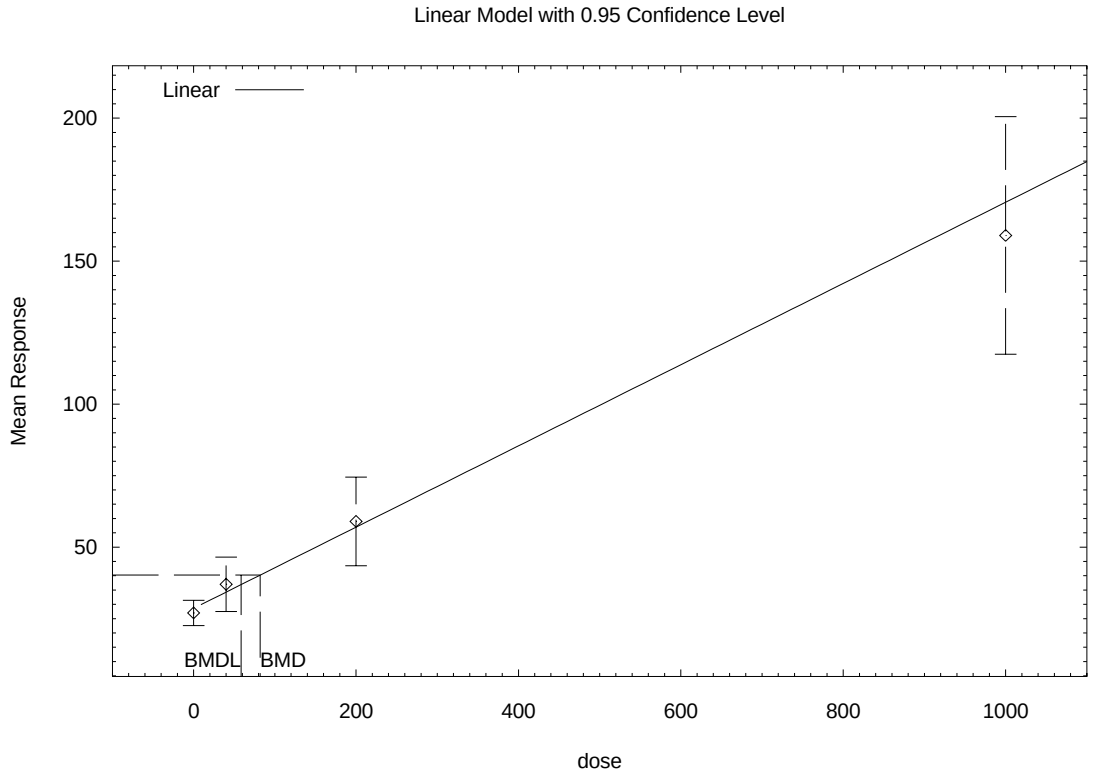
Benchmark Dose Computation
Specified effect = 1

Risk Type = Estimated standard deviations from the control mean

Confidence level = 0.95

BMD = 81.8426

BMDL = 58.3727



13:00 05/09 2006

```
==========================================================================
        Polynomial Model. Revision: 2.2  Date: 9/12/2002
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.plt
                                      Tue May 09 13:01:37 2006
 ==========================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = beta_0 + beta_1*dose + beta_2*dose^2 + ...


   Dependent variable = MEAN
   Independent variable = COLUMN1
   The polynomial coefficients are restricted to be positive
   The variance is to be modeled as Var(i) = alpha*mean(i)^rho

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008
```

Default Initial Parameter Values

alpha =	1
rho =	0
beta_0 =	28.5738
beta_1 =	0.160635
beta_2 =	0

Parameter Estimates

Variable	Estimate	Std. Err.	95.0% Wald Confidence Interval Lower Conf. Limit	Upper Conf. Limit
alpha	0.0983241	0.116485	-0.129982	0.32663
rho	2.15231	0.291636	1.58072	2.72391
beta_0	28.6439	2.33865	24.0602	33.2276
beta_1	0.141692	0.0184799	0.105472	0.177912
beta_2	0	NA		

NA - Indicates that this parameter has hit a bound
implied by some inequality constraint and thus
has no standard error.

Asymptotic Correlation Matrix of Parameter Estimates

	alpha	rho	beta_0	beta_1
alpha	1	-0.99	0.06	-0.17
rho	-0.99	1	-0.065	0.18
beta_0	0.06	-0.065	1	-0.39
beta_1	-0.17	0.18	-0.39	1

The following parameter(s) have been estimated at a
boundary
point or have been specified. Correlations are not
computed:

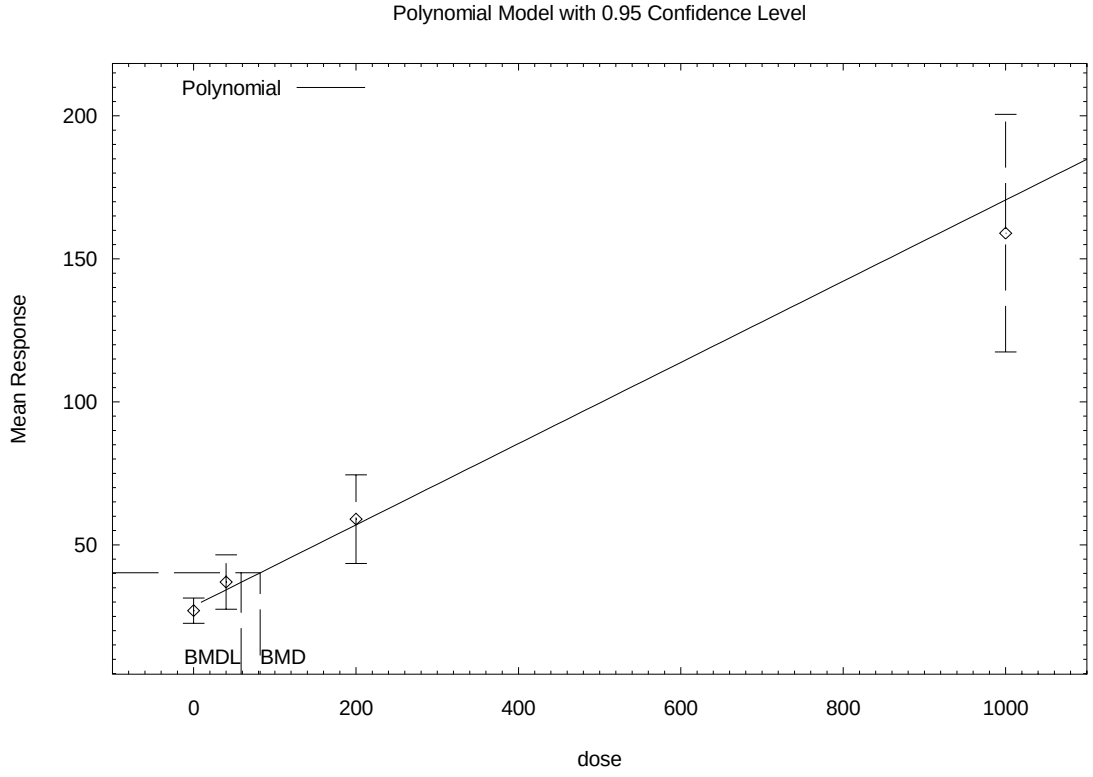
beta_2

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	15	27	8	28.6	11.6	-0.549
40	15	37	17.2	34.3	14.1	0.739

```
 200    15         59           28           57         24.3          0.321
1000    15        159           75          170           79         -0.556


 Model Descriptions for likelihoods calculated


 Model A1:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma^2

 Model A2:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = Sigma(i)^2

 Model A3:        Yij = Mu(i) + e(ij)
          Var{e(ij)} = alpha*(Mu(i))^rho

 Model  R:         Yi = Mu + e(i)
           Var{e(i)} = Sigma^2


                      Likelihoods of Interest

            Model      Log(likelihood)   DF        AIC
              A1         -250.943933       5     511.887866
              A2         -216.540867       8     449.081734
              A3         -217.537818       6     447.075636
           fitted        -219.838125       4     447.676251
              R          -281.663312       2     567.326624


                   Explanation of Tests

 Test 1:  Does response and/or variances differ among Dose
levels?
          (A2 vs. R)
 Test 2:  Are Variances Homogeneous? (A1 vs A2)
 Test 3:  Are variances adequately modeled? (A2 vs. A3)
 Test 4:  Does the Model for the Mean Fit? (A3 vs. fitted)

                     Tests of Interest

   Test    -2*log(Likelihood Ratio)  Test df        p-value

   Test 1             130.245          6          <.0001
   Test 2             68.8061          3          <.0001
   Test 3              1.9939          2           0.369
   Test 4             4.60062          1         0.03196

The p-value for Test 1 is less than .05.  There appears
to be a
difference between response and/or variances among the
dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  A
non-homogeneous variance
```

```
model appears to be appropriate

The p-value for Test 3 is greater than .05.  The
modeled variance appears
 to be appropriate here

The p-value for Test 4 is less than .05.  You may want
to try a different
model


 Benchmark Dose Computation
Specified effect =            1

Risk Type        =     Estimated standard deviations from the control mean


Confidence level =          0.95

             BMD =       81.8426


            BMDL =       58.3727
```



```
 ================================================================
        Power Model. $Revision: 2.1 $ $Date: 2000/10/11 20:57:36 $
        Input Data File: C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.(d)
        Gnuplot Plotting File:  C:\DOCUMENTS AND SETTINGS\HCLYNCH\MY
DOCUMENTS\_CYANO TOX REV\MODELING\FAWELL_MALE_ALT.plt
                                        Tue May 09 13:03:23 2006
 ================================================================

 BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

   The form of the response function is:

   Y[dose] = control + slope * dose^power


   Dependent variable = MEAN
   Independent variable = COLUMN1
   The power is restricted to be greater than or equal to 1
   The variance is to be modeled as Var(i) = alpha*mean(i)^rho

   Total number of dose groups = 4
   Total number of records with missing values = 0
   Maximum number of iterations = 250
   Relative Function Convergence has been set to: 1e-008
   Parameter Convergence has been set to: 1e-008



                 Default Initial Parameter Values
                        alpha =      1692.21
                          rho =            0
                      control =           27
                        slope =     0.519763
                        power =     0.801589


           Asymptotic Correlation Matrix of Parameter Estimates

                  alpha          rho      control        slope        power

     alpha            1        -0.99         0.13        -0.46         0.48

       rho        -0.99            1        -0.12         0.41        -0.43

   control         0.13        -0.12            1        -0.76         0.74

     slope        -0.46         0.41        -0.76            1           -1

     power         0.48        -0.43         0.74           -1            1


                          Parameter Estimates
```

Variable	Estimate	Std. Err.
alpha	0.098324	0.141722
rho	2.15231	0.343016
control	28.6439	3.51452
slope	0.141692	0.218496
power	1	0.231677

Table of Data and Estimated Values of Interest

Dose	N	Obs Mean	Obs Std Dev	Est Mean	Est Std Dev	Chi^2 Res.
0	15	27	8	28.6	11.6	-0.142
40	15	37	17.2	34.3	14.1	0.191
200	15	59	28	57	24.3	0.083
1000	15	159	75	170	79	-0.144

Model Descriptions for likelihoods calculated

Model A1: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma^2

Model A2: Yij = Mu(i) + e(ij)
Var{e(ij)} = Sigma(i)^2

Model A3: Yij = Mu(i) + e(ij)
Var{e(ij)} = alpha*(Mu(i))^rho

Model R: Yi = Mu + e(i)
Var{e(i)} = Sigma^2

Likelihoods of Interest

Model	Log(likelihood)	DF	AIC
A1	-250.943933	5	511.887866
A2	-216.540867	8	449.081734
A3	-217.537818	6	447.075636
fitted	-219.838125	5	449.676251
R	-281.663312	2	567.326624

Explanation of Tests

Test 1: Does response and/or variances differ among Dose levels? (A2 vs. R)
Test 2: Are Variances Homogeneous? (A1 vs A2)
Test 3: Are variances adequately modeled? (A2 vs. A3)
Test 4: Does the Model for the Mean Fit? (A3 vs. fitted)

```
                    Tests of Interest

   Test    -2*log(Likelihood Ratio)    d.f        p-value

   Test 1              130.245          6         <.00001
   Test 2              68.8061          3         <.00001
   Test 3               1.9939          2           0.369
   Test 4              4.60062          1         0.03196

The p-value for Test 1 is less than .05.  There appears to be a
difference between response and/or variances among the dose levels
It seems appropriate to model the data

The p-value for Test 2 is less than .05.  A non-homogeneous variance
model appears to be appropriate

The p-value for Test 3 is greater than .05.  The modeled variance appears
 to be appropriate here

The p-value for Test 4 is less than .05.  You may want to try a different
model


 Benchmark Dose Computation
Specified effect =             1

Risk Type        =     Estimated standard deviations from the control mean

Confidence level =          0.95

             BMD =       81.8426


            BMDL =       58.3727
```