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Toxicological Reviews of Cyanobacterial Toxins: Anatoxin-A

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LIST OF ACRONYMS

ALT	Alanine aminotransferase
ASP	Aspartate aminotransferase
BMD	Benchmark dose
CCL	Contaminant Candidate List
CI	Confidence interval
ED_{50}	Effective dose in 50% of subjects
EPA	Environmental Protection Agency
FEL	Frank effect level
GD	Gestation day
GGT	Glutamyl transpeptidase
LD_{50}	Dose lethal to 50% of population
LOAEL	Lowest-observed-adverse-effect level
NOAEL	No-observed-adverse-effect level
PND	Postnatal day
RfC	Reference concentration
RfD	Reference dose
SDWA	Safe Drinking Water Act
UF	Uncertainty factor
VFDF	Very Fast Death Factor

PREFACE

The Safe Drinking Water Act (SDWA), as amended in 1996, requires the Environmental Protection Agency (EPA) to publish a list of contaminants that, at the time of publication, are not subject to any proposed or promulgated national primary drinking water regulations, are known or anticipated to occur in public water systems, and 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. Anatoxin-a was identified at this meeting as being a toxin of high priority based on those criteria.

The National Center for Environmental Assessment has prepared this Toxicological Review of Cyanobacterial Toxins: Anatoxin-a 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 anatoxin-a 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 anatoxin-a.

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 anatoxin-a.

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1. INTRODUCTION

This toxicological review presents background and justification for hazard and dose-response assessments of anatoxin-a. U.S. Environmental Protection Agency (EPA) toxicological reviews may include oral reference doses (RfD) and inhalation reference concentrations (RfC) 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. The RfD (expressed in units of mg/kg-day) is defined as an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime. The inhalation RfC (expressed in units of mg/m^3) is analogous to the oral RfD, but provides a continuous inhalation exposure estimate. The inhalation RfC considers toxic effects for both the respiratory system (portal-of-entry) and for effects peripheral to the respiratory system (extrarespiratory or systemic effects). Reference values are generally derived for chronic exposures (up to a lifetime), but may also be derived for acute (24 hours), short-term (up to 30 days), and subchronic (up to 10% of average lifetime) exposure durations, all considered to be daily exposures, continuously or intermittently, throughout the duration specified.

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 anatoxin-a has followed the general guidelines for risk assessment as set forth by the National Research Council (NRC, 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 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 Studies* (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), *Science Policy Council Handbook: Risk*

Characterization (U.S. EPA, 2000b), *Benchmark Dose Technical Guidance Document* (U.S. EPA, 2000c) and *Supplementary 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).

Literature searches were conducted for studies relevant to the derivation of toxicity and carcinogenicity values for anatoxin-a. The following databases were searched: MEDLINE (PubMed), TOXLINE, BIOSIS, CANCERLIT, TSCATS, CCRIS, DART/ETIC, EMIC, GENETOX, HSDB and RTECS. The relevant literature was thoroughly reviewed through May 2006.

2. CHEMICAL AND PHYSICAL INFORMATION

Anatoxin-a is a naturally occurring toxin produced by some strains of *Anabaena* (particularly *Anabaena flos-aquae*) and at least four other genera of freshwater cyanobacteria (commonly referred to as blue-green algae), including *Aphanizomenon*, *Microcystis*, *Planktothrix* and *Oscillatoria* (Fawell et al., 1999; Viaggiu et al., 2004). A structural analog, homoanatoxin-a (methylene-anatoxin-a), has been isolated from a strain of *Oscillatoria formosa* (Skulberg et al., 1992). Anatoxin-a has a semi-rigid bicyclic secondary amine structure, 2-acetyl-9-azbicyclo[4:2:1]non-2-ene, a molecular formula of $C_{10}H_{15}NO$ and a molecular weight of 165.26 (Lewis, 2000). Of the two enantiomeric forms of anatoxin-a, only (+)-anatoxin-a is produced in nature. The pKa of (+)-anatoxin-a is 9.4 (Valentine et al., 1991), indicating that the molecule is almost completely protonated at acidic and neutral pH (e.g., in natural water). The structure of the protonated molecule is shown below.

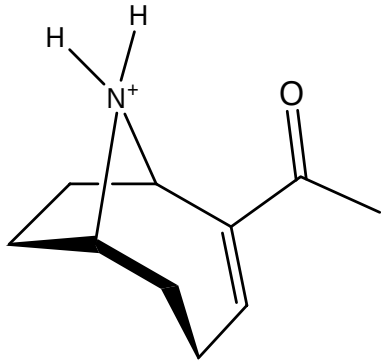

Figure 2-1. Chemical Structure of Anatoxin-a (protonated state)

Chemical and physical property data are not available in the open literature for anatoxin-a as the free base. Anatoxin-a is commercially produced as both hydrochloride and fumarate salts on a small scale for research purposes (A.G. Scientific Inc., 2006; BIOMOL International LP, 2006; MacPhail et al., 2005). Both of these salts have been used in toxicity studies of anatoxin-a because they readily ionize in water. Available experimental property data for racemic (+/-) anatoxin-a hydrochloride and anatoxin-a fumarate are provided in Table 2-1 (Sigma, 1995); data were not located for the (+)-enantiomer.

Table 2-1. Experimental Property Data for Racemic (+/-)-Anatoxin-a Hydrochloride and Anatoxin-a Fumarate

Name	(+/-)-Anatoxin-a Hydrochloride	(+/-)-Anatoxin-a Fumarate
Molecular Formula	$C_{10}H_{15}NO{\cdot}HCl$	$C_{10}H_{15}NO{\cdot}C_4H_4O_4$
Molecular Weight	187.67	281.31
Appearance	Information not available	Light brown hydroscopic solid; $[\alpha]^{20}_D$=+28°(c=0.29, MeOH)
Melting Point	Information not available	124-126°C (decomposes)
Solubility in Water	Information not available	1.51×10^4 mg/mL

3. TOXICOKINETICS

3.1. ABSORPTION

No quantitative data were located regarding the rate or extent of absorption of anatoxin-a in humans or animals. Acute oral toxicity studies in animals indicate that anatoxin-a is rapidly absorbed from the gastrointestinal tract, as shown by the occurrence of clinical signs of neurotoxicity including loss of coordination, muscular twitching, and death from respiratory paralysis within several minutes of exposure (Fitzgeorge et al., 1994; Stevens and Krieger, 1991).

3.2. DISTRIBUTION

No information regarding the tissue distribution of anatoxin-a was identified in the materials reviewed for this assessment.

3.3. METABOLISM

No information regarding the metabolism of anatoxin-a was identified in the materials reviewed for this assessment.

3.4. ELIMINATION

No information regarding the elimination of anatoxin-a was identified in the materials reviewed for this assessment.

3.5. PHYSIOLOGICALLY BASED TOXICOKINETIC MODELS

No physiologically based toxicokinetic models have been developed for anatoxin-a.

4. HAZARD IDENTIFICATION

Investigations into the characterization of anatoxin-a effectively began in 1961 following the deaths of cows that had ingested water from a lake containing an algal bloom in Saskatchewan, Canada (Carmichael and Gorham, 1978; Carmichael et al., 1975; Devlin et al., 1977; Moore, 1977). Toxin-producing and non-toxin-producing unialgal colony isolates of *A. flos-aquae* were isolated from specimens of the bloom in 1964, and toxicity testing showed that the cultured toxin-producing isolates elicited the same physiological effects as the parent bloom. The toxin was termed Very Fast Death Factor (VFDF) because intraperitoneal (i.p.) injection of toxin-producing cells or cell culture filtrates into mice induced paralysis, tremors, mild convulsions and death within 2-7 minutes. VFDF was chemically isolated and purified from lyophilized cells of one of the colony isolates (NRC-44h) in 1966, the structure of VFDF was determined and re-named anatoxin-a in 1977, and methods were subsequently developed to synthesize both racemic anatoxin-a and optically pure (+)-anatoxin-a. Experimental studies on the health effects of anatoxin-a began in the 1970s in response to poisoning outbreaks in animals that drank water from lakes, ponds and reservoirs containing blooms of *A. flos-aquae*. Although some preliminary studies were performed using suspensions of cultured cells, essentially all of the pertinent toxicity studies, which are summarized below, used anatoxin-a that was purified from cell extracts or synthesized *de novo*. Interest in anatoxin-a has continued due to concern for possible negative impacts on human drinking and recreational water quality and because it has been found to be a particularly useful molecule for characterizing the properties of acetylcholine receptors in the nervous system.

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

Cases of non-lethal human poisonings, predominantly manifested as acute gastrointestinal disorders (e.g., nausea, vomiting and diarrhea), have been attributed to the ingestion of lake water containing unspecified species of *Anabaena* and *Microcystis* (Schwimmer and Schwimmer, 1968). A number of these cases were documented by the detection of *Anabaena*, either alone or with *Microcystis*, in the feces. An allergy to *Anabaena* was demonstrated in a young woman who developed skin papulo-vesicular eruptions whenever she swam in a lake containing a bloom of the algae (Schwimmer and Schwimmer, 1968).

Anatoxin-a was implicated in the death of a 17-year-old boy who died 2 days after swallowing water while swimming in a pond containing an algal bloom (Behm, 2003). The boy went into shock and suffered a seizure before dying from heart failure. A companion teenage boy who also swallowed some of the pond water while swimming later became sick with severe diarrhea and abdominal pain but survived. Three other teenage boys who swam in the pond at the same time as other two, but had not been fully submerged in the water, developed only unspecified minor symptoms. Tests of stool samples from the two affected boys revealed the presence of *A. flos-aquae* cells (Behm, 2003). Results of initial analyses of liver, blood and ocular (vitreous) fluid samples from the boy who died indicated the presence of anatoxin-a but were negative for other cyanobacterial toxins (microcystins, cylindrospermopsins and saxitoxins) (Carmichael et al., 2004). The coroner concluded that anatoxin-a was the most reasonable cause

of the death based on the available information, but a definitive diagnosis was confounded by the delay between exposure and overt toxicity and the lack of other anatoxin-a -related human fatalities for a temporal comparison (Behm, 2003). In particular, the time of death is inconsistent with what is known about anatoxin-a toxicity as determined from laboratory animal studies (i.e., that signs of neurotoxicity and death typically occur within minutes to several hours of exposure). More recent (unpublished) analyses determined that the compound detected in the body fluids and liver tissue samples was not anatoxin-a but the amino acid phenylalanine (Carmichael et al., 2004), further confounding the diagnosis.

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

4.2.1. Oral Exposure

4.2.1.1. Acute Studies

An acute oral (single dose gavage) LD_{50} value of 16.2 mg/kg (95% confidence interval [CI]: 15.4-17.0) was determined for synthetic (+)-anatoxin-a hydrochloride (commercial product, ≥98% pure) in male Swiss Webster ND-4 mice (Stevens and Krieger, 1991). This LD_{50} is equivalent to 13.3 mg anatoxin-a/kg (95% CI: 12.8-14.1). A single dose gavage LD_{50} of >5 mg/kg was determined for anatoxin-a in newly weaned CBA/BalbC mice of unspecified sex (Fitzgeorge et al., 1994); the anatoxin-a in this study was a commercial product in a "suitably purified" but unspecified form. Deaths occurred within 2 minutes of gavage administration and were due to neurotoxicity, with manifestations that included loss of coordination, muscular twitching and death by respiratory paralysis (Fitzgeorge et al., 1994). A single dose gavage LD_{50} value of 6.7 mg anatoxin-a/kg was determined for male Swiss Webster ND-4 mice administered the toxin as a lysate solution of lyophilized *A. flos-aquae* (NRC-44-1) cells (Stevens and Krieger, 1991).

Anatoxin-a has been implicated in case reports of poisonings and deaths in dogs, livestock and waterfowl that consumed water containing blooms of toxin-producing cyanobacteria (Carmichael and Gorham, 1978; Edwards et al., 1992; Gunn et al., 1992; Pybus et al., 1986). Signs of toxicity were predominantly neurologic, with deaths due to respiratory paralysis. Quantitative exposure data were not reported.

4.2.1.2. Short-Term Studies

A 5-day oral toxicity study was performed in which groups of two male and two female Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in daily doses of 1.5, 3, 7.5 or 15 mg/kg (equivalent to 1.2, 2.5, 6.2 or 12.3 mg anatoxin-a/kg) (Fawell and James, 1994; Fawell et al., 1999). This is a range-finding study that was conducted to determine the maximum tolerated dose to be used in the 28-day study summarized below. The dosing of the 6.2 and 12.3 mg/kg-day groups commenced approximately 24 hours after the dosing of the 1.2 mg/kg-day group. The 2.5 mg/kg-day group was established 5 days after dosing of the 6.2 and 12.3 mg/kg-day groups as an intermediate level due to toxicity at these dosages (discussed below). No control group was

included. Clinical signs, body weight and food consumption were assessed, and surviving animals were necropsied. All high-dose mice and one female mouse in the 6.2 mg/kg-day group died within 5 minutes of dosing during the first 4 days. Males in the 6.2 mg/kg-day dose group were hyperactive following the third dose; no other signs of neurotoxicity were reported and none of the other surviving animals had any abnormal clinical signs. No changes in body weight or food consumption or unusual necropsy findings were observed in any animals. The highest no-observed-adverse-effect level (NOAEL), 2.5 mg/kg-day (3 mg anatoxin-a hydrochloride/kg-day), was selected as the maximum tolerated dose for the 28-day main study.

In the main study, groups of 10 male and 10 female Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in daily doses of 0 (vehicle control), 0.12, 0.6 or 3 mg/kg (0, 0.1, 0.5 or 2.5 mg anatoxin-a/kg) for 28 days (Fawell and James, 1994; Fawell et al., 1999). Endpoints that were examined included general condition and behavior (daily), body weight (weekly), food consumption (weekly), ophthalmoscopic condition (final week), hematology (final week; erythrocyte count, packed and mean cell volumes, hemoglobin concentration, mean cell hemoglobin, mean cell hemoglobin concentration, total and differential leukocyte counts and platelet counts) and blood chemistry (final week; blood urea nitrogen, glucose, alkaline phosphatase, alanine and aspartate aminotransferases [ALT and ASP, respectively], total protein, albumin, albumin/globulin ratio, sodium, chloride, potassium, calcium, inorganic phosphorus, total bilirubin, creatinine and cholesterol). Gross pathology and organ weights (liver, kidneys, adrenals and testes) were evaluated in all animals at the end of the study. Comprehensive histological examinations were performed on the control and high dose groups, on animals that died or were sacrificed during the study and on gross lesions from all animals. Histology was evaluated in the following tissues: adrenals, aorta, brain, cecum, colon, duodenum, epididymides, eyes (including optic nerves), femur (including marrow), heart, jejunum, kidneys, liver (including gall bladder), lungs (including mainstem bronchi), mammary gland, mesenteric lymph node, esophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, stomach, submandibular lymph node, testes, thymus, thyroid, parathyroid, trachea, urinary bladder and uterus.

There were three deaths during the course of the study. One death was not treatment-related: a male in the 0.1 mg/kg-day group was humanely sacrificed after being attacked by its cage mates. One 0.5 mg/kg-day male and one 2.5 mg/kg-day female died within 2.5 hours of dosing on days 10 and 14 of treatment, respectively. Both of these animals were clinically unremarkable prior to death, and the postmortem examinations were unable to establish the cause of death, leading the authors to conclude that a possible relationship to treatment could not be ruled out. The only other effects reported in treated animals were several minor hematology and blood chemistry changes that were not considered to be toxicologically significant. These alterations included statistically significant ($p<0.05$) increases in mean cell hemoglobin in males at >0.1 mg/kg-day, mean cell hemoglobin concentration in females at >0.5 mg/kg-day and serum sodium in females at $\geq$0.5 mg/kg-day. The alterations also included a nonsignificant increase in mean serum ASP in males at $\geq$0.5 mg/kg-day and sporadic changes in a few other blood chemistry indices. The study authors concluded that the NOAEL was 0.1 mg/kg-day based on the two deaths that occurred at the higher dose levels. This conclusion was based on their inability to determine the cause(s) of death (i.e., to completely rule out a relationship with

treatment), and they indicated that the true NOAEL may actually be 2.5 mg/kg-day. Due to the low incidences of mortality that showed no dose-response or gender consistency (1/10 males at 0.5 mg/kg-day and 1/10 females and 2.5 mg/kg-day), the lack of characteristic clinical signs of acute neurotoxicity in the two animals that died, and the absence of toxicologically significant effects in the surviving mice, as well as the lack of effects at 2.5 mg/kg-day in mice as reported in the 5-day study discussed above and a developmental toxicity study (discussed below) (Fawell and James, 1994; Fawell et al., 1999), EPA concludes that the deaths are likely to be incidental and that the actual NOAEL is 2.5 mg/kg-day.

4.2.1.3. Subchronic Studies

Groups of 20 female Sprague-Dawley rats were administered anatoxin-a in the drinking water in concentrations of 0, 0.51, or 5.1 ppm for 7 weeks (Astrachan and Archer, 1981; Astrachan et al., 1980). The anatoxin-a used in this study was extracted from the culture media of *A. flos-aquae* (NRC-44-1) cells and partially purified; purity was not quantified, but the toxin had a UV absorbance spectrum that qualitatively indicated that anatoxin-a was the principal UV-absorbing component. The authors assumed that the test rats consumed 0.1 mL/g body weight-day (based on a preliminary water consumption study), indicating that the estimated daily intakes of anatoxin-a in the low and high dose rats were 0.05 and 0.5 mg/kg-day, respectively. Endpoints evaluated throughout the study included clinical signs, food consumption, body weight (weekly), red and total white blood cell counts (weekly) and serum enzyme activities (alkaline phosphatase, ALT, gamma glutamyl transpeptidase [GGT] and cholinesterase) (weekly). Endpoints assessed at the end of the exposure period included hepatic mixed function oxidase activity (aldrin epoxidation *in vitro*), organ weights (liver, kidneys, spleen), gross pathology and histology (liver, kidneys, spleen, adrenals, heart, lungs and brain). Additional information regarding the design of this study was not reported. No treatment-related effects were observed, indicating a free-standing NOAEL of 0.5 mg/kg-day.

4.2.1.4. Chronic Studies

No information regarding the chronic effects of oral exposure to anatoxin-a in animals was identified in the materials reviewed for this assessment.

4.2.2. Inhalation Exposure

No information regarding the inhalation toxicity of anatoxin-a was identified in the materials reviewed for this assessment.

4.3. REPRODUCTIVE/DEVELOPMENTAL STUDIES - ORAL AND INHALATION

4.3.1. Oral Exposure

A developmental toxicity screening study was conducted in which groups of 10 and 12 pregnant Crl:CD-1(ICR)BR mice were administered aqueous (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by gavage in doses of 0 (vehicle control) or 3 mg/kg-day (0 or 2.5 mg anatoxin-a/kg), respectively, on gestation days (GD) 6-15 (Fawell and

James, 1994; Fawell et al., 1999). Clinical signs and body weight were recorded until day 18 of gestation, at which time the maternal animals were sacrificed and necropsied. Developmental endpoints appear to have been limited to numbers of implantations (live and dead) and live fetuses, post implantation loss and fetal body weight, sex ratio and external abnormalities. No treatment-related maternal or fetal effects were observed although it was noted that mean fetal weight (male, female and total) in the treated group was marginally lower than in controls (data not reported). The lack of adverse effects in dams and fetuses identifies 2.5 mg/kg-day as a free-standing NOAEL for maternal and developmental toxicity.

4.3.2. Inhalation Exposure

No information regarding the reproductive or developmental effects of inhalation exposure to anatoxin-a was identified in the materials reviewed for this assessment.

4.4. OTHER STUDIES

4.4.1. Neurotoxicity by Parenteral Exposure

Acute (single dose) i.p. LD_{50} values have been determined in mice; values include 0.25 mg/kg (95% CI: 0.24-0.28) for (+)-anatoxin-a hydrochloride (commercial product, ≥98% pure) (0.21 mg anatoxin-a/kg) (Stevens and Krieger, 1991) and 0.375 mg/kg for commercial anatoxin-a (form and purity not reported) (Fitzgeorge et al., 1994). Lethal i.p. doses were characterized by neurotoxic effects that included loss of coordination, muscular twitching and death by respiratory failure within 2 minutes (Fitzgeorge et al., 1994). Another study compared acute lethality in male BalbC mice that were administered single i.p. injections of (+)-, racemic or (-)-anatoxin-a hydrochloride (all >95% pure) and observed for 30 minutes following dosing (Valentine et al., 1991). LD_{50} values were determined to be 386 μg/kg (95% CI: 365-408) for (+)-anatoxin-a hydrochloride (0.32 mg anatoxin-a/kg) and 913 μg/kg (95% CI: 846-985) for racemic anatoxin-a hydrochloride (0.76 mg anatoxin-a/kg). No deaths or clinical signs occurred in mice treated with doses of (-)-anatoxin-a hydrochloride as high as 73 mg/kg (i.e., doses 189 times higher than (+)-anatoxin-a hydrochloride). The approximately 2-fold potency difference between (+)-anatoxin-a and the racemic mixture and the lack of toxicity with (-)-anatoxin-a is consistent with mechanistic data indicating that (+)-anatoxin-a is the biologically active enantiomer (see Section 4.5.2).

An incompletely reported 2-day study in mice was performed to determine the doses for a neurodevelopmental study (Rogers et al., 2005). Doses of anatoxin-a (commercial product, ≥90% purity) in distilled water ranging from 10 to 400 μg/kg were administered by i.p. injection to female CD-1 mice for 2 consecutive days. Another study by the same laboratory identified the same commercial product and lot number as racemic (+/-)-anatoxin-a hydrochloride (MacPhail et al., 2005). Individual dose levels of anatoxin-a hydrochloride included 10, 100, 200, 250, 300 and 400 μg/kg (0.008, 0.08, 0.17, 0.21, 0.25 and 0.33 mg anatoxin-a/kg); however, it was not reported if these were the only levels tested. The authors noted that the study was conducted with 18 mice, but it is unclear if this refers to total number of animals or group size. Endpoints other than survival and clinical signs of toxicity were not evaluated. After one dose, mortality in the 0.08, 0.17, 0.21, 0.25 and 0.33 mg/kg groups was 0, 0, 50, 100 and 100%,

respectively; all rats that received a second dose survived. Observations in mice administered lethal doses (≥0.21 mg/kg-day) included decreased motor activity, altered gait, difficulty breathing and convulsions. The onset of clinical signs was noted after 5-6 minutes and death occurred within 10 minutes. Similar clinical signs (decreased motor activity level, altered gait and breathing difficulties) occurred at 0.17 mg/kg and in the 0.21 mg/kg mice that survived, but the convulsion stage was never reached and recovery occurred by 15-20 minutes. Additional information on this study, including results for doses lower than 0.08 mg/kg, was not reported.

In the neurodevelopmental study, groups of 8-11 time-pregnant CD-1 mice were administered (+/-)-anatoxin-a hydrochloride (commercial product, ≥90% purity) via i.p. injection in distilled water in doses of 0, 125 or 200 µg/kg-day (0, 0.10 or 0.17 mg anatoxin-a/kg-day) on GD 8-12 or 13-17 (Rogers et al., 2005). All mice were allowed to give birth and body weight and viability of the pups were determined on postnatal days (PND) 1 and 6. Neurodevelopmental maturation was assessed by testing righting reflex, negative geotaxis and hanging grip time on PND 6, 12 and/or 20 in pups from dams exposed on GD 13-17. These behavioral tests were only conducted in the pups exposed on GD 13-17 because this gestational interval follows the onset of neurogenesis in the mouse brain (Rice and Barone, 2000). The litters from the dams exposed on GD 13-17 were normalized to eight pups (four males and four females) on PND 6, and a randomly selected male and female pup from each litter was evaluated on each test day. Righting reflex was tested on PND 6 and 12 by gently turning the pup over and holding it on its back and then quickly releasing it and measuring the time for the pup to return to an upright position with all feet on the horizontal surface. Negative geotaxis was tested on PND 6, 12 and 20 by placing the pup on an inclined screen facing downhill and determining the time to rotate to facing up the incline. Hanging grip time was tested on PND 12 and 20 by holding the pups by the base of the tail above the work surface and allowing them to grasp a bar with their front feet, releasing them to hang and measuring the time until each pup let go.

Maternal toxicity was observed at 0.17 mg/kg-day, as shown by decreased motor activity immediately after treatment (additional details not reported) (Rogers et al., 2005). There were no effects on pup viability (number of live pups) on PND 1 or 6 in mice treated on GD 8-12 or 13-17 or on pup body weight on PND 1 or 6 in mice treated on GD 8-12. Pups treated on GD 13-17 showed a statistically significant dose-related trend for reduced body weight on PND 1 ($p<0.05$) but not on PND 6 ($p<0.07$). Body weight on PND 1 in the pups exposed on GD 13-17 was 7.1 and 8.7% less than controls at 0.10 and 0.17 mg/kg-day, respectively, and the differences between the treated and control groups were not significant. Although the trend data could have been interpreted as a treatment-related effect during the latter part of gestation, the investigators believed that the marginal effect on pup weight was due to random variability in litter size: the litter size of the GD 13-17 controls was noticeably smaller than the treated groups ($p=0.09$), and a difference in litter size would impact both birth weight and growth on PND 1-6 because pups in smaller litters are larger at birth (McCarthy, 1967) and grow more rapidly postnatally (Rogers et al., 2003). Almost all of the results of the righting reflex, negative geotaxis and hanging grip time tests showed no statistically significant differences between exposed and control groups or dose-related trends, indicating a lack of postnatal neurotoxicity. Findings in the righting reflex test included a non-significant ($p<0.086$) dose-related trend towards slower righting in males on PND 6 and a significantly (p value not reported) slower reflex in females than males in all treatment groups on PND 6 but no treatment or gender differences in righting reflex were

observed on PND 12. The negative geotaxis test was complicated by control values (turning times) that did not decrease from PND 6 to 20 as expected and by many control and dose groups in which 1-4 pups fell off the screen before turning. Although the latter finding was a confounding factor because only data from mice that stayed on the inclined screen could be evaluated, there were no significant differences across treatment groups in either the number of fallen mice or the average turning times. There also were no treatment-related differences in hanging grip time on either test day. Hang time increased significantly from PND 12 to 20 in females, as expected, although males did not show the expected increase in hang time. The investigators observed that gender differences are usually not evident at this age, indicating that random variability in the tested population may account for the finding in the male pups.

The mouse pups that were exposed to anatoxin-a on GD 13-17 in the Rogers et al. (2005) study were subsequently tested as adults to determine the effect of prenatal exposure to anatoxin-a on the motor activity of adult mice and their responses to nicotine challenge (MacPhail et al., 2005). Motor activity was measured in 30-minute sessions using a photocell device when the offspring were approximately 8 months old. Preliminary testing was performed in which groups of 12 male and 12 female mice were subcutaneously administered a single 0, 0.1, 0.3, 1.0 or 3.0 mg/kg dose of nicotine in saline approximately 5 minutes before testing motor activity. These mice were taken from litters that received saline vehicle on GD 8-12 or 13-17 (Rogers et al., 2005) and were assigned to the nicotine dose groups regardless of gestational period. Dose-related decreases in both horizontal and vertical activity were observed and 0.65 mg/kg was estimated to be the effective dose in 50% of subjects (ED_{50}) for nicotine in both sexes. Mice exposed to 0, 0.10 or 0.17 mg anatoxin-a/kg-day on GD 13-17 were then given the nicotine ED_{50} or saline vehicle approximately 5 minutes before testing motor activity. The nicotine ED_{50} and saline vehicle treatments were separated by 1 week. Group sizes were 10 per gender, except for the high-dose anatoxin-a female group, which contained nine mice. There were no differences in horizontal or vertical motor activity between the anatoxin-a-treated mice and the controls. The report presents the results of the activity tests in bar graphs but provides no indication that the comparisons were based on a statistical evaluation of the data.

Additional information on neurobehavioral effects of anatoxin-a is available from intravenous and subcutaneous injection studies. Mice that were administered a single dose of (+)-anatoxin-a hydrochloride (commercial product, purity not reported) by intravenous injection were evaluated using the Irwin Screen and rota-rod tests at levels of 10-100 μg/kg (8-83 μg anatoxin-a/kg) and 30-60 μg/kg (25-50 g anatoxin-a/kg), respectively (Fawell and James, 1994; Fawell et al., 1999). The Irwin Screen is a standard functional observational battery used to characterize CNS effects, including motor activity, behavioral changes, coordination and sensory/motor reflex responses. The rota-rod test assesses sensorimotor coordination by evaluating the animal's ability to remain on a rotating rod. There were no exposure-related effects in either of these tests although the highest doses caused clinical signs of neurotoxicity and death within 1 minute of exposure. The clinical signs of neurotoxicity included increased respiration, salivation, micturition, hyperactivity and Straub tail (contraction of the sacrococcygeus muscle, resulting in vertical erection of the tail). Testing in rats that were administered aqueous (+)-anatoxin-a fumarate by subcutaneous injection showed that a single dose of 0.1 mg/kg (0.06 mg anatoxin-a/kg) caused decreased locomotor activity as well as a partial nicotine-like discriminative stimulus effect in animals trained to discriminate nicotine

from saline in an operant conditioning procedure (Stolerman et al., 1992). As reported in an abstract, anatoxin-a also decreased response and reinforcement rates in multiple-schedule operant performance tests in rats treated by subcutaneous injection, although substantial tolerance developed upon repeated administration (Jarema and MacPhail, 2003).

4.4.2. Other Effects by Parenteral Exposure

The short-term parenteral toxicity of anatoxin-a was evaluated in groups of 18 female Sprague-Dawley rats that were administered daily doses of 0 or 0.062 mg/kg via i.p. injection for 21 days (Astrachan and Archer, 1981; Astrachan et al., 1980). The anatoxin-a used in this study was extracted from the culture media of *A. flos-aquae* (NRC-44-1) cells and partially purified; purity was not quantified, but the toxin had a UV absorbance spectrum that qualitatively indicated that anatoxin-a was the principal UV-absorbing component. Endpoints included body weight, food consumption, serum enzyme activities (alkaline phosphatase and ALT), cholinesterase activity (whole blood and brain) and gross pathology and histology (scope not specified). No exposure-related effects were observed.

The developmental toxicity of i.p. injected anatoxin-a was evaluated in groups of five or six golden hamsters that received doses of 0.2 mg/kg once a day on gestation days 8-14 or 0.125 or 0.2 mg/kg 3 times a day on gestation days 8-11 or 12-14 (Astrachan et al., 1980). The anatoxin-a used in this study was extracted from the culture media of *A. flos-aquae* (NRC-44-1) cells and partially purified; purity was not quantified, but the toxin had a UV absorbance spectrum that qualitatively indicated that anatoxin-a was the principal UV-absorbing component. Vehicle (not reported) and untreated control groups were included in the study. Maternal animals were sacrificed on gestation day 15 for assessment of endpoints that were limited to numbers of implantations and resorptions, fetal body weight and numbers of fetuses with malformations (external, visceral and skeletal). No maternal toxicity was observed in any of the treated animals (scope of examinations not reported). Fetal weights were significantly reduced in the groups exposed to 0.2 mg/kg-day on gestation days 8-14 (18% less than controls), 0.125 or 0.2 mg/kg 3 times a day on gestation days 8-11 (9% less than controls at both dose levels) and 0.125 mg/kg 3 times a day on gestation days 12-14 (24% less than controls). The only other reported effect was hydrocephaly that was not likely to be chemical related; it occurred in all 10 fetuses in one out of six total litters in the 0.125 mg/kg group treated 3 times a day on gestation days 12-14.

4.4.3. Effects by Intranasal Instillation

An acute LD_{50} value of 2 mg/kg was determined for intranasal instillation of anatoxin-a (commercial product, form and purity not reported) in mice (Fitzgeorge et al., 1994). Additional information regarding this study was not reported.

4.4.4. *In Vitro* Studies

In vitro developmental toxicity was evaluated in groups of 9-13 mouse whole embryos (GD 8, mouse strain not reported) that were exposed to 0, 0.1, 1.0, 10 or 25 μM of (+/-)-anatoxin-a hydrochloride (0, 0.08, 0.8, 8.3 and 20.8 μM anatoxin-a) (commercial product, ≥90%

purity) in culture medium for 26-28 hours (Rogers et al., 2005). The range of concentrations included some with no adverse effects. At the end of the culture period the embryos were evaluated for dysmorphogenesis; endpoints included somite number, normal morphology and yolk sac dysmorphology. Perturbations in yolk sac vasculature, i.e., a decrease in large caliber vessels and a reduction in arborization, occurred in 0, 0, 44.4, 100 and 100% of the conceptuses at 0, 0.08, 0.8, 8.3 and 20.8 μM, respectively.

An *in vitro* amphibian toxicity test of (+/-)-anatoxin-a hydrochloride (commercial product, ≥90% purity) was conducted using toad embryos (*Bufo arenarum*) beginning at life cycle Stage 18 (muscular response) or Stage 25 (complete operculum) (Rogers et al., 2005). Stage 18 embryos were exposed to 0.03, 0.3, 3.0 or 30 mg/L concentrations of anatoxin-a hydrochloride (0.025, 0.25, 2.5 or 25 mg anatoxin-a/L) for 10 days, and Stage 25 embryos were exposed to 30 mg/L of anatoxin-a hydrochloride (25 mg anatoxin-a/L) for 10 days. Embryos were monitored for viability and functional impairments during the 10-day exposures and for 3 days post-exposure. Main effects included narcosis and mortality; loss of equilibrium and edema were also noted. The narcosis was transient (times of occurrence not reported), dose-dependent and affected ≥70% of the embryos at 25 mg/L in both embryonic stages. The mortality was delayed in both embryonic stages, occurring after 6-10 days of exposure. Mortality in Stage 18 embryos occurred at 0.25-25 mg/L on days 10-13 (i.e., up to 3 days after cessation of exposure). At 25 mg/L, mortality was 20% on day 8 and reached 100% between days 10 and 13. In Stage 25 embryos, mortality was initially observed at day 6 and reached 100% by day 9. Additional information regarding the results of this study was not reported.

4.4.5. Interactions With Other Cyanotoxins

Possible interactions between anatoxin-a and other cyanotoxins are of interest because algal blooms can contain multiple cyanotoxin-producing algal species and many toxin-producing species produce more than one toxin. Potential synergism was tested in CD-1 mice (number and sex not reported) that were administered a single gavage dose of 0, 500 or 1000 μg/kg (0, 0.5 or 1 mg/kg) of microcystin-LR (commercial product, ≥98% purity) in distilled water, followed 50 minutes later by similar administration of 0, 500, 1000 or 2500 μg/kg (+/-)-anatoxin-a hydrochloride (0, 0.4, 0.9 or 2.5 mg anatoxin-a/kg) (commercial product, ≥95% purity) (Rogers et al., 2005). Mice were monitored for clinical signs of acute intoxication and changes in body weight (mice were weighed prior to treatment and 3 hours later). No deaths or definitive signs of intoxication (decreased motor activity, rough hair coat, altered gait, convulsions or failure to eat) occurred in any group, and there were no differences in pre- to post-treatment changes in body weight.

4.5. MECHANISTIC DATA AND OTHER STUDIES IN SUPPORT OF THE MODE OF ACTION

4.5.1. Mechanisms of Neurotoxicity

In vitro studies have clearly demonstrated that (+)-anatoxin-a mimics the action of acetylcholine at neuromuscular nicotinic receptors (Aronstam and Witkop, 1981; Biggs and Dryden, 1977; Carmichael et al., 1975, 1979; Swanson et al., 1986) and is significantly more

potent than acetylcholine and nicotine as an agonist (see Section 4.5.2). Anatoxin-a has become a very useful agent for investigating nicotinic acetylcholine receptors because it is resistant to enzymatic hydrolysis by acetylcholinesterase and because it is 100-fold more selective for nicotinic acetylcholine receptors than for muscarinic acetylcholine receptors (Aronstam and Witkop, 1981). When acetylcholine is released at the neuromuscular junction of motor neurons, it binds to muscle cell receptor molecules consisting of a neuromuscular binding site and an ion channel, which triggers ionic currents that induce muscle cell contraction. Extracellular acetylcholinesterase acts on acetylcholine by degrading the neurotransmitter to prevent overstimulation of the muscle cells. Because anatoxin-a is not degraded by cholinesterase or any other known cellular enzymes, muscle cells continue to be stimulated, causing muscular twitching, fatigue and paralysis. Severe overstimulation of respiratory muscles may result in respiratory arrest and rapid death, as observed in acute lethality studies in animals (Carmichael et al., 1975, 1977; Devlin et al., 1977; Stevens and Krieger, 1991).

Anatoxin-a also acts as a nicotinic cholinergic agonist at receptors in the cardiovascular system of rats, resulting in increased blood pressure and heart rate (Adeyemo and Sirén, 1992; Dube et al., 1996; Sirén and Feuerstein, 1990), as well as in rat and human brain neurons (Durany et al., 1999; Thomas et al., 1993; Zhang et al., 1987). Anatoxin-a is a potent agonist of the secretory response of bovine adrenal chromaffin cells, presumably via neuronal-type nicotinic receptor activation (Molloy et al., 1995).

Anatoxin-a is capable of eliciting the release of neurotransmitters from presynaptic neuromuscular and brain cell terminals. Incubation of guinea pig ileum longitudinal muscle-myenteric plexus preparations with anatoxin-a resulted in dose-dependent release of acetylcholine (Gordon et al., 1992). Anatoxin-a stimulated the release of dopamine from rat striatal synaptosomes in a dose-dependent manner (Clarke and Reuben, 1996; Rowell and Wonnacott, 1990; Soliakov et al., 1995; Wonnacott et al., 2000). These findings indicate that anatoxin-a can bind to presynaptic nicotinic receptors to trigger neurotransmitter release. Increased neurotransmitter release could contribute to increased stimulation of postsynaptic receptors.

4.5.2. Structure-Activity Relationships

Anatoxin-a is produced as the natural stereoisomer, (+)-anatoxin-a, by some strains of *Anabaena* (particularly *A. flos-aquae*) and at least four other genera of freshwater cyanobacteria, including *Aphanizomenon*, *Microcystis*, *Planktothrix* and *Oscillatoria* (Devlin et al., 1977; Fawell et al., 1999; Huber, 1972; Viaggiu et al., 2004). As discussed in Section 4.5.1, (+)-anatoxin-a is a nicotinic acetylcholine receptor agonist that exerts its effects at both peripheral and central cites in the nervous system. It is generally believed that nicotinic agonists form hydrogen bonds in the planar region and contain a bulky cationic group approximately 5.9 Å from the hydrogen bond (Beers and Reich, 1970; Chothia and Pauling, 1970; Spivak and Albuquerque, 1982); anatoxin-a exhibits these characteristics. The importance of stereospecificity was demonstrated in assays of contracture potency in frog rectus abdominis muscle preparations; natural (+)-anatoxin-a exhibited at least a 2.5- and 150-fold greater potency than racemic and (-)-anatoxin-a, respectively (Spivak et al., 1983; Swanson et al., 1986). Similar potency differences were demonstrated in *in vivo* lethality assays in mice (Valentine et al., 1991).

As discussed in Section 4.4.1, acute i.p. LD_{50} values of 386 and 913 μg/kg were determined for (+)-anatoxin-a hydrochloride and racemic anatoxin-a hydrochloride, respectively. No clinical signs or deaths occurred in mice that were similarly treated with doses of (-)-anatoxin-a hydrochloride as high as 73 mg/kg. These findings indicate that (+)-anatoxin-a was 2.4 and at least 189 times as potent as racemic and (-)-anatoxin-a, respectively.

(+)-Anatoxin-a is significantly more potent than acetylcholine and nicotine as an agonist at neuromuscular nicotinic acetylcholine receptors. Anatoxin-a has been shown to bind to the nicotinic acetylcholine receptor with an affinity approximately 3.6 times greater than acetylcholine (Swanson et al., 1986). Following complete inhibition of acetylcholinesterase activity in frog rectus abdominis muscle preparations, anatoxin-a exhibited an 8-fold greater potency by contracture than acetylcholine (Swanson et al., 1986). Anatoxin-a was 7-136 times more potent than nicotine in a series of *in vitro* acetylcholine receptor (guinea pig ileum, rat phrenic nerve, chick biventer cervicis muscle) and mouse intravenous (neuropharmacological signs) screening studies (Fawell and James, 1994; Fawell et al., 1999). Additionally, the agonist potency of (+)-anatoxin-a was 3-50 times more than nicotine and approximately 20 times more than acetylcholine at neuronal nicotinic acetylcholine receptors from rat hippocampal synaptosomes, fetal rat hippocampal neurons, mouse M10 cells and frog (*Xenopus*) oocytes (Thomas et al., 1993).

Neuromuscular and neuronal assays of structure activity relationships indicate that N-methylation of anatoxin-a greatly reduces the acetylcholine-mimicking effect at nicotinic cholinergic receptors (Aracava et al., 1987; Costa et al., 1990; Kofuji et al., 1990; Stevens and Krieger, 1990; Swanson et al., 1989, 1991; Wonnacott et al., 1991).

4.5.3. Other Studies

Anatoxin-a caused apoptosis in rat thymocytes and monkey kidney (Vero) cells that was characterized by DNA fragmentation and apparently mediated by generation of reactive oxygen species and caspase activation (Rao et al., 2002). Anatoxin-a also caused cytotoxicity in cultured mouse B- and T-lymphocytes, but apoptosis was not induced; the cytotoxic action appeared to be non-selective and non-specific, and the mechanism remains to be elucidated (Teneva et al., 2005).

4.6. SYNTHESIS AND EVALUATION OF MAJOR NONCANCER EFFECTS

4.6.1. Oral

The main oral toxicity studies of anatoxin-a are summarized in Table 4-1. A limited amount of information is available on the health effects of anatoxin-a in humans. One report surveyed several cases of nonlethal human poisonings caused by ingestion of lake water containing *Anabaena sp*. (Schwimmer and Schwimmer, 1968). The most prominent and best documented effects were acute gastrointestinal disorders. In a more recent report, anatoxin-a was implicated in the poisonings of two teenage boys who swallowed water from a pond containing an algal bloom (Behm, 2003). One of the boys suffered a seizure and died from heart failure 2 days after swallowing the water, and the other boy became sick with severe diarrhea

Table 4-1. Summary Results of Major Oral Toxicity Studies of Anatoxin-a in Experimental Animals								
Species	Sex	Average Daily Dose (mg/kg-day)	Exposure Duration	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Responses	Comments	Reference
Acute Exposure								
No suitable acute studies are available								
Short-term Exposure								
Mouse	M,F	1.2, 2.5, 6.2, 12.3	5 days	2.5	6.2*	FEL = 6.2 mg/kg-day due to mortality in the two highest dose groups.	No clinical signs or effects on body weight, food consumption, survival or necropsy at ≤2.5 mg/kg-day. Other endpoints not examined. No control group and small group sizes (2/sex/dose).	Fawell and James, 1994; Fawell et al., 1999
Mouse	M,F	0, 0.1, 0.5, 2.5	28 days	2.5	ND	Two deaths at ≥0.5 mg/kg-day; it was unclear whether these deaths could be attributed to compound administration. Minor hematology and blood chemistry changes at ≥0.1 mg/kg-day were not clearly exposure-related and/or toxicologically significant.	Well-designed study that investigated clinical signs, body weight, food consumption, ophthalmic condition, hematology, blood chemistry, gross pathology, organ weights and histology (comprehensive). 10 mice/sex/dose.	Fawell and James, 1994; Fawell et al., 1999
Mouse	F	0, 2.5	9 days; gestation days 6-15	2.5	ND	No exposure-related adverse maternal or fetal effects. Mean fetal weight was marginally reduced at 2.5 mg/kg-day (data not reported) but not considered to be toxicologically significant.	Maternal endpoints included clinical signs, body weight and necropsy. Developmental endpoints included numbers of implantations and live fetuses, post implantation loss, body weight, sex ratio and external abnormalities. No fetal internal examinations. 10-12 mice/dose.	Fawell and James, 1994; Fawell et al., 1999

Table 4-1 cont.								
Species	Sex	Average Daily Dose (mg/kg-day)	Exposure Duration	NOAEL (mg/kg-day)	LOAEL (mg/kg-day)	Responses	Comments	Reference
Subchronic Exposure								
Rat	F	0, 0.05, 0.5	7 weeks	0.5	ND	None	Limited number of endpoints: clinical signs, food consumption, body weight, red and total white blood cell counts, serum enzyme activities (alkaline phosphatase, ALT, GGT, cholinesterase), hepatic MFO activity (aldrin epoxidation *in vitro*), organ weights (liver, kidneys, spleen), gross pathology and histology (liver, kidneys, spleen, adrenals, heart, lungs and brain). 20 rats/dose.	Astrachan and Archer, 1981; Astrachan et al., 1980
Chronic Exposure								
No suitable chronic studies are available								

* FEL
FEL = Frank effect level
LOAEL = Lowest-observed-adverse-effect level
ND = not determined
MFO = mixed function oxidase

and abdominal pain but survived. Testing of stool samples from both boys revealed the presence of *A. flos-aquae* cells, and analyses of blood, liver tissue and ocular fluid from the boy who died found a compound initially identified as anatoxin-a. A definitive diagnosis of anatoxin-a as the cause of death was confounded by the apparent delay between exposure and overt toxicity (Behm, 2003), and subsequent analysis determined that the detected compound was not anatoxin-a (Carmichael et al., 2004). Relevant dose-response information, including estimated amounts of water or toxin ingested, was not provided in either of the above reports.

Anatoxin-a has also been implicated in cases of animal poisonings following consumption of water containing blooms of *A. flos-aquae* (Carmichael and Gorham, 1978; Edwards et al., 1992; Gunn et al., 1992; Pybus et al., 1986) although no quantitative exposure data are available. The preponderance of experimental studies on anatoxin-a are *in vitro* and pertain to its mode of neurotoxic action (see Section 4.5.1).

Information on the *in vivo* effects of anatoxin-a in orally-exposed laboratory animals is available from several acute and short-term studies and one subchronic study that provide a limited amount of dose-response data on systemic toxicity and developmental toxicity, as summarized below.

Acute toxicity data for anatoxin-a are essentially limited to the results of lethality assays in mice that determined a single-dose LD_{50} value of 13.3 mg anatoxin-a/kg and identified neurotoxicity as the cause of death (Fitzgeorge et al., 1994; Stevens and Krieger, 1991).

Information on the short-term oral toxicity of anatoxin-a is available from 5- and 28-day systemic toxicity studies in mice (Fawell and James, 1994; Fawell et al., 1999) and a developmental toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999). The 5-day mouse study used four dose levels (1.2, 2.5, 6.2 and 12.3 mg/kg-day by gavage) but is limited by small numbers of animals (2/sex/dose), lack of controls and few endpoints (clinical signs, body weight, food consumption, and necropsy). Based on dose-related mortality at 6.2 mg/kg-day (1/4 mice) and 12.3 mg/kg-day (4/4 mice), the NOAEL and FEL are 2.5 and 6.2 mg/kg-day, respectively.

The 28-day mouse study used three dose levels (0.1, 0.5 and 2.5 mg/kg-day by gavage) and was generally comprehensive in that hematology, blood chemistry, gross pathology and histology were included in the evaluations (Fawell and James, 1994; Fawell et al., 1999). The only remarkable findings were one death (1/10 males) at 0.5 mg/kg-day and one death (1/10 females) at 2.5 mg/kg-day that occurred within 2.5 hours of dosing on days 10 and 14 of treatment. The deaths were not preceded by any clinical signs of distress and postmortem examination of the animals did not identify a cause of death. Accordingly, the authors could not completely rule out a possible relationship to anatoxin-a exposure and concluded that the NOAEL was 0.1 mg/kg-day, although they indicated that the actual NOAEL may be 2.5 mg/kg-day. Considering the lack of clinical signs (anatoxin-a is an acute neurotoxin with characteristic clinical signs before death), very low incidences of mortality that showed no dose-response or gender consistency, absence of toxicologically significant effects in surviving mice and lack of effects at 2.5 mg/kg-day in the 5-day and developmental toxicity studies, EPA concludes that the

deaths in the 28-day study are likely to be incidental and that the actual NOAEL is 2.5 mg/kg-day.

The developmental toxicity study in mice was limited by use of only one dose level (2.5 mg/kg-day by gavage) and fetal assessments that lacked internal soft tissue and skeletal examinations (Fawell and James, 1994; Fawell et al., 1999). No maternal effects (clinical signs, body weight, necropsy) or developmental effects (numbers of implantations and fetuses, fetal body weight and sex ratio, external abnormalities) were observed. Although this study provides insufficient information on possible fetal abnormalities, a developmental toxicity study of intraperitoneally-injected anatoxin-a in hamsters found no external, soft tissue or skeletal changes in fetuses at doses high enough to cause reduced fetal weight (Astrachan et al., 1980). Additionally, there were no effects on postnatal neurodevelopmental maturation, as shown by righting reflex, negative geotaxis and hanging grip time tests on PND 6-20, in offspring of mice that were gestationally administered anatoxin-a at intraperitoneal doses high enough to cause decreased maternal motor activity (Rogers et al., 2005). The lack of effects on fetal weight and other endpoints in the oral mouse study indicates that 2.5 mg/kg-day is a free-standing NOAEL for maternal toxicity and developmental toxicity by oral exposure.

As indicated above, the 28-day toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999) is the best-designed short-term oral study of anatoxin-a. The 2.5 mg/kg-day NOAEL for systemic toxicity in this study is supported by the 2.5 mg/kg-day NOAEL in the 5-day mouse study (Fawell and James, 1994; Fawell et al., 1999) and the 2.5 mg/kg-day NOAEL for maternal and developmental toxicity in mice (Fawell and James, 1994; Fawell et al., 1999). No adverse effect levels were identified in the 28-day and developmental toxicity studies although 6.2 mg/kg-day was a FEL in the 5-day study. Considering the evidence for the 2.5 mg/kg-day NOAEL from two studies and its apparent proximity to the threshold for adverse effects (as indicated by the 6.2 mg/kg-day FEL), the NOAEL is an adequate basis for quantitative assessment of short-term noncancer risks of anatoxin-a (see Section 5.2.3). The lack of adverse effects at doses below those causing death is consistent with the acute neurotoxic nature of the chemical.

Information on the subchronic oral toxicity of anatoxin-a is available from a 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980). This study is limited by the use of only two dose levels (0.05 and 0.5 mg/kg-day) and non-comprehensive examinations, particularly for hematology (two indices), blood chemistry (four serum enzymes) and histology (seven tissues). No treatment-related effects were found, indicating that 0.5 mg/kg-day is a subchronic NOAEL. The small number of endpoints and lack of an adverse effect level to provide information on proximity of the NOAEL to the toxicity threshold are problematic, but data from the short-term studies are supportive of the subchronic NOAEL. In particular, the NOAELs of 2.5 mg/kg-day in the 5-day, 28-day and developmental toxicity studies and FEL of 6.2 mg/kg-day in the 5-day study (Fawell and James, 1994; Fawell et al., 1999) provide indications that the 0.5 mg/kg-day NOAEL in the 7-week study is a reliable value and a sufficient basis for subchronic risk assessment.

No information is available on the chronic oral toxicity of anatoxin-a.

4.6.2. Inhalation

No information is available on the inhalation toxicity of anatoxin-a.

4.6.3. Mode of Action Information

The main known toxic effect of anatoxin-a is acute neurotoxicity that is manifested as progressive clinical signs that include loss of coordination, muscular fasciculations, convulsions and death by respiratory paralysis. It is well documented that anatoxin-a acts by mimicking the action of acetylcholine at neuromuscular nicotinic receptors (Aronstam and Witkop, 1981; Biggs and Dryden, 1977; Carmichael et al., 1975, 1979; Swanson et al., 1986). As an agonist that is significantly more potent than acetylcholine and is not degraded by cholinesterase (Swanson et al., 1986; Thomas et al., 1993), (+)-anatoxin-a interacts with the nicotinic acetylcholine receptors to cause persistent stimulation of the muscle cells.

4.7. WEIGHT-OF-EVIDENCE EVALUATION AND CANCER CHARACTERIZATION

4.7.1. Summary of Overall Weight-of-Evidence

No cancer or genotoxicity studies, no information on potential modes of carcinogenic action nor other carcinogenicity data are available for anatoxin-a. In accordance with the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), the weight of evidence descriptor for the carcinogenic hazard potential of anatoxin-a is "Inadequate Information to Assess Carcinogenic Potential."

4.7.2. Synthesis of Human, Animal and Other Supporting Evidence

No information regarding carcinogenicity in humans or animals or on possible carcinogenic processes and mode(s) of action of anatoxin-a was identified in the materials reviewed for this assessment.

4.8. SUSCEPTIBLE POPULATIONS AND LIFE STAGES

4.8.1. Possible Childhood Susceptibility

There is no information on the degree to which children might differ from adults in the disposition of, or response to, anatoxin-a.

4.8.2. Possible Gender Differences

There is no information on possible gender differences in the disposition of, or response to, anatoxin-a.

4.8.3. Other Possible Susceptible Populations

Anticholinergic agents have been recommended for the treatment of various medical conditions, but therapeutic uses are mainly in four areas: atony of the smooth muscle of the intestinal tract and urinary bladder, glaucoma, myasthenia gravis and termination of the effects of competitive neuromuscular blocking agents (Taylor, 1996). It is conceivable that people using anticholinergic agents for therapeutic purposes could be at risk of experiencing an increase in unwanted side effects if exposed to anatoxin-a due to the potential for additivity of adverse effects.

5. DOSE-RESPONSE ASSESSMENTS

5.1. NARRATIVE DESCRIPTION OF THE EXTENT OF THE DATABASE

Studies on the absorption, tissue distribution, metabolism and elimination of anatoxin-a have not been performed. Acute oral toxicity studies in animals indicate that anatoxin-a is rapidly absorbed from the gastrointestinal tract as shown by clinical signs of neurotoxicity and death within several minutes of exposure.

The only information on the toxicity of anatoxin-a in humans consists of two reports implicating ingestion of lake or pond water containing *Anabaena sp.* in several cases of non-lethal gastrointestinal poisonings and in one death. Anatoxin-a has also been implicated in cases of domestic and wild animal neurotoxicity and death following consumption of water containing blooms of *A. flos-aquae*. Details regarding most of these human and non-laboratory animal exposures and effects were not reported and doses were not estimated.

The acute *in vivo* neurotoxicity of anatoxin-a in animals is well-documented and characterized by tremors, altered gait, convulsions and death by respiratory paralysis. Little information is available on *in vivo* neurotoxicity at sublethal doses; findings include no effects of gestational intraperitoneal exposure on postnatal neurodevelopmental maturation in mice and no effects of acute intravenous exposure on motor activity, coordination, sensory/motor reflexes and other central nervous system responses in mice. The preponderance of experimental studies of anatoxin-a are *in vitro*, pertain to its mode of neurotoxic action and have established that it is a nicotinic acetylcholine receptor agonist that exerts its effects at both peripheral and central sites in the nervous system. *In vitro* studies also indicate that anatoxin-a can affect non-neuronal cells, causing effects that include apoptosis via production of reactive oxygen species and caspase activation in rat thymocytes and monkey kidney cells, and cytotoxicity without apoptosis in mouse lymphocytes.

Information on the *in vivo* effects of anatoxin-a in orally exposed laboratory animals is available from single-dose lethality assays in mice, 5- and 28-day studies in mice, a 7-week study in rats and a developmental toxicity study in mice. These studies only provide a limited amount of dose-response data on systemic toxicity and developmental toxicity due to limitations in experimental design and reporting, including insufficient numbers of dose levels and study endpoints. In particular, the oral database is limited by a preponderance of NOAELs and no LOAELs; information on adverse effects essentially consists of FELs for neurotoxicity-induced mortality in the single-dose and 5-day studies. The 28-day and 7-week studies provide bases for derivation of short-term and subchronic oral RfDs, but the remaining oral data are insufficient for deriving acute and chronic RfDs.

No information is available on the inhalation toxicity of anatoxin-a.

5.2. ORAL REFERENCE DOSE (RfD)

5.2.1. Data Considered in Deriving Reference Values

Data considered in deriving oral reference values for each duration of exposure are summarized in Table 4-1 (see Section 4.6.1).

5.2.2. Acute Duration

5.2.2.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

The available acute duration oral toxicity data for anatoxin-a are inadequate to support derivation of an acute RfD. Cases of non-lethal human poisonings, manifested mainly as acute gastrointestinal disturbances, have been attributed to ingestion of lake or pond water containing anatoxin-a-producing *Anabaena sp.* (Behm, 2003; Schwimmer and Schwimmer, 1968). Anatoxin-a was implicated in the death of a person who suffered a seizure and heart failure 2 days after swallowing pond water containing *A. flos-aquae* in an algal bloom (Behm, 2003; Carmichael et al., 2004). None of these case reports provide dose information or unequivocally establish anatoxin-a as the causal agent. Acute oral experimental data for anatoxin-a in animals are essentially limited to the results of two lethality assays in mice that determined a single-dose LD_{50} value of 13.3 mg anatoxin-a/kg and identified neurotoxicity as the cause of death (Fitzgeorge et al., 1994; Stevens and Krieger, 1991). Derivation of an acute oral RfD based on the human or animal data is precluded by insufficient information on sensitive endpoints and associated dose-response relationships.

5.2.3. Short-Term Duration

5.2.3.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Information on the short-term oral toxicity of anatoxin-a is available from 5- and 28-day systemic toxicity studies in mice (Fawell and James, 1994; Fawell et al., 1999) and a developmental toxicity study in mice (Fawell and James, 1994; Fawell et al., 1999), as discussed in Sections 4.2.1.2 and 4.6.1. The best designed of these studies is the 28-day study in mice, which tested groups of 10 mice/sex at dose levels of 0, 0.1, 0.5 and 2.5 mg/kg-day and identified an apparent NOAEL of 2.5 mg/kg-day. The authors concluded that the NOAEL was 0.1 mg/kg-day due to deaths in 1/10 males at 0.5 mg/kg-day and 1/10 females at 2.5 mg/kg-day. This conclusion was based on an inability to determine the cause(s) of death and completely rule out a relationship with treatment, but the study authors indicated that the true NOAEL may actually be 2.5 mg/kg-day. EPA concludes that the actual NOAEL is 2.5 mg/kg-day due to the low mortality incidences that showed no dose-response or gender consistency, a lack of clinical signs of acute neurotoxicity prior to death (the animals died within 2.5 hours of dosing on days 10 and 14), a lack of toxicologically significant effects in the surviving animals (comprehensive evaluations were performed that included hematology, clinical chemistry and histology) and supporting NOAELs of 2.5 mg/kg-day in the 5-day and developmental toxicity studies. The

5-day study identified an FEL of 6.2 mg/kg-day for mortality but no LOAEL, and no LOAEL or FEL was identified in the developmental toxicity studies. The proximity of the 6.2 mg/kg-day FEL to the 2.5 mg/kg-day NOAEL indicates that the NOAEL is close to the toxicity threshold region, and therefore, is an appropriate basis for RfD assessment.

5.2.3.2. Methods of Analysis - Including Models (Physiologically Based Pharmacokinetic [PBPK], Benchmark Dose [BMD], etc.)

The NOAEL/LOAEL approach is used to derive the RfD due to limitations in the available studies. BMD analysis is precluded by lack of appropriate dose-response data (adverse effects other than mortality were not observed) and by predominantly qualitative reporting of results.

5.2.3.3. RfD Derivation - Including Application of Uncertainty Factors (UFs)

To derive the short-term RfD, the 2.5 mg/kg-day NOAEL for systemic toxicity was used as the point of departure (POD). Dividing the POD of 2.5 mg/kg-day by a composite uncertainty factor (UF) of 1000 results in a short-term RfD for anatoxin-a of $3x10^{-3}$ mg/kg-day.

Short-Term RfD	=	NOAEL ÷ UF
	=	2.5 mg/kg-day ÷ 1000
	=	**0.0025 mg/kg-day or $3x10^{-3}$ 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 is used to account for the interspecies variability in extrapolating from laboratory animals (mice) to humans. No relevant information is available on the toxicity of anatoxin-a in humans, and data on toxicokinetic differences between animals and humans in the disposition of ingested anatoxin-a are not available.

- An intraspecies UF of 10 is used to account for variations in sensitivity within human populations because 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 anatoxin-a.

- A 10-fold UF is used to account for deficiencies in the database. There is no information on the short-term toxicity of anatoxin-a in orally-exposed humans. The 2.5 mg/kg-day NOAEL is based on a generally well-designed 28-day study, but no adverse effect level was identified and some uncertainty in the NOAEL exists due to the low incidence of deaths that the study authors could not rule out as being possibly exposure-related. The 2.5 mg/kg-day NOAEL in the 28-day study is supported by NOAELs of 2.5 mg/kg-day in the 5-day and developmental toxicity studies, but neither of these studies was comprehensive or identified a LOAEL. Although the 6.2 mg/kg-day FEL for mortality in the 5-day study suggests that the 2.5 mg/kg-day NOAEL is close to the toxicity threshold

region, the 5-day study is limited by very small numbers of animals, indicating that the lack of a LOAEL is an important limitation of the database. The developmental toxicity study is limited by use of only one dose level and fetal assessments that lacked internal soft tissue and skeletal examinations. The database for short-term oral exposure is additionally limited by lack of reproductive toxicity data as well as toxicity testing in a second species.

5.2.4. Subchronic Duration

5.2.4.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Information on the subchronic oral toxicity of anatoxin-a is available from a 7-week drinking water study in rats (Astrachan and Archer, 1981; Astrachan et al., 1980). As discussed in Sections 4.2.1.3 and 4.6.1, this study identified a NOAEL of 0.5 mg/kg-day but is limited by insufficiencies that include two dose levels, a minimal number of endpoints and the lack of an adverse effect level. Although the study provides no information on proximity of the 0.5 mg/kg-day NOAEL to the toxicity threshold, due to the lack of a LOAEL or FEL, results of the short-term studies discussed in Section 5.2.3.1 are supportive of the subchronic NOAEL. In particular, the NOAELs of 2.5 mg/kg-day in the 5-day, 28-day and developmental toxicity studies and FEL of 6.2 mg/kg-day in the 5-day study (Fawell and James, 1994; Fawell et al., 1999) indicate that the 0.5 mg/kg-day NOAEL in the 7-week study is a reliable value and a sufficient basis for RfD derivation.

5.2.4.2. Methods of Analysis - Including Models (Physiologically Based Pharmacokinetic [PBPK], Benchmark Dose [BMD], etc.)

The NOAEL/LOAEL approach is used to derive the RfD due to limitations in the available study. BMD analysis is precluded by lack of appropriate dose-response data (adverse effects were not observed) and predominantly qualitative reporting of results.

5.2.4.3. RfD Derivation - Including Application of Uncertainty Factors (UFs)

To derive the subchronic RfD, the 0.5 mg/kg-day NOAEL for systemic toxicity was used as the point of departure (POD). Dividing the POD of 0.5 mg/kg-day by a composite uncertainty factor (UF) of 1000 results in a subchronic RfD for anatoxin-a of $5x10^{-4}$ mg/kg-day.

Subchronic RfD	=	NOAEL ÷ UF
	=	0.5 mg/kg-day ÷ 1000
	=	**0.0005 mg/kg-day or $5x10^{-4}$ 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 is used to account for the interspecies variability in extrapolating from laboratory animals (rats) to humans. No relevant information is available on the toxicity of anatoxin-a in humans, and data on toxicokinetic differences between animals and humans in the disposition of ingested anatoxin-a are not available.

- An intraspecies UF of 10 is used to account for variations in sensitivity within human populations because 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 anatoxin-a.

- A 10-fold UF is used to account for deficiencies in the database. Only one subchronic study was conducted and it is limited by deficiencies that include two dose levels, a minimal number of endpoints (e.g., two hematology indices, four clinical chemistry indices, seven tissues for histological examination) and lack of an adverse effect level. Some supporting data are provided by short-term systemic and developmental toxicity studies, but these studies have limitations, as discussed in Section 5.2.3.3. Additionally, the database for subchronic oral exposure is limited by the lack of reproductive toxicity data as well as toxicity testing in a second species.

5.2.5. Chronic Duration

5.2.5.1. Choice of Principal Study and Critical Effect - with Rationale and Justification

Insufficient data are available to support derivation of a chronic duration oral RfD for anatoxin-a. No chronic oral studies have been performed and use of the subchronic study for chronic RfD estimation by extrapolation across exposure durations is precluded by the study limitations discussed in Section 5.2.4.

5.2.6. Route-to-Route Extrapolation

Derivation of RfD values for anatoxin-a by route-to-route extrapolation could not be considered due to a lack of inhalation data.

5.3. INHALATION REFERENCE CONCENTRATION (RfC)

No information is available on the inhalation toxicity of anatoxin-a.

5.4. CANCER ASSESSMENT

There is no information on carcinogenicity in humans or animals or on possible carcinogenic processes and mode(s) of action for anatoxin-a. Under the *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005a), the database is inadequate for an assessment of human carcinogenic potential.

6. MAJOR CONCLUSIONS IN THE CHARACTERIZATION OF HAZARD AND DOSE RESPONSE

6.1. HUMAN HAZARD POTENTIAL

Anatoxin-a is a naturally occurring chemical produced by *Anabaena* (particularly *A. flos-aquae*) and at least four other genera of freshwater cyanobacteria. Toxicokinetic studies of anatoxin-a have not been performed, although the rapid onset of clinical signs in toxicity studies indicate that it is quickly absorbed from the gastrointestinal tract. Anatoxin-a is a well documented acute neurotoxin based on observations in domestic and wild animals that consumed water containing blooms of *Anabena flos-aquae* and results of oral and parenteral toxicity studies in laboratory animals. Mode of action studies indicate that anatoxin-a is a nicotinic acetylcholine receptor agonist that exerts effects at both peripheral and central cites in the nervous system, presumably via action of the parent compound. Acute gastrointestinal effects in humans (e.g., nausea, vomiting and diarrhea) and potentially one death have been attributed to the ingestion of lake water containing *Anabaena* and *Microcystis.*

The acute clinical effects of high doses of anatoxin-a are mainly neuromuscular in nature and characteristically include signs such as muscular twitching, gasping respiration, convulsions and death from paralysis of respiratory muscle. The database is limited in the number and quality of studies on effects of anatoxin-a following oral exposure to sublethal levels. No adverse effects other than frank neurotoxicity and death have been observed in acute, short-term and subchronic oral studies in animals. These studies identified a preponderance of NOAELs and no LOAELs for systemic and developmental toxicity. No information is available on the reproductive toxicity, chronic toxicity or carcinogenicity of anatoxin-a by any route of exposure. Testing following inhalation has not been performed.

There is inadequate evidence to evaluate the carcinogenicity of anatoxin-a.

6.2. DOSE RESPONSE

The available data were sufficient for derivation of short-term and subchronic oral RfDs. Based on a NOAEL of 2.5 mg/kg-day for systemic toxicity in mice exposed to anatoxin-a for 28 days (Fawell and James, 1994; Fawell et al., 1999), a short-term RfD of $3x10^{-3}$ mg/kg-day was derived by dividing the NOAEL by an uncertainty factor of 1000. The uncertainly factor comprises component factors of 10 for interspecies extrapolation, 10 for interindividual variability and 10 for database deficiencies. Based on a NOAEL of 0.5 mg/kg-day for systemic toxicity in rats exposed to anatoxin-a for 7 weeks (Astrachan and Archer, 1981; Astrachan et al., 1980), a subchronic RfD of $5x10^{-4}$ mg/kg-day was derived by dividing the NOAEL by an uncertainty factor of 1000. The uncertainly factor comprises component factors of 10 for interspecies extrapolation, 10 for interindividual variability and 10 for database deficiencies.

Acute and chronic oral RfDs could not be derived due to inadequate data.

Inhalation RfC derivation is precluded by the lack of data for this route of exposure.

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