



RISK ASSESSMENT OF ANATOXIN-A

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ABSTRACT

Current natural conditions as well as human activities have been promoting changes and increasing stress in the freshwater and marine environment. One of the consequences is the worldwide occurrence of cyanobacterial (blue-green algae) blooms, which is considered a serious environmental and economic problem (ARAOZ, et al 2005).

Risk assessment of cyanotoxins is made more difficult by the lack of scientifically-sound toxicological and epidemiological studies. The available animal data is limited, principally chronic or long term effects. The lack of data is reflected in the fact that a WHO guideline has been agreed only for one group of cyanotoxins (microcystins) (CHORUS and BARTRAM, 2003). Until now, there has been paid little attention to the assessment of risk to drinking water consumers of anatoxin-a, mainly because the suspicion of the rapid excretion of this toxin from the body, no evidence of residual effects and low free-water concentrations in lakes (FALCONER and HUMEPAGE, 2005).

Therefore, the aim of this work is to accomplish the risk assessment of anatoxin-a, by approaching three main steps of this process: hazard identification and characterization, exposure assessment, and risk characterization, since the presence of *Anabaena sp.* blooms capable to produce anatoxin-a are common in São Paulo reservoirs, mainly in spring and summer seasons. Additionally, previous data has shown that this toxin can be found in other Brazilian freshwater reservoirs.

Keywords: cyanotoxins, anatoxin-a, risk assessment

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1. INTRODUCTION

Cyanobacteria presence in the worldwide biota is normal. As very ancient life-form they occupy many ecological niches; however, their abundance is limited by nutrient and light availability (FALCONER, 2005). There is a strong relationship between phosphorus concentration in water and cyanobacterial numbers and a similar linked relationship between dissolved nitrate/ammonia and cyanobacteria too (MUR, 1983). The increase in the aquatic nutrients (called eutrophication) leads to the uncontrolled growth of cyanobacteria phenomenon named as bloom. The concern with cyanobacteria is because of the toxic properties of some strains which may produce toxic secondary metabolites called cyanotoxins. They have been extensively investigated in fresh, brackish, and marine waters, including Europe, Americas, Australia, Africa, and Asia (CARMICHAEL et al. 1977, CODD et al. 2005, BITENCOURT-OLIVEIRA et al. 2005). Cyanotoxins have been related to many poisoning incidents of livestock, wildlife, domestic animals, and even humans (JOCHIMSEN et al. 1998, BALLOT et al. 2005, GUGGER et al. 2005, MOLICA et al. 2005). These toxins cause predominantly hepatotoxic and neurotoxic effects. Among the neurotoxins are the group of anatoxins, represented by anatoxin-a, homoanatoxin-a, and anatoxin-a(S) (QUILLIAM et al. 2000). Anatoxin-a is produced by several cyanobacterial strains as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Oscillatoria*, *Microcystis*, *Raphidiopsis mediterranea*, *Planktothrix*, *Arthrospira*, *Nostoc* and *Phormidium* (OSSWALD et al. 2007). Anatoxin-a occurrence is the most common among the anatoxins around the world.

2. PHYSICAL AND CHEMICAL DATA

Common name:

Anatoxin-a

Chemical name:

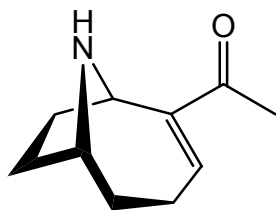


2-acetyl-9-azabicyclo[4.2.1]non-2-ene

CAS Registry number

285-06-9

Structural formula:



Molecular formula

C₁₀H₁₅NO

Relative molecular mass:

165.232

pKa: 9,4, at physiological pH it exists in the protonated form.

Physico-chemical proprieties (source: Chemdraw® 8.0)

Boiling point: 561° K (288° C)

Melting point: 395° K (122° C)

Octanol/water partition coefficient: LogP_{ow}: 0,21

Stability: Anatoxin-a is instable under natural conditions of sunlight and alkaline medium. First order decay kinetics of anatoxin-a in sunlight is both pH and light intensity dependent. In the solutions examined by Stevens and Krieger, which represented expected biological conditions, the half-life of anatoxin-a was on the order of 1-2 hr. However, in the absence of sunlight and even in the presence of metal ions the half-live is on the order of several days. Depending on environmental conditions, it may be partially or totally



degraded to non-toxic products called dihydroanatoxin-a and epoxyanatoxin-a (STEVENS & KRIEGER, 1991). As it is showed in the table 1, Stevens & Krieger proved that anatoxin-a photodegradation is dependent of oxygen, but not by photo-oxidation.

Table 1 - Anatoxin-a half-life (minutes or days) under different pH, light and atmosphere conditions (from STEVENS & KRIEGER, 1991)

	Sunlight photolysis at lower intensity	Sunlight photolysis at higher intensity	Nitrogen atmosphere	Oxygen atmosphere	Oxygen atmosphere and 10 μ m Cu ²⁺ in solution
Aqueous anatoxin-a	∞ at pH 2; 151 min at pH 6; 96 min at pH 9	330 min at pH 9; 270 min at pH 12	10 days at pH 9	5 days at pH 9	3.8 days at pH 9
Anatoxin-a in algae lysate	96 min at pH 9	-----	-----	-----	-----

Source: Osswald et al. 2007

3. KINETIC AND METABOLISM DATA

Because of the difficulties to obtain enough quantities of standards, there is a lack of toxicokinetic information of several cyanotoxins, principally the neurotoxins as anatoxin-a.

Currently, there are known routes of exposure of anatoxin-a for animals such as oral, dermal and respiratory tract, while for the man intravenous exposure during hemodialysis may be added (OSSWALD et al. 2007). The most of the published studies about anatoxin-a's toxicology have been done using the intraperitoneal route in rodents, maybe because the lower dose necessary to produce the characteristic effects.

Researchers supervised by Carmichael and Stolerman observed that anatoxin-a is rapidly absorbed after oral administration, afterwards, it may reach the brain, which possibly contributes to its rapid lethal effects (CARMICHAEL et al. 1977;

STOLERMAN et al. 1992). One aspect that must be considered is the differences of sensitivity to the toxin among different animals, particularly avian species.



Carmichael and Biggs (1978) observed a higher resistance to anatoxin-a of the ring-necked pheasant than the Mallard duck. These differences in the same taxonomic group should be carefully considered especially in environmental risk evaluation of anatoxin-a.

Table 2 – Shows sub lethal and pre-death clinical symptoms and onset time of different animal species intoxicated with anatoxin-a.

Test animal	Time post-treatment (min)	Effects and symptoms	Reference
Mouse	2	Gasp, tremors, mild convulsions, paralysis, no detectable changes upon autopsy	GORHAM et al., 1964
Calf	4	Staggers, convulsions, muscle fasciculation (shoulder and limbs), loss of muscle coordination,	CARMICHAEL et al., 1975, 1977
Rat	14		
Mouse	4		
Goldfish	2	breathing abdominal (animal down), ptosis, collapse from respiratory arrest, no detectable changes upon autopsy.	
Mallard duck	15	Latency followed by twitching, gasping and convulsion. Latency followed by muscle rigidity, opened mouth contracted. Opisthotonus (appearance of fowl botulism "limberneck"), muscle rigidity.	
Mouse	60	Decrease locomotor activity.	STOLERMAN et al., 1992
Mouse	1	Increased respiration, salivation, micturition, hyperactivity, Straub tail.	LILLEHEIL et al., 1997
Mouse	5-6	Decreased motor activity, altered gait, difficult breathing, and convulsions.	ROGERS et al., 2005
Mouse		Decrease in pup weight on post-natal day 1.	MACPHAIL et al., 2005

Source: Osswald et al. 2007.



4. TOXICITY DATA AND TOXICITY EVALUATION

General toxicity

Anatoxin-a's target is the cholinergic synapse, specially the places where nicotinic receptors are in abundance. It acts as a potent postsynaptic depolarizing neuromuscular blocking agent. Anatoxin-a binds to the nicotinic acetylcholine receptor at the neuromuscular junction, causes persistent stimulation and consequently a block on further electrical transmission. At enough high doses it may lead to paralysis, asphyxiation and death (CARMICHAEL et al. 1975). Death is often preceded by leaping movements in smaller laboratory animals, while in field cases collapse and sudden death is observed in larger animals (CARMICHAEL, 2001). According to Alkondon and Albuquerque (1995) anatoxin-a is more potent than nicotine or acetylcholine in evoking type 1A or type 2 current responses in rats neurons. Besides, it may evoke the secretion of endogenous catecholamines in bovines (MOLLOY et al. 1995).

Toxicity studies in animals

4.1. Acute toxicity

4.1.1. Acute toxicity- Oral studies

The oral anatoxin-a's LD₅₀ values in many species range from 1 to 10 mg/kg bw with a latent period after administration followed by symptoms of intoxication such as twitching, gasping, convulsions and death. In mice it was observed a LD₅₀ greater than 5000 µg/kg bw (ASTRACHAN et al. 1980). As well, Fawell and collaborators (1999) observed mice recovered rapidly and completely at a single sub lethal i.v. dose.

4.1.2. Acute toxicity- Intraperitoneal studies

The intraperitoneal LD₀ (lowest dose causing death) in mice for anatoxin-a is 250 µg/kg bw and the LD₅₀ 350 µg/kg bw (CHORUS and BARTRAM, 2003). There are also have been reported values of LD₅₀ in mice of less than 100 µg/kg bw and from 200 to 250 µg/kg bw (WOLF & FRANK, 2002). The World Health Organization adopted the value of 350 µg/kg bw as LD₅₀ for this compound.



4.1.3. Acute toxicity– Intravenous studies

By this route, anatoxin-a LD₅₀ in mice is < 100 µg/kg bw (CHORUS & BARTRAM, 2003).

4.1.4. Acute toxicity– Intranasal studies

The intranasal LD₅₀ in mice for anatoxin-a is 2000 µg/kg bw (CHORUS & BARTRAM, 2003).

4.2. Subacute toxicity (repeated dosing)

4.2.1. Subacute toxicity –oral studies

Astrachan et al (1980) administered anatoxin-a in rats orally in the drinking water for 54 days at 0.51 or 5.1 ppm (equivalent to 51 and 510 µg/kg bw per day, respectively). No toxicity as deaths due to treatment, significant changes in body weight gain, hematology or clinical chemistry was observed.

Fawell et al. 1999 treated orally by gavage groups of two male and two female mice with 1.5, 3, 7.5 or 15 mg of anatoxin-a hydrochloride/kg bw for 5 days. After the treatment, they observed all mice at 15 mg/kg bw and one mouse at 7.5 mg/kg bw died within 5 min over the first 4 days of the experiment. No signs of clinical toxicity or changes in the body weight related to the treatment were observed. No treatment related changes were seen at the necropsy.

Anatoxin-a hydrochloride was administered in groups of 10 male and female mice by gavage at dose levels of 0, 120, 600 or 3000 µg/kg bw for four weeks. During the experiment, one male receiving 600 µg/kg bw per day and one female receiving 3000 µg/kg bw per day died. There were no signs of clinical toxicity or histopathological abnormalities in those animals, and no cause of death could be identified. The authors concluded that those deaths may not be excluded as treatment-related, although this was considered to be unlikely. There were no other treatment-related findings.



4.2.2. Sub acute toxicity (repeated dosing)–intraperitoneal studies

During 21 days, female rats were treated daily by i.p. injection 0 or 16 µg of anatoxin-a/rat (approximately 0 or 80 µg/kg bw). There were no deaths neither effects on body weight gain, hematology or clinical chemistry (ASTRACHAN et al. 1980).

4.2.3. Sub acute toxicity (repeated dosing) – subcutaneous studies

Recently, Jarema et al. (2008) treated subcutaneously groups of male Long Evans rats with anatoxin-a fumarate (0.05, 0.075, 0.1, 0.15 and 0.2 mg/kg) and nicotine (0.125, 0.3, 0.6, 1.2 and 1.8 mg/kg) during four weeks. The animals were trained to respond under a multiple variable-ratio 30-response variable-interval 60-s (mult VR-30 VI-60) schedule of food reinforcement. When initially administered, each compound decrease response and reinforcement rates in both components of the multiple schedules. Tolerance to anatoxin-a's effects was developed, although to a lesser degree than nicotine. The results suggest that the behavioral effects of anatoxin-a and nicotine are similar, but not identical, and the episodic administration of anatoxin-a may cause tolerance.

4.3. Reproductive effects

Hamsters received intraperitoneally one or three times per day anatoxin-a doses of 200 or 125 µg/kg bw, respectively at days 12 to 14 of pregnancy (after organogenesis), at day 15 dams were sacrificed. Fetal malformation (hydrocephaly) in all fetuses in one of 10 litters and stunted growth in almost all litters were caused by the treatment given three times per day. Once per day treatment produced stunted growth. No maternal toxicity was observed. (ASTRACHAN et al. 1980)

Fawell and James (1994) did another study where groups of 10 and 12 time-mated female mice received anatoxin-a hydrochloride by gavage at 0 or 3000 µg/kg bw (equivalent to 2460 µg/kg bw of anatoxin-a) respectively on the 6th to 15th day of pregnancy. No effects related to the treatment were observed in the dams of offsprings, even so there was a slight decrease in fetal weight compared with controls.



4.4. Genotoxicity and mutagenicity

Currently, no data on genotoxic neither mutagenic potential of anatoxin-a are available.

4.5. In vitro studies

Lakshmana et al. (2002) treated cultured rat thymocytes and African green monkey kidney cells (Vero) with anatoxin-containing cell-free extracts from *Anabaena flos-aquae* and purified anatoxin-a. They observed the toxin-induced cytotoxicity was characterized by loss of viability, lactate dehydrogenase leakage, loss of mitochondrial function and DNA fragmentation. Besides, thymocytes showed dose- and time-dependent toxin-induced generation of reactive oxygen species. So, those facts suggested anatoxin-a induce apoptosis which is possibly mediated by generation of reactive oxygen species and caspase activation.

A more recent research was conducted by Teneva et al. (2005), they investigated the influence of microcystin-LR (an hepatotoxic cyanotoxin) and anatoxin-a on mouse B- and T-lymphocyte subpopulations *in vitro*. Both cyanotoxins significantly decreased the cells viability after 4 and 24 h when compared to the untreated control. Anatoxin-treated splenocytes viability dropped to 57%. Afterwards, anatoxin-a showed cytotoxic effects on both lymphocyte subpopulations (T and B), apparently this action appears to be non-selective and non-specific.

4.6. Effects on humans

No data about effects of anatoxin-a in humans are available.

5. EXPOSURE

Annual or even permanent blooms of toxic cyanobacteria are becoming increasingly common in drinking water reservoirs. For example the three main reservoirs supplying Brisbane in Australia all present abundant population of toxic *Cylindroespermopsis raciborskii*, other examples are the main drinking water supply reservoirs in Lodz-Poland and



São Paulo-Brazil which contain heavy blooms of the toxic *Mycrocystis aeruginosa* strain (FALCONER and HUMEPAGE, 2005).

Occurrence of anatoxin-a has already been reported in all the five continents, principally in developed countries as Finland, Norway, Germany, Italy, Spain, France, Ireland, United Kingdom, North Korea, Japan, EEUU and Canada.

Until now, there has been no clear evidence of human poisoning with anatoxin-a, just one suspicious case in 2003 when a coroner from Wisconsin reported that one teenager male, who was diving and playing in a pond containing neurotoxic *Anabaena* strain, died as a consequence of ingestion of that cyanobacteria (BEHM, 2003). To confirm anatoxin-a intoxication as the cause of death, it was analyzed the stomach content of the victim by LC-MS technology to identify anatoxin-a as confirmatory result. However, even it was initially reported as anatoxin-a-produced death, after it was discovered that there was a misidentification with phenylalanine amino acid also present in the stomach content. That is why there is no official published report of the presence of anatoxin-a in humans. By contrast, anatoxin-a was confirmed in stomach content of two dogs (a Yorkshire terrier weighting 2,5 kg and a Dogue of Bordeaux weighting 25 kg) in France (GUGGER et al., 2005). Both dogs showed similar clinical symptoms (vomiting, paralysis of the muscles of hind legs and respiratory failure) after drinking water from the La Loue River. Even so anatoxin-a stability in sunlight and acidic conditions is no far than 2-3 hours, the cited report demonstrated it was enough to produced the dogs death after eating decaying lumps of cyanobacteria on the lakeside (the smaller dog day immediately and the bigger one after 5 hours). From this cases, it may be concluded that anatoxin-a risk of exposure is principally from consumption of contaminated drinking and contact with the aerosol during recreational aquatic activities.

Because is too difficult to evaluate the population exposure to anatoxin-a worldwide, in this case we considered a specific population supplied by freshwater from one reservoir which has presented cyanobacteria, as for example, Billings reservoir in São Paulo-Brazil (Figure 2). We outlined a scenario during a bloom occurrence of toxic cyanobacteria. The table 3 shows some of the parameters related to cyanobacteria presence reported in the São Paulo State Freshwater Quality Report of 2007.



Figure 2 - Billings Reservoir photo (source: <http://www.sabesp.com.br>)

Some important data of Billings Reservoir:

Drainage área: 1560 km²

Location: Santo André, São Bernardo do Campo, Diadema, Ribeirão Pires, Town- São Paulo State

Storage volume: 995 million of m³

Flow: 4,7 m³/s

Table 3 - Some of water quality parameters monitored and published in the São Paulo State Freshwater Quality Report of 2007

Parameter	Unit	Reference Value	Billings reservoir - Point code		
			01SP06100 BILL02100	01SP06100BI LL02100	01SP06635 BILL02900
pH	U.pH	6-9	7,5	7,36	8,15
Total phosphorus	mg/l	maximum 0,3	0,13	0,05	0,02
Ammoniac nitrogen	mg/l	maximum 3,7	0,53	0,29	0,26
Nitrate	mg/l	maximum 10	0,61	1,64	0,49
Nitrite	mg/l	maximum 1	0,43	0,03	0,02
Chlorophyll-a	mg/l	maximum 30	49,74*	36,32*	16,02
Cyanobacteria cell	Cell number/ml	maximum 50000	212562,5*	98016*	115053*
Mycrocistins	µg/mL	maximum 1 µg/mL	-----	-----	0,47

*Outside of the reference value

Source: CETESB São Paulo State Freshwater Quality Report of 2007



Considering the worst case approach, at least 700 thousand people live around this reservoir. It is used as a freshwater supplier and for recreational purposes, therefore they may be exposed to anatoxin-a by oral and respiratory route. If at least 50% of the cyanobacteria cells reported in the São Paulo State Freshwater Quality Report were anatoxin-a-producers strains we may outline two situations.

Situation1.

This scenario is based on the oral exposure to anatoxin-a. There may be considered:

Body weight: 60 kg for adults and 15 kg for children (U.S. ENVIRONMENTAL PROTECTION AGENCY, 1997).

Volume of water drunk by day: approximately 2 L/day

Anatoxin-a concentration in water: Difficult to established. It will depend of environmental conditions. Nevertheless, we may consider the oral LD₅₀ of 350 µg/kg bw in rats.

Situation 2

This scenario is based on the respiratory exposure to anatoxin-a aerosol. They should be considered: body weight (60 kg for adults and 15 kg for children)*, respirable fraction (a respirable fraction of 34.4% for the particles with a diameter of 5 µm is expected)* and inhalation (inhalation rate for adults, considering the rest state, is 9330 cm³/min, corresponding to 0,56 m³/h)* (*EUROPEAN COMMISSION DG-ENVIRONMENT, 2002). The oral LD₅₀ is 2000 µg/kg bw in rats.

6. TOXICITY, HAZARD AND RISK ESTIMATION

Generally, hazard is understood as the propriety of a substance (or activity) to cause harm. Many substances are hazardous but will not necessarily lead to harm unless circumstances lead to human exposure. Still after exposure, an adverse health outcome is not necessarily certain, but rather probably. So, a hazard may be defined as an intrinsic propriety of a biological, chemical or physical agent to cause adverse health effects. Risk refers to a probability that exposure to a hazard will lead to a specific (adverse) health

