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Research Paper

STUDY ON CULTURAL ASPECTS AND ACUTE TOXICITY OF MICROCYSTIS *AERUGINOSA*, *ANABAENA VARIABILIS* AND *ANABAENA CIRCINALIS* TO DAPHNIDS

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Abstract

The bloom of toxic Cyanobacteria in water bodies is generally related with environmental factors and nutrient concentrations. This research article summarizes effect of cyanotoxins of *M. aeruginosa A. variabilis*, *A. circinalis* and *Phormidium* sp. to Daphnids. Isolation, characterization of toxic cyanobacterial species, feeding and rearing of Daphnids and toxicity to Daphnids study was conducted in the Laboratory. Different culture media BG-11 for Cyanobacteria and Becker's medium for *Scenedesmus obliquus* (nontoxic feed for Daphanids) used for algal growth. Daphnids were reared feeding with artificial feed. in semi hard water in the laboratory. Using pure cultures, sonicated pure cultures of M. *aeruginosa, A. variabilis* and *A. circinalis* were used in percentage mortality occurred in Daphnids during acute toxicity assay. In our observation, *M. areginosa* of 10^5 cells concentration leading to mortality in Daphnids however at lower concentration 10^3 cause morbidity and blocking effect in Daphnids. Cyanobacterial toxins possibly protect cells against planktivores.

Key words: *Cyanobacterial diversity*, *Microcystins*, *Daphnids*, *Acute LC_{50} toxicity assay*.

INTRODUCTION

The Cyanobacterial toxins are classified as microbial secondary metabolites [1]. *Microcystis aeruginosa* and *Planktothrix agarrdhi*, are known for production of heapatotoxin (Microcystins) other genera *Nodularia spumigena* contains Nodularin, *Aanabaena variabilis*, *Anabaena circinalis*, *Phormidium* sp., contain neurotoxin (anatoxin-a homoanatoxin-a anatoxin-a(s) and Lyngbya sp. are known for shellfish poisoning saxitoxins. Toxic Cyanobacteria cause illnesses ranging from acute pneumonia and gastroenteritis. Toxic Cyanobacteria cause many animal deaths and have also been implicated in cases of human illness in the U.S.A., Australia, China, Brazil and Great Britain. Dinoflagellates and algal species produce saxitoxins (a toxin from *Aphanizomenon* sp*., L. birgei*) which are known as causative toxin for paralytic shell fish poisoning (PSPs) exclusively in Australian waters [2]. Toxins classified in to two

categories (I) Cytotoxins; one of the cytotoxin is a alkaloid from *Cylindrospermopsis raciborskii* (a tropical species) which occurs in river, lakes and water supply reservoirs. Microcystin structure with different X - Z amino acid variants (-D-Ala-L-X-erythro-*β*-methyl-D-iso Asp-L-Y-ADDA-D-isoGlu-*N*-methyldehydro-Ala) (**Fig.1**). The amino acids and Adda (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid) are considered responsible for hepatotoxicity. Variations occur primarily at positions 1 and 2. Microcystin-LR contains the amino acids leucine (L) and arginine (R) at positions 1 and 2 respectively[3]. Quantity of microcystin-LR typically comprised 0.1-1.0 % of the dry weight of toxic species of *M. aeruginosa*. Cylindrospermopsin is a guanidine alkaloid linked to hydroxymethyl uracil and is stable to boiling temperature of water. It inhibit protein synthesis although also cause liver and extensive kidney injury[4, 5]. (II) Biotoxins, toxins further divided in to hepatoxins and neurotoxins and are produced by strains within the genera. Anatoxin-a is a low molecular weight alkaloid (MW = 165), a secondary amine, 2-acetyl- 9-azabicyclo (4-2-1) non-2-ene (**Fig.2**)[6]. Anatoxin-a(S) is a unique phosphate ester of a cyclic N-hydroxyguanine (MW=252) produced by Anabaena flos-aquae strain NRC 525-17 (**Fig.3**)[7].

The algal bloom are reported to source of live stock poisoning and paralytic shell fish poisoning (PSPs) including saxitoxin, neosaxitoxin and ganyautoxin alkaloids that are potent sodium channel blocker in nerve axons resulting in progressive paralysis and death due to respiratory failure[8]. MCs are reported directly lethal to zooplankton, reduce the feeding activity, or influence the community structure of zooplankton; however dose dependent effect is highly variable and inconsistent between zooplankton species[9]. *Daphnia* sp. has preferentially fed over green algae and the diatom *Melosira granulata* seldom feeding on toxic Cyanobacteria under depletion of edible food. Water safety guideline for drinking water USEPA that drinking water containing less than 1µg of microcystins has been approved drinking water purpose[10]. Cyanobacterial toxins act as defense or deterrent agents against planktivores.

Environmental factors

Light: Light effect on *Microcystis aeruginosa* culture was studied. We used a range 21-205 µE m^{-2} s^{-1} and found toxicity was lowest at the minimum light intensity and further declined at the highest light intensity**[11]**. Production of Microcystin-LR varies markedly between toxic species. Light is limiting factor for *M. aeruginosa* culture**[12]**. No significant difference of toxic *M. aeruginosa* M-228 was grown at either 30 or 75 µE m^{-2} s^{-1} (LD_{50} 9.65 and 9.95 mg Kg^{-1} of mice; LD_{50} is the median dose in milligrams of dry algae per kilogram of mice), but low light intensity of 7.5 µE m^{-2} s^{-1} produced a culture 4 time less toxic LD_{50} 36.9 mg/ Kg^{-1}. Similarly *Anabaena* strain 202 A1 and its toxin concentrations were saturated at low light as microcystin-LR concentration of strain 90 at irradiances of 2 to 25 µmol m^{-2} s^{-1}.

Temperature: Maximum toxicity of *M. aeruginosa* was found to be varied from 18 to 25 °C (LD_{50} ca. 11 mg Kg^{-1}) decreasing by ~40% at 32 °C. In two strains of Anabaena, temperature and nitrogen increase the proportion of toxins of demethylated an amino acid 3 of microcystin-LR as compared to their corresponding methylated variants (Mdha).

pH: Maximum toxicity of *M. aeruginosa* influenced by pH varied from pH 7.9 to 9.4 and toxicity was generally increased with increasing pH.

Aeration: Increasing the aeration rate from 100 to 1000 ml min-1 in *M. aeruginosa* NRC culture, the toxicity was more than doubled of the culture otherwise as in unchanged culture.

Nutrients: Effect of different nutrient concentrations and toxicity in growing the M. *aeruginosa* NRC-1. The toxicity of cells grown in the standard BG-11 culture medium was shown LD_{50} 45.3 mg kg^{-1}. Toxicity more or less doubled LD_{50} ranging from 19.8 to 25.6 mg Kg^{-1} when cells were grown at the 0.5X, concentrations of nitrogen, phosphorus, calcium and micronutrients solutions, 2.0X phosphorous, magnesium, iron concentrations and 3.0X of phosphorous, and ferric citrate concentrations[13]. The only treatment that did not produce any increase in toxicity was At high light levels hepatotoxic strains and their toxin levels did not decrease as did the levels of anatoxin-a. Lack of phosphorus had no effect on anatoxin-a level but decreased the amount of hepatotoxins produced[15], the half strength magnesium sulphate. Microcystin-LR was most abundant toxin among isolates from hypertrophic reservoir[14]. Increasing phosphorus concentrations caused increase in the levels of all the toxins. Compared to neurotoxin-anatoxin producing *Anabaena* strains, the most pronounce difference between hepatotoxic *Anabaena* strains depends upon light and phosphorus.

MATERIALS AND METHODS

Cultural aspects of Cyanobacteria: *M. aeruginosa*, (isolated from lake water) *A. variabilis* and *A. circinalis* (anabaena cultures were obtained from IARI, New Delhi) and were cultured in BG-11 medium. Green algae *Scenedesmus obliquus* (nontoxic feed for Daphnids) in Becker's medium for rearing of Daphnids in the laboratory. Both media were autoclaved 121ºC for 15 minute. Algal cultures were maintained in 100 ml of medium in 250 ml Erlenmeyer conical flasks less than 40 watt white fluorescent light with the light intensity reaching to cultures was about 25-75 µE m^{-2} s^{-1} in culture racks. Laboratory culture and rearing of Daphnids *in vitro* is possible because shorter life span of clones.

Extraction of Chlorophyll 'a': Chlorophyll 'a' was determined in modified Lorenzen's method[16]. The modification consists of the extraction of chlorophyll 'a' using the cyanobacterial biomass. Culture was filtered through 0.45µ membrane filter paper with the addition of $MgCO_3$ over the filter than filter paper was kept overnight in 90% acetone at 4ºC in refrigerator and Spectrophotometrically determined.

Extraction of Microcystins: Biomass of *M. aeruginosa* and *A. variabilis* and *A. circinalis* were grown in BG-11[17]. *M. aeruginosa* was suspended in 5% glacial acetic acid. Microcystin was extracted from *M. aeruginosa*, and anatoxins form *A. variabilis and A. circinalis*. Wet weight of biomass was taken after filtration through GF-C filter paper. Prior to analysis, cell dry weight was determined by drying 100ml filtered culture on pre-weighed filter paper at 85ºC in a petri-dish until constant weight. About 75mg wet weight of *M. aruginosa* and 100mg of *A. circinalis* and 100mg of *A. variabilis* cells suspended in the solvent (Water : Methanol : Butanol) 75ml : 20ml : 53ml (once) and subsequently 3ml (twice) centrifuged three times (centrifugation at 8500rpm/15min) in append off tubes. Microcystin was extracted in Methanol : Butanol : Water, (5ml : 20ml : 75ml) from the crude aqueous extract was filtered through 0.45µm mambrane filter paper. Another set, all the three samples were sonicated 6X30s, 150/10min. for sequential extraction the sample extracted 3 times with aqueous methanol: butanol followed by three extractions with 1.5 ml de-ionized distilled water. All fractions were centrifuged 10,000 rpm for 15 minute after centrifugation supernatant kept separately three times in de-ionized distilled water. After centrifugation the supernatants were blown to dryness by purging N_2 gas[18].

Reverse Phase HPLC: The pallet resolved in 50% aqueous methanol prior to HPLC analysis[19]. Detection of microcystin was performed with solvent delivery system 501,

Injector waters U6K and Detector, Waters Lambda-Max-481. Extracts of *M. aeruginosa,* was separated in RP-18 (4.6 mm ID x 250mm length x 19µm pore size) of ODS – octadecyl saline column (Applied Bio-system USA) using a gradient of three solvent mobile phases (I) 15% acetonitrile with 85% 0.1M phosphate buffer (pH-6.2) (II) 35% acetonitrite with 8mM ammonium acetate buffer (pH-6.5) (III) Methanol with 0.05 M phosphate buffer (pH-3) in 38:42 proportion. The samples were injected 10µL,25 µl volumes in to injector and mobile phase acetonitrile (ACN) with buffers passed through ODS column with FR-0.2ml/min to 1 ml/min and peaks were detected at 238 nm of 0.05 to 0.20, 0.25, 0.50AU sensitivity with retention time varying from 2 min to 15 Min. Microcystins peaks were detected. The quantification wavelength was 238nm and spectra of microcystin were plotted on plotter compared with the Microcystin standard (Sigma Aldrich) for identification of toxins. Anatoxins crude extract did not analyze for HPLC.

Daphnids cultural study: Laboratory culture and rearing of Daphnids *in vitro* is possible because shorter life span of clones. Growth and feeding experiments were conducted using laboratory reared species of Daphnids collected from lake water. Before the toxicity experiments the animals were cultured using prepared semi hard water from distilled water contained $NaHCO_3$-96mg/l $CaSO_4.2H_20$-60mg/l $MgSO_4$-60 mg/l and KCl-4mg/l pH 7.4-7.8, water hardness ranging from 80-100mg $CaCO_3$/l at alkalinity 60-70 mg $CaCO_3$/l for 21 days (at least 2 generation at 28 to 31ºC, under dim continuous light source) Feed given during the rearing of Daphnids was trout chow pallet suspension prepared i.e. 6.3g trout chow pallets, 2.6g dried yeast and 0.5g dried alfa alfa in blender jar. Add 500ml de-ionized water and mixed at 1000 rpm for 5 minutes, allowed to settle in a refrigerator for 1 hour, decanted and save top 300 ml and discarded reminder. Freeze 30 to 50 ml portion in small (50 to 100 ml) polyethylene bottles with screw caps and thaw portions as and when needed[20]. Periodically 5-10 ml trout chow suspension of feed was given to Daphnids reared in 5 liter glass aquarium.

RESULTS

Different toxic Cyanobacteria cultures biomass obtained after 15 days in the laboratory. *M. aeruginosa* cell numbers and wet weight was varied from 2.13±0.7X10^5/ml and 68.7±0.5mg/L to 194.7±0.5mg/L in culture flasks. In another set, *M. aeruginosa* cell numbers 5.80±0.8X10^5/ml *and* wet weight from 5.25±0.8mg/L to 601±0.8mg/l was observed in culture flasks. *M.aeruginosa* cell numbers 2.13±0.7X10^5 corresponds to value of Chlorophyll 'a' content 47.95mg/l was determined in the Laboratory. Wet weight of *A. variabilis* and *A. circinalis* were varied from 485.4± 0.5 to 543.6± 0.5/L and 423.3± 0.5 to 441.8± 0.5/L in replicates culture flasks (**Table-1**).

Ingestion Experiments: Zooplankton exhibits both physiological and behavioral adaptations that enhance their ability to coexist with toxic Cyanobacteria. Acute toxicity of *M. aeruginosa*, *A. circinalis* and *A. variabilis* cyanotoxin strains has shown positive tests for microcystin and anacystin-a in *Daphnids*. Typically a strain of Cyanobacteria is considered toxic if zooplankton dies more quickly in the presence of Cyanobacterium than without any food, considered for acute toxicity[21]. The effective concentration (EC) was assessed for qualitative working definition of toxicity. *M.. aeruginosa* toxicity to *Bosmina* sp. was assessed and only up to 40% mortality was observed in the experiment tubes(**Table-2**). In higher animals, microcystin-LR induces the liver tumor, particularly in hepatocytes. It has similar toxic effect but more potent than cell permeant okadaic acid. LC_{50} value (concentration giving 50% mortality of population) of 9.6 to 21.4µg microcystin-LR per ml have observed.. In the present laboratory study, acute toxicity of

M. aeruginosa producing microcystins caused LC_{50} 9.6 - 21.4µg ml^{-1} in Daphnids. *D. pulex* mortality found over 48-hr exposure to *M. aeruginosa* cells.

Survival experiments of *C. rignandi* conducted from fraction of purified toxin, cell extracts and readily ingested Cyanobacteria. *M. aeruginosa* toxicity to *C. rignandi* was assessed and up to 100% mortality was observed in the experiment tubes (**Table-3**). Response to hepatotoxin to test organisms induced symptoms toxins *M. aeruginosa* ($5.8x10^5$/ml - 1ml) was found toxic to *D. pulex* and up to 50% mortality was observed in the experimental tubes (**Table-4**). Poor survival among *C. rignandi* was associated with greater physiological sensitivity and nearly uninhibited feeding on toxic *Microcystis* sp. *Anabaena* strains contain very potent and fast-acting nerve blocking agent. *Anabaena* and *Phormidium* strains contain very potent and fast-acting nerve blocking agent. Physiological effects are at least neurotoxic partly responsible for the rapid inhibition of feeding by Daphnids. Earlier studies have shown that even exudates form toxic *Anabaena* can inhibit feeding by cladocerans and copepods. *A. variabilis* and *A. circinalis* have produced up to 70% mortality in *C. rignandi* (**Table-5**).

Table-1: Different toxic cyanobacteria cultures biomass obtained after 15 days.

S. No.	Cyanobacteria species	Wet weight mg/L	Count/ml and Chlorophyll 'a' content
1.	*M. aeruginosa* (i) (ii) (iii) (iv)	99.9±0.5 68.7±0.5 138.8±0.5 194.7±0.5	Cell numbers 2.13± 0.7X10^5/ml Corresponds to value of Chlorophyll 'a' content 47.95mg/L
2.	*M. aeruginosa* (i) (ii) (iii)	525±0.5 591±0.5 601±0.5	5.80±0.8X10^5/ml
4.	*A. variabilis* (i) (ii)	485.4±0.5 543.6±0.5	
5.	*A. circinalis* (i) (ii)	423.3±0.5 441.8±0.5	

Table-2: Toxicity of *M. aeruginosa* to *Bosmina* sp.

Sr. No.	Algal count/ml	Toxicity experiment of *Bosmina* sp. kept in Semi hard water	Toxicity EC_{50}, 96 hrs.		Percentage of Daphnids mortality
			survival	mortality	
1.	Control	Semi hard water	10	-	1.
2.	2.13X10^5/ml of *M. aeruginosa*	1ml suspension of *M. aeruginosa*	8	2	2.
3.	*M. aeruginosa* of 2.13X10^5/ml	1ml suspension of *M. aeruginosa*	8	2	3.
4.	*M.aeruginosa* of 2.13X10^5/ml	1ml sonicated suspension of *M. aeruginosa*	6	4	4.

Table-3: Toxicity of *M.. aeruginosa* to *C. rignandi*

Sr. No.	Algal count/ml	Toxicity experiment of *C. rignandi* kept in Semi hard water	Ceriodephnia rignandi		Acute Toxicity EC_{50} - 96 hrs.		Percentage of mortality	
			1st set	2nd set	1st set	2nd set	1st set	2nd set
1.	Control	Semi hard water	10	10	-	1	-	10
2.	1ml of $2.13X10^5$/ml of *M.aeruginosa*	*M. .aeruginosa*	10	10	10	10	100	100
3.	2ml of $2.13X10^5$/ml of *M.aeruginosa*	*M. .aeruginosa*	10	10	10	10	100	100
4.	2ml of $2.13X10^5$/ml of *M.aeruginosa*	*M. .aeruginosa*	10	10	10	6	100	60
5.	1ml sonicated suspension of *M.aeruginosa*	*M. .aeruginosa*	10	10	10	5	100	50

Table-4: Toxicity of *M. aeruginosa* to *Daphnia pulex*

Sr. No.	Toxic Algae count/ml	Toxicity experiment of *D.pulex.* kept in Semi hard water	Acute Toxicity EC_{50} -96 hrs.		Percentage of mortality
			Survival	Mortality	
1.	Reference culture	*D. pulex*	10	0	-
2.	(i)*M.aeruginosa* $5.8x10^5$/ml (1ml)	*D. pulex*	5	5	50
3.	(ii) *M.aeruginosa* $5.8x10^5$/ml (1ml)	*D. pulex*	6	4	40
4.	(i)Sonicated *M.aeruginosa* cell extract, $5.8x10^5$/ml (1ml)	*D. pulex*	8	2	20
5.	(ii)Sonicated *M.aeruginosa* cell extract, $5.8x10^5$/ml (1ml)	*D. pulex*	7	3	30

Table-5: Toxicity of *A. variabilis* and *A. circinalis* to *Ceriodephnia rignandi*

Sr. No.	Algal weight mg/L	Toxicity experiment of *C. rignandi* kept in Semi hard water	*C. rignandi*		Acute Toxicity EC_{50} -96 hrs.		Percentage of mortality	
			1st set	2nd set	1st set	2nd set	1st set	2nd set
1.	Control	*C. rignandi*	10	10	-	-	-	-
2.	1ml of *A. variabilis* 514±0.4mg/L	*C. rignandi*	10	10	7	7	70	70
3.	1ml of *A. circinalis* 432±0.5mg/L	*C. rignandi*	10	10	7	3	70	30

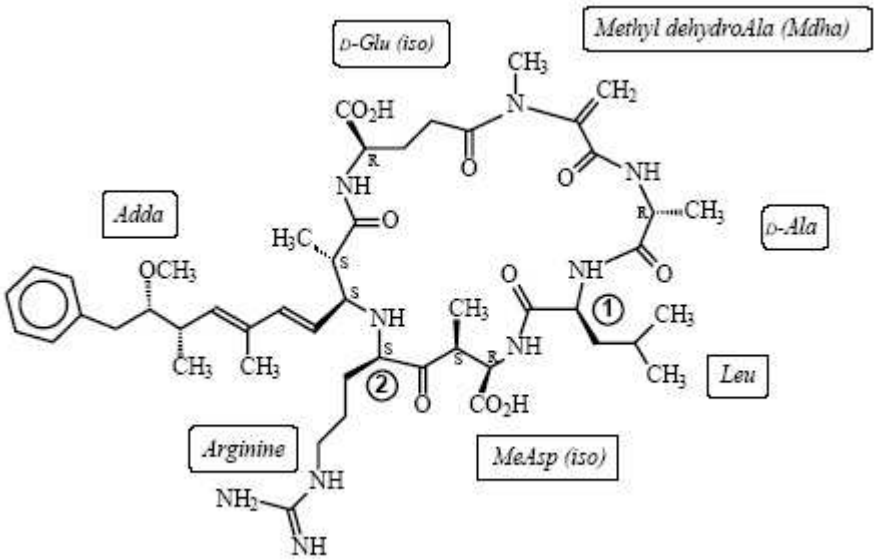

Fig. 1: Heptapeptide structure of a microcystin, (Carmichael, 1992).

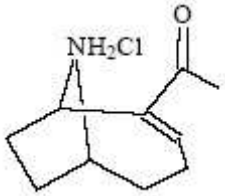

Fig. 2: Anatoxin-a, a secondary amine, 2-acetyl- 9-azabicyclo (4-2-1) non-2-ene (Falconer, 1999).

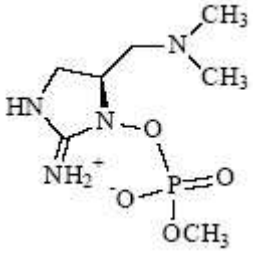

Fig. 3: Anatoxin-a(S), a cyclic N-hydroxyguanine (Falconer, 1999)

DISCUSSION

Cultured mouse hepatocytes cells, microcystin-LR causes the effect on cyatokinesis, ploidy and apoptosis. Microcystin content of *M. aeruginosa* can account for proportionate chlorophyll 'a' (i.e. 0.1 to 0.2 µg of microcystin per µg of chlorophyll-'a',). Toxicity of microcystins is mediated through active transport of microcystins in to hepatocytes by bile acid anion transport system subsequently inhibition of eukaryotic serine / threonine protein phosphatases 1 and 2A in the liver cells. Protein phosphatases control cell structure and function. Internal changes from hepatotoxins resulted into intrahepatic haemorrhages and hypovolaemic shock. Hepatocyte necrosis with destruction of sinusoidal endothelium followed by intrahepatic haemorrhages is based on increase in liver weight as a fraction of body weight (up to 100% in small animals tested in the laboratory) as well as on hepatic haemoglobin and iron concentrations that account for blood loss sufficient to induced irreversible shock (tumor formation). In animals that live longer i.e. a few days, hepatic insufficiency may develop to a degree that becomes incompatible with life. Death occurs within a few hours to a few days after initial exposure and may be preceded by coma, muscle tremors and force expiration of air. Neurotoxin symptoms of toxicosis in field cases of wild and domestic animals include staggering, muscle fasciculation gasping, convulsion and opistothonos (birds). Anatoxin-a(s) is an irreversible inhibitor of cholinesterase and toxicity compared to organophosphate (OP), di-isopropil- fluorophosphates (DFP) and showed that antx-a(s) is about 22 times more potent than DFP. Death is most probably due to respiratory arrest and occurs within minute or a few hours vary with doses in the species and prior food consumption. Toxicity of hepatotoxin should be treated with cyclosporine-A, rifampin and silymarine and neurotoxin is curable by Atropine[22].

Physiological effects are at least neurotoxic partly responsible for the rapid inhibition of feeding by Daphnids, producing the neurotoxic alkaloid anatoxin-a[23]. The most sensitive species *(S. pectinata*) was inhibited at a concentration of 0.2µg ml^{-1} and the least sensitive species (*B. calyciflorus*) was inhibited at a concentration of 5µg ml^{-1}. Previous studies have shown that even exudates form toxic Anabaena can inhibit feeding by cladocerans and copepods.

Feeding with *Scenedesmus* sp. and increasing concentration of toxic algae in the diet of Daphnids, inhibit the growth and reproduction[24]. Zooplankton-cyanobacterial study is probably a consequence of practical difficulties in quantifying dose dependent responses and separating the effects of toxicity and physical interference on feeding. The blooming event of *M. aeruginosa* showed best correlation with У-glutamyl transferase, indicator of microcystin poisoning in test animals[25]. *D pulicaria* showed least while *D .pulex* showed strongest inhibition of growth and reproduction and other three species of Daphnids exhibited intermediate sensitivity. Poor survival-ship among *D. Pulex* was associated with greater physiological sensitivity and nearly uninhibited feeding on toxic *Microcystis*. Earlier observation on feeding of toxic *M.aeruginosa* by Daphnids have shown two type of response- grazing >5 cells colony increasing to 10^5 cells concentration leading to death of organisms however at lower concentration 10^3 cause blocking effect i.e. inhibition of feeding by Daphnids. Toxic concentration and survival data is based on pair wise comparisons between a starvation treatment (no feed added) and each concentration of toxic and nontoxic *Microcystis* sp. and *planktothrix* sp. experienced rapid mortality at (10^6 cells/ml) and intermediate (10^5 cells/ml). However, very high concentrations of dissolved concentrations of dissolved toxin are needed to cause mortality in Daphnids, (equivalent to ~10µg/ml dry weight of toxic microcystis) but much lower density of toxic cells can strongly inhibit feeding

10,000 cell/ml of toxic *Microcystis* sp.: 0.2 µg/ml dry wt.) and caused mortality when ingested[26]. Cyanotoxin occurred at an equivalent microcystin-LR concentration (0.001µg/ml) is four orders of magnitude lower than that obtained with purified microcystin-LR in water[27].

Human and animal health hazards:

The findings summarized toxins are becoming increasingly recognized as human and animal health hazards. Symptoms of toxicity of hepatotoxin include weakness, anorexia, pallor of mucous membranes, vomiting, cold extremities and diarrhea.

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