Bull. Environ. Contam. Toxicol. (2001) 67:339–346

DOI: 10.1007/s00128-001-0130-y

Bulletin of Environmental Contamination and Toxicology

Toxin Production in Batch Cultures of Freshwater Cyanobacterium *Microcystis aeruginosa*

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Received: 2 December 2000/Accepted: 30 May 2001

Toxic cyanobacteria (blue-green algae) found in eutrophic, municipal and residential water supplies are an increasing environmental hazard in several parts of the world (Carmichael 1992, 1994; Rao et al. 1994; Rao and Bhattacharya 1996; Rao et al. 1998; Dawson 1998) . Cyanobacteria produce lethal toxins, and domestic and wild animal deaths are caused by drinking water contaminated by these toxins (Chorus et al. 2000; Hitzfeld et al. 2000). The toxins produced by cyanobacteria include alkaloid neurotoxins and peptide hepatotoxins viz. microcystins and nodularin. Among the species causing death of livestock, blooms of *Microcystis aeruginosa* are the most common. More than 60 microcystins have been isolated to date and they are the most abundant cyanobacterial toxins. Hazards to human health may result from chronic exposure via contaminated water supplies. Microcystins are powerful tumour promoters and inhibitors of protein phosphatase 1 and 2A and they are suspected to be involved in the promotion of primary liver cancer in humans (Bell and Codd 1994; Nishiwaki-Matsushima et al. 1992). Recently, there were reports on the death of 50 patients in Brazil due to the presence of microcystin in the water used for haemodialysis (Jochimsen et al. 1998; Pouria et al. 1998). Cyanobacteria can undergo rapid growth under certain environmental conditions to form blooms in marine, brackish and freshwater bodies. The environmental or abiotic factors which are known to influence toxic bloom formation are; temperature, pH, light intensity, and nutrient concentrations especially nitrogen and phosphorous levels (van der Westhuizen and Eloff 1985; Sivonen 1990; Hee-Mock Oh et al. 2000). These factors are also known to influence toxin production in laboratory cultures of several cyanobacteria (Codd et al. 1989). More information is needed on physical, physiological and genetic factors which regulate the toxin production by cyanobacteria in order to understand the dynamics of toxic blooms in nature .

The aim of the work described here was to study the toxin production and toxicity in relation to (a) nutrient media , (b) culture duration and (c) growth characteristics of *Microcystis aeruginosa* (PCC 7820) cells grown in static batch cultures .

MATERIALS AND METHODS

The axenic cultures of *Microcystis aeruginosa* (PCC 7820) used in the present study were obtained from Pasteur Culture Collection, Institute Pasteur, France. In all growth experiments 300 ml of culture medium were placed in 750 ml Erlenmeyer flasks. Static batch cultures were maintained for different culture

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durations at a controlled room temperature of 25±2°C at 1000 lux light intensity and 16 h photoperiod. Replicate cultures were maintained without agitation and aeration. The growth of *M. aeruginosa* in batch cultures was compared in four different chemically defined media, viz. MA (Ichimura 1979), B12 (Nakagawa et al. 1987), A_3M_7 (Carmichael et al. 1988), and CB (Shirai et al. 1989) . Cells from stock cultures were inoculated at a concentration of approximately 5 x 10^5 cells/ml. Cultures were maintained for up to 7 weeks. The cells were harvested at weekly interval by centrifugation and lyophilized. Growth characteristics were studied in terms of cell count as determined by haemocytometer and the chlorophyll-a (chl-a) content according to the method of Hassmann (1973). The biomass increase between weekly intervals (0-7 weeks) was calculated as the specific growth rate/week (μ) according to the formula: Specific growth rate (μ^{w-1}) = /n (X_2/X_1) / (t_2-t_1) where X_2 was the cell number at the end of a selected time interval (1-7 weeks); X_1 was the cell number at the beginning of a selected time interval (0 day), and (t_2-t_1) was the time elapsed between selected time intervals. The macromolecular composition of the cultured cells was determined in terms of total protein, carbohydrate, and lipids (Kochert 1978b).

The bioassay for toxicity was carried out as described earlier (Rao et al. 1994). Acute toxicity was measured in terms of mouse units (Berg and Soli 1985), which represented the minimum dose required to kill a 20 g mouse in four hours by intraperitoneal administration. Log concentrations were examined to arrive at the mouse unit and expressed as mouse units/g of dry cells. For experiments on the effect of media on toxicity, the culture duration for the individual media was selected on the basis of the highest chl-a content. The effect of culture duration on toxicity was determined using cells grown in MA medium for 1-6 weeks. Microcystin content in the cultured cells was determined by HPLC according to the method of Harada et al. (1988). Dried cells (about 100 mg) were extracted three times with 10 ml of 5% acetic acid for 30 min while stirring. The extract was centrifuged at 9300 X g and the supernatant was applied to activated C18 cartridge. The cartridge was rinsed with water, and eluted with 10 ml of methanol. The methanol fraction was dissolved in 1-5 ml of methanol and 10 μl of the solution was injected into a high performance liquid chromatograph equipped with a constant-flow pump and a variable wavelength U.V. detector operated at 238 nm. The separation was performed on Polygosil C18 column (150 x 4.0 mm) with methanol, 0.05% trifluoroacetic acid-water (11:9) at 2 ml/min flow rate. The toxin content was quantified by using purified microcystin-LR standard.

The data presented in Table 1 was analysed by two way analysis of variance with Student Newman Keul's multiple range test for finding the significant difference between various media and time periods. A probability of < 0.05 was taken as significant. For the statistical analysis a SigmaStat program (Jandel Scientific Corporation, U.S.A.) was used.

RESULTS AND DISCUSSION

The first set of experiments were carried out to compare the growth characteristics of *M. aeruginosa* in four different media at various culture durations. The results on cell count, chl-*a* content and specific growth rate/week for different duration are summarised in Figure 1. The exponential growth was initiated at week 2 for MA, CB and B12 and reached maximum cell number at sixth week in MA and CB and fourth week in B12 (Fig 1A). But in A_3M_7 medium it was initiated at week 4 and reached maximum at week 6. In B12

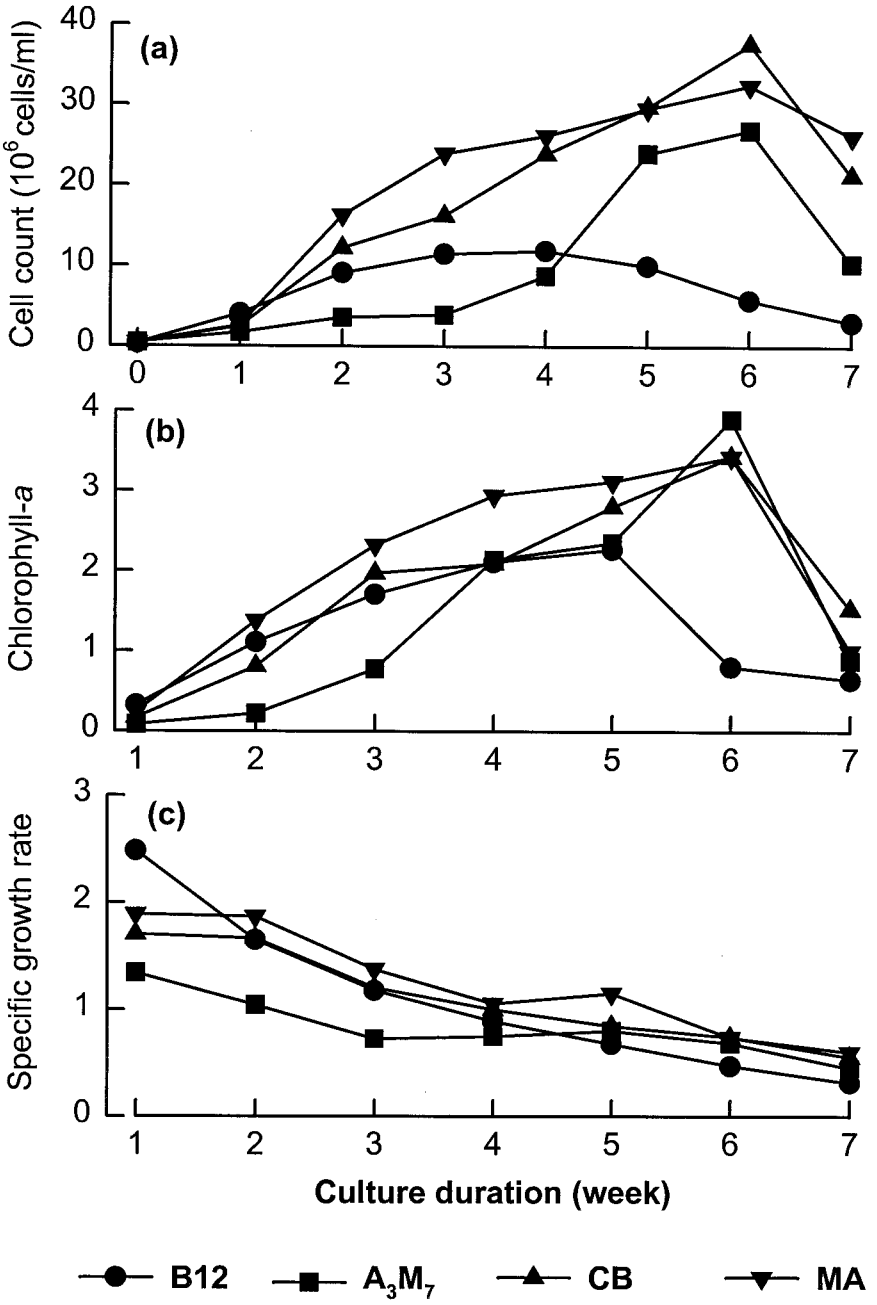

Figure 1. The growth characteristics of *M. aeruginosa* cells grown in batch cultures in different media and duration.

medium there was no significant difference in cell density from 2-5th week. Unlike MA and CB, there was notable decrease in cell number at 7th week in A_3M_7 and B12 media. The chl-a content of cells grown in all the media showed 2 to 5 fold increase between week 1 and 2 (Fig. 1b). The chl-a content increased with duration till sixth week in all the media except B12. A sharp decline in chl-a content was noticed in all the media at 7th week. The specific growth rate/week was highest during first week in all the media and declined with duration.There was no significant difference with media and culture duration in subsequent weeks.

The macromolecular composition of cells grown for 1-6 weeks is summarised in Table 1. Total carbohydrate level showed culture duration dependent increase in all the media. But in terms of carbohydrate content cells grown in MA and CB media showed highest concentration at week 5 and 6 as compared to A_3M_7 and B12 media. Similar profile was observed with respect to protein content of cultured cells. In all the four media there was significant increase in protein

Table 1. Effect of culture media and duration on macromolecular composition of *Microcystis aeruginosa* cells grown in static batch cultures.

Duration (Weeks)	Culture media MA	CB	A_3M_7	B12
CARBOHYDRATES*				
1	11.1 ± 0.42^{b}	10.3 ± 0.39^{b}	3.1 ± 0.22^{a}	16.0 ± 0.53^{c}
2	23.1 ± 1.87^{c}	10.6 ± 1.08^{ab}	5.3 ± 0.82^{a}	16.2± 3.60^{b}
3	25.3 ± 0.78^{b}	26.2 ± 1.53^{b}	15.3 ± 1.28^{ac}	14.0 ± 1.38^{a}
4	128.1 ± 4.49^{b}	32.1 ± 1.64^{d}	20.9 ± 0.93^{c}	12.1 ± 1.47^{a}
5	192.5 ± 4.81^{b}	201.2 ± 2.16^{b}	42.4 ± 0.88^{a}	75.0 ± 5.30^{c}
6	327.0 ± 3.39^{c}	283.5 ± 1.69^{b}	39.3 ± 0.91^{a}	43.6 ± 2.19^{a}
PROTEINS				
1	10.9 ± 0.20^{d}	9.5 ± 0.38^{cd}	3.4 ± 0.13^{a}	7.4 ± 0.18^{bc}
2	27.5 ± 1.43^{d}	13.3 ± 0.45^{b}	4.2 ± 0.39^{a}	17.8 ± 2.42^{c}
3	59.5 ± 2.45^{d}	39.1 ± 1.16^{c}	10.9± 0.31^{a}	18.2 ± 4.81^{b}
4	113.9 ± 4.15^{c}	91.9 ± 2.67^{b}	35.0 ± 1.75^{a}	31.0 ± 4.81^{a}
5	128.5 ± 6.68^{c}	113.3 ± 2.30^{c}	80.4 ± 5.14^{b}	34.9 ± 6.22^{a}
6	193.2 ± 2.45^{d}	156.2 ± 2.52^{c}	137.5± 2.21^{b}	63.5 ± 0.31^{a}
LIPIDS				
1	326.8 ± 3.22^{b}	301.2 ± 6.34^{a}	303.5 ± 2.53^{a}	379.3 ± 1.02^{c}
2	523.2 ± 1.65^{a}	537.5 ± 4.50^{a}	569.5 ± 2.06^{b}	534.5 ± 5.69^{a}
3	563.7 ± 7.98^{a}	576.5 ± 3.30^{a}	565.0 ±11.90^{a}	741.5 ±9.10^{b}
4	657.5 ± 6.29^{b}	662.5 ± 4.78^{b}	580.0 ± 4.08^{a}	594.5 ± 4.85^{a}
5	640.0 ± 4.08^{c}	657.5 ± 4.78^{c}	617.5 ± 10.1^{b}	580.0 ± 4.08^{a}
6	726.5 ± 3.59^{b}	720.0 ± 4.08^{b}	760.0 ± 3.55^{c}	580.0 ± 5.77^{a}

* In µg/ml of cells.

Values are mean ± SE of four replicates. In each row means followed by the same superscript are not significantly different at $p > 0.05$ by Student Newman Keul's multiple range test.

content in cultured cells with duration reaching maximum by sixth week. The protein content was highest in MA medium followed by CB, A_3M_7 and B12. Unlike carbohydrate and protein, the lipid content was similar in the four media. Except B12 medium where lipid content reached highest on week 3 in the other three media there was duration dependent increase in concentration.

The relative toxin content in cultured cells was determined in terms of mouse units/g cyanobacterial cells. The effect of media on toxicity is shown in Figure 2a. The duration for a given media was selected on the basis of highest chl-a content which was six weeks for MA, CB and A_3M_7 and 5 weeks for B12. The mouse units varied from 800-2500/g of dry cells. The highest toxicity was observed in MA medium followed by CB, A_3M_7 and B12. For the effect of culture duration (1-6 weeks) on toxin content, MA medium was selected (Fig. 2b). There was nearly two fold increase in toxicity after two weeks of growth. No increase in toxicity was observed from 3-6 weeks. The microcystin-LR content quantitatively estimated by HPLC for cells grown in the four media and different duration are summarized in figure 2c and 2d. The toxin content varied from 1.3 -3.0 µg/mg dry cells. Similarly cells grown in MA medium for 1-6 weeks showed marginal increase in toxin content with culture duration.

The effects of several environmental factors on growth and toxin production of cyanobacteria have been studied in batch and continuous culture. The important factors influencing toxin production include nutrients, pH, light, temperature and culture age. Many reports have noted the variable toxicity of samples from cyanobacterial (blue-green algal) water-blooms with regard to site, season, week or even day of collection (Codd and Bell 1985). However, information regarding microcystin production related to the nutrient status in cells or in ambient circumstances is still insufficient.

In this study we evaluated the effect of four culture media viz. MA, CB, A_3M_7 and B12 and different culture duration on growth characteristics, macromolecular composition and toxicity in batch cultures of a microcystin producing strain of *M. aeruginosa* (PCC 7820). We did not test the effects of varying temperature, light, pH or aeration. The culture medium influenced growth in terms of cell count and chl-a content. The macromolecular composition of cultured cells viz. carbohydrate, protein and lipid showed significant variations due to media and culture duration. The effect of interactions due to media and duration was also significant. These results indicate a clear influence of culture media on biomass growth, chl-a content, and macromolecular composition in interactions with culture duration.

Our results concerning the effect of various media on toxicity have shown that cells grown in MA medium produced maximum toxicity followed by CB , A_3M_7 and B12 in decreasing order. Microcystin-LR concentration in cultured cells in different media and duration varied from 1.3-3.0 µg/mg dry cells. There was only marginal increase in toxin concentration with duration. This variation may be attributed to differences in the composition of the media and in growth parameters. Toxicity of cells grown in MA medium increased significantly at week 3 and remained constant till week 6. There appears to be a positive correlation with increase in chl-a content to toxin content . Carmichael et al. (1988) showed that toxin production in *Nodularia spumigena* did not parallel the biomass increase but correlated with chl-a content. With additional nitrogen both chl-*a* content and toxin content increased. These results indicate that toxin production might be closely related to the primary energy processes of the cell.

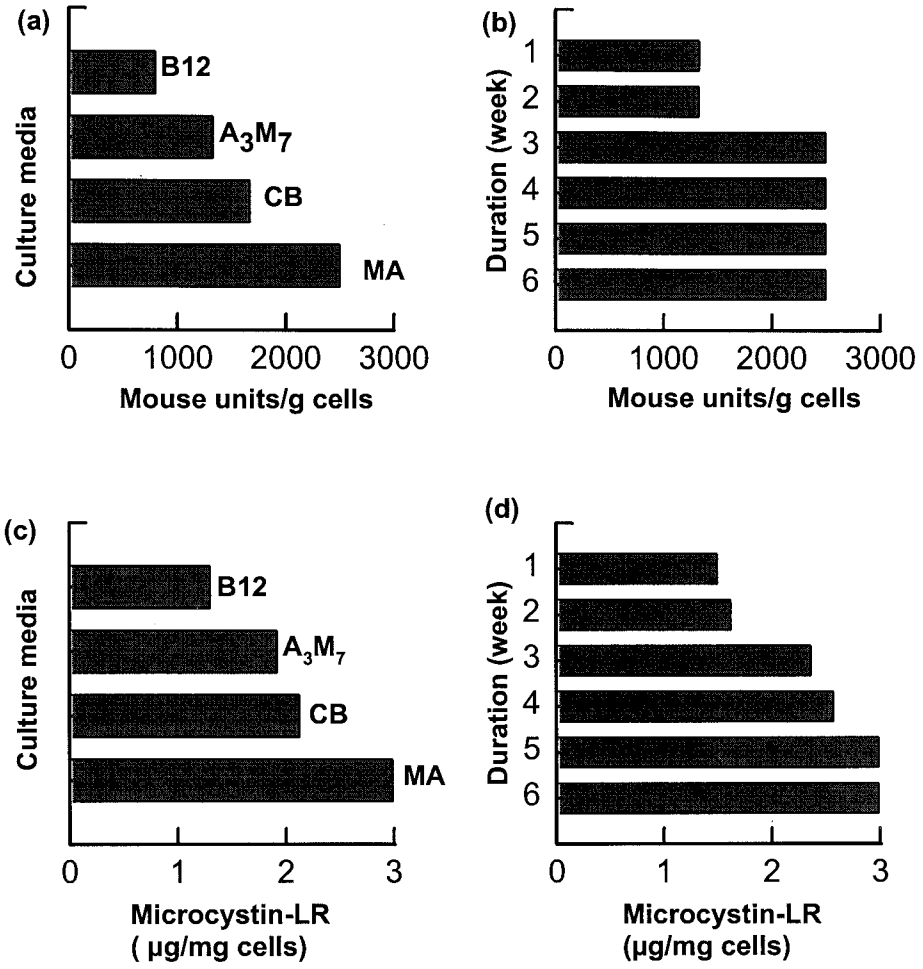

Figure 2. Effect of culture media and duration on toxicity (a,b) and toxin content (c, d) in batch cultures of *M. aeruginosa.*

The role of toxins in cyanobacteria is still not understood. Microcystins, the potent inhibitors of protein phosphatases, have been suggested to act as protective compounds against grazing zooplankton or as intracellular chelators inactivating free cellular Fe^{2+} (Utkilen and Gjølme 1995), or to have some specific cell regulatory function . It is still not understood as to why some species of certain cyanobacteria produce toxins whereas others do not. It is not known whether there are environmental factors which convert a non-toxin producing organism into a toxin producer. Hee-Mock Oh et al. (2000) showed that phosphorus was an important factor in the control of both the production of microcystin and the type of microcystin produced. Reduction of phosphorus in eutrophic waters may lower the growth and microcystin producing rate of *M. aeruginosa*, resulting in the reduction of toxic bloom formation. In conclusion, the present investigation demonstrates the definitive effect of media composition

and culture duration on growth, macromolecular composition and toxicity of *M. aeruginosa* in laboratory conditions. The mechanism behind the differences in toxicity is not fully understood . More detailed studies on various factors influencing toxic bloom formation and degradation would be useful in understanding the effects of toxins in relation to human activities and use.

Acknowledgments. The authors thank Dr. R. Vijayaraghavan, Head of the Division of Pharmacology and Toxicology for his help in statistical analysis, and Dr. R.V. Swamy, Director, DRDE, for his support and keen interest. Nidhi Gupta is thankful to Defence Research and Development Organization (DRDO), for the award of Research Fellowship.

REFERENCES

Bell SG, Codd GA (1994) Cyanobacterial toxins and human health. Rev Med Microbiol 5:256-264

Berg K, Soli NE (1985) Toxicity studies with *Oscillatoria agardhii* from two eutrophic Norwegian lakes. Acta Vete Scandinavia 26:363-373

Carmichael WW, Eschedor JT, Patterson GML, Moore RE (1988) Toxicity and partial structure of hepatotoxic peptide produced by the cyanobacterium *Nodularia spumigena* Mertens emend. L575 from New Zealand. Appl Environ Microbiol 84:2253-2257

Carmichael WW (1992) Cyanobacteria secondary metabolites - cyanotoxins. J Appl Bact 72:445-459

Carmichael WW (1994) The toxins of cyanobacteria. Sci Amer 270:78-86

Chorus I, Falconer IR, Salas HJ, Batram J (2000) Health risks caused by freshwater cyanobacteria in recreational waters. J Toxicol Environ Health 3:323-347

Codd GA, Bell SG (1985) Eutrophication and toxic cyanobacteria in freshwaters. J Water Pollut Cont 34:225-232

Codd GA, Bell SG, Brooks WP (1989) Cyanobacterial toxins in water. Water Sci Tech 21:1-13

Dawson RM (1998) The toxicology of microcystins. Toxicon 36:953-962

Harada K-I,Suzuki M, Dahlem AM, Beasley VR, Carmichael WW, Rinehart KL (1988) Improved method for purification of toxic peptides produced by cyanobacteria. Toxicon 26:433-439

Hassmann E (1973) Pigment analysis. In: Stein JR (ed) Handbook of Phycological Methods, Culture Methods and Growth Measurements. Cambridge University Press, Cambridge, p 359-368

Hee-Mock OH, Lee SG, Jang M-H, Yoon B-D (2000) Microcystin production by *Microcystis aeruginosa* in a phosphorus-limited chemostat. Appl Environ Microbiol 66:176-179

Hitzfeld BC, Hoger SJ, Dietrich DR (2000) Cyanobacterial toxins: Removal during drinking water treatment, and human risk assessment. Environ Health Perspect 108:113-122

Ichimura T (1979) Media for blue-green algae. In: Nishizawa M, Chihara M (eds) Methods in Algology, Kyoritsu, Tokyo, p 294

Jochimsen EM, Carmichael WW, Jisi An, Carod DM, Cookson ST, Holmes CEM, Bernadete M, Antunes C, Djalma A, Filho DM, Lyra TM, Spinell V,Barreto MD, Sandra MFO, Azevedo, Jarvis WR (1998) Liver failure and death after exposure to microcystins at a haemodialysis centre in Brazil. New England J Med 338:873-878

Kochert G (1978b) Quantitation of macromolecular components of microalgae. In: Hellebust JA, Craige JS (eds) Handbook of Phycological Methods:

Physiological and Biochemical Methods. Cambridge University Press, Cambridge, p 189
Nakagawa MY, Takamura, Yagi O (1987) Isolation of the slime from a cyanobacterium, *Microcystis aeruginosa* K-3A. Agric Biol Chem 51:329-337
Nishiwaki-Matsushima R, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Soc 114:7941-7942
Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VTS, Ward CJ, Preiser W, Poon GK, Neild GH, Codd GA (1998) Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. The Lancet 352:21-26
Rao PVL, Bhattacharya R, Dasgupta S (1994) Isolation, culture and toxicity of cyanobacterium (blue-green algae) *Microcystis aeruginosa* from a fresh water source in India. Bull Environ Contam Toxicol 52:878-885
Rao PVL, Bhattacharya R (1996) Cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver in vivo. Toxicology 114:29-36
Rao PVL, Bhattacharya R, Parida MM, Jana AM, Bhaskar ASB (1998) Freshwater cyanobacterium *Microcystis aeruginosa* (UTEX 2385) induced DNA damage in vivo and in vitro. Environ Toxicol Pharmacol 5:1-6
Shirai M, Matsumara K, Ohotake A, Takamura Y, Aida T, Nakano M (1989) Development of a solid medium for growth and isolation of axenic microcystin strain (cyanobacteria) Appl Environ Microbiol 55:2569-2571
Sivonen K (1990) Effects of light, temperature, nitrate, orthophosphate and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. Appl Environ Microbiol. 56:2658-2666
Utkilen H, Gjølme (1995) Iron-stimulated toxin production in *Microcystis aeruginosa*. Appl Environ Microbiol 61:797-800
van der Westhuizen A J, Eloff JN (1985) Effect of temperature and light intensity on the toxicity of and growth of blue-green alga *Microcystis aeruginosa* (UV-006). Planta 163:*55-59*