

## Detection of microcystins in the coastal lagoon La Albufera de Valencia, Spain by an enzyme-linked immunosorbent assay (E.L.I.S.A.).

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### ABSTRACT

Over the last twenty years, cyanobacteria have become the predominant phytoplankton in La Albufera de Valencia (coastal lagoon). Dense populations of *Planktothrix* (*Oscillatoria*) have been observed, sometimes in excess of  $3.10^5$  individuals/ml. This genus has been commonly found to produce microcystins, a hepatotoxin. In order to assess the presence or absence of these toxins in La Albufera, samples were taken in July and August of 1999. Physico-chemical and biological parameters were assessed by standard techniques and microcystin concentrations were measured with an E.L.I.S.A. Concentrations of microcystin were found to range from 5.0 to 2102.9 ng/l (average 300.1 ng/l). Microcystin levels appeared unrelated to the abundance of known microcystin producers (i.e. *Planktothrix*, *Microcystis*), but were generally higher when *Planktolyngbya contorta* was abundant and lower during *Chroococcus dispersus* dominance. This study constitutes the first report of microcystins in Spain.

Keywords: microcystin, Albufera de Valencia, phytoplankton, eutrophication, Valencia, Spain, *Planktolyngbya contorta*

### RESUMEN

*Durante los últimos veinte años, las cianofíceas filamentosas han sido el grupo dominante en el fitoplancton de La Albufera de Valencia (laguna costera). Importantes poblaciones de Planktothrix (Oscillatoria) han sido observadas en este periodo, llegando en ocasiones a  $3.10^5$  de individuos por mililitro. Este género ha sido encontrado comunmente como productor de microcistinas, una hepatotoxina. Para evaluar la presencia o ausencia de estas toxinas en La Albufera de Valencia, se tomaron muestras en Julio y en Agosto de 1999.*

*Los parámetros físicoquímicos y biológicos fueron analizados con métodos estandar y la medición de microcistinas se realizó mediante la técnica E.L.I.S.A. Se detectaron concentraciones de microcistinas desde 5.0 hasta 2102.9 ng/l (300.1 ng/l de media). Los niveles de microcistinas no aparecían en relación a la cantidad de productores de microcistinas conocidos (Planktothrix, Microcystis), pero tendían a ser mayores durante el incremento en la población de Planktolyngbya contorta, y menores durante la dominancia de Chroococcus dispersus. Este estudio constituye la primera cita de microcistinas en España.*

*Palabras clave: microcistinas, Albufera de Valencia, fitoplancton, eutrofización, Valencia, España, Planktolyngbya contorta*

### INTRODUCTION

La Albufera de Valencia is a shallow, hypertrophic, oligohaline lagoon located 10 km south of Valencia along the Mediterranean coast of Spain (39°20'N, 0° 20'W). It is located within La Albufera Natural Park in a densely populated urban area. In the early 1970's, diatoms dominated the system, with increases in blue green algae

populations occurring in the summer. Chlorophyll a levels between 1972 and 1974 increased from 13 µg/l in 1972 to 54 µg·l<sup>-1</sup> in 1974 (Dafauce, 1975), indicating the start of eutrophication. Population growth of surrounding towns and the explosion of industries along the banks of La Albufera (almost a ten-fold increase in factories from the 1970's to the 1980's) lead to massive and sustained increases in sewage and industrial

discharges to the lagoon (Vicente & Miracle, 1992). These additional inputs lead to a dramatic change in trophic status and phytoplankton composition by the 1980's. In 1981 and 1982, cyanobacteria represented 75-95% of the phytoplankton community in nearly every month sampled (Garcia *et al.*, 1984; Miracle *et al.*, 1984). Chlorophyll a concentrations at these sampling times (1 14-646 µg/l) indicated a state of hypereutrophication.

From 1980 to 1988, filamentous blue-green algae accounted for 80-90% of the total phytoplankton abundance and biomass in La Albufera, (Romo & Miracle, 1994). Among the dominant cyanobacteria was *Planktothrix agardhii*. This species was found in 99% of the samples taken over this period. Mean density was circa 51 000 individuals·ml<sup>-1</sup> (Vicente & Miracle, 1992), yielding up to 740 mg/l wet weight (Romo & Miracle, 1993).

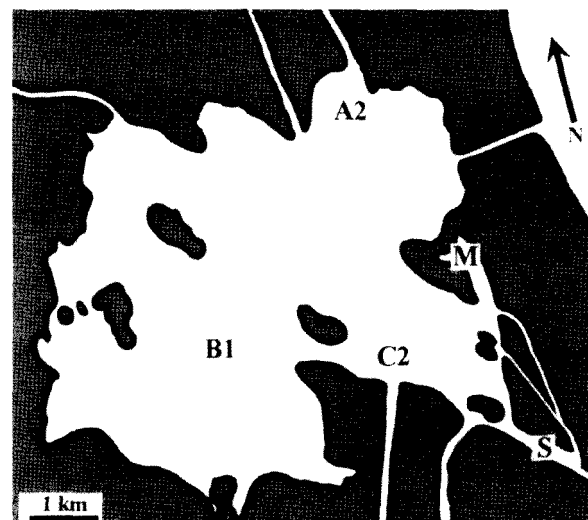
*P. agardhii* has been found to produce microcystins (Eriksson *et al.*, 1988; Bruno *et al.*, 1992; Luukkainen *et al.*, 1993), a group of hepatotoxins found in several genera of colonial and filamentous cyanobacteria, including *Nostoc*, *Anabaena* and *Microcystis*, (Sivonen *et al.*, 1990a; Namikoshi *et al.*, 1992; Vasconcelos *et al.*, 1995). Even within cyanobacterial species shown to produce microcystins, toxin production is variable. *Microcystis aeruginosa* isolated from a single lake, for instance, was found to have both toxic and non-toxic strains (Skulberg *et al.*, 1984, 1993). It is estimated that 25% to 95% of algal blooms are toxic (Repavich *et al.*, 1990; Sivonen *et al.*, 1990b; Baker & Humpage, 1994; Pearson, 1994). The effects of microcystins on organisms vary widely; aquatic invertebrates experience decreased feeding rates and reduced reproductive fitness (Hietala *et al.*, 1995; Lauren-Maatta *et al.*, 1997), while vertebrates can be subject to mild illness, tumor promotion and in extreme cases, death (Slatkin *et al.*, 1983; Galey *et al.*, 1987; Falconer & Humpage, 1996; Jochimsen *et al.*, 1998).

Blooms of hepatotoxic cyanobacteria have become a global concern (Codd *et al.*, 1999b). Toxic blooms have been reported throughout

southern Europe, including Portugal (Vasconcelos, 1994; Vasconcelos *et al.*, 1995; Vasconcelos, 1999) and France (Michard *et al.*, 1996; Vezie *et al.*, 1997). In Spain, blooms of toxic dinoflagellates in marine and freshwater systems (Mariño *et al.*, 1998, Prego *et al.*, 1998), and incidents of potentially-toxic cyanobacterial blooms in freshwater systems (Alvarez *et al.*, 1998) have been reported. However, no reports of the detection of hepatotoxins in Spanish freshwater systems exist to date. The current study represents a first survey of a cyanobacteria-dominated system for presence of microcystins.

## MATERIALS AND METHODS

Samples were taken on three dates (July 14, August 4 and August 26, 1999) at five locations (Fig. 1) in La Albufera (Valencia, Spain, 39°20'N, 0° 20'W). Phytoplankton and physico-chemical sampling were performed simultaneously by taking samples at 0.5 m below the surface with 2 litre PVC containers. Conductivity, dissolved oxygen, temperature and pH were measured in situ with a WTW MultiLine P4 multiprobe.



**Figure 1.** Sampling sites for La Albufera (Valencia, Spain). Site "M" indicates the Mata de Fang and site "S" indicates Sequiota. *Puntos de muestreo en La Albufera (Valencia, España). El punto "M" indica La Mata del Fang y el punto "S" indica La Sequiota*

Chlorophyll a concentrations (Shoaf & Lium, 1976; Burnison, 1980) were determined in the laboratory. Water samples for microcystin analysis were kept cool in the field, then stored at -20°C. Phytoplankton counts were performed for points A2, B1 and C2 (Fig. 1) with an inverted microscope (Olympus IM) according to Utermohl (1958) and reported as individuals per

milliliter. Phytoplankton classification was based on Germain (1981), Huber-Pestalozzi (1983), Anagnostidis & Komarek (1988), and Komarek & Anagnostidis (1999).

Whole lake water (WLW) samples, stored in HDPE sample cups, were subjected to three freeze-thaw cycles to lyse the cells. Samples were thoroughly mixed and 15 mL transferred into a 30-mL borosilicate-glass serum bottle. The bottles were frozen at a 30° angle to maximize the exposed surface area of ice. Frozen samples were lyophilized in a Labconco Freezone 4.5 (-50°C, 30' 10-3 mbar) and then rehydrated with 1.5 mL of Milli-Q water. This 10-fold concentration was used to bring the WLW samples into a range that was detectable by the E.L.I.S.A. procedure used. Each sample was filtered through a Whatman PTFE syringe filter (13 mm diam, 0.2 mm pore diameter) into a polypropylene centrifuge tube, which was then frozen until E.L.I.S.A. analyses were performed.

Microcystin analysis was done using an enzyme-linked immunosorbent assay (E.L.I.S.A.). A 96-well microcystin plate kit was used (Envirologix Inc., Portland, ME). Subsamples of the three MC standards provided with the kit (160, 500, and 1600 ng/l) were diluted with Milli-Q water to make up four additional standards at concentrations of 15, 25, 53, and 100 ng/l to extend the sensitivity of the assay. Each of the seven standards was run in duplicate. Due to considerable variation in the 15 ng/l standard, the

**Table 1.** Concentrations of microcystins in ng·l<sup>-1</sup> for all sampling points and dates. Values in parentheses are under the detection limit of the assay. *Concentración de microcistinas en ng·l<sup>-1</sup> para cada punto de muestreo. Los valores entre paréntesis no llegan al límite de detección.*

Date	Site	Microcystin concentration (ng·l <sup>-1</sup> )
July 14, 1999	A2	76.7
	B1	(1.7)
	c 2	132.1
	Mata de Fang Sequiota	2102.9 (0.7)
August 3, 1999	A2	5.0
	B1	103.6
	c 2	69.3
	Mata de Fang Sequiota	52.2 934.5
August 26, 1999	A2	623.0
	B1	222.3
	c 2	(2.0)
	Mata de Fang Sequiota	160.8 14.5

**Table 2.** Percent composition of phytoplankton from Abufera samples. *Porcentaje de los grupos del fitoplancton de La Abufera (Valencia, Spain).*

Date	Site	Cyanophyceae	Chlorophyceae	Bacillariophyceae	Cryptophyceae	Euglenophyceae
July 14, 1999	A2	97.7	2.3	<0.1	0.1	
	B1	97.2	2.8			
	c 2	97.5	2.3	0.1	0.1	<0.1
August 3, 1999	A2	96.8	3.2	-		<0.1
	B1	97.0	2.8		0.1	<0.1
	c 2	98.2	1.7	-	0.1	
August 26, 1999	A2	78.8	8.1	11.2	1.9	
	B1	96.6	3.0	0.0	0.3	<0.1
	c 2	96.4	3.3	0.2	0.1	<0.1

25 ng/l standard was considered the lowest sensitivity of the assay. Thus, the lowest detectable microcystin concentration is 2.5 ng/l, because of the 10-fold concentration performed on the samples.

Methodology followed the Enviroligix protocol provided in each kit. Optical densities (OD) of the wells were read on a Bio-tek (Winooski, VT) EL800 Plate Reader (sensitivity off 0.010 Abs) at a wavelength of 450 nm. KC Junior, a companion program to the plate reader, was used to record the OD from each well and calculate microcystin concentrations based on a cubic log-log standard curve derived from the standards. Wells were also read at a dual wavelength of 630 nm as a reference to remove any interference that may have been caused from bubbles in the sample or scratches on the plate.

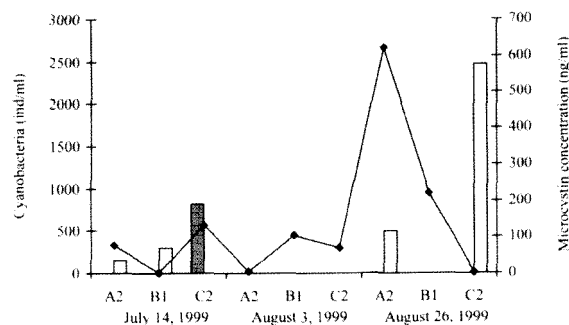
## RESULTS

Microcystin concentrations varied widely, ranging from below detectable limits (b.d.l.) to 2102.9 ng/l (mean = 300.1 ng/l) (Table 1). Variation explained (i.e.  $r^2$ ) by E.L.I.S.A. standard curves ranged between 95.6-99.5%. The results of the E.L.I.S.A. were below the detection limit of our methods (i.e. 2.5 ng/l) in four. Cyanobacteria

were the dominant group in all samples, constituting 78.8-97.7% of total phytoplankton abundance (Table 2). The concentration of microcystins did not correlate well with the presence of known microcystin producers, such as *M. aeruginosa* and *P. agardhii* (Fig. 2). However, microcystin levels tended to be lower when *C. dispersus* was abundant, and higher when *Pl. contorta* was present in abundance (Fig. 3).

Chlorophyll *a* concentrations (67-300  $\mu\text{g}/\text{l}$ , mean 199  $\mu\text{g}/\text{l}$ ) indicate that La Albufera is currently less eutrophic than during the 1980's. In spite of this, cyanobacteria dominated the phytoplankton at all sampling locations on all three sampling dates (Table 2). *C. dispersus* and *P. contorta* were the most common cyanobacteria and together accounted for 74%-87% of cyanobacteria total abundance. *M. aeruginosa* and *P. agardhii* were occasionally found, but never accounted for more than 0.1% of total cyanobacteria.

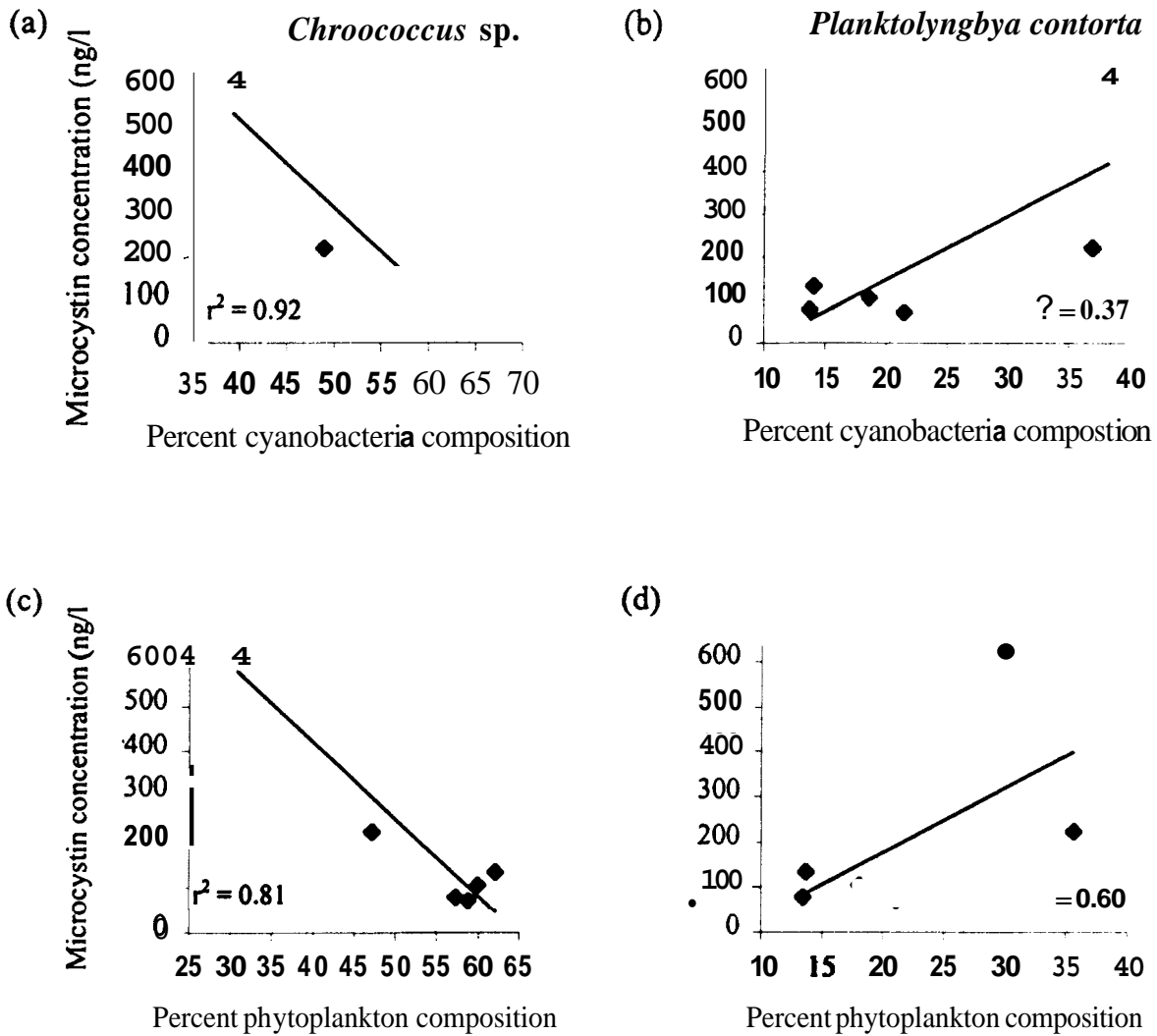
No significant relationships were found between microcystin concentrations and any of the biological and chemical characteristics measured. Also, microcystin concentrations did not correlate with the presence of known microcystin-producing species (Fig. 2), indicating that the *M. aeruginosa* and *P. agardhii* found in the water column were not directly responsible for the microcystins detected. However, a shift in dominance between *C. dispersus* and *P. contorta* correlated with changing microcystin levels (Fig. 3). Microcystin concentrations increased with increasing *P. contorta* concentrations, while the opposite trend was observed with *C. dispersus*.



**Figure 2.** Concentration of microcystin in water samples from La Albufera against densities of known hepatotoxin producers *Microcystis aeruginosa* (grey columns) and *Planktothrix agardhii* (white columns). *Concentracidn de microcistinas en las muestras de agua de La Albufera frente a concentraciones de conocidos productores de hepatotoxinas Microcystis aeruginosa (columnas grises) y Planktothrix ugaudhii (columnas blancas).*

## DISCUSSION

Microcystins were detected in La Albufera at relatively low levels (i.e. b.d.l to 2102.9 ng/l). In a study of 17 Wisconsin lakes, McDermott *et al.*, (1995) used an E.L.I.S.A. to test for microcystins. The reported concentrations (maximum = 56 000 ng-l, average = 16 085 ng/l) were much higher than those found in La Albufera. Ueno *et al.* (1996) tested lakes and reservoirs across Japan, Thailand, Germany and Portugal



**Figure 3.** Relationships between microcystin concentration in Albufera and percent composition of *Chroococcus dispersus* and *Planktolyngbya contorta*. Graphs (a) and (b) are based on percent composition in relation to all phytoplankton, while (c) and (d) are based on composition of cyanobacteria only. *Correlucion entre las concentraciones de microcistinas del agua de la Albufera y el porcentaje de Chroococcus dispersus y Planktolyngbya contorta. Las gráficas (a) y (b) estan basadas en el porcentaje de fitoplancton total, mientras (c) y (d) estan basadas en el porcentaje de ciunobacterius.*

with an E.L.I.S.A. assay from 1993-1995. Maxim varied widely (i.e. Japan: 1.300.000 ng/l, Thailand: 200 ng/l, Germany: 36000 ng/l, Portugal: 37000 ng/l), but in general were at least an order of magnitude greater than the highest level detected in the current study.

However, the maximum microcystin concentration in La Albufera (2102.9 ng/l) was higher than

that in the majority of the microcystin-containing lakes surveyed in both studies (Wisconsin - 63%, Japan - 53%, Germany - 64%, Portugal - 54%).

It appears that *P. contorta* was responsible for most microcystin production in La Albufera, although no direct evidence was found as phytoplankton samples were not isolated and tested. Production by this species would fit reports of

toxin production by Oscillatoriales (Eriksson *et al.*, 1988; Bruno *et al.*, 1992, Luukkainen *et al.*, 1993, Brittain *et al.*, 2000). Although the correlation between % total abundance of *P. contorta* and microcystin concentrations was relatively high ( $r^2 = 0.60$ ) (Fig. 3), a great deal of the variation in microcystin levels is still unaccounted for. *C. dispersus* as a percentage of total phytoplankton exhibited a strong correlation with microcystin concentrations ( $r^2 = 0.81$ ). When regressions were based on percent of this species over total cyanobacteria, the effect of *P. contorta* decreased ( $r^2 = 0.37$ ), while that of *C. dispersus* increased ( $r^2 = 0.92$ ). It may be that *C. dispersus* displaces microcystin-producing cyanobacteria, thus acting to limit microcystin concentrations when dominant. It could also be possible that the conditions which favour the growth of *C. dispersus* do not favour the production of microcystins.

There are several possible explanations for the unexplained variation in microcystin production seen in La Albufera. One could be the lack of comprehensive knowledge of all genera and species of cyanobacteria capable of microcystin production. The focus of many microcystin studies has been large, colonial, bloom-forming genera and species. Studies continually add to the list of microcystin producers, and have begun to include smaller, less studied species (Domingos *et al.*, 1999). In addition, even known microcystin producers, such as *M. aeruginosa*, can be present in both toxic and non-toxic strains within the same lake (Skulberg *et al.*, 1984, 1993).

The amount of variation may also be attributed to the physical characteristics of La Albufera. Many forms of cyanobacteria have a tendency to float to the surface of lakes under certain conditions, while under others, cyanobacterial cells may accumulate in the benthos, through sedimentation or as a benthic phase of their life cycle (Barbiero & Welch, 1992; Threlkeld, 1994). Not only could cyanobacteria be found throughout the water column and/or in the sediments of La Albufera, they may also be frequently displaced from what would constitute a "normal" location. Since the lagoon is large (i.e. 21 km<sup>2</sup>, 7 km across) and quite shallow (i.e. average depth

0.75 m), wind and lack of thermal stratification provide a great deal of mixing. The concentration and location of microcystins are related to the length of time the toxin is present in La Albufera after its production. While some reports attest to the good stability of microcystins through their resistance to various types of chemical and physical degradation (Watanabe *et al.*, 1992; Tsuji *et al.*, 1994), other studies have exhibited microbial degradation of microcystins (Jones *et al.*, 1994, Rapala *et al.*, 1994, Inamori *et al.*, 1998). Most of these studies found that toxins are still detectable after 15-30 days of degradation. Microcystins have been found to interact with other substances in the water and bind to particulate matter (Rapala *et al.*, 1994, Lahti *et al.*, 1997). Therefore, even if cyanobacterial cells did not precipitate, free microcystin could be bound to other particles and settle out of the water column. In all cases, i.e. free microcystins in the water, inside of floating cells, inside precipitating cells, adsorbed to particulate matter, it is possible they are being constantly redistributed throughout the water column and benthic zone in a well-mixed system such as la Albufera.

Since La Albufera was historically more eutrophic and contained greater populations of known microcystin producers, such as *P. agardhii* (Vicente & Miracle, 1992; Romo & Miracle, 1993), it is possible that microcystin concentrations were greater in the 1980's than today. However, Jones *et al.* (1994) suggested that biodegradation of microcystins may commence more quickly in bodies of water which experience frequent exposure to microcystin-producing cyanobacteria. Therefore, it is possible that La Albufera may contain a large community of organisms ready to degrade the toxin. This could help explain the relatively low concentration of microcystins relative to the extremely high concentrations of cyanobacteria and chlorophyll *a*. In all samples, except for the July 14 sample of the Mata de Fang, microcystin concentrations were below standards proposed by the World Health Organization (1998) for microcystin-LR in drinking water (i.e. 1 µg/l), and more recent standards by Falconer *et al.* (1999) suggested for

"relatively mild and/or low probabilities of adverse health effects" (2-4 µg/l).

Even though current levels in La Albufera likely pose little acute risk to humans, there is a moderate potential for chronic effects. Although not used as a drinking water source, thus minimizing human exposure, even low level exposure to microcystins has been found to promote liver cancer (Ueno *et al.*, 1996). It is more probable that wildlife (fish, birds, amphibians) could be affected, because of their direct contact with the lake water, and resultant higher concentrations due to bioaccumulation. In addition, microcystins have been found to accumulate in lettuce when microcystin-containing water was used for their cultivation (Codd *et al.*, 1999a). A slight possibility may exist for accumulation of microcystins in plants in rice fields surrounding La Albufera. However, the potential accumulation in rice grains appears to be smaller than that in lettuce leaves.

La Albufera provides an opportunity for the study of microcystins in an oligohaline environment. Few studies on cyanobacterial toxicity have been conducted in brackish environments. Although La Albufera is oligohaline, thus only slightly saline relative to sea water, it provides a system which is more saline than the vast majority of lake systems, but fresh enough to allow for cyanobacterial dominance (Paerl 1988, 1996; Codd, 1998). Comprehensive studies are needed on the seasonal dynamics of microcystins in La Albufera to better assess conditions of this unique system as well as potential impacts to animal and human populations.

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