Effects of a bloom of *Planktothrix rubescens* on the fish community of a Spanish reservoir

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ABSTRACT

During the spring of 2000 and 2001 there were two cyanobacterial bloom episodes in El Atazar reservoir (central Spain). They were attributed to the toxic cyanobacteria *Planktothrix rubescens*. The goal of the present study was to determine the possible effects and toxicity of these blooms on the fish community of the reservoir. The main objectives were to analyse the presence and concentration of toxins in fish liver and to carry out histopathological analyses of liver tissue. In addition, recent data on water quality of the reservoir were inspected to ascertain the likely causes of the blooms. The analysis of water quality data and weather conditions showed that there was not a single key cause of the *P. rubescens* bloom. Toxin analyses showed that microcystins were absent or in very low concentrations in fish liver extracts obtained from the reservoir. This finding should not be considered as definitive since liver pathologies were detected in the fish studied, which were probably due to exposure to hepatotoxins. Histological analyses revealed the occurrence of pigmented macrophage aggregates (MAs) in the liver sections of nearly all fish exposed to the bloom. MAs are known to change in number, size and pigment content in relation to fish health and environmental degradation. The average MAs per specimen showed a great variability among samples, and their distribution within the liver tissue was highly heterogeneous. However, further research is needed to give an insight into the pathogenesis for the cyanobacteria-associated liver pathology.

Keywords: Cyanobacterial bloom, freshwater fish, histopathology, microcystins, pigmented macrophage aggregates, *Planktothrix rubescens*

RESUMEN

Durante la primavera de 2000 y 2001 se produjeron dos proliferaciones masivas de una cianobacteria tóxica identificada como Planktothrix rubescens *en el embalse de El Atazar (centro de España). En el presente estudio se ha realizado una investigación sobre los posibles efectos y toxicidad de esta proliferación en la comunidad de peces del embalse. Los principales objetivos fueron analizar la presencia y concentración de toxinas en muestras de hígado, así como llevar a cabo un análisis histopatológico del tejido hepático. También se analizaron datos recientes sobre la calidad del agua del embalse para detectar los posibles cambios que pudieron desencadenar la proliferación de la cianobacteria. El análisis de la calidad del agua y de las condiciones meteorológicas mostró la existencia de múltiples factores que pudieron influir en las proliferaciones de* P. rubescens*. En los análisis de las extracciones realizadas a partir de las muestras de hígado de los peces del embalse no se detectaron toxinas o estuvieron presentes en concentraciones muy bajas. Estos resultados no se pueden considerar definitivos, ya que se detectaron afecciones hepáticas en los individuos estudiados que probablemente fueron debidas a la ingestión de toxinas por los peces. La mayor parte de los peces afectados por la proliferación presentaron centros melanomacrofágicos (MAs) en el hígado. El número, tamaño y contenido en pigmentos de los MAs suele depender de la salud del pez y del grado de degradación ambiental. El número medio de MAs presentó una notable variación entre los individuos analizados y su distribución en el hígado fue bastante heterogénea. Sin embargo, se necesita un estudio más detallado para profundizar en los mecanismos que han originado la patología observada.*

Palabras clave: *Peces continentales, centros melanomacrofágicos, histopatología, microcistinas,* Planktothrix rubescens*, proliferación de cianobacterias*

INTRODUCTION

Cyanobacterial blooms occur in response to a combination of climatic and hydrographic events and to the availability of nutrients, trace metals and vitamins (Bruno *et al*., 1989; Chorus & Bartram, 1999; Paerl *et al*., 2001). They are usually caused by an increase of nutrients in water, mainly phosphorus and nitrogen, that favours the growth of cyanobacteria (Smith, 1977; Mason, 1991; Chorus & Bartram, 1999). Maximum growth rates for cyanobacteria have been observed around 25ºC (Robarts & Zohary, 1987). Therefore, these blooms usually take place in eutrophic waters at high temperature.

During the spring of 2000 and 2001 a substantial bloom of *Planktothrix rubescens* occurred in El Atazar reservoir (central Spain). Blooms of *Planktothrix* spp. have been previously reported mainly from northern lakes and reservoirs in Europe (e.g. Ostensvik *et al*., 1981; Leeuwangh *et al*., 1983; Skulberg *et al*., 1984; Berg *et al*., 1986; Lindholm *et al*., 1989). Cyanobacteria of the genus *Planktothrix* are capable of producing several potent hepatotoxins (Chorus & Bartram, 1999), called microcystins (Carmichael, 1992; Skulberg *et al*., 1993). Bioaccumulation of microcystins in aquatic vertebrates and invertebrates has been formerly reported by Eriksson *et al*. (1989), Tencalla *et al*. (1994), Carbis *et al*. (1994), Vasconcelos (1994, 1995), Beattie *et al*. (1998) and Thostrup & Christoffersen (1999). Therefore, high cyanobacteria concentrations in an aquatic system could deteriorate water quality and increase the risk of animal toxicity (Freitas de Magalhaes *et al*., 2001).

Fish are considered to be appropriate test organisms for the assessment of harmful xenobiotic chemicals as microcystins, because of their systematic position as vertebrates and as an end-point of the aquatic food chain (Baganz *et al*., 1998). The main pathological effects of microcystins on fish are damage to the gills, skin, heart, kidney, spleen and liver, as well as growth inhibition and mortality (Phillips *et al*., 1985; Rahbergh *et al*., 1991; Rodger *et al*., 1994; Tencalla *et al*., 1994). Furthermore, microcystins can also affect embryos (Oberemm *et al*., 1997) and fish behaviour (Baganz *et al*., 1998). These damages have been observed both in experimental studies (Gaete *et al*., 1994; Bury *et al*., 1996) and field conditions (Eriksson *et al*., 1986; Rodger *et al*., 1994). However, previous studies indicate that microcystins act specifically on liver cells (Eriksson *et al*., 1990; Rahbergh *et al*., 1991). The exclusive targeting of microcystin toxicity to liver reflects the hepatic accumulation of the toxin, since following dosing of animals with radioactively labelled microcystin derivatives significant amounts of microcystins are only found in this organ (Runnegar *et al*., 1986,1993; Falconer *et al*., 1986; Robinson *et al*., 1991).

The final aim of the present study was to determine the effects and toxicity of the bloom of *Planktothrix rubescens* on the fish community of El Atazar reservoir located in central Spain. Our main objectives were to analyse the presence and concentration of toxins in fish liver and to carry out histopathological analyses of liver tissue. In addition, recent data on the water quality of the reservoir were inspected to ascertain the likely causes of the blooms.

MATERIAL AND METHODS

Study area

The study was conducted in El Atazar reservoir, a deep (100 m) oligo-mesotrophic water body located in central Spain (UTM: 30TVL458545338) which is fed by the River Lozoya. This river is a tributary of the River Tagus and have soft, infertile waters arising from granite and metamorphic grounds. The reservoir has an area of 10.7 km^2 and holds an average of 425 hm^3 of water, being the largest and most important water supply reservoir of the Madrid city area. The River Madarquillos (UTM: 30TVL451145427) was chosen as a reference site, since it was not affected by the bloom. It is a tributary of the River Lozoya located further upstream of the reservoir.

Water analyses

Data on water quality of the reservoir during the spring-summer periods of 2000 and 2001 were analysed. These data are based on weekly analyses made by "Canal de Isabel II", from the Regional Environmental Agency of Madrid. Chemical water parameters were analysed according to American Public Health Association (APHA) (1985). Chlorophyll *a* was determined by fluorometric analysis. Microcystins were estimated using ELISA assay that is based upon the polyclonal antibody method described by Chu *et al*. (1990). Microcystin standards were used to construct regression relationships to calculate sample toxin concentration.

Fish sampling

During June 2001, fish were captured in the reservoir using trammel nets in areas affected by the bloom. Additionally, control fish were collected in the reference site by means of electrofishing using a 220 W DC generator. A total of 38 specimens were captured, most of which were common barbel *Barbus bocagei*. There was also one goldfish *Carassius auratus* and one largemouth bass *Micropterus salmoides*. Each fish was packed in a plastic bag and frozen immediately after capture on dry ice until analysed in the laboratory. Fish were measured (fork length, to the nearest mm), weighted (to the nearest 0.1 g) and examined for external lesions or parasites which could be related to the presence of the toxic cyanobacteria in the water. While still partly frozen fish were dissected, and liver samples were collected and preserved for histopathological and toxin analyses. The fish from the affected area mainly consisted of similar-sized barbel (mean length, 40.8±22.6 cm; range, 36.5-45.6), whereas in the reference site fish captured were also barbel but smaller (mean length, 10.1±15.5; range, 7.8-11.6).

Histology

The liver samples were embedded in Tissue-Tec® and frozen in liquid nitrogen. Then, twelve sections of 10 µm per specimen were cut with a cold microtome (Cryocut). Sections were stained with haematoxylin and eosin, and mounted for later pathological assessment by light microscopy (Disbrey & Rack, 1970).

Microcystins analysis

To analyse toxins in liver tissue, six samples from El Atazar and two from the reference site were extracted twice with 100% methanol. The

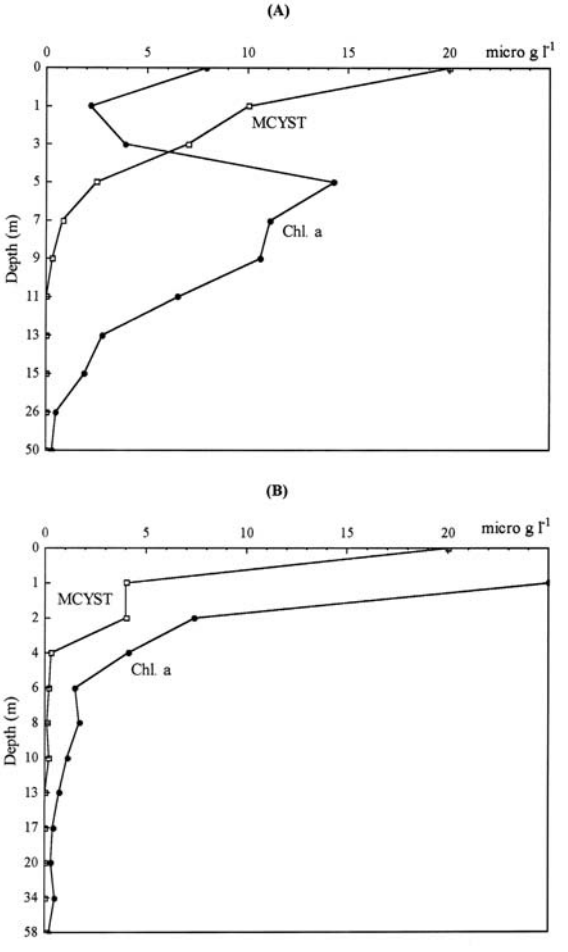

Figure 1. Vertical distribution of chlorophyll *a* (closed circles) and microcystins (open squares) in El Atazar reservoir during the bloom of *P. rubescens* in A) June 1 2000 and B) May 31 2001. *Distribución vertical de clorofila* a *(círculos rellenos) y microcistinas (cuadrados vacíos) en el embalse de El Atazar durante la proliferación de* P. rubescens *en A) 1 de junio de 2000 y B) 31 de mayo de 2001.*

methanol extract was mixed three times with equal volumes of hexane. Hexane layers were discarded and the methanol extract was dried and redissolved in deionized water. This extract was loaded into a C18 cartridge which was washed and eluted with 30 ml of 20% methanol and 50 ml of 100% methanol, respectively. The 100% methanol fraction was dried and redissolved in 1 ml of 100% methanol:water (1:1 v/v). Analyses for microcystin detection were performed by HPLC with a Lichrospher 100 RP-18 reverse phase column (5µm-Merck). Chromatography was carried out under isocratic conditions with a mobile phase of 20 mm ammonium acetate, pH 5.0 and acenonitrile (7:3), for 10 min. Volume injected was 20 µl with a flow rate of 1 ml min^{-1}. UV detection was done at 238 nm and the absorption espectrum of each peak was analysed over the range of 190-300 nm (Freitas de Magalhaes *et al*. 2001).

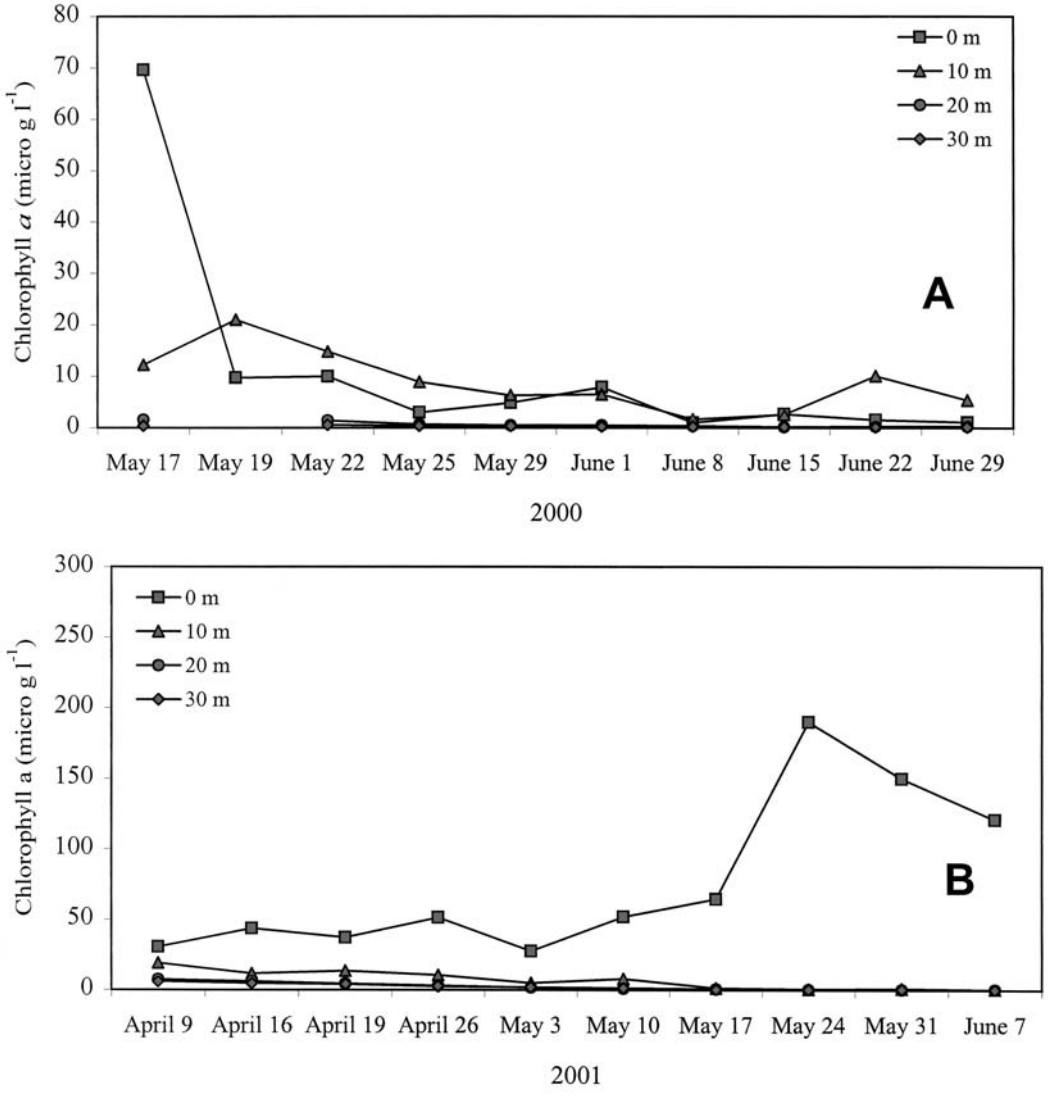

Figure 2. Vertical distribution of chlorophyll *a* (µg l^{-1}) in El Atazar reservoir during the spring of 2000 and 2001. *Distribución vertical de clorofila* a *(µg l^{-1}) en el embalse de El Atazar durante la primavera de 2000 y 2001.*

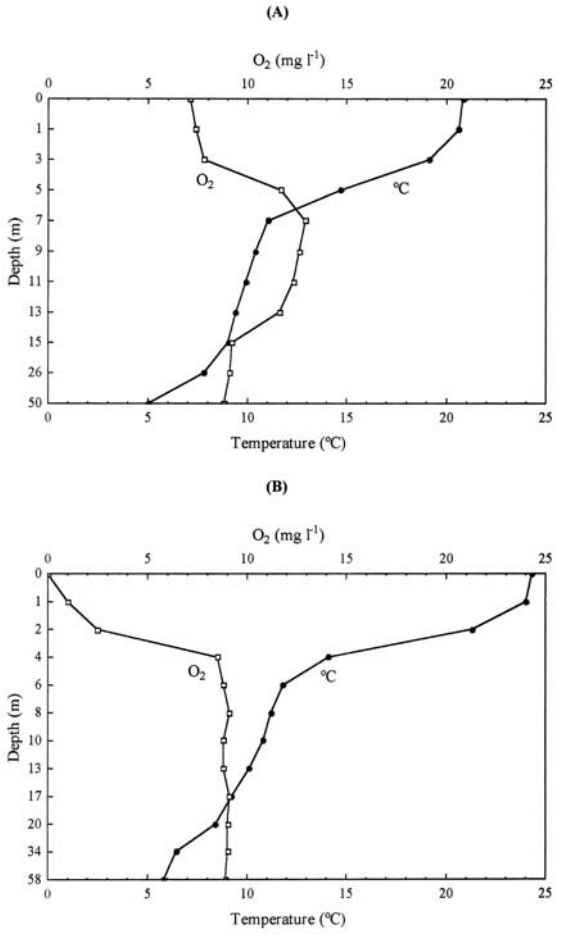

Figure 3. Hydrographical conditions (closed circles: water temperature; open squares: dissolved oxygen) of El Atazar reservoir during the bloom of *P. rubescens* in (A) June 1 2000 and (B) May 31 2001. *Condiciones hidrográficas (círculos rellenos: temperatura del agua; cuadrados vacíos: oxígeno disuelto) del embalse de El Atazar durante la proliferación de* P. rubescens *en (A) 1 de junio de 2000 y (B) 31 de mayo de 2001.*

RESULTS

During the spring of 2000 and 2001 a surface bloom of *P. rubescens* was reported in El Atazar, an oligo-mesotrophic reservoir from central Spain. These blooms were firstly associated with the beginning of the thermal stratification of the reservoir. The spring rainfall in the area was higher than usual during those years, thus increasing the run-off of nutrients into the catchment. Moreover, rainfall events were alternated with exceptionally warm weather conditions, resulting in a sharp increase of water temperature and the onset of the stratification process in the reservoir.

The bloom of the year 2000 took place in the epilimnion from mid-May until the beginning of June, which caused high levels of chlorophyll *a* (69.6 µg l^{-1} during the peak of the bloom) (Figs. 1 and 2). This was joined with high pH values (mean pH, 9.2±0.2) and oxygen saturation (mean O_2 saturation, 133.9±4.3 %). Then, the red layer due to *P. rubescens* moved downwards to 5-10 m where it remained during the summer. The chlorophyll *a* values rapidly decreased and mainly occurred at 5-10 m (mean Chl *a*, 11.0±5.3 µg l^{-1}) (Fig. 2). Below 10 m the water was relatively clear.

In spring 2001 the bloom was more intensive than the year before and lasted for a longer period (April to June). By the end of May the bloom reached a peak, which corresponded to a sharp increase in chlorophyll *a* (mean Chl *a*, 153.7±34.6 µg l^{-1}) (Fig. 1 and 2), ammonia (mean NH_4^+, 1.1±0.2 mg l^{-1}) and nitrite (mean NO_2^-, 0.003±0.001 mg l^{-1}) concentrations, as well as to a drop in dissolved oxygen which reached levels of 0 mg l^{-1} (Fig. 3). In addition, a substantial increase of surface water temperature was detected both years in the reservoir during the bloom (Figs. 3 and 4).

The data on water quality showed an increment of microcystin levels in the surface of the reservoir just after the beginning of the bloom in 2000 (Fig. 1). During the spring of 2001, MCYST concentrations in the surface were fluctuating and reached peaks above 1µg l^{-1}, whereas in 2000 MCYST were below this value except for the last 15 days of May.

The HPLC-mass spectrometer results indicated that only one sample of fish liver from the reservoir contained low amounts of molecules with molecular weight typical for the microcystins (mw 1024). The rest of samples did not contain microcystins of that mass. If at all, there were only very low concentrations of microcystins in the liver extracts from El Atazar.

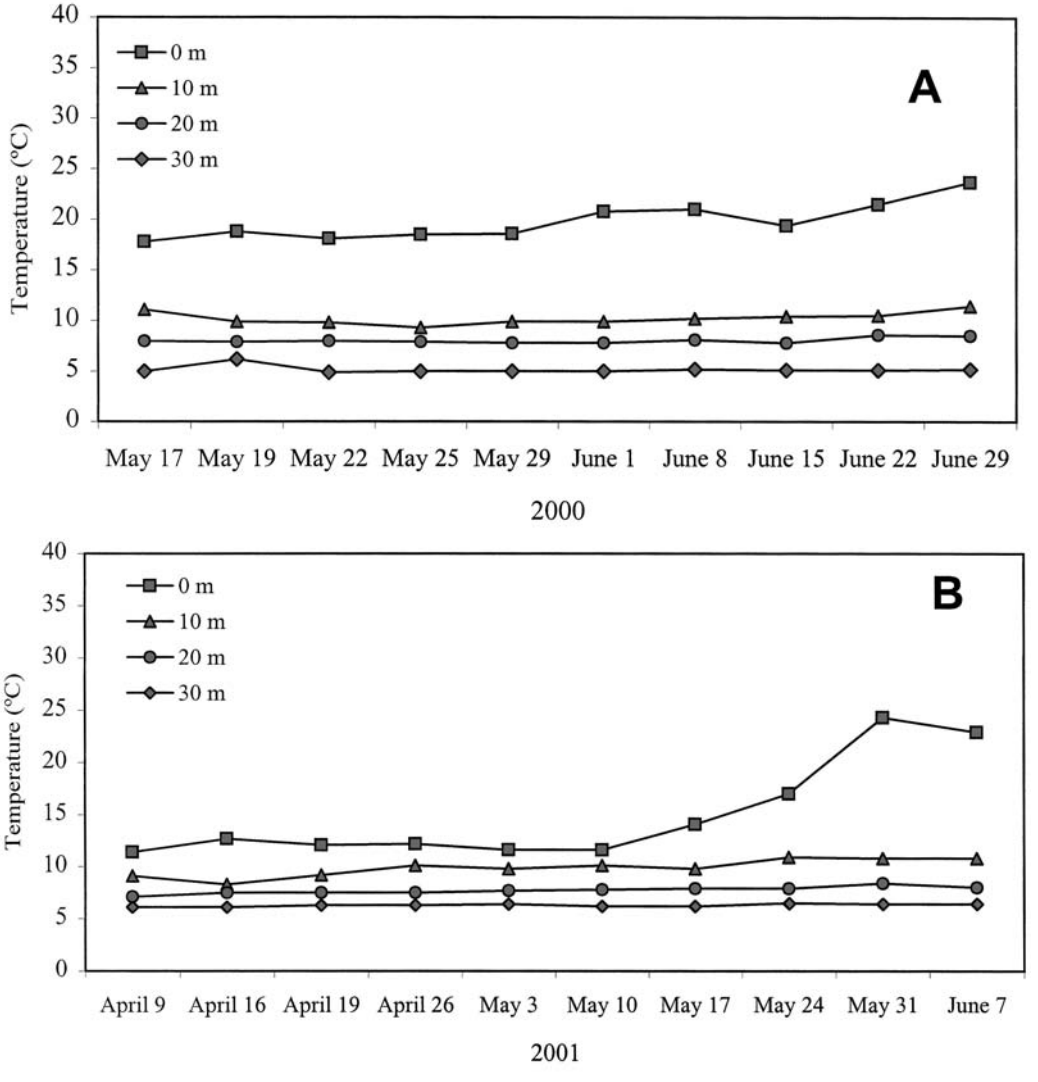

Figure 4. Vertical distribution of temperature (ºC) in El Atazar reservoir during the spring of 2000 and 2001.*Distribución vertical de la temperatura (ºC) en el embalse de El Atazar durante la primavera de 2000 y 2001.*

Liver sections revealed that 93% of the fish affected by the bloom had pigmented macrophage aggregates (MAs), which were not observed in the liver of control fish (Fig. 5). The mean number of MAs per specimen showed a large variability among samples, ranging from 0 to 131.2 (mean, 29.6±33.0) (Table 1). The distribution of MAs within the liver tissue was highly heterogeneous. Thus, the mean coefficient of variation (CV, %) within each sample ranged between 14 and 88% (mean, 38.2±20.8) (Table 1). No other histopathological abnormalities were found in the liver.

Table 1. Average number (± SD), range and coefficient of variation (CV, %) of pigmented macrophage aggregates (MAs) per individual found within the histological sections of liver from fish captured in El Atazar reservoir during the 2001 bloom. *Número medio (± SD), rango y coeficiente de variación (CV, %) de centros melanomacrofágicos (MAs) por individuo encontrado en las preparaciones histológicas de tejido hepático de peces capturados en el embalse de El Atazar durante la proliferación de 2001.*

Mean ± SD	Range	CV (%)
1.2 ± 1.0	0-3	88.3
1.5 ± 1.3	0-3	87.6
4.3 ± 2.2	2-7	51.4
9.9 ± 4.9	8-18	49.1
11.7 ± 4.8	9-16	41.2
11.8 ± 6.0	3-18	50.8
12.0 ± 8.8	9-15	73.0
14.8 ± 5.3	10-23	35.9
15.2 ± 4.6	9-21	30.5
16.0 ± 7.8	12-20	48.7
19.4 ± 5.0	17-26	25.9
20.0 ± 7.4	13-27	36.9
20.4 ± 12.6	11-31	61.7
20.7 ± 6.2	14-27	29.8
21.2 ± 4.0	18-24	18.8
23.5 ± 9.1	12-31	38.7
23.8 ± 9.4	17-33	39.7
25.3 ± 6.3	13-30	24.9
26.9 ± 5.5	22-34	20.5
31.5 ± 6.9	25-39	21.9
52.4 ± 7.5	48-58	14.4
56.5 ± 12.7	46-70	22.5
59.6 ± 9.6	46-70	16.1
82.4 ± 18.5	53-91	22.5
116.9 ± 21.7	101-137	18.6
131.2 ± 32.8	100-161	25.0

DISCUSSION

The likely cause of *P. rubescens* blooms in El Atazar reservoir seemed to be the exceptional weather conditions during 2000 and 2001. First, the rapid increase of temperature during spring resulted in the reservoir stratification, which occurred just before the blooms. On the other hand, the increment of nutrients in the epilimnion caused by run-off during spring rainfall could have favoured cyanobacterial growth. The spring of 2001 was exceptionally rainy, which probably increased the nutrient levels of the surface layers in the reservoir and lead to the observed long-lasting bloom.

The occurrence of cyanobacterial blooms in two consecutive years suggests that *P. rubescens* remains latent at low densities until the conditions which favour its growth are present. At first, the cyanobacterial population probably displayed a rapid growth until it reached a threshold of maximum biomass, when nutrients presumably started to impoverish. As a result, massive death of cyanobacteria occurred, producing large amounts of dead organic matter and high MCYST concentrations in the reservoir. Also, the increment of suspended organic matter likely caused a sharp drop of dissolved oxygen and an increase of turbidity.

The accumulation and persistence of microcystins in fish liver during cyanobacterial bloom conditions have been recently reported by Freitas de Magalhaes *et al.* (2001). However, microcystins were not clearly found in the liver tissue of fish captured in the reservoir. This finding should not be considered as definitive since liver pathologies were detected in the fish studied. Thus, previous studies where fish were exposed to microcystins showed evidence of toxin accumulation and tissue damage in the liver (Phillips *et al*., 1985; Rahbergh *et al*., 1991; Tencalla *et al*., 1994; Carbis *et al*., 1996; Williams *et al*., 1997a; Fischer & Dietrich, 2000). Freitas de Magalhaes *et al.* (2001) suggested that the method used in the present study for toxin extraction clearly underestimates microcystins concentration in the liver. Likewise, Williams *et al.* (1997a,b) found that only 24% of the total microcystins from fish liver was extractable with methanol. On the other hand, several studies have shown that microcystins can be excreted rapidly (Eriksson *et al.*, 1989; Robinson *et al.*, 1991; Vasconcelos, 1995). Additionally, toxins could be metabolised to a less toxic compound through a detoxification system, as suggested by Wiegand *et al*. (1999) and Freitas de Magalhaes *et al.* (2001).

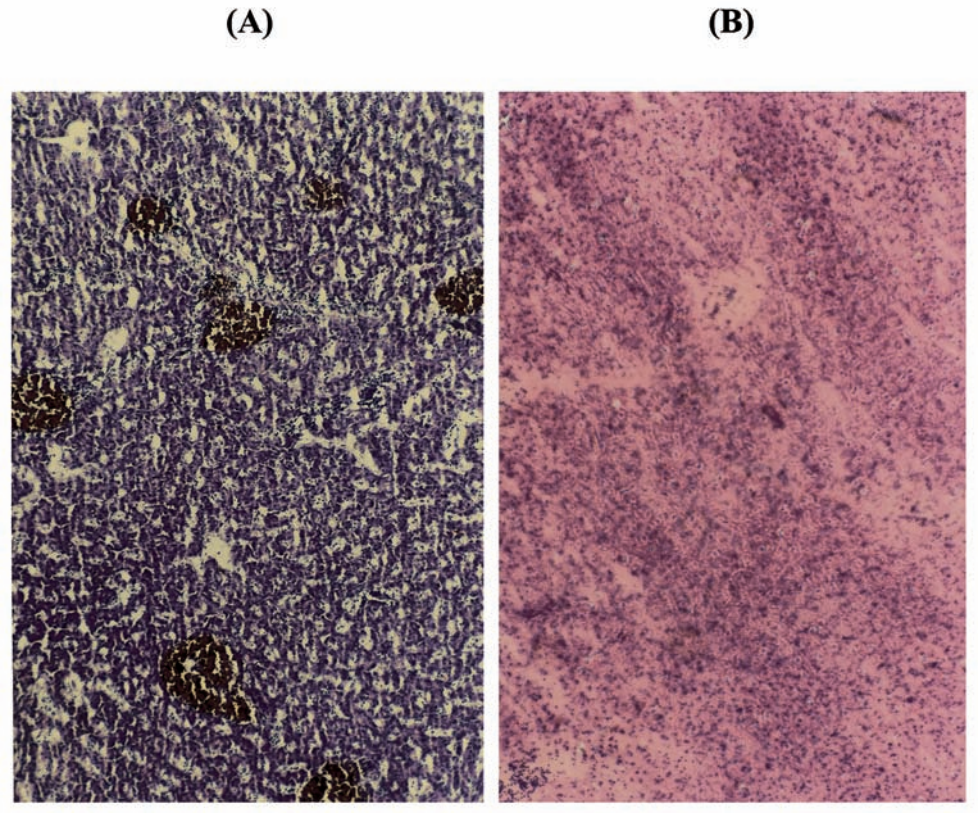

Figure 5. Histological sections of liver from fish (*Barbus bocagei*) captured in (A) El Atazar reservoir, showing several pigmented macrophage aggregates, and (B) River Madarquillos (reference area), showing a normal liver of control fish (H&E, x10). *Preparaciones histológicas de tejido hepático de peces (*Barbus bocagei*) capturados en (A) Embalse de El Atazar, donde se observan varios centros melanomacrofágicos, y (B) Río Madarquillos (área de referencia), donde se observa un hígado normal de peces utilizados como control (H&E, x10).*

The liver pathology found in the present study has similarities to the hepatic changes observed in fish from contaminated sites elsewhere (Ericson *et al*., 1999; Davis *et al*., 1999; Marty *et al*., 1999). Hence, the presence of pigmented macrophage aggregates in liver tissue samples from fish captured in the reservoir indicates that the hepatic pathology may be resulted from the direct ingestion or absorption of hepatotoxins from the water. The macrophage aggregates are usually found in kidney and spleen of teleost fish, but they can also appear in the liver as a result of certain pathologies (Wolke, 1992; A. Zapata, personal communication). The general function of MAs is the centralisation of foreign materials and cellular debris for destruction, detoxification or recycling (Vogelbein *et al.*, 1987; Wolke, 1992; Zapata *et al.*, 1996). Moreover, MAs are repositories of pigments that likely reflect previous tissue injury with accumulation of membrane breakdown products (Davis *et al*., 1999). Many factors are known to affect the accumulation and/or proliferation of MAs, including nutritional status (Agius & Roberts, 1981) and infectious diseases (Herráez & Zapata, 1987; Vogelbein *et al*., 1987). Further, MAs are known to change in number, size and pigment content in relation to fish health and environmental degradation (Wolke, 1992; Fournie *et al*., 2001). Therefore, some authors (Wolke, 1992; Fournie *et al*., 2001) have indicated that MAs can be used as histopathological

bioindicators to monitor fish health and environmental quality. Moreover, the use of MAs as environmental biomarkers has been extensively documented during the last decades (e.g. Barker *et al*., 1994; Blazer *et al*., 1987,1994; Khan *et al*., 1994; Couillard & Hodson, 1996).

In conclusion, the present study has shown that fish affected by the cyanobacterial bloom exhibited a liver pathology probably due to exposure to hepatotoxins. However, further research using electron microscopy techniques is needed to identify the accumulated pigments within the MAs. This could give an insight into the pathogenesis for the cyanobacteria-associated liver pathology. Additionally, alternative extraction methods are needed to improve the detection and quantification of toxins in the liver tissue, although an efficient liver detoxification can reduce the accumulated amount.

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