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# The presence of microcystins in fish *Cyprinus carpio* tissues: a histopathological study

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## Abstract

The occurrence of heavy cyanobacterial blooms has become a worldwide problem, as a consequence of eutrophication of the aquatic ecosystems; furthermore, 60% to 75% of these blooms have been found to be toxic. Microcystins (MCYSTs), the predominant toxins of cyanobacterial blooms, are associated with mortality and illness in both animals and humans. Laboratory-controlled experiments studying the effects of different microcystins on the common carp (*Cyprinus carpio*) have revealed various histopathological alterations. The aim of the present study is to investigate the effect of chronic or subchronic exposure of fish to microcystins under natural environmental conditions by examining the possible histopathological changes associated with a dense cyanobacterial bloom and determining the microcystin contents of fish tissues. Common carps (*C. carpio*) were caught from Lake Karla (Greece), during a dense cyanobacterial bloom. The concentration of MCYSTs in the fish liver, kidney and muscle tissues was measured by enzyme-linked immunosorbent assay. The pseudogaster contents were analysed, and a histopathological examination was performed using light and electron microscopy. Severe alterations were detected in the liver and the kidney, suggesting that the toxic effects were caused by various pollutants that were particularly associated with microcystins. The histopathological findings are also discussed, taking into consideration the health conditions of the common carp as a commercial fish species. The mechanisms of expansion of the microcystins and the poisoning of aquatic organisms (e.g. fish) are not yet known in the Lake Karla ecosystem. Future research may focus on identifying the changes caused by microcystins and other factors that exert similar effects on fish tissues, as well as on establishing the overall combined effect of all these factors on fish health.

**Keywords:** Cyanobacterial bloom, Liver, Kidney, Muscle, Tissues, *Cyprinus carpio*, Lake Karla (Greece)

## Background

The frequent occurrence of cyanobacterial blooms has been regarded as a serious global public health problem and a major environmental issue. Cyanobacteria can produce several toxic metabolites known as cyanotoxins (Ressom et al. 1994; Blaha et al. 2009). Many species of cyanobacteria produce toxic metabolites, peptides and alkaloids that are a serious threat to the various uses of freshwater lakes and reservoirs. Toxin-producing cyanobacteria that are widely distributed in freshwaters include the planktonic N<sub>2</sub>-fixing genera of *Anabaena*, *Aphanizomenon*, *Nostoc*, *Cylindrospermopsis* and

the non N<sub>2</sub>-fixing genera, such as *Microcystis*, *Planktothrix* and *Oscillatoria* (Welker et al. 2004). Among these cyanobacteria, microcystins (MCYSTs) are the most abundant group. Exposure to MCYSTs imposes a health risk on aquatic organisms, wild life, domestic animals and humans through ingesting the cyanobacteria present in the water or the food (Duy et al. 2000; Malbrouck and Kestemont 2006).

Fish, a group of organisms that represents one of the main inhabitants of the aquatic systems, are frequently exposed to MCYSTs both directly and passively, leading to their poisoning and/or their subsequent mortality (Carbis et al. 1996; Ernst 2008).

Fish typically either ingest cyanobacteria or prey that has already fed on cyanobacteria (Tencalla et al. 1994; Fisher and Dietrich 2000). To a lesser extent, they can also absorb the toxins directly from the water (Phillips et al. 1985; Smith et al. 2008). Currently, there is a large body of literature describing experimental studies of cyanobacterial toxins in aquatic systems and their acute toxicity effects; however, there are gaps about the toxicological effects of these toxins under field conditions in environmentally relevant concentrations. Although acute toxicity experiments are useful in the study of toxicokinetics and histopathological changes, such experiments do not represent the mode of exposure in a natural environment (Ferrão-Filho and Kozlowsky-Suzuki 2011). Because the chronic and subchronic effects in aquatic organisms might be more relevant than the acute lethal effects, studies addressing the exposure of biota in cyanobacteria-rich lakes have attracted substantial attention (Ibelings and Chorus 2007; Ibelings and Havens 2008).

It is well known that one cyano-toxicogenic mechanism is the potent inhibition of the protein phosphatases 1 and 2A; this inhibition results in the abrogation of the protein phosphorylation status, which is directly associated with the cytotoxic effects and tumour-promoting activity of cyanotoxins (Carmichael 1994; Hooser 2000). In addition, studies of fish pathology have shown that MCYST exposure can produce histopathological effects in the gills, intestine and heart (Atencio et al. 2008; Jiang et al. 2011). Hepatic tumours and severe hepatic haemorrhages, as well as the disruption of the hepatic cytoskeleton and the subsequent, progressive liver necrosis and apoptosis, have been widely reported in fish (Tencalla et al. 1994; Fischer et al. 2000). The degree of these MCYST-induced effects depends on the exposure route. However, most of the studies on MCYSTs have been performed using an IP injection or an oral uptake as the exposure route.

*Cyprinus carpio* (common carp) is a widespread species found in various freshwater habitats, including almost all freshwater Greek lakes. The cyano-toxicological effects on the common carp are thought to be quite important given its commercial importance as a product of inland fisheries and aquaculture. Planktivorous fish, such as the carp, intentionally ingest cyanobacteria and, in the process, are also exposed to cyanobacterial metabolites through the aquatic food web (Malbrouck and Kestemont 2006).

Although these species are frequently exposed to cyanotoxins in their natural environment, they are also considered to be among the most tolerant in terms of survival. No evidence exists regarding the sublethal effects of cyanotoxins, including pathological alterations. Studies on endemic fish species in Europe, Australia and USA have shown that fish liver histopathology is a useful biomarker of exposure to various contaminants (Au 2004; Feist et al. 2004).

The primary aim of each of these studies was to identify histological changes in selected target organs of bio-indicator fish species with regard to one or more specific contaminants. Regarding cyanotoxins, in most cases, the liver and renal histological

alterations were highly important (Fisher and Dietrich 2000; Molina et al. 2005; Lang et al. 2006, Li et al. 2007a; Gutierrez-Praena et al. 2011).

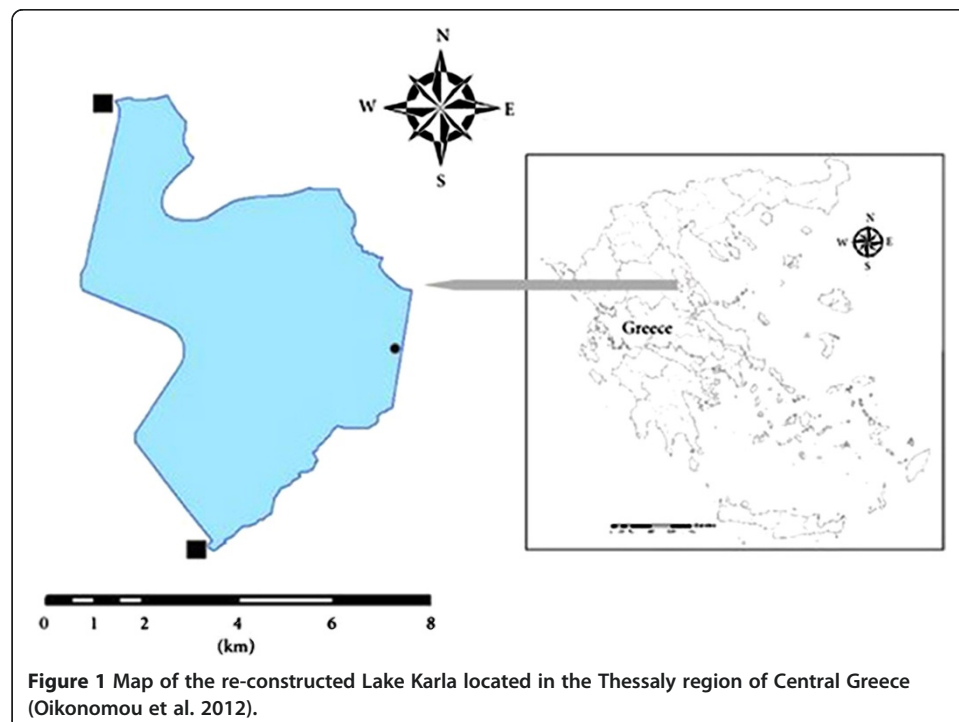
In this context, the purpose of the present study is to assess the occurrence and distribution of MCYST in the tissues of the fish species *C. carpio*, as well as to evaluate the histopathological alterations under cyanobacteria-rich conditions. The histopathological findings were revealed by light and electron microscopy. To our knowledge, this is the first study evaluating the histopathology of a freshwater biomarker under natural conditions to provide information about the public health issue arising from human consumption.

Lake Karla (Central Greece) is a unique example (in Europe) of a shallow lake ecosystem that was dried in the 1960s and has been reconstructed, establishing a 'new' ecosystem. During the past 2 years of the lake's rehabilitation (2009 to 2011), frequent and extensive cyanobacterial blooms have occurred that were dominated by toxin-producing species (Oikonomou et al. 2012). During the same period, many episodes of fish mortality were also reported. These events have received intense media coverage due to the lake's importance as a major water reservoir in Greece. Fishing is also among the traditional economic activities in the area, and the common carp is considered to be the main commercial fish species.

## Methods

### Lake Karla: limnological features of the study area

Lake Karla occupied the lowest part of the Thessaly plain and was considered to be one of the most important wetlands in Greece until 1962. Surface runoff from the watershed and the floodwaters of the Pinios River (discharging via a constructed ditch) supplied the lake with large quantities of freshwater. In 1962, the lake was completely drained to create more land for agriculture. The reconstructed Lake Karla is located on the lower depression of the Thessaly plain, a region of Central Greece (Figure 1). The



**Figure 1** Map of the re-constructed Lake Karla located in the Thessaly region of Central Greece (Oikonomou et al. 2012).

lake lies between latitude 39°26'49" and 39°32'03" N and longitude 22°46'47" and 23°51'50" E and has a surface area of 38 km<sup>2</sup> with a perimeter of 228 km. The hydrological regime of the lake is determined by the inputs (the rainfall on the lake and the tributary inflows) and the outputs (evaporation). The lake has no natural outflow because the constructed tunnel draining into the Pagasitikos bay is currently closed. Considering the present environmental conditions, Papadimitriou et al. (2011) reported on the water chemistry, and an in-depth analysis of the water column and sediment interactions in Lake Karla has also been published by Jouni (2011). Both studies reported on the highly eutrophic conditions of the lake and highlighted its nutrient-rich content and high chlorophyll-a values. Moreover, there was also an evidence of phosphorous mobilisation from the sediment to the water column. In the same period, Oikonomou et al. (2012) confirmed the occurrence and dominance of toxic cyanobacteria species of *Anabaenopsis* and *Planktothrix*, as well as a diverse microbial community indicative of a hypertrophic status.

#### **Fish collection and MCYST detection**

Thirty specimens of *C. carpio* with a mean body weight and a mean body length of 1,032 ± 121 g and 30 ± 4.2 cm, respectively, were collected from Lake Karla via two surveys during a period of dense cyanobacterial bloom (May and June 2011) using a trammel net that has a 60-mm inner and 300-mm outer mesh size. Ten *C. carpio* fish were also obtained from a fish farm (Hatchery station of river Louros-Ioannina, Hellenic Ministry of Agriculture) and were used as controls. To extract the toxins from the fish organs, the fish were sacrificed, and the liver, kidney and muscle tissues were excised, weighed and immediately frozen. Subsequently, all of the tissues were homogenised separately, extracted in 100% methanol (Magalhães et al. 2001), stirred overnight at room temperature and then centrifuged at 1,300 g for 15 min using a Universal 32 centrifuge (DJB Labcare Ltd., Buckinghamshire, England, UK). The supernatants were collected and stored overnight at 4°C. A 5-ml aliquot of each supernatant was concentrated under nitrogen stream to 350 µl to remove the organic solvent. A 100-µl aliquot of the concentrated sample extract was diluted with 900 µl of distilled water according to Sipia et al. (2002). The final sample was clarified using membrane filters (pore size of 0.45 µm and diameter of 4 mm). The sample solutions were immediately analysed by enzyme-linked immunosorbent assay (ELISA). For each ELISA assay, the negative control and the four standards were assayed at least in duplicate. The results are expressed in nanogrammes of MCYST-LR equivalents per gramme of fish tissue.

MCYSTs were also measured in the water of Lake Karla. Two forms of MCYSTs were investigated: dissolved in water (extracellular) and cell-bound in seston (intracellular). For the latter, 1,000 mL of water was filtered on a Whatman GF/C filter (Sigma Aldrich, St. Louis, MO, USA) which was immediately frozen at -20°C. MCYSTs were extracted from the filter after placement in 100% methanol and stirring overnight at room temperature followed by centrifugation at 1,300×g for 15 min. This extraction procedure was repeated three times, and the three supernatants were pooled. The organic solvent was removed by placing the extract under nitrogen stream. The remaining concentrated sample was subjected to ELISA. The results are

expressed as microgrammes of cellular MCYST-LR equivalents per litre. For analysis of dissolved MCYSTs, the filtered water was applied directly to ELISA. A commercial Abraxis Microcystin ELISA kit was used (Warminster, PA, USA) following the instructions of the manufacturer.

The recovery of the method was determined by analysing the samples before and after the addition of the pure MCYST and then subtracting the concentration of the MCYSTs present in the sample prior to spiking. The matrix effect (i.e. the effect of animal tissue) was checked by spiking the control tissues with the MCYST-LR standard (2 µg/g). The response was compared to that of the 100% methanol spiked with the same amount of the standard.

### **Microscopic analyses**

Histological samples from the excised tissues, including the liver and the kidney, were subjected to light and electron microscopy. For light microscopy, the samples were initially fixed in a 10% formalin buffer for 24 h at 4°C and were then immediately dehydrated in a graded series of ethanol, immersed in xylene and embedded in paraffin wax using an automatic processor. Sections of 5 to 7 µm were then mounted. After deparaffinisation, the sections were rehydrated, stained with hematoxylin and eosin (Humason 1972) and mounted with Cristal/Mount (Sigma Aldrich, St. Louis, MO, USA). Subsequently, all of the tissues were examined microscopically, and their histological abnormalities were recorded.

For electron microscopy, tissue specimens were prefixed in 2.5% glutaraldehyde diluted in a 0.1-M sodium cacodylate solution for 24 h at 4°C. The specimens were washed in the same buffer before and after fixation. Post-fixation was performed with 1% osmium tetroxide in 0.1-M sodium cacodylate for 2 h at 4°C. The specimens were washed in the same buffer before and after post-fixation and then rinsed in distilled water. Then, the specimens were dehydrated in a graded ethanol series and were subsequently immersed and left overnight in a 1:1 mixture of propylene oxide and the embedding resin. The final step of the embedding took place in capsules containing agar resin. Polymerisation of the resin was completed after 48 h at 60°C. Ultrathin sections (60 to 80 nm) were cut with a Reichert Supernova ultramicrotome (DeKalb, IL, USA). The sections were mounted on a copper grid and stained with uranyl acetate and lead citrate. The tissue sections were examined in a Philips CM10 electron microscope (Amsterdam, The Netherlands).

The common carps are stomachless fish. The short oesophagus connects the posterior pharynx to the anterior part of the intestine, the pseudogaster. Microscopic analysis of the pseudogaster contents was also performed on the examined fish specimens. The pseudogaster was removed from fresh carps, and samples of its contents were collected and kept at 1°C to 5°C until further analysis. Two aliquots of each sample were taken, and one of the aliquots was diluted in distilled water. Dilution was performed in a glass test tube by mixing 330 µl of the sample with 330 µl of distilled water. The non-diluted pseudogaster contents (330 µl) were placed on one end of the slide. The cover slip was placed at a 45° angle on top of the sample to avoid creating air bubbles. Similarly, 330 µl of the diluted pseudogaster contents was placed on the other end of the slide, and optical microscopy images were obtained from both sides of the slides.

## Statistics

The data are expressed as the means  $\pm$  SD. A two-way analysis of variance (ANOVA) was used to compare the means of the MCYSTs contents between the tissues and the sampling periods. The results were considered statistically significant when  $P < 0.05$ . When the ANOVA results indicated a significant effect, Tukey's multiple comparison test was used to compare the differences between the means. All statistical analyses were performed with SPSS vers. 20.0 for Windows (Chicago, IL, USA).

## Results

### MCYST in the fish tissues and lake water

All fish samples of *C. carpio* examined during the present study contained MCYSTs (Table 1). According to the statistical analysis, there were significant differences between the tissues ( $n = 20$ ,  $F = 12.53$ ,  $P < 0.05$ ). However, there were no significant differences in MCYST concentration between the sampling periods ( $n = 20$ ,  $F = 0.05$ ,  $P > 0.05$ ). The highest MCYST concentration was found in the liver ( $732 \pm 350$  ng/g), followed by the kidney ( $362 \pm 207$  ng/g), while the lowest concentrations were detected in the muscle tissues ( $114 \pm 25$  ng/g) (Table 1). During the second survey, in June, the kidney had a higher concentration of MCYST than the liver tissues ( $696 \pm 258$  and  $346 \pm 156$  ng/g, respectively); again, the lowest MCYST values were found in the muscle tissues ( $108 \pm 33$  ng/l) (Table 1). The examined tissues of the control *C. carpio* individuals contained no MCYSTs when examined by ELISA.

The extracellular MCYST values were  $2.03 \pm 0.32$   $\mu$ g/l and  $3.01 \pm 0.41$   $\mu$ g/l for May and June, respectively. The respective intracellular MCYST values were  $4.19 \pm 0.33$   $\mu$ g/l and  $5.5 \pm 0.29$   $\mu$ g/l (Table 1) for the same time periods as above.

The recovery of MCYST that we obtained from the spiked samples was  $77 \pm 2.5\%$  for liver,  $73 \pm 3.2\%$  for kidney and  $72.6 \pm 2.12\%$  for muscle. The matrix effect was negligible (from 0.08% to 5.2% of the differences between the matrix and the methanol results, with an average of  $1.9 \pm 0.7\%$ ).

### Mortality and macroscopic observations

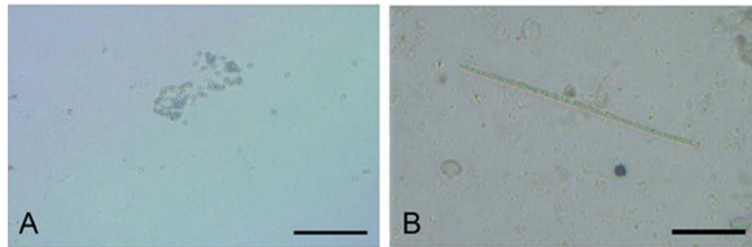
No fish mortalities were observed during the studied period, and the appearance of the examined organisms was macroscopically normal. All of the examined samples (100%) of the fish pseudogaster contents contained various cyanobacterial species, with the dominant species being *Microcystis aeruginosa* and *Planktothrix agarrdii* (Figure 2). While examining the test organs, macroscopic lesions were observed in the liver and

**Table 1 MCYST concentrations in *C. carpio* tissues (ng/g), extracellular MCYST( $\mu$ g/l) and intracellular MCYST ( $\mu$ g/l) in May and June 2011**

	May 2011	June 2011
<i>C. carpio</i> liver	732 $\pm$ 350	346 $\pm$ 156
<i>C. carpio</i> kidney	362 $\pm$ 207	696 $\pm$ 258
<i>C. carpio</i> muscle	114 $\pm$ 25	108 $\pm$ 33
Extracellular	2.03 $\pm$ 0.32	3.01 $\pm$ 0.41
Intracellular	4.19 $\pm$ 0.33	5.5 $\pm$ 0.29

Values are given as mean  $\pm$  SD. In all of the control *C. carpio* individuals' examined tissues, there were zero levels of MCYST.



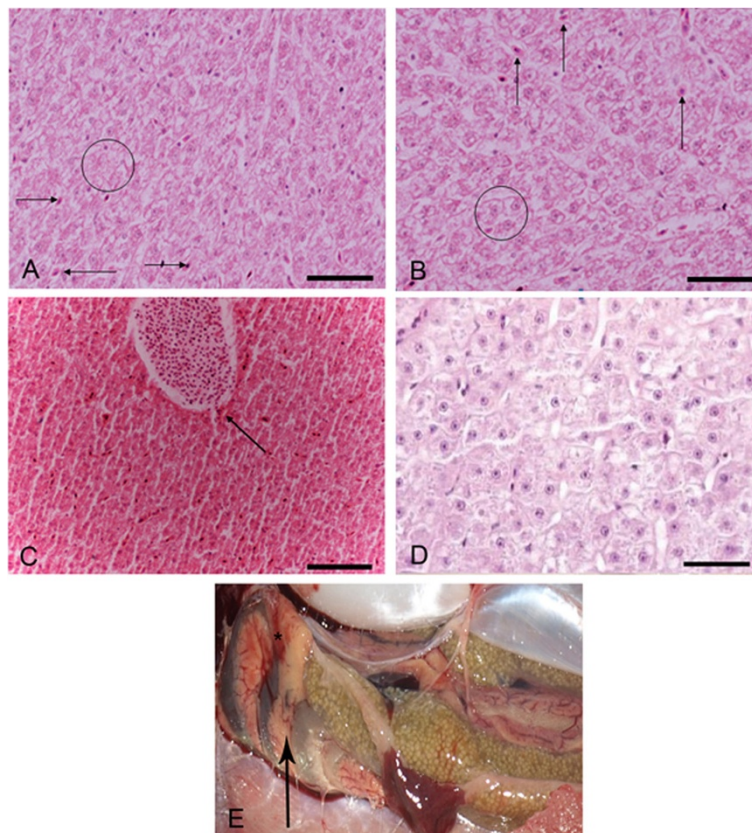


**Figure 2** Microscopic examination of *Cyprinus carpio* stomachic contents. (A) *Microcystis aeruginosa* and (B) *Planktothrix agardii*. Bar = 25  $\mu$ m.

the kidney. Morphological changes included liver discoloration and brittleness, as well as ecchymoses and petechiae (Figure 3). The kidney appeared brittle and normal in colour. Neither the organ displayed appeared enlarged. No pathological changes were observed in the control samples.

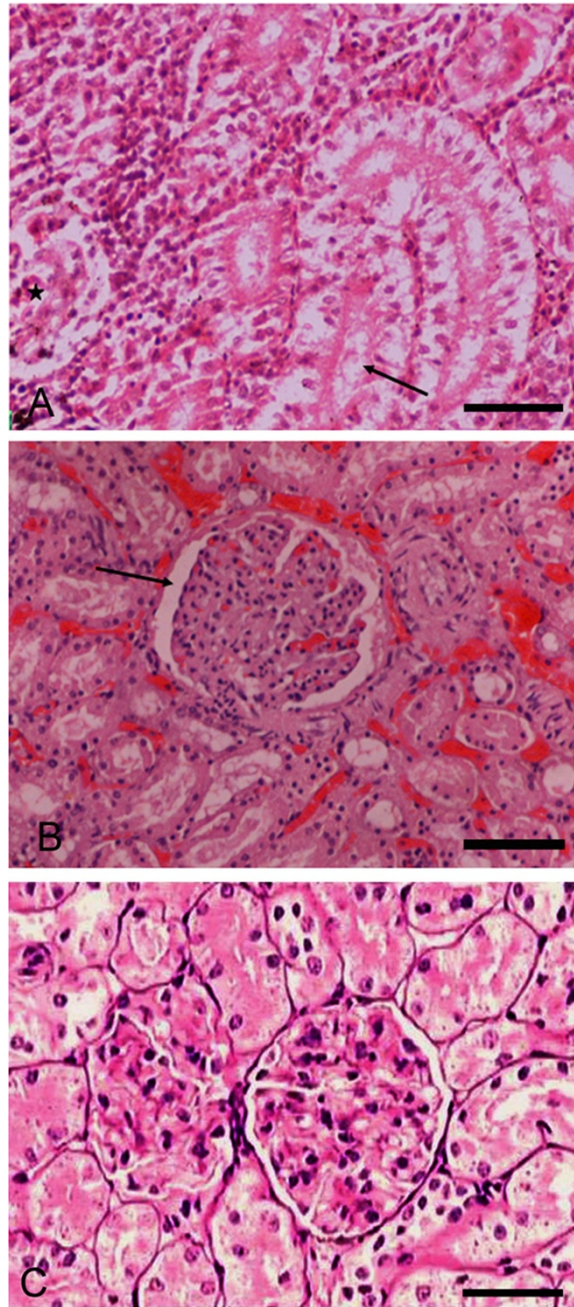
#### Histopathology under light microscopy

Histopathological changes were observed in the liver and the kidney of the fish specimens in comparison with the control ones. However, no differences were observed



**Figure 3** *Cyprinus caprio* liver. (A) Hydropic degeneration, granular glycogen (circle) and sporadic haemorrhagic symptoms (arrows). (B) Onion-like cells (circle) and sporadic haemorrhagic symptoms (arrows). (C) Capillary fracture (arrow). (D) Control, there are no histopathological degenerations. Bar=62.5  $\mu$ m. (E) Macroscopic degenerations of the liver. Discoloration (arrow) and petechiae (star) were observed.

among the sampling surveys. Histopathological lesions were detected in the liver and the kidney and were characterised by hydropic degeneration and necrosis. Pathological changes in the fish liver included loss of the architecture structure, onion-like cells, focal necrosis, granular glycogen, nuclei pyknosis (Figure 3) and sporadic haemorrhagic symptoms. The kidney pathology was characterised by the degeneration of the renal tubule, glomerulopathy, glomerular atrophy and a dilated Bowman's capsule (Figure 4). No pathological changes were observed in the muscles.



**Figure 4** *Cyprinus carpio* kidney. (A) Degeneration of the renal tubular (arrow) and glomerular atrophy (star). Bar = 62.5  $\mu$ m. (B) Glomerulopathy and dilation of Bowman's capsule (arrow). (C) Control, there are no histopathological degenerations. Bar = 43.48  $\mu$ m.

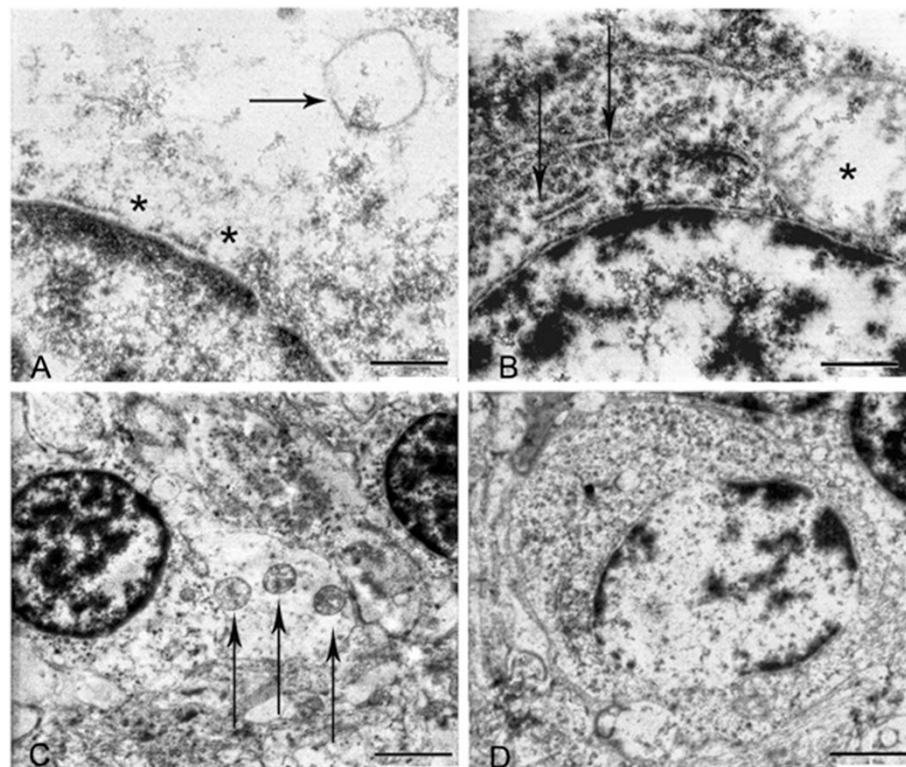


### Ultrastructural histopathological observations

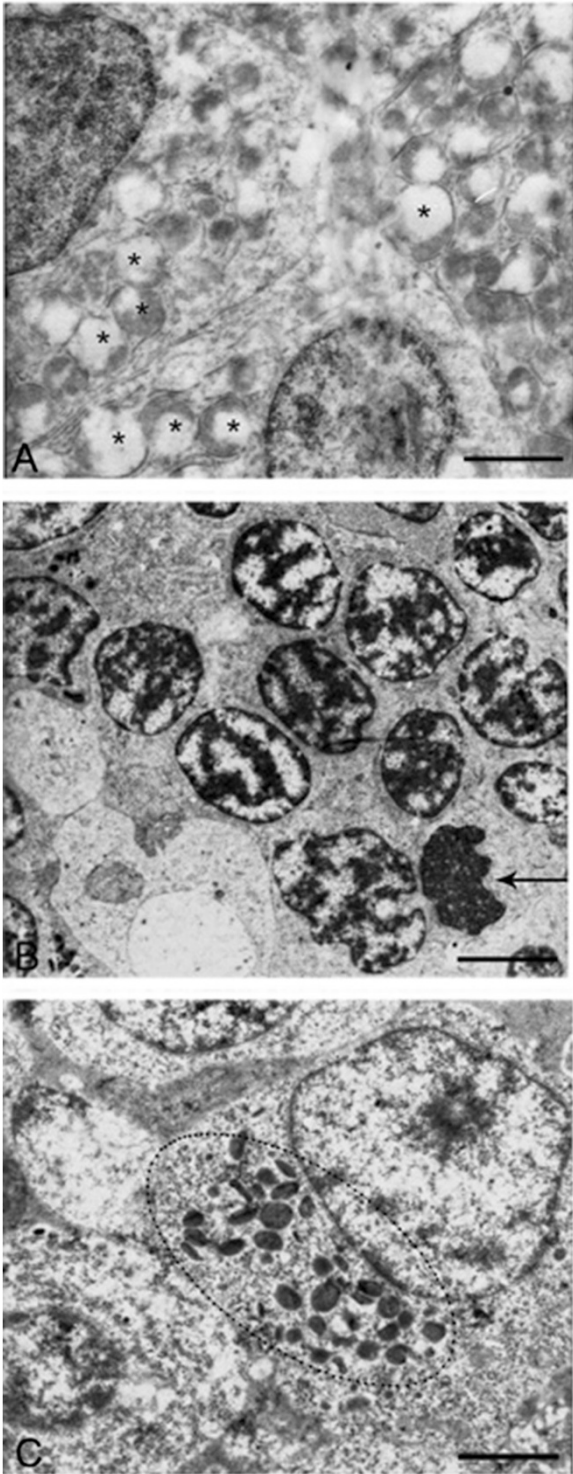
Histopathological alterations were observed in the liver and the kidney of all fish samples except for the control ones. Both organs appeared swollen, with a loss of the parenchymal architecture. An apparent lysis of the membranes of the hepatic cells resulting in necrotic cells was observed, along with vacuolisation and the presence of lipid droplets. The fragmentation of the nuclear envelope, the swelling of mitochondria, the fragmentation and vesicularisation of the endomembrane system, the pyknosis of nuclei and karyorrhexis were also observed in the liver (Figure 5). Nuclear pyknosis, nuclear envelope fragmentation and proliferation of the lysosomes were observed in the kidney (Figure 6).

### Discussion

The only study before Lake Karla dried out was by Ananiadis (1956). He reported signs of eutrophication and occurrence of few cyanobacterial species. Our data concerning lake water, suggest that the re-constructed Lake Karla is experiencing the occurrence of significant MCYST concentrations. Papadimitriou et al. (2011) previously reported the presence of MCYST concentrations in Lake Karla ranging between 0.75 and 3.90  $\mu\text{g/l}$ , while Oikonomou et al. (2012) and Papadimitriou et al. (2013) confirmed the occurrence of the toxic cyanobacterial species of *Anabaenopsis*, *Planktothrix* and *Microcystis*



**Figure 5** Ultrastructural observation of *Cyprinus carpio*'s liver tissue. (A) Nuclear envelop fragmentation (stars) and a swelling mitochondrion (arrow). Bar = 0.37  $\mu\text{m}$ . (B) Fragmentation of endoplasmic reticulum (arrows) and mitochondria internal membrane system (star). Bar = 0.60  $\mu\text{m}$ . (C) Lysis of the hepatic cell's plasma membrane. Mitochondria (arrows) in the extracellular space. Bar = 1.74  $\mu\text{m}$ . (D) Karyorrhexis. Bar = 1.74  $\mu\text{m}$ .



**Figure 6** Ultrastructural observation of *Cyprinus carpio*'s kidney tissue. (A) Lipid droplets (stars) are present. Bar = 1.74  $\mu\text{m}$ . (B) Pyknotic nuclei (arrow). Bar = 3.33  $\mu\text{m}$ . (C) Proliferation of lysosomes in the cell (circle). Bar = 1.74  $\mu\text{m}$ .

in Lake Karla. The above cyanobacterial species found in Lake Karla have been shown to produce a variety of toxins and alkaloids with toxic activity in many lakes worldwide (Sivonen and Jones 1999; Sabour et al. 2005; Kurmayer et al. 2005; Hisem 2008). MCYST concentrations (intracellular and extracellular) found in Lake Karla are similar to those reported for other Mediterranean lakes in Turkey (Albay et al. 2003) and in Portugal (Vasconcelos et al. 1996), but generally, lower to those of the temperate lakes in Canada (Kotak et al. 1996) or in Finnish lakes (Lindholm et al. 2003) and also lower than those reported for Brazil lagoons (Magalhães et al. 2001). Extracellular and intracellular MCYST concentrations found in Lake Karla are comparable to those found in another shallow lake in Greece, Lake Pamvotis (Papadimitriou et al. 2012a). It is worth noting that the comparable MCYST concentrations found in Lake Pamvotis, were associated with the bioaccumulation of MCYST in tissues of aquatic organisms (Papadimitriou et al. 2012a).

Studies addressing the chronic effects resulting from the exposure of cyprinids to sublethal concentrations of MCYSTs are quite limited. Our study focuses on the histopathological alterations observed in the *C. carpio* species found in the eutrophic Lake Karla, thus addressing the effects of the environmentally relevant MCYST concentrations. This study revealed that tissues from all fish samples caught during the sampling surveys contained MCYSTs. This result suggests that the MCYST content in the aquatic environment was adequate to promote MCYST accumulation in the exposed fish. It is well documented that the primary target organ for MCYST accumulation is the liver (Ernst et al. 2006; Papadimitriou et al. 2012a; Moutou et al. 2012), whereas muscle usually presents the lowest MCYST content. The preferential accumulation in the liver may be attributable to the high density of the organic anionic transporters on the surface of hepatocytes, while the process known as presystematic hepatic elimination could prevent or at least minimise the distribution of MCYSTs to other parts of the body (Fischer et al. 2005). However, there is also an evidence that the carp's kidney is the first organ to be affected by MCYST in terms of pathological changes (Fisher and Dietrich 2000).

MCYSTs produced by cyanobacteria in an aquatic environment have been shown to exert adverse effects on fish. Fish are exposed to MCYSTs directly during feeding and/or passively through their continuous contact with their aquatic environment; cyanobacterial metabolites are known to be transported by the aquatic food web (Xie et al. 2004; Smith et al. 2008). Cyanotoxicity can result in two types of structural changes. One is the direct toxic effect of the pollutant, which leads to tissue degeneration and necrosis, and the other is the development of compensatory mechanisms, such as cellular hyperplasia, to address the stressor (Li et al. 2007b). The histopathological changes observed in tissues are the result of histological-level reactions of the fish; thus, histopathology represents a useful tool to assess the degree of toxicity.

According to Palikova et al. (2011), cyanobacteria are regular components of the cyprinid diet, and it appears that *M. aeruginosa* comprises a significant portion of the carp diet when a bloom formation occurs (Carbis et al. 1997). Nevertheless, carps are not able to avoid the ingestion of toxic cyanobacteria and their toxins in eutrophicated lakes (Tencalla et al. 1994). *M. aeruginosa* and *P. agarrdii* found in the pseudogasters of the examined fish were also found in the water of Lake Karla according to the studies of Papadimitriou et al. (2013). This confirms the direct exposure of fish originated

from Lake Karla to cyanobacteria and to MCYSTs through feeding. Many previous studies have demonstrated the acute toxicological effects induced by MCYSTs in fish using carps as bioassay models. Laboratory-controlled experiments using different MCYSTs, doses and routes of administration in the common carp (*C. carpio*) have shown severe histopathological effects (Rabergh et al. 1991; Fisher and Dietrich 2000; Jiang et al. 2011). Jiang et al. (2011) exposed *C. carpio* individuals to 10 µg/l MC-LR for 14 days, and the results included some liver histopathological changes, such as slight swelling of hepatic cells and partially dissolved parenchymal architecture characterised by vacuolar degeneration with damage of the nuclei (pyknosis, karyolysis). Although the dose of 10 µg/l MC-LR is higher than the MCYST concentrations found in Lake Karla, the histopathological observation found in common carps from Lake Karla are similar. This may be explained by the effect of chronic or subchronic exposure of fish to cyanobacterial blooms containing MCYSTs.

Our histopathological observations seem to be in agreement with previous reports concerning cyanobacterial toxicity (Carbis et al. 1996; Fisher and Dietrich 2000; Malbrouck and Kestemont 2006; Qui et al. 2007; Jiang et al. 2011). Concerning the liver histopathology, the fish exhibited hydropic degeneration and necrosis, in agreement with the findings reported by Rabergh et al. (1991), Snyder et al. (2002), Sugaya et al. (1990), Tencalla et al. (1994), Fisher and Dietrich (2000) and Liu et al. (2002). In contrast to mammals, only a few studies have demonstrated haemorrhagic effects due to cyanobacteria and MCYSTs in fish (Tencala and Dietrich 1997; Tencalla et al. 1994; Jiang et al. 2011). Kidney alterations included degeneration of the renal tubule, glomerulopathy and dilation of Bowman's capsule. According to Carbis et al. (1996), Fischer and Dietrich (2000) and Kotak et al. (1996), the MCYST-induced renal pathology appears to be generally restricted to the proxima in the posterior part of the kidney. Our results seem to confirm these previously published findings.

Very few *in vivo* studies have been performed regarding the toxic effects of MCYSTs on the ultrastructures of the hepatocytes in the fish, and very little knowledge is available about the kidney's ultrastructure histopathology (Li et al. 2001; Atencio et al. 2008; Trinchet et al. 2011). Our main histological findings were loss of the parenchymal architecture, hepatocyte necrosis with vacuolisation of the cytoplasm and the presence of lipid droplets. Vacuolisation has been reported in isolated hepatocytes from the common carp (Li et al. 2001) after oral exposure to MCYSTs. According to Atencio et al. (2008) and Molina et al. (2005), vacuolisation might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of release of these substances into the systemic circulation. Atencio et al. (2008) have observed glomerulopathy with thickening of the basal membrane, an increased number of lysosomes in the proxima tubules and generalised vacuolisation. We also found degenerations that were mainly associated with the irregularity of the endomembrane system, the swelling of mitochondria and the fragmentation of the mitochondria internal membrane system; these findings are more or less consistent with the observations made by Carbis et al. (1996) after exposing common carps to the MCYST.

It is important to note that the cyanobacterial bloom usually contains many different toxins that have been found to be more toxic than the individually purified component toxin(s) (Ibelings and Chorus 2007). Thus, our histopathological observations provide information on a natural model of disease based on the synergies between the toxicity



of the various toxins and the other factors, rather than on the quantities of the individual compounds. Moutou et al. (2012) studied the response of the oxidative system in the same fish species under MCYST-rich conditions. They reported that under natural conditions, the response of the GSH/GSSH system and the catalase activity may also depend on other factors besides continuous exposure.

Lang et al. (2006) suggested a categorisation system for the diagnosis of liver histological alterations in terms of the toxicological relevance of these alterations based on the findings in flatfish species. Recently, van Dyk et al. (2011) have applied this system to evaluate pollution-related histopathological changes in the catfish. These categories include non-specific lesions, early toxicopathic non-neoplastic lesions, pre-neoplastic lesions and neoplasms. The alterations listed within each of these categories are widely accepted as useful indicators for monitoring the biological effects of contaminants on fish (Feist et al. 2004). Using the proposed categories as guidelines, it is clear that many of these alterations were also observed in *C. carpio* during the present study, such as early toxicopathic, non-neoplastic lesions, including hepatocellular necrosis, nuclei pyknosis, vacuolisation and steatosis.

In conclusion, common carps from Lake Karla that were exposed to a cyanobacterial bloom contained high amounts of MCYSTs in the examined tissues. Regarding the risk that MCYST toxicity poses to public health, the World Health Organization (World Health Organization 1998) proposes 0.04 µg MC-LR equivalent/kg per day as the tolerable daily intake for MCYSTs. The estimated daily intake for *C. carpio* samples from Lake Karla exhibited MCYST amounts above the WHO guideline (World Health Organization 1998); thus, there might be potential harmful effects on human health from the lifelong consumption of contaminated fish that cannot be overlooked.

In general, our results confirmed the hypothesis that chronic exposure to a cyanobacteria-rich environment and, thus, to MCYST can lead to pathological changes in the exposed carps. The potential ecological risk for the species *C. carpio*, which always dominates eutrophic, hypertrophic lakes and reservoirs, is also demonstrated. However, because MCYSTs almost never occur in nature as single species but rather occur as a mixture of toxins, the present toxicological profile could obviously be the result of multiple cyanotoxins that were present in the lake water and exerted a synergistic effect on the health of *C. carpio*. In our study, fish were collected during the spring and the early summer; therefore, it is unlikely that the histopathological differences identified would be related to the seasonal variations. Furthermore, we did not estimate the fish age, but all fish samples were of similar body size; thus, it is likely that all fish were of the same age class. However, taking into consideration the different accumulation rates in the different age classes (Papadimitriou et al. 2012b), there is a need for further research regarding the factors, such as seasonal variation and fish age, when histopathological observations are evaluated. Given the number of contradictory results regarding the effects of cyanotoxins in various biomarker studies, histopathological observations could be quite sensitive biomarkers that reflect the cyanotoxicity effects at the cellular level. The results of the current study may contribute to supporting the cause-effect relationship between fish pathology and the pollution levels in the freshwater environment.

Finally, studies about the effects of cyanobacteria and the accumulation of their toxins in the Mediterranean region are of great importance, as the incidence and

persistence of toxic blooms in this region are greater than those observed in temperate regions. Monitoring programmes, specifically at environmentally sensitive sites such as the protected Lake Karla, should include the establishment of baseline histopathological data for the endemic and bio-indicator fish species of the region.

## Conclusions

The results of the present study contribute to our knowledge on the accumulation of microcystins in fish tissues under algal bloom rich conditions. Carps from Lake Karla that were exposed to a cyanobacterial bloom contained high amounts of MCYSTs in the examined tissues appearing also histopathological changes.

Our results also confirmed that the liver accumulated the highest MCYST concentrations, while the lowest concentrations were detected in the muscle tissues. Histopathological findings could serve as sensitive signals concerning cyanotoxicity. In terms of public health the consumption of carps as lifelong dietary items might cause harmful effects on human health.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

AM was responsible for the sampling, analysis and laboratory handling. IK deployed the research protocol, contributed to the analysis of the data, discussion, writing, and reviewing. NP was responsible for the histopathological observations with the optical microscope and contributed in results evaluation. PB was responsible for electron microscopy and ultrastructural histopathological diagnosis, contributed to the data analysis, discussion, writing, and reviewing. EM was responsible for the fish sampling, contributed in analysing the data, discussion of the results, writing and editing. TP made the MCYST detection, contributed to sampling, statistical analysis, discussion, writing and reviewing. All authors read and approved the final manuscript.

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