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Assessment of microcystins in lake water and fish (Mugilidae, *Liza* sp.) in the largest Spanish coastal lake

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Abstract Cyanobacteria dominance and cyanotoxin production can become major threats to humans and aquatic life, especially in warm shallow lakes, which are often dominated by cyanobacteria. This study investigates the occurrence and distribution of microcystins (MCYST) in water, cell-bound and in the tissues of the commercial mugilid *Liza* sp. in the largest, coastal, Spanish Mediterranean lake (Albufera of Valencia). This is the first report concerning microcystin accumulation in tissues of mugilid fish species. Considerable amounts of microcystins were found in the water and seston, which correlated with development of *Microcystis aeruginosa* populations in the lake. The MCYST concentrations found in Lake Albufera (mean 1.7 and 17 μg/L and maximum 16 and 120 μg/L in water and seston, respectively) exceeded by one to two orders of magnitude the guideline levels proposed by the World Health Organization and were higher than that reported in other lakes of the Mediterranean zone. The presence of MCYST was found in all the fishes studied and accumulated differently among tissues of the commercial species *Liza* sp. Toxin accumulation in fish tissues showed that although the target organ for MCYST was the liver, high concentrations of microcystins were also found in other analysed tissues (liver>intestine>gills>muscle). Human tolerable daily intake for microcystins is assessed relative to the WHO guidelines, and potential toxicological risks for humans, wildlife and related ecosystems of the lake are discussed.

Keywords Cyanobacteria · *Microcystis* · Toxicology · Mediterranean · Conservation · Fish tissues

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Introduction

In many countries cyanobacterial blooms and cyanotoxins have become major water quality problems. There has been long awareness of the risk but only recently have systematic empirical data been collected together (Chorus 2001; Huisman et al. 2005). Assessments of cyanotoxin occurrence and distribution in organisms in natural lakes are still scarce compared with reservoirs used for drinking water. Nevertheless, the importance of toxicological assessment in natural lakes is unquestionable both regarding human uses, as

well as wildlife and conservation (Chorus and Bartram 1999; WHO 2003).

The relative biomass of cyanobacteria in the phytoplankton is often high in warm, shallow lakes, partly mediated by nutrient loading, poor underwater light and low zooplankton grazing (Romo et al. 2004; Kosten et al. 2011). Higher temperatures may increase the stability of the water column, which may be advantageous for some buoyant cyanobacteria like *Microcystis* (Visser et al. 2005). The Mediterranean climate, with high irradiance and temperature, and low rainfall, promotes conditions for cyanobacterial growth all year (Beklioglu et al. 2007), which likely increases the risks of environmental toxicity. A recent survey on cyanotoxins in Greek lakes has reported a widespread presence and increase of cyanobacteria and cyanotoxins (Cook et al. 2004; Gkelis et al. 2005; Papadimitriou et al. 2009). Nevertheless, the information on cyanotoxins in the Mediterranean zone is still scarce (see reviews by Cook et al. 2004; Kardinaal and Visser 2005).

Cyanobacteria can produce neurotoxins, hepatotoxins, cytotoxins and skin irritants, all of which can threaten humans, other vertebrates and invertebrates (Chorus and Bartram 1999). A wide range of bloom-forming cyanobacteria (e.g. *Microcystis*, *Anabaena*, *Oscillatoria* and *Nostoc*) are potentially microcystin producing. Microcystins are hepatotoxic to mammals and fish through inhibition of protein phosphatases (MacKintosh et al. 1990; Xu et al. 2000) and in drinking water can poison human populations (Chorus and Bartram 1999). Fish consumption is also a common means of transfer through food to humans. Fish can be exposed to toxins during direct feeding and/or passively when the toxins pass through the gills during breathing and other activities (Malbrouck and Kestemont 2006). Microcystins are accumulated in the liver, but can pass to muscles, kidney and brain (Fisher and Dietrich 2000; Kagalou et al. 2008). The mechanisms of uptake and accumulation in different fish species are yet scarcely investigated, as is the distribution of microcystins in different body tissues or organs, which could limit uses of fish as human food. Most of the studied fish species are commercial or cultivated (Andersen et al. 1993; Bury et al. 1996; Carbis et al. 1997; Tencalla and Dietrich 1997; Fisher and Dietrich 2000; Ernst et al. 2001; Magalhães et al. 2001; Mohamed et al. 2003; Soares et al. 2004; Xie et al. 2005; Zhao et al. 2005; Cazenave et al. 2005; Zhang et al. 2006; Smith and Haney 2006; Gkelis et al. 2006; Kagalou et al. 2008; Papadimitriou et al. 2009). Among them only a few of these studies were from the Mediterranean area (Mohamed et al. 2003; Gkelis et al. 2006; Kagalou et al. 2008; Papadimitriou et al. 2009). In general, knowledge on this topic is fragmentary and more quantitative studies are needed to understand better the accumulation and effects of microcystins in aquatic food webs (Malbrouck and Kestemont 2006; Gkelis et al. 2006).

This study assess the levels of microcystin in different tissues of a commercial mugilid species (*Liza* sp.), in the largest, coastal, Spanish Mediterranean lake (Lake Albufera). To our knowledge, this is the first report on the occurrence and tissue distribution of microcystins in mugilid fish species. Mugilids comprise around 100 fish species distributed in tropical and temperate zones in the world and many of them have a commercial value. The presence of microcystins in the water and seston was studied for 2 years to determine possible plankton dynamics affecting pathways of cyanotoxin accumulation. Human tolerable daily intake (TDI) for microcystins is assessed relative to international guidelines, and potential toxicological risks for humans, wildlife and related ecosystems of the lake are discussed. This investigation is also one of the few studies concerning cyanobacteria and cyanotoxicity in natural Spanish lakes.

Materials and methods

Study area

Lake Albufera is the largest Spanish coastal lake with a surface area of 23.2 km^2. It is a shallow (mean depth of 1.2 m), polymictic and oligohaline (salinity 1–2‰) lake located in the Natural Park of the Albufera (210 km^2) on the Mediterranean Spanish coast (39 20° N, 0 21° W). The Park is a wetland of international importance, protected by the Ramsar Convention and the European

Habitat list NATURA 2000. Since the eighteenth century, rice has been cultivated intensively in the areas surrounding the lake, and the lake water level is regulated for irrigation by sluice gates situated at its three outlet channels which flow into the Mediterranean Sea. The lake is eutrophic and dominated by cyanobacteria and the high nutrient loading affects the whole food web (Romo et al. 2005). The Albufera has been in a eutrophic state since the 1960s (Villena and Romo 2003), but since 2002, there has been a general trend of increase in harmful cyanobacteria (*Microcystis aeruginosa* and *Cylindrospermopsis raciborski*, Romo et al. 2008). Fisheries are still an economic activity in the lake, and about 200 tons of mugilids (mainly *Liza* sp. and *Mugil cephalus*) are removed annually for human consumption. More detailed information about fish species composition and captures is given elsewhere (Blanco et al. 2003; Romo et al. 2005; Blanco and Romo 2006).

Sampling and phytoplankton analyses

Samples were taken at three representative points in the lake dependent on water inputs (Romo and Miracle 1993). Water samples were taken from the upper 50 cm of the water column. A total of 43 water samples were collected between November 2005 and December 2007 at weekly and monthly intervals. Phytoplankton community composition was determined according to Romo and Miracle (1993) and algal biovolume according to Hillebrand et al. (1999). Biovolume of *M. aeruginosa* colonies was calculated as the sum of cell volumes per colony to avoid overestimation when biovolume is estimated considering colony volume. Chlorophyll *a* was extracted with 95% acetone after filtration with GF/F filters and measured spectrophotometrically (APHA 1992).

Toxin extraction

Dissolved and cell-bound microcystins were analysed. Water samples were filtered onto fiberglass filters GF/F and frozen for subsequent total microcystins (MCYST) analysis. For analysis of dissolved microcystins, the filtered lake water was analysed directly by enzyme-linked immunosorbant assay (ELISA). Cell-bound microcystins were extracted from the filters with methanol (90%) followed by vacuum centrifugation to remove the organic solvent. The extract was suspended in distilled water and the supernatant after centrifugation was subjected to ELISA assay in duplicate or triplicate (Fastner et al. 1998). For each ELISA test the commercial EnviroGard microcystin kit was used and the control and six calibrates were assayed. The ELISA is an indirect-competitive method using the b-amino acid 6E-ADDA as the epitope for antibody recognition for the quantitative analysis of all the microcystin analogues and nodularins. The limit of quantification was of 0.1–1.6 ppb and each kit was tested against a standard spiked at 1 ppb for recovery value. Results are expressed as micrograms of cellular MCYST equivalents per litre (Kotak et al. 1995).

Fish samples and extraction from fish tissues

Fish samples were randomly taken from captures made by professional fishermen in the Albufera Lake during weekly intervals in October 2007, at the starting of the fish campaign and after confinement of the fish populations in the lake during summer. Fishermen used trammel nets with 30-mm mesh size. The sampled number of fishes was estimated statistically within a 95% confident level. One hundred and three specimens of *Liza* sp. were sampled during the study. To extract the toxin from the fish organs, liver, intestine, gills and muscle tissues were cut out, weighed and frozen for subsequent analyses. Tissues were separately homogenized in a mortar with quartz sand and extracted with 90% methanol followed by vacuum centrifugation to remove the organic solvent (Fastner et al. 1998; Magalhães et al. 2001). The extract was suspended in distilled water and the supernatant after centrifugation was analysed by ELISA test in duplicate or triplicate. When necessary, the extract was diluted before the assay. The results are expressed as nanograms of MCYST equivalents per gram of fish tissue (Magalhães et al. 2001). The MCYST concentration in each tissue was divided by the total body weight of each specimen in order to estimate the tissue concentration to body mass ratio for

a subsample of 21 individuals (Malbrouck and Kestemont 2006; Kagalou et al. 2008).

Tolerable daily intake of MCYST in the diet was calculated according to Kuiper-Goodman et al. (1999). Determinations were made for a standardized adult human weight of 60 kg and an average ingestion in the diet of about 300 g of *Liza* sp. muscle tissue. It was assumed that all the MCYST analogues determined by ELISA have equivalent toxicity to MC-LR (Kotak et al. 1995).

Statistical analyses

Data were log-transformed if necessary for statistical normality and otherwise analysed by a non-parametric test. Spearman correlation coefficient was used to determine significant relationships between pairs of variables. The general linear model was used to examine significant differences between tissues in relation to the microcystins concentrations. Differences of MCYST concentrations in lake water and seston between sampling points were analysed by an ANOVA and Kruskal–Wallis' test. Data were explored using the statistical package SPSS 17.0 for Windows.

Results

Microcystin concentrations in water and seston

There were no significant differences in the concentration of microcystins in the lake water and cell-bound among the three sampling points during the study period ($p > 0.05$), thus the average values of the sampling points are reported.

All samples contained MCYST in the lake water and seston, and among them 79% of the samples had values above 1 μg/L. In the water samples mean MCYST concentration was of 1.7 ± 0.52 μg/L and ranged between 0.04 and 16 μg/L for the study period. The mean MCYST concentration that was cell-bound was 17 ± 4.37 μg/L and varied between 0.06 and 120.5 μg/L for the study period. A significant correlation was found between MCYST concentration in water and in the seston ($r = 0.394$, $p < 0.05$). Seston samples contained tenfold higher MCYST concentrations than the dissolved fraction, and in general, dissolved MCYST appeared in the lake water after peaks in the seston (Fig. 1a).

There were no significant correlations between chlorophyll *a* and MCYST concentrations in the water or the cell-bound fraction ($p > 0.05$). However, abundance and biovolume of chroococcal cyanobacteria significantly correlated with MCYST (water: $r = 0.34$ and 0.44; seston: $r = 0.74$ and 0.60, $p < 0.05$, respectively) and they were higher than the correlations of MCYST with total cyanobacteria (MCYST water: $r = 0.41$ and 0.39; MCYST seston: $r = 0.69$ and 0.58, $p < 0.05$, respectively). Total cyanobacteria averaged 75% (maximum 97%) of total phytoplankton biovolume during the study period.

There was a high positive correlation between abundance and biovolume of *M. aeruginosa* and

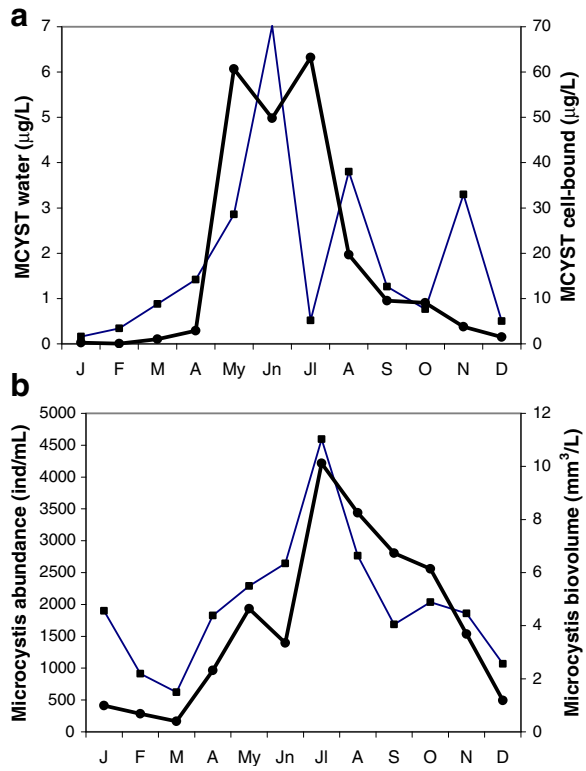

Fig. 1 **a** Seasonal changes of MCYST concentrations in lake water (*thin line*) and seston (*thick line*) in Lake Albufera (East Spain). **b** Seasonal changes in the abundance (*thin line*) and biovolume (*thick line*) of *Microcystis aeruginosa* populations. Data represent monthly means for the study period (November 2005 to December 2007)

MCYST in the seston ($r = 0.55$ and $r = 0.68$, $p < 0.001$, respectively). Populations of *M. aeruginosa* represented up to 14% of total biovolume during the study period and developed mainly during summer–autumn at the time of longer water residence in the lake (Fig. 1b). There was no significant difference among the sampling points for *M. aeruginosa* abundance and biovolume ($p > 0.05$).

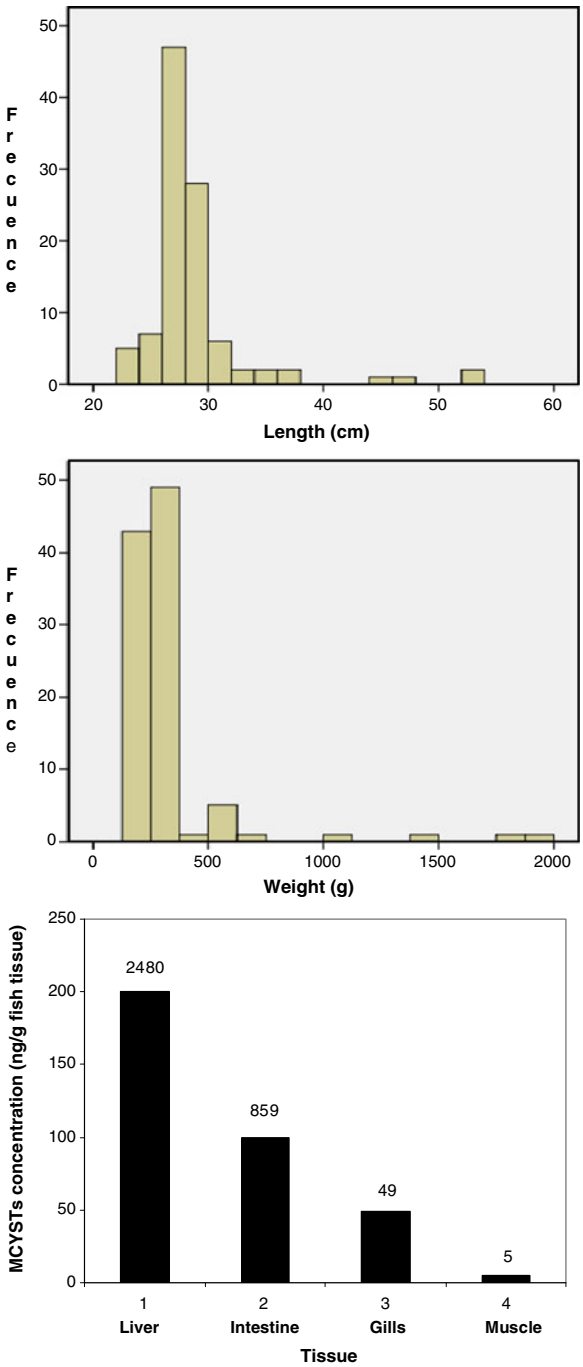

Fig. 2 Histograms of the length and weight class distribution of the individuals of *Liza* sp. (Mugilidae) and mean MCYST concentrations in different tissues of this species analysed from Lake Albufera (Spain; $n = 103$ specimens)

Microcystin concentration in fish tissues

The size of the fishes studied had a mean length of 28 ± 0.23 cm (range 23–54 cm) and a mean weight of 300 ± 11 g (range 150–2,100 g) and the size distribution was bias toward a few size classes as expected for commercial captures (Fig. 2). All the tissue samples of *Liza* sp. examined contained MCYST. There were significant differences among the concentrations of MCYST in the different tissues ($F = 225$, $p < 0.001$; Fig. 2). Among tissues, the increasing order of MCYST concentrations was liver>intestine>gills>muscle. The liver had the greatest content of MCYST with a mean value of 2,480 ± 360 ng/g fish tissue, followed by the intestine (859 ± 128 ng/g fish tissue), gills (49 ± 25 ng/g fish tissue) and muscle (5.21 ± 0.59 ng/g fish tissue). The MCYST concentrations in the liver and intestine correlated with MCYST levels in gills ($r = 0.50, r = 0.46$, $p < 0.05$, respectively). Furthermore, bigger individuals of *Liza* sp. had higher concentrations of MCYST in the liver and intestine ($r = 0.47, r = 0.45$, $p < 0.05$, respectively). It was observed during sampling that some of the studied specimens showed liver damages. The MCYST concentration in muscle was not related to other tissue MCYST concentrations or to fish body weight. The mean MCYST concentration in muscle corresponded to a daily intake of 0.025 μg/kg/day and 13% of the analysed specimens had TDI values above 0.04 μg/kg/day. The mean size of these individuals was 29.5 ± 0.8 cm and 365 ± 36 g.

Discussion

Microcystin in water and seston

Microcystins were present in lake water and seston in all the study samples, which confirmed potential toxicity risks all year round. In general, cell-bound samples contained one order of magnitude higher MYCST concentrations than the

dissolved fraction and toxins appeared in dissolved form after peaking in the seston. Microcystins are considered endotoxins with the majority of the toxin allocated within the cells (Kotak et al. 1996), but are released into the water after algal lysis by several factors, such as senescence, presence of chemicals that inhibit new cell synthesis, enzymatic reactions or photosynthesis (Lam et al. 1995; Kotak et al. 1996).

In the present study, there was a positive correlation between populations of *M. aeruginosa* and MCYST. Furthermore, toxic genotypes of this species were identified in some strains isolated from Lake Albufera during the study (Ouahid and Fernández 2009). Isolated cultures of some of the Oscillatorial species that are also present in the lake, such as *Pseudanabaena* and *Geitlerinema*, showed no MCYST in ELISA assays. Likewise, low dissolved MCYST concentrations in the Albufera lake water were reported in summer 1999 (Bradt and Villena 2002) and found also in February–March 2002 (Romo, S., unpublished data), when filamentous cyanobacteria dominated the phytoplankton. There were no significant differences among the sampling points, either for the concentrations of MCYST in the water and seston or *M. aeruginosa* abundance and biovolume. This suggests that any part of the lake has similar risk for holding toxic cyanobacteria and microcystins, which can further affect uses and restoration measures.

Abundance and biovolume of algae were more sensitive variables than chlorophyll *a* for relationships between MCYST concentrations and phytoplankton. Several authors have discussed the restrictions of using chlorophyll *a* values as estimation of cyanobacteria biomass (Reynolds 1997). In several studies, MCYST concentrations were not related to the phytoplankton biomass when estimated as chlorophyll *a* (Kotak et al. 1996; Lindholm et al. 2003). This seems relevant when selecting variables for a monitoring programme. According to WHO (2003), moderate risk at MCYST guideline values of 10–40 μg/L is equivalent to 10^5 cyanobacterial cells per millilitre or approximately 50 μg/L chlorophyll *a*, if cyanobacteria, and especially *Microcystis*, dominate. Although quantification of cyanobacteria by a variety of methods can be used to estimate the toxic risk, it is clear that it cannot replace toxin monitoring. Furthermore, the toxin concentrations per cell may vary among strains of the same cyanobacteria species and between species (Kurmayer et al. 2002; Kardinaal and Visser 2005).

The MCYST concentrations found in Lake Albufera (mean 1.7 and 17 μg/L and maximum 16 and 120 μg/L in water and cell-bound, respectively) exceeded one to two orders of magnitude the guideline level for microcystin-LR proposed by the World Health Organization (1 μg/L, WHO 2003). According to international standards, relatively low probabilities of adverse health effects (e.g. irritant or allergenic effects) in recreational waters are in the cell-bound MCYST range of 2–10 μg/L, while values between 10 and 20 (−40 μg/L) represent a moderate risk and values over 20–40 μg/L have probabilities of high-risk health effects (WHO 2003). The Albufera Lake would be in the moderate–high risk range.

The total MCYST levels assessed in this study are higher than those reported for other Mediterranean lakes, such as in Turkey (1.0–3.65 μg/L, Albay et al. 2003), Portugal 1.0–37.0 μg/L, (Ueno et al. 1996; Vasconcelos et al. 1996), France (0–5.2 μg/L, Briand et al. 2002), Algeria (0.05–29 μg/L, Nasri et al. 2007) and Greece (0.1–16 μg/L, Kagalou et al. 2008; Papadimitriou et al. 2009), but lower than some maximum values reported for some Australian lakes (3–1,800 μg/L, Jones and Orr 1994) and for a Brazil lagoon (1–980 μg/L, Magalhães et al. 2001).

Agricultural products irrigated with water containing MCYST represent also a potential hazard to human health and wildlife (McElhiney et al. 2001; Crush et al. 2008). Farmers are potentially in danger from the inhalation of droplets containing toxins or through dermatological contact (Falconer et al. 1999). Paddy fields surrounding the Albufera Lake are sometimes flooded during the year with water that could have high levels of cyanotoxins, which further increase risks for humans and biota in this Natural Park. Accumulation in the rice plants and fauna (mainly birds) has not been surveyed, but should be considered in further studies. The uptake and fate of microcystins and other cyanotoxins by food plants and their persistence on plant surfaces and other plant

parts need in general further research (Chorus and Bartram 1999). Furthermore, Lake Albufera, like other coastal lakes, is located in a transitional zone which links epicontinental and marine waters. Contaminants and cyanotoxins are more than an isolated problem, because they can pollute the related ecosystems, such as the aforementioned ricefields, associated wetland, nearby beaches and marine water. Overall, there is need for a more intense monitoring programme to assess toxicological risks in the related aquatic systems and within the Albufera Natural Reserve.

Microcystins in fish tissues

Fish can be exposed to MCYST either during feeding or passively when the toxins pass through gills during breathing and other activities (Xie et al. 2005). Knowledge of the effects of MCYST on fish, and their response to cyanotoxins in sub-lethal or chronic exposure is limited (Soares et al. 2004; Magalhães et al. 2001; Fisher and Dietrich 2000; Kagalou et al. 2008; Papadimitriou et al. 2009). For some cyprinids the presence of toxins can limit their feeding rate and body growth (Beveridge et al. 1993; Keshavanath et al. 1994). In eutrophic lakes, however, most fish species are unable to avoid ingestion of toxic algae during feeding, and the digestive tract is one of the main routes for fish uptake of MCYST (Tencalla et al. 1994).

In this study, the digestive track and intestine of *Liza* sp. was often filled with cyanobacteria mixed with lake sediment, which confirms the benthopelagic and omnivorous habits of this species in Lake Albufera (Blanco et al. 2003). Our results revealed MCYST in all the fishes analysed and differences in their partitioning among fish tissues. The highest MCYST concentrations were found in the liver>intestine>gills>muscle. MCYST is thought to be taken up by a bile-acid transporter in intestinal and liver cells, with part of the toxin being extracted by faeces and another part accumulated in the liver (Sahin et al. 1996; Malbrouck and Kestemont 2006). Hepatic elimination prevents or minimizes the distribution of foreign chemicals to other parts of the body but overwhelming this process during fish exposure to toxins may explain MCYST accumulation in the other organs (Klaassen and Watkins 1984; Malbrouck and Kestemont 2006). It seems that the tissues and organ distribution of MCYST is partly governed by organic anion transporters (Fisher and Dietrich 2000). Some studies have reported the presence of MCYST in the gastrointestinal tract, kidneys, gills and/or brain (Fisher and Dietrich 2000; Kagalou et al. 2008; Malbrouck and Kestemont 2006). It assumed that high MCYST concentrations in fish liver (maximum average of 31,100 ng/g tissue) could cause dead and severe damage to some fish species (Tencalla et al. 1994). In our study, liver accumulated the highest amounts of MCYST and some of the specimens of *Liza* sp. showed hepatic lesions.

The high levels of MCYST observed in gills during the present study, with tenfold times greater concentration than in muscles, suggest that *Liza* sp. populations in the lake are steadily exposed to toxins in the water. Several authors have shown damage to gills by dissolved MCYST in tilapia and trout (Gaete et al. 1994; Bury et al. 1996).

The mean amounts of MCYST found in muscle of *Liza* sp. in this study was lower than that reported for other fish species, such as tilapia, common carp and *Carassius gibelio* (Malbrouck and Kestemont 2006; Kagalou et al. 2008; Papadimitriou et al. 2009). Since the studied mugilid species in Lake Albufera (*Liza* sp.) is a commercial species, it was relevant to determine the tolerable daily intake of MCYST in the human diet (Kuiper-Goodman et al. 1999). The mean concentration of MCYST in muscle tissue of *Liza* sp. corresponded to daily intake of 0.025 μg/kg of body weight per day, but 13% of the analysed specimens had values above the recommended guideline concentration of 0.04 μg/kg of body weight per day proposed by WHO (Kuiper-Goodman et al. 1999). Guideline values for MCYST are based on a standardized adult weight of 60 kg, while it is obvious that children, females and other individuals within the human population may be target groups with lower TDI and therefore exposed to higher toxicological risks. For Lake Albufera, a mean of 200 tons of *Liza* sp. is sold yearly, which is equivalent to about 350 mg of MCYST, based on the mean MCYST value found in fish muscle in this study (5 ng/g

fish muscle). This amount may be three orders of magnitude greater if we consider MCYST passed to birds and other wildlife that can feed not only on fish muscle but also on other parts of the fish. This is relevant for a Natural Reserve, catalogued as an International Protected Bird Zone and as said above, further studies are needed to assess the impact of cyanotoxins in the food webs of the Albufera Natural Park.

Conclusions

1. All water and seston samples analysed in Lake Albufera contained MCYST and among them 79% had values above 1 μg/L. The MCYST concentrations (mean 1.7 and 17 μg/L, and maximum 16 and 120 μg/L in water and seston, respectively) were one to two orders of magnitude higher than the levels recommended by the international standards and than those reported in some Mediterranean lakes. The lake is in the moderate–high health risk range for recreational activities, agriculture, fisheries and wildlife. Populations of *M. aeruginosa* were correlated with MCYSTs. Abundance and biovolume of algae were more sensitive variables than chlorophyll *a* for monitoring relationships between MCYST concentrations and phytoplankton.
2. The presence of MCYST was also found in all the fishes studied and accumulated differently among tissues of the commercial species *Liza* sp. High levels in the liver, intestine and gills denoted that populations are steadily exposed to toxins. The mean concentration of MCYST in muscle corresponded to a daily intake of 0.025 μg/kg of body weight per day, but 13% of the analysed specimens had values above the recommended guideline concentration of 0.04 μg/kg of body weight per day. This international guidance value has to be applied with caution depending on several factors, such as, age distribution of the local human population. Systematic control of cyanotoxins in the commercial fish captures of the lake is needed, with special care on larger individuals, as well as control of MCYST content in the water and seston. Reduction of the lake eutrophication is necessary.
3. Comprehensive and further studies on other compartments of the food webs and transference of toxins to the related ecosystems (freshwater and marine) of the Albufera Natural Park need attention in order to deal with management and conservation of this international protected zone.

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