

Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions

Ángeles Jos^{a,*}, Silvia Pichardo^a, Ana I. Prieto^a, Guillermo Repetto^{a,b},
Carmen M. Vázquez^c, Isabel Moreno^a, Ana M. Cameán^a

^a Area of Toxicology, Faculty of Pharmacy, University of Seville, Profesor García González no. 2, 41012 Seville, Spain

^b National Institute of Toxicology and Forensic Sciences, Seville, Spain

^c Area of Physiology, Faculty of Pharmacy, University of Seville, Profesor García González no. 2, 41012 Seville, Spain

Received 4 October 2004; received in revised form 13 January 2005; accepted 18 January 2005

Abstract

The effects of microcystins from cyanobacterial cells on various oxidative stress biomarkers in liver, kidney and gill tissues in freshwater tilapia fish (*Oreochromis* sp.) were investigated under laboratory conditions. Microcystins are a family of cyclic peptide toxins produced by species of freshwater cyanobacteria (blue-green algae). Fish were exposed to the cyanobacterial cells in two ways: mixed with a commercial fish food or crushed into a commercial fish food so that the toxins were released. Two different exposure times were studied: 14 and 21 days. The oxidative status of fish was evaluated by analyzing the level of lipid peroxidation (LPO), as well as the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). The findings of the present investigation show that microcystins induce oxidative stress in a time-dependent manner and that the type of administration of the cyanobacterial cells influences the extent of these effects. Thus, the crushed cyanobacterial cells (released toxins) induced the antioxidant defences studied and increased the LPO level to a greater extent than the non-crushed cells. The liver was the most affected organ followed by kidney and gills. These results together with reports that fish can accumulate microcystins mean that cyanobacterial blooms are an important health, environmental and economic problem.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Cyanobacteria; Microcystin; Tilapia; Fish; Oxidative stress

1. Introduction

Aerobic organisms generate reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$)

* Corresponding author. Tel.: +34 954 556762;
fax: +34 954 233765.

E-mail address: angelesjos@us.es (Á. Jos).

as a result of oxidative metabolism. •OH can initiate lipid peroxidation (LPO) in tissues (Halliwell and Gutteridge, 1984). To minimize the negative effects of ROS, fish, like other vertebrates, possess an antioxidant defence (AD) system, which utilizes enzymatic and non-enzymatic mechanisms. Some of the most important AD enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), while the non-enzymatic defenses include Vitamins E, C and A, glutathione, carotenes and ubiquinol₁₀ (Filho, 1996). When the activity of these antioxidant defence systems decreases or ROS production increases, oxidative stress may occur (Packer, 1995).

Mitochondria are the sites where reactive oxygen species are mainly produced, and red muscles are the most important source of mitochondria in endotherms. Therefore, this tissue is considered to be the main contributor to ROS generation in mammals and birds. However, in most fish red muscle makes up only a small proportion of tissues, and other tissues such as liver, kidney and also gills (as the first tissue in contact with the xenobiotics in the water) are more important in this regard (Filho et al., 2000).

Oxidative stress biomarkers are becoming increasingly important in the field of ecotoxicology. The high number of pollutants can disturb the equilibrium between ROS and the AD system. It has been suggested that they could also be used in environmental monitoring programs (Pandey et al., 2003).

Various pesticides (Sayeed et al., 2003), pulp mill effluents (Filho et al., 1997) and metals (Almeida et al., 2002) are some of the pollutants that have been reported to elicit oxidative stress in aquatic organisms but naturally occurring toxicants such as microcystins (MCs) are also capable of doing so (Wiegand et al., 1999; Li et al., 2003). MCs are a family of cyclic peptide toxins produced by species of freshwater cyanobacteria (blue-green algae), primarily *Microcystis aeruginosa* (Dawson, 1998). Anthropogenic activities lead to the eutrophication of the water, which in turn means that these algae can grow in massive quantities. This generates a so called cyanobacterial bloom (Mankiewicz et al., 2001). It seems that every country in the world will have water bodies that support blooms of toxic cyanobacteria at some time or another (Sivonen and Jones, 1999). The intact cells as well as the toxins released after cellular lysis can

be responsible for the toxic effects observed in both animals and humans (Carmichael and Falconer, 1993; Moreno et al., 2004a), and they have been associated with fish kills (Zimba et al., 2001). In response to the increase in health-related problems on a global scale, the World Health Organisation (WHO) has established safe guidelines for drinking water at 1.0 µg MC-LR/L (Falconer et al., 1999). Microcystins are cyclic heptapeptide molecules containing both L- and D-amino acids and an unusual hydrophobic C₂₀ D-amino acid commonly termed ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid). There are over 70 different MCs, which differ mainly in the two L-amino acids at positions 2 and 4. The most common, and also the most extensively studied, are MC-LR, MC-RR and MC-YR (Fastner et al., 2002).

MCs have proved to be highly potent hepatotoxins in mammals and fish (Fisher and Dietrich, 2000; Towner et al., 2002). It is well recognized that among their toxicogenic mechanisms they are potent inhibitors of protein phosphatase 1 and 2A that cause increased protein phosphorylation, which is directly related to their cytotoxic effects and tumor-promoting activity (Hooser et al., 1989; Carmichael, 1994; Hooser, 2000). Some evidence suggest that oxidative stress may play a significant role in the pathogenesis of microcystin toxicity in mammals (Hermansky et al., 1991; Ding et al., 1998, 2001; Guzman and Solter, 1999; Towner et al., 2002; Ding and Ong, 2003; Moreno et al., 2003; Bouaïcha and Maatouk, 2004; Zegura et al., 2004), but data of in vivo experiments in fish are still scarce.

Little work has been undertaken on the possible transference of MCs along food chains, and some studies suggest that fish farms should be monitored for the presence of toxic cyanobacterial blooms to minimize the exposure of fish to potent hepatotoxins (Magalhaes et al., 2001; Lawrence and Menard, 2001; Mohamed et al., 2003).

This study aims to investigate the effects of microcystin-LR in cells from toxic cyanobacterial blooms on oxidative stress biomarkers including LPO and several antioxidant enzymatic activities such as CAT, GPx, GR and SOD in liver, kidney and gill tissues from tilapia fish (*Oreochromis* sp.). *Oreochromis* sp. is an important group of fish species in commercial fisheries in South America and it is also being introduced in Europe. Moreover, they are commonly found in brackish water in estuaries around the world and re-

spond promptly to environmental alterations (Almeida et al., 2002). Fish were exposed to the cyanobacterial cells through their diet at different exposure times and the different administrations were compared.

2. Materials and methods

2.1. Experimental setup and acclimation of fish

Studies were conducted using male *Oreochromis* sp. (Nile tilapia, Perciformes: Cichlidae) with mean weight 49.92 ± 9.1 g. They were obtained from a fish hatchery in Córdoba and transferred to the laboratory where they were held in aquariums (8 individuals/aquarium) with 96 L of fresh water. Exposure to chlorine was minimized by filling the tanks at least 3 days before the fish were introduced. The aquariums were also set up with continuous system of water filtration and aeration (Eheim Liberty 150 and Bio-Espumador cartridges (Bio-Espumador)) and the temperature was kept constant (21 ± 2 °C). Dissolved oxygen values were maintained between 6.5 and 7.5 mg/L. Mean values for additional parameters of water quality were: pH 7.6 ± 0.2 , conductivity 292 μ S/cm, Ca^{2+} 0.60 mM/L and Mg^{2+} 0.3 mM/L. Fish were fed with commercial fish food and were acclimatized for 15 days before the beginning of the experiments.

2.2. Collection of *Microcystis* waterbloom and determination of cyanobacterial toxins

MCs from two different *Microcystis* waterblooms from the river Guadiana (Mértola, Portugal) were extracted from dried cell material using the method of Moreno et al. (2004b). The lyophilised cells (50 mg) were extracted three times with 10 mL 0.1 M acetic acid and 20 mL of a mixture of methanol:chloroform (1:1 v/v). The cell suspension was sonicated in an ultrasound bath for 15 min, stirred for 30 min at room temperature, and then centrifuged at $4500 \times g$ for 15 min. The upper aqueous phases were combined and concentrated in a rotary evaporator. The residue was resuspended in 0.5 mL methanol for chromatographic separation.

The LC system used to analyse the toxin contents was a Varian 9012 equipped with a Varian ProStar 330 Diode Array Detector. Chromatographic data were

processed with a Star Chromatography Workstation (Varian Technologies). Chromatographic separation of MCs was performed according to Moreno et al. (2004b) on a 250 mm \times 4.6 mm i.d., 5 μ m, LiChrosphere C18 column purchased from Merck (Darmstadt, Germany).

Microcystin standards were supplied by Calbiochem-Novabiochem (Nottingham, UK). Standard solutions of microcystins MC-LR, MC-YR and MC-RR were prepared in methanol (500 μ g/mL) and diluted as required with methanol for use as working solutions (0.5–5.0 mg/L of each toxin). Only MC-LR was identified in the cyanobacterial cell extracts analyzed and the peak area of the MC-LR standard solution was applied for quantification. The concentrations of MC-LR obtained from both lyophilised cells were similar, 3230 μ g/g MC-LR and 3340 μ g/g MC-LR.

2.3. Exposure

Tilapia fish were exposed to MC-LR by feeding with cyanobacterial cells under laboratory conditions for two different periods of time (14 and 21 days) and two different types of toxin administration (crushed and non-crushed cyanobacterial cells). Two experiments were designed as follows. A control group of fish ($n = 8$) in both experiments was administered only the commercial fish food for the period that exposed groups received food-containing MC-LR:

- *Experiment 1.* Fish in a test aquarium ($n = 8$) were fed with commercial fish food plus lyophilised cyanobacterial cells, containing 3230 μ g/g MC-LR. *M. aeruginosa* cells were fed to the fish by manually crushing mixture of both components (fish food and toxic cells) in a mortar followed by sonication. This procedure resulted in small sticky pellets and was designed to replicate the type of exposure that may occur when a bloom of cyanobacteria undergoes lysis under field conditions. The pellets were placed in the tank and drifted to the bottom for the fish to take. It was ensured that all the pellets were eaten within an hour. The amount of commercial fish food administered per fish was 0.3 g/day and the quantity of cyanobacterial cells was selected in order to dose approximately 60.0 μ g MC-LR/fish/day (determined from HPLC analysis), for 14 days.
- *Experiment 2.* It was carried out using two test aquaria with eight fish in each. The exposure time

to cyanobacterial cells was 21 days. In accordance with the dose of toxin chosen (approximately 60.0 µg MC-LR/fish/day) and the concentration of microcystin contained in the cyanobacterial cells (3340 µg/g MC-LR), we determined the quantity of lyophilised cells that had to be added to the aquarium. The lyophilised cells were administered in two different ways. In one of the test aquariums, the fish received the toxins in the same way as in Experiment 1 (in pellets containing commercial fish food plus crushed lyophilised cyanobacterial cells). In the second test aquarium, the fish were fed with commercial fish food (0.3 g/fish/day) and lyophilised *Microcystis* cells (non-crushed cells) dusted on the water surface, daily for 21 days. The uptake of lyophilised cells by the fish was completed within 3 h in both treatments.

2.4. Preparation of post-mitochondrial supernatant (PMS)

At the end of the experiments, the fish were anaesthetized in tricaine methane sulphonate (MS-222) before they were killed by transection of the spinal cord. The liver, kidney and gills were removed, weighed, rinsed with ice-cold saline and kept at -85°C until analysis. The gill filaments of both sides were trimmed from the gill arches and the arches were discarded. The tissues were homogenized in chilled TRIS buffer (100 mM, pH 7.8; 1:10 w/v) using an Ultra-Turrax® tissue homogenizer. The homogenates were centrifuged at $10\,500 \times g$ for 20 min at 4°C to obtain the post-mitochondrial supernatant (PMS) for various biochemical analyses.

2.5. Lipid peroxidation

The malondialdehyde (MDA) concentration, an index of lipid peroxidation, was measured following the method described by Esterbauer and Cheeseman (1990) on homogenized tissues. The homogenized tissue (0.5 mL), previously treated with 25 µL of butylhydroxytoluene 1% v/v in glacial acetic, was mixed with 0.2 mL of sodium laurylsulphate (8%), 1 mL of acetic acid (20% v/v) and 1 mL of 0.8% thiobarbituric acid. This mixture was then heated at 95°C for 30 min. The resulting chromogen was extracted with 3 mL of *n*-butyl alcohol and, after centrifugation ($1500 \times g$ for

10 min), the absorbance of the organic phase was determined at 532 nm. 1,1,3,3-Tetramethoxypropan was used as a standard. Values were presented as µmol MDA formed/g tissue.

2.6. Antioxidant enzymes

Catalase (CAT; EC 1.11.1.6) activity was assayed by the method of Beers and Sizer (1952). The reduction of hydrogen peroxide was followed spectrophotometrically at 240 nm, using 1.0 mL quartz cuvettes with a light path of 1.0 cm. Results are expressed in terms of nmol H_2O_2 consumed/min/mg protein.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured using the xanthine oxidase–cytochrome *c* method as described by McCord and Fridovich (1969). The reactions between xanthine and xanthine oxidase, and 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) led to superoxide radicals which reacted to form a red coloured formazan. This was used to determine the SOD activity. In the presence of SOD in the medium, superoxide radicals were removed and the formation of formazan was therefore inhibited. SOD activity was measured spectrophotometrically at 505 nm and calculated as inhibition percent of formazan formation.

Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed by following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase. The specific activity was determined using the extinction coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ (Lawrence and Burk, 1976). Glutathione reductase (GR; EC 1.6.4.2) activity was determined spectrophotometrically by measuring NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975).

2.7. Protein estimation

Protein contents in the samples were estimated by the method of Bradford (1976) using bovine γ -globuline as standard.

2.8. Statistical analysis

Results are presented as the mean \pm S.E. The differences between the data from the different types of cyanobacterial cell administration for 21 days were analyzed by one-way analysis of variance (ANOVA).

When significance was found, unpaired two-tailed Student's *t*-test was used and differences were considered significant from $p < 0.05$. All other comparisons were performed by the unpaired *t*-test.

3. Results

No fish died during the exposure periods of 14 or 21 days. Visual inspection throughout the experiments revealed that the fish behaved similarly to the control group. Likewise, the behavior of the fish was unchanged in the two different ways of administration. Moreover, no external alterations were observed in any of the experiments.

3.1. Effect of cyanobacterial cells on lipid peroxidation

After 14 days, no changes were observed in MDA in exposed fish when compared to the control group in any of the organs studied (Fig. 1). However, a longer exposure time significantly increased this biomarker in liver ($p < 0.01$), kidney ($p < 0.01$) and gills ($p < 0.05$) in fish subjected to the crushed cyanobacteria (Experiment 2). The liver was the most affected organ. The induction of lipid peroxidation was also evident in kidney of fish exposed to the non-crushed cyanobacteria for 21 days.

3.2. Effect of cyanobacteria cells on antioxidant enzymes

SOD activity did not change significantly in the liver, kidney or gills of fish that had been exposed to crushed cyanobacteria for 14 days (Fig. 2). The longer exposure resulted in a significant increase in the SOD activity in liver ($p < 0.01$, both diets) and in gills ($p < 0.05$, non-crushed food) but induction in kidney was not significant.

No discernible effects were observed in CAT activity of liver or kidney after 14 days of exposure, but activity increased 2.5-fold in liver ($p < 0.01$) and kidney ($p < 0.05$) after 21 days of treatment (Fig. 3). Both diets had similar effects. The basal CAT activity level in gills was much lower than that in the other organs and was not induced in any of the studies performed.

GPx activity was maintained at its basal level after the shorter exposure time (Fig. 4). After the longer ex-

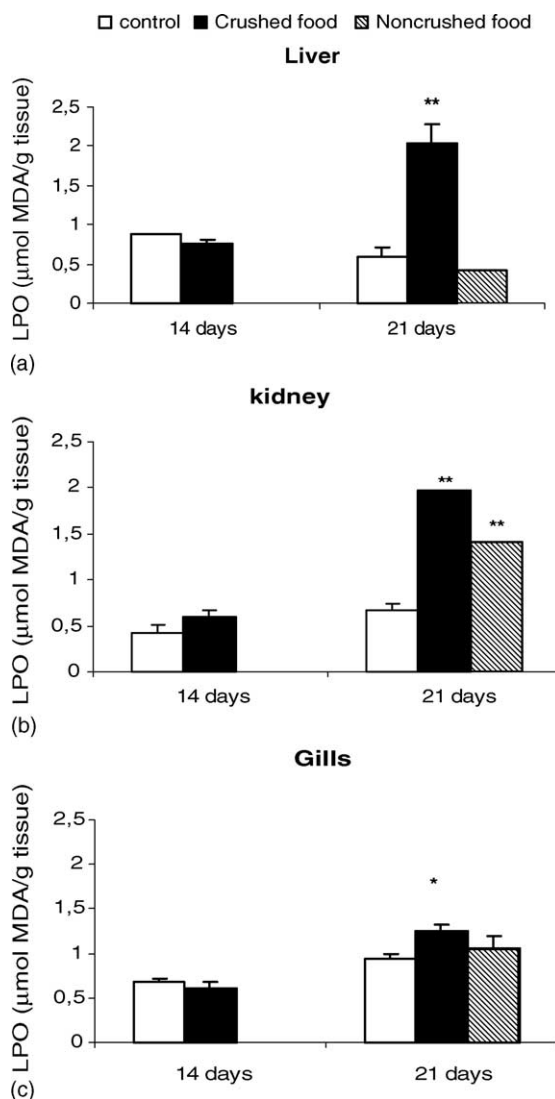


Fig. 1. Lipid peroxidation values in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to cyanobacterial cells (MC-LR offered to fish = 60.0 μg fish/day) for two different types of administration: crushed and non-crushed food. The values are expressed as mean ± S.E. ($n = 8$). LPO values are expressed as μmol MDA/g tissue. The significance levels observed are * $p < 0.05$ and ** $p < 0.01$ in comparison to control group values.

posure (21 days) there was a significant induction of GPx activity in liver ($p < 0.01$ crushed diet; $p < 0.05$ non-crushed diet) and kidney ($p < 0.05$ both diets). However, GPx activity showed a significant decrease in gills ($p < 0.01$) with both diets.

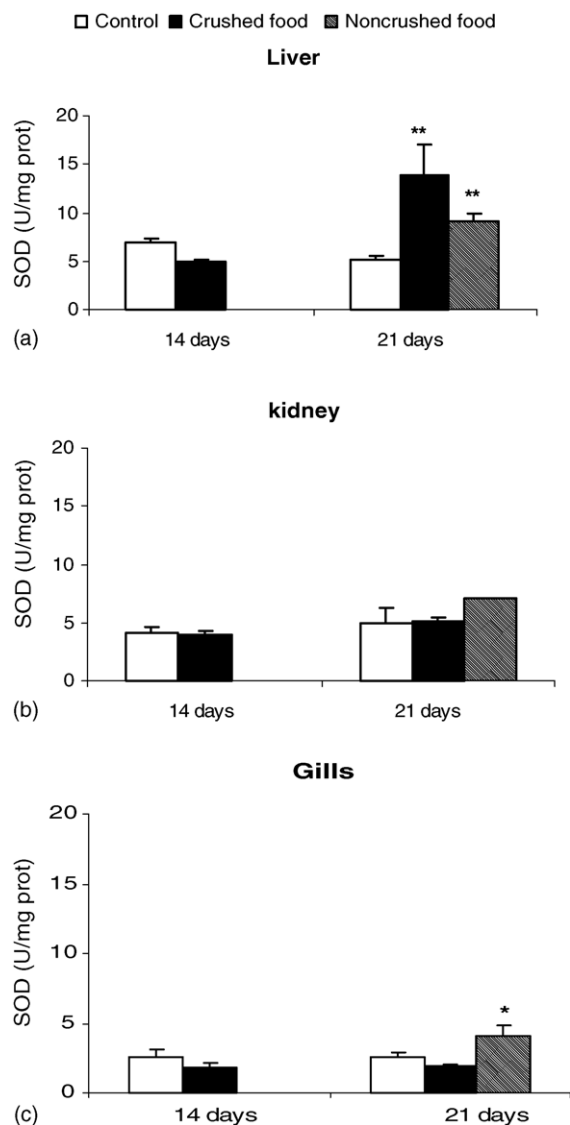


Fig. 2. Superoxide dismutase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to cyanobacterial cells (MC-LR offered to fish = 60.0 μg fish/day) for two different types of administration: crushed and non-crushed food. The values are expressed as mean \pm S.E. ($n=8$). SOD activity is expressed as U/mg protein. The significance levels observed are * $p < 0.05$ and ** $p < 0.01$ in comparison to control group values.

After 14 days of exposure GR behaved just like the previous biomarkers, and showed no significant changes (Fig. 5). GR activity was significantly induced after 21 days of exposure in liver ($p < 0.01$ crushed diet; $p < 0.05$ non-crushed diet) and kidney ($p < 0.01$ in both

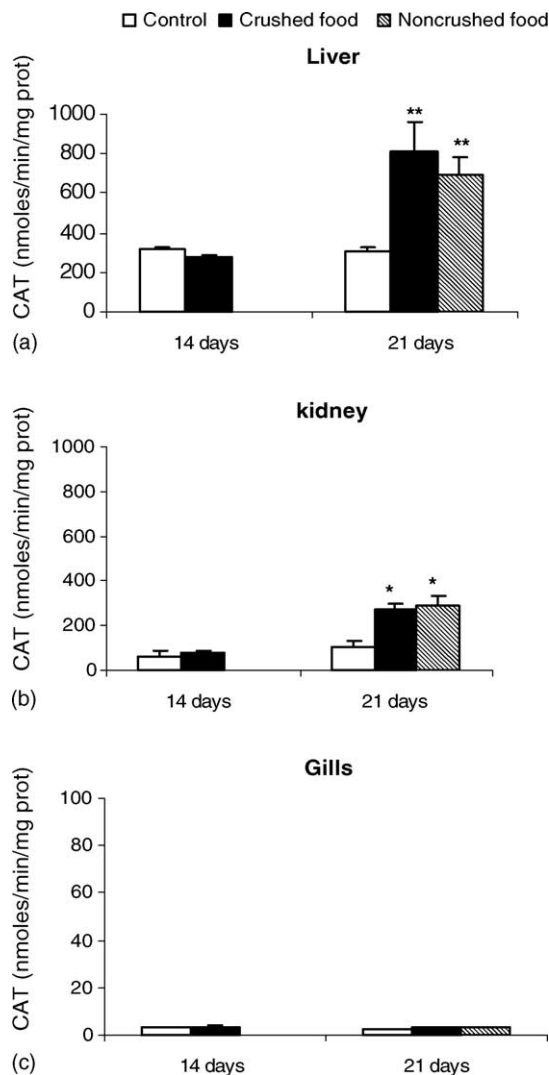


Fig. 3. Catalase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to cyanobacterial cells (MC-LR offered to fish = 60.0 μg fish/day) for two different types of administration: crushed and non-crushed food. The values are expressed as mean \pm S.E. ($n=8$). CAT activity is expressed as nmol H_2O_2 consumed/min/mg protein. The significance levels observed are * $p < 0.05$ and ** $p < 0.01$ in comparison to control group values.

diets) and showed no significant changes in gills. In liver, changes in GR and GPx activities were more pronounced when the cyanobacterial cells were crushed with the fish food.

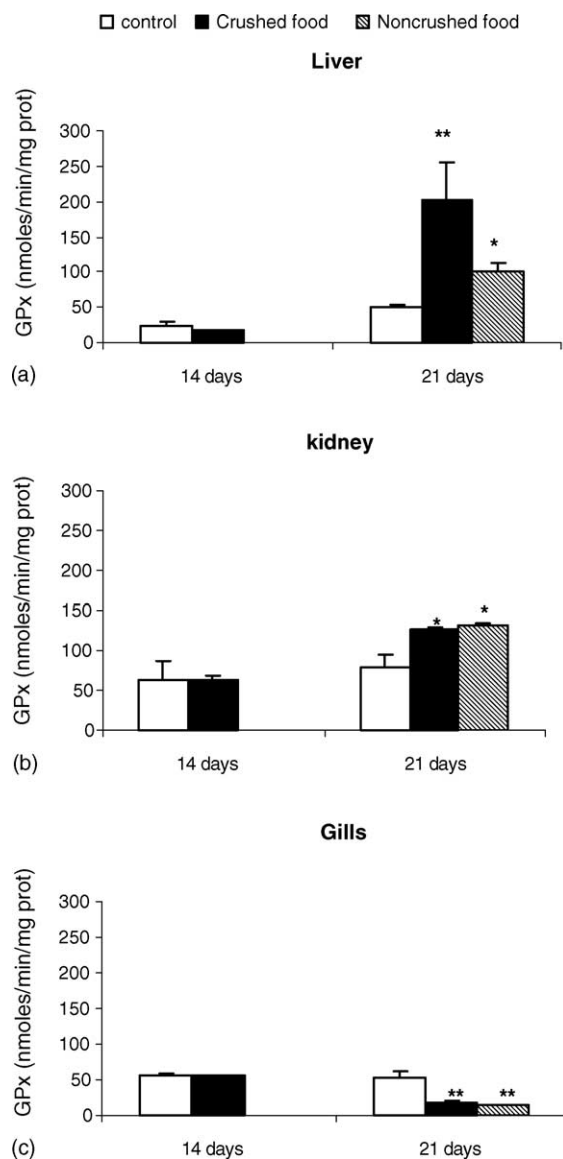


Fig. 4. Glutathione peroxidase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to cyanobacterial cells (MC-LR offered to fish = 60.0 μg fish/day) for two different types of administration: crushed and non-crushed food (intact cyanobacterial cells). The values are expressed as mean \pm S.E. ($n = 8$). GPx activity is expressed as nmol NADPH oxidized/min/mg protein. The significance levels observed are * $p < 0.05$ and ** $p < 0.01$ in comparison to control group values.

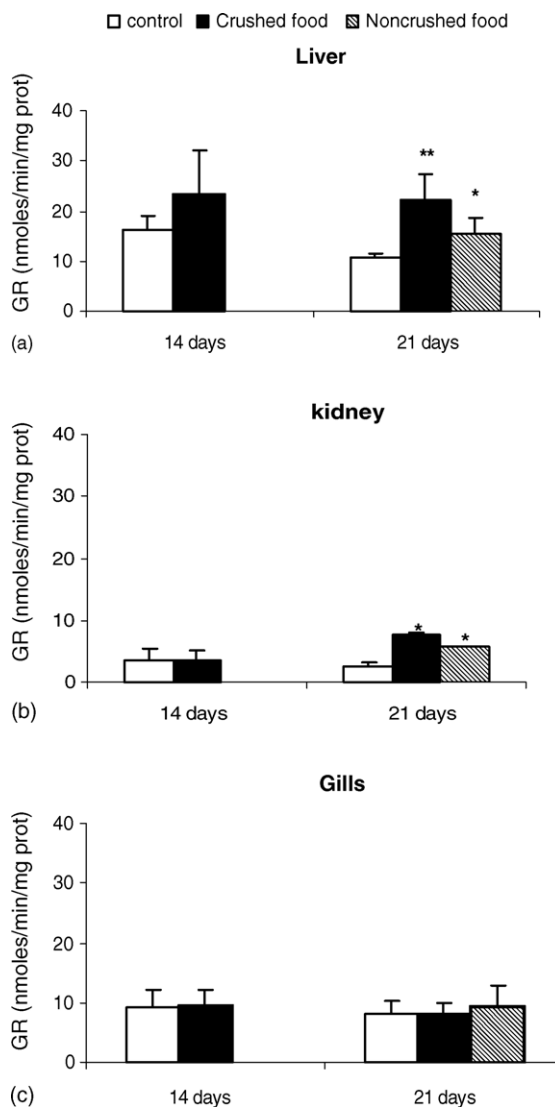


Fig. 5. Glutathione reductase activity in (a) liver, (b) kidney and (c) gills of control fish and fish exposed to cyanobacterial cells (MC-LR offered to fish = 60.0 μg fish/day) for two different types of administration: crushed and non-crushed food. The values are expressed as mean \pm S.E. ($n = 8$). GR activity is expressed as nmol NADPH oxidized/min/mg protein. The significance levels observed are * $p < 0.05$ and ** $p < 0.01$ in comparison to control group values.

4. Discussion

This study revealed that the microcystin-LR contained in cyanobacterial blooms induce oxidative stress in tilapia (*Oreochromis* sp.) that are exposed to re-

peated doses of toxins. The antioxidant status of the fish changed although no observable effects were detected in this species.

Toxic cyanobacterial blooms can be associated with fish mortality (Zimba et al., 2001). Some authors have evaluated the intraperitoneal exposure of such fish species as common carp (Rabergh et al., 1991; Carbis et al., 1997) and rainbow trout (Kotak et al., 1996) to microcystins, with particular emphasis on histopathological aspects. All of these studies showed necrosis and degeneration in liver and kidney, and Carbis et al. (1997) also indicated that MCs may damage the gill epithelium.

Similar changes have been recorded when fish are orally exposed to microcystins. Carps exposed to MCs underwent damage to renal proximal tubular cells and hepatocytes (Fisher and Dietrich, 2000). Carps taken from lake Mokoan (Australia) suffer from liver and gill damage (Carbis et al., 1997). Best et al. (2001) demonstrated cardiac alterations, and Bury et al. (1995) observed ionic imbalance and reduced growth in brown trout after chronic exposure.

Very few studies have been made on the subchronic oral toxicity of MCs in fish under laboratory conditions. Soares et al. (2004) observed the accumulation of MCs in *Tilapia rendalli* and Li et al. (2004) showed growth inhibition and severe damage to hepatocytes in MC-treated carps. Both these studies administered the toxin in a similar way to the present paper. However, neither of them focused on the oxidative stress induced by microcystins.

Cyanobacteria can be a part of the diet of several species of fish (Bowen, 1982) and high numbers of toxic *Microcystis* cells have been recorded in fish guts, which confirms that tilapia (*Oreochromis niloticus* L.) do graze on toxic cyanobacteria (Mohamed et al., 2003). The way in which the microcystins were administered – whether the cyanobacterial cells were non-crushed or crushed with the commercial fish food – affected diverse oxidative stress biomarkers. The latter produced major significant effects in most of the organs studied. When the cyanobacterial cells are mixed with the commercial food and the mixture thoroughly crushed, the toxins are apparently released and become easily bioavailable.

Moreover, the results show that microcystin-LR contained in cyanobacterial blooms induce oxidative stress in a time-dependent manner. After the 14-day

treatment, none of the biomarkers studied had been induced in either liver, kidney or gills. After 21 days of exposure, on the other hand, oxidative stress was evident by the induction of lipid peroxidation and the increase in antioxidant enzymatic activities.

The extent of LPO was determined by the balance between the production of oxidants, and the removal and scavenging of those oxidants by antioxidants. The MDA level generally increased in the organs studied after 21 days of exposure. Many studies have demonstrated that lipid peroxidation and oxidative stress increases in tissues of different species of aquatic organisms, as a result of being exposed to environmental stressors (Wiston and DiGiulio, 1991), but this is the first study to focus on microcystins. However, it is by no means a general rule that exposure to a pollutant increases the MDA level. Some authors have shown lowered MDA levels in fish sampled in a site contaminated by metal and organic compounds (Rodríguez-Ariza et al., 1993), and others have shown that fish show no response when exposed to azinphosmethyl and 2,4-dichlorophenoxyacetic acid (Oruc and Üner, 2000). The increase in the MDA level observed in the three organs of the tilapia studied agreed with previous investigations carried out with rats that had been intraperitoneally exposed to microcystins (Moreno et al., 2003).

As far as SOD and CAT activities are concerned, a simultaneous induction response is usually observed after exposure to pollutants (Dimitrova et al., 1994). However, in the present study no such relationship was observed in kidney and gills of the organs evaluated. Fish exposed to the crushed diet for 21 days underwent a 2.6-fold increase in CAT activity in liver, very similar to the 2.7-fold increase observed in SOD activity. Li et al. (2003) also reported enhanced SOD and CAT activities in the hepatocytes of common carp (*Cyprinus carpio* L.), induced by MC-LR. CAT activity increased 2.7 times also in kidney while the induction of SOD activity was not significant and gills showed no statistical change in the activity of CAT, although SOD was significantly increased after 21 days (non-crushed food), but the enzyme activities were very low in the kidney and gills compared to the liver.

The increase in GPx activity observed, predominantly in liver and kidney, is similar to the results reported by Li et al. (2003) who studied the responses of the antioxidant systems in the hepatocytes of common

carp (*Cyprinus carpio* L.) to microcystin-LR. The increase in GPx activity probably reflects an adaptation to the oxidative conditions to which the fish had been exposed (Lenartova et al., 1997). However, GPx activity decreased in gills after the longer exposure time, corroborating the LPO increase in this organ; this may be explained because gills are less efficient than kidney and liver at neutralizing the impact of peroxidative damage (Sayeed et al., 2003; Ahmad et al., 2004).

The GR enzyme has an important role in cellular antioxidant protection because it catalyzes the regeneration of GSH from GSSG. Its induced activity after 21 days of exposure reveals an enhanced production of GSSG predominantly in liver and kidney. In general, Stephensen et al. (2000) demonstrated that fish from polluted sites have high GR activity because of higher peroxidative components in the polluted aquatic site.

The results suggest that microcystins cause oxidative stress and that the antioxidant enzymes are induced as a defence mechanism. However, they fail to prevent lipid peroxidation. The liver was the most affected organ in all cases because microcystins are potent hepatotoxins in mammals and fish (Fisher and Dietrich, 2000; Towner et al., 2002). The kidney was the next most affected organ. This agrees with the results obtained by other authors who reported kidney lesions induced by MC-LR in fish (Rabergh et al., 1991; Kotak et al., 1996; Fisher and Dietrich, 2000). Finally, gills were the least affected organs probably due to the more important role of the oral exposure in this study. Rodger et al. (1994) reported severe gill damage in brown trout after the lysis of a cyanobacterial bloom at Loch Leven, Scotland and there are even studies that consider that gills are the organs that are most sensitive to the LPO induced by xenobiotics, because their antioxidant potential is weaker than that of other organs (Sayeed et al., 2003). However, some laboratory studies have shown that the gill and skin epithelia of freshwater fish form a barrier to microcystin transport (Tencalla et al., 1994; Bury et al., 1995) and, consequently, ingestion may be the most probable route for microcystin uptake in fish (Bury et al., 1998).

Data about the induction of oxidative stress by microcystins in fish are scarce, because most of the studies on microcystins in fish focus on histopathological aspects, as has been stated above. Bury et al. (1996) studied the stress responses of brown trout, *Salmo trutta* L., to *Microcystins aeruginosa* and analyzed plasma cor-

tisol, glucose and ion (Na^+ and Cl^-) levels. They concluded that fish had a classic stress response. Li et al. (2003) studied the responses of antioxidant systems in the hepatocytes of common carp to the toxicity of MC-LR. They showed that SOD, CAT and GPx activities increased and concluded that the toxicity mechanisms of MC-LR on fish hepatocytes were similar to those on mouse hepatocytes. Other authors, however, have observed that lesions in the liver and kidney of fish exposed to MC-LR are considerably different than those previously reported for mammals (Kotak et al., 1996).

5. Conclusion

The results of this study showed that when tilapia fish were exposed to cyanobacterial cells under laboratory conditions (60.0 μg MC-LR/fish/day) the endogenous antioxidant defense system were altered in a time-dependent manner. Simultaneously, the lipid peroxidation level increased significantly in the liver, kidney and gills of the fish treated with MC-LR for 21 days, particularly when fish were exposed to crushed cyanobacterial cells. These findings suggest that oxidative stress plays an important role in in vivo MC-LR induced toxicity in tilapia fish.

Acknowledgements

Lyophilised cyanobacteria from blooms were kindly supplied by Dr. Susana Franca and Dr. Paulo Pereira (Lisboa, Portugal). The authors wish to thank the CI-CYT (AGL 2002-02622) for the financial support for this study.

References

- Ahmad, I., Pacheco, M., Santos, M.A., 2004. Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbor water exposure. *Ecotox. Environ. Safe.* 57, 290–302.
- Almeida, J.A., Diniz, Y.S., Marques, S.F.G., Faine, L.A., Ribas, B.O., Burneiko, R.C., Novelli, E.L.B., 2002. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. *Environ. Int.* 27, 673–679.

- Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 195, 133–140.
- Best, J.H., Eddy, F.B., Codd, G.A., 2001. Effects of purified microcystin-LR and cell extracts of *Microcystis* strains PCC 7813 and CYA 43 on cardiac function in brown trout (*Salmo trutta*) alevine. *Fish Physiol. Biochem.* 24, 171–178.
- Bouaïcha, N., Maatouk, I., 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured rat hepatocytes. *Toxicol. Lett.* 148 (1–2), 53–63.
- Bowen, S.H., 1982. Feeding and digestion and growth-qualitative considerations. In: Pullin, R.S.V., Lowe-McConnell, R.H. (Eds.), *The Biology and Culture of Tilapias*. International Center for Living Aquatic Resources Management (ICLARM), Manila, pp. 141–156.
- Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 72, 248–254.
- Bury, N.R., Eddy, F.B., Codd, G.A., 1995. The effects of the cyanobacterium *Microcystis aeruginosa*, the cyanobacterial toxin microcystin-LR and ammonia on growth rate and ionic regulation of brown trout (*Salmo trutta* L.). *J. Fish Biol.* 46, 1042–1054.
- Bury, N.R., Eddy, F.B., Codd, G.A., 1996. Stress responses of brown trout, *Salmo trutta* L., to the cyanobacterium, *Microcystis aeruginosa*. *Environ. Toxicol. Water Qual.* 11 (3), 187–193.
- Bury, N.R., Newlands, A.D., Eddy, F.B., Codd, G.A., 1998. In vivo and in vitro intestinal transport of H-microcystin-LR, a cyanobacterial toxin, in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 42, 139–148.
- Carbis, C.R., Rawlin, G.T., Grant, P., Mitchell, G.F., Anderson, J.W., McCauley, I., 1997. A study of feral carp, *Cyprinus carpio* L., exposed to *Microcystis aeruginosa* at Lake Mokoan, Australia, and possible implications for fish health. *J. Fish Dis.* 20, 81–91.
- Carlberg, I., Mannervik, B., 1975. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* 250, 5475–5480.
- Carmichael, W.W., 1994. The toxins of cyanobacteria. *Sci. Am.* 270, 78–86.
- Carmichael, W.W., Falconer, I.R., 1993. Diseases related to freshwater blue green algal toxins, and control measures. In: Falconer, I.R. (Ed.), *Algal Toxins in Seafood and Drinking Water*. Academic Press, London, pp. 187–209.
- Dawson, R.M., 1998. The toxicology of microcystins. *Toxicol.* 36 (7), 953–962.
- Dimitrova, M.S.T., Tsinova, V., Velcheva, V., 1994. Combined effects of zinc and lead on the hepatic superoxide dismutase–catalase system in carp (*Cyprinus carpio*). *Comp. Biochem. Physiol.: Part C* 108, 43–46.
- Ding, W.X., Shen, H.M., Shen, Y., Zhu, H.G., Ong, C.N., 1998. Microcystic cyanobacteria causes mitochondrial membrane potential alteration and reactive oxygen species formation in primary cultured rat hepatocytes. *Environ. Health Persp.* 106, 409–413.
- Ding, W., Shen, H., Ong, C., 2001. Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. *J. Toxicol. Environ. Health A* 64 (6), 507–519.
- Ding, W., Ong, C., 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett.* 220 (1), 1–7.
- Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonetal. *Meth. Enzymol.* 186, 407–421.
- Falconer, I., Bartram, J., Chorus, I., Kuiper-Goodman, T., Utkilen, H., Burch, M., Codd, G.A., 1999. Safe levels and safe practices. In: Chorus, I., Bartram, J. (Eds.), *Toxic Cyanobacteria in Water – A Guide to Their Public Health Consequences, Monitoring and Management*. E & FN Spon, London, pp. 155–178.
- Fastner, J., Codd, G.A., Metcalf, J.S., Woitke, P., Wiedner, C., Utkilen, H., 2002. An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. *Anal. Biochem. Chem.* 374, 437–444.
- Filho, W., 1996. Fish antioxidant defenses. A comparative approach. *Braz. J. Med. Bio. Res.* 29, 1735–1742.
- Filho, W., Baptista, L., Soares, C., Pedrosa, R., 1997. The effect of pulp mill effluent on two fish species. In: *Proceedings of the Fifth Brazilian Symposium on Chemistry of Lignins and Other Wood Components*, Curitiba, Brazil, 15–17 October, pp. 612–619.
- Filho, W., Torres, M.A., Marcon, J.L., Fraga, C.G., Boveris, A., 2000. Comparative antioxidant defences in vertebrates – emphasis on fish and mammals. *Trends Comp. Biochem. Physiol.* 7, 33–45.
- Fisher, W.J., Dietrich, D.R., 2000. Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* 164, 73–81.
- Guzman, R.E., Solter, P.F., 1999. Hepatic oxidative stress following prolonged sublethal microcystin LR exposure. *Toxicol. Pathol.* 5, 582–588.
- Halliwell, B., Gutteridge, J.M.C., 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219, 1–14.
- Hermansky, S.J., Stohs, S.J., Eldeen, Z.M., Roche, V.F., Mereish, K.A., 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. *J. Appl. Toxicol.* 11, 65–73.
- Hooser, S.B., 2000. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. *Toxicol. Pathol.* 28, 726–733.
- Hooser, S.B., Beasley, V.R., Lovell, R.A., Carmichael, W.W., Haschek, W.M., 1989. Toxicity of microcystin LR, a cyclic heptapeptide hepatotoxin from *Microcystis aeruginosa*, to rats and mice. *Vet. Pathol.* 26, 246–252.
- Kotak, B.J., Semalulu, S., Friyzt, D.L., Prepas, E.E., Hrudehy, S.E., Coppock, R.W., 1996. Hepatic and renal pathology of intraperitoneally administered microcystin-LR in rainbow trout (*Oncorhynchus mykiss*). *Toxicol.* 34, 517–525.
- Lawrence, A., Burk, R.F., 1976. Glutathione peroxidase activity in selenium deficient rat liver. *Biochem. Biophys. Res. Commun.* 71, 952–958.
- Lawrence, J.F., Menard, C., 2001. Determination of microcystins in blue-green algae, fish and water using liquid chromatography with ultraviolet detection after sample clean-up employing

- immunoaffinity chromatography. *J. Chromatogr. A* 922, 111–117.
- Lenartova, V., Holovska, K., Pedrajas, J.R., Martínez-Lara, E., Peinado, J., López-Barea, J., Rosival, I., Kosuth, P., 1997. Antioxidant and detoxifying fish enzymes as biomarkers or river pollution. *Biomarkers* 2, 247–252.
- Li, X., Liu, Y., Song, L., Liu, J., 2003. Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. *Toxicol.* 42, 85–89.
- Li, X., Chung, I., Kim, J., Lee, J., 2004. Subchronic oral toxicity of microcystin in common carp (*Cyprinus carpio* L.) exposed to *Microcystis* under laboratory conditions. *Toxicol.* 44, 821–827.
- Magalhaes, V.F., Soares, R.M., Azevedo, S.M., 2001. Microcystin contamination in fish from the Jacarepagua Lagoon (Rio de Janeiro Brazil): ecological implication and human health risk. *Toxicol.* 39, 1077–1085.
- Mankiewicz, J., Tarczynska, M., Fladmark, K.E., Doskeland, S.O., Walter, Z., Zalewski, M., 2001. Apoptotic effect of cyanobacterial extract on rat hepatocytes and human lymphocytes. *Environ. Toxicol.* 16, 225–233.
- McCord, J.M., Fridovich, I., 1969. Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein). *J. Biol. Chem.* 244, 6049–6055.
- Mohamed, Z.A., Carmichael, W.W., Hussein, A.A., 2003. Estimation of microcystins in the freshwater fish *Oreochromis niloticus* in an Egyptian fish farm containing a *Microcystis* bloom. *Environ. Toxicol.* 18, 137–141.
- Moreno, I., Mate, A., Repetto, G., Vázquez, C.M., Cameán, A.M., 2003. Influence of microcystin-LR on the activity of membrane enzymes in rat intestinal mucosa. *J. Physiol. Biochem.* 59 (4), 293–300.
- Moreno, I.M., Maraver, J., Aguete, E.C., Leao, M., Gago-Martínez, A., Cameán, A.M., 2004a. Decomposition of microcystin-LR, microcystin-RR, and microcystin-YR in water samples submitted to in vitro dissolution tests. *J. Agric. Food Chem.* 52, 5933–5938.
- Moreno, I.M., Pereira, P., Franca, S., Cameán, A., 2004b. Toxic cyanobacteria strains isolated from blooms in the Guadiana river (southwest of Spain). *Biol. Res.* 37, 405–417.
- Oruc, E., Üner, N., 2000. Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of *Oreochromis niloticus*. *Comp. Biochem. Phys. C* 127, 291–296.
- Packer, J.E., 1995. Oxidative stress, antioxidants, aging and disease. In: Gutter, R.G., Packer, L.B., Eratum, J., Mori, A. (Eds.), *Oxidative Stress and Aging*. Birkhauser Verlag, Basle, pp. 152–163.
- Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., Raisuddin, S., 2003. Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl & Schn.). *Sci. Total Environ.* 309, 105–115.
- Rabergh, C.M.I., Bylund, G., Eriksson, J.E., 1991. Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium (blue-green algae) *Microcystis aeruginosa*, on common carp (*Cyprinus carpio* L.). *Aquat. Toxicol.* 20, 131–146.
- Rodger, H.D., Turnbull, T., Edwards, C., Codd, G.A., 1994. Cyanobacterial (blue-green algal) bloom associated pathology in brown trout (*Salmo trutta* L.) in Loch Leven. Scotland *J. Fish Dis.* 17, 177–181.
- Rodríguez-Ariza, A., Peinado, J., Pardo, C., López-Barea, J., 1993. Biochemical indicators of oxidative stress in fish from polluted littoral areas. *Can. J. Fish Aquat. Sci.* 50, 2568–2573.
- Sayeed, I., Parvaez, S., Pandey, S., Bin-Hafeez, B., Haque, R., Raisuddin, S., 2003. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. *Ecotoxicol. Environ. Safe.* 56, 295–301.
- Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, M.J. (Eds.), *Toxic Cyanobacteria in Water. A Guide to Their Public Health Consequences, Monitoring and Management*. E & FN Spon, London, pp. 41–111.
- Soares, R.M., Magalhaes, V.F., Azevedo, S., 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (Cichlidae) under laboratory conditions. *Aquat. Toxicol.* 70, 1–10.
- Stephensen, E., Svavarsson, J., Sturve, J., Ericson, G., Adolfsson-Erici, M., Forlin, L., 2000. Biochemical indicators of pollution exposure in shorthorn sculpin (*Myoxocephalus scorpius*), caught in four harbours on the southwest coast of Iceland. *Aquat. Toxicol.* 48, 431–442.
- Tencalla, F.G., Dietrich, D.R., Schattler, C., 1994. Toxicity of *Microcystis aeruginosa* peptide toxin to yearling rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 30, 215–224.
- Towner, R.A., Sturgeon, S.A., Hore, K.E., 2002. Assessment of in vivo oxidative lipid metabolism following acute microcystin-LR-induced hepatotoxicity in rats. *Free Radic. Res.* 36 (1), 63–71.
- Wiegand, C., Pflumacher, S., Oberemm, A., Meems, N., Beattie, K.A., Steinberg, C.E.W., Codd, G.A., 1999. Uptake and effects of microcystin-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). *Environ. Toxicol.* 14, 89–95.
- Wiston, G.W., DiGiulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 19, 137–161.
- Zegura, B., Lah, T.T., Filipic, M., 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology* 200, 59–68.
- Zimba, P.V., Khoo, L., Gaunt, P., Carmichael, W.W., Brittain, S., 2001. Confirmation of catfish mortality from *Microcystis* toxins. *J. Fish Dis.* 24, 41–47.