Phenological and Liver Antioxidant Profiles of Adult Nile Tilapia (*Oreochromis niloticus*) Exposed to Toxic Live Cyanobacterium (*Microcystis aeruginosa* Kützing) Cells

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Blue-green algae (cyanobacteria) constitute the greater part of the phytoplankton. *Microcystis aeruginosa* is amongst the most ubiquitously distributed cyanobacterial species, and almost invariably produces cyclic heptapeptide toxins called microcystins (MCs). The present study was designed to investigate the phenological and liver antioxidant profiles of the Nile tilapia *Oreochromis niloticus* chronically exposed to toxic live *M. aeruginosa* cells. Fish were grown in the absence and presence of *M. aeruginosa* in three different concentrations for seven days, and subsequently reared for another 30 days in the absence of the cyanobacteria. While cyanobacteria did not cause any fish mortality, there was a progressive development of yellowish discolouration in the livers of exposed fish. In the livers, the activities and levels of superoxide dismutase (SOD), lactate dehydrogenase (LDH), glutathione (GSH), and lipid peroxidation products like malondialdehyde (MDA) were elevated in response to the concentration of *M. aeruginosa.* Moreover, DNA fragmentation and DNA-protein cross-links were measured. These parameters can thus be considered potential biomarkers for the fish exposure to *M. aeruginosa*. The present study sheds light on cyanobacterial blooms like health, environmental, and economic problem, respectively.

Key words: Cyanobacteria, *Oreochromis niloticus*, Oxidative Stress

Introduction

Eutrophication in water bodies is linked to anthropogenic influences such as climate change, poor agricultural practices, and the global nutrient run off from human waste disposal (Milinkovitch *et al*., 2011). Phytoplankton communities of eutrophic lakes are often dominated by cyanobacteria that can form dense blooms (Gragnani *et al*., 1999). Many cyanobacteria including the genera *Microcystis* and *Anabaena* produce toxic bioactive compounds like the hepatotoxic microcystin-LR and anatoxin-a (Fischer *et al*., 2000; El-Sheekh *et al.*, 2010; Ibrahem *et al.*, 2012). *Microcystis* spp. cyanobacteria grow in marine, brackish, and freshwater bodies (Fleming and Stephen, 2001). Blooming of toxic cyanobacteria has been detected in many ponds in Egypt (Shakweer and Gharib, 2005).

Cyanobacteria are harmful to both humans (Carmichael, 2001) and fish (Malbrouck and Kestemont, 2006). They cause direct intoxication of animals and humans through contact with water bloom or indirect poisoning by consumption of contaminated food (Mohamed *et al*., 2003). Besides being a source of nutrition for aquatic life, cyanobacterial blooms cause health hazards for terrestrial vertebrates. Toxic cyanobacteria in surface water ecosystems (Briand *et al.*, 2003) cause hypoxia and clogging of gills of fish, and mortality in wild and cultured fish has been associated with mass occurrence of cyanobacteria. Uptake of cyanobacterial toxins by fish occurs primarily via oral ingestion of toxic cyanobacterial cells and, to a lesser or even negligible extent, from toxin uptake by the gill epithelium (Bury *et al.*, 1995).

Microcystins are produced inside the cyanobacterial cells, and when cells lyse upon death the toxin is released into the water. Although microcystins can be broken down by some bacterial proteases, the toxin can persist for months

or even years in natural water bodies, when bacteria producing such proteases are absent (Rapala, 2005).

Fish are exposed to the toxin either directly or by ingestion of cyanobacterial cells or a prey contaminated with cyanobacteria (Fischer *et al.*, 2000). The liver functions in the biodegradation and biotransformation of toxins and exhibits an adaptive response. Microcystins are actively taken up by the liver of fish where they disrupt normal cellular activity by inhibiting all protein phosphatases (Meier-Abt, 2007). Inhibition of these enzymes in fish can ultimately result in widespread cellular death and loss of the liver structure (Malbrouck and Kestemont, 2006). Protein phosphatases are particularly important during fish embryonic development because they regulate critical developmental processes (Gotz, 2000).

Most of the previously conducted studies were restricted to the acute, rather than chronic exposure to toxin or bloom. In addition, the exposure routes in most previous studies did not realistically reflect environmentally relevant situations.

The aim of this study was to investigate the chronic exposure of Nile tilapia (*Oreochromis niloticus*) to *M. aeruginosa* with regard to phenological alterations and oxidative stress parameters.

Material and Methods

Fish

A total of 60 male *Oreochromis niloticus* of (40.0 ± 0.5) g were netted, collected alive from an unpolluted semi-intensive fish farm, and transported in water tanks to the Fish Disease and Management Laboratory (FDML), Cairo University, Cairo, Egypt. Fish were acclimated to laboratory conditions during 2 weeks prior to the experiments. They were maintained in a tank containing chlorine-free aquarium water and acclimatized in a temperature-controlled environment, with a 12-h light/12-h dark cycle at (26 ± 2) °C and a pH value of about 7.0. Ammonia-nitrogen was monitored once a week; it was less than 0.5 mg L^{-1}, and residual chloride was less than 0.01 mg L^{-1} (APHA, 1992). Fish were fed twice a day with a balanced commercial pelleted fish diet with 30% protein (Zoocontrol Company, Cairo, Egypt) and starved 48 h before an experiment to clear their gut contents.

Microcystis aeruginosa

Microcystis aeruginosa was originally isolated, according to the procedure of Stein (1973), from a water sample from a River Nile channel near Tanta City, Egypt. One or two drops of the collected water sample were streaked on agar plates containing Allen and Stanier (1968) medium. The plates were then incubated for one week under controlled laboratory conditions [irradiance, 80 μmol m^{-2} s^{-1}; temperature, (25 ± 2) °C] in a culture chamber. The cultures of *M. aeruginosa* were grown axenically according to Venkataraman (1969). After one week, a single colony was transferred from an agar plate to an 1-L flask containing 500 mL of Allen and Stanier medium and incubated for 1–2 weeks. The cultures were then aerated with sterilized air and grown under the same conditions as during isolation. *M. aeruginosa* was identified according to Prescott (1978).

The growth of *M. aeruginosa* was measured by following the optical density at 750 nm according to Lerfort-Tran *et al.* (1988) and at the end of the exponential growth phase, the cells were harvested.

In a previous study by El-Sheekh *et al.* (2010), intracellular microcystin-LR (MC-LR) was quantified in *M. aeruginosa* during growth by high-performance liquid chromatography (HPLC) based on an MC-LR standard (Alexis Corporation, Lausen, Switzerland). The intracellular MC-LR concentration was 6.65 μg (25 ml of cells)$^{-1}$ which was equivalent to 65.5 μg g dry weight^{-1}, and the toxicity of MC-LR was confirmed in the brine shrimp (*Artemia salina*) bioassay according to Kiviranta *et al.* (1991).

Experimental design

Four groups of the previously acclimated *O. niloticus*, each containing 15 fish, were assigned for the experimental study. Three replicates, each consisting of 5 fish per group, were run. Each 5 fish were separately held in a glass aquarium containing 10 L of de-chlorinated water. A stock culture of *M. aeruginosa* containing $8 \cdot 10^8$ cells mL^{-1} was used to prepare cultures of $8 \cdot 10^4$ cells mL^{-1} (group 1), $16 \cdot 10^4$ cells mL^{-1} (group 2), and $24 \cdot 10^4$ cells mL^{-1} (group 3), respectively, in three aquaria for each concentration.

During the 7-d exposure trial, fish were dependent on *M. aeruginosa* as source of food, a fourth group was kept without any treatment,

assigned as the control (C), and fed a balanced diet (30% protein) throughout the test period. Except for siphoning off the faeces from the bottom of the jars, water was not changed but well aerated. By the end of one week, fish from each group were transferred separately to aquaria containing chlorine-free tap water and fed a fish diet (30% protein) for 30 d. The test was run in duplicate.

Mortality, clinical, and post-mortem investigations

All fish were monitored closely during the experimental period. Behavioural responses, clinical signs, and mortalities were recorded daily. Post-mortem examination was carried out at the end of the experimental period according to Amlacher (1970).

Organ samples

At the end of the experimental period, fish were killed by a sharp blow on the head followed by cervical dislocation. Livers were removed immediately frozen in liquid nitrogen, and stored at –80 °C for further investigations. Liver homogenate was prepared in PBS (phosphate-buffered saline) using a glass homogenizer. The homogenates were centrifuged at 13,000 x *g* for 10 min at 4 °C, and the supernatants were stored at –80 °C until analysed.

Determination of reduced glutathione (GSH) level

GSH level was determined according to Ellman (1959). Briefly, liver samples were homogenized with 25% (v/v) trichloroacetic acid (TCA) and centrifuged at 7,500 x *g* for 10 min. The supernatant (50 μL) was mixed with 230 μL of 0.8 M Tris-HCl [tris(hydroxymethyl)aminomethane-HCl], 0.02 M EDTA (ethylenediaminetetraacetic acid), pH 8.9, and 20 μL of 0.01 M DTNB (2,2'-dinitro-5,5'-dithiobenzoic acid). The reaction mixture was incubated for 5 min at room temperature, before the absorbance of the formed GSH-DTNB conjugate was determined at 412 nm.

Determination of lactate dehydrogenase (LDH) activity

LDH activity was determined using a kit from Spectrum Diagnostics (Cairo, Egypt) according to the manufacturer's instruction.

Determination of superoxide dismutase (SOD) acitivity

SOD activity was determined according to the procedure described by Giannopolitis and Ries (1977). Briefly, 1 mL of 50 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer, pH 7.8, was added to 30 μL of 0.1 mM NaEDTA, 1 mL of 50 mM Na_2CO_3, 150 μL of 10 mM methionine, 150 μL of 63 μM NBT (nitroblue tetrazolium), and 30 μL of 1.3 μM riboflavin. The total volume was made up to 3 mL with sample; then the absorbance was read at 560 nm.

Determination of lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined as the thiobarbituric acid reactive substance (TBARS) as described by Albro *et al.* (1986). In a centrifuge tube, 2.5 mL of 10% TCA were added to 0.5 mL of tissue homogenate and then put in a boiling water bath for 15 min. Tubes were cooled under tap water and centrifuged at 2,500 x *g* for 10 min. Then, 2 mL of the supernatant were transferred to a test tube, and 1 mL of 0.67% thiobarbituric acid (TBA) was added. Tubes were shaken and kept in a boiling water bath for 20 min, then cooled under tap water, and the optical density was read at 532 nm against the blank reagent TBA.

Determination of protein

Protein contents in the samples were determined by the method of Bradford (1976).

DNA fragmentation assay

The DNA fragmentation assay was conducted according to Sellins and Cohen (1987). Tissues were lysed in 1 mL buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% Triton X-100). The pellets containing total intact DNA (designated P) and the supernatants containing smaller fragments of DNA (designated S) were treated separately with 0.5 mL of 25% TCA. Both sets were

left overnight at 4 °C, and precipitated DNA was collected by centrifugation. Each pellet was treated with 80 μL of 5% TCA followed by heat treatment at 90 °C for 15 min. One mL freshly prepared diphenylamine (DPA) reagent was added to each sample, tubes were allowed to stand overnight at room temperature, and the optical density was recorded at 600 nm. Percentage DNA fragmentation was calculated as follows:

% DNA fragmentation = $[S/(S + P)] \cdot 100$, where S is the optical density of the supernatant and P the optical density of the pellet.

DNA-protein cross-links

DNA-protein cross-links were determined according to the procedure described by Zhitkovich and Costa (1992). Briefly, 1.5 mL of 2% sodium dodecyl sulfate (SDS) was added to 1.5 mL of liver homogenate. The mixture was kept frozen at –20 °C for 12 h. The sample was thawed and incubated at 65 °C for 15 min. Then, 1.5 mL of 200 mM KCl was added and the mixture cooled on ice for 5 min and centrifuged for 10 min at 150 x *g*. The upper phase that contained free DNA was removed. The pellets, containing protein-bound DNA, were re-suspended in an equal amount of KCl and SDS, and precipitation was repeated three times. Proteinase K was added to digest the DNA-protein cross-links, and samples were incubated for 3 h at 50 °C, then centrifuged for 10 min at 1,500 x *g*. The supernatant containing bound DNA was removed. One mL of a solution containing 0.88 M DPA in a solution containing 98% (v/v) glacial acetic acid, 1.5% (v/v) sulfuric acid, and 0.5% (v/v) of 1.6% acetaldehyde solution was added to 0.5 mL of extracted DNA (free and bound). The absorbance was read at 578 nm. The percentage of DNA-protein cross-links was calculated according to:

% DNA-protein cross-links = $[OD$ bound$/(OD$ free + OD bound$)] \cdot 100$, where OD bound is the optical density of protein-bound DNA in the pellet, and OD free is the optical density of free DNA in the upper phase.

Statistical analysis

Values were expressed as mean ± SE (standard error). The statistical analysis was performed using one way ANOVA (mean at significance level of $p \leq 0.05$) according to Duncan (1955).

Results

Mortality, clinical, and post-mortem investigations

No mortality occurred in any of the aquaria throughout the test duration (30 days). The recorded abnormal manifestations were directly correlated with the concentration of *M. aeruginosa* cells. The fish in groups 2 and 3 (exposed to $16 \cdot 10^4$ and $24 \cdot 10^4$ cells mL^{-1}, respectively, of *M. aeruginosa*) showed a gradual loss of appetite and lacked signs of escape reflex. No behavioural changes or any mortality was recorded in the control group throughout the period of the exposure. Behavioural, clinical, and post-mortem changes were noticeable among fish of groups 2 and 3, but not in fish of group 1 exposed to $8 \cdot 10^4$ cells mL^{-1}. Post-mortem examination revealed obvious skin paleness in fish of groups 2 and 3. Visual inspection of the gastrointestinal tract at the end of the experiment revealed a progressive development of yellowish discolouration in the livers of treated fish.

Determination of GSH level

Fish exposed to different concentrations of *M. aeruginosa* cells had significantly elevated levels of GSH in comparison with the negative control group. This elevation was significant in groups 2 and 3 (Fig. 1).

Determination of LDH activity

The LDH activity was significantly increased in groups 1, 2, and 3 compared to the control group (Fig. 2).

Determination of SOD activity

The SOD activity was significantly increased in groups 2 and 3 (Fig. 3).

Determination of lipid peroxidation

Significant changes were observed in the MDA level in exposed fish corresponding to the different concentrations of *M. aeruginosa* cells. The highest MDA level was detected in group 3 (Fig. 4).

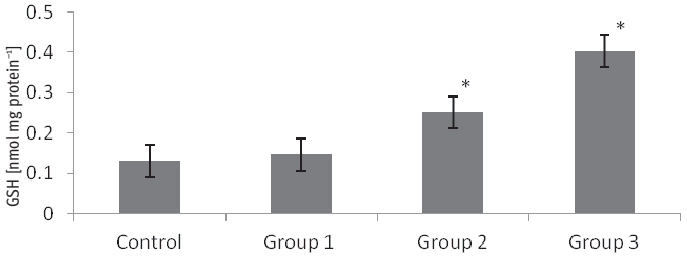

Fig. 1. Glutathione (GSH) level in livers of control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as means ± SE (n = 5). GSH levels are expressed as nmol mg protein^{-1}. * Significantly different from the control group ($p \leq 0.05$).

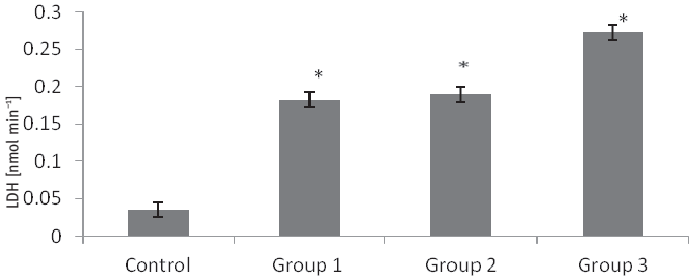

Fig. 2. Lactate dehydrogenase (LDH) activity in livers of control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as means ± SE (n = 5). LDH activity is expressed as nmol min^{-1}. * Significantly different from the control group ($p \leq 0.05$).

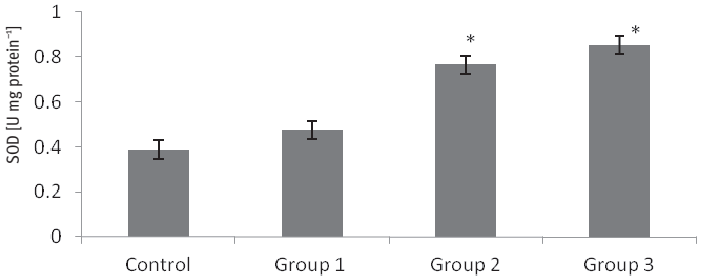

Fig. 3. Superoxide dismutase (SOD) activity in livers of control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as means ± SE (n = 5). SOD activity is expressed as U mg protein^{-1}. * Significantly different from the control group ($p \leq 0.05$).

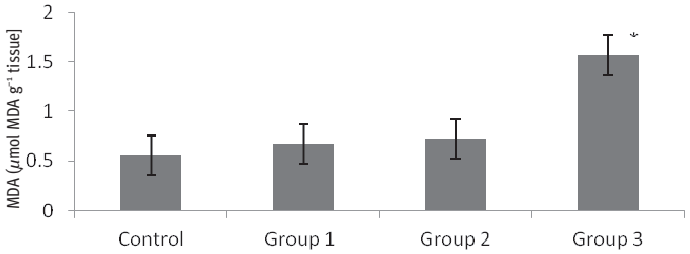

Fig. 4. Malondialdehyde (MDA) levels, as indicator of lipid peroxidation, in livers of control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as means ± SE (n = 5). Lipid peroxidation values are expressed as μmol MDA g tissue^{-1}. * Significantly different from the control group ($p \leq 0.05$).

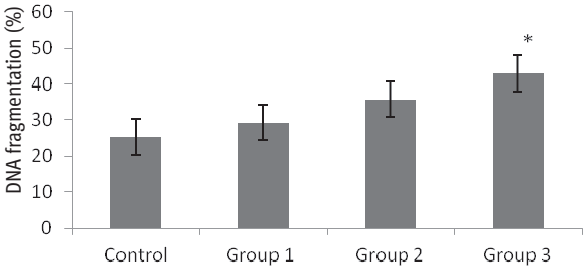

Fig. 5. DNA fragmentation assay in control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as percentage of total DNA means ± SE ($n = 5$). *Significantly different from the control group ($p \leq 0.05$).

Determination of DNA fragmentation

The DNA fragmentation percentage showed a significant elevation in group 3 in comparison to the negative control and other treated groups (Fig. 5).

Determination of DNA-protein cross-links

The percentage of DNA-protein cross-links was significantly increased in group 3 (Fig. 6).

Discussion

The population increase and the consequent intensification of agricultural and industrial activities have led to an increasing eutrophication of water bodies. Thus there are worldwide more frequent cyanobacterial blooms, and more fish are exposed to such blooms (Fleming and Stephen, 2001). Cyanobacteria can constitute part of the diet of several species of fish (Bowen, 1982), and high numbers of toxic *Microcystis* cells have been recorded in tilapia (*Oreochromis niloticus* L.) guts, which confirms that tilapia do graze on toxic cyanobacteria (Mohamed *et al.*, 2003).

Very few studies have been conducted on the effects of *Microcystis* cells at concentrations being chronically toxic to *O. niloticus* L. under laboratory conditions. Thus, we focused on studying the effects of exposing *O. niloticus* L. to water polluted with graded levels of *M. aeruginosa* for 30 days.

No mortality occurred in any of the fish groups during the entire test period. Clinical and behavioural abnormalities were observed in fish exposed to water containing $16 \cdot 10^4$ cells mL^{-1} and $24 \cdot 10^4$ cells mL^{-1} of *M. aeruginosa*. Gross morphological inspection of livers revealed a progressive development of yellowish discolouration in exposed fish. The results of the current study agreed with those of Tencalla and Dietrich (1997) on rainbow trout which were exposed to freeze-dried toxic cells of *M. aeruginosa* strain PCC 7806 at acutely toxic doses of 5700 μg (g microcystin · kg body weight)$^{-1}$. Jos *et al.* (2005) found that no *O. niloticus* died during exposure to intact and broken cyanobacterial cells containing 3230 μg g microcystin-LR^{-1}.

Microcystins in the cyanobacterial cells are responsible for the increase of cellular oxidative stress which subsequently can trigger apoptotic processes (Song *et al.*, 2006; Zhang *et al.*, 2008). Elevation of the GSH levels reflects stimulation of the detoxification metabolism as reported for aquatic organisms by Best *et al.* (2002).

There have been several laboratory and field studies documenting fish impairment associated with cyanobacterial blooms (Zimba *et al.*, 2001; Malbrouck and Kestemont, 2006), and some of them have shown that impairment is a function of the dose and time of exposure to microcystins (Prieto *et al.*, 2007; Zhang *et al.*, 2008).

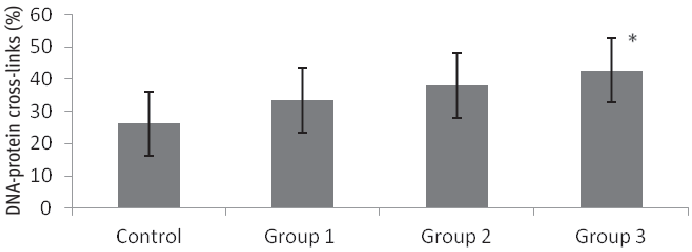

Fig. 6. DNA-protein cross-links in control fish and fish exposed to different concentrations of *M. aeruginosa* cells. The values are expressed as mean percentages ± SE ($n = 5$). *Significantly different from the control group ($p \leq 0.05$).

In the current study, the GSH levels were markedly elevated with increasing concentrations of *M. aeruginosa* cells (Fig. 1). An elevation of GSH and MDA levels, important biomarkers of oxidative stress, was detected in silver carp (*Hypophthalmichthys molitrix* Val.) grown in *M. aeruginosa* water blooms (Blaha *et al.*, 2004). Zegura *et al. (*2006) verified an increase in the levels of reduced GSH during the exposure to MC-LR. Concomitant with this increase was the expression of glutamate-cysteine ligase (GCL), the rate-limiting enzyme of GSH synthesis, indicating an increased rate of the *de novo* synthesis of GSH. Li *et al.* (2008) reported an increase in GST (glutathione *S*-transferases) mRNA abundance in liver, kidney, and intestine of goldfish intraperitoneally injected with cyanobacterial crude extracts at doses of 50 and 200 μg MC-LR kg body weight^{-1}.

Exposure of fish to cyanobacterial bloom induced DNA damage accompanied by an increased production of reactive oxygen species (ROS) and elevated LDH activity in the liver (Fig. 2).

The lipid peroxidation level was significantly increased only in group 3 (Fig. 4). This process of lipid peroxidation is controlled by an efficient cellular system involving SOD (Shika, 1996), which ensures the maintenance of cell integrity, optimum metabolism, and functional performance (Ernster, 1993). Changes in the levels of SOD were detected in fish exposed to different concentrations of *M. aeruginosa* cells such that the activity of SOD increased from group 1 to 3 (Fig. 3). SOD is inducible in mammals, plants, and microorganisms, and the level of the enzyme increases with an increased need of protection against ROS production (Fridovich, 1986).

DNA damage caused by oxidative stress was assessed by the DNA fragmentation assay using the DPA method. The magnitude of DNA fragmentation was a function of the concentration of the cyanobacteria that the fish had been exposed to (Fig. 5). The induction of DNA fragmentation has been explained by a genotoxic effect of MC-LR that may trigger apoptosis by endonuclease activation (Chen *et al.*, 2005).

Proteins interacting with specific DNA sequences to turn genes on or off, *i.e.* transcriptional regulators (Hunter and Karin, 1992), can lead to deletion of DNA sequences during DNA replication which may cause cell death if the cross-link is not repaired (Tsapakos *et al.*, 1981). DNA protein cross-linkage was significantly elevated in group 3 (Fig. 6). These results agree with those of Leao *et al.* (2008) who reported an elevation in DNA protein cross-links in polychaetes treated with microcystins.

The current study recorded potentially cumulative adverse effects (increases in SOD, LDH, GSH, and MDA) of increasing concentrations of toxic live *M. aeruginosa* cells on *O. niloticus*. These parameters can thus be considered potential biomarkers for exposure of fish to *M. aeruginosa.*

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