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# Toxic Response of *Daphnia magna* to *Microcystis aeruginosa*

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Microcystins produced by *M. aeruginosa* have adverse effects on aquatic organisms and are recognized as potent stress factors in aquatic ecosystems. As an ideal model organism for aquatic toxicology research, *D. magna* has been selected in this study to assess the adverse effects of microcystins in incipient stage of cyanobacteria *M. aeruginosa* bloom. The results show that the contents of total protein in *D. magna* of experimental groups have no obvious difference compared to the control groups during seven days, nor do the other physiological and biochemistry indicators such as albumin, alkaline phosphatase and total cholesterol. However, the alanine aminotransferase and glycerin trilaurate in *D. magna* decrease rapidly in experimental groups. Large amounts of lipid droplets are also observed in the body of *D. magna* fed on *M. aeruginosa*. The RAPD assay of genome experiment indicates both microcystin-LR and *M. aeruginosa* will induce DNA changes at the second and third generation of *D. magna*. The results suggest that *M. aeruginosa* will affect the *D. magna* on both DNA and metabolism.

**Key Words: *Microcystis aeruginosa*, Microcystins, Physiological and biochemical effect, DNA changes, *Daphnia magna*.**

## INTRODUCTION

Eutrophication of aquatic ecosystem is still a severe problem in some countries and regions. In recent years, people have been beset by cyanobacteria (*e.g. M. aeruginosa*) bloom commonly occurred in the Tai Lake and the Dian Lake in China. The cyanobacteria are low nutritional value food to plankton and toxic to zooplankton and fish[1-5]. The most commonly detected cyanobacteria toxins are microcystins, which contain microcystin-RR, microcystin-LR and so on[1,3]. The microcystins can cause internal injuries and even death to aquatic organisms. They are specific inhibitors of protein phosphatase 1 and protein phosphatase 2A, which have effects on tumor promotion[6]. They also cause extensive liver damage due to the destruction of the sinusoidal endothelium followed by a massive intrahepatic hemorrhage, which in turn leads to the death of animals[4,7,8].

*D. magna* is an important species in freshwater food chains. As they are primary consumers in aquatic ecosystem, they ingest suspended particles and phytoplankton including cyanobacteria, green alga and so on. Numerous studies have showed that microcystins have strong adverse effects on *Daphnia*, such as increasing mortality, decreasing growth rate, delaying maturation and decreasing offspring production[9-11]. *M. aeruginosa* and microcystins impair the crustaceans by the survivals and growth[12,13].

Many investigations have showed that physiological metabolism of clodecera are affected by contaminations in aquatic ecosystem including inhibiting enzyme activities, changing fatty acid composition, *etc.*[14-16]. For example, De Schamphelaere *et al*. reported that the concentrations of total fatty acids and total ω3-PUFA with dietary copper would decline obviously. Also the microcystins (from trace concentration to 200 μg $L^{-1}$) could inhibit the enzyme activities of *D. magna*[17]. But few researches have reported the impact of protein and fatty acids of *D. magna* when the organisms are exposed to microcystins.

Many investigations focus on environmental organic pollutants and heavy metals which affect the DNA duplication and repairment of *D. magna*[18]. For example, after exposed to benzo(*a*)pyrene [B(*a*)P] for 3 or 6 days, the DNA of *D. magna* showed changes in RAPD profiles[19] and DNA methylation of daphnia decreased significantly in the off spring when parental generation exposed to Zn[20]. So far no report has been found on DNA alterations of *D. magna* after *M. aeruginosa* treating.

In this study we discuss the physiological responses and genotoxicity of *D. magna* when exposed to microcystins. In most of the chronic studies, exposures were performed with one generation of Daphnids and lasted for around 3 weeks or

1 month[21], while in this study, we incepted the samples according to generations. We simulated the environment as preliminary stages of *M. aeruginosa* bloom when the concentration of MC-LR and *M. aeruginosa* individuals are at low levels to avoid the acute toxicity or death to *D. magna*[22].

## EXPERIMENTAL

**Test organisms and MC-LR:** *D. magna* were descended from laboratory clonal cultures and fed with *Chlorella vulgaris* and yeast. In the following experiments, the culture started from a single *Daphnia* to ensure that all the experimental individuals were genetically identical. The culture was maintained at 23 ± 1 ºC in a temperature controlled room. *M. aeruginosa* was used as the toxic source to *D. magna* and cultured in CT medium as the same as *C. vulgaris*. MC-LR standard substance was purchased from National CRM/RM information center (China). Analysis of MC-LR was performed using Shimadzu 20A HPLC system with a 5 μm 4.6 × 250 mm $C_{18}$ column in an isocratic run (1.0 mL/min) with 65 % of methanol and 35 % ultrapure water (containing 0.15 % trifluoroacetic acid). Column temperature was maintained at 38 ºC and the injection volume was 20 μL.

For physiological and biochemistry test, uniformity *D. magna* separation from the same generation were cultured in 40 beakers at 23 ± 1 ºC. Each beaker contained more than 50 individuals. Twenty beakers of cultures were fed with *M. aeruginosa* (1.0-5.0 × $10^5$ cell/mL), while the others were set as control groups fed with *C. vulgaris* (1.0 × $10^6$ cell $mL^{-1}$) and yeast. We chose 20 individuals randomly from one beaker of experimental or control groups for biochemistry analyses every 2 h in the first 12 h. Then samples were acquired each day until the 7th day when crustacean bred. The residual crustaceans were cultured to observe the breed situation of fecundity.

To analyze DNA alterations, we set groups as following.

• In control group C, *D. magna* was fed with *C. vulgaris* and yeast only. The first three generations of organisms in control groups were referred as $C_{F1}$, $C_{F2}$ and $C_{F3}$.

• In experimental group S, MC-LR (20 ± 5 ng $mL^{-1}$) was dissolved in the solution and the other status was similar to the control groups. Acute toxicity experiments with purified MC-LR have showed that the 48 h $LC_{50}$ range from 9.6-21.4 μg $mL^{-1}$ to *Daphnia* and 0.45-1.0 μg $mL^{-1}$ to *Diaptomus*[22], so we set the concentration of MC-LR at 20 ng $mL^{-1}$ for chronic experiment and then detected with HPLC system every day to make sure that the concentration is stable. The first three generations of organisms in this group were referred as $S_{F1}$, $S_{F2}$ and $S_{F3}$.

• In experimental group m, the organisms were fed on *M. aeruginosa* (1.0-5.0 × $10^5$ cell $mL^{-1}$). The first three generations of organisms in this group were referred as $m_{F1}$, $m_{F2}$ and $m_{F3}$.

When plenty of offspring were produced, old individuals were removed from beakers and 10 of them were collected and prepared for DNA profiles analysis.

**Measurement of biochemical indicators:** *D. magna* were harvested in a plastic tube and homogenized with a pestle. After centrifugated at 10,000 rpm for 3 min, the supernatant was reserved for further analysis of total protein (TP), alkaline phosphatase, alanine aminotransferase (ALT), albumin (ALB), glycerin trilaurate (TG) and total cholesterol (TC) by clinical chemistry analyzer (Mindary BS200, China).

**Determinations of *Daphnia magna* DNA profiles:** Genomic DNA from *D. magna* was extracted and purified using a conventional phenol/chloroform method. Ten *D. magna* individuals were homogenized in 500 μL of sperm lysis buffer (10 mM *Tris*-HCl, pH 8.0; 0.1 M ethylenediaminetetra acetic acid (EDTA), pH 8.0; 0.5 % SDS), added with 20 μL protein K and kept at 55 ºC for 2 h. The DNA was extracted using phenol, chloroform and isoamyl alcohol (25:24:1) mixture and precipitated in two volumes of ice-cold ethanol with the presence of 3 M sodium acetate (10 % of the DNA volume) at -20 ºC for 0.5 h. Precipitated DNA was finally collected by centrifugation, dried in clean air and dissolved in sterile analytical grade water.

The DNA profiles of *D. magna* were generated in RAPD reactions performed in a reaction volume of 25 μL. Profiles were amplified with primer OPB9 (GGGTAACGCC) and OPB7 (GGTGACGCAG). PCR amplification was then carried out as follows: 5 min at 95 ºC for predenaturation, followed by 40 cycles each consisting of denaturation at 95 ºC for 1 min, annealing at 50 ºC for 1 min and extension at 74 ºC for 1 min, with the final extension at 74 ºC for 10 min by using a DNA thermal cycler, at last 4 ºC stored prior to use. From each reaction tube, 10 μL of PCR products was separated by electrophoresis on 1.2 % (w/w) agarose gel in TAE buffer for 40 min.

**Statistical analysis:** All biochemical measurements were repeated three times and data were recorded as the mean with relatively standard deviation (RSD). Significant differences between control and test groups were assessed using a one-way analysis of variance (ANOVA) with Turkey's multiple comparisons, the limit of significance was set at $p = 0.05$.

## RESULTS AND DISCUSSION

**Physiological tests of *D. magna*:** The physiological and biochemical test lasted for 7 days. The average concentrations of total protein (Fig. 1a) and albumin (Fig. 1b) in experimental groups were 35.8 ± 3.4 μg $mg^{-1}$ and 12.0 ± 2.9 μg $mg^{-1}$ (wet weight and the same below), while 35.2 ± 3.0 μg $mg^{-1}$ (TP) and 10.0 ± 1.5 μg $mg^{-1}$ (ALB) in control groups, which showed no obvious changes in 7 days. But the percentage of albumin to total protein in experimental groups at the first day were higher than that in control groups and the difference was significantly at 2, 4, 8 and 10 h (Fig. 1c). No regular changes were observed as for the concentration of total cholesterol along with the experimental time (Fig. 1d).

Comparing with the enzyme alkaline phosphatase (Fig. 2 a), the activity of enzyme ALT (Fig. 2b) declined from the 8th h to the end of experiment. The enzyme alkaline phosphatase did not show significant trend in 7 days' experiment (Fig. 2a). The content of TG gradually declined in the experimental group except for the time from 2 h to 6 h (Fig. 3).

Individuals of *D. magna* were observed under microscope in the period of physiological and biochemical test. It was shown that the crustacean fed with *C. vulgaris* and yeast had lots of brown chyme in alimentary canal (Fig. 4a) and no

orbicular algae cells were observed. A lot of lipid droplets appeared in the body of organisms, which were fed with *M. aeruginosa* (Fig. 4b, c and d). Most lipid droplets appeared near the alimentary canal whose colour was green under microscope (Fig. 4c).

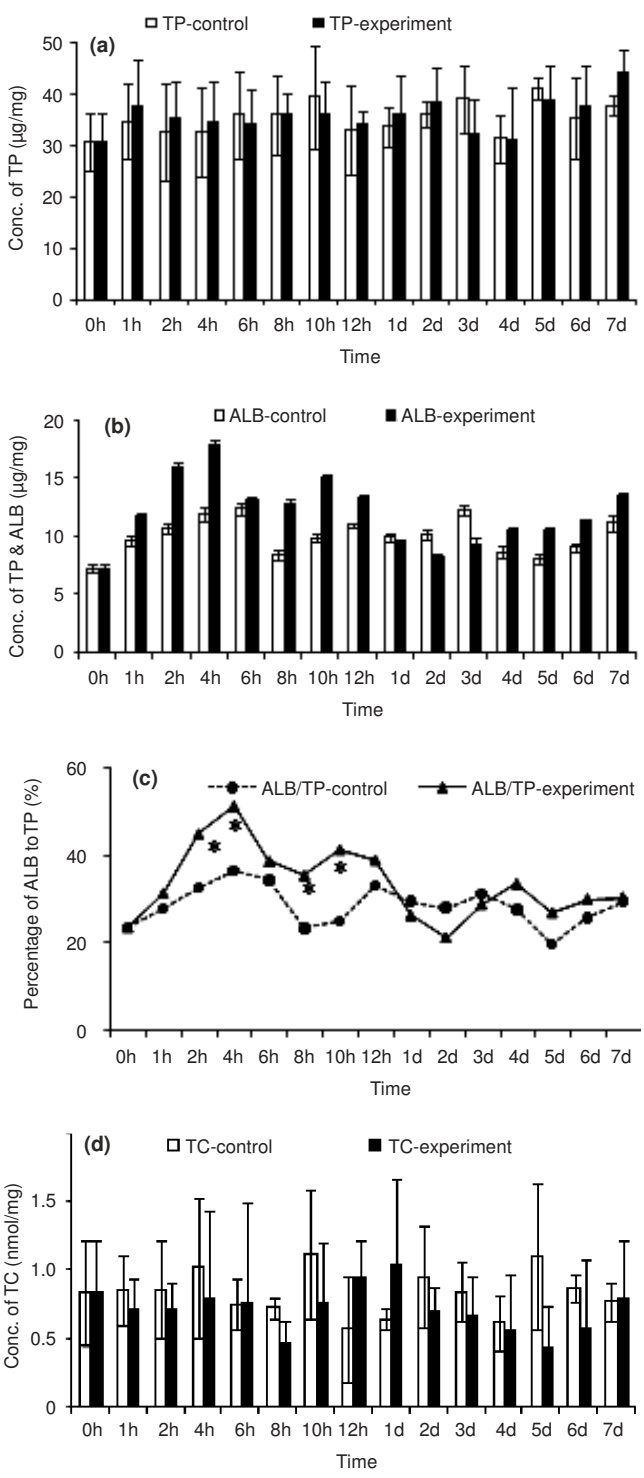


Fig. 1. Quantity of total protein and albumin did not change significantly in 7 days' exposure to *M. aeruginosa*. The concentrations of albumin in experimental groups were more than that in control groups in the first day. The proportion of albumin to total protein were significantly (*$p < 0.05$) at time 2, 4, 8 and 12 h. The concentrations of total cholesterol kept fluctuation in 7 days

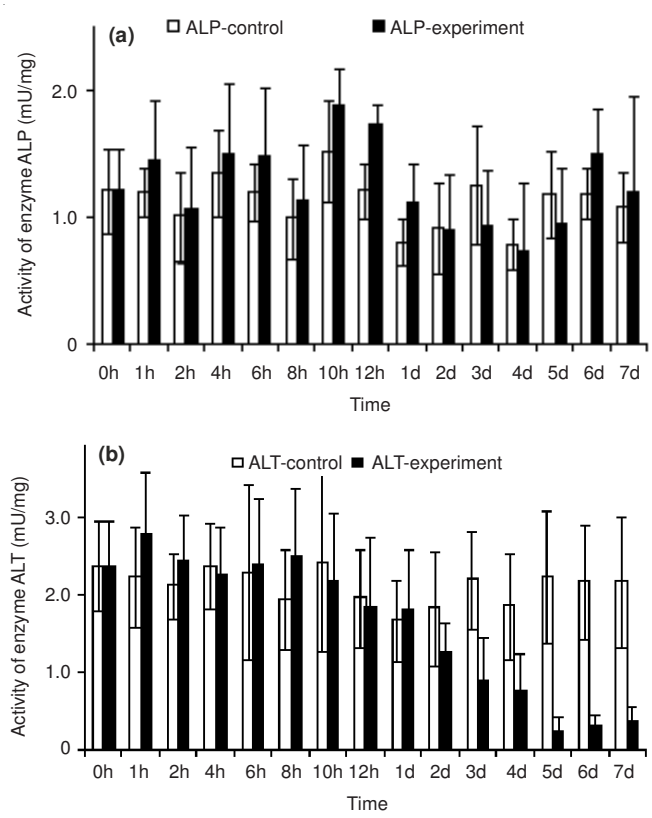


Fig. 2. Activity of enzyme alkaline phosphatase in experimental group kept fluctuation and was higher than that in control group as a whole. The activity of ALT decreased rapidly and was much lower than that in control group from the 8th h to the end

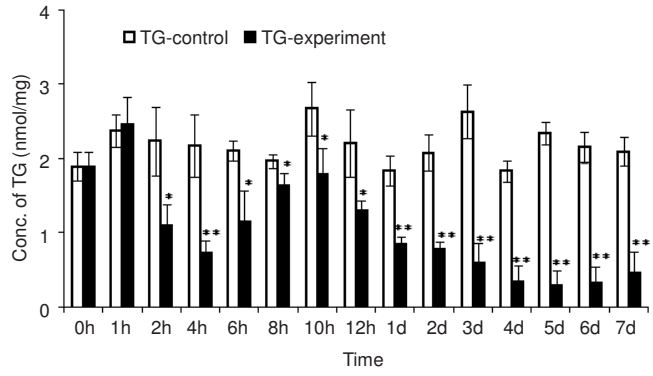


Fig. 3. Concentration of TG varied in the first 10 h in *M. aeruginosa* treated groups and was significant lower than the control group since then (*$p < 0.05$, **$p < 0.01$)

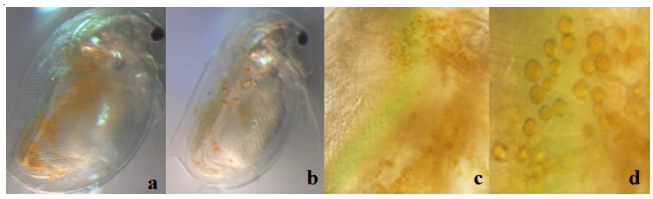


Fig. 4. *D. magna* fed with *C. vulgaris* and yeast (a) showed amount of chyme in alimentary canal. The crustacean (b) fed with *M. aeruginosa* had lots of lipid droplets (diameter around 10 μm) especially near the alimentary canal and undigested cells of *M. aeruginosa* were visible (c, d)

**RAPD analysis:** It took 7-8 days for the first generation of *D. magna* to reproduce and the time was extended to 8-10

days for the next generation and 9-12 days for the third generation. Repetitions of RAPD tests were performed and all the results of the experiments did not show any genetic damage in first generation of *D. magna* raised under normal environment, or exposed to MC-LR standard substance and *M. aeruginosa* (Fig. 5). As for the second generations, band F2-1 and F2-2 appeared in group S treated with MC-LR, while electrophoregram of group m displayed two bands (F2-3, F2-4) with the primer mentioned above, which were differ from control group (Fig. 5). When compared to control groups, the results on RAPD of experimental groups still showed changes in the third generation (Fig. 5).

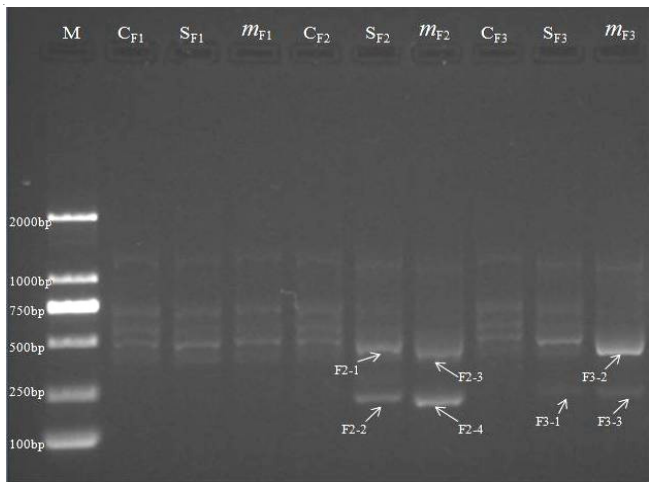


Fig. 5. Results of RAPD electrophoregram of three generations of *D. magna* after exposure to MC-LR standard substance and *M. aeruginosa* respectively. The patterns were obtained using primers OPB7 and OPB9. $C_{F1}$, $C_{F2}$ and $C_{F3}$ were controls; $S_{F1}$, $S_{F2}$ and $S_{F3}$ were treated with MC-LR; $m_{F1}$, $m_{F2}$ and $m_{F3}$ were treated with *M. aeruginosa*. F1 signified the first generation of *D. magna*, F2 and F3 signified the second and third generation respectively. The molecular sizes (bp) of DNA marker was shown on the left and changes of DNA were indicated by arrows

Most researches of toxin microcystins on *Daphnia* have shown that the toxins can injure the cladocera even to death. Crustacean denutrition would occur when *M. aeruginosa* blooming happened in eutrophicated aquatic environment[2,5]. This study has concluded that the total protein of experimental organisms treated with *M. aeruginosa* showed no significant changes comparing with control group (Fig. 1). The TG of experimental group was lower than control through all the time of experiment except the 1st h. The average concentration of TG on day 5-7 was 0.3 nmol/mg which was much lower than control which average concentration was 2.18 nmol/mg. Combined with the lipid droplets in *D. magna* fed with *M. aeruginosa*, low content of TG may indicate that *M. aeruginosa* is a deleterious food for *D. magna* and will affect the normal metabolism of crustacean. ALT is an enzyme associated with protein metabolism. It will decrease significantly after *M. aeruginosa* treating, while total protein hasn't shown regular change in this study. In addition, no research has reported the change of total protein and ALT during the period of *D. magna* breeding, so it's difficult to conclude whether the change of total protein is correlated to ALT decreasing and further study is necessary. In Yang's study on the toxicity of $C_{60}$ nanoparticles to *D. magna*[23], large amounts of lipid droplets were observed within the epithelium. Large lipid droplets appeared within mucosal cells in the gill of fluoranthene affected Pimephales promelas[24]. In this study, large amounts of lipid droplets were also appeared in organisms feed with *M. aeruginosa*, which was similar to other studies on pollutants' toxicity to aquatic organisms.

The DNA mutation experiment by RAPD showed the effect of microcystins to *D. magna* started on second generation either exposed to MC-LR standard substance or fed with *M. aeruginosa*. The DNA aberrant still appeared on third generation of both experimental groups (Fig. 5). These results suggest that DNA adducts, DNA breaks, point mutations and genomic rearrangements may occur under the microcystins treatment[25]. However DNA mutation did not appear in adult period of first generation, suggesting that the damage might occur during the period of germ cells or larva when more fertilized eggs were exposed to microcystins. It is concluded that damage of lower population growth rate and reproductive and nutritional deficiency of crustacean *D. magna* appeared when *M. aeruginosa* bloom in water environment[26-28] and this study also indicates that both the damage of DNA mutations and change of enzyme activities on crustacean have occurred when *D. magna* expose to cyanobacteria.

In brief, *M. aeruginosa* feeding will impact the growth of *D. magna*, reduce the content of TG and activity of enzyme ALT and induce anomaly of DNA in common with MC-LR. *M. aeruginosa* and MC-LR inhibit the growth and delay the reproduction of cladocera *D. magna*. It will be interesting to study the reason why the lipid droplets appear under low nutrition status and whether the DNA changes can be repaired in normal environment and fed with plenty of palatable food.

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