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Using the nematode *Caenorhabditis elegans* as a model animal for assessing the toxicity induced by microcystin-LR

LI Yunhui[1,2,3], WANG Yang[2,3], YIN Lihong[1], PU Yuepu[1,*], WANG Dayong[2,3,*]

1. School of Public Health, Southeast University, Nanjing 210009, China. E-mail: lyh1216@126.com
2. Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Nanjing 210009, China
3. Department of Genetics and Developmental Biology, Southeast University, Nanjing 210009, China

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Abstract

Among more than 75 variants of microcystin (MC), microcystin-LR (MC-LR) is one of the most common toxins. In this study, the feasibility of using *Caenorhabditis elegans* to evaluate MC-LR toxicity was studied. *C. elegans* was treated with MC-LR at different concentrations ranging from 0.1 to 80 μg/L. The results showed that MC-LR could reduce lifespan, delay development, lengthen generation time, decrease brood size, suppress locomotion behavior, and decreases *hsp-16-2-gfp* expression. The endpoints of generation time, brood size, and percentage of the population expressing *hsp-16-2-gfp* were very sensitive to 1.0 μg/L of MC-LR, and would be more useful for the evaluation of MC-LR toxicity. Furthermore, the tissue-specific *hsp-16-2-gfp* expressions were investigated in MC-LR-exposed animals, and the nervous system and intestine were primarily affected by MC-LR. Therefore, the generation time, brood size, and *hsp-16-2-gfp* expression in *C. elegans* can be explored to serve as valuable endpoints for evaluating the potential toxicity from MC-LR exposure.

Key words: microcystin-LR; toxicity assessment; brood size; stress response; *Caenorhabditis elegans*
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Introduction

Eutrophic freshwaters and cyanobacteria (blue-green algae, BGA) occur in many countries. They decrease water quality, and are increasing environmental problem worldwide (Bell and Codd, 1994; Falconer, 1999; Haider *et al*., 2003). Some cyanobacterial genera can produce toxic secondary metabolites, neurotoxins, and hepatotoxins, including microcystins (MCs) and nodularin (Gupta *et al*., 2003). MCs contain a family of potent cyclic heptapeptide toxins (Bell and Codd, 1994). Among more than 75 variants of MCs that have been reported thus far, limited information is available on the toxicity profile of MC variants other than microcystins-LR (MC-LR) (Dawson, 1998; Moreno *et al*., 2005). In addition, previous studies have focused on the adverse effects of acute, subchronic and chronic MC-LR exposure (Gupta *et al*., 2003). Acute MC-LR uptake can cause rapid disorganization of the hepatic architecture, breakdown of sinusoidal structures, and pooling of blood in liver (Rao *et al*., 1995; Dawson, 1998), whereas chronic poisoning could result in hepatocyte degeneration with necrosis, progressive fibrosis, and mononuclear leukocyte infiltration (Hitzfeld *et al*., 2000; Gupta *et al*., 2003).

Usually, a guideline value (GV) is selected and used to ensure drinking water safety throughout the lifetime consumption. The no-observed-adverse-effect level (NOAEL) value of MC-LR, 1.0 μg/L was proposed as the GV for adults by the World Health Organization (WHO). This implies that MC-LR at concentrations ⩽ 1.0 μg/L consumed throughout life would not present a significant health risk (WHO, 1996). The environmental availability of MC-LR can be determined by chemical reactions. However, environmental bioavailability is also important and should be paid attention to for assessment of MC-LR toxicity. The toxicity bioassay technique, as a powerful approach for environmental assessment and toxicity testing, has become a fundamental tool to evaluate the early warning signs of potential environmental pollutants that are hazardous to the health of humans and wild animals (Gerhardt *et al*., 2002; Han and Choi, 2005). However, the organism should meet the following criteria before it is selected as a bioindicator. It should have endpoints sensitive to the tested pollutants, can operate rapidly and easily, can allow the testing of a large number of samples in a small volume, and is inexpensive (Gomot, 1998; Han and Choi, 2005). The free-living nematode *Caenorhabditis elegans* has been already proved to be an ideal organism for the evaluation of several biological effects of various environmental chemicals or toxins (Candido and Jones, 1996; Dhawan *et al*., 1999; Dengg and van Meel, 2004). *C. elegans*, a free-living

* Corresponding author. E-mail: ypp@seu.edu.cn; dayongw@seu.edu.cn

nematode, is a fully differentiated organism with a nervous system, specialized muscles, digestive, and reproductive systems (Brenner, 1974; Boehm and Slack, 2005). It is an excellent model organism because of its short lifespan (approximately 3 weeks at 20°C under optimal conditions), simple cell lineage and can be easily cultivated. The various requirements for culturing, such as illumination, feeding and medium changing, can be eliminated for *C. elegans*. The LC_{50} range for the 96-h of metal exposure in *C. elegans* is comparable to that of *Daphnia* (Williams and Dusenbery, 1990). All these features and a series of acute aquatic toxicity assays for different toxins suggested that nematode is a sensitive bio-indicator, and can be used for testing water and soil pollution (Mutwakil *et al*., 1997; Graves *et al*., 2005), such as heavy metals and organophosphate-induced mammalian neurotoxicity (Mutwakil *et al*., 1997; Cole *et al*., 2004; Power and Pomerai, 1999; Chu and Chow, 2002; Swain *et al*., 2004). Bioavailability is the degrees to which a toxin is free for uptake into or sorption onto an organism. But to our knowledge, no reports of similar experiments with terrestrial nematodes have appeared, although such nematodes are more numerous and species-rich than earthworms.

The objective of this study is to determine the feasibility of using *C. elegans* for the evaluation of MC-LR toxicity. *C. elegans* was used in this experiment to systematically analyze the multiple defects and behavioral toxicities caused by MC-LR exposure. The endpoints were evaluated with regard to lifespan, development, reproduction and locomotion behavior. Because the availability of recombinant technology can make it possible to improve the sensitivity of assays by transgenesis, we simultaneously investigated the stress responses in embryos and in the nervous system, muscle, and intestine of adults by the stable transgenic line *hsp-16-2-gfp*. The KC136 strain, carrying a transgene of green fluorescent protein (GFP) reporter driven by the *hsp-16-2* heat shock promoter, can reflect the presence of toxic effect or stress within 5 h of exposure when animals are examined under a fluorescent microscope (Chu and Chow, 2002). The induced GFP fluorescent signal at different tissues is strong enough which allow a distinct identification of induced expression in nematodes (Chu and Chow, 2002; Wang *et al*., 2007).

1 Materials and methods

1.1 Chemicals

MC-LR is the gift from the National Institute for Environmental Studies, Japan. MC-LR was dissolved in minimum amount of methanol (0.1%) and maintained at 4°C. A range of different concentrations of exposure solutions were prepared before being used in different media and sterilized by filtration through a 0.22-mm Millipore filter. After replacing the previous medium, the exposure solutions were added to the assay systems, and incubated for anticipated exposure time period. Methanol solutions served as control groups. All other chemicals were obtained from Sigma-Aldrich (St. Louis, USA).

1.2 Preparation of nematode cultures

The strains used in the current study were wild-type N2 and CL1234, originally obtained from the *Caenorhabditis* Genetics Center (Minneapolis, USA), and KC136 (gift from Dr. King L. Chow). They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described by Brenner (1974). Age synchronous populations of L4-larva stage nematodes were obtained by the collection as described by Donkin and Williams (1995). The L4-larva stage animals were washed with double-distilled water twice, followed by washing with modified K medium (mmol/L) (NaCl 50, KCl 30, NaOAc 10, pH 5.5) (Williams and Dusenbery, 1990). Exposures were performed in sterile culture plates (glass). Approximately 100 nematodes were transferred into 5 μL exposure solution in the micropipetter. The exposures time was 2-d and were carried out in incubator at 20°C in the absence of food.

1.3 Life span assay

The control and MC-LR exposed nematodes were, respectively, picked onto the normal NGM plates and the time was recorded as $t = 0$. About thirty of them were placed onto a single plate and adult animals were transferred every 2 d to fresh plates during the brood period. The numbers of survivors were counted every day. Nematodes that failed to respond to repeated touch stimulation were considered as death. The experiments had been conducted at least three times before the data were collected.

1.4 Body size assay

After 2-d exposure, MC-LR exposed and control nematodes were picked out for body size measure. Body size was determined by measuring the flat surface area of nematodes using the Image-Pro® Express software. For each test, at least 30 nematodes were picked.

1.5 Brood size and generation time assay

The experiments were performed as described by Swain *et al*. (2004). Briefly, brood size was assayed by placing single MC-LR exposed or control nematodes onto individual culture plates. The parental nematodes were transferred to a new well every 1.5 d. Progeny were counted on the day after the transfer. The generation time refers to the time from parental egg to the first egg of the next generation. For both the brood size and the generation time test, at least 30 replicates were performed for statistical purposes.

1.6 Head thrash and body bend assay

The head thrash was assayed as previously described (Tsalik and Hobert, 2003). The MC-LR exposed and control nematodes were washed with the double-distilled water, followed by washing with K medium. Every one was transferred into a microtiter well containing 60 μL of K medium on the top of agar. After a 1-min recovery, the head thrashes were counted for 1 min. A thrash was defined as a change in the direction of bending at the mid body. To assay the body bends, the nematodes were placed onto a

second plate and scored for the number of body bends in an interval of 20 s as a change in the direction of some of them corresponding to the posterior bulb of the pharynx along the *y* axis, assuming that the animal was traveling along the *x* axis. Thirty nematodes were examined for each concentration of MC-LR exposure.

1.7 Analysis of transgenic strain

The method was performed as described previously (Chu and Chow, 2002; Wang *et al*., 2007). In the stress tests, the synchronized L4-stage of the transgenic KC136 nematodes displaying the most prominent response was used. The synchronized nematodes were washed off the plates and resuspended at a concentration of the 5000 nematodes/mL without addition of *E. coli* OP50. The appropriate concentrations of MC-LR were added to the test tubes. The tubes were tilted on a supported surface with constant shaking at room temperature (20°C) for 5 h. To analyze the changes of *hsp-16-2-gfp* expression patterns, the treated KC136 animals were allowed to settle for 10 min, and then pipetted onto an agar pad on a glass slide, mounted and observed for the fluorescent signals at vulva, intestine, muscle and nervous systems. More than 50 nematodes were counted for the statistical analysis.

1.8 Statistical analysis

All data in this article were expressed as means ± SD. One-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test was used to determine the significance of the differences between the groups. The probability levels of 0.05 and 0.01 were considered statistically significant.

2 Results

2.1 Effect of MC-LR exposure on life span of *C. elegans*

First, we evaluated the possibly toxic effects of MC-LR exposure on the life span of nematodes. Concentrations of 0, 1, 10, 20, 40, and 80 μg/L were selected for the lifespan assay. As shown in Fig. 1, the remarkable difference in maximum lifespan was only shown at 40 and 80 μg/L compare to control ($P < 0.05$). Analysis of the mean lifespan of nematodes exposed to MC-LR supported this conclusion. The mean lifespan of nematodes exposed to 40 and 80 μg/L MC-LR were significantly different from that in control nematodes ($P < 0.05$). Therefore, the concentrations of 0, 1, 10, and 40 μg/L were selected for other aspects of toxicity evaluation.

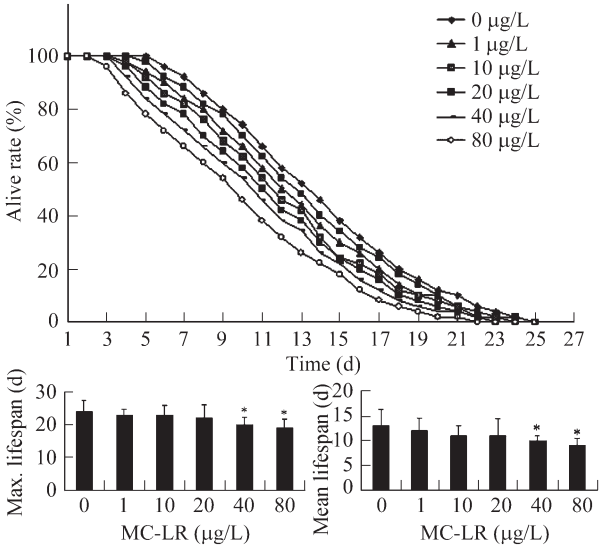

Fig. 1 Life spans of animals exposed to different concentrations of MC-LR. Bars represent SD; * $P < 0.05$.

2.2 Effect of MC-LR exposure on body size in *C. elegans*

Compared to that in control, only MC-LR exposure at 40 μg/L resulted in obvious defects in body size (Fig. 2). In addition, we did not observe any apparent developmental abnormalities in nematodes exposed to different concentrations of MC-LR (data not shown). Therefore, MC-LR cannot be considered as teratogenic in *C. elegans*.

2.3 Effect of MC-LR exposure on reproduction in *C. elegans*

We further investigated the toxic effects of MC-LR exposure on the generation time of *C. elegans*. As shown in Fig. 3a, the animals exhibited prolonged generation time as the MC-LR concentration increased. The MC-LR concentration in the range of 1–40 μg/L could cause significantly severer defects in generation time compared to that in control nematodes (1 and 10 μg/L, $P < 0.05$; 40 μg/L, $P < 0.01$).

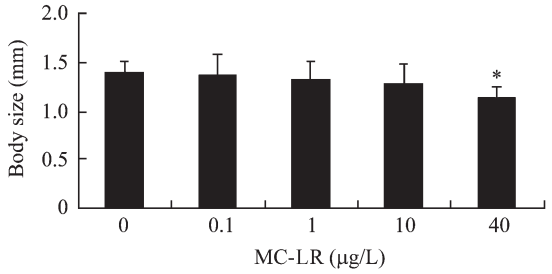

Fig. 2 Body sizes of animals exposed to different concentrations of MC-LR. Bars represent SD; * $P < 0.05$.

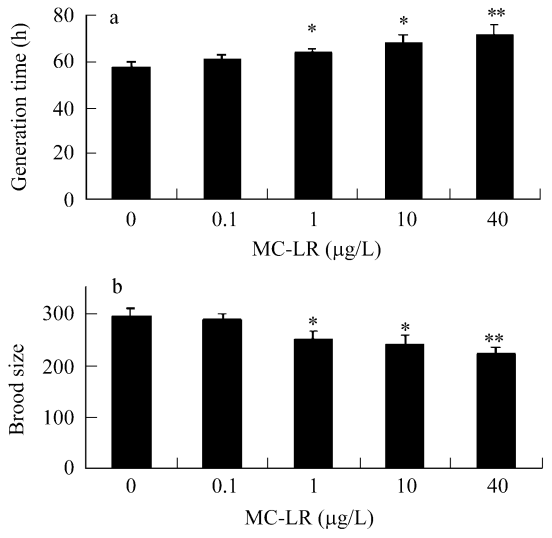

Fig. 3 Generation times and brood sizes of animals exposed to different concentrations of MC-LR. Bars represent SD; * $P < 0.05$, ** $P < 0.01$.

Reproductive speed is evaluated based on the endpoint of generation time, and brood size can reflect the reproductive capacity. We also examined the possible effects of MC-LR exposure on brood sizes in *C. elegans*. The brood sizes were gradually reduced as the increase of MC-LR concentrations. All concentrations caused significantly severe defects in brood sizes compared to control (1 μg/L, $P < 0.05$; 10 μg/L, $P < 0.05$; 40 μg/L, $P < 0.01$) (Fig. 3b). Therefore, the endpoints of generation time and brood size were found to be very sensitive for evaluating the MC-LR reproduction toxicity.

2.4 Effect of MC-LR exposure on locomotion behaviors in *C. elegans*

As shown in Fig. 4, only the head thrashed and body bends of animals exposed to 10 and 40 μg/L of MC-LR exhibited significant ($P < 0.05$) differences from that in control. Exposure to 0.1 and 1 μg/L of MC-LR would had toxic effects on head thrashed and body bends compared to control. Therefore, the endpoints of head thrash and body bend were not sensitive enough to assess the locomotion behavior toxicity from 1 μg/L of MC-LR exposure.

2.5 Evaluation of the sensitivity to MC-LR exposure with endpoints of generation time and brood size

Figure 5 shows that the MC-LR exposure with concentrations less than 1 μg/L would not result in significantly severe defects of generation time and brood size. Compared to this, exposure to 1 μg/L (GV value of microcystin in drinking water) (Majsterek *et al.*, 2004) of MC-LR resulted in significantly ($P < 0.05$) severe defects in generation time and brood size.

2.6 Stress responses for MC-LR toxicity in *C. elegans*

As shown in Fig. 6, MC-LR exposure significantly ($P < 0.01$) increased the percentage of the population expressing *hsp-16-2-gfp* compared to control even at a concentration of 1 μg/L, whereas exposure to MC-LR at concentrations less than 1 μg/L would not cause significant

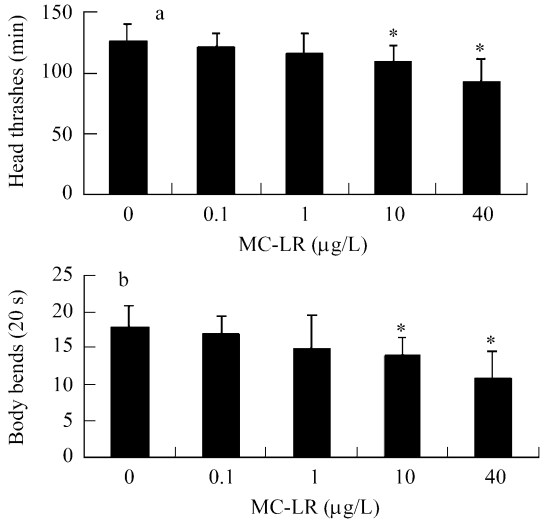

Fig. 4 Head thrashes and body bends of animals exposed to different concentrations of MC-LR. Bars represent SD; * $P < 0.05$.

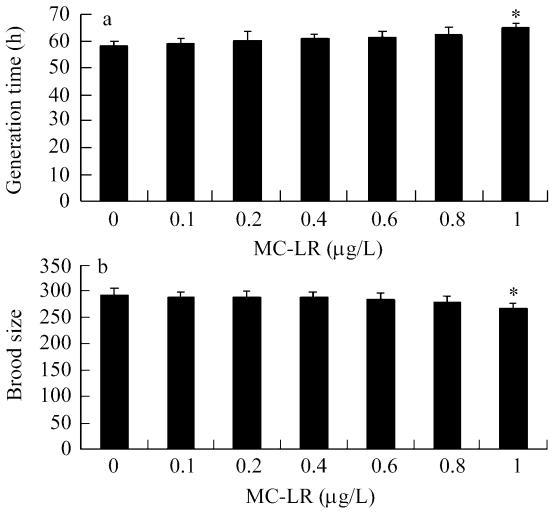

Fig. 5 Assessment of the sensitivity from endpoints of generation time and brood size to MC-LR toxicity. Bars represent SD; * $P < 0.05$.

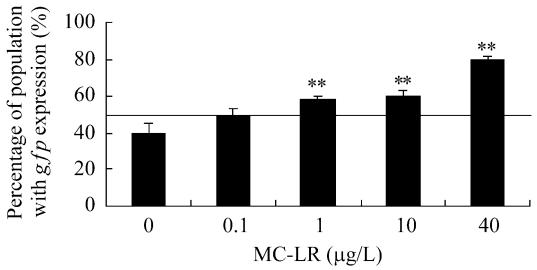

Fig. 6 Stress response assay in MC-LR exposed transgenic nematodes. Significant induction of *gfp* expression (50% of a population, above the dotted line) was observed in animals exposed to MC-LR. Bars represent SD; ** $P < 0.01$.

defects in the percentage of the population expressing *hsp-16-2-gfp* (data not shown). In addition, to exclude the possible attenuation of *hsp-16-2-gfp* expression from MC-LR exposure, strain of CL1234, constitutively expressing *gfp*, was also assayed, and no changes of *gfp* expression were observed after MC-LR exposure (data not shown). Therefore, besides the generation time and brood size, percentage of the population expressing *hsp-16-2-gfp* is also a sensitive endpoint for evaluating MC-LR toxicity in nematodes.

As shown in Fig. 7, for adult nemadotes exposed to different MC-LR concentrations (0.1, 1, 10, and 40 μg/L), the fold changes in *gfp* expression in the muscle system were always around 1.0, whereas those in *gfp* expression in the entire nervous system and intestine systems were always greater than 1.0. In addition, the fold changes in *gfp* expression in the vulva region were similar to those of *gfp* expression in the intestine and nervous systems.

3 Discussion

The first goal of this study was to determine whether *C. elegans* have endpoints sensitive to MC-LR toxicity. The endpoints of lifespan, development, reproduction, locomotion behavior, and *hsp-16-2-gfp* expression were considered for the assays, since they have been extensively

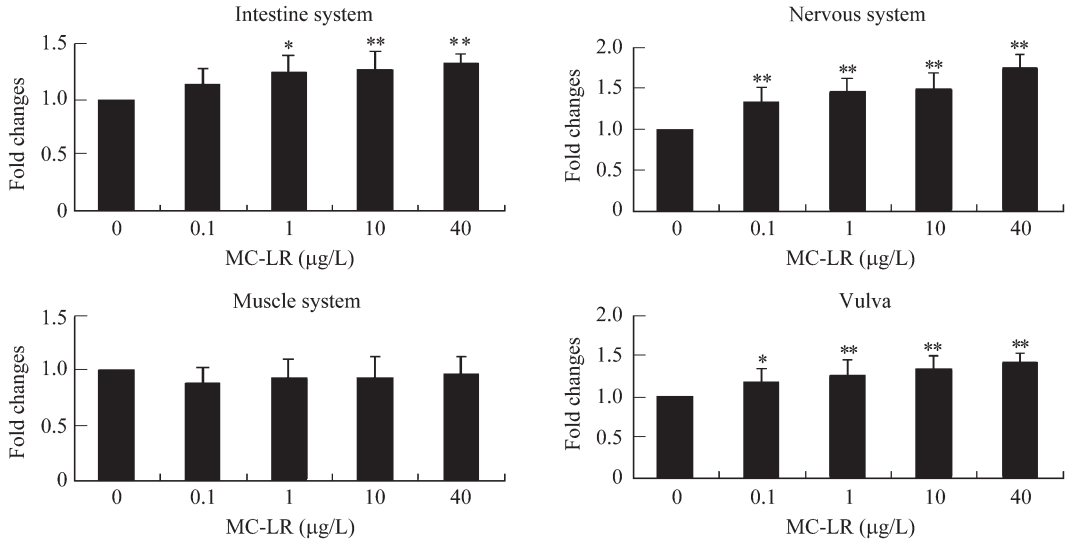

Fig. 7 Tissue-specific *hsp-16-2-gfp* expressions in adult nemadotes exposed to MC-LR. The *gfp* expression in nervous system was tested mainly at the region of nerve ring.

used in the evaluation of toxicity from metal exposure in *C. elegans* (Power and Pomerai, 1999; Chu and Chow, 2002; Swain *et al*., 2004; Wang *et al*., 2007; Wang and Yang, 2007). Our data suggest that MC-LR exposure can result in numerous severe defects in *C. elegans*. It reduces life span, delays development, lengthens generation time, decreases brood size, suppresses locomotion behavior, and decreases *hsp-16-2-gfp* expression. Therefore, *C. elegans* may be a suitable organism for evaluating MC-LR toxicity and sensitive to the MC-LR exposure.

We further identify the possible endpoints that may be mostly sensitive to MC-LR exposure. In the current work, the endpoints of lifespan and body size were found to be significantly affected in nematodes exposed to relatively high concentrations of MC-LR (40 and/or 80 μg/L). In contrast, the endpoints of generation time, brood size, locomotion behavior (head thrash and body bend), and percentage of the population expressing *hsp-16-2-gfp* were also significantly affected when the animals were exposed to relatively low MC-LR concentration (10 μg/L). In particular, the animals tended to exhibit remarkably severe defects in generation time, brood size, and percentage of the population expressing *hsp-16-2-gfp* when exposed even to 1 μg/L of MC-LR. Therefore, the endpoints of generation time, brood size, and percentage of the population expressing *hsp-16-2-gfp* will be more useful for the evaluation of MC-LR toxicity.

The second goal of this study was to analyze the possible reasons inducing MC-LR toxicity in *C. elegans*. Cyanobacterial toxins are usually grouped into neurotoxins, hepatotoxins, cytotoxins, and irritants and gastrointestinal toxins according to the physiological systems, organs, tissues, or cells that they affect (Codd *et al*., 2005). Recent evidence suggests that oxidative stress may play a significant role in the pathogenesis of MC toxicity in mammals (Jayaraj *et al*., 2006). The spatial distribution of *hsp*-16 expression in response to a number of metal and paraquat has been reported (Stringham and Candido, 1994). Therefore, we investigated tissue-specific *hsp-16-2-gfp* expression in animals exposed to MC-LR, since *hsp-16* induction has been suggested as an early marker of oxidative stress (Polla *et al*., 1996). The fold changes in the *gfp* expression in the intestine and nervous system were always found to be greater than 1.0 in adult exposed to different concentrations of MC-LR. This implies that in nematodes the nervous and intestine system may be primarily affected by MC-LR toxicity. Oxidative stress occur either due to a decrease in the cellular antioxidant levels or due to the overproduction of ROS (Weng *et al*., 2007). Thereby, overproduction of ROS may occur in nervous and intestine systems of MC-LR exposed nematodes. Moreover, the fold changes in *gfp* expression in the vulva were also greater than 1.0 in adult exposed to MC-LR. Therefore, oxidative stress, possibly accompanied by neurotoxicity or endocrine toxicity, occurred in the vulva region, could largely account for the obvious reproductive defects induced by MC-LR toxicity. Nevertheless, more research is still required on the possible neurotoxicity or endocrine toxicity due to MC-LR exposure in nematodes.

Moreover, considering the fact that there are more than one inducers in polluted water or soil, the relatively sensitive endpoints (generation time, brood size, and percentage of the population expressing *hsp-16-2-gfp*) suggests that in the current work might also be able to be induced by other stresses. Therefore, we should use the combination of these endpoints in *C. elegans* with the environmental availability assay and other bioindicators in other organisms for the toxicity evaluation from MC-LR exposure, such as the cow thymus mitochondrial oxidase complex (Majsterek *et al*., 2004) and human liver mitochondrial aldehyde dehydrogenase (Chen *et al*., 2006). The endpoints of a specific bioindicator alone may still not completely enough for early monitoring the risks from toxic cyanobacteria.

In addition, exposure to cyanotoxins can occur by different routes including dermal, inhalation, oral, and intravenous (de Magalhães *et al*., 2001). The cuticle of

nematodes probably protects them well from toxins. It is more likely that oral exposure is responsible for MC-LR toxicity, once nematodes are put into the liquid where they are forced to swim in and drink the toxin continuously.

4 Conclusions

Caenorhabditis elegans can serve as a valuable bioindicator for evaluating MC-LR toxicity. Especially, the generation time, brood size, and percentage of the population expressing *hsp-16-2-gfp* can be exploited as useful endpoints to assess the potential toxicities from MC-LR exposure. In addition, the functions of nervous system and intestine system may be primarily effect by MC-LR exposure in *C. elegans*.

Acknowledgments

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