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Rapid Bioassay for Microcystin Toxicity Based on Feeding Activity of *Daphnia*

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Microcystins (MCs) are hepatotoxin produced by bloom-forming cyanobacteria, primarily *Microcystis* species, in eutrophic lakes and reservoirs. These toxins are soluble cyclic heptapeptide and are known to be poisonous to many kinds of aquatic organisms. MCs may be directly lethal to zooplankton, reduce the feeding activity, or influence the community structure of zooplankton (Peñaloza et al. 1990; DeMott et al. 1991; Carmichael 1994; Jungmann and Benndorf 1994). However, the effect is highly variable and inconsistent between zooplankton species.

Daphnia magna Straus (Cladocera, Crustacea) have been widely used as an acute and chronic test organism for cyanobacterial toxins because of its sensitivity to toxicants (Caspers 1998), rapid reproduction rate, short-term lifetime, and easy culturing and handling (Adema 1978). Acute toxicity tests with daphnids use immobilization as an end-point and require 24 hr or 48 hr as an exposure time, whereas chronic toxicity tests based on the reproduction rate require at least 14 d (OECD 1984). Short-term acute toxicity to heavy metals based on the suppression of the feeding activity of *Ceriodaphnia dubia* has also been explored (Bitton et al. 1995, 1996; Lee et al. 1997). In this case, a 1-hr or 6-hr feeding activity suppression assay as an end-point was found to be similar in sensitivity to the traditional 48-hr acute bioassay. However, previous studies on the development of a short-term acute bioassay for MC using *Daphnia* are rare (DeMott et al 1991; Jungmann and Benndorf 1994), and no rapid bioassay for MC-LR using the feeding activity of *Daphnia* has yet been reported. MC-LR including leucine and arginine as variable amino acid is the most common variant of the MCs reported to date (Rinehart et al. 1994). Accordingly, the aim of the current study was to develop a rapid and simple toxicity test for MC-LR based on the suppression of the feeding activity of *D. magna.*

MATERIALS AND METHODS

Daphnia magna Straus were obtained from KRICT (Korea Research Institute of Chemical Technology) from an original culture maintained by the GSF-Institute of Ecological Chemistry (Gesellschaft für Strahlen und Umwelt Forschung-Institut für Ökologische Chemie, Neuherberg, Germany). *D. magna* were cultured

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in a chamber at 20 ± 1℃ on a 16-hr light : 8-hr dark photoperiod under cool white fluorescence lamps at an average light intensity of 20 μmol/m^2/sec (OECD 1984; Caspers 1998). Fifteen adult daphnids were held in a 3-L glass beaker containing 2 L of a hard-constituted water medium. The green alga *Scenedesmus subspicatus* was supplied as food once a day at about 1.0×10^5 cells/mL (US EPA 1991). The composition of the hard-constituted water medium was as follows: 192 mg $NaHCO_3$, 120 mg $CaSO_4{\cdot}2H_2O$, 120 mg $MgSO_4$, and 8 mg KCl in 1 L double-distilled water (US EPA 1991). Total hardness, alkalinity, dissolved oxygen, and pH of the medium during the test were within a range of 160-180 mg/L, 110-120 mg/L, 5.0-5.9 mg/L, and 8.0-8.5, respectively. A new culture was carried out on every Tuesday, and a brood of 4-wk-old daphnids was substituted for neonate daphnids. The neonate daphnids aged less than 24 hr, obtained from laboratory stock cultures, were used as the test organisms. The *S. subspicatus* was grown in an Allen medium (1968) and maintained at 25 ± 1℃ on a 12-hr light : 12-hr dark photoperiod. All glassware and other materials used for culturing were acid-washed and autoclaved at 121℃ before use.

Standard MC-LR (Sigma, USA) was used to prepare the stock solution with methanol as the carrier solvent. The toxin solutions were prepared by diluting the MC-LR stock solution (250 mg/L) with hard-constituted water, and the toxin concentration ranged from 10^{-3} to 10 mg/L. The methanol and water used in preparing the test and stock solutions of the toxin were HPLC grade (Merck, Germany). The methanol content in the toxin dilutions was less than 1% except for 4% in 10 mg/L toxin solution. To clarify the effect of methanol, we carried out two control treatments in every experiments as follows: one treatment with only hard-constituted water medium and the other treatment with hard-constituted water medium plus 4% methanol. We could not find any difference in the feeding activities by *Daphnia* between two control treatments. Therefore, it was concluded that methanol concentration below 4% had no effect on the feeding activity by *Daphnia*.

The feeding experiments were carried out in 20-mL glass vials, each containing 10 mL of a hard-constituted water medium at 20 ± 1℃ in the dark. Prior to the test, the neonate daphnids were starved for 4 hr, then groups of 10 daphnids were placed in 4 different concentrations of *S. subspicatus* ranging from 2.0×10^4 to 2.5×10^6 cells/mL. The daphnids were removed from the vial after being allowed to feed for selected time periods (10 to 60 min). The concentration of the remaining algae in each test vial was measured by *in vivo* fluorescence using a fluorometer (Tuner 450) and enumerated by a particle counter (Coulter Z1) (Mayer et al. 1997). The reduced algal biomass was computed as the number of algal cells ingested by each daphnid per unit time. The optimum feeding time for measuring the feeding rate by *D. magna* was determined based on the feeding data. Food ingestion by the daphnids was examined in triplicate.

For the toxicity test, the daphnids were starved for 4 hr, then groups of 10 daphnids were exposed to a series of MC-LR dilutions, each containing 10 mL of

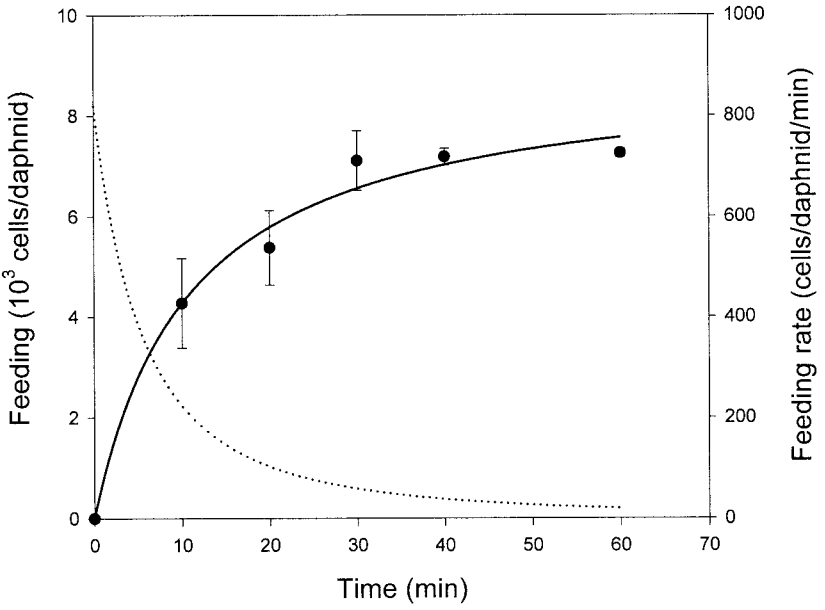

Figure 1. Feeding (solid line) and feeding rate (dotted line) of *Daphnia magna* as function of time. The concentration of *Scenedesmus subspicatus* as the food was 5.0×10^5 cells/mL. Each point represents mean ± SD (n = 3).

the toxin solution in a 20-mL glass vial, for selected time periods (1, 3, and 24 hr) at 20 ± 1℃ in the dark. About 5.0×10^5 cells/mL of *S. subspicatus* was added to each test vial 30 min before the end of the selected exposure times. The daphnids were removed from the vial after being allowed to feed for 30 min. The remaining algal biomass in the toxin dilutions was measured by *in vivo* fluorescence, and then the reduced algal biomass was computed as the feeding rate of the daphnids. The acute toxicity test of MC-LR for each exposure time was carried out in triplicate.

The EC_{50} (median effective concentration) was defined as the MC-LR concentration that inhibited ingestion of 50% of the supplied food by the daphnids The EC_{50} was computed for each replicate test and determined according to the method of Litchfield and Wilcoxon (1949).

RESULTS AND DISCUSSION

The feeding and feeding rate of the neonate daphnids as a function of time are shown in Figure 1. The feeding increased linearly with time until about 30 min and then reached a maximum. The feeding curve fitted to the data exhibited a hyperbolic curve.

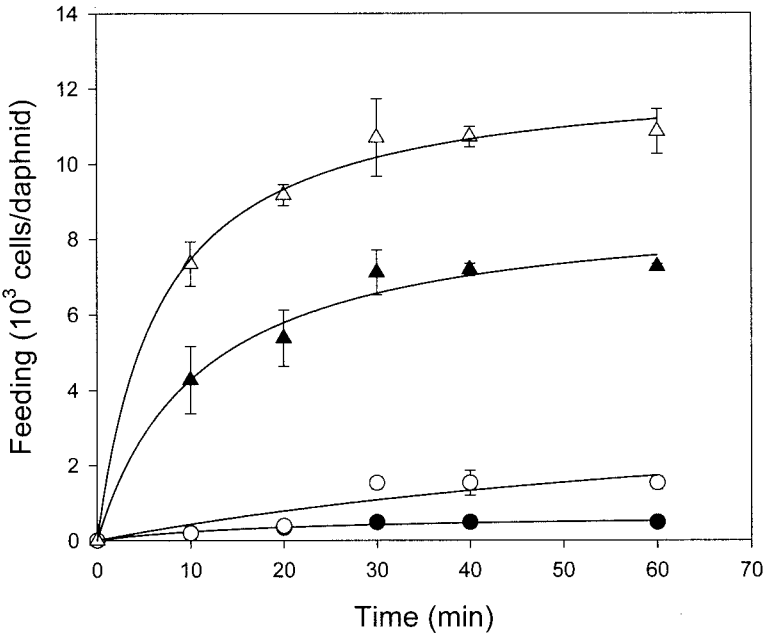

Figure 2. Effect of food concentration on feeding of *Daphnia magna*. *Scenedesmus subspicatus* was supplied as the food. Symbols are: ●, $2.0x10^4$; ○, $1.0x10^5$; ▲, $5.0x10^5$; △, $2.5x10^6$ cells/mL. Each point represents mean ± SD (n = 3).

The feeding rate calculated from the fitted-feeding curve was highest during the initial 10 min of the feeding period and thereafter continuously decreased to nearly zero after 30 min. As such, 30 min was determined as the appropriate feeding time as this seemed to be sufficient for the *D. magna* neonates. Although slightly longer than 20 min required for yeast ingestion (Bitton et al. 1995), this time period was still shorter than the 35 to 40 min for algae ingestion by *C. dubia* neonates (Lee et al. 1997).

The effect of the food concentration on the feeding of *D. magna* is shown in Figure 2. The feeding of *D. magna* on *S. subspicatus* continuously increased to a concentration of 2.5×10^6 cells/mL. On the basis of the best-fitted feeding curve, the feeding rate also increased from 16 ± 1 to 354 ± 35 cells/daphnid/min with the algal concentrations ranging from 2.0×10^4 to 2.5×10^6 cells/mL for 30 min. However, the feeding rate of up to 354 cells/daphnid/min at 2.5×10^6 cells/mL was higher than the net uptake rate of yeast by *C. dubia* at 200 cells/daphnid/min based on a concentration of 2.5×10^6 cells/mL (Bitton et al. 1995).

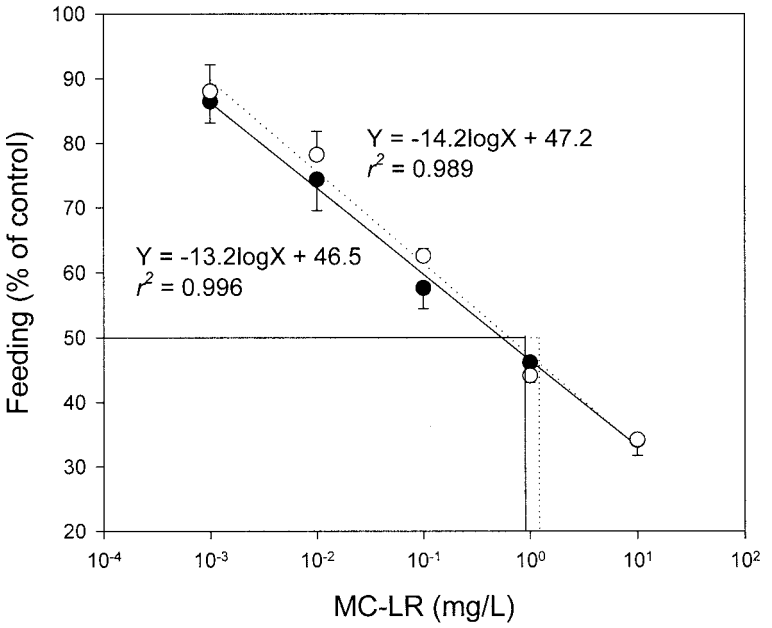

Figure 3. Suppressed feeding of *Daphnia magna* with 3-hr exposure (●, solid) and 24-hr exposure (○, dotted) to MC-LR. The EC_{50} was calculated from the regression line. Each point represents mean ± SD (n = 3).

The feeding of *D. magna* during 3 hr was lower than that for 1 hr (data not shown) at all the examined concentrations, yet nearly similar to that during 24 hr (Figure 3). No significant difference was observed in the feeding activities between the 3-hr and 24-hr exposure ($F_{(1,20)}$ = 2.22, P = 0.151). The feeding of *D. magna* decreased with an increasing MC concentration ranging from 10^{-3} to 10 mg/L.

The linear regression lines fitted to the feeding of *D. magna* based on the 3-hr and 24-hr exposure to MC-LR exhibited very similar slopes (-13.2 and -14.2) and intercepts (46.5 and 47.2). In other words, the 3-hr EC_{50} for MC-LR with *D. magna* was 0.60 ± 0.11 mg/L, which was not much different from 0.65 ± 0.04 mg/L after a 24-hr exposure (Figure 3). In addition, the correlation between the feeding of the daphnids during the 3-hr and 24-hr exposure to MC-LR was highly significant (Y = 0.972X + 0.493, r^2 = 0.990). Consequently, the rapid bioassay based on the feeding of *D. magna* during the 3-hr exposure produced very similar results to those obtained during the 24-hr exposure as regards both the EC_{50} and the suppression pattern.

Several studies have already investigated the effect of the exposure time on toxicant solutions with *C. dubia* and *D. magna* (Janssen and Persoone 1993; Bitton et al. 1995, 1996; Lee et al. 1997). The 1-hr EC_{50}s for 11 chemical compounds, based on the inhibition of *D. magna* β-galactosidase, were found to be highly correlated with their 24-hr EC_{50}s (r = 0.97; $P < 0.05$) (Janssen and Persoone 1993). In addition, the 1-hr and 6-hr EC_{50}s values for heavy metals and organic compounds, respectively, based on the suppressed feeding activity of *C. dubia,* also correlated well with their 48-hr EC_{50}s (Bitton et al. 1995, 1996). Plus, a *Ceriodaphnia* algal uptake suppression test (CAUST), based on the feeding behavior of *C. dubia,* only required a 1-hr contact time between *C. dubia* and the toxicant (Lee et al. 1997).

The 3-hr EC_{50} obtained in the current study was compared with other 24-hr or 48-hr acute bioassays for MC (Table 1). DeMott et al. (1991) reported that the 24-hr LC_{50}s for MC-LR changed significantly within a range of 0.98 - 34.2 mg/L depending on the tested species of zooplankton. The 3-hr EC_{50} with *D. magna* examined in the current study was 0.60 mg/L, which placed between the 24-hr and 48-hr LC_{50}s with the copepod *Diaptomus birgei.*

Table 1. Microcystin (MC)-LR toxicity determined by acute bioassay with zooplankton

Species	Acute bioassay (mg/L)			Remark	References
	3 hr	24 hr	48 hr		
Daphnia magna	0.60	0.65	-	EC_{50}	Current study
D. hyalina	-	34.2	11.6	LC_{50}	DeMott et al. 1991
D. pulex	-	10.7	9.6	LC_{50}	DeMott et al. 1991
Diaptomus birgei	-	0.98	0.45	LC_{50}	DeMott et al. 1991

Although a direct comparison of the EC_{50} and LC_{50} is difficult, the 3-hr rapid bioassay seemed to be more sensitive than the results obtained by 24-hr or 48-hr acute assays. Accordingly, it would appear that a bioassay based on the suppressed feeding activity of *D. magna* with a 3-hr exposure can be effectively used as a rapid and easy toxicity test for MC instead of the traditional 24-hr acute bioassay based on immobilization.

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