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Fluorescence Intensity Assay Based on Suppression of *Daphnia Magna* Feeding on Dry Algae (Chlorella and Spirulina)

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***Abstract*— We investigated a new method to measure aquatic toxicant concentrations using reductions in *Daphnia magna* feeding rates as a surrogate. As toxicant concentration increases, *D. magna* filter-feeding decreases, and fluorescence of unconsumed algal particles intensifies. Thus, fluorescence of algal food particles was measured in assays of normally filtering control *D. magna* versus *D. magna* exposed to various concentrations of potassium dichromate to describe the correlation between toxicant concentration and fluorescence intensity of unfiltered algal food particles. Using spirulina and chlorella powder to feed *D. magna*, we found that fluorescence of spirulina more closely correlated with toxicant concentrations when *D. magna* was exposed to potassium dichromate. This method is an effective, rapid way to measure aquatic toxicant concentrations requiring less time, cost, and labor than traditional direct measures of chemical concentrations. Furthermore, it incorporates the biological impact of a toxicant, even unknown compound, in contaminated water.**

***Keywords*— Daphnia feeding suppression, Spirulina and chlorella powder, Fluorescence intensity**

I. INTRODUCTION

Daphnia magna shows widespread occurrence, ecological significance (broad distribution and important link in pelagic food chains), parthenogenetic reproduction, short life cycle, and sensitivity to a broad range of chemicals and environmental pollutants [2; 7; 3]. In natural freshwater systems, the chemicals presented are usually only detectable during the first 24h after application to adjacent areas. For mesocosm and microcosm studies, this period is too brief to determine the potential effects of chemicals [22]. Although mortality is an ultimate and non-specific endpoint in short invertebrate bioassay, recording movement patterns, such as average speed, turns, and circling movements, is relatively widely used [25; 8]. Life-history traits, such as somatic (individual) growth [6], time to first reproduction, development of parthenogenetic eggs [18; 13], and number of newborns [15], can also be studied for this purpose.

Daphnia feeding studies conducted with juvenile and adult stages also found that many toxicant effects on feeding occurred at lower concentrations that impair survival [1; 5; 21; 12]. In fact, in *Daphnia* and many other aquatic organisms, feeding is physiologically linked to growth and reproduction; hence, the effect on feeding usually translates to population response [3].The results obtained by Barata et al.,2008 [4] denoted a greater sensitivity of the pre-exposure feeding bioassay (bioluminescence bacteria, algae growth, and *D. magna* acute and *Danio rerio* fish acute tests) in 51 out of 65 tests performed with an average sensitivity 50-fold greater than that of the standardized tests.

The rate of filtration measured either as an ingestion rate or as the rate of movement of thoracic appendages above incipient limiting concentration (ILC) was no longer proportional to concentration but remained constant; for *Daphnia*, smaller particle sizes had higher ILC [17]. Matorin et al., 2009 [16] estimated the consumption of living chlorella cell by *Daphnia* according to the decrease in constant fluorescence that was directly proportional to the increase in the concentration of the algae cells in the solution.

The objective of our present study was to design a simple acute feeding test that would be more sensitive and would require less time than the commonly used 24-hour *D. magna* lethality test. For this purpose, we carried out the short time (3 and 6 h) feeding experiments with food substance that was less costly: spirulina and chlorella dry powder. We have measured decreased feeding/ingestion rate by fluorescence photometer based on altering the algae powder solution concentrations according to the consumption of algae powder solution by daphnids.

For calibration purposes, the results were compared with those of the standard immobility test as described by the ISO 6341:1996 standard [23].

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II. Material And Methods

D. magna was cultured in an incubator at 20±1 °C using spring water from the well near Kogakuin University (pH=6.34, hardness 50–70 mg/L of calcium carbonate, and oxygen=6.01 mg/L). *D. magna* was cultivated according to the literature [23] and fed daily with spirulina powder (GRAS, http://www.bunkajin. com/shop ping). In acute feeding experiments, we used *D. magna* neonates not older than 24 h.

Stock solution of potassium dichromate (Kanto Chemical Corporation, Japan) (50 mg/L) were prepared using MilliQ water (Organo Corporation, Japan). As a control, vial water was prepared with moderately hard water (JIS 0229/1992). The stock solutions for the preparation of toxicant concentrations were as follows: $NaHCO_3$– 7.5×10^{-4}, KCl – 7.5×10^{-5}, $MgSO_4\cdot7H_2O$ – 5.0×10^{-4}, and $CaCl_2\cdot2H_2O$ – 2.0×10^{-5}mol/L (pH 7.8±0.2 and hardness 250±25 mg/L of calcium carbonate).Stock solution of 100 mg/L of hydrogen peroxide (H_2O_2):0.33mL of 30% (w/v) H_2O_2(Wako Pure Chemical Industries Co.,Ltd., Japan) was dissolved in 1L of water.

Acute lethality bioassay was carried out according to the standard method [23]. For standard tested toxicant (potassium dichromate), five randomly selected neonates were transferred into 20 mL glass vials containing 5 mL of 1 out of 5 toxicant concentrations (0.0010–10 mg/L),and one container held control water. The number of motile/living and immobilized daphnids was counted after 24 h exposure to potassium dichromate. Five toxicant concentrations were calculated according to the formula: $a_n=a_1\times R_{n-1}$(USEPA, 1985).The 5 toxicant concentrations and control sample were tested in triplicate in both lethality 24 h acute feeding tests. In the final three replicates of the experiment, the concentrations ranging from 0.7 to 2.1mg/L of potassium dichromate were prepared to estimate the EC_{50} lethality endpoint. The experiment was conducted under normal laboratory condition [22].

Acute feeding bioassay. Suppression of *D. magna* thoracic legs from moving due to exposure to toxic substance was used as an endpoint in acute feeding bioassay. To calculate the decreased feeding endpoint after exposure to potassium dichromate, we used the least square method of linear regression (y=ax+b) to calculate the formula: y=a log (toxicant concentration) + b (Fig.1). From this equation, the y axis=0, i.e., $\log[(F_0-F_n)/F_n]=0$, which indicated EC_{50}, where toxic value corresponding to y=0 represented the half toxic value measured for control "Median fluorescence intensity" means the stable value analyzed by fluorescence photometer.

An EC_{50} for both the lethality bioassay and the feeding suppression bioassay was computed for each replicate series of dilutions, hence three EC_{50}s were computed for each sample. To calculate suppression in food uptake of *D. magna* due to exposure for 3 and 6 h of potassium dichromate, we have measured fluorescence intensity of spirulina (DIC Raihutekku Co.,Ltd., Japan) and chlorella (Genryouyadottokomu Co.,Ltd.) food powder solutions using a Shimadzu RF-5300PC fluorescence spectrophotometer (UV-2450;cell size of 1×1×5cm filled until 3 mL of the solution). We prepared calibration curves of chlorella or spirulina powder solution taken at different concentrations (0–100 mg/L) to show which powder solution has greater sensitivity by fluorescence intensity measurement (Fig.2).

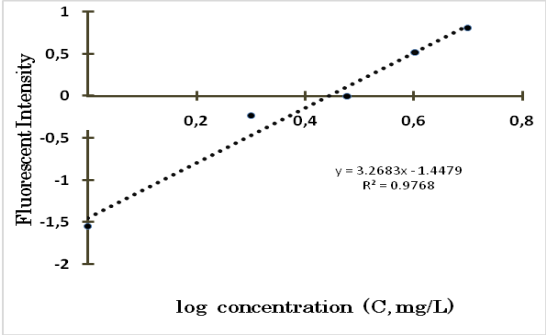

Fig. 1The linear equation for potassium dichromate: y=a log[$K_2Cr_2O_7$] + b; y axis=0, i.e., $\log[(F_0-F_n)/F_n]=0$, this indicates EC_{50}, i.e., half of the fluorescence intensity of F_0 (control).

For feeding bioassay, we investigated an experimental design as follows: we exposed 10 neonates to 20 mL glass container simultaneously filled with 2.5 mL potassium dichromate solution and 2.5 mL of spirulina or chlorella solution. The concentration of dry algae powder stock solution was 100 mg/L, and 50 mg/L was placed into 20 mL glass container. We have prepared 5 toxicant concentrations of potassium dichromate ranging from 0 to 5 mg/L ("0" value was measured as fluorescence intensity for control sample where no toxic substance was found). In the simultaneous exposure, we allowed 10 daphnids to infiltrate into the chlorella and spirulina powder solutions for 2, 4, 6, 8, 10, and 12 h with potassium dichromate as standard toxicant solution. For both chlorella and spirulina solutions, the concentrations used were 40, 50, and 60 mg/L (Fig.3).

After simultaneously exposing the experiments, we have also conducted a bioassay to allow *Daphnia* feeding in algae powder for only 30 min.

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Ten daphnids were exposed to 20 mL vials containing 5 mL of potassium dichromate solution (concentration ranging from 0 to 5 mg/L) for 3 and 6 h. Then, daphnids were washed and transferred into 5 mL of 50 mL algae powder solution (Table 1).

III. Results

A. *Acute lethality assay of Daphnia.*

The estimated EC_{50} lethality endpoint was 1.24±0.07 mg/L concentration obtained as the EC_{50} endpoint for immobilization test.

B. *Acute feeding assay of D. magna with potassium dichromate*

The results of prepared calibration curves of chlorella or spirulina powder were shown in Fig. 2.We can obtain higher value of median fluorescence intensity by using spirulina powder solution than that by using chlorella powder solution.

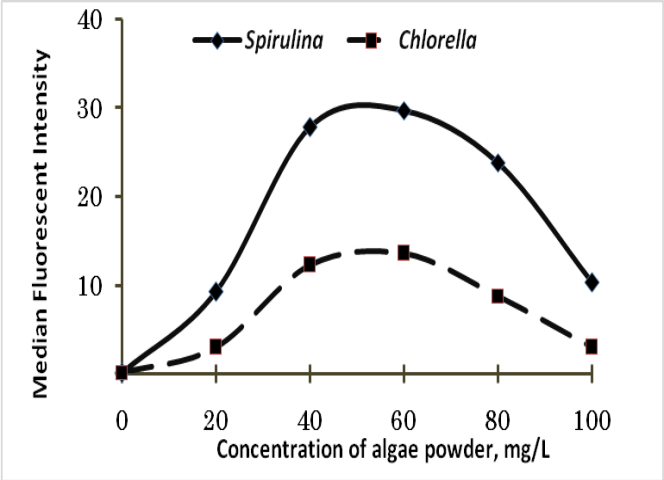

Fig.2Effect (control) of spirulina (E_x=580nm and E_m=640nm) and chlorella (E_x=439 nm and E_m=679nm) concentrations (mg/L) on fluorescence intensity after10 daphnids are exposed for 30 min to different concentrations of algae powder solution; peak value of fluorescence intensity is observed at 50 mg/L.

For spirulina and chlorella powder solutions, the maximum fluorescence intensity was obtained by measuring chlorella and spirulina solutions at50 mg/L (Fig.3, a-b). At higher concentrations, fluorescence intensity decreased, because there is a critical concentration (incipient limiting level) for food particles; below that concentration, the filtration rate of *D. magna* increases proportional to food particles [17].

Then, we measured median fluorescence intensity according to simultaneous exposure to algae and toxicant. As for spirulina powder solution with median fluorescence intensity, a sharp/linear increase in the measured values until 6 h of exposure was shown and then plateaued. For both chlorella and spirulina solutions, we have observed a plateau of median fluorescence intensity values from 6 h to 12 h (Fig.3, a-b). In our previous study [11] using the flowing system and simultaneously exposing toxicants and *Chlorella vulgaris* cells for 180 min, we had also obtained a linear relationship between increased fluorescence intensity and increased toxicant concentration.

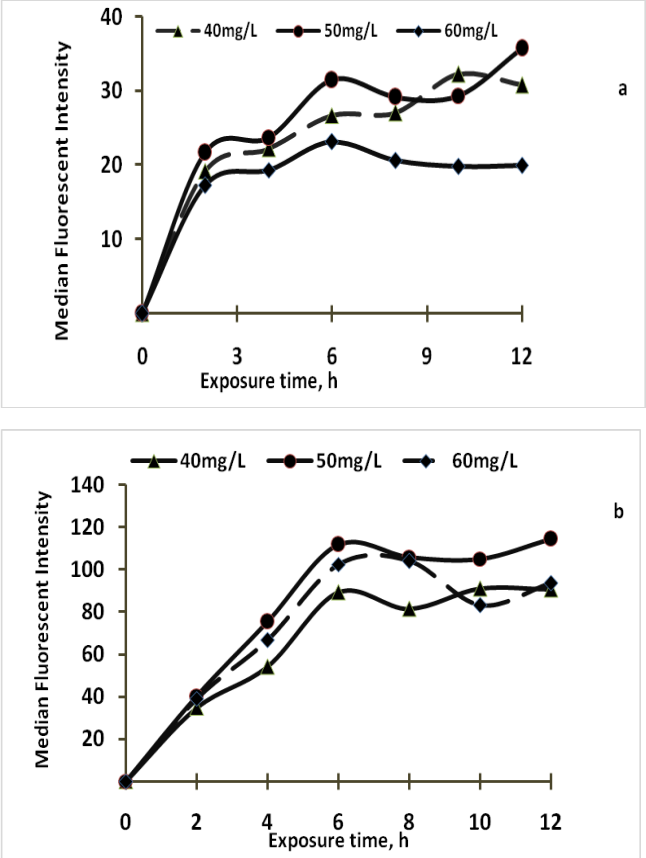

Fig.3Effect of exposure time (hours) on fluorescence intensity of 10 neonate daphnids exposed to three concentrations (mg/L) of a) chlorella powder solution and b) spirulina powder solution.

We have observed the precipitation of spirulina and chlorella powder solutions to better understand why there is a difference in measured value of fluorescence intensity between two solutions.

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We have prepared 50 mg/L of both spirulina and chlorella solutions and observed the precipitation mechanism in 200 mL glass containers. In Fig.4, photos were captured at 15-minute intervals over a 45-minute time period. From the figure, we can see that the particles of the chlorella solution were precipitated more quickly than those of the spirulina solution. It was likely that fast particle precipitation of chlorella solution was due to low values of fluorescence intensity.

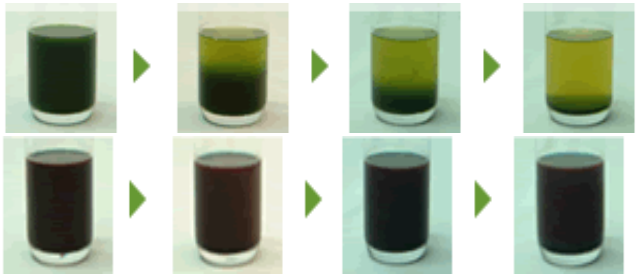

Fig.4Comparison of precipitation of two powder solutions (chlorella and spirulina) for a period of 45 min.

In the feeding suppression experiment, we can consider two variants of toxicant exposure: simultaneously dissolved in food substance and separate procedure of feeding after exposure. In the case of simultaneous food exposition for long periods, the effect of precipitation of toxic food substance might influence the procedure of filtration and thus fluorescence intensity measurement. In the case of separation of feeding procedure, we can expose daphnids in toxicant solution for longer period; however, there is a drawback that daphnids may recover their ability to feed more normally when placed in solution without toxicant.

In Table 1, we have showed the comparison results of the EC_{50}endpoints calculated for 3 and 6 h feeding bioassay (chlorella and spirulina) at two variants of exposure experiments: simultaneous toxicant and feeding exposure and pre-exposure to toxicant and separate feeding exposure. As seen in Table 1, $EC_{50}$for 3 h versus pre-exposure experiment showed more similar sensitivity in comparison with the standard 24 h immobility test (1.24 mg/L). In the case of 6 h exposure to toxicant EC_{50} concentration, the values for two variants of exposure experiments were approximately the same and had similar sensitivity in comparison with the immobility test. However, the standard deviation values for 3 and 6 h versus the simultaneous experiment were less than those versus the pre-exposure experiment.

Table I
Comparison Of EC50 Endpoint Measurement (± SD) Obtained In Two Variants Of Suppressed Feeding Bioassays Of Two Dry Algae Powder For Standard Toxicant Exposure

Hr-s	EC_{50} [mg/L]			
	Exposure to toxicant and feeding solution at the same time		Exposure to toxicant before 30 min feeding	
	Chlorella	Spirulina	Chlorella	Spirulina
3	2.77±0.45 (R^2=0.9768)	3.14±0.23 (R^2=0.9906)	1.71±0.61 (R^2=0.8981)	1.41±0.54 (R^2=0.8981)
6	1.72±0.35 (R^2=0.9175)	2.20±0.15 (R^2=0.9693)	1.93±1.12 (R^2=0.8235)	1.72±0.75 (R^2=0.8575)

C. Acute feeding assay of D. magna with H_2O_2

We have carried out feeding assay to obtain EC_{50} for 3 h simultaneous exposure of 10 daphnids at different concentrations of hydrogen peroxide and 50 mg/L of spirulina powder solution. The EC_{50} endpoint concentration was 0.13 mg/L showing more sensitive evaluation than the immobilization test. For example, the LC_{50} of D. magna lethality test for H_2O_2 was 5.6mg/Las reported by Reichwaldet al., 2012 [19].

IV. Discussion

The best results from our previous studies measuring decreased *Daphnia* feeding on fluorescence intensity were obtained for toxic evaluation of heavy metal ions [11; 14; 9].

It is known that food intake by daphnids is highly dependent on food availability as well as on the quality of food and size of particles. McMachon and Rigler [17] suggested that if care is taken to provide less than the incipient limiting concentration, a measurement of filtering rate in only one concentration of food might be enough to show whether foods were toxic or inhibitory Matorin et al. [16] have shown that in higher algae cell concentrations, daphnids are unable to filter more food and, therefore, loose the "excessive" filtered cells back to the solution. *D.magna* filtration rate remained constant at chlorella cell concentration ranging from 30 to 300×10^3 cells/mL [16]. On the other hand, Filenko et al., [10] investigated that *Daphnia* was unable to meet its food intake at algae concentrations less than 5×10^3cells/mL.

In our previous study [11], in the flowing system for 3 h of toxicant solution with ingested *C.vulgaris* cells by *Daphnia*, we observed that fluorescence intensity value could be used as a sensitive indicator of decreased feeding: the obtained EC_{50} at 1.23mg/L for 3 h exposure to potassium dichromate was comparable to EC_{50} obtainedat 1.71 mg/L for 24 h immobility test. As in the study by Ishii et al. [11], simultaneous exposure up to 3h to toxicants and *C.vulgaris* did not show any effect on fluorescence intensity measurement.

Rodriguez et al., 2006 [20] used chlorella cells to evaluate decreased feeding of daphnids to potassium dichromate and after 4 h of exposure obtained similar sensitivity of endpoint in comparison with the standard lethality test.

In our present study, in the 30 min exposure of 10 daphnids to spirulina and chlorella powder solutions, the fluorescence intensity increased proportional to the concentration of algae solution approximately 40 mg/L, remained constant at a range of 40–60 mg/L, and then decreased from 70 to 100 mg/L. If we measured filtration rate as fluorescence intensity, it is expected that the concentration of algae solutions at a range of 40–60 mg/L is close to the incipient limiting concentration (ILC) and allows us to easily measure fluorescence intensity, because we have obtained max values.

In the present study for simultaneous exposure, the EC_{50} values showed more similar result to 24 h lethality endpoint only after 6 h. In the case of feeding daphnids only 30 min of spirulina and chlorella powder, we obtained EC_{50} for 3 h exposure period at the same level of sensitivity as EC_{50} obtained for 24 h immobilization test; however, indexes of R^2 and standard deviation values (Table 1) for this design showed less values than those in design when daphnids were allowed feeding during all periods of 3 or 6 h exposure.

In our studies to measure fluorescence intensity for evolution feeding activity of *Daphnia*, we usually used neonates (1.35 mm) instead of juveniles or adults to allow an actual comparison of the performance of *Daphnia* feeding assay with that of standardized acute lethal *Daphnia*, which is conducted with 1-day born neonates. However, Matorin et al., 2009 [16] investigated that the filtration rate in 1-day neonates (<1mm) did not exceed 0.3 mL/h and was characterized by a great variation (CV=42%). The most stable *F* (CV=21%) was found in 6–7-day old juvenileswith3.5–4 mm size and consisted of 3.5–4 mL/h. In the next experimental design, we would like to use juveniles instead of neonates for the evaluation of decreased feeding.

Feeding rate is one sensitive endpoint that could be measured as fluorescence intensity value at incipient limiting level concentration of food particles.

Using filtration rate as fluorescence intensity value for 6 h of daphnid simultaneous exposure to algae powder solution allows us to measure EC_{50} concentration as one sensitive physiological endpoint to mimic environmentally relevant conditions. Using spirulina and chlorella powder solutions of 50 mL/L for 6 h toxicant exposure to these solutions, we obtained EC_{50} 2.20 and 1.72 mg/L respectively. Spirulina powder solution concentration of potassium dichromate curves showed more distinct correlation between median fluorescence intensity and toxicant concentration.

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