Engineering of a New Acute Bioassay Design Based on Feeding Behavior of *D. magna* with Fluorescent Microbeads

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Abstract — A rapid acute-toxicity assay based on feeding behavior of *Daphnia magna* was developed and evaluated. Lethality is a toxicity endpoint for standard acute *D. magna* bioassays; however, the feeding activities of daphnids provide a more sensitive endpoint for the acute test. Since the amount of particles ingested by *D. magna* decreased when we exposed daphnids to toxic materials, the suppression of feeding activity is very useful for developing new feeding acute toxicity tests. We used polystyrene fluorescent particles with a diameter of 0.2 μm instead of food particles for daphnids. The amount of microbeads ingested by daphnids after toxicant exposure was calculated from the fluorescent intensity of the homogenized solution of daphnids. The effective concentration (EC50) equaled the concentration that caused a 50 % decrease in fluorescence intensity compared to the control. We tested 4 toxicants at 4-, 8- and 12-h exposure times and compared EC50 concentrations obtained from subsequent feeding bioassay with EC50 obtained from a 24 h lethality bioassay. We found that 8 h of exposure to potassium dichromate, nickel sulfate and pentachlorophenol sodium salt followed by 8 min of feeding time resulted in EC50 not significantly different from the standard 24 h lethality test, however zinc sulfate required either longer exposure time or longer feeding time to produce a comparable EC50. Our results indicate that feeding-suppression (i.e. reduction in fluorescent intensity) following exposure to toxicants can be used to rapidly assess EC50 of a toxicant within one working day.

Keywords — *Daphnia magna*, feeding suppression, fluorescent microbeads, toxicity bioassay, potassium dichromate, zinc sulfate, nickel sulfate, pentachlorophenol.

I. INTRODUCTION

Acute toxicity tests using freshwater invertebrates is required to support registration of a pesticide intended for outdoor application, establish water quality criteria, and provide aquatic safety assessment for chemicals (USEPA, 1985). Acute 24 h *D. magna* bioassay requires lethality for daphnids as an experiment endpoint. Instead of lethality, the use of daphnid feeding activity as criteria in acute testing would be more rapid and sensitive as an endpoint. The rationale for using daphnid feeding behavior as an endpoint in acute toxicity test include rapid assessment, and physiological and ecological relevance (Sibly, 1996; Calow & Sibly, 1990; De Coen & Janssen, 1998). Exposure to any toxic substance may reduce resource acquisition (supply-side effect; 2000), reduce offspring production, and increase the age at first reproduction. To quantify the suppression of daphnid feeding activity after toxic exposure, a method of calculating the uptake of food particles ingested by daphnids must first be determined. For cladocerans, Allen et al (1995) suggested direct counts of yeast or algae cell quantities before and after feeding activity. Another approach described by Bitton et al. (1995) involved fluorescent microscopy for observation of gut conditions after the feeding period: whether or not the daphnid guts were filled by yeast or algae cells labeled with any fluorescent substance. 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 and less labor than the feeding bioassays described above. *D. magna* as a filter-feeder prefers algae, but any suspended particles may be retained on their filtering appendages and then ingested (Brandelberger, 1985). Further, we designed two stages of *D. magna* exposure to be performed separately: “toxicant exposure” and “feeding exposure” to avoid adsorption of heavy metal ions or any toxicants on feeding materials during experiments. Furthermore, the food filtration of daphnids has great effect on reproduction rate, growth and mortality rate, and therefore on their population level (Barata & Baird, 2000) and it becomes a very useful acute endpoint to provide safety assessment for chemicals.
II. MATERIALS AND METHODS

A. Daphnia magna Culture

*Daphnia magna* was cultured in an incubator at 21-22 °C using spring water from the well near Kogakuin University (pH=6.34, hardness 50-70 mg/L of calcium carbonate, oxygen=6.01 mg/L). *D. magna* was cultivated according to the literature (ISO, 1989; J.I.S., 1992) and fed daily with Spirulina powder (GRAS, http://www.bunkajin.com/shopping). In both acute experiments, lethality and feeding, we used *D. magna* neonates not older than 24 h (the mean size of neonates was 1.35 mm) and in the first instar.

B. Microbeads Stock Solution

The fluorescent microbeads stock solution (d=0.2 μm; 2% FluoSphere carboxylate modified microsphere F8805; invitrogen.com) contained 4.55×10^12 particles per mL. It was prepared using MilliQ water (Organo corporation) and diluted to a final concentration 1.37×10^10 particles/mL with moderately hard water containing 0.6 mol/L sodium hydro carbonate (7.7×10^{-4}), 0.06 mol/L potassium chloride (7.7×10^{-5}), 0.4 mol/L magnesium sulfate (5.0×10^{-4}), 1.6 mol/L calcium chloride (2.0×10^{-3}); (J.I.S. K 0229/1992).

C. Stock Solution of Toxicants

Stock solutions of potassium dichromate, zinc sulfate, copper sulfate and pentachlorophenol sodium salt were prepared using MilliQ water (Organo Corporation). The standard toxicant test solution of potassium dichromate (50 mg/L), zinc sulfate (1000 mg/L), nickel sulfate (1000 mg/L) and pentachlorophenol sodium salt were diluted to six different concentrations of toxicant. One control vial was prepared with moderately hard water J.I.S. 0229/1992.

D. Microbead Uptake

The uptake of fluorescent microbeads by *D. magna* neonates was observed using fluorescent microscopy (Olympus Company). To establish a baseline of the relationship between fluorescent intensity and exposure time under normal feeding conditions, we exposed neonates to microbeads solution (1.37×10^{10} particles/mL) for 2, 4, 8, 12, 16, 20, 24 and 28 minutes without any toxicant exposure and then measured the amount of ingested microbeads/fluorescent intensity according the method described below. After holding neonates into microbeads solution the excess of microbeads solution carefully removed from the outer surface of neonates (three times washed with deionized water) and then 10 neonates were transferred into 1.5-ml micro tubes contend 1 ml of deionized water and prepared homogenized solution with Azuwa’ homogenizer for 2 minutes. Fluorescence intensity of 1 ml the homogenized solution was measured using a fluorescence spectrophotometer (by wavelengths: Ex=365 nm, Em=413 nm).

E. The 30-minute Potassium Dichromate Feeding-Suppression Test

Experiments of the effect of toxicant exposure on fluorescent intensity/amount of ingested microbeads we conducted out using 30 minutes exposure of neonates (mean body size is 1.35 mm) in potassium dichromate diluted to 0.1, 0.2, 0.5, 1.0, 3, 8 and 18 mg/L.

F. Acute Lethality Bioassays

Acute lethality bioassay was carried out according to the standard method (ISO-6341-1989; J.I.S. 0229/1992). These experiments were conducted within an incubator at a set temperature of 22 °C. For each tested toxicant (potassium dichromate, nickel sulfate, zinc sulfate and pentachlorophenol) five randomly picked neonates were transferred into 20 ml glass containers containing 5 ml of one of 6 toxicant concentrations and one container with control water (J.I.S. 0229/1992). The number of motile and immobilized daphnids was counted after 4, 8, 12, 20 and 24 h for potassium dichromate and nickel sulfate, and after 24 h for zinc sulfate and pentachlorophenol. Toxicant concentrations used in lethality experiments were calculated according with formula: 
\[
\frac{\text{Exposure Time (min)}}{\text{Feeding/microbead exposure time (min)}} = \frac{\text{R}^{\text{final concentrations} - \text{initial and final concentrations} \text{CHOSEN by experimental way}}}{\text{Exposure Time (min)}}
\]

Once we established the minimum time required to fluoresce normal daphnids from the feeding/microbead exposure experiment as 8 min (Fig 1), we repeated the feeding/microbead exposure experiment with daphnids that had been pre-exposed to the 4 toxicants.
The suppression of feeding activity, and thus fluorescent intensity, corresponded to toxicant exposure. For the feeding-activity suppression bioassay, 10 neonates were randomly selected from the mother culture and transferred into 20 ml glass containers with 5 ml of 4 test toxicants and one container with control water (J.I.S. 0229/1992). Exposure time for toxicant solutions was 4, 8, and 12 h for potassium dichromate and nickel sulfate and 4 and 8 h for zinc sulfate and pentachlorophenol sodium salt. Six different concentrations for four toxicants were calculated according formula described above; initial and final concentrations for each exposure period were chosen by experimental way: 1.0, 1.3, 1.7, 2.3, 3.0 and 4.0 mg/L for 4 h exposure, 0.3, 0.4, 0.5, 0.7, 0.9 and 1.3 mg/L for 8 h exposure, and 0.2, 0.3, 0.4, 0.5, 0.7 and 1.0 mg/L for 12 h exposure. For nickel sulfate, the concentrations tested were, 20, 30, 40, 54, 72, 97 and 130 mg/L for 4 h exposure, 30, 37, 45, 54, 66 and 80 mg/L for 8 h exposure, and 10, 13, 17, 23, 30 and 40 mg/L for 12 h exposure. For nickel sulfate, the concentrations tested were 30, 40, 50, 60, 80 and 100 mg/L for 4 h exposure, and 20, 25, 30, 40, 55 and 70 mg/L for 8 h exposure. For pentachlorophenol sodium salt the concentrations tested were 0.2, 0.3, 0.45, 0.68, 1.0 and 1.5 mg/L for 4 h exposure and 0.15, 0.25, 0.35, 0.55, 0.85 and 1.25 mg/L for 8 h exposure. The temperature during all feeding suppression experiments was 22 ºC. As for control vessel the exposure times in each experiment for four chemicals were set up the same as for six vessels of toxicant. After the exposure period in toxicant solution, the 10 neonates were washed with deionized water and transferred into a glass container with 5 ml of microbead solution for 8 minutes. The endpoint concentration EC50 during the feeding suppression experiment was calculated as the amount of ingested beads that corresponded to a 50 % decrease in fluorescent intensity compared to the control (Kamaya et al, 2011).

H. Statistical Analysis

An EC50 for both the lethality bioassay and the feeding suppression bioassay were computed for each replicate series of dilutions, hence three EC50s were computed for each sample. EC50 obtained from the feeding-suppression assays were compared to the standard 24 h lethality EC50 using t-tests with Bonferroni adjustment in OriginPro 7.5. Linear regression and correlation coefficient with corresponding 95% confidence limit for feeding-suppression versus lethality bioassays were calculated using the analysis package in a Microsoft Excel spreadsheet.

III. RESULTS

A. Microbeads Uptake Kinetics

The uptake of fluorescent microbeads by D. magna neonates was observed using fluorescent microscopy. The intestinal tract of the neonates became approximately 50 % filled with microbead particles after only 3 minutes and nearly full and brightly glowing after 5 minutes of feeding time when it started the defecation of microbead particles. We also measured fluorescent intensity over a period of 28 minutes of ingestion time (Fig. 1).

![Figure 1. Fluorescent intensity/amount of ingested microbeads over time by neonate daphnids under normal feeding conditions.](image-url)

The period of ingestion time from 4-20 minutes was characterized by a gradual increase in fluorescent intensity. During the period of time from 16-24 minutes, a plateau of approximately constant values of fluorescent intensity was reached. After 20 minutes of fluorescent microbead ingestion, there was a period of smoothly decreasing fluorescent intensity. The smallest variation between minimum and maximum fluorescent intensity was observed up to 12 minutes of exposure time and the largest variation was observed 16-24 minutes. So, 8 min was used for feeding suppression part of experiment as a point when fluorescent intensity goes up and has the smallest variation between min and max.

We also investigated the effect of toxicant exposure on fluorescent intensity/amount of ingested microbeads using 30 min exposure in potassium dichromate (Fig.2).
Figure 2. Effect of standard toxicant (K$_2$Cr$_2$O$_7$) concentrations on fluorescent intensity of daphnids that were subsequently exposed to a microbead solution (1.37×10$^{10}$ particles/mL) for 8 min.

At the lower concentrations of potassium dichromate, fluorescent intensity decreased with increasing toxicant concentrations; however, at a certain toxicant concentration (about 4 mg/L), fluorescent intensity reached a plateau. The point at which the curve reached a plateau (4 mg/L of potassium dichromate) was used as the highest concentration of potassium dichromate for the feeding-suppression bioassays.

**TABLE I**

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>4 h EC$_{50}$ (mg/L)</th>
<th>8 h EC$_{50}$ (mg/L)</th>
<th>12 h EC$_{50}$ (mg/L)</th>
<th>20 h EC$_{50}$ (mg/L)</th>
<th>24 h EC$_{50}$ (mg/L)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dichromate</td>
<td>8.85±2.00*</td>
<td>2.28±0.09</td>
<td>1.79.±0.09</td>
<td>0.95±0.07</td>
<td>0.77±0.02</td>
</tr>
<tr>
<td>Nickel sulfate</td>
<td>2602.5±867.1</td>
<td>867.4±72.0</td>
<td>352.9±135.4</td>
<td>91.4±13.8</td>
<td>62.07±1.1</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>15.05±3.1</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.36±0.13</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>4 h EC$_{50}$ (mg/L)</th>
<th>8 h EC$_{50}$ (mg/L)</th>
<th>12 h EC$_{50}$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dichromate</td>
<td>1.21 ± 0.11</td>
<td>0.75 ± 0.03</td>
<td>0.45 ± 0.08</td>
</tr>
<tr>
<td>Nickel sulfate</td>
<td>70.66 ± 2.57</td>
<td>62.07 ± 2.81</td>
<td>45.83 ± 2.54</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>42.70 ± 13.6</td>
<td>36.09 ± 3.90</td>
<td>n/a</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>0.64 ± 0.11</td>
<td>0.48 ± 0.11</td>
<td>n/a</td>
</tr>
</tbody>
</table>
The feeding-suppression 8 h test was equivalent to the standard 24 h lethality test in sensitivity for potassium dichromate, nickel sulfate and pentachlorophenol (p<0.05). The feeding suppression 12 h test was more sensitive than the lethality test for potassium dichromate and nickel sulfate (p<0.05). For zinc sulfate, the 24 h lethality test was more sensitive than the 4 and 8 h feeding-suppression tests; was noticed that coefficient of variation of EC 50 s for 4 and 8 h feeding tests and 24 h lethality test in zinc sulfate had greater values compared with other 3 studied toxicants. For nickel sulfate at the p<0.05 level there was no significant difference between the EC50 means of 8 h feeding-suppression tests and the 24 h lethality test.

C. Correlation of Lethality- Versus Feeding-Suppression Test Results.

The EC50 obtained via lethality tests were correlated (p<0.05) with EC50 obtained via 4 and 8 h feeding-suppression tests for all four toxicants (Fig. 3). The correlation coefficient for this regression was 0.93.

![Graph showing relationship between mean EC50 for 24 h lethality bioassay versus 4- and 8 h feeding-suppression bioassays for potassium dichromate, nickel sulfate, zinc sulfate and pentachlorophenol.](image)

Therefore it is likely that the fluorescent microbeads as non-digestible particles pass through the intestinal tract of daphnids faster than digestible cells of yeast, algae or bacteria. This phenomenon (gut passing time) allowed us to shorten the “feeding” exposure time for daphnids in microbeads solution to 8 minutes. As filter-feeders, daphnids sieve large quantities of water to collect suspended particles, and although their preferred food is phytoplankton, they will ingest any suspended particle that can be retained by their filtering appendages (i.e. > 0.45 μm; Brendelberger, 1985). On the other hand, D. magna was classified by Geller and Muller (1981) as a fine filter feeder that is able to actively filter particles as small as 200 nm, although this is an estimate based upon the size/gap between the setules of daphnids. Probably, during filter-feeding in the microbeads solution, D. magna was not able to distinguish whether ingested particles were digestible or non-digestible, especially for this short time of 8 minutes. The separate exposure of daphnids to toxicant and to microbeads solution allowed us to construct these short-time bioassays (i.e. for whole body metal accumulation). Although it is not standard practice to clear the gut of planktonic organisms before analyzing their tissues, some investigators do transfer animals to clean algae to purge their gut after they have been fed metal-contaminated algae. For instance, Munger and Hare (1997) fed clean algae to Ceriodaphnia dubia for 30 min after exposing them to Cd-contaminated algae. Hooke and Fisher (2001) transferred C. dubia to clean algae for 4 h after exposure to Ag-labeled algae, and Barata et al. (2002) transferred D. magna to clean algae for 8 h to purge Cd-labeled algae.

The concentration of fluorescent microbeads used in our feeding tests was 1.37×10^10 particles/mL, much more than the concentration of yeast, bacteria or algae used in previous feeding experiments with D. magna (e.g. 7.5×10^4 yeast cells/ml; Bitton et al, 1995). Generally above a critical food concentration (incipient limiting concentration, ILC), food uptake is constrained by the rates of either ingestion (mandibular rate) or digestion (gut volume, gut passage time) (Rigler, 1961). Philippova and Postnov (1988) found that the costs of feeding for daphnids varied depending on food concentration. At high food concentrations they found food processing (digestion and biochemical processing) to be the most costly aspect of feeding. Probably, ingestion of fluorescent microbeads was a less energy consuming process compared to biochemical processing of algae/bacteria and for this reason we were able to use such high concentration of microbeads to facilitate filtering procedure for our bioassays.
In our previous study (Kamaya et al., 2011), we had measured the depression of *D. magna* feeding activity through only 30 minutes of exposure to toxicant solution; however, before toxicant exposure, we had to leave neonates taken from an in-house culture in moderately hard water for 2 hours to get a higher filtration rate of neonates. The starved daphnids consume more food in 1 hour than fed animals when both are in non-limiting concentrations of *Chlorella vulgaris* (McMahon and Rigler, 1965). Starvation led to a behaviorally mediated decrease in appendage beat rate, but starved daphnids consistently showed an almost immediate increase in their appendage beat rate once food was added (Plath, 1998). In this study we prolonged exposure to the toxicant from 30 minutes to 4, 8 and 12 h and obtained the increase in neonate appendage beat rate after 4-12 h of starvation. Increased beat rate resulted in faster uptake of fluorescent microbeads and therefore shortened the optimal feeding time to just 8 minutes and allowed the bioassays to be completed in less time and do not influence uptake of toxicants. Furthermore, as a results of these tests we could gain EC50 for feeding tests that was comparable to EC50 of standard 24 h lethality test.

V. CONCLUSION

Based on a limited number of toxicants, it appears that an 8 h feeding-suppression test can give results that compare closely to those of a standard 24 h acute lethality tests. This new method exploiting the decrease in fluorescent intensity after exposure to a toxicant allows for more rapid assessment of the EC50 of a toxicant. Eight h of exposure to potassium dichromate, nickel sulfate and pentachlorophenol followed by 8 min of feeding in the microbead solution produced results not significantly different from the 24 h lethality bioassay. Because up to 8 h of exposure to zinc sulfate did not produce an EC50 comparable to the 24 h lethality bioassay, we recommend further study with zinc sulfate to find a longer feeding time in the microbead solution that would increase the sensitivity of the feeding-suppression bioassay. We are investigating possibilities for further optimizing the feeding-suppression bioassay by decreasing the length of toxicant exposure (from 8 to 6 h) and prolonging the feeding period to 12-15 min, a feeding time that resulted in greater fluorescent intensity (Fig.1). Our new methodology can be applied to other potential toxicants to establish the optimum combination of toxicant exposure time with feeding time for each toxicant. This new method allows rapid assessment of toxicants within one working day.

Acknowledgments

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REFERENCES

