Chronic Toxicity Tests with *Daphnia magna* for the Examination of River Water Quality

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**ABSTRACT**

The toxicological effects of river water on the reproduction of the water flea *Daphnia magna* were assessed with the standard 21-d chronic test (OECD TG211) at four river sites (St. 1–4) in rural districts of the Kanto Plain (Ibaraki Prefecture, Japan) at monthly intervals from April 2007 to March 2010. Over the 3 years, we recorded the following average numbers of live *D. magna* offspring after the 21-d tests: 17–233, 14–223, 29–210, and 84–209 in St. 1, 2, 3, and 4, respectively. The average numbers in August 2007, May and September 2008, and April 2009 at St. 1; August 2007 and May 2008 at St. 2, and June 2007 and April 2009 at St. 3 were significantly lower than those of the control (ANOVA, $P < 0.01$). The results of the above tests suggest that *D. magna* may be affected not only by the overall pesticide toxicity in the river water, but also by other factors that were not clarified in the present study. In addition, significant positive correlations were observed at each site between the body length of *D. magna* and the total number of offspring at 7 d ($r^2 = 0.783$), 14 d ($r^2 = 0.772$), and 21 d ($r^2 = 0.931$) after the test began on 10 May 2008. These findings suggest that the body length of *D. magna* might be used to evaluate the toxicological effects of river water, even at 7 d, by using the standard chronic toxicity test.

**Key words:** *Daphnia magna*; chronic toxicity; reproduction; river; body length

1. **INTRODUCTION**

Pesticides are designed to kill insects (insecticides), weeds (herbicides), or fungi (fungicides). *Daphnia* and shrimps seem to be highly sensitive to insecticides while some plants and algae seem to be highly sensitive to herbicides (and possibly fungicides). Many kinds of pesticides are permitted for use on field and rice paddies in Japan, and some of them have been detected in river water (Tanabe *et al.*, 2001; Sudo *et al.*, 2002; Nakano *et al.*, 2004; Iwafune *et al.*, 2011; Phong *et al.*, 2012; Yamamoto *et al.*, 2012). Animals and plants living in the rivers are exposed to these pesticides, acute studies such as Palma *et al.* (2010) in Portugal and Kikuchi *et al.* (2000, 2008) in Japan and those outside of Japan but few studies have examined the toxicological effects of river water using the standard chronic test (Hatakeyama *et al.*, 1994; 1997) in Japan.

Some other researchers have been published their studies investigating chronic toxicity of river water using Green algae and shrimp (e.g.,
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Hatakeyama *et al.*, 1994, 1997), *Ceriodaphnia dubia* (e.g., Vigano *et al.* 1996; Werner *et al.*, 2000), and *Daphnia magna* (e.g., Sakai *et al.*, 2001, 2002).

The effects of herbicides on algal production in the Kokai River were assessed using a *Selenastrum* growth inhibition test. The algal production in the river was affected by the single or joint action of herbicides (Hatakeyama *et al.*, 1994). The shrimp mortality in the river water samples was also caused by the overall pesticide toxicity (Hatakeyama *et al.*, 1997; 1999).

The River Lambro (Italy) demonstrated variable toxicity in different time periods, although the most frequent effects were sublethal, on both reproduction and growth of *C. dubia*, Ammonia, nickel, and zinc can be indicated as possible toxicants using the standardized U. S. Environmental Protection Agency (EPA) freshwater toxicity test (EPA, 1994) with *C. dubia* (Vigano *et al.* 1996).

To detect and characterize the spatial extent, severity, frequency, and causes of potential toxicity caused by anthropogenic pollutants, a monitoring study was conducted over a period of two years (1993-1995) (Werner *et al.*, 2000). Sites (California's Sacramento–San Joaquin Delta, USA) were monitored on a monthly basis using the 7-d toxicity tests (EPA) with *C. dubia* (Vigano *et al.* 1996).

Sakai (2001, 2002) reported that river water sampled in the Maioka River located in Yokohama, Japan in May 1999, and May and June 2000 was subjected to chronic tests with *D. magna* to investigate the harmful effects of pesticides.

The water flea *Daphnia* (Cladocera, Crustacea), whose relatives include other arthropods such as lobsters and crabs, are filter-feeding planktonic crustaceans that can be found in almost any permanent body of water.

*D. magna* has been used extensively as the standard laboratory testing organism to determine the toxicity of effluents and specific toxicants, and it has been demonstrated to be sensitive to many environmental contaminants (Hoffman *et al.*, 2003). The standard chronic toxicity test (TG211) of the Organization for Economic Cooperation and Development (OECD, 1998) assesses the reproduction of *D. magna* with food provided in a temperature- and light-controlled incubator for 21 d. This has led the developed countries to collect chronic toxicity data using *D. magna*. Acute and chronic toxicity tests using *D. magna* have been extensively used to study pesticide effects on nontarget aquatic invertebrates because of this species’ high sensitivity and fast growth and reproductive rates (Gersich, 1985; Day and Kaushik, 1987; Munzinger and Monicelli, 1992; Sakai, 2001).

The objective of the present study was to evaluate the toxicological effects of river water on the reproduction of *D. magna* over 3 years, as assessed by the standard 21-d chronic test (OECD TG211) at four rivers in central Japan. In addition, we compared *D. magna* growth and reproduction at 7, 14, and 21 d after the tests began and examined the relationship between growth and reproduction of *D. magna* when individuals were exposed in river water.
2. MATERIALS AND METHODS

2.1 Study sites and water sampling

This study was carried out from April 2007 to March 2010 at four rivers in the rural districts of Kanto Plain (Ibaraki Prefecture, Japan). As shown in Fig. 1, four sampling sites were chosen. St. 1 was in the lower reach of Sakura River (63.4 km), which originates from the foothills of Mt. Tsukuba in Makabe Town, Ibaraki Prefecture, and flows through north eastern rim of Kanto Plain to Lake Kasumigaura, where it joins Tone River and finally flows to the Pacific Ocean. St. 2 was in the upper reach of Hanamuro River (14 km), which originates from a small pond in Tsukuba, Ibaraki Prefecture, and flows into Lake Kasumigaura south of where the Sakura River meets the lake. St. 3 was in the middle reach of Yata River (18 km), which originates from rice paddy fields in Tsukuba, flows to Lake Ushiku, and then joins Tone River. St. 4 was in the lower reach of Kokai River (111.8 km) originates from Pond Kokaigaike in Nasu-karasuyama, Tochigi Prefecture, flows across Kanto Plain, and joins the Tone River.

These sites were lined by rice paddy fields and orchards, with scattered houses. The river width and depth at each site were as follows: 20–30 m and 50–60 cm at St. 1, 4–5 m and 30–40 cm at St. 2, 4–5 m and 30–40 cm at St. 3, and 50–60 m and 40–50 cm at St. 4.

On the first or second Thursday of every month, a 3-L water sample was collected at each site and filtered through a plankton net (mesh size, 63 µm) into a 3-L glass bottle. Samples were brought to the laboratory in an ice chest within 2 h of collection and kept in the dark room.
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2.2 Physicochemical features

On every sampling site, water temperature, electric conductivity, and pH were measured at each site with a portable conductivity meter and a pH meter (SC-51 and PH-81, Yokogawa Electric Works, Tokyo, Japan) between 9 and 11am.

2.3 Test animal

NIES-strain *Daphnia magna* was used in this study. This strain has been maintained for more than 10 years in the National Institute for Environmental Studies (Tsukuba, Japan). Animals were maintained in 1-L glass beakers of dechlorinated tap water (hardness 60±5 mg L\(^{-1}\)) at a density of ~ 50 adults L\(^{-1}\) for several generations before experiments for acclimatization. Animals were fed daily with the green algae *Chlorella vulgaris* at a final concentration of ~ 1 \(\times\) 10\(^6\) cells ml\(^{-1}\) and were transferred to fresh medium by pipette every other day. More than 100 neonates were transferred to a new medium to establish the next generation, which took two weeks. The density of animals was reduced before they matured. All cultures were kept in a room automatically controlled under a photoperiod of 14:10 h light:dark at 22±0.5 °C.

2.4 Toxicity tests

Each water sample was decanted into five replicate 100-ml glass beakers, and the water temperature was adjusted to room temperature. The 21-d chronic toxicity tests with *D. magna* were performed according to the OECD method (OECD 1998). Fifty milliliters of test solution, or dechlorinated tap water for the control, was poured into a beaker, and a test animal (<24 h old) was added. Cultures were kept in the other automatically controlled room above for toxicity tests at a temperature of 22±0.5°C (water temperature 21±0.5°C) and a photoperiod of 14:10 h light:dark. The test solution was renewed at 7 d (first hatch) after the test began and then three times per week until the test ended on day 21. The *D. magna* were fed *C. vulgaris* daily, at a carbon level of 0.2 mg C per animal, as measured with a TOC meter (915B, Beckman Coulter Inc., Brea, CA, USA). As offspring were born, they were counted and then discarded.

2.5 Measurement of body lengths of the test animals

The body length of each test animal was measured using an ocular micrometer of a binocular microscope at 7, 14, and 21 d after the beginning of the test. Body length is defined as the distance from the top of the head capsule to the base of the tail spine. The animals were returned to their respective experimental medium immediately after image capture.

2.6 Statistical analysis

Data from the chronic tests and the body lengths of *D. magna* were analyzed using a one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test (StatView, ver. 5.0, SAS Institute, Cary, NC, USA), which allowed us to identify whether differences in the total number of offspring between the control and the river water samples were significant (\(P < 0.01\)) in cases where there are more than two groups.

3. RESULTS

3.1 Seasonal change of physicochemical features

The ranges of water temperatures were 2.7–
25.6 °C at St. 1, 4.1–24.5 °C at St. 2, 5.5–25.4 °C at St. 3, and 5.6–27.4 °C at St. 4 (Fig. 2). The pH of the water at each site varied over time, ranging from 6.0 to 7.9 across all sites. The electric conductivity of the water ranged from 186 to 331 µS cm−1 at St. 1, from 177 to 299 µS cm−1 at St. 2, from 235 to 341 µS cm−1 at St. 3, and from 152 to 301 µS cm−1 at St. 4. The pH and specific conductivity of dechlorinated tap water (i.e., the control) were 7.9±0.1 and 300±10 µS cm−1, respectively.

3.2 The 21-d changes in total number of *D. magna* offspring

The 21-d changes in total numbers of live *D. magna* offspring in the control and river water samples for tests that began on 10 January and 8 May 2008 are especially shown in Figure 3. For the test that began on 10 January, the average numbers of live offspring in river water samples were almost the same as the control at 7-d (first hatch), 14-d and 21-d, respectively.

For the test that began on 8 May, in contrast, there were only 8 live offspring in the control and 2–6 in river water samples at 7-d and 103 in the control and 20–83 in river water at 14-d. The total number of offspring increased to 173±18, 58±9, 75±25, 116±41, 137±12 in the control, Sts. 1, 2, 3, and 4 water samples, respectively by the end of the test (21 d).

The offspring hatched consistently every 3 d in the control and all river water samples in the January tests, and the total numbers of offspring at day 21 were similar among treatments (Fig. 3 A). In the May tests (Fig. 3 B), in contrast, hatching occurred every 3 d in the control, Sts. 3 and 4 while every 1 to 3 d in Sts. 1 and 2, and the total numbers of offspring were significantly less in St. 1 and 2 water than in the control (ANOVA, *P* < 0.01 in both cases).
3.3 Seasonal changes in the reproduction of *D. magna*

Figure 4 illustrates the seasonal changes in the average total number of live *D. magna* offspring at the end of the chronic toxicity tests from April 2007 to March 2010. The average (±SD) values ranged between 53±50 (August 2007) and 209±10 (February 2009) in the control, 17±14 (August 2007) and 233±23 (February 2009) in St. 1 water samples, 14±16 (August 2007) and 223±19 (January 2010) in St. 2 water, 29±20 (April 2009) and 210±22 (October 2009) in St. 3 water, and 84±42 (July 2008) and 209±7 (March 2009) in St. 4 water.

In the control, the average total number of live *D. magna* offspring at the end of the chronic toxicity tests were relatively low in July and August 2007 (53–124), June and August 2008 (113–138), and August and October 2009 (104–124). The average value also decreased in March and December 2009 and February 2010 compared to those of all sampling sites. Certain average values at St. 1 (17±15 in August 2007, 58±9 in May 2008, 66±30 in August 2008, 19±19 in September 2008, and 37±31 in April 2009), St. 2 (14±16 in August 2007 and 75±25 in May 2008), and St. 3 (34±8 in June 2007 and 29±20 in April 2009) were significantly lower than the corresponding values in the control (154±9 in June 2007, 53±50 in August 2007, 173±18 in May 2008, 134±20 in August 2008, 156±9 in September 2008, and 158±13 in April 2009) (ANOVA, \( P < 0.01 \) in all cases). The onset of reproduction of *D. magna* was delayed for one day (Fig3 B).

3.4 Relationship between growth and reproduction of *D. magna*

The average (±SD) values of body lengths of live *D. magna* offspring ranged between 4.5±0.2 (July 2007) and 5.2±0.0 (May 2008) in the control, 3.8±1.6 (December 2007) and 5.2±0.1 (April 2008) in St. 1 water samples, 3.2±0.1 (August 2007) and 5.3±0.2 (March 2008) in St. 2 water, 3.8±0.0 (June 2007) and 5.3±0.0 (March 2008) in St. 3 water, and 4.5±1.2 (December 2007) and 5.2±0.2 (February 2008) in St. 4 water by the end of the test (21 d). Certain average values at St. 1 (4.2±0.1 in May 2008), St. 2 (3.2±0.1 in August 2007 and 4.5±0.1 in May 2008), and St. 3 (3.8±0.0 in June 2007) were significantly smaller than the corresponding values in the control (5.0±0.0 in June 2007, 5.2±0.5 in August 2007, 5.2±0.0 in May 2008) (ANOVA, \( P < 0.01 \) in all cases).
The average body lengths and the numbers of live offspring for the chronic toxicity tests that began on 10 May 2008 are especially listed in Table 1. At each site, we observed positive correlations between *D. magna* body length and the total number of living offspring per female at 7 d ($r^2 = 0.783$, $P < 0.01$), 14 d ($r^2 = 0.772$, $P < 0.01$), and 21 d ($r^2 = 0.931$, $P < 0.01$) after the tests began on 10 May 2008 (Fig. 5).

### 4. DISCUSSION

Pesticides, linear alkylbenzene sulfonates (LAS) (Garcia *et al*., 2009; Lürling *et al*., 2011), and pharmaceuticals (Nakada *et al*., 2007; Ji *et al*., 2012) account for most of the toxic compounds in Japanese rivers. Most rivers in the rural districts of Japan have paddies on both sides, which are sprayed with many kinds of pesticide during the rice-planting and growing season from April to August. Pesticides flow easily into rivers from paddy fields, which are directly connected to rivers via drainage canals (Maru, 1991; 1993). Several herbicides (e.g., simetryn, oxadiazon, pretilachlor) and insecticides (e.g., fenobucarb, fenithion, fenitrothion) were detected in river water between April and June, and April and August, respectively in the Koise River system, which flows east and south of Mt. Tsukuba (Hatakeyama *et al*., 1999). Studies have shown that paddy pes-

### Table 1. Average (±SD) body lengths and the numbers of live *Daphnia magna* offspring for the chronic toxicity tests that began on 10 May 2008 ($n= 5$).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>Period since test began</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7 d</td>
</tr>
<tr>
<td>Average body length (mm)</td>
<td>Control</td>
<td>3.8±0.0</td>
</tr>
<tr>
<td></td>
<td>St. 1</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td></td>
<td>St. 2</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td></td>
<td>St. 3</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td></td>
<td>St. 4</td>
<td>3.4±0.1</td>
</tr>
<tr>
<td>Number of live offspring</td>
<td>Control</td>
<td>7.8±5.0</td>
</tr>
<tr>
<td></td>
<td>St. 1</td>
<td>2.4±3.6</td>
</tr>
<tr>
<td></td>
<td>St. 2</td>
<td>3.8±3.6</td>
</tr>
<tr>
<td></td>
<td>St. 3</td>
<td>5.6±4.6</td>
</tr>
<tr>
<td></td>
<td>St. 4</td>
<td>3.4±3.1</td>
</tr>
</tbody>
</table>

Fig. 5  Correlation between the average (±SD) *Daphnia magna* body length and number of live offspring at 7 d (A), 14 d (B), and 21 d (C) after the test began on 10 May 2008.
ticides are detected in river water at higher frequencies (Hatakeyama et al., 1994; 1997; 1999) and higher concentrations than are pesticides applied to upland fields (Tanabe et al., 2001). The pesticides reach peak concentrations within a short period, ranging from several hours to several days, and then decrease within a short period (Hatakeyama et al., 1990; Iwakuma et al., 1993; Tada and Shiraishi, 1994; Hatakeyama and Yokoyama, 1997; Tada, 2002; Iwafune et al., 2011). If pesticides do not disappear from river water within 1 or 2 d of entering the river, then acute testing is not sufficient for investigating water quality, so chronic testing should be needed. The continuity of discharge for some pesticides and the hazardous effects on the maintenance of population can be better modeled by the chronic assay than acute assay. For some compounds, reproduction and other chronic endpoints are targeted. Few chronic toxicity studies have been conducted (Sakai 2001, 2002), however, and most studies on the harmful effects of pesticides have been performed using acute toxicity tests with daphnids (Galassi et al., 1992; Ernst et al., 1994; Hosokawa et al., 1995; Kikuchi et al., 2008).

A simple and rapid screening method based on the immobilization of D. magna (acute toxicity) was developed for detecting pesticide pollution. Laboratory testing of the toxicity of 11 organophosphate insecticides to D. magna determined the concentrations at which the mobility of 50% of individuals was inhibited after 48 h of exposure (i.e., 48-h EC_{50}), which ranged from 0.19 to 2.6 μg L^{-1} (Kikuchi et al., 2000). The mobility of D. magna was inhibited in several river and stream water samples collected from May 1995 to February 1998 in Tokyo (Kikuchi et al., 2000).

Kikuchi et al. (2008) investigated the cause of water quality problems in the Naka River, which runs through Saitama Prefecture to Tokyo metropolitan area, from April 1994 to September 2004. Mobility inhibition tests of the river water using D. magna clarified that acutely toxic chemicals existed in the river in the mid-1990s. The pollution of Naka River with chemicals acutely toxic to D. magna continued until 2000, but after 2001 the pollution markedly decreased. The mobility inhibition of D. magna was shown to be caused by organophosphate insecticides.

Thirty-nine paddy pesticides were monitored in the Sakura River (at St. 1) during the rice cultivation season in 2007 and 2008 (Iwafune et al., 2011). The organophosphate insecticides fenitrothion, fenthion, and diazinon and the carbamate insecticide fenobucarb were detected in the river. Fenitrothion was detected between April and July 2007 and in May and August 2008, at a peak concentration of 0.4 μg L^{-1}. The no observed effect concentration (NOEC) (0.009 μg L^{-1}), LC_{50} (0.067 μg L^{-1}) of 24 h and EC_{50} (0.067 μg L^{-1}) of 48 h for D. magna, were reported (Ferrando et al., 1996, Kikuchi et al., 2000). In the present study, the average total number of offspring after 21 d in river water samples from St. 1 significantly decreased to 32% that of the control in August 2007, 34% in May 2008, and 49% in August 2008 (Fig. 3). Acute toxicity was observed in two test organisms in August 2007 and four in August 2008; these individuals died before the first hatch, about 2 to 3 d after the tests began.

To determine the causative chemical(s), TIEs were conducted on 23 toxic samples (Werner et al., 2000). These included eight follow-up samples taken to determine whether toxicity at the respective site persisted. Werner et al. (2000) concluded that analysis of data from the follow-up samples suggested that toxicity may have persisted over periods of several days to weeks. In
the present study, *D. magna* survival and reproduction were affected by toxicity during three weeks in river water samples.

In the Maioka River, two insecticides (fenitrothion and dichlorvos) affected *D. magna* significantly, because harmful effects were observed in chronic tests using reconstituted water to which the appropriate levels of pesticides had been added. Additionally, these results also indicate that factors other than the two insecticides may be involved in the harmful effects (Sakai, 2002). The concentrations of 17 pesticides were measured three times a week from April to August 1993 in the Kokai River (Hatakeyama et al., 1994). Hatakeyama et al. (1994) concluded that the shrimp mortality in the river water samples was caused by the overall pesticide toxicity, although their concentrations were low and varied independently.

The results of our tests suggest that *D. magna* may be affected not only by the overall pesticide toxicity in the river water, but also by other factors that were not clarified in the present study. To measure the concentrations of chemicals (e.g., insecticides) and conduct TIEs for the causative chemicals should be needed for the furthermore study.

Dechlorinated tap water was used for the control of the toxicity tests. Elendt M4 or Elendt M7 currently recommended for OECD testing was not used for the present study because of the high-hardness (~250 mg L⁻¹ CaCO₃) media. In general, the river water in Japan is soft water (the average of the hardness: 30 mg L⁻¹ CaCO₃) (Kitano, 2009). The quality (e.g., hardness) of the tap water changes as well as the river water seasonally. In the control (dechlorinated tap water), the average total number of live *D. magna* offspring at the end of the chronic toxicity tests were relatively low in July and August 2007, June and August 2008, and August and October 2009 (Fig. 3). Especially, the smallest average number of neonates was 53±50 in August 2007, which was below the criteria of the valid OECD TG211 of 60. The reproduction of *D. magna* in the control might be affected by the hardness of tap water.

*C. dubia* for conducting freshwater toxicity test (whole effluent toxicity test: WET) have been used to evaluate ambient water quality (de Vlaming, et al., 2000). WET takes only 7 or 8 days for *C. dubia* reproduction tests in US and Canada, which can significantly save time and cost compared to *D. magna* TG211. On the other hand, TG211 is the standard method in the world for evaluating chronic toxicity of pollutants and waste water to aquatic invertebrates (OECD, 1998). *D. magna* is often used for the monitoring of the water quality for the high sensitivity of toxicant and the ease of culturing and operating for the chronic test. Because of the body length of neonate of *D. magna* is large (1 mm) enough as compared with that of *C. dubia*.

But the requirement of a 21-d exposure period makes this a costly test. In addition, numerous laboratories have reported problems with the lack of consistent and sustained reproduction as well as inadequate survival over the 21-d period. These problems often detract from the overall credibility of *Daphnia* test data and the utility of the test species (Adams and Hedolph, 1985). Tong et al. (1996) compared the results of 14- and 21-d *D. magna* survival and reproduction tests. For the two pollutants tested in the study (acrylonitrile and acetonitrile), the sensitivity of reproduction and survival in the 14-d tests was the same as that in the 21-d tests. Therefore, they suggested that the standard pro-
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tocol for the *D. magna* chronic toxicity test be changed to a 14-d period of exposure (Tong et al., 1996). This proposal was supported by the results of other study as well (Gersich, 1990).

In this study, at each site we observed a positive correlation between *D. magna* body length and the number of offspring at 7, 14, and 21 d after the test began on 10 May 2008 (Fig. 5). These findings suggest that *D. magna* body length could be used to evaluate the toxicological effects of river water, even at 7 or 14 d from the beginning of a chronic toxicity test.

5. CONCLUSIONS

Toxicological effects of river water on the reproduction of *D. magna* were assessed with the standard 21-d chronic test at four river sites in rural districts. The average numbers of live *D. magna* offspring in river water samples were almost the same as the control at 7-d (first hatch), 14-d and 21-d, respectively by the tests that began on 10 January 2008. For the test that began on 8 May 2008, however, it increased to 173±18 in the control, 58±9 in St. 1 water, 75±25 in St. 2 water, 116±41 in St. 3 water, and 137±21 in St. 4 water, and the total numbers of offspring were significantly less in St. 1 and 2 water than in the control.

The seasonal changes for over the three years in the average total number of live *D. magna* offspring at the chronic toxicity tests were 53–209 in the control, 17–233 in St. 1 water samples, 14–223 in St. 2 water, 29–210 in St. 3 water, and 84–209 in St. 4 water. The average numbers in May and September 2008, and April 2009 at St. 1; May 2008 at St. 2, and June 2007 and April 2009 at St. 3 were significantly lower than those of the control.

In addition, we observed significant positive correlations between *D. magna* body length and the total number of living offspring per female at 7 d, 14 d, and 21 d after the tests began on 10 May 2008 at each site.

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