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RESEARCH ARTICLE

Burrowing mayfly *Ephemera orientalis* (Ephemeroptera: Ephemeridae) as a new test species for pesticide toxicity

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Abstract The potential of mayfly *Ephemera orientalis* McLachlan eggs and first-instar larvae in ecotoxicological testing was investigated. Both stages of *E. orientalis* showed high tolerance to various environmental variables, such as water temperature, pH, water hardness, and dissolved organic carbon. Toxicological assays were conducted with three insecticides (emamectin benzoate, endosulfan, and cypermethrin), one fungicide (mancozeb), and one herbicide (paraquat dichloride). The two toxicity endpoints for the assay were the 14-day egg median hatching rate (EHC_{50}) in static and renewal exposure systems and 24-h median larval mortality (LC_{50}). Cypermethrin was the most toxic to both eggs (EHC_{50} in static system = 36.9 μg/L; EHC_{50} in renewal system < 0.15 μg/L) and larvae (LC_{50} = 4.5 μg/L), and paraquat dichloride was the least toxic to eggs (EHC_{50} in static system = 54,359.8 μg/L; EHC_{50} in renewal system = 49541.3 μg/L) and larvae (LC_{50} = 9259.5 μg/L). The results were compared to literature data of *Daphnia magna* Straus and *Cloeon dipterum* Linnaeus to determine its relative sensitivity to pesticides. These three species had different toxicities to the tested pesticides, especially according to the exposure system. *E. orientalis* eggs in the static system were found to be less sensitive were *D. magna* and *C. dipterum*, but eggs in the renewal system and larvae had similar or higher sensitivities to the tested pesticides. The results revealed that this species has potential for use in ecotoxicological testing of pesticides. Because of its geographic distribution, *E. orientalis* may be used as an alternative or complementary test species for ecotoxicological studies in Northeast Asian countries, where natural populations of the international standard species, *D. magna*, are rarely found.

Keywords Bioindicator · Ecotoxicology · Environmental variable · Novel test organism

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Introduction

Many organisms are used in aquatic ecotoxicological studies, and each organism has unique strengths and weaknesses as a test species (Traunspurger and Drews 1996). Among the criteria for test organism selection, it is generally agreed that the species should be native for greater ecological relevance, is sensitive to the contaminant, and is important in the food chain (Chapman 2002). In addition, the test species should be highly adaptable to various environmental variables, such as temperature, pH, dissolved organic carbon (DOC), and water hardness (Nunes et al. 2006).

In the USA and EU, international standard test species such as *Daphnia magna* Straus and *Chironomus riparius* Meigen have been used to establish water quality requirements (US EPA 2002; OECD 2004). Although *D. magna* and *C. riparius* have several advantages in ecotoxicological studies, their suitability as representative test organisms for other aquatic species requires further investigation. Furthermore, their low population densities in natural habitats, especially outside Europe and North America, limit their ecological relevance

(Rasmussen 1984; De Gelas and De Messter 2005). Use of ecologically irrelevant species may produce incorrect conclusions in ecological risk assessments. To compensate for these disadvantages, the parallel use of a standard test species with a common, ecologically relevant species is recommended in ecological risk assessment programs (Traunspurger and Drews 1996).

The burrowing mayfly, family Ephemeridae, is almost cosmopolitan in distribution and consists of 96 species in seven genera (Hwang et al. 2007). Burrowing mayflies are important members of aquatic ecosystems, where they convert organic detritus and its contained biota into food for many fish species, and have useful environmental applications, such as in biomonitoring and ecotoxicology (Fremling and Mauck 1980; Edsall 2001). *Hexagenia limbata* Serville and *H. rigida* McDunnough, which are burrowing mayfly species native to the near arctic region and widespread in Canada and the USA (Giberson and Resenberg 1994), have been commonly used for ecotoxicity tests, particularly to assess heavy metal toxicities (ASTM 1992; US EPA 2000b). However, these species have been rarely used in pesticide tests and are not distributed naturally in Northeastern Asia.

Ephemera orientalis McLachlan is a member of the family Ephemeridae and is a common burrowing mayfly distributed in temperate East Asia, including Northeastern China, Mongolia, the Russian Far East, the Korean Peninsula, and the Japanese Islands (Hwang et al. 2007). *E. orientalis* inhabits relatively clean lowland streams and rivers and emerges in large quantities for at least 6 months of the year (May–October) in temperate climate (Lee et al. 2008). This species has served as a useful indicator of aquatic ecosystem health in Korea (Lee et al. 1997; Hwang et al. 2007), but no attempt has been made to use this species for ecotoxicological research. Mo et al. (2013) reported that this species has two possible advantages for ecotoxicity testing: first, a single *E. orientalis* female produces more than 2000 eggs in one reproductive cycle, and second, eggs are produced individually and not as an egg mass, making treatment or distribution more convenient. So far, it has not been established for successive culture, but we are trying various tests to rear this species in the lab, such as proper substrate, food, space for mating, etc.

Pesticides represent an important stressor for freshwater ecosystems and can affect many groups of organisms (Schäfer et al. 2007), although several organisms are not affected or even benefit from pesticide exposure (Liess and Von Der Ohe 2005). In Korea, pesticides are major sources of contamination in rural streams and rivers (Lee et al. 1997; Kim et al. 1998; Khim et al. 2001). This study aimed to evaluate the potential use of *E. orientalis* as a new test species for pesticide toxicity assessment in aquatic ecosystems. The acute toxicity of five pesticides of different pesticide classes on *E. orientalis* eggs and larvae was examined. Toxicity endpoints were median egg hatching concentrations (EHC_{50}s) for eggs and median larval lethal concentrations (LC_{50}s) for larvae. In addition, the tolerance ranges of the eggs and larvae to environmental variables—such as temperature, pH, water hardness, and DOC—were examined using the same endpoints as the toxicity tests. The responses of *E. orientalis* eggs and larvae to pesticides were primarily compared with published data on the standard species, *D. magna*. Also, the sensitivities were compared with those of *Cloeon dipterum* (L. 1761) (Ephemeroptera: Baetidae), the common wetland mayfly in Korea.

Materials and methods

Test organism

The *E. orientalis* eggs and larvae used in all bioassays originated from field-collected adult females because standard laboratory culture protocols for this species have not yet been established (Fig. 1). Hwang et al. (2009) reported that there are two cohorts of *E. orientalis*, S1 and S2, in Korea. The life cycles of S1 and S2 are from July to next May and from October to next September, respectively. Using a light trap, adult females were collected from Wangsuk Stream (37° 42 26 N, 127° 10 17 E), South Korea, in May–September 2012. Collected adult females were placed in a plastic collecting box (30 × 25 × 25 cm) and returned to the laboratory within 30 min.

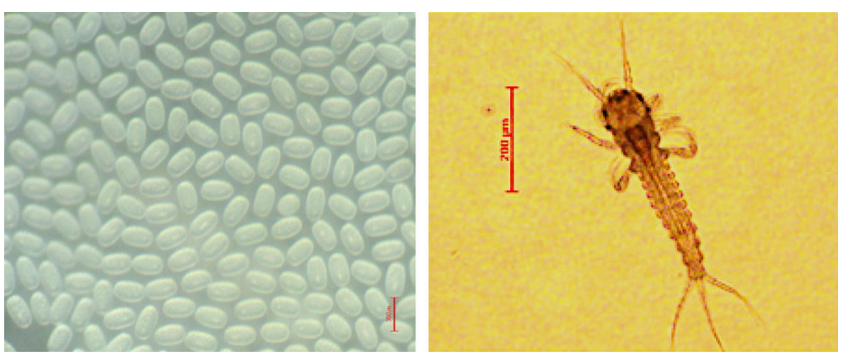

Fig. 1 Tested eggs and larvae of *Ephemera orientalis*

Collected females were induced to lay eggs by soaking their abdominal tips in deionized water. A batch of eggs from at least five females was prepared and mixed thoroughly. The egg tests were conducted immediately after eggs were laid because the eggs secrete mucus when they start to develop and, consequently, stick to each other. To obtain a sufficient number of age-synchronized first-instar larvae for the larval bioassays, at least 15,000 eggs from five adult females were incubated at 20 °C with a photoperiod of 16:8 (L/D) for 14 days. The resulting first-instar larvae were used for the larval test. All larvae used in the experiments were <12-h-old first instars.

Pesticide selection and analytical procedures

Five pesticides were tested; these included three insecticides (cypermethrin, emamectin benzoate, and endosulfan), one fungicide (mancozeb), and one herbicide (paraquat dichloride) from the list in the Agrochemicals Year Book in Korea (Korea Crop Protection Association 2011). Formulated products of each pesticide were purchased from pesticide companies, and the formulations and label instructions for each pesticide are described in Table 1. These pesticides were chosen because they are frequently used in many agricultural settings in Korea, and their toxic effects against *D. magna* have been determined. All pesticides tested in this study are highly toxic to *D. magna* and considered a potential risk to aquatic ecosystems (Nebeker et al. 1983; Mayer and Ellersieck 1986; US EPA 2000a, 2007).

Among the pesticides, endosulfan was banned by law in Korea in 2012, but toxicity assessment of this pesticide is necessary because it has been widely used in developing countries and will continue to be used for the near future because of its high efficacy, low cost, and environmental stability (Moon and Chun 2009). Also, a considerable amount of data regarding endosulfan toxicity toward various organisms is available.

Analytical quantification of the formulated pesticides

Actual concentrations of the formulated pesticides used in the bioassay studies were determined. Selected concentrations of pesticide solutions including cypermethrin (45 μg/L), emamectin benzoate (187 mg/L), endosulfan (376 μg/L), mancozeb (75 mg/L), and paraquat dichloride (168 mg/L) were measured for comparison to nominal concentrations. Each sample was mixed at the time of test initiation in a 100-mL Erlenmeyer flask. All pesticide standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Five data points were set to get linearity of pesticide standards. All linearity was over 0.99 and its *P* values were less than 0.0001, respectively.

Cypermethrin was quantified using a gas chromatograph (GC) (Agilent 7890, Agilent Technologies, Palo Alto, CA, USA) equipped with a mass selective detector (MSD) (Agilent 5975, Agilent Technologies). Separations were carried out on a DB-5MS column (30-m length, 0.25-mm inside diameter, 0.25-μm film thickness; J&W Scientific Products, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. The MSD was operated in electron impact (EI, 70 eV) selected ion monitoring (SIM) mode. The ions for cypermethrin analysis were monitored with an m/z of 181 for quantification and 163–165 for confirmation (Nguyen et al. 2008).

Endosulfan was mixed with internal standard (1 μg of 2-fluorobiphenyl for GC-MSD and TCMX for GC-ECD) and added 2 mL of hexane to substitute the solvent. And then, endosulfan was quantified using a Shimadzu GC-2100 gas chromatograph (Shimadzu Corp., Tokyo, Japan) equipped with a 63Ni electron capture detector and an automatic sampler. The capillary column was a DB-5MS (J&W Scientific Products). The oven temperature was programmed from an initial temperature of 60 °C (hold 5 min) to 300 °C (hold 5 min) at a rate of 10 °C/min. Nitrogen gas was used as the carrier gas and make-up gas at a flow rate of 1 mL/min.

Table 1 Formulated pesticides used for toxicity tests on *Ephemera orientalis*

Pesticide	Class	Target organism	AI (%) and formulation[a]	RFC	Manufacturer
Cypermethrin	Insecticide	Leaf roller caterpillar, apple leafminers, aphid	5.0 EC[a]	25–50 μL/L	Dongbu HiTek, Korea
Emamectin benzoate	Insecticide	Thrips, leafminers	2.1 EC	11 μL/L	Syngenta, Switzerland
Endosulfan	Insecticide	Oriental tobacco budworm	35.0 EC	700 μL/L	Bayer CropScience, Germany
Mancozeb	Fungicide	Anthracnose, black rot, downy mildew, late blight	75.0 WP[b]	1240–1500 mg/L	Hankook Samgong, Korea
Paraquat dichloride	Herbicide	Nonselective herbicide	8.2 EC	613–1022 μL/L	Dongbu HiTek, Korea

The active ingredient (AI), target organism, and manufacturer's recommended field concentration (RFC) for each pesticide are as shown

[a] Emulsifiable concentrate

[b] Wettable powder

The concentrations of emamectin benzoate and paraquat dichloride were determined using a high-performance liquid chromatograph (HPLC) (Varian 940-LC, Agilent Technologies, Palo Alto, CA, USA) equipped with a UV detector. A reversed-phase C18 (Varian Pursuit XRs C18, 5 μm, 4.6 × 250 mm) column was used. The mobile phases were methanol/water (80:20 by volume) containing ammonium acetate for emamectin benzoate analysis and acetonitrile/water (25:75 by volume) containing 0.01 M heptane sulfonic acid (pH 3.2) for paraquat dichloride analysis at a flow rate of 1.0 ml/min. The identifications and quantifications for emamectin benzoate and paraquat dichloride were achieved with UV detections at 245 and 254 nm based on peak areas, respectively.

Mancozeb concentration was determined according to the method described in the literature (Nguyen et al. 2008). Mancozeb concentrations were analyzed as carbon disulfide (CS_2) by gas-liquid chromatographic headspace. Trapped CS_2 was injected directly onto the Agilent Technologies Model 5890 Series II gas chromatographer using a gastight syringe and was detected with a flame photometric detector (FPD) operating in sulfur mode. Hydrogen was used as the carrier gas at a linear velocity of 42 cm/s and nitrogen 9.5 analytical degrees as make-up gas for the FPD at a flow rate of 25 mL/m. The injection port was set at 200 °C and the detector at 250 °C. The analytical column was 5 % phenyl and 95 % dimethyl polysiloxane. The initial oven temperature was set at 40 °C, held for 1 min, followed by heating at 10 °C min^{-1} to a final temperature of 180 °C, at which it was held for 1 min.

Pesticide degradation in water during experimental periods

A separate study was conducted to evaluate the stability of the pesticides during the 14-day egg hatching bioassays. Formulated pesticides were added to deionized water at the nominal concentrations of each formulated pesticide. The concentration was checked in 1 L of sample fortified with 1.0 mg of active ingredient/L of each pesticide. Samples, prepared in duplicate, were maintained in the dark at room temperature. Three 10-mL aliquots of each sample were taken and analyzed on the same day as fortification (day 0) and at 1, 3, 7, and 13 days after fortification using the procedure reported above. The degradation rate was expressed as the percentage of the recovered concentration divided by the nominal concentration on each checking day.

General bioassay procedures

Both eggs and first-instar larvae were tested to assess the effects of various environmental variables and five pesticides. For the egg bioassays, 20 freshly laid eggs (<12 h old) were placed in a Petri dish (52 mm in diameter and 12 mm in height) prepared for each environmental variable or in a 25-mL glass beaker (Duran®, Germany) for the pesticides. All the tests were conducted at 20 °C with a photoperiod of 16:8 (L/D) and 1000 lux light intensity.

Egg viability (hatching) in each test vessel containing 10 mL of solution was monitored at 14 days after exposure to the environmental variables or pesticides. The 14-day time frame for observation was chosen because it provides sufficient time to complete two life cycle events, embryogenesis and hatching. According to a preliminary study, >95 % of eggs hatched and developed to the first larval stage within 14 days under various environmental conditions. Few eggs had hatched after 14 days of exposure.

For the larval test, 20 first-instar larvae (<12 h old) were placed in a 25-mL glass beaker, and the larval mortality was assessed 24 h after exposure to the environmental variables and the pesticides. This test duration was chosen because the freshly hatched larvae survive no more than 3 days in fresh water without feeding in preliminary larval tests. In addition, this duration enables us to compare the results of *Daphnia magna* with ours because *D. magna* is frequently tested with exposure durations of 24–48 h. The death of mayfly larva was judged when there was no movement in response to prodding with a fine needle under a microscopic observation.

Tolerance of eggs and larvae to environmental variables

The effect of four environmental variables—including pH, water hardness, dissolved organic carbon (DOC), and temperature—which are considered critical in water quality analysis (US EPA 1980; Hart 1982; Schubauer-Berigan et al. 1993; Howe et al. 1994) on the egg-hatching rate and larval mortality of *E. orientalis* were evaluated. Eggs were exposed to four environmental variables for 14 days and the first instar larvae for 24 h under static conditions without replacement of test solution.

Test ranges of pH, water hardness, and DOC were selected within ranges representing natural surface waters; pH varied from 4.0 to 8.0, hardness from 3.1 to 200 mg/L (as $CaCO_3$), and DOC from 4 to 62 mg/L (Kim et al. 2006; Korean Ministry of Environment 2016). The pH was adjusted by adding 0.1 N NaOH or 0.1 N HCl solution to deionized water. The water hardness level was adjusted by adding the amount of concentrated hardness solution (2.94 g/L $CaCl_2 \cdot 2\ H_2O$ and 1.23 g/L $MgSO_4 \cdot 7\ H_2O$) needed to obtain the required hardness. The concentrated DOC solution was prepared by adding 5 g of artificial humic acid (humic acid sodium salt; Sigma-Aldrich, St. Louis, MO, USA) to 1 L of deionized water. The DOC concentration of the filtered (0.45 μm filter) stock solution was measured by infrared spectrophotometry (TOC-5000A, Shimadzu Corp., Kyoto, Japan) and was added to deionized water until reaching the required DOC content.

The temperature effects were studied at 15, 20, 25, and 30 °C using deionized water (pH 6.8) as a test medium.

Acute toxicity to pesticides

Chemical concentrations of five pesticides were prepared by serial dilution from newly prepared stock solutions in 100 mL of US EPA soft water (US EPA 1994) with pH 7.2–7.6 and water hardness of 40–48 mg/L as $CaCO_3$. Five serial dilutions with a logarithmic series for each pesticide were prepared to determine the 50 % effective concentration to inhibit egg hatching (EHC_{50}) or 50 % lethal concentration to kill the first-instar larvae (LC_{50}). The range of concentrations causing 0–95 % mortality on the eggs or the larvae was initially determined.

The nominal concentrations for the egg tests ranged from 0.001 to 0.32 μL/L for cypermethrin, 0.01 to 3.2 μL/L for emamectin benzoate, 0.1 to 80 μL/L for endosulfan, 0.0.1 to 8.0 mg/L for mancozeb, and 0.1 to 160 μL/L for paraquat dichloride. For the larval tests, the nominal concentrations ranged from 0.001 to 0.062 μL/L for cypermethrin, 0.008 to 0.134 μL/L for emamectin benzoate, 0.016 to 1.296 μL/L for endosulfan, 0.250 to 2.250 mg/L for mancozeb, and 0.001 to 0.0817 μL/L for paraquat dichloride. Prepared pesticide solutions in 200-mL brown amber glass bottles were conditioned for 24 h at 4 °C before toxicity analysis.

For the egg tests, two systems of exposure, static and renewal, were performed to identify the effects of each pesticide on egg hatching. In the renewal system, the solution was replaced periodically every 48 h, for a total of five times. This renewal period was chosen to simulate pesticide overuse to control pests. However, the larvae were tested under a static system only as the test duration was very short (24 h). There were four replications for each concentration, and the entire experiment was replicated four times using freshly prepared stock solutions. The control groups for both egg and larval tests were exposed to only US EPA soft water.

Data analysis

For the analysis of the effects of environmental variables on eggs and first-instar larvae, all proportional data were arcsine-root transformed to stabilize variances (Zar 1999). Subsequently, differences in egg-hatching rates and larval mortalities between treatments were subjected to one-way analysis of variance (ANOVA) using SAS 9.3 (SAS Institute 2010). For factors significant in ANOVAs ($P < 0.05$), each treatment group was compared to the controls using Tukey's test ($\alpha = 0.05$).

Percentages of egg hatching and larval mortality, compared with the controls, were used to calculate EHC_{50} and LC_{50}, followed by probit analysis using SAS 9.3 (SAS Institute 2010). The actual measured concentrations, not the nominal concentrations, of each pesticide determined by the quantitative analysis described above were used to calculate the EHC_{50} estimates for eggs and the LC_{50} estimates for larvae; the toxicity values were expressed in units of micrograms per liter.

The EHC_{50} and LC_{50} values were compared using a Wilcoxon two-sample test (PROC NPAR1WAY; SAS Institute 2010) to determine if the toxicity rankings of the EHC_{50} and LC_{50} values differed according to *E. orientalis* stage (egg or larva). In addition, EHC_{50} values from static and renewal systems were compared using the same statistical method described above.

Results

Actual pesticide concentrations used in toxicity studies and their degradation in water

The measured concentrations of the five formulated pesticides were generally lower than the nominal concentrations, with the exception of paraquat dichloride (Table 2). The measured concentrations of paraquat dichloride were significantly higher than the nominal concentrations: recovery ranged from 107 to 114 %, and the mean measured concentrations were 110 % of the nominal concentrations. For cypermethrin and mancozeb, the measured concentrations were considerably lower than the nominal concentrations; their mean concentrations were 55 and 75 %, respectively, of the nominal concentrations. Chemical analyses of emamectin benzoate and endosulfan showed that the nominal and measured concentrations

Table 2 Nominal and measured concentrations with SE of the five commercial pesticides tested in this study

Pesticide	Nominal concentration	Measured concentration	Recovery ratio (%)[a]
Cypermethrin	45 μg/L	24.8 ± 5.1	55.0 ± 11.3
Emamectin benzoate	187 mg/L	181.0 ± 1.0	96.8 ± 0.5
Endosulfan	376 μg/L	343.2 ± 40.3	91.3 ± 1.1
Mancozeb	75 mg/L	56.2 ± 14.2	74.9 ± 18.9
Paraquat dichloride	168 mg/L	185.2 ± 5.8	110.2 ± 3.4

[a] Measured concentration/nominal concentration × 100

were comparable, with mean percentage recovery rates of 97 and 91 %, respectively.

Degradation of the formulated pesticides was evaluated within a 13-day time frame. The degradation of each pesticide was significantly different ($F=23.81$, $df=4$, $P<0.001$) (Fig. 2). Cypermethrin, endosulfan, mancozeb, and paraquat dichloride degraded rapidly: less than 50 % remained 1 day after exposure (39.0, 49.8, 36.1, and 18.1 %, respectively), and 2.1, 5.7, 6.0, and 0.4 %, respectively, remained 13 days after exposure. However, the degradation of emamectin benzoate was significantly ($P<0.001$) slower than that of the other pesticides: 91.9 and 68.6 % remained after 1 and 13 days, respectively. These degradation results were not very different from other published data (Kosinski and Merkle 1984; US EPA 1989; Singh et al. 1991; Guerin and Kennedy 1992; APVMA 1998; EU biocides CAR 2010)

Effects of environmental variables on egg hatchability and larval mortality

The effect of temperature on the egg hatching rate of *E. orientalis* was not significant within the test ranges ($P>0.05$); hatching rates ranged from 83 % at 15 °C to 97 % at 30 °C. Similarly, water hardness and DOC did not appear to have a significant effect on egg hatchability. Hatching rates were at least 85 %, regardless of the test treatment. The effect of pH was significant only at pH 4.0 ($P<0.05$). Egg hatching increased slightly as pH increased and reached a maximum (~90 %) at pH ≥6.0. The lowest egg-hatching rate was 78 % at pH 4.0.

Similar to the results of the egg tests, the effects of pH, water hardness, temperature, and DOC on 24-h larval mortality were not significant within the test ranges of environmental variables ($P>0.05$). All survival rates after 24 h of exposure were ≥95 %.

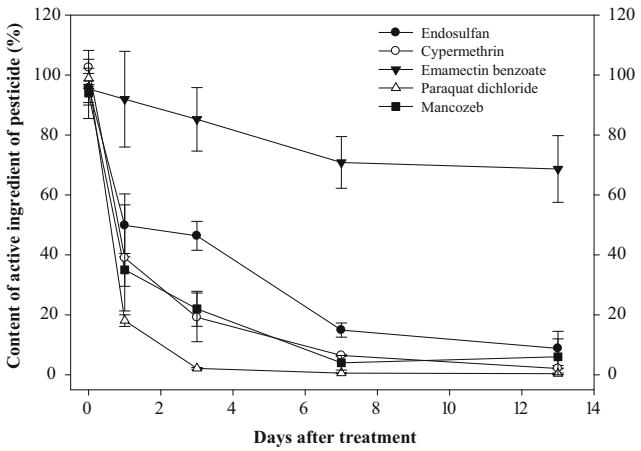

Fig. 2 Degradation of active ingredients of five pesticides (endosulfan, cypermethrin, emamectin benzoate, mancozeb, and paraquat dichloride) in water for 2 weeks

Acute toxicity of pesticides to eggs and larvae

In static system of the eggs, two of the insecticides, cypermethrin and emamectin benzoate, were the most toxic; their EHC_{50} values were 36.9 and 473.9 μg/L, respectively (Table 3). Interestingly, the fungicide, mancozeb ($EHC_{50}=808.6$ μg/L) was very toxic to the eggs, and even more so than endosulfan ($EHC_{50}=39077.4$ μg/L). As expected, the herbicide paraquat dichloride exhibited the lowest toxicity.

Using the renewal system, the toxicities to egg hatching were higher compared to static system, but the EHC_{50} values of cypermethrin and mancozeb could not be estimated because the hatching rate was lower than 50 % at the lowest concentration applied. Toxic ratios (EHC_{50} from the static system/EHC_{50} from the renewal system) differed markedly among the pesticides (Table 3). The toxicities of emamectin benzoate and paraquat dichloride were similar between the two exposure system, but the toxicities of cypermethrin, endosulfan, and mancozeb increased greatly to <0.15, 143.1, and <3.3 μg/L, respectively. These represent at least 245-fold increases.

In the larval test, cypermethrin was the most toxic ($LC_{50}=4.5$ μg/L), followed by emamectin benzoate, mancozeb, endosulfan, and paraquat dichloride (Table 4). Generally, the larvae were more sensitive to the pesticides than to the eggs in the static system, with the exception of the fungicide mancozeb. Mancozeb had similar toxic effects on eggs and larvae ($EHC_{50}=808.6$ μg/L; $LC_{50}=1076.2$ μg/L). When sensitivity was compared with the eggs in renewal system, the larvae were more sensitive to emamectin benzoate and paraquat dichloride, but less sensitive to cypermethrin, endosulfan, and mancozeb (Tables 3 and 4).

The associations of the toxicity measurements using the two exposure systems (static and renewal in egg tests) and life stages (eggs and larvae) were determined by comparing the orders of toxicity. Although significant toxic ratios were observed, the order of toxicity did not differ significantly according to the exposure system (Wilcoxon two-sample test, $P=0.2417$) or life stage ($P=0.3235$ for eggs in static with larvae, and $P=0.6859$ for eggs in renewal with larvae).

Discussion

Although burrowing mayflies play a key role in aquatic ecosystems, only two species in Ephemeridae, *H. limbata* and *H. rigida*, have been widely used for ecotoxicity test species to date (ASTM 1992; US EPA 2000b; Harwood et al. 2014). In the present study, *E. orientalis* was tested to confirm its potential as an ecotoxicological test species and to assess the acute toxicity of pesticides in an ambient water system.

Table 3 The 14-day median egg-hatching concentrations (EHC_{50}s: μg/L) with 95 % confidence intervals (CIs) in static and renewal exposure system, respectively, for *Ephemera orientalis* exposed to five pesticides

Pesticide	Static system	Renewal system	Ratio[a]
Cypermethrin	36.9 (32.1–43.5)	<0.15	>246
Emamectin benzoate	473.9 (400.2–613.6)	446.2 (385.3–528.8)	1.1
Endosulfan	39,077.4 (29,302.0–52,195.9)	143.1 (77.6–197.7)	273.1
Mancozeb	808.6 (704.9–946.2)	<3.3	>245
Paraquat dichloride	54,359.8 (39,444.9–92,525.3)	49,541.3 (44,152.4–56,161.4)	1.1

[a] EHC_{50} of static system/EHC_{50} of renewal system

The selection criteria for ecotoxicity test species include species distribution, ecological importance, toxicant sensitivity, and practicality (Maltby et al. 2005). The first problem for laboratory toxicity testing using a new test species is determining the effects of natural environmental variables on toxicity. Laboratory toxicity data are obtained under constant conditions that do not represent those in the aquatic environment, which leads to misleading test results. Thus, the identification and quantification of the relationship between toxicity effects and natural environmental variables are priorities in the development of any toxicological assay and should be conducted before responses can be ascribed to contaminant effects (ASTM 1992; Thomas 1993). Heijerick et al. (2003) suggested that toxicities can change based on the physicochemical properties of test medium or site-specific factors of a test organism. This study shows that *E. orientalis* eggs and larvae have a high tolerance to wide ranges of environmental factors, such as temperature, pH, water hardness, and DOC. These observations have important implications for *E. orientalis* as an ecotoxicological test organism. First, there is considerable tolerance to a wide range of temperature, i.e., >85 % hatching rate from 15 to 30 °C. In terms of high temperature, *E. orientalis* eggs have an advantage over *D. magna* because most cladocerans are unable to live at temperatures >26 °C (Dodson and Frey 1991; Heugens et al. 2003). Second, *E. orientalis* eggs and larvae are highly resistant to a wider range of pH conditions (pH 4.0–8.0) and water hardness values (up to 200 mg as $CaCO_3$/L) than other aquatic invertebrates. Almer et al. (1974) reported that the *Daphnia* group is not found at pH values below 5.8, and Heijerick et al. (2003) reported that *D. magna* favors a medium with hardness between 150 and 250 mg/L as $CaCO_3$ for reproduction. The total hardness of surface water ranges from 60.2 to 127.2 mg/L as $CaCO_3$ in Korea (Kim et al. 2006), and this range did not affect the 14-day hatching rate or 24-h larva mortality. Thus, *E. orientalis* eggs and first-instar larvae can be used to monitor the quality of natural surface water, which has wide ranges of physicochemical properties.

Table 4 The 24-h median larval lethal concentrations (LC_{50}s: μg/L) with 95 % confidence intervals (CIs) for *Ephemera orientalis* exposed to five pesticides

Pesticide	24 h LC_{50} (CI)
Cypermethrin	4.5 (3.4–6.0)
Emamectin benzoate	36.8 (32.5–41.9)
Endosulfan	224.0 (171.5–292.9)
Mancozeb	1076.2 (885.0–1358.3)
Paraquat dichloride	9259.5 (7275.6–11694.2)

After confirming tolerance to environmental variables, toxicant sensitivity was examined by means of a pesticide test. The toxicities of five pesticides tested in this study differed according to *E. orientalis* life stage (egg and larva) and exposure system (static and renewal). Generally, *E. orientalis* eggs in static system were less sensitive to the tested pesticides than the first-instar larvae, but the eggs were more sensitive in the renewal system (Tables 3 and 4). Interestingly, mancozeb, a fungicide, is highly toxic to *E. orientalis* eggs, even more so than endosulfan, a persistent insecticide. Mancozeb is a dithiocarbamate that contains chelated metals (Mn and Zn) and can be metabolized to release free Mn (and/or Zn) as well as ethylene thiourea. Research on the toxicity of mancozeb has demonstrated its harmful effects on a wide variety of living creatures. The disturbances may occur not only in invertebrates but also in vertebrates, including humans (Adamski and Ziemnicki 2004). Because of these facts, more information is needed in several areas to establish effective criteria for the protection of sensitive species and wildlife against mancozeb due to its heavy use globally (Transparen cymarketresearch.com 2015).

Higher egg toxicities with the renewal system can be explained by pesticide degradation during the 14-day exposure period. The actual concentrations of pesticides used in the egg tests were markedly lower than the nominal concentrations: >50 % of cypermethrin, endosulfan, mancozeb, and paraquat dichloride was hydrolyzed within 24 h (Fig. 2). Although emamectin benzoate and paraquat dichloride had similar toxicities to eggs in the two exposure systems (Table 3), the degradation patterns and final degraded amounts differed markedly: emamectin benzoate was degraded very slowly and the level of degradation product was 31.4 % at 13 days, but paraquat dichloride degraded rapidly, and the final level was <1 %. The reason for this difference is unclear but is likely because the toxicity of these two pesticides is determined at the time of first exposure. In contrast, increasing toxicities of cypermethrin, endosulfan, and mancozeb in renewal exposure

were likely due to cumulative toxic effects as test solution was replaced with fresh pesticide solution. Considering the usage pattern of pesticides in agriculture, multiple exposures to pesticides may have a greater impact on aquatic invertebrates than expected from the results of laboratory testing.

The results of this study suggest that the mechanisms of toxicity against *E. orientalis* eggs differ among the pesticides, and subsequently, each pesticide would have a different impact on aquatic ecosystems. Insect eggs are often more resistant to pollution than larvae are (Williams et al. 1987). Klowden (2013) stated that the insect eggshell, or chorion, is a critical structure because its basic role is to serve as a two-way barrier to prevent the loss of egg contents to the environment and minimize the disturbance of those contents by environmental hazards. To date, the toxicity mechanisms of synthetic pesticides to insect eggs are unclear. Thus, further study is needed to examine the reason for the different toxicities between exposure systems, including egg morphology and physiology.

To investigate the ecotoxicological relevance of *E. orientalis* as a test organism, the response of *E. orientalis* to the five pesticides was compared with published data for *D. magna* and *C. dipterum* (Table 5). Since no published data on pesticide toxicities are available for the burrowing mayflies *H. limbata* and *H. rigida* to date, *C. dipterum* was used as a surrogate species for the comparison. *C. dipterum* is one of the most common and abundant mayflies, being distributed throughout temperate areas of the Eurasian continent, including Europe, Siberia, Mongolia, and Northeast Asia (Lee et al. 2013). Although direct comparisons between this study and published data are not feasible due to the differences in exposure systems (static system and flow-through system), test species, and exposure durations, *E. orientalis* eggs in static tests were less sensitive than eggs of *D. magna* and *C. dipterum*. However, *E. orientalis* larvae had similar or higher sensitivities to the tested pesticides, with the exception of cypermethrin (Table 5). The LC_{50} value for

Table 5 Comparison of five pesticide-acute toxicities and summary of test methods on *Daphnia magna* and *Cloeon dipterum* (unit: μg/L)

Species	Pesticide	Life stage or Age	Test type	Exposure period	Toxic concentration	Reference or database
Daphnia magna	Cypermethrin	<24 h	FTS[b]	48 h	$EC_{50}=0.45$	National Site for the Regional IPM Centers [d]
		<24 h	SS[c]	48 h	$EC_{50}=1.01$	National Site for the Regional IPM Centers National Site for the Regional IPM Centers Stephenson, 1982
		<24 h	SS	24 h	$EC_{50}=2.00$	
		<24 h	SS	24 h	$EC_{50}=2.00$	
	Emamectin benzoate	<24 h	FTS	48 h	$EC_{50}=1.00$	National Site for the Regional IPM Centers
		<24 h	SS	48 h	$EC_{50}=728$	US EPA (2000b)
	Endosulfan	1st instar	SS	48 h	$EC_{50}=166$	National Site for the Regional IPM Centers Ernst et al. (1991)
		NR[a]	SS	24 h	$LC_{50}=620$	
	Mancozeb	NR	SS	48 h	$EC_{50}=580$	National Site for the Regional IPM Centers
		NR	SS	48 h	$EC_{50}=1000$	US EPA (2000b)
	Paraquat dichloride	1st instar	SS	48 h	$EC_{50}=3800$	National Site for the Regional IPM Centers National Site for the Regional IPM Centers National Site for the Regional IPM Centers Benijts-Claus and Persoone (1975)
		NR	SS	48 h	$EC_{50}=8000$	
		<24 h	SS	48 h	$EC_{50}=1250$	
		<24 h	SS	24 h	$LC_{50}=6100$	
Cloeon dipterum	Cypermethrin	Larvae	SS.	24 h	$LC_{50}=0.60$	Stephenson (1982)
		Larvae	FTS	96 h	$LC_{50}=0.01$	National Site for the Regional IPM Centers National Site for the Regional IPM Centers National Site for the Regional IPM Centers
		Larvae	FTS	96 h	$LC_{50}=0.02$	
		Larvae (<24 h)	SS	24 h	$EC_{50}=2.00$	
	Emamectin benzoate	NR	NR.	NR.	NR	
	Endosulfan	Larvae	SS.	48 h	$LC_{50}=160$	Hashimoto and Nishiuchi (1981)
	Mancozeb	Larvae	NR	24 h	$LC_{50}\geq 40{,}000$	Nishiuchi and Asano (1979)
	Paraquat dichloride	Larvae	NR	24 h	$LC_{50}\geq 40{,}000$	Nishiuchi and Asano (1979)

[a] Not recorded

[b] Flow-through system

[c] Static system

[d] National Site for the Regional IPM Centers (http://www.ipmcenters.org/index.cfm/ipm-databases)

E. orientalis larvae is 4.5 μg/L, but the reported toxic doses ranged from 0.45 to 2.00 μg/L in *D. magna* and from 0.02 to 2.00 μg/L in *C. dipterum*. Among the insecticide groups, cypermethrin exhibited the greatest toxicity to the eggs and larvae of *E. orientalis*, followed by emamectin benzoate and endosulfan. Similar toxicity levels for *D. magna* and *C. dipterum* were found in the published data. However, *E. orientalis* eggs with renewal system had higher sensitivities than *D. magna* and *C. dipterum* did, indicating that *E. orientalis* eggs can be used as bioindicators of pesticide toxicities in situ, under conditions in which multiple exposures to pesticides occur. After establishment of rearing system of *E. orientalis* in the lab, which is another important study for us, this species can be a useful assessment test species for ecotoxicity. Finally, *E. orientalis* could be a test species complementing the sensitivity profile of existing test organisms in the future. Especially the effect of neonicotinoids on *E. orientalis*, which is not very toxic to *D. magna*, will be studied soon or late.

Conclusion

Few ecotoxicological studies have used mayfly species, although their ecological significance and sensitivity to environmental changes and even climate change has been investigated (Fialkowski et al. 2003; Harper and Peckarsky 2006; Lee et al. 2008). The development of indigenous test species and standardization of test protocols is important for maximizing the comparability, replicability, and reliability of pollutant toxicity assays (De Zwart 2002). In this study, *E. orientalis* showed tolerance to wide ranges of environmental variables and sensitivity to pesticides using different exposure systems and life stages. From this perspective, the current study highlights the potential of *E. orientalis* as a test species for ecotoxicological assessments.

The following would improve the applicability of *E. orientalis* as a standard test species. First, a standard rearing system should be established to provide test organisms of stable quantity and quality. Second, standard test protocols should be developed for acute and chronic tests with proper endpoints. In particular, studies on artificial food and substrates for *E. orientalis* are necessary for chronic tests. Finally, comparative studies with other international standard test species using diverse contaminants are required.

This study was limited to the acute toxicity of pesticides on *E. orientalis*. Studies of feeding conditions, artificial habitat, exposure condition, and endpoint selection are in progress for chronic toxicity testing using *E. orientalis* larvae. Our work will be a foundation for future ecotoxicological testing protocols using *E. orientalis* and the investigation of the chronic effects of contaminants, including pesticides, on aquatic ecosystems.

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