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Differences in susceptibility of five cladoceran species to two systemic insecticides, imidacloprid and fipronil

Daisuke Hayasaka · Tomoko Korenaga · Kazutaka Suzuki · Francisco Sánchez-Bayo · Koichi Goka

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Abstract Differences in susceptibility of five cladocerans to the neonicotinoid imidacloprid and the phenyl-pyrazole fipronil, which have been dominantly used in rice fields of Japan in recent years, were examined based on short-term (48-h), semi-static acute immobilization exposure tests. Additionally, we compared the species sensitivity distribution (SSD) patterns of both insecticides between two sets of species: the five tested cladocerans and all other aquatic organisms tested so far, using data from the ECOTOX database of U.S. Environmental Protection Agency (USEPA). The sensitivity of the test species to either imidacloprid or fipronil was consistent, spanning similar orders of magnitude (100 times). At the genus level, sensitivities to both insecticides were in the following descending order: *Ceriodaphnia* > *Moina* > *Daphnia*. A positive relationship was found between body lengths of each species and the acute toxicity (EC_{50}) of the insecticides, in particular fipronil. Differences in SSD patterns of imidacloprid were found between the species groups compared, indicating that test cladocerans are much less susceptible than other aquatic species including amphibians, crustaceans, fish, insects, mollusks and worms. However, the SSD patterns for fipronil indicate no difference in sensitivity between cladocerans tested and other aquatic organisms despite the greater exposure, which overestimates the results, of our semi-static tests. From these results, *Ceriodaphnia* sp. should be considered as more sensitive bioindicators (instead of the standard *Daphnia magna*) for ecotoxicological assessments of aquatic ecosystems. In addition, we propose that ecotoxicity data associated with differences in susceptibility among species should be investigated whenever pesticides have different physicochemical properties and mode of action.

Keywords Acute toxicity · *Ceriodaphnia* · *Daphnia* · Moina · Pesticide · Species sensitivity distribution · Zooplankton

D. Hayasaka (✉) · T. Korenaga · K. Suzuki · K. Goka
National Institute for Environmental Studies (NIES),
16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
e-mail: hayasaka.daisuke@nies.go.jp; awayotou@hotmail.com

F. Sánchez-Bayo
Department of Environmental Sciences, Centre for Ecotoxicology, University of Technology Sydney,
C/-P.O. Box 29, Lidcombe, NSW 1825, Australia

Introduction

Pesticides are developed to protect crops against pests, and are indispensable to assure agricultural quality and productivity. However, pesticides can have adverse impacts on some non-target organisms in the aquatic ecosystem. Especially, rural areas including paddies play an important role as habitats for many species (Bambaradeniya and Amerasinghe 2003). Even if complex experimental systems such as micro- and mesocosms (e.g., Chang et al. 2005; Sánchez-Bayo and Goka 2006a; Beketov et al. 2008) are essential for effective higher-tier ecological risk assessment to pesticides (Campbell et al. 1999), acute ecotoxicity data still play an important role in first-tier risk assessments for regulatory purposes.

In Japan, as in most developed countries, the ecotoxicity of pesticides to aquatic organisms is estimated using only laboratory single-species tests based on the OECD guidelines (1982). These guidelines recommend using three test species: a zooplankton crustacean (typically *Daphnia*

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magna), a small fish (e.g. *Oryzias latipes*) and an aquatic algae (e.g. *Pseudokirchneriella subcapitata*). In particular, since zooplankton are prey to fish and aquatic insects while being consumers of phytoplankton, zooplankton organisms are important links in the aquatic food chain and the function of freshwater ecosystems (Chang et al. 2005; Steiner et al. 2005). It is also important to take species sensitivities into consideration for a proper evaluation of laboratory acute toxicity tests. Different species can vary significantly in their sensitivity to toxic contaminants (Wogram and Liess 2001; Posthuma et al. 2002). However, information on the susceptibility among zooplankton species to many modern pesticides such as neonicotinoid and phenyl-pyrazole is deficient, and most of our knowledge is based on carbamate insecticides and metallic compounds (e.g. Sakamoto et al. 2005; Vesela and Vijverberg 2007; Mano et al. 2010), whereas most ecotoxicity data refers to *Daphnia magna* (Sánchez-Bayo 2006).

In this study, we examined the relative sensitivities of five cladoceran species to two new systemic insecticides imidacloprid and fipronil, which belong to the neonicotinoid and phenyl-pyrazole chemical classes, respectively, and have different chemical properties. Our comparison is based on the 48-h acute toxicity test, taking their body size into account (Gliwicz 1990; Mano et al. 2010). In addition, we compared the sensitivity of the five test cladocerans and that of other species of aquatic vertebrates and invertebrates, using species sensitivity distribution (SSD) curves for the respective insecticides. The concept of SSD is to statistically predict the safe environmental concentration of a toxicant that is protective of most species (usually above 95% in a community) (Posthuma et al. 2001).

Table 1 Physicochemical properties and acute toxicity of imidacloprid and fipronil

	Imidacloprid	Fipronil
Physicochemical properties		
Water solubility at 20°C (mg/l)	610[a]	3.78[c]
Octanol: water partition coefficient at 20°C (logPow)	0.57[c]	4[c]
Hydrolysis half-life at 25°C (days)	>30[b]	>100[d]
Aqueous photolysis half-life at 25°C (days)	0.0398[b]	0.33[d]
Sorption in soil (Koc)	132–310[b]	542–1176[e]
Acute toxicity		
Crustaceans (48-h LC_{50}: µg/l)		
Daphnia magna	10440–64873[f]	>100[f]
Fish (96-h LC_{50}: µg/l)		
Lepomis macrochirus (bluegill)	>105000[f]	25–83[f]
Oncorhynchus mykiss (rainbow trout)	83000–229100[f]	39–246[f]

[a] Data from Tomlin (2001–2002)

[b] Data from Kollman and Segawa (1995)

[c] Data from Japan Plant Protection Association (2005)

[d] Data from Gunasekara et al. (Gunasekara et al. 2007)

[e] Data from Ying and Kookana (2001)

[f] Data from ECOTOX database (http://cfpub.epa.gov/ecotox/)

Materials and methods

Physicochemical properties and acute toxicity of target insecticides

Physicochemical data of imidacloprid and fipronil are given in Table 1. Imidacloprid has high water solubility, and though the active ingredient disappears quickly from surface waters (Kollman and Segawa 1995), it is more persistent in underground water environments (Felsot et al. 1998; Nemeth-Konda et al. 2002). By contrast, fipronil has low water solubility, is more stable and it is adsorbed more strongly onto soil (USEPA 1996; Ying and Kookana 2001; US Geological Survey 2006; Gunasekara et al. 2007) than imidacloprid. The penetration rates of imidacloprid and fipronil, which are dominantly used in rice fields of Japan, are 18.9 and 24.8%, respectively (Ministry of Agriculture, Forestry and Fisheries 2005). Since these insecticides can be absorbed by rice seedlings and stored in their tissues, they are usually applied to nursery boxes in granular formulation before planting, to protect crops against pests. From the acute toxicity data, it appears that fipronil is 100–1000 times more toxic than imidacloprid to *Daphnia magna* and two species of fish (Table 1).

Test species

All test cladocerans in this study (*Ceriodaphnia dubia*, *Ceriodaphnia reticulata*, *Daphnia magna*, *Daphnia pulex* and *Moina macrocopa*) were obtained from the National Institute for Environmental Studies, Tsukuba, Japan. Except for non-indigenous species such as *D. magna* and *C. dubia*, all others occur commonly in freshwater environments in Japan, including rice fields (Hanazato 1998). These stock cultures have been maintained for 30 years at the institute. Stock cultures were kept at a constant temperature of 22 ± 1°C with a light:dark cycle of 16:8-h. The five cladocerans were separately cultured in 1 l glass beakers filled with dechlorinated tap water and fed daily, using green alga *Chlorella vulgaris* as their exclusive diet. Parameters of the tap water used are follows: pH 7.8; turbidity, <0.1; water hardness, 76 mg/l; and total organic carbon (TOC), 0.9 mg/l.

Toxicity bioassays (immobilization test, 48-h EC_{50})

In this test, we used wettable powders of imidacloprid and fipronil to make the insecticidal solutions. Commercial imidacloprid [Admire® Flowable, imidacloprid/water and surfactant (20:80, v/v)] was obtained from BASF Japan Ltd. and fipronil [Prince® Flowable, fipronil/water and surfactant (5:95, v/v)] from Kumiai Chemical Industry Co., Ltd., both from Tokyo, Japan.

The bioassays were performed following OECD guideline no. 202 (1984, 2004) for acute immobilization tests and good laboratory practice. Female neonates (<24-h old) from the second or later broods were used in all tests. The nominal concentrations of imidacloprid and fipronil, and number of tests for each species/treatment are shown in Table 2. The concentration ratio between successive solutions in all the tests was 2.0. Nominal chemical concentrations were prepared by serial dilution with dechlorinated tap water of stock solutions in distilled water. For each concentration, four replicates were used, each replicate beaker containing five neonates of the same species, which were placed in 50 ml of the test solutions. Each species was tested separately. Controls were prepared in the same way but using only dechlorinated tap water. No food was provided during the test period. Because of the fast aqueous photolysis of both insecticides (Table 1), the acute immobilization test in this study was semi-static, with chemical solutions being renewed daily according to the test guideline for longer exposure tests (OECD 1984). This means our results may be slightly overestimated when compared to those from static 48-h tests. The test beakers were kept at 21 ± 1°C with a light:dark cycle of 16:8-h for 48-h. The endpoint used for all bioassays was immobility, i.e., the inability to swim within 15 s after gentle agitation of the test container. Test organisms were checked after 48-h from the beginning of the tests.

Abiotic factors such as pH and dissolved oxygen (DO) were measured at the beginning and end of the tests in the controls and beakers with the highest test substance concentrations. Water pH and DO were measured by a portable multi-meter (DM-32P; TOA DKK-TOA Corporation, Tokyo, Japan).

Initial values of pH were 7.92 ± 0.07, and though they decreased slightly after 48-h (7.84 ± 0.06), the change was not significant. The values of DO at the start and the end were 8.30 ± 0.20 and 8.04 ± 0.15, respectively.

Body length of neonates of the five test cladocerans

To clarify the relationship between the EC_{50} values of imidacloprid and fipronil and the body sizes of the test species, we measured body lengths of their neonates (Table 3). Prior to the bioassays, 30–40 female neonates, randomly selected from the stock culture of each organism, were preserved in formalin (4%). Body lengths, from the crown of the head to the base of the tail spine (Mano et al. 2010), were measured using graphic software (IE-500, Leica Microsystems AG, Switzerland) under a dissecting microscope (Leica DFC490, Leica, Wetzlar, Germany).

Data analysis

All observations were recorded at 48-h exposures to determine the corresponding acute EC_{50} (immobilization), which was calculated by the Probit method (Finney 1971) using the program EcoTox-Statics ver. 2.5 (http://www.intio.or.jp/jset/ecotox.htm). The relationship between estimated EC_{50} values of imidacloprid and fipronil among each test species and their body lengths was analyzed by Pearson's correlation coefficient.

To examine differences in the patterns of other aquatic organisms (i.e., except cladocerans) to imidacloprid and fipronil, we compared our results with the acute toxicity data (LC_{50} and EC_{50}) from the ECOTOX database (http://cfpub.epa.gov/ecotox/), using all data available for these insecticides to amphibians, crustaceans, fish, insects, mollusks and worms. Species sensitivity distributions (SSDs) of each insecticide were used to this purpose. Based on laboratory single-species acute toxicity tests, SSDs are constructed by fitting a cumulative density function to a plot of species toxicity data against rank-assigned percentiles (Aldenberg and Jaworska 2000). From the distribution of such data the 5% hazardous concentration (HC5) of each insecticide was calculated, which would indicate the

Table 2 Nominal concentrations of imidacloprid and fipronil used in the acute tests

Species	Imidacloprid		Fipronil	
	Range (μg/l)	Number of treatments	Range (μg/l)	Number of treatments
Ceriodaphnia dubia (C.dub)	390.63–6250	5	0.39–12.5	6
Ceriodaphnia reticulata (C.ret)	781.25–50000	7	0.39–100	9
Daphnia magna (D.mag)	12500–400000	6	9.77–625	7
Daphnia pulex (D. pul)	6250–200000	6	9.77–625	7
Moina macrocopa (M. mac)	6250–20000	6	6.25–200	6

Table 3 Acute toxicity (immobilization), of imidacloprid and fipronil to five cladocerans

Species	Sample size (*n*)	Mean body length (mm)	Imidacloprid 48-h EC_{50} (μg/l)	Fipronil 48-h EC_{50} (μg/l)
Ceriodaphnia dubia	34	0.34 ± 0.06	571.62 ± 289.6–841.2	0.99 ± 0.62–1.42
Ceriodaphnia reticulata	30	0.37 ± 0.07	5552.9 ± 4213.3–7387.8	8.83 ± 5.52–17.57
Daphnia magna	25	1.11 ± 0.06	43265 ± 34302–53592	88.30 ± 64.20–141.12
Daphnia pulex	32	0.73 ± 0.10	36872 ± 28399–48106	40.392 ± 30.04–53.50
Moina macrocopa	41	0.56 ± 0.07	45271 ± 34378–62218	29.57 ± 16.83–9.E + 7

Body length is indicated by the mean and standard deviation

concentration that has a negligible effect on natural biocenosis. Differences in SSD patterns between the five test cladocerans and other aquatic organisms to the two insecticides were analyzed by paired *t*-test. The statistical analysis was conducted using SPSS ver 11.5 J (SPSS Japan, Tokyo, Japan).

Results

Calculated acute toxicities values (48-h EC_{50}) of the test organisms to imidacloprid and fipronil are given in Table 3. In this study, the values of *D. magna* for imidacloprid and fipronil were 43,265 and 88.3 μg/l, respectively. These values are in the same range as reported for this species on the ECOTOX database (6,029–85,200 μg/l for imidacloprid, and 29–190 μg/l for fipronil).

Clear differences in susceptibility among the cladocerans tested were found for both imidacloprid and fipronil. The degree of sensitivity of the species to the two insecticides spanned similar orders of magnitude: about 100 times from the most sensitive (*Ceriodaphnia dubia*) to the least. Toxicity of imidacloprid was in the following decreasing order: *C. dubia* > *C. reticulata* > *D. pulex* > *D. magna* > *M. macrocopa*. For fipronil: *C. dubia* > *C. reticulata* > *M. macrocopa* > *D. pulex* > *D. magna*. *Ceriodaphnia dubia* and *C. reticulata* showed the highest sensitivities to the two insecticides, and *D. magna* exhibited the lowest (Table 3). At the genus level, *Ceriodaphnia* spp. are more sensitive, whereas *Daphnia* spp. and Moina sp. are less susceptible to either imidacloprid or fipronil (Table 3).

The relationship between mean body lengths and EC_{50} values of the species tested to the two insecticides are shown in Fig. 1. Although a weak relationship between the two factors was found in the case of imidacloprid ($r^2 = 0.585$, $P = 0.132$), the acute toxicity of fipronil was significantly correlated with body length ($r^2 = 0.989$, $P < 0.001$). On the other hand, there were no clear differences in susceptibility to the insecticides between indigenous (*C. reticulata*, *D. pulex* and *M. macrocopa*) versus non-indigenous species (*C. dubia* and *D. magna*) (Fig. 1).

Comparative results of SSD patterns between the test five species of cladocerans tested in this study and other aquatic organisms are shown in Fig. 2. For imidacloprid, the 5% hazardous concentration (HC5) values calculated from the ECOTOX database (all aquatic organisms except cladocerans) and our data (five cladoceran species) to imidacloprid were 0.67 and 513.68 μg/l, respectively. Those of fipronil were 0.10 and 0.88 μg/l, respectively. In the case of imidacloprid, a significant difference in SSD patterns was found between the cladocerans and other aquatic organisms ($t = -3.112$, $P < 0.01$), with cladocerans being less sensitive than other species. However, similar SSD patterns were found between the two species groups compared in the case of fipronil ($t = 1.239$, $P = 0.231$).

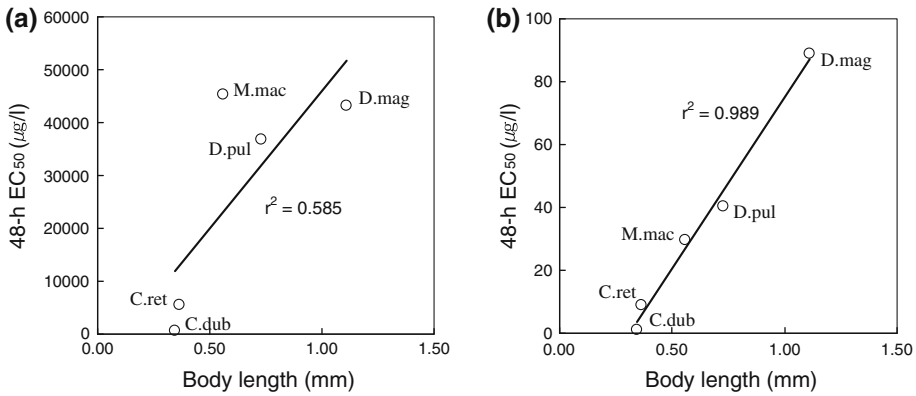

Fig. 1 Relationships between the acute toxicity of insecticides (48-h EC_{50} in μg/l) and body size of five cladoceran species: **a** imidacloprid, **b** fipronil. Abbreviations of the test species: *C.dub Ceriodaphnia dubia*; *C.ret Ceriodaphnia reticulata*; *D.mag Daphnia magna*; *D.pul Daphnia pulex* and *M.mac Moina macrocopa*. *Solid line* indicates a regression line of the relationship between the two factors

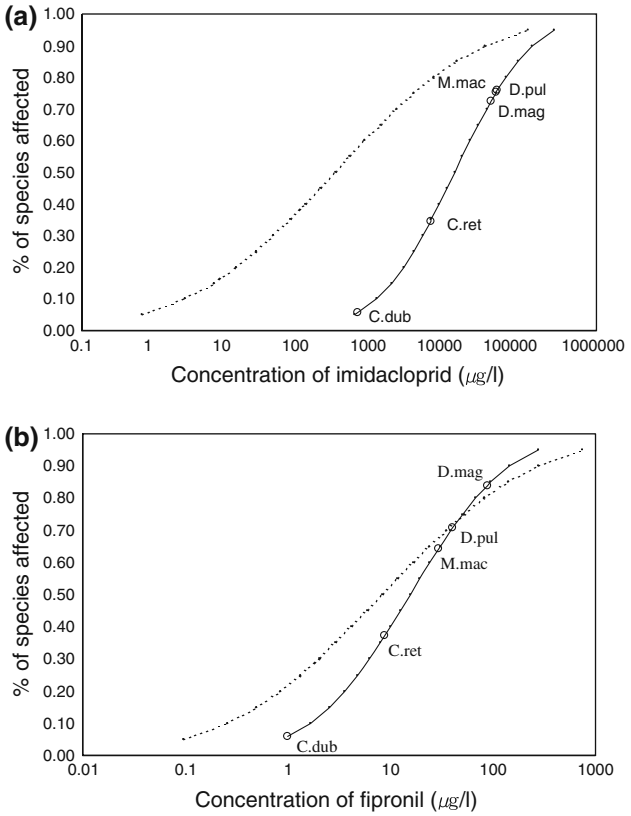

Fig. 2 Species sensitivity distribution (SSD) of five cladoceran species in this study (*solid line*) and other aquatic organisms (*dotted line*) using data from ECOTOX database for **a** imidacloprid and **b** fipronil. Abbreviations of the test species as in Fig. 1. Other aquatic organisms include amphibians, crustaceans, fish, insects, mollusks and worms

Discussion

Sensitivities of the five cladoceran species tested here to imidacloprid and fipronil varied depending on the body size of species and taxonomic (genus) level rather than species status (indigenous and non-indigenous) (Fig. 1; Table 3). Our results showed the wide interspecific variation in the susceptibility of test cladocerans to both insecticides. Similar finding is reported by Hose and Van den Brink (2004) that the sensitivity of organisms to toxicants is independent of their geographic origin. Body size was positively related to the capacity of these organisms to withstand the stress caused by the two insecticides (Fig. 1), since smaller species tend to be more sensitive to toxic stress than the larger ones (Wong et al. 2009). Similar findings have been reported by other researchers when testing for metals and cholinesterase-inhibitor insecticides (e.g. Sakamoto et al. 2005; Vesela and Vijverberg 2007), whereas a review by Hanazato (1998) indicates that larger cladoceran species are more sensitive to insecticides than smaller zooplankton species such as rotifers. A comprehensive comparative study by Sánchez-Bayo (2006) found that there is no significant effect of size on the sensitivities of zooplankton crustaceans to most toxic chemicals. In fact, positive correlations with size appear to be the exception (16% of toxicants) rather than the norm, and are found with preference among chemicals with specific mode of action such as insecticides, which are usually the most toxic (Vaal et al. 1997). In our study, fipronil showed a clear correlation with the size of the five species tested ($r^2 = 0.989$), but that of imidacloprid was not as strong ($r^2 = 0.585$). Thus, differences in the insecticide impacts on biocenosis may depend on the mode of action of the chemicals as well as the cladoceran species composition (Mano et al. 2010). Previous studies reported the high tolerance capability of *M. macrocopa* (Hatakeyama and Sugaya 1989; Mano et al. 2010) and by contrast the high sensitivity of *Ceriodaphnia* sp. (Hatakeyama et al. 2010; Mano et al. 2010) to carbamate pesticides. The bioassay tests results shown here, which consider different chemical classes of insecticides (neonicotinoid and phenyl-pyrazole) also showed a similar tendency (Table 3). *Ceriodaphnia reticulata* is known to consume micro-organisms such as bacteria more efficiently than other cladocerans, perhaps because of its small size (Geller and Müller 1981).

From these results, because of their high sensitivity to these two insecticides, *Ceriodaphnia* spp. may be more suitable bioindicators of ecological disturbance by imidacloprid and fipronil in aquatic ecosystems than the current OECD surrogate species, *Daphnia magna*. Low sensitivity of *D. magna* to neonicotinoid thiacloprid was also found by Beketov and Liess (2008). Mano et al. (2010) indicate that a decrease in the abundance of *Ceriodaphnia* spp., in particular *C. reticulata* by carbamate insecticides such as carbaryl and methomyl may reduce the energy flow through the microbial loop, since heterotrophic micro-organisms such as bacteria are consumed by zooplanktons.

Species sensitivity distribution (SSD) of ecotoxicological data is one of the most effective approaches for ecological risk assessment to pesticides because it aims at protecting biodiversity (Posthuma et al. 2001; Nagai et al. 2011). Clear differences in SSD patterns of the two insecticides tested here, in particular imidacloprid, were found between the five cladocerans used in this study and other aquatic organisms (Fig. 2). Among the zooplanktons, cladocerans are more sensitive than rotifers and copepods to a large range of pollutants (Hanazato and Yasuno 1990; Sierzen and Lozano 1998; Wong et al. 2009), and have been attractive test organisms also due to their short generation cycle and ease of culture and maintenance in laboratories (Benfield and Buikema 1980). In addition, Dodson et al. (1995) reported that prey zooplankton such as cladocerans are more sensitive to toxicants than their

predators, and therefore are preferred as sentinel bioindicators of the ecosystem (Sakamoto et al. 2005). The significant differences in HC5 values for imidacloprid between the two groups compared (Fig. 2a) suggests, however, that imidacloprid residues in water can have larger adverse effects on aquatic organisms other than cladocerans: indeed, most aquatic taxa are about 500 times more sensitive to imidacloprid than cladocerans. In particular, ostracods are two to three orders of magnitude more susceptible to imidacloprid than cladocerans (Sánchez-Bayo and Goka 2006b). By constant, the sensitivity of cladocerans to fipronil is no different from that of other aquatic taxa (Fig. 2b). The SSD patterns shown here are in agreement with the finding reported by Vaal et al. (1997), who documented that reactive and specific mode of action chemicals such as insecticides usually have the largest intraspecific variation, as shown by the less steep slope of a SSD curve of toxicity data from aquatic species.

On the other hand, Hose and van den Brink (2004) indicate that arthropod taxa in mesocosm were less sensitive than in laboratory tests, which suggests that laboratory single-species data used on SSDs may be overprotective of field populations. However, Hayasaka et al. (2011) report that imidacloprid in paddy mesocosms can have adverse effects on zooplankton, neuston, nekton and benthic communities at concentrations well below the HC5 protective value of the test cladocerans, whereas small impacts of fipronil on the same aquatic organisms were found. This discrepancy may not be surprising because many researchers have shown similar tendencies with other insecticides (e.g., Liess and von der Ohe 2005; Schäfer et al. 2007). However, the results from the semi-static tests in this study may be regarded as slightly overestimated due to the greater exposure.

As mentioned above, ecotoxicological assessment protocols for aquatic organisms are standardized by the OECD guidelines. Harmon et al. (2003) and Wu et al. (2007) indicate that although the regulating authorities accept the test organisms and protocols, they do not always reflect local taxa or site-specific conditions. For instance, Wu et al. (2007) have suggested that *Daphnia carinata* is a more suitable test species for tropical and subtropical regions, where *D. magna* is not found, while other authors have criticized the use of *D. magna* on size considerations (Koivisto 1995).

The strong differences in susceptibility among cladocerans to the pesticides imidacloprid and fipronil were clarified in this study, and our findings agree well with interspecific differences shown by many other authors using insecticides. Therefore, we conclude that toxicological data associated with differences in susceptibility among species should be investigated whenever pesticides have different physicochemical properties and mode of actions. Such information may help define uncertainty factors to extrapolate from laboratory acute toxicity tests based on OECD test guidelines (i.e. *Daphnia magna*) to other species with similar ecological function in the ecosystems.

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