Paddy and Water Environment (2019) 17:1–11
https://doi.org/10.1007/s10333-018-0678-3

ARTICLE

Exposure to anti-mosquito insecticides utilized in rice fields affects survival of two non-target species, *Ischnura elegans* and *Daphnia magna*

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Received: 1 December 2017 / Revised: 1 September 2018 / Accepted: 17 September 2018 / Published online: 21 September 2018
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Abstract

Insecticides are commonly utilized to control mosquito larvae in rice fields. They can, however, have negative effects on both vertebrates and non-target invertebrate species. In this study, we examined the effects of pulse exposition to different concentrations of cypermethrin (0.15, 0.015, 0.0015 mg/L) and diflubenzuron (0.15, 0.015, 0.0015 mg/L) on egg hatching rate, larval growth, and larval survival in a damselfly, *Ischnura elegans*, and on survival of a crustacean, *Daphnia magna*. Insecticide exposure had significant negative effects on hatching rate in damselfly eggs. Exposed damselfly larvae also grew less and showed a higher mortality than control larvae. In *Daphnia,* the acute toxicity test (ISO 6341 in Water quality—determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)—acute toxicity test, Int Organ Stand Geneve, Geneva, 2012) showed an increased inhibition of mobility in the presence of insecticides. We observed a proportional response in relation to insecticide concentration, such that the highest exposure levels showed the largest reduction of vital performances. Our highest tested values correspond to those currently employed in agriculture. This study suggests that exposure to two common insecticides strongly affects non-target invertebrates even at very low concentration levels (cypermethrin 0.0015 mg/L and diflubenzuron 0.0015 mg/L).

Keywords Cypermethrin · Diflubenzuron · Odonate · Damselfly · Water flea

Introduction

Rice fields are widespread throughout the world, cover over 1.5 million km^2, and are the primary source of calories for over half of the human population (Elphick 2007). Due to the large decline of natural wetland areas, this agricultural environment has been considered as vicariates for natural wetlands for waterbirds (Fasola and Ruiz 1996; Lourenço and Piersma 2009) and other vertebrates (van Dijk et al. 2004). Besides, aquatic invertebrates are relatively abundant in flooded rice fields with densities of 6.3–31.7 kg/ha reported in North America (Stafford et al. 2007).

However, the possibility for rice fields to act as an environmental substitute for wetland fauna can be hampered by an excessive use of chemical products to increase food production and combat weed, fungi, and undesirable animals. In particular, there are a variety of products available on the market for mosquito control. Mosquito larvicides are chemicals designed to be applied directly to water to control mosquito larvae, but they can have negative effects on non-target species, including human beings (Carvalho et al. 2014).

The effect of the application of chemicals on animals can be examined considering the whole faunal community (Crossland and La Point 1992; Williams et al. 2002), or the effects on single species (Beketov 2004; Carvalho et al. 2014). In general, invertebrates can be rather tolerant to some organic nutrients, i.e. ammonium, nitrates, and phosphates, but are highly sensitive to pesticides (Pal et al. 2010).

Several animal species have been used to assess the presence and effects of pollutants in the aquatic environment,

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including the invertebrates [mostly insects, crustaceans, and molluscs (Negri et al. 2013; Malaj et al. 2014)]. Insects in particular exhibit selective tolerance for the pollutants and their larval, aquatic stages are ideal to test the effect of insecticides for this reason and obtain information on the toxic properties of substances, solutions, or mixtures (Tang and Siegfried 1996; Beketov 2004). For their role of intermediate predators in the food chain of lentic and lotic waters, the Odonata are excellent indicators of environmental health (Sahlén and Ekestubbe 2001; D'Amico et al. 2004; Smith et al. 2007; Cordoba-Aguilar 2008; Funk et al. 2009) and can be used to assess habitat quality with the use of Odonate indexes (Chovanec and Waringer 2001; Chovanec et al. 2015). However, Odonates are still little used in ecotoxicology (Beketov 2004; Tollett et al. 2009; Buckland-Nicks et al. 2014), and there are few studies concerning a single target species (Stewart 1996; Chang et al. 2009). Indeed, in the last few decades only some studies tested the effects of herbicides and pesticides on Odonate larval stages, e.g. azinphos-methyl and carbayl (Hardersen and Wratten 2000), diflubenzuron (Hurd et al. 1996), avermectin, imidacloprid, fipronil (Chang et al. 2007; Jinguji et al. 2013), and PFOS (Van Gossum et al. 2009).

Odonates, and particularly the damselflies (suborder Zygoptera), are particularly suitable as environmental pollution indicators, because their behaviour is well known (Corbet and Brooks 2008), and they are also representative for other aquatic taxa who share many characteristics (Stoks et al. 2015), are widespread, have limited mobility and long life cycles, and can be reared in laboratory conditions (Hardersen and Wratten 2000). The damselflies' high sensitivity to chemicals can be measured not only in terms of mortality, but also by their body accumulation (Heintzman et al. 2015), effects on growth, bilateral symmetry, and impaired behavioural responses to predators or when feeding (Chang et al. 2009; Van Gossum et al. 2009; Che Salmah et al. 2012).

The *Daphnia magna* is a common water flea widely distributed in freshwater habitats (Barry 1996; Barata et al. 2005), that can be utilized as an indicator for zooplankton condition (LeBlanc 2016). This species has been used to evaluate antioxidant enzymes and oxidative stress in relation to UV and oxygen concentration (Borgeraas and Hessen 2002), and to assess the toxicity of several chemicals (Christensen et al. 2005; Beketov and Liess 2008; Toumi et al. 2013). This crustacean is an important non-target species, since it is sensitive both at individual and community levels to pesticides utilized to combat agricultural pest species (Friberg-Jensen et al. 2003; Wendt-Rasch et al. 2003). *Daphnia magna* is placed at an intermediate level in the freshwater ecosystem trophic chain (Christensen et al. 2005), hence a reduction of its abundance (Abe et al. 2014) or its mobility and ability to capture food (Duchet et al. 2011) will end in a cascade of effects on the whole environment with increased phytoplankton and water turbidity (Friberg-Jensen et al. 2003). The species can be reared and tested in the lab using standard protocols (ISO 6341 2012), and it is employed routinely by most environmental agencies.

In this study, we tested the effects of two anti-mosquito insecticides on two non-target species, the Blue-tailed damselfly *Ischnura elegans* (Odonata: Coenagrionidae) and the Water flea *Daphnia magna* (Crustacea: Cladocera). We applied pulse exposition to different concentrations of two anti-mosquito insecticides commonly utilized in rice fields, i.e. cypermethrin (0.15, 0.015, 0.0015 mg/L) and diflubenzuron (0.15, 0.015, 0.0015 mg/L), and then we examined egg hatching rate, larval growth, and larval survival in *Ischnura elegans* and inhibition of mobility as a standard index (ISO 6348) of survival in *Daphnia magna*.

Materials and methods

The cypermethrin [Cyano-(3-phenoxyphenyl)methyl]3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate, also known as Contest® (BASF), is an insecticide used in the anti-mosquito prophylaxis. It is a broad spectrum, highly active pyrethroid, which is able to act very quickly and very effectively against parasites in farms, residential areas, and areas of productive activities. This insecticide acts as a neurotoxin and is highly stable in daylight and at ambient temperature (up to 1 year in soil, 5–7 days in water; Agnelli et al. 2015).

Diflubenzuron, *N*-[(4-Chlorophenyl)carbamoyl]-2,6-difluorobenzamide, is an insecticide used in mosquito prophylaxis acting as a growth regulator. It is able to inhibit chitin synthase enzyme, thus preventing the deposition of chitin during larval moulting (Miyamoto et al. 1993; EPA 1997).

The trials with diflubenzuron were carried out at concentrations 0.15, 0.015, and 0.0015 mg/L. The test concentrations were obtained using 1, 0.1, and 0.01 µL/L, respectively, of the Device SC-15® of Chemtura, UK. Diflubenzuron was applied in three different experiments: in the first the insecticide was administered to the eggs of *Ischnura elegans*, in the second experiment it was administered to larvae of *Ischnura elegans* 2 days after hatching, and in the third experimental set-up we tested the effects on *Daphnia magna* through the inhibition of mobility test (ISO 6341 2012). Similarly, we carried out three experiments (insecticide administered to *Ischnura* eggs and to larvae and the immobility test for *Daphnia*) with the alfa-cypermethrin at concentrations 0.0015, 0.015 and 0.15 mg/L. The test concentrations were obtained using 1, 0.1, and 0.01 mg/L, respectively, of the Contest® product by BASF AGRI, France. For both chemicals, the higher concentration, equal to 0.15 mg/L, is equivalent to the dose normally used in rice fields (IPCS 1992; European Union Competent Authority Report 2007), and the other doses are then diluted respectively 10 and 100 times. Finally, we conducted three experiments (on *Ischnura* eggs, *Ischnura* larvae and *Daphnia*, respectively) using a mixture of the two insecticides, both dosed at the lowest concentration levels, i.e. 0.0015 mg/L.

As recently reviewed, the biological effect of insecticides can vary according to the concentration but also to the time of exposure, i.e. chronic or pulse contact (Tennekes and Sánchez-Bayo 2013). Our protocol included a single pulse exposure and was designed to simulate realistic field exposure conditions where administration of the chemicals to rice fields typically occurs only once by small aircrafts or vehicles from the ground. In our experimental setup, the administration of insecticides took place only once, without any subsequent addition. In the experiment with eggs, chemicals were added to water 1 day after the oviposition, while in the experiment with larvae, they were administered 2 days after hatching.

Maintenance of *Ischnura elegans*

Blue-tailed damselflies can be easily reared (Piersanti et al. 2015) and utilized in eco-toxicological studies (Van Praet et al. 2012, 2014a, b, c). Pairs of mating damselflies (a male and female that were found joined in the typical wheel shape) were collected with a hand net in the field from the grass vegetation near the edge of a small lake (formerly a quarry site), located close to our lab in Alessandria, NW Italy. The lake is located in an agricultural area with fields of wheat and corn and is located about 15 km from the nearest paddy fields area. No larvae were collected directly in the field. In the lab, mating pairs were first kept in a large cage, and then when copulation and male clasping ended, the males were released while each female was inserted in a separate cup with water and a wet filter paper sheet. In the following hours, laying females readily utilized the wet paper as a substrate for oviposition of fecundated eggs. After laying, females were released and eggs were counted. On average, each female laid a mean of 120.1 ± 12.8 SE eggs. The filter paper containing the entire clutch was cut into four portions containing an approximately equal numbers of eggs, and each portion was randomly allocated to a different experimental dose of insecticide (cypermethrin: 0.15, 0.015, 0.0015 mg/L, and control group; diflubenzuron: 0.15, 0.015, 0.0015 mg/L, and control group).

Each group of eggs was maintained in a tray (23 × 30 cm) filled with spring water up to 2 cm depth. We utilized mountain spring water that had been bottled by a commercial company for human drinking (Fontebracca. pH: 7.6; EC: 424 µS/cm; TOC: 15.0 mg/L; nitrates: 1.6 mg/L). Eggs were counted at the beginning and checked for hatching each day. After 15–18 days, the larvae emerged from the eggs and were kept in individual dishes (20 cm diameter) to prevent cannibalism. According to the report (De Block and Stoks 2008), *Artemia* nauplii were the only food item provided throughout the rearing period. In order to assess the effects of insecticides on growth and mortality, we carried out counts and measurements of the larvae twice a week.

Daphnia magna: acute immobilization test

The test followed the ISO 6341 (2012) standard specifications for water quality (utilized water: pH 7.46; bicarbonates 296 mg/L, calcium 51.4 mg/L; magnesium 29.7 mg/L, nitrates 9 mg/L; sodium 6 mg/L; sulphates 4.2 mg/L; chlorides 2.6 mg/L; potassium 0.97 mg/L; fluorides < 0.1 mg/L) and the standard OECD (2004) No 202 acute immobilization test. Ten newly hatched daphnids (aged less than 24 h) were placed in glass beakers (100 ml) and exposed to the pesticides at 20 ± 2 °C, with a 16/8 h light–dark cycle. Four replicates were made per treatment (i.e. 40 animals per each pesticide concentration and 40 animals in the control group). The number of immobile animals was counted after 24 and 48 h.

Statistics

The software R (R Core Team 2016) was used in all statistical analyses. Hatching rate is a binomial variable, with 0 and 1 values for unhatched and live individuals respectively. Hatching rate in control versus the three experimental groups was compared using a logistic model, with hatching as a binomial dependent variable, insecticide concentration as a fixed effect, and female ID as a random component (package *lme4*). Mortality rate was compared for each larval group tested using a standard survival analysis, with Kaplan–Meier estimates. We utilized the survival analysis implemented in the *survival* packages (Fox and Carvalho 2012; Therneau 2015) for R Commander (Fox 2005). In the models, female ID was entered as a random effect using the “frailty” function. To identify significant effects of pesticide concentration on growth, we used a mixed model ANOVA. Insecticide concentration with four levels (0.15, 0.015, 0.0015 mg/L) was inserted as the fixed component of the models, whereas the random component was female ID. Differences in response of *Daphnia* to an acute immobilization test were verified by comparison of the values measured in the experimental groups with respect to control values (Mann–Whitney U test).

Results

Cypermethrin

Treatment on eggs

The survival of larvae hatched from eggs treated with cypermethrin was low during the first 6 days after hatching. The three different insecticide concentrations had a significant influence (all $P < 0.01$) on survival (Table 1), resulting in a higher value in larvae treated with a lower dose and an almost total mortality within the first 8 days regarding the larvae treated with the highest concentration (Fig. 1a).

Larvae of the control group grew slightly but not significantly faster than larvae pertaining to the 0.0015 mg/L group ($F_{1,640} = 1.289$; $P = 0.19$). Growth of the other two experimental groups cannot be compared, because the very high mortality reduced their number and prevented the possibility of performing any statistical comparison.

Treatment on larvae

Figure 1b shows the survival of larvae that were treated with cypermethrin after their hatching. Survival was significantly lower in the groups treated with the insecticide with respect to the control group (Table 1). Along the first 20 days, larvae treated with the low dose of insecticide had a lower mortality than the larvae treated with the middle and high concentration, but afterwards, mortality was almost total for all groups (Fig. 1b).

Table 1 Mortality of *Ischnura elegans* larvae treated with different concentrations of the cypermethrin insecticide

Effect	Coef.	SE	Chi square	*df*	*P*
Treatment at the egg stage (*N* = 4583 larvae, evaluation period = 13 days)					
Cypermethrin (0.0015)	0.549	0.0409	180.2	1	< 0.001
Cypermethrin (0.015)	1.492	0.0455	1073.2	1	< 0.001
Cypermethrin (0.15)	1.853	0.0539	1180.1	1	< 0.001
Female ID			183.9	26.25	< 0.001
Treatment at the larval stage (*N* = 1061 larvae, evaluation period = 90 days)					
Cypermethrin (0.0015)	1.108	0.1710	41.97	1	< 0.001
Cypermethrin (0.015)	2.786	0.2271	150.49	1	< 0.001
Cypermethrin (0.15)	2.830	0.1240	520.84	1	< 0.001
Female ID			307.53	16.78	< 0.001

Coef. coefficient, *SE* standard error, *df* degrees of freedom, *P* probability

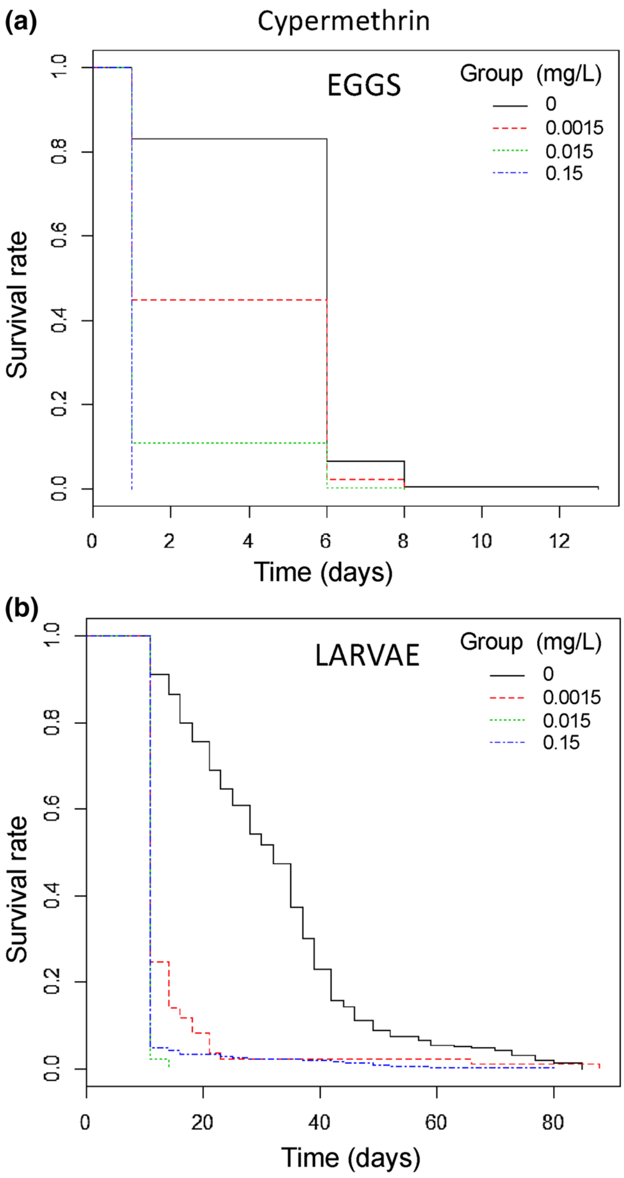

Fig. 1 Cypermethrin: effect of different concentrations on *Ischnura elegans* larval mortality. Pulse application of the insecticide at: **a** egg stage, or **b** larval stage. Survival rate indicates the ratio between number of surviving larvae to all hatched larvae

Treatment on *Daphnia magna*

The immobilization test showed a negative effect of the treatment with cypermethrin (Mann–Whitney U tests, $P < 0.01$), with a dose-dependent pattern (Fig. 2a). The negative effect was significant within 24 h in the 0.015 and 0.15 mg/L groups and was observed in all three experimental groups after 48 h (Fig. 2a).

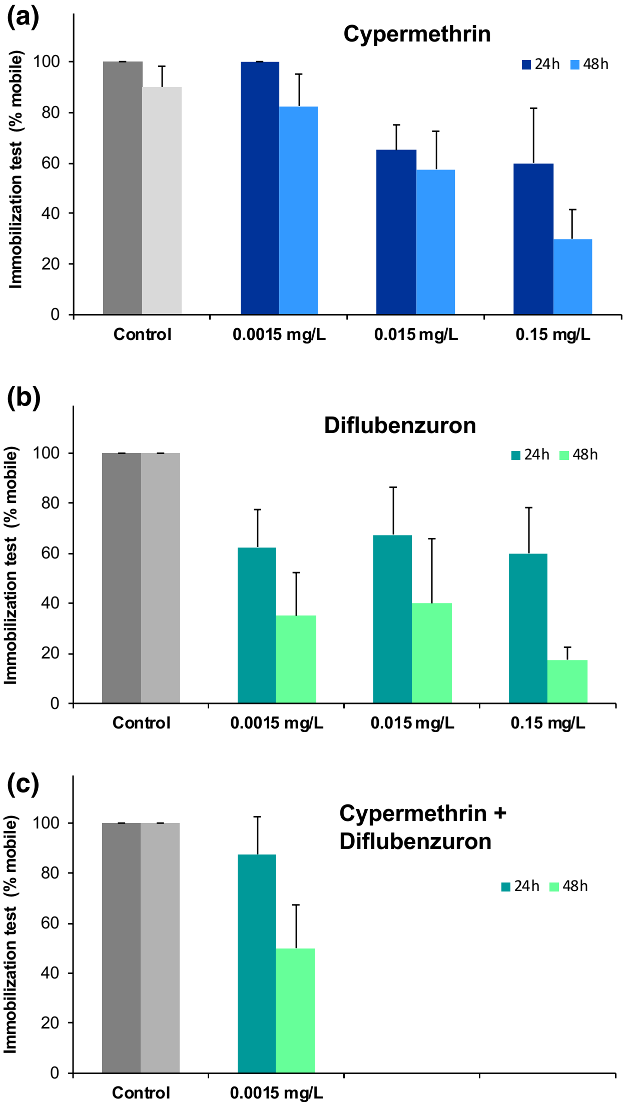

Fig. 2 Immobility test for *Daphnia magna*: effects of different concentrations of **a** cypermethrin, **b** diflubenzuron, **c** mixture of the two insecticides

Diflubenzuron

Treatment on eggs

Hatching rate was significantly influenced by the presence of diflubenzuron; the effect was dose dependent (Table 2).

The survival of larvae hatched from eggs treated with diflubenzuron was low following the first days after hatching. Different insecticide concentrations had a significant influence on survival (Table 3), resulting in a higher value in the larvae treated with a lower dose and an almost total mortality within the first 10 days regarding the larvae treated with the highest concentration (Fig. 3a).

Table 2 Hatching rate of *Ischnura elegans* eggs treated with different concentrations of the diflubenzuron insecticide

Effect	Estimate	SE	*z* value	*P*
Intercept	2.273	0.335	6.781	< 0.001
Diflubenzuron (0.0015)	− 1.065	0.186	− 5.736	< 0.001
Diflubenzuron (0.015)	− 1.644	0.171	− 9.631	< 0.001
Diflubenzuron (0.15)	− 2.553	0.170	− 15.021	< 0.001

During the first 8 days, larvae of the control group grew significantly faster than larvae pertaining to the treated groups ($F_{3,202} = 27.84$; $P < 0.001$). Afterwards, growth cannot be compared, because mortality prevented the possibility of performing any statistical comparison.

Treatment on larvae

Figure 3b shows the survival of larvae that were treated with diflubenzuron after their hatching. Survival was significantly lower in the groups treated with the insecticide with respect to the control group (Table 3). Along the first 14 days, larvae treated with the lower and the middle dose of insecticide had a slightly higher survival than the larvae treated with the highest concentration, but by day 25 mortality was complete for all treated groups (Fig. 3b).

During the first 25 days, larvae of the control group grew significantly faster than larvae pertaining to the treated groups ($F_{3,435} = 23.73$; $P < 0.001$). Afterwards, growth cannot be compared, because mortality prevented the possibility of performing any statistical comparison.

Table 3 Mortality of *Ischnura elegans* larvae treated with different concentrations of the diflubenzuron insecticide

Effect	Coef.	SE	Chi square	*df*	*P*
Treatment at the egg stage (*N* = 1412 larvae, evaluation period = 60 days)					
Diflubenzuron (0.0015)	1.269	0.1002	160.2	1	< 0.001
Diflubenzuron (0.015)	2.330	0.0988	555.9	1	< 0.001
Diflubenzuron (0.15)	2.913	0.1090	714.0	1	< 0.001
Female ID			163.2	10.43	< 0.001
Treatment at the larval stage (*N* = 1352 larvae, evaluation period = 30 days)					
Diflubenzuron (0.0015)	1.824	0.1049	302.1	1	< 0.001
Diflubenzuron (0.015)	1.785	0.1044	292.3	1	< 0.001
Diflubenzuron (0.15)	1.735	0.0963	324.6	1	< 0.001
Female ID			48.5	12.52	< 0.001

Coef. coefficient, *SE* standard error, *df* degrees of freedom, *P* probability

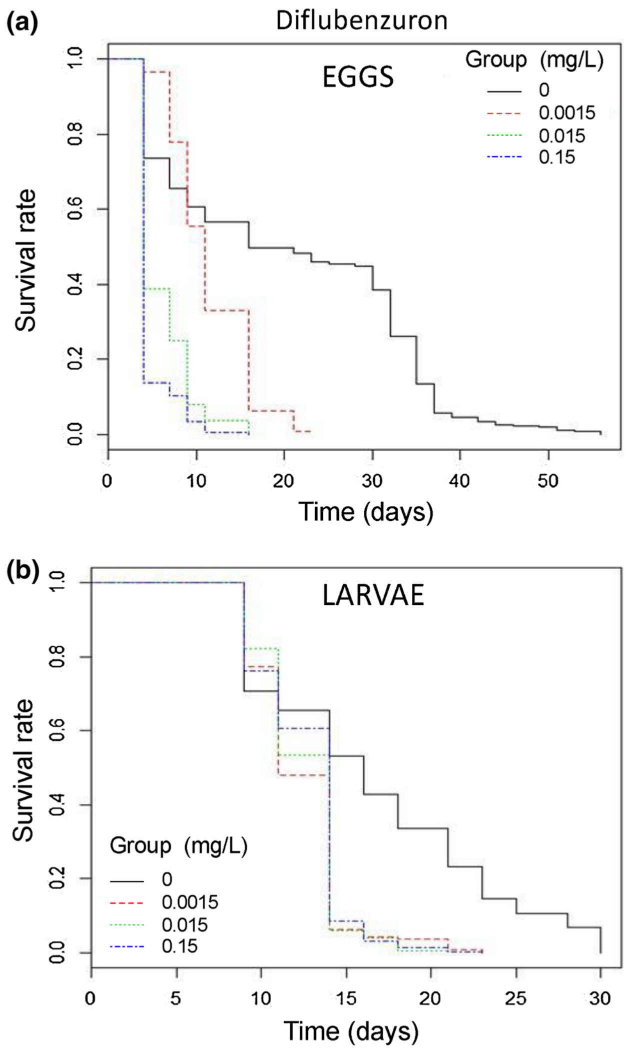

Fig. 3 Diflubenzuron: effect of different concentrations on *Ischnura elegans* larval mortality. Pulse application of the insecticide at: **a** egg stage, or **b** larval stage. Survival rate indicates the ratio between number of surviving larvae to all hatched larvae

Treatment on *Daphnia magna*

The immobilization test showed a negative effect of the treatment with cypermethrin (Mann–Whitney U tests, $P < 0.01$) and was similar regardless of the insecticide concentration (Fig. 2b). The negative effect appeared within 24 h in all groups and was stronger after 48 h (Fig. 2b).

Mixture of cypermethrin and diflubenzuron

Treatment on eggs

In the experimental group, the mixture of cypermethrin and diflubenzuron caused the death of all the larvae hatched from the treated eggs within 24 h after hatching.

Treatment on larvae

In the experimental group, the mixture of cypermethrin and diflubenzuron caused the death of all the larvae within 24 h after application.

Daphnia magna

The immobilization test showed a significantly negative effect of the treatment with cypermethrin plus diflubenzuron (Mann–Whitney U tests, $P < 0.01$). The negative effect appeared within 24 h and was stronger after 48 h (Fig. 2c).

Discussion

In this study, we investigated the effects of exposure to several different concentrations of two insecticides, cypermethrin and diflubenzuron, on hatching rate, growth, and survival of the Blue-tailed damselfly, *Ischnura elegans*, and on the inhibition of mobility in the Water flea, *Daphnia magna*. We found that insecticide exposure elicited significant negative effects on all examined biological parameters.

Cypermethrin is a pyrethroid used in agricultural applications and in consumer products for domestic purposes. The molecule acts as a neurotoxin on insects. The diflubenzuron is an insecticide used in mosquito prophylaxis. As a growth regulator, it is able to inhibit the chitin synthetase enzyme, thus preventing the deposition of chitin during larval moulting (Korytko and Scott 1998). Several insecticides can affect the abundance of species and the structure of invertebrate communities (Hurd et al. 1996; Suhling et al. 2000; Friberg-Jensen et al. 2003; Berenzen et al. 2005; Schäfer et al. 2007). Larvae from the dragonfly *Sympetrum infuscatum* exposed to imidacloprid, a neurotoxic neonicotinoid, showed a significant increase in mortality soon after administration. Besides, the insecticide caused a decrease in zooplankton resulting in an indirect negative effect (Jinguji et al. 2013). The hatching rate of the damselfly *Xanthocnemis zealandica* was reduced by organophosphates and carbammates (Hardersen and Wratten 2000). The impact of organophosphate sublethal doses can involve the impairment of predator and antipredator behaviours (Van Dinh et al. 2014), as well as food intake which in turn can delay the emerging date and reduce body mass (Janssens et al. 2014), with an overall decrease in fitness (Stoks and Cordoba-Aguilar 2012).

During the field application of insecticides, the chemicals can reach the non-target invertebrate species when they are at the egg or larval stages. However, little is known about effects at the egg stage. In this study, we found a decrease in hatching rate after application of diflubenzuron to eggs. The coat enveloping the eggs derives from the chorion, and performs an adhesive function plus a protective role during egg segmentation (Gaino et al. 2008). The protection provided by the egg coat of *Ischnura elegans* was not adequate enough to defend the developing embryo from the negative effect of chemicals, as mirrored by the lower hatching rate and lower survival of the larvae derived from the few hatched eggs. A high sensitivity of Odonates at the egg stage has been reported in a few other cases with effects spanning from immediate effects during exposure (Hardersen and Wratten 2000; Bots et al. 2010; Andrew 2012) to even stronger delayed postexposure effects on larvae (Debecker et al. 2017; Fontana-Bria et al. 2017).

When insecticides were applied at the *Ischnura elegans* larval stage, again we observed a negative effect of chemicals with a higher mortality in exposed larvae with respect to the controls. The growth of the few larvae that survived was lower compared to the growth of control group; this is an indication of long lasting effects from a single pulse application of insecticides. Our results are similar to those reported on the same species for other pollutants (Van Praet et al. 2014b) and to those reported for our same two insecticides in other arthropods (Savitz et al. 1994; Chen et al. 2008; Seccacini et al. 2008; Weston et al. 2015), or on insect communities (Sundaram et al. 1991; Schäfer et al. 2007).

The results obtained from testing of the two insecticides on *Daphnia magna* indicate that immobility in individuals treated with the two substances is linked to the presence of the chemicals and to the level of concentration administered. In particular, the results show that the highest inhibition of mobility is linked to the higher concentrations. The mixture of the two insecticides had a negative effect even at the lowest concentration. Similar negative effects of cypermethrin on *Daphnia* were found in other studies through tests performed with acute, pulse, and chronic administration (Lakota et al. 1989; Kim et al. 2008). The immobility is a good predictor of mortality. The first effect of a reduced mobility of *Daphnia* in the presence of harmful chemicals is a decrease in feeding efficiency mirrored by a low content of chlorophyll pigments in the gut (Christensen et al. 2005). The test can be used to detect negative effects besides other sublethal endpoints previously used in other studies, including clutch size and adult size as indices of fecundity and growth rate (Kashian and Dodson 2002).

Concerning diflubenzuron, other authors found a negative effect of this chemical on *Daphnia magna* (Kashian and Dodson 2002), suggesting that application of the insecticide will end in an increased environmental risk for the species (Abe et al. 2014). Alongside the acute consequences mirrored by the immobility test, long-term effects of diflubenzuron on *Daphnia* were also verified through the reproduction test (Duchet et al. 2011).

Similarly to cypermethrin, other pyrethroids used in agriculture were found to have negative effects on *Daphnia*. An analysis of acute and chronic toxic effects of deltamethrin detected consequences for survival correlated with the delivered concentration resulting in an increase in embryonic deformations; deltamethrin was found to be an endocrine disruptor that interferes with sex determination and leads to significant abnormalities in development (Toumi et al. 2013). Another study found a dose-dependent response in the treatment of *Daphnia* with the pyrethroid fenvalerate; in particular, it was noted that this substance delays the age of first reproduction, and this delay is associated at lower doses to a reduction in the number of young per female and at higher concentrations to an inhibition of population growth (Reynaldi and Liess 2005).

The dose sufficient to determine a negative effect on Odonates can be very small, particularly when considering the effects on behaviour, that represents a highly sensitive and integrated response to internal and external conditions (Van Gossum et al. 2009). There are no previous studies testing cypermethrin and diflubenzuron in damselflies. However, it is known that the effects of other pyrethroids was significant at concentration as low as 0.01 µg/L (Beketov 2004) or 0.1 µg/L (Schroer et al. 2004; Reynaldi and Liess 2005), i.e. a range of values similar to those found in our study.

The concentration of insecticides that was found to significantly affect *Daphnia* depends on the biological parameter considered. Mortality EC50-values were 0.87 µg/L and 0.36 µg/L in two studies for cypermethrin (Lakota et al. 1989; Westergaard et al. 2012), while sublethal effects were observed between 0.05 and 0.6 µg/L (Christensen et al. 2005). Likewise, the diflubenzuron showed a high toxicity to *D. magna* (0.03–0.01 µg/L), indicating that the use of the substance could result in a high environmental risk for this species (Abe et al. 2014). Results from our study confirm that even a low dose of these two insecticides can have a significant effect on the water flea.

Concentrations tested in this study for cypermethrin and diflubenzuron lie within the concentration range occurring in freshwater systems after pesticide application (Pistocchi et al. 2009; Feo et al. 2010b; Aznar et al. 2017). The doses tested are environmentally relevant concentrations that reflect a realistic risk for the two insecticides, or mixtures of the two chemicals, to non-target species (Fischer and Hall 1992; Konstantinou et al. 2006; Feo et al. 2010a). The exact concentration in freshwater environments depends on several factors, among others the degradation time in the water phase, the condition and chemistry of soil, and the intensity and duration of light hours (Lewis et al. 2016). Although the degradation rate of the insecticides used in this study is fast (2/3 days; cypermethrin: IPCS 1992; diflubenzuron: IPCS 1995), it should be noted that, as mentioned above, the concentrations detected in paddy waters are similar to those tested in this study. Therefore, our data on the additive/synergic effects of diflubenzuron and cypermethrin clearly indicate a real possibility of risk for non-target freshwater organisms. This fact is relevant when considering that, in water samples collected in our study area, tens of different pesticides have been identified to be present (IPSRA 2018). Moreover, the simple evaluation of each pesticide concentration doesn't consider the fact that at least some of their degradation products could maintain a certain level of toxicity. The study of ecotoxicity of the pesticide's degradation products is only at an initial stage and may represent an important aspect of the future research development in this field (Fenner et al. 2013).

Conclusions

Our study shows that insecticides utilized in rice-fields to control mosquitoes have negative effects on non-target species. Insecticides dispersed in the freshwater environment are relatively mobile and wash into water bodies near agricultural areas. Even if major damages are likely to occur using the more persistent molecules (Diana et al. 2000), this study shows that even pulse applications of short-living chemicals can negatively affect freshwater species. This negative effect is of particular importance for non-targeted species, such as the Odonates, an invertebrate group that is of particular conservation concern and includes several species listed as endangered in European directives (Boudot and Kalkman 2015). As the effects of compounds with a similar mode of action were found to be comparable (Van Wijngaarden et al. 2005), this study can help ameliorate the use of chemicals in an agricultural environment, and contribute to enhancing the protection of non-target invertebrates.

Acknowledgements We thank Emiliano Tambornini and Daria Prini for assistance in damselfly rearing. The research is original and was supported by an Università del Piemonte Orientale "Ricerca locale 2016" Grant.

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