Laboratory studies on the effects of a neonicotinoid-containing seed treatment product on non-target soil animals

Anikó SERES[1] – Krisztina HRÁCS[1] – Gergő GYURCSÓ[1,2] – Miklós SÁROSPATAKI[1] – Judit SZAKÁLAS[1] – Péter I. NAGY[1]

1: Department of Zoology and Animal Ecology, Szent István University, H-2103 Gödöllő, Páter Károly u. 1., Hungary E-mail: seres.aniko@mkk.szie.hu

2: Agro-Environmental Research Institute, National Agricultural Research and Innovation Centre, H-1022 Budapest, Herman Ottó u. 15., Hungary

Abstract: Cruiser 350 FS (Syngenta) is a widely applied thiamethoxam-containing seed treatment product. Despite of this fact, little is known about its side effects on non-target organisms other than bees. In this study, the effects of Cruiser 350 FS mixed in soil in different concentrations (according to OECD standards) were investigated on the mortality and reproduction of a Collembola species (*Folsomia candida*). On the basis of springtail mortality data, an LC_{50} of 223.6 mg/kg and a NOEC of 24.5 mg/kg were determined for *Folsomia candida*. The following toxicological limits were obtained on the basis of the reproduction data from the springtail test: EC_{50}: 61.73 mg/kg, NOEC: 12.27 mg/kg. Moreover, mortality tests were performed in microplates on two nematode species of different feeding and reproductive strategies. After 24 hours of exposure, treatment had no effect on mortality up to a concentration of 35 g of thiamethoxam/l in the r-strategist bacterivorous *Panagrellus redivivus*; while the species-specific LC_{50} was determined to be 0.19 g/l for *Xiphinema vuittenezi*. Our results proved the K-strategist plant-feeding *X. vuittenezi* to be more sensitive than the r-strategist bacterivore *P. redivivus*. Our results highlight the difference in the sensitivity of nematodes of different feeding and functional groups, suggesting the importance of a more sophisticated study approach.

Keywords: side effect, thiamethoxam, Collembola, Nematoda

Introduction

The main environmental problem related to plant protection products is that they may pose a risk to non-target organisms, thereby decrease the diversity of species and functions.

The use of insecticide neurotoxins that are similar to plant-based nicotine and belong to the class of neonicotinoids has become widespread in the past two and a half decades. The most problematic issue is their sublethal effect on non-target organisms (Blacquière et al. 2012). These agents have received attention because of their effect on bees (Apidae), the most important pollinators. An association was found between the use of neonicotinoids and Colony Collapse Disorder (CCD), a syndrome that occurs in Western honeybees (*Apis mellifera* L.) (Pisa et al. 2015), and probably at bumblebees (*Bombus* spp.), which is the second most studied group (Laycock et al. 2012). Neonicotinoids are effective against insect pests both in the case of direct contact and ingestion. Their mode of action is blocking acetylcholine receptors on the post-synaptic side, thus disrupting stimulation, leading to the death of the insect (Honda et al. 2006). These agents can interfere with the very precise coordination between the neural and the hormonal systems of an insect, disrupting the series of behavioural and physiological events that lead to egg laying (Desneux et al. 2007). Neonicotinoids are effective at very low concentrations, provided that the duration of exposure is sufficiently long (Tennekes 2010). Findings of Whitehorn et al. (2012) show that imidacloprid reduced bumblebee colony growth and the rate of egg laying by queens under laboratory conditions. For this reason, after a moratorium period of two years in 2013-2015, the EU introduced a ban on the use of neonicotinoid pesticides. Despite of this fact, the side effects of these compounds in terrestrial systems are still rather poorly known.

Thiamethoxam-containing seed treatment products reach the soil, these may affect soil biota that live there and play a central role in soil decomposition and nutrient turnover

processes. Despite this, hardly any literature data are available concerning the effects of these agents on soil fauna (Bonmatin et al. 2015). Springtails are important regulators of decomposition processes in soil through the selective consumption of soil microbes (Fountain and Hopkins 2005; Seres et al. 2007; 2009). El-Naggar et Zidan (2013) studied the effects of thiamethoxam on soil fauna under field conditions. Soil samples were obtained from two depths following seed treatment with a 70% Cruiser WG (2 g/kg seeds) and spraying with 25% Actara WG (20 g/100 l). The number of individuals belonging to the following soil animal groups was determined weekly for five weeks: Collembola, Psocoptera, Oribatida, Actinedida, Gamasida. When thiamethoxam-containing plant protection products were used, a clear increase was noted in the number of individuals of soil animals studied, mainly that of springtails. This phenomenon was probably due to death of predatory mites. For springtails, one single study was found to investigate the effects of Cruiser 350 FS, a thiamethoxam-containing insecticide under laboratory conditions (Alves et al. 2014). In acute test, it was found that the product was lethal to the springtail *Folsomia candida* only at the highest concentration tested (1,000 mg/kg). In the chronic test, thiamethoxam had no effect on reproduction of springtails at the concentrations tested (0.06 mg/kg, 0.12 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 1.0 mg/kg). The following toxicological limits were derived from the study: a NOEC of 500 mg/kg in the acute test and a NOEC of 1 mg/kg thiamethoxam in the chronic test for reproduction.

Nematodes are a part of soil microfauna and are important participants in soil processes due to their high numbers, diverse feeding types and life history strategies. As for the effects of neonicotinoid insecticides to free-living nematodes, scientific literature is restricted to the sensitivity of entomopathogenic nematodes (EPN) to these compounds (Koppenhöfer et al. 2003; 2015).

Usually, bacterivorous r-strategist nematodes that are easy to breed in the laboratory are used in toxicological studies. The species most commonly used as test animals are *Caenorhabditis elegans* (Maupas, 1900) and *Panagrellus redivivus* (Linné, 1767). However, several results show that there may be huge differences in the sensitivity to xenobiotics of nematodes with different life history and feeding strategies (Bongers and Bongers 1998; Nagy 2009). Therefore, apart from performing tests on *Panagrellus redivivus*, we aimed to involve a further test species of considerably different feeding type and life strategy as compared to the usually applied bacterial feeder test species.

Springtails and free-living nematodes applied in ecotoxicology were used to explore the effects of the seed treatment product on soil animals. The questions to be answered during the research were:

(i) At what concentrations and at what rate does the tested insecticide cause mortality in the springtail species (*Folsomia candida*)?

(ii) Does thiamethoxam have an effect on the reproduction of the tested springtail species?

(iii) What is the mortality rate caused by the insecticide in two nematode species with different feeding type and life strategy (*Panagrellus redivivus*, *Xiphinema vuittenezi*) in the acute mortality test at different concentrations?

Materials and methods

Collembolan reproduction test

The test was performed on the basis of OECD 232 protocol (OECD, 2009). The parthenogenetic species, *Folsomia candida* (Willem 1902) was used as test animal. Synchronised cultures were established first because same aged individuals (9–12 days) are required for the test. Test agent was Cruiser 350FS, a thiamethoxam-containing seed treatment product. Seven concentrations were set in the test (3.5 g/l, 1.75 g/l, 0.875 g/l, 0.437 g/l, 0.219 g/l, 0.109 g/l, and 0.055 g/l) on the basis of results from a previous range finding test (unpublished data). Four replicates were applied for each concentration and 8 in the negative control without pesticide. These

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concentrations were equal to 786 mg/kg, 393 mg/kg, 196 mg/kg, 98 mg/kg, 49 mg/kg, 24 mg/kg, and 12 mg/kg, respectively, as calculated to dry soil weight. Water holding capacity of soil was determined before the test and soils were moistened to 60% thereof. Standard soil was mixed at proportions according to the protocol as follows: 5% sphagnum peat, 20% kaolin clay, 74% sand and 1% calcium carbonate. An amount of 24.5 g of OECD soil was placed into plastic test vessels. They were moistened with 5.5 ml of distilled water and a water solution of the insecticide at a given concentration. Ten randomly selected 10-12 days old specimens of *Folsomia candida* were introduced into each of the test vessels with an aspirator. The animals were provided baker's yeast at the start of the test and once weekly thereafter. Duration of the test was 28 days, during which test vessels were kept in an incubator (TS 606CZ/4-Var) at 20 ± 1°C. Test validity criteria in the control groups were: adult mortality should not exceed 20%, the number of juveniles should reach 100 and the coefficient of variation calculated for the number of offspring should not exceed 30%. At the end of the test, the number of adults and juveniles was counted. Animals were washed from soil in the vessels and were counted after transferring them into a Petri dish and adding blue ink for better visibility. The endpoints of the test were mortality, i.e. the number of dead individuals, and reproduction, i.e. the number of offspring.

Acute mortality test on nematodes

The test was performed involving two free-living soil nematode species. One of these is the r-strategist bacterivorous *Panagrellus redivivus*. The other species is the K-strategist plant-feeding nematode, *Xiphinema vuittenezi* (Luc, Lima, Weischer and Flegg, 1964). The test is not standardised but *P. redivivus* has been commonly used since decades as its testing is simple, quick and does not require special equipment (Samoiloff 1987). Endpoint of the tests was mortality, with an exposure time of 24 hours. We also considered the nematode tests valid if mortality in the control treatment did not exceed 20%. Thiamethoxam concentrations used in the first two range-finding tests for both species were as follows: 350 g/l, 35 g/l, 3.5 g/l, 0.35 g/l and 0.035 g/l. Eight replicates were applied for each concentration and 16 in negative control without pesticide. Tests were performed in disposable microtiter plates with 8x12 wells (Bioster). An amount of 370 µl test solution, or in the control distilled water were pipetted into each well. Subsequently, five *P. redivivus* and three *X. vuittenezi* specimens were introduced with a micropipette to the holes of the microtiter plates. Adult female individuals were randomly selected from synchronised cultures in the case of *P. redivivus*. *X. vuittenezi* females were extracted from samples collected from grapevine soil in a garden in Isaszeg, Hungary (GPS coordinates: 47.52945, 19.39419). It is difficult to maintain cultures of this species under laboratory conditions. For soil extractions we used a modified version of Cobb's decanting and sieving method (Brown and Boag 1988), which is based on the positive hydrotaxic response of the animals. Nematodes were stored in a refrigerator at 4°C until the initiation of testing. The third test was set on the basis of the previous ones. Based on the results of the range finding tests, the definitive test was carried out only in *X. vuittenezi* in 6 replicates. Apart from concentrations and numbers of replicates, the definitive test was identical to the *Xiphinema* range finding test. The used concentrations were as follows: 3.5 g/l, 1.75 g/l, 0.875 g/l, 0.437 g/l, 0.219 g/l, 0.109 g/l, and 0.055 g/l of thiamethoxam. Subsequently, plates were placed into a temperature-controlled incubator (TS606-CZ/4-WAR) until the end of the test at 20 ± 1°C. Test plates were checked under a microscope (Olympus SZH 10). Immobility and no response to physical stimulations are signs of mortality in nematodes. Moreover, in case of *X. vuittenezi*, open C-shaped body posture is a typical form of dead animals. In contrary *P. redivivus* individuals become straight after death, similarly to other small bacterivorous species.

Statistical analysis

The results were analysed using R software

package (R Core Team 2013). After confirming that the conditions of the utility, one-way ANOVA and a post-hoc Dunnett's test were used. ToxRat statistical software was used in order to determine NOEC, LOEC, LC_{50} an EC_{50} levels.

Results

Collembolan reproduction test

The test satisfied validity criteria as follows: mean mortality of adults in the control group was 1.25%; mean number of offspring in the control group was 376±65.38 individuals, variation coefficient: 17.39. Based on ANOVA results, treatment had a strongly significant effect on both mortality ($F=77.39$, $p<0.001$) and reproduction (37.69, $p<0.001$). Dunnett's test revealed a significant difference in the mortality of springtails as compared with control at 393 mg/kg (0.875 g/l) concentration and at higher concentrations (Figure 1). On the basis of mortality data, LC_{50} was defined as 223.6 mg/kg (0.996 g/l) while mortality NOEC level was found to be 24.5 mg/kg (0.109 g/l). Reproduction differed from the control group at 49.1 mg/kg concentration level and at higher concentrations (Figure 2). The following toxicological limits were determined on the basis of reproduction data from springtail test: EC_{50}: 61.73 mg/kg, (0.275 g/l), NOEC: 12.27 mg/kg (0.055 g/l).

Acute mortality test in nematodes

In the case of the bacterivorous species, *P. redivivus,* the highest concentration resulted in 100% mortality, while lower concentrations lead to significant differences in two cases: at 0.35 g/l and at 3.5 g/l (Table 1). However, mortality did not differ from the control group when the 35 g/l concentration was used. In test with *Xiphinema* species, the two highest concentrations resulted in 100% mortality and high mortality was observed at the next two concentration levels as well (3.5 and 0.35 g/l) (Table 1). A significant difference was found in each case compared to the control, except

Figure 1. Mean mortality of adult *Folsomia candida* individuals (± SD) on a logarithmic scale. Different letters indicate significantly different results ($p<0.001$) according to Dunett's test results.

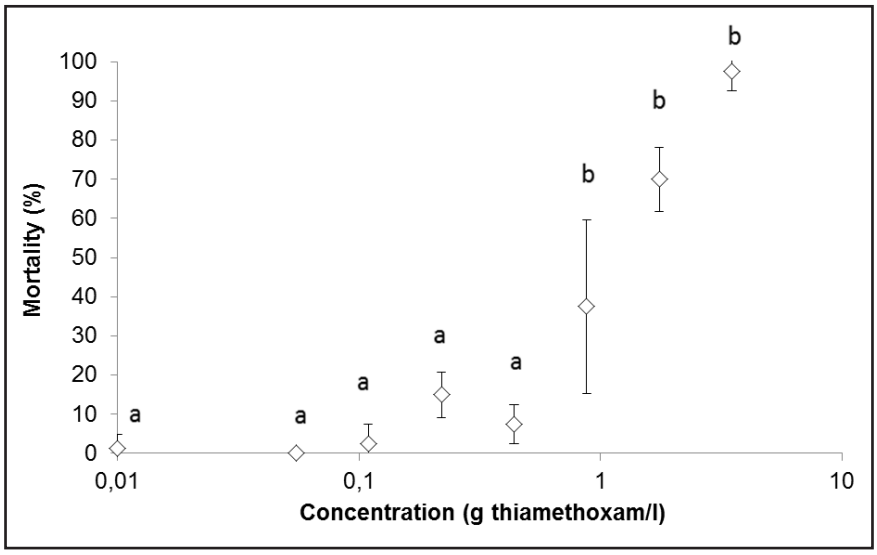

Table 1. Mortality of *Panagrelus redivivus* and *Xiphinema vuittenezi* individuals (%) in range-finding test (mean of 8 replicates ± SD).

	Concentration (g/l)					
	Control	0.035	0.35	3.5	35	350
P. redivivus	0 ± 0	2.5 ± 6.2	17.5 ± 19.9	22.5 ± 14.7	0 ± 0	100 ± 0
X. vuittenezi	12.5 ±15.2	4.2 ± 10.4	83.3 ± 22.2	91.7 ± 20.8	100 ± 0	100 ± 0

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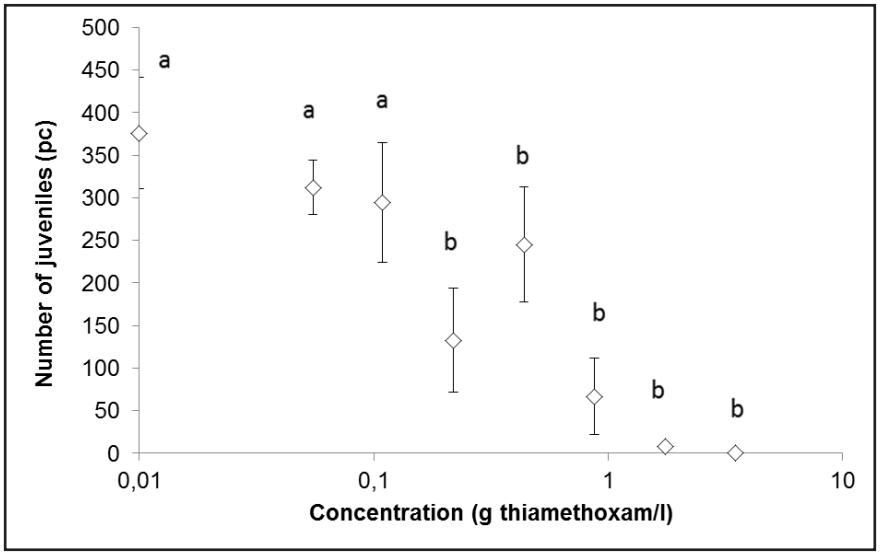
Figure 2. Mean number of *Folsomia candida* offsprings during each treatment (± SD) on a logarithmic scale. Different letters indicate significantly different results ($p<0.001$) according to Dunett's test results.

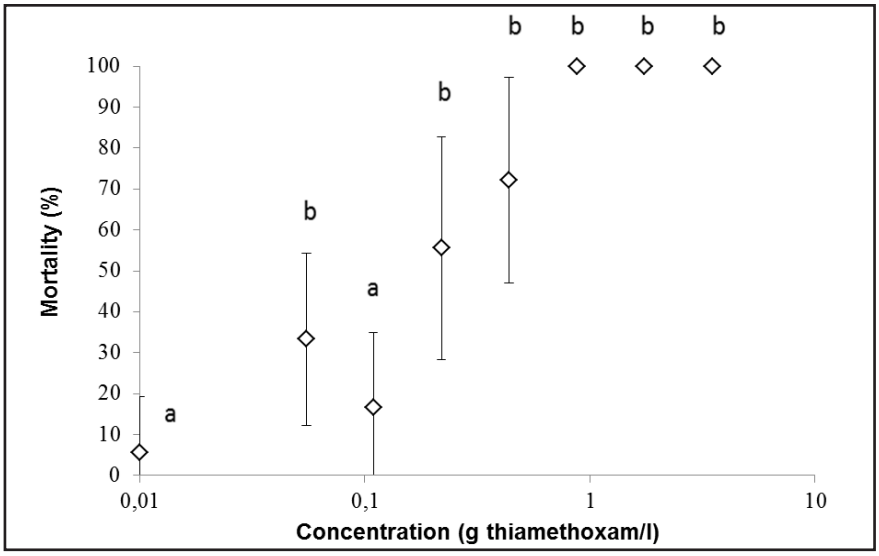


the lowest concentration. The second test with *X. vuittenezi* yielded more specific results regarding sensitivity of the species (Figure 3). A mortality of 100% was experienced at the three highest concentration levels. Dunnett's test showed a significant difference ($F=2.81$, $p<0.05$) in comparison with control group already at the lowest concentration (0.055 g/l). The next concentration level (0.109 g/l) did not differ statistically from the control group, but higher concentrations resulted a strong significant difference in each case ($p<0.001$). The species-specific LC_{50} was determined to be 0.19 g/l.

Discussion

The results from the OECD reproduction test with springtails show that the applied pesticide caused a complete mortality only at a very high concentration (786 mg/kg), which is not realistic under field conditions of normal agricultural practices (Bonmatin et al. 2015). Mortality rate differed significantly from that observed in the control group at concentrations of 196 mg/kg and above where a mortality of 37.5% was experienced. A significant effect on reproduction was found at a lower concentration, i.e. 49.1 mg/kg. When the above toxicity indices

Figure 3. Mean mortality of adult *Xiphinema vuittenezi* females (± SD) on a logarithmic scale. Different letters indicate significantly different results ($p<0.001$) according to Dunett's test results.



are compared with the results by Alves et al. (2014), it is to be noted that lower mortality NOEC and LC_{50} values were obtained, thus springtails were found to be more sensitive to thiamethoxam in the present experiment. This may be due to the fact that Alves et al. (2014) examined mortality during a two-week subacute test, while in our study a four-week chronic test was used. Regarding inhibition of reproduction, Alves et al. (2014) determined a NOEC of 1 mg/kg. In the present test a higher NOEC of 12.27 mg/kg was calculated. The difference in results is explained by the test concentrations chosen. These concentrations are well above that are used and potentially present in the soil under realistic circumstances. PEC of the thiamethoxam-containing Cruiser F350 is 0.201 mg/kg based on literature data (Alves et al. 2013). Field concentrations used in practice most probably do not have a negative side effect on springtails, but discharge of this agent into the soil in high concentrations (for example, in the case of an accident or inadequate application) poses a risk to this group of soil animals. Our results explain the phenomenon seen in studies by El-Naggar et al. (2013). The number of springtails increased at the expense of other soil-dwelling microarthropods because these animals are not sensitive to thiamethoxam and their number increased after the death of their predators.

The tests performed with the two free-living nematode species showed that, following 24 hours of exposure, treatment had no mortality effect up to a concentration of 35 g of thiamethoxam/l in the r-strategist bacterivorous *Panagrellus redivivus*; however, a 100% mortality rate was observed at a concentration of 350 g/l. Hardly any studies that specifically examine the side effects of neonicotinoids on free-living nematodes were found in literature. Only the effectiveness of entomopathogenic nematode (EPN) species has been tested, with the focus on their ability to control insect pest as influenced by the insecticide treatment. According to Kopenhöffer et al. (2015), neonicotinoid treatments usually showed a synergistic interaction when applied in combination with several EPN species (e.g. *Steinernema glaseri*, *Heterorhabditis bacteriophora*). The combined effects of thiamethoxam and entomopathogenic nematodes of the order Rhabditida against pest insects were investigated both under field and laboratory conditions. During the tests performed by Koppenhöfer et al. (2003), thiamethoxam did not have a negative effect on the reproduction of entomopathogenic nematodes. This supports the results of our study concerning *P. redivivus*, which also belongs to the order Rhabditida, i.e. nematodes of this group were not sensitive to thiamethoxam. As for the mortality of *Xiphinema vuittenezi*, the difference versus the control group was significant even at the lowest concentration after the exposure period of 24 hours. Mortality rate was 100% when the test concentration of 0.875 g/l was used. According to our results obtained so far, the sensitivity of the K-strategist plant-feeding *X. vuittenezi* considerably exceeds that of *P. redivivus*. This finding makes it worthwhile to carry out further tests on the sensitivity of different plant-feeding nematodes for the environmentally realistic concentration levels of neonicotinoid compounds. Furthermore, our results highlight the difference in the sensitivity of nematodes belonging to different feeding and life history groups. This underlines the importance of a more sophisticated study approach than the generally applied use of few r-strategist bacterial feeder species only.

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