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Ecotoxicity of pesticide formulations and their mixtures: the case of potato crops in Costa Rica

Michael Méndez-Rivera[1] · Didier Ramírez-Morales[1] · José R. Montiel-Mora[1] · Carlos E. Rodríguez-Rodríguez[1]

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Abstract

Despite their environmental implications, ecotoxicological information regarding pesticide mixtures is relatively scarce. This study aimed to determine the ecotoxicity of individual pesticide formulations and their mixtures (insecticides and fungicides), which are applied during the production cycle of potato, according to agricultural practices from a Latin American region in Costa Rica. Two benchmark organisms were employed: *Daphnia magna* and *Lactuca sativa*. First, the evaluation of individual formulations (chlorothalonil, propineb, deltamethrin+imidacloprid, ziram, thiocyclam and chlorpyrifos) revealed differences between available EC_{50} for active ingredients (a.i.) and their respective formulations toward *D. magna*; on the contrary, no information could be retrieved from scientific literature for comparison in the case of *L. sativa*. In general, acute toxicity was higher toward *D. magna* than *L. sativa*. Moreover, interactions could not be determined on *L. sativa*, as the chlorothalonil formulation was not toxic at high levels and the concentration-response to propineb could not be fitted to obtain an IC_{50} value. The commercial formulation composed of deltamethrin+imidacloprid followed the concentration addition model (when compared with parameters retrieved from individual a.i.) and the other three mixtures evaluated (I: chlorothalonil-propineb-deltamethrin+imidacloprid; II: chlorothalonil-propineb-ziram-thiocyclam; III: chlorothalonil-propineb-chlorpyrifos) produced an antagonistic effect on *D. magna*, thus suggesting less acute toxicity than their individual components. Subsequent chronic studies showed that one of the most toxic mixtures (II) negatively affected *D. magna* reproduction at sublethal concentrations indicating that this mixture poses a risk to this species if these pesticides co-exist in freshwater systems. These findings provide useful data to better estimate the impact of real agricultural practices related to the use of agrochemicals.

Keywords Fungicide · Insecticide · Toxicity · Potato · Pesticide mixture · Chronic toxicity

Introduction

Pesticides are chemical substances used worldwide, intentionally created to fight crop pests and diseases. An estimated 2 million tons of pesticides were utilized in fields across the world in 2019, led by herbicides, insecticides and then fungicides (Sharma et al. 2019). In particular, Costa Rica used an estimated 11.5 kg/ha of active ingredient (a.i.) during 2019 (SFE 2020). According to the classification by the World Health Organization (WHO), the imports of pesticides in Costa Rica in the period 2000–2015 can be classified from moderately dangerous to extremely and highly dangerous (Bravo et al. 2015); moreover, 67,370 tons of a.i. with potential acute toxicity hazard were imported in this period. Crops of special attention in the country due to the large quantities of pesticides applied during their production include melon, coffee, chayote, banana, pineapple, rice, sugarcane (Bravo et al. 2013), and potato (Ramírez-Muñoz et al. 2014).

Potato (*Solanum tuberosum*) represents a product of high economic value and high demand at the national level. In the year 2015, Costa Rica had 3674 hectares planted with potato and produced 90,576 tons (SEPSA 2016). In addition, the potato agricultural sector accounts for larger areas compared to other vegetables such as carrots, broccoli, cauliflower and cilantro. For example, in the highlands in North Cartago, up

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✉ Carlos E. Rodríguez-Rodríguez
carlos.rodriguezrodriguez@ucr.ac.cr

[1] Centro de Investigación en Contaminación Ambiental (CICA), Universidad de Costa Rica, 2060 San José, Costa Rica

to three quarters of the producers (38% of land use) are dedicated to potato production (Fournier et al. 2010).

Potatoes are planted all year round, generally in two cycles comprising the dry and rainy seasons. Pesticides are applied during all planting stages (seed storage, seed preparation, maintenance and harvesting), and application programs include herbicides, insecticides, fungicides and their mixtures (Ramírez-Muñoz et al. 2014). It is estimated that some of these farms make use of 54.7 kg a.i./ha/cycle and up to 31 different a.i. (Fournier et al. 2010); moreover, in areas dedicated to seed production, the application of pesticides can reach values of up to 88.3 kg a.i./ha/cycle (Ramírez-Muñoz et al. 2014).

An intensive use of pesticides is usually related to environmental contamination issues; during the application of agrochemicals a carryover of pesticide residues takes place from the crops to the surrounding surface water bodies (Aguilar 2014). The high number of a.i., as well as the pesticide mixtures (fungicides, insecticides and herbicides) applied on potato crops (Ramírez-Muñoz et al. 2014), increase the risk of adverse effects on non-target organisms in the nearby ecosystems (Fournier et al. 2010). Even though the ecotoxicological parameters of individual pesticides are widely known, information regarding the effects of mixtures is largely unstudied; moreover, available ecotoxicological data refer mainly to a.i., while the data for formulations is scarce, although they represent a more realistic environmental scenario.

The presence of pesticide mixtures in surface water is a matter of global concern (Schreiner et al. 2016), as it is frequent to find combinations of these a.i. and their metabolites. Chiu et al. (2016) mention the need to evaluate the temporary effects of pesticide cocktails on stream ecosystems. The environmental exposure to these mixtures can result in adverse effects to human and general biota at different organization levels, depending on the compounds present, the dose and the extent of acute or chronic exposure (Rizzati et al. 2016). Taking into account the extensive use of pesticides in potato production and their unknown environmental fate, the aim of this work was to determine the ecotoxicity of selected pesticide formulations, individually and in mixtures, applied in potato crops in Costa Rica toward two benchmark organisms (the microcrustacean *Daphnia magna* and lettuce, *Lactuca sativa*), in order to draw attention to the potential risk they pose to nearby ecosystems. *D. magna* was selected as a widely employed bioindicator of pollution in aquatic compartments, as pesticide application practices and the management of their residues at field level may easily result in the contamination of water streams; *L. sativa*, a terrestrial plant, was chosen given that potentially polluted water streams may be used for irrigation purposes (including crop fields). Three mixtures composed of representative formulations were considered according to the pesticide application cycle locally used in a region of Costa Rica. This study provides novel information regarding the potential effects and risk on environmental communities, derived from local and realistic agricultural practices related to pesticide application. Furthermore, the use of formulations instead of a.i. alone reflects a more accurate panorama of the environmental risk. This work suggests a need to perform similar studies for extensive crops in Latin American farmlands.

Materials and methods

Pesticide commercial formulations

Commercial formulations of chlorothalonil (Bravonil 72 SC®, 72% w/v), propineb (Antracol 70 WP®, 70% w/w), ziram (Zetaran 76 WG®, 76% w/w), chlorpyrifos (Lorsban 48 EC®, 48% w/v), deltamethrin+imidacloprid (Muralla Delta 19 OD®, 15 and 4% w/v, respectively) and thiocyclam (Evisect 50 SP®, 50% w/w) were purchased from a local distributor. Working solutions were prepared by diluting commercial formulations with deionized water at most 1 day before the setup of ecotoxicological bioassays (1/100 dilution at the most). Later dilutions were prepared with moderately hard reconstituted water.

Evaluation of pesticide mixtures

Mixture ratios and selection of the compounds

The selection of pesticide formulation mixtures was done according to the description of the pesticide application cycle in potato crops determined by Aguilar (2014) in Tierra Blanca, northern region of Cartago province, Costa Rica. Mixtures composed of at least three different pesticide formulations were selected according to the following criteria: (a) pesticides with higher application frequency or simultaneously applied; and (b) pesticides with higher individual toxicity toward *D. magna*. Therefore, the research was carried out with three mixtures, Mixture I (33.4% chlorothalonil [Bravonil]) + 64.9% propineb [Antracol] + 1.4% deltamethrin [Muralla Delta] + 0.4% imidacloprid [Muralla Delta]), Mixture II (18.7% chlorothalonil [Bravonil] + 36.5% propineb [Antracol] + 39.6% ziram [Zetaran] + 5.2% thiocyclam [Evisect]), Mixture III (30.5% chlorothalonil [Bravonil] + 59.3% propineb [Antracol] + 10.2% chlorpyrifos [Lorsban]) (Table 1). According to the application cycle for potato production, mixture I is the first one applied; followed by mixture II (1 month later), and finally mixture III (3 weeks later) (Aguilar 2014). A total of seven a.i. were contained within these formulations: chlorothalonil, propineb, ziram, chlorpyrifos, deltamethrin, imidacloprid and thiocyclam. The application rate in potato crops used by farmers (Aguilar 2014) was used to determine the a.i.

Table 1 Description of the active ingredients tested in the present study: commercial formulation, mode of action, aqueous hydrolysis degradation time (DT_{50}) and working nominal concentrations (mg/L) in each mixture, following the application practices of farmers in potato production in Cartago, Costa Rica

Active ingredient	Type of pesticide	Commercial formulation	Mixture I (mg/L)	Mixture II (mg/L)	Mixture III (mg/L)	Mode of action	Aqueous hydrolysis DT_{50} (days)[b]
Chlorothalonil	Fungicide	Bravonil 72 SC®	2264	2264	2264	Multi-site contact activity. Avoids ATP production by inactivation of cell sulfhydryl enzymes (van Scoy and Tjeerdema 2014)	29.6
Chlorpyrifos	Insecticide	Lorsban 48 EC®	0	0	755	Acetylcholinesterase inhibitor (Lewis et al. 2016)	53.5
Deltamethrin	Insecticide	Muralla Delta 19 OD®[a]	94	0	0	Nerve impulse transmission inhibitor. Sodium channel modulator (Lewis et al. 2016)	Stable
Imidacloprid	Insecticide	Muralla Delta 19 OD®[a]	25	0	0	Acetylcholine nicotinic receptor (nAChR) agonist (Lewis et al. 2016)	Stable
Propineb	Fungicide	Antracol 70 WP®	4403	4403	4403	Multi-site contact activity and possibly impairment of central neurotransmitter pathways (Marinovich et al. 2002; FRAC 2021)	1.5
Thiocyclam	Insecticide	Evisect 50 SP®	0	629	0	A nereistoxin analogue insecticide. Acetylcholine nicotinic receptor (nAChR) agonist (Lewis et al. 2016)	5
Ziram	Fungicide	Zetaran 76 WG®	0	4780	0	Multi-site contact activity; neurological impairments, inhibition of brain cholinesterase and brain neurotoxic esterase (EPA 2002; FRAC 2021)	0.7

[a]Deltamethrin and imidacloprid are a.i. of the same formulation Muralla Delta 19 OD (4% of deltamethrin and 15% of imidacloprid)

[b]Values from the Pesticide Properties DataBase (PPDB, Lewis et al. 2016)

concentrations in the mixtures (ratios) to be used in the ecotoxicological tests (Table 1).

Model for mixture toxicity assessment

The expected joint toxicity of the three mixtures based on the *D. magna* acute toxicity test, was determined by the Concentration Addition model (CA) and the Independent Action model (IA). The CA model considers the relative toxicities of the a.i.; Eq. (1) was employed to predict the expected toxicity values of the mixtures, according to the CA model (Faust et al. 2000):

$$ECx_{mix} = \left(\sum_{i-1}^{n} \frac{Pi}{ECxi}\right)^{-1} \quad (1)$$

where ECx_{mix} is the effect concentration of the mixture, Pi indicates the proportion of component i in the mixture, and $ECxi$ indicates the concentration of component i acting by itself that would cause x effect. Additionally, the model deviation ratio (MDR) (Belden et al. 2007) was obtained to evaluate the difference between the expected and observed toxicity. The MDR is calculated by dividing the expected EC_{50} by the observed EC_{50}. If the result is $0.5 \leq MDR \leq 2$ the mixture follows the CA model; a result > 2 indicates that the mixture interaction corresponds to synergism and, if the result is <0.5, the interaction is considered as antagonism (Cedergreen 2014).

The IA model is based on the combination of probabilities of suffering the adverse effect measured as endpoint, following the Eq. (2) (Escher et al. 2020) expressed as a percentage:

$$Effect\ of\ mixture = \left[1 - \prod_{i=1}^{n} (1 - effect(C_i)\right] x100 \quad (2)$$

where the percentage of effect of the mixture is calculated from the effect of each individual component (effect (Ci)). For each mixture, the observed individual concentrations were estimated from the observed EC_{50} values of the mixture; then the effect of each observed individual concentration was obtained from the dose-response curve for individual pesticides. The individual effects were used to estimate the expected effect of the mixture; synergy is present if expected effect is lower than 50% and antagonism if expected effect is higher than 50%.

Ecotoxicological assays

Daphnia magna acute immobilization test

Acute and chronic tests were carried out in the Laboratory of Ecotoxicology (CICA-Universidad de Costa Rica).

Table 2 Ecotoxicity of pesticide commercial formulations and their mixtures, as determined with an acute test in *D. magna* and seed germination tests in *L. sativa*

a.i. from tested commercial formulations and pesticide mixtures	*D. magna* test		*L. sativa* test
	EC_{50} 48 h (mg/L a.i) (C.I.)[a]	EC_{50} (mg/L) for a.i. standard[b]	IC_{50} 144 h (mg/L a.i.) (C.I.)[a]
Chlorothalonil	0.070 (0.0044–0.14)	0.054	No response
Chlorpyrifos	0.0012 (0.00082–0.0015)	0.0001	125 (91–161)
Deltamethrin- imidacloprid	0.0028 (0.00060–0.0051	Deltamethrin = 0.00056; Imidacloprid = 85[c]	262 (228–301)
Propineb	2.1 (1.4–2.8)	1.5	Not determined
Thiocyclam	0.61 (0.44–0.77)	2.01	2.2 (2–2.6)
Ziram	0.020 (0.017–0.023)	0.172	67.5 (40.2–120.2)
Mixture I	0.29 (0.21–0.36) (dilution to 0.0042%)	Not reported	129.8 (124–136.2) (dilution to 2.75%)
Mixture II	0.13 (0.087–0.181)(dilution to 0.0011%)	Not reported	119.5 (109.7–130.8) (dilution to 1.86%)
Mixture III	0.15 (0.13–0.17) (dilution to 0.002%)	Not reported	4346 (3369–5206) (dilution to 47.7%)

[a]C.I., 95% confidence intervals, are shown in parenthesis

[b]Values from the Pesticide Properties DataBase (PPDB, Lewis et al. 2016)

[c]Values for individual a.i. contained in the formulation are shown

D. magna Straus culture is maintained in moderately hard reconstituted water (hardness of 160–180 mg/L as $CaCO_3$) according to EPA (2002), plus B12 vitamin complex. Culture is kept at 21 ± 1 °C with a photoperiod of 12 h (light):12 h (darkness) and fed with the microalgae *Raphidocelis subcapitata*.

The acute toxicity test was performed according to EPA (2002); triplicate glass vials containing 10 neonates (<24 h) were exposed to 20 mL of proper dilutions of each commercial formulation individually and in mixtures and incubated in darkness at 21 °C. The proper dilutions and the control were prepared with moderately hard reconstituted water without B12 vitamin complex; starting concentrations are indicated in Table 1. After a 48 h exposure period, the immobilization of neonates was determined and assumed as equivalent to mortality. The concentration that produced immobilization in 50% of the exposed daphnids (EC_{50}) was calculated; the concentration-response data were fitted with a three parameters log-logistic model using the software R, version 4.1.1 (R Core Team 2021) and the package "drc" (Ritz et al. 2015). The acute toxicity interpretation (acute 48 h EC_{50}) for aquatic invertebrates from the PPDB database (Lewis et al. 2016) was employed to classify our results: $EC_{50} > 100$ mg/L = low acute toxicity; EC_{50} between 0.1 and 100 mg/L = moderate acute toxicity; and $EC_{50} < 0.1$ mg/L = high toxicity.

D. magna chronic reproduction test

The semi-static 21-d chronic reproduction bioassay in *D. magna* was adapted from the OECD test No. 211 (OECD 2012), according to Ruíz-Hidalgo et al. (2016). The mixture II (see Table 1) was selected since it exhibited a high toxicity in acute tests (Table 2); therefore, sublethal concentrations of 0.0112, 0.0056, 0.0028, 0.0014 and 0.0007 mg/L from the mixture were prepared as the test's treatments, based on results achieved in the previous acute test. Ten female neonates (<24 h) were individually exposed by triplicate to 30 mL of each dilution and the control (moderately hard reconstituted water with vitamin B12 complex); the conditions of 21 ± 1 °C and photoperiods of 12 h (light):12 h (darkness) were kept. The number of offspring (alive or dead) for each daphnid was daily recorded for 21 d as the test endpoint. Data were analyzed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test, both with the software R. A p value < 0.05 was considered to be statistically significant.

Seed germination test in *Lactuca sativa*

Phytotoxicity of each individual formulation and the three mixtures was determined by seed germination tests with lettuce (*L. sativa*, var. Georgia) (USEPA 1996). The relative seed germination (SG), relative root elongation (RE) and germination index (GI) were obtained using triplicates of 10 seeds exposed to 5 mL of proper dilutions of the individual pesticides or mixtures, by comparison to a control exposed to moderately hard reconstituted water. Tests were performed in petri dishes, incubated in darkness at 22 °C for 6 d; the parameters were calculated as described elsewhere (Huete-Soto et al. 2017). The concentration producing 50% of inhibition (IC_{50}) in the GI, was calculated with a log-logistic regression using the package “nplr” in R (Commo and Bot 2016).

Results and discussion

Acute toxicity in *D. magna* and effects on seed germination in *L. sativa* after exposure to single pesticide formulations

The 48 h EC_{50} values corresponding to the immobilization tests in *D. magna* exposed to the insecticide and fungicide formulations, as well as their mixtures are shown in Table 2 (raw data is shown in Supplementary material, Table S1). The four most toxic products (EC_{50} values based on the concentration of a.i.) corresponded to the formulations containing chlorpyrifos (Lorsban 48 EC®, $EC_{50} = 0.0012$ mg/L of a.i., dilution of 0.00025% from commercial formulation), deltamethrin+imidacloprid (Muralla Delta 19 OD®, $EC_{50} = 0.0028$ mg/L of a.i. [0.0004 mg/L of deltamethrin + 0.0014 mg/L of imidacloprid], dilution of 0.0015% from commercial formulation), ziram (Zetaran 76 WG®, $EC_{50} = 0.020$ mg/L of a.i., dilution of 0.0026% from commercial formulation) and chlorothalonil (Bravonil 72 SC®, $EC_{50} = 0.070$ mg/L of a.i., dilution of 0.0097% from commercial formulation); these four products are considered highly toxic to *D. magna* (Lewis et al. 2016). Then, thiocyclam (Evisect 50 SP®, $EC_{50} = 0.61$ mg/L of a.i., dilution of 0.12% from commercial formulation) and the least toxic formulation propineb (Antracol 70 WP®, $EC_{50} = 2.1$ mg/L of a.i., dilution of 0.33% from commercial formulation) are considered moderately toxic to this aquatic invertebrate (Lewis et al. 2016).

Acute toxicity parameters of individual a.i. from the Pesticide Properties DataBase (PPDB, Lewis et al. 2016) (Table 2) show slightly higher toxicity for chlorpyrifos ($EC_{50} = 0.0001$ mg/L) and lower toxicity for thiocyclam and ziram ($EC_{50} = 2.01$ mg/L and 0.172 mg/L, respectively), with respect to our results, while the reported values for chlorothalonil and propineb (0.054 mg/L, and 1.5 mg/L, respectively) fall within our estimated 95% confidence intervals (C.I.). Some authors highlight the increase in toxicity toward non-target organisms when exposed to commercial formulations (compared to the pure a.i.), basically by the effects of the inert compounds like surfactants, adjuvants and other additives (Joly et al. 2013; Pereira et al. 2009; Nagya et al. 2020), which might be the reason for the higher toxicity determined for thiocyclam and ziram formulations in *D. magna*, although information about their inert components is not available.

Muralla Delta® is a particular formulation as it combines two a.i. to improve the control of target organisms; when compared to the individual toxicities of deltamethrin and imidacloprid ($EC_{50} = 0.00056$ and $EC_{50} = 85$ mg/L, respectively [Lewis et al. 2016]), the CA model was able to predict joint effects for this binary mixture (Table 3), i.e., no apparent synergy/antagonism is observed in the toxicity for *D. magna* when combining these two a.i.. (Table 3). This result must be taken cautiously as the binary mixture is contained within the commercial formulation (which includes non-active co-formulants), while the ecotoxicological parameters for individual deltamethrin and imidacloprid were obtained from the pure a.i.. Among the scarce ecotoxicological data related to this commercial binary mixture, its exposure to the thrips *Corynothrips stenopterus*, *Franklinellia invasor* and *Selenothrips rubrocinctus* (Thysanoptera) resulted in $LD_{50} = 0.10$ mg/L, $LC_{50} = 5.4$ mg/L and $LD_{50} = 3.16$ mg/L, respectively (Walter et al. 2018; Walter et al. 2020). Comparing to our results, the Muralla Delta® formulation seems to be more toxic for daphnids than thrips. Individually tested, deltamethrin or imidacloprid have shown to be lethal to other arthropods like the mirid bug *Hyaliodes vitripennis* (Bostanian et al. 2001) and the honeybee *Apis mellifera* (Decourtye et al. 2004), and to the rotifer *Brachionus calyciflorus* (Gharaei et al. 2020).

Even though none of the formulations tested were herbicides, we hypothesized that phytotoxicity at some extent may be associated with the insecticide and fungicide formulations; for this reason, the germination test on the non-target organism *L. sativa* was included. According to our results, seedling emergence and root growth effects were observed (Fig. 1) and the highest toxicity was obtained with the formulation composed of thiocyclam ($IC_{50} = 2.2$ mg/L a.i.), followed by equally toxic ziram ($IC_{50} = 67.5$ mg/L a.i.) and chlorpyrifos ($IC_{50} = 125$ mg/L a.i.) and then the less toxic deltamethrin+imidacloprid ($IC_{50} = 262$ mg/L) (Table 2). On the contrary, no toxic effect was determined for chlorothalonil even at very high concentrations, while some (concentration independent) inhibition was observed for propineb, which is also the reason why the determination of IC_{50} values and synergistic/antagonistic interactions could not be performed. Information about *L. sativa* seedling emergence inhibition due to these a.i. is scarce; an IC_{90} (96 h) of 1.05 mg/L for chlorpyrifos (Iannacone et al. 2000) was the only comparable value found in scientific literature with respect to *L. sativa* germination. Nevertheless, effects such as the reduction in the whole cucumber and tomato growth (Ebel et al. 2000) and the decrease in the number of emerging sugar beet seedlings (Dewar et al. 1997) have been described for imidacloprid. Moreover, toxicological parameters to other non-target plants exposed to chlorothalonil ($EC_{50} > 4.2$ mg/L) and deltamethrin ($EC_{50} = 0.0004$ mg/L) are reported (Lewis et al. 2016). To the best of the authors' knowledge, this is the first report of phytotoxicity related to thiocyclam and ziram.

Acute and chronic toxicity in *D. magna* and effects on seed germination in *L. sativa* after exposure to mixtures of pesticide formulations

This study describes for the first time the ecotoxicological evaluation of realistic ternary (I and III) and quaternary (II) mixtures of pesticide formulations applied in potato crops, according to application practices from Costa Rica. Mixture II and III were similarly toxic to *D. magna* ($EC_{50} = 0.13$ mg/L and $EC_{50} = 0.15$ mg/L, respectively) and mixture I was slightly less toxic ($EC_{50} = 0.29$ mg/L) (Table 2; raw data is shown in Supplementary material, Table S2); the three mixtures are considered as moderately toxic to *D. magna* (Lewis et al. 2016). On the other hand, according to the CA model applied to the acute toxicity data and the IA model, the interaction between the formulations showed an antagonistic effect in all the pesticide mixtures evaluated (for CA model: MDR < 0.5; and for IA model: expected percentage of immobilization > observed percentage of immobilization i.e., 50%) (Table 3).

Despite the toxicity demonstrated on non-target species at low concentrations of the realistic mixtures, the overall antagonism means that the observed effects achieved when combining these pesticide formulations and applying them according to agricultural practices are lower from the ecotoxicological point of view, compared to the effect of individual formulations. Antagonistic effects have also been observed on *D. magna* during the exposure of the combination of imidacloprid and propineb (Escobar-Chávez et al. 2019) and on *Ceriodaphnia dubia* exposed to the ternary mixture of chlorothalonil, permethrin and atrazine (Phyu et al. 2011). On the contrary, synergistic effects have been reported on *Ctenopharyngodon idellus* cells (Li et al. 2021) and *A. mellifera* (Tomé et al. 2017) from the simultaneous exposure to chlorothalonil and imidacloprid, on soil microorganisms during co-exposure of chlorothalonil and chlorpyrifos (Xiaoqiang et al. 2008), and also on *Nilaparvata lugens* exposed to the binary mixture of chlorpyrifos and imidacloprid (Xu et al. 2020).

Phytotoxicity effects were observed on *L. sativa* after exposure to the three mixtures of fungicides and insecticides, which highlight the potential ecological impact that could be exerted on communities of non-target plants on the margins of crops and streams (Sobrero and Ronco 2004) when exposed to these pesticide mixtures. Contrary to the results obtained for *D. magna*, mixtures I and II presented similar toxicity toward *L. sativa* ($IC_{50} = 129.8$ mg/L and $IC_{50} = 119.5$ mg/L, respectively), while mixture III was considerably less toxic ($IC_{50} = 4346$ mg/L). The IC_{50} values obtained in the germination test were approximately three to four orders of magnitude higher than the EC_{50} determined for *D. magna* (Table 2). As an important result of this research, we demonstrated phytotoxicity of mixtures of

Table 3 Determination of combined effects for the binary, ternary and quaternary mixtures according to concentration addition model (CA) and independent action model (IA), as determined with an acute test on *D. magna*

Mixtures	Observed EC_{50} (mg/L)	Expected EC_{50} according to CA model (mg/L)	MDR	Combined effect[a]	Expected immobilization according to IA model (%)[b]
Deltamethrin – imidacloprid[c]	0.0028	0.0027	0.96	Concentration addition	NA
Mixture I: chlorothalonil - propineb - deltamethrin – imidacloprid	0.29	0.090	0.31	Antagonistic	92.1%
Mixture II: chlorothalonil - propineb - thiocyclam – ziram	0.13	0.045	0.34	Antagonistic	99.8%
Mixture III: chlorothalonil - propineb - chlorpyrifos	0.15	0.0070	0.047	Antagonistic	99.8%

[a] Mixture follows the CA model if $0.5 \le MDR \le 2$; synergism if MDR is >2; and antagonism if MDR < 0.5

[b] Parameter calculated using Eq. (2), as described in the section *Model for mixture toxicity assessment*

[c] This mixture corresponds to the commercial formulation Muralla Delta 19 OD®. No IA calculation is performed as it is not possible to obtain individual toxicity from the components of the commercial formulation

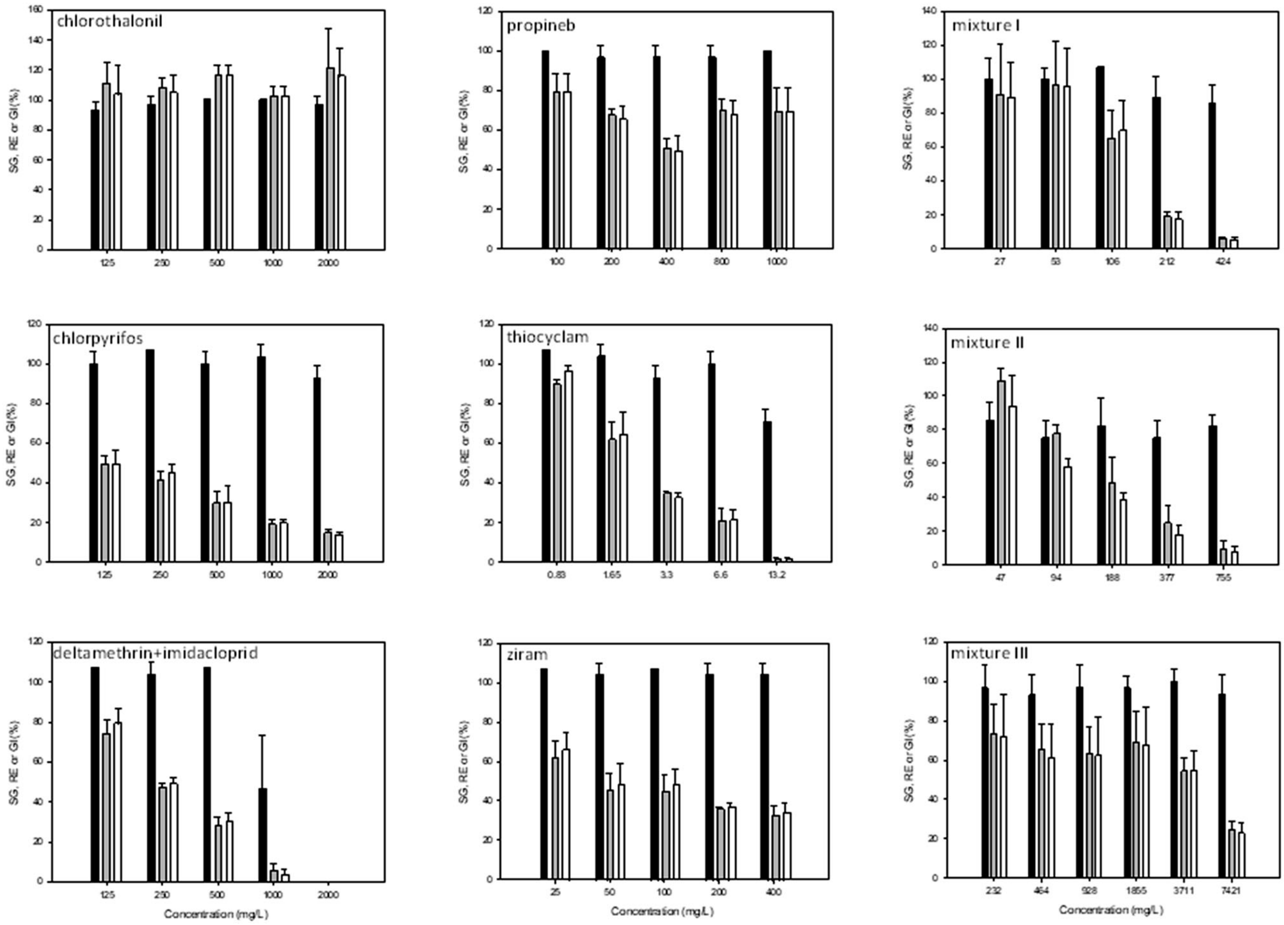

Fig. 1 Seed germination test with lettuce (*L. sativa*) as an indicator of phytotoxicity of pesticide mixtures and their individual a.i. Relative seed germination (SG, ■), relative root elongation (RE, ■) and germination index (GI, □). Bars = standard deviation

fungicides and insecticides (Fig. 1) on the seedling emergence and root growth of the bioindicator *L. sativa*, for which adverse effects are expected to occur mainly in the presence of herbicides (de Jong et al. 2008).

According to pesticide application cycles for potato production, mixture I is the first one applied; followed by mixture II (1 month later), and finally mixture III (3 weeks later) (Aguilar 2014). Considering the transport of pesticides from agricultural activities to the environment (soil, air, surface waters) (Loewy et al. 2011; Ramírez-Morales et al. 2021), this application schedule translates into a repeated release of these pesticide mixtures to the crops, eventually resulting in continuous exposure of surrounding ecosystems. Furthermore, due to their steep slope topography, some areas used for potato cultivation in Costa Rica are classified as areas of runoff risk; consequently, usual runoff events might favor the transport of pesticides from agricultural lands to streams (Fournier et al. 2010; Aguilar 2014), thus leading to deleterious effects on aquatic ecosystems and surroundings at very low concentrations of these pesticide mixtures, as demonstrated for *D. magna* and *L. sativa*.

Environmental monitoring in areas influenced by potato crops in the northern region of Cartago (from where the application cycle was retrieved) has revealed the presence of chlorothalonil (0.01–3.6 μg/L), chlorpyrifos (0.01–14 μg/L) and imidacloprid (0.12 μg/L) in surface waters, as well as in sediment samples (chlorpyrifos from 8 to 12 μg/kg) (Chin-Pampillo et al. 2012; Fournier et al. 2010; Ramírez-Morales et al. 2021; Pérez-Villanueva et al. 2021). The absence of environmental records on the area regarding other a.i. employed in this work is likely related to the lack of frequent on-site monitoring, since these compounds are also applied in nearby crops, such as beetroot, broccoli, cabbage, carrot, cauliflower, coriander and grasses (Fournier et al. 2010; Ramírez-Muñoz et al. 2014). Regarding the detected a.i., there is a real risk to non-target species, as environmental concentrations levels are close, or in the case of chlorpyrifos above, the EC_{50} values determined in this study. Monitoring from other regions of Costa Rica reported concentrations ranging from 0.05 to 2.06 μg/L for chlorothalonil (Diepens et al. 2014; Arias-Andrés et al. 2018; Rämö et al. 2018), and from 0.026 to 1.42 μg/L for chlorpyrifos (Echeverría-Sáenz et al. 2012; Arias-Andrés

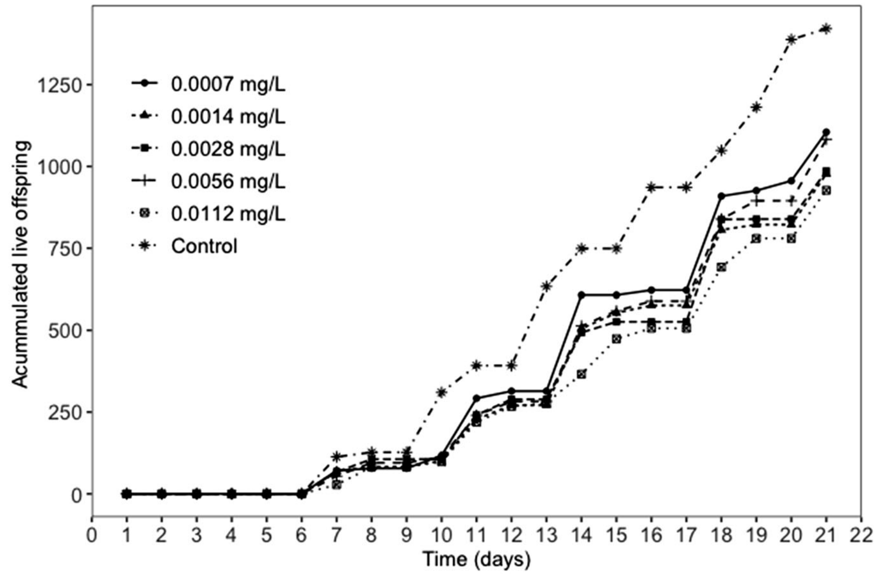

Fig. 2 Effect of different concentrations of Mixture II on the cumulative live offspring using *D. magna* as bioindicator, during an exposure time of 21 d

et al. 2018; Carazo-Rojas et al. 2018; Rämö et al. 2018) in water samples; similarly, levels ranging from 0.26 to 18.2 µg/kg for chlorpyrifos and 1.52 µg/kg for deltamethrin have been reported in sediment samples (Carazo-Rojas et al. 2018; Rodríguez-Rodríguez et al. 2021). No local information was retrieved for the other pesticides.

To demonstrate the adverse effects due to low concentrations of pesticide mixtures, a chronic test with *D. magna* was performed to evaluate the reproduction effects when the microcrustaceans were exposed to non-lethal concentrations of the mixture II (one of the most toxic in acute assays). After the 21 d exposure period, no mortality was observed in the control group and the mean number of offspring exceeded 60 daphnids (test quality control criterion). In the treatments as well, no mortality was present, and the accumulated live offspring per treatment during the test are shown in Fig. 2. Our results showed a highly significant effect on the reproduction of *D. magna* ($F = 8.24$, $\mathrm{Df} = 5$, $p = 0.00001$) due to the exposure to different concentrations of the pesticide mixture. The post-hoc Dunnett test indicated that the quantity of offspring obtained in the control was significantly higher with respect to the five mixture concentrations (See Fig. 3): 0.0112 mg/L ($p = 0.00005$), 0.0056 mg/L ($p = 0.033$), 0.0028 mg/L ($p = 0.000003$), 0.0014 mg/L ($p = 0.0004$) and 0.0007 mg/L ($p = 0.0006$).

Similar results were found by other authors when analyzing the effects of pesticides on the reproduction of *D. magna*. Song et al. (2017) exposed daphnids to sublethal concentrations of the fungicide chlorothalonil and observed negative effects on the total fecundity per female, the first pregnancy time, first brood time and number of neonates in the first brood. Regarding insecticides, a decrease in mean total offspring per female and the brood size was obtained

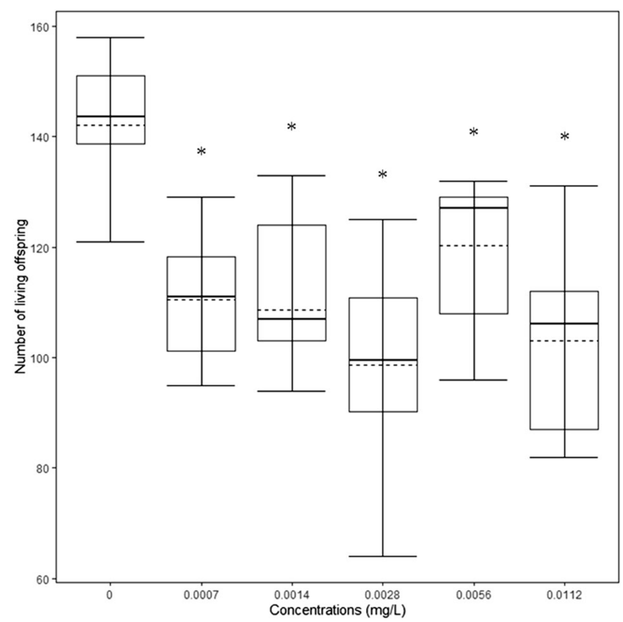

Fig. 3 Effect of different concentrations of Mixture II on the reproduction of *D. magna*: the dotted line marks the mean, the solid line within the box indicates the median, inferior and superior boundaries of the box mark the 25th and 75th percentiles, respectively, and whiskers represent the minimum and maximum values. Mean values significantly different from the control are marked with an asterisk

after a chronic exposure of *D. magna* to endosulfan (Fernández-Casalderrey et al. 1993), diazinon (Fernández-Casalderrey et al. 1995) and fenitrothion (Ferrando et al. 1996). On the other hand, 21 d of exposure to the herbicides thiobencarb (Sancho et al. 2001) and propanil (Villarroel et al. 2003) resulted in a reduction in the mean total offspring per female and the mean brood size.

It is known that aquatic macroinvertebrate communities are sensitive to pesticides from conventional agriculture

(Rizo-Patrón et al. 2013; Echeverría-Sáenz et al. 2018). As we demonstrated, chronic exposure of *D. magna* to sublethal concentrations of mixture II caused a significant decrease in the number of live neonates during the 21 d assay. Therefore, if this mixture of insecticides and fungicides reaches water bodies, even at very low concentrations, it would represent a significant ecological risk for the aquatic invertebrates that inhabit the areas surrounding the crops, by affecting their fertility and consequently their populations. Moreover, quality analysis of streams near the area of potato production considered for this study, revealed unsuitable conditions for aquatic biota in this ecosystem due to factors like the presence of pesticide residues, fertilizers and discharge of organic material (Chin-Pampillo et al. 2012).

Conclusions

The ecotoxicity of simultaneously applied commercial insecticide/fungicide formulations (alone and in mixtures) employed in potato crops was analyzed. In both cases, acute toxicity was higher in *D. magna*, compared to the less employed benchmark organism *L. sativa*; moreover, no toxic effect was determined for the formulation containing chlorothalonil on the latter organism. Differences in the available EC_{50} values (on *D. magna*) for individual pesticide a.i. and their respective formulations showed the importance of assaying the toxicity in the formulations, as they will be in the end, the ones applied on crops to consequently reach different environmental compartments.

Realistic mixtures of pesticide formulations commonly applied in potato crops resulted in antagonistic acute effects on *D. magna*, when compared to CA and IA models, which suggests that their ecotoxicological effect as mixtures is lower than their effect as individual formulations. The binary mixture formulation containing deltamethrin+imidacloprid and commercialized as Muralla Delta® followed the CA model, when compared with ecotoxicological parameters determined for individual pure a.i. The negative impact on *D. magna* reproduction observed at chronic sublethal concentrations of mixture II (chlorothalonil - propineb - ziram – thiocyclam), supports the concern that should prevail in case these pesticide mixtures reach aquatic environments, as they represent a real risk for non-target species in aquatic systems surrounding the potato crop field.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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