Single and mixture toxicity of abamectin and difenoconazole to adult zebrafish (*Danio rerio*)

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HIGHLIGHTS

- LC50 values were 59 μg/L (abamectin) and 1.4 mg/L (difenoconazole).
- The pesticide mixture showed synergetic effects to zebrafish.
- Implications for pesticide risk assessment in tropical countries are discussed.

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ABSTRACT

Concerns have been raised in recent years on the potential risks related with pesticide mixtures that are likely to be present in agricultural edge-of-field waterbodies. Despite the high use of pesticides in tropical countries like Brazil, studies evaluating pesticide mixtures are especially scarce in the tropics. The insecticide abamectin and the fungicide difenoconazole are the main pesticides intensively used in Brazilian strawberry crop and are hence likely to occur simultaneously. The aim of the present study was therefore to evaluate the toxicity of abamectin, difenoconazole and their mixture to the tropical fish *Danio rerio*. Laboratory toxicity tests with the individual pesticides indicated 48 h-LC_{50} values of 59 μg L^{-1} for abamectin and 1.4 mg L^{-1} for difenoconazole. Mixtures of the two pesticides revealed a synergistic deviation of the independent action model. Implications of study findings for the aquatic risk assessment of pesticide mixtures, especially in tropical countries and indications for future research are discussed.

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1. Introduction

Agricultural production systems in tropical countries are generally under a greater pest pressure than their temperate counterparts due to favourable environmental conditions for insect pests and weeds to proliferate (Lewis et al., 2016). Intensification of agriculture and expansion of agricultural frontiers with concomitant increases in pesticide use have therefore been evident in Latin America since the late 1990s (Carriquiriborde et al., 2014). Brazil, for example, became the world's top pesticide market consumer in 2008, and currently accounts for approximately 20% of the total world use (Albuquerque et al., 2016).

Despite this high use of pesticides in tropical countries like Brazil, there is still relatively little knowledge about the fate and toxicity of pesticides in tropical aquatic ecosystems as compared to temperate systems (Daam and Van den Brink, 2010; Carriquiriborde et al., 2014; Lewis et al., 2016). Sensitivity comparisons of tropical and temperate species to pesticides have not demonstrated a consistent greater or lesser sensitivity of tropical species as compared to their temperate counterparts, although such comparisons are based on a relatively small tropical dataset

(e.g. Maltby et al., 2005; Kwok et al., 2007; Rico et al., 2011). Studies evaluating mixtures of pesticides likely to occur in tropical edge-of-field are even scarcer (Lewis et al., 2016; Moreira et al., 2017).

The main Brazilian strawberry crop area in the municipality of Bom Repouso (Minas Gerais) has a tropical climate by altitude, and can be classified as a monsoon-influenced humid subtropical climate according to the Köppen climate classification. It is an agricultural area with intensive use of pesticides and previous field studies in this area identified the insecticide/acaricide Kraft® 36 EC (a.i. abamectin) and the fungicide Score® 250 EC (a.i. difenoconazole) as the main pesticides used in this area (Nunes, 2010; Nunes and Espindola, 2012). Abamectin is an avermectin that acts on the gamma-aminobutyric acid (GABA) receptors in both invertebrates and vertebrates (Novelli et al., 2016). Difeneconazole is an azole fungicide known to act by interfering with the ergosterol biosynthesis in target fungi leading to morphological and functional changes of the fungal cell membrane (Campbell, 1989; EC, 2006a.) Since both pesticides are intensively used throughout the year, they are likely to occur simultaneously in edge-of-field water bodies in this region and this pesticide mixture may have greater toxic effects to aquatic life in these ecosystems than the individual compounds. Moreira et al. (2017), for example, noted synergistic effects of high binary mixture concentrations containing abamectin and difenoconazole to the Neotropical cladoceran *Macrothrix flabelligera*. In addition, these authors noted that water from microcosms receiving runoff water from experimental soil plots applied with the recommended doses of these individual pesticides did not produce mortality in the test organisms. Microcosms that received runoff water containing the pesticide mixture, however, did cause a short-term effect on mobility of *M. flabelligera* (Moreira et al., 2017).

The aim of the present study was to evaluate the toxicity of abamectin and difenoconazole to the zebrafish *Danio rerio*. These two compounds have already been tested on this species individually but not in mixtures. Besides that, the species chosen is widely known and used in ecotoxicological studies and it has been recommended as a test species for tropical regions (OECD, 1998; Daam and Van den Brink, 2010). Methods for laboratory cultivation and toxicity testing have previously been developed for this species (e.g. OECD, 1992; ABNT, 2011). Acute laboratory toxicity tests were conducted with the individual compounds to establish their respective toxicity thresholds. Mixtures of both compounds were also tested to test their combined effect and to evaluate its underlying mechanism.

2. Materials and methods

2.1. Test organisms and acclimatisation

Adult zebrafish (*Danio rerio*) were acquired from a local commercial hatchery in São Carlos (SP, Brazil). The organisms were acclimatised and maintained in our laboratory (NEEA/CRHEA) under controlled temperature (25 ± 2 °C) and photoperiod (12 h light:12 h dark; light intensity ± 1000 lx). Fish were kept in 90-L glass aquaria containing reconstituted water prepared according to standard ABNT (2011) with pH 7.0–7.6 and hardness 40–48 mg CaCO$_3$/L. Water was constantly aerated (dissolved oxygen > 6,0 mg L^{-1}) and fish were fed *ad libitum* with Tetramin® on a daily basis until one day before the start of the test. Fish were not fed during the experiment and initial fish size (2.8 ± 0.4 cm) and weight (0.4 ± 0.1 g) were determined based on 10% of the fish in each lot used.

2.2. Toxicity tests

Acute toxicity tests (test duration: 48 h; endpoint: mortality)

were conducted with technical abamectin (PESTANAL®, Sigma-Aldrich - CAS Number: 71751-41-2; purity 98,6%) and difenoconazol (PESTANAL®, Sigma-Aldrich - CAS Number: 119446-68-3; purity 99,7%). Stock solutions of abamectin (1 mg L^{-1}) and difenoconazole (10 mg L^{-1}) were prepared by diluting the technical substances in acetone. Subsequently, these stock solutions were diluted with reconstituted water (ABNT, 2011) to obtain five test concentrations for abamectin (20, 40, 60, 80 and 100 μg L^{-1}) and difenoconazole (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L), besides an untreated control (only reconstituted water) and a solvent control (reconstituted water with 0.025% v/v acetone). The test concentrations of the two compounds were based on the results of preliminary tests (and hence expected mortality rates) of the two compounds.

Toxicity tests with the mixture of the two compounds were conducted with all 36 possible concentration combinations using a factorial design (Fig. 1), which with the solvent control totalized 37 treatments. All treatments with the individual compounds and mixtures were conducted simultaneously to avoid any influence of eventual differences in sensitivity of test organisms used and experimental conditions. Each treatment was conducted in triplicate with each replicate consisting of a 1-L non-toxic polypropylene plastic container (PRAFESTA®) containing three fish each, in accordance with ABNT (2011) recommendations. The experimental conditions during the test were the same as those described for fish acclimatisation. Physicochemical parameters [temperature, pH and dissolved oxygen (DO)] in the test water were measured at the beginning and at the end of the test. Temperature and pH were measured with a Micronal B374 potentiometer, whereas dissolved oxygen (DO) was quantified using a YSI DO meter (YSI 55-25FT). A reference test with KCl was conducted to confirm good physiological conditions of the fish.

2.3. Chemical analysis of the test substances

To confirm nominal test concentrations, stock solutions were analyzed with high-performance liquid chromatography (HPLC/MS/MS Agilent® 6490 series). The chromatographic analysis conditions were: Agilent Zorbax ODS C18 column (250 mm × 4.6 mm × 5 mm) and temperature of 25 °C. The isocratic mobile phase utilized was acetonitrile and water (0.1% formic acid; 90:10 v/v) for 6 min, at an injection volume of 20 μL and a flow rate of 1.0 mL min−1. Analyses were carried out in three replicates. Based on absorbance signals observed in the Diode-Array Detection (DAD)
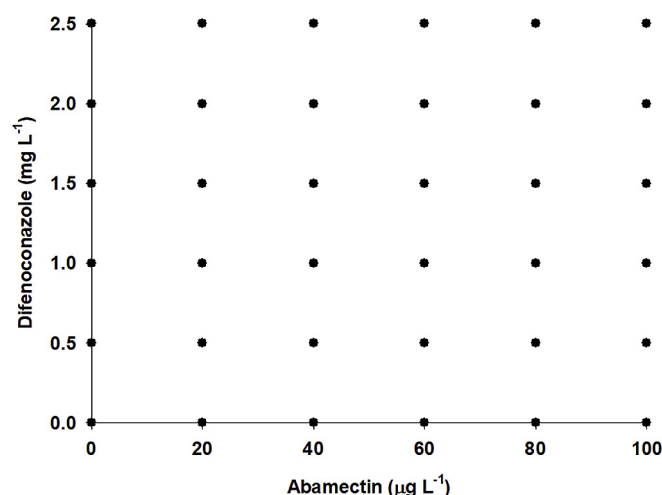
Fig. 1. Factorial design adopted to select the test concentrations in the single and mixture toxicity tests with abamectin and difenoconazole.

spectrum of the standard solutions, abamectin and difenoconazole were detected and quantified at 246 nm and 230 nm with retention times of 2.9 and 2.2 min, respectively. The precision in terms of repeatability, expressed as relative standard deviation (RSD), was 2.05% for abamectin and 1.88% for difenoconazole. Detection limits were 22.2 μg/L (abamectin) and 27.2 μg/L (difenoconazole).

2.4. Data analysis

The 48 h-LC_{50} values for the acute toxicity tests conducted with the single compounds were calculated based on confirmed nominal test concentrations by nonlinear regression using the three-parameter logistic curve through the software Statistica version 7 (Statsoft, 2004). Mortality data from the toxicity tests with the pesticide mixtures were analysed through the conceptual models of concentration addition (CA) and independent action (IA). Initially, the observed mortality data were compared with the combined expected effect calculated from the individual exposures using the MIXTOX tool (Jonker et al., 2005). The analysis was then extended, as described in Jonker et al. (2005) and the three deviations from the reference models synergetic/antagonistic interactions (S/A), deviation dose ratio-dependent (DR) and dose level-dependent (DL) were modelled by adding two parameters ("a" and "b"; Table 1). The parameter "a" becomes negative and positive in synergistic and antagonistic deviations, respectively. To describe the dose level-dependent (DL) deviation, another parameter (BDL) is included in addition to the parameter "a". In the latter case, the value of "a" indicates the deviation in high and low doses and the value of BDL indicates at what dose level the deviation changes. Further details on the deviation functions can be obtained from Jonker et al. (2005). The data were verified for these conceptual models and deviations, and the best fit was chosen through the maximum likelihood method. After identifying the statistically most appropriate model for the description of the deviation, the pattern of toxicity was deduced directly from the parameter values (Table 1) and the maximum deviation could be calculated in terms of effect level (Jonker et al., 2005; Freitas et al., 2014).

3. Results and discussion

3.1. Test performance

Physical–chemical water conditions were overall stable during the experimental period of the tests and also comparable between the different treatments: Water temperature = 24 ± 0.4 °C; pH = 7.6 ± 0.2 (0 h) and 7.2 ± 0.3 (48 h); DO = 7.2 ± 0.2 μg L-1 (0 h) and 5.4 ± 0.6 mg L-1 (48 h). Although DO slightly decreased throughout the experimental period, it remained within the

reference level as set in) (>5.0 mg L^{-1}). The reference test conducted with KCl revealed an LC_{50} of 1.0 ± 0.2 mg/L (mean ± SD; range 0.7–1.4 mg/L) and as such fulfilled the acceptance criteria set in ABNT (2011).

3.2. Single compound toxicity

The LC_{50} values obtained for the individual compounds were in agreement with those reported in the literature for *D. rerio* exposed to these compounds. For abamectin, the 48 h-LC_{50} value of 59 μg L^{-1} (95% C.I.: 54–63 μg L^{-1}) calculated in the present study is comparable to that previously established in our laboratory (33 μg L^{-1}; Novelli et al., 2012). The 48 h-LC_{50} obtained for difenoconazole in the present study was 1.41 mg L^{-1} (95% C.I.: 1.40–1.43 mg L^{-1}) so it was approximately 24 times less toxic to the fish than abamectin. The 96 h-LC_{50} reported by Mu et al. (2013; 1.45 mg L^{-1}) is the same as the one we report here, despite the longer exposure used by those authors.

3.3. Mixture toxicity

Pesticide mixtures with similar modes of action are generally considered to act through concentration addition, whereas the independent action (IA) model is used with mixtures of pesticides with different modes of action (e.g. Jonker et al., 2005; Loureiro et al., 2010; Altenburger et al., 2013; Silva et al., 2015). Subsequently, the IA model was applied in the present study to evaluate the response of *D. rerio* exposed to mixtures of abamectin and difenoconazole, and deviations from this model were evaluated using the methodology developed by (Jonker et al,. 2005; Table 1). As anticipated, the derived mixture toxicity data indeed fitted the IA model, producing a sum of the squares of the residuals (SS) of 71.92 ($p < 0.05$; $r^2 = 0.29$; Table 2).

The results of the toxicity tests are visualised in Figs. 2 and 3. The concave lines in the isobologram (Fig. 2) clearly indicate a synergistic effect of the two compounds on *D. rerio*. When the modelled LC_x and obtained LC_x are plotted against one another (Fig. 3), a synergistic interaction between the pesticide mixture and the test species is apparent. These visual observations are also confirmed with the analysis of deviations from the IA model following the method of (Jonker et al., 2005; Table 2). After the addition of the parameters "a" and "b" to the IA model, the synergistic deviation of the IA model was indeed noted to best describe the data, as can be deduced from the decrease in the SS value to 47.90 and the obtained correlation coefficient ($p < 0.05$; $r^2 = 0.53$; Table 2). The toxic effects of mixtures include both toxicokinetic and toxicodynamic aspects and it is therefore necessary to evaluate the underlying species- and compound-related toxicity and clearance mechanisms to understand the synergistic effects as observed in the present

Table 1
Interpretation of the additional parameters ("a" and "b") that define the functional form of the standard deviations from the independent action model (IA); adapted from Jonker et al. (2005).

Standard Deviation	Parameter "a"	Parameter "b"
Synergism/antagonism (S/A)	a > 0 - antagonism a < 0 - synergism	
Dose ratio-dependent (DR)	a >0 - antagonism, except for those proportions of mixtures where a value of significant negative b indicates synergism a <0 - synergism, except for those proportions of mixtures where a significant positive value of b indicates antagonism	b_i > 0 - antagonism where the toxicity of the mixture is mainly caused by the toxic agent i b_i < 0 - synergy where the toxicity of the mixture is mainly caused by toxic i
Dose level-dependent (DL)	a >0 - low dose level antagonism and synergy in high dose level a <0 - low dose level synergism and antagonism in high dose level	b_{DL} > 2 - change in level of dose lower than EC50 b_{DL} = 2 - change in EC_{50} 1 < b_{DL} < 2 - change in level of dose greater than EC50 b_{DL} < 1 - no change, but the magnitude of S/A is dependent on the level of effect

Table 2
Summary of the analysis of the acute toxicity tests evaluating mixtures of abamectin and difenoconazole to *Danio rerio*.

	IA	S/A	DR	DL
max	0,98	0,98	0,98	0,98
$\beta_{Abamectin}$	2,68	3,70	0,98	9,39
$\beta_{Difenoconazole}$	9,11	9,15	9,39	11,96
LC_{50} to Abamec	0,03	0,03	11,96	0,06
LC_{50} to Difeno	1,05	1,11	0,06	1,23
a	–	−13,37	1,24	−9,00
$b_{DR/DL}$	–	–	−12,42	0,19
SS	71,92	47,90	45,68	46,42
r^2	0,29	0,53	0,55	0,54
χ^2 or test F	29,75	24,02	2,22	1,48
df	–	1	1	1
p (χ^2/F)	$5,5 \times 10^{-6}$	$9,5 \times 10^{-7}$	0,14	0,22

max = maximum value of the response; β = slope the individual response dose curve; LC_{50} = median effective concentration; a, b_{DR} e b_{DL} = s function parameters; SS = sum of the squares of the residuals; r^2 = regression coefficient; Test χ^2 or F = statistical test; df = degree of freedom; p (χ^2/F) = level of significance for the statistical test. IA + independent action model, S/A = deviation synergism/antagonism, DR = dose ratio-dependent deviation and DL = dose level dependent deviation.
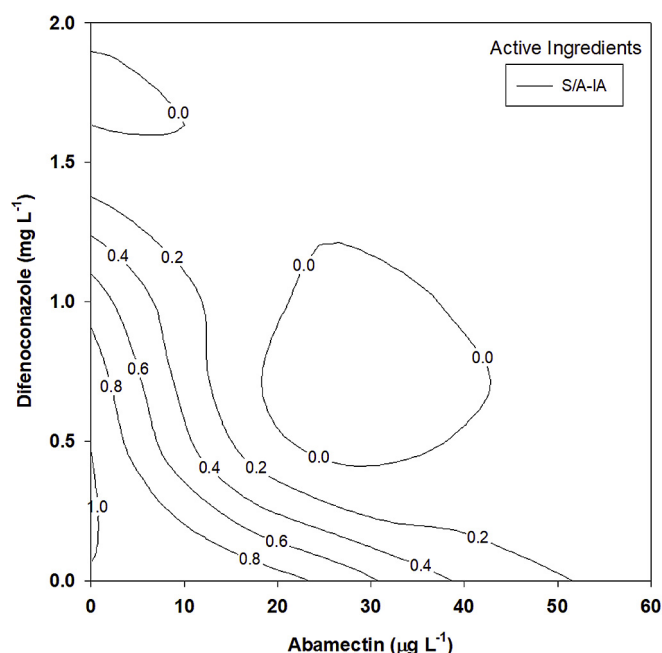

Fig. 2. Isobologram of the interactive effects of the pesticide mixtures on *Danio rerio* survival, demonstrating a synergism deviation from the independent action (IA) model that was analysed. Linear, concave (numbers within the isoboles < 1) and convex (numbers within the isoboles > 1) isoboles in isobolograms represent no interaction, synergy and antagonism, respectively (Ryall and Tan, 2015).

study (Loureiro et al., 2010).

Unlike in mammals, abamectin can cross the blood–brain barrier in fish and cause toxicity (Høy et al., 1990). Abamectin is known to act on the gamma-aminobutyric acid (GABA) receptors in both invertebrates and vertebrates, and also on glutamatergic receptors in the chloride channels of invertebrates (Novelli et al., 2016). After abamectin exposure, the increase in chloride ions hyperpolarizes the nerve and muscle cells, ultimately interfering with neuromuscular transmission, leading to death (Campbell, 1989).

Difenoconazole is a triazole fungicide that is known to act by interfering with the ergosterol biosynthesis in fungi by inhibition of the C-14-demethylation of sterols, which leads to morphological and functional changes of the fungal cell membrane (EC, 2006a).
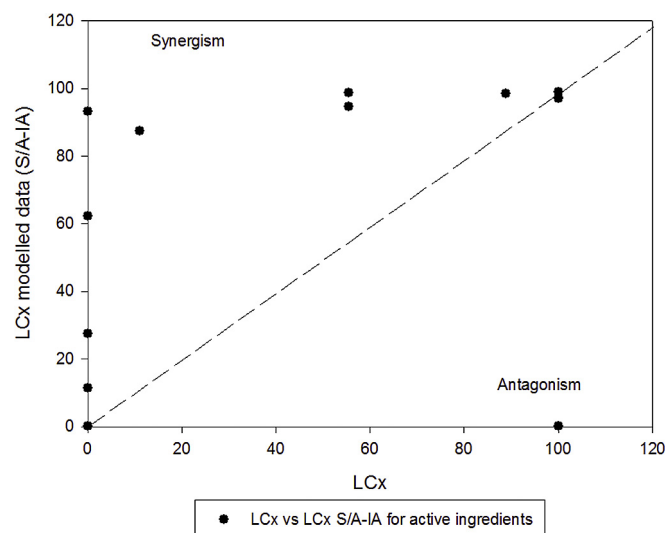

Fig. 3. Modelled versus obtained LC_x values, demonstrating a synergistic interaction between the pesticide mixture and *Danio rerio*.

Interestingly, it has been discussed that there is no indication that toxic effects of difenoconazole in fish result from a specific mode of action other than general or systemic toxicity (EC, 2006a).

After reviewing mixture toxicity studies, Cedergreen (2014) concluded that synergistic interactions involving azole fungicides are most likely all examples of cases where the metabolization of the pesticides is inhibited by the azole. Azole fungicides are known inhibitors of a wide range of P450 monooxygenases, which are enzymes responsible for the phase I metabolization of lipophilic compounds, together with a range of biosynthesis processes in both plants and animals (Guengerich, 2008; Walker, 2009). Hence, the toxicity of insecticides is often severely enhanced when mixed with azole fungicides (Rasmussen et al., 2012; Cedergreen, 2014), as was observed in the present study.

3.4. Implications for risk assessment and concluding remarks

Studies in Brazil and elsewhere have reported the simultaneous use of formulated products containing difenoconazole and abamectin in agricultural areas near adjacent water bodies (e.g. Milhome et al., 2009; Nunes, 2010; Thuy et al., 2012). Azole fungicides and pyrethroid insecticides are also often applied in tank mixtures to agricultural fields (Rasmussen et al., 2012). Various studies have proven that azole fungicides synergise the effect of insecticides, although such studies appear to be limited to invertebrates and primary producers (c.f. Rasmussen et al., 2012; Cedergreen, 2014). For example, Moreira et al. (2017) previously demonstrated the synergetic effects of higher mixture concentrations of abamectin and difenoconazole on the cladoceran *Macrothrix flabelligera*. To the best of our knowledge, however, there are no other studies that have demonstrated synergetic effects of binary mixtures containing azole fungicide and insecticide on fish.

This study demonstrated that mixtures of the two selected pesticides caused greater toxicity (synergism) to the fish *D. rerio* than when tested individually. Prospective environmental risk assessments and environmental quality standards (EQS) based on individual exposures may hence not adequately protect aquatic ecosystems. In practice, however, this will only be the case if predicted or measured environmental concentrations (PEC and MEC, respectively) may be expected to exert non-acceptable risks. For example, in the European prospective risk assessment of abamectin

and difenoconazole, the worst-case tier-4 PECs are 0.43 μg L^{-1} and 0.44 μg L^{-1}, respectively (EC, 2006a, 2006b). These PECs are slightly below the acute regulatory acceptable concentration (RAC) for abamectin (0.66 μg L^{-1}) but well below the RAC for difenoconazole (6.5 μg L^{-1}) (EC, 2006a, 2006b). From an effect assessment perspective, the procedure used with individual compounds would not be protective if effects for mixtures containing pesticide concentrations at their respective RAC levels would exert unacceptable effects. From a risk assessment perspective, if mixtures at exposure levels corresponding to their PEC or MEC exert unacceptable risks, the procedure based on individual compounds would not be protective. Subsequently, although several studies have noted synergistic effects (e.g. as reviewed by Cedergreen, 2014), this does not necessarily mean that risk assessments based on individual compounds are not protective. In the present study, for example, the lowest test concentrations (20 μg L^{-1} and 500 μg L^{-1} for abamectin and difenoconazole, respectively) were well above both PECs and RACs indicated above. Future studies including treatments with concentrations considered protective (RAC, EQS) and those equalling predicted (PEC) or measured (MEC) levels would enable evaluating to what extent mixture toxicity needs to be included in effect and risk assessments. At such low test concentrations, these future studies should also include sublethal endpoints.

The RAC and PEC values above were derived for a European ERA and could have little applicability for tropical countries like Brazil. Sensitivity comparisons between temperate and tropical species (e.g. Maltby et al., 2005; Kwok et al., 2007; Rico et al., 2011) and communities (e.g. Daam and Van den Brink, 2011 and references therein) have not demonstrated consistent differences. Subsequently, toxicity data and RACs based on taxa from both climatic regions may also be expected not to differ in a significant, or at least consistent, matter. Peak exposures of edge-of-field water bodies to pesticides, however, may be expected to be greater in tropical than in temperate agroecosystems due to higher expected levels of runoff and spray drift as well as more intensive pesticide use in the tropics (Moreira et al., 2017). On the other hand, dissipation of pesticides may be expected to be faster under warm tropical conditions (e.g. Sanchez-Bayo and Hyne, 2011). Subsequently, PEC and MEC values may be expected to differ to a significant extent between the climatic regions. Field studies in tropical farms measuring pesticide concentrations and environmental parameters are needed to increase our knowledge on pesticide exposure profiles in tropical edge-of-field waterbodies. Besides providing MECs, such studies would also aid in developing and calibrating pesticide fate simulation models that enable determining PECs. Semi-field experiments may also aid in underpinning the importance of different pesticide entry routes under a more controlled experimental settings. For example, in previous studies we treated aquatic microcosms by direct overspray and with runoff from an experimental agricultural field to evaluate the differential toxic effects caused through these exposure routes (Novelli et al., 2016; Moreira et al., 2017). In that way, Moreira et al. (2017) demonstrated that microcosms receiving runoff water only showed toxicity to the cladoceran tested (*Macrothrix flabelligera*) when obtained from the experimental plots that were treated with both pesticides. In addition, the microcosms that were treated by direct overspray of both pesticide formulations showed the most pronounced toxic effects (Moreira et al., 2017).

Although there has been a great increase in research on mixture toxicity over the past few years, several other authors have also concluded that additional information is required to develop practical criteria for selecting pesticide mixtures that require additional (Legislative) attention based on their likelihood to exert synergistic responses at concentrations likely to occur in the field (e.g. Altenburger et al., 2013; Coors et al., 2013; Silva et al., 2015).

Toxicokinetic—toxicodynamic (TKTD) models are increasingly used in the analysis of toxicity data for single-chemical exposure (e.g. EFSA, 2013). However, models of this type are also absolutely essential for a more mechanistic understanding of mixture ecotoxicology (Jager et al., 2014).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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