

# Acute effects of binary mixtures of Type II pyrethroids and organophosphate insecticides on *Oreochromis niloticus*

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**Abstract** Pyrethroid and organophosphate insecticides have been used for more than 20 years worldwide to control a variety of insect pest in different settings. These pesticides have been detected in a variety of environmental samples, including surface waters and sediments and therefore there is significant concern about their potential toxic effects on non-target organisms. Mixtures of compounds from these groups of pesticides have been found to frequently show enhanced toxicity but it has been a challenge to predict whether or not enhanced toxicity will occur for a given combination of compounds. This study therefore studied the effects of binary pyrethroid-organophosphate mixtures using cypermethrin, deltamethrin and dimethoate in an acute toxicity test system with *Oreochromis niloticus*. The 96 h LC50s for individual insecticides were 9.13 µg/l, 9.42 µg/l and 45.52 mg/l for cypermethrin, deltamethrin and dimethoate respectively. These showed that the pyrethroid insecticides were highly toxic to *Oreochromis niloticus* and were far more toxic than dimethoate. All mixtures were also more toxic than single insecticides throughout the concentration-response curve with mixtures resulting in mortality at concentrations which the individual pesticides in the mixture were below their respective NOECs. In addition, observed mixture toxicities deviated from the predicted mixture effects based either on the Concentration Addition (CA) or Independent Action (IA) models independent of mixture ratio. However, the extent of observed mixture mortality deviation was dependent on the effect

level. Significant deviations ( $MDR > 2.0$ ) were observed at lower concentrations indicating synergistic effects at lower and possibly environmentally relevant concentrations. This is not unexpected since organophosphate insecticides are known to inhibit acetylcholinesterase as well as inactivate esterase, resulting in reduced detoxification of pyrethroid insecticides and consequently greater toxicity than would be expected. This has important implications for risk assessment of mixtures since the risk of pyrethroid-organophosphate mixtures may be underestimated if either the CA or IA model is employed.

**Keywords** Mixture toxicity · Pyrethroids · Organophosphate · Insecticides · *Oreochromis niloticus* · Synergism · Concentration addition

## Introduction

Numerous man-made chemicals are concurrently used in any given area and most of these substances and/or their degradation products are finally emitted into the environment (Backhaus and Faust 2012). Chemical monitoring provides ample evidence that organisms in their environment are exposed to complex cocktails of contaminants rather than individual chemicals (Altenburger et al. 2003; Backhaus and Faust 2012; Altenburger et al. 2013). Amongst these chemicals, pesticides are unique in the sense that they are made to be toxic and are intentionally placed in the environment in huge quantities (Lydy et al. 2004). There are about 1000 pesticide formulations in use throughout the world today (Yadav 2010). In addition, about 5 million tons of pesticides are applied annually in the world, of which

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about 70% is used for agriculture and the remainder by public health agencies and government agencies for vector control and by home owners (Yadav 2010). Dimethoate is an organophosphorous insecticide that is used worldwide in agriculture and urban areas due to its high efficacy and rapid environmental degradation. In the US roughly 816,466 kg of active ingredient is applied annually on agricultural sites with the highest applications being on alfalfa, wheat, cotton, and corn (Van Scoy et al. 2016). Dimethoate is highly water soluble and has low soil persistence. Due to these two factors, the potential to runoff into surface waters and/or leaching into groundwater is high (Van Scoy et al. 2016). Synthetic pyrethroid insecticides have been used for more than 20 years to control insect pests in a variety of crops, as well as for nurseries, golf courses, urban structural and landscaping sites, residential home and garden pest control, and mosquito abatement (Maund et al. 2001; Rasmussen et al. 2013; DeLorenzo et al. 2014). They are among the most potent insecticides known (Khalili et al. 2012), and have become increasingly popular following outright bans or limitations on the use of cholinesterase-inhibiting insecticides (Palmquist et al. 2012; Prusty et al. 2015). Combinations of several pesticides are common in the surface water in agricultural areas, with the exact type of substance depending on the dominant crops in an area (Deneer 2000; Nowell et al. 2014). Pyrethroids have been detected in a variety of environmental samples, including surface waters and sediments (DeLorenzo et al. 2014) and have generated public concerns due to their increasing use and potential effects on aquatic ecosystems (Palmquist et al. 2012).

The behavior of chemicals in a mixture may not correspond to that predicted from data on the pure compounds. Interactions of components in a mixture can cause complex and substantial changes in the apparent properties of its constituents (Altenburger et al. 2003). Despite this common occurrence of chemical mixtures in the environment, even modern legislations such as REACH (European Regulations on Registration, Evaluation, Authorisation and Restriction of Chemicals) focus almost exclusively on the assessment of individual chemicals. This may result in risk underestimations and it has been put forward by the Council of the European Environmental Ministers as a major shortcoming that needs attention (Backhaus and Faust 2012). The fundamental question of whether knowledge of the individual toxicities of the mixture components would allow the prediction of their combined effect remains largely unanswered. Thus, the challenge emerges on how to assess potential combined effects of mixture exposures (Altenburger et al. 2013).

Concentration Addition (CA) and Independent Action (IA) are the two classical concepts that allow calculating the expected mixture toxicity, based on the toxicities of the individual compounds and their concentrations in the mixture (Backhaus and Faust 2012). The main feature of CA is

that the compounds act as dilutions of each other, and the overall effective concentration can be calculated by adding up all the effective concentrations (expressed as fractions of the effect concentrations, or Toxic Units) of all compounds (Deneer 2000; Altenburger et al. 2003; Nowell et al. 2014). For a mixture of  $n$  components, the concept can be mathematically expressed as:

$$\sum_{i=1}^n \frac{c_i^*}{EC_{x_i}} = 1$$

where  $c_i^*$  is the concentration (or dose) of the  $i^{\text{th}}$  component in an  $n$ -compound mixture which elicits  $x\%$  total effect and  $EC_{x_i}$  denotes the concentration of that substance which provokes  $x\%$  effect if applied singly. Every fraction  $c_i/EC_{x_i}$  (also termed a “toxic unit”) gives the concentration of a compound in the mixture scaled for its relative potency (Backhaus and Faust 2012). If for a mixture the sum of the TUs equals 1 at  $y = 50\%$  the mixture is considered additive under CA. If the sum is less than 1, the mixture is said to act “greater than additive”, i.e. synergistic; if the sum of TUs is greater than 1, the mixture is said to act “less than additive”, i.e. antagonistic (De Laender et al. 2009).

The IA model (also called response addition or Bliss independence) assumes that all mixture components affect the same end point. But in contrast to CA, Independent Action assumes that compounds in the mixture act independently, which, in toxicological terms, is often understood in the sense that compounds having different molecular acceptor sites may act on different physiological systems within the exposed organisms and that impaired subsystems affect the end point under observation independently of each other (Altenburger et al. 2000; Backhaus and Faust 2012; Nowell et al. 2014). The combined effect is therefore calculated according to the probability of non-excluding processes (Nowell et al. 2014) according to the following mathematical formulation when a response parameter (such as e.g. mortality) increases with increasing concentrations:

$$E(C_{mix}) = 1 - \prod_{i=1}^n (1 - E(C_i)) \quad (1)$$

where  $E(C_{mix})$  denotes the total predicted effect (scaled 0–1) of an  $n$ -compound mixture,  $C_i$  is the concentration of the  $i^{\text{th}}$  compound, and  $E(C_{mix})$  is the effect of that concentration if the compound is applied singly (Belden et al. 2007; Backhaus and Faust 2012; Altenburger et al. 2013).

While CA and IA both assume that there are no interactions between the components in a mixture (Backhaus and Faust 2012), mixtures of chemicals with the same mode of action (MOA) are usually described by the CA model and the IA model may underestimate effects (Altenburger et al. 2000; Backhaus and Faust 2012). Belden et al. (2007)

showed good predictive power of the CA model for most (88%) pesticides mixtures studied with aquatic organisms. However, despite all progress in predicting mixture toxicity from knowledge on their components there are cases of synergism/antagonism between contaminants, that is, clear deviations from the predictions derived from CA or IA (Belden et al. 2007; Altenburger et al. 2013). Efforts of identifying patterns of combined effects for different types of mixtures led to the idea of comparatively testing the predictive power of the general concepts of CA and IA. (Altenburger et al. 2013). For regulatory purposes it is worthwhile to identify combinations of compounds which give rise to deviations from CA or IA—predicted toxicity (Deneer 2000). Combinations identified as producing the largest number of deviations from CA were the combination of an organophosphorus ester or a carbamate with either another organophosphorus ester or a synthetic pyrethroid. These, unfortunately, are groups of pesticides which are commonly used in agricultural practice (Deneer 2000; Laetz et al. 2009; DeLorenzo et al. 2014). Although numerous toxicity studies have been performed with pesticide mixtures, the amount of data available is small compared to the large number of mixtures that occur in the environment. Knowledge about interactions in aquatic organisms of specific compounds belonging to these groups is quite sparse and predicting whether or not enhanced toxicity will occur for a given combination of compounds is therefore often not straightforward (Deneer 2000; Belden et al. 2007). Direct observations from laboratory experiments with pesticide mixtures are also necessary to completely understand the potential joint action and interactions for specific mixtures that previously have not been studied (Backhaus and Faust 2012). In order to enhance our understanding of the interactions between and among individual pesticides and combinations of pesticides, more studies of mixture toxicities are needed and these studies should assess the mixtures of greatest potential importance (occurrence and toxicity) in aquatic ecosystems including the testing of more species and acute and chronic endpoints (Belden et al. 2007). The main objective of the present research was therefore to assess the effects of binary mixtures of pyrethroid (cypermethrin, deltamethrin) and organophosphate (dimethoate) insecticides on the freshwater fish, *Oreochromis niloticus*, in order to determine if the type of joint action involved can be adequately predicted by CA or IA.

## Materials and methods

### Study species and chemicals

The Nile tilapia, *Oreochromis niloticus*, is a native brackish and freshwater cichlid of commercial value in several African

countries (Van den Bergh and Teugels 1998; Ndiwa et al. 2014). It is also one of the most common freshwater fish used in toxicological studies (Jiraungkoorskul et al. 2002; Fai and Fagade 2005; Boateng et al. 2006; Ayoola 2008; Osman 2012; Fai et al. 2015), because it presents a number of characteristics that may make it an appropriate model that can be used as indicator species in biomonitoring programs (Osman 2012). One month old *Oreochromis niloticus* fingerlings weighing  $1.8 \pm 0.05$  g were obtained from the Institute of Agronomic Research and Development (IRAD) in Fombot, West Region of Cameroon. Once in the laboratory the fingerlings were transferred into 40 L glass holding tanks containing aerated tap water and acclimatized for at least 7 days at constant aeration under ambient laboratory conditions. The holding water had a temperature of  $21.5 \pm 2$  °C, dissolved oxygen of 4 mg/l and a pH of 7.2–7.5. During this period the water quality parameters were monitored twice a week, fingerlings were fed thrice a week and their waste siphoned out daily. Fingerlings were considered to be fully acclimatized when no death was observed for four consecutive days. Any deaths observed during the tests were therefore considered to be due to the effect of the pesticides.

In order to obtain results that are relevant to real world situations, insecticide formulations were used as opposed to pure active ingredients in the present study and the choices of formulations were based on those currently used by farmers in tropical African countries (Matthews et al. 2003; Matthews 2008; Ntow 2008; Abang et al. 2013; MINADER 2013; Jepson et al. 2014). The dimethoate formulation used was Dimeforce 40% EC (40 g dimethoate/L) manufactured by Sino Agro-Chemical Industries Ltd, Guangdong China. The deltamethrin formulation was Decis 25 EC (25 g deltamethrin/L) produced by Bayer Crop Science. The cypermethrin formulation used was Cypercot 100 EC (containing 100 g cypermethrin/L) manufactured by Meghani Organics Limited, India. Pesticide formulations were obtained from a local pesticides store. All pesticide concentrations reported are nominal and calculated based on the analytical information provided by the respective manufacturers of the various formulations which were all well within their active period. Although these are hydrophobic compounds, they have been formulated such that water dilutions can be made from them for field application by farmers.

### Single toxicity tests

The individual toxicities of the three insecticides were determined by exposing the *Oreochromis niloticus* fingerlings to several concentrations of each insecticide in separate standard 96 h static non-renewal test systems according to standard OECD protocol (OECD 1992). Fingerlings were not fed during the test period and no aeration was done in test aquaria. The following six deltamethrin concentrations were tested: 2.5, 5, 8.19, 10.0, 12.8 and 15.0 µg

deltamethrin/l while the cypermethrin concentrations tested were: 4, 8, 16, 25 and 35 µg cypermethrin/l. In the case of dimethoate the tested concentrations were 19.0, 38.1, 42.8, 47.6 and 57.1 mg dimethoate/l. Seven fingerlings were used per concentration in 13 L of water (control) or test solution and each concentration was tested in triplicate. Each concentration was prepared separately by measuring the pesticide formulation and mixing with aerated water. This was then distributed into the three glass aquaria making up the triplicate. The controls which were also in triplicate only contained aerated water since no solvent was used in any of the test solutions. The fingerlings were then introduced using a hand net starting from the controls and followed by the test concentrations from lowest to highest. The number of dead fingerlings were recorded every 24 up to 96 h and used to establish the respective concentration-response curves. Various lethal concentrations were calculated using the Microsoft Excel macro, REGTOX, which models the concentration–response relationship with the non-linear Hill equation ([http://eric.vindimian.9online.fr/en\\_index.html](http://eric.vindimian.9online.fr/en_index.html)). The concentration of each pesticide was then normalized to the respective LC50 concentration for that individual insecticide as in (Laetz et al. 2009). The LC50-normalized concentration is described as the Toxic Unit (TU) by Rasmussen et al. (2013). All data for the three pesticides expressed in the form of LC50 normalized concentrations were subsequently combined and fitted with a single non-linear (3-Parameter Logistic) regression as in Laetz et al. (2009). The 3-Parameters Logistic (3PL) non-linear regression model is commonly used for curve-fitting analysis in bioassays such as dose-response curves. It is characterized by its classic “S” or sigmoidal shape that fits the top plateaus of the curve, the EC50, and the slope factor (Hill’s slope). The curve is symmetrical around its inflection point. The 3PL equation is given by:

$$y = \frac{a}{1 + e^{-\left(\frac{x-x_0}{b}\right)}} \tag{2}$$

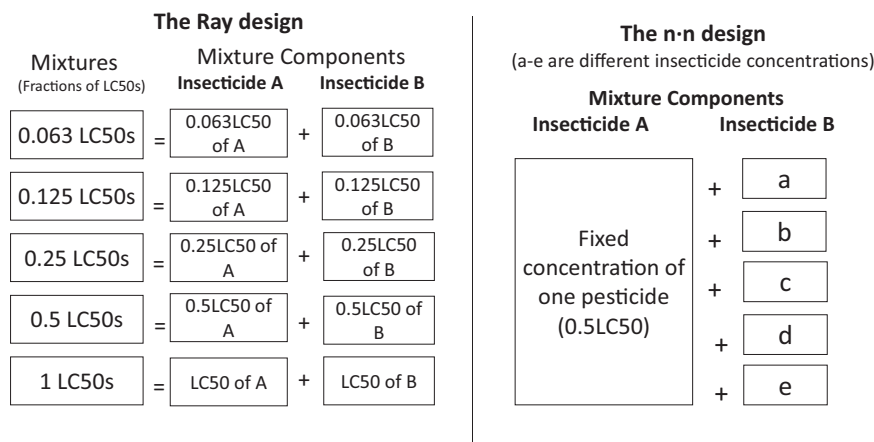
where  $y$  = effect (i.e. % mortality);  $x$  = Concentrations,  $a$  = maximum effect (which was defined to be  $\leq 100$ ),  $x_0$  = LC50;  $1/b$  = gradient at the linear part of the curve and  $e$  = the natural logarithm base. Since all concentrations in this study were normalized to respective LC50 values, this implies that all concentrations ( $x$ ) in the equation are LC50 normalized concentrations or TU.

The resulting regression equation obtained from the combination of individual pesticides concentration-response curves in the present study was later used to predict mixture mortalities based on the CA and IA models to determine which of them gave a better prediction.

**Mixture toxicity tests**

Mixture toxicity tests were carried out following a composite experimental design in the same standard test system described for single insecticides. This design combines aspects of the n-n design and Ray design for covering any possible interactions at various mixture ratios (Altenburger et al. 2003). Firstly, the n-n design was used to determine the concentration-response relationship of mixtures containing a fixed concentration (0.5 LC50) of a pyrethroid insecticide and increasing concentrations of the organophosphate insecticide (dimethoate) in the binary mixtures and vice versa. Secondly, the Ray design was used where equal mixture ratios, in terms of fractions of LC50s, were tested (Fig. 1). Preparations of the LC50s obtained from single pesticide concentration-response curves were tested alongside mixtures in the Ray design experiments. Following normalization for potency, the concentrations of all mixture components can be summed to obtain a value that can be used to predict toxicity (Altenburger et al. 2003). Effective mixture concentrations were obtained by normalizing each mixture component to its respective LC50 and adding up the normalized concentrations in the case of CA (Laetz et al. 2009) or using Eq. 1 in the case of IA.

**Fig. 1** Ray and n-n experimental designs for mixture experiments



**Table 1** Effective concentrations of mixtures tested in the n-n design based on the Concentration Addition (CA) and Independent Action (IA) models

Mixtures	Concentrations of mixture components		Concentrations of mixture components normalized to respective LC50s		Effective mixture concentrations	
	Dimethoate Conc (mg/l)	Deltamethrin ( $\mu$ g/l)	Dimethoate	Deltamethrin	CA	IA
0.5LC50 Dimethoate + Deltamethrin concentrations	22.75	2.25	0.50	0.25	0.75	0.70
	22.75	5.00	0.50	0.55	1.05	0.92
	22.75	8.19	0.50	0.90	1.40	0.97
	22.75	10.00	0.50	1.10	1.60	1.01
	22.75	12.80	0.50	1.40	1.90	1.06
0.5LC50 Deltamethrin + Dimethoate concentrations	19.00	4.50	0.42	0.49	0.91	0.70
	38.10	4.50	0.84	0.49	1.33	0.92
	42.86	4.50	0.94	0.49	1.43	0.97
	57.10	4.50	1.25	0.49	1.75	1.13
Mixtures	Dimethoate Conc (mg/l)	Cypermethrin ( $\mu$ g/l)	Dimethoate	Cypermethrin	Effective Mixture Conc (CA)	Effective Mixture Conc (IA)
0.5LC50 Cypermethrin + Dimethoate concentrations	19.00	4.20	0.42	0.48	0.90	0.70
	38.10	4.20	0.84	0.48	1.32	0.92
	42.80	4.20	0.94	0.48	1.42	0.97
	46.60	4.20	1.02	0.48	1.51	1.01
	51.10	4.20	1.12	0.48	1.61	1.06
0.5LC50 Dimethoate + Cypermethrin concentrations	22.30	2.00	0.50	0.23	0.73	0.62
	22.30	4.00	0.50	0.46	0.96	0.73
	22.30	8.00	0.50	0.92	1.42	0.96
	22.30	16.00	0.50	1.84	2.34	1.42

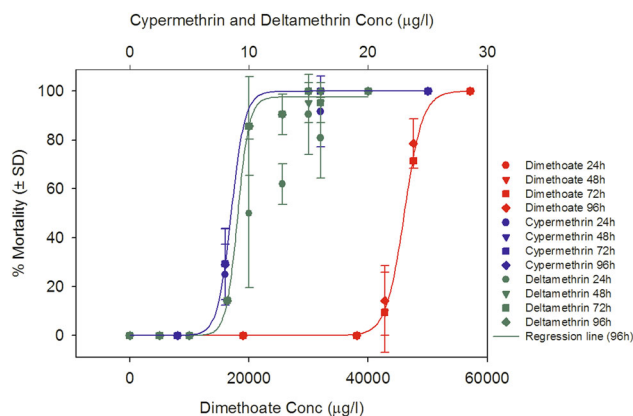
**Table 2** Concentrations of mixtures tested in the Ray design

Concentrations of mixture components		concentrations of mixture components normalized to respective LC50s		Effective mixture concentrations	
Dimethoate (mg/l)	Deltamethrin ( $\mu$ g/l)	Dimethoate	Deltamethrin	CA	IA
2.84	0.57	0.06	0.06	0.12	0.12
5.7	1.1	0.125	0.125	0.25	0.24
11.4	2.3	0.25	0.25	0.5	0.44
22.75	4.6	0.5	0.5	1	0.75
45.5	9.13	1	1	2	1.00
Dimethoate (mg/l)	Cypermethrin ( $\mu$ g/l)	Dimethoate	Cypermethrin	Effective Mixture Conc (CA)	Effective Mixture Conc (IA)
5.7	1.1	0.125	0.125	0.25	0.24
11.4	2.1	0.25	0.24	0.49	0.44
22.75	4.2	0.5	0.48	0.98	0.75

Mixtures tested in the n-n and Ray designs are shown in Tables 1 and 2 respectively.

Concentration-response curves from the n-n design mixture study were compared with single pesticide concentration response curves while results from the Ray design experiments were compared with effects of tested

LC50s of single insecticides. The regression equation derived from the combination of individual data sets of the 3 tested insecticides was used to calculate predicted mortalities for each mixture and plotted. To determine the precision of predictions, the 95% confidence intervals were calculated using Microsoft Excel (Brown 2001) and plotted



**Fig. 2** Comparing individual 24–96 h concentration-response curves for deltamethrin, dimethoate and cypermethrin

using SigmaStat 4.0. The predicted mixture concentration-response curves according to the CA and IA models were then compared with the observed mixture concentration-response curve in order to determine how well the mixture effects could be predicted by the CA and IA models. Deviations from the model were quantified using the model deviation ratio (MDR), which is defined as:

$$MDR = \frac{Expected}{Observed} \tag{3}$$

where Expected = effective concentration of the mixture that would be predicted by the model and Observed = effective concentration for the mixture obtained from toxicity testing. An MDR of 1.0 indicates perfect fit to the model (Belden et al. 2007). Greater than 1 indicates that the model predicts a higher effective mixture concentration than is observed for the same effect level (less toxicity predicted than observed), while less than 1 indicates more observed toxicity than predicted. In addition, one sample t-tests were performed between observed results and predictions to determine the significance of deviations. All graphs were plotted using Sigmaplot 2000.

## Results

### Single toxicity tests

The individual concentration-response curves obtained for cypermethrin, deltamethrin and dimethoate over the 96 h exposure period are shown on Fig. 2 from which it can be seen that maximum mortality occurred within 24 h of exposure in all insecticides except for deltamethrin where the mortality increased up to 48 h after which no further increase in effects occurred until 96 h. This is confirmed by

the median lethal concentrations (LC50s), obtained from the concentration-response relationships at various exposure periods and presented on Table 3 in which the LC50s for cypermethrin and dimethoate did not change significantly from 24 to 96 h (all 95% Confidence Intervals (CI) overlap) while in the case of deltamethrin there is a clear although small difference between the 24 h LC50 and those from 48 to 96 h (no overlap of 24 h LC50 CIs with the others). However, Cypermethrin and deltamethrin were equitoxic since the confidence limits around their LC50s overlap at all exposure times when compared to each other (Table 3). On the other hand, there was an enormous difference between the LC50s of the pyrethroid insecticides and that of dimethoate with deltamethrin and cypermethrin being respectively 4986 and 5232 times more toxic than dimethoate. In addition, all curves had very steep slopes. The highest tested concentrations that did not result in any mortality (NOECs) for cypermethrin, deltamethrin and dimethoate were respectively 4.0 µg/l, 5.0 µg/l and 38.1 mg/l. Due to the steep concentration-response curves for these pesticides, these NOEC values were higher than half of the respective LC50s (>0.5 LC50s). The concentration-response curves of the three insecticides were compared by fitting each of the 96 h data with a non-linear regression curve (Fig. 2). Given the huge differences in toxicities the concentrations of the two pyrethroid insecticides were plotted on a different x-axis from that of dimethoate. The slopes of the three concentration-response curves are clearly seen to be parallel to each other showing that a single relationship can be used to describe all curves. All concentrations were therefore normalized to the respective LC50s and the data sets from all three insecticides combined and plotted together (Fig. 3). A single 3-parameter non-linear regression curve together with its corresponding 95% CIs were then fitted to the entire dataset and a regression equation describing the relationship was obtained (Eq. 4).

$$y = \frac{98.53}{1 + e^{-\left(\frac{x-1.0058}{0.057}\right)}} \tag{4}$$

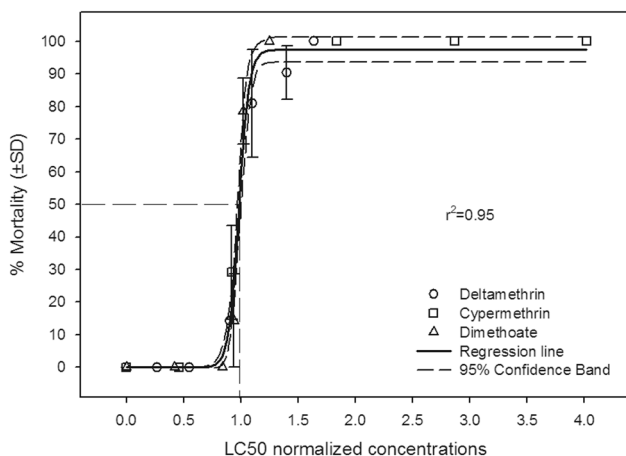
Equation 4 gives the LC50 value as 1.0058. In addition, the 95% confidence interval around the linear part of the slope was computed to be 0.019. This indicates a high level of confidence implying that Eq. 4 will give accurate predictions of mixture effects and was therefore used to statistically predict mixture effects (Laetz et al. 2009).

### Mixture toxicity tests

Effective mixture concentrations were used for comparison of mixture effects in the n-n design mixture experiments with LC50 normalized concentrations of single insecticides. Figure 4 shows dimethoate-deltamethrin mixture effects

**Table 3** Lethal concentrations at 24 to 96 h and associated 95% lower and upper confidence intervals

Exposure time (h)	Deltamethrin			Cypermethrin			Dimethoate		
	LC <sub>50</sub> (ug/l)	95% Confidence interval (CI)		LC <sub>50</sub> (ug/l)	95% Confidence interval (CI)		LC <sub>50</sub> (mg/l)	95% Confidence interval (CI)	
24	10.53	9.87	11.38	9.93	8.6	11.4	46.16	45.05	47.13
48	9.14	8.92	9.46	8.7	8.4	9.3	46.16	45.26	47.13
72	9.14	8.89	9.42	8.7	8.4	9.3	46.16	45.23	47.13
96	9.13	8.86	9.42	8.7	8.4	9.3	45.52	44.56	46.47

**Fig. 3** Combined dose-response curve for deltamethrin, cypermethrin and dimethoate at 96 h

while Fig. 5 shows dimethoate-cypermethrin mixture effects based on both CA and IA models. In all cases the mixtures caused higher mortalities compared with corresponding single insecticides. This can be seen as shifts of the mixture concentration-response curves to the left of the single insecticide curves. In the case of dimethoate-cypermethrin mixtures containing increasing cypermethrin concentrations (Fig. 5b) the increase in mortality was so drastic that there were no intermediate mortalities and therefore the concentration-response curve could not be established. Figures 4 and 5a also show that at lower mixture concentrations (up to about 0.5 LC<sub>50</sub>) there was hardly any difference in the mixture effects calculated either by CA or IA but as the mixture concentrations increased there was increasing difference in mixture effects depending on the model used to calculate effective mixture concentrations with IA mixture concentration-response curves deviating more from the single pesticide curves.

In the Ray design mixture experiments binary mixtures containing equivalent (equipotent) fractions of LC<sub>50</sub>s of either cypermethrin or deltamethrin with dimethoate were tested side by side individual LC<sub>50</sub> preparations of deltamethrin, cypermethrin and dimethoate. Results show that the single pesticides at their respective LC<sub>50</sub>s gave mean

mortalities within the expected 50% mortality range (Fig. 6). However, no mortality was observed in all deltamethrin-dimethoate mixtures up to 0.5 LC<sub>50</sub>s (Fig. 6a) while for cypermethrin-dimethoate mixtures mortality was observed right from 0.25LC<sub>50</sub> (Fig. 6b). In all cases, 100% mortality was observed in mixtures of 1LC<sub>50</sub> instead of 50% mortality that is expected based on the CA model.

All observed mixture toxicity data obtained from both n-n and Ray design toxicity tests were then combined, and regression curves based on the CA and IA models fitted with the maximum y value set at 100 (that is “a ≤ 100” in Eq. 2) (Fig. 7) giving Eqs. 5 and 6 respectively.

$$y = \frac{100.00}{1 + e^{-\left(\frac{x-0.6062}{0.1243}\right)}} \quad (5)$$

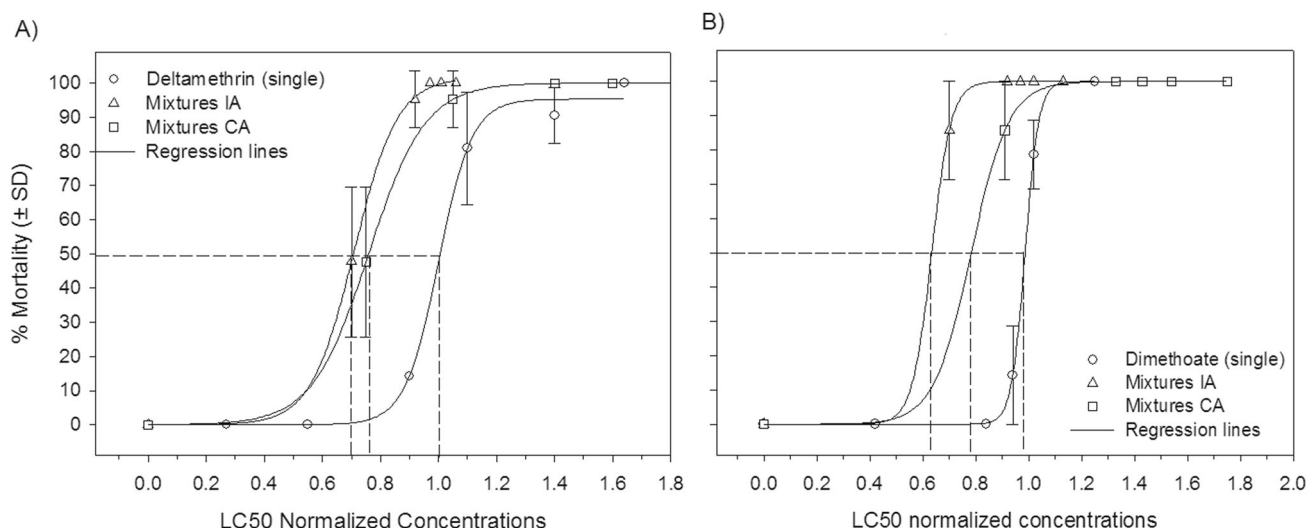
$$y = \frac{99.86}{1 + e^{-\left(\frac{x-0.508}{0.089}\right)}} \quad (6)$$

Equation 4 was used to calculate predicted mortalities based on CA and IA models and single regression curves fitted separately on the entire CA and IA model datasets (Fig. 7). The regression equations obtained following predictions from CA and IA models are shown in Eqs. 7 and 8 respectively.

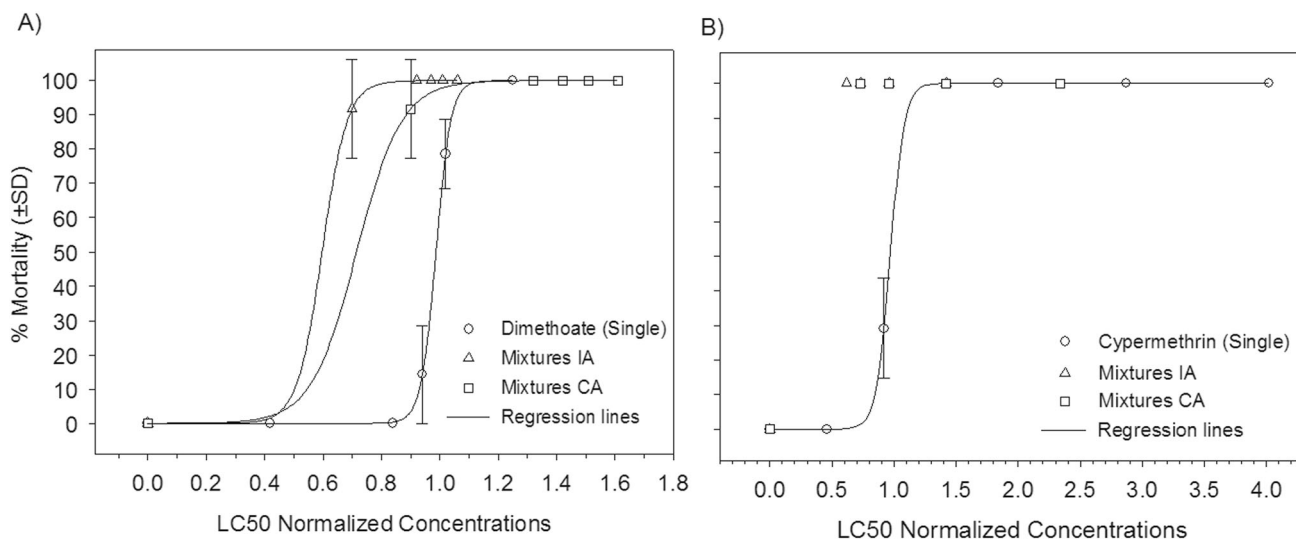
$$y = \frac{98.52}{1 + e^{-\left(\frac{x-1.005}{0.05767}\right)}} \quad (7)$$

$$y = \frac{97.97}{1 + e^{-\left(\frac{x-1.004}{0.05424}\right)}} \quad (8)$$

The calculated 95% confidence intervals of the CA and IA predicted effects were respectively ±0.006 and ±0.002 showing a high level of precision in the model predictions. It can be seen from Fig. 7 that both the CA and IA model concentration-response curves aligned with each other although IA predicted relatively higher effects. This is not unexpected given that both predictions were based on the common concentration-relationship described by Eq. 4. Therefore differences in the model fitness came from the deviations from their respective observed effects. Equations 5 to 8 were used to calculate observed and predicted mixture concentrations corresponding to various effects ranging from 5 to 95% mortality (Table 4) in order to



**Fig. 4** Effects of Dimethoate-Deltamethrin mixtures compared with respective single insecticides at 96 h. **a** mixture effects compared with deltamethrin. **b** mixture effects compared with dimethoate

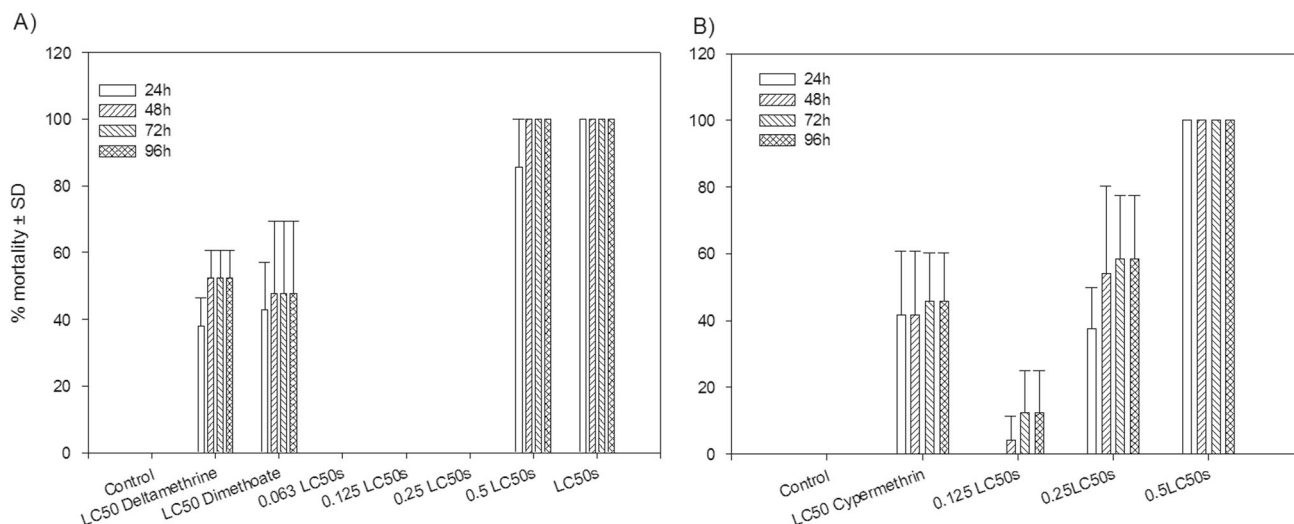


**Fig. 5** Effects of Dimethoate-Cypermethrin mixtures compared with respective single insecticides at 96 h ( $n = 21$  per concentration, Error bars =  $\pm 1SD$ ). **a** mixture effects compared with dimethoate. **b** mixture effects compared with cypermethrin

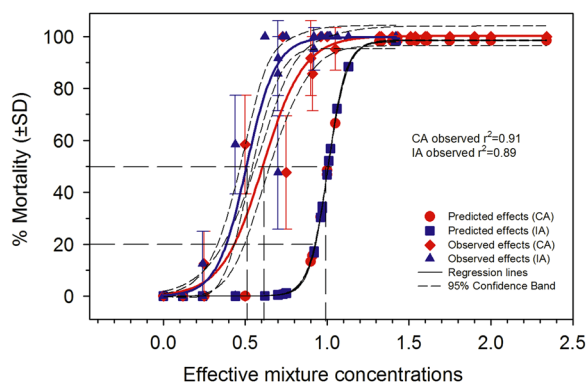
determine deviations of observed mortalities from the predicted values based on the CA and IA models using Model Deviation Ratios (MDRs) (Eq. 3). These are presented on Table 4 from which it can be seen that in all cases the MDRs were greater than 1 showing that mixture concentrations predicted by both models were higher than the respective observed concentrations for the same mixture effect level. This indicates that both models predicted less toxicity than was observed throughout the concentration-response curves as seen in the plots of MDRs values against % mortality (Fig. 8). This figure also shows that both CA and IA model deviations were lower at higher mortalities (which correspond to higher concentrations) and increased progressively as mortality reduces (i.e. reducing

concentrations). It was essential to determine the significance of these model deviations. An MDR of 2 implies that the predicted effect concentration is double the observed value. From Fig. 8 it is seen that the IA model MDRs were  $\geq 2$  at all concentrations almost up to LC50% while the CA model had MDRs  $\geq 2$  only up to about LC30. In addition Fig. 7 shows that the 95% confidence intervals of the CA and IA curves overlap at lower effect concentrations but split up just before 50% effect and remain separate until effects greater than 95%, which is the region of maximum effects. These imply that at low concentrations both CA and IA give similar predictions but as the mixture concentration increases the difference between predictions from the two models become more and more significant





**Fig. 6** Effects of equipotent mixtures compared with single pesticide effects at their respective LC50s at various exposure times from 24 h to 96 h ( $n = 21$  per concentration, Error bars =  $\pm 1SD$ ). **a** Deltamethrin-dimethoate mixtures. **b** Cypermethrin-dimethoate mixture



**Fig. 7** Comparing observed mixture concentration-response curves from CA and IA models to predicted mortalities at 96 h ( $n = 21$  per concentration, Error bars =  $\pm 1SD$ )

with CA giving a relatively better prediction overall. T-tests also showed an overall highly significant difference ( $P = 5.8 \times 10^{-7}$ ) between IA predicted and observed effects while the difference was not significant ( $p = 0.06$ ) for CA. This corroborates the MDR results where the IA model gave higher deviations than corresponding CA. Despite this, the CA model was not a good predictor of mixture effects as there was greater than additive effects observed indicating synergism particularly at lower concentrations.

## Discussion

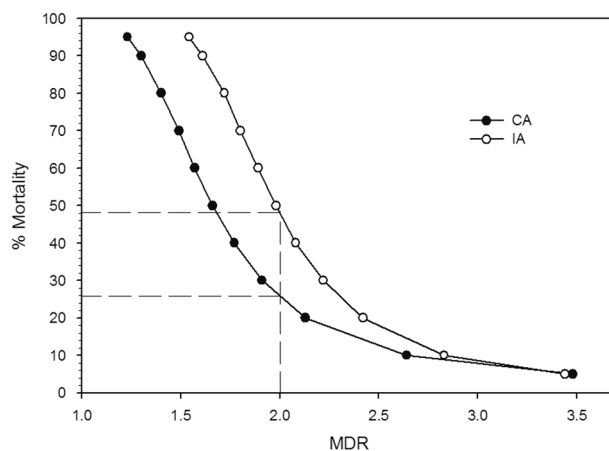
Several pesticide combinations that are frequent contaminants in surface water have not been tested despite the predictability of their co-occurrence due to applications in the same crop settings (Altenburger et al. 2013). The present

study has assessed the effects of binary mixtures of pyrethroid (cypermethrin, deltamethrin) and organophosphate (dimethoate) insecticides using *Oreochromis niloticus*.

Individual LC50s obtained for cypermethrin and deltamethrin in the present study show that these pyrethroid insecticides are highly toxic to *Oreochromis niloticus* in agreement with published literature which demonstrate that pyrethroid insecticides are highly toxic to fish and aquatic invertebrates (Boateng et al. 2006; Schäfer et al. 2007; Khalili et al. 2012; Prusty et al. 2015). In addition, similar to DeLorenzo et al. (2014), we observed very steep concentration-response curves for all three insecticides tested with most of the observed toxicity occurring within 24 h of exposure. This could be due to the fact that pyrethroid insecticides are strongly hydrophobic and as such, the water-soluble fraction of pyrethroids introduced into an aquatic system will be short-lived and quickly reduced (Palmquist et al. 2012). However, contrary to the findings of DeLorenzo et al. (2014), cypermethrin and deltamethrin had similar levels of toxicity. The two pyrethroid insecticides were by far more toxic than dimethoate. This can be explained by the fact that cypermethrin and deltamethrin, being Type II pyrethroids, are very potent neurotoxins with toxicity being solely attributed to alpha-cyano groups which have been shown to have increased biological activity (greater nerve membrane depolarization and block of sensory and motor axons) (DeLorenzo et al. 2014; Prusty et al. 2015). On the other hand, although dimethoate inhibits acetylcholinesterase (AChE) like other organophosphates (Van Scoy et al. 2016), it may have a low affinity for the acetylcholinesterase enzyme in *Oreochromis niloticus*. The dynamics of interaction of organophosphate compounds with their target site has been shown to depend largely upon

the affinity of a particular insecticide for the enzyme (Tortelli et al. 2006) and marked differences have been shown in the inhibition kinetic parameters between several fish species (Silva Filho et al. 2004). In addition, Svendsen et al. (2010) reported higher toxicity of organophosphates (Chlorpyrifos and Diazinon) relative to the pyrethroid (permethrin) and attributed their results to the more effective interaction of organophosphates with their target (acetylcholinesterase) in *C. elegans*. This is contrary to our results where the pyrethroids were more toxic than the organophosphate. However, permethrin is a Type I pyrethroid and according to Prusty et al. (2015) Type II ( $\alpha$ -cyano) pyrethroids are more potent neurotoxicants than Type I (non cyano) pyrethroids.

Pesticide mixtures continue to pose major challenges for natural resource agencies including the data gaps that exist for many individual chemicals, experimental design difficulties, poorly understood pathways for chemical interaction, potential differences in response among species, and the need for more sophisticated statistical tools for analyzing complex data (Laetz et al. 2009). The approach used in the present study to explore mixture effects of the studied insecticides involved a composite experimental design which has the advantage of combining both the n-n and Ray designs in order to cover all possible interactions at various mixture ratios (Altenburger et al. 2003). This approach removed the limitation of only testing equivalent fractions of insecticide LC50s and made it possible to test a wider range of mixture ratios, which is a much more realistic scenario as chemical mixtures in the environment do not necessarily exist in equipotent ratios. Because of the very steep concentration-response curves of the tested insecticides, concentrations equivalent to half of their respective LC50s (0.5LC50) were lower than the NOECs and therefore fell within the range of concentrations which did not result in any mortality, thus the choice of this particular



**Fig. 8** Comparing deviations (MDR) of the CA and IA model predictions at various mixture effect levels

concentration for the mixture studies in the n-n design. Our results show that binary pyrethroid-organophosphate insecticides mixtures at all levels led to increased mortality compared to the individual insecticides. This implies that mixtures of the insecticides at concentrations at which neither of the components in the mixture were toxic resulted in mortality. This is in agreement with the assertion that compounds present in the field at concentrations far below their individual median effective concentration or no observed effect concentration (NOEC), may still contribute to substantial effects (Altenburger et al. 2003).

The combined effect of insecticides may be without interaction, or it may be interactive (i.e., synergistic or antagonistic). Having established that the observed toxicity of all mixtures was higher than that for single insecticides irrespective of which of the two models was used, it was necessary to determine whether or not there was interaction taking place in the mixtures. Analysis of joint action of chemical mixtures is based on comparing the observed

**Table 4** Predicted and observed mixture lethal concentrations and associated model deviation ratios (MDR)

% Mortality	Concentration addition			Independent action		
	Observed mixture concentration	Predicted mixture concentration	MDR	Observed mixture concentration	Predicted mixture concentration	MDR
5	0.2402	0.8361	3.48	0.2461	0.8455	3.44
10	0.3331	0.8792	2.64	0.3126	0.8861	2.83
20	0.4339	0.9261	2.13	0.3848	0.9302	2.42
30	0.5009	0.9574	1.91	0.4328	0.9596	2.22
40	0.5558	0.9831	1.77	0.4721	0.9839	2.08
50	0.6062	1.0067	1.66	0.5082	1.0062	1.98
60	0.6566	1.0306	1.57	0.5444	1.0288	1.89
70	0.7115	1.0568	1.49	0.5838	1.0538	1.80
80	0.7785	1.0894	1.40	0.6320	1.0850	1.72
90	0.8793	1.1410	1.30	0.7048	1.1355	1.61
95	0.9722	1.1950	1.23	0.7726	1.1920	1.54

effects to a reference model (Altenburger et al. 2003). CA still serves as a useful reference point to predict or describe the joint action of compounds, even if theoretical considerations indicate that CA is not expected (Deneer 2000). In the present research observed effects were compared to predictions from both the CA and IA models. Based on the CA concept, when toxicants have a similar mode of action, they act jointly in an additive manner after normalizing for potency while the IA concept assumes that compounds in the mixture act independently (Belden et al. 2007; Nowell et al. 2014). Interactions are therefore discovered as deviations from the CA or IA models. Our results demonstrated that there was a deviation of observed mixture mortalities from predictions based on either the CA or IA models with predicted mixture effects in all cases being lower than observed effects. Synergism is a comparative statement, as it describes a mixture toxicity that is substantially higher than expected. Its use, therefore, requires a definition of the expected response of a mixture. Only this allows an unambiguous use, by comparing experimental observations with the expected response (Laetz et al. 2009; Altenburger et al. 2013). Small deviations that are not considered to be biologically important are not statistically significant (Mumtaz et al. 2010). In order to avoid leaving judgement of significance to the discretion of the investigator or risk assessor, Belden et al. (2007) have proposed a scale whereby observed model deviation ratio (MDR) values that fall within a factor of 2 of the predicted value (between 0.5 and 2.0) are not considered to be significantly different from the model. Although arbitrary, this range provides a benchmark for determining the significance of deviation of the model (Belden et al. 2007). In our study MDRs above a factor of 2 were obtained with the IA model at all effect concentrations up to almost LC<sub>50</sub> while MDRs greater than 2 were only obtained below LC<sub>30</sub> with the CA model. This implies that the deviation from both models was significant at lower mixture concentrations (<LC<sub>30</sub>) but not significant at higher concentrations. It is interesting to note that t-test results largely corroborated the MDR results as it gave an overall significant difference for deviations from the IA predicted effects while overall deviation from the CA model predictions was not significant. Therefore, the arbitrary limits for significance of MDR values set by Belden et al. (2007) are statistically relevant, even if the biological relevance has not been demonstrated. MDR has the additional benefit of being able to distinguish the significance of deviations at various points on the concentration-response curves. Consequently, MDR has proven to be an important parameter for determining the significance of model deviation. Based on the MDRs, both models predicted lower toxicities than was observed. This is contrary to reports that CA usually predicts either a slightly higher mixture toxicity (Altenburger

et al. 2000; Backhaus and Faust 2012) or CA and IA predict virtually identical mixture toxicities (Backhaus and Faust 2012). Cedergreen et al. (2008) reported no convincing difference between CA and IA models in the predictability of the joint effect of mixtures with binary data from bacteria, daphnia, and algae. While this was true at lower effect levels in our study, we have shown a clear difference in the predictions from the two models at higher effect levels. This indicates that both models may be equally relevant when predicting mixture effects at environmentally relevant concentrations. However neither model proved to be a good predictor of effects in the present study and the pyrethroid-organophosphate mixtures studied were synergistic, particularly at lower effect concentrations.

Our findings also showed that for any given pair of pyrethroid-organophosphate insecticides prediction of the combined effects were independent of mixture ratio but dependent on the effective mixture concentration. This finding has important implications for risk assessment of these insecticide mixtures as the CA model which is proposed as the default model will underestimate mixture toxicity at environmentally relevant concentrations. Interestingly, the IA model gave worse predictions than CA showing that while the compounds in the mixtures did not act as dilutions of each other, they did not act independently of each other either. There was therefore interaction of mixture components resulting in greater than additive (synergistic) mixture effects particularly at lower mixture concentrations.

The synergistic effects of mixtures containing pyrethroid and organophosphate insecticides may be largely explained by their modes of action. Although pyrethroid and organophosphate insecticides are all neurotoxins, they have different modes of action. Organophosphate pesticides are known to selectively inhibit cholinesterase activity. The inhibition creates a buildup of acetylcholine at the nerve synapses disabling the enzyme cholinesterase that is vital for a functioning central nervous system. The concentration of acetylcholine in the synapses results in continuous stimulation of the muscles eventually leading to seizures, exhaustion and possibly death (Tortelli et al. 2006; Van Scoy et al. 2016). On the other hand, the principal mechanism of action of pyrethroids is the disruption of sodium channel function in the nervous system. They react with voltage-gated sodium channels on nerves, prolonging the time during which the channels are open. This results in altered nerve function, which manifests either as a series of short bursts or a prolonged burst, and is caused by repetitive discharge of nerve signals or stimulus-dependent nerve depolarization. In the case of Type 2 pyrethroids, which includes cypermethrin and deltamethrin, this manifests as hyperactivity, incoordination, convulsions and writhing (Palmquist et al. 2012; DeLorenzo et al. 2014). Therefore,

while organophosphate insecticides act on the central nervous system of exposed animals, pyrethroid insecticides disrupt the normal function of the peripheral nervous system (Palmquist et al. 2012). They clearly have different modes of action but the outcomes at a higher level in the organism are very similar implying that mixtures should produce additive effects. However, this is not the case since organophosphate insecticides also inactivate esterase, causing reduced detoxification of pyrethroid insecticides, which results in greater toxicity than would be expected (Svendsen et al. 2010; Laetz et al. 2014). Pyrethroids and organophosphate pesticides like dimethoate are primarily metabolized by monooxygenases, after which they are conjugated and excreted. For the pyrethroids, first phase metabolism results in metabolites that are less toxic than the parent compound (Svendsen et al. 2010). However, for dimethoate and many other organophosphates, first-phase metabolism produces the oxon metabolite which is more toxic than the parent compound and is the main active form (Svendsen et al. 2010). Inactivation of esterase would therefore not increase dimethoate toxicity but will slow down pyrethroid breakdown. Consequently the synergistic effects in our study is likely to be due mainly to increased toxicity of the pyrethroid insecticides in the mixture. Mixtures of pyrethroid and organophosphate pesticides is therefore likely to be mostly synergistic irrespective of the individual potencies of the mixture components.

## Conclusion

There is significant concern over the toxic effects of pyrethroid and organophosphate insecticides to non-target aquatic organisms exposed simultaneously to low concentrations of these chemicals because they are highly used and their combinations are among those identified as producing the largest number of deviations from CA (Deneer 2000). Knowledge about interactions in aquatic organisms of specific compounds belonging to these groups is, however, quite sparse and predicting whether or not enhanced toxicity will occur for a given combination of compounds is therefore often not straight forward. For regulatory purposes it is worthwhile to identify combinations of compounds which give rise to deviations from CA-predicted toxicity (Deneer 2000). In the present study a composite experimental study design has proved to be a useful method to explore a wide range of mixture ratios in assessing the joint effects of binary pyrethroid-organophosphate insecticide mixtures on *Oreochromis niloticus*. Our results show that binary pyrethroid-organophosphate insecticide mixtures at all levels led to increased mortality compared to the individual insecticides. In addition, mixtures made up of insecticides at concentrations lower than their respective

NOECs caused mortality. This implies that mixtures of the insecticides at concentrations at which neither of the components in the mixture were toxic led to mortality. The CA and IA models both predicted significantly lower mixture effects than observed. Although mixture effects were independent of mixture ratios, MDRs indicated greater deviations of both models at lower concentrations. This implies that the deviation from both models was significant at lower mixture concentrations but not significant at higher concentrations showing that model predictions were dependent on effect level. Pyrethroid-organophosphate insecticide mixtures therefore showed synergistic behavior especially at lower mixture concentrations which may be environmentally relevant. Therefore, both the CA and IA models may underestimate risk if used for certain pyrethroid-organophosphate insecticide mixtures.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no competing interests.

**Ethical Approval** Institutional guidelines for the care and use of animals have been followed.

**Informed Consent** This article does not contain any studies with human participants performed by any of the authors.

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