 doi:10.20944/preprints202103.0752.v1

Article

Assessing combined effects for mixtures of similar and dissimilar acting neuroactive substances on zebrafish embryo movement

Afolarin O. Ogungbemi [1,3,*], Riccardo Massei [2], Rolf Altenburger [1], Stefan Scholz [1] and Eberhard Küster [1],

[1] Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research - UFZ, Permoserstraße 15, 04318 Leipzig, Germany

[2] Department of Effect-Directed Analysis, Helmholtz Centre for Environmental Research - UFZ, Permoserstraße 15, 04318 Leipzig, Germany

[3] Institute for Environmental Sciences, University of Koblenz-Landau, Fortstraße 7, 76829 Landau, Germany

* Correspondence: afolarin.ogungbemi@ufz.de

Received: date; Accepted: date; Published: date

Abstract: Risk assessment of chemicals is usually conducted for individual chemicals whereas mixtures of chemical are occurring in the environment. Considering that neuroactive chemicals are a group of contaminants that dominate in the environment, it is then imperative to understand the combined effects from mixtures. The commonly used models to predict mixture effects, namely concentration addition (CA) and independent action (IA), are thought suitable for mixtures of similarly or dissimilarly acting components, respectively. For mixture toxicity prediction, one important challenge is to clarify whether to group neuroactive substances based on similar mechanisms of action, e.g. same molecular target or rather similar toxicological response, e.g. hyper- or hypoactivity (effect direction). We addressed this by using the spontaneous tail coiling (STC) of zebrafish embryos, which represents the earliest observable motor activity in the developing neural network, as a model to elucidate the link between mechanism of action and toxicological response. Two questions were asked: 1.) Can the mixture models CA or IA be used to predict combined effects for neuroactive chemical mixtures when the components share a similar mode of action (i.e. hyper- or hypoativity) but show different mechanism of action? 2.) Will a mixture of chemicals where the components show opposing effect directions result in an antagonistic combined effect? Results indicate that mixture toxicity of chemicals such as propafenone and abamectin as well as chlorpyrifos and hexaconazole that are known to show different mechanisms of action but similar effect directions were predictable using CA and IA models. This could be interpreted with the convergence of effects on the neural level leading to either a collective activation or inhibition of synapses. We also found antagonistic effects for mixtures containing substances with opposing effect direction. Finally, we discuss how the STC may be used to amend risk assessment.

Keywords: Mixture toxicity; Neurotoxicity; Antagonism; Organophosphate; Acetylcholinesterase inhibitors; GABA; Behavior; Risk assessment; Spontaneous movement activity;

1. Introduction

Chemicals typically occur as mixtures in the environment and hence, organisms are exposed to a combination of these chemicals. However, prospective risk assessment is conducted for single chemicals and may not account for combined effects [1]. Since it is practically impossible to test all the possible combinations of chemical exposure, modeling of mixture toxicity allows to at least predict an expected effect of several chemicals from their individual effects.

Two common mixture toxicity models are concentration addition (CA) and independent action (IA). CA is based on the notion that mixture toxicity can be predicted by the addition of the fractions of exposure and effect concentrations for the mixture components. In addition, the single components

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

of the mixture should cause a similar effect or target a similar receptor in the organism [2]. On the other hand, IA may be applied when compounds are acting independently [3] which has been interpreted as acting on different target sites in the organism [4]. Both models have been found to be reasonably predictive in several studies exposing unicellular organisms to bioactive compounds with known mechanisms of action [5–7]. Nevertheless, these models cannot predict interaction of chemicals at the physical, toxicokinetic or toxicodynamic level [8]. In this case CA and IA models may be used to evaluate observations as antagonistic (less effect than predicted) or synergistic (higher effect than predicted) and to quantify such deviations.

Neuroactive chemicals are often found in insecticidal and pharmaceutical products. Here they represent active ingredients designed to interact with specific targets and receptors of the nervous system. Busch et al. [9] found that neuroactive substances are the largest group (13%) of chemicals detected in European surface waters. Despite neuroactive substances being often detected in the environment, only few studies have explored how neuroactive substances act in mixtures to induce combined neurotoxicity (eg. Corbel et al. [10]; Yang et al. [11]) and how to use mode of action knowledge to group them for mixture effect prediction using CA and IA models.

Zebrafish embryos are considered as an alternative model to animal testing since they are considered to feel less pain or distress [12]. Due to behavioral patterns already established in embryonic stages, embryos are also frequently used as a model for neurotoxicity assessment. Several behavioral test methods have been developed such as spontaneous tail coiling (STC), photomotor response (PMR) and locomotor response (LMR) (reviewed in Ogungbemi et al. [13]). Despite the potential of non-lethal endpoints such as behavior for ecotoxicology research, the applicability of CA and IA models to such endpoints for mixture effect prediction is not well studied. Hence, it is valuable to investigate the applicability of CA and IA models for such experimental systems to predict and understand how mixtures of neuroactive substances may act in the environment.

To implement mixture models, bioassays capable of quantitatively detecting impact on the nervous system are required. In this study we explored the spontaneous tail coiling (STC) of zebrafish embryos, one frequently used assay for assessing neuroactivity. STC represents the earliest motor activity observed in developing zebrafish embryos. It is the result of the innervation of the muscles by the primary motor neurons and can be observed using embryos beginning at 17 hours post fertilization (hpf) [14,15]. Measurement of the STC frequency has been proposed as an indicator for the function and development of the nervous system [13,16] and has been used to study the effects of diverse neuroactive chemicals [17–20]. Until now the STC has not been used as a test method to measure mixture neurotoxicity based on a chemical's mode or mechanism of action.

In this study, we define mechanism of action as the interaction of neuroactive chemicals with specific molecular targets such as acetylcholinesterase (AChE) and gamma aminobutyric acid (GABA) activated ion channels. While mode of action is defined here as the series of key events (including the mechanism of action) in the nervous system leading to a measurable toxicological response such as hyper- or hypoactivity behavior phenotypes (referred to as effect direction onwards). Hypoactivity refers to a decrease in the STC frequency, while hyperactivity refers to the increase with respect to the level in non-exposed embryos.

The STC test has been shown to discriminate movement activity changes due to exposure to chemicals with different modes of action causing either hyper- or hypoactivity but not those with different mechanisms of action [13,17]. This led to the STC neuroactivity hypothesis which states that a neuroactive substance will induce increased STC (hyperactivity) in zebrafish embryos if its mechanism of action directly or indirectly leads to activation of the neuronal synapse and vice versa for hypoactivity. For example, different mechanisms of action such as AChE inhibition and GABA antagonism may both enhance neuronal activation potential in the neuromuscular synapses by inducing the inflow of sodium ions and blocking the inflow of chloride ions respectively [21]. Both mechanisms are expected to cause hyperactivity response regardless of the different target receptors. Similarly, compounds activating GABA receptors or blocking sodium channels may cause hypoactivity through enhancing the inhibitory synapses [22].

Based on such prior knowledge about the link between mechanism of action and toxicological response, we defined two levels of similarity for our mixture toxicity expectation: (i) The mixture

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

components are known to have similar target receptors or mechanism of action and (ii) they show similar toxicological response (i.e. effect direction: hyper- or hypoactivity) in the STC test.

Previous studies, have shown that effect direction in the STC test is related to the mechanism of action. In contrast, it is still open if this also works for mixture exposure. Therefore, the goal of the present study is to address the following questions: 1.) Can the additivity models CA or IA be used to predict combined effects for neuroactive chemical mixtures when the components share a similar mode of action (hyper- or hypoativity) but show different mechanism of action? 2.) Will a mixture of chemicals where the components show opposing effect direction result in an antagonistic combined effect? We demonstrate that mixtures of neuroactive substances with different mechanisms of action follow the additivity concept and we propose ways to use the STC test in risk assessment.

2. Materials and Methods

2.1. Test organism

Zebrafish embryos were raised from an in-house hybrid strain (OBI-WIK strain, F3 generation). The adults were cultured under 14 h light/10 h dark photoperiod in 120 L aquaria (tap water, 26.5 ± 1 °C). Spawning was initiated by inserting spawning trays 4–6 h before the end of the light cycle prior to the spawning day. Eggs were collected and cleaned 1 h after the onset of light. Fertilized embryos were selected according to Kimmel et al. [23] with a microscope and embryos between the 16^{th} and 128^{th} cell stage were used to start the exposure.

2.2. Chemicals

Chlorpyrifos (99.9 %, CASRN 2921882), hexaconazole (CASRN 79983-71-4), abamectin (100 %, CASRN 71751412) and propafenone-hydrochloride (CASRN 34183-22-7) were purchased from Sigma-Aldrich. Carbamazepine (99 %, CASRN 298464) was purchased from Acros Organics™ and chlorpyrifos-oxon (97.9 %, CASRN 5598152) from Dr. Ehrenstorfer GmbH. Stock solutions were prepared in 100% dimethyl-sulfoxide (DMSO) and diluted in ISO water as specified in ISO 7346-3 (1996) [80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl].

2.3. Mixture testing in the STC test

Mixture components were selected according to their mechanism of action and effect direction (hyper- or hypoactivity) as follows: Mixture A - compounds with same mechanism of action and same effect direction; Mixture B - compounds with different mechanism of action but same effect direction; Mixture C – compounds in A and B; Mixture D – compounds with different mechanism of action and different effect direction. Mixtures A and B are binary while C and D are ternary. The exposure concentration of the mixtures are based on the effect concentrations of the single substances given in Table 1 and 2. The detailed procedures for STC testing have been previously reported in detail [24]. Briefly, twenty fertilized embryos were exposed in 20 mL of the mixture solution prepared from DMSO stock solution (0.1% maximum concentration) of the components, within a 60 mm glass crystallization dish covered with a watchmaker glass. Two replicates per concentration and at least 2 independent experiments were conducted. The exposed embryos were incubated at 28 °C under 14 h light/10 h dark photo-period for 21 ± 1 h. On the next day, at 24 hpf, exposed embryos were removed from the incubator and allowed to acclimatize to room temperature for at least 30 min. Videos of normally developed embryos were recorded for 60 s. Collected videos were analyzed for STC counts by means of a workflow using the KNIME® Analytical Platform [24,25].

Table 1. Effect of single substances in the STC test

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

Substance	Mechanism of action[a]	Expected activity i.e. effect direction	STC EC_{50} (µmol/L)[b]	Slope of crc[b]
Chlorpyrifos	Acetylcholinesterase inhibitor*	Hyperactivity	1.85 (1.95)	1.30
Chlorpyrifos-oxon	Acetylcholinesterase inhibitor*	Hyperactivity	0.32 (0.44)	1.00
Hexaconazole	Ergosterol biosynthesis inhibitor*	Hyperactivity	4.03 (3.63)	1.80
Abamectin	Activation of GABA-gated chloride channel[$]	Hypoactivity	0.06 (0.09)	1.70
Carbamazepine	Sodium channel blocker[#]	Hypoactivity	271	2.28
Propafenone	Sodium channel blocker[#]	Hypoactivity	32 (46)	1.94

[a]Mechanism of action was obtained from different sources including [#]http://drugbank.com, *pesticide properties database (https://sitem.herts.ac.uk/aeru/ppdb/index.htm) and [$]Sánchez-Bayo, (2012). [b]Data obtained from Ogungbemi et al., (2020), the minimum and maximum of the concentration response curves (crc) were set to 0 and 100, respectively. Values in parenthesis were obtained from independent experiments and were used for the mixture modelling.

Table 2. Summary of the mixture design and toxicity predictions

Mixture type	Substance mixture	Observed activity	Mix ratio	Highest exposure (µmol/L)	Predicted EC_{50} (µmol/L)		Observed EC_{50} (µmol/L)
					CA	IA	
Mixture A	Chlorpyrifos & chlorpyrifos oxon	Hyperactivity	0.816:0.184	5	1.19	1.16	1.25
	Carbamazepine & propafenone	Hypoactivity	0.86:0.14	320	159	207	132
Mixture B	Hexaconazole & chlorpyrifos	Hyperactivity	0.65:0.35	15	2.79	3.69	2.79
	Abamectin & propafenone	Hypoactivity	0.002:0.998	90	23	27.6	17.4
Mixture C	Chlorpyrifos, hexaconazole & chlorpyrifos-oxon	Hyperactivity	0.603:0.324:0.073	12	2	2.19	1.95
Mixture D	Chlorpyrifos, hexaconazole & abamectin	Hyper & Hypoactivity	0.34:0.64:0.02	5	-*	-	-

*no mixture and toxicity predictions

2.4. Mixture modeling

Mixture toxicity modeling was based on the concentration addition (CA) and independent action (IA) models. Effect data for the single substances used for mixture modelling was obtained from a previous study [17]. The CA mixture modeling is based on the effect concentration of the individual chemicals and it considers chemicals in a mixture to be a dilution of each other [5]. It is used to predict mixture toxicity of chemicals with a similar mechanism of action.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

$$ECx_{Mix} = \sum_{i=1}^{n} \frac{P_i^{-1}}{ECx_i} \qquad (1)$$

Equation 1 shows the mathematical representation of the CA model where ECx_{Mix} is the total concentration of the mixture provoking x effect (i.e. 50 % effect), P_i is the fraction of component i which represents the concentration of component i in the mixture, ECx_i is the concentration of component i provoking x effect, when applied singly.

The IA mixture modeling is based on the effect induced by individual chemicals in a mixture. It is usually applied to predict the mixture toxicity of chemicals with dissimilar mechanism of action.

$$EC_{Mix} = 1 - \prod_{i=1}^{n}(1 - EC_i) \qquad (2)$$

Equation 2 shows the mathematical representation of the IA model where EC_{Mix} is the total effect of the mixture and EC_i is the effect of component i in the mixture when applied singly. Mixture toxicity modeling was performed using an in-house excel sheet and the mixtox package in R [27].

2.5. Concentration response modeling

Data from the mixture experiment were obtained as STC count per minute (STC frequency). The mean STC frequency was estimated for the exposed 20 embryos. The absolute STC frequency varied between the independent experiments. To combine results from independent experiments, mean percentage change in STC frequency with respect to unexposed embryos from independent experiments was estimated. Concentration-response modeling of the percentage change in STC frequency was performed using the 4-parameter logistic function (LL.4) of the drc package in R [28].

$$y = c + \frac{(d - c)}{1 + \left(\frac{x}{e}\right)^b} \qquad (3)$$

Equation 3 shows the dose response model where b is the slope; c and d are the minimum and maximum STC response set to 0 and 100, respectively; and e is the inflection point e.g. the EC_{50}.

In cases of hyperactivity, the maximum effect of STC frequency was different for the three tested hyperactive chemicals - chlorpyrifos, chlorpyrifos-oxon and hexaconazole (see Figure 1). Mixture prediction using different maximal of the percentage STC effect would have been based on a non-equitoxic mixture ratio of EC_{50}, EC_{41} and EC_{24} for hexaconazole, chlorpyrifos and chlorpyrifos-oxon respectively. To equalize the mixture ratio and maximum effect, the percentage STC change was standardized by dividing with the maximum effect for each chemical to obtain a standardized percentage hyperactivity effect leading to 100 % maximum effect for all hyperactive chemicals (Figure 1). Skipping this hyperactivity standardization step would have led to unpredictability of mixture effects higher than that of the chemical with the least maximal effect. Scholze et al. [29] used the toxic unit extrapolation approach to equalize and extend the dose response curves for partial agonists. However, the observed hyperactivity effect in this study is usually followed by hypoactivity (possibly due to paralysis) at higher concentrations and this could indicate a saturated hyperactive effect. This appears not to support partial agonism but rather, the differential maximal effect of the chemicals could be an indication of different mechanism of hyperactive action. A partial agonist is expected to act as an antagonist in the presence of a full agonist [30] but this was not observed in the present study. Consequently, we consider the standardized percentage hyperactivity effect to be more representative of the observations and for mixture modeling in this study. The effect concentration causing 50% increase or decrease of the STC was estimated from the concentration-response curve and the confidence interval was estimated as 2 times the standard error.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

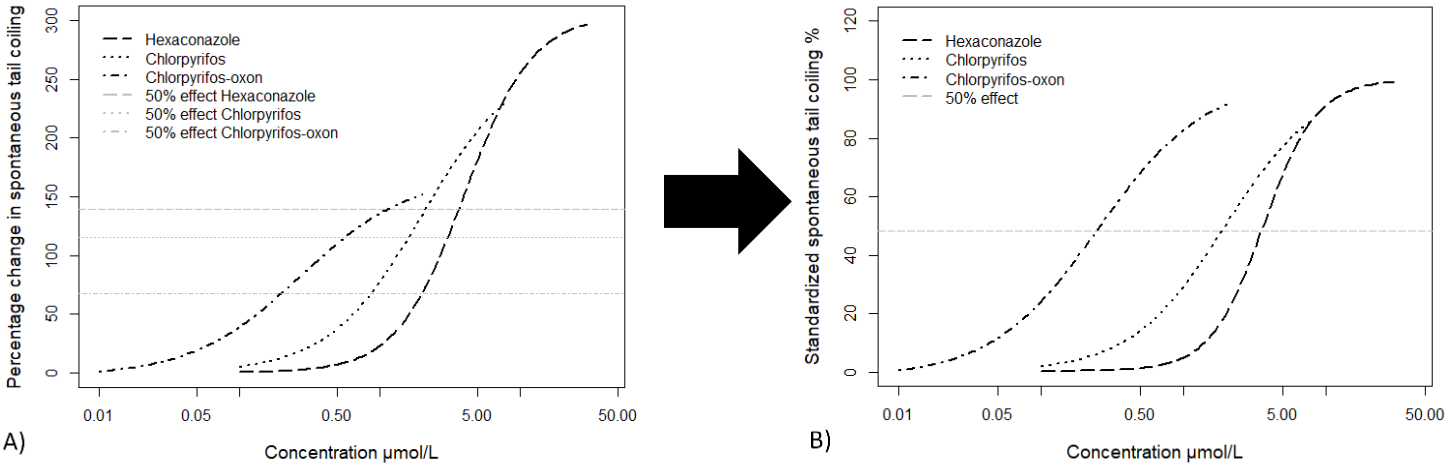

Figure 1. Visual representation of the data transformation for hyperactivity inducing chemicals: A) Concentration response curves showing different maximal for the hyperactivity inducing substances. The horizontal lines shows EC_{50}, EC_{41} and EC_{24} which corresponds to the 50 % effect for hexaconazole, chlorpyrifos and chlorpyrifos-oxon respectively; B) Standardized concentration response curves for the hyperactivity substances. The horizontal line shows the same 50 % effect for the 3 substances after standardization.

2.6. Measurement of the exposure concentrations

For quantifying chlorpyrifos/chlorpyrifos-oxon and chlorpyrifos/hexaconazole mixtures, chemical analyses were conducted using an HPLC system (Merck-LaChrom) with diode array (model L7450) detector. One mL of the exposure solution for each concentration of the respective mixtures was sampled and 30 µL was injected directly. A reversed-phase column (Lichrospher 60 Reverse Phase (RP) select B, Merck, C-8), with a particle size of 5 µm was used. The column temperature was set to 40 °C and the flow rate was adjusted to 0.5 mL/min. Different mobile phase ratios of AcN:water were used for chlorpyrifos/chlorpyrifos-oxon (57:43 %, elution time of 15 min) and chlorpyrifos/hexaconazole (65:35 %, elution time of 12 min). The substances were detected at an absorbance of 207 nm. For quantifying carbamazepine/propafenone and abamectin/propafenone mixtures, chemical analyses were performed on a linear ion trap/Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Scientific). Samples were diluted 100 (carbamazepine/propafenone) and 10 (abamectin/propafenone) times with ISO water before injection. An Agilent 1200 series HPLC system with a Kinetex C18 column (100 x 3 mm, 2.6 µm particle size, Phenomenex) was used for chromatographic separation after injection of 10 µL of sample. We used 0.1% formic acid and methanol containing 0.1% formic acid as mobile phases at a column temperature of 40 °C and a flow rate of 0.4 mL/min. The analysis was conducted in full scan mode with a mass range of m/z 100-1000 in negative and positive mode ESI with a nominal resolving power of 100,000 (referenced to m/z 400). For peak integration, compound calibration, and compound quantification, the software program TraceFinder 3.2 (Thermo Scientific) was used.

3. Results

3.1. Chemical analysis

Results of the chemical analysis are shown in Table 3. Measured concentration were close to the nominal concentration, typically with a maximum deviation of about 20 % for the highest

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

concentrations (propafenone 3 and 37 %), carbamazepine (8.8 %), chlorpyrifos (20 and 20 %), chlorpyrifos-oxon (19 %) and hexaconazole (15 %). Abamectin was below detection limit in all the measurements. Reasons might be due to losses or rather adsorption to the test vessels because of its high lipophilicity ($logD_{pH7.4(ACD/Labs)}$ of 5.85). It is important to note that chlorpyrifos concentrations in DMSO stock solutions declined by 25 - 40 % after 2 months of storage. However, this reduction in concentration did not lead to significant difference in the STC effect (data not shown). Therefore, we used the nominal concentrations for further mixture toxicity evaluations based on the assumption that a 20 % difference between nominal and measured concentrations will not cause a significant change in the observed effect.

Table 3. Measured concentrations of single substances in each mixture in micromole/liter. Values in round brackets are the percentage change of the measured concentrations with respect to the nominal concentrations.

Hyperactive Mixture A		**Hypoactive Mixture A**		**Hyperactive Mixture B**		**Hypoactive Mixture B**	
Chlorpyrifos	**Chlorpyrifos-oxon**	**Carbamazepine**	**Propafenone**	**Chlorpyrifos**	**Hexaconazole**	**Abamectin**	**Propafenone**
<MDL [0.25]	<MDL [0.05]	92.2 (+36)	22.1 (+120)	<MDL [0.2]	0.4 (-4)	<MDL [0.009]	6.0 (+37)
0.2 (-59)	<MDL [0.1]	128.0 (+20)	33.1 (+89)	0.2 (-50)	0.8 (+5)	<MDL [0.018]	11.4 (+31)
0.7 (-32)	0.5 (+109)	190.8 (+11)	47.7 (+70)	0.6 (-37)	1.8 (+10)	<MDL [0.035]	20.2 (+15)
1.8 (-12)	0.6 (+39)	250.7 (-8.8)	61.3 (+37)	1.4 (-23)	3.6 (+10)	<MDL [0.07]	31.4 (-10)
3.2 (-20)	1.1 (+19)			2.8 (-20)	7.5 (+15)	<MDL [0.14]	68.0 (-3)

MDL = Method detection limit. Chlorpyrifos MDL = 0.4 µM, Chlorpyrifos-oxon MDL = 0.1 µM, Hexaconazole MDL = 0.3 µM, Carbamazepine MDL = 0.0045 µM, Propafenone MDL = 0.0034 µM, Abamectin MDL = 0.0005 µM, squared brackets = nominal concentrations

3.2. Description of mixture effect in comparison to CA and IA model

The effects of single substances used in the mixture testing have already been described in Ogungbemi et al. [17] and are summarized in Table 1. The mixture effects exceeded those of the single substances for all mixtures. Concentration response curves for the observed and predicted mixture effects as well as those for the single substances are shown in Figure 2. Observed and predicted EC_{50} values are also shown in Table 2.

Hyperactive mixture A (chlorpyrifos and chlorpyrifos-oxon) induced hyperactivity at EC_{50} of 1.25 µM. The CA and IA models were similar and they both predicted the EC_{50} of the mixture (Table 2). The prediction curves were within the confidence boundary of the tested mixture at low and mid concentrations but both models slightly deviated and overestimated the effect at higher concentrations (Figure 2A). The hypoactive mixture A (carbamazepine and propafenone) caused hypoactivity at an EC_{50} of 132 µM. The EC_{50} of Both CA (159 µM) and IA (207 µM) underestimated the mixture effect. Nevertheless, CA was predictive at low and medium high concentrations (50-150 µM) while IA was less predictive and underestimated the hypoactivity effects except at lowest concentration range up to 100 µM (Figure 2B). Overall the estimation difference was always below a factor of 2 for CA and IA.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

Hyperactive mixture B (chlorpyrifos and hexaconazole) showed hyperactivity at EC_{50} of 2.79 µM (Table 2). CA could predict the exact observed EC_{50} of the mixture but IA slightly underestimated the mixture effect [EC_{50} = 3.69 µM] (Figure 2C). Hypoactive mixture B (abamectin and propafenone) showed hypoactivity at EC_{50} of 17.4 µM. Both CA and IA slightly underestimated the mixture toxicity with EC_{50}s of 23 and 27.6 µM respectively. CA aligned with the confidence boundary of the observed mixture effect while IA deviated from the observed concentration response curve (Figure 2D). Further, we tested a ternary mixture (Mixture C comprising of chlorpyrifos, chlorpyrifos-oxon and hexaconazole). Both CA and IA models showed similar predictions and were predictive of the observed mixture effect (Figure 3). In general, we observe a trend where CA and IA could very well predict mixture hyperactivity effects but to a slightly lesser extent for the hypoactivity effects - though these differences were minor.

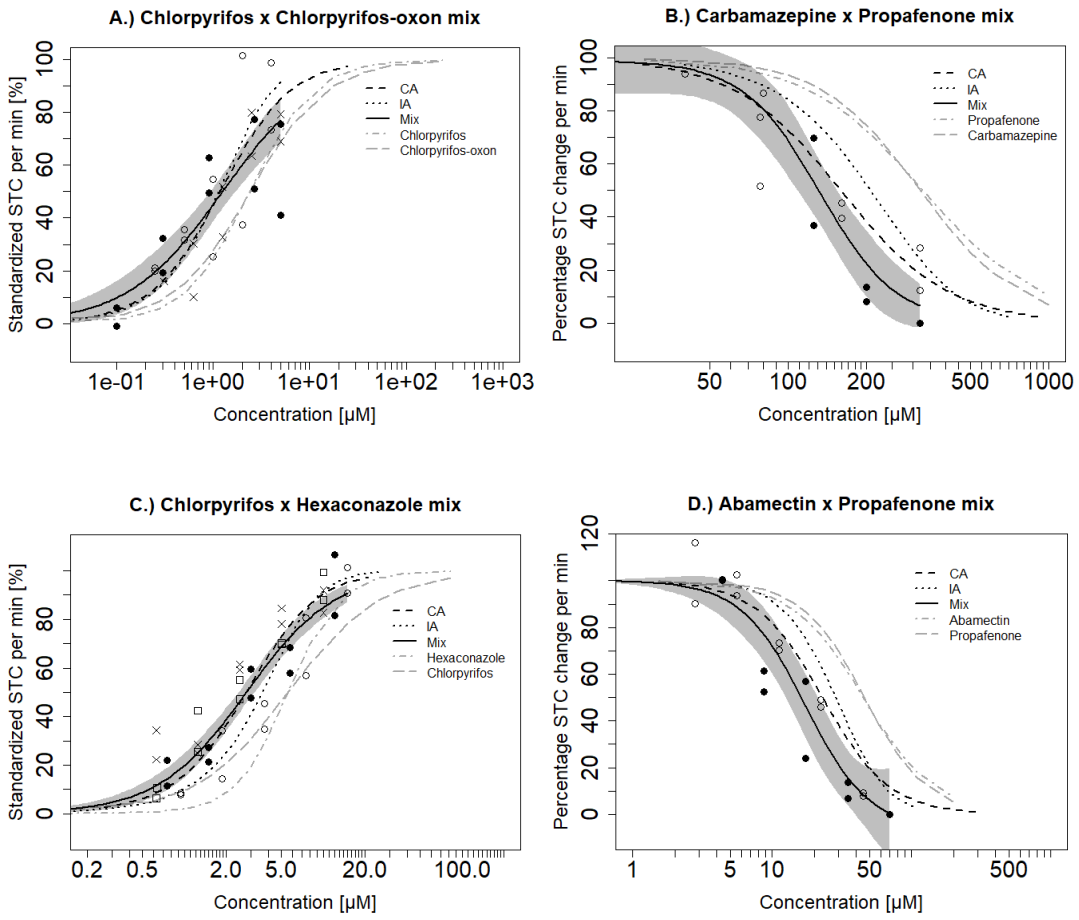

Figure 2. Comparison of observed mixture effects of binary mixtures with predicted mixture effects based on the concentration addition (CA) and independent action (IA) models, as well as, with effect levels expected from the single substances in the STC test. Grey shaded area represents the confidence interval of the fitted mix model for the observed effect. Different symbols represent observed effect data from independent mixture experiments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

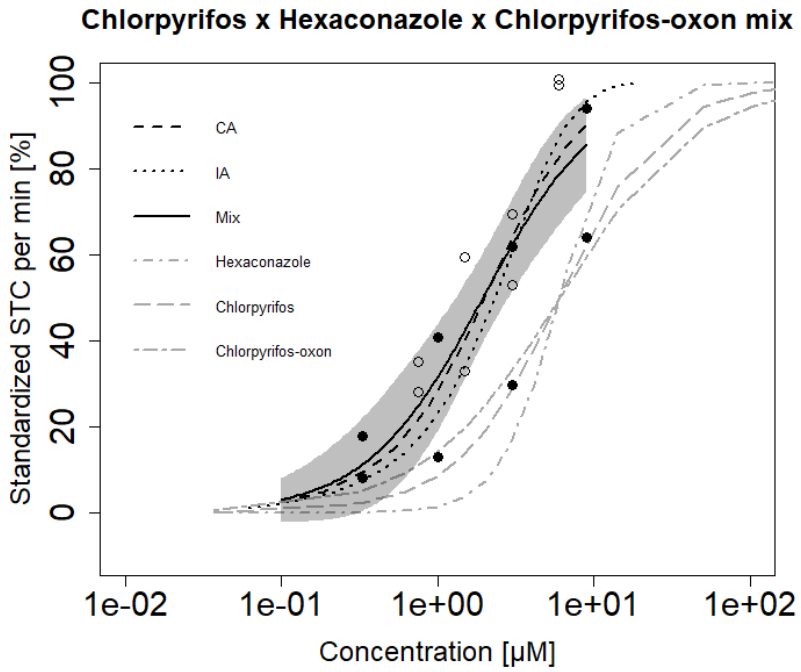

Figure 3. Comparison of observed mixture effects of a ternary mixture with predicted mixture effects based on the concentration addition (CA) and independent action (IA) models, as well as, with effect levels expected from the single substances in the STC test. Grey shaded area represents the confidence interval of the fitted mix model for the observed effect. Different symbols represent observed effect data from independent mixture experiments.

To test if the simple case assumption of CA i.e. substances are a dilution of each other and an equitoxic concentration of one can replace another [5], holds true for combined neurotoxicity effects in the STC test, we performed dilution experiments with the ternary mixture to simulate the hyperactivity mixtures A and B (chlorpyrifos and hexaconazole as well as chlorpyrifos and chlorpyrifos-oxon respectively). A portion of chlorpyrifos was replaced with an EC_{50} equitoxic portion of chlorpyrifos-oxon in mixture A and hexaconazole in mixture B (Figure 4 A and B). An EC_{50} of 2.13 µM was estimated for hexaconazole and (chlorpyrifos + chlorpyrifos-oxon) which was lower than the hexaconazole and chlorpyrifos mix by only a factor of 1.3. The mixture of chlorpyrifos-oxon and (chlorpyrifos + hexaconazole) showed an EC_{50} of 1.77 µM which was higher than that of chlorpyrifos-oxon and chlorpyrifos mixture by only a factor of 1.4.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

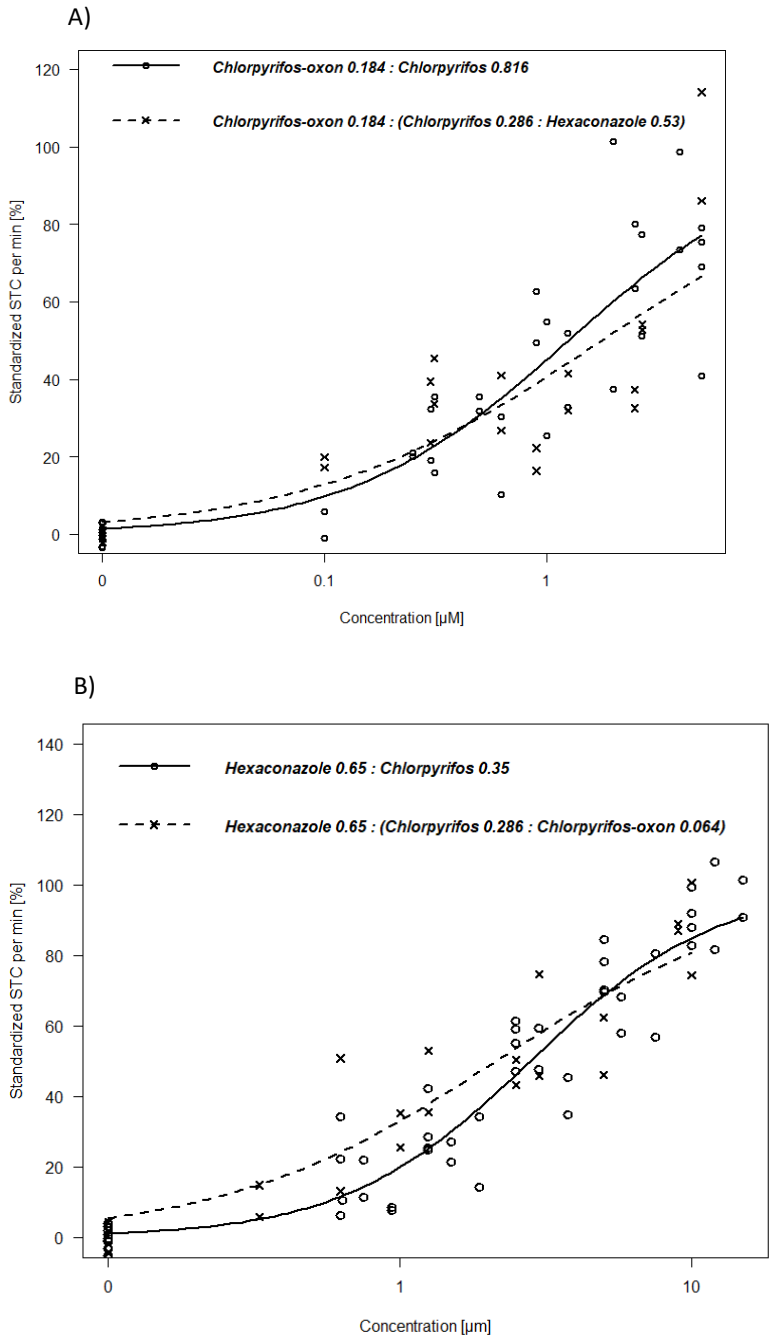

Figure 4. A ternary mixture is used to simulate a binary mixture by replacing a portion of one of the binary components with an equitoxic proportion of another substance: A) Concentration response curves for hyperactive Mixture A containing chlorpyrifos-oxon and chlorpyrifos. Portions of chlorpyrifos was replaced with hexaconazole; B) Concentration response curves for hyperactive Mixture B containing hexaconazole and chlorpyrifos. Portions of chlorpyrifos was replaced with chlorpyrifos-oxon.

3.3. Antagonistic mixture effects in the STC test

To understand if and how compounds with different mechanisms of action and opposing effect direction would interact in the STC test, we exposed a ternary mixture of substances (Mixture D) with different toxicological response (i.e. hyper- and hypoactivity). Mixtures were designed to reflect an unequitoxic scenario (0.33 hypoactivity : 0.66 hyperactivity) by mixing the hypoactivity causing abamectin with two hyperactivity causing substances (chlorpyrifos and hexaconazole). The

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 31 March 2021 doi:10.20944/preprints202103.0752.v1

result shows that the antagonistic effect of abamectin significantly decreased the hyperactivity effect expected from hexaconazole and chlorpyrifos (hyperactive mixture B) [Figure 5].

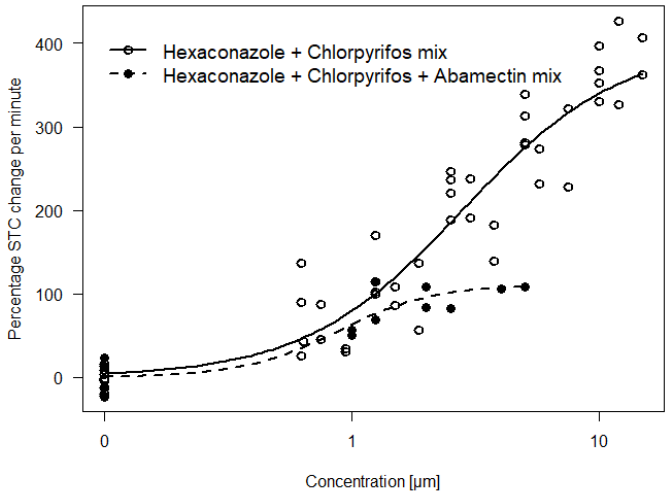

Figure 5. Antagonistic mixture effect in the STC test. Concentration response curves showing the antagonistic effect of abamectin on the expected hyperactivity due to chlorpyrifos and hexaconazole.

4. Discussion

In order to evaluate mixture toxicity of neuroactive compounds, two main challenges have to be considered regarding the application of prediction models: 1.) Neuroactive chemicals in mixtures interact with different biochemical targets. To capture the effects of such a mixture, a possibility is to measure the effects at converging key events 2.) Mixtures may comprise of neuroactive chemicals with opposing effects. Consequently, we explored 1) whether mixture effects of neuroactive substances with similar effect directions (whether hyper- or hypoactivity) but different mechanisms of action would be additive and if concentration addition (CA) or independent action (IA) models can predict such mixture effect and 2) if mixtures of neuroactive substances with different mechanisms/modes of action and opposing effect direction would induce observable antagonistic effects. In order to address these challenges we used an established behavior test, the spontaneous tail coiling (STC) of zebrafish embryos. It is responsive to diverse mechanisms of actions that finally translate to increased or reduced frequency of spontaneous movements as a result of either activation or inhibition of the neuronal synapse leading to hyper- or hypoactivity respectively (STC neuroactivity hypothesis). Accordingly, we hypothesized in the present study that neuroactive chemicals inducing the same response (either hyper- or hypoactivity) in the STC test can be predicted from CA or IA models. In contrast, compounds with mode of actions with opposing effects would result in antagonistic effects if compared to individual compounds.

4.1. Mixture components with different mechanisms of action but similar effect direction can act in an additive way

The first goal of the present study was focused on addressing the question – "*Can additivity be assumed for a mixture of substances with the same mode of action (e.g. antiandrogenic) but not the same mechanism of action (e.g. receptor-blocking and inhibition of androgenproduction)?*" which was posed in Kortenkamp et al. [31]. Based on theory, CA model is adequate to predict mixture toxicity of similarly acting components (i.e. similar mechanisms of action) while IA is assumed to hold for dissimilarly acting chemicals. However, CA may also be applied to predict the effect of chemicals showing similar toxicological responses (i.e. hyper- or hypoactivity) or modes of action [32]. We

 doi:10.20944/preprints202103.0752.v1

hypothesized that irrespective of the mechanism of action, compounds inducing the same toxicological response (whether hyper- or hypoactivity) would also lead to an additive response in the STC. This allows to define the similarity/dissimilarity of mixture components based on a combined knowledge of both mechanism of action and toxicological response. For example, mixture components of different mechanisms of action - an acetylcholinesterase (AChE) inhibitor (chlorpyrifos) and a suspected gamma aminobutyric acid (GABA) receptor inhibitor (hexaconazole) were expected to individually enhance axonal action potential thereby leading to a combined hyperactivity effect (Figure 2C). The combined effect was reflected in the STC behavior endpoint and was predictable using the CA and IA models. Predictions of the IA model were very close to those of CA and this is not surprising for a binary mixture considering that the differences between the models increases with more mixture components [33]. CA and IA models could also predict combined effect of pyrethroids and organophosphates in a *D. magna* immobility assay [34].

The predictability of the mixture models for differing neuro-mechanisms as observed in zebrafish embryos and daphnids may not be applicable in other test systems or endpoints with different levels of complexity or specificity [35]. For instance, CA and IA are expected to give different predictions for simpler but specific neuro-endpoints such as neural electric signal which may not reflect an integrated output as the STC but this remains to be investigated. Therefore, it is dependent on the mechanistic understanding of the test endpoint if neuroactive substances acting on different targets in the nervous system should be considered as similarly or dissimilarly acting components [34]. This also indicates that the assessment of similarity/dissimilarity of mixture components should go beyond knowledge of molecular targets and should consider other factors such as toxicological response and secondary mode of action [36].

4.2. Mechanistic understanding of the predictability power of CA and IA

The STC is presumed to be generated by depolarizations which trigger action potentials in the synapses of the primary motor neurons [37]. Consequently, it is not farfetched to consider different target interaction or mechanisms of action as similarly acting in so far as they result in the same key-event (activation or inhibition of neuronal synapses) and same toxicological response (hyper- or hypoactivity). In this case, we may consider neuroactivity via the STC endpoint to be an integrated effect on neuronal synapses and CA might be more appropriate to predict mixture effects of chemicals in the STC. We showed in the present study the capacity of CA to predict mixture B (substances with different mechanisms of action but similar effect direction). This is consistent with previous studies on nervous system related endpoints. For example, Wolansky et al. [38] found that CA was a good predictor of the mixture neurotoxicity of different pyrethroids on the motor activity of rats and Gonçalves et al. [39] reported that CA was adequate to predict mixture effect of PAHs on fish behavior.

Further, the accuracy of IA model in complex organisms such as zebrafish embryos has been questioned due to converging signaling pathways and inter-dependent subsystems [31,35,40]. For instance, Corbel et al. [10] found that a carbamate and pyrethroid had a converging effect on acetylcholine concentration in the synapse even though they have different mechanisms of action. Estrogen receptor activation was also seen as an integrated effect of different cascading steroidal receptor signaling [29]. In addition, we could simulate concentration additive mixtures by replacing a portion of the mixture component with another similar acting substance (similar effect direction but different mechanism of action) [Figure 4A and B]. This adds credence to the CA assumption that components can be described as a dilution of each other in the STC test. However, the results of mixture assessment with STC do not allow to favor one of the models as the differences between CA and IA were quite small.

Mixture toxicity prediction using CA and IA models assume that the mixture components do not interact to affect the uptake, distribution, metabolism and elimination of each other [8,41]. Mixture interaction of neuroactive substances may occur via the biotransformation pathways due to the reduced activation or competition for biotransformation sites [42]. Organophosphates were found to be a major synergistic group due to their ability to inhibit esterases which are responsible for phase

 doi:10.20944/preprints202103.0752.v1

2 biotransformation of chemicals [43]. However, we did not observe synergistic interaction of a mixture of chlorpyrifos and its oxon metabolite in the present study and this could be due to potential limited biotransformation capacity of early stages of the zebrafish embryo [44] or the sensitivity of our test system. Other mixture neurotoxicity studies have shown interaction effects. For example, a mixture of chlorpyrifos and nickel on zebrafish embryo was found to be antagonistic [45] and mixture of atrazine and chlorpyrifos was assessed as synergistic [46]. However, 120 and 96 hpf embryos which should have higher rates for biotransformation into the active oxon metabolite were used in these studies.

4.3. Mixture components with different mechanisms of action and opposing effect direction are antagonistic

We investigated the STC outcome for mixtures comprising of different mechanism of action as well as opposing effect direction. The results show that mixtures with both hyper- and hypoactivity inducing components will lead to antagonistic interaction (Figure 5). Our results corroborate the recommendation of a chemical grouping for mixture analysis based on common adverse outcomes (hyper- and hypo-activity in this case) with less emphasis on similarity of mechanism of action [31]. Information on common adverse outcomes such as hyper- and hypoactivity will be useful to qualitatively predict mixture outcomes of multicomponent/complex mixtures as well as to understand deviations from additivity. For instance, the antagonistic effects of abamectin on the hyperactivity level of the mixture of chlorpyrifos and hexaconazole (Figure 5B) would have been unexplainable if only mechanism of action based classification was used.

Hyper- and hypoactivity response could also be used as an effect-based strategy for bio-monitoring of complex environmental mixtures which can facilitate the identification of chemicals inducing mixture neurotoxicity that would not have been detected with analytical chemical measurements [47,48]. However, equitoxic ratio of substances with opposing effect direction could lead to no observed effects or effects occurring at control level. This counteracting effects could be a huge challenge for diagnostic risk assessment. Therefore, effect evaluation with STC as converging key event of a complex environmental mixture may only indicate an effect size related to the amount of neuroactive components if they show effect in the same direction (i.e. hyper- or hypoactivity). With opposing effects in the STC, effect evaluation may not relate to the cumulative exposure levels. However, this may present a better evaluation of the exposure level regarding the relevant biological effects and potential hazard.

Nevertheless, a solution could be to spike environmental mixtures with a positive control such that deviations from the known effect size of the positive control could be an indication of inherent effect of the mixture. In prospective mixture evaluation, one solution could be to employ a non-equitoxic mixture ratio design (eg. 25% compound A and 75% compound B or vice versa) for opposing acting substances such that the strength of the counteracting effects is weakened. However, this approach may lead to hidden effects and could give a false perspective of effect assessment. Regardless, it is necessary to elaborate when effect normalization is an acceptable ecological risk.

5. Conclusions

We found that mixtures of neuroactive substances with different mechanisms of action but similar effect direction are additive and could be predicted using CA or IA models. Convergence and integration of effects in the nervous system provides a mechanistic understanding to support similarity classification of neuroactive compounds not only based on mechanisms of action but also considering the toxicological response or effect direction (whether hyper- or hypoactivity). Consequently, we recommend to consider toxicological response or effect direction as an additional grouping factor when applying CA and IA models. On the other hand, mixtures of substances with different mechanism of action and opposing effect direction are antagonistic. Being able to detect neurotoxicity within an environmental sample (complex mixture) is relevant since neuroactive chemicals are usually dominating concentrations of contaminants in the environment and may be major drivers of mixture toxicity. Since established effect based tools may overlook or may not

capture neurotoxicity, we propose in this study a way to use the STC test for risk assessment despite counteracting effects which could complicate proper evaluation.

Data Availability Statement: The data presented in this study are openly available in Zenodo at [10.5281/zenodo.4640059].

Author Contributions: Conceptualization, A.O., R.M., R.A., S.S., and E.K.; methodology, A.O., R.M., S.S., and E.K.; formal analysis, A.O., R.A., and E.K.; investigation, A.O.; writing—original draft preparation, A.O.; writing—review and editing, A.O., R.M., R.A., S.S., and E.K.; visualization, A.O.; supervision, E.K., S.S.; project administration, E.K.; funding acquisition, E.K.; All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the German Federal Ministry of Education and Research (BMBF) within the Neurobox Project (FKZ 02WRS1419F). In addition parts of the study were co-financed by the UFZ integrated project Exposome.

Acknowledgments: Firstly, we would like to acknowledge the high-spirited patronage by the late Dr. Tamara Grummt throughout the whole project! We also appreciate Gianina Jakobs for the discussions during the design of the experiments. We highly acknowledge the help by Nicole Schweiger for fish culture and embryo production.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Faust, M.; Backhaus, T.; Altenburger, R.; Dulio, V.; van Gils, J.; Ginebreda, A.; Kortenkamp, A.; Munthe, J.; Posthuma, L.; Slobodnik, J.; et al. Prioritisation of water pollutants: the EU Project SOLUTIONS proposes a methodological framework for the integration of mixture risk assessments into prioritisation procedures under the European Water Framework Directive. *Environ. Sci. Eur.* **2019**, *31*, doi:10.1186/s12302-019-0239-4.
2. Belden, J.B.; Gilliom, R.J.; Lydy, M.J. *How well can we predict the toxicity of pesticide mixtures to aquatic life?*; 2007; Vol. 3;.
3. Bliss, C.I. the Toxicity of Poisons Applied Jointly. *Ann. Appl. Biol.* **1939**, *26*, 585–615, doi:10.1111/j.1744-7348.1939.tb06990.x.
4. Hewlett, P.S.; Plackett, R.L. A Unified Theory for Quantal Responses to Mixtures of Drugs: Non-Interactive Action. *Biometrics* **1959**, *15*, 591, doi:10.2307/2527657.
5. Altenburger, R.; Backhaus, T.; Boedeker, W.; Faust, M.; Scholze, M.; Grimme, L.H. Predictability of the toxicity of multiple chemical mixtures to Vibrio fischeri: Mixtures composed of similarly acting chemicals. *Environ. Toxicol. Chem.* **2000**, *19*, 2341–2347, doi:10.1002/etc.5620190926.
6. Backhaus, T.; Altenburger, R.; Boedeker, W.; Faust, M.; Scholze, M.; Grimme, L.H. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. *Environ. Toxicol. Chem.* **2000**, *19*, 2348–2356, doi:10.1002/etc.5620190927.
7. Faust, M.; Altenburger, R.; Backhaus, T.; Blanck, H.; Boedeker, W.; Gramatica, P.; Hamer, V.; Scholze, M.; Vighi, M.; Grimme, L.H. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquat. Toxicol.* **2001**, *56*, 13–32, doi:10.1016/S0166-445X(01)00187-4.
8. Cedergreen, N.; Svendsen, C.; Backhaus, T. Toxicity Prediction of Chemical Mixtures. *Encycl. Environ. Manag.* **2013**, 2572–2581, doi:10.1081/E-EEM-120046684.
9. Busch, W.; Schmidt, S.; Kühne, R.; Schulze, T.; Krauss, M.; Altenburger, R. Micropollutants in

European rivers: A mode of action survey to support the development of effect-based tools for water monitoring. *Environ. Toxicol. Chem.* **2016**, *35*, 1887–1899, doi:10.1002/etc.3460.

10. Corbel, V.; Stankiewicz, M.; Bonnet, J.; Grolleau, F.; Hougard, J.M.; Lapied, B. Synergism between insecticides permethrin and propoxur occurs through activation of presynaptic muscarinic negative feedback of acetylcholine release in the insect central nervous system. *Neurotoxicology* **2006**, *27*, 508–519, doi:10.1016/j.neuro.2006.01.011.
11. Yang, Y.; Ma, H.; Zhou, J.; Liu, J.; Liu, W. Joint toxicity of permethrin and cypermethrin at sublethal concentrations to the embryo-larval zebrafish. *Chemosphere* **2014**, *96*, 146–154, doi:10.1016/j.chemosphere.2013.10.014.
12. Strähle, U.; Scholz, S.; Geisler, R.; Greiner, P.; Hollert, H.; Rastegar, S.; Schumacher, A.; Selderslaghs, I.; Weiss, C.; Witters, H.; et al. Zebrafish embryos as an alternative to animal experiments-A commentary on the definition of the onset of protected life stages in animal welfare regulations. *Reprod. Toxicol.* **2012**, *33*, 128–132, doi:10.1016/j.reprotox.2011.06.121.
13. Ogungbemi, A.; Leuthold, D.; Scholz, S.; Küster, E. Hypo - or hyperactivity of zebrafish embryos provoked by neuroactive substances : a review on how experimental parameters impact the predictability of behavior changes. *Environ. Sci. Eur.* **2019**, doi:10.1186/s12302-019-0270-5.
14. Saint-Amant, L.; Drapeau, P. Time course of the development of motor behaviors in the zebrafish embryo. *J. Neurobiol.* **1998**, *37*, 622–632, doi:10.1002/(SICI)1097-4695(199812)37:4<622::AID-NEU10>3.0.CO;2-S.
15. Kimmel, C.B.; Patterson, J.; Kimmel, R.O. The development and behavioral characteristics of the startle response in the zebra fish. *Dev. Psychobiol.* **1974**, *7*, 47–60, doi:10.1002/dev.420070109.
16. Selderslaghs, I.W.T.; Hooyberghs, J.; De Coen, W.; Witters, H.E. Locomotor activity in zebrafish embryos: A new method to assess developmental neurotoxicity. *Neurotoxicol. Teratol.* **2010**, *32*, 460–471, doi:10.1016/j.ntt.2010.03.002.
17. Ogungbemi, A.O.; Teixido, E.; Massei, R.; Scholz, S.; Küster, E. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. *Neurotoxicol. Teratol.* **2020**, *81*, 106918, doi:10.1016/j.ntt.2020.106918.
18. Zhang, K.; Liang, J.; Brun, N.R.; Zhao, Y.; Werdich, A.A. Rapid Zebrafish Behavioral Profiling Assay Accelerates the Identification of Environmental Neurodevelopmental Toxicants. *Environ. Sci. Technol.* **2021**, *55*, 1919–1929, doi:10.1021/acs.est.0c06949.
19. Vliet, S.M.; Ho, T.C.; Volz, D.C. Behavioral screening of the LOPAC1280 library in zebrafish embryos. *Toxicol. Appl. Pharmacol.* **2017**, *329*, 241–248, doi:10.1016/j.taap.2017.06.011.
20. de Oliveira, A.A.S.; Brigante, T.A.V.; Oliveira, D.P. Tail coiling assay in zebrafish (Danio rerio) embryos: Stage of development, promising positive control candidates, and selection of an appropriate organic solvent for screening of developmental neurotoxicity (DNT). *Water (Switzerland)* **2021**, *13*, 119, doi:10.3390/w13020119.
21. Casida, J.E.; Durkin, K.A. Neuroactive insecticides: Targets, selectivity, resistance, and secondary effects. *Annu. Rev. Entomol.* **2013**, *58*, 99–117, doi:10.1146/annurev-ento-120811-153645.
22. Söderpalm, B. Anticonvulsants: Aspects of their mechanisms of action. *Eur. J. Pain* **2002**, *6*, 3–9, doi:10.1053/eujp.2001.0315.
23. Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B.; Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn. an Off. public* **1995**, *203*, 253–310, doi:10.1002/aja.1002030302.
24. Ogungbemi, A.O.; Teixido, E.; Massei, R.; Scholz, S.; Küster, E. Automated measurement of the spontaneous tail coiling of zebrafish embryos as a sensitive behavior endpoint using a workflow in KNIME. *MethodsX*.
25. Teixido, E.; Klüver, N.; Ogungbemi, A.O.; Küster, E.; Scholz, S. Evaluation of Neurotoxic effects in zebrafish embryos by automatic measurement of early motor behaviors. In Experimental Neurotoxicology Methods. In *Neuromethods*; Springer.

26. Sánchez-Bayo, F. Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. *J. Environ. Anal. Toxicol.* **2012**, *s4*, doi:10.4172/2161-0525.s4-002.
27. Zhu, X.W.; Chen, J.Y. Mixtox: An r package for mixture toxicity assessment. *R J.* **2016**, *8*, 422–434, doi:10.32614/rj-2016-056.
28. Ritz, C.; Streibig, J.C. *Bioassay analysis using R*; 2005; Vol. 12;.
29. Scholze, M.; Silva, E.; Kortenkamp, A. Extending the applicability of the dose addition model to the assessment of chemical mixtures of partial agonists by using a novel toxic unit extrapolation method. *PLoS One* **2014**, *9*, doi:10.1371/journal.pone.0088808.
30. Jackson, A. Partial Agonist. In *Encyclopedia of Psychopharmacology*; Stolerman, I.P., Ed.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2010; pp. 959–960 ISBN 978-3-540-68706-1.
31. Kortenkamp, A.; Backhaus, T.; Faust, M. State of the Art Report on Mixture Toxicity. *Report* **2009**, *Contract N*, 1–391, doi:http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf.
32. Cleuvers, M. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicol. Lett.* **2003**, *142*, 185–194, doi:10.1016/S0378-4274(03)00068-7.
33. Drescher, K.; Boedeker, W. Assessment of the Combined Effects of Substances: The Relationship between Concentration Addition and Independent Action. *Biometrics* **1995**, *51*, 716, doi:10.2307/2532957.
34. Rose, S.; Altenburger, R.; Sturm, A. Mixture toxicity effects of sea louse control agents in Daphnia magna. *Chemosphere* **2016**, *144*, 599–606, doi:10.1016/j.chemosphere.2015.08.053.
35. Jakobs, G.; Krüger, J.; Schüttler, A.; Altenburger, R.; Busch, W. Mixture toxicity analysis in zebrafish embryo: a time and concentration resolved study on mixture effect predictivity. *Environ. Sci. Eur.* **2020**, *32*, 143, doi:10.1186/s12302-020-00409-3.
36. Cedergreen, N.; Christensen, A.M.; Kamper, A.; Kudsk, P.; Mathiassen, S.K.; Streibig, J.C.; Sørensen, H. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environ. Toxicol. Chem.* **2008**, *27*, 1621–1632, doi:10.1897/07-474.1.
37. Drapeau, P.; Saint-Amant, L.; Buss, R.R.; Chong, M.; McDearmid, J.R.; Brustein, E. Development of the locomotor network in zebrafish. *Prog. Neurobiol.* **2002**, *68*, 85–111, doi:10.1016/S0301-0082(02)00075-8.
38. Wolansky, M.J.; Gennings, C.; DeVito, M.J.; Crofton, K.M. Evidence for dose-additive effects of pyrethroids on motor activity in rats. *Environ. Health Perspect.* **2009**, *117*, 1563–1570, doi:10.1289/ehp.0900667.
39. Gonçalves, R.; Scholze, M.; Ferreira, A.M.; Martins, M.; Correia, A.D. The joint effect of polycyclic aromatic hydrocarbons on fish behavior. *Environ. Res.* **2008**, *108*, 205–213, doi:10.1016/j.envres.2008.07.008.
40. Schmidt, S.; Busch, W.; Altenburger, R.; Küster, E. Mixture toxicity of water contaminants-effect analysis using the zebrafish embryo assay (Danio rerio). *Chemosphere* **2016**, *152*, 503–512, doi:10.1016/j.chemosphere.2016.03.006.
41. Altenburger, R.; Nendza, M.; Schüürmann, G. Mixture toxicity and its modeling by quantitative structure-activity relationships. *Environ. Toxicol. Chem.* **2003**, *22*, 1900–1915, doi:10.1897/01-386.
42. Corbett, J.R. The biochemical mode of action of pesticides. *Acad. Press* **1974**, *330 p.*, doi:10.1042/bst0021391.
43. Cedergreen, N. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. *PLoS One* **2014**, *9*, doi:10.1371/journal.pone.0096580.
44. Kühnert, A.; Vogs, C.; Aulhorn, S.; Altenburger, R.; Küster, E.; Busch, W.; Kühnert, A.; Vogs, C.; Altenburger, R.; Hollert, H.; et al. Biotransformation in the zebrafish embryo –temporal gene transcription changes of cytochrome P450 enzymes and internal exposure dynamics of the AhR binding xenobiotic benz[a]anthracene. *Environ. Pollut.* **2017**, *230*, 1–11, doi:10.1016/j.envpol.2017.04.083.
45. Kienle, C.; Köhler, H.R.; Gerhardt, A. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. *Ecotoxicol. Environ. Saf.*

2009, *72*, 1740–1747, doi:10.1016/j.ecoenv.2009.04.014.

46. Pérez, J.; Domingues, I.; Monteiro, M.; Soares, A.M.V.M.; Loureiro, S. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish Danio rerio. *Environ. Sci. Pollut. Res.* **2013**, *20*, 4671–4680, doi:10.1007/s11356-012-1443-6.
47. Bal-Price, A.; Crofton, K.M.; Leist, M.; Allen, S.; Arand, M.; Buetler, T.; Delrue, N.; FitzGerald, R.E.; Hartung, T.; Heinonen, T.; et al. International STakeholder NETwork (ISTNET): creating a developmental neurotoxicity (DNT) testing road map for regulatory purposes. *Arch. Toxicol.* **2015**, *89*, 269–287, doi:10.1007/s00204-015-1464-2.
48. De Baat, M.L.; Kraak, M.H.S.; Van der Oost, R.; De Voogt, P.; Verdonschot, P.F.M. Effect-based nationwide surface water quality assessment to identify ecotoxicological risks. *Water Res.* **2019**, *159*, 434–443, doi:10.1016/j.watres.2019.05.040.