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Toxicological Characterization and Screening of Neuroactive Chemicals and Mixtures using Zebrafish Embryo Behavior

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Toxicological Characterization and Screening of Neuroactive Chemicals and Mixtures using Zebrafish Embryo Behavior

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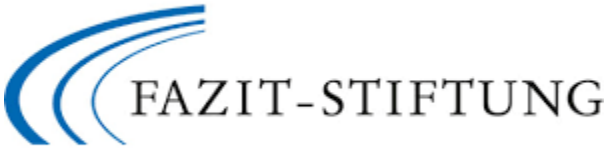
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Summary

Despite the significant presence of neuroactive substances in the environment, bioassays that allow to detect diverse groups of neuroactive mechanisms of action are not well developed and not properly integrated into environmental monitoring and chemical regulation. Therefore, there is a need to develop testing methods which are amenable for fast and high-throughput neurotoxicity testing. The overall goal of this thesis work is to develop a test method for the toxicological characterization and screening of neuroactive substances and their mixtures which could be used for prospective and diagnostic hazard assessment.

In this thesis, the behavior of zebrafish embryos was explored as a promising tool to distinguish between different neuroactive mechanisms of action. Recently, new behavioral tests have been developed including photomotor response (PMR), locomotor response (LMR) and spontaneous tail coiling (STC) tests. However, the experimental parameters of these tests lack consistency in protocols such as exposure time, imaging time, age of exposure, endpoint parameter etc. To understand how experimental parameters may influence the toxicological interpretation of behavior tests, a systematic review of existing behavioral assays was conducted in **Chapter 2**. Results show that exposure concentration and exposure duration highly influenced the comparability between different test methods and the spontaneous tail coiling (STC) test was selected for further testing based on its relative higher sensitivity and capacity to detect neuroactive substances (**Chapter 2**).

STC is the first observable motor activity generated by the developing neural network of the embryo which is assumed to occur as a result of the innervation of the muscle by the primary motor neurons. Therefore, STC could be a useful endpoint to detect effect on the muscle innervation and also the on the whole nervous system. Consequently, important parameters of the STC test were optimized and an automated workflow to evaluate the STC with the open access software KNIME® was developed (**Chapter 3**).

To appropriately interpret the observed effect of a single chemical and especially mixture effects, requires the understanding of toxicokinetics and biotransformation. Most importantly, the biotransformation capacity of zebrafish embryos might be limited and this could be a challenge for assessment of chemicals such as organophosphates which require a bioactivation step to effectively inhibit the acetylcholinesterase (AChE) enzyme. Therefore, the influence of the potential limited biotransformation on the toxicity pathway of a typical organophosphate, chlorpyrifos, was investigated in **Chapter 5**. Chlorpyrifos could not inhibit AChE and this was attributed to possible lack of biotransformation in 24 hpf embryos (**Chapter 5**).

Since neuroactive substances occur in the environment as mixtures, it is therefore more realistic to assess their combined effect rather than individually. Therefore, mixture toxicity was predicted using the concentration addition and independent action models. Result shows that mixtures of neuroactive substances with different mechanisms of action but similar effects can be predicted with concentration addition and independent action (**Chapter 4**). Apart from being able to predict the combined effect of neuroactive substances for prospective risk assessment, it is also important to assess in retrospect the combined neurotoxic effect of environmental samples since neuroactive substances are the largest group of chemicals occurring in the environment. In **Chapter 6**, the STC test was found to be capable of detecting neurotoxic effects of a wastewater effluent sample. Hence, the STC test is proposed as an effect based tool for monitoring environmental acute and neurotoxic effects.

Overall, this thesis shows the utility and versatility of zebrafish embryo behavior testing for screening neuroactive substances and this allows to propose its use for prospective and diagnostic hazard assessment. This will enhance the move away from expensive and demanding animal testing. The information contained in this thesis is of great potential to provide precautionary solutions, not only for the exposure of humans to neuroactive chemicals but for the environment at large.

Table of Contents

List of Figures

List of Tables

Abbreviations

STC	Spontaneous tail coiling
LMR	Locomotor response
PMR	Photomotor response
LMR-L/D	Light-dark locomotor response
AChE	Acetylcholinesterase
ACh	Acetylcholine
GABA	Gamma aminobutyric acid
nAChr	Nicotinic acetylcholine receptor
mAChr	Muscarinic acetylcholine receptor
PTZ	Pentylenetetrazole
AcN	Acetonitrile
MoA	Mode of action
hpf	Hours post fertilization
LOEC	Lowest observed effect concentration
CA	Concentration addition
IA	Independent action
DNT	Developmental neurotoxicity
LCMS	Liquid chromatography mass spectroscopy
LC-HRMS	Liquid chromatography – high resolution mass spectroscopy
REF	Relative enrichment factor
LC	Lethal concentration
EC	Effect concentration
WWTP	Wastewater treatment plant
EBM	Effect based method

Chapter 1: Introduction

1.1 Background

In recent times, there has been an increase in the number of people suffering from neurological diseases and incidents of nervous system-related diseases are increasingly associated with exposure to pesticides and pharmaceuticals (Brown et al. 2005; Huhn et al. 2021). These chemicals may interfere with the functioning of the nervous system and very little disruptions can lead to long term neurological diseases such as Parkinson and autism (Betarbet et al. 2000). The concern for neuroactive chemical exposure is also supported by environmental data. Busch et al. (2016) showed that chemicals with neuroactive modes of action represent the largest group (13%) of chemicals detected in European rivers (Figure 1.1). Schreiner et al. (2016) also reported that insecticides (mostly neuroactive) are among the most frequently detected pesticides after herbicides. Furthermore, neuroactive insecticides have been identified as the main driver of biodiversity loss in aquatic invertebrate communities (Liess et al. 2021). This indicates the need to emphasize on screening neuroactive chemicals from environmental samples as well as the assessment of neurotoxic risk of new chemicals to protect human and environmental health.

Currently, testing guidelines exist for risk assessment of neurotoxic effects using rodents (OECD 424 and 426) but these guidelines are rarely applied due to high complexity and only required for chemicals with indications of neuroactive mode of action or structures related to known neurotoxicants (Masjosthusmann et al. 2018). In addition, this regulation is focused on human exposure and fails to consider effects on non-target organisms in the environment (Legradi et al. 2018). In reality, there is no regulation for environmental neurotoxic risk despite the significant presence of neuroactive substances in the environment. Further, effect based methods for neurotoxic endpoints are also not yet integrated into chemical monitoring (Busch et al. 2016; Schmidt et al. 2017). Therefore, there is a need to develop bioassays which are amenable for fast, high throughput neurotoxicity screening.

Apart from neurotoxic effects towards humans and non-target organisms, developmental neurotoxicity (DNT) has been identified as a major concern due to the particular vulnerability of the developing nervous system to toxic effects (Grandjean and Landrigan 2014; Fritsche et al. 2018). Apparently, DNT testing is also conducted in rodents (OECD 426) and due to the laborious and expensive test system, the DNT status of several chemicals is still unknown. Thus, there is a need to develop cheaper and efficient alternative test systems for DNT testing (Crofton et al. 2012; Legradi et al. 2018).

To develop new and alternative approaches to animal testing, the European regulatory agencies (ECHA and EFSA), with their American counterpart (US-EPA) have stimulated and encouraged scientific discourse towards appropriate in-vitro, in-silico and alternative in-vivo methods (Embry et al. 2010; Crofton et al. 2012; Scholz et al. 2013b; Aschner et al. 2017; Masjosthusmann et al. 2018; Sobanska et al. 2018). With such collaborative efforts amongst the government, industry and academia, it is evident that the future of chemical safety assessment is in the direction of alternative and mechanistic endpoints rather than laborious animal testing.

The overall goal of this thesis work is to contribute to the advancement of alternative and mechanistic testing by exploring the use of zebrafish embryos (which is considered an alternative test system) to investigate the toxicity of neuroactive substances and their mixtures. The study shows the utility and versatility of zebrafish embryos and allows to propose its use for prospective and diagnostic hazard assessment.

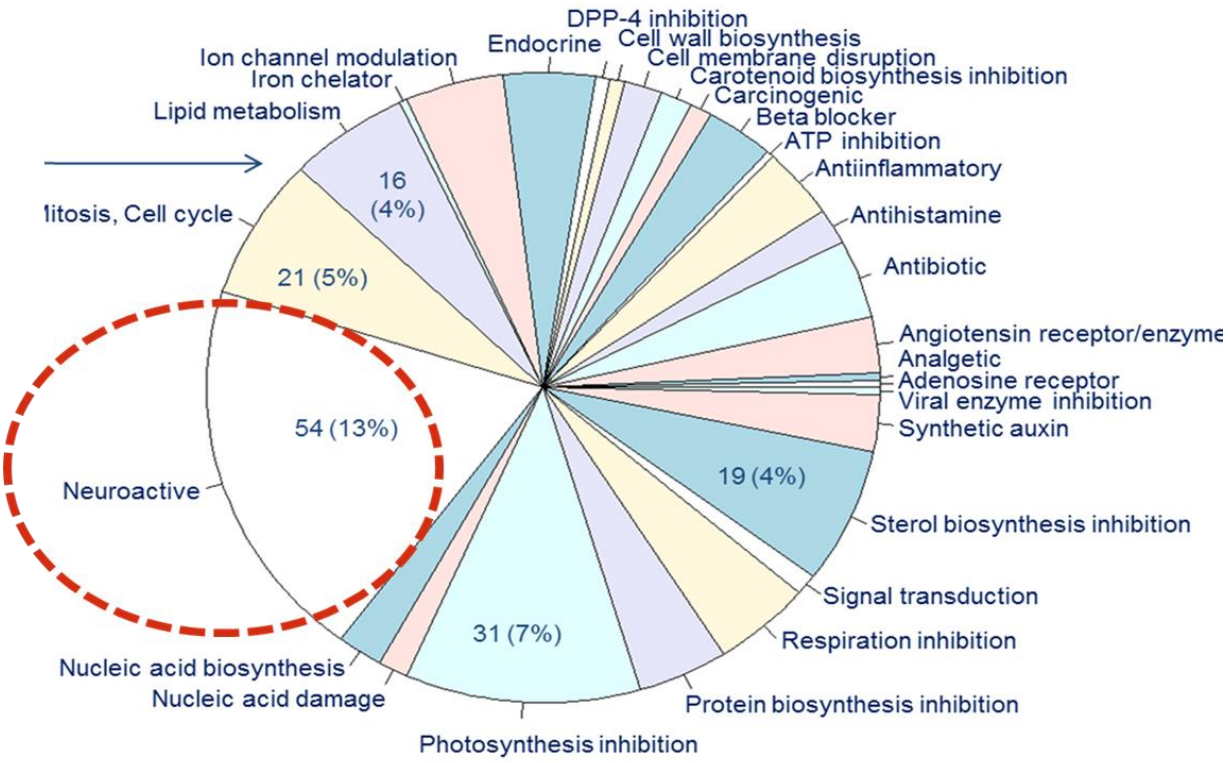

Figure 1.1: Different modes of action for chemicals detected in European surfaces waters. Neuroactive substances (red circle) represent the largest group of mode of action. Figure reproduced from Busch et al. (2016) with slight modification.

1.2 Neuroactive substances

Neuroactive substances are chemicals with capacity to disrupt the functioning of the nervous system. Most of the known neuroactive substances were primarily designed as pesticides or pharmaceuticals to interact with specific parts of the nervous system. For example, chlorpyrifos is an insecticide which disturbs cholinergic neurotransmission by inhibiting acetylcholinesterase enzyme (Casida and Durkin 2013), while carbamazepine is an anticonvulsant drug used to control seizures by blocking voltage gated sodium channels (Söderpalm 2002). Besides these substances with known neuroactive mechanisms of action, many existing chemicals may have unknown secondary or side neuroactive mechanisms of action. For example, hexaconazole, a fungicide was found to cause neurotoxic effects in a poisoned human (David

et al. 2008). Moreover, neurotoxic effects of industrial chemicals (e.g. flame retardants) which were not primarily designed to interact with the nervous system have also been reported (Noyes et al. 2015; Oliveri et al. 2018). Furthermore, endocrine disrupting substances have been reported to induce neurotoxic effects (Wang et al. 2018; Walter et al. 2019) and such effects were attributed to the disruption of neuroendocrine processes such as synthesis of neuropeptides, neurotransmitters, or other receptors (Waye and Trudeau 2011; Lupu et al. 2020).

Neuroactive substances can induce a range of effects with different levels of damage in living organisms. Therefore, the 2 commonly used terms to describe the effect of neuroactive substances were distinguished in this thesis. Neurotoxicity is defined as an interaction of a substance with the nervous system primarily leading to a structural change (e.g. axonal deformation or inhibition of neurite outgrowth), while neuroactivity is a functional interaction with specific nervous receptors leading to a change without necessarily being accompanied by structural changes (e.g. alteration of neurotransmission or blockage of nervous receptor). Nevertheless, neuroactivity may also cause structural and morphological changes over a long exposure duration or higher concentration. In this chapter, neuroactive was used as the word to describe or qualify chemicals while neurotoxic was used as the word to describe the adverse effect.

Neuroactive substances target several receptors in living organisms leading to diverse mechanisms of action. The major mechanisms of action discussed in this thesis include: acetylcholinesterase inhibition, nicotinic acetylcholine receptor activation, Gamma aminobutyric acid activation/inhibition and voltage gated sodium channel blockage (See Table 1.1 for information on the hypothesized action of these mechanisms).

1.3 The use of the zebrafish embryo in neurotoxicity testing

Animal models such as rodents and fishes are usually used for neurotoxicity testing. However, exposure of animals to chemicals may inflict pain and distress, hence there is an increased pressure to develop alternatives to animal tests because of ethical reasons, as well as to reduce time and cost of these tests (Braunbeck et al. 2005; Scholz et al. 2008). Consequently, the use of animals in toxicity testing is gradually being discouraged in favor of promoting the 3R principle: reduction, refinement and replacement (Russell and Burch 1959). Zebrafish embryos prove to be a valid alternative due to the following reasons including; 1.) Embryonic stages of vertebrates are not regulated and are considered alternative to animal testing. 2.) Zebrafish embryo test utilizes low volume of chemical solutions, hence reducing chemical waste. 3.) The small embryo size also enables high-throughput studies in multi well plates. 4.) Zebrafish embryos can be made available in high numbers since adult zebrafish have high fecundity and are capable of laying 200-300 eggs per day. 5.) The transparency of zebrafish embryos enables non-invasive evaluation of developmental toxicity. 6.) Zebrafish embryos have a fast development and the organogenesis of main organs is completed at 5dpf. 7.) Zebrafish embryos are highly versatile with high diagnostic capacity and can be used for evaluating diverse effects such as heart rate, developmental toxicity, neurotoxicity, endocrine disruption etc. (Hill et al. 2005; Ali et al. 2011a; Sobanska et al. 2018).

Besides, zebrafish embryos have been shown to be a good predictor for toxicity in adult fish and rodents. Acute toxicity results from the fish embryo test were found to mostly correlate with those from the adult fish test (Nagel 2002; Lammer et al. 2009; Belanger et al. 2013; Scholz et al. 2013a; Lillicrap et al. 2020) and with those from rodent tests (Ali et al. 2011b; Ducharme et al. 2015).

Even though the toxicity of zebrafish embryos may overall correlate well with that of adult fish and mammals, deviations have been observed for certain types of chemicals. For example, Klüver et al. (2015) identified several neurotoxic chemicals that had significantly higher lethal effect concentrations (less toxicity) in the fish embryo compared to the adult fish. Surprisingly, they found an up to 660-fold decrease in effect concentrations in the embryo when behavioral endpoints were measured. They concluded that the application of embryo tests for neuroactive chemicals should be based on behavior testing since behavioral endpoints were observed at concentrations that did not induce malformations and lethality. This indicates that behavior can be considered as a biomarker for neuroactive chemicals and the use of behavioral tests has since been intensified to screen neuroactive chemicals.

There are other limitations of using zebrafish embryos for neurotoxicity testing including: possibly limited biotransformation capacity in early developmental stages for compounds requiring metabolic activation (Kühnert et al. 2013, 2017) and possibly limited toxicokinetics for high molecular weight and high lipophilic substances due to the chorion barrier and yolk storage respectively (Sobanska et al. 2018; Halbach et al. 2020). Nevertheless, zebrafish embryo behavior can be used within a weight of evidence approach along with other relevant information and particularly, they could be used to cover the limitation of the fish embryo test to detect substances with neuroactive modes of action (Sobanska et al. 2018). Subsequent chapters of this thesis show the strength of zebrafish embryo behavior for screening neuroactive substances and their mixtures.

1.4 Zebrafish embryo behavioral test methods

Measurement of animal behavior is a widely accepted indicator of toxicity and has been used for environmental biomonitoring (Melvin and Wilson 2013; Bae and Park 2014). However, behavior endpoints are relatively less popular than other sublethal endpoints such as reproduction and growth (Scott and Sloman 2004). Behavioral endpoints are usually driven by a direct interaction of chemicals disrupting the function or development of the nervous system. Alternatively, indirect effects of chemicals on the nervous system may also occur via effects on other biological systems leading to secondary behavior effects (Tierney 2011). Since behavior integrates physiological and ecological processes, methods which measure effects on behavior are increasingly being employed as an ecologically relevant tool and early warning signal to detect pollution (Scott and Sloman 2004; Hellou 2011). Behavior methods are known to offer increased sensitivity when compared to lethality; increased ease of measurement when compared to reproduction and increased relevance for population effects when compared to growth metrics (Gerhardt 2007; Robinson 2009).

In recent times, there has been an increase in the use of the zebrafish model for behavior testing and this is partly due to the advancement in technology for measuring and automating behavior chemicals (Gerhardt 2007). In particular, the behavioral response of zebrafish embryos is a good alternative to

animal testing to detect neurotoxic effects. Consequently, several behavioral test methods have been developed including photomotor response (PMR), locomotor response (LMR) and spontaneous tail coiling (STC) etc. (Irons et al. 2010; Selderslaghs et al. 2010; Kokel et al. 2010; Fitzgerald et al. 2021). Although these methods are distinct in their application, most of their experimental parameters lack consistency in protocols such as exposure time, imaging time, endpoint parameter etc. This inconsistent use of experimental parameters may cause inaccurate interpretation of behavioral response (e.g. hyper- and hypoactivity referring to increased and decreased activity respectively) of zebrafish during toxicity diagnosis.

To understand how experimental parameters may influence the hyper- and hypoactivity behavior of zebrafish embryos, we conducted a systematic review of existing behavioral assays to ask these questions in **Chapter 2**: 1.) Which experimental parameters mostly influence the observed effects in commonly used behavioral test methods and is it possible to rank these parameters? 2.) How often is the observed hypo- or hyperactivity of zebrafish embryos in literature consistent with the expected mode of action of a chemical substance? 3.) Which of the behavioral test methods could be selected for further use to test neuroactive substances in the current study?

The literature review shows that exposure concentration, exposure duration, endpoint parameter and developmental stage highly influenced the comparability between different test methods. Combination of these parameters caused inaccurate prediction of expected hyper- and hypoactivity and hence mode of action. Due to its short duration and potential higher capacity to detect hyperactivity, the STC test was selected for further testing in the subsequent studies of this thesis work.

1.5 The spontaneous tail coiling of zebrafish embryos

Spontaneous tail coiling (STC) is the earliest observable motor activity generated by the developing neural network of zebrafish embryos and it consists of single or multiple rhythmic contractions (Kimmel et al. 1974). STC can be observed as early as 17-19 hpf and are assumed to be induced by the innervation of the muscle by the primary motor neuron (Saint-Amant and Drapeau 1998; Richendrfer et al. 2014). Therefore, STC could be a useful endpoint to detect effects on the muscle innervation and generally the nervous system.

Based on the review conducted in Chapter 2, STC was selected for further testing because of: 1.) Short exposure duration of 24 h or less. 2.) Possibility of assessing effects in early developmental stages. 3.) Proximity to an important neural function – neurotransmission. 4.) Potential to reveal or distinguish between different modes of action.

STC has already been used in some studies to detect the effects of neuroactive chemicals (Selderslaghs et al. 2010; Watson et al. 2014; Raftery et al. 2014; Vliet et al. 2017; Weichert et al. 2017). These neuroactive chemicals target specific parts of the nervous system including acetylcholinesterase (AChE), nicotine receptors, Gamma aminobutyric acid (GABA) receptors, sodium channels etc. Based on the hyper or hypoactivity response, STC appears to be able to predict the excitatory or inhibitory action (i.e. flow of sodium or chloride ions into the cell respectively) resulting from chemical interaction with these neural targets (Figure 1.2). For instance, hyperactivity effect (referring to increased STC frequency) may be

correlated to activation potential in nerve cells while hypoactivity (decreased STC) could be a result of the inhibitory potential.

Hence, the STC neuroactivity hypothesis was postulated 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 (Table 1.1 describes the STC neuroactivity hypothesis for different mechanisms of action). Therefore, the goals of the study in **Chapter 3** are: 1.) to develop an optimized STC test for screening neuroactive compounds and 2.) to investigate the capacity of the optimized STC test to rapidly and efficiently screen substances with different modes of action.

Important experimental parameters such as imaging and acclimation duration were optimized and an automated workflow to analyze the STC in the open access software KNIME® was developed. A detailed and already published experimental protocol of the STC is given in Appendix 3 (Ogungbemi et al. 2021). The STC test had 75 % accuracy to detect neuroactive substances and neuroactivity was also found for substances which are not primarily known to be neuroactive. These results reveal the capacity of the STC test to capture early effects and to predict neuroactive mechanisms of action within a short duration testing framework. This could be of high relevance for prospective and diagnostic hazard assessment.

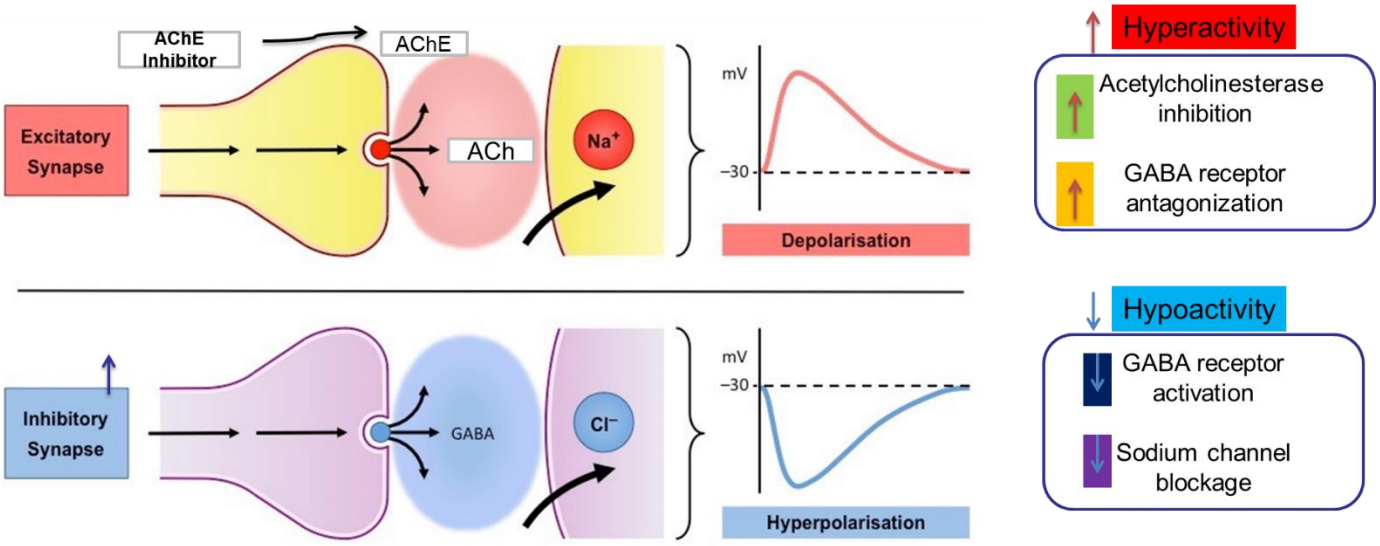

Figure 1.2: Schematic diagram of the excitatory and inhibitory neurotransmission. Flow of Na+ and Cl- into the cell modulates the cell potential leading to either depolarization (excitatory synapse) or hyperpolarization (inhibitory synapse) respectively. Acetylcholine (ACh) and GABA are released into the synaptic cleft and activate receptors which keep the channel open for Na+ and Cl- to flow into the neuron cell and this induces the excitatory and inhibitory synapses leading to hyper and hypoactivity respectively. Substances that inhibit Acetylcholinesterase (AChE) or antagonize GABA receptor may induce hyperactivity while substances that activate GABA receptor or block NA channels may cause hypoactivity in the STC test. Reproduced with slight modifications from: https://ib.bioninja.com.au/options/option-a-neurobiology-and/a5-neuropharmacology/synaptic-transmission.html

Table 1.1: Examples of neuroactivity mechanisms of action and corresponding neuroactivity hypotheses of how the mechanisms may induce hyper- or hypoactivity in the STC test.

Mechanism of action*	Chemical examples	Hypothesized activity in the STC	Explanation of hypothesis
Acetylcholinesterase inhibition	Organophosphates i.e. chlorpyrifos, diazinon	Hyperactivity	Acetylcholinesterase inhibition leads to accumulation of acetycholine in the synaptic junction and therefore keeps the acetylcholine gated channel opens for more sodium ions to flow into the cell. This enhances the excitatory synapse and hence hyperactivity
GABA receptor antagonization	Organochlorines i.e. chlordane, endosulfan	Hyperactivity	Antagonization of GABA receptors would block the flow of chloride ions into the nerve cell and therefore limiting the inhibitory synapse. The absence or reduction of the inhibitory potantial would enhance or make the excitatory synapse to be more pronounce.
Activation of voltage gated sodium channel	Pyrethroids i.e. permethrin, bifenthrin	Hyperactivity	Activation of voltage gated sodium channels allows more sodium ions to flow into the cell and hence a release of acetycholine leading to excitation synapse of neurotransmission and hence hyperactivity
Nicotinic acetycholine receptor agonist	Neonicotinoids i.e. imidacloprid, thiacloprid	Hyperactivity	Agonization of nicotinic acetycholine receptors allows more sodium ions to flow into the cell leading to excitation synapse of neurotransmission and hence hyperactivity
Activation of GABA or glutamate gated chloride channel	Avermectins i.e. abamectin, emamectin	Hypoactivity	Activation of GABA gated chloride channels enhances the flow of chloride ions into the cell leading to inhibition of neurotransmission and hence hypoactivity
Sodium channel blocker	Indoxacarb, Metaflumizone	Hypoactivity	Blockage of the volateg gated sodium channels inhibits the flow of sodium ions into the cell and therefore prevents excitatory synapse. Hence the inhibitory neurotransmission would be enhanced causing hypoactivity.

*Information obtained from IRAC online mode of action classification https://irac-online.org/modes-of-action/

1.6 Mixture toxicity of neuroactive chemicals

As much as it is important to be able to differentiate neuroactive modes of action for single chemicals using behavioral tests, it is also necessary to investigate the additive, synergistic and antagonistic effects of neuroactive chemical mixtures. Generally, chemicals occur in the environment as mixtures and it is therefore important to assess the toxicity of chemical mixtures rather than only single chemicals. To predict mixture neurotoxicity or to conduct a comprehensive neurotoxic assessment of an environmental sample, it is important to be able to differentiate neuroactive modes of action for single chemicals within a mixture of other chemicals.

Concentration addition (CA) and independent action (IA) models have been used to predict toxicity of similarly acting and dissimilarly acting chemical mixtures, respectively (Altenburger et al. 2000; Backhaus et al. 2000). CA is based on the definition that mixture toxicity can be predicted by the addition of the effect concentration of single compounds causing a similar effect or targeting a similar receptor in the organism. On the other hand, IA is based on the multiplication of fractional effects when single compounds are acting on different target sites in the organism.

While only few studies have studied behavioral effects of mixtures (e.g. Kienle et al. 2009), most studies on zebrafish embryos were based on lethal and sublethal endpoints (e.g. Schmidt et al. 2016). Since behavioral endpoints are more sensitive to detect neuroactive chemicals (Klüver et al. 2015), and also more relevant for ecological effects, then it is probably more effective to encourage the use of behavioral tests to predict neuroactive chemical mixtures.

As described previously, the STC test has the potential to distinguish between substances with differential neuroactivity mechanisms of action leading to either hyper or hypoactivity behavior. Thus, it could be used to evaluate the mixture toxicity of substances with similar or dissimilar mechanisms of action. Therefore, the goal of the study in **Chapter 4** is to address the following questions: 1.) Can the mixture models CA or IA be used to predict neuroactive chemical mixtures of similar and dissimilar mechanisms of action? 2.) Will a combination of chemicals which show both hyper and hypoactivity result in an antagonistic interaction effect?

It was shown that mixtures of neuroactive substances with similar and dissimilar mechanisms of action can be predicted with CA and IA models when substances in the mixture induce only hyper or hypoactivity. Further, mixtures of substances showing both hyper and hypoactivity are antagonistic and we discuss the implication of this for the use of STC test as an effect based method in environmental monitoring.

1.7 Toxicokinetics of neurotoxic chemicals in zebrafish embryo

To appropriately interpret the observed effect of a single chemical and especially mixture effects, it is required to understand the relationship between exposure and internal concentration during specific time points, referred to as toxicokinetics. Toxicokinetics comprises processes including uptake or absorption of chemicals into the organism; distribution or circulation within the organism; biotransformation or metabolism leading to either activation or deactivation of the chemical and excretion from the organism.

A combination of these processes determines the potential amount of the chemical that reaches the target site for a toxic response to be initiated.

The zebrafish embryo model used in this thesis work develops rapidly and major organs are differentiated during the 5-day exposure period. This could lead to differences in the toxicokinetics and hence, effectiveness for chemicals with different physico-chemical properties (Brox et al. 2016). Knowledge gaps exist regarding the toxicokinetics of chemicals in zebrafish embryos and only few studies have been conducted to understand the biotransformation capacity of zebrafish embryos (i.e. Yang et al. 2011; Kühnert et al. 2013, 2017).

Toxicokinetics of zebrafish embryos is important for elucidating chemical mixtures in which interaction between substances can influence the exposure dynamics (Altenburger et al. 2012). Toxicokinetics may also be instrumental for selecting an appropriate zebrafish behavioral endpoint and exposure duration. For instance, the 96 h locomotor response test should be preferred over the 24 h STC test for chemicals with slow uptake kinetics. Toxicokinetics have also been used to investigate the influence of the embryo chorion on toxicity outcome (Brox et al. 2014). The chorion is a permeable layer which protects the embryo in early life stages and it acts as a barrier to high molecular weight chemicals (Sobanska et al. 2018).

Furthermore, the biotransformation capacity of zebrafish embryos is considered to increase with development stage and this capacity might be limited at 24 hpf (Kühnert et al. 2013, 2017). This could be a challenge for assessment of chemicals such as organophosphates which require a bioactivation step to become more effective. In such cases, toxicokinetics could be used to study their biotransformation which allows to make adequate toxicity inferences. For example, Kühnert et al. (2013) could understand the biotransformation and toxicity dynamics of the polyaromatic hydrocarbon (benzo-a-pyrene) by investigating its uptake and elimination kinetics in zebrafish embryos. Despite the numerous advantages of toxicokinetics, it is usually not investigated in routine toxicity tests because of time and resources constraints.

In **Chapter 5**, the study was focused on: 1) understanding the influence of the potential limited biotransformation of organophosphates in the early stages of zebrafish embryos and 2) comparing the toxic mechanism of action of an organophosphate, which requires biotransformation, to its bioactivated product. The study reveals the need to include toxicokinetics in toxicity testing in order to enhance proper interpretation of toxicity data of single chemicals and mixtures.

1.8 Effect based methods for neurotoxicity monitoring

Effect based methods (EBMs) are bioassays capable of detecting effects of chemicals caused by short-term exposure to environmental samples. EBMs have been recommended as a complementary tool to chemical analysis monitoring in order to enhance the protection of aquatic resources (Escher and Leusch 2011).

In the European water framework directive, it is stipulated to monitor a certain amount of priority substances and only chemical monitoring techniques are used. However, an alternative approach for risk

assessment requires appropriate EBMs to detect specific effects such as neurotoxic and endocrine effects, as well as, to identify risks induced by chemical mixtures occurring in low concentrations which are undetectable by chemical monitoring (Brack et al. 2019). Therefore, it is important to develop novel strategies for environmental monitoring. For example, the effect directed analysis allows the identification and prioritization of potential toxic chemicals in a chemical mixture (Brack et al. 2016). In-vitro receptor assays and in-vivo zebrafish embryo assays also allow to measure endocrine disrupting and acute toxicity effects respectively (Altenburger et al. 2019).

Neuroactive substances are frequently detected in the environment (Busch et al. 2016) and they appear to be a major group of chemicals driving ecological impairment (Malaj et al. 2014; Liess et al. 2021). Despite, effect based methods for neurotoxic effects are not yet well developed and still lacking (Legradi et al. 2018). To date, only a few studies have used zebrafish embryos as an EBM for neurotoxicity testing. For example, Massei et al. (2019) used the AChE activity and locomotor response (LMR) of zebrafish as EBM to detect neurotoxicity in environmental samples while Ribeiro et al. (2020) used the STC test.

In this thesis, we also used the STC and LMR tests as EBMs to screen a wastewater effluent sample for acute effects and neurotoxicity. The specific aim of the study in **Chapter 6** were: 1) to compare the capacity of the STC and LMR tests for environmental monitoring purposes and 2) to investigate the capacity of these tests to identify effect drivers. The utility of zebrafish embryo behavior tests as EBMs to screen neurotoxicity alongside the standard fish embryo test for acute toxicity detection was demonstrated in this study.

1.9 Overview of objectives, research questions and methodology

The overarching goal of this thesis work is the toxicological characterization and screening of neuroactive substances and their mixtures using zebrafish embryos (Figure 1.3).

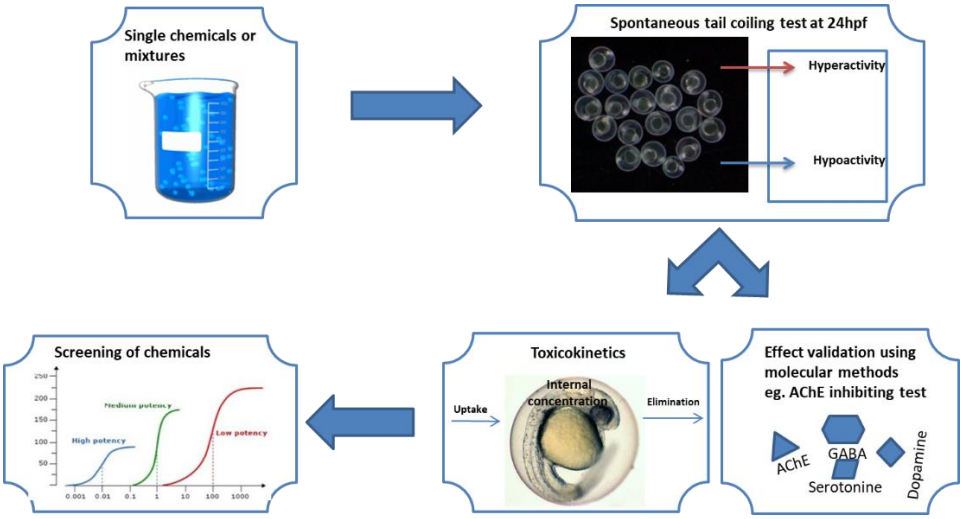

Figure 1.3: Overview of this thesis work. Neuroactive substances and their mixtures were tested in the STC test. To confirm the neuroactive mechanism of action, further approaches such as AChE activity test and toxicokinetics were performed to give a proper characterization and screening of neuroactive substances.

Objective 1: The goal in **Chapter 2** is to identify and select a zebrafish embryo behavioral test that can detect and characterize the effects of single neuroactive chemicals and their mixtures

Research questions

1.) Which experimental parameters mostly influence the observed effects in commonly used behavioral test methods and is it possible to rank these parameters?
2.) Is the expected mode of action of a chemical consistent with its observed hypo or hyper-activity in zebrafish embryo behavioral tests?
3.) Which of the behavioral test methods could be selected for further use to test neuroactive substances in the current study?

These questions were answered by conducting a systematic literature review on published behavioral studies. The behavioral test which is more consistent in predicting neuroactive mechanisms of action was selected for further test verifications.

Objective 2: The goal in **Chapter 3** is to optimize the experimental parameters of the behavior test method selected in **Chapter 2**. Another goal is to prove that the selected method can adequately screen a diverse group of neuroactive compounds with different modes of action.

Research questions

1.) Which experimental parameters affect the response of the selected behavior test method and can these parameters be optimized?
2.) Can the selected behavior test method efficiently screen a diverse group of neuroactive compounds with different modes of action?

These questions were answered by thoroughly evaluating the important experimental parameters discovered in **Chapter 2** and investigating the effect of diverse chemicals to understand the sensitivity, specificity and reproducibility of the test method.

Objective 3: The goal in **Chapter 4** is to understand the mixture toxicity of similar and dissimilarly acting neuroactive chemicals using the concentration addition and independent action models.

Research questions

1.) Can the mixture models, concentration addition and independent action be used to predict neuroactive chemical mixtures of similar and dissimilar mechanisms of action?
2.) Will a chemical mixture containing compounds which induce both hyper and hypoactivity result in an antagonistic interaction effect?

These questions were answered by preparing different mixtures of similarly and dissimilarly acting chemicals based on previously estimated effect concentrations for behavior effects. Further the mixture effects will be predicted using concentration addition and independent action models by using single chemical effect concentrations estimated in **Chapter 3.**

Objective 4: The goal in **Chapter 5** is to understand the influence of the potential limited biotransformation in the early stages of zebrafish embryos on the toxicity propagation of single and mixtures of neuroactive substances.

Research questions

1.) Does chlorpyrifos and its bioactivated oxon metabolite – chlorpyrifos-oxon have similar mode of action in the STC test?
2.) Is chlorpyrifos taken up fast and biotransformed in the 24 hpf zebrafish embryos?

These questions were answered by using abamectin, an hypoactivity inducing chemical, to antagonize the effect of chlorpyrifos and its oxon metabolite. Experiments were conducted to investigate if both organophosphates inhibit acetylcholinesterase. Furthermore, internal concentration was quantified using an appropriate chemical analysis method. Biotransformation was investigated by comparing the internal concentration across various developmental stages.

Objective 5: The goal in **Chapter 6** is to evaluate the use of the spontaneous tail coiling (STC) test for environmental monitoring purposes and to validate the use of STC as a diagnostic tool for chemical identification and prioritization.

Research questions

1.) Which behavior test is more suitable to detect neurotoxicity effects, STC or LMR tests?
2.) Can the effect drivers be identified using a molecular test i.e. acetylcholinesterase inhibition test?

These questions were answered by collecting wastewater treatment plant effluent samples and exposing it to zebrafish embryos in the STC and LMR tests. Further acetylcholinesterase inhibition analyses were conducted to understand the observed STC effects and potential effect drivers.

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Chapter 2: Hypo- or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes*

Abstract

Tests with zebrafish embryos have gained wide acceptance as an alternative test model for drug development and toxicity testing. In particular, the behavioral response of the zebrafish embryo is currently seen as a useful endpoint to diagnose neuroactive substances. Consequently, several behavioral test methods have been developed addressing various behavioral endpoints such as spontaneous tail coiling (STC), photomotor response (PMR), locomotor response (LMR) and alternating light/dark induced locomotor response (LMR-L/D). Although these methods are distinct in their application, most of their protocols differ quite strongly in the use of experimental parameters and this is usually driven by different research questions. However, if a single mode of action is to be diagnosed, then varying experimental parameters may cause incoherent behavioral responses (hypo- or hyperactivity) of zebrafish during toxicity assessment. This could lead to inconclusiveness of behavioral test results for use within a prospective and diagnostic risk assessment framework.

To investigate the influence of these parameters, we conducted a review of existing behavioral assays to address the following two questions: 1.) To what extent do varying experimental parameters influence observed effects in published behavioral test methods? 2.) Is the observed behavior change (hypo- or hyperactivity) of zebrafish embryos consistent with the expected mode of action of a chemical? We compiled a set of 18 substances which are anticipated to be neuroactive. We found that behavioral changes are not only affected by chemicals but variation in the use of experimental parameters across studies seems to have a high impact on the outcome and thus comparability between studies. Four parameters i.e. exposure concentration, exposure duration, endpoint parameter and developmental stage were the most influential parameters. Varying combinations of these parameters caused a non-reproducible outcome for the hyperactivity expected for the organophosphates; chlorpyrifos and diazinon.

We highlighted that the STC test shows a higher capacity to predict the hyperactivity of organophosphates while PMR and LMR-L/D were more suitable to predict the hypoactivity expected for anticonvulsants. We provide a list of recommendations which, when implemented, may help to exclude the risk of bias due to experimental parameters if similar goals are desired.

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2.1 Background

Many chemical substances released into the environment exhibit neuroactive properties and may have negative consequences on human and environmental health (Legradi et al. 2018). In fact, most of these substances are designed to interact with the nervous system. For example, insecticides target the nervous system of invertebrates while some pharmaceuticals are designed to treat neurodegenerative diseases in humans (Damstra 1978; Söderpalm 2002). Although chemical monitoring techniques are commonly used, a holistic approach for risk assessment requires appropriate effect based tools to detect neurotoxic effects (Brack et al. 2019). Busch et al. (2016) showed that neuroactive substances represent the largest group (13%) of chemicals with known mode of action (MoA) detected in European rivers. These neuroactive substances co-exist in the environment with other chemicals (Legradi et al. 2018) and substance-receptor relationships could be useful to identify these neuroactive substances within a complex mixture. Typically, neuroactive substances target specific parts of the nervous system such as acetylcholinesterases (AChE), nicotine receptors (nAChr), Gamma-aminobutyric acid receptors (GABA) and sodium channel receptors. Despite the widespread occurrence of neuroactive chemicals in the environment and their ability to disrupt the nervous system, standardized methods for assessing the risks of these substances are lacking (Legradi et al. 2015). Hence, we conducted a review of the commonly used behavior test methods in zebrafish embryo which are used to assess neurotoxicity. We collated behavior testing of 18 substances from different studies. This review evaluates the comparability of the experimental parameters used in these studies as a means to optimize them for assessing and detecting neuroactive substances within a prospective and diagnostic risk assessment framework.

Importance of zebrafish embryo behavior

Testing of the adverse effects of chemicals to humans and the environment relies at present to a large extent on animal models such as rodents and adult fish (Lillicrap et al. 2016). It is however known that the exposure of animals to chemicals may inflict pain and distress. Hence there is an increased need to develop alternatives to animal tests because of ethical reasons, as well as to reduce time and cost of these tests (Braunbeck et al. 2005; Doke and Dhawale 2015). Consequently, the use of animals in toxicity testing has been highly discouraged in favor of promoting the 3R principle: reduction, refinement and replacement (Russel and Burch 1959; Embry et al. 2010). In turn, the use of cell lines are encouraged as an alternative due to their ability to identify mechanisms underlying toxic effects (Schirmer 2006). However, the inability of cell lines to integrate the interaction of various tissues within a multicellular system is a major disadvantage (Scholz et al. 2008). Alternatively, zebrafish embryos proved to be a promising model due to its capacity to predict fish and rodent toxicity (Hill et al. 2005; Scholz et al. 2013b). Further, the behavior of zebrafish embryos can be used to distinguish between different neurotoxic MoA such as beta-adrenergic receptor agonists, dopamine agonists and adenosine receptor antagonists (Kokel et al. 2010).

In fish, functional interference with the cardiovascular and nervous system, particularly demonstrated for AChE inhibition, leads to the respiratory failure syndrome resulting in enhanced mortality due to oxygen limitations (Russom et al. 2014). In contrast, fish embryos appear to lack the respiratory failure syndrome because oxygen in embryos is mainly supplied via skin diffusion (Rombough 2002; Klüver et al. 2015). As a result, embryos only show a weak mortality for neurotoxic substances. However, it has been shown that

some neuroactive substances exhibit effects on behavior at concentrations well below the lethal range (Klüver et al. 2015). Hence, the observation of behavior changes at sublethal concentration ranges in embryos may provide an indicator for neuroactivity and/or could be used to infer adverse effects. Therefore, the aim of this review is to identify experimental parameters for behavior assays that could support the unbiased diagnosis of different neuroactive mode of actions.

Types of behavioral tests considered in this review

The potential to identify interactions of chemicals with the nervous system using behavioral assays in zebrafish has been recognized and several behavioral test methods have been developed. In this review, we focus on the most commonly utilized tests and their endpoints including spontaneous tail coiling (STC), photomotor response (PMR), locomotor response (LMR) and alternating light and dark induced locomotor response (LMR-L/D) [Table 2.1].

Table 2.1: Characteristics of different behavioral tests.

	Spontaneous tail coiling test (STC)	**Photomotor response test (PMR)**	**Locomotor response test (LMR)**	**Alternating light and dark induced locomotor response test (LMR-L/D)**
Applied stimuli	non	High intensity light	non	Alternating light/dark
Endpoint	Number or frequency of tail coilings	Movement activity or motion index	Swimming distance, duration, and speed	Swimming distance, duration, and speed
Exposure duration	0-28hpf	0-42hpf	0-120hpf	0-120hpf
Age of embryo at time of measurement	19-28 hpf	28-42hpf	72-120hpf	72-120hpf

Spontaneous tail coiling (STC) is the first motor activity generated by the developing neural network which occurs as a result of the innervation of the muscle (Kimmel et al. 1974). This event is assumed to be important for the hatching of the embryo from its chorion but evidence for a role of STC for hatching has not yet been provided (Kimmel et al. 1974; Saint-Amant and Drapeau 1998). Frequency changes of the STC has been used as a tool to detect the effects of neuroactive chemicals in developing embryo (Weichert et al. 2017; Vliet et al. 2017). The photomotor response (PMR) is an embryonic movement induced by a high-intensity light stimulus (wavelength between 300 and 700 nm). This response is independent from

light perception by the eyes and mediated through photoreceptors in the developing hindbrain (Kokel et al. 2010; Reif et al. 2016). The PMR can be divided into four broad phases, a pre-stimulus background phase, latency phase, excitation phase and refractory phase. The visualization of these PMR phases has been used for chemical classification and drug screening (Kokel et al. 2010). Both STC and PMR represent endpoints measured in pre-hatching embryo-stages (19 – 42 hpf) of zebrafish.

The locomotor response can either be spontaneous (LMR) or induced with alternating light/dark periods (LMR-L/D) and these can be measured in the post-hatching embryo stages (>48 hpf) of zebrafish. In LMR-L/D, zebrafish embryos exhibit weak movement when illuminated by light but exhibit an increase in activity when switched from light to dark (Irons et al. 2010; Selderslaghs et al. 2010). Therefore, light-dark cycles are applied to monitor this behavior. LMR is estimated by recording various swimming activity endpoints such as swimming time, swimming distance, swimming speed (calculated from distance and time) and swimming angle, while LMR-L/D measures the stated parameters under alternating light/dark cycles. The LMR assessment is similar to behavioral monitoring studies that use adult fish, e.g. for online (bio-) monitoring of waste- or surface-water, also known as “fish toximeters”. In these toximeters for example alteration of swimming activity is often used as an indicator of potential adverse effects due to chemical exposure (Bae and Park 2014).

Influence of experimental parameters

Although these behavioral test methods, particularly LMR and PMR are widely used and have been applied in large drug and chemical screens (Reif et al. 2016; Bugel and Tanguay 2018), they differ largely in experimental parameters between different labs such as exposure duration and selection of endpoint parameter etc. Several authors have studied the influence of experimental parameters on zebrafish embryo behavior. For example, distance moved by zebrafish embryo varies with age of embryos and size of exposure vessel (Padilla et al. 2011; De Esch et al. 2012); light/dark response is usually affected by duration of cycles and the number of repeats (MacPhail et al. 2008); and the observed effect is highly dependent on the exposure concentration or endpoint selected (De Esch et al. 2012; Ingebretson and Masino 2013). Legradi et al. (2015) reviewed the literature to compare behavioral test methods. They found that for LMR assessment, experimental parameters such as duration of behavior assay and developmental stage of embryos varied significantly among studies. It is still largely unknown how this variability influences the outcome of behavioral studies. The need to harmonize and report experimental parameters has previously been discussed for zebrafish embryotoxicity test (Beekhuijzen et al. 2015) and in-vitro neurotoxicity testing (Crofton et al. 2011). However, such critical discussion and analysis for neurotoxicity testing using zebrafish embryo behavior is still lacking.

The use of hypo- and hyperactivity as a predictor

In some studies the differentiation between hypo- and hyperactivity has been suggested as a potential indicator for neuroactivity MoA (Kokel et al. 2010; Ellis et al. 2012; Fraser et al. 2017). With respect to embryonic behavior of unexposed embryos, hypoactivity refers to a decrease in the spontaneous or induced movement of embryos, while hyperactivity refers to the opposite. In this study, we define neurotoxicity as an interaction of a substance with the nervous system primarily leading to a structural change (eg. axonal deformation or inhibition of neurite outgrowth) while neuroactivity is a functional interaction with specific nervous receptors leading to a change without necessarily being accompanied by

structural changes (eg. alteration of neurotransmission or blockage of nervous receptor). Nonetheless, neuroactivity may lead secondarily also to structural and morphological changes over a longer duration or higher concentration of chemical exposure. It is assumed that neuroactive substances are able to modulate nervous receptors leading to hypo- or hyperactivity behavior (Tierney 2011). For example, Vliet et al. (2017) used the STC response as a metric to screen a library of 1,280 pharmacologically active compounds for neuroactivity. Reif et al. (2016) used the hypo- or hyperactivity observed in the different phases of the PMR to characterize a suite of 1060 chemicals; and Bugel and Tanguay (2018) were able to negate a GABA mode of action for a suite of 24 flavonoids in the LMR based on the induction of hypo- or hyperactivity.

The rationale behind the above mentioned screening studies was that substances with the same or similar mode of action would only induce either hypo- or hyperactivity. However, it is also possible that both hypo- and hyperactivity (biphasic activity) may be induced by the same substance depending on the concentration level or duration of exposure. For example, chlorpyrifos-oxon and aldicarb-sulfoxide stimulate nerve cells by inhibiting acetylcholinesterase, thereby inducing hyperactivity and increased heartbeat of embryos, respectively (Küster and Altenburger 2007; Watson et al. 2014). At higher concentrations, the over-excitation of the cholinergic system may result in paralysis caused by seizures and thus leading to hypoactivity (Stehr et al. 2006). Alternatively, abamectin induces hypoactivity due to its inhibitory action when it activates the GABA gated chloride channel (Raftery and Volz 2015). Such distinct characteristics of neuroactive substances suggests that the hypo- or hyperactivity of zebrafish embryos may be used to identify MoA when experimental parameters are adequately controlled (Basnet et al. 2019).

Aims and approach of this review

We aimed to investigate the influence of experimental parameters on the hypo- or hyperactivity response of zebrafish embryos by reviewing existing literature. We first created a collection of pharmaceutical and pesticide substances with known MoA, for which sufficient information on effect concentrations and experimental parameters were available, to address the following questions:

1.) Which experimental parameter(s) mostly influence the observed effects in the four above mentioned behavioral test methods and is it possible to rank these parameters?

2.) How often is the observed hypo- or hyperactivity of zebrafish embryos in literature consistent with the expected mode of action of a chemical substance?

These questions are based on the hypo- or hyperactivity hypothesis i.e. whether the behavioral response (hypo- or hyperactivity in moved distance, tail coiling or else) of zebrafish embryos predicts the mode of action of a neuroactive chemical and vice versa. This review provides information that will support the selection of a combination of appropriate behavioral tests within a prospective risk assessment framework. We also present critical evaluations on how to use hypo- and hyperactivity detection as a tool to improve the identification of neuroactive mode of action in a complex mixture within a diagnostic risk assessment framework.

2.2 Method

2.2.1 Literature selection

Literature search was mainly undertaken by searching the "web of science" database (www.webofknowledge.com) and the search results were filtered in a KNIME® (www.knime.com) workflow. A few additional papers were selected by cross-referencing of citations. Figure 2.1 shows a summary of the literature selection procedure.

1.) Keyword search on "web of science" database: The following keywords combinations were searched for representation in either title, abstract or keywords: ("zebrafish embryo" OR "zebrafish larvae" OR zebrafish) AND (*throughput* or locomotor OR LMR OR "spontaneous activity" OR STC OR Photomotor OR PMR) AND (behav* OR hyperactivity OR hypoactivity OR neurotoxicity OR movement). Only studies published between year 2000 and 2018 were retrieved. The search was conducted on 17th August 2018. The search was repeated on 29th July 2019 to include additional hits of chemicals that were already included in the analysis.

2.) Apply exclusion criteria by screening the abstract of the study hits: The following exclusion criteria were applied to reduce the variability between studies and to increase the quality of data collection.

   a.) Organism: Not zebrafish, or a mutant zebrafish strain was used

   b.) Age: Juvenile or adult stages were tested

   c.) Method: Other assays than STC, PMR, LMR and LMR-L/D were used.

   d.) Chemical: Only a mixture, an inorganic compound or a natural, undefined product was used.

   e.) Exposure: Oral exposure

   f.) Effect: No report or indication of hypo- or hyperactivity

3.) Regrouping of study hits into chemical hits:

   a.) A KNIME® workflow was used to aggregate and regroup the study hits according to the test chemical. The KNIME® workflow is shown in Appendix 1 - Figure S1.

   b.) The KNIME® workflow was also used to exclude chemicals with less than 3 entry hits

4.) A further exclusion criterion was applied to the chemical hits to increase the quality of the collected data. I.e. chemicals with unknown or unclear mode of action were excluded.

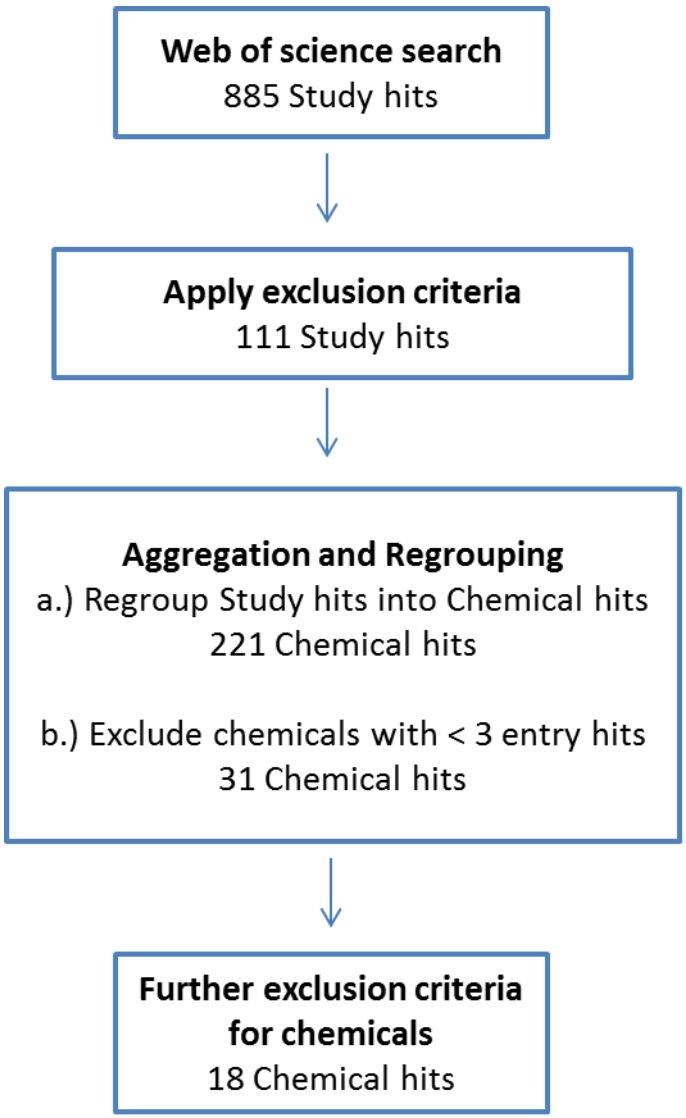

Figure 2.1: Flowchart describing literature search strategy and selection of studies.

2.2.1.1 Data collection and analysis

After selecting the appropriate studies based on the criteria above, we proceeded to analyze data reported in these studies. Data collected included: chemical type, mode of action, effect concentration (for hyper- and hypoactivity), exposure duration, analysis duration, exposure well size, developmental stage at exposure, zebrafish strain, etc.

2.3 Results and discussion

In the first part of the results, we address the question - which experimental parameters are mostly influencing toxicity outcome of behavioral tests and we considered parameters related to exposure design and effect measurement for this analysis.

In the second part of the results, we address the question - how often the observed hypo- or hyperactivity of zebrafish embryos after exposure to a chemical is consistent with the expected mode of action of this chemical. We compared observed and expected activities at three levels:

a) Individual chemical comparison i.e. individual chemicals were selected and the found literature results were compared on the level of observed behavior effect;

b) Chemical class comparison i.e. does organophosphates for example, always show the same activity change in all behavior assays, even if test parameters were different;

c) Behavioral method comparison i.e. whether different behavioral test methods or contrasting experimental parameters give similar results?

For the ranking of the influence of each parameter, we analyzed the percentage concordance of hyperactivity between expected and observed activity for each behavioral test method. This was estimated by dividing the number of studies in which hypo- or hyperactivity was observed by the total number of studies for a certain chemical expected to cause hypo- or hyperactivity. Any observed non-concordance was attributed to experimental parameters as a risk of bias factor.

2.3.1 Influence of experimental parameters on behavior analysis

Thirteen experimental parameters were identified in the literature survey with potential impact on the outcome of different behavior tests. The parameters were sub-grouped into biological (or intrinsic) and technical (or extrinsic) experimental factors. Developmental stage, zebrafish strain, malformations, rearing conditions, time of day for behavior analysis and the selected endpoints were considered as biological factors. Exposure duration, exposure concentration, duration of behavior analysis, exposure well size, material used for exposure vessel, light conditions and solvent concentration were considered as technical factors. Table 2.2 shows the potential influence of the 13 experimental parameters which are more precisely explained in the text below.

Table 2.2: Summary of the Influence and optimization of experimental parameters on behavioral test performance.

Parameter Type	Parameter	Influence on:	Potential measures to exclude bias
Biological or intrinsic	Developmental stage	Biotransformation, effect concentration, Receptor availability Stage specific behavior	Unify developmental stage for comparability; report on incubation temperature
	Time of day	Performance of control group, test sensitivity	Measure behavior at similar time of the day
	Developmental malformations	Effect concentration, effect direction (hypo- or hyperactivity), swimming ability	Malformations should be analyzed in parallel with behavioral testing to indicate at which concentration they may contribute to the observed behavioral effects
	Endpoint parameter	Effect concentration, effect direction (hypo- or hyper-activity)	Compare the same endpoints within a particular assay, i.e. distance moved for LMR and frequency for STC
	Rearing conditions during exposure	Performance of control group, test sensitivity	Report all rearing conditions
	Zebrafish strain	Control performance, test sensitivity	Consider strain effects during result comparison and interpretation
Technical or extrinsic	Exposure concentration	Effect concentration type (LOEC versus ECx) and accuracy, effect direction (hypo- or hyperactivity)	Avoid single concentration and include a full concentration-response analysis
	Exposure duration	Effect concentration, Internal bioavailable concentration, biotransformation, effect direction (hypo- or hyper-activity), observation of (neuro)developmental effects	Consider toxicokinetics/toxicodynamics and its influence on chemical concentration during test design
	Duration of behavior analysis	Performance of unexposed group, test sensitivity	Differences in the duration of light-dark cycles may impact on the sensitivity and outcome in LMR-L/D
	Exposure well size	Effect direction (hypo- or hyperactivity), Performance of control group, test sensitivity	Select and use exposure wells of same dimension for reproducibility i.e. 96 well for neurotoxicity testing and 24 well for diagnosing neuroactivity MoA
	Material used for exposure vessel	Effect concentration, test sensitivity	Avoid plastic for lipophilic chemicals, measure/predict exposure concentration and install measures to ensure a stable exposure concentration if required.
	Light conditions	Performance of control group, test sensitivity	Light intensity, duration, and sequence of photo stimuli should be reported
	Solvent concentration	Exposure concentration, side effects	Avoid DMSO and other solvents or use 0.01% concentration when necessary. Test solvent control

2.3.1.1 Biological (intrinsic) factors

Developmental stage

Padilla et al. (2011) investigated the influence of development stage on distance moved in alternating light and dark periods. They compared the behavior of 4, 5 and 6 day old non-exposed embryos/larvae in the light period and found that older embryos had increased movement, indicated by the distance moved, than younger ones. On the other hand, the influence of age in the dark period was not obvious except for differences in the pattern of movement. The observed influence of age on behavior in the light period may be due to differences in retinal maturation as the retina ganglia cells of younger larvae may not respond to light as much as older larvae (Padilla et al. 2011). These findings are corroborated by De Esch et al. (2012), who compared 5, 6 and 7 day old embryos/larvae and Leuthold et al. (2019) who compared 4 and 5 day old embryos. Fraser et al. (2017) reported increased movement for older embryos (120 > 100 > 96 hpf) when raised in constant darkness. In contrast, Ingebretson and Masino (2013), while considering total distance moved in constant light, reported that 7 day old larvae moved less than 4 day old embryos. However, the impact of age on the distance moved was only observed when embryos were analyzed in deep but not in shallow wells. The developmental stage may not only impact on control behavior but can impact on the observed responses to exposure of chemicals that require biotransformation such as organophosphates (Kristofco et al. 2016). Furthermore, some neurotransmitters such as histamine and 3-methoxytyramine may not be present in early developmental stages and this could lead to differences in observed effects due to target availability (Rico et al. 2011; Tufi et al. 2016). Principally, differences in the uptake kinetics of chemicals may also impact on behavior at specific developmental stages. However, so far, there is weak evidence for considerable uptake differences (Massei et al. 2015). Nevertheless, for pre-hatched stages and high molecular weight compounds, the chorion may represent a barrier (Scholz et al. 2008; Pelka et al. 2017). These findings indicate that the developmental stage at which the analysis is conducted may be influential. Relative effects by chemicals may not necessarily be disturbed if they are measured at the same stage.

Time of the day for analysis

Kristofco et al. (2016) investigated the influence of day-timing on behavior analysis in alternating light/dark test (LMR-L/D). They found that activity of embryos and larvae measured daily (between 4-10 dpf) were significantly lower in the early hours of the day at 9 and 10 a.m. and more variable during these periods, while activity was higher and less variable in the afternoon. In contrast, MacPhail et al. (2008) measured locomotion and reported higher movement for 6dpf larvae at 10 and 11 a.m. in the morning while lower movement was recorded in the afternoon. The two studies used the same 14/10 light/dark cycle. MacPhail et al. (2008) recorded behavior only for the dark period. Both authors concluded that activity was less variable when measured in the afternoon. In contrast to both studies, Fitzgerald et al. (2019) reported that time of day did not change the activity of 5,6 and 7 dpf larvae in the dark period of LMR-L/D measured at 9 a.m and 2 p.m., and activity was more variable in the afternoon rather than in the morning. However, a different temperature (26°C versus 28°C of the other studies) was used as rearing temperature and the controversial finding may have been caused by differences in growth and developmental stage. Furthermore, the influence of day-time may rather relate to the time after the onset of light, but details on light-cycles are missing in some articles. These results suggest that diurnal rhythm

of fish embryos and the impact on responses may have to be controlled by measuring behavior at similar time of the day and that impacts of growth and developmental as potential confounding factors should be considered.

Developmental malformations

Padilla et al. (2011) reported that malformed zebrafish in control solution showed hypoactivity in both light and dark periods while healthy control animals remained unaffected in behavior. Hence, reduced movement of activity may represent a secondary effect following malformations when embryos are exposed to chemicals. Consequently, malformations should be analyzed in parallel and concentration-response data for phenotypic effects and behavior should be compared to identify potential secondary effects. By comparing the effect concentration for behavior and malformation the specificity of the behavioral effects could be assessed. However, subtle alterations at sub-organism level (e.g. muscle structure) not easily detectable by microscopical observations may also impact on the behavior.

Endpoint parameter

De Esch et al. (2012) compared 3 different endpoints in LMR-L/D, namely distance moved, duration of movement and swimming velocity (calculated from distance and time), among 3 different developmental stages. They found that results for swimming velocity were negatively correlated to the other endpoints i.e. high velocity was correlated to lower distance moved and shorter duration of movement. This was attributed to the fact that the increased velocity was caused by short movements and hence, only a short distance was moved. Therefore, they concluded that endpoints should be selected cautiously since swimming velocity might not completely represent other endpoints. Alternatively, Ingebretson and Masino (2013) advocated the use of more than one endpoint as an integrated approach to maximize diagnostic capacity of behavioral activity. In the case of STC, two different endpoints were typically reported; frequency of STC and percentage of embryos showing spontaneous activity (Yozzo et al. 2013; Raftery et al. 2014). Since the latter endpoint only reveals the numbers of embryos showing STC, it may not be able to demonstrate effects that mainly affect the frequency of STC. For comparative assessment, the same endpoint parameter within a particular assay i.e. distance moved for LMR and frequency for STC test should be used.

Rearing conditions

Rearing conditions may also be considered as an extrinsic technical factor. However, since rearing conditions (raising and/or exposure in groups versus raising of individuals, choice of rearing temperature) are known to impact on development in general and behavior patterns it was formally considered as intrinsic factor. Zellner et al. (2011) observed that zebrafish embryos raised in groups were more active than those raised individually after the first 5 days of development. They proposed that rearing condition before behavior measurements could probably influence the effects of exposure to neuroactive substances. This seems consistent with the knowledge that swarm fish like zebrafish show shoaling and schooling and this (i.e. raising as a group) decreases their overall stress level under certain conditions (Ramsay et al. 2006). This peer inclusion has also been reported to increase stimulation and may facilitate neuron connection (Lazic et al. 2006). A guidance document on fish rearing delineates the density as being important (Lawrence 2007) but at the moment it is not clear whether crowding is important for early stages of zebrafish and what group size would be the appropriate rearing size (Parker et al. 2012; Ribas et

al. 2017). However, when embryos were exposed to valproate by Zellner et al. (2011), hyperactivity was recorded but no significant difference was observed for embryos raised singly or in-groups. Whether embryos were exposed singly or in-groups may also impact on results due to the requirement for different types of data analysis. E.g. individual tracking of movement is difficult when exposure is conducted with group of animals.

Rearing temperature represents an important factor as well, since it influences the rate of development and therefore the developmental stages at which behavior may be analyzed (Kimmel et al. 1995). Other rearing conditions such as pH and salinity which could also impact on the behavior analysis were not discussed due to insufficient data or lack of reporting. Therefore, it is recommended to report specific rearing conditions.

Zebrafish strain

In the literature, a discussion is ongoing whether different zebrafish strains may differ in their susceptibility to chemicals and how much is based on genetically based differences, physiological adaptations to cultivation or other parameters (Diekmann and Nagel 2005; Guryev et al. 2006). For behavioral effects, De Esche et al. (2012) investigated the influence of different strains (TL and AB) on locomotion in alternating light and dark periods. They found significant differences in distance moved but only in the dark periods. Lange et al. (2013) also compared 5 different strains of 6 dpf larvae (AB, TU, WIK, Casper and Ekkwill) and found that Casper and Ekkwill strains moved less than the other strains. They also compared AB strains from different laboratories and their results show that these AB strains moved similar distances but had different swimming times. They concluded that the strain might influence locomotor activity of zebrafish. Similarly, Liu et al. (2015) reported behavioral differences of strains (TL, TLAB and AB) when measuring locomotion during abrupt changes in light cycles. Strain differences were also found in a study on survival and neurocranial effects of ethanol (Loucks and Carvan 2004). Further, strain differences have been reported during chemical exposure. 5D strain exposed to haloperidol showed hyperactivity while the AB strain showed no effect (Oliveri and Levin 2019). These differences in strain behavior are probably related to genetic differences, albeit the factors and differences leading to strain variability have not yet been identified. Hence, possible influence on behavior by the strain should be considered and also between laboratories.

2.3.1.2 Technical (extrinsic) factors

Exposure concentration

Exposure concentration is obviously one of the most important experimental parameters in toxicity studies. Hamm et al. (2019) identified that the renewal or non-renewal of exposure solution could influence toxicity testing, particularly in case of volatilization, degradation and/or adsorption to exposure vessels, resulting in a decline of exposure concentrations. The exposure concentration could represent a factor of high relevance in high-throughput studies that only examine a single, selected concentration for a given test chemical. Depending on the selected concentration, hypo- or hyperactivity may be provoked and this can have an impact on diagnostic patterns if obtained with a single or a limited range of concentrations. For example, ethanol causes hyperactivity at concentrations below 2 % and hypoactivity at concentrations above 4 % (Irons et al. 2010; De Esch et al. 2012). Zebrafish exposed to the

cholinesterase inhibitor paraoxon showed hyperactivity in a lower exposure range (31 – 500 nM) and became hypoactive with a 100 fold increase in concentrations [3.1 – 50 µM] (Yozzo et al. 2013; Raftery et al. 2014). The differential response may be associated with excitation of nerve signaling at lower concentrations due to acetylcholinesterase inhibition and seizure-driven paralysis at higher concentrations (Stehr et al. 2006). However, organophosphates have also been shown to affect axonal morphology of motor neurons at high concentrations (>500 nM) (Yang et al. 2011) and this could probably explain the hyperactivity observed at low concentrations rather than hypoactivity (Yozzo et al. 2013). Therefore, behavioral tests should include a concentration-response analysis.

Exposure duration

In a STC study by Vliet et al. (2017), exposure duration was reduced from 23 h (2-25hpf) to 2 h (23-25hpf) to eliminate false positives associated with malformations. This decreased the number of STC hits from 43 to 15. However, this decrease in hits could be compromised by a slow uptake of certain chemicals. Internal exposure analyses have indicated that for many chemicals, time to equilibrium with average internal concentrations can exceed 24 h depending on the compound's characteristics (Brox et al. 2014b). In behavior assays that allow longer exposure durations such as the LMR, the same exposure concentration that causes hyperactivity in the STC may lead to axonal defects, malformations and/or paralysis resulting in hypoactivity in these long duration tests (Kristofco et al. 2016). This could explain the opposite effect direction observed in STC tests (hyperactivity) and LMR tests (hypoactivity) for chlorpyrifos (Watson et al. 2014). For similar reasons, differences in the effect direction (hypo- or hyperactivity) may also be observed within the same assay when different exposure durations are used. For instance, Leuthold et al. (2019) have applied a 24 h exposure regime in the LMR-L/D and differences to studies conducted with longer exposure durations may be associated with the factor described above. This means that significant differences in exposure duration could influence the internal concentration of a chemical. Hence – if that is a possible confounding factor for the goal of the experiment, behavioral test designs should ideally measure or try to model the toxicokinetics/toxicodynamics of the chemical for appropriate result interpretation e.g. short duration for identifying neuroactivity MoA while long duration may be preferred for assessing developmental neurotoxicity.

Duration of behavior analysis

Zebrafish embryos are sensitive to alternating light/dark periods. Non-exposed zebrafish were found to be more active in dark than light periods. Therefore, by purpose, various studies have utilized cycles of light and dark periods to improve detection of hypo- and hyperactivity (eg. Irons et al. 2010; Kristofco et al. 2016; Leuthold et al. 2019; Zindler et al. 2019). However, the dynamics of analysis duration has been shown to affect test outcome. Exposing 6dpf larvae after a dark acclimation period of 10 mins and a subsequent extended (40 mins) period in light (or dark) showed contrasting behavior - Activity increased to a maximum in the 10 min dark acclimation period, followed by a decline which continued to either a stable low level in the subsequent dark period or to a stable high level in the light period (MacPhail et al. 2008). Interestingly, in a light/dark preference test by Steenbergen et al. (2011), zebrafish prefer light and the increased movement in the dark is hypothesized to relate to a behavior such as escaping from a predator (De Esch et al. 2012). Alternately, it was discussed that foraging in zebrafish depend on their visual system to find food and therefore increased activity in the dark may be related to a light-searching behavior (Horstick et al. 2017). For toxicity tests, the basal activities during light and dark periods are

important to detect hypo- and hyperactivity. While it might be difficult to detect hypoactivity in light periods given the low activity level, detection of hyperactivity could be more relevant during this period. The duration and frequency of light/dark cycles could also be of high relevance in a toxicity testing setup. Dark acclimation of zebrafish (10 mins), followed by different light periods of either 5 or 15 mins showed a higher magnitude of increased activity in the subsequent dark period for the larvae exposed to 15 mins light than that of 5 mins (MacPhail et al. 2008). Different dark acclimation periods of 10 and 20 mins did not affect activity in subsequent light and dark periods. Another study by Liu et al. (2015), found that analysis of short periods of 30 seconds before and after light/dark transition amplified behavior changes. Taken together, these results suggest that extended periods of light or dark can impact on the activity of zebrafish embryos and hence may affect the sensitivity and outcome of the study.

Exposure well size

Velki et al. (2017) compared the total distance moved for zebrafish embryo/larvae exposed in 24- or 96 well-plates in the dark period. They reported an average distance of 600-700 mm moved in 24 well-plates, whereas those in 96 well moved 40% less. Similarly, Padilla et al. (2011) found that larval movement was approximately 4 times higher in 24 well than in 96 well-plates and distance moved in 48 well plate was not statistically different from that of 96 well plate. They elucidated that the distance moved is mainly influenced by the circumference of the well rather than the area since embryos tend to swim more around the circumference of the well (Colwill and Creton 2011). In contrast, Ingebretson and Masino (2013) found no difference in distance moved when different well diameters of 12, 20 and 30mm were compared [approximately referring to the diameter of 48- (10.9mm), 24- (15.5mm) and 6 well-plates (35 mm). A potential bias by rearing conditions can probably be excluded since embryos were raised and tested in the same wells in Padilla et al. (2011) but transferred to well plates after exposure for behavioral analysis in all other studies.

From the analyzed literature it is not yet clear if the decreased distance in smaller wells is associated with a lower sensitivity of the assay. For test reproducibility and uniformity, it is recommended to use exposure wells of same dimension. As an alternative, one could conduct experiments in different well sizes to ensure maximal confidence of the sensitivity of the assay used.

Light conditions

Padilla et al. (2011) investigated the influence of light intensity on the behavior of 6dpf zebrafish larvae. They found an increased activity when light levels were decreased and this increased activity was dependent on the magnitude of the decreased light intensity. Therefore, light intensity, duration, and sequence of photo stimuli should be recorded in different experimental setups and their impact on the effects obtained by exposure of chemicals should be investigated.

Material used for exposure vessels

The sorption of lipophilic substances to plastic material used for exposure is well known (Schreiber et al. 2008; Fischer et al. 2017). Sorption could decrease the exposure concentration leading to underestimation of effects if based on nominal concentrations. Raftery et al. (2014) exposed embryos to the highly hydrophobic glutamate channel blocker - abamectin. They found an effect concentration for the spontaneous tail coiling (STC, conducted in 384 well plates) which is 12 fold higher than that reported in a subsequent study (Raftery and Volz 2015) in which embryos were exposed in glass beakers (both used

nominal concentrations). Similarly, Vliet et al. (2017) who also exposed embryos to abamectin in 384 well plates found an effect concentration which is 6 fold higher than that observed in Raftery and Volz (2015). Beside the test container material, the ratio of surface area to volume could also induce variability. The reported effect concentration for emamectin is over 40 times lower when embryos were exposed in 24 well-plates (Weichert et al. 2017) compared to exposure in 384 well plates (Raftery et al. 2014). This could be the result of higher well absorption area with respect to volume in 384 well plates leading to higher adsorption. Hence, as would be appropriate for any other test and endpoint, the sorption of chemicals to exposure vessels should be considered by: 1.) determine or predict the real exposure concentration; 2.) estimate a possible loss of the chemical instead of using nominal concentrations only (Gülden et al. 2001; Gülden and Seibert 2005; Fischer et al. 2018).

Solvent concentration

Solvents such as dimethyl sulfoxide (DMSO) are often used to accelerate dissolutions or to store stock solution for toxicity tests. At high concentrations, solvents may also impact the uptake of chemicals and provoke toxicity. Therefore, OECD guidance for aquatic toxicity tests recommends using a maximum solvent concentration of 0.01% (v/v). In concordance with these recommendation, Kais et al. (2013) reported that DMSO concentration of 0.1% and 1% increased uptake of hydrophobic dyes into zebrafish embryos while 0.01% had no effect. DMSO has also been found to increase the movement of 24 and 144hpf embryo/larvae at 0.1 and 0.01 % respectively (Chen et al. 2011; Huang et al. 2018). These results showed that DMSO may be a source of confounding effects in behavioral tests. It is highly recommended to avoid solvents, but if necessary, a solvent control should always be utilized and a range of solvent concentrations could be tested to characterize and exclude possible confounding effects.

2.3.1.3 Summary of influence of experimental parameters

The studies cited above showed the possible impact of different biological and technical experimental parameters on the behavioral response of zebrafish exposed to a chemical. Thus, experimental parameters may influence changes in behavior induced by chemicals. We ranked the importance of experimental parameters based on the frequency at which they explained inconsistencies (risk of bias factors) in chemicals as follows (Figure 2.2): exposure duration (11) > exposure concentration (10) > endpoint parameter (8) > developmental stage (7) > light conditions (2) > material used for exposure vessel (1) = exposure well size (1) = duration of behavior analysis (1) = zebrafish strain (1). This ranking does not consider the behavioral test method as a factor.

Depending on the goal of the research question, the reproducibility of behavioral tests may be improved by developing protocols with harmonized experimental parameters. However, while there is evidence for the impact of intrinsic and extrinsic parameters affecting behavior, only few studies have conducted a systematic assessment how this may impact on the detection of chemical effects. One of such studies found that developmental stage, light conditions during rearing and size of well plate affected the locomotor response of zebrafish larvae exposed to bisphenol A and tetrabromobisphenol A (Fraser et al. 2017). Although the evidence that effects of exposure to chemicals may not be detected due to the choice of experimental parameters is not clear, the observed effects in unexposed groups are sufficient to motivate the harmonization of behavioral tests for specific hypotheses.

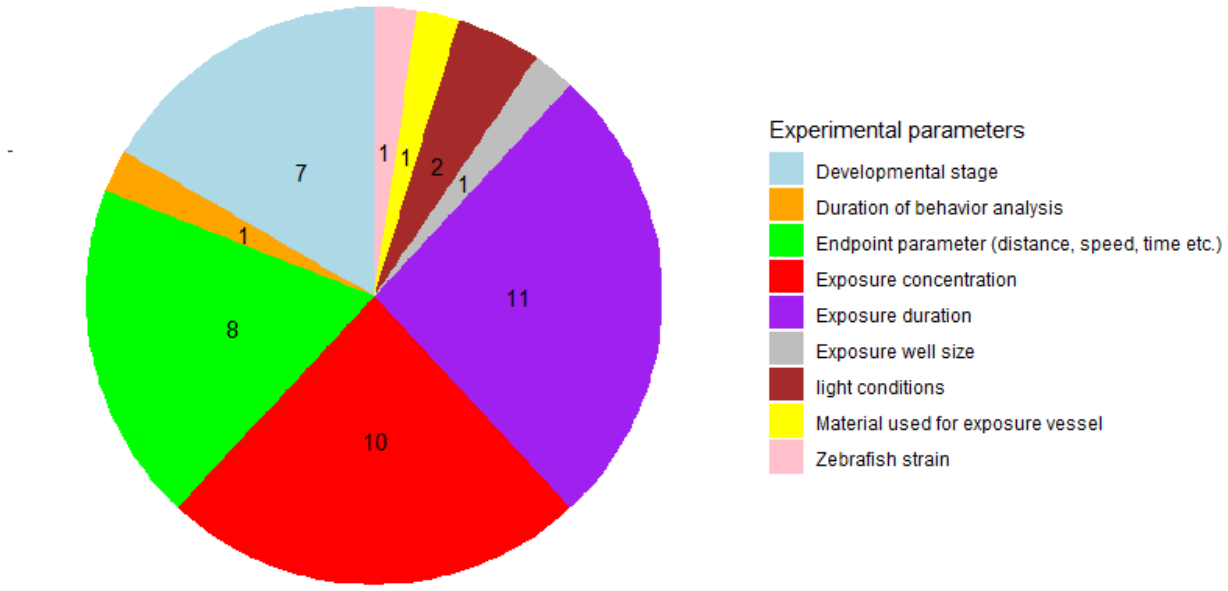

Figure 2.2: Pie chart showing the weight of influence of the analyzed experimental parameters. The numbers within each pie represents the weight of each parameter. The weight analysis was done by ranking each parameter according to the number of times it occurred as an assumed reason (risk of bias) to explain inconsistencies within chemical comparison. The risk of bias factors is recorded in Table 2.3 for each chemical.

2.3.2 Comparative assessment of observed activity (hypo- and hyperactivity) in zebrafish and expected activity based on mode of action of chemicals

The review above describes the influence of biological and technical factors on behavioral assays. This outcome was then utilized to prioritize which experimental factors may be considered to influence results when comparing observed activity in zebrafish to expected activity based on relation to the mode of action (MoA). Therefore, a collection of publications was analyzed with respect to chemical type, mode of action, effect concentration (for hyper- and hypoactivity), endpoint parameter, exposure duration, duration of behavior analysis, exposure well size, developmental stage at exposure and zebrafish strain used in zebrafish embryo behavior tests. Figure 2.1 shows the flowchart that summarizes how the collected papers were processed. In the first step, 885 studies were obtained in the "web of science" search and these were reduced to 111 studies after a manual abstract screening process based on the exclusion criteria. Second, the study hits were transformed through a series of aggregation and regrouping processes (to exclude chemicals with less than 3 study hits) in KNIME® to obtain 31 chemical hits. The chemical hits were subjected to a further exclusion criterion to eliminate non-pharmaceutical and non-pesticide chemicals and to retain only chemicals with well described mode of action. Finally, a total of 18 chemical hits were analyzed in this review.

In order to analyze the association of the expected mode of action of a chemical to its observed effects (i.e. hypo- or hyperactivity) in zebrafish embryos, a comparison was done in relation to individual substances with known neuroactive mode of action or substance classes with different neuroactive mode of action. Furthermore, different behavioral test methods were compared for the same compounds.

2.3.2.1 Comparison of individual substances with known neuroactive mode of action

Neuroactive substances with at least 3 entries in the established literature collection were compared to estimate whether in zebrafish embryos:

(1) Similar behavioral methods resulted in a consistent behavioral response across studies with regard to anticipated activity (hypo- or hyperactivity),

(2) Different methods (STC, PMR, LMR, LMR-L/D) gave consistent anticipated activity (hypo- or hyperactivity),

(3) The observed activity was consistent with the anticipated activity regardless of the method used,

(4) The respective effect concentrations in the different studies are similar - in cases when hypo- or hyperactivity is consistent between the studies.

Eighteen different comparative assessments were conducted. These were organized into 3 groups according to the expected effect, based on the MoA – hyperactive, hypoactive and unclear. We discussed all 18 substances but only show here pentylenetetrazole (PTZ) and abamectin as representative substances for hyperactivity and hypoactivity respectively. Detailed discussions and corresponding figures for the remaining substances can be found in Appendix 1. Nonetheless, results for all substances are summarized in Table 2.3. A comprehensive overview of all data is shown in Figure 2.3. The effect concentrations for all 18 chemicals span over 8 orders of magnitude and individual chemicals range over 2-3 orders of magnitude. Such high variation in effect concentrations reveals the heterogeneity of the results obtained which may be attributed to the use of different experimental protocols and parameters. For instance, an inconsistent activity trend (hyper- or hypoactivity) can be seen for chemicals expected to cause hyperactivity and this is probably influenced by high variability of exposure duration (Figure 2.3).

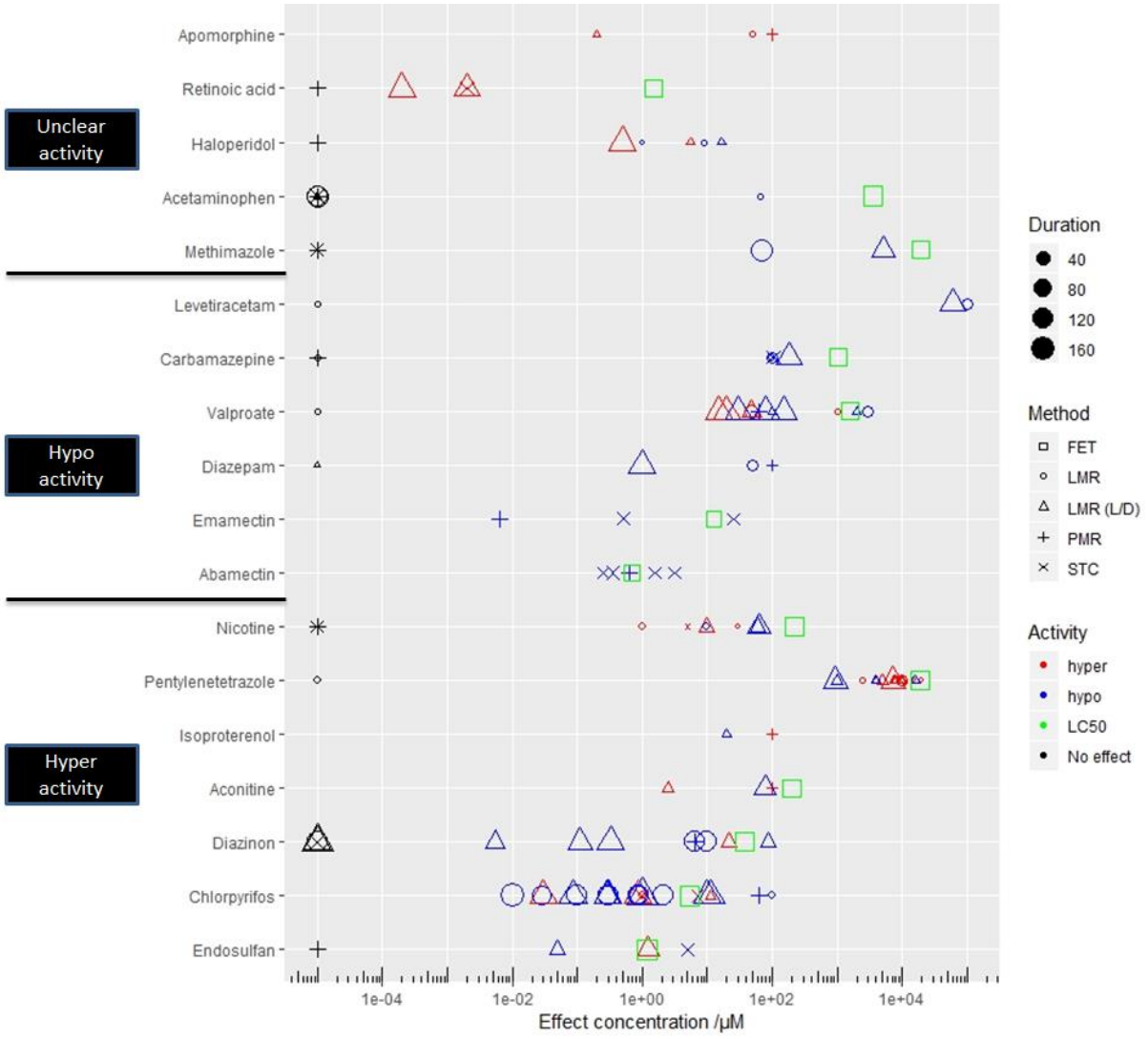

Figure 2.3: Comparison of lowest observed effect concentrations for all 18 chemicals. The plot shows the sensitivity for different behavioral methods, different exposure durations (hpf) and different effect activity represented as shape, size and color respectively. No effect concentration values are shown at 1^{e-05} µM for visibility. The anticipated activities of the chemicals in zebrafish based on mode of action are shown in the black box on the left of the y-axis. LC_{50} data were collected from different sources shown in Appendix 1 - Table S1.

Table 2.3: Summary of Comparative analysis for individual substances that are expected to provoke hyperactivity, hypoactivity and unclear activity.

Chemical	Number of entries per Chemical	[3]Mode of action (MoA)	Expected activity based on MoA	Q1:Do similar methods give consistent hypo- or hyperactivity?	Q2: Do different methods give consistent hypo- or hyperactivity?	[2]Q3: Regardless of the method used, is the observed activity consistent with the expected activity?	Q4: Are effect concentrations varying within a factor of 10 when hypo- or hyperactivity is consistent?	[1]Risk of Bias factors	Behavioral test method	References
Aconitine	3	Activation of voltage gated sodium ion channel	Hyperactivity	No	Yes	Yes: LMR(L/D)=1/2 ; PMR=1/1	Yes	Exposure duration	PMR; LMR (L/D)	(Kokel et al. 2010; Ali et al. 2012; Ellis et al. 2012)
Chlorpyrifos	22	Acetylcholinesterase Inhibitor	Hyperactivity	Yes for STC	No	Yes: STC=2/2; PMR=0/1; LMR=1/10; LMR(L/D)=2/9	No	Exposure duration; Exposure concentration; Endpoint parameter	STC; PMR; LMR(L/D); LMR	(Levin et al. 2004; Kienle et al. 2009a; Selderslaghs et al. 2010; Yen et al. 2011; Richendrfer et al. 2012; Watson et al. 2014; Jarema et al. 2015; Jin et al. 2015; Oliveri et al. 2015; Reif et al. 2016; Sun et al. 2016; Glazer et al. 2017; Bugel and Tanguay 2018; Cao et al. 2018; Li et al. 2018b; Dach et al. 2019)
Diazinon	12	Acetylcholinesterase Inhibitor	Hyperactivity	No: Some studies show no effect	No	Yes: STC=0/2; PMR=0/1; LMR=0/3; LMR(L/D)=1/6	No	Exposure concentration; Developmental stage; Endpoint parameter; Exposure duration	STC; PMR; LMR; LMR(L/D)	(Scheil et al. 2009; Yen et al. 2011; Watson et al. 2014; Kristofco et al. 2016; Reif et al. 2016; Velki et al. 2017; Cao et al. 2018; Steele et al. 2018; Leuthold et al. 2019; Schmitt et al. 2019)

Chemical	Number of entries per Chemical	[3]Mode of action (MoA)	Expected activity based on MoA	Q1:Do similar methods give consistent hypo- or hyperactivity ?	Q2: Do different methods give consistent hypo- or hyperactivity ?	[2]Q3: Regardless of the method used, is the observed activity consistent with the expected activity?	Q4: Are effect concentrations varying within a factor of 10 when hypo- or hyperactivity is consistent?	[1]Risk of Bias factors	Behavioral test method	References
Endosulfan	4	GABA-gated chloride channel antagonist	Hyperactivity	Yes	No	Yes: STC=0/1; PMR=0/1; LMR(L/D)=2/2	No	Exposure duration; Endpoint parameter; developmental stage	PMR; STC; LMR(L/D)	(Raftery and Volz 2015; Reif et al. 2016; Dale et al. 2017; Leuthold et al. 2019)
Isoproterenol	4	Beta-adrenergic receptor agonists	Hyperactivity	Yes	No	Yes: PMR=3/3; LMR(L/D)=0/1	Yes	Developmental stage; Exposure concentration	PMR; LMR(L/D)	(Kokel et al. 2010; Copmans et al. 2016; Gauthier and Vijayan 2018)
Nicotine	7	Nicotinic acetycholine receptor agonist	Hyperactivity	Yes for LMR	Yes	Yes: STC=1/2; PMR=0/1; LMR=2/2; LMR(L/D)=1/2	No	Exposure duration; Endpoint parameter; Analysis duration	STC; PMR; LMR; LMR(L/D)	(Thomas et al. 2009; Ali et al. 2012; Raftery et al. 2014; Reif et al. 2016; Mora-Zamorano et al. 2016; Bugel and Tanguay 2018; Leuthold et al. 2019)
Pentylenetetrazole (PTZ)	16	Inhibiting GABAa receptor	Hyperactivity	Yes	Yes	Yes: LMR=6/7; LMR(L/D)=9/9	Yes	Light conditions; Exposure concentration	LMR; LMR(L/D)	(Baraban et al. 2005; Berghmans et al. 2007; Baxendale et al. 2012; Ellis et al. 2012; Ellis and Soanes 2012; Afrikanova et al. 2013; Long et al. 2014; Peng et al. 2016; Torres-Hernández et al. 2016; Yang et al. 2017; Steele et al. 2018; Bugel and Tanguay 2018; Li et al. 2018a)

Chemical	Number of entries per Chemical	[3]Mode of action (MoA)	Expected activity based on MoA	Q1:Do similar methods give consistent hypo- or hyperactivity ?	Q2: Do different methods give consistent hypo- or hyperactivity ?	[2]Q3: Regardless of the method used, is the observed activity consistent with the expected activity?	Q4: Are effect concentrations varying within a factor of 10 when hypo- or hyperactivity is consistent?	[1]Risk of Bias factors	Behavioral test method	References
Abamectin	5	Activation of GABA gated chloride channel; Glutamate gated chloride channel	Hypoactivity	Yes	Yes	Yes: STC=4/4; PMR=1/1	No	Material used for exposure vessel	STC; PMR	(Raftery et al. 2014; Raftery and Volz 2015; Reif et al. 2016; Weichert et al. 2017; Vliet et al. 2017)
Carbamazepine	6	Sodium channel blocker	Hypoactivity	Yes	Yes	Yes: STC=1/1; PMR=0/1; LMR=2/3; LMR(L/D)=1/1	Yes	Exposure duration; Exposure concentration	STC; PMR; LMR; LMR(L/D)	(Berghmans et al. 2007; Beker van Woudenberg et al. 2014; Reif et al. 2016; Weichert et al. 2017; Bugel and Tanguay 2018; Martinez et al. 2018)
Diazepam	5	Stimulates GABA receptor	Hypoactivity	Yes	Yes	Yes: PMR=2/2; LMR=1/1; LMR(L/D)=1/2	No	Exposure concentration; Exposure duration; Endpoint parameter	PMR; LMR; LMR(L/D)	(Berghmans et al. 2007; Kokel et al. 2010; Steenbergen et al. 2011; Copmans et al. 2016; Dach et al. 2019)
Emamectin Benzoate	3	Activation of GABA gated chloride channel; Glutamate gated chloride channel	Hypoactivity	Yes	Yes	Yes: STC=2/2; PMR=1/1	No	Exposure well size; Endpoint parameter	STC; PMR	(Raftery et al. 2014; Reif et al. 2016; Weichert et al. 2017)
Levetiracetam	3	Inhibiting voltage dependent calcium channel	Hypoactivity	No	Yes	Yes: LMR=1/2; LMR(L/D)=1/1	Yes	Exposure duration; Developmental stage; Exposure concentration; Light conditions	LMR; LMR(L/D)	(Berghmans et al. 2007; Beker van Woudenberg et al. 2014; Martinez et al. 2018)

Chemical	Number of entries per Chemical	[3]Mode of action (MoA)	Expected activity based on MoA	Q1:Do similar methods give consistent hypo- or hyperactivity ?	Q2: Do different methods give consistent hypo- or hyperactivity ?	[2]Q3: Regardless of the method used, is the observed activity consistent with the expected activity?	Q4: Are effect concentrations varying within a factor of 10 when hypo- or hyperactivity is consistent?	[1]Risk of Bias factors	Behavioral test method	References
Valproate	12	Inhibition of GABA transminase or voltage gated sodium channel	Hypoactivity	No	Yes	Yes: PMR=1/1; LMR=1/4; LMR(L/D)=5/7	No	Exposure concentration; Exposure duration	PMR; LMR(L/D); LMR	(Berghmans et al. 2007; Zellner et al. 2011; Cowden et al. 2012; Beker van Woudenberg et al. 2014; Zimmermann et al. 2015; Bailey et al. 2016; Reif et al. 2016; Torres-Hernández et al. 2016; Bugel and Tanguay 2018; Li et al. 2018a; Martinez et al. 2018; Dach et al. 2019)
Acetaminophen	9	cyclooxygenase Inhibitors	Not clear	No	No	NA	NA	Exposure duration; Exposure concentration; developmental stage	STC; PMR; LMR; LMR(L/D)	(Selderslaghs et al. 2013; Reif et al. 2016; Reuter et al. 2016; Xia et al. 2017; Dach et al. 2019)
Apomorphine	4	stimulation of post-synaptic dopamine D2-type receptors	Not clear	Yes	Yes	NA	No	Light conditions; Developmental stage	PMR, LMR, LMR(L/D)	(Kokel et al. 2010; Irons et al. 2013; Copmans et al. 2016; Ek et al. 2016)
Haloperidol	4	Dopamine receptor antagonist	Not clear	Yes	No	NA	Yes	Endpoint parameter; Exposure concentration; Developmental stage; zebrafish strain	LMR; LMR(L/D); PMR	(Giacomini et al. 2006; Irons et al. 2013; Reif et al. 2016; Oliveri and Levin 2019)

Chemical	Number of entries per Chemical	[3]Mode of action (MoA)	Expected activity based on MoA	Q1:Do similar methods give consistent hypo- or hyperactivity ?	Q2: Do different methods give consistent hypo- or hyperactivity ?	[2]Q3: Regardless of the method used, is the observed activity consistent with the expected activity?	Q4: Are effect concentrations varying within a factor of 10 when hypo- or hyperactivity is consistent?	[1]Risk of Bias factors	Behavioral test method	References
Methimazole	4	binds to thyroid peroxidase to inhibit conversion of iodide to iodine	Not clear	NA	Yes	NA	No	Exposure duration	STC; PMR; LMR; LMR(L/D)	(Selderslaghs et al. 2013; Fetter et al. 2015; Reif et al. 2016)
Retinoic acid	4	Retinoic acid receptor agonist	Not clear	Yes	Yes	NA	Yes	Endpoint parameter	STC; PMR; LMR(L/D)	(Wang et al. 2014; Bailey et al. 2016; Reif et al. 2016)

[1]Experimental parameters related to exposure design and effect measurement were considered to identify risk of bias factors that may lead to deviating outcomes. The number of times each experimental parameter occurred as a risk of bias was used to estimate the most influential parameters. [3]Mode of action was obtained from different sources including www.drugbank.ca and published literature. [2]The numerator is the number of consistent results while the denominator is total number of results. Results obtained in Question 3 was used to estimate the percentage concordance for organophosphates, avermectins and anticonvulsants.

Abamectin

Abamectin is an avermectin insecticide expected to cause hypoactivity by activating GABA gated chloride channel (Casida and Durkin 2013). Five studies were compared. All studies reported hypoactivity (Figure 2.4). Effect concentration for hypoactivity reported in all studies are within a factor of 10 (0.36 – 3.13 µM) except the STC study by Raftery and Volz (2015) which reported an effect at 0.25 µM. This lower effect concentration could be due to conducting exposure in glass beakers instead of plastic well-plates as exposure vessel. Abamectin is highly hydrophobic ($logD_{pH7.4(ACD/Labs)}$ of 5.85) and hence has higher affinity to bind to plastic than glass. Therefore, abamectin may be highly bioavailable to the embryos in a glass container leading to effects occurring at lower concentration.

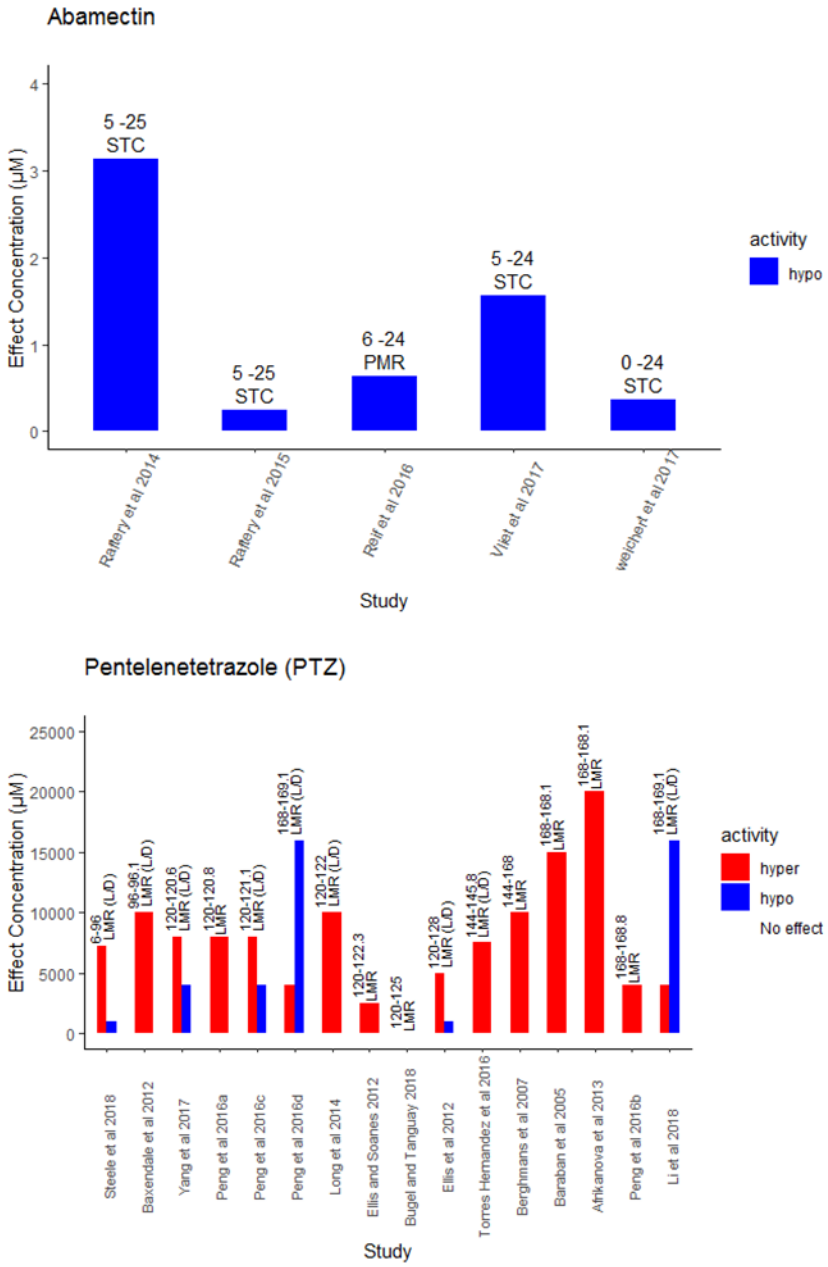

Figure 2.4: Effect concentrations for pentylenetetrazole and abamectin that are expected to provoke hyperactivity and hypoactivity respectively. Bars show the magnitude of the effect concentrations which represents lowest effect concentrations as deduced from each study. When there is no bar, it indicates no effect observed within the tested concentration range. When two different bars are depicted for one study, it indicates effect concentrations for both hypo- and hyperactivity. The text written on top of each bar represents the behavioral test method while the numbers represents the exposure duration (hpf).

Pentylenetetrazole (PTZ)

PTZ is a convulsant drug and it is expected to cause hyperactivity by binding to GABA receptors (Squires et al. 1984). Sixteen studies were compared. PTZ showed hyperactivity effects in all the studies except the LMR study by Bugel and Tanguay (2018) which reported no effect (Figure 2.4). However, in this study exposure concentrations 2.5 fold below the effect concentration of the other studies were used and this was probably below the effective range of PTZ. Even though the effect concentrations for hyperactivity were within a factor of 10 in all studies, hypoactivity was also reported, at different concentrations and light periods, as an additional effect to hyperactivity in some LMR-L/D studies. The effect of PTZ may be enhanced under alternating light-dark periods and PTZ has been reported to cause a reversal of the observed activity in control treatment i.e. higher activity in dark and lower activity in light phase for non-exposed embryos (Ellis et al. 2012; Torres-Hernández et al. 2016). Consequently, it is likely that PTZ induces a differential response in light and dark phases and this effect is only observed under alternating light conditions. Hence, the use of different light conditions during measurement could be a limiting factor for comparing the output from different behavior methods. Nevertheless, alternating light conditions could give important insights on how a substance modulates behavior.

2.3.2.2 Comparison of substance classes with same/similar mode of action

Neuroactive chemicals which emerge from the same chemical class can be anticipated to exhibit similar mode of action. The aim of this comparison is to evaluate the consistency of the observed activity of chemicals in zebrafish to the expected activity within a certain class of chemicals (organophosphates, avermectins and anti-convulsant drugs) e.g. do all chemicals within a class conform to the expected mode of action (hypo- or hyperactivity) in zebrafish embryos?

Organophosphates

Organophosphates act by inhibiting the acetylcholinesterase enzyme (AChE) which breaks down acetylcholine and therefore keeps the acetylcholine gated sodium channels open for more ions to flow into the cell leading to an action potential (Casida and Durkin 2013). Hence, it is expected for organophosphates to cause hyperactivity in zebrafish at lower concentrations but hypoactivity at higher concentrations due to over-excitation resulting in paralysis induced by abnormal mechanical stress (Stehr et al. 2006) or axonal deformation (Yang et al. 2011). Additionally, the interaction of exposure concentration and duration plays a major role. For instance, chlorpyrifos caused hyperactivity at a low concentration of 0.03 µM and long duration of 120 h in an LMR-L/D study (Figure 2.5). Inversely, a high concentration of 11 µM and short duration of 4 h also resulted in hyperactivity (Jarema et al. 2015; Oliveri et al. 2015). A summary of the data for organophosphates (Chlorpyrifos and Diazinon) is shown in Figure 2.5 and in Appendix 1. Although hyperactivity is expected for lower test concentrations, it was only observed in 18% of the studies. The percentage concordance of observed to expected activity was estimated to be 50(n=4), 0(n=2), 7.7(n=13) and 20(n=15) % for STC, PMR, LMR and LMR-L/D respectively (number of studies per method in parenthesis). Considering that STC has the highest percentage of consistency, we could deduce that STC may be the most-sensitive method to detect the hyperactive effect of organophosphates. This could be due to the assumed capability of the STC to measure basic response of the primary motor neurons (Saint-Amant and Drapeau 1998; Richendrfer et al. 2014) rather than the secondary neurons measured in LMR. Many organophosphates require biotransformation for highest

inhibition capacity (Chambers and Oppenheimer 2004), and a limited biotransformation in embryos would represent a confounding factor and might interfere with the detection of behavioral effects in early embryo stages. Interestingly, the LMR-L/D study by Leuthold et al. (2019) was the only one that showed hyperactivity for diazinon (Figure 2.5). This could be due to the use of a combination of older developmental stage of 96 hpf (with potential higher biotransformation) and a short exposure duration of 24 h (with potential no over-excitation or paralysis effect). However, not only the oxon-metabolite but also the parent compound of chlorpyrifos induced hyperactivity in the STC at an earlier developmental stage of 24 hpf (with potential limited biotransformation). This suggests the influential AChE inhibiting activity of chlorpyrifos or a high efficacy of the low amount of the oxon-metabolite resulting from the limited biotransformation (Watson et al. 2014; Weichert et al. 2017). Reif et al. (2016) also found a relatively lower effect concentration (0.64 µM) for chlorpyrifos-oxon compared to chlorpyrifos (64 µM) and this supports the limited biotransformation of chlorpyrifos in younger developmental stages. Hence, for neuroactive compounds such as organophosphates which require bioactivation, the stage-dependent bioactivation or the internal concentrations related to the exposure time may influence the effect concentrations.

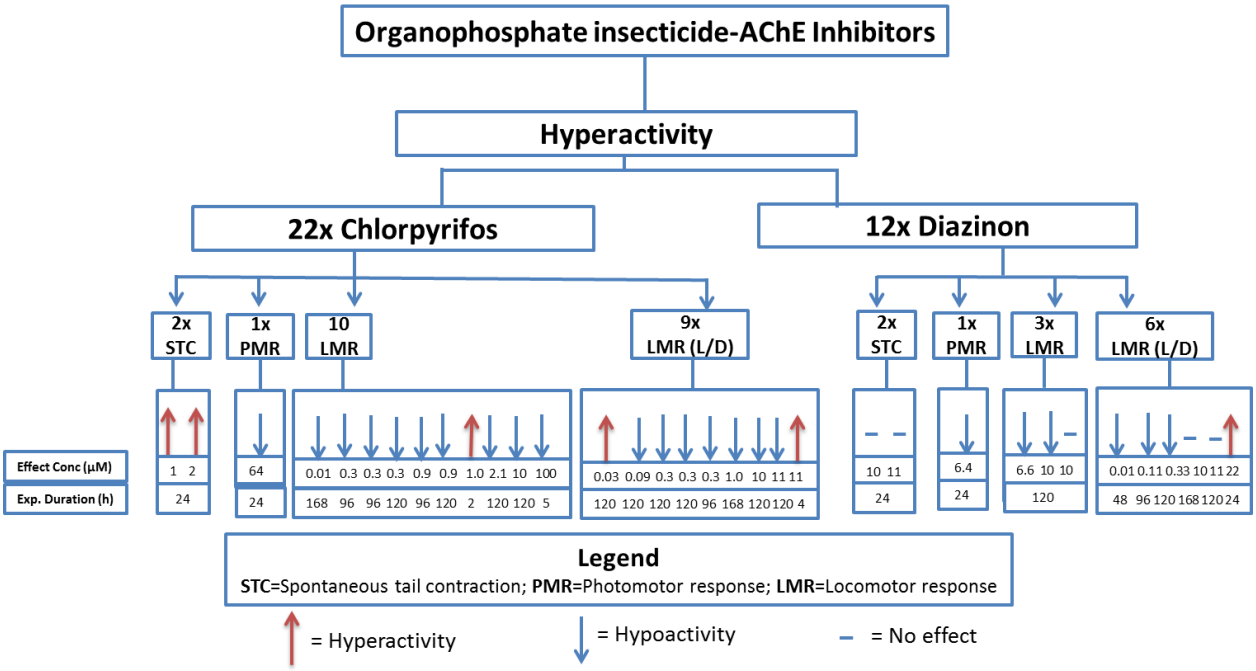

Figure 2.5: Flowchart showing the consistency of two organophosphates to hyperactivity. The number in front of the substance and the method shows the total number of chemical hits and the chemical hits per method, respectively. The arrows indicate hyper-, hypo- or no activity. The numbers below each arrow indicates the effect concentration in µmol/L. The arrows are organized with increasing effect concentrations from left to right.

Avermectins

Avermectins act by activating the GABA-gated chloride channel and/or glutamate-gated chloride channel leading to an inhibitory potential and hence hypoactivity (Casida and Durkin 2013). Both avermectin chemicals considered in this study; emamectin and abamectin, showed hypoactivity which is consistent with their mode of action. A summary of the data for avermectins shows that 100% hypoactivity effect was reported. Percentage concordance of observed to expected activity was estimated to be 100(n=6) and 100(n=2) % for STC and PMR respectively (number of studies in parenthesis). This shows that avermectins can be reasonably detected in short duration embryo tests. See Appendix 1 for more details on abamectin and emamectin.

Anticonvulsant drugs

Anticonvulsants are a class of drugs used for controlling seizure activity. They propagate their action on the nervous system via different mechanisms including: 1.) Blockage of the sodium gated channels. 2.) Indirect or direct enhancement of inhibitory GABA neurotransmission. 3.) Inhibition of excitatory glutamatergic neurotransmission (Söderpalm 2002). Based on their mode of action, it is expected that anticonvulsants will cause hypoactivity in zebrafish embryo. A summary of the data for anticonvulsants (Figure 2.6) shows that despite using different methods, 62% hypoactivity effect was reported. Percentage concordance of observed to expected activity was estimated to be 100(n=1), 75(n=4), 50(n=10) and 64(n=11) % for STC, PMR, LMR and LMR-L/D, respectively (number of studies in parenthesis). STC was not considered since only one study was found. Hence, we can deduce that PMR and LMR-L/D may be the most-sensitive method to detect anticonvulsants and this could be due to the light stimulation utilized in both methods which may interact with complex nervous processes in the brain.

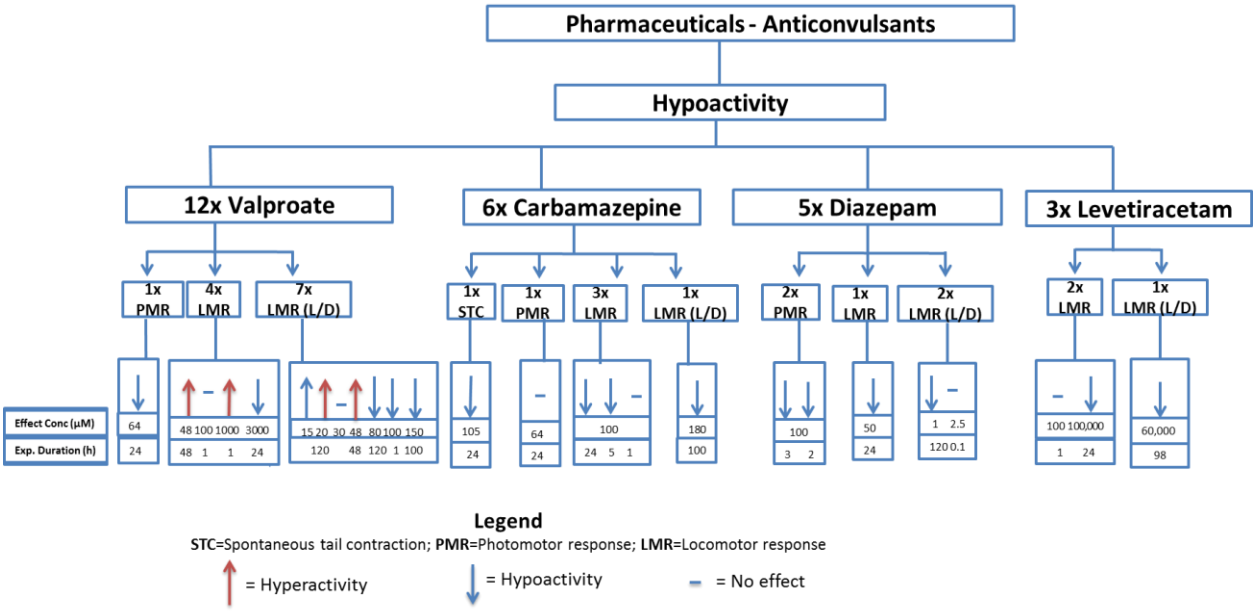

Figure 2.6: Flowchart showing the consistency of anticonvulsants to hypoactivity. The number before the chemical and the method shows the total number of chemical hits and the chemical hits per method. The arrows indicate hyper-, hypo or no activity. The numbers after each arrow indicates the effect concentration in µmol/L. The arrows are organized with increasing effect concentrations from left to right.

2.3.2.3 Comparison of different behavioral methods

In the last part of this results section, we tried to estimate whether different behavioral measurement methods or contrasting experimental parameters give similar results. For that we identified case studies that reported different methods or setups for the same chemical or different chemicals. Here, the goal is to compare two behavioral methods using substance(s) tested in both methods.

Effects of abamactin in the STC

Case study: Raftery et al. (2014); (2015); Vliet et al. (2017); Weichert et al. (2017)

For abamectin, an enhancer of GABA- or glutamate gated chloride channel, 5 different studies have reported the effects in the STC (Figure 2.4). While the material used for exposure vessels varied to some extent, developmental stage, exposure duration and duration of behavior analysis were very similar for these studies. The effect concentrations of the different studies were within a factor of 10 (except Raftery and Volz 2015) and in all studies hypoactivity was observed. As described earlier (see comparison of individual substances), the minor differences in effect concentrations may be attributed to the use of different exposure vessels. For instance, the lowest effect concentration (0.25µM) was reported in a study that used glass exposure vessels (Raftery and Volz 2015). Moreover, exposures in 24 well plates, with lower surface area, resulted in lower effect concentrations compared to 384 well plates (Raftery et al. 2014; Vliet et al. 2017). Abamectin has a high log kow of 5.85 and this suggests that the lower effect concentrations in glass and 24 well vessels could be due to higher bioavailability. This may be attributed to higher adsorption to plastic wells (relative to glass) and especially 384 well plates (relative to 24 well plates). In general, the results of STC were in line with the mode of action and largely consistent between studies and differences attributed to the adsorption of the compound to exposure vessels. However, Vliet et al. (2017) concluded that STC might not be capable of distinguishing between modes of action because they observed only hypoactivity even for chemicals with hyperactive MoA. Possible reasons for their observed hypoactivity include the use of a single exposure concentration and use of a different endpoint (percentage of embryos showing STC), which might be inherently biased (see section “endpoint parameter” for more details).

Comparison of STC and PMR

Case study: Fipronil, emamectin benzoate, carbamazepine, abamectin

Comparison of results between STC and PMR for some chemicals showed a lack of consistency in the observed effect concentrations. There are 2 exceptions. First, abamectin comparison in which the STC studies (Raftery and Volz 2015; Weichert et al. 2017) showed similar effect concentrations to another PMR study (Reif et al. 2016). Second, behavioral effects were observed for carbamazepine in the STC test (Weichert et al. 2017) at a concentration not tested in the PMR (Reif et al. 2016). The chemicals that show inconsistencies includes; fipronil, chlorpyrifos and emamectin. For fipronil, an effect concentration of 25 µM was reported for the STC study by Raftery and Volz (2015) while the PMR study by Reif et al. (2016) showed no effect at concentrations up to 64 µM. This difference could be due to the use of plastic well plates in the study of Reif et al. (2016) leading to a possible adsorption and hence decreased exposure and effect concentrations. For emamectin, the STC study by Weichert et al. (2017) reported a hypoactivity effect at 1.03 µM while the PMR study by Reif et al. (2016) reported 0.0064 µM. Even though STC and PMR have many similar experimental parameters; there is a major difference in their endpoint parameter.

While PMR measures movement of the whole embryo under light stimulation, STC only measures the spontaneous tail contractions. This difference could lead to major differences in the behavioral outcome of both tests (see Additional file 1 for more details).

STC/LMR Comparison

Case study: Chlorpyrifos in Selderslaghs et al. (2010); (2013)

Selderslaghs et al. (2010, 2013) exposed zebrafish embryos to different chemicals and both the LMR and STC were conducted to identify neuroactive effects or effects on neurodevelopment. STC captured effects of more substances and this sensitivity difference could be due to a different range of exposure concentrations. Higher exposure concentrations were used for the STC test because exposure concentrations were based on the highest non teratogenic concentration estimated at exposure durations of 24 and 144 h respectively (Selderslaghs et al. 2013). Even though, different concentration ranges were used, effects were observed at an overlapping concentration of 1.8µM and 2.1µM for chlorpyrifos in STC and LMR respectively (Selderslaghs et al. 2010). However, the effects were not consistent – hyperactivity in STC and hypoactivity in LMR. This activity difference may be due to the influence of exposure duration (steady state not reached in shorter exposure), and/or developmental-stage dependent metabolic activation. As discussed earlier (see sections on exposure duration and organophosphates), longer exposure might increase the internal concentration of the substance and thus increase effects. Vice versa, a short exposure might need higher external and thus internal concentrations to get the same effects. In the case of chlorpyrifos one could assume that the longer exposure caused over-excitation of acetycholine receptors due to the irreversible inhibition of AChE and thus the resulting paralysis translates to hypoactivity. Therefore, similar exposure durations should be used for a comparison of the sensitivity of both assays (albeit differences in toxicokinetics and targeted receptors of the tested developmental stages may still apply). Moreover, STC measures the response of the primary motor neurons in the embryo while LMR measures the response of both primary and secondary motor neurons in free swimming embryo (Grunwald et al. 1988; Yozzo et al. 2013). Hence, differences in the results could occur because these assays partially target different neuronal structures.

PMR/PMR Comparison

Case study: Isoproterenol, Apomorphine and Diazepam in Kokel et al. (2010) and Copmans et al. (2016)

Kokel et al. (2010) and Copmans et al. (2016) were selected for this comparison because crucial experimental parameters including exposure age, exposure duration and analysis time were similar. Also, 3 chemicals namely isoproterenol, apomorphine and diazepam were tested in both studies. Despite the fact that a single concentration was tested in both studies, similar effect concentrations and activity were obtained. Embryos were hyperactive to isoproterenol and apomorphine while hypoactivity was observed for diazepam (Table 2.3). These similar effects observed for different studies indicate the reproducibility of PMR tests if experimental parameters are similar. The fact that the observed activity of embryos for the 3 chemicals was consistent with the expected activity assumed from the MoA suggests that PMR can be a valuable test to detect mode of action of neuroactive substances if steady state of concentrations can be achieved within exposure window and the respective receptor is available.

Comparison of LMR with different light regimes

Case study: Pentylenetetrazole (PTZ)

There are 2 major types of LMR reported in the literature: The non-stimulus LMR either conducted in constant dark or light phase only and the LMR using alternating light-dark cycles (LMR-L/D). A comparative assessment of the different types of LMR was difficult due to the use of different endpoint parameters such as total distance moved, time spent on locomotion, swimming speed, mean turn angle etc. PTZ was the only chemical for which sufficient data could be identified to enable a comparative assessment (Figure 2.4). In the LMR-L/D, PTZ induces a behavior which is opposite to that observed in control embryos i.e. high activity in dark period and low activity in light period for embryos in untreated solution (Ellis et al. 2012; Torres-Hernández et al. 2016). Therefore, all hyperactivity effects were recorded during the light phase in the LMR-L/D tests with PTZ. In the LMR, hyperactivity was recorded irrespective of the analysis being conducted in continuous light or dark phase. Interestingly, effect concentrations for hyperactivity reported for both setups in all the studies were within a variation factor of 10. This shows that the hypoactivity observed only in the LMR-L/D tests was mainly driven by alternating light/dark cycles. This suggests that PTZ might react differentially under alternating light conditions and the LMR-L/D could be utilized as an extensive diagnostic tool for such epileptic effects in zebrafish.

2.4 Conclusions

Based on the assessment of published zebrafish embryo behavior studies it was possible to identify major factors impacting on the magnitude and type of response in behavioral assays. Exposure duration, exposure concentration, endpoint parameter and developmental stage were the most influential parameters. Understanding and controlling these factors and potentially revising/harmonizing protocols would help reduce variability, and interpretation of results for hazard assessment of chemicals.

The review was motivated by the hypothesis that the MoA of chemicals may be reflected by the type of response in behavioral assays, i.e. whether hypo- or hyperactivity is induced by the exposure. The data indicated that a clear association of the response with the mode of action was difficult (eg. 18 and 62 % consistency for organophosphates and anticonvulsants respectively), partially also caused by experimental limitations and diversity of protocols used. Despite the low number of STC studies, the STC test appears to reveal the most consistent results with respect to the expected hyperactivity of a substance (especially for organophosphates). However limited biotransformation capacity and uptake of chemicals into the embryos may affect the detectability and sensitivity of hyperactivity in the STC as was shown by the effects of the organophospate compounds, chlorpyrifos and diazinon. The PMR also shows great potential to predict neuroactive MoA, however, the use of single exposure concentrations in many PMR studies limited the appropriate evaluation of its possible potential. LMR (L/D) showed 64 % of the expected hypoactivity related to the MoA of anticonvulsants. However, the anticipated hyperactivity for organophosphates could not be shown in most cases and which could be partially attributed to long exposure duration (e.g. 3-5 days). Long exposure durations may impact on the neuronal development due to axonal defects or seizure-induced paralysis (Stehr et al. 2006; Yang et al. 2011). Hence, compromising the function/structure of the nervous system and indirectly resulting in hypoactivity. The different LMR test methods may only acquire the ability to predict hyperactivity if the exposure duration is significantly

reduced as shown in the PTZ studies and as reported in Leuthold et al. (2019). The possibility to discriminate neuro-developmental effects from direct functional effects may also improve MoA prediction. Finally, it is evident that behavioral tests are capable of screening neuroactive substances and a combination of the four tests considered in this review will be more powerful and reliable than the individual tests alone. Nonetheless, the full potential of these methods for risk assessment of chemicals cannot be realized until the impacts of experimental parameters are addressed more systematically in comparative studies.

2.4.1 Recommendations

The experimental design is always strongly related to the research question. Hence, the type of assays and conditions used for behavioral tests may be different depending on the goal of the study. The perspective in this manuscript was to analyze the comparability and reproducibility of behavioral test results for identifying neuroactivity MoA within a prospective and diagnostic risk assessment framework. The recommendations given below are based on this particular goal but may be different for other research questions. With respect to this focus and the results of this review, we suggest to particularly address the most important experimental parameters in behavioral assays:

1.) Exposure duration could have a strong impact on behavioral outcomes for several reasons such as biotransformation rates or overall kinetics. From the data used in this review, it appears that functional neuroactivity can be provoked already by relative short exposure periods, while developmental neurotoxicity is rather detected by long-term exposure exceeding 24 hours and including early developmental stages. Hence, MoA-specific neuroactivity may rather be detected when using short term exposure scenarios (< 24-32 h of exposure) provided that uptake and biotransformation are not limiting the availability of the compound at the target site.

2.) Behavioral tests are sensitive to exposure concentrations and responses may change within a range of low to high concentrations (e.g. due to seizure paralysis caused by high overstimulation or interfering of developmental toxicity at high exposure concentrations). Therefore, test design should include a range of concentrations that allow capturing of the potential transition from low dose hyperactivity to high dose hypoactivity effects. Concentration-response relationships should also be related to lethal effect concentrations at defined exposure periods and to predicted concentrations causing baseline toxicity. This will indicate whether behavioral effects may have been caused by unspecific secondary responses to overt toxicity.

3.) Zebrafish embryos develop rapidly and their normal patterns of embryonic movements changes with developmental stage. Hence, harmonization of protocols with regard to the developmental stages used for assessment is likely to increase reproducibility and reliability of results. Factors such as biotransformation requirement of the substance and availability of target receptor should be considered during experimental design.

4.) For uniformity reasons, it seems from the analyzed literature that it is generally desirable to select distance moved for LMR and LMR-L/D and frequency of STC as optimal endpoint parameters. Other parameters could be used additionally until proof of usability.

5.) Based on the review, we suggest to consider the following parameters to be used for the different behavioural assays.

STC:

- Exposure duration: 2 – 26 hpf (± 2). Shorter durations maybe used for MoA analysis, for indirect assessment of toxicokinetics or to distinguish between acute and developmental effects;
- Selection of exposure concentration should relate to lethal or sublethal concentrations such as the LC_{50} at 24 hpf as the highest test concentrations and a full concentration-response analysis should be performed;
- Endpoint parameter: Frequency or number of STC;
- Developmental stage: Measurement should be conducted between 23-25 hpf (based on the age at which maximum STC is observed) to account for the stage dependency of the frequency.

PMR:

- Exposure duration: 2 – 32 hpf (± 2). Shorter durations within this period maybe used for MoA analysis or indirect assessment of toxicokinetics or to distuingish between acute and developmental effects;
- Selection of exposure concentration should relate to lethal or sublethal concentrations such as the LC_{50} at 24 or 48 hpf as the highest test concentrations and a full concentration-response analysis should be performed;
- Endpoint parameter: Movement activity as used in Kokel et al. (2010) and Copmans et al. (2016);
- Analysis duration: 30 seconds measurement from 30 hpf.

LMR or LMR-L/D:

- Exposure duration: 2-120 hpf for developmental neurotoxicity assessment. Short durations, e.g. from 96-120 hpf may be appropriate for identifying acute neuroactivity effects not related to neurodevelopmental toxicity;
- Selection of exposure concentration should relate to lethal or sublethal concentrations such as the LC_{50} at 72 or 96 hpf as the highest test concentrations and a full concentration-response analysis should be performed;
- Endpoint parameter: Total distance moved and other endpoints such as swimming duration and velocity could be used as additional.

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Chapter 3: Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo*

Abstract

Neuroactive chemicals are frequently detected in the environment. At sufficiently high concentrations or within mixtures, they could provoke neurotoxic effects and neurological diseases to organisms and humans. Fast identification of such neuroactive compounds in the environment could help in hazard assessment and risk mitigation. Behavior change is considered as an important endpoint and might be directly or indirectly connected to a neuroactive mode of action. For a fast evaluation of environmental samples and pure substances, we optimized the measurement of a behavioral endpoint in zebrafish embryos - the spontaneous tail coiling (STC). Evaluation of results is automated via the use of a workflow established with the KNIME® software. Analysis duration and developmental stage were optimized to 1 minute and 25 ± 1 hpf respectively during measurement. Exposing the embryos in a group of 10 or 20 and acclimatizing for 30 min at room temperature proved to be reliable. The optimized method was used to investigate neurotoxic effects of 18 substances with different modes of action (MoA). The STC test accurately detected the effect of 8 out of 11 neuroactive substances (chlorpyrifos, chlorpyrifos-oxon, diazinon, paraoxon-methyl, abamectin, carbamazepine, propafenone and diazepam). Aldicarb and nicotine showed subtle effects which were considered to be conditional and imidacloprid showed no effect. For substances with unknown neuroactive MoA, 3 substances did not provoke any effect on the STC (pyraclostrobin, diuron and daunorubicin-hydrochloride) while 4 other substances provoked an increased STC (hexaconazole, aniline, dimethyl-sulfoxide and 3,4-dichloroaniline). Such unexpected effects indicate possible neuroactive side effects or unknown mechanisms of action that impact on the STC. In conclusion, the optimized STC parameters and the automated analysis in KNIME® indicate opportunities for the harmonization of the STC test and further development for prospective and diagnostic testing.

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3.1 Introduction

Neuroactive substances are frequently detected in the environment and environmental concentrations may induce adverse effects such as neurological damage in humans and in the ecosystem (Busch et al. 2016). To prevent neurotoxic hazard, it is necessary to develop new, fast and sensitive toxicological tests to screen neuroactive substances. Behavior tests such as locomotor activity are considered to be sensitive and specific to detect neurotoxic effects since it is anticipated that behavior is directly or indirectly related to the function of the nervous system. Such behavior tests have been utilized for both drug development and toxicity testing in animals such as rodents, fish and amphibians (OECD 2007a; OECD 2007b; Parker 2016; Tierney 2011). However, alternative techniques are required to reduce the time, cost and number of animals in developmental neurotoxicity testing (Bal-Price et al. 2015). Currently early life stages of fish are particularly gaining wide acceptance for use in behavior testing due to the non-protection of these stages as well as possibility for small-scale and high throughput testing (Basnet et al. 2019; Braunbeck et al. 2005; Legradi et al. 2015; Ogungbemi et al. 2019; Scholz et al. 2013).

In particular, zebrafish embryos represent an attractive toxicity testing model for several reasons: its small size allows the use of low quantity exposure solution, its fast development makes it amenable to short duration testing and its transparency enables the assessment of developmental effects and protocols for the assessment of early behavioral features such as spontaneous tail coiling are available (Hill et al. 2005; Scholz et al. 2013). Furthermore, due to the conservation of principal mechanisms of neurotoxicity in animals, testing of zebrafish embryos also allows extrapolation to other species including humans. A previous review of different zebrafish embryo behavior tests (Ogungbemi et al. 2019) had indicated that the spontaneous tail coiling (STC) of zebrafish embryos could represent a reliable endpoint to detect neurotoxicity and hence this endpoint was selected for further optimization in the present study. The STC consists of single or alternating tail coilings which can be observed as early as 19 hours post fertilization (hpf) in the developing embryo (Kimmel et al.1974; Saint-Amant and Drapeau 1998). The observed tail coilings are assumed to occur as a result of innervation of the muscle by the primary motor neurons and therefore, measurement of the STC frequency could be a good indicator of adverse effects to the function and development of the muscle innervation or generally the nervous system.

In previous studies, the STC test has been used to analyze effects of neuroactive chemicals such as abamectin, chlorpyrifos, carbamazepine etc. (Cheng et al. 2017; Selderslaghs et al. 2010; Vliet et al. 2017; Weichert et al. 2017). STC response of these chemicals relative to negative control appears to be a promising technique to predict either the stimulatory or inhibitory mode of action (MoA) of neuroactive compounds (Ogungbemi et al. 2019). For example, the hyperactivity (referring to increased STC) effect of chlorpyrifos-oxon may be correlated to its stimulatory action when it inhibits acetylcholinesterase enzyme while the hypoactivity (decreased STC) effect of abamectin may be linked to its inhibitory action when it activates Gamma aminobutyric acid (GABA) receptors (Raftery and Volz 2015).

However, reports on the use of the STC test method vary in their experimental protocol and how effects are estimated. This may lead to lack of reproducibility and usability of results for the identification of specifically acting neuroactive substances. For instance, differences in effect concentration for abamectin may be attributed to the use of different exposure material (Ogungbemi et al. 2019; Raftery et al. 2014);

different effect concentrations for dichlorvos may relate to the use of different endpoint – frequency or duration of STC (Watson et al. 2014; Zindler et al. 2019); inconsistent effects (hyper- or hypoactivity) were also reported for paraoxon and this could be attributed to estimating the endpoint in different ways – percentage of embryos showing STC versus frequency of STC (Ogungbemi et al. 2019; Yozzo et al. 2013); and different substances were indicated as potential neurotoxic depending on a short (2 h) or a long (23 h) exposure duration (Vliet et al. 2017). Other experimental parameters such as age or developmental stage of embryo, duration of behavioral analysis and sample size could influence the STC result leading to incoherent interpretations. Richendrfer et al. (2014) also showed that variation in the age of embryo in reported STC studies could influence behavioral analysis. Hence, there is a need to optimize these experimental parameters for appropriate interpretation of neurotoxicity. Crofton et al. (2011) suggests a list of guidelines to develop alternative test methods for developmental neurotoxicity testing. These recommendations could also facilitate validation of the STC test for the use in hazard assessment and effect-based environmental monitoring.

The aim of the present study was to investigate the influence of experimental parameters on the STC response and to develop an optimized STC test for screening neuroactive compounds. We optimized important experimental parameters and created an automated workflow to measure the STC in the open access software KNIME® (Berthold et al. 2009). Subsequently, we implemented the guidelines recommended by Crofton et al. (2011) to establish an optimized STC protocol. We tested the new protocol on 18 chemicals with different modes of action - either with an expected activation or inhibition of movement or without any expected effect.

3.2 Materials and method

3.2.1 Test organism

Fish cultivation, feeding and embryo collection was conducted as described previously (Massei et al. 2015). Briefly, two strains of adult zebrafish (OBI and WIK strains) were crossed to produce a hybrid strain (OBI-WIK strain, F3 generation) in order to avoid inbred effects. The strain was cultured under 14h light/10h dark photoperiod in 120 L aquaria (tap water, 26.5±1 °C). Spawning trays were inserted on the afternoon 4-6 hours before the end of the light cycle. To initiate spawning, lights were automatically switched on at 8am the following day and eggs were collected at 9am inside a rectangular glass dish covered with a stainless steel sieve. Fertilized and normal embryos were selected according to Kimmel et al. (1995) with a binocular microscope and embryos between 16 and 128 cell stage were used for the experiments.

3.2.2 Media and chemicals

Information about the purity and manufacturer of all chemicals are shown in Appendix 2 - Table S1. Stock solutions were prepared either 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] or in 100 % dimethyl-sulfoxide (DMSO). All chemicals were dissolved in DMSO except; imidacloprid, 3,4-dichloroaniline, aniline, daunorubicin-hydrochloride,

diazinon and nicotine. For preparation in ISO water, test chemicals (except liquid substances) were prepared a day before exposure and left to stir overnight for dissolution. The DMSO stock solutions were diluted to lower concentrations in ISO water during exposure and the DMSO concentrations varied along the dilution series but never exceeded 0.1% (v/v) in diluted solutions.

3.2.3 Chemical exposures

The chemicals were grouped by their expected effects in the STC in relation to their known mode of action: chlorpyrifos, chlorpyrifos-oxon, diazinon, paraoxon-methyl, aldicarb, imidacloprid and nicotine were anticipated to represent hyperactive chemicals; abamectin, carbamazepine, diazepam and propafenone were considered to represent hypoactive chemicals; chemicals with unknown neuroactive mode of action or without any expected effect to the STC were represented by diuron, aniline, pyraclostrobin, hexaconazole, daunorubicin-hydrochloride, DMSO and 3,4-dichloroaniline. Exposure concentrations are given in Appendix 2 - Table S1 and these were selected based on mortality data from published literature or in-house unpublished mortality data. Briefly, twenty fertilized embryos (1-3 hpf) were exposed in 20 mL of diluted stock solution or ISO water as control, within a 60 mm glass crystallization dish covered with a watchmaker glass. A solvent control was used when the substance was dissolved in DMSO. The exposed embryos were incubated at 28^{o}C under 14h light/10h dark photoperiod for 21 ± 1h. The exposure was conducted using 2 technical parallel replicates and at least 2 independent replicates to get sufficient amount of data for the concentration-response modelling. pH of the highest concentration and control solution were measured before and after the experiment to control for possible changes within the exposure time.

3.2.4 Measurement of the spontaneous tail coiling (STC)

Detailed description of the STC measurement is reported in Appendix 3. At 24 hpf, exposed embryos were removed from the incubator and allowed to acclimatize to room temperature for at least 30 min. Embryos were inspected for lethality/malformations and affected embryos were separated. Samples with less than 20% affected embryos were considered valid for STC assessment. Videos of normally developed embryos were recorded for 60 s (frame rate of 2 frames per second) with a video camera (Olympus DP21, Hamburg, Germany) mounted to an Olympus SZX7 stereomicroscope (0.8x magnification). The embryos were recorded in groups of 20 using a black background and dark field transmitted light at the base of the microscope, with an ISO speed of 400, time of exposure of 1/80 and image size of 400x300 pixels. Collected videos were analyzed for STC counts by means of a workflow using the KNIME® Analytical Platform (Berthold et al. 2009). Occasionally two tail coilings appear very close together. In such cases the camera setting of 2 frames per second could not resolve them as individual coilings, these were counted manually. Influence of experimental parameters.

3.2.4.1 Exposure duration and developmental stage of analysis

To investigate the optimal exposure duration or developmental stage during behavior analysis for zebrafish embryos in the STC test, 20 embryos (<3hpf) were exposed in ISO water and STC was measured hourly starting from 21 hpf to 31 hpf. This experiment was conducted with 3 technical replicates.

3.2.4.2 Acclimation duration

STC measurement and video recording were not undertaken in temperature controlled chambers. As a result, to investigate the influence of temperature changes during acclimation time (after removal from the incubator at 28 °C and before STC measurement) on the STC response, 20 embryos per treatment were exposed in ISO water and temperature was measured during acclimation. Treatment 1 = control (no acclimation); Treatment 2 = 15 minutes acclimation at room temperature; Treatment 3 = 30 minutes acclimation at room temperature. After incubation, treatment 2 and 3 were removed from the incubator at 15 and 30 minutes respectively before STC measurement. Treatment 1 was not acclimatized under room temperature but measured as immediately possible. Three technical replicates were used for all treatments and STC measurement was conducted between 24 – 25 hpf for all embryos.

3.2.4.3 Sample size

To evaluate the effect of simultaneously reducing sample size (20 to 10 embryos per replicate) and increasing the number of replications (3 to 5 replicates) on variability of the STC response, two treatments were considered. In the first treatment, 20 embryos of 3 replicates were exposed in ISO water. 10 embryos of 5 replicates were used in the second treatment. The experiment was repeated thrice and STC measurement was conducted between 24 – 25 hpf. Additionally, previosly collected and analyzed STC control data were reanalyzed by estimating the mean of 10 embryos in comparison to the mean of 20 embryos per sample.

3.2.4.4 Analysis duration

The impact of reducing the analysis duration of the STC was investigated. STC data for abamectin and chlorpyrifos were re-analyzed in the KNIME® workflow in which the recorded video of 60s was segmented into different time bins of 60, 30, 20 and 10s.

3.2.4.5 Rearing condition

To test if the movement of one embryo might stimulate the movement of other nearby embryos and therefore accidentally influence outcome, we reared embryos with ISO water in single or group conditions. In single condition, 10 embryos were individually placed in 10 glass crystallization dishes and in 2 replicates (one embryo per dish per 10 dishes and a total of 20 dishes). Group condition was implemented by placing 10 embryos in a group within the same dish (10 embryos per dish and 2 replicates per dish). STC measurement was conducted between 24 – 25 hpf.

3.2.4.6 Image analysis parameters

To optimize the image analysis of STC in KNIME®, we investigated the influence of parameters like threshold (thrs) and the so-called smoothing parameter (spar) used for identification of peaks within the R-snippet node in KNIME®. Threshold is the value beyond which the STC counts as one. Any response below this value was attributed to noise. The higher the threshold, the lower the sensitivity. Smoothing parameter is responsible for the smoothing of the response peak signal. Smoothing removes small peaks assumed to represent signal noise. The higher the smoothing parameter, the lower the peak signal, and hence the lower the sensitivity to detect small peaks or the higher the possibility that smaller peaks will be counted as noise. These parameters were manipulated or changed in an R script (function

smooth.spline and test peaks within dcpR package) embedded in KNIME®. Manipulated threshold values were - 0.001, 0.002, 0.003, 0.004 and 0.005 while smoothing parameter values – of 0.1, 0.2 and 0.3 were applied. The analysis was done by varying the threshold parameter for each level of the smoothing parameter. Three independent experiments were conducted for untreated embryos. The resulting STC response in KNIME® was then compared to a manual STC count.

3.2.5 Data analysis

STC was expressed as the number of STCs per minute (frequency) for one embryo. The mean STC frequency was estimated for a group of 20 embryos that were subject to the same treatment. The absolute STC frequency varied between the independent experiments while the trend provoked by treatments was conserved. To combine results from independent experiments, a normalized percentage mean STC frequency was obtained by dividing the mean STC frequency by the respective mean STC frequency for control embryos and multiplying by 100. Data for hypoactivity modeling were further treated by adding 100 to convert the negative values to positive. Concentration-response modeling of the percentage STC frequency was performed using the 4-parameter logistic function (LL.4) of the drc package in R (Ritz and Streibig 2005).

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

Where b is the slope function; c and d are the minimum and maximum STC response respectively; and e is the EC_{50}.

In cases of hyperactivity, the maximum parameter d in the model was fixed as the highest hyperactivity response. The effect concentration causing 10 and 50% increase or decrease of the STC was estimated from the concentration-response curve. Some compounds showed biphasic response (i.e. initial hyperactivity and declining hypoactive response at higher concentrations). The hypoactivity at higher concentration could be a result of strong seizures due to over-excitation or represent a result of subtle malformation and overt toxicity (Behra et al. 2002; Stehr et al. 2006). Hence, these data were not included in constructing concentration-response models. Hypothesis testing was used to check for differences in experimental parameters. Shapiro test and Bartlett test were used to check for normality and homogeneity of variance, respectively. Analysis of variance or Friedman test were used to test for statistical differences between treatment groups. Bonferroni adjusted Wilcoxon signed-rank test was used as a post-hoc test. Statistical difference was considered when the p-value < 0.05. Sensitivity ratio (SR) was calculated by dividing the available LC_{50} data with the STC EC_{50} data (Bittner et al. 2019). SR > 1 means the STC EC_{50} is more sensitive than LC_{50} i.e. STC effect is observed at a factor (factor of SR) lower concentrations than lethal effect and vice versa when SR < 1. Low SRs close to 1 indicate that the effect on STC was observed close to mortality.

3.3 Results

3.3.1 Influence of experimental parameters

The spontaneous tail coiling (STC) frequency depends on the developmental stage used for the assessment. This has been reported previously (Cheng et al. 2017; Saint-Amant and Drapeau 1998) and was confirmed for our experimental setup. A weak STC frequency (1 count per minute) was observed at 21 and 22 hpf, with maximum values (3.5 counts per minute) at 23 and 24 hpf, followed by a gradual decline until 31 hpf (Figure 3.1). Acclimation duration does not affect the STC response when acclimation under room temperature is ≤ 30 minutes. After removal of the exposure dish from the incubator (28°C), the measured temperature of the solution was ≈ 25°C and this declined to a stable value of 22.8°C after 30 minutes acclimation under room temperature (Appendix 2 - Table S2 and S3). There were no statistical differences (p-value = 0.542) in STC response between control (no acclimation), 15 minutes acclimation and 30 minutes acclimation. Sample size manipulation did not seem to affect the variability of the STC after reducing the number of embryos in a dish from 20 to 10, and simultaneously increasing the number of replicates from 3 to 5. The means and standard deviations of the different setups were similar (Appendix 2 - Table S4). Additionally, analyzing a sample size of 10 and 20 embryos from the same dish resulted in no observable differences (Appendix 2 - Figure S1). Single or group rearing conditions did not seem to influence the STC response. A comparison of standard deviations shows there is no difference between both setups and this suggests that group exposure does not probably cause contagious stimulation of STC in neighboring embryos (Appendix 2 - Figure S2). To evaluate the influence of analysis duration on STC response, we selected typical hyperactive (chlorpyrifos) and hypoactive (abamectin) substances. Comparing the STC frequency for different analysis duration of 60, 30, 20 and 10 s shows a slightly declining STC trend from 60 to 10 s in all the dataset considered (Figure 3.2). However, this decline was not statistically significant.

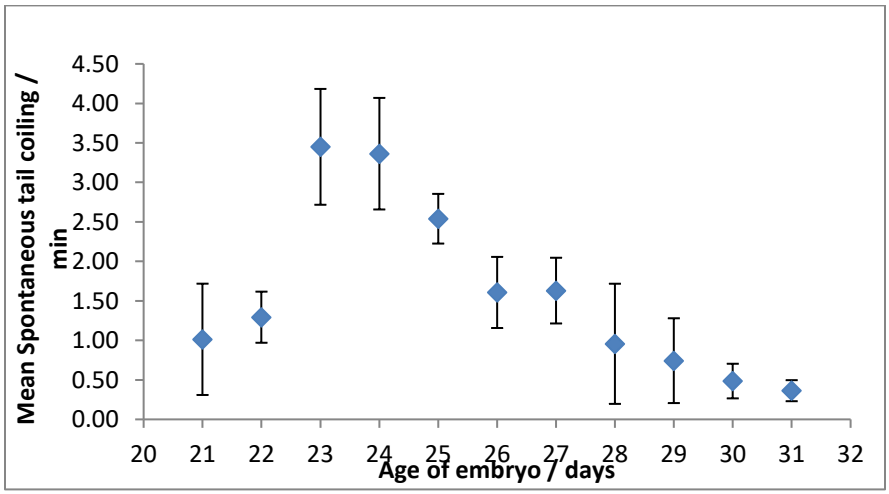


Figure 3.1: Effect of exposure duration (or developmental stage) on STC response for untreated embryos. Embryos were incubated at 2hpf at 28°C and monitored at 21hpf hourly till 31hpf. Twenty embryos were measured per replicate. Data points show mean value of 3 replicates and error bars represent standard deviation.

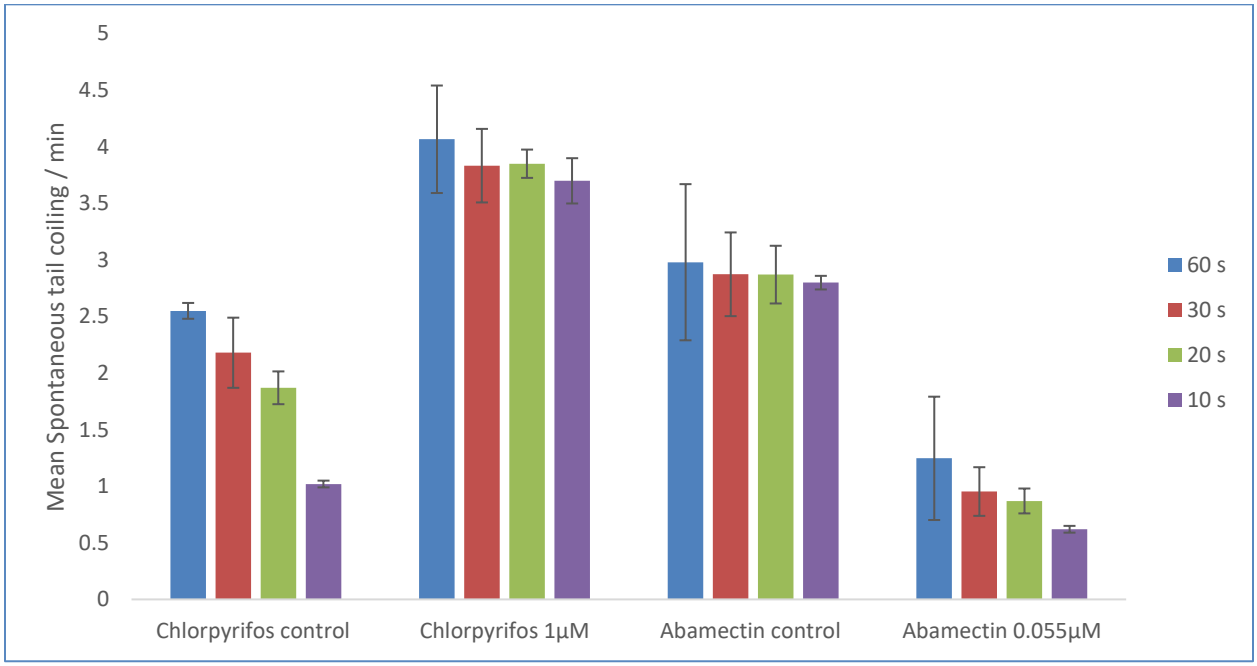
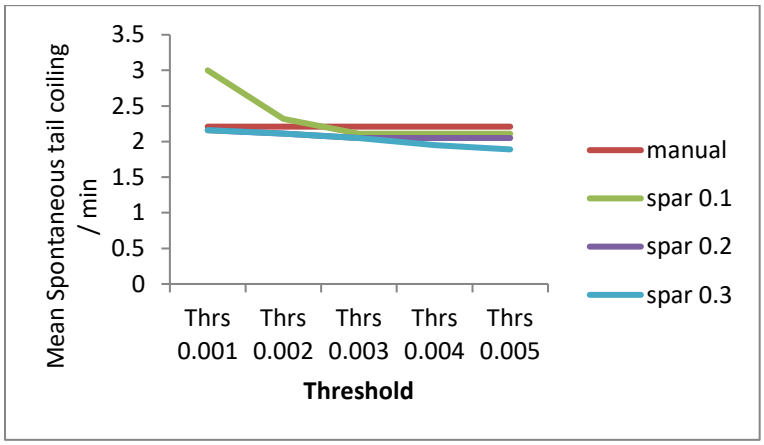


Figure 3.2: Comparison of STC frequency from different analysis duration of 60, 30, 20 and 10s. Analysis was done for chlorpyrifos and abamectin at specific concentrations showing effect on the STC. Chlorpyrifos control and abamectin control refer to DMSO solvent control. Data points show mean value of 3 replicates and error bars represent standard deviation. A Friedman test showed no statistically significant difference (p-values of 0.042, 0.72, 0.80, 0.085) between the analysis duration of each treatment (chlorpyrifos control and 1um; abamectin control and 0.055 respectively). A further Wilcoxon sign-rank post-hoc test for chlorpyrifos control showed no statistical significance.

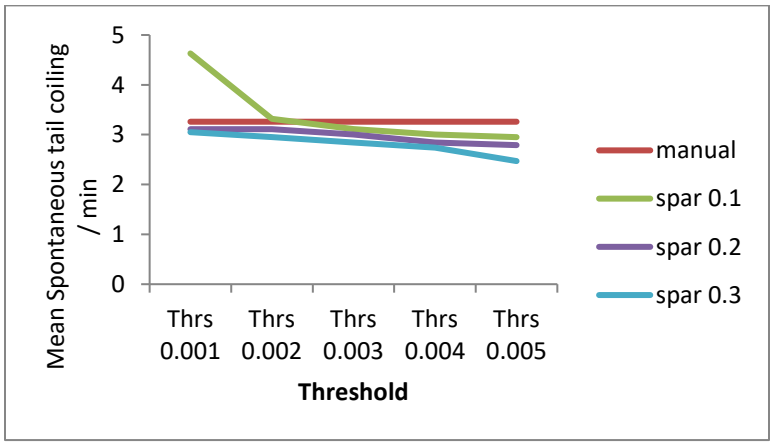
3.3.2 Influence of image analysis parameters

Two parameters used for image analysis namely threshold (thrs) and smoothing-parameter (spar) can particularly influence the calculation of STC counts in the KNIME® workflow. The comparison of different threshold and smoothing parameters show an inverse relationship between STC response and threshold or smoothing-parameter (Figure 3.3). This trend was most obvious for the smoothing parameter of 0.1. To obtain optimal parameter setting with results similar to manual STC count, smoothing- and threshold were selected as 0.0025thrs/0.1spar, 0.002thrs/0.1spar and 0.0035thrs/0.2spar for the 3 independent replicates respectively. Based on visual observation of the graphs (Figure 3.3) we selected 0.003thrs/0.1spar parameters for all subsequent analysis given that these parameters were showing the highest concordance with manual analysis.

A)



B)



C)

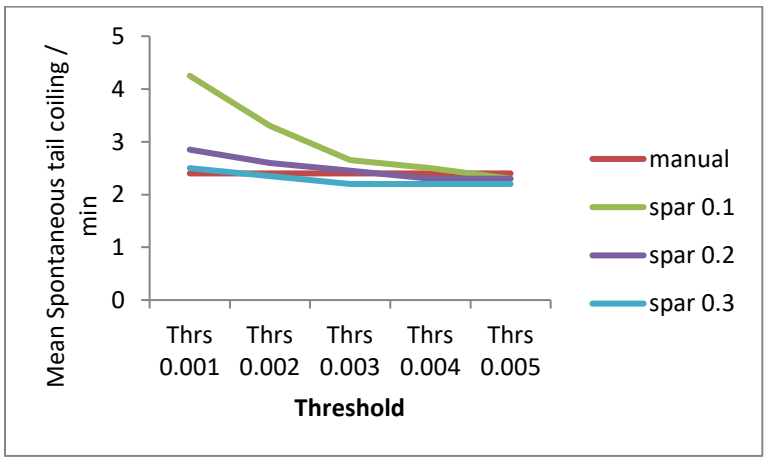


Figure 3.3: Effect of threshold (thrs) and smoothing parameter (spar) for comparison of results to manual counting of STCs are shown. A, B and C represent 3 independent experiments. Increase in threshold or spar leads to a decrease in the STC response. For subsequent analysis, parameters were selected that resulted in highest concordance between manual and automated assessment of STC frequency.

3.3.3 Effect of chemicals in the STC test

Effect concentrations of all chemicals are reported in Table 3.1. Observed STC effects for each chemical were compared to the expected effect based on the chemical's mode of action. Among chemicals which are expected to cause hyperactivity; diazinon, chlorpyrifos, chlorpyrifos-oxon and paraoxon-methyl displayed a clear hyperactivity response with EC_{50}s of 5.24, 1.85, 0.32 and 4.13 µM respectively (Figure 3.4). Additionally, the hyperactivity for chlorpyrifos-oxon peaked at 1 µM and started to decline at 1.76 µM. Diazinon caused up to 50% mortality at 10 µM while paraoxon-methyl at 100 µM caused sublethal effects such as incomplete tail coiling and reduced-resorption of the yolk sac (Appendix 2 - Figure S3). Nicotine and aldicarb also showed subtle hyperactivity at EC_{50}s of 0.97 and 29.6 µM respectively (Figure 3.4). However, these hyperactivity effects were not consistent and highly variable, hence we considered them as conditional effects. To test the influence of exposure duration as an explanation for lack of clear nicotine effect, embryos were exposed to nicotine for 20 mins between 24-25hpf. In contrast to the longer duration exposure in which only mild effects were observed, nicotine induced clear hyperactivity in all tested concentrations of 10, 20, 30, 40 µM (Appendix 2 - Figure S4). Imidacloprid showed no effect in the STC test up to 2000 µM.

Among chemicals which are expected to cause hypoactivity; abamectin, carbamazepine, diazepam and propafenone all caused hypoactivity with EC_{50}s of 0.055, 271, 20.9 and 31.6 µM respectively (Figure 3.4). Additionally, diazepam at 50 and 100 µM induced sublethal effects such as reduced-resorption of the yolk sac and edema of the pericard.

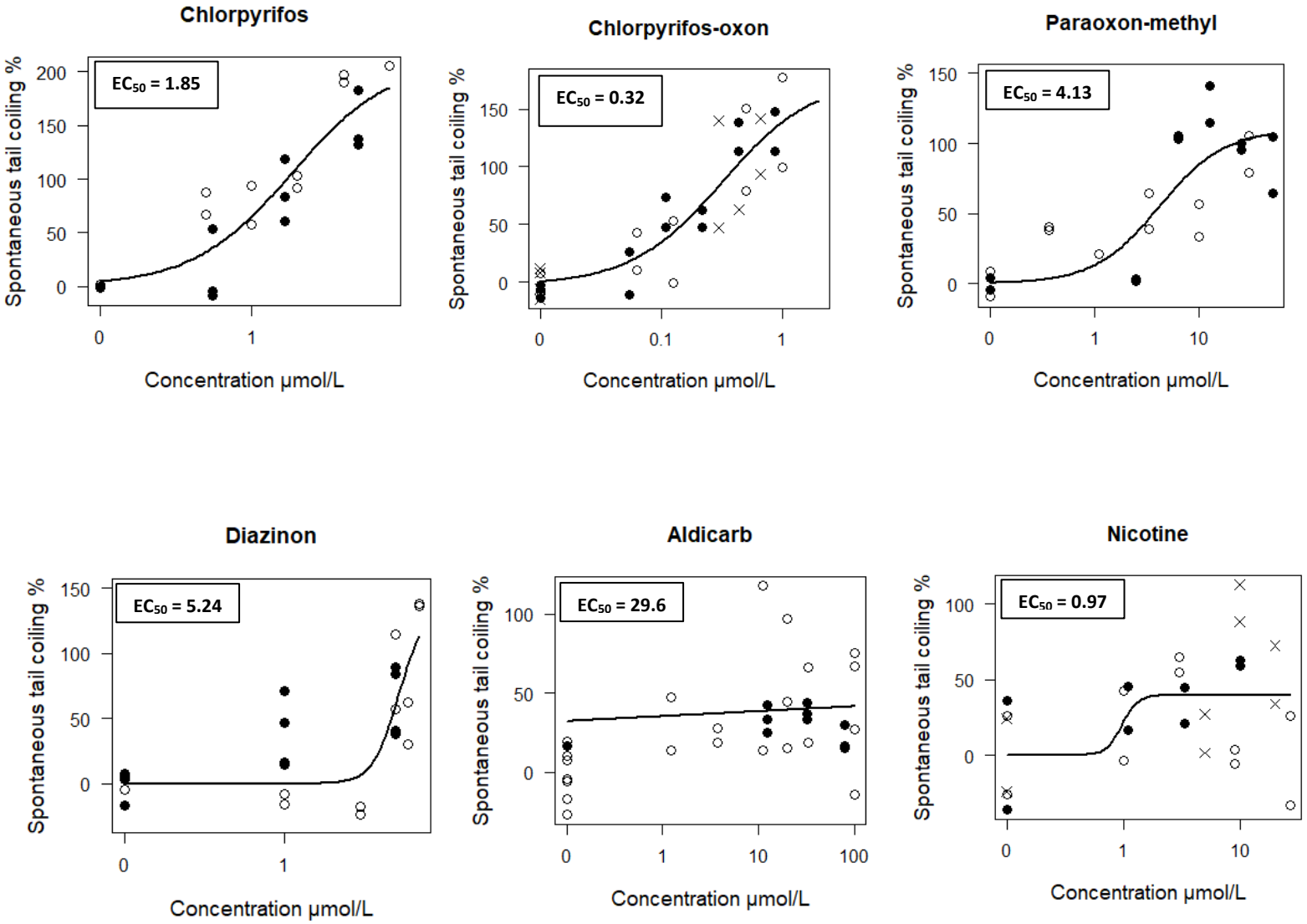
In search for negative control substances, different chemicals which do not have a known neuroactive mode of action were tested. Diuron, an herbicide, showed no significant effect up to 8 µM and caused 100% mortality at 16 µM. Daunorubicin-hydrochloride, an antimitotic drug showed no STC effect up to 50 µM. Pyraclostrobin, a fungicide showed no STC effect up to 0.14 µM (Appendix 2 - Figure S5). Higher concentrations of 0.2 and 0.25 µM caused sublethal effects, such as reduced-resorption of the yolk sac, no tail detachment and no clear formation of the head, which could be indications of developmental delay (Appendix 2 - Figure S3), while 0.4 µM caused between 50 – 100% mortality. Aniline, a known baseline toxic/narcotic substance caused hyperactivity at EC_{50} of 832 µM while 3000 µM induced 100% mortality. 3,4-dichloroaniline, a precursor and metabolite of diuron also caused hyperactivity at EC_{50} of 5.79 µM. Hexaconazole, a fungicide, caused hyperactivity (EC_{50} = 4.03 µM) up to a maximum concentration of 15 µM and higher concentration of 25 µM caused a decline of the activity towards control level (Figure 3.4). DMSO, a commonly used solvent induced hyperactivity at EC_{50} of 275455 µM (1.96%

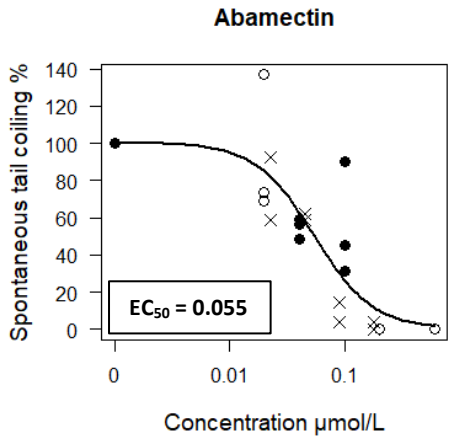
Table 3.1: Summary of STC effect characterization for all chemicals exposed to zebrafish embryos. Data collected in the present study are effect concentrations and confidence intervals (In parenthesis). Expected activity was inferred from the mode of action of each chemical.

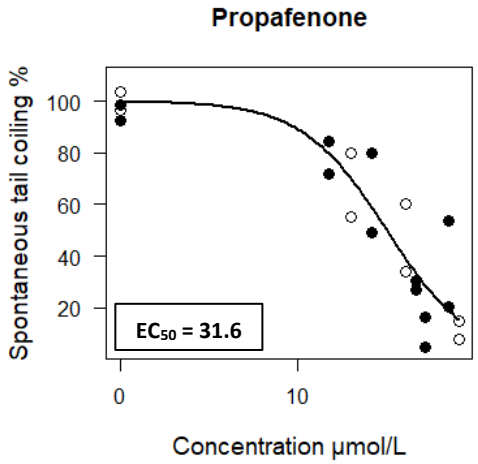
Substance	Mode of Action[m]	Expected activity	Observed activity	STC EC_{10} (µM)	STC EC_{50} (µM)	0-48 hpf LC_{50} (µM)	Baseline toxicity[t] (µM)	Sensitivity Ratio LC_{50}/EC_{50}
Chlorpyrifos	Acetylcholinesterase inhibitor	Hyperactivity	Hyperactivity	0.35 (0.11-0.59)	1.85 (1.37-2.33)	5.4[+d]	1.85	2.9
Chlorpyrifos oxon	Acetylcholinesterase inhibitor	Hyperactivity	Hyperactivity	0.047 (0.003-0.09)	0.32 (0.2-0.43)	1.5[w]	54.1	4.7
Diazinon	Acetylcholinesterase inhibitor	Hyperactivity	Hyperactivity	3.46 (2.3-4.6)	5.24 (4.58-5.9)	19.7[d]	17.7	3.7
Paraoxon-methyl	Acetylcholinesterase inhibitor	Hyperactivity	Hyperactivity	0.81 (-2.12-3.74)	4.13 (1.36-6.9)	230[d]	1097	55.7
Aldicarb	Acetylcholinesterase inhibitor	Hyperactivity	Hyperactivity	-	29.6[#] (-2.16-2.75)	279.9[+k]	7967	9.4
Nicotine	Nicotinic acetylcholine receptor agonist	Hyperactivity	Hyperactivity	0.69[#] (-1.79-3.19)	0.97[#] (0.09-1.85)	3353[e]	6792	3456
Imidacloprid	Nicotinic acetylcholine receptor agonist	Hyperactivity	No effect	-	-		28556	-

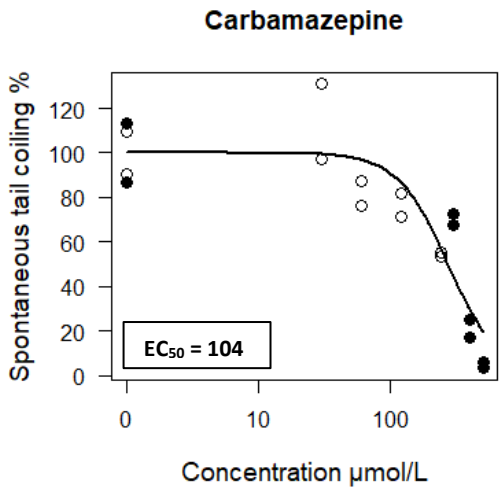
Substance	Mode of Action[m]	Expected activity	Observed activity	STC EC_{10} (µM)	STC EC_{50} (µM)	0-48 hpf LC_{50} (µM)	Baseline toxicity[t] (µM)	Sensitivity Ratio LC_{50}/EC_{50}
Abamectin	Activation of GABA-gated chloride channel; glutamate-	Hypoactivity	Hypoactivity	0.015 (0.0039-0.026)	0.055 (0.035-0.074)	0.7[w]	4.61	12.7
Propafenone	Sodium channel blocker	Hypoactivity	Hypoactivity	9.5 (2.8-16.3)	31.6 (23-40)	81[d]	45.1	2.56
Carbamazepine	Sodium channel blocker	Hypoactivity	Hypoactivity	104 (-0.99-209)	271 (193-350)	263[d]	393.1	0.97
Diazepam	GABA receptor agonist	Hypoactivity	Hypoactivity	14.8 (6.4-23.2)	20.9 (15.3-26.5)		169.1	8.1
Pyraclostrobin	Respiration inhibitor	No activity	No effect	-	-	0.26*[b]	9.14	-
Diuron	Photosystem II inhibitor	No activity	No effect	-	-	12.6*[d]	233	-
Aniline	Narcosis	No activity	Hyperactivity	736 (583-890)	832 (734-930)	1910*[b]	8929	2.3
Daunorubicin HCl	Topoisomerase II inhibitor	No activity	No effect	-	-	110[e]	2029	-
Hexaconazole	Inhibits ergosterol biosynthesis	No activity	Hyperactivity	1.18 (-0.106-2.47)	4.03 (1.78-6.28)	65[d]	22.2	16
3,4 dichloroaniline	Metabolite of diuron	No activity	Hyperactivity	2.18 (-0.4-4.75)	5.79 (2.53-9.05)	15.2*[d]	222.3	2.6
Dimethyl sulfoxide	Solvent	No activity	Hyperactivity	275455 (232094-318817)	213851 (-83686-511389)	454755[d]	2272479	1.65

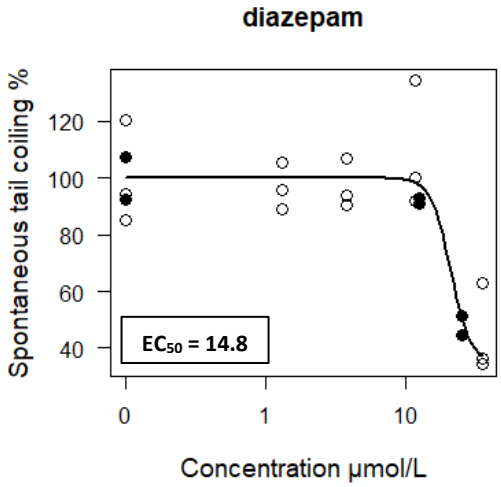
*[#]Conditional effect due to inconsistency between replicates. *data for 0-24hpf. [+]data for 0-96hpf. [k]data from Klüver et al 2015. [b]data from Birke and Scholz 2019. [d]unpublished data of the Helmholtz Centre for Environmental Research. [w]data from Weichert et al. 2017. [m]Mode of action was obtained from different sources including http://drugbank.ca, pesticide properties database and published literature. [t]Baseline toxicity is the lethal concentration predicted from lipophilicity estimated from Klüver et al. 2016.*

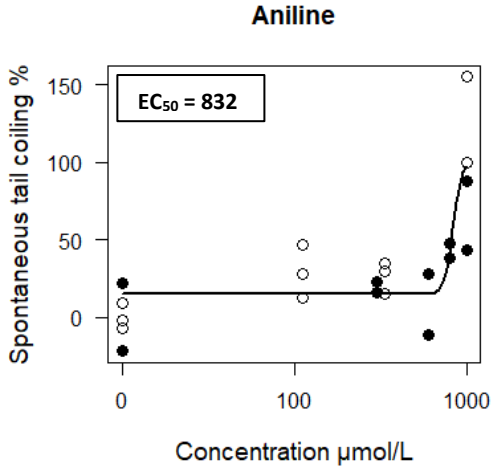

Chlorpyrifos
EC50 = 1.85
Spontaneous tail coiling %
Concentration µmol/L
Chlorpyrifos-oxon
EC50 = 0.32
Spontaneous tail coiling %
Concentration µmol/L
Paraoxon-methyl
EC50 = 4.13
Spontaneous tail coiling %
Concentration µmol/L
Diazinon
EC50 = 5.24
Spontaneous tail coiling %
Concentration µmol/L
Aldicarb
EC50 = 29.6
Spontaneous tail coiling %
Concentration µmol/L
Nicotine
EC50 = 0.97
Spontaneous tail coiling %
Concentration µmol/L

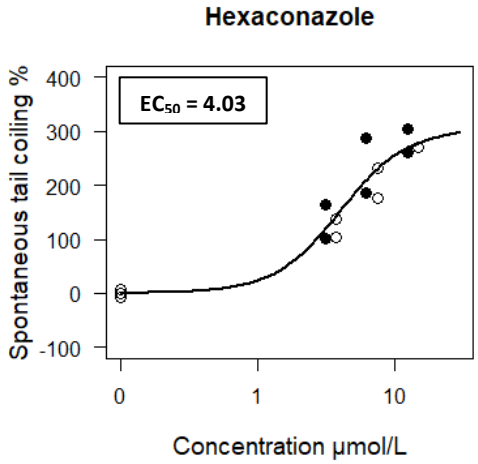

Abamectin
Spontaneous tail coiling %
140
120
100
80
60
40
20
0
EC50 = 0.055
0
0.01
0.1
Concentration µmol/L


Propafenone
Spontaneous tail coiling %
100
80
60
40
20
EC50 = 31.6
0
10
Concentration µmol/L


Carbamazepine
Spontaneous tail coiling %
120
100
80
60
40
20
0
EC50 = 104
0
10
100
Concentration µmol/L


diazepam
Spontaneous tail coiling %
120
100
80
60
40
EC50 = 14.8
0
1
10
Concentration µmol/L


Aniline
Spontaneous tail coiling %
150
100
50
0
EC50 = 832
0
100
1000
Concentration µmol/L


Hexaconazole
Spontaneous tail coiling %
400
300
200
100
0
-100
EC50 = 4.03
0
1
10
Concentration µmol/L

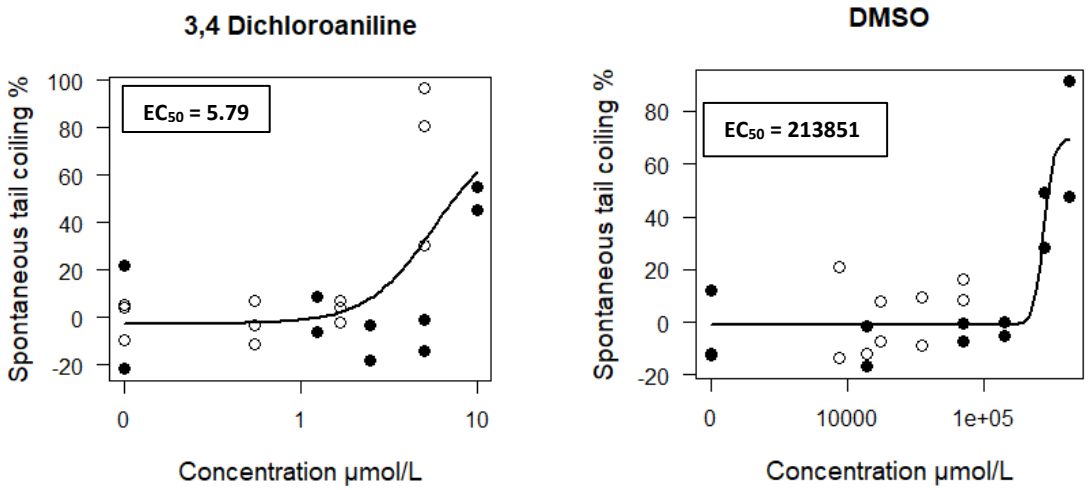


Figure 3.4: Concentration-response curves for chemicals impacting on the frequency of spontaneous tail coiling. Y-axis represents spontaneous tail coiling normalized to control and X-axis shows the exposure concentration. Different symbols represent independent experiments. Upward curves indicate hyperactivity effect with respect to controls while downward curves indicate hypoactivity effect.

3.4 Discussion

Screening and detection of neuroactive substances is a major challenge in environmental protection. Assessment of animal behavior as an integrative endpoint appears to be a very promising approach to screen for compounds with diverse neuroactive mode of actions. In fact, zebrafish embryo behavior tests are considered to fill the gap for the probable insufficient capacity of the fish embryo test (FET) to screen neuroactive compounds (Sobanska et al. 2018; Klüver et al. 2015). However, systematic assessment of the predictivity and reliability of behavior endpoints are lacking. Available behavioral methods such as the locomotor response test, spontaneous tail coiling test and photomotor response test (reviewed in Ogungbemi et al. 2019) are either not sufficiently specific to detect only neuroactive substances or they are restricted in their diagnostic capacity to detect a wide range of neuroactive substances. Behavior tests used in regulation also require conduction of experiments with adult animals which are subject to ethical concern and are cost- and labor-intensive (OECD 2007a and b). To exploit alternatives to animal testing, we explored the reliability of the STC test as an alternative screening system for the detection of (developmental) neurotoxic compounds. The STC test represents one of the available fish embryo behavior tests and has been proposed to detect chemicals interfering with motor neurons. However, a limited diagnostic capacity of the STC could occur because of 1.) Possible incapability to reveal responses in the brain due to effects being majorly propagated from the spinal cord; 2.) Possible limited biotransformation capacity of early stages of the embryo; 3.) Probable low internal concentration of chemicals that are slowly taken up (e.g. charged or hydrophobic compounds); and 4.) Possible limited uptake of high molecular weight substances due to the chorion (pore-size) barrier. At present, it is difficult to estimate the diagnostic capacity given that diverse protocols are used for STC assessment. Hence, it is necessary to characterize the extent of sensitivity and specificity of different test setups and associated parameters. Crofton et al. (2011) described a set of guidelines for developing and optimizing alternative tests for developmental neurotoxicity. We used these guidelines to characterize the capacity of the STC test to detect neuroactive substances. In the present study, we assessed the influence of experimental parameters on the variability and reproducibility of the STC response. An optimized experimental protocol was then validated using 11 chemicals known to interact with the nervous system and 7 others which are not primarily known to disrupt or affect the nervous system.

3.4.1 Discussion of the STC test performance in relation to guidance for (developmental) neurotoxicity testing

Key event of neurodevelopment - Endpoints should model key aspects of neurodevelopment

Spontaneous tail coiling (STC) represents the first motor activity generated by the developing neural network which occurs as a result of the innervation of the muscle and is assumed to support hatching of the embryo from its chorion (Kimmel et al. 1974; Saint-Amant and Drapeau 1998). The STC is presumed to be generated by depolarizations which trigger action potentials in the synapses of the primary motor neurons (Drapeau et al. 2002). These synapses leading to STC are assumed to be mainly due to an electrically coupled network in the spinal cord (Saint-Amant and Drapeau 2000). This raises uncertainties about the contribution of chemical neurotransmitters to mediate the observed STC or if they are present at this early stage of development. Tufi et al. (2016) measured different neurotransmitters including

acetylcholine and Gamma aminobutyric acid in 24 hpf embryos and hence the presence of neurotransmitters at early stages of development is established. Some other studies have shown significant involvement of neurotransmitter – receptor interaction. Acetylcholine and nicotine induced hyperactive STC in 28 hpf embryos and this is considered to be a result of activation of nicotinic acetylcholine receptors (nAChRs) (Thomas et al. 2009). STC response was also abolished (hypoactivity) in a sodium channel knockdown mutant in 24 hpf embryos (Chen et al. 2008). Spasmodic STC behavior and later on paralysis was observed in an acetylcholinesterase (AChE) knockdown mutation in 27 hpf embryos and this could be due to the over-excitation of the acetylcholine receptors by undegraded acetylcholine (Behra et al. 2002). Moreover, embryonic response was abolished by cholinergic blockers - bungarotoxin and d-tubocurarine in 28 hpf embryos (Grunwald et al. 1988; Saint-Amant and Drapeau 1998). These results suggest that both electrical and chemical induced synapses at least play a part in mediating the STC response and hence, the STC endpoint is able to reveal effects of neuroactive chemicals on the synapses at an early zebrafish embryo age of 24 hpf.

Endpoint measurement - Correct and accurate measurement of the endpoint

Measurement of the STC can be conducted by manually counting the coiling frequency or by analyzing videos with an automated workflow in KNIME®. Counts of the STC are normalized against control embryos to infer hyper- or hypoactivity. A detailed analysis was undertaken to compare the output of the automated analysis in KNIME® with manual counting. The results shown in Figure 3.3 indicates the accuracy of the automated analysis in KNIME®. Nevertheless, it is recommended to implement a correction protocol (as in Appendix 3) to control for potential errors.

Dynamic range - Determination of the extent of measurable change

The STC`s provide a dynamic range that allows to detect hyper- and hypoactivity effects relative to the control within the same assay. These effects can be quantified using hypothesis testing or dose-response modeling. The average STC count for untreated embryos can vary between 2-5 counts/min between experiments. Figure 3.5 shows the distribution of negative and solvent controls for all chemicals tested. An average STC count of 3.3 ± 0.85 /min was estimated for a pool of 94 replicates measured on different days. However, the trend of exposures is conserved. Therefore, we have normalized all data with respect to individual control from independent experiments and this could demonstrate reproducibility of the effects and allows for extensive concentration-response modelling.

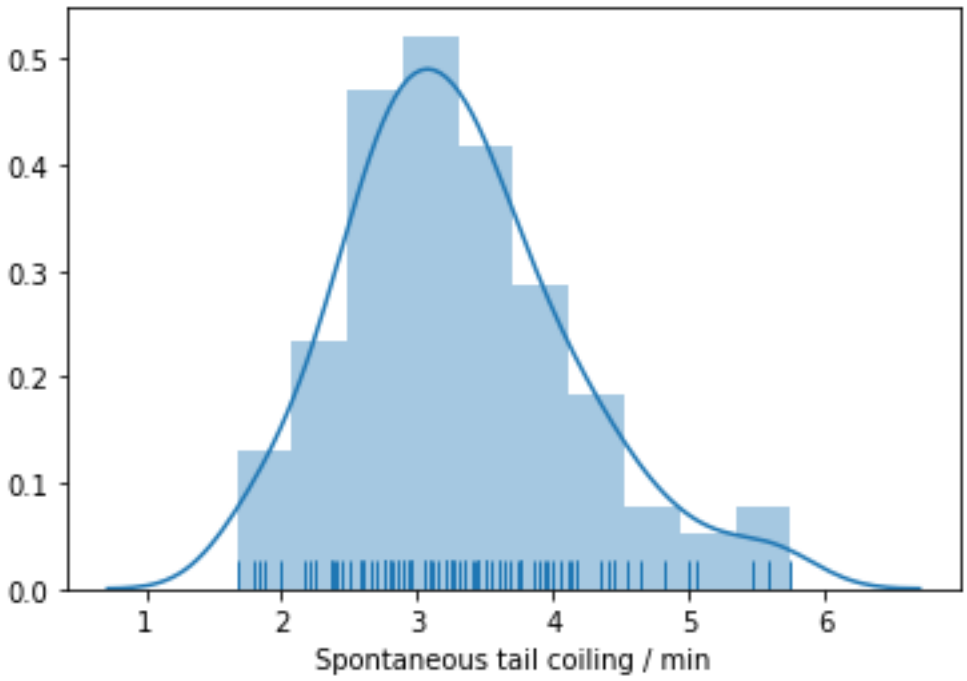


Figure 3.5: Histogram and density plot showing the distribution of 94 negative/solvent controls measured on different days.

Parametric controls - Assay parameters that predictably change the endpoint

Effect of development stage

To characterize the intrinsic behavior of a specific zebrafish strain, it is important to investigate the optimal STC response across different developmental stages or ages for that particular strain. Varying developmental stages from 21 hpf till 31 hpf showed an initial low response which then rapidly increased and peaked around 23 and 24 hpf, followed by a gradual decline until 31 hpf (Figure 3.1). To explore a high sensitivity of STC test, it is beneficial to measure during the peak response (23-25 hpf) in untreated embryos. Nevertheless, the full dynamic range and diagnostic capacity can be explored by measuring during a wider range of development stage (19-28 hpf). Similar to our result, Chen et al. (2012) reported control STC peak of 5 counts/min at 22, 23 and 24 hpf. Saint Amant and Drapeau (1998) characterized STC in dechorionated embryos and they did not only find significantly higher frequency (60/min), but peak STC was observed at 19 hpf. A similar high frequency of ≈35/min when embryos were dechorionated at 24 hpf was observed in Ogungbemi et al. (2020). Thomas et al. (2009) also reported peak STC at 19 hpf and higher STC counts for dechorionated embryos. This discrepancy in STC counts for dechorionated embryos could be due to the excessive stimulation as a result of direct contact with ionic media containing potassium chloride (Thomas et al. 2009). To obtain robust toxicological information, it is recommended to measure the STC of the fish strain at use over several time points to understand the intrinsic variability of that strain.

Effect of analysis duration

Shorter analysis duration may allow to increase the throughput of STC tests. Therefore, we investigated the effect of different analysis duration of 60, 30, 20 and 10 s. The results show a trend in which the STC frequency slightly declined across the durations from 60 to 10 s (Figure 3.2). Even though the decline was not statistically significant, it could mean a loss of STC peak information when shorter durations are used. Raftery et al. (2014) utilized lower duration of 6 seconds and they reported that lower sensitivity observed could be due to short duration. Shorter durations could be problematic especially for hypoactivity effects in which an embryo could give only one peak which could occur at any time-point within a duration of 60 s. In such cases, a 60 s duration may be more robust to capture the STC response. Nevertheless, shorter durations of 30 and 20 s also appear to be mildly robust and could be used within a miniaturized setup.

Effect of acclimation, sample size and rearing conditions

Some experimental parameters did not seem to influence the STC response. For example, acclimation time did not cause any change in STC counts within a duration of 30 minutes, even though the temperature declined from incubation temperature of 28 °C to room temperature of 22.8 °C (Appendix 2 - Table S3). Vliet et al. (2017) also found no effect of acclimation temperature on STC response when embryos were acclimatized for 1 h at different temperatures. However, Saint Amant and Drapeau (1998) reported 40 % decline in STC after acclimatization to room temperature. They did not state the duration of acclimation and a confounding effect of developmental stage or the use of dechorionated embryos could be responsible for their observed decline in STC response. Nevertheless, we implemented an acclimation period of 30 min before measurement in our STC protocol. Manipulation of sample size by reducing number of embryos in a dish from 20 to 10 and increasing number of replicates from 3 to 5 did not seem to affect the variability of the STC (Appendix 2 - Table S3). Additionally, mean of 10 embryos appear to have similar STC response with mean of 20 embryos and therefore, 10 embryos could be used within a miniaturized setup or when lower exposure volume is required. Studies on rearing conditions show that group exposure conditions do not cause contagious stimulation of the STC due to movement of neighboring embryos (Appendix 2 - Figure S1). However, older embryos raised in groups showed a higher locomotor activity than those raised individually after the first 5 days of development (Zellner et al. 2011).

Effect of image analysis parameters

To determine the optimal parameters for automated video-based STC analysis consistent with manual STC counting, we investigated the influence of threshold (thrs) and smoothing-parameter (spar) in peak detection analysis. Results show that both factors are equally influential such that an increase in one parameter needs to be balanced by the decrease in the other to obtain results consistent with manual counts. This is obvious because an increase in the smoothing-parameter will reduce the signal and a decrease in threshold will capture a reduced signal. Balance ratio of smoothing-parameter to threshold of 40 (0.1/0.0025), 50 (0.1/0.002) and 57 (0.2/0.0035) revealed similar STC counts in comparison to manual counts for untreated zebrafish embryos. It is important to note that some other confounding factors can influence the STC peak analysis. For example, uncontrolled events such as strong signal from movement of whole embryo, unstable videos with background changes in pixels, many weak peaks close to the threshold and inaccurate accountability of fast multiple peaks may influence the STC response

(Ogungbemi et al. 2021). The use of 0.003thrs/0.1spar parameterization can handle some of these challenges. To ensure high quality STC data, it is also recommended to re-check the video and peaks for these potential errors and correct them accordingly in the initial setup. For example, our recommended KNIME® parameter produced 10% deviation from the true count in one of the independent replicates because of the strong effects of moving embryos. In such cases, manual correction will be more effective than changing the KNIME® parameters. A possible correction workflow can be: 1) Check for moving embryos; 2) Visually inspect the peaks for errors. Irregular shaped and wide peaks are suspects; 3) Manually count problematic embryos or peak areas (Ogungbemi et al. 2021).

Response characterization - Level of change determined to be an effect

The STC response which is considered to be a significant effect can be characterized using hypothesis testing or fitting a dose response model for ECx estimation. A response in hypothesis testing is defined as probability value below the threshold of 0.05. In this study, we used dose-response modeling to estimate EC_{10} and EC_{50} responses. This method was capable of accurately characterizing hyper- and hypoactivity responses in the STC test. In this study we did not consider or characterize the amplitude of STC or differentiate between strong and weak STC response.

Concentration range - Methods must be designed to allow determination of concentration-response

It is generally recommended to test a minimum of 5 concentrations to enable concentration-response modeling (OECD 236; Crofton et al. 2011). The STC test as devised in this study allows the convenient assessment of 15-20 dishes within a duration of ≈30 minutes by a single person. STC assessment within a 30 minutes time-frame reduces possible influences of changes in developmental stage on the STC response. In order to detect an STC effect, it is also essential that the concentration range covers the effective range of the chemical. Hence, no fixed concentration range should be applied. In contrast concentration ranges need to be adjusted for individual chemicals. For example, carbamazepine was only effective in the STC test after extending the concentration range from 0 – 80 µM to 0- 500 µM. Crofton et al. (2011) recommends 5 logs below the solubility limit of the chemical and we recommend to use, depending on the data available, maximum lethal concentrations of LC50/2 or LC10 as starting concentrations to avoid unspecific sublethal effects. Additionally, conducting an initial range-finding test may allow to consider lab-specific factors such as zebrafish strain and rearing conditions.

Endpoint selectivity - Discrimination of the endpoint of concern from non-specific outcomes

It is possible to assess non-specific outcomes such as developmental malformations during STC measurement. The effects on STC should be compared to effect concentrations for malformations or lethality in order to estimate the specificity of the effects. This ensures that observed behavior effects are not driven by morphological effects since malformed embryos could show hypoactivity (Padilla et al. 2011). It is worthy to note that chemicals causing hyperactivity such as organophosphates, may induce hypoactivity at high concentrations in non-deformed embryos. This could be due to over-excitation of the neuron cell leading to axonal defects or paralysis and this does not necessarily lead to observable phenotypes (Behra et al 2002; Stehr et al 2006, Piña-Crespo et al 2014). This biphasic response could be accounted for by testing an extensive concentration range covering both the hypo- and hyperactivity effects.

Endpoint selective controls - Chemicals known to reliably and consistently alter the endpoint at a mechanistic level

Abamectin and chlorpyrifos were identified as hypo- and hyperactivity controls respectively, while diuron and pyraclostrobin could represent suitable negative controls in the STC test. Abamectin consistently caused hypoactivity at an EC_{50} of 0.055µM. Hypoactivity effects (i.e. LOEC) were also found for abamectin by Raftery et al. (2014) [3.1µM], Raftery et al. (2015) [0.25µM], Weichert et al. (2017) [0.72µM] and Vliet et al. (2017) [1.56µM]. The variation in hypoactivity effect concentrations for abamectin could be due to the use of hypothesis testing rather than dose-response modeling used in this study. Hyperactivity was also recorded for chlorpyrifos at an EC_{50} of 1.85µM and this was consistent with the effects (LOEC) of Watson et al. (2014) [1µM] and Selderslaghs et al. (2010) [1.8µM]. The reproducibility of chlorpyrifos and abamectin, as demonstrated by comparing our studies to literature studies indicates the usability of these chemicals as positive controls in the STC test. However, a mechanistic level investigation is still required to verify how these chemicals alter the endpoint. Diuron did not induce any effect within the concentration range tested (1 - 8µM). However, diuron caused hypoactivity in another STC test at 16.3µM (Velki et al. 2017). This same concentration could not be assessed in the present study because it caused 100% lethality. Velki et al. (2017) did not only report hypoactivity at 16.3µM, but also incomplete tail coiling which could represent unspecific effects due to overt toxicity.

Training set of chemicals - Proof-of-concept that the test method can rapidly and efficiently screen moderate numbers of chemicals

The STC test as devised in this study for MoA identification takes approximately 2 mins for measuring a single glass dish. This means a single chemical with 5 concentrations and 2 replicates will last approximately 20 mins. The required time can be reduced for rapid screening of chemicals in which lower number of concentrations and replicates are used. Furthermore, high resolution cameras and well plates can be applied in screenings to achieve a higher throughput. A total of 18 chemicals were tested in this study to evaluate the capability of the STC test to detect neuroactive substances. The chemicals were classified based on their known mode of action to be hyperactive, hypoactive and not-active. Hyperactive chemicals are expected to activate neuronal synapse while hypoactive ones are expected to inhibit neuronal signal transduction, thereby causing increase and decrease in the STC respectively. Seven of the exposed chemicals were expected to cause hyperactivity. The STC test detected hyperactivity for chlorpyrifos, chlorpyrifos-oxon, paraoxon-methyl and diazinon with sensitivity ratios (LC_{50}/EC_{50}) of 2.9, 4.7, 55.7 and 3.7 respective to their 48 or 96h LC50 (Table 3.1). The hyperactivity effect of these substances could be related to their proven capacity to inhibit acetylcholinesterase in zebrafish embryos (Kais et al. 2015; Küster 2005; Yang et al. 2011; Yen et al. 2011). This was revealed in the fact that chlorpyrifos was about 6 times less toxic than chlorpyrifos-oxon which is the readily potent form to inhibit acetylcholinesterase. Additionally, chlorpyrifos-oxon induced a biphasic effect i.e hyperactivity at low concentrations and hypoactivity at higher concentration of 25 µM which could be an indication of axonal deformation or over-excitation of nerve cells resulting in paralysis (Behra et al 2002; Ogungbemi et al. 2019). Paraoxon-methyl also induced sublethal effects (incomplete tail coiling and reduced-resorption of yolk sac) at high concentration of 100 µM which could be indications of developmental delay (Appendix 2 - Figure S3). Teixidó et al. (2013) also found developmental delay (reduced head-trunk angle and tail length) for embryos exposed from 48-52 hpf to 20 µM paraoxon. Despite that hyperactivity has been

reported for aldicarb (Kokel et al. 2010) and nicotine (Leuthold et al. 2019; Thomas et al. 2009) in short exposure behavior tests, both chemicals showed only a subtle and highly variable hyperactivity in the present study (Figure 3.4). This may be attributed to low hydrophobicity (Log Kow of 1.2) which may lead to quick attainment of steady state (Kühnert et al. 2013) and hence a relatively long exposure of 24 h could lead to degradation/detoxification or a desensitization effect of these compounds. In particular, 30 µM nicotine was found to reach steady state in 10 min for 23 hpf embryos. This then desensitized the nicotinic acetylcholine receptors even after a 2 h depuration (Thomas et al. 2009). To further investigate this possible desensitization of nicotine, we conducted an additional short duration exposure (20 mins) for nicotine. Similar to the study by Thomas et al. (2009), we found a clear hyperactivity for nicotine at different concentrations (10, 20, 30, 40 µM) which became minimal in a long duration exposure (Appendix 2 - Figure S4). This result suggests short duration tests could be implemented as a second-tier or alongside long duration tests to improve the diagnostic capacity of the STC, especially for substances with fast uptake kinetics. Imidacloprid up to 2000 µM did not induce effect in the STC test. Despite imidacloprid has been thought to be selective to insect nicotinic acetylcholine receptors (nAChRs), some studies have reported effects of imidacloprid on locomotor activity of 5dpf zebrafish (Leuthold et al 2019; Crosby et al 2015). Absence of effect of imidacloprid in the present study may be due to specific effect of imidacloprid on the brain nAChRs rather than the neuromuscular receptors which the STC measures.

Hypoactivity was detected for all four chemicals; abamectin, carbamazepine, diazepam and propafenone with sensitivity ratios of 12.7, 0.97, 8.1 and 2.56 respective to their 48 h LC50 or baseline toxicity (Table 3.1). The hypoactivity effect of these substances could be related to their proven capacity to inhibit neuronal synapses by activating GABA gated chloride channels or blocking sodium channels (Söderpalm 2002). Carbamazepine induced hypoactivity (EC_{50} = 195 µM) in Weichert et al. (2017) and this was only 1.4 fold lower than EC_{50} of 271 µM obtained in the present study. Both values are in the same range as the 48 h LC_{50} (263 µM) and this low sensitivity of the STC for carbamazepine could be due to similar issues related to low hydrophobicity and quick attainment of steady state as discussed for nicotine above (Halbach et al 2020).

In search for non-active chemicals, we exposed 6 chemicals with unknown or no reported neuroactive mode of action. The ideal negative controls are chemicals that induce effect on other biological systems, but are not expected to disrupt the nervous system (Aschner et al 2017). Birke and Scholz (2019) classified aniline and pyraclostrobin to be narcotic substances based on their toxic ratio (defined as the ratio of a chemical's LC_{50} estimated from a QSAR for baseline toxicity and the experimental LC_{50}) value of 5.4 and 3.1 respectively. Other negative substances were selected based on unknown neurotoxic MoA. Only pyraclostrobin, daunorubicin-hydrochloride and diuron did not cause STC effect (Appendix 2 - Figure S5). No STC effect for pyraclostrobin has already been reported up to 0.76 µM (Raftery et al 2014). Interestingly, the STC test detected hyperactivity for hexaconazole, aniline and 3,4-dichloroaniline with sensitivity ratios of 16, 2.3 and 2.6 respectively to their 24 or 48 hpf LC_{50} (Table 3.1). We consider hyperactivity to represent a specific effect on STC since unspecific secondary effects caused by cytotoxicity and/or malformation may rather result in hypoactivity. In fact, an hexaconazole containing product has been reported to cause neurotoxic effects such as trembling, jittering and shaking in a poisoned human (David et al. 2008) and hexaconazole is classified as neurotoxic to the human nervous system (Grandjean

and Landrigan 2014). Similar to our findings, hexaconazole also induced hyperactivity in the zebrafish embryo photomotor response test (Reif et al. 2016). Hexaconazole also decreased thyroxine (T4) levels while increasing triiodothyronine (T3) in 120 hpf zebrafish embryos (Yu et al. 2013). Hyperactivity effects of T3 and T4 on light/dark induced locomotor response of 120 hpf zebrafish embryo have also been reported (Walter et al. 2019). Subsequently, the hyperactivity induced in the STC test by hexaconazole may be associated to thyroid hormone disruption impacting the proper development of the motor neurons. An alternative hypothesis is hexaconazole may induce hyperactivity by blocking GABA receptors similar to its structurally related pentylenetetrazole (Squires et al. 1984). Aniline is classified as neurotoxic in the pesticide properties database and tremor manifestations was associated to aniline exposure (National research council 2008). Interestingly, a commonly used solvent, DMSO, also induced hyperactivity in the STC test despite that it has been listed as a potential negative control for developmental neurotoxicity (Aschner et al 2017). Following from these results, we can consider the STC test valuable to indicate indirect effects on the nervous system.

Specificity and sensitivity - Analysis to determine ability to correctly differentiate active and non-active chemicals

The STC test was able to accurately detect 8 out of 11 neuroactive substances, amounting to 73% sensitivity. However, the results from this study are too few to reliably estimate specificity and sensitivity. Moreover, it is difficult to estimate the specificity of the test because substances which do not have a known neuroactive mode of action may have unknown or indirect neuroactive side-effects like in the case of hexaconazole or aniline. Similarly, chemicals which show neurotoxic effect may induce this effect via non-neural organs or receptors.

High throughput - Test system and endpoint should be amenable to automation

The STC test can be considered to be a mid-high throughput test because of its short test duration of 24 h compared to other behavior tests, short video acquisition duration and possible automated workflows for estimating the STC frequency. It may be further optimized to comply with analysis in 96-well plates which could further improve throughput. However, utilizing plastic 96-well plates may compromise effect concentrations due to sorption of lipophilic compounds to plastic wells.

Documentation - Full and published documentation of the test method Resources

Full documentation of the STC test as used in the current study can be found within the method section and within the complementary method paper associated to this study (Ogungbemi et al. 2021).

Transferability - Resources for use should be available for any laboratory

The required resources for easy implementation of the STC test are accessible and widely available. The test organism, zebrafish, is a model organism and can be easily reared in indoor aquaria. Moreover, the eggs obtained from the adults can be synchronized by cell stage. The glass crystallization exposure dish can be readily purchased. The assessment tools; microscope and camera are regularly used resources in most biology laboratories and can be easily purchased and set up. Most especially, we provide a workflow for automated STC counting within the KNIME® platform. This workflow is freely available by searching for “spontaneous tail coilings detection in zebrafish” on https://hub.knime.com/ and can be easily implemented by following basic instructions outlined in the associated method paper (Ogungbemi et al.

2021) or by watching this video - https://youtu.be/wgJN71zTvRw. This means that laboratories that cannot afford commercially available software can still maximize the capacity of the STC test.

3.5 Conclusion

In this study, we optimized the STC test and investigated the effect of 18 chemicals with different MoA. We show that developmental stage and analysis duration can influence the STC response. Based on this, we selected 24-25 hpf and 1 min as the optimal developmental stage and analysis duration for testing. Other parameters such as acclimation duration (within 30 mins), sample size and rearing conditions had no observable impact. Consequently, we selected a sample size of 20 embryos, group rearing condition and acclimatized the sample at room temperature for 30 min before analysis. Apart from a MATLAB® tool (González-Fraga et al. 2019) which still requires a paid version of MATLAB®, our KNIME® workflow is the only available freeware for STC analysis. The optimized STC test showed high sensitivity by detecting 8 out of 11 neuroactive substances at concentrations below their acute or baseline lethality. Interestingly, the STC test could also detect effects for substances with unknown neuroactive MoA which indicates possible neuroactive side effects or unknown mechanisms of action that impact on the STC. Two of the chemicals tested in this study (chlorpyrifos and nicotine) are classified as reference compounds for developmental neurotoxicity (Aschner et al 2017). In conclusion, we show the high potential of the STC test to screen developmental neurotoxicity for hazard assessment and for effect-based environmental monitoring. Therefore, a desired next step will be to harmonize and validate the STC test for prospective and diagnostic testing.

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Chapter 4: Assessing combined effects for mixtures of similar and dissimilar acting neuroactive substances on zebrafish embryo movement*

Abstract

Risk assessment of chemicals is usually conducted for individual chemicals whereas mixtures of chemicals 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 of 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. Our objective was to answer the following two questions: 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 hypoactivity) 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.

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4.1 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 (Faust et al. 2019). 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 of the mixture should cause a similar effect or target a similar receptor in the organism (Belden et al. 2007). On the other hand, IA may be applied when compounds are acting independently (Bliss 1939) which has been interpreted as acting on different target sites in the organism (Hewlett and Plackett 1959). Both models have been found to be reasonably predictive in several studies exposing unicellular organisms to bioactive compounds with known mechanisms of action (Altenburger et al. 2000; Backhaus et al. 2000; Faust et al. 2001). Nevertheless, these models cannot predict interaction of chemicals at the physical, toxicokinetic or toxicodynamic level (Cedergreen et al. 2013). 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 in which they represent active ingredients designed to interact with specific targets and receptors of the nervous system. Busch et al. (2016) 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 (e.g. Corbel et al. 2006; Yang et al. 2014) 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 (Strähle et al. 2012). 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. 2019). 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 beginning at 17 hours post fertilization (hpf) (Kimmel et al. 1974; Saint-Amant and Drapeau 1998). Measurement of the STC

frequency has been proposed as an indicator of adverse effects on the function and development of the nervous system which could lead to population and ecosystem effects (Selderslaghs et al. 2010; Ogungbemi et al. 2019). Consequently, the STC has been used to study the effects of diverse neuroactive chemicals (Vliet et al. 2017; Ogungbemi et al. 2020; de Oliveira et al. 2021; Zhang et al. 2021). 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. On the other hand, 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 (Ogungbemi et al. 2019, 2020). Based on previous results in Chapter 3 (Ogungbemi et al. 2020), we postulate 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 (Casida and Durkin 2013). 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 (Söderpalm 2002).

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 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. Therefore, we selected mixture components based on the above factors. Compounds expected to induce hyperactivity were chlorpyrifos, chlorpyrifos-oxon and hexaconazole while abamectin, carbamazepine and propafenone are anticipated to induce hypoactivity in the STC test.

The link between effect direction and mechanism of action has been shown for single substances. In contrast, it is still open if this also works for mixture components with similar or dissimilar mechanisms of action. 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 hypoactivity) 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? CA or IA cannot be used to predict the opposing effects and therefore we define antagonistic effect in this case as a counteracting effect and not a lower effect than predicted by

CA or IA. 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.

4.2 Materials and methods

4.2.1 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). Adult fish were fed twice a day either with commercial dry food flakes or *Artemia* sp. and physicochemical parameters of the aquaria water were frequently measured (pH 7–8; water hardness 2–3 mmol/L, conductivity 540–560 µS/cm, nitrate < 2.5 mg/L, nitrite < 0.025 mg/L, ammonia < 0.6 mg/L, oxygen saturation 87–91%). 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. (1995) with a microscope and embryos between the 16^{th} and 128^{th} cell stage were used to start the exposure.

4.2.2 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]. The properties, effect concentrations and model parameters for single substances used in mixture modeling are given in Table 4.1.

Table 4.1: Properties and effects of single substances in the STC test.

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

4.2.3 Mixture testing in the STC test

Several mixtures were designed to investigate the appropriate classification for similar and dissimilar neuroactive substances which is suitable for mixture effect prediction using CA or IA models. Mixture components were selected according to their mechanism of action and effect direction (hyper- or hypoactivity) as follows (Figure 4.1): 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 concentrations of the mixtures given in Table 4.2 are based on mixture ratios of the single substances calculated as molar fraction of their effect concentrations (EC_{50}). The EC_{50} concentration was selected to ensure that all components in the mixture contribute to the effect. Mixture D was particularly designed to understand if and how dissimilar compounds with different mechanisms of action and opposing effect direction would interact in the STC test. Although components of mixture D are equitoxic (in terms of EC_{50} ratio), the mixture was designed to reflect an un-equitoxic scenario with respect to effect direction (0.33 hypoactivity : 0.66 hyperactivity).

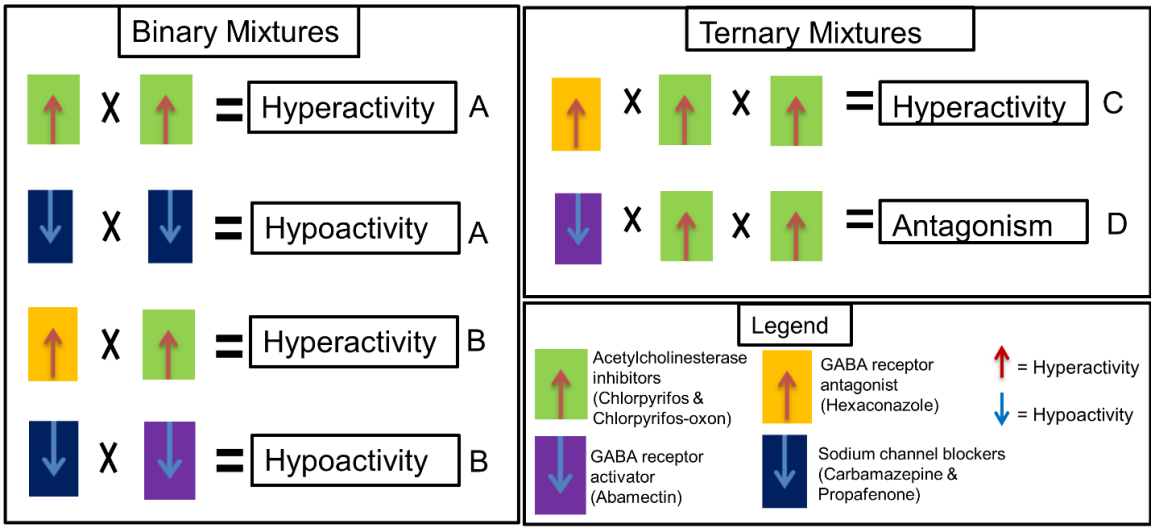

Figure 4.1: Mixture design scheme representing the hypotheses of this study. The letters A, B, C and D represent the mixture design according to Table 4.2. Each equation scheme for mixtures A, B and C represents a hypothesis whether CA or IA models could predict the hyper- or hypoactivity effects expected for mixtures with similar and dissimilar mechanisms of action. Equation for mixture D represents an antagonistic effect hypothesis.

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 (Altenburger et al. 2000), 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 (Table 4.2).

Table 4.2: Summary of the mixture design, observed toxicity and predicted toxicity.

Mixture	Substances	Observed activity	Mixture ratio[a]	Exposure concentration (µmol/L)[b]	Predicted EC_{50} (µmol/L)		Observed EC50 (µmol/L)
					CA	IA	
Mixture A	Chlorpyrifos & chlorpyrifos-oxon	Hyperactivity	0.816:0.184	0, 0.25, 0.5, 1, 2, 4 0, 0.1, 0.3, 0.9, 2.7, 5 0, 0.313, 0.625, 1.25, 2.5, 5	1.19	1.16	1.25
	Carbamazepine & propafenone	Hypoactivity	0.86:0.14	0, 40, 80, 160, 320 0, 78, 125, 200, 320	159	207	132
Mixture B	Hexaconazole & chlorpyrifos	Hyperactivity	0.65:0.35	0, 0.94, 1.87, 3.75, 7.5, 15 0, 0.75, 1.5, 3, 5.73, 12 0, 0.625, 1.25, 2.5, 5, 10 0, 0.625, 1.25, 2.5, 5, 10	2.79	3.69	2.79
	Abamectin & propafenone	Hypoactivity	0.002:0.998	0, 2.8, 5.6, 11.3, 22.5, 45 0, 4.38, 8.75, 17.5, 35, 70	23	27.6	17.4
Mixture C	Chlorpyrifos, hexaconazole & chlorpyrifos-oxon	Hyperactivity	0.603:0.324 :0.073	0, 0.75, 1.5, 3, 6, 12 0, 0.33, 1, 3, 9	2	2.19	1.95
Mixture D	Chlorpyrifos, hexaconazole & abamectin	Hyper & Hypoactivity	0.34:0.64 :0.02	0, 1.25, 2.5, 5 0, 1, 2, 4	-*	-	-
Simulation of Hyperactive Mixture A	Chlorpyrifos-oxon, (chlorpyrifos & hexaconazole)	Hyperactivity	0.184: (0.286 :0.53)	0, 0.313, 0.625, 1.25, 2.5, 5 0, 0.1, 0.3, 0.9, 2.7	-	-	-
Simulation of Hyperactive Mixture B	Hexaconazole, (chlorpyrifos & chlorpyrifos-oxon)	Hyperactivity	0.65: (0.286 :0.064)	0, 0.625, 1.25, 2.5, 5, 10 0, 0.33, 1, 3, 9	-	-	-

**no mixture and toxicity predictions; [a]Mixture ratios are calculated as molar fraction of the total concentration. The ratio in the mixture is defined by the ratio of EC50s. [b]The given exposure concentrations refer to the exposure range of independent experiments. In subsequent experiments, often different ranges were used to promote a better description of concentration-response curves. All concentration ranges were combined for concentration-response modelling.*

The detailed procedures for STC testing have been previously reported in detail (Ogungbemi et al. 2021). 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 photoperiod 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 (without any obvious malformation) were recorded for 60 s. Collected videos were analyzed for STC counts per minute (STC frequency) by means of a workflow using the KNIME® Analytical Platform (Teixido et al.; Ogungbemi et al. 2021).

4.2.4 Mixture modeling

Mixture toxicity modeling was performed to investigate the capacity of concentration addition (CA) and independent action (IA) models to predict combined effect of similar and dissimilar neuroactive substances. Effect data for the single substances used for mixture modelling was obtained from a previous study (Ogungbemi et al. 2020). 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 (Altenburger et al. 2000). It is used to predict mixture toxicity of chemicals with a similar mechanism of action.

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

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 (Zhu and Chen 2016).

4.2.5 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 was estimated for independent experiments. 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 (Ritz and Streibig 2005).

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

Equation 3 shows the concentration (x)-response (y) 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 4.2). 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 (obtained by normalizing to control) was standardized by dividing with the maximum percentage effect for each chemical to obtain a standardized percentage hyperactivity effect leading to 100 % maximum effect for all hyperactive chemicals (Figure 4.2). This allowed to obtain a similar half-maximum effect (EC_{50}) for the 3 chemicals. 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. (2014) 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 3 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 (Jackson 2010) 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.

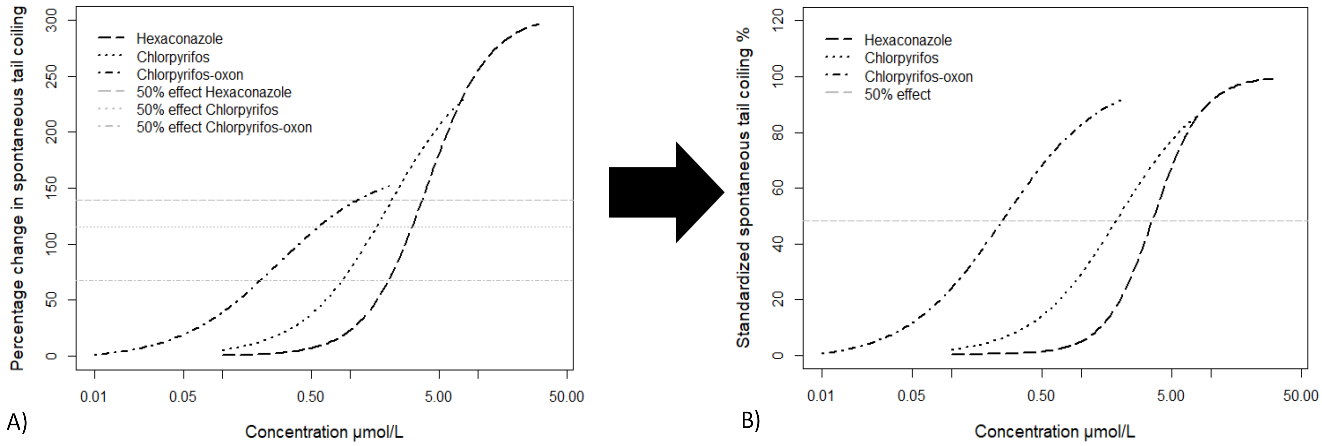

Figure 4.2: Visual representation of the data transformation for hyperactivity inducing chemicals: A) Concentration response curves showing different maximal for the hyperactivity inducing substances. The horizontal lines show EC_{50}, EC_{41} and EC_{24} which corresponds to the 50 % effect for hexaconazole, 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. Data taken from Ogungbemi et al. (2020).

4.2.6 Measurement of the exposure concentrations

Measurement of exposure concentrations was conducted to verify that test compounds were present in adequate concentrations in the test. Chemical measurement was performed only for one independent experiment of the binary mixtures since the same relation of measured and nominal concentrations were expected for other independent experiments and also for the ternary mixture. 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 was 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.

4.3 Results

4.3.1 Chemical analysis

Results of the chemical analysis are shown in Table 4.3. Measured concentrations were close to the nominal concentrations, typically with a maximum deviation of about 20 % for the highest tested concentrations for propafenone (+37 in Hypoactive Mixture A and -3 % in Hypoactive Mixture B), carbamazepine (-8.8 %), chlorpyrifos (-20 and -20 % in both mixtures), chlorpyrifos-oxon (+19 %) and hexaconazole (+15 %). Measured concentrations of abamectin were below detection limit (MDL) in all 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. 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 4.3: 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 while values in squared brackets are nominal concentrations which are below detection limit.

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.

4.3.2 Description of mixture effect in comparison to CA and IA models

The effects of single substances used in the mixture testing have already been described in Ogungbemi et al. (2020) and are summarized in Table 4.1. 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 4.3. Observed and predicted EC_{50} values are also shown in Table 4.2.

Hyperactive Mixture A (chlorpyrifos and chlorpyrifos-oxon) [see section 4.2.3 for definition of the mixture name] induced hyperactivity with an EC_{50} of 1.25 µM. The CA and IA models were similar and they both predicted the EC_{50} of the mixture (Table 4.2). 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 4.3A). The Hypoactive Mixture A (carbamazepine and propafenone) caused hypoactivity with an EC_{50} of 132 µM. Both CA and IA (EC_{50} of 159 µM and 207µM, respectively) 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 4.3B). Overall the estimation difference was always below a factor of 2 for CA and IA.

Hyperactive Mixture B (chlorpyrifos and hexaconazole) showed hyperactivity with an EC_{50} of 2.79 µM (Table 4.2). CA could predict the exact observed EC_{50} of the mixture but IA slightly underestimated the mixture effect [EC_{50} = 3.69 µM] (Figure 4.3C). Hypoactive Mixture B (abamectin and propafenone) showed hypoactivity with an EC_{50} of 17.4 µM. Both CA and IA slightly underestimated the mixture toxicity with EC_{50} values 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 4.3D). 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 4.4). 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.

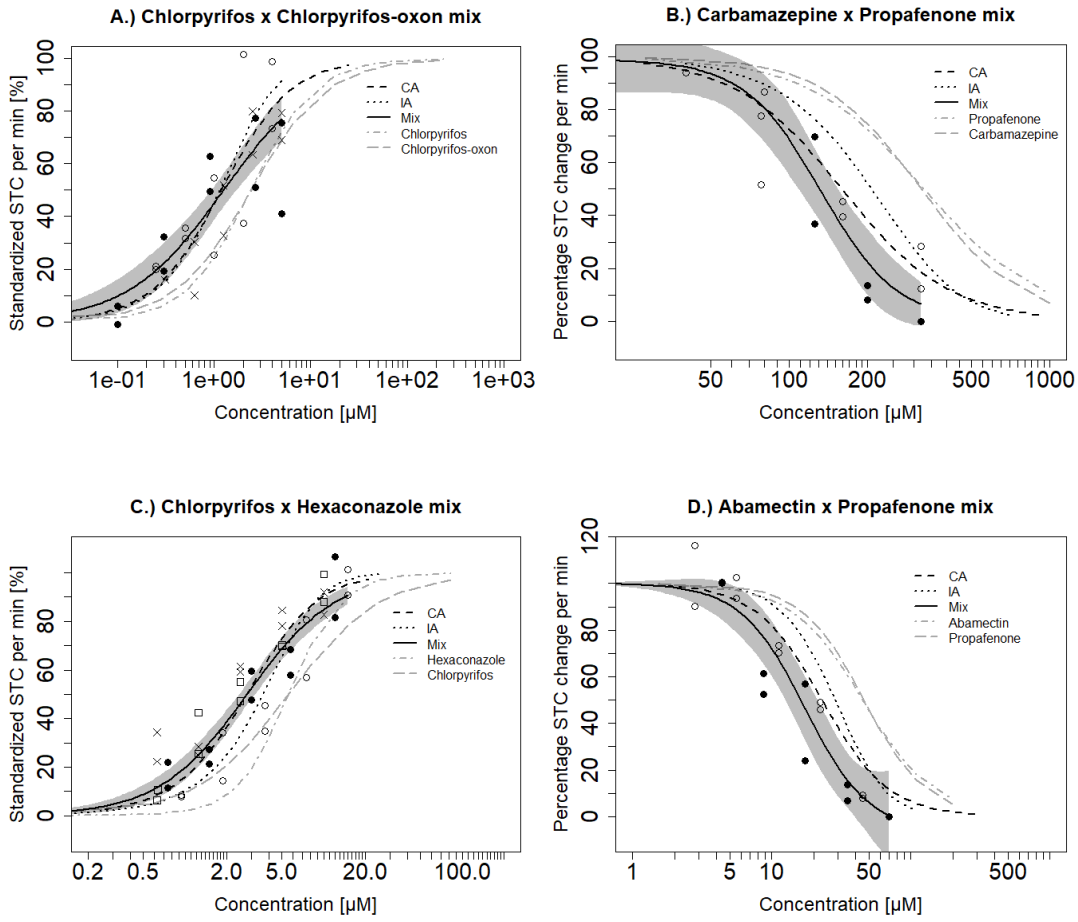

Figure 4.3: Comparison of observed (Mix) versus predicted effects of binary mixtures based on the concentration addition (CA) and independent action (IA) models in the STC. Furthermore, mixture effects are compared to single substances effects: A.) Hyperactivity Mixture A; B.) Hypoactivity Mixture A; C.) Hyperactivity Mixture B; C.) Hypoactivity Mixture B. Grey shaded areas represent the confidence interval

of the fitted mixture model for the observed effect. Different symbols represent observed mean of STC effect for 20 embryos exposed in independent mixture experiments.

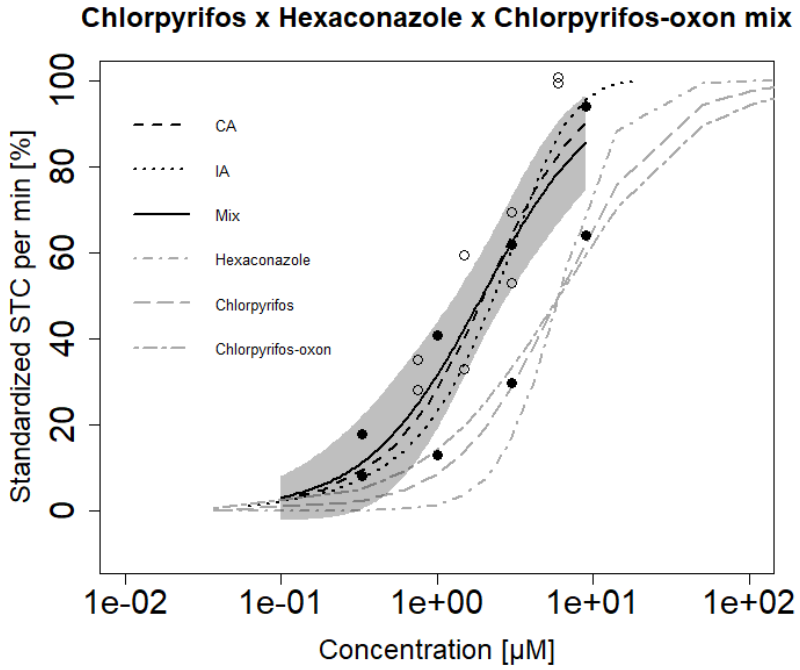

Figure 4.4: Comparison of observed (Mix) versus predicted effects of a ternary mixture based on the concentration addition (CA) and independent action (IA) models for mixture C. Furthermore, mixture effects are compared to single substances effects: Grey shaded areas represent the confidence interval of the fitted mixture model for the observed effect. Different symbols represent observed mean of STC effect for 20 embryos exposed in independent mixture experiments.

Further, we investigated the CA assumption that substances are dilution of each other. Results show that substituting portions of chlorpyrifos in the Hyperactivity Mixtures A and B with hexaconazole and chlorpyrifos-oxon respectively, induced similar concentration response curves as the non-substituted mixture (Figure 4.5 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.

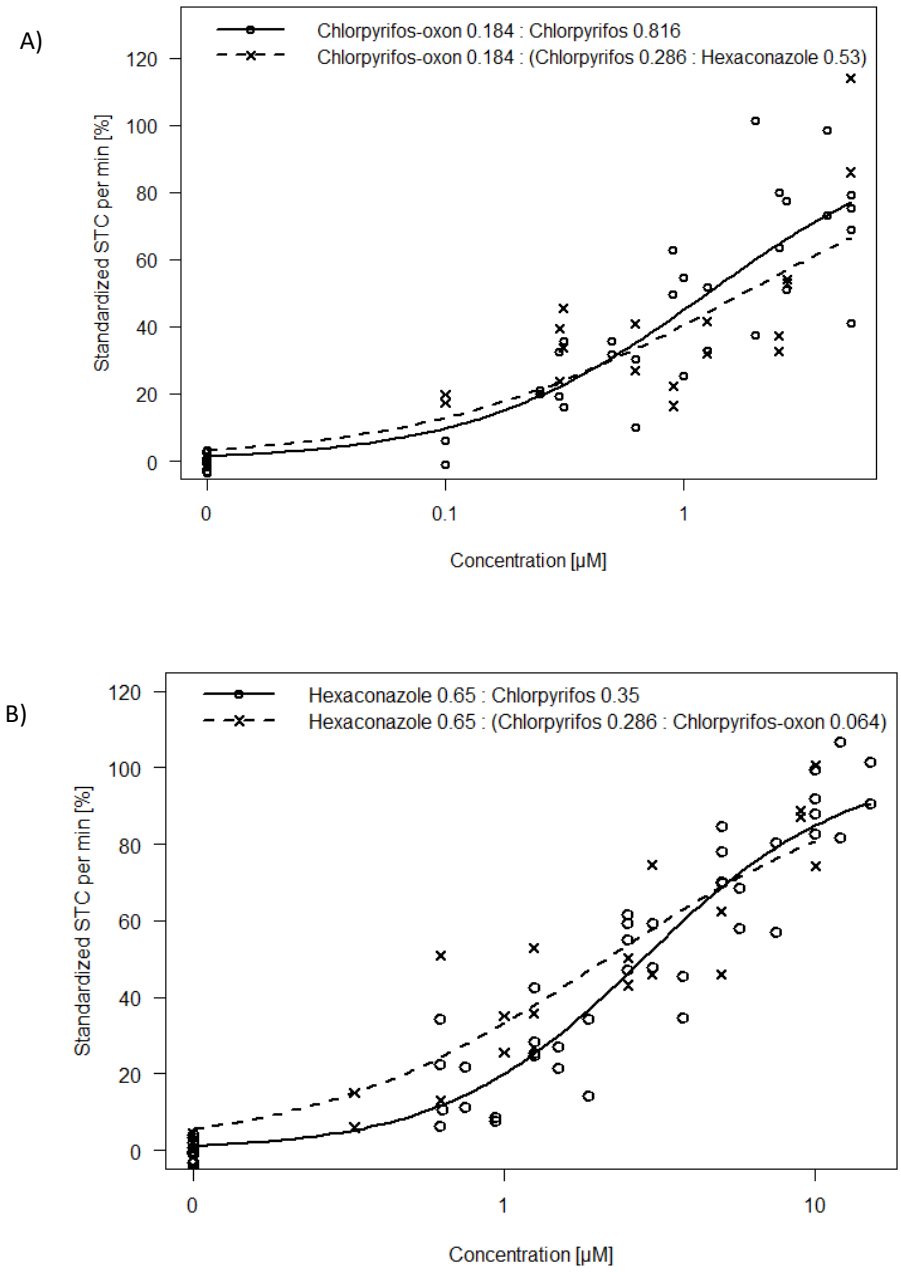

Figure 4.5: A ternary mixture is used to simulate a binary mixture by replacing a portion of one of the binary components with an equitoxic proportion of another substance: A) Concentration response curves for Hyperactive Mixture A containing chlorpyrifos-oxon and chlorpyrifos. Portions of chlorpyrifos were replaced with hexaconazole; B) Concentration response curves for Hyperactive Mixture B containing hexaconazole and chlorpyrifos. Portions of chlorpyrifos were replaced with chlorpyrifos-oxon.

4.3.3 Antagonistic mixture effects in the STC test

Exposure of substances inducing opposing effect direction may induce antagonistic or counteracting effects. Therefore, we exposed a ternary mixture of dissimilar substances (Mixture D) with different mechanisms of action and opposing effect directions (i.e. hyper- and hypoactivity). Mixtures were designed to reflect an un-equitoxic scenario (0.33 hypoactivity : 0.66 hyperactivity; with respect to the corresponding EC_{50} values) by mixing the hypoactivity causing abamectin with two hyperactivity causing substances (chlorpyrifos and hexaconazole). The result shows that the antagonistic effect of abamectin significantly decreased the hyperactivity effect expected from hexaconazole and chlorpyrifos (Hyperactive Mixture B). Apart from this antagonistic effect, a biphasic effect (initial hyperactivity followed by hypoactivity) was observed for the mixture with abamectin (Figure 4.6).

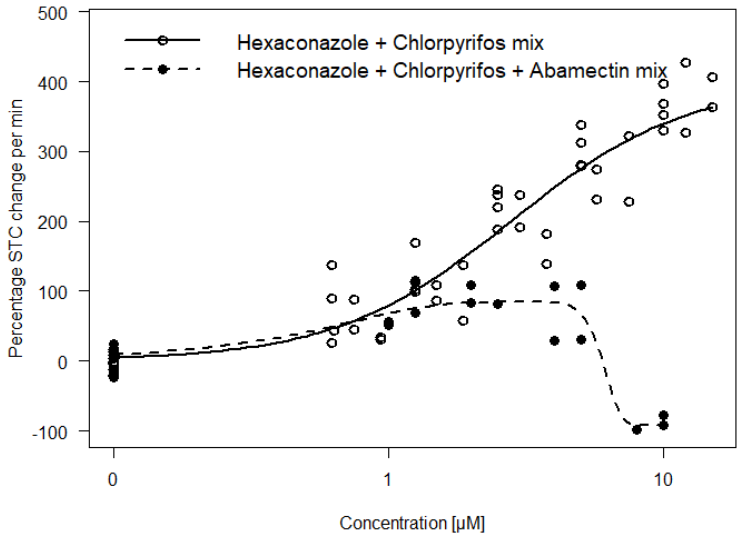

Figure 4.6: Comparison of concentration response curves for hexaconazole and chlorpyrifos (Hyperactive Mixture B) with or without the addition of abamectin. Addition of abamectin decreases the hyperactivity effect (i.e. indicating an antagonistic effect) observed for the mixture without abamectin.

4.4 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 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.

4.4.1 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 androgen production)?" which was posed in Kortenkamp et al. (2009). 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 (Cleuvers 2003). We 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. Results from the current study indicate that mixture toxicity of chemicals such as propafenone and abamectin as well as chlorpyrifos and hexaconazole that are known to induce different mechanisms of action but similar effect directions were predictable using CA and IA models. (Figure 4.3C and D). 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 increase with more mixture components (Drescher and Boedeker 1995). However, there was also no difference in the prediction of CA and IA for the ternary Mixture C (Figure 4.4). CA and IA models could also predict combined effect of pyrethroids and organophosphates in a D. magna immobility assay (Rose et al. 2016). 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 (Jakobs et al. 2020). 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 (Rose et al. 2016). 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 (Cedergreen et al. 2008).

4.4.2 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 (Drapeau et al. 2002). Consequently, it is not farfetched to consider different target interaction or mechanisms of action as similarly acting, so far 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. (2009) found that CA was a good predictor of the mixture neurotoxicity of different pyrethroids on the motor activity of rats and Gonçalves et al. (2008) reported that CA was adequate to predict mixture effect of PAHs on fish behavior.

Based on the confidence interval of the experimental mixture, the IA model was slightly less predictive (a factor of about 1.6 % deviation) for hypoactivity effects (Figure 4.2B and D). This could be due to unspecific effects such as axonal deformation and malformations which might contribute additional effect to the primary hypoactivity of the embryo (Ogungbemi et al. 2020). Such additional effects would likely be captured as an integrative hypoactivity effect in the CA model. 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 (Kortenkamp et al. 2009; Schmidt et al. 2016; Jakobs et al. 2020). For instance, Corbel et al. (2006) found that a carbamate and pyrethroid had a converging effect on acetylcholine concentration in the neuronal 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 (Scholze et al. 2014). 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 4.4A 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 (Altenburger et al. 2003; Cedergreen et al. 2013). Mixture interaction of neuroactive substances may occur via the biotransformation pathways due to the reduced activation or competition for biotransformation sites (Corbett 1974). Organophosphates were found to be a major synergistic group due to their ability to inhibit esterases which are responsible for phase 2 biotransformation of chemicals (Cedergreen 2014). 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 (Kühnert et al. 2017) 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 (Kienle et al. 2009) and mixture of atrazine and chlorpyrifos

was assessed as synergistic (Pérez et al. 2013). However, 120 and 96 hpf embryos which should have higher rates for biotransformation into the active oxon metabolite were used in these studies.

4.4.3 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 (Mixture D). The results show that mixtures with both hyper- and hypoactivity inducing components will lead to antagonistic interaction (Figure 4.6). 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 (Kortenkamp et al. 2009). 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 4.6) would have been unexplainable if only mechanism of action-based classification was used. This particularly apply to endpoints with opposing effect directions such as locomotor activity or even gene response. For such endpoints, chemicals which primarily induce hyperactivity at low concentrations may cause hypoactivity at higher concentrations due to seizures and paralysis (Ogungbemi et al. 2019). The use of chemicals inducing such biphasic activity as component in a mixture without considering the primary effect direction could lead to misinterpretation of its impact on the combined effect. This biphasic activity was also observed for Mixture D in the current study and could be due to the relatively higher counteractive potency of abamectin (EC_{50} of 0.06 µM) induced at high mixture concentrations in comparison to the hyperactivity effect of chlorpyrifos and hexaconazole with much higher EC_{50}s (Figure 4.6).

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 (Bal-Price et al. 2015; De Baat et al. 2019). However, equitoxic ratio of substances with opposing effect direction could lead to no observed effects or effects occurring at control level. This counteracting effect 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 number 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. This non-equitoxic design was useful to evaluate Mixture D in the current study. 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.

4.5 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.

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Chapter 5: Differential mechanism of action of chlorpyrifos and its oxon metabolite in early stages of zebrafish embryo: implications of biotransformation capacity

Abstract

Recently, new test systems with zebrafish embryos have been developed to screen for potential neuroactive modes of action. However, measuring effects of neuroactive substances, which require metabolic activation or biotransformation using zebrafish embryos could be limited by a reduced biotransformation capacity. For instance, organophosphates such as chlorpyrifos require biotransformation to efficiently inhibit acetylcholinesterase (AChE). Nevertheless, we demonstrated in a previous study the hyperactivity effect for chlorpyrifos in the spontaneous tail coiling test at an early stage of 24 hpf, albeit, at higher effect concentrations in comparison to the active metabolite chlorpyrifos-oxon. We hypothesized that this lower hyperactivity potency of chlorpyrifos, relative to chlorpyrifos-oxon, could be due to: 1) the intrinsic AChE inhibiting capacity of chlorpyrifos; 2) weak biotransformation efficacy at this stage for generating the oxon metabolite or 3) a different mechanism of action responsible for the hyperactivity effect of the parent compound. Therefore, we investigated which of these factors could be mainly responsible for the hyperactivity induced by chlorpyrifos. Results show that chlorpyrifos-oxon but not chlorpyrifos inhibited AChE in 24 hpf embryos and this could be due to lack of biotransformation. Furthermore, we show that chlorpyrifos but not chlorpyrifos-oxon could antagonize the hypoactivity induced by abamectin, which activates Gamma aminobutyric acid receptor (GABAr). Finally, we found that hexaconazole (a suspected GABAr antagonist) induces similar hyperactivity patterns as chlorpyrifos while not inhibiting AChE. Based on these findings and literature studies, which show the similarity of action of both chlorpyrifos and hexaconazole to pentylenetetrazole (a GABA antagonist), we suggest a potential GABAr antagonism mechanism of action for chlorpyrifos in early embryonic stages.

5.1 Introduction

Neuroactive substances are one of the most frequently detected chemical groups in the environment (Busch et al. 2016). For appropriate hazard screening and prediction of mixture effects, it is necessary to understand the mechanism of action for neuroactive substances in model organisms. Organophosphate insecticides are a major chemical group of neuroactive substances mainly used as pesticides targeting the nervous system. Hence, they were found to be highly toxic to vertebrates because of their main mode of action, which is to inhibit the activity of acetylcholinesterase enzyme [AChE] (Ware and Whitacre 2004). Chlorpyrifos is a well-known organophosphate extensively used for controlling agriculture and household pests all over the world. Its use is currently restricted in many countries and a total ban is being discussed due to its potential to induce developmental neurotoxicity in humans (Grandjean and Landrigan 2014; Silva 2020). Chlorpyrifos and many other organophosphates usually require metabolic activation or biotransformation to the oxon-metabolite to effectively inhibit AChE (Fukuto 1990). This biotransformation occurs via cytochrome P450 based monooxygenases in an intact organism leading to the oxidation of the phosphorothionate group in the parent compound.

The use of zebrafish embryos (0-96 hpf) as an alternative to animal testing is rapidly growing. However, a potential limited biotransformation capacity of zebrafish embryos could be a confounding factor and might interfere with detecting the effects of neuroactive substances in early embryo stages particularly when they require metabolic activation (Ogungbemi et al. 2019). It has been suggested that the biotransformation capacity of zebrafish embryos increases with developmental stage and the unhatched embryo stage (0-24 hpf) may have a weaker biotransformation capacity (Yang et al. 2011; Kühnert et al. 2017). Therefore, hazard assessment of neuroactive chemicals such as organophosphates, which require metabolic activation, in zebrafish embryos may lead to less sensitivity and effects occurring at high concentrations due to a possibly limited biotransformation capacity. There is still a research gap in understanding how biotransformation capacity of different developmental stages of zebrafish may influence the toxicity of neuroactive substances, which need metabolic activation.

In recent times, the behavior of zebrafish embryo has been gaining wide acceptance as an endpoint for neurotoxicity diagnosis and are assumed to be directly or indirectly related to the function of the nervous system (Legradi et al. 2015; Ogungbemi et al. 2019). However, behavioral endpoints integrate other system processes and may therefore indicate also non-neural alterations. For example, adverse effects on the thyroid system may indirectly impact neurodevelopment and such effects might be captured in a behavior test (Aschner et al. 2017; Wang et al. 2018; Walter et al. 2019).

Among the repertoire of available behavior tests, the spontaneous tail coiling (STC) test is the earliest observed motor activity of the developing embryo (Fitzgerald et al. 2020). The observed tail coilings are assumed to occur as a result of innervation of the muscle by the primary motor neurons (Saint-Amant and Drapeau 1998). Therefore, measurement of the STC frequency could be a good indicator of adverse effects on nervous system function (i.e. neuroactivity) or impact on the structure and development of the nervous system and muscle innervation [i.e. neurotoxicity] (Selderslaghs et al. 2013; Ogungbemi et al. 2020). In previous studies, we showed that a model organophosphate, chlorpyrifos and its oxon metabolite (chlorpyrifos-oxon) increased STC frequency (hyperactivity) in 24 hpf embryos with EC_{50}s of 1.85 and 0.32µM respectively (Ogungbemi et al. 2020). Other studies have also found behavior effects of

chlorpyrifos in early developmental stages of zebrafish (Selderslaghs et al. 2010; Watson et al. 2014; Zhang et al. 2021). The observed differences in effect concentrations for chlorpyrifos and its metabolite in the STC test suggests potential limited biotransformation capacity in early stages of the embryo (24 hpf) leading to ineffective AChE inhibition of the parent compound. Therefore, we hypothesized that the weaker STC effect for chlorpyrifos at 24 hpf is due to 1) the intrinsic but weaker ACHE Inhibition activity of the parent compound; 2) a weak biotransformation to the oxon-metabolite or 3) a different mechanism of action (i.e. Gamma aminobutyric acid receptor [GABAr] antagonism) leading to hyperactivity in the STC (Figure 5.1).

Therefore, the aim of this study was to investigate possible differences in the mechanism of action of chlorpyrifos and its oxon metabolite as well as to understand the influence of biotransformation dynamics on the propagation of the hyperactivity effect in 24 hpf embryos. To test the above hypotheses, test methods or tools that allow to provide mechanistic understanding of effects are required. Therefore, we utilized AChE inhibition test and toxicokinetics measurements to further understand the toxicity of chlorpyrifos and its oxon-metabolite in zebrafish embryos at 24 hpf. We also conducted exvivo receptor blocking experiments using a typical inhibitory chemical to understand the neurotransmitters and neural receptors involved in the hyperactivity effect of chlorpyrifos and its metabolite. Investigations were conducted in parallel for hexaconazole (a suspected GABAr anatagonist, which also induces hyperactivity) as an alternative hypothesis for the mechanism of action of chlorpyrifos. This study shows the importance of considering the potential limited biotransformation capacity of zebrafish embryos on chemical effects.

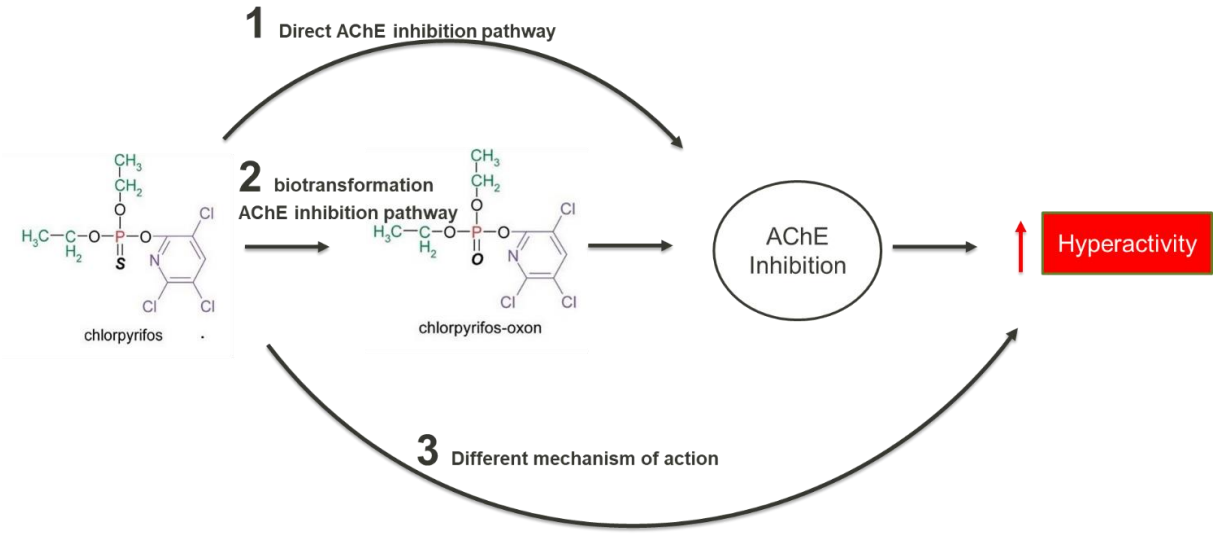


Figure 5.1: Schematic representation of the hypotheses in this study.

5.2 Materials and method

5.2.1 Test organism

Two strains of adult zebrafish (OBI and WIK strains) were crossed to produce a hybrid strain (OBI-WIK strain, F3 generation) in order to avoid inbred effects. The fish was maintained under 14 h light/10 h dark photoperiod in 120 L aquaria (tap water, 26.5 ± 1 °C) and feeding was conducted twice a day either with commercial dry food flakes or Artemia sp. Spawning trays with artificial plants were inserted in the afternoon 4–6 h before the end of the light cycle. Eggs were collected 1 hour after the onset of the light cycle on the next day and cleaned thoroughly by rinsing with ambient tank water and transferred to exposure medium. Fertilized and normal embryos were selected with a binocular microscope and embryos between 16 and 128 cell stages were used for the experiments.

5.2.2 Media and chemicals

Chlorpyrifos (99.9 %, CAS RN 2921882), hexaconazole (CAS RN 79983-71-4) and abamectin (100 %, CAS RN 71751412) were purchased from Sigma-Aldrich. Chlorpyrifos-oxon (97.9 %, CAS RN 5598152) was obtained 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]. Solutions used for testing did not exceed 0.1 % DMSO (v/v).

5.2.3 AChE measurement

To investigate the difference in the capacity of chlorpyrifos and its oxon metabolite to inhibit AChE, we measured AChE activity as described by Küster (2005) with slight modifications. After STC measurement, embryos were transferred into a 2 mL Eppendorf tube and washed twice with Milli-Q water. Twenty µL of phosphate buffer (pH 7.5) per embryo and glass beads were added. Embryos were homogenized using a FastPrep (FastPrep-24 5G, MP Biomedicals, 6.5 U×S-1, 3×35 s). Homogenized samples were immediately centrifuged at 4 °C for 30min at 13,000 g. The supernatant was collected and stored at −20 °C until analysis. During analysis, protein concentration of the samples was determined by transferring 5 µL of sample in quadruplicate into 96-well plate. Standard reagents were added using a commercial kit (DC Protein Assay, BioRad, Munchen, Germany) and Bovine serum albumin, fraction V was prepared as calibration standards. Samples were measured at 750 nm in a photometer.

AChE activity was determined by transferring 50 µL of sample in quadruplicate into 96-well plate. 50 µL and 100 µL of phosphate and 1mM DTMB were added respectively. The sample was mixed and incubated under darkness for 10 min. Subsequently, 100 µL of 1.35 mM acetylthiocholine iodide was added and the samples were measured at 412 nm in a photometer (SpectraMax 250 Photometer, Molecular Devices, USA). Four technical replicates were used per concentration and 2 independent experiments were conducted.

5.2.4 Chemical analysis and toxicokinetics

Chemical analysis was conducted for determining the real concentrations of the chemicals in the exposure solutions and the concentrations taken up by the embryo. For quantifying concentrations in the exposure

media, 1 mL sample was collected at time 0, 7, 24, 48, 72, 96 and 118 hours post exposure (hpe). Samples (1 mL) were also collected for the AChE experiments. For quantifying internal concentrations in the embryo, 20 embryos were collected at time 24, 48, 72, 96 and 118 hpe, transferred into a 2mL Eppendorf tube and washed twice with Milli-Q water. The embryos were dried, snap frozen with liquid nitrogen after adding glass beads. Subsequently, the embryos were thawed on ice and 800 µL of acetonitrile (AcN) was added. Embryos were homogenized and later centrifuged at 4 °C for 30min at 13,000 g. The supernatant was collected and measured immediately or stored at −80 °C until measurement (not exceeding 2 days). To determine the efficiency of this extraction method, a spike and recovery assessment was performed. Untreated embryos were spiked with 4 µM and 8 µM chlorpyrifos in AcN and the extraction was performed as described above.

The exposure and internal concentration of chlorpyrifos was measured by injecting 30 µL of sample on an HPLC system (Merck-LaChrom) with diode array (model L7450) detector. A reversed-phase column (Lichrospher 60 Reverse Phase (RP) select B, Merck, C-8), length 60cm, with a particle size of 5 m was used. The column temperature was set to 40 °C and the flow rate was adjusted to 0.5 mL/min. Mobile phase of AcN:water (65:35 %, elution time of 12 min) was used and detection was done at an absorbance of 207 nm.

5.2.5 Sequential exposure to demonstrate antagonistic effects

A sequential exposure was conducted to investigate whether the hyperactivity induced by chlorpyrifos and chlorpyrifos-oxon follows a similar mechanism of action and if both chemicals can reverse the hypoactivity induced by abamectin, which is a Gamma aminobutyric acid receptor (GABAr) agonist. A suspected GABAr antagonist (hexaconazole) was tested in parallel to further understand the antagonistic action of a different hyperactivity chemical. The sequential exposure was selected over a combined exposure design because we wanted to investigate the antagonistic effects at a single concentration (EC_{50}). Simultaneous exposure of the substances at their respective EC_{50} concentrations could induce additive effects not related to the STC neuroactive mode of action e.g. axonal deformation or paralysis leading to hypoactivity effect. Such hypoactivity effects at high concentrations would mask the initial hyperactivity effect, which is of interest (Ogungbemi et al. 2019, 2020). Although the sequential exposure allowed to avoid unspecific effects, at least partial depuration of internal concentration after transferring embryos into the second solution can be expected (El-Amrani et al. 2012). The sequential exposure design is depicted in Figure 5.2. First, zebrafish embryos (2-3 hpf) were exposed for 18 h to chlorpyrifos, chlorpyrifos-oxon, hexaconazole and abamectin at their STC EC_{50} determined earlier by Ogungbemi et al. (2020). Subsequently, embryos exposed in the hyperactivity substances were washed and transferred to the hypoactivity-inducing abamectin (and vice versa) for 2 h. At 24 ± 1 hpf (i.e. after 21 h total exposure), embryos were videotaped for STC measurement as described below. Two technical replicates were used for the sequential exposure and 2 independent experiments were conducted. A positive control of each chemical was used and ISO water with the appropriate DMSO concentration was used as negative control.

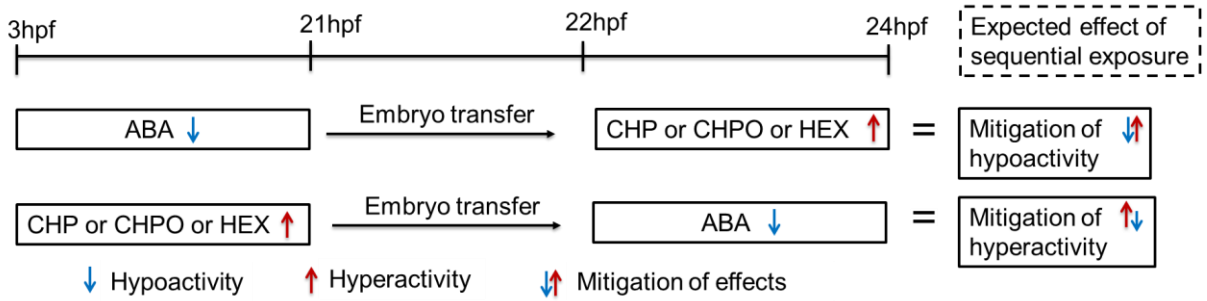


Figure 5.2: Sequential exposure design. 3 hpf embryos were exposed to chemicals for 18 h. At 21hpf, the embryos were washed and transferred into a different chemical for 2 h until 24hpf. It is expected that the hyper- and hypoactivity effects from the single chemicals will mitigate/cancel/antagonize each other leading to an overall sequential effect approaching that of unexposed embryos. ABA = Abamectin; CHP = Chlorpyrifos; CHPO = Chlorpyrifos-oxon; HEX = Hexaconazole; Hpf = hours post fertilization.

5.2.6 STC measurement

The detailed procedures for STC testing have been previously reported (Ogungbemi et al. 2021b). Briefly, twenty fertilized embryos were exposed in 20 mL of the chemical solution within a 60 mm glass crystallization dish covered with a watchmaker glass. 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 ± 0.5 hpf, exposed embryos were allowed to acclimatize to room temperature for at least 30 min before videotaping. Malformed and dead embryos were removed and 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 (Teixido et al. In press).

5.2.7 Data analysis

Data for AChE activity was normalized to control to obtain a percentage change of negative control. A concentration-response curve was fitted to AChE activity for chlorpyrifos-oxon using a 4-parameter log-logistic model (Ritz and Streibig 2005). Effect concentration for inhibiting AChE (IC_{50}) was estimated from the fitted concentration-response model. All analyses were performed in R (RStudio Team 2018). For the toxicokinetics analysis, number of moles per sample was estimated for the measured internal concentrations (equation 1). Internal concentration was estimated by dividing number of moles by the volume of 20 pooled embryos at different developmental stages obtained from Halbach et al. (2020) (equation 2). Concentration for one embryo was calculated by dividing the internal concentration for 20 embryos by 20.

$$n = C\ x\ v \qquad (1)$$

$$C = n/\ v \qquad (2)$$

Where n is number of moles per sample. C in equation 1 is the measured concentration while C in equation 2 is the estimated internal concentration per embryo. v in equation 1 is the volume of sample after embryo extraction (800 µL) while v in equation 2 is the volume of 20 embryos.

5.3 Results

5.3.1 Validation of exposure and internal concentrations

Concentrations of the substances in the STC and AChE tests were measured (Table 5.1A). All measured concentrations were within 68 to 122 % of the nominal concentrations, except for chlorpyrifos concentrations at 1.95 µM with only 15%. Since overall the measured concentrations were close to the nominal concentrations, we used nominal concentrations for analysis of effects. Recovery analysis was done to determine efficiency of the extraction process for measuring internal concentrations in the embryos (Table 5.1B). The measured values at 24 and 48 hpf time point were within a 20 % deviation of the spiked concentrations and the measured values at 4 and 8 µM were not consistent at 72 and 118 hpf.

Table 5.1: Nominal and measured concentrations in µM: A) starting exposure concentrations for chlorpyrifos, chlorpyrifos-oxon and hexaconazole in the STC and AChE tests; B) recovery of the internal concentration of chlorpyrifos during extraction.

A

Substance	Nominal	Measured	% of nominal
Chlorpyrifos	1	0.8	80
	1.95	0.3	15
	4	2.7	68
Hexaconazole	3.63	4.2	115
	14.52	17.7	122
Chlorpyrifos-oxon	0.44	0.51	116
	1.25	1.24	99

B

Time (hpf)	Nominal	Measured	% of nominal
24	4	4.38	109.38
24	8	8.54	106.71
48	4	3.50	87.44
48	8	8.99	112.37
72	4	7.32	182.88
72	8	11.04	138.04
96	8	13.15	164.43
118	4	6.06	151.47
118	8	6.07	75.90

5.3.2 Chlorpyrifos-oxon but not chlorpyrifos inhibits AChE

We conducted AChE inhibition test to investigate the capacity of chlorpyrifos (parent compound) to inhibit AChE in comparison to chlorpyrifos-oxon (biotransformation product). Chlorpyrifos-oxon caused a concentration dependent inhibition of AChE with EC_{10} and EC_{50} of 0.0153 (CI = 0.01-0.02) µM and 0.0913 (CI = 0.077-0.105) µM respectively (Figure 5.3C). The AChE inhibition induced by chlorpyrifos-oxon was more sensitive than the corresponding STC effect by a factor of 3.5 at the EC_{50} level. On the other hand, chlorpyrifos and hexaconazole did not inhibit AChE but induced significant STC hyperactivity effect (Figure 5.3A and B). This indicates the hyperactivity induced by chlorpyrifos in 24 hpf embryos may not be related to AChE inhibition.

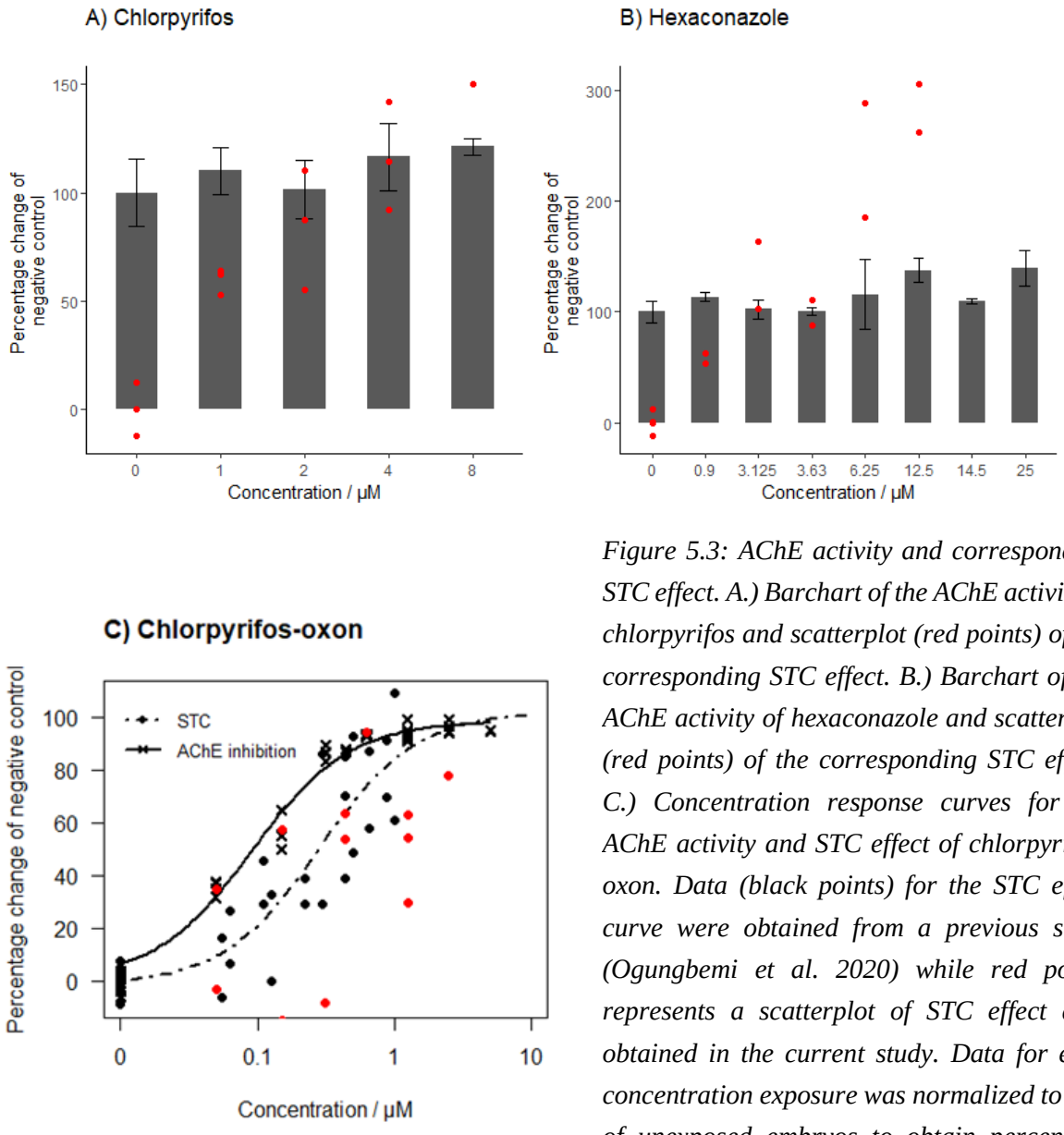
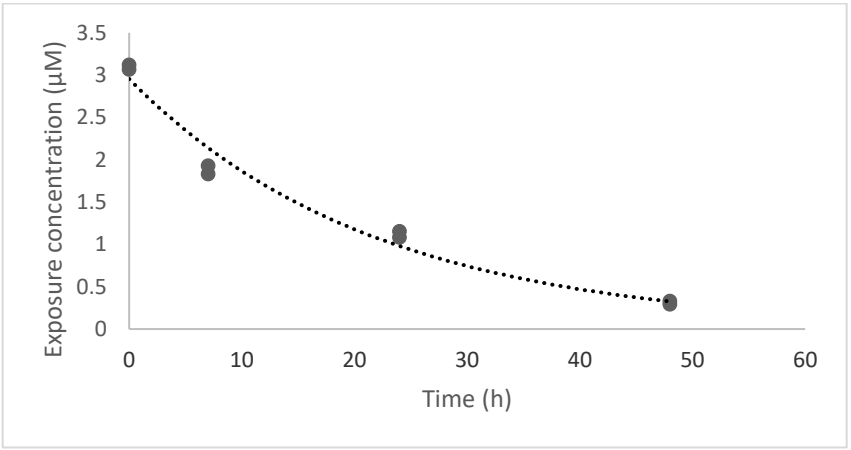


Figure 5.3: AChE activity and corresponding STC effect. A.) Barchart of the AChE activity of chlorpyrifos and scatterplot (red points) of the corresponding STC effect. B.) Barchart of the AChE activity of hexaconazole and scatterplot (red points) of the corresponding STC effect. C.) Concentration response curves for the AChE activity and STC effect of chlorpyrifos-oxon. Data (black points) for the STC effect curve were obtained from a previous study (Ogungbemi et al. 2020) while red points represents a scatterplot of STC effect data obtained in the current study. Data for each concentration exposure was normalized to that of unexposed embryos to obtain percentage change represented on the y-axis.

5.3.3 Chlorpyrifos is accumulated fast but does not reach steady state

To exclude that a limited biotransformation and uptake of chlorpyrifos into embryo was responsible for the lack of AChE inhibition in 24 hpf embryos, we investigated its time dependent uptake and biotransformation in zebrafish embryos. Chlorpyrifos concentration in the exposure media declined rapidly to about 50% after 7 h exposure and was undetectable (detection limit of 0.4 µM) after 72 h exposure (Figure 5.4A). Correspondingly, the internal concentration in the embryo increased to a maximum at 48 hpf and declined afterwards till 120 hpf (Figure 5.4B). Although biotransformation products were not directly measured, the inability of chlorpyrifos to reach a steady state and the declining internal concentration could be an indication of biotransformation at later stages beyond 48 hpf. However, it cannot be excluded that the decline in internal concentration at later stages could be due to elimination processes or instability of the exposure concentration.

A



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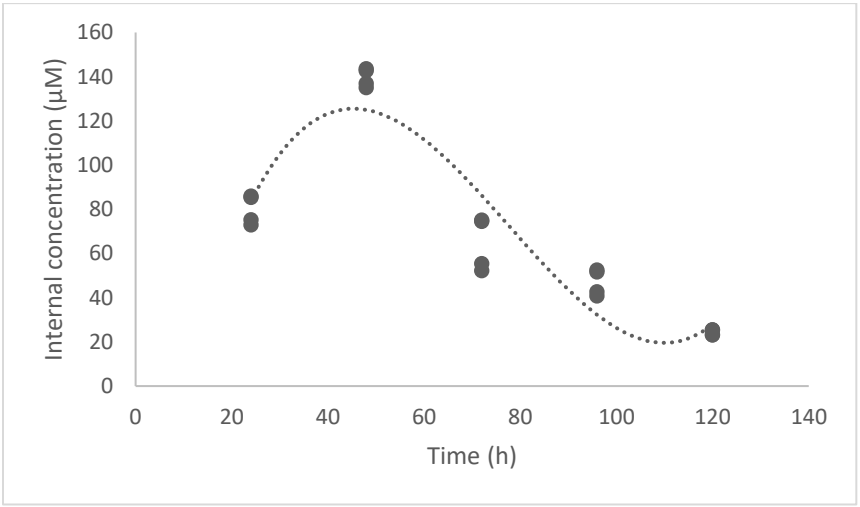

Figure 5.4: Toxicokinetics of chlorpyrifos in zebrafish embryos. A) Exposure concentrations - time profiles. B) Internal concentrations – time profiles per embryo. 2 and 4 technical replicates per time were used for exposure and internal concentration measurements respectively.

5.3.4 Chlorpyrifos but not chlorpyrifos-oxon antagonizes abamectin

Differences in the antagonistic capacities of the organophosphates, chlorpyrifos and chlorpyrifos-oxon against a typical hypoactivity inducing chemical, abamectin, may be an indication of differences in their hyperactivity mechanism of action. Therefore, we conducted a sequential exposure experiment by initially exposing embryos to abamectin followed by a second phase of exposure in the hyperactivity inducing substances (chlorpyrifos, chlorpyrifos-oxon and hexaconazole) and vice versa (Figure 5.2). The results show that chlorpyrifos and hexaconazole were capable to antagonize or mitigate the initial hypoactivity induced by abamectin and vice-versa (Figure 5.5A and B). In contrast, chlorpyrifos-oxon could not antagonize the hypoactivity of abamectin, even though abamectin was effective to antagonize the hyperactivity of chlorpyrifos-oxon (Figure 5.5C). Since chlorpyrifos-oxon has a lower log kow (3.3) than chlorpyrifos (4.96), it could have a lower accumulation in zebrafish embryos and perhaps the 2 h sequential exposure might be insufficient to induce its hyperactivity and to properly antagonize abamectin. Therefore, we performed a longer duration exposure of 8 h in which the embryos were sequentially transferred to chlorpyrifos-oxon from abamectin at 16 hpf instead of 22 hpf. Similarly, chlorpyrifos-oxon could not antagonize the hypoactivity of abamectin. Moreover, embryos exposed to the positive control of chlorpyrifos-oxon following the longer duration regime (exposure from 16 h to 24 hpf) induced hyperactivity in the 8 h exposure experiment (Figure 5.5D). This indicates that the inability of chlorpyrifos-oxon to antagonize abamectin like chlorpyrifos might be due to differences in their mechanism of action and not toxicokinetic issues.

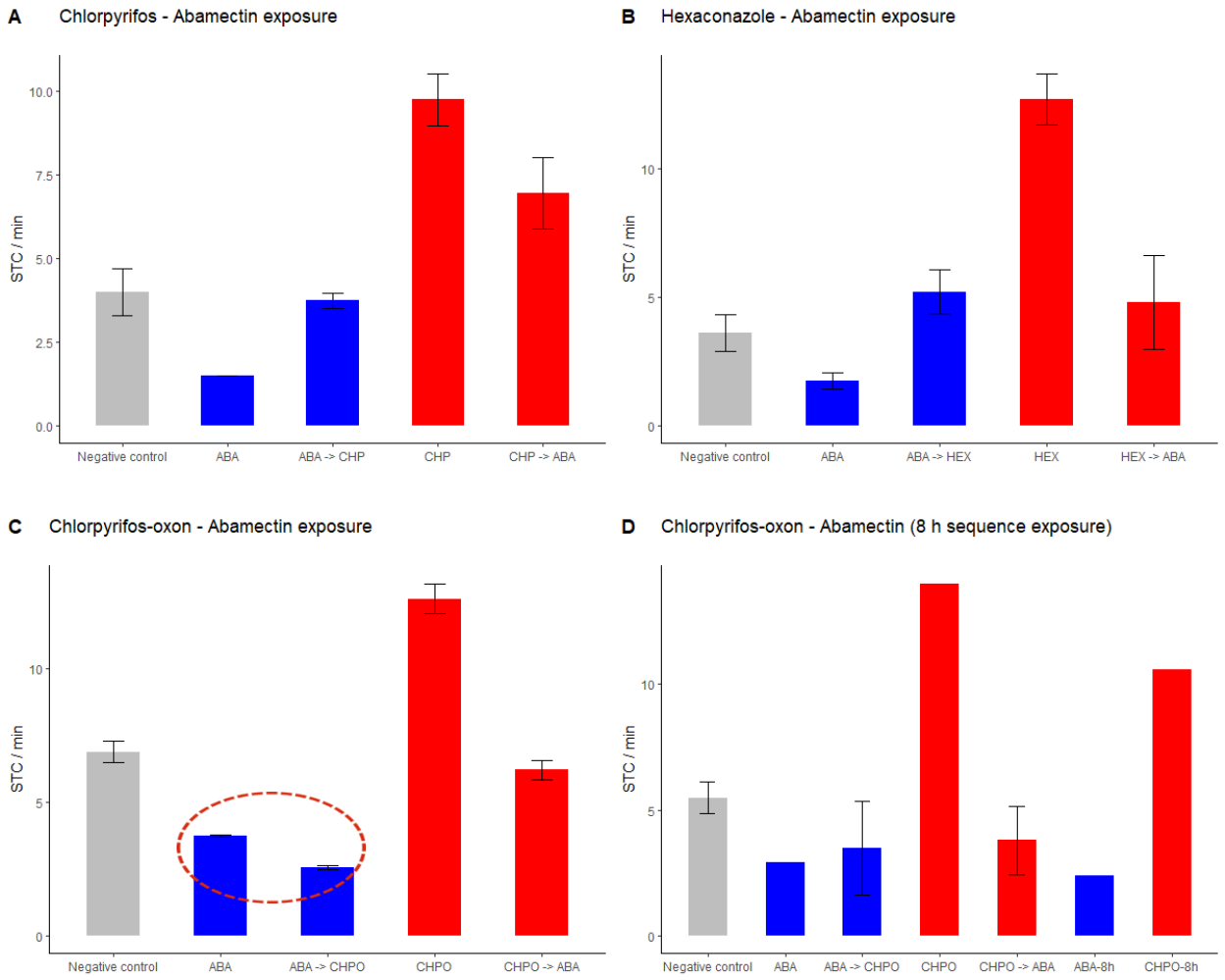

Figure 5.5: STC effect after sequential exposure. A) Chlorpyrifos (CHP) and Abamectin (ABA). B) Hexaconazole (HEX) and abamectin (ABA). C) Chlorpyrifos-oxon (CHPO) and Abamectin (ABA). D) Chlorpyrifos-oxon (CHPO) and Abamectin (ABA) after 8 h sequence exposure. ABA-8h and CHPO-8h represent the positive control for the 8 h exposure duration. The blue color indicates initial exposure to abamectin leading to hypoactivity; red color indicates initial exposure leading to hyperactivity; grey color indicates negative control for all experiments.

5.4 Discussion

In this paper we sought to understand the differences in the mechanism of hyperactivity action induced by chlorpyrifos and its oxon metabolite - chlorpyrifos-oxon, as well as how this difference may be mediated by potential limited biotransformation in early stages of the embryo. This investigation was conducted using the spontaneous tail coiling (STC) response of zebrafish embryos at 24 hpf. First, we measured the capacity of the organophosphates to inhibit acetylcholinesterase (AChE) at 24 hpf. Second,

we measured the internal concentration – time profiles of chlorpyrifos to understand the influence of biotransformation capacity on the mediation of the STC effect and AChE activity. Finally, we conducted sequential exposure experiments to evaluate whether the organophosphates have similar capacity to antagonize abamectin, an hypoactivity inducing substance.

Organophosphates inhibit AChE thereby leading to accumulation of acetylcholine (ACh) in the synaptic junction and overstimulation of the acetylcholine-gated sodium channels (Casida and Durkin 2013). This is assumed to be the basis of their hyperactivity response in the STC test. However, organophosphates such as chlorpyrifos require biotransformation for effective inhibition of AChE (Fukuto 1990). Moreover, early developmental stages (0-24 hpf) of zebrafish embryos may be limited in their biotransformation capacity due to lack of some biotransformation enzymes (Kühnert et al. 2017). This was indicated for certain chemicals due to observation of bell-shaped internal exposure profiles. It has yet not been shown whether this limited biotransformation also applies to organophosphates and if there are differences between different stages. However, differences in the hyperactivity effect concentration between the parent chlorpyrifos (1.85 µM) and its oxon-metabolite (0.32 µM) suggest that the biotransformation activity for organophosphates may be weak in embryonic stages (Reif et al. 2016; Ogungbemi et al. 2019, 2020). The observed hyperactivity of chlorpyrifos could be due to: 1) an intrinsic AChE inhibiting capacity of chlorpyrifos albeit weaker than its oxon metabolite; 2) a weaker biotransformation in early stages leading to lower internal concentration of the active metabolite in earlier stages and hence higher STC effect concentrations and 3) a different mechanism of action leading to hyperactivity effect (Figure 5.1). Therefore, we sought to understand which of these factors is mainly responsible for the hyperactivity induced by chlorpyrifos.

5.4.1 Chlorpyrifos does not inhibit AChE

To investigate the AChE inhibiting capacity of chlorpyrifos and chlorpyrifos-oxon, we conducted AChE inhibition experiments. Chlorpyrifos (up to 8 µM) could not inhibit AChE in 24 hpf embryos (Figure 5.3) but chlorpyrifos-oxon inhibited AChE (EC_{50} = 0.09 µM). This is consistent with other studies showing the ability and inability of chlorpyrifos-oxon and chlorpyrifos respectively to inhibit AChE (Jacobson et al. 2010; Yang et al. 2011; Yen et al. 2011). Yen et al. (2011) also found that chlorpyrifos (0.3 µM) significantly inhibited AChE at later stages of 72 and 96 hpf and this indicates possible bioactivation of chlorpyrifos at later stages. Therefore, a role of limited (i.e. no or very low) biotransformation of chlorpyrifos at earlier embryonic stages cannot be excluded (Yang et al. 2011).

5.4.2 Chlorpyrifos is likely not biotransformed in early stages of zebrafish embryos

Biotransformation processes transform a compound to a more soluble form for excretion. Despite making a compound water soluble for easy elimination, biotransformation may also lead to the formation of a more toxic compound (Schlenk et al. 2008). Biotransformation often reduces the concentration of a toxic substance by transforming it to more water soluble and potential less toxic compounds. Internal concentration – time profiles may help to understand biotransformation capacity of zebrafish embryos (Brox et al. 2016a). For example, Kühnert et al. (2013) showed that the inability of benz[a]anthracene to reach steady state and its declining concentration over time in the zebrafish embryos is likely attributed to biotransformation.

We measured internal concentration – time profiles of chlorpyrifos to investigate if the ability of chlorpyrifos to induce hyperactivity is based on the oxon-metabolite resulting from the biotransformation in the embryos. We show that maximum internal concentration was reached at 48 hpf without steady state and declined at higher developmental stages until a minimum concentration was reached at 118 hpf (Figure 5.4B). El-Amrani et al. (2012) showed similar results in which a steady state was not achieved after 48 h exposure in 72 hpf embryos. Lack of steady state for internal concentration of chlorpyrifos could be due to relatively higher log Kow (5.0) leading to slower uptake. Although we did not measure transformation products, the decline in internal concentration over time could be an indication of possible biotransformation at 72, 96 and 118 hpf. However, it is important to note that this decline could also be attributed to elimination of chlorpyrifos due to lack of stable exposure concentrations in our experiment. Nevertheless, a potential limited biotransformation at 24 hpf is plausible and supported by the lack of chlorpyrifos to inhibit AChE. Hence, the hyperactivity induced by chlorpyrifos in the STC test might not be due to an oxon-metabolite resulting from a biotransformation process.

5.4.3 Chlorpyrifos but not chlorpyrifos-oxon blocks hypoactivity action of abamectin

To further understand the mechanism of chlorpyrifos in the STC test, we investigated the last hypothesis that hyperactivity effect induced by chlorpyrifos is based on a different mechanism of action and not AChE inhibition. The STC response has been linked to the action of neurotransmitters on synaptic transmission (Behra et al. 2002; Thomas et al. 2009). Thus, a blockage of neurotransmission or receptors could help to understand the mechanism of hyperactivity action for chlorpyrifos. Abamectin acts by activating Gamma aminobutyric acid (GABA) gated chloride channels leading to inhibitory synapses and hence hypoactivity (Casida and Durkin 2013; Raftery and Volz 2015). Within a mixture, it is expected that abamectin and chlorpyrifos at equitoxic concentrations will antagonize each other leading to a reduced effect if compared to single exposures. However, higher mixture concentrations may also induce nonspecific hypoactivity, which could be due to axonal deformation and paralysis. To avoid such unspecific effects at high mixture concentrations, we conducted a sequential exposure of both chemicals at their EC_{50} values. Chlorpyrifos could antagonize abamectin but this was not the case for chlorpyrifos-oxon (Figure 5.5). The inability of chlorpyrifos-oxon to antagonize abamectin could be due to low accumulation since it has a relatively lower lipophilicity than chlorpyrifos. Hence, we repeated the experiment by increasing the exposure duration from 2 h to 8 h. Interestingly, the hyperactivity of chlorpyrifos-oxon (exposed for 8 h) was confirmed in the repeated test but not its antagonistic capacity (Figure 5.5D). This indicates that abamectin was still active (i.e. not fully depurated) to block the hyperactivity action of chlorpyrifos-oxon in the sequential exposure despite removing the embryos from abamectin solution.

Based on the assumption of converging signaling pathways and interdependent systems, which leads to addition of mixture effects, which was already shown in Chapter 4 for similar effect direction i.e. either hyper- or hypoactivity (Kortenkamp et al. 2009; Ogungbemi et al. 2021a), we expect that hyperactivity resulting from AChE inhibition (chlorpyrifos-oxon) should block or at least mitigate the inhibitory hypoactivity signal resulting from GABA receptor activation (abamectin). Lack of this occurrence suggests that antagonistic effects may only be driven when similar receptors or related mechanisms are involved (hypothesis for non-convergence of unrelated antagonistic mechanisms) [Figure 5.6A]. However, this hypothesis seems to not apply to hypoactivity antagonistic effect since abamectin could block the

hyperactivity induced by chlorpyrifos-oxon. An argument for the supposed antagonistic effect of abamectin against chlorpyrifos-oxon could be related to the quick depuration of chlorpyrifos-oxon leading to pure hypoactivity effect of abamectin rather than antagonism. Similar to our study, Raftery and Volz (2015) found that endosulfan and fipronil, which are GABA antagonists could block the action of abamectin but strychnine, a glycine receptor antagonist failed to block the action of abamectin. Corbel et al. (2006) also showed that atropine, an antagonist of muscarinic acetylcholine receptor (mAChR) [mAChR functions to modulate/reduce acetylcholine (ACh) concentration via negative feedback], blocked the action of propoxur (an AChE inhibitor) on mAChR to reduce ACh concentration via negative feedback. Despite primarily acting on different targets, atropine could antagonize propoxur due to a secondary effect of propoxur on mAChr (Figure 5.6B). The hypothesis we proposed above that unrelated antagonistic mechanisms may not converge appears to hold true in these studies and therefore corroborates the findings in the present study that the mechanism of hyperactivity action of chlorpyrifos might not be based on AChE inhibition. Our study was not based on molecular observations, therefore, further studies are required to make a conclusion on this hypothesis.

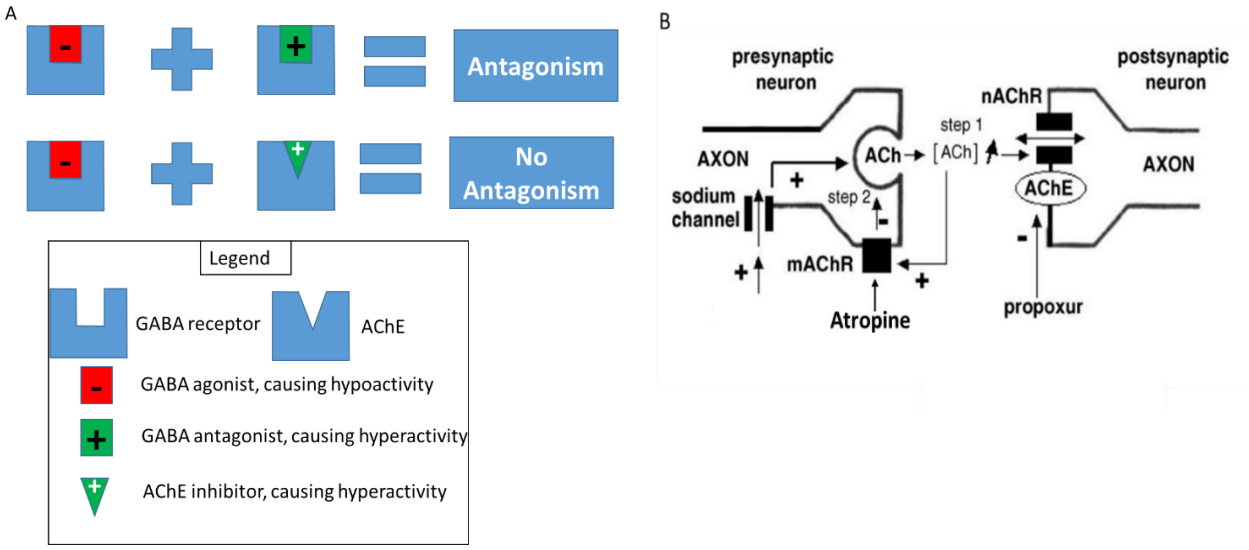

Figure 5.6: Schematic representation of the hypothesis for non-convergence of unrelated antagonistic mechanisms. A) A GABA receptor antagonist can antagonize the action of a GABA receptor agonist but an AChE inhibitor may not be able to antagonize a GABA receptor agonist due to unrelated receptor mechanisms. B) Atropine acts by directly blocking mAChR and this leads to an indirect blocking of the action of propoxur (an AChE inhibitor) to initiate negative feedback of ACh via the mAChR. Figure B was adapted/modified from Corbel et al (2006).

5.4.4 Potential interaction of chlorpyrifos with GABA receptors

To investigate if another mechanism of action (i.e. via GABA receptors) is responsible for the hyperactivity of chlorpyrifos in the STC test, we conducted sequential exposure of abamectin also with the fungicide hexaconazole, which is suspected to act via GABA receptor (Grandjean and Landrigan 2014; Ogungbemi

et al. 2020). Similar to chlorpyrifos, hexaconazole antagonized abamectin and did not inhibit AChE. It has been reported that endosulfan and fipronil, which are GABA antagonists, could block the action of abamectin in the STC test (Raftery and Volz 2015). Hence, it appears GABA antagonism pathway could be a potential pathway for the antagonistic action of chlorpyrifos and hexaconazole against abamectin. In line with this, pentylenetetrazole (PTZ), which is also a GABA antagonist has been shown to induce hyperactivity in other zebrafish behavior studies (Squires et al. 1984; Ellis and Soanes 2012; Afrikanova et al. 2013). Due to structural similarity of hexaconazole (triazole) and PTZ (tetrazole), the hyperactivity of hexaconazole in the STC test has been suggested to be via GABAr antagonization (Sabbah et al. 2012; Ogungbemi et al. 2020). Most importantly, chlorpyrifos has also been reported to mimic the anxiogenic action of PTZ in a rat discriminative test and this could be due to blockage of GABA (Sánchez-Amate et al. 2002). This is consistent with metabolomics studies in adult zebrafish where chlorpyrifos exposure induced lower concentrations of GABA neurotransmitters (Gómez-Canela et al. 2017). However, chlorpyrifos-oxon also reduced GABA concentrations, hence, measuring GABA concentrations may not distinguish between the mechanism of action of both compounds (Gómez-Canela et al. 2018). Similar to results obtained in the present study, albeit in pre-weaning developing stages of rats, chlorpyrifos did not inhibit AChE activity and was found to induce increased spontaneous motor activity and upregulated GABA receptor genes respectively (Gómez-Giménez et al. 2018; Perez-Fernandez et al. 2020). Nevertheless, our results in zebrafish embryos could be comparable with rat studies since zebrafish share common neurotransmitter systems with mammals (Rico et al. 2011; Horzmann and Freeman 2016). In-vitro studies have also shown that the dominant mechanism of action for chlorpyrifos could be inhibition of voltage gated calcium channels rather than AChE inhibition, which is dominant for chlorpyrifos-oxon (Meijer et al. 2014). Based on these findings which show similarity of action of both chlorpyrifos and hexaconazole to PTZ, as well as, findings from the present study showing the similar hyperactivity pattern for the 2 compounds, we therefore propose a GABA pathway for the hyperactivity induced by chlorpyrifos in 24 hpf embryos (Figure 5.7). However, further molecular studies are required to confirm the exact mechanism of action of chlorpyrifos, whether via GABA or others.

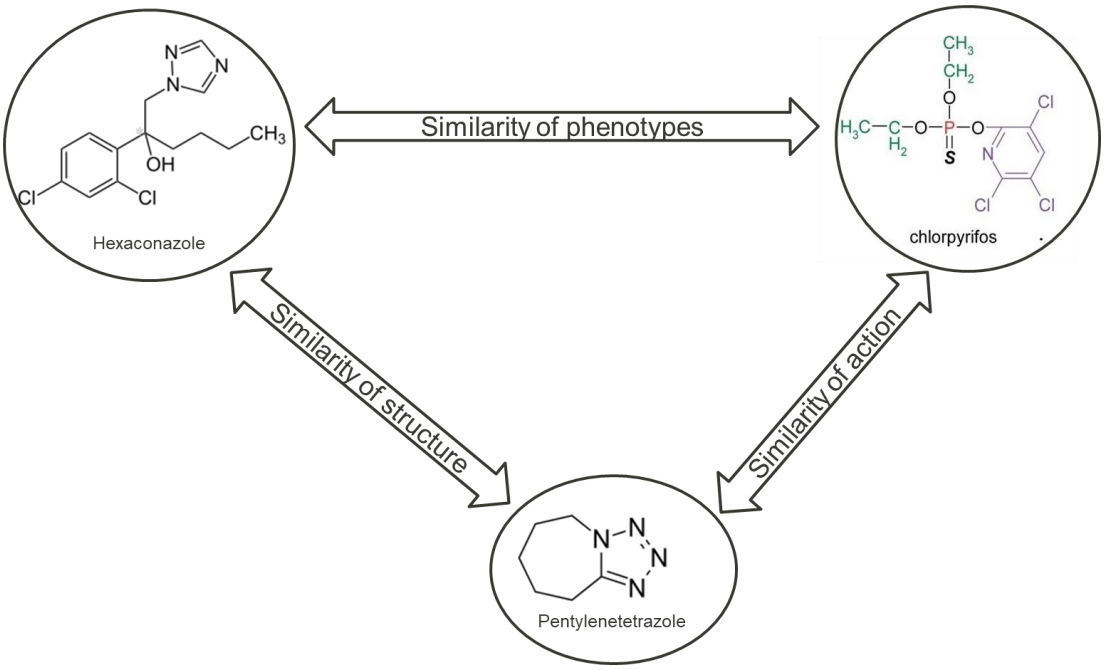

Figure 5.7: Schematic diagram showing the triangle of knowledge hypothesis for the mechanism of hyperactivity action of chlorpyrifos in the STC test. Chlorpyrifos and hexaconazole show similar phenotypes in the STC and AChE tests. Hexaconazole and PTZ have similar structures. Chlorpyrifos and PTZ show similarity of action in a rat study.

5.5 Conclusion

In conclusion, we showed that chlorpyrifos and its oxon-metabolite, chlorpyrifos-oxon, do not have similar mechanism of hyperactivity action in the STC test and this is probably due to a lack of bioactivation of chlorpyrifos to the more active oxon-metabolite leading to inability of chlorpyrifos to inhibit AChE. We supported these results by showing that chlorpyrifos but not chlorpyrifos-oxon could antagonize the hypoactivity induced by abamectin in the STC test. Finally, we showed that hexaconazole induces similar hyperactivity and AChE patterns as chlorpyrifos. Based on results in this study and previous studies, we suggest that 1) chlorpyrifos and hexaconazole may have the same mechanism of action due to similar behavior phenotypes in zebrafish embryos; 2) hexaconazole and PTZ may have similar mechanism of action due to similarity of structure and 3) chlorpyrifos and PTZ may have the same mechanism of action due to similarity of observed effects in rats (Figure 5.7). With this triangle of knowledge, we therefore propose the mechanism of action for chlorpyrifos to involve GABA or another pathway rather than AChE inhibition.

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Chapter 6: Application of zebrafish embryo behavior tests as effect based methods for toxicity assessment of a wastewater treatment plant effluent

Abstract

Wastewater Treatment Plants (WWTP) represent one of the major sources of anthropogenic contamination in the environment. Several studies demonstrate a negative impact of WWTP discharges on biodiversity and essential ecosystem functions. It is therefore necessary to evaluate the ecotoxicological impact of WWTP effluent, to identify the toxic components and thus to enable a prospective WWTP management.

Although chemical analyses techniques are commonly employed for environmental monitoring, combined effects of chemical mixtures are not considered and therefore a holistic approach to capture the undetected chemicals within a mixture is needed. Effect based methods (EBMs) can overcome and complement the limitations of chemical target monitoring and reveal information regarding the whole-mixture toxicity. In this context, zebrafish embryo test has been recommended as one of the EBM for acute toxicity testing of environmental samples such as WWTP effluent. Apart from the standard fish embryo test, behavioral assays can also be applied to evaluate ecological impact of pollutants and they have potential to be used to screen neurotoxicity. In particular, the spontaneous tail coiling (STC) and the locomotor response (LMR) tests are two commonly used zebrafish embryo behavior assays.

The aim of this study is to evaluate the use of STC and LMR tests as EBMs. The research goals include: 1) To compare the sensitivity of the short duration STC test to the long duration LMR test. 2) To use a biochemical assay such as acetylcholinesterase (AChE) inhibition to assess if the observed behavior effects are associated to neurotoxicity

The LMR test shows an increase in locomotor activity at relative enrichment factor (REF) of 1 and all concentrations did not inhibit AChE activity. On the other hand, the STC results show that the WWTP effluent at REF 7.5 and 10 caused a progressive hyperactivity in 24 hpf zebrafish embryos and AChE was inhibited at REF 10. Target chemical analysis was conducted for the WWTP effluent. Five compounds (organophosphates and carbamates) were selected as possible inhibitor of AChE and potential contributors to the observed behavior and AChE effects. This study shows the potential of zebrafish embryo behavior tests to be used as EBMs for detecting both acute toxicity and neurotoxicity.

6.1 Introduction

Wastewater Treatment Plant (WWTP) effluent represents a major source of chemical discharge into receiving water bodies (Prasse et al. 2015; Le et al. 2017). This could lead to pollution of surface and ground water leading to environmental and health risks (Malaj et al. 2014). Several toxic effects resulting from exposure of WWTP effluent extract to model organisms have been reported (Välitalo et al. 2017; Ribeiro et al. 2020).

The Water Framework Directive is an European legislation created to address pollution levels in surface waters including also discharges from WWTPs (EU 2000). This directive stipulates to monitor the concentrations of 45 priority compounds and benchmarking their measured concentration against an environmental quality standard. However, this monitoring system does not take into consideration the combined effect of these substances and others occurring at concentrations below detection limit (Altenburger et al. 2019). However, effects in the environment are propagated as combined effects of several substances. Therefore, the use of effect based methods (EBMs) with capacity to measure effect of whole environmental samples has been recommended as an additional tool to complement chemical measurement for adequate diagnostic risk assessment of water bodies (Wernersson et al. 2015; Brack et al. 2019).

However, the use of EBMs alone for monitoring and assessment of water bodies has its challenges because most EBMs have limited diagnostic capacity for identifying chemical groups. One proposed solution is to integrate EBMs with chemical measurements. Such approaches have been implemented in some studies using EBMs and chemical fractionation (Brack et al. 1999; Qu et al. 2011) or EBMs and chemical monitoring (Massei et al. 2019; Neale et al. 2020). An alternative solution is to develop a battery of EBMs with specific endpoints capable of identifying different chemical modes of action (Välitalo et al. 2017; De Baat et al. 2020). For example, in-vitro receptor assays have been recommended for endocrine disrupting effects and in-vivo zebrafish embryo assays for acute toxicity screening (Brack et al. 2019).

Despite neuroactive substances are frequently occurring in the environment, bioassays for neurotoxicity assessment are largely missing among recommended EBMs (Busch et al. 2016; Schmidt et al. 2017). The behavior of zebrafish embryos has been shown to be altered by neuroactive substances, however, some uncertainty exists regarding their sensitivity and specificity (Legradi et al. 2015; Leuthold et al. 2019; Ogungbemi et al. 2019). Nevertheless, behavior tests are useful as EBMs due to ease of measurement with automated techniques and potential capacity to capture diverse effects including both acute toxicity and neurotoxicity. In particular, the spontaneous tail coiling (STC) and the locomotor response (LMR) tests are two commonly used zebrafish embryo behavior assays. STC is measured in the early stages (19-28 hpf) and assumed to be induced by the innervation of the muscle through the synaptic neurotransmission in primary motor neurons. In contrast, LMR is measured in later stages (72-120 hpf) and induced by both primary and secondary motor neurons (Ogungbemi et al. 2019). Therefore, measurement of the STC or LMR could be good indicators of adverse effects in the nervous system and could potentially reveal effects in complex environmental mixtures.

The goals of this study are: 1) To compare the STC and the LMR test in order to evaluate their capacity as EBMs for neurotoxicity screening. 2) To use a biochemical assay i.e. acetylcholinesterase (AChE) inhibition

to assess if the observed behavior effects in STC and LMR tests are associated to neurotoxicity. The test sample was collected from a WWTP effluent next to the Mulde river, which has been reported to receive untreated chemical effluents and therefore contaminated with a diverse range of chemicals (Kuballa et al. 1995; Brack et al. 1999). We used a combination of chemical analysis and AChE inhibition test to identify specific risk drivers. We show the capacity of zebrafish embryo behavior tests to screen neurotoxicity alongside the standard fish embryo test for acute toxicity detection.

6.2 Materials and method

6.2.1 Sampling and sample preparation

A water sample was collected in February 2018 in front of the outlet of an industrial wastewater treatment plant (WWTP) discharging into the Mulde river (Bitterfeld, state of Saxony-Anhalt, Sachsen-Anhalt, Germany). Sampling was conducted using a large volume solid phase extraction (LV-SPE) as described in Schulze et al. (2017). Briefly, 100 liters of water were filtered on-site through a chromabond HRX, a hydrophobic polystyrene-divinylbenzene copolymer (Macherey-Nagel, Düren, Germany). After sampling, the LV-SPE device was brought back to the laboratory (UFZ, Leipzig, Germany) and the cartridge dried under nitrogen overnight. The day after, the sorbent was extracted using a mixture of LC-MS grade ethyl acetate (EtAc) and methanol (MeOH) (50:50 v/v). The sample was then concentrated to a final volume of 100 mL in order to achieve a relative enrichment factor (REF) of 1000 (Escher et al. 2014) and stored at -20 °C until further analyses.

6.2.2 Chemical analysis of the extracts

A list of 491 organic compounds likely to occur in the environment was selected for chemical analyses. Among these compounds, candidate AChE inhibitors were selected based on information retrieved from different sources. Extract and blank were analyzed by LC-HRMS using Thermo Ultimate 3000 LC system, coupled to a hybrid linear ion trap Orbitrap MS (LTQ Orbitrap XL, Thermo Scientific) conformed by Heated Electrospray Ionization (HESI). In order to maintain the same ratio of the LC mobile phase (70: 30, water: methanol), 60 µL of purified water (LC-MS grade) and 30 µL of methanol (LC-MS grade) were added to 100 µL aliquots of the extracts. Additionally, 10 µL of reference 19 isotope-labeled internal standards were added for matrix effect correction (10 ng/mL). The final relative enrichment factor (REF) before injection was 500.

The injected volume was 10 µL and the column used was a Phenomenex Kinetex™ (C18, Core Shell, TMS, pore size 100 Å, 2.6 u) running under a temperature of 40 °C. The mobile phase comprised of water and methanol, at a flow rate of 300 µL/min. Analyses were conducted in separate positive and negative mode runs using ESI combining a full scan experiment (100 - 1000 m/z) at a nominal resolving power of 70000 (referenced to m/z 200) and data-independent MS/MS experiments (DIA) at a nominal resolving power of 35000. Compounds were quantified using a method matched calibration with 11 points within a range from 0.1 to 1000 ng/L using the software TraceFinder (version 3.2). Method detection limits (MDLs) were determined based on a replicate analysis of calibration standards based.

6.2.3 Zebrafish behavior tests

The fish (UFZ-OBI strain) was originally established from a wild type strain purchased from a local supplier (OBI hardware store, Leipzig) and maintained at the UFZ for more than 13 generations. Adult fish was cultured under 14 h light/10 h dark photoperiod in 120 L aquaria (tap water, 26.5 ± 1 °C) and feeding was conducted twice a day either with commercial dry food flakes or Artemia sp. To initiate spawning, spawning trays with artificial plants were inserted in the afternoon 4–6 h before the end of the light cycle. Eggs were collected 1 hour after the onset of the light cycle on the next day and cleaned thoroughly by rinsing with ambient tank water and transferred to exposure medium. Fertilized and normal embryos (2hpf) were selected with a microscope.

The WWTP extract concentrated to a relative enrichment factor (REF) of REF 1000 was diluted to give lower concentrations of REF 10, 7.5, 5, 2.5 and 1, which were used in the behavior testing. Behavioral testing was performed based on a prior lethal concentration (LC_{10}) assessment, which was used as the highest concentration (REF 10). Two commonly used behavior tests, spontaneous tail coiling (STC) and locomotor response (LMR) tests were used. For the evaluation of STC, twenty fertilized embryos were exposed in 20 mL solution of the respective REF concentrations and a negative control. The exposed embryos were incubated at 28 °C for 21 ± 1 h. On the next day, at 24 ± 0.5 hpf, exposed embryos were videotaped for 1 min. Malformed and dead embryos were removed and not included in the videotaping. Collected videos were analyzed for STC counts by means of a workflow using the KNIME® Analytical Platform (Ogungbemi et al. 2021). For the evaluation of LMR, after 96 h exposure, 16 embryos were transferred to a 96 well plate (one embryo per well) in 500 µL of the respective REF concentration and negative control. Embryos were acclimatized for 10 min and LMR was measured for 40 min at a light/dark regime of 10 min dark, 20 min light and 20 min dark. Video tracking was done using the ZebraBox video tracking system (Viewpoint, Lyon, France) at a temperature of 28 ± 1 °C. Both STC and LMR experiments were performed in triplicates.

6.2.4 AChE measurement

AChE measurement was performed according to Küster, (2005) with few modifications. After STC and LMR measurement, embryos were transferred into a 2 mL Eppendorf tube and washed twice with Milli-Q water. Phosphate buffer (20 µL per embryo, pH 7.5) and glass beads were added. Embryos were homogenized using a FastPrep (FastPrep-24 5G, MP Biomedicals, 6.5 U×S-1, 3×35 s) and centrifuged at 4 °C for 30min at 13,000 g. The supernatant was collected and stored at −20 °C until analysis. AChE activity was determined by pipetting 50 µL of sample in quadruplicate into 96-well plate. 50 µL and 100 µL of phosphate buffer and 1mM DTMB were further added. After a 10 min incubation in darkness, 100 µL of 1.35 mM acetylthiocholine iodide was added and the samples were measured at 412 nm in a photometer (SpectraMax 250 Photometer, Molecular Devices, USA).

6.2.5 Data analysis

STC was expressed as the number of STCs per minute (frequency) for one embryo. The mean STC frequency was estimated for a group of 20 embryos that were subject to the same treatment. LMR was obtained as total distance moved per 40 min for one embryo. Mean distance moved was estimated for 16

embryos exposed individually per well. The mean distance moved was normalized to the negative control to obtain a normalized LMR effect. Hypothesis testing was used to check for differences in the effect induced by different concentrations. Shapiro and Bartlett tests were used to check for normality and homogeneity of variance, respectively. Analysis of variance or Friedman test were used to test for statistical differences. Bonferroni adjusted Wilcoxon signed-rank test was used to further find differences in effect between concentrations. Statistical difference was considered when the p-value < 0.05.

6.3 Results

6.3.1 Behavior and AChE inhibition tests for WWTP effluent extract

To evaluate the utility of zebrafish behavior assays for testing WWTP effluent, we conducted the spontaneous tail coiling (STC) and locomotor response (LMR) tests. The results show that the WWTP effluent at relative enrichment factor (REF) of 1 induced hyperactivity in the LMR test while REF 7.5 and 10 induced hyperactivity effect in the STC test (Figure 6.1). To investigate if the hyperactivity was driven by AChE inhibition, AChE activity of the embryos exposed to the extract was measured after STC (24 hpf) and LMR (96 hpf). AChE activity of embryos after LMR test at 96 hpf was not affected. In contrast, after 24 hpf, at the stage of STC analysis, the highest concentration (REF 10) inhibited AChE activity (Figure 6.2).

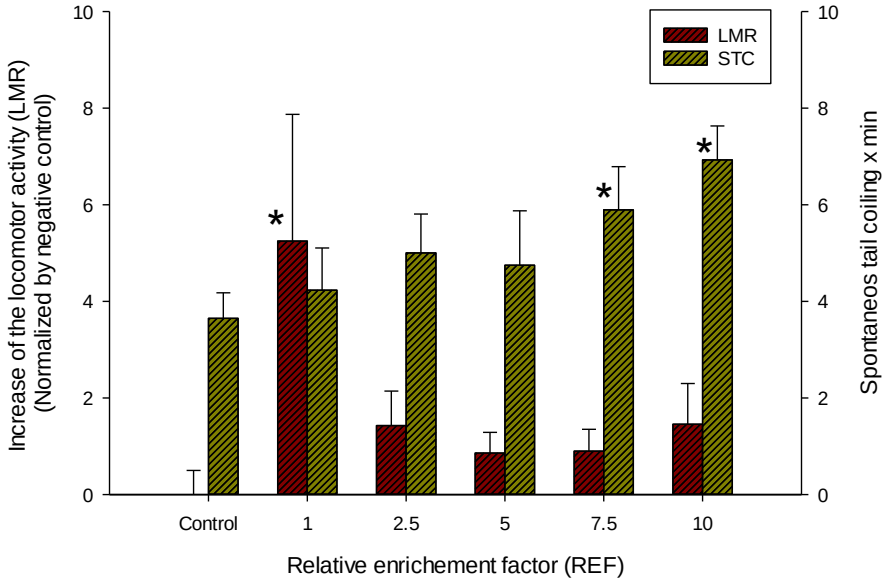

Figure 6.1: STC and LMR effect in 24 hpf and 96 hpf zebrafish embryos respectively. Only data for LMR (red bars) was normalized by the negative control

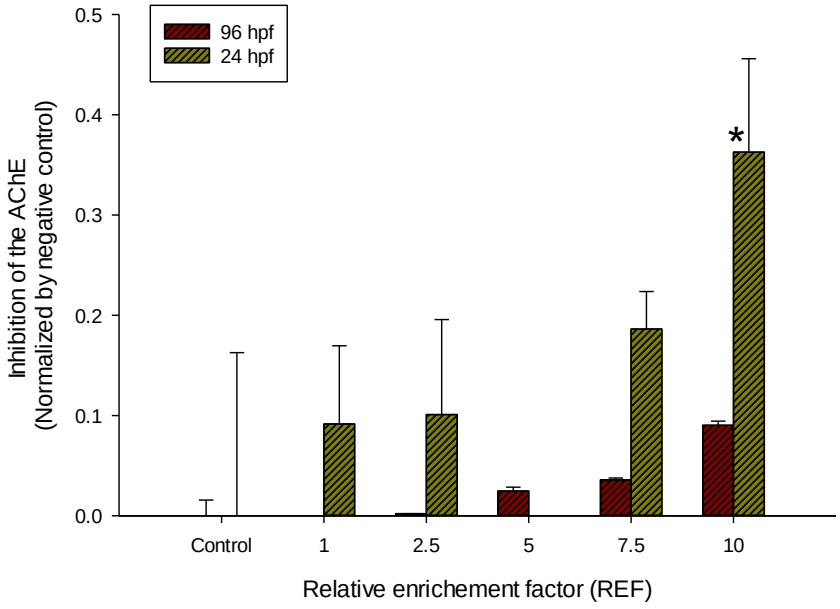

Figure 6.2: Inhibition of AChE activity of embryos exposed to WWTP effluent extract in the STC and LMR tests at 24 and 96 hpf respectively. Data for AChE inhibition at both 24 and 96 hpf were normalized by negative control.

6.3.2 Behavior test for single compounds

To further diagnose the WWTP effluent extract for possible effect drivers of the observed behavior and AChE inhibiting effects, organophosphate and carbamate compounds (i.e. potential AChE inhibitors) detected during chemical analysis were selected for further testing in the STC test. Out of the 126 organic micropollutants detected in the WWTP effluent extract, five substances were selected as potential AChE inhibitors (carbendazim, dimethoate, propamocarb, triethylphosphate and diphenylphosphate). An overview on the chemical concentrations are given in Table 6.1. More information about detected chemicals and further analysis can be found in Massei et al. (in preparation). Three tested chemicals, propamocarb, triethylphosphate and diphenylphosphate did not induce any STC effect up to 1mg/L (Figure 6.3). Carbendazim induced hyperactivity at 0.63 mg/L but 25 % mortality was also recorded at this concentration. Dimethoate showed a trend of hyperactivity at 0.33 mg/L but no effect was observed at higher concentration of 1 mg/L (Figure 6.3).

Table 6.1: Concentrations of the suspected AChE inhibitors detected in the WWTP effluent extract

Compound	Concentration (ng/L)	Chemical/Usage Class	Reference for AChE inhibition
Carbendazim	140	Benzimidazole-carbamate fungicide	Andrade et al. (2016)*; Janakidevi et al. (2013)[ǂ]
Dimethoate	992	Organophosphate Insecticide	De Mel and Pathiratne, (2005)[#]
Propamocarb	114	Carbamate Fungicide	Liu et al (2020)*
Triethylphosphate	45989	Organophospate flame retardant	Suspected as weak AChE inhibitor based on organophosphate chemical structure
Diphenylphosphate	155	Organophosphate flame retardant	Suspected as weak AChE inhibitor based on organophosphate chemical structure

**study with zebrafish larvae [#]study with fish species [ǂ]study with other aquatic species*

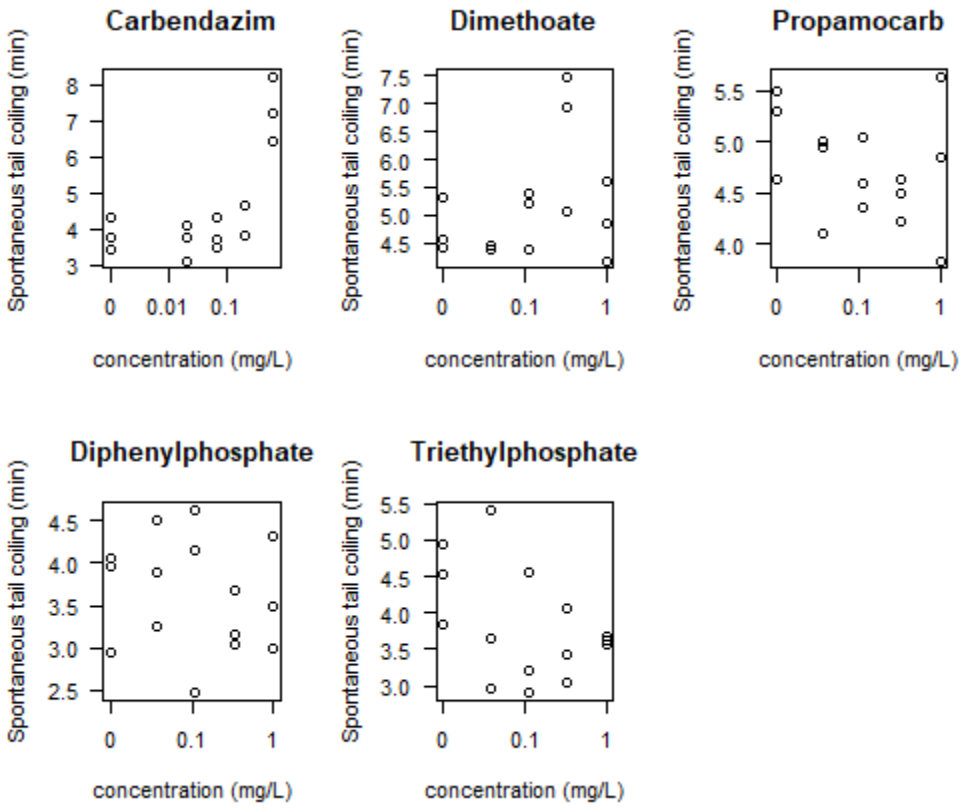

Figure 6.3: Scatterplot for STC effect of the suspected AChE inhibitors detected in the WWTP effluent extract. One data point shows the mean of 20 embryos for one replicate.

6.4 Discussion

Effect based methods (EBMs) are recommended to complement chemical analysis in environmental monitoring. Diverse batteries of EBMs have been recommended to detect specific and acute effects (Brack et al. 2019). There is still a need to develop sensitive, specific, high-throughput EBMs capable to detect a diverse range of effects. In this study, we evaluated the use of 2 commonly used zebrafish embryo behavior tests for screening both acute toxicity and neurotoxicity in environmental samples.

6.4.1 Comparison of STC and LMR in relation to AChE inhibition

In the current study, we found that the WWTP effluent induced hyperactivity effect at a relative enrichment factor (REF) of 1 in the LMR test after an exposure of 96h. Similar hyperactivity effect was found in the STC test at higher concentrations of REF 7.5 and 10 but shorter exposure duration of 24 hours. This suggests an impact on the nervous system since hyperactivity is considered as a specific effect and unspecific secondary effects caused by cytotoxicity and/or malformation may rather result in hypoactivity (Ogungbemi et al. 2020). Other studies have reported neurotoxic effects induced by WWTP effluents. For example, Ribeiro et al., (2020) found hyperactivity in the STC test for WWTP effluent from Brazil and Massei et al., (2019) reported effects on LMR for sediments collected from sites with input of WWTP effluent.

Acetylcholinesterase (AChE) inhibition test is a widely accepted biomarker for neurotoxicity, and substances which inhibit AChE are expected to disturb neurotransmission possibly leading to hyperactivity (Walker 1995; Küster 2005). Therefore, we measured AChE activity in embryos exposed to WWTP effluent extract to investigate if the hyperactivity observed in the STC and LMR tests is related to neurotoxicity via AChE inhibition. Interestingly, REF 10 which induced hyperactivity in the STC test also inhibited AChE at 24 hpf while no AChE inhibition effect was induced following the LMR test at 96 hpf. Different pattern of AChE inhibition for STC and LMR could be related to the differential development stage. For instance, the availability of more receptor targets (neuro or non-neuro receptors) in the advanced development stage of 96 hpf could increase the competition for binding the AChE inhibitors, thus leading to a lower enzymatic AChE inhibition at 96 hpf. An alternative argument for the differential AChE inhibition pattern at 24 and 96 hpf could be metabolic degradation of the substances at 96 hpf, which might be limited in early stages of the embryo (Yang et al. 2011; Kühnert et al. 2017). Moreover, the consistency in results between STC and AChE tests indicates the proximity of the STC test to molecular events in neuronal synapse and this could be due to the short exposure duration and endpoint specificity of the STC test.

6.4.2 Hyperactivity effect could be related to presence of AChE inhibiting substances

Organophosphate and carbamate compounds are known to be chemical groups with capacity to inhibit AChE (Casida and Durkin 2013). Inhibition of AChE causes the accumulation of acetylcholine in the neuronal synapses leading to prolonged activation potential and therefore possibly hyperactivity behavior. Three organophosphates (dimethoate, triethylphosphate and diphenylphosphate) and two carbamates (carbendazim and propamoarb) were detected in the WWTP effluent extract and suspected to be drivers of the hyperactivity effect in the STC as well as the corresponding AChE inhibition at 24 hpf. Only carbendazim and dimethoate induced hyperactivity effect in the STC when exposed individually to

the embryos (Figure 6.3). Consistent with our result, carbendazim induced increased swimming distance and time of zebrafish embryos in the LMR test but only enhanced AChE activity (Andrade et al. 2016). In contrast, carbendazim was found to inhibit AChE in juvenile African catfish (Ezeoyili et al. 2019). Similarly, dimethoate inhibited AChE in fish (Frasco and Guilhermino 2002; De Mel and Pathiratne 2005). These reports of the AChE inhibiting capacity of dimethoate and carbendazim supports our hypothesis that they may play a role in the hyperactivity found in the current study. However, we cannot exclude that these AChE inhibiting substances may not play a role in the observed hyperactivity. For instance, early stages of zebrafish embryo may have limited biotransformation capacity (Yang et al. 2011; Kühnert et al. 2017) and therefore organophosphates such as dimethoate, which require biotransformation to efficiently inhibit AChE may not be responsible for the AChE inhibition found in embryos at an early stage of 24 hpf.

Triethylphosphate, diphenylphosphate and propamocarb did not induce STC effect up to a limit concentration of 1mg/L. The limit concentration was imposed to avoid exceeding a maximum solvent concentration of 0.1%. Consequently, it is likely that the effective concentration of these chemicals was not reached in the short duration STC test. For instance, 1 mg/L propamocarb caused increased LMR and inhibited AChE in zebrafish larvae after a long exposure duration of 7 days (Liu et al. 2020). Based on the principle of something from nothing (Silva et al. 2002), it is possible that these suspected AChE inhibitors are existing in the WWTP effluent extract below their effective concentrations along with other non-detected chemicals. This may, however, lead to combined AChE inhibition and hyperactivity effects as a result of mixture exposure. Further, Organophosphate esters such as triethylphosphate and diphenylphosphate have been found to interfere with thyroid hormone and such endocrine disturbance could affect the nervous system leading to behavior defects (Hill et al. 2018; Walter et al. 2019). Therefore, it is also possible that hormonal disruption contributed to the combined hyperactivity effects of the WWTP effluent sample.

6.5 Conclusion

Effect based methods are proposed as an early detection tool and to capture mixture effects, which cannot be detected by chemical monitoring. We show in the current study that behavior tests of zebrafish embryos could detect acute effects and potential neurotoxicity induced by the WWTP effluent extract. Based on the differences in the effect pattern observed at 24 and 96 hpf, we therefore encourage the use of both LMR and STC to capture variations of effects. Nevertheless, the STC test may be used for sample prioritization and identification of effect drivers due to its short exposure duration, fast assessment, endpoint specificity and linkage to molecular effects i.e. AChE inhibition. Additionally, we suspect that the AChE inhibiting substances in the extract are potential drivers of the hyperactivity effect. Nevertheless, other tests e.g. molecular biology or biochemical tests are required to further confirm the effects observed since the effect could also be related to other mechanisms arising from the complex environmental mixture.

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Chapter 7: General discussion and future perspective

In this thesis, the focal question was whether it is possible to screen neuroactive substances using the behavior response of zebrafish embryos, with a particular focus on the spontaneous tail coiling (STC) assay. This question led to further sub questions: (1) How does experimental parameters affect zebrafish behavior and which behavior test can be selected for further testing (**Chapter 2**); (2) can the selected spontaneous tail coiling (STC) test efficiently screen a group of neuroactive substances with diverse mechanisms of action (**Chapter 3**); (3) can the concentration addition and independent action mixture models be used to predict mixture toxicity of neuroactive substances in the STC test (**Chapter 4);** (4) does potential limited biotransformation in early stages of the embryos influence the toxicity and mechanism of action of compounds such as organophosphates which require bioactivation (**Chapter 5**); (5) can the STC test be used as an effect based method to monitor and screen neurotoxic effects in environmental samples (Chapter 6).

7.1 Main outcomes and discussion

7.1.1 Exposure concentration and duration mainly influences the outcome of behavior tests

The literature review conducted in **Chapter 2** showed that exposure duration, exposure concentration, endpoint parameter and developmental stage were the most influential parameters affecting the outcome and interpretation of behavior tests. Discussion on standardizing or harmonizing these parameters has been on-going in the literature (Gerhardt 2007; Legradi et al. 2015). Recently, a collaborative trial on neurotoxicity was launched to evaluate the applicability of different zebrafish embryo behavioral tests to a specific set of water pollutants (Legradi et al. 2021). The 6 laboratories that participated in the trial used different behavioral tests and experimental parameters. Although the results were consistent to an extent, it is clear that a standardized protocol and experimental parameters would enhance the comparability and reproducibility of behavior testing for regulatory purposes (Ford et al. 2021).

Another goal of the literature review in **Chapter 2** was to understand the linkage between neuroactive mechanisms of action and behavioral response i.e. whether hypo- or hyperactivity is consistent with the expected mechanism of action of a chemical. The data showed only 18 and 62% consistency for organophosphates and anticonvulsants respectively, which are the largest group of chemicals evaluated in the study. The low consistency was attributed to experimental limitations. For example, the expected hyperactivity phenotype for organophosphates was not largely realized in the locomotor response test (LMR) due to long exposure duration (0-96 hpf) while the short duration spontaneous tail coiling test (STC) could detect this expected hyperactivity. However, the LMR could also reveal the expected hyperactivity of organophosphates if shorter exposure durations or lower exposure concentrations are used (Leuthold et al. 2019). This is because longer durations or higher concentrations could induce alterations at the sub-organism level (i.e. non-visible axonal malformations) leading to paralysis or weaker movements (**Chapter 2**)

7.1.2 STC is capable of screening neuroactive substances

Recent studies have shown the capacity of the STC test to screen neurotoxicity (Selderslaghs et al. 2010; Watson et al. 2014; Raftery et al. 2014; Vliet et al. 2017; Weichert et al. 2017). Therefore, the STC test was selected for further optimization and used during the course of this thesis work. However, some concerns were raised about its capacity to discriminate neuroactive modes of action (Vliet et al. 2017). According to the conclusion in **Chapter 2**, different experimental parameters can influence the behavior outcome and the capacity of the STC test to distinguish modes of action. For example, the expected effect of nicotine was not observed in Raftery et al. (2014) probably due to short video acquisition duration of 6 s and the anticipated hyperactivity of paraoxon was also not realized, probably due to the use of a different STC endpoint **(Chapter 2**). Additionally, the inability of the STC to distinguish modes of action in Vliet et al. (2017) could be due to the use of single exposure concentrations. Thus, **Chapter 3** was focused on optimizing important STC parameters in order to improve its capacity to discriminate modes of action. Developmental stage and video acquisition duration were found to be influential and 24–25 hpf and 1 min were selected as optimal for these parameters respectively. An optimized developmental stage allows the STC measurement to be performed at the most sensitive stage in which the STC is most detectable. Use of short analysis duration of 10 s and below could lead to loss of STC information and hence a biased outcome. Other parameters such as acclimation duration, sample size and rearing conditions were not influential. The optimized STC test was able to detect 8 out of 11 neuroactive substances and could also detect effects for substances with unknown neuroactive mechanism of action. Two of the neuroactive substances detected in this study, chlorpyrifos and nicotine, are already classified as reference compounds for developmental neurotoxicity (Aschner et al. 2017). This shows the capacity of the STC test to screen neuroactive substances when experimental parameters are properly controlled.

Another goal of **Chapter 3** was to develop a novel image analysis workflow to evaluate the STC video files using the KNIME software. This KNIME workflow and the optimized STC protocol (Published as a method in Ogungbemi et al. 2021, Appendix 3) is one of the few STC analysis freeware available alongside a MATLAB® tool (González-Fraga et al. 2019) and an Image J macro (Zhang et al. 2021). Despite the tremendous utility of the STC protocol developed in this thesis, the video acquisition process, which involves individual video recordings of single glass vials could limit the through-put. However, works are currently ongoing to further develop the STC protocol to be amenable to 96-well plates.

7.1.3 Concentration addition and even Independent action could predict mixture effects of neuroactive substances

Neuroactive substances are frequently occurring in the environment and organisms are usually exposed to a combination of these chemicals. However, risk assessment is usually conducted for single chemicals and it is largely unknown how mixtures of neuroactive substances interact to alter behavior (Legradi et al. 2021). It was already established in **Chapter 3** that neuroactive substances could induce similar STC effect direction (hyper or hypoactivity) despite having different mechanisms of action. Therefore, the goal of **Chapter 4** was to investigate the predictability of neuroactive mixture effects as regarding similarity/dissimilarity of mechanisms of action. Neuroactive substances with different mechanisms of action but similar effect direction combined in an additive way and mixture effects were predictable using both concentration addition and independent action models. In contrast, substances with different

mechanisms of action and opposing effect direction were antagonistic. The results in **Chapter 4** indicate that classifying the similarity/dissimilarity of chemicals based on both mechanism of action and effect direction (hyper or hypoactivity) instead of only mechanism of action proved to be reliable to predict and understand mixture outcomes. Hence, the assessment of similarity/dissimilarity of mixture components should go beyond surface knowledge of mechanism of action and should consider other factors such as toxicological response and toxicity pathway (Cedergreen et al. 2008).

7.1.4 Limited biotransformation in zebrafish embryos may influence the toxicity pathway

A potential limited biotransformation capacity in early stages of zebrafish embryos have been reported (Yang et al. 2011; Kühnert et al. 2013, 2017). Although the strength and utility of the STC test for neurotoxicity screening has been shown in preceding chapters, it is also necessary to evaluate its weakness regarding potential limited biotransformation. Biotransformation is particularly relevant for the assessment of neuroactive substances such as organophosphates, which require biotransformation to the oxon-metabolite in order to effectively inhibit acetylcholinesterase enzymes. The goal of **Chapter 5** was to investigate the influence of biotransformation on the mechanism of action of the organophosphate, chlorpyrifos. The results show that chlorpyrifos and its oxon-metabolite (chlorpyrifos-oxon) may not have similar mechanism of hyperactivity action in the STC test mainly due to possible lack of biotransformation of chlorpyrifos in 24 hpf embryos. Thus, leading to inability of chlorpyrifos to inhibit AChE like chlorpyrifos-oxon. A further probe on the mechanism of action for chlorpyrifos showed that chlorpyrifos but not chlorpyrifos-oxon could antagonize the hypoactivity induced by abamectin in the STC test. Therefore, another mechanism (e.g. GABA receptor pathway) was suggested as a possible mechanism of action for chlorpyrifos rather than AChE inhibition. The implication of this finding is that the STC test may not be capable of screening neuroactive substances, which require biotransformation and has no secondary mechanism of action like chlorpyrifos. This could perhaps limit the application domain of the STC test but its strength can be utilized within a battery of other behavior tests.

7.1.5 STC can be used as an effect based tool for monitoring neurotoxic effects

Effect based methods (EBMs) have been recently proposed as a complimentary tool to support chemical analysis for water quality monitoring (Brack et al. 2019). Despite neuroactive substances are largely occurring in surface waters, adequate EBMs targeted at neurotoxic effects are missing (Busch et al. 2016; Schmidt et al. 2017; Legradi et al. 2018). One of the goals of this thesis work is to develop a testing method that can also be used as an EBM to screen neurotoxic effects. The optimization and validation of the STC test for neurotoxicity testing was discussed in **Chapters 3, 4 and 5**. Therefore, the effort in **Chapter 6** was focused on assessing the capacity of the STC test as an EBM to screen neurotoxicity and to identify effect drivers based on hyper and hypoactivity response. The STC test detected hyperactivity for a wastewater effluent and a subsequent acetylcholinesterase (AChE) inhibition effect of the effluent sample was confirmed. Therefore, AChE inhibitors such as organophosphates and carbamates were suspected to be the drivers of the observed hyperactivity in the STC test. The STC test has great potential to be used for both acute toxicity and neurotoxicity screening, as well as, identification of effect drivers due to its short exposure duration, fast assessment, endpoint specificity and linkage to molecular events. Nevertheless, it is important to note that behavior tests may not realize their full potential as EBMs for neurotoxicity

(especially for identification of neurotoxic effect drivers) if molecular tests are not included in the toolbox. In any case, behavior testing could be used as reliable EBMs for acute toxicity screening and prioritization of samples.

7.2 Future perspectives

7.2.1 Behavior testing and mechanism of action investigation

One major goal of this thesis work was to correlate behavior effect patterns to neuroactive mechanisms of action. This is anticipated to enhance the use of behavior testing for identifying effect drivers as well as predicting chemical hazard. For example, hyper- or hypoactivity response of zebrafish was linked to the expected mechanisms of action in **Chapter 2**; hyper- or hypoactivity response in the STC test was linked to the innervation of the muscle by the motor neurons (in neuromuscular junctions) in **Chapter 3**; mixture effects were elucidated based on hyper and hypoactivity response as well as mechanism of action in **Chapter 4**; and potential effect drivers in a wastewater effluent were identified using hyper or hypoactivity response in **Chapter 6**. Surprisingly, many previous behavior studies only considered general behavior effect and only few studies focused on linking behavior to mechanisms of neurotoxic action (e.g. Drummond et al. 1986; Kokel et al. 2010; Raftery and Volz 2015; Leuthold et al. 2019; Myers-Turnbull et al. 2020). Moreover, it appears efforts are in the direction of expanding the number of endpoints that can be obtained from a test (i.e. distance, speed, duration). Indeed, increasing number of endpoints may enable to derive patterns to classify substances into certain mechanisms but a mechanistic understanding of how a substance may act to induce a certain behavior effect is also very relevant. For behavior testing in zebrafish to contribute to the 3R (replacement, reduction and refinement) of animal testing, it is necessary to improve the mechanistic understanding of behavior tests and to link responses to specific parts of the nervous system, at least for a high proof of evidence (Legradi et al. 2015; Ford et al. 2021). This is certainly a way to enhance the use of zebrafish behavior testing for regulatory purposes and drug development. Hence, future development of behavior tests should include mechanistic understanding of endpoints for discriminating mechanisms of action alongside measuring behavior effects.

7.2.2 Behavior effects in ecological risk assessment

It is known from the literature that behavior endpoints are often more sensitive than lethality i.e. effects may occur at lower concentrations in behavior tests (Gerhardt 2007; Robinson 2009; Klüver et al. 2015). Even though exceptions have been reported (i.e. Leuthold et al. 2019), there is still a general conclusion that behavior testing is more sensitive or similarly sensitive relative to lethality. Nevertheless, behavior endpoints are not commonly used in risk assessment. Apart from this possible greater sensitivity than lethality, behavior endpoints are also relevant for ecological effects such as population fitness. Only recently has behavior endpoints gained increased attention for neurotoxicity screening but not for general or acute effect assessment. This raises a question: if behavior endpoints could also be used to assess sublethal effects within an ecological risk assessment framework since they seem to provide more toxicological information and require similar time and resource costs in comparison to lethality. It is acknowledged, however, that the parameters and protocols for behavior testing are currently not standardized and this could lead to lack of reproducibility and misinterpretation of results. Calls for

harmonization of protocols within the scientific community are currently gaining momentum and would definitely enhance the regulatory acceptability of behavior testing. A recent review of behavior ecotoxicology by Ford et al. (2021) also gave the following recommendations to improve the use of behavior testing in a regulatory context: "1) Improve the mechanistic understanding of contaminant-Induced behavioral alterations; 2) develop new and adapt existing standard toxicity tests to include behavior; 3) develop an integrative approach to environmental risk assessment, which includes behavior; 4) improve the reliability and reproducibility of behavioral end points; 5) develop guidance and training on the evaluation and reporting of behavioral studies; 6) better integrate human and wildlife behavioral toxicology"

7.2.3 Use of STC in mixture toxicity assessment

Chemicals occur as mixtures in the environment and it is logical to evaluate the risk of chemical mixtures rather than single chemicals as it is currently done. However, the challenge is that it is almost impossible to test all the possible variations of chemical combination as they would occur in the environment. In this regard, mixture prediction using classical models of concentration addition and independent action have been in continuous development for the last decade and could be close to being implemented in regulation (More et al. 2019). One focal point of this thesis was to gain a better understanding of how neuroactive substances combine to induce joint effect. This would allow a better implementation of the component based and whole mixture approaches for prospective and diagnostic neurotoxic hazard assessment respectively. The study in **Chapter 4** revealed that not only the mechanism of action information but also the toxicological response (i.e. hyper- and hypoactivity) is important to adequately predict mixture neurotoxicity in the STC test. This finding is in line with the recommendation by Kortenkamp et al. (2009) that chemical grouping for mixture analysis should be based on common adverse outcomes (hyper and hypo-activity in this case) with less emphasis on similarity of mechanisms. Information on common adverse outcomes will be very useful to predict mixture effects of complex mixtures and to understand deviations. For instance, the resulting effect of a mixture with both hyper and hypoactivity inducing components would be unexplainable if classification based on only mechanism of action was used rather than a combination of mechanisms and adverse effect. It can be argued that adverse effect grouping using the STC test is logical and must be done since the STC effect is bi-directional. Nevertheless, this concept is also relevant for other behavior or neurotoxicity endpoints (e.g. locomotor activity) in which reduced movement is the dominant response even for chemicals expected to induce hyperactivity. This dominant hypoactivity is usually a result of long-term exposure to high concentrations. Combined exposure occurring at low concentrations may directly reveal the expected hyper or hypoactivity and influence the mixture outcome. Even though adverse effect knowledge of single substances shows huge promise for understanding mixture toxicity, acquiring such data for different biological systems and endpoints would be highly demanding. Nevertheless, the point is not to acquire such enormous data but to develop working assumptions/hypotheses of the adverse effect of neuroactive substances based on their mechanism of action and literature data, which could then be used to interpret deviations from concentration addition and independent action models.

7.2.4 Use of STC as effect based method

The perspective on common adverse effects as described above also applies to the whole mixture approach used for environmental monitoring. In this case, a hyperactivity response would indicate a potential enhancement of neuronal synapses while hypoactivity would mean potential inhibitory synapse. These hyper and hypoactivity information could then be used in further screening to prioritize molecular tests for identifying dominant mechanisms of action for effect drivers. This was applied in **Chapter 6** of this thesis where AChE inhibiting test was used to further diagnose the hyperactivity induced by an environmental sample.

Furthermore, it is possible to observe no-effect when testing environmental samples using the STC test. This could be due to counteraction of hyper and hypoactivity within the sample leading to equivalent antagonism or normalization of effects. For example, no STC effect may be observed in environmental mixtures due to effect canceling or mitigation of opposing acting effects. This could become a huge challenge for diagnostic risk assessment. One solution is to spike complex 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. It is clear that the STC test and other behavior test methods are of great potential for use as effect based methods but further work is required to develop these tools for optimal use.

7.2.5 Limitations and way forward: Proposal of a tiered or combined behavior testing

Despite the value and utility of the STC test for neurotoxicity screening shown in this thesis work, there are limitations that might hinder its applicability domain. First, the STC endpoint is assumed to be mediated by spinal neurons and might not be able to detect effects occurring in the brain. Second, the STC is assumed to occur as a result of neurotransmission leading to innervation of the muscle by the primary motor neurons. Therefore, the STC test may only capture the effect of chemicals on signal transmission at the neuromuscular junction. However, the effect of chemicals on other neuronal structures such as axon-dendrite synapses may also induce an STC effect. Nevertheless, such structural or developmental effects are expected to occur at higher concentrations leading to hypoactivity and could be spotted or identified. Third, the developmental stage at which STC is measured is confounded by a potential limitation in biotransformation capacity. This could lead to false negatives for chemicals which require biotransformation such as organophosphates. Fourth, the chorion of the embryos may act as a barrier for chemical uptake, especially for high molecular weight compounds. Such compounds can however be tested using dechorionated embryos but the chorion could still be a challenge for diagnostic hazard assessment in which the mixture components would be largely unknown. Nevertheless, it seems that neurotoxic substances are mostly not high molecular weight substances, therefore, the limitation of the chorion as barrier for high molecular weight substances may be less relevant. Fifth, during the relative short exposure duration in the STC test (24 h or less), equilibrium internal concentrations may not have been reached and could lead to overestimation of effect concentrations (=underestimation of toxicity). Nonetheless, the short duration has been recognized as a feature that confers specificity for neuroactivity since long duration tests could affect neuronal structures leading to a non-specific hypoactivity response.

To improve detection capacity of the STC test, one option could be to increase the number of STC endpoints (i.e. coil angle, coil time and coil speed), which could allow classifying of chemicals using

different effect patterns (Zindler et al. 2019; Zhang et al. 2021). An alternative option is to complement the STC test by using other established behavior assays, which have a different application domain. For example, the limited application domain, biotransformation and toxicokinetics of the STC may not fully affect the locomotor response (LMR) test. Inversely, limitations of the LMR test such as lack of specificity, mechanistic understanding and diagnostic capacity could be supported by the STC test. In this light, it could be beneficial to develop a battery of behavioral assays, which could be organized into tiers based on applicability domain and complexity. Recent development in technology and software also allows to measure and evaluate the results of several behavior endpoints within a single pipeline. For instance, several behavior endpoints (e.g. STC, PMR, LMR, startle response, habituation etc.) can be measured within the same experiment when using multi-well plate exposure. It is clear that the value of behavior testing would not be fully realized for both regulatory and research purposes without some level of test standardization and organization of tests into tiers.

7.3 References

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Appendix

8.1 Appendix 1: Supporting information – chapter 2

Table S1: Sources of fish embryo test lethality data used in Figure 3 of main text.

Substance	Data Source	LC50 (µM)	Exposure duration (hpf)
Valproate	Selderslaghs et al 2012	1570	72
Carbamazepine	Van den brandhofs and montforts 2010	1037	72
Endosulfan	*Biotox database	1.2	96
Chlorpyrifos	*Biotox database	5.4	96
Diazinon	Steele et al 2018	37.5	96
Aconitine	Ali et al 2012	200	72
Pentylenetetrazole	Steele et al 2018	19153	96
Nicotine	Ali et al 2012	220	96
Abamectin	Weichert et al 2017	0.7	48
Emamectin	Weichert et al 2017	12.6	48
Methimazole	Selderslaghs et al 2012	19120	72
Acetaminophen	Selderslaghs et al 2012	3539	96
Retinoic acid	Selderslaghs et al 2012	1.47	72

**Data retrieved from internal database maintained at Department of bioanalytical ecotoxicology, Helmholtz center for environmental research, Leipzig*

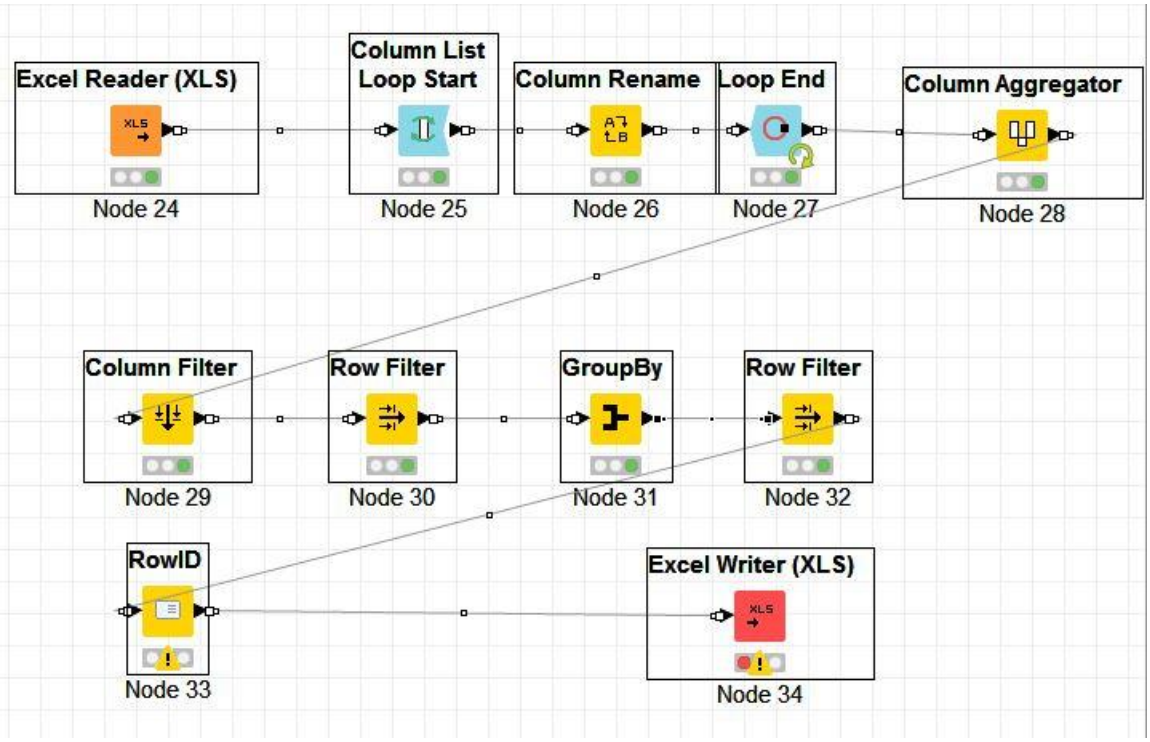


Figure S1: Summary of the literature selection process in KNIME

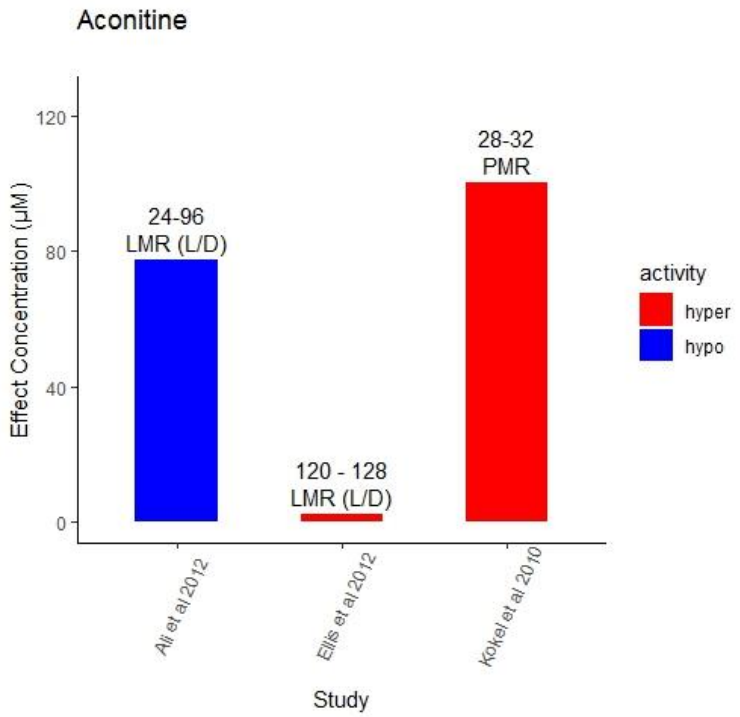
Comparison of individual substances with known neuroactive mode of action

Comparison of effect concentrations for all chemicals considered in this review are described in the text and the corresponding figures for each chemical depicted below. Bars show the magnitude of the effect concentrations which represents lowest effect concentrations as deduced from each study. When there is no bar, it indicates no effect observed within the tested concentration range. When two different bars are depicted for one study, it indicates effect concentrations for both hypo- and hyperactivity. The text written on top of each bar represents the behavioral test method while the numbers represents the exposure duration (hpf)

Substances with an expected hyperactivity effect on zebrafish embryos

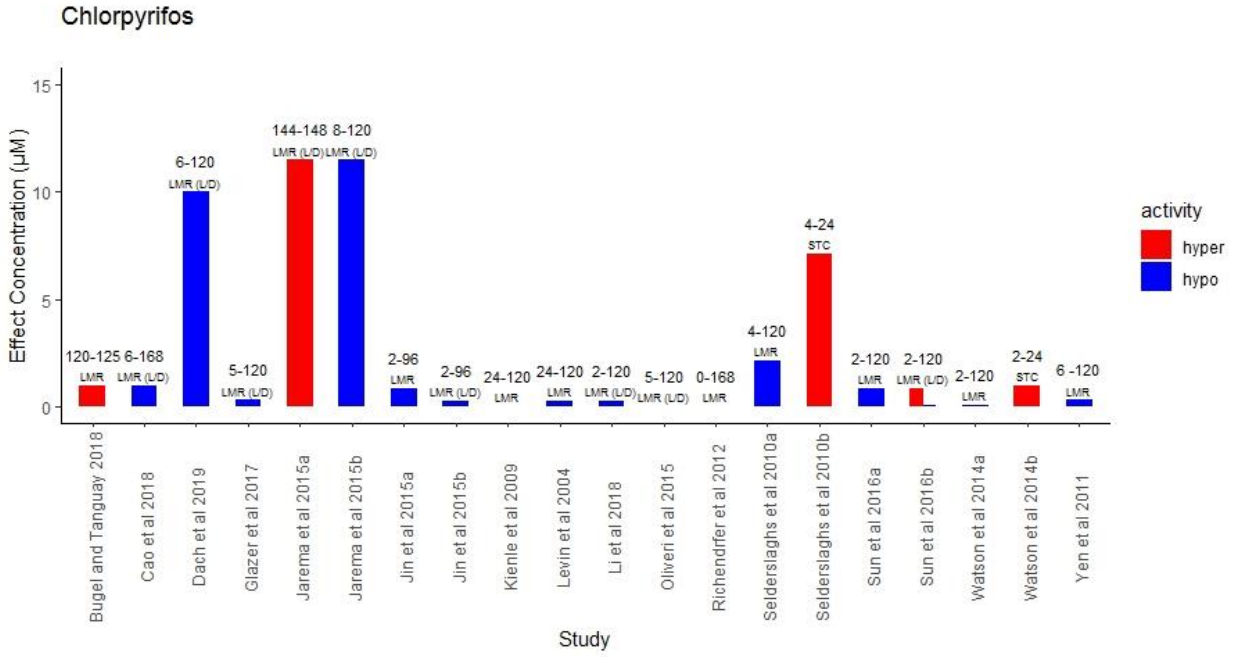
Aconitine

Aconitine is expected to stimulate nerve cells by activating the voltage gated sodium channel, hereby causing hyperactivity (Gutser et al. 1997). Three studies were compared. Hyperactivity was observed in the LMR-L/D study by Ellis et al. (2012) and PMR study by Kokel et al. (2010). The LMR-L/D study by Ali et al. (2012) showed hypoactivity and this could be due to the long exposure duration leading to over-excitation of the nerve cells and hence paralysis.



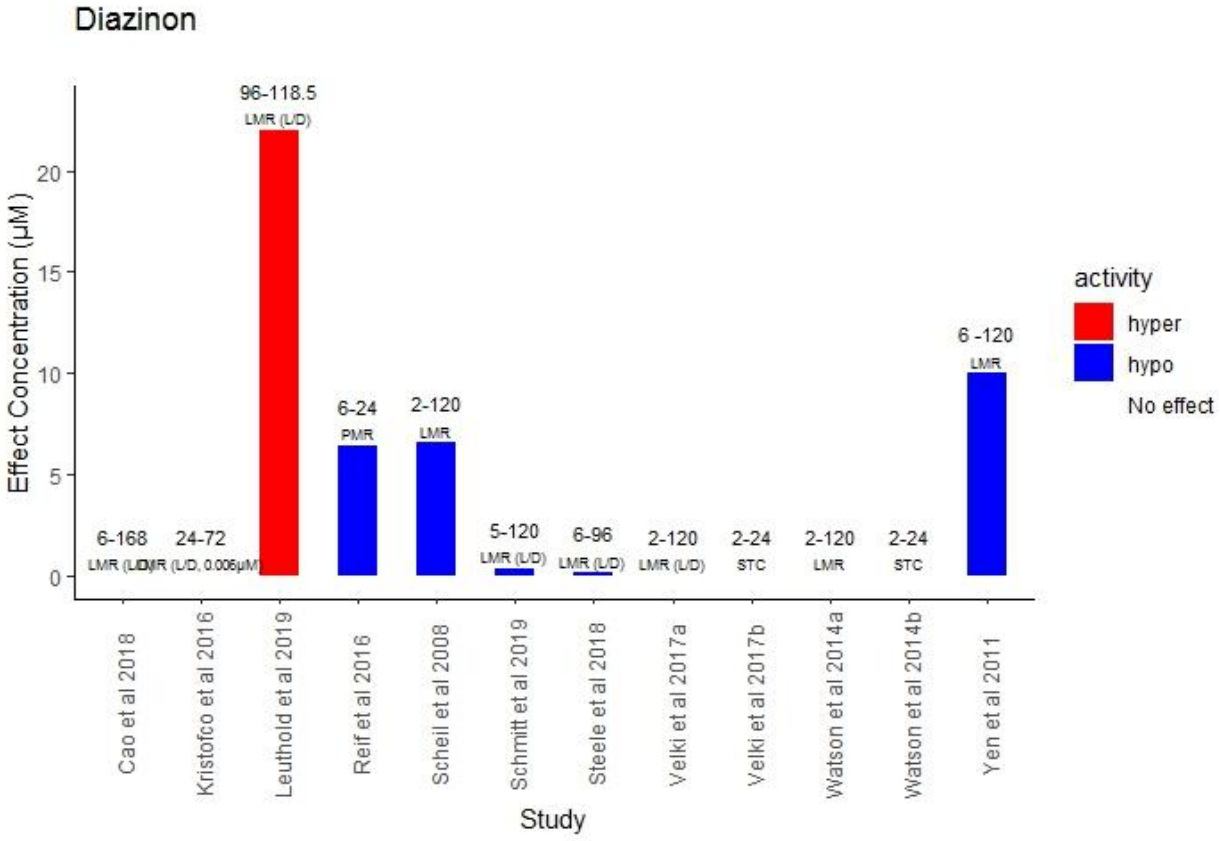
Chlorpyrifos

Chlorpyrifos is an organophosphate expected to cause hyperactivity at lower doses by inhibiting acetylcholinesterase enzyme after it is bioactivated i.e. transformed into the corresponding oxon metabolite (Casida and Durkin 2013). Twenty-two studies were compared. Five studies that reported hyperactivity were short exposure duration tests (2-20 h) except the LMR-L/D study by Oliveri et al. (2015) in which a lower concentration (0.03µM) was exposed for 115 h. This suggests that chlorpyrifos might be taken up fast in the embryo and therefore its neurotoxic effect may be visible at low concentration and short exposure. Consequently, the hypoactivity which is mostly observed at higher exposure concentrations and long duration may be due to over-excitation of the neuron cells leading to axonal defects or seizures and subsequent paralysis. In addition, endpoint parameter varies across the tests and this could also contribute to the risk of bias.



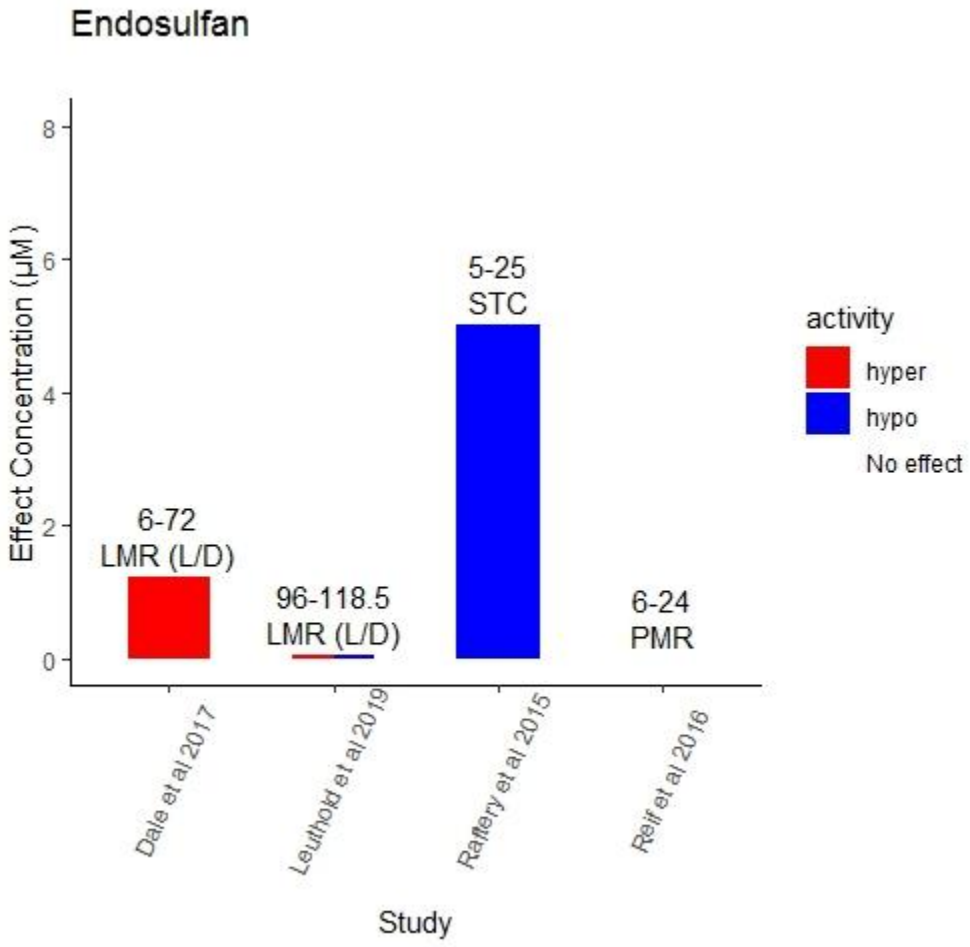
Diazinon (see Figure 3 and S2)

Diazinon is an organophosphate expected to cause hyperactivity by inhibiting acetylcholinesterase after it is bioactivated i.e. transformed into its –oxon metabolite (Casida and Durkin 2013). Twelve studies were compared. Hyperactivity was observed only in a single LMR-L/D study (Leuthold et al. 2019). The other 10 studies either showed hypoactivity or no effect. Interestingly, the LMR-L/D study by Leuthold et al. (2019) was the only one that employed a combination of an older developmental stage (96 hpf) and a short exposure duration (24 h) and the anticipated hyperactivity of diazinon was observed at low exposure concentrations while hypoactivity was observed at high concentrations. The lack of effect observed in only short duration studies (STC and PMR) with younger stages could be due to slow uptake or a possible bioactivation limitation of the early life-stage of the developing embryo. Therefore, the hypoactivity effect could be due to the narcotic effect of diazinon at relatively high concentrations. Different endpoint parameters were also used and this made it difficult to compare effects in some cases.



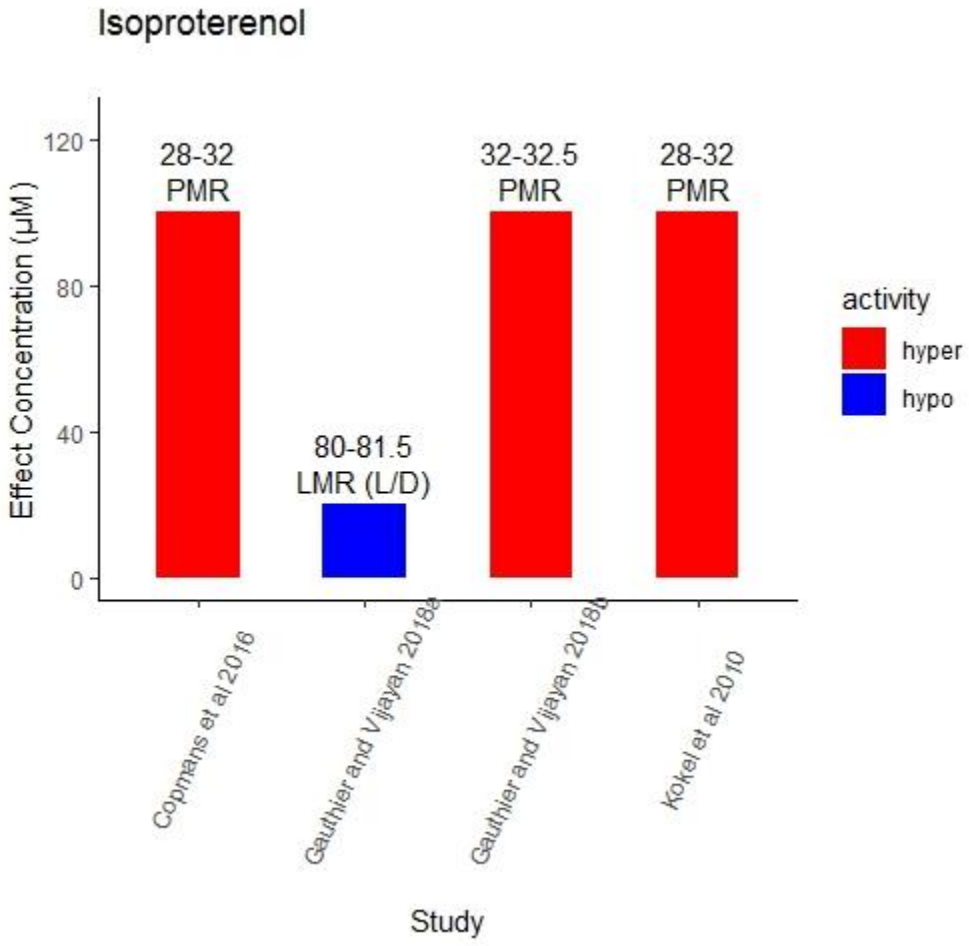
Endosulfan

Endosulfan is an organochlorine insecticide which is expected to cause hyperactivity by blocking GABA-gated chloride channel (Casida and Durkin 2013). Four studies were compared. Zebrafish embryos reacted with hyperactivity to endosulfan exposure in an LMR-L/D study by Dale et al. (2017) [long exposure duration] and Leuthold et al. (2019) [short exposure duration], but hypoactivity was observed in the STC study by Raftery and Volz (2015) [short exposure duration]. This could be due to limited uptake and perhaps limited biotransformation in the early development stage. Moreover, the STC endpoint (see selected behavioral endpoints) used in Raftery and Volz (2015) might be biased towards hypoactivity since endosulfan blocked abamectin induced hypoactivity in the same study (see section endpoint parameters above). The fourth studies, a PMR study by Reif et al. (2016) did not show any effect and this is also attributed to differences in endpoint parameter between STC and PMR.



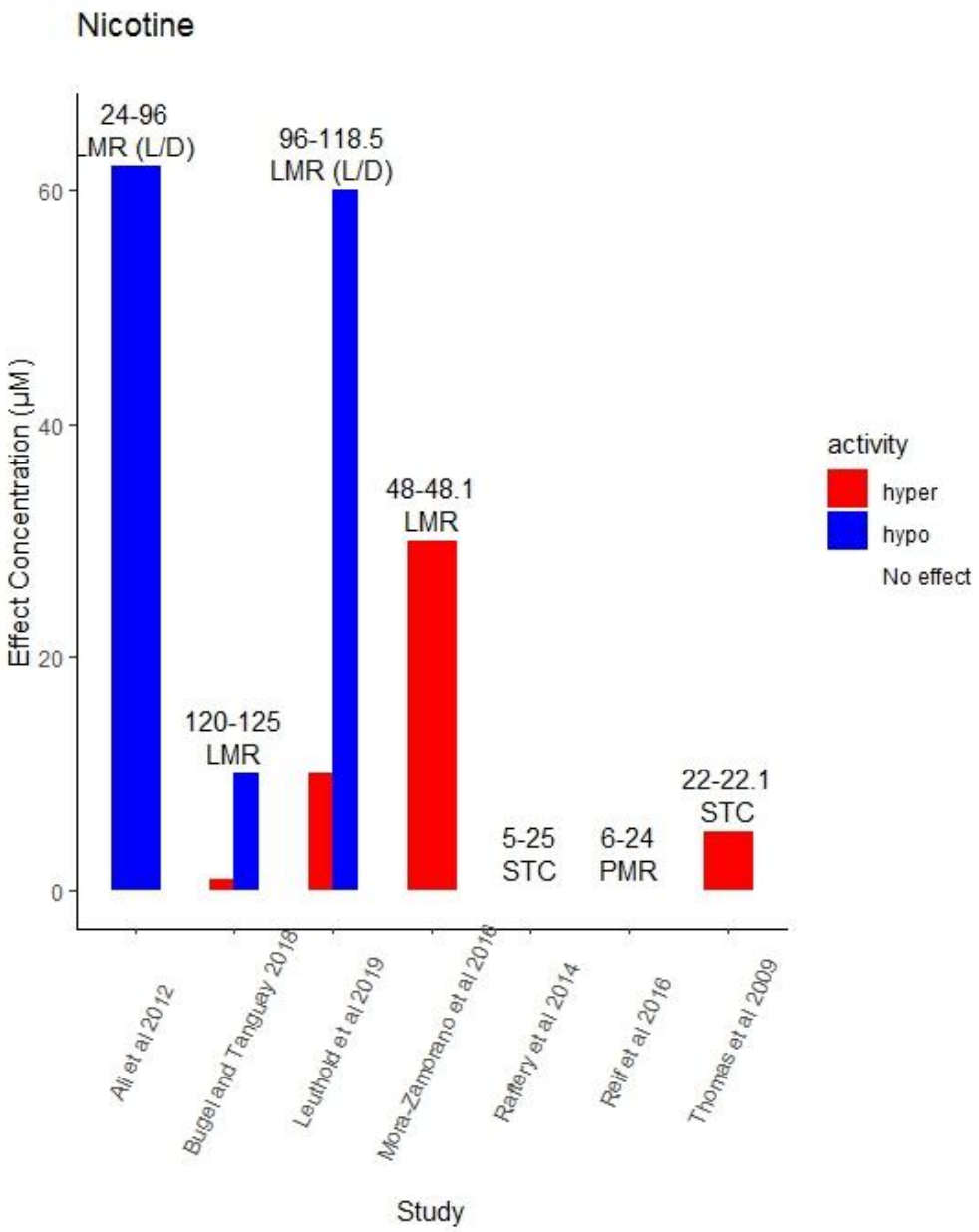
Isoproterenol

Isoproterenol is a pharmaceutical expected to cause hyperactivity by agonizing beta-adrenergic receptors (www.drugbank.ca). Four studies were compared. Isoproterenol showed consistency in its hyperactivity effects observed in 3 PMR studies. The only exception is the hypoactivity reported in the LMR study by Gauthier and Vijayan (2018). This hypoactivity could be related to side effects of isoproterenol in older developmental stages which probably could possess more molecular receptors, thus increasing susceptibility. Additionally, exposure concentration seems to be a limiting factor for adequate comparison. The LMR study exposed at a single concentration of 20µM which is 5 times lower than the effect concentration (100µM) reported in the other PMR studies



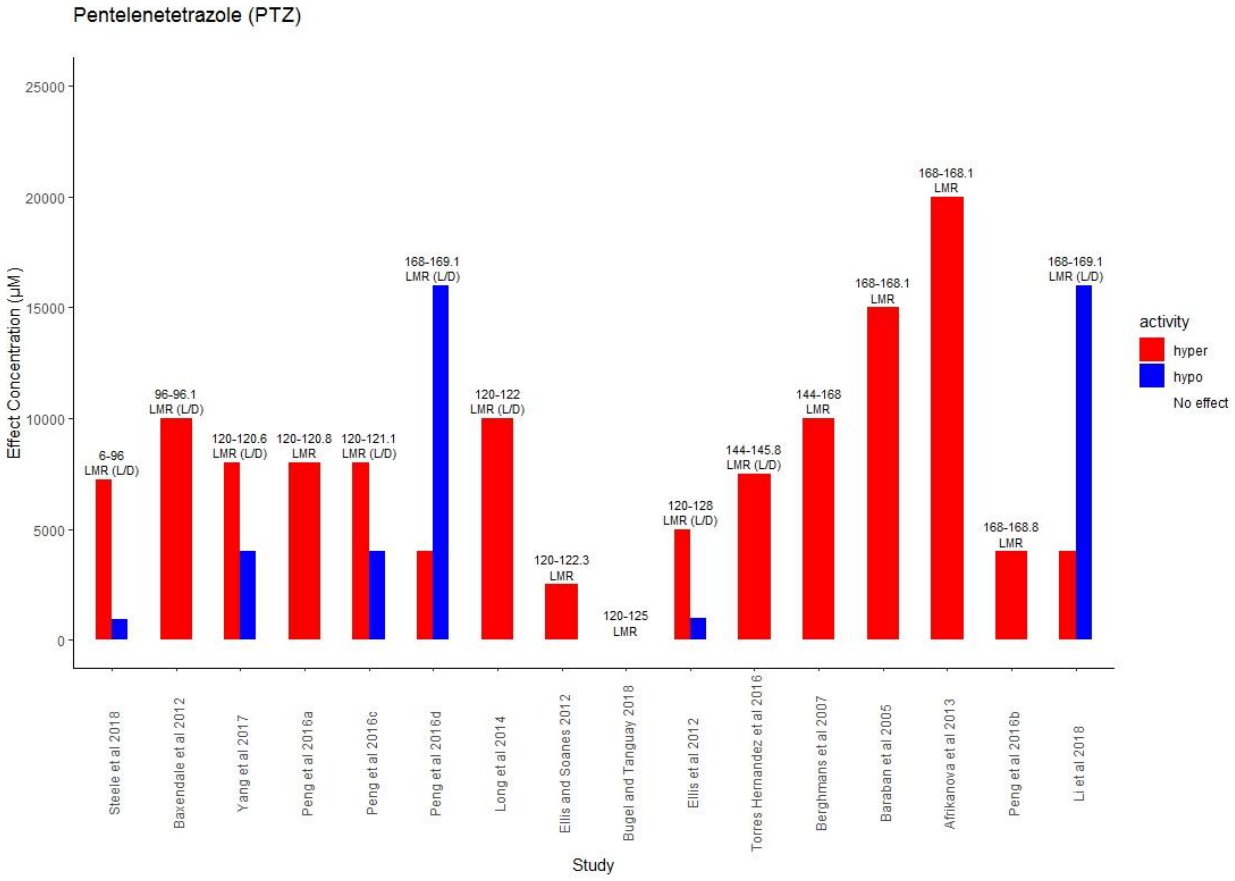
Nicotine

Nicotine is an insecticide that acts by agonizing the nicotinic acetylcholine receptors, thereby causing hyperactivity (Casida and Durkin 2013). Seven studies were found and compared. A trend can be observed in which the short exposure duration tests showed hyperactivity. Nicotine is taken up fast in zebrafish embryos and equilibrium is reached after 10 mins (Thomas et al. 2009). This suggests that the hypoactivity reported in the long duration test by Ali et al. (2012) could be due to over-excitation and paralysis. Furthermore, the STC test by Raftery et al. (2014) did not report any effect. This might probably due to short analysis duration of 6 seconds and the different endpoint parameter which might be inherently biased against detecting hyperactivity. Similarly, the PMR study by Reif et al. (2016) did not show any effect and this is also attributed to differences in endpoint parameter.



Pentylenetetrazole

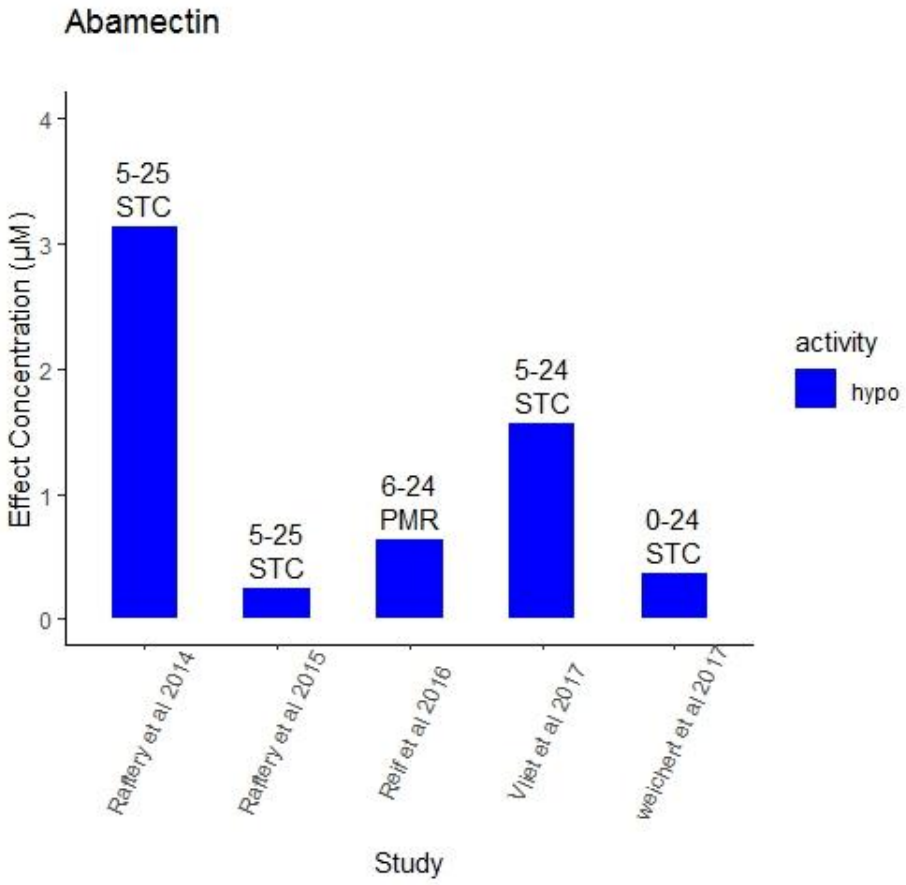
PTZ is a convulsant drug and it is expected to cause hyperactivity by binding to GABA receptors (Squires et al. 1984). Sixteen studies were compared. PTZ showed hyperactivity effect in all the studies except the LMR study by Bugel and Tanguay (2018) which reported no effect. This is possibly due to the use of low exposure concentrations which are probably below the effective range of PTZ. Even though the effect concentrations for hyperactivity were within a factor of 10 in all studies, hypoactivity was also reported, at different concentrations or light period, as an additional effect to hyperactivity in some LMR-L/D studies. The effect of PTZ may be enhanced under alternating light-dark periods and PTZ has been reported to cause a reversal of the observed activity in control treatment i.e. higher activity in dark and lower activity in light phase (Ellis et al 2012; Torres-Hernandez et al 2016). Consequently, it is likely that PTZ is biphasic and this effect is only observed under alternating light conditions. Hence, the use of different light conditions during measurement could be a limiting factor for comparing different behavior methods.



Substances with an expected hypoactivity effect on zebrafish embryos

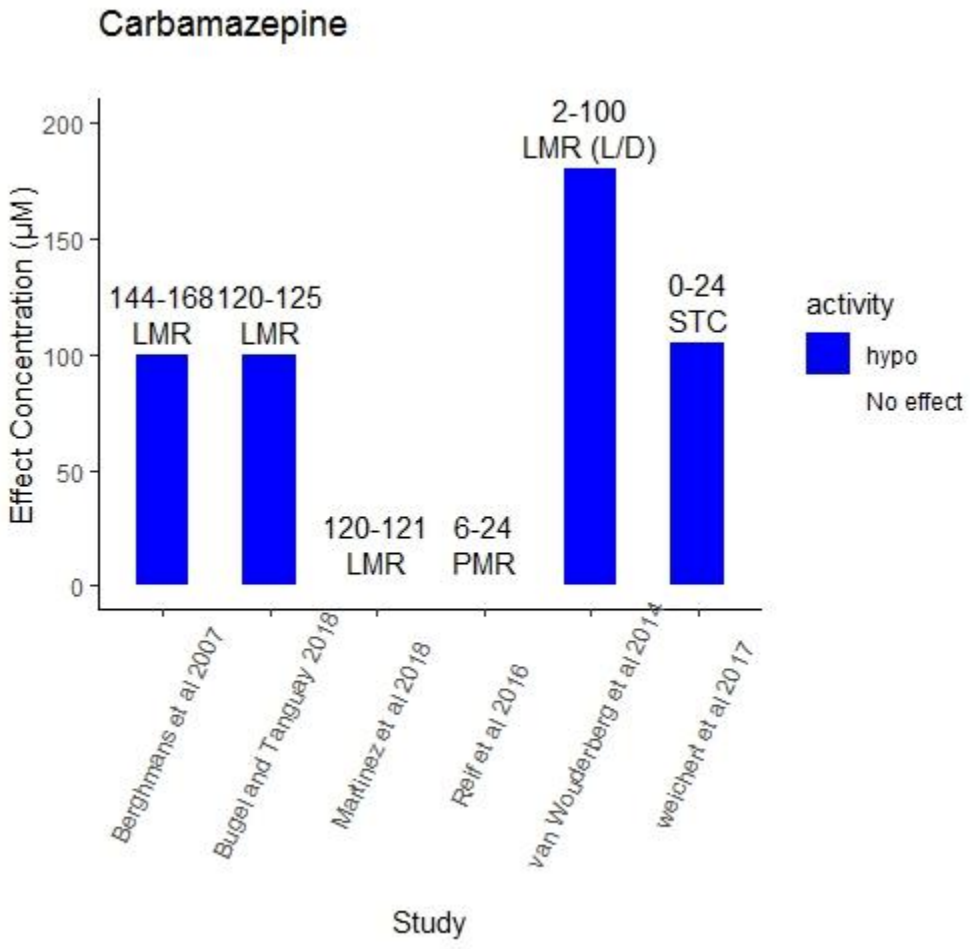
Abamectin

Abamectin is an avermectin insecticide expected to cause hypoactivity by activating GABA gated chloride channel (Casida and Durkin 2013). Five studies were compared. All studies reported hypoactivity. Effect concentration for hypoactivity reported in all studies are within a factor of 10 (0.36 – 3.13µm) except the STC study by Raftery and Volz (2015) which reported an effect at 0.25µm. This lower effect concentration could be due to conducting exposure in glass beakers instead of plastic titer-plates as exposure vessel. Abamectin is highly lipophilic ($logD_{pH7.4(ACD/Labs)}$ of 5.85) and hence has more affinity to bind to plastic than glass; therefore, abamectin may be highly bioavailable to the embryos in a glass container leading to effects occurring at lower concentration (see above results section on "nature of test container").



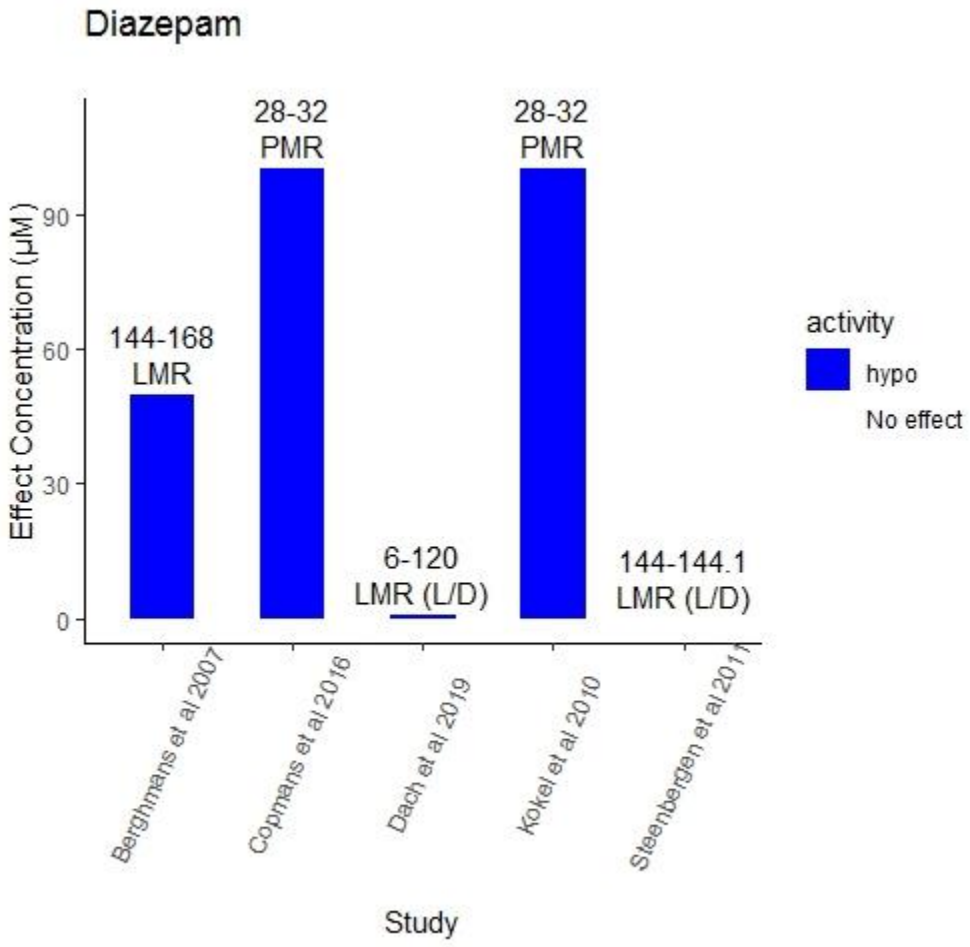
Carbamazepine

Carbamazepine is an anticonvulsant drug which is assumed to act by blocking sodium channels (www.drugbank.ca), therefore causing hypoactivity. Six studies were compared. Four out of six studies reported hypoactivity at similar effect concentrations (100 - 180µM) except the PMR study by Reif et al. (2016) and the LMR study by Martinez et al. (2018) which reported no effect. This could be due to exposure concentrations lower than observed effective range in the PMR study. The LMR study utilized an exposure duration of 1 hour and this could lead to low uptake of carbamazepine and therefore negligible effects or effects not yet developed to an extent that could be observed in the LMR test.



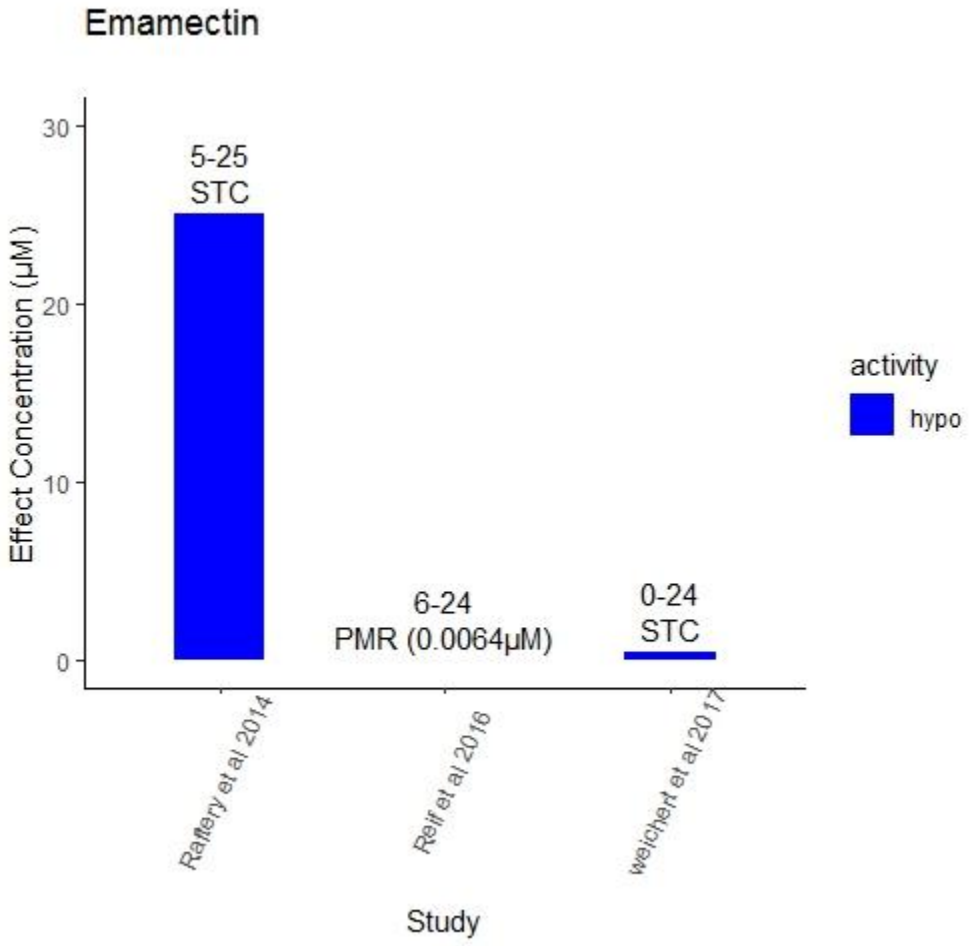
Diazepam

Diazepam is an anticonvulsant and anxiolytic drug which acts by stimulating the GABA receptor (www.drugbank.ca) and it is expected to cause hypoactivity. Five studies were compared. Hypoactivity was reported in all studies considered except the LMR-L/D study by Steenbergen et al. (2011) which reported no effect. This could be attributed to the use of a different endpoint parameter and single exposure concentration (2.5µM). The effect concentration for hypoactivity reported by Dach et al. 2019 was lower than the others and this could be due to longer exposure duration (114 h) employed.



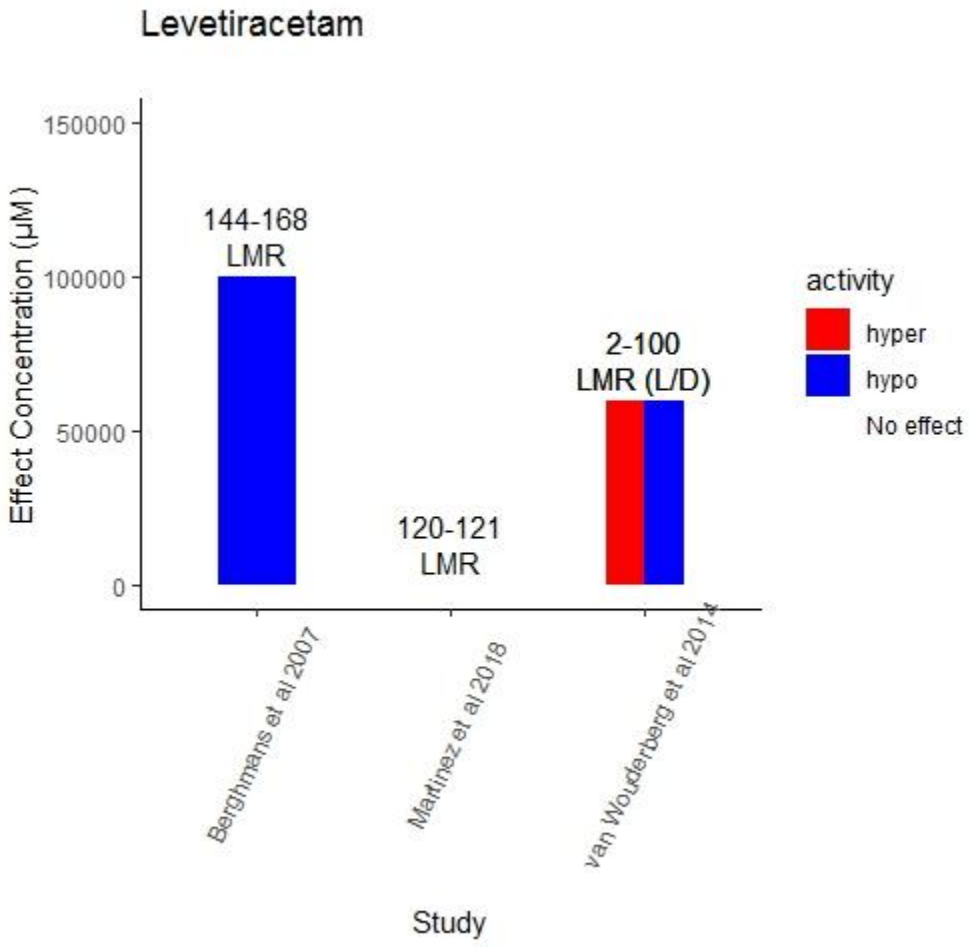
Emamectin benzoate

Emamectin is an avermectin insecticide and it is expected to cause hypoactivity by activating GABA gated chloride channels (Casida and Durkin 2013). Three studies were compared. Although all three studies reported hypoactivity for emamectin, the effect concentrations (0.0064, 1.03 and 25 µM) were not within a factor of 10. A possible explanation is the use of different exposure well sizes. The 384 well-plates used in Raftery et al. (2014) with effect concentration of 25 µM could cause a higher adsorption of emamectin ($logD_{pH7}$ of 5) compared to the 24 well-plates used in Weichert et al. (2017) [effect concentration =1.03 µM]. This could lead to an overall decrease in bioavailability and hence toxicity in the former (see section exposure well size above). Additionally, different endpoint parameter between the STC and PMR could be responsible for the divergent effect concentrations.



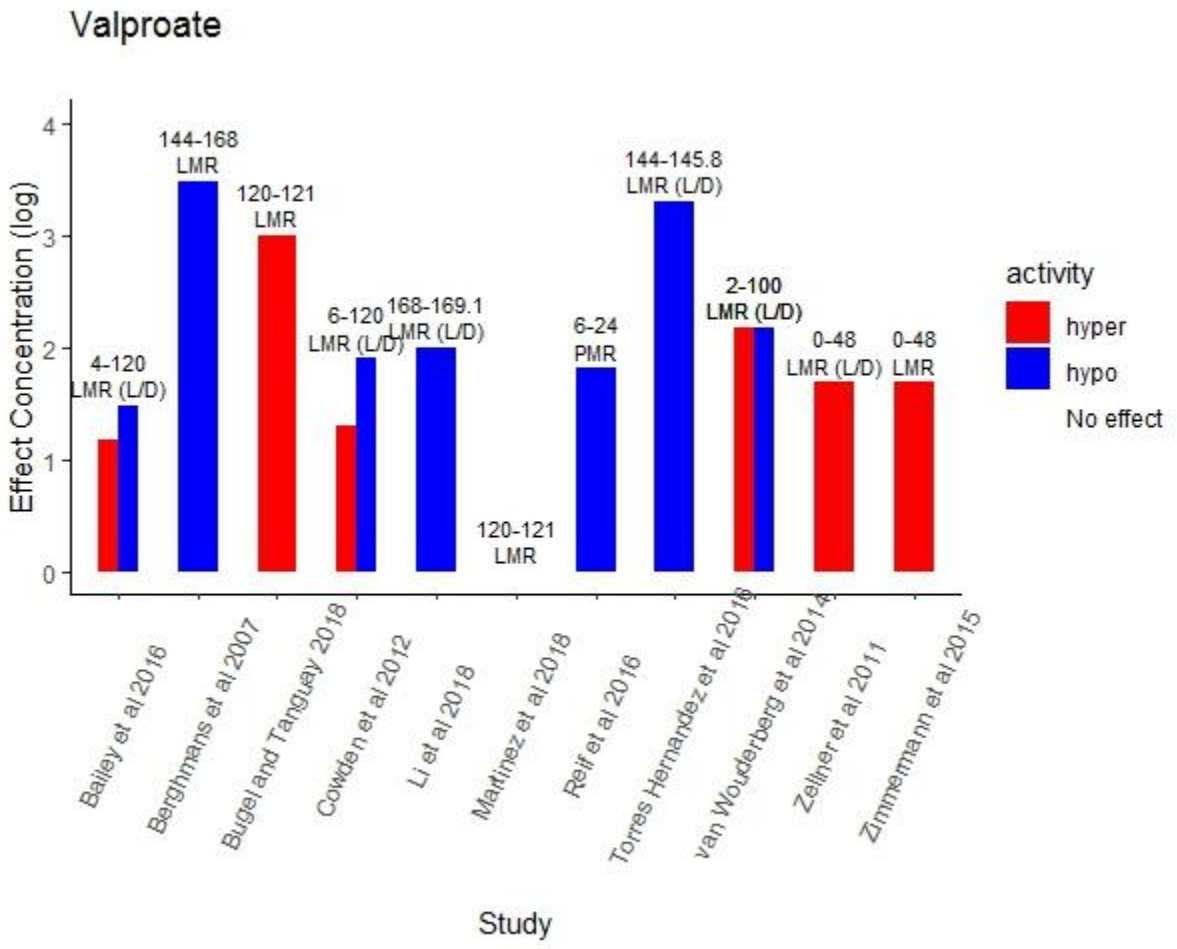
Levetiracetam

Levetiracetam is an anticonvulsant drug which is assumed to selectively prevent hypersynchronization of epileptiform burst firing by inhibiting voltage dependent calcium channel (www.drugbank.ca). It is expected to cause hypoactivity in zebrafish embryos based on its ability to reduce the release of neurotransmitters into the synaptic cleft. This is supported by effectively inhibiting the movement in PTZ (GABA receptor blocker) induced hyperactivity (Berghmans et al. 2007). Three studies were compared. It was probably impossible to detect an effect in the LMR study by Martinez et al. (2018) because the exposure concentration used was a factor of 1000 below that used in the other studies and this suggests that these concentrations might not be in the effective range of levetiracetam. Even though the LMR study by Berghmans et al. (2007) and the LMR(L/D) study by Beker van Woudenberg et al. (2014) shows hypoactivity at a similar concentration, the latter study also shows hyperactivity at higher concentrations. This might be attributed to the differences in exposure duration, developmental stage and light conditions between the studies.



Valproate

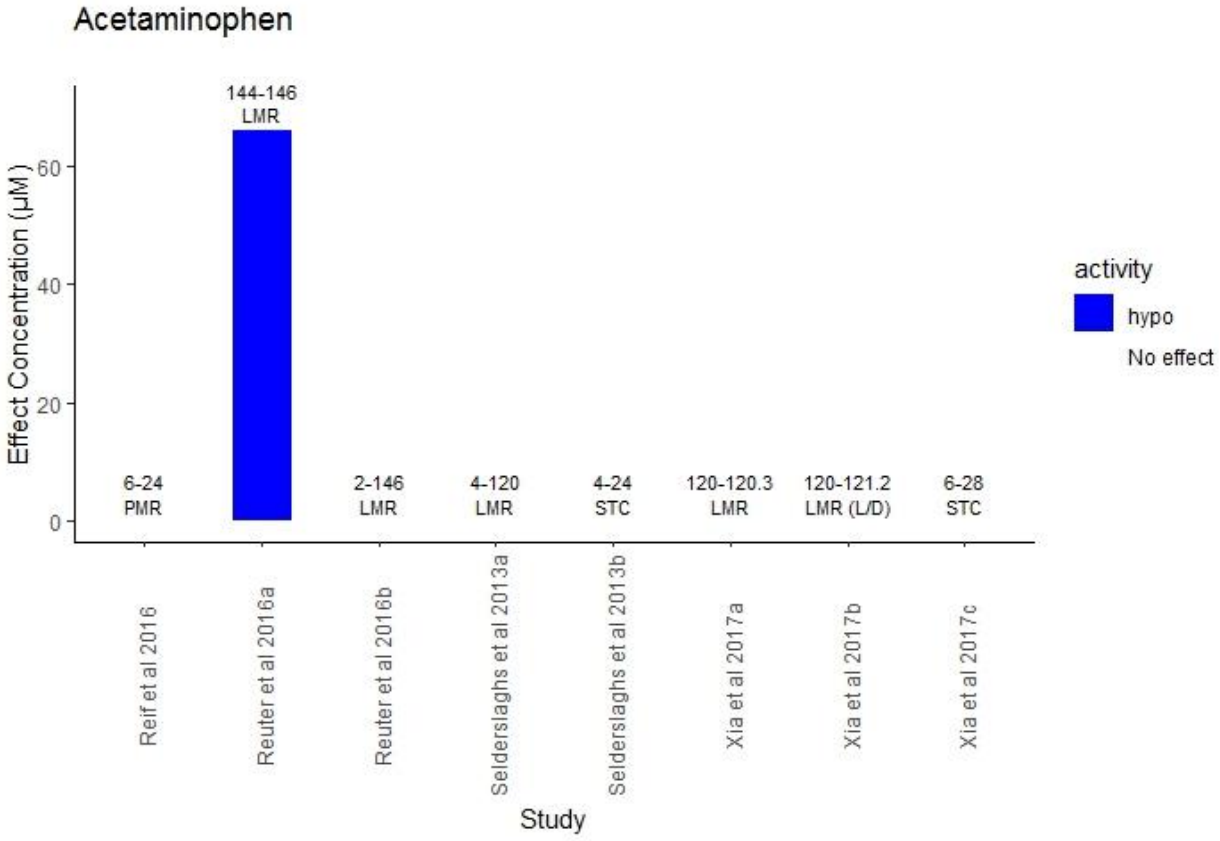
Valproate is an anticonvulsant drug and it is expected to cause hypoactivity by inhibiting GABA transaminase (www.drugbank.ca). This assumption is supported by Baraban et al. (2005) who reported that valproate reduced PTZ invoked seizures and epileptic activity. Twelve studies were compared. In six studies, valproate showed a trend in which hyperactivity was reported mostly at low concentrations (Zellner et al. 2011; Cowden et al. 2012; Beker van Woudenberg et al. 2014; Zimmermann et al. 2015; Bailey et al. 2016; Bugel and Tanguay 2018). It is important to note that the LMR studies by Zellner et al. (2011) and Zimmermann et al. (2015) only exposed from 0 – 48 hpf and measured behavior at 144 hpf. This kind of exposure regime might influence the internal concentration of valproate. Brox et al. (2016) showed that equilibrium concentration is reached after 72hrs of exposing valproate to zebrafish embryos (0-120 hpf exposure) and that it is possible that the elimination is complete at 6 days when behavior was measured in Zellner et al. (2011) - this might partly explain the results. Therefore, exposure duration, which might influence the toxicokinetics, is a possible limiting factor for an adequate comparison. On the other hand, hypoactivity was mostly observed at high concentrations in seven studies. This suggests that valproate might act biphasic on behavior. Nonetheless, single exposure concentrations used in some studies (eg. Zellner et al. 2011; Zimmermann et al. 2015) could cause a comparison bias.



Substances with a mode of action that does not allow anticipating hyper- or hypoactivity

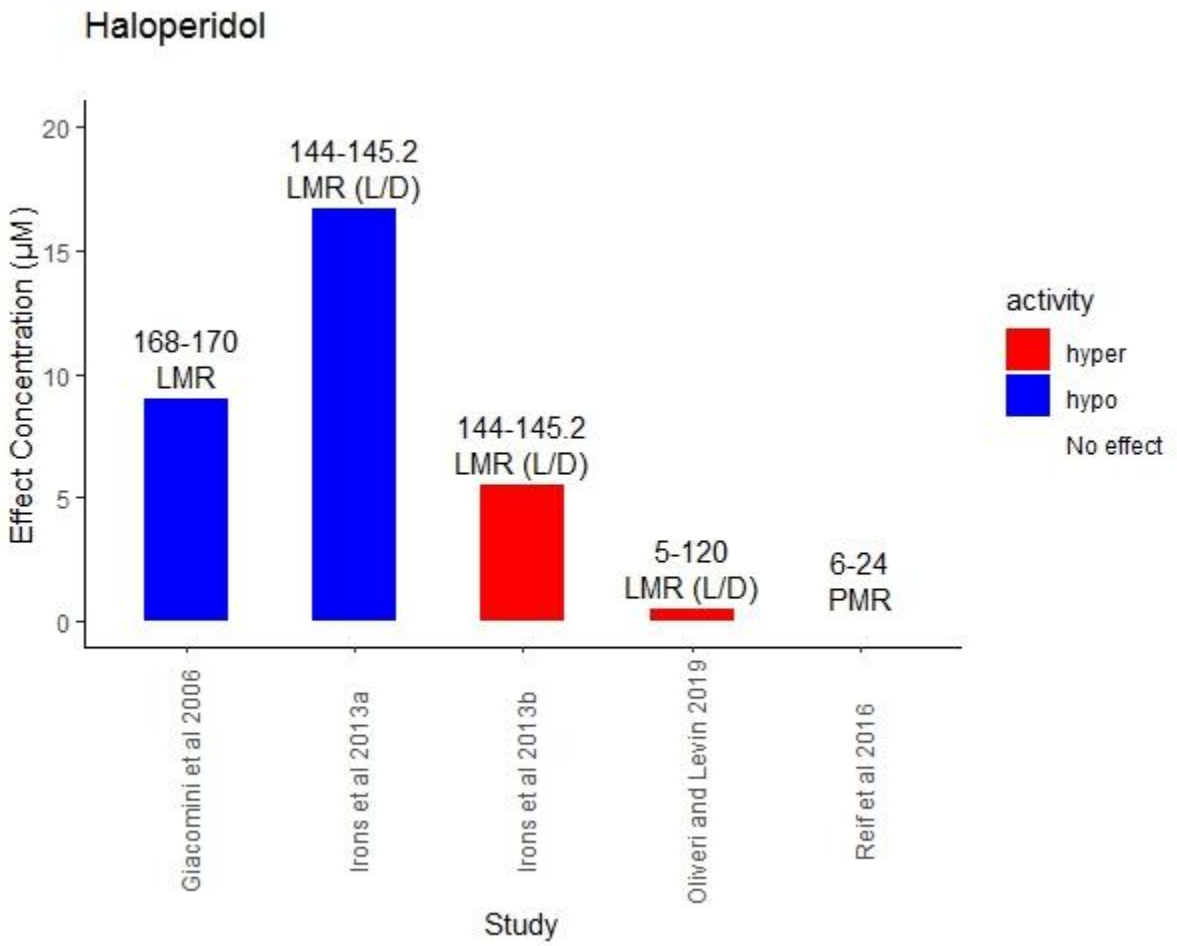
Acetaminophen

Acetaminophen is a cyclooxygenase inhibitor and an analgesic drug (www.drugbank.ca). Its expected activity in zebrafish embryo based on this mode of action is not clear. Nine studies were compared. All studies considered did not report an effect except the hypoactivity effect [66.2 - 6620µM] reported in the LMR study by Reuter et al. (2016). This study is the only one that utilized a combination of higher developmental stages (144hpf) and short exposure duration (2 h). This could indicate the lack of target receptors at lower developmental stages. Additionally, LC_{50}s of 10120, 9920, 7870 and 3710 µm at 24, 48, 72 and 144 hpf respectively were reported for acetaminophen (Selderslaghs et al 2012). Hence, it is possible that the exposure concentration and hence, the internal concentration in the other studies may be excessively below the effective range of acetaminophen.



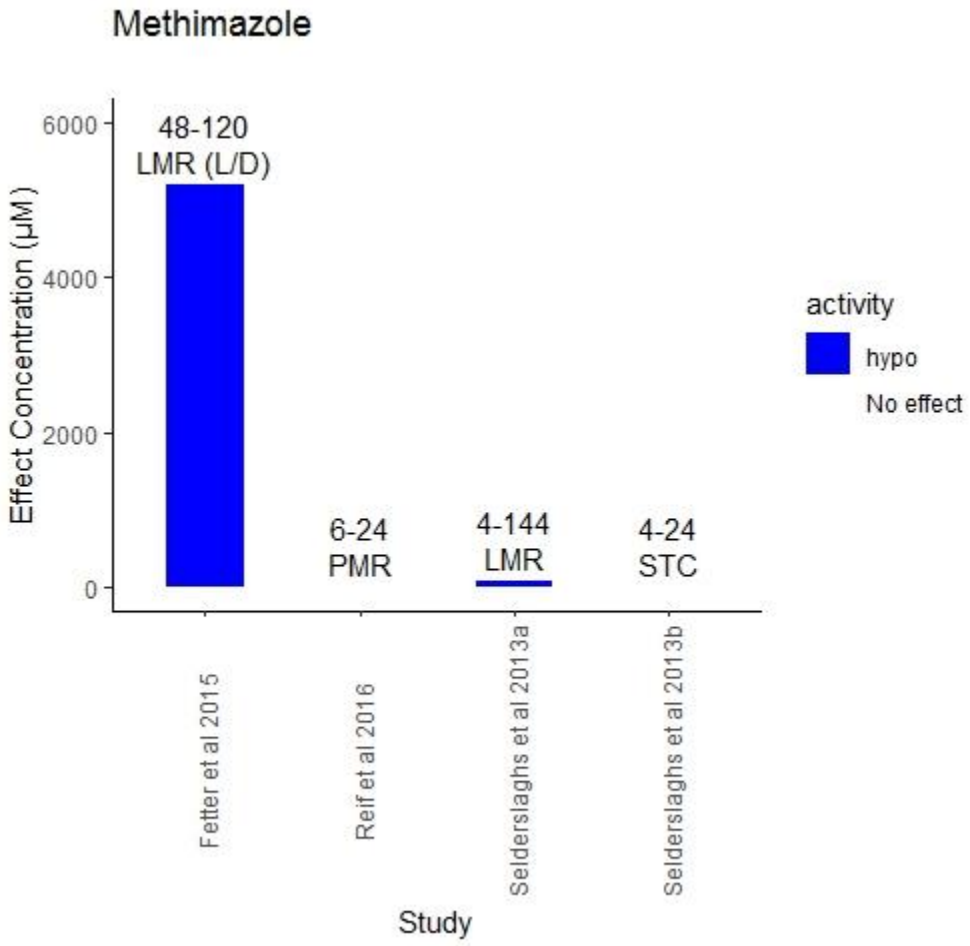
Haloperidol

Haloperidol is -beside other uses- an antipsychotic drug acting as a dopamine receptor antagonist and its expected activity in zebrafish embryo based on this mode of action is not clear. Based on known side effects in humans (www.drugbank.ca) both hypo- and hyper-activity could be expected. Five studies were compared. The LMR study by Giacomini et al. (2006) reported hypoactivity while the LMR-L/D study by Irons et al. (2013) reported a biphasic activity. The former study used only one exposure concentration and swimming speed was the endpoint parameter. This does not allow appropriate comparison with the latter study which assessed distance moved. Oliveri and Levin (2019) compared 2 different zebrafish strains (AB and 5D) and found hyperactivity effect for the 5D strain only. Furthermore, the PMR study by Reif et al. (2016) did not report any effect and this is probably due to basic differences in exposure design, especially developmental stage at exposure initiation.



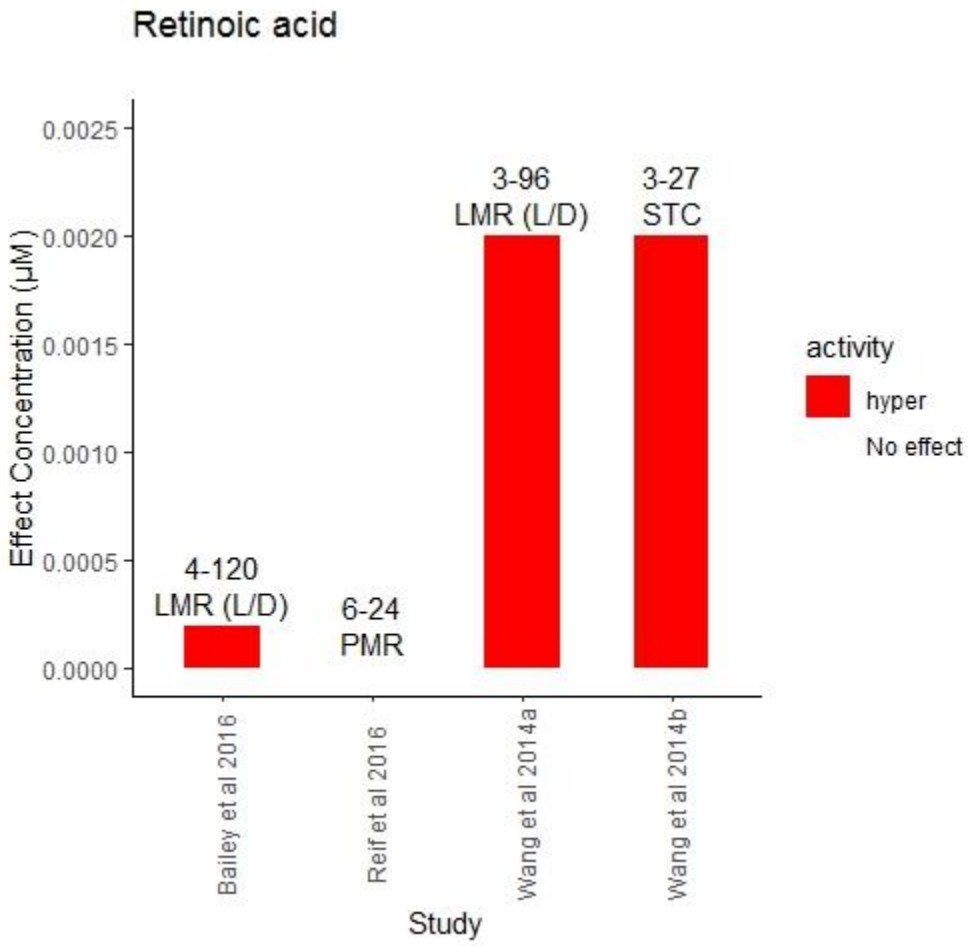
Methimazole

Methimazole is an antithyroid drug that inhibits the conversion of iodide to iodine (www.drugbank.ca). Its expected activity in zebrafish embryo based on this mode of action is not clear. Four studies were compared. Methimazole shows hypoactivity effect in only the long duration studies; LMR study by Selderslaghs et al. (2013) (4-144hpf) and LMR-L/D study by Fetter et al. (2015) (48-120hpf). The short duration studies (0-24hpf), comprising of STC and PMR, show no effect. This suggests that exposure duration, and hence kinetics could be a limiting factor in the propagation of the effect of methimazole.



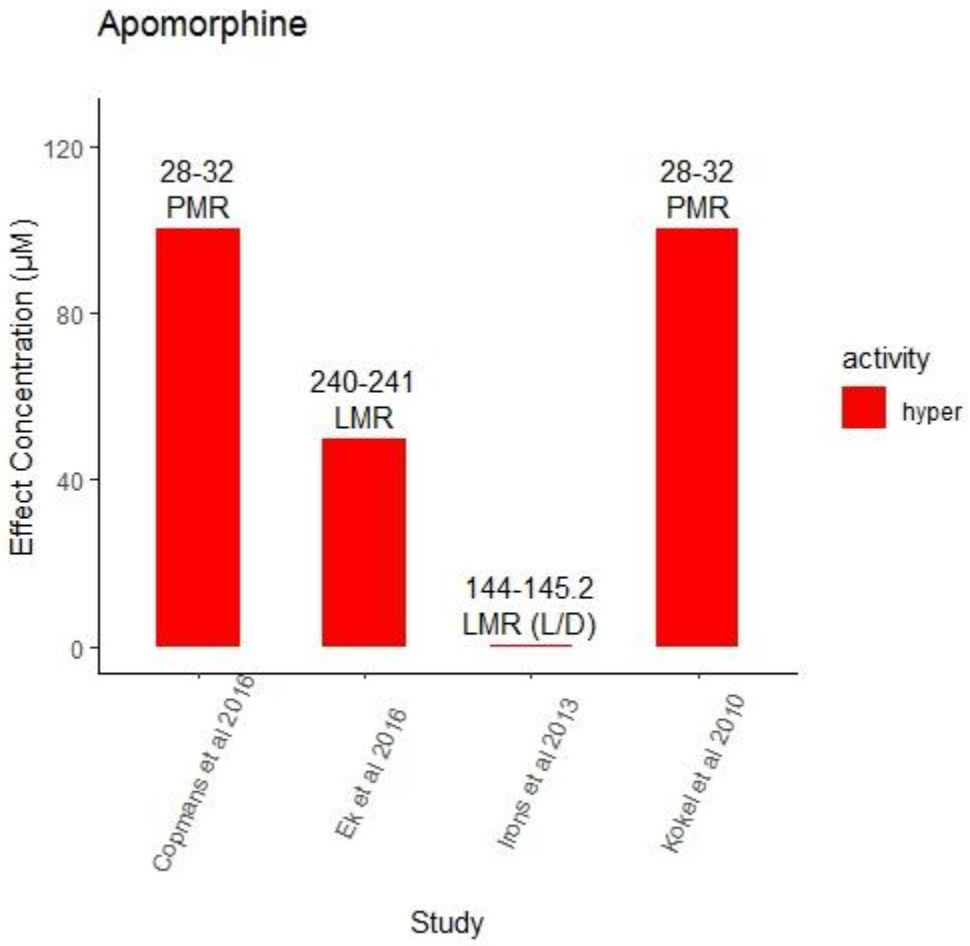
Retinoic acid

Retinoic acid is a retinoic acid receptor agonist (www.drugbank.ca) and its expected activity in zebrafish embryo is not clear. Four studies were compared. All studies except the PMR study by Reif et al. (2016) showed hyperactivity and the effect concentrations were within a variation factor of 10. The reason for the inactivity reported in Reif et al. (2016) is probably related to the different endpoint parameter used in the PMR method.



Apomorphine

Apomorphine acts by stimulating post-synaptic dopamine D2-type receptors within the brain (www.drugbank.ca). Its expected activity is not clear. All four studies showed hyperactivity. However, the effect concentration for the LMR-L/D study by Irons et al. 2013 was below a factor of 10 variation of the other studies. This could be due to the higher sensitivity of zebrafish at the developmental stage of 6dpf. The LMR-L/D method could also be more sensitive than the LMR due to the induced alternating light conditions.



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8.2 Appendix 2: Supporting information – chapter 3

Table S1: Manufacturer details and exposure concentrations of substances analyzed in the STC test

Substance	**CAS number**	**Purity %**	**Company**	***Exposure concentration range µM**
Dmso	67-68-5	100	Sigma-Aldrich	0, 0.0625, 0.1, 0.125, 0.25, 0.5 0, 0.1, 0.5, 1, 2, 3
Chlorpyrifos	2921882	99.9	Sigma-Aldrich	0, 0.5, 1, 2, 4, 8 0, 0.55, 1.66, 5.00
Chlorpyrifos-oxon	5598152	97.9	Dr Ehrenstorfer GmBH	0, 0.0625, 0.125, 0.25, 0.5, 1 0, 0.055, 0.11, 0.22, 0.44, 0.88, 1.76 0, 0.3, 0.44, 0.66
Diazinon	333-41-5	99	Sigma-Aldrich	0, 1, 3, 5, 6, 7, 0, 1, 5
Paraoxon-methyl	950356	99.5	Dr Ehrenstorfer GmBH	0, 0.37, 1.11, 3.33, 10, 30 0, 2.5, 6.25, 12.5, 25, 50
Aldicarb	116063	98	Sigma-Aldrich	0, 20, 100, 500 0, 12.5, 32, 80, 200
Nicotine	54115	98.7	Sigma-Aldrich	0, 1, 3, 9, 27, 40 0, 1.11, 3.33, 10, 30, 0, 5, 10, 20, 40, 100
Abamectin	71751412	100	Sigma-Aldrich	0, 0.02, 0.04, 0.1, 0.2, 0.6 0, 0.025, 0.05, 0.1, 0.2
Propafenone-hydrochloride	34183-22-7		Sigma-Aldrich	0, 9.5, 19, 38, 76.0 0, 20, 40, 80
Carbamazepine	298464	99	ACROS organics	0, 30, 60, 120, 240 0, 300, 400, 500
Diazepam	439-14-5			0, 1.3, 3.8, 11.6, 35 0, 12.5, 25
Pyraclostrobin	175013-18-0		Sigma-Aldrich	0, 0.01, 0.04, 0.14 0, 0.0375, 0.075, 0.15 0, 0.05, 0.1
Diuron	330541	99.5	Sigma-Aldrich	0, 1, 4 0, 1, 2, 4, 8
Aniline	62533	99	Sigma-Aldrich	0, 111, 333, 1000 0, 300, 600, 800, 1000
Daunorubicin-hydrochloride	23541-50-6	98.5	Sigma-Aldrich	0, 0.40, 2.00, 10, 50 0, 3.75, 7.50, 15, 30, 60
Hexaconazole	79983-71-4		Sigma-Aldrich	0, 3.75, 7.50, 15, 30

				0, 3.125, 6.25, 12.5, 25
3,4 dichloroaniline	95761	99.9	Sigma-Aldrich	0, 0.55, 1.67, 5 0, 1.25, 2.50, 5.00, 10
Imidacoprid	138261-41		Sigma-Aldrich	0, 16, 80, 400, 2000 0, 69.3, 138.6, 277.3, 554.6, 1109, 2219

**The given exposure concentration ranges refer to the exposure range of independent replicates. In subsequent replicates often different ranges were used to promote a better description of critical concentration ranges. All concentration ranges/replicates were combined for concentration-response modelling*

Table S2: STC response of zebrafish embryos acclimatized for 0, 15 and 30 minutes under room temperature of 22.8ºC. Three replicates were measured per treatment and there was no significant difference between treatments (p-value = 0.542)

Treatment	Replicate	STC
control	1	3.11
control	2	2.84
control	3	2.15
15mins	1	3.2
15mins	2	3.32
15mins	3	2.5
30mins	1	3.32
30mins	2	2.95
30mins	3	2.9

Table S3: Temperature measurement showing decline of temperature over a 30 minutes duration during STC assessment

Treatment	Replicate number	Time	Temperature ºC	Temperature status
Treatment 1_control	1	9:23	23.9	Immediately after removal from incubator
Treatment 1_control	2	9:23	25	Immediately after removal from incubator
Treatment 1_control	3	9:23	24.7	Immediately after removal from incubator
Treatment 1_control	1	9:32	23.3	after STC measurement
Treatment 1_control	2	9:32	23.9	after STC measurement
Treatment 1_control	3	9:32	23.8	after STC measurement
Treatment 2_15mins	1	8:50	24.8	Immediately after removal from incubator
Treatment 2_15mins	2	8:50	25.1	Immediately after removal from incubator
Treatment 2_15mins	3	8:50	24.6	Immediately after removal from incubator
Treatment 2_15mins	1	9:10	22.8	15mins after removal and just before STC measurement
Treatment 2_15mins	2	9:10	23.4	15mins after removal and just before STC measurement
Treatment 2_15mins	3	9:10	23.3	15mins after removal and just before STC measurement
Treatment 2_15mins	1	9:20	22.8	after STC measurement
Treatment 2_15mins	2	9:20	23	after STC measurement
Treatment 2_15mins	3	9:20	22.8	after STC measurement
Treatment 3_30mins	1	8:30	25.1	Immediately after removal from incubator
Treatment 3_30mins	2	8:30	25.3	Immediately after removal from incubator
Treatment 3_30mins	3	8:30	25.2	Immediately after removal from incubator
Treatment 3_30mins	1	8:45	23.4	15mins after removal from incubator
Treatment 3_30mins	2	8:45	23.4	15mins after removal from incubator
Treatment 3_30mins	3	8:45	22.8	15mins after removal from incubator
Treatment 3_30mins	1	9:00	23.1	30mins after removal and just before STC measurement
Treatment 3_30mins	2	9:00	23	30mins after removal and just before STC measurement
Treatment 3_30mins	3	9:00	22.6	30mins after removal and just before STC measurement
Treatment 3_30mins	1	9:10	22.8	after STC measurement
Treatment 3_30mins	2	9:10	22.7	after STC measurement
Treatment 3_30mins	3	9:10	22.6	after STC measurement

Table S4: STC response for 2 different sample-size setups. Values represent mean ± standard deviation. The experiment was performed with 3 biological replicates.

Set-up	10 embryos & 5 replicates	20 embryos & 3 replicates
Trial 1	1.89 ± 0.13	2.77 ± 0.30
Trial 2	2.82 ± 0.70	3.11 ± 0.50
Trial 3	3.11 ± 0.55	2.88 ± 0.76

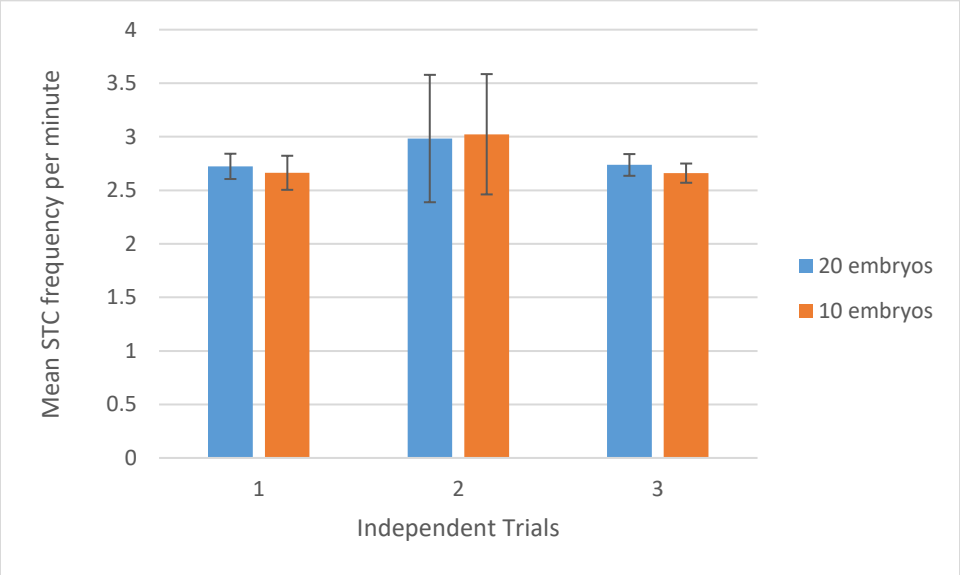


Figure S1: Comparison of STC response for 10 and 20 embryos exposed to ISO water within the same dish. Analysis was done for 3 independent replicates. Error bars represent standard deviation.

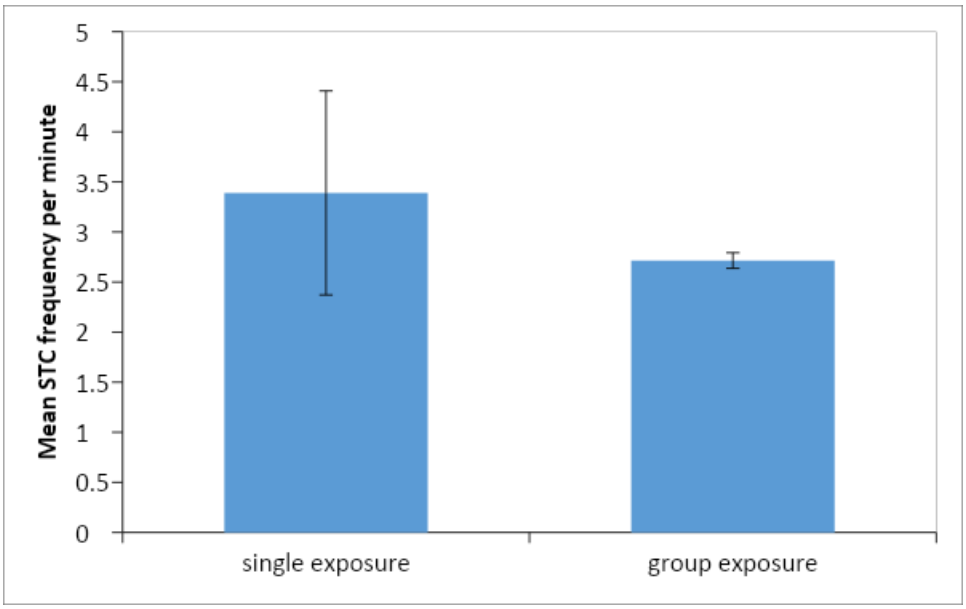
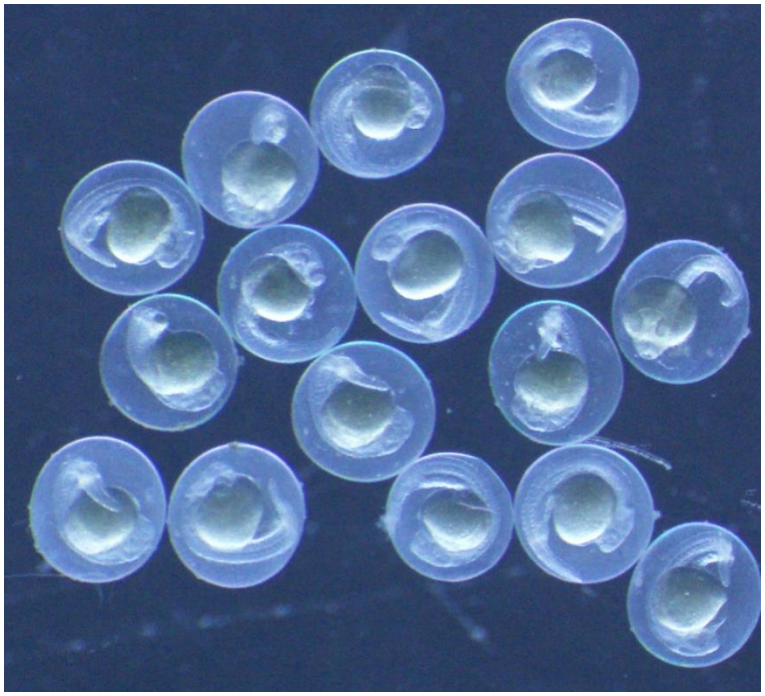
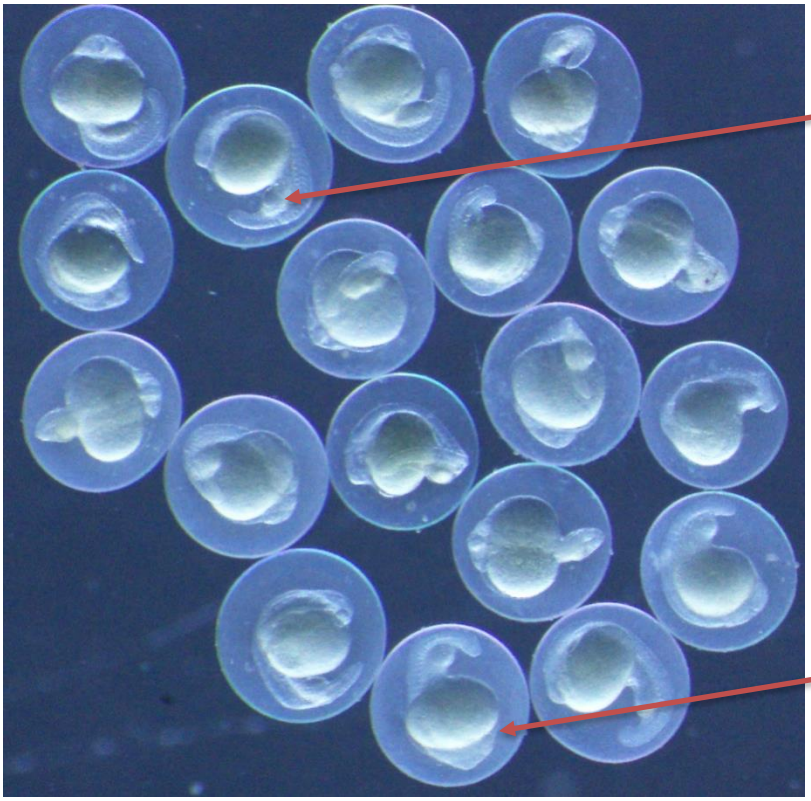


Figure S2: Influence of single and group rearing conditions on STC response. No effect of contagious stimulation of STC among embryos in group exposure is observed.



A)



B)

Reduced-resorption of yolk sac

No clear formation of head and eye

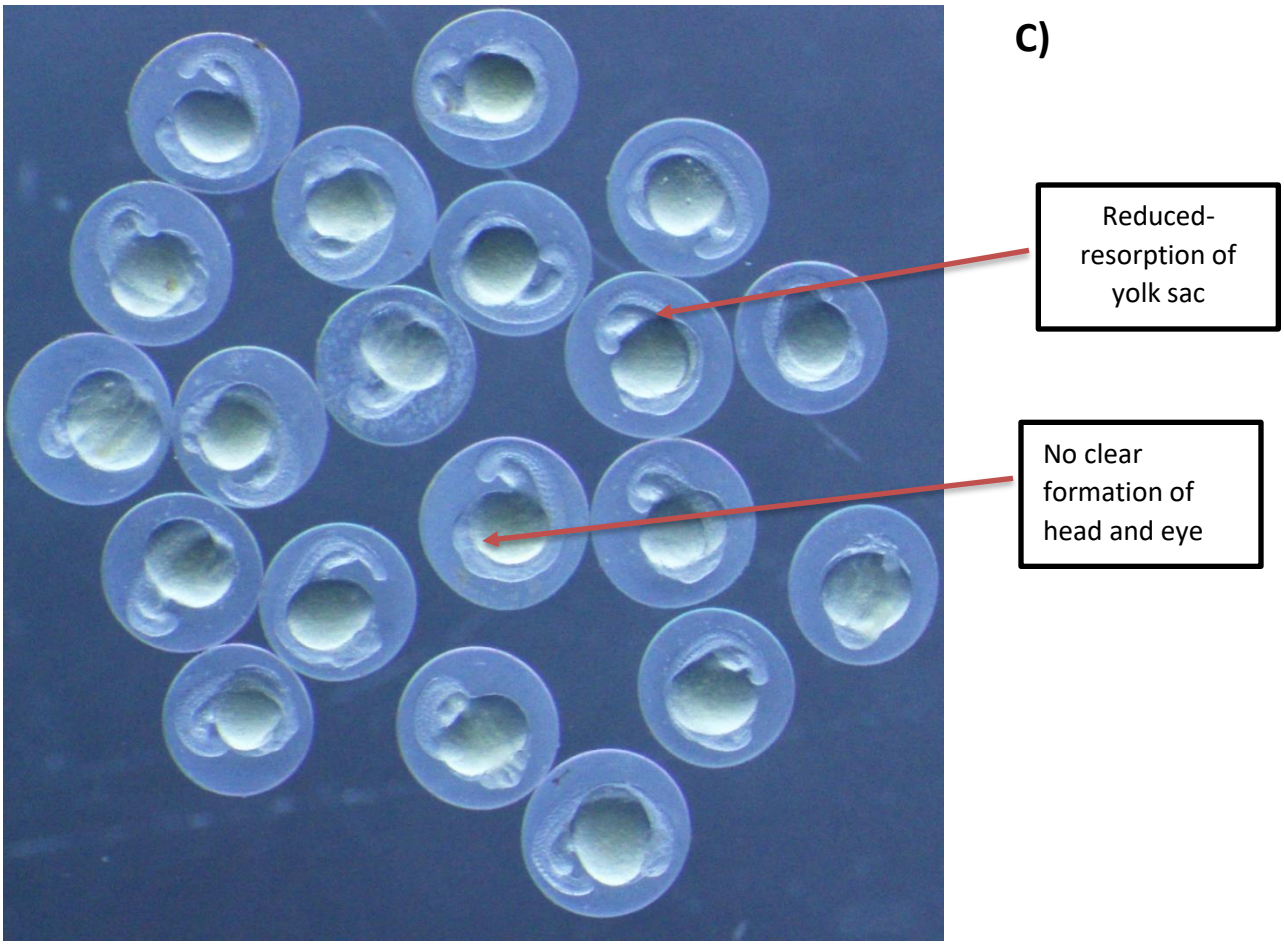


Figure S3: Sublethal effects observed in 24hpf zebrafish embryos exposed to ISO water (control) (A), 100 µM paraoxon-methyl (B) and 0.2 µM pyraclostrobin (C). The observed effects indicate signs of developmental delay. Based on Kimmel et al 1995, the affected embryos seem to be between ages 18-19 hpf.

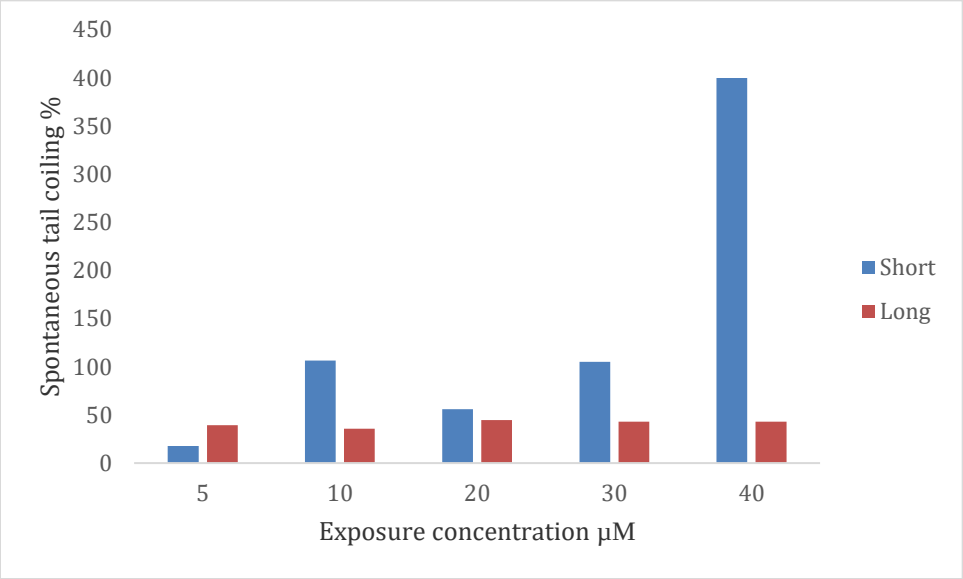


Figure S4: Comparison of nicotine exposed to zebrafish embryos in a short duration (20 min) and long duration (21 h) exposure setup.

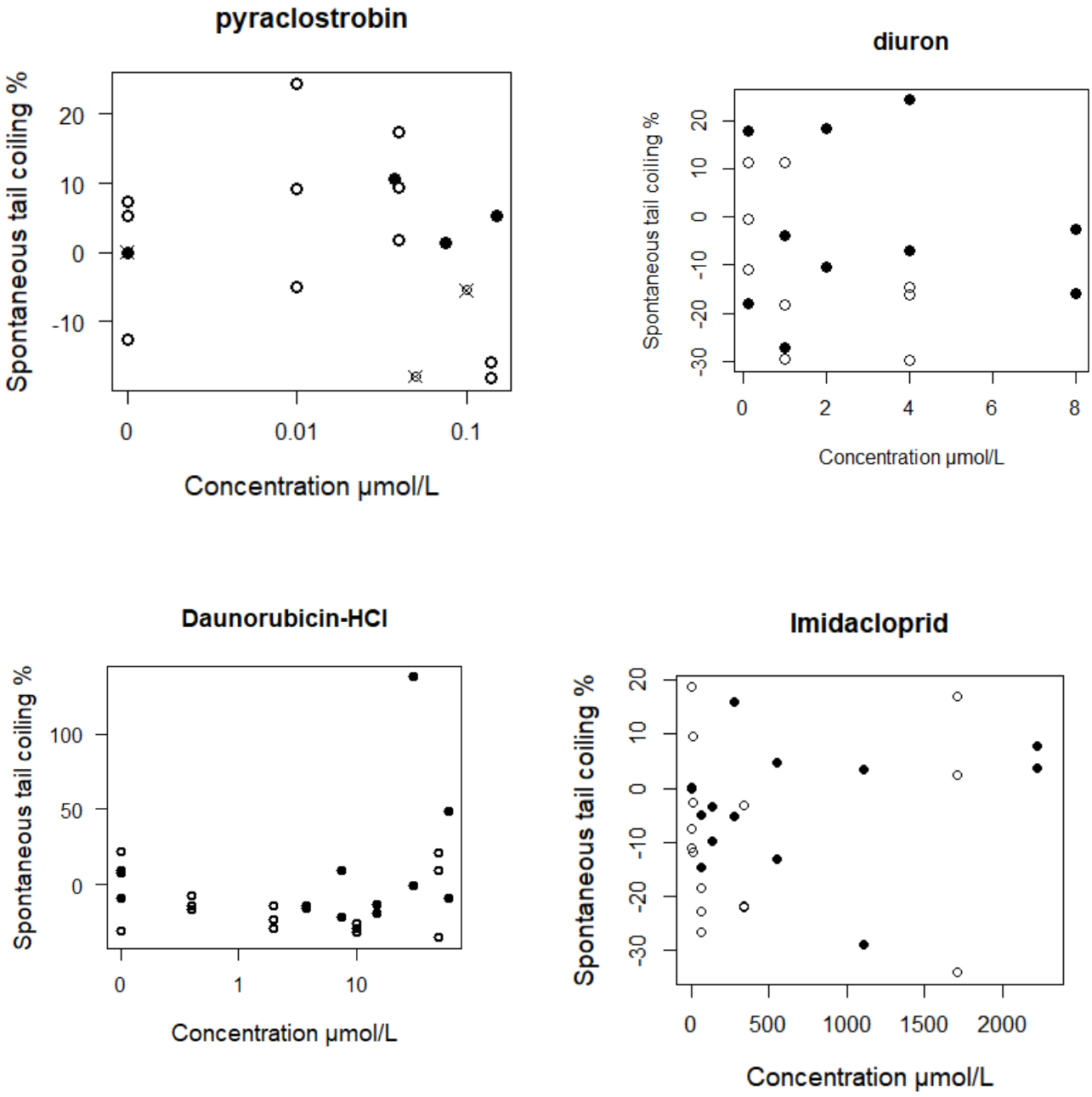


Figure S5: Concentration-response curves for chemicals not showing effect on the frequency of spontaneous tail coiling. Different symbols represent independent experiment

8.3 Appendix 3 - Automated measurement of the spontaneous tail coiling of zebrafish embryos as a sensitive behavior endpoint using a workflow in KNIME*

ABSTRACT

Neuroactive substances are the largest group of chemicals detected in European surface waters. Mixtures of neuroactive substances occurring at low concentrations can induce adverse neurological effects in humans and organisms in the environment. Therefore, there is a need to develop new screening tools to detect these chemicals. Measurement of behavior or motor effects in rodents and fish are usually performed to assess potential neurotoxicity for risk assessment. However, due to pain and stress inflicted on these animals, the scientific community is advocating for new alternative methods based on the 3R principle (reduce, replace and refine). As a result, the behavior measurement of early stages of zebrafish embryos such as locomotor response, photomotor response and spontaneous tail coiling are considered as a valid alternative to adult animal testing. In this study, we developed a workflow to investigate the spontaneous tail coiling (STC) of zebrafish embryos and to accurately measure the STC effect in the KNIME software. We validated the STC protocol with 3 substances (abamectin, chlorpyrifos-oxon and pyracostrobin) which have different mechanisms of action. The KNIME workflow combined with easy and cost-effective method of video acquisition makes this STC protocol a valuable method for neurotoxicity testing.

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Background

The spontaneous tail coiling (STC) represents the earliest motor activity observed in the developing neural network of zebrafish embryos. It is assumed to be mediated by the innervation of the muscle by the primary motor neurons, which are first present at around 17 hpf (Kimmel et al. 1974; Saint-Amant and Drapeau 1998; Richendrfer et al. 2014). These motor neurons are known to originate in the spinal cord. In contrast, another early motor behavior, the photomotor response, requires a high-intensity light stimulus which is mediated in the hindbrain (Reif et al. 2016). A comparison between the PMR test and the STC test has been previously published (Ogungbemi et al. 2019). Counting the average STC in the embryos is considered to be a fast and reliable behavioral endpoint which finds its application in the screening of neuroactive substances or for general toxicological screening (Ogungbemi et al. 2020). A previous review has also identified the STC test to be more sensitive to detect organophosphate insecticides in comparison to other commonly used behavior tests such as locomotor activity and photomotor response (Ogungbemi et al. 2019). Different software are available to measure the STC, however, most of them are expensive and may not allow for a manual correction or quality check of the assessment (i.e. identification of embryos not labelled appropriately). As a result, the actual count of the STC may be inadequately assessed. The aim of the current method paper is to describe a way to accurately and automatically count the STC using a workflow in the open and free KNIME software.

Embryo selection and exposure

Adult zebrafish (OBI and WIK strains) were obtained from a local commercial breeder and crossed to obtain a hybrid strain (OBI-WIK, F3 generation). Fish were cultured under 14 h light/10 h dark photoperiod in 120 L aquaria. Spawning trays were placed in the tanks on the afternoon 4-6 hours before the end of the light cycle. The following day, lights were automatically switched on at 8am to initiate the spawning and eggs were collected at 9am. In order to remove dirt and debris, the eggs were washed several times with ISO water. After washing, fertilized eggs between 2-3 hpf were selected under a stereomicroscope. Twenty embryos were exposed to 20 mL of test chemical in 40 mL glass petri-dish (60 mm diameter, Carl Roth GmbH, Karlsruhe Germany) and covered with a glass lid to prevent evaporation or cross-contamination. Glass dishes were incubated at 28 ºC till the next day. Exposure was conducted with 14 glass dishes representing 7 concentrations and 2 replicates including the negative and positive control (Fig. 1). The glass dish exposure system allowed high resolution video-taping and was therefore preferred over multi-well plates. However, provided that a sufficient resolution can be provided for video recording of the entire well or individual wells, the protocol could be adopted for multi-well plates.

<u>Specific procedure:</u>

1. ISO water was prepared according to 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. Chemical stock solutions were prepared a day before in ISO water or were already standing on the bench when prepared in dimethyl sulfoxide (DMSO). The stock solution was diluted to give lower concentrations in 50 mL standard volumetric flasks.
3. The glass petri-dishes were labelled with the necessary experiment information according to the number of treatments and replicates

4. Twenty fertilized embryos (2-3 hpf) were selected and transferred into each labelled dish. Embryos were transferred into the dishes systematically i.e. first replicate for all treatments starting from lowest to highest concentration were filled and followed by the second replicate. This system models the video acquisition format (see Fig. 1). This ensures that similar time offset is transferred from the embryo selection to the video acquisition phase.
5. The selected embryos were exposed to each chemical concentration and ISO water as negative control. Water was first removed using a pipette and 20 mL of the respective exposure solution was added into the dishes. A solvent control should be used when solvents are used to prepare the chemical solution.
6. The dishes were covered and Incubated at 28°C overnight.

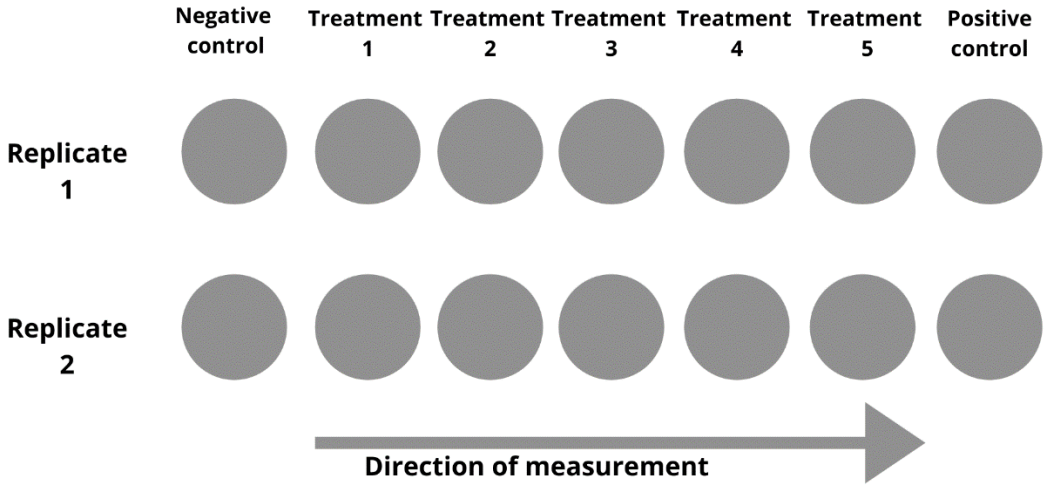

Fig 1. Visual representation of the arrangement of the exposure glass dishes and the direction of measurement during video acquisition. The measurement starts with all treatments of replicate 1 followed by replicate 2.

Video Acquisition

On the next day, the microscope and camera were setup for video acquisition. The embryos were removed from the incubator and allowed to acclimatize at room temperature for at least 30 mins. The acclimatization period aimed to ensure a constant measuring temperature since the microscope was not contained within an incubator. We found in a previous study (Ogungbemi et al. 2020) that this equilibration temperature of 30 mins enabled the reproducible assessment of STC. Beginning at 9AM ± 0.25 h, each glass dish was videotaped for 1 min. The videotaping was done systematically such that the first replicate of each concentration starting from negative controls to highest concentration was recorded, followed by the second replicate (Fig 1). Additionally, the negative controls could be recorded again at the end to ensure the stability of the STC measurement.

Specific procedure:

1) The computer screen, camera (Olympus DP21) and stereomicroscope (Olympus SZX7) were turned-on. Light source was from a LED illumination base of the microscope.
2) The magnification of the microscope was set to 0.8X and the background base was tuned to dark background to create a contrast against the transparent embryos.
3) Camera settings were ISO = 400; shutter speed = 1/80, image size = 400x300 pixels and image resolution = 1600x1200 pixels. Other types of camera including a mobile phone camera may be used to collect videos if quality requirements are met.
4) Embryos at ≈ 24-25 hours post fertilization were removed from the incubator and equilibrated at room temperature for at least 30 min.
5) Embryos were assessed for developmental malformations and lethality under the stereomicroscope. Deformed or dead embryos were removed or separated. Number of removed embryos were recorded in a data sheet.
6) All normal embryos were clustered to the center by slightly swirling the dish and forceps were used to improve the clustering when required. Embryos were placed side by side and not super imposed on each other (Fig. 2a).
7) Embryos were videotaped for 1 min using a stop clock. Embryos could be monitored or observed during video acquisition via the computer screen. It is important to keep the table holding the camera still during video recording.
8) Video acquisition was completed within a period of ≈ 30 min in order to avoid confounding effects of developmental stage within an experiment i.e. 2 min per dish or 28 min for 7 treatments and 2 replicates.
9) A second chemical exposure may be conducted in parallel by following the same procedure as for the first chemical i.e. preparation of chemical solutions, embryo selection may be conducted simultaneously but video acquisition should be performed in blocks of independent experiments.
10) The videos were stored in a mobile drive and transferred to a local or cloud drive for further analysis.

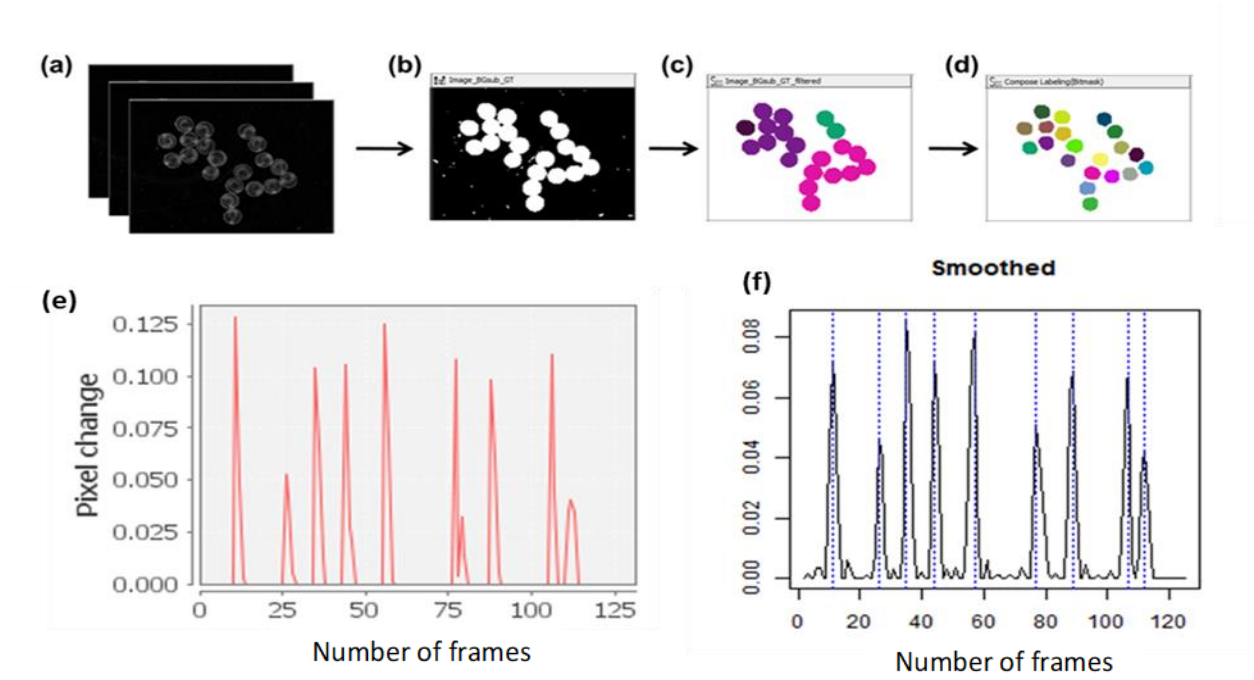

Fig 2. Automated workflow for STC analysis in KNIME. (a) Video files (AVI format) are converted to image stacks. (b) A threshold is applied to identify the location of the embryos in the image, (c) the binary image is segmented and (d) adjacent touching embryos are separated using morphological operations. (e) Variance of pixels between frames are identified to indicate movement. The graph shows the variance of one selected embryo. (f) Each peak (indicated by a dashed vertical line) represents an individual tail flip. Peaks were identified using an R script embedded in a KNIME workflow. The plot shows the STC peaks of one embryo within a duration of 60 s or 120 frames.

KNIME setup and analysis

Description of the KNIME® workflow

The workflow (Fig 3) computes how many times an embryo moves by image analysis using the video recordings. The workflow is divided in several sections using "metanodes" representing a collection of several other nodes, each responsible for a specific calculation (basic description of the KNIME® Analytical Platform can be consulted in Berthold et al. 2009; Copmans et al. 2016).

The workflow requires video recordings in AVI format as input and iterates over all video files in the selected folder. The video files are converted to stacks of images using the FFmpeg tool (2016) at a sample rate of 120 frames per minute. The program is executed by using the external tool node in KNIME®. The individual embryos in the image are detected by applying a threshold for conversion to binary images. Briefly, for each frame a median filter is applied to smooth the image and the background is subtracted using the ImageJ macro node (SubstractBackground function). Then a threshold is automatically set and applied using a variable node. The threshold is based on the mean pixel intensity of all frames, but it can be adjusted depending on the characteristics of the video files. The image with the applied threshold should display embryos completely filled in white and the background in black (Figure 2b). Then images

are segmented to label each embryo independently. In order to separate connected labels of adjacent embryos, the Waehlby cell clump splitter node in KNIME® is used (Waehlby et al 2004). Subsequently, various morphological image operations (e.g. erosion) are applied to optimize segmentation of individual embryos (Figure 2 c-d).

Subsequently the variance of gray values of embryo labels of two successive images is compared to identify movements. Therefore, a lag column is created and the difference of pixel across each video frame stamp is calculated using the image calculator node. Then a threshold is applied and the variance in pixel is extracted using the image segment features node for each labeled embryo. This threshold was set by verifying the concordance between the final KNIME® output and the visual count of the STCs.
Embryo labels between subsequent frames were associated using the centroid of the label. Because embryos may slightly move between each frame, a distance of 15 pixels was allowed between label centroids of individual frames.

Finally, the frequency of movements of each embryo is analyzed by iterating over each label and using as input the pixel variance over all the frames. An increase in variance indicates tail coiling. Therefore, during the time series, peaks representing tail coilings were identified using the function 'findpeaks' of the R package 'quantmod' (Ryan et al 2017) embedded in the KNIME workflow by means of an R snippet node. Figure 2 e-f shows an example of a graph obtained and the identified peaks for each embryo. The STC frequency per minute is calculated taking into account the total duration of the video recordings and at the end, an Excel® file with the same name as the video is automatically saved with the output results.
Note that we included a metanode ("Check for error") after the "position binner" metanode. This node serves as an internal control to detect when there is an error during the image analysis, for example in case that embryos were not correctly identified and labeled. That would require to repeat the analysis of the specific video by adjusting the first threshold value (the one that allows identifying each individual embryo) or correcting the maximum distance of centroid allowed between individual frames.

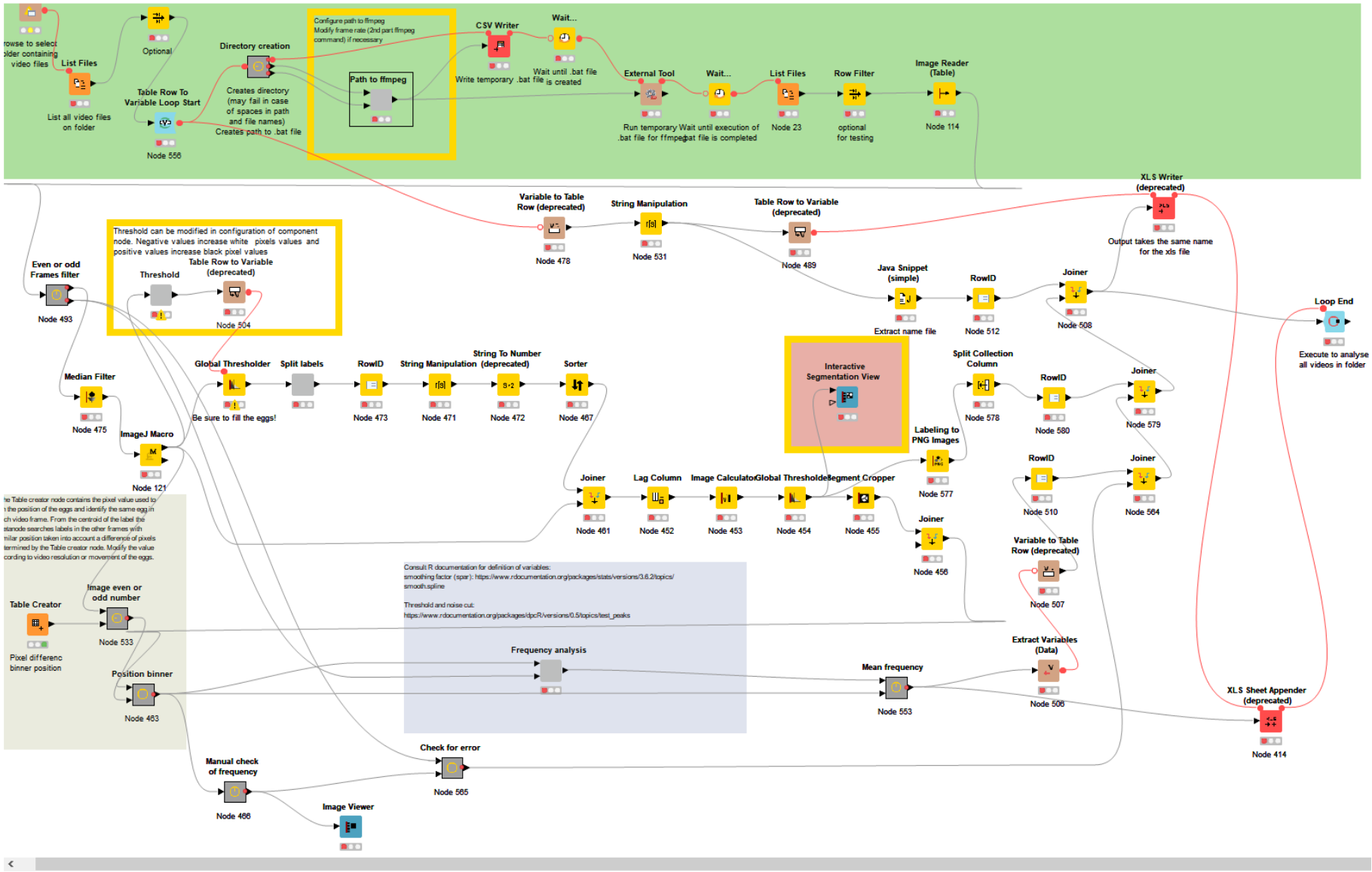

Figure 3: The STC workflow in KNIME

Specific procedure

More details on how to implement the workflow are shown in the youtube video:
https://youtu.be/wgJN71zTvRw

1. Download KNIME (https://www.knime.com/downloads) and install on your PC.
2. Also download the STC workflow and ffmpeg software from KNIME hub and import it into your KNIME software. Open the KNIME software and set the workspace to your preferred folder.
3. Install all necessary KNIME extensions (math formula, quickforms, community image analysis nodes, external tool node) for the STC workflow and restart KNIME.
4. Configure ffmpeg software by setting up the path to its location on your PC using the bin folder as the end of the path.
5. Open R software and install the required packages (quantmod, dpcR and Rserve). If necessary, change the file path of R to the correct location. R error messages can be diagnosed by clicking Eval script in KNIME and then installing the missing packages
6. The video file or folder containing the video files to analyze should be inserted in the KNIME workspace folder, use the Explorer browser node to select the folder containing the video. Alternatively, you can use the List files node to select folders outside of the workspace folder. Specific video files from the folder can be selected using the row filter node next to list files node.
7. The workflow can be executed by clicking the double green arrow in the top menu to start the loop.
8. The workflow creates a folder with the same name as the video file analyzed which contains the stack of images generated. After the analysis they should be removed to save disk space.
9. Changing KNIME parameters:
   a.) Frames per minute can be changed in the ffmpeg component configuration and in the Frequency analysis component configuration. Default is set to 120 frames per minute. Both configurations should have the same frame rate for a correct analysis.
   b.) R parameters for threshold and smoothing can be changed in the Frequency analysis component configuration. Default is set to 0.003 and 0.1 for threshold and smoothing respectively.
   c.) Global threshold for detecting embryo can be changed by subtracting or adding a number in the Threshold component configuration. This controls the global threshold node.

Data treatment

Data was obtained from the KNIME workflow as the number of STCs per minute or STC frequency for one embryo. Fig 2f shows an example of the peak count of an embryo with 9 STC counts per min. The workflow automatically calculates the STC frequency per min based on the length of the video. However, it is also possible to manually calculate the STC frequency especially when a correction is required. A correction protocol may be implemented when the user makes observations that may potentially influence the outcome of the STC analysis i.e. uncontrolled events such as strong signal from movement of whole embryo, many weak peaks close to the peak defining threshold and inaccurate accountability of fast multiple peaks may influence the STC frequency.

Correction protocol

Inspect the peaks: A possible correction protocol could be to visually inspect the peaks for errors e.g. by comparing unsmoothed and smoothed peaks (Fig 4). Irregular shaped, wide peaks and very small peaks are suspects. For example, the suspected wide and weak peaks shown in Fig 4 can be confirmed by inspecting the unsmoothed peaks which display the shape of the erroneous peaks more clearly.

Suspect peaks: Suspected peaks should be verified using the annotated label of the embryo to locate and check embryo movement in the original video (Fig 5). The user would be able to identify errors without checking the original video after a period of video training to identify error peaks. A visual comparison of automated and corrected STC counts for control measurements in different independent experiments shows that the differences are not significant (Fig 6). Therefore, the results from the automated analysis may be used for fast screening of chemicals and correction may only be required for a thorough analysis such as mode of action identification analysis.

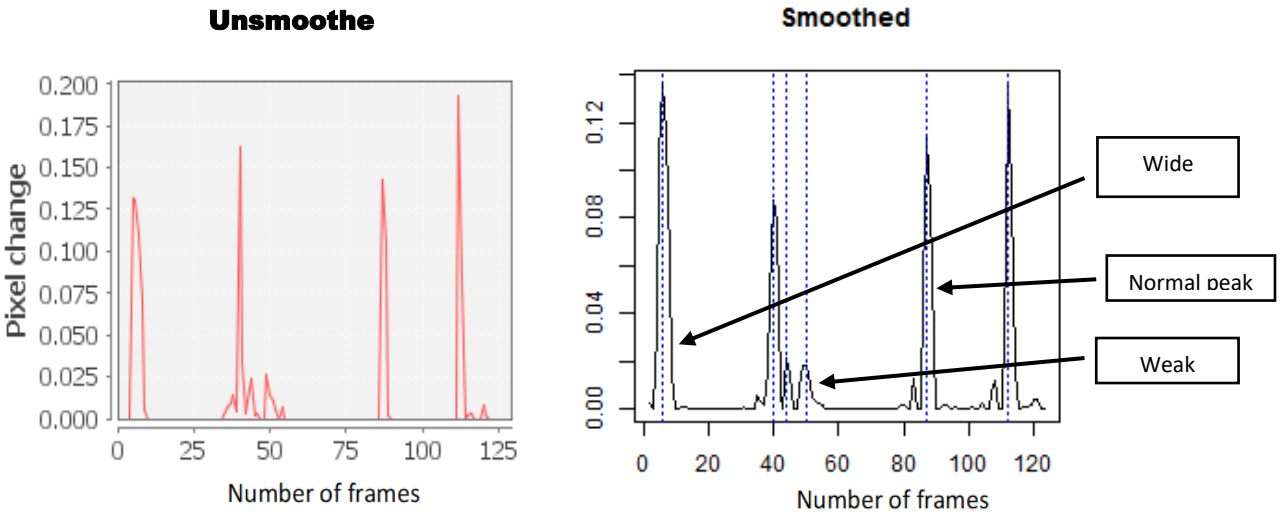

Fig 4. Smoothed and unsmoothed STC peaks. Unsmoothed peaks show the raw peaks without any processing and can be used to validate errors. The wide peaks could be due to fast multiple coils while the weak peaks could be due to movement of whole embryo.

Data analysis

The STC frequency of the individual embryos, the mean of all embryos, the STC peaks and the annotated embryos are compiled as results within an output Excel file from the KNIME workflow. The mean STC frequency (3.3 ± 0.85/min) for untreated embryos varied between independent experiments (Fig 6). To obtain comparable STC results for independent experiments of the same chemical, we had to normalize the STC frequency for different concentrations to that of the untreated embryos to obtain a normalized percentage mean STC frequency.

To determine the actual effect of a chemical, it is possible to analyze the data using concentration-response modelling or hypothesis testing. We performed concentration-response modeling to estimate the EC_{50} – the concentration at which the percentage STC is half-maximum relative to the untreated embryos. Hypothesis testing may also be used when sufficient technical replicates are tested. Shapiro test and Bartlett test could be used to check for normality and homogeneity of variance, respectively. In case normality of the data is not met, non-parametric test such Kruskal-Wallis or Dunnet tests could be used to test for statistical differences between treatment groups. Statistical difference was considered when the p-value < 0.05.

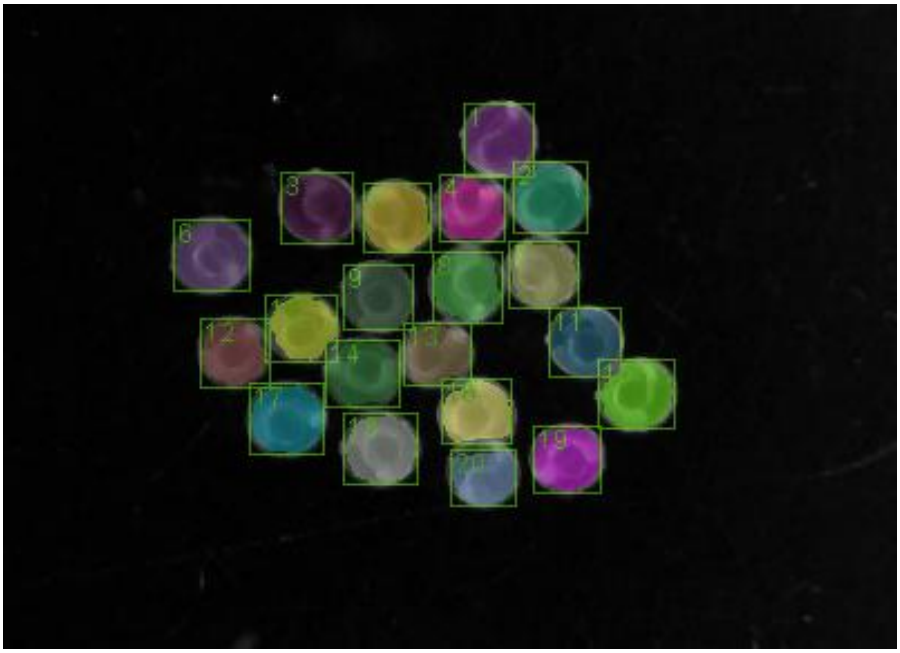

Fig 5. Labelling and annotation of individual embryos. Labels can be used to identify embryos in the video to check for suspected erroneous peaks. Note: Labels in the excel sheet start from 0 while labelled images start from 1. Therefore, embryo 0 in the excel sheet will be embryo 1 in the labelled image.

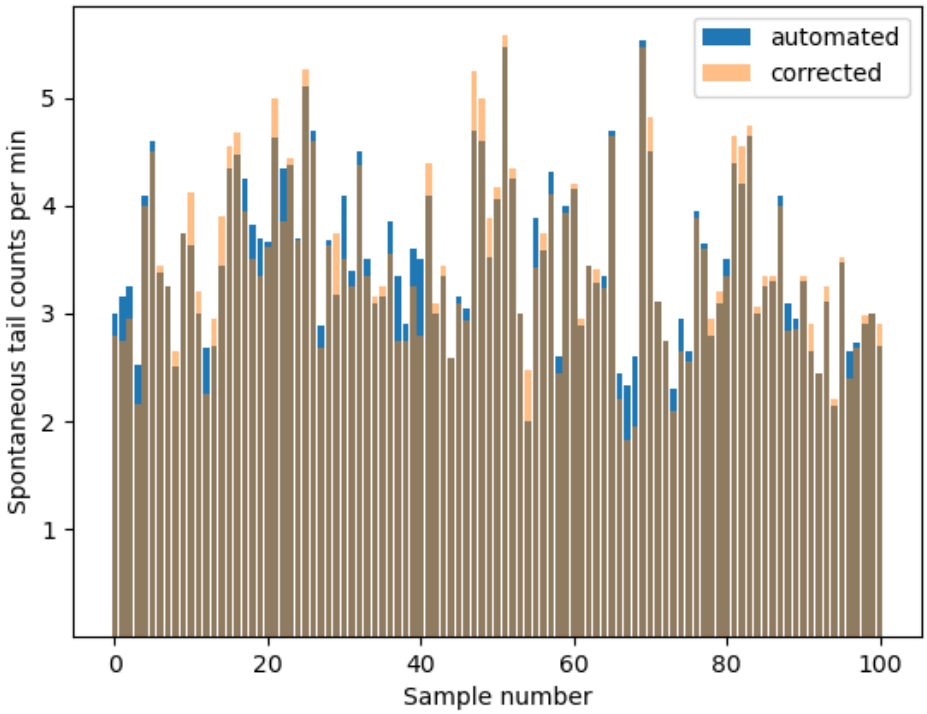

Fig 6. Comparison of automated and corrected STC counts for 100 independent control measurements. Each bar or peak represents the mean STC count for 20 embryos. Dark brown portion of the bars represent areas where automated and corrected counts are the same. Blue portion of the bar represents tests in which automated counts are higher than corrected while yellow portion represents tests in which corrected are higher.

Method validation

The STC test as devised in this study can be used to screen neuroactive chemicals based on the hyper and hypoactivity response of zebrafish embryos. In addition, the STC test may also be used to screen non-neuroactive substances assuming that behavior endpoints are usually more sensitive than lethality. The STC test method described in the current paper have already been applied to screen a range of 18 test chemicals with different modes of action (Ogungbemi et al. 2020). Here we give 3 examples of chemicals with typical modes of action either with an expected hyperactivity or hypoactivity or without any expected effect.

Abamectin is an avermectin insecticide expected to cause hypoactivity by activating Gamma aminobutyric acid-gated chloride channel. Abamectin induced hypoactivity in the STC test at an EC_{50} of 0.055 µM. Four other studies reported hypoactivity for abamectin in the STC test but the reported lowest observed effect concentrations (LOEC) were higher than the EC_{50} found in the current study (Raftery et al. 2014; Raftery and Volz 2015; Vliet et al. 2017; Weichert et al. 2017). This could be due to conducting exposure in plastic well-plates rather than glass as exposure vessel. The only study (Raftery and Volz 2015) that conducted exposure in glass had the least deviation (factor of 4) from our study. Abamectin is highly lipophilic (logDpH7.4(ACD/Labs) of 5.85) and hence has more affinity to bind to plastic than glass, therefore,

abamectin may be more bioavailable to the embryos in a glass container leading to effects occurring at lower concentration. The use of a different endpoint (percentage of organisms showing hypoactivity) could be an additional reason for the deviations in effect concentrations (Ogungbemi et al. 2019). The only study (Weichert et al. 2017) that used the same endpoint (STC frequency) as in the present study had the second least deviation of a factor 6. Further, the analysis duration used in these studies were lower than the 1 min used in the present study and this could also be the cause for inconsistent effect concentrations.

Chlorpyrifos-oxon is a metabolite of chlopyrifos which is an organophosphate insecticide. It acts by inhibiting acetylcholinesterase enzyme which breaks down acetylcholine, and therefore keeps the nicotinic acetylcholine receptors open for sodium ions to flow into the cell leading to an action potential and hence potential hyperactivity. In our study, chlorpyrifos-oxon induced hyperactivity in the STC test at an EC_{50} and EC_{10} of 0.32 and 0.05 µM respectively and this is consistent (the EC_{10}) with the LOEC of 0.03 µM reported by Weichert et al. (2017). These effect concentrations are significantly lower (or more toxic) than that of the parent compound – chlorpyrifos which might be due to the limited bioactivation in the early stages of zebrafish embryo (Ogungbemi et al. 2020).

Pyraclostrobin is a fungicide and expected to not impact on the STC due to its classification as a narcotic or baseline toxic in quantitative structure and activity relationship (QSAR) for zebrafish (Birke and Scholz 2019). Pyraclostrobin did not induce any effect in the STC test up to a concentration of 0.15 µM and similar absence of STC effect up to 0.76 µM were reported by Raftery et al 2014. Figure 7 shows the concentration-response relationships for abamectin, chlorpyrifos-oxon and pyraclostrobin. These results validate the STC test method for screening chemicals.

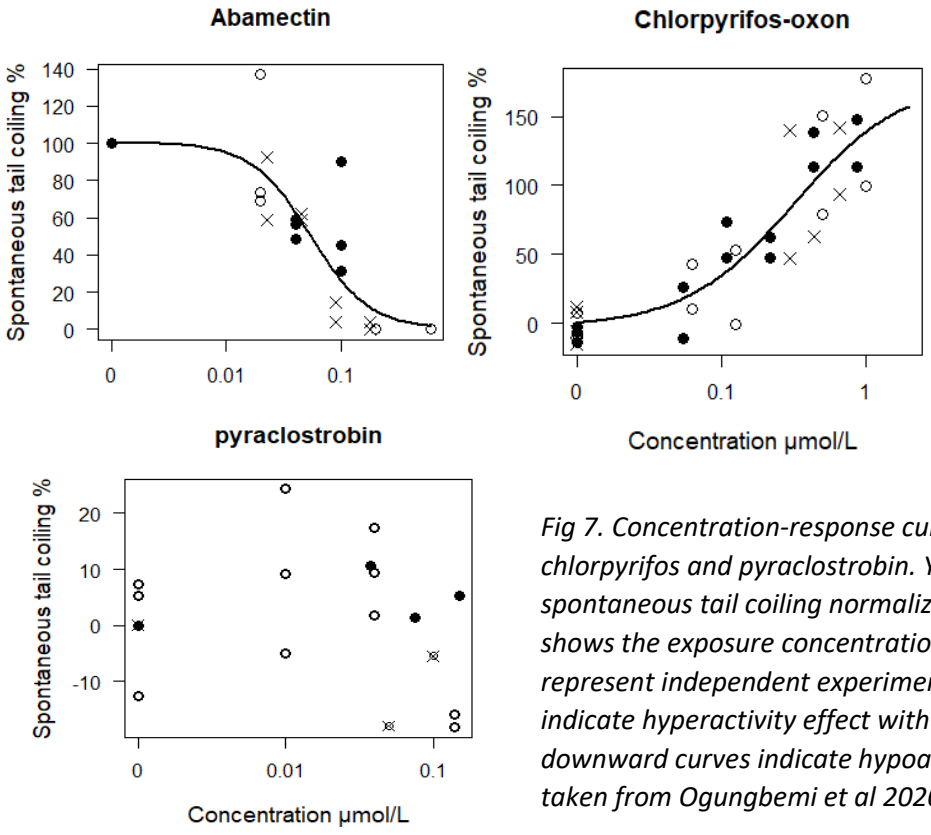

Fig 7. Concentration-response curves for abamectin, chlorpyrifos and pyraclostrobin. Y-axis represents spontaneous tail coiling normalized to control and X-axis shows the exposure concentration. Different symbols represent independent experiments. Upward curves indicate hyperactivity effect with respect to controls while downward curves indicate hypoactivity effect. Figures taken from Ogungbemi et al 2020.

Conclusion

In this paper, we described a protocol for measuring spontaneous tail coiling in zebrafish embryos. First, we gave exhaustive guidelines on how to conduct the experiment based on optimized experimental parameters. Second, we detailed how to automatically analyze the collected video recordings in a workflow with the open source KNIME software. Third, we then validated the described method using three chemicals with different modes of action. The STC test can now be used for the assessment of neuro (developmental) toxicity and testing of both neuro and non-neuroactive compounds. This automated analysis in KNIME provides an easier way to analyze STC over the laborious manual counting in (Weichert et al. 2017). The correction protocol utilized in the current study could enable more accurate estimation of the STC counts than other advanced behavioral tools that may not allow for real-time inspection and correction of STC peaks (eg. Raftery et al. 2014). However, costume tools which are free and solely targeted at STC analysis are recently being developed. In this regard, the KNIME workflow developed in the current study alongside a MATLAB® tool (González-Fraga et al. 2019) and an Image J macro (Zhang et al. 2021) are the current available freeware for STC analysis to our knowledge. Due to video resolution of multi-well system, our STC assessment was developed for a microscope set-up which might reduce throughput. However, we are recently applying improved video resolutions and modified workflows to enable also the assessment of STC in multi-well plates (i.e. Teixido et al. in press). It is also important to recognize that the STC workflow presented in this article does not require an expensive imaging device and can be easily implemented with a camera mounted on a standard dissection microscope. This provides a cost-effective solution for laboratories that would like to add a new, simple and sensitive method to their repertoire of testing tools.

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Declaration of contributions by other scientists to publications and the thesis

I, Afolarin Ogungbemi (AO) was the main author of the publications. The publications were co-authored by David Leuthold (DL); Riccardo Massei (RM); Elisabet Teixido (ET); Rolf Altenburger (RA), Stefan Scholz (SS) and Eberhard Küster (EK).

General contribution to the thesis

EK and SS supervised the whole work and contributed to all publications

RA was part of a yearly PhD committee meeting and contributed to publication 3

DL contributed to Publication 1

RM was part of the funded project and contributed to Publications 2, 3 and 4

ET contributed to the method development and publications 2 and 3

Contribution (approximate) to the publications

Publication 1: Ogungbemi AO,* Leuthold D, Scholz S, Küster E (2019) Hypo-or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. Environmental Sciences Europe 31:88. https://doi.org/10.1186/s12302-019-0270-5

Conceptualization: AO 70 %, EK 20 %, SS 10 %

Methodology: AO 70 %, EK 15 %, SS 15 %

Data processing and analysis: AO 80 %, EK 10 %, SS 10 %

Data Interpretation: AO 70 %, EK 10 %, SS 10 %, DL 10 %

Writing original draft: AO 100 %

Review and editing: All authors

Publication 2: Ogungbemi AO,* Teixido E, Massei R, Scholz S, Küster E (2020) Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. Neurotoxicology and Teratology 81:106918. https://doi.org/10.1016/j.ntt.2020.106918

Conceptualization: AO 90 %, EK 10 %

Methodology: AO 80 %, ET 10 %, RM 10 %

Data processing and analysis: AO 80 %, ET 20 %

Data Interpretation: AO 80 %, EK 10 %, SS 5 %, ET 5 %

Writing original draft: AO 100 %

Review and editing: All authors

Publication 3: Ogungbemi AO,* Massei R, Altenburger R, Scholz S, Küster E (2021) Assessing combined effects for mixtures of similar and dissimilar acting neuroactive substances on zebrafish embryo movement. Toxics *9*(5):104.

Conceptualization: AO 70 %, EK 15 % RA 5 %, RM 5 %, SS 5 %

Methodology: AO 80 %, RM 20 %

Data processing and analysis: AO 90 %, EK 5 %, RA 5 %

Data Interpretation: AO 70 %, EK 10 %, SS 10 %, RA 10 %

Writing original draft AO 100 %

Review and editing: All authors

Publication 4: Ogungbemi AO,* Teixido E, Massei R, Scholz S, Küster E (2020) Automated measurement of the spontaneous tail coiling of zebrafish embryos as a sensitive behavior endpoint using a workflow in KNIME. MethodsX 8:101330.

Conceptualization: AO 80 %, EK 5 %, ET 5 %, SS 5 %, RM 5 %

Methodology: AO 80 %, ET 20 %

Data processing and analysis: AO 50 %, ET 30 %, SS 20 %

Data Interpretation AO 70 %, EK 10 %, SS 10 %, ET 10 %

Writing original draft AO 100 %

Review and editing: All authors

DECLARATION

I, Afolarin Ogungbemi, declare that this PhD thesis entitled 'Toxicological Characterization and screening of Neuroactive Chemicals and Mixtures using zebrafish embryo behavior' has been independently drawn up by me and the contribution of other scientists have been clearly documented. I have also acknowledged all aids and sources used and have cited these in the reference section. I received no paid assistance whatsoever by any sort of PhD service agencies. This dissertation has not been submitted for scientific examination at any other university or faculty in Germany or in another country. I am aware that the PhD title can be revoked and legal consequences are possible as a result of failure to regard any of the aforementioned points.

Landau, 25.05.2021

Signature of student

Afolarin Ogungbemi

Afolarin Ogungbemi

Education

2017-08 - Current

Ph.D. Ecotoxicology

University of Koblenz-Landau - Landau, Germany

Thesis at Helmholtz Centre for Environmental Research, Leipzig, Germany: Toxicological Characterization and Screening of Neuroactive Chemicals and Mixtures using Zebrafish Embryo Behavior

2014-10 - 2017-05

Master of Science: Ecotoxicology

University of Koblenz-Landau - Landau, Germany

Thesis at Vrije Universiteit Amsterdam, Netherlands: The Influence of Soil Organic Matter Content and the Role of Soil Porewater on the Toxicity of Imidacloprid to the Springtail *Folsomia candida*

2012-02 - 2014-02

Master of Science: Analytical Chemistry

University of Ibadan - Ibadan, Nigeria

Thesis: Decontamination of Automobile Workshop Soils containing Heavy Metals and PAHs using Chelating Agents

2006-05 - 2009-11

Bachelor of Science: Chemistry

University of Ibadan - Ibadan, Nigeria

Thesis: Analysis of heavy metals and total petroleum hydrocarbons in sediments

Publications

***Corresponding author**

1. **Ogungbemi, A.O.** and van Gestel, C.A.M.,* 2018. Extrapolation of imidacloprid toxicity between soils by exposing *Folsomia candida* in soil pore water. *Ecotoxicology*, 27(8), pp. 1107-1115. https://doi.org/10.1007/s10646-018-1965-x

2. **Ogungbemi, A.O.,*** Leuthold, D., Scholz, S. and Küster, E., 2019. Hypo-or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. *Environmental Sciences Europe*, 3 1(1), p.88. https://doi.org/10.1186/s12302-019-0270-5

3. **Ogungbemi, A.O.,*** Teixido, E., Massei, R., Scholz, S. and Küster, E., 2020. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. *Neurotoxicology and Teratology*, p. 106918. https://doi.org/10.1016/j.ntt.2020.106918

4. Ipeaiyeda, A.R. and **Ogungbemi, A.O.,*** 2020. Decontamination of Automobile Workshop Soils containing Heavy Metals and PAHs using Chelating Agents. *International Journal of Environmental Pollution and Remediation*, *8*, pp.37-45.

5. **Ogungbemi, A.O.,*** Teixido, E., Massei, R., Scholz, S. and Küster, E., 2020. Automated measurement of the spontaneous tail coiling of zebrafish embryos as a sensitive behavior endpoint using a workflow in KNIME. *MethodsX*, *8*, p.101330.

6. **Ogungbemi, A.O.,*** Massei, R., Altenburger, R., Scholz, S. and Küster, E., 2021. Assessing combined effects for mixtures of similar and dissimilar acting neuroactive substances on zebrafish embryo movement. *Toxics*, *9*(5), p.104.

7. Teixidó, E.,* Klüver, N., **Ogungbemi, A.O.,** Küster, E., Scholz, S., 2021. Evaluation of Neurotoxic effects in zebrafish embryos by automatic measurement of early motor behaviors. In: Experimental Neurotoxicology Methods. pp. 381-397. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1637-6_17

Conference presentations

1. **Ogungbemi A.O.,** Lima C., van Gestel C.A.M., 2017. Predicting imidacloprid toxicity for the springtail *Folsomia candida* in soil from pore-water exposure scenarios. **Oral presentation**. SETAC Young Environmental scientists meeting, 16-20 February 2017, Stockholm, Sweden.

2. **Ogungbemi A.O.,** Kenngott K., Altendorfer K., Kurtz S., Schaumann G.E., Brühl C., Jager T., van Gestel C.A.M., Kurtz M.P., 2017. Age Sensitivity Distribution of the Springtail *Folsomia candida*: An aquatic test approach. **Poster Presentation**. SETAC Europe Annual meeting, 7 – 11 May 2017, Brussels, Belgium.

3. **Ogungbemi A.O.,** and van Gestel C.A.M., 2017. Proposal of a tiered effect assessment for chemicals in the soil: A regulatory perspective. **Oral presentation**. SETAC Africa Biennial meeting, 17 – 19 October 2017, Calabar, Nigeria.

4. **Ogungbemi A.O.,** Leuthold D., Scholz S., and Küster, E., 2018. Comparability of zebrafish behavioral tests: A need for standardization of experimental parameters. **Poster Presentation**. SETAC Europe Annual meeting, 13 – 17 May 2018, Rome, Italy.

5. **Ogungbemi A.O.,** Leuthold D., Scholz S., and Küster, E., 2018. Hypo/hyper-behavioral response of zebrafish embryo to chemicals: Analysis of comparability and predictability of mode of action. **Oral Presentation**. International Symposium on Fish and amphibian embryos as alternative models in toxicology and teratology, 29-30 November 2018, Paris, France.

6. **Ogungbemi, A.O.,** Teixido, E., Massei, R., Scholz, S. and Küster, E., 2019. Detection of hyper- and hypoactivity in zebrafish embryo using the spontaneous tail coiling test. **Poster Presentation**. SETAC Young Environmental scientists meeting (SETAC), 5-10 February 2019, Ghent, Belgium.

7. **Ogungbemi A.O.,** Leuthold D., Teixido, E., van Gestel C.A.M., Scholz S., and Küster, E., 2019. The use of behavioral screening in pollution control: Locomotion response as a sensitive endpoint in fish and invertebrates. **Oral Presentation**. SETAC Africa Biannual meeting, 6-8 May 2019, CapeTown, South Africa.

8. **Ogungbemi, A.O.,** Massei, R., Altenburger, R., Scholz, S. and Küster, E., 2019. Determination of mixture neurotoxicity using the hyper- and hypoactivity behavior of zebrafish embryo in the spontaneous tail coiling test. **Oral Presentation**. SETAC North-America annual meeting, 3-7 November 2019, Toronto, Canada.

9. **Ogungbemi A.O.,** Scholz S., and Küster, E., 2021. Does the organophosphate chlorpyrifos and its bioactivated oxon-metabolite have similar mechanism of action in early stages of zebrafish embryo? **Oral Presentation**. SETAC Young Environmental scientists meeting, 22 -26 February 2021, Virtual.

10. **Ogungbemi, A.O.,** Scholz, S., Küster, E., and Massei, R., 2021. Effect-based monitoring of a wastewater effluent using the spontaneous tail coiling (STC) of zebrafish embryos. **Oral Presentation**. SETAC Europe annual meeting, 3-6 May 2021, Virtual.

Grant and Awards

Successful

- FAZIT completion scholarship, FAZIT Stiftung, Germany (2021)
- SETAC World Council Presidential Citations Award (2020)
- Best oral presentation prize, Society of Environmental Toxicology and Chemistry, Calabar, Nigeria (2017)
- Conference grant, Society of Environmental Toxicology and Chemistry (SETAC), Brussels, Belgium (2017) and Toronto, Canada (2019)
- Scholarship for outstanding Master's student (Aufland-Nachwuchsstipendium), University of Koblenz-Landau, Germany (2016)
- DAAD PROMOS Scholarship, German Academic Exchange Services, Germany (2016)
- Erasmus mobility Grant, EUservice point, Germany (2015)
- International Student's Scholarship Award, University of Koblenz-Landau, Germany (2014)
- Tutorial Assistantship Award, University of Ibadan, Ibadan, Nigeria (2013)

Declined

- EU Marie Skłodowska-Curie Actions Individual Fellowships, 2021. **Ranked 78%**
- DAAD Postdoctoral Researchers International Mobility Experience (PRIME) 2021. **Ranked top 17% out of 182 applicants.**

Volunteer Experience

- Chair person, Students Advisory Council, Society of Environmental Toxicology and Chemistry (SETAC Africa). 2019 till present.
- Member, SETAC World Congress scientific committee. 2020
- Student representative, Executive council, Society of Environmental Toxicology and Chemistry (SETAC Africa). 2017 – 2019
- Member, HIGRADE Graduate school conference planning committee, Helmholtz Centre for Environmental Research, Leipzig, Germany. 2018
- Chair, Intercultural party organizing committee, University of Koblenz-Landau, Germany. 2016
- General Secretary, Students Chemical Society of Nigeria (SCSN, Uni-Ibadan branch). 2008 - 2009

Language skills

- English Language: Expert C2 level
- German Language: Advanced B2 level
- French Language: Beginner A1 level

There is positivity in every negativity. In the face of challenges, hold your heads up, look towards the brighter side and never give up because there is light at the end of the tunnel

These words kept me going when I felt like giving up

THE END

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