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PHD DISSERTATION 4 | 2020

David Leuthold

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Helmholtz Centre for Environmental Research – UFZ
Department of Bioanalytical Ecotoxicology

Fakultät für Mathematik, Informatik und Naturwissenschaften | RWTH AACHEN UNIVERSITY

Zebrafish Locomotor Activity as a Sensitive Effect-Based Tool for the Assessment of Environmental Chemicals and Mixtures

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

vorgelegt von

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Tag der mündlichen Prüfung: 09. Juli 2020

Glaube denen, die die Wahrheit suchen, und zweifle an denen, die sie gefunden haben.

André Gide

Abstract

Chemical contamination poses a risk to the aquatic environment. Chemicals can affect the biological quality of surface waters, i.e. the composition of aquatic communities. The preservation or restoration of a good ecological status of surface water bodies requires the determination of the chemical status and the assessment of its effects on biological quality. Current regulatory assessments are restricted to single priority chemicals. This approach does not account for the entirety of ecologically relevant chemicals, transformation products, or the joint effects of chemical mixtures that are ubiquitously present in water bodies.

Various toxicity tests are used to assess biological effects of environmental chemicals on aquatic organisms. However, acute toxicity tests, such as the fish embryo acute toxicity test (OECD test no. 236), lack sensitivity to detect sub-lethal chemical toxicity, particularly in the realm of chemicals that target the developing nervous system. Therefore, new approaches are required to quantify the effects of chemicals and mixtures on neurotransmission and neurodevelopment. Embryo-larval stages of zebrafish (*Danio rerio*) represent an excellent model system for the detection of chemical neurotoxicity, particularly during early development which represents one of the most vulnerable life stages to chemical exposure. The functionality of the zebrafish nervous system can be determined using automated behavior assays. However, key variables that influence chemical-dependent behavioral effects are not well characterized. Therefore, this thesis seeks to refine the execution, computational analyses, and applicability domain of an automated light-dark transition zebrafish behavior assay to ultimately delineate distinct behavioral responses following exposure to environmental chemicals and mixtures.

From an ecological perspective, adequate behavior is indispensable for biological functions such as migration, feeding, predator avoidance, and mating. It is widely accepted that exposure to chemicals can alter behavior in various species including *D. rerio*. For example, exposure to a wide array of neuroactive chemicals such as certain pesticides (e.g. diazinon, aldicarb, or chlorpyriphos) or pharmaceuticals (e.g. anxiolytic drugs like oxazepam) can produce hyper- and/or hypolocomotion via disruption of distinct signaling pathways such as cholinergic or GABAergic neurotransmission, respectively. In addition, alterations in behavior related to exposure to neuroactive chemicals partially occur at concentrations well below those that cause lethality. Altered locomotor behavior therefore represents a sensitive and ecologically relevant outcome that can be used to assess exposure to chemicals and mixtures with widespread environmental occurrence. However, there are several knowledge gaps that hinder the use of automated zebrafish behavior assays for the identification of neuroactive and developmentally neurotoxic chemicals and mixtures. These include (i) a

complete understanding of the experimental variables that influence and potentially confound chemical testing, (ii) the unbiased identification of environmental chemicals that harbor the capacity to disrupt locomotor behavior, (iii) the illumination of underlying biological mechanisms by which environmental chemicals alter locomotion, and (iv) the application of single chemical exposure phenotypes to predict and diagnose the effects of chemicals on zebrafish locomotor behavior in defined mixtures.

The main goal of this work was to evaluate and advance the use of a behavior-based assay in zebrafish for the ecotoxicological assessment of chemicals and mixtures. This work was carried out in four steps. In step one, a reproducible light-dark transition assay was established and the influence of intrinsic and extrinsic parameters (e.g. developmental stage or light regimens) was determined (**Chapter 2**). In the second step, behavioral profiling and concentration-response modeling were used to differentiate and assess the effects of 18 environmental chemicals with different suspected modes of action (**Chapter 3**). In **Chapter 4**, biological mechanisms, particularly acetylcholinesterase inhibition, were explored as essential pathways that cause chemical-induced hyperactivity. Finally, the ability to predict the effect of chemical mixtures on locomotion based on locomotor phenotypes obtained with single chemical constituents was investigated. In addition, the diagnostic capacity of behavioral phenotyping in the identification of putative chemical drivers of mixture toxicity was evaluated (**Chapter 5**).

The current work revealed several key findings. First, different exposure regimens during embryo-larval zebrafish development significantly influence behavioral responses, including hypo- and hyperactive phenotypes. In particular, chemical exposure throughout development was shown to affect morphological features relevant for proper locomotion such as swim bladder inflation, and may therefore mask or mimic neurodevelopmental outcomes. Thus, to avoid chemical-induced malformations that confound locomotor behavior, acute exposure can be employed to assess the direct effects of chemicals on neurotransmission (**Chapter 2**). Second, a suite of compounds with known neuroactive properties were compared to a set of environmentally relevant chemicals with non-neurotoxic modes of action. Novel computational analyses revealed enhanced sensitivity (>10 fold) in the behavior assays relative to mortality for chemicals with proposed neuroactive modes of action. In contrast, behavior-based and lethal effect concentrations were indistinguishable for compounds with non-neurotoxic modes of action. Exposure to certain chemical classes such as acetylcholinesterase inhibitors produced shared behavioral phenotypes. However, for the majority of chemicals evaluated, behavioral profiling revealed partially similar phenotypes for distinct mode of action classes (**Chapter 3**). From a mechanistic perspective, while inhibition of acetylcholinesterase activity and/or reduced heart rate were initially considered as

potential causes of chemical-dependent hyperlocomotion, a clear link between those endpoints could not be confirmed. Instead, multiple zebrafish models of epileptic seizures suggest that a complexity of molecular initiating events and/or key events may lead to hyperlocomotion in individuals exposed to different environmental chemicals (**Chapter 4**). Furthermore, behavioral phenotypes determined with an environmentally relevant five-component mixture were found to resemble the locomotor patterns determined with distinct mixture constituents (**Chapter 5**).

This work represents the basis for the efficient application of an automated locomotor activity test as a sensitive, sub-lethal, predictive, and diagnostic measure of the biological activity of single chemicals and mixtures *in vivo*. Overall, this work confirmed that various experimental parameters determine behavioral outcomes (**Chapter 2**). Furthermore, exposure to a multiplicity of environmental chemicals can alter zebrafish behavior at concentrations below those that elicit lethality (**Chapter 3**). Specific behavioral patterns, such as light-induced hyperlocomotion, can indicate the disturbance of specific molecular or physiological pathways (**Chapter 4**). Finally, resulting behavioral phenotypes possess valuable diagnostic information in the assessment of chemical mixtures (**Chapter 5**). These findings are important to optimize and standardize automated behavior assays for the identification of neuroactive environmental chemicals, determine the applicability domain of behavioral tests, verify behavioral alterations as a sensitive outcome of chemical exposure, and establish how behavioral patterns can be used as a diagnostic tool. This work raises further questions relevant to utilize the presented approaches in ecotoxicological assessments such as: What are the underlying biological mechanisms by which chemicals cause locomotor hyperactivity (and also other distinct phenotypes)? How do multiple complex modes of action converge to disrupt behavior in individuals exposed to environmentally relevant mixtures? Taken together, this work represents new material knowledge to address the design, execution, and interpretation of locomotor activity assays for the identification and characterization of neurotoxicity outcomes in zebrafish exposed to chemicals and mixtures.

Zusammenfassung

Chemische Verschmutzung stellt eine Gefahr für die aquatische Umwelt dar. Dabei können sich Chemikalien auf die biologische Qualität von Gewässern, d.h. die Zusammensetzung aquatischer Lebensgemeinschaften, auswirken. Um den guten ökologischen Zustand von Oberflächengewässern zu erhalten oder wiederherzustellen muss deren chemischer Zustand erfasst und dessen Auswirkung auf die biologische Qualität bewertet werden. Derzeitige regulatorische Maßnahmen zur Bewertung des chemischen Zustandes beschränken sich auf einzelne, prioritäre Chemikalien. Dieser Ansatz berücksichtigt weder die Gesamtheit ökologisch relevanter Chemikalien und Transformationsprodukte, noch die Kombinationseffekte chemischer Mischungen, welche ubiquitär in Gewässern verbreitet sind.

Um die biologische Wirkung von Umweltchemikalien auf aquatische Organismen zu bewerten, werden verschiedene Toxizitätstests verwendet. Jedoch mangelt es akuten Toxizitätstests, wie z.B. dem akuten Fischembryotoxizitätstest (OECD Test Nr. 236), an Sensitivität um subletale chemische Toxizität, insbesondere von Chemikalien die auf das sich entwickelnde Nervensystem abzielen, zu detektieren. Daher bedarf es neuer Ansätze um die Wirkung von Chemikalien und Mischungen auf die neuronale Reizweiterleitung und Neuronalentwicklung zu quantifizieren. Das embryo-larvale Stadium des Zebrabärblings (*Danio rerio*), welches eines der empfindlichsten Lebensstadien für chemische Exposition darstellt, repräsentiert ein herausragendes Modellsystem für die Bestimmung chemischer Neurotoxizität. Die Funktionalität des Zebrafisch-Nervensystems kann mittels automatisierter Verhaltenstests untersucht werden. Jedoch sind Schlüsselvariablen, die chemikalienabhängige Verhaltenseffekte beeinflussen, bisher unzureichend charakterisiert. Daher ist es das Ziel dieser Dissertation die Durchführung, Computer-gestützte Auswertung und den Anwendungsbereich eines automatisierten Hell-Dunkel-Verhaltenstests im Zebrafisch weiterzuentwickeln um schließlich verschiedene Verhaltensantworten nach der Exposition gegenüber Chemikalien und Mischungen zu charakterisieren.

Aus ökologischer Sicht ist adäquates Verhalten unabdingbar für biologische Funktionen wie Migration, Ernährung, Vermeidung von Fressfeinden, sowie Paarung. Es ist weithin anerkannt, dass Chemikalienexposition das Verhalten diverser Spezies, einschließlich *D. rerio*, verändern kann. Zum Beispiel führt die Exposition gegenüber eines breiten Spektrums neuroaktiver Chemikalien, wie etwa Pestiziden (z.B. Diazinon, Aldicarb, oder Chlorpyriphos) oder Pharmaka (z.B. Angst lösende Mittel wie Oxazepam), zu Hyper- und/oder Hypoaktivität durch die Störung bestimmter Signalwege wie etwa cholinerger beziehungsweise GABAerger Neurotransmission. Darüber hinaus treten Verhaltensveränderungen aufgrund der Exposition gegenüber neuroaktiven Chemikalien teilweise in subletalen

Konzentrationsbereichen auf. Verändertes lokomotorisches Verhalten stellt daher eine sensitive, ökologisch relevante Wirkung dar, welche genutzt werden kann um die Exposition gegenüber in der Umwelt weitverbreiteten Chemikalien und Mischungen zu bewerten. Jedoch gibt es einige Wissenslücken welche die Nutzung automatisierter Verhaltenstests mit Zebrabärblingen zur Identifizierung neuroaktiver und entwicklungs-neurotoxischer Chemikalien und Mischungen erschweren. Diese beinhalten: (i) ein vollständiges Verständnis experimenteller Parameter welche die Chemikalientestung beeinflussen und potenziell beeinträchtigen, (ii) die Identifizierung von Umweltchemikalien die in der Lage sind lokomotorisches Verhalten zu stören, (iii) die Aufklärung zugrundeliegender biologischer Mechanismen durch die Umweltchemikalien Fortbewegung beeinflussen, und (iv) die Anwendung von, durch einzelne Substanzen hervorgerufenen, Phänotypen zur Vorhersage und Diagnose chemischer Mischungseffekte auf das lokomotorische Verhalten von Zebrabärblingen.

Das Hauptziel dieser Arbeit war es, die Nutzung verhaltensbasierter Testverfahren in Zebrabärblingen für die ökotoxikologische Bewertung von Chemikalien und Mischungen zu beurteilen und weiterzuentwickeln. Diese Arbeit wurde in vier Schritten durchgeführt. Im ersten Schritt wurde ein reproduzierbarer Hell-Dunkel-Test etabliert und der Einfluss intrinsischer und extrinsischer Variablen (z.B. Entwicklungsstadium oder Lichtverhältnisse) bestimmt (**Kapitel 2**). Im zweiten Schritt wurde die verhaltensbasierte Charakterisierung und Konzentrations-Wirkungs-Modellierung genutzt um die Effekte von 18 Umweltchemikalien mit unterschiedlichen Wirkmechanismen zu differenzieren und zu bewerten (**Kapitel 3**). In **Kapitel 4** wurden biologische Mechanismen, insbesondere die Inhibierung der Acetylcholinesterase, als grundlegende Wirkungspfade untersucht, die zu chemisch induzierter Hyperaktivität führen. Schließlich wurde die Vorhersagbarkeit chemischer Mischungseffekte auf das Bewegungsverhalten, auf Grundlage lokomotorischer Phänotypen hervorgerufen durch Einzelchemikalien, untersucht. Darüber hinaus wurde das diagnostische Potenzial verhaltensbasierter Phänotypisierung bei der Identifizierung vermeintlicher Treiber chemischer Mischungstoxizität bewertet (**Kapitel 5**).

In der vorliegenden Arbeit wurden verschiedene, wesentliche Erkenntnisse erzielt. Erstens, unterschiedliche Expositionsszenarien während der embryo-larvalen Entwicklung des Zebrabärblings haben einen wesentlichen Einfluss auf die Verhaltensantworten, sowohl bei hypo- also auch bei hyperaktiven Phänotypen. Insbesondere wurde gezeigt, dass eine chemische Exposition im Verlauf der Entwicklung für die Fortbewegung relevante morphologische Merkmale, wie die Schwimmblasenfüllung, beeinträchtigt und daher Auswirkungen auf die Entwicklung des Nervensystems maskiert oder imitiert. Um chemisch verursachte Deformationen, welche das lokomotorische Verhalten beinträchtigen können, zu

vermeiden, kann eine akute Exposition angewandt werden, um die direkte Wirkung von Chemikalien auf die neuronale Impulsübertragung zu detektieren (**Kapitel 2**). Zweitens wurde eine Reihe von Stoffen mit bekannten neuroaktiven Eigenschaften mit einer Gruppe von umweltrelevanten Chemikalien mit nicht-neurotoxischen Wirkmechanismen verglichen. Eine neue, Computer-gestützte Analysemethode offenbarte eine, im Vergleich zu Letalität, erhöhte Sensitivität (>10-fach) des Verhaltenstests für neuroaktive Chemikalien. Im Gegensatz dazu bewegten sich verhaltens- und letalitätsbasierte Effektkonzentrationen für Chemikalien mit nicht-neurotoxischer Wirkung in einem ähnlichen Konzentrationsbereich. Eine Exposition gegenüber bestimmten Chemikalienklassen, wie Acetylcholinesterase-Inhibitoren, verursachte ähnliche Verhaltens-Phänotypen für verschiedene Substanzen. Jedoch zeigte die verhaltensbasierte Charakterisierung für die Mehrzahl der untersuchten Chemikalien teilweise ähnliche Phänotypen für unterschiedliche Wirkmechanismen (**Kapitel 3**). Aus mechanistischer Sicht wurden zunächst die Inhibierung der Acetylcholinesterase-Aktivität und/oder eine reduzierte Herzschlagrate als mögliche Gründe für Hyperaktivität in Betracht gezogen. Jedoch konnte kein klarer Zusammenhang dieser Endpunkte hergestellt werden. Stattdessen weisen mehrere Zebrabärblings-basierte Epilepsiemodelle darauf hin, dass verschiedene molekulare und/oder Schlüsselereignisse zur Hyperaktivität bei Individuen führen die gegenüber unterschiedlichen Umweltchemikalien exponiert sind (**Kapitel 4**). Des Weiteren wurde festgestellt dass Verhaltensphänotypen die mit einer umweltrelevanten Fünf-Stoff-Mischung bestimmt wurden, den lokomotorischen Mustern einzelner Mischungskomponenten ähneln.

Diese Arbeit repräsentiert die Grundlage für die effiziente Anwendung eines automatisierten Verhaltenstests als sensitives, subletales, prädiktives und diagnostisches Maß der biologischen Wirkung einzelner Chemikalien und Mischungen *in vivo*. Insgesamt bestätigte diese Arbeit dass verschiedene experimentelle Parameter beobachtete Verhaltenseffekte maßgeblich beeinflussen können (**Kapitel 2**). Außerdem führt die Exposition gegenüber einer Vielzahl von Umweltchemikalien bei Zebrabärblingen zu Verhaltensveränderungen in subletalen Konzentrationsbereichen (**Kapitel 3**). Spezifische Bewegungsmuster, wie etwa lichtabhängige Hyperaktivität, können die Störung spezifischer molekularer und physiologischer Signalwege anzeigen (**Kapitel 4**). Schließlich besitzen Verhaltensphänotypen einen nützlichen, diagnostischen Informationsgehalt bei der Bewertung chemischer Mischungen (**Kapitel 5**). Diese Ergebnisse sind wesentlich um automatisierte Verhaltenstests für die Identifizierung neuroaktiver Umweltchemikalien zu optimieren und standardisieren, den Anwendungsbereich von Verhaltenstests zu bestimmen, Verhaltensänderungen als sensitiven Effekt chemischer Exposition zu verifizieren und um spezifische Bewegungsmuster als diagnostisches Werkzeug zu etablieren. Basierend auf den Ergebnissen dieser Arbeit ergeben sich weitere Fragen, welche relevant für den Einsatz

der hier beschriebenen Ansätze im Rahmen ökotoxikologischer Bewertung sind, z.B.: Was sind die zugrundeliegenden biologischen Mechanismen durch die Chemikalien lokomotorische Hyperaktivität (und andere spezifische Phänotypen) hervorrufen? Wie konvergieren verschiedene Wirkmechanismen bei Individuen die umweltrelevanten Mischungen ausgesetzt sind auf Verhaltensebene? Insgesamt bietet diese Arbeit neue Fachkenntnisse bezüglich Design, Durchführung und Interpretation von Verhaltenstests für die Identifizierung und Charakterisierung neurotoxischer Effekte von Chemikalien und Mischungen im Zebrabärbling.

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% v/v	volume percent
4C	4-cell stage
AChE	acetylcholinesterase
AFT	acute fish toxicity
ALD	aldicarb
ANOVA	analysis of variance
AO	adverse outcome
AOP	adverse outcome pathway
BCF	bioconcentration factor
bpm	beats per minute
CA	concentration addition
CAS RN	Chemical Abstracts Service registry number
CI	confidence interval
CNS	central nervous system
COX	cyclooxygenase
CTL	control
CYP	cyprodinil
DIC	diclofenac
DIU	diuron
DZN	diazinon
dpf	days post fertilization
EBT	effect-based tool
ECx	median effect concentration at which x% of the tested population is affected
E-R	estrogen receptor
EY50	total effect, i.e. absolute difference in distance moved between treatment and control at EC50
FET	Fish embryo acute toxicity
GABA	gamma-aminobutyric acid
GABA-R	gamma-aminobutyric acid receptor
hpf	hours post fertilization
IA	independent action
L4	larval stage, day 4
LCx	median lethal concentration at which x% of the tested population die
LMR	locomotor response
LOEC	lowest observed effect concentration

MDS	multidimensional scaling
MEC	measured environmental concentration
MetS	methionine synthesis
MIE	molecular initiating event
MOA	mode of action
MTP	metoprolol
MW	molecular weight
MX3	three-component mixture
MX4	four-component mixture
MX5	five-component mixture
nACh-R	nicotinic acetylcholine receptor
NAP	naproxen
NMDA	N-methyl-d-aspartate
NOEC	no observed effect concentration
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
OECD	Organization of Economic Cooperation and Development
P6	prim 6 stage
PMR	photomotor response
PSII	photosystem II
PTZ	pentylenetetrazol
QSAR	quantitative structure-activity relationship
REACh	Registration, Evaluation, Authorization and Restriction of Chemicals
rpm	revolutions per minute
SD	standard deviation
SEM	standard error of the mean
SNR	serotonin-norepinephrine reuptake
SSR	selective serotonin reuptake
Sw	limit of water solubility
TPP	triphenylphosphate
VER	verapamil
ZFE	zebrafish embryo

Background and motivation

1.1 Chemicals in the aquatic environment

Water bodies are of outstanding ecological and economical importance as source for drinking and service water as well as ecosystems. For the Europe-wide protection of surface waters and groundwater the EU water framework directive (2000/60/EG) was constituted (EU 2000). The aim of this directive is to preserve and restore the good ecological and chemical status of European waters. Out of a total of 9900 surface water bodies, i.e. sections of rivers, lakes, transitional and coastal waters, only 8.7% are in “very good” or “good ecological state” (BMU 2017). The assessment of surface water bodies’ ecological status is based on biological, chemical, physicochemical, and hydromorphological quality components (BMU 2017). Because the ecological status of a surface water body is predominantly dependent on biological quality components (composition of aquatic communities including phytoplankton, other flora components, invertebrates, and fish fauna), chemical conditions have to ensure the existence of aquatic communities in particular (BMU 2017). Although the majority of surface waters in Germany are in “good chemical state” (Umweltbundesamt 2017), the classification of surface water bodies’ chemical status is carried out on the basis of defined maximum concentrations (environmental quality standards) of single priority chemicals, other pollutants, and nitrate only (EU 2000). Not monitored but toxicologically relevant environmental chemicals, transformation products, and mixtures are potentially overlooked with chemical analytics alone (Altenburger et al. 2015). Previous studies show that chemicals of anthropogenic origin, in particular pesticides, pharmaceuticals, and industrial chemicals, occur with great diversity in aquatic ecosystems (Malaj et al. 2014; Moschet et al. 2014). Although the multitude of pollutants is usually present at low environmental concentrations, their combined effect poses a potential risk to aquatic ecosystems (Schwarzenbach et al. 2006). Consequently, chemical monitoring of a restricted number of pollutants alone does neither allow for a definite statement about the potential translation of chemical mixture exposure into adverse biological effects, nor whether or not measures must be taken to reduce their environmental impact. To allow for adequate risk assessment it is vitally important to know which chemical mixtures occur and which biological combination effects are associated (Altenburger et al. 2015). Several discussions and demands highlight the opportunity to apply bioanalytical tests that can bridge the gap between chemical exposure and related biological effects (Busch et al. 2016; Neale et al. 2017; Wernersson et al. 2015). In future, the application of such tests could link chemical exposure with environmental

protection goals more directly. Organism-based bioassays using fish embryos, daphnia, and alga have been routinely applied for single compound risk assessment and are required under certain legislations (e.g. REACh). Their suitability for the assessment of chemical mixtures has been demonstrated recently (Altenburger et al. 2018). Given the availability of operative methods the complementation of chemical analytical approaches with effect-based methods could allow for a more appropriate assessment of both the ecological and chemical status of surface waters (Brack et al. 2019).

1.2 Fish embryo acute toxicity (FET) test: Advantages, applications, and limitations

Bioanalytical tests cover various trophic levels relevant to ecosystem function including primary producers, as well as secondary and tertiary consumers. Examples include the freshwater alga and cyanobacteria growth inhibition test (OECD test no. 201; OECD 2011), the *Daphnia* sp. acute immobilization test (OECD test no. 202; OECD 2004), and the fish acute toxicity test (OECD test no. 203; OECD 2019). As the only primarily aquatic class of vertebrates fish are considered as indispensable part of ecotoxicological research. However, conventional testing on adult fish is viewed as ethically questionable (Scholz et al. 2013). Furthermore, animal testing is time-consuming, cost-intensive, and provides very little information on underlying mechanisms of toxicity. In accordance with the reduction, refinement and replacement of animal testing (3-R-principle; Russell and Burch 1959), embryonic and early larval stages of the zebrafish (*Danio rerio*) offer an alternative to conventional vertebrate models. Early developmental stages of *D. rerio* are not considered animals up to 120 h post fertilization (hpf) (Strähle et al. 2012). They offer numerous advantages such as transparency, high reproduction rate, and small size which allow for the evaluation of morphological and physiological features as well as high-throughput applications. Moreover, it has been demonstrated that fish embryo acute toxicity is highly correlated with the fish acute toxicity test (Belanger et al. 2013; Klüver et al. 2015; Lammer et al. 2009). The fish embryo acute toxicity (FET) test represents a well-established, largely harmonized (OECD test no. 236; OECD 2013) bioanalytical tool to determine the acute toxicity of chemicals on embryonic stages of the zebrafish. In the context of German standard methods for the examination of water, waste water and sludge (DIN EN ISO 15088), the FET test is already routinely applied to the assessment of the toxic potential of chemical mixtures to the embryonic development of zebrafish. The determination of acute toxicity is usually based on apical endpoints indicative for lethality. However, such observations on lethality do not allow for adequate ecotoxicological assessment of potential sub-lethal and chronically relevant toxicity of chemicals and mixtures. Hence, the

determination of lethal endpoints alone does not allow for the assignment of mechanisms that should potentially be considered for risk assessment of chemicals.

1.3 Neuroactive chemicals: Outside the applicability domain of the FET test

Certain modes of action (MOAs) are associated with high acute-to-chronic ratios. A recent meta-analysis of fish early life stage tests (OECD test no. 210; OECD 2013) revealed that non-narcotic MOAs like endocrine disruption, methemoglobinemia, and particularly neuromuscular toxicity elicit increased chronic toxicity in comparison with acute toxicity tests including FET (Scholz et al. 2018; Sobanska et al. 2018). In addition, even though the FET test correlates with the fish acute toxicity test (Belanger et al. 2013; Lammer et al. 2009), a comparatively weak sensitivity of the FET test was particularly found with neurotoxic compounds (Klüver et al. 2015). With regard to environmental risk assessment these findings appear even more relevant since a previous study showed that neuroactive chemicals are the second largest group of xenobiotics in European rivers after chemicals with unknown MOA (Busch et al. 2016). In this context, diverse chemical classes with distinct neuroactive MOAs that target different neurotransmitter pathways such as GABAergic, serotoninergic, and cholinergic neurotransmission were identified as environmentally relevant due to the occurrence of respective chemicals in numerous European surface water bodies (Busch et al. 2016). However, even though neuroactive chemicals are often elusive with acute lethal toxicity in the FET test, the analysis of embryo-larval behavior in terms of locomotor activity was demonstrated to increase sensitivity towards levels of other more sensitive toxicity tests (Klüver et al. 2015). For example, an increased sensitivity by a factor of 660 was found for aldicarb, a carbamate insecticide with anti-acetylcholinesterase MOA when comparing effect concentrations obtained with a behavior assay, namely the locomotor-response test, with its lethal effect concentration (Klüver et al. 2015). These findings highlight both, the potential and the relevance of behavior-based approaches in the assessment of neuroactive environmental chemicals. This is not least because behavior represents an endpoint of potential ecological relevance.

1.4 Behavior in embryonic and early larval stage of zebrafish as potential endpoint in ecotoxicology

From an ecological point of view, adequate behavior assures the survival at the level of the individual and beyond (reviewed in Hellou (2011)). Examples include feeding, predator avoidance, mating, and migration. Behavior integrates responses to both internal

(physiological) and external (environmental) factors (Evans 1994). Olfactory, gustatory, visuo-, and mechanosensory structures perceive environmental signals like odor, taste, light, acoustics, pressure, hydrodynamics, and tactile stimuli, respectively. The sensory system transduces external signals into information processible by the nervous system. In simple terms, neural function translates to behavior via a signal transduction cascade including receptor stimulation, ion channel activation, membrane polarization, and action potential creation (e.g. reviewed in Legradi et al. (2018)). Besides exogenous factors behavior is the result of endogenous processes including both neuronal development and internal signaling (Coecke et al. 2007).

Zebrafish demonstrate a set of stereotypic behaviors evolving during embryonic and early larval development. As early as 17 h post fertilization (hpf) the first motor behavior, spontaneous tail contractions, is observed. Subsequently, touch-evoked coiling and swimming occur at 21 and 27 hpf, respectively (Saint-Amant and Drapeau 1998). Spontaneous movements neither require supraspinal input (Downes and Granato 2006) nor several neurotransmitter systems including acetylcholine, GABA, NMDA, and glutamate (Saint-Amant and Drapeau 2000). Instead, the developing spinal cord alone generates the three motor patterns, likely with distinct roles of glycine-mediated neurotransmission (Downes and Granato 2006). The photomotor response (PMR) is the first photic behavior in zebrafish at 30 to 40 hpf (Kokel et al. 2013). A series of two high-intensity light flashes elicits a sequence of distinct motor behaviors in dark-adapted zebrafish embryos (Kokel et al. 2010). The PMR is a non-visual photobehavior mediated by light-sensitive neurons (opsin-based photoreceptors) in the hindbrain (Kokel et al. 2013). Probably, multiple neurotransmitter pathways such as adrenergic, dopaminergic, and serotonergic pathways contribute to PMR regulation (Kokel et al. 2010). By 40 hpf the Mauthner cell, a pair of neurons located in the hindbrain, drives vigorous spontaneous tail flips, also triggered by a tactile-vibrational stimulus. Mauthner cell mediated spikes are likely the eventual cause of hatching around 3 d post fertilization (Eaton et al. 1977). From around 3 d post fertilization (dpf), zebrafish acquire the ability to sense chemical cues such as amino acids and bile acids (Li et al. 2005). For example, exposure to the amino acid L-cysteine results in an olfaction-mediated, aversive response. This manifests itself in a spatial displacement and an increase in locomotor activity (Vitebsky et al. 2005). At 3 to 4 dpf the zebrafish larva inflates its swim bladder. This transition marks the onset of active swimming and increased responsiveness to stimuli (Fero et al. 2011). The first known visual motor behavior caused by light on the retina includes the visual body twitch (distinction of light and dark across time) and the optokinetic reflex (distinction of light and dark across space) between 68 and 79 hpf. Visuomotor behavior relies on the integrity of a neural circuit composed of photoreceptors, brain, and muscles (Easter and Nicola 1996; Easter and Nicola 1997). From day four, behavior in

zebrafish is controlled by circadian rhythmicity (Hurd and Cahill 2002). However, light and dark directly regulate locomotor activity in zebrafish larvae and can override circadian activity levels. It was proposed that dark-induced hyperactivity serves to accelerate navigation to a well-lit environment (Burgess and Granato 2007a; Burgess et al. 2010). In contrast to directional light-seeking behavior, dark photokinesis is an undirected hyperactivity in response to darkness mediated by deep brain photoreceptors independent of the retina or pineal (Fernandes et al. 2012). Besides photic behavior, larval zebrafish respond to acoustic/vibrational stimuli. Acoustic stimuli can elicit Mauthner cell-mediated startle responses of short latency and high speed that override ongoing movements. Hence, in contrast to light mediated behavior, acoustic responses are “true” startle responses (Burgess and Granato 2007b). Responses to acoustic stimuli are likely triggered via deflection of inner ear otoliths (Burgess and Granato 2007b; Kohashi and Oda 2008). However, the lateral line may play a role as well (Fero et al. 2011).

Neuronal function and spatiotemporal neurodevelopmental processes like proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis are sensitive to chemical exposure (Rice and Barone 2000). As highlighted above, the diverse behavioral repertoire of embryo-larval stages of zebrafish led to the development of numerous behavior-based tests that, *inter alia,* intend to assign chemicals to biological function. For example, spontaneous tail contractions are able to identify potent neuroactive chemicals but may not be specific to modes of action (Vliet et al. 2017). In contrast, with the method of PMR diverse classes of neuroactive chemicals were found to cause specific patterns of behavior that clustered with functional similarity (Kokel et al. 2010). Furthermore, a battery of acoustic and visual assays in larval zebrafish identified and classified multitarget antipsychotic-like compounds (Bruni et al. 2016).

1.5 Aim of the thesis: Behavior-based identification of environmentally relevant chemicals and related modes of action

As outlined above, numerous (mostly pharmacological) studies highlight the use of zebrafish as a behavioral model to study mechanisms of chemical neuroactivity and neurotoxicity. In addition, behavior-based endpoints were found to increase the sensitivity of zebrafish larvae to neuroactive chemicals beyond lethal acute toxicity (Klüver et al. 2015). Therefore, behavioral profiling of environmental chemicals and mixtures using the zebrafish model could potentially inform about both, adverse biological effects at organism level and underlying mechanisms in a non-targeted manner.

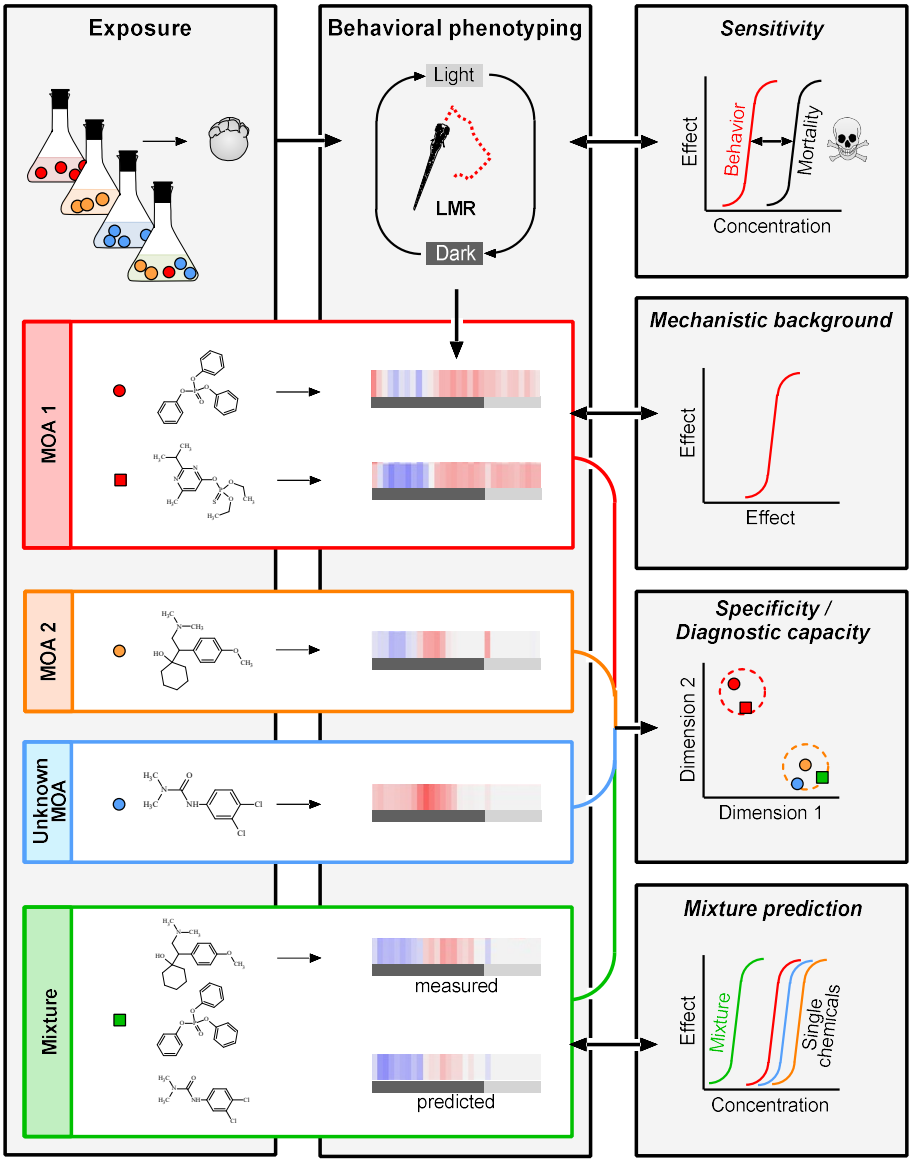

Figure 1.1. Schematic representation of the envisioned approach of the present work including the determination of behavior-derived endpoints for the effect-based assessment of environmental chemicals and mixtures. LMR, locomotor response; MOA, mode of action.

A striking argument supporting the implementation of behavior-based endpoints into the assessment of single environmental chemicals and mixtures is the potential to holistically evaluate effects from an ecotoxicological and mechanistic perspective on a sub-lethal scale. The analysis of behavior-based endpoints could complement chemical monitoring with information on the biological activity of respective components. The aim of this thesis was, therefore, to develop an efficient, sensitive, and specific bioanalytical tool which can be applied to the mechanism-oriented analysis of chemicals and mixtures.

Figure 1.1 serves as a graphical abstract of the approach of this thesis and shows a schematic overview on the envisioned approach. It includes that behavioral profiles should be derived from zebrafish larvae exposed to model chemicals that represent environmentally

relevant MOAs. The phenotypic similarity of profiles shall be used to aggregate chemicals according to their behavior. In addition, this approach should assist the identification of mechanisms of chemicals with unknown MOA. Finally, such MOA-based phenotypes might be used for the prediction of combined effects as well as the identification of such patterns in chemical mixtures. The underlying hypothesis is based on the assumption that distinct behavioral phenotypes are MOA-specific. Finally, the implementation of behavioral endpoints in effect assessment might increase the sensitivity of the embryo-larval zebrafish model compared with the hereto only considered lethal endpoints. In order to follow this approach, first a robust assay is needed, i.e. a reproducible, accurate test with high resolution which allows for the detection of as far as possible specific behavioral responses to chemical exposure. Second, the sensitivity and specificity of such an assay needs to be investigated. For example, it needs to be shown whether chemicals with distinct MOAs cause different behavioral phenotypes. Furthermore, the mechanistic background of specific profiles as well as the applicability of the approach for the investigation and characterization of effects of chemical mixtures need to be considered.

The following four chapters address several, detailed steps and questions related to the outlined approach, which is explained and introduced in more detail below.

1.5.1 Establishment of a light-dark transition assay in larval zebrafish: Which parameters need to be considered?

There are certain prerequisites which must be met in order to determine behavioral profiles indicative of a chemicals' MOA. These include considerations of concentration, temporal, developmental, and statistical aspects in the test design. Therefore, the following questions were the focus of **Chapter 2** of this thesis:

- Which test design provides the dual advantage of increased throughput and data robustness in combination with content-rich behavioral responses?
- Which period of exposure allows for the determination of preferably exclusively neuroactivity-related responses along with the time course of development?
- In which range of lethal effect concentrations can alterations in locomotor behavior actually be expected?

Here, an important issue is the design of the test itself. Due to diversity in test design the inter-comparability of behavioral assays is often compromised (Legradi et al. 2015). There are numerous different protocols of which some may largely cover potential compound-specific behavioral phenotypes while others may not. This can be seen as a result of test

duration, temporal resolution, and light regime. As it is known that the light-dark transition test is very sensitive to external influences and internal factors (e.g. time of day, strain), a careful evaluation of such parameters is of vital importance. In the end, the optimization of the test procedure represents a compromise between throughput and content-richness of the behavioral response.

Another point is the consideration of toxicokinetic and toxicodynamic aspects. On one hand, a chemical needs to be bioavailable, i.e. exposure should be extended to a period long enough to take account of the time required to enter the organism and to access to relevant target sites. This, in turn, presumes the presence of corresponding receptors as a matter of development. Some of them, such as acetylcholinesterase, develop early (Küster 2005) whereas others, such as $GABA_A$ receptors, are not functional before 2 d post fertilization (Monesson-Olson et al. 2018). On the other hand, adverse outcomes at cellular, organ, and organism scale which potentially affect locomotion need to be ruled out as far as possible. Such side effects can impact on morphological features relevant to locomotion.

One last aspect to consider is the concentration dependence of behavioral responses. Many large-scale studies on behavioral profiling are restricted to single or only a few test concentrations (e.g. Kokel et al. 2010). However, it has to be assumed that a chemicals' behavioral profile changes with concentration. In a first attempt, applied exposure concentrations were, therefore, anchored to lethal effect concentrations derived from FET tests to reveal potential concentration dependent differences in observed phenotypes.

1.5.2 Behavioral profiling of environmental chemicals: Are locomotor responses to chemical exposure indicative of modes of action?

Having established a robust test protocol for the detection of behavioral changes in zebrafish due to chemical exposure the next step could then be the investigation on the diagnostic value of behavioral responses for distinct MOAs. The questions attached thereto include:

- Can neuroactive chemicals be distinguished from others?
- Are there patterns of locomotion specific to distinct modes of action?
- Which periods of exposure offer a maximum discriminatory power?
- To which extend does the use of behavioral endpoints increase sensitivity beyond acute lethal toxicity?

These questions are addressed in **Chapter 3**.

In order to verify the specificity of the locomotor response test for neuroactive chemicals, compounds which provide both, similar and dissimilar mechanisms of neuroactivity, need to be selected on one hand. On the other hand, specificity needs to be confirmed using reference substances anticipated to act via mechanisms distinct from neuroactivity. With regard to environmental risk assessment this is vitally important due to the co-occurrence of neuroactive and differently acting chemicals in aquatic environments (Busch et al. 2016).

Furthermore, it needs to be investigated whether chemicals with similar molecular targets, i.e., similar MOAs, cause similar behavioral responses at organism scale. This approach appears necessary because environmental exposure rather occurs with multiple chemicals, partly having equivalent effects, instead of single entities considered separately (Busch et al. 2016; Loos et al. 2013). Behavioral assays may contribute to the assessment of an ecologically relevant impact of chemicals given a discriminatory power with respect to distinct MOAs. They may assist the identification of potential drivers of mixture toxicity at sub-lethal scale, meaning both single chemicals as wells as additively acting mixtures. Currently, studies on behavior are frequently restricted to evaluation in a rather descriptive manner (significant differences in control versus treatment). However, in order to achieve a maximum discriminatory power, locomotor signals need to be captured in their entire complexity. Consequently, a methodology needs to be developed that enables comparability of results originating from separately conducted experiments among each other. Therefore, a method previously applied in behavior-based drug discovery, namely behavioral barcoding (Jordi et al. 2018; Kokel et al. 2010), needs to be adapted here. This would facilitate the evaluation of behavioral responses in a qualitative and quantitative manner, meaning extend of up- and down-regulation of locomotor activity.

In order to verify to which extend patterns of locomotion are indicative of a certain MOA several factors including concentration and time dependence of behavioral responses need to be considered. Both time and concentration are an integral part in the determination of, as far as possible, compound/MOA-specific patterns because initially molecular mediated responses may switch to less specific outcomes over time as a result of toxicodynamic and toxicokinetic processes (Rozman and Doull 2000). Therefore, a time- and concentration-resolved experimental design seems indispensable to identify appropriate patterns.

As outlined above, the value of behavioral assays may not only be restricted to diagnostic applications but also the potential increase in sensitivity in comparison with conventional lethal endpoints. Therefore, concentration-response relationships are necessary which inform about respective effect concentrations in a communicable and comparable manner.

1.5.3 Evaluation of underlying mechanisms: What is the biological background of chemical-induced hyperactivity?

In advance of findings in **Chapter 3**, namely the similarity of behavioral phenotypes observed with chemicals of anticipated distinct and partially non-neuroactive MOAs, the following questions are addressed in **Chapter 4**:

- Are observations on similar phenotypes of hyperlocomotion related to similar mechanisms at molecular (inhibition of acetylcholinesterase) or physiological scale (heart rate)?
- How do acetylcholinesterase inhibition, heart rate, and hyperactivity depend on each other?
- Are there other similarities in the mechanistic background of different chemicals which may explain hyperlocomotion?

The presumed MOA of a chemical is usually related to its application (e.g. pharmaceuticals, pesticides) while others are elusive in vertebrate species (e.g. herbicides) or living organisms in general (e.g. industrial chemicals). Consequently, the unintentional release of chemicals into the environment represents a challenge to the evaluation of mechanisms in non-target species. For example, the herbicide diuron is considered to act via inhibition of the photosystem in plant species. In contrast, the precise MOA in vertebrates remains elusive. *Vice versa*, there are chemicals, such as pharmaceuticals, whose molecular mechanisms are most likely conserved across vertebrate species and therefore of potential relevance to related aquatic organisms. In pharmacology, the discovery of novel neuroactive drugs is usually based on the observation of behavioral phenotypes (Rennekamp and Peterson 2013). In chapter 4 it is explored whether the knowledge of certain biological functions of chemicals can be applied to unravel the mechanisms involved in the behavioral activity of environmental chemicals with previously unknown MOA in zebrafish.

1.5.4 Combined effects of chemicals on locomotor activity: Is a combined effect on locomotion predictable from single compounds' effects and can individual behavioral profiles be recovered from profiles of mixture exposure?

The potential scope of application of behavioral tests to environmental mixture assessment includes two domains, namely prediction and diagnostic purposes (e.g. identification of drivers of mixture toxicity). Both rely on the previous knowledge of locomotor phenotypes of single chemicals (see **Chapters 3** and **4**) while diagnosis may be extended to chemicals with

expected similar mechanisms and outcomes at organism level, respectively. The related questions addressed in **Chapter 5** include:

- Do chemicals with distinct molecular background but similar behavioral phenotype elicit additive effects on locomotion?
- Can combined effects on locomotor activity be predicted using common concepts of mixture toxicity prediction including concentration addition (CA) and independent action (IA)?
- Is it possible to identify compound/mechanism-specific phenotypes in a synthetic mixture of environmental relevance?

There are two major concepts available for the prediction of combined effects, namely CA and IA (Altenburger et al. 2013). These concepts have successfully been applied to the prediction of apical endpoints, particularly lethality, but have not yet been proved applicable to motor endpoints. The transferability of mixture models to the prediction of locomotor responses is confronted with some complications. This is because, first, locomotor activity is regulated bidirectional, i.e., in dependence on chemicals' MOA and concentration, locomotor activity can be up- and down-regulated, respectively. Furthermore, the non-monophasic character of locomotor responses in dependence on concentration has been demonstrated with numerous chemicals (Ali et al. 2012). Second, locomotor activity is unrestrained in its upper limit. I.e. the maximum activity is not known and may vary considerably between chemicals. Therefore, measured responses need to be scaled appropriately in order to allow for comparability among chemicals and prediction of their joint effect.

As outlined above, in order to identify relevant chemicals/MOAs from a mixture we need to rely on as far as possible unique/specific motor patterns distinct from other chemicals. Such behavioral profiles could then be compared with respective profiles of a mixture/environmental sample to identify components driving the observed effect. Additionally, the previous knowledge about MOAs of single substances can assist to obtain information on underlying mechanisms in a mixture. In order to evaluate the applicability of behavioral profiling to the assessment of environmental exposure, several properties of environmental mixtures such as occurring chemicals, measured environmental concentrations, and relative proportion need to be considered. This was achieved in a proof of principle study on a synthetic mixture under consideration of the mentioned aspects.

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Establishment of a light-dark transition assay in larval zebrafish for the assessment of environmental chemicals

Abstract

The emergence of neuroactive chemicals in the aquatic environment raises concerns about human and environmental health. Current approaches that use lethality-based endpoints to assess chemical effects, such as the fish embryo acute toxicity (FET) test (OECD test guideline no. 236), fail to cover the anatomical and physiological complexity of the nervous system, its interaction with chemicals, and potential adverse outcomes at sub-lethal scale. Alterations in behavior have been proposed as appropriate readout of exposure to neuroactive chemicals. The light-dark transition test in larval zebrafish (*Danio rerio*) represents a widely applied behavioral assay and allows for the determination of effects of chemicals on neuronal signaling and (neuro)-developmental processes, respectively. However, due to diversities in test design, i.e., variability in exposure and light regimens, the inter- and intra-comparability as well as the information content of behavioral tests is compromised. Here, we report the development and application of a locomotor response assay to measure alterations in photo-, i.e. light/dark-, dependent responses of larval zebrafish after chemical exposure. We aimed at establishing a sensitive, mechanism-specific, and reproducible endpoint at the sub-lethal level. Therefore, different periods of exposure during early embryonic and larval development were systematically examined and lethality-based anchoring was used to adjust applied concentrations. Model compounds of different functional classes, including inhibitors of acetylcholinesterase and cyclooxygenase as well as anticipated narcotic substances, differentially regulated locomotor activity in dependence on the period and level of exposure. Early developmental exposure affected morphological features relevant for larval locomotion as observed in the case of swim bladder inflation. Therefore, we suggest the acute, short-term exposure of larval stages in order to specifically detect interferences of chemicals with neuronal function instead of impairments of locomotor behavior due to other toxic effects throughout development (e.g. lack of swim bladder inflation). Furthermore, we conclude that the use of longer photoperiods (e.g. 10-20 min) may contribute to increase the diagnostic capacity based on a more comprehensive representation of potentially mechanism-specific locomotor responses.

2.1 Introduction

The assessment of waterborne chemical exposure and related potential adverse effects on aquatic organisms is crucial to the protection of environmental health (Altenburger et al. 2019; Brack et al. 2019a). While regulatory legislation only requires the surveillance of few priority chemicals (2013/39/EU), advancements in chemical analytical approaches increasingly facilitate the identification of new classes of environmental chemicals (Brack et al. 2019b; Gavrilescu et al. 2015). So-called chemicals of emerging concern have the potential to cause adverse biological effects even in the nanomolar concentration range (Colón-Cruz et al. 2018). Among them, particularly neuroactive chemicals, i.e. substances that alter nervous system function, have been identified as one of the most abundant classes of chemicals in European surface water bodies (Busch et al. 2016). Neuroactive chemicals, such as certain pharmaceuticals, pesticides, and industrial chemicals, have been associated with increased toxic and acute-to-chronic ratios (Scholz et al. 2018; Vaal et al. 2000). However, traditionally, ecotoxicology relies on the assessment of a limited number of endpoints, such as reproduction and lethality, which have severe implications to the survival of a species (Guilhermino et al. 2000; Padilla et al. 2012). The evaluation of other, potentially ecologically relevant effects at sub-lethal scale has not been implemented in the routine assessment of chemicals so far. Thus, the development of more sensitive and/or specific endpoints is needed in order to determine subtle changes in physiology indicative of potential adverse chronic effects. Moreover, new approaches are required to address the complexity of the nervous system and related disorders (Bal-Price et al. 2008). Alterations in behavior have been proposed as an appropriate readout of exposure to neuroactive chemicals (Gerhardt 2007; Robinson 2009).

Embryonic and early larval stages of the zebrafish (*D. rerio*) show a functional and comparably simple nervous system (Burgess and Granato 2007a; Stahl 1977). Together with a high reproduction rate, rapid *ex utero* development, small size, pharmacological and genetic manipulability, as well as morphological, genetic, and behavioral homology to other vertebrate species including humans, the developing zebrafish emerged as valuable vertebrate model in developmental toxicity and behavioral research (Kalueff et al. 2014; Teixido et al. 2019). According to the European animal directive (2010/63/EU) embryo-larval stages of the zebrafish are not considered animals up to 5 d post fertilization (dpf) and, thus, represent an alternative to conventional animal testing. The fish embryo acute toxicity test allows for the assessment of lethal endpoints up to 5 dpf (OECD test no. 236; OECD, 2013) but also of visible malformations occurring during embryonic development (Lammer et al. 2009). In addition, it has been demonstrated that (neuro)-toxic effects on behavior can occur at remarkably lower concentration levels than lethality and malformations (Klüver et al. 2015;

Selderslaghs et al. 2010). Thus, alterations in behavior represent a sensitive measure of subtle changes in physiology.

Developing zebrafish undergo a rapid neurodevelopment with the onset of neurogenesis around 6 h post fertilization (hpf), i.e. during gastrulation, and almost complete maturation of the nervous system by 96 hpf, i.e. at larval stage (Blader and Strähle 2000). As the developing nervous system is often more vulnerable to chemical exposure than the matured one (Grandjean and Landrigan 2006) mechanisms of action and related adverse outcomes can differ depending on both, the time point and duration of exposure. Hence, exposure settings need to be adjusted carefully in order to address distinct research questions properly. On the one hand, exposure throughout development or specific developmental processes, i.e. developmental exposure, is indispensable to assess developmental (neuro)-toxicity (Padilla et al. 2012; Selderslaghs et al. 2010). In contrast, other studies focus on acute (short-term) exposures at distinct stages of development in order to determine the direct interaction/interference with pathways of neurotransmission, i.e. neuroactivity (Bruni et al. 2016; Kokel et al. 2010). In any case it needs to be considered that developmental toxicity can affect morphological features (e.g. swim bladder, muscles) which, in turn, may lead to alterations in behavior not necessarily related to neurotoxicity and –activity, respectively. As a consequence, it is crucial to preclude such effects through adequate exposure settings in order to assess direct neuroactivity instead of measuring side effects on behavior e.g. due to malformations.

Manifold assays are described to determine chemical mediated behavioral alterations in embryo-larval stages of zebrafish using different stimuli and endpoints, respectively, such as spontaneous tail contractions (Raftery et al. 2014; Vliet et al. 2017), responsiveness to tactile stimuli (Stanley et al. 2009), and photo-dependent motor activity (Irons et al. 2010; Kokel et al. 2010). Here, we focus on the light-dark transition test which represents a widely used assay not least because of the commercial availability of automated video tracking technologies which enable the unbiased and efficient quantification of alterations in larval movement. The test is based on alternating light and dark periods which cause a resting state in the light followed by increased locomotion in dark conditions (Burgess and Granato 2007a). Alterations in photo-dependent locomotor activity after chemical treatment have successfully been used to classify pharmaceuticals according to biological activity (Bruni et al. 2016; Jordi et al. 2018). However, it is worth to note that there is remarkable variability between experimental procedures (Basnet et al. 2019; Legradi et al. 2015). Variations include the stage of zebrafish used and the length of experiments including light and dark conditions (Basnet et al. 2019). Applied light-dark transitions differ in temporal aspects, i.e. duration and sequence, and can be in the range of seconds (Burgess and Granato 2007a) to

minutes (Ali et al. 2012; Irons et al. 2010), hours (Burgess and Granato 2007a), and even days (Rihel et al. 2010), respectively. Consequently, there are remarkable differences in the biological background of locomotor responses to different light regimens. For example, single light and dark flashes (500 ms) trigger startle-like escape responses in larval zebrafish (Burgess and Granato 2007a). Post-dark-flash activity has also been used as a measure of lethargy (Jordi et al. 2018). Alterations in the range of minutes induce light-seeking behavior probably attributable to chasing strategy (Burgess and Granato 2007a). In contrast, long-term changes in photo regimens address diurnal rhythmicity and reflect rest/wake activity in zebrafish larvae (Rihel et al. 2010). Besides the length of photoperiods the sequence and frequency of photic stimuli are of relevance for locomotor behavior. For example, rapid (10 Hz), continuous alterations in photoperiods cause freezing behavior (strobe light response) in 7 dpf zebrafish larvae which has been associated with predator avoidance (Rennekamp et al. 2016). Overall, the manifold photo-dependent locomotor responses of larval zebrafish demonstrate that it is imperative to specify and control respective test variables carefully.

In order to exploit the dual advantage of the light-dark transition test as a sensitive readout of neuroactivity in connection with its diagnostic potential to indicate underlying mechanisms of action a thorough investigation of factors which potentially impact on the outcome of the test is indispensable. With the present study a set of such influential factors including different exposure and light regimens is addressed. Therefore, we first examined the impact of different photo cycle periods and sequences on locomotor activity of untreated individuals. Furthermore, we tested a set of chemicals with anticipated distinct mechanisms of action including inhibitors of acetylcholinesterase (diazinon, triphenylphosphate) and cyclooxygenase (diclofenac, naproxen), as well as a fungicide (cyprodinil) and an herbicide (diuron) with unknown toxicity mechanisms in larval zebrafish. We compared outcomes among different exposure regimens, i.e. levels and periods of exposure during different stages of embryonic and larval development. Our aim was to establish a robust locomotor response assay able to specifically detect alterations in neurotransmission due to chemical exposure and in the absence of potential confounding factors such as morphological alterations (e.g. lack of swim bladder inflation).

2.2 Materials and methods

2.2.1 Fish culture and embryo collection

Zebrafish (*Danio rerio*) of an UFZ in-house strain ("OBI") were raised and maintained at 26.5 ± 1 °C. A photoperiod of 14:10 h light:dark was used including onset and switching off of light at 8:00 AM and 10:00 PM, respectively. Commercial dry food and *Artemia* sp. were fed once and twice a day, respectively. Fish were cultured and used according to German and European animal protection standards. Fish culture was approved by the Government of Saxony (Landesdirektion Leipzig, file number 75–9185.64). At 9:00 AM, spawn from group matings was collected within stainless steel sieve-covered glass dishes. Fertilized eggs were selected by means of microscopy. If not exposed instantly, collected eggs were subsequently transferred into oxygen-aerated (≥ 24 h) and pH-adjusted (pH 7.4±0.1) standard dilution water as specified in ISO 7346-3 (80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl) with a density of one egg per 2 mL (28 °C, 14:10 h light:dark).

2.2.2 Chemicals and stock preparation

Chemicals and purities purchased from Sigma-Aldrich (Steinheim, Germany) were cyprodinil (99.9%, CAS RN 121552-61-2), diclofenac sodium (≥98.0%, CAS RN 15307-79-6), naproxen sodium (98.0-102.0%, CAS RN 26159-34-2) and triphenylphosphate (≥99.0%, CAS RN 115-86-6). Diazinon (98.5%, CAS RN 333-41-5) and diuron (≥98.0%, CAS RN 330-54-1) were purchased from Sigma-Aldrich (Buchs, Switzerland). Chemicals were dissolved in pure methanol (≥99.8%, CAS RN 67-56-1, J.T. Baker®, Avantor Performance Materials Inc, Pennsylvania, USA). Methanolic stocks were then dissolved in standard dilution water. The final concentration of methanol was 0.1% v/v in each exposure solution including controls.

2.2.3 Determination of fish embryo acute toxicity and concentration-response modelling

Static chemical exposure was performed in the period from 4-cell (~1 hpf) and prim 6 stage (~25 hpf) to larval stage at 4 dpf, respectively. Developmental stages were indexed according to Kimmel et al. (1995). 7.5-mL gas chromatography vials were equipped with three individuals and 6 mL of test solution in triplicate (26 °C, 75 rpm, Edmund Bühler SM-30 Control, 14:10 h light:dark). At 4 dpf, lethal toxicity was determined as described in OECD test number 236, fish embryo acute toxicity (FET) test, including coagulation, lack of somite

formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat (OECD, 2013). Concentration-response modelling was performed using a 4-parameter logistic function implemented in R (Ritz and Streibig 2005)

$$y = E_0 + \frac{E_{max} * x^h}{{LC_{50}}^h + x^h} \qquad \textbf{Equation 2.1}$$

with y being the modeled mortality, E_0 and E_{max} are the minimum and maximum effect, i.e. zero and 100% lethality, respectively. x refers to exposure concentration, LC_{50} is the concentration causing 50% mortality, and h (Hill factor) represents the slope of the tangent in the inflection point of the model.

2.2.4 Locomotor response assay

Fish embryo lethal effect concentrations (1, 10, and 90%) were administered to zebrafish of different developmental stages including 4-cell, prim 6, and larval day 4, respectively. The total time of exposure was 96, 72 and 1.5 h, respectively. Sixteen individuals per treatment were distributed separately into wells of clear flat-bottom 96-square-well plates (Whatman™ microplate devices, uniplate®, GE Healthcare UK Limited, Buckinghampshire, UK) along with 400 µL exposure medium. The batch was incubated at 28 °C (14:10 h light:dark) before subsequent locomotor response tests at 4 dpf. Plates were transferred to a ZebraBox monitoring device with corresponding software in tracking mode (ViewPoint Life Sciences, Lyon, France). An incubator was used to maintain a constant temperature of 28 °C. Locomotor activity data were recorded as total distance moved per unit of time, i.e. per hour and per minute, respectively. Subsequently, individuals were checked for mortality and swim bladder inflation. Dead individuals were excluded from data analysis.

2.2.5 Statistical analyses and hierarchical clustering

Statistical analyses were performed using GraphPad Prism 8.1.1 (GraphPad Software, Inc.). Significant differences ($p<0.05$) between photoperiods were determined with an unpaired t test. Comparisons of treatment versus control were conducted with an ANOVA including Kruskal-Wallis test ($p<0.05$). Significant results were classified as “hyperactivity” and “hypoactivity” if the mean rank difference of treatment versus control was >0 and <0, respectively. Otherwise, non-significant results were classified as “normal”. Hierarchical clustering was performed using the heatmap.2 function from the R package gplots version 2.13.0 (Warnes et al. 2015).

2.3 Results

2.3.1 The diurnal rhythm of newly hatched zebrafish larvae

To investigate the developmental time course of behavior in newly hatched zebrafish larvae along with a diurnal rhythm of 14:10 h light:dark, we monitored locomotor activity between three and four days post fertilization (dpf). We discovered that activity rises approximately linearly with time (R^2=0.63). In derogation of this, we found that locomotor activity was particularly increased at sudden light-dark transition. *Vice versa*, a slight but significant alteration was also observed in response to abrupt onset of light at 4 dpf (**Figure 2.1A**). In order to exploit the locomotor potential of 4 dpf zebrafish larvae, we investigated light-dark transition behavior in more detail.

2.3.2 Experimental parameters alter light-dark transition behavior

Second, we asked whether different photo regimens and repeated application of photo stimuli alter the diurnal response in 4 dpf larvae. As expected, each photo setting elicited light-dark responsiveness (**Figure 2.1B**). However, we found that responsiveness to dark periods decreased with increasing number of iterations. In particular, we observed that repeated application of relatively short dark:light cycles with 4:4 and 2:2 minutes reduced the maximum response relative to the first round by 2% (R^2=0.85) and 4% (R^2=0.45) per iteration, respectively. In contrast, a dark:light regimen of 20:10 minutes attenuated dark responsiveness (R^2=0.54) by just 1.5% (**Figure 2.1C**). To take advantage of increased throughput, reproducibility, and a more robust data situation, we decided to further apply 4:4 minute light:dark cycles in order to determine potential behavioral alterations after chemical exposure.

2.3.3 Chemical-induced behavioral alterations depend on various exposure settings

To determine how different chemicals affect photo-, i.e. light/dark-, dependent behavior in zebrafish, we examined different exposure periods starting from early (4-cell; 4C), middle (prim 6; P6), and late (larval, day 4; L4) time points in development, respectively, up to 4 dpf. I.e. for each of the three scenarios the locomotor response (LMR) was tested on day four (**Figure 2.2A**). To adjust applied exposure levels, we performed lethality-based anchoring, i.e. low (LC01), medium (LC10), and high doses (LC90) applied in the light-dark transition test were based on effect concentrations derived from fish embryo acute toxicity (FET) data

determined with exposures from 0 to 4 dpf (red), and 1 to 4 dpf (blue), respectively (**Figure 2.2A** and **B**, **Table 2.1**). To examine how different chemicals interfere with behavior, we included various model compounds with anti-acetylcholinesterase (AChE) activity (diazinon;

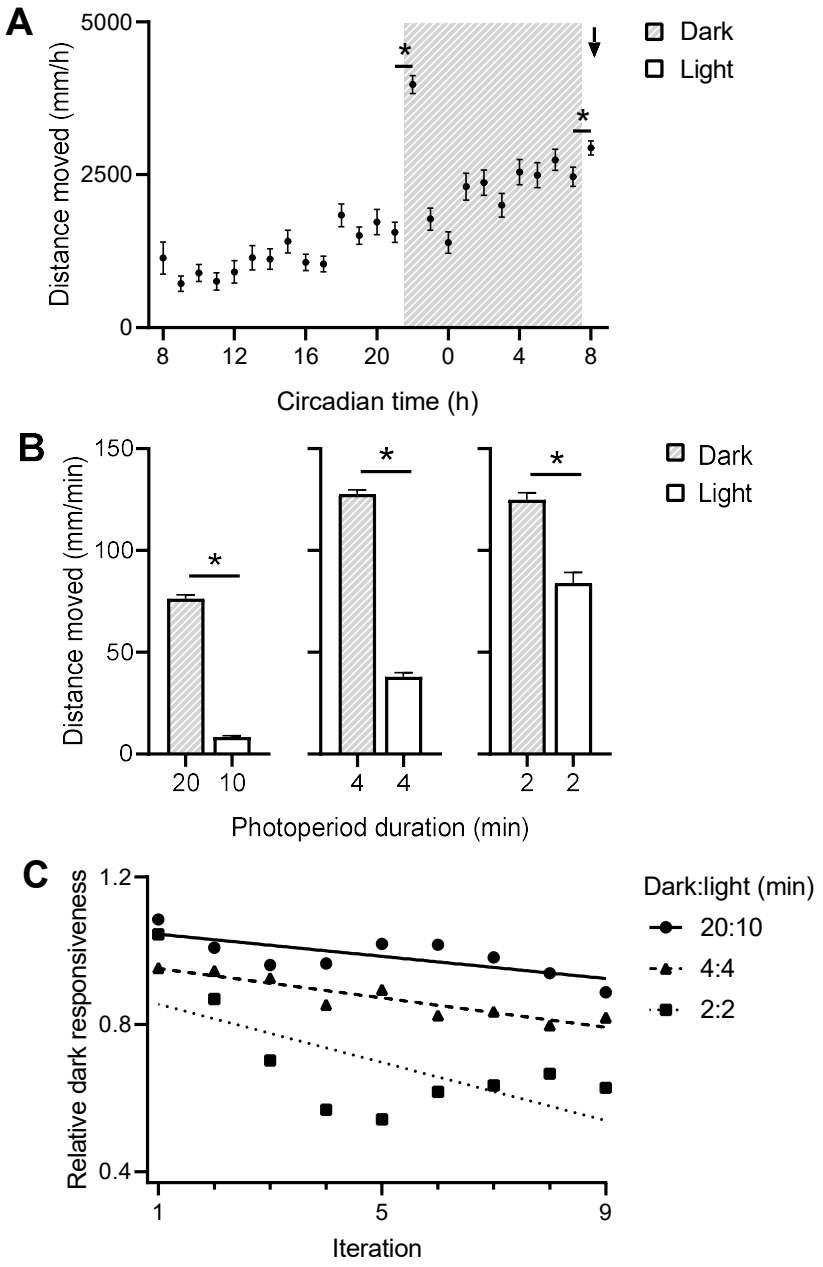

Figure 2.1. Light-dark transitions trigger stereotyped locomotor responses in larval zebrafish. (**A**) Development of locomotor activity between three and four days post fertilization along with a 14:10 h light:dark cycle. The difference in locomotor activity before and after photo transitions is significant: *p<0.05. *n*= 96 individuals. Data are mean ± SEM. Arrow indicates 4 dpf. (**B**) Different photo regimens elicit significant light-dark responsiveness in 4 dpf zebrafish: *p<0.05. *n*= 96 individuals. Data are mean ± SEM. (**C**) Linear regression showing that repeated application of photic stimuli decreases dark responsiveness relative to the first photo cycle (shown in B).

DZN, triphenylphosphate; TPP), inhibitors of cyclooxygenase (diclofenac; DIC, naproxen; NAP), and two substances unknown for potential mechanisms in vertebrates including the photosynthesis inhibitor diuron (DIU), and cyprodinil (CYP), a fungicide acting via protein synthesis inhibition (**Table 2.1**).

Table 2.1. Selected model chemicals and fish embryo acute toxicity.

Chemical	ID	LC_{01}(0-4 dpf)	LC_{10}(0-4 dpf)	LC_{90}(0-4dpf)	LC_{01}(1-4 dpf)
Diazinon	DZN	5.1	10.8	41.9	57.8*
Triphenylphosphate	TPP	2.8	4.0	7.8	5.5*
Diclofenac	DIC	1.2	3.3	20.2	2.4
Naproxen	NAP	135.5	205.7	441.6	189.1
Diuron	DIU	5.4	8.3	18.4	4.6
Cyprodynil	CYP	0.9	1.7	4.7	7.5

Effect concentrations are given in µM. *LC_{01} was replaced by $LC_{0.1}$ and $LC_{0.01}$ because of concentration response curve slopes >10 and >100 determined with diazinon and triphenylphosphate, respectively.

As mentioned above, we applied 4:4 minute light:dark cycles including an initial acclimation period followed by four repetitive photo sequences. A stereotypic locomotor activity profile of untreated zebrafish larvae at 4 dpf comprised of immediate, high locomotor output subsequent to light-dark transition and transient decrease in response to abrupt light emission (**Figure 2.2C**). **Figure 2.2D** shows an exemplary activity profile of zebrafish larvae exposed to a medium concentration of DZN after L4-treatment, i.e. 1.5 h post exposure. When aggregating every minute observations (see **Figure 2.2C** and **D**) by light and dark, as well as control versus treatment, respectively, significant reduction in response to light becomes apparent with a medium dose of DZN (**Figure 2.2E**). On the contrary, dark responsiveness remained unaffected (**Figure 2.2F**). Intriguingly, no such alteration was observed with the same medium exposure level after developmental 4C-exposure (**Figure 2.2E** and **F**). A high dose of DZN in the late L4 exposure scenario increased light dependent locomotor activity (**Figure 2.2E**) but decreased locomotion in the dark phase (**Figure 2.2F**). Furthermore, significant alteration in dark responsiveness was observed with low DZN exposure initiated at P6 stage (**Figure 2.2F**).

Analogous to DZN, we performed analyses with various exposure settings for each of the six model compounds (**Supporting figure A2.1**). To ease data accessibility, we categorized behavioral responses into hypoactivity, normal, and hyperactivity, respectively. Overall, in 40% (24/60) of the cases no behavioral effect was observed. The majority (45%; 27/60) of observed alterations were represented by hypoactive responses. Hyperactivity occurred in 15% (9/60) of all cases. In more detail, behavioral alterations were evenly distributed across photoperiods (**Figure 2.3A**). Similarly, early (4C) and late (L4) exposure periods showed the

same trend in the reduction (33-38%) and increase of locomotor activity (8%), respectively, whereas P6-exposure caused a higher number of alterations (**Figure 2.3B**). Increasing exposure levels were found to trigger a higher degree of both hypo- and hyperactivation.

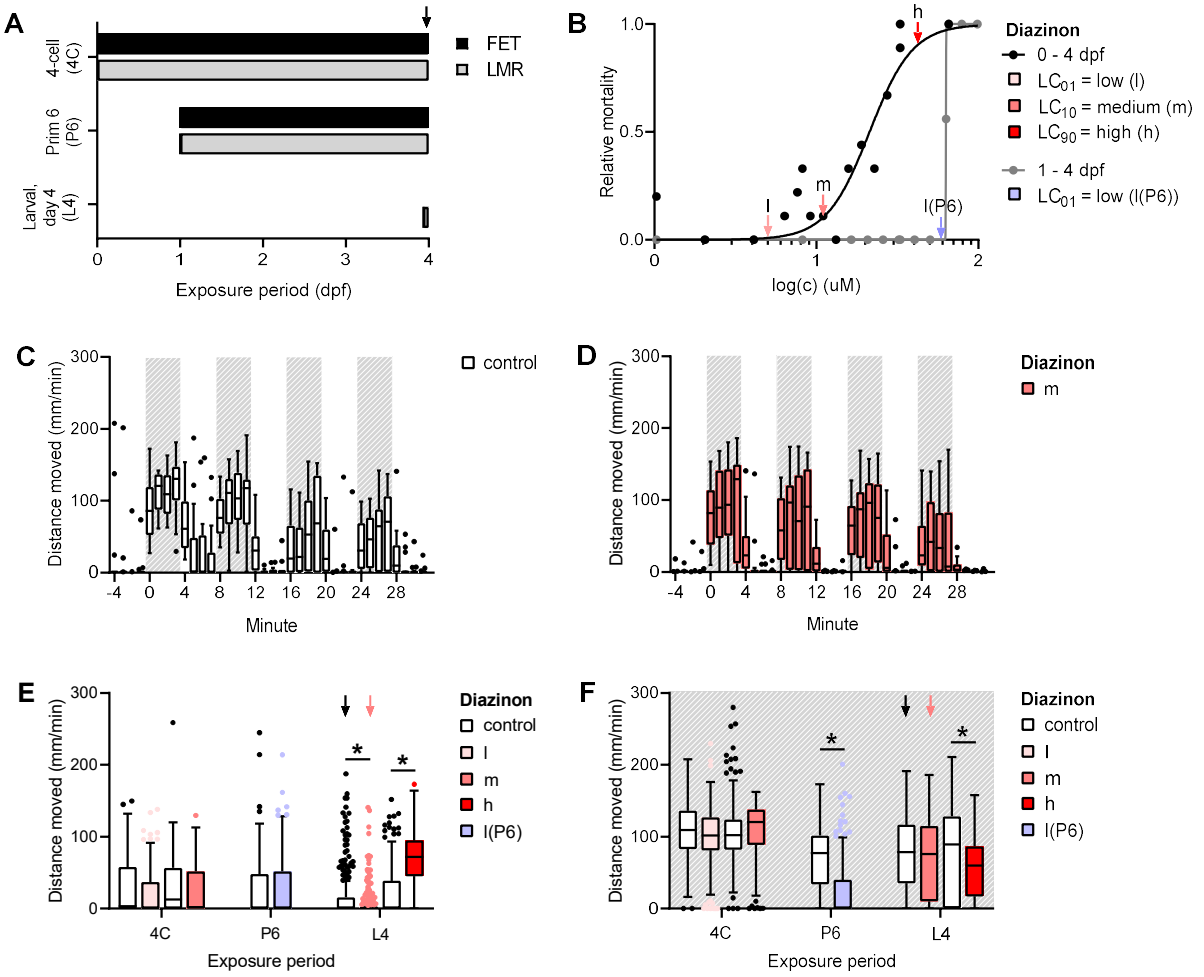

Figure 2.2. Experimental procedure used to assess the potential effect of chemicals on locomotor behavior. (**A**) Bar plot showing early (4-cell), middle (prim 6), and late (larval, day 4) exposure periods used in the fish embryo acute toxicity (FET) test and locomotor response (LMR) test, respectively. Arrow indicates conduction of LMR tests at 4 dpf. (**B**) Low (LC01), medium (LC10), and high (LC90) exposure levels applied in the LMR test were derived from FET concentration response curves. Colored arrows indicate effect concentrations tested with the LMR assay. (**C** and **D**) Tukey box plots showing exemplary locomotor activity profiles (one-minute increments) of (**C**) untreated larva and (**D**) individuals treated with a medium (m) dose of diazinon at larval, day 4 stage. White: light phases, grey; dark phases. n≤16 individuals per condition. (**E** and **F**) Tukey box plots show (**E**) light and (**F**) dark dependent alterations of locomotor activity with different exposure periods (4C=4-cell, P6=prim 6, L4=larval day 4) and exposure levels of diazinon (l=low, m=medium, h=high). Black and red arrows indicate data shown in C and D, respectively. Differences between control and treatment are significant as indicated: *p<0.001.

Again, treatment with low dose, i.e. LC01(1-4 dpf), which was exclusively applied to the P6 stage, caused the highest number of significant alterations in locomotor activity, namely in three quarters of all cases (**Figure 2.3C**). Finally, highest variability was found among different chemicals. In descending order of the number of observations on hypoactivity, NAP was followed by TPP, CYP, DZN, and DIC. In contrast, in the case of DIU exclusively increased locomotion was found in 60% of the cases. Furthermore, DZN, TPP, and DIC each caused activation in locomotion in 10% of all cases (**Figure 2.3D**).

To get a more cohesive overview, we used hierarchical clustering to organize chemicals as well as exposure settings based on similar behavioral phenotypes. Starting with similarities amongst chemicals (y-axis in **Figure 2.3E**), we found that CYP and NAP elicited comparable patterns of reduction in locomotor activity including P6 low dose treatment and light as well as dark dependent hypoactivity in the 4C and L4 exposure scenarios, respectively.

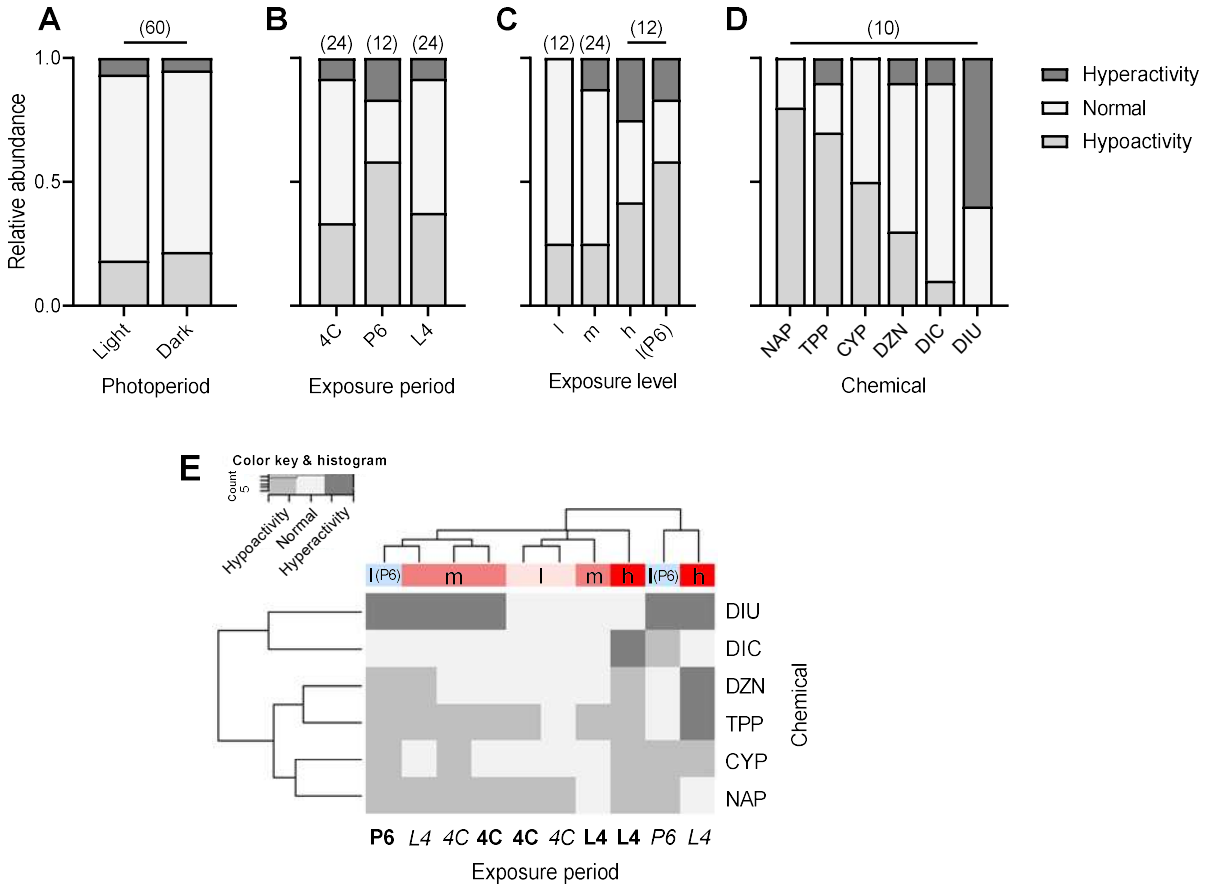

Figure 2.3. Various factors determine chemical-induced behavioral alterations. (**A** to **D**) Hypo- and hyperactivity are determined by (**A**) photoperiod, (**B**) exposure period (4C=4-cell, P6= prim 6, L4=larval day 4), (**C**) exposure level (l=low, m=medium, h=high, l(P6)=low prim 6), and (**D**) chemical (see **Table 2.1** for IDs). Values in brackets represent the total number of cases. (**E**) Hierarchical clustering of exposure settings (x axis) and chemicals (y axis) based on phenotypic similarity. Bolt=dark phase, italic=light phase.

Furthermore, hierarchical clustering revealed matches between the AChE inhibitors DZN and TPP. Both chemicals increased light dependent locomotion in the L4 exposure scenario along with the maximum applied exposure level. In addition, responsiveness to light as well as dark stimuli was reduced at some low, medium, and high exposure levels during P6 and L4 treatment, respectively. In contrast, DIU and DIC differed from each other in all respects and rather clustered according to dissimilarities than similarities. Finally, at the level of exposure settings (x-axis in **Figure 2.3E**), a coherent set of similarities was found between P6 dark- and L4 light responsiveness to low and medium exposure levels, respectively, as well as with the 4C exposure scenario at medium doses. Similarly but distinct from that cluster, low exposure levels applied to the 4C stage clustered in both photoperiods. Furthermore, responsiveness to dark stimuli in the L4 setup in combination with medium and high levels of exposure, respectively, differed from each other as the latter triggered a higher number of behavioral responses. A high number of behavioral alterations was also observed with light periods in the P6 and L4 exposure scenario at low and high levels of exposure, respectively. Therefore, these settings clustered and where largely distinguishable from others (**Figure 2.3E**).

2.3.4 Developmental exposure affects zebrafish morphology

Finally, we sought to consider a potential impact of morphological defects on observed behavioral phenotypes. Therefore, we focused on a feature relevant for adequate locomotion, namely swim bladder inflation. We found that swim bladder inflation was only affected if exposure was initiated at early (4C) and middle (P6) stages of development (**Figure 2.4**). Low and medium exposure levels suppressed swim bladder inflation in the 4C exposure scenario including NAP, CYP, and DZN. Inflation of the swim bladder was also affected with low level exposure of the P6 stage in the case of NAP, CYP, DZN, and DIC. Interestingly, except for CYP (low and medium 4C exposure), a lack of inflation was accompanied by a reduction in dark and/or light responsiveness as soon as more than half of the individuals were affected. In contrast, hyperactivity was exclusively observed if the swim bladder was inflated in the whole test group as exemplified by DIU and high exposure levels of the AChE inhibitors TPP and DZN (**Figure 2.4**).

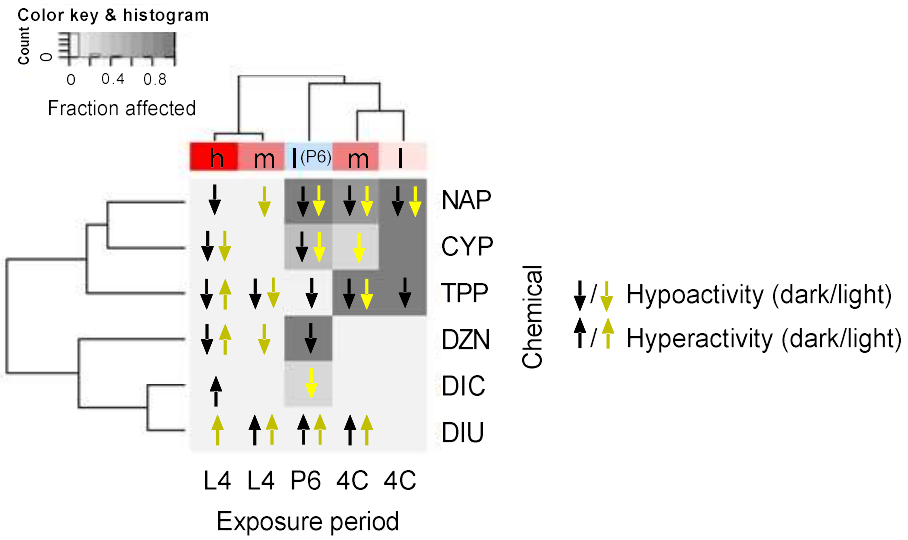

Figure 2.4. Developmental exposure affects swim bladder inflation in 4 dpf zebrafish. Hierarchical clustering of exposure settings (x axis) and chemicals (y axis) based on test group fraction showing absence of swim bladder inflation. Exposure periods; 4C=4-cell, P6=prim 6, L4=larval day 4. Exposure levels; l=low, m=medium, h=high, l(P6)=low prim 6. Chemicals; see **Table 2.1** for IDs.

2.4 Discussion

The light-dark transition test represents a widely applied behavioral assay that allows for the determination of effects of chemicals on neuronal signaling and (neuro)-developmental processes, respectively. Different photo and exposure regimens may provide varying information content suitable to distinct research questions. Here, we strived for a solution which provides the dual advantage of a sensitive readout of direct neuroactivity (as opposed to developmental toxicity) in connection with the diagnostic potential to indicate underlying mechanisms of action. Therefore, we addressed a set of influential factors and examined different photo and exposure regimens for their impact on the outcome of a light-dark transition test in larval zebrafish.

Age, time of day, and photo regimen affect zebrafish behavior

Embryonic and early larval stages of the developing zebrafish show various stereotyped behaviors that serve different biological functions. The first visual, light-driven behavior occurs as early as 68 hpf. An abrupt decrease in light intensity evokes a body twitch in larval zebrafish (Easter and Nicola 1996). Photic regulation of locomotor activity in zebrafish larvae has been proposed to serve spatial navigation toward an illuminated environment optimal for feeding (Burgess and Granato 2007a; Burgess et al. 2010)(). Here, we investigated a

circadian light:dark rhythm of 14:10 h. We found that locomotor activity increases gradually and independent of lighting conditions between 3 and 4 dpf. I.e., at the same time of day (8:00 AM) but different age, locomotor activity increased by more than two and a half times. Furthermore, responsiveness to abrupt changes in illumination, in particular a light-dark transition in the evening (10:00 PM), increased motor activity by factor 2.5.

Acute light exposure overwrites endogenous circadian rhythms in diurnal animals - a phenomenon known as masking (Burgess and Granato 2007a). Accordingly, in larval zebrafish, we found that different lighting regimens mimic circadian rhythms on the scale of minutes. However, repeated application of photic stimuli reduced dark responsiveness, particularly with relatively short photo cycles. Decreased responsiveness to the repeated experience of non-aversive stimuli has also been reported with habituation of the acoustic startle response (Burgess and Granato 2007b). Furthermore, when applying relatively long and repeated photo cycles, a potential impact of age and time of day on larval behavior needs to be considered as reported previously (de Esch et al. 2012). In conclusion, age, time of day, and photo regimens affect zebrafish behavior. Hence, these parameters apparently limit the inter-comparability of different studies. Given that the duration of photo cycles is sufficiently long and the number of iterations is kept relatively low, repeated application of photic stimuli can improve data robustness and proof reproducibility. In addition, responses to short repeated photo cycles can be used as a measure of habituation.

Various exposure settings determine behavioral alterations

Behavioral assays such as the light-dark transition test can be used to assess whether and to which extent chemicals interfere with the developing as well as mature nervous system. However, most toxicological studies of behavior use widely varying experimental setups since assessment of behavior represents a non-standardized procedure (Legradi et al. 2015). Here, we systematically investigated different periods of exposure and used lethality-based anchoring to adjust exposure levels. In doing so, we also considered developmental-stage specific susceptibility. Moreover, we used various chemicals such as AChE (DZN, TPP) and cyclooxygenase inhibitors (DIC, NAP), as well as anticipated narcotic chemicals (CYP, DIU) to determine potential chemo-specific behavioral responses. In dependence on the various settings including period and level of exposure, we determined significant alterations in locomotor activity for each of the six model chemicals. Thereof, the majority of observed responses showed a down-regulation of locomotor activity. Deviating from that, DIU caused hyperactivity with any exposure scenario including early, middle, as well as late exposure to low, medium, and high levels, respectively, ranging from 4.6 to 18.4 µM. Our results are in line with previous reports on increased locomotion with DIU concentrations as low as 4.3 µM, i.e. below the NOEC(0-4 dpf) for mortality (Velki et al. 2017). Velki et al.

(2017) determined light-dark transition behavior at 5 dpf after 118 h of exposure using a twice repeated photo cycle of 4 min per photoperiod. The same study did not find any differences between control and treatment with DZN up to the LC_{10}(0-4 dpf) of 11.5 µM (Velki et al. 2017). Our results confirm these findings. However, we found that the 4-cell stage is remarkably more susceptible to DZN than the prim 6 stage. Lethal effect concentrations differ by one order of magnitude between the different exposure periods. A similar stage-specific susceptibility was observed with the organophosphorus AChE inhibitor azinphos-methyl (Massei et al. 2015). Massei et al. (2015) identified the early cleavage period between 0 and 4 hpf as the most sensitive developmental stage whereas initiation of exposure at 24 hpf (~prim 6) and 72 hpf did not elicit any lethal toxicity. Indeed, we observed decreased locomotion with DZN concentrations of 57.8 and 41.9 µM when exposure was initiated at prim 6 and larval stage on day four, respectively. Interestingly, short term treatment of the larval stage with a high dose of DZN caused a light dependent hyperactive phenotype. Both observations match the expected mode of action (MOA) of AChE inhibition. Accumulation of the neurotransmitter acetylcholine at neuromuscular junctions results in overstimulation of muscarinic and nicotinic acetylcholine receptors. Depending on the degree of severity, i.e. level and period of exposure, spasms and myopathy-like phenotypes manifest, respectively (Behra et al. 2002; Fukuto 1990). Similar to DZN, TPP caused a hyperactive phenotype specific to exposure of the larval stage. Such distinctions in stage-specific susceptibility to AChE inhibitors may be attributed to the versatile roles of AChE in developmental processes such as neuronal and muscular development (Behra et al. 2002). Furthermore, the biotransformation capacity of zebrafish increases with the time course of development (Brox et al. 2016). Hence, the higher transformation rate of e.g. organic phosphorothioates like DZN into more potent oxon metabolites may explain higher behavioral efficacy in later developmental stages.

Differential behavioral effects of cyclooxygenase inhibitors

Besides anti-AChEs, we examined non-steroidal anti-inflammatory drugs (NSAIDs) as a further class of chemicals with distinct MOA namely COX inhibition. Mechanistically, NSAIDs repress synthesis of prostaglandins relevant to numerous functions such as sleep-wake regulation (Hayaishi 1991; Urade and Hayaishi 2011). However, we determined remarkable differences in behavioral efficacy between the COX inhibitors DIC and NAP depending on the period of exposure. While DIC exclusively increased dark responsiveness with acute exposure at larval stage and reduced light dependent locomotion when exposure was initiated at prim 6, NAP decreased locomotion at each of the examined exposure periods. Although both chemicals are non-selective COX inhibitors, the two differ in selectivity for different COX isoforms. DIC has a two times higher selectivity for the inducible COX-2

whereas NAP has a three-fold increased selectivity for COX-1 compared with respective isoforms (reviewed in Rao and Knaus 2008). Like NAP, NSAIDs more selective for COX-1 including diflunasil, piroxicam, and fenoprofen but also other chemicals with anti-inflammatory properties such as steroidal glucocorticoids and phosphodiesterase inhibitors were found to increase waking activity in larval zebrafish by day but not at night. In contrast, DIC did not alter any of the two parameters but increased rest latency, i.e. the timing of a first rest bout after a light-dark transition (Rihel et al. 2010). While differential target selectivity may explain differences in observed behavioral phenotypes between the NSAIDs, inter-chemical variation between exposure periods is left unexplained. Not least because both DIC and NAP are poorly taken up by the zebrafish embryo but nevertheless elicit stable or even slightly increasing internal concentrations over time, respectively (Schüttler et al. 2019).

Together, the present findings suggest that anchoring of exposure levels based on lethal effect concentrations may not necessarily capture behaviorally relevant effect levels of chemicals such as drugs because therapeutic effective doses may be of relevance for changes in behavior and might therefore occur well below acute toxicity levels. In addition, high toxic ratios, i.e. the ratio of a chemical's LC_{50} estimated from a QSAR for baseline toxicity and its experimental LC_{50}, are also expected for some pesticides that act highly specific on nervous system relevant targets (Vaal et al. 2000). For example, the carbamate insecticide aldicarb was found to alter locomotor activity (EC_{50}(0-4 dpf)) of zebrafish 660 times below its respective LC_{50}(0-4 dpf) (Klüver et al. 2015). Account must also be taken of non-monophasic dose response relationships in studies of behavior. In a study by Ali et al. (2012) more than half out of sixty chemicals of various groups caused a biphasic concentration response. Nevertheless, lethality-based anchoring may provide a reasonable starting point to determine behavioral alterations at sub-lethal levels of exposure. Furthermore, regarding the specificity of behavioral responses, numerous studies use widely varying photo regimens (Legradi et al. 2015) and may therefore miss behaviorally and toxicologically relevant alterations. For example, apomorphine, a dopamine receptor agonist, particularly extended darkness-mediated hyperactivity in subsequent minutes after light-dark transition (Irons et al. 2013). Hence, mechanism-specific differentiation of chemicals on the basis of behavioral phenotypes may be improved by using prolonged photo cycles and a higher temporal resolution.

Developmental exposure affects swim bladder inflation and locomotor activity

Inflation of the swim bladder at 3 to 4 dpf represents a key transition in locomotor behavior as larvae shift from lying motionless aside into active swimming accompanied by increased responsiveness to stimuli (Fero et al. 2011). Noteworthy, in a large-scale genetic screen of 525 zebrafish mutants, most mutations caused lethality by 5 dpf while almost all surviving

mutants failed to inflate their swim bladder which certainly died by two weeks of age (Amsterdam et al. 2004). Hence, the swim bladder may represent one of the most sensitive targets for chemicals in developing zebrafish. Along those lines, we found that inflation of the swim bladder was in many cases affected if exposure was initiated in the early cleavage phase and at prim 6 stage, respectively. This applies to NAP and CYP whereas TPP exclusively prevented inflation with 4-cell exposure. On the other hand, zebrafish embryos exposed to DZN and DIC where only affected when exposure was initiated at prim 6 stage. Interestingly, failure of swim bladder inflation was accompanied by a hypoactive locomotor phenotype. In contrast, excessive locomotor activity was exclusively observed if the swim bladder was developed properly as seen with late short term exposure of the larval stage to DIC, DZN, and TPP. Furthermore, DIU which did not affect swim bladder inflation with any exposure scenario caused a consistently hyperactive phenotype. The differential sensitivity of early developmental stages of the zebrafish with regard to the COX inhibitors NAP and DIC may also explain their diverging behavioral phenotypes. In conclusion, it seems as if it is probably more than coincidence that the inflated swim bladder represents an indispensable morphological feature allowing for proper larval locomotion. To overcome potential limitations due to morphological defects such as a lack of swim bladder inflation, exposure of larval stages may be considered as a reasonable alternative. To compensate for potentially lower internal concentrations due to a reduction in exposure time, the estimation of the bioconcentration factor in adult fish (OECD 2012) can be used to correct aqueous exposure concentrations (see **Supporting information A2.2**). Not least because fish acute toxicity test and FET test elicit nearly one-to-one correlation (Belanger et al. 2013; Klüver et al. 2015; Knöbel et al. 2012; Lammer et al. 2009).

Concluding remarks

In summary, first, the assessment of alterations in locomotor behavior can improve sensitivity beyond levels of acute lethal toxicity. Effects on locomotion can be detected at effect levels as low as LC_{01}. Moreover, it appears necessary to cover a wide concentration range to address specific neuroactive effects at sub-lethal effect levels. In this context, lethality-derived effect concentrations provide a reasonable upper starting point. Second, although underlying mechanisms of locomotor regulation by anticipated narcotic chemicals, such as DIU and CYP, remain to be elucidated, such chemicals need to be considered in the assessment of neuroactivity. Thus, further research is needed to determine whether alterations in behavior are specific to (known and unknown) neuroactive chemicals or whether altered locomotion only represents the manifestation of unspecific/secondary effects on nervous and/or locomotor system. Third, exposure throughout development can induce morphological defects which in turn may affect larval locomotion as observed in the case of

swim bladder inflation. Therefore, we suggest the exposure of larval stages in order to specifically detect interferences of chemicals with neuronal function instead of measuring unspecific side effects on locomotion due to developmental toxicity. Finally, according to existing literature and in line with our own observations, the temporal resolution, i.e. the time increments of aggregation of locomotor activity on one hand, and sufficiently long photoperiods on the other hand may improve the specificity of behavioral responses to chemical exposure. Such more detailed setups offer additional information on changes in activity within specific periods during light and dark conditions. Furthermore, as we proofed a limited number of iterations of light-dark transitions to be reproducible, the repetition of photo cycles can improve the data situation/robustness for subsequent analyses. It is now clear how to design experiments with behavioral alterations as readout for direct effects of chemicals on the nervous system as opposed to developmental defects. Whether the proposed measures apply to the differentiation and sensitive detection of distinct MOA classes of chemicals remains to be investigated.

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Chapter 3

Can environmentally relevant neuroactive chemicals be detected specifically with the locomotor response assay in zebrafish larvae? [1]

Abstract

Chemicals considered as neuroactive, such as certain pesticides, pharmaceuticals and industrial chemicals are among the largest groups of bioactive substances recently detected in European rivers. However, the determination of nervous system-specific effects has been limited using *in vitro* tests or conventional endpoints including lethality. Thus, neurobehavioral tests using *in vivo* models (e.g. zebrafish embryo) have been proposed as complementary approaches. To investigate the specificity and sensitivity of a light-dark transition locomotor response (LMR) test in 4 to 5 days post fertilization zebrafish with respect to different modes of action (MOAs), we analyzed a set of 18 environmentally relevant compounds with various anticipated MOAs. We found that exposure-induced behavioral alterations were reproducible and dependent on concentration and time. Comparative and quantitative analyses of the obtained locomotor patterns revealed that behavioral effects were not restricted to compounds primarily known to target the nervous system. A clear distinction of MOAs based on locomotor patterns was not possible for most compounds. Furthermore, chemicals with an anticipated same MOA did not necessarily provoke similar behavioral phenotypes. Finally, we determined an increased sensitivity (≥10-fold) compared to observed mortality in the LMR assay for 5 of 8 neuroactive chemicals as opposed to non-neuroactive compounds.

3.1 Introduction

Aquatic ecosystems face the contamination with multiple anthropogenic chemicals including pesticides, pharmaceuticals and industrial chemicals. An analysis of chemical monitoring data from major European river catchments revealed that besides compounds with unknown modes of action (MOAs), especially neuroactive chemicals are representing the largest group of detectable compounds with known MOA (Busch et al. 2016). Although *in silico* target prediction approaches for neurotoxicity are a desirable long term goal, their development requires large amounts of bioactivity data (Llorens et al. 2012). However, due to the limited availability of *in vitro* assays able to capture effects relevant for the nervous system (Agid et al. 2007), the detection of neuroactive chemicals and the assessment of their

[1] Published in a slightly modified form as: Leuthold D, Klüver N, Altenburger R, Busch W. 2019. Can environmentally relevant neuroactive chemicals specifically be detected with the locomotor response test in zebrafish embryos? Environ Sci Technol 53(1): 482-493.

acute and sub-lethal toxicity is challenging (Altenburger et al. 2018). Using *in vivo* assays such as the acute fish embryo toxicity (FET) test with zebrafish (*Danio rerio*) (OECD 2013) as a potential alternative for the acute fish test with adults (OECD 1992), it has been shown that certain neuroactive compounds provoke lower acute toxicity in early life stages of fish compared to adult stages (Klüver et al. 2015; Lammer et al. 2009; Scholz et al. 2016). Behavior-based assays such as the light-dark transition locomotor response (LMR) test have therefore been described of being specifically sensitive for neuroactive compounds and to increase sensitivity beyond acute toxicity levels (Klüver et al. 2015). Beyond that, the diagnostic capacity of behavioral tests in embryonic and larval zebrafish has been demonstrated in neuroactive drug discovery (Bruni et al. 2016; Kokel et al. 2010). Conversely, whether behavioral profiling has the potential to support the assessment of complex chemical mixtures such as environmental samples, e.g. through identification of mixture toxicity drivers, needs to be elucidated. However, behavior assays, such as the LMR test, lack a uniform test design (Legradi et al. 2015) and it is known that minor methodological changes (e.g. age, incubation conditions, light driving, plate format) can alter its outcome (Fraser et al. 2017). Consequently, comparisons between results from different studies are difficult.

To apply behavioral tests for the diagnosis and assessment of neuroactive chemicals, specificity and sensitivity measures need to be determined. Therefore, we performed behavior-based toxicity tests on a set of 18 chemicals representing various MOAs to investigate whether the obtained LMR patterns are sufficiently specific to potentially indicate the anticipated MOAs. Therefore, an improved, extended protocol as outlined in **Chapter 2** was used. In addition, based on results presented in **Chapter 2** it was already substantiated that certain chemicals not considered as neuroactive can cause behavioral alterations. Here, we, furthermore, hypothesized that neuroactive chemicals should provoke neurobehavioral effects at concentration ranges well below acute toxicity levels as opposed to non-neuroactive chemicals. Considering concentration and time dependency of toxicity during zebrafish development and investigating lethal and behavioral effects we could not clearly show a general specificity of the applied LMR assay for the investigated neuroactive compounds but found an increased sensitivity for certain chemicals of this group.

3.2 Materials and methods

3.2.1 Fish cultivation, embryo collection, chemicals and stock preparation

Details on fish cultivation, embryo collection, chemicals and stock preparation are given in **Supporting information B3.1**.

3.2.2 Experimental design and determination of exposure concentrations

Exposure was initiated at 96 h post fertilization (hpf) (up to 120 hpf) in order to avoid exposure-induced developmental abnormalities potentially affecting larval movement. Because no mortality data were available for this time window, concentrations initially selected for testing were based on toxicity data obtained in earlier studies using exposure windows of 24-96 hpf and 0-96 hpf, respectively. These tests were conducted according to OECD test guideline no. 236 (OECD 2013) adapted to 7.5-mL glass vials using three individuals per 6 mL test solution in triplicate (26 °C, 75 rpm, Edmund Bühler SM-30 Control, 14:10 h light:dark). Subsequently, for behavioral experiments, a dilution series was calculated for each compound considering mortality, bioconcentration (see also **Supporting information B2.2**) and maximum water solubility, yielding 7 to 17 test concentrations per compound (**Supporting table B3.1**).

3.2.3 Exposure and light-dark transition locomotor response assay

Sixteen 96 hpf zebrafish embryos (ZFEs) per treatment were exposed to a linear concentration series of a single chemical. Subsequently, individuals were transferred separately to single wells of 96-well polystyrene plates which were covered with cell culture test plate lids and sealed with laboratory film. Until the end of the test at 120 hpf, plates were incubated at 28 °C in the dark. ZFEs were analyzed in a light-dark transition locomotor response (LMR) test after 1.5, 6.0, and 22.5 h of exposure (i.e. at 97.5, 102, and 118.5 hpf), using a ZebraBox monitoring enclosure with corresponding software in tracking mode (ViewPoint Life Sciences, Lyon, France). The total measurement time comprised 80 min with a photoperiod sequence of 10min dark, 10 min light, and a twice repeated dark-light transition of 20 and 10 min, respectively. The first dark and light phase were used for acclimation and were not considered for quantitation. Subsequent to each LMR test, mortality was evaluated based on absence of heartbeat and coagulation of the larva. Dead individuals

were excluded from behavioral data analysis at the respective time point of investigation. More details are given in **Supporting information B3.1**.

3.2.4 Analysis of locomotor activity data

Locomotor activity was recorded in terms of total distance moved and was integrated every single minute for each treatment (T) and control group (C) (*n* = 16 larvae each). Photoperiods repeated twice were treated as replicates. I.e. data were aggregated as median total distance covered per minute yielding 30 min in total (20 min dark, 10 min light). Differences between C and T were considered significant in case the confidence intervals (CIs) of C and T did not overlap (**Supporting information B3.1**).

3.2.5 Multidimensional scaling (MDS) and cluster analysis

MDS coordinates were calculated (KNIME Distance Matrix Calculate node) using the median distance moved per minute for each treatment and control group. Heatmap generation and hierarchical clustering were conducted using the heatmap.2 function from the R package gplots (version 2.13.0, Warnes et al. 2015). The latter was performed on a selected subset of data (**Supporting information B3.1**).

3.3 Results

In this study, we analyzed a set of 18 environmentally relevant compounds representing eight MOA groups with at least two model compounds per group in order to investigate their corresponding behavioral phenotypes and the specificity and sensitivity of the LMR assay. We included anticipated neuroactive compounds (e.g. insecticides, specific pharmaceuticals) representing four different mechanisms of nervous system interaction as well as substances without known neuroactive properties (e.g. anti-inflammatory pharmaceuticals, industrial chemicals) and chemicals with unknown MOAs in fish (e.g. herbicides, fungicides) (**Table 3.1**).

To prevent developmental toxicity-induced morphological alterations, potentially affecting larval locomotion, we did not initiate exposure before 96 hpf. Behavioral phenotypes were recorded after 1.5, 6.0, and 22.5 h of exposure in a concentration-dependent manner. Furthermore, we assumed that the surveillance of behavioral alterations throughout the complete time window of exposure should allow for the identification of appropriate exposure

Table 3.1. Modes of action and model compounds selected for locomotor response analyses.

	Molecular target	Mode of action *	Compound	Substance group	Use	MW (g/mol)	logD (pH7.4)
Neuroactive	Acetylcholinesterase	AChE inhibition[a,b]	Diazinon	Organothiophosphate	Pesticide (insecticide, acaricide, repellent, veterinary substance)	304.35	3.80
	(AChE)	AChE inhibition[c]	Triphenylphosphate	Organophosphate	Flame retardent, plasticizer	326.28	4.12
	Serotonin reuptake	Selective serotonin reuptake (SSR) inhibition[d]	Citalopram hydrobromide	Nitrile	Drug (antidepressant)	405.311	1.27
	(SSR/SNR)	Serotonin-norepinephrine reuptake (SNR) inhibition[d]	D,L-Venlafaxine	Tertiary amino compound	Drug (antidepressant)	277.4	1.43
	Nicotinic acetylcholine receptor	Competitive nACh-R agonism[a,b]	(-)-Nicotine	Pyrimidine	Pesticide (insecticide)	162.23	0.37
	(nACh-R)	Competitive nACh-R agonism[a,b]	Imidacloprid	Neonicotinoid	Pesticide (insecticide, veterinary substance)	255.66	0.29
	Gamma-aminobutyric acid receptor	GABA-gated Cl^--channel antagonism[a,b]	Endosulfan	Organochlorine	Pesticide (insecticide, acaricide)	406.93	3.87
	(GABA-R)	GABA-gated Cl^--channel antagonism[a,b]	Fipronil	Phenylpyrazole	Pesticide (insecticide, veterinary substance)	437.15	3.71
Non-neuroactive	Cyclooxygenase	COX inhibition, inhibition of leukocyte migration[d]	Diclofenac sodium	Monocarboxylic acid	Drug (nonsteroidal anti-inflammatory agent)	318.13	1.37
	(COX)	COX inhibition[d]	Naproxen sodium	Methoxynaphtalene	Drug (nonsteroidal anti-inflammatory agent)	252.25	0.45
	Photosystem II	PSII inhibition[a]	Isoproturon	Urea	Herbicide	206.28	2.45
	(PSII)	Inhibition of photosynthesis[a]	Diuron	Phenylurea	Herbicide	233.1	2.75
		Methaemoglobin production[e]	3,4-Dichloroaniline	Unclassified	Metabolite	162.02	2.60
	Estrogen receptor	E-R agonism[f]	4-n-Nonylphenol	Alkyl phenol	Surfactant (adjuvant, other substances)	220.35	5.13
	(E-R)	E-R agonism[g]	Bisphenol A	Bisphenol	Plasticizer, pesticide (fungicide)	228.29	3.63
	Protein synthesis	Methionine synthesis (MetS) inhibition[a]	Cyprodinil	Anilinopyrimidine	Pesticide (fungicide)	225.29	3.62
	(MetS)	Methionine synthesis (MetS) inhibition[a]	Pyrimethanil	Anilinopyrimidine	Pesticide (fungicide)	199.25	3.18
Solvent	Multiple	Narcotic, metabolic acidosis, ocular damage[h]	Methanol	Alcohol	Solvent	32.04	-0.52

* MOA information were compiled from (a) Pesticide Properties Database, (b) Insecticide Resistance Action Committee database, (c) Mc Millan et al. 1991, (d) Drugbank, (e) McMillan et al. 1991, (f) Hill et al. 2003, (g) LeFol et al. 2017, and (h) Liesivuori et al. 1991 (relevance of MOA in fish is not necessarily considered here). Molecular weight (MW) according to manufacturer description. logD (pH 7.4) was estimated using the PhysChem Profiler module of ACD/Percepta (ACD/Labs, build 2726. 27 Nov 2014). Chemical structures of the tested compounds are provided in **Supporting figure SB3.1**.

periods that exclusively reveal the primary behavioral mechanism of a compound in the absence of morphological alterations.

3.3.1 Concentration and time dependency of behavioral profiles

The underlying principle of the light-dark transition test is the rapid alteration from basal to sharply increasing locomotor activity induced by an immediate switch from visible to invisible light. Prior to the finally applied experimental setup we conducted tests on the design, time point and duration of measurement. Within the established setup, we recognized that the behavioral phenotype of untreated ZFEs was reproducible at each time point but changed with age, especially between 102 and 118.5 hpf. Within this temporal frame larvae became more active (**Figure 3.1**). Hence, the performed LMR assay proved to be robust and suitable in order to display potentially distinct behavioral phenotypes in a time dependent manner.

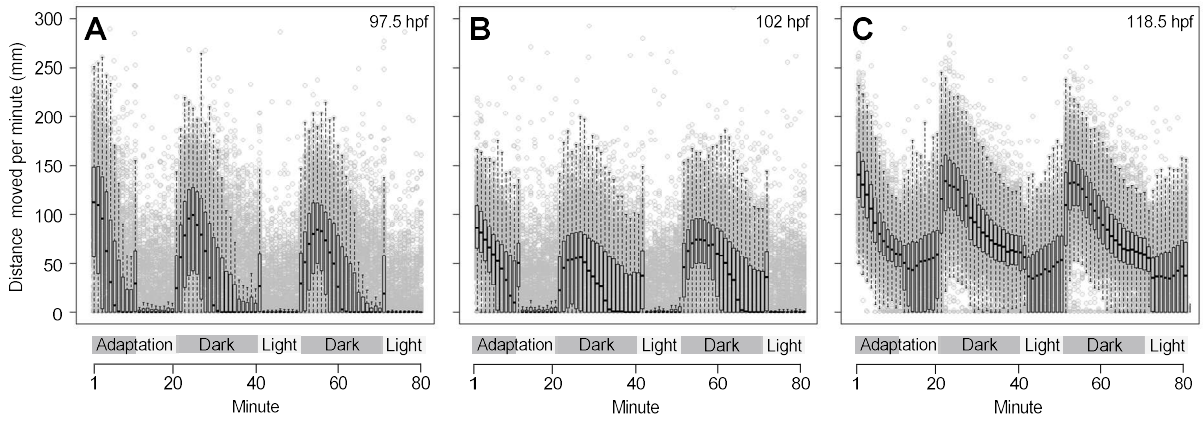

Figure 3.1. Locomotor activity is developmental stage-specific. Boxplots show the distance moved per minute of untreated zebrafish larvae (*n*=688) of different age: (**A**) 97.5, (**B**) 102.0 and (**C**) 118.5 hpf, respectively.

We found that the age-specific profiles changed after chemical treatment depending on concentration. Using the example of 3,4-dichloroaniline, we show how swimming behavior was altered after treatment with three different concentrations for 1.5 and 22.5 h (**Figure 3.2A** and **B**). The activity profiles changed with exposure time and concentration, e.g. from constant locomotion (**Figure 3.2A**; red line) to complete inhibition of movement (**Figure 3.2B**; red line) and *vice versa* (**Figure 3.2A** and **B**; yellow line). Normalizing the observed profiles to control levels using differences in activity between exposed larvae and untreated controls translated into a heatmap indicating hypo- (blue) and hyperactivity (red), respectively, for overall ten analyzed concentrations of 3,4-dichloroaniline. We observed that high and low concentrations caused hypolocomotion compared to control levels whereas median concentrations caused hyperactivity in ZFEs (**Figure 3.2C** and **D**). Additionally, looking at

e.g. a concentration of 271 µM, 3,4-dichloraniline caused hyperactivity at 97.5 hpf but hypoactivity at 118.5 hpf (1.5 and 22.5 h post exposure, respectively; **Figure 3.2C** and **D**). Our observations on concentration but also time dependent behavioral phenotypes were not restricted to 3,4-dichloroaniline but rather showed up for all of the investigated compounds (**Supporting figure B3.2**).

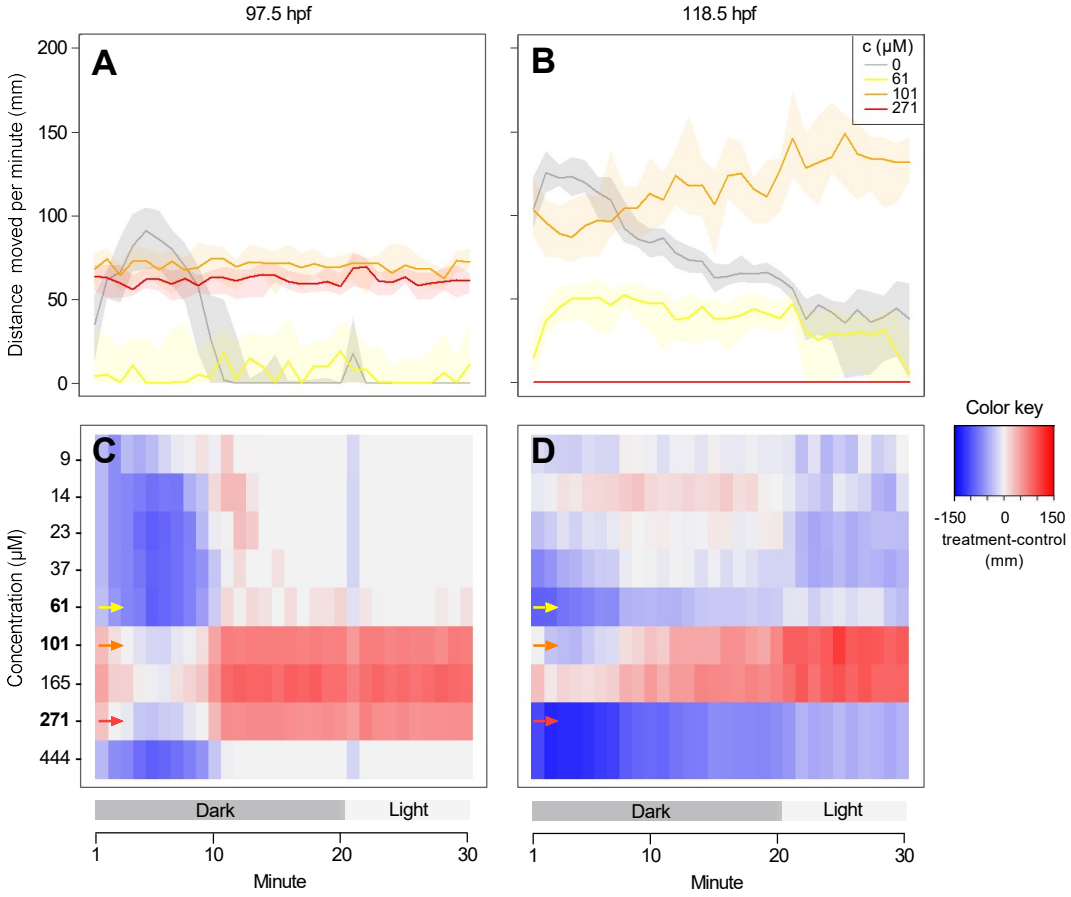

Figure 3.2. Behavioral profiles of 3,4-dichloroaniline are concentration and time dependent. Grey (control) and colored lines (treatment): median distance moved per minute (*n*=16 individuals per treatment) at (**A**) 97.5 (1.5 h of exposure) and (**B**) 118.5 hpf (22.5 h of exposure), respectively. Shaded areas: respective 95% CIs. (**C, D**) Heatmaps indicate the difference in activity between treatment and control for all tested concentrations.

3.3.2 Behavioral phenotype-based clustering of chemicals

In order to gain an overview on the relation between behavioral profiles obtained with our assay, we calculated pairwise Euclidean distances between all locomotor patterns of each of the 18 compounds and every concentration at each investigated time point (1.5, 6.0, 22.5 h of exposure between 96 and 118.5 hpf). We, therefore, used MDS considering all recorded behavioral profiles in a joint analysis. Results are presented in three plots separated

according to exposure duration. Additionally, warm and cold colors indicate anticipated neuroactive and other MOAs, respectively (**Figure 3.3**). As described above, the movement pattern of controls was subjected to age-specific changes as it can also be seen from the MDS representations in which the control cluster moves from the top right corner to the top left side over time (**Figure 3.3**; grey shaded areas). We found that behavioral alterations were not restricted to neuroactive compounds but also comprised the other investigated MOA classes. Depending on the level and duration of exposure each of the 18 tested compounds altered locomotion.

After 1.5 h of exposure (**Figure 3.3A**) we observed the most pronounced alterations in movement (largest spatial distance to controls) at various concentrations of AChE inhibitors (22-87 µM diazinon and 5-11 µM triphenylphosphate, yellow points and triangles) as well as for diuron (34-83 µM, light blue triangles), 3,4-dichloroaniline (101-271 µM, turned light blue triangles) and naproxen (668-1236 µM, dark blue triangles). These data points have a close spatial proximity, indicating that these compounds shared a similar behavioral phenotype at the given concentrations. In contrast to the tested AChE inhibitors, isoproturon (83-215 µM, light blue points) and diclofenac (1-12 µM, dark blue points) did not show close spatial relation to their MOA analogues and formed separate, compound-specific clusters (**Figure 3.3A**). Additionally, endosulfan (0.1-0.2 µM, pink circles) and nicotine (10.1 µM, 20.2-30.3 µM, red circles) provoked patterns distinct from other compounds (**Figure 3.3A**). Besides MOA- and compound-related clusters, we found that despite citalopram, venlafaxine, imidacloprid, diclofenac, naproxen, isoproturon, and diuron, 11 chemicals converged towards a common data point (MDS coordinates ~[66, 2]) in at least one concentration (**Figure 3.3A**). The characteristic behavioral profile behind was the complete inhibition of movement throughout the LMR test which was often indicative for mortality at a later time point of investigation.

After 6 h of exposure (**Figure 3.3B**), we observed that diazinon (34-58 µM, yellow triangles) and triphenylphosphate (7-11 µM, yellow points) did not remarkably change their spatial position indicating temporally stable behavior profiles for these concentrations, whereas lower concentrations of triphenylphosphate (5 µM) and higher concentrations of diazinon (87 µM) converged closer to control levels. This was also observed for naproxen (**Figure 3.3B**; 491-908 µM, dark blue triangles). This time dependent shift led to a separation of diuron (light blue triangles) and 3,4-dichloroaniline (turned light blue triangles) forming a more independent cluster (**Figure 3.3B**). However, data points of AChE and PSII inhibitors are located close to each other, indicating that the underlying behavioral phenotypes were not unique and specific for the respective MOAs. In contrast, a unique pattern for endosulfan

was observed (0.05-0.20 µM, pink points), being, however, different from the pattern of its tested MOA analogue fipronil (pink triangles) (**Figure 3.3B**).

As mentioned above, at 118.5 hpf (**Figure 3.3C**) we observed the most pronounced shift in locomotor activity by control groups as compared to previous time points of investigation (also see **Figure 3.1**). Furthermore, similarities of the tested SSR/SNR inhibitors citalopram (22-172 µM, orange points) and venlafaxine (56-225 µM, orange triangles) became more obvious after 22.5 h of exposure (**Figure 3.3C**). They converged in a concentration-dependent manner towards the MDS region described above as being characteristic for absolute inactivity (**Figure 3.3C**). Additionally, exposure to endosulfan (0.05-0.79 µM, pink points) and 3,4-dichloroanilline (101-165 µM, turned light blue triangles) led to the most pronounced alterations in behavior (large spatial distance from the control cluster) (**Figure 3.3C**). At a concentration of 61 µM, 3,4-dichloroaniline (turned light blue triangle) formed a cluster with its MOA analogues diuron (153 µM, light blue triangle) and isoproturon (215-340 µM, light blue points) (**Figure 3.3C**). However, this cluster could not be identified as MOA-specific since behavioral profiles of triphenylphosphate (7-11 µM, yellow triangles) and nonylphenol (12 µM, dark green point) were located in the same area as the PSII inhibitors (**Figure 3.3C**).

In order to identify phenotypes potentially indicative for a certain MOA we systematically selected specific patterns (**Supporting information B3.1**) for each compound and performed hierarchically clustering. The results are shown in a heatmap in **Figure 3.4**.

Most behavioral profiles changed remarkably over time. For example naproxen (c7=668 µM), led to an overall activation in movement after 1.5 h of exposure (**Figure 3.4A**) which turned into hypoactive behavior with peak hyperlocomotion at the transition from dark to light after 22.5 h of exposure which was exclusively observed for naproxen (**Figure 3.4B**). After 22.5 h of exposure, two main clusters were observed, whereof the bottom cluster consisted of behavioral profiles mainly representing hypoactivity throughout the test period (**Figure 3.4B**). These profiles mainly included concentrations which induced lethality (>5%) at this time point (**Figure 3.4B**). We only identified endosulfan (c9=0.8 µM) and pyrimethanil (c6=185 µM) to cause both hyperactivity and lethality at 118.5 hpf (**Figure 3.4B**). Please note that only vital larvae were considered for LMR data analyses. Furthermore, we found several combined profiles where activity was increased in the dark phase, but decreased in the light phase (e.g. methanol, c9=720 mM) and *vice versa* (e.g. diuron, c6=83 µM). Some compound-related clusters indicating a decrease in activity were found for bisphenol A (c6=38 µM-c8=77 µM) (**Figure 3.4A**) and fipronil (c5=1 µM-c10=9 µM) (**Figure 3.4B**).

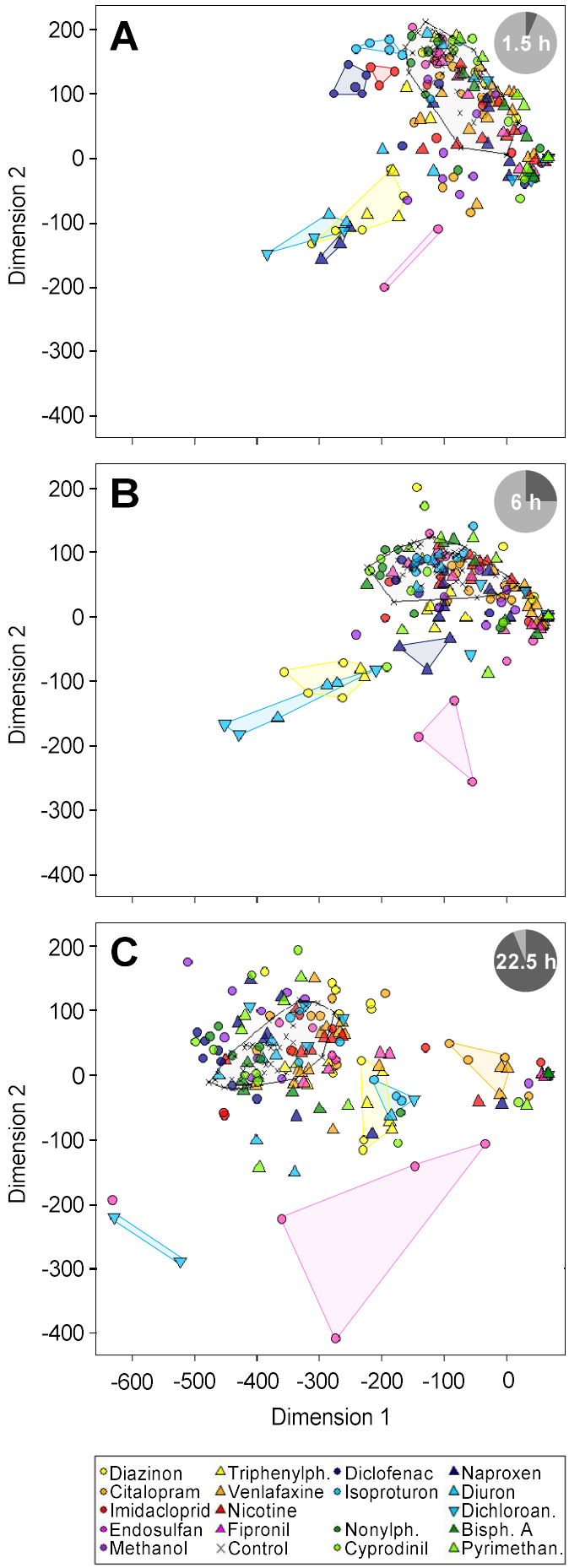

Figure 3.3. Multidimensional scaling (MDS) plots of behavioral profiles. Same color: same mode of action group. Each symbol refers to a specific concentration. Rows: exposure duration: (**A**) 1.5 h, (**B**) 6 h, and (**C**) 22.5 h. Light grey areas: spatial expansion of control groups in the two-dimensional space. Clusters discussed in the main text are highlighted. Each of the three plots is directly comparable with each other since MDS was based on a common distance matrix calculated from median moved distances per minute within a measurement period of 30 min in total (20 min dark, 10 min light).

Overall, clusters did not show clear differentiation of response patterns of neuroactive compounds from other MOA classes. Independent of exposure duration, we could not clearly identify MOA-specific clusters. We merely found that e.g. 3,4-dichloroaniline (c7=165 µM, c8=271 µM) and diuron (c6=83 µM) clustered according to behavioral profiles mainly characterized by hyperactivity (**Figure 3.4A**). However, e.g. the neuroactive diazinon (c10=58 µM) also caused a behavioral phenotype similar to the two PSII inhibitors (**Figure 3.4A** and **B**). Furthermore, diazinon (c11=87 µM) grouped with its MOA analogue triphenylphosphate (c5=5 µM) after 1.5 but not after 22.5 h of exposure (**Figure 3.4A** and **B**). Also the SNR/SSR inhibitors venlafaxine (c6=0.2 µM) and citalopram (c9=22 µM) exclusively clustered 1.5 h post exposure (**Figure 3.4A**).

3.3.3 Sensitivity of locomotor responses in relation to lethal acute toxicity

In order to assess the sensitivity of behavioral responses in relation to mortality, we conducted concentration response analysis of both endpoints and compared EC50 and LC50 after 1.5, 6.0 and 22.5 h of exposure. **Figure 3.5** additionally shows the contribution of hypo- and hyperactivity to the total observed effect and how these changed over time. All concentration-response curves are shown in **Supporting figure B3.3**.

For example, **Figure 3.5A** shows that exposure to diazinon for 1.5 and 6.0 h induced behavioral alterations mainly driven by hyperactivity (red pies), whereas 22.5 h exposure led to hypoactivity-dominated phenotypes (blue pie). Additionally, we determined a reduced effect intensity in terms of difference in the total distance moved at EC50 (smaller pie chart size) after 22.5 h of exposure compared to the previous two time points. Furthermore, behavioral and lethal effects in terms of EC50 (pie chart centers) and LC50 (triangles), converged over time. Along those lines we found that the sensitivity of behavioral responses in many cases decreased over time or that at least sensitivity did not remarkably increase with exposure duration. In contrast, citalopram and the investigated GABA-receptor antagonists endosulfan and fipronil led to lower EC50 values after prolonged exposure, i.e. 6.0 and/or 22.5 h (**Figure 3.5C**, **G**, and **H**).

Similar to diazinon, we observed time dependent changes in behavioral phenotypes for numerous other compounds characterized by initial hyperactivity followed by hypoactive behavior after 6.0 and/or 22.5 h of exposure. Also, the intensity of observed behavioral effects was subjected to temporary changes. For e.g. diclofenac we observed a slight but significant hyperactivation in movement (EY50=75.5 mm) after 1.5 h of exposure, which turned into an even weaker response (EY50=10 mm) after 6.0 h and finally completely disappeared after 22.5 h of exposure (**Figure 3.5J**). On the other hand, we found that the

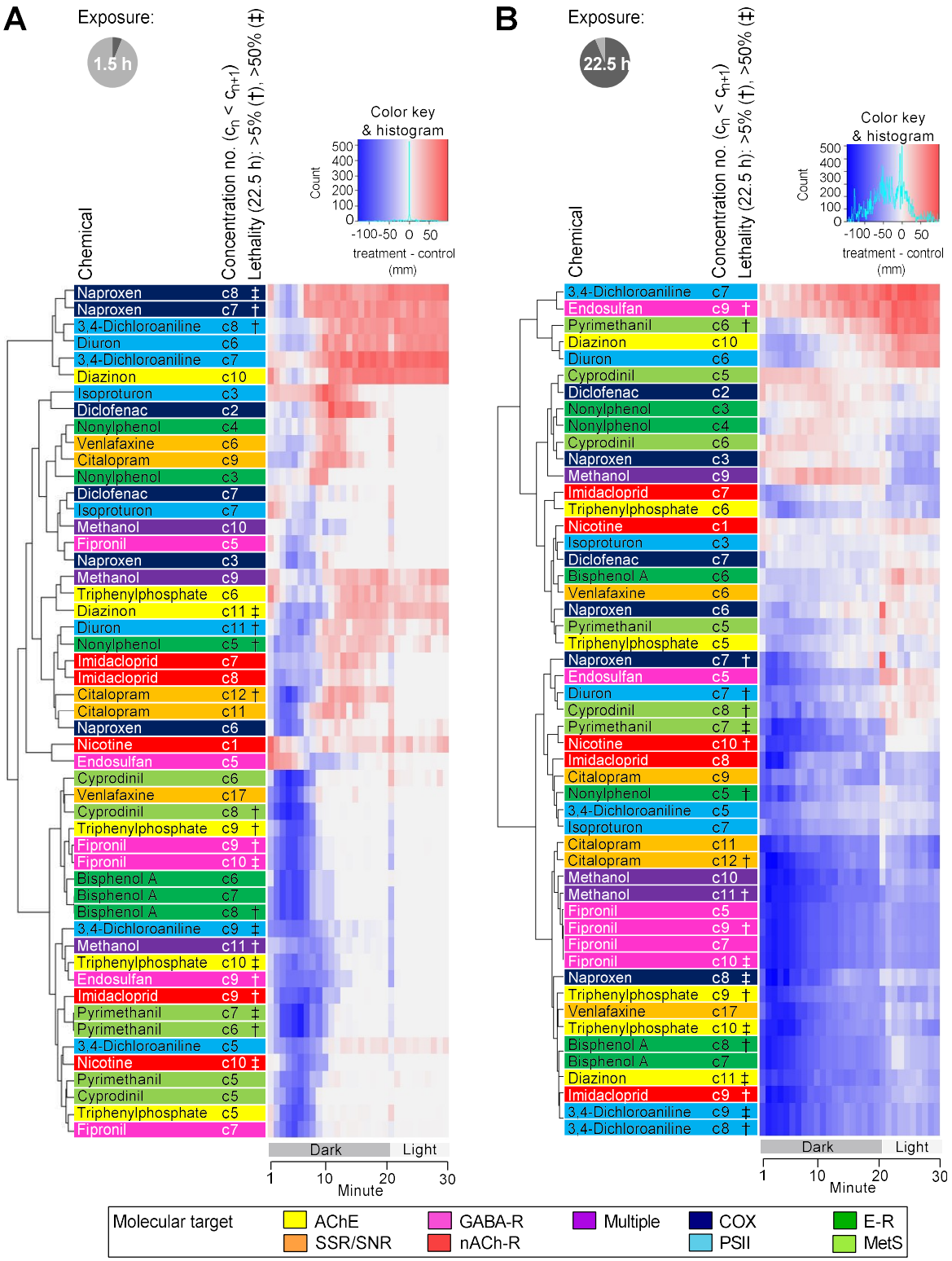

Figure 3.4. Behavioral phenotype-based clustering of chemicals with various modes of action after (**A**) 1.5 and (**B**) 22.5 h of exposure for selected concentrations (systematic data selection is described in Supporting Information). Concentrations are listed in **Supporting table B3.1**. Color key and histogram indicate the difference in median distance moved between treatment and control and the distance frequency distribution of shown profiles, respectively.

two investigated serotonin reuptake inhibitors, the two nACh-R agonists (**Figure 3.5C-F**), as well as triphenylphosphate, fipronil, methanol and bisphenol A (**Figure 3.5B**, **H**, **I**, and **P**) remarkably increased effect intensity over time.

We additionally compared behavioral and lethal effect concentrations and found that exposure to diclofenac led to the most sensitive behavioral response (EC50(1.5 h)=0.7 µM) of all investigated compounds and time points being two orders of magnitude more sensitive than its corresponding LC50(1.5 h)=165 µM. However, we did not observe behavioral alterations after 22.5 h of exposure but increasing mortality (LC50(22.5 h)=77 µM) (**Figure 3.5J**). Except for venlafaxine and isoproturon which caused behavioral alterations in the absence of acute toxicity, we determined that behavioral and lethal effect concentrations increasingly converged with exposure duration. In this context, we merely found few compounds leading to an at least 10-fold increase in sensitivity (compared to mortality at the respective time point) for the respective exposure time window, including citalopram (22.5 h) and endosulfan (22.5 h). In addition, the ratio of LC50(22.5 h)/EC50(1.5 h) was >10 for imidacloprid and diuron (see **Supporting table B3.2** for effect values).

Since the exposure window applied here (96-118.5 hpf) deviates in duration and developmental stage from the one applied in the acute FET test according to OECD test guideline 236 where exposure is performed from 0-96 hpf, we compared our results with acute FET data obtained in toxicity tests conducted from 0-96 hpf and from 0-120 hpf, the latter covering the developmental stage we used for exposure in our study. To get an idea of whether certain compounds and MOAs have a higher toxicity in adult life stage, we also included acute fish toxicity (AFT) test (OECD 1992) data previously summarized by Scholz et al. (2018). **Figure 3.6** summarizes the data that we had available for this comparison (**Supporting table B3.2**). The figure indicates that LC50 values obtained after 96 and 120 h of larval exposure are nearly identical. No mortality was detected for imidacloprid, endosulfan, fipronil and venlafaxine in the 96 h FET test. We therefore compared our results to the limit of water solubility (Sw) as a reference value. Except for diuron and 3,4-dichloraniline, our LC50 values for 22.5-h exposure (between 96 and 118.5 hpf) are in most cases slightly higher due to shorter exposure but are within a range of one order of magnitude compared to acute toxicity after 0-96 and 0-120 hpf exposure, respectively. The even weaker acute toxicity of diuron and 3,4-dichloroaniline indicates a higher susceptibility of the early embryonic developmental stage to these compounds. Behavior-based EC50 values, determined after 22.5 h of exposure, were in most cases in a similar range as acute FET data. However, deviations by at least factor 10 from the reference value (96-h LC50 or Sw) could be observed for citalopram, venlafaxine, endosulfan, fipronil, nicotine, and 3,4-dichloroaniline. At the same time, we observed an increasing sensitivity in mortality for three

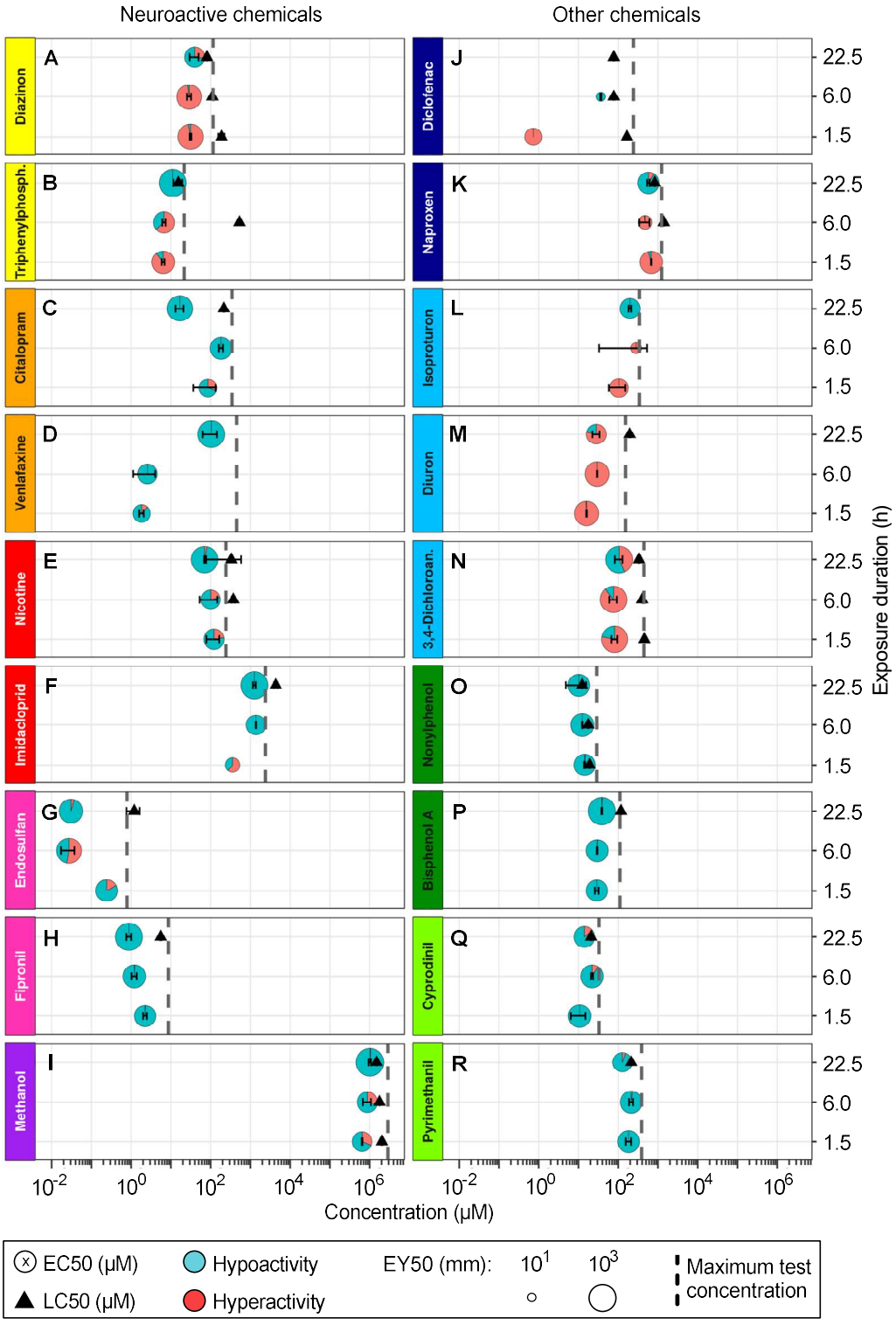

Figure 3.5. Sensitivity of behavioral responses (EC50) and their relation to lethal effect concentrations (LC50) over time. Center of pie chart, EC50 (LMR test); size of pie chart, EY50 (total effect, i.e. absolute difference in distance moved between treatment and control at EC50); blue/red pies, contribution of hypo- /hyperactive behavior to overall observed effect; LC50 at respective time point (same exposure duration), black triangles; maximum tested concentration (LMR assay), dashed vertical lines; error bars, 95% CI (n≥160 individuals per compound). LC50s above maximum tested concentration were extrapolated.

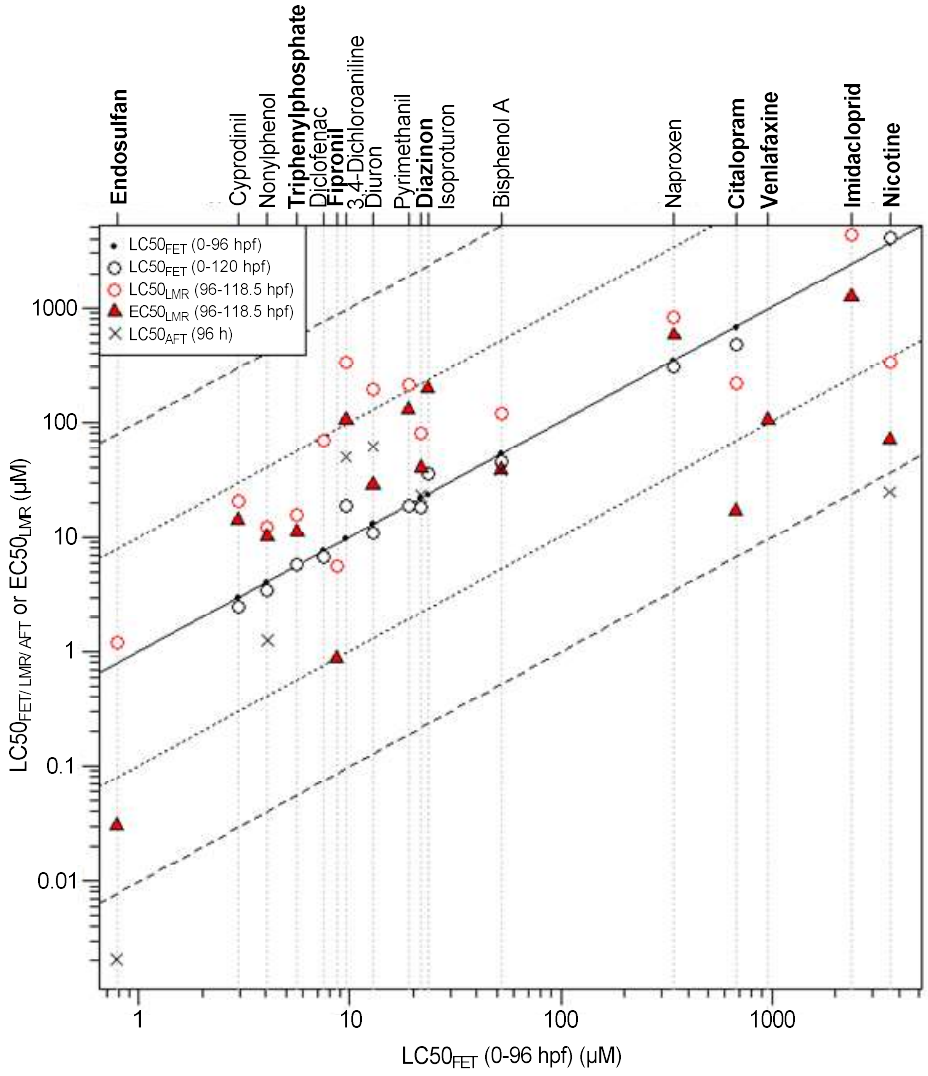

Figure 3.6. Comparison of acute fish embryo toxicity (FET, circles), acute fish toxicity (AFT, crosses) and locomotor response effects (LMR, triangles) for selected exposure time windows. In case no mortality was observed within 96 h, the water solubility limit of the respective compound was used as reference point. Broken lines indicate orders of magnitude deviations from LC50 (0-96 hpf). Methanol not shown (see **Supporting table B3.2** for effect concentrations).

of these five compounds (fipronil, citalopram, nicotine) when exposure was initiated at 96 hpf. Considering this, a ratio between lethality and locomotor effects larger than 10 was only found for the SSR/SNR inhibitors citalopram, and venlafaxine, as well as for the GABA-R antagonist endosulfan, while venlafaxine did not lead to lethality at all. In comparison with the AFT test our study shows that results obtained with the FET test are in a similar range for 5 out of 7 available datasets except for the neuroactive substances endosulfan and nicotine, where the adult fish has a higher sensitivity. For such compounds a supplementation of the FET test with behavioral responses can increase the sensitivity of the fish embryo in the context of animal alternative testing.

3.4 Discussion

The occurrence of a variety of chemicals potentially acting as neurotoxicants in the aquatic environment has been shown (Busch et al. 2016; Massei et al. 2018). Organisms are expected to be exposed to mixtures of potentially neuroactive and other compounds. Next to neuroactive pharmaceuticals, which show a low acute toxicity but high pharmacological efficacy by design (reviewed e.g. by Khetan and Collins (2007)), also insecticides and compounds with unintended neuroactivity are a matter of concern due to their environmental occurrence. The detection of neurotoxicity is difficult as availability of acute *in vitro* assays is limited and compounds with a neurotoxic MOA have previously been demonstrated to show weak toxicity in the acute FET test (Klüver et al. 2015). Hence, ecologically relevant endpoints such as behavior have been proposed for improving the detection of ecotoxicological and potentially adverse effects of neurochemicals (Crane et al. 2006; Klüver et al. 2015).

In the present study we applied a light-dark transition test in larval zebrafish (4-5 d post fertilization, dpf) with a time and dose-resolved experimental design in order to investigate the specificity and sensitivity of this assay in comparison to lethality measures. Therefore, we analyzed substances with an anticipated neuroactive MOA as well as compounds presumably exerting other MOAs.

Larval zebrafish demonstrate robust, age-specific locomotor activity

First, we demonstrated that ZFEs between 4 and 5 dpf display a reproducible locomotor pattern which is specific to their developmental stage. Our finding is directly in line with a previous study of de Esch et al. (2012) who found that the motor activity pattern of *D. rerio* (5-7 dpf) is influenced by fish age. Different from 4 dpf old individuals, 5 dpf old ZFEs were characterized particularly by a sharp light-dark response and a comparatively higher level of basal locomotor activity under light conditions. Additionally, we found that the variability in activity between individuals is also stage-dependent and lowest when using the ~5-dpf stage. Another aspect which suggests the use of stages ≥4 dpf derives from the fact, that the inflation of the first posterior chamber of the swim bladder which represents a vital organ regulating buoyancy takes place around 4.5 dpf (Winata et al. 2009).

Exposure regimens determine the outcome of the light-dark transition assay

The dependency of motor responses from the duration of exposure has been demonstrated for e.g. certain dopaminergic drugs (Irons et al. 2013). As this assay is not standardized, exposure regimes vary in literature ranging from early developmental exposures to late

embryonic short-term exposures (Legradi et al. 2015). Taking into consideration that the nervous system of *D. rerio* is still developing during embryogenesis (reviewed e.g. by Schmidt et al. 2013), the use of different developmental stages and exposure scenarios may lead to significant differences in the results of behavior studies. Variable and complex processes of embryogenesis provide varying targets for chemicals and interactions may lead to adverse outcomes directly or via a cascade of effects. Consequently, observed behavioral alterations may have different causes including developmental (neuro)-toxicity and morphological defects.

The developmental stage-specific expression of nervous system receptors during embryogenesis poses a challenge for neurobehavioral research. For example, 23 different $GABA_A$ receptor subunits were identified in zebrafish (Cocco et al. 2017). Monesson-Olson et al. (2018) investigated the expression of eight α subunit-encoding genes for their spatial and temporal expression at 1, 2, and 4 dpf and found that two out of eight genes were not expressed before 1 dpf and one gene even not before 2 dpf. Transcripts for all α subunit-encoding genes were only detected at 4 dpf (Monesson-Olson et al. 2018). This finding ties in well with our observations wherein the anticipated GABA-gated chloride channel antagonists endosulfan and fipronil did not cause mortality within 0-4 dpf, while mortality emerged during a 4-5 dpf exposure. Hence, the availability of molecular targets and their physiological functionality is essential in order to provoke MOA-related effects detectable at organism level. Therefore, it seems more reasonable to assume that neurobehavioral effects related to the primary MOA in certain cases may be found rather in advanced life stages due to the further developed functionality of corresponding receptor systems. Therefore, we applied a 24-h short-term exposure scenario starting 4 dpf and including multiple time points of measurement. Our results on behavioral responses to 18 compounds occurring as soon as 1.5 h post chemical treatment confirm that short-term exposure is a reasonable choice for the detection of anticipated MOA-specific effects. Furthermore, we observed in case of e.g. AChE inhibitors time-dependent shifts of the behavioral phenotype from hyper- to hypoactivity. Contrary to the absent expression of $GABA_A$ receptors during 1 and 2 dpf (Monesson-Olson et al. 2018), AChE is already present during early embryonic development of *D. rerio* (Küster 2005; Teixidó et al. 2013). Primarily, it is involved in the termination of signal transmission mediated by the neurotransmitter acetylcholine at neuromuscular junctions and cholinergic brain synapses (reviewed e.g. by Soreq and Seidman 2001). Hence, the partial inhibition of AChE and subsequent accumulation of acetylcholine can be expected to produce a hyperactive locomotor phenotype. The results of our experiments on the AChE inhibitors diazinon and triphenylphosphate found clear support for this hypothesis. However, we showed that hyperactivity was only found after 1.5 and 6.0 h but not after 22.5 h of exposure, although we still detected significant inhibition of AChE at that time point

(**Supporting figure B3.4**). Furthermore, besides its primary biological role in acetylcholine-mediated neurotransmission, AChE has been shown to be involved in the neuronal and muscular development of the zebrafish embryo (Behra et al. 2002). Consequently, the duration and the time point of exposure initiation influences the way of interference and observed behavioral phenotypes. Particularly, alterations in neuronal development not visible morphologically but at a behavioral level can distort the recognition of a specific MOA. In this context, Yang et al. (2011) demonstrated that 48-h exposure of zebrafish (24-72 hpf) to chlorpyrifos oxon altered the touch-evoked swimming responses which were, however, accompanied by significant inhibition in outgrowth of sensory and motoneurons below exposure levels inducing mortality or obvious developmental defects. For the SNR inhibitor venlafaxine it was found that exposure (0-120 hpf) reduced the larval swimming activity in the dark and also promoted neurogenesis. These two examples indicate that potential secondary side effects not visible by eye can impact on behavioral phenotypes when applying long-term exposures. Considering this in connection with our findings, we propose to use short-term exposure scenarios of older embryonic/larval stages when aiming at capturing molecular initiating events and their directly related outcomes at organism level. Overall these findings are in accordance with the experimental design used by e.g. Kokel et al. (2010) and Bruni et al. (2016) who applied chemical treatments for 1-10 h for phenotypical differentiation of specifically acting drugs.

Diagnostic capacity of locomotor phenotypes for distinct modes of action

The specificity of the LMR assay towards distinct MOAs was of interest for this study. Therefore, we selected and investigated anticipated neuroactive compounds as well as compounds with other anticipated MOAs. In line with previous studies we found that neuroactive chemicals including AChE inhibitors, SSR/SNR inhibitors, nACh-R agonists, and GABA-R antagonists cause behavioral effects detectable with the LMR assay. Moreover, we demonstrate that behavioral alterations are not restricted to chemicals known to be neuroactive. Here we show that e.g. herbicides, anti-inflammatory drugs and fungicides can alter locomotor activity, too. This is consistent with what has been reported in some studies showing that e.g. photosynthesis inhibitors such as diuron (Velki et al. 2017a) and atrazine (Liu et al. 2016b), certain COX inhibitors (Xia et al. 2017), or fungicides such as imazalil (Jin et al. 2016) are able to disturb embryonic and larval swimming behavior in the absence of acute toxicity and obvious morphological defects. An explanation provided for the observations made for the fungicide imazalil and the herbicide atrazine was their ability to inhibit AChE in zebrafish (Jin et al. 2016; Liu et al. 2016b). A similar result was obtained in a study by Bretaud et al. (2000) who found significant inhibition of brain AChE in juvenile goldfish (*Carassius auratus*) after 24 h of exposure to 2.1 µM diuron. By contrast, Velki et al.

(2017b) did not detect AChE inhibition by the herbicide diuron in zebrafish exposed from 2-98 hpf up to a concentration of 8.6 µM. However, they found significant changes in thigmotactic and locomotor behavior in 120 hpf ZFEs at a concentration of 4.3 µM (Velki et al. 2017a). Apart from the studies cited here, the availability of literature investigating behavioral effects and underlying molecular mechanisms of chemicals that are not assumed to be neuroactive is scarce. This highlights that little is known about the neuroactive potential of most compounds in organisms like fish. Hence, the limited knowledge about the transferability of MOAs that were defined in biological models other than *D. rerio* calls for more detailed investigation of underlying mechanisms. In addition, zebrafish are capable to biotransform xenobiotics already during early embryogenesis (Brox et al. 2016). Hence, observed temporal changes in the behavioral phenotype may in certain cases rather be attributed to unknown transformation products than parent compounds.

The observed behavioral profiles in our study comprised of both hypo- and hyperactivity in ZFEs for the majority of investigated compounds. Activity patterns changed with increasing concentrations meaning that there is not one single behavioral profile being representative for a certain compound. At high exposure levels, which we assume to induce a general disturbance of homeostasis shortly before apparent toxicity (similar to cytotoxicity in cell-based *in vitro* assays) the majority of chemicals caused complete inhibition of locomotion which indicated lethality. Frequently, we observed multiphasic concentration-response patterns especially for those compounds which induced hyperactivity at low concentrations while higher concentrations caused hypoactivation. Such biphasic concentration response patterns were also reported by Ali et al. (2012) who analyzed a set of 60 water-soluble compounds using a light-dark transition assay in 120 hpf ZFEs exposed from 24 hpf. The multiphasic behavioral effects of certain compounds may reflect their ability to impact on different neurotransmitter systems or other molecular targets in a dose dependent manner. However, based on the lacking specificity of observed behavioral responses, we cannot distinguish such patterns. Furthermore, some chemicals may have multiple MOAs potentially affecting behavior in different ways. For example, chronic exposure to the AChE inhibitor triphenylphosphate has been shown to alter hormone levels in zebrafish (Liu et al. 2016a). However, mechanisms of acute developmental and chronic (neuro)-toxicity are not necessarily covered by our approach.

Evidence for MOA-indicative clusters, at least temporarily, could be identified for some substances including those assigned as AChE and SSR/SNR inhibitors. Especially the SSR/SNR inhibitors citalopram and venlafaxine were different compared to other compounds and MOAs at 118.5 hpf (22.5 h of exposure) due to their systematic concentration-dependent decrease of locomotor activity which could be observed with concentrations from around 1 to

>100 µM. For all other compounds behavioral effects ranged within one order of magnitude. Our findings on venlafaxine are directly in line with recent results of Thompson et al. (2017) who found a significant dose-related reduction in the activity level of zebrafish (120 hpf) in the dark. We also found phenotypic similarities between the hyperactivity-dominated profiles of the AChE inhibitors diazinon and triphenylphosphate which were, however, also similar to the effects found for the PSII inhibitor diuron and its degradation product 3,4-dichloroaniline. So far, however, there is no evidence that diuron or 3,4-dichloroaniline inhibit AChE in embryonic or larval zebrafish which means that the underlying concept of similarity between those behavioral phenotypes remains to be elucidated (see **Chapter 4**).

Behavioral profiles of single compounds being distinct from MOA analogues were observed for diclofenac, isoproturon, endosulfan, and fipronil. This might be explained by the differences in chemical structures compared to anticipated MOA analogues. For example, the insecticides endosulfan and fipronil are assigned as GABA-gated chloride channel antagonists but belong to two different chemical classes, namely organochlorines and phenylpyrazoles, respectively. In addition, endosulfan is thought to specifically act on certain arachnids whereas fipronil is known as a specific disruptor of the central nervous system in insects. In our study, endosulfan caused an anti-cyclic light-dark response whereas fipronil provoked a completely different hypoactive phenotype. There are indications that fipronil does not (primarily) act as a GABA-R antagonist in ZFEs but rather inhibits glycine receptors (Stehr et al. 2006). In contrast, known GABA-R antagonists like pentylenetetrazole and picrotoxin were found to reverse the normal light-dark response in zebrafish larvae (Ellis et al. 2012; Yang et al. 2017) as endosulfan did in our study. Hence, GABA-R antagonists may be identified via this specific phenotype.

Finally, our results demonstrate that behavioral alteration alone cannot be seen as a specific parameter exclusively indicating effects of anticipated neuroactive substances. A refined investigation of specificity using a larger set of chemicals and including compounds with distinct known neuroactive MOAs (e.g. certain pharmaceuticals) that serve as a phenotypical reference may further improve and establish LMR tests as diagnostic tools. In addition, a sophisticated test design consisting of a more complex application of stimuli as used by Bruni et al. (2016) in the context of neuropharmaceutical discovery might be necessary for behavior-based distinction of chemicals according to their MOAs. Furthermore, besides using the total distance moved as endpoint, an additional integration of other parameters and endpoints such as time spent moving or direction of movement as demonstrated by Palmér et al. (2017) may refine the resulting patterns and may help to increase specificity.

Relationship between behavioral responses and lethality

As a measure for sensitivity of locomotor activity-based endpoints we compared ratios of effect concentrations for acute toxicity and behavioral alteration. Our results demonstrate that significant behavioral effects mostly occurred throughout a narrow concentration range at the borderline to lethality, in most cases covering only one order of magnitude or less. This holds true for some anticipated neuroactive but also for the other compounds. A higher sensitivity of the LMR assay was identified for the SSR/SNR inhibitors citalopram and venlafaxine with ratios ≥9.3 after 22.5 h of exposure as well as for the GABA-R antagonist endosulfan. We found that the behavioral responses and the acute toxicity converged with increasing exposure duration except for venlafaxine and isoproturon which did not cause lethality up to the limit of water solubility. In contrast to previous findings showing an increased sensitivity through behavioral endpoints using exposure from 2-98 hpf (Klüver et al. 2015), we could not confirm that this type of LMR test in general provides a substantial increase in sensitivity beyond the acute toxicity levels when exposure is initiated at later embryonic stages. Klüver et al. (2015) investigated e.g. the AChE inhibitor aldicarb and found a 660-fold increase in sensitivity compared to mortality in the 4-day acute FET test when using a light-dark transition test at 98 hpf. By contrast, we detected a merely 2-fold increase in sensitivity for the AChE inhibitors diazinon and triphenylphosphate with our short-term exposure regime in older stages. These differences might, however, be explained by unspecific activities of diazinon and triphenylphosphate as we detected AChE inhibition at levels close to baseline toxicity (**Supporting figure B3.4**). For endosulfan an EC50 (LMR assay) of 0.015 µM after 96 h of exposure was reported by Klüver et al. (2015) without mortality up to the limit of water solubility. Here we determined a similar EC50 of 0.031 µM within a four times shorter exposure window (96-118.5 hpf) but, in contrast to the previous study, we already observed mortality after 22.5 h of exposure and extrapolated an LC50 (96-118.5 hpf) of 1.2 µM. We assume that the observed hyperactivity due to the interaction of endosulfan with the GABA-R leads to increased energy consumption and allocation and finally to mortality. Especially at 5 dpf this appears critical because internal energy resources in form of yolk are exhausted (Kimmel et al. 1995). The limited GABA-R availability before 96 hpf (Monesson-Olson et al. 2018), therefore, may prohibit an acute toxicity of endosulfan in earlier stages of embryonic development. These findings indicate that toxicity testing of compounds with molecular targets not expressed in the early embryo might be supported by additional LMR analyses at 4 dpf or should be performed in later embryo-larval stages.

Concluding remarks and future perspective

In summary, first, the finding that various environmentally relevant chemicals including both, anticipated neuroactive chemicals as well as chemicals with other, partially unknown

mechanisms, alter locomotor activity in larval zebrafish highlights the necessity to additionally consider chemicals with previously unknown neuroactive or other properties to interfere with behavior in future studies. Moreover, second, given the phenotypic similarity determined among chemicals of different anticipated MOA classes, an in-depth analysis of actual underlying mechanism in zebrafish is indispensable in order to further validate the diagnostic power of the light-dark transition test. Therefore, a subset of chemicals which were demonstrated to cause hyperlocomotion will be examined in more detail for potentially underlying mechanisms in **Chapter 4**. In this context, the present study may give a first indication since such a phenotype, e.g., observed with diazinon and triphenylphosphate, could already be associated with the inhibition of AChE. Third, as it was found that numerous neuroactive chemicals elicit increased behavioral efficacy in comparison with lethal acute toxicity, including both FET test (0-4 dpf) and the exposure period between 4 and 5 dpf, this work once more substantiates that behavioral alterations represent a sensitive measure of sub-lethal chemical exposure, particularly for neuroactive chemicals. Finally, the present study highlights that the larval zebrafish in combination with acute short-term exposures represents an efficient model system to study the immediate effects of various chemicals on behavior.

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Co-occurrence of acetylcholinesterase inhibition and bradycardia is associated but not causally linked with hyperlocomotion in zebrafish larvae after chemical exposure

Abstract

The release of anthropogenic chemicals into the environment poses a risk to aquatic organisms. For the purpose of environmental risk assessment, information on mechanisms of action of chemicals is of relevance but detailed mechanistic information is often elusive, particularly in non-target species. In this chapter we strived to elucidate the underlying mechanisms of hyperlocomotion previously observed in the light-dark transition test with larval zebrafish (*Danio rerio*) after exposure to diverse environmental chemicals including the anti-acetylcholinesterase pesticide diazinon, the industrial chemical triphenylphosphate, and the herbicide diuron as well as its degradation product 3,4-dichloroaniline. Using molecular, physiological, and phenocopy approaches the mechanistic background of hyperlocomotion was investigated. It was found that inhibition of acetylcholinesterase, reduction in heart rate, and narcosis are factors of influence but are not sufficient to explain observed changes in behavior. Instead, a comparison of results with findings from literature indicate that hyperlocomotion is related to complex disorders of the brain since observed behavioral phenotypes are similar to those reported for pharmacological zebrafish models of epilepsy. The findings of this study provide a basis for further research necessary to unravel details on underlying mechanisms and causalities between certain molecular and physiological key events leading to hyperlocomotion in zebrafish larvae after chemical exposure.

4.1 Introduction

The occurrence of anthropogenic chemicals in the aquatic environment raises concerns about adverse biological effects. So called effect-based tools (EBTs) such as the fish embryo acute toxicity test (OECD test no. 236, OECD 2014), *Daphnia* sp. acute immobilization test (OECD test no. 202, OECD 2004), freshwater alga growth inhibition test (OECD test no. 201, OECD 2011) and *in vitro* bioassays have been proposed to detect, identify, and/or quantify interactions between chemicals and biological targets, respectively (Altenburger et al. 2015; Altenburger et al. 2019; Wernersson et al. 2015). According to their level of biological organization, EBTs differ in complexity and specificity for chemical-biological interdependencies (Busch et al. 2016). For example, *in vitro* assays provide specific, target-

based information (e.g. activation of the aryl hydrocarbon or estrogen receptor) but fail to capture complex interactions (e.g. between tissues and organs within an organism) and related outcomes at higher levels of biological organization (Agid et al. 2007; Paul et al. 2010). In contrast, phenotype-based approaches, using cell cultures or whole organisms, are rather untargeted and do not require *a priori* assumptions about specific molecular interactions (Stockwell 2000). However, it has been challenging to link adverse outcomes (AOs) at higher levels of biological organization to underlying mechanisms of action at the molecular, cellular, and organ level. Consequently, actual mechanisms of toxicity are often elusive, particularly in non-target species (Busch et al. 2016). The adverse outcome pathway (AOP) framework has been developed for knowledge-based safety assessment of chemicals (Ankley et al. 2010). It relies on the understanding of toxicity mechanisms, rather than the simple observation of AOs at the apical scale (Bal-Price et al. 2015). The AOP concept covers molecular initiating events (MIEs), i.e. a chemicals' interaction with a molecular target (e.g. a receptor), followed by a sequential series of key events, i.e. alterations in biological processes at different levels ranging from cell to organism, that ultimately result in an AO at individual and/or population scale (Ankley et al. 2010). Such structured evidence of mechanistic toxicity is anticipated to guide a more effective (prospective) risk assessment and regulatory decision making (Ankley et al. 2010; Collins et al. 2008; Legradi et al. 2018).

Among various xenobiotics, particularly neuroactive chemicals pose a challenge to environmental risk assessment. This is because the functional and structural complexity of the nervous system provides a variety of targets for neurotoxicants (Bal-Price et al. 2015). Furthermore, neurotoxicity does not necessarily result in acute lethal toxicity (Klüver et al. 2015). A previous literature study revealed that a neuroactive MOA is associated to nearly 30% of chemicals detected in European surface water bodies (Busch et al. 2016). Furthermore, it has been estimated that up to ~30,000 commercially used chemicals may have an unknown neuroactive potential (Tilson et al. 1995). Nevertheless, there is only a limited number of AOPs related to neurotoxicity and developmental neurotoxicity, respectively. This is due to a number of challenges such as a general lack of understanding of certain MIEs and the limited number of well-defined pathophysiological outcomes which restrict the development and use of the AOP framework for neurological outcomes (Bal-Price et al. 2015). Even though there are on-going efforts to develop cellular screens for developmental neurotoxicity (Bal-Price et al. 2012; Bal-Price et al. 2010; Coecke et al. 2007; Crofton et al. 2011; Lein et al. 2007), *in vivo*-based screens have been proposed as more suitable readout of neuroactivity given their ability to depict the anatomical and functional heterogeneity of the nervous system (MacRae and Peterson 2015).

As one of several EBTs zebrafish (*Danio rerio*) emerged as indispensable vertebrate model for *in vivo* analysis of developmental and acute toxicity mechanisms. In particular, early developmental stages enable high-throughput, as well as high-content approaches due to their small size and represent an alternative to conventional animal testing (MacRae and Peterson 2015; Scholz et al. 2013; Teixidó et al. 2019). Moreover, alterations in gene expression, physiology, morphology and behavior have been used to unravel underlying mechanisms of bioactivity and toxicity, respectively (Dubińska-Magiera et al. 2016). For some time now, phenotype-based screens, aiming at the determination of mechanistic similarities between chemicals of known and unknown bioactivity, proved to be a powerful tool for the identification of mechanisms (Kokel et al. 2010; Lehár et al. 2008). In particular, behavior-based approaches have been used to discover new targets and functions of chemicals (Bruni et al. 2014). Among them, the light-dark transition test has become established as a widespread method to determine alterations in behavior as the integrated response to chemical exposure.

In **Chapter 3** it was found that diverse neuroactive as well as anticipated differently acting chemicals alter the locomotor response to light-dark transitions in larval zebrafish and that certain chemicals elicit similar phenotypes within specific concentration ranges. In particular, it was discovered that chemicals known to inhibit acetylcholinesterase (AChE), such as diazinon and triphenylphosphate, as well as the herbicide diuron and its degradation product 3,4-dichloroaniline cause similar phenotypes of hyperlocomotion (Leuthold et al. 2019). In this study, the aim was to investigate the background of these observations at the molecular and organ level. Based on previous observations we hypothesized that the inhibition of AChE may be associated with the observed behavioral phenotype.

4.2 Materials and methods

4.2.1 Fish cultivation and embryo collection

A hybrid of wild-type (Wild India Kolkate, “WIK”) and an in-house *D. rerio* strain, “UFZ-OBI” (generation F2 and F3), were cultured in 120-L aquaria (activated carbon-filtered tap water, 26.5±1 °C) with a photoperiod of 14:10 h light:dark. Commercial dry food and *Artemia* sp. were fed once and twice a day, respectively. Cultivation and use were conducted according to German and European animal protection standards. Fish culture was approved by the Government of Saxony (Landesdirektion Leipzig, file number 75–9185.64). Spawn was collected within stainless steel sieve-covered glass dishes. Fertilized eggs were selected by means of microscopy and were transferred into oxygen-aerated (≥ 24 h) and pH-adjusted

(pH 7.4±0.1) standard dilution water as specified in ISO 7346-3 (80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl) with a density of 1 egg per 2 mL. Subsequently, zebrafish embryos were raised at 28 °C with a 14:10 h light:dark cycle until initiation of exposure at 4 d post fertilization (dpf).

4.2.2 Chemicals and stock preparation

Chemicals and purities purchased from Sigma-Aldrich (Steinheim, Germany) were 3,4-dichloroaniline (99.9%, CAS RN 95-76-1), triphenylphosphate (≥99.0%, CAS RN 115-86-6), and verapamil hydrochloride (≥99.0%, CAS RN 152-11-4). Chemicals and purities purchased from Sigma-Aldrich (Buchs, Switzerland) were aldicarb (≥98.0%, CAS RN 116-06-3), diuron (≥98.0%, CAS RN 330-54-1), and diazinon (98.5%, CAS RN 333-41-5). Metoprolol tartrate (CAS RN 56392-17-7) was purchased from MP Biomedicals (Illkirch, France). Chemical stocks were either prepared directly in standard dilution water with a final concentration of 0.1% v/v methanol immediately prior to testing or chemicals were dissolved in pure methanol (≥99.8%, CAS RN 67-56-1, J.T. Baker®, Avantor Performance Materials Inc, Pennsylvania, USA). Test solutions were then produced from serial dilution of stock solutions prior exposure. The final concentration of methanol was 0.1% v/v in every exposure solution.

4.2.3 Exposure and pre-incubation

4 dpf zebrafish were exposed to linear concentration series of the respective chemicals. Immediately after exposure was initiated, individuals were transferred separately to single square-shaped flat bottom wells of a 96-well clear polystyrene plate (Whatman™ microplate devices, uniplate®, GE Healthcare UK Limited, Buckinghampshire, UK). The volume of test solution within each well was 400 µL. Subsequently, plates were sealed with a self-adhesive polyester film (Th. Geyer, Wertheim, Germany). Additionally, plates were covered with cell culture test plate lids (Techno Plastic Products, Trasadingen, Switzerland) and sealed with laboratory film (Pechiney Plastic Packaging, Chicago, Illinois, USA). Until test initiation at 1.5 h post exposure plates were incubated at 28 °C in the dark.

4.2.4 Light-dark transition assay and analysis of locomotor activity data

Light-dark transition tests were performed as described in **Chapter 3**. Briefly, 1.5 h post exposure locomotor activity, i.e. the distance moved per minute, was evaluated using an automated tracking device (ZebraBox, Viewpoint, Lyion, France). The sequence of applied

photoperiods was 10’ D, 10’ L, (20’ D, 10’ L) × 2 (D, dark; L, light). The latter two repeated phototransitions were aggregated minute-wise for the respective time increments per photo cycle. Behavioral profiles were then calculated by subtraction of median locomotor activity in treatment and control. Heat map representations were created using the heatmap.2 function of R (gplots, version 2.13.0).

4.2.5 Quantification of acetylcholinesterase activity

Subsequent to the locomotor response assay, i.e. ~3 h post exposure, larvae were sampled from well plates for analysis of *in vivo* acetylcholinesterase (AChE) activity. The photometric determination of enzyme activity was performed according to Ellmann et al. (1961). In addition, samples were examined for total protein content as previously described (Lowry et al. 1951). Further details are given in **Supporting information C4.1**. Subsequently, the change in optical density per minute was normalized by the total amount of protein (OD/min/mg). Results were expressed as relative AChE activity referring the activities of treated samples to the respective mean activity of controls. Concentration-response curves were fit to the mean relative AChE activity using a Hill 4-parameter logistic model (see **Equation B3.3** in **Appendix B**) to derive the half maximum effect concentration, i.e., EC50 (R package “drc”, Ritz and Streibig 2005). Results are shown in **Supporting figure C4.3**. No (NOEC) and lowest and observed effect concentration (LOEC) of AChE activity (OD/min/mg) were determined using a two-tailed t-test with $p<0.05$ (GraphPad Prism 8.2.0). *In vitro* AChE activity was determined as described above but using 4 dpf zebrafish whole body homogenates spiked with the respective chemicals dissolved in phosphate buffer and incubated for 3 h (Thermomixer comfort, Eppendorf, Hamburg, Germany, 28 °C, 300 rpm). Significance analysis was performed using an ordinary one-way ANOVA followed by Dunett’s multiple comparisons test (GraphPad Prism 8.2.0).

4.2.6 Heart rate determination

Exposure was conducted according to **4.2.3**, with the difference that crystallization dishes were used as incubation vessels. The volume of exposure medium was 10 mL per eight individuals. 3 h post exposure individuals were anesthetized using 0.5 mL tricain (6 g/L, 98%, CAS RN 886-86-2, Sigma Aldrich, Steinheim, Germany) per vessel. Heart beats were then counted manually by means of stereo microscopy for a total of 15 s. Relative heart rate was derived from the ratio of individual heart rates (beats per minute, bpm) of equally treated larvae relative to the mean heart rate in the respective control group. The mean value thereof

was used for concentration-response analysis. Curves were fit using a Hill 4-parameter logistic model as described in **Equation B3.3** (**Appendix B**). Corresponding data is shown in **Supporting figure C4.4**. Statistically significant differences (NOEC, LOEC) in heart rate (bpm) between treatment and control were determined using a two-tailed t-test with $p<0.05$ (GraphPad Prism 8.2.0).

4.2.7 Hierarchical clustering and multidimensional scaling

Hierarchical clustering and multidimensional scaling were performed as described in **Chapter 3**. In summary, heat maps based on the difference in median distance moved between treatment and control group were generated and clustered using the heatmap.2 function included in the R package gplots, version 2.13.0 (Warnes et al. 2015). Multidimensional scaling coordinates were calculated in terms of pairwise Euclidian distances (KNIME Distance Matrix Calculate node) based on the median distance moved per minute for each treatment and control group.

4.3 Results

4.3.1 Acetylcholinesterase inhibitors and herbicides cause a hyperactive locomotor phenotype

As a first step, we aimed to record light-dark-transition phenotypes of a set of chemicals previously reported to cause hyperactivity in larval zebrafish including 3,4-dichloroaniline (DCA), diuron (DIU), diazinon (DZN), and triphenylphosphate (TPP) (Leuthold et al. 2019). We applied ten to eleven concentrations per compound to capture the responsive range. At the end of the locomotor response test, i.e. ~3 h post exposure, no malformations or lethality in terms of coagulation were observed for any of the tested concentrations. Indeed, in dependence on concentration, we determined behavioral profiles with high phenotypic similarity across the four compounds (**Supporting figure C4.1A**). In particular, locomotor activity was constantly increased over time and independent of photoperiods, i.e., larvae rest in a steady state of hyperactivity. This effect was most pronounced with DCA (139.7-209.5 µM) and DZN (31.1-51.7 µM) but was less strong with DIU (28.4 µM) and TPP (8.0-10.0 µM) (**Figure 4.1A-E**). Additionally, hierarchical clustering performed on single time increments of light and dark periods revealed a redundancy of certain minutes (**Supporting figure C4.1B**). We found that different chemicals caused specific, multiphasic patterns of locomotor activity during distinct phases of photoperiods. Compound-specific bi- to triphasic

concentration response behavior was observed at photo transitions, throughout the first and second half of the dark period, respectively, as well as within the light phase (**Supporting figure C4.2**).

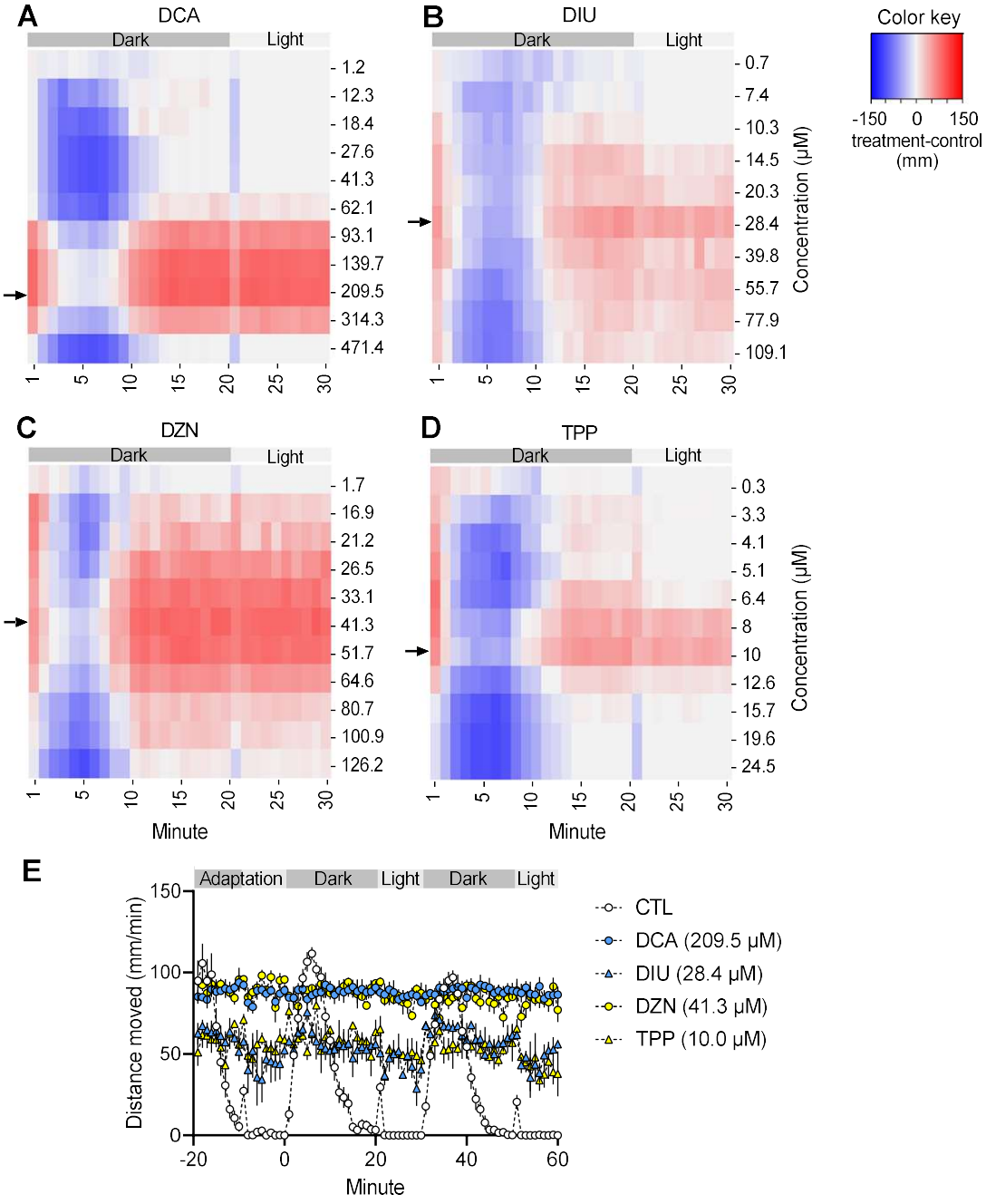

Figure 4.1. Chemicals cause distinct patterns of locomotion in consecutive minutes of dark and light phases (x axis) and in dependence on concentration (y axis). Heat map representations of locomotor activity in 4 dpf zebrafish larvae treated with (**A**) 3,4-dichloroaniline, (**B**) diuron, (**C**) diazinon, and (**D**) triphenylphosphate. Colors represent deviation from median control phenotype: red, higher activity; blue, lower activity. (**E**) Representative behavioral profiles of most pronounced hyperlocomotion per chemical (mean ± SEM) indicated with arrows in A-D. *n*≥64 larvae per condition.

4.3.2 Inhibition of acetylcholinesterase is not correlated with hyperlocomotion

We hypothesized that the observed phenotypic similarity in behavior is due to similar interactions of the four chemicals at the molecular level. Since DZN and TPP were shown to inhibit AChE (Leuthold et al. 2019), we speculated that this enzyme may also be inhibited by DCA and DIU. To investigate this, zebrafish larvae were sampled following the behavioral test, i.e. 3 h post exposure, and *in vivo* AChE inhibition of the whole body homogenate was measured. It could be confirmed that DZN (≥16.9 µM) and TPP (≥15.7 µM) in fact inhibit AChE, however, neither DCA nor DIU significantly reduced AChE activity up to concentrations of 471.4 and 109.1 µM, respectively. The EC50(3 h) for enzyme inhibition was 30.8 µM for DZN. With a calculated EC50(3 h) of 57.2 µM the half maximum AChE inhibition determined for TPP was above the highest concentration tested (**Table 4.1**, **Supporting figure C4.3A-D**). Additionally, whole body homogenates of four day old zebrafish were spiked with the respective chemicals and AChE inhibition *in vitro* was determined. DZN significantly decreased *in vitro* AChE activity with doses of 10 and 101 µM. Surprisingly, TPP did not alter *in vitro* AChE activity at all indicating bioactivation *in vivo*. DCA and DIU inhibited AChE only at the highest doses of 109 and 314 µM, respectively (**Figure 4.2**). The discrepancy between *in vivo* and *in vitro* inhibition may be related to toxicokinetic processes since DIU and DCA have low to moderate bioconcentration factors whereas bioconcentration of DZN and particularly TPP is high, respectively (**Table 4.1**).

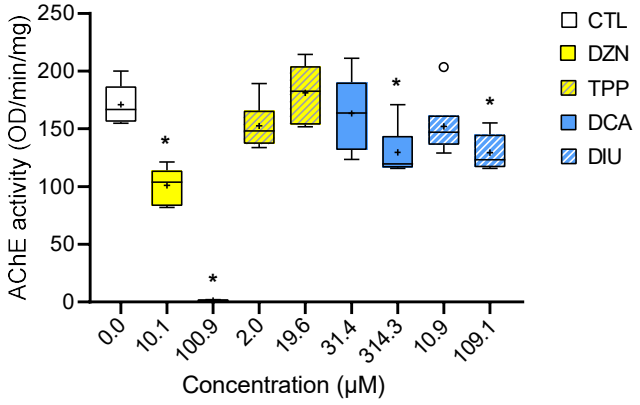

Figure 4.2. Tukey boxplots show AChE activity *in vitro*. CTL, control; DZN, diazinon; TPP, triphenylphosphate; DCA, 3,4-dichloroaniline; DIU, diuron, asterisk; p<0.01.

With LC50(96 h) values for fish of 17.4 and 3.1 µM (**Table 4.1**), respectively, DZN and TPP both elicited effects on locomotion and AChE in the range of predicted baseline toxicity. To determine whether the observed hyperlocomotion is either related to narcosis or AChE deactivation, we additionally investigated aldicarb (ALD), a carbamate insecticide whose action is based on AChE inhibition too. In contrast to DZN and TPP, and with an EC50(0-

Table 4.1. Toxicity, physicochemical properties, and effect concentrations of acetylcholinesterase inhibition and decrease in cardiac activity of the investigated chemicals.

Chemical	ID	LC50‡ (µM)	logD	BCF	AChE inhibition (EC in µM)			Heart rate decrease (EC in µM)		
					NOEC	LOEC	EC50	NOEC	LOEC	EC50
3,4-Dichloroaniline	DCA	386.2	2.60	55.3	≥471.4	>471.4	>471.4	62.1	93.1	217.5
Diuron	DIU	204.4	2.75	1.0	≥109.1	>109.1	>109.1	39.8	55.7	171.1†
Diazinon	DZN	17.4	3.80	453.7	1.7	16.9	30.8	1.7	16.9	36.8
Triphenylphosphate	TPP	3.1	4.12	791.8	12.6	15.7	57.2*	0.3	3.3	5.9
Aldicarb	ALD	3.1×10^{3}	1.36	6.4	0.1	0.3	0.8	97.8	309.2	4.7×10^{3}†
Verapamil	VER	2.5*	2.38*	16.0*	n.d.	n.d.	n.d.	13.0	19.5	32.8
Metoprolol	MTP	1.5×10^{3}*	-0.25*	1.0*	n.d.	n.d.	n.d.	5.2	52.0	880.7

logD, octanol-water partition coefficient at pH 7.4; BCF, bioconcentration factor at pH 7.4 (ACD/Percepta, ACD/Labs, build 2726. 27 Nov 2014); NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; EC50, 50% effect concentration; n.d., not determined; *, neutral state; †, effect concentration beyond tested range. ‡, Baseline toxicity (neutral organic structure-activity relationship) in terms of fish LC50(96 h) determined with ECOSAR v1.11.

4 dpf) of 0.1 µM (Klüver et al. 2015), ALD was found to alter behavior four orders of magnitude below predicted baseline toxicity (LC50(96 h)=3.1 mM). In correspondence with these findings, we determined hyperactivity dominated behavioral profiles for ALD (9.8-309.2 µM) comparable with those of DCA, DIU, DZN, and TPP (see **Figure 4.1A-D**). With the experimental setup applied here, this characteristic effect was still detectable ≥10 times below the predicted baseline toxicity (**Figure 4.3**). Hence, it appears that the observed hyperactive behavior is not related to a narcotic effect.

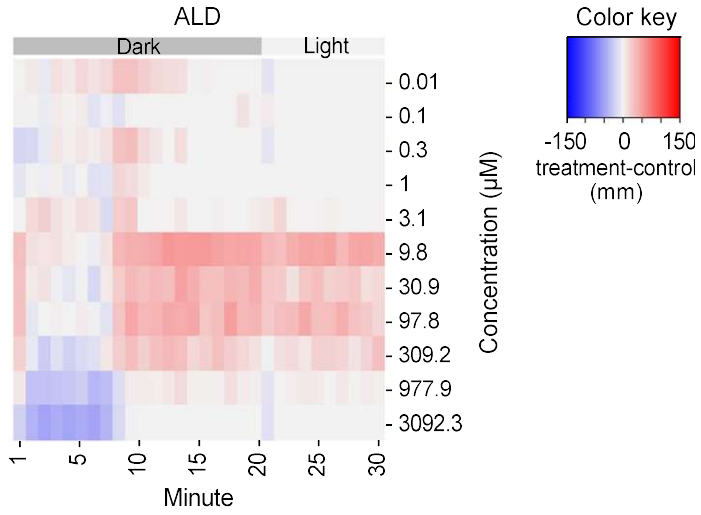

Figure 4.3. Aldicarb (ALD) causes a hyperlocomotor phenotype comparable with 3,4-dichloroaniline, diuron, diazinon, and triphenylphosphate. Colors represent deviation from median control phenotype: red, higher activity; blue, lower activity. *n*=32 larvae per condition.

Finally, we examined whether the degree of AChE inhibition correlates with the stimulation of locomotor activity (**Figure 4.4**). Therefore, those chemicals tested positive for *in vivo* anti-AChE activity were considered including DZN, TPP, and ALD (**Table 4.1**, **Supporting figure C4.3C-E**). Because exposure of zebrafish larvae to light causes a low level of motor activity, the mean distance moved in the light period (minute 22-30, see also **Supporting figure C4.1B**) was used as a measure of hyperactivity. Here, the maximum hyperactive effect (+82.7 ± 3.4 mm; mean ± SD) was observed with 41.3 µM DZN. In comparison, the corresponding maximum effect on locomotion determined for ALD and TPP was up to 50% lower. **Figure 4.4** shows that DZN, TPP, and ALD differed from each other in relative AChE activity levels, namely 0.4, 0.1 and 0.9, respectively, at which maximum relative hyperactivity occurred. Intriguingly, in the case of ALD, severe hyperactive behavior only occurred with inhibition levels ≥90% (≥9.8 µM) whereas ALD related inhibition in the range of 15 to 75% (0.3-3.1 µM) exclusively extended darkness mediated responsiveness (see **Figure 4.3**). No such pattern was observed with DZN and TPP (see **Figure 4.1C** and **D**). Thus, it can be

concluded that the inhibition of AChE cannot (DCA, DIU) or at least not only (DZN, TPP, ALD) be responsible for the hyperactive phenotype observed with the respective compounds.

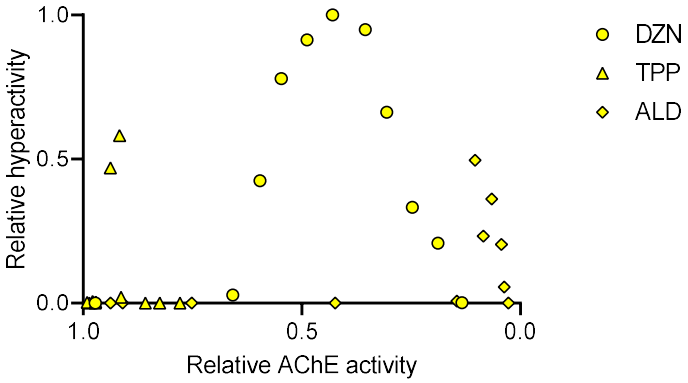

Figure 4.4. Hyperlocomotion is not correlated with AChE inhibition. Effect-effect relationships of hyperactivity (y axis) versus AChE activity *in vivo* (x axis) reveal compound specific differences. Hyperactivity data normalized to maximum effect observed with DZN. AChE activity relative to control. DZN, diazinon; TPP, triphenylphosphate; ALD, aldicarb.

4.3.3 Acetylcholinesterase inhibitors and herbicides cause bradycardia

In the absence of evidence that hyperactivity is directly related to AChE inhibition we were further searching for possible causes that may explain excessive locomotor activity. Finally, we discovered that each of the chemicals examined so far led to bradycardia. To quantify these observations heart rate was recorded in a concentration dependent manner 3 h post exposure. A significant decrease in heart rate was found with DCA, DZN, and TPP at concentrations of 93.1, 16.9, and 3.3 µM, respectively. The corresponding EC50(3 h) values were 217.5, 36.8, and 5.9 µM, respectively. Significant bradycardia was also found with ALD (≥309.2 µM) and DIU (≥55.7 µM). However, the respective EC50(3 h) values of 4.7 mM and 171.1 µM, respectively, were beyond the tested concentration ranges (**Table 4.1**, **Supporting figure C4.4A-D**). Noteworthy, the relationship between cardiac activity and inhibition of AChE was rather compound-specific than -independent. For TPP and DZN the half maximum decrease in heart rate was found at distinct relative AChE levels of 0.9 and 0.5, respectively. In the case of ALD, heart rate remained unaffected up to a relative AChE activity ≤0.15 (**Supporting figure C4.5**).

4.3.4 Cardioselective drugs only partially copy hyperactive phenotypes

To figure out whether bradycardia is related to the observed behavioral phenotype or just a side effect, we sought to test the antiarrhythmic drug verapamil (VER), a calcium antagonist and calcium channel blocker, and the antihypertensive drug metoprolol (MTP), a beta blocker known to decrease heart rate activity in zebrafish (Bittner et al. 2018). If a decreased heart rate is related to hyperlocomotion, these drugs, considered as selectively acting on the cardiovascular system, should also induce the hyperactive phenotype observed with the chemicals above. As expected, VER and MTP both decreased heart rate at concentrations ≥19.5 and ≥52.0 µM, respectively. The EC50(3 h) was 32.8 and 880.7 µM for VER and MTP, respectively (**Table 4.1**, **Supporting figure C4.4F** and **G**). However, while VER indeed caused excess locomotor activity in the range of 29.3 to 98.8 µM, MTP exclusively suppressed locomotion up to 2.0 mM (**Figure 4.5**).

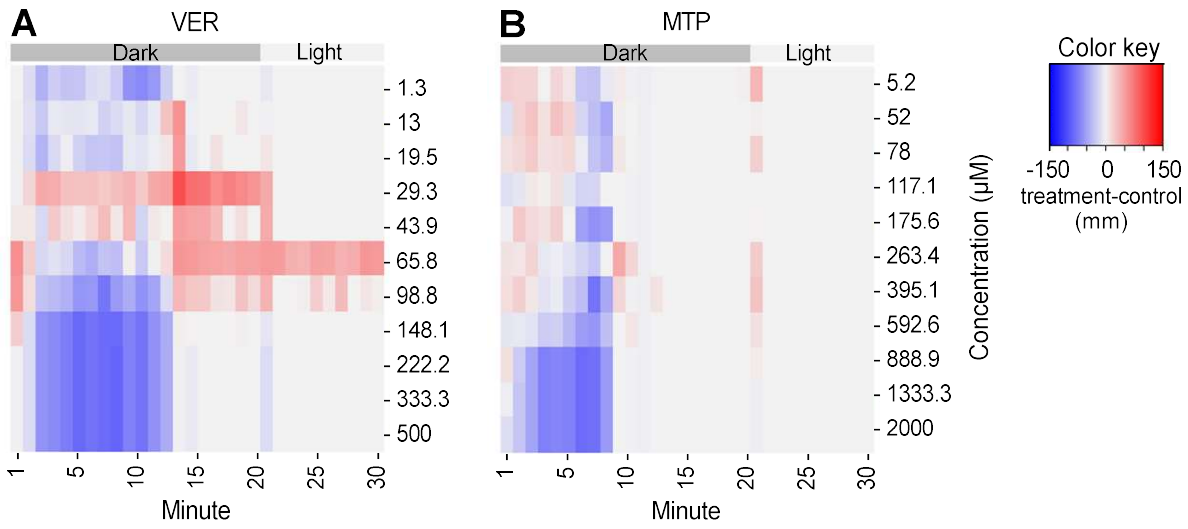

Figure 4.5. Cardioselective drugs cause different patterns of behavior. (**A**) The calcium channel blocker verapamil (VER) elicits distinct patterns of hyperlocomotion. (**B**) The ß-blocker metoprolol (MTP) decreases locomotor activity with increasing concentration. *n*=8 individuals per condition.

In order to reveal similarities and dissimilarities between the cardioactive drugs and the considered AChE inhibitors as well as the herbicide DIU and its degradation product DCA more comprehensively, we performed multidimensional scaling (MDS) on the entirety of concentration dependent behavioral profiles (**Figure 4.6**). Indeed, behavioral profiles of VER at 65.8 (MDS coordinates [-124, 167]) and 98.8 µM (MDS coordinates [18, 83]) show close spatial proximity to numerous profiles obtained with DZN, TPP, ALD, as well as DCA and DIU, indicating a high degree of phenotypic similarity. It is also obvious that the AChE inhibitors cluster across several concentrations in quadrant IV. Furthermore, this cluster is

interspersed with profiles of DCA (e.g. 314.3 µM, MDS coordinates [-153, 177]) and DIU (e.g. 28.4 µM, [-165, 145]), respectively. In contrast to VER (65.8-98.9 µM), behavioral profiles of MTP constitute a compound specific cluster distinct from responses to other chemicals. Similar to MTP, VER elicited rather unique profiles in the range of 1.3 to 43.9 µM. Noteworthy, the spatial concordance of profiles around the point with the MDS coordinates [131, 22] in quadrant III can be considered as unspecific as it represents complete inhibition of locomotion which was observed with each of the investigated compounds (see **Figures 4.1**, **4.3** and **4.5**).

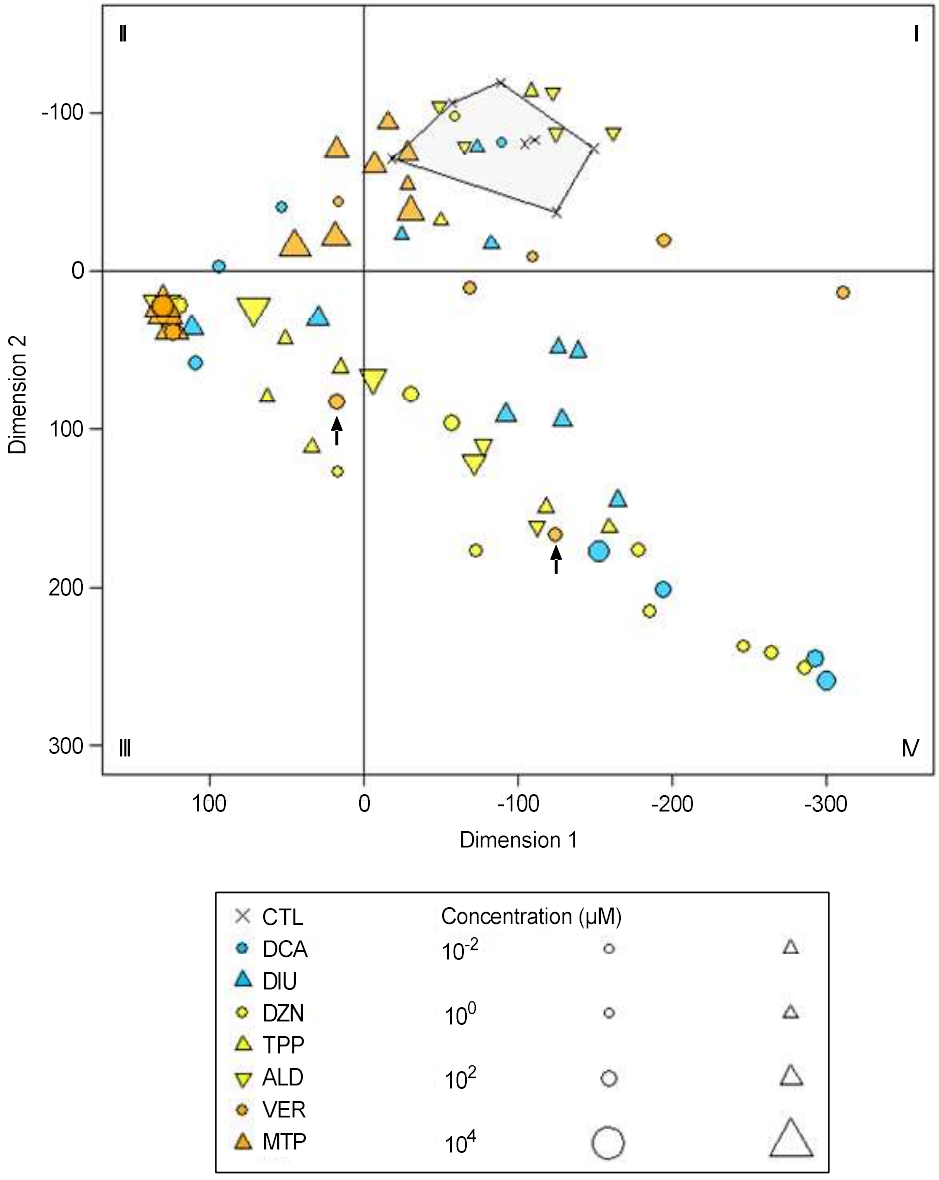

Figure 4.6. Cardioselective drugs and other cardioactive chemicals cause distinct alterations in zebrafish behavior. Multidimensional scaling representation of pairwise distances between behavioral profiles of zebrafish larvae treated with the indicated chemicals. Symbol size, concentration; gray area, control (CTL); arrows, behavioral profiles of verapamil similar to other chemicals. See **Table 4.1** for chemical IDs.

4.3.5 Heart rate and locomotor activity are partially co-regulated

Next, we examined whether the increase in locomotor activity and the decrease in heart rate correlate quantitatively. DCA and DZN showed a similar maximum in locomotor activity at similar levels of relative heart rate of 0.6 and 0.5, respectively. The increase in locomotion was less pronounced with DIU, ALD, and TPP but respective maxima were found at comparable levels of relative heart rate in the range of 0.9 to 0.6. Interestingly, except for TPP and VER, the half maximum hyperactive effect was observed with a distinct reduction in cardiac activity of 12 to 29%. Unfortunately, data variability prevented an appropriate correlation analysis for TPP. In contrast, VER showed high cardioselectivity with a maximum in locomotor activity observed close to cardiac arrest (**Figure 4.7**). A clear correlation between heart rate and hyperactivity cannot be concluded from the data shown. Hence, it remains to be elucidated how the observed phenotypes are linked to each other.

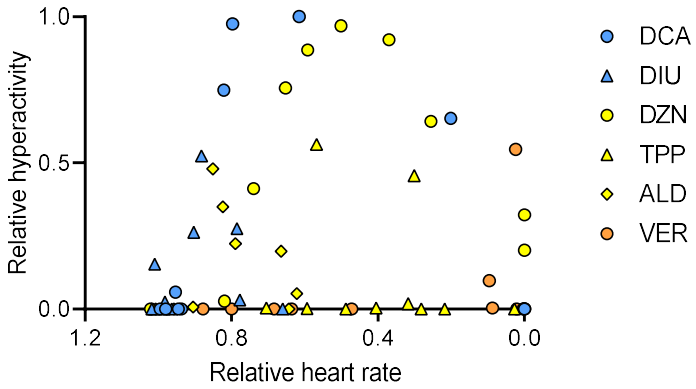

Figure 4.7. Hyperactivity in response to chemical exposure occurs with reduction in heart rate. Relative effect on locomotion (y axis) derived from mean activity during light period (minutes 22-30) versus heart rate (x axis). Activity levels were normalized to maximum hyperactive effect (+85.3 ± 1.4 mm; mean ± SD) observed with 209.5 µM 3,4-dichloroaniline (DCA). Heart rate is relative to control. See **Table 4.1** for chemical IDs.

4.4 Discussion

The understanding of chemicals' interaction with biological functions and related outcomes at phenotypic scale, and *vice versa*, is of relevance in several respects. For example, behavioral phenotyping can assist the determination of underlying mechanisms at subordinate levels of biological organization (Bruni et al. 2016; Kokel et al. 2010). Although molecular and cellular approaches, such as receptor-binding and *in vitro* assays may be more meaningful in terms of mechanism-specificity, these approaches are of limited ecological relevance as long as the linkages between molecular responses and adverse

outcomes at the organism level are not sufficiently understood. In contrast, behavioral phenotyping in larval zebrafish provides the dual advantage of high-throughput in combination with ecologically more relevant outcomes at organism level additionally indicative of potentially complex interactions at molecular, cellular, and organ level. *Vice versa*, it is necessary to validate the diagnostic power of behavior-based approaches through the interconnection of phenotypes and underlying mechanisms. From an environmental perspective, the knowledge about the mechanism of chemicals in non-target species can assist a more realistic assessment of adverse environmental impacts by means of a combined assessment of biologically similar acting chemicals instead of treating such compounds as separate, independent components as performed currently. Last but not least, changes in behavior represent a sensitive, sub-lethal marker of chemical exposure relevant to environmental risk assessment but legally not considered yet.

In the present study behavioral phenotyping was used as a basis to examine effect-effect-relationships between changes in locomotor activity and potential underlying mechanisms. In particular, we hypothesized that similar hyperlocomotor phenotypes caused by different chemicals with partially unknown mechanisms may be based on similar actions at sub-organism level. Therefore, we examined the interdependence of alterations in behavior and subordinate structural elements including AChE activity and heart rate.

Hyperlocomotion is not correlated with AChE inhibition

Contrary to our initial assumption, we found clear evidence that hyperlocomotion observed with DCA, DIU, DZN, and TPP is not or at least not only related to inhibition of AChE. This is supported by the fact that, first, in contrast to previous reports on anti-cholinergic activity in brain of adult goldfish (Bretaud et al. 2000), DIU is not an inhibitor of AChE in larval zebrafish. This also applies to its degradation product DCA. Second, chemicals we proved to be effective inhibitors of AChE, including DZN, TPP, and ALD, differed remarkably in their potency to elicit hyperlocomotion, i.e., there was no quantitative effect-effect-relationship between locomotor and enzyme activity among the different chemicals. From a mechanistic perspective it could be assumed that the behavioral phenotype determined with ALD, namely an extended dark responsiveness, due to decelerated acetylcholine degradation and prolonged neurotransmission, is more likely to be a representative phenotype of partial AChE inhibition since continuous hyperlocomotion was only observed with inhibition levels ≥90%. Another explanation for observed differences in behavioral phenotypes of AChE inhibitors may be the distinct spatial distribution of chemicals (see discussion below). Furthermore, compound-specific inhibition kinetics could be an explanation for differences in locomotor activation.

In addition, our results substantiate that hyperlocomotion is not necessarily subject to narcosis because it was found with ALD concentrations ≥10 times below predicted baseline toxicity. Since each of the examined chemicals caused paralysis at high exposure levels, it appears more reasonable to assume hypolocomotion as the behavioral outcome of narcosis. However, we cannot exclude that altered membrane permeability enhances the effect of hyperlocomotion due to increased accessibility of molecules to related target sites.

Hyperlocomotion and bradycardia co-occur but are not directly correlated

Furthermore, we found that bradycardia is rather a co-occurring effect than the actual cause of hyperlocomotion. In contrast to AChE inhibition, we found that, except for DIU (<55.7 uM), in each of the examined cases, hyperlocomotion was associated with bradycardia. *Vice versa*, bradycardia did not necessarily result in hyperlocomotion as for example seen with the β-blocker MTP. Here, a concentration dependent, monotonic down-regulation of both locomotor activity and heart rate was observed. Hence, the mutual interdependence of behavioral alteration (i.e., both, the increase and decrease in locomotor activity) and bradycardia is undermined by the fact that, first, maximum locomotor activity observed with DIU occurred in the absence of bradycardia, second, locomotor responses were bidirectional depending on the applied chemical and, third, there were quantitative differences in the effect-effect-relationship of locomotion and heart rate: The antiarrhythmic drug VER elicited hyperlocomotion close to cardiac arrest whereas DCA, DIU, DZN, TPP, and ALD were found to up-regulate locomotor activity along with a relative heart rate in the broad range of 0.9 to 0.5. Since VER regulates heart rate via blockage of calcium channels, observed hyperlocomotion may also result from non-cardioselective interactions with other calcium-related processes. Our observations on bradycardia may be related to cardiotoxic effects like cardiomyopathy, direct toxicity on cardiomyocytes, or toxic effects on the vascular system (extensively reviewed by (Mladenka et al. 2018). Cardiotoxicity in terms of bradycardia and atrial failure was for example observed in 2 dpf zebrafish embryos treated for 3 h with TPP concentrations as low as 10 µM (Alzualde et al. 2018). Another explanation for the simultaneous observation of locomotor regulation along with bradycardia might be the involvement of a superordinate structural element such as the central nervous system (CNS). This assumption is supported by the fact that the CNS can regulate cardiovascular activity by modulation of excitatory and inhibitory processes that affect autonomic discharge (Korner 1971). Moreover, the CNS “has the capacity to alter cardiovascular activity as a result of or in anticipation of behavioral events” (reviewed in Talman 1985). However, owing to complementary functions of central, peripheral, and autonomic nervous system, the impairment of none of these can be precluded as a potential cause of abnormal locomotor and cardiac activity. For example, in the AOP framework, AChE inhibitors are known to affect

heart rate through atrioventricular block and bradycardia (Russom et al. 2014). In this context, acetylcholine stimulates the parasympathetic nervous system and lowers the heart rate (Gordan et al. 2015). However, differences in the effect-effect-relationship of reduction in heart rate and inhibition of AChE suggest that observed bradycardia was not or not exclusively related to anti-cholinergic activities of ALD, DZN, and TPP. One possible explanation for these observations could be that the inhibitors differ in their spatial distribution and, as a consequence, elicit organ-specific activities. Noteworthy, most studies have demonstrated tachycardia secondary to CNS stimulation during seizure activity in vertebrates like cat (Doba et al. 1975), primates (Delgado et al. 1960), and human (Leutmezer et al. 2003; Opherk and Hirsch 2002) whereas ictal bradycardia occurs in less than 2% of seizures in human (reviewed in Devinsky 2004). However, a clinical study by Nashef et al. (1996) reports later ictal bradycardia secondary to tachycardia. Consequently, it remains to be elucidated whether simultaneous alterations in locomotor and cardiac activity are mutually interdependent in certain cases or whether both are secondary to stimulation or lesions of the CNS.

Hyperlocomotion indicates seizure-like behavior

Intriguingly, the behavioral phenotype reported here is consistent with zebrafish models of epilepsy. Epilepsy is a complex neurological disorder marked by recurrent seizures as a result of an imbalance of excitatory and inhibitory processes in the brain (Afrikanova et al. 2013; Baxendale et al. 2012; Stewart et al. 2012). A common chemoconvulsant-based model of epilepsy is the acute pentylenetetrazol (PTZ) zebrafish seizure model. Zebrafish larvae exposed to the $GABA_A$ receptor antagonist PTZ show hyperlocomotion, seizure-like behavior, and abnormal electrophysiological responses (Afrikanova et al. 2013). Besides PTZ, there are several other known convulsant drugs, e.g. picrotoxin ($GABA_A$ receptor antagonist), pilocarpine (acetylcholine receptor agonist), kainate (glutamate receptor agonist), and caffeine (adenosine receptor antagonist) (reviewed in Stewart et al. 2012). The diversity of neurotransmitter pathways involved in seizure-like states highlights the complexity of neurological disorders like epilepsy. Noteworthy, there are differences in seizure-like phenotypes among different proconvulsants probably attributed to the location and extent of abnormal brain activity. For example, the convulsant agent cyclotrimethylenetrinitramine evokes corkscrew-swimming (Williams et al. 2012), whereas kainate *inter alia* causes head-shaking (Alfaro et al. 2011). To this end, it appears appropriate to assume that DCA, DIU, DZN, and TPP elicit convulsant activity via interference with brain functions. This is not least because these or related chemicals (i.e., constitutional isomers, chemicals with related functional groups) are able to penetrate the blood-brain barrier and to accumulate in the brain of fish due to their lipophilicity (Bretaud et

al. 2000; Cravedi et al. 2001; Vale 1998; Wang et al. 2016). Along those lines, although our analyses initially indicated a high degree of phenotypic similarity, a closer examination revealed slight differences in locomotor phenotypes between both, different chemicals as well as concentration dependent behavioral profiles of individual compounds. This may indicate that, first, DCA, DIU, DZN, and TPP do not necessarily interfere with the same neurotransmitter pathways, and second, a superimposition of distinct mechanisms may occur with increasing exposure level leading to more or less comparable outcomes at behavioral level. For example, DCA elicited an anesthetic effect prior to hyperlocomotion at higher concentrations. The co-occurrence of these presumably independent processes, currently unknown of their molecular background, possibly enhances hyperlocomotion. Furthermore, even though both DZN and ALD decreased AChE activity, maximum hyperlocomotion occurred with distinct levels of inhibition (DZN<ALD) which suggests the involvement of mechanisms independent of DZNs' anti-cholinergic activity. However, differences in behavioral efficacy may also originate from, e.g., tissue-specific distribution (e.g. brain/specific brain regions, neuromuscular junction). In contrast to DZN, TPP mediated hyperactivity was less pronounced and occurred in the absence of AChE inhibition. Additionally, our results demonstrate that TPP is not an AChE inhibitor *in vitro* whereas its anti-cholinergic activity *in vivo* indicates bioactivation. Supporting this, we previously found that one-day exposure (4-5 dpf) of larval zebrafish leads to complete deactivation of AChE (Leuthold et al. 2019) whereas incomplete inhibition was observed after 3 h of exposure. In addition, TPP is able to cross the blood-brain barrier whereas metabolites like diphenylphosphate do not permeate the brain (Wang et al. 2016). Furthermore, already at the lowest tested concentration TPP particularly increased responsiveness to light-dark-transitions, a phenomenon which was not observed with any of the other tested compounds. Taken together, these results indicate that the underlying mechanism of hyperactivity observed with TPP is distinct from DZN and DCA. A comparable, weak activation in locomotion was observed with DIU. A possible explanation for this may be its low bioaccumulation potential. A recent transcriptome study demonstrated that DIU interferes with genes involved in the phototransduction pathway, retinal photoreceptor layer, and neuronal system (Schüttler et al. 2019).

Potential consequences of seizure-like hyperlocomotion

In **Chapter 3** it was found that severe cases of initial hyperlocomotion often include death at adjacent higher doses and over time, i.e., for periods of 24 h or even less (Leuthold et al. 2019), a phenomenon also reported in previous studies (Caillouet Jr 1967; Wood et al. 1983). Here, it was confirmed that, except for DIU (maximum water solubility prevented increase of exposure), high doses of DCA, DZN, and TPP lead to cardiac arrest, a lethal

endpoint according to OECD test no. 236 (OECD 2013). There are several theories how hyperactivity translates into lethality. One of them is the impact of toxicants on skeletal muscles, i.e. muscular toxicity: An increase of calcium levels in muscles due to receptor over-stimulation causes myopathy via excitotoxicity (Brennan et al. 2005; Lefebvre et al. 2004). This could explain why paralysis and acute myocardial infarction occur immediately after excess locomotion. Another possible explanation for lethal outcomes as a result of hyperlocomotion may be found in the 'blood lactic acid' hypothesis. Post-exercise mortality has been related to excessive blood lactic acid accumulation (Black et al. 1966; Caillouet Jr 1967). The increase in lactic acid leads to reduction in oxygen capacity of blood (Black and Irving 1938; Root 1931; Root and Irving 1943; von Buddenbrook 1938), probably due to erythrocyte deformation (von Buddenbrook 1938). As a result, hypoxia occurs in tissues of fish after hyperactivity (Black et al. 1962). Under aerobic conditions lactic acid is particularly oxidized by the heart (Guyton 1961). However, under anoxic conditions muscular activity mainly depends on glycolysis which results in the formation of lactic acid from muscle glycogen (Needham 1960; Wood et al. 1983). There are doubts on the 'blood lactic acid' hypothesis. For example, Wood et al. (1983) proposed that intracellular acidosis may be the actual cause of death after severe exercise. Nevertheless, it is proven that hyperactivity in fish can result in hypoxia (Black et al. 1962). Therefore, another, related hypothesis is anoxia of the CNS. Neurons are highly sensitive to oxygen deficiency. The high level of energy demand results from the necessity to maintain ion gradients by means of active, energy consuming transport mechanisms which represent the basis for nerve impulse transmission. Brain hyperactivity as in the case of epileptic seizures increases the demand for energy tremendously. An exhaustion of energy results in (irreversible) damage to the brain after persistent states of anoxia (Marquardt and Schäfer 1994). In the context of the present study, given the simultaneous occurrence of conditions that favor anoxia including hyperlocomotion and reduced circulatory efficiency due to bradycardia, this hypothesis on anoxia as a potential cause of death appears plausible and demands for further investigation.

Concluding remarks and future perspectives

In the present study different environmental chemicals were characterized which elicit similar phenotypes of hyperlocomotion in larval zebrafish and we strived for the identification of underlying mechanisms. It was found that inhibition of AChE and reduction in heart rate co-occur with hyperlocomotion but cannot causally be linked to the observed hyperactivity. Instead, it is assumed that hyperlocomotion represents seizure-like behavior related to complex disorders of the brain. In this context, slight differences in hyperlocomotor patterns of the respective chemicals were determined which may hint at different underlying mechanisms, nevertheless leading to similar outcomes at the behavior phenotypic level. To

better characterize different seizure-like states, behavioral phenotyping may, e.g., benefit from 3D video-tracking approaches using side- and top-views and dissection of movement patterns based on locomotion parameters (e.g. velocity, turn angle) (Stewart et al. 2012). Besides motor seizures and abnormal electrical activity in the brain, a thorough investigation of circuitry involved in seizures using *c-fos* as a regional biomarker of neuronal activation appears promising too (Bullitt 1990; Chan et al. 1993; Hoffman et al. 1993). In addition, phenotype rescue has proven useful in both the elucidation of toxicity mechanisms and the characterization of mechanisms of action of antidotes. For example, calcium channel blockers (e.g. nimodipine, nitrendipine, suloctidil) were identified as inhibitors of both PTZ-induced *fos* expression and hyperlocomotion probably due to their ability to prevent neural and muscular activity by blockage of calcium influx (Baxendale et al. 2012). In the same way antidotes against hypotension and bradycardia (e.g. atropine, adrenaline) may be applied to narrow down the relationship between cardiotoxicity and neurotoxicity. In order to more precisely dissect neurotransmitter pathways involved in hyperlocomotion and behavioral alterations in general, metabolomic approaches proved to be a helpful tool. For example, Jin et al. (2013) developed a high-throughput zebrafish screen to identify antidotes of organophosphate-induced lethality. A metabolite profiling approach enabled determination of antidote-specific mechanisms of action such as histamine, acetylcholine, or NMDA pathways (Jin et al. 2013). Similar to metabolomics, transcriptomic approaches can improve our understanding of underlying biology. In this context, Schüttler et al. (2019) recently discovered that behavioral alterations observed with DIU may be related to genes involved in the phototransduction pathway, retinal photoreceptor layer, and neuronal system. Overall, this study provides a basis for further investigation necessary to finally identify the potential key events on molecular or physiological level that lead to the observed specific behavioral phenotype of hyperlocomotion and subsequent adverse outcomes. Finally, from an environmental perspective there is reasonable concern about additive effects potentially caused by environmental chemicals acting similarly on behavior e.g. in terms of hyperlocomotion. In **Chapter 5** it is, therefore, examined how mixtures of respective chemicals influence the observed hyperlocomotor phenotype and whether combined effects occur.

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Combined effects of chemical mixtures on locomotor activity: Prediction and diagnostic potential of behavioral alterations in larval zebrafish

Abstract

Under real environmental conditions chemicals rather occur in mixtures than as individual constituents. Nevertheless, the assessment of chemicals' adverse effects on aquatic organisms is currently conducted for individual compounds merely. While a predictive assessment of joint chemical effects is applicable to, e.g., certain outcomes at molecular or apical level, such as receptor activation or lethality, this has not yet been demonstrated with behavioral alterations. In this study it was, therefore, investigated whether effects on light-dark transition behavior of larval zebrafish (*Danio rerio*) obtained with various environmental chemicals combine in mixture exposure. On a quantitative scale, we show that (i) combination effects occur at the level of locomotor behavior and that (ii) concentration-response modelling as well as mixture effect prediction, using the mixture concept of concentration addition (CA), are applicable to behavioral responses based on data obtained with individual components. Moreover, we found that mixtures composed of chemicals that cause hyperlocomotion can even exceed predictions made with CA. Qualitatively, we found that behavioral profiles of a chemical mixture resembles distinct phenotypes of its individual components. Thus, behavioral profiling of chemical mixtures provides a great potential in the quantitative and qualitative assessment of mixture toxicity. The other way around, we also observed that certain phenotypes cannot be recovered from a particular mixture which potentially indicates antagonism of certain ingredients. Overall, the presented results emphasize the relevance of mixture assessment beyond evaluation of individual chemical substances, also at behavioral scale.

5.1 Introduction

The widespread use of pesticides, pharmaceuticals, and industrial chemicals results in their release into the aquatic environment, e.g., via wastewater treatment plant effluents, industrial discharge, or agricultural runoff (Altenburger et al. 2019). Consequently, under realistic environmental conditions, chemicals rather occur in mixtures than as individual elements. However, assessment of chemicals' effects on living organisms is usually rather conducted on single constituents than mixtures (Brack et al. 2019). Even though the existence of drivers

of mixture toxicity has been demonstrated (Hashmi et al. 2018; Muschket et al. 2017), the co-occurrence of a variety of chemicals in numerous surface water bodies suggests that actual mixture effects exceed those presumed for individual components (Altenburger et al. 2018; Busch et al. 2016). *Inter alia*, it is assumed that multiple mixture constituents simultaneously act on a common biological function in a similar manner, e.g., via same molecular initiating events (e.g. interaction with a receptor) or via convergence of key events related to higher levels of biological organization. To predict such combination effects there are various established mixture concepts including concentration addition (CA) and independent action (IA). CA is used to assess synergy/antagonism of combinations of similarly acting chemicals (Howard and Webster 2009). In contrast, IA is used to model combinations of chemicals with dissimilar modes of action (MOAs). Both concepts require concentration response model-based parameters of individual constituents as input. In this context, behavior-based concentration response relationships were already established in **Chapter 3** (Leuthold et al. 2019). However, although the applicability of concepts to the prediction of chemical combination effects on apical endpoints, such as lethality, is established (Altenburger et al. 2018), the joint action of chemicals at the sub-lethal scale, such as impairment of behavior, has rarely been investigated yet. However, this is relevant to environmental risk assessment, not least because of the simultaneous presence of multiple neuroactive chemicals with distinct but also identical MOAs, e.g. in European surface water bodies, that are known to affect different as well as similar pathways of neurotransmission in aquatic organisms such as fish (Busch et al. 2016). Moreover, it has been demonstrated that adverse outcomes associated with exposures to neuroactive chemicals are frequently related to alterations in behavior due to increased acute-to-chronic ratios (Klüver et al. 2015; Scholz et al. 2018).

Another challenge in environmental toxicology is the occurrence of unknown chemicals whose potential contribution to mixture toxicity may be overlooked with targeted chemical-analytical approaches as respective chemicals cannot be included in predictions of potential combined effects. Therefore, effect-based tools such as the fish embryo acute toxicity test (OECD 2013) or the *Daphnia* sp. acute immobilization test (OECD 2004) have been proposed as non-target approaches to capture biological outcomes of combined exposures in a comprehensive manner (Brack et al. 2019). In addition, behavior-based approaches such as the light-dark transition test in larval zebrafish may be used to assess the sub-lethal effects of mixtures. Furthermore, behavioral profiling may additionally inform on underlying mechanisms of mixture toxicity as the indicative value of locomotor responses in zebrafish has been demonstrated in, e.g., the discovery of novel neuroactive drugs (Bruni et al. 2016; Kokel et al. 2010; Rihel et al. 2010). However, the applicability of behavioral profiling to the diagnostic assessment of chemical mixtures has not been examined yet.

In the present study it was investigated whether chemical mixtures cause combined effects on locomotor activity in larval zebrafish. Moreover, a quantitative assessment of the applicability of mixture concepts including CA and IA on the prediction of locomotor responses was performed. Therefore, chemicals previously identified for similar behavioral phenotypes of hyperlocomotion (see **Chapter 4**) were combined in artificial mixtures. In addition, a multi-component mixture of partly dissimilarly acting chemicals was used to qualitatively evaluate whether supposed compound-specific or MOA-specific behavioral profiles could be recovered from the respective mixture.

5.2 Materials and methods

5.2.1 Fish cultivation and embryo collection

A hybrid of wild-type (Wild India Kolkate, “WIK”) and an in-house *D. rerio* strain, “UFZ-OBI” (generation F2 and F3), were cultured in 120-L aquaria (activated carbon-filtered tap water, 26.5±1 °C) with a photoperiod of 14:10 h light:dark. Commercial dry food and *Artemia* sp. were fed once and twice a day, respectively. Cultivation and use were conducted according to German and European animal protection standards. Fish culture was approved by the Government of Saxony (Landesdirektion Leipzig, file number 75–9185.64). Spawn was collected within stainless steel sieve-covered glass dishes. Fertilized eggs were selected by means of microscopy and were transferred into oxygen-aerated (≥ 24 h) and pH-adjusted (pH 7.4±0.1) standard dilution water as specified in ISO 7346-3 (80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl) with a density of 1 egg per 2 mL. Subsequently, zebrafish embryos were raised at 28 °C with a 14:10 h light:dark cycle until initiation of exposure at 4 d post fertilization (dpf).

5.2.2 Chemicals and stock preparation

Chemicals and purities purchased from Sigma-Aldrich (Steinheim, Germany) were 3,4-dichloroaniline (99.9%, CAS RN 95-76-1), diclofenac sodium (≥98.0%, CAS RN 15307-79-6), naproxen sodium (98.0-102.0%, CAS RN 26159-34-2), and triphenylphosphate (≥99.0%, CAS RN 115-86-6). Chemicals and purities purchased from Sigma-Aldrich (Buchs, Switzerland) were diuron (≥98.0%, CAS RN 330-54-1), and diazinon (98.5%, CAS RN 333-41-5). Chemical stocks were either prepared directly in standard dilution water with a final concentration of 0.1% v/v methanol immediately prior to testing or chemicals were dissolved in pure methanol (≥99.8%, CAS RN 67-56-1, J.T. Baker®, Avantor Performance Materials

Inc, Pennsylvania, USA). Test solutions were then produced from serial dilution of stock solutions prior exposure. The final concentration of methanol was 0.1% v/v in every exposure solution.

5.2.3 Mixture design and mixture effect prediction

To test the applicability of mixture concepts to the prediction of behavioral responses, a tertiary mixture of diuron, diazinon, and triphenylphosphate as well as a quaternary mixture additionally including 3,4-dichloroaniline were designed. The selection of model chemicals was based on similar behavioral phenotypes of hyperlocomotion previously determined in **Chapter 3** and **4**. First, concentration response models (see **Equation B3.3** in **Appendix B**) for individual chemicals were created (R package 'drc'; Ritz and Streibig 2005). Therefore, locomotor activity in terms of differences in median distance moved per minute between control and treated groups were used. Locomotor endpoints (data taken from **Chapter 4**) were normalized to an effect range from 0 to 1 using the maximum response determined with the respective chemicals in order to scale models in a uniform way, assuming equal minimum (=0) and maximum (=1) responses for each chemical. Output parameters of chemical specific concentration response models including slope and EC50 served as input for mixture models including CA and IA. Predictions were performed as described in Altenburger et al. (2018). Briefly, CA predictions were calculated for a multi-component mixture according to

$$EC_X(mixture) = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{X,i}} \right)^{-1} \qquad \textbf{Equation 5.1}$$

with $EC_x(mixture)$ being the mixture concentration that results in an effect X for a combination of n individual concentrations c_i. $EC_{X,i}$ are the concentrations of the individual components that on their own produce the same effect X as the respective mixture. p_i represents the ratio of the i^{th} mixture component ($p_i = c_i/(c_1+...+c_n)$).

Based on the assumption that different dissimilarly acting components of a mixture are not correlated with each other (Bliss 1939), IA is defined as

$$E(c_{mixture}) = \prod_{i=1}^{n} E(c_i) \qquad \textbf{Equation 5.2}$$

where $E(c_{mixture})$ is the total effect of the mixture. $E(c_i)$ denotes the effects of individual components c_i.

The relative concentration ratio of mixture components was constant. Concentration and dilution factor selection for both mixtures were performed with the intention to cover the concentration range predicted with CA and IA, i.e., to receive multiple data points that encompass the gradient of the respective curve predictions.

A third, quinary mixture composed of diclofenac, diuron, diazinon, naproxen, and triphenylphosphate was designed based on measured environmental concentrations (MEC) summarized in Busch et al. (2016). Therefore, reported 95% percentiles (MEC95) from every analyzed monitoring study were considered per compound. Since diclofenac was detected within all of the seven monitoring studies, the MEC95 of diuron, diazinon, naproxen, and triphenylphosphate, respectively, was divided by the MEC95 of diclofenac per study. Finally, the mean MEC95 ratio was calculated from all study-specific ratios with diclofenac being set to one.

5.2.4 Exposure, locomotor response assay, and data analysis

Detailed information on test design and evaluation strategy is given in **Chapter 4**. In summary, 4 dpf zebrafish were exposed to linear concentration series of single chemicals and their mixtures, respectively. The locomotor response test was initiated at 1.5 h post exposure. Photoperiods were applied as follows: 10’ D, 10’ L, (20’ D, 10’ L)×2 (D, dark; L, light). The two repeated phototransitions were aggregated minute-wise for the respective time increments per light cycle. Behavioral profiles were then derived from subtraction of median locomotor activity in treatment and control. Heat map representations were created using the heatmap.2 function in R (gplots, version 2.13.0).

5.2.5 Multidimensional scaling

Multidimensional scaling (MDS) was performed analogues to **Chapter 3** and **4**. Behavioral profiles of chemicals presented in **Chapter 4** were included in MDS representations of the respective mixture.

5.2.6 Quantification of acetylcholinesterase activity, heart rate, and morphological features

Quantification of *in vivo* acetylcholinesterase activity and heart rate was conducted as described in **Chapter 4**. Shortly, subsequent to the locomotor response test, i.e. ~3 h post

exposure, whole-body homogenates of individuals treated with the respective mixture (MX5) were analyzed for residual enzyme activity as previously reported (Ellman et al. 1961). Enzyme activity was normalized to the total protein amount determined according to Lowry et al. (1951). Another batch of individuals was anesthetized using tricain (98%, CAS RN 886-86-2, Sigma Aldrich, Steinheim, Germany). Subsequently, heart beat was counted manually for 15 s. Both acetylcholinesterase activity and heart rate were normalized to mean control level. Respective effect concentrations were derived from a four parameter logistic model. No (NOEC) and lowest observed effect concentration (LOEC) were determined using an ordinary one-way ANOVA wit $p<0.0001$ (GraphPad Prism 8.2.0). Additionally, morphological defects were assessed by means of microscopy.

5.3 Results

5.3.1 Classical concentration response models are applicable to locomotor activity

In **Chapter 4** it was substantiated that certain environmental chemicals provoke a similar phenotype of hyperlocomotion marked by recurrent seizures. Here, we further hypothesized that a mixture of chemicals whose single constituents cause such hyperlocomotion, acts additively on locomotor activity. In order to verify this hypothesis, a quaternary mixture (MX4) was designed composed of chemicals that provoke the respective phenotype, including 3,4-dichloroaniline (DCA), diuron (DIU), diazinon (DZN), and triphenylphosphate (TPP). Exemplarily, one single minute was selected in which the hyperactive response to the four single chemicals was maximized. In consideration of the various chemicals, concentrations, and integration periods the lowest common dominator was found with minute 20, i.e., at the end of the dark phase. Noteworthy, according to cluster analysis, minute 20 is highly correlated with several other periods of integration including the second part of the dark phase (minutes 12-19) and minutes 22-30 within the light phase (see **Supporting figure C4.1B**). Therefore, locomotor activity in minute 20 can be considered representative for hyperactive responses in the respective periods. To model the stimulation of locomotor activity as a function of concentration, data on hyperlocomotion was first normalized to the maximum response which was observed with DCA. Then, a four parameter logistic (logit) model was fitted to the data obtained for each of the four chemicals (**Figure 5.1**). The respective EC50(3 h) values determined with TPP, DIU, DZN, and DCA were 8.4, 22.8, 23.3, and 80.3 µM, respectively. Furthermore, curve slopes were in a comparable range of 5.1 to 5.4 μM^{-1} for TPP, DIU, and DCA, whereas the curve determined with DIU was less steep with a slope of 2.7 (**Table 5.1**).

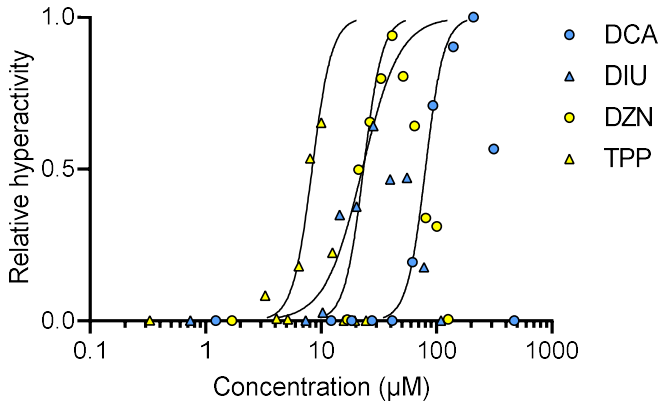

Figure 5.1. Environmental chemicals cause concentration dependent hyperlocomotion well reflected by the logistic regression model. The increase in locomotor activity in minute 20 relative to control (y axis) is shown as a function of concentration (x axis). Hyperactivity is relative to maximum effect (+85.3 ± 1.4 mm, mean ± SD) observed with 209.5 µM DCA. Concentration-response models only consider data up to the maximum response per chemical. *n*=64 individuals per condition. See **Table 5.1** for chemical IDs.

Table 5.1. Effect concentrations of hyperlocomotion calculated for individual chemicals on the basis of data obtained in minute 20 of the locomotor response test.

Chemical	ID	EC50(3 h) (µM)	Hill-slope (μM^{-1})
3,4-Dichloroaniline	DCA	80.3	5.4
Diuron	DIU	22.8	2.7
Diazinon	DZN	23.3	5.3
Triphenylphosphate	TPP	8.4	5.1

5.3.2 Mixtures of chemicals act additively on locomotor activity

In a second step we performed predictions on combined effects of the investigated chemicals using the concepts of concentration addition (CA) and independent action (IA). In order to not let one of the components dominate the mixture, a mixture design equitoxic at EC50(3 h) was used, i.e., each component equally contributes to the half maximum response (**Table 5.2**, **Supporting figure D5.1A**). This equtoxic concentration of 134.8 µM (total molarity) was used as maximum concentration for testing. The molar mixture ratios were 0.60:0.17:0.17:0.06 for DCA:DIU:DZN:TPP. This four-component mixture is hereinafter referred to as MX4. To cover the whole range of concentrations potentially affecting locomotor activity as predicted with CA and IA, we interspersed two dilution series each with a dilution factor of 1.9 to span a concentration range of three orders of magnitude. Finally, when testing MX4, we found mixture profiles characterized by excess locomotion and

therefore comparable with its single components (see **Figure 4.1** in **Chapter 4**) in the range of total molarity from 28.7 to 37.5 µM (**Figure 5.2A**). Again, minute 20 was used as a proxy of hyperlocomotion to derive a concentration response relationship of measured increase in locomotor activity and prediction, i.e., CA and IA. The measured EC50(3 h) of MX4 is approximately consistent with the one predicted by CA and is underestimated by factor 1.3 whereas IA underestimates the actual result by factor 3.5. This meets our expectations as all components elicit similar phenotypes of hyperlocomotion. In addition, as expected, the combination effect of MX4 outweighed the effect of its single constituents, i.e., the EC50(3 h) was decreased by factor ~5 compared to the individual components (=EC50(component$_n$)/EC50(mixture)×fraction(component$_n$)). Surprisingly, the curve slope of 4.3 µM^{-1} predicted with CA does not fit the actual one of 14.1 µM^{-1} whereas it is better reflected by IA, namely 5.2 µM^{-1} (**Table 5.2**). Noteworthy, none of the slopes calculated for the individual chemicals (see **Table 5.1**) implies such a steep increase in locomotor activity as actually observed with MX4. Consequently, CA overestimates the actual activation in locomotion at concentrations ≤19.8 µM but underestimates the combination effect with higher concentrations. This may imply that (some of) the individual components of MX4 act independently/oppositely at concentrations up to 19.8 µM whereas higher concentrations elicit a strong synergistic effect on locomotion.

Table 5.2. Effect concentrations of hyperlocomotion calculated for chemical mixtures on the basis of data obtained in minute 20 of the locomotor response test.

Mixture (molar ratio)	ID	EC50(3 h) (µM)			Hill-slope (µM^{-1})		
		MX	CA	IA	MX	CA	IA
DIU+DZN+TPP (0.42:0.43:0.15)	MX3	10.6	18.1	38.4	4.1	4.0	4.9
DCA+DIU+DZN+TPP (0.60:0.17:0.17:0.06)	MX4	26.0	33.6	89.9	14.1	4.3	5.2

MX, measured with respective mixture; CA, predicted with concentration addition; IA, predicted with independent action. See **Table 5.1** for chemical IDs.

As opposed to the other three constituents of MX4, DCA elicits an anesthetic effect in the range of 12.3 to 41.4 µM, i.e., at concentrations below which a hyperactive response was observed (see **Figure 4.1A** and **Supporting figure C4.2A** in **Chapter 4**). This anesthesia is likely to antagonize hyperlocomotion at concentrations <28.7 µM (see **Figure 5.2A**). Therefore, both concepts, IA as well as CA, more or less failed to precisely predict the effect of MX4. Thus, DCA was removed from the mixture. Analogues to the procedure outlined above but under exemption of DCA, we now performed prediction on a tertiary mixture (MX3) of DIU, DZN, and TPP (**Supporting figure D5.1B**). The relative ratio of the three

constituents present in both MX3 and MX4 is the same. This time behavioral profiles characterized by increased locomotor activity were found in the range of 10.3 to 24.6 µM and, therefore, at lower total molarity compared to MX4 (**Figure 5.3A**). However, there is still a discrepancy between prediction and observation. With EC50(3 h) values of 18.1 and 38.4 µM, CA and IA underestimate the actual EC50(3 h) of 10.6 µM by factor 1.7 and 3.6, respectively (**Table 5.2**). In contrast, measured (4.1 μM^{-1}) and predicted (4.0 - 4.9 μM^{-1}) concentration response slopes are in good agreement. It is worth mentioning that the maximum response observed with MX3 reached a plateau in relative hyperactivity of 0.61 ± 0.05 (mean ± SD) in the range of 10.3 to 24.5 µM. Although DZN was found to increase locomotor activity beyond this maximum level observed with MX3 (see **Figure 5.1**), the actual response does not exceed this threshold. Furthermore, it could be observed that alterations in locomotor activity in terms of decreased dark responsiveness were still detectable at the lowest tested concentration of MX3 (see **Figure 5.3A**). These alterations were found with a total molarity as low as 2.7 µM, i.e., a molarity of single ingredients as low as 0.4-1.2 µM.

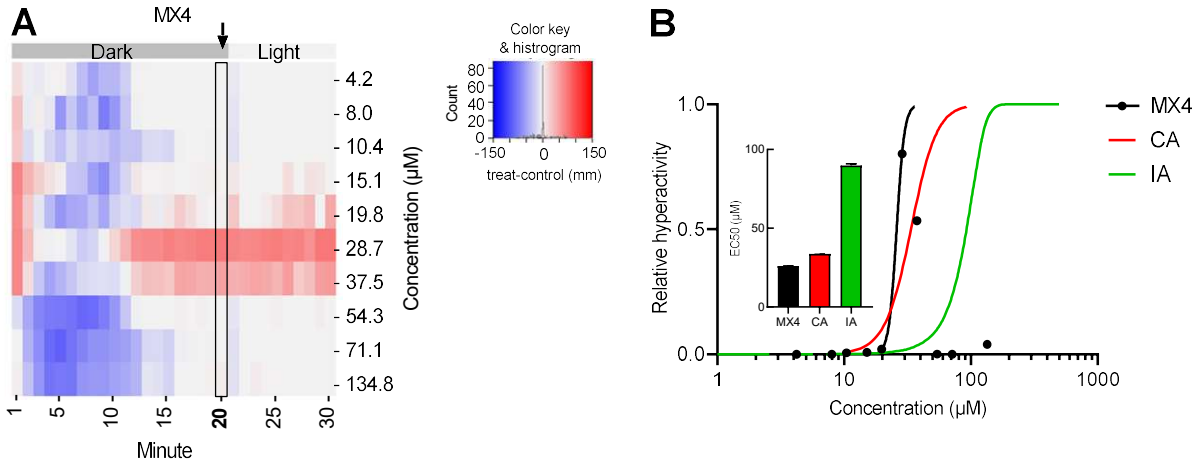

Figure 5.2. A quartary mixture (MX4) composed of 3,4-dichloroaniline, diuron, diazinon, and triphenylphosphate causes hyperlocomotion as approximately predicted by concentration addition (CA). (**A**) Heat map representation of behavioral profiles of MX4. Colors show deviation from median control activity: red, higher activity; blue, lower activity. Arrow: time increment used for comparison with predictions shown in B. (**B**) Compliance of measured and predicted behavioral effects in minute 20. Hyperactivity is relative to maximum effect observed with 3,4-dichloroaniline. Insert: EC50(3 h); error bars, 95% CI. *n*=16 individuals per condition. IA, independent action.

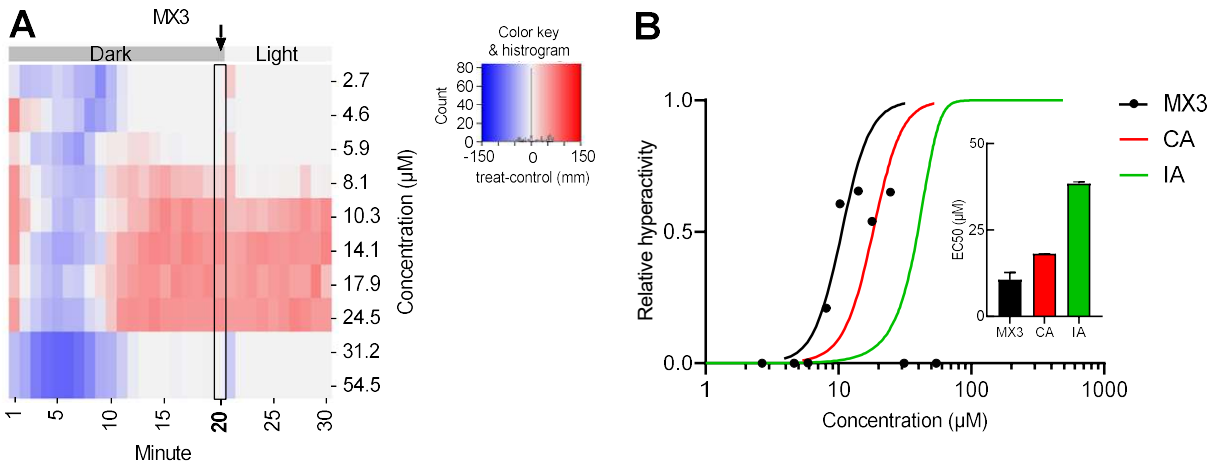

Figure 5.3. A tertiary mixture (MX3) composed of diuron, diazinon, and triphenylphosphate causes hyperlocomotion as approximately predicted by concentration addition (CA). (**A**) Heat map representation of behavioral profiles of MX3. Colors show deviation from median control activity: red, higher activity; blue, lower activity. Arrow: time increment used for comparison with predictions shown in B. (**B**) Compliance of measured and predicted behavioral effects in minute 20. Hyperactivity is relative to maximum effect observed with 3,4-dichloroaniline. Insert: EC50(3 h); error bars, 95% CI. *n*=16 individuals per condition. IA, independent action

5.3.3 Design of an environmentally relevant mixture to examine the diagnostic potential of locomotor response patterns

Next to the questions whether chemicals elicit combined effects on locomotor activity and whether mixture concepts apply to the prediction of locomotor responses in a mixture, we aimed to evaluate the applicability of behavioral profiling as a diagnostic tool in the assessment of bioactivity of chemical mixtures. This means: Could a known behavioral profile be recovered in a more complex exposure scenario? To answer this, first, a chemical mixture was constituted that considers the environmental composition, i.e. the ratio, of selected chemicals to one another in order to represent a simplified but as far as possible realistic exposure scenario based on monitoring data reported in Busch et al. (2016). Therefore, a set of environmentally relevant chemicals was used including DIU, DZN, and TPP as well as the cyclooxygenase (COX) inhibitors diclofenac (DIC) and naproxen (NAP). This selection is, *inter alia*, based on distinct behavioral COX phenotypes determined in **Chapter 3**. The 95 percentiles of mean environmental concentrations (MEC95) reported in Busch et al. (2016) are 2.78, 0.49, 0.06, 1.70, and 0.23 nM for DIC, DIU, DZN, NAP, and TPP, respectively (**Figure 5.4A**). Subsequently, the relative proportions of co-occurring chemicals per monitoring study were used to derive a mean ratio of the respective chemicals

to one another. The molar ratio of the constituents was adjusted relative to the ubiquitous DIC whose relative proportion was, therefore, set to one. Consequently, the proportions of the respective chemicals in the final mixture were 0.27:0.15:1.82:0.12 for DIU:DZN:NAP:TPP (**Figure 5.4B**). This yields a relative mixture ratio of 0.30:0.08:0.05:0.54:0.04 for DIC:DIU:DZN:NAP:TPP (**Figure 5.4C**). This five-component mixture is hereinafter referred to as MX5.

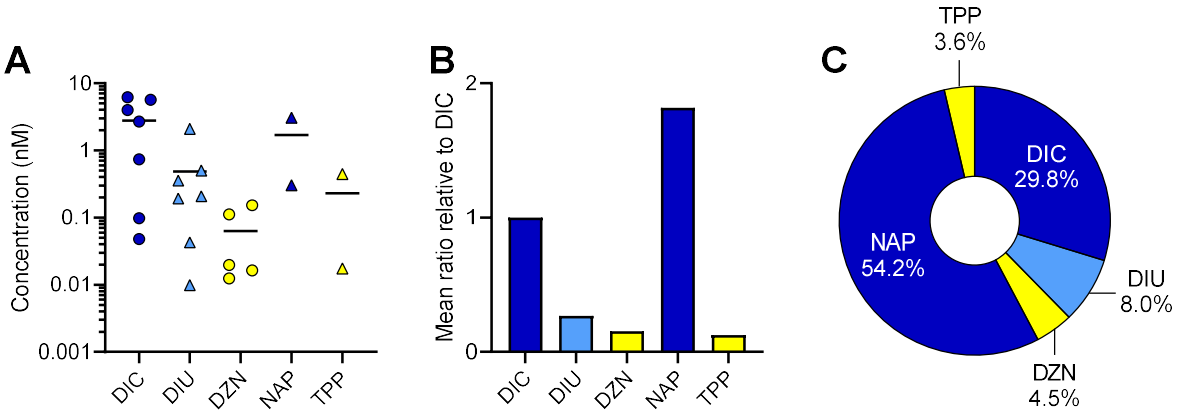

Figure 5.4. Design of a quinary mixture (MX5) of chemicals based on measured environmental concentrations. (**A**) Measured environmental concentrations (MEC95) reported from single chemical monitoring studies summarized in Busch et al. (2016). Horizontal bars, mean. (**B**) Mean ratio of environmental chemicals relative to diclofenac (DIC). (**C**) Molar composition of MX5. See **Table 5.1** for chemical IDs.

5.3.4 Determination of COX inhibitor-specific behavioral phenotypes

Prior to testing the quinary mixture (MX5) of the selected chemicals, we determined their individual behavioral phenotypes in a concentration dependent manner in detail. Phenotypes of DIU, DZN, and TPP are reported in **Chapter 4** (see **Figure 4.1B-D**). DIC and NAP were found to alter locomotor activity at the lowest tested concentrations of 0.1-3.3 and 7.3 µM, respectively. At the given concentrations both COX inhibitors caused similar behavioral profiles characterized by increased activity within the second half of the dark period whereas no alteration was observed for peak and basal activity in dark and light phase, respectively (**Figure 5.5**). With further increasing concentration DIC and NAP both reduced locomotor activity. However, while DIC (5.9-200.0 µM) almost completely prevented locomotion, individuals treated with high doses of NAP (765.3 µM, 1.1 mM) were found to be hyperactive especially within the light period and showed light cycle independent hyperactivity at 1.5 mM (**Figure 5.5B**). Hence, behavioral profiles of the two COX inhibitors are distinct from those of

DIU, DZN, and TPP. This is an important prerequisite in order to recover compound- or even MOA-specific motor patterns in more complex chemical mixtures.

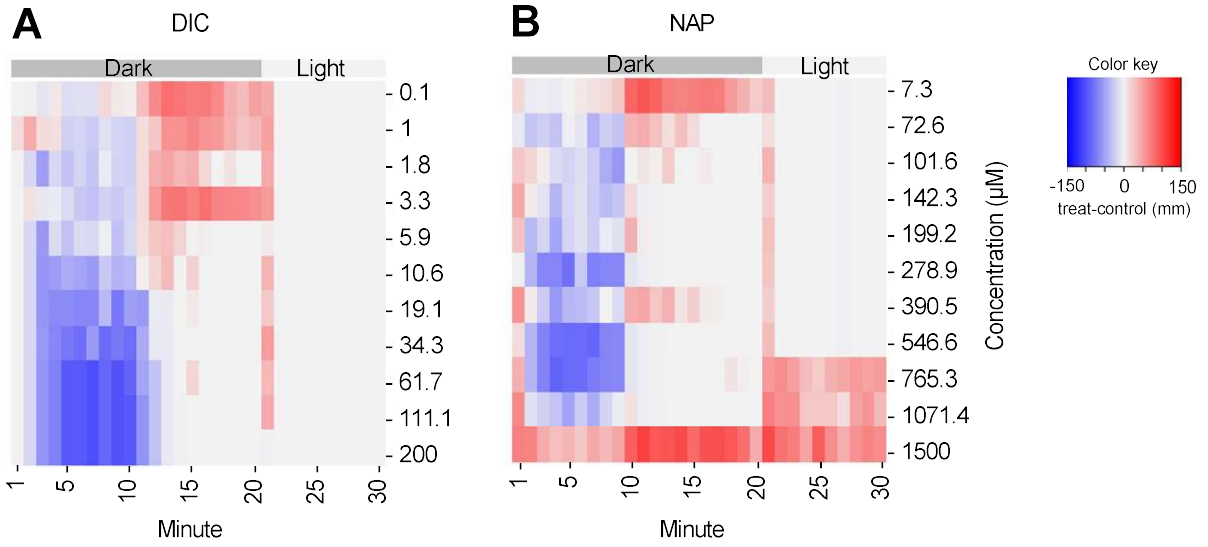

Figure 5.5. COX inhibitors elicit similar behavioral phenotypes at low concentrations. (**A**) Diclofenac (DIC) extends dark responsiveness with lower concentrations and acts sedative with high doses. (**B**) Behavioral profiles of naproxen (NAP) vary widely with increasing concentration and resemble DIC-specific phenotypes at 7.3 µM. Colors show deviation from median control activity: red, higher activity; blue, lower activity. *n*=8 individuals per condition.

Besides altered behavior, morphological defects were observed with DIC at 200.0 µM including somite degeneration and distortion of notochord and body axis (**Supporting figure D5.5A**). Furthermore, a significant decrease in heart rate was found at ≥111.1 µM. The corresponding EC50(3 h) was 94.0 µM (**Supporting figure C5.2A**). Significant bradycardia was also observed with NAP at 1.1 mM whereas no morphological abnormalities were observed up to the maximum tested concentration of 1.5 mM. Here, the heart rate EC50(3 h) was 2.2 mM which was beyond the maximum concentration tested of 1.5 mM (**Supporting figure C5.2B**). Noteworthy, impairment of heart rate was in the range of predicted baseline toxicity with LC50(96 h) values of 127.1 and 839.6 µM for DIC and NAP, respectively (Ecosar v.11, U.S. Environmental Protection Agency). In summary, behavioral phenotypes observed with DIC and NAP were not related to alterations in morphology and heart rate up to concentrations of ≤61.7 and ≤765.3 µM, respectively.

5.3.5 Behavioral phenotypes determined with a quinary mixture of chemicals resemble distinct profiles of individual components

When testing the environmentally relevant, quinary mixture of DIC, DIU, DZN, NAP, and TPP, not only the relative proportion of components was considered but also their respective environmental concentrations. This means, MX5 was tested across six orders of magnitude of total molarity, ranging from 3.6 nM to 336.0 µM, whereby exposure in the nanomolar range may be considered as environmentally relevant (see **Figure 5.4A**). Indeed, remarkable differences in behavior between treated and untreated groups were found at a concentration as low as 0.011 µM (**Figure 5.6**). Up to a concentration of 10.6 µM behavioral profiles were characterized by slightly decreased but prolonged dark responsiveness, though no effect was observed during the light period. In the concentration range from 33.6 up to 336.0 µM locomotor activity was increasingly suppressed. Intriguingly, even though MX5 contained ingredients which caused consistent hyperactivity both, individually and in combination (e.g. see **Figure 5.3A**), such a pattern was not observed in the case of MX5. To make a quantitative statement on the absence of hyperlocomotion in MX5 but observed with some of its individual constituents including DIU, DZN, and TPP as well as their tertiary mixture (MX3, see **Figure 5.3A**) a prediction on their combined effects in MX5 was performed (**Supporting figure D5.3**). Again, predictions were based on locomotor activity in minute 20 which was demonstrated to be highly correlated with hyperlocomotion determined with the second part of the dark phase as well as the whole light period (see **Supporting figure C4.1B**). DIC and NAP were still considered in the total molarity of MX5 but were assumed to not produce hyperlocomotion comparable to the other three ingredients as shown above (compare **Figure 5.5A** and **B** with e.g. **Figure 5.3A**). Furthermore, the mixture ratio of constituents of MX5 was constant (see **Figure 5.4C**). Interestingly, CA (which proved to be robust in the prediction of hyperlocomotion) predicted an EC50(3 h) of 103.1 µM. However, behavioral profiles determined with adjacent test concentrations including 33.6, 106.3, and 336.0 µM did not produce the predicted hyperactive phenotype (**Figure 5.6**).

To find out how behavioral profiles of single constituents and their quinary mixture relate to each other, we used multidimensional scaling (MDS) to visualize phenotypic similarities and dissimilarities (**Figure 5.7**). The MDS representation reveals that the single constituents of MX5 elicit distinct locomotor phenotypes over a certain concentration range (highlighted in **Figure 5.7**). This is the case for DIC (0.1-5.9 µM), NAP (72.6-199.2 µM, 390.5 µM), and DIU (14.5-55.7 µM). Behavioral profiles of DZN and TPP share phenotypic features and both span quadrant I and IV. Most interesting, MX5 (0.03-10.6 µM) resembles phenotypes of both, DIC and NAP. However, MX5 is not an exact phenocopy. Instead, the locomotor phenotype

of MX5 apparently represents a hybrid of behavioral profiles of DIC and NAP. This suggests that the COX inhibitors drive the behavioral effect observed with MX5.

In addition to behavioral alterations, multiple outcomes at various levels of biological organization were assessed for MX5. At molecular level, inhibition of AChE was found to be significant at concentrations ≥33.6 µM (**Supporting figure D5.4A**). Heart rate was affected in a similar range: ≥106 µM (**Supporting figure D5.4B**). EC50(3 h) values of 213.6 and 87.2 µM were determined for reduction in AChE activity and heart rate, respectively. At morphological scale effects similar as observed with DIC, i.e. altered somite structure as well as deformations of notochord and body axis, were observed at the maximum concentration of 336 µM (**Supporting figure D5.5B**). Thus, locomotor phenotypes identified specific for MX5 are neither affected by AChE inhibition nor bradycardia or morphological abnormalities.

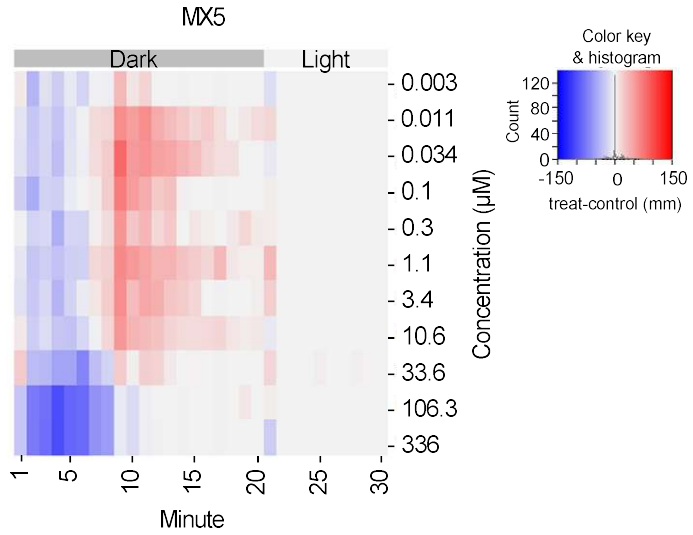

Figure 5.6. A quinary mixture (MX5) composed of diclofenac, diuron, diazinon, naproxen, and triphenylphosphate elicits extended dark responsiveness across two orders of magnitude in concentration (0.01-10.8 µM).

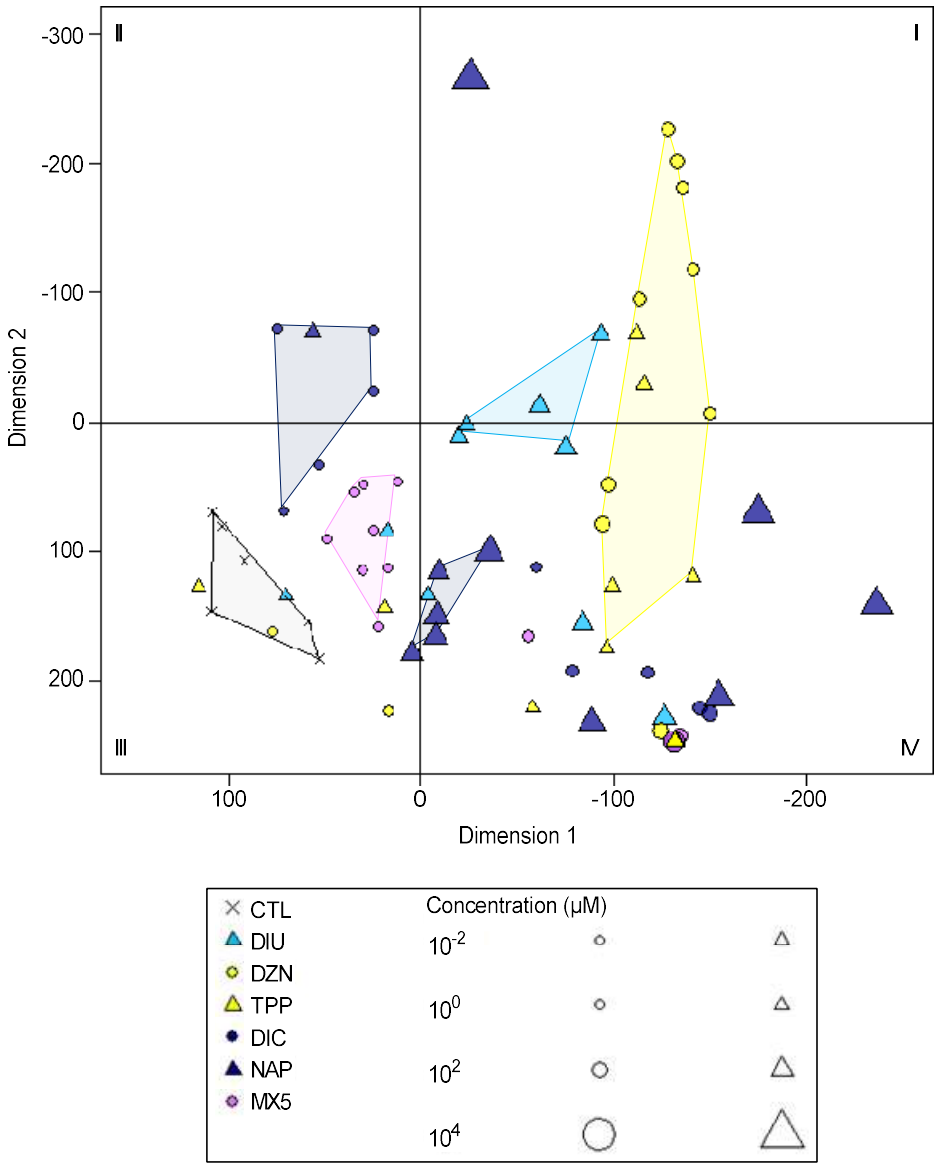

Figure 5.7. Multidimensional scaling representation of pairwise distances between behavioral profiles of zebrafish larvae treated with the indicated chemicals and their mixture (MX5). Symbol size, concentration; gray area, control (CTL). See **Table 5.1** for chemical IDs.

5.4 Discussion

The co-occurrence of chemicals in the aquatic environment is proven (Liška et al. 2015; Loos et al. 2013; Moschet et al. 2014). Numerous chemicals are expected to have a joint effect on aquatic organisms as they potentially target both distinct but also similar biological functions (Busch et al. 2016; Schwarzenbach et al. 2006). Hence, the individual assessment of single environmental chemicals, as carried out currently, is likely to underestimate actual biological effects under realistic environmental conditions (Brack et al. 2019). Therefore, effect-based tools such as the fish embryo acute toxicity (FET) test have been proposed as approaches

complementary to chemical analytical surveillance (Brack et al. 2019; Connon et al. 2012). However, the FET test has been demonstrated to offer weak sensitivity towards chemicals with certain MOAs (Scholz et al. 2018). At the same time, it has been shown that the locomotor response of larval zebrafish represents a valuable endpoint to assess the sub-lethal efficacy of specifically acting chemicals like neuroactive ones (Klüver et al. 2015). While concepts of mixture toxicity prediction have successfully been applied to lethal endpoints (Altenburger et al. 2018), their applicability to sub-lethal endpoints, such as e.g. changes in behavior, has not yet been evaluated.

Chemical combination effects occur at the level of behavioral alterations

First of all, we demonstrated that chemical combination effects occur at the level of behavioral alterations. In more detail this means that the combination effect of artificial mixtures of similarly acting chemicals including MX4 and MX3 remarkably outweighed the effect of individual constituents. Moreover, the actual combination effect even outweighed the one predicted with CA, even though CA adds up concentrations of individual components scaled by a factor of efficacy. To our knowledge this is the first report on the prediction of behavioral responses to chemical mixture exposure, furthermore, showing increased sensitivity with regard to anticipated outcomes. Although predictions on combined effects on behavior have not been reported yet, the increased behavioral efficacy of combined exposures is known. For example, exposure during embryonic development (2-50 hpf) to environmentally relevant concentrations of eight priority pharmaceuticals and their mixture led to behavioral alterations in larval zebrafish at 118 hpf (Zhou et al. 2019). These findings emphasize the relevance of mixture assessment beyond evaluation of individual chemical substances. This is true not only for lethal endpoints (Altenburger et al. 2018), but also applies to alterations in behavior.

More particularly, the present findings shed further light on underlying mechanisms of respective environmental chemicals which cause hyperlocomotion in larval zebrafish. In **Chapter 4** it was already substantiated that similar phenotypes of hyperlocomotion observed with environmental chemicals like DCA, DIU, DZN, and TPP probably represent seizure-like behavior potentially related to a complex neurological disorders of the central nervous system. In particular, diverse neurotransmitter pathways are involved in such seizure-like states including GABAergic, cholinergic and glutamatergic neurotransmission (Stewart et al. 2012). Consequently, the present finding on increased behavioral susceptibility to combined exposure may further hint that certain mixture constituents target different as well as similar neurotransmitter pathways as the actually measured effect exceeded the one predicted by CA.

Classical concentration response models are applicable to locomotor activity

Furthermore, we demonstrated for the first time that classical concentration response models, such as the four-parameter logit (Hill) model are well-suited to model the locomotor response to individual chemicals. Here, in contrast to a more general determination of effect concentrations valid for the overall alteration in locomotor activity throughout a whole light-dark transition test as performed in **Chapter 3**, we strived for a more detailed description of motor patterns. In this context, it was demonstrated that, based on locomotor activity during distinct time increments (here, minute 20) as part of the complete behavioral fingerprint of a chemical, such detailed, model-based description of locomotor activity is feasible. Even though there are few studies that employ locomotor activity to derive effect concentrations, our results go beyond previous reports, showing that more abstract quantification methods, such as the overlapping area approach (Klüver et al. 2015; Selderslaghs et al. 2010), can be extended by the method presented here. It provides the dual advantage to additionally inform about both the directional regulation and the degree of alteration in locomotor activity. Although we focused on phenotypes of hyperlocomotion, the model-based description of hypolocomotor phenotypes follows the same principle and can be applied accordingly. The great potential of such a concentration response modelling approach with high temporal resolution lies in the parametrization of whole locomotor profiles. A detailed, model-based description of respective phenotypes could be used to inter- and extrapolate locomotor activity across concentrations. Additionally, the ability to model concentration depended locomotor responses provides the basis for predictions on combined effects of chemicals on motor activity.

Common mixture concepts are applicable to the prediction of mixture effects on locomotor responses with regard to similarly acting chemicals

In a further step we verified for the first time that commonly used concepts for the prediction of chemical mixture effects including CA and IA are applicable to the prediction of behavioral alterations. Indeed, as expected from the phenotypic similarity of individual constituents, both examined mixtures (MX4, MX3) caused a concertation dependent phenotype of hyperlocomotion which was approximately predicted with CA. However, in case of MX4, while the EC50(3 h) was slightly underestimated with CA, the actual slope was steeper than predicted with both CA and IA. *Vice versa*, IA predicted the absence of effects at low concentrations more appropriately than CA whereas the EC50(3 h) was remarkably underestimated. Intriguingly, the actual slope determined with MX4 was remarkably steeper than the slope of concentration response curves determined with its individual constituents. These findings hint that locomotor alterations, e.g., observed with MX4 are related to multiphasic dose response features of one or more mixture components which results in

concentration dependent antagonism and synergism effects, respectively. This theory follows the observation that DCA initially causes sedation which is likely to counteract hyperlocomotion. DCAs' locomotor activation effect is secondary to anesthesia on concentration scale (see **Supporting figure C4.2A**). Our hypothesis was confirmed as DCAs' exclusion from MX4 resulted in a more accurate prediction of both EC50(3 h) and slope. The EC50(3 h) predicted with CA only differed by less than factor two from the actual effect concentration determined with MX3. Additionally, the actual slope was nearly identical to the one predicted with CA. In conclusion, the concept of CA appears appropriate in order to predict monophasic relationships provided that mixture components cause a unidirectional behavioral response. Although the results presented here provide a first, promising evidence for the applicability of mixture concepts to the prediction of combined effects of chemicals on locomotor activity it needs to be recognized that the current data situation is too scarce to allow for a final, definite conclusion. Therefore, a repetition of the respective experiments, *inter alia*, including a higher concentration resolution, is indispensable to take account of data variability and to finally verify the statistical significance of the present findings. Moreover, the prediction of locomotor alterations is confronted with some complications. For example, the multiphasic, bidirectional character of locomotor responses observed with chemicals like DCA, complicates the predictions of behavioral profiles and demands for further adaption of prediction models. Limitations which need to be overcome in order to more precisely depict motor phenotypes include the non-monophasic character as well as differences in the magnitude of locomotor responses. To overcome such limitations, Scholze et al. (2014) developed a solution for *in vitro*-based receptor assays which considers mixtures of partial agonists with differing maximal effects using a toxic unit extrapolation approach. The proposed approach accurately predicted responses with a mixture of 21 estrogenic chemicals whose concentration response varied widely in shape, slope and maximal effect (Scholze et al. 2014). Additionally, a fitting procedure for dose-response curves with multiphasic features due to the presence of combined agonist and antagonist effects of individual chemicals was published recently (Di Veroli et al. 2015). The combination of these approaches, each restricted to the application to *in vitro* data at the moment, offers great potential for future application in behavior-based concentration response modelling and the prediction of mixture effects. Another approach, distinct from the one presented here, represents the modelling of larval locomotor activity. Gauthier and Vijavan (2018) fit larval light-dark activities by an asymmetric Ricker-beta function to estimate the magnitude of activity and temporal aspects. Characteristics of the phenotype include mean and maximum activity, duration of excitatory period, and time at maximum rate of increase in activity. The nonlinear mixed-model was capable of detecting mixture effects in larval behavior (Gauthier and Vijayan 2018). Even though the modelling of locomotor activity has not yet been applied

to the prediction of combination effects, the parametrization of larval behavior is another promising starting point.

Putative MOA-specific patterns of locomotion can be recovered from chemical mixtures of environmentally relevant composition

In a final step of the present study, we strived to exploit the diagnostic capacity of behavioral profiling with regard to its application in the identification of mixture toxicity drivers. Behavioral profiling of individual chemicals and their quaternary mixture (MX5) revealed distinct locomotor phenotypes. A high degree of phenotypic similarity was found among DIC, NAP, and MX5. Intriguingly, behavioral profiles determined with MX5 were not exactly identical with the non-steroidal anti-inflammatory drugs (NSAIDs) but rather represented a hybrid of their individual behavioral profiles. Therefore, we suggest that the COX inhibitors drive the behavioral effect observed with MX5. Supporting this, morphological abnormalities observed with high doses of DIC including altered somite structure as well as deformations of notochord and body axis were also found with high doses of MX5. It needs to be considered that the MX5-specific behavioral profiles were not affected by deformations, bradycardia, and inhibition of AChE.

It is perhaps not surprising that DIC and NAP dictated the phenotype determined with MX5 given the higher molarity as compared to DIU, DZN, and TPP. In addition, NSAIDs like DIC and NAP exhibit a high pharmacological efficacy (Khetan and Collins 2007; Rao and Knaus 2008). However, based on predictions performed on MX5-constituents that elicit hyperlocomotion including DIU, DZN, and TPP, a respective phenotype would have been anticipated but was not observed. Nevertheless, as the artificial mixture considered the environmental ratio of chemical constituents, our finding highlights the potential of behavioral profiling in the diagnostic assessment of chemical mixtures and the determination of subtle physiological effects related to the specificity and efficiency of certain compounds such as pharmaceuticals to interfere with distinct molecular targets and pathways relevant for behavior at low, sub-lethal concentrations.

Whether behavioral phenotypes of DIC and NAP are indeed related to inhibition of COX is beyond the scope of this study. However, it is known is that numerous COX inhibitors alter light dependent rest/wake regulation in larval zebrafish in a similar manner (Rihel et al. 2010). Thus, the behavioral efficacy at low concentrations in combination with a high pharmacological efficacy and a low bioaccumulation in developing zebrafish (Schüttler et al. 2019) indicate that the observed locomotor pattern may be related to the actual MOA of these NSAIDs.

Mixtures composed of multiple components with distinct mechanisms (such as MX5) could be compared with multitarget chemicals such as psychiatric drugs which rather act via polypharmacology on multiple targets (Roth et al. 2004). In this context, a battery of behavioral assays in larval zebrafish was shown to provide sufficient phenotypic resolution to identify multitarget drugs, thereby, combining behavioral phenotypes with receptor targets and binding affinities (Bruni et al. 2016). Therefore, it appears surprising that, although MX5 also comprised of constituents which caused hyperlocomotion both individually (DIU, DZN, TPP; see also **Chapter 4**) and in combination (MX3), no such phenotype was observed with MX5. In addition, exposure to MX5 concentrations ≥33.6 µM decreased both heart rate and AChE activity but resulted in paralysis instead of hyperlocomotion as observed in **Chapter 4**. Interestingly, in a screen for compounds with anti-convulsant properties using the acute pentylenetetrazole (PTZ)-based zebrafish model of epileptic seizures, the COX inhibitors NAP and mefenamic acid suppressed PTZ-induced expression of *c-fos*, a biomarker of neuronal activation, in 50 hpf embryos (Baxendale et al. 2012). Similar anticonvulsant properties of NAP and mefenamic acid were also reported for PTZ-treated mice (Dhir et al. 2005; Wallenstein 1991). Zebrafish larvae exposed to the $GABA_A$ receptor antagonist PTZ show hyperlocomotion/seizure-like behavior (Afrikanova et al. 2013) similar to DIU, DZN, and TPP. In conclusion, COX inhibitors, such as NAP, may elicit neuroprotective functions superordinate to convulsant activities.

Concluding remarks

In summary, clear evidence was found for additive effects of environmental chemicals at behavioral level and the commonly applied concept of CA was found to be applicable for the prediction of combined effects of similarly acting chemicals. In order to more comprehensively depict locomotor behavior, further research should elaborate on multiphasic models which also consider differences in the magnitude of locomotor responses. In addition, for the first time it was demonstrated that behavioral profiling of environmental chemicals in larval zebrafish offers the potential to identify drivers of mixture effects and related modes of action. Both predictive and diagnostic approaches presented here provide great potential for future applications in behavior-based evaluation of chemicals' biological efficacy and prediction of their combined effect on locomotion. Besides the diagnostic potential of behavioral profiling, our results once more substantiate that behavioral alterations occur with environmentally relevant concentrations, i.e., in the nanomolar range. Given the great diversity of environmental chemicals, these results appear to be even more severe. To assess the environmental risk at sub-lethal scale, further research should focus on combined effects of chemicals and the related ecological relevance of acute behavioral alterations.

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Conclusions and future perspectives

Neuroactive environmental chemicals are often associated with increased acute-to-chronic ratios (Scholz et al. 2018) and, thus, demand innovative approaches which go beyond the acquisition of lethal acute toxicity. Numerous studies highlight the potential of behavior assays in embryo-larval stages of zebrafish to assess both, the sub-lethal effects and underlying mechanisms of action of neuroactive chemicals (Bruni et al. 2016; Klüver et al. 2015; Kokel et al. 2010). Nevertheless, a number of obstacles need to be overcome in order to routinely apply behavior-based tests to a sensitive acquisition of effects and the mechanistic assessment of chemicals and mixtures as well as the prediction of combined effects as envisioned in **Section 1.5**. These include the standardization of test protocols and evaluation strategies in order to facilitate the inter-comparability of different studies. In the course of the present dissertation I arrived at the following key findings:

- A review of related literature revealed that there are manifold behavior-based approaches to assess chemical neuroactivity and –toxicity, respectively. Consistent with findings from previous studies, it was shown that experimental parameters such as test design (duration, sequence, and repetition of photoperiods), developmental stage (e.g. age-specific locomotor activity, developmental toxicity), and exposure (time point of initiation, duration, concentration) can significantly impact on the outcome of a light-dark transition test. Consequently, the inter-comparability of such behavioral tests is compromised. In this thesis, a locomotor response assay in larval zebrafish was refined for the purpose of assessing the neuroactive potential of environmental chemicals and mixtures. The respective methodology is published in Leuthold et al. (2019).

- The developed behavioral barcode represents the biological fingerprint of a chemical and its mechanism of action. It could be shown that behavioral barcoding of chemicals, i.e., the integration of responses observed in treated and untreated test groups, represents a valuable approach to quantify changes in behavior in a condensed, meaningful and communicable manner. It does not only allow for comparability of compound- and concentration-dependent signals but also of results originating from independent experiments.

- Compound- and mode of action (MOA)-specific behavioral profiles can be used to identify chemicals with related mechanisms according to phenotypic similarity.

However, in the present work the precise underlying mechanisms of characteristic locomotor phenotypes, such as seizure-like hyperlocomotion, have not yet been finally determined. Nevertheless, it was demonstrated that such specific responses could allow for the identification of key effect drivers from chemical mixtures.

- The developed locomotor activity quantification approach enables the establishment of concentration-response relationships which, in turn, allow for the derivation of effect concentrations (ECs). Locomotor-derived ECs can now be used to assess the sensitivity of behavioral responses in comparison with, e.g., lethal concentrations (LCs). In the framework of this study it could be shown that certain neuroactive chemicals but also other environmental chemicals (e.g. diclofenac) elicit increased (factor ≥10) behavioral susceptibility in larval zebrafish compared to lethal acute toxicity (LC50) determined with the fish embryo acute toxicity (FET) test.

- Mixture toxicity concepts such as concentration addition (CA) can be applied to the prediction of combined effects of chemicals with similar behavioral phenotypes. Furthermore, the concept of independent action (IA) can inform about mixture effects of dissimilarly acting chemicals. Hitherto, this has not been investigated or reported at all.

- As it was demonstrated that chemicals previously unknown for their potential to affect behavior indeed cause alterations in locomotion, it is reasonable to extend the scope of application of locomotor response tests to a wider range of chemicals previously not considered as bioactive at a behavioral scale.

The above outlined points highlight implications for future research which are discussed in the following.

6.1 Behavioral endpoints in the effect-based assessment of chemicals and mixtures

The assessment of potentially adverse long-term effects of chemicals and chemical mixtures is often elusive with regard to acute toxicity assays such as the FET test (OECD test no. 236, OECD 2013). Although, the FET test allows for the evaluation of other apical endpoints in addition to lethality, subtle changes in physiology and anatomy due to chemical exposure are often hard to determine using conventional procedures, namely subjective observations. In contrast, behavior-based endpoints represent an integrated response to chemical exposure whose assessment is rather unbiased and more efficient due to the availability of automated screening technologies. This gives the opportunity to extend the FET test with behavioral measures. The advantage of a behavior-based complementation is twofold and includes the

use of behavioral responses as a sensitive, sub-lethal measure of chemical exposure as well as the acquisition of additional (behavior-based) endpoints to increase the diagnostic capacity of an extended FET test.

In this work, it has been demonstrated that a complementation of the FET test with the light-dark transition test, indeed, contributes to determine increased sensitivity for certain environmental chemicals below levels of lethal acute toxicity. Moreover, similar LC50 and EC50 values of FET and light-dark transition test, respectively, were determined with numerous compounds (see **Chapter 3**) and indicate the potential use of behavioral outcomes of short-term chemical treatments as a proxy of lethal acute toxicity. In addition, the established approach proofed to be robust and reproducible but differs in design from the 'classical' FET test. This is because the application of exposure settings as described in OECD test no. 236 (OECD 2013), namely exposure throughout embryo-larval development, adds remarkable complexity to the differentiation of purely neurologically mediated and developmental toxicity related alterations in behavior. Therefore, developmental (neuro)-toxicity testing should always be complemented with phenotypic analyses at morphological and physiological scale. As envisioned by Rennekamp and Peterson (2015), advances in imaging and automation will improve chemical screens, increase the number of screened chemicals, and reduce costs. In addition, automated morphological phenotyping in zebrafish allows for quantitative assessments and reduces subjective bias (Teixidó et al. 2019). It must be taken into account that developmental toxicity assays such as the FET test and behavior-based approaches specifically aiming at neuroactivity are not necessarily compatible. Based on the results obtained here, it has become clear that the larval zebrafish represents a competent model system to study the immediate effects of acute short-term chemical exposure on the mature nervous system.

In order to routinely apply behavioral tests in the assessment of chemicals and chemical mixtures, uniform, efficient test designs as well as appropriate evaluation strategies are required. Here, the focus should be on capturing behavioral alterations in an as far as possible comprehensive manner in order to provide a maximum discriminatory power. A major challenge in the establishment of uniform light-dark transition tests for chemicals is the heterogeneity of applied test designs (e.g. sequence, duration of photoperiods) and the variability in data processing. Accordingly, different procedures may differ in robustness, reproducibility, specificity, and sensitivity. Unfortunately, the majority of studies rarely report on motives for the choice of employed test conditions and appear rather random than scientifically justified. It is beyond the realms of possibility of this work to propose *the* optimal approach (insofar one exists at all, given the diversity of research questions) which provides maximum sensitivity and specificity. Nevertheless, the present work revealed that certain,

potentially compound/MOA-specific phenotypes would have been overlooked if, first, photoperiods would have been reduced in time, second, the temporal resolution of responses would have been lower than applied, and, third, time and concentration would have been ignored as important variables of toxicity. Hence, more minimalistic test designs, i.e. setups that, *inter alia*, lack detailed temporal and concentration resolution, should be reviewed critically. Furthermore, given the potential diversity of existing locomotor phenotypes, further optimization of the light-dark transition test cannot be achieved without the examination of untreated individuals in parallel with representative chemicals that interfere with major neurotransmitter pathways, e.g. glutamatergic, GABAergic, serotoninergic, cholinergic neurotransmission (Horzmann and Freeman 2016). Concrete proposals for appropriate reference compounds have already been made (see e.g. Aschner et al. 2017). Last but not least, a comprehensive meta-analysis of existing results appears indispensable. However, the limited public availability of respective data renders such analyses. In the future, publically available databases should be established which precisely report on experimental conditions (e.g. exposure and light regimens) and interindividual variability in treatments and control groups (e.g. quantiles of locomotor activity) or ideally, provide raw data files.

Regarding evaluation strategies, there are numerous adequate options. In a regulatory context, one important prerequisite is probably the provision of ECs that allow for comparability among different studies, chemicals, other endpoints (e.g. LC50), as well as comparisons with thresholds such as environmental quality standards. In this work, a common four-parameter concentration-response model was successfully adapted to derive motor activity-based EC50 values which inform on both, directionality (up- or downregulation) and intensity of locomotor responses. Therefore, the distance moved per unit of time proved to be a sensitive and robust measure of locomotor activity. A matter of future discussion may be which light phases or parts of it are most suitable to derive ECs. In this context, this work clearly showed that both, photo periods and distinct periods in between, provide valuable information on compound-specific behavioral alterations and should, therefore, be considered in chemical assessments. In addition, further computational work is required to allow for an adequate representation of multiphasic dose-response patterns and varying maximum effects. Approaches from receptor assays appear promising to be adapted in future (e.g. see Di Veroli et al. 2015; Scholze et al. 2014).

The prediction of combined effects in a chemical mixture based on behavioral profiles of its single components represents a novel field of application in behavioral research. Currently, (the few) behavior-based mixture studies are restricted to the observation of increased sensitivity towards certain mixtures compared with efficacy of respective constituents (e.g.

Zhou et al. 2019). In the present work it has been demonstrated that the combined effect of chemicals that elicit similar behavioral phenotypes is feasible using common concepts of mixture toxicity prediction such as CA. However, the prediction of combination effects on locomotion becomes increasingly complicated as soon as chemicals elicit opposite locomotor activity (i.e. hyper- and hypoactivity, respectively) and frequently observed multiphasic concentration-response behavior. Therefore, suitable models should be established which address both, multiphasic and bidirectional behavior. Nevertheless, the current work shows that combination effects of chemicals on locomotion occur and need to be considered.

In order to implement behavior measures in a regulatory context it is necessary to determine the relevance of behavioral responses with regard to adverse effects at the organism and/or population level. It is recognized that chemicals can alter behaviors with ecological consequences (Brodin et al. 2013). However, as behavior is a complex, integrated response, not every change in locomotion may necessarily be associated with adverse ecological effects. Therefore, behavioral alterations, or more precisely, distinct phenotypes need to be hardwired to ecologically relevant outcomes such as survival, feeding, predator avoidance, and reproduction (Mittelbach et al. 2014). For example, in this work, numerous observations on various chemicals and mixtures hint that paralysis may be indicative for narcosis which in turn is related to survival. Furthermore, it was found that severe forms of hyperlocomotion are often associated with subsequent lethality (see **Chapters 3** and **4**). These results demonstrate the predictive potential of short-term behavioral tests for acute lethality at a later stage. The interconnection of behavioral outcomes with other environmental outcomes appears in reach. For example, Jordi et al. (2018) successfully combined a multibehavioral phenotyping approach in larval zebrafish with food intake using fluorescently labelled live paramecia. Thereby, it was demonstrated that certain neuroactive drugs modify a variety of zebrafish behaviors and modulate appetite (Jordi et al. 2018).

6.2 Diagnostic capacity of behavioral phenotypes

Next to an increase in sensitivity compared to lethal toxicity, a great potential of behavioral phenotyping lies in the identification of underlying toxicity mechanisms of single chemicals and mixtures such as environmental samples. Since diverse embryo-larval patterns of behavior underlie distinct neurobiology, i.e. pathways of neurotransmission, as highlighted in **Section 1.4**, behavior-based profiling of chemicals provides a great opportunity to interconnect phenotypes with underlying mechanisms. Numerous studies have demonstrated that behavioral profiling of chemicals in zebrafish contributes the evaluation of mechanisms, e.g. via comparison of phenotypes of chemicals with known and unknown molecular

targets/MOAs (e.g. Bruni et al. 2016; Kokel et al. 2010; Rihel et al. 2010). In the present work, it has been shown that more or less distinct locomotor phenotypes can be obtained with different chemicals and anticipated MOA classes using the light-dark transition test in larval zebrafish. Thus, further elaboration is required in order to determine the actual underlying mechanisms leading to alterations in light-dark transition behavior. In particular, it needs to be figured out why chemicals expected to act similarly based on same anticipated MOAs do not cause similar locomotor phenotypes. *Vice versa*, the unexpected phenotypic similarity of certain chemicals either indicates (unknown) similar molecular initiating events or a convergence of key events which result in similar behavioral outcomes at the organism level. Furthermore, this thesis highlights that, against the naïve initial assumption, there is not one representative phenotype of a chemical/MOA but rather a multitude of concentration and time dependent responses which potentially reflect interference with multiple receptor systems, transmission pathways and/or other targets. In addition, within the framework of this work, it was achieved for the first time to disclose the potential of behavioral profiling to interconnect phenotypes determined with individual compounds with locomotor patterns observed in mixtures of the respective chemicals. This finding indicates a tremendous potential to apply behavioral profiling to diagnostic applications in the assessment of chemical mixtures. Nevertheless, although, high-throughput approaches, such as the light-dark transition test, may serve as a first indication of mechanisms involved in phenotypic alterations, a combination of behavioral tests and additional methods able to capture complex toxicity pathways in a time and concentration dependent manner (e.g. metabolomics, transcriptomics) may contribute in-depth analyses to definitely identify the underlying machinery.

Diverse neurological disorders with distinct pathophysiology result in similar phenotypes, and *vice versa*, diverse outcomes result from similar pathophysiology (Bal-Price et al. 2015). Therefore, in order to exploit the diagnostic potential of behavior-based tests for chemical mechanisms, it seems indispensable to design more sophisticated assays capable to unravel the potentially complex underlying mechanisms of interference with neurotransmission. As outlined in **Section 1.4**, the behavioral repertoire of embryo-larval zebrafish stages comprises of more than just responsiveness to abrupt changes in lighting conditions. Moreover, light-dark transitions only touch upon a limited number of behaviorally relevant receptors, in particular the eye. The implementation of further endpoints (e.g. acoustic stimuli addressing the lateral line) may further improve the diagnostic capacity of behavioral readouts. Furthermore, a battery of behavioral assays comprising of various stereotyped behaviors may extend isolated phenotypic observations to the field of chemobehavioral phenomics (Kokel and Peterson 2008).

This thesis shows that behavioral assays, such as the light-dark transition test, can increase sensitivity beyond lethal acute toxicity and, therefore, represent a sensitive sub-lethal readout of chemical exposure which, nevertheless, needs to be interconnected with ecotoxicologically relevant outcomes in future. Furthermore, this work guides on how behavior based tests and evaluation strategies can be further optimized and harmonized in order to provide a maximum of sensitivity and specificity. In addition, behavioral profiling of environmental chemicals and mixtures can be used to derive understanding of underlying mechanism via comparison with behavioral phenotypes of compounds with known MOAs. The present work also highlights that joint effects of different chemicals with anticipated neuroactive and other MOAs in a mixture occur on the behavior level which can be identified and calculated from individual, specific locomotor phenotypes. However, behavior-based assays alone do not allow for definite statements on underlying mechanisms and need, therefore, to be complemented with other, e.g. molecular, approaches in future. Finally, the high-throughput capacity of behavioral screens in connection with high sensitivity offers a great potential to perform screens on hundreds of thousands of chemicals, partly not yet characterized for their sub-lethal and potentially ecologically relevant outcomes.

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Appendix

Appendix A

Supporting information – Chapter 2

A2.1 Materials and methods

Estimation of the bioconcentration factor. Fish and fish embryo acute toxicity are strongly correlated (Belanger et al. 2013; Klüver et al. 2015; Knöbel et al. 2012; Lammer et al. 2009). However, a toxicokinetic model for the estimation of internal concentrations in the fish embryo is not available yet. Hence, to compensate for potentially lower doses due to a reduction in exposure time, the estimation of the bioconcentration factor in adult fish (OECD 2012) can be used to correct aqueous exposure concentrations applied in the fish embryo acute toxicity (FET) test.

If the aqueous concentration c_w is constant, the internal concentration c_f is described by

$$c_f = BCF \times c_w(1 - e^{-k_2 \times t}) \qquad \textbf{Equation A2.1}$$

with *BCF* being the bioconcentration factor, k_2 (day^{-1}) being the elimination rate constant, and *t* (day) being the duration of exposure. An estimate of k_2 can be obtained with the following empirical relationship:

$$k_2 = 10^{1.47 - 0.414 \times logD} \qquad \textbf{Equation A2.2}$$

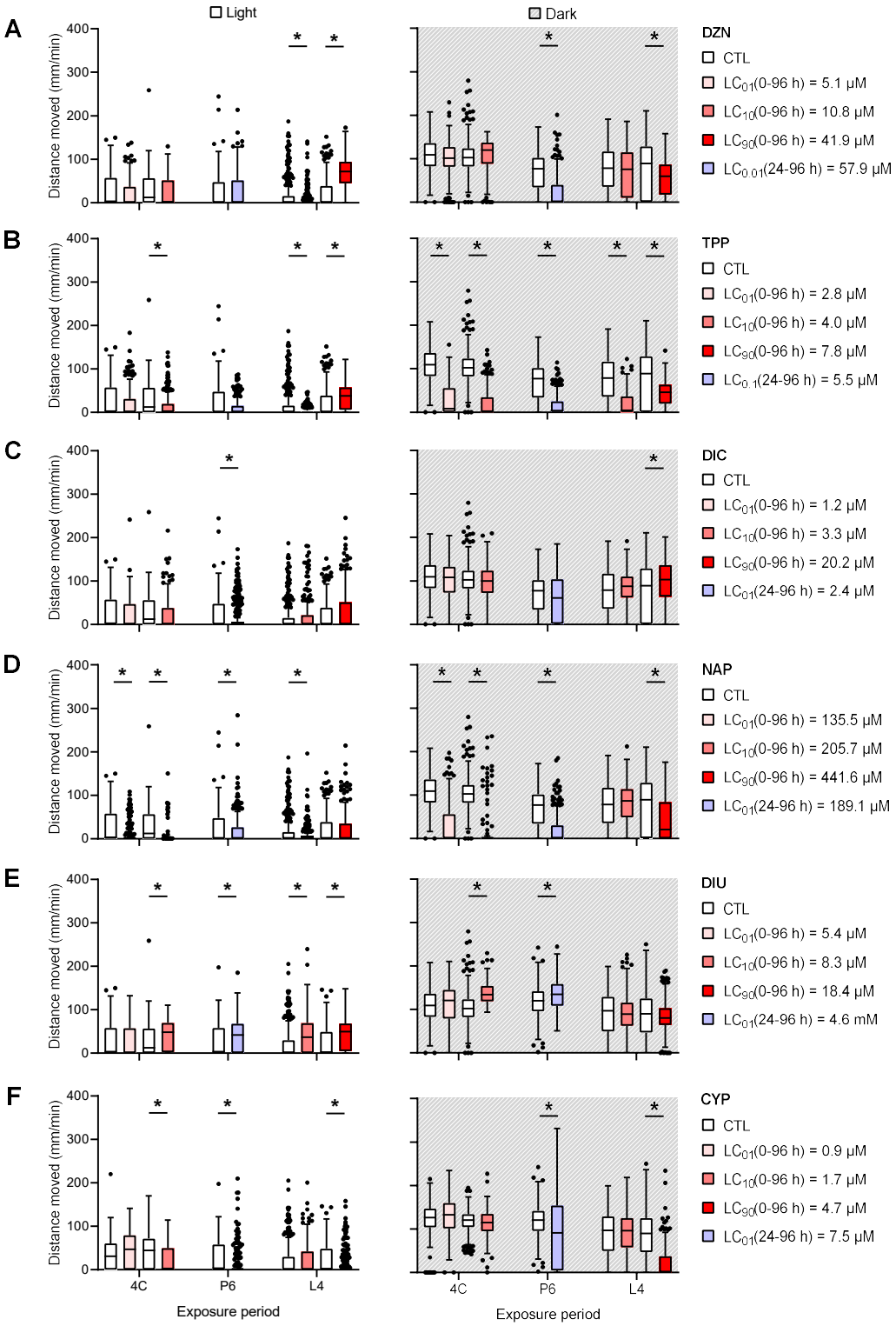
log*D* represents the common logarithm of the octanol-water distribution coefficient *D* at a given pH. The internal concentration c_f causing half maximum lethality in the test population, i.e. the LC_{50}, is given by

$$c_f = BCF \times LC_{50} \qquad \textbf{Equation A2.3}$$

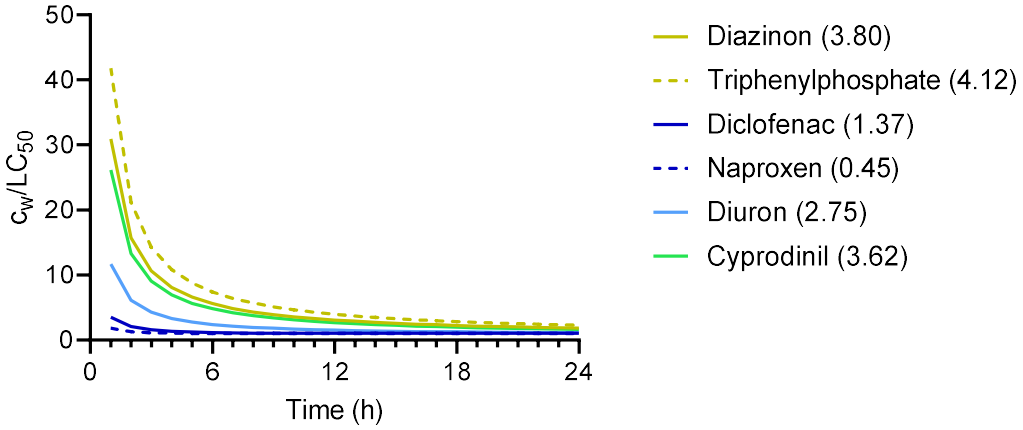
By substitution of k_2 and c_f from **Equation A2.2** and **A2.3**, respectively, in **Equation A2.1**, the aqueous concentration c_w causing half maximum lethality within a given period of time *t* and in dependence on *D* is obtained with

$$c_w(LC_{50}, D, t) = \frac{LC_{50}}{1 - e^{-(10^{1.47 - 0.414 \times logD}) \times t}} \qquad \textbf{Equation A2.4}$$

The ratio c_w/LC_{50} represents a correction factor which specifies an aqueous concentration that obtains 50% mortality within a certain period of time. A graphical representation of the corresponding results for the six model compounds is shown in **Supporting figure A2.2**. For example in the case of diazinon, c_w needs to be increased by factor 1.8 in order to cause half maximum lethality within 24 h. In contrast, with diclofenac half maximum lethality is already reached by 95% after 9 h of exposure without correction of LC_{50}. The proposed approach is accompanied by several limitations such as the assumption that acute toxicity is exclusively driven by hydrophobicity. Hence, factors, such as mechanism and stage-specific susceptibility are not considered.



Supporting figure A2.1. Locomotor responses in 4 dpf zebrafish are dependent on compound, and the level and period of exposure. Tukey box plots show light (left column) and dark (right column) dependent alterations of locomotor activity with different exposure periods (4C=4-cell (0-96 hpf), P6=prim 6 (24-96 hpf), L4=larval day 4 (94.5-96 hpf)) and exposure levels of (**A**) diazinon, (**B**) triphenylphosphate, (**C**) diclofenac, (**D**) naproxen, (**E**) diuron, and (**F**) cyprodinil. Differences between control and treatment are significant as indicated: *p<0.001. n≤16 individuals per condition. CTL, control.



Supporting figure A2.2. Estimation of corrected aqueous exposure concentrations to compensate for exposure reduced in time using the bioconcentration factor estimation approach as specified in OECD test guideline 305 (OECD 2012). Graph shows the correction factor c_w/LC_{50} (y axis) in dependence on the duration of exposure (x axis). Values in brackets represent estimates of logD at pH 7.4 (ACD/Percepta, PhysChem Profiler, ACD/Laboratories, build 726. Nov 7, 2014).

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Appendix B

Supporting information – Chapter 3

B3.1 Materials and methods

Fish cultivation and embryo collection. Adult zebrafish (*D. rerio*) of the strain UFZ-OBI (generation F14) were kept in 120-L aquaria (activated carbon-filtered tap water, 26.5±1 °C) with a photoperiod of 14:10 h light:dark. Commercial dry food and *Artemia* sp. were fed once and twice a day, respectively. Fish were cultured and used according to German and European animal protection standards and fish culture was approved by the Government of Saxony (Landesdirektion Leipzig, file number 75–9185.64). Zebrafish eggs were collected within stainless steel sieve-covered glass dishes. Fertilized eggs were selected by means of microscopy and were subsequently transferred into oxygen-aerated (≥ 24 h) and pH-adjusted (pH 7.4±0.1) standard dilution water as specified in ISO 7346-3 (80 mM $CaCl_2 \cdot 2H_2O$, 20 mM $MgSO_4 \cdot 7H_2O$, 31 mM $NaHCO_3$, 3.1 mM KCl) with a density of 1 egg per 2 mL. Afterwards, zebrafish embryos were incubated at 28 °C with a 14:10 h light:dark cycle until initiation of exposure at 96 hpf (hours post fertilization).

Chemicals and stock preparation. Chemical stocks were either prepared directly in standard dilution water with a final concentration of 0.1% v/v methanol the day prior to testing or chemicals were dissolved in pure methanol (≥99.8%, CAS RN 67-56-1, J.T. Baker®, Avantor Performance Materials Inc, Pennsylvania, USA). Test solutions were then produced from serial dilution of stock solutions shortly before exposure. The final concentration of methanol was 0.1% v/v in every exposure solution. pH and oxygen levels of exposure (highest concentration tested per plate) and control media at initiation of exposure were 7.6±0.1 and 91.1±3.5%, and 7.5±0.1 and 92.6±3.5% (mean±standard deviation), respectively. Chemicals and purities purchased from Sigma-Aldrich (Steinheim, Germany) were (-)-nicotine (98.7%, CAS RN 54-11-5), 3,4-dichloroaniline (99.9%, CAS RN 95-76-1), bisphenol A (≥99.0%, CAS RN 80-05-7), cyprodinil (99.9%, CAS RN 121552-61-2), diclofenac sodium (≥98.0%, CAS RN 15307-79-6), endosulfan (99.4%, CAS RN 115-29-7), fipronil (97.9%, CAS RN 120068-37-3), naproxen sodium (98.0-102.0%, CAS RN 26159-34-2) and triphenylphosphate (≥99.0%, CAS RN 115-86-6). Chemicals and purities purchased from Sigma-Aldrich (Buchs, Switzerland) were diazinon (98.5%, CAS RN 333-41-5), diuron (≥98.0%, CAS RN 330-54-1), imidacloprid (≥98.0%, CAS RN 138261-41-3), isoproturon (99.8%, CAS RN 34123-59-6) and pyrimethanil (99.9%, CAS RN 53112-28-0). Furthermore, 4-n-nonylphenol (99.9%, CAS RN 104-40-5, Riedel de Haën, Honeywell Specialty Chemicals, Seelze, Germany), citalopram hydrobromide (95.0%, CAS RN 59729-32-7, Fluorochem Ltd Hadfield, UK), and D,L-venlafaxine (98.0%, CAS RN 93413-69-5, Santa Cruz Biotechnology Inc Dallas, Texas, USA) were used as model compounds.

Exposure. Immediately after exposure was initiated, individuals were transferred separately to single square-shaped flat bottom wells of a 96-well clear polystyrene plate (Whatman™ microplate devices, uniplate®, GE Healthcare UK Limited, Buckinghampshire, UK). The volume of test solution within each well was 400 µL. Subsequently, plates were covered with cell culture test plate lids (Techno Plastic Products, Trasadingen, Switzerland) and sealed with laboratory film (Pechiney Plastic Packaging, Chicago, Illinois, USA). In case of volatile compounds (3,4-dichloroaniline, diazinon, endosulfan, methanol, nicotine), plates were additionally sealed with a self-adhesive polyester film originally intended for real time PCR (Th. Geyer, Wertheim, Germany).

Light-dark transition locomotor response test. An incubator surrounding the ZebraBox was used to maintain a constant temperature of 28 °C (27.4±0.9 °C; mean±standard deviation). Tracking was initiated right after placing the plate within the measuring chamber. Light intensities during dark and light photoperiods were 0 and172 µmol/s/m^2 per µA, respectively. Tracking was conducted with continuous infrared illumination and recorded via an infrared camera. Videos were recorded at a rate of 25 images per second and data was binned into 1-min intervals. The detection threshold was set to 0.2 mm/s defining larvae as being inactive below this level. Besides mortality, apparent morphological abnormalities were recorded subsequent to each LMR test. Observed malformations included brain necrosis, axis distortion, edema formation and fin deformation. However, morphological alterations showed up at concentrations subsequently causing mortality within the observation interval (≤120 hpf). The influence of time of day on the outcome of the LMR assay was excluded by measuring at the same time, i.e. at ~08:00 AM, 02:00 PM, and 06:00 AM, respectively.

Analysis of locomotor activity data. In order to identify significant differences between control (C) and treatment (T), a 95% confidence interval (CI) per minute for both was calculated using

$$CI_{2.5} = \frac{n}{2} - \frac{1.96\sqrt{n}}{2} \qquad \textbf{Equation B3.1}$$

as lower limit and

$$CI_{97.5} = 1 + \frac{n}{2} + \frac{1.96\sqrt{n}}{2} \qquad \textbf{Equation B3.2}$$

as upper limit, with *n* being the number of embryos tested per concentration and 1.96 being the approximate value of the 97.5% percentile. An approximate 95% confidence interval for the sample median is given by the n^{th} ranked values obtained with **Equation B3.1** and **B3.2**. For the calculation of CI(C) all controls per tested compound were included. More specifically, T was considered hypoactive if the lower control limit ($CI_{2.5}$(C)) was above the

upper treatment limit ($CI_{97.5}$(T)) and hyperactive if the upper control limit ($CI_{97.5}$(C)) was below the lower treatment limit ($CI_{2.5}$(T)), respectively. Finally, absolute distances in mm between the CIs of C and T for each minute were summed up per test concentration as a measure of the total effect. Subsequently, a concentration response curve was fitted to the total effect and, as a measure of sensitivity, an EC50 was calculated for each compound (**Equation B3.3**). Because multiphasic concentration response patterns were observed for many of the examined compounds, modelling merely incorporated data up to the maximum observed total effect but not beyond to account for mode of action-unrelated secondary effects. Additionally, to determine the relative contribution of hypo- and hyperactive behavior to the total effect, the respective distances between CIs considered for concentration response modelling were summed up. This approach was performed for every compound and every measurement time point separately. Statistically significant differences between the measurement time points were not addressed here.

Concentration-response modelling. Concentration-response modelling of behavioral effect and lethality data was performed using a 4-parameter logistic function

$$y = E_0 + \frac{E_{max} * x^h}{EC_{50}{}^h + x^h} \qquad \textbf{Equation B3.3}$$

with *y* being the modeled effect, E_0 and E_{max} are the minimum and maximum effect set at 0 and the corresponding maximum observed effect per compound, respectively. I.e. 0 to 100% lethality and 0 to the maximum difference in distance moved between treatment and control in mm, respectively. *x* refers to exposure concentration, EC_{50} is the concentration causing a half maximum effect, and *h* (Hill factor) represents the slope of the tangent in the inflection point of the model.

Multidimensional scaling. A distance matrix was calculated (KNIME Distance Matrix Calculate node) using the median distance moved per minute for each treatment and control group. Euclidean distances were used for ordination in two-dimensional space in which the relative distance between single samples reflects their similarity in terms of behavior. Hence, samples located close to each other show a high degree of similarity.

Cluster analysis. Due to the circumstance that overall 179 concentrations were tested and in order to visualize clusters more clearly, a maximum of four test concentrations per compound was included in cluster analysis. Therefore, three categories were formed based on the modeled mortality after 22.5 h of exposure (96-118.5 hpf): category 1: mortality < 5%, category 2: 5% ≤ mortality ≤ 50%, and category 3: 50% < mortality < 100%. Since one dead individual out of 16 used per treatment yields 6% mortality, category 1 describes behavioral effects in the absence of mortality. For each category the concentrations causing the most pronounced and significant (see ‘Analysis of locomotor activity data’ in this section (S1))

effect across all three time points (1.5, 6.0, 22.5 h of exposure) were chosen for cluster analysis (largest sum of distances spent in hypo- and hyperactivity, respectively, for category 1; largest overall sum of distances spent in hypo- and hyperactivity for category 2 and 3, respectively). Consequently, each of the time points of the investigation are comparable since the selection of test concentrations for each of them is the same. For the selected concentrations the differences between the median distance moved per minute of treatment and control were calculated and used for hierarchical clustering. The applied distance and cluster functions were ‘euclidean’ and ‘complete’, respectively.

Supporting table B3.1. Tested concentrations in µM and their identification number.

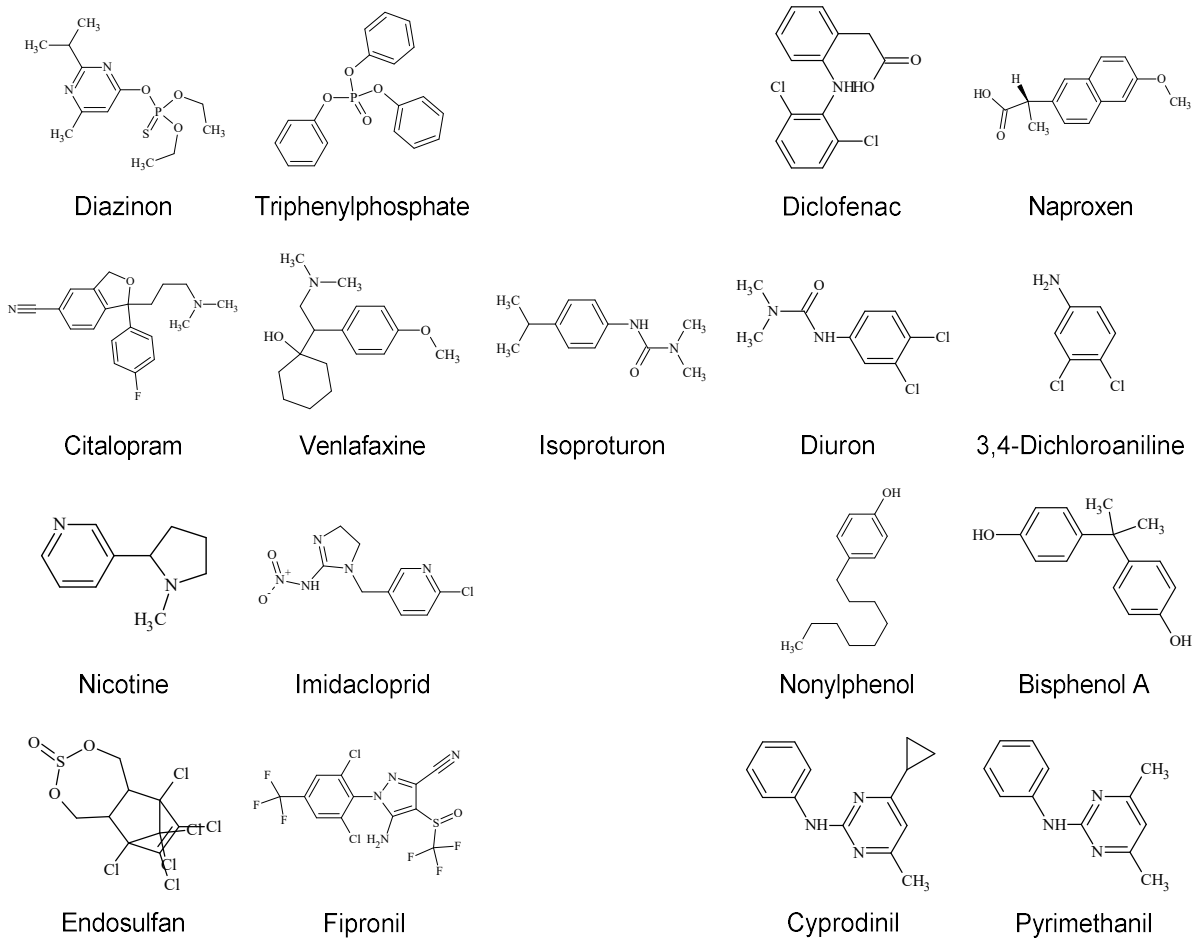
Concentration no.	Diazinon	Triphenylphosphate	Citalopram	Venlafaxine	Nicotine	Imidacloprid	Endosulfan	Fipronil	Methanol	Diclofenac	Naproxen	Isoproturon	Diuron	3,4-Dichloroaniline	Nonylphenol	Bisphenol A	Cyprodinil	Pyrimethanil
c1	2.7	0.9	0.1	0.007	10.1	9.3	0.003	0.2	33738.8	0.6	105.6	82.6	0.9	8.5	0.7	6.3	0.8	53.8
c2	3.6	1.3	0.2	0.014	15.1	18.6	0.006	0.4	67477.7	1.2	143.6	104.9	2.3	13.9	1.5	9.0	1.2	68.8
c3	5.4	1.8	0.3	0.028	20.2	37.3	0.012	0.5	89979.3	2.6	195.3	133.2	5.6	22.8	3.6	12.9	2.0	88.1
c4	7.2	2.7	0.7	0.055	30.3	74.6	0.025	0.7	134955.4	5.5	265.7	169.2	13.8	37.4	8.2	18.5	3.1	112.8
c5	10.8	3.6	1.3	0.110	40.4	149.1	0.049	1.1	179949.5	11.7	361.3	214.9	33.9	61.3	11.1	26.4	5.0	144.4
c6	14.4	5.4	2.7	0.220	60.6	298.2	0.098	1.4	269910.8	25.0	491.4	271.9	82.9	100.6	14.5	37.8	8.0	184.8
c7	21.6	7.2	5.4	0.440	80.8	596.5	0.197	2.2	359881.0	53.2	668.2	340.3	152.7	165.0	19.0	54.0	12.8	236.5
c8	28.9	10.8	10.8	0.881	121.1	1193.0	0.393	2.9	539821.5	113.4	908.8	-	-	270.5	25.0	77.2	20.5	302.7
c9	43.3	14.4	21.6	1.761	161.5	2385.0	0.786	4.3	719762.3	241.5	1236.0	-	-	443.7	28.8	110.4	32.8	387.5
c10	57.7	21.6	43.1	3.522	242.2	-	-	8.6	1079643.0	-	-	-	-	-	-	-	-	-
c11	86.6	-	86.2	7.044	-	-	-	-	1439524.0	-	-	-	-	-	-	-	-	-
c12	115.4	-	172.5	14.089	-	-	-	-	2879048.1	-	-	-	-	-	-	-	-	-
c13	-	-	345.0	28.178	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c14	-	-	-	56.356	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c15	-	-	-	112.712	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c16	-	-	-	225.424	-	-	-	-	-	-	-	-	-	-	-	-	-	-
c17	-	-	-	450.848	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Each compound was tested down to a concentration where no obvious difference to the control movement pattern was visible anymore (manual inspection of raw data plots), and up to a level where ideally, at least at the last time point of assessment (118.5 hpf), ≤100% mortality were observed in order to allow for concentration-response modelling. Therefore, the R package drc (version 2.3-96,) implemented in KNIME Analytics Platform (version 3.2.1, August 19, 2016, KNIME GmbH, Konstanz, Germany) was used, applying a 4-parameter logistic function (**Equation B3.3**).

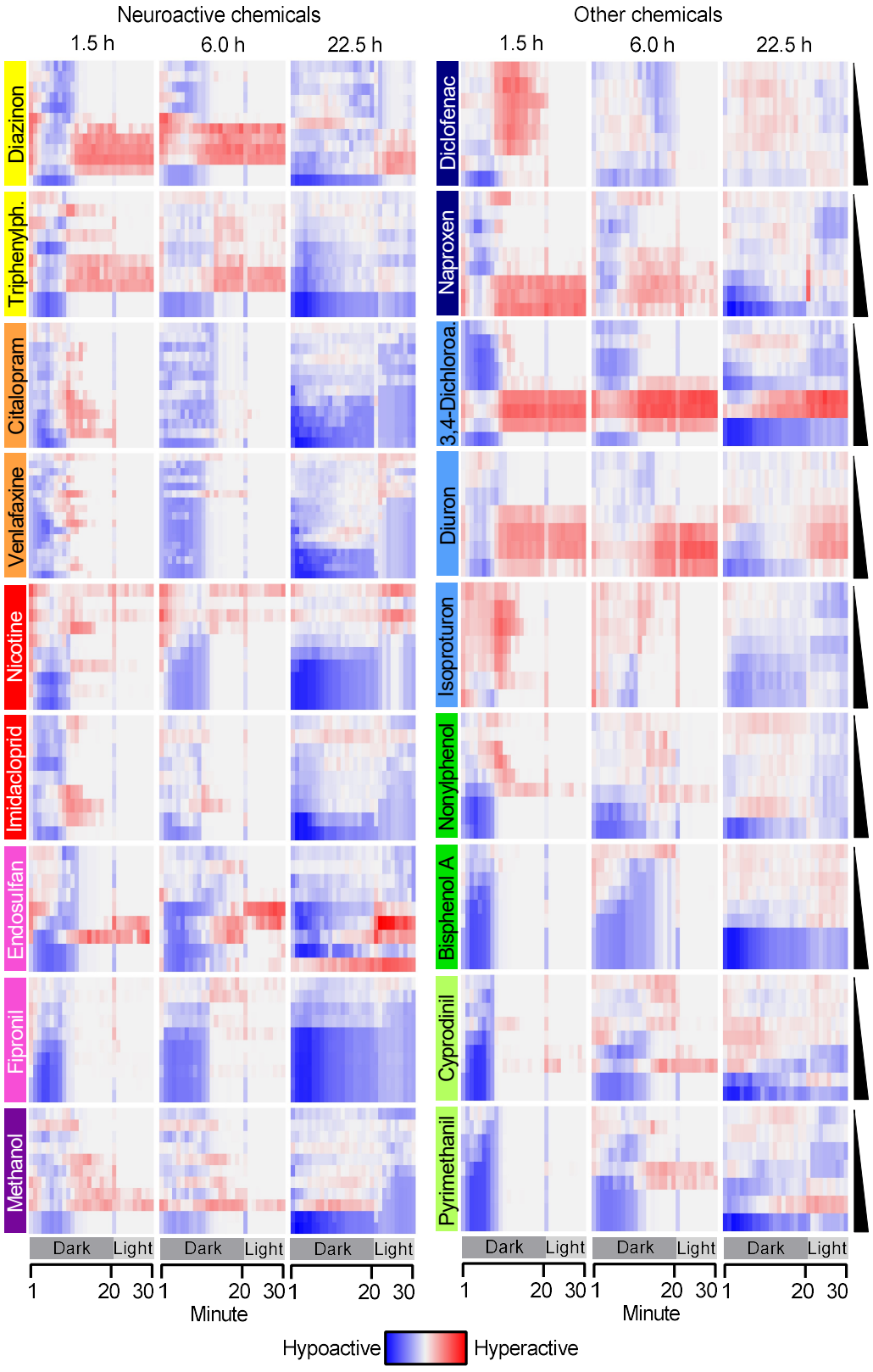
Supporting table B3.2. Toxicity data used for comparative analysis of acute fish embryo toxicity and LMR data.

Compound	LC50 (µM)					EC50 (µM)			Ratio LC50/EC50
	0-96 hpf	0-120 hpf	96-97.5 hpf	96-102 hpf	96-118.5 hpf	96-97.5 hpf	96-102 hpf	96-118.5 hpf	96-118.5 hpf
Diazinon	21.7	18.2	188.5	110.4	80.8	31.2	28.8	39.7	2.0
Triphenylphosphate	5.6	5.7	no effect	531.3	15.4	6.4	6.7	11.2	1.4
Citalopram	674.4	476.8	no effect	no effect	215.0	85.4	182.6	16.9	12.7
Venlafaxine	961.4[a]	>Sw	no effect	no effect	no effect	1.8	2.6	103.5	9.3
Nicotine	3624.4	4143.9	no effect	372.5	330.6	121.3	100.1	70.3	3.5
Imidacloprid	2386.0[a]	>Sw	no effect	no effect	4360.6	355.7	1379.9	1262.6	4.7
Endosulfan	0.79[a]	>Sw	no effect	no effect	1.2	0.24	0.03	0.03	40.0
Fipronil	8.6[a]	>Sw	no effect	no effect	5.6	2.2	1.2	0.9	6.2
Methanol	0.6×10^6	0.6×10^6	2.0×10^6	1.8×10^6	1.5×10^6	0.6×10^6	0.9×10^6	1.0×10^6	1.5
Diclofenac*	8.4	7.4	165.5	77.4	77.4	0.7	36.5	no effect	-
Naproxen	344.4	301.4	no effect	1398.3	832.0	675.9	474.1	576.6	1.4
3,4-Dichloroaniline	9.6	18.5	454.5	399.3	333.5	81.1	76.2	105.2	3.2
Diuron	12.9	11.0	no effect	no effect	194.4	16.0	29.5	28.1	6.9
Isoproturon	23.4	35.2	no effect	no effect	no effect	104.2	281.8	198.0	-
Nonylphenol	4.1	3.5	19.2	17.7	12.4	14.3	12.5	10.2	1.2
Bisphenol A	52.0	45.5	no effect	no effect	117.3	29.0	29.5	38.7	3.0
Cyprodinil	2.9	2.5	no effect	no effect	20.8	10.7	22.0	14.1	1.5
Pyrimethanil	18.9	19.0	no effect	no effect	212.8	182.7	211.9	127.6	1.7

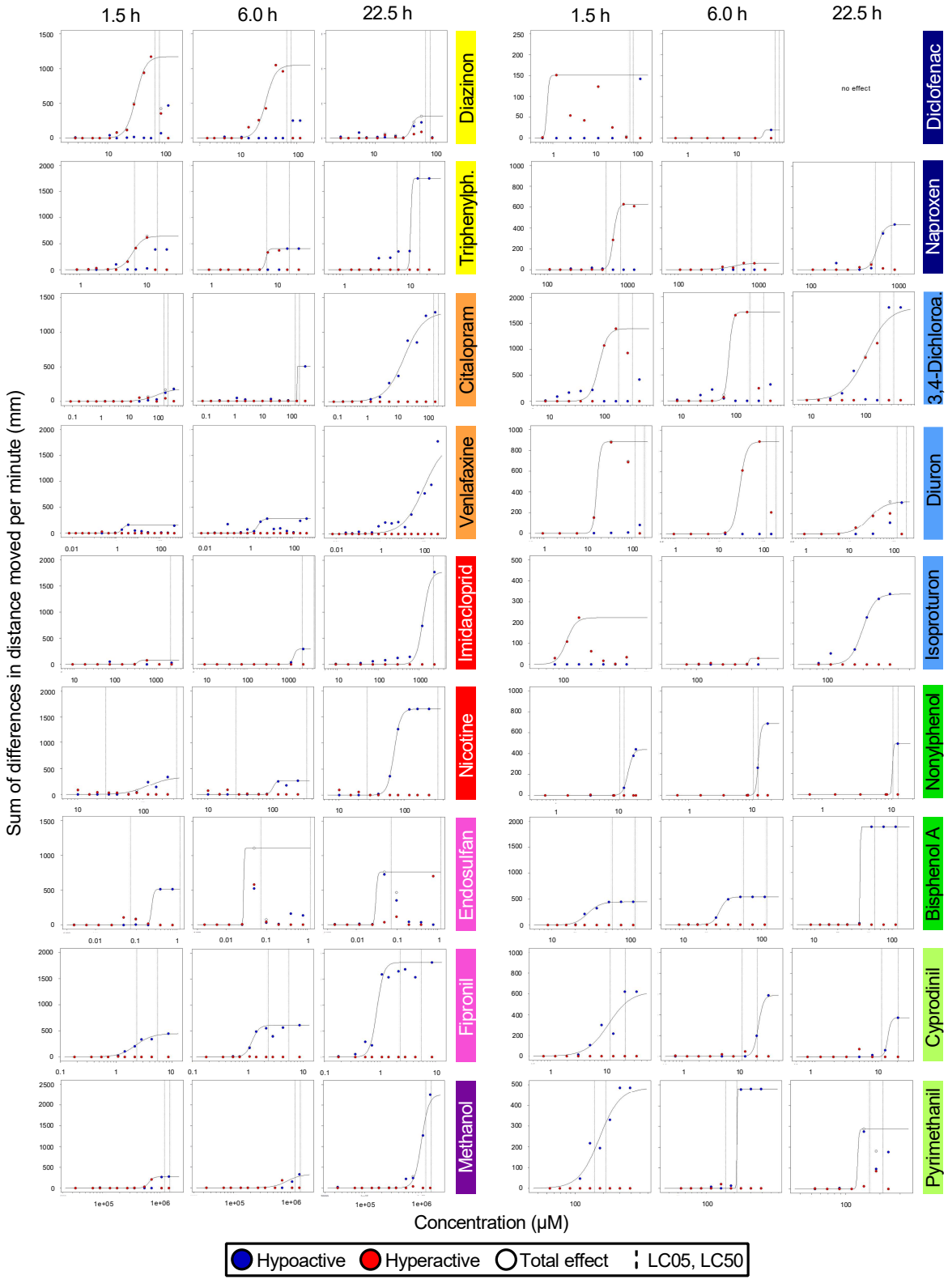
Sw = Limit of water solubility, a = Maximum water solubility used because no mortality was observed. *Effect concentrations for diclofenac were reduced by 10% in **Figure 3.6** of **Chapter 3** to avoid overlap of data points.



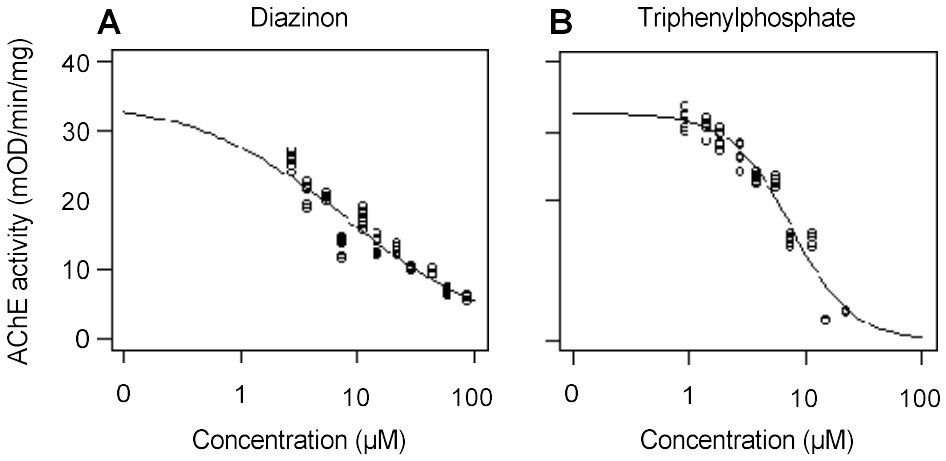
Supporting figure B3.1. Chemical structures of the tested compounds.



Supporting figure B3.2. Overview on behavioral profiles of the 18 investigated compounds recorded after 1.5, 6.0 and 22.5 h of exposure. Profiles were calculated from the difference in median distance moved per minute of treatment and control. Increasing concentrations are indicated by black triangles on the right hand side. Compounds with an anticipated same mode of action are indicated by uniform color.



Supporting figure B3.3. Concentration-response curves for alterations in locomotor activity after 1.5, 6.0 and 22.5 h of exposure (96-118.5 hpf). The maximum observed total effect (sum of distances spent hypo- and hyperactive) was used as the maximum of each model. Blue and red data points, significant differences in the distance moved per minute between treatment and control; broken vertical lines, LC05 and LC50 (22.5 h of treatment).



Supporting figure B3.4. Acetylcholinesterase (AChE) activity in 120 hpf zebrafish larvae after 24 h of exposure with (A) diazinon and (B) triphenylphosphate, respectively. AChE activity was determined photometrically and was normalized to the total protein content of each sample (*n*=4 with ≤8 larvae) as previously described (Küster 2005). Enzyme activity in controls was 34.4±3.5 and 32.5±1.8 mOD/min/mg (mean±SD) for diazinon and triphenylphosphate, respectively.

References

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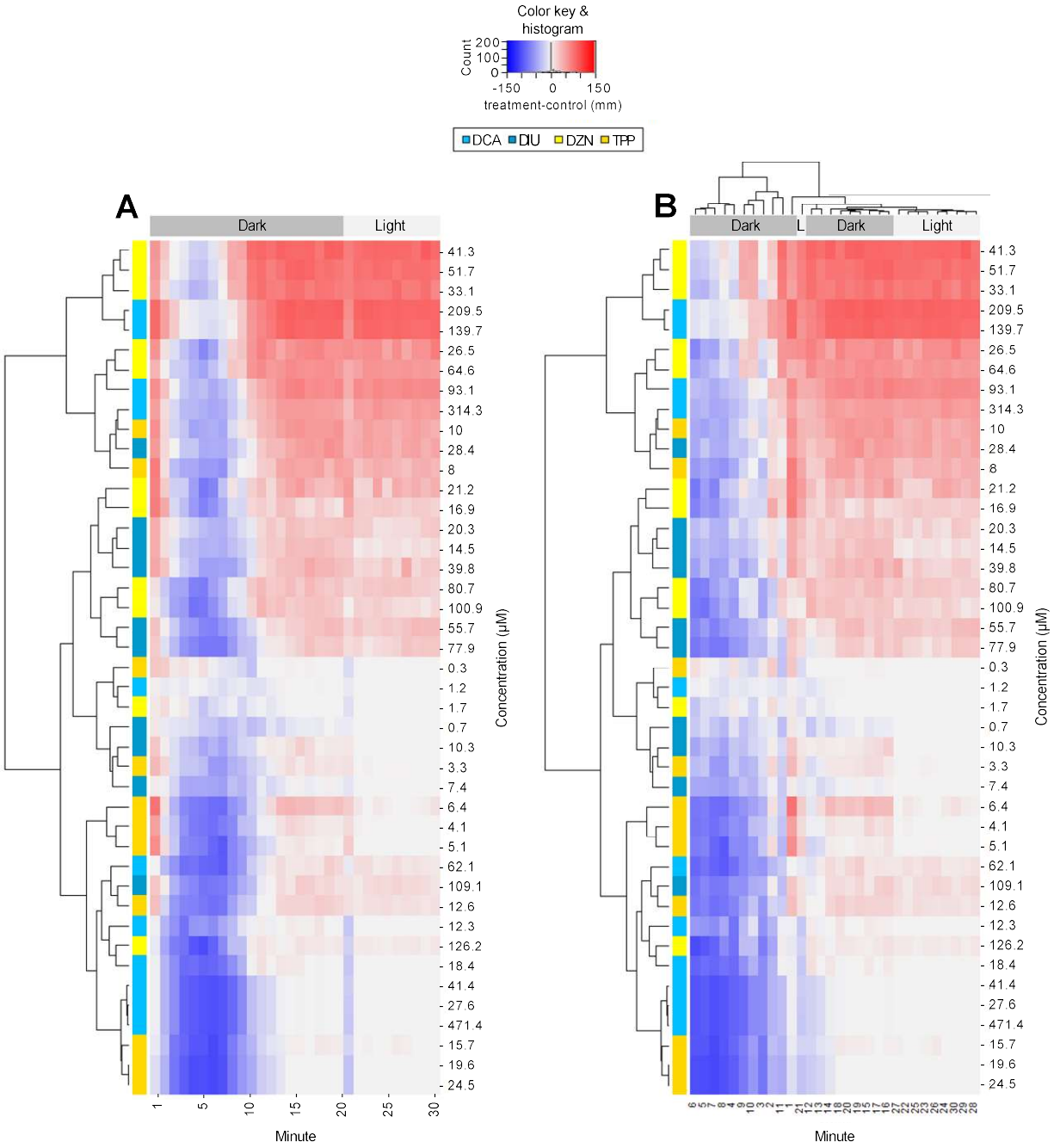
Appendix C

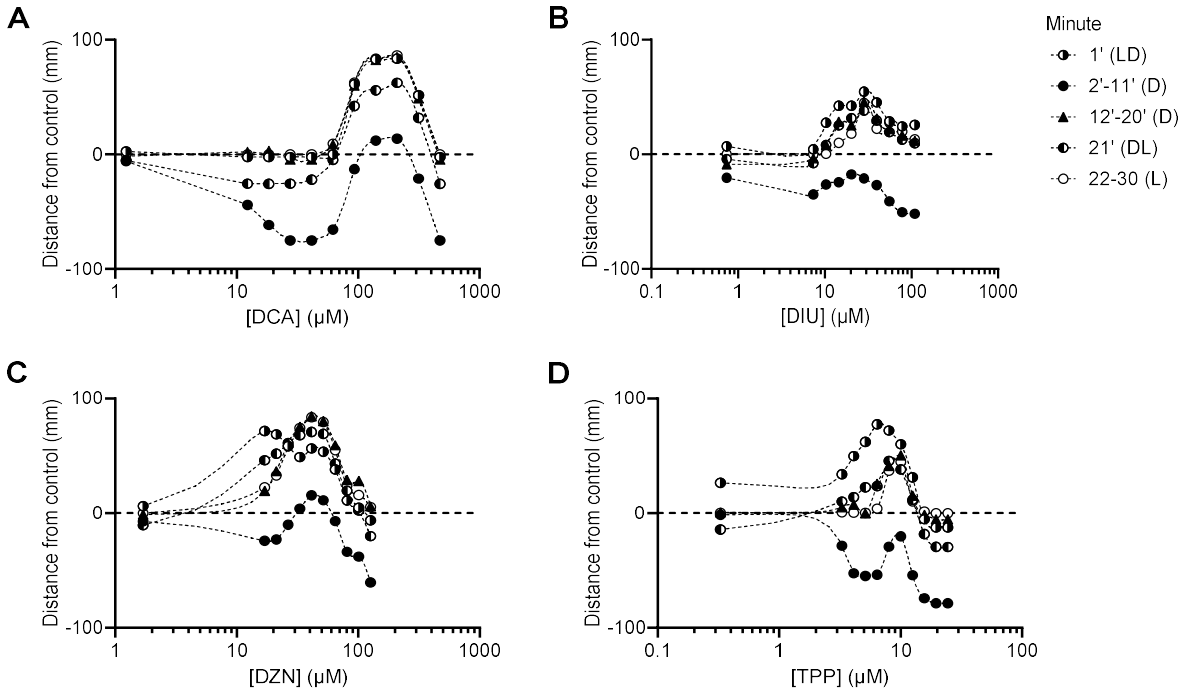
Supporting information – Chapter 4

C4.1 Materials and methods

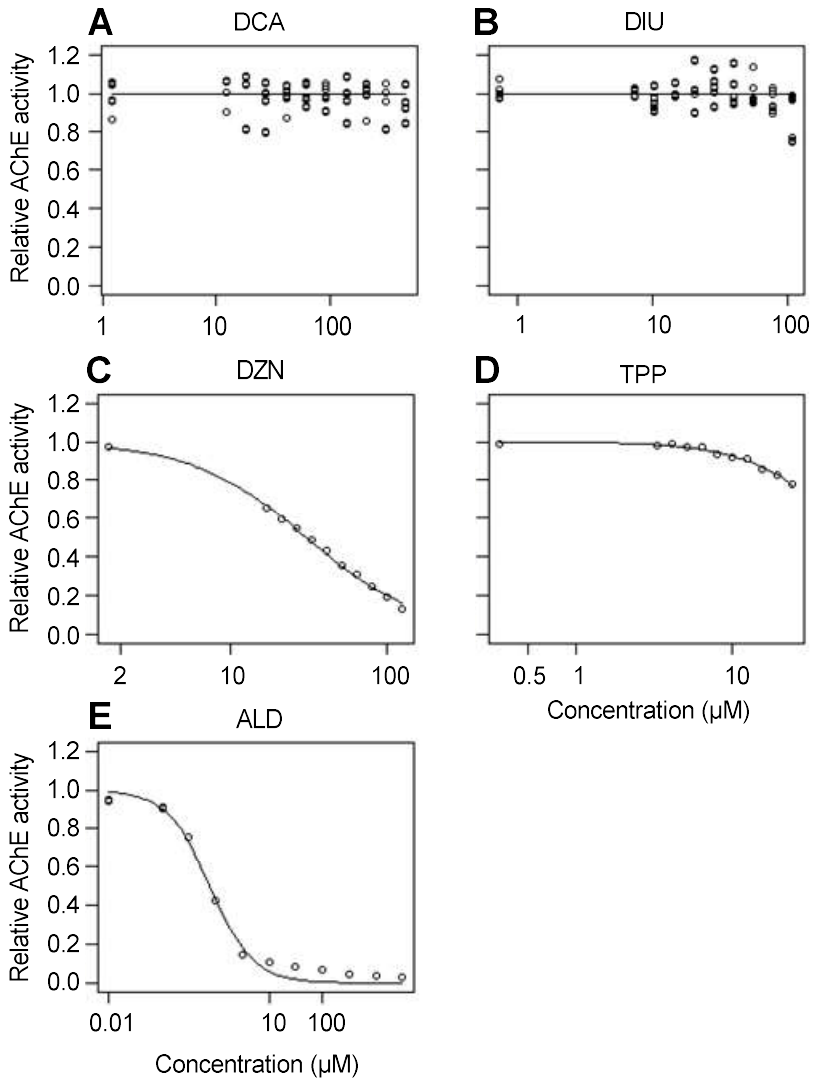
Quantification of acetylcholinesterase activity. Eight individuals were pooled per condition in quadruplicates and were transferred to FastPrep© tubes. The supernatant was removed and larvae were rinsed for ~10 s with 2 mL ultra-pure water. Immediately, larvae were snap-frozen in liquid nitrogen and were subsequently stored at -80 °C. Storage did not exceed four weeks. For protein isolation, samples were thawed on ice and were homogenized in 200 µL ice-cold phosphate buffer (pH 7.5, 0.1 M $NaH_2PO_4 \times H_2O$; 0.1% v/v Triton X-100, Calbiochem, Darmstadt, Germany, molecular biology grade, CAS RN 9002-93-1) using a high-speed benchtop homogenizer (FastPrep®-24, MP Biomedicals, 6.0 U/s, 30 s). Cell disruption was accomplished by multidirectional, simultaneous beating of previously added glass beads (1 mm diameter). The homogenate was then centrifuged (10 min, 13000 rpm, 4 °C). The protein-containing supernatant was used for protein measurement and colorimetric determination of acetylcholinesterase activity. The latter was performed according to the method of Ellman (Ellman et al. 1961). Therefore, microtiter wells of a 96-well polystyrene plate were loaded with 45 µL sample in triplicates. Wells were filled up with 50 µL phosphate buffer, 100 µL 5,5'-dithio-bis-2-nitrobenzoate (1 mM; Sigma, ≥98%, CAS RN 69-78-3) and 100 µL acetylthiocholine iodide (1.35 mM; MP Biomedicals, Illkirch, France, CAS RN 1866-15-5). For blank value determination, eight wells were equipped in the same way despite substitution of the sample volume by 45 µL phosphate buffer. Immediately, the reaction mixture was positioned within a plate reader (SpectraMax 250 Photometer, Molecular Devices, Sunnyvale, CA, USA). The change in optical density per minute was measured at 412 nm and data were recorded every 14 s and repeated intermediate mixing for a total duration of 5 min. The total protein amount was measured as follows: 5 µL sample were pipetted into a 96-well plate in triplicate. As blank, the same volume of phosphate buffer was applied eight-fold. A calibration function was derived from a 1:2-dilution series (ranging from 10.0 to 0.6 mg/L) of albumin bovine fraction v (Roth, Karlsruhe, Germany, ≥98%, CAS RN 9048-46-8) dissolved in phosphate buffer. Each dilution step was applied to the well plate in triplicates. A modified, commercial Lowry assay (Lowry et al. 1951), the DC protein assay (BioRad, Munich, Germany), was used. 5 mL of alkaline copper tartrate solution (reagent A) and 100 µL surfactant solution (reagent S) were mixed and 25 µL of this mixture was added to each well. Additionally, 200 µL dilute Folin reagent (reagent B) were added. The reaction batch was mixed using the auto mixing function of the plate reader and was incubated for 20 min at room temperature. Subsequently, the optical density at 750 nm was measured.



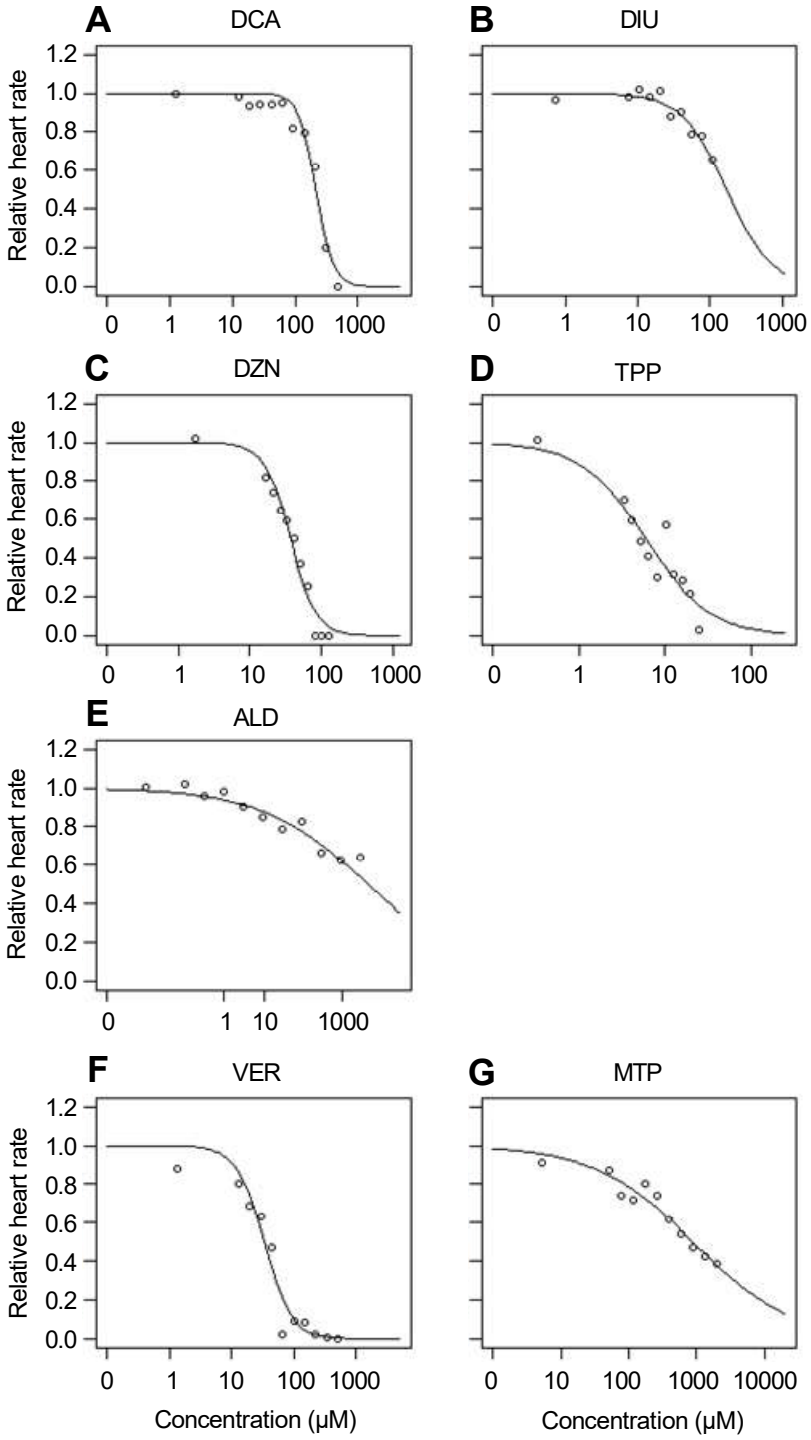
Supporting figure C4.1. Hierarchical clustering reveals functional similarities between chemicals. (**A**) Heatmap of locomotor activity profiles over time clustered by phenotypic similarity (y axis). (**B**) Clustering performed on single time increments (x axis) reveals redundancy of certain minutes. DCA, 3,4-dichloroaniline; DIU, diuron; DZN, diazinon; TPP, triphenylphosphate.



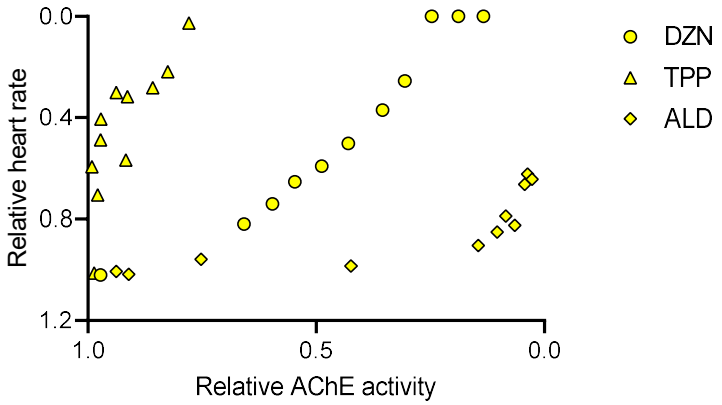
Supporting figure C4.2. Chemicals cause specific, multiphasic patterns of locomotor activity during distinct phases of photoperiods. Graphs show concentration dependent deviation from median control activity (zero line) for (**A**) 3,4-dichloroaniline, (**B**) diuron, (**C**) diazinon, and (**D**) triphenylphosphate. Aggregation of time increments according to **Supporting figure C4.1B**. Dashed lines, spline curves; L, light; D, dark; LD, light-dark transition; DL, dark-light transition.



Supporting figure C4.3. Environmental chemicals differ in their potential to decrease acetylcholinesterase (AChE) activity. Relative specific acetylcholinesterase activity (y axis) as a function of concentration (x axis). (**A**-**E**) DCA, 3,4-dichloroaniline; DIU, diuron; DZN, diazinon; TPP, triphenylphosphate; ALD, aldicarb. Data normalized to mean control activity. Concentration-response models were fit to mean.



Supporting Figure C4.4. Chemicals of different functional classes cause bradycardia in 4 dpf zebrafish. Reduction in heart rate (y axis) as a function of concentration (x axis). (**A**-**G**) DCA, 3,4-dichloroaniline; DIU, diuron; DZN, diazinon; TPP, triphenylphosphate; ALD, aldicarb; VER, verapamil; MTP, metoprolol. Data normalized to control. Concentration-response curves were fit to mean.



Supporting figure C4.5. Variability among chemicals reveals that bradycardia is not directly correlated with reduction in AChE activity. Heart rate and AChE activity are relative to control. DZN, diazinon; TPP, triphenylphosphate; ALD, aldicarb.

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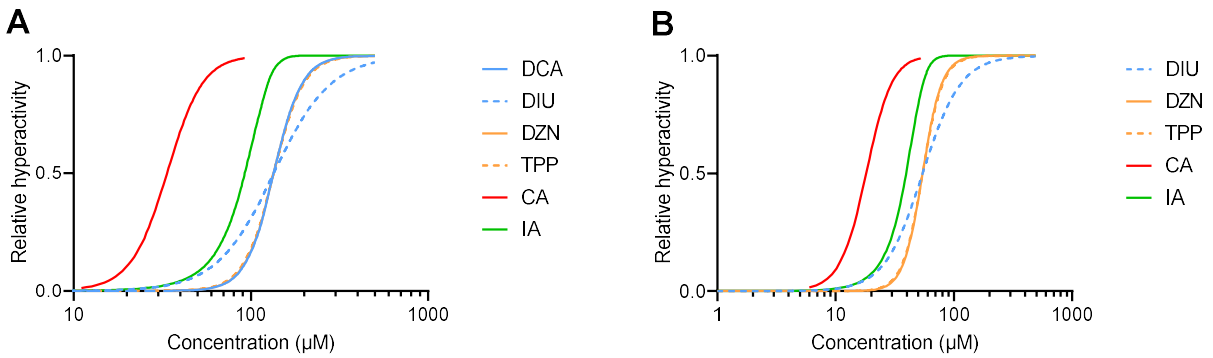
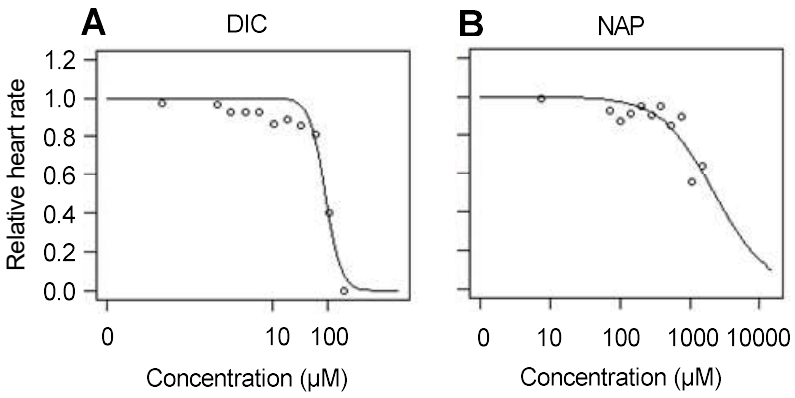
Appendix D

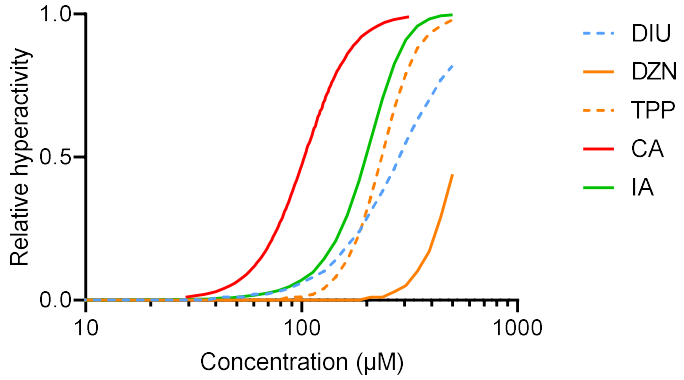
Supporting information – Chapter 5



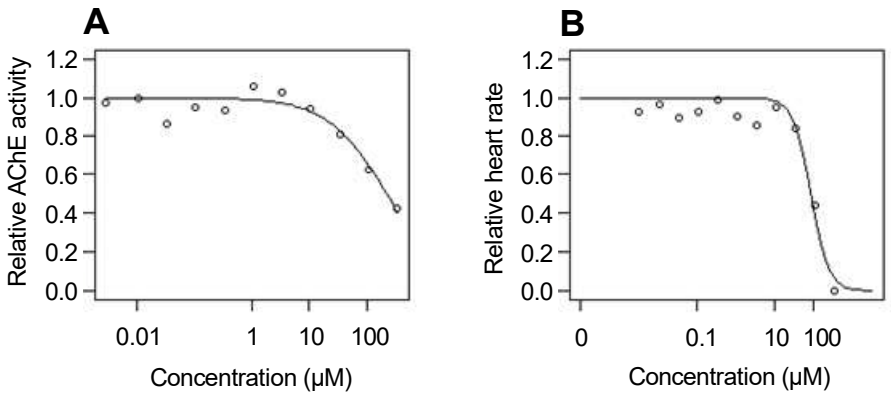
Supporting figure D5.1. Equitoxic design of two chemical mixtures and related predictions based on concentration addition (CA) and independent action (IA). (**A**) Quartary mixture (MX4). (**B**) Tertiary mixture (MX3). Mind that curves of DCA, DZN, and TPP overlap. DCA, 3,4-dichloroaniline; DIU, diuron; DZN, diazinon; TPP, triphenylphosphate.



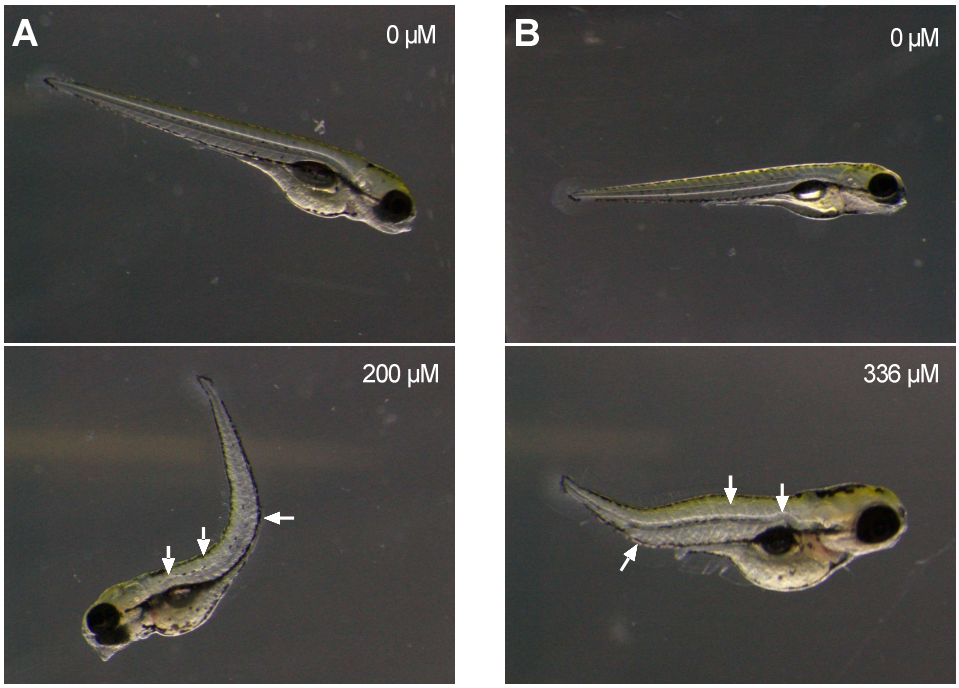
Supporting Figure D5.2. Diclofenac (DIC) and naproxen (NAP) only cause bradycardia with high concentrations. (**A**) DIC decreases heart rate with concentrations ≥111.1 µM. (**B**) NAP decreases heart rate with concentrations ≥1.1 mM. Heart rate is relative to control mean. *n*=8 individuals per condition.



Supporting figure D5.3. Prediction of combined effects of diuron (DIU), diazinon (DZN), and triphenylphosphate (TPP) on hyperlocomotion in a quinary mixture additionally including diclofenac and naproxen. Predictions based on normalized locomotor activity in minute 20 of locomotor response test. Concentration represents total molarity of the five-component mixture. CA, concentration addition; IA, independent action.



Supporting figure D5.4. Molecular and physiological alterations observed with a quinary mixture of diclofenac, diuron, diazinon, naproxen, and triphenylphosphate. (**A**) Inhibition of acetylcholinesterase (AChE). (**B**) Decrease in heart rate. Data is relative to control mean. *n*=8 individuals per condition.



Supporting figure D5.5. A quinary mixture of diclofenac, diuron, diazinon, naproxen, and triphenylphosphate causes morphological abnormalities similar to those observed with diclofenac alone. Morphological defects observed with exposure to (**A**) 200 µM diclofenac and (**B**) 336 µM of the quinary mixture. Top, control; bottom, treatment. Arrowheads mark altered somite structure as well as deformations of notochord and body axis.

Acknowledgement

I want to express my gratitude to my supervisors Prof. Dr. Rolf Altenburger and Dr. Wibke Busch. Without your guidance and persistent help this dissertation would not have been possible. Thanks for all of your advice, support, inspiration and motivation throughout the past three years – beginning from ideas for a scholarship proposal through to innumerable discussions and comments that have shaped this work. Special thanks also for being extraordinarily tolerant, although, once again, it took longer than expected. Thanks for your long lasting support and you always believed in me. In addition, Prof. Dr. Henner Hollert is acknowledged for the generous support provided by the university.

Thanks to all of the members of the Department Bioanalytical Ecotoxicology for providing such an inspiring environment. Advice and comments given by Dr. Nils Klüver, Dr. Stefan Scholz, and Dr. Till Luckenbach have been a great help in the development of this thesis. I owe a great thanks to the whole laboratory team. I am particularly grateful for the assistance given by Janet Krüger. Probably no one else would have been willing to expose zebrafish at six in the morning three times a week. Special thanks also to Nicole Schweiger, Gianina Jakobs, Joanke van Dijk, Jelena Fix, and Jona Schulze for their support in the conduction of numerous experiments. I owe my deepest gratitude to Dr. Andreas Schüttler and Stefan Lips. I appreciate your support in the struggle with computational data analyses and data visualization which saved me a lot of time and nerves. I would like to extent my thanks and appreciation to our iTox team including Wibke Busch, Rolf Altenburger, Janet Krüger, Andreas Schüttler, Gianina Jakobs, and Stefan Krämer. I appreciate our illuminating discussions. Furthermore, I want to thank Dr. Tamara Tal and Alice Teipelke for proof reading and translating parts of the manuscript.

I would also like to express my gratitude to the “Deutsche Bundesstiftung Umwelt” for their financial support. I am particularly grateful to Dr. Volker Wachendörfer and Verena Exner. I have greatly benefited from the inspiring scholarship holders’ meetings all over Germany that allowed a view beyond my scientific horizon. Thanks to Sabine Dannhauer for the always open and friendly communication.

Last but not least, my heartfelt appreciation goes to my family and friends for all the motivation and warmly welcome distractions during my time as a PhD student. Special thanks goes to Julia for your endless patience and the numerous sacrifices you made in the course of the last years. Special thanks also to my daughter Nela who has given me a lot of strength to finish this thesis. This work is dedicated to my beloved grandpa. I am sure you take care of us from the heaven above.

Erklärung

Die vorliegende Dissertation wurde am Helmholtz-Zentrum für Umweltforschung – UFZ im Department Bioanalytische Ökotoxikologie in Zusammenarbeit mit dem Institut für Umweltforschung (Biologie V) der RWTH Aachen University unter Betreuung von Herrn Prof. Dr. Rolf Altenburger angefertigt.

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe. Alle Textauszüge und Grafiken, die sinngemäß oder wörtlich aus veröffentlichten Schriften entnommen wurden, sind durch Referenzen gekennzeichnet.

Leipzig, den 16. Januar 2020

David Leuthold

Contributions to manuscripts and chapters

This thesis was funded by the Deutsche Bundesstiftung Umwelt (DBU). The experimental work was conducted at the Helmholtz-Centre for Environmental Research – UFZ (Leipzig) with contributions of members of the Department Bioanalytical Ecotoxicology. A detailed explanation of my contributions to each of the chapters of my dissertation is given in the following.

Chapter 1

Conception of this chapter was done by DL and WB. DL wrote the chapter. WB revised the chapter.

Chapter 2

DL, WB, NK and RA designed the study. DL performed the fish embryo cultivation, preparation of chemical stock solutions, chemical exposure and locomotor response assays including subsequent mortality and morphology assessments. Fish embryo mortality data was generated by members of the Department Bioanalytical Ecotoxicology (UFZ, Leipzig) in the framework of the EU project SOLUTIONS. DL conducted the concentration-response analysis on the mortality raw data to derive lethal effect concentrations applied in the behavior tests. Data were analyzed and visualized by DL. DL wrote the chapter. WB and RA revised the chapter.

Chapter 3

This chapter is published as scientific article in Environmental Science and Technology. DL, WB and NK designed the study. As in Chapter 2, DL performed all experiments except for fish embryo acute toxicity tests which were previously conducted at the UFZ within the SOLUTIONS project. Additional fish embryo acute toxicity tests were conducted by JK under DL's guidance, i.e., DL selected the chemicals and respective concentration ranges to be tested. NS analyzed samples provided by DL for acetylcholinesterase activity. AS visualized data for the comparison of sensitivity of behavioral responses and acute lethality (Figure 3.5). DL analyzed and merged the data for the manuscript. DL wrote the manuscript. All authors read, revised and approved the final manuscript.

Chapter 4

DL and WB designed the study. DL planned and carried out all experiments as well as computational data analyses and statistics. DL wrote the chapter. WB revised the chapter.

Chapter 5

DL and WB designed the study. DL carried out all experiments as well as computational data analyses and statistics. Mixture effect predictions were made by DL using an Excel template provided by MS. DL wrote the chapter. WB revised the chapter.

Chapter 6

Conception of this chapter was done by DL and WB. DL wrote the chapter. WB revised the chapter.

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Legradi JB, Di Paolo C, Kraak MHS, van der Geest HG, Schymanski EL, Williams AJ, Dingemans MML, Massei R, Brack W, Cousin X, Begout M-L, van der Oost R, Carion A, Suarez-Ulloa V, Silvestre F, Escher BI, Engwall M, Nilén G, Keiter SH, Pollet D, Waldmann P, Kienle C, Werner I, Haigis A-C, Knapen D, Vergauwen L, Spehr M, Schulz W, Busch W, **Leuthold D**, Scholz S, vom Berg CM, Basu N, Murphy CA, Lampert A, Kuckelkorn J, Grummt T, Hollert H. 2018. An ecotoxicological view on neurotoxicity assessment. Environ Sci Eur 30(1):46.

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* Publication is part of this PhD thesis.

Conference contributions

Leuthold D, Krüger J, Altenburger R, Busch W. 2016. The Zebrafish Embryo Toxicity Test as Effect-Based Tool – Validation for Testing Environmental Samples (poster). Fish and amphibian embryos as alternative models in toxicology and teratology. Paris, France.

Leuthold D, Scholz S, Altenburger R, Busch W. 2018. Behavioral Profiling of Environmental Contaminants using a Zebrafish (*Danio rerio*) Locomotor Response Assay (poster). SETAC North America Focused Topic Meeting: High-Throughput Screening. Durham, North Carolina, USA.

Leuthold D, Klüver N, Altenburger R, Busch W. 2019. Behavioral Profiling of Environmentally Relevant Chemicals in the Zebrafish Embryo (poster). SOT 58th annual meeting, Baltimore, USA.

Leuthold D, Klüver N, Altenburger R, Busch W. 2019. Can environmentally relevant neuroactive chemicals specifically be detected with the locomotor response test in zebrafish embryos? (platform presentation). SETAC Europe 29th annual meeting. Helsinki, Finland.

ISSN 1860-0387

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