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Title:

Can environmentally relevant neuroactive chemicals specifically be detected with the locomotor response test in zebrafish embryos?

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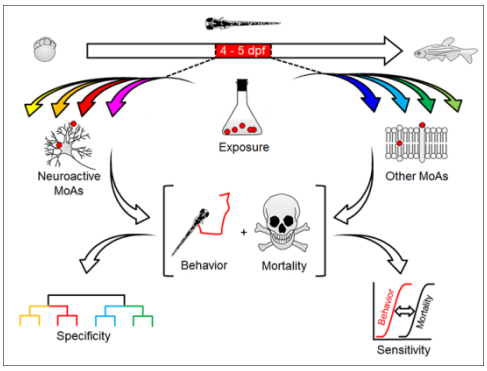
Abstract

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.

Graphical Abstract



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 [1]. Although *in silico* target prediction approaches for neurotoxicity are a desirable long term goal, their development requires large amounts of bioactivity data [2]. However, due to the limited availability of *in vitro* assays able to capture effects relevant for the nervous system [3], the detection of neuroactive chemicals and the assessment of their acute and sublethal toxicity is challenging [4]. Using *in vivo* assays such as the acute fish embryo toxicity (FET) test with zebrafish (*Danio rerio*) [5] as a potential alternative for the acute fish test with adults [6], it has been shown that certain neuroactive compounds provoke lower acute toxicity in early life stages of fish compared to adult stages [7-9]. 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 [9]. Beyond that, the diagnostic capacity of behavioral tests in embryonic and larval zebrafish has been demonstrated in neuroactive drug discovery [10, 11]. 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 [12] and it is known that minor methodological changes (e.g. age, incubation conditions, light driving, plate format) can alter its outcome [13]. 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. Furthermore, we 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.

2. Materials and methods

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 (S1).

2.2. Experimental design and determination of exposure concentrations

Exposure was initiated at 96 hours post fertilization (hpf) (up to 120 hpf) in order to avoid exposure-induced developmental abnormalities potentially affecting embryonic 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 [5] adapted to 7.5-mL glass vials using 3 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 and maximum water solubility, yielding 7 to 17 test concentrations per compound (Supporting Table S1).

2.3. Exposure and light-dark transition locomotor response test

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 embryo. Dead individuals were excluded from behavioral data analysis at the respective time point of investigation. More details are given in Supporting Information S1.

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 embryos 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 S1).

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, [14]). The latter was performed on a selected subset of data (Supporting Information S1).

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 1).

117 **Table 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

118 *MoA information were compiled from (a) Pesticide Properties Database, (b) Insecticide Resistance Action Committee database, (c) [15], (d)

119 Drugbank, (e) [16], (f) [17], (g) [18], and (h) [19] (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 S1.

To prevent developmental toxicity-induced morphological alterations, potentially affecting embryonic 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 durations that exclusively reveal the primary behavioral mechanism of a compound in the absence of morphological alterations.

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 embryos became more active (Figure 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.

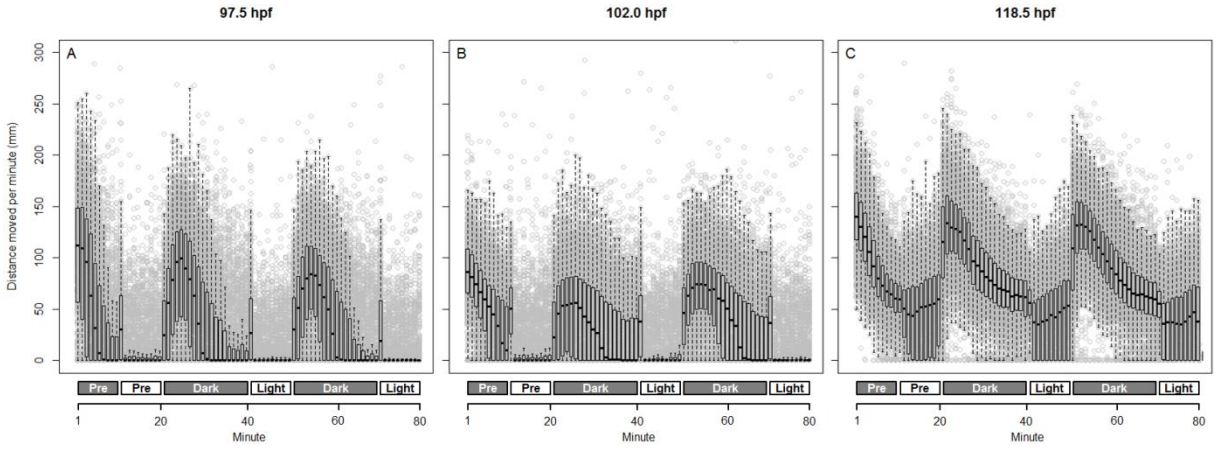


Figure 1. Locomotor activity is developmental stage-specific. Boxplots show the distance moved per minute of untreated zebrafish embryos (*n*=688) of different age: (A) 97.5, (B) 102.0 and (C) 118.5 hpf, respectively.

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 2A, B). The activity profiles changed with exposure time and concentration, e.g. from constant locomotion (Figure 2A; red line) to complete inhibition of movement (Figure 2B; red line) and vice versa (Figure 2A, B; yellow line). Normalizing the observed profiles to control levels using differences in activity between exposed embryos and untreated controls translated into a heatmap indicating hypo- (blue) and hyperactivity (red), respectively, for overall ten analyzed concentrations of 3,4-dichloroaniline (Figure 2C, D). We observed that high and low concentrations caused hypolocomotion compared to control levels whereas median concentrations caused hyperactivity in ZFEs (Figure 2C, 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 2C, 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 S2).

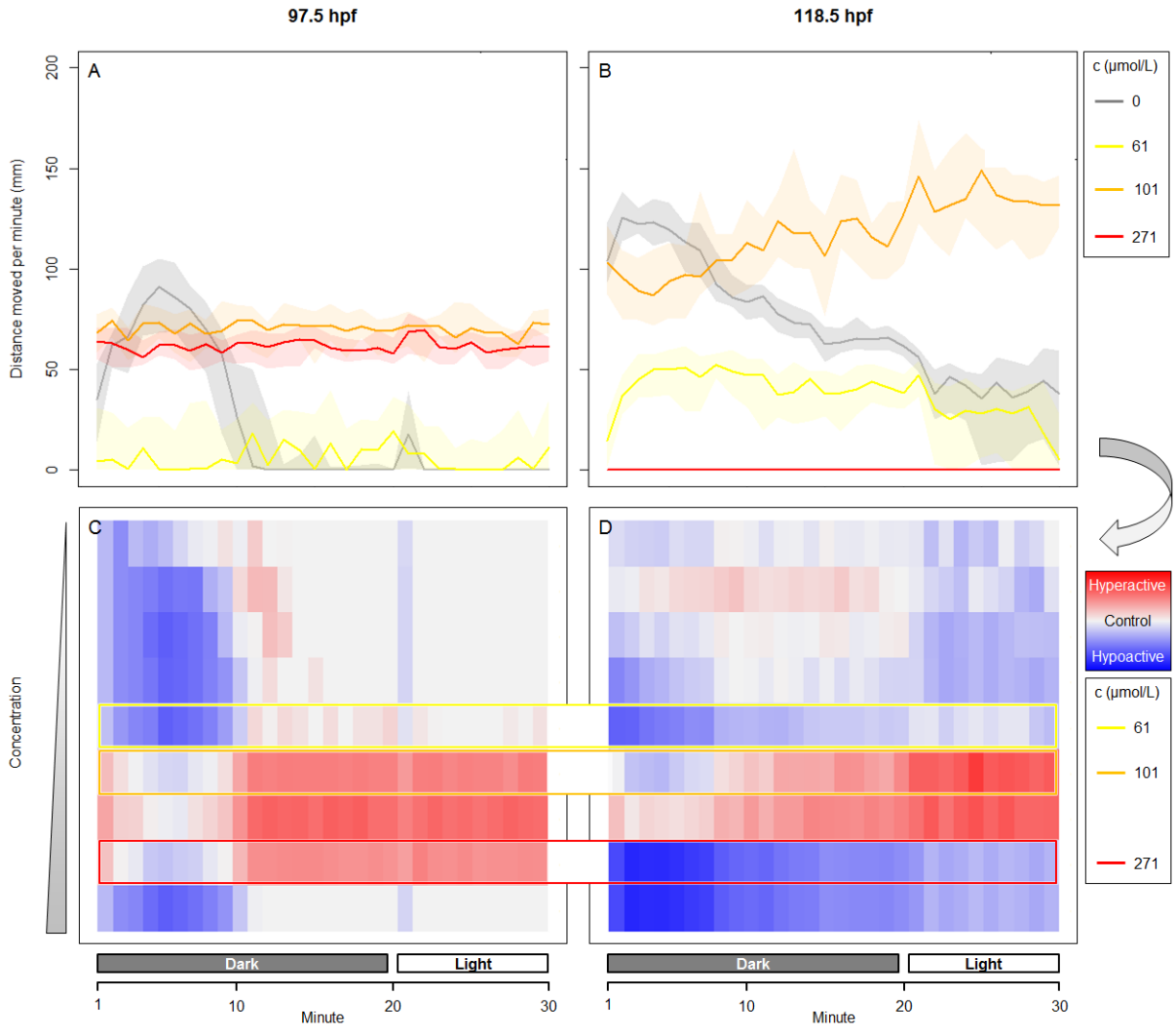


Figure 2. Behavioral profiles of 3,4-dichloroaniline are concentration and time dependent. Grey (control) and colored lines (treatment): median distance moved per minute (*n*=16 individuals per treatment) at (A) 97.5 (1.5 h of exposure) and (B) 118.5 hpf (22.5 h of exposure), respectively. Shaded areas: respective 95% CIs. (C, D) Heatmaps indicate the difference in activity between treatment and control for all tested concentrations.

3.2. 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).

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; grey shaded areas).

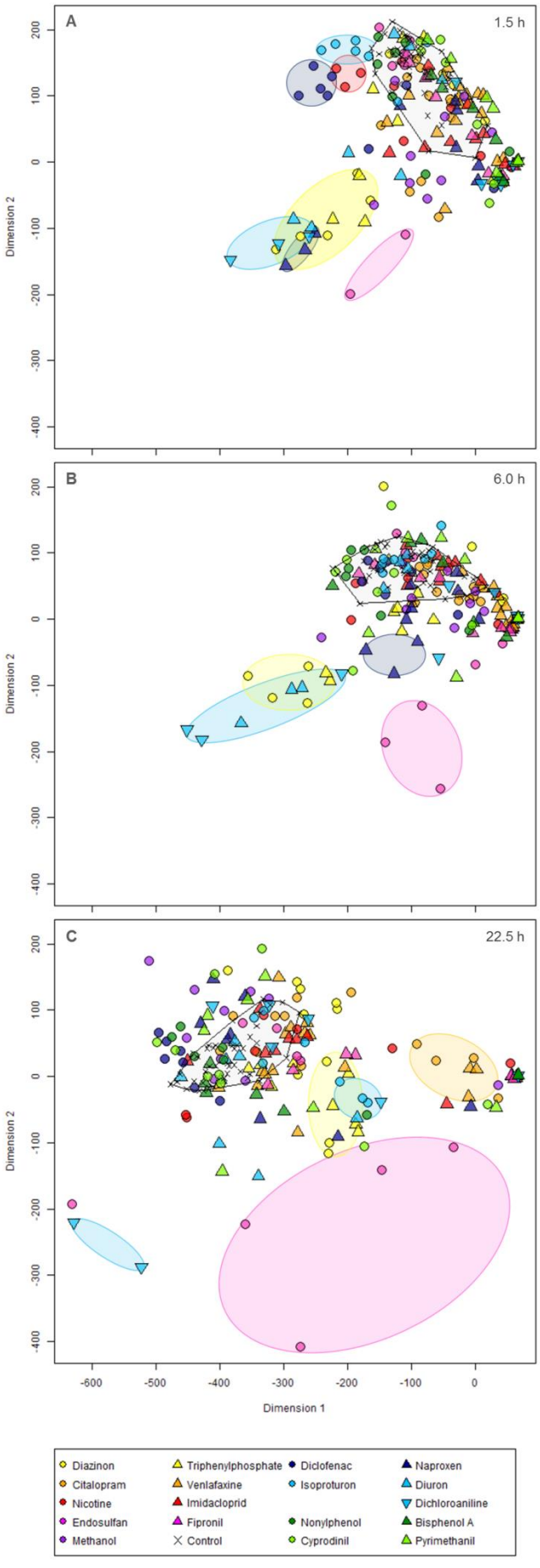
We found that behavioral alterations were not restricted to neuroactive compounds but also comprised the other investigated MoA classes. Depending on the level and duration of exposure each of the 18 tested compounds altered locomotion.

After 1.5 h of exposure (Figure 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 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 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 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 3B), we observed that diazinon (34-58 µM, yellow triangles) and triphenylphosphate (7-11 µM, yellow points) did not remarkably change their spatial

196 position indicating temporally stable behavior profiles for these concentrations, whereas
197 lower concentrations of triphenylphosphate (5 µM) and higher concentrations of diazinon
198 (87 µM) converged closer to control levels. This was also observed for naproxen (Figure 3B;
199 491-908 µM, dark blue triangles). This time dependent shift led to a separation of diuron
200 (light blue triangles) and 3,4-dichloroaniline (turned light blue triangles) forming a more
201 independent cluster (Figure 3B). However, data points of AChE and PSII inhibitors are
202 located close to each other, indicating that the underlying behavioral phenotypes were not
203 unique and specific for the respective MoAs. In contrast, a unique pattern for endosulfan was
204 observed (0.05-0.20 µM, pink points), being, however, different from the pattern of its tested
205 MoA analogue fipronil (pink triangles) (Figure 3B).

206 As mentioned above, at 118.5 hpf (Figure 3C) we observed the most pronounced shift in
207 locomotor activity by control groups as compared to previous time points of investigation
208 (also see Figure 1). Furthermore, similarities of the tested SSR/SNR inhibitors citalopram
209 (22-172 µM, orange points) and venlafaxine (56-225 µM, orange triangles) became more
210 obvious after 22.5 h of exposure (Figure 3C). They converged in a concentration-dependent
211 manner towards the MDS region described above as being characteristic for absolute
212 inactivity (Figure 3C). Additionally, exposure to endosulfan (0.05-0.79 µM, pink points) and
213 3,4-dichloroanilline (101-165 µM, turned light blue triangles) led to the most pronounced
214 alterations in behavior (large spatial distance from the control cluster) (Figure 3C). At a
215 concentration of 61 µM, 3,4-dichloroaniline (turned light blue triangle) formed a cluster with
216 its MoA analogues diuron (153 µM, light blue triangle) and isoproturon (215-340 µM, light
217 blue points) (Figure 3C). However, this cluster could not be identified as MoA-specific since
218 behavioral profiles of triphenylphosphate (7-11 µM, yellow triangles) and nonylphenol
219 (12 µM, dark green point) were located in the same area as the PSII inhibitors (Figure 3C).


A
1.5 h
B
6.0 h
C
22.5 h
Dimension 2
Dimension 1
200
100
0
-100
-200
-300
-400
-600
-500
-400
-300
-200
-100
0
Diazinon
Citalopram
Nicotine
Endosulfan
Methanol
Triphenylphosphate
Venlafaxine
Imidacloprid
Fipronil
Control
Diclofenac
Isoproturon
Nonylphenol
Cyprodinil
Naproxen
Diuron
Dichloroaniline
Bisphenol A
Pyrimethanil

220

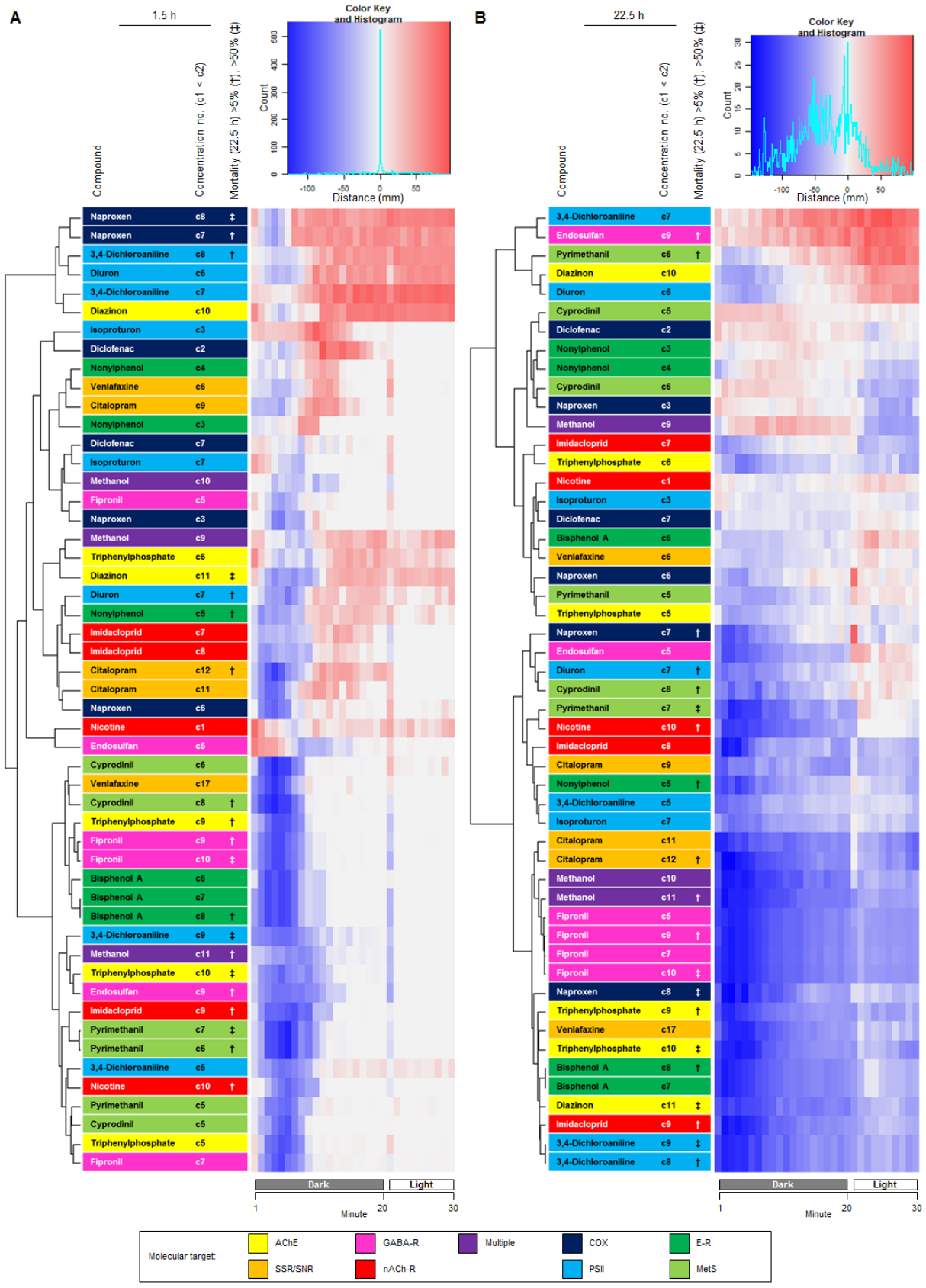
221 **Figure 3.** Multidimensional scaling plots of behavioral profiles. Same color: same mode of
222 action group. Each symbol refers to a specific concentration. Rows: exposure duration. Light
223 grey areas: spatial expansion of control groups in the two-dimensional space. Clusters
224 discussed in the main text are highlighted. Each of the 3 plots is directly comparable with
225 each other since multidimensional scaling was based on a common distance matrix
226 calculated from median moved distances per minute within a measurement period of 30 min
227 in total (20 min dark, 10 min light).

228 In order to identify phenotypes potentially indicative for a certain MoA we systematically
229 selected specific patterns (Supporting Information S1) for each compound and performed
230 hierarchically clustering. The results are shown in a heatmap in Figure 4.

231 Most behavioral profiles changed remarkably over time. For example naproxen (c7=668 µM),
232 led to an overall activation in movement after 1.5 h of exposure (Figure 4A) which turned into
233 hypoactive behavior with peak hyperlocomotion at the transition from dark to light after 22.5 h
234 of exposure which was exclusively observed for naproxen (Figure 4B). After 22.5 h of
235 exposure, two main clusters were observed, whereof the bottom cluster consisted of
236 behavioral profiles mainly representing hypoactivity throughout the test period (Figure 4B).
237 These profiles mainly included concentrations which induced lethality (>5%) at this time point
238 (Figure 4B). We only identified endosulfan (c9=0.8 µM) and pyrimethanil (c6=185 µM) to
239 cause both hyperactivity and lethality at 118.5 hpf (Figure 4B). Please note that only vital
240 embryos were considered for LMR data analyses. Furthermore, we found several combined
241 profiles where activity was increased in the dark phase, but decreased in the light phase (e.g.
242 methanol, c9=720 mM) and *vice versa* (e.g. diuron, c6=83 µM). Some compound-related
243 clusters indicating a decrease in activity were found for bisphenol A (c6=38 µM-c8=77 µM)
244 (Figure 4A) and fipronil (c5=1 µM-c10=9 µM) (Figure 4B).

245 Overall, clusters did not show clear differentiation of response patterns of neuroactive
246 compounds from other MoA classes. Independent of exposure duration, we could not clearly
247 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 4A). However, e.g. the neuroactive diazinon (c10=58 µM) also caused a behavioral phenotype similar to the two PSII inhibitors (Figure 4A, 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 4A, B). Also the SNR/SSR inhibitors venlafaxine (c6=0.2 µM) and citalopram (c9=22 µM) exclusively clustered 1.5 h post exposure (Figure 4A).



255

256 **Figure 4.** Behavioral phenotype-based clustering of chemicals with various modes of action

257 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 S1. Color key and histogram indicate the difference in median distance moved between treatment and control and the distance frequency distribution of shown profiles, respectively.

3.3. Sensitivity of LMR in relation to acute toxicity

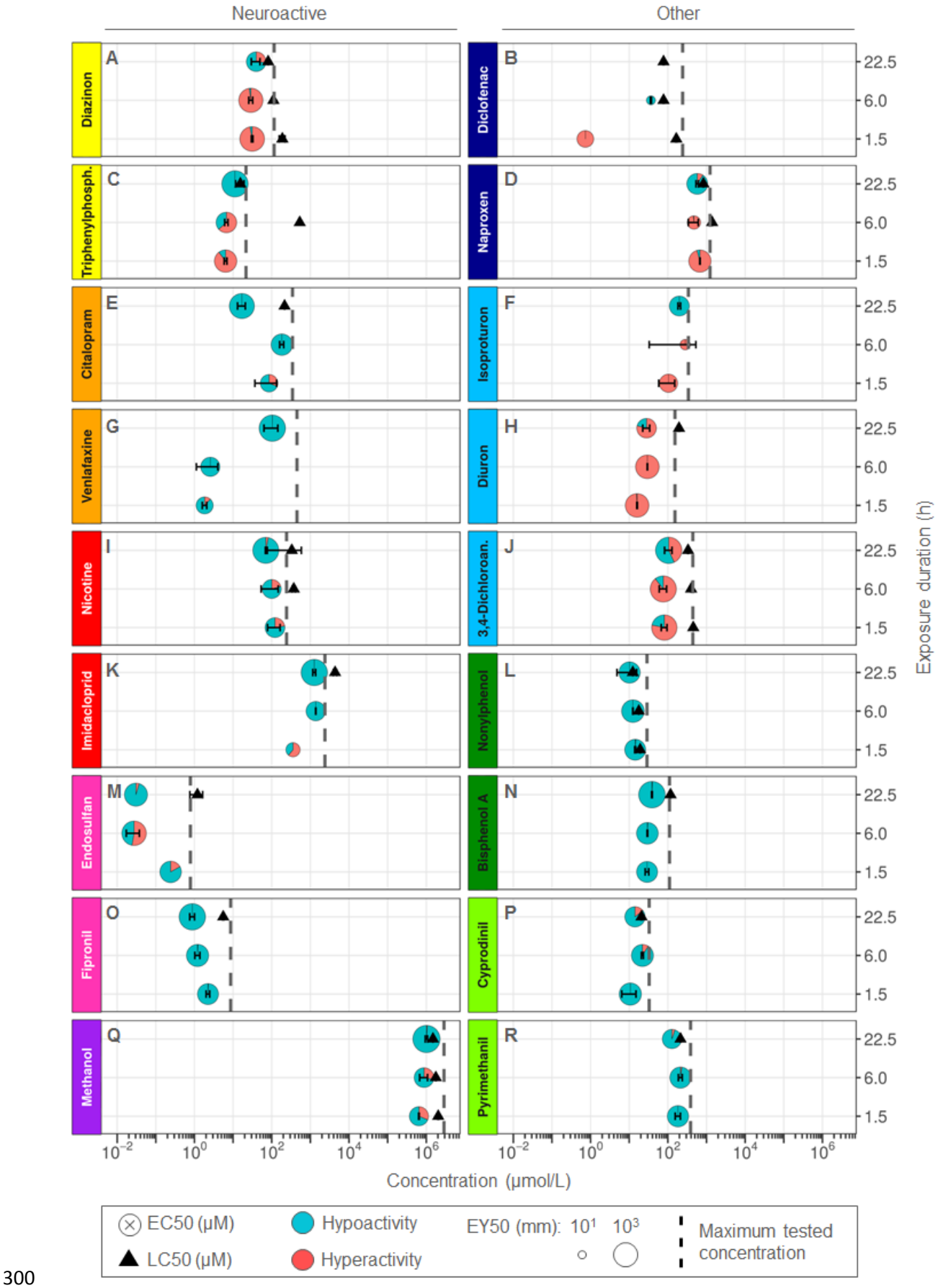
In order to assess the sensitivity of behavioral responses in relation to mortality, we conducted concentration response analysis of both endpoints and compared EC50 and LC50 after 1.5, 6.0 and 22.5 h of exposure. Figure 5 additionally shows the contribution of hypo- and hyperactivity to the total observed effect and how these changed over time. All concentration-response curves are shown in Supporting Figure S3.

Figure 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 5E, M, O).

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 5B). On the other hand, we found that the two

investigated serotonin reuptake inhibitors, the two nACh-R agonists, as well as triphenylphosphate, fipronil, bisphenol A and methanol 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). 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 S2 for effect values).


Neuroactive
Other
Diazinon
Diclofenac
Triphenylphosph.
Naproxen
Citalopram
Isoproturon
Venlafaxine
Diuron
Nicotine
3,4-Dichloroan.
Imidacloprid
Nonylphenol
Endosulfan
Bisphenol A
Fipronil
Cyprodinil
Methanol
Pyrimethanil
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
22.5
6.0
1.5
Exposure duration (h)
10^{-2} 10^{0} 10^{2} 10^{4} 10^{6}
Concentration (µmol/L)
EC50 (µM)
LC50 (µM)
Hypoactivity
Hyperactivity
EY50 (mm): 10^{1} 10^{3}
Maximum tested concentration

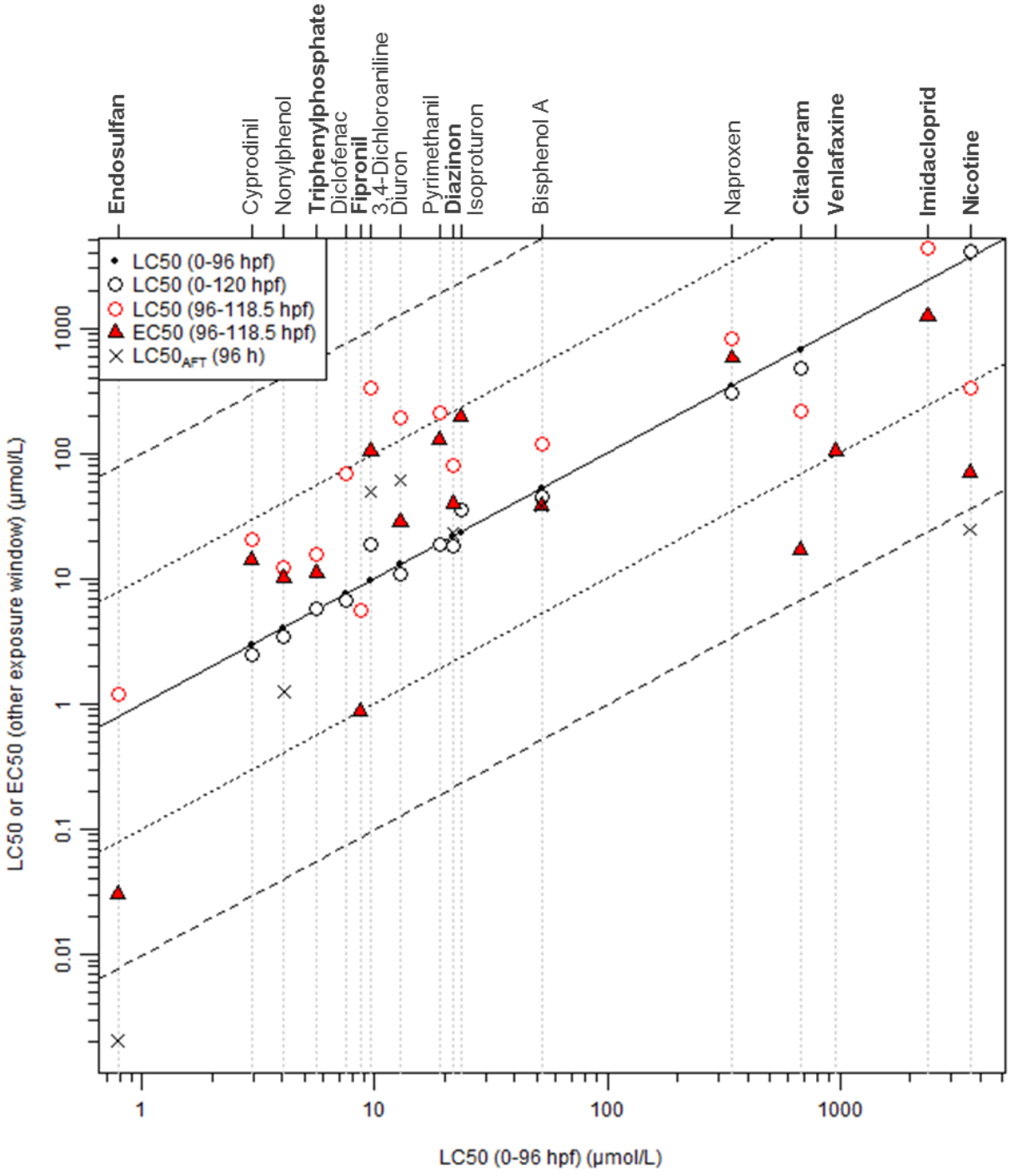
300

Figure 5. Sensitivity of behavioral responses (EC50) and their relation to lethal effect concentrations (LC50) over time. The center of each pie chart represents the EC50 of the LMR test. Size of pie chart: EY50 (total effect, i.e. absolute difference in distance moved between treatment and control at EC50). Blue and red pies indicate contribution of hypo- and hyperactive behavior to the overall observed effect, respectively. LC50 at the respective time point (same exposure duration) is indicated by black triangles and the maximum tested concentration (LMR assay) by dashed vertical lines. Error bars indicate 95% CI ($n \geq 160$ individuals per compound). LC50 values above the maximum tested concentration were extrapolated.

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 [6] data previously summarized by Scholz et al. [20]. Figure 6 summarizes the data that we had available for this comparison (Supporting Table S2).

The figure indicates that LC50 values obtained after 96 and 120 h of embryo 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 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.



340

341 **Figure 6.** Comparison of acute fish embryo toxicity (FET, circles), acute fish toxicity (AFT,
342 crosses) and locomotor response effects (LMR, triangles) for selected exposure time
343 windows. In case no mortality was observed within 96 h, the water solubility limit of the
344 respective compound was used as reference point. Broken lines indicate orders of
345 magnitude deviations from LC50 (0-96 hpf). Methanol not shown (see Supporting Table S2
346 for effect concentrations).

4. Discussion

The occurrence of a variety of chemicals potentially acting as neurotoxicants in the aquatic environment has been shown [1, 21]. 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 [22]), 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 [9]. Hence, ecologically relevant endpoints such as behavior have been proposed for improving the detection of ecotoxicological and potentially adverse effects of neurochemicals [9, 23].

In the present study we applied a light-dark transition test on ZFEs (4-5 days 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.

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. [24] 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 [25].

The dependency of motor responses from the duration of exposure has been demonstrated for e.g. certain dopaminergic drugs [26]. As this assay is not standardized, exposure regimes vary in literature ranging from early developmental exposures to late embryonic short-term exposures [12]. Taking into consideration that the nervous system of *D. rerio* is still developing during embryogenesis (reviewed e.g. by Schmidt et al. [27]), 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 [28]. Monesson-Olson et al. [29] 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 [29]. 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 [29], AChE is already present during early embryonic development of *D. rerio* [30, 31]. 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 [32]). 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 S4). 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 ZFE [33]. 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. [34] 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 [35]. 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 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. [11] and Bruni et al. [10] who applied chemical treatments for 1-10 h for phenotypical differentiation of specifically acting drugs.

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 [36] and atrazine [37], certain COX inhibitors [38], or fungicides such as imazalil [39] are able to disturb embryonic 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 [37, 39]. A similar result was obtained in a study by Bretaud et al. [40] 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. [41] 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 [36]. 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, ZFEs are capable to biotransform xenobiotics already during early embryogenesis [42]. Hence, observed temporal changes in the behavioral phenotype may in certain cases rather be attributed to unknown transformation products than parent compounds.

456 The observed behavioral profiles in our study comprised of both hypo- and hyperactivity in
457 ZFEs for the majority of investigated compounds. Activity patterns changed with increasing
458 concentrations meaning that there is not one single behavioral profile being representative
459 for a certain compound. At high exposure levels, which we assume to induce a general
460 disturbance of homeostasis shortly before apparent toxicity (similar to cytotoxicity in cell-
461 based *in vitro* assays) the majority of chemicals caused complete inhibition of locomotion
462 which indicated lethality. Frequently, we observed multiphasic concentration-response
463 patterns especially for those compounds which induced hyperactivity at low concentrations
464 while higher concentrations caused hypoactivation. Such biphasic concentration response
465 patterns were also reported by Ali et al. [43] who analyzed a set of 60 water-soluble
466 compounds using a light-dark transition assay in 120 hpf ZFEs exposed from 24 hpf. The
467 multiphasic behavioral effects of certain compounds may reflect their ability to impact on
468 different neurotransmitter systems or other molecular targets in a dose dependent manner.
469 However, based on the lacking specificity of observed behavioral responses, we cannot
470 distinguish such patterns. Furthermore, some chemicals may have multiple MoAs potentially
471 affecting behavior in different ways. E.g. chronic exposure to the AChE inhibitor
472 triphenylphosphate has been shown to alter hormone levels in zebrafish [44]. However,
473 mechanisms of acute developmental and chronic (neuro)toxicity are not necessarily covered
474 by our approach.

475 Evidence for MoA-indicative clusters, at least temporarily, could be identified for some
476 substances including those assigned as AChE and SSR/SNR inhibitors. Especially the
477 SSR/SNR inhibitors citalopram and venlafaxine were different compared to other compounds
478 and MoAs at 118.5 hpf (22.5 h of exposure) due to their systematic concentration-dependent
479 decrease of locomotor activity which could be observed with concentrations from around 1 to
480 >100 µM. For all other compounds behavioral effects ranged within one order of magnitude.
481 Our findings on venlafaxine are directly in line with recent results of Thompson et al. [35] who
482 found a significant dose-related reduction in the activity level of zebrafish (120 hpf) in the
483 dark. We also found phenotypic similarities between the hyperactivity-dominated profiles of

484 the AChE inhibitors diazinon and triphenylphosphate which were, however, also similar to the
485 effects found for the PSII inhibitor diuron and its degradation product 3,4-dichloroaniline. So
486 far, however, there is no evidence that diuron or 3,4-dichloroaniline inhibit AChE in the ZFE
487 which means that the underlying concept of similarity between those behavioral phenotypes
488 remains to be elucidated.

489 Behavioral profiles of single compounds being distinct from MoA analogues were observed
490 for diclofenac, isoproturon, endosulfan, and fipronil. This might be explained by the
491 differences in chemical structures compared to anticipated MoA analogues. For example, the
492 insecticides endosulfan and fipronil are assigned as GABA-gated chloride channel
493 antagonists but belong to two different chemical classes, namely organochlorines and
494 phenylpyrazoles, respectively. In addition, endosulfan is thought to specifically act on certain
495 arachnids whereas fipronil is known as a specific disruptor of the central nervous system in
496 insects. In our study, endosulfan caused an anti-cyclic light-dark response whereas fipronil
497 provoked a completely different hypoactive phenotype. There are indications that fipronil
498 does not (primarily) act as a GABA-R antagonist in ZFEs but rather inhibits glycine receptors
499 [45]. In contrast, known GABA-R antagonists like pentylenetetrazole and picrotoxin were found
500 to reverse the normal light-dark response in zebrafish larvae [46, 47] as endosulfan did in our
501 study. Hence, GABA-R antagonists may be identified via this specific phenotype.

502 Finally, our results demonstrate that behavioral alteration alone cannot be seen as a specific
503 parameter exclusively indicating effects of anticipated neuroactive substances. A refined
504 investigation of specificity using a larger set of chemicals and including compounds with
505 distinct known neuroactive MoAs (e.g. certain pharmaceuticals) that serve as a phenotypical
506 reference may further improve and establish LMR tests as diagnostic tools. In addition, a
507 sophisticated test design consisting of a more complex application of stimuli as used by Bruni
508 et al. [10] in the context of neuropharmaceutical discovery might be necessary for behavior-
509 based distinction of chemicals according to their MoAs. Furthermore, besides using the total
510 distance moved as endpoint, an additional integration of other parameters and endpoints

511 such as time spent moving or direction of movement as demonstrated by Palmér et al. [48] may
512 refine the resulting patterns and may help to increase specificity.

513 As a measure for sensitivity of locomotor activity-based endpoints we compared ratios of
514 effect concentrations for acute toxicity and behavioral alteration. Our results demonstrate that
515 significant behavioral effects mostly occurred throughout a narrow concentration range at the
516 borderline to lethality, in most cases covering only one order of magnitude or less. This holds
517 true for some anticipated neuroactive but also for the other compounds. A higher sensitivity
518 of the LMR assay was identified for the SSR/SNR inhibitors citalopram and venlafaxine with
519 ratios ≥9.3 after 22.5 h of exposure as well as for the GABA-R antagonist endosulfan. We
520 found that the behavioral responses and the acute toxicity converged with increasing
521 exposure duration except for venlafaxine and isoproturon which did not cause lethality up to
522 the limit of water solubility. In contrast to previous findings showing an increased sensitivity
523 through behavioral endpoints using exposure from 2-98 hpf [9], we could not confirm that this
524 type of LMR test in general provides a substantial increase in sensitivity beyond the acute
525 toxicity levels when exposure is initiated at later embryonic stages. Klüver et al. [9] investigated
526 e.g. the AChE inhibitor aldicarb and found a 660-fold increase in sensitivity compared to
527 mortality in the 4-day acute FET test when using a light-dark transition test at 98 hpf. By
528 contrast, we detected a merely 2-fold increase in sensitivity for the AChE inhibitors diazinon
529 and triphenylphosphate with our short-term exposure regime in older stages. These
530 differences might, however, be explained by unspecific activities of diazinon and
531 triphenylphosphate as we detected AChE inhibition at levels close to baseline toxicity
532 (Supporting Figure S4). For endosulfan an EC50 (LMR assay) of 0.015 µM after 96 h of
533 exposure was reported by Klüver et al. [9] without mortality up to the limit of water solubility.
534 Here we determined a similar EC50 of 0.031 µM within a 4 times shorter exposure window
535 (96-118.5 hpf) but, in contrast to the previous study, we already observed mortality after
536 22.5 h of exposure and extrapolated an LC50 (96-118.5 hpf) of 1.2 µM. We assume that the
537 observed hyperactivity due to the interaction of endosulfan with the GABA-R leads to
538 increased energy consumption and allocation and finally to mortality. Especially at 5 dpf this

539 appears critical because internal energy resources in form of yolk are exhausted [49]. The
540 limited GABA-R availability before 96 hpf [29], therefore, may prohibit an acute toxicity of
541 endosulfan in earlier stages of embryonic development. These findings indicate that toxicity
542 testing of compounds with molecular targets not expressed in the early embryo might be
543 supported by additional LMR analyses at 4 dpf or should be performed in later embryonic
544 stages.

Supporting Information

S1. Materials and Methods

Supporting Table S1. Tested concentrations in µM and their identification number

Supporting Table S2. Toxicity data used for comparative analysis of acute fish embryo toxicity and LMR data

Supporting Table S3. Median moved distances per minute for the 18 investigated compounds

Supporting Figure S1. Chemical structures of the tested compounds

Supporting Figure S2. Overview on behavioral profiles of the 18 investigated compounds recorded after 1.5, 6.0 and 22.5 h of exposure

Supporting Figure S3. Concentration-response curves for zebrafish embryo locomotor alteration

Supporting Figure S4. Acetylcholinesterase activity in 120 hpf zebrafish embryos after 24 h of exposure with diazinon and triphenylphosphate, respectively

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Supporting Information

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Can environmentally relevant neuroactive chemicals specifically be detected with the locomotor response test in zebrafish embryos?

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14

Figures:

Supporting Figure S1, Supporting Figure S2, Supporting Figure S3, Supporting Figure S4

Tables:

Supporting Table S1, Supporting Table S2, Supporting Table S3 (EXCEL file)

S1. 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

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

as lower limit and

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

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. 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 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 MoA-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

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

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 S1. 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	-	-	-	-	-	-	-	-	-	-	-	-	-	-

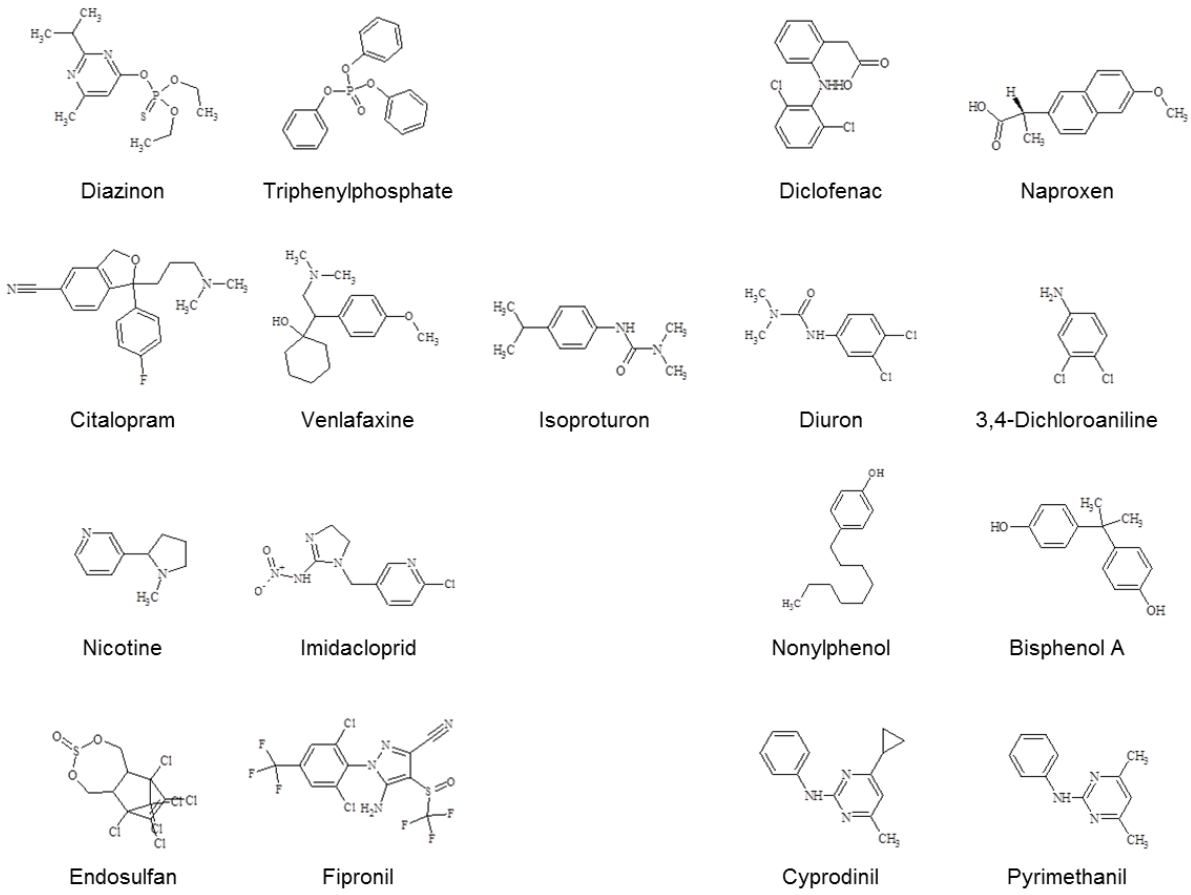
Each compound was tested down to a concentration where no obvious difference to the control movement pattern was visible anymore (manual inspection of raw data plots), and up to a level where ideally, at least at the last time point of assessment (118.5 hpf), ≤100% mortality were

observed in order to allow for concentration-response modelling. Therefore, the R package drc (version 2.3-96, [1]) 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 3).

Supporting Table S2. Toxicity data used for comparative analysis of acute fish embryo toxicity and LMR data.

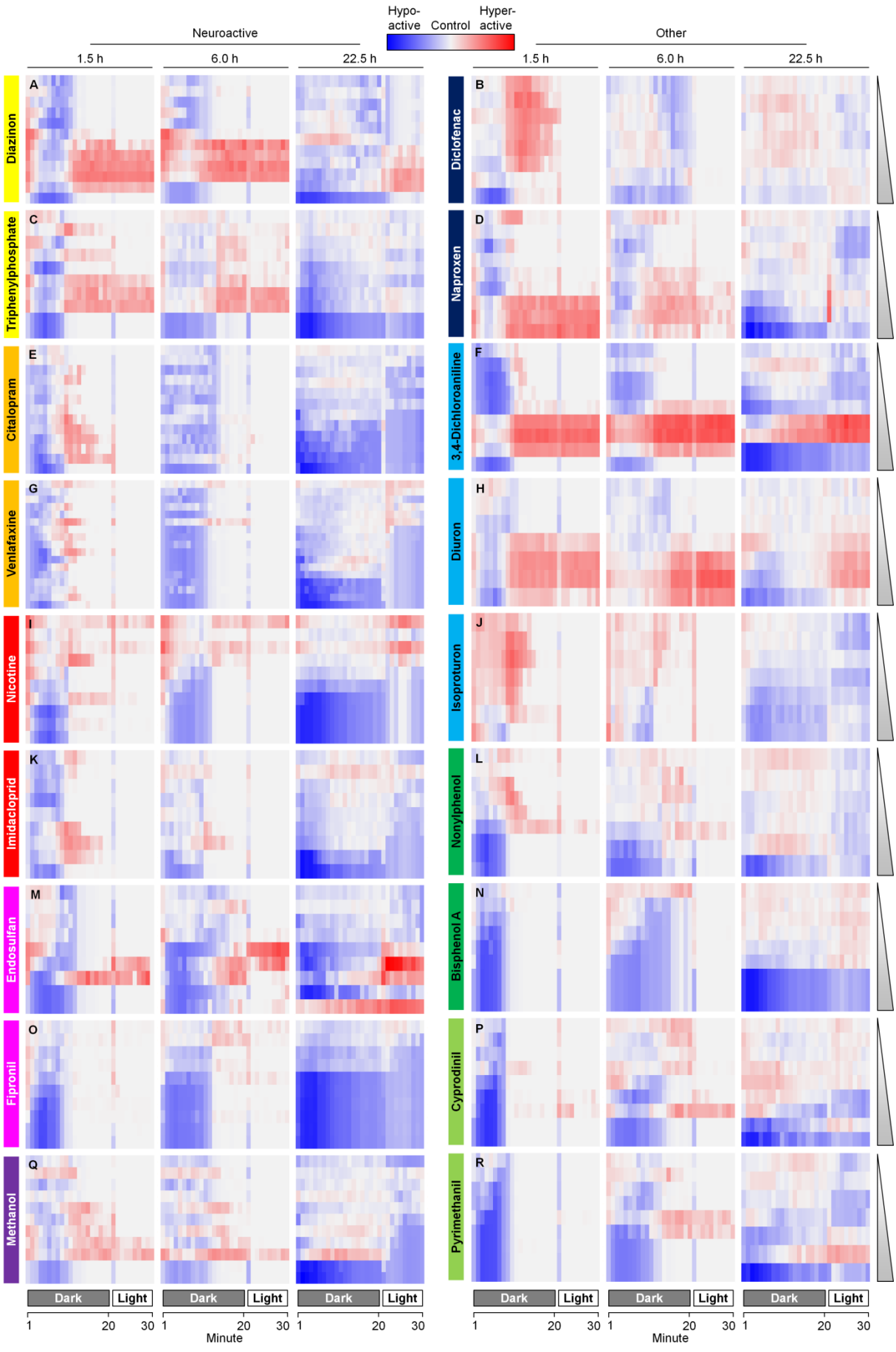
Compound	LC50 (µmol/L)					EC50 (µmol/L)			Ratio LC50/EC50
	0-96 hpf	0-120 hpf	96-97.5 hpf	96-102 hpf	96-118.5 hpf	96-97.5 hpf	96-102 hpf	96-118.5 hpf	96-118.5 hpf
Diazinon	21.7	18.2	188.5	110.4	80.8	31.2	28.8	39.7	2.0
Triphenylphosphate	5.6	5.7	no effect	531.3	15.4	6.4	6.7	11.2	1.4
Citalopram	674.4	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 6 of the main article to avoid overlap of data points.



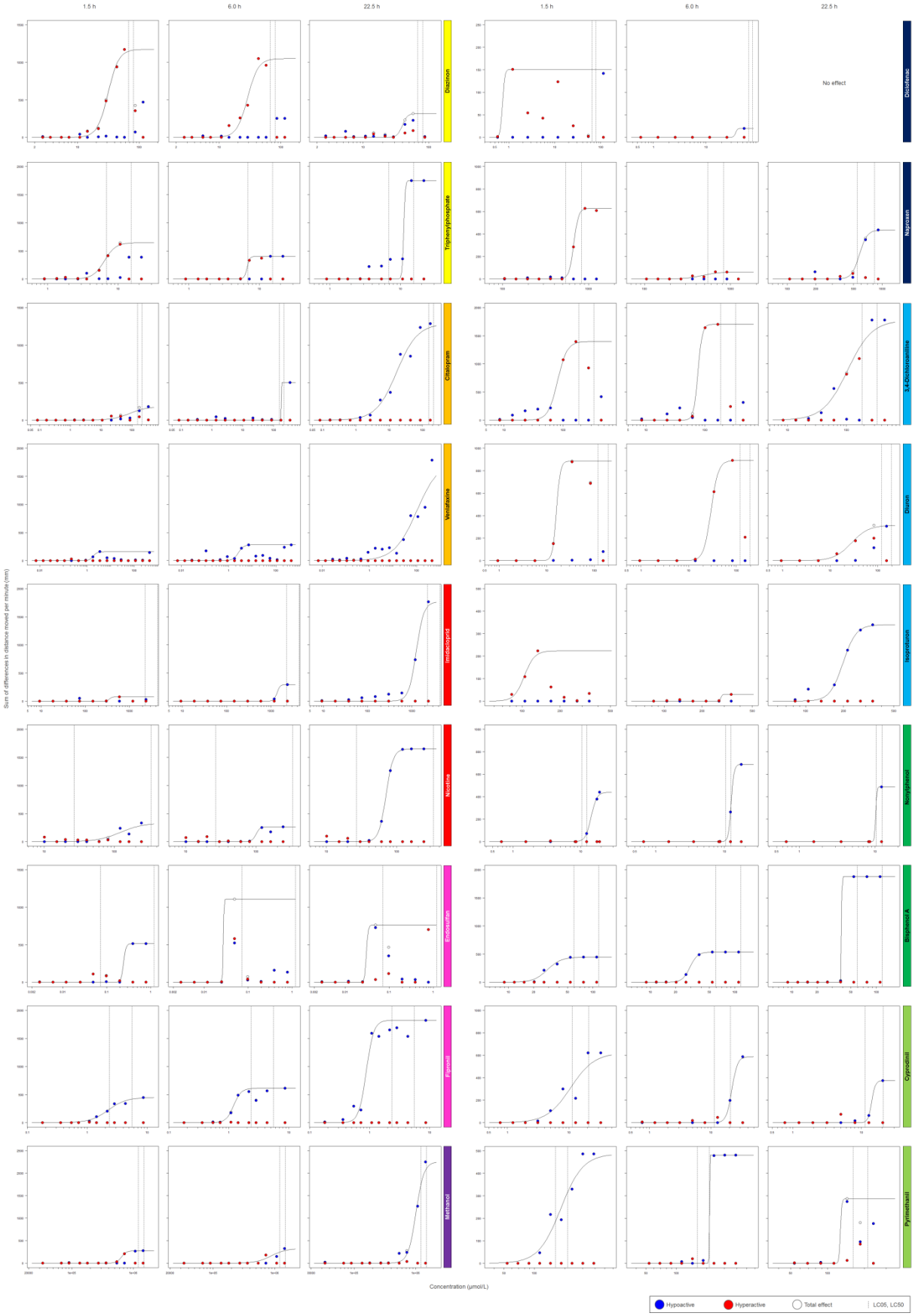
133

134 **Supporting Figure S1.** Chemical structures of the tested compounds.


Hypo-active
Control
Hyper-active
Neuroactive
Other
1.5 h
6.0 h
22.5 h
Diazinon
Triphenylphosphate
Citalopram
Venlafaxine
Nicotine
Imidacloprid
Endosulfan
Fipronil
Methanol
Diclofenac
Naproxen
3,4-Dichloroaniline
Diuron
Isoproturon
Nonylphenol
Bisphenol A
Cyprodinil
Pyrimethanil
Dark
Light
1
20
30
Minute

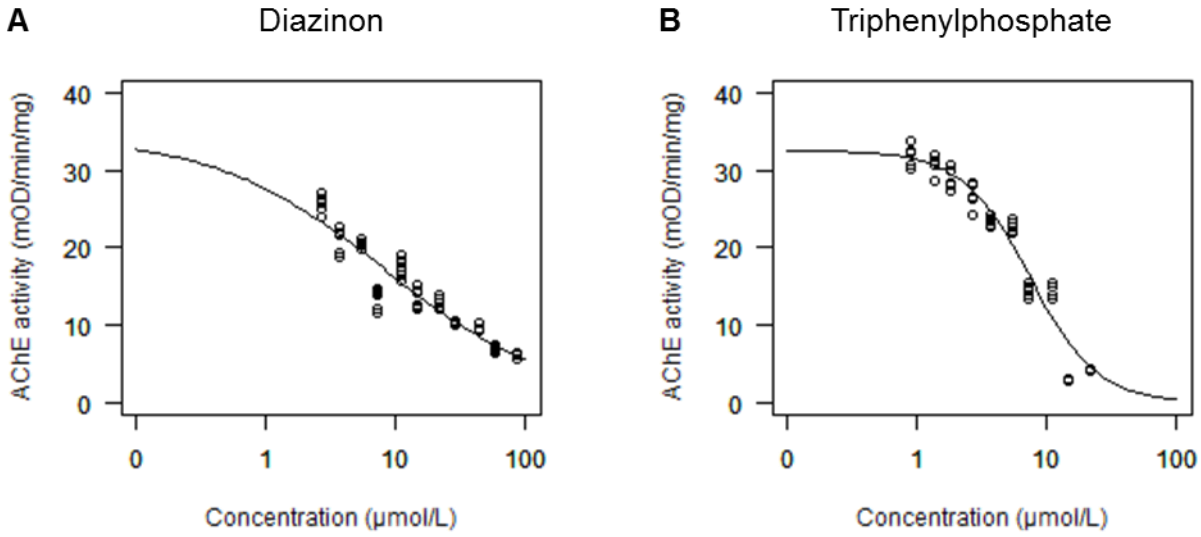
135

Supporting Figure S2. 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 grey triangles on the right hand side. Compounds with an anticipated same mode of action are indicated by uniform color.


1.5 h
6.0 h
22.5 h
1.5 h
6.0 h
22.5 h
Diazinon
No effect
Diclofenac
Triphenylphosphate
Naproxen
Chlorpyrifos
3,4-Dichloroaniline
Venlafaxine
Diuron
Imidacloprid
Isoproturon
Nicotine
Norglyphosate
Bisphenol A
Fipronil
Cyprodinil
Methanol
Pyrimethanil
Sum of differences in distance moved per minute (mm)
Concentration (µmol/L)
Hypoactive
Hyperactive
Total effect
LC05, LC50

141

Supporting Figure S3. Concentration-response curves for zebrafish embryo locomotor alteration after 1.5, 6.0 and 22.5 h of exposure (96-118.5 hpf). Blue and red data points represent significant differences in the distance moved per minute between treatment and control, respectively. The maximum observed total effect (sum of distances spent hypo- and hyperactive) was used as the maximum of each model. Broken vertical lines represent LC05 and LC50, respectively, after 22.5 h of treatment.



Supporting Figure S4. Acetylcholinesterase (AChE) activity in 120 hpf zebrafish embryos (ZFEs) 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 ZFEs) as previously described [2]. Enzyme activity in controls was 34.4±3.5 and 32.5±1.8 mOD/min/mg (mean±SD) for diazinon and triphenylphosphate, respectively.

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