Ortmann et al.:

Photomotor Response Data Analysis Approach to Assess Chemical Neurotoxicity with the Zebrafish Embryo

Supplementary Material

Text S1: Chemical stability

Chemical stability of azinphos-methyl, propoxur, tricaine and 3,4-dichloroaniline was determined in three independent experiments by measuring UV/VIS – absorption at 231 nm, 270 nm, 312 nm and 297 nm, respectively, using a nanodrop nd-1000 photometer (peQlab Biotechnology GmbH, Erlangen, Germany). For each compound standard curves were prepared, and the concentrations of the test compounds were measured after exposures at 26°C for 24 h and 48 h in 24 well plates with one embryo in 2 mL per well and 10 wells per concentration. Within 48 h of exposure, declines of the nominal concentrations were found to be below 1% (azinphos-methyl, tricaine), 5% (propoxur) and 15% (3,4-dichloroaniline). Due to the little changes in concentrations over time, biological effects were related to nominal exposure concentrations for these test chemicals. For the lipophilic pyrethroids esfenvalerate and flucythrinate, the exposure concentrations were below the detection limit for UV/VIS analysis. For esfenvalerate, a strong deviation from nominal concentrations and a rapid concentration decline within 24 h exposure has previously been observed in a semistatic exposure setup (Klüver et al., 2015). Due to the similarities in structure and physico-chemical properties to esfenvalerate, we assume that the actual concentration of flucythrinate in the wells also strongly deviated from nominal concentrations. However, as we lack analytical data, we relate observed biological effects to nominal concentrations of these compounds and need to note that actual concentrations were possibly lower so that, therefore, calculated effect concentrations may be overestimated for these compounds.

Text S2: Zebrafish maintenance, embryo collection, exposure conditions and FET tests

Zebrafish maintenance, embryo collection, exposure conditions

Zebrafish eggs were obtained from the strain “UFZ-OBI” (F5) that was established from a broodstock of fish originally purchased from a local OBI hardware store. Parental fish were maintained in local tap water (conductivity 540-560 mS/cm, water hardness 2-3 mM divalent ions, pH 7-8, oxygen saturation 87-91%) in a circulating tank system with a central biological filter unit. About 1% of the tank water was replaced per h by fresh dechlorinated tap water. Nitrate (< 2.5 mg/L), nitrite (< 0.025 mg/L) and ammonium (< 0.6 mg/L) concentrations were checked weekly and were below detection limits. The water temperature was adjusted to 26°C ± 1°C and monitored daily. The photoperiod was set to 14 h:10 h (light:dark). Twenty-five to 30 fish were kept per 30 L tank with a 2:1 male to female ratio. The age of fish in spawning tanks was between 6 and 18 months. Fish were fed daily twice with live Artemia. Spawning was stimulated by placing glass trays covered with a 3 mm mesh and artificial plants into the breeding tanks the evening before fertilized eggs were needed. Eggs were collected from spawning trays within 1 h after lights were switched on in the aquarium room and poured into a 100 mm mesh. Prior to exposures in the FET test or for PMR measurements, embryos were rinsed several times with tank water.

Determination of fish embryo toxicity (FET; LC_{50})

FET tests were performed with 3,4-dichloroaniline, azinphos-methyl, esfenvalerate, flucythrinate, propoxur and tricaine with few modifications according to the OECD TG 236 and a static exposure setup without renewal of exposure solutions. Fertilized eggs (embryos at 4-32 cell stage) were transferred with a pipette to 24-well plates (Cellstar Greiner Bio-One, Frickenhausen, Germany) (one embryo per well, ten wells per exposure concentration). Exposures were performed in three independent experiments on different days for 96 h from 2-98 hpf, and lethal effects were recorded at 48 and 96 hpf. To detect dead embryos, the criteria given in the OECD TG 236 were applied, i.e., either coagulation, missing heartbeat, failure to develop somites, or a lack of detachment of the tail-bud from the yolk sac were considered indicators of lethality. A 4-parameter logistic regression model (Eq. 1; see below) was fitted to the data using JMP11 (JMP; Cary NC; USA).

Retrieval of AFT data (LC_{50})

Lethal effect concentrations for adult or juvenile zebrafish were not available for all selected test compounds. Therefore, given the overall high correlation of fathead minnow with zebrafish AFT data (Belanger et al., 2013), the respective AFT LC_{50} values for fathead minnow (*Pimephales promelas*) were retrieved from a US-EPA database (Russom et al., 1997). For azinphos-methyl, propoxur, flucythrinate, tricaine and 3,4-dichloroaniline, those values are 0.20 µM, 42.0 µM, 1.0 nM, 302.0 µM and 47 µM, respectively (Table 1). For esfenvalerate, the LC_{50} of the structurally similar fenvalerate was used, which differs from esfenvalerate by stereoisometric composition (3.6 nM).

doi:10.14573/altex.2004021s

Equation for concentration response modeling

$$E(c_i) = max + \frac{\max - min}{1+\left(\frac{c_i}{EC_{50}}\right)^{-p}} \quad \text{Eq. 1}$$

$E(c_i)$:	is the effect at concentration ci
c_i:	is the concentration of substance i
max:	is the effect in controls
min:	is the maximum effect at curve saturation
p:	is the slope of the curve
EC_{50}:	is the constant describing the concentration of substance causing 50% of the maximum effect

The model was fitted to the concentration-effect data, and the model parameters were estimated using the program JMP (SAS, Marlow, UK) for fish toxicity test data or BDMS (available at https://www.epa.gov/bmds) for PMR data. A 4-parameter logistic regression model was used for analysis of FET test results (min and max were set to 0% and 100%); a 4-parameter regression model was used for PMR effects based on the 2D-density approach (min was set to 19%). Effect concentrations and model parameters are summarized in Table S1.

Residual analysis

To evaluate how well the model fits the PMR data, we calculated for each data point (representing a PMR effect at a certain substance concentration) the standardized residuals (Eq. 2) and plotted them against the corresponding prediction estimate (Eq. 1, see above). The scatter of the residuals is concentration-independent and showed no heteroscedasticity (Fig. S1). Therefore, we concluded that the logistic model explains the PMR effect adequately for all five neuroactive substances (azinphos-methyl, propoxur, esfenvalerate, flucythrinate and tricaine). The PMR effects measured in three independent experiments showed no difference in the standardized residuals, which was a strong indication that the measurements of the PMR effects were reproducible. The residual standard error (RSE; Eq. 4) for the regression ranged from 6% for propoxur to 10% for tricaine, which means that the average response deviated from the regression line by a maximum of 10% of PMR effect. The standardized residuals were found to be in a range of ± 2 for all compounds and the controls (mean of 0 ± 0.2 and standard deviation of maximal 1.6) (Fig. S1).

$$Residual_i = E(c_i)_{measured} - E(c_i)_{predicted} \quad \text{Eq. 2}$$

$$Standardized\ Residual_i = \frac{Residual_i}{\sqrt{E(c_i)_{predicted}}} \quad \text{Eq. 3}$$

$$RSE = \sqrt{\frac{\sum_{n=1}^{i}\left(E(c_i)_{predicted} - E(c_i)_{measured}\right)^2}{i-2}} \quad \text{Eq. 4}$$

$Residual_i$:	is the residual of an effect at a concentration c_i
$Standardized\ Residual_i$	is the standardized residual of an effect at a concentration c_i
RSE:	is the residual standard error
i:	is the amount of data points used for model fitting

Text S3: Control variability

The PMR is characterized by an intrinsic variability, which can partially be minimized by controlling certain parameters, such as the time of day when the analysis is conducted and the temperature. These parameters were controlled in the present study. While the typical response pattern was observed in all replicates, some variability in the magnitude of the motion index could still be observed. Figure S1A shows the raw data time course of the motion index that was observed in the present study. As indicated by the ratio of the motion indices for the excitation versus the pre-pulse phases, the motion index (Fig. S1B and Tab. S1) can be normalized by the motion index of the refractory phase. The use of surface density areas as performed for the assessment of PMR data in this study is another way to normalize the motion indices, since for each replicate the surface density areas are compared to the respective replicate controls (for details refer to Section 2 in the main article).

Text S4: PMR effect quantification based on the OA-approach

The PMR effect quantification based on the OA-approach was performed as described in the Material and Methods section in the main article. The distribution of the motion index values, detected during 5-25 s of a PMR measurement, were described by fitting a density function to the data. The OA of density curves for control and respective treatment groups is a measure for the similarity of the movement activity. One minus the OA is used as PMR effect parameter based on this so-called OA-approach. Concentration-dependent PMR effects were modeled using a 4-parameter logistic regression model (Eq. 1, see above). The regression curves (Fig. S2) and residual plots (Fig. S3) are shown for the five neuroactive compounds azinphos-methyl, propoxur, flucythrinate, esfenvalerate and tricaine, and the narcotic reference compound 3,4-dichloroaniline. The resulting parameter estimates and the benchmark concentrations are shown in Table S2.

Text S5: Morphological effects

Morphological effects were analyzed by automated image assessment (see main article for details) for the exposure of embryos to two selected concentrations, i.e., the effect concentration of the PMR (either EC_{50} or benchmark concentration) and the LC_{50} (or maximum water solubility if no lethality was observed). The compound-specific results of the quantitative assessment of the various morphological features can be found in the supplementary excel file[2]. To summarize the data, the means and standard deviations between controls and exposed embryos were compared (Tab. S6). Except for small differences in the contour-yolk distance of propoxur and several endpoints for tricaine, no morphological effects for embryos exposed to PMR effect concentrations were observed. Slightly more changes in morphology were observed for higher effect concentrations (at the LC_{50} or maximum water solubility). Hence, except for tricaine, potential secondary effects on the PMR caused by morphological changes can be excluded.

Tab. S1: Parameter estimates of the 4-parameter logistic regression curve (Eq. 1) fitted to concentration-dependent photomotor response (PMR) effect data

PMR effects were estimated with the 2D-density approach for the neuroactive substances azinphos-methyl, propoxur, flucythrinate, esfenvalerate and tricaine, and the narcotic reference compound 3,4-dichloroaniline. The standard errors, lower and upper confidential limits are listed. The benchmark concentrations and the benchmark concentration limits of the effect curves are shown.

Compound	Variable	Estimate	Std. err.	Lower conf. lim.	Upper conf. lim.
Azinphos-methyl	alpha	27.9	5.6	17.0	38.8
	max-min effect [%]	27.5	2.2	23.2	31.7
	slope	3.7	1.3	1.1	6.3
	EC_{50} [µM]	0.7	0.1	0.5	0.8
	Benchmark concentration [µM]	0.4			
	Benchmark concentration limit [µM]	0.3			
Propoxur	alpha	37.5	7.2	23.4	51.7
	max-min effect [%]	19.9	3.3	13.5	26.3
	slope	1.6	0.7	0.3	2.9
	EC_{50} [µM]	9.8	3.4	3.0	16.5
	Benchmark concentration [µM]	6.0			
	Benchmark concentration limit [µM]	3.2			
Flucythrinate	alpha	33.4	6.8	20.0	46.8
	max-min effect [%]	46.9	2.5	42.0	51.9
	slope	1.8	0.5	1.0	2.7
	EC_{50} [nM]	12.1	1.6	8.9	15.3
	Benchmark concentration [nM]	4.2			
	Benchmark concentration limit [nM]	2.5			
Esfenvalerate	alpha	55.6	11.4	33.4	77.9
	max-min effect [%]	35.3	4.8	26.0	44.6
	slope	2.4	1.0	0.4	4.4
	EC_{50} [nM]	1.1	0.2	0.6	1.5
	Benchmark concentration [nM]	0.6			
	Benchmark concentration limit [nM]	0.4			
Tricaine	alpha	186.1	38.0	111.6	260.5
	max-min effect [%]	67.4	15.2	37.6	97.2
	slope	1.0	NA		
	EC_{50} [µM]	58.3	32.1	-4.6	121.2
	Benchmark concentration [µM]	14.8			
	Benchmark concentration limit [µM]	8.5			
3,4-Dichloroaniline	alpha	22.9	4.9	13.2	32.6
	max-min effect [%]	16.8	3.5	10.0	23.6
	slope	18.0	NA		
	EC_{50} [µM]	4.7	0.2	4.3	5.2
	Benchmark concentration [µM]	4.5			
	Benchmark concentration limit [µM]	3.9			

[2] doi:10.14573/altex.2004021s2

Tab. S2: Parameter estimates with standard errors and confidence intervals for concentration-PMR response relationships estimated with the overlapping area (OA) approach (min = 19%)

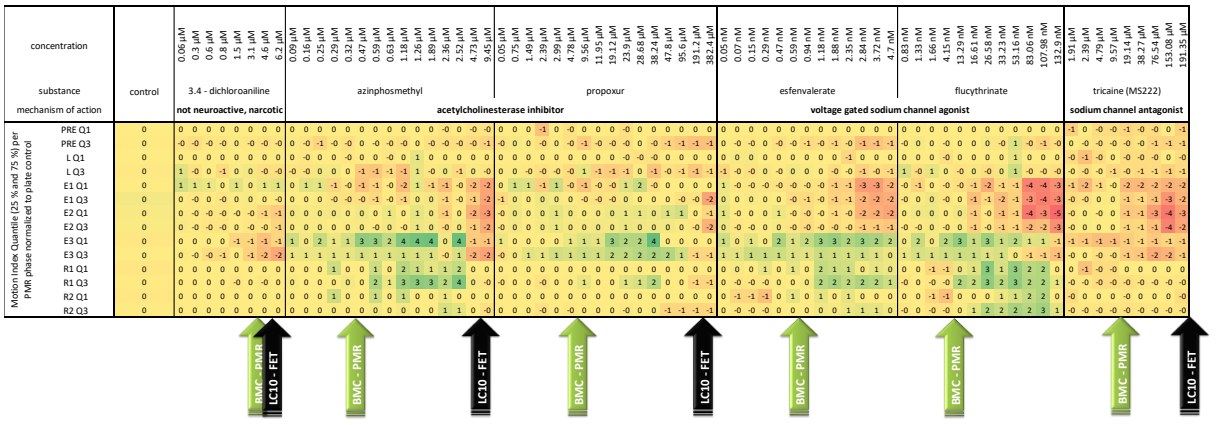
Substance	Variable	Estimate	Std. err.	Lower conf. limit	Upper conf. limit	N	RSE
Azinphos-methyl	alpha	13.3	2.7	8.1	18.4	50	3.1
	min effect [%]	6.8	0.6	5.5	8.1		
	max-min effect [%]	10.3	2.1	6.2	14.5		
	slope	4.5	4.2	-3.7	12.7		
	EC_{50} [µM]	0.8	0.2	0.3	1.3		
	benchmark concentration [µM]	0.7					
	benchmark concentration limit [µM]	0.5					
Propoxur	alpha	13.1	2.5	8.2	18.1	54	3.45
	min effect [%]	6.7	0.7	5.2	8.1		
	max-min effect [%]	4.5	1.6	1.3	7.7		
	slope	1.6	1.6	-1.6	4.8		
	EC_{50} [µM]	3.1	4.1	-4.9	11.1		
	benchmark concentration [µM]	7.6					
	benchmark concentration limit [µM]	computation failed					
Flucythrinate	alpha	16.3	3.3	9.8	22.8	48	3.92
	min effect [%]	7.2	0.7	5.7	8.6		
	max-min effect [%]	15.7	1.3	13.2	18.3		
	slope	18.0	NA				
	EC_{50} [nM]	13.6	0.5	12.7	14.5		
	benchmark concentration [nM]	12.8					
	benchmark concentration limit [nM]	11.2					
Esfenvalerate	alpha	17.4	3.6	10.5	24.4	48	3.34
	min effect [%]	6.7	0.7	5.3	8.1		
	max-min effect [%]	9.1	1.4	6.4	11.8		
	slope	18.0	NA				
	EC_{50} [nM]	1.0	0.1	0.9	1.1		
	benchmark concentration [nM]	1.0					
	benchmark concentration limit [nM]	0.9					
Tricaine	alpha	43.2	8.8	25.9	60.5	48	5.94
	min effect [%]	7.6	1.4	4.8	10.4		
	max-min effect [%]	22.6	3.8	15.2	30.0		
	slope	1.0	NA				
	EC_{50} [µM]	14.0	9.8	-5.2	33.2		
	benchmark concentration [µM]	5.7					
	benchmark concentration limit [µM]	2.2					
3,4-Dichloroaniline	alpha	10.3	2.2	6.0	14.7	43	
	min effect [%]	6.4	0.5	5.4	7.4		
	max-min effect [%]	5.7	2.3	1.1	10.3		
	slope	18.0	NA				
	EC_{50} [µM]	4.5	0.5	3.4	5.5		
	benchmark concentration [µM]	4.5					
	benchmark concentration limit [µM]	3.4					

Tab. S3: FET test results: model parameters of concentration effect curves (Fig. S5)

Substance	Exposure duration	Parameter	Estimate	Standard error	Lower CI	Upper CI	N	RMSE
Propoxur	0-48 hpf	LC_{50} [µM]	509.3	36.3	448.8	621.6	24	17.2
		slope	4.1	1.2	2.0	8.7		
		LC_{10} [µM]	296.9					
	0-96 hpf	LC_{50} [µM]	480.0	19.3	445.0	525.6	33	16.1
		slope	6.2	1.5	3.7	11.0		
		LC_{10} [µM]	336.2					
Azinphos-methyl	0-48 hpf	LC_{50} [µM]	13.6	0.5	12.4	14.7	26	13.3
		slope	4.9	0.9	3.2	7.4		
		LC_{10} [µM]	8.7					
	0-96 hpf	LC_{50} [µM]	8.0	0.1	7.7	8.2	16	6.9
		slope	6.0	0.6	4.8	7.4		
		LC_{10} [µM]	5.5					
Tricaine	0-48 hpf	LC_{50} [µM]	458.4	38.5	380.6	543.8	42	13.6
		slope	2.5	0.5	1.6	4.6		
		LC_{10} [µM]	190.3					
	0-96 hpf	LC_{50} [µM]	444.7	31.1	384.8	519.3	19	8.4
		slope	3.0	0.6	2.0	5.6		
		LC_{10} [µM]	214.0					
3,4-Dichloroaniline	0-48 hpf	LC_{50} [µM]	12.4	0.8	10.8	14.0	14	12.1
		slope	3.8	0.8	2.3	5.4		
		LC_{10} [µM]	7.0					
	0-96 hpf	LC_{50} [µM]	9.6	0.6	8.5	10.7	14	11.5
		slope	4.3	1.0	2.5	6.2		
		LC_{10} [µM]	5.8					

Tab. S4: Concentration-dependent movement patterns of zebrafish embryos caused by the five neuroactive compounds applied here

Concentration is increasing from left to right. Colors indicate reduced (orange) or increased movement (green) with respect to controls. The arrows indicate the corresponding FET LC_{10} (96 h; black arrows) or PMR benchmark concentrations (BMC; green arrows). The positioning of arrows between two adjacent concentrations indicates that the corresponding value (which represents a modeled effect concentration) is between these test concentrations (see Tab. S2).

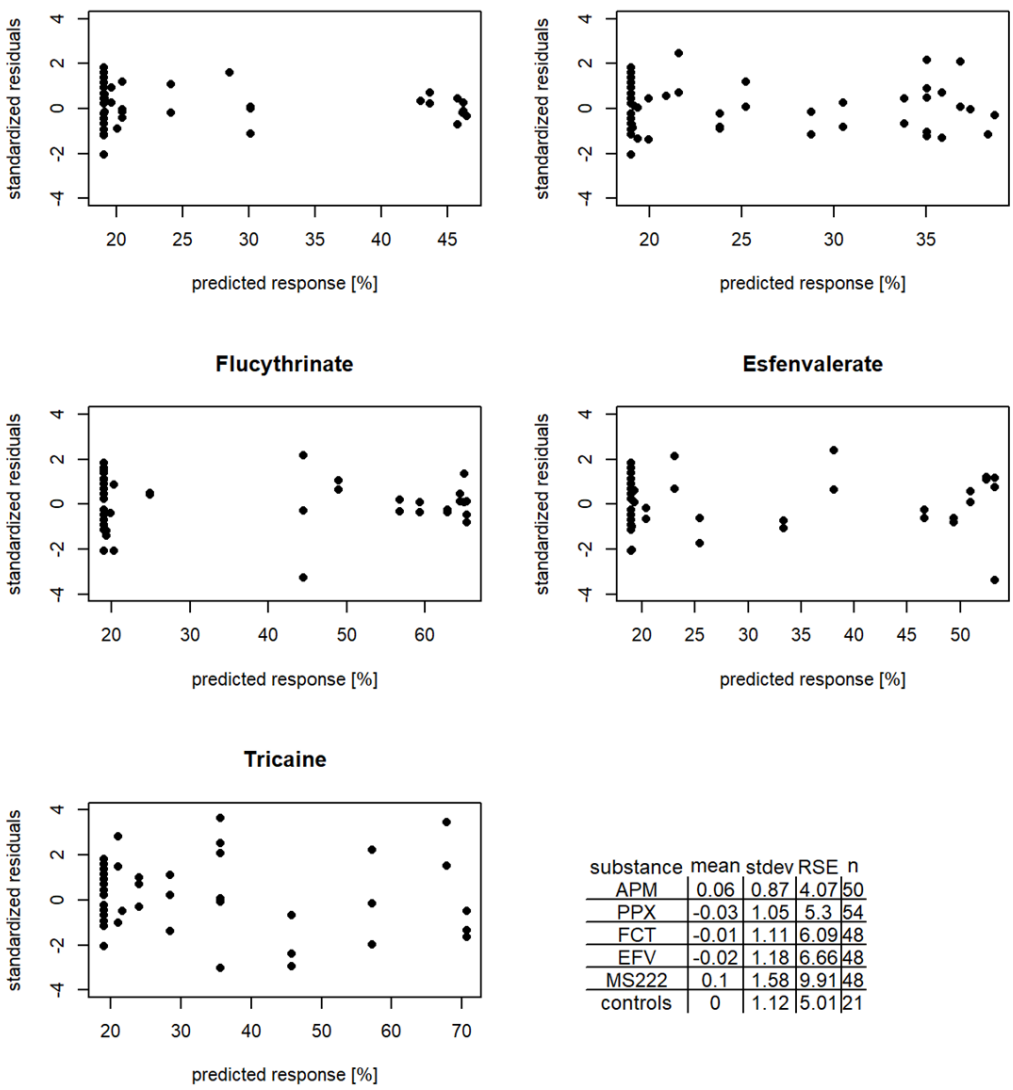


Tab. S5: Descriptive statistics of PMR phases (control embryos)
The values represent the sum of the motion index inside a specific phase of the PMR.

Parameter	Minimum	Maximum	Mean	Median	Standard deviation
Prepulse	106	608	350	342	121
Excitation	110	1230	679	682	278
Refractory	37.0	159	67.3	59.7	25.0
Excitation/prepulse	0.593	2.63	1.91	1.97	0.444

Tab. S6: Overview of the determined morphological effects upon exposure of zebrafish embryos to the test compounds, determined at 30 hpf
Each compound was tested at two concentrations: (1) a PMR effect concentration (EC_{50} or benchmark concentration – BMC) and (2) a concentration in the lethal range (around FET LC_{50} (48 h)) or the maximum water solubility if no mortality was observed. A shaded field with a "1" in the table indicates that for the given compound and test concentration the standard deviation of the measured morphological endpoint did not overlap with the standard deviation from controls and hence the deviation was considered different from controls.

Compound	Concentration	Unit	Distance cont-yolk_mm	Eye size_mm2	Head size_mm2	Head-trunk angle	Length_mm	Max tail curvature	Otolith-eye distance_mm	Pericard size_mm2	Yolk sac size_mm2	Effect concentration
3,4-DCA	4.5	µM	0	0	0	0	0	0	0	0	0	BMC PMR
3,4-DCA	11.8	µM	0	1	0	0	0	0	0	0	0	~LC50
APM	0.7	µM	0	0	0	0	0	0	0	0	0	EC50 PMR
APM	13.6	µM	0	1	0	0	0	0	0	0	0	LC50
Esfenvalerate	1.1	nM	0	0	0	0	0	0	0	0	0	EC50 PMR
Esfenvalerate	15	nM	0	0	0	0	0	0	0	0	0	Maximum water solubility
Flucythrinate	12.1	nM	0	0	0	0	0	0	0	0	0	EC50 PMR
Flucythrinate	89	nM	0	0	0	0	0	0	0	0	0	Maximum water solubility
Propoxur	9.8	nM	1	0	0	0	0	0	0	0	0	EC50 PMR
Propoxur	509.3	µM	0	1	0	0	0	1	1	0	0	LC50
Tricaine	58.3	µM	1	1	1	0	0	0	0	0	1	EC50 PMR
Tricaine	387.4	µM	1	0	1	0	0	1	1	1	0	~LC50



substance	mean	stdev	RSE	n
APM	0.06	0.87	4.07	50
PPX	-0.03	1.05	5.3	54
FCT	-0.01	1.11	6.09	48
EFV	-0.02	1.18	6.66	48
MS222	0.1	1.58	9.91	48
controls	0	1.12	5.01	21

Fig. S1: Residual plots
A 4-parameter logistic regression model was fitted to concentration-dependent PMR effect data estimated with the 2D-density approach (Eq. 1., min set to 19%). Standardized residuals depending on predicted PMR effect were calculated and are shown for the 5 neuroactive chemicals: azinphos-methyl, propoxur, flucythrinate, esfenvalerate and tricaine. Mean, standard deviation of standardized residuals and residual square error were calculated, and the total amount of data points (n) are shown.

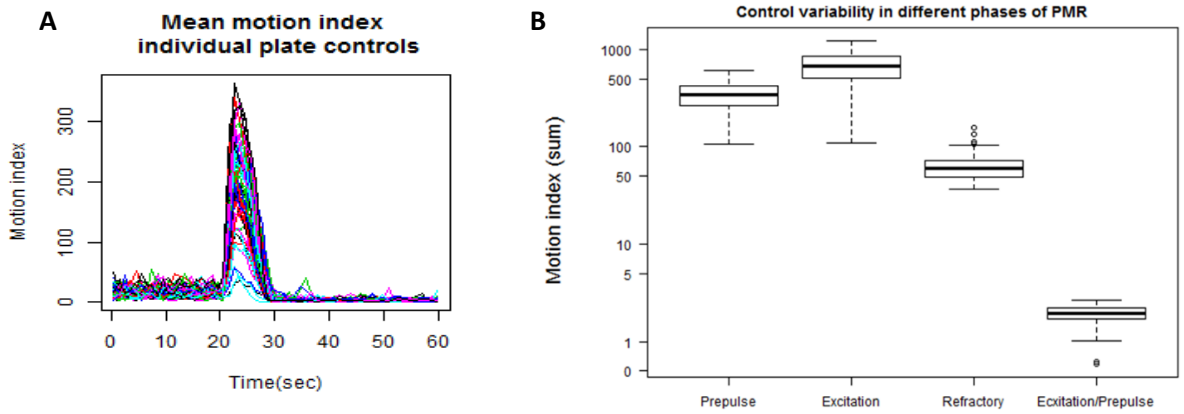


Fig. S2: Raw data plots of the PMR of zebrafish embryos
A) Each graph represents the time course of the mean motion index per 0.5 s bins (the motion index is recorded in 0.04 s intervals, but the mean of 0.5 s bins is shown) for control. The analysis was conducted in 96-well plates. For a total of 62 plates, the 6 wells with non-exposed control embryos were analyzed (with 5 embryos per each well). B) Box plot analysis of PMR phases (sum of motion index within a specific PMR phase) of controls from 62 plates. The boxes represent the median and the 25 and 75% percentiles. Whiskers represent 1.5 times the interquartile range, and the dots refer to any data outside of the interquartile range. Pre-pulse – 1-20 s, Excitation – 21-25 s, Refractory – 40-60 s.

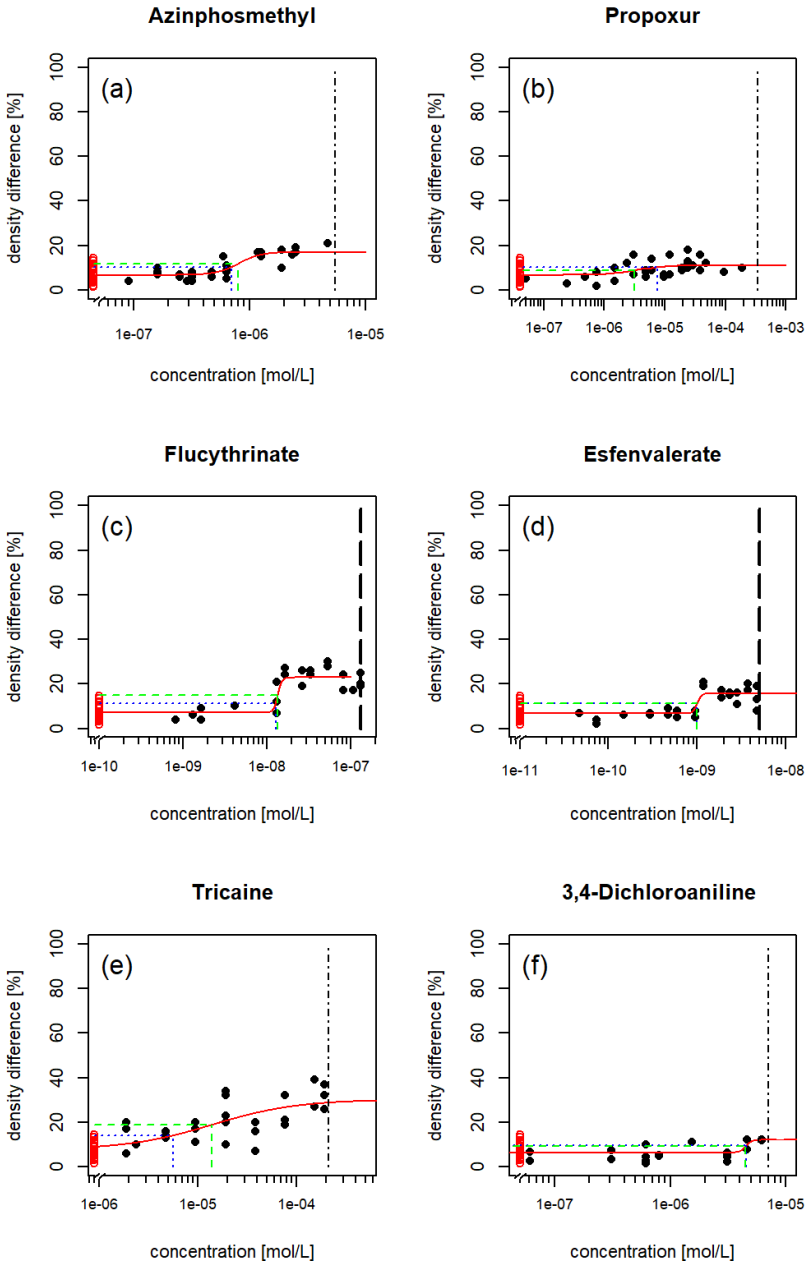
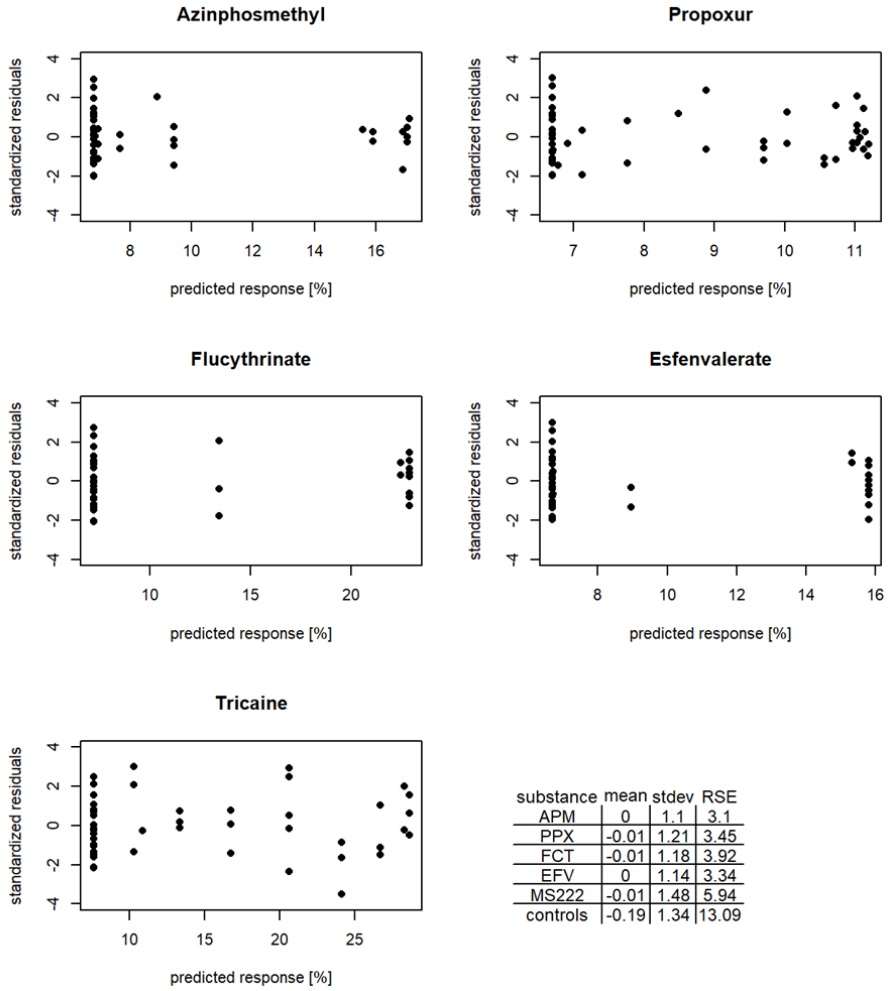


Fig. S3: Concentration response curves for PMR effects (●) in zebrafish embryos (30-35 hpf) exposed to different concentrations of the five neuroactive compounds, acetylcholine esterase inhibitors azinphos-methyl (a) and propoxur (b); voltage gated sodium channel agonists flucythrinate (c) and esfenvalerate (d), and the sodium channel antagonist tricaine. 3,4-Dichloroaniline (f) served as a non-neuroactive reference compound with a narcotic mode of action
Data points (●) represent differences in the overlapping area (OA) of the activity parameter density between control and the respective chemically treated embryos (OA approach). The control variability of the PMR is indicated by open circles (○). A logistic model was fitted to the data and used to calculate EC_{50} values and benchmark concentrations (BMC). The LC_{10} for fish embryos at 48 hpf is indicated by a vertical line (— • —). In the case that no mortality was observed, the maximum water solubility level is indicated by a vertical line (▬ ▬).



substance	mean	stdev	RSE
APM	0	1.1	3.1
PPX	-0.01	1.21	3.45
FCT	-0.01	1.18	3.92
EFV	0	1.14	3.34
MS222	-0.01	1.48	5.94
controls	-0.19	1.34	13.09

Fig. S4: Residual plots for PMR effects estimated by the overlapping area (OA) approach
Standardized residuals depending on predicted response is shown for the five neuroactive chemicals azinphos-methyl, propoxur, flucythrinate, esfenvalerate and tricaine. Mean, standard deviation of standardized residuals, and residual square error for each compound were calculated.

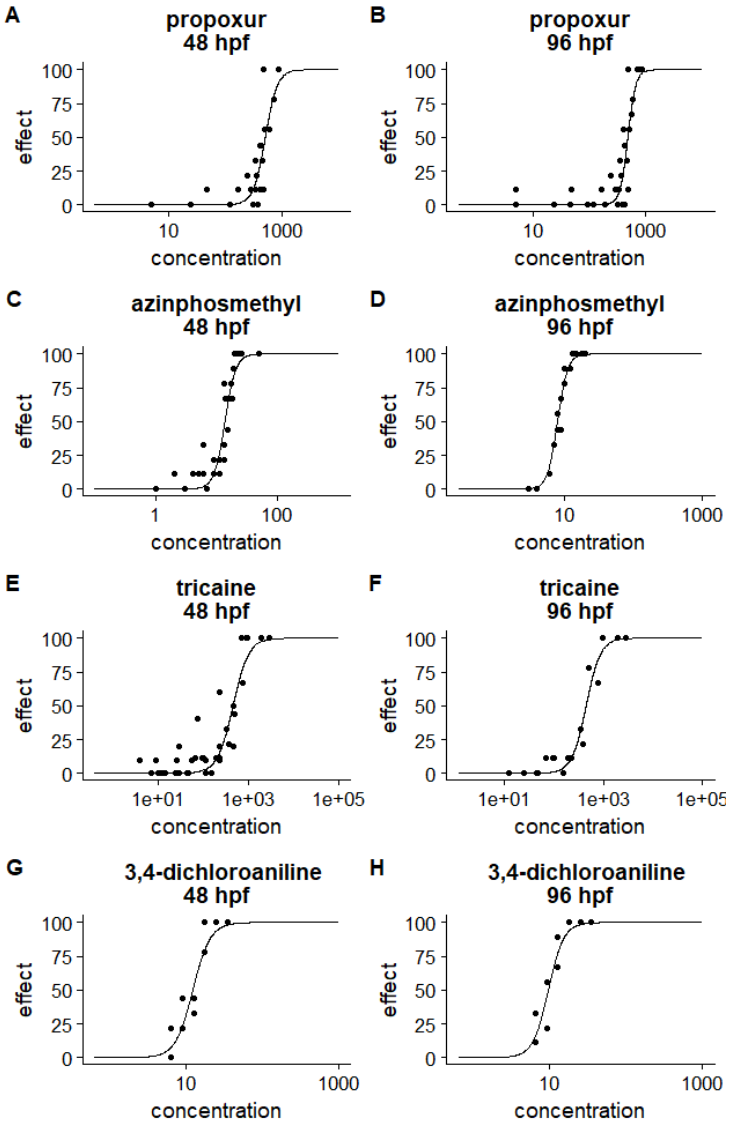


Fig. S5: Lethal effects of the test compounds in the FET test
Effect - % mortality, concentration - µM

References

Belanger, S. E., Rawlings, J. M. and Carr, G. J. (2013). Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Environ Toxicol Chem 32*, 1768-1783. doi:10.1002/etc.2244

Klüver, N., König, M., Ortmann, J. et al. (2015). Fish embryo toxicity test: Identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. *Environ Sci Technol 49*, 7002-7011. doi:10.1021/acs.est.5b01910

Russom, C. L., Bradbury, S. P., Broderius, S. J. et al. (1997). Predicting modes of toxic action from chemical structure: Acute toxicity in the fathead minnow (*Pimephales promelas*). *Environ Toxicol Chem 16*, 948-967. doi:10.1002/etc.5620160514