Neurotoxicology and Teratology 32 (2010) 460–471

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

# Locomotor activity in zebrafish embryos: A new method to assess developmental neurotoxicity

Ingrid W.T. Selderslaghs [a,b,*], Jef Hooyberghs [a,c], Wim De Coen [b], Hilda E. Witters [a]

[a] VITO, Flemish Institute for Technological Research, Unit of Environmental Risk and Health, Division of Toxicology, Boeretang 200, 2400 Mol, Belgium
[b] University of Antwerp, Department of Biology, Laboratory for Ecophysiology, Biochemistry and Toxicology, Groenenborgerlaan 171, 2020 Antwerp, Belgium
[c] Hasselt University, Department WNI, Agoralaan-Building D, 3590 Diepenbeek, Belgium

ARTICLE INFO

*Article history:*
Received 14 October 2009
Received in revised form 4 January 2010
Accepted 1 March 2010
Available online 6 March 2010

*Keywords:*
Zebrafish
Developmental neurotoxicity
Locomotor activity

ABSTRACT

Currently, neurotoxicity testing defined by OECD and FDA is based solely on *in vivo* experiments, using large numbers of animals, being expensive, time-consuming and unsuitable for screening numerous chemicals. The great demand for thousands of chemicals yet to be evaluated, urges the development of alternative test methods which are cheaper, faster and highly predictive for developmental neurotoxicity. In this study, we developed a new method to assess locomotor activity in early life stage of zebrafish at 24 h post fertilization (hpf), in comparison to locomotor activity of zebrafish larvae at 96 to 192 hpf. We hypothesized that this endpoint at early life stages could be used to predict the developmental neurotoxic potential of chemicals and performed exposure studies with chlorpyrifos to demonstrate this. Furthermore, the case study with chlorpyrifos was used to critically evaluate behavioral data analysis and improve method sensitivity. The approach for data analysis using distribution plots for parameters on locomotor activity, next to mean values allowed to obtain more accurate information from the same set of behavioral data, both for embryos and larvae. Embryos exposed to chlorpyrifos, within the range 0.039 to 10 mg/l, exhibited a significant concentration-dependent increase in the frequency and total duration of their spontaneous tail coilings at 24–26 hpf. Larvae exhibited altered swimming activity, as evidenced by a significant decrease in the total duration of movement and an increase in mean turn angle in the range 0.18 to 0.75 mg/l chlorpyrifos. Methodological evaluation showed that locomotor effects in larvae were most pronounced and reproducible at 96 hpf, compared to older individuals (120, 144, 168 and 192 hpf). These new methods based on locomotor activity at early life stages of zebrafish allowed to classify chlorpyrifos as a developmental neurotoxicant. Further research to judge the validity of these alternative methods is currently performed with an extended set of expected positive or negative chemicals for developmental neurotoxicity.



## 1. Introduction

The developing human brain is inherently more susceptible to injury caused by toxic agents than is the brain of an adult [21]. Many agents can cause developmental neurotoxicity as a result of interferences with the developmental processes of the nervous system, such as proliferation, migration, differentiation, synaptogenesis, gliogenesis, myelination and apoptosis [20]. Neurotoxicity detection induced by chemicals represents a major challenge due to the physiological and morphological complexity of the central and peripheral nervous system [1]. Currently, regulatory authorities such as the Organisation for Economic Cooperation and Development (OECD) [17] and the U.S. Environmental Protection Agency (U.S. EPA) [26] use mammalian *in vivo* methods solely for both adult and developmental neurotoxicity testing (DNT). These methods, OECD TG 426 [16] and OPPTS 870.6300 [28] for the evaluation of developmental neurotoxicity and TG 424 [15] and OPPTS 870.6200 [27] for neurotoxicity assessment in adults in which the rat is the preferred species, are mainly based on a neurobehavioral evaluation of cognitive, sensory and motor functions accompanied by neuropathological studies. Although the current DNT guidelines generate useful data for risk assessment purposes [13], many of the recommended tests are complex and expensive in terms of scientific resources, time and animal use [11].

Of the thousands of chemicals on the market, fewer than half have been subjected to even basic laboratory testing for toxicity assessment. Nearly 3000 of these substances are produced in quantities of almost 500 ton every year, but for nearly half of these high-volume chemicals no basic toxicity data are publicly available. Moreover, for 80% of these chemicals, information is lacking with regard to developmental or pediatric toxicity [7]. Due to the actual need to test large sets of compounds for specific regulatory requirements in Europe and the U.S. (e.g. the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) policy and the High Production Volume Programme (HPVP)), there is high pressure to develop

* Corresponding author. VITO, Flemish Institute for Technological Research, Unit Environmental Risk and Health, Division of Toxicology, Boeretang 200, 2400 Mol, Belgium. Tel.: +32 14 335261; fax: +32 14 582657.
*E-mail address:* hilda.witters@vito.be (H.E. Witters).

0892-0362/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ntt.2010.03.002

alternative test strategies which are more rapid, economically feasible and have an acceptable predictive capacity [2].

Cell culture techniques have been developed to address key biochemical and functional features of developmental neural biology, such as cell migration, formation of neuronal networks, synaptogenesis and neuron–glia interaction [4]. However, due to the complexity of the nervous system, none of the existing *in vitro* models entirely reflects the *in vivo* situation as, once isolated, most of the neuronal culture systems represent cells that are no longer part of any integrated neural network [1]. Moreover, *in vitro* models cannot recapitulate more complex endpoints such as behavior, which is the ultimate result of neuronal development and signaling [4]. Simple whole organism systems such as the nematode, fruit fly or zebrafish, which enable assessment of integrative effects together with knowledge of the organism's complete genome, may offer a more immediately viable and ultimately more powerful approach to DNT alternatives [11].

In the search for alternative models for DNT, the zebrafish offers many advantages: low cost, high throughput, an almost limitless range of morphological and functional assays, and an apparently high degree of similarity with mammalian systems [18]. Moreover, the fundamental processes of neurodevelopment in zebrafish are homologous to those that occur in humans [25].

In this study we explored the possibility to assess the developmental neurotoxic potential of a compound in zebrafish embryos and larvae after exposure to a known developmental neurotoxicant. We therefore developed and optimized two new methods based on the locomotor activity in zebrafish, namely spontaneous tail coilings in embryos and swimming activity in larvae. Chlorpyrifos (CPF), a widely employed organophosphate pesticide, was selected as the model compound for the development and standardization of screening procedures since evidence of the developmental neurotoxic potential of this compound is extensive [5].

## 2. Materials and methods

### 2.1. Test animals and collection of eggs

Adult, wild type, zebrafish were obtained from a commercial supplier (Aquaria Antwerp, Aartselaar, Belgium) and were kept at the VITO laboratory facility at least three weeks prior to the first intended spawning. The adult fish were maintained in large 60–70 l aquaria with a maximum density of 1 g fish/l water at 25 ± 2 °C with a constant light–dark (14–10 h) cycle. The water was continuously aerated and renewal of the water occurred in a semi-static manner. Fish were regularly provided with a varied diet, consisting of commercial dry flake food, young water flea and live brine shrimp. The health condition of the fish was checked daily, except weekends.

Prior to spawning, males and females were housed separately for a minimum of five days. The day before eggs were required, males and females were placed in breeding tanks with a 6:4 male:female ratio. The breeding tanks were equipped with a spawning tray, which consisted of a fine net with an appropriate mesh size for eggs to fall through, close to the bottom of the tank. The fish were left undisturbed overnight and eggs could be collected 1 hour after the light was turned on the next morning. Eggs were rinsed in 0.0002% methylene blue (CAS # 7220-79-3, Sigma-Aldrich), diluted in medium (see below) and placed into large petri dishes. At the start of exposure to test conditions, embryos were transferred to microtiter plates which were placed in the incubator where they were maintained on a 14:10 light:dark cycle at 28.5 ± 0.5 °C. If relevant for the experiments, exposure was continued in the incubator in static conditions without external feeding until free swimming larval stages at the age of 192 hpf.

At the end of experiments, embryos and larvae were placed in a 0.05% solution of 2-phenoxy-ethanol for the purpose of euthanasia. The procedures described in this study were approved by the local ethical committee.

### 2.2. Medium and test substance

Fish water, reconstituted as described in the test guideline OECD 203, annex 2 was used as the dilution medium for all the experiments. Water quality parameters were regularly checked and measurements were in the following ranges: pH 7.5–8.0, conductivity 632–676 µS/cm², hardness 217–235 mg/l $CaCO_3$, oxygen 92–98%.

Chlorpyrifos (CAS# 2921-88-2) was obtained from Sigma-Aldrich. Stock solutions with concentrations of 25, 10 and 0.75 mg/ml were prepared in 100% dimethylsulfoxide (DMSO) and kept refrigerated. Test solutions were freshly prepared in fish water with a final DMSO concentration of 0.1% (v/v). For all test solutions, pH was checked (range 6.8–8) and oxygen levels of the solutions were always higher than 80%.

### 2.3. Determination of the highest non-teratogenic concentration (HNTC)

To determine an appropriate concentration regimen for further evaluation of locomotor activity, teratogenic endpoints induced by CPF were evaluated as described in our former work [24]. Briefly, embryos were exposed within 2 h post fertilization (hpf) to a dilution series of CPF in the range of 0.05 to 25 mg/l, as derived from preliminary experiments. For each concentration tested as well as negative and vehicle controls, a minimum of 15 embryos were placed in a solution in a 6-well plate. Starting at 6 hpf, fertilization success was assessed and twelve fertilized eggs were transferred to a 24-well plate, one embryo per well containing corresponding test solutions of CPF or control solutions. Exposure was static and continuous without feeding. Morphological characteristics of each individual were evaluated at 24, 48, 72 and 144 hpf by means of a light microscope. The characteristics evaluated were selected with respect to normal developmental processes and included the presence and morphological development (as appropriate) of otoliths, eyes, somites, tail detachment, heart beat, circulation, pigmentation, hatching, kinks in the tail, sidewise position and active swimming behavior of larvae. Mortality and the presence of malformations were assessed for each concentration and time point of development. Final experiments were conducted three times ($n = 3$) and based on these data, concentration–response curves were created for each age for which an evaluation was performed for both embryotoxicity and teratogenicity. The highest non-teratogenic concentration (HNTC) was defined as the highest concentration at which no malformations or lethality were observed in at least 90% of the individuals in three consecutive experiments. The HNTC was determined for 24 and 144 hpf, and these were the highest test concentrations used for experiments on locomotor behavior for embryos and larvae respectively.

### 2.4. Evaluation of spontaneous tail coilings in zebrafish embryos

Within 2 hpf, a minimum of 60 zebrafish embryos were placed in a well of a 6-well plate containing either a 0.1% DMSO solution (vehicle control) or a CPF solution. Five concentrations of CPF, starting at the HNTC at 24 hpf (see Results, Section 3.1) and further diluted by a factor of 4, were tested in separate experiments, each time with corresponding controls. During exposure, embryos were placed at 28.5 ± 0.5 °C in a temperature and light controlled incubator with a constant light–dark (14–10 h) cycle. Starting at 6 hpf, fertilization success was assessed (range 80–100%) and a minimum of 48 fertilized embryos were transferred to fresh solutions.

Spontaneous tail coilings were evaluated in embryos aged 24 to 26 hpf. Embryos, which did not show any malformations, were transferred to a 96-well plate with round bottom (Iwaki brand, Asahi Glass Co., LTD, Japan) a few minutes before the evaluation, four embryos per well in 200 µl of solution. Vehicle controls and CPF exposed embryos were placed in an alternating manner on the plate. Tail coilings were detected with a high resolution movie file (mpeg2) with a duration of 5 min (at a maximum of 25 frames per second),

obtained with a camera mounted on an inversed light microscope (Olympus IX81, magnification 2.5×). Subsequently, mobility of each embryo was evaluated by means of the EthoVision software (Noldus, Wageningen, The Netherlands). Movie files were evaluated at a sample rate of five frames per second and detection occurred based on gray values of the embryo. For each embryo, a separate arena was defined around the embryo and optimal detection variables (low and high limit for gray values) were set as to guarantee optimal detection. EthoVision detected the changing status of individual pixels occurring between two subsequent samples. The thresholds for the detection of tail coilings, defined as strong mobility, and total immobility were set by verifying concordance between EthoVision output and visual assessment of the number of spontaneous tail coilings. Intermediate changes of pixels, which did not correspond to strong mobility, nor total immobility, were defined as 'mobility'.

After evaluation of movie files for mobility of each embryo, raw data and values for frequency and total duration of the different states of mobility (immobile, mobile, strong mobile) for each individual were exported from EthoVision. Based on raw data, a quality control check was performed before analysis. The raw data included the total surface area (the number of pixels which had a gray value within the defined range) and the changed surface area (number of pixels with changed status) for each sample. The ratio changed surface area / total surface area was plotted against time for the total duration of the trial and for each embryo. Quality of the data for each embryo was assessed visually based on those plots which had to comply with criteria defined in advance. Tracks from embryos which did not met those criteria were discarded from the final dataset. Furthermore, due to the occasional presence of missing samples, the exact total duration of the tracks was calculated as the sum of the total duration of immobility, mobility and strong mobility. Values for frequency and total duration of the tail coilings (=strong mobility only) were normalized to the exact duration of the tracks instead of the theoretical 300 s. Further analysis was performed on the normalized data.

### 2.5. Zebrafish larval locomotor activity assay

Within 2 hpf, zebrafish embryos were placed in 6-well plates containing either a 0.1% DMSO solution (vehicle control) or a CPF solution. A series of five concentrations of CPF was tested, starting at the HNTC at 144 hpf (see Results, Section 3.1) and further diluted by a factor of 2. Each treatment group, consisting of minimum 60 embryos, had a corresponding vehicle control group. After exposure, embryos were placed at 28.5 ± 0.5 °C in a temperature and light controlled incubator with a constant light–dark (14–10 h) cycle. Starting at 6 hpf, fertilization success was assessed and fertilized embryos were placed in a 48-well plate with flat bottom (Iwaki brand, Asahi Glass Co., LTD, Japan), one embryo per well in 1 ml of solution, corresponding to the treatment group in the 6-well plates. Two 48-well plates were used for each concentration tested, and they each contained 24 control as well as 24 exposed embryos. Exposure was continuous, the plates were covered with sealing tape to minimize volatilization and solutions were not renewed. Embryos were allowed to hatch within the plates and larvae were allowed to use up the remainder of their yolk and were left undisturbed without external feeding in the incubator until their swimming activity was assessed.

Prior to the assessment of the swimming activity of the larvae, they were screened for teratogenic effects, as described above. Larvae which displayed malformations, although in low numbers as the highest test concentration was the HNTC at 144 hpf, were excluded for locomotor analysis.

In order to select an optimal observation condition, the swimming activity of larvae was evaluated at consecutive ages of 96, 120, 144, 168 and 192 hpf for the same individuals. This was achieved using movie files (mpeg 2) with a duration of 5 min obtained with the Tracksys Tower Filming System (www.tracksys.co.uk). This equipment was composed of an infra-red light source, a high resolution digital infrared video camera (at a maximum of 25 frames per second) and a hood to exclude external light. Well plates were placed under the hood and recording was started immediately. Subsequently, measurement of the swimming activity was performed with the EthoVision locomotion tracking software at 25 frames per second. Swimming movements were captured within predefined arenas (one arena corresponded to one well, with an internal diameter approximating 10 mm), by means of subtraction (i.e. differences in contrast between samples of the movie and a single reference image from that movie). For each movie file, the detection variables were set as to guarantee optimal detection of all larvae. After tracking, a quality control measure was applied as wells showing incorrect tracking (e.g. detection of the edge of the well instead of the larva) were discarded from the final dataset. Thereafter, the EthoVision software analyzed the data and provided values for activity parameters, characterizing the swimming activity of each individual.

### 2.6. Data analysis

#### 2.6.1. Data handling

For each concentration tested in the embryo assays, three independent experiments were performed with corresponding control conditions. The data obtained for the mobility status of embryos from all separate experiments were normalized and then combined as to constitute a complete concentration series. In this way, three data sets i.e. concentration series were created to be handled as three experimental runs. As the output from the larval swimming assays contained concentration series in each experiment, this remained unchanged.

Outliers were removed from each of the datasets, where outliers were defined as measured values that were not within the range mean ±2*standard deviation of the mean (range 0–8.3% outliers present/dataset).

#### 2.6.2. Quantification of effect

The quantification of exposure effect on an activity parameter $x$, was established as follows. Consider the distributions of a large sample of control individuals (C) and exposed individuals (E), as shown for a hypothetical example in Fig. 1A. In this study, we used the overlap area (OA) of the two distributions as a measure for the exposure effect (Fig. 1D). In this section we will show that the OA is a meaningful effect measure since it is directly related to the minimal classification error of a Bayesian classification model with a flat prior distribution.

In this approach we recognize that the separation of the two distributions in Fig. 1A, is related to the ability to make a classification model from this data. Suppose one is presented a sample of new individuals together with their activity parameters $x$, but with unknown E or C. How can one build a classification model with a minimal average error in allocating these animals to E or C based on $x$?

First, for notational simplicity assume that a classifier can be identified with the choice of one threshold value $x_T$ (see Fig. 1B), then the probability of misclassification is written as $P_{error} = \int_{-\infty}^{x_T} P(x,C)dx + \int_{x_T}^{+\infty} P(x,E)dx$ which is the sum of the probability to allocate an individual to $E$ while it is $C$ and vice versa. Here $P(x,E)$, $P(x,C)$ is the joint probability that an individual has activity parameter $x$ and is $E$ or $C$ respectively. Next denote the class-conditional probabilities as $P(x|E)$, $P(x|C)$ which correspond to the two probability distributions from Fig. 1A and can be estimated from experiments with known $E$ and $C$ samples. Next we assume to have no prior knowledge on an individual that needs to be classified, hence the a priori probability is equal: $P(E) = P(C) = 1/2$, i.e. a flat prior distribution. Since $P(x,E) = P(x|E) \cdot P(E)$, $P(x,C) = P(x|C) \cdot P(C)$ one gets $P_{error} = \frac{1}{2}\left[\int_{-\infty}^{x_T} P(x|C)dx + \int_{x_T}^{+\infty} P(x|E)dx\right]$ which is one half of the shaded area shown in Fig. 1C. Next $P_{error}$ can be minimized using

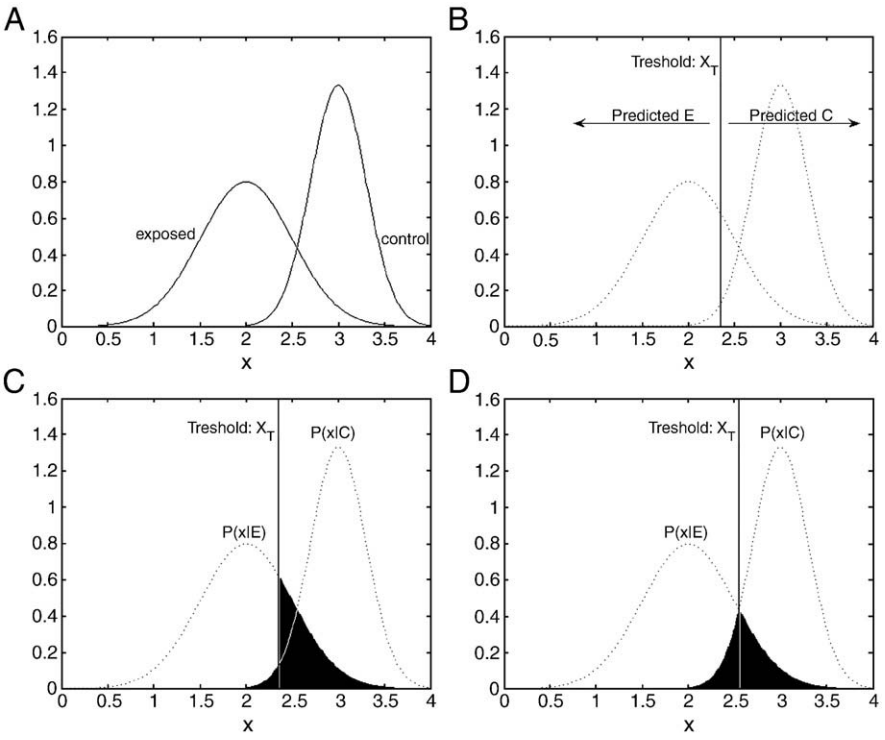


**Fig. 1.** (A) Hypothetical distribution or histogram for activity parameter $x$ in the exposed and control group (B) threshold based classification model (C) graphical interpretation of (two times) the misclassification probability and (D) graphical interpretation of (two times) the minimal misclassification probability as the overlap area of the class conditional distributions.

a Bayesian approach. What is needed are the posterior probabilities $P(E|x)$, $P(C|x)$. The classification is optimal with the allocation of an individual to $E$ if $P(E|x)>P(C|x)$ and C otherwise. These posteriors can be inferred from the class conditional ones by "The Bayes theorem": $P(E|x) = \frac{P(x|E).P(E)}{P(x)}$, $P(C|x) = \frac{P(x|C).P(C)}{P(x)}$. Using again the flat prior distribution one immediately arrives at the optimal selection rule: $E$ if $P(x|E)>P(x|C)$ and $C$ otherwise. The final graphical interpretation of the minimal classification error, using a flat prior, is $(P_{error})_{\min} = \frac{1}{2}OA$ with OA the overlap area as shown in Fig. 1D.

The percentage of exposed individuals that demonstrate locomotor activity which is very distinct from that of controls, and thus do not coincide with controls (Fig. 1D), then equals 1-OA or 1-2$P_{error}$. This percentage is further referred to as 'effect percentage'.

The above described analysis was performed for the activity parameters frequency and total duration of strong mobility of embryos, and total distance moved, mean velocity, mean turn angle and frequency and total duration of movement of larvae. Effect percentages calculated were plotted versus the logarithm of the concentration and thus creating concentration–response curves.

*2.6.3. Interpretation of results*

In order to be able to distinguish the effects originating from the biological variability between individuals from effects resulting from exposure, the biological variability within a population of controls was estimated by dividing the control group of a concentration series into two randomly composed groups, to which the above described method of analysis was applied. This was repeated 1000 times and the mean and standard deviation of the acquired effect percentages for controls were calculated. This was done separately for each of the endpoints in each of the experiments. If the effect percentages acquired for the comparison of controls versus exposed were higher than the one acquired for the comparison controls versus controls, the effect was attributed to interference by the compound tested and not to biological variability.

*2.6.4. Statistical significance*

Supplementary to fore mentioned method of analysis, a two-tailed Student's $t$-test was performed on the raw data for controls and exposed individuals, which assumed equal variance of the data. Differences were considered significant if $p<0.05$.

## 3. Results

### 3.1. Teratogenic endpoints observed

At 24 hpf, lethality was observed in approximately 20% of zebrafish embryos exposed to 25 mg/l CPF. The remaining individuals in this exposure group showed pericardial and yolk sac edema. There were no such effects observed at lower concentrations CPF tested. At 144 hpf, the lowest concentration inducing lethality (65% of individuals) corresponded to 6.25 mg/l CPF. Further, skeletal deformities and sidewise position of larvae were observed at 144 hpf with exposures to 1 mg/l CPF and higher. The HNTCs of CPF, to be used in the locomotor assays, were determined to be 10 and 0.75 mg/l at 24 and 144 hpf respectively.

### 3.2. Spontaneous tail coilings in zebrafish embryos

*3.2.1. Methodological evaluation*

At first, thresholds set in EthoVision for the detection of, and discrimination between, immobility, mobility and strong mobility were verified. In order to do so, detection of the different states of mobility was compared with a visual assessment. Whenever EthoVision detected strong mobility, one could observe a spontaneous tail coiling. No tail coilings could be observed for which detection did not occur. This goes for every embryo, selected at random from different experiments, for which this assessment was carried out. Thus, the threshold set for strong mobility, enabled accurate and reliable tracking. For immobility and mobility, however, inaccurate tracking was observed. This could be attributed to the background noise, present in the movie files, as the

percentage of changed pixels between two samples never reached zero although the embryo was completely immobile upon visual observation. Moreover, there was only a small difference between immobility and mobility to start with as compared to strong mobility and thus detection in EthoVision sporadically switches between immobile and mobile without any observable movement. This was also reflected by a higher degree of variability in the distribution of control embryos between different experiments as illustrated in Fig. 2 for frequency of the different states. As also demonstrated in Fig. 2 this led to a shift of the calculated mean for the frequency of especially immobility and mobility. Moreover, standard deviations of the mean for immobility and mobility were substantially higher in comparison to those calculated for strong mobility, in contrast to the relatively small difference in standard deviation of the mean across experiments for the different mobility states (Fig. 2). For further analysis of the effect of exposure to chlorpyrifos, only data obtained for strong mobility were thus considered.

### 3.2.2. Effects observed for chlorpyrifos

Chlorpyrifos was able to induce changes in the locomotor activity of zebrafish embryos at 24–26 hpf. The frequency as well as the total duration of the spontaneous tail coilings (referred to as strong mobile) increased as evidenced by the clear right hand shift of the distributions of exposed embryos (Fig. 3). Although data for controls originated from independent experiments, the range of the obtained data was relatively constant across experiments and showed high repeatability, as can be derived from Figs. 2 and 3. This implicated that effects observed after exposure to different concentrations of chlorpyrifos as well as those observed in independent repeat experiments, could be compared with each other. In this respect, concentration–response curves were created based on three experimental runs (Fig. 4). Standard deviations of the mean effect percentages, as shown in Fig. 4, were relatively small and did thus demonstrate that also data for embryos exposed to the respective concentrations of CPF, were relatively constant and showed high repeatability. In addition, estimation of the biological variability showed a mean effect percentage with standard deviation of $14 \pm 0.5\%$ for both frequency and total duration of strong mobility. Effect percentages, exceeding 14.5%, were considered to be due to exposure. Moreover, when a Student's *t*-test was performed on raw data for controls versus exposed embryos for the different concentrations of CPF tested (Table 1), no significant differences were found for the concentrations for which effect percentages were at or close to the level of biological variability of controls. Higher concentrations of CPF

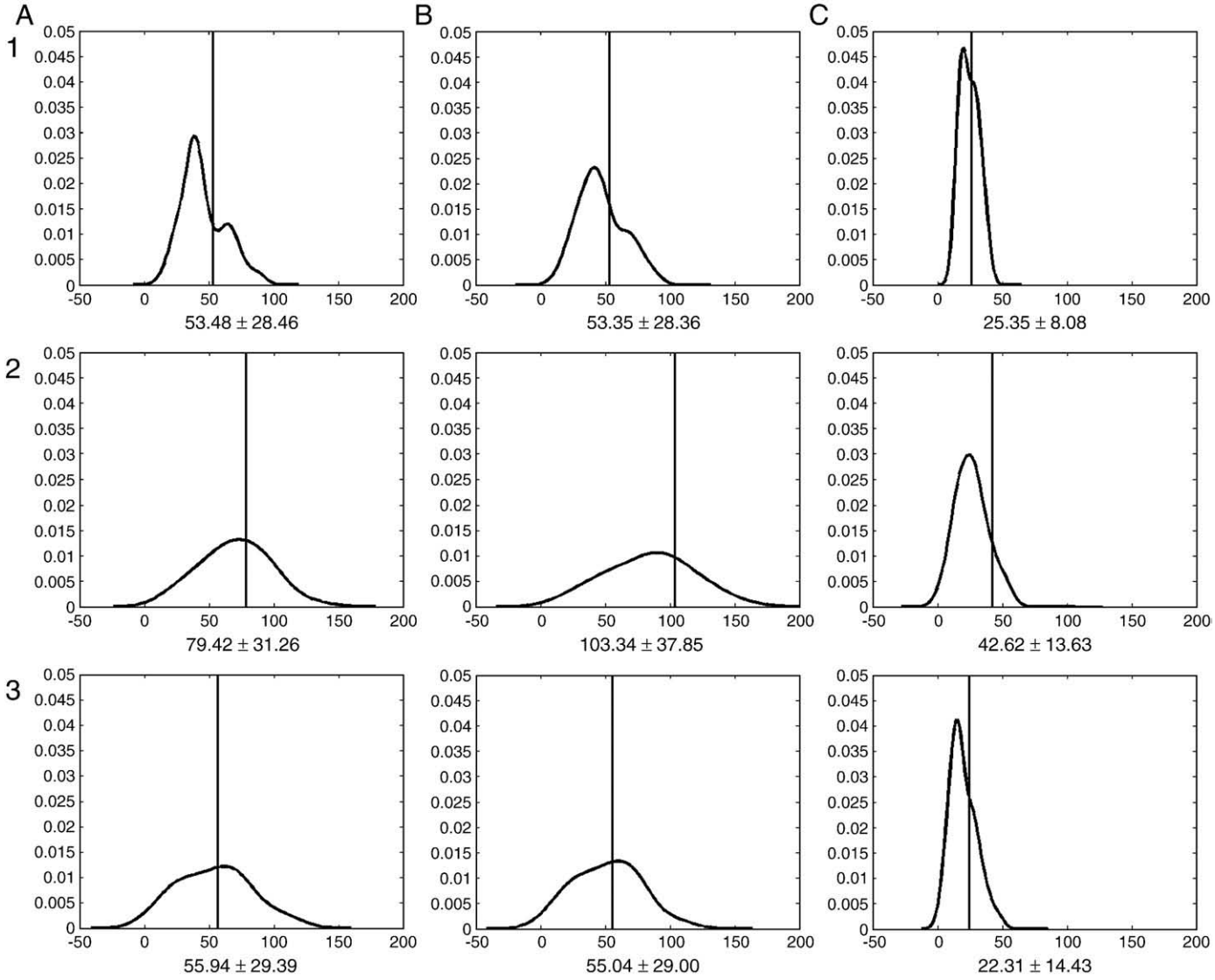


**Fig. 2.** Distributions (measured values for the parameter indicated in *X*, plotted against the incidence of this value being measured in the population in Y), obtained for control embryos from 3 independent experiments (1–3) for frequency of (A) immobility, (B) mobility and (C) strong mobility. Calculated mean frequencies are indicated by the vertical lines. Mean and standard deviation values for frequency are given beneath the graphs.

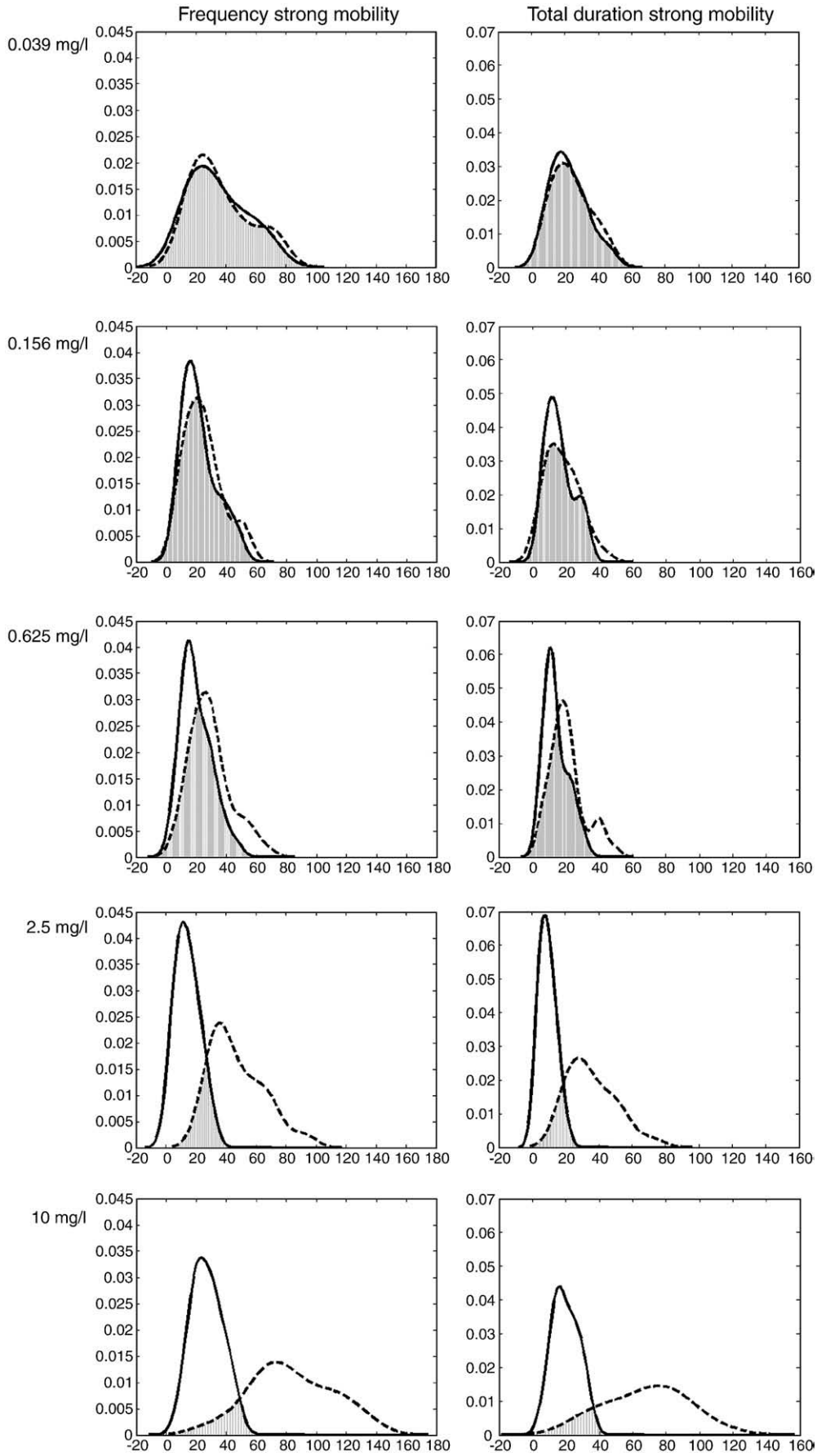


**Fig. 3.** Distributions (measured values in *X*, plotted against the incidence of this value being measured in the population in *Y*) for frequency (left) and total duration (right) of strong mobility from controls (full line) and embryos exposed to chlorpyrifos (dotted line), originating from independent experiments. The overlap area is indicated in grey.

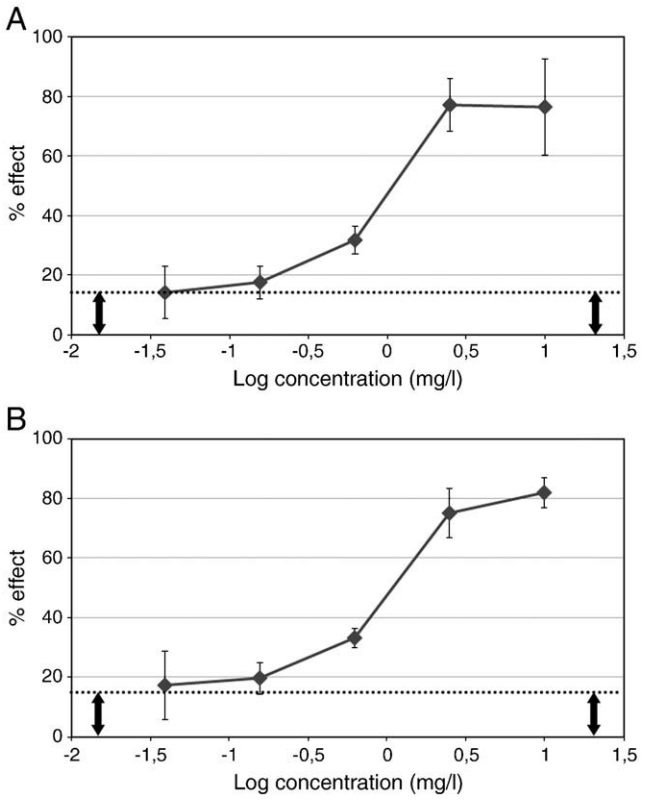


**Fig. 4.** Concentration–response curves for (A) frequency and (B) total duration of strong mobility ($n=3$ experiments) in zebrafish embryos after exposure to chlorpyrifos (0.039, 0.156, 0.625, 2.5 and 10 mg/l) at 24–26 hpf. % effect (mean ± SD) is shown versus the logarithm of the concentrations tested (mg/l). The mean biological variation compared to 0 in control embryos is indicated by the dotted line.

showed effect percentages which were clearly higher than the level of biological variability and highly significant ($p$-values<0.01) as listed in Table 1.

### 3.3. Locomotor activity in zebrafish larvae

#### 3.3.1. Methodological evaluation

Initially, as no prior knowledge concerning the swimming behavior of zebrafish larvae was available, swimming behavior was assessed at five ages, namely 96, 120, 144, 168 and 192 hpf for the same individuals. Data from control larvae were analyzed in order to obtain insight into the 'normal' ranges for the different parameters assessed. As shown in Fig. 5, the total distance moved for control larvae for the duration of a trial, differed at different ages. At 168 and 192 hpf, larvae covered less distance during the whole duration of the trial. As their total distance moved approached zero at these ages, we considered them inappropriate for further consideration for the reason that potential left hand shifts of the distributions of exposed larvae, might be missed. At 96, 120 and 144 hpf, normal ranges for total distance moved were farther away from zero when compared to 168 and 192 hpf, and thus accurate assessment of the effect of an exposure, either left or right hand shift of the distribution of exposed larvae, was more likely. Nevertheless, differences were observed for control larvae when one compared activity to earlier developmental stages, e.g. 96 versus 120 hpf. As a consequence, corresponding controls for each time point of CPF exposure had to be included and evaluated to guarantee an adequate experimental design.

In addition, we assessed the variability amongst control larvae in the same experiment but on different plates. As mean values for controls (data not shown) tended to differ between plates, within the same experiment, we decided to have a set-up containing within-plate controls in order to accurately evaluate the effects of exposure to chlorpyrifos.

#### 3.3.2. Effects observed for chlorpyrifos

The effect of exposure to CPF on the swimming behavior of zebrafish larvae was thus assessed at ages 96, 120 and 144 hpf. This assessment was based on the evaluation of five parameters descriptive for the locomotor activity, namely, total distance moved, mean velocity, mean turn angle, frequency of movement and total duration of movement. Not all of these parameters were affected at all or affected to an equal extent by CPF exposure, nor were they affected to an equal extent at all ages evaluated. The presence of a concentration–response was used as the acceptance criterion in order to distinguish between random effects and effects induced by exposure and to finally retain reliable parameters.

As data obtained for total distance moved, mean velocity and frequency of movement did not meet the acceptance criterion, they were not taken into consideration for the determination of effects exerted by CPF on the swimming behavior of larvae. On the other hand, data obtained for mean turn angle (degrees) did meet the acceptance criterion by displaying clear right hand shifts of the distributions for larvae exposed to CPF in a concentration-dependent manner at 96, 120 as well as 144 hpf (data not shown). Therefore, concentration–response curves could be created at 3 time points of larval development for the parameter mean turn angle (Fig. 6). The biological variability observed in the population of control larvae at 96, 120 and 144 hpf was estimated to be 13.2 ± 1.1, 15.1 ± 1.3 and 14.2 ± 0.8% respectively. Effect percentages obtained for larvae exposed to 0.75 mg/l (HNTC at 144 hpf) were clearly higher than the estimation of the biological variability at all time points evaluated. Moreover, for larvae exposed to this concentration, $p$-values<0.01 were obtained with a Student's $t$-test (Table 2). At 96 hpf, statistical significant results ($p<0.05$ obtained with a Student's $t$-test) (Table 2) and effect percentages higher than the estimated biological variability were also obtained for larvae exposed to 0.1875 and 0.375 mg/l CPF.

Besides mean turn angle, the parameter total duration of movement also complied with the acceptance criterion. At 96 hpf, CPF induced a decrease in the total duration of movement (left hand shift of distribution of exposed larvae). This was most pronounced after exposure to the two highest concentrations tested as evidenced by effect percentages higher than the biological variability and $p$-values<0.05 (Table 3). Contrary to observations at 96 hpf, no reproducible concentration-dependent effects of exposure were observed at 120 hpf (Table 3). Effect percentages obtained, were the result of a redistribution of the histograms of exposed larvae where no horizontal shifts were present and thus no statistical significant results ($p<0.05$) were obtained. At 144 hpf, left hand shifts of the distributions of larvae exposed to the two highest concentrations tested were observed in two out of three independent experiments (Table 3). Effect percentages for the highest concentration tested were higher than the biological variation and significantly different from the control group with $p<0.05$ for those two experiments.

## 4. Discussion

In this study, we describe two new methods to assess the locomotor activity in early life-stage zebrafish after exposure to a developmental neurotoxicant. Locomotor activity, namely spontaneous tail coilings in embryos at 24–26 hpf and swimming activity in larvae at 96, 120 and 144 hpf, was selected as an endpoint in these assays as a measure for developmental neurotoxicity. Research in the zebrafish has already shown that the motor network starts with a very simple circuit and builds complexity by adding layers of organization. Each new layer increases the range of possible motor acts from purely reflexive to volitional behaviors [22]. The first motor activity that is generated by the developing motor network in zebrafish is presented by spontaneous tail coilings. It has been shown by others that perturbation of the early

**Table 1**
Effect percentages, *p*-values and direction of the shift of distributions for exposed embryos, obtained for frequency and total duration of strong mobility at 24–26 hpf, for 3 independent experiments (cfr Figs. 3 and 4) in comparison to control group.

| | | | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution |
| Frequency strong mobility | Controls (mean ± SD) | | 13.85 ± 5.90 | | | 14.33 ± 5.94 | | | 14.51 ± 6.52 | | |
| | Concentration CPF (mg/l) | 0.039 | **23.80** | **0.013** | → | 11.95 | 0.075 | – | 6.54 | 0.483 | – |
| | | 0.156 | **22.58** | 0.884 | – | **18.46** | 0.626 | → | 11.73 | 0.339 | – |
| | | 0.625 | **30.09** | **$4.7^{*}10^{-4}$** | → | **37.06** | **$3.6^{*}10^{-4}$** | → | **28.18** | **0.011** | → |
| | | 2.5 | **67.76** | **$9.5^{*}10^{-14}$** | → | **85.32** | **$9.2^{*}10^{-16}$** | → | **78.40** | **$8.9^{*}10^{-13}$** | → |
| | | 10 | **80.06** | **$8.5^{*}10^{-17}$** | → | **90.50** | **$1.3^{*}10^{-18}$** | → | **58.87** | **$4.8^{*}10^{-16}$** | → |
| Total duration strong mobility | Controls (mean ± SD) | | 14.31 ± 5.98 | | | 13.53 ± 6.23 | | | 14.43 ± 6.32 | | |
| | Concentration CPF (mg/l) | 0.039 | **28.39** | **0.004** | → | **17.82** | 0.085 | – | 5.5 | 0.898 | – |
| | | 0.156 | **20.28** | 0.633 | – | **24.54** | 0.964 | → | 14.03 | 0.182 | – |
| | | 0.625 | **36.39** | **$2.6^{*}10^{-4}$** | → | **33.04** | **$6.4^{*}10^{-4}$** | → | **30.01** | **0.005** | → |
| | | 2.5 | **65.58** | **$9.8^{*}10^{-13}$** | → | **78.75** | **$1.9^{*}10^{-13}$** | → | **80.96** | **$4.4^{*}10^{-13}$** | → |
| | | 10 | **76.18** | **$1.6^{*}10^{-14}$** | → | **85.38** | **$1.7^{*}10^{-17}$** | → | **84.38** | **$7.7^{*}10^{-17}$** | → |

Notes:
Effect percentages, exceeding the estimated biological variation of the control group and *p*-values < 0.05 are shown in bold.
Symbols used: → the distribution of exposed embryos shifted to the right in comparison to the distribution of control embryos, – the distribution of exposed embryos did not shift in comparison to the distribution of control embryos.

pattern of spontaneous activity affects motor axon guidance in the chick embryo [8] and disturbs the assembly of spinal motor networks in the mouse [14]. Although no records were found that similar events occur in zebrafish, we studied this first pattern of activity. This spontaneous locomotor behavior has, by our knowledge, not yet been considered by others as an endpoint for toxicity studies and thus might be a new approach in zebrafish behavioral studies. Here, we evaluated whether chlorpyrifos is able to affect this spontaneous motor activity in zebrafish embryos or at larval stages of zebrafish development.

### 4.1. Analysis of the methodology

We exposed zebrafish embryos to CPF and evaluated spontaneous tail coilings in zebrafish embryos at 24–26 hpf and swimming activity in larvae at 96, 120 and 144 hpf. In order to ascertain that effects observed on locomotor activity, are due to a disturbance in the motor network of the nervous system rather than to morphological defects, the highest concentration tested, corresponded to the highest non-teratogenic concentration at the embryonic and larval stage for the respective assays. Malformations observed at 144 hpf, including skeletal deformities and pericardial edema, were also reported by others [10], although the concentration reported in their study was lower (malformations occurring after exposure to 0.25 mg/l at 96 and 120 hpf) than the HNTC of 0.75 mg/l reported here.

In order to evaluate the results of neurobehavioral studies, it is necessary to understand the observed variability of the parameters being measured as this has implications for the study design, conduct, analysis and finally interpretation of any apparently treatment-related effects. Variability is considered as being of two basic types, intrinsic and extrinsic variability [19]. Intrinsic variability is the natural 'background' variability among individuals (controls) that is inherent in each parameter that is being measured. Here, it is termed 'biological' variability. As this variability cannot be controlled, neither ruled out, one must consider that the magnitude of this variability should be known [19]. In the present study, the biological variability was estimated by determining effect percentages amongst control individuals and thus intrinsic variability was taken into account in interpreting data obtained. Moreover, the method of analysis reported can also detect changes in variability that may be due to exposure to the test chemical.

The quantification of exposure effect on an activity parameter, poses some practical problems. First, the effect may influence the distribution in an a priori unknown manner, it can be an effect on mean, variance, skewness, … which makes the choice for a specific statistical test less trivial. Secondly, the use of a significance value of a statistical test e.g. a Student's *t*-test, is not straightforward since it depends on sample size which can vary in practice [9]. To deal with these two problems we used instead the overlap area (OA) of the distributions of control and exposed individuals as a measure for the exposure effect. In addition, our method allows to estimate the number of exposed individuals which display activity that is distinct from that of controls based on the distribution plots. The latter has advantages compared to the use of a significance value which can only demonstrate the absence or presence of a difference between groups [9]. Moreover, the estimation of the number of affected individuals enables us to create concentration–response curves. We thus believe that our new method of analysis using the distributions has clear advantages by providing supplementary information. Furthermore, for comparability to usual data analysis methods, we complemented our method with a Student's *t*-test to obtain a reference statistical value. In order to avoid fluctuations for *p*-values, sample sizes were kept as constant as possible and large enough in both assays. As can be derived from Tables 1–3, both methods of evaluation provide the means to conclude that CPF has an effect on locomotor activity of zebrafish embryos and larvae. When the distribution of exposed individuals displays a clear horizontal shift in comparison to the distribution obtained for controls, this is reflected by an effect percentage exceeding the estimated biological variability as well as a *p*-value < 0.05. Moreover, results in Tables 1–3 consistently show that the lowest concentration for which an effect could be observed, based on our method of analysis, is systematically lower than the one observed, based on the *p*-values obtained with the Student's *t*-test. This also means that subtle effects on locomotor activity are faster detected using our new method. This means that, for those studies where one should depend solely on a Student's *t*-test, changes in the intrinsic variability will likely not be detected at all (Table 3). With the higher sensitivity of our method, the likelihood to observe false positive effects may increase (see Table 2; higher effect percentages at 144 hpf after exposure to a low concentration), but we anticipated to this by including the criterion that concentration dependent effects should be seen.

### 4.2. Comparison of embryo and larval assays related to their sensitivity

After exposure to the HNTC of CPF, effects on locomotor activity were observed in both the embryonic and larval assays.

Effects of CPF exposure obtained for the swimming activity of larvae, were the most pronounced at 96 hpf in comparison to 120 and

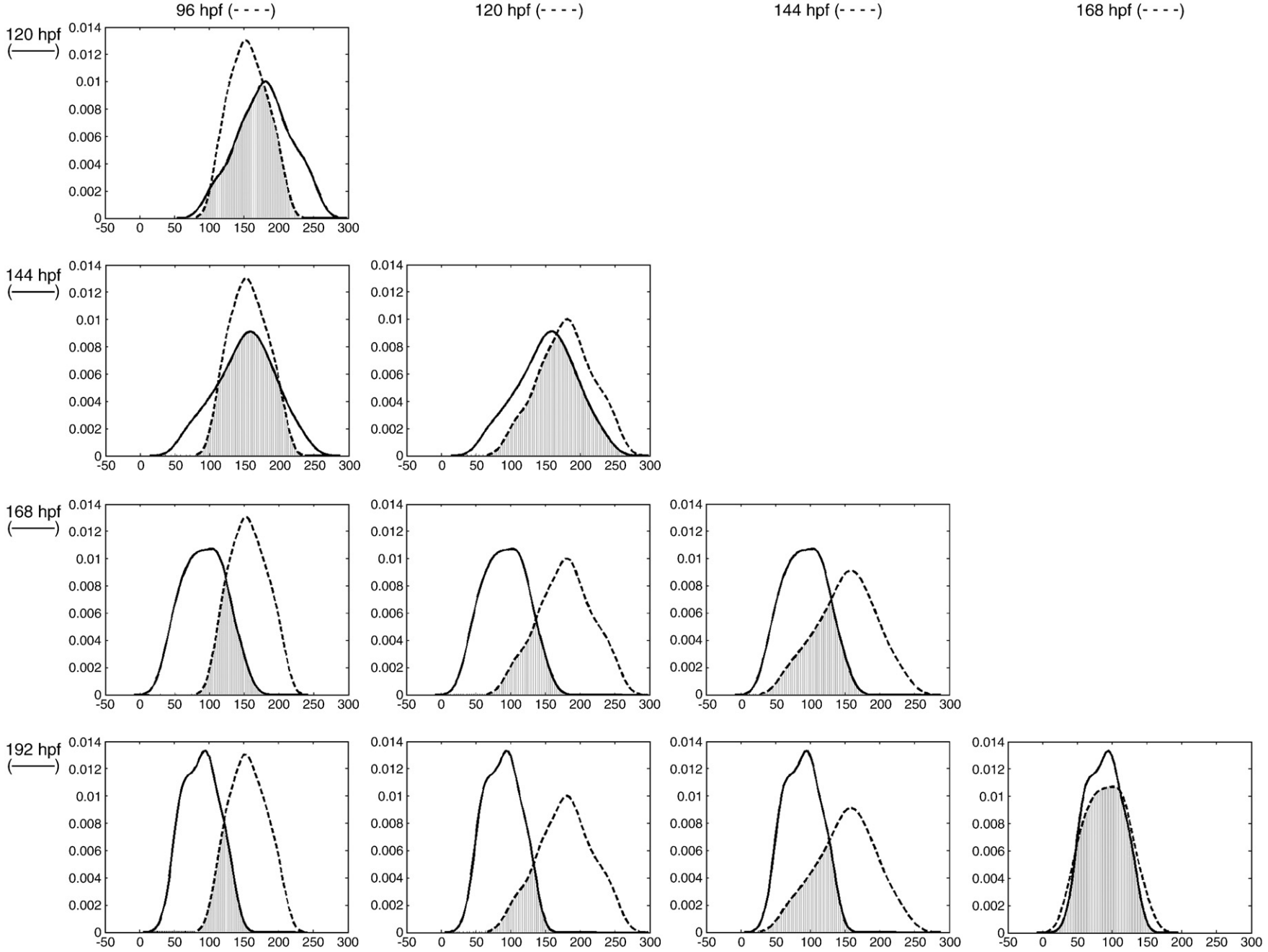


**Fig. 5.** Comparison of distributions of measured values for total distance moved ($X$), plotted against the incidence ($Y$) of larvae from the control population which display the corresponding value, at different time points of evaluation. Time points, aligned horizontally, are represented by the distributions with a dotted line; time points, aligned vertically, are represented by the distributions with a full line.

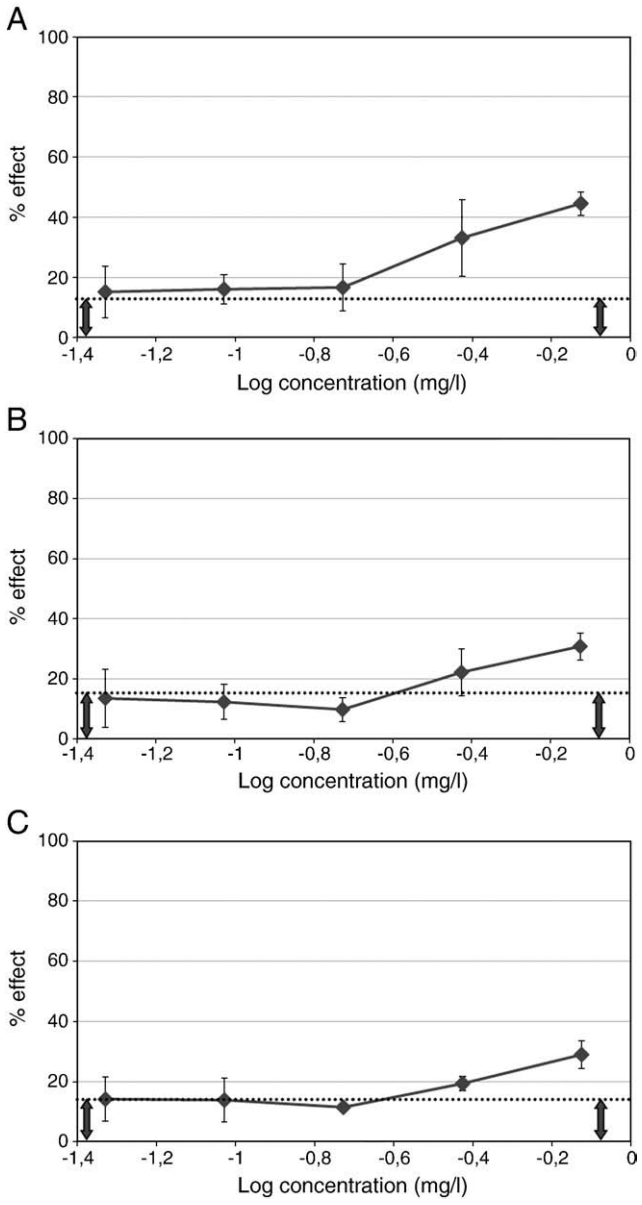


**Fig. 6.** Concentration–response curves for mean turn angle (°) at (A) 96, (B) 120 and (C) 144 hpf ($n = 3$ experiments) of zebrafish larvae after exposure to chlorpyrifos (0.0469, 0.0938, 0.1875, 0.375 and 0.75 mg/l). % effect (mean ± SD) is shown versus the logarithm of the concentrations tested (mg/l). The mean biological variation in control larvae is indicated by the dotted line.

144 hpf. This is evidenced by the slope of the concentration–response curves (Fig. 6) which are more steep at 96 hpf as well as the higher effect percentages obtained (Table 3). Furthermore, results obtained at 120 hpf seem to be less robust than those obtained at 96 and 144 hpf (Table 3). Consequently, for CPF, analysis of swimming activity could be limited to 96 hpf in order to determine whether the compound can affect swimming in zebrafish larvae. But, as no prior knowledge regarding other compounds to be tested with the reported assay is available, we suggest not to limit future evaluations with other chemicals to a single moment in development.

Although different concentration series for CPF were tested in the embryonic versus the larval assay, one can observe that for similar concentrations (e.g. 0.625 mg/l in the embryo and 0.75 mg/l in the larval assay), similar effect percentages were obtained. This indicates a comparable sensitivity for both assays. The lowest concentration of CPF for which still effects on locomotor activity could be observed in a minimum two out of three experiments, corresponds to 0.039 and 0.047 mg/l for the total duration of strong mobility in the embryonic assay and total duration of movement at 96 hpf in the larval assay respectively. This also demonstrates that our reported methods of analyzing effects on locomotor activity in both embryos and larvae, are more sensitive than the one reported by Levin et al. in which effects were only observed after exposure to 0.1 mg/l at 144 hpf [12]. For the method described by Kienle et al. [10], however, the reported lowest effective dose of CPF for larvae aged 120 hpf corresponded to 0.01 mg/l.

### 4.3. Biological relevance of effects observed

In embryos, 24–26 hpf, we demonstrated an increase in locomotor activity after exposure to CPF. This effect is caused by an increase in both frequency and total duration of spontaneous tail coilings. At the moment of spontaneous tail coiling, which starts at 17 hpf, the spinal cord is still immature and only four types of neurons are present at this time point [23]. Primary motoneurons show repetitive spontaneous periodic depolarizations. Sodium channels, gap junctions and calcium activated potassium channels have been implicated in the generation, propagation and termination of these periodic depolarizations respectively. Moreover, it has been shown that the average frequency of depolarization in motoneurons from embryos, matches the average frequency of the spontaneous coiling behavior [22]. To our knowledge, there are no reports in which effects of CPF or a metabolite of CPF on sodium channels, gap junctions and calcium activated potassium channels in motoneurons available. Therefore, we cannot provide a plausible explanation for the increases in locomotor activity in embryos in this study. However, as the frequency of the coils in embryos peaks at 1 Hz at 19 hpf and slowly decreases to 0.1 Hz by 26 hpf [22], one can suggest that exposure to CPF induces a delay in the development of embryos although this possible delay, likely subtle, was not apparent in the teratological screening performed.

Larvae displayed a decrease in locomotor activity at control conditions as shown for total distance moved (Fig. 5) with age (168 and 192 hpf). To our knowledge, this fact has not been previously reported. Although we cannot confirm, we suggest it may be due to a decreasing access to energy supply as the yolk sac is almost depleted from nutrients at this time while they received no external feeding. After exposure to CPF, a decrease in swimming activity was observed in larvae at 96, 120 and 144 hpf as also previously reported [10,12]. As CPF is a known cholinesterase inhibitor [5] and as for mammals, acetylcholinesterase is critical to the normal development of the zebrafish nervous system [3], this may be the cause of the impaired swimming activity observed.

### 4.4. Conclusions

In this study, we describe two new methods with which we explored the possibility to assess the developmental neurotoxic potential of a compound in zebrafish embryos and larvae after exposure to a known developmental neurotoxicant, namely chlorpyrifos. These two new screening methods based on the locomotor activity in zebrafish, namely spontaneous tail coilings in embryos and swimming activity in larvae were thoroughly optimized and standardized in our lab. The method described to analyze the obtained data is unique as it does not solely consider a significance value, obtained with a statistical test, to determine whether there are effects present due to exposure but adds up new information by consideration of distribution of data for control and exposed groups. We have shown that this method has numerous advantages and in addition is more sensitive than a Students's *t*-test.

After exposure to CPF, a well known developmental neurotoxicant, significant effects on the selected endpoints, namely spontaneous tail coilings in embryos and swimming activity in larvae were observed. We propose that these endpoints can be used in the screening of developmental neurotoxicants, other than CPF and these methods are suited for medium-throughput screening purposes. Shared principles of locomotor network organization have been described from invertebrates to vertebrates, including mammals. The segmented

**Table 2**
Effect percentages, *p*-values and direction of the shift of distributions for larvae exposed to chlorpyrifos, obtained for mean turn angle at 96, 120 and 144 hpf for 3 independent experiments in comparison to control group.

| | | | | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution |
| Mean turn angle | 96 hpf | Controls (mean ± SD) | | 11.93 ± 5.20 | | | 13.64 ± 5.83 | | | 14.00 ± 6.27 | | |
| | | Concentration CPF (mg/l) | 0.0469 | **15.20** | 0.516 | – | 6.49 | 0.697 | – | **23.52** | **0.044** | → |
| | | | 0.0938 | **21.05** | 0.509 | → | 11.39 | 0.073 | – | **15.43** | 0.549 | – |
| | | | 0.1875 | **24.34** | **0.002** | → | **16.54** | **0.047** | → | 8.77 | **0.172** | – |
| | | | 0.375 | **28.05** | **0.001** | → | **47.58** | **$1.1^{*}10^{-5}$** | → | **23.57** | **0.019** | → |
| | | | 0.75 | **42.83** | **$6.1^{*}10^{-5}$** | → | **41.63** | **$5.9^{*}10^{-8}$** | → | **49.00** | **$8.3^{*}10^{-8}$** | → |
| | 120 hpf | Controls (mean ± SD) | | 13.58 ± 5.40 | | | 15.76 ± 6.53 | | | 15.89 ± 6.62 | | |
| | | Concentration CPF (mg/l) | 0.0469 | 10.77 | 0.623 | – | 5.48 | 0.829 | – | **24.20** | 0.432 | ↑ |
| | | | 0.0938 | 12.61 | 0.988 | – | 6.28 | 0.593 | – | **17.93** | 0.322 | ↑ |
| | | | 0.1875 | 11.95 | 0.248 | – | 5.15 | 0.753 | – | 12.13 | 0.663 | – |
| | | | 0.375 | **25.56** | 0.263 | → | 13.19 | 0.331 | → | **27.78** | 0.581 | → |
| | | | 0.75 | **35.96** | **$1.3^{*}10^{-5}$** | → | **28.72** | 0.186 | → | **27.74** | **0.008** | → |
| | 144 hpf | Controls (mean ± SD) | | 13.32 ± 5.94 | | | 14.87 ± 5.66 | | | 14.31 ± 5.75 | | |
| | | Concentration CPF (mg/l) | 0.0469 | 6.38 | 0.567 | – | **20.68** | 0.736 | ↓ | **15.19** | 0.162 | → |
| | | | 0.0938 | **22.18** | 0.717 | ↑ | 9.05 | 0.504 | – | 10.18 | 0.232 | – |
| | | | 0.1875 | 12.30 | 0.975 | – | 10.19 | 0.343 | – | 11.64 | 0.409 | – |
| | | | 0.375 | **18.21** | 0.914 | ↑ | **21.93** | **0.049** | → | **17.66** | 0.704 | – |
| | | | 0.75 | **23.72** | **$4.8^{*}10^{-4}$** | → | **30.55** | **0.007** | → | **32.48** | **0.001** | → |

Notes
Effect percentages, exceeding the estimated biological variation of the control group and *p*-values < 0.05 are shown in bold.
Symbols used: → the distribution of exposed larvae shifted to the right in comparison to the distribution of control larvae, – the distribution of exposed larvae did not shift in comparison to the distribution of control larvae, ↑/↓ the base of the distribution of exposed larvae is smaller/broader corresponding to a smaller/larger range of obtained values in comparison to the distribution of control larvae.

hindbrain and spinal cord for example, are important locomotor regions in all vertebrates, that are highly homologous in structure across species [6]. This encourages the view that at the earliest stages of development, insights gained from model vertebrates such as zebrafish may shed light on common developmental principles and that zebrafish embryos and/or larvae may also be excellent models for studying toxicant-induced impaired development of the nervous system and behavioral dysfunction. Whether the methods proposed, are able to reveal the developmental neurotoxic potential of other compounds, will be the subject of further research.

**Table 3**
Effect percentages, *p*-values and direction of the shift of distributions for larvae exposed to chlorpyrifos, obtained for total duration of movement at 96, 120 and 144 hpf for 3 independent experiments in comparison to control group.

| | | | | Experiment 1 | | | Experiment 2 | | | Experiment 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution | % effect | *p*-value | Shift distribution |
| Total duration of movement | 96 hpf | Controls (mean ± SD) | | 14.69 ± 6.64 | | | 12.59 ± 5.73 | | | 14.45 ± 6.73 | | |
| | | Concentration CPF (mg/l) | 0.0469 | **18.99** | 0.384 | ← | **18.19** | 0.611 | – | 10.42 | 0.635 | ← |
| | | | 0.0938 | 13.38 | 0.941 | – | **21.12** | **0.042** | ← | **18.71** | 0.597 | ↑ |
| | | | 0.1875 | **18.17** | 0.064 | ← | **21.52** | **0.042** | ← | **29.22** | 0.932 | ↑ |
| | | | 0.375 | **19.31** | **0.014** | ← | **32.31** | **8.4*10 − 5** | ← | **16.33** | 0.062 | ← |
| | | | 0.75 | **35.17** | **3.4*10 − 4** | ← | **47.75** | **0.003** | ← | **32.91** | **8.1*10 − 4** | ← |
| | 120 hpf | Controls (mean ± SD) | | 18.04 ± 7.29 | | | 15.77 ± 7.18 | | | 16.32 ± 6.48 | | |
| | | Concentration CPF (mg/l) | 0.0469 | **26.01** | 0.231 | ↑ | **17.84** | 0.391 | – | 13.43 | 0.145 | ↓ |
| | | | 0.0938 | 16.14 | 0.498 | ↑ | 10.84 | 0.449 | – | **25.01** | 0.598 | ↑ |
| | | | 0.1875 | **19.72** | 0.400 | ↑ | **25.56** | 0.116 | ↓ | 10.02 | 0.981 | – |
| | | | 0.375 | **23.81** | 0.921 | – | 4.38 | 0.883 | – | 5.88 | 0.869 | – |
| | | | 0.75 | **27.41** | 0.158 | ↓ | **22.52** | 0.279 | ↓ | **28.39** | 0.127 | ↓ |
| | 144 hpf | Controls (mean ± SD) | | 16.53 ± 6.86 | | | 13.95 ± 5.97 | | | 11.59 ± 5.63 | | |
| | | Concentration CPF (mg/l) | 0.0469 | 10.63 | 0.782 | – | 8.18 | 0.533 | – | 6.89 | 0.368 | ← |
| | | | 0.0938 | 13.29 | 0.623 | ↑ | 12.6 | 0.288 | – | 10.25 | 0.944 | – |
| | | | 0.1875 | 12.43 | 0.555 | ↑ | 11.13 | 0.892 | – | 5.12 | 0.758 | – |
| | | | 0.375 | **16.54** | 0.954 | ↑ | 10.35 | 0.245 | ← | **14.66** | 0.449 | ← |
| | | | 0.75 | **23.25** | 0.088 | ↓ | **19.17** | **0.008** | ← | **20.17** | **0.004** | ← |

Notes
Effect percentages, exceeding the estimated biological variation of the control group and *p*-values < 0.05 are shown in bold.
Symbols used: ← the distribution of exposed larvae shifted to the left in comparison to the distribution of control larvae, – the distribution of exposed larvae did not shift in comparison to the distribution of control larvae, ↑/↓ the base of the distribution of exposed larvae is smaller/broader corresponding to a smaller/larger range of obtained values in comparison to the distribution of control larvae.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Acknowledgements

Ingrid Selderslaghs was supported by a VITO fellowship. This work was supported by grants from the John Hopkins Center for Alternatives to Animal Testing (CAAT 2006-28 and 2007-22) and Henkel-Phenion. For excellent advice the authors are grateful to An Van Rompay and for the technical assistance to Francis Boonen and Guy Geukens.

## References

[1] A.K. Bal-Price, H.T. Hogberg, L. Buzanska, S. Coecke, Relevance of in vitro neurotoxicity testing for regulatory requirements: challenges to be considered, Neurotoxicol Teratol 32 (2010) 36–41.
[2] A.K. Bal-Price, C. Sunol, D.G. Weiss, E. van Vliet, R.H. Westerink, L.G. Costa, Application of in vitro neurotoxicity testing for regulatory purposes: symposium III summary and research needs, Neurotoxicology 29 (2008) 519–530.
[3] M. Behra, X. Cousin, C. Bertrand, J.L. Vonesch, D. Biellmann, A. Chatonnet, U. Strahle, Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo, Nat. Neurosci. 5 (2002) 111–118.
[4] S. Coecke, A.M. Goldberg, S. Allen, L. Buzanska, G. Calamandrei, K. Crofton, L. Hareng, T. Hartung, H. Knaut, P. Honegger, M. Jacobs, P. Lein, A. Li, W. Mundy, D. Owen, S. Schneider, E. Silbergeld, T. Reum, T. Trnovec, F. Monnet-Tschudi, A. Bal-Price, Workgroup Report: incorporating *in vitro* alternative methods for developmental neurotoxicity into international hazard and risk assessment strategies, Environ. Health Perspect. 115 (2007) 924–931.
[5] T. Colborn, A case for revisiting the safety of pesticides: a closer look at neurodevelopment, Environ. Health Perspect. 114 (2006) 10–17.
[6] P. Drapeau, L. Saint-Amant, R.R. Buss, M. Chong, J.R. McDearmid, E. Brustein, Development of the locomotor network in zebrafish, Prog. Neurobiol. 68 (2002) 85–111.
[7] P. Grandjean, P.J. Landrigan, Developmental neurotoxicity of industrial chemicals, Lancet 368 (2006) 2167–2178.
[8] M.G. Hanson, L.T. Landmesser, Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules, Neuron 43 (2004) 687–701.
[9] R.R. Holson, L. Freshwater, J.P.J. Maurissen, V.C. Moser, W. Phang, Statistical issues and techniques appropriate for developmental neurotoxicity testing: a report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints, Neurotoxicol. Teratol. 30 (2008) 326–348.
[10] C. Kienle, H.R. Kohler, A. Gerhardt, Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae, Ecotoxicol. Environ. Saf. 72 (2009) 1740–1747.
[11] P. Lein, E. Silbergeld, P. Locke, A.M. Goldberg, In vitro and other alternative approaches to developmental neurotoxicity testing (DNT), Environ. Toxicol. Pharmacol. 19 (2005) 735–744.
[12] E.D. Levin, H.A. Swain, S. Donerly, E. Linney, Developmental chlorpyrifos effects on hatchling zebrafish swimming behavior, Neurotoxicol. Teratol. 26 (2004) 719–723.
[13] S.L. Makris, K. Raffaele, S. Allen, W.J. Bowers, U. Hass, E. Alleva, G. Calamandrei, L. Sheets, P. Amcoff, N. Delrue, et al., A retrospective performance assessment of the developmental neurotoxicity study in support of OECD test guideline 426, Environ. Health Perspect. 117 (2009) 17–25.
[14] C.P. Myers, J.W. Lewcock, M.G. Hanson, S. Gosgnach, J.B. Aimone, F.H. Gage, K.F. Lee, L.T. Landmesser, S.L. Pfaff, Cholinergic input is required during embryonic development to mediate proper assembly of spinal locomotor circuits, Neuron 46 (2005) 37–49.
[15] OECD, Test guideline 424, OECD guideline for testing of chemicals, Neurotoxicity Study in Rodents, 1997.
[16] OECD, Test guideline 426, OECD guideline for testing of chemicals, Developmental Neurotoxicity Study, 2008.
[17] Organization for economic cooperation and development, www.oecd.org.
[18] R.T. Peterson, R. Nass, W.A. Boyd, J.H. Freedman, K. Dong, T. Narahashi, Use of non-mammalian alternative models for neurotoxicological study, Neurotoxicology 29 (2008) 546–555.
[19] K.C. Raffaele, J.E. Fisher Jr, S. Hancock, K. Hazelden, S.K. Sobrian, Determining normal variability in a developmental neurotoxicity test A report from the ILSI Research Foundation/Risk Science Institute expert working group on neurodevelopmental endpoints, Neurotoxicol. Teratol. 30 (2008) 288–325.
[20] D. Rice, S. Barone Jr., Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models, Environ. Health Perspect. 108 (Suppl 3) (2000) 511–533.
[21] P.M. Rodier, Vulnerable periods and processes during central nervous system development, Environ. Health Perspect. 102 (Suppl 2) (1994) 121–124.
[22] L. Saint-Amant, Development of motor networks in zebrafish embryos, Zebrafish 3 (2006) 173–190.
[23] L. Saint-Amant, P. Drapeau, Time course of the development of motor behaviors in the zebrafish embryo, J. Neurobiol. 37 (1998) 622–632.
[24] I.W. Selderslaghs, A.R. Van Rompay, W. De Coen, H.E. Witters, Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo, Reprod. Toxicol. 28 (2009) 308–320.
[25] V. Tropepe, H.L. Sive, Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav. 2 (2003) 268–281.
[26] U.S. Environmental Protection Agency, www.epa.gov.
[27] U.S. E.P.A., Health effects test guidelines OPPTS 870.6200 Neurotoxicity Screening Battery, (1998).
[28] U.S. EPA, Health effects test guidelines OPPTS 870.6300 Developmental Neurotoxicity study, (1998).