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COILING TAIL ACTIVITY IN ZEBRAFISH EMBRYO: A PROTOCOL FOR AN EARLY WARNING SYSTEM OF NEUROTOXIC SUBSTANCES

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ABSTRACT

Thousands of chemical substances daily reach the aquatic environment, mainly deriving from industrial, urban, and agricultural activity. Such chemical substances can form mixtures that are difficult to detect with routine chemical analysis. Many of them exhibit neuroactive properties and they are considered an emerging issue for both human and environmental health. The real impact of exposure to neurotoxic contaminants on the ecosystem is not yet well known but there is evidence that they can cause important changes in organism behavior with a long-term impact on biodiversity and human health. Humans can be indirectly exposed to neurotoxicants for example through ingestion of fishery products and drinking waters. Neurotoxicity was identified as one of the most emerging Modes of Action (MoAs) in the aquatic environment. An improvement to bridge the gap of knowledge in this sector is needed and new standardized methods are required.

The aim of this study is to verify the feasibility of the Coiling Activity Test (CAT) with the zebrafish embryo model applied on environmental samples and chemical substances. Spontaneous movements of the tail in embryos occurred earlier at 17 hours post-fertilization (hpf) originate from a single neural circuit and are influenced by contaminant exposure. The count of bursts and their intensity are important parameters of the neurotoxic effect and specific software allows rapid measurement of them.

We applied the CAT on different environmental samples and chemical substances at different laboratory conditions in order to recommend a rapid, cost-effective operating protocol for environmental analysis. In parallel, we have carried out the 96 hours Fish Embryo Acute Toxicity (FET) test (OECD, 236) to add more information on the samples and to verify the sensibility between the two tests.

The spontaneous tail movement in zebrafish is demonstrated to be a very powerful tool in eco-neurotoxicological studies as it provides in few hours important screening information on the presence of dangerous neurotoxic substances in the ecosystem. This test could give also a valid contribution to implementing the regulatory guidelines for the identification of neurotoxic risks in the ecosystems.

KEYWORDS:
Zebrafish embryo, coiling activity, neurotoxicity, early warning system

INTRODUCTION

The number of neuroactive compounds released into the ecosystems has been increasing over the past years, and there is, therefore, a growing interest in assessing the related potential risks for both ecological and human health [1]. Indeed, many chemical pollutants commonly exhibit neurotoxic effects on a wide range of living organisms, including invertebrates [2], fish [3], birds [4], and humans [5]. Legradi *et al*., in recent years, have prompted the use of the term 'environmental neurotoxicity' when referring to human exposure neurotoxicity [1]. It has been estimated that up to 30% of all commercially used chemicals may have neurotoxic potential, around 30.000 chemicals in total. Moreover, the chemicals in the environment can be present at low concentrations, escaping the limits of instrumental detectability, and can also form mixtures with unknown effects. In this context, the ecotoxicological evaluation is a solid help in bridging the knowledge gap together with the chemical analysis [6]. The Joint Research Centre (JRC) of the European Commission (EU) has recently drawn up the third Technical Report [7] under the EU Water Framework Directive (WFD) [8]that includes a list of emerging contaminants that are worthy of major attention to establishing whether they have to be classified as 'priority substances' on the basis of a risk assessment procedure that includes European exposure data. Different substances listed in this Report, e.g. the antibiotic ciprofloxacin and the fungicide tebuconazole, are suspected to be neurotoxicants [9, 10].

With the aim to adequately address the need for legal requirements on neurotoxic effects to be consistently in place to ensure that risks from simultaneous exposure to multiple chemicals are effectively and systematically taken into account across chemicals-related policy areas. As it is currently not realistic nor economically feasible to specifically monitor, assess and regulate an almost infinite number of possible combinations of chemicals, the scientific consensus is emerging that the detection of effects

through Effect Based Methods (EBMs) need to be taken into account [11]. It is important to know which are the real effects caused by the sum of the chemical substances in the aquatic environment (including emerging pollutants, metabolites and transformation products) and to link the observed effects with cost-effective management objectives. The report of the European Environmental Agency (EEA) of 2018 clearly states that EBMs could represent great support for the identification of effects caused by mixtures of pollutants and not monitored substances [12].

To investigate the effects of chemical pollutants with neurotoxic potential both in terms of ecological and human health, the early life stages of animals represent a powerful tool [1, 13]. In this framework, fishes have proved extremely beneficial [14, 15]. Behavioural analyses, such as the study of locomotion, have been proposed as critical in detecting potential neuroactive effects [16]. Furthermore, the spontaneous tail coiling showed by the embryos of zebrafish (*Danio rerio*) might represent another important endpoint in neurotoxicity assessment [17]. This method allows a very quick screening since it can be performed within 24 hours [18].

In this study, we assessed the capability and reliability of the coiling activity test (CAT) based on the spontaneous tail movements, i.e. coiling activity test applied to zebrafish embryos in detecting potential neurotoxic effects in different environmental water samples. Specifically, we aimed to evaluate the results provided by this promising method in combination with the Fish Embryo Acute toxicity (FET) Test [19].

MAJOR NEUROTOXICITY ENDPOINTS FOR ZEBRAFISH

In the last 15 years, zebrafish has been widely used as a non-mammal vertebrate model for toxicology and drug research [20], and today it is considered a powerful and useful tool for neurotoxicity assessment [14, 21, 22]. In this perspective, different endpoints have been proposed with the aim of investigating potential neurotoxicity in zebrafish embryos and early larvae. Such endpoints require different timing across zebrafish development depending on the parameter to be used. However, all these analyses must be concluded within 120 hours after egg fertilisation (hpf) [23, 24]. A summary of the main neurotoxicity endpoints for zebrafish is reported below.

a. Locomotor activity. Locomotor activity, i.e. swimming behaviour, can be an important endpoint in neurotoxicity detection in zebrafish, with responses in most cases comparable to locomotion analysis in mammals: as in mammals, ethanol-treated zebrafish embryos showed altered locomotor activity [14]. Moreover, many chemicals already known for their neurotoxic potential for mammals showed a similar mode of action (MoA) when tested on zebrafish [25]. Selderslaghs *et al*. proposed the new method for locomotor analysis in 2010 [26]. The larval activity was evaluated at consecutive ages of 96, 120, 144, 168 and 192 hpf in 48-well plates with the help of a camera paired to a tracking system. In this case, behavioural tracking software was used in order to evaluate the embryo activity. It has to be noted that particular attention should be placed on the selection of the plates, as several studies observed how embryos locomotor activity can be affected by the depth of the wells [27, 28].

b. Acetylcholinesterase (AChE). Several studies support the use of zebrafish as a screening tool for neurotoxicity induced by chemicals on the cholinergic system [20, 29-31]. The cholinergic system is associated with several cognitive functions and cognitive processes. The concentration of Acetylcholine (ACh), the neurotransmitter involved in the cholinergic system, are regulated by two different cholinesterases, acetylcholinesterase (AChE) (E.C. 3.1.1.7) and butyrylcholinesterase (BuChE) (E.C. 3.1.1.8) [31]. In this context, zebrafish is a useful and simple model for neurotoxic studies, as it has a well-conserved amino acidic sequence for the AChE gene [32]. Moreover, Butyrylcholinesterase is not encoded in the zebrafish genome [30], so that it only expresses acetylcholinesterase (AChE), without any butyrylcholinesterase activity [29]. This is a useful characteristic as it makes the evaluation of the AChE concentrations simpler [32, 33].

c. Lateral tail movements. Lateral tail movements represent the first spontaneous behaviour observed in zebrafish embryos; at 28.5°C this phenomenon starts at 17 hpf [34], while at 26°C its occurrence has been observed since 23 hpf [18]. The tail coiling test, based on this principle, consists of the evaluation of spontaneous tail coiling frequency in zebrafish embryos, usually aged 24 to 26 hpf, as a possible indicator of neurotoxicity [26]. Several studies have been focused on this test in recent years and thanks to its sensitivity, the coiling test is overall considered a promising and efficient tool [25, 35]. It has to be noted, however, that in some cases different studies have been carried out at different temperatures, ranging from 26 °C [18] to 28.5°C [26] and using different types of multi-well plates for exposure and observation, ranging from 96 wells [26] to 24 [18]. This lack of uniformity could in some cases affect the results, and it has been observed that differences in temperature can modify the tail coiling behaviour and occurrence [18]. For these reasons, a more accurate method standardisation may probably be required in the next coming years [18].

MATERIALS AND METHODS

Proposal for an operational protocol of coiling activity test (CAT). The method proposed in this study is based on the principle that the spontaneous activity of the embryo's tail occurs at the highest frequency at around 23 hpf [18]. Eggs are collected and selected under a stereomicroscope in order to keep only embryos at the stage between 16/32 cells, as required also in the FET test [19].
The operational protocol is developed into three steps:

a. Embryo selection and set up of the plates
b. Recording and video capture
c. Video analysis

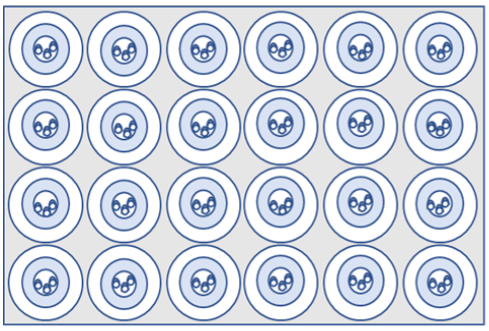
a. **Embryo selection and set up of the plates.** The test was conducted in 24-well plates filled with 2 ml of test solution per well. With the aim to facilitate the video acquisitions, some modifications were made to the test setup of the standardised FET test. Indeed, to minimise light transmission interference during image acquisition and to prevent the eggs from moving inside the well, an inert plastic washer was placed in the bottom middle of each well. The washers have been obtained using an inert plastic tube (PTFE), commonly used in aquariology, with an internal diameter of 4 mm and an external diameter of 8 mm and cutting it with a cutter into about 1/1.5 mm thick slices. The washers obtained were kept in 70% ethyl alcohol for 30 minutes and vigorously rinsed five times with distilled water in a 50 ml test tube, finally placed in standard freshwater. The washers must be dry on bibulous paper and left for a few minutes in the corresponding test solutions before placing them in the wells with a tweezer.
Finally, three embryos were delicately housed inside the washer using a glass pipette with a rounded tip (Figure 1).

Standard freshwater was used as a negative control, while two positive control were needed as hyperactivity and hypoactivity reference, respectively ethanol 1% and ethanol 5% (Figure 2). Two different concentrations were selected since ethanol causes hyperactivity at concentrations below 2% and hypoactivity at concentrations above 4% [17].

Plates were finally incubated at 26±1°C in the dark for a period of approximately 22 hours until the embryos reached the 23 hpf stage.

b. **Recording and video capture.** The recording was made with a Basler acA1300-60gm camera with a frame rate of 30 frames per second (fps) and a resolution of 1024x768 pixels. Videos were then analysed and the embryos tracked based on changes in greyscale values of each pixel with DanioScope® 1.2 (Noldus). Therefore, for the tracking system to work properly, high contrast between the eggs and the surrounding environment should be ensured in the recording process. The plate was left under the stereomicroscope for 5 minutes before starting the video acquisition. Each time we moved on to a new well, we waited 30 seconds before starting the video recording. If an embryo moves outside the frame area, it can be gently repositioned: in that case, we waited for another 15 seconds before restarting the video recording for reducing biases. Each recording lasted about 1 minute.

c. **Video analysis.** Videos were analysed with the DanioScope® software provided by Noldus that is based on changes in the grayscale values of each pixel. For this reason, it is able to detect embryos and analyse the movements individually as independent subjects and at the same time, it can exclude those embryos that are not suitable for analysis of the respective video. DanioScope® can evaluate three main measurement parameters to assess the CAT: Burst activity, Mean burst duration and Burst count/minute.



C4	C4	C4	C4	C4	C4
C5	C5	C5	C5	C5	C5
Et1	**Et1**	**Et1**	**Et1**	**Et1**	**Et1**
Et5	**Et5**	**Et5**	**Et5**	**Et5**	**Et5**

C1	C1	C1	C1	C1	C1
C2	C2	C2	C2	C2	C2
C3	C3	C3	C3	C3	C3
C0	**C0**	**C0**	**C0**	**C0**	**C0**

FIGURE 1
Scheme setup of a 24-well plate with washers and embryos (left). Plate filling example for a substance with five concentrations (right).
C0: negative control; Et1: ethanol at 1% positive hyperactive substance control; Et5: ethanol at 5% positive hypoactive substance control

Burst activity describes the percentage of the measurement duration in which embryos were scored active, Mean burst duration is the average duration of total events when embryos were scored active, and burst count/minute is the total number of events when embryos were scored active divided by the total measurement duration and is expressed in number/minute. It is possible to reduce background levels avoiding the count of a false movement for all coiling parameters with change the onset and offset level.

At the end of the test, it is possible to carry out a FET test by removing the washer from the wells and re-incubating the plate for 72h.

EXAMPLES OF CAT APPLICATIONS

After testing different parameters for the development of the method (i.e. the number of embryos, presence of washers, type of plate, exposure time), the most suitable and fastest operating conditions were selected. Afterwards, the proposed protocol was applied together with the FET test on different environmental river samples (R1, R2, R3) and three decreasing concentrations of a known compound (C1, C2, C3) in order to test the feasibility of the method.

The effect percentage for the CAT parameters were calculated with the following equation:

$$\% \text{ Effect} = 100 \times (A-B)/A$$

Where "A" is the effect measured on the control and "B" is the effect on each sample.

Table 1 shows the main results of the two methods. The environmental samples did not show acute toxicity with FET (no toxicity for values ≤10%), while it has been detected the presence of neurotoxic hypoactive substances (percentages values > 0) applying CAT on the same samples. Related to chemical sample FET and CAT trace well the decreasing concentrations, even if CAT is more sensitive and it highlights a hyperactive neurotoxic property of the substance (values <0). Acute toxicity test does not give specific information on which contaminants are responsible for lethality, especially when the percentage is very high, while in this case, the CAT test has been demonstrated more sensitive in less time.

For each parameter, the statistical distribution in each sample was provided (**Figure 2**). About 60 measurements for each sample have been analyzed. The boxplots were built in R 4.0.5 for the three tested parameters.

RESULTS AND DISCUSSION

The observation time proposed in this study for this test is set at 23 hpf, since it is suggested in the literature that early spontaneous coiling activity in zebrafish embryos might lack the potential to discriminate the different modes of action of potent neuroactive chemicals, due to life-stage specific sensitivities and the absence of neurotransmitter-related signalling [36]. While at a time greater than 30 hpf that coiling activity proved negligibly low [34]. Compared to the FET test, our version of the CAT presents some advantages (Table 2) that make it an interesting alternative in the short-term assessment of neurotoxicity: the total exposure time for the embryos is much shorter, and this allows the detection of a neurotoxic compound more rapidly. Moreover, we use the same 24-wells plates used in the FET test, but we load three eggs in each well, compared to only one egg in the standard FET test. Since the sample quantity in each well is the same between the two tests, this allows using in the CAT much less sample and much fewer plates for the same number of embryos. However, it has to be pointed out that this test is specifically focused on neurotoxic substances, and cannot be used to detect any other MoAs. Moreover, the lack of a standardised protocol can make it sometimes difficult to this day to compare the results produced from different studies.

TABLE 1

Main results of FET and CAT applied to environmental samples (R1, R2, R3) and a chemical substance at three concentrations (C1, C2, C3) for the feasibility of the proposed method.

Sample	CAT % effect on Burst Activity	CAT % effect on Mean Burst Duration	CAT % effect on Burst Count/minute	FET % lethality
R1	23.0	11.4	14.0	10.0
R2	61.0	35.0	43.0	10.0
R3	34.0	30.0	7.0	5.0
C1	-12.5	65.0	-202.7	100.0
C2	-87.0	5.0	-125.0	10.0
C3	-29.5	5.0	-55.0	10.0

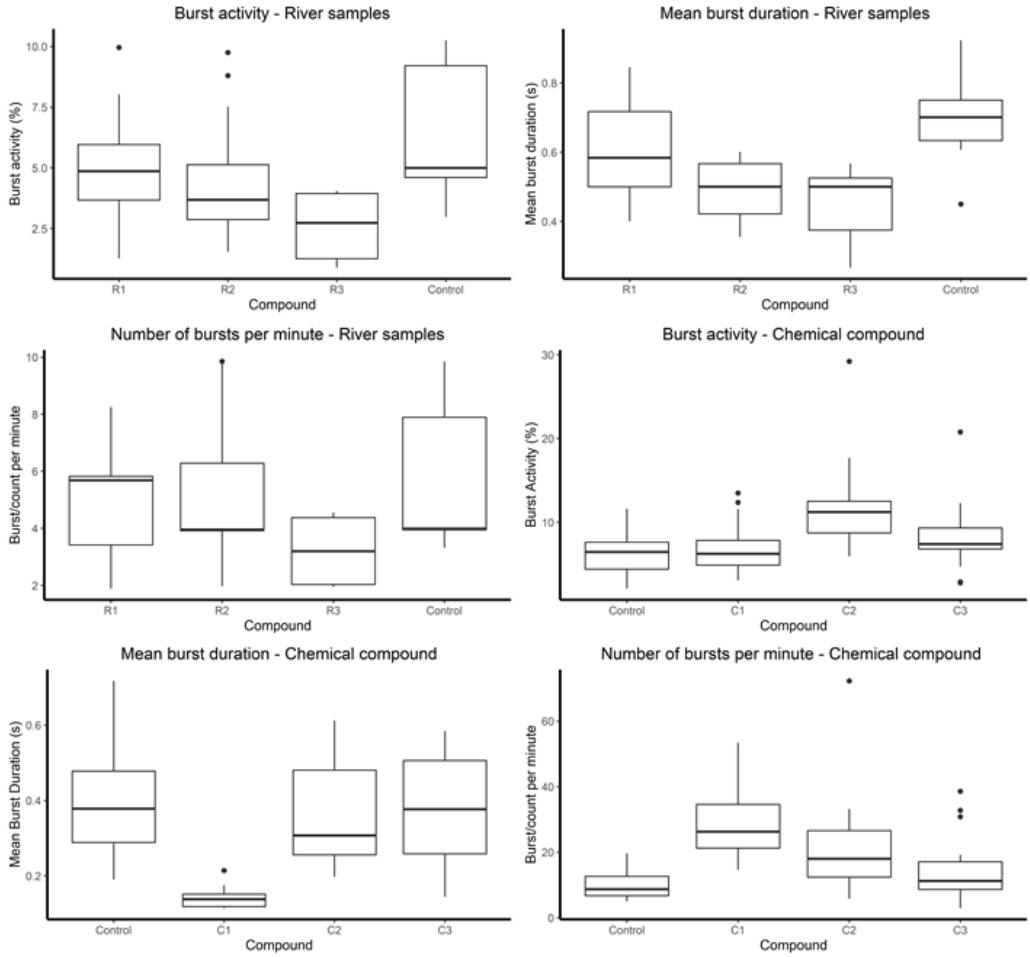


FIGURE 2
Boxplots of each parameter obtained by the CAT analysis in both river and chemical compound samples. Dark dots indicate outliers within the distribution.

TABLE 2
Comparison of parameters of Coiling Activity Test (CAT) and Fish Embryo Test (FET).

Parameters	CAT	FET
Exposure time	23 hours	96 hours
Endpoints	Burst activity, Mean burst duration, Burst count/minute	Lethality
N° of exposed embryos	3 embryos/well, 54 embryos/plate	1embryos/well, 20 embryos /plate
Cell stage at exposure	16/32 cells	16/32 cells
Test plate type	24 wells	24 wells
N° of plates for a complete test	2	7
Sample volume	12 ml (2 ml/well)	40 ml (2 ml/well)
Light condition	No	Not necessary
Temperature	26±1°C	26±1°C

CONCLUSION

The CAT is overall considered a promising, smart and efficient tool due to its sensitivity [25, 35], although a more accurate method standardisation may be required [18]. In our study, the CAT was demonstrated as a valid and rapid early warning system for neurotoxicity. Since it is estimated that neurotoxic compounds are very likely to be released into the environment [1], this test can be extremely useful for both the detection of neurotoxicity in the monitoring process and for the evaluation of emerging substances, for which information on MoAs is not

yet available; early warning systems are also key tools for the detection of effects caused by climate changes. Furthermore, the CAT could be improved through the knowledge on the different patterns of chemical substances combining this method with other EBMs mentioned in this article, e.g. AChE, Locomotion and FET test.

Finally, the CAT could make a valuable contribution to implementing EU regulatory guidelines and strategies [37] for identifying neurotoxic risks in the ecosystem and for this reason its forthcoming harmonization and standardization of method would be required.

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