Preliminary final report

NORMAN WG-2 Bioassays
JPA COLLABORATIVE TRIAL ON BIOASSAYS FOR NEUROTOXICITY TESTING

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on behalf of all the participants of the JPA

Since a face-to-face meeting in Sept 2020 on the discussion of the results obtained by the interlab study in a broader context and the publication on the results had to be postponed due to the COVI-19 situation to Summer 2021 this report is only a preliminary final report on the JPA

Abstract

Currently there is no regulatory framework for neurotoxicity assessment for aquatic systems. At the same time, neuroactive / neurotoxic aquatic contaminants are attracting increasing attention. To address this gap, a Collaborative Trial (CT) on neurotoxicity was launched in 2018 by NORMAN WG-2 under the leadership of RWTH, Aachen. The aim was to promote the use and application of bioassays for the assessment of the neuroactive and neurotoxic potential of chemicals and environmental samples. All participating laboratories applied different zebrafish embryo behavioural tests. 6 laboratories participated in the study. Laboratories received the samples (2 compounds and one spiked environmental water Extract) in October 2018 and they were asked to test them blindly using their neurotoxicity assay(s) according to their own test protocols, including appropriate controls, and quality assurance procedures. Analysis and statistical evaluation of raw data was performed by each laboratory. The final results were reported to RWTH. This study did not involve any quantitative comparison of the performance of each testing procedure. The final aim was to evaluate the applicability which behavioural assayis responsive to a representative set of water pollutants and may thus be suitable for water quality monitoring. The advantage of the zebrafish embryo behavioural test is it's usability for human and ecotoxicity testing. As part of the Ringtest an overview article on neurotoxicity for ecotoxicity testing was published in ESEU journal.

Introduction

Within the current European chemical regulation, (developmental) neurotoxicity is assessed using only in vivo tests with rodents, preferable rats. Neurotoxicity and developmental neurotoxicity (DNT) tests are not generally required but need to be performed if other tests or in silico data indicate that a compound has DNT or neurotoxic potential. The current guideline is very time, cost and extremely animal consuming, with around 1400 animals per test (Rovida and Hartung, 2009). It is assumed that the costs and animal numbers influence the decision if such a test is necessary and that if in question the test is more likely not performed. This is in contradiction with some estimates that up to 10% of all chemicals might be neurotoxic and a fast majority of those compounds might also be developmental neurotoxic (Rovida and Hartung, 2009). Considering the increased incidences of neurological diseases and the impact of such diseases on individual, families and society, DNT and neurotoxicity tests should be done on a regular base. However it is obvious that the current testing strategy is not able fulfil these demands. To solve that problem, a workshop was held by the European Food Safety Authority (EFSA) and the OECD to establish an improved method for developmental and neurotoxicity testing using alternative bioassays. Since developmental neurotoxicity is assumed to be worse than neurotoxicity due to its lifelong effects, priority has been given to developmental neurotoxicity.

The workgroup suggested the following tiered approach (Fritsche, 2016):

Tier 0: toxicokinetic modelling;

Tier 1: in vitro tests using human cells;

Tier 2: alternative model organism testing;

Tier 3: in vitro tests on rodent cells; and

Tier 4 (optional): rodent in vivo tests.

To further develop this approach several expert groups were started. An in vitro assay OECD/EFSA working group was started to further develop tier. Within this group 136 potential or known DNT compounds were selected and tested in a variety of different in vitro assays. The results of these tests will be publicly available beginning of 2020. For the development of tier 2, behavioural tests with zebrafish embryos are suggested.

<u>Objectives of the collaborative trial</u>

The initial objective of this trial was comparing the performance of different bioassays for neurotoxicity and related mechanisms for evaluation of chemical water quality. Due to the recent developments within the OECD, the NORMAN collaborative trial was focused on behavioural tests with zebrafish embryos applicable for Tier 2. Interestingly most of the ongoing neurotoxicity studies focus solely on the human relevance and ignore potential impacts on the ecosystem. But especially for the context of water quality monitoring the ecological relevance of neurotoxicity is also of interest. Therefore we further specified our objectives into those two individual objectives:

Obj.1 Test the applicability of commonly applied behavioural tests performed with zebrafish embryos for water quality testing.

Obj.2 Investigate and describe the ecotoxicological relevance of neurotoxicity.

Description of the collaborative trial (Obj.1)

Beginning of 2018, participants were actively contacted to contribute in the trial. Therefore laboratories known to perform behavioural tests with zebrafish embryos were asked to participate. Additionally flyers were distributed during relevant conferences (e.g. SETAC 2018). End of July 2018 a group of six laboratories was finally included in the trial.

Name of participating laboratories

1. RWTH Aachen (Prof. Dr. Henner Hollert, Michael Gundlach)
2. VU Amsterdam (Dr. Jessica Legradi, Ann-Cathrin Haigis)
3. Eawag/Umwelttoxikologie (Dr. Collette vom Berg, Sarah Könemann, Anze Zupanic)
4. UFZ - Umweltforschungscentrum (Dr. Riccardo Massei, Afolarin Ogungbemi, David Leuthold)
5. MTM Research Centre, Örebro University (Dr. Steffen Keiter, Norina Pagano)
6. Ifremer (Dr. Xavier Cousin)

As part of the trial every laboratory was asked to test two known substances and one environmental Extract spiked with an unknown substance. All test samples were distributed in September 2018. Every laboratory could choose their behavioral test and exposure design. Important all tests should be performed with zebrafish not older than 5 days post fertilization to comply with the alternative animal testing restrictions.

The test substances were (send pure or DMSO solution):

1. Diazinon (20 mg - CAS Number: 333-41-5, state: liquid)
2. Diazoxon (active metabolite of Diazinon; 20 mg - CAS Number: 962-58-3, state liquid)
3. Spiked environmental Extract (Mix of different environmental samples spiked with Diazinon 15 mM; solved in 200 µl DMSO, in S1 the method how the environmental Extract was made is described)

Since the focus of the trial was on the behavioral tests additional information on the general toxicity was given to the participants. This is the information which was shared:

"As a guidance with a Diazinon exposure in zebrafish, from 2hpf till 120 hpf, with 0.01% DMSO, not refreshing the medium during the exposure, I could not see with our fishline visual changes at 10 uM, whereas some visual malformations where there starting from 15 uM and higher.

For the Diazinon-Oxon, same exposure design, 1uM was ok (NOEC visual) and 5 uM and higher gave visual effects.

For the Extract no further information was given."

Results (Obj.1)

Ifremer reported some unusual effects with the first tests maybe due to shipment issues of the send samples. Due to the limited amount of available test substances the tests could not be repeated. Therefore those results were not in included in the comparative analysis.

Exposure setup

Each participant was requested to use their in-house established exposure procedure. An overview of the differences between the exposure protocols is presented in table 1. Differences in exposure volume, plate material, can be seen.

Table1. Overview of the exposure setups per participant.

	UFZ	EAWAG	RWTH	VU	MTM
Preconditioned plates	no	yes (no change of exposure solution)	no	no	no
exposure time	72-96hpf	2-120hpf	2- 96hpf	2-120hpf	1.5-96hpf
Platematerial	glass	plastic	glass	plastic	glass
#embryos/ volume	20 /20 ml	1/500 ul	24/50 mL	5/2.5 mL after 48 hpf 1/250 µL	
Temperature in ˚C	28	26	26	26	26
Max. solvent	0.1% DMSO	no solvent for Diazinon/Diazoxon; 0.01% DMSO for Extracts	0.05% DMSO	0.1% DMSO	0.01% DMSO
Medium	OECD236	OECD236	OECD236	OECD236	OECD236
Light/dark	14:10	14:10	14:10	14:10	14:10

Concentrations

In table 2 the test concentrations selected by each participant are shown. In some cases those concentrations are based on additional tests performed by the participant. In most cases the given information was used for the selection. All partners tested at least 3 concentrations. In general there was a good overlap in the concentration range with EAWAG having the lowest and UFZ the widest test concentration range.

Table 2. The selected test-concentration per participant

	UFZ	EAWAG	RWTH	VU	MTM
Diazinon in µM	1; 3.2; 10; 31.6; 100	0.1; 1; 10	0.7; 3.0; 6.2; 9.5; 11.8	1;5;10;15	0.06; 0.125; 0.25; 0.5, 1
Diazoxon in µM	0.1; 0.3; 1; 3.2; 5; 10	0.01; 0.1; 1	0.35; 0.7; 1.4; 2.8; 4.2	0.5; 1;2;4	0.006; 0.0125; 0.025; 0.05; 0.1
Extract	1:1k; 1:3.2k; 1:10k; 1:33k; 1:100k	1:10k; 1:100k; 1:1000k	1:2.5k; 1:5k; 1:10k	1:1k, 1:10k, 1:100k, 1:1000k	1:10k highest conc. (5 conc. and 1:2 series)

Behavioral tests

Most participants performed the Light-dark-transition test. Therefore we will base our main comparison on that test. In addition tests at different life stages as well with no or other stimuli as light were done. An overview of the performed tests is given in table 3.

Table 3. List of the behavioral tests performed by each participant

UFZ	**Light-dark-transition-test,** Spontaneous movement
EAWAG	**Light-dark-transition-test**
RWTH	**Light-dark-transition-test**
VU	**Light-dark-transition-test**; PMR; Touch Response; Spontaneous movement; Locomotion
MTM	Tapping test

Comparison of the Light-dark-transition test

The Light-dark-transition test is the most applied behavior test done with zebrafish embryos. For the test embryos are distributed in multiwell plates (24 or 96 well) and subjected to alternating short light and dark phases. During the light phase embryos display a calm steady activity (figure 1). When the light is switched off, embryos start to move hectically. Overtime this high activity is slowly decreasing (figure 1).

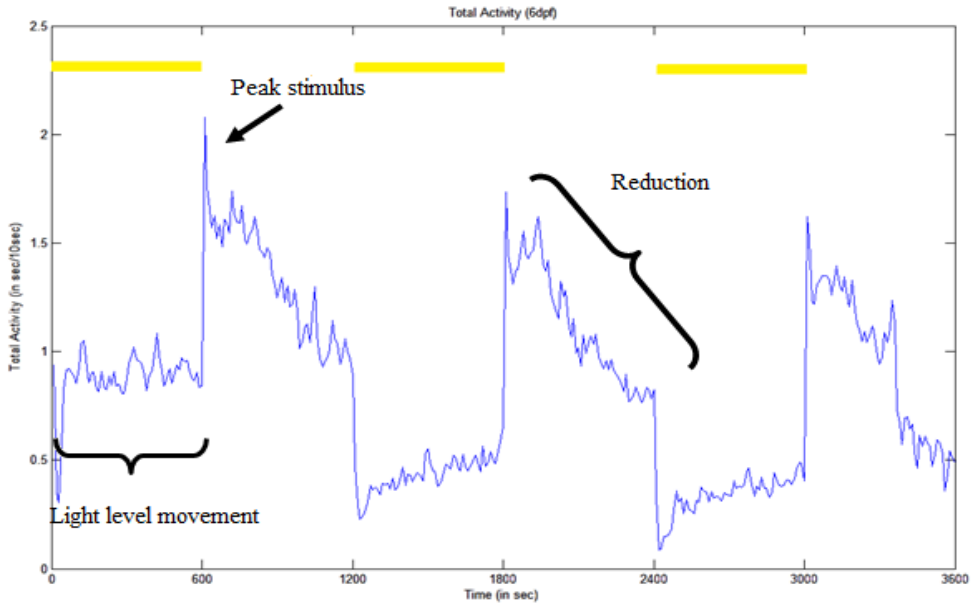

Figure 1. Total activity (time moved) of zebrafish embryos during the Light-dark-transition test (Legradi *et al.*, 2014).

Similar to the exposure protocols differences in the protocols for the Light-dark-transition test were found. All groups measured distance moved and used at least 2 light and 2 dark phases.

Duration of the phases was in most cases 10 minutes. Tests were performed at 96hpf or 120hpf. All participants performed 3 independent tests with 8-16 embryos per replicate. In all cases different concentrations were selected. A detailed overview of the test procedures is given in table 4.

Table 4. Comparison of the protocols used for the Light-dark-transition test for each participant.

	UFZ	EAWAG	RWTH	VU
Preincubation	dark; 10 min	light; 40min	dark; 10min	light; 10 min
Temperature in ˚C	27	26	26	26
Total measure time in min	80	80	30	
Dark phases (min)	3 (10:20:20)	2 (10:10)	2 (10:10)	2(10:10)
Light phases (min)	3 (10:10:10)	2 (10:10)	1 (10)	2 (10:10)
# embryos	16	8-12	24	12
exclution of effected fish	yes (dead fish were excluded)	yes (dead fish were excluded)	yes (malformed or dead)	yes (malformed or dead)
measureds value	distance moved (mm)	distance moved (cm)	distance moved (cm/10min)	distance moved (cm/10min)
replicates	3	3	3	3
age of embryos	96 hpf	120 hpf	96 hpf	120 hpf
test system	Viewpoint	Noldus	Noldus	Viewpoint
exposure volume	500 uL	500 uL	300 ul	250 µL
plate format	96 well plate	48	96	96

The results of the individual groups are presented in table 5. The data graphs of each participant and test can be found in the supplement S2.

In general, for Diazinon a slight (non-significant) decrease of activity in the dark phase was seen for concentrations lower as 10 µM, a significant decrease of activity around 10 µM, a significant increase of activity on the light phase at 32 µM and a significant decrease of activity during all light phases at 100 µM.

For Diazoxon all groups saw a decrease in activity in the dark phase. The effect was indicative for concentrations lower as 2 µM and significant for concentrations between 2 and 5 µM.

For the Extracts results were more diverse. High test concentrations between 1:1k and 1:2.5k gave significant effects. Mostly an increase in activity in all light phases was reported. Interestingly the effects were different than observed with the pure compounds. Also the concentrations were effects were seen were lower with the environmental Extract. This could indicate additive or synergistic effects between Diazinon and the environmental Extract. Due to the limited amount of sample the unspiked Extract could unfortunately not be tested.

Table 5. Overview of the results for the three different samples per participant. Red indicates significant effects, orange corresponds to no significant effects but trends, green indicates no effects.

	UFZ	EAWAG	RWTH	VU
Diazinon	significant effects in all phases for 100 uM and the light phases for 32 uM	No significant differences were found for Diazinon, however, a slight decrease in activity (dark phase) was measured for the highest concentration of 10 µM	significant effect in dark phases at 11.8 uM	no significant effects, however in dark periods tendency towards decreased movement in 5, 10 and 15 µM
Diazoxon	significant effects for the dark phases at 5 uM and 3.2 uM (Dark 1)	No significant differences were found for Diazoxon, however, a slight decrease in activity (dark phase) was measured for the highest concentration of 1 µM	significant effects in dark phases at 2.8 and 4.2 uM	significant effects (less distance) at 2 and 4 µM in dark periods, tendency towards reduced movement 0.5 and 1 µM
Extract	effects for the light and dark phase at 1k	No significant differences were found for the Extract, however, the larvae´s activity slightly increased for all three tested concentrations when compared to the control as well as the solvent control	significant effects in dark phases at 1:2.5k	no significant effects on behavior, high mortality in 1:1k dilution, effects only in early embryonic development

Results from other behavioral tests

As mentioned before besides the Light-dark-transition test also other behavioral tests were performed. As those tests were done only by individual participants no comparative analyses could be done.

Tapping at 96hpf:

For the investigation of behavioral changes after tapping, a tapping Device located inside the DanioVision Observation Chamber (DVOC-0040/T, Noldus, The Netherlands) was used. It is based on a metal core twisted with an electromagnetically inductive coil. The metal core is connected to a push pin which hits the plate support where the well plate is kept on (Coenders). Before measurement zebrafish larvae (96 hpf) were investigated for deformation or coagulation. Coagulated eggs and larvae possess severe deformations were removed and not considered for

measurements and calculation using Ethovision software XT 13. For acclimatization of the larvae, they were kept in the chamber under light for 1 minute without any other stimulus (at 26 ±1 °C). After the acclimatization, the vibrational stimuli were initiated using the tapping device. Based on results of a previous investigations (data not published) ten consecutive tapping were used with a time span of 120 sec between the single tappings. The measured endpoint was distance of motion (travelled distance), one second before and one second after each tapping.

Negative and solvent controls don't show any significant alteration of the behavior. In contrast, all tested samples show statistically significant alterations of the behavior using 10 consecutive vibrational stimuli. For Diazinon all concentrations except of 0.625 µM show a significant change of the behavior. Exposure to Diazoxon cause for all concentrations tested a significant change of the traveled distance in comparison to the solvent control. However, comparison between the two substances shows concentrations of Diazoxon are 10-times lower than for Diazinon. Likewise, to tested compounds, for the provided Extract (sample 3) all concentrations show a significant alteration of the behaviour using 10 consecutive vibrational stimuli.

Spontaneous movement

Spontaneous movement occurs during motor neuron innervation from the spinal cord and is characterised by periodic tail flipping of the embryo. Prior to the assay malformed or dead embryos were removed. Embryonic movement was assessed at 28hpf and over 10 minutes, using a Zebrabox from Viewpoint and the corresponding software zebralab. Exposure to Diazoxon lead to significantly reduced coiling events at 0.5, 2 and 4 µM compared to solvent and negative control. For Embryos exposed to Diazinon, no significant reduction was seen in tested concentration, but differences between negative and solvent control were also comparably high. Exposure to the Extract reduced the mean number of coiling´s at 1:1K, 1:100K and 1:1000K dilutions. However, reduced coiling's at 1:1k dilutions are due to high mortality in this concentration.

Photomotor response (PMR)

The PMR is a non-visual response of embryonic zebrafish and occurs from ca. 30-42hpf. In the PMR assay the response of the embryos to a strong light flash was monitored. Normally developing embryos are supposed to respond to this light flash (500 ms) with strongly increased coiling. To enhance this response, embryos were kept in darkness at least 10 minutes prior to the PMR assay. Embryos exposed to Diazoxon at 0.5, 2 and 4 µM displayed a tendency to less coiling's, whereas for Diazinon no differences between exposed and control embryos was seen. Exposure to the Extract sample lead to less coiling in exposed embryos compared to control embryos. Again, the result for the 1:1K dilution is due to high mortality in this concentration. Coiling frequency and duration were recorded using Viewpoint's Zebrabox and the zebralab software.

Locomotion

The locomotion assay was done with free swimming embryos (96hpf) and analyses the embryonic behaviour under continuous light. Embryonic swimming activity was tracked over 15 minutes using the mean distance moved as endpoint. Tracking was done with the zebrabox and the Zebralab software from Viewpoint (VU). Exposure to 10 µM Diazinon significantly reduced movement in embryos compared to the solvent control. Yet, the mean distance moved in the negative control was also significantly lower, compared to the solvent control. 4 µM Diazoxon exposure lead to an increased embryonic activity compared to the solvent control. However,

differences were not statistically significant. For the Extract no effect on locomotion was detected.

Touch response

Embryos (96hpf) were exposed to a physical stimulus to the head and the tail. Behaviour upon this stimulus was filmed and analysed via a scoring system. Embryos exposed to 10 and 15 µM Diazinon achieved a smaller mean score as did embryos from the negative and solvent control. The same was seen for Diazoxon, where embryos exposed to 2 and 4 µM also reached a lower mean score. For the Extract no influence on the touch response was recorded. Results from 1K dilution are again due to high mortality in this concentration.

Discussion and conclusion (Obj. 1)

Despite the differences in the exposure protocols no big differences in the effect concentrations were observed. The selected test compounds are known to be stable and not attach to the surface of plastic plates. Also the uptake of those compounds was not expected to be an issue. For such compounds no standardized exposure protocol seems to be necessary. But since not all compounds share those characteristics a standardized exposure protocol would be recommended. Especially the selection of the exposure concentrations seems to be critical. Testing a wider range might be favorable to avoid false negatives. Although no impact of the temperature on the results was seen in our study, differences in temperature have a direct impact on the "speed" of the development of zebrafish embryos. The same holds for the exposure time. According to EU-regulations tests with zebrafish embryo can be performed till 120hpf (at 28˚C) as non-animal test. At 120hpf the organs as the nervous system is further developed as at 96hpf. This could impact the results of a behavioral test.

When looking at behavioral tests other than the Light-dark-transition test a clear impact of the metabolic capability of the embryo was seen (Table 6). Diazinon needs to be metabolized by the liver to induce toxicity. The embryo liver starts working around 48hpf. Therefore no effects for Diazinon in tests performed at the early development were seen. Whereas Diazoxon induced effects already early in development. This is important especially if effects should be translated to species where embryos develop inside the mother, so an active metabolism.

Table 6. Qualitative overview of the results for all performed tests. + = significant effect; (+) = non-significant effect; - = no effect

	Diazinon	Diazoxon	Extract
Spontaneous movement	-	+	+
Photomotor response	-	+	+
Locomotion	+	(+)	-
Touch response	+	+	-
Light-Dark-transition	++(+)(+)	+++(+)	+(+)+-
Tapping	+	+	+

The effects from the spiked Extract were rather diverse throughout the tests and participants. If this is due to the differences in exposure protocols is unclear. What also should be considered is that a neurotoxic substance can increase and decrease behavior depending on the exposure concentration. Water Extracts can consist of a number of different pollutants. So far little is

known how mixtures of neurotoxic compounds interfere with each other and ultimately alter behavior.

Description and Results of the collaborative trial (Obj.2)

To improve the understanding of the impact of neurotoxicity on ecological systems an expert performed. Therefore leading experts in different relevant fields e.g. ecosystem monitoring, eco-toxicologists, regulators and Insilco toxicologists were asked to participate. In total 38 people from different international institutes participated. The review was published 12/2018 in ESEU under the title: An ecotoxicological view on neurotoxicity assessment (https://doi.org/10.1186/s12302-018-0173-x).

Discussion and conclusion (Obj. 2)

In the review it was eminent that neurotoxicity is relevant not only for humans but also for ecosystems. An array of methods applying different model species from different trophic levels already exists and could be easily applied for standard water quality testing. In addition AOPs could be used to identify in vitro tests which could help screening the increasing number of chemicals found in water systems for their neurotoxic potential.

Conclusion of the collaborative trial

Although only a limited amount of behavioral tests were performed the results indicated the usability of behavioral tests with zebrafish embryos can be used for neurotoxicity assessment. Despite different protocols similar results could be observed. For regulatory testing a standardized exposure and behavioral protocol would improve the comparability and reproducibility of the results especially for compounds with a difficult chemical characteristic (e.g. stability). An OECD expert panel is at the moment working on a standardized protocol for the Light-dark-transition test. The protocol is expected to be published Mid 2020. The protocol is designed and tested for individual pure substances. It's usability for complex mixtures like water samples is not evaluated. Based on our results more information on the mixture effects of neurotoxic substances is needed to be able to further develop current testing protocols to be applicable for water quality monitoring. Furthermore to our knowledge no protocols for other behavioral tests like the photomotor response test are in preparation. A combination of different test might further improve the detectability of neurotoxic substances in complex mixtures.

Although the focus at the moment is on developing assessment strategies for detecting potential human neurotoxicants our results show that the impact on ecosystems should not be neglected. Zebrafish embryos offer the great opportunity to be applied for human and ecosystem neurotoxicity testing. In order to improve the usability of zebrafish embryo behavioral data for ecotoxicity testing more information is needed to better understand the links of the used behavioral tests (e.g. Light-dark-transition test) and real life behaviors like predator escape or mating.

References

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