OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

Fish Embryo Toxicity (FET) Test

INTRODUCTION

1. This Test Guideline describes a Fish Embryo Toxicity (FET) test with the zebrafish (*Danio rerio*). This test is designed to determine acute toxicity of chemicals on embryonic stages of fish. The FET-test is based on studies and validation activities performed on zebrafish (1)(2)(3)(4)(5)(6)(7)(8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19).

2. Definitions used in this Test Guideline are given in Annex 1.

PRINCIPLE OF THE TEST

3. The test starts with chemical exposure of newly fertilised zebrafish eggs and has a duration of 96 hrs. Every 24 hrs, up to four apical observations are recorded as indicators of lethality (6): (i) coagulation of fertilised eggs, (ii) lack of somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv) lack of heartbeat. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC50 is calculated.

INITIAL CONSIDERATIONS

4. Useful substance-specific information and properties includes structural formula, molecular weight, purity, stability in water and light, pKa and Kow, water solubility and vapour pressure as well as results of a test for ready biodegradability (OECD TG 301)(20). A reliable analytical method for the quantification of the substance in the test solutions with known and reported accuracy and limit of detection should be available. Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses of the test substance may occur. In case of testing difficult substances, refer to OECD GD 23 (21).

5. For bioreactive substances additional information on metabolism might be necessary. If there are indications that metabolites or other transformation products of relevance may be more toxic than the parent compound, it is also recommended to perform the test with these metabolites/ transformation products and to also use these results when concluding on the toxicity for the test substance.

TEST ACCEPTANCE CRITERIA

6. For the test results to be acceptable, the following conditions should apply:

   a. The fertilisation rate of the eggs should be ≥70%.

   b. At the beginning of the test, the dissolved oxygen concentration in the negative control and highest test concentration should be ≥80% of saturation.

c. The water temperature should be maintained at 26±1 °C in test chambers at any time during the test.

d. Overall survival of embryos in the negative control, and, where relevant, in the solvent control should be ≥90% until the end of exposure.

e. Exposure to the positive control (*e.g.*, 4.0 mg/L 3,4-dichloroaniline for zebrafish) should result in a minimum mortality of 30% at the end of the exposure.

f. Hatching rate in the negative control should be ≥80% at the end of 96 hrs exposure.

LIMITATION OF THE TEST METHOD

7. Some substances may cause delayed hatch which will preclude or reduce the post-hatch exposure. In this case, other toxicity tests might be more appropriate.

DESCRIPTION OF THE METHOD

8. An overview of relevant maintenance and test conditions is available in Annex 2.

Apparatus

9. The following equipment is needed:

a. Fish tanks made of chemically inert material (*e.g.*, glass) and of a suitable capacity in relation to the recommended loading;

b. Inverted microscope and/or binocular with at minimum 30-fold magnification. If the room used for recording observations cannot be adjusted to 26±1 °C, a temperature-controlled cross movement stage is necessary;

c. Test chambers; *e.g.*, 24-well plates (see "Test chambers");

d. Self-adhesive foil to cover the 24-well plates or vapour-dense lids provided with plates;

e. Incubator or air-conditioned room maintained at 26±1 °C;

f. pH-meter;

g. Oxygen meter;

h. Equipment for determination of hardness of water and conductivity;

i. Spawn trap: instrument trays of glass, stainless steel or other inert materials; Wire mesh (grid size 2±0.5 mm) of stainless steel or other inert material indented about 1 cm into the tray; spawning substrate (*e.g.*, plant imitates of inert material) (OECD 229, Annex 4a (22));

j. Pipettes with widened openings to collect eggs;

k. Glass vessels to prepare different test concentrations and dilution water (beakers, graduated flasks, graduated cylinders and graduated pipettes) or to collect zebrafish embryos (*e.g.*, beakers, crystallization dishes).

Test chambers

10. Glass or polystyrene exposure plates with a 2.5 - 5 ml filling capacity should be used (*e.g.*, 24-well plates). In case adsorption to polystyrene is suspected, inert materials (glass) should be used. Test chambers should be randomly positioned.

Water

11. For the embryo toxicity test, dilution water should be prepared from reconstituted water (23)(24). The resulting degree of hardness should be equivalent to 100-300 mg/L $CaCO_3$.Other well-characterised surface or well water may be used. The reconstituted water may be adapted to maintenance water of low hardness by dilution with deionised water up to a ratio of 1:5 resulting in a hardness of around 30-35 mg/L $CaCO_3$. The water is aerated to oxygen saturation prior to addition of the test substance. Temperature should be kept at 26±1 °C throughout the test. The pH should be adjusted to a range between pH 6.5 and 8.5, and not vary by more than 1.5 during the course of the test. Use of HCl and NaOH to correct pH is recommended.

Test solutions

12. Test solutions of the selected concentrations can be prepared, *e.g.*, by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test substance in the dilution water by mechanical means (*e.g.*, stirring or ultrasonification). If the test substance is difficult to dissolve in water, procedures described in the OECD GD 23 for handling difficult substances should be followed (21). The use of solvents or dispersants (solubilizing agents) should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. In addition to the examples of suitable solvents given in (21), dimethylsulfoxide (DMSO) might be useful. Where a solubilizing agent is used to assist in stock solution preparation, its final concentration should not exceed 100 µl/L and should be the same in all test vessels.

Maintenance of brood fish

13. A breeding stock of unexposed, wild-type zebrafish with well-documented fertilisation rate of eggs is used for egg production. Fish should be free of macroscopically discernible symptoms of infection and disease and should not have undergone any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Breeding fish are maintained in aquaria with a loading capacity of a minimum of 1 L water per fish and a fixed 12 – 16 hour photoperiod (3)(5)(6)(25)(26)(27)(28)(29). Females and males are continuously held together, *e.g.*, in a ratio of 1 to 2. Optimal filtering rates should be adjusted; excess filtering rates causing heavy perturbation of the water should be avoided. For feeding conditions, see Annex 2. Surplus feeding should be avoided, and water quality and cleanness of the aquaria should be monitored regularly and be reset to the initial state, if necessary.

Proficiency Testing

14. A reference substance such as 3,4-dichloroaniline (used in the validation studies (1)(2)), should be tested as a means to check the sensitivity of the fish embryos and strain used, preferably twice a year.

Egg production

15. Zebrafish eggs may be produced via spawning groups (in individual spawning tanks) or via mass spawning (in the maintenance tanks). In the case of spawning groups, males and females are placed in spawning tanks a few hours before the onset of darkness on the day prior to the test. Since spawning groups of zebrafish may occasionally fail to spawn, the parallel use of at least three

spawning tanks is strongly recommended. To avoid genetic bias, eggs are collected from a minimum of three breeding pairs or groups, mixed and randomly selected.

16. For collection of eggs, spawn traps are placed into the spawning tanks or maintenance tanks before the onset of darkness on the day prior to the test or before the onset of light on the day of the test. To prevent predation of eggs by adult zebrafish, the spawn traps are covered with stainless steel mesh of appropriate mesh size (approx. 1 - 2 mm). If considered necessary, artificial plants made of green plastic or glass can be fixed to the mesh as spawning stimulus (3)(4)(5)(30)(31). Mating, spawning and fertilisation take place within 30 min after the onset of light in the morning and the spawn traps with the collected eggs can be carefully removed.

Egg differentiation

17. At 26°C, fertilised eggs undergo the first cleavage after about 15 min and the consecutive synchronous cleavages form 4, 8, 16 and 32 cell blastomers, respectively (see Annex 3)(31). At these stages, fertilised eggs can be clearly identified by the development of a blastula.

PROCEDURE

Conditions of exposure

18. 20 Embryos per concentration are exposed to the test substance with semi-static renewal technique, unless there is evidence that the concentrations of the test substance in solution can be satisfactorily maintained under static exposure. Alternatively, flow-through systems can be used. In any case, care should be taken to minimise any stress to the embryos. Test chambers should be saturated at least for 24 hrs with the test solutions prior to test initiation, unless it is known that no absorption to test chambers will occur.

Test concentrations

19. Normally, five concentrations of the test substance spaced by a constant factor not exceeding 2.2 are required to meet statistical requirements. Justification should be provided, if fewer than five concentrations are used. The highest concentration tested should preferably result in 100% lethality, and the lowest concentration tested should preferably give no observable effect. A range-finding test before the definitive test allows an appropriate choice of the appropriate concentration range. The following instructions refer to performing the test in 24-well plates. If different test chambers (*e.g.*, small Petri dishes) are used or more concentrations are tested, instructions have to be adjusted accordingly.

20. Details and visual instructions for allocation of concentrations across 24-well plates are available in paragraph 26 and Annex 5, Fig 1.

Controls

21. Pure dilution water is used as a negative control. Negative controls are required both as negative control and as internal plate controls. If more than 1 dead embryo is observed in the internal plate control the plate is rejected.

22. A positive control at a fixed concentration of 4 mg/L 3,4-dichloroaniline is performed at every testing occasion.

23. In case a solubilising agent is used, an additional group of 20 embryos is exposed to the solubilising agent on a separate 24-well plate, thus serving as a solvent control (21). To consider the test acceptable, the solvent or solubilising agent should be demonstrated to have no significant effects on survival, nor produce any other adverse effects on the embryos (cf. paragraph 6d).

Start of exposure and duration of test

24. The test is initiated as soon as possible after fertilisation of the eggs and terminated after 96 hours of exposure. The embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or at latest in the 16 cell-stage. To start exposure with minimum delay, at least twice of the number of eggs needed per treatment group are randomly selected and transferred into the respective concentrations and controls (e.g., in 100 ml crystallisation dishes; eggs should be fully covered) not later than 60 minutes post fertilisation (onset of light).

25. Viable fertilised eggs should be separated from unfertilised eggs and be transferred to 24-well plates pre-saturated for 24 hrs and refilled with 2 ml/well freshly prepared test solutions within 180 minutes post fertilisation (past the onset of light). By means of stereomicroscopy (preferably ≥30-fold magnification), fertilised eggs undergoing cleavage and showing no obvious irregularities during cleavage (*e.g.*, asymmetry, vesicle formation) or injuries of the chorion are selected. For egg collection and separation, see Annex 5, Fig 2.

Distribution of eggs over 24-well plates (see Annex 5, Fig 1)

26.

- 20 eggs on one plate for each test concentration;
- 20 eggs as solvent control on one plate (if necessary);
- 20 eggs as positive control on one plate;
- 4 eggs as internal plate control on each of the above plates
- 24 eggs as negative control on one plate

Observations

27. Apical observations include: coagulation of embryos, lack of somite formation, non-detachment of the tail, lack of heartbeat (Table 1). These observations are used for the determination of lethality: Any positive outcome in one of these observations means that the zebrafish embryo is dead. Additionally, for checking the quality of the eggs hatching is recorded. Observations are recorded every 24 hrs, until the end of the test at 96 hours after the start of exposure..

Table 1. Apical observations of acute toxicity in zebrafish embryos 24 - 96 hrs post fertilisation.

	Exposure times			
	24 hrs	48 hrs	72 hrs	96 hrs
Coagulated embryos	+	+	+	+
Lack of somite formation	+	+	+	+
Non-detachment of the tail	+	+	+	+
Lack of heartbeat		+	+	+

28. *Coagulation of the embryo:* Coagulated embryos are milky white and appear dark under the microscope (see Annex 4, Fig. 1). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

29. *Lack of somite formation:* At 26±1°C, about 20 somites have formed after 24 hrs (see Annex 4, Fig. 3) in a normally developing zebrafish embryo. A normally developed embryo shows spontaneous movements (side-to-side contractions). Spontaneous movements indicate the formation of somites. The absence of somites is recorded after 24, 48, 72 and 96 hrs.

30. *Non-detachment of the tail:* In a normal developing zebrafish embryo, detachment of the tail (see Annex 4, Fig. 2) from the yolk is observed following posterior elongation of the embryonic body. Absence of tail detachment is recorded after 24, 48, 72 and 96 hrs.

31. *Lack of heartbeat:* In a normally developing zebrafish embryo at 26±1 °C, the heartbeat is visible after 48 hrs (see Annex 4, Fig. 4). Particular care should be taken when recording this endpoint, since irregular (erratic) heartbeat should *not* be recorded as lethal. Moreover, visible heartbeat without circulation in aorta abdominalis is considered non-lethal. The observation time to record an absence of heartbeat should be at least of 1 minute with a minimum magnification of 80×. Absence of heartbeat is recorded after 48, 72 and 96 hrs.

32. Hatching rates of negative controls should be recorded and reported.

33. Detailed description of the normal (31) and examples of abnormal development of zebrafish embryos is illustrated in Annex 4.

Analytical measurements

34. At the beginning and at the end of the test, dissolved oxygen concentrations, pH, total hardness and conductivity in the control(s) and in the highest test substance concentration are measured. The dissolved oxygen concentration in the negative controls and highest text concentration should be in compliance with the test requirements (see paragraph 6). The temperature is usually measured in control vessels and it should be recorded preferably continuously during the test or, as a minimum, daily.

35. As a minimum, the concentration of the test substance should be measured in the highest and lowest test concentration, but preferably in all treatments, at the beginning and end of the test. In case of insufficient volume for analysis merging of test solutions or use of surrogate chambers being of the same material and having the same volume to surface area ratios as 24-well plates may be useful. It is strongly recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained

within 20% of the nominal or measured initial concentration throughout the test, results can be based on nominal or measured initial values.

LIMIT TEST

36. Using the procedures described in this guideline, a limit test may be performed at 100 mg/L of test substance or up to its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the LC50 is greater than this concentration. The limit test should be performed using 20 embryos in the treatment, the positive control and – if necessary - in the solvent control and 24 embryos in the negative control. If the percentage of lethality exceeds 10% and there is no mortality in the negative control at the end of the test, a full study should be conducted. Any observed effects should be recorded.

DATA AND REPORTING

Treatment of results

37. The percentages of embryos for which at least one of the apical observations is positive at 48 and 96 hrs respectively are plotted against test concentrations. For calculation of the slopes of the curve and LC50-values and the confidence limits (95%) appropriate statistical methods should be applied (32)(33)(34)(35)(36)(37) and the OECD Guidance Document No. 54 should be consulted (38).

38. Where the data obtained are inadequate for the use of standard methods of calculating the LC50, the geometric mean between the highest concentration causing no statistically significant mortality and the lowest concentration producing 100 % mortality should be used as an approximation for the LC50.

Test report

39. The test report should include the following information:

Test substance:

- physical nature, purity and where relevant, physicochemical properties (including isomerisation);
- any special precautions taken that are based on the physical or chemical characteristics of the test substance;
- identification data and Chemical Abstract Services Registry Number, if known.

Test organisms:

- scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.

Test conditions:

- test procedure used (*e.g.*, semi-static renewal)*;*
- photoperiod;
- test design (*e.g.*, number of test chambers, types of controls);

- water quality characteristics in fish stock maintenance (*e.g.*, pH, hardness, temperature, conductivity, dissolved oxygen);
- dissolved oxygen concentration, pH, total hardness, temperature and conductivity of the test solutions at the start and after 96 hrs (in semi-static renewal systems the pH should be measured prior to and after water renewal);
- method of preparation of stock solutions and test solutions as well as frequency of renewal;
- justification for use of solvent and justification for choice of solvent, if other than water;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of quantification (LoQ) should also be reported;
- evidence that controls met the overall survival acceptance criteria
- fertilisation rate of the eggs.

Results:

- cumulative mortality for each concentration at the recommended observation times;
- the LC50 values at 48 and 96 hrs for mortality with 95% confidence limits, if possible;
- maximum concentration causing no statistically different mortality within the period of the test;
- minimum concentration causing 100% mortality within the period of the test;
- graph of the concentration-mortality curve at the end of the test;
- mortality in the controls (negative controls, internal plate controls, as well as positive control and any solvent control used);
- data on the outcome of each of the four apical observations;
- incidence and description of morphological and physiological abnormalities, if any;
- incidents in the course of the test which might have influenced the results;
- statistical analysis and treatment of data (*e.g.*, probit analysis, logistic regression model or geometric mean for LC50)
- slope and confidence limits of the regression of the (transformed) concentration-response curve.

Discussion and interpretation of results.

LITERATURE

1. OECD (2011) Validation Report (Phase 1) for the Zebrafish Embryo Toxicity Test: Part I and Part II. Series on Testing and Assessment No. 157, OECD, Paris. Available: [http://www.oecd.org/document/46/0,3746,en_2649_34377_47786926_1_1_1_1,00.html]
2. OECD (2012) Validation Report (Phase 2) for the Zebrafish Embryo Toxicity Test: Part I and Part II. Series on Testing and Assessment No. XX, OECD, Paris. Available: [http://www.oecd.org/document/46/0,3746,en_2649_34377_47786926_1_1_1_1,00.html]
3. Braunbeck, T., Böttcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M. and Seitz, N. (2005) Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species - an update. ALTEX 22: 87-102.
4. DIN (2001) German standard methods for the examination of water, waste water and sludge – Subanimal testing (group T) – Part 6: Toxicity to fish. Determination of the non-acute-poisonous effect of waste water to fish eggs by dilution limits (T 6). DIN 38415-6; German Standardization Organization.
5. Nagel, R. (2002) DarT: The embryo test with the zebrafish (*Danio rerio)* - a general model in ecotoxicology and toxicology. ALTEX 19: 38-48.
6. Schulte, C. and Nagel, R. (1994) Testing acute toxicity in embryo of zebrafish, *Brachydanio rerio* as alternative to the acute fish test - preliminary results. ATLA 22, 12-19.
7. Bachmann, J. (2002) Development and validation of a teratogenicity screening test with embryos of the zebrafish (*Danio rerio*). PhD-thesis, Technical University of Dresden, Germany.
8. Lange, M., Gebauer, W., Markl, J. and Nagel, R. (1995) Comparison of testing acute toxicity on embryo of zebrafish (*Brachydanio rerio),* and RGT-2 cytotoxicity as possible alternatives to the acute fish test. Chemosphere 30/11: 2087-2102.
9. Kammann, U., Vobach, M. and Wosniok, W. (2006) Toxic effects of brominated indoles and phenols on zebrafish embryos. Arch. Environ. Contam. Toxicol., 51:97-102, in press.
10. Groth, G., Kronauer, K. and Freundt, K.J. (1994) Effects of *N*,*N*-diemethylformamide and its degradation products in zebrafish embryos. Toxicol. *In Vitro* 8: 401-406.
11. Groth, G., Schreeb, K., Herdt, V. and Freundt, K.J. (1993) Toxicity studies in fertilized zebrafish fish eggs treated with *N*-methylamine, *N*,*N*-dimethylamine, 2-aminoethanol, isopropylamine, aniline, *N*-methylaniline, *N*,*N*-dimethylaniline, quinone, chloroacetaldehyde, or cyclohexanol. Bull. Environ. Contam. Toxicol. 50: 878-882.
12. Roseth, S., Edvardsson, T., Botten, T.M., Fuglestadt, J., Fonnum, F. and Stenersen, J. (1996) Comparison of acute toxicity of process chemicals used in the oil refinery industry, tested with the diatom *Chaetoceros gracilis*, the flagellate *Isochrysis galbana*, and the zebrafish, *Brachydanio rerio*. Environ. Toxicol. Chem. 15: 1211-1217.
13. Versonnen, B.J. and Janssen, C.R. (2004) Xenoestrogenic effects of ethinylestradiol in zebrafish (*Danio rerio*). Environ. Toxicol. 19: 198-206.
14. Versonnen, B.J., Roose, P., Monteyne, E.M. and Janssen, C.R. (2004) Estrogenic and toxic effects of methoxychlor on zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 23: 194-201.
15. Ferrari, B., Paxeus, N., Lo Giudice, R., Pollio, A. and Garric, J. (2003) Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibric acid, and diclofenac. Ecotoxicol. Environ. Saf. 55: 359-70.
16. Hallare, A.V., Kohler, H.R. and Triebskorn, R. (2004) Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56: 659-66.

17. Nguyen, L.T. and Janssen, C.R. (2001) Comparative sensitivity of embryo-larval toxicity assays with African catfish (*Clarias gariepinus*) and zebra fish (*Danio rerio*). Environ. Toxicol. 16: 566-71.

18. Wiegand, C., Krause, E., Steinberg, C. and Pflugmacher, S. (2001) Toxicokinetics of atrazine in embryos of the zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 49: 199-205.

19. Cheng, S.H., Wai, A.W.K., So, C.H. and Wu, R.S.S. (2000) Cellular and molecular basis of cadmium-induced deformities in zebrafish embryos. Environ. Toxicol. Chem. 19: 3024-3031.

20. OECD (1992) Ready Biodegradability. Test Guideline No. 301. Guidelines for Testing of Chemicals, OECD, Paris. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

21. OECD (2000) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Series on Testing and Assessment No. 23, ENV/JM/MONO8200)6, OECD, Paris. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

22. OECD (2009) Fish Short Term Reproduction Assay.Test Guideline No. 229, Guidelines for the Testing of Chemicals, OECD, Paris. Available : [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html]

23. ISO (1996) International Organization for Standardization. Water quality - Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346-3: Flow-through method. Available: [http://www.iso.org].

24. ISO (1997) Water Quality Sampling, ISO 5667, Part 16. Guidance on biotesting of samples, Wiley-VCH, Weinheim-New York. Available: [http://www.iso.org].

25. Laale, H.W. (1977) The biology and use of zebrafish, *Brachydanio rerio*, in fisheries research. A literature review. J. Fish Biol. 10: 121-173.

26. Westerfield, M. (2000) The zebrafish book: A guide for the laboratory use of zebrafish (*Brachydanio rerio*). 3rd edition. Eugene, University of Oregon Press, Institute of Neuroscience, USA.

27. Canadian Council on Animal Care (2005) Guidelines on: the Care and Use of Fish in Research, Teaching and Testing, ISBN: 0-919087-43-4 [http://www.ccac.ca/Documents/Standards/Guidelines/Fish.pdf]

28. European Commission 2007: Commission recommendation 2007/526/EC of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (notified under document number C(2007) 2525) [http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:197:0001:0089:EN:PDF]

29. European Union 2010 – Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union, L 276:33-79; 20.10.2010 [http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF]

30. Nagel, R. (1986) Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio*, Ham.-Buch.). J. Appl. Ichthyol. 2: 173-181.

31. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. and Schilling, T.F. (1995) Stages of embryonic development of the zebrafish. Dev. Dyn. 203: 253-310.

32. Litchfield, J.T. and Wilcoxon, F. (1949) A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exper. Ther. 96: 99-113.

33. Sprague, J.B. (1969) Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res. 3: 793-821.

34. Sprague, J.B. (1970) Measurement of pollutant toxicity to fish. II. Utilizing and applying bioassay results. Water Res. 4: 3-32.

35. Stephan, C.E. (1977) Methods for calculating an LC50. In Aquatic Toxicology and Hazard Evaluation (edited by Mayer, F.I. and Hamelink, J.L.). ASTM STP 634, pp 65-84, American Society for Testing and Materials.

36. Finney, D.J. (1978) Statistical Methods in Biological Assay (3rd Edition). Charles Griffin & Company Ltd, London, U.K.

37. ISO (2006) International Organization for Standardization. Water quality - Guidance on statistical interpretation of ecotoxicity data ISO TS 20281. Available: [http://www.iso.org].

38. OECD (2006) Guidance Document on Current Approaches in the Statistical Analysis of Ecotoxicity Data: a Guidance to Application. Series on Testing and Assessment No. 54.. OECD, Paris. Available: [http://www.oecd.org/document/22/0,2340,en_2649_34377_1916054_1_1_1_1,00.html].

ANNEX 1

DEFINITIONS

Blastula: The blastula is a cellular formation around the animal pole that covers a certain part of the yolk.

Median Lethal Concentration (LC50) is the concentration of a test substance that is estimated to be lethal to 50% of the test organisms.

Semi-static renewal test is a test without continuous flow of test solutions, but with regular batchwise renewal of the test solutions after defined periods (*e.g.*, every 24 hrs).

Static test is a test in which no flow of test solution occurs. Solutions remain unchanged throughout the duration of the test.

Flow-through test is a test with continued flow of test solutions through the test system during the duration of exposure.

Somite: In the developing vertebrate embryo, somites are masses of mesoderm distributed laterally to the neural tube, which will eventually develop dermis (dermatome), skeletal muscle (myotome), and vertebrae (sclerotome).

Internal Plate Control: Internal control consisting of 4 wells filled with dilution water per 24-well plate to identify potential contamination of the plates by (a) the manufacturer or (b) by the researcher during the procedure.

ANNEX 2

Maintenance, breeding and typical conditions for zebrafish embryo toxicity tests (1)

Zebrafish (*Danio rerio*)		
Origin of species		India, Burma, Malakka, Sumatra
Sexual dimorphism		Females: protruding belly, when carrying eggs Males: more slender, orange tint between blue longitudinal stripes (particularly evident at the anal fin)
Feeding regime		Dry flake food (max. 3% fish weight per day) 3 - 5 times daily; from three days before spawning, plus frozen adult brine shrimp (*Artemia* spec.) nauplii or small daphnids of appropriate size obtained from an uncontaminated source twice daily (ad libitum). To guarantee for optimal water quality, excess feces should be removed approx. one hour after feeding.
Approximate weight of adult fish		Females: 0.65±0.13 g Males: 0.5±0.1 g
Maintenance of parenthal fish	Illumination	Fluorescent bulbs (wide spectrum); 10 - 20 µmol/m^2/s, 540 - 1080 lux, or 50 - 100 ft-c (ambient laboratory levels); 12 - 16 hrs photoperiod
	Water temperature	26±1 °C
	Water quality	O_2 ≥80% saturation, hardness: e.g., ~ 30 - 300 mg/L $CaCO_3$, NO_3^-: ≤48mg/L, NH_4^+ and NO_2^-: <0.001 mg/L, residual chlorine <10 µg/L, total organic chlorine <25 ng/L, pH = 6.5 - 8.5
	Further water quality criteria	Particulate matter <20 mg/L, total organic carbon <2 mg/L, total organophosphorus pesticides <50 ng/L, total organochlorine pesticides plus polychlorinated biphenyls <50 ng/L
	Tank size for maintenance	*e.g.*, 180 L , 1 fish/L
	Water purification	Permanent (charcoal filtered); possible are combinations with semi-static renewal maintenance or flow-through system with continuous water renewal
Recommended male to female ratio for breeding		2:1 (or mass spanwing)
Breeding tanks		*e.g.*, 4 L tanks equipped with steel grid bottom and plant dummy as spawning stimulant; external heating mats, or mass spawning within the maintenance tanks
Egg structure and appearance		Stable chorion, highly transparent, non-sticky, diameter ~ 0,8 – 1,2 mm
Spawning rate		A single mature female spawns at least 50 - 80 eggs per day. Depending on the strain, spawning rates may be considerably higher. The fertilization rate should be ≥70%. In case first time spawning fish are used, fertilisation rates of the eggs may be lower in the first few spawns.
Test type		Static, semi-static renewal, flow-through, 26±1 °C, 24-well plates (2 ml per cavity)

Annex 3

Normal zebrafish development

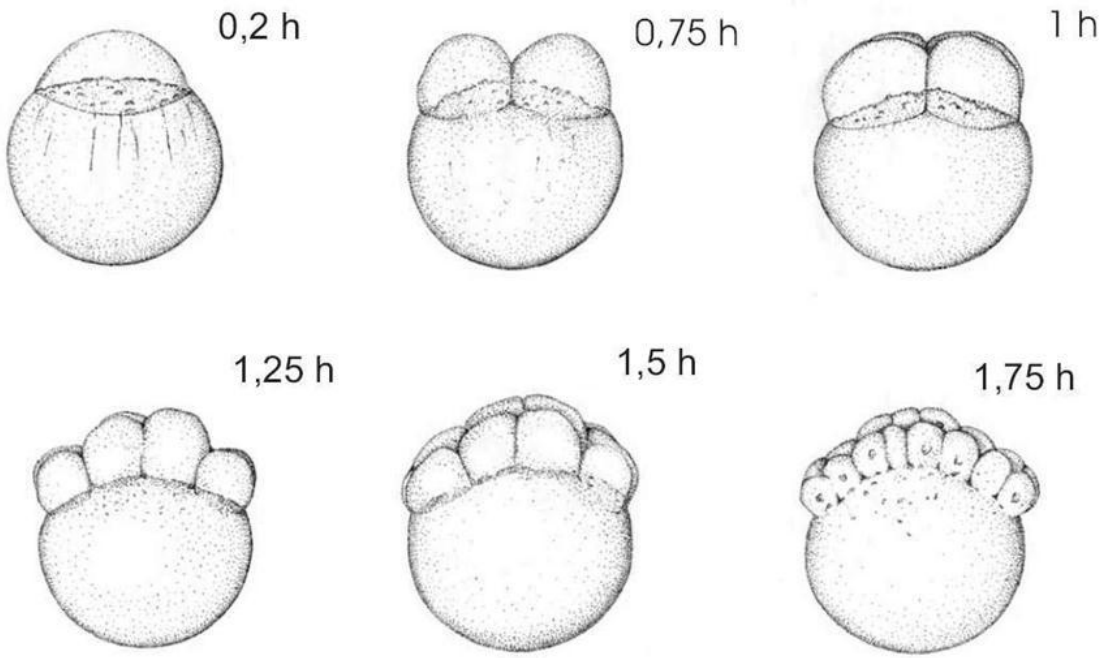


Fig. 1: **Selected stages of early zebrafish (*Danio rerio*) development**: 0.2 – 1.75 h post-fertilization (from Kimmel *et al.*, 1995). The time sequence of normal development may be taken to diagnose both fertilization and viability of eggs (see paragraph 25: Selection of fertilized eggs).

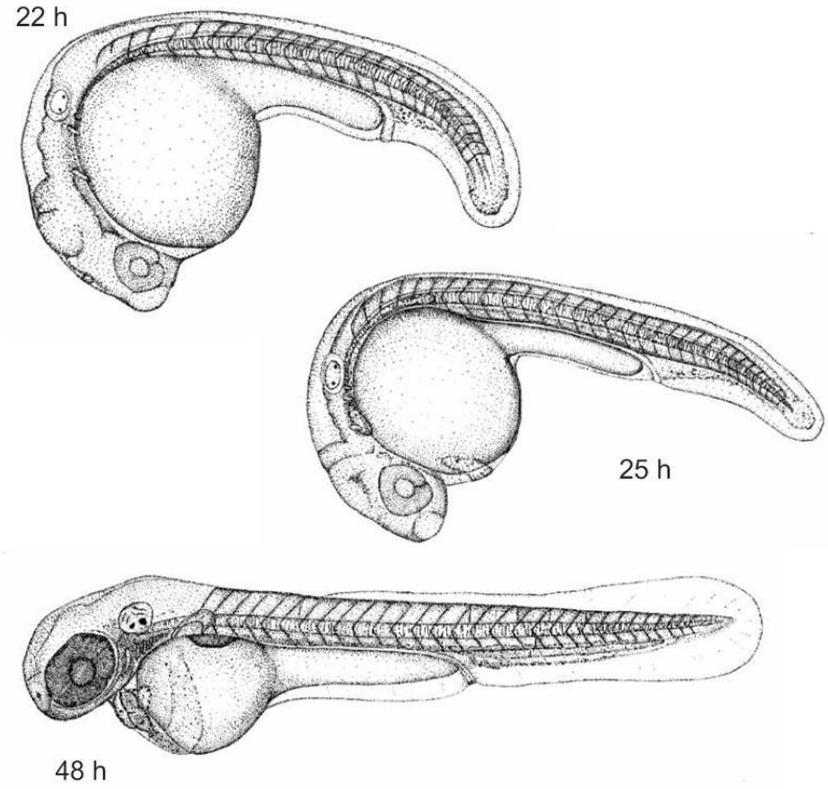


Fig. 2: **Selected stages of late zebrafish (Danio rerio) development**: 22 - 48 h after fertilization (from Kimmel *et al.*, 1995).

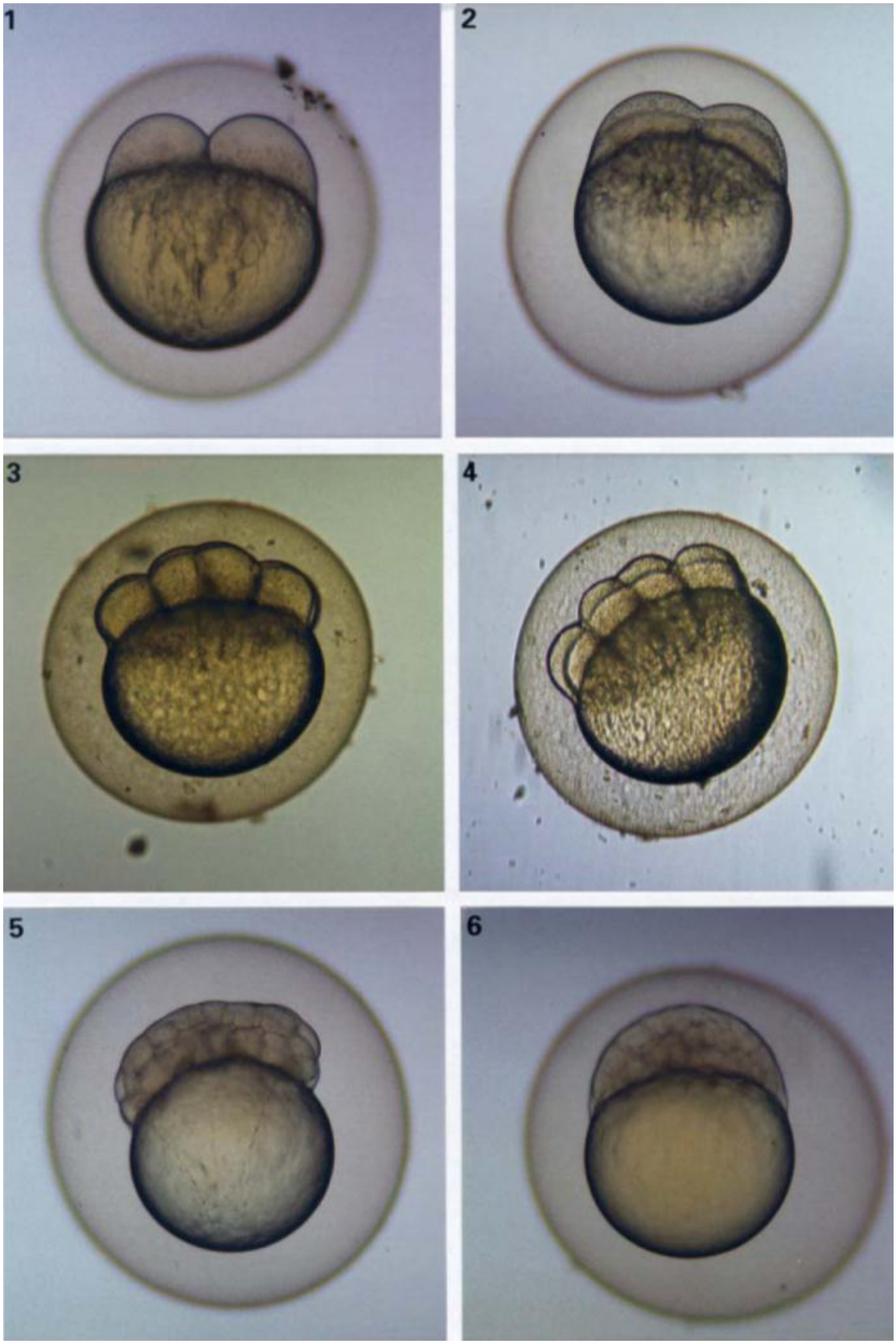


Fig. 3: **Normal development of zebrafish (*Danio rerio*) embryos** I: (1) 0.75 h, 2-cell stage; (2) 1 h, 4-cell stage; (3) 1.2 h, 8-cell stage; (4) 1.5 h, 16-cell stage; (5) 4.7 h, beginning epiboly; (6) 5.3 h, approx. 50 % epiboly (from Braunbeck & Lammer 2005).

Annex 4

Atlas of lethal endpoints for the Zebrafish Embryo Toxicity Test

The following apical endpoints indicate acute toxicity and, consequently, death of the embryos: coagulation of the embryo, non-detachment of the tail, non-formation of somites and non-detection of the heartbeat. The following micrographs have been selected to illustrate these endpoints.

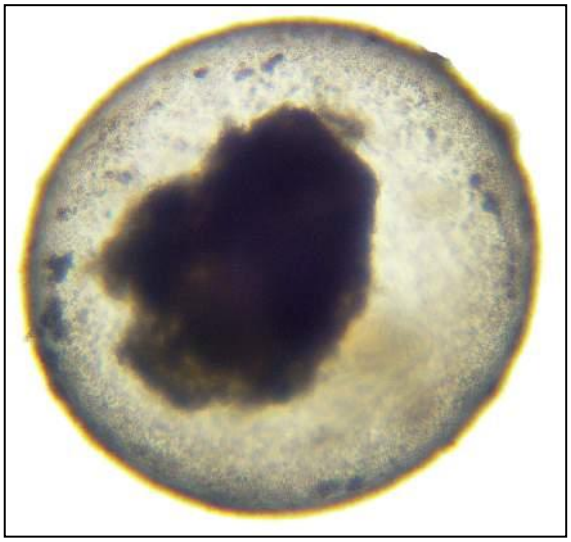


Fig. 1: **Coagulation of the embryo**: Under bright field illumination, coagulated zebrafish embryo show a variety of intransparent inclusions.

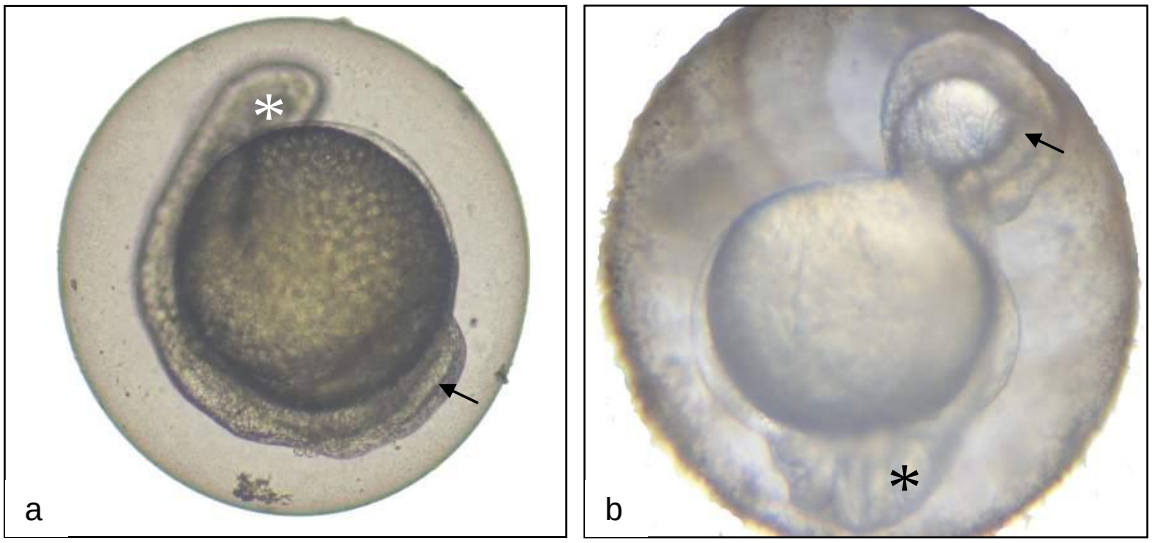


Fig. 2: **Non-detachment of tail** bud in lateral view (a: →; 96 h old zebrafish embryo) and frontal rear view (b: →; 96 h old zebrafish embryo). Note also the lack of the eye bud (*).

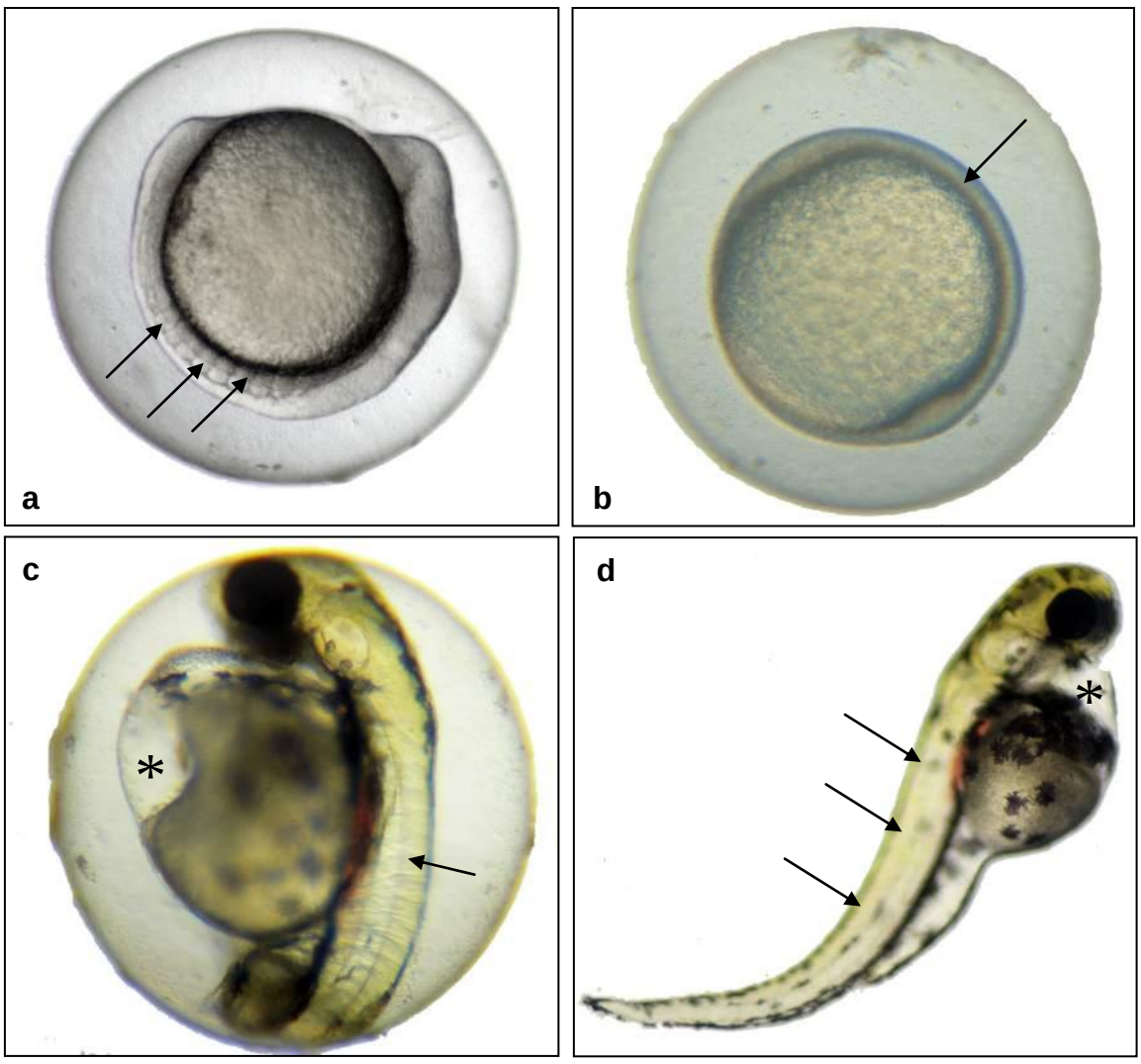


Fig 3: **Non-formation of somites**: Although retarded in development by approx. 10 h, the 24 h old zebrafish embryo in (a) shows well-developed somites (a: →), whereas the embryo in the right micrograph does not show any sign of somite formation (b: →). Although showing a pronounced yolk sac edema (*), the 48 h old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 h (!) old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial edema in the embryo shown in Fig. (d), see also figure A2d.

.

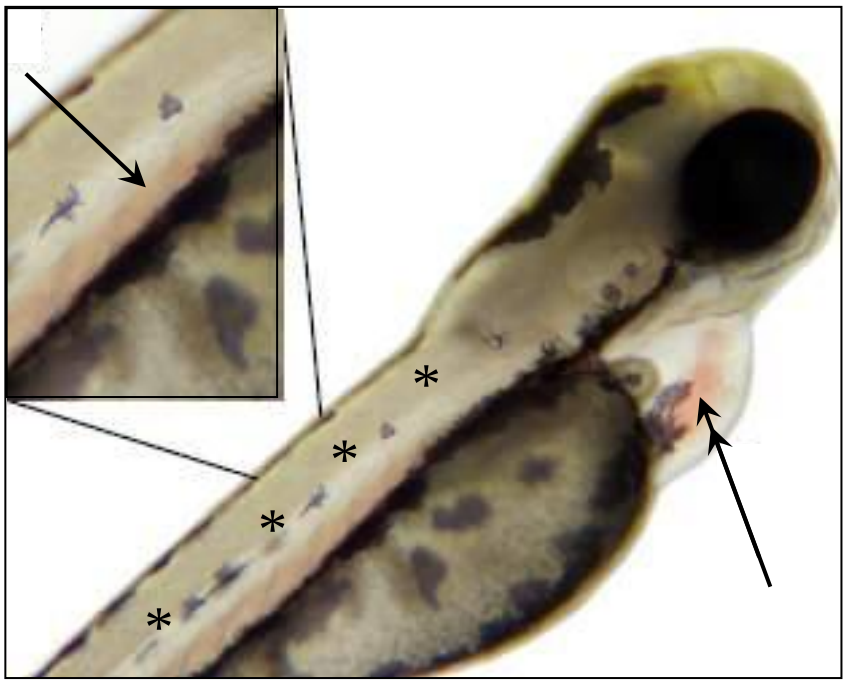
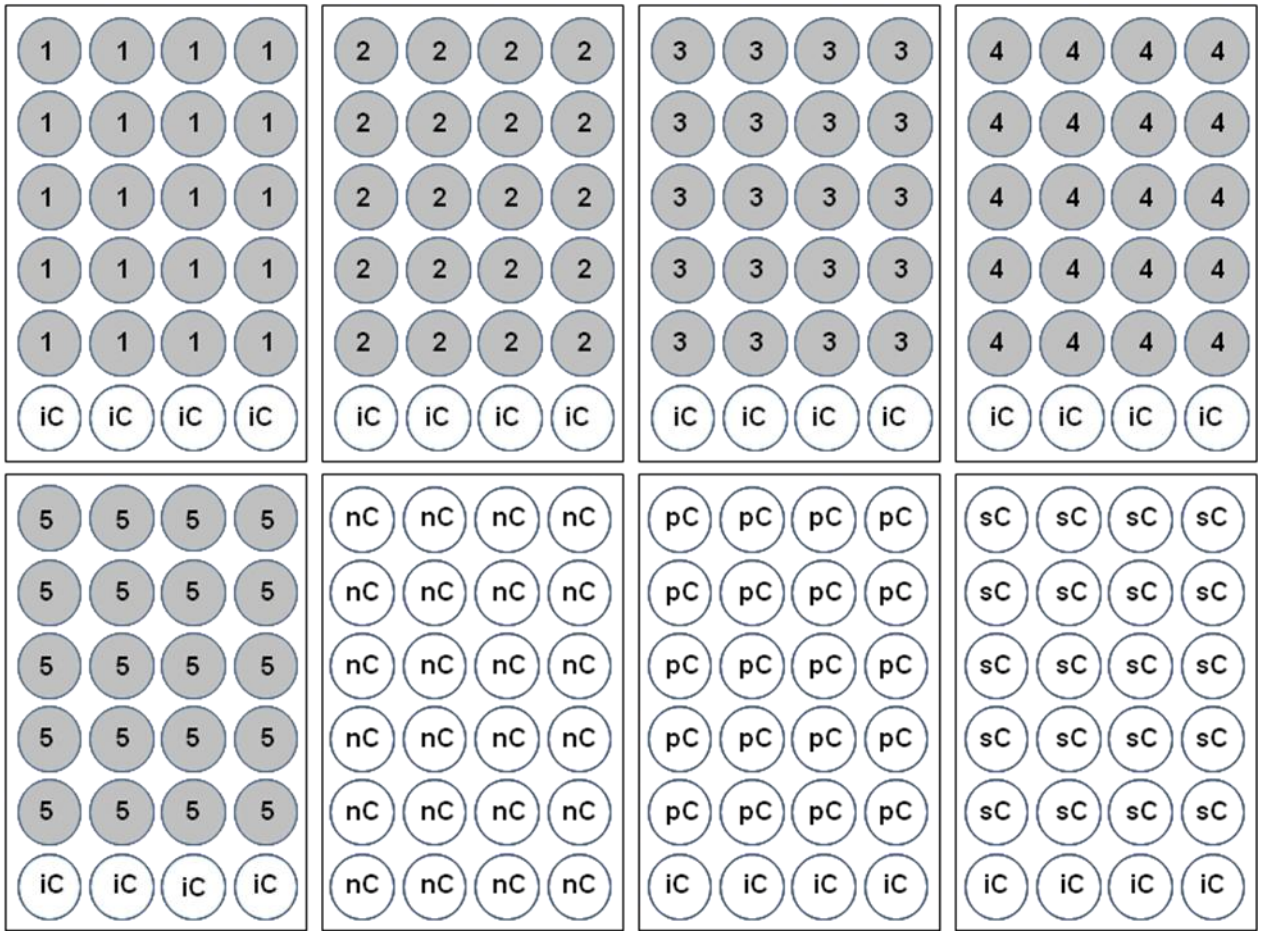


Fig. 4: **Lack of heart beat** is, by definition, difficult to illustrate in a micrograph. Lack of heart beat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, e.g., the aorta abdominalis (→ in insert) is not an indicator for lack of heart beat. Note also the lack of somite formation in this embryo (*, homogenous rather than segmental appearance of muscular tissues). The observation time to record an absence of heart beat should be at least of 1 min with a minimum magnification of 80×.

Annex 5

Fig 1: Layout of 24-well plates



1-5 = five test concentrations / chemical; nC = negative control (dillution water); iC = internal plate control (dilution water); pC = positive control (3,4-DCA 4mg/L); sC = solvent control

Fig. 2: Scheme of the zebrafish embryo toxicity test procedure (from left to right): collection of the eggs, pre-exposure immediately after fertilisation in glass vessels, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised eggs into 24-well plates prepared with the respective test concentrations/controls, n = number of eggs required per test concentration/control (here 20)