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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Fish Embryo Acute Toxicity (FET) Test

INTRODUCTION

1. This Test Guideline (TG) 236 describes a Fish Embryo Acute 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). The FET-test has been successfully applied to a wide range of substances exhibiting diverse modes of action, solubilities, volatilities, and hydrophobicities (reviewed in 15 and 16).

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

PRINCIPLE OF THE TEST

3. Newly fertilised zebrafish eggs are exposed to the test chemical for a period 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 LC_{50} is calculated.

INITIAL CONSIDERATIONS

4. Useful information about substance-specific properties include the structural formula, molecular weight, purity, stability in water and light, pK_a and K_{ow}, water solubility and vapour pressure as well as results of a test for ready biodegradability (OECD TG 301 (17) or TG 310 (18)). Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur. 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.

5. If the Test Guideline is used for the testing of a mixture, its composition should, as far as possible, be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (see paragraph 4). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

6. Concerning substances that may be activated *via* metabolism, there is evidence that zebrafish embryos do have biotransformation capacities (19)(20)(21)(22). However, the metabolic capacity of embryonic fish is not always similar to that of juvenile or adult fish. For instance, the protoxicant allyl

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alcohol (9) has been missed in the FET. Therefore, if there are any 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 of the test chemical, or alternatively perform another test which takes metabolism into further account.

7. For substances with a molecular weight ≥ 3kDa, a very bulky molecular structure, and substances causing delayed hatch which might preclude or reduce the post-hatch exposure, embryos are not expected to be sensitive because of limited bioavailability of the substance, and other toxicity tests might be more appropriate.

VALIDITY OF THE TEST

8. For the test results to be valid, the following criteria apply:

a) The overall fertilisation rate of all eggs collected should be ≥ 70% in the batch tested.

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

c) Overall survival of embryos in the negative (dilution-water) control, and, where relevant, in the solvent control should be ≥ 90% until the end of the 96 hrs exposure.

d) 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 96 hrs exposure.

e) Hatching rate in the negative control (and solvent control if appropriate) should be ≥ 80% at the end of 96 hrs exposure.

f) At the end of the 96 hrs exposure, the dissolved oxygen concentration in the negative control and highest test concentration should be ≥80% of saturation.

DESCRIPTION OF THE METHOD

9. An overview of recommended maintenance and test conditions is available in Annex 2.

Apparatus

10. 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 (see "Maintenance of brood fish", paragraph 14);

b) Inverted microscope and/or binocular with a capacity of at least 80-fold magnification. If the room used for recording observations cannot be adjusted to 26 ± 1 °C, a temperature-controlled cross movement stage or other methods to maintain temperature are necessary;

c) Test chambers; *e.g.*, standard 24-well plates with a depth of approx. 20 mm. (see "Test chambers", paragraph 11);

d) *e.g.*, self-adhesive foil to cover the 24-well plates;

e) Incubator or air-conditioned room with controlled temperature, allowing to maintain 26 ±1 °C in wells (or test chambers);

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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 to protect the eggs once laid; spawning substrate (*e.g.*, plant imitates of inert material) (OECD 229, Annex 4a (23));

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 eggs (*e.g.*, beakers, crystallisation dishes);

l) If alternative exposure systems, such as flow-through (24) or passive dosing (25) are used for the conduct of the test, appropriate facilities and equipment are needed.

Test chambers

11. Glass or polystyrene test chambers should be used (*e.g.*, 24-well plates with a 2.5 – 5 ml filling capacity per well). In case adsorption to polystyrene is suspected *(e.g.,* for non-polar, planar compounds with high K_{OW}*)*, inert materials (glass) should be used to reduce losses due to adsorption (26). Test chambers should be randomly positioned in the incubator.

Water and test conditions

12. Dilution of the maintenance water is recommended to achieve hardness levels typical of a wide variety of surface waters. Dilution water should be prepared from reconstituted water (27). The resulting degree of hardness should be equivalent to 100-300 mg/L $CaCO_3$ in order to prevent excessive precipitation of calcium carbonate. 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 to a minimum hardness of 30-35 mg/L $CaCO_3$. The water is aerated to oxygen saturation prior to addition of the test chemical. Temperature should be kept at 26 ± 1 °C, in the wells, throughout the test. The pH should be in a range between pH 6.5 and 8.5, and not vary within this range by more than 1.5 units during the course of the test. If the pH is not expected to remain in this range, then pH adjustment should be done prior to initiating the test. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test chemical is caused. Use of hydrogen chloride (HCl) and sodium hydroxide (NaOH) to correct pH in the solutions containing the test chemical is recommended.

Test solutions

13. 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 chemical in the dilution water by mechanical means (*e.g.*, stirring and / or ultra-sonification). If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance Document No. 23 for handling difficult substances should be followed (28). The use of solvents should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution. Where a solvent 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. When a solvent is used, an additional solvent control is required.

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Maintenance of brood fish

14. 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 recommended loading capacity of 1 L water per fish and a fixed 12 – 16 hour photoperiod (29)(30)(31)(32)(33). 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

15. As a reference substance, 3,4-dichloroaniline (used in the validation studies (1)(2)), should be tested in a full concentration-response range to check the sensitivity of the fish strain used, preferably twice a year. For any laboratory initially establishing this assay, the reference chemical should be used. A laboratory can use this chemical to demonstrate their technical competence in performing the assay prior to submitting data for regulatory purposes.

Egg production

16. 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 (*e.g.*, at a ratio of 2:1) in a breeding group 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 recommended. To avoid genetic bias, eggs are collected from a minimum of three breeding groups, mixed and randomly selected.

17. For the 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 inert wire mesh of appropriate mesh size (approx. 2±0.5 mm). If considered necessary, artificial plants made of inert material (*e.g.*, plastic or glass) can be fixed to the mesh as spawning stimulus (3)(4)(5)(23)(35). Weathered plastic materials which do not leach (*e.g.*, phthalates) should be used. Mating, spawning and fertilisation take place within 30 min after the onset of light and the spawn traps with the collected eggs can be carefully removed. Rinsing eggs with reconstituted water after collection from spawning traps is recommended.

Egg differentiation

18. 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 (see Annex 3)(35). At these stages, fertilised eggs can be clearly identified by the development of a blastula.

PROCEDURE

Conditions of exposure

19. Twenty embryos per concentration (one embryo per well) are exposed to the test chemical. Exposure should be such that ± 20% of the nominal chemical concentration are maintained throughout the test. If this is not possible in a static system, a manageable semi-static renewal interval should be applied (*e.g.*, renewal every 24 hrs). In these cases exposure concentrations need to be verified as a minimum in the highest and lowest test concentrations at the beginning and the end of each exposure interval (see

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paragraph 36). If an exposure concentration of ± 20% of the nominal concentrations cannot be maintained, all concentrations need to be measured at the beginning and the end of each exposure interval (see paragraph 36). Upon renewal, care should be taken that embryos remain covered by a small amount of old test solutions to avoid drying. The test design can be adapted to meet the testing requirements of specific substances (*e.g,*. flow-through (24) or passive dosing systems (25) for easily degradable or highly adsorptive substances (29), or others for volatile substances (36)(37)). In any case, care should be taken to minimise any stress to the embryos. Test chambers should be conditioned at least for 24 hrs with the test solutions prior to test initiation. Test conditions are summarised in Annex 2.

Test concentrations

20. Normally, five concentrations of the test chemical 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, as defined in paragraph 28. A range-finding test before the definitive test allows selection of the appropriate concentration range. The range-finding is typically performed using ten embryos per concentration. 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.

21. Details and visual instructions for allocation of concentrations across 24-well plates are available in paragraph 27 and Annex 4, Figure 1.

Controls

22. Dilution water 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, thus reducing the number of concentrations used to derive the LC_{50}. If an entire plate is rejected the ability to evaluate and discern observed effects may become more difficult, especially if the rejected plate is the solvent control plate or a plate in which treated embryos are also affected. In the first case the test must be repeated. In the second one the loss of an entire treatment group(s) due to internal control mortality may limit the ability to evaluate effects and determine LC_{50} values.

23. A positive control at a fixed concentration of 4 mg/L 3,4-dichloroaniline is performed with each egg batch used for testing.

24. In case a solvent is used, an additional group of 20 embryos is exposed to the solvent on a separate 24-well plate, thus serving as a solvent control. To consider the test acceptable, the solvent should be demonstrated to have no significant effects on time to hatch, survival, nor produce any other adverse effects on the embryos (cf. paragraph 8c).

Start of exposure and duration of test

25. The test is initiated as soon as possible after fertilisation of the eggs and terminated after 96 hrs of exposure. The embryos should be immersed in the test solutions before cleavage of the blastodisc commences, or, at latest, by the 16 cell-stage. To start exposure with minimum delay, at least twice 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 90 minutes post fertilisation.

26. Viable fertilised eggs should be separated from unfertilised eggs and be transferred to 24-well plates pre-conditioned for 24 hrs and refilled with 2 ml/well freshly prepared test solutions within 180 minutes post fertilisation. By means of stereomicroscopy (preferably ≥30-fold magnification), fertilised

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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 3, Fig. 1 and 3 and Annex 4, Fig. 2.

Distribution of eggs over the 24-well plates

27. Eggs are distributed to well plates in the following numbers (see also Annex 4, Fig. 1)

- 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 in dilution water as internal plate control on each of the above plates;
- 24 eggs in dilution water as negative control on one plate.

Observations

28. Apical observations performed on each tested embryo include: coagulation of embryos, lack of somite formation, non-detachment of the tail, and 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, hatching is recorded in treatment and control groups on a daily basis starting from 48 hrs. Observations are recorded every 24 hrs, until the end of the test.

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		+	+	+

29. *Coagulation of the embryo:* Coagulated embryos are milky white and appear dark under the microscope (see Annex 5, Fig. 1). The number of coagulated embryos is determined after 24, 48, 72 and 96 hrs.

30. *Lack of somite formation:* At 26±1°C, about 20 somites have formed after 24 hrs (see Annex 5, Figure 2) 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. Non-formation of somites after 24 hrs might be due to a general retardation of development. At latest after 48 hrs, the formation of somites should be developed. If not, the embryos are considered dead.

31. *Non-detachment of the tail:* In a normally developing zebrafish embryo, detachment of the tail (see Annex 5, Figure 3) 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.

32. *Lack of heartbeat:* In a normally developing zebrafish embryo at 26±1 °C, the heartbeat is

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visible after 48 hrs (see Annex 5, Figure 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. To record this endpoint, embryos showing no heartbeat should be observed under a minimum magnification of 80x for at least one minute. Absence of heartbeat is recorded after 48, 72 and 96 hrs.

33. Hatching rates of all treatment and control groups should be recorded from 48 hrs onwards and reported. Although hatching is not an endpoint used for the calculation of the LC_{50}, hatching ensures exposure of the embryo without a potential barrier function of the chorion, and as such may help data interpretation.

34. Detailed descriptions of the normal (35) and examples of abnormal development of zebrafish embryos are illustrated in Annexes 3 and 5.

Analytical measurements

35. At the beginning and at the end of the test, pH, total hardness and conductivity in the control(s) and in the highest test chemical concentration are measured. In semi-static renewal systems the pH should be measured prior to and after water renewal. The dissolved oxygen concentration is measured at the end of the test in the negative controls and highest test concentration with viable embryos, where it should be in compliance with the test validity criteria (see paragraph 7f). If there is concern that the temperature varies across the 24-well plates, temperature is measured in three randomly selected vessels. Temperature should be recorded preferably continuously during the test or, as a minimum, daily.

36. In a static system, the concentration of the test chemical should be measured, as a minimum, in the highest and lowest test concentrations, but preferably in all treatments, at the beginning and end of the test. In semi-static (renewal) tests where the concentration of the test chemical is expected to remain within ± 20% of the nominal values, it is recommended that, as a minimum, the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal. For tests where the concentration of the test chemical is not expected to remain within ± 20% of nominal, all test concentrations must be analysed when freshly prepared and immediately prior to renewal. 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. When the concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be expressed relative to the geometric mean of the measured concentrations; see Chapter 5 in the OECD Guidance Document No. 23 for more details (28).

LIMIT TEST

37. Using the procedures described in this guideline, a limit test may be performed at 100 mg/L of test chemical or at its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the LC_{50} 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 at the concentration tested exceeds the mortality in the negative control (or solvent control) by 10%, a full study should be conducted. Any observed effects should be recorded. If mortality exceeds 10% in the negative control (or solvent control), the test becomes invalid and should be repeated.

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DATA AND REPORTING

Treatment of results

38. In this test, the individual wells are considered independent replicates for statistical analysis. The percentages of embryos for which at least one of the apical observations is positive at 48 and/or 96 hrs are plotted against test concentrations. For calculation of the slopes of the curve, LC_{50} values and the confidence limits (95%), appropriate statistical methods should be applied (38) and the OECD Guidance Document No. 54 should be consulted (39).

Test report

39. The test report should include the following information:

Test chemical:

Mono-constituent substance

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate).

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

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 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;
- 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

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of the test chemical 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 validity criteria;
- fertilisation rate of the eggs;
- hatching rate in treatment and control groups.

Results:

- maximum concentration causing no mortality within the duration of the test;
- minimum concentration causing 100 % mortality within the duration of the test;
- cumulative mortality for each concentration at the recommended observation times;
- the LC_{50} values at 96 hrs (and optionally at 48 hrs) for mortality with 95% confidence limits, if possible;
- 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 (see examples provided in Annex 5, Figure 2);
- incidents in the course of the test which might have influenced the results;
- statistical analysis and treatment of data (probit analysis, logistic regression model and geometric mean for LC_{50});
- slope and confidence limits of the regression of the (transformed) concentration-response curve.

Any deviation from the Guideline and relevant explanations.

Discussion and interpretation of results.

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ANNEX 1

DEFINITIONS

Apical endpoint: Causing effect at population level.

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

Epiboly: is a massive proliferation of predominantly epidermal cells in the gastrulation phase of the embryo and their movement from the dorsal to the ventral side, by which entodermal cell layers are internalised in an invagination-like process and the yolk is incorporated into the embryo.

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

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 the manufacturer or by the researcher during the procedure, and any plate effect possibly influencing the outcome of the test (e.g. temperature gradient).

IUPAC: International Union of Pure and Applied Chemistry

Maintenance water: Water in which the husbandry of the adult fish is performed.

Median Lethal Concentration (LC_{50}) is the concentration of a test substance that is estimated to be lethal to 50% of the test organisms within the test duration.

Semi-static renewal test is a test with regular renewal of the test solutions after defined periods (*e.g.*, every 24 hrs).

SMILES: Simplified Molecular Input Line Entry Specification

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).

Static test is a test in which test solutions remain unchanged throughout the duration of the test.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials

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ANNEX 2

MAINTENANCE, BREEDING AND TYPICAL CONDITIONS FOR ZEBRAFISH EMBRYO ACUTE TOXICITY TESTS

		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; additionally brine shrimp (*Artemia* spec.) *nauplii* and / or small daphnids of appropriate size obtained from an uncontaminated source. Feeding live food provides a source of environmental enrichment and therefore live food should be given wherever possible. To guarantee for optimal water quality, excess food and 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 parental fish	Illumination	Fluorescent bulbs (wide spectrum); 10 - 20 µE/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); other possibilities include 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 spawning)
Spawning 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

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Egg structure and appearance	Stable chorion (*i.e.* highly transparent, non-sticky, diameter ~ 0.8 – 1.5 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 fertilisation rate should be ≥70%. For first time spawning fish, 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 hrs conditioned test chambers (*e.g.*, 24-well plates 2.5 - 5 ml per cavity)

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ANNEX 3

NORMAL ZEBRAFISH DEVELOPMENT AT 26°C

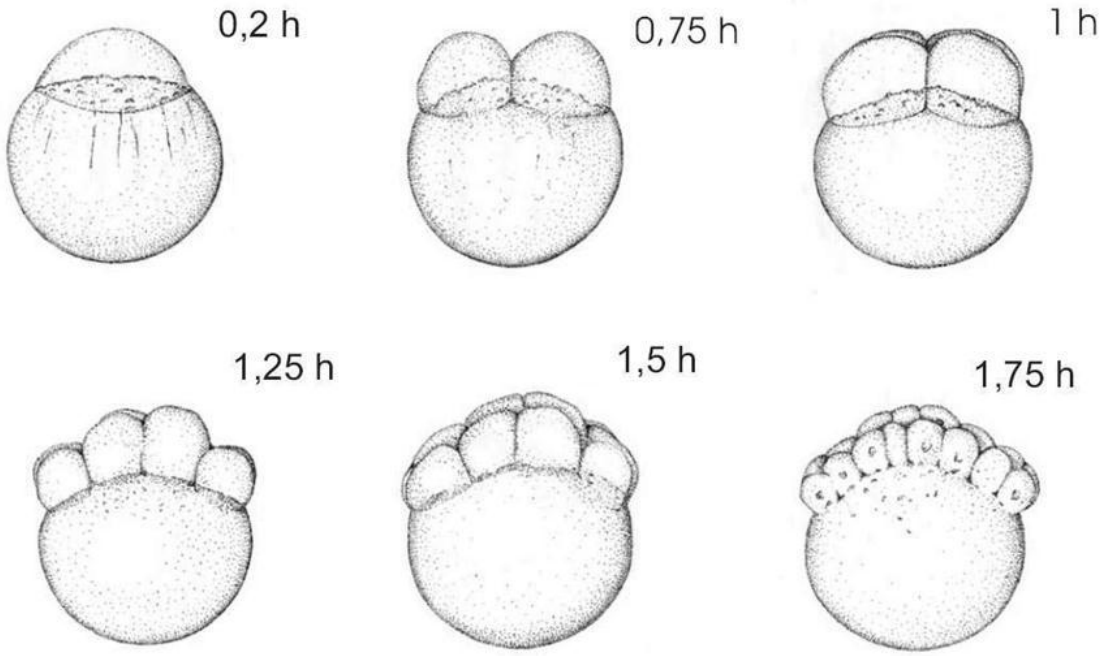

Fig. 1: **Selected stages of early zebrafish (*Danio rerio*) development**: 0.2 – 1.75 hrs post-fertilisation (from Kimmel *et al.*, 1995). The time sequence of normal development may be taken to diagnose both fertilisation and viability of eggs (see paragraph 26: Selection of fertilised eggs).

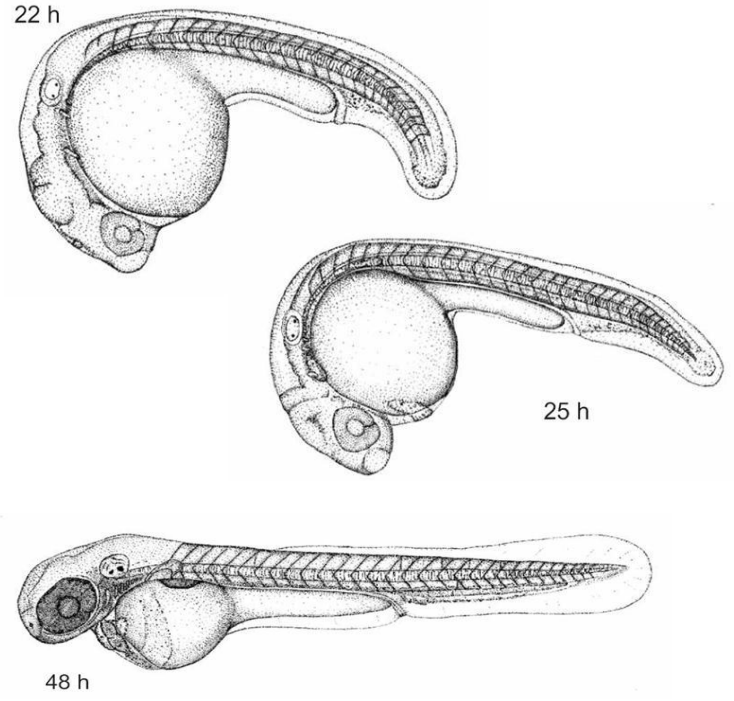

Fig. 2: **Selected stages of late zebrafish (Danio rerio) development (de-chorionated embryo to optimise visibility)**: 22 - 48 hrs after fertilisation (from Kimmel *et al.*, 1995 (35)).

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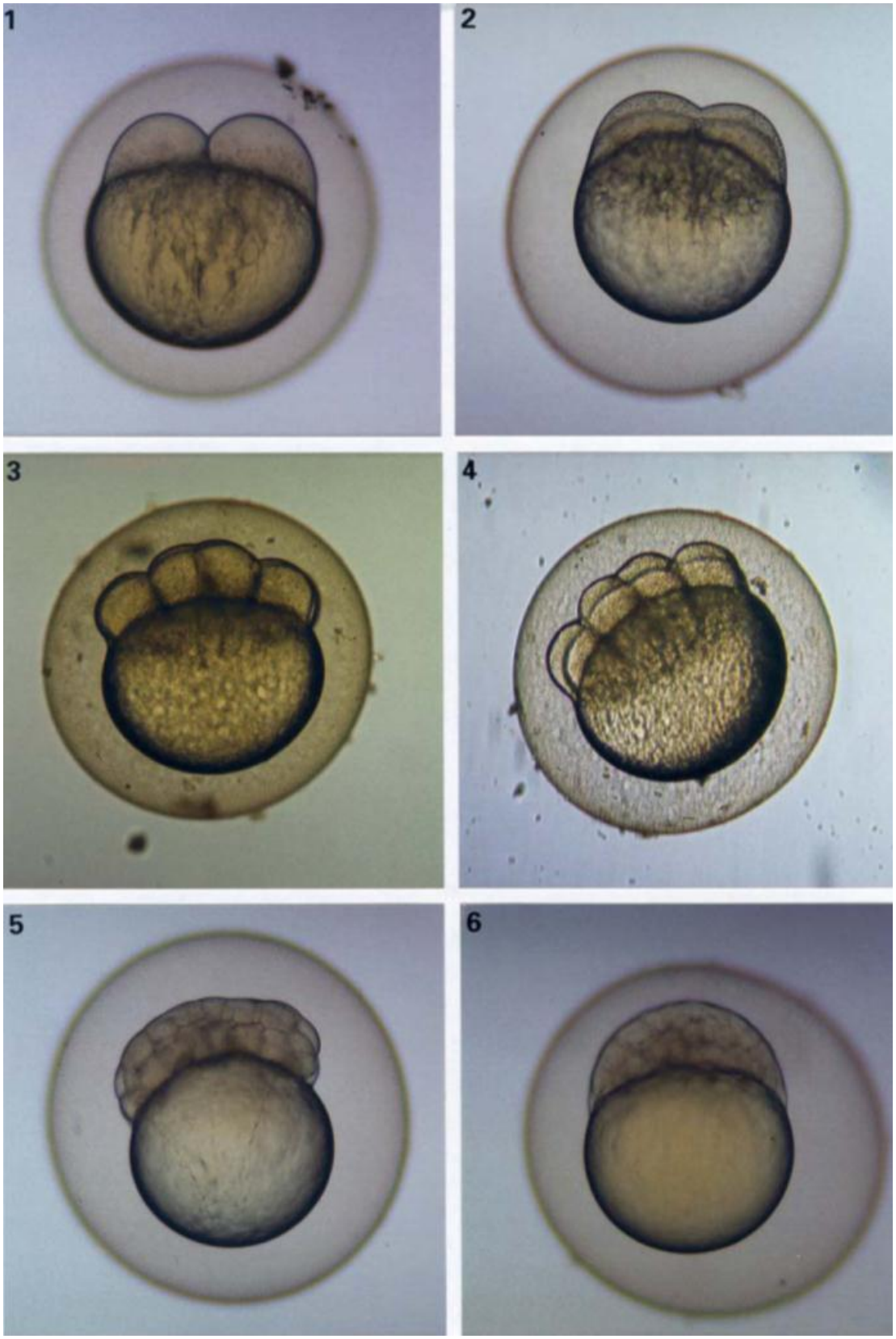

Fig. 3: **Normal development of zebrafish (*Danio rerio*) embryos**: (1) 0.75 hrs, 2-cell stage; (2) 1 hr, 4-cell stage; (3) 1.2 hrs, 8-cell stage; (4) 1.5 hrs, 16-cell stage; (5) 4.7 hrs, beginning epiboly; (6) 5.3 hrs, approx. 50 % epiboly (from Braunbeck & Lammer 2006 (40)).

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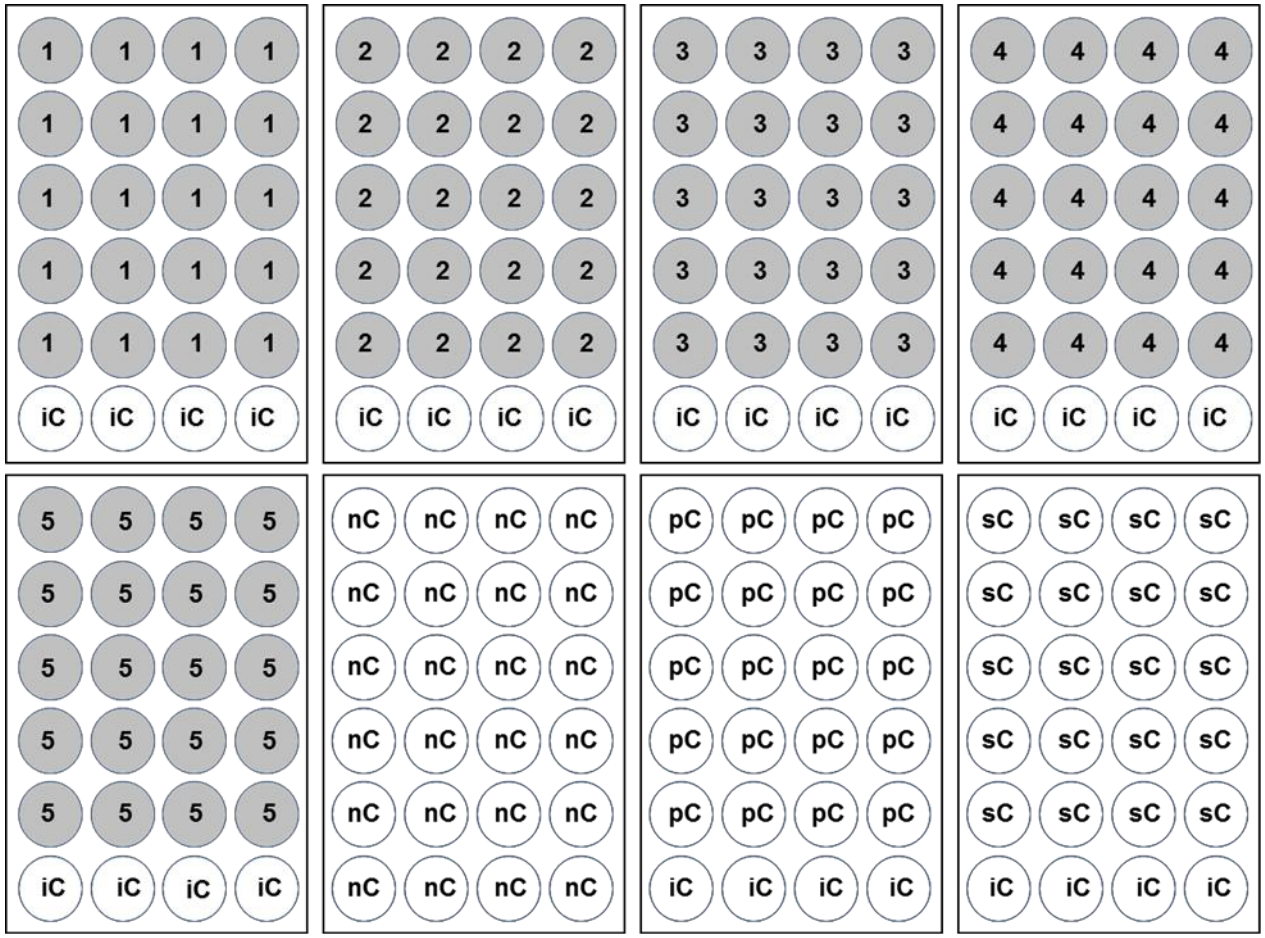
ANNEX 4

Fig. 1: **Layout of 24-well plates**



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

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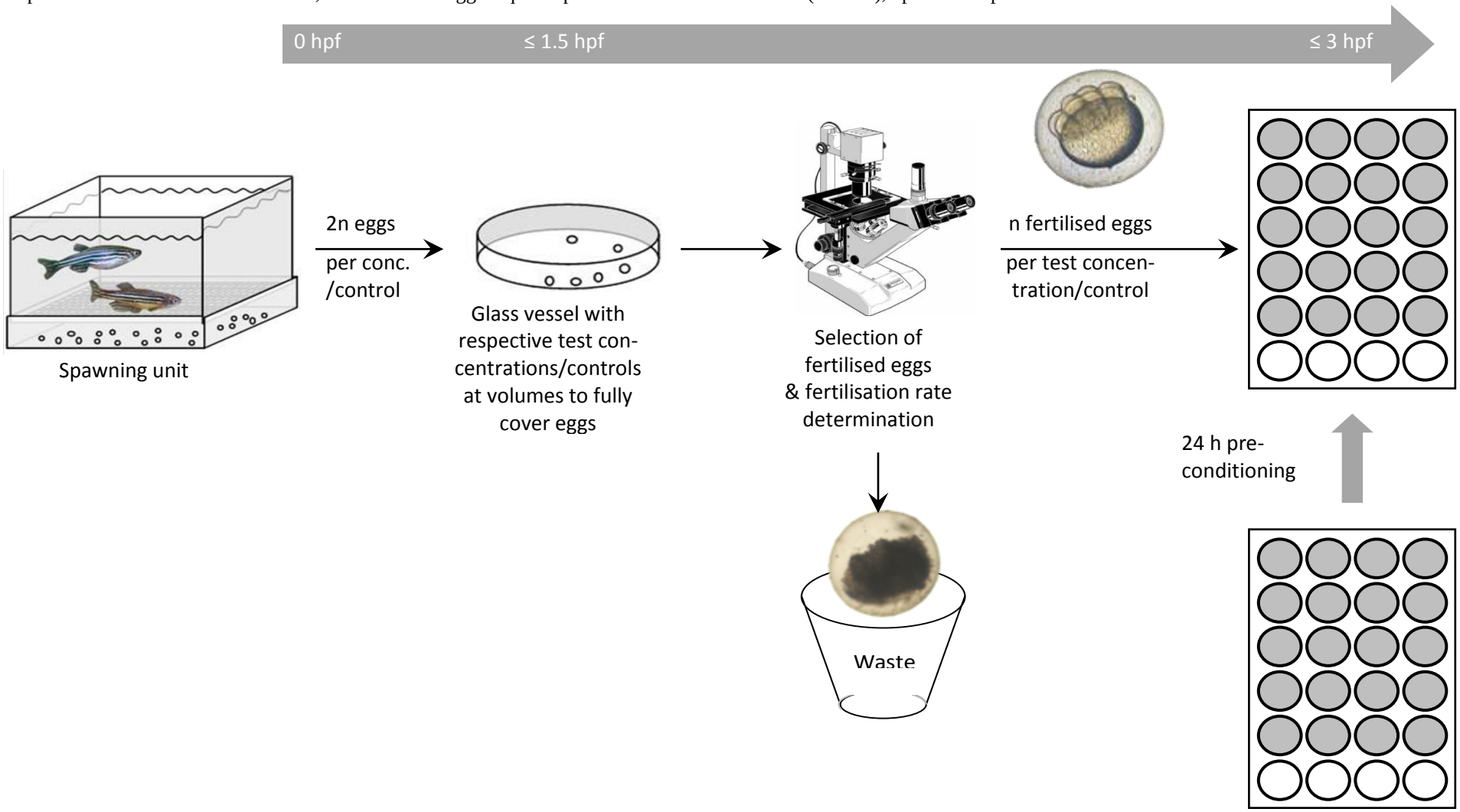
Fig. 2: **Scheme of the zebrafish embryo acute toxicity test procedure (from left to right):** production of eggs, collection of the eggs, pre-exposure immediately after fertilisation in glass vessels, selection of fertilised eggs with an inverted microscope or binocular and distribution of fertilised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



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ANNEX 5

ATLAS OF LETHAL ENDPOINTS FOR THE ZEBRAFISH EMBRYO ACUTE TOXICITY TEST

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

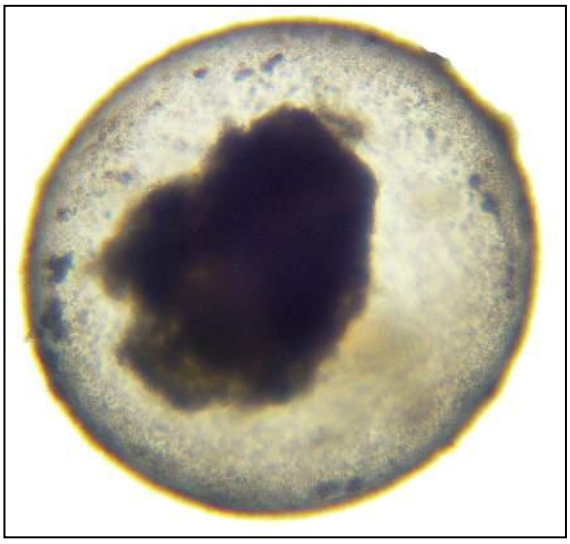

Fig. 1: **Coagulation of the embryo**: Under bright field illumination, coagulated zebrafish embryos show a variety of intransparent inclusions.

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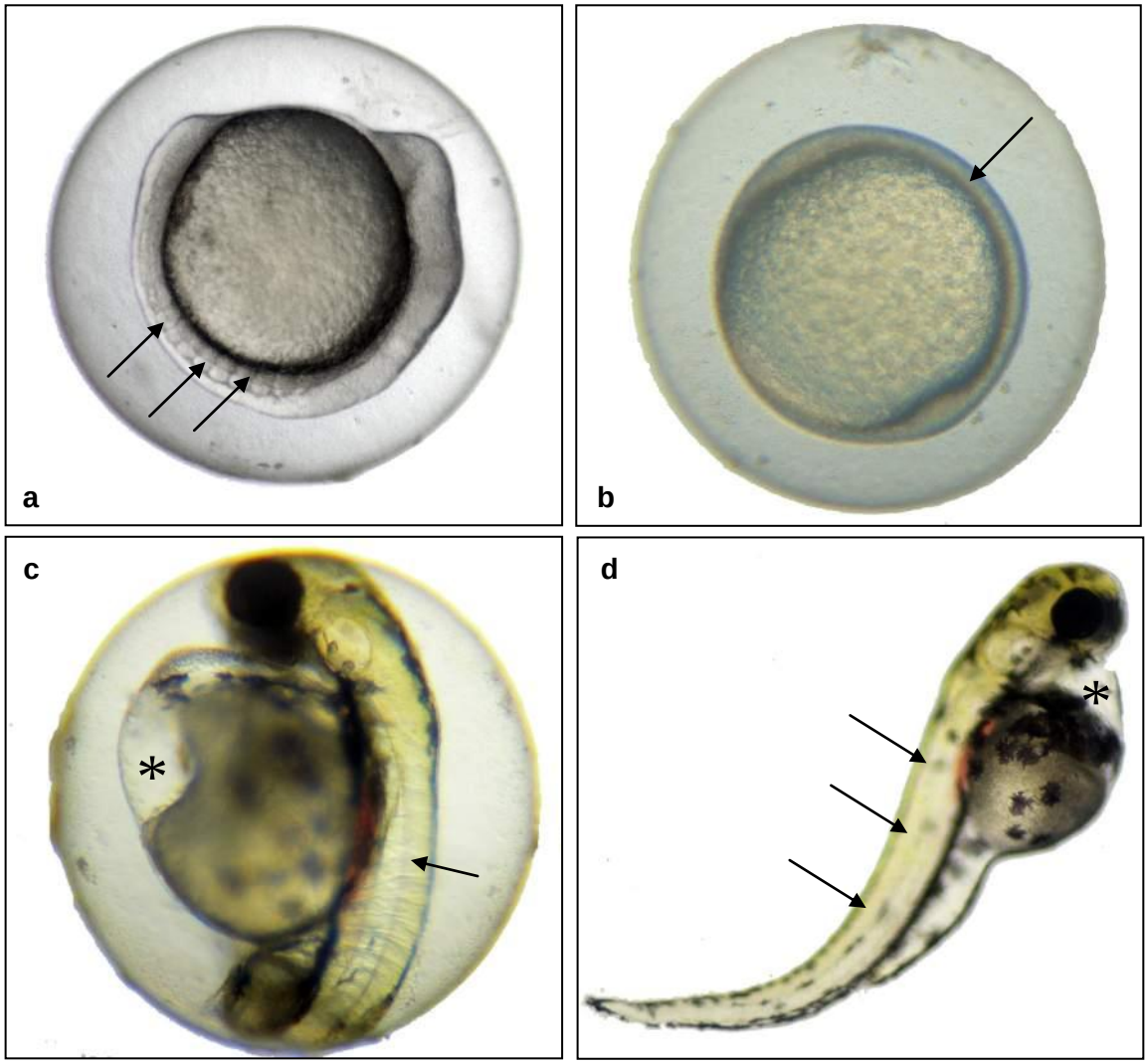

Fig. 2: **Lack of somite formation**: Although retarded in development by approx. 10 hrs, the 24 hrs old zebrafish embryo in (a) shows well-developed somites (→), whereas the embryo in (b) does not show any sign of somite formation (→). Although showing a pronounced yolk sac oedema (*), the 48 hrs old zebrafish embryo in (c) shows distinct formation of somites (→), whereas the 96 hrs old zebrafish embryo depicted in (d) does not show any sign of somite formation (→). Note also the spinal curvature (scoliosis) and the pericardial oedema (*) in the embryo shown in (d).

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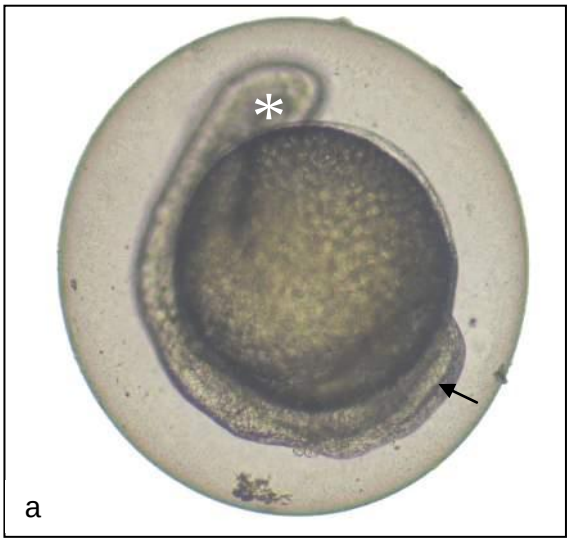

Fig. 3: **Non-detachment of the tail** bud in lateral view (a: →; 96 hrs old zebrafish embryo). Note also the lack of the eye bud (*).

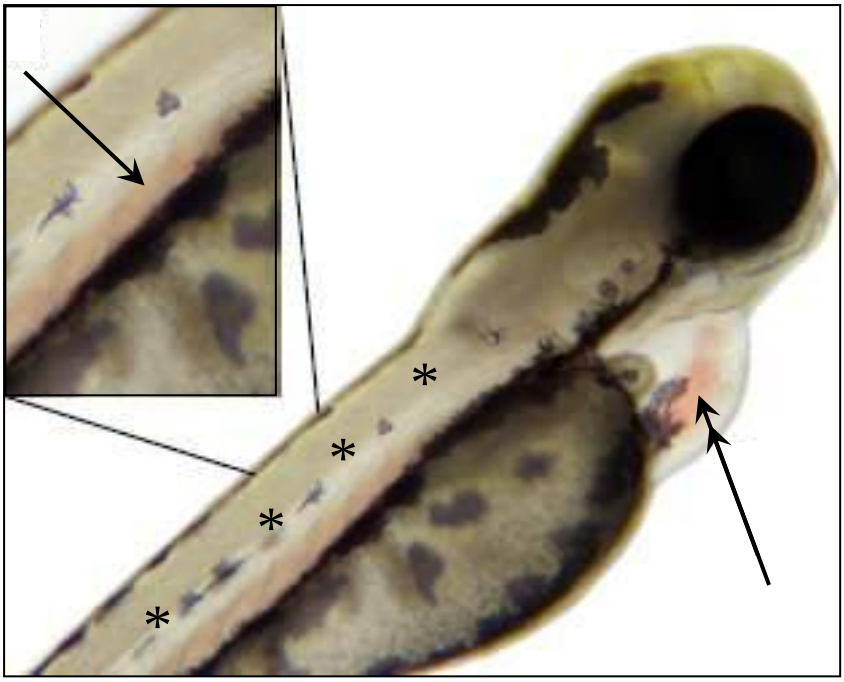

Fig. 4: **Lack of heartbeat** is, by definition, difficult to illustrate in a micrograph. Lack of heartbeat is indicated by non-convulsion of the heart (double arrow). Immobility of blood cells in, *e.g.*, the aorta abdominalis (→ in insert) is not an indicator for lack of heartbeat. Note also the lack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

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