LABPLAS

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Land-Based Solutions for Plastics in the Sea

D6.2. SOPs for adapted TG236 and TG201 to test small micro- and nano-plastics

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Nº	Participant name	Acronym	Country	Type
1	UNIVERSIDADE DE VIGO	UVI	SPAIN	HES
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3	Bundesanstalt fuer Gewaesserkunde	BfG	GERMANY	RTO
4	LABORATORIO IBERICO INTERNACIONAL DE NANOTECNOLOGIA	INL	PORTUGAL	RTO
5	KATHOLIEKE UNIVERSITEIT LEUVEN	KUL	BELGIUM	HES
6	HELMHOLTZ ZENTRUM FUR OZEANFORSCHUNG KIEL	GEOMAR	GERMANY	RTO
7	NATIONAL OCEANOGRAPHY CENTRE	NOC	UNITED KINGDOM	RTO
8	SORBONNE UNIVERSITE	SU	FRANCE	HES
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11	ASSOCIACAO PARA O DESENVOLVIMENTO DO ATLANTIC INTERNATIONAL RESEARCH CENTRE	AC	PORTUGAL	RTO
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Executive summary:	This document corresponds to Deliverable 6.2 *SOPs for adapted TG236 and TG201*. Current test guidelines for aquatic toxicity assessment are described for chemicals but not adapted to nanoparticles or nanoplastics. Their intrinsic properties (particulate material, high specific surface, usual low dispersibility in aqueous solutions) make new or adapted guidelines necessary and start to appear for standardization. In this deliverable, we propose the adaptation of two test guidelines (TG201 and TG236) to obtain more relevant, meaningful and accurate results on the hazard assessment of small micro- and nanoplastics (SMNPs) on freshwater organisms using two models: *Danio rerio* embryos and *Microcystis aeruginosa* cyanobacteria.

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2.0	27/05/2022	Revised and formatted version (open for modification)
3.0	13/02/2023	Reviewed with comments from the reviewer and PO addressed

Disclaimer

The views and opinions expressed in this document reflect only the authors' views, and not necessarily those of the European Commission.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

Abbreviation / Acronym	Description
DAD	Diode-Array Detection
EC$_{50}$	Half maximal effective concentration
ELISA	Enzyme-Linked Immunosorbent Assay
hpf	hours post fertilization
HPLC	High-Performance Liquid Chromatography
LoQ	Limit of Quantification
NTA	Nanoparticle Tracking Analysis
OECD	The Organization for Economic Co-operation and Development
SOP	Standard Operating Procedure
STDV	Standard Deviation
TG	Test Guideline

1 INTRODUCTION

Plastic is pouring from land into our oceans at a rate of nearly 10 million tonnes a year. Once in the sea, plastics fragment into particles moving with the currents and ocean gyres before washing up on the coastline. The smaller the size the higher the risk posed by these particles to organisms and human health. Because small, micro- and nano-plastics (SMNP) cannot be removed from oceans, proactive action regarding research on plastic alternatives and strategies to prevent plastic from entering the environment should be taken promptly. The **LABPLAS** project is a 48-month project whose vision is to develop new techniques and models for the detection and quantification of SMNP. Specifically, **LABPLAS** will determine reliable identification methods for a more accurate assessment of the abundance, distribution, and toxicity determination of SMNP and associated chemicals in the environment. It will also develop practical computational tools that should facilitate the mapping of plastic-impacted hotspots and promote scientifically sound plastic governance.

This document corresponds to Deliverable 6.2 *SOPs for adapted TG236 and TG201 to test small micro- and nano-plastics*. This deliverable supplements Deliverable 6.1, where the full LABPLAS toxicity testing scheme is described. This scheme is organized in two steps. In Tier I leachates from SMNP are tested. In the Tier II test organisms are exposed to SMNP themselves. We refer to D6.1 for further details on the procedures and particle sizes used in each step.

Current test guidelines for aquatic toxicity assessment are described for chemicals but not adapted to plastic particles, especially those of smaller sizes such as nanoparticles or nanoplastics. Their intrinsic properties (particulate material, high specific surface, usual low dispersibility in aqueous solutions) make new or adapted guidelines necessary and start to appear for standardization. In this deliverable, we propose the adaptation of two test guidelines (TG201 and TG236) to obtain more relevant, meaningful and accurate results on the hazard assessment of small micro- and nanoplastics (SMNPs) on freshwater organisms using two standard models: *Danio rerio* embryos and *Microcystis aeruginosa* cyanobacteria. Particular attention is paid to:

i) the colloidal stability of the SMNP dispersion in test media
ii) possible sub-lethal effects of SMNPs on embryonic development with particular attention to endpoints related to metabolic/hormonal disruption such as lipid metabolism
iii) implications of the presence of SMNPs in the production of biotoxins (microcystins) by the toxinogenic cyanobacteria *M. aeruginosa*

These SOPs will be used during the LABPLAS project to evaluate the toxicity of SMNPs on freshwater organisms.

2 STANDARD OPERATING PROCEDURES

2.1 Preparation and characterization of small micro- and nanoplastics dispersions obtained from plastic particles for ecotoxicological tests

2.1.1 Purpose

The purpose of this SOP is to document the procedures to prepare and characterize small micro- and nanoplastics dispersions obtained from plastic particles. The dispersions are to be used in ecotoxicological tests and they should be prepared in the medium used for the tests (i.e. test medium).

2.1.2 Equipment and material

The following equipment and material are needed to perform the test:

- Mill
- 20 µm metallic sieve
- Ultrapure water
- Test medium
- Watch glass (one per sample)
- Glass flasks and vials
- Balance ± 0,00001g
- Metallic spatula
- 1 mL glass syringes

2.1.3 Preparation of nanoplastics

1. Grind the plastics until they are below 20 µm.
2. Manually sieve (with the help of a brush) the plastic particles by a metallic 20 µm sieve. An automatic sieve column may also be used.

2.1.4 Initial physicochemical characterization of the plastics

Optimize the dispersion conditions and characterize them:

1. In a glass vial, prepare a dispersion of 50 mg/L of the small micro- and nanoplastics in ultrapure water and test medium. Homogenize the dispersion by gently shaking the vial. Sonication or mechanical stirring should be avoided to preserve the original characteristics of the plastics.
2. Analyze the particles by nanoparticle tracking analysis (NTA) to get their size, size distribution and the concentration of particles in the dispersion (particles/mL). Load a 1 mL glass syringe with the as-prepared plastic dispersion and adjust the pumping rate to 50 uL/min (>1x107 particles/mL). A minimum of 5 videos with a duration of at least 60 seconds should be recorded (total frames analyzed = 1498). Rinse the NTA with 1 mL of ultrapure water between samples.
3. Tween-20 can be used to stabilize the particles if sedimentation or poor colloidal stability is observed. If used, the final concentration in the toxicology test should not produce toxicity and the respective control should be included in the test.

Observation: Sample concentration, flow rate and duration of the recorded videos might require optimization depending on the nature and size of the small micro-and nanoplastics.

For the ecotoxicological tests, the stock solution should be prepared according to the conditions defined in this step.

2.1.5 Long-term evaluation of small micro-and nanoplastics stability

Evaluate the stability of the small micro- and nanoplastics in ultrapure water and test medium for up to 7 days.

1. Prepare triplicates of 100 mL of the 50 mg/L small micro- and nanoplastics dispersions in ultrapure water and test medium.
2. Collect 1 mL aliquots from the first half of each dispersion in the following time points: 0 h, 2 h, 4 h, 6 h, 8 h, 12 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, and 7 days. Samples should remain undisturbed to evaluate the possible sedimentation of the particles. If sedimentation is observed, be careful not to disturb the dispersion when collecting the aliquots at each time point.
3. Analyze the collected samples by NTA to obtain their size, size distribution and the concentration of particles in the dispersion (particles/mL). Evaluate if the results are trustable or not via standard deviation (STDV) calculated in every measurement.
4. At 0 h and 7 days, collect samples to analyze by electron microscopy (transmission and/or scanning).

2.2 *Microcystis aeuriginosa* growth inhibition test and toxin production test

2.2.1 Purpose

The purpose of this SOP is to document the procedures of the *Microcystis aeruginosa* growth inhibition test and toxin production test.

2.2.2 Objective

The objective of this test is to evaluate the medium-term exposure toxicity of small micro- and nanoplastics in the *Microcystis aeruginosa* and if the presence of SMNPs induces any change in the toxin (microcystins) production and release.

2.2.3 Validity of the test

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

- The biomass in the control cultures should have increased exponentially by a factor of at least 16-fold during the test period.
- The mean coefficient of variation for section-by-section specific growth rates in the control cultures must not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures must not exceed 10%.

2.2.4 Description of the method

2.2.4.1 Equipment and material

The following equipment and material are needed to perform the test:

- Glass Erlenmeyers with air-permeable stoppers
- Light measurement instrument
- Apparatus to determine algal biomass such as microscope with counting chamber
- Micropipettes
- Vessels for the preparation of test concentrations
- Equipment for determination of pH

2.2.4.2 Test organisms

Test organisms should be the freshwater cyanobacteria *Microcystis aeruginosa* (toxin-producing strain).

2.2.4.3 Test chambers

Test chambers should be glass Erlenmeyers with air-permeable stoppers that allow sufficient mass transfer of CO_2 from the atmosphere.

2.2.4.4 Growth medium

Test water should be filtered Z medium. The constitution of the medium can be found in table 1.

Component	Concentration in medium (mg/L)	Component	Concentration in medium (mg/L)
NaNO$_3$	467	*Trace metals solution*	
Ca(NO$_3$)$_2$.4H$_2$O	59	(NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O	0.00704
K$_2$HPO$_4$	31	KBr	0.00952
MgSO4.7H2O	25	KI	0.00664
Na$_2$CO$_3$	21	ZnSO$_4$.7H$_2$O	0.02296
Fe-EDTA solution		Cd(NO$_3$)$_2$.4H$_2$O	0.01232
FeCl$_3$.6H$_2$O	9.0	Co(NO$_3$)$_2$.6H$_2$O	0.01168
Na$_2$EDTA	9.3	CuSO$_4$.5H$_2$O	0.01
Trace metals solution		NiSO4(NH4)2SO4.6H2O	0.01584
H$_3$BO$_3$	0.248	Cr(NO3)2.7H2O	0.00296
MnSO$_4$.4H$_2$O	0.1784	V$_2$O$_4$(SO$_4$)$_3$.16H$_2$O	0.0028
Na$_2$WO4.2H$_2$O	0.0024	Al$_2$(SO$_4$)$_3$K$_2$SO$_4$.24 H$_2$O	0.03792

Table 1. Constitution of the Z medium

2.2.4.5 Initial biomass concentration

The initial biomass should be 1-2x106 cells/mL.

2.2.4.6 Duration of test

The duration of the test may vary given that it should only terminate when the biomass in the control cultures has increased exponentially by a factor of at least 16-fold during the test period.

2.2.5 Procedure

2.2.5.1 Controls

Negative control of Z medium and positive control of 25 mg/L 3,5-dichlorophenol are used.

If a solvent/stabilizer is used to disperse the small micro- and nanoplastics a solvent /stabilizer control is added.

2.2.5.2 Test concentrations

When possible, five test concentrations should be used separated by a factor of 10. This range can be decreased if the toxicity is not well distributed within the concentrations. The small micro- and nanoplastics stock solution should be dispersed as required and the test concentrations prepared in a filtered Z medium whenever possible.

2.2.5.3 Replicates

At least three replicates should be included for each concentration.

2.2.5.4 Inoculum culture

An inoculum culture should be prepared 3-4 days before the start of the test to ensure that the *Microcystis* are in exponential growth when used. The initial concentration of the culture should be above $1.5x10^6$ cells/mL.

2.2.5.5 Incubation

The test must be incubated at 25ºC (± 2ºC), photoperiod of 14:10 (L:D) and light intensity of 1500-2000 lux.

The flasks should be placed randomly and repositioned daily in the incubator. After collecting the sample for counting, the flasks should be shaken before being returned to the incubator.

2.2.5.6 Evaluations

The algae biomass in each flask should be assessed continuously during the test period. A small amount should be removed from each replicate and the number of cells counted by microscope or assessed by chlorophyll fluorescence (if a correlation can be demonstrated).

Any abnormal appearance of the algae should be registered. If another method than the microscope counting is chosen, the appearance of the *Microcystis* should be checked at the end of the test.

The pH must be measured at the beginning and the end of the test in all concentrations.

2.2.5.7 Small micro- and nano plastics quantification

The real concentration of small micro- and nanoplastics should be assessed at all concentrations (unless below the LoQ for the analytical method). This quantification should be performed at the beginning and end of the test.

2.2.6 Data and statistical analysis

2.2.6.1 Response variables

Two response variables are evaluated:

2.2.6.1.1 Average specific growth rate

The average specific growth rate for a specific period is calculated as the logarithmic increase in the biomass from the equation for every single vessel of controls and treatments,

where:

$$\mu_{i-j} = \frac{ln\ ln\ X_j\ - ln\ ln\ X_i}{t_j - t_i}\ (day - 1)$$

$\mu_{i\text{-}j}$ is the average specific growth rate from the time i to j;

X_i is the biomass at the time i;

X_j is the biomass at the time j

For each treatment group and control group, calculate a mean value for growth rate along with variance estimates.

Calculate the average specific growth rate over the entire test duration. Assess, also the section-by-section growth rate, calculated as the specific growth rates for each day during the course of the test (e.g. 0-1, 1-2, etc.).

Calculate the percent inhibition of growth rate for each treatment replicate from the following equation:

$$\%Ir = \frac{\mu C - \mu T}{\mu C} \times 100$$

where:

%Ir is the percent inhibition in average specific growth rate;

µC is the mean value for the average specific growth rate (µ) in the control group;

µT is the average specific growth rate for the treatment replicate.

When solvents are used to prepare the test solutions, the solvent controls rather than the controls without solvents should be used in the calculation of inhibition percentage.

2.2.6.1.2 Yield

Yield is calculated as the biomass at the end of the test minus the starting biomass for each replicate of controls and small micro- and nanoplastics concentrations. For each test concentration and control, calculate a mean value for yield along with variance estimates. The percent inhibition in yield (%Iy) may be calculated for each treatment replicate as follows:

$$\%Iy = \frac{Yc - YT}{Yc} \times 100$$

where:

% Iy is the percent inhibition of yield;

YC is the mean value for yield in the control group;

YT is the value for yield for the treatment replicate.

2.2.6.2 Toxin production

Microcystins' production is evaluated by quantifying microcystins in the cyanobacteria samples before and after the experiment. In brief, cells are disrupted by probe ultrasonication (300 watts, 1 min with pulses of 5 s on/ 5 s off), treated samples are filtered through 1µm filters to remove the debris and quantification of the toxin in the filtrate by ELISA, HPLC-DAD or protein phosphatase-1 inhibition test.

2.2.6.3 Statistical analysis

Appropriated statistical methods should be applied to detect differences between the controls and the concentrations of exposure for both response variables. When possible the EC$_{50}$ should be calculated.

2.3 Semi-static zebrafish embryo toxicity test

2.3.1 Purpose

The purpose of this SOP is to document the procedures of the semi-static zebrafish embryo toxicity test.

2.3.2 Objective of the test

The objective of this test is to evaluate the toxicity of small micro- and nanoplastics in the embryos of zebrafish in a semi-static exposure, including the evaluation of sub-lethal endpoints in embryo development.

2.3.3 Validity of the test

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

- Mortality in the negative control is not higher than 10% at the end of the test.
- Mortality in the positive control is higher than 30% at the end of the test.
- Hatching in the negative control is higher than 80% at the end of the test.

2.3.4 Description of the method

2.3.4.1 Equipment and material

The following equipment and material are needed to perform the test:

- Glass tanks
- Marbles
- Heater
- Plastic tubing and aeration stones
- Fish net
- Inverted microscope
- Equipment to take photographs of eggs/embryos
- Equipment to control time
- Plastic petri dish
- 24-well plates
- Pipettes
- Containers for the preparation of test concentrations
- Equipment for the determination of water parameters such as pH, temperature, conductivity, hardness and dissolved oxygen.

2.3.4.2 Test organisms

Test organisms should be zebrafish embryos (*Danio rerio*).

2.3.4.3 Test chambers

Test chambers should be 24-well plates.

2.3.4.4 Water and test conditions

Test water should be filtered reconstituted freshwater with a conductivity of at least 300 µS/cm. Plates should be maintained at 27ºC (± 1ºC) and a photoperiod of 14:10 (L:D).

2.3.4.5 Duration of test

The test has a duration of 80 hours post fertilization (hpf).

2.3.5   Egg production

Female and male adult fish (5F:5M) are transferred into breeding cages on the day before the test. At least two cages should be set to assure a sufficient number of eggs. Measures should be taken to prevent predation of the eggs by the adult fish (e.g. placing marbles at the bottom of the tanks; additional cage with a net bottom).

One hour after the onset of light the eggs are carefully collected from the breeding cages and cleaned with filtered reconstituted freshwater.

2.3.6   Procedure

2.3.6.1   Controls

Negative control of freshwater and positive control of 4 mg/L 3, 4-dichloroaniline are used.

If a solvent/stabilizer is used to disperse the nanomaterials a solvent /stabilizer control is added.

2.3.6.2   Test concentrations

When possible, five test concentrations should be used separated by a dilution factor of 10. The nanomaterial stock solution should be dispersed as required and the test concentrations prepared in filtered reconstituted fresh water whenever possible.

2.3.6.3   Conditions of exposure

The embryos should be exposed to the test concentrations as soon as possible.

Ten embryos are distributed in each well according to the layout shown in figure 1. Each well should be filled with 2 mL of the respective concentration.
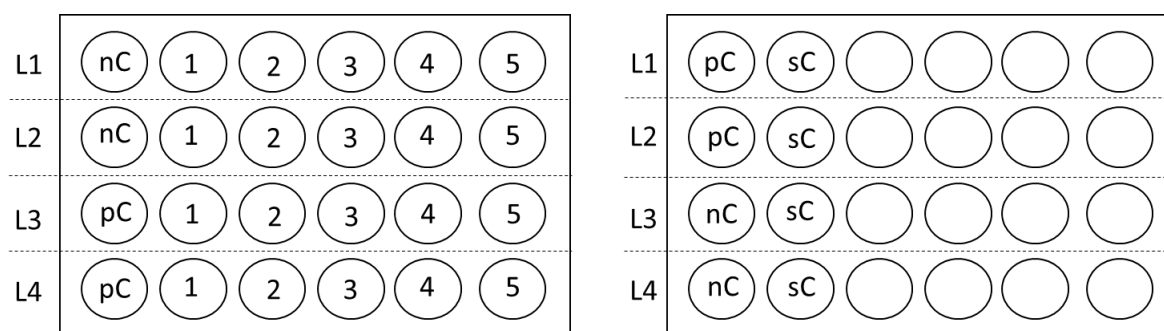

Figure 1. Layout of the 24-well plates. nC: negative control; pC: positive control; 1-5: nanomaterials concentrations; sC: solvent control; L1 – L4: line number of the plate.

2.3.6.4   Exposure concentrations renewal

The exposure medium should be renewed after 24, 48 and 72 hours. In every renewal, the number of dead embryos should be verified and removed from the wells.

2.3.6.5 Evaluations

Several parameters should be evaluated during the test, taking into consideration the development of the embryos described by Kimmel et al. (1995). Photographs should be taken at some of the time points for posterior analysis. The defined time points and evaluated parameters are as follow (Table 2):

Time point	Observations
8 hpf	Mortality
	Take photos of L1 and L3 (every embryo)
32 hpf	Mortality
	Hatching
	Heartbeat for 10 seconds (5 eggs/well in L2 and L4)
	Spontaneous movement for 30s (L2 and L4)
	Lack of somite formation (whole plate)
	Non-detachment of the tail (whole plate)
	Take photos of L2 and L4 (5 eggs/well)
56 hpf	Mortality (whole plate)
	Hatching (whole plate)
	Heartbeat 10 seconds (5 eggs/well in L1 and L3)
	Lack of somite formation (whole plate)
	Non-detachment of the tail (whole plate)
	Take photos of L1 and L3 (5 eggs/well)
80 hpf	Mortality (whole plate)
	Hatching (whole plate)
	Free swimming (whole plate)
	Lack of somite formation (whole plate)
	Non-detachment of the tail (whole plate)

Table 2. Time points and evaluations during the test

Always register and take photos of all malformations. Photos from different evaluation stages can be found in the figure below (Fig 2).
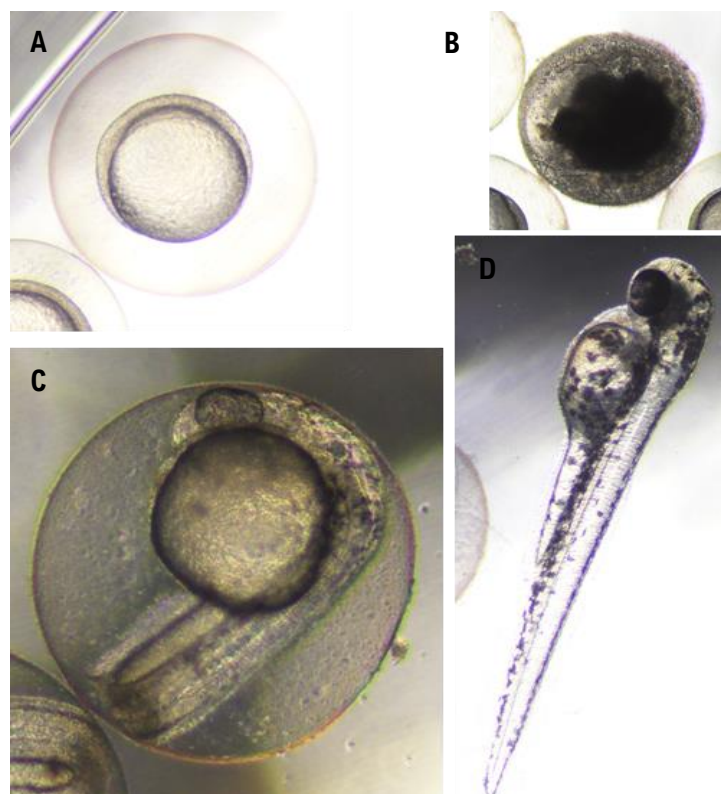

Figure 2. Zebrafish evaluation stages; A: embryo at 8hpf, B: dead embryo at 8hpf, C: larvae at 32hpf, D: larvae at 56 hpf.

2.3.6.6   Water quality measurements

The following parameters should be measured for each concentration at the beginning and at the end of the test: temperature, pH, conductivity, alkalinity and dissolved oxygen. Whenever possible the parameters should be assessed in each test well.

The parameters should be maintained within the following ranges (table 3):

Parameters	Range
Temperature	27ºC ± 1ºC
pH	6.5 - 8.5
Dissolved oxygen	≥ 80 %

Table 3. Acceptable ranges for the water parameters

2.3.6.7 Nanomaterials quantification

The real concentration of nanomaterials in each well should be assessed at all concentrations (unless below the limit of detection- LoQ for the analytical method). This quantification should be performed at the beginning and end of the test and also in the medium collected from the daily renewal.

2.3.6.8 Photograph analysis

The data that should be obtained from the photographs taken at each time point can be found in table 4, as well as details of measurements in figure 3.

Time point	Observations
8 hpf *(fig. 3)*	Yolk and egg diameter (equatorial and polar)
	Epibolic arc perimeter
32 hpf *(fig. 4)*	Eye and pupil diameter (equatorial and polar)
	Yolk and egg diameter (equatorial and polar)
	Head-trunk angle
56 hpf *(fig. 5)*	Eye and yolk diameter (equatorial and polar)
	Larvae length
	Yolk extension

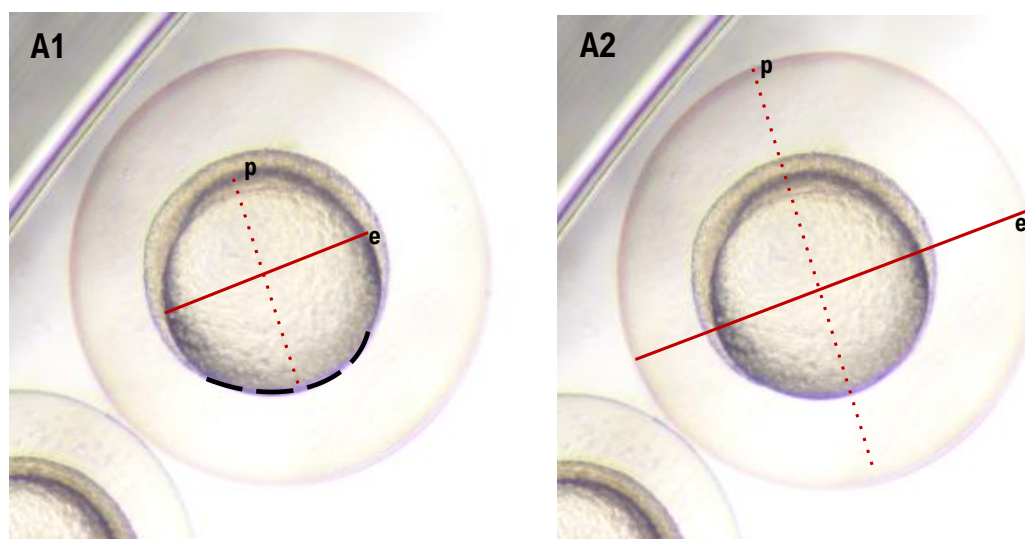*Table 4. Parameters obtained from the photographs*

Figure 3. Measurements obtained from embryos at 8hpf. A1: yolk diameter (e-equatorial; p-polar) and epibolic arc perimeter; A2: yolk and egg diameter (e – equatorial; p – polar)
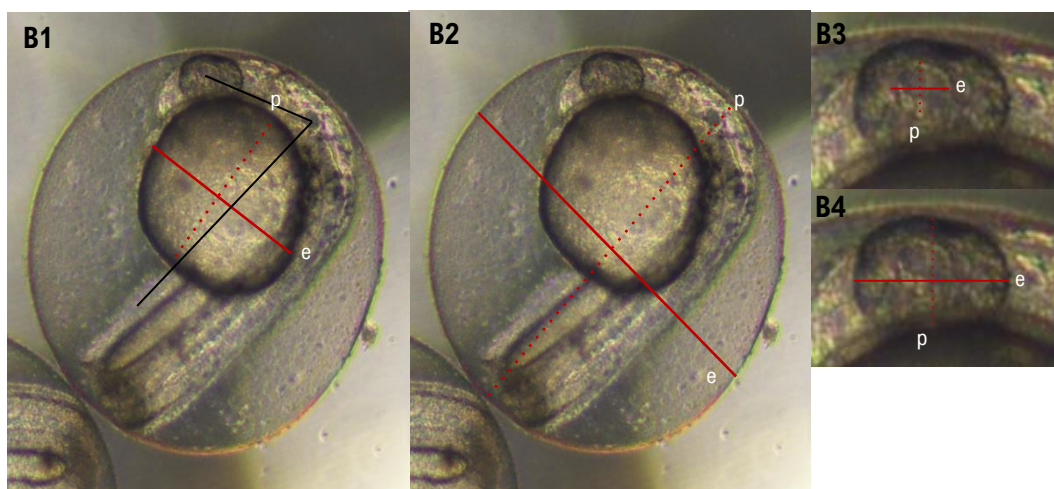
Figure 4. Measurements obtained from embryos at 32hpf. B1: yolk diameter (e – equatorial; p - polar) and head-trunk angle (black line); B2: egg diameter (e – equatorial; p - polar); B3: pupil diameter (e – equatorial; p - polar); B4: eye diameter (e – equatorial; p - polar); red dot line – polar measurement; red solid line – equatorial measurement.
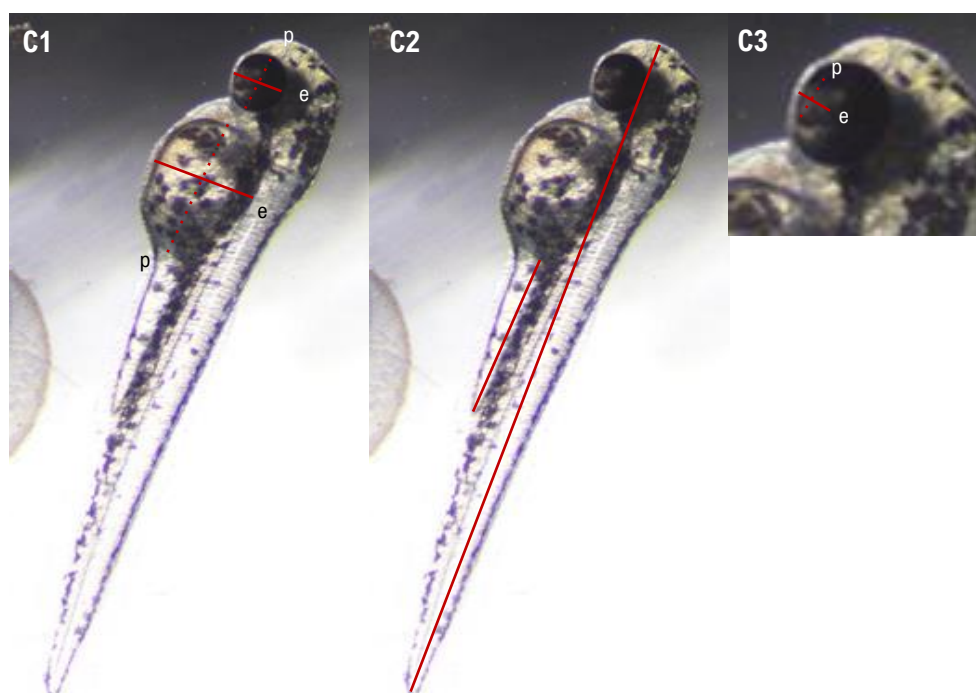

Figure 5. Measurements obtained from a larva at 56 hpf. C1: yolk diameter (e – equatorial; p - polar) and eye diameter (e – equatorial; p - polar); C2: larvae and yolk length; C3: pupil diameter (e – equatorial; p - polar)

2.3.7   Data and statistical analysis

Calculate the percentage of the following parameters for each concentration: epiboly at 8 hpf, spontaneous movement at 32 hpf, hatching at 56 hpf, survival and free-swimming at 80 hpf.

Calculate the values of the following parameters for each concentration: yolk volume and egg volume at 8 and 32 hpf, pupil surface, eye surface and head-trunk angle index at 32 hpf, cardiac frequency at 32 and 56 hpf and larvae length and yolk volume and extension at 56 hpf.

Appropriated statistical methods should be applied to detect differences between the control and the concentrations of exposure for the parameters evaluated. Moreover, an analysis of covariance should be applied for: yolk volume vs egg volume, pupil surface vs eye surface and larvae length vs yolk extension.

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