LABPLAS

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Land-Based Solutions for Plastics in the Sea

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D6.1 Test battery and protocols of plastic toxicity assessment for terrestrial, freshwater and marine ecosystems

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4	LABORATORIO IBERICO INTERNACIONAL DE NANOTECNOLOGIA	INL	PORTUGAL	RTO
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6	HELMHOLTZ ZENTRUM FUR OZEANFORSCHUNG KIEL	GEOMAR	GERMANY	RTO
7	NATIONAL OCEANOGRAPHY CENTRE	NOC	UNITED KINGDOM	RTO
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Executive summary:	This document corresponds to Deliverable 6.1 *Compilation of test battery and protocols of plastic toxicity for terrestrial, freshwater and marine ecosystems to be applied in Task 6.1 of the LABPLAS project.* It covers a selection of standard toxicity tests using ecologically relevant endpoints and species representative of terrestrial, freshwater, and marine habitats, suitable to be adapted to test the environmental toxicity of any plastic according to standard reproducible methods, hereafter referred to as the **LABPLAS Plastic Toxicity Testing Scheme.**

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TABLE OF CONTENTS

PROJECT INFORMATION 1
DELIVERABLE DETAILS 2
TABLE OF CONTENTS 3
LIST OF FIGURES 5
LIST OF TABLES 6
ABBREVIATIONS AND ACRONYMS 7
1. SCOPE 8
2. LABPLAS PLASTIC TOXICITY TESTING SCHEME 9
3. SAMPLE PRETREATMENT 12
3.1. STANDARDIZING PARTICLE SIZE BY GRINDING 12
3.2. LIXIVIATION 13
3.2.1. Materials and equipment 13
3.2.2. Procedure 14
3.2.3. Expression of results 15
3.3. TESTING THE EFFECTS OF PARTICLES 15
3.3.1. Material and equipment 15
3.3.2. Procedure 15
3.3.3. Expression of results 16
4. TEST SPECIES 17
4.1. TERRESTRIAL 17
4.1.1. Protocol for assessing the toxicity of plastic particles by conducting the earthworm (Eisenia andrei) reproduction test 17
4.1.2. Protocol for assessing the toxicity of plastic particles by conducting the plant germination test in *Lepidium sativum* 19
4.1.3. Protocol for assessing the toxicity of plastic particles by conducting the soil microorganisms' carbon and nitrogen transformation test 21
4.2. FRESHWATER 23
4.2.1. Protocol for assessing the toxicity of small micro and nanoplastic particles (SMNP) by conducting the *Microcystis* growth inhibition test 23
4.2.2. Protocol for assessing the toxicity of plastic materials in *Daphnia magna* 26
4.2.3. Protocol for assessing the toxicity of small micro and nanoplastic particles (SMNP) by conducting the fish embryo toxicity (FET) test 27
4.3. SEDIMENT 29

4.3.1. Protocol for assessing the toxicity of plastic materials in *Lumbriculus variegatus* 29
4.4. MICROORGANISMS 31
4.4.1. Protocol for assessing the toxicity of plastic materials by conducting the luminescent bacterium *Vibrio fischeri* test 31
4.5. MARINE 32
4.5.1. Protocol for assessing the toxicity of plastic materials by conducting the marine microalgal growth inhibition test 32
4.5.2. Protocol for assessing the toxicity of plastic materials by conducting the *Acartia tonsa* marine copepod survival test 33
4.5.3. Protocol for assessing the toxicity of plastic materials by conducting the Sea-urchin Embryo Test (SET) 34
4.5.4. Protocol for assessing the toxicity of plastic materials by conducting the *Cyprinodon variegatus* embryo-larval development test 39
4.6. NON-STANDARD TESTS 41
ANNEXES 43
ANNEX I: RANGE FINDING TEST FOR *EISENIA ANDREI* REPRODUCTION TEST 43
ANNEX II: PROPOSED CONCENTRATION RANGE AND REPLICATES FOR PLANT GERMINATION TEST 44
ANNEX III: PROPOSED CONCENTRATION RANGE AND REPLICATES FOR SOIL MICROORGANISMS TEST 45
RANGE-FINDING TEST 45
DEFINITIVE TEST 45
ANNEX IV: *C. VARIEGATUS* EMBRYO-LARVAL DEVELOPMENT (PHOTOS: A. VILAS, ECOTOX, UVI) 46
REFERENCES 49

LIST OF FIGURES

Figure 1. LABPLAS plastic toxicity test battery applicable to conduct ERA for plastics within the REACH Regulation framework. 8
Figure 2. a. LABPLAS plastic toxicity testing scheme **b.** Flowchart indicating the main steps in the LABPLAS plastic toxicity testing scheme 10
Figure 3. Range of particle sizes reported being ingested by different aquatic invertebrates and small fish model species. All invertebrates readily ingest particles below 20 µm 11
Figure 4. 48h embryos 46
Figure 5. DEAD FERTILIZED EGGS. They differ from UNFERTILIZED ovules in that they have remnants of cell masses inside (normally one of these masses is more brown and the other black). 46
Figure 6. DEAD larva. Lacks mobility and the heart does not beat. 47
Figure 7. Larvae with a strongly curved spine and abnormal swimming. They are identified by having curved tails and by swimming in circles 47
Figure 8. Larva with heart in OPAQUE MASS. These larvae usually do not reach the end of the bioassay. ... 48
Figure 9. Larva with swollen yolk sack. The 9-day-old larva has almost no yolk sac, in this case, the size is so large that it makes it difficult for the larva to swim normally. 48
Figure 10. Egg with two formed embryos. Separation in the middle part of the spine. Two well-differentiated heads. 48

LIST OF TABLES

Table 1. Approximate particle size and sample amount needed for each test 11
Table 2. Specific requirements for some Tier I tests depending on the test organisms. 14
Table 3. Table of dilutions 16
Table 4. Assessment criteria to be developed to obtain 4 categories of toxicity for small micro and nanoplastics using the *Microcystis* test 25
Table 5. Assessment criteria to be developed to obtain 4 categories of toxicity for small micro and nanoplastics using the FET 29
Table 6. Physico-chemical parameters acceptable for incubation of the SET 36
Table 7. Preparation of leachate dilutions for testing using ASW 36
Table 8. Table of dilutions 37
Table 9. Proposed assessment criteria to obtain 4 categories of toxicity for 1g/L and 10 g/L lixiviates 39
Table 10. Lixiviate dilutions 40
Table 11. Proposed concentration range and replicates, based on the OECD guideline and current knowledge of plastic pollution in soils for Eisenia andrei reproduction test 43
Table 12. Proposed concentration range and replicates, based on the OECD guideline and current knowledge of plastic pollution in soils for plant germination test 44
Table 13. Proposed concentration range and replicates, based on the OECD guidelines and current knowledge of plastic pollution in soils for soil microorganisms test 45

ABBREVIATIONS AND ACRONYMS

Abbreviation / Acronym	Description
ASTM	American Society for Testing and Materials
ASW	Artificial Seawater
DO	Dissolved Oxygen
EC_{50}	Median Effective Concentration
EC_{10}	10% Effect Concentration
ED_{50}	Median Effective Dilution
ELS	Early Life Stages
ERA	Environmental Risk Assessment
FET	Fish Embryo Toxicity
FPM	Fine Particulate Materials
FSW	Natural Filtered Seawater
GF/F	Glass Fiber Filter
HC	Haemocytometer
ISO	The International Organization for Standardization
ICES	International Council for the Exploration of the Sea
LC_x	Lethal Concentration
LOEC	Lowest Observed Effect Concentration
MP	Micro Plastics
NOEC	No Observed Effect Concentration
NTA	Nanoparticle Tracking Analysis
OECD	The Organisation for Economic Co-operation and Development
PNR	Percentage Net Response
PVC	Poly Vinyl Chloride
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SET	Sea-urchin Embryo Test
SMNP	Small-Micro and Nano Plastics
SOP	Standard Operating Procedure
SR	Sedgewick Rafter
STDV	Standard Deviation
TRAP	Toxicity Relationship Analysis Program
TU	Toxic Units
US-EPA	United States Environmental Protection Agency
WP	Work Package

1. SCOPE

Plastic is pouring from land into our oceans at a rate of nearly 10 million tons 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 6.1 *Compilation of test battery and protocols of plastic toxicity for terrestrial, freshwater, and marine ecosystems to be applied in Task 6.1 of the LABPLAS project.* Task 6.1. in LABPLAS WP6 focuses on the **selection of standard toxicity tests** using ecologically relevant endpoints and species representative of terrestrial, freshwater, and marine habitats, suitable **to be adapted to test the environmental toxicity of any plastic according to standard reproducible methods**; hereafter referred to as **LABPLAS Plastic Toxicity Testing Scheme**. Those tests should be suitable to assess through ecotoxicological experimentation the effects of plastic samples obtained from terrestrial, freshwater, and marine samples from the areas studied in WP2 (Tasks 6.2 and 6.3), as well as samples of commercial plastic materials, disregarding size or origin. The toxicity of these field samples (WP2) should be related to their physicochemical profile (WP3) to model impact as a function of chemical composition, and eventually to establish scientifically sound methods for **Environmental Risk Assessment** (ERA) applicable to plastic pollution (Task 6.4). Most of these tests should also be useful for Task 5.3. (WP5), consisting of the evaluation of the ecotoxicological impact of the degradation products from different natural and synthetic biodegradable polymeric materials.

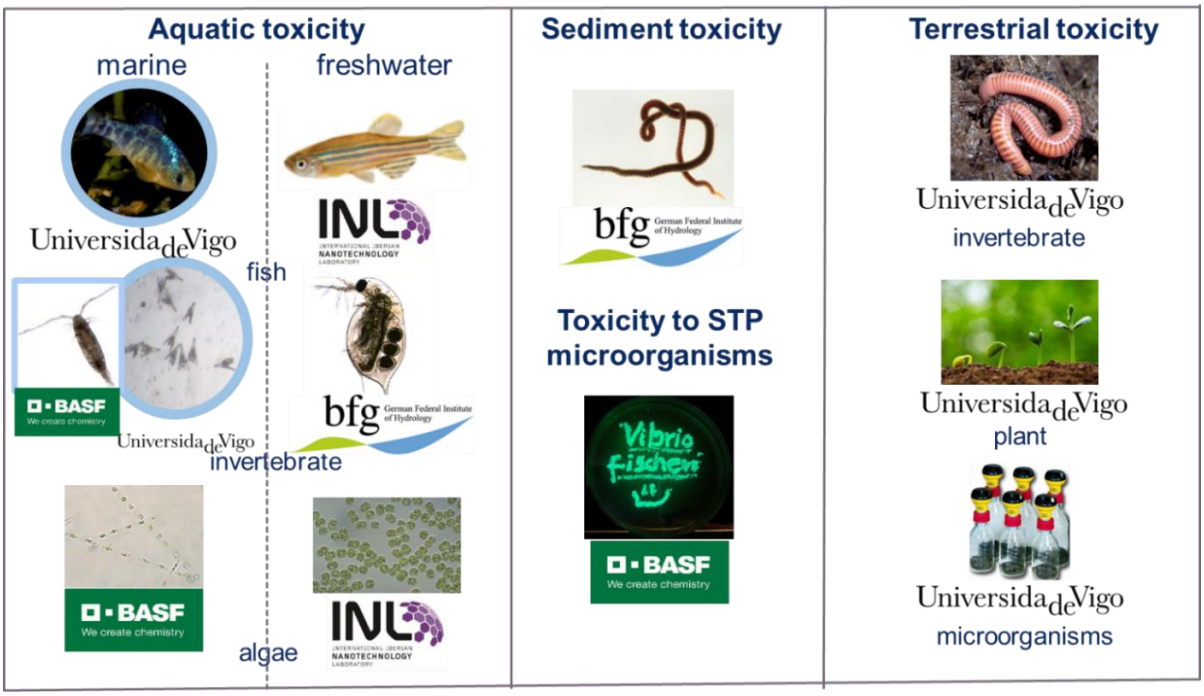


Figure 1. *LABPLAS plastic toxicity test battery applicable to conduct ERA for plastics within the REACH Regulation framework.*

Therefore, the **LABPLAS Plastic Toxicity Testing Scheme** must be applicable to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials employing a tiered approach (described in the next section) where potential effects of leached chemicals, ingestion and/or contact with plastic particles are assessed within the micro size range and if needed within the nano plastics range.

Organisms selected for the **LABPLAS Plastic Toxicity Testing Scheme** and endpoints recorded must be in harmony with international standards for environmental toxicity testing, such as those developed by ISO, US-EPA, ICES or ASTM. However, since most standard methods for regulatory use are not designed for particulate matter, they should be adapted to make them suitable to test a particulate phase and/or its lixiviate. The protocols included in the present deliverable should explain these in detail for each model species these procedures, adapting standards to test particles of SMNP or their lixiviates. This may include issues such as dosing, checking actual concentrations in suspension, obtaining a liquid phase (lixiviate) for testing, positive controls, non-polymeric particle controls (e.g. minerals), interpretation of results and assessment criteria. Description of the standard ISO, US-EPA, ICES or ASTM toxicity test procedures themselves are beyond the scope of this document and in fact, cannot be reproduced for copyright issues. Early life stages and sublethal but fitness-relevant endpoints are preferred to maximize the sensitivity of the tests and provide a low level of detection in short-term exposures.

2. LABPLAS Plastic Toxicity Testing Scheme

The scheme for testing the potential toxicity of plastic materials on aquatic organisms is depicted in Figs. 2*a* and *b*. Two routes of exposure are considered: (1) waterborne chemicals leached from the plastics and (2) plastic particles in contact and/or ingested by the organisms. If the formation of plastic particles in the nano-size range is suspected, nanoplastic effects can also be assessed. Therefore, toxicity testing can be organized as a tiered procedure.

Tier I exposes the organisms to serial dilutions of a **leachate** obtained according to a standard protocol described below. This allows the detection of effects caused by chemicals leached from the plastics into the water. These chemicals may be part of the original plastic composition or, in the case of environmental plastics, sorbed from the surrounding aquatic medium. Leachates are chemically characterized in Task 4.4.

Tier II exposes the organisms to plastic **particles** of a standard size suitable to be ingested by the test species. This size depends on the model organism. For most zooplanktonic organisms such as invertebrate larvae, an upper size limit of 20 µm is suited. Adult filter feeders and small fish can ingest larger particles. The size range of particles that different marine organisms can ingest is compiled in Figure 3.

Leachates obtained as described below (Sect. 3.2) may include non-aggregated particles passing through the GF/F filters such as nanoparticles. In addition, Tier II (Sect. 3.3) tests the effects of all particles below 20 µm (or other specific thresholds adapted to the test organism), including those within the nano range. In some cases, particularly when assessing plastic materials such as biopolymers that during environmental degradation may release nanoplastics, we might be interested to try a third step, Tier III, exposing the organisms to plastic particles within the nano-size range (<1 µm) only. This step is technically challenging, demands prefiltering by 20 µm (recording by using NTA before and after 20 µm filtration of the 1000-1nm fraction), and may be limited by the amount of nanoplastics available for testing.

In aquatic tests, experimental designs for testing the effects of particles (Tier II and, if attempted, Tier III) must include checking actual particle concentrations in suspension throughout the exposure period. This can be achieved by using laser or electric counters or NTA, depending on the size range.

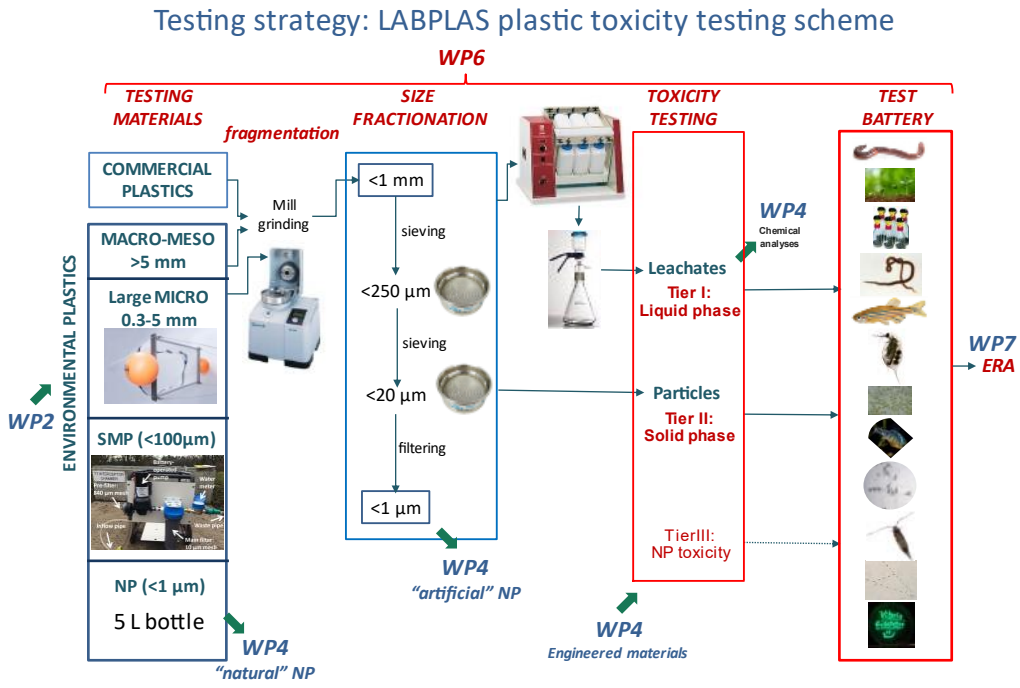
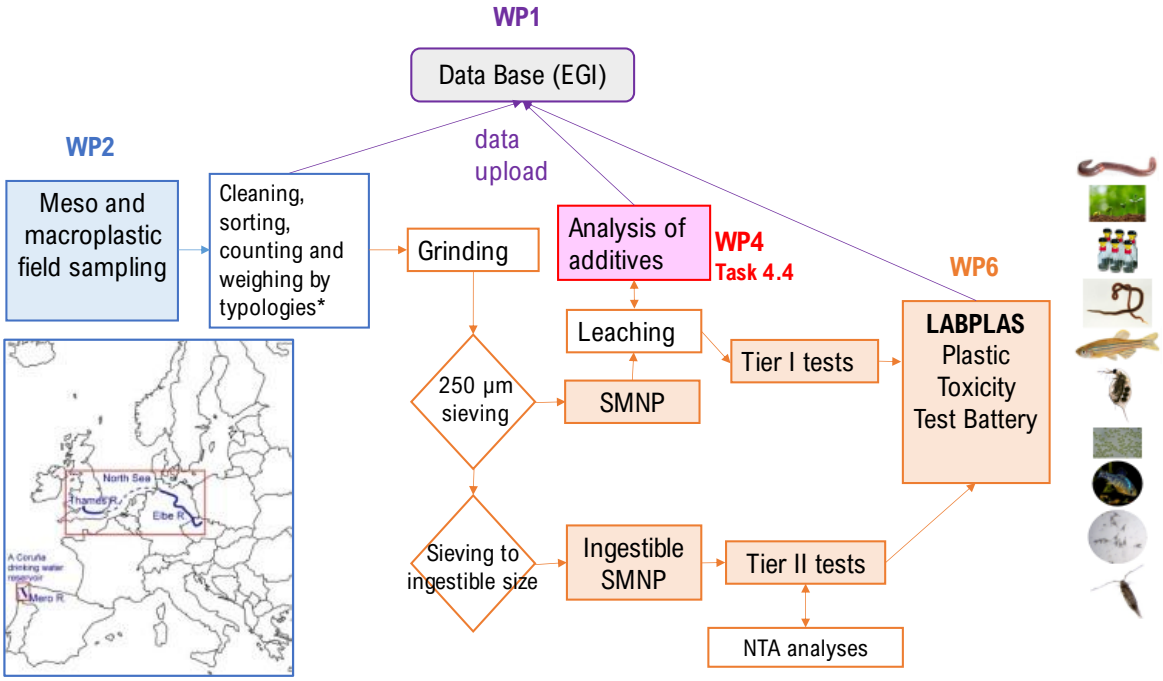


Figure 2a. *LABPLAS plastic toxicity testing scheme proposed in WP6, including Tier I intended to detect waterborne chemicals, Tier II to assess the effects of SMNP particles themselves, and if needed Tier III specifically targeting nanoparticles. Links with other WPs are also shown.*



* For typologies see OSPAR Agreement 2020-02 and JRC Tech Rep EUR 30348 EN (2021)

Figure 2b. *Flowchart indicating the main steps in the LABPLAS plastic toxicity testing scheme. Particle sizes used in Tier II depend on test species and are shown in Table 1. For typologies see OSPAR Agreement 2020-02 and JRC Tech Rep EUR 30348 EN (2021)*

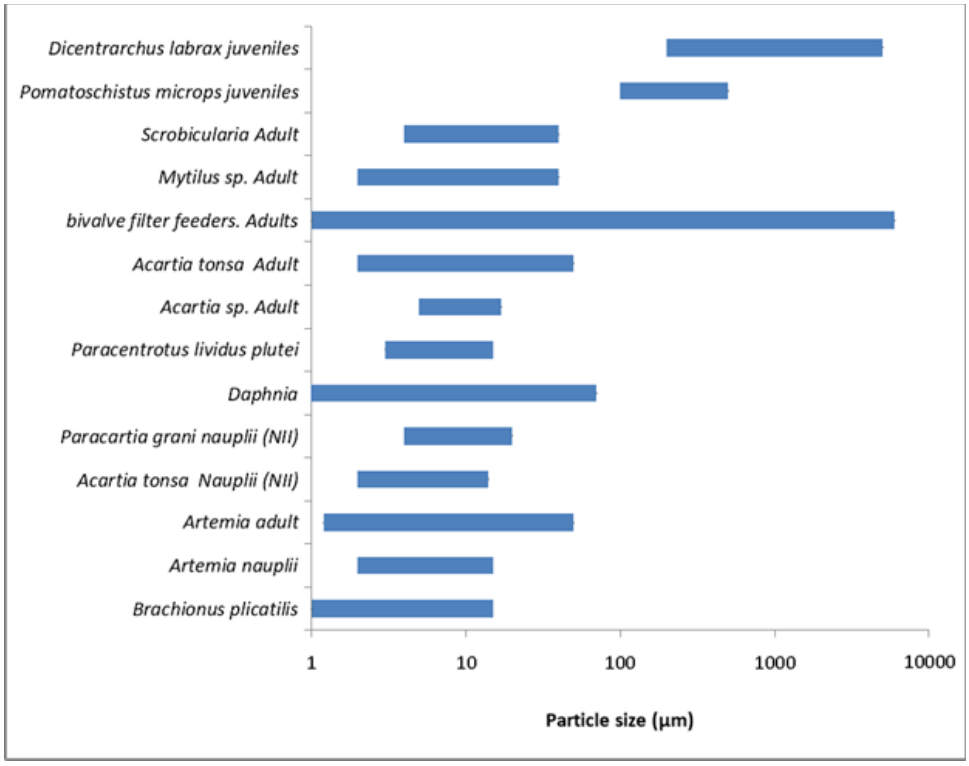


Figure 3. *Range of particle sizes reported being ingested by different aquatic invertebrates and small fish model species. All invertebrates readily ingest particles below 20 µm.*

Table 1 shows the amount of sample needed for each test and the particle size used. In the case of Tier II particle sizes must be suitable to be ingested by each model species and thus the differences in each test. For marine organisms, this is reviewed in Fig. 3. Terrestrial earthworms can ingest larger particles.

test	habitat	Test/sp	standard	Tier I	Tier II	Partner
4.1.1	soil	*Eisenia* chronic Rep.	OECD 222	10 g <250 µm	35 g <250 µm	UVI-GEA
4.1.2	soil	Plant seedling emergence	OECD 208	5 g <250 µm	5 g <250 µm	UVI-GEA
4.1.3	soil	Soil microorg N,C transfer	OECD 216/217	10 g <250 µm	50 g <250 µm	UVI-GEA
4.2.1	freshwater	*Microcystis*	OECD 201	7 g <250 µm	2 g <20 µm	INL
4.2.2	freshwater	*Daphnia (21 d)*	OECD 211	10 g <250 µm	10 g <64 µm	BfG
4.2.3	freshwater	*Zebrafish (ELS)*	OECD 236	5 g <250 µm	1 g <20 µm	INL
4.3.1	freshwater sediment	*Lumbriculus*	OECD 225	50 g <250 µm	50 g <100 µm	BfG
4.4.1	WWTP bacteria	*Vibrio fischeri*	ISO 11348	1 g < 250 µm	1 g < 250 µm	BASF
4.5.1	marine water	Marine microalgae	ISO 10253	10 g	-	BASF
4.5.2	marine water	*Acartia*	ISO 16778/ISO 14669	1 g < 250 µm	0,2 g <20 µm	BASF
4.5.3	marine water	*Paracentrotus (larva)*	ICES TIMES 51	1 g < 250 µm	0,2 g <20 µm	UVI-EC
4.5.4	marine water	*Cyprinodon (ELS)*	US EPA 821-R02-014	26.5 g (5.3x5)	-	UVI-EC

Table 1. *Approximate particle size and sample amount needed for each test*

Ultimately, methods must yield robust dose-response relationships allowing fitting toxicity models (e.g. logistic, probit) and estimating toxicity parameters (EC_{50}, EC_{10}, NOEC) useful in quantitative risk assessment for a better understanding of the full range of potential impacts of plastics in the environment. In order to achieve this, dosage should include treatments causing levels of effect close to 10% and 90% and also intermediate levels close to 50%. For materials with low toxicity, like most plastics, this may require testing levels above common environmental concentrations. Testing such environmentally not-relevant levels is not an issue here, since we target to rank the hazard potentially posed by materials/samples/sites including those with no effects at concentrations reported in the environment.

Attention must be paid to **quality assurance and quality control** (QA/QC) to produce robust, reproducible results. This includes positive controls to assess sensitivity, acceptability criteria to assess the quality of the biological material, particle controls to make the difference between the effects of particles in general or effects specific to plastics, and particle characterization to ensure homogeneous particulate materials and exposure conditions. The positive control will consist of a reference plastic material of well-known composition and toxicity (e.g. additivated PVC). Unfortunately, different stocks of the same material may show different ecotoxicological properties, which restricts the validity of positive controls to samples from the same stock. Comprehensive efforts have been recently devoted to developing microplastic reference materials (e.g. JPI-Oceans BASEMAN project) with, to the best of our knowledge, limited success.

For Tier I tests, the positive control may simply consist of the incubation medium spiked with known concentrations of a reference toxicant, such as potassium dichromate, copper, 3,5-dichlorophenol, etc., or a single or mixture of additives of known effects for the endpoint tested.

For Tier II tests, particle controls may be useful to discriminate between effects specifically due to synthetic polymers from those generally caused by turbidity or physical effects of particles. Depending on the test, the particle control may consist of mineral particles with similar size and shape to tested SMNP (e.g. kaolin) or reference inert particles (e.g. latex particles) of well-known lack of toxicity. Properties that may affect the effect of particles on organisms (e.g., size, polymer type, shape, surface charge), must be considered in order to generate reproducible and comparable data. **Particle characterization** can be conducted with different instrumentation according to the size range of particles. Useful instruments are laser diffraction particle analysers (e.g. LS I3 320 by Beckman Coulter), electric Coulter counters (with a more restricted range of analysable sizes), and for smaller sizes Nanoparticle Tracking Analysis (NTA).

Reproducible results in water column tests require also a homogeneous dispersion of the plastic particles in the exposure medium, providing adequate stirring to keep particles in suspension, available for ingestion and contact with the organisms without causing mechanical stress. If suitable for the experimental set-up, a plankton wheel set at 1 rpm may provide adequate homogeneous SMNP suspensions during testing. Again, **recording actual particle density** in the exposure media at relevant time scales is an important QA/QC requirement.

3. Sample Pretreatment

3.1. Standardizing particle size by grinding

The **LABPLAS Plastic Toxicity Testing Scheme** is intended to be applicable to any plastic material disregarding size. However, the size of the plastic particles is a key aspect affecting the effects of both leachates and the particles themselves. Concerning the effects of chemicals carried by the plastic, desorption kinetics from the polymeric matrix is greatly influenced by particle size. In addition, when the effects of ingested plastics are targeted, the experimental design demands a particle size range suitable to be ingested by the testing

organisms. Particle size standardization is then a pre-requirement to warrant the comparability of results. Pretreatment of all samples to obtain particles of a standard size is needed to allow comparability of results, except when the testing material already conforms to a homogeneous size distribution (e.g. engineered micro or nanospheres).

Pretreatment in the **LABPLAS Plastic Toxicity Testing Scheme** consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size. The task leader laboratory (ECOTOX, University of Vigo) uses sieving through **250 µm** as a common procedure. This protocol will be described below, and it can be easily adapted to other final particle sizes suitable for standardization, such as 1 mm. In fact, current ongoing research intended to compare the effects of leachates obtained at 250 µm and 1000 µm is in progress. If the sensitivity of the test does not decrease, the use of the <1 mm fraction may allow obtaining larger amounts of test material in a less time-consuming fashion. The choice of the standard size may also depend on logistic issues or the test species and will not be discussed in this document.

Large plastic materials must be, when needed, manually cut to pieces not larger than 5 mm by using strong metallic scissors or pruning shears. These pieces are then ground at 18000 rpm in an **ultra-centrifugal mill** (e.g. Retsch ZM200) fitted with a 250 µm stainless steel ring mesh. Before feeding the material into the grinding chamber it is mixed with dry ice at an approximate proportion of 4 parts of dry ice per 1 part of the sample. Finer final particle sizes can be achieved by using a Retsch **CryoMill** fed with liquid nitrogen.

After grinding, the material is sieved by a certified 250 µm metallic sieve. Sieving may be manually conducted with the help of a brush, or an automatic sieve column shaker may be used.

3.2. LIXIVIATION

The present protocol describes the production of a 10 g/L solid-to-liquid ratio leachate and further testing of serial dilutions of the leachate, according to previously published methods (Beiras et al. 2018).

3.2.1. Materials and equipment

1. Plastic particles sieved by 250 µm. The amounts needed for each test are shown in Tab. 1.
2. Incubation medium: ASW or FSW for marine testing; M4 medium for daphnia testing
3. Pyrex glass bottles with Teflon-lined screw caps (one per sample). Volumes depend on test species.
4. Glass beakers (one per sample) to manipulate leachates and test water quality parameters
5. Incubation chamber (20 ± 0.2 °C) or isothermal room (20 ± 1 °C)
6. Weighing scale ± 0.00001g, metallic spatulas and watch glasses
7. Rotatory wheel / Overhead rotator (1 rpm)
8. Filter system (glass Büchner flask and vacuum pump)
9. 0.7 µm (GF/F, Whatman) filters (one per sample)
10. Volumetric flask and automatic pipettes to make the serial dilutions. Volumes depend on test species. 25 mL is suitable in most cases.
11. Glass vials for incubations. Volumes depend on test species. 5 mL is suitable in most cases.
12. Thermosalinometer, oximeter and pH meter to check water quality

3.2.2. Procedure

Day 1: Preparing the leachate (10 g/L, < 250µm plastic particles)

1. Weigh using a 0.00001 g precision weighing scale, the watch glass and the spatula, the amount of <250 µm MP required for each test, according to Table 2.
2. Transfer the MP into a 50 mL glass bottle and add ASW to the brim. Close and shake strongly.
3. Place each bottle inside a dark plastic container that fits into the rotatory wheel.
4. Stir at 1 rpm for 24 h in the dark at 20 ºC.

Day 2: Filtration and preparation of testing dilutions

1. Stop the wheel and take out the bottles.
2. Place a GF/F filter on the filtration system and rinse with abundant distilled water. Discard the distilled water. This step avoids false positives due to filter leachates.
3. Filter 100 mL of ASW to obtain the filter control. Recover the liquid in a 250 mL glass beaker.
4. Filter each bottle's content through a new rinsed GF/F filter.
5. Record in the lixiviate the essential water quality variables (temperature, salinity, DO, pH) and make sure ranges lie within optimal values of the test organisms. If values depart from tolerance limits correct them. If salinity is too low or too high correct with a hypersaline solution or distilled water. If DO is too low aerate the lixiviate for 10 min. Using ASW, pH departures are not expected.

Test	Medium	MP mass	Leachate vol.	Dilution vol.	Incubation vol.
Daphnia	Elendt M4	10 g (x9)	1 L	500 mL	50 mL (x10)
SET	ASW (35 ppt)	650 mg	65 mL (50 mL bottle)	25 mL	4 mL (x4)
Cyprinodon	FSW (28 ppt)	5.3 g (x5)	530 mL (500 mL bottle)	250 mL	50 mL (x4)
Zebrafish	FSW	1 g	100 mL	40 mL	2 mL (x4) x 4 renovations
Microcystis aeruginosa	Z8	2 g	200 mL	120 mL	40 mL (x3)

Table 2. *Specific requirements for some Tier I tests depending on the test organisms. For semistatic tests, the total amounts of MP mass needed are those needed for a single leachate stock times water renewal occasions. Leachate volumes correspond to Pyrex bottles filled to the rim with no head space.*

6. Make up the lixiviate dilutions in geometric scale (e.g. undiluted, 1/3, 1/10, 1/30 ...) using volumetric flasks of the volume needed (Dilution vol. In Tab. 2), and transfer them to the incubation vessels. Begin with the highest dilution to allow using the same flask and beaker for each lixiviate. If after reading the results the 1/30 dilution shows toxicity, repeat the experiment with higher dilutions (x1/100, x1/300, …) until a NOEC is found.
7. Add the organisms to the incubation vessels, including all controls.

3.2.3. Expression of results

1. Response (survival, growth, reproduction, normal development) recorded in each treatment (R) is corrected by mean control response (R_C) to obtain the Net Response (NR): $NR = R / R_C$
2. NR data are fit to a dose:response sigmoidal model to estimate with the aid of some statistical software (e.g. Graph Pad Prism or SPSS) the ED_{50} and the ED_{10} values, which must be expressed along with their corresponding 95% confidence intervals. The ED_{50} can be estimated only when some treatments showed a decrease >50% in the endpoint recorded. Common models recommended are logistic or probit.
3. The toxicity of each sample may be now expressed in Toxic Units (TU):

$$TU = 1/ED_{50}$$

4. When none of the dilutions had a level of effect >50% then the result is expressed as <1 TU.
5. For comparative purposes, from the ED_{50} values (expressed in dilution factors) EC_{50} values (expressed in mass per volume units) can be calculated taking into account the solid-to-liquid ratio used to make up the leachate; in this case 10 g/L:

$$EC_{50}\ (mg/L) = 10{,}000 \times ED_{50}$$

However, since the toxicity of undiluted plastic leachates does not linearly increases with the solid-to-liquid ratio used, EC_{50} values can be compared only among experiments using the same plastic load to make up the leachate (in this case 10 g/L).

3.3. TESTING THE EFFECTS OF PARTICLES

3.3.1. Material and equipment

- Plastic particles sieved by 20 µm. The amounts needed for each test are shown in Tab. 1.
- Incubation medium: ASW or FSW for marine testing; M4 medium for daphnia testing; Z medium for *Microcystis* testing
- Balance ± 0.00001g, metallic spatulas and watch glasses
- Tween®20
- Pyrex glass bottles, volumetric flasks, glass measuring cylinders, glass beakers and glass vials of the needed volumes
- Glass funnel and glass Pasteur pipettes
- Incubation chamber (20 ± 0.2 °C) or isothermal room (20 ± 1 °C)
- Rotatory wheel / Plankton wheel (1 rpm)
- Instrumentation to check actual particle densities (Coulter counter, laser particle analyzer, NTA)

3.3.2. Procedure

Preparation and characterization of the SMNP stock suspension

1. Mix 1L medium and 3 µL of Tween®20 in a Pyrex bottle with a Teflon-lined cap. Manually shake.
2. Weigh using a 0,00001 g precision balance, a metallic spatula and a watch the needed amount of <20 µm SMNP.
3. Prepare a SMNP stock suspension by adding the needed amount of SMNP to the needed volume of the medium-Tween mixture in a new vessel, with the help of a measuring cylinder, glass pipette and a funnel.
4. Characterize the SMNP dispersion by Nanoparticle Tracking Analysis (NTA) or Coulter Counter, as described in 4.2

Making up dilutions

1. Using the medium-Tween® mixture and volumetric flasks, dilute the stock suspension to the target final volume. For dilution volumes of 50 mL proceed according to this table:

Treatment	300 mg/L stock volume (mL)	Final volume (mL)
1 mg/L	0.168	50
3 mg/L	0.5	50
10 mg/L	1.67	50
30 mg/L	5	50
100 mg/L	16.7	50

Table 3. *Table of dilutions*

   It is very important to shake the stock before adding it to the volumetric flask before each dilution.

2. Fill the 5 mL glass vials with no head space (7.8 mL in our vials) except egg vials which are filled with just 4 mL:
   - egg vials (ASW) (fill with just 4 mL)
   - ASW control vials
   - 8 Filter control vials (ASW +Tween)
   - vials with 1 mg/L
   - vials 3 mg/L
   - vials 10 mg/L
   - vials 30 mg/L
   - 4 vials with 100 mg/L
3. Fertilize the eggs and conduct SET according to std methods I-LB-BE-001.
4. Fix the egg vials at t=0 with 6 drops of 40% formalin and take them apart.
5. Place the remaining vials inside dark containers padded with soft material to avoid glass breaking during stirring.
6. Place the containers in the wheel at 1 rpm for 48 h in the dark at 20 ºC.

End of the bioassay.

1. After 48h stop the wheel and take the vials out of the dark containers and place them on a grid.
2. Unscrew the caps except for the egg vials and add 6 droplets of formalin to each vial.

3.3.3. Expression of results

1. Mean length increase for n=35 individuals per vial minus mean egg size data are analyzed using the Dunnet test to calculate NOEC and LOEC.
2. Data are control corrected and fit to a sigmoidal curve using a log-logistic or probit model to estimate the EC10 and EC50 (mg/L) and corresponding 95% CI.

4. TEST SPECIES

4.1. TERRESTRIAL

4.1.1. Protocol for assessing the toxicity of plastic particles by conducting the earthworm (Eisenia andrei) reproduction test

4.1.1.1. Scope

This protocol describes the procedures needed to test the toxicity of plastic particles to earthworms (*Eisenia andrei)*, using an ecotoxicological test, based on the standard test guidelines provided by OECD (OECD 222), with adaptations. These adaptations reflect the current knowledge on the use of artificial soil and/or substrate as ideal conditions for earthworm development[1]. It applies to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials.

Pre-treatment of samples to obtain particles of a standard size is needed to allow comparability of results. Pre-treatment consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size. Pre-treatment procedures are described in section 3 of this document. To uniformize this protocol with others within the project and based on the literature, a size of 250 µm was selected.

4.1.1.2. Definitions

EC_{50}: theoretical concentration of plastic particles (mg/kg dry soil or number of particles/dry soil) corresponding to a 50% reduction of the endpoint recorded

NOEC: the lowest concentration with no observable effect

Toxic Units: $1/EC_{50}$

4.1.1.3 Equipment and Materials

Species: *Eisenia andrei* (adult earthworms with clitellum – at least 2 months old)

Endpoints recorded: Survival (after 28 days) and reproduction (after 56 days)

Exposure Media: Spent coffee grounds with 75% humidity, aged for at least one month at air temperature

List of laboratory material

- 450 mL glass vials (ø = 6.7 cm; h = 16.9 cm)
- 0.5 mm pore size mesh sieves
- Metal spoons
- Weighing scale
- White plastic trays
- Bowl made of inert plastic
- Petri dishes (100x15 mm and 150x15 mm)
- Filter paper
- Tweezers
- Sterile gauze (ideally 20 threads per cm^2)
- Acclimatized chamber with ventilation

4.1.1.4 Feeding and Maintenance

1. Spent coffee grounds are used both as an exposure matrix and food source for *E. andrei*
2. Test vessels are replenished with spent coffee grounds and water every week, according to the vessel weight variation observed, considering the water loss in test conditions to be around X% of total substrate weight.
3. After two weeks, control test vessels are emptied, and earthworms are checked for mortality and any morphological damage that may compromise test validity criteria.

4.1.1.5 Protocol

1. Conduct the Earthworm Reproduction Test (*Eisenia andrei*) according to standard procedures from OECD 222[3]. Use glass vials to ensure no interference from other plastics, covered with sterile gauze to allow proper aeration. The standard procedures are adapted, with the use of spent coffee grounds as substrate and the test should run at 20 ± 2 °C.
2. Plastic particles added to 200 g of the substrate are thoroughly mixed to ensure a homogeneous distribution, according to the nominal concentration and aim (see Annex I).
3. Adult earthworms with clitellum (n=10) are pre-acclimated to spent coffee grounds one week prior to starting the test and are introduced to the glass vials at t=0. Both, substrate depth and vial weight are recorded.

Day 28: Survival Endpoint Evaluation

1. The exposure matrix (substrate) from test vials is transferred to a clean tray, to remove adult worms.
2. Prior to weighting, earthworms are washed with deionized water. The excess water is removed by placing the worms on filter paper.
3. Count and weigh them.

Day 56: Reproduction Endpoint Evaluation

1. After 4 additional weeks, the exposure matrix (substrate) is thoroughly distributed on top of a 1 mm pore-size mesh, with bulk sections being ground by hand.
2. The juveniles are expected to pass through the mesh pores and are retrieved in a tray for counting. Juveniles that may still be in the debris on the top are retrieved with tweezers.
3. Cocoons are retrieved using tweezers, transferred to a petri dish with little water and counted.

4.1.1.6. Expression of results, acceptability criteria and assessment criteria

1. Survival data should be presented as the mean number of adults after 28 days and reproduction data as the mean number of juveniles.
2. The data obtained in the control should follow the validity criteria presented in the OECD guideline:
   - less than 10% mortality after 28 days.
   - ≥ 30 juveniles after 56 days per replicate.
   - the coefficient of variation of reproduction to be less than 30% in control.
3. Statistical analysis for NOEC determination will follow the OECD protocol guidelines, Annex I.
4. Estimation of ECx (e.g., EC_{10}, EC_{20}, EC_{50} and EC_{80}) for each parameter (survival or reproduction) is performed using the Toxicity Relationship Analysis Program (TRAP), with a preference to use a two-

parameter logistic model estimation. ECx values should be presented with the corresponding 95% confidence intervals.

5. For each plastic tested, for each parameter, the toxicity of each sample may be now expressed in Toxic Units (TU): TU = $1/EC_{50}$, allowing the comparison between plastics and with samples retrieved from the field.

4.1.2. Protocol for assessing the toxicity of plastic particles by conducting the plant germination test in *Lepidium sativum*

4.1.2.1. Scope

This protocol describes the procedures needed to test the toxicity of plastic particles and lixiviates obtained from the same plastic particles to plants (*Lepidium sativum)*, using an ecotoxicological test, based on the standard test guidelines provided by OECD (OECD 208), with adaptations[1]. It applies to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials.

Pre-treatment of samples to obtain particles of a standard size is needed to allow comparability of results. Pre-treatment consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size. Pre-treatment procedures are described in section 3 of this document. To uniformize this protocol with others within the project and based on the literature, a size of 250 µm was selected.

In addition, lixiviates from plastic particles will be obtained following the same protocol as the lixiviation in saltwater, replacing the artificial saltwater with distilled water, to mimic the freshwater systems.

4.1.2.2. Definitions

EC_{50}: theoretical concentration of plastic particles (mg/kg dry soil or number of particles/dry soil) corresponding to a 50% reduction of the endpoint recorded

NOEC: the lowest concentration with no observable effect

Toxic Units: $1/EC_{50}$

4.1.2.3. Equipment and Materials

Species: *Lepidium sativum* (common garden cress)

Endpoints recorded: Germination (8h, 24h, 48h, 72h) and Root Growth (72h)

Exposure Media: Standard LUFA 2.3 soil, soil-water extract, water

List of laboratory material

- Glass Petri dishes (100x15 mm size)
- Grade 1 Whatman Filter paper
- Metal spoons
- Weight balance
- Acclimatized chamber with ventilation and light exposure
- Digital calliper
- Microscope

4.1.2.4. Protocol

Conduct the Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test, according to standard procedures from OECD 208[3]. The usage of glass Petri dishes will ensure no interference from other plastics. The standard procedures are adapted, with the use of LUFA 2.3 soil to prepare soil water extracts for *L. sativum* exposure in a more realistic scenario. The test should run at 20 ± 2 °C.

Soil-Water Extraction

1. Extracts from the soil, either spiked with or solution, are obtained by thoroughly mixing soil samples with ultrapure water in a ratio of 1:5 (soil weight:water volume), placed in a mixer for 15 minutes and allowed to rest for a minimum of 2h and a maximum of 24h to ensure the separation between particles in the solid and liquid phase.
2. The liquid phase is carefully extracted, with the use of a micropipette and transferred to an empty vial for use and storage.

Spiking

Plastic particles as a powder

Plastic particles as powder are added to 4 mL of soil extracts in a Petri dish per replicate and thoroughly mixed to ensure a homogeneous distribution, and volume is adjusted with 1 mL of ultrapure water, according to the nominal concentration and aim (see Annex II).

Plastic particles lixiviates as aqueous solution:

Serially diluted solutions containing lixiviates obtained from a 10 g plastic particle / L stock solution are added as 1 mL of aqueous solution to 4 mL of soil extracts, according to the nominal concentration and aim (see Annex II).

Seed exposure

1. *L. sativum* seeds are exposed to plastic particles through interaction with exposure media over 7 days.
2. The number of germinated seeds is assessed after 8h, 24h, 48h and 72h by counting the number of opened seed covers in each replicate.
3. Assessment of root growth is performed after 72h and 7 days, by measuring root length with a digital calliper in a microscope. The details for each exposure media are as follows:
4. 30 *L. sativum seeds* are distributed throughout a Grade 1 Whatman Filter paper and inserted into the petri dish containing a soil water extract spiked with plastic particles or lixiviates.

4.1.2.5. Expression of results, acceptability criteria and assessment criteria

The data retrieved from seed germination and root length can be presented under the form of the following parameters, calculated through the respective equations:

Germination Percentage (GP):

$$\frac{N\ germinated\ seeds}{N\ total\ seeds}$$

Relative Seed Germination (RSG):

$$\frac{N\ seeds\ in\ exposed\ condition}{N\ seeds\ in\ control} \times 100$$

Relative Root Growth (RRG):

$$\frac{Average\ root\ length\ in\ exposed\ condition}{Average\ root\ length\ in\ control} \times 100$$

Germination Index (GI) as a percentage (%):

$$\frac{RRG \times RSG}{100}$$

Estimation of ECx (e.g., EC_{10}, EC_{20}, EC_{50} and EC_{80}) for each parameter is performed using the Toxicity Relationship Analysis Program (TRAP)[5], with a preference to use a two-parameter logistic model estimation. ECx values should be presented with the corresponding 95% confidence intervals.

4.1.3. Protocol for assessing the toxicity of plastic particles by conducting the soil microorganisms' carbon and nitrogen transformation test

4.1.3.1. Scope

This protocol presents the methodology used to test the toxicity of plastic particles and lixiviates obtained from the same particles to microorganisms and their respective carbon and nitrogen transformation, based on standard test guidelines provided by OECD (OECD 216 and 217) and current protocols for carbon and nitrogen transformation assessment. Detailed technical methods can be further found in the literature. It applies to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials.

Pre-treatment of samples to obtain particles of a standard size is needed to allow comparability of results. Pre-treatment consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size. Pre-treatment procedures are described in section 3 of this document. To uniformize this protocol with others within the project and based on the literature, a size of 250 µm was selected.

In addition, lixiviates from plastic particles will be obtained following the same protocol as the lixiviation in saltwater, replacing the artificial saltwater with distilled water, to mimic the freshwater systems.

4.1.3.2. Definitions

EC_{50}: theoretical concentration of plastic particles (mg/kg dry soil or number of particles/dry soil) corresponding to a 50% reduction of the endpoint recorded

NOEC: the lowest concentration with no observable effect

4.1.3.3. Materials and Equipment

Endpoints recorded: Basal respiration, nitrate and ammonia concentration

Exposure Media: Standard LUFA 2.3 soil

List of laboratory material

- Glass flasks (volume 200 mL, with ø = 71,8 mm)
- Metal spoons
- Weighing scale
- Acclimatized chamber with ventilation and light exposure
- Whatman Filter Paper
- K_2SO_4

4.1.3.4. Protocol

Conduct the Soil Microorganisms transformation test, following the standard procedures from OECD 216 for Nitrogen and OECD 217 for Carbon. The usage of glass Petri dishes will ensure no interference from other plastics. The standard procedures are adapted, with the use of LUFA 2.3 soil as a well-known and characterized soil. The test should run at 20 ± 2 °C for a maximum of 28 days, with sampling points at 0, 7, 14 and 28 days.

Soil Spiking

Plastic particles as a powder

Plastic particles as a powder are added to 100 g pre-moistened soil in a glass flask per replicate and thoroughly mixed to ensure a homogeneous distribution, according to the nominal concentration and aim (see Annex III). Afterwards, water is added until reaching 50% of the water-holding capacity of the soil.

Plastic particles lixiviates as aqueous solution

Serially diluted solutions containing lixiviates obtained from a 10 g plastic particle / L stock solution are added as aqueous solution to 90 g dry soil, in a volume that should not exceed 15% of the total dry mass, according to the nominal concentration and aim (see Annex III). Afterwards, water is added until reaching 50% of the water-holding capacity of the soil

Carbon Transformation Assessment

1. Carbon transformation is assessed as the soil basal respiration, being measured as the amount of carbon dioxide (CO_2) produced by microorganisms in the soil each sampling time (0, 7, 14, 28 days).
2. Measurements are performed using a titration method, well-described in the literature, although other methodologies can be used, e.g. commercially available OxiTop.

Nitrogen Transformation Assessment

1. Nitrogen transformation is assessed by the conversion of soil nitrate ions (NO_3^-) into ammonium ions (NH_4^+) and vice-versa due to microorganism activity.
2. NO_3^- and NH_4^+ quantification measurements are performed in soil extracts, prepared by dissolving soil samples from each replicate in a solution of 0.5M K_2SO_4 in a ratio of 1 : 10 (weight : volume) and mixed for one hour in an orbital shaker at 200rpm, before filtration through a Whatman Filter Paper, recovering the solution for nitrate and ammonia analysis. This should be done for 3 replicates at each timepoint.
3. Measurements are performed following standard operating procedures (SOP), based on photometric assays, as described and validated in literature, although other methodologies can be used.

4.1.3.5. Expression of results, acceptability criteria and assessment criteria

The data retrieved regarding the carbon and nitrogen transformation is measured as the concentration of CO_2, NO_3^- and NH_4^+ respectively. Estimation of EC_x (e.g., EC_{10}, EC_{20}, EC_{50} and EC_{80}) for each parameter is performed using the Toxicity Relationship Analysis Program (TRAP), with a preference to use a two-parameter logistic model estimation. EC_x values should be presented with the corresponding 95% confidence intervals.

4.2. FRESHWATER

Preparing leachates with freshwater

1. Grind and sieve plastic materials to obtain particles <250 µm.
2. Weigh the required amount of particles.
3. Transfer MP into a glass bottle (e.g., 1 L) and add medium (ISO or Elendt M4) to the brim not letting any headspace.
4. Place each bottle in a rotatory shaker (1 rpm) for 24 hours at 20°C in the dark.
5. Vacuum-filtrate the suspension (GF/F filter).
6. Make lixiviate dilutions using volumetric flasks.
7. For leachate controls, apply steps 3 to 5 without any particles.

4.2.1. Protocol for assessing the toxicity of small micro and nanoplastic particles (SMNP) by conducting the *Microcystis* growth inhibition test

4.2.1.1. Scope

This protocol describes the procedures to prepare small micro and nanoplastics dispersions from plastic particles and test their toxicity in *Microcystis aeruginosa* using the standard Freshwater Alga and Cyanobacteria growth inhibition test. It applies to any polymeric material obtained from environmental samples, commercial items or engineered raw materials. Pre-treatment of all samples is needed to obtain particles below 20 µm in size. Pre-treatment consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size.

Species: *Microcystis aeruginosa*

Endpoint recorded: Average specific growth rate and yield

4.2.1.2. Definitions

NTA: Nanoparticle Tracking Analysis

EC_{50}: the concentration that results in a 50% effect.

Biomass: number of cells per millilitre.

Average growth rate: logarithmic increase in biomass in a specific period.

Yield: biomass at the end of the test minus the starting biomass.

Toxic Units: $1/EC_{50}$

4.2.1.3. Materials and Equipment

- Mill
- 20 µm metallic sieve
- Ultrapure water
- Z medium
- Watch glass (one per sample)
- Glass flasks and vials
- Weighing scale ± 0.00001g
- Metallic spatula
- 1 mL glass syringes

4.2.1.4. Procedure

Preparation of the SMNP

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

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 Z 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. For the nano-range particles, 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 (>$1x10^7$ 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. For the micro-range particles analyse the suspension by using an electric or laser Coulter Counter.
4. 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.

Obs: 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.

Long-term evaluation of SMNP stability

Evaluate the stability of the SMNP in ultrapure water and Z 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 Z 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).

Preparation of testing dispersions

1. Prepare a stock solution of the SMNP according to the conditions defined previously.
2. If previous information on SMNP toxicity is not available, five concentrations spaced by a constant factor of 10 can be used (e.g. 0.01; 0.1; 1; 10; 100 mg/L). This range can be decreased if the toxicity is not well distributed within the concentrations.
3. At the beginning and the end of exposure, analyze the dispersions used in the test by NTA.
4. Conduct the Freshwater Alga and Cyanobacteria growth inhibition test with *Microcystis aeruginosa* for each small micro and nanoplastic according to the OECD 201.

4.2.1.5. Expression of results and assessment criteria

1. The endpoints recorded are the average growth rate and yield after 72 hours of exposure, expressed as a percentage of inhibition. When the effect is higher than 50% in some concentrations the EC_{50} can be calculated, otherwise, only EC_{10} is obtained. The most sensitive endpoint will be considered for the EC_{50} and EC_{10} calculations.
2. Assessment criteria to be developed will allow translating results into a scale of colors with 4 categories:

EC10 (mg/L)	Category
>100	No toxicity
100-10	Slight toxicity
10-0.1	Relevant toxicity
<0.1	High toxicity

Table 4. *Assessment criteria to be developed to obtain 4 categories of toxicity for small micro and nanoplastics using the Microcystis test*

4.2.2. Protocol for assessing the toxicity of plastic materials in *Daphnia magna*

4.2.2.1. Scope

This protocol describes the procedures to test the toxicity of plastic materials to the water flea *Daphnia magna*. This experiment is based on the standard guideline OECD 211 with some minor adaptations addressing experimental issues related to particle toxicities. It is applicable for plastic materials with a higher density than freshwater obtained from environmental samples, commercial items or engineered raw materials.

Pre-treatment of the plastic material is needed to obtain particle size distributions that are relevant for daphnids. This includes milling and sieving of the respective materials. In general, particles <64 µm are needed to ensure an interaction during the development of daphnids.

4.2.2.2. Definitions

EC_{50}: theoretical concentration of plastic particles or leachates corresponding to a 50% reduction of the endpoint recorded

NOEC: the highest concentration with no observable effect

4.2.2.3. Procedure

General recommendations:

- 7 replicates per treatment
- 5-6 particle or leachate concentrations
- 3 different concentrations of particle controls, at least
- 1 analytical replicate for each treatment
- 21 days exposure at 20±1°C room temperature and 16:8 h light:dark
- Renew medium 2 times a week by transferring daphnids into freshly prepared vessels
- Daily counting and removal of neonates
- At 0 days, measure the length of 20 neonates
- After 21 days, remove and preserve adult daphnids (ethanol) for length measurements

For leachates:

1. Leachates are prepared as described previously.
2. Fill 50 mL leachate in each replicate (100 mL glass beaker).
3. Add algae (food) and 1 neonate per replicate.
4. Cover beakers with watch glasses to reduce evaporation.

For plastic particles:

1. Grind and sieve plastic materials to obtain particles < 64 µm.
2. Prepare stock solutions by suspending the required amount (weight) of plastic or natural particles in Elendt M4 medium (e.g., 0.03-1 g/L). Homogenize the stock solutions for 24 h in a rotatory shaker. Tween-20 may be added to stabilize suspensions.
3. Use glass vials with lids as test vessels and add stock solution, algae (food) and M4 to obtain the final concentration.
4. Add 1 neonate (<24 h) per replicate.
5. Place the closed vials in a rotatory shaker (1-3 rpm).
6. Optional: include 2 treatments with leachates to increase comparability between leachate (tier I) and particle (tier II) exposure.

Endpoints recorded: Mortality, reproduction and growth (21 days).

Results: Expression of results (e.g., the mean number of neonates) and statistical analysis (NOEC, EC_X) follow the OECD guideline 211.

4.2.3. Protocol for assessing the toxicity of small micro and nanoplastic particles (SMNP) by conducting the fish embryo toxicity (FET) test[1]

4.2.3.1. Scope

This protocol describes the procedures to prepare small micro and nanoplastics dispersions from plastic particles and test their toxicity using the standard fish embryo toxicity (FET) test. It applies to any polymeric material obtained from environmental samples, commercial items or engineered raw materials. Pre-treatment of all samples is needed to obtain particles below 20 µm in size. Pre-treatment consists of grinding the materials with the help of a mill and sieving the so-obtained powder through a metallic mesh of a standard size.

Species: *Danio rerio* embryos

Endpoint recorded: 96 hours mortality and hatching

4.2.3.2. Definitions

NTA: Nanoparticle tracking analysis

LC_{50}: the concentration that results in 50% mortality.

Toxic Units: $1/LC_{50}$

4.2.3.3. Materials and Equipment

- Mill
- 20 µm metallic sieve
- Ultrapure water
- Artificial freshwater
- Watch glass (one per sample)
- Glass flasks and vials
- Weighing scale ± 0.00001g
- Metallic spatula
- 1 mL glass syringes

4.2.3.4. Procedure

Preparation of the nanoplastics

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

[1] originally a separate Deliverable D6.2

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 artificial freshwater. 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 (>$1x10^7$ 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.

Obs: 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.

Long-term evaluation of small micro and nanoplastics stability

Evaluate the stability of the small micro and nanoplastics in ultrapure water and artificial freshwater 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 artificial freshwater.
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).

Preparation of testing dispersions

1. Prepare a stock solution of the small micro and nanoplastics according to the conditions defined previously.
2. If previous information on the small micro and nanoplastic toxicity are not available, five concentrations spaced by a constant factor of 10 can be used (e.g. 0.01; 0.1; 1; 10; 100 mg/L). This range can be decreased if the toxicity is not well distributed within the concentrations.
3. Conduct the fish embryo toxicity test for each small micro and nanoplastic according to the OECD 236.
4. Analyze by NTA the dispersions used at the beginning of the test and after each renewal in the old medium.
5. An embryo should be considered dead if it is positive for at least one of these parameters: coagulation of the embryo, lack of somite formation, lack of detachment of the tail and lack of heartbeat.

4.2.3.7. Expression of results and assessment criteria

1. The endpoint recorded is mortality after 96 hours of exposure expressed in percentage. When the mortality is higher than 50% in some concentrations the LC_{50} can be calculated, otherwise, only LC_{10} is obtained.
2. Assessment criteria to be developed will allow translating results into a scale of colors with 4 categories:

LC10 (mg/L)	Category
>100	No toxicity
100-10	Slight toxicity
10-1	Relevant toxicity
<1	High toxicity

Table 5. *Assessment criteria to be developed to obtain 4 categories of toxicity for small micro and nanoplastics using the FET*

4.3. SEDIMENT

4.3.1. Protocol for assessing the toxicity of plastic materials in *Lumbriculus variegatus*

4.3.1.1. Scope

This protocol describes the procedures to test the toxicity of plastic materials in the freshwater oligochaete *Lumbriculus variegatus*. This experiment is based on the standard guideline OECD 225 with some minor adaptations addressing experimental issues related to particle toxicities. It is applicable for plastic materials with a higher density than freshwater obtained from environmental samples, commercial items or engineered raw materials.

Pre-treatment of the plastic material is needed to obtain particle size distributions that are relevant for *L. variegatus*. This includes milling and sieving of the respective materials. In general, particles <100 µm are needed to ensure an interaction.

4.3.1.2. Definitions

EC_{50}: theoretical concentration of plastic particles or leachates corresponding to a 50% reduction of the endpoint recorded

NOEC: the lowest concentration with no observable effect

4.3.1.3. Procedure

General recommendations:

- 4 replicates per treatment
- 5 leachate (tier I) or particle (tier II) concentrations
- 3 different concentrations of particle controls, at least
- 1 analytical replicate for each treatment
- 50 g artificial sediment and 200 mL medium per replicate
- 28 days exposure at 20±1°C room temperature and16:8 h light:dark

Sediment composition:

Sediments consist of washed quartz sand, ground and sieved leaves of *Urtica dioica* and *Alnus glutinosa* (< 250 µm, 1:1 mixture based on mass) as carbon source and either MP or kaolin (particle control) as fine particulate materials (FPM). Sediments for each replicate are individually prepared and mixed in 250 mL glass beakers. Each exposure vessel contains 50 g sediment (dry weight) consisting of quartz sand, 1.6 % leaves and increasing proportions of SMNP or kaolin. The sediments are homogenised and 200 mL ISO medium or leachate is carefully added per replicate to prevent suspension of particulate matter. Control treatments consist of sediments without fine particulate materials. For leachates, kaolin is used as FPM to increase the comparability between tier I and tier II experiments. Here, the amount of kaolin depends on the concentration of the leachate.

For leachates:

1. Leachates are prepared as described previously.
2. Sediments contain a leachate-related amount of kaolin as FPM (e.g., 10 g MP/L leachate → 2 g MP/ 200 mL leachate → 2 g kaolin as FPM).
3. Fill 200 mL leachate in each replicate (250 mL glass beaker).
4. After a 5-days incubation, aeration is stopped, and specimens are added (10 per beaker). Aeration is continued the next day.
5. After 28 days, worms are removed from sediments, counted and dried for weighting (dry weight per replicate).

For plastic particles:

1. Grind and sieve plastic materials to obtain particles < 100 µm.
2. Prepare sediments as described above.
3. Fill 200 mL ISO medium in each replicate (250 mL glass beaker).
4. After a 5-days incubation, aeration is stopped, and specimens are added (10 per beaker). Aeration is continued the next day.
5. After 28 days, worms are removed from sediments, counted and dried for weighting (dry weight per replicate).
6. Optional: include 2 treatments with leachates to increase comparability between leachate (tier I) and particle (tier II) exposure.

Endpoints recorded: Number of worms and dry weight (28 days).

Results: Expression of results (e.g., mean number of worms) and statistical analysis (NOEC, EC_X) follow the OECD guideline 225.

4.4. MICROORGANISMS

4.4.1. Protocol for assessing the toxicity of plastic materials by conducting the luminescent bacterium *Vibrio fischeri* test

4.4.1.1. Scope

The luminescent bacterium *Vibrio fischeri* will be exposed to the leachate originating from the biodegradation of polymers. Serial dilutions of the leachate will be prepared for testing. The test is carried out with a LUMIStox photometer and a LUMIStherm incubation unit of the company Dr Lange.

4.4.1.2. Species

The marine water luminescent bacterium *Vibrio fischeri*. The natural habitat of *Vibrio fischeri* is seawater, but it can also be found in freshwater.

The test organism is cultured according to the ISO 11348 Part 2 using liquid-dried bacteria.

4.4.1.3. Preparation of samples

The preparation of samples for the determination of adverse effects to the marine water luminescent bacterium *Vibrio fischeri* exposed to the leachate from biodegradation tests of polymers is as follows:

- **Control sample:** *Vibrio fischeri* not exposed to the test material in medium with NaCl
- **Test sample:** *Vibrio fischeri* exposed to the test material
- **Reference sample:** *Vibrio fischeri* exposed to the reference material (3,5 Dichlorophenol), as well as NaCl (blind value).

Turbid samples should be allowed to settle for 1 hour or centrifuged, for example for 10 minutes at 5000g, or should be filtered using filter paper.

4.4.1.4. Calculation of percentage inhibition and evaluation of results

The calculation of percentage inhibition of light emission (bioluminescence) of samples (see Annex IV, chapter A.1) is performed according to ISO 11348:

Hcs = percentage inhibition of control sample (see Annex IV, chapter A.1) after 30 min

Hts = percentage inhibition of test sample (see Annex IV, chapter A.1) after 30 min.

Hrs = percentage inhibition of reference sample (see Annex IV, chapter A.1) after 30 min.

The results are expressed as EC_{20}, EC_{50} or EC_{80} values. The EC value corresponds to an inhibitory concentration at which inhibition of 20, 50 or 80 % is present.

4.4.1.5 Validity of the test

The test is considered valid if the validity criteria laid down in ISO 11348 are fulfilled.

In addition, the percentage inhibition of the light emission (bioluminescence) of the control sample (*Hcs*) shall be ≤25%.

If the percentage inhibition of the control sample (*Hcs*) is ≥25%, then the test is considered invalid. In this case, the control should be diluted appropriately, and the measurements should be repeated. If the dilution samples give a *Hcs* of ≤25% for the first time, this dilution series shall be used to determine the percentage inhibition of the test sample (*Hts*).

4.5. MARINE

4.5.1. Protocol for assessing the toxicity of plastic materials by conducting the marine microalgal growth inhibition test

4.5.1.1. Scope

ISO 10253 2016 - Marine algal growth inhibition test with *Skeletonema* sp., applicable to *Tisochrysis lutea* (formerly *Isochrysis galbana*)

Species: *Tisochrysis lutea*

Endpoint recorded: EC(r)$_{50}$ for each time interval and the 72h EC(r)$_{90}$ and NOEC

4.5.1.2. Definitions

Lixiviation: extraction with seawater of the hydrosoluble components of a solid phase, in this case, plastic particles.

Lixiviate or leachate: liquid phase obtained by lixiviation.

NOEC: the lowest dilution with no observable effect

Toxic Units: 1/ED$_{50}$

4.5.1.3. Material and Equipment

- The diluent and culture medium for all preparation and testing will be treated/filtered natural seawater and enriched with trace minerals, vitamins and chelating agents as specified in ISO 10253 2016.
- Each treatment is inoculated with cells from the pre-cultures in the exponential growth phase to give an initial cell density of approximately 2000 cells per ml.
- The test will be conducted in100 ml Erlenmeyer borosilicate glass flasks. Test vessels are continuously shaken to keep the cells in free suspension to facilitate CO_2 mass transfer from air to water, and in turn, reduce pH shift.
- To avoid toxic effects due to extreme acidity or alkalinity, the test stocks at 0h should have a pH of 8 The pH will be adjusted with either 1M HCl or NaOH.
- Tests are conducted in a temperature-controlled room, with a white fluorescent light providing continuous even illumination.
- The effects of test substances will be quantified by measuring cell number or fluorescence. Cell number may be quantified by microscope using either a Haemocytometer (HC) or Sedgewick Rafter (SR) or determined by a fluorometer

4.5.1.4. Procedure

The method estimates the effect of chemicals on the growth rate of laboratory cultures of marine unicellular algae. Toxicity is expressed as a proportionate reduction in growth rate with respect to control growth rate over a period of 72h at a temperature of 20±2 ºC.

4.5.1.5. Expression of results

Growth rates will be calculated for each replicate individually and the growth rate for each replicate will be compared with the controls to determine the result of the test. The EC(r)50 for each time interval and the 72h

EC(r)90 and NOEC will be calculated (with 95 % confidence limits, if possible) using an appropriate statistical method from the CETIS version 1.8 software.

4.5.1.6. Validity criteria

- Control growth rate >0.9 d-1
- Control growth rate variation coefficient ≤7 % at 72h
- Control pH range during test ±1

4.5.1.7. Assessment criteria

See 4.5.3. SET

4.5.2. Protocol for assessing the toxicity of plastic materials by conducting the *Acartia tonsa* marine copepod survival test

4.5.2.1. Scope

ISO 14669: 1999: Determination of acute lethal toxicity to marine copepods (Copepoda; Crustacea).

Species: *Acartia tonsa*

Endpoint recorded: 24h LC50 and 48h LC50, LC90 and NOEC values

4.5.2.2. Definitions

Lixiviation: extraction with seawater of the hydrosoluble components of a solid phase, in this case, plastic particles.

Lixiviate or leachate: liquid phase obtained by lixiviation.

NOEC: the lowest dilution with no observable effect

Toxic Units: $1/ED_{50}$

4.5.2.3. Materials and Equipment

- The diluent medium for all preparation and testing will be clean natural seawater which will be UV treated and 0.2 µm-filtered before use.
- The species *Acartia tonsa* will be used with this procedure. Test organisms will be obtained from an age-standardised laboratory culture maintained in accordance with ISO 14669: 1999: Determination of acute lethal toxicity to marine copepods (Copepoda; Crustacea). The culture will be maintained at 20 (±2 °C). Only adult organisms of stage 5 or above between 13 and 25 days of age at test commencement will be used.
- The test is conducted in 100 ml borosilicate glass crystallising dishes. During the test, the dishes will be covered with soda watch glass covers of a diameter at least 10 mm greater than that of the dishes.
- The test will be conducted in a controlled-temperature facility maintained at 20 ±2 °C. The Study will be conducted under a 16h light, 8h dark photoperiod
- To avoid toxic effects due to extreme acidity or alkalinity, the test stocks at 0h will be adjusted with either 1M HCl or NaOH.

4.5.2.4. Procedure

The method estimates the effect of chemicals on the mortality or immobilisation of adult copepods over a period of 48h at a temperature of 20 ±2 °C

4.5.2.5. Expression of results

The total number of animals affected (dead or immobile) in each concentration at each time point will be expressed as a proportion of the total number recorded as present at that concentration at the end of the test.

The 24h LC_{50} and 48h LC_{50} and LC_{90} values, with 95 % confidence limits, 48h NOEC will be calculated using the CETIS software

4.5.2.6. Validity criteria

- Control Mortality <10 %
- Dissolved oxygen >4 mg/l
- Reference - 3,5-DCP LC50 0.5 - 1.5 mg/l

4.5.2.7. Assessment criteria

See 4.5.3.6. SET

4.5.3. Protocol for assessing the toxicity of plastic materials by conducting the Sea-urchin Embryo Test (SET)

4.5.3.1. Scope

This protocol describes the procedures needed to test the toxicity of lixiviates from plastic particles or the toxicity of plastic particles themselves using the standard marine test, SET. It applies to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials. Pretreatment of all samples to obtain particles of a standard size is needed to allow comparability of results. Pretreatment is described in section 3, which reflects the procedure of grinding down to <250 µm used at the ECOTOX laboratory (UVI). Other particle sizes may also be acceptable.

Biological material: *Paracentrotus lividus* fertilized eggs

Endpoint recorded: 48-h size increase

4.5.3.2. Definitions:

ED_{50}: theoretical dilution of the lixiviate corresponding to a 50% reduction of the endpoint recorded

Lixiviation: extraction with seawater of the hydrosoluble components of a solid phase, in this case, plastic particles.

Lixiviate or leachate: liquid phase obtained by lixiviation.

NOEC: the lowest dilution with no observable effect

Toxic Units: $1/ED_{50}$

4.5.3.3. Material

For the leachate test:

1. 650 mg (for 10 g/L load) or 65 mg (for 1 g/L load) of microplastics <250 µm.
2. ASW
3. Pyrex glass bottles (50 mL) with Teflon lined screw caps (one per sample) Total volume of liquid with no air inside: 65 mL
4. Watch glass (one per sample)
5. Glass 100 mL beaker (one per sample)
6. Glass 50 mL beaker (one per sample)
7. 250 µm metallic sieve
8. Incubation chamber (20 ± 0.2 °C)
9. Weighing scale ± 0.00001g
10. Metallic spatula
11. Glass and plastic Pasteur pipettes (one per sample)
12. Dark 500 mL plastic flasks (one per sample).
13. Rotatory wheel (1 rpm)
14. Filter system (glass Büchner flask and vacuum pump)
15. 450 ºC furnace
16. 0.7 µm (GF/F, Whatman) filters (one per sample)
17. 25 mL volumetric flask (one per sample)
18. mL glass vials (4 vials for eggs + 8 filter controls+ 4 ASW controls+ 4xMPx4 dilutions)
19. Thermosalinometer
20. Oximeter
21. pH meter

For the SMNP particle test:

1. 3.75 mg of microplastics <20 µm
2. ASW
3. Tween®20
4. One 125 mL and one 1 L Pyrex glass bottles (one per sample)
5. Glass funnel
6. 25 mL and 100 mL glass measuring cylinders
7. Watch glass (one per sample)
8. 100 mL glass beaker (one per sample)
9. Incubation chamber (20 ± 0,2 °C) or isothermal room (20± 1 °C)
10. Balance ± 0,00001g
11. Metal spatula
12. Plastic and glass Pasteur pipettes (two per sample)
13. Dark vessels (500 mL) 2 per treatment.
14. Plankton wheel / Rotatory shaker (1 rpm)
15. 25 mL volumetric flask (one per sample)
16. 5 mL glass vials (6xsamplex4 dilutions)

4.5.3.4. Procedure for the leachate test

Day 1: Preparing the leachate (10 g/L and 1 g/L, < 250µm particles)

1. Manually sieve (with the help of a brush) the plastic particles through a metallic 250 µm sieve. An automatic sieve column may also be used.
2. Weigh using a 0.00001 g precision weighing scale, the watch glass and the spatula, 650 mg of <250 µm MP for a 10 g/L leachate. Alternative, if testing material is limiting use 65 mg to make up a 1 g/L leachate.
3. Transfer the MP into a 50 mL glass bottle and add ASW to the brim. Close and shake strongly.
4. Place each bottle inside a dark plastic container that fits into the rotatory wheel.
5. Stir at 1 rpm for 24 h in the dark at 20 ºC.

Day 2: Filtration and preparation of testing dilutions

1. Stop the wheel and take out the bottles.
2. Place a GF/F filter on the filtration system and rinse with abundant distilled water. Discard the distilled water. This step avoids false positives due to filter leachates.
3. Filter 100 mL of ASW to obtain the filter control. Recover the liquid in a 250 mL glass beaker.
4. Filter each bottle content through a new rinsed GF/F filter.
5. Record the variables of the lixiviate listed in Table 6. If values depart from tolerance limits for the sea-urchin and bivalve larvae correct them. If salinity is <30‰ correct with a hypersaline solution. If DO <5mg/L aerate the lixiviate for 10 min. Using ASW, pH departures are not expected.

Variable	
Temperature	20°C
Salinity	> 30‰
Dissolved oxygen	> 5 mg/L
pH	8.5 ≥ x > 7.5

Table 6. *Physico-chemical parameters acceptable for incubation of the SET*

6. Make up the lixiviate dilutions using a 25 mL volumetric flask, and transfer them to a 50 mL glass beaker to further deliver them into the incubation vials. Begin by the highest dilution to allow using the same flask and beaker for each lixiviate. If we do not have previous information on the leachate toxicity we can choose the following dilutions:

	Leachate volume (mL)	Final volume
X1/30	0.833	25
x1/10	2.5	25
x1/3	8.33	25
x1	16	-

Table 7. *Preparation of leachate dilutions for testing using ASW*

If after reading the results the 1/30 dilution shows toxicity, repeat the experiment with higher dilutions (x1/100, x1/300, …) until a NOEC is found.

7. Fill the 5 mL glass vials with 4 mL of each treatment:
   - vials for eggs
   - vials for ASW control
   - up to 8 (*) vials for the filter control
   - vials x 1/30
   - vials x 1/10
   - vials x 1/3
   - vials x 1

(*) Note: If more than 2 samples are tested then 8 filter controls are recommended, 4 at the beginning of the filtration and 4 at the end.

8. Conduct the sea-urchin embryo test according to standard procedures (e.g. ICES TIMES N. 51, 2012) adapted to 5 mL glass vials, as described above.
9. Fix the egg vials at t=0 with 6 droplets of 40% formalin using a fume cupboard. Caution!! Only the egg vials.
10. Close all the vials and place them at 20 ºC for 48 h incubation in the dark.

Day 4: End of the bioassay.

Take the vials out of the incubation chamber, unscrew and fix with 6 droplets of 40% formalin using a fume cupboard.

4.5.3.5. Procedure for the SMNP particle test

Preparation and characterization of the SMNP stock suspension

1. Mix 1L ASW and 3 µL of Tween®20 in a Pyrex bottle with a Teflon lined cap. Manually shake.
2. Weigh using a 0,00001 g precision balance, a metallic spatula and a watch glass 3.75 mg of <20 µm SMNP.
3. Prepare a SMNP stock suspension in a 125 mL Pyrex bottle by adding 3.75 mg of SMNP to 100 mL of the ASW-Tween mixture, flushig the watch glass with further 25 mL of ASW-Tween mixture, with the help of a measuring cylinder, Pasteur pipette and glass funnel.
4. Characterize the SMNP dispersion by Nanoparticle Tracking Analysis (NTA) or Coulter Counter, as described in 4.2.

Making up doses

5. Using the ASW-Tween® mixture and 50 mL volumetric flasks, dilute the stock suspension to the target final volume. For dilution volumes of 50 mL proceed according to this table:

Treatment	300 mg/L stock volume (mL)	Final volume (mL)
1 mg/L	0.168	50
3 mg/L	0.5	50
10 mg/L	1.67	50
30 mg/L	5	50
100 mg/L	16.7	50

Table 8. *Table of dilutions*

It is very important to shake the stock before adding it to the volumetric flask before each dilution.

6. Fill the 5 mL glass vials with no head space (7.8 mL in our vials) except egg vials which are filled with just 4 mL:
   - egg vials (ASW) (fill with just 4 mL)
   - ASW control vials
   - 8 Filter control vials (ASW +Tween)
   - vials with 1 mg/L
   - vials 3 mg/L
   - vials 10 mg/L
   - vials 30 mg/L
   - 4 vials with 100 mg/L
7. Fertilize the eggs and conduct SET according to std methods I-LB-BE-001.
8. Fix the egg vials at t=0 with 6 drops of 40% formalin and take them apart.
9. Place the remaining vials inside dark containers padded with soft material to avoid glass break during stirring.
10. Place the containers in the wheel at 1 rpm for 48 h in the dark at 20 ºC.

End of the bioassay

11. After 48h stop the wheel and take the vials out of the dark containers and place them on a grid.
12. Unscrew the caps except for the egg vials and add 6 droplets of formalin to each vial.

4.5.3.6. Expression of results, acceptability criteria and assessment criteria

6. The endpoint recorded is the maximum dimension of 35 individuals per vial (L).
7. Mean length per vial is expressed as length increase by subtracting the average size of eggs (L_0): $\Delta L' = L - L_0$
8. A bioassay is considered acceptable when control larval length increases, ΔLc >218 µm (Saco-Álvarez e col., 2010)
9. Length increase data is compared for each treatment with the control using the Dunnet test to obtain NOEC and LOEC.
10. Length increase data is corrected by the control to obtain the Percentage Net Response (PNR):

$$PNR = \frac{\Delta L'}{\Delta L_C}$$

11. PNR data are fit to a dose:response sigmoidal model to estimate the ED_{50}, with the aid of some statistical software, e.g. Graph Pad Prism or SPSS. This can be done only when some treatments showed a decrease >50% in the endpoint recorded. Common models recommended are logistic or probit. ED_{50} values must be listed along with the corresponding 95% confidence intervals. 50%.
12. The toxicity of each sample may be now expressed in Toxic Units (TU):

$$TU = 1/ED_{50}$$

13. When none of the dilutions had a level of effect >50% then the result is expressed as <1 TU.
14. Assessment criteria allow translating results into a scale of colors with 3 categories:

10 g/L	
TU	**Category**
<1	No toxicity
1 ≤ TU< 5	Slight toxicity
5 ≤ TU< 25	Relevant toxicity
≥ 25	High toxicity

1 g/L	
TU	**Category**
<1	No toxicity
1 ≤ TU< 2	Slight toxicity
2 ≤ TU< 10	Relevant toxicity
≥ 10	High toxicity

Table 9. *Proposed assessment criteria to obtain 4 categories of toxicity for 1g/L and 10 g/L lixiviates*

4.5.4. Protocol for assessing the toxicity of plastic materials by conducting the *Cyprinodon variegatus* embryo-larval development test

4.5.4.1. Scope

This protocol describes the procedures needed to test the toxicity of a seawater lixiviate obtained from plastic particles using the *Cyprinodon variegatus* embryo-larval development test. It applies to any polymeric material obtained either from environmental samples, commercial items or engineered raw materials. Pretreatment of all samples to obtain particles of a standard size is needed to allow comparability of results. Pretreatment is described in Section 3, which reflects the procedure of grinding down to <250 µm used at the ECOTOX laboratory (UVI). Other particle sizes may also be acceptable.

4.5.4.2. Materials and Equipment

Biological material: *Cyprinodon variegatus* fertilized eggs (n=200 per leachate)

Lab ware:

- 1 µm ASW sterilized by UV
- yoghurt glass vessels as test vessels
- 100 mL glass beakers (n=20 for 4 dilutions plus control per quadruplicate)
- 63 µm and 300 µm sieves
- Plastic trays for egg selection
- 25 ± 1 °C; 16:8 h light:dark isothermal room
- Weighing scale ± 0.00001g

- Metal spatula
- Pasteur pipettes to catch the eggs
- 55 mm Petri dishes
- Overhead rotator
- 250 mL volumetric flasks (1 per dilution)
- 28 ‰ seawater in a squeezing bottle
- Stock of ca 20 L ASW preconditioned at 28 ppt and 25ºC
- Thermosalinometer
- Oximeter
- pH meter
- 1 mL, 5 mL and 10 mL automatic pipettes and tips
- Binocular microscope
- Dissecting needles
- 50 ml measuring cylinder

Endpoints recorded: survival and normal development

4.5.4.3. Definitions:

ED_{50}: theoretical dilution of the lixiviate corresponding to a 50% reduction of the endpoint recorded

Lixiviation: extraction with seawater of the hydrosoluble components of a solid phase, in this case, plastic particles.

Lixiviate or leachate: liquid phase obtained by lixiviation.

NOEC: the lowest dilution with no observable effect

Toxic Units: $1/ED_{50}$

Survival: within the context of this protocol, the survival rate is the proportion of individuals that at the end of the incubation period present visual evidence to be alive and do not show any of the morphological abnormalities described below.

4.5.4.4. Procedure

Day 1: Prepare a 10 g/L leachate according to 3.2.2. but scaling up to the following amounts: weigh 5.3 g of plastic particles <250 µm and put in a 500 Pyrex bottle filled to the brim with FSW.

Day 2:

1. Prepare experimental dilutions according to 3.2.2. upscaled to the following volumes:

Dilutions	Leachate Vol (mL)	FSW (up to mL)
x1/30	8.33	250
x1/10	25	250
x1/3	83.33	250
x1	200	-
TOTAL:	316.66	750

Table 10. *Lixiviate dilutions*

2. Use a 250 mL volumetric flask to make the dilutions according to Table 8, using ASW. Dilutions to be tested depend on the material toxicity. If no previous information is available test the dilutions reflected in Table 8. If after testing 1/30 dilution shows significant toxicity higher dilutions (x1/100, x1/300, etc) must be tested until a NOEC is found. If none of the tested dilutions shows effects between the 10% and 90% levels, intermediate dilutions must be tested.
3. Conduct the *C. variegatus* embryo-larval test according to standard procedures (US-EPA 2002) adapted as described below.
4. Select under the binocular microscope 200 fertilized eggs ca. 48h old (Figure 4, Annex V).
5. Collect the eggs in n=10 pools in the yoghurt vessels with the aid of a Pasteur pipette.
6. Fill the glass beakers with 50 mL of the testing dilutions (per quadruplicate)
7. Transfer 10 eggs from each yoghurt vessel to the beaker.

Days 1-9. Medium renewal and water quality check.

1. Record T°, pH and oxygen of the water stock before incubation (day 1) and before each water renewal (days 3, 5 and 7). Use the 63 µm sieve to retain the eggs and change the medium. Keep a minimum of old water to avoid drying eggs or larvae.
2. Record dissolved oxygen in each replicate. For other variables (T°, salinity, pH) a single replicate per treatment may be enough.
3. Record at days 1, 3, 5 and 7 the number of living and dead individuals, discarding the latter. An individual is considered dead (Fig. 2) when the heart does not beat.

Day 9: End of the test.

1. Record survival and abnormalities. The survival rate in control must be ≥ 80 %.
2. Morphological and behavioural abnormalities include:
   - Normal swimming but slightly bent spine
   - Abnormal swimming and markedly bent spine (Figure 7 Annex IV)
   - Missing appendages (Figure 6, Annex IV)
   - Opaque mass in the heart cavity (Figure 8, Annex IV)
   - Swollen yolk sac and abnormal swimming (Figure 9, Annex IV)

4.5.4.5. Expression of results

Survival data per vessel after angular transformation ($Arcsen(\sqrt{survival})$) are used to calculate NOEC and LOEC by using ANOVA and Dunnett test. If transformed data does not meet homoscedasticity and normality non-parametric tests must be used.

Secondly, responses are control corrected and fit to a sigmoidal dose:response model (log-logistic or probit), for EC_{50} calculation and 95% confidence intervals. That can be done only when treatments with a level of effect >50% are obtained.

4.6. NON-STANDARD TESTS

Specific mechanisms of toxicity due to plastic components may be further explored by the use of non-standard toxicity tests tailored to identify a given adverse outcome pathway. These non-standard tests are normally costly and labour demanding, so they are not included as routine assessment tools, but may be useful when certain effects (such as endocrine disruption) are suspected.

Non-standard terrestrial toxicity tests include the assessment of molecular biomarkers such as stress enzymes activities and gene expression in *Eisenia* spp. and other earthworm species, or effects on the composition of soil bacterial communities by using 16S and 18S rRNA–V4 amplicons and sequence variant analysis.

Non-standard freshwater toxicity tests may include the influence on production and adsorption of microcystins in toxinogenic vs non-toxinogenic strains of *Microcystis aeruginosa*, and sublethal endpoints in zebrafish embryos.

Useful non-standard tests applicable to marine habitats include the identification of gene expression alterations in biomarkers of an endocrine disruption using the fish model *Cyprinodon variegatus*.

ANNEX I: RANGE FINDING TEST FOR *EISENIA ANDREI* REPRODUCTION TEST

RANGE-FINDING TEST

To assess the adequate interval of concentrations to be used in a definitive test, a range-finding test must be performed as proposed in Table 7.

DEFINITIVE TEST

A definitive test should contain a control plus 5 concentrations, that may affect 20%, 50% and 80% of the population exposed, according to the results from the Range-finding test. The number of replicates per condition remains the same (8 per control, 4 per concentration).

Concentration (mg/kg)	Replicates (N)
0	8
50	4
100	4
225	4
450	4
900	4
1800	4
2700	4
3600	4

Table 11. *Proposed concentration range and replicates, based on the OECD guideline and current knowledge of plastic pollution in soils for Eisenia andrei reproduction test*

ANNEX II: PROPOSED CONCENTRATION RANGE AND REPLICATES FOR PLANT GERMINATION TEST

RANGE-FINDING TEST

To assess the adequate interval of concentrations to be used in a definitive test, a range-finding test must be performed as proposed in Table 8.

DEFINITIVE TEST

A definitive test should contain a control plus 5 concentrations, that may affect 20%, 50% and 80% of the population exposed, according to the results from the Range-finding test. The number of replicates per condition remains the same.

Plastic particles added as a powder		Plastic lixiviates added as a solution (from a 10g plastic/L stock)	
Plastic particle concentration (mg/L)	Replicates (N)	Plastic particle concentration (mg/L)	Replicates (N)
0	5	0	5
10	5	2	5
20	5	5	5
45	5	10	5
90	5	20	5
180	5	50	5
360	5	100	5
540	5	200	5
720	5	400	5

Table 12. *Proposed concentration range and replicates, based on the OECD guideline and current knowledge of plastic pollution in soils for plant germination test*

ANNEX III: PROPOSED CONCENTRATION RANGE AND REPLICATES FOR SOIL MICROORGANISMS TEST

RANGE-FINDING TEST

To assess the adequate interval of concentration to be used in a definitive test, a range-finding test must be performed, encompassing as proposed in Table 9.

DEFINITIVE TEST

A definitive test should contain a control plus 5 concentrations, that may affect 20%, 50% and 80% of the population exposed, according to the results from the Range-finding test. The number of replicates per condition remains the same.

Plastic particles added as powder		Plastic lixiviates added as solution (from a 10g plastic/L stock)	
Plastic particle concentration (mg/kg)	Replicates (N)	Plastic particle concentration (mg/kg)	Replicates (N)
0	12	0	12
100	12	10	12
250	12	25	12
500	12	50	12
1000	12	100	12
2000	12	250	12
4000	12	500	12
8000	12	1000	12

Table 13. *Proposed concentration range and replicates, based on the OECD guidelines and current knowledge of plastic pollution in soils for soil microorganisms test*

ANNEX IV: *C. VARIEGATUS* EMBRYO-LARVAL DEVELOPMENT (PHOTOS: A. VILAS, ECOTOX, UVI)

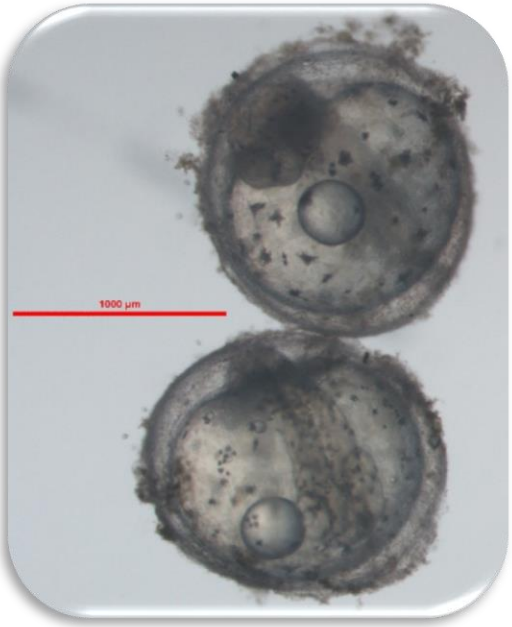


Figure 4. *48h embryos*

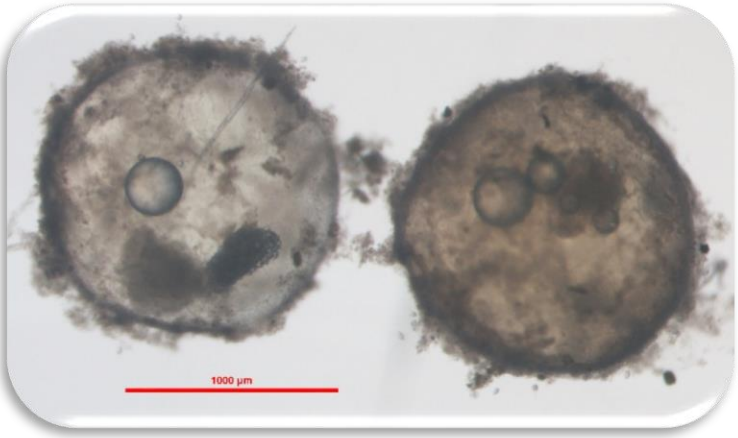


Figure 5. *DEAD FERTILIZED EGGS. They differ from UNFERTILIZED ovules in that they have remnants of cell masses inside (normally one of these masses is more brown and the other black).*

Figure 6. *DEAD larva. Lacks mobility and the heart does not beat.*

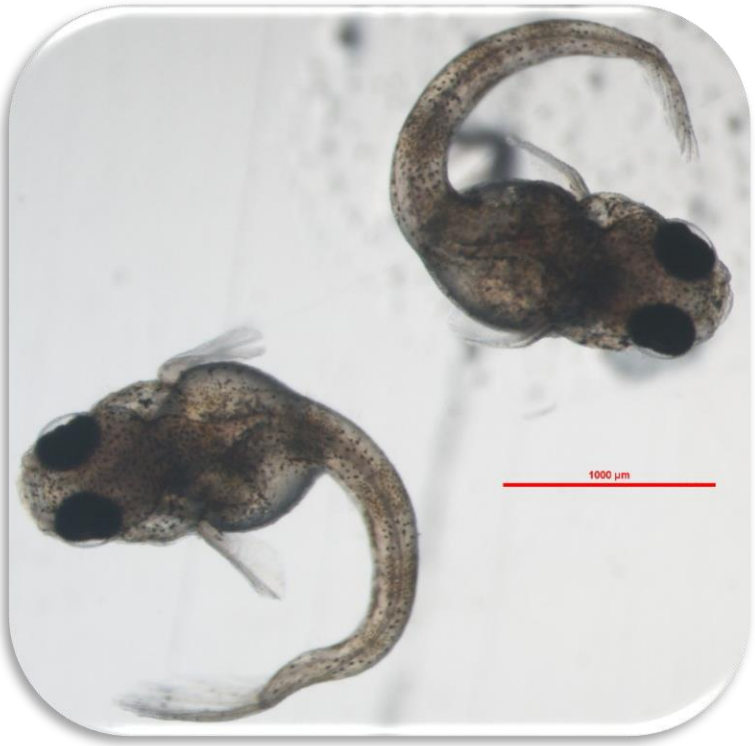


Figure 7. *Larvae with a strongly curved spine and abnormal swimming. They are identified by having curved tails and by swimming in circles*

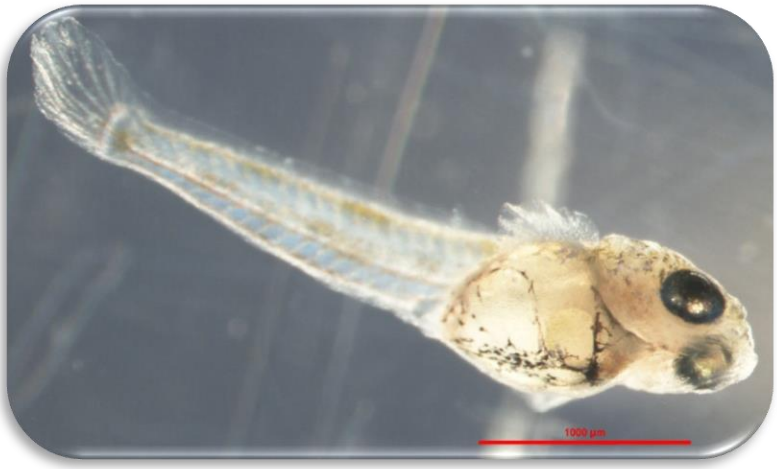


Figure 8. *Larva with heart in OPAQUE MASS. These larvae usually do not reach the end of the bioassay.*

Figure 9. *Larva with swollen yolk sack. The 9-day-old larva has almost no yolk sac, in this case, the size is so large that it makes it difficult for the larva to swim normally.*

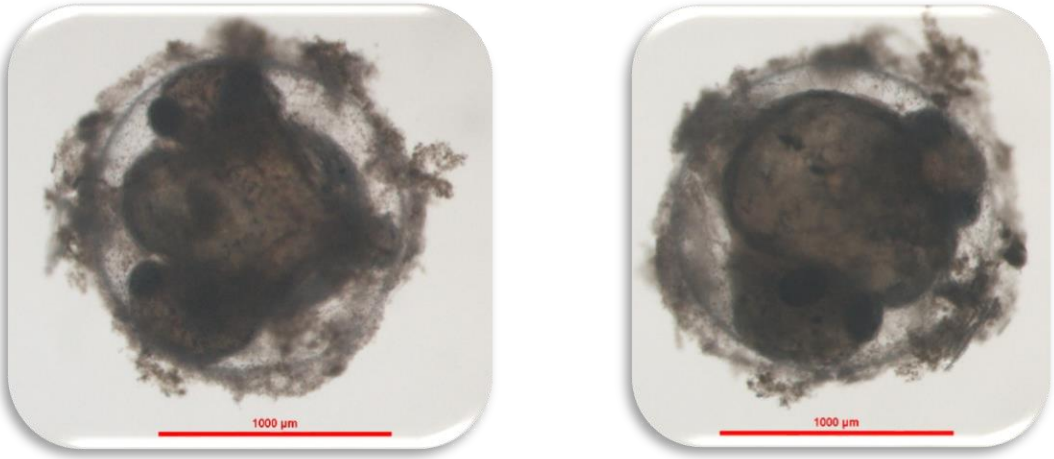


Figure 10. *Egg with two formed embryos. Separation in the middle part of the spine. Two well-differentiated heads.*

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