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TECHNICAL REPORT: BIODEGRADATION AND ECOTOXICITY TESTS

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Table of contents

I. BACKGROUND 4

II. MATERIALS & METHODS 4

a. Description of the study samples 4

b. Study of ultimate biodegradability in the marine environment 4

i. Preparation of tested materials 5

ii. Incubation medium: natural seawater 5

iii. Respirometric monitoring method 7

iv. Evaluation of results: mathematical smoothing equation 8

c. Ecotoxicological impact study: operating procedures 8

i. Preparation of test samples 9

ii. Microalgae *Phaeodactylum tricornutum* 9

iii. Crustacean *Artemia salina* 13

iv. Sea urchin larva *Paracentrotus lividus* 16

III. RESULTS : BIODEGRADABILITY STUDY 17

a. Physico-chemical and biological parameters 17

b. Cinetics of biodegradability in the marine environment 18

IV. RESULTS: ECOTOXICOLOGICAL STUDY 19

a. Microalgae *Phaeodactylum tricornutum* 19

i. Determination of the Fluorescence-cell density correlation equation 19

ii. Growth inhibition observed after exposure to study materials 20

b. Crustacean *Artemia salina* 21

c. Sea urchin larva *Paracentrotus lividus* 22

i. Sensitivity to the reference toxicant 23

ii. Results obtained for aged samples 25

V. DISCUSSIONS & OUTLOOKS 27

TABLE OF FIGURES

Figure 1: Technical and specification standards associated with biodegradability in the marine environment 5
Figure 2: Natural seawater and sediment sampling site for biodegradability tests 6
Figure 4: Malassez cell counting system 11
Figure 5: Example of Dose-Response data generated using the RegTox macro 13
Figure 6: Simplified diagram of the toxicity assessment test on the Artemia salina model 15
Figure 8: Biodegradation rates observed for samples selected in phase 2 18
Figure 11: Correlation equation between fluorescence and microalgal cell density 20
Figure 12: Inhibition of algal growth at 72H depending on the material tested at a concentration of 150 mg/L 21
Figure 13: Dose-response curve for exposure of A. salina to copper sulphate after 48 hours 22
Figure 14: Mortality observed in A. salina nauplii after 48H exposure to environments contaminated by particles from the materials tested 22
Figure 15 - Rate of larval non-development in the copper range 23
Figure 16 - Rate of non-development and malformation in the copper range 24
Figure 17 - Rate of non-development as a function of the sample tested 25
Figure 18 - Rate of non-development and malformations as a function of the sample tested 26
Figure 19 - Toxicity gradient for the nine samples tested 26
Figure 20: Toxicity debt (credit : Rillig et al. 2021, Env Sci Techn) 29

Table 1: Samples studied 4
Table 2: Mother nutrient solutions 10
Table 3: Chemical composition of synthetic seawater 10
Table 4: Physico-chemical and biological parameters 17
Table 5: Correspondence between codes for samples received and samples tested 19
Table 6: Criteres de validites de l'essai 21

I. BACKGROUND

Biodegradable materials can be broken down naturally by biological processes, such as the action of living organisms or micro-organisms, into simple, non-toxic elements. They offer a more environmentally friendly alternative to traditional materials, which can evolve for centuries in the environment without decomposing.

These particular characteristics make them particularly attractive in today's environmental protection challenges, such as reducing plastic waste, conserving resources and protecting marine ecosystems.

If we look at the fishing industry, it's worth turning to these alternatives, which have a more controlled environmental impact. Biodegradable fishing gear would present several challenges by reducing marine waste, limiting the accumulation of floating debris and ghost fishing.

However, challenges remain, particularly in terms of the performance and durability of the gear, which must be strong enough to withstand marine conditions and fishing activity. The cost and accessibility of biodegradable fishing gear can also pose problems, as can the establishment of appropriate standards and certifications to guarantee their effectiveness and biodegradability, and the assessment of the toxicological impact according to the time scale considered.

II. MATERIALS & METHODS

a. Description of the study samples

A total of 12 samples was submitted to the study and, following all the tests carried out (performance, physico-chemical characterisation, etc.) on the upstream deliverables, a selection of three formulations was made: two mono-filaments (fine mesh) and one multi-filament (catining mesh), described below (Table 1)

Nom échantillon	Correspondance
INd_AC(M)	MONO_1_AC
INd_AH(V)	MONO_2_AH
INd-Y	MULTI_2

Table 1: Samples studied

b. Study of ultimate biodegradability in the marine environment

There are several standards that are specific to the area under study (Figure 1).

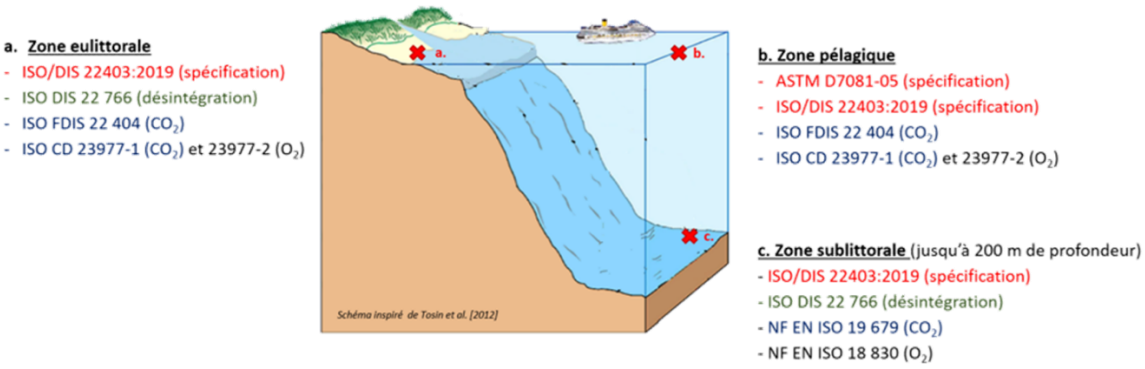


Figure 1: Technical and specification standards associated with biodegradability in the marine environment

Biodegradability is assessed based on NF EN ISO 19679:2017 Plastics - Determination of aerobic biodegradation of non-floating plastics at the seawater/sediment interface - Method by analysis of released carbon dioxide. The methodology varies from the standard by using NaOH as the CO_2 absorber instead of KOH. The reaction equations are given in section II.b.iii of this document.

i. Preparation of tested materials

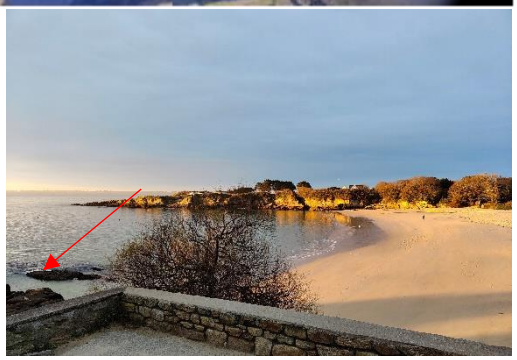
The materials tested were supplied in the form of 2 to 3 mm granules. All the granules were ground under liquid nitrogen (IK A10 Basic, Werke GmbH & Co, Germany) to limit the impact of the temperature rise in the chamber during grinding, thereby guaranteeing the integrity of the properties of the materials studied. The crushed material obtained was then sieved on a 200µm mesh sieve. The exact granulometry of the sieved fraction is not measured. This fraction is then used for the biodegradability tests.

The total organic carbon content of each study sample and the positive reference is determined by elemental analysis (Vario Cube, Elementar), which is used to determine the total quantity of sample to be submitted to the study and to determine the maximum theoretical quantity of CO_2 released by the material during the process.

ii. Incubation medium: natural seawater

The natural seawater used for the experiments is taken from the coastal site Le Perello, 56270 PLOEMEUR (coordinates 47.69974136352539, -3.4431159496307373) (Figure 2)

Figure 2: Natural seawater and sediment sampling site for biodegradability tests



The direct environment of the sampling site is free from industrial activity and the quality of the water is suitable for carrying out the tests. The water and sediment taken are sent directly to the laboratory for testing. To avoid any conservation, the tests are initiated directly, and the microorganism load is checked afterwards.

Controlling the micro-organism load:

The concentration of microorganisms was determined by depositing 100µL of the natural seawater solution and spreading successive dilutions onto nutrient agar (on Petri dishes). The agar is prepared using 37.5 g of Marine Broth medium (Sigma-Merck, Marine Broth 2216) with 1.5% agar by mass for 1 litter of deionized water. The Petri dish was incubated at 20°C in a thermostatic chamber for 48 to 72 hours before counting the colony-forming units.

Natural seawater preparation before incubation:

To compensate for deficiencies in the elements needed to maintain good environmental quality, natural seawater is supplemented with 1 mM ammonium chloride solution (NH_4Cl) and 0.1 mM potassium phosphate solution (K_2HPO_4). The elements phosphorus and nitrogen are necessary for the micro-organisms, so it is imperative not to reach limiting conditions in N and P.

Sample inoculation and incubation:

In a Durand washer bottle (tared) with a capacity of 250 mL, 30 grams of sediment were deposited at the bottom, then 100 mL of natural seawater amended for phosphorus and nitrogen were added. A second wash bottle was filled with 30mL of a 0.2N NaOH solution. The system is then connected so that the whole system forms a sealed device with oxygen re-circulation and capture of the CO_2 emitted.

This configuration is representative of the respiration control batches, which correspond to the activity of the microorganisms in the absence of a test substance or reference substance.

Bioassay system50 mg of carbon equivalent for each sample was added to the various test batches, while the positive reference control batches were spiked with 123.15 mg of Avicell microcrystalline cellulose. All weighing was carried out using a 0.1 mg precision balance (Radwag Wagi Elektroniczne, Poland).

The air pump system was sealed and checked by dosing CO_2 into the system over a period of 15 days prior to the experiment. The quantity must not exceed the quantity naturally found in the air in relation to the quantity of air present in the system, i.e. approximately 0.5 L.

Incubation is carried out in a water bath (Memmert GmbH, Germany) maintained at a temperature of 20°C ± 2°C and the air pump is switched on for 15 minutes every 2 hours to recirculate oxygen and force the capture of CO_2 in the absorbent solution.

iii. Respirometric monitoring method

Monitoring is carried out every two to three weeks to obtain enough measurement points to trace the kinetics. Throughout the incubation period, the micro-organisms will, as far as possible, bio-assimilate the carbon present in the sample under study and mineralise it, producing various elements including CO_2. By measuring the CO_2 emitted between two measurements, it is possible to determine the quantity of carbon bio-assimilated and therefore biodegraded. This CO_2 is then trapped in the container containing the absorbent solution (0.2N NaOH) according to the following reaction mechanisms:

The CO_2 is trapped by the absorbent solution according to the following equation:

$$2NaOH + CO_2 \rightarrow Na_2CO_3 + H_2O$$

Addition of 5 mL of a solution of barium chloride ($BaCl_2$) at a concentration of 246 g/L

$$Na_2CO_3 + BaCl_2 \rightarrow 2\ NaCl + BaCO_3 \text{ (milky white precipitate)}$$

As there is an excess of absorbent solution, it is possible to measure the excess by adding 0.1N hydrochloric acid and displaying the colour change (pH=7) using a coloured indicator, thymolphthalein (change from a blue solution to a white solution when the colour change zone is reached).

$$NaOH + HCl + \text{thymolphtalein} \rightarrow NaCl + H_2O$$

The volume of HCl poured can be used to determine the quantity of NaOH in excess and the quantity of NaOH that reacted with the CO_2.

Since the percentage of carbon in the sample is known, it is possible to determine the theoretical maximum quantity of CO_2 released ($ThCO_2$) by the sample in the case of complete mineralisation. The percentage of biodegradation is therefore calculated as follow :

$$\%\ biodegradation = \frac{\text{mg } CO_2 \text{ (sample)} - \text{mg } CO_2 \text{ (respirometric control)}}{ThCO_2}\ x\ 100$$

For each measurement, the bottles were left open for 1 minute with the pumps running to force the renewal of oxygen in the system. The opening of the bottles results in an increase in the CO2 content of the renewed air, which is considered by subtracting the measured mass of CO2 released by the control batches.

Each vial was also weighed and compared with its tare weight in order to quantify any evaporation of the incubation medium. In the event of evaporation, deionised water is added to the flask to preserve the initial salinity of the natural seawater used in the study.

iv. Evaluation of results: mathematical smoothing equation

The results obtained must express the rate of biodegradation as a function of incubation time. The kinetics thus obtained are compared with those obtained for the reference material (AVICELL microcrystalline cellulose, Grosseron).

The data was smoothed using DataFit9 software (Oakdale Engineering). The data sets are integrated as input and, depending on the type of raw graph obtained during respirometric monitoring, then smoothed mathematically using Hill's equation.

It is not possible to extrapolate the data obtained and it is important to consider only the time zone covered by the measurements.

c. Ecotoxicological impact study: operating procedures

The tests were carried out on a selection of three formulations: MONO 1 AC PE, MONO 2 AH VF, MULTI 1 BOBINE 2. Each of these formulations was aged in air or seawater.

The standards used in the toxicological assessment of substances on organisms in the marine environment are associated with soluble molecules. In the tests carried out, the particles are not soluble, and the test needs to be adjusted. One possibility is to assess the toxicity of seawater from ultimate aerobic biodegradability tests at the seawater-sediment interface. As this condition was not feasible given the number of samples tested in the biodegradation stage and the volumes involved (100mL per replicate) compared with the volumes required for all the toxicological tests (>1L), it was decided to carry out the tests on the basis of material aged in a climatic chamber (see deliverable T3.1.2 Technical report on the study of the ageing of new fishing gear) and to work on homogenising the particles before testing and testing the seawater used to age the particles.

i. Preparation of test samples

The samples, aged under different conditions (see deliverable T3.1.2), were supplied to IRMA in the form of microparticles-macroparticles. The study samples were suspended in a volume of nutrient-enriched artificial seawater to obtain a concentration of 150 mg of particles/L of seawater. The solutions were kept under agitation to optimise the homogeneity of the insoluble particles in the container.

ii. Microalgae *Phaeodactylum tricornutum*

Phaeodactylum tricornutum (class: *Bacillariophyceae*; order: *naviculales*; familly: *Phaeodactylaceae*) is a marine diatom used in ecotoxicology as a model for assessing water quality. The tests are based on the international standard ISO 10253:2016 Water quality: Marine algae growth inhibition test with Skeletonema costatum and *Phaeodactylum tricornutum*. The *P. tricornutum* strain used in the tests came from the culture collection of the University of Caen Normandie (Algobank-Caen) under strain number AC590-CCAPP1052/1. On receipt, the microalgae are preserved in F/2 medium, which is a specific nutrient medium equivalent to the preparation solution described in standard ISO 10253:2016 (§5.3, 5.4, 7.1).

The test consists of exposing a culture of the microalga *P. tricornutum* to the study solutions and recording the differences in growth between a control batch and the test batches and validating the sensitivity of the diatom using a reference toxic substance.

The test consists of 6 stages:

Preparation of growth media:

A pre-culture is prepared 2 to 4 days before the start of the test, allowing for the exponential phase of growth. The growth medium is artificial seawater (Table 3) enriched with nutrient solutions (Table 2) and is made up as follows: 15 mL of nutrient stock solution 1, 0.5 mL of nutrient stock solution 2 and 1 mL of nutrient stock solution 3, then made up to 1L in a volumetric flask using the artificial seawater solution. The pH of the solution is adjusted to 8.0 ± 0.2 using a dilute solution of hydrochloric acid or sodium hydroxide.

NB: the solutions are filtered through a 0.22 µm membrane to guarantee sterility of the experiment.

Nutrients	Concentrations	Concentrations in the final solution
Solution 1		
$FeCl_3.6H_2O$	48 mg/L	149 µg/L (Fe)
$MnCl_2.4H_2O$	144 mg/L	605 µg/L (Mn)
$ZnSO_4.7H_2O$	45 mg/L	150 µg/L (Zn)
$CuSO_4.5H_2O$	0,157 mg/L	0,6 µg/L (Cu)
$CoCl_2.6H_2O$	0,404 mg/L	1,5 µg/L (Co)
H_3BO_3	1 140 mg/L	3,0 mg/L (B)
Na_2EDTA	1 000 mg/L	15,0 mg/L
Solution 2		
Thiamin Chlorhydrate	50 mg/L	25 µg/L
Biotine	0,01 mg/L	0,0005 µg/L
B12 Vitamin	0,1 mg/L	0,05 µg/L
Solution 3		
K_3PO_4	3,0 g/L	0,438 mg/L (P)
$NaNO_3$	50 g/L	8,24 mg/L (N)
$Na_2SiO_3.5H_2O$	14,9 g/L	1,97 mg/L (Si)

Table 2: Mother nutrient solutions

Salts	**Compounds concentration in artificial seawater g/L**
NaCl	22
$MgCl_2.6H_2O$	9,7
Na_2SO_4 (anhydrous)	3,7
$CaCl_2$ (anhydrous)	1,0
KCl	0,65
$NaHCO_3$	0,20
H_3BO_3	0,023

Table 3: Chemical composition of synthetic seawater

Preparation of preculture and inoculum:

The preculture is prepared by adding a sufficient volume of the mother culture to obtain a cell density of between $2,10^3$ cells/mL and 10^4 cells/mL. In order to respect the validity criteria indicated below, it is necessary to start from a density of 10^4 cells/mL, otherwise an average specific growth rate of greater than 0.9 day^{-1} over the duration of the test will not be achieved.

The pre-culture is prepared 3 to 4 days before the start of the experiment and incubated under the same conditions as the test.

Cell density:

There are many ways of counting the number of cells in the different solutions (precultures, mother culture, test solutions). As part of the Interreg INdIGO project, it was decided to proceed in two stages:

firstly, by counting on Malassez cells (Figure 4) and then by establishing a correspondence by measuring the fluorescence emitted by *P. tricornutum*. Microalgal density was monitored by measuring chlorophyll a (chla) fluorescence (λexcitation=633 nm, λemission=638- 720 nm) using a multi-well plate reader (Tecan Austria GmbH, Grödig, Austria).

This enables the fluorescence = f(concentration) relationship to be determined, allowing the microalgal density to be measured more quickly during the assays.

The Malassez cell count is carried out on 10 zones squared vertically and horizontally, the count is reduced to a number of cells/zone and then related to a working volume calculated from the metric data of the cell, i.e. a calculation of the microalgal density expressed in cells / mL determined as follows:

$$cell\ density = \frac{\Sigma\ cells\ for\ X\ zones}{X\ zones}\ x\ 10^5$$

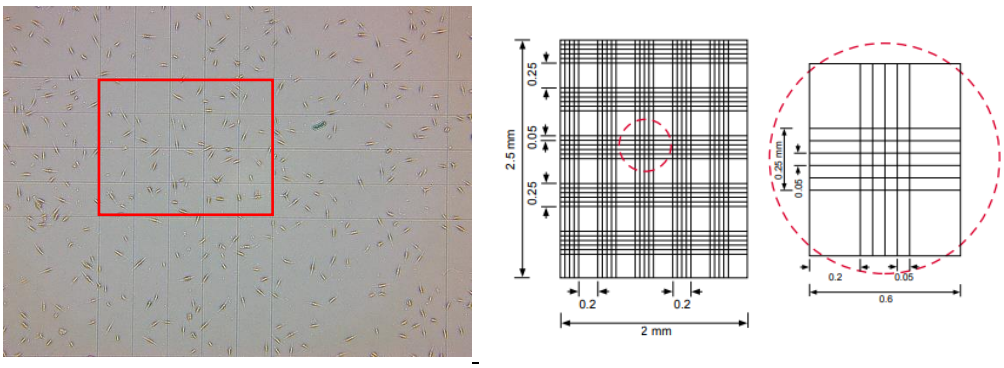


Figure 3: Malassez cell counting system

Test concentrations:

A pre-test on a concentration of 150 mg particles / L of nutrient-enriched artificial seawater (as described in § Preparation of the growth medium) is carried out. If a toxic response is recorded during this test, it is necessary to expose the microalgae to a series of 5 concentrations in a geometric range of reason less than or equal to 3.2 (for example 1.0 mg/L, 1.8 mg/L, 3.2 mg/L, 5.6 mg/L ...). The range thus chosen must include at least one inhibitory response above and below the EC50 parameter (e.g., one response above the EC_{90} and one below the EC_{10}).

Incubation :

Incubation was carried out in a HELIOS 600 phytotronic chamber (Cryotek, France) where the temperature was maintained at 20°C ± 2°C, and followed a 16H/8H day-night cycle with a light intensity equal to 80 µmol.m^{-2}.s^{-1}. The total incubation time was 72H ± 2H.

Assessment of the percentage of inhibition:

The growth inhibition percentage represents the difference between the average specific growth rate of the control batches and that of the test batches over the duration of the test.

The average specific growth rate, μ, is calculated as follows:

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0}$$

with :

t_0 start of the assay

t_L end of the assay

N_0 cell density t0

N_L cell density at the end of the assay

Once the average specific growth rate has been calculated for each of the containers in the test, the percentage of inhibition, Iμi, in each vial is determined using the calculation method below:

$$I_{\mu i} = \frac{\bar{\mu}_C - \mu_i}{\bar{\mu}_C} \, x \, 100$$

with:

- μi : the growth rate for the test vial i
- $\bar{\mu}_C$: the average growth rate for the control batches

The raw data thus obtained are entered into an Excel spreadsheet called RegTox (University of Lyon 1), which has a macro developed for dose-response models. Running the macro generates the graph Iμi = f (concentration) and indicates on it the parameters EC_{10}, EC_{50} and EC_{90} where possible (example of data generated by RegTox in Figure 5).

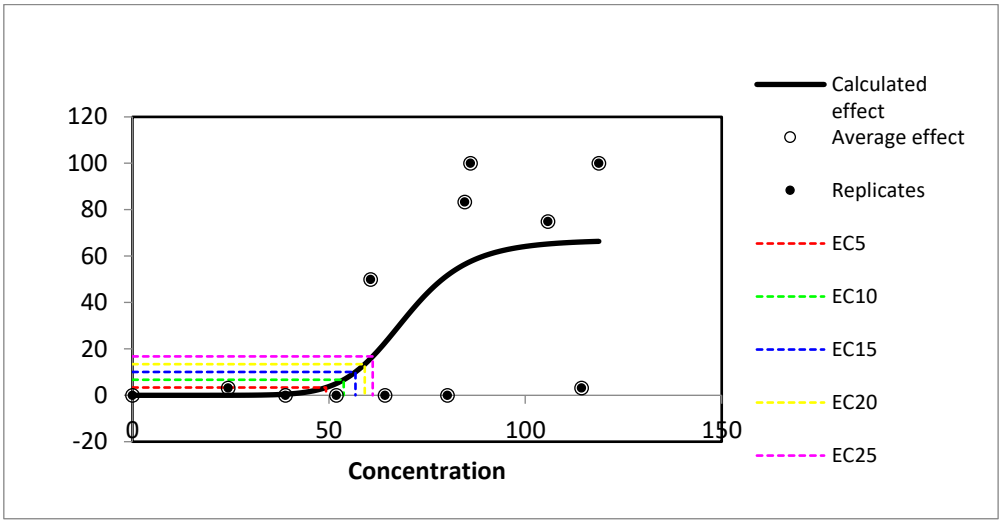


Figure 4: Example of Dose-Response data generated using the RegTox macro

Validity of the trial:

The test is considered valid if the following conditions are met for the growth control batches:

- The average cell density increased by a factor of 16 in 72 hours (i.e. an average specific growth rate of 0.9 d^{-1}).
- The coefficient of variation of the specific growth rates is less than 7%.
- The variation in pH was less than or equal to 1 unit.

iii. Crustacean *Artemia salina*

Artemia salina (class: *branchiopodes*, order: *anostracés*, familly: *artemiidae*) is a model organism in ecotoxicology (Sorgeloos et al 1978). The tests were carried out according to an adaptation of standard FD ISO 14669:2003-08: Determination of acute lethal toxicity to marine copepods. The standard provides for tests on a model organism, Acartia tonsa, which requires populations to be maintained generation after generation, making it a more difficult study model to use in toxicological assessments. The test organisms are presented in the form of dehydrated cysts (supplier Philanima, manufacturer JBL) whose hatching rate, which must be greater than 80%, is determined before the start of each series of experiments in order to check the quality of the biological material.

The trial consists of three stages: hatching, exposure, and evaluation.

Cyst hatching:

Dehydrated *A. salina* cysts are rehydrated by immersion in artificial seawater produced from a pre-constituted mixture (InstantOcean, Aquarium Systems) at a salinity equal to 33 PSU. Oxygenation of the medium is provided by a mini-pump connected to a capillary immersed in the pre-culture container

(beaker with a capacity of 1L), thereby homogenising the medium. The cysts hatch within 72 hours under the following physico-chemical conditions: temperature equal to 20°C ± 2°C, pH equal to 8.0 ± 0.3, dissolved oxygen concentration greater than or equal to 80%, salinity equal to 33 ± 3 PSU, 16H/8H day/night cycle). As hatching usually takes place from 48H onwards under the specified conditions, the tests are carried out on larvae or nauplii at the Instar I stage, so that the difference in age between the oldest and youngest nauplii does not exceed 6H.

Cyst exposition:

Exposures are carried out in 12-well multiwell plates (Cellstar® Greiner Bio-one, Cells in suspension, capacity of 4mL per well). The first step is to expose the undiluted test solutions. Each test solution tested, including the control batch, is inoculated onto a multi-well plate, giving a total of 12 wells and around 60 to 70 exposed individuals.

The control batch represents the exposure of nauplii to artificial seawater prepared according to ISO 10253:2006 §5.3 Seawater (Table 3). This is the reference medium for the assessment of mortality and morbidity presented in step 3 "Assessment of mortality and behaviour".

The test batches represent *A. salina* populations exposed to undiluted test solutions prepared in synthetic reference seawater equivalent to that used for the control batches.

A toxic reference batch, copper sulphate ($CuSO_4.5H_2O$), was also added to the study in order to determine the sensitivity of the nauplii to a substance for which the concentration causing 50% mortality of the exposed population (LC_{50}) was determined beforehand, compared with the literature and then used in subsequent tests.

Each of the test wells must contain 5 to 7 nauplii with a minimum volume per individual greater than or equal to 0.5mL/nauplii. As the working volume in the wells is 4mL, it is imperative not to exceed a total of 8 individuals per well.

NB: observation by counting dead/live/morbid individuals is made difficult when the number of nauplii exceeds 7 individuals.

Exposure was carried out by adding a volume of 100 µL containing the required number of *A. salina* to each well containing 4 mL of synthetic seawater (control batch well), 4 mL of the test solution (test batch wells) and 4 mL of the reference toxic solution $CuSO_4$ at the different concentrations evaluated.

The nauplii were sampled from the initial culture medium using a pipette with a tip diameter compatible with the size of the nauplii so as not to damage them, which would affect the results obtained. The volume of 100 µL taken brings the final volume in each well to 4.1 mL, resulting in a dilution of 0.025. In the case of the reference toxic batches, the actual exposure concentration was recalculated using this dilution factor.

The exposure time was 48 hours, allowing the larvae to use their vitelline reserves as an energy source, limiting the need to feed them. The plates were kept in a thermostatic chamber at 20 ± 2°C with a day/night photoperiod of 16H/8H.

A simplified representation of the trial is shown in Figure 6.

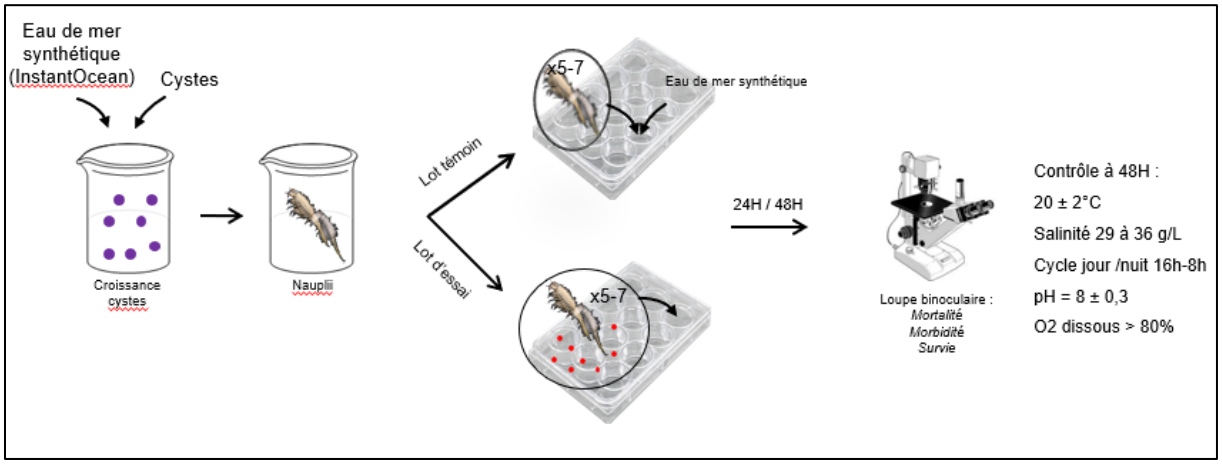


Figure 5: Simplified diagram of the toxicity assessment test on the Artemia salina model

Mortality and behaviour assessment:

Observation with a binocular magnifying glass was carried out after 24 and 48 hours of exposure and the behaviour of the larvae reported as follows:

- **Healthy** : the nauplius behaves normally
- **Morbid** : the nauplius has abnormal behaviour ranging from reduced swimming ability to a moribund state
- **Dead** : absence of movement of the nauplius for at least 20 seconds

In order to preserve the optimum quality of the incubation medium and reduce the risk of metabolic waste, nauplii counted dead after 24 hours were removed.

The mortality rate for each test batch is determined by the ratio of the number of dead individuals to the sum of all observed states (alive, dead, morbid).

In the same way as for the tests on the microalga *P. tricornutum*, the raw data are entered into RegTox in order to obtain the dose-response model and, if feasible, the parameters. EC_{10}, EC_{50} et EC_{90}.

Assay validity:

The test is considered valid when the mortality rate in the control batch is less than 10%. If this is not the case, the test must be discarded, and a new iteration carried out.

The pH, dissolved oxygen and salinity parameters are monitored at the start and end of the test.

iv. Sea urchin larva *Paracentrotus lividus*

The use of sea urchins is of particular interest in ecotoxicology, due to their high sensitivity in the early stages of their development. As a result, and thanks to our expertise in echiniculture, which enables us to obtain mature broodstock throughout the year, sea urchin larvae are used according to a standardised method (S. Pétinay et al., C. R. Biologies 332 (2009)) to assess the quality of seawater, or to determine the impact of certain products on the survival and development of these larvae.

Biological materials:

The Paracentrotus Lividus sea urchin larvae used come from broodstock reared at SMEL (Synergie Mer et Littoral) in Blainville-sur-Mer (50, Manche). They are reared in semi-closed circuits in tanks with water temperature controlled at 18°C. They are fed with Laminaria Digitata until they reach a sufficient size of around 40mm, i.e. around two years. The broodstock then undergo a maturation cycle lasting around four months, as follows:

- A fasting phase at 18°C with a short-day photoperiod (8 a.m. to 4 p.m.), which enables the gonads to be emptied through weight loss and thus homogenise the broodstock batch.
- A one-month re-feeding phase with maize and kelp. At the same time, the water temperature is gradually lowered to 8°C.
- This temperature is maintained for a month, and the broodstock are fed with Laminaria Digitata. At the same time, the photoperiod was gradually increased to a long day (16H/8H).
- Finally, the water temperature was gradually raised by 1°C every 48 hours, until it reached 18°C. These conditions were maintained for a month.
- Each day, the faeces are removed, and the water is renewed by 50%.

Gamete release is triggered by injecting 0.5 mL of potassium chloride (KCl) into the perioral membrane, the injection being repeated a maximum of three times. The gametes are released into reconstituted seawater, and fertilisation is achieved by adding sperm to the water containing the oocytes. Microscopic observation of the fertilised oocytes (100µL on a slide) is used to ensure that spawning has been successful. The fertilisation rate must be over 95%. For the experiment, around 300 fertilised oocytes are placed in each 5mL sample to be tested.

Observation method:

The samples were left for 72 hours in a room at 18°C to allow the larvae to develop to the pluteus stage. The larvae were not fed in order to limit the bias caused by the addition of external elements.

At the end of this incubation period, the larvae were fixed with formaldehyde (200µL for 5mL, i.e. a final concentration of 0.75%) to halt their development.

Out of one hundred larvae observed per sample, the following are assessed:

- The rate of undeveloped larvae (fertilised egg, blastula, gastrula).
- The rate of malformation (at the echinopluteus stage).

Fifteen larvae per sample are measured:

- Spicule lengths (± IC_{95})

The series of samples were validated by a negative control, in this case seawater (EDM) taken offshore from the Chausey Islands archipelago, in quintupla, and a positive copper control (10µgCu/L; 30µgCu/L; 50µgCu/L; 70µgCu/L; 90µgCu/L).

Measurements were taken using a microscope (x100), combined with software for automatic image acquisition (Zen).

Statistical analysis method:

The averages of the rates of non-development (%), malformation (%) and spicule length (µm) are calculated, along with the standard deviations and confidence intervals (IC_{95}). These averages, depending on the concentrations, are then used to determine the median lethal concentration (LC_{50}) at 24H and 72H, i.e. the concentration of a substance that causes 50% mortality in the population tested at 24H and 72H of exposure. The concentration of the commercial product in the seawater used will be used here. To determine the significant differences between the biocides tested, the rates of non-development, malformation and spicule length were analysed using an ANOVA and a post-hoc test (Student-Newman-Keuls test, α=0.05), using StatView software.

Validity of the copper-positive control:

The sensitivity of larvae is validated by exposure to copper. ($CuSO_4.5H_2O$) according to a concentration range consistent with the methodology presented in the literature (S. Pétinay et al., (2009), p.5). Concentrations are expressed as Cu^{2+} equivalent per unit volume (concentration in µg $_{Cu2+}$/L $_{ASW}$).

III. RESULTS : BIODEGRADABILITY STUDY

a. Physico-chemical and biological parameters

The pH, salinity and temperature of the seawater sampled are reported in Table 4 for the control batches.

Parameters	Start of trial	End of trial
pH (lab measurment)	8,09	7,99
Temperature (lab measurment)	13,6*	21,5
Salinity (lab measurment)	32,1	32,6
Micro-organisms density (cultivables)	$3,2.10^3$	NC

Table 4: Physico-chemical and biological parameters

(*) temperature of natural seawater after sampling and before stabilisation in the thermostated water bath.

As measurements are still in progress for some of the samples, the measurement taken at the end of the trial corresponds to the measurement taken in March 2023 on the control batch. There was a slight acidification of the control medium at the end of the experiment.

b. Cinetics of biodegradability in the marine environment

Three of the materials tested were selected on the basis of multiple criteria (biodegradability, physico-chemical properties, mechanical properties, durability, etc.): INd_AH(V), INd_Y and INd_AC(M). The biodegradation kinetics are shown in Figure 8. Biodegradation of samples AC(M) and AH(V) begins after immersion for 2 and 3 months respectively, compared with cellulose, for which biodegradation begins very quickly. AC(M) reaches a plateau of complete biodegradation (≥100%) after 6 months of immersion, while AH(V) reaches a plateau of around 30% after 5 months of immersion. The plateau continues for the duration of the experiment. The INd_Y sample showed constant biodegradability over a period of 6 months before the appearance of a decrease in the rate, which could indicate a form of ecotoxicity. It is necessary to compare this observation with the toxicological study, as various phenomena could explain this behaviour, in particular a fault in the electrical box supplying the pumps responsible for oxygenating the environment, or a fault in the pumps themselves. As the device is not connected to any data logger, it is not possible to provide any information on this type of fault.

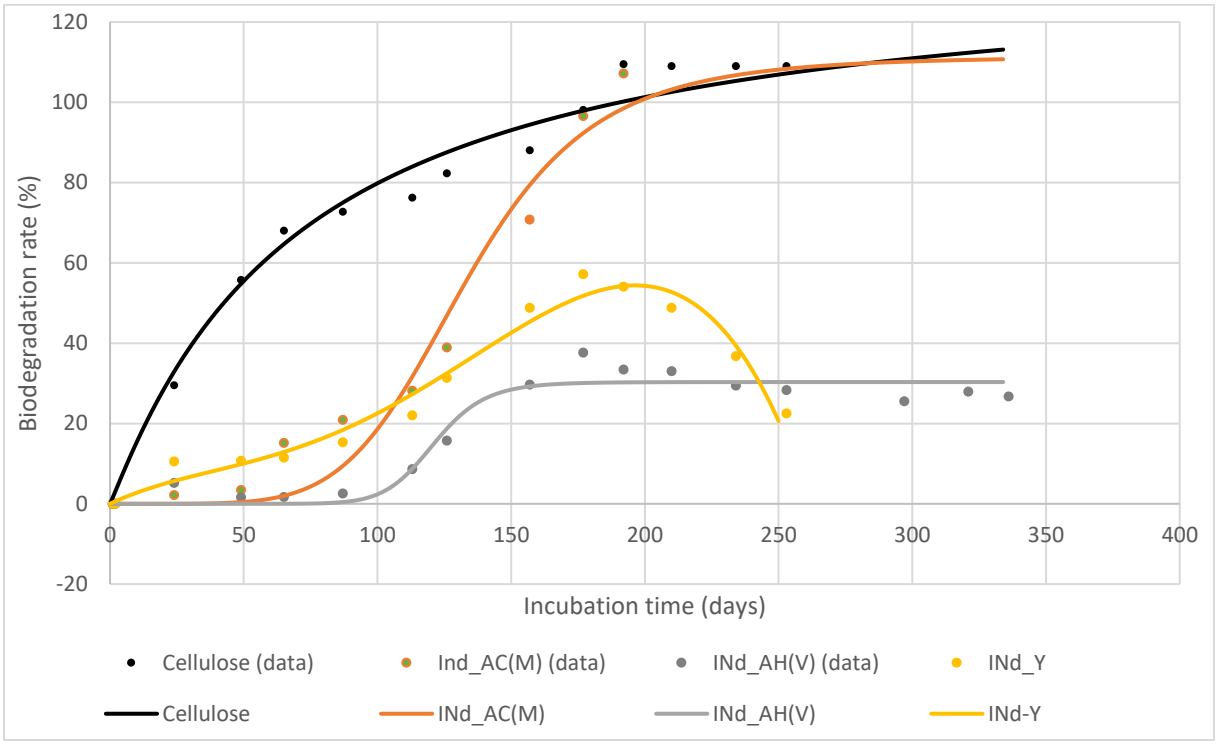


Figure 6: Biodegradation rates observed for samples selected in phase 2

IV. RESULTS: ECOTOXICOLOGICAL STUDY

For the toxicological tests on the *P. tricornutum* and *A. salina* models, the samples were renamed as follows (Table 5):

CODIFICATION SAMPLE	CODIFICATION TRIAL	MEDIA
Mono 1 AC PE Powder aged in air	MONO AC	Air
Mono 1 AC PE Powder aged in seawater		Seawater
Mono 1 AC PE Powder control		Control
Mono 2 AH VF Powder aged in air	MONO AH	Air
Mono 2 AH VF Powder aged in seawater		Seawater
Mono 2 AH VF (PE x1.5) control		Control
Multi bobine 2 aged in air	MULTI	Air
Multi bobine 2 aged seawater		Seawater
Multi bobine 2 control		Control

Table 5: Correspondence between codes for samples received and samples tested

a. Microalgae *Phaeodactylum tricornutum*

i. Determination of the Fluorescence-cell density correlation equation

Successive dilutions from an aliquot of the mother culture allow fluorescence measurements to be linked to counts made on a counting cell (Malassez type). The affine line passing through 0 with equation $y = 119.32\,x$ (Figure 11) shows an excellent correlation with a coefficient of determination $R^2 = 0.9991$. The advantage of establishing such a correlation lies in the speed with which cell densities can be read with random control on the counting cell to confirm the validity of the measurements obtained by fluorescence.

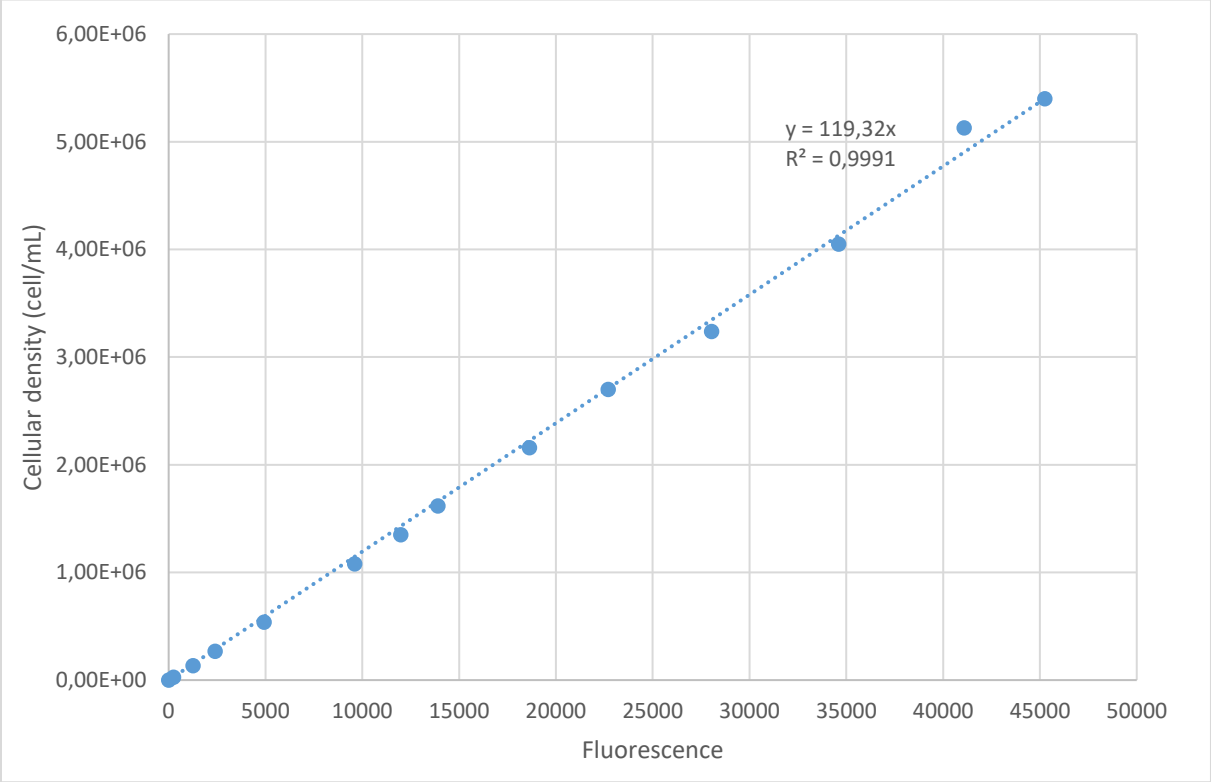


Figure 7: Correlation equation between fluorescence and microalgal cell density

ii. Growth inhibition observed after exposure to study materials

The ISO 10253: 2006 standard, Seaweed Growth Inhibition Test, indicates an IC_{50} (concentration resulting in 50% inhibition of algal growth) for the reference toxicant $K_2Cr_2O_7$ of 20.1 ± 5.3 mg/L with a coefficient of variation equal to 26%. The concentration of $K_2Cr_2O_7$ in the test was chosen based on these data and set at 20 mg/L. Figure 12 shows that exposure of *P. tricornutum* to potassium dichromate resulted in an average inhibitory response of between 45% and 65%, thus validating the sensitivity of the model in the trial.

All the inhibitory responses recorded for the control materials and the two ageing conditions show a toxicity below the IC_{10} for an exposure concentration of around 150 mg/L. In some cases, a response favouring algal growth was recorded (MONO_AC_Air, MONO_AC_Seawater, and MULTI_Control and MULTI_Seawater).

A Student's t-test on all the data showed on the one hand:

- A significantly different growth inhibition compared with the growth control batch (indicated by * in Figure 12)

- A significant difference in the growth inhibition of the aged materials compared with their control (indicated by ** in Figure 12)

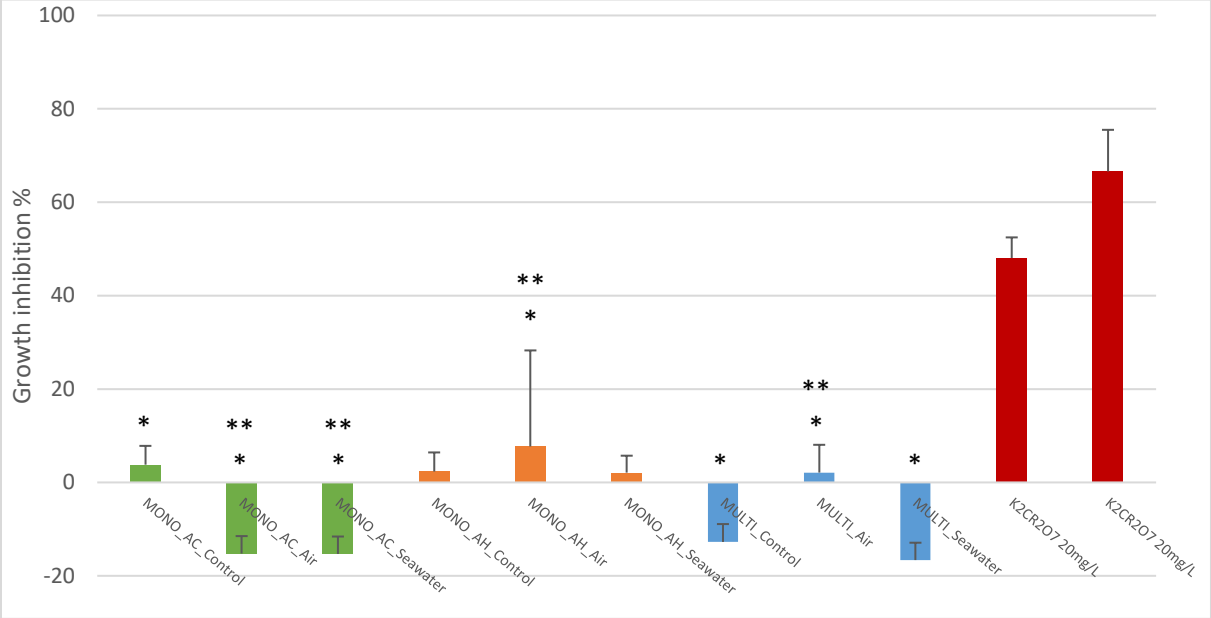


Figure 8: Inhibition of algal growth at 72H depending on the material tested at a concentration of 150 mg/L

New tests are currently being carried out on the MONO_AH_Air sample, which shows a wide range of values. The insolubility of the material in the incubation medium could be the cause of the discrepancies observed.

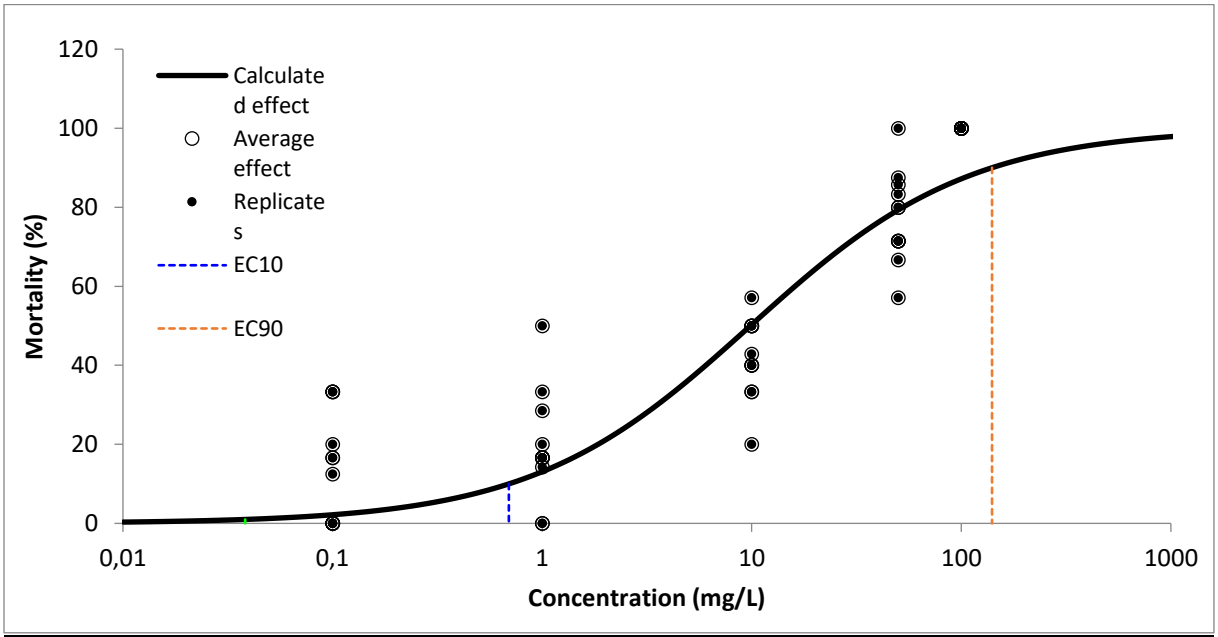
b. Crustacean *Artemia salina*

Data on the sensitivity of the biological model are presented in Figure 13 and show the response of specimens when exposed to the reference toxic substance: copper sulphate ($CuSO_4$).

The percentages of mortality after 48 hours for the different exposure media are shown in Figure 14. Very low mortality was observed in crustaceans exposed to a nominal concentration of 150 mg particle/L, with behaviour similar to that observed in the control. Since the validity criteria were met (Table 6), the test is considered valid. In addition, exposure to a concentration of 10 mg/L of copper resulted in a response equal to approximately 44% (close to the theoretical response of 50% - $LC_{50\text{-}48H}$).

CRITERIA	VALIDITY	T 0	T 48H
pH	= 8,0 ± 0,3	8,00	8,01
O_2 dissolved	> 4 mg/L	> 4 mg/L	> 4 mg/L
% control mortality	< 10%	-	2,25

Table 6: Critères de validités de l'essai



$CL_{10\text{-}48H}$	$CL_{50\text{-}48H}$	$CL_{90\text{-}48H}$
0,69 mg Cu^{2+}/L	9,86 mg Cu^{2+}/L	140,16 mg Cu^{2+}/L

Figure 9: Dose-response curve for exposure of A. salina to copper sulphate after 48 hours

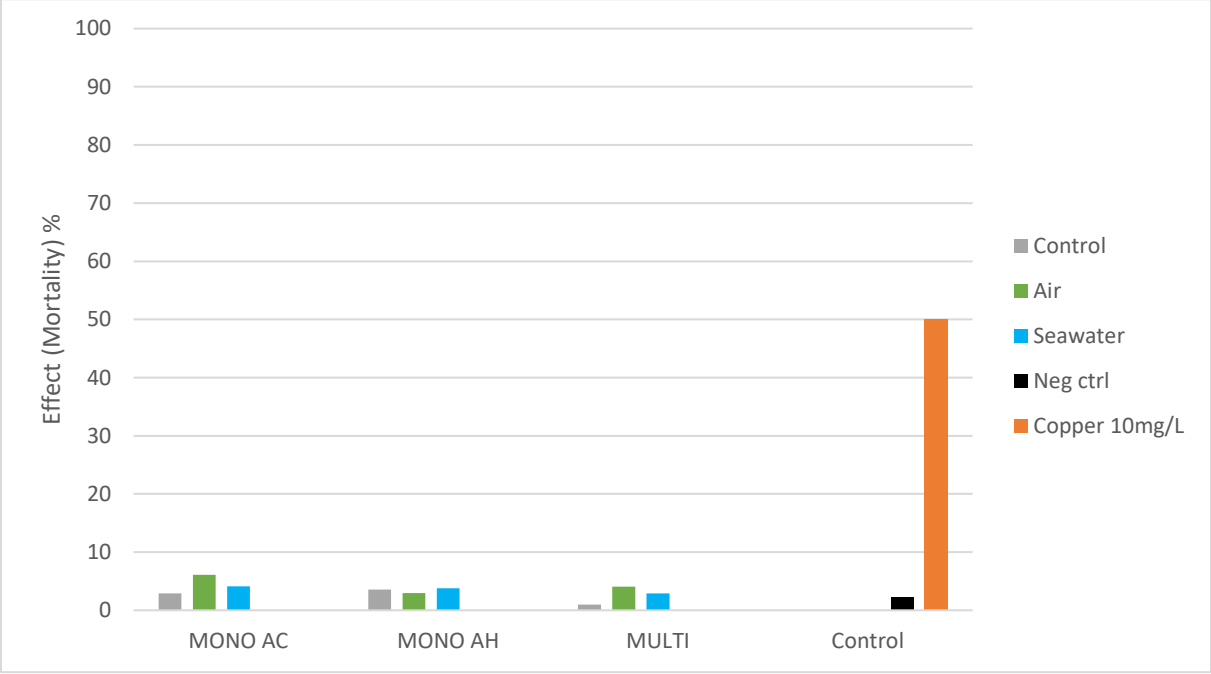


Figure 10: Mortality observed in A. salina nauplii after 48H exposure to environments contaminated by particles from the materials tested

There does not appear to be any toxic effect of the particles tested on the copepod *A. salina*.

Based on the data obtained, it does not seem appropriate to carry out a dilution range of the samples tested, as the responses recorded for a concentration of 150 mg/L are below the $LC_{10\text{-}48H}$.

c. Sea urchin larva *Paracentrotus lividus*

i. Sensitivity to the reference toxicant

The results of tests carried out on the reference substance copper sulphate ($CuSO_4$) are presented below and segmented according to the observation criteria considered:

Percentage of undeveloped larvae (Figure 11)

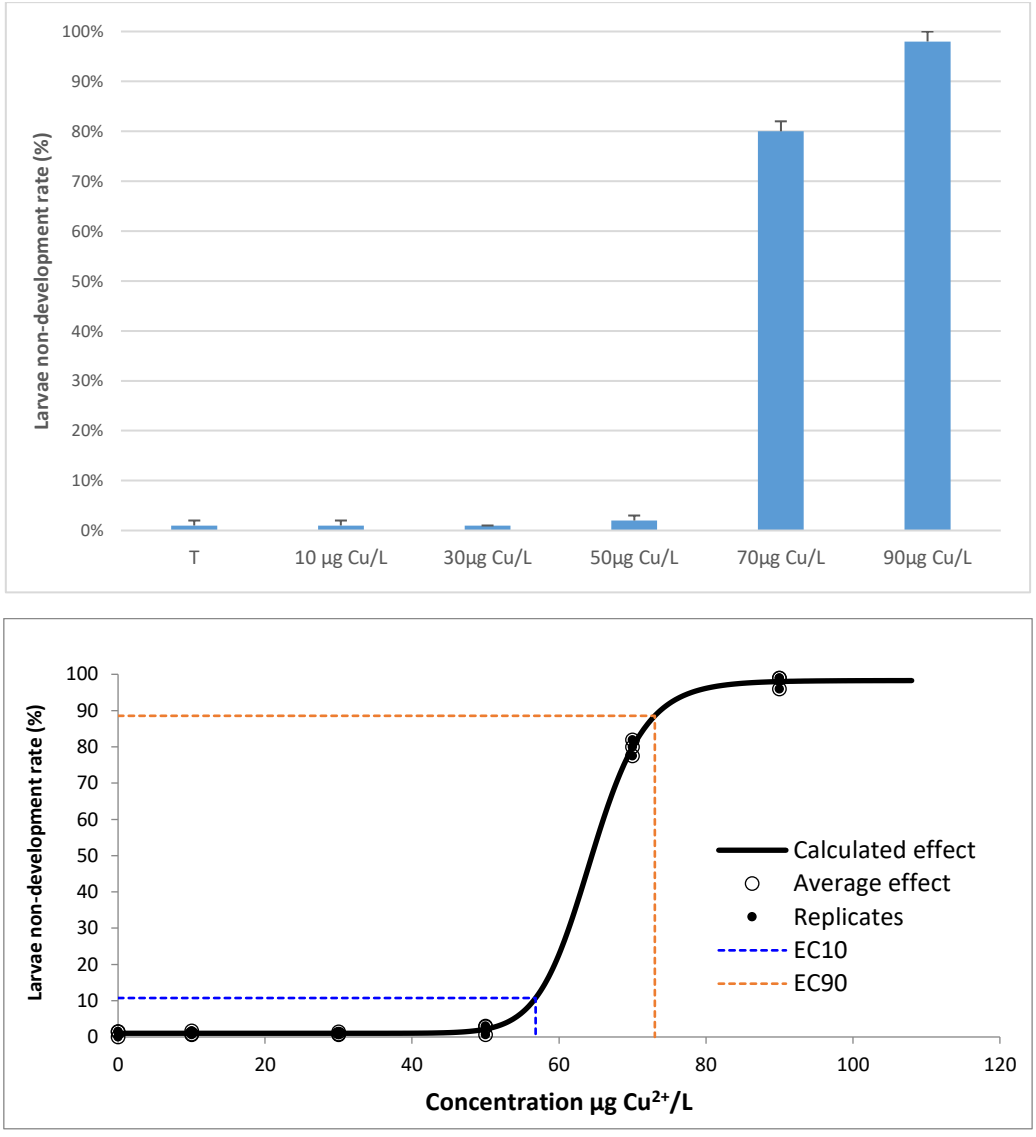


Figure 11 - Rate of larval non-development in the copper range

The product is lethal at concentrations above 70 µg Cu^{2+}/L. Lethal concentration at 24H ($LC_{50\text{-}24h}$) is estimated by Hill modelling at 64,4 µg Cu^{2+}/L. Parameters of LC_{10} and LC_{90} are valued at 56,82 µg Cu^{2+}/L and 73,04 µg Cu^{2+}/L.

Percentage of undeveloped and malformed larvae (Figure 12):

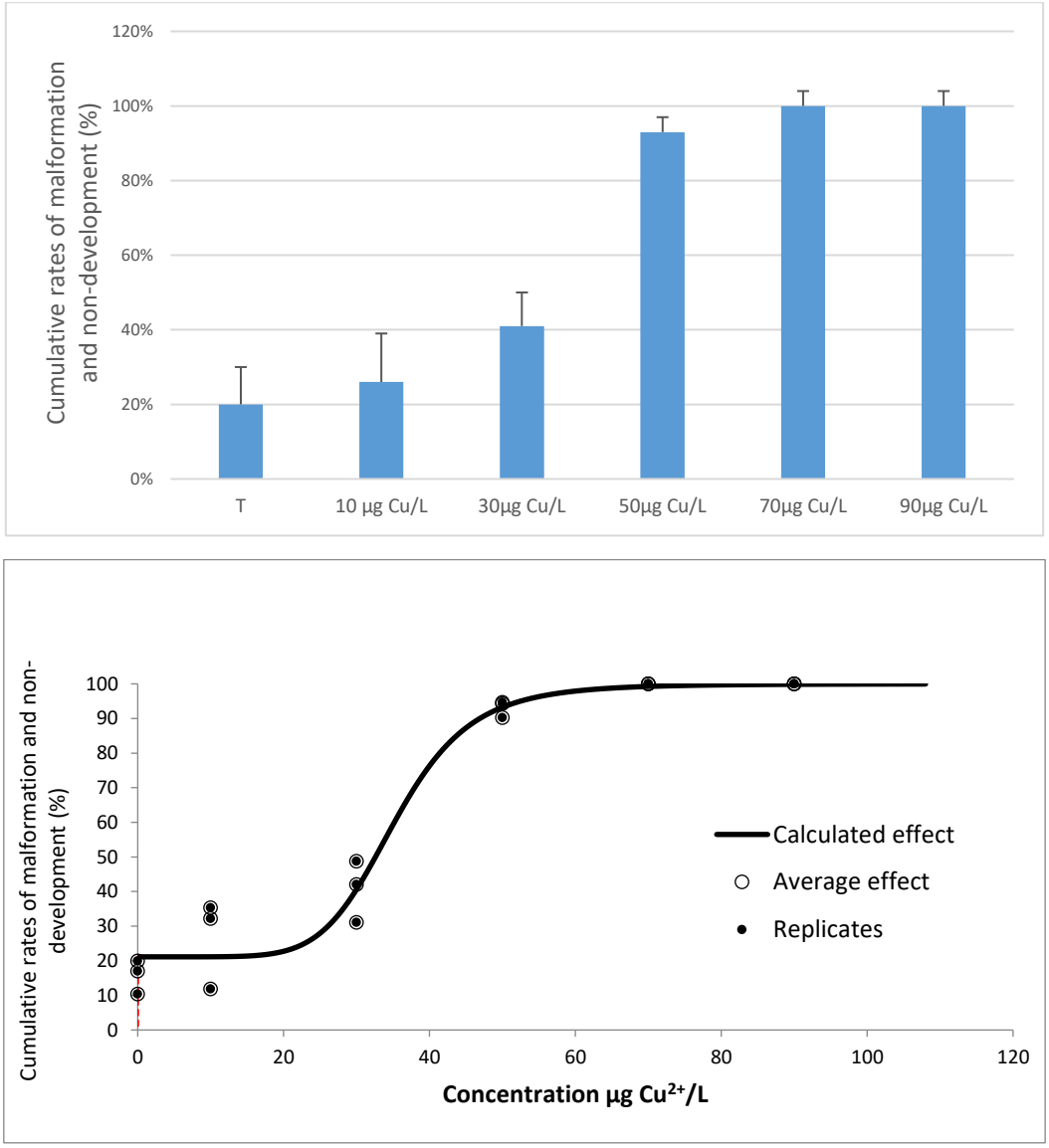


Figure 12 - Rate of non-development and malformation in the copper range

The product is lethal at concentrations equal to or greater than 70 µg Cu^{2+}/L. The $LC_{50\text{-}72H}$ was estimated by Hill modelling to be 35.32 µg Cu^{2+}/L. Based on the measurements taken, the cumulative percentages of malformation and non-development of larvae in the control were 15% ± 5.5%, remaining below 20%. However, this does not allow the LC_{10} parameter to be determined in the study. The LC_{90} for the cumulative observation of malformations and non-development of larvae was evaluated at 48.77 µg Cu^{2+}/L.

Validity of the copper-positive control:

Malformations at a rate significantly different from the control were observed in larvae from 30 µg Cu^{2+}/L (40%). At concentrations equal to and greater than 50 µg Cu^{2+}/L, the cumulative rates of non-development and malformations exceeded 90% and were significantly different from the seawater control ($p < 0.05$). Similar effects were observed in the copper toxicity assessment phase of the standardisation method used (S. Pétinay et al., (2009), p.5). These various elements validate the positive control of this bioassay and attest to the sensitivity of the P. lividus model during the study.

ii. Results obtained for aged samples

In the same way as the tests carried out on *A. salina* and *P. tricornutum*, the samples were previously aged and compared with their non-aged counterparts to differentiate them with respect to the toxicity that would be linked to the release of molecules by the material studied.

The sample codes for the toxicity tests on sea urchin larvae are given in Table 5.

Percentage of undeveloped larva (Figure 13)*:*

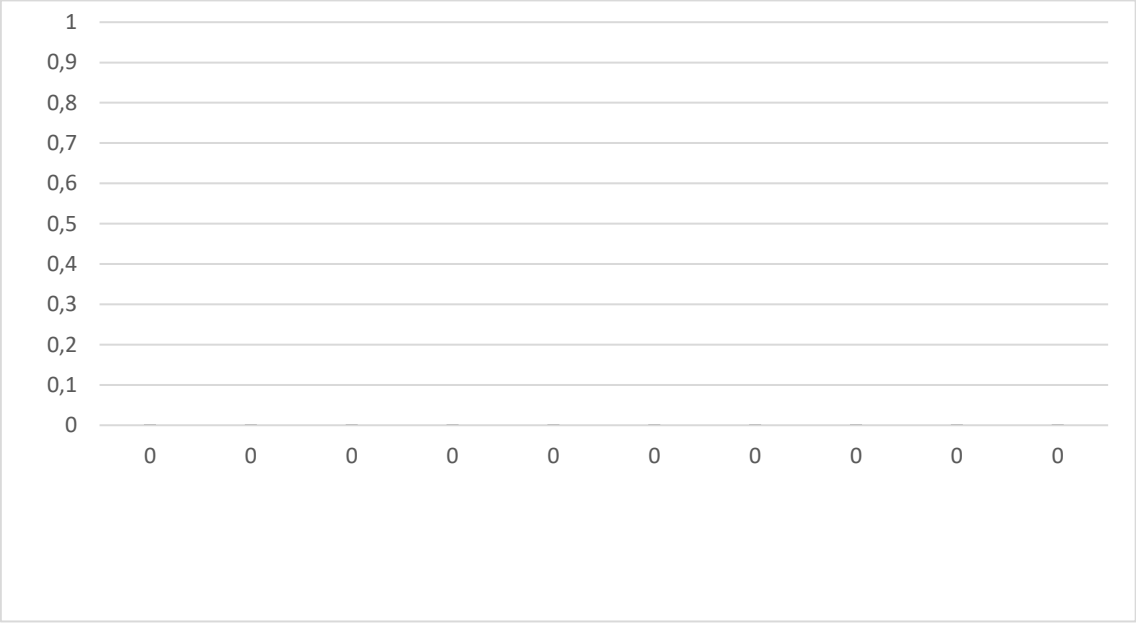


Figure 13 - Rate of non-development as a function of the sample tested

All the samples tested had a non-development rate of less than 15%, meaning that almost all the larvae had developed beyond 24 hours. The non-development rate for the control batch was 0.33%.

Percentage of undeveloped and malformed larvae *(*Figure 14*)*

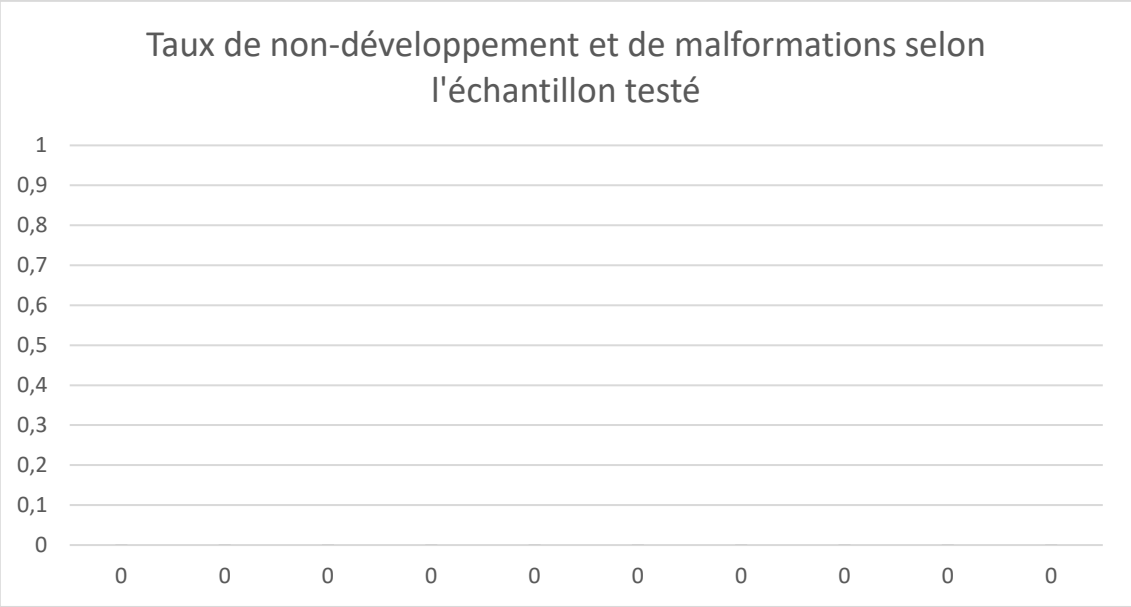


Figure 14 - Rate of non-development and malformations as a function of the sample tested

Samples Air/Seawater for MONO_AC et MONO_AH and Control/Air for MULTI, showed cumulative rates of non-development and malformations greater than 50% and were significantly different from the control. Control samples from MONO_AC and MONO_AH as well as MULTI_Seawater show cumulative rates of non-development and malformations of less than 25% and are not significantly different from the control.

Toxicity gradient and bio-test conclusion:

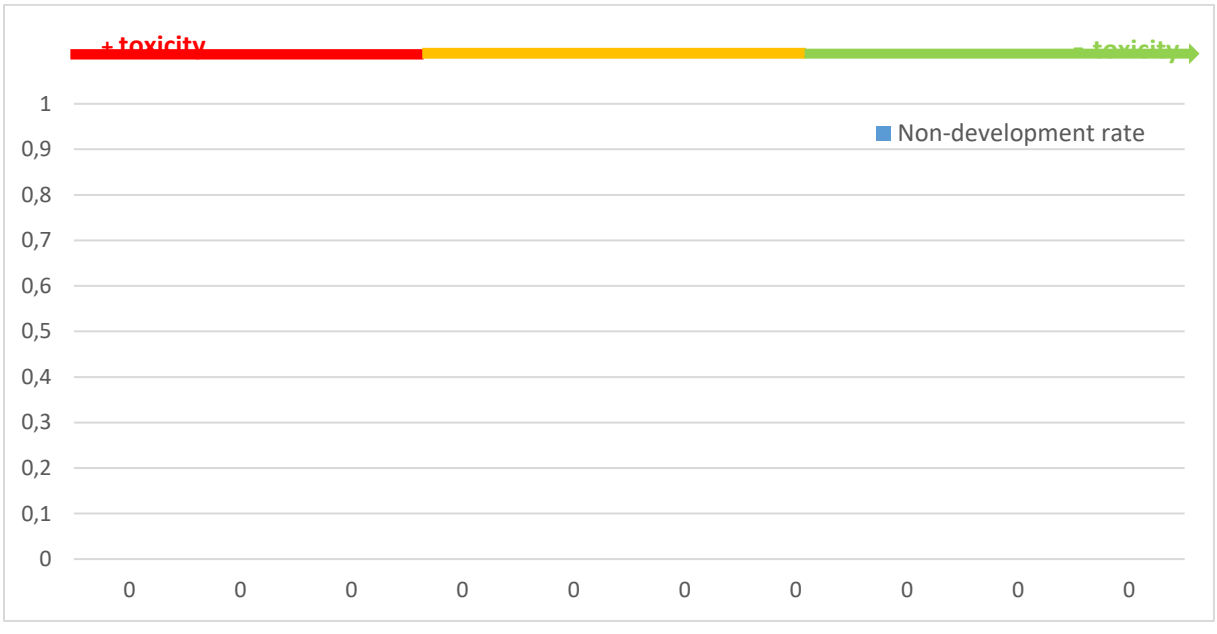


Figure 15 - Toxicity gradient for the nine samples tested

The rate of non-development of the larvae is the most important criterion for establishing a toxicity gradient for the biocides tested (Figure 19). Larvae that remain in the fertilised egg, blastula or gastrula stage are dead before 24 hours (Falugi and Angelini, 2002). According to this indication, the higher the average non-development rate, the more toxic the sample is for the larvae.

The non-development rate added to the malformation rate is the second important criterion for refining the gradient. Larvae with malformations are larvae with absent or incomplete spicules or crossed or spread spicules. (Carballeira et al., 2012).

Based on these indicators, it can be determined that samples 6 (Mono 1 AC PE Powder aged in seawater - 630mg) and 1 (Mono 1 AC PE Powder aged in air - 750mg) have the greatest impact on the larvae, with rates of non-development and malformations of over 80%. Samples 4 (Mono 2 AH VF Powder aged in seawater - 700mg), 5 (Mono 2 AH VF (PE x1.5) control powder - 350mg), 2 (Mono 1 AC PE Powder control 100m - 750mg) and 7 (Multi bobine 2 aged seawater - 600mg) are at an intermediate level but still between 60% and 70%. Finally, samples 9 (Multi bobine 2 control powder - 320mg), 8 (Multi bobine 2 aged in air powder - 520mg) and 3 (Mono 2 AH VF Powder aged in air - 760mg) are the least harmful for the larvae, each presenting cumulative rates of non-development and malformations of less than 25%.

However, these results should be interpreted with caution. The samples were tested in powder form, and the water was not filtered before coming into contact with the larvae. The debris created by the powders in suspension may therefore be the cause of the malformations observed, affecting the larvae more than the chemical molecules from the samples. If this experiment were to be repeated, it would be wise to filter the water before bringing it into contact with the larvae, to be able to validate or refute this hypothesis.

V. DISCUSSIONS & OUTLOOKS

The study of the biodegradability of the different formulations showed different behaviours, ranging from complete ultimate aerobic biodegradation in 6 months (MONO_1_AC) to the appearance of threshold plateaus at 40%-60% biodegradability (MONO_2_AH, MULTI_2). However, this type of experiment is carried out over a period of 2 years, which is difficult to reconcile with the duration of collaborative projects, particularly the Interreg INdIGO project (46 months), for which there were several delays due to the health crisis. The results presented to date can therefore under no circumstances be extrapolated to predict biodegradation over a period longer than that of the trial. In addition, the current specification standards, which set the thresholds to be reached to qualify the biodegradability of a material, are not suitable for studying materials whose expected lifetime exceeds the maximum duration of exposure to the test environment. For example, the production of a biodegradable fishing net with a life expectancy of more than 2 years would not be able to validate the thresholds indicated by the standards. larvae. The debris created by the powders in suspension may therefore be the cause of the malformations observed, affecting the larvae more than the chemical molecules from the samples. If this experiment were to be repeated, it would be wise to filter the water before bringing it into contact with the larvae, to be able to validate or refute this hypothesis.

In the case of the INdIGO project, two different types of gear were tested: a mono-filament fine net and a multi-filament catline net.

In the first case, the final prototype had to retain satisfactory mechanical properties to meet the needs of users and remain as fishable as the traditional alternatives. This constraint alone means that the net must disintegrate slowly, and biodegradability kinetics must be controlled.
In the second case, it is necessary for the net to disintegrate completely after 6 months to remove a stage of interaction between the user and the net and to enhance the value of this biodegradable alternative compared with its traditional counterpart.
The study of biodegradability makes it possible to determine the behaviour of the microparticles produced during the material's degradation stages. It is essential to distinguish between these two stages, which are concomitant and depend on parameters such as temperature, the density of micro-organisms and the granulometry of the exposed material....
The biodegradability data obtained to date is encouraging and shows that the formulations produced can meet the requirements set out in the specifications. However, the harmlessness of the materials tested on model organisms from the marine environment still needs to be validated.

The toxicological data obtained showed that the three formulations studied were safe. The compounds used in the composition of the different formulations were chosen for their qualities, such as food contact certification. When carrying out a complete ecotoxicological study, it is important to consider the notion of risk, which depends on the evaluation of multiple criteria such as:

- The concentration of chemical substance(s) in the marine environment (in water, sediments, or the tissues of marine organisms). This key element also implies the ability to quantify these elements.
- Acute toxicity, which measures the immediate effects on marine organisms during short but intense exposure. Acute toxicity tests generally assess mortality and sublethal effects.
- Chronic toxicity measures the long-term effects of a chemical substance (or cocktail of substances) on marine organisms. Chronic toxicity tests assess the effects on reproduction, growth and development of organisms exposed over a prolonged period.
- Persistence in the marine environment gives an indication of its lifespan. Persistent substances can accumulate in marine ecosystems and have long-term effects.
- Bioaccumulation, which assesses the capacity of a chemical substance to accumulate in the tissues of living organisms over time. Substances that accumulate can reach toxic concentrations in marine organisms and disrupt biological functions.
- The transport and dispersion of substances in the marine environment to understand their spread and potential exposure to organisms. This includes the study of ocean currents, tides, surface currents and mixing processes.
- Effects on ecosystems that go beyond the organism alone and consider the effects on marine ecosystems as a whole. This includes the assessment of ecological interactions, biodiversity, ecosystem functions and resilience.

The tests carried out in the INdIGO project focus on acute and semi-chronic toxic effects. In the case of soluble chemical substances, it is easy to assess toxicity in the various compartments of the marine environment. On the other hand, in the case of plastic polymer materials, it becomes difficult to carry out this assessment due to the insoluble nature of the material, the leaching time of releasable substances that could present a toxicity and therefore the ageing period at which the tests must be carried out. If we take toxicological assessment in industrial composting as a reference, it is carried out on the compost that has been used to certify the disintegrating nature of the material, and therefore

after a certain degree of ageing of the material. However, no quantitative measurements are taken (data not required by current standards), so it is not possible to link toxicity to the physico-chemical state of the material at the time of testing. This is currently the main problem, as a material can be disintegrated without releasing sufficient substances into the test environment (notion of plastic debt Figure 20) to cause a toxicological response.

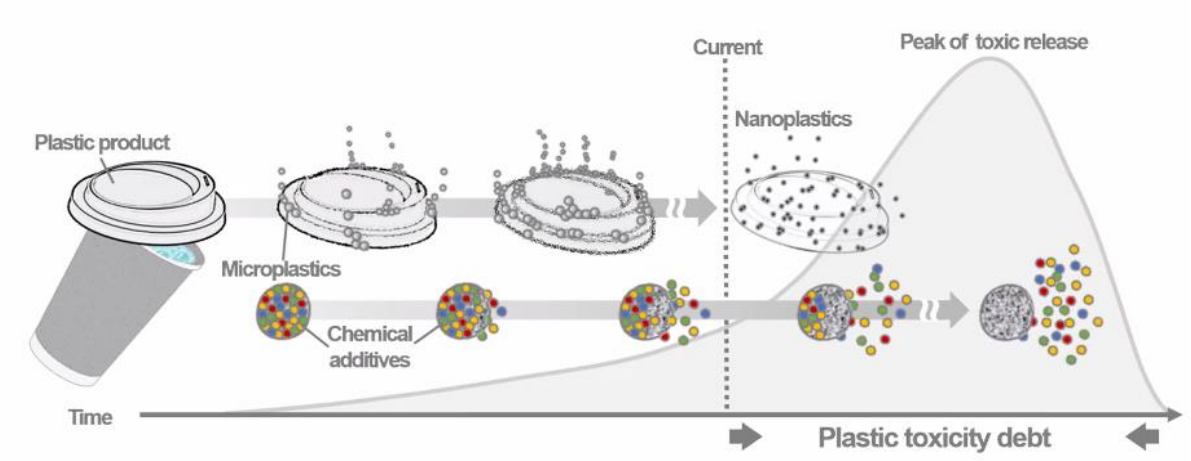


Figure 16: Toxicity debt (credit : Rillig et al. 2021, Env Sci Techn)

This notion of toxic debt is reflected in negative toxicological responses on trophic levels such as crustaceans and microalgae, for which the quantities of substances present in the environment would be well below the quantities required to observe a toxic response, and this is linked to the possibility that the microparticles produced during the degradation of the material have not reached the critical size stage inducing the release of potentially impacting substances. Finally, in the tests carried out, the aged material is non-soluble in seawater and the plastic particles visible to the naked eye have sedimented. This behaviour has an influence because the availability of the material tested is not optimal. Nevertheless, this initial toxicological approach remains promising, and it seems appropriate to continue the analyses by looking at the effects observed over the long term and, if possible, assessing the toxicological risk.

All these data show the importance of adapting scientific tools to the issue of the impact of plastic materials on the marine environment, and biodegradable plastics in particular. To date, the tools used to obtain the information needed to draw up a Life Cycle Assessment (LCA) have a very poor understanding of toxicological indicators and do not allow biodegradable solutions to be evaluated when considering Global Warming factors linked to the quantities of CO_2 emitted. Since the biodegradation process emits CO_2, among other things, this represents a negative impact according to the criteria used for the LCA.