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Test guidelines for ecotoxicity and impact of biopolymers and biocomposites in marine biota

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Abstract

The dependence of fossil-based polymers in human society has led to a series of environmental issues, such as accumulation of plastic debris in the oceans. In marine environments, due to UV irradiation and other physicochemical stresses, plastic debris can break down into microplastic, as well as leaching chemicals to aquatic environments. Concerns on the persistence and potential negative impacts to marine organisms of microplastics and leachates, together with concerns over the carbon footprint of plastics, have increased the demand to create more sustainable alternatives such as biopolymers and biocomposites. These are composite materials made from a natural-sourced feedstock, with a potential lower environmental impact. However, the current knowledge on their degradation and ecotoxicological impacts in the marine environment remains limited, and there is a need of systematic standardised protocols for the impact assessment.

In this output 7.1 of the SeaBioComp project, clear test guidelines and a method are provided to assess the ecotoxicity and marine impacts of biopolymers and biocomposites by modifying and integrating current standard ecotoxicology assays (e.g. ISO, OECD) and reliable methods reported in peer-reviewed literature. In a first section, the assessment of microplastic formation under UV radiation is discussed. In the second section, we provide guidance on assessing the ecotoxicity of leachates from biocomposites. We anticipate these test guidelines will contribute to a sound assessment of the marine impacts of biopolymers and biocomposites one of the goals of the SeaBioComp project.

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List of abbreviation

ISO: International Organization for Standardization

OECD: Organisation for Economic Co-operation and Development

ASTM: American Society for Testing and Materials

MP: Microplastic

UV: Ultraviolet

µFTIR: Fourier Transform Infrared Spectrography

SEM: Scanning Electron Microscopy

QA/QC: Quality Assurance and Quality Control

EC50: Half Maximal Effective Concentration

EC10: 10% Maximal Effective Concentration

LOEC: Lowest Observed Effective Concentration

NOEC: No Observed Effect Concentration

1 Introduction

The use of fossil fuel-based polymers in human society has brought not only various benefits throughout their applications, but their omnipresence also led to a series of environmental issues, such as plastic debris accumulation in the environment. Once plastics items enter marine ecosystems they undergo a series of changes in their surface, and break down to smaller sizes due to the environmental conditions, such as exposure to Ultraviolet (UV) radiation (Jahnke et al., 2017). Microplastic (MP), i.e. plastic particles smaller than 5 mm in diameter, are ubiquitous in the global ocean and potentially cause harmful effects on marine organisms and potentially in human health (SAPEA, 2019). The leaching substances from plastic items, i.e. additives and plastic monomers, are released from the polymer matrix to the marine environment, and can lead to further chemical-led, rather than particle induced, toxicological effects in organisms (e.g. Bejgarn et al., 2015; Capolupo et al., 2020). There is consensus that society needs to improve the recycling procedures and waste management, but also decrease the dependence on fossil fuel-based polymer products (Whitacre, 2014). Polymers made from a natural-sourced feedstock, like polylactic acid (PLA) and thermoplastic starch (TPS), known as biopolymers, are seen as potential sustainable alternatives, with a lower carbon and environmental footprint. However, our knowledge remains limited on the biopolymer's degradation and ecotoxicological impacts in the marine environment.

Standardised ecotoxicity tests provide a great tool to evaluate a compound's hazard potential. Test species for these experiments are usually selected from a set of model species which are meant to be representative of ecosystem functional species. Despite various standardised assays available, there is the need to modify and integrate these assays into systemic guidelines considering the varied nature of MP particles (wide range of sizes, polymers, shapes) and leachates (wide variety of chemicals). For fossil-based polymers, a first effort has been made in the guidelines of OECD 317 (OECD, 2020), where systemic guidance has been given on assessing the aquatic and sediment ecotoxicity of small-sized materials (i.e. nanomaterials). With respect to biocomposites, however, such standardised test guidelines are still lacking.

2 Scope

This document provides guidance for assessing ecotoxicity and marine impact of biopolymers and biocomposites. Biopolymers and biocomposites are polymers and composites produced from biological products such as corn or sugar cane. Typical examples of biopolymers and biocomposites include polylactic acid (PLA) and thermoplastic starch (TPS). To enable a fair environment assessment of the biocomposites, a comparison between the impact of the biocomposites and fossil-based

polymers has to be made. The results of the fossil-based polymer can then be used as benchmarking indicator. However, note that the selection of reference fossil-based polymer is beyond the scope of this document.

In the first section, this document focuses on assessing the microplastic formation of biocomposites and the reference fossil-based polymer during their photo-degradation. Test specimens will be exposed to seawater and UV irradiation. Other stresses (e.g. mechanical forces by waves and sediments) in the marine environment may also lead to the release of microplastics, however, the latter is beyond the scope of these guidelines.

In the second section, the ecotoxicity of leachates from biocomposites and the reference fossil-based polymer will be assessed. The leachate from biocomposites and reference fossil-based polymer will be extracted in saltwater medium. If the actual study is interested in the monomers and oligomers leached due to photo-degradation, the biocomposites and the reference fossil-based polymer should be subjected to UV radiation prior to leachate extraction. Once leachate solutions obtained, their ecotoxicological impacts on marine organisms will be assessed using standardised aquatic ecotoxicity assays. Sediment assays are also important, but out of the scope of these guidelines. Meanwhile, chemical analysis will be performed to identify and quantify leached substances. Mainly organic compounds are focused, inorganic compounds may be detected but beyond the scope.

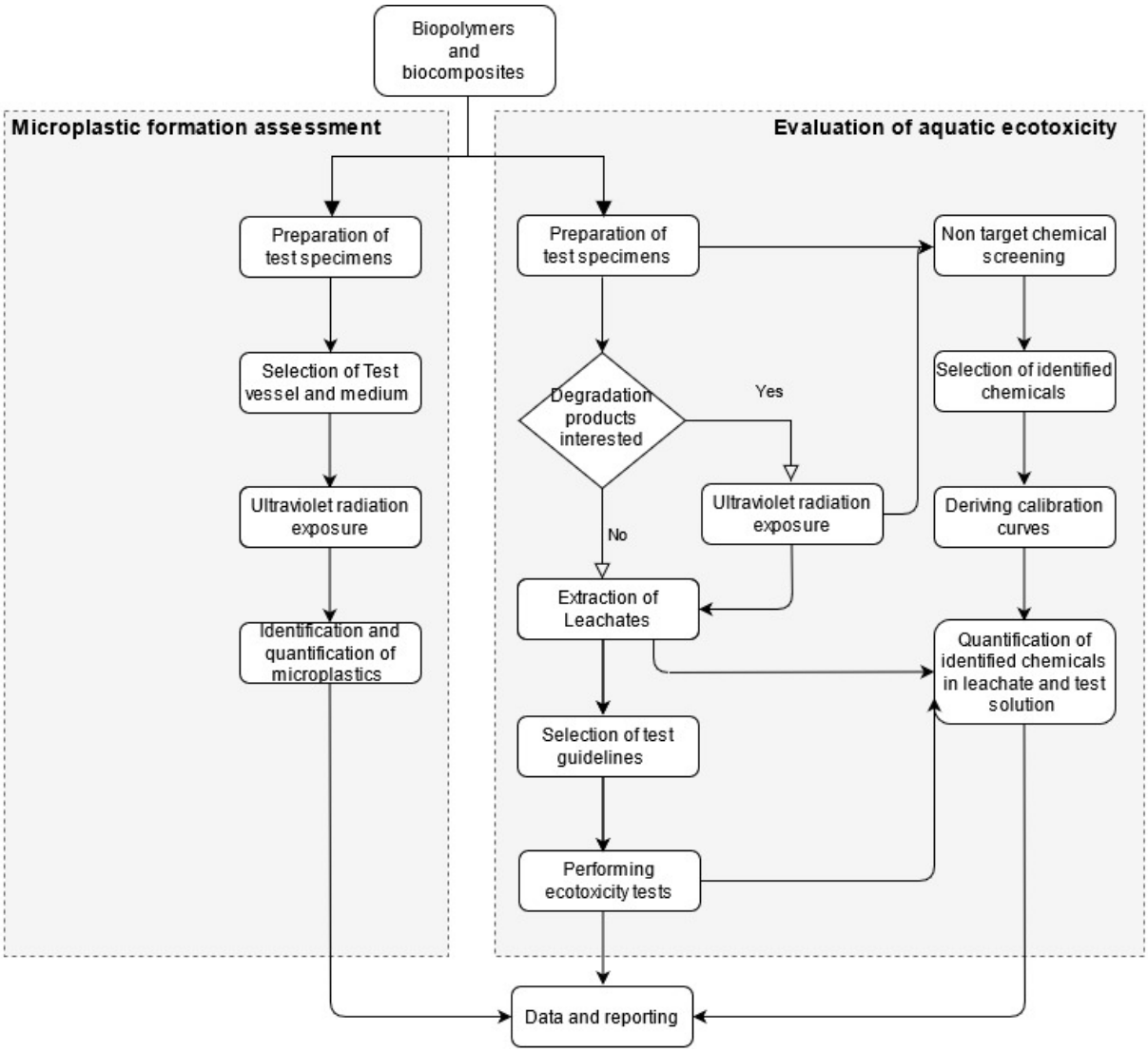

Figure 1. An overall flowchart of the procedures included in these guidelines.

3 Methodology

3.1 Evaluation of Microplastic formation

3.1.1 Principle of the test

Test specimens of biocomposites and the reference fossil-based polymer are exposed in seawater and intensive UV irradiation. The no-UV controls should be covered in aluminium foil and incubated together with treatments for the same period. After exposure, the seawater is filtered and microplastic (MP) particles will be visualised and characterised. In doing so, the MP formation of biocomposite and reference polymers can be assessed by quantifying and comparing the number of MP particles formed after UV radiation.

3.1.2 Test procedures

3.1.2.1 Preparation of test specimens

Test specimens of biocomposite and the reference fossil-based polymer should be prepared in appropriate size, shape and form [described in ISO 4892-1:2016 (ISO, 2016a)]. Adaptations may be made depending on actual purpose but mechanical stress (e.g. by cutting) on specimens should be avoided.

3.1.2.2 Test vessels and water medium

The use of quartz made vessels is recommended as quartz does not absorb UV radiation, in opposition to glass. Vessels made of other materials may be used, but care must be taken for their UV absorbance. For no-UV control, the test vessels should be covered with aluminium foils to block UV radiation (e.g. Lambert and Wagner, 2016).

Both filtered artificial seawater and filtered natural seawater can be used as water medium, but the final selection depends on the purpose of the study. The artificial seawater should be prepared based on standardised protocols [e.g. ISO 10253:2016 (ISO, 2016b)] for comparability. The seawater should then be filtered through a 0.2 µm sterile filter to remove microplastic contamination and microorganisms, and to prevent biodegradation. Water physio-chemical parameters (e.g. pH, salinity) should be measured and reported.

3.1.2.3 Ultraviolet exposure

Ultraviolet exposure should be performed in a state-of-the-art weathering chamber equipped with a fluorescent UV lamp. The selection and use of fluorescent UV lamps are described in ISO 4892-3:2016 (ISO, 2016c). A climate room/chamber with appropriate ventilation may be necessary to achieve environmentally realistic exposure temperatures. During the UV exposure, no-UV controls should be also incubated in the same chamber while covered in aluminium foil.

The dose of UV radiation can be expressed as total UV irradiance (KWh) and simulated number of exposure days (d):

- Total irradiation dose: These equations are sourced from Gewert et al. (2018). The total irradiance for the exposure periods is calculated using the experimental intensity (W/m^2) and hours of exposure:

$$Total\ irradiance\ exposed = Intensity\ \left[\frac{W}{m^2}\right] \times hours\ of\ exposure\ [h]$$

- Simulated sunlight exposure days: The simulated number of exposure days under mean European UV irradiance is calculated using a European mean irradiance ≈ 1200 kWh / (m² year), 5% of which is considered UV light giving a mean UV irradiance of 60 kWh/ (m² year).

$$Simulated\ days\ = \frac{Total\ irradiance\ exposed}{Mean\ European\ UV\ irradiance\ (or\ other\ reference\ data)} \times 365$$

3.1.2.4 Characterization of microplastic

After UV exposure, samples should be filtered through an appropriate filter (e.g. glass filter or stainless steel) to separate degraded MPs and media. The filter pore size shall be selected based on the actual need to collect formed MP fragments. The specimens can be discarded or preserved separately if further analysis needs (e.g. SEM analysis). The polymer composition of MP (> 20µm) can be identified using micro Fourier Transform Infrared Spectroscopy and Nile red staining (e.g. Catarino et al., 2018; Maes et al., 2017). To quantify MP fragments, methods proved reliable can be applied and the selection of methods may be based on actual purpose and conditions in laboratories.

3.1.2.5 Quality assurance and quality control criteria

At least four Quality Criteria and Quality Control (QA/QC) measures should be implemented during the experimental procedures to avoid contamination of the samples by airborne fibres and other particles:

- All glassware should be pre-cleaned and sterilised.
- Potential sources of MP contamination should be minimised by avoiding the use of any plastic equipment and using only prewashed glass and metal items.
- All filtration manipulations should be performed in a clean flow cabinet.
- Procedural blanks, observation of filtered water samples without plastic items to detect contamination, should be carried out throughout the analysis.
- To calculate the recovery rate of MP fragments during filtration, positive controls with known amount of MP fragments of biocompsoites and the reference fossil-based polymer should be performed prior to samples.

3.1.2.6 Data and test reporting

Outcomes for each polymer are expressed as number of MP particles per surface area of specimen. The formation of MP will be confirmed if significant number of MPs detected in samples than in no UV control. The relative resistance of releasing MPs under UV radiation by biocomposites and the reference fossil-based polymer will be assessed by comparing the number of MPs per surface area formed after the same UV exposure.

This test report shall contain at least the following information (Table 1).

Table 1. Test reporting checklist for microplastic formation assessment.

Category	Details
Materials	• Test specimens: Polymer compositions (both biocomposites and the reference fossil-based polymer), size, form, shape, weight per specimen, synthesis technique, UV radiation dose (if applicable). • All instruments involved. • Glassware and consumables • All chemicals involved (name, grade, supplier, and quantity) • Seawater and other water medium: Source (or preparation methods), physio-chemical parameters.
UV radiation exposure	• Procedures: wave length of the UV lamp, light intensity, chamber temperatures, black panel temperatures (if applicable), duration. • Results: UV radiation dose
Characterization of microplastics	• Procedures: protocol followed, validity of the method, lowest detectable particle size. • Results: identification of microplastic (number and correct rate), quantity of microplastic of each sample.
Quality assurance and quality control	• Procedures: All measures applied. • Results: recovery rate to tested biocomposites and the reference fossil-based polymer, background loads of MP contamination.

3.2 EVALUATION OF AQUATIC ECOTOXICITY

3.2.1 Principle of the test

This section focuses on assessing chemical ecotoxicology of biocomposites. To do so, leachate solution in saltwater is extracted from solid specimens of biocomposite and a reference fossil-based polymer. Then, selected standardised ecotoxicity assays on marine organisms are performed. The outcome will be expressed as dose-response relationship and toxicological values (e.g. EC50,EC10, LOEC, NOEC) for each polymer. Meanwhile, the concentrations of identified leached substances will be measured. In doing so, the chemical ecotoxicity of biocomposites will be evaluated by comparing the toxicological values with refence fossil-based polymer.

3.2.2 Preparation of test specimen

The size, form and shape of specimen can be decided based on actual purpose and should be consistent between biocomposites and the reference fossil-based polymer. If degradation products are of interests, test specimens shall be UV weathered prior to leachate extraction (methods described in 3.1.2.3).

3.2.3 Extraction of leachates

The established standard leaching protocols (e.g. DIN 38414-S4 and Minnesota test) have some limitations, such as extracting compounds in distilled water, and for short periods of time (< 48 h), making them less representative for the leaching of substances that occurs in the marine environment. To prepare leachate solution for ecotoxicity test, more environmentally relevant leaching tests under marine conditions (i.e. saltwater media) with longer duration (> 48 h) are recommended. Some methods in peer-reviewed recent ecotoxicological assessments can be adapted (Table 2). Adaptations may be made based on the specific ecotoxicity tests, but the conditions of leaching tests (solid-liquid ratio, type of saltwater, duration, etc.) should be reported.

After leaching, the leachate solution should be filtered through 0.2 µm sterile filter to separate specimens and to remove potential microplastic particles. The water physicochemical parameters (e.g. pH and salinity) of leachate solutions should be measure before and after leaching test.

Ideally, the ecotoxicity test should be performed immediately after fresh leachate obtained. In case any delay is foreseen, the leachate solution should be stored at - 20 ºC in dark and conditioned for 24 h prior to ecotoxicity test. Water samples should be taken for chemical analysis.

Table 2. A summary of methods of leachate preparation in seawater for ecotoxicity tests.

Solid/liquid ratio	Illumination	Duration	Temperature	References
Kg/L			°C	
0.1	-	96 h	Room temperature	Bejgarn *et al.* (2015)
0.008-0.025 (HDPE); 0.000125-0.005(PVC)	continuous illumination	5 d	22	Tetu *et al.* (2019)
0.08	no	14 d	25	Capolupo *et al.* (2020)
0.25	UV A+B light irradiation	96 h	20~30	Rummel *et al.* (2019)

3.2.4 Test Procedures

3.2.4.1 Selection of the test guidelines

The ecotoxicity of leachate from biocomposites and reference fossil-based polymer are recommended using standardised protocols (Table 3). The selection of these guidelines can be based on actual purpose, e.g. acute tests serve as a first step and indication to assess the ecotoxicity; but chronic tests are expected to contribute more for an eventual environmental risk assessment.

Table 3. Examples of marine aquatic ecotoxicity standard assays.

Exposure type	Organism	Species	Test guideline	Tittle
Acute	Phytoplankton	*Skeletonema sp*; *Phaeodactylum tricornutum*	ISO 10253:2016	Water quality — Marine algal growth inhibition test with *Skeletonema sp*. and *Phaeodactylum tricornutum*
Acute	Invertebrate	*Nitokra spinipes*	ISO 18220: 2016	Water quality — Larval development test with the harpacticoid copepod *Nitokra spinipes*
Chronic	Invertebrate	*Amphiascus tenuiremis*	OECD, TG 201, 2014	New guideline document on harpacticoid copepod development and reproduction Test with amphiascus
Acute	Invertebrate	*Crassostrea gigas; Crassostrea virginica; Mercenaria mercenaria; Mytilus edulis*	ASTM-E724-98:2012	Standard Guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs

3.2.4.2 Modification of test procedures and systems

As most leached substances are hydrophobic, the use of plastic products and contamination of microplastic should minimized. If intermediate solvents are used to extract leachate, extra controls with only solvent should be implemented.

3.2.4.2.1 Preparation of test solution

Considering the complexity in chemical-composition of leachates, nominal dilution series (e.g. %) instead of absolute concentrations (e.g. mg / L) will be used during the ecotoxicity test. A preliminary range-finding test should be performed to determine the dilution series. To ensure enough power to confidently report effects, a power analysis is highly recommended to quantify the number of replicates needed with a certain power and theoretical effect size.

Water samples of each dilution level should be taken and the absolute concentration of identified substances should be quantified. Specifically, for tests like algal growth inhibition test (ISO 10253: 2016), the leachate solutions are prepared in seawater without nutrients. The three nutrient stocks are then added to filtered leachate solution (the decrease in leachate concentration should be considered).

3.2.4.2.2 Controls

Negative controls (i.e. only control medium) with sufficient number of replicates should be performed. If intermediate solvents are used to extract leachate, extra controls with solvent and control medium should be implemented. To increase validity and inter-studies comparability, positive controls with reference toxic substances (if available, e.g. potassium dichromate) should be included at relevant concentrations.

3.2.4.2.3 Water renewal and feeding

Water renewal for dilution series of leachates should be performed if required in specific test guidelines. For chronic exposure, the leachate solution should be stored appropriately and its quality should be concerned. A preliminary test can be performed to monitor changes in physical-chemical parameters and concentration of identified substances of leachate solutions during the test. For feeding, the decrease in leachate concentration (%) caused each time should be concerned and below 2%.

3.2.4.2.4 Concentration of identified substances

It is recommended that, as a minimum, absolute concentrations of identified substances in the highest and lowest dilutions are quantified when freshly prepared — at the start of the test and immediately prior to renewals (if applicable) and at the end of the test. For tests where the absolute concentration of identified substances is not expected to remain within ± 20% of the nominal concentration, it is necessary to sample all test concentrations (including control), when freshly prepared and at renewal.

3.2.5 Chemical analysis

Regarding the complexity in chemical-composition of leachates, cluster chemical analysis will be performed (Figure 1). For the first step, a non- target chemical screening will be performed with solids to identify potential leached substances. Then calibration curves of these identified substances are derived for quantification. After all steps, the absolute concentrations of identified substances in test solutions will be measured [further information see in **Deliverable report D 3.3.1**(Catarino et al., 2021)].

3.2.6 Data and test reporting

Exposure-response relationship and toxicological values (e.g. EC50, EC10, LOEC, NOEC) for each polymer should be expressed as dilutions (%). When reporting effect levels, the absolute concentrations of identified substances should be given. This test report shall contain at least the following information (Table 4).

Table 4. Test reporting checklist for microplastic formation assessment.

Category	Details
Materials	• Test specimens: Polymer compositions (both biocomposites and the reference fossil-based polymer), size, form, shape, weight per specimen, synthesis technique, UV radiation dose (if applicable). • All instruments involved. • Glassware and consumable • All chemicals involved (name, grade, supplier, and quantity) • Seawater and other water medium: Source (or preparation methods), physio-chemical parameters.
Extraction of leachates	• Procedures: Test protocols followed, solid-liquid ratio, incubation condition (e.g. illumination, temperature, if shaking induced), duration, filtration method (e.g. pore size of filters), if organic solvent added, • Results: water physio-chemical parameters before and after leaching, concentrations of identified substances.

Ecotoxicity test	• Procedures: Test guidelines followed, test species, preparation of test solution, dilutions series of leachate tested, number of replicates and power analysis, if positive controls included • Results: raw toxicity data, dose-response curve (including method used), water physio-chemical parameter measurement, concentrations of identified substances at each dilution levels.
Chemical analysis	• Procedures: Protocols and methods; internal or external standards; lowest detection limit. • Results: Spectrums of non-target chemical screening; concentrations of identified substances in leachate and test solutions.

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