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BIOUPTAKE - Technical and ecotoxicological analysis of biobased materials

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Foreword

This CEN Workshop Agreement (CWA 18370:2026) has been developed in accordance with the CEN-CENELEC Guide 29 “CEN/CENELEC Workshop Agreements — A rapid way to standardization” and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN Workshop BIOUPTAKE — Technical and ecotoxicological analysis of biobased materials, the secretariat of which is held by Asociación Española de Normalizacion, UNE consisting of representatives of interested parties on 2025-12-03, the constitution of which was supported by CEN following the public call for participation made on 2025-10-10. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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Introduction

This CEN Workshop Agreement is a result of the European R&I project Bio-Uptake funded by the European Union under the grant agreement number 101057049.

The transition to a circular bioeconomy is a cornerstone of the European Green Deal, aiming to replace fossil-based materials with sustainable bio-based alternatives while minimizing environmental impacts. In 2022, bio-based plastics production in Europe reached approximately 2,2 million tonnes, representing 1,6 % of total plastics but projected to grow to 5,6 million tonnes by 2027 (16 % market share), driven by policies like the Renewable Energy Directive and Circular Economy Action Plan. However, rapid innovation in bio-based materials — such as bioplastics (e.g., PLA, PHA), fibers, and composites — raises concerns about potential ecotoxicity from additives, impurities, or degradation products, necessitating robust safety assessments under REACH and CLP regulations.

Bio-based products must undergo life-cycle ecotoxicological evaluation to verify environmental safety, including leachates/elutriates testing for aquatic hazards via standardized batteries (e.g., OECD TGs 201, 202, 203, 236). Yet, fragmented protocols across innovation stages — from raw feedstocks (lignocellulose, algae) to intermediates (pellets, composites) and end-products — hinder consistent hazard characterization. Existing standards like EN 16785 (bio-based content) and EN 13432 (compostability) focus on physicochemical properties but lack harmonized ecotoxicity roadmaps.

The BIOUPTAKE project addresses this by developing a roadmap for preparing diverse bio-based substances and materials for ecotoxicity testing along the value chain. This includes extraction protocols (e.g., EN 12457-4 elutriates, EN 15863 leachates), solvent limits (≤ 0,01% DMSO per OECD), and multispecies assays representing multiple trophic levels (*Raphidocelis subcapitata, Daphnia magna, Danio rerio embryos*). By integrating technical preparation with hazard profiling, BIOUPTAKE ensures bio-products comply with EU sustainability criteria, supporting claims under the Sustainable Products Initiative and preventing microplastic/ecotoxin releases.

This CWA describes BIOUPTAKE methodologies, implemented by the consortium, for the characterization of the freshwater ecotoxicity of bio-based developed substances, materials and intermediate composites. This complementary approach will allow materials and products to be validated in terms of environmental requirements for their marketing.

The proposed methodology mainly addresses the ecotoxicological testing conditions for the materials and their leachates.

This CWA will not define requirements related to safety aspects.

1 Scope

This CWA establishes a tests methodology for the ecotoxicity characterization of bio-based substances and materials and benchmark them with the corresponding fossil-based that are currently marketed.

The characterization process described in this CWA can be applied to bio-based epoxy resins; bio-based fibers from wood and carbon materials, bio-based polymers in the form of pellets and intermediate bio-based composite formats.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 15863:2015, *Characterisation of waste — Leaching behaviour test for basic characterisation — Dynamic monolithic leaching test with periodic leachant renewal, under fixed test conditions*

EN 12457-4:2002, *Characterisation of waste — Leaching - Compliance test for leaching of granular waste materials and sludges — Part 4: One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 10 mm (without or with size reduction)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1
elutriate
aqueous solution obtained after adding water to a solid substance (e.g., sediment, tailings, drilling mud, or dredging spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant

3.2
solution – A
liquid or solid phase containing more than one substance, when for convenience one (or more) substance, which is called the solvent, is treated differently from the other substances, which are called solutes

Note 1 to entry: When, as is often but not necessarily the case, the sum of the mole fractions of solutes is small compared with unity, the solution is called a dilute solution. A superscript attached to the symbol for a property of a solution denotes the property in the limit of infinite dilution.

4 Symbols and abbreviations

1,6-H	1,6-Hexanediamine
4,4'-D	4,4'-Dithiodiamine
4-AFD	4-aminophenyl difsulfide
CF	Carbon Fiber
CYS	Cystamine
DGEBA	Diglycidyether of bisphenol-A
DGEVA	Diglycidylehter of vanillyl alcohol
DMSO	Dimethyl Sulfoxide
PA	Polyamide
PCL	Polycaprolactone
PHTE	Phloroglucinol trisepox
PLA	Polylactic acid
WF	Wood Fiber

5 Preparation of solutions, elutriates, and leachates

5.1 Epoxy resins

Stock solutions of epoxy resins should be prepared by directly dissolving the resins in the standard test medium designated for the ecotoxicity assay with each target freshwater species. Special precautions are required during resin handling to prepare these stock solutions, as certain resins are oxygen-sensitive: exposure to oxygen can inhibit curing or polymerization via free radical scavenging, or induce oxidative degradation. In such cases, manipulation must be conducted under an inert gas atmosphere (e.g., argon). Additionally, resin solubility in the aqueous test medium must be considered; where necessary, stock solutions may first be prepared in a compatible organic solvent (e.g., dimethylsulfoxide) prior to the preparation of the test concentrations.

The concentration ranges tested in the ecotoxicity assays should be selected to enable determination of the median lethal concentration (LC_{50}) or median effective concentration (EC_{50}) for each resin in the target biological models, where feasible (noting that these parameters cannot be calculated if resins prove non-toxic).

For example, a stock solution of 34 mg/mL could be prepared by dissolving the resin in dimethyl sulfoxide (DMSO), ensuring the final solvent concentration does not exceed 0,01 % (v/v) at the highest tested concentration. OECD guidelines recommend limiting solvent to ≤ 0,01 % (100 µL/L or 100 mg/L) as the solvent control threshold in standard assays with freshwater species (e.g., OECD TG 203, 236) to preclude confounding effects on survival, development, or behavior; higher levels (up to 1 % to 2 %) may be acceptable in specific cases, but require rigorous validation against solvent controls exhibiting ≤ 10 % malformation or mortality.

The highest tested concentration of each resin does not need to surpass 100 mg/L, if no effects are observed. based on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

5.2 Bio-based fibers and polymers in the form of pellets

For the bio-based fibers (carbon and wood) and polymers in the form of pellets (e.g., PLA, PA, and PCL), 24-h elutriates shall be prepared according to standard EN 12457-4 in a 10:1 ratio (V:W), to simulate the potential release of substances and materials from solid matrices into aqueous phases.

The elutriates are prepared in distilled water and allowed to stir for a 24 h period at room temperature.

The elutriates obtain after 24 h are then filtered (0;22 µm pore membrane) and afterwards supplemented with several salts, according to the requirements of each test medium, to avoid effects induced by osmotic stress in the tested biological models.

5.3 Intermediate composite formats

For the intermediate composite formats, leachates shall be prepared following the standard EN 15863, by using a liquid-to-surface area ratio of 8 cm^3/cm^2.

These leachates are prepared by stirring the composite formats in distilled water for a 24 h period at room temperature, followed by filtration through 0;22-µm pore-size membrane. The resulting leachates are then supplemented with several salts, according to the requirements of each test medium, to avoid any osmotic stress and effect in the biological models.

For the use in ecotoxicity assays, the pH and conductivity of the solutions elutriates and leachates (100 % without salt supplementation, 100 % with salts supplementation, and respective dilutions) shall be measured at the beginning of all assays. For algae and rotifers, due to a very low amount of volume of the suspensions and dilutions prepared these parameters may only be measured at the beginning of the assay.

6 Ecotoxicological assays

6.1 General

To account for varying chemical and material sensitivities among freshwater species, as well as their ecological relevance, the ecotoxicological assessment should employ monospecific bioassays with at least six representative freshwater taxa spanning key trophic and functional guilds, the following are suggested: two producers (*Raphidocelis subcapitata* and *Lemna minor*, the latter providing an additional uptake route and greater structural complexity); two primary consumers (*Brachionus calyciflorus* and *Daphnia magna*, representing small and large grazers, respectively); and two secondary consumers (embryonic stages of *Danio rerio* and *Xenopus laevis*). All assays should follow standardized guidelines (e.g., OECD, ISO). The following standard ecotoxicity assays as per OECD guidelines are recommend to be performed for the prepared solutions, elutriates and leachates.

6.2 72-h growth inhibition test *Raphidocelis subcapitata*

The growth inhibition assay with the freshwater microalgae *Raphidocelis subcapitata* should be conducted in accordance with the OECD Guideline 2011-201, adapted to 24-well microplate. The experiments shall begin with inocula from exponentially axenic growing cultures.

The control consists of MBL medium alone. A minimum of three replicates per control and tested concentration or dilution shall be performed, each should contain 1800 µL of the solution, elutriate, or leachate desired or control medium (MBL), to which 200 µL of microalgae inoculum (at an initial concentration of 10^5 algae cells mL^{-1}, to ensure that assays would begin with an initial algae concentration of 10^4 cells mL^{-1}) must be added,

The test conditions shall be controlled ((23 ± 1) °C of temperature and constant illumination of 100 µE m^2 s^{-1}), and all wells shall be resuspended daily to prevent algal settling and shading.

To account for potential interference of the solutions, elutriates and leachates, additional wells of each solution, elutriate, and leachates dilution, without no algae, shall be conducted and exposed to identical conditions.

Cell density (CD, cells mL^{-1}) is determined spectrophotometrically by measuring absorbance (ABS) at 440 nm. ABS values shall be corrected by subtracting readings from the corresponding blanks (for solution, elutriate, or leachate) and then converted to CD using a pre-established calibration curve (Annex A).

This must be curve prepared prior to the assays by quantifying microalgae cell concentrations (e.g., via Neubauer chamber counts) across a range of dilutions and measuring their corresponding absorbances at 440 nm.

The CD data is used to calculate *R. subcapitata* yield (Y, biomass produced during the assay, cells mL^{-1}) and growth rate (r, day^{-1}) for each solution, elutriate, leachate dilution treatment and control, as described in OECD Guideline 2011-201.

6.3 7-days *Lemna minor* Growth Inhibition test

The 7-days *Lemna minor* growth inhibition assays should be performed by following the OECD guidline 2006-221. Three colonies with 4 fronds each of *L. minor* shall be exposed for 7 days to a control and to the range of concentrations or dilutions of the tested solutions, elutriates and leachates.

Three replicates shall be established for each treatment and the control, each replicate containing 8 mL of solution. The control consisted of Steinberg medium alone.

The test conditions shall be controlled ((23 ± 1) °C of temperature and constant illumination of 100 μE m^2 s^{-1}).

At the end of the assay, the total number of fronds shall be recorded for yield determination (Y, based on the number of fronds) and the growth rate (r, day^{-1}). The fronds fresh weight (mg) shall be assessed immediately, and the dry weight (mg) after lyophilization.

6.4 24-h acute test with *Brachionus calyciflorus*

The acute assay with the freshwater rotifer *Brachionus calycliforus* should be performed according to the protocol acute Rotoxkit F (ROTOX-F), that conforms to ISO 19827:2016. Five replicates shall be assembled per treatment and control.

Each replicate shall contain 1 mL of each test solution, elutriate, or leachate or control medium (no food shall be added during the assay) and five newly hatched neonates shall be assigned per replicate.

After 24 h of incubation, at 23 °C in the dark, the number of dead rotifers shall be recorded

6.5 48-h Immobilisation test with *Daphnia magna*

The 48-h immobilisation test with the freshwater cladoceran *D. magna* shall be performed according to OECD guideline 2004- 202. In brief, neonates with less than 24-h old neonates, collected from 3rd to 5th broods, shall be exposed to a series of concentrations or dilutions .

Four replicates, each with 10 mL of test solution or dilution, shall be assembled by treatment or control, with five randomly assigned neonates. During the 48-h exposure, no food shall be added nor the medium renewed. The controls consist of standard ASTM hardwater alone.

Assays run under the controlled temperature of (20 ± 1) °C and 16 hL: 8 hD photoperiod.

Mortality shall be checked at 24 and 48 h by gentle prodding of the organisms, considering it dead if no movement was observed within a 15-sec period.

6.6 96-h Fish Embryo Acute Toxicity test with *Danio rerio*

The 96-h Fish Embryo Acute Toxicity test with *D. rerio* should be performed accordingly to OECD guideline 2025-236. This test is recommended, instead of the juvenile OECD Test Guideline 203 (Fish, Acute Toxicity Test) to avoid conducting Animal experimentation. The embryos used in the assays shall be collected within one hour after fish naturally mating and carefully screened under a stereomicroscope to discard injured and coagulated eggs. Before assigning the embryos to the assay, they shall be carefully washed twice with clean, sterilized maintenance water from the fish facility.

For the 96-h assay, ten embryos (~3 hours post-fertilization) shall be assigned per concentration or dilution and control. Assays shall be assembled in 24-well microplates, with one single egg assigned per well, filled with 2 mL of test solution or water, under controlled temperature and light conditions ((26 ± 1) °C and 16 hL: 8 hD photoperiod). The control consists of zebrafish maintenance water solely (carbon filtered water at a conductivity of (750 ± 50) µS/cm).

Every 24 h, mortality and the developmental stage of the embryos shall be recorded.

Table 1 — Resume of the raw materials and formats and of the methods used to prepare the test solutions, elutriates, and leachates

Chemical/ Material group		Acronym	Methods for preparation of solutions or 24-h elutriates or 24-h leachates
Epoxy resins	**Coatings**	Bisphenol-free diglycidyl ether of vanillyl alcohol **(DGEVA)** Phloroglucinol triglycidyl ether **(PHTE)** Diglycidyl ether of Bisphenol A* **(DGEBA)** 1,6-Hexanediamine* **(1,6-H)** 4,4'-Dithiodiamine* **(4,4'-D)**	Stock solution of 34 mg/mL; Dimethyl sulfoxide as solvent (DMSO 0,3 %), except for CYS and 1,6-H; dilution of the stock solution with the different test media CYS and 1,6-H directly dissolved in the different test media
	Hardener	Cystamine **(CYS)**	
Bio-based fibers		Carbon fibers **(CF)** Wood fibers **(WF)**	Standard EN 12457-4 in a 10:1 ratio (V:W), filtered 0,22 µm pore membrane, supplemented afterwards
Bio-based pellets		Polyamide 10-10 **(PA 10-10)** Polylactic acid 1 **(PLA1)** Polylactic acid 2 **(PLA2)** Polylactic acid 3 **(PLA3)** Polylactic acid 4 **(PLA4)** Polycaprolactone **(PCL)**	Standard EN 12457-4 in a 10:1 ratio (V:W), filtered 0,22 µm pore membrane, supplemented afterwards
Bio-based intermediate formats	**Flax fibers reinforced** **Mono- and bicomponent filaments**	BUP PRP04 BUP PRP05 PLA 50216 PLA-PLA1-PLA2 (1- and 5-mm thickness) PLA-PLA3-PLA4 (3 mm thickness) PLA-PLA1-PLA4 (5 mm thickness)	Standard EN 15863 in a proportion of 8 cm^3cm^{-2}, filtered 0,22 µm pore membrane, supplemented afterwards
	Bio-based thermoplastic material with carbon and wood reinforcement	PA10 5% carbon PA10 5% wood	

Annex A
(informative)

Calibration curve

Concentration (cells ml^{-1}) = –17107,5 + (ABS × 7925350), where ABS is the absorbance at 440 nm.

Available at: Venâncio, C., Anselmo, E., Soares, A. and Lopes, I., 2017. Does increased salinity influence the competitive outcome of two producer species?. Environmental Science and Pollution Research, 24(6), pp.5888-5897. https://doi.org/10.1007/s11356-016-8346-x

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Venâncio C., Anselmo E., Soares A., Lopes I. Does increased salinity influence the competitive outcome of two producer species? *Environ. Sci. Pollut. Res. Int*. 2017, 24 (6) pp. 5888–5897 https://doi.org/10.1007/s11356-016-8346-x

ISO 19827:2016, *Water quality — Determination of chronic toxicity to Brachionus calyciflorus — Short-chronic protocol*

ASTN D1126, *Standard Test Method for Hardness in Water*