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Characterization of waste - Preparation of waste samples for ecotoxicity tests

Caractérisation des déchets - Préparation des échantillons de déchets en vue d'essais écotoxicologiques

Charakterisierung von Abfällen - Herstellung von Abfallproben für ökotoxikologische Untersuchungen

This European Standard was approved by CEN on 27 June 2005.

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Foreword

This European Standard (EN 14735:2005) has been prepared by Technical Committee CEN/TC 292 “Characterization of waste”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2006, and conflicting national standards shall be withdrawn at the latest by February 2006.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Introduction

Ecotoxicity can be estimated using two approaches: a chemical-specific approach and a toxicity-based approach. Chemical analyses are compared, in the first case, to quality criteria or threshold values to estimate ecotoxicity. In the second one, ecotoxicity is measured directly using biological tests. These two approaches complement each other, however, determination of pollutants in complex mixtures of unknown composition, that is a characteristic of many wastes, does not allow a relevant estimation of ecotoxicity. For such samples, the toxicity based approach is usually recognised to be appropriate to assess potential toxicity. Bioassays integrate, indeed, the effects of all contaminants including additive, synergistic and antagonistic effects. They are sensitive to the bioavailable fraction of the contaminants only. Finally, bioassays integrate the effects of all contaminants, including those, not considered or detected by chemical analyses.

Ecotoxicity tests can be applied to wastes to identify their potential hazardous properties with respect to the environment for classification purposes or to assess the risk related to a site-specific exposure scenario.

Identification of properties potentially hazardous to the environment for classification purposes

A classification system, based on the assessment of intrinsic properties, should be independent of an exposure scenario. The main requirement, in order to establish a relevant system for classifying wastes and for assessment of hazard properties, is to obtain comparable test results. This can only be obtained if the ecotoxicity tests on wastes are carried out according to a unique procedure describing more or less conventional test conditions (an exclusive dilution medium for terrestrial tests, a unique L/S ratio for preparation of water extracts, a unique liquid / solid separation step etc). This procedure should be applicable to a very wide range of waste materials whatever their physical properties are.

Any strategy for the assessment of properties potentially hazardous to the environment used in a classification system should include test organisms representing the terrestrial and the aquatic compartment. Both types of tests should be considered because they expand the range of effect expression due to differences in species sensitivity and exposure. For this specific purpose, the water extracts preparations for toxicity testing do not simulate leaching from wastes under environmental conditions but measure the water available fraction of the toxic components of the wastes.

Site-specific exposure scenario

The second application of ecotoxicity tests to wastes refers to a risk assessment approach. In this particular case, the test strategy should model site specific exposure conditions and should take into account the transfer of contaminants via the food chain and to surface and ground water by run-off or leaching. This application concerns firstly the definition of generic scenarios frequently encountered (e.g. wastes deposit in stockpiles, re-use of wastes) and focus on the relevant way of exposure to terrestrial and aquatic organisms.

〈AC$_1$〉 This European Standard describes the necessary steps to be performed before carrying out ecotoxicity tests on wastes within the context of assessment of ecotoxic properties for classification purposes. 〈AC$_1$〉

1 Scope

This European Standard describes the necessary steps to be performed before carrying out ecotoxicity tests on wastes. The purpose of this European Standard is to provide guidance on the taking of the sample, transport, storage of wastes and to define preparation, for the determination of ecotoxicological properties of wastes under the conditions specified in this European Standard by biological testing either as raw wastes or water extracts from wastes. Sample preparation for other applications (e.g. assessment of waste effects on aquatic and terrestrial organisms in a disposal scenario) is not considered.

Specifying a test battery to characterize ecotoxicological properties of wastes is not in the scope of this European Standard.

This European Standard is applicable to solid and liquid wastes.

2 Normative references

The following referenced documents are indispensable for the application of this European Standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

prEN 14899, *Characterization of waste - Sampling of waste materials - Framework for the preparation and application of a Sampling Plan*

EN 12457-2:2002, *Characterization of waste – Leaching – Compliance test for leaching of granular waste materials and sludges – Part 2: One stage batch test at a liquid to solid ratio of 10 l/kg for materials with particle size below 4 mm (without or with size reduction)*

EN ISO 5667-3, *Water quality - Sampling - Part 3: Guidance on the preservation and handling of water samples (ISO 5667-3:2003)*

ISO 10390, *Soil quality – Determination of pH*

ISO 11268-1, *Soil quality – Effects of pollutants on earthworms (Eisenia fetida) – Part 1: Determination of acute toxicity using artificial soil substrate*[1)]

ISO 11465, *Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method*

ISO 14238:1997, *Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes*

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1) Definition of soil substrate.

3 Terms and definitions

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

3.1
dilution medium
[AC1] liquid or solid used for the preparation of control vessels and the preparation of test mixtures [AC1]

3.2
ecotoxicological properties
potential adverse effects to biological systems which a waste has an inherent capacity to cause

3.3
eluate
solution recovered from a leaching test

[EN 12457-2:2002]

3.4
granular waste
waste not being monolithic, nor a liquid, a gas or a sludge

[EN 12457-2:2002]

3.5
laboratory sample
sample or subsample(s) sent to or received by the laboratory (IUPAC definition)

NOTE 1 When the laboratory sample is further prepared (reduced) by subdividing, mixing, grinding or by combinations of these operations, the result is the test sample. When no preparation of the laboratory sample is required, the laboratory sample is the test sample. A test portion is removed from the test sample for the performance of the test or for analysis. The laboratory sample is the final sample from the point of view of sample collection but it is the initial sample from the point of view of the laboratory.

NOTE 2 Several laboratory samples can be prepared and sent to different laboratories or to the same laboratory for different purposes. When sent to the same laboratory, the set is generally considered as a single laboratory sample and is documented as a single sample.

3.6
leachant
liquid used in a leaching test

NOTE For the purpose of this European Standard the leachant is water as specified in Clause 4.

3.7
leaching test
test during which a material is put into contact with a leachant and some constituents of the material are extracted

3.8
liquid sludge
sludge that has the characteristic of a liquid as specified in the definition of a [AC1] liquid waste (3.9) [AC1]

3.9
liquid waste
waste that completely flows out of a calibrated opening, down to the upper level of the opening within a limited period of time (see Annex B of EN 12457-2:2002)

3.10
monolithic waste
material which has dimensional, physical and mechanical properties that comply with the criteria defined in an on going document

3.11
paste-like material
material of soft plastic or wet cement consistency – usually smooth

3.12
sludge
mixture of liquid and solid separated from various types of liquid as a result of natural or artificial processes

[EN 12457-2:2002]

3.13
test mixture
mixture of the test portion (waste or water extract) with the dilution medium

3.14
test portion
amount or volume of the test sample taken for measurement of ecotoxicological properties by biological testing and/or other properties of interest, usually of known weight or volume (adapted from IUPAC definition)

NOTE 1 The test portion can be taken from the laboratory sample directly if no preparation of sample is required (e.g. with liquids), but usually it is taken from the prepared test sample.

NOTE 2 A unit or increment of proper homogeneity, size and fineness, needing no further preparation, can be a test portion.

3.15
test sample
sample, prepared from the laboratory sample, from which test portions are removed for biological testing or analysis (adapted from IUPAC definition)

NOTE The preparation of the test sample can include particle size reduction, preparation of water extract etc.

3.16
water extract
solution obtained from a leaching test, a liquid/liquid extraction and a liquid/solid separation (centrifugation)

4 Equipment and reagents

Usual laboratory equipment and the following.

4.1 Sieving equipment with sieves of 4 mm square mesh.

NOTE Due to sieving, contamination of the sample may occur to an extent that affects the leaching of some constituents of concern e.g. cobalt and tungsten from tungsten carbide equipment or chromium, nickel and molybdenum from stainless steel equipment.

4.2 Crushing equipment: jaw crusher or cutting device.

NOTE Due to crushing, contamination of the sample may occur to an extent that affects the leaching of some constituents of concern e.g. cobalt and tungsten from tungsten carbide equipment or chromium, nickel and molybdenum from stainless steel equipment.

4.3 Balance of accuracy of at least 0,1 g.

4.4 End-over tumbler (5 rpm to 10 rpm) **or rollertable** rotating at about 10 rpm.

NOTE Other shaking or mixing devices can be used provided that they are proven to be equivalent.

4.5 Centrifuge operating at 2 500 g.

4.6 Filtering apparatus, either a vacuum filtration device (between 30 kPa and 70 kPa) (300 mbars to 700 mbars) or a high pressure filtration apparatus (< 0,5 MPa) (5 bars).

4.7 Mixer.

4.8 pH meter.

4.9 Glass or high density polyethylene(HDPE)/polypropylene(PP) bottles in accordance with EN ISO 5667-3, glass bottles having caps of inert material, for example polytetrafluoroethylene. Rinsing is compulsory.

NOTE For inorganic constituents HDPE/PP bottles are preferred, except for samples tested for mercury.

4.10 Distilled water, demineralized water or deionized water with a conductivity < 5 µS/cm.

5 Taking of laboratory sample

Obtain a laboratory sample of a quantity sufficient for the number of tests to be performed and in accordance with the requirements of biological standardised methods to be used.

The laboratory sample should be obtained according to the guide to the preparation of a sampling plan for liquid and granular waste materials including paste-like materials and sludge, under development by CEN/TC 292/WG 1.

Special precautions should be taken to avoid any contamination of laboratory samples by material of sampling devices and/or storage equipment, according to prEN 14899.

NOTE [AC1] Sampling devices are described in a Technical Report under development (prCEN/TR 15310-2). [AC1]

Some wastes are subject to chemical, physical and biological changes as soon as they are collected (e.g. wastes that are fermentable, subject to oxidation or carbonation and wastes that contain volatile substances). Possible changes shall be considered and sampling conditions shall be designed accordingly in order to limit the effects of such changes on the results of ecotoxicity tests.

However, addition of preservatives (e.g. acids, basic solutions, biocides) in order to retard chemical and biological activity is prohibited.

6 Transport

Transport of laboratory samples shall be performed in the dark, in tight containers fully filled with the waste to be tested. However, special precautions should be taken for transport and storage of sludge or other microbial active wastes. Containers can become pressurised due to gas production and explosions may occur. For such laboratory samples, containers should not be completely filled. Nevertheless, headspace shall not exceed 10 % of the total capacity of the container. Manual release of pressure during and after transport may be necessary.

The container material shall be appropriate. The container, in which the waste laboratory sample is transported, and the stopper shall not react with the constituents of the sample and shall not be a

cause of contamination. Wastes shall be stored in polyethylene, polypropylene, polytetrafluoroethene (PTFE) or glass containers. However, security aspects shall be considered, including the risk of explosion due to gas generation (for example glass vessels are not suitable for sludge samples).

Transport of waste laboratory samples should be as short as possible. Possible changes shall be considered and transport conditions shall be designed accordingly in order to limit the effects of such changes on the results of ecotoxicity tests. Transport time shall be regarded as part of storage time.

〚AC1〛 A transport time of less than 48 h and/or low temperature conditions (4 ± 2) °C are appropriate to maintain the properties of laboratory samples. 〚AC1〛

7 Storage

7.1 General

Storage should be carried out in the containers defined in Clause 6. Possible changes shall be considered and storage conditions shall be designed accordingly in order to limit the effects of such changes on the results of ecotoxicity tests.

7.2 Waste sample

Storage time starting from reception of laboratory sample and ending with the start of definitive tests should be as short as possible.

〚AC1〛 A storage time of less than two months and low temperature conditions (4 ± 2) °C are appropriate to maintain the properties of waste samples. 〚AC1〛

NOTE Freezing may induce changes of characteristics of the waste sample.

7.3 Water extracts

Water extracts should be stored at (4 ± 2) °C in polyethylene, polypropylene, polytetrafluoroethene (PTFE) or glass containers. Before testing, the containers shall be filled with a headspace less than 5 %.

NOTE 1 Freezing may induce changes of characteristics of water extracts.

It is recommended to minimise the time between the start of the different tests to be performed on the same laboratory sample in order to minimise its changes.

Ecotoxicological tests shall start immediately after production of water extract as specified in the applicable standard for the considered ecotoxicity test and in no case later than 72 h after production of water extract. If a range-finding test and a definitive test are performed, the definitive test shall be completed within 10 days after production of the water extract.

For longer tests (e.g. semi-static chronic tests), several water extracts shall be produced and used within 10 days after production.

If definitive test results are not in accordance with the range-finding test, water extraction shall be repeated and the test shall be performed on the new water extract.

NOTE 2 It could be possible to extend the duration of storage if it has been proved that no modification of toxicity occurred within the storage period (e.g. carry out the same ecotoxicity test immediately after extraction period and at the end of storage period).

8 Waste characterization

The following characteristics shall be determined prior to the performance of ecotoxicity tests:

— pH, according to ISO 10390 for granular waste, monolithic waste, paste-like waste and sludge;

— dry matter content, according to ISO 11465 for granular waste, monolithic waste, paste-like waste and sludge (see Clause 9);

— water holding capacity, according to Annex A of ISO 14238:1997 for granular waste, monolithic waste, paste-like waste and sludge (see note).

NOTE [AC1] The method described in Annex A of ISO 14238:1997 has been found to be appropriate for most kinds of waste. [AC1]

9 Waste pre-treatment : particle size reduction (granular waste, monolithic waste, paste-like waste and sludge)

Both ecotoxicological and leaching tests are performed on material which originally and after pretreatment has a particle size less than 4 mm.

The tests shall be made on material with a grain size of at least 95 % (mass) less than 4 mm. Therefore, the laboratory sample shall be sieved (4.1). If oversized material exceeds 5 % (mass) the entire oversized fraction shall be crushed with crushing equipment (4.2). On no account shall the material be finely ground. Non-crushable material (e.g. metallic parts such as nuts, bolts, scrap) in the sample shall be separated and the weight and nature of the material shall be recorded. The method of size-reduction applied shall be documented and recorded in the test report. Irrespective of any necessary size reduction, the separate fractions with the exception of the non-crushable material shall be mixed to constitute the test sample. If the laboratory sample cannot be crushed or sieved because of its moisture content, it is allowed, only in this case, to dry the laboratory sample. The drying temperature shall not exceed 40 °C.

NOTE 1 Fibrous materials, paste-like waste and plastics can often be size-reduced after cryogenic treatment. The sample is usually plunged into liquid nitrogen (- 196 °C) just before crushing to make it fragile and brittle. It also limits the overheating during crushing. As a result, the sample obtained is fine and perfectly homogeneous.

NOTE 2 In order to minimise the possible contamination during the sieving, fragmentation and splitting, it is recommended, before preparing the test sample, to process a portion of the laboratory sample through the devices for sieving, fragmentation and splitting and to discard such material thereafter. This recommendation does not cover the situation described in the notes under 4.1 and 4.2.

For this European Standard, any other waste pre-treatment is excluded ; especially, the test sample which shall not be further dried. The determination of the dry matter content ratio and the moisture content ratio shall be determined on a dedicated test portion. The moisture content of the test sample shall be determined at (105 ± 5) °C. It shall be taken into account when adjusting the L/S ratio in leaching test. The dry mass of the sample shall be determined at (105 ± 5) °C according to ISO 11465 and the dry matter content ratio is calculated as follows:

$$DR = 100 \times \frac{M_{\mathrm{D}}}{M_{\mathrm{W}}} \qquad (1)$$

where

DR is the dry matter content ratio (%);

M_{D} is the mass of the dried test portion (kg);

M_{W} is the mass of undried test portion (kg).

The moisture content ratio is calculated as follows:

$$MC = 100 \times \frac{\left(M_{\mathrm{W}} - M_{\mathrm{D}}\right)}{M_{\mathrm{D}}} \qquad (2)$$

where

MC is the moisture content ratio (%).

NOTE 3 The basis for the calculation of the moisture content is the mass of the moisture content of the dry residue in this European Standard, as specified in ISO 11465 (for the determination of the water content of soil). It should be noted that in EN 12880 (for the determination of water content of sludge), the water content is calculated on the basis of the raw mass.

NOTE 4 The above moisture content determination could be not accurate enough in some cases (e.g. large amount of volatile or unstable compounds). In such cases a direct determination of the true water content should be performed and the moisture content calculated accordingly.

10 Tests performed on terrestrial organisms

10.1 General considerations

The determination of ecotoxicological properties of wastes under conventional conditions requires using a dilution medium as inert as possible. This dilution medium shall allow the survival and the good development of organisms during the test period. Both requirements may be difficult to reconcile particularly considering plant growth inhibition tests and microbial tests (tests that required indigenous population of micro-organisms).

In order to fulfil theses requirements, the dilution medium called "artificial soil" shall be used unless otherwise specified in the standardized terrestrial test methods. The same medium shall be used for both control and dilution.

Moreover, preparation of a medium should be reproducible to allow comparison of ecotoxicity tests results.

Several standardized ecotoxicity tests were considered to establish the following conditions for testing wastes on terrestrial organisms. This compilation of tests is given in Annex B.

Preparation of test mixtures may differ according to the type of waste and according to the ecotoxicity tests to be performed. Preparation of the different test mixtures is summarized in Annex A.

10.2 Dilution medium

The dilution medium shall have the following composition (such as defined in ISO 11268-1):

— sphagnum peat finely ground and with no visible plant remains: 10 % (percentage expressed on dry mass basis);

— kaolinite clay containing not less than 30 % kaolinite: 20 % (percentage expressed on dry mass basis);

— industrial quartz sand (more than 50 % of particle size from 0,05 mm to 0,20 mm): 70 % (percentage expressed on dry mass basis).

Calcium carbonate ($CaCO_3$), pulverised and of recognised analytical grade is added to bring the pH of the wetted substrate to 6,0 ± 0,5 (generally between 0,5 % and 1 % of the mass of the dry ingredients).

Water (4.10) or mixture of water extract with water is added to the dilution medium to reach the percentage of the total water holding capacity recommended for each test organism.

10.3 Introduction of waste into the dilution medium

10.3.1 General

Preparation of test mixtures differs according to the waste to be tested (see Annex A). The different methods of preparation are described below.

10.3.2 Monolithic waste, granular waste, paste-like waste and sludge

Different methods can be applied to introduce the test portion into the dilution medium. Several parameters influence the selection of introduction method such as physical properties of waste or amounts to be tested. The following methods are recommended:

— *for small amounts*, introduce the test portion in the water (or in part of it) necessary to wet the dilution medium, then mix this suspension thoroughly with the dilution medium;

— *for large amounts*, mix the test portion thoroughly with the already hydrated dilution medium;

— *for hydrophobic waste*, mix the test portion thoroughly with the dilution medium, then add the water necessary to wet this mixture.

Test mixtures are expressed in percentages (dry mass of waste per total dry mass of test mixture).

10.3.3 Liquid sludge

Introduce the test portion in the water (or in part of it) necessary to wet the dilution medium, then mix this suspension thoroughly with the dilution medium in order to obtain the test mixture to perform the considered ecotoxicological test. Test mixtures are expressed in percentages (mass of waste per total dry mass of test mixture).

The volume of the liquid sludge to be added is limited by the percentage of water holding capacity of the test mixture recommended in the test methods.

10.3.4 Liquid waste miscible with water

Introduce the test portion in the water (or in part of it) necessary to wet the dilution medium, then mix this suspension thoroughly with the dilution medium.

Some liquid waste may not contain any water and does not hydrate the test mixture, in which case it is necessary to add water to allow survival of test organisms within the given test period.

The maximum quantity of liquid waste and water to be added shall correspond to the water holding capacity of the dilution medium recommended for each test method. The structure achieved after adding the liquid waste should meet the requirements of the test organisms.

10.3.5 Liquid waste non miscible with water

The following methods are recommended.

a) *For small amounts:*

— introduce by ultrasonic dispersion the test portion in the water (or in part of it) necessary to wet the dilution medium, then mix this suspension thoroughly with the dilution medium; or

— prepare a mixture of quartz sand (see 10.2) and the quantity of test portion required to obtain the desired amount (a ratio of 10 g of sand per kilogram of soil is usually recommended). Mix with the dilution medium thoroughly, then add the water necessary to wet this mixture.

b) *For large amounts:*

— mix the test portion thoroughly with the dilution medium already hydrated; or

— mix the test portion thoroughly with the dilution medium, then add the water necessary to wet this mixture.

Test mixtures are expressed in percentages (mass of waste per total dry mass of test mixture).

10.4 Water extracts of waste

Introduce the water extract (see Clause 11) in the water (or in part of it) necessary to wet the dilution medium, then mix this suspension thoroughly with the dilution medium.

The maximum quantity of water extract to be added shall correspond to the water holding capacity of the dilution medium recommended for each test method.

Test mixtures are expressed in percentages (mass of water extract per total dry mass of test mixture).

10.5 pH

Tests shall be carried out without pH adjustment of the test portion.

pH of all test mixtures is measured at the beginning and at the end of the test and reported.

NOTE 1 pH of test mixtures may significantly differ from pH of test portion according to the selected dilution range and according to buffer capacity of test medium or test portion.

NOTE 2 If toxic effects are observed in the dilutions where pH is not compatible with the survival of the organisms, the test(s) can be repeated with pH adjustment of the test portion.

10.6 Addition of test organisms

The test procedure is selected according to the aim of the study: determination of a dose-effect relationship or limit test at a given dilution.

Test organisms shall be added as soon as possible after test mixture preparation and on no account later than 24 h after preparation.

11 Tests performed on aquatic organisms

11.1 General considerations

Ecotoxicological properties of wastes are assessed under conventional conditions (and not according to specific scenarios) and a unique procedure is specified, for each material status, to produce the water extract in order to obtain comparable ecotoxicity tests results performed on different wastes.

Water extract preparation is influenced by several parameters such as physical properties of waste and required volume of water extract. This last parameter may become crucial when chronic ecotoxicity tests are performed.

The L/S = 10 ratio is recommended, since it is applicable to a very wide range of wastes. The volume of leachant provided is suitable to obtain a water extract even for wastes with high water holding capacities (e.g. sludge). The volume of water extract obtained is consistent with the implementation of chronic ecotoxicity tests.

NOTE Deviation from an L/S ratio of 10 leads to test results not comparable with test results obtained with a ratio of 10.

Several standardized ecotoxicity tests were considered to establish the following conditions for testing wastes on aquatic organisms. This compilation of tests is given in Annex B.

Preparation of test mixtures may differ according to the type of waste and according to the ecotoxicity tests to be performed. Preparation of the different test mixtures is summarized in Annex A.

11.2 Monolithic waste, granular waste, paste-like waste and sludge

11.2.1 Leaching procedure

The following procedure is based on EN 12457-2. The following text includes the modifications required by the tests listed in Annex B.

Place the test portion with the total mass M corresponding to (90 ± 5) g of dry mass M_D in a bottle (4.9) with a nominal volume of 1 l to minimise headspace.

Ecotoxicological tests usually require several litres of eluate leading to an adjustment of mass of the test portion. When a large volume of eluate is required, it is possible to perform the leaching test in a vessel of appropriate capacity into which the corresponding number of test portions [AC1] [a dry mass of (90 ± 5) g] [AC1] are introduced or to perform parallel leaching tests at the same time with an appropriate number of vessels of 1 l capacity each (see above). In that case, all the individual eluates shall be mixed in order to obtain the test sample. When the leaching test is performed in one vessel, the size of the vessel shall be appropriate to minimise the headspace (≤ 5 % of the total capacity of the vessel).

Add an amount of leachant (L) establishing a liquid to solid ratio (L/S) = 10 l/kg ± 2 % during the extraction. Care should be taken to obtain good mixing of solid and liquid.

$$L = \left(10 - \frac{MC}{100}\right) \times M_{\mathrm{D}} \qquad (3)$$

where

L is the volume of leachant used (in l);

M_{D} is the dry mass of the test portion (in kg);

MC is the moisture content ratio (in %).

Place the capped bottle in an agitation device (an end-over-end tumbler 5 rpm to 10 rpm or rollertable rotating at about 10 rpm). Agitate for (24 ± 0,5) h at a temperature within the range 15 °C to 25 °C.

During the extraction care should be taken to prevent settlement of solids in the bottle.

Excessive abrasion leading to significant particle size reduction shall be avoided.

NOTE Some wastes generate gas when they are wetted. Examples are waste incineration fly ash and sand blasting waste that may contain metallic particles. If gas emission occurs, careful opening of the bottle a few times during the leaching can prevent too high pressure. Such opening should be documented in the test report.

11.2.2 Liquid/solid separation procedure

As ecotoxicological properties of wastes are assessed under conventional conditions, the eluates shall be filtrated through 0,45 µm membrane filter. It is recommended to use PTFE or nylon filters instead of acetate or nitrate cellulose filters. This filtration has the advantage that several bioassays, which may be influenced by suspended materials in the test mixture (determination of inhibitory effects on the light emission of *Vibrio fischeri*, algal growth inhibition test etc), may be performed.

The separation of the solid and the liquid phases is carried out as follows in the same temperature conditions as defined above.

Allow the suspended solids to settle for (15 ± 5) min. When the separation of the solid and liquid phases is not achieved within this time period, the mixture is centrifuged for 30 min at 2 500 g.

Filter the eluate over a 0,45 µm membrane filter using a vacuum or pressure filtration device (4.6). Rinsing of the filter with water or another solvent is not allowed after filtration.

NOTE When volatile components are analysed, vacuum filtration should not be used.

Determine the volume of eluate VE.

Measure immediately conductivity (in µS/cm), pH (and optionally redox potential E_{h} in mV and temperature) of the eluate.

11.2.3 pH

Tests shall be carried out without pH adjustment of the test portion.

pH of all test mixtures is measured at the beginning and at the end of the test and reported.

NOTE 1 pH of test mixtures may significantly differ from pH of test portion according to the selected dilution range and according to buffer capacity of test medium or test portion.

NOTE 2 If toxic effects are observed in the dilutions where pH is not compatible with the survival of the organisms, the test(s) can be repeated with pH adjustment of the test portion.

11.3 Liquid sludge

11.3.1 Procedure

Large particles should be removed from sludge by centrifugation (for 30 min at 2 500 g) and the water extract is filtrated through a 0,45 µm membrane filter. It is recommended to use PTFE or nylon filters instead of acetate or nitrate cellulose filters.

11.3.2 pH

See 11.2.3.

11.4 Liquid waste miscible with water

11.4.1 Procedure

No specific procedure is needed, the liquid waste miscible with water should be used for aquatic toxicity testing after filtration (see 11.2.2).

Measure conductivity (in µS/cm), pH (and optionally redox potential E_h in mV and temperature) of the liquid waste.

11.4.2 pH

See 11.2.3.

11.5 Liquid waste non miscible with water

11.5.1 Procedure

A liquid/liquid extraction is performed by bringing water in contact with the test portion. In order to compare liquid waste with the other waste materials, the parameters used for this procedure should be the same as those defined in other extraction procedures. The liquid/liquid extraction is performed as follows.

Place the test portion with the total mass M corresponding to (90 ± 5) g of dry mass M_D in a bottle (4.9) with a nominal volume of 1 l to minimise headspace.

Ecotoxicological tests usually require several litres of water extract leading to an adjustment of mass of the test portion. When a large volume of water extract is required, it is possible to perform the liquid/liquid extraction in a vessel of appropriate capacity into which the corresponding number of test portions (a mass of (90 ± 5) g) are introduced or to perform parallel liquid/liquid extraction at the same time with an appropriate number of vessels of 1 l capacity each (see above). In that case, all the individual water extracts shall be mixed in order to obtain the test sample. When the liquid/liquid extraction is performed in one vessel, the size of the vessel shall be appropriate to minimise the headspace (≤ 5 % of the total capacity of the vessel).

Add an amount of leachant establishing a ratio of 10 during the extraction.

Place the capped bottle in an agitation device (an end-over-end tumbler 5 rpm to 10 rpm or rollertable rotating at about 10 rpm). Agitate for (24 ± 0,5) h at a temperature within the range 15 °C to 25 °C.

11.5.2 Liquid/liquid separation

Allow the two phases to separate for (15 ± 5) min in the same temperature conditions as defined above. When separation of the two liquid phases is not achieved within this time period, the mixture is centrifuged for 30 min at 2 500 g.

Filter the water extract over a 0,45 µm membrane filter using a vacuum or pressure filtration device (4.6). Rinsing of the filter with water or another solvent is not allowed after filtration.

11.5.3 pH

See 11.2.3.

11.6 Preparation of test mixtures

The water extract is mixed thoroughly with the dilution medium in order to obtain homogenous test mixtures. The water extract is diluted with the dilution medium specified in the selected ecotoxicity test method.

Test mixtures are expressed in percentages (volume of water extract per total volume of test mixture).

The test procedure is selected according to the aim of the study: determination of a dose-effect relationship or limit test at a given dilution.

12 Test report

The test report shall include the following information:

a) reference to this European Standard;

b) waste characterization (pH, dry matter content, water holding capacity, granularity etc);

c) complete description of sampling conditions;

d) complete description of transport and storage conditions (temperature, duration, containers etc);

e) complete description of waste pre-treatment, including method of size reduction, fraction above 4 mm, fraction of non crushable material (if relevant information);

f) complete description of water extraction procedure including type, size and material of the vessel being used; description of the liquid-solid separation procedure; volume of water extract;

g) description of storage conditions of water extract (if relevant information);

h) presentation of the results and the test conditions according to the ecotoxicity test standards.

Annex A
(normative)

Preparation of test mixtures according to the ecotoxicity tests to be performed

Table A.1 — Preparation of test mixtures (3.13) according to the ecotoxicity tests to be performed

Category of waste	Preparation of water extract	Test mixtures for tests performed on aquatic organisms	Test mixtures for tests performed on terrestrial organisms
Waste with particle size below 4 mm (originally or after pre-treatment)	Leaching test according to EN 12457-2	Eluate is diluted with the dilution medium of the selected ecotoxicity tests.	- Waste is mixed with the dilution medium [a]. - Eluate is added to the water used to wet the dilution medium.
Sludge	Leaching test according to EN 12457-2	Eluate is diluted with the dilution medium of the selected ecotoxicity tests.	- Waste is mixed with the dilution medium [b]. - Eluate is added to the water used to wet the dilution medium.
Liquid sludge	Separation of liquid and particulate matter by centrifugation	The water extract is diluted with the dilution medium of the selected ecotoxicity tests.	The water extract is added to the water used to wet the dilution medium.
Liquid waste (miscible with water)	Not relevant	The waste is diluted with the dilution medium of the selected ecotoxicity tests.	Waste is added to the water used to wet the dilution medium.
Liquid waste (non miscible with water)	Liquid/liquid extraction (Liquid/liquid ratio = 10)	The water extract is diluted with the dilution medium of the selected ecotoxicity tests.	- Waste is mixed with the dilution medium. - The water extract is added to the water used to wet the medium.

[a] For paste-like waste, the range of concentrations may be limited for waste with a high moisture content ratio.

[b] The range of concentrations may be limited for waste with a high moisture content ratio.

Annex B
(informative)

Ecotoxicity tests considered to establish this European Standard

B.1 General

The ecotoxicity tests listed in the following tables were considered to establish this European Standard. These test methods are commonly used for hazard assessment of chemicals, water or soil quality. All the test methods, listed in the following tables, are standardized, at least at a national level; or more often, these test methods are internationally harmonized by CEN, ISO or OECD. As such, these ecotoxicity tests have gained a scientific credibility.

These ecotoxicity tests fulfill the following criteria: ecological relevance, sensitivity to toxicants, reproducibility of results, practicability, reliability and robustness.

Each table describes briefly the test protocol and provides information about its application on waste or water extracts from waste. It also highlights possible limitations of the test method.

This compilation of tests is not exhaustive and does not constitute a recommendation on tests to be performed in order to characterize ecotoxicological properties of wastes. Other standardized ecotoxicity test methods could also be used for characterization of wastes.

B.2 Terrestrial tests methods

B.2.1 Earthworms – Acute toxicity

1. Title of the test	Effects of pollutants on earthworms (*Eisenia fetida*) – Part 1: Determination of acute toxicity using artificial soil substrate
2. Harmonization	International
3. References	ISO 11268-1, OECD 207
4. Principle	Determination of the percentage of mortality of adult earthworms placed in a defined substrate containing the test substance.
5. Test type	Acute, static
6. Test organism	Earthworm
Breeding stocks	*Eisenia fetida* Savigny, *Eisenia andrei* Bouché
Age	> two months
Feeding	No
7. Dilution medium	Artificial soil
Volume	500 g (dry mass) / container
8. Test conditions	
Test chamber	Enclosure capable of being controlled
Temperature	(20 ± 2) °C
pH	6,0 ± 0,5
Light intensity/quality	400 lux to 800 lux
Photoperiod	12 h/12 h or 16 h/8 h (day/night)
Soil moisture	40 % to 60 % WHC
9. Number of replicates	Four (10 worms per replicate)
10. Test duration/incubation	14 days
11. Control	Artificial soil
12. Validity criteria	Control: mortality < 10 %, biomass loss ≤ 20 %
13. Reference substance	Chloroacetamide, LC 50 14 days: 20 mg/kg to 80 mg/kg
14. Statistics	Multiple t-test
15. Test parameter(s)	Mortality, biomass
16. Endpoints	LC 50
17. Application to wastes and water extracts from wastes: limitations and comments — The test originally was designed for testing substances added to an artificial soil. Due to its endpoint the sensitivity is limited, but is sufficient for screening purposes. — The test organism is not sensitive to modifications of the test mixture granulometry.	

B.2.2 Earthworms – Effects on reproduction

1. Title of the test	Effects of pollutants on earthworms (*Eisenia fetida*) – Part 2: Determination of effects on reproduction
2. Harmonization	International
3. References	ISO 11268-2
4. Principle	Determination of the percentage of mortality, of effects on growth and reproduction of adult earthworms placed in a defined substrate containing the test substance
5. Test type	Subchronic, static
6. Test organism	Earthworm
Breeding stocks	*Eisenia fetida* Savigny, *Eisenia andrei* Bouché
Age	> 2 months, < 1 year
Feeding	Cow dung
7. Dilution medium	Artificial soil
Volume	500 g to 600 g (dry mass) / container
8. Test conditions	
Test chamber	Enclosure capable of being controlled
Temperature	(20 ± 2) °C
pH	6,0 ± 0,5
Light intensity/quality	400 lux to 800 lux
Photoperiod	12 h/12 h or 16 h/8 h (day/night)
Soil moisture	40 % to 60 % WHC
9. Number of replicates	Four (10 worms per replicate)
10. Test duration/incubation	8 weeks
11. Control	Artificial soil
12. Validity criteria	Control: 30 juveniles/container, CV ≤ 30 %, adult mortality ≤ 10 %
13. Reference substance	Carbendazim: 1 ≤ LOEC ≤ 5 mg/ai Carbendazim
14. Statistics	Multiple t-test, u – test, regression analysis
15. Test parameter(s)	Mortality, growth, reproduction
16. Endpoints	EC 50, NOEC
17. Application to wastes and water extracts from wastes: limitations and comments — Acute and reproduction test can be combined (reproduction is the most sensitive parameter). — Effects expression is strongly influenced by pH deviation from the given range (reproduction test). — The test organism is not sensitive to modifications of the test mixture granulometry.	

B.2.3 Collembola – Effects on reproduction

1. Title of the test	Effects of pollutants on collembola (*Folsomia candida*) – Method for the determination of effects on reproduction
2. Harmonization	International
3. References	ISO 11267
4. Principle	Determination of the effect on reproduction of springtails incubated over a four week test period
5. Test type	Static subchronic
6. Test organism	Springtails
Breeding stocks	*Folsomia candida* Willem 1902
Age	10 days to 12 days
Feeding	Dry yeast
7. Dilution medium	Artificial soil
Volume	30 g (wet weight) / container
8. Test conditions	
Test chamber	Enclosures
Temperature	(20 ± 2) °C
pH	6,0 ± 0,5
Light intensity/quality	400 lux to 800 lux
Photoperiod	12 h/12 h or 16 h/8 h (day/night)
Soil moisture	40 % to 60 % of total WHC
9. Number of replicates	Four (10 springtails per replicate)
10. Test duration/incubation	28 days
11. Control	Artificial soil
12. Validity criteria	Control: mortality < 20 %, minimum reproduction ≥ 100 juveniles, CV ≤ 30 %
13. Reference substance	E 605 forte (a.i. 507,5 g/l parathion): effects on reproduction in the range 0,10 mg/kg to 0,18mg/kg. Betanal plus (a.i. 160 g/l phenmedipham) effects on reproduction in the range 100 mg/kg to 200 mg/kg
14. Statistics	Multiple t-test, u-test, regression analysis
15. Test parameter(s)	Mortality of adults, inhibition of reproduction
16. Endpoints	EC 50, NOEC (EC_x)

17. Application to wastes and water extracts from wastes: limitations and comments

— The test originally was designed for testing substances added to an artificial soil. The collembolan species F. candida prefer air-filled soil pores. A crumbly structure of the test substrate is therefore achieved throughout all waste dilutions used in the test.

— Effects expression is strongly influenced by pH deviation from the given range (reproduction test).

B.2.4 Coleoptera – Acute test

AC1

1. Title of the test	Effects of pollutants on insect larvae (*Oxythyrea funesta*) – Determination of acute toxicity using artificial soil substrate
2. Harmonization	International
3. References	ISO 20963; NF X 31-260
4. Principle	Determination of the percent mortality of *Cetoniinae* larvae during growth period after 10 days
5. Test type	Static acute
6. Test organism	Third instar larvae of the species *Oxythyrea funesta*
Breeding stocks	
Age	≈ 15 days, wet mass 100 mg to 200 mg
Feeding	Dried and finely ground cow-dung
7. Dilution medium	Artificial soil
Volume	300 g (dry weight) /container
8. Test conditions	
Test chamber	Enclosures
Temperature	(26 ± 1) °C
PH	6,0 ± 0,5
Light intensity/quality	Darkness
Photoperiod	-
Soil moisture	50 % of total WHC
9. Number of replicates	Three (10 larvae per replicate)
10. Test duration/incubation	10 days
11. Control	Artificial Soil
12. Validity criteria	Control: mortality < 10 %, biomass increase in the controls > 80 %
13. Reference substance	ISO 20963: 2,4,5 trichlorophenol, 60 ≤ LC 50 ≤ 180 mg/kg NF X31-260:$HgCl_2$, 15 mg/kg ≤ LC 50 ≤ 45 mg/kg (as Hg^{2+})
14. Statistics	Multiple t-test, u-test, regression analysis
15. Test parameter(s)	Mortality, growth (optional)
16. Endpoints	LC 50, NOEC (EC_x)
17. Application to wastes and water extracts from wastes: limitations and comments The test organism is not sensitive to modifications of the test mixture granulometry.	

AC1

B.2.5 Enchytraeid – Reproduction test

1. Title of the test	Enchytraeid reproduction test
2. Harmonization	International
3. References	ISO 16387 , OECD test guideline n°220
4. Principle	Adult enchytraeid worms are exposed to a test substance mixed in artificial soil. After a test period of 6 weeks the effect on the sublethal parameter reproduction is determined. The test design includes the investigation of possible lethal effects (mortality) on the parental enchytraeids.
5. Test type	Subchronic, static
6. Test organism	Enchytraeids
Breeding stocks	*Enchytraeus albidus* Henle 1837 and other *Enchytraeus sp.*
Age	Adult worms with eggs in the clitellum region
Feeding	Rolled oats
7. Dilution medium	Artificial Soil
Volume	20 g (dry mass) / container
8. Test conditions	
Test chamber	Enclosure capable of being controlled
Temperature	(20 ± 2) °C
pH	6,0 ± 0,5
Light intensity/quality	400 lux to 800 lux
Photoperiod	Preferably 16 h/8 h (day/night)
Soil moisture	40 % WHC to 60 % WHC
9. Number of replicates	Two to four depending on the test design (NOEC/EC_x); 6 to 8 control vessels (10 enchytraeids per replicate)
10. Test duration/incubation	6 weeks (final test)
11. Control	Artificial soil
12. Validity criteria	Control: Mortality ≤ 20 %, minimum number of juveniles 25/vessel, CV ≤ 50 %
13. Reference substance	Carbendazim: EC 50 1,2 mg a.i./kg ± 0,8 mg a.i./kg
14. Statistics	Multiple t-test, regression analysis, probit analysis
15. Test parameter(s)	Mortality, reproduction
16. Endpoints	LC 50, NOEC, EC_x
17. Application to wastes and water extracts from wastes: limitations and comments — The test organism is sensitive to modifications of the test mixture granulometry. — Effects expression is strongly influenced by pH deviation from the given range (reproduction test). — Other species are appropriate for other pH range.	

B.2.6 Soil Flora – Inhibition of root growth

1. Title of the test	Determination of the effects of pollutants on soil flora – Part 1: Method for the measurement of inhibition of root growth
2. Harmonization	International
3. References	ISO 11269-1
4. Principle	Growth of pregerminated seeds under controlled conditions. Differences in the root lengths of seedlings grown in any test medium compared to the controls are indicative of an effect.
5. Test type	Acute, static
6. Test organism	Barley (*Hordeum vulgare L.)*
Breeding stocks	Variety CV triumph or other varieties
Age	Seeds
Feeding	No
7. Dilution medium	Good quality soil of the same textural class as the soil under test, sand
Volume	500 g (dry mass) /container
8. Test conditions	
Test chamber	Growth cabinet
Temperature	Day (20 ± 2) °C ; night (16 ± 2) °C
pH	
Light intensity/quality	25 000 lm/m^2
Photoperiod	12 h/12 h or 16 h/8 h (day/night)
Soil moisture	70 % WHC ± 5 % WHC
9. Number of replicates	Three (6 seeds per replicate)
10. Test duration/incubation	7 days (Germination of seeds two days; incubation time 5 days)
11. Control	Good quality soil of the same textural class as the soil under test, sand
12. Validity criteria	None
13. Reference substance	None
14. Statistics	Multiple t-test
15. Test parameter(s)	Root elongation
16. Endpoints	NOEC, LOEC

17. Application to wastes and water extracts from wastes: limitations and comments

— The method is applicable to all soils, soil materials, wastes or chemicals which may be applied to soil except where the contaminant is highly volatile or only affects photosynthesis. The method can be used to compare soils or to determine the effect of added substances. The method is not intended for use as a measure of the ability of the soil to support sustained plant growth. In the case of contaminated soil, the individual chemicals are unidentified and therefore correct information on their properties cannot be selected. No incorporation is required, but it may be necessary to dilute with uncontaminated soil or sand before testing.

— The proposed plant test is not suitable for dilutions of waste with a very disturbed structure (e.g. rubble). In these cases an inhibition may result without relevant contamination.

B.2.7 Effects on emergence and growth

1. Title of the test	Soil quality – Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants
2. Harmonization	International
3. References	ISO 11269-2 (under revision)
4. Principle	Emergence and early growth response of a variety of terrestrial plant species to various concentrations of a chemical added to the test soil
5. Test type	Subchronic, static
6. Test organism	Monocotyledonous and dicotyledonous plants
Breeding stocks	Various species
Age	Seeds
Feeding	No
7. Dilution medium	Soil [pH 5 to 7,5 ; C.O. < 1,5 % ; fine particles (less than 20 µm) < 20 %]
Volume	Pot with top internal diameter 85 mm to 95 mm
8. Test conditions	
Test chamber	Phytotron, plant growth room, green house
Temperature	Suitable for normal growth
pH	
Light intensity/quality	
Photoperiod	
Soil moisture	
9. Number of replicates	Four (10 seeds per replicate)
10. Test duration/incubation	14 days to 21 days after 50 % emergence in the control pots
11. Control	Soil [pH 5 to 7,5 ; C.O. < 1,5 % ; fine particles (less than 20 µm) < 20 %]
12. Validity criteria	7 healthy seedlings per control pot
13. Reference substance	Sodium trichloroacetate, boric acid
14. Statistics	
15. Test parameter(s)	Emergence, growth (seedlings fresh mass or seedlings dry mass)
16. Endpoints	NOEC; LOEC, ECx

17. Application to wastes and water extracts from wastes: limitations and comments

— Same as for ISO 11269-1.

— For test mixtures including high percentage of waste, inhibition of growth may occur due to nutrient deficiency.

— For small seeds (e.g. lettuce), an inhibition of emergence may occur for test mixtures with a very disturbed structure even if the waste does not contain contaminant.

— As with other bioassays proposed, tests with higher plants are designed to consider the pollutant situation in the soil and the bioavailability of pollutants to the test organisms which are not detected by chemical analysis. By applying a test period of at least 14 days, short-term changes in soil by the test plant itself are included.

— The accumulation of pollutants in soils, their metabolism and effects on consumers are not investigated in the test. They also do not apply for assessment of soil fertility and productivity.

B.2.8 Ammonium oxidation – Rapid test

1. Title of the test	Ammonium oxidation – Rapid method to test potential nitrification in soil
2. Harmonization	International
3. References	ISO 15685
4. Principle	Autotrophic ammonium oxidising bacteria in soil are exposed to ammonium sulphate in a soil slurry buffered at pH 7,2. The accumulation rate of the nitrite during 6 h of incubation is taken as an estimate of the activity.
5. Test type	
6. Test organism	Autotrophic ammonium oxidising bacteria present in the test soil
Breeding stocks	Does not apply to this test
Age	Does not apply to this test
Feeding	Does not apply to this test
7. Dilution medium	Soil slurry; soil treated according to ISO 10381-6
Volume	25 g moist soil in 100 ml medium
8. Test conditions	
Test chamber	Glass flasks (of appropriate volume) on an oscillating table
Temperature	25 °C
pH	Approximately 7,2
Light intensity/quality	Not specified
Photoperiod	-
Soil moisture	Not applicable
9. Number of replicates	Two
10. Test duration/incubation	6 h
11. Control	None
12. Validity criteria	Ammonium-oxidising activity of soil (200 ngN/g soil/hour to 800 ngN/g soil/hour)
13. Reference substance	None
14. Statistics	Mean, standard deviation
15. Test parameter(s)	Rate of ammonium oxidation
16. Endpoints	In tests of chemicals EC 10, EC 50
17. Application to wastes and water extracts from wastes: limitations and comments — The test is a rapid method to determine the potential rate of ammonium oxidation, the first step in the autotrophic nitrification in nitrifying soils. The measurement can be taken as an assessment of the potential activity of nitrifying populations at the time of sampling. It can be used as a rapid screening test for monitoring of soil quality, and is suitable for testing the effects of waste materials in soil. Wastes with limited water solubility require special attention. — This test method is not applicable for the determination of a dose-effect relationship. — Other limitation: pH buffered at 7,2.	

B.2.9 Mineralization and nitrification

1. Title of the test	Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
2. Harmonization	International
3. References	ISO 14238
4. Principle	The rates or extent of N-mineralization in aerobic soils are determined by measuring the concentrations of ammonium, nitrite and nitrate released during mineralization of nitrogen contained in the soil organic matter, or during mineralization of an added nitrogenous organic compound.
5. Test type	Basic mineralization test, toxicity test
6. Test organism	Microbial organisms present in a test soil
Breeding stocks	Does not apply to this test
Age	Does not apply to this test
Feeding	Does not apply to this test
7. Dilution medium	Field soil treated according to ISO 10381-6
Volume	50 g to 100 g recommended; or bulk incubation with sub-sampling
8. Test conditions	
Test chamber	Appropriate container; soil layer < 3 cm.
Temperature	(20 ± 2) °C
pH	Intrinsic pH of the soil
Light intensity/quality	Darkness (toxicity test)
Photoperiod	-
Soil moisture	40 % WHC to 60 % WHC or ca. 0,02 MPa suction pressure (toxicity test)
9. Number of replicates	Three
10. Test duration/incubation	28 days
11. Control	Soil
12. Validity criteria	None
13. Reference substance	None
14. Statistics	Regression analysis
15. Test parameter(s)	Mineralization rate, nitrification rate
16. Endpoints	Concentration of mineral N; inhibitory dose (ID %)
17. Application to wastes and water extracts from wastes: limitations and comments — The International Standard describes laboratory procedures in different soils, or for comparison of N-mineralization in one soil collected at different times of the year. — To determine the influence of chemicals or wastes on N-mineralization a simplified test design can be used allowing for the establishment of dose-effect relationships. However, this method is not applicable for testing mixtures including high percentagse of waste. — The experience on monitoring the soil quality of polluted soils or the characterization of waste diluted with soil is limited. Care should be taken to collect unpolluted control soil.	

B.2.10 Juvenile land snails (*Helix aspersa*)

1. Title of the test	Effects of pollutants on juvenile land snails (Helicidae)- Determination of the effects on growth by soil contamination.
2. Harmonization	International
3. References	ISO/DIS 15952
4. Principle	Determination of the effect on survival and growth of juvenile terrestrial snails exposed four weeks to a defined substrate containing the material under test.
5. Test type	Semi-static (substrate can be renewed each week) or static; subchronic
6. Test organism	Snails
Breeding stocks	*Helix aspersa aspersa* Müller (recommended species) and other species of the Helicidae family
Age	Three to five weeks
Feeding	Flour-based feed
7. Dilution medium	Artificial soil (or natural soil)
Volume	≈ 150 g (dry mass artificial substrate)/container
8. Test conditions	
Test chamber	Enclosures
Temperature	(20 ± 2) °C
pH	6 ± 0,5
Light intensity/quality	50 lx to 100 lx
Photoperiod	18 h/6 h (day/night)
Soil moisture	50 % of total WHC to 60 % of total WHC
9. Number of replicates	Three (5 snails per replicate)
10. Test duration/incubation	4 weeks
11. Control	Artificial soil (or natural soil)
12. Validity criteria	Control: mortality ≤ 10 %; mean mass multiplied by 4
13. Reference substance	$CdCl_2$: 398 mg/kg < EC50 < 622 mg/kg (expressed as Cd^{2+})
14. Statistics	Regression analysis; multiple t-test
15. Test parameter(s)	Growth inhibition, survival
16. Endpoints	EC 50 (ECx), NOEC, LC 50
17. Application to wastes and water extracts from wastes: limitations and comments The method is applicable to soils, soil materials, waste or chemicals which may be applied to soil (soils and solid waste samples are sieved at 4 mm).	

B.3 Aquatic tests methods

B.3.1 *Daphnia magna* – Inhibition of mobility

1.	**Title of the test**	Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea)
2.	**Harmonisation**	International
3.	**References**	EN ISO 6341
4.	**Principle**	Determination of the effect of chemicals, water samples and wastewater on mobility of young daphnids.
5.	**Test type**	Acute, static/semi-static
6.	**Test organism**	Daphnids
	Breeding stock	*Daphnia magna* Straus
	Age of test organism	< 24 h
	Feeding	None
7.	**Dilution medium**	Freshwater or synthetic reconstituted medium
	Volume	10 ml
8.	**Test conditions**	
	Test chamber size	20 ml
	Temperature	(20 ± 2) °C
	pH	7,8 ± 0,2
	Light intensity/quality	Darkness
	Photoperiod	
9.	**Number of container, number of replicates**	5 daphnids per vessel, four replicates
10.	**Test duration**	24 h/48 h
11.	**Control**	Freshwater or synthetic reconstituted medium
12.	**Validity criteria**	Control mortality ≤ 10 % ; 0_2 concentration ≥ 2 mg/l ; sensitivity to $K_2Cr_2O_7$
13.	**Reference substance**	$K_2Cr_2O_7$: 0,6 mg/l ≤ EC 50 24h ≤ 2,1 mg/l
14.	**Statistics**	Regression, probits
15.	**Test parameter(s)**	Immobilisation
16.	**Endpoints**	EC 50

17. Application to wastes and water extracts from wastes: limitations and comments

— Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).

Survival may be affected by low oxygen content.

B.3.2 *Daphnia magna* – Inhibition of reproduction

1.	**Title of the test**	Determination of long term toxicity of substances to *Daphnia magna* Straus (Cladocera crustacea)
2.	**Harmonisation**	International
3.	**References**	ISO 10706 ; OECD guideline n°211
4.	**Principle**	Inhibition of reproduction and survival of *Daphnia magna*
5.	**Test type**	Chronic, semi-static
6.	**Test organism**	*Daphnia magna* from the second to the fifth brood obtained by acyclical parthenogenesis
	Breeding stock	*Daphnia magna* Straus
	Age of test organism	< 24h
	Feeding	⟨AC1⟩ Unicellular algae (*Chlorella* sp., *Pseudokirchneriella subcapitata* or *Scenedesmus subspicatus*). 0,1 mg carbon/animal/day to 0,2 mg carbon/animal/day ⟨AC1⟩
7.	**Dilution medium**	ELENDT M4 or M7 synthetic medium ; ASTM reconstituted hard freshwater
	Volume	50 ml to 100 ml
8.	**Test conditions**	
	Test chamber size	50 ml to - 100 ml beakers
	Temperature	Within 18 °C to 22 °C, variations within less than 2 °C
	pH	6,0 to 9,0
	Light intensity/quality	< 1 200 lux
	Photoperiod	16 h light
9.	**Number of container, number of replicates**	5 concentrations × 10 replicates (one animal per vessel is recommended)
10.	**Test duration**	21 days
11.	**Negative control dilution**	ELENDT M4 or M7 ; ASTM
12.	**Validity criteria**	Mortality of adults or living males ≤ 20 % in the control, mean number of living offspring per parent ≥ 60 in the control, coefficient of variance for control fecundity < 20 %
13.	**Positive control/reference toxicant, mean EC (and CV)**	The Daphnid culture may be controlled using $K_2Cr_2O_7$ (effect on mobility)
14.	**Statistics**	Dunnett or Williams test and regression
15.	**Test parameter**	Mortality of adults, inhibition of reproduction
16.	**End points**	ECx, NOEC

17. Application to wastes and water extracts from wastes: limitations and comments

— This test is mainly used for pure substances.
— Bioavailability of metals ions can be reduced by complexation with EDTA from the test medium (ELENDT M4 or M7).
— Turbid and coloured samples may disturb mother and neonates behaviour and counting.
— When the sample is likely to interfere with the test (protozoa, micro-organisms…) a filtration or centrifugation of the sample can be performed.
— Other limitations: volume of water extract necessary to perform the test and test duration.
— Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).

B.3.3 *Ceriodaphnia dubia* reproduction test

1.	**Title of the test**	Determination of chronic toxicity to *Ceriodaphnia dubia* in 7 days – Population growth inhibition test
2.	**Harmonisation**	National (ISO standard in preparation)
3.	**References**	NF T 90-376 ; ISO/NWI 20665 (American and Canadian standards)
4.	**Principle**	Inhibition of reproduction and mortality of *Ceriodaphnia dubia*
5.	**Test type**	Chronic, semi-static
6.	**Test organism**	*Ceriodaphnia dubia* obtained by acyclical parthenogenesis for at least three generations
	Breeding stock	*Ceriodaphnia dubia* (over 6 days and less than 14 days old)
	Age of test organism	< 24 h ; taken from a brood comprising at least 8 neonates
	Feeding	*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* (previously known as *Selenastrum capricornutum*) and fish food
7.	**Dilution medium**	ELENDT M4 synthetic medium
	Volume	50 ml
8.	**Test conditions**	
	Test chamber size	100 ml containers
	Temperature	(25 ± 1) °C
	pH	8,0 ± 0,3
	Light intensity/quality	< 300 lux
	Photoperiod	16 h light
9.	**Number of container, number of replicates**	At least 5 concentrations (separation factor < 3,2) ; 10 replicates (one animal per vessel)
10.	**Test duration**	7 days
11.	**Control**	ELENDT M4 synthetic medium
12.	**Validity criteria**	Mortality of adults ≤ 20 % in the control batch; proportion of males in the parent generation < 20 %; average number of offspring per live mother ≥ 15 in the control batch; 60 % of mothers alive produce a minimum of three broods in the control batch.
13.	**Reference substance**	Sodium pentachlorophenate: 170 µg/l to 330 µg/l (EC_{c50}); Pentahydrate copper sulfate: 135 µg/l to 311 µg/l (EC_{c50} expressed as Cu^{2+}).
14.	**Statistics**	Logistic model
15.	**Test parameter(s)**	Mortality of mothers, population growth inhibition
16.	**End points**	ECx, NOEC (optional)

17. Application to wastes and water extracts from wastes: limitations and comments

— Bioavailability of metal ions can be reduced by complexation with EDTA from the test medium. This population growth inhibition test can be carried out using other test medium.

— Turbid and coloured samples may disturb mother and neonates behaviour and counting.

— When the sample is likely to interfere with the test (protozoa, micro-organisms…), a filtration or centrifugation of the sample can be performed.

— Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).

— Survival and reproduction may be affected by low oxygen content.

B.3.4 *Brachionus calicyflorus* reproduction test

1.	**Title of the test**	Determination of chronic toxicity to *Brachionus calyciflorus* in 48 h– Population growth inhibition test
2.	**Harmonisation**	National (ISO standard in preparation)
3.	**References**	NF T 90-377 ; ISO/NWI 20666
4.	**Principle**	Inhibition of population growth of *Brachionus calyciflorus*
5.	**Test type**	Chronic, static
6.	**Test organism**	*Brachionus calyciflorus*
	Breeding stock	*Brachionus calyciflorus*
	Age of test organism	< 2 h
	Feeding	*Chlorella vulgaris*
7.	**Dilution medium**	Synthetic reconstituted medium
	Volume	1 ml
8.	**Test conditions**	
	Test chamber size	Microplate
	Temperature	(25 ± 1) °C
	pH	7,6 ± 0,3
	Light intensity/quality	Darkness
	Photoperiod	
9.	**Number of replicates**	At least 5 concentrations (separation factor < 3,2); 8 replicates (one animal per vessel)
10.	**Test duration**	48 h
11.	**Control**	Synthetic reconstituted medium
12.	**Validity criteria**	Reproduction of *Brachionus* is observed in at least 7 replicates out of 8; the average number of live female *Brachionus* counted per well ≥ 3 in the control batch.
13.	**Reference substance**	Sodium pentachlorophenate: EC_{c50} average 548 µg/l; SD 232 Pentahydrate copper sulfate: EC_{c50} average 53,5 µg/l; SD 17,7 (EC_{c50} expressed as Cu^{2+})
14.	**Statistics**	Logistic model
15.	**Test parameter(s)**	Population growth inhibition
16.	**Endpoints**	ECx, NOEC (optional)

17. Application to wastes and water extracts from wastes: limitations and comments

— Turbid and coloured samples may disturb mother and neonates behaviour and counting.

— When the sample is likely to interfere with the test (protozoa, micro-organisms…), a filtration or centrifugation of the sample can be performed.

— Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).

— Survival and reproduction may be affected by low oxygen content.

B.3.5 *Vibrio fischeri* – Luminescent bacteria test

1.	**Title of the test**	Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)
2.	**Harmonisation**	International
3.	**References**	EN ISO 11348-1, 2, 3
4.	**Principle**	Short term inhibition of effect of toxicants on bacterial luminescence
5.	**Test type**	Acute, static
6.	**Test organism**	*Vibrio fischeri* (saltwater luminescent bacteria)
	Breeding stock	*Vibrio fischeri* NRRL B-11177 freshly prepared (EN ISO 11348-1), liquid-dried (EN ISO 11348-2), freeze-dried (EN ISO 11348-3)
	Age of test organism	
	Feeding	None
7.	**Dilution medium**	NaCl 2 %
	Volume	1 ml
8.	**Test conditions**	
	Test chamber size	test tubes
	Temperature	(15 ± 1) °C
	pH	6,0 to 8,5
	Light intensity/quality	Darkness
	Photoperiod	None
9.	**Number of container, number of replicates**	At least 5 concentrations; one or two replicates
10.	**Test duration**	15 min and 30 min
11.	**Control**	NaCl 2 %
12.	**Validity criteria**	Correction factor within the range 0,6 to 1,8 (30 min incubation time); variation between two measurements < 3 % of mean value sensitivity to reference toxicants; sensitivity to reference substances.
13.	**Reference substance**	3,5-dinitrophenol, $ZnSO_4$, $K_2Cr_2O_7$
14.	**Statistics**	Regression
15.	**Test parameter(s)**	Inhibition of luminescence
16.	**Endpoints**	EC 20 ; EC 50

17. Application to wastes and water extracts from wastes: limitations and comments

— Turbid samples may interfere with measurement of bacterial luminescence.

— If the pH of the sample is outside the range 6,0 to 8,5, pH is adjusted to 7,0 ± 0,2.

— The range of sensitivity for reference substances differs according to the preparation of bacteria (freshly prepared, liquid-dried, freeze-dried).

— Highest tested concentration: 80 %.

B.3.6 *Pseudomonas putida* growth inhibition test

1.	**Title of the test**	Water quality – *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test)
2.	**Harmonisation**	International
3.	**References**	EN ISO 10712
4.	**Principle**	Determination of inhibitory effects of water, wastewater samples, chemicals on the growth of *Pseudomonas putida*
5.	**Test type**	Chronic
6.	**Test organism**	*Pseudomonas putida*
	Breeding stock	*Pseudomonas putida* (strain Migula ; strain NCIB 9494)
	Age of test organism	Inoculum from an exponentially growing pre-culture (5 h ± 0,5 h) ; 5 FTU in final volume
	Feeding	Nutrient solutions
7.	**Dilution medium**	Deionized water
	Volume	100 ml
8.	**Test conditions**	
	Test chamber size	250 ml conical flasks
	Temperature	(23 ± 1) °C
	pH	
	Light intensity/quality	Darkness
	Photoperiod	-
9.	**Number of container, number of replicates**	At least 5 concentrations; three replicates ; 6 controls
10.	**Test duration**	16 h ± 1 h
11.	**Control**	Nutrient solutions (10 %), deionised water (90 %)
12.	**Validity criteria**	Control: multiplication factor ≥ 60; sensitivity to reference substance
13.	**Reference substance**	3,5 dichlorophenol: 10 mg/l ≤ EC 50 ≤ 30 mg/l
14.	**Statistics**	Regression model
15.	**Test parameter(s)**	Multiplication of cells
16.	**Endpoints**	EC 10 ; EC 50
17. Application to wastes and water extracts from wastes: limitations and comments		
—	Coloured samples may interfere with measurement of bacterial growth.	
—	High particle density in the sample may disturb growth measurements.	
—	Volatile substances may be stripped by agitation of the tests flasks.	
—	Highest tested concentration: 80 %.	

B.3.7 Freshwater algal growth inhibition test

1.	**Title of the test**	Water Quality – Freshwater algal growth inhibition test with *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata*
2.	**Harmonisation**	International
3.	**References**	OECD 201; EEC method C3 ; ISO 8692
4.	**Principle**	Determination of effect on growth of a population of unicellular algae
5.	**Test type**	Chronic, static
6.	**Test organism**	Planktonic freshwater unicellular algae
	Breeding stock	*Desmodesmus subspicatus (previously Scenedesmus subspicatus)* or *Pseudokirchneriella subcapitata (*previously *Selenastrum capricornutum)*
	Age of test organism	Inoculum from an exponentially growing pre-culture ;10^4 cells/ml in final volume
	Feeding	Nutrient concentrate (10 % of final volume)
7.	**Dilution medium**	Deionized water
	Volume	~ 100 ml (alternatives on small volumes)
8.	**Test conditions**	
	Test chamber size	250 ml conical flasks
	Temperature	(23 ± 2) °C
	pH	8,3 ± 0,2 (nutrient concentrate)
	Light intensity/quality	35 photons/m²/s to 70 x 10^{18} photons/m²/s (400 nm to 700 nm) ; 6 000 lux to 10 000 lux
	Photoperiod	Continuous light
9.	**Number of container, number of replicates**	at least 5 concentrations (three replicates) + control (6 replicates)
10.	**Test duration**	72 h
11.	**Control**	Nutrient concentrate (10 %) + deionised water (90 %)
12.	**Validity criteria**	Control population increase > 16 within 72 h (OECD); > 67 (ISO); CV of growth rate in controls < 5 % : pH increase in controls < 1,5
13.	**Reference substance**	$K_2Cr_2O_7$ EC 50 growth rate 0,92 mg/l to 1,46 mg/l (*P. subcapitata*) 3,5 dichlorophenol EC 50 growth rate 2,08 mg/l to 4,68 mg/l (*P. subcapitata*)
14.	**Statistics**	Multisample comparison, regression
15.	**Test parameter(s)**	Growth rate or biomass integral
16.	**Endpoints**	NOEC and ECx (x = 10, 20, 50)

17. Application to wastes and water extracts from wastes: limitations and comments

— Chemicals absorbing light in the range 400 mm to 700 nm may interfere with algal growth for physical reasons rather than by toxic action.
— High particle density in the sample may disturb growth measurements (particle counter, spectrophotometer).
— Bacteria, algae from other species and algal predators may interfere with algal growth.
— Bioavailability of metals ions can be reduced by complexation with EDTA from the test medium.
— Volatile substances may be stripped by agitation of the tests flasks.
— See ISO 14442 for information on difficult substances management.
— Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).

B.3.8 *Lemna minor* – Growth inhibition test

1.	**Title of the test**	Water quality – Duckweed growth inhibition – Determination of the toxic effect of water constituents and waste water to duckweed
2.	**Harmonisation**	International
3.	**References**	ISO/DIS 20079, OECD test guideline n°221
4.	**Principle**	Determination of effect on growth of the aquatic plant *Lemna minor*
5.	**Test type**	Chronic, static
6.	**Test organism**	Monocotyledonous, free-floating angiosperm
	Breeding stock	*Lemna minor*
	Age of test organism	Inoculum from culture at least 7 days to 10 days adaption to test conditions; quality criteria: growth rate ≥ 0,275 d^{-1}
	Feeding	Nutritive mineral medium (mod. Steinberg)
7.	**Dilution medium**	Water
	Volume	≥ 100 ml
8.	**Test conditions**	
	Test chamber size	≥ 150 ml cylindrical vessels
	Temperature	(24 ± 2) °C
	pH	5,5 ± 0,2
	Light intensity/quality	85 µE/m^2/s to 125 µE/m^2/s (400 nm to 700 nm) ± 15 %, at level of water; light from side (under water surface) and bottom excluded, neutral white
	Photoperiod	Continuous light
9.	**Number of container, number of replicates**	≥ 10 fronds (two or three per plant)/container Three replicates per concentration, 6 controls
10.	**Test duration**	7 days
11.	**Control**	Deonized water + concentrated mineral medium
12.	**Validity criteria**	Growth rate within (0,25 d^{-1} to 0,35 d^{-1}) Growth rate of frond number growth rate ≥ 0,275 d^{-1}
13.	**Reference substance**	3,5-Dichlorophenol E_rC_{50} (growth rate, frond number) within 1,8 mg/l to 3,6 mg/l
14.	**Statistics**	Regression
15.	**Test parameter(s)**	Growth; obligatory observation parameter: 1. frond number 2. frond area or dry weight or chlorophyll
16.	**Endpoints**	E_rCx, lowest ineffective dilution (LID)
17. Application to wastes and water extracts from wastes: limitations and comments		
—	No interference with light absorbing substances (400 nm to 700 nm) due to exclusion of reflection light.	
—	Special considerations for substances enriched at the water surface.	
—	Amount of EDTA minimised in nutrient medium to minimise complexation of metals.	
—	Freshwater organisms may be affected by test mixtures that contain high amount of salts. This may occur when testing low dilutions of water extracts (e.g. dilutions factors 1/1, 1/2, 1/4).	

B.3.9 Freshwater fish acute toxicity test

1.	**Title of the test**	Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Danio rerio* Hamilton Buchanan (Teleostei, Cyprinidae)]
2.	**Harmonisation**	International
3.	**References**	ISO 7346 ; OECD 203
4.	**Principle**	Effect on survival of *Danio rerio*
5.	**Test type**	Acute (ISO 7346-1 = static, ISO 7346-2 = semi-static, ISO 7346-3 = continuous renewal).
6.	**Test organism**	Zebra fish
	Breeding stock	*Danio rerio* Hamilton-Buchanan
	Age of test organism	Adults (30 mm ± 5 mm ; 0,3 g ± 0,1 g)
	Feeding	None
7.	**Dilution medium**	Freshwater or synthetic reconstituted medium
	Volume	1 l/g of fish
8.	**Test conditions**	
	Test chamber size	up to 10 l
	Temperature	(23 ± 1) °C
	pH	7,8 ± 0,2
	Light intensity/quality	Normal laboratory illumination
	Photoperiod	12 h to 16 h day light
9.	**Number of container, number of replicates**	At least 7 fish per vessel, one vessel per concentration
10.	**Test duration**	96 h
11.	**Control**	Water
12.	**Validity criteria**	Dissolved O_2 > 60 % saturation, control fish mortality < 10 %, no abnormal behaviour
13.	**Reference substance**	$K_2Cr_2O_7$
14.	**Statistics**	Regression, probit
15.	**Test parameter(s)**	Mortality
16.	**Endpoints**	LC_{50}
17. Application to wastes and water extracts from wastes: limitations and comments		
— Volume of water extract necessary to perform test.		
— The recommended species: *Poecilia reticulata* (guppy) can be adapted to salty medium.		

B.3.10 Marine copepods – Acute toxicity test

1.	**Title of the test**	Water quality - Determination of acute lethal toxicity to marine copepods (Copepoda, crustacea)
2.	**Harmonisation**	International
3.	**References**	ISO 14669
4.	**Principle**	Determination of effects of toxicants on survival of marine copepods
5.	**Test type**	Acute, static / semi-static
6.	**Test organism**	Marine copepods
	Breeding stock	*Acartia tonsa* (Dana), *Tisbe battagliai* (Volkmann-Rocco), *Nitocra spinipes* (Bœck)
	Age of test organism	*A. tonsa*: copepodids stage 5 or adults, *T. battagliai*: copepodids (6 days ± 2 days), *N. spinipes*: adults three to four weeks
	Feeding	None
7.	**Test substrate**	Natural or synthetic seawater
	Volume	*A. tonsa*: 5 ml/animal, others 0,5 ml/animal
8.	**Test conditions**	
	Test chamber size	Depending on the number of animals par vessel
	Temperature	(20 ± 2) °C
	pH	8,0 ± 0,3
	Light intensity/quality	not specified
	Photoperiod	16 h day/8 h night
9.	**Number of replicates**	At least four replicates of 5 animals par concentration
10.	**Test duration/incubation**	48 h (96 h optional)
11.	**Control**	Dilution seawater
12.	**Validity criteria**	Dissolved oxygen at end of test > 4 mg/l, control mortality < 10 %; sensitivity to the reference substance
13.	**Reference substance**	3,5 dichlorophenol
14.	**Statistics**	Regression
15.	**Test parameter(s)**	Mortality of animals
16.	**Endpoints**	LC_{50}

17. Application to wastes and water extracts from wastes: limitations and comments

— Turbid and coloured samples may disturb mother and neonates behaviour and counting.

— When the sample is likely to interfere with the test (protozoa, micro-organisms…) a filtration or centrifugation of the sample can be performed.

— Saltwater organisms should be preferred when testing water extracts from wastes that contain high amounts of salts.

B.3.11 Marine algal growth inhibition test

1.	**Title of the test**	Water quality - Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*
2.	**Harmonisation**	International
3.	**References**	EN ISO 10253 (under revision)
4.	**Principle**	Determination of effect on growth of a population of marine algae
5.	**Test type**	Chronic, static
6.	**Test organism**	Unicellular algae
	Breeding stock	*Skeletonema costatum* or *Phaeodactylum tricornutum*
	Age of test organism	Inoculum from an exponentially growing pre-culture ; $2x10^3$ cells/ml to 10^4 cells/ml in final volume
	Feeding	Nutrient medium
7.	**Dilution medium**	Synthetic seawater or natural seawater (*Skeletonema costatum*)
	Volume	100 ml
8.	**Test conditions**	
	Test chamber size	250 ml conical flasks
	Temperature	(20 ± 1) °C
	pH	8 ± 0,2
	Light intensity/quality	35 photons/m²/s to 70 x 10^{18} photons/m²/s (400 nm to 700 nm) ; 6 000 lux to 10 000 lux
	Photoperiod	Continuous light
9.	**Number of container, number of replicates**	Three replicates per concentration, 6 replicates for control
10.	**Test duration**	72 h
11.	**Control**	Nutrient medium + seawater
12.	**Validity criteria**	Control population increase > 16 within 72 h corresponding to a control growth rate of 0,04/h
13.	**Reference substance**	$K_2Cr_2O_7$, 3,5-dichlorophenol
14.	**Statistics**	Multisample comparison, regression
15.	**Test parameter(s)**	Growth rate or biomass integral
16.	**Endpoints**	NOEC and ECx (x = 10, 50)

17. Application to wastes and water extracts from wastes: limitations and comments

— Chemicals absorbing light in the range 400 nm to 700 nm may interfere with algal growth for physical reasons rather than by toxic action.

— High particle density in the sample may disturb growth measurements (particle counter, spectrophotometer).

— Bacteria, algae from other species and algal predators may interfere with algal growth.

— Bioavailability of metals ions can be reduced by complexation with EDTA from the test medium.

— Volatile substances may be stripped by agitation of the tests flasks.

— See ISO 14442 for information on difficult substances management.

— Saltwater organisms should be preferred when testing water extracts from wastes that contain high amounts of salts.

B.3.12 Salmonella / Microsome test

1.	**Title of the test**	Determination of the genotoxicity of water and waste water using the Salmonella/microsome test
2.	**Harmonisation**	International
3.	**References**	ISO/DIS 16240
4.	**Principle**	The possible mutagenic activity of sample is detected by comparing for the respective bacterial strain and the respective activation condition the number of mutant colonies on plate treated with the negative control and on plates treated with the test sample.
5.	**Test type**	Genotoxicity
6.	**Test organism**	*Salmonella typhimurium*
	Breeding stock	Mutants of *Salmonella typhimurium* LT2 strains TA 100 and TA 98
	Age of test organism	-
	Feeding	Bacto agar + mineral medium
7.	**Dilution medium**	Sterile deionized water
	Volume	2,6 ml (tube containing sample + bacteria + soft agar + S9 mix)
8.	**Test conditions**	Metabolic activation (S9 mix) / No metabolic activation
	Test chamber size	Petri dishes filled with 25 ml of agar
	Temperature	(37 ± 1) °C
	PH	7,0 ± 0,2
	Light intensity/quality	Darkness
	Photoperiod	-
9.	**Number of container, number of replicates**	Two plates for each test conditions; three plates for negative control
10.	**Test duration**	48 h to 72 h
11.	**Control**	Negative control; positive control (reference substances)
12.	**Validity criteria**	Mutant colonies per plate in negative control (TA 100: 80 –180; TA 98: 15 – 40); mean number of mutant colonies in positive controls
13.	**Reference substance**	Nitrofurantoin, 4-Nitro-1,2-phenylene diamine, 2-aminoanthracene
14.	**Statistics**	-
15.	**Test parameter(s)**	Number of mutant colonies
16.	**Endpoints**	Induction rate of mutant colonies; decisive D value

17. Application to wastes and water extracts from wastes: limitations and comments

— Samples containing solids should be centrifuged.

— All samples should be sterilized.

B.3.13 UMU test

1.	**Title of the test**	Determination of the genotoxicity of water and waste water using the umu test
2.	**Harmonisation**	International
3.	**References**	ISO 13829
4.	**Principle**	The genotoxicity of the test sample is detected by comparing the induction of the gene umuC of the test sample to the spontaneous activation of the negative control.
5.	**Test type**	Genotoxicity
6.	**Test organism**	*Salmonella typhimurium*
	Breeding stock	*Salmonella typhimurium* TA 1535 / psK1002
	Age of test organism	≤ 12 h (O.D. 600 nm ≥ 800 FTU)
	Feeding	Synthetic medium (TGA)
7.	**Dilution medium**	Deionized water
	Volume	-
8.	**Test conditions**	Metabolic activation (S9 mix) / No metabolic activation
	Test chamber size	Microplate
	Temperature	(37 ± 1) °C ; (28 ±1) °C
	pH	7,0 ± 0,2
	Light intensity/quality	Darkness
	Photoperiod	-
9.	**Number of container, number of replicates**	Three replicates per concentration
10.	**Test duration**	4 h
11.	**Control**	Negative control; positive control (reference substances)
12.	**Validity criteria**	Induction rate ≥ 2 in positive controls; minimal growth in negative control = 140 FTU
13.	**Reference substance**	4-Nitro-quinoline-N-oxide, 2-aminoanthracene
14.	**Statistics**	-
15.	**Test parameter(s)**	-
16.	**Endpoints**	LID
17. Application to wastes and water extracts from wastes: limitations and comments		
— Coloured samples may interfere with measurements.		
— High particle density in the sample may disturb measurements.		

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