INTERNATIONAL STANDARD

ISO 15799

Second edition
2019-03

Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials

Qualité du sol — Lignes directrices relatives à la caractérisation écotoxicologique des sols et des matériaux du sol

Reference number
ISO 15799:2019(E)

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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

This second edition cancels and replaces the first edition (ISO 15799:2003), which has been technically revised. The main changes compared to the previous edition are as follows:

— standardized forms of recommended test systems in Annex A have been amended and updated (e.g. ISO 20963 deleted and ISO 18311, ISO 18187 added).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Most of the existing ecotoxicological test methods (biotests) that are being internationally harmonized were developed to describe the ecotoxic potential of a test substance when added to a soil/soil material. These methods can be used with some modifications for the ecotoxicological characterization of soils and soil materials with respect to their function depending on the intended use. For substances with properties resulting in toxic effects, biotests are a complement to conventional chemical analysis. Results from chemical analysis can be used for ecotoxicological assessments based on information on the substances identified, including properties of the chemicals, e.g. their bioaccumulation potential. This information is often scarce (if it exists at all) and it does not include possible interactions (synergy/antagonism) between chemicals and the complex soil matrix. Furthermore, an exhaustive identification and quantification of substances is impractical. Therefore, ecotoxicological testing of soils can be used for investigating the potential toxicity of complex chemical mixtures. The extrapolation from laboratory tests to field conditions requires adequate consideration of important environmental factors within the test conditions and the selection of suitable ecotoxicological end points.

Soil quality — Guidance on the ecotoxicological characterization of soils and soil materials

1 Scope

This document is one of a family of International Standards providing guidance on soils and soil materials in relation to certain functions and uses including conservation of biodiversity. It applies in conjunction with these other standards. It provides guidance on the selection of experimental methods for the assessment of the ecotoxic potential of soils and soil materials (e.g. excavated and remediated soils, refills, embankments) with respect to their intended use and possible adverse effects on aquatic and soil dwelling organisms.

NOTE This is a reflection of the maintenance of the habitat and retention function of the soil. In fact, the methods listed in this document are suitable for usage in a TRIAD approach, i.e. for an ecological assessment of potentially contaminated soils (see ISO 19204).

This document does not cover tests for bioaccumulation.

The ecological assessment of uncontaminated soils with a view to natural, agricultural or horticultural use is not within the scope of this document. Such soils can be of interest if they can serve as a reference for the assessment of soils from contaminated sites.

The interpretation of results gained by applying the proposed methods is not in the scope of this document.

2 Normative references

There are no normative references in this document.

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 https://www.iso.org/obp

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

3.1 Types of soil and other soil materials

3.1.1
soil
upper layer of the Earth's crust composed of mineral particles, organic matter, water, air and organisms

[SOURCE: ISO 11074:2015, 2.1.11, modified — The definition has been slightly modified and the Note 1 to entry has been deleted.]

3.1.2
soil material
material which includes *excavated soil* (3.1.3), dredged materials, manufactured soils, treated soils and fill materials

3.1.3
excavated soil
any natural material excavated from ground including top-soil, sub-soil, altered parent rock and parent rock itself

Note 1 to entry: Excavated soil typically arises during construction works.

[SOURCE: ISO 15176:2002, 3.1.5]

3.1.4
standard soil
field collected soil whose main properties (e.g. pH, texture, organic matter content) are within a known range

Note 1 to entry: An example for standard soils is "Eurosoils" (see Reference [28]).

3.2 Terms relating to soil characteristics

3.2.1
habitat function
ability of *soils* (3.1.1)/*soil materials* (3.1.2) to serve as a habitat for microorganisms, plants, soil living animals and their interactions (biocenoses)

3.2.2
retention function
ability of *soils* (3.1.1)/*soil materials* (3.1.2) to adsorb *pollutants* (3.2.3) in such a way that they cannot be mobilised via the water pathway and translocated into the food chain

Note 1 to entry: The habitat and retention functions include the following soil functions according to ISO 11074:2015:

— control of substance and energy cycles as components of ecosystems;

— basis for the life of plants, animals and man;

— carrier of genetic reservoir;

— basis for the production of agricultural products;

— buffer inhibiting movement of water, contaminants or other agents into the ground water.

3.2.3
pollutant
substance which due to their properties, amount or concentration cause impacts on the soil function or soil use

Note 1 to entry: See also *contaminant* (3.2.4) and *potentially harmful substance* (3.2.5).

[SOURCE: ISO 11074:2015, 3.4.18, modified — Wording has been slightly modified and Note 1 to entry has been added.]

3.2.4
contaminant
substance or agent present in the *soil* (3.1.1) as a result of human activity

Note 1 to entry: There is no assumption in this definition that harm results from the presence of the contaminant: see also *pollutant* (3.2.3) and *potentially harmful substance* (3.2.5).

[SOURCE: ISO 15176:2002, definition 3.2.6, modified — The wording in the Note 1 to entry has been slightly modified.]

3.2.5
potentially harmful substance
substance which when present in sufficient concentration or amount may be harmful to humans or the environment

Note 1 to entry: It may be the result of human activity [*contaminant* (3.2.4)] or naturally occurring.

[SOURCE: ISO 15176:2002, 3.2.8, modified — A Note 1 to entry has been added.]

3.3 Land and sites

3.3.1
re-use
useful and harmless utilisation of soil materials

Note 1 to entry: In the context of this International Standard, re-use means the transfer of soil materials to another location for use in agriculture, horticulture, forestry, gardens, recreational areas and construction sites.

[SOURCE: ISO 15176:2002, 3.4.1]

4 Field of application

4.1 Soils and areas of soil use where ecotoxicity tests should be considered

Ecotoxicity tests should be considered in the following soils and areas of soil use:

— Assessment of the ability of a soil to sustain a natural biocenosis or agriculture.

— Assessment of the combined ecotoxicity of all bioavailable contaminants present in soils or soil materials.

— Assessment of the ecotoxicity of potentially harmful substances in cases where the soil/soil material can affect the ground and surface water.

— Identification of soils or soil materials (refills, embankments) with a low degree of contamination usually within a depth of 1 m, which can remain at the site without further treatment.

— Detection of potential ecotoxicity which could not be traced by chemical analysis.

— Monitoring and control of the success of soil treatment (off-site, on-site/*in situ*).

— Monitoring and control of soils/soil materials, which have been decontaminated and are to be applied at the surface.

4.2 Soils and areas of soil use where ecotoxicological tests are not necessary

Provided that groundwater contamination can be excluded, ecotoxicological testing is not necessary in the following cases.

— Contaminated soils which are classified as hazardous waste or can be characterized clearly by chemical/analytical parameters. In such cases, ecotoxicological testing may be useful for a final investigation after remediation and for process control during biological remediation.

— Commercially/industrially used areas with no prospect of horticultural/agricultural use.

— Soil materials or backfilled materials in an area which is to be effectively sealed by covering with buildings or other forms of low permeability cover such as concrete or tarmacadam or asphalt.

5 Selection of tests according to the use/re-use of soils and soil materials and soil functions

5.1 Use of ecotoxicity tests

Toxicants can affect different species (and in some cases genotypes) present within ecosystems at different concentrations. The ideal approach for the precise ecotoxicological characterization of the soil toxicity is to use a battery of tests with several species belonging to different taxonomic and trophic groups to avoid false-negative results due to an adaption of a test system (genotypic shift) to a specific contaminant as compared to uncontaminated soils. Studies using field or semi-field investigations are rarely carried out and may be very expensive.

The ideal scheme can be rendered more practicable by the adoption of simpler testing strategies and the application of safety factors to the results obtained. If, however, testing is performed on one species or function only, the high diversity in the sensitivity of species to toxicants will result in a high level of uncertainty. It is therefore recommended to test at least a microbial process, a species from the plant kingdom, and one from the animal kingdom, usually a saprophagous/detritivorous species. If more than one animal species are tested, a predatory species should be included in the test battery. The minimum number of species to be tested depends on the regulations to which the test strategy shall comply. This document only gives the basic principles for their use. Further considerations to the selection of tests using soil organisms are given in 5.3.

5.2 General criteria for selection of tests

Criteria for the selection of ecotoxicity tests were established in the context of hazard assessment and classification of chemicals. These criteria should also apply for the ecotoxicological characterization of contaminated soils. Criteria reviewed were scientific validity, ecological significance, practicability and acceptability (see References [27] and [28]).

Basic requirements which test protocols shall fulfil in order to be laid down in International Standards include reproducibility, statistical validity, general acceptance and performance.

The importance of a criterion is relative to the specific situation. Decisions have to be made between which criteria are most important or tests which may have to be modified by more practical considerations, such as easy culturing of test organisms in the laboratory or the availability of life stages required for a test throughout the year.

The test methods recommended (see Annex A) in this document were originally designed for hazard assessment of chemicals and were in most cases internationally harmonized e.g. by OECD, EU or ISO. In most of them provisions have been made to adapt the test design for the purposes within the scope of this document. In addition, the selection of ecotoxicological test methods for the assessment of soils/soil materials depends on their intended use/re-use and on the soil functions to be protected, in particular the retention and habitat functions.

Table 1 gives an example of a decision scheme based on the relevant function.

Table 1 — Relevance for ecotoxicological testing to the intended re-use of the soil

Re-use of soils	Soil function		
	Retention function	Habitat function	
	Aquatic organisms	Plant growth	Soil biocenoses
	Detection of biological effects		
Below sealed areas	low[a]	low	low
Commercially and industrially used un-sealed areas	high	low	low
Landfill covering	high	high	low
Green areas, parks and recreation areas	high	high	high
Areas used in horticulture or agriculture	high	high	high

[a] Applies only to the unsatured soil zone.

5.3 Considerations for the examination of soil functions

5.3.1 Retention function

Transport via water of soluble, colloidal or particle fractions play a dominant role in the risk assessment of contaminated soils. This is true not only because water can mobilize contaminants, but also because contaminants and metabolites in the water phase potentially have a severe effect on microorganisms, plants and soil fauna.

Aqueous eluates (for preparation see Clause 6, ISO 18772, EN 14735) are useful for testing ecotoxic effects on organisms exposed via the water mediated transport. It should be taken into account that substances mobilized via water can be subjected to different types of changes, such as metabolism or hydrolysis when transported into the groundwater and from there into surface waters, and that their concentrations are reduced by dilution. Moreover, substances can be mobilised over time due to environmental changes (e.g. pH, chemical and biological transformation). Eluates can serve as early indicators for the contamination of pore and ground water prior to the exposure of surface and drinking water.

With these aspects, the investigation of groundwater and eluates is of utmost importance regardless of the proposed soil use.

For ecotoxicology tests working with aqueous soil extracts and aquatic test organisms it shall be considered that nutrient ions and compounds are easily dissolved in water (at least easier than hydrophobic pollutants) and can substantially interfere during the test.

5.3.2 Habitat function

5.3.2.1 General

The suitability of the soil for living organisms can best be examined by means of test methods which are selected to include organisms and processes representative of different taxonomic and ecological groups.

5.3.2.2 Soil material used as control for bioassays on solid matrices

As a general principle in ecotoxicological testing, any end point measured in a treatment is compared with the one measured in the control(s).

In order to evaluate the suitability of the soil for soil-dwelling organisms, it is a prerequisite to compare the contaminated soil or soil material with a control material, which may also be used for preparing dilution series with the contaminated sample.

Several types of control material can be used:

— an uncontaminated soil with comparable pedological properties to the sample being tested;

— an inert material (e.g. quartz sand);

— a certified natural soil (e.g. standard soil);

— a standardized artificial soil (see ISO 11268-1 and ISO 11268-2, ISO 11267).

The choice between these control materials depends entirely on the aims of the ecotoxicological assessment, the type of biological test being carried out and the requirements of the test organism. This recommendation cannot be generalized for all biological tests. Adding sand to a soil or a soil material can create a compact mixture which is incompatible with the growth and development of many organisms (e.g. plant growth tests). It is preferable to use a more complex control material (such as artificial soil) for dilution where this would have the advantage of reproducing more closely the natural environment of the organisms and even if it may interact with pollutants. Placing an organism in a medium which does not match the most important characteristics of its natural habitat may cause stress.

— If a dose-response curve is needed, one of the control materials mentioned above may be used to dilute the contaminated substrate.

— If the aim is to classify each sample of soil or soil materials in terms of ecotoxicity hazard, it is preferable to use an inert material (e.g. quartz sand) which will not interact with the pollutants present in the sample, and whose composition and granulometry can be rigorously standardized.

The requirements of the control material shall take into account the different soil uses and the type and origin of the soil (e.g. undisturbed soil, refilling material, excavated soil, remediated soil). Nutrient deficiency, as well as physical conditions, can cause differences in plant growth and animal behaviour that need not necessarily be caused by the pollutant situation and the hazard potential.

— If the aim is to evaluate the ecotoxicity of a soil or soil material sample from a contaminated site, the preferred method would be to use an uncontaminated control material that is similar to the sample being tested.

— If the aim is to evaluate the ecotoxicity of soils or soil materials which may be re-employed for certain specific uses, the preferred method would be to use as a control material any material which may in future be mixed with soil or soil material.

5.3.2.3 Soil as substrate (medium) for soil microorganisms

The soil microflora comprises on average 80 % of the mass of organisms living in soil. In combination with the microfauna, the main functions of the microflora are the decomposition and degradation of complex organic substances to easily available nutrients thereby maintaining the natural substance cycles of carbon, nitrogen, phosphorus and sulfur.

Substrate-induced respiration provides an indicator of the microbial population density.

Nitrifying bacteria which are responsible for the oxidation of ammonium to nitrite and from nitrite to nitrate, are a very sensitive group of microorganisms. Decreased nitrification need not necessarily lead to significant changes in the ecosystem but can be used as a sensitive indicator for the inhibition of an essential soil process.

The purpose of determining the microbial biomass or other microbial processes in soils is to allow assessment of the continued maintenance of soil fertility, the potential ability to degrade organic compounds, and the effects of added materials on the soil microbial community.

5.3.2.4 Soil as substrate for plant growth

After microorganisms, plant roots constitute the largest biological surface in soil. Their contact area with soil particles is increased by the presence of root hairs and mycorrhizal associations (VA-mycorrhiza with cultivated plants and additional ectomycorrhizal with woody plants).

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

The accumulation of pollutants in plants, their metabolism and their effects on consumers are not investigated in these tests. They do not apply to the assessment of soil fertility and productivity.

5.3.2.5 Soil as substrate for soil-inhabiting fauna

Soil animals generally fulfil the following four functions:

— mechanical activities (drainage, aeration, mixing, mechanical comminution);

— chemical changes (enhanced availability of nitrate and phosphate from excrements and accelerated formation of clay-humus complexes, after the substrate has passed the gut);

— biological changes (distribution of microorganisms in the soil matrix, synergistic effects through stimulation of microbial activity and organic matter decomposition);

— significant links in the food web.

Short-term and long term tests are available for examination of the effects of pollutants on soil fauna. For testing the habitat function, characterization by sublethal test parameters is particularly recommended.

Since a single test method cannot adequately represent the vast number of very diverse invertebrates, a test battery should be used. When selecting the individual test species, the following criteria should be considered:

— trophic level: e.g. saprophagous and predatory species should be included;

— taxonomic/physiological groups: in order to cover the biodiversity of soil communities, at least representatives of Annelida and Arthropoda have to be selected;

— size class/exposure pathway: species of the micro-, meso- and macrofauna do not only represent various size classes but also different life-styles and therefore exposure routes (e.g. pore water versus food uptake);

— ecological role: at least soil-dwelling and litter-inhabiting species are important to be considered.

Only internationally standardized methods should be used.

6 Sampling, transport, storage and sample preparation

Before soil quality is assessed by any of the methods proposed, soil samples need to be collected from the site under investigation (see ISO 23611-6). Soil sampling should be carried out by trained personnel with sufficient knowledge of sampling, handling of samples and safety measures at contaminated sites and sampling locations. The sampling strategy and handling should be determined by the site to be investigated, the kind of contamination and the aim of the biological tests (e.g. quantities of soil samples can vary between 100 g and 100 kg depending on the tests selected).

Record all data concerning sampling, transport and sample preparation. Instructions on the design of sampling programmes, sampling techniques, safety, investigations of natural, cultivated, urban and industrial sites and on the collection, handling and storage of soil for the assessment of biological functional and structural end points in the laboratory are given in ISO 18400-206. For the preparation of eluates for testing the retention function with aquatic test methods ISO 18772 and EN 14735 are recommended.

7 Limitations of proposed biotests for soils/soil materials

Biological test systems are only suitable to a limited extent for volatile pollutants. Other methods should be developed for this purpose. Similarly the impact of organic contaminants, which are easily degradable under aerobic conditions, may be detected incompletely by the methods described. In this case alternative methods for sampling and sample preparation should be applied.

The proposed terrestrial and aquatic test methods in A.1 and A.2 were developed to assess the ecotoxic potential of chemicals. The characterization of soils or soil eluates was not their primary goal. Therefore, the methods shall be adapted to the specific requirements of soil and site assessment.

Annex A
(informative)

Standardized forms of recommended test systems

A.1 Terrestrial test methods

A.1.1 Soil fauna

A.1.1.1 Collembola — Effects on reproduction

1.	**Title of the test:**	Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil contaminants
2.	**Harmonization**	International
3.	**References**	ISO 11267
4.	**Principle**	Determination of the effect on reproduction of springtails incubated over a four weeks test period
5.	**Test type**	Static subchronic
6.	**Test organism**	Springtails
	Breeding stocks	Folsomia candida Willem 1902
	Age	10 d to 12 d
	Feeding	Dry yeast
7.	**Test substrate**	Artificial Soil, contaminated soil
	Volume	30 g (wet mass)/container
8.	**Test conditions**	
	Test chamber	Enclosure capable of being temperature-controlled
	Temperature	20 °C ± 2 °C
	pH	6 ± 0,5 for testing contaminated soils do not adjust pH
	Light intensity/quality	Between 400 lx and 800 lx
	Photoperiod	12 h: 12 h or 16 h: 8 h
	Soil moisture	40 % to 60 % of total water holding capacity
9.	**No. Replicates**	At least 4
10.	**Test duration/incubation**	28 d
11.	**Neg. control/ dilution soil**	Reference or standard soil (e.g. artificial soil)
12.	**Validity criteria**	Control: mortality < 20 %, min. reproduction 100 juveniles, CV ≤ 30 %
13.	**Pos. Control/reference toxicant Mean EC50, CV**	Boric acid or Betosip (a.i. 157 g/l Phenmedipham)
14.	**Statistics**	ANOVA, multiple *t*-test, u-test, regression analysis

15. **Test parameter(s)**	Mortality of adults, inhibition of reproduction
16. **End points**	EC_x (x = % effect level, e.g. 10, 50), NOEC, NOER
17. **Limitations/Comments**	Originally the test was designed for testing substances added to an artificial soil. The test is also applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, industrial, agricultural or other sites under concern as well as waste materials.

A.1.1.2 Earthworms — Acute toxicity

1. **Title of the test:**	Soil quality — Effects of pollutants on earthworms — Part 1: Determination of acute toxicity to *Eisenia fetida/Eisenia andrei*
2. **Harmonization**	International
3. **References**	ISO 11268-1
4. **Principle**	The percent mortality of adult earthworms (species: *Eisenia fetida* or *Eisenia andrei*) exposed to the test soil are compared to those observed for test organisms exposed to a control soil.
5. **Test type**	Acute, static
6. **Test organism**	Earthworms
Breeding stocks	*Eisenia fetida* Savigny, *E. andrei* Bouché
Age	> 2 months
Feeding	No
7. **Test substrate**	Test soil, standard soil (e.g. Artificial Soil)
Volume	500 g (dry mass)
8. **Test conditions**	
Test chamber	Enclosure capable of being controlled
Temperature	20 °C ± 2 °C
pH	6 ± 0,5, Field-collected soils, soil or waste materials: Soil pH should not be corrected as it can influence bioavailability of soil contaminants.
Light intensity/quality	400 lx to 800 lx
Photoperiod	Between 12 h: 12 h or 16 h: 8 h
Soil moisture	40 % to 60 % water holding capacity
9. **No. Replicates**	4
10. **Test duration/ incubation**	14 days
11. **Neg. control/dilution soil**	Standard soil or artificial soil
12. **Validity criteria**	Control: mortality < 10 %, biomass loss ≤ 20 %
13. **Positive control/ reference toxicant**	Boric acid. Significant effects on mortality should be observed between 3 000 mg and 4 500 mg of boric acid per kilogram of artificial soil (dry mass).
14. **Statistics**	Fisher's exact test ANOVA

15. **Test parameter(s)**	Mortality, biomass
16. **End points**	LC50 – 14 d
17. **Limitations/Comments**	Originally the test was designed for testing substances added to an artificial soil. The test is also applicable to soils and soil materials of unknown quality, e.g. from contaminated sites, amended soils, soils after remediation, agricultural or other sites concerned, and waste materials. In a new Annex the test performance under tropical conditions is described. The test has been used successfully for the assessment of wastes.

A.1.1.3 Earthworms — Effects on reproduction

1. **Title of the test:**	Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction of *Eisenia fetida*/*Eisenia andrei*
2. **Harmonization**	International
3. **References**	ISO 11268-2
4. **Principle**	The effects on reproduction of adult earthworms (species: *Eisenia fetida* or *Eisenia andrei*) exposed to the test soil are compared to those observed for test organisms exposed to a control soil.
5. **Test type**	Subchronic, static
6. **Test organism**	Earthworms
Breeding stocks	*Eisenia fetida* Savigny, *E. andrei* Bouché
Age	between 2 months and 1 year
Feeding	Cow dung
7. **Test substrate**	Test soil, standard soil (e.g. Artificial Soil)
Volume	500 g to 600 g dry mass
8. **Test conditions**	
Test chamber	Enclosure capable of being controlled
Temperature	20 °C ± 2 °C
pH	6 ± 0,5 Field-collected soils, soil or waste materials: Soil pH should not be corrected as it can influence bioavailability of soil contaminants.
Light intensity/quality	400 lx to 800 lx
Photoperiod	Between 12 h: 12 h or 16 h: 8 h
Soil moisture	40 % to 60 % water holding capacity
9. **No. Replicates**	4
10. **Test duration/ incubation**	8 weeks
11. **Neg. control/ dilution soil**	Standard soil or artificial soil
12. **Validity criteria**	Control: 30 juveniles/container, CV ≤ 30 %, adult mortality ≤ 10 %
13. **Pos. Control/reference toxicant**	Boric acid. Significant effects on mortality should be observed between 3 000 mg and 4 500 mg of boric acid per kilogram of artificial soil (dry mass).
14. **Statistics**	Multiple *t*-test, u – test, regression analysis

15. **Test parameter(s)**	Mortality, growth, reproduction
16. **End points**	EC50, NOEC
17. **Limitations/Comments**	Same as for ISO 11268-1. In a new Annex the test performance under tropical conditions is described. The test has been used successfully for the assessment of wastes.

A.1.1.4 Enchytraeid — Effects on reproduction

1. **Title of the test**	Soil quality — Effects of contaminants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction
2. **Harmonization**	International
3. **References**	ISO 16387
4. **Principle**	The effects on reproduction of adult enchytraeids (species: *Enchytraeus sp.*) exposed to the test soil are compared to those observed for test organisms exposed to a control soil.
5. **Test type**	Subchronic, static
6. **Test organism**	Enchytraeids
Breeding stocks	*Enchytraeus albidus* Henle and other species of the *Enchytraeus sp.*, in particular *E. crypticus* see Reference [37]
Age	Adult worms with eggs in the clitellum region
Feeding	Rolled oats
7. **Test substrate**	Test soil, field soils or Artificial soil
Volume	20 g dry mass/container
8. **Test conditions**	
Test chamber	Enclosure capable of being controlled
Temperature	20 °C ± 2 °C
pH	6 ± 0,5
Light intensity/ quality	400 lx to 800 lx
Photoperiod	Preferably 16 h: 8 h
Soil moisture	40 % to 60 % water holding capacity
9. **No. Replicates**	2 to 4 depending on the test design (NOEC/EC_x)
10. **Test duration/ incubation**	6 weeks (final test)
11. **Neg. control/ dilution soil**	Artificial soil, field soil (e.g. LUFA 2.2)
12. **Validity criteria**	Control: Mort. ≤ 20 %, min. no. of juveniles 25/vessel CV ≤ 50 %
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Carbendazim EC_{50} (1,2 ± 0,8) mg a.i./kg
14. **Statistics**	Multiple *t*-test, regression analysis, probit analysis
15. **Test parameter(s)**	Mortality, reproduction
16. **End points**	LC50, NOEC, EC_x
17. **Limitations/ Comments**	The test has been used successfully for the assessment of wastes.

A.1.1.5 Nematoda — Effects on growth, fertility and reproduction

1. **Title of the test**	Water quality and soil quality — Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda)
2. **Harmonization**	International
3. **References**	ISO 10872
4. **Principle**	The effects on growth, fertility and reproduction of juvenile nematods (species: *C. elegans*) exposed to the test soil are compared to those observed for test organisms exposed to a control soil.
5. **Test type**	Chronic, static
6. **Test organism**	Nematods
Breeding stocks	*Caenorhabditis elegans* (Maupas, 1899)
Age	First stage juveniles (J1; exposed test organisms)
Feeding	Defined food medium, containing the bacterium *Escherichia coli*
7. **Test substrate**	Field soils
Volume	0,500 ± 0,010 g (dry mass) per test well
8. **Test conditions**	
Test chamber	Multidishes, with 12 wells, 3,5 cm^2/well
Temperature	20 °C ± 2 °C (room temperature)
pH	Not specified (5,4 to 6,5 values show the range of control soils)
Light intensity/ quality	Not specified
Photoperiod	In the dark
Soil moisture	80 % water holding capacity
9. **No. Replicates**	At least 4
10. **Test duration/ incubation**	96 h
11. **Neg. control/ dilution soil**	LUFA 2.2 or Artificial soil
12. **Validity criteria**	Mean recovery of exposed test organisms from the control is ≥ 80 % and ≤ 120 %; Mean percentage of males in the control is ≤ 10 %; the percentage of males in a single control replicate is ≤ 20 %; Mean fertility in the control is ≥ 80 %; Mean reproduction in the control is ≥ 30 offspring per exposed test organism.
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Benzylcetyldimethylammonium chloride monohydrate (BAC C16), tested in water. At 15 mg/l growth should be inhibited in a range of 20 % to 80 %. Additionally, the EC_{50} of BAC shall be determined at least every 12 months. The EC_{50} (growth) in water shall be in the range of 8 mg to 22 mg BAC/l
14. **Statistics**	Not specified.

15. **Test parameter(s)**	Growth, fertility, reproduction
16. **End points**	Deviation from control; EC_x
17. **Limitations/ Comments**	Recovery of exposed test organisms from the control sediment or control soil is an indicator of accuracy when adding test organisms and accuracy of nematode separation. The determination of ≥ 80 % and ≤ 120 % recovery as validity criteria ensures certain accuracy for the calculations of reproduction. The test has been used successfully for the assessment of wastes.

A.1.1.6 Snails — Effects on growth

1. **Title of the test:**	Soil quality — Effects of pollutants on juvenile land snails (Helicidae) — Determination of the effects on growth by soil contamination
2. **Harmonization**	International
3. **References**	ISO 15952
4. **Principle**	The effects of soil contaminants on growth and survival of young snails, usually *Helix aspersa aspersa*, are determined in artificial or natural soil and are compared to those observed for test organisms exposed to a control soil.
5. **Test type**	Semi-static
6. **Test organism**	Snails (Gastropoda)
Breeding stocks	*Helix aspersa aspersa* (= *Cornu aspersum*) Müller (1774); other species of the Helicidae possible.
Age	Juveniles (3 weeks to 5 weeks old); fresh mass of (1 ± 0,3) g; shell diameter of (15,5 ± 1) mm
Feeding	Flour-based feed comprising cereals, forage, mineral salts and vitamins (commercially available)
7. **Test substrate**	Artificial Soil or natural soils (not specified)
Volume	140 g (dry mass) in case of Artificial Soil; the bottom of the test container has to be covered up to one centimetre.
8. **Test conditions**	
Test chamber	Container: 24 cm (length) ± 10,5 cm (width) ± 8 cm (height)
Temperature	20 °C ± 2 °C (room temperature)
pH	Artificial Soil: 6 ± 0,5; natural soils not specified
Light intensity/ quality	50 lx – 100 lx
Photoperiod	Day-night photoperiod of 18 h to 6 h
Soil moisture	50 % to 60 % of its total water-holding capacity
9. **No. Replicates**	At least 3
10. **Test duration/ incubation**	28 days (plus a nursery period of 3 weeks to 5 weeks)
11. **Neg. control/ dilution soil**	Artificial Soil or natural soil (not specified)
12. **Validity criteria**	Percentage of mortality: ≤ 10 % at the end of the test; Coefficient of variation (growth) ≤ 40 %; Mean body mass: increase at least by factor of 4 throughout the test duration; Mean shell diameter: increase by at least by a factor of 1,5;

13. **Pos. Control/ reference toxicant Mean EC50, CV**	EC_{50},mass (28 days) of $CdCl_2$: 350 mg – 650 mg of Cd per kg of dry test substrate; EC_{50}, diameter (28 days) of $CdCl_2$: 500 mg – 800 mg of Cd per kg of dry test substrate.
14. **Statistics**	Appropriate methods (e.g. the logistic model); examples are given.
15. **Test parameter(s)**	Mortality, body mass, shell diameter
16. **End points**	For body mass and shell diameter: growth coefficient per replicate and the mean percentage of growth inhibition. Graphic presentation of the results of the test, focusing on dose-response relationship for the effects
17. **Limitations/ Comments**	The test has successfully been performed with waste materials.

A.1.1.7 Bait lamina — Acute effects

1. **Title of the test**	Soil quality — Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test
2. **Harmonization**	International
3. **References**	ISO 18311
4. **Principle**	Determination of the feeding activity of soil invertebrates using perforated plastic strips filled with an artificial food mixture (= bait)
5. **Test type**	Chronic, static
6. **Test organism**	Natural field invertebrate community
Breeding stocks	N. a.
Age	N. a.
Feeding	N. a.
7. **Test substrate**	Natural soil in undisturbed condition
Volume	N. a.
8. **Test conditions**	
Test chamber	N. a. (field sites)
Temperature	N. a. (field conditions)
pH	Not defined
Light intensity/quality	N. a. (field conditions)
Photoperiod	N. a. (field conditions)
Soil moisture	50 % water holding capacity
9. **No. Replicates**	3
10. **Test duration/ incubation**	Depending on climate: Tropics (about 7 d); Temperate (continental): 10 d to 20 d (spring/autumn), up to 112 d (incl. summer)
11. **Neg. control**	Soil (untreated, uncontaminated)
12. **Validity criteria**	Control: at least 30 % of baits pierced at one of the 16 depth levels.
13. **Pos. Control**	N. a.
14. **Statistics**	e.g. GLM analysis for binominal distribution (depends on design)
15. **Test parameter(s)**	Number of “fed” apertures including their vertical distribution
16. **End points**	Percentage of pierced baits and their vertical distribution
17. **Limitations/ Comments**	Active soil invertebrate community is needed at the test site

A.1.2 Soil flora

A.1.2.1 Soil flora — Inhibition of root growth

1. **Title of the test**	Soil quality — 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**	Crop plants
Breeding stocks	Barley (*Hordeum vulgare L.*), oat (*Avena sativa L.*), wheat (*Triticum aestivum*)
Age	Seeds
Feeding	No
7. **Test substrate**	soil, control soil, sand
Volume	≤ 500 g dry mass /container
8. **Test conditions**	
Test chamber	Phytotron, plant growth room, greenhouse
Temperature	Day: 20 °C ± 2 °C; Night: 16 °C ± 2 °C
Light intensity/ quality	≥ 2 500 lx
Photoperiod	Day: 12 h to 16 h: Night 8 h to 12 h
Soil moisture	(70 ± 5) % water holding capacity
9. **No. Replicates**	3
10. **Test duration/ incubation**	approximately exposure: 4 d; pregermination: 2 d to 3 d according to the species
11. **Neg. control/ dilution soil**	Standard soil, artificial soil, reference soil, sand
12. **Validity criteria**	Root elongation in sand control within a defined range; CV < 20 % in sand control
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Nickel sulfate; Boric acid
14. **Statistics**	ANOVA Dunnet test, any suitable statistical procedures to calculate the EC_x (10, 20, 50) with confidence limits (P = 0,95).

15. **Test parameter(s)**	Root elongation
16. **End points**	LC50, NOEC, EC_x
17. **Limitations/ Comments**	The method is applicable to all soils, soil materials, waste or chemicals which may be applied to soil except where the contaminant is highly volatile or only affects photosynthesis. The method may be used to compare soils to monitor changes in their activity or to determine the effect of added substances. The method is not intended as a measure of the ability of the soil to support sustained plant growth. In the case of contaminated soil, it may be necessary to dilute the test substrate with uncontaminated soil or sand before testing. The proposed plant test is not suitable for soil samples with a very disturbed structure (e.g. mixtures of soil and rubble). In these cases an inhibition may result without relevant contamination.

A.1.2.2 Soil flora — 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 contaminated soils on the emergence and growth of higher plants
2. **Harmonization**	International
3. **References**	ISO 11269-2
4. **Principle**	The test measures emergence and early growth of at least two terrestrial plant species (one monocotyledonous and one dicotyledonous). The test compares the development of plants in a test soil and/or a series of mixtures with a control soil.
5. **Test type**	Subacute, static
6. **Test organism**	Crop plants (monocotyledonous and dicotyledonous species)
Breeding stocks	Oat (*Avena sativa*), turnip rape (*Brassica rapa*) or wild turnip (*Brassica rapa* ssp. *rapa*)
Age	Seeds
Feeding	Dilution of commercially available liquid fertilizer
7. **Test substrate**	Soil, soil material, amended soils (compost, sludge, waste)
Volume	500 g
8. **Test conditions**	
Test chamber	Phytotron, plant growth room, green house
Temperature	(23 ± 3) °C
pH	pH (KCl): 5,0 to 7,5
Light intensity/ quality	7 000 lx
Photoperiod	Day: 16 h; night 8 h
Soil moisture	
9. **No. Replicates**	4
10. **Test duration/ incubation**	14 days to 21 days after 50 **%** emergence in the control pots
11. **Neg. control/ dilution soil**	Standard soil, artificial soil, reference soil

12. **Validity criteria**	In control pots: Emergence sufficient to provide 7 healthy seedlings per pot; mean survival of emerged seedlings is at least 90 % for the duration of the study; the No visible phytotoxic effects (e.g. chlorosis, necrosis, wilting, leaf and stem deformations) and the plants exhibit only normal variation in growth and morphology for the selected species
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Sodium trichloroacetate, Boric acid
14. **Statistics**	Analysis of variance and multiple comparisons
15. **Test parameter(s)**	Emergence, growth
16. **End points**	NOEC, LOEC, EC_{20}, EC_{50}, ER_{20}, ER_{50}
17. **Limitations/ Comments**	Same as for ISO 11269-1 (see A.1.2.1) As with other bioassays proposed, tests with higher plants are designed to consider the pollutant situation and bioavailability of pollutants 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 requirements of the control soil shall take into account the different soil uses and the type and origin of the soil (e.g. undisturbed soil, refilling material, excavated soil, remediated soil). Different soil compaction and nutrient deficiency as well as differences in the water-holding capacity and pore volume can cause differences in plant growth.

A.1.2.3 Soil flora — Effects on emergence and growth

1. **Title of the test:**	Soil quality — Chronic toxicity in higher plants
2. **Harmonization**	International
3. **References**	ISO 22030
4. **Principle**	The inhibition of the growth and reproductive capability of two species of higher plants is determined under controlled conditions. The test compares the development of plants in a test soil and/or a series of mixtures with a control soil.
5. **Test type**	Chronic, static
6. **Test organism**	Crop plants (monocotyledonous and dicotyledonous species)
Breeding stocks	Oat (*Avena sativa*), rapid cycling variety turnip rape (*Brassica rapa* CrGC syn. Rbr)
Age	Seeds
Feeding	Dilution of commercially available liquid fertilizer
7. **Test substrate**	Soil, soil material, amended soils (compost, sludge, waste)
Volume	500 g
8. **Test conditions**	
Test chamber	Phytotron, plant growth room, green house
Temperature	(23 ± 3) °C
pH	pH (KCl): 5,0 to 7,5
Light intensity/ quality	(13 000 ± 2 000) lx
Photoperiod	Day: 16 h; night 8 h.

Soil moisture	The soil should be wet (but not highly soaked): At the start of the test 80 % of the WHC_{max} for *Avena sativa* and 60 % of the WHC_{max} for *Brassica rapa* (ISO 11269-2) are sufficient.
9. **No. Replicates**	4
10. **Test duration/ incubation**	First harvest: 14 days to 21 days after 50 **%** emergence in the control pots. *A. sativa* should be harvested after the inflorescences in the control treatment have emerged (after 7 weeks to 8 weeks) and for the rapid cycling turnip rape when seed pods have developed (after 5 weeks to 6 weeks) in the control treatment.
11. **Neg. control/ dilution soil**	Standard soil, artificial soil, reference soil
12. **Validity criteria**	The emergence rate of the control plants has to be at least 75 % (mean of all replicates); Healthy plants should not etiolate and flowers should appear during the first three weeks (rapid cycling turnip rape) or 8 weeks respectively (oat). Not more than one of the emerged plants per pot shall die during the test.
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Sodium trichloroacetate, boric acid or zinc sulfate.
14. **Statistics**	Analysis of variance and multiple comparisons
15. **Test parameter(s)**	Seedling emergence. After 14 days: occurrence of visible flower buds per plant and number of flowers per plant (only *B. rapa*); fresh weight per plant; proportion of living plants. Final harvest: growth stadium according to the BBCH scheme, number of all flowers per plant (only *A. sativa*); number of seed pods carrying fertile seeds (visibly swollen) (*B. rapa* only); fresh mass of shoots (*A. sativa*: without inflorescences; *B. rapa*: without seed pods); fresh mass of inflorescences (*A. sativa*) or seed pods (B. rapa); water content of the shoots, inflorescences and seed pods of each pot (replicate), dry mass of shoots; dry mass of inflorescences (*A. sativa*) or seed pods (*B. rapa*); proportion of dead plants (percentage of plants compared to the number after thinning out).
16. **End points**	NOEC, LOEC, EC_{20}, EC_{50}, ER_{20}, ER_{50}
17. **Limitations/ Comments**	Same as for ISO 11269-1 and ISO 11269-2.

A.1.2.4 Soil flora — *Vicia faba* micronucleus test

1. **Title of the test:**	Soil quality — Assessment of genotoxic effects on higher plants — *Vicia faba* micronucleus test
2. **Harmonization**	International
3. **References**	ISO 29200
4. **Principle**	The test is based on the detection of micronuclei in the cells of the secondary root tips of *Vicia faba* (broad bean). The micronucleus frequency is determined in the control root cells and in those which have been exposed to the soil (or soil material) or the water extract of the soil being tested. A statistical test then enables to determine the significativeness of the test.
5. **Test type**	Genotox test
6. **Test organism**	Broad bean
Breeding stock	*Vicia faba*
Age of test organism	Seeds
Feeding	no

7. **Test substrate**	Field soils
Volume/mass	200 g, 200 ml (minimum for water extracts)
8. **Test conditions**	
Test chamber size	Climatic chamber, Phytotron
Temperature	24 °C ± 1 °C
pH	pH (water) 5,0 – 8,0
Light intensity/quality	≥ 5 000 lx
Photoperiod	16 h/8 h day/night
Other	70 % WHC
9. **No./container, No. replicates**	3
10. **Test duration**	48 h
11. **Neg. Control. dilution soil**	Natural soil (e.g. LUFA), Reference soil
12. **Validity criteria**	For each concentration, the micronucleus frequency is reliable if the mean mitotic index is greater than 20 cells in division for 1 000 observed cells. A positive response is obtained with the reference substance.
13. **Positive control/reference toxicant, mean EC (and CV)**	Maleic hydrazide is recommended as a reference substance. The positive control is carried out at the concentration of 10 mol/l to 5 mol/l, respectively 1,12 mg/kg and 1,12 mg/l for solid-phase and liquid-phase exposures.
14. **Statistics**	The use of a non-parametric method (e.g. the Kruskal-Wallis test followed by Dunn's multiple comparison test) is recommended in order to highlight the significant differences between the control and test concentrations.
15. **Test parameter**	Average number of micronuclei per 1 000 cells observed
16. **End points**	NOEC, LOEC, EC_x
17. **Limitations/Comments**	This method allows the assessment of genotoxicity (toxicity for genetic material) of soils and soil materials like compost, sludge, waste, fertilizing matters, and chemicals. It can be also used for the detection of genotoxic substances in soil eluates.

A.1.2.5 Soil flora — Germination and early growth of higher plants

1. **Title of the test:**	Soil quality — Determination of the toxic effects of pollutants on germination and early growth of higher plants
2. **Harmonization**	International
3. **References**	ISO 18763

4. **Principle**	This method compares the seed germination and early growth of monocotyledonous and dicotyledonous plants in a test soil and/or a series of mixtures with a control soil. This method may also be used for the testing of compost, sludge or waste. Seeds of one monocotyledonous plant and two dicotyledonous plants are exposed to the test material under controlled conditions. After (72 ± 1) h, the number of germinated seeds is recorded and the length of the roots of the test plants is measured in the test soil and in the control soil. At the end of the incubation period, the length of each root (and shoot, if wished) can be measured directly with a ruler and recorded. Calculation of the shoot/root length ratio is a possible additional effect parameter.
5. **Test type**	Acute, static
6. **Test organism**	Crop plants (monocotyledonous and dicotyledonous species)
Breeding stock	*Sorghum saccharatum* (L.), *Lepidium sativum* L., *Sinapis alba* L.
Age of test organism	Seeds
Feeding	No
7. **Test substrate**	Test soil, amended soils (compost, sludge, waste)
Volume/mass	90 cm^3 of test soil
8. **Test conditions**	Seeds are positioned on a black filter paper placed on top of the hydrated soil.
Test chamber size	Transparent plates in polyvinylchloride (PVC) allowing to hold approximately 90 cm^3 of test soil.
Temperature	25 ± 1 °C
pH	
Light intensity/quality	No light
Photoperiod	No
Other	
9. **No./container, No. replicates**	3
10. **Test duration**	(72 ± 1) h
11. **Neg. Control, dilution soil**	Natural soil (e.g. LUFA), artificial soil
12. **Validity criteria**	70 % of the seeds of the three test species shall have germinated in the negative controls at the end of the three days exposure period. The mean root length in the negative controls shall be at least 30 mm for the three test species, and 40 mm in case the measurements are based on the mean length of the longest root.
13. **Positive control/ reference toxicant, mean EC (and CV)**	Boric acid (250 mg/kg)
14. **Statistics**	No information (reference to ISO/TS 20281)
15. **Test parameter**	Germinated seeds, root length, shoot height (optional)
16. **End points**	Inhibition of germination and elongation compared with a control
17. **Limitations/Comments**	The seeds and roots are not in direct contact with the hydrated soil sample (separation by a black filter). The soil treatment prior to testing (air-drying, sieving and addition of water to reach 100 % WHC) may modify significantly the soil structure.

A.1.3 Soil microorganisms

A.1.3.1 Mineralization and nitrification

1. **Title of the test:**	Soil quality — Biological methods — 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**	
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. **Test substrate**	Field soil treated according to ISO 18400-206
Volume/mass	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	Dark (toxicity test)
Photoperiod	—
Soil moisture	40 % to 60 % water holding capacity or approximately 0,02 MPa suction pressure (toxicity test)
9. **No. Replicates**	3
10. **Test duration/ incubation**	28 days
11. **Neg. control/dilution soil**	Soil
12. **Validity criteria**	Not mentioned
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Not mentioned
14. **Statistics**	Regression analysis
15. **Test parameter(s)**	Mineralization rate, nitrification rate
16. **End points**	Concentration of mineral N; Inhibitory dose (ID %)
17. **Limitations/ Comments**	The document 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 on N-mineralization a simplified test design can be used allowing for the establishment of dose-response relationships.

A.1.3.2 Ammonium oxidation — Rapid test

1. **Title of the test:**	Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation
2. **Harmonization**	International
3. **References**	ISO 15685
4. **Principle**	Autotrophic ammonium oxidising bacteria in soil are exposed to ammonium sulfate 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. **Test substrate**	Soil slurry; soil treated according to ISO 18400-206
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. **No. Replicates**	2
10. **Test duration/ incubation**	6 h
11. **Neg. control/ dilution soil**	None
12. **Validity criteria**	Ammonium-oxidising activity of soil 200 ng N/g soil/h to 800 ng N/g soil/h
13. **Pos. Control/ reference toxicant Mean EC50, CV**	None
14. **Statistics**	Mean, standard dev.
15. **Test parameter(s)**	Rate of ammonium oxidation
16. **End points**	In tests of chemicals EC_{10}, EC_{50}
17. **Limitations/ 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 both chemical substances in soil and the effects of cultivation methods. Test substances with limited water solubility require special attention.

A.1.3.3 Soil respiration

1. **Title of the test:**	Soil quality — Determination of the activity of the soil microflora using respiration curves
2. **Harmonization**	International
3. **References**	ISO 17155
4. **Principle**	The CO_2 production or O_2 consumption (respiration rate) from unamended soils as well as the decomposition of an easily degrading substrate (glucose + ammonium + phosphate) is monitored regularly (e.g. every hour). From the CO_2 production or O_2 consumption data the different microbial parameters (basal respiration, substrate-induced respiration, lag time) can be calculated.
5. **Test type**	
6. **Test organism**	Microorganisms 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. **Test substrate**	Field soil treated according to ISO 18400-206
Volume/mass	The sub-samples should contain 1 g of organic matter. If mineral soils are used the sub-samples should not be less than 20 g.
8. **Test conditions**	
Test chamber	Appropriate container of a respirometer
Temperature	20 °C
pH	Intrinsic pH of the soil
Light intensity/ quality	Not specified
Photoperiod	—
Soil moisture	< 400 % of the organic matter content
9. **No. Replicates**	3 for each level of contamination
10. **Test duration/ incubation**	Approximately 5 days
11. **Neg. control/ dilution soil**	Does not apply
12. **Validity criteria**	Not mentioned
13. **Pos. Control/ reference toxicant Mean EC50, CV**	Not mentioned
14. **Statistics**	Mean values for each sample. The microbial parameters should be plotted against the concentration of the contaminating substance and evaluated by regression analysis.
15. **Test parameter(s)**	Basal respiration, substrate induced respiration, lag time
16. **End points**	In tests of chemicals EC_{10}, EC_{50}
17. **Limitations/ Comments**	The test can be used in field- and laboratory contamination studies. It is suitable for the A_o or more layers of podzolic forest soils and arable soils. For the use of mineral soils, complementary studies of suitable moisture content and sample size will have to be made. The test can be also used for soils of unknown quality and for soils sampled along contamination gradients. In contaminated soils the quotient of basal respiration/substrate induced respiration, is much higher than in uncontaminated soils. Contaminated soils show much longer lag times than uncontaminated soils.

A.1.3.4 Enzyme activities using a microplate method with lyophilised fluorogenic substrates

1. **Title of the test:**	Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates
2 Harmonization	International
3. **References**	ISO/TS 22939
4. **Principle**	The method is based on the use of soil samples diluted into buffer, which are incubated in multi-well plates containing lyophilised fluorogenic substrates. After the incubation the enzyme activities are measured as fluorescence (355 nm / 460 nm) with a plate reading fluorometer.
5. **Test type**	—
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. **Test substrate**	Soil slurry; soil treated according to ISO 18400-206
Volume/mass	4 g of soil is added to 120 ml of the selected buffer; 200 µl of the final dilution 1:100 or 1:1 000 is used
8. **Test conditions**	
Test chamber	96-wells microplates on a shaker
Temperature	(30 ± 2) °C
pH	5,5 (acetate buffer) or at soil sample pH (modified universal buffer)
Light intensity/ quality	Not specified
Photoperiod	—
Soil moisture	Not applicable
9. **No. Replicates**	4
10. **Test duration/ incubation**	3 h
11. **Neg. control/ dilution soil**	Does not apply
12. **Validity criteria**	—
13. **Pos. Control/ reference toxicant Mean EC50, CV**	—
14. **Statistics**	mean
15. **Test parameter(s)**	Rate of release of 4-methylumbelliferon (arylsulphatase, α-glucosidase, cellobiosidase, ß-xylosidase, ß-glucosidase, phosphodiesterase, chitinase, phosphomonoesterase) or 7-amino-4-methylcoumarine (leucine-aminopeptidase, alanine-aminopeptidase) from artificial fluorogenic substrates.
16. **End points**	Concentration of 4-methylumbelliferon or 7-amino-4-methylcoumarine
17. **Limitations/ Comments**	Preparation of microplates with freeze-dried substrates requires an instrument for lyophilisation. A laboratory homogenizer or ultrasonic disaggregator is used for preparation of soil slurry.

A.1.3.5 Dehydrogenase activity using triphenyltetrazolium chloride (TTC)

1. **Title of the test:**	Soil quality — Determination of dehydrogenase activity in soils — Part 1: Method using triphenyltetrazolium chloride (TTC)
2 Harmonization	International
3. **References**	ISO 23753-1
4. **Principle**	TTC solution is added to a soil sample and the mixture is incubated. The triphenylformazan (TPF) released is extracted with acetone and determined by photometry at a wavelength of 485 nm.
5. **Test type**	—
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. **Test substrate**	Soil slurry, soil treated according to ISO 18400-206
Volume/mass	5 g of naturally moist soil in 5 ml medium; for L, F, H horizons, soil portions of 0,5 g or 1 g are recommended
8. **Test conditions**	
Test chamber	Test tubes
Temperature	25 °C
pH	7,8 for acidic soil (pH less than 6), 7,6 for neutral soil (pH 6 to 7), 7,4 for carbonate-rich soil (pH greater than 7)
Light intensity/ quality	Dark
Photoperiod	—
Soil moisture	Not applicable
9. **No. Replicates**	3
10. **Test duration/ incubation**	16 h
11. **Neg. control/ dilution soil**	Does not apply
12. **Validity criteria**	—
13. **Pos. Control/ reference toxicant Mean EC50, CV**	—
14. **Statistics**	mean
15. **Test parameter(s)**	Rate of production of TPF
16. **End points**	Concentration of TPF
17. **Limitations/ Comments**	In the case of soil having reducing characteristics (e.g. waterlogged soil), dehydrogenase activity should not be used as a measure of the biological activity in the soil. Abiotic components, such as iron(II) compounds or sulfides, can reduce TTC.

A.1.3.6 Dehydrogenase activity using iodotetrazolium chloride (INT)

1. **Title of the test:**	Soil quality — Determination of dehydrogenase activity in soils — Part 2: Method using iodotetrazolium chloride (INT)
2 Harmonization	International
3. **References**	ISO 23753-2

4.	**Principle**	INT solution is added to a soil sample and the mixture is incubated. The iodonitrotetrazolium formazan (INTF) released is extracted with acetone (in the case of humic soil) and determined by photometry at a wavelength of 485 nm.
5.	**Test type**	—
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.	**Test substrate**	Soil slurry, soil treated according to ISO 18400-206
	Volume/mass	2,00 g to 5,00 g portions of naturally moist soil in 2 ml to 5 ml medium
8.	**Test conditions**	
	Test chamber	Test tubes
	Temperature	30 °C
	pH	7,7 for soil having a carbonate content exceeding 2 % or 7,9 for soil having a carbonate content not exceeding 2 %
	Light intensity/ quality	Dark
	Photoperiod	—
	Soil moisture	Not applicable
9.	**No. Replicates**	4
10.	**Test duration/ incubation**	18 h
11.	**Neg. control/ dilution soil**	does not apply
12.	**Validity criteria**	—
13.	**Pos. Control/ reference toxicant Mean EC50, CV**	—
14.	**Statistics**	mean
15.	**Test parameter(s)**	Rate of production of INTF
16.	**End points**	Concentration of INTF
17.	**Limitations/ Comments**	In the case of soil having reducing characteristics (e.g. waterlogged soil), dehydrogenase activity should not be used as a measure of the biological activity in the soil. Abiotic components, such as iron(II) compounds or sulfides, can reduce INT.

A.1.3.7 *Arthrobacter globiformis* — Solid contact test using dehydrogenase activity

1.	**Title of the test:**	Soil quality — Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*
2.	**Harmonization**	International
3.	**References**	ISO 18187
4.	**Principle**	Inhibition of *A. globiformis* dehydrogenase activity using the redox dye resazurin
5.	**Test type**	Acute, static
6.	**Test organism**	*Arthrobacter globiformis* (Soil bacterium)
	Breeding stock	*Arthrobacter globiformis* strain number ATCC 8010
	Age of test organism	Inoculum from culture at the beginning of the exponential phase

	Feeding	None
7.	**Test substrate**	Soil and soil materials (wastes)
	Volume/mass	(600 ± 6) mg
8.	**Test conditions**	
	Test chamber size	24-well microplates
	Temperature	(30 ± 1) °C (incubation)
	pH	7,0 ± 2,0
	Light intensity/quality	Not applicable
	Photoperiod	Not applicable
	Other	Agitation: 150 min^{-1}
9.	**No./container, No. replicates**	6 treatments *per* microplate, 4 replicates *per* treatment
10.	**Test duration**	3 h (includes 1 h of enzyme activity measurement)
11.	**Neg. Control. dilution soil**	Reference soil, natural standard soil (e.g. Lufa 2.2), artificial soil or quartz sand, depending on the test substrate
12.	**Validity criteria**	The absolute value of the average relative fluorescence of the negative control increases by a factor of > 5 within the measuring time 0 min to 60 min. The reference substance induces an average inhibition between 30 % and 80 % at 600 mg C16-BAC kg^{-1} Lufa 2.2 soil dry mass. The coefficient of variation for the average slope of relative fluorescence in the negative control replicates is less than 15 %.
13.	**Positive control/ reference toxicant, mean EC (and CV)**	C16-BAC, 600 mg C16-BAC kg^{-1} Lufa 2.2 soil dry mass induces an average inhibition of *ca.* 58 %
14.	**Statistics**	Linear regression analysis (e.g. logistic regression) One-way analysis of variances followed by a multiple comparison *post-hoc* test (e.g. Tukey or Dunnett's tests)
15.	**Test parameter**	Dehydrogenase activity
16.	**End points**	EC_{50} (95 % confidence limits), NOEC, LOEC Threshold value: 30 % inhibition of dehydrogenase activity (comparatively to the negative control)
17.	**Limitations/Comments**	Not applicable for samples with pH < 5 or > 9 Soils containing coloured substances or high organic matter percentage may interfere with the dye and/or affect the fluorescence level Not applicable to test samples containing volatile contaminants.

A.1.3.8 Biomass — SIR method

1.	**Title of the test:**	Soil quality — Determination of soil microbial biomass — Part 1: Substrate induced respiration method
2.	**Harmonization**	International
3.	**References**	ISO 14240-1
4.	**Principle**	Soil is amended with a series of increasing concentrations of glucose until a maximum respiration rate is reached. From this rate, the active biomass is estimated.
5.	**Test type**	—
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.	**Test substrate**	Field soil treated according to ISO 18400-206
	Volume/mass	Not specified
8.	**Test conditions**	
	Test chamber	An appropriate container of a respirometer
	Temperature	(22 ± 1) °C
	pH	The intrinsic pH of the test soil
	Light intensity/ quality	Not specified
	Photoperiod	—
	Soil moisture	The intrinsic soil moisture of the test soil
9.	**No. Replicates**	3
10.	**Test duration/ incubation**	6 h
11.	**Neg. control/ dilution soil**	Does not apply
12.	**Validity criteria**	None
13.	**Pos. Control/ reference toxicant Mean EC50, CV**	None
14.	**Statistics**	None
15.	**Test parameter(s)**	Respiration/CO_2 evolution
16.	**End points**	Soil microbial carbon
17.	**Limitations/ Comments**	The document for the determination of microbial biomass offers different incubation systems. The document gives a method for the estimation of active microbial biomass in soil. Method for the determination of substrate induced respiration is described in ISO 16072.

A.1.3.9 Biomass — FE method

1.	**Title of the test:**	Soil quality — Determination of soil microbial biomass — Part 2: Fumigation – extraction method
2.	**Harmonization**	International
3.	**References**	ISO 14240-2
4.	**Principle**	Through fumigation of the soil sample, intact microbial cells are lysed and the microbial organic matter released. The organic carbon extracted is determined for fumigated and unfumigated samples. The difference is used to determine microbial biomass.
5.	**Test type**	
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.	**Test substrate**	Field soil treated according to ISO 18400-206

Volume/mass	25 g to 50 g (dry mass)
8. **Test conditions**	
Test chamber	Glass beaker or petri dish
Temperature	(25 ± 2) °C
pH	The intrinsic pH of the test soil
Light intensity/ quality	Not specified
Photoperiod	—
Soil moisture	Minimum 30 % water holding capacity
9. **No. Replicates**	3
10. **Test duration/ incubation**	22 h to 24 h
11. **Neg. control/ dilution soil**	Does not apply
12. **Validity criteria**	None
13. **Pos. Control/ reference toxicant Mean EC_{50}, CV**	None
14. **Statistics**	None
15. **Test parameter(s)**	Extractable organic carbon
16. **End points**	Soil microbial carbon
17. **Limitations/ Comments**	The document gives a method for the estimation of microbial biomass of soils by measurement of total biomass of extractable organic material mainly from freshly killed microorganisms. The $CHCl_3$ fumigation also affects soil fauna. But the contribution of carbon from these organisms can be neglected (<5 %) and therefore it is referred to as microbial biomass. The method is applicable to aerobic and anaerobic (e.g. water logged or paddy) soil over the whole range of soil pH. Biomass can also be measured in soils containing actively decomposing substrates and soils supersaturated with K_2SO_4 solution.

A.1.3.10 Determination of soil microbial diversity (PLFA and PLEL analyses)

1. **Title of the test:**	Soil quality — Determination of soil microbial diversity — Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis Soil quality — Determination of soil microbial diversity — Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA
2. **Harmonization**	International
3. **References**	ISO/TS 29843-1, ISO/TS 29843-2
4. **Principle**	Lipids are extracted using the Bligh and Dyer extraction procedure. Lipid extracts are separated by liquid chromatography using a silica column (Si-column). Phospholipids are transformed into fatty acid methyl esters (FAME) by mild alkaline hydrolysis and into phospholipid ether lipids (PLEL) by acid hydrolysis and methylation. Separation of FAME into saturated (SATFA), mono-unsaturated (MUFA), poly-unsaturated (PUFA), hydroxy-substituted (PLOH), non-ester-linked unsubstituted (NEL-UNSFA) and non-ester-linked hydroxy-substituted (NEL-UNOH) fatty acids is achieved on solid-phase extraction columns. The different FAME are measured using gas chromatography/mass spectrometry (GC-MS).

5. **Test type**	Lipid based procedure
6. **Test organism**	—
Breeding stock	—
Age of test organism	—
Feeding	—
7. **Test substrate**	Soil, soil material
Volume/mass	10 g to 25 g (dry mass)
8. **Test conditions**	—
Test chamber size	—
Temperature	—
pH	—
Light intensity/quality	—
Photoperiod	—
Other	—
9. **No./container, No. replicates**	—
10. **Test duration**	—
11. **Neg. Control. dilution soil**	—
12. **Validity criteria**	—
13. **Positive control/ reference toxicant, mean EC (and CV)**	—
14. **Statistics**	—

15. **Test parameter**	(EL-)PLFA (ester-linked) phospholipid fatty acid(s) — SATFA saturated fatty acid(s) — MUFA mono-unsaturated fatty acid(s) — PUFA poly-unsaturated fatty acid(s) — PLOH hydroxy-substituted fatty acid(s) — NEL-PLFA non-ester-linked phospholipid fatty acid(s) — UNSFA unsubstituted fatty acid(s) — UNOH hydroxy-substituted fatty acid(s) — PLEL phospholipid ether lipid(s)
16. **End points**	—
17. **Limitations/Comments**	Phospholipids are essential components of membranes of all living cells, and their fatty acid (PLFA: phospholipid fatty acids) or ether-linked isoprenoid side chains (PLEL: phospholipid ether lipid) allow for taxonomic differentiation within complex microbial communities. The approach described in Part 1 is well established in soil ecology and serves as a phenotypic and thus complementary tool to genotypic (molecular genetic) approaches for determining microbial diversity. Part 2 specifies a simple method for the extraction of only phospholipid fatty acids (PLFA) from soils. In contaminated soils/soil materials the ratio MUFA/SATFA is > 1. (see References [38] and [39])

A.1.3.11 Determination of microbial group abundance in soil by quantitative PCR

1. **Title of the test**	Soil quality — Method to directly extract DNA from soil samples and Soil quality — Estimation of abundance of selected microbial gene sequences by quantitative PCR from DNA directly extracted from soil
2. **Harmonization**	International
3. **References**	ISO 11063 (DNA extraction) and ISO 17601 (quantitative PCR)
4. **Principle**	DNA is extracted according to ISO 11063 based on a direct extraction procedure using SDS and bead beating for cell lysis and ethanol for DNA precipitation. Marker genes are quantified using quantitative real time PCR based on ISO 17601-chromatography using a silica column (si-column). Phospholipids are transformed into fatty acid methyl esters (FAME) by mild alkaline hydrolysis and into phospholipid ether lipids (PLEL) by acid hydrolysis and methylation. Separation of FAME into saturated (SATFA), mono-unsaturated (MUFA), poly-unsaturated (PUFA), hydroxy-substituted (PLOH), non-ester-linked unsubstituted (NEL-UNSFA) and non-ester-linked hydroxy-substituted (NEL-UNOH) fatty acids is achieved on solid-phase extraction columns. The different FAME are measured using gas chromatography/mass spectrometry (GC-MS).
5. **Test type**	DNA based procedure
6. **Test organism**	Microorganisms harbouring the respective marker genes

Breeding stock	—
Age of test organism	—
Feeding	—
7. **Test substrate**	Soil, soil material
Volume/mass	0,5 g to 5 g (dry mass)
8. **Test conditions**	Direct extraction from soil; storage of soil samples after sampling at –20 °C or –80 °C until processing; Storage of extracted DNA at –80 °C
Test chamber size	—
Temperature	—
pH	—
Light intensity/quality	—
Photoperiod	—
Other	—
9. **No./container, No. replicates**	—
10. **Test duration**	—
11. **Neg. Control. dilution soil**	Blank extraction for DNA extraction; no template control for PCR
12. **Validity criteria**	Standard curves should be prepared using the marker gene cloned in vector systems, which will be used in different dilutions.
13. **Positive control/reference toxicant, mean EC (and CV)**	—
14. **Statistics**	—
15. **Test parameter**	So far marker genes are mentioned in the reference for the estimation of — total bacterial communities, — denitrifiers, — nitrifiers.
16. **End points**	—
17. **Limitations/Comments**	The direct extraction of DNA from soil samples does not a discrimination between living cells and free DNA in soil released from dead microbes. Thus acute toxic effects cannot be measured by this approach. The number of marker genes present in a sample cannot be directly linked to the cell number due to multiple operons in one cell. The list of marker genes can be extended to any gene of interest.

A.2 Aquatic test methods

A.2.1 *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. **Harmonization**	International
3. **References**	ISO 6341
4. **Principle**	Determination of the effect of water samples (effluents, waste water, chemicals, fresh water, aqueous extracts, eluates) 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 and should not be first brood progeny
Feeding	None
7. **Test substrate**	Natural water, reconstituted water, dechlorinated tap water
Volume	Sufficient to provide at least 2 ml of test solution for each animal (most often a volume of 10 ml for 5 Daphnia per test container)
8. **Test conditions**	
Test chamber size	
Temperature	20 °C ± 2 °C
pH	7,8 ± 0,2
Light intensity/quality	Photoperiod 16 h/8 h or darkness
Photoperiod	
9. **No./container, No. Replicates**	At least 20 animals, preferably divided into four groups of five animals each
10. **Test duration**	48 h
11. **Neg. Control. dilution water**	Natural water, reconstituted water, dechlorinated tap water
12. **Validity criteria**	Control mortality is less than or equal to 10 %; 24 h – EC_{50}; of the potassium dichromate is within the range of 0,6 mg/l to 2,1 mg/l.
13. **Positive control/ reference toxicant, mean EC (and CV)**	$K_2Cr_2O_7$ 24h – EC_{50}: 0,6 mg/l to 2,1 mg/l
14. **Statistics**	Probit analysis, moving average, binomial methods…
15. **Test parameter**	Immobilisation
16. **End points**	EC_{50}; minimum concentration corresponding to 100 % immobilization; maximum concentration corresponding to 0 % immobilization.
17. **Limitations/Comments**	

A.2.2 Freshwater algal growth inhibition test

1. **Title of the test**	Water quality — Freshwater algal growth inhibition test with unicellular green algae
2. **Harmonization**	International
3. **References**	ISO 8692
4. **Principle**	Effect on unicellular algae growth, inoculum from a culture in exponential growth phase
5. **Test type**	Chronic, static
6. **Test organism**	Unicellular green algae
Breeding stock	*Pseudokirchneriella subcapitata*; *Desmodesmus subspicatus*
Age of test organism	Exponentially growing pre-culture (2 d to 4 d)
Feeding	—
7. **Test substrate**	Growth medium
Volume	Usually 100 ml (alternatives on small volumes)
8. **Test conditions**	
Test chamber size	250 ml erlenmeyer flasks

Temperature	(23 C ± 2) °C
pH	8,3
Light intensity/quality	35 to 70 × 10^{18} photons/m^2/s (400 nm to 700 nm); 6 000 lx to 10 000 lx (for light-measuring instruments calibrated in lux)
Photoperiod	Continuous light
9. **No./container, No. replicates**	at least 3 replicates × 5 concentrations + 6 replicates of control
10. **Test duration**	72 h ± 2 h
11. **Neg. Control.**	Growth medium
12. **Validity criteria**	The average growth rate in the control replicates shall be at least 1,4 d^{-1} corresponding to an increase in cell density by a factor 67 in 72 h. The variation coefficient of the growth rate in the control replicates shall not exceed 5 %. The pH in the control shall not have increased during the test by more than 1,5 relative to the pH of the growth medium.
13. **Positive control/ reference toxicant, mean EC (and CV)**	$K_2Cr_2O_7$: EC_{50} growth rate *D. subspicatus*: 0,84 mg/l (0,12) EC_{50} growth rate *P. subcapitata*: 1,19 mg/l (0,27) 3,5 dichlorophenol: EC_{50} growth rate *D. subspicatus*: 6,42 mg/l (2,38) EC_{50} growth rate *P. subcapitata*: 3,38 mg/l (1,30)
14. **Statistics**	Multisample comparison or regression
15. **Test parameter**	Growth rate
16. **End points**	NOEC or EC_x (x = 10, 20, 50)
17. **Limitations/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. — Metals may not be bio-available by complexation with EDTA from the test medium — Volatile substances may be stripped by aeration in the tests flasks. See ISO 14442 for information on difficult substances management.

A.2.3 Freshwater fish acute toxicity test

1. **Title of the test:**	Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]
2. **Harmonization**	International
3. **References**	ISO 7346
4. **Principle**	Effect on survival of *Danio rerio*
5. **Test type**	Acute, (Part 1 = static, 2 = semi-static, 3 = continuous renewal).
6. **Test organism**	Zebra fish
Breeding stock	*Danio rerio* Hamilton-Buchanan

	Age of test organism	Adults
	Feeding	None
7.	**Test substrate**	Fresh water
	Volume	1 l per g of fish
8.	**Test conditions**	
	Test chamber size	up to 10 l
	Temperature	23 °C ± 1 °C
	pH	7,8 ± 0,2
	Light intensity/quality	usual laboratory illumination
	Photoperiod	12 h to 16 h day light
9.	**No./container, No. replicates**	At least 7 fish per vessel, 1 vessel per concentration
10.	**Test duration**	96 h
11.	**Neg. Control dilution**	Water
12.	**Validity criteria**	Dissolved > 60 % saturation, control fish mortality < 10 %, no abnormal behaviour
13.	**Positive control/ reference toxicant, mean EC (and CV)**	$K_2Cr_2O_7$
14.	**Statistics**	Regression
15.	**Test parameter**	Mortality
16.	**End points**	LC_{50}
17.	**Limitations/Comments**	Volume of eluate necessary to perform the test. The implementation of the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

A.2.4 Fish egg test

1.	**Title of the test:**	Water quality — Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)
2.	**Harmonization**	International
3.	**References**	ISO 15088
4.	**Principle**	Acute effects on survival of fertilised eggs
5.	**Test type**	Acute
6.	**Test organism**	Zebra fish
	Breeding stock	*Danio rerio* Hamilton-Buchanan (healthy spawners free from externally visible diseases and aged between 6 months and 24 months)
	Age of test organism	Fertilized eggs from the 4-cell stage to the 128-cell stage
	Feeding	None
7.	**Test substrate**	Waste water, effluents etc.
	Volume	2 ml (1 egg)
8.	**Test conditions**	
	Test chamber size	2,5 ml to 5 m (usually 24 well microplate)
	Temperature	26 °C ± 1 °C
	pH	7,0 ± 0,2
	Light intensity/quality	No recommendation
	Photoperiod	16 h/8 h (light/dark) or 12/12 h

9. **No./container, replicates No.**	10 eggs per test condition
10. **Test duration**	48 h
11. **Neg. Control dilution**	Dilution water
12. **Validity criteria**	At least 90 % of the embryos in the negative dilution water control survive after the 48 h incubation time. The results of the positive control are within the defined range (3,7 mg/l of reference substance should cause an effect > 10 %)
13. **Positive control/ reference toxicant, mean EC (and CV)**	3,4-dichloroaniline
14. **Statistics**	No information (reference to ISO/TS 20281)
15. **Test parameter**	Number of coagulated eggs, tail detachment, heartbeat
16. **End points**	LID, EC_{50}
17. **Limitations/Comments:**	

A.2.5 Marine algal growth inhibition test

1. **Title of the test**	Water quality — Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*.
2. **Harmonization**	International
3. **References**	ISO 10253
4. **Principle**	Algal population growth inhibition
5. **Test type**	Chronic, static
6. **Test organism**	Unicellular algae; inoculum from a culture in exponential growth phase
Breeding stock	*Skeletonema costatum* or *Phaeodactylum tricornutum*
Age of test organism	Inoculum from a population
Feeding	Nutritive medium
7. **Test substrate**	Seawater
Volume	approximately 100 ml
8. **Test conditions**	
Test chamber size	250 ml
Temperature	20 °C ± 1 °C
pH	6 to 8,5
Light intensity/quality	35 to 70 × 10^8 photons/m^2/s (400 nm to 700 nm)
Photoperiod	Continuous light
9. **No./container, replicates No.**	3 replicates per concentration, 6 replicates for control
10. **Test duration**	72 h
11. **Neg. Control. dilution**	Seawater
12. **Validity criteria**	The control cell density shall have increased by a factor of more than 16 h in 72 h, corresponding to a specific growth rate of 0,9 d^{-1}. The variation coefficient of the control specific growth rates should not exceed 7 %. The control pH shall not have increased by more than 1,0 during the test.

13. **Positive control/ reference toxicant, mean EC(and CV)**	$K_2Cr_2O_7$: EC_{50} growth rate *S. costatum:* 2,5 mg/l (1,1) EC_{50} growth rate *P. tricornutum*: 20,1 mg/l (5,3) 3,5 dichlorophenol EC_{50} growth rate *S. costatum*: 1,6 mg/l (0,3) EC_{50} growth rate *P. tricornutum*: 2,7 mg/l (0,2)
14. **Statistics**	Comparison and regression
15. **Test parameter**	Population growth inhibition
16. **End points**	NOEC and EC_x
17. **Limitations/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. — Metals may not be bio-available by complexation with EDTA from the test medium. — Volatile substances may be stripped by aeration in the tests flasks. See ISO 14442 for information on difficult substances management.

A.2.6 Daphnia magna reproduction test

1. **Title of the test:**	Determination of long term toxicity of substances to *Daphnia magna* Straus *(Cladocera crustacea)*
2. **Harmonization**	International
3. **References**	ISO 10706
4. **Principle**	Inhibition of reproduction and survival of *Daphnia magna*
5. **Test type**	Chronic, static/semi-static
6. **Test organism**	Water flea
Breeding stock	*Daphnia mag*na Strauss *D. magna* at least third generation obtained by acyclical parthenogenesis.
Age of test organism	< 24 h
Feeding	Unicellular algae (*Chlorella* sp., *Pseudokirchneriella subcapitata* or *Scenedesmus subspicatus*) 0,1 mg to 0,2 mg carbon/animal/day
7. **Test substrate**	Aqueous test medium
Volume	50 ml to 100 ml
8. **Test conditions**	
Test chamber size	100 ml to 200 ml beakers
Temperature	within 18 °C to 22 °C, variations within less than 2 °C
pH	7,8 ± 0,2
Light intensity/quality	< 1 200 lx
Photoperiod	16 h light
9. **No./container, No. replicates**	5 concentrations × 10 replicates (1 animal per vessel is recommended)
10. **Test duration**	21 days
11. **Neg. Control. dilution**	Water

12. **Validity criteria**	Mortality of adults or living males < 20 % in the control, mean number of offspring per parent > 60 in the control
13. **Positive control/ reference toxicant, mean EC(and CV)**	The Daphnid culture may be controlled using acute $K_2Cr_2O_7$ test
14. **Statistics**	Dunnett or Williams test and regression
15. **Test parameter**	Mortality of adults, inhibition of reproduction or population growth
16. **End points**	EC_x, NOEC
17. **Limitations/Comments**	This test is mainly used for pure substances, short-term alternatives exist, for instance using *Ceriodaphnia dubia*.

A.2.7 Chronic toxicity to Ceriodaphnia dubia

1. **Title of the test:**	Water quality — Determination of chronic toxicity to *Ceriodaphnia dubia*
2. **Harmonization**	International
3. **References**	ISO 20665
4. **Principle**	*Ceriodaphnia dubia*, less than 24 h old at the beginning of the test, are exposed individually to a range of concentrations of the sample under test for a period of (7 ± 1) d. The test typically ends after 7 d when 60 % of the control organisms have produced their third brood. The mortality of the adult females and their reproduction are monitored throughout the exposure time.
5. **Test type**	Chronic, semi-static
6. **Test organism**	*Ceriodaphnia dubia*
Breeding stock	*Ceriodaphnia dubia* neonates are obtained by parthenogenesis from adult females for at least three generations under the conditions of temperature, photoperiod and food identical to those in the test. The *Ceriodaphnia dubia* used for the test shall be < 24 h old and shall have been taken from a brood comprising at least eight newly born animals.
Age of test organism	< 24 h
Feeding	1) *Chlorella vulgaris, Pseudokirchneriella subcapitata* and fish food suspension, or 2) Yeast/Cerophyll/trout chow and *Pseudokirchneriella subcapitata*
7. **Test substrate**	Eluates, effluents, waste water, freshwater, chemicals
Volume	15 ml to 50 ml
8. **Test conditions**	
Test chamber size	—
Temperature	(25 ± 2) °C
pH	No recommendations
Light intensity/quality	100 lx to 600 lx
Photoperiod	16 h of daylight / 8 h of darkness
9. **No./container, No. replicates**	10 (1 *C.dubia* per replicate)
10. **Test duration**	7 ± 1 d
11. **Neg. Control. dilution**	Aqueous test medium: Elendt M4 or moderately hard water

12. **Validity criteria**	The mean mortality rate of the adult females at the end of the test does not exceed 20 %; The proportion of adult males does not exceed 10 %; 60 % or more of the adult females produce three broods by the end of the test; The mean number of offspring born per alive adult female at the end of the test is greater than or equal to 15.
13. **Positive control/ reference toxicant, mean EC(and CV)**	Sodium pentachlorophenolate, copper sulfate pentahydrate, sodium chloride or zinc sulfate.
14. **Statistics**	regression
15. **Test parameter**	Mortality of adults, inhibition of reproduction or population growth
16. **End points**	EC_x, e.g. EC_{10}, EC_{20} or EC_{50}
17. **Limitations/Comments**	If the raw sample or the decanted supernatant is likely to interfere with the test (due to the presence of microcrustaceans, residual suspended matter, protozoa, microorganisms, etc.), it is filtered through a 0,45-µm-membrane filter or centrifuged.

A.2.8 Chronic toxicity to *Brachionus calyciflorus* in 48 h

1. **Title of the test:**	Water quality — Determination of chronic toxicity to *Brachionus calyciflorus* in 48 h
2. **Harmonization**	International
3. **References**	ISO 20666
4. **Principle**	Female *Brachionus calyciflorus*, less than 2 h old at the beginning of the test, are exposed individually to a range of concentrations of the sample under test for a period of 48 h. The test focuses on the population growth of planktonic rotifers by parthenogenetic reproduction. At the end of the test, the number of female rotifers is determined and, by comparison with the control, the population growth inhibition percentages are determined for each concentration.
5. **Test type**	Chronic, static
6. **Test organism**	Rotifer (*Brachionus calyciflorus*)
Breeding stock	Females of the species *Brachionus calyciflorus* are obtained from a laboratory culture or born from commercially available cysts (If cysts are used, the first generation obtained by hatching of cysts is selected for the test).
Age of test organism	The animals used for the test shall be less than 2 h old, the hatching should therefore be supervised as from 17 h of incubation, then every half hour.
Feeding	*Chlorella vulgaris* or *Pseudokirchneriella subcapitata*
7. **Test substrate**	Eluates, effluents, waste water, freshwater, chemicals
Volume	1 ml
8. **Test conditions**	
Test chamber size	24 well microplate
Temperature	(25 ± 1) °C
pH	No recommendations
Light intensity/quality	No light
Photoperiod	No