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Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei —

Part 1: Evaluation of genotoxicity using amphibian larvae

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National foreword

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The UK participation in its preparation was entrusted by Technical Committee EH/3, Water quality, to Subcommittee EH/3/5, Biological methods, which has the responsibility to:

— aid enquirers to understand the text;

— present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep UK interests informed;

— monitor related international and European developments and promulgate them in the UK.

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Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei —

Part 1: Evaluation of genotoxicity using amphibian larvae

Qualité de l'eau — Évaluation de la génotoxicité par le mesurage de l'induction de micronoyaux —

Partie 1: Évaluation de la génotoxicité à l'aide de larves d'amphibiens

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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.

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The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 21427-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 21427 consists of the following parts, under the general title *Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei*:

— *Part 1: Evaluation of genotoxicity using amphibian larvae*

— *Part 2: Mixed population method using the cell line V79*

Introduction

Environmental protection calls for taking into account the genotoxic hazards likely to concern the different populations making up ecosystems. Since such hazards may emerge within waters, it is essential to assess them by means of laboratory tests that allow the evaluation, within aqueous environments, of the genotoxicity of a water, effluent, substance or preparation with respect to organisms living in aquatic environments.

This part of ISO 21427 describes a test method that is likely to provide information in this field. It allows the highlighting, within aqueous environments, of the genotoxic effects on larvae of the two amphibian species, *Xenopus laevis* and *Pleurodeles waltl*.

The choice of the Xenopus is recommended because of several advantages offered by this species: high hatching rates, available all year round after hormone treatment of the breeders, rapid larval development, easy feeding of larvae, widespread distribution within research or breeding centres. However, the described method is, basically, also applicable to Pleurodeles larvae. The provisions specific to Pleurodeles are described in Annex A.

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Water quality — Evaluation of genotoxicity by measurement of the induction of micronuclei —

Part 1:
Evaluation of genotoxicity using amphibian larvae

WARNING — Persons using this part of ISO 21427 should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this part of ISO 21427 be carried out by suitably trained staff.

1 Scope

This part of ISO 21427 specifies a method for assessing genotoxicity using Amphibia larvae (*Xenopus laevis* and *Pleurodeles waltl*). The provisions specific to Pleurodeles are described in Annex A.

The method is applicable to:

— aqueous effluents;

— aqueous leachates;

— eluates of soils;

— eluates of industrial waste;

— eluates of sludges from sewage treatment;

— surface and ground water;

— water-soluble substances.

2 Normative references

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

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 7346-1, *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (*Teleostei, Cyprinidae*)] — Part 1: Static method*

ISO 7346-2, *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (*Teleostei, Cyprinidae*)] — Part 2: Semi-static method*

ISO 7346-3, *Water quality — Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio *Hamilton-Buchanan (*Teleostei, Cyprinidae*)] — Part 3: Flow-through method*

EN 12457-2, *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 with particle size below 4 mm (without or with size reduction)*

NF T 90-305, *Testing water — Determination of the acute toxicity of a substance to* Salmo gairdneri *— Static and flow through methods*

3 Terms and definitions

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

3.1
micronuclei
small particles consisting of acentric fragments of chromosomes and/or entire chromosomes which lag behind at anaphase stage of cell division and form, after telophase, single or multiple micronuclei in the cytoplasm

3.2
sample under test
substance, effluent, surface water, ground water, aqueous effluent, leachate, percolate or eluate submitted to testing

3.3
test medium
mixture of test water (6.2), the sample under test and food

3.4
test solution
mixture of test water (6.2) and the sample under test

4 Principle

The test organisms are exposed during 12 d [3] to a range of concentrations of the sample under test. A control test without a sample (negative control) and a control test with a genotoxic reference substance (positive control) are carried out in parallel under the same conditions. The positive control allows the quality of the biological reagent to be checked and the test to be validated.

During cell division, chromatid fragments without centromers do not move to the nuclei of the daughter cells and stay within the cytoplasma. Some of the chromosomal aberrations induced by the test material are due to chromatid fragments without centromer and are therefore not incorporated in the nuclei of the daughter cells. In addition, spindle disorders may lead to chromosomes which are not incorporated into the nucleus. They are not nuclei and they are formed in the cell cytoplasm not in the plasma. The rate (per thousand ‰) of erythrocytes with micronuclei is determined for each concentration of the sample under test and for the control solutions. The rate of erythrocytes with micronuclei for each concentration is compared to that of the negative control in order to determine the concentrations that induce a positive genotoxic effect.

NOTE The rate of erythrocytes with micronuclei is defined as the level of erythrocytes including one or more micronuclei scored in a sample of 1 000 erythrocytes observed in one blood smear.

5 Test environment

All of the tests and handling operations as well as the breeding of the adults and larvae shall be performed in a room in which the atmosphere is free from toxic dusts and vapours.

The test is conducted under lighting (artificial light or natural light without direct sunshine) according to a 12 h light/12 h dark cycle, in a thermostatically-controlled chamber (7.3) so as to maintain the bottles at a temperature of 22 °C ± 1 °C.

6 Reagents

6.1 Biological reagent, *Xenopus laevis*[1)] designated hereafter by the common name Xenopus.

The tests shall start on larvae at stage 50 of the Nieuwkoop and Faber [1] chronological table of development. At this stage, the animals measure 20 mm to 27 mm in length and present a constriction at the base of the hind leg.

The larvae shall be kept in vessels (7.2) for a period of at least 8 d before the start of the test under temperature and lighting conditions identical to those of the test. The larvae shall be free of diseases and malformations.

For a given test, the batches of treated larvae and control larvae shall stem from the same hatching process. Each batch is made up of at least 15 test vessels (7.2).

6.2 Test water.

The water used for the test shall meet the criteria described in the first paragraph of Annex B. If water other than the breeding water is used, the organisms shall be maintained in this water during at least 8 d before starting the test.

6.3 Food.

The food employed is that usually intended for aquarium fish[2)]. It shall be ground to a powder just prior to use. Freeze-dried watercress powder may also be used.

6.4 Reference substance, Cyclophosphamide monohydrate ($C_7H_{15}Cl_2N_2O_2P,H_2O$), of recognized analytical quality, at a concentration of 20 mg/l.

6.5 Intermediate solvent, dimethyl sulfoxide (DMSO) or any other appropriate water-miscible solvent.

The genotoxicity and general toxicity of the solvent being used shall be established beforehand.

6.6 Anaesthetic, tricaine methane sulfonate.

6.7 Heparin, 200 µg/l solution of powdered heparin in a 7 g/l aqueous solution of sodium chloride.

6.8 Methanol, CH_3OH, of recognized analytical quality.

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1) Xenopus can be obtained from the Service d'élevage de Xénopes du C.N.R.S., UPRES A 6026 C.N.R.S., Université de Rennes I, Avenue du Général Leclerc, 35042 Rennes Cedex, France. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) Tetraphyll is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6.9 Stains [16]

6.9.1 Masson's haemalum.

Composition	
Haematin	2 mg/ml
Potassium alum	10 %

6.9.2 Groat's haematoxylin.

Composition	
Ammonium ferric sulfate (Iron alum)	1,0 g
Haematoxylin	0,5 g
Sulfuric acid concentrated	0,8 ml
Ethanol	50 ml
Water	50 ml

7 Apparatus

Ordinary laboratory apparatus in glass or chemically inert material and in particular the following.

7.1 Aquariums, 25 l or 50 l capacity.

7.2 Glass containers, e.g. bottles, 5 l capacity, capable of being hermetically closed.

Conical flasks with ground-glass stoppers or rubber bungs covered with a tetrafluorocarbon film are suitable.

7.3 Thermostatically-controlled chamber, of sufficient capacity to hold all the bottles corresponding to the different batches of one complete test – test sample, reference substance and negative control – and able to maintain the bottles (7.2) at a temperature of 22 °C ± 1 °C.

7.4 Binocular magnifying glass.

7.5 Microscope, fitted with an immersion lens (total magnification × 1 500).

7.6 Heparinized micropipettes.

NOTE To be heparinized, the micropipettes are stretched and opened, then the sharp size of the micropipette is filled with a 200 µg/ml solution of heparin in a 7 g/l NaCl solution.

8 Treatment and preparation of samples

8.1 Waters, effluents, leachates and eluates to be tested

It is recommended to carry out the sampling, transportation and storage of the samples in accordance with the general procedures as specified in ISO 5667-16.

The interval between the collection of the sample and its receipt by the laboratory shall not exceed 24 h.

For solid samples (waste, soils, sludge), conduct, within 1 month following the receipt of the sample by the laboratory, a leaching test according to the protocol specified in EN 12457-2. However, the obtained aqueous eluates shall not be filtered prior to testing. The obtained eluates shall be kept in the dark at a temperature of 4 °C ± 3 °C until the test is performed. The latter shall be started at the latest 24 h after the leaching stage.

The samples of waters, effluents or leachates, contained in bottles made from chemically inert materials, shall be kept in the dark at a temperature of 4 °C ± 3 °C until the test is performed. The latter shall be conducted at the latest 24 h after receipt of the sample by the laboratory.

At the time of testing, homogenize the sample to be analysed. The quantities of sample (3.2) required for the daily renewals of test medium (3.3) are brought up to the temperature of 22 °C ± 1 °C beforehand.

If the pH of the sample is not between 6 and 8 inclusive, adjust it to 7 ± 1 using hydrochloric acid or sodium hydroxide and homogenize the sample under test (3.2).

8.2 Substances to be tested

8.2.1 Preparation of the stock solutions of substances to be tested

The stock solution of the substance to be tested is prepared by dissolving a known quantity of substance in a specified volume of test water (6.2). It shall be prepared at the time of use. However, if the stock solution of the substance is stable in the dark and at 4 °C, it may be prepared in advance and stored under these conditions.

8.2.2 Preparation of the test solutions of substances to be tested

The test solutions are prepared just prior to use by diluting the stock solution in the test water (6.2) in order to obtain the necessary concentrations.

In the case of substances that are slightly soluble or insoluble in water, an intermediate water-miscible solvent (6.5) may be used. In this case, the solvent concentration shall be the same in each container and shall not exceed 100 mg/l. The test water (6.2) is shaken when introducing the intermediate solution, which generally leads to the formation of a microsuspension. If a precipitate forms, the test cannot be carried out. If the use of an intermediate solvent is unavoidable, a negative control batch containing the same solvent concentration shall be included in the test.

9 Procedure

9.1 Selection of concentrations

The test shall comprise at least five concentrations of the sample under test selected within a geometric range with a separation factor not exceeding two. These concentrations constitute the test solutions (3.4).

For the substances, example concentrations are:

10 mg/l; 5 mg/l; 2,5 mg/l; 1,25 mg/l; 0,62 mg/l; 0,31 mg/l.

For the samples of waters, effluents, leachates or eluates, example concentrations are:

1 000 ml/l; 500 ml/l; 250 ml/l; 125 ml/l; 62,5 ml/l.

This range of concentrations is prepared by diluting the sample with the test water (6.2).

Each test shall include a negative control batch without any sample under test.

NOTE The test concentrations are proposed as examples. The full-scale definitive test can be found from the previous literature on the tested substance or predicted from the results of a range finding test [3], [8], [9], [13].

9.2 Conducting the test

For each concentration and for the negative control batch, introduce into a 5 l bottle (7.2) the test solution (9.1) at the rate of 100 ml per larva, the larvae (15 to 20) and the food (6.3).

Produce, parallel to each test, a positive control with the reference substance (6.4).

Stopper the bottles and place them in the thermostatically-controlled chamber (7.3).

Each container shall receive the same quantity of food. The entire quantity of food shall be consumed. After a few hours, check whether the food is being consumed at the same speed in all the bottles. If this is not the case, mention it in the test report.

NOTE A negative test result obtained from a batch of larvae exposed to the sample in test and which are not feeding cannot be validated.

Every 24 h, renew in each bottle, under a fume cupboard, the test solution (9.1) and the food (6.3).

In the case of highly polluted waters, the test medium may require to be oxygenated by bubbling air through it. During the course of the test, the test medium shall have a dissolved oxygen concentration maintained at 60 % of the saturation value.

Record daily the mortality rate and any behavioural disturbances of the larvae (e.g. feeding interrupted, growth interrupted, abnormal motor activity).

Stop the test at the end of 12 d. Store for the analysis at least the 3 strongest concentrations which do not give rise to any acute toxicity.

For the containers corresponding to the selected concentrations, anaesthetize the animals by immersion in a 0,2 g/l solution of tricaine methane sulfonate (6.6).

For each animal, take a blood sample under binocular magnifying glass (7.4) by intracardiac puncture using a heparinized micropipette (7.6). Take one smear per animal using a ground glass slide spreading over degreased histological slides.

Dry the preparations in air and fix them with methanol (6.8) for 3 min. The smears can be stored as such.

9.3 Reading of the histological preparations

Colour the blood smears either with Masson's haemalum (6.9.1) for about 7 min or with Groat's haematoxylin (6.9.2) for about 15 min. Wash the slides in running water for 10 min, then dry them.

Carry out the reading of the slides using the immersion microscope (7.5), determining for each slide the proportion of erythrocytes with micronuclei and the mitotic index on the basis of the examination of at least 1 000 erythrocytes.

If necessary, note as additional information the proportions of erythrocytes with 1, 2, 3, 4 micronuclei or more.

10 Expression of results

10.1 Presentation of results

For each of the test and control batches, present the results in the form of a table (proportion per thousand erythrocytes with micronuclei) corresponding to a minimum of 12 correctly performed smears, a single smear being obtained from each of at least twelve different animals, and classified in ascending order of the values.

10.2 Processing of results

For each batch, the distribution of the obtained values does not generally obey normal distribution (Laplace-Gauss distribution).

Use the appropriate statistical method in order to compare the results of the processed batches with those of the control batches. Annex C provides an example with the use of a statistical method

For a given concentration, a result is considered positive (i.e. that at this concentration, the sample being studied gives rise to the formation of micronuclei), when the proportion per thousand erythrocytes with micronuclei is greater than that of the negative control and the difference is statistically significant.

Indicate the result for each concentration (positive or negative).

10.3 Interpretation of results

10.3.1 Positive test

The positivity of a test can be based on the detection, with respect to the negative control, of a statistically significant response for at least one of the test concentrations.

10.3.2 Negative test

The test is considered negative if:

— with respect to the negative control, no statistically significant positive response is observed for the concentrations submitted to testing;

— the maximum concentration which does not give rise to an acute toxicity has been determined and tested.

11 Validity of the test

The test is considered valid if the following conditions are met.

— The mortality rate in the positive and negative controls shall not exceed 15 % of the number of animals processed, for a given batch.

— A significant positive response with the positive control batch is obtained.

— The rate of the micronuclei in the control batches shall not exceed 1 % for *Xenopus laevis* and 2 % for *Pleurodeles waltl*.

— The mitotic index in the tested batches is not nil and is not different from the mitotic index scored in the control groups.

12 Test report

The test report shall make reference to this part of ISO 21427 and shall include the following information:

a) the origin and characteristics of the biological reagent;

b) the breeding conditions of the biological reagent;

c) all data required for the identification of the sample or substance under test;

d) the sample preparation methods;

   — for effluents, waters, eluates or leachates, the method and the storage time of the samples, the pH of the initial sample, if need be, the conditions in which a possible adjustment of the pH was carried out;

   — for chemical substances, the method of preparation of the stock and test solutions;

e) the observations of acute toxicity conducted during the course of the test;

f) the test results in accordance with Clause 10, specifying the statistical method employed;

g) all operating details not specified in this part of ISO 21427 and incidents likely to have influenced the results.

Annex A
(normative)

Data specific to *Pleurodeles waltl*

A.1 Reagents

A.1.1 Biological reagent, *Pleurodeles waltl*[3] designated hereafter by the common name Pleurodele.

The tests shall begin on larvae at a stage between 52b and 53 of Gallien and Durocher's chronological table of development [2]. At that time, the animals are about 35 mm long and present the anlage of the fifth toe of the hind leg.

A.1.2 Food.

The food is made up of live prey according to choice. Use, preferably, chironome, daphnia, tubifex larvae or if this is not possible, Artemia larvae (*Artemia salina*) having hatched in salt water less than 24 h earlier, and having been filtered and concentrated in breeding water.

A.2 Apparatus

A.2.1 Thermostatically-controlled chamber, of sufficient capacity to hold all the bottles corresponding to the different batches of one complete test – test sample, reference substance and negative control – and able to maintain the bottles at a temperature of 20 °C ± 1 °C.

A.2.2 Microscope, fitted with an immersion lens (total magnification × 1 000).

A.3 Test method

Distribute the food (A.1.2) all at once when the test solution is renewed and in sufficient quantity so that there remains some unabsorbed food 7 h after distribution.

If Artemia larvae are used, give half the ration at the time of renewal of the test solution and the remainder 7 h later.

Bring the quantities of sample required for the daily renewals of the test medium up to the temperature of 20 °C ± 1 °C beforehand.

Conduct, parallel to each test, a positive control with the reference substance (6.4) at a concentration of 2 mg/l.

3) Pleurodele can be obtained from the Centre de Biologie du Développement, UMR 5547, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Annex B
(normative)

Breeding

The larvae and the eggs of the selected species develop best in a water which satisfies the traditional potability criteria and which is chlorine-free such as: mains water treated with activated charcoal in order to eliminate all traces of chlorinated derivatives, ground water, mineral water or dilution water used for acute toxicity testing on fish (see ISO 7346-1, ISO 7346-2, ISO 7346-3 or NF T 90-305).

The lighting may be distributed in the form of artificial or natural light without direct sunshine, according to a 12 h light/12 h dark cycle. The temperature shall be maintained between 18 °C and 23 °C.

Xenopus larvae feed on industrial feeding stuff usually supplied for aquarium fish, ground to a powder prior to use, or on freeze-dried watercress powder.

Annex C
(informative)

Example of a statistical method for processing results

C.1 Scope

The Mac Gill statistical method [5] is recommended for analysing the results of this method. It allows, in particular, the processing of the results obtained on small samples ($n \geqslant 7$) for which the distribution of the values does not obey normal distribution (Laplace-Gauss distribution).

A sample is made up of n values corresponding to the number of smears carried out per batch.

C.2 Principle

Determine the ranks of the median and of the quartiles as follows.

If the sample comprises an even number of values $2p$, the ranks of the median and of the quartiles are defined using the following equations.

$$\text{Median} = \frac{2p+1}{2}$$

$$\text{Lower quartile} = \frac{p+1}{2}$$

$$\text{Upper quartile} = p + \frac{p+1}{2}$$

If the sample comprises an odd number of values $2p+1$, the ranks of the median and of the quartiles are defined using the following equations.

$$\text{Median} = p+1$$

$$\text{Lower quartile} = \frac{p}{2}+1$$

$$\text{Upper quartile} = \frac{3p}{2}+1$$

From this, deduce the values of the median, the lower quartile and the upper quartile for each of the batches.

Determine the confidence interval, for a probability of 95 %, of the median of each sample according to the equation:

$$\text{Confidence interval} = M \pm 1{,}57 \times \frac{I_{QR}}{\sqrt{n}}$$

where

M is the observed median;

n is the number of smears per batch;

I_{QR} is the interquartile range, i.e. the value of the upper quartile minus that of the lower quartile.

The difference between the medians of two samples is significant if their confidence intervals do not overlap.

The result of a processed batch recorded as being positive signifies that in the erythrocytes of the animals making up this batch, the sample under test gives rise to the formation of micronuclei at a rate statistically higher than that of a control batch with which it is compared.

C.3 Example of application

C.3.1 Raw data

The proportions per thousand items of erythrocytes with micronuclei classified per batch and per ascending order of the values are given in Table C.1.

Table C.1 — Raw data

Rank	Batch 1 Negative control ($n = 15$)	Batch 2 Positive control ($n = 15$)	Batch 3 Test batch ($n = 14$)
1	1	2	5
2	1	5	5
3	1	5	6
4	1	6	8
5	2	7	8
6	2	9	8
7	2	10	9
8	2	12	12
9	3	13	12
10	3	14	14
11	6	14	14
12	6	14	15
13	7	15	16
14	7	15	17
15	7	17	

C.3.2 Processing and interpretation of the results

Table C.2 — Calculations

	Batch 1		Batch 2		Batch 3	
	Rank	**Value M_1**	**Rank**	**Value M_2**	**Rank**	**Value M_3**
Median	8	2	8	12	7,5	10,5
Lower quartile	4,5	1,5	4,5	6,5	4	8
Upper quartile	11,5	6	11,5	14	11	14
Confidence interval		1,82		3,04		2,57
NOTE $M_1 = 2 \pm 1{,}82$; $M_2 = 12 \pm 3{,}04$; $M_3 = 10{,}5 \pm 2{,}57$.						

The confidence intervals of the medians M_1 and M_3 do not overlap. The difference between the medians of the two samples is therefore significant. The result of batch 3 is recorded as being positive. The sample under test is genotoxic.

The test is validated, the result of batch 2 is recorded as being positive.

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