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Water quality — Determination of acute toxicity of water samples and chemicals to a fish gill cell line (RTgill-W1)

Qualité de l'eau — Détermination de la toxicité aiguë d'échantillons d'eau et de produits chimiques vis-à-vis de la lignée cellulaire de branchies de poissons (RTgill-W1)

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Foreword

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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).

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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 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

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

Millions of fish are used annually to test the acute toxicity of water samples, such as effluents or chemicals. Using an alternative model of testing would not only reduce the need for animals, but would have added benefits, such as much faster testing, using smaller volumes and creating less waste. Using embryos of zebrafish prior to independent feeding has partly filled this need for alternative fish acute toxicity testing.

This document describes a procedure that assesses fish acute toxicity using a permanent fish cell line. Comparative work with both the zebrafish embryo and the cell line has shown that they are expected to yield similar results, i.e. within approximately a 10-fold range based on measured concentrations. They also have a common limitation, i.e. a limited ability to detect neurotoxic compounds. Resource needs, however, differ. For example, while the use of the cell line omits any need for fish and the time from exposure to obtaining the test results is reduced, it does require sterile culture techniques. Thus, the choice of the assay may be guided by the available resources and needs.

The fish cell line in the procedure described in this document is the RTgill-W1 cell line[1] established from rainbow trout (*Oncorhynchus mykiss*) gill. It is commercially available as ATCC® CRL-2523™[1)]. Two similarly structured procedures are described: one for water samples, such as effluents, and one for chemical testing.

The standards ISO 15088[2] and OECD 236[3] are also related to prediction of waste water or chemical fish acute toxicity, relying on zebrafish embryos.

1) ATCC® CRL-2523™ is the trademark of a product supplied by ATCC, US. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

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WARNING — Working with chemicals or water samples requires precautionary safety measures for handling. This document does not purport to address the safety problems associated with its use. It is the responsibility of the user to establish appropriate health and safety practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

1 Scope

This document specifies a method for the determination of fish acute toxicity using the permanent cell line from rainbow trout (*Oncorhynchus mykiss*) gill, RTgill-W1. Cells in confluent monolayers in 24-well tissue culture plates are exposed to water samples, such as surface waters or different kinds of effluents, or to chemicals for 24 h and, thereafter, cell viability is assessed based on fluorescent cell viability indicator dyes (see 4.1). Data are then expressed as a percentage of unexposed control and toxicity quantified based on the percentage of cell viability versus the percentage of effluent or the chemical concentration in response curves (see Clause 9).

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
L-15/ex
protein-free medium, containing the same amounts of salts, galactose and pyruvate as Leibovitz L-15 medium[4], used for exposure ("/ex") of RTgill-W1 cells to chemicals or water samples

Note 1 to entry: See 6.3.7.

3.2
whole-water sample/ex
w-ws/ex
water sample with adjusted osmolality due to the addition of salts, galactose and pyruvate similar to *L-15/ex* (3.1)

Note 1 to entry: See 6.3.8, 6.3.9 and 8.3.2.3.

3.3
negative control
exposure medium *L-15/ex* (3.1) without test chemical

3.4
positive control
well-characterized reference substance that, when evaluated by a specific test method, demonstrates the suitability of the test system to yield a reproducible, appropriate response

Note 1 to entry: The protocols presented in this document were validated using 3,4-Dichloroaniline (3,4-DCA) as a positive control because it is easy to handle, and were used as positive control in ISO 15088[2] and OECD 236[3] (see Introduction). Other substances can be suitable as positive control, as long as they yield a reproducible, appropriate response in the test system.

3.5
solvent control
exposure medium [*L-15/ex* (3.1)] plus the respective concentration of the used co-solvent, e.g. dimethyl sulfoxide (DMSO), if required

Note 1 to entry: Solvent control applies in case a co-solvent, such as DMSO, is required.

3.6
w-ws/ex solvent control
whole-water sample/ex (3.2) plus the respective concentration of the used solvent, for example dimethyl sulfoxide (DMSO), for dosing the *positive control* (3.4) chemical, if required

3.7
no cells-control
two wells per test plate without cells, one receiving *L-15/ex* (3.1) and the other receiving either 100 % *whole-water sample/ex* (3.2) or *L-15/ex* (3.1) with the highest concentration of the test chemical, to determine background fluorescence

Note 1 to entry: See 5.2.

3.8
effective concentration
EC_x
concentration of the test material that causes an x percentage change in cell viability compared to the *negative* (3.3) or *solvent control* (3.5) during a specified time interval

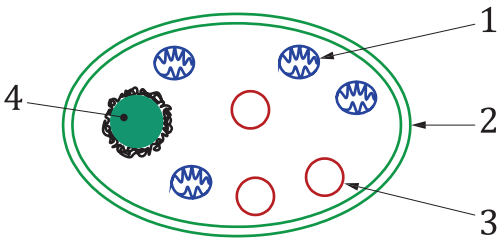
Note 1 to entry: EC_{50} is the concentration at which the cell viability is 50 % compared to the (solvent) control (see Clause 9).

4 Principle

4.1 Cell viability assay

Water samples and chemicals may cause toxic effects to cell cultures. The assay described in this document allows the detection of three different toxicity end points on the same set of cells. The assay is evaluated photometrically by measuring fluorescence of dyes indicating the toxicity and the results are expressed as percentage-cell viability in comparison to an untreated control group.

The assay is based on the combination of three fluorescent indicator dyes: alamarBlue, CFDA-AM and neutral red, which measure, respectively, metabolic activity, integrity of the cell membrane and integrity of the lysosomal membrane, see Figure 1[5].



Key

1 metabolic activity
2 integrity of cell membrane
3 integrity of lysosomal membrane
4 nucleus

Figure 1 — Three fluorescent dyes measuring cytotoxicity based on different targets

alamarBlue™[2] is a commercial preparation of the dye resazurin[6] and all procedures in this document are based on alamarBlue. Other comparable resazurin-based dyes are commercially available, such as PrestoBlue®[3], which can be used interchangeably without any adaptations. Resazurin enters the cells in its non-fluorescent form and is converted to the fluorescent product, resorufin, by mitochondrial, microsomal or cytoplasmic oxidoreductases. A reduction in alamarBlue-fluorescence indicates a decline in cellular metabolism, including disruption of mitochondrial membranes.

5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) rapidly diffuses into the cells and is converted by non-specific esterases of the plasma membrane of living cells to the fluorescent product, 5-carboxyfluorescein. The product diffuses out of intact fish cells slowly[4]. Therefore, a decline in CFDA-AM fluorescence indicates disturbance of plasma membrane integrity.

Neutral red diffuses into the cells and accumulates in lysosomes[7]. Disruption of lysosomes therefore results in a decrease in neutral red fluorescence.

4.2 Key differences in water sample and chemical testing procedure

In the case of testing water samples, first osmolality is adjusted to ensure isotonic exposure conditions when diluting the water sample in cell exposure medium. Thereafter, the sample is filtered in order to avoid interferences by microorganims (see 8.3.2.3). Cell viability is then quantified as described in 4.1.

In the case of chemical testing, the cell exposure medium is sampled at the onset and the end of the exposure period and chemical concentrations quantified to determine actual exposure concentrations. A reliable analytical method for the quantification of the test chemical with reported accuracy and limit of detection should be available. This allows derivation of effective concentrations causing 50 % of effects (EC_{50} value) based on measured concentrations.

5 Interferences

5.1 Matrix effects by effluent samples

The undefined matrix of an effluent (or generally water) sample may limit the capability of the cell line assay to detect toxicants (i.e. if the toxicant is masked by the matrix). For this reason, each effluent

2) alamarBlue™ is the trademark of a product supplied by Thermo Fisher Scientific, USA. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

3) PrestoBlue® is the trademark of a product supplied by Thermo Fisher Scientific, USA. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

sample is tested simultaneously in the presence of a reference chemical as positive control and as the effluent alone. For effluent testing with the positive control, the reference chemical, i.e. 3,4-DCA, is spiked to the effluent in a concentration range that is expected to yield a full concentration response curve and EC_{50} values as indicated in Clause 10.

If the effluent itself is toxic, with a clear concentration-response curve, the positive control effluent with 3,4-DCA would also not result in a clear concentration-response curve and is, therefore, obsolete (e.g. see B.2, Table B.2 and Figure B.1, B, sample 6/site E). However, as many effluents are not acutely toxic, obtaining a concentration-response curve with 3,4-DCA will indicate proper functioning of the assay procedure if little or no toxicity stems from the effluent. A shift of the 3,4-DCA concentration response curve in the effluent matrix compared to L-15/ex to the right (i.e. lower part of curve is missing — 0 % cell viability can no longer be observed) would indicate that the matrix lowers availability of the chemical to the cells but that the assay procedure worked (e.g. see B.2, Table B.2 and Figure B.1, A, sample 3/site B). A shift of the concentration-response curve to the left (i.e. upper part of the curve is missing — 100 % cell viability can no longer be observed) would indicate that the assay procedure worked but that the effluent by itself elicits a certain toxicity to the cells that is enhanced by the additional stress of the 3,4-DCA (e.g. see B.2, Table B.2 and Figure B.1, D, sample 17/site O).

In some cases, precipitates may form upon addition of salts (see 8.3.2.3). This by itself does not preclude testing of the water sample as the formation of precipitates does not per se interfere with the assay (see also Reference [8]). If cell viability is affected by such a water sample, however, whether the effect is caused solely by the precipitates or linked to toxicants in the samples cannot be clearly distinguished.

5.2 Interferences of water constituents or chemicals with fluorescent dye assays

Two no cells-control wells per 24-well test plate are necessary to quantify the background fluorescence of the dyes. These cell-free wells are treated in the same way as the wells containing cells. To one of the two no cells-control wells, 2 ml of L-15/ex are added. Inasmuch as undefined water constituents or also chemicals can yield a background fluorescence, a second no-cells control well is included for either water sample or chemical testing to detect possible interferences between whole-water sample/ex or test chemical and the fluorescent dyes. To this well, 2 ml of 100 % whole-water sample/ex or the highest chemical concentration is added (see 8.3.2.6, 8.3.2.9 and 8.3.3.5). If an interference is detected (i.e. a fluorescence higher/lower by 20 % as compared to the other no cells-control well), a no cells-control reference plate is required in an additional test with this particular water sample or chemical including all dilutions (cell free plate treated in the same way as the exposure plate). Such interference has, as of yet, not been detected, thus appears rare. If no interference is detected, both no cells-control wells are treated as no cells-control values (see Clause 9).

6 Reagents

6.1 General

As far as available, use only cell culture tested grade chemicals.

6.2 Ready-for-use purchased reagents

6.2.1 Bovine serum, fetal bovine serum (FBS).

Do not heat inactivate the FBS.

6.2.2 Gentamicin, 10 mg/ml ($C_{20}H_{40}N_4O_{10}$, 496,56 g/mol, CAS-No: 49863-47-0).

6.2.3 Trypsin, 0,25 % in Phosphate Buffered Saline (PBS) w/o, Ca^{2+}, Mg^{2+} (CAS-No: 9002-07-7).

6.2.4 Versene, 0,2 g/l EDTA(Na4) in PBS ($C_{10}H_{14}N_2Na_4O_9$, 398,19 g/mol, CAS-No: 194491-31-1).

6.2.5 Leibovitz L-15 medium, with glutamine and without phenolred.

6.2.6 Formaldehyde, 37 % (w/v) (CH_2O, 30,03 g/mol, CAS-No: 50-00-0).

6.2.7 Acetic acid, ≥ 99,5 % ($C_2H_4O_2$, 60,05 g/mol, CAS-No: 64-19-7).

6.2.8 alamarBlue solution, DAL1100; Invitrogen **or PrestoBlue**, A13262; Invitrogen (Resazurin: $C_{12}H_7NO_4$, 229,19 g/mol, CAS-No: 550-82.3)[4].

6.2.9 CFDA-AM, ($C_{28}H_{20}O_{11}$, 532,46 g/mol, CAS-No: 124412-00-6).

6.2.10 Neutral red solution, ($C_{16}H_{17}IN_4$, 288,78 g/mol, CAS-No: 553-24-2).

6.2.11 Dulbecco's PBS (PBS), 10x, with Ca^{2+} and Mg^{2+}.

6.2.12 Dimethyl sulfoxide (DMSO), ≥ 99,9 % (C_2H_6SO, 78,13 g/mol, CAS-No: 67-68-5).

6.2.13 Ethanol, absolute, for analysis (C_2H_6O, 46,07 g/mol, CAS-No: 64-17-5).

6.2.14 Calcium chloride, ≥ 96 % ($CaCl_2$, 110,98 g/mol, CAS-No: 10043-52-4).

6.2.15 Sodium chloride, ≥ 99 % (NaCl, 58,44 g/mol, CAS-No: 7647-14-5).

6.2.16 Potassium chloride, ≥ 99 % (KCl, 74,55 g/mol, CAS-No: 7447-40-7).

6.2.17 Magnesium sulfate, ≥ 98 % ($MgSO_4$, 120,37 g/mol, CAS-No: 7487-88-9).

6.2.18 Magnesium chloride, ≥ 97 % ($MgCl_2$, 95,21 g/mol, CAS-No: 7786-30-3).

6.2.19 Galactose, ≥ 96 % ($C_6H_{12}O_6$, 180,16 g/mol, CAS-No: 59-23-4).

6.2.20 Sodium pyruvate, ≥ 99 % ($C_3H_3O_3Na$, 110,04 g/mol, CAS-No: 113-24-6).

6.2.21 Sodium phosphate dibasic, ≥ 99 % (Na_2HPO_4, 141,96 g/mol, CAS-No: 7558-79-4).

6.2.22 Potassium phosphate monobasic, ≥ 99 % (KH_2PO_4, 136,09 g/mol, CAS-No: 7778-77-0).

6.2.23 Deionized water, resistivity: ≤ 18,2 MΩ*cm.

6.2.24 3,4-Dichloroaniline ($C_6H_5Cl_2N$, 162,02 g/mol, CAS-No: 95-76-1).

Use only analytical standard grade.

4) These are examples of suppliers able to provide suitable chemicals for the assay performance. This information is given for the convenience of the users of this document and does not constitute an endorsement by ISO of this supplier. Similar products from other providers are available.

6.3 Freshly prepared solutions

6.3.1 L-15 complete culture medium.

Add to 500 ml L-15:

— 25 ml FBS;

— 2,5 ml Gentamicin.

The storage time should not exceed three months at (4 ± 1) °C.

6.3.2 20x salt solution A (for L-15/ex and w-ws/ex).

— 80 g NaCl;

— 4,0 g KCl;

— 0,98 g $MgSO_4$;

— 0,94 g $MgCl_2$.

Fill up with deionized water to 300 ml and autoclave.

The storage time should not exceed six months at room temperature.

6.3.3 30x salt solution B (for L-15/ex and w-ws/ex).

— 1,4 g $CaCl_2$.

Fill up with deionized water to 33,33 ml and autoclave.

The storage time should not exceed six months at room temperature.

6.3.4 30x salt solution C (for L-15/ex and w-ws/ex).

— 1,9 g Na_2HPO_4;

— 0,6 g KH_2PO_4.

Fill up with deionized water to 100 ml and autoclave.

The storage time should not exceed six months at room temperature.

6.3.5 30x galactose solution (for L-15/ex and w-ws/ex).

— 9,0 g galactose.

Fill up with deionized water to 33,33 ml.

Filter-sterilize (0,2 µm) and prepare 3,33 ml aliquots.

The storage time should not exceed six months at (–20 ± 1) °C.

6.3.6 30x sodium pyruvate solution (for L-15/ex and w-ws/ex).

— 5,5 g sodium pyruvate.

Fill up with deionized water to 33,33 ml.

Filter-sterilize (0,2 µm) and prepare 3,33 ml aliquots.

The storage time should not exceed six months at (–20 ± 1) °C.

6.3.7 L-15/ex (prepare aseptically).

— 30 ml 20x salt solution A;

— 3,33 ml 30x salt solution B;

— 10 ml 30x salt solution C;

— 3,33 ml 30x galactose solution;

— 3,33 ml 30x sodium pyruvate solution.

Fill up with sterilized deionized water to 1 000 ml.

The storage time should not exceed three months at room temperature.

6.3.8 Whole-water sample/ex (100 % salts) (prepare aseptically to keep stock solutions sterile).

— 3 ml 20x salt solution A;

— 0,333 ml 30x salt solution B;

— 1 ml 30x salt solution C;

— 0,333 ml 30x galactose solution;

— 0,333 ml 30x sodium pyruvate solution.

Fill up with water sample to 100 ml.

Prepare freshly every time.

6.3.9 Whole-water sample/ex (80 % salts) (prepare aseptically to keep stock solutions sterile).

— 2,4 ml 20x salt solution A;

— 0,266 ml 30x salt solution B;

— 0,8 ml 30x salt solution C;

— 0,266 ml 30x galactose solution;

— 0,266 ml 30x sodium pyruvate solution.

Fill up with water sample to 100 ml.

Prepare freshly every time.

6.3.10 CFDA-AM, 4 mM stock solution.

— 5 mg CFDA-AM;

— 2,32 ml DMSO.

6.3.11 PBS

— 900 ml deionized water;

— 100 ml 10x Dulbeccos PBS.

Stir and adjust the pH to 7,1 (with 10 N NaOH).