INTERNATIONAL STANDARD

ISO 21115

First edition
2019-04

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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Reference number
ISO 21115:2019(E)

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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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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.

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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.

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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.

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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

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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].

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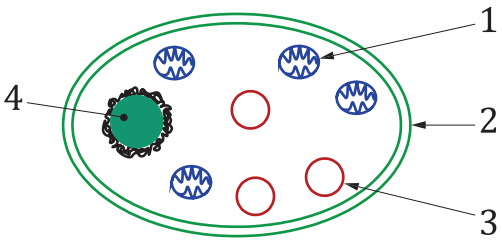

Key

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

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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).

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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.

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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.

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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.

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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).

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6.3.12 alamarBlue and CFDA-AM working solution for one 24-well plate (5 % volume fraction, alamarBlue, 4 µM CFDA-AM in PBS).

— 11,4 ml PBS;

— 600 µl alamarBlue;

— 12 µl CFDA-AM stock solution.

Prepare freshly for testing of cytotoxicity and protect from light.

6.3.13 Neutral red working solution for one 24-well plate.

— 11,82 ml PBS;

— 180 µl neutral red solution.

Prepare freshly for testing of cytotoxicity and protect from light.

6.3.14 Fixative (for neutral red assay).

— 5 g $CaCl_2$;

— 6,75 ml 37 % (w/v) formaldehyde.

Fill up with deionized water to 1 000 ml.

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

6.3.15 Extraction solution (for neutral red assay).

— 500 ml ethanol (abs.);

— 10 ml acetic acid (≥99,5 %).

Fill up with deionized water to 1 000 ml.

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

7 Apparatus and material

7.1 General equipment

7.1.1 Biosafety cabinet.

7.1.2 Incubator, temperature-controlled or climatization of the room to (19 ± 1) °C, without illumination during cell incubation.

7.1.3 Microscope.

7.1.4 Vacuum pump.

7.1.5 Pasteur pipettes, 230 mm, sterile.

7.1.6 Glass pipettes or serological plastic pipettes, 5 ml, 10 ml, 20 ml, sterile.

7.1.7 Pipetting help.

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7.1.8 Pipette box to sterilize glass and Pasteur pipettes.

7.1.9 Pipette tips, 10 µl, 100 µl, 1 ml, 2 ml, 5 ml, 10 ml, sterile.

7.1.10 Pipettes, 10 µl, 100 µl, 1 ml, 2 ml, 5 ml, 10 ml.

7.1.11 Multi-channel pipette, 50 µl to 1 200 µl.

7.1.12 Pipette tips for multi-channel pipette, 50 µl to 1 200 µl, sterile.

7.1.13 Reagent reservoirs, autoclavable or sterile.

7.1.14 Vortex.

7.1.15 pH meter.

7.2 Whole-water sample/ex preparation

7.2.1 Osmometer.

7.2.2 Glass-filter apparatus.

7.2.3 Durapore membrane filter hydrophilic, pores 0,22 µm.

7.3 Cell seeding and plate dosing

7.3.1 Cell culture flasks, 75 cm^2 with vent screw cap.

7.3.2 Centrifuge, temperature-controlled at 18 °C to 21 °C, 875 g.

7.3.3 Plastic centrifuge tubes, 15 ml, 50 ml, sterile.

7.3.4 Cell counting chamber, e.g. Neubauer chamber (improved).

7.3.5 Cell counter, Coulter counter.

7.3.6 Multi-well plates, 24-well, sterile, with hydrophilic surface (TC surface treatment) for improved cell adhesion, high clarity and low autofluorescence.

7.4 Preparation/dosing of stock solutions

7.4.1 Scale.

7.4.2 Amber glass vials and screw caps, 4 ml, 15 ml, screw caps with polytetrafluoroethylene (PTFE) liner, sterile.

7.4.3 Vial shaker, orbital shaker.

7.4.4 Duran glass bottles, 100 ml, 250 ml, 500 ml, 1 l.

7.4.5 Plastic pipette, 50 ml.

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7.4.6 Measuring cylinder, 500 ml, sterile.

7.4.7 Adhesive foil to seal multi-well plates, polyester film with acrylic adhesive.

7.5 Sampling for chemical analysis

7.5.1 Sample vials, 1,5 ml short thread vial.

7.5.2 Vial caps with septa, PTFE lined.

7.5.3 Solvent, depending on the analytical method.

7.6 Detection of cytotoxicity

7.6.1 Fluorescent multiwell plate reader.

7.6.2 Plastic centrifuge tubes, 50 ml.

7.6.3 Plate shaker.

7.6.4 Aluminium foil.

7.6.5 Kitchen paper.

8 Procedure

8.1 Cell line used

The cell line used for the cytotoxicity assay is RTgill-W1[1]. The maintenance is described in Annex A. It is commercially available at ATCC® [Nr. CRL-2523™[5)]].

8.2 Seeding cells into 24-well plates

8.2.1 General

One confluent 75 cm^2 cell culture flask of RTgill-W1 contains around 10 million cells. 350 000 cells per well are seeded into a 24-well plate. Thus, one plate can be generated out of one confluent cell culture flask of RTgill-W1. To test one water sample, two cell plates are needed. For testing one chemical, one cell plate is needed. In case of chemical testing, a separate test plate with the positive control 3,4-DCA needs to be prepared in L-15/ex every five tests (i.e. every sixth test plate). The same procedure is recommended for water samples. For any laboratory initially establishing this assay, 3,4-DCA is suggested as reference chemical.

8.2.2 Preparation of working materials and solutions for cell seeding

Thaw the trypsin. Adjust trypsin, Versene (6.2.3 and 6.2.4) and the L-15 complete culture medium (6.3.1) to 19 °C in the incubator for at least 1 h before performing the cell seeding. Turn the biosafety cabinet on, wipe the cabinet with disinfection according to the sterile procedures in the facility and let the air flow for approximately 10 min to 15 min. Clean the pipette boxes [Pasteur (7.1.5) and glass (7.1.6)],

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trypsin, Versene, L-15 complete culture medium bottles and the confluent cell culture flasks according to the sterile procedures in the facility and put them under the biosafety cabinet.

Depending on the cell counting method you use, prepare a cell counting chamber for manual cell counting or prepare the electric cell counter (Coulter counter) for automatic counting.

8.2.3 Seeding cells: step-by-step performance

Description for seeding of one 24-well plate (one confluent cell culture flask):

a) check cells in the cell culture flask under the microscope;

b) if confluency is reached (see Figure 2), prepare cells for seeding as follows:

   1) draw off the media using sterile Pasteur pipette and vacuum pump;

   2) gently add 1 ml Versene (do not pipette Versene directly onto cells, detachment of cells may occur) (6.2.4);

   3) wash cells by gentle agitation of the cell culture flask;

   4) draw off the Versene and repeat washing step: 2), 3) and 4);

   5) after drawing off Versene for the second time, add 0,7 ml trypsin (6.2.3);

   6) promote the cell detachment by gently tapping and agitating the cell culture flask (check detachment visually);

   7) add 5 ml L-15 complete culture medium (6.3.1) to stop the action of trypsin;

   8) suspend cell clumps and remove remaining attached cells by gently pipetting the solution several times up and down and also over the surface of the cells (strictly avoid the formation of foam – shear forces can disrupt the cells);

   9) transfer the cell solution into a plastic tube (15 ml centrifuge tube) (7.3.3);

   10) centrifuge for 3 min with 875 g at 18 °C to 21 °C;

   11) draw off the supernatant as low as possible without removing the cell pellet;

   12) dissolve the cell pellet in 5 ml L-15 complete culture medium by gently pipetting the solution several times up and down or against the tube wall for better cell suspension without foam formation;

   13) determine the cell number of the cell suspension in two independent runs with the counting method of choice;

   14) if the difference between the two independent measurements of the cell number with the chosen cell counting method differ by more than 10 %, take another count and use the average of the two closest values;

   15) calculate the volume of the cell suspension prepared under 12) required to prepare 25 ml of a cell suspension with a cell density of 350 000 cells/ml;

   16) transfer the respective volume of cell suspension into a new 50 ml plastic tube (7.3.3) and fill up with L-15 complete culture medium (6.3.1) to 25 ml (seeding solution); mix by gently pipetting the solution up and down at least three to four times;

   17) transfer the seeding solution into a sterile reagent reservoir (7.1.13) and transfer 1 ml of the solution into each well of a 24-well plate, except for the “no cells-control” wells (see pipetting scheme in Figure 3); take care to mix the cells continuously during the seeding procedure by gently pipetting the cell suspension up and down in the medium reservoir and gently swaying

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the reservoir back and forth after every second row of seeded cells; close the well plate with the lid;

18) incubate the plate at (19 ± 1) °C for 24 h.

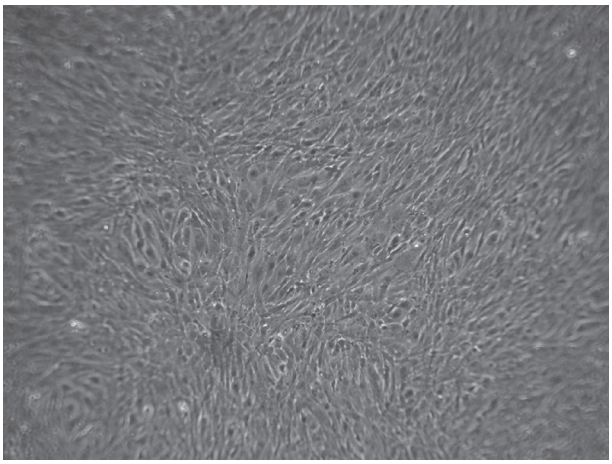

Figure 2 — Confluent monolayer of RTgill-W1 cells

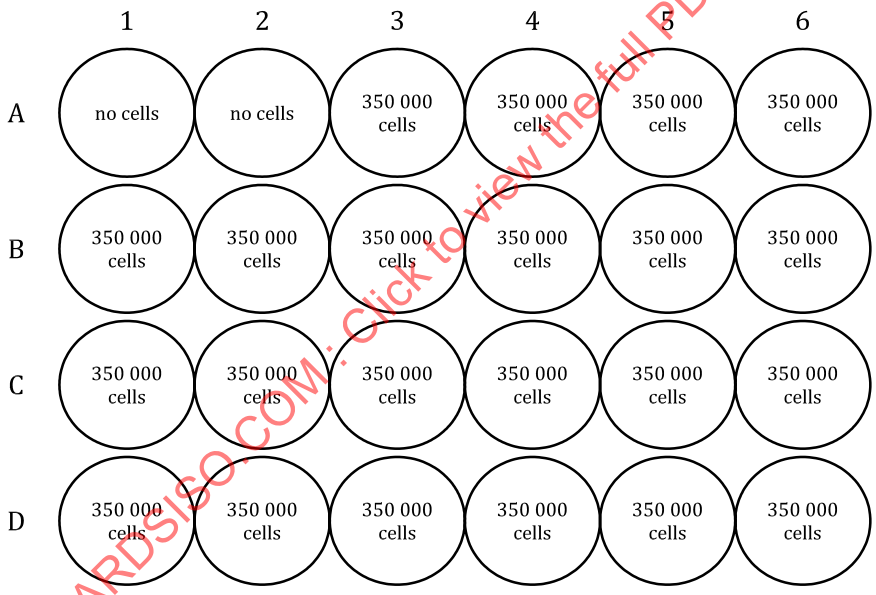

Figure 3 — Pipetting scheme for cell seeding into 24-well plates (1 ml seeding volume)

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8.3 Cell exposure

8.3.1 General

This subclause describes two ways of cell exposures. 8.3.2 specifies the procedure for the testing of water samples, where one cell plate is exposed to a dilution series of the water sample and a second test plate to the water sample along with the positive control 3,4-DCA to test for potential interferences of the matrix of the water sample with chemicals (see 5.1). 8.3.3 specifies the procedure for the testing of individual chemicals, where one cell plate is needed per testing of one chemical and samples for chemical analysis have to be taken from the exposure media out of the wells at the beginning and end of exposure.

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8.3.2 Exposure of cells to water samples

8.3.2.1 General

In 8.3.2, it is described how the cytotoxicity assay with the fish gill cell line, RTgill-W1, can be used for the direct testing of water samples without extraction, adapted and slightly modified from Reference [8]. Since the osmolality of fresh water samples is usually below the osmolality typically required by animal cells, the water samples need to be processed by adding liquid amounts of salts, galactose and pyruvate to the water sample to give concentrations and osmolalities equivalent to the exposure medium, L-15/ex. A standard osmometer is used to verify osmolality of the water samples before and after salt addition. After doing so, the water sample is referred to as "whole-water sample/ex" (abbreviated as "w-ws/ex") and in combination with the exposure medium L-15/ex, a dilution series of the whole-water sample/ex can be tested.

Further, as a positive control, a concentration range of 3,4-Dichloroaniline in the whole-water sample/ex is used to test whether a full concentration response is obtained by the chemical or whether the water sample matrix interferes with chemical testing by, e.g. masking toxicity such that the exposure may result in a reduced toxicity of the chemical (see 5.1).

A general overview about the whole water sample test procedure is given in Figure 4. An explanation is given in Table 1.

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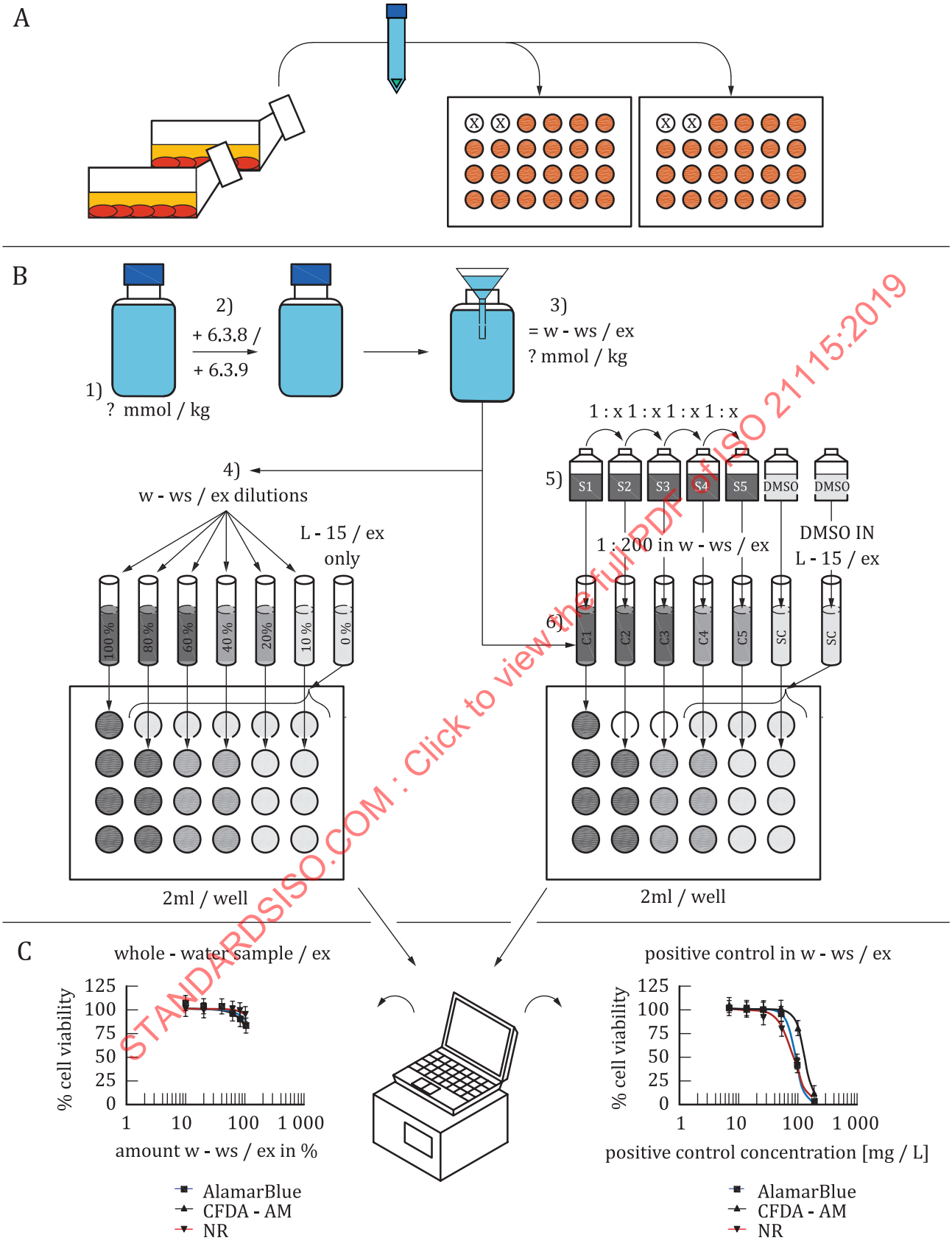

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Figure 4 — General overview about the water sample testing procedure

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Table 1 — Explanation to Figure 4

A – Day 1	Cell seeding	Two well plates per water sample — 350 000 cells per well in 1 ml except for well A1 and A2 (x)
B – Day 2	Whole-water samples/ ex preparation and cell exposure	1) Measure osmolality of raw water sample
		2) Addition of L-15/ex stock solutions (see 6.3.8 or 6.3.9)
		3) Filtration and measurement of Osmolality of w-ws/ex
		4) Preparation of w-ws/ex dilution series with L-15/ex and cell exposure with 2 ml per well
		5) Dilution series of positive control stock solution in DMSO
		6) Preparation of dosing mixtures (10 ml w-ws/ex + 50 µl respective positive control stock solution or DMSO) and cell exposure with 2 ml respective dosing mixture per well
C – Day 3	Measurement of cytotoxicity	Measurement of cytotoxicity of the two well plates per water sample

8.3.2.2 Preparation of DMSO stock solutions of the positive control

Using DMSO as a solvent, the chemical stock solutions need to be 200-times higher concentrated than the final exposure concentrations of the chemical to gain a final DMSO concentration in the exposure media of 0,5 % volume fraction. The highest exposure concentration of 3,4-DCA in the wells is 100 mg/l. Therefore, prepare a stock of 20 g/l in DMSO by weighing the appropriate amount of 3,4-DCA in a 4 ml amber glass vial and addition of the corresponding DMSO volume. Mix this first stock solution extensively by using a shaker for at least 15 min. This stock solution is now the starting point for a serial dilution in DMSO as depicted in Figure 4, B, 5. 3,4-DCA should be tested in five concentrations spaced by a constant factor of two. These concentrations were established to obtain a full concentration response curve in L-15/ex, leading to 0 % and 100 % cell viability compared to the control for the highest and lowest concentration, respectively. The 3,4-DCA dilution series shall be prepared freshly for each experimental day.

Special attention should be drawn to keep the DMSO free of contaminations. It is recommended to use a new bottle and take aliquots, which are then used to prepare the chemical dilution series.

8.3.2.3 Preparation of the whole-water sample/ex

In a first step, the osmolality of the raw water sample is measured in triplicates using a standard osmometer (see Figure 4, B, 1). If the raw osmolality is below 90 mmol/kg, a total of 5 ml of 6.3.8 is added to 95 ml of water sample (see Figure 4, B, 2). If the raw osmolality is above 90 mmol/kg, a total of 4 ml of 6.3.9 is added to 96 ml of the water sample (see Figure 4, B, 2). By adding the solutions (6.3.8 and 6.3.9) as described above, the water sample is being diluted by 4 % or 5 %. In some cases, precipitates may form upon addition of salts (see 5.1).

After adding the respective amount of salts to the water sample, the sample is now referred to as "whole-water sample/ex" and the whole-water sample/ex is filtered through a 0,22 µm membrane filter in a glass-filter apparatus (see Figure 4, B, 3) before the final osmolality is measured in triplicates. The final osmolality of the whole-water sample/ex should be within the acceptable range between 290 mmol/kg and 360 mmol/kg.

8.3.2.4 Preparation of the dilution series of the whole-water sample/ex

Steps 8.3.2.4 and 8.3.2.5, as well as 8.3.2.7 and 8.3.2.8, are performed under sterile conditions. Thus, turn the biosafety cabinet on and let the air flow for approximately 10 min to 15 min. Clean the cabinet and the material needed for preparation of dosing mixtures and exposure according to the sterile procedures of the facility and put them under the cabinet.

For the preparation of the dilution series of the whole-water sample/ex, place seven sterilized 15 ml glass vials under the biosafety cabinet. Add to each glass vial the respective amount of L-15/ex and

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whole-water sample/ex to obtain 10 ml each of the following dilutions: 100 %, 80 %, 60 %, 40 %, 20 %, 10 % and 0 % (see Figure 4, B, 4), where 100 % contains whole-water sample/ex only and 0 % contains L-15/ex only. Mix them by gently pipetting up and down.

8.3.2.5 Whole-water sample/ex exposure: step-by-step performance

Twenty-four hours after plating (see 8.2.3), cells should have formed a confluent monolayer within each well (see Figure 2). This is observed by microscopy. If confluence is not yet reached after 24 h of incubation, the plate can be incubated another 24 h before whole-water sample/ex exposure.

Even distribution of the cells across the whole well should be observed. RTgill-W1 cells grow in monolayer. Once a confluent monolayer is formed, they can still increase in cell number, though very slowly, which leads to tighter packing of cells. The cells do not form multi-layers. As well, if in L-15/ex, the cells no longer grow.

a) Draw off the L-15 complete culture medium from each well. Tilt the plate to allow for easier removal and take care not to touch the cells with the Pasteur pipette. Alternatively, the plate can be aspirated and dabbed dry onto kitchen paper. Immediately proceed to the next step.

b) Gently add 1 ml L-15/ex (6.3.7) to each well using the multi-channel pipette (do not pipette directly onto the cells, otherwise cells will detach from the bottom) and wash wells by manual, gentle swaying of the plate.

c) Draw off L-15/ex or aspirate the plate and dab dry onto paper.

d) Add 2 ml of the respective whole-water sample/ex dilution (see 8.3.2.4) into each well (see Figure 5). Do not pipette directly onto the cells, otherwise cells will detach from the bottom. During the dosing procedure, put the lid on the plate and open only the wells to pipette in. Doing so prevents that the cell layer in not yet dosed wells dries out.

e) Cover the plate with adhesive foil (7.4.7).

f) Incubate the plate for 24 h at (19 ± 1) °C.

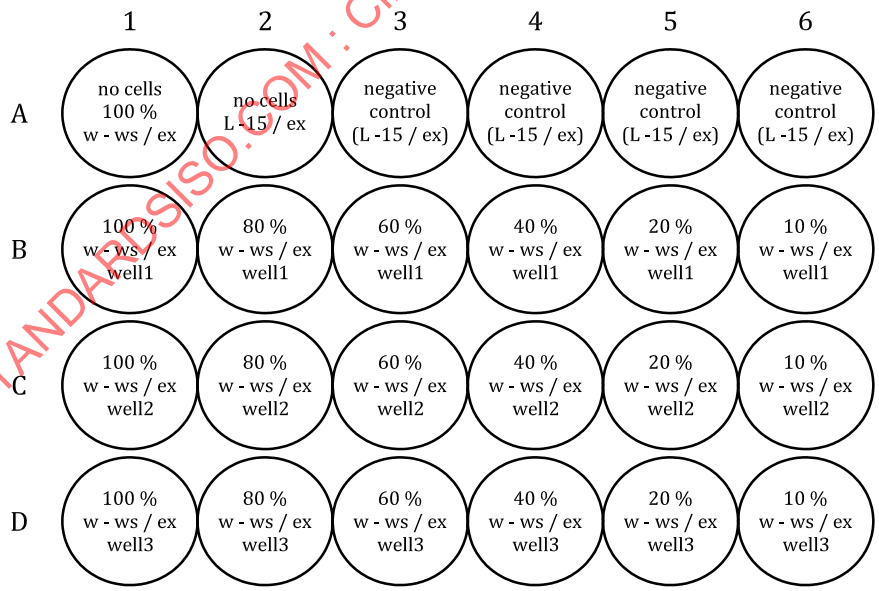

Figure 5 — Pipetting scheme for whole-water sample/ex exposure [one water sample (whole-water sample/ex), six test dilutions and three replicates per plate]

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8.3.2.6 "No cells-control" wells

These are prepared for the purpose and in the manner as described in 5.2, where 2 ml of 100 % whole-water sample/ex is added to well A1 and 2 ml of L-15/ex are added to well A2.

8.3.2.7 Preparation of dosing mixtures of the positive control in whole-water sample/ex

Dosing mixtures are prepared by diluting the stock solutions in the previously prepared whole-water sample/ex (see 8.3.2.3) under sterile conditions and the use of sterile 15 ml amber glass vials. The dosing mixtures should be prepared freshly immediately before dosing onto the cells.

For the preparation of the chemical dosing mixtures from the DMSO stock solutions, place seven sterilized 15 ml glass vials (7.4.2) under the biosafety cabinet. Add to six of the seven glass vials 10 ml whole-water sample/ex (see 8.3.2.3) and 50 µl of the respective 3,4-DCA-DMSO dilution or DMSO (see 8.3.2.2, Figure 4, B, 6). As a further solvent control, prepare one vial with 10 ml L-15/ex and 50 µl DMSO. Shake the dosing mixture vigorously for 10 min using an orbital shaker.

8.3.2.8 Positive control in whole-water sample/ex exposure: step-by-step performance

After 24 h incubation, cells should have formed a confluent monolayer within each well (see Figure 2). This is observed by microscopy. If confluence is not yet reached after 24 h of incubation, the plate can be incubated another 24 h before chemical exposure.

a) Draw off the L-15 complete culture media from each well. Tilt the plate to allow for easier removal and take care not to touch the cells with the Pasteur pipette. Alternatively, the plate can be aspirated and dabbed dry onto kitchen paper. Immediately proceed to the next step.

b) Gently add 1 ml L-15/ex (6.3.7) to each well using the multi-channel pipette (do not pipette directly onto the cells, otherwise cells will detach from the bottom) and wash wells by manual, gentle swaying of the plate.

c) Draw off L-15/ex or aspirate the plate and dab dry onto kitchen paper.

d) Add 2 ml of the respective dosing mixture (see 8.3.2.7) into each well (see Figure 6). Do not pipette directly onto the cells, otherwise cells will detach from the bottom. During dosing procedure, put the lid on the plate and open only the wells to pipette in. Doing so prevents that the cell layer in not yet dosed wells dries out.

e) Cover the plate with adhesive foil (7.4.7).

f) Incubate the plate for 24 h at (19 ± 1) °C.

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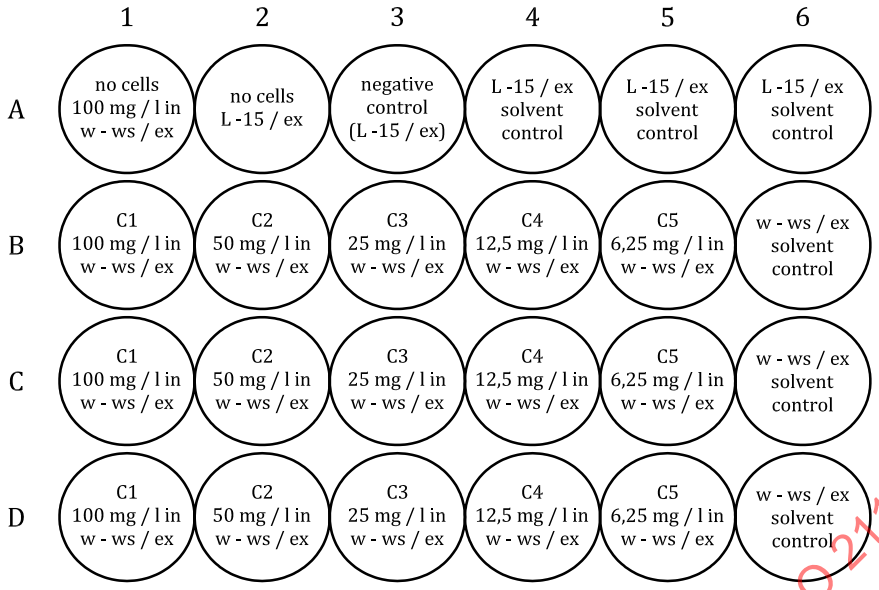

Figure 6 — Pipetting scheme for positive control 3,4-Dichloroaniline in w-ws/ex (one chemical, five test concentrations and three replicates per plate)

8.3.2.9 "No cells-control" wells

These are prepared for the purpose and in the manner as described in 5.2, where 2 ml of the dosing mixture in whole-water sample/ex containing the highest 3,4-DCA test concentration is added to well A1 and 2 ml of L-15/ex are added to well A2.

8.3.3 Exposure of cells to chemicals

8.3.3.1 General

The procedures for cell exposure to chemicals are similar to the testing of the positive control in the water sample exposure parts 8.3.2.2, 8.3.2.7 and 8.3.2.8 with the difference that chemical stock solutions are diluted in the exposure medium L-15/ex instead of whole-water sample/ex. For analytical confirmation of chemical exposure concentrations, samples are taken at the beginning and end of the test out of the exposure wells containing cells, with the exception of the negative control well A3 (Figure 6).

A general overview about the entire chemical test procedure is given in Figure 7. An explanation is given in Table 2.

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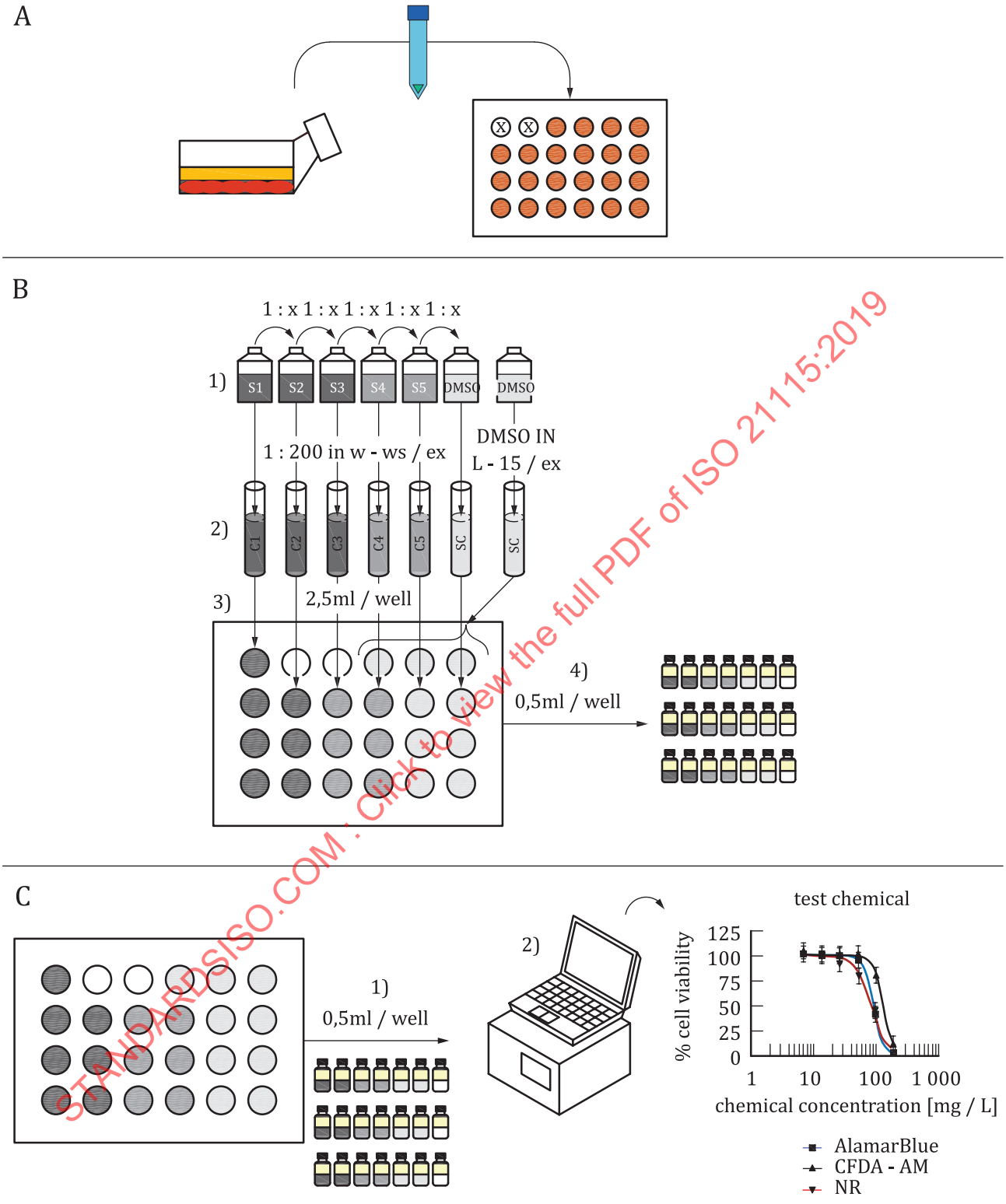

Figure 7 — General overview about the chemical testing procedure

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Table 2 — Explanation to Figure 7

A – Day 1	Cell seeding	One well plate per test chemical — 350 000 cells per well in 1 ml except for well A1 and A2 (x)
B – Day 2	Cell exposure and sampling	1) Dilution series of chemical stock solution in DMSO
		2) Preparation of dosing mixtures (12 ml L-15/ex + 60 µl respective stock solution or DMSO)
		3) Cell exposure with 2,5 ml respective dosing mixture per well
		4) Sampling of 0,5 ml per well at start of exposure
C – Day 3	Sampling and measurement of cytotoxicity	1) Sampling of 0,5 ml per well at end of exposure
		2) Measurement of cytotoxicity

8.3.3.2 Preparation of chemical stock solutions in DMSO

Procedures for preparation of the chemical stock solutions in DMSO follow exactly the preparation of DMSO stock solutions of the positive control, described in 8.3.2.2, with the difference that six instead of five dilutions are prepared (Figure 7, B, 1) with a constant dilution factor not exceeding 2,5.

If the test chemical has a high solubility in water (≥100 mg/l), it can also be dissolved directly in L-15/ex, thereby avoiding the need of a co-solvent. The highest concentration tested should preferably result in 0 % cell viability with regard to the respective control (for chemical testing using co-solvent compare to solvent control, for chemical testing without co-solvent compare to negative control, see Clause 9). The lowest concentration tested should preferably give no effect and therefore result in 100 % cell viability with regard to the respective control. A range-finding test before the definitive test allows the selection of the appropriate concentration range. The range finding test is performed similar to the definitive test, but with concentrations starting at water solubility and respective dilutions with a spacing factor of 10.

8.3.3.3 Preparation of dosing mixtures in L-15/ex

Steps 8.3.3.3 and 8.3.3.4 are performed under sterile conditions. Thus, turn the biosafety cabinet on and let the air flow for approximately 10 min to 15 min. Clean the cabinet and the material needed for preparation of dosing mixtures and exposure according to the sterile procedures of the facility and put them under the cabinet.

Dosing mixtures are prepared by diluting the stock solutions in L-15/ex exposure medium (6.3.7) under sterile conditions and the use of sterile 15 ml amber glass vials (7.4.2). The dosing mixtures should be prepared freshly immediately before dosing onto the cells.

For the preparation of the chemical dosing mixtures from the DMSO stock solutions, place seven sterile 15 ml glass vials under the biosafety cabinet. Add to each glass vial 12 ml L-15/ex and 60 µl of the respective chemical-DMSO dilution or DMSO (Figure 7, B, 2). Shake the dosing mixture vigorously for 10 min using an orbital shaker.

8.3.3.4 Chemical exposure and sampling for chemical analyses: step-by-step performance

24 h after plating (see 8.2.3), cells should have formed a confluent monolayer within each well (Figure 2). This is observed by microscopy. If confluence is not yet reached after 24 h of incubation, the plate can be incubated another 24 h before chemical exposure.

a) For the validation of the exposure concentrations by chemical analysis, prepare the required number of 1,5 ml sampling vials to take samples later on out of each exposure and solvent control well. The sampling vials might be pre-loaded with solvent according to the analytical protocol.

b) Draw off the L-15 complete culture media from each well. Tilt the plate to allow for easier removal and take care not to touch the cells with the Pasteur pipette. Alternatively, the plate can be aspirated and dabbed dry onto kitchen paper. Immediately proceed to the next step.

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c) Gently add 1 ml L-15/ex (6.3.7) to each well using the multi-channel pipette. Do not pipette directly onto the cells, otherwise cells will detach from the bottom. Wash wells by manual, gentle swaying of the plate.

d) Draw off L-15/ex or aspirate the plate and dab dry onto paper.

e) Add 2,5 ml of the respective dosing mixture (8.3.3.3) into each well, except for the negative control with L-15/ex and the no cells-control wells (8.3.3.5) where 2 ml are added (see Figure 8). Do not pipette directly onto the cells, otherwise cells will detach from the bottom. During dosing procedure, put the lid on the plate and open only the wells to pipette in. Doing so prevents that the cell layer in not yet dosed wells dries out.

f) Immediately after dosing the plate, take a 500 µl sample from each of the three wells per concentration and solvent control. Remove 500 µl of the exposure medium from the well and transfer it into the respective sampling vial, pre-loaded or not, with a solvent according to the analytical protocol. Immediately close the vials tightly.

g) Cover the plate with adhesive foil (7.4.7).

h) Incubate the plate for 24 h at (19 ± 1) °C.

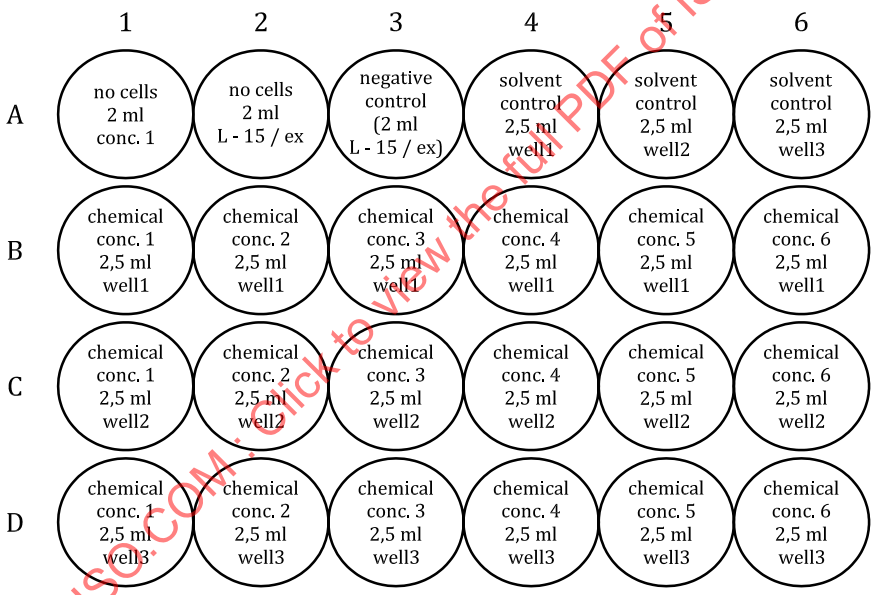

Figure 8 — Pipetting scheme for test chemicals (one chemical, six test concentrations and three replicates per plate)

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8.3.3.5 "No cells-control" wells

These are prepared for the purpose and in the manner as described in 5.2, where 2 ml of the dosing mixture in L-15/ex containing the highest chemical test concentration is added to well A1 and 2 ml of L-15/ex are added to well A2.

8.4 Determination of cytotoxicity

8.4.1 General

The steps given in 8.4.2 to 8.4.6 apply to both the water sample and to chemical testing.

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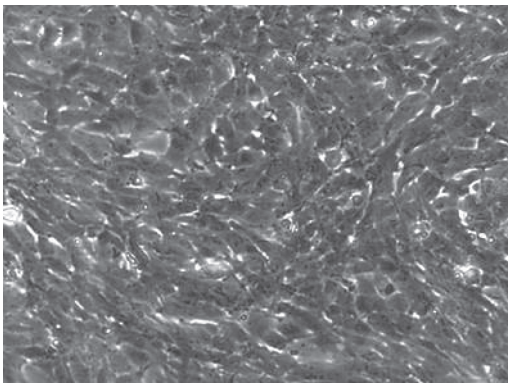
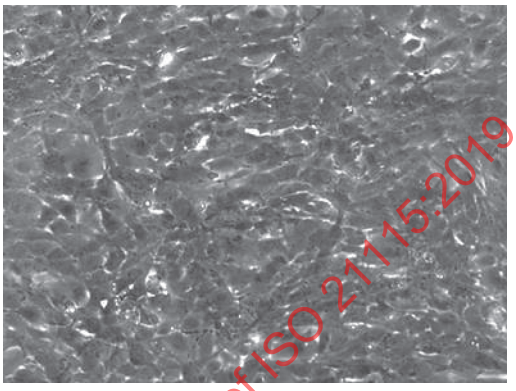
8.4.2 Visual control of cell damage

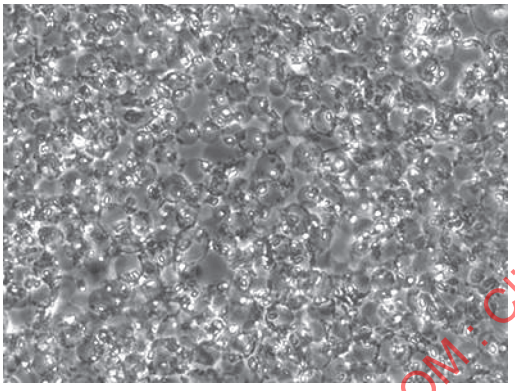
Before measuring cytotoxicity, cells in the 24-well plate should be visually observed (without taking off the adhesive foil), paying particular attention to the degree of damage as a first control of the test performance. In Figure 9, cells are shown with a different grade of damage after exposure to the positive control 3,4-Dichloroaniline in the exposure medium L-15/ex. These images serve as a guide to ensure optimal test performance.



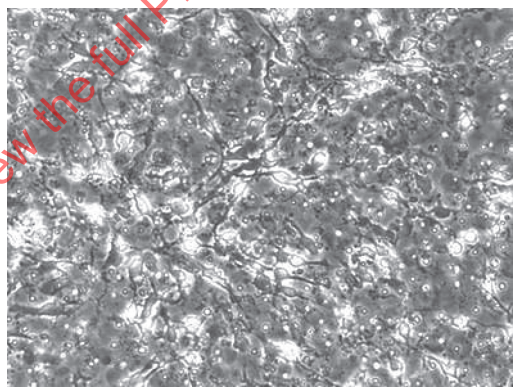
a) Negative control (100 % metabolic activity)



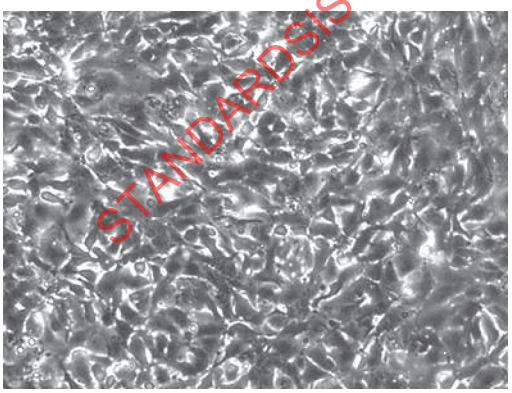
b) Solvent control (100 % metabolic activity)



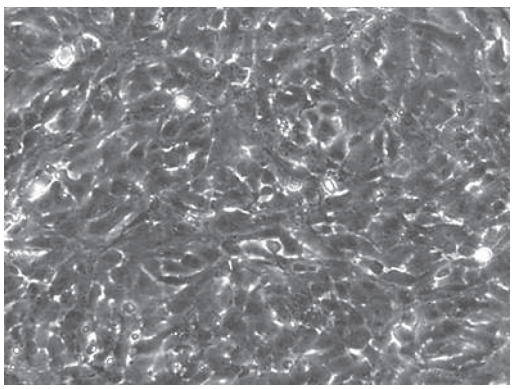
c) 100 mg/l 3,4-DCA (2 % metabolic activity)



d) 50 mg/l 3,4-DCA (12 % metabolic activity)



e) 25 mg/l 3,4-DCA (64 % metabolic activity)



f) 12,5 mg/l 3,4-DCA (81 % metabolic activity)

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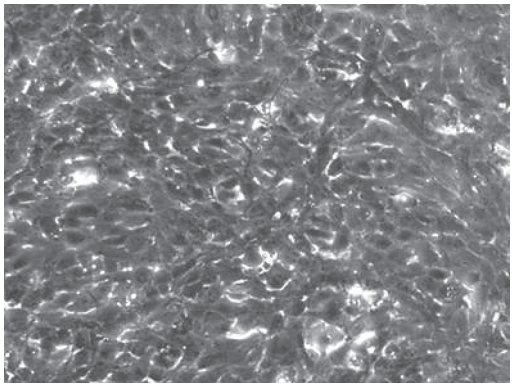
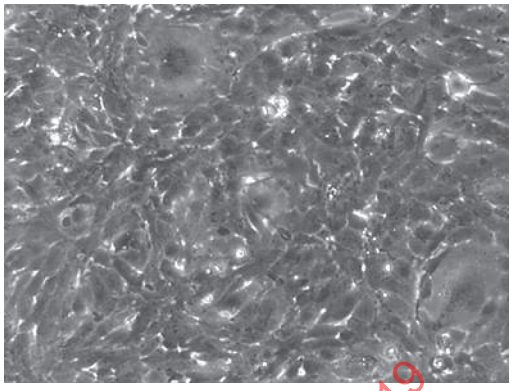


g) 6,25 mg/l 3,4-DCA (91 % metabolic activity)



h) 3,13 mg/l 3,4-DCA (96 % metabolic activity)

NOTE Percentages refer to the percentage of metabolic activity measured by alamarBlue on these cells compared to the control.

Figure 9 — Images of RTgill-W1 cells with different grades of damage after 24 h of exposure to the positive control 3,4-Dichloroaniline

8.4.3 Preparation of working materials and solutions for cytotoxicity measurement

Prepare the alamarBlue/CFDA-AM and the neutral red working solutions (see 6.3.12 and 6.3.13). For measuring several plates at the same time, prepare respective multiple volumes of the dye working solutions.

8.4.4 Measuring cytotoxicity — Step-by-step performance

Steps a) and b) specify the sampling step necessary for the determination of medium exposure concentrations in case of chemical testing. In case of water sample testing, skip a) and b) and start with step c) after removing the adhesive foil.

a) For the validation of the exposure concentrations by chemical analysis, prepare the required number of 1,5 ml sampling vials to take samples later on out of each exposure and solvent control well. The sampling vials might be pre-loaded with solvent according to the analytical protocol.

b) Remove the adhesive foil from the test plate and take samples of exposure medium at the end of the exposure (see Figure 8 for plate set-up). Remove 500 µl of the exposure medium from the well and transfer it into the respective sampling vial. Immediately close the vials tightly.

c) Discard the exposure solution.

d) Wash each well with 1 ml PBS (6.2.11). Do not pipette directly onto the cells, otherwise cells will detach from the bottom.

e) Discard the washing solution.

f) Add to each well 400 µl of the alamarBlue/CFDA-AM working solution (see 8.4.3). Do not pipette directly onto the cells, otherwise cells will detach from the bottom.

g) Cover the plate with the plate lid and incubate at (19 ± 1) °C for 30 min in the dark (e.g. wrap the plates in aluminium foil).

h) Measure first the fluorescence of alamarBlue (for wavelengths see 8.4.5) and after that the fluorescence of CFDA-AM (for wavelengths, see 8.4.5) with the fluorescent plate reader.

i) Discard the alamarBlue/CFDA-AM working solution.

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j) Add to each well 400 µl neutral red working solution (see 8.4.3). Do not pipette directly onto the cells, otherwise cells will detach from the bottom.

k) Cover the plate with the plate lid and incubate at (19 ± 1) °C for 60 min in the dark (e.g. wrap the plates in aluminium foil).

l) Discard the neutral red working solution.

m) Wash each well with 400 µl fixative (6.3.14) and discard the fixative.

n) Add to each well 400 µl extraction solution (6.3.15), place the plate lid and wrap plates in aluminium foil.

o) Incubate for 10 min at room temperature and gently shake on a plate shaker.

p) Measure the fluorescence of neutral red (for wavelengths, see 8.4.5) with the fluorescent plate reader.

8.4.5 Parameters for the fluorescence measurement

Excitation and emission wavelengths for the three different cell viability indicator dyes are as follows:

alamarBlue:	exc: 530 nm	em: 595 nm
CFDA-AM:	exc: 493 nm	em: 541 nm
neutral red:	exc: 530 nm	em: 645 nm

8.4.6 Chemical analyses of the test samples

In the case of chemical testing, perform the chemical analysis of the samples taken at the start and at the end of exposure out of each solvent control and exposed wells according to the analytical protocol.

9 Preparation and expression of results

The raw data gained is used to analyse the toxicity values as a percentage of cell viability compared to the respective control (see below). From this, calculate the EC_{50} value[9].

Test item	Respective control
Water sample testing	negative control
Water sample positive control testing	solvent control
Chemical testing using co-solvent	solvent control
Chemical testing without co-solvent	negative control

a) Subtraction of the corresponding average “no cells-control” fluorescence units from the average fluorescence units of the corresponding test plate (see 5.2).

b) The average values of fluorescence units of each control and tested water sample dilution or chemical concentration, respectively, of each test plate are determined (average of three).

c) These values are used to calculate the percentage of cell viability with regard to the respective control. Therefore, the fluorescence units of the controls are set as 100 % (100 % of the cells are vital).

d) Calculate the corresponding cell viability of each whole-water sample/ex dilution or chemical test concentration (in %), as shown by Formula (1):

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$$\%\ \text{ctrl} = \frac{(FU_{\text{TI}} \times 100\ \%)}{FU_{\text{ctrl}}} \quad (1)$$

where

ctrl is the % of respective control;

FU_{TI} is the fluorescent units of test item (i.e. either chemical or w-ws / ex dilution);

FU_{ctrl} is the fluorescent units of respective control.

e) For chemical testing, use the geometric mean based on measured concentrations of each test concentration at the start and the end of the test and the percentage of cell viability values for EC_{50} calculation.

f) For water sample testing, use the respective test dilutions of whole-water sample/ex and the tested concentrations of the positive control 3,4-DCA and their respective percentage of cell viability values for EC_{50} calculation.

NOTE As described in Clause 4, neutral red measures the integrity of the lysosomal membranes. The dye accumulates in lysosomes[7] and a disruption of the lysosomes results in a decrease in neutral red fluorescence. In case of ammonia containing water samples, however, massive vacuolisation was observed in RTgill-W1 cells accompanied by increased uptake of neutral red[10] with little change in plasma membrane integrity or metabolism, correlating closely with fish toxicity. Therefore, this test set-up also allows for the detection of ammonia toxicity, a major fish toxicant, entering streams and water bodies via waste water treatment plants.

10 Validity criteria of the test

10.1 No cells-control wells

The variation of fluorescence between the no cells-control wells should not exceed 20 %. If an interference is detected (e.g. a fluorescence higher/lower than 20 % as compared to the other no cells-control well, see 5.2), a no cells-control reference plate is required in an additional test (cell free plate treated in the same way as the exposure plate) for concentration-dependent fluorescence background subtraction of the dyes.

10.2 Positive control in L-15/ex

In the case of chemical and water sample testing, for every five tests a separate plate carrying a concentration range of the positive control 3,4-Dichloroaniline in the exposure medium L-15/ex should be tested (i.e. every sixth test plate should be for 3,4-DCA). The mean EC_{50} value (for each cell viability indicator dye) based on nominal concentrations (without analytical determination of exposure concentration) should lie between two-and-a-half standard deviations (SD) from the EC_{50} values stated below.

— alamarBlue: 42,9 mg/l ± 5,7 mg/l ($2\frac{1}{2}$ SD range: 28,7 mg/l to 57,1 mg/l)

— CFDA-AM: 62,6 mg/l ± 24,9 mg/l ($2\frac{1}{2}$ SD range: 0,4mg/l to 124,9 mg/l)

— Neutral red: 48,8 mg/l ± 10,0 mg/l ($2\frac{1}{2}$ SD range: 23,7 mg/l to 73,8 mg/l)

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10.3 Positive control in w-ws/ex

If the results of the positive control plate with 3,4-DCA for water sample testing are outside the two-and-a-half SD from the given EC_{50} values (see 10.2), strong interferences of chemicals with the matrix of the water sample are indicated (see 5.1 and B.3).

10.4 Solvent control

In the case of chemical testing, the L-15/ex solvent control should not be more than 10 % lower than the negative control. Otherwise, troubleshooting shall be done as, for example, the DMSO stock could be contaminated or there could be other procedural problems (e.g. contaminated glass vials for stock or dosing mixture preparation) that need to be fixed.

In the case of water sample testing, the whole-water sample/ex solvent control of the positive control test plate should not be more than 10 % lower than the L-15/ex solvent control. Otherwise, it indicates a toxicity of the water sample towards the cells. If this effect is not observed in the plate carrying the water sample in dilutions, troubleshooting needs to be done as, for example, the DMSO stock could be contaminated or there could be other procedural problems (e.g. contaminated glass vials for stock or dosing mixture preparation) that need to be fixed.

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Annex A
(informative)

Routine cell culture of RTgill-W1

A.1 General

This annex describes the routine cell culture procedure (sub-culturing, freezing and thawing of cells) of the gill cell line from rainbow trout, RTgill-W1.

A.2 Recurrent quality assurance measures

Every third month, all cell cultures should be tested for the presence of mycoplasma. In suspicious cases (e.g. decreasing sensitivity of the test system), cell cultures should be tested immediately.

Permanent cell lines are a common target of mycoplasma contamination. Mycoplasma interfere with the cell cycle and metabolism of the cells and subsequently may have an influence on the sensitivity of the cytotoxicity test. Therefore, every culture shall be checked regularly every third month for mycoplasma.

A.3 Basics of RTgill-W1 cell culturing

RTgill-W1 cells grow adherent and are cultivated as monolayer cultures.

The cell line is cultivated in cell culture flasks at (19 ± 1) °C, without any additional CO_2 supply or steam saturation.

Cells are able to grow to a defined degree of confluence. Further cultivation will lead to the mortality of the cells. Therefore, it is necessary to propagate the cells on a regular basis. After reaching 150 passages, the cells will be discarded and new cells of a lower passage number should be thawed.

The cells should be split as soon as they reach confluency, i.e. when the cell culture surface is completely covered. They are routinely split 1:2 (i.e. to generate 2 flasks out of 1). Under these conditions, cells can be passaged every 10 d to 12 d.

The presence of bacteria, fungi and yeast is mostly indicated by an opaque and muddy culture medium and dying/detaching cells. In that case, all contaminated cell culture flasks and associated media and solutions should be eliminated immediately and the working place cleaned thoroughly with disinfection according to facility standards (e.g. 70 % volume fraction ethanol or isopropyl alcohol).

A.4 Reagents

A.4.1 Ready-for-use purchased solutions

A.4.1.1 Bovine serum, fetal bovine serum (FBS).

FBS shall be tested for compatibility. This can be done by culturing the cells for three passages in culture medium with this FBS and observing cell viability and growth by microscopy. If the FBS does not work, the cells grow more slowly, i.e. cells split 1:2, need more than 12 d to reach confluency or even start detaching. If a suitable FBS is found, several bottles of this respective batch should be ordered to ensure the use of the same FBS throughout the testing period. Do not heat inactivate FBS.

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A.4.1.2 Gentamicin, 10 mg/ml.

A.4.1.3 Trypsin, 0,25 % in PBS w/o Ca^{2+}, Mg^{2+}.

A.4.1.4 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).

A.4.1.5 Leibovitz L-15media, with Glutamine and without Phenolred.

A.4.1.6 Dimethyl sulfoxide (DMSO), cell culture tested grade.

A.4.2 Self-prepared solutions

L-15 complete culture medium:	add to 500 ml L-15:	
	+25 ml FBS	
	+2,5 ml Gentamicin solution	
	store at (4 ± 1) °C (storage time should not exceed three months)	
Cell culture freezing solution:	add to L-15 media (4 °C):	e.g. 10 ml
	10 % volume fraction FBS	+1 ml
	10 % volume fraction DMSO	+1 ml
	adjust to (4 ± 1) °C when freshly prepared	

A.5 Apparatus and material

A.5.1 Biosafety cabinet.

A.5.2 Incubator, temperature-controlled or climatization of the room to (19 ± 1) °C, without illumination during cell incubation.

A.5.3 Cell culture flasks, 75 cm^2 and 25 cm^2 with vent screw cap.

A.5.4 Glass pipettes or serological plastic pipettes, 5 ml, 10 ml and 20 ml, sterile.

A.5.5 Pasteur pipettes, 230 mm, sterile.

A.5.6 Pipetting help.

A.5.7 Pipette box, to sterilize Pasteur or glass pipettes.

A.5.8 Microscope.

A.5.9 Vacuum pump.

A.5.10 Pipette tips, 10 µl, 100 µl, 1 ml, 2 ml, 5 ml and 10 ml, sterile.

A.5.11 Pipettes, 10 µl, 100 µl, 1 ml, 2 ml, 5 ml and 10 ml.

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A.5.12 Centrifuge, temperature-controlled to 18 °C to 21 °C, 875 g.

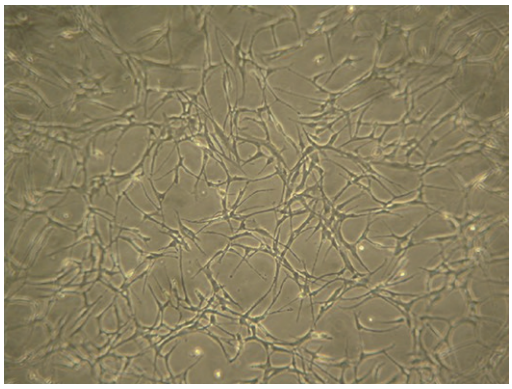
A.5.13 Plastic centrifuge tubes, 15 ml and 50 ml, sterile.

A.5.14 Cryo vials, 1,2 ml.

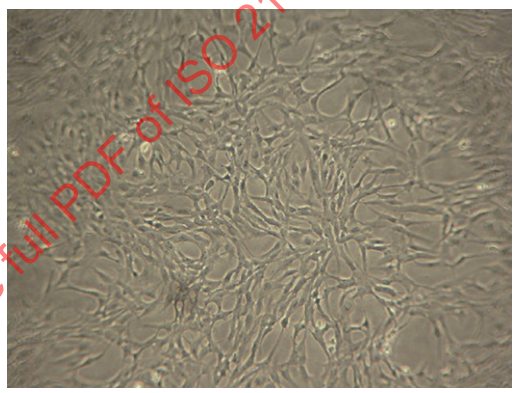
A.6 Procedure

A.6.1 Cell line used

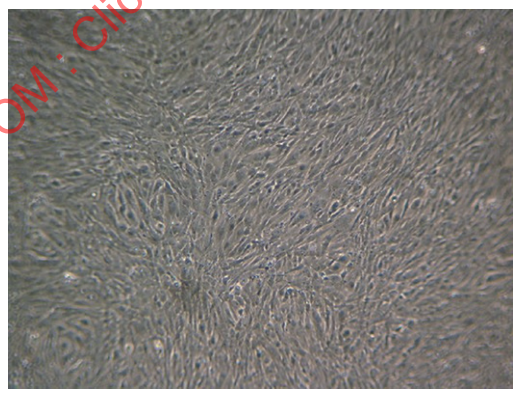
The RTgill-W1 cell line is commercially available as Nr CRL-2523™[1]. The cell line has the following morphological characteristics: morphology of RTgill-W1: at low density the cells have an irregular spindly shape, but with reaching confluence the cells exhibit a polygonal shape and are tightly packed (Figure A.1)[1].



a) Non-confluent — 1 d after passaging



b) Non-confluent — 5 d after passaging



c) Confluent — 12 d after passaging

Figure A.1 — Monolayers of RTgill-W1 cells

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A.6.2 Preparation of working materials and solutions

Thaw the trypsin. Adjust trypsin, Versene and the L-15 complete culture medium to (19 ± 1) °C in the incubator for at least 1 h before performing the sub-culturing. Turn the biosafety cabinet on, wipe the cabinet with disinfection according to the sterile procedures in the facility and let the air flow for approximately 10 min to 15 min. Clean the pipette boxes (Pasteur and glass), trypsin, Versene, L-15 complete culture medium bottle and the confluent cell culture flasks according to the sterile procedures in the facility and put them under the cabinet.

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A.6.3 Step by step performance — Sub-culturing

The sub-culturing is performed at room temperature and therefore should be done quickly so the cells are not exposed to high temperatures for too long. Do not sub-culture the cells at room temperatures of ≥25 °C.

Description for sub-culturing one cell culture flask:

a) check cells in the cell culture flask under the microscope;

b) if confluency is reached [see Figure A.1 c)], prepare the cells for sub-culturing as follows:

   1) draw off the media using sterile Pasteur pipette and vacuum source;

   2) gently add 1 ml Versene (do not pipette Versene directly onto cells, detachment of cells may occur);

   3) wash cells by gentle agitation of the cell culture flask;

   4) draw off the Versene and repeat washing step (2, 3 and 4);

   5) after drawing off Versene for the second time, add 0,7 ml trypsin.

   The cell detachment after adding the trypsin occurs quite fast (within 1 min). Therefore, it is recommended to not split more than eight cell culture flasks at once so to avoid long a handling time during trypsination, which can lead to cell digestion by the trypsin.

   6) promote the cell detachment by gently tapping and agitating the cell culture flask (check detachment visually);

   7) add 5 ml L-15 complete culture medium to stop the action of trypsin;

   8) dissolve cell clumps and remove remaining attached cells gently by pipetting the solution several times up and down and also over the surface of the cells (strictly avoid the formation of foam; shear forces can disrupt the cells);

   9) transfer the cell solution into a plastic tube (15 ml or 50 ml centrifuge tube);

   10) centrifuge for 3 min with 875 g at (18 to 21) °C;

   11) draw off the supernatant as much as possible without removing the cell pellet;

   12) dissolve the cell pellet in 10 ml L-15 complete culture medium by pipetting the solution several times up and down or against the tube wall (strictly avoid the formation of foam; shear forces can disrupt the cells);

   13) sub-culture the cells in two cell culture flasks (cell culture flasks can be used for two passages of cells):

      — add 5 ml of the cell solution to each cell culture flask;

      — add 5 ml complete culture medium to reach a final volume of 10 ml;

      — close the cell culture flasks and incubate the cells at (19 ± 1) °C in the dark.

Labelling of the cell culture flasks: cell line (RTgill-W1), passage number (P xx), date, dilution factor (1:2).

Cells should be checked regularly (at least every three days) by microscopy regarding the morphology and for microbial contaminations. The minimum/maximum cultivation temperature should be controlled every working day and deviations beyond ± 1 °C should be documented. Cells should be sub-cultivated approximately every 10 d to 12 d. An exchange of culture medium should be performed after 7 d.

The sub-culturing procedure is described for using one cell culture flask. If several flasks are sub-cultured at once (make sure to use only cells from the same passage), the amount of Versene and trypsin

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