The Use of Fish-Derived Cell Lines for Investigation of Environmental Contaminants: An Update Following OECD's Fish Toxicity Testing Framework No. 171

Vivian R. Dayeh,[1] Niels C. Bols,[1] Katrin Tanneberger,[2] Kristin Schirmer,[2] and Lucy E. J. Lee[3]

[1]Department of Biology, University of Waterloo, Waterloo, Ontario, Canada
[2]Department of Environmental Toxicology, Eawag: Swiss Federal Institute of Aquatic Science and Technology, Dübendorf, Switzerland
[3]Department of Biology, University of the Fraser Valley, Abbotsford, British Columbia, Canada

ABSTRACT

Protocols for evaluating chemical toxicity at the cellular level using fish cell lines are described in this unit. Routine methodologies for growing salmonid cell lines, and using them in aquatic toxicology studies that support the mandate of the Organization for Economic Co-operation and Development (OECD) to reduce the use of whole animals in toxicity testing, are presented. Rapid, simple, cost-effective tests evaluating viability of cells with three indicator dyes per sample provides a broad overview of the sensitivity of cells to chemical contaminants. This fluorometric assay involves: (1) alamar blue for metabolic activity, (2) CFDA-AM for membrane integrity, and (3) neutral red for lysosomal function. These protocols are conveniently performed in semi-unison within the same multiwell plates and read at three different wavelengths. Detailed step-by-step descriptions of the assays, parameters to consider, troubleshooting, and guidelines for data interpretation are provided as essential tools for investigating environmental aquatic contaminants at the cellular level. *Curr. Protoc. Toxicol.* 56:1.5.1-1.5.20. © 2013 by John Wiley & Sons, Inc.

Keywords: fish cell lines • in vitro toxicity • fluorescent indicator dyes • alamar Blue • CFDA-AM • neutral red • toxicity test • cell viability

INTRODUCTION

In light of a new "Fish Testing Framework" recently released by the Organization for Economic Co-operation and Development (OECD), and alternative bioassay tests being advocated to reduce whole animal testing in fish toxicity assays (OECD, 2012), this unit describes protocols for growing salmonid cell lines and using them in in vitro toxicology studies. Cell viability of cultures is assessed with three indicator dyes: alamar blue for metabolic activity, CFDA-AM for membrane integrity, and neutral red for lysosomal activity. These protocols are essential tools for investigating environmental toxicity at the cellular level.

Fish-derived cell lines are being used for at least three basic purposes in the investigation of environmental contaminants. One is in ascertaining the mechanisms by which contaminants exert their toxicity; a second is in determining the relative toxicity of different chemical contaminants; and a third application is in evaluating the toxicity of environmental samples. These three goals are interrelated, as toxic mechanisms give insight into

Current Protocols in Toxicology 1.5.1-1.5.20, May 2013
Published online May 2013 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/0471140856.tx0105s56

the basis of toxicity rankings and allow the risk of untested compounds and of environmental samples to be estimated. The endpoints that are used in these studies can be specific or general. A specific response evaluates a particular function, which might be expressed by only certain fish-derived cell lines and is caused by just some classes of chemical contaminants. General responses assess fundamental cellular activities, which would be expressed by all fish cell lines and affected by a wide range of contaminants.

In this unit, three assays for assessing a change in cell viability, a general response, are described. Each assay is done on cells in microwell cultures and uses a different fluorescent indicator dye, but the results are quantified in arbitrary fluorescent units (FU) with a common instrument—a fluorescent plate reader (O'Connor et al., 1991; Schirmer et al., 1997). Cell viability is compared in toxicant-treated cultures by expressing their FU as a percentage of the FU for control cultures. The use of these assays is illustrated with some members of an important class of environmental contaminants, the polycyclic aromatic hydrocarbons (PAHs), which can act as toxicants and as phototoxicants (Schirmer et al., 1998a,b).

This unit describes a protocol on how to culture fish cell lines, which provide a continuous supply of cells. These cells are used to initiate microwell cultures for direct or indirect exposure to environmental toxicants in the dark and in the presence of UV radiation, and cell viability is assessed using the fluorescent indicator dye alamar blue (see Basic Protocol). Cell viability is also assessed using other fluorescent indicator dyes, such as CFDA-AM to assess membrane integrity (see Alternate Protocol 1) and neutral red to assess lysosomal activity (see Alternate Protocol 2).

BASIC PROTOCOL

EVALUATION OF TOXICITY IN FISH-DERIVED CELL LINES USING ALAMAR BLUE TO ASSESS METABOLIC ACTIVITY

This protocol describes the routine maintenance of salmonid cell lines, although very similar procedures could likely be used for cell lines from other groups of fish. Approximately 45 different fish cell lines are available in total from the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Cultures (ECACC), although many more have been described in the literature. A comprehensive list of fish cell lines available from ATCC is provided in Lee et al. (2009), while the ECACC holds approximately 25 fish cell lines, less than half of which are unique to ECACC. The rainbow trout gill cell line, RTgill-W1, which is available from ATCC as CRL-2523, is used as an example in this protocol for growing fish cells.

This protocol outlines the steps for setting up confluent monolayers of cultures of fish-derived cell lines in 24-, 48-, or 96-well microwell plates (6-, and 12-well plates are also available and can be used), which will then be exposed to toxicants in the presence or absence of UV irradiation. This allows the detection of directly cytotoxic compounds, or those that need the presence of UV irradiation to become toxic, in which case the compound is called photocytotoxic (Schirmer et al., 1998a,b). Upon termination of the experiment, cell viability as measured by metabolic activity is assessed by using the fluorescent indicator dye alamar blue, which may also be analyzed spectrophotometrically (O'Brien et al., 2000). Other fluorescent dyes are used to assess membrane integrity (CDFA-AM; see Alternate Protocol 1) and lysosomal activity (neutral red; see Alternate Protocol 2).

Alamar blue is a commercial preparation of the dye resazurin (O'Brien et al., 2000) and is increasingly being used in pharmacology to screen for compounds toxic to mammalian cells (Evans et al., 2001). Resazurin, which is not fluorescent, becomes fluorescent resorufin upon reduction by oxidoreductases of living cells.

Materials

70% ethanol solution
Confluent culture of RTgill-W1 cells (ATCC #CRL-2523) in a 75-cm^2 flask
0.53 mM versene (EDTA; Invitrogen/Life Technologies) diluted 1:5000 (1× 0.2 g tetrasodium EDTA/liter in PBS)
Trypsin solution (see recipe) *or* 0.25% trypsin solution in PBS without Ca^{2+}, Mg^{2+}, *or* phenol red (Biowest, Biochrom, or Invitrogen/Life Technologies) *or* TrypLE (Invitrogen/Life Technologies; see notes below)
Leibovitz's (L-15) complete medium containing FBS (see recipe)
Test compounds (toxicants), stock solutions
DMSO
L-15/ex solution (see recipe)
Alamar blue (Immunocorp, Invitrogen/Life Technologies)

Laminar flow hood, either horizontal or vertical
Inverted phase-contrast microscope
Vacuum aspirator
15-ml or 50-ml centrifuge tubes, sterile
24-, 48- or 96-well tissue-culture treated microwell plate
75-cm^2 tissue culture flask
Incubator (see Critical Parameters and Troubleshooting)
Catch basin (a plastic container at least slightly larger than the size of the microwell plate)
Positive-displacement digital microdispenser (e.g., Nichiryo model 800) or adjustable-volume micropipet (e.g., Eppendorf Reference 0.1 to 2.5 μl)
Glass pipet tips for digital microdispenser (Nichiryo) or plastic micropipet tips (e.g., Eppendorf)
Multichannel pipettor (e.g., Eppendorf Research plus, 100 to 1000 μl, or 30 to 300 μl)
Parafilm
Cover foil (non-breathable; Nunc)
Radiation exposure chamber containing UV-A and/or UV-B fluorescent lamps (Southern New England Ultraviolet) and a fan
Spectroradiometer (e.g., InstaSpec II photodiode array spectroradiometer, Oriel)
Transformer to modulate UV intensity
Fluorometric microwell plate reader

Additional reagents and equipment for counting cells (*APPENDIX 3B*)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and aseptic technique should be used accordingly. Work >6-in. from the front of the vertical laminar flow hood, as the sterile zone begins there.

NOTE: EDTA and trypsin steps can be replaced with TrypLE, a recombinant form of trypsin available from Invitrogen that works without the EDTA rinse step and which is a much gentler dissociating solution.

Prepare fish-derived cell cultures

1. Adjust all solutions (medium, versene, trypsin) to 18° to 22°C or to room temperature for approximately 1 hr before use.
2. Turn on the laminar flow hood, and wipe all surfaces with 70% ethanol solution.
3. Examine the confluent culture of RTgill-W1 cell flask under an inverted phase-contrast microscope.

Note the general appearance of the culture, checking for inadvertent microbial contamination or unexpected rounding and detachment of the fish-derived cells. Cultures to be passaged should appear normal and confluent (i.e., covering the bottom of the flask completely) or close to confluence (90%).

4. Under the laminar flow hood, aspirate the old medium and add 1.5 ml of 0.53 mM versene to the flask. Swirl it around gently, leave for 1 min, and aspirate it off. Repeat rinse with 1 ml of versene and remove.

   This step can be omitted if, rather than using trypsin solution as indicated in step 5 below, TrypLE (Invitrogen) solution is added directly onto the cells.

5. Add 1 ml trypsin solution to the flask, replace the cap, and observe under the inverted phase-contrast microscope. Do not leave the cells in trypsin for >5 min, as cellular digestion and cell death may ensue.

   The cells will begin to detach from the culture surface in ∼1 to 3 min. Older cultures that have not been passaged for a long period of time are difficult to detach. Ideally, the cells will detach individually, and form a single-cell suspension.

6. Add 3 ml Leibovitz's complete medium containing FBS to the flask. Pipet the medium up and down, directing the stream towards the bottom of the flask, to make sure that all cells are dislodged and resuspended in the medium.

   Trypsin inhibitors in FBS stop the action of the trypsin.

7. Transfer the cell suspension to a sterile 15-ml centrifuge tube and centrifuge 3 min at 200 × *g*, room temperature, in a tabletop centrifuge.

8. Aspirate the supernatant from the 15-ml centrifuge tube, being careful not to aspirate the cell pellet. Leave a small amount of supernatant (0.25 ml) over the cell pellet. Flick the centrifuge tube to resuspend the cells in the small volume of medium.

Plate cells to maintain cell line

9a. Add 10 ml fresh Leibovitz's complete medium containing FBS to the centrifuge tube and transfer 5 ml to each of two 75-cm^2 tissue culture flasks. Add 5 ml medium to each flask and close flasks tightly.

10a. Examine flasks using a phase-contrast microscope. Note whether cells have detached as single cells or as clumps and whether the suspension has been distributed equally between the two flasks.

11a. Allow the cells to grow at 18° to 22°C. When the cultures are confluent (7 to 10 days), split 1:3 or harvest to use for an experiment.

Plate cells in microwell plates for toxicant exposure

9b. Add 5 ml Leibovitz's complete medium containing FBS. Determine the cell density using a hemacytometer (*APPENDIX 3B*). Using fresh medium, adjust cell density to 1.5×10^5 cells/ml if using 24-, 48- or 96-well plates.

   The cell number counted should optimally be between 50 and 100. To assure a reasonable density, the cell pellet needs to be resuspended in 5 ml or more of complete medium. If more than one flask is used, increase the volume to add accordingly.

10b. If using a 24-well tissue culture microwell plate, add 1.5×10^5 RTgill-W1 cells in 1000 µl of Leibovitz's complete medium to 22 wells. Use the remaining two wells as blanks by adding Leibovitz's complete medium alone. If using a 48-well tissue-culture microwell plate, add 500 µl of the respective cell suspension (contains 7.5×10^4 RTgill-W1 cells in 500 µl of Leibovitz's complete medium) to 45 wells of a 48-well tissue-culture microwell plate. Use the remaining three wells as blanks by adding Leibovitz's complete medium alone. If using a 96-well tissue-culture

microwell plate, add 3×10^4 RTgill-W1 cells in 200 µl of Leibovitz's complete medium to 84 wells of a 96-well tissue culture plate. Use the remaining twelve wells as blanks by adding Leibovitz's complete medium alone.

11b. Allow the cells to grow for 3 to 4 days in the dark at 18° to 22°C to form a confluent cell monolayer for the 24- and 48-well plates and for 2 to 3 days for the 96-well plates.

Growing cells over a 2- to 4-day period allows for a more consistent cell density and better adherence of cells than newly initiated, dense cultures.

Expose fish-derived cell lines to putative toxicants

12. Turn on the laminar flow hood, and wipe all surfaces with 70% ethanol solution.

13. Examine the plated microwell plate under the inverted phase-contrast microscope.

Note the general appearance of the cell culture, making sure that the bottom of the wells contain confluent monolayers and inadvertent microbial contamination is absent.

14. Make serial dilutions (working solutions) from test compound stock solutions in carrier solution (e.g., DMSO) or directly in L-15/ex exposure medium at final test concentrations.

If the carrier solution is an organic solvent, prepare working solutions such that the concentrations are at least 200 times the final concentrations desired in the culture wells.

Serial dilutions are necessary to ensure that the solvent is diluted sufficiently in the culture medium in the wells in order to minimize interference due to the solvent with cell viability and/or toxicant uptake. For more water-soluble compounds, a dilution series can be prepared directly in exposure medium. However, many organic toxicants, especially the hydrophobic ones with log Kow > 2 to 3, will require the use of an organic carrier solvent.

15. Remove growth medium from plates by inverting over a catch basin. Drain plates further for a few seconds on a small stack of paper towels.

Alternatively, aspirate each well using a Pasteur pipet attached to a vacuum aspirator.

16. Rinse each culture well with 1000 µl L-15/ex solution if using a 24-well plate, 500 µl L-15/ex solution if using a 48-well plate, or 200 µl L-15/ex solution if using a 96-well plate. Proceed to either step 17a or step 17b.

Alternatively, aspirate each well using a Pasteur pipet attached to a vacuum aspirator.

Adding toxicants via direct dosing (aseptic)

17a. Expose cells to the test compound in a vertical laminar flow hood. Remove rinsing medium from wells of cell culture plate by inverting over a catch basin. Add 1000 µl L-15/ex solution if using a 24-well plate, 500 µl L-15/ex solution to each well if using a 48-well plate, or 200 µl L-15/ex solution if using a 96-well plate. In cases where the chemical solutions were prepared in an organic solvent (such as DMSO) at 200× the final concentration required, pipet 5 µl/well test compound to each well containing 1000 µl/well L-15/ex solution using a positive-displacement digital micropipet, when 24-well plates are used. For 48-well plates, pipet 2.5 µl test compound to each well containing 500 µl/well L-15/ex solution using a positive-displacement digital micropipet. If using a 96-well plate, pipet 1.0 µl test compound dissolved in DMSO to each well containing 200 µl/well L-15/ex solution.

Because cells in L-15/ex solution are particularly sensitive to DMSO, tilt the plates before adding the test compound, to increase the volume of medium above the cells as a protective layer. Dispense the 5 µl, 2.5 µl or 1 µl of test compound above the level of the medium, and then touch the droplet to the surface of the medium to aid in dispersion. In addition, reduce the light level in the flow hood to avoid irradiation of cells in the

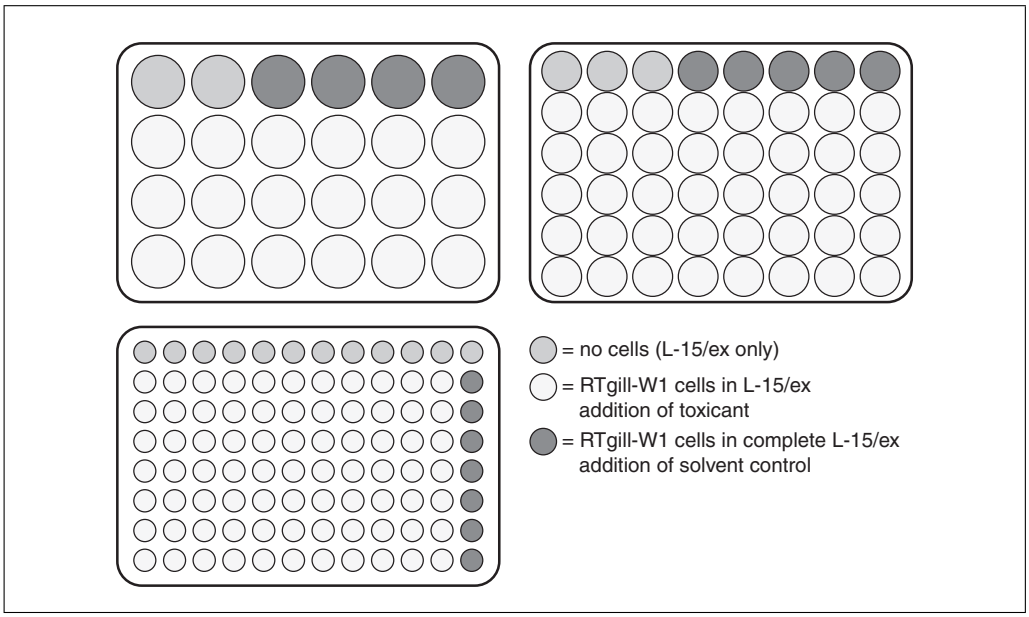

Figure 1.5.1 Schematic representation of the 24-, 48-, and 96-well plate configuration. Light-shaded circles represent wells without cells, white circles represent wells with a confluent monolayer of RTgill-W1 exposed to the putative toxicant in L-15/ex, and dark-shaded circles represent wells with a confluent monolayer of RTgill-W1 exposed to solvent in L-15/ex.

presence of the toxicant. After dosing, gently agitate the plate to assure a homogenous mixture of exposure medium, solvent, and chemical.

Adding toxicants via indirect dosing (aseptic)

17b. Expose cells to the test compound in a vertical laminar flow hood. Place seven (for 24-well plates), nine (for 48-well plates), or twelve (for 96-well plates) sterile 4-ml amber glass vials under the laminar flow hood and aseptically add 3.5 ml L-15/ex into each vial. Afterwards add 17.5 µl (when 24-well plates are used), 8.75 µl (when 48-well plates are used), or 3.5 µl (when 96-well plates are used) test compound using a positive-displacement digital micropipet. Close amber glass vials tightly and shake vigorously for 10 min. Remove rinsing medium from wells of cell culture plate by inverting over a catch basin. Dose dosing mixture into the respective wells. Therefore, dispense 1000 µl/well for 24-well plates, 500 µl/well for 48-well plates or 200 µl/well for 96 well plates.

18b. In a 24-well plate, dose three wells for each of the six concentrations and the solvent control (to the two blank control wells dose only the highest applied test concentration). In a 48-well plate, dose five wells (in the three blank control wells dose only the highest applied test concentration) for each of seven concentrations of compounds and the DMSO control. In a 96-well plate, dose eight wells (seven wells with confluent cell monolayers and one without cells to serve as a blank) for each of eleven concentrations of compounds and the DMSO control (Fig. 1.5.1). Wrap plates in Parafilm to prevent evaporation during the exposure period.

The use of plate-sealing foils, which are commercially available, e.g., from Nunc or Greiner, is recommended in order to minimize well-to-well transfer and losses due to evaporation of more volatile toxicants.

For exposure of fish-derived cells to toxicants in the dark

19a. For exposure in the dark, incubate plates up to 48 hr at 18° to 22°C.

20a. If not specifically desired (see below), avoid exposure of the plates to light.

Although L-15/ex is unlikely to be affected by irradiation, many toxicants may be altered chemically.

21a. At the end of the dark exposure, remove plates from the incubator and continue with the cytotoxicity assay for assessing metabolic activity with the fluorescent dye alamar blue (proceed to step 24).

For membrane integrity assessment, use CDFA-AM (see Alternate Protocol 1) or for lysosomal activity assessment, use neutral red (see Alternate Protocol 2).

For exposure of fish-derived cells to toxicants in the presence of irradiation

19b. Turn on the lamps of the radiation exposure chamber at least 15 min prior to use in order to allow lamps to warm up and emit a stable radiation.

The radiation chamber should contain at least two fixtures to hold lamps, e.g., one UV-A and one UV-B fluorescent lamp, and it should be shielded from any radiation outside the chamber. Ideally, lamps should be linked to a transformer in order to allow for easy manipulation of the radiation output.

20b. Place the appropriate unit of the spectroradiometer into the radiation exposure chamber in order to perform UV radiation measurements, as described in the user manual of the spectroradiometer.

Ensure that the appropriate unit of the spectroradiometer is positioned the same distance from the UV lamps as the culture plate would be. Also, place the lid of the culture plate on top of the unit to measure the amount of UV irradiation passing through the lid of the plate.

21b. Using the transformer, adjust UV intensities as desired.

For example, a 10:1 ratio of UV-A to UV-B may be desirable in order to mimic the ratio of these two UV components in nature. If no transformer is available, radiation intensity may be adjusted by varying the distance between the exposure tray and the lamps, but this method is somewhat cumbersome.

22b. Once the radiation exposure chamber has been set up, place tissue culture plates into the chamber in an atmosphere of air for the required exposure period. Place a second tissue culture plate into the chamber at the same time but protected from radiation.

This second plate serves as the dark control.

23b. At the end of the UV exposure, remove plates from the chamber and proceed with the cytotoxicity assay for assessing metabolic activity using alamar blue, go to step 24 (for membrane integrity using CDFA-AM, see Alternate Protocol 1; or for lysosomal activity using neutral red, see Alternate Protocol 2).

Assess metabolic activity using alamar blue

24. Prepare a 5% (v/v) working solution of alamar blue in L-15/ex solution.

Alamar blue is purchased as a ready-to-use solution in quantities of 25 ml and 100 ml. When stored in the dark at 2° to 8°C and kept aseptically, alamar blue can be used for at least 1 year.

25. Remove exposure medium from plates by inverting over a catch basin. Drain plates further for a few seconds on a small stack of paper towels.

26. Add 400 µl (24-well plate), 100 to 150 µl (48-well plate), or 50 to 100 µl (96-well plate) of 5% alamar blue working solution to each well of the respective plates.

The general rule is to at least cover the growth surface of the wells, although some plate readers may require slightly larger volumes for accurate readings.

27. Incubate the plates in the dark at 18° to 22°C for 30 min.

Although longer incubation times are also possible, the yield of fluorescent units from alamar blue can decline if the incubation period is too long (O'Brien et al., 2000).

28. Measure alamar blue on the fluorometric plate reader at excitation and emission wavelengths of 530 and 590 nm, respectively.

ALTERNATE PROTOCOL 1

EVALUATION OF TOXICITY IN FISH-DERIVED CELL LINES USING CFDA-AM TO ASSESS MEMBRANE INTEGRITY

Although esterase substrates have been used as a measure of cell membrane integrity since the 1960s (Rotman and Papermaster, 1966), 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is an example of one that was developed to improve this application (Haugland, 1996). CFDA-AM diffuses into cells rapidly and is converted by nonspecific esterases of living cells from a nonpolar, nonfluorescent dye into a polar, fluorescent dye, 5-carboxyfluorescein (CF), which diffuses out of cells slowly. In this protocol, fish cells in microwell cultures are exposed for a period of time to a putative toxicant, and, after removal of the putative toxicant, CFDA-AM is added and the capacity of the cells to produce CF is measured.

Additional Materials *(also see Basic Protocol)*

4 mM 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, see recipe)
RTgill-W1 cells in a 24-well, 48-well or 96-well plate exposed to toxicants (see Basic Protocol, steps 1 to 23)

1. Prepare a working solution of 4 μM CFDA-AM by diluting the 4 mM CFDA-AM stock solution 1:1000 in L-15/ex solution.
2. Remove exposure medium from RTgill-W1 cells in a 24-well, 48-well, or 96-well plate exposed to toxicants by inverting over a catch basin. Drain plates further for a few seconds on a small stack of paper towels.
3. Add 400 μl (24-well plate), 100 to 150 μl (48-well plate), or 50 to 100 μl (96-well plate) of 4 μM CFDA-AM working solution to each well of the respective plates and incubate the plate in the dark for 30 to 120 min at 18° to 22°C.
4. Measure CF fluorescence on the fluorometric plate reader at respective excitation and emission wavelengths of 485 and 530 nm.

Alamar blue (see Basic Protocol, steps 24 to 28) and CFDA-AM can be added together to perform the two assays in a single step (Schirmer et al., 1997) because the fluorescent products of the two indicator dyes can be detected at different emission wavelengths without interfering with each other. The advantage of doing so is the conservation of material and time, as fluorescent readings are taken on the same culture wells. Thus, in order to perform the two assays together, add the appropriate amount of alamar blue to make a 5% (v/v) working solution in L-15/ex solution and then dilute the CFDA-AM stock solution (4 mM in DMSO) 1:1000 in that same volume of L-15/ex solution. Proceed with the incubation period as described above in step 3.

Inasmuch as alamar blue and CFDA-AM do not affect the viability of cells, fluorescent dyes can be removed after fluorescent measurement and replaced by culture medium to allow the cells to recover for a period of time, or be re-exposed, after which the indicator dyes can be re-applied.

ALTERNATE PROTOCOL 2

EVALUATION OF TOXICITY IN FISH-DERIVED CELL LINES USING NEUTRAL RED TO ASSESS LYSOSOMAL ACTIVITY

Although first used to evaluate cell viability in virology, neutral red (NR) has been utilized most intensively in in vitro toxicology. The principle is that viable cells accumulate NR (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) in lysosomes (Borenfreund and Puerner, 1984). NR can be applied before or after the exposure of cell cultures to toxicants, so that the measured endpoint represents either the release or uptake of

the dye (Borenfreund and Puerner, 1984; Reader et al., 1990). Measurements can be done either spectrophotometrically (Borenfreund and Puerner, 1984) or fluorometrically (Essig-Marcello and van Buskirk, 1990). In this protocol, the use of NR after the exposure of fish cells to toxicants and the fluorometric measurement of any subsequent changes in NR uptake are described.

***Additional Materials** (also see Basic Protocol)*

Neutral red (NR) solution (0.33% w/v in DPBS; Sigma Aldrich, or see recipe for stock solution in Reagents and Solutions)
RTgill-W1 cells in a 24-well, 48-well or 96-well plate exposed to toxicants (see Basic Protocol, steps 1 to 23)
Neutral red fixative solution (see recipe)
Neutral red extraction solution (see recipe)
Orbital shaker

1. Prepare NR working solution by adding 180 µl neutral red solution to 11.82 ml L-15/ex, or prepare a 33 µg/ml neutral red working solution by diluting the neutral red stock solution 1:100 in L-15/ex solution.
2. Remove exposure medium from RTgill-W1 cells in a 24-well, 48-well or 96-well plate exposed to toxicants by inverting over a catch basin. Drain plates further for a few seconds on a small stack of paper towels.
3. Add 400 µl (for 24-well plate), 100 to 150 µl (for 48-well plate), or 50 to 100 µl (for 96-well plate) of NR working solution to each well of the respective plates and incubate the plate in the dark for 60 min at 18° to 22°C.
4. Remove the neutral red working solution by inverting over a catch basin and drain a few seconds on a small stack of paper towels.

   It is critical to remove all the neutral red working solution from each well, especially in a 96-well plate.

5. Rinse wells once with 400 µl/well (24-well plate) 100 µl/well (48- and 96-well plate) of neutral red fixative solution.

   The rinsing step removes any excess neutral red that has not been localized in lysosomes.

6. Add 400 µl/well (for 24-well plate) or 100 µl/well (for 48- or 96-well plate) of neutral red extraction solution to solubilize the lysosomal neutral red. Place plates on an orbital shaker and shake at ~40 rpm for 10 min.
7. Measure neutral red fluorescence on the fluorometric plate reader at excitation and emission wavelengths of 530 and 645 nm, respectively.

   In the interest of conserving material, and for performing cell viability assays on the same set of cells, the neutral red assay can be performed on the same plate previously used to assess alamar blue (see Basic Protocol) and/or CFDA-AM fluorescence (see Alternate Protocol 1). After termination of the alamar blue and CFDA-AM exposures, remove the dye solution(s) and start by adding neutral red as described above. However, inasmuch as the neutral red assay will terminate the cell culture because cells will be fixed, a separate plate needs to be used if the alamar blue/CFDA-AM plates are to be used to study recovery or repeated exposure (Schirmer et al., 1998a,b).

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

CFDA-AM stock solution, 4 mM

Dissolve the 5 mg vial of CFDA-AM (Molecular Probes) in 2.35 ml of sterile anhydrous DMSO (final concentration 4 mM) in a laminar flow hood. Dispense into sterile 1.5-ml microcentrifuge tubes in 50-µl aliquots to prevent degradation from thawing and refreezing. Wrap each microcentrifuge tube in aluminum foil to prevent light degradation. Store desiccated up to 1 year at –20°C to avoid ester hydrolysis due to moisture.

L-15/ex solution

Development of this modified medium is outlined in Schirmer et al. (1997) and is based on the constituents of basal medium, L-15 (Leibovitz, 1963). Make up all components from cell culture–grade reagents (Sigma) and prepare in cell-culture grade, distilled water.

Salt solution A: In 600 ml water, dissolve: 80 g NaCl, 4.0 g KCl, 2.0 g $MgSO_4$, and 2.0 g $MgCl_2$
Salt solution B: In 100 ml water, dissolve: 1.4 g $CaCl_2$
Salt solution C: In 300 ml water, dissolve: 1.9 g Na_2HPO_4 and 0.6 g KH_2PO_4
Autoclave each solution separately and store up to 1 year at room temperature

Sodium pyruvate solution: In 100 ml of water, dissolve 5.5 g sodium pyruvate. Filter sterilize through a 0.2-µm filter, dispense in 5.7-ml aliquots, and store up to 1 year at –20°C.

Galactose solution: In 100 ml of water, dissolve 9.0 g galactose. Filter sterilize through a 0.2-µm filter, dispense in 5.7-ml aliquots, and store up to 1 year at –20°C.

To prepare L-15/ex solution:
To 500 ml of sterile cell culture-grade, distilled water, add aseptically:
34.0 ml salt solution A
5.7 ml salt solution B
17.0 ml salt solution C
5.7 ml sodium pyruvate solution
5.7 ml galactose solution
Store L-15/ex solution up to 1 year at room temperature

L-15/ex can also be commercially purchased as a convenient powder from U.S. Biological, (cat. no. L1501 http://www.usbio.net/).

Leibovitz's L-15 complete medium containing FBS

To 500 ml of Leibovitz's L-15 medium (Sigma), aseptically add 50 ml fetal bovine serum (FBS; Sigma). The final FBS concentration in culture medium is 8.9%, which is usually referred to as 10%. Aseptically add 10 ml of 50× penicillin/streptomycin (final concentrations 100 IU/ml penicillin, 100 µg/ml streptomycin; Sigma). Store up to 1 year at 4°C.

Neutral red extraction solution

Prepare 1% (v/v) acetic acid and 50% (v/v) ethanol in deionized, distilled water. Store for up to 1 year at room temperature in the dark.

Neutral red fixative solution

Prepare 0.5% (v/v) formaldehyde and 1% (w/v) $CaCl_2$ in deionized, distilled water. Store for up to 1 year at room temperature in the dark.

Neutral red stock solution

Dissolve 3.3 mg of neutral red powder (Sigma) per ml of Dulbecco's PBS (DPBS; Sigma or Invitrogen/Life Technologies) in an amber vial. Store up to 1 year at 4°C.

Alternatively, neutral red stock solution can be purchased dissolved in DPBS at a concentration of 3.3 mg/ml (Sigma).

Trypsin solution

Dissolve 100 mg trypsin (Sigma) in 10 ml of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Sigma) to make a trypsin stock solution. Dispense 0.5 ml of this solution into 9.5 ml of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (e.g., Invitrogen/Life Technologies). Store up to 1 year at –20°C.

The alternative TrypLE (Invitrogen, cat no. 12605-010 or -013, with or without phenol red, respectively; either may be used) can be kept and used at room temperature, substituting for trypsin without the need of a chelating agent like EDTA.

COMMENTARY

Background Information

The many in vitro toxicology tests can be divided into two types: general (basal) cytotoxicity tests and tests of differentiated cell function (Flint, 1990). The general type consists of tests that measure cytotoxic phenomena only, e.g., the inhibition of cell proliferation. One premise of this approach is that all toxic phenomena are fundamentally related to an impairment of some aspect of cellular activity in vivo. Therefore, toxicity in vivo should be expected if the test agent is bioavailable to a target tissue at concentrations that are observed to impair cell viability in vitro. A potential weakness of this approach is the observation that, rather than being a general phenomenon of all tissues, toxicity in vivo is often limited to a small group of organs and cells within these organs. Thus, specific toxic effects might occur at concentrations well below those causing general cytotoxicity. The second class of in vitro tests attempts to overcome this weakness by monitoring a cellular function specific for the differentiated state of the cultured cell. From a risk-assessment point of view, the tests for differentiated cell functions are usually more valuable (Flint, 1990). To date, mostly general cytotoxicity tests have been used in fish toxicology, and are described in this unit.

Many general cytotoxicity tests have been described. They measure impairment of cellular activities by potentially toxic treatments. However, since their first introduction, assays of cytotoxicity and/or cell viability have been criticized as to their meaning and have aroused debate as to which assay is most appropriate (Schrek, 1965; Shaw, 1994). Cytotoxicity assays that monitor reproductive capacity have been described as being the most comprehensive because they integrate the soundness of the entire cellular machinery (Shaw, 1994). However, proliferation assays are not ideal for all purposes. They usually reveal little about the specific cellular events that lead to impaired proliferation and can miss subtle, transitory effects. They give little insight into the potential short-term impact of an effect on cells on the integrity of an organ or tissue. Also, colony formation and proliferation rates are impractical endpoints with most fish cell lines, because the cells grow slowly. As an alternative to proliferative endpoints, assays of cell viability and cell injury can be performed (Shaw, 1994), such as the ones described here.

Although numerous assays of cell viability have been developed, those that utilize fluorometric indicator dyes are perhaps best. First, more and more dyes are becoming commercially available to evaluate distinct cellular parameters. Second, the development of fluorometric multiwell plate readers has made the use of fluorometric dyes easy and rapid, such as for the assays in this unit. The microwells conserve material resources by reducing the number of cells needed and increasing the number of replicates. The plate readers have the potential for high interlaboratory reproducibility and can be coupled to robots for dosing and to computers for managing large amounts of data quickly and easily.

Critical Parameters and Troubleshooting

Temperature

The choice of temperature for growth, exposure, and assay of fish cell cultures is flexible

and can be dictated by practical considerations or by scientific objectives. This is because fish cells can be grown over a wide temperature range, e.g., from 5° to 25°C for salmonid cells (Bols et al., 1992). The medium used in this unit is based on Leibovitz's L-15, which does not need a 5% CO_2 atmosphere in order for the pH to be buffered (Leibovitz, 1963). As a result, the "incubator" for fish cell cultures in CO_2-independent media, such as L-15, can be a desk drawer at room temperature, or a temperature-regulated chamber, such as a conventional incubator or a refrigerator. For all the protocols that are described in this unit with salmonid cells, temperatures from 18° to 22°C give consistent results, and fluctuations within this range have little or no effect on the outcome.

Toxicant preparation

The acts of dissolving compounds and of adding them to cell cultures can be the source of several problems. Unfortunately, these are difficulties that cannot really be solved, but are important to consider when interpreting results. The problems are most severe for hydrophobic environmental contaminants, such as the PAHs. Several alternative strategies for toxicant delivery have to be considered. One is whether or not to use carrier solutions to dissolve the compounds. The second is whether or not to completely remove the medium that was used to initiate the cell cultures from the microwells and replace it with an equivalent volume of toxicant solution. For compounds that are going to be presented without the use of a carrier, there is usually no choice—the original medium has to be replaced with the toxicant solution. Except for extremely water-soluble compounds, the highest doses in dose-response curves cannot be achieved by adding small aliquots of a concentrated solution. By contrast, high concentrations of hydrophobic compounds can be achieved in carrier solutions, allowing them to be added to cultures in small aliquots. Potential problems with carriers, exposure media, and presentation strategies for toxicants are further discussed below.

Carriers such as dimethylsulfoxide (DMSO) have nearly always been used to dissolve environmental contaminants for toxicity testing, but they potentially can influence the results. For example, fluoranthene that was dissolved in L-15/ex solution with DMSO was slightly more photocytotoxic to RTgill-W1 cells than fluoranthene dissolved in L-15/ex solution without DMSO (Schirmer et al., 1997). Differences can also be found between carriers. DDT in DMSO was more cytotoxic to tilapia brain cells than DDT in acetone (Parkinson and Agius, 1987). Ibuprofen in DMSO but not in ethanol was cytotoxic to RTL-W1, and the cells were more sensitive to ibuprofen when the final DMSO concentration was 0.5% rather than 0.05% (Schnell et al., 2009). The induction of 7-ethoxyresorufin *o*-deethylase (EROD) activity in RTL-W1 cells was better after exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in DMSO than in isooctane (Clemons et al., 1994). Also, the carrier can influence the induction potency of some compounds (Yu et al., 1997).

Exposure medium

The medium in which cells are incubated during exposure to environmental contaminants is another variable that can influence results. An extremely simple one is used in this unit, which contains salts, galactose, and pyruvate at specified concentrations in the basal medium, Leibovitz's L-15 (Leibovitz, 1963), which is termed L-15 exposure or L-15/ex (Schirmer et al., 1997). Commercially available L-15/ex in powder form (U.S. Biological) has previously been used to evaluate the toxicity testing capabilities of RTgill-W1 in biochips (Brennan et al., 2012). L-15/ex has several advantages. For photocytotoxicity studies, the absence of vitamins and aromatic amino acids prevents the inadvertent generation of toxicants from these compounds during the UV treatment. L-15/ex is also advantageous for detecting toxicants that cause cytotoxicity through the generation of reactive oxygen species (ROS). This is because expression of their toxicity should be aided by the absence in L-15/ex of most antioxidants. The one exception might be the presence of pyruvate. For mammalian cells, pyruvate is part of an antioxidant defense (O'Donnell-Tormey et al., 1987). A limitation of L-15/ex is that exposure times are restricted to several days, as nutrient deprivation will ultimately cause cell death. The short exposure times mean that toxicants acting by inducing particular cellular processes, such as xenobiotic metabolism and causing accumulative damage, might be missed. Yet, RTgill-W1 cells survive in L-15/ex for at least 100 hr (Schirmer et al., 1997). Although addition of fetal bovine serum (FBS) to the medium extends the life of cell cultures, the antioxidants of FBS prevent the detection of fluoranthene photocytotoxicity (Schirmer et al., 1997). Also, FBS can alter

the bioavailability of toxicants (Schirmer et al., 1997; Hestermann et al., 2000).

Dosing strategies

Chemicals can be in applied in several ways to the cells. The two most commonly used dosing strategies are indirect and direct dosing. For indirect dosing, the medium that was used to initiate the cell cultures needs to be removed completely from the microwells and an equivalent volume of solution with the toxicant under study added. Both steps present difficulties. The medium-removal step has two problems. First, removal must be done quickly without damaging cells, which can be done by aspirating off the medium. But, this sometimes causes cell death. Death usually appears within minutes of aspiration and occurs in large patches over the culture surface. Routinely examining cultures with an inverted phase-contrast microscope shortly after aspiration easily identifies this problem. Alternatively, the medium can be removed by inverting the plate over a catch basin and blotting with a paper towel. This medium removal-technique has negligible contamination issues if done carefully in a laminar flow hood for exposures of short duration (as described in this unit). This is the recommended medium-removal technique, because the cells are not subjected to the same force as aspiration may involve. Second, complete removal of the initiating medium over the cell cultures has the potential to change the physiology of cell cultures. An example of this is the rapid induction of 7-ethoxyresorufin *o*-deethylase (EROD) activity upon the removal of medium from cultures of the rainbow trout liver cell line RTL-W1 (Segner et al., 2000). Replacing the medium with toxicant solution is problematic for hydrophobic compounds. A concentrated solution of toxicant in a carrier such as DMSO must be serially diluted in an aqueous solution to prepare concentrations for dose-response curves. Hydrophobic compounds have a tendency to stick to the walls of containers and pipets used to prepare these solutions. For volatile compounds, the number of pipetting steps increase the risk of evaporation of the test compounds. Thus, the final toxicant concentrations that the cultures receive can be lower than the concentrations apparently added. This problem can be solved by measuring the concentrations through analytical methods, as recently demonstrated for exposures in 24-well plates (Tanneberger et al., 2010, 2013), although these techniques may not be readily available to a cell culture laboratory. However, for some compounds, concentrations in the culture wells may change so quickly that accounting for available toxicant concentrations may also be difficult by analytical means alone. Potential alternatives in these cases are passive dosing systems (Kramer et al., 2010; Smith et al., 2010).

The second presentation strategy, direct dosing, uses micropipets to add small volumes ($\leq$10 µl) of the toxicant in carrier solvent to the medium over the cells in microwells. Dosing in this manner must be done very carefully with carriers such as DMSO. The small volume of DMSO sometimes falls as a blob directly onto the cells, immediately killing them. Again, examining cultures with an inverted phase-contrast microscope easily identifies this problem (Schnell et al., 2009). As mentioned, the difficulty can be avoided by keeping the pipet tip close to the medium surface and allowing the surface tension to disperse the DMSO rapidly and evenly through the culture. This second presentation strategy has at least two advantages. Changing the medium is avoided, which also prevents any possible changes in the cells caused by this act. Final culture concentrations are closer to the apparent toxicant concentration that is added. In extreme cases, a compound might appear to be toxic with this dosing method, but not by the method of preparing the toxicant in exposure medium and using this solution to replace the growth medium of cell cultures. Schnell et al. (2009) dissolved ibuprofen in DMSO and found a 20-fold higher toxicity when dosed directly instead of indirectly. A similar observation was made by Tanneberger et al. (2010), whereby the toxicity of 1,2-dichlorobenzene was 10-fold higher when the DMSO stock solutions was applied directly onto the cells. However, this phenomenon could only be observed when DMSO was used as solvent, and seems to appear only when hydrophobic and/or volatile compounds are used. When 1,2-dichlorobenzene was dissolved in methanol, the cytotoxicity triggered by both dosing methods was indistinguishable (Tanneberger et al., 2010). If the results of the two different dosing strategies are profoundly different, they should be reported as such, as this will aid others in replicating the results in any future studies and will stress the subtlety of the cytotoxicant's actions.

UV irradiation

For the concurrent exposure of cultures to environmental contaminants and UV light, radiation exposures can be expressed in different ways, sometimes causing confusion. The

most important distinction is between energy units, which refer to the wave-like character of radiation, and quantum units, which reflect the corpuscular character. The conversion from energy content to photon content is defined by Planck's equation. Fluence rates are used to refer to the quantity of radiation per area per time. Thus, Wm^{-2} and $Jm^{-2}\ sec^{-1}$ are expressions of energy fluence rates, whereas $mol_{(photons)}m^{-2}sec^{-1}$ is an expression of photon fluence rates. In contrast to the fluence rates, the total amount of radiation received by the cells can be expressed as fluence, that is quantity per area. Thus, the energy fluence ($Jm^{-2} = Wm^{-2}sec$), or the photon fluence ($mol_{(photons)}m^{-2}$) can be calculated by multiplying the fluence rate by the time of irradiation (in seconds). For example, a photon fluence rate of UV-B at 313 nm of 1.4 $\mu mol(m^{-2})sec^{-1}$, which has been shown to be environmentally relevant (Oris and Giesy, 1987), is equivalent to an energy fluence rate at that wavelength of 53 μWcm^{-2}(or 53 $\mu Jcm^{-2}sec^{-1}$). If irradiation is performed for 2 hr, the energy fluence that the cells are exposed to is 0.4 $Wcm^{-2}sec$ (or 0.4 Jcm^{-2}).

Inasmuch as UV radiation exposure decreases with increasing distance from the UV source, it is important to measure UV radiation at the position at which culture plates will be placed. In order to account for absorption of UV radiation by culture plate lids, place a lid between the UV source and the radiation measurement device. The irradiation of cells in the presence of tissue culture plate lids is highly recommended for two reasons. First, the lids ensure sterility during the illumination process. Second, the plate lids absorb any radiation below a wavelength of 290 nm, a filtering process which, under natural conditions, is carried out by stratospheric ozone (Schirmer et al., 1997).

While measurements of UV irradiance should be done frequently to ensure that UV intensities of the fluorescent lamps are as required, initial measurements should confirm that the culture medium covering the cells does not detrimentally affect UV penetration. To study this, a lid-covered tissue culture plate with and without culture medium should be placed between the UV source and the spectroradiometer, and the values compared. If measurements are the same, it can be concluded that the culture medium used has no discernible effect on UV penetration. In contrast, lower UV intensities measured in the presence of culture medium indicate that cells obtain less UV radiation than anticipated. For example, the authors found that UV intensities were not significantly affected upon passage through a 4.7 mm path of L-15/ex medium, which equals 500 µl L-15/ex medium in a 48-well tissue culture plate. In contrast, the same passage of UV in the presence of 10% FBS led to a reduction of UV readings of 27% for UV-B and 9% for UV-A (Schirmer et al., 1997). Another factor to consider is temperature. In the presence of a small fan and a distance of the tissue culture plates from the UV lamps of at least 15 cm, the authors did not find temperature to rise in the tissue culture medium within 2 hr of UV irradiation at a photon fluence rate of 10 µmol m^{-2} sec^{-1}UV-A and 1 µmol $m^{-2}sec^{-1}$UV-B (Schirmer et al., 1998b). However, longer exposures or higher UV intensity may potentially lead to an increase in temperature.

Toxicant removal

Termination of toxicant exposures prior to the addition of the fluorescent indicator dyes to cell cultures must be done carefully and consistently. Inverting plates over a catch basin is recommended over aspiration. Removal must be done rapidly but without damaging cells. Viewing control cultures with an inverted phase-contrast microscope will identify any problems. Of course, at this stage, the loss of cells from toxicant-treated cultures can be expected.

Fluorescent microwell plate readers

A number of manufacturers (e.g., Molecular Devices, Applied Biosystems) make fluorescent plate readers, and likewise, microwell plates are produced by several companies (e.g., Falcon, Costar, Nunc). Although all fluorescent plate readers are likely suitable, the crucial issue is to make sure the microwell plate correctly matches the plate reader. Under most circumstances, only the appropriate settings need to be chosen, as the plate readers have been designed to accept most plates from various manufacturers. However, sometimes the dimensions for a particular microwell plate must be obtained from the manufacturer and entered into the plate reader.

As mentioned earlier, the minimum volume of fluorescent indicator dyes needed for accurate measurement might vary for different microwell plate readers. Thus, initial experiments should determine the most suitable volume.

Anticipated Results

Data analysis: Calculation of EC_{50}

The raw fluorescent units resulting from the cell viability assays are used to evaluate the

toxicity of the chemical being tested. Cell viability is expressed as a percent of non-toxicant-exposed cells (% of control). For the highest test concentration (in case of 24- and 48-well plates) or for each concentration (for 96-well plates) of toxicant, there is one well that contains no cells (no cell control). Both the well with no cells and the wells with cells are exposed to the toxicant. Prior to calculating percent of control, subtract the fluorescent units (FU) for wells without cells from the experimental (ex) and control (con) values with cells. To calculate the cell viability (% of control) use the following formula:

$$\%\ \text{of control} = (FU_{\text{ex cells}} - FU_{\text{ex no cells}}) \times 100/(\text{average } [FU_{\text{con}} - FU_{\text{con no cells}}])$$

Data for each well of each concentration are expressed as a percent of control. Then, the average and standard deviation for each concentration are calculated. These values are used to calculate the EC_{50} for the toxicant.

Dose-response data typically follow a sigmoidal relationship and can be analyzed by nonlinear regression in most graphing software such as SigmaPlot (Jandel Scientific) or GraphPad Prism (GraphPad Software). The data are fitted to the four-parameter logistic function for continuous response data. The logistic function is:

$$y(\mathrm{d}) = Y_{\min} + (Y_{\max} - Y_{\min})\backslash\{1 + \exp[-g(\ln(d) - \ln(\mathrm{EC50}))]\}^{-1}$$

where $y(d)$ is the % cell viability at the dose d, $Y_{\min}$ is the minimum percent cell viability, $Y_{\max}$ is the maximum percent cell viability, g is a slope parameter, and EC_{50} is the dose that produces 50% of cell viability.

Inasmuch as cell viability data are expressed on a 0% to 100% basis, the four-parameter equation is simplified to a two-parameter equation because $Y_{\max}$ and $Y_{\min}$ are constants of 100% and 0%, respectively:

$$y(d) = 0\% + (100\% - 0\%)\ \{1 + \exp[-g(\ln(d) - \ln(EC_{50}))]\}^{-1}$$

Interpretation of results

With each fluorescent indicator dye (alamar blue, CFDA-AM, and NR), a reduction in fluorescent unit readings in experimentally treated cultures relative to the readings in control cultures indicates cytotoxicity or a loss of cell viability. The use of multiple dyes has the potential of revealing the mechanism(s) behind the cytotoxicity. However, the results and the interpretation can be straightforward or complicated, depending on the toxicant under study. Both a simple (Fig. 1.5.2) and a complex example (Fig. 1.5.3) are presented.

When the dose-response curves for the three indicator dyes are identical or very similar for cell cultures after short exposures to toxicants, e.g., illustrated in Figure 1.5.2 for 1,4-dimethyl naphthalene, this indicates that the toxic mechanism is general membrane damage, which includes impairment of organelle membranes, such as those for mitochondria and lysosomes, as well as the plasma membrane. Because this loss of cell viability occurs quickly, it has come to be known as direct cytotoxicity, to indicate that cellular metabolism of the toxicant is unlikely to be involved (Schirmer et al., 1998a). The terms ultra-fast cell death, or less preferably, necrosis, have been suggested for cell death that occurs quickly in mammalian cell cultures in response to strong stimuli (Blagosklonny, 2000). Ultra-fast cell death appears before the activation of caspase, which is a characteristic of apoptosis (Blagosklonny, 2000). For mammalian cells, Blagosklonny (2000) has suggested that a time frame for the development of a decline in cell viability can be used to distinguish ultra-fast cell death (2 to 16 hr), apoptosis (16 to 36 hr), and slow cell death (>36 hr). As cellular phenomena take longer to develop in fish cells being grown at 18° to 22°C than in mammalian cells at 37°C, the time frame for these in fish cell lines might be increased considerably.

Alternatively, the dose-response curves with the different indicator dyes can be unlike one another. Several examples already have appeared in the literature. One combination of outcomes is a decline in cell viability as measured with alamar blue being accompanied by little or no change in cell viability as measured with CFDA-AM. Such results have been seen with benzo[a]pyrene (BaP) and 6, 12-BaP quinone (Schirmer et al., 2000). As the reduction of alamar blue to a fluorescent product is now thought to indicate cellular metabolism rather than specifically mitochondrial activity (O'Brien et al., 2000), the results are interpreted as indicating that these compounds impair metabolism without impacting plasma membrane integrity. Another outcome combination is that of a greater decline in cell viability as measured with neutral red than in the cell viability monitored with alamar blue and CFDA-AM. This was seen in studies on the photocytoxicity of acenaphthylene, acenaphthene, phenanthrene, fluoranthene, pyrene, anthracene, and benzo[g,h,i]perylene (Schirmer

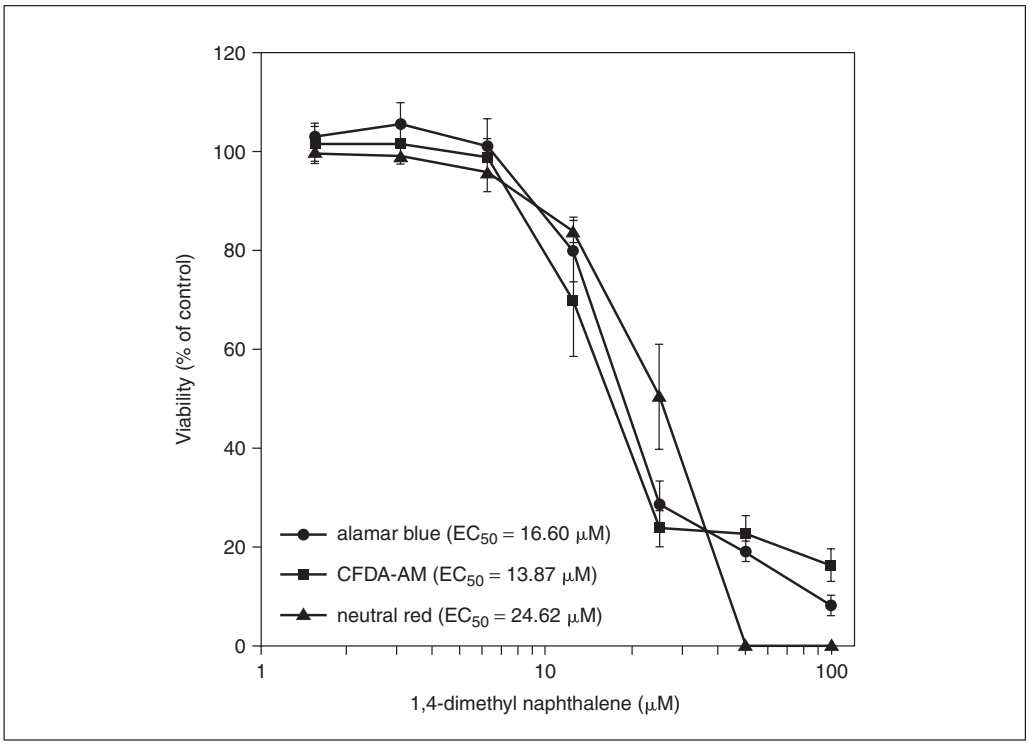

Figure 1.5.2 Effect of 1,4-dimethyl naphthalene on viability of RTgill-W1 cultures. After the cultures had been exposed for 2 hr, cell viability was assessed with alamar blue (circles), CFDA-AM (squares), and neutral red (triangles). Results were expressed as a percentage of the readings in control wells exposed to L-15/ex solution with DMSO.

et al., 1998b). The interpretation of these results is that specific lysosomal damage has occurred immediately after concurrent exposure to these compounds and UV radiation, with little or no impairment of plasma membranes and cellular metabolism.

The CFDA-AM assay appears to monitor impairment to plasma membranes, but Figure 1.5.3A illustrates a perplexing outcome. A decline in fluorescent readings occurs at lower concentrations of 2-ethyl phenanthrene with CFDA-AM than with alamar blue, resulting in a lower EC_{50} for this toxicant. If the CFDA-AM measures membrane integrity, then cellular metabolism evaluated with alamar blue would not be expected to continue with little or no impairment while the integrity of the plasma membrane has been lost. An explanation can be advanced for this apparent anomaly. When carried out as described here, a decrease in fluorescent readings with CFDA-AM actually measures a decline in the total esterase activity within a microwell cell culture. The decrease in esterase activity with toxicant treatment could be achieved in two general ways: the loss of plasma membrane integrity and/or specific inhibitory actions on cellular esterases. In turn, the loss of plasma membrane integrity could decrease culture esterase activity in two slightly different ways. The first of these would be the complete or partial lysis of the cells upon toxicant exposure so that the esterases are released into the medium and lost when the medium is removed and replaced with the CFDA-AM solution. Another possible cause for the diminution of esterase activity is a change in plasma membrane integrity so that cytoplasmic constituents are lost to the medium but the esterases remain contained within the cells, which are still attached to the surface of the microwells. This change in the cytoplasmic milieu would be less able to support maximal esterase activity. Alternatively, the toxicant treatment could leave membrane integrity unimpaired but specifically interfere with cellular esterases, causing activity to decline. Examples of this would be a toxicant interfering with the uptake of the substrate, CFDA-AM, across the plasma membrane, or inhibiting the catalytic activity of the esterases. The results in Figure 1.5.3A, in which increasing 2-ethyl phenanthrene concentrations cause a more precipitous decline in CFDA-AM readings than in alamar blue readings, are likely an example of a toxicant impairing esterase activity rather than the plasma membrane.

Additional information into mechanisms of toxicity can be obtained by applying the

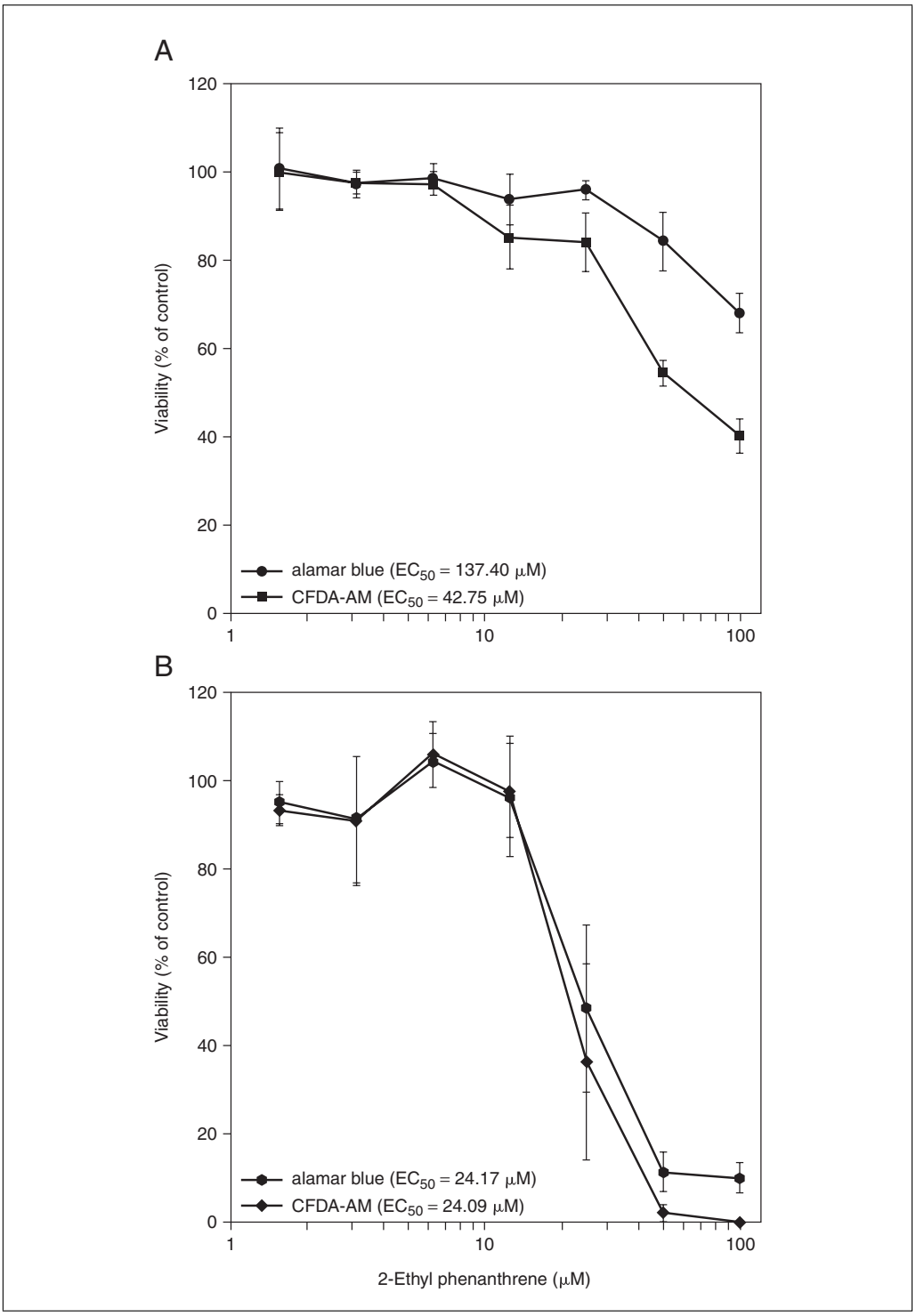

Figure 1.5.3 Effect of 2-ethyl phenanthrene on viability of RTgill-W1 cultures. After the cultures had been exposed for 2 hr (**A**), cell viability was assessed with alamar blue (circles) and CFDA-AM (squares). The exposure medium was replaced with complete medium and the cells were incubated for an additional 24 hr (**B**), after which viability was reassessed with alamar blue (hexagons) and CFDA-AM (diamonds). Results were expressed as a percentage of the readings in control wells exposed to medium with DMSO.

indicator dyes both immediately after terminating toxicant exposure and later after a period of potential recovery. The assays with alamar blue and CFDA-AM are nontoxic and can be applied on the same microwell cultures for both time points. For the NR assay, which requires cultures to be extracted, separate microwell cultures must be used. Differences between dose-response curves immediately and 24 hr after toxicant exposures have been found, suggesting impairments at specific cellular sites and the capacity of cells to repair them. For example, alamar blue readings decreased immediately after the end of exposures to BaP and 6, 12 BaP quinone, but recovered 24 hr later (Schirmer et al., 2000). As the CFDA-AM readings showed no changes, these results suggested that short exposures to these compounds transitorily disrupted cellular metabolism, although continuous exposure would ultimately lead to cell death. By contrast, when cell cultures are exposed to increasing 2-ethyl phenanthrene concentrations, rinsed 2 hr later, and evaluated for cell viability either immediately or 24 hr later, the highest concentrations cause some loss of cell viability immediately after ending the exposure to 2-ethyl phenanthrene but cause a profound loss 24 hr later (Fig. 1.5.3). This suggests that during the 2-hr exposure period at high concentrations, 2-ethyl phenanthrene initiates a damaging process that continues over the next 24 hr in the absence of the test agent. Therefore, under these circumstances, 2-ethyl phenanthrene is causing irreparable cellular damage. The damage can likely be attributed to some 2-ethyl phenanthrene being retained within the cells, despite the 2 hr exposure being terminated, and over the next 24 hr either acting directly but slowly or being metabolized into more cytotoxic compounds.

The above discussion illustrates some of the complexities that might be anticipated. Likely, not all of the possible scenarios for cellular responses to toxicants as measured with these three indicator dyes have been described. Several additional complicated scenarios will likely be revealed only by examining more compounds with these indicator dyes. For example, under some circumstances, neutral red readings might increase (Zhang et al., 1990; Dayeh et al., 2009). Overall, the methods described in this unit allow the rapid and inexpensive screening of toxicants for fish cells and at the same time give potential insight into their mechanism(s) of toxicity. In the future, better understanding of the cellular function(s) being monitored with each indicator dye will improve their utility in identifying toxicity mechanisms.

Time Considerations

One must consider the time to culture the fish-derived cell lines in preparation for exposure to the toxicants. The time between subculturing of the stock culture is between 7 and 14 days if a 1:2 split is used routinely. Once the flask is confluent, the cells are transferred to a microwell culture plate, allowing the cells to attach and become a confluent monolayer on the bottom of each well, which will take ~2 to 3 days depending on the use of 24-well, 48-well, or 96-well plates. The times for exposure to a toxicant can vary. Initially they might be short, for example 2 hr, and if no change in viability is detected, increased to 24 or 48 hr. In some cases, exposures as long as 7 days might be considered if a slowly developing mechanism of toxicity is expected.

Literature Cited

Blagosklonny, M.V. 2000. Cell death beyond apoptosis. *Leukemia* 14:1502-1508.

Bols, N.C., Mosser, D.D., and Steels, G.B. 1992. Temperature studies and recent advances with fish cells in vitro. *Comp. Biochem. Physiol.* 103A:1-14.

Borenfreund, E. and Puerner, J.A. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays. *J. Tissue Cult. Methods* 9:7-12.

Brennan, L.M., Widder, M.W., Lee, L.E., and van der Schalie, W.H. 2012. Long-term storage and impedance-based water toxicity testing capabilities of fluidic biochips seeded with RTgill-W1 cells. *Toxicol. In Vitro* 26:736-745.

Clemons, J.H., van den Heuvel, M.R., Stegeman, J.J., Dixon, D.G., and Bols, N.C. 1994. A comparison of toxic equivalent factors for selected dioxin and furan congeners derived using fish and mammalian liver cell lines. *Can. J. Fish. Aquat. Sci.* 51:1577-1584.

Dayeh, V.R., Schirmer, K., and Bols, N.C. 2009. Ammonia-containing industrial effluents lethal to rainbow trout vacuolization and neutral red uptake in the rainbow trout gill cell line, RTgill-W1. *ATLA* 37:77-87.

Essig-Marcello, J.S. and van Buskirk, R.G. 1990. A double-label in situ cytotoxicity assay using the fluorescent probes neutral red and BCECF-AM. *In Vitro Toxicol.* 3:219-227.

Evans, S.M., Casartelli, A., Herreros, E., Minnick, D.T., Day, C., George, E., and Westmoreland, C. 2001. Development of a high throughput in vitro toxicity screen predictive of high acute in vivo toxic potential. *Toxicol. In Vitro* 15:579-584.

Flint, O.P. 1990. In vitro toxicity testing: Purpose, validation and strategy. *Altern. Lab. Anim.* 18:11-18.

Haugland, R.P. 1996. Handbook of Fluorescent Probes and Research Chemicals. pp. 365-398, 549. Molecular Probes, Inc., Eugene, Oregon.

Hestermann, E.V., Stegemann, J.J., and Hahn, M.E. 2000. Serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in cell culture bioassays. *Toxicol. Sci.* 53:316-325.

Kramer, N.I., Busser, F.J.M., Oosterwijk, M.T.T., Schirmer, K., Escher, B.I., and Hermens, J.L.M. 2010. Development of a partition-controlled dosing system for cell assays. *Chem. Res. Toxicol.* 23:1806-1814.

Lee, L.E.J., Dayeh, V.R., Schirmer, K., and Bols, N.C. 2009. Applications and potential uses of fish gill cell lines: Examples with RTgill-W1. *In Vitro Cell Dev. Biol. Anim.* 45:127-134.

Leibovitz, A. 1963. The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Am. J. Hygiene* 78:173-180.

O'Brien, J., Wilson, I., Orton, T., and Pognan, F. 2000. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* 267:5421-5426.

O'Connor, S., McNamara, L., Swerdin, M., and Van Buskirk, R.G. 1991. Multifluorescent assays reveal mechanisms underlying cytotoxicity-phase I CFTA compounds. *In Vitro Toxicol.* 4:197-207.

O'Donnell-Tormey, J., Nathan, C.F., Lanks, K., DeBoer, C.J., and de la Harpe, J. 1987. Secretion of pyruvate: An antioxidant defense of mammalian cells. *J. Exp. Med.* 165:500-514.

OECD. 2012. Fish Testing Framework. Series on Testing and Assessment. N° 171. http://search.oecd.org/officialdocuments/displaydocumentpdf/?cote=ENV/JM/MONO(2012)16&doclanguage=en.

Oris, J.T. and Giesy, J.P. 1987. The photo-induced toxicity of polycyclic aromatic hydrocarbons to larvae of the fathead minnow (*Pimephales promelas*). *Chemosphere* 16:1395-1404.

Parkinson, C. and Agius, C. 1987. The effects of solvents on the toxicity of DDT to fish cells. *Arch. Toxicol. Suppl.* 11:240-242.

Reader, S.J., Blackwell, V., O'Hara, R., Clothier, R.H., Griffin, G., and Balls, M. 1990. Neutral red release from pre-loaded cells as an in vitro approach to testing for eye irritancy potential. *Toxicol. In Vitro* 4:264-266.

Rotman, B. and Papermaster, B.W. 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. U.S.A.* 55:134-141.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., and Bols, N.C. 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. *Toxicol. In Vitro* 11:107-119.

Schirmer, K., Greenberg, B., Dixon, D.G., and Bols, N.C. 1998a. Ability of 16 priority PAHs to be directly cytotoxic to a cell line from the rainbow trout gill. *Toxicology* 127:129-141.

Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., and Bols, N.C. 1998b. Ability of 16 priority PAHs to be photocytotoxic to a cell line from the rainbow trout gill. *Toxicology* 127:143-155.

Schirmer, K., Chan, A.G.J., and Bols, N.C. 2000. Transitory metabolic disruption and cytotoxicity elicited by benzo[a]pyrene in two cell lines from rainbow trout liver. *J. Biochem. Mol. Toxicol.* 14:262-276.

Schnell, S., Kawano, A., Porte, C., Lee, L.E.J., and Bols, N.C. 2009. Effects of ibuprofen on the viability and proliferation of rainbow trout liver cell lines and potential problems and interactions in effect assessment. *Environ. Toxicol.* 24:157-165.

Schrek, R. 1965. In vitro methods for measuring viability and vitality of lymphocytes exposed to 45°C, 47°C and 50°C. *Cryobiology* 3:322-328.

Segner, H., Behrens, A., Joyce, E.M., Schirmer, K., and Bols, N.C. 2000. Transient induction of 7-ethoxyresorufin-*o*-deethylase (EROD) activity by medium change in the rainbow trout liver cell line, RTL-W1. *Mar. Environ. Res.* 50:489-493.

Shaw, A.J. 1994. Defining cell viability and cytotoxicity. *A.T.L.A.* 22:124-126.

Smith, K.E.C., Oostingh, G.J., and Mayer, P. 2010. Passive dosing for producing defined and constant exposure of hydrophobic organic compounds during in vitro toxicity tests. *Chem. Res. Toxicol.* 23:55-65.

Tanneberger, K., Rico-Rico, A., Kramer, N.I., Busser, F.J.M., Hermens, J.L.M., and Schirmer, K. 2010. Effects of solvents and dosing procedure on chemical toxicity in cell-based *in vitro* assays. *Environ. Sci. Technol.* 44:4775-4781.

Tanneberger, K., Knöbel, M., Busser, F.J.M., Sinnige, T.L., Hermens, J.L.M., and Schirmer, K. 2013. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. *Environ. Sci. Technol.* 47:1110-1119.

Yu, K.O., Fisher, J.W., Burton, G.A., and Tillitt, D.E. 1997. Carrier effects of dosing the H4IIE cells with 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) in dimethyl sulfoxide or isooctane. *Chemosphere* 35:895-904.

Zhang, S.Z., Lipsky, M.M., Trump, B.F., and Hsu, I.C. 1990. Neutral red (NR) assay for cell viability and xenobiotic-induced cytotoxicity in primary cultures of human and rat hepatocytes. *Cell. Biol. Toxicol.* 6:210-234.

Key References

Bols, N.C. and Lee, L.E.J. 1994. Cell lines: Availability, propagation and isolation. *In* Biochemistry and Molecular Biology of Fishes, vol. 3. (P.W. Hochachka andT.P. Mommsen, eds.) pp. 145-159. Elsevier Science, Amsterdam.

An overview of protocols for obtaining and growing fish cell lines.

Ganassin, R.C., Schirmer, K., and Bols, N.C. 2000. Cell and tissue culture. *In* The Laboratory Fish (G.K. Ostrander, ed.) pp. 631-651. Academic Press, San Diego.

Gives technical protocols for using fish cell lines in toxicology.

Babich, H and Borenfreund, E. 1991. Cytotoxicity and genotoxicity assays with cultured fish cells: A review. *Toxicol. In Vitro* 5:91-100.

Bols, N.C., Dayeh, V.R., Lee, L.E.J. and Schirmer, K. 2005. Use of fish cell lines in toxicology of fish. *In* Biochemistry and Molecular Biology of Fishes-Environmental Toxicology. Vol. 6. (T.W. Moon andT. P. Mommsen, eds) pp. 43-84. Elsevier Science, Amsterdam.

Fent, K. 2001. Fish cell lines as versatile tools in ecotoxicology: Assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicol. In Vitro* 15:477-488.

Schirmer, K. 2006. Proposal to improve vertebrate cell cultures to establish them as substitutes for the regulatory testing of chemicals and effluents using fish. *Toxicology* 224:163-183.

The above four articles provide a broad overview on use of fish cell lines in toxicology and ecotoxicology.