15. RAINBOW TROUT GILL CELL LINE MICROPLATE CYTOTOXICITY TEST

VIVIAN R. DAYEH
Department of Biology
University of Waterloo, Waterloo
Ontario N2L 3G1, Canada
vrdayeh@sciborg.uwaterloo.ca

KRISTIN SCHIRMER
Department of Cell Toxicology
UFZ-Centre for Environmental Research
Permoserstr, 15
04318, Leipzig, Germany
kristin.schirmer@ufz.de

LUCY E.J. LEE
Department of Biology
Wilfrid Laurier University, Waterloo
Ontario N2L 3C5, Canada
llee@wlu.ca

NIELS C. BOLS
Department of Biology
University of Waterloo, Waterloo
Ontario N2L 3G1, Canada
ncbols@sciborg.uwaterloo.ca

1. Objective and scope of the RTgill-W1 cytotoxicity test method

This chapter describes a rapid, inexpensive *in vitro* test for evaluating the toxicity of water samples and a potential alternative to the use of fish in routine toxicity testing. The overall objective of the procedure is to assess water samples for acute or basal cytotoxicity to fish cells. Basal cytotoxicity refers to impairment of cellular activities that are shared by all or most cells. The basic procedure can also be used to evaluate the acute cytotoxicity of individual chemicals and to understand the mechanism(s) behind their toxicity. The speed and cost-saving features arise from assessing the viability of fish cells in 96-well microplates. This reduces the cost of disposables to be used and of shipping large volumes of water samples to a central testing facility.

C. Blaise and J.-F. Férard (eds.), Small-scale Freshwater Toxicity Investigations, Vol. 1, 473-503.

The described protocol is for whole-water samples, but water extracts could also be examined. Theoretically, any water could be tested, but samples containing copious amounts of microbes or particulate matter may require a filtration step, which could also lead to an inadvertent removal of some toxicants.

2. Summary of the RTgill-W1 cytotoxicity test procedure

The test procedure can be considered as the integration of three basic protocols. The first involves routinely growing a fish cell line in flasks and using cells from these culture vessels to initiate test cultures in either 48- or 96-well microplates. Each culture well receives approximately 5 x 10^4 or 3 x 10^4 cells and is confluent in approximately 3 days at which time the cells are exposed to the test solution. The second protocol describes how water samples are prepared for application to the microwell cultures of fish cells. The key preparative step is adding medium components to the water samples in order to achieve an osmolality appropriate for fish cells. The third protocol evaluates basal cytotoxicity in fish cell cultures after a 24 h exposure to the water samples. This is done with cell viability assays that utilize fluorometric dyes to monitor different cellular activities. The results are read in a fluorometric plate reader and expressed as a percentage of the control. These three basic protocols are given in detail in Sections 6 to 9 and have been described in different formats in other publications (Bols and Lee, 1994; Ganassin et al., 2000; Dayeh et al., 2003b). Table 1 summarizes the general characteristics of the test procedure.

3. Overview of applications reported with the toxicity test method

As described here, measuring basal cytotoxicity in microplate cultures of fish gill cells is a means of evaluating the toxicity of water samples. Fish cells have been used widely in microwell plates for toxicological studies (Bols et al., 2005; Castaño et al., 2003; Dayeh et al., 2002; Fent, 2001), but the procedure of this chapter has several special features. The most unique one is the addition of very few medium components to water samples as solids to make up a solution that will maintain the cells and allow the cytotoxicity of the water to be evaluated (see Section 4.2). Another is the employment of a continuous gill epithelial cell line, RTgill-W1 (Fig. 2). A third one is to monitor changes in cell viability with three fluorescent indicator dyes that can be measured with a fluorometric multiwell plate reader (Fig. 3). To date, the procedure has been applied to effluent from a paper mill (Dayeh et al., 2002) and currently is being evaluated for its usefulness in testing the toxicity of mining effluent (Dayeh et al., 2003a). In addition an early variation of the procedure was successful with oil refinery effluent (Schirmer et al., 2001). The procedure also can be used to rank the cytotoxicity of individual chemicals for the general purposes of identifying compounds that have the potential to be acutely toxic *in vivo*. Data on the basal cytotoxicity of a range of chemicals to RTgill-W1 have been obtained. This includes polycyclic aromatic hydrocarbons (PAHs) (Schirmer et al., 1998a, b),

surfactants (Dayeh et al, 2002; 2004) and metals (Dayeh et al., 2003a). This information can help to interpret results obtained with whole-water samples.

Table 1. Rapid summary of RTgill-W1 cytotoxicity test.

Test organism	- Rainbow trout gill cell line, RTgill-W1
Type of test	- Acute toxicity test (24 h exposure); static
Test format	- 48 or 96-well tissue-culture treated flat bottom microwell plates
Well volume contents	- 500 µL for 48-well plates and 200 µL for 96-well plates
Initial cell plating density	- 5 x 10^4 for 48-well plates and 3 x 10^4 for 96-well plates grown until confluent monolayer has formed (~ 3 days)
Lighting	- Cell culturing in ambient lighting; dosing in reduced lighting; test exposure in darkness*
Temperature	- Cell growth and exposure at ambient room temperature (20 ± 2ºC)
Experimental configuration	- 48-well plates: 5 control wells, 7 serial dilutions of test solution, each with 4 replicates with cells and one no-cell control - 96-well plates (two configurations available) 1. same as above for 48-well plates (able to conduct two separate compounds) 2. 7 control wells, 11 serial dilutions of test solution, each with 6 replicates and one no-cell control
Measurement of cell viability	- Fluorescent indicator dyes quantified on a multiwell fluorescent plate reader - alamar Blue – metabolic activity - CFDA-AM – cell membrane integrity - Neutral red – lysosomal activity
Endpoints determined	- EC50, based on % of control cells
Reference toxicants	- Abietic acid (85% purity – Acros Organics through Fisher Scientific)

* allows additional evaluation of photo-cytotoxicity in the presence of UV irradiation.

4. Advantages of conducting the toxicity test method

Each of the three basic protocols that make up the test procedure brings advantages to the overall procedure. This begins with the use of cells in culture, the kind of cell culture, and the choice of cell type.

4.1 ADVANTAGES OF CELL CULTURES AND CELL LINES

Cell cultures in general offer several advantages over fish as assay tools for environmental samples (Bols et al., 2005; Castaño et al., 2003; Dayeh et al., 2002; Fent 2001). Commonly, results are obtained more rapidly and at less cost with the cell assays than with intact animals. The small volume of sample needed for cell assays provides convenience and saves money. For example, in Canada the pulp and paper industry has to pay for shipping large volumes of effluent, often from remote sites, to a central facility for the rainbow trout 96 h lethality test. Finally, assays with cell cultures satisfy a societal desire to reduce the use of animals in toxicology testing (see Box 1).

Two general types of cultures can be used to study animal cells *in vitro*. One is the primary culture; the other, cell lines. Primary cultures are initiated directly from the cells, tissues or organs of fish and typically last for only a few days. The two are interrelated because cell lines are developed from primary cultures. By convention (Schaeffer, 1990), the primary culture ends and the cell line begins upon subcultivation or splitting of the primary culture into new culture vessels. The cell line can continue to be propagated by repeating the cycle of allowing cell number to increase through cell proliferation followed by splitting the cell population into new culture vessels, usually flasks. This cycle of growth and splitting, which is often referred to as passaging, might be possible for only a limited number of population doublings, which is a finite cell line, or done indefinitely, which is a continuous cell line. In the case of fish, the cell lines almost always appear to be continuous or immortal (Bols et al., 2005).

Box 1. Main advantages of using RTgill-W1 cytotoxicity assay.

- Continuous supply of cells
- Low volume sample requirement
- Detection of multiple endpoints of cell viability
- Large numbers of samples can be tested
- Detection of mechanism(s) of toxicity
- Ease of sample preparation
- Rapid exposure time (24 hours)

As to the choice of cell culture type in environmental assays, cell lines have several advantages over primary cultures. Cell lines are a much more reproducible and convenient source of cells because, once established, cell lines are fairly homogeneous and can be cryopreserved indefinitely. Although a single preparation from an organ or pooled organs can yield identical primary cultures, there is the cost of maintaining the fish and of the labor involved in repeatedly initiating new primary cultures. Once a cell line is developed no further animals need to be consumed. Thus cell line assays better satisfy the desire to use fewer animals in toxicity tests.

Although much more is known about mammalian than piscine cell lines and mammalian cell lines have been used to monitor water quality (Richardson et al., 1977; Mochida, 1986), ultimately piscine cell lines should be superior in assays of water quality for several reasons. Firstly, the whole animal tests that are used to assay water employ fish, making fish cells more appropriate as alternatives. Secondly, the toxicants can be applied to fish cells at temperatures more typical of the temperatures to which fish would be exposed. A wide range of exposure temperatures can be utilized for the testing including the temperature normally used for whole fish. Thirdly, the cells of a piscine cell line should better reflect the properties of the fish from which they were derived than the cells of a mammalian cell line. Finally, fish cells tolerate being maintained in culture for a day or two in a simple exposure medium. Such a medium is L-15/ex, which was developed for studying the photocytotoxicity of polycyclic aromatic hydrocarbons (PAHs) to the rainbow trout gill cell line, RTgill-W1 (Schirmer et al., 1997). This medium has only salts, pyruvate and galactose. The simplicity favors expression of cellular responses to toxicants because protective molecules such as antioxidants are absent. As well, the medium is much less expensive than complete cell culture medium. Thus whole-water samples can be applied to fish cell cultures by being used to make up L-15/ex.

Finally, as the overall endpoint of the described procedure is basal cytotoxicity, any fish cell line might be suitable. However, the recommended cell line, RTgill-W1 has advantages besides being derived from an appropriate species, which is discussed in Section 5. RTgill-W1 cells remain attached firmly to microwell plates under a variety of culture conditions and after repetitive rinsing of the cultures and changes in solutions, which are necessary to perform the assays. RTgill-W1 is available from the American Type Culture Collection (ATCC CRL-2523), which assures quality of the line and continuity of the supply. Additionally, the cell line was derived from the gill, which is often the organ that fails during acute fish toxicity tests. However, the extent to which RTgill-W1 expresses gill epithelium properties is unknown, but future studies might identify them, which would allow the development of assays that monitor differentiated or tissue-specific functional endpoints.

4.2 ADVANTAGES OF WHOLE-WATER SAMPLES

Testing whole-water samples on cells offers several important advantages. Applying the whole sample to cultures assures that little or no toxicant is lost in any processing steps. As well, the total toxicity of the sample, encompassing all potential synergistic, antagonistic and additive interactions, is measured. The cost and time of testing is reduced because labor-intensive extraction procedures with expensive organic solvents are eliminated. As mentioned previously, the cost of shipping large

volumes of effluent samples from distant sources to testing facilities is reduced because relatively small volumes are needed for testing. Finally, whole-water samples are more analogous to the protocol used to test the toxicity of water samples to fish.

Preparation of whole-water samples in L-15/ex has several advantages over the use of complete culture medium for applying whole-water samples to cells in culture. The simplicity of this medium, which contains only salts, pyruvate and galactose, favors expression of cellular responses to any toxicants that might be present because protective molecules, such as antioxidants, are absent. The medium is much less expensive than complete cell culture medium. As well, the amount of L-15/ex components can be varied easily to account for any big differences in the osmolality of whole-water samples. Finally, the simplicity of the medium reduces the growth of any microbial contamination during the 24 h of presentation of the whole-water sample to cells in culture.

4.3 ADVANTAGES OF MULTIPLE FLUORESCENT ASSAYS FOR CELL VIABILITY

Although numerous assays of cell viability have been developed, those that focus on the integrity of the plasma membrane and metabolism and utilize fluorescent dyes to indicate impairment in these cellular parameters are perhaps best. The tests can be performed on cultures after relatively brief exposure to putative toxicants. In the procedure of this chapter, the exposure is kept short (24 h) to reduce overgrowth by any microbes in the whole-water sample and to provide information about the status of the water sample as quickly as possible. One potential drawback is that toxicants inducing a particular cellular process, such as the xenobiotic metabolism, or by causing cumulative damage might be missed. More and more fluorescent dyes are becoming available commercially to evaluate different cellular parameters, including the integrity of metabolism and the plasma membrane. As well, the development of fluorometric multiwell plate readers has made the use of fluorometric dyes easy and rapid. 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 interlab reproducibility and can be coupled to computers to rapidly and easily manage data, which can allow for multiple assays. Such assays with slightly different cellular endpoints can be more sensitive than a single test and also reduce the chance of recording a false negative. Multiple cellular endpoints also have the potential of revealing the mechanisms behind the cytotoxicity of a water sample. In the procedures described in this chapter three assays are used: membrane integrity is monitored with 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM); energy metabolism, with alamar Blue (AB) or resazurin; and lysosomal activity, with neutral red (NR).

4.3.1 Plasma membrane integrity and CFDA-AM

The integrity of the plasma membrane in cultures of fish cell lines has been assayed in a variety of ways, but most assays can be considered to be one of two types (Bols et al., 2005). Methods that measure the ability of the plasma membrane to exclude large bulky, charged molecules, such as dyes, constitute one type. The classic dye

exclusion technique is trypan blue, which has been applied to fish cells, but can often be tricky and tedious to use because the results must be scored under the light microscope. The alternative to dye exclusion is the capacity of the plasma membrane to retain a marker molecule. The marker can be the appearance in the medium of an intracellular molecule, such as an intracellular enzyme like lactate dehydrogenase (LDH), which can be complicated by several factors (Putnam et al., 2002).

In this chapter an esterase substrate (5-carboxyfluorescein diacetate acetoxymethyl ester, CFDA-AM) is used to measure cell membrane integrity, with the fluorescent product being the marker retained (Schirmer et al., 1997; 1998a; 1998b; 2000). CFDA-AM diffuses into cells rapidly and is converted by non-specific esterases of living cells from a nonpolar, nonfluorescent dye into a polar, fluorescent dye, 5-carboxyfluorescein (CF), which diffuses out of cells slowly. Although the CFDA-AM assay appears to monitor impairment to plasma membranes, the test as described in this chapter could result in more complex explanations. When the CFDA-AM is applied to fish cells in microwell plates after having been exposed to the test solution and read sometime later without removing the dye, the fluorescent readings or units (FU) constitute the CF both inside and outside the cells. In this case a decrease in FU with CFDA-AM actually measures a decline in the total esterase activity within a microwell cell culture (Ganassin et al., 2000; Dayeh et al., 2003b).

The decrease in esterase activity with toxicant treatment could be achieved in two general ways: interference with plasma membrane integrity or with cellular esterase activity. A loss of plasma membrane integrity would decrease 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 in 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 left 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. These potential complexities can be overcome by carrying out the other viability assays.

4.3.2 Metabolic impairment and alamar Blue (AB)

Although metabolism by fish cell cultures has been monitored by measuring their ATP content or their ability to reduce either 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (Segner, 1998) or resazurin; the reduction of resazurin has some convenient features. Resazurin can be purchased as a commercial solution called alamar Blue (AB). AB reduction can be measured either spectrophotometrically or fluorometrically. Recovery from metabolic impairment can be evaluated by repeatedly applying the dye to the same culture over a period of days (Ganassin et al., 2000). Originally, AB was thought to be reduced by mitochondrial enzymes (De Fries and Mistuhashi, 1995), but now enzymes, such as diaphorases, with both cytoplasmic and mitochondrial locations, are thought to be responsible for

dye reduction (O'Brien et al., 2000). Thus a decline in AB reduction indicates an impairment of cellular metabolism rather than specific mitochondrial dysfunction.

4.3.3 Lysosomal activity and neutral red (NR)

Neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) measures plasma membrane integrity after exposure to putative toxicants (Babich and Borenfreund, 1991; Segner, 1998), but as well, NR can detect injury specific to lysosomes. The general principle behind the use of this dye is that only viable cells accumulate NR into lysosomes (Borenfreund and Puerner, 1984). In the procedure of this chapter, NR is applied after the exposure to water samples, so the endpoint is the lysosomal accumulation of NR rather than NR retention. NR can be measured either spectrophotometrically (Borenfreund and Puerner, 1984) or fluorometrically (Essig-Marcello and van Buskirk, 1990). Although accumulating specifically in lysosomes, NR accrual and retention is dependent on an intact plasma membrane, adequate energy metabolism, and a functioning lysosome. Under most circumstances, the NR assay likely detects impairment to all three cellular parameters and the results are commonly similar to the results with other viability assays. However, hints of specific lysosomal damage have been seen. For example with the RTgill-W1 cell line, Schirmer et al. (1998b) found that immediately after UV irradiation in the presence of either acenaphthylene, acenaphthene or phenanthrene, photocytotoxicity was detected with NR but not with other indicator dyes, which suggests that lysosomes were being impaired before cell viability was lost.

5. Test species

The recommended test subject is a continuous epithelial cell line, RTgill-W1, from the gill of rainbow trout (Bols et al., 1994). Rainbow trout or *Oncorhynchus mykiss*, formerly *Salmo gairdneri*, is widely available and easily maintained. This has led to the species being used intensively in toxicology, and in some instances, to being referred to as the piscine 'white rat' (Wolf and Rumsey, 1985). As a result, more is likely known about the toxicology of rainbow trout than any other aquatic vertebrate, and rainbow trout have become incorporated into standardized toxicology tests, such as the 96 h acute lethality test (Environment Canada, 1990). In Canada, legislation requires that effluent from pulp and paper mills be assessed routinely by the 96 h rainbow trout lethality test (Environment Canada, 1989). Thus for the cell culture approach, rainbow trout is an excellent species to obtain cells from and to use in toxicity tests because the *in vitro* results can be compared to the enormous amount of *in vivo* data. In addition, as an *in vitro* alternative to rainbow trout in routine toxicity testing, cells from the same species would intuitively appear to be more suitable than cells from other species. The advantages of RTgill-W1 over other types of cell cultures and lines have been described in the previous sections.

6. Culture/maintenance of fish cell lines in the laboratory

6.1 LAB FACILITIES REQUIRED

Like any other tissue culture facility, the fish cell culture lab should also emphasize the need to maintain sterile conditions (Freshney, 2000). The ideal tissue culture facility should have an area for preparing primary cultures separate from the maintenance and experimental areas to prevent contamination. If this is not feasible a minimum of two laminar flow hoods located as far away as possible from each other is desirable (and never facing each other). One flow hood can be designated for work involving preparation of primary cultures and the other for routine maintenance and testing. Ideally, the latter should be a level 2 biosafety cabinet as opposed to the primary hood which can be a level 1 cabinet.

In addition to the working hoods, the facility must have a sink located near the entrance of the room for washing, and a low bench ideally in the middle of the room where an inverted phase contrast microscope can be placed. A centrifuge, fridge, freezer and aspirator are also needed. An incubator is desirable but not needed as most fish cells grow well at room temperature (Bols and Lee, 1991; 1994).

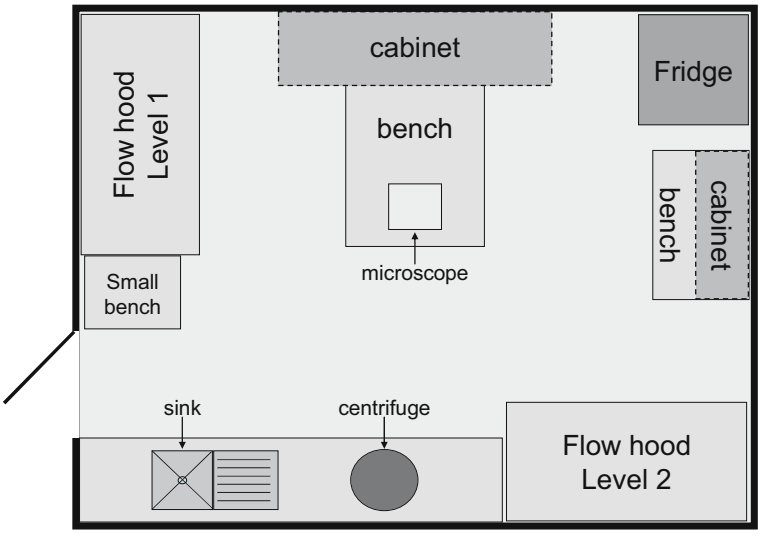

Figure 1. Minimal requirements for a small self-contained fish tissue culture lab facility.

Glassware washing and autoclaving facilities should be located nearby. Figure 1 depicts a small self-contained fish tissue culture lab. The laboratory should have restricted access and have the basic requirements to be designated Containment Level 2 as indicated in the Laboratory Biosafety Guidelines (Health Canada, 1996). These are the optimal laboratory requirements and are the same as for mammalian cell lines, but fish cell lines are easier to maintain than mammalian lines because the

lower temperature requirements means incubators are not absolutely needed and microbial contamination is less frequent.

6.2 MATERIALS

Box 2. Required materials for cytotoxicity testing with RTgill-W1.

- RTgill-W1 cell culture (CRL-2523, ATCC) in a 75 cm^2 tissue culture flask.
- Leibovitz's L-15 complete medium (with FBS – see Section 6.5.1 for recipe).
- 0.53 mM Versene (EDTA; Life Technologies) diluted 1:5000 (1 x 0.2 g tetrasodium EDTA/L in PBS).
- Trypsin solution (see Section 6.5.1 for recipe).
- 75 cm^2 tissue culture flask.
- 15 mL centrifuge tubes.
- 9" Pasteur pipettes to be stored in autoclavable pipette boxes.
- 10 mL transfer pipettes (glass or sterile disposable – graduated) to be stored in autoclavable pipette boxes.
- Digital micropipette and glass capillary tubes for toxicant dispensing.
- 70% ethanol solution.
- Microwell plates (sterile, disposable): either 48- or 96-well format.

6.3 EQUIPMENT

Box 3. Equipment required to culture fish cells in the laboratory.

- Laminar flow hood, either horizontal or vertical
- Inverted phase-contrast microscope
- Vacuum aspirator
- Incubator
- Centrifuge
- Pipettor
- Hemacytometer
- Fridge

6.4 WASHING OF GLASSWARE

Glassware for cell culture must be washed in a mild laboratory detergent and should be scrubbed thoroughly and rinsed 5–7 times with hot tap water and then rinsed 3–5 times in deionized water. The glassware is then left to air dry and is sterilized with an

autoclave for 30 minutes at a temperature of 121ºC and pressure of 20 PSI. The autoclaved glassware is then further dried for an additional 3 hours at 90ºC to remove all condensation within the glassware.

Glassware that contains toxicants and is to be reused must be washed after soaking the glassware in an acid detergent for 24 hours. This is then followed by rinses as done for cell culture glassware with autoclaving and drying as described above.

6.5 PREPARATION OF REAGENTS AND CELL CULTURE MEDIA

6.5.1 Culture maintenance

Leibovitz's L-15 complete medium containing FBS. RTgill-W1 cells are grown on the plastic surfaces of flasks and microwells in the basal medium, Leibovitz's L-15, supplemented with fetal bovine serum (FBS). This is prepared by adding aseptically 50 mL of FBS (Sigma) to a 500 mL bottle of L-15 (Sigma), which gives a solution that is commonly described as being 10% FBS. The growth medium is completed by adding 10 mL of penicillin/streptomycin (100 IU/mL penicillin, 100 µg/mL streptomycin; Sigma) to this solution and can be stored at 4ºC for months.

Versene solution. A rinse with versene (0.53 mM ethylenediaminetetraacetic acid or EDTA) is used to begin the process of removing RTgill-W1 cells from the plastic growth surface, which is necessary to initiate new cultures either in flasks or microwells. Versene can be purchased as a ready to use solution (Life Technologies). Rinsing with versene chelates and removes divalent cations, allowing trypsin to function.

Trypsin solution. Trypsin detaches the RTgill-W1 cells from the growth surface. The trypsin solution is prepared by aseptically dissolving 100 mg of trypsin (Sigma) into 10 mL of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Sigma) to make up a trypsin stock solution. Once dissolved, dispense 0.5 mL of the stock solution into 9.5 mL of Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution. Keep the trypsin solution sterile, store at –20ºC for up to 1 year.

7. Preparation of RTgill-W1 for toxicity testing

7.1 ROUTINE CELL CULTURING

Since the fish cell lines are immortal they can be continuously cultured so that there is an endless number of cells/flasks that can be used to continue the stock culture as well as to initiate the toxicity tests.

(1) Switch on the laminar flow hood (either horizontal or vertical). All surfaces must be sprayed with a 70% ethanol solution and wiped clean. Make sure that the laminar flow hood is functioning properly with suitable air flow to ensure maximal sterility. Place all needed

equipment in the laminar flow hood and wipe each item with a 70% ethanol soaked paper towel.

(2) Under an inverted phase-contrast microscope examine the confluent flask of RTgill-W1 cells. Check that the flask is free of contamination or unexpected rounding and detachment of cells. The cells that are to be passaged should have a normal morphology and are a confluent culture (see Fig. 2).

(3) In the laminar flow hood, remove the cap of the culture flask and aspirate the old medium using a Pasteur pipette attached to a vacuum aspirator. Dispense 1.5 mL of versene to the flask and swirl around gently to cover the entire bottom of the flask. Leave on for 1 minute and aspirate off.

(4) Add 1 mL of versene and 1 mL of the trypsin solution to the flask, replace the cap and swirl around gently to cover the bottom of the flask. Observe the cells detaching under the inverted phase-contrast microscope periodically tapping on the side of the flask to assist in detachment. Do not leave the cells in the trypsin solution for greater than 5 minutes as the enzymes may cause cellular digestion resulting in cellular death.

(5) Once the cells have detached add 3 mL of complete Leibovitz's L-15 medium containing FBS. Pipette the medium up and down over the bottom of the flask ensuring that all the cells are detached and resuspended in the medium.

(6) Transfer the cell suspension to a sterile 15 mL centrifuge tube and centrifuge for 5 minutes at 200 x *g*.

(7) After centrifugation, aspirate the supernatant from the 15 mL centrifuge tube leaving a small amount of media (~ 0.25 mL) above the pellet, being careful not to aspirate the cell pellet. Re-suspend the pellet into the remaining media by flicking the centrifuge tube.

(8) Add 10 mL of fresh L-15 medium to the centrifuge tube and dispense 5 mL to each of two 75 cm^2 tissue culture flasks and add a further 5 mL of medium to each flask.

(9) Observe the flasks under the phase-contrast microscope to check if the culture has been divided equally and that the cells are in a single cell suspension.

(10) Place the flasks in an incubator at 18° to 22°C. When the cultures are confluent (7 to 10 days) the cells can be subcultured or harvested for use in an experiment.

7.2 PREPARATION FOR TOXICITY TESTING

Cells from a confluent flask can be used to initiate cultures for a toxicity test using multiwell tissue-culture plates. Follow the first 7 steps of the routine cell culturing protocol up to re-suspension of the cells in the 15 mL centrifuge tube and continue with the following steps.

(1) To the resuspended cells in the centrifuge tube, add 4 mL of complete L-15 medium with FBS and ensure that the cells are evenly distributed throughout the tube. Count cells using a hemacytometer to determine the density of the cells. Adjust the cell density to 1 x 10^5 cells/mL if using 48-well plates and to 1.5 x 10^5 cells/mL if using 96-well plates using fresh medium.

(2) If using a 48-well plate, add 5 x 10^4 cells in 500 µL of L-15 complete medium with FBS to 40 of the 48 wells, add L-15 complete medium alone to the remaining eight wells. If using a 96-well plate, add 3 x 10^4 cells in 200 µL of L-15 complete medium with FBS to 84 of the 96 wells, add L-15 complete medium alone to the remaining twelve wells (see Fig. 4).

(3) Once plated, allow the cells to grow for three to four days in the dark at 18° to 22°C to form a confluent cell monolayer for 48-well plates and for two to three days for 96-well plates.

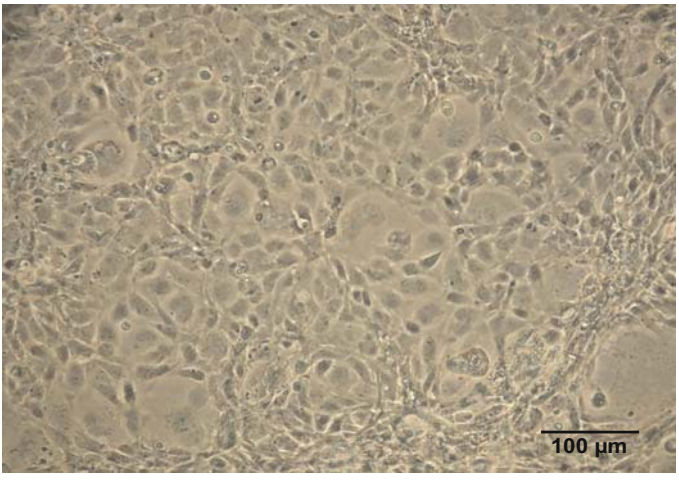

Figure 2. Confluent culture of RTgill-W1 under normal growth conditions viewed under phase contrast microscopy (100 X magnification).

8. Testing procedure

8.1 INFORMATION/GUIDANCE REGARDING TEST SAMPLES PRIOR TO CONDUCTING BIOASSAYS

8.1.1. Chemicals

All chemicals tested should be dissolved in a carrier suitable for the characteristics for that particular chemical. Dissolving chemicals in water or culture medium is ideal; however the use of ethanol or DMSO as a carrier for compounds that have low water-solubility may be necessary. When using an organic solvent, the working solutions must be at least 200 times the final concentration desired for exposure. Care must be taken when dosing cells with chemicals dissolved in DMSO/ethanol to prevent damage to the cells by the carrier alone.

8.1.2 Whole-water samples

Storage and preparation of whole-water sample. Upon receipt from the sample source, the sample should be kept at 4°C in the dark. The sample should be tested as soon as possible upon receipt due to possible degradation of potential toxicants.

The osmolality of raw whole-water samples is too low to support viability of the RTgill-W1 cell cultures, and needs to be increased to the levels of culture media (~300 $mOsmkg^{-1}$). In order to raise the osmolality, the salts, galactose and pyruvate of L-15 medium are added to the sample (Figure 3; Dayeh et al., 2002). This minimal medium is known as L-15/ex (Schirmer et al., 1997). At least 100 mL of sample is required because the amount of solid L-15 constituents required for this volume is the smallest that can be weighed out conveniently and accurately. See Table 2 for the amounts needed for 250 mL. Osmolality can be measured in the laboratory with an osmometer. Some work on the principle of freezing point depression; others, vapor pressure. We have routinely used the Westcor 5001B vapor pressure osmometer (Westcor, Utah, USA). Measure the raw osmolality of the whole-water sample. If the osmolality is below 90 $mOsmkg^{-1}$, the sample receives the normal salt concentrations of the constituents of L-15/ex (Tab. 2). Samples that have an osmolality above 90 $mOsmkg^{-1}$ (up to a maximum of 120 $mOsmkg^{-1}$) receive 80% of the normal salt concentrations (Table 2).

Table 2. Measurements of L-15/ex salt constituents to add to 250 mL of raw whole-water sample.

L-15/ex salt constituents	***For samples below 90 $mOsmkg^{-1}$ (in grams)***	***For samples above 90 $mOsmkg^{-1}$ (max. of 120 $mOsmkg^{-1}$) (in grams)***
NaCl	2.0	1.6
KCl	0.1	0.08
$MgSO_4$	0.05	0.04
$MgCl_2$	0.05	0.04
$CaCl_2$	0.035	0.028
Na_2HPO_4	0.0475	0.038
KH_2PO_4	0.015	0.012
Galactose	0.225	0.18
Pyruvate	0.1375	0.11

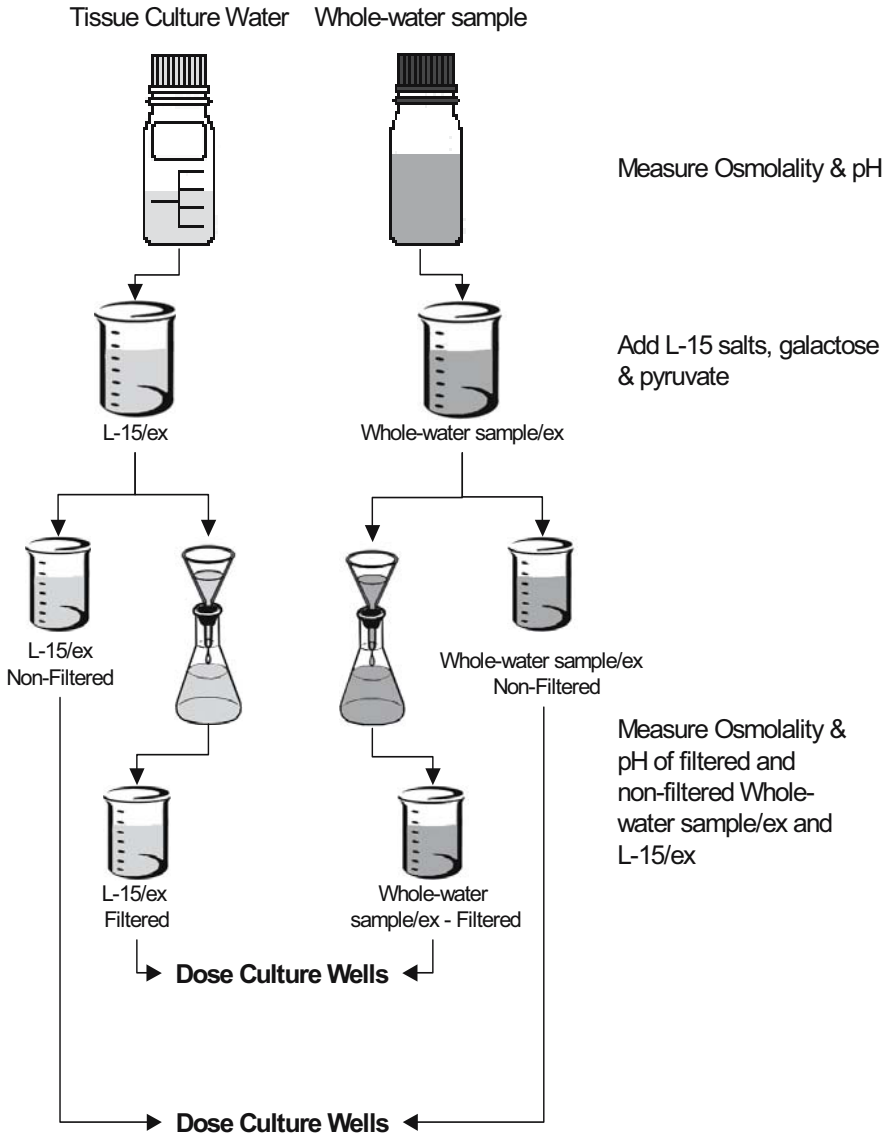

Figure 3. Scheme of L-15/ex and whole-water sample/ex preparation. Tissue culture salts are dissolved into commercial tissue culture water to give rise to L-15/ex and into whole-water samples to give rise to whole-water sample/ex. These solutions are used to dose confluent cultures of RTgill-W1 in wells of either 48-well or 96-well microplates (from Dayeh et al., 2002).

8.2 EXPERIMENTAL CONFIGURATION/DESIGN

The experimental configuration chosen for undertaking the RTgill-W1 cytotoxicity assay depends on the number of wells in the multiwell plate (see Fig. 4). Both 48- and 96-microwell plates can be used as these can be accepted by several multiwell plate readers. For both 48- or 96-well microplates a no-cell control is recommended which receives the various concentrations of sample in the absence of cells. This is done to observe if there are any interactions between the samples and the fluorescent indicator dyes used to determine the endpoint measurement of cell viability. The no-cell control also allows detection of some microbial contamination in whole-water samples. A positive control is also needed for each test configuration. The configuration described for 48-well plates allows eight concentrations of toxicant with four replicates including a control with four replicates. One row of the 48-well plate is dedicated to eight concentrations of a positive control toxicant including a control. The template for 96-well plates allows either twelve concentrations of toxicant including a control with six replicates each. Twelve concentrations of a positive control toxicant including a control are also included in this plate configuration. A 96-well plate can also be sub-divided into two 48-well configurations with eight concentrations of toxicant with four replicates including a control with four replicates. In this configuration two different samples can be tested on the same microwell plate. Two rows of the 96-well plate can be used for exposure to eight concentrations of a positive control toxicant.

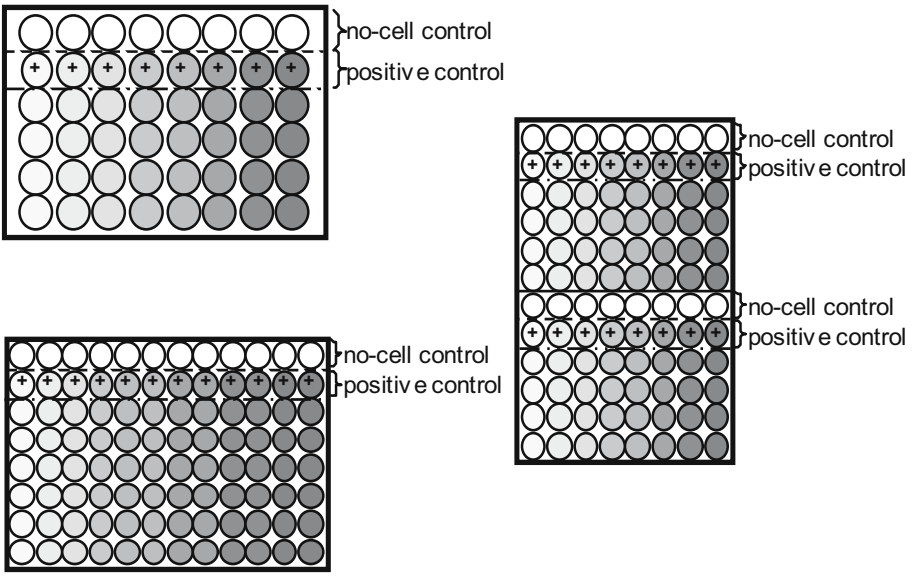

Figure 4. Suggested experimental configuration of 48-well and 96-well microplates for fish cell toxicity testing.

8.3 TEST SAMPLE CONCENTRATIONS

8.3.1 Chemicals

The optimal test concentration will vary depending on the chemical that is being investigated. When testing a new chemical whose toxicity is not known a good starting point is to test a broad concentration range. In order to determine the optimal concentration of the chemical to test conduct exposures on a log scale of concentrations (*i.e.,* 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100, etc.). Testing on this scale will narrow the range of exposure concentrations to be used for further experiments. Dissolving the chemicals in water or tissue culture solution is ideal and diluting in the wells is not necessary. However, when using an organic solvent, the working solutions must be at least 200 times the final concentration desired for exposure (*i.e*., 1 µL of the 200 times concentrated test chemical into 200 µL medium in the well). A concentrated stock solution is necessary only when the chemical to be tested is dissolved in an organic solvent or carrier, which may damage the cells if the concentration of the solvent in the well is too high.

Abietic acid dissolved in L-15/ex medium can be used as a positive control for the chemical tests. This will allow the comparison of cell response between plates/runs/days. Dissolve abietic acid (85% purity, Acros Organics through Fisher Scientific) into the L-15/ex medium at a concentration of 100 µg/mL. The concentration series for the positive control should be as follows: 0, 15, 30, 45, 60, 75, 90 and 100%.

*8.3.2 Whole-water sample*s

The highest concentration of a whole-water sample that can be tested is 100%. This is due to the addition of L-15/ex constituents as solids to the whole-water sample. It is recommended that a concentration series of increments of 15% be tested (*i.e.,* 0, 15, 30, 45, 60, 75, 90 and 100%) for the whole-water sample; however, other dilution series can be used such as 0, 5, 10, 30, 50, 70, 90 and 100%. These are prepared in sterile glass vials, with dilutions prepared using previously made L-15/ex. Cells that are not exposed to the whole-water sample will serve as the control cells (*i.e.,* 0%). Also a positive control should be used when exposing cells to a whole-water sample (Fig. 5; Dayeh et al., 2002). Dissolve abietic acid into the 100% whole-water sample at a concentration of 100 µg/mL. A concentration series for the positive control should follow that of the whole-water sample (*i.e.,* 0, 15, 30, 45, 60, 75, 90 and 100%).

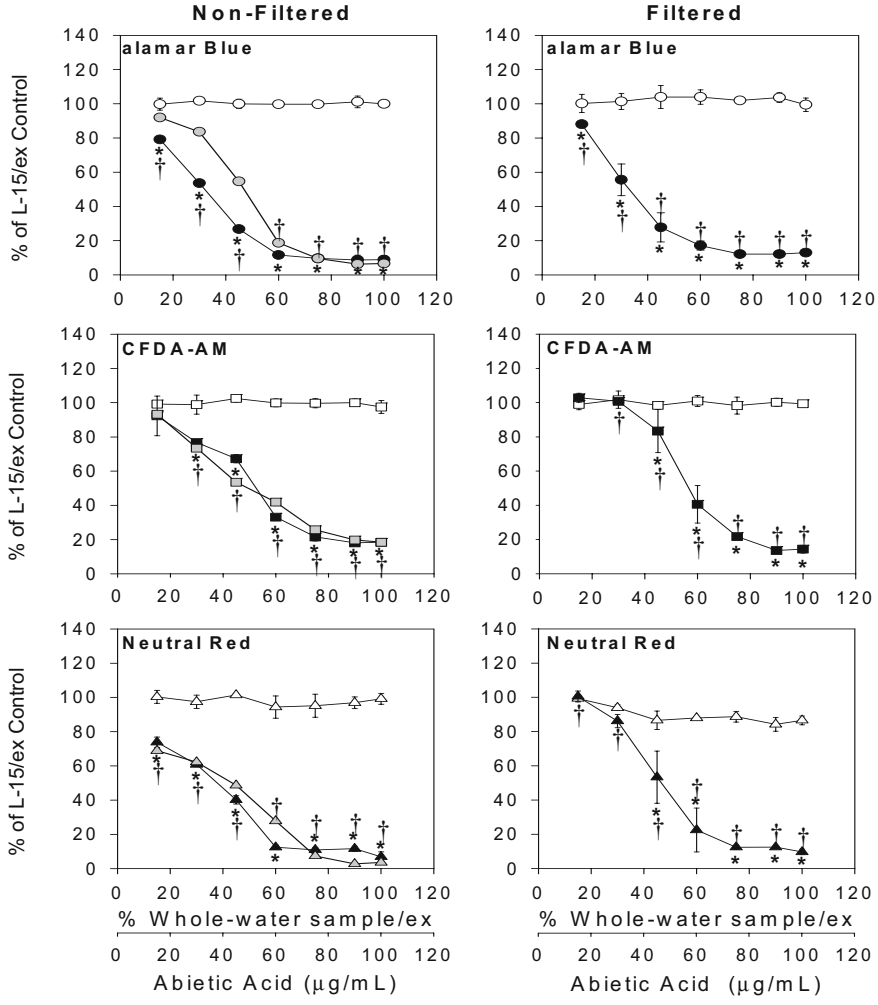

Figure 5. Viability of RTgill-W1 cultures after 24 h in abietic acid. A non-toxic whole-water sample was spiked with 100 µg/mL abietic acid in DMSO (closed symbols) or with DMSO alone (open symbols) and either filtered (right panels) or not (left panels). Abietic acid in L-15/ex was tested simultaneously (shaded symbols). Whole-water sample preparations were mixed in culture wells with various volumes of L-15/ex in order to obtain a dose-range of abietic acid-spiked whole-water sample/ex (closed symbols) or appropriate DMSO control (open symbols). Cell viability was assessed with three indicator dyes, alamar Blue (circles), CFDA-AM (squares) and neutral red (triangles). Asterisks denote the % of abietic acid-spike whole-water sample/ex that resulted in fluorescence units different than those in L-15/ex controls (one-way ANOVA followed by Dunnett's test, $\alpha = 0.05$). The † symbol indicates the % abietic acid spiked whole-water sample/ex that yielded fluorescence unit readings significantly different from DMSO-spiked control (unpaired t-test, $\alpha = 0.05$) (from Dayeh et al., 2002).

8.4 DISPENSING SAMPLE, RTgill-W1 AND EXPOSURE SOLUTIONS

8.4.1 Sample dispensing

The sample is dispensed in either a 48- or 96-microwell plates that have a confluent monolayer of RTgill-W1 cells. The cell will have been growing for approximately 3 days after plating to achieve confluency. These cultures of RTgill-W1 cells will be exposed to either dilutions of concentrated putative toxicant or the prepared whole-water sample.

8.4.2 Chemicals

(1) Turn on the vertical laminar flow hood and wipe all surfaces down with 70% ethanol solution. Place all needed equipment in the laminar flow hood and wipe each item with a 70% ethanol soaked paper towel.

(2) Remove the L-15 complete medium, which the cells have been plated in by inverting over a catch basin and blotting on a stack of paper towels.

(3) The remaining L-15 media must be removed with an L-15/ex rinse. To each well add 500 µL of L-15/ex to wells of a 48-well plate or 200 µL in a 96-well plate. Remove the L-15/ex rinse by inverting the plate over a catch basin and blotting on a stack of paper towels.

(4) The cells will be exposed to the chemicals in the L-15/ex medium. Thus, add 500 µL/well of L-15/ex to wells of a 48-well plate or 200 µL/well in a 96-well plate.

(5) Dose the cells with a 200 times concentrated working solutions of the desired test chemical when dissolved in an organic solvent. Using a positive displacement micropipette with a glass capillary tip, add 2.5 µL to wells of a 48-well plate or 1 µL to wells of a 96-well plate. When dosing, dispense the test compound above the level of the liquid in each well. The surface tension will ensure that the test chemical is dispersed evenly within the well and avoid any damage to the cells due to the organic solvent. Larger volumes for dosing can be used when the chemical is dissolved in either water, tissue culture medium or L-15/ex medium.

(6) Once all the wells have been dosed with the appropriate test compound and controls, wrap the edges of the plate with a thin strip of Parafilm M® to seal the edges and minimize evaporation.

(7) Expose the plates in the dark at 18° to 22°C for a period of 24 hours. Longer exposures up to 96 hours can also be conducted with this technique.

(8) Assess viability after the exposure using the alamar Blue/CFDA-AM and Neutral Red indicator assays as described below.

8.4.3 Whole-water samples

(1) Turn on the vertical laminar flow hood and wipe all surfaces down with 70% ethanol solution. Place all needed equipment in the laminar flow hood and wipe each item with a 70% ethanol soaked paper towel.

(2) Remove the L-15 complete medium, which the cells have been plated in by inverting over a catch basin and blotting on a stack of paper towels.

(3) The remaining L-15 media must be removed with an L-15/ex rinse. To each well add 500 μL of L-15/ex to wells of a 48-well plate or 200 μL in a 96-well plate. Remove the L-15/ex rinse by inverting the plate over a catch basin and blotting on a stack of paper towels.

(4) Using the dilution series of the prepared whole-water sample/ex, add 500 μL/well to wells of a 48-well plate or 200 μL/well in a 96-well plate. Add L-15/ex to each well that will serve as the control wells.

(5) Once all the wells have been dosed with the whole-water sample and controls, wrap the edges of the plate with a thin strip of Parafilm M® to seal the edges and minimize evaporation.

(6) Expose the plates in the dark at 18° to 22°C for a period of 24 hours. Longer exposures up to 96 hours can also be conducted with this technique.

(7) Assess viability after the exposure using the alamar Blue/CFDA-AM and Neutral Red indicator assays as described below.

9. Post-exposure observations/measurements and endpoint determinations

9.1 MICROSCOPIC OBSERVATIONS

Examine the cell cultures upon termination of the experiment. The cell cultures in the tissue culture plate can be observed using an inverted phase contrast microscope. Note the general appearance of the cultures across the various concentrations of toxicant tested taking note of any morphological changes.

9.2 MEASUREMENT ENDPOINT DETERMINATION

Viability of the cell cultures after exposure to a potential toxicant is measured using fluorescent indicator dyes. Due to the use of multiwell plates, fluorescence levels are determined using a fluorescent multiwell plate reader. There are a few manufacturers of fluorescent multiwell plate readers, which have either fixed excitation and emission filters (such as the CytoFluor, Applied Biosystems) or varying excitation and emission filters (such as the SpectraMax Gemini, Molecular Devices). These plate readers are designed to accept multiwell plates from various manufacturers. As well, the plates can be read either with or without a lid when read using multiwell

plate reader configured as a bottom reader (*i.e*., the CytoFluor 4000). However, if the multiwell plate reader is configured as a top reader (*i.e.,* the SpectraMax) the plate lid must be removed before reading the plate.

Three fluorescent indicator dyes are used to measure the viability of RTgill-W1 cultures after treatment to a toxicant. These are alamar Blue for metabolic activity, 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for membrane integrity, and neutral red for lysosomal function. These three dyes can be used with cells in one microwell plate, this allows for three endpoint determinations on the same set of cells.

9.2.1 Alamar Blue assay

Alamar Blue (Immunocorp) is a commercial preparation of the dye resazurin (O'Brien et al., 2000) and is used to assess metabolic activity in cell cultures. Resazurin is a non-fluorescent dye that once reduced by metabolically active cells becomes the fluorescent product resorufin. It comes in pre-mixed solutions of 25 mL and 100 mL volumes ready to be prepared as a working solution to be applied to cells (alamar Blue and CFDA-AM dyes can be mixed into one working solution as these two fluorescent dyes have different excitation and emission wavelengths).

(1) Turn on the laminar flow hood and wipe all surfaces with 70% ethanol solution. Keep the light off in the flow hood.

(2) Make a 5% (v/v) working solution of alamar Blue in L-15/ex. Keep in an amber glass vessel to prevent light degradation of the dye.

(3) Remove the exposure medium from the plates. This can be done by inverting the plate over a catch basin and blotting on a stack of paper towels to drain the plates further, or careful aspiration of each well using a Pasteur pipette with a vacuum aspirator. It is recommended to invert over a catch basin if the entire plate is to be assessed at one time.

(4) Add 100 to 150 µL of the 5% alamar Blue working solution to each well of a 48-well plate, or 50 to100 µL to each well of a 96-well plate. Volumes will depend on the type of fluorescent multiwell plate reader used. In general, the bottom of the culture well must be completely covered with the working solution.

(5) Incubate the plates in the dark for 30 min at 18° to 22°C.

(6) Place plate in plate carrier of multiwell plate reader. Assess fluorescence of alamar Blue using excitation and emission filters of 530 and 590 nm respectively. Depending on the plate reader, removal of the plate lid may be necessary.

9.2.2 CFDA-AM assay

The 5'-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM, Molecular Probes) is used to measure cell membrane integrity. CFDA-AM rapidly diffuses into cells and is converted from a non-polar, non-fluorescent dye into a polar, fluorescent dye 5'-carboxyfluoroscein (CF) by non-specific esterases present in living cells

(CFDA-AM and alamar Blue dyes can be mixed into one working solution as these two fluorescent dyes have different excitation and emission wavelengths).

(1) Turn on the laminar flow hood and wipe all surfaces with 70% ethanol solution. Keep the light off in the flow hood.

(2) Dissolve CFDA-AM in sterile DMSO to make a 4 mM stock solution. Dispense in small aliquots in sterile 0.5 mL microcentrifuge tubes to prevent degradation from thawing and refreezing. Wrap in aluminum foil to prevent light degradation. Store in a -20°C defrost cycle free freezer in a dessicator to prevent ester hydrolysis due to moisture for up to 1 year.

(3) Prepare a 4 μM working solution of CFDA-AM by diluting the 4 mM CFDA-AM stock solution 1:1000 in L-15/ex. Keep in a glass amber vessel to prevent light degradation of the dye.

(4) Remove the exposure medium from the plates. This can be done by inverting the plate over a catch basin and blotting on a paper towel to drain the plates further, or aspiration of each well using a Pasteur pipette with vacuum aspiration. It is recommended to invert over a catch basin if the entire plate is to be assessed at one time.

(5) Add 100 to 150 μL of the 4 μM working solution to each well of a 48-well plate, or 50 to 100 μL to each well of a 96-well plate. Volumes will depend on the type of fluorescent multiwell plate reader used. In general, the bottom of the culture well must be completely covered with the working solution.

(6) Incubate the plates in the dark for 30 min at 18° to 22°C.

(7) Place plate in plate carrier of multiwell plate reader. Assess fluorescence of CF using excitation and emission filters of 485 and 530 nm respectively. Depending on the plate reader, removal of the plate lid may be necessary.

9.2.3 Alamar Blue and CFDA-AM assay

As these two dyes have different excitation and emission wavelengths, they can be combined together to assess two endpoints of cell viability concurrently (Ganassin et al., 2000). To perform these two assays together, prepare a 5% (v/v) working solution of alamar Blue in L-15/ex and then dilute the CFDA-AM stock solution in DMSO (4 mM) 1:1000 in the prepared alamar Blue working solution. Add this working solution to the cells as described above.

9.2.4 Neutral red assay

Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a weakly basic fluorescent dye that is used to measure lysosomal function. Neutral red accumulates in acidic compartments such as lysosomes and can be applied before or after toxicant exposure (as described here) to measure neutral red release or uptake respectively. Note again that this assay can be done on a separate set of cells or on

the same cells that have previously been investigated using alamar Blue and CFDA-AM. However, inasmuch as cell cultures are terminated during the NR assay, this assay always has to be carried out last.

(1) Turn on the laminar flow hood and wipe all surfaces with 70% ethanol solution. Keep the light off in the flow hood.

(2) Dissolve 3.3 mg of neutral red powder (Sigma) per mL of Dulbecco's PBS (D-PBS; Sigma or Life Technologies) in a glass amber vial. Pass dissolved neutral red through a 0.2 μm filter. Store this stock solution for up to 1 year at 4°C. Neutral red can also be purchased as a 3.3 mg/mL stock solution in D-PBS (Sigma).

(3) Prepare a 33 μg/mL working solution of neutral red by diluting the stock solution 1:100 in L-15/ex. Keep in a glass amber vessel to prevent light degradation of the dye.

(4) Remove the exposure medium from the plates. This can be done by inverting the plate over a catch basin and blotting on a stack of paper towels to drain the plates further, or aspiration of each well using a Pasteur pipette with vacuum aspiration. It is recommended to invert over a catch basin if the entire plate is to be assessed at one time.

(5) Add 100 to 150 μL of the 33 μg/mL working solution to each well of a 48-well plate, or 50 to 100 μL to each well of a 96-well plate. The bottom of the culture well must be completely covered with the working solution.

(6) Incubate the plates in the dark for 60 min at 18° to 22°C.

(7) Invert the plate over a catch basin and blot on a stack of paper towels to remove the neutral red working solution. Ensure removal of excess neutral red in each well.

(8) Rinse wells once with 100 to 150 μL to each well of a 48-well plate, or 50 to100 μL to each well of a 96-well plate of the neutral red fixative solution: 0.5% (v/v) formaldehyde and 1% (w/v) $CaCl_2$ in deionized, distilled water; stored in the dark for up to 1 year. Remove neutral red fixative after 1 min by inverting the plate over a catch basin and blot on a stack of paper towels.

(9) Add 100 to 150 μL to each well of a 48-well plate, or 50 to100 μL to each well of a 96-well plate of the neutral red extraction solution: 1% (v/v) acetic acid and 50% (v/v) ethanol in deionized, distilled water; stored in the dark for up to 1 year. Volumes will depend on the type of fluorescent multiwell plate reader used. In general, the bottom of the culture well must be completely covered with the working solution.

(10) Place plate on an orbital shaker and shake at ~ 40 rpm for 10 min. This will ensure solubilization of the neutral red accumulated in the lysosomes.

(11) Place plate in plate carrier of multiwell plate reader. Assess fluorescence of neutral red using excitation and emission filters of 530 and 645 nm respectively. Depending on the plate reader, removal of the plate lid may be necessary.

9.2.5 Data analysis – calculation of EC50

Upon completion of the cell viability assays the raw fluorescent units 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 each concentration of toxicant, there is one well that has no cells in it (no-cell control) whereas all the remaining wells have cells in them. Prior to calculating % of control, subtract the fluorescent units (FU) for wells without cells from the experimental (ex.) and control (con.) values with cells. Cell viability (as % of control) can be calculated using the following formula:

$$\% \text{ of control} = (FU_{ex.cells} - FU_{ex.no\ cells}) \times 100 / (\text{Average } [FU_{con.} - FU_{con.no\ cells}]) \quad (1)$$

Data for each well of each concentration are expressed as a % of Control. Then, the average and standard deviation for each concentration is calculated. The data can then be plotted as % control on the y-axis versus concentration on the x-axis. These values are used to calculate the EC50 for the toxicant.

A sigmoid relationship is characteristic of dose-response data and thus can be analyzed by a nonlinear regression in most graphing software such as SigmaPlot (Jandel Scientific). The data is fitted to the four-parameter logistic function for continuous response data. The logistic function is:

$$y(d) = Y_{min} + (Y_{max} - Y_{min}) \{1 + \exp[-g(\ln(d) - \ln EC50)]\}^{-1} \quad (2)$$

where y(d) is the % cell viability at the dose d, Y_{min} is the minimum % cell viability, Ymax is the maximum % cell viability, g is a slope parameter, EC50 is the dose that produces 50 % of cell viability.

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

$$y(d) = 0\ \% + (100\ \% - 0\ \%) \{1 + \exp[-g(\ln(d) - \ln EC50)]\}^{-1}$$

10. Factors capable of influencing performance of rtgill-w1 test

10.1 EXPOSURE MEDIUM

The recommended exposure medium is L-15/ex. The RTgill-W1 cell line can survive in L-15/ex for at least 101 hours (Schirmer et al., 1997). Exposure in complete L-15 medium with or without a serum supplementation can reduce the toxicity due to

the reduced bioavailability of the chemical that is being evaluated (Hestermann et al., 2000; Schirmer et al., 1997; Dayeh et al., 2003a).

10.2 DOSING METHOD FOR CHEMICALS

As numerous chemicals need to be dissolved in solvents such as DMSO or ethanol, care must be taken when dosing RTgill-W1 cultures with these solutions. Presentation of these toxicants to the cells must be conducted in such a manner as to not damage the cells due to the carrier solvent alone. This can be accomplished by using micropipettes to add small volumes (≤10 μL) of the toxicant in carrier solvent to the medium over the cells in microwells. Dispense the droplet of the carrier solution from the micropipette above the level of the medium surface and touch this droplet to the surface. This allows the surface tension to disperse the carrier solvent rapidly and evenly throughout the culture well. Failing to do this near the surface can result in a blob of DMSO falling directly onto the cell monolayer and causing the immediate death of all or part of the monolayer.

10.3 WHOLE-WATER SAMPLES

Complex samples might limit the application of whole-water samples to RTgill-W1 cultures. Complexities could include excessive microbes, precipitates, suspended particulates, and colour. Most of these problems might be overcome by adding a filtration step, which could have the detrimental effect of removing toxicants. Another problem would be hyperosmotic samples, which would necessitate diluting the sample. As mentioned in Section 11 on case studies, the full range of problems that might arise from the whole-water approach has yet to be identified.

11. Application in a case study

Testing for cytotoxicity to RTgill-W1 cells can be used to compare the toxic potency of individual chemicals and to evaluate the toxicity of whole-water samples. The methods for these two purposes are very similar. The procedure for single compounds has been presented in a previous publication (Dayeh et al., 2003b), whereas the procedure for whole-water samples is detailed in this chapter. Presented below are the chemical classes that have been examined and a discussion of a case study with paper mill effluent.

11.1 CHEMICALS

RTgill-W1 cells have been used to evaluate the cytotoxicity of PAHs, phenolics, and the surfactants, abietic acid and Triton X-100 (Schirmer et al., 1998a; Dayeh et al., 2002; 2004). An advantage of performing cytotoxicity tests in L-15/ex is being able to also test compounds for their potential to be photocytotoxic without interference from medium components. The killing of cells by concurrent exposure to a chemical and ultraviolet light (UV) is photo-cytotoxicity, and L-15/ex contains no medium components that by themselves are photo-cytotoxic. RTgill-W1 cells have been used

to determine the photocytotoxicity of PAHs and creosote (Schirmer et al., 1998b; Schirmer et al., 1999). Six of sixteen PAHs were photo-cytotoxic at concentrations theoretically achievable in water (Schirmer et al., 1998b). In all these studies, toxicity has been evaluated using the alamar Blue, CFDA-AM and neutral red viability assays and calculating EC50s in order to compare the results.

11.2 WHOLE-WATER SAMPLES

The RTgill-W1 cell line bioassay has been used successfully to evaluate the toxicity of samples collected from a paper mill over a year of operation (Dayeh et al., 2002). In total, thirty-one whole-water samples were tested for their cytotoxicity to RTgill-W1 cells. Of these thirty-one samples, eleven were also tested by the conventional 96-h whole rainbow trout lethality bioassay, eighteen, by the *Daphnia* lethality bioassay. There was no correlation between the *Daphnia* and the RTgill-W1 test results. Eleven samples were toxic to *D. magna* but not to the gill cell line. Thus the Daphnia test has a greater sensitivity to something in the water samples, perhaps heavy metals, than RTgill-W1 test. Only one sample, number 28, was toxic to rainbow trout as evaluated by the 96-h lethality bioassay. This was the only sample of thirty-one that was cytotoxic to RTgill-W1 (Fig. 6). Thus the correlation between tests with rainbow trout and the rainbow trout cell line was excellent, suggesting that the fish cell line bioassay is a promising alternative to the use of whole fish in the routine toxicity testing of whole-water samples. However, this successful case study raises a number of issues and suggestions for future developments.

Firstly, the cytotoxicity of sample 28 was complex (Fig. 6, Dayeh et al., 2002). All three viability assays indicated that sample 28 was cytotoxic, but the results with neutral red had a high standard deviation and indicated more cytotoxicity than the other two assays. Surprisingly, when the sample was filtered, the neutral red assay no longer detected a decline in cell viability with an increasing % of whole-water sample/ex. On the other hand, alamar Blue detected more cytotoxicity in the filtered sample. This complexity is hard to explain, although some possible mechanisms were advanced in Dayeh et al. (2002). The results suggest two recommendations for future screens of industrial effluent. Firstly, more than one endpoint of cell viability should be tested. Secondly, both filtered and non-filtered sample should be tested. In this way, if a toxicant is removed by filtration, it should be detected with the non-filtered sample. Secondly, RTgill-W1 seems less sensitive than rainbow trout to the one toxic sample, number 28. All 10 rainbow trout died in the 96-h lethality test, whereas the reduction in RTgill-W1 cell viability was at the most only by about 55%. One possible explanation for this difference is that the toxicant(s) require more time than the 24-h of the *in vitro* tests to elicit their full toxicity. Another possibility is that the particular toxicant(s) in this sample are more potent at the organism level than the cellular level. Toxicants that target specific organ systems, such as the nervous system, might fit into this category.

Several avenues of research could be explored in the future to improve the sensitivity. One would be to expose RTgill-W1 cultures to samples for a longer period. However, as a routine practice, this is not desirable because microbial contamination is more likely to appear and overwhelm the fish cell cultures. Sensitivity might also be improved by using different or additional cellular endpoints

for evaluating cell viability. A long-term solution might be to genetically engineer RTgill-W1 to be more sensitive to cytotoxicants.

Sample preparation also could be the key to sensitivity, but this will likely vary with the nature of the sample. The amount of microbes in the water sample will dictate on how essential a filtration step is. In turn, how much of a potential toxicant is adsorbed to filterable particulates will determine how filtration interferes with sensitivity. A surprising feature of the paper mill study is that the only toxic sample was from the 'clean water bypass', which is the water that is used in cooling the plant and will ultimately receive effluent, although the mechanisms behind its cytotoxicity might be complex (Dayeh et al., 2002).

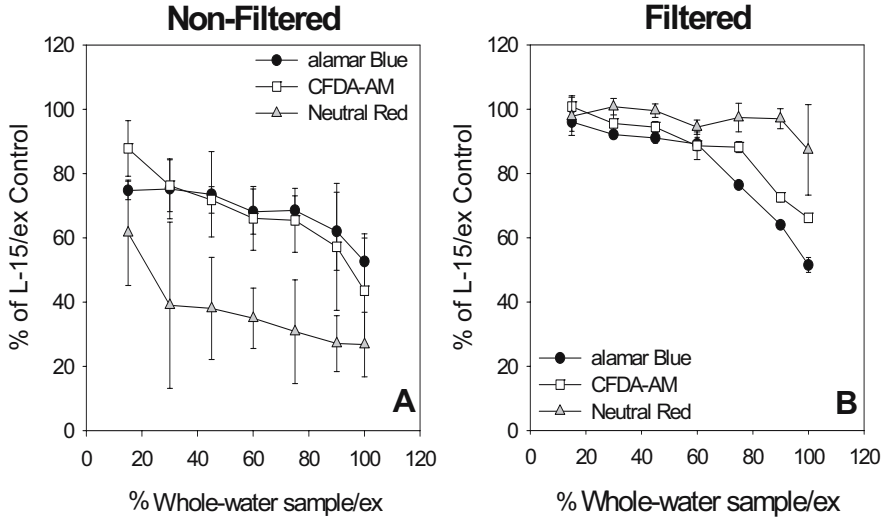

Figure 6. Viability of RTgill-W1 cultures after 24 h in whole-water sample 28 from a paper mill. The sample had been filtered through a 0.2 μm filter in Panel B but not in Panel A. Cell viability was assessed with three indicator dyes, alamar Blue, CFDA-AM and Neutral Red (from Dayeh et al., 2002).

12. Accessory/miscellaneous test information

Although a single cell viability assay might be considered as time and resources would be saved, multiple cell-viability assays are recommended because some endpoints might be less sensitive to certain toxicants than others. For example, when RTgill-W1 cells were exposed to pentachlorophenol, a dose-dependent decline in cell viability was observed with alamar Blue and neutral red, but not with CFDA-

AM (Dayeh et al., 2002). In this case pentachlorophenol seemed to impair energy metabolism more profoundly than plasma membrane integrity.

Some non-filtered samples increased alamar Blue readings as the percentage of whole-water sample increased (Dayeh et al., 2002). Although the magnitude was not large, the increase was statistically significant. These results occurred with samples that were not toxic to *Daphnia*, rainbow trout, or RTgill-W1. Why the values increased is a matter of speculation, but microbial contamination seems a likely source. Whether such increases could interfere with the detection of cytotoxicity with alamar Blue is unclear at this time.

13. Conclusions/prospects

Evaluating the toxicity of water samples by measuring their capacity to cause cytotoxicity in microwell cultures of the rainbow gill cell line, RTgill-W1, has several advantages and successes with some kinds of samples. The attractive features include cost. This method requires milliliters instead of tens of liters of effluent shipped from distant industries to central testing facilities. The time for the response of exposure to the effluent is only 24 hours as opposed to 96 hours, which in turn reflects the cost of labour. The approximate cost per sample is around $15 - 20 Canadian. Furthermore, routine cell culturing techniques done in house will keep an endless supply of cells to complete the tests instead of having to purchase rainbow trout. The use of fish cell cultures as an alternative to whole fish also satisfies the societal goal to reduce the use of animals in toxicity testing. To date, the procedure has been used successfully with paper mill samples (Dayeh et al., 2002). With these samples, the RTgill-W1 test would be a powerful tool in a program of toxicity identification evaluation (TIE).

Additional studies are needed in order to understand and validate the capability of the RTgill-W1 procedure. More samples that are toxic to rainbow trout need to be examined with the RTgill-W1 cells. In this way, enough examples could be obtained to allow a statistical test of the correlation between toxicity to rainbow trout with cytotoxicity to RTgill-W1. Different types of whole-water samples need to be examined with the RTgill-W1 procedure. Some kinds of effluents might be less successful because of the amount of particulate material or microbes or both and procedures to overcome these problems will have to be developed. As well, some effluents might need to be concentrated or extracted in order for cytotoxicity to be detected. Ultimately, with validation, the RTgill-W1 procedure could be combined with other microplate assays of this book to be part of a battery of tests to routinely appraise the quality of water samples.

Acknowledgements

The research was supported by the Natural Science and Research Council of Canada (NSERC), Centre for Research in Earth & Space Technologies (CRESTech), and the Canadian Water Network (CWN).

The authors wish to thank the copyright holder for permission to reproduce the following: Figures 3, 5 and 6, Elsevier Science, Amsterdam, Netherlands. Reproduced from: Dayeh, V.R., Schirmer, K. and Bols, N.C. (2002) Applying whole-water samples directly to fish cell cultures in order to evaluate the toxicity of industrial effluent. *Water Research* **36,** 3727-3728 (Figures 1, 4 and 6, unaltered).

References

Babich, H. and Borenfreund, E. (1991) Cytotoxicity and genotoxicity assays with cultured fish cells: a review, *Toxicology In vitro* **5,** 91-100.

Bols, N.C. and Lee, L.E.J. (1991) Technology and uses of cell cultures from the tissues and organs of bony fish, *Cytotechnology* **6,** 163-187.

Bols, N.C. and Lee, L.E.J. (1994) Cell lines: availability, propagation and isolation. in: P.W. Hochachka and T.P. Mommsen (eds.), *Biochemistry and Molecular Biology of Fishes,* Vol. 3. Elsevier Science, Amsterdam, Netherlands, pp. 145-149.

Bols, N.C., Barlian, A., Chirino-Trejo, M., Caldwell, S.J., Goegan, P. and Lee, L.E.J. (1994) Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills, *Journal of Fish Diseases* **17,** 601-611.

Bols, N.C., Dayeh, V.R., Lee, L.E.J. and Schirmer, K. (2005) Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology, in T.W. Moon and T.P Mommsen (eds.), *Biochemistry and Molecular Biology of Fishes*, Vol. 6, Elsevier Science, Amsterdam, Netherlands (in press).

Borenfreund, E. and Puerner, J.A. (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays, *Journal of Tissue Culture Methods* **9**, 7-12.

Castaño, A., Bols, N.C., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L.E.J., Mothersill, C., Part, P., Repetto, G., Sintes, J.R., Rufli, H., Smith, R., Wood, C. and Segner, H. (2003) The use of fish cells in ecotoxicology –The report and recommendations of ECVAM workshop 47, *Alternatives to Laboratory Animals* **31**, 317-351.

Dayeh, V.R., Schirmer, K. and Bols, N.C. (2002) Development and testing of methods for applying industrial effluent directly to fish cell cultures in order to evaluate effluent toxicity, *Water Research* **36**, 3727-3738.

Dayeh, V.R., Lynn, D.H. and Bols, N.C. (2003a) Assessment of metal toxicity to protozoa and rainbow trout cells in culture, in G. Spiers, P. Beckett and H. Conroy (eds.), *Sudbury 2003 Mining and the Environment Conference Programme,* Laurentian University Centre for Continuing education, Sudbury, ON, Canada, pp. 205-211.

Dayeh, V.R., Schirmer, K., Lee, L.E.J. and Bols, N.C. (2003b) The use of fish-derived cell lines for investigation of environmental contaminants, in *Current Protocols in Toxicology*, John Wiley & Sons, New York, NY, USA, pp. 1.5.1-1.5.17.

Dayeh, V.R., Chow, S.L., Schirmer, K., Lynn, D.H. and Bols, N.C. (2004) Evaluating the toxicity of Triton X-100 to Protozoan, Fish and Mammalian Cells using fluorescent dyes as indicators of cell viability, *Ecotoxicology and Environmental Safety* **57**, 375-382.

de Fries, R. and Mitsuhashi, M. (1995) Quantification of mitogen-induced human lymphocytes-proliferation - comparison of alamar Blue™ assay to 3H-thymidine incorporation assay, *Journal of Clinical Laboratory Analysis* **9**, 89-95.

Environment Canada (1989) The development document for the effluent monitoring regulation for the pulp and paper sector, Queen's Printer for Ontario, Environment Canada, pp. 1-55.

Environment Canada (1990) Biological test method: acute lethality test using rainbow trout, Environment Protection Series, EPS 1/RM/9, pp. 1-51.

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 Toxicology* **3**, 219-227.

Fent, K. (2001) Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P450, An induction potential and estrogenic activity of chemicals and environmental samples, *Toxicology In vitro* **15**, 477-488.

Freshney, R.I. (2000) Culture of Animal Cells: A Manual of Basic Technique, 4th ed. John Wiley & Sons, New York, NY, USA, pp. 1-577.

Ganassin, R.C., Schirmer, K. and Bols, N.C. (2000) Methods for the use of fish cell and tissue cultures as model systems in basic and toxicology research, in G.K. Ostrander (ed.), *The Laboratory Fish,* Academic Press, San Diego, CA, USA, Chapter 38, pp. 631-651.

Health Canada (1996) The Laboratory Biosafety Guidelines, available from http://www.hc-sc.gc.ca/pphb-dgspsp/publicat/lbg-ldmbl-96/index.html

Hestermann, E.V., Stegeman, J.J. and Hahn, M.E. (2000) Serum alters the uptake and relative potencies of halogenated aromatic hydrocarbons in cell culture bioassays, *Toxicological Sciences* **53**, 316-25.

Mochida, K. (1986) Aquatic toxicity evaluated using human and monkey cell culture assays, *Bulletin of Environmental Contamination and Toxicology* **36**, 523-526.

O'Brien, P.J., Wilson, I., Orton, T. and Pognan, F. (2000) Investigation of the alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity, *European Journal of Biochemistry* **267**, 5421-5426.

Putnam, K.P., Bombick, D.W. and Doolittle, D.J. (2002) Evaluation of eight *in vitro* assays for assessing the cytotoxicity of cigarette smoke condensate, *Toxicology In vitro* **16**, 599-607.

Richardson, D., Dorris, T.C., Burks, S., Browne, R.H., Higgins, M.L. and Leach, F.R. (1977) Evaluation of a cell culture assay for determination of water quality of oil-refinery effluents, *Bulletin of Environmental Contamination and Toxicology* **18**, 683-690.

Schaeffer, W.I. (1990) Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics, *In vitro Cellular and Developmental Biology* **26**, 97-101.

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, *Toxicology In vitro* **11**, 107-119.

Schirmer, K., Greenberg, B.M., 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., Herbrick, K., Greenberg, B.M., Dixon, D.G. and Bols, N.C. (1999) Use of fish gill cells in culture to evaluate the cytotoxicity and photocytotoxicity of intact and photomodified creosote, *Environmental Toxicology and Chemistry* **18**, 1277-1288.

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, *Journal of Biochemical and Molecular Toxicology* **14**, 262-276.

Schirmer, K., Tom, D.J., Bols, N.C. and Sherry, J.P. (2001) Ability of fractionated petroleum refinery effluent to elicit cyto- and photocytotoxic responses and to induce 7-ethoxyresorufin-o-deethylase activity in fish cell lines, *The Science of the Total Environment* **271**, 61-78.

Segner H. (1998) Fish cell lines as a tool in aquatic toxicology, in T. Braunbeck, D.E., Hinton and B. Streit (eds.), *Birkhäuser, Basel, Ecotoxicology*, pp. 1-38.

Wolf, K. and Rumsey, G. (1985) The representative research animal: Why rainbow tout (*Salmo gairdneri* Rich.)? *Zeitschrift fur angewandte Ichthyologie* **3**, 131-138.

Abbreviations

AB	alamar Blue
ATP	Adenosine tri-phosphate
CF	5-carboxyfluorescein
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
con.	control
D-PBS	Dulbecco's Phosphate-Buffered Saline
DMSO	Dimethyl sulfoxide
EC50	Concentration that causes an effect in 50% of the cells
ex.	experimental
FBS	Fetal Bovine Serum

FU	Fluorescent Units
IU	International Units
L-15	Leibovitz's L-15 medium
L-15/ex	L-15 exposure medium
LDH	lactate dehydrogenase
$mOsmkg^{-1}$	milli-osmole per kilogram
MTT	either 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
NR	Neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride)
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate buffer saline
PSI	Pounds per Square Inch.