Assessment of Metal Toxicity to Protozoa and Rainbow Trout Cells in Culture

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Abstract

Methodology has been developed for the use of fish cell lines as alternatives to the use of whole fish in the toxicity testing of industrial effluents. Relative to conventional fish tests, this should reduce the number of fish consumed in testing and save money by cutting the volume of effluent that must be shipped from distant sources to central testing facilities. The effectiveness of this methodology was illustrated recently by screening effluent samples from a paper mill. Potentially, the same approach could be applied to the mining sector. As a result, the toxicity of various metals [Cu, Zn, Cd, Fe and Ni] to fish cell lines was studied to determine the effectiveness of these cells as a screening tool for mining effluent. The cell lines were RTL-W1 from the liver and RTgill-W1 from the gill of rainbow trout. Cellular viability was measured using three fluorometric assays. These were alamar Blue™ (AB) for metabolic activity, CFDA-AM for membrane integrity and neutral red (NR) for lysosomal activity. The toxicity of the metals was affected by exposure medium. Little or no toxicity occurred when the cells were exposed to metals in the basal medium, L-15, or in L-15 with fetal bovine serum (FBS). However, toxicity was observed for all metals except Ni when exposures were done in the minimal medium L-15/ex, which contains only physiological salts, galactose and sodium pyruvate. In L-15/ex, the rank order of toxicity from least to most toxic was Ni <Fe < Cd < Zn < Cu. Research was also begun on using the free-living protozoan, *Tetrahymena thermophila* as another potential test cell to screen for metal toxicity. The results suggest that with a little further research, inexpensive and sensitive cell tests can be developed to evaluate the toxicity of environmental samples from the mining sector.

Introduction

The effluent from several industries is evaluated routinely for toxicity. In Canada, government legislation mandates that manufacturers of pulp and paper follow a regular schedule of testing their effluent for toxicity (Environment Canada, 1989). Recently, the mining sector has begun environmental effects monitoring (EEM) (Environment Canada, 2002).

One of the toxicity checks required by law is the 96 h rainbow trout lethality test (LC_{50}) (Environment Canada, 1990). In one form of this assay, fish are placed in 100 % effluent. If five or more fish are alive at the end of 96 h, the effluent passes. Despite its commendable simplicity, the assay has drawbacks. The volume of effluent needed for a test is large and the cost of shipping large volumes to a central testing facility from remote sites can be considerable. The seasonable availability and expense of fish also can be problematic. Finally, segments of society find the use of animals in toxicity testing unacceptable. For all these reasons, alternative toxicity evaluations are being sought. Testing on animal cell cultures is one alternative (Ahne, 1985) and has the advantage of requiring small amounts of effluent and being quick and inexpensive.

Recently, we have had success using cultures of fish cells to test the toxicity of paper mill effluent (Dayeh *et al.*, 2002a). The procedure began by adding as solids the salts and energy sources for the basal growth medium, L-15, to effluent samples. This modified effluent sample was then applied to microwell cultures of the rainbow trout gill cell line, RTgill-W1. The viability of the cultures was monitored 24 h later, using three fluorescent indicator dyes, alamar Blue, CFDA-AM and neutral red. These dyes monitor respectively metabolic activity, plasma membrane integrity, and lysosomal function. The data were quantified by reading the multiwell cultures in a fluorescent plate reader. When effluent from a paper mill was evaluated over approximately a

year of operation, results from tests with rainbow trout and RTgill-W1 showed a good congruence.

In order to see if this approach could be developed for effluent from the mining sector, we have begun to explore the sensitivity of rainbow trout cell lines and the ciliated protozoan *Tetrahymena thermophila* to metals. The protozoan is being investigated because as well as having the advantages of animal cells, this unicellular eukaryote could have a unique convenience. The sample could be applied directly to *T. thermophila,* without the trouble of getting the effluent in a form suitable for presentation to animal cells. Recently, we showed that fluorescent viability assays could be applied to *T. thermophila* (Dayeh *et al.*, 2002c). This should make evaluating a detrimental impact on this organism more rapid than the use of growth as an endpoint, which has been done commonly in past toxicology studies with *T. thermophila* (Larsen *et al.*, 1997; Schultz, 1997).

Materials and Methods

Fish cell cultures

The cell lines employed in this study were the rainbow trout (*Oncorhynchus mykiss*) liver cell line, RTL-W1 and the gill cell line, RTgill-W1 (ATCC # CRL 2523). Cells were routinely cultured in 75 cm^2 culture flasks at 20ºC in Leibovitz's L-15 culture medium (Sigma-Aldrich Ltd., Oakville, ON, Canada), with a 5% and 10% fetal bovine serum (FBS; Sigma) supplement for the RTL-W1 and RTgill-W1 cell lines respectively and 2% penicillin streptomycin (100 µg/mL streptomycin, 100 IU/mL penicillin; Sigma). Culture supplies and subcultivation procedures were as previously described (Bols and Lee, 1994; Schirmer *et al.*, 1994).

Stock solutions of $CuCl_2$, $CuSO_4$, $CdCl_2$, $ZnCl_2$, $ZnSO_4$, $FeSO_4$, and $NiCl_2$ (Sigma) were prepared in tissue culture grade water (Sigma). Cells were added to 96-well tissue culture plates (Falcon, Becton Dickinson, Franklin Lakes, NY, USA) at a density of 5 X 10^4 cells per 200 µL L-15 with FBS and grown to confluency at which time the growth medium was replaced with 200 µL of exposure medium. The exposure medium was either L-15 with FBS, L-15 or L-15/ex, which contains only the physiological salts, galactose and sodium pyruvate of L-15. A 1 µL aliquot of metal stock solution was added to 200 µL of exposure medium in each well of the 96 well plates. After 24 h, the medium was removed and the cultures assayed for viability as described below.

Protozoan Cultures

The ciliate *Tetrahymena thermophila* (Oligohymenophorea; Tetrahymenidae) was cultured axenically in 10 mL of proteose peptone yeast extract medium (PPYE) (Gilron *et al.* 1999). A modification of the protocol by Gilron *et al.* (1999) was used to prepare cultures for testing. Ten mL of a 24-hour-old culture were transferred to 50 mL of sterile PPYE in a 250 mL Erlenmeyer flask and grown on an orbital shaker (50 rpm) for 1-2 days at room temperature. The culture was then harvested by centrifugation (1,200 rpm for 5 min) and washed 3 times with spring water (Aberfoyle Springs, Aberfoyle, Ontario, Canada). After the final centrifugation cells were resuspended in 10 mL of spring water. The cells were counted using a Coulter Z2 particle counter and adjusted to a cell density of 500,000 cell/ml (± 10%) with spring water.

One mL of *T. thermophila* at a density of 500,000 cells/mL in spring water was added to each microcentrifuge tube (1.5 mL size). After centrifugation at 1,200 rpm for 5 min, the spring water was removed by aspiration and the cells resuspended in the microcentrifuge tube in 1 mL of different concentrations of $CuSO_4$ in spring water. After 24 h, the cells were spun down and assayed for viability as described below.

Fluorometric indicator dyes

Three dyes were used to monitor cell viability. These were Alamar Blue (AB) (Medicorp. Science Inc., Montreal, PQ, Canada); 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) (Molecular Probes, Eugene, OR, USA); and neutral red (NR) (3-amino-7-dimethylamino-2-methylphenanzine hydrochloride; Sigma). This laboratory has published step-by-step protocols on how to

perform cell viability assays on animal cell lines with AB and CFDA-AM (Ganassin *et al.*, 2000) and with neutral red (Dayeh *et al*., 2002b). Protocols for using these dyes with *T. thermophila* have also been developed (Dayeh *et al*., 2002c). For the fish cell lines the metal exposure solutions were removed and working solutions of the dyes were added directly to the wells of the 96 well plate. For *T. thermophila,* cells were centrifuged to remove the metal exposure solutions and resuspended in the working solutions of the dyes. 100 µL aliquots of the cells were then transferred to each well of a 96 well plate.

Quantifying cell viability

For all dyes, fluorescence as fluorescent units (FUs) was quantified with the CytoFluor Series 4000 microplate reader (Applied Biosystems, Foster City, CA, USA) at respective excitation and emission wavelengths of 530 and 595 nm for AB, 485 and 530 nm for CFDA-AM, 530 nm and 645 nm for NR. Cell viability was expressed as a percentage of the control cells.

Results and Discussion

With the possible exception of neutral red (NR), the fluorometric indicator dyes were able to detect the toxicity of metals to both fish cells and *T. thermophila* (Figs. 1 & 2). NR did not indicate a decline in viability for the protozoan after Cu exposure even though alamar Blue and CFDA-AM did (Fig. 2). Sporadically, NR also gave puzzling results with fish cells after exposure to Cu but not the other metals. At very high Cu concentrations NR values increased rather than declined (panel C, Fig. 1). These observations suggest that an interaction between NR and Cu independent of cells accounts for the increase in fluorescent readings, but so far this has not been possible to demonstrate experimentally.

The toxicity of Cu and Zn to the fish cells was strongly dependent on the exposure medium (Table 1 & Fig. 1). No toxicity was observed in L-15 and in L-15 with FBS. L-15 has higher concentrations of cysteine than many other basal media and cysteine likely chelates these metals making them unavailable to elicit toxicity. FBS would also bind metals, reducing their availability. Therefore L-15/ex was used to study the toxicity of the other metals.

The results from testing five metals on RTgill-W1 in L-15/ex are summarized in Table 1. The most toxic metal as judged by the metal ion EC_{50} was copper, followed by zinc, cadmium, iron and nickel. The same rank order of toxicity and similar EC50s were found with the other cell line, RTL-W1 (data not shown), suggesting no particular metal sensitivity between lines. The rank order was similar but not identical to the rank order described by others with different cell lines, exposure conditions, and cytotoxic endpoints (Maracine and Segner, 1998). Usually cadmium was found to be more toxic than copper. The difference might due to the use of a simplified exposure medium in the current study. Enhancing sensitivity to copper is beneficial for the purpose of screening mining effluent, as copper is usually the more abundant contaminant.

Several issues need to be studied further to demonstrate the utility of cells as a screening tool for metals. Sensitivity should be enhanced, as the concentrations that were cytotoxic in the current study are too high to be expected very commonly in environmental samples. For copper the fish cell lines appear more responsive than *T. thermophila*. One possible way of enhancing sensitivity is to reduce the intracellular levels of glutathione, which is a cellular defense mechanism against metal stress in both fish and protozoa (Marcine and Segner, 1998; Wagner, 1989). Other issues to explore are the response of cells to mixtures of metals or to mixtures of metals and other contaminants, such as ammonia, which might be expected in mining effluent.

Acknowledgements

This research was supported by CRESTech (Centre for Earth and Space Technology). Thanks to G. Gilron, Dennis Farara, and R. Scroggins for advice and support.

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Table 1: Viability of RTgill-W1 cells after 24 h exposures to metals in variations of the basal medium, L-15

Exposure Medium	Metal Salt (highest concentration tested)	Metal concentration causing a 50% reduction (EC_{50}) in cell viability as measured in 3 assays					
		alamar Blue		CFDA-AM		Neutral red	
		Metal Salt (mg/L)*	Metal Ion (mg/L)*	Metal Salt (mg/L)*	Metal Ion (mg/L)*	Metal Salt (mg/L)*	Metal Ion (mg/L)*
L-15 & 10% FBS	$CuCl_2 \cdot 2H_2O$ (10 mg/L)	—	—	—	—	—	—
	$ZnCl_2$ (15 mg/L)	—	—	—	—	—	—
	$CdCl_2$ (183 mg/L)	76.46 (n=1)	48.73 (n=1)	55.63 (n=1)	34.11 (n=1)	75.59 (n=1)	46.36 (n=1)
	$FeSO_4 \cdot 7H_2O$	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
	$NiCl_2$	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
L-15	$CuCl_2 \cdot 2H_2O$ (10 mg/L)	—	—	—	—	—	—
	$ZnCl_2$ (3.8 mg/L)	—	—	—	—	—	—
	$CdCl_2$	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
	$FeSO_4 \cdot 7H_2O$	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
	$NiCl_2$	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
L-15/ex	$CuCl_2 \cdot 2H_2O$ (10 mg/L)	1.72 ± 1.1 (n=7)	0.64 ± 0.4 (n=7)	2.99 ± 1.8 (n=7)	1.11 ± 0.7 (n=7)	—	—
	$CuSO_4 \cdot 5H_2O$ (10 mg/L)	3.69 (n=1)	0.92 (n=1)	6.56 (n=1)	1.67 (n=1)	—	—
	$ZnCl_2$ (15 mg/L)	4.37 ± 0.9 (n=5)	1.98 ± 0.2 (n=5)	5.13 ± 0.2 (n=2)	2.40 ± 0.1 (n=2)	5.16 ± 1.2 (n=5)	2.48 ± 0.6 (n=5)
	$CdCl_2$ (183 mg/L)	7.60 ± 5.7 (n=2)	4.66 ± 3.5 (n=2)	16.42 ± 8.9 (n=2)	10.07 ± 5.4 (n=2)	8.17± 5.6 (n=2)	5.01 ± 3.4 (n=2)
	$FeSO_4 \cdot 7H_2O$ (174 mg/L)	80.63 ± 11.5 (n=3)	16.20 ± 2.3 (n=3)	105.07 ± 11.6 (n=3)	21.13 ± 2. 3 (n=3)	84.41 ± 13.6 (n=2)	16.96 ± 2.7 (n=2)
	$NiCl_2$ (250 mg/L)	—	—	—	—	—	—

* The "—" indicates viability did not decrease 15% relative to the control

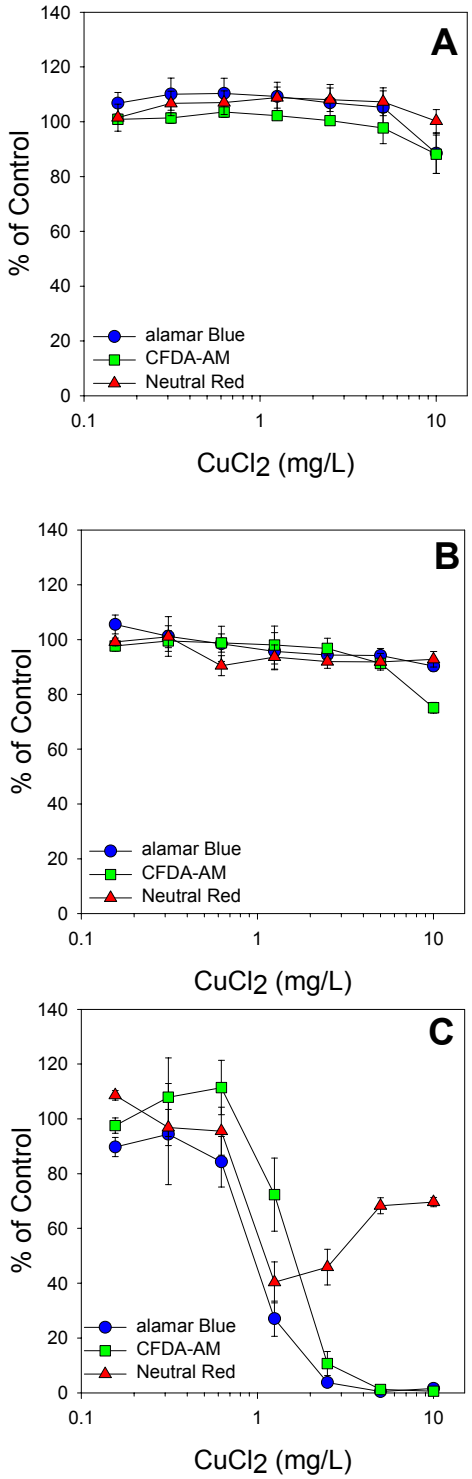

Figure 1: Viability of RTgill-W1 cells after 24 h exposures to $CuCl_2$ in variations of the basal medium, L-15. Cells in microwell plates were exposed in L-15 with fetal bovine serum (FBS) (panel A), L-15 (panel B) and L-15/ex (panel C). For each medium, control cultures received no $CuCl_2$. Cell viability was measured with fluorescent indicator dyes: alamar Blue (circles), CFDA-AM (squares), neutral red (triangles). Results were expressed as a percentage of the readings for controls. Data points represent the mean of 5 cultures with standard deviation.

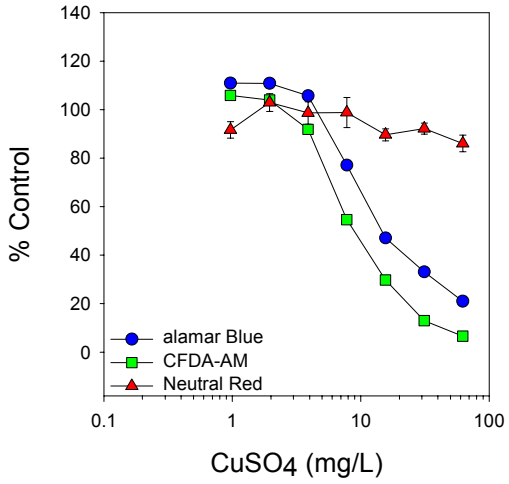

Figure 2: Viability of *Tetrahymena thermophila* after 24 h exposures to $CuSO_4$ in spring water. Cells were in microcentrifuge tubes for the $CuSO_4$ exposures and afterwards transferred to microwell plates for quantification of viability. Controls were in spring water alone. Cell viability was measured with fluorescent indicator dyes: alamar Blue (circles), CFDA-AM (squares), neutral red (triangles). Results were expressed as a percentage of the readings for the controls. Data points represent the mean of 5 cultures wells with standard deviation.