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## IMPACTS OF LOW-DOSE GAMMA-RADIATION ON GENOTOXIC RISK IN AQUATIC ECOSYSTEMS

**Cheryl L. Cassidy** □ Medical Sciences, McMaster University, 1280 Main Street West, Hamilton Ontario, Canada

**Jennifer A. Lemon, Douglas R. Boreham** □ Medical Physics and Applied Radiation, McMaster University, Hamilton Ontario, Canada

□ Chinook salmon cells were exposed to gamma radiation and chromosome damage was assessed using the micronucleus assay. The salmon cells were resistant to radiation at all doses compared to human and mammalian cells. We used an indirect approach to determine if prior low dose exposures at environmental dose levels might alter the consequences of radiation exposures to high doses of radiation (adaptive response). The cells adapted but only at doses which were above levels that might be expected environmentally. The “adaptive response” endpoint was useful to show biological responses to exposure, however, under these conditions it might not help in risk assessment of aquatic organisms since the cells seem to be very resistant and environmental radiation levels are typically extremely low. Preliminary experiments were conducted on two other fish cell model systems (Rainbow Trout and Medaka) to optimize conditions for the micronucleus assay for future environmental radiation studies. Since fish cells appear to be more radiation resistant than mammalian cells, we postulate that radiation risk in the whole organism may also be lower. Therefore whole body studies designed to test effects with the specific aim of assessing relative risk between species are in process.

*Keywords: Low Dose, Fish, Radiation, Adaptive Response*

### INTRODUCTION

Canada’s nuclear power industry consists of 22 CANDU reactors (17 of which are in service) that produce approximately 15% of Canada’s electricity needs. The reactors are located in Ontario, Quebec, and New Brunswick. Since Canada’s CANDU reactors are situated near aquatic systems, it is important for the nuclear industry to understand the risk from low-dose radiation exposures on aquatic organisms. Historically, the International Commission on Radiological Protection (ICRP) recommended that the protection of humans from radiation will adequately protect other organisms in the environment (ICRP 1991). This paradigm comes from the fact that humans are considered to be the most radiation sensitive organism (Harrison and Anderson 1996). In Canada, dose limits for nuclear energy workers and the public are controlled by the Canadian Nuclear Safety Commission (CNSC) and in practice these lim-

Address correspondence to Douglas Boreham, Medical Physics and Applied Radiation, McMaster University, 1280 Main Street West, Hamilton Ontario, Canada L8S4K1. Tel: +1-905-525-9140 ext. 27538; fax: +1-905-522-5982; e-mail: boreham@mcmaster.ca

its assume all other living organisms are protected. Protection limits for humans are currently based on the linear no-threshold hypothesis, which extrapolates low-dose radiation risk from observed high-dose effects. There is concern, however, that the low-dose protection models estimated for man may not offer the adequate protection for some non-human biota, like fish. The ICRP has recently proposed to change their current philosophy from protection of man to include environmental protection of reference animals and plants.

Man-made sources of ionizing radiation include nuclear fuel cycle products, nuclear weapon testing fall out, and nuclear medicine techniques. The radiation that we are most concerned with is the nuclear fuel cycle products and the impact that this radiation can have on fish species that live near nuclear power plants in Canada. Radionuclides of important environmental concern that can be released from CANDU reactors include tritium and carbon-14. The effects from low-dose chronic exposures of these radionuclides to ecosystems are currently unknown.

Our radiation biology laboratory utilizes a Cs-137 source that produces gamma rays. The absorption of these photons into cells occurs by the Compton process. In this process, the photons interact with free electrons and transfer most of their energy to the electron. The photon is then deflected from its path with the remaining energy and the end result is the production of fast moving electrons. It is these fast moving electrons that interact with other atoms/molecules in a cell to produce free radicals (very reactive species with an unpaired orbital electron) that damage the cell's bio-molecules to produce a chemical/biological effect. This process is known as an indirect action of radiation. DNA is the principal target of radiation damage and its biological effects. It can be damaged many ways but when the double helix back bone is broken, single strand breaks (SSB) or double strand breaks (DSB) can result. Once a cell's DNA has been damaged by radiation, it can be processed by one of three pathways; (a) the damage can be fixed correctly; (b) the damage can be fixed incorrectly; or (c) the cell can destroy itself as a result of the type of damage (apoptosis). SSB can be fixed relatively easily as the cell will use the complement strand of DNA as a template to repair the broken piece, whereas DSB are much harder for the cell to fix. DSB can be repaired by either homologous or non-homologous recombination. Homologous recombination is error-free repair that occurs in mammalian cells and uses a complementary DNA strand as a template for filling in the gap of damaged DNA. Non-homologous repair does not use a template to fix the damaged DNA and as a result, this process is error-prone.

There are many studies being conducted to ensure the adequate protection of man from ionizing radiation. Work has been done on the induction of chromosomal aberrations in human cells resulting from the exposure of ionizing radiation (Dolling *et al.* 1997; Plan *et al.* 2005) and on the

effects of chronic low-dose exposure on producing micronuclei in blood lymphocytes in nuclear power workers (Sari-Minodier *et al.*, 2002; Hadjidekova *et al.* 2003; Joseph *et al.* 2004). This work has been facilitated by the amount of time and resources dedicated to developing molecular techniques and blood assays for humans. If the ICRP past recommendation is correct, then protection of man from radiation will ensure the safety of other living organisms. However, this paradigm is just an assumption and little work has been done on the effects of ionizing radiation on ecosystems and non-human biota. The development of molecular techniques for non-human biota is starting to emerge with chromosome paints now developed for turtles (Ulsh *et al.* 2000) and lake trout (Phillip *et al.* 2001). It is very important to more thoroughly investigate this field as the use of nuclear power in Canada and the world is expected to increase with time.

In our studies, a common cytogenetic biomarker of radiation exposure (frequency of micronuclei) will be used to examine chromosomal effects in three fish species, Chinook Salmon (*Oncorhynchus tshawytscha*), Rainbow Trout (*Oncorhynchus mykiss*), and Medaka (*Oryzias latipes*). The cytokinesis-block micronucleus (MN) assay (Fenech 2000) is useful for quantifying chromosome aberrations induced by ionizing radiation and has been used on human (Dolling *et al.* 2000; Muller and Rode 2002) and non-human (Takai *et al.*, 2004; Ulsh *et al.* 2004) cell types. The methodology for this assay is simple and quick, which makes it an economical and worthwhile procedure. This technique utilizes a chemical called cytochalasin B to block cells from dividing into two new cells, thus preventing the loss of genetic material as a result of DNA damage or improper chromosome segregation. Cells that have completed one nuclear division can be identified under fluorescence microscopy as binucleated cells. Any DNA damage that has not been repaired by the cell will be visible in binucleated cells and classified as micronuclei. Micronuclei are identical to the cell's main nucleus but are smaller and contain whole chromosomes or chromosome pieces. The use of this endpoint has been shown to be sensitive enough to measure radiation exposures and represents the ability of cells to repair chromosome breaks.

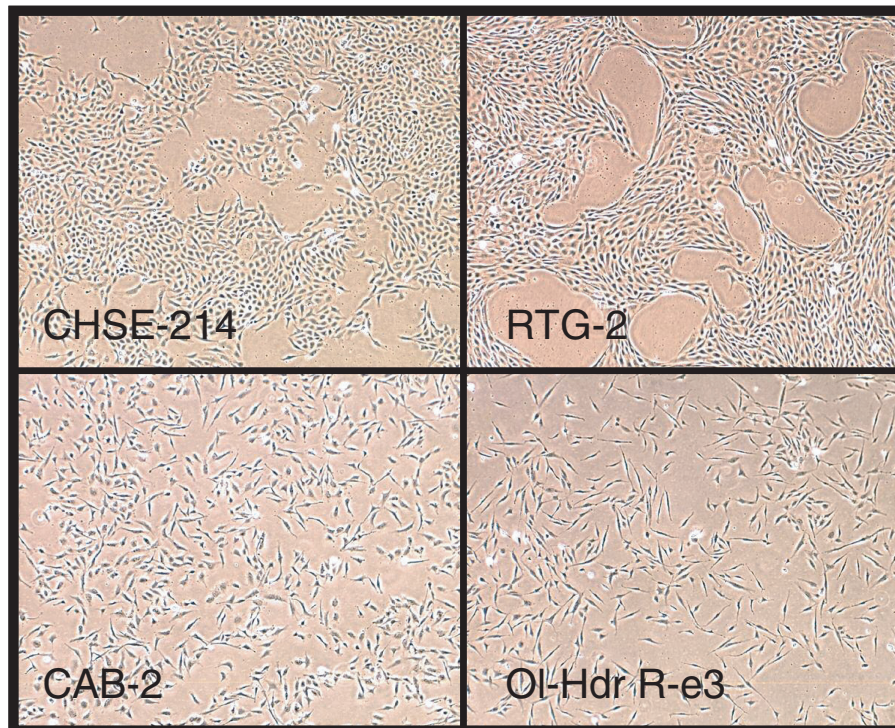
Our study had three objectives: (1) to establish optimal conditions for each of the four cell lines for the micronucleus assay, (2) determine the radiation sensitivity of a fish cell line relative to human cells using the micronucleus assay, and (3) determine if fish cells have the capacity to adapt to low doses of radiation similar to human cells.

## MATERIALS AND METHODS

### Fish cell lines

Four different fish cells lines were used in these experiments; Chinook salmon embryonic cells (CHSE-214), Rainbow Trout gonad

cells (RTG-2), adult Medaka fin cells (CAB-2), and Medaka embryo cells (Ol-Hdr R-e3). The cell culture morphology of exponentially growing cell culture from each cell line is shown in Figure 1. Chinook salmon embryonic cells (CHSE-214) were epithelial cells received from Dr. Carmel Mothersill of McMaster University and were grown in a 19° C incubator and cultured in DMEM-F12 media supplemented with 15% fetal bovine serum, 2.5 % HEPES buffer, 1% L-glutamine, 1% penicillin streptomycin and 1% hydrocortisone. Rainbow Trout gonad cells (RTG-2) were fibroblast cells purchased from the American Type Culture Collection (CCL 55) and were grown in a 19° C incubator and cultured in  $\alpha$ -MEM media supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin streptomycin. Adult Medaka fin cells (CAB-2) were epithelial cells derived from a CAB strain of Medaka fish and Medaka embryo cells (Ol-Hdr R-e3) were epithelial cells derived from an Hd-rR strain of Medaka fish. Both Medaka cell lines were received as a generous gift from Dr. Hiroshi Mitani of the University of Tokyo. Medaka fish cell lines were grown in a 32° C incubator and cultured in L-15 media supplemented with 1% HEPES, 25% fetal bovine serum, 1.25% penicillin streptomycin and 1.25% kanamycin sulfate.



**FIGURE 1.** Exponentially growing cell culture from Chinook salmon embryonic cells (CHSE-214), Rainbow Trout gonad cells (RTG-2), adult Medaka fin cells (CAB-2), and Medaka embryo cells (Ol-Hdr R-e3).



### **Cytochalasin B responses**

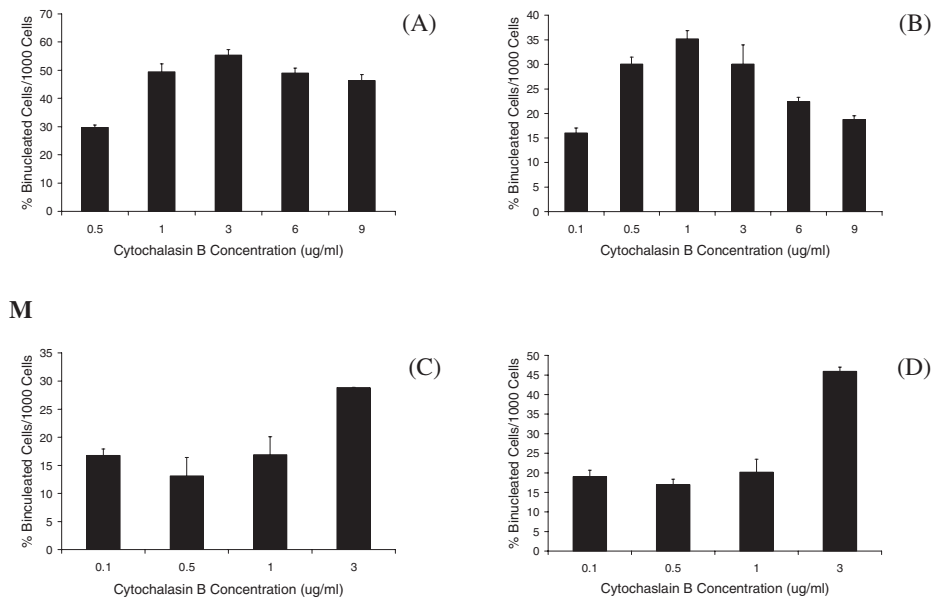
Optimal cytochalasin B concentrations and incubation time experiments were conducted on each cell line. NUNC flaskettes were seeded with 72 000 cells in 3ml of media per flaskette. CHSE-214 and RTG-2 cells were incubated at 19°C for 2 days prior to the addition of cytochalasin B, while Ol-Hdr R-e3 and CAB-2 cells were incubated at 32°C for 3 days. Cytochalasin B was added to each flaskette at various concentrations (0.1, 0.5, 1.0, 3.0, 6.0, 9.0 ug/ml) and cells were incubated for either 24 hours (Rainbow Trout and Medaka) or 48 hours (Chinook salmon). After each time point, the cells were given a hypotonic treatment for 5 minutes (2ml 0.075 M KCl) to swell them and then preserved by adding a soft fix (2ml 3:1 methanol: glacial acetic acid) for 15 minutes to the hypotonic solution followed by removal of the soft fix and addition of a hard fix (4ml 3:1 methanol: glacial acetic acid) for 10 minutes. Slides were air-dried overnight and stained for 10 seconds in acridine orange (10mg/ml). The stained cells were then covered with a glass slip and viewed under a fluorescence microscope. Percentage of mono-, bi-, tri- and tetra-nucleated cells in 1000 cells was scored and spontaneous micronuclei were scored in 1000 binucleated using criteria from Fenech (2000). Approximately 3 independent experiments were conducted on each fish cell line with 9 slides scored for each concentration and time point.

#### **Dose response: CHSE-214**

NUNC flaskettes were seeded at a concentration of 72,000 cells per flask in growth media. The flaskettes were incubated at 19° C for 48 hours and then exposed to 0, 10, 20, or 30 Gy of <sup>137</sup>Cs gamma radiation (1.0 Gy/min) at 0°C in sterile phosphate buffer saline (PBS) solution. After irradiation, the PBS was removed and 3 ml of complete fresh media was added. Cytochalasin B was added at the determined optimal concentration of 3 ug/ml to arrest cellular cytokinesis and the flaskettes were incubated at 19°C for 48 hours. The cells were lysed, fixed, stained and analyzed the same as for the cytochalasin B response experiments. For the adaptive response experiments, cells were exposed to a low dose priming exposure of 0.5, 1.0, or 4.0 Gy (1.0 Gy/min) and allowed to incubate under normal growth conditions for 3 hours prior to being exposed to a 30 Gy (1.0 Gy/min) challenge dose. The micronucleus assay was performed as described above.

### **RESULTS**

Cytochalasin B response experiments showed that each cell line has an individual optimal cytochalasin B concentration with an optimal incubation time (Fig 2). Chinook Salmon cells had a maximum response of 55% binucleated cells with 3ug/ml cytochalasin B after a 48 hour incu-

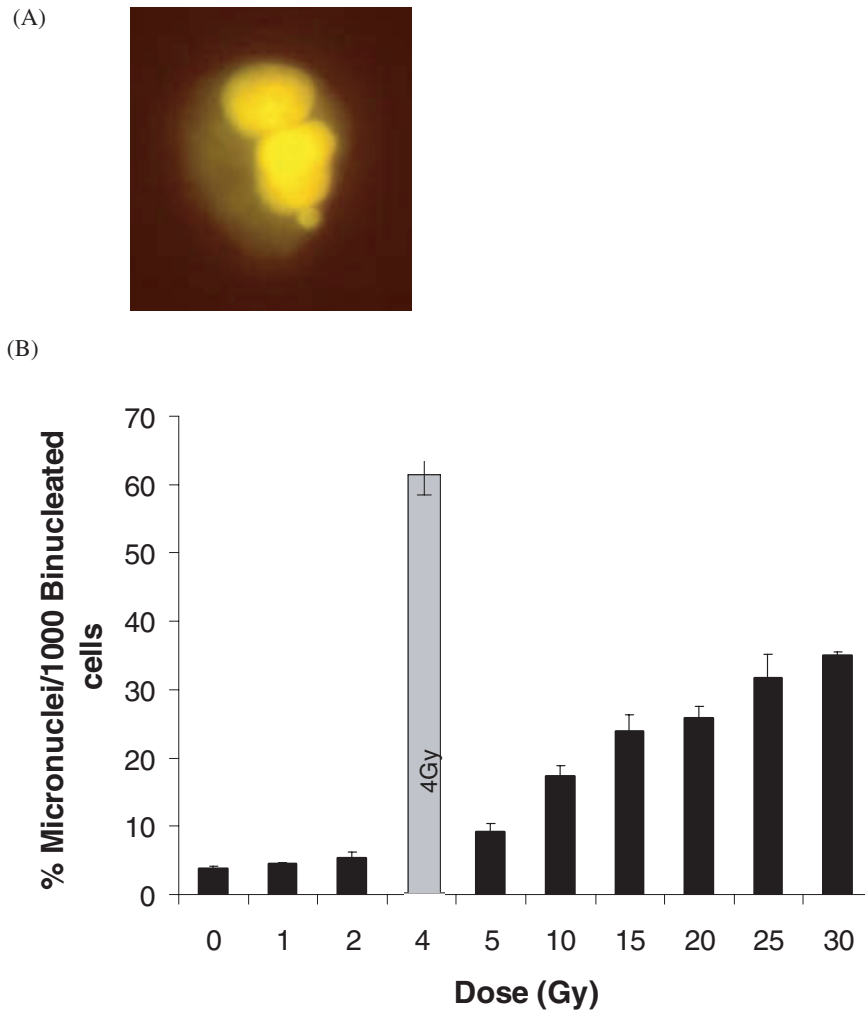


**FIGURE 2.** Dose response to determine optimal concentration of cytochalasin B in (A) CHSE-214, (B) RTG-2, (C) CAB-2 and (D) Ol-Hdr R-e3 cell lines. Optimal cytochalasin B concentration was determined as the concentration producing the maximum number of binucleated cells while minimizing the number of spontaneous micronuclei.

bation (Fig 2A), while the Rainbow Trout cells had a maximum response of 37% binucleated cells with 1ug/ml cytochalasin B following a 24 hour incubation (Fig 2B). Both the CHSE-214 and RTG-2 cell lines had < 3% spontaneous micronuclei formation even at the highest cytochalasin B concentration (9ug/ml) and the longest incubation time of 72 hours (data not shown). In contrast, a lower binucleated cell percentage (28%) and a higher spontaneous micronuclei formation (36%) was observed in the adult Medaka fin (CAB-2) cell line after an incubation of 24 hours (Fig 2C). Medaka embryo cells (Ol-Hdr R-e3) had a response of 46% binucleated cells with 3ug/ml cytochalasin B after a 24 hour incubation with a high spontaneous micronuclei formation of 26% (Fig. 2D).

Radiation dose response experiments conducted on Chinook salmon cells showed a linear dose response at high doses (10-30 Gy) for micronucleus induction (Fig.3B). The percentage of micronuclei increased up to 30 Gy but above this dose the cells exhibited extreme cell cycle delay, had relatively few binucleated cells and were difficult to score (data not shown). In comparison to Chinook salmon cells, human cells are much more radiation sensitive using micronucleus formation as the endpoint (see Fig. 3B). Radiation sensitivity studies on the dose response in Rainbow Trout and Medaka fish cells are in progress.

The adaptive response experiments showed a significant ( $p < 0.05$ ) reduction in micronucleus formation (adaption) induced by a 30 Gy



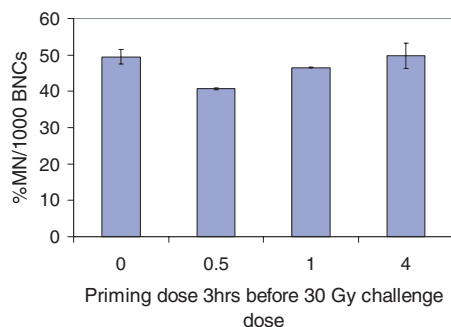
**FIGURE 3.** (A) A micronucleus (small circle) in a binucleated (two larger circles) Chinook salmon cell after exposure to gamma radiation. (B) The effect of radiation dose on the percentage of micronuclei formation in CHSE-214 fish cells. The bar at 4 Gy (shaded) are results for micronucleus formation in human cells (Broome et al. 2002). Each data point in (B) represents an average of 9 slides from 3 independent experiments (open bars).

challenge dose when cells were first exposed to a 0.5 Gy priming dose three hours prior to the challenge dose (Fig. 4). There was no significant adaption when cells were primed with 1.0 or 4.0 Gy and subsequently challenged with 30 Gy (Fig. 4).

### DISCUSSION

Cytochalasin B concentration response experiments demonstrated that each cell line has its own optimal cytochalasin B concentration and incubation time. The optimal concentration and time were considered to





**FIGURE 4.** Cells were exposed to priming doses (0.5, 1.0, 4.0 Gy) of gamma-radiation 3 hours prior to a large 30 Gy challenge dose. The frequency of micronucleus formation is given as percentage per 1000 binucleate cells (BNCs) scored.

be the amount of cytochalasin B that produced the most binucleated cells with the least amount of spontaneous micronuclei formation during the shortest incubation time. The difference in optimal cytochalasin B concentration and incubation time may result from the difference in cell type (epithelial versus fibroblast) or from the different growth kinetics and doubling times of each cell line. Cytochalasin B had relatively no effect on the formation of spontaneous micronuclei formation in Chinook salmon and Rainbow Trout cells whereas it had a large effect in both the Medaka cell lines. This was likely observed as the Medaka cell lines grow at a much quicker rate than the Chinook salmon and Rainbow Trout cells. The micronucleus assay is a measure of chromosome damage that is visible after a cell completes one cell division. Therefore, since the Medaka cell lines have a shorted cell cycle, they may show more chromosome damage than the Chinook salmon and Rainbow Trout cells as more cells will be completing the cell cycle in the incubation times.

Our radiation dose response results show that the micronucleus assay is sensitive enough to evaluate micronuclei formation induced by high doses of ionizing radiation in Chinook salmon cells. However, at environmental low-dose exposure levels, the micronucleus assay is not sensitive enough to measure genomic damage directly. Studies of this nature have been conducted on mammalian and human cell lines (Dolling *et al.* 2000; Ulsh *et al.* 2004). Typically these experiments use radiation exposures of up to 4 Gy to induce a high percentage of micronuclei formation. In our studies, the Chinook salmon cells required a much larger dose of gamma radiation of at least 10 Gy to produce any noticeable change in micronuclei formation different from baseline spontaneous micronuclei. This would suggest that these cells are more resistant to larger doses of radiation compared to mammalian and human cells. Reasons for the observed increase in radiation resistance in Chinook salmon cells as compared to human cells include: (a) the size difference in DNA content, (b)

the difference in DNA repair processes, or (c) the difference in cell cycle kinetics and cell growth. DNA content is an important factor that accounts for radiation sensitivity because cells that have a larger DNA content (mammalian & human cells) have a larger target for radiation to interact with and cause damage (Hall 1994). The fish cells used in our studies have a smaller DNA content than human and mammalian cells and this may account for the observed increase in radiation resistance. Differences in cell cycle kinetics and cell growth may also explain the difference in radiation sensitivity. Our Chinook Salmon cells grew at a very slow rate (cell doubling time of 48 hours) in a 19°C incubator while human and mammalian cells often grow at a much quicker rate (cell doubling time of 20-28 hours) in a 37°C incubator (Fenech 2000; Gebel *et al.*, 2002). Cells that grow slower have more time for DNA repair and therefore should have less DNA damage.

We have shown the fish cells can adapt to low doses of radiation and become resistant to subsequent larger doses. This has been observed in mammalian cells and now has been demonstrated in poikilothermic vertebrate cells. In the experiments performed here, 0.5 Gy seemed to induce the largest adaptive response. This is a relatively large adapting dose compared to mammalian cells, where doses as low as 1.0 mGy have been shown to adapt cells (Broome *et al.* 2002). This large adapting dose might be necessary because of the high constitutive radiation resistance in this cell type as explained above. The cells were allowed to incubate for 3 hours after the priming dose before the large challenge dose was given. Therefore, the maximum level of resistance developed by the 0.5 Gy priming dose may be a reflection of kinetics. In other words, perhaps there was not enough time for the other two larger priming doses to cause the cells to adapt maximally. However, it has been shown in mammalian cells that the magnitude of the priming dose is independent of maximal adaptive response (Broome *et al.* 2002).

The results of these studies demonstrate that using the micronucleus assay for environmental biodosimetry of radiation exposure is thus far only valid for Chinook salmon cells at high doses, since radiation induced micronuclei were only observed above control levels at doses higher than 10 Gy. Since Chinook salmon and Rainbow Trout can be found in Lake Huron near nuclear power plants, it is critical to determine an appropriate cytogenetic marker to evaluate chronic low-dose exposures. Future experiments conducted on Chinook salmon, Rainbow Trout and Medaka cells will determine the validity of this methodology between various fish cells. Since fish cells appear to be much more radiation resistant than mammalian cells, we postulate that radiation risk in the whole organism may also be lower. Therefore, studies designed to test effects in whole organisms with the specific aim of assessing relative risk between species are in progress.

## ACKNOWLEDGMENTS

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