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# Comparing a ciliate and a fish cell line for their sensitivity to several classes of toxicants by the novel application of multiwell filter plates to *Tetrahymena*

Vivian R. Dayeh [a], Sherri Grominsky [a], Stephanie J. DeWitte-Orr [a], Dana Sotornik [a], Christine R. Yeung [a], Lucy E.J. Lee [b], Denis H. Lynn [c], Niels C. Bols [a,*]

[a] *Department of Biology, University of Waterloo, Waterloo, ON, N2L 3G1, Canada*
[b] *Department of Biology, Wilfrid Laurier University, Waterloo, ON, N2L 3C5, Canada*
[c] *Department of Zoology, University of Guelph, Guelph, ON, N1G 2W1, Canada*

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**Abstract**

Although ciliated protozoa such as *Tetrahymena* have many desirable properties as toxicological test organisms, their attributes would be better realized if multiple cultures could be simultaneously exposed to toxicants, quickly washed to terminate toxicant exposure, and conveniently evaluated for changes in cellular functions. Therefore, multiwell filter plates (MWFPs), manufactured primarily for biochemical applications, were used to expose *Tetrahymena thermophila* to copper, Triton X-100, and gliotoxin and compared to results of exposure in microcentrifuge tubes (MCTs). For MWFP, removal of toxicant solutions and retention of *Tetrahymena* in wells was done by placing plates on a manifold and applying pressure with a vacuum pump. Retained cells were resuspended in the same wells and their functions assessed with the fluorescent indicator dyes, Alamar blue to measure energy metabolism, and 5′-carboxyfluorescein diacetate acetoxymethyl ester to evaluate membrane integrity. For MCTs, exposures were terminated by centrifugation, and resuspended *Tetrahymena* were transferred to conventional multiwell plates for viability assessment with the same fluorescent dyes. Results were measured with a fluorescent multiwell plate reader and dose-response curves were obtained successfully with both procedures. However, MWFPs were much more convenient and rapid, potentially allowing 96 cultures to be processed at a time. Exposing *Tetrahymena* in MWFPs also allowed the ciliate and a rainbow trout gill cell line, RTgill-W1, to be compared for their sensitivity to toxicants under similar conditions of exposure and by common viability assays. Both cell systems showed toxic responses to Triton X-100 and copper at similar concentrations, but RTgill-W1 was more sensitive to gliotoxin.


*Keywords:* *Tetrahymena thermophila*; Ciliate; RTgill-W1; Multiwell filter plate; Resazurin; Cell viability assay; Copper; Triton X-100; Gliotoxin

## 1. Introduction

*Tetrahymena*, free-living ciliates in the class Oligohymenophorea, subclass Hymenostomatia [18], have been used in toxicology for over 30 years, but the toxicological endpoints have usually been restricted to a measure of growth inhibition rather than of cell viability [28]. Through the decades, motility and light microscopic appearance of the cells have been used periodically as indicators of viability [6]. However, in the last few years, several tests from the animal cell literature have been tried. These include ATP content and reduction of a tetrazolium dye, 3-(4,5-dimethylthizol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) [10,20], as well as several tests with fluorescent indicator dyes. Calcein-AM and ethidium homodimer-1 were used together (Calcein-AM/EthD-1 test) to evaluate membrane

* Corresponding author.
*E-mail address:* ncbols@sciborg.uwaterloo.ca (N.C. Bols).

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integrity, with the results being analyzed by fluorescence microscopy [11]. Recently, a fluorescent microwell plate reader and several fluorescent dyes were used to measure the viability of *Tetrahymena thermophila* after Triton X-100 exposures [9]. The successful dyes were alamar blue (AB), neutral red (NR), and propidium iodide (PI), which respectively monitored energy metabolism, lysosomal activity, and membrane integrity. A fourth dye, 5′-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), which measured cell membrane integrity in fish cells [13], was problematic, as CFDA-AM failed to detect a decline in *Tetrahymena* viability under circumstances in which the other dyes did.

Although fluorescence viability tests can be read conveniently in a multiwell plate reader, the potential ease of the procedure is not fully realized because exposure of *Tetrahymena* to the toxicant must be done in another container or vessel. An additional vessel is necessary because the toxicant test solution must be removed rapidly before exposing the cells to the fluorescent dyes in order to evaluate viability. This cannot be done in conventional multiwell plates (MWPs) because the ciliates do not adhere to the surface and are lost when the test solution is removed. Therefore, toxicant exposures have been done in microcentrifuge tubes (MCTs), which allow the toxicant exposure to be terminated quickly by centrifugation [9]. The test solution, which is the supernatant, is discarded and the cells, which constitute the pellet, are transferred to 96-well plates for viability tests. This is a cumbersome, time-consuming step and seriously limits the number of samples that can be processed at one time. Therefore, an alternate strategy was sought that utilized multiwell filter plates. Multiwell filter plates (MWFPs) are manufactured for a variety of procedures, primarily biochemical, and come with a range of filter types and sizes, but they appear never to have been used to manipulate ciliates.

MWFPs could also aid toxicology studies aiming to compare *Tetrahymena* with animal cells. For several different purposes, *Tetrahymena* and the cells of vertebrates increasingly have been compared for their sensitivity to toxicants. One reason has been to make use of *Tetrahymena* as a model cellular system for studying the toxicology of pharmaceuticals for human medicine [5]. Another is the use of *Tetrahymena* as a bioassay tool to evaluate the safety of products for human consumption [1,24]. Finally the comparison has been in studies with an environmental or ecotoxicological purpose in order to consider the impact of ecotoxicants on different groups of organisms [9,25,26]. Usually the comparison has been made with mammalian cells. These have included human lymphocytes [17], and murine L929 cell lines [24,25]. Recently, the comparison has been with a gill cell line, RTgill-W1, from rainbow trout, which is an important species in aquatic toxicology [9]. For these animal cell/*Tetrahymena* comparisons, the methods for exposing cells to toxicants and evaluating cellular responses often have been quite different, as befitting such different groups. However, exposing *Tetrahymena* to toxicants in MWFPs would be as close as currently possible to exposing animal cells in conventional MWPs, allowing similar volumes and cell numbers to be used and cell viability assays to be performed.

In the present study, methodology has been developed to allow *Tetrahymena thermophila* to be exposed to toxicants and evaluated for viability in the wells of MWFP. This was accomplished in three steps. The first step was to find a MWFP that would retain *Tetrahymena* in wells after removal of the test solution through filtration. The second step was to determine whether a fluorescence multiwell plate reader would detect fluorescent indicator dyes in MWFPs. The third step was to perform fluorescence viability assays on *T. thermophila* after exposure to test agents in MWFPs and in MCTs, a procedure developed previously to study Triton X-100 [9]. The test agents were Triton X-100 and $CuSO_4$, which are ecotoxicants that have been studied previously in *Tetrahymena* by a variety of methods [19,21], and gliotoxin, which is a mycotoxin produced by various fungal species but not previously studied on ciliates [31]. Finally, the three compounds were compared for their toxicity to *Tetrahymena* and to the fish cell line, RTgill-W1, which was exposed in conventional MWPs and evaluated for viability by the same procedures used to monitor *Tetrahymena*.

## 2. Materials and methods

### 2.1. Tetrahymena cultures

*T. thermophila* was maintained axenically in 10 ml of proteose peptone yeast extract (PPYE) medium [14]. Preparation of the stock cultures for testing was conducted as previously described [9]. Briefly, 10 ml of a 24-h-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 2 days at room temperature. The culture was then harvested at mid-to-late log phase by centrifugation (200 *g* for 5 min) and washed 3 times with one of the three exposure solutions. After the final centrifugation cells were resuspended in 10 ml of the exposure solution (see below). The cells were counted using a Coulter Z2 particle counter and adjusted to a cell density of 500 000 cell/ml (±10%).

### 2.2. RTgill-W1 cultures

RTgill-W1 is a cell line derived from the gill epithelium of rainbow trout (*Oncorhynchus mykiss*) and is available from the American Type Culture Collection (ATCC # CRL 2523) [4]. The cell line was routinely cultured in 75-$cm^2$ culture flasks at 21°C in Leibovitz's L-15 culture medium supplemented with 10% fetal bovine serum (FBS–Sigma) and 2% penicillin-streptomycin (100 µg/ml streptomycin, 100 IU/ml penicillin, Sigma) [8]. Confluent flasks were