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Development of Toxicity Index to Evaluate the Level of Water Contamination by Using Cellular Responses ⋆

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Abstract: Conventional tests in environmental monitoring have been performed by quantifying levels of toxicity of specific substances such as pesticides or drugs, and then comparing with known toxicity thresholds. These tests are conducted using analytical chemistry methods and can only be used for targeted substances, often missing unexpected toxicants. This shortcoming underlines the need for novel tests for rapid assessment of toxicity of environmental sample mixtures, followed by more detailed and expensive laboratory analysis as required. In order to evaluate the level of toxicity in water contamination, a mathematical model to predict the toxicity index is developed based on time-dependent cellular response curves (TCRCs). First, the water sample is diluted to a series of strength (80%, 60%, 40%, 30% 20% and 10%) to get the multiple concentrations. Then, the living cells are exposed to those water samples and the corresponding dynamic cytotoxicity response curves are collected via xCELLigence real-time cellular analyzer for high throughput (RTCA HT) system. A synthetical index, based on the calculation of area under curve (AUC) of the negative control, is proposed to evaluate the level of toxicity in water contamination. The proposed index also takes the variation of biological experiment into consideration. The biological experiment demonstrates the effectiveness of the proposed toxicity index to measure the level of toxicity in water contamination.

Keywords: Cytotoxicity, Area under the negative control line (*AUC*), Concentration-response curve, Time-dependent cellular response curve (TCRC)

1. INTRODUCTION

Toxicity testing for environmental monitoring has been conventionally performed by characterizing the specific substance, quantifying its level, and then comparing it to known regulatory guidelines. A wide range of analytical techniques are being used to achieve this goal (Janet et al., 2012; Edmund and Tandeka, 2009). However, this approach can only identify targeted substances and the unexpected toxicants, which the analysis is not designed to identify, are missed. Furthermore, the environmental contaminants are usually present as mixtures. The synergistic effects cannot be simply done by summing up the toxicity from individual substance. Currently, little is known on the mixed cytotoxic effects caused by multiple environmental chemicals and their related human health risks. There is an urgent need for novel tests that can be used for the screening and monitoring of a wide range of toxic contaminants in the environment, and then a more detailed and expensive laboratory analysis as required.

The development of *in vitro* toxicity assays in environmental analysis would play a valuable role in such assessments (Ibrahim et al. 2010). By using human cell lines, the toxicity data will be more relevant in assessing human health risks (Kavlock et al. 2008)). In order to evaluate the level of toxicity in water contamination, we introduce a novel high-throughput cell electronic sensing technology to monitor the real time cellular response to the water samples (Boyd et al. 2008). An *AUC*-effect index is proposed for identifying and analyzing the level of harmful effect.

2. MATERIALS

2.1 Cell Lines

Human hepatocellular carcinoma cells (HepG2) (Order# HB-8065, Cat.# 30-2003, ATCC, Manassas, VA) were routinely maintained in EMEM (Eagle's minimal essential medium) supplemented with 10% (v/v) fetal bovine serum

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(FBS) at 37°C incubator with 100% relative humidity and 5% CO_2.

2.2 Controls

Controls are the subjects closely resembling the experiment subjects but not receiving the treatment and thereby serving as a comparison group when treatment results are evaluated.

Postive Control: Arsenics have been a concern in Alberta ground waters since the early 1990s. They were monitored and found at relatively high levels in some ground water samples. Therefore, **Arsenic III** ($500\mu M$ and $50\mu M$) and a mixture of the trace elements were chosen as positive controls for the cytotoxicity assay.

Negative Control: The negative control contains the target cells, the culture medium and the maximum concentration of solvent used to dissolve chemicals. In this experiment, H_2O was included as the negative control for environmental water analysis.

2.3 Water Samples

Three types of samples were selected as representatives of the water bodies including domestic wells, storm ponds, and recreational lakes.

Well Samples: Private domestic wells are the drinking and household water sources for rural families. The samples were analyzed for routine chemistry, trace elements, as well as cytotoxicity.

Storm Pond samples: Storm water ponds are frequently built into urban areas in North America to provide storm water flow control and improve water quality. They also collect suspended sediments, which are often found in high concentrations in storm water due to upstream construction and sand applications to roadways. Storm water ponds could be chemical soups of pesticides, fertilizers, pet wastes, oil, grease and other contaminants. The samples were analyzed for routine chemistry, trace elements, pesticides, VOCs, as well as cytotoxicity.

Lake samples: Water samples were collected from lakes. The samples were analyzed for routine water chemistry, trace elements, total microcystins, as well as the cytotoxicity.

Dilution Rule: Each water sample was diluted to a series of strength (80%, 60%, 40%, 30% 20% and 10%) to get the multi-concentration cellular response curve.

2.4 RTCA HT system

The xCELLigence Real-time Cellular analyzer for High Throughput (RTCA HT) system was used as the platform to facilitate this study (Huang and Xing, 2006; Boyd et al., 2008). The system has been developed by the ACEA Biosciences Inc. (San Diego, USA) in the 96x well plate format. The system measures the electronic impedance as the results of cell contacting biocompatible microelectrode fabricated at the bottom of well of tissue culture plate (E-Plate).

Using ACEA's proprietary algorithm, the impedance value is converted to Cell Index (CI), which closely reflects not only cell growth and cell death, but also cell morphology (attachment and spreading/shrinking) and adhesion. Toxic phenotypes other than cell death can be identified in the system. As the measurement is non-invasive and label free, the system can continuously monitor the cells from the time when cells are seeded. Quality control of the cells (e.g. doubling time, attachment) becomes a built-in feature of the system. More importantly, unlike end-point assays, cell responses from minutes to days after substance addition are recorded, which ensure that no meaningful time points are missed for analysis.

In this study, we use xCELLigence MP system with six 96x E-plate format to conduct the experiment. Two water samples with six dilutions are arranged in the two sides of each 96x E-plate, and the positive control and negative control are arranged in the middle of E-plate (shown in Fig.1). Each dilution of each water sample has been repeated four times. As a result, 12 water samples are monitored by the RTCA HT system in each experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	s1 80%	s1 80%	s1 80%	s1 80%	Negative control	Negative control	s2 80%	s2 80%	s2 80%	s2 80%	PBS
C	PBS	S1 60%	S1 60%	S1 60%	S1 60%	Negative control	Negative control	S2 60%	S2 60%	S2 60%	S2 60%	PBS
D	PBS	S1 40%	S1 40%	S1 40%	S1 40%	Positive control 1	Positive control 1	S2 40%	S2 40%	S2 40%	S2 40%	PBS
E	PBS	S1 30%	S1 30%	S1 30%	S1 30%	Positive control 1	Positive control 1	S2 30%	S2 30%	S2 30%	S2 30%	PBS
F	PBS	S1 20%	S1 20%	S1 20%	S1 20%	Positive control 2	Positive control 2	S2 20%	S2 20%	S2 20%	S2 20%	PBS
G	PBS	S1 10%	S1 10%	S1 10%	S1 10%	Positive control 2	Positive control 2	S2 10%	S2 10%	S2 10%	S2 10%	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Fig. 1. Design of Experiment.

3. METHOD

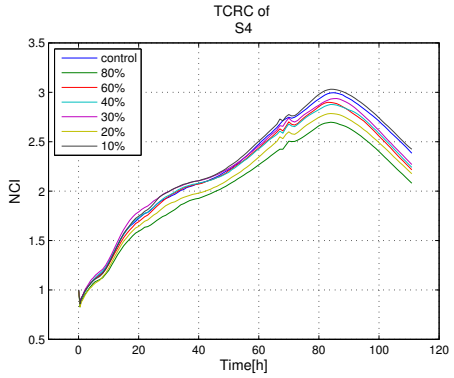
3.1 Cell Index

Continuously monitored cell substrate impedance in real-time has been considered to produce very specific time-dependent cellular response curves upon treatment with biologically active compounds. Based on the measured impedance, a dimensionless parameter termed Cell Index (CI) is derived to provide quantitative information about the physiological and pathological responses of the living cells to a given chemical compound. In our analysis, normalized CI (NCI) is considered. NCI is the ratio of cell index at a particular time point to the cell index at the time of exposure as shown in the following equation:

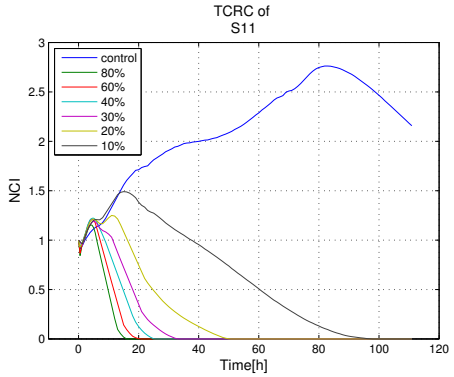
$$NCI(k) = \frac{CI(k)}{CI(0)}, \quad k = 1, 2, \cdots, K \tag{1}$$

where k is the number of the frequency points at which the impedance is measured.

The example is shown in Fig.2. The real-time concentration-specific growth reflects the growth pattern change in comparison with the negative control. Some of them are close to the negative control (Fig.2(a)), which do not show the toxicity of the sample. Others depart from the negative control, which demonstrates the toxicity at a given concentration level.



(a) TCRC of water sample 4.



(b) TCRC of water sample 11.

Fig. 2. The TCRCs of water samples in HepG2 cells.

The observed TCRCs reflect the dynamic evolution of cell proliferation status. The patterns show the following properties:

1. Time kinetic: the variation in $NCI(k)$ with respect to time $t(k)$, and

2. Dependence on concentration: the TCRCs of test substances varied at different concentration levels.

3.2 Area under the negative control line (AUC)

It is well known that the cells in culture media alone (negative controls) demonstrate typical cell growth curve with four phases (lag-phase, log-phase, plateau-phase, decline-phase) without exposure to test substances. The number of viable cells declines due to the natural path of the cell cycle and shortage of nutrient supplements at the decline-phase. If the decline-phase is included into the toxicity assay, the assessment will not be credible, because of the ambiguity whether the cell death is due to nutrient deficiency or is caused by the compound toxicity during this phase. In order to keep the toxicity assay within the cell proliferation boundary and to have the same evaluation baseline, the log-phase was selected for the cell-based *in vitro* assay, i.e.

$$N = \min \left\{ \arg\max_{k} \left\{ NCI_{c,j}(k) \right\} \right\}_{j=1}^{6} \tag{2}$$

where $NCI_{c,j}(k)$ is the value $NCI(k)$ of the negative control at the time $t(k)$ in the j^{th} E-plate, $j = 1, 2, \cdots, 6$ in six experiments (each experiment only includes two water samples) and k is the index of sampling time.

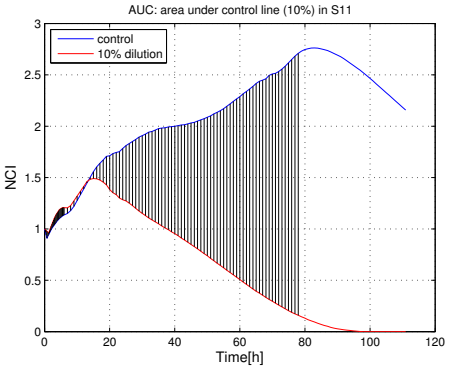
As shown in Fig.2, the *distance* between the negative control and TCRC demonstrates the level of toxicity; however, it varies with different concentration levels and different exposure time. In order to evaluate the overall toxicity effect, a metric based on the area between the control line and the TCRC corresponding to a particular concentration is developed as follows.

AUC_j, the area between the control line and the j^{th} TCRC is defined as:

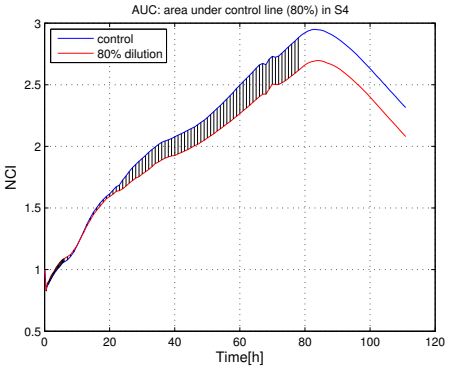
$$AUC_j = \sum_{k=2}^{N} \frac{\left(\Delta NCI(k) + \Delta NCI(k-1)\right)\left(t(k) - t(k-1)\right)}{2} \tag{3}$$

where $\Delta NCI(k) = NCI_c(k) - NCI_j(k)$ are the cell index of negative control and j^{th} concentration of a water sample respectively.

The AUC_j reflects the cumulative difference in affected cell population and the negative control (or normal) cell population over the log-phase, which evaluates the toxic effects on cellular growth inhibition or cell killing with this concentration (shown in Figure 3). Here, the trapezoid rule is used to numerically approximate the integral.



(a) AUC of water sample 11 with 10% dilution.



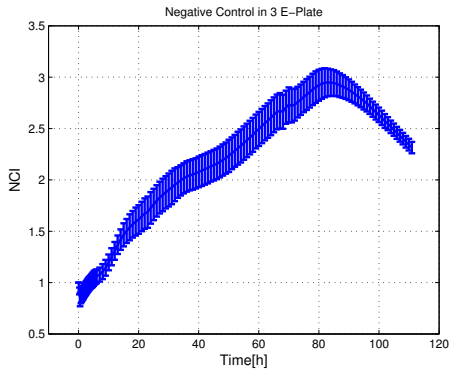
(b) AUC of water sample 4 with 80% dilution.

Fig. 3. Area under negative control curve.

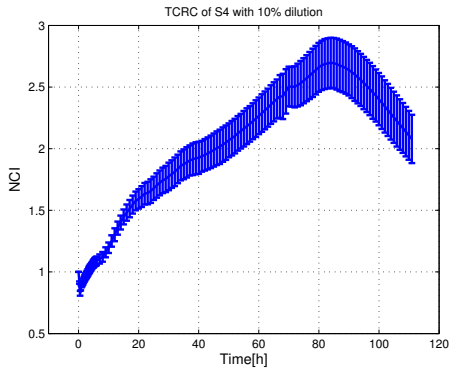
3.3 Toxicity Index

Although AUC_j evaluates the toxic effect when HepG2 cells are exposed to a water sample with j^{th} different dilutions, it does not consider the variation of the equipment. Due to the limitation of various sensors, the difference

of wells, the cell health, and the disturbance of injection etc., there are some differences among the quadruplicate curves. As illustrated in Fig.4(a), the variation of negative control is greater than the AUC_j of water sample 4 with 10% dilution (shown in Fig.3(b)). Thus only using AUC_j without considering the experimental variation, may not give the correct assessment.



(a) Variation in negative control (quadruplicate).



(b) Variation in water sample 4 with 80% dilution (quadruplicate).

Fig. 4. Variation using the median response with standard deviation in the experiment.

In order to remove the variation effect, AUC_j is compared with the standard deviation of the controls and the j^{th} TCRC. The mean $\mu_j(k)$ and standard deviation $\sigma_j(k)$ of j^{th} TCRC at each sampling time index k are calculated as follows:

$$\begin{cases} \mu_j(k) = \dfrac{1}{I-1}\displaystyle\sum_{i=1}^{I} NCI_{j,i}(k) \\ \sigma_j(k) = \sqrt{\dfrac{1}{I-1}\displaystyle\sum_{i=1}^{I}(NCI_{j,i}(k)-\mu_j(k))^2} \end{cases} \tag{4}$$

where I is the number of repeated TCRC (here $I = 4$) and $j = 1, 2, \cdots, 6$ is the index of dilution of a water sample. Similarly, the mean $\mu_c(k)$ and standard deviation $\sigma_c(k)$ of negative control line can also be calculated by the Eq.(4).

Then, a toxicity index to evaluate individual sample toxicity at a certain concentration is proposed as in the following equation.

$$\Gamma_j = \frac{AUC_j}{n \times \left(\displaystyle\sum_{k=1}^{N}\sigma_c(k) + \sum_{k=1}^{N}\sigma_j(k)\right)} \tag{5}$$

where $n = 1, 2, 3$ is the amplification of standard deviation, which is similar to confidence levels at 68.27%, 95.45%, or nearly all (99.73%) respectively. In this study, n is set as 2.

Γ_j evaluates the level of toxicity for the j^{th} concentration of a water sample, which changes with the dilution factor w_j (here, $w_j \in \{0.8, 0.6, 0.4, 0.3, 0.2, 0.1\}$). Thus each concentration is weighted differently. In order to give a synthetic toxicity index, a weighted water toxicity index is calculated for each water sample combining all concentration responses as in Equation (6).

$$\bar{\Gamma} = \frac{\displaystyle\sum_{j=1}^{6} w_j \times AUC_j}{\displaystyle\sum_{j=1}^{6} w_j} \tag{6}$$

where $\bar{\Gamma}$ is the synthetic toxicity index, w_j is the diluted factor.

If $\bar{\Gamma}$ is greater than 1 (threshold), which means the AUC is beyond the experimental inherent variation, the the water sample can be regarded as a harmful water sample.

4. RESULT AND DISCUSSION

4.1 Result

In this paper, 12 water samples included one process water sample, two tap water samples, two distilled water samples, three ground water samples and four surface water samples (shown in Table.1). We first implemented the routine water chemistry analysis. Among the parameters in routine water chemistry analysis (alkalinity, bicarbonate, calcium, carbonate, chloride, conductivity, fluoride, hardness, hydroxide, magnesium, nitrate, arsenic, nitrite, pH, potassium, iron, sodium, sulfate, TDS), all values were within the normal range. Then, the selected cells were exposed to the twelve water samples. Data from 6 experiments and a total of 12 water samples were selected to validate the proposed method. We compared the quadruplicate cellular responses and excluded the unreasonable responses (outliers), and statistical analysis associated with the remaining responses was used in the subsequent analysis.

First, the mean value and standard deviation of each cellular response were calculated. Then, the proposed toxicity index Γ_j were calculated as shown in Fig.5(a) and Fig.5(b). The red horizontal line is the defined threshold. The indices indicate the level of toxicity in each water sample at each concentration. The warmer the color, the more cytotoxicity (Fig.5(b)). The sample 10 and sample 11 showed cytotoxic response in HepG2 cells.

Beyond that, the synthetical toxicity index was calculated for each water sample combining all concentration responses (shown in Fig.5(c)). The $\bar{\Gamma}$ value of sample 10 and 11 were far greater than 1, which reconfirmed the effectiveness of the proposed method. The example also

Table 1. Summary of the tested water samples

ID	Description	Note
S1	process water	from AEW
S2	tap water	from the lab
S3	tap water	from home
S4	single distilled water	from the lab
S5	double distilled water	ThermoNanopure system
S6	ground water	
S7	ground water	
S8	ground water	
S9	surface water	from Lake #1
S10	surface water	from Lake #2
S11	surface water	from Lake #3
S12	surface water	from Lake #4

Table 2. Comparison with Microtox and the proposed method

ID	Microtox ($IC50$)	the proposed method ($\bar{\Gamma}$)
S1	71	0.81
S2	$-$*	0.31
S3	> 91	0.08
S4	> 91	0.17
S5	$-$	0.11
S6	> 91	0.35
S7	> 91	0.49
S8	> 91	0.18
S9	> 91	0.63
S10	1.1	13.30
S11	56	12.79
S12	> 91	0.39

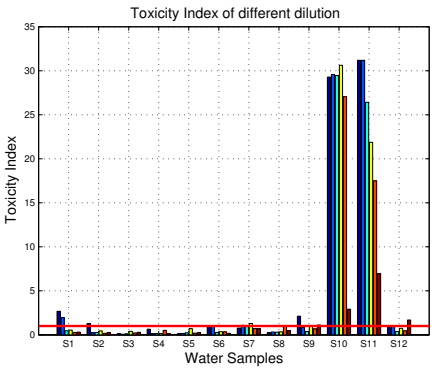
* - not tested due to the limited sample volume

demonstrated the general usefulness of this approach as a screening tool.

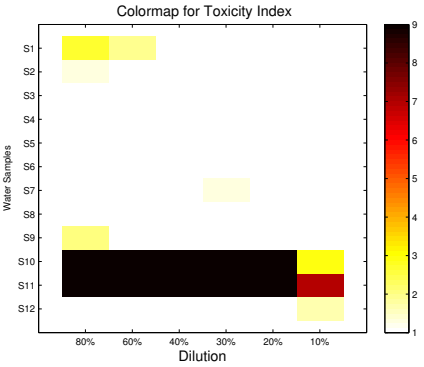
In order to evaluate the performance of the proposed method, the twelve water samples were sent to Hydroqual for Microtox analysis. Microtox is a standardized toxicity test system which is rapid, sensitive, reproducible, ecologically relevant and cost effective. Microtox measures the decrease in respiration, and subsequent light output, of the marine luminescent bacterium Vibrio fischeri as the toxic response. Increased levels of toxicity are highly correlated to decreased rates of bioluminescence. Since the Microtox assay is a kind of end-point detection methods, the $IC50$ at the half an hour is used as toxicity index in this paper. The results are shown in Table.2. The $IC50$ values of sample 10 and 11 have small values and show the toxicity. To give further explanation, the correlation analysis between Microtox and the proposed method is implemented as shown in Fig.6. The test results (10 water samples exclude sample 2 and 5) shows high correlation ($R^2 = 92.1891\%$).

4.2 Discussion

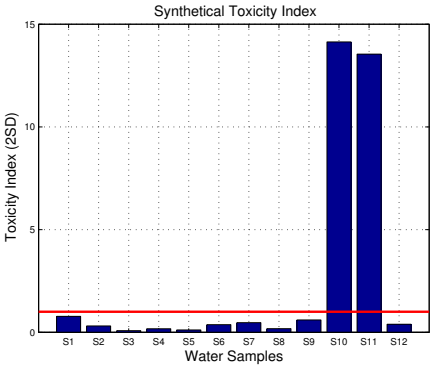
Although numerous toxicity indices have been developed for the quantization of time-dependent cells growth inhibition and cells killing curves, the design of *in-vitro* studies, data treatment, and data interpretations remain as a problem. The primary goal of this work is to explore a cell-based *in-vitro* assay strategy in which TCRCs can be used to assay the toxicity of environmental toxicants. The success of the proposed method is based on the concept



(a) Histogram of toxicity index Γ_j for 12 water samples with 6 dilutions.



(b) Hot colormap of toxicity index Γ_j for 12 water samples with 6 dilutions.



(c) The synthetic toxicity index $\bar{\Gamma}$ for 12 water samples.

Fig. 5. Water toxicity index.

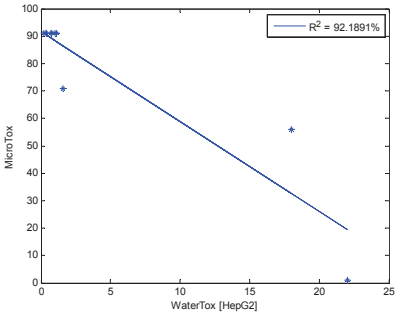


Fig. 6. Correlation Analysis for Microtox and the proposed method.

that the area between the negative control line and the TCRCs reflects the cumulative toxicity effect over the log-phase.

A challenge exists when maintaining low assay variance involving inter/intra-plate. Here, the coefficient of variation ($CV\%$), where CV is the ratio of standard deviation to mean, is used to provide a quantitative indicator of the repeatability of the tests. In Fig.7, the $CV\%$ are all less than 20%, which can be considered acceptable.

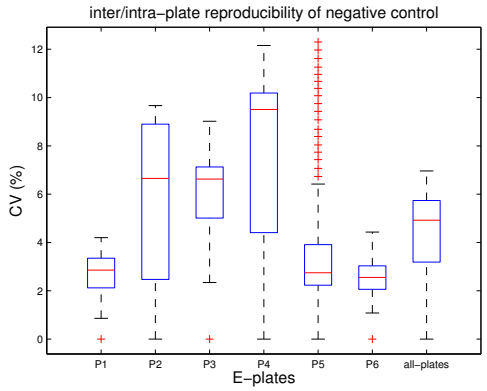


Fig. 7. Inter/Intra-plate reproducibility assessment for negative control.

5. CONCLUSION

In order to assess human health risk to environmental toxicants, the use of human cell based assay is the most relevant approach. The real-time cell electronic sensing system not only provides the high-throughput assay platform, but also collects multi-dimensional data that allows complex data analysis, clustering and profiling. In this study, a novel method is proposed for the assessment of environmental toxicity. Twelve samples from ground water, storm ponds, and lake water were analyzed by the methods. Preliminary data demonstrates the sensitivity of the assay and the range of cytotoxicity that may be expected from environmental samples.

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