Application Note

Cytotoxicity/
Environmental Toxicity

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Assessing Water Cytotoxicity with an Impedance-Based, Real-Time, and Label-Free Cellular Assay

In vitro testing using an Agilent xCELLigence real-time cellular analyzer (RTCA) Multiple Plate (MP) system

Authors

Dorothy Yu Huang and
David W. Kinniburgh
Alberta Centre for Toxicology,
University of Calgary,
Alberta, Canada

Stephan Baumann
Agilent Technologies, Inc.
USA

Abstract

Chronic adverse effects due to exposure to hazardous chemicals are subtle but are recognized by occupational health studies. There is growing evidence that adverse effects and even chronic diseases can occur at very low concentrations after prolonged exposure.[1] This realization is helping to drive awareness of water toxicity screening among environmental managers and public health decision makers.

Traditionally, toxicity analysis relied on animal testing.[2] This application note demonstrates a label-free, real-time cellular assay for source water monitoring developed at the Alberta Centre for Toxicology. This *in vitro* cytotoxicity assay uses the impedance-based Agilent xCELLigence real-time cell analyzer (RTCA). This approach allows noninvasive and continuous monitoring of the perturbation of cellular growth following exposure to the cumulative toxicants present in water samples. The assay can identify trends and environmental hotspots using the water toxicity index (WTI), percentage of biological effect (PoE), and cell growth inhibition (AUC). It is a noninvasive assay system that can work with many adherent cell types and potentially be applied to a broad range of *in vitro* assays.

Introduction

Source water monitoring is an essential environmental public health service. Water toxicity screening is of great importance to environmental managers and public health decision makers.

There are several existing examples of cellular assays being used for toxicity screening. For example, the mitochondrial impact of chemical exposure has been validated by the Irish Environmental Protection Agency (EPA), in conjunction with Luxcel Biosciences, now a part of Agilent.[3] Similarly, the nontargeted analysis approach to chemical risk assessment has been validated by the US EPA.[4] Therefore, this study focuses on using the Agilent xCELLigence real-time cellular analyzer (RTCA) multiple plate (MP) instrument to measure water cytotoxicity.

High-throughput techniques such as effect-directed analysis (EDA) enhance the understanding of the occurrence and biological activity of chemicals in exposure-related samples and their impact on the environment. Environmental toxicology testing is increasingly using EDA to supplement multiple/whole organism testing. High-throughput methods such as EDA require less time and money than traditional testing, which is a benefit for risk assessments and routine monitoring. Also, EDA can potentially provide insight into the underlying mechanism of toxicity. This application note shows how EDA helps identify and prioritize anthropogenic compounds found in the environment and biological samples.

Workflow overview

The steps in a complete EDA workflow[5] include:

1. The cytotoxicity of water, air, or soil samples are evaluated for bioactivity.
2. Samples that demonstrate cytotoxicity are analyzed by mass spectrometry.
3. High-quality features can be extracted from complex GC/MS and LC/MS data as part of a chemical analysis.
4. Chemometrics can be applied with nontargeted data. These tools allow users to statistically identify suspect hits.
5. Suspect hits can be evaluated to see if they impact a known adverse outcome pathway.
6. The suspect hits with no known bioactivity can re-enter the biological assay step to evaluate the cytotoxicity of individual compounds to help determine causality.

This application note covers the first two steps of the workflow. However, it is important to place the work in the context of the whole EDA process, which is shown in Figure 1.

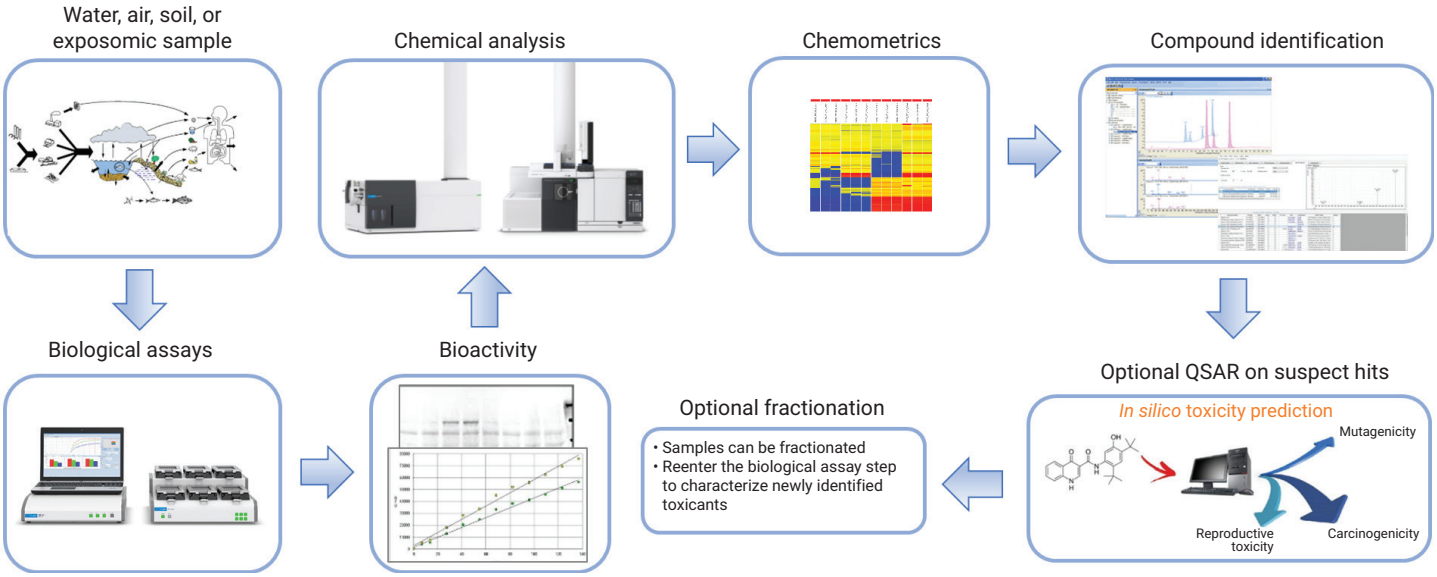


Figure 1. Generic EDA workflow. Suspect hits can be searched in the EPA's DSSTox Database with 875,000 entries. These suspect hits can be isolated and evaluated for bioactivity.

Experimental

Instrumentation

An Agilent xCELLigence RTCA MP instrument was used in this study.

Samples and sample preparation

A total of 436 river water samples were collected during the open water season from 2012 to 2014 for assessment of water cytotoxicity.

- The water samples were taken from rivers that flow in Northern Alberta.
- The assay was performed using HepG2 (human hepatocarcinoma) cells.
- Cytotoxicity response was tested at dilutions of 80%, 60%, 40%, 30%, 20%, and 10% of the original water samples.
- Hourly readings were automatically taken throughout the 96-hour exposure period.
- The advantage of this method is that it can achieve the time-concentration-response profile that provides rich data.[6]

Arsenic III and a mixture of trace elements were chosen as positive controls for the cytotoxicity assay. The negative controls consisted of water with target cells, and culture medium.

Cell culture prepared under laboratory conditions is free of biological contaminants such as bacteria, mold, yeast, virus, protozoa, and mycoplasma. These biological contaminants can achieve high densities, altering the growth and characteristics of the culture, and potentially leading to inaccurate and erroneous results in the cell-based assay. Therefore, it is important to have sterile cell culture. No bacteria or mycoplasma contaminations were observed under current assay conditions.[7]

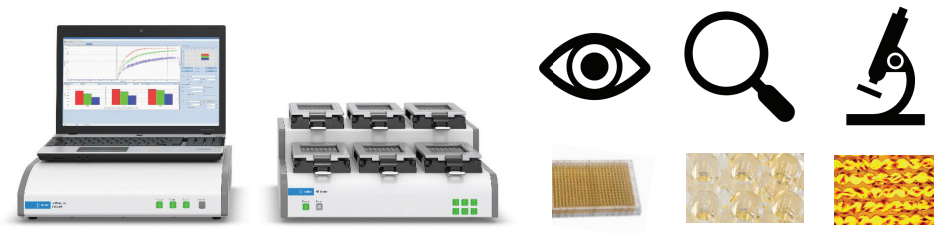


Figure 2. xCELLigence RTCA MP system with plates and wells under magnification, showing the electrodes in detail.

Results and discussion

The RTCA system allows noninvasive and continuous monitoring of the perturbation of HepG2 cell growth following exposure to the combined toxic effect present in water samples. Three hazard parameters including the WTI, the PoE, and the area under the cellular response profile (AUCRP) were developed to assess the cytotoxicity of source and other waters.

The negative controls (red curve in Figure 3) follow a typical cell growth curve because they are only exposed to culture media and dilute solvent. The negative control curves display four distinct phases: lag-phase, log-phase, plateau-phase, and decline-phase.[8] The number of viable cells declines due to the natural cycle exhibited by cells and a shortage of nutrient supplements at the decline-phase. If the decline-phase is included into the assay, the results will not be deemed credible, since there would be uncertainty in the cause of death. Therefore, the AUCRP is calculated using the log-phase portion of the curve.

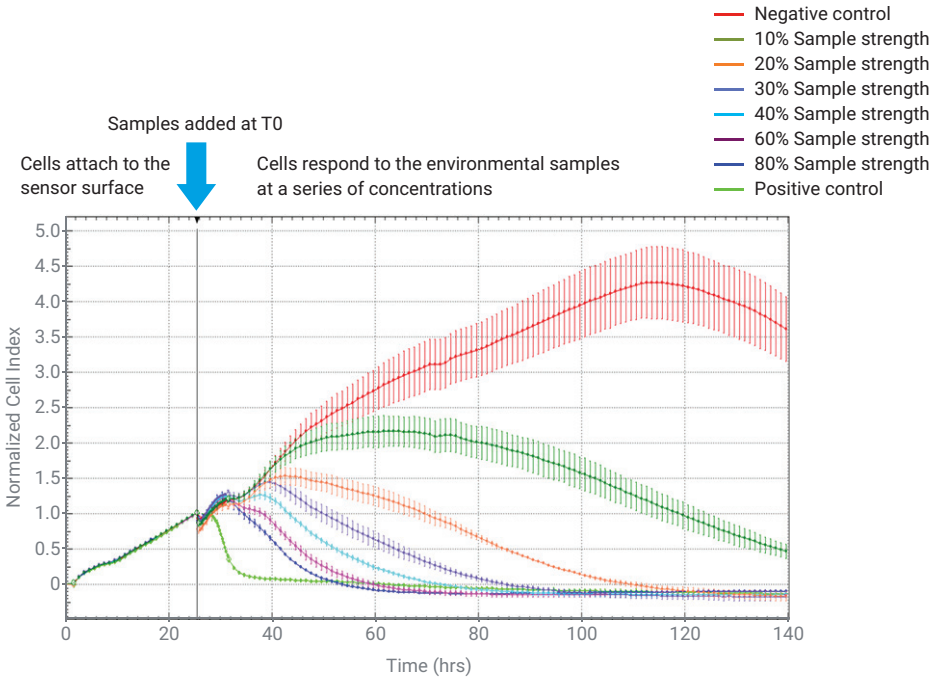


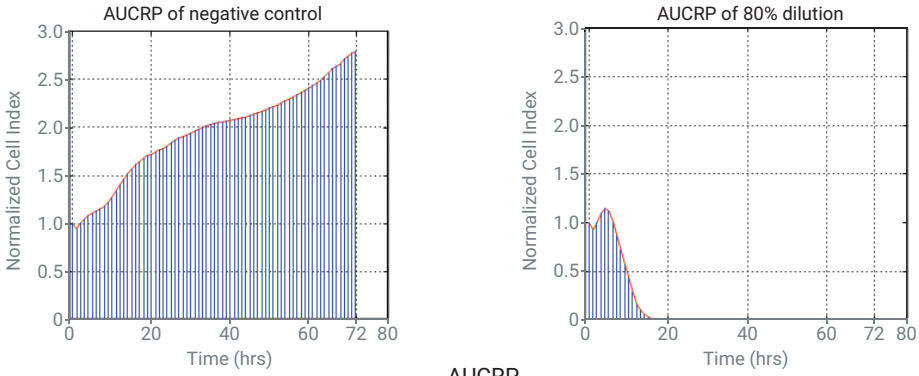
Figure 3. Agilent xCELLigence RTCA time- and dose-dependent cytotoxicity response curves. The time between T_0 and the apex at 114 minutes is where the biological effect was monitored.

The value of PoE directly measures the biological activity of a water sample with the selected concentration. It reflects the cumulative biological effect within the definitive time range.

To better comprehend exposure-related effects, a concentration-response curve based on PoE was introduced, where x denotes the concentration, p1, p2, and p3 are parameters independent of water sample concentrations, and PoE(x) denotes the PoE value when the cells are treated with x concentration of a water sample.[6]

$$PoE(x) = p1 \times x^{p2} + p3$$

Figure 4A shows the untreated negative control, and Figure 4B shows the PoE curve of sample 11 that has strong biological activity. These data can also be shown as a heatmap or a dose-response curve as in Figure 5. In this case, relatively benign samples, such as sample 4, have little cytotoxicity even at high concentrations while the PoE of sample 11 shows cytotoxicity starting at low dilutions.



$$PoE_{i,j} = \frac{AUCRP_{i,j}}{AUCRP_{m,NC}} \times 100$$

Figure 4. AUCRP is a measure of cell growth inhibition where $PoE_{i,j}$ is the activity index of i^{th} water sample with j^{th} concentration. $AUCRP_{m,NC}$ is the AUCRP of negative control in the m^{th} E-Plate.

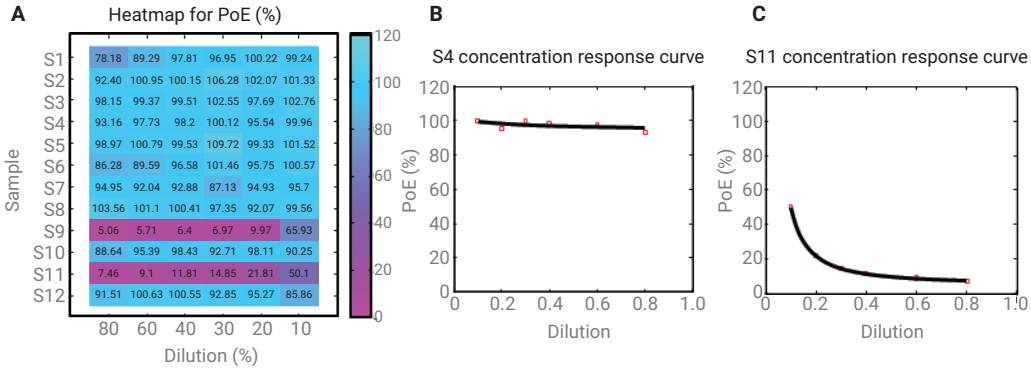


Figure 5. (A) histogram of activity index PoE for 12 water samples at six dilutions. (B) PoE dose-response of a water sample with very little cytotoxicity. (C) PoE dose-response for a water sample with high cytotoxicity.

Since PoE is specific to a particular exposure time, and because some water samples are not toxic enough to cause 20 or 50% growth inhibition (as shown in Figure 6), a single metric was introduced to represent the relative cytotoxicity. To give a synthetic toxicity index, a weighted WTI is calculated by combining the cumulative responses at all the dilutions using the dilution factors as weights.[9]

Before calculating the WTI, the mean value and standard deviation of each cellular response must be calculated. Then individual $\Gamma_{i,j}$ values are calculated, as shown in Figure 7. These values are then combined into a cumulative response using the dilution factors as weights.

As shown in Table 1, the WTI of Northern Alberta river water ranged from 0.19 to 13.72 over the three-year period. Overall, 57% of samples had a WTI greater than the cutoff value (i.e., 1), indicating potential toxicity. Broken down by year, 96% of the sites had at least one sample being cytotoxic in 2012, 93% in 2013 (Figure 8), and 90% in 2014. The minimum WTI was similar from year to year (~0.20), indicating a potential baseline value. The maximum toxicity was observed in 2012 and decreased over the years 2013 and 2014. Based on the WTI, PoE, and AUC values, possible hotspots of cytotoxicity were identified.

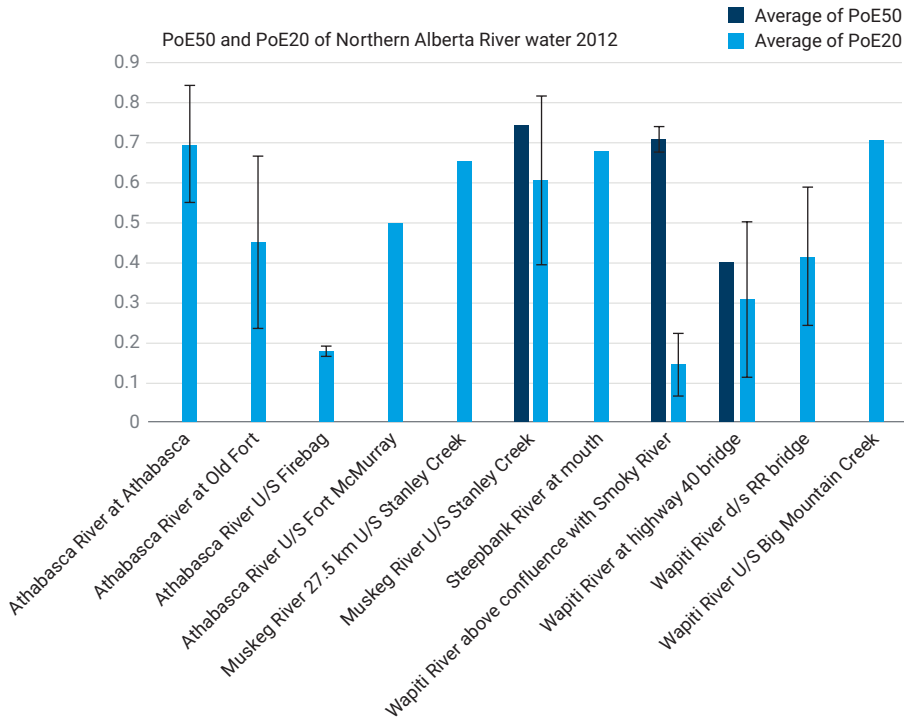


Figure 6. The concentrations that cause 20% and 50% growth inhibition (PoE20 and PoE50) were interpolated from the concentration response curve. Nearly all the sites had at least one sample being cytotoxic in 2012.

$$\Gamma_{i,j} = \frac{Area_{i,j}}{\sum_{k-1}^{K} \sigma_{i,k}}, \quad i = 1,2, \ldots ,12, j = 1,2, \ldots 6$$

$$\tilde{\Gamma}_i = \frac{[\Gamma_{i,1}, \Gamma_{i,2}, \ldots, \Gamma_{i,6}][0.8, 0.6, 0.4, 0.3, 0.2, 0.1]^T}{0.8 + 0.6 + 0.4 + 0.3 + 0.2 + 0.1}, i = 1,2, \ldots ,12$$

Figure 7. Γ is the WTI, σ is the standard deviation of the negative control, i is the number of water samples, and j corresponds to the concentrations of each sample. A WTI value greater than 1 was set as the cut-off for a meaningful biological response.

Table 1. The WTI of Northern Alberta River water.

			Water Toxicity Index								
Year	Sites	Samples	Max	Min	Median	Mean	Geo Mean	<1	1 < 5	5 < 10	10 < 15
2012	25	110	13.72	0.19	1.21	1.59	1.16	44	64		2
SD								0.22	0.78		2.04
2013	29	263	9.84	0.23	1.19	1.68	1.24	115	139	9	
SD								0.21	0.9	1.59	
2014	21	62	5.56	0.26	1.35	1.63	1.25	26	35	1	
SD								0.22	0.99		

Conclusion

A previous study showed the endpoint results using the Agilent xCELLigence RTCA MP system being consistent with traditional Microtox test and cellular responses.[6] In this experiment, it was demonstrated that it can also be used as a high-throughput screening tool to monitor environmental water for cytotoxicity. From this study, results showed that:

- Water toxicity index is a useful metric that incorporates the cumulative responses for all the dilutions into a single value.
- The baseline water toxicity index values for the Northern Alberta rivers are approximately 0.2.
- Water toxicity varied by geography and time, peaking in May and June 2013.
- The Wapiti River was identified as a potential hotspot for future in-depth investigation.

However, there is still a need to fully characterize the most toxic water samples via the adverse outcome pathway approach to classify the toxicants that are causing cytotoxicity.

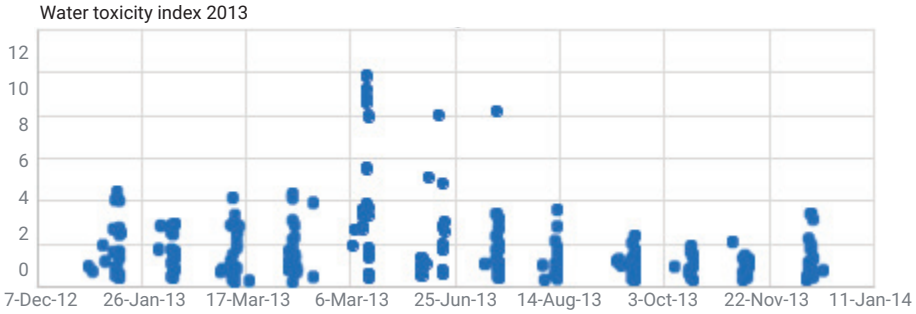


Figure 8. Temporal distribution of water toxicity in the Northern Alberta rivers in 2013.

References

1. Stiborová, M. *et al.* Balkan Endemic Nephropathy: an Update on its Aetiology. *Archives of Toxicology* **2016**, *90(11)*, 2595–2615.
2. White, K. B.; Liber, K. Chronic Toxicity of Surface Water from a Canadian Oil Sands End Pit Lake to the Freshwater Invertebrates Chironomus dilutus and Ceriodaphnia dubia. *Arch. Environ. Contam. Toxicol.* **2020**, *78*, 439–450.
3. Papkovsky, D. B. *et al.* Development of a Novel Environmental Monitoring System based on Optical Oxygen Sensing and Respirometry, (AT-04-01-01) EPA IE, **2009**.
4. Sobus, J. R. *et al.* Integrating Tools for Non-Targeted Analysis Research and Chemical Safety Evaluations at the US EPA, *J. Expo. Sci. Environ. Epidemiol*. **2018**, *28*, 411–426.
5. Burgess, R. M. *et al.* Effects-Directed Analysis (EDA) and Toxicity Identification Evaluation (TIE): Complementary but Different Approaches for Diagnosing Causes of Environmental Toxicity. *Environ. Toxicol. Chem.* **2013**, *32*, 1935–1945.
6. Pan T. *et al.* High-throughput Screening Assay for the Environmental Water Samples Using Cellular Response Profiles. *Ecotoxicol. Environ. Saf.* ***2015***, *114*, 134–142.
7. Alberta Health, Water Toxicity Testing Technical Report, Edmonton, Alberta, Canada, **2012**.
8. Davis, J. Animal Cell Culture: Essential Methods, Wiley-Blackwell, West Sussex, UK, 2011.
9. *IFAC Proceedings Volumes* **2013**, *46(31)*, 309–314.

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