

# Induction of Mammalian Cell Chronic Cytotoxicity and Acute Genomic DNA Damage by Drinking Water Disinfection Byproducts

Michael J. Plewa, Elizabeth D. Wagner  
University of Illinois at Urbana-Champaign

Illinois Water Conference 2008



*water*CAMPUS

# Safe Drinking Water: Benefits and Risks

- 💧 Drinking water disinfection was a major public health triumph of the 20th century. The disinfectants greatly reduced the incidence of typhoid, cholera and other waterborne diseases.
- 💧 Each day water utilities in the U.S.A. produce over  $1.3 \times 10^{10}$  liters of high quality, safe drinking water to over 270 million people.

Richardson SD, Simmons JE, Rice G. 2002. Disinfection by-products: the next generation. Environ Sci Technol 36:1198A-1220A.



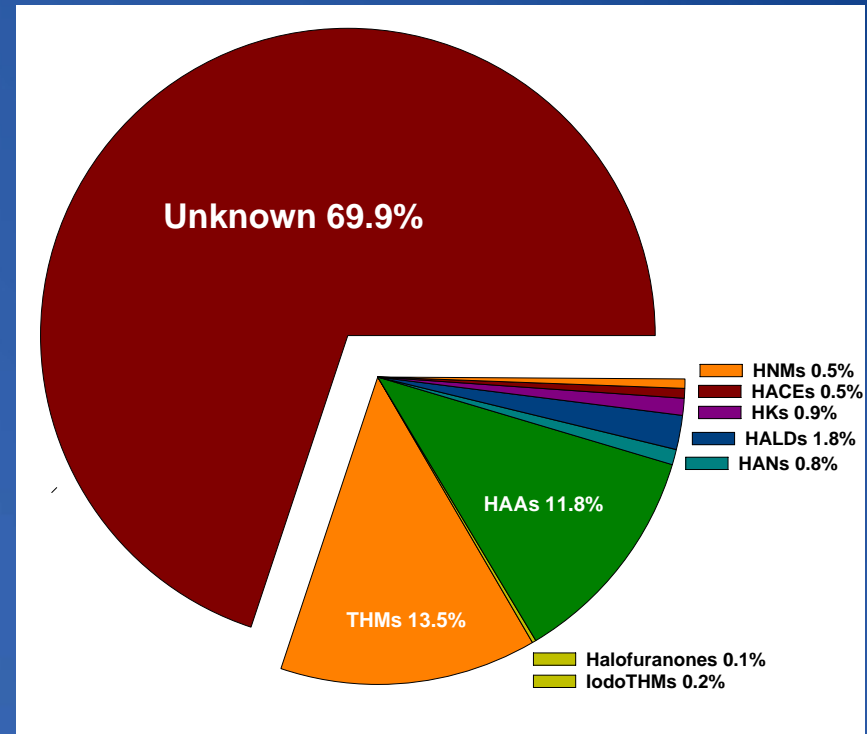
# Safe Drinking Water: Benefits and Risks

- ⦿ However, there is an unintended consequence of disinfection, the generation of **disinfection by-products (DBPs)**.
- ⦿ **DBPs are toxic compounds formed during drinking water disinfection as a result of the reaction between organic materials and disinfectants.**



# After 34 Years How Can DBPs Be Emerging Contaminants?

- 💧 Humic/fulvic structure is undefined; you cannot predict DBPs to close the TOX gap.
- 💧 Analytical chemists dream!
  - Difficult search to identify missing DBPs
  - >600 DBPs identified
  - Regulatory nightmare



Summary distribution of DBP chemical classes in water analyzed in the U.S. EPA Nationwide Occurrence Study as a component of TOX. Data summarized by S. Krasner.



# After 34 Years How Can DBPs Be Emerging Contaminants?

- ◆ Decreasing supplies of pristine waters → use of impaired waters
  - Algal impacts
  - Wastewater impacts and wastewater use (CA and FL)
  - Use of impaired waters → more organic-N → Nitrogenous DBP precursors

Schreiber IM and Mitch WA. 2006. Occurrence and fate of nitrosamines and nitrosamine precursors in wastewater impacted surface waters using boron as a conservative tracer. *Environ Sci Technol* 40:3203-3210.



# After 34 Years How Can DBPs Be Emerging Contaminants?

- 💧 U.S. EPA Stage 2 DBP Rule to reduce THMs/HAA5
  - Utilities switching to ozonation and chloramination.
  - **Unexpected consequences** → Promote N-DBP and Iodo-DBP formation.
- 💧 U.S. EPA and AWWA recommend more research in N-DBPs, I-DBPs, and I-N-DBPs.
  - EPA Workshop 2005; AWWA meeting 2005, 2006; Gordon Research Conference 2006.
  - The **first comprehensive review of DBP occurrence, genotoxicity and carcinogenicity**: *Mutation Research, 2007*.

Richardson SD, Plewa MJ, Wagner ED, Schoeny R, DeMarini DM. 2007. Occurrence, genotoxicity, and carcinogenicity of emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutation Res* 636:178-242 .



# Solutions to the Impediments for a Comparative Toxicology of DBPs

- 💧 Structure-Function Activity studies to define high priority DBPs.
- 💧 EPA Nationwide Occurrence Study.
- 💧 New analytical biological tools to integrate analytical toxicology with the analytical chemistry of DBPs.

Woo Y-T, Lai D, McLain JL, Manibusan MK, Dellarco V. 2002. Use of mechanism-based structure-activity relationships analysis in carcinogenic potential ranking for drinking water disinfection by-products. *Environ Health Perspect* 110 (Supp 1):75-87.

Weinberg HS, Krasner SW, Richardson SD, Thruston, AD, Jr. 2002. *The Occurrence of Disinfection By-Products (DBPs) of Health Concern in Drinking Water: Results of a Nationwide DBP Occurrence Study*, U.S. Environmental Protection Agency, National Exposure Research Laboratory, Athens, GA. EPA/600/R-02/068; [www.epa.gov/athens/publications/EPA\\_600\\_R02\\_068.pdf](http://www.epa.gov/athens/publications/EPA_600_R02_068.pdf)

Plewa MJ, Kargalioglu Y, Vankerk D, Minear RA, Wagner ED. 2002. Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. *Environ Molec Mutagenesis* 40:134-142.

# Research Objectives

- Obtain samples (~50 mg) of synthesized, analytical grade DBPs from U.S. EPA.
- Analyze the direct-acting cytotoxicity and genomic genotoxicity of the individual DBPs with Chinese hamster ovary (CHO) cells.
- Determine the cytotoxic and genotoxic rank order of the DBPs.
- Develop a quantitative and comparative DBP toxicity database.

Plewa MJ, Wagner ED. 2004. Quantitative Comparative Mammalian Cell Cytotoxicity and Genotoxicity of Selected Classes of Drinking Water Disinfection By-Products. AwwaRF Grant #3089.





# Chinese Hamster Ovary Cells

CHO Cells, AS52, Clone 11-4-8



- ◆ CHO cells are widely used in toxicology research.
- ◆ Clone 11-4-8 was isolated by our laboratory and expresses a stable chromosome complement.
- ◆ CHO AS52 cells express functional p53 protein and are competent for DNA repair.
- ◆ The cells exhibit normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci.

Wagner ED, Rayburn AL, Anderson D, Plewa MJ. 1998. Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation in an isolated clone of Chinese hamster ovary cells. *Environ Molec Mutagenesis* 32:360-368.

Rundell MS, Wagner ED, Plewa MJ. 2003. The comet assay: Genotoxic damage or nuclear fragmentation? *Environ Molec Mutagenesis* 42:61-67.



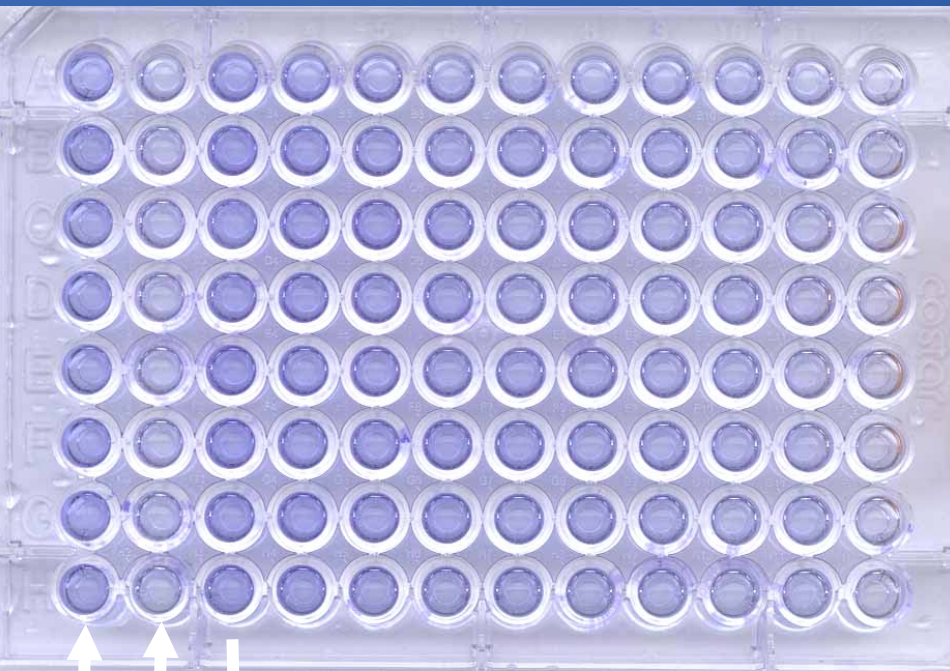
# Mammalian Cell Chronic Cytotoxicity Assay

- Standard plating methods to measure toxicity are laborious, time consuming, expensive and require large amounts of sample.
- We developed a rapid, semi-automated, microplate-based, chronic cytotoxicity assay that measures the impact of a specific DBP on CHO cells to grow over a 72 h (~3 cell cycles) period.

Plewa MJ, Kargalioglu Y, Vankerk D, Minear RA, Wagner ED. 2000. Development of quantitative comparative cytotoxicity and genotoxicity assays for environmental hazardous chemicals. *Water Sci Technol* 42:109-116.



# Cytotoxicity Absorbancy Data: Dibromoacetamide



control

blank

DBP concentrations

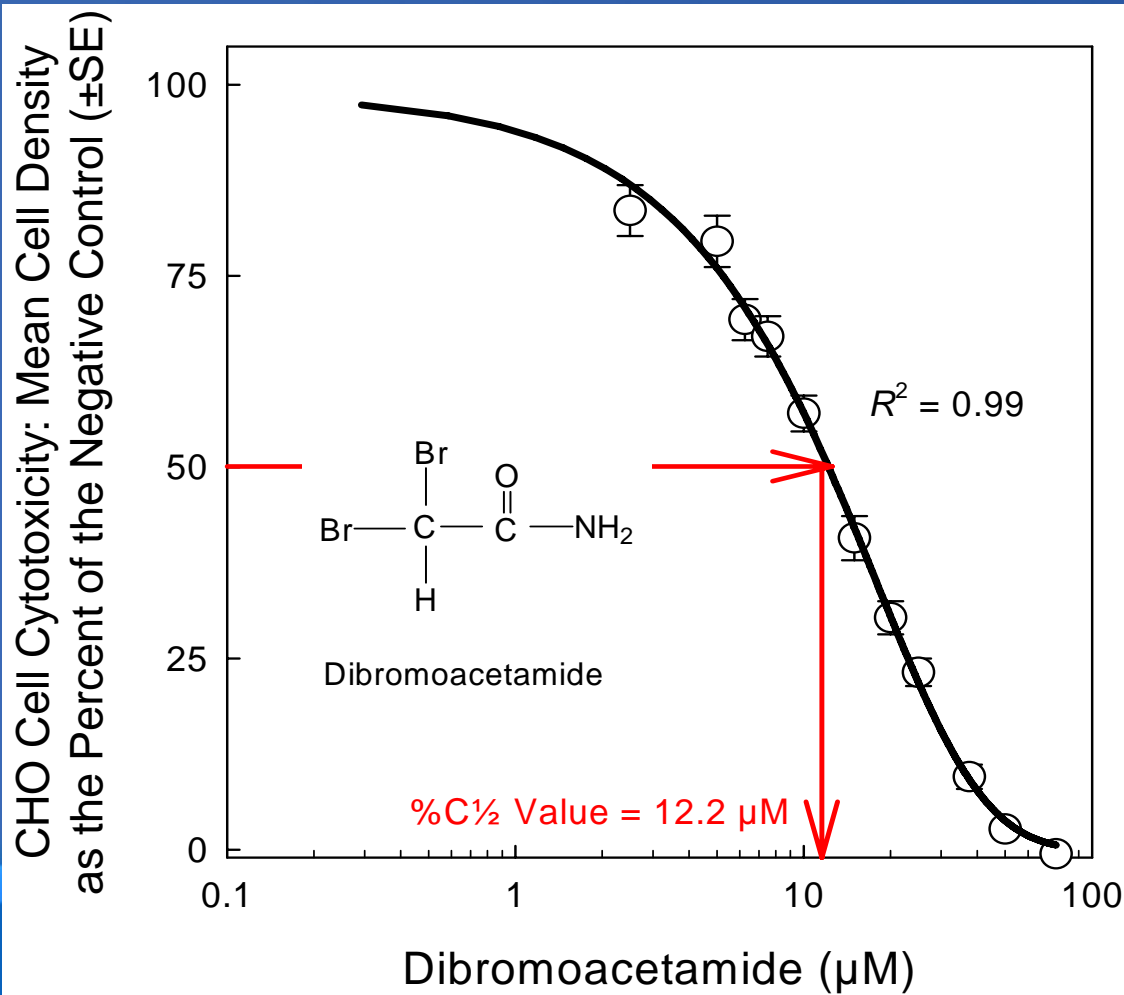
Wash cells, fix with MeOH, stain, wash and scan on microplate reader at 595 nm.

Blank correct data

Normalize data to a percentage of the concurrent negative control

Save data as a spreadsheet file

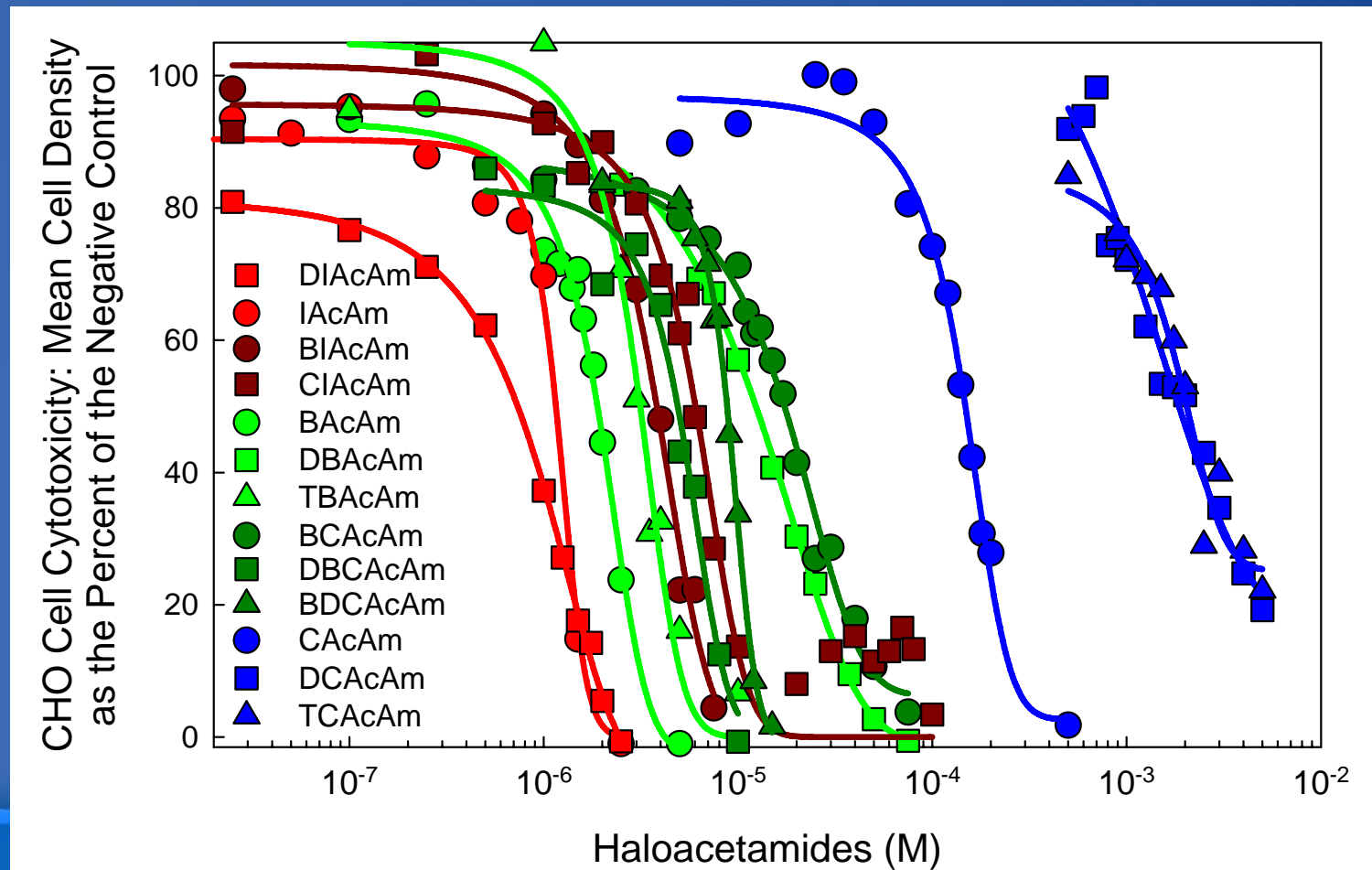
# CHO Cell Chronic Cytotoxicity of Dibromoacetamide: %C<sub>1/2</sub> Value



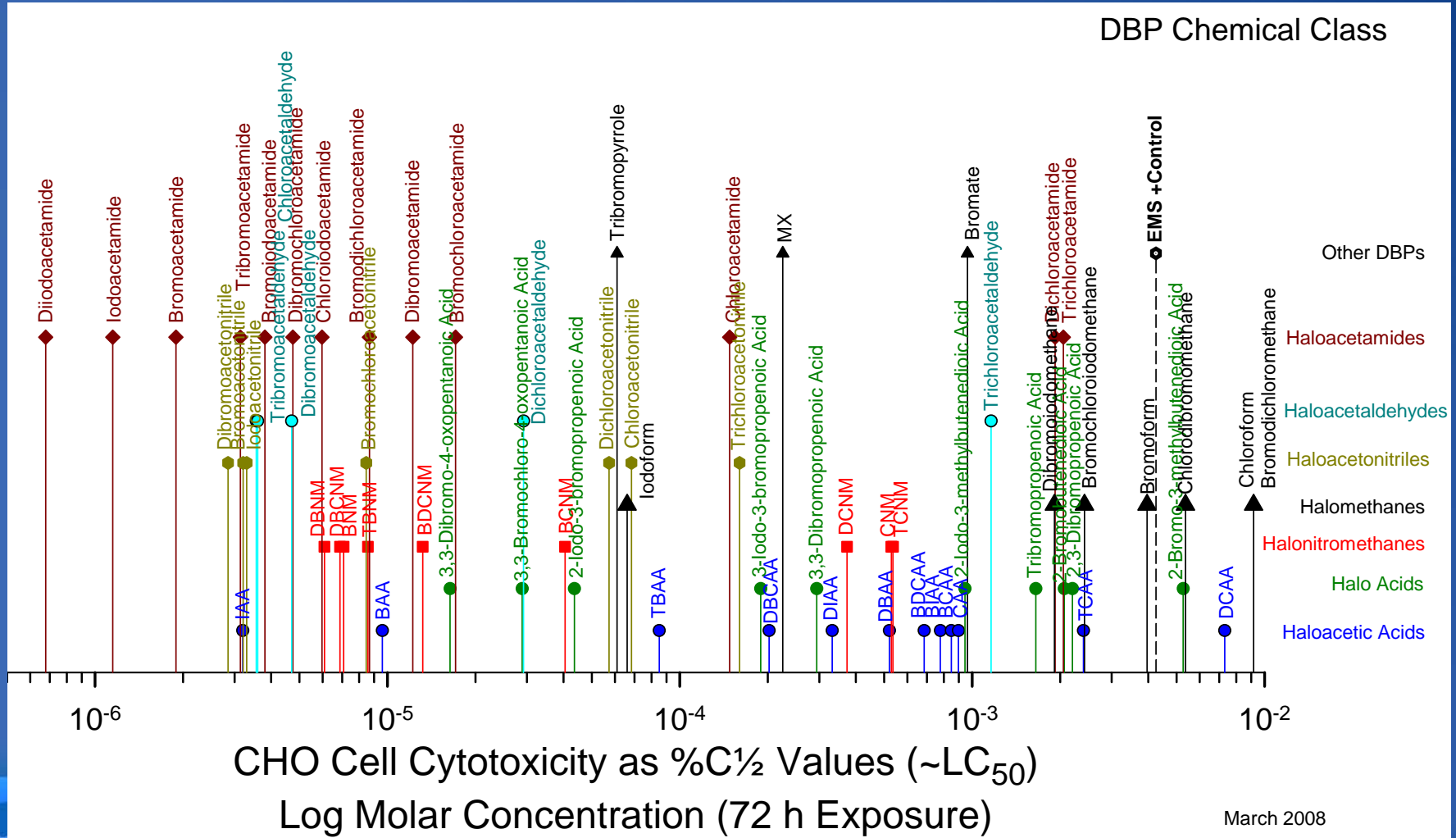
The %C<sub>1/2</sub> value is the concentration of each test agent that reduced the CHO cell density by 50% as compared to the negative control.

The %C<sub>1/2</sub> value is analogous to the LC<sub>50</sub> measurement.

# Comparative Cytotoxicity for the Haloacetamides



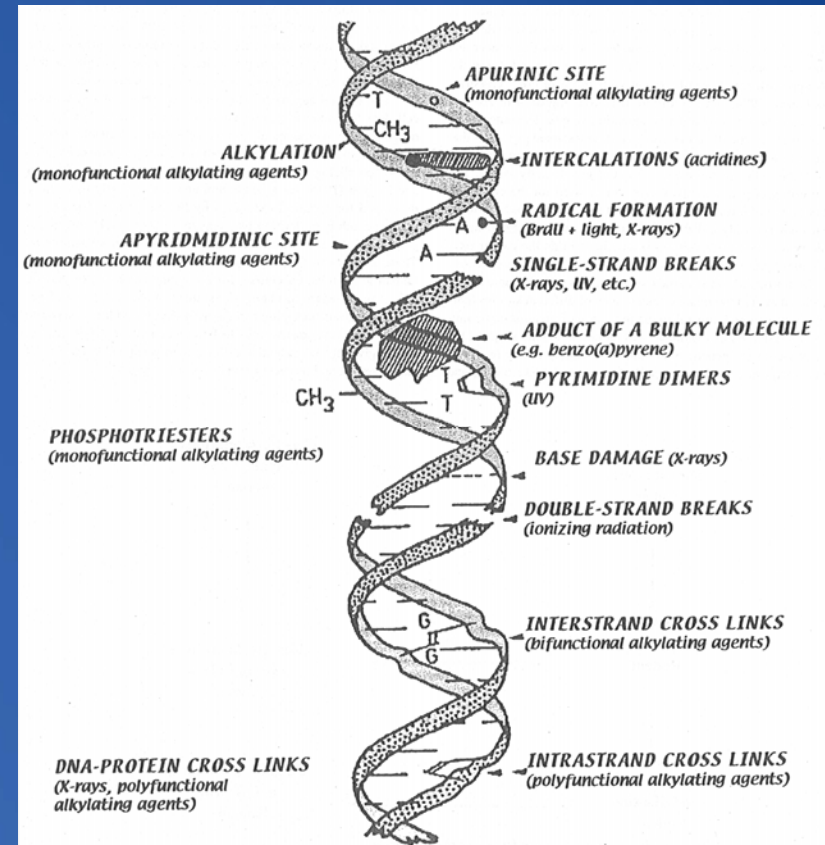
# Comparative DBP CHO Cell Chronic Cytotoxicity Database



# Genomic DNA Damage Induced by Drinking Water Disinfection By-Products

## Single Cell Gel Electrophoresis

The target is the genome, not just a gene.



Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu J-C, Sasaki YF. 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Molec Mutagenesis* 35:206-221.

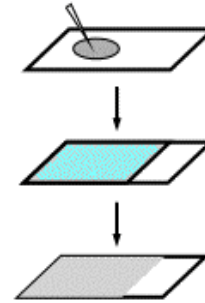
# Single Cell Gel Electrophoresis

- CHO cells grown in microplate wells were treated with a test agent for 4h.
- The cells were harvested and placed in an agarose sandwich upon a SCGE microscope slide – the microgel.
- The cell membranes were lysed and the DNA in the nuclei were denatured.
- The naked nuclei were electrophoresed at a pH >13.5.
- The microgels were neutralized and stained with ethidium bromide.
- Each microgel was analyzed with a fluorescence microscope with a CCD computer interface.
- The data were stored in computer spreadsheets.

## COMET ASSAY PROTOCOL

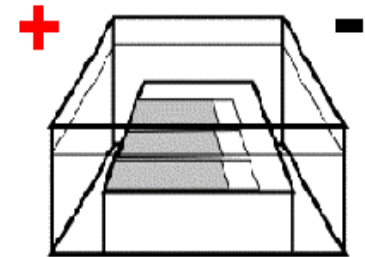
### Making slides

Nuclei + agarose



### DNA unwinding and electrophoresis

300 mM NaOH, pH 13.5

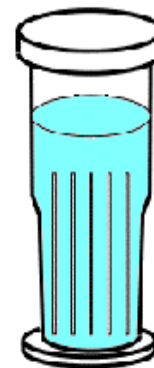


### Neutralization

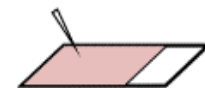


### Lysing

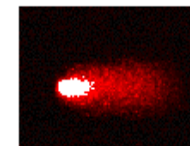
2.5 M NaCl, 10 % DMSO



### Dying

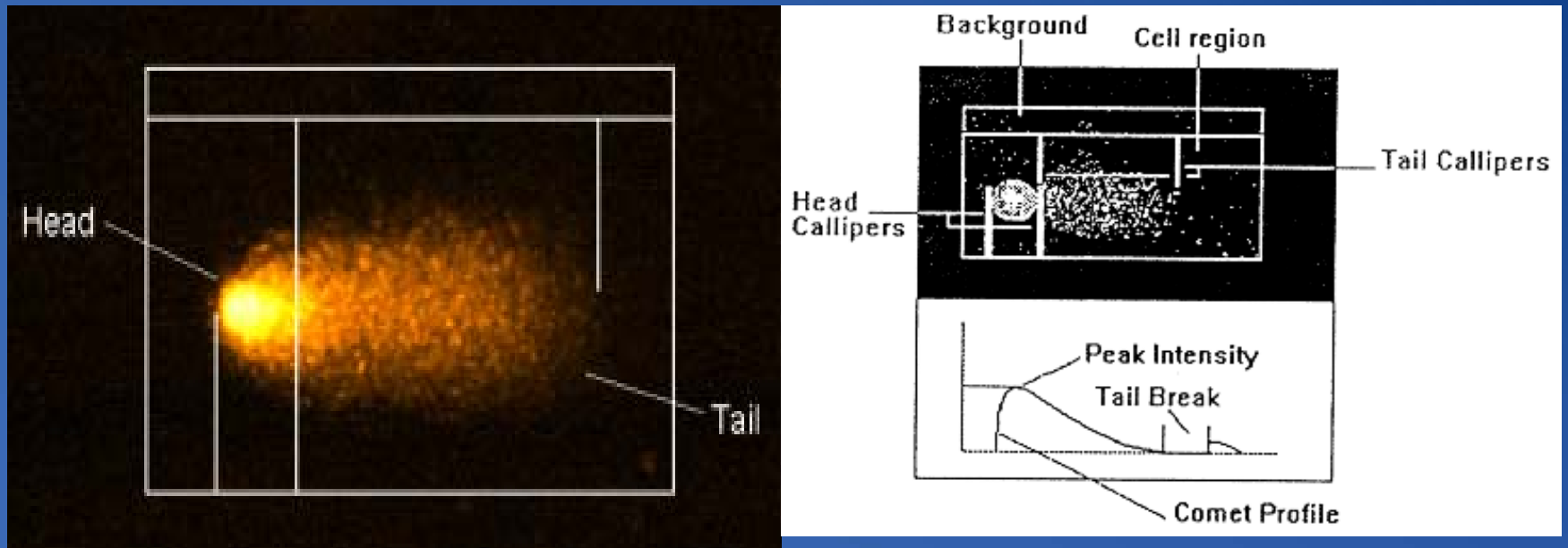


### Comet analysis



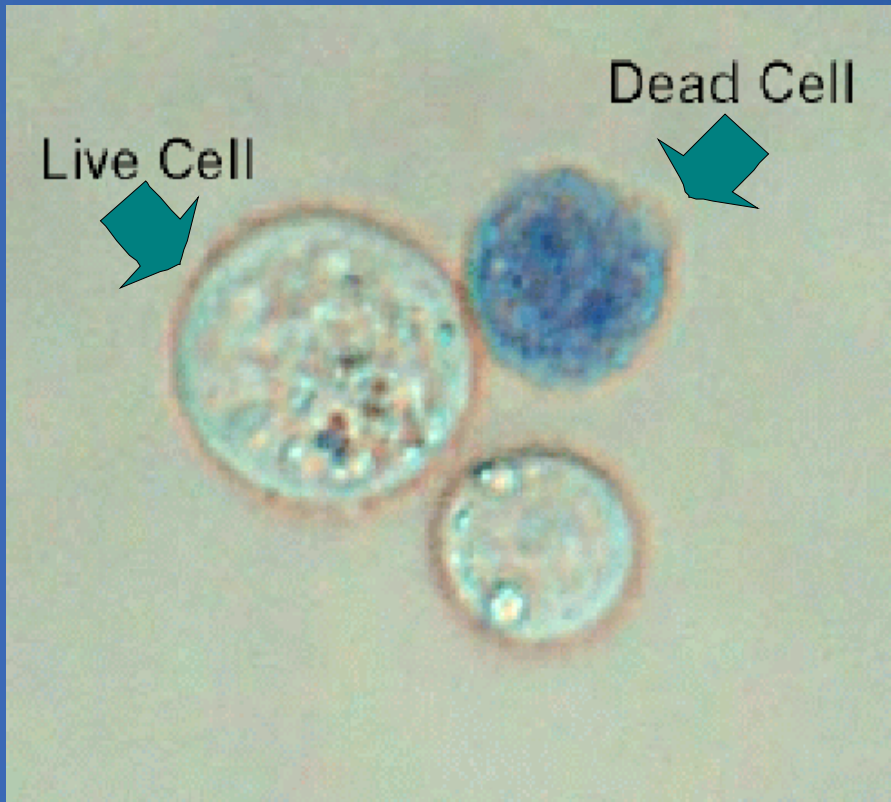


# Computer Analysis of SCGE Images



- The nuclei were analyzed with a Zeiss fluorescence microscope using an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. A computerized image analysis system was employed to measure various Comet parameters. (Komet 3.1, Kinetic Imaging Ltd).
- The **tail moment** is the integrated value of DNA density multiplied by the migration distance. The **% tail DNA** is the amount of DNA that has migrated into the gel from the nucleus.

# Acute Cytotoxicity

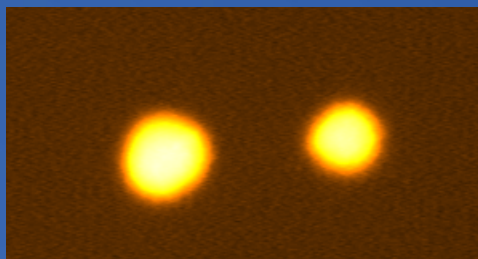


- From each treated cell suspension a 10  $\mu$ l aliquot was stained with 10  $\mu$ l of 0.05% trypan blue vital dye in PBS.
- The percent survival for each treatment group was determined by counting the dead cells (blue) and the live cells (clear).

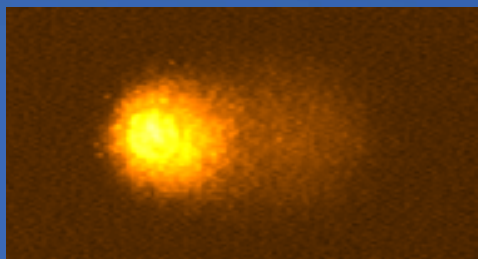


# Genomic DNA Damage Induced by Dibromonitromethane

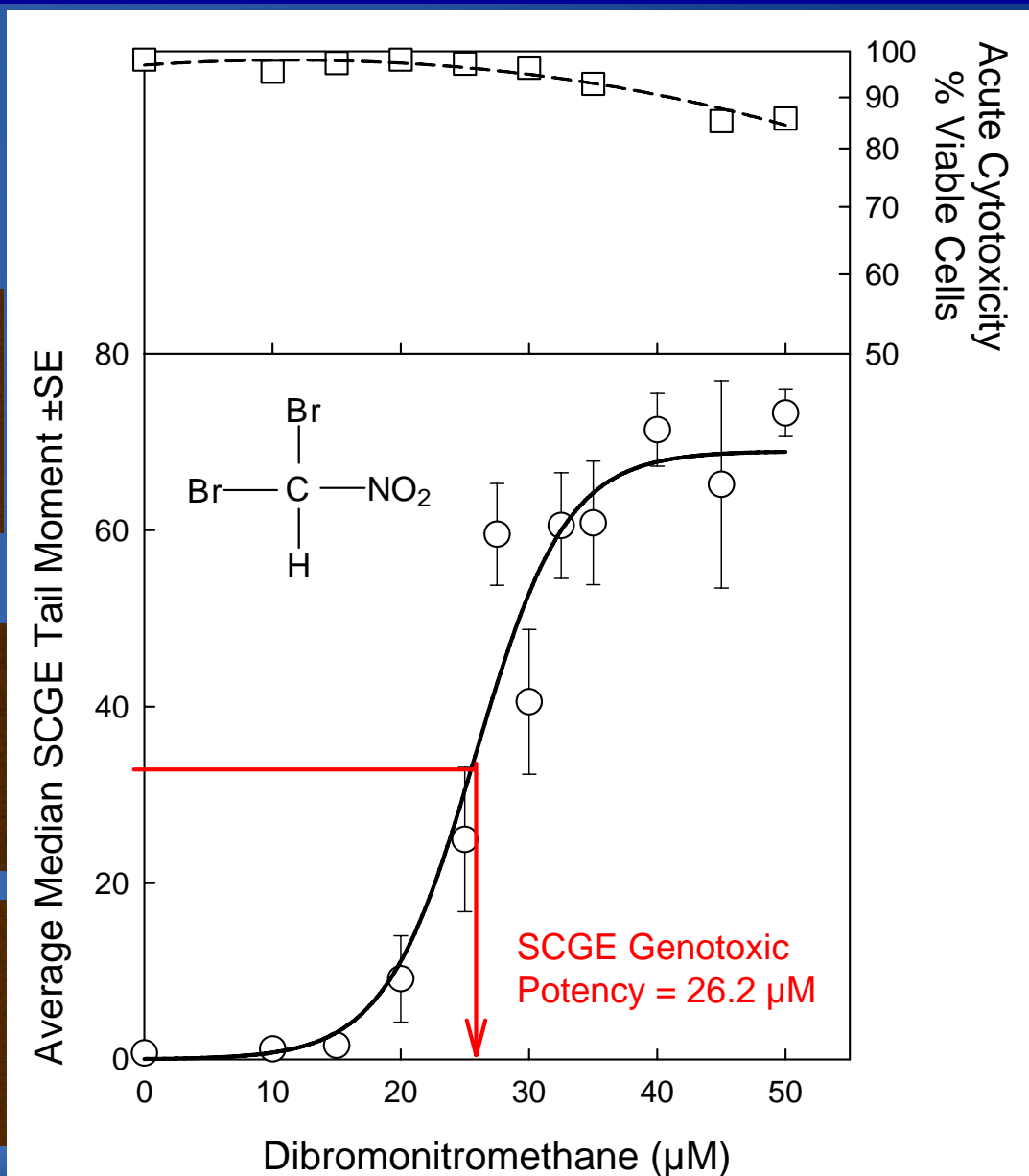
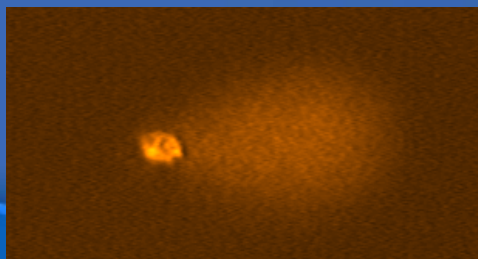
Control



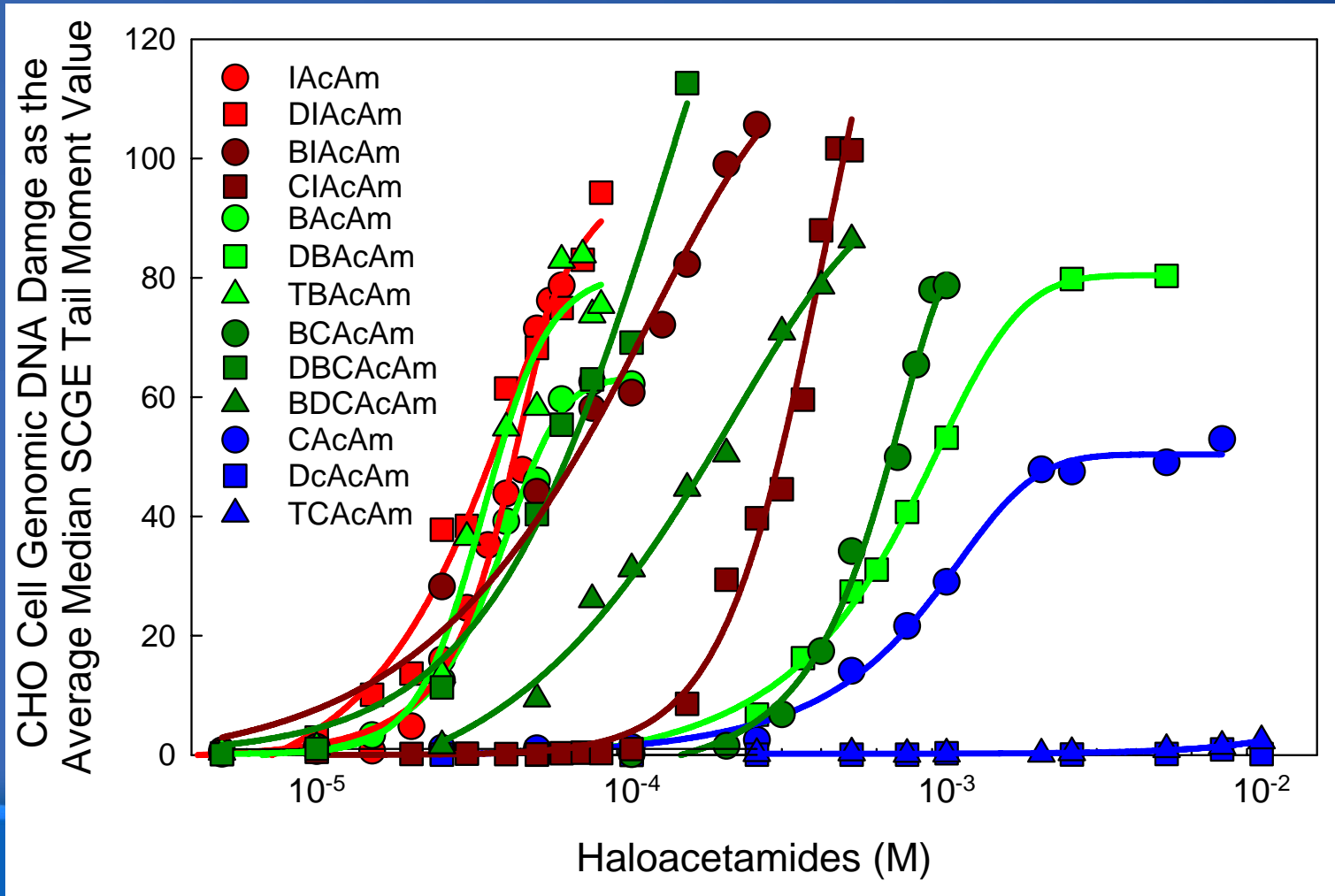
30  $\mu\text{M}$   
DBNM



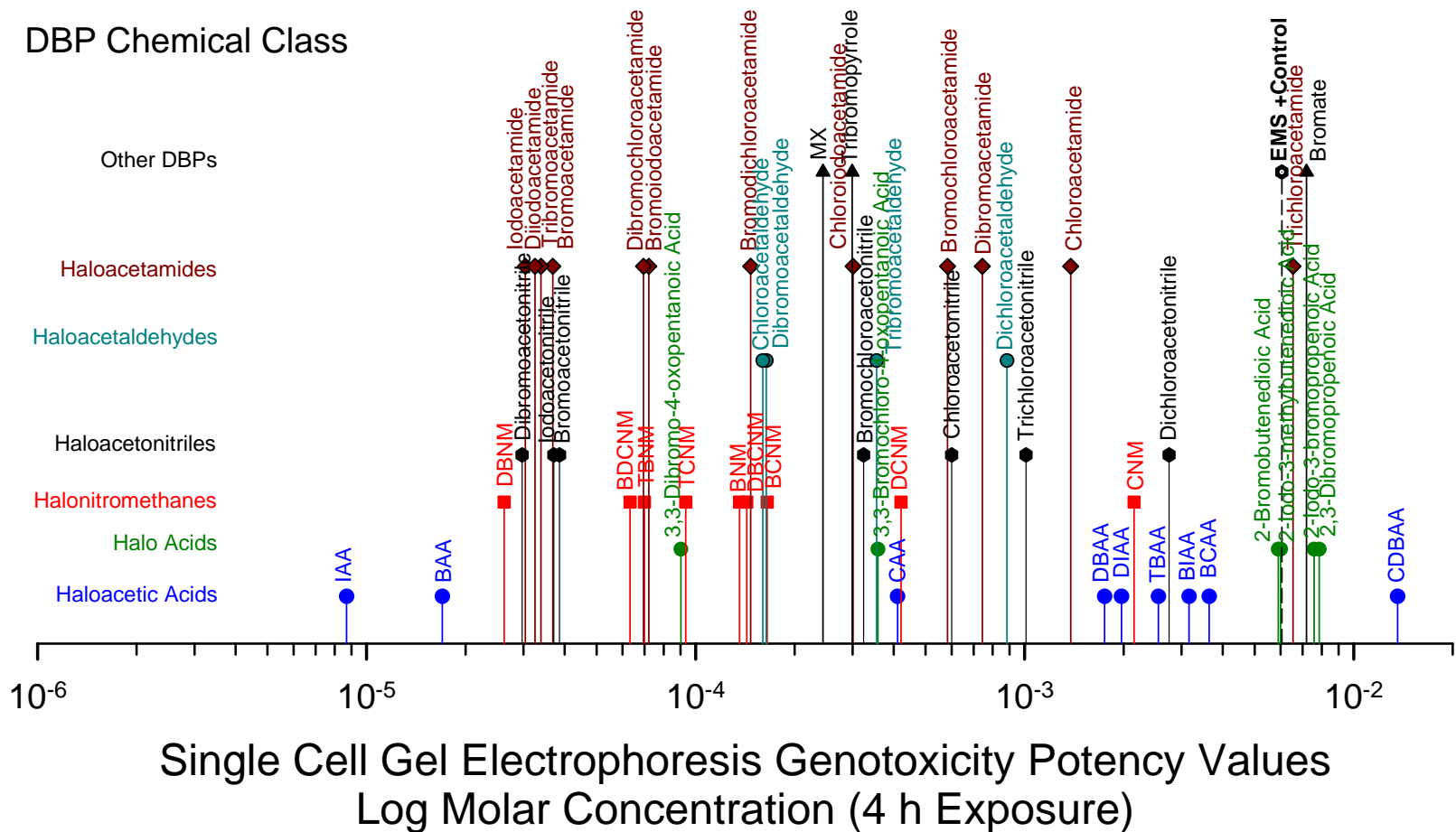
40  $\mu\text{M}$   
DBNM



# Comparative Genotoxicity of the Haloacetamides



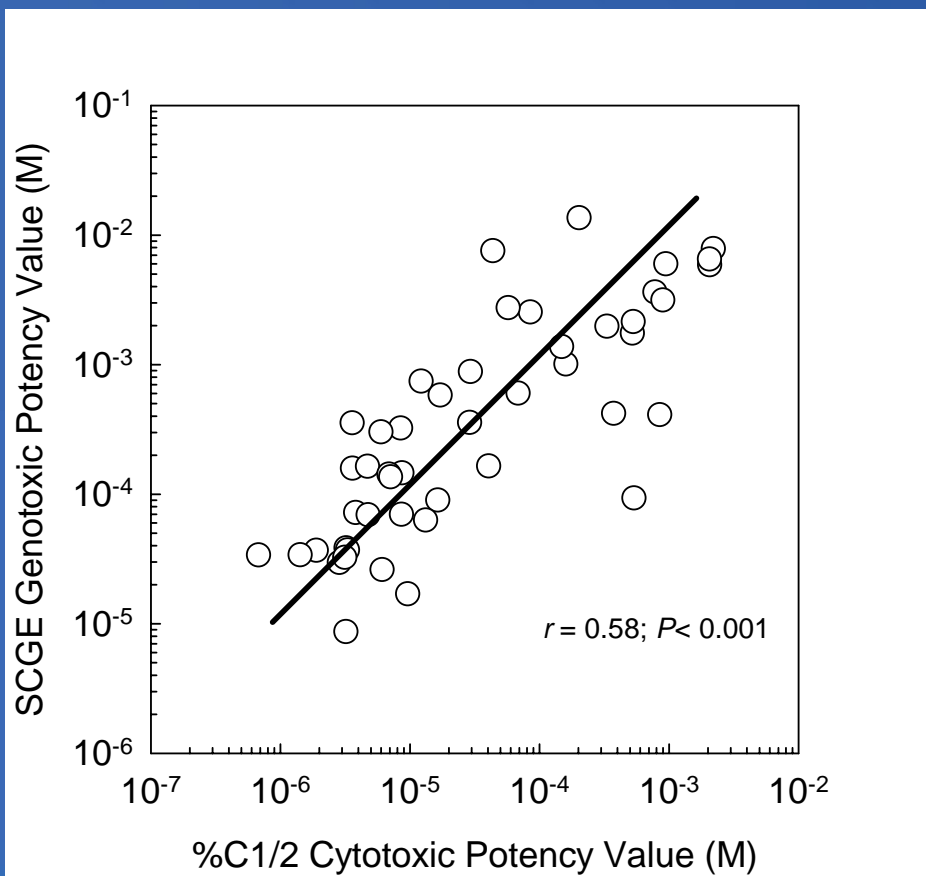
# Comparative DBP CHO Cell Acute Genotoxicity Database



**Not Genotoxic:** DCAA, TCAA, BDCAA, Dichloroacetamide, Chloroform, Chlorodibromomethane, Bromoform, Iodoform, Bromochloriodomethane, Dibromiodomethane, Bromodichloromethane, 3,3-Dibromopropenoic Acid, 3-Iodo-3-bromopropenoic Acid, 2,2,3,3-Tribromopropenoic Acid, trans-2-bromo-3-methylbutenedioic Acid, Trichloroacetaldehyde

March 2008

# Comparison Between CHO Cell Cytotoxicity and Genotoxicity of DBPs



- A large dataset was available for a correlation test.
- This consisted of six chemical classes of DBPs and related compounds that induced significant chronic cytotoxicity and acute genotoxicity.
- A direct, highly significant ( $P \leq 0.001$ ) correlation was observed ( $r = 0.58$ ).

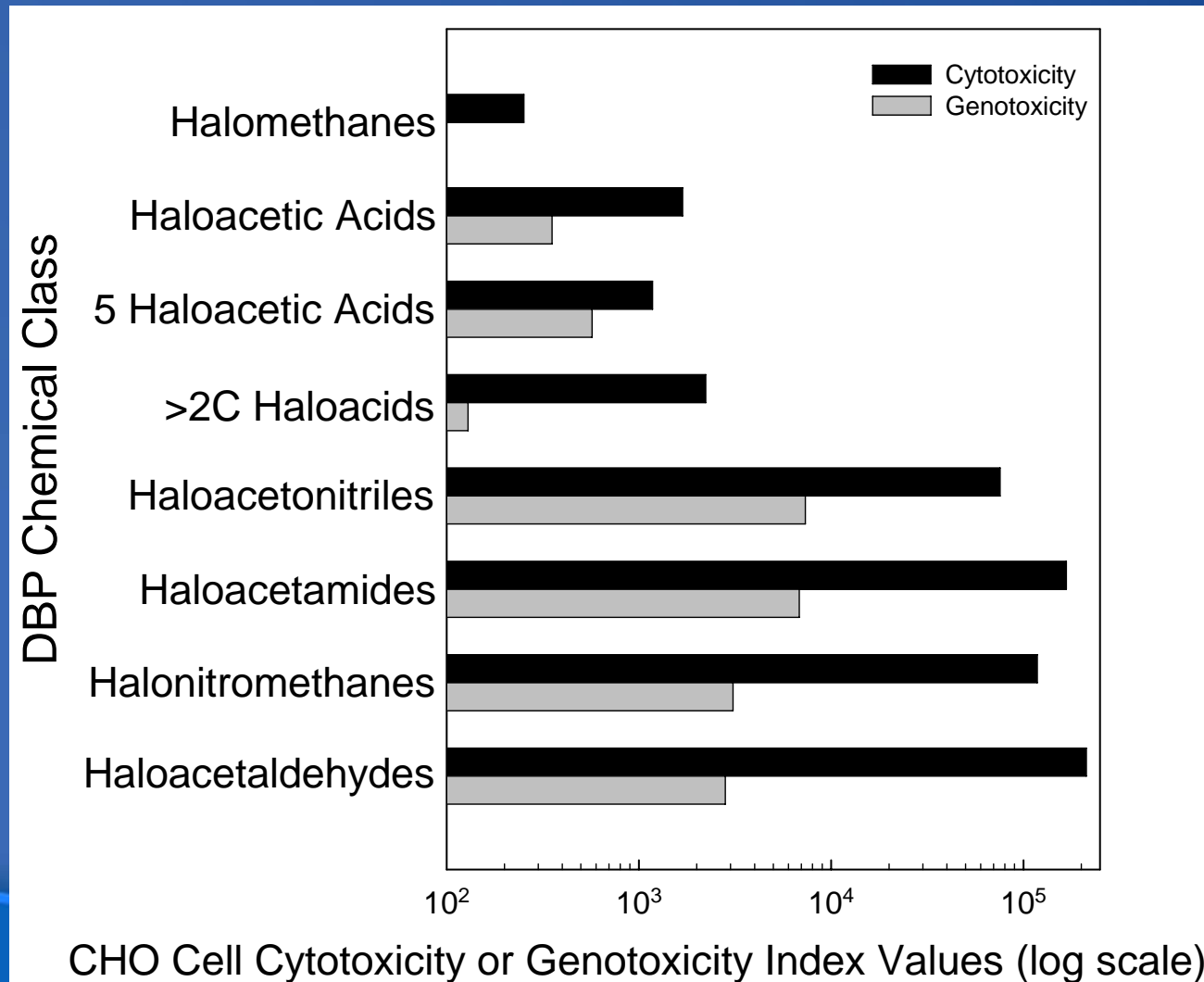
Plewa, M. J.; Wagner, E. D., *Quantitative Comparative Mammalian Cell Cytotoxicity and Genotoxicity of Selected Classes of Drinking Water Disinfection By-Products*. Am. Water Works Res. Foundation: Denver, CO, 2008 (In Press).

# Conclusions I

- 💧 With our current database, ~70 DBPs were compared on a level toxicological playing field.
- 💧 We can quantitatively compare the cytotoxicity of DBPs using their %C<sub>1/2</sub> values.
- 💧 We can quantitatively compare the genotoxicity of DBPs using the SCGE Genotoxic Potency values.
- 💧 We can compare classes or specific groups of DBPs based on their **Toxicity Index**. Within a class, the reciprocal of the averaged median %C<sub>1/2</sub> values is the **cytotoxicity index value** and the reciprocal of the averaged median genotoxic potency values is the **genotoxicity index value**.

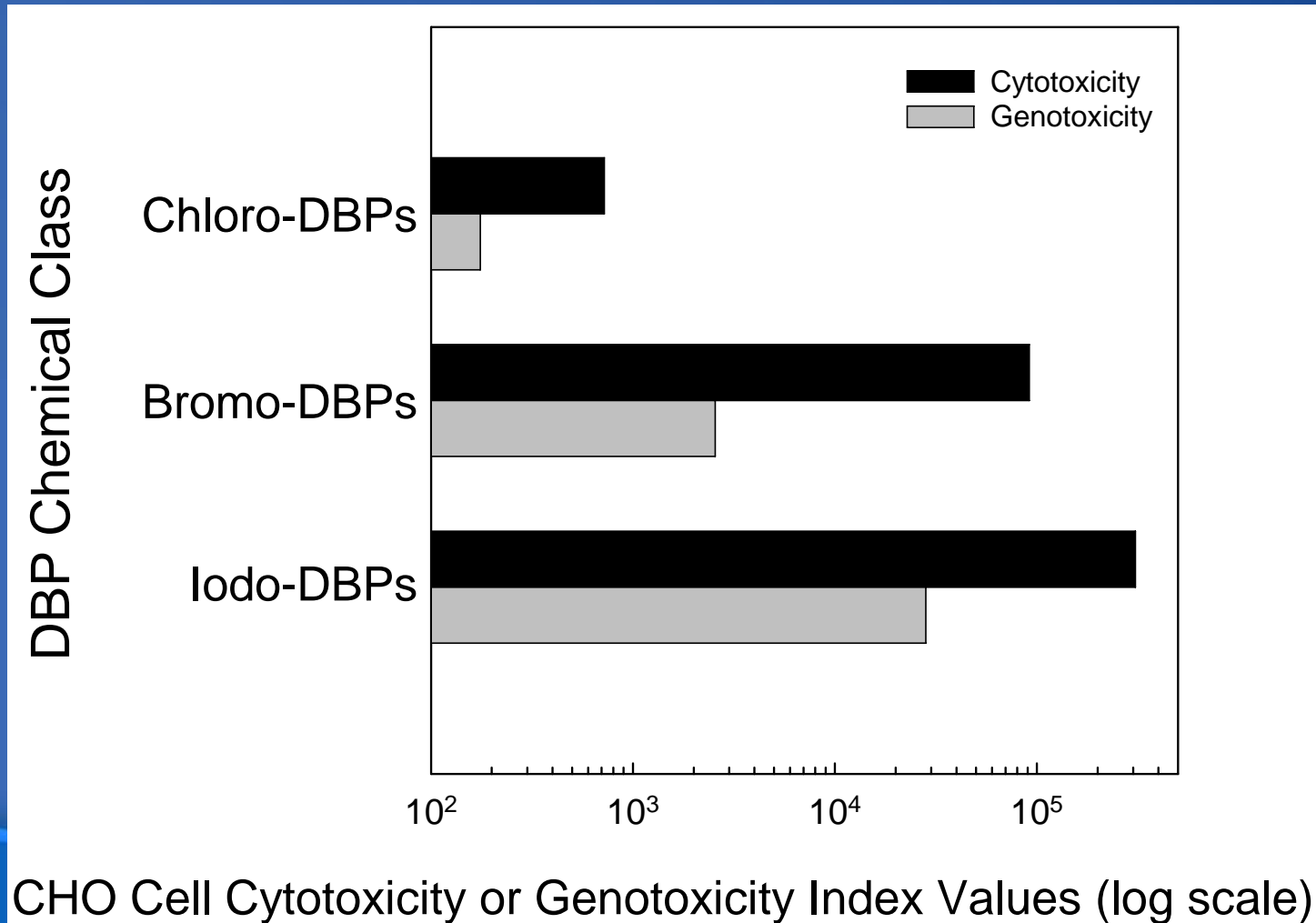


# Comparison of the Cytotoxicity and Genotoxicity of DBP Classes

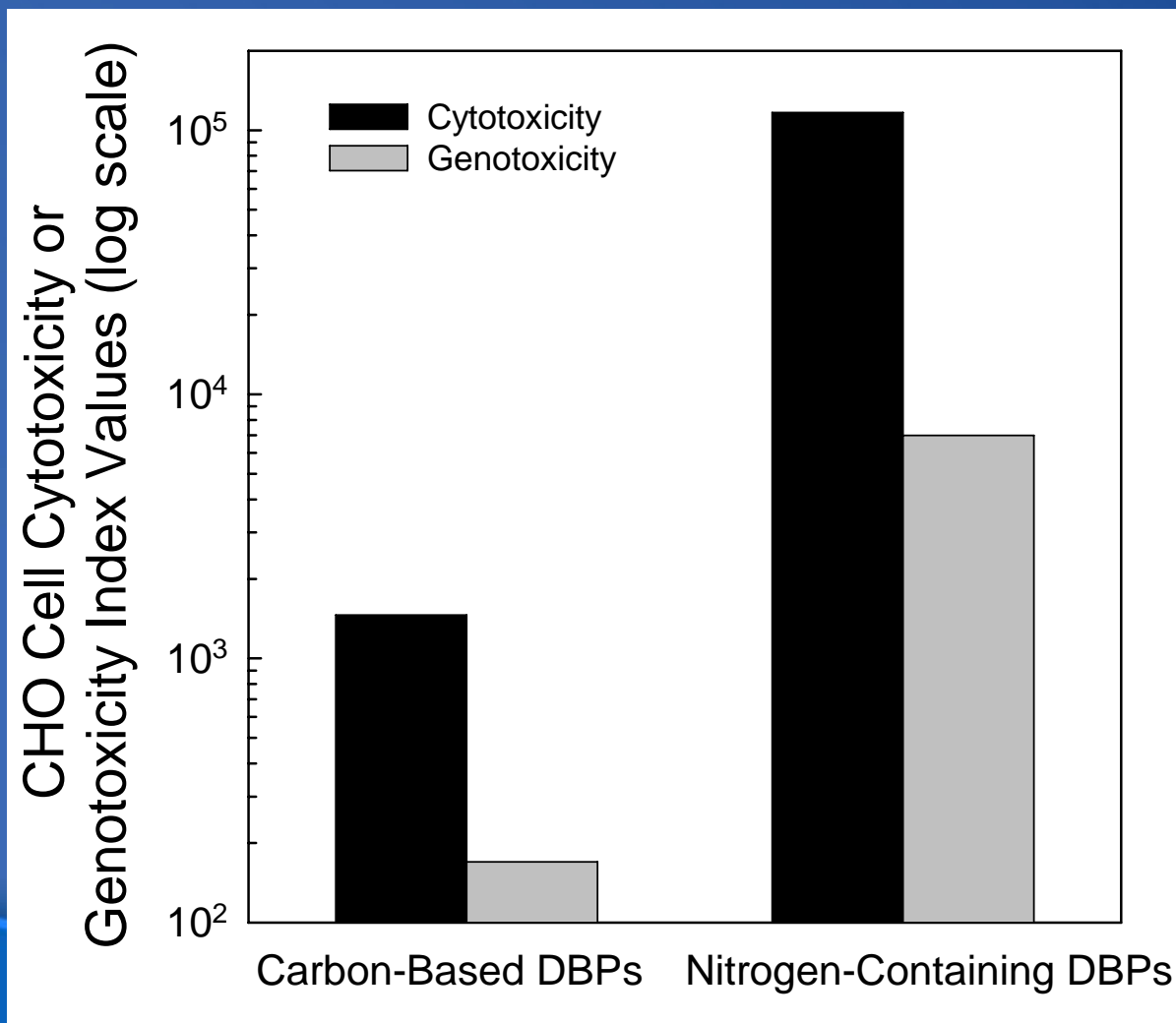




# Toxicity Index of DBP Classes: (impact of the halogen leaving group)



# Toxicity Index of DBP Classes: (C-DBPs versus N-DBPs)



# Conclusions II

- The current U.S. EPA-regulated DBP classes (THMs and HAAs) are substantially less toxic than emerging DBPs.
- Iodinated-DBPs are far more toxic than their brominated and chlorinated analogs.
- N-DBPs are much more toxic than C-DBPs.
- The occurrence of these emerging DBPs are on the rise because of changes in source water quality and the increased use of alternative water disinfectants.
- These emerging DBPs may pose adverse health risks.



# Future Studies

- Expand the number of DBP classes analyzed (cyanogen halides, aldehydes ketones, nitrosamines etc.)
- Use the database to guide the analysis of drinking water for emerging DBPs.
- Improve the resolution of the *in vitro* toxicity assays and determine the mechanism of toxic action by the use of human toxicogenomic analysis.



# Students and Colleagues

- 💧 Dr. Yahya Kargalioglu
- 💧 Dr. Mark Muellner
- 💧 Ms. Nancy Hsu
- 💧 Dr. Eduardo Cemeli
- 💧 Mr. Justin Pals
- 💧 Ms. Jessica Wallace
- 💧 Ms. Yukako Kumada
- 💧 Dr. Susan Richardson
- 💧 Dr. Yin-Tak Woo
- 💧 Dr. Bruce McKague
- 💧 Dr. Matt Hudson
- 💧 Dr. Benito Mariñas

Funded in part by AwwaRF Grants 554 & 3089, U.S. EPA Grant CR83069501, Illinois-Indiana Sea Grant R/WF-09-06, and WaterCAMPWS NSF Center Grant CTS-0120978.

M. Muellner was supported by T32 ES07326 NIH Predoctoral Fellowship.

