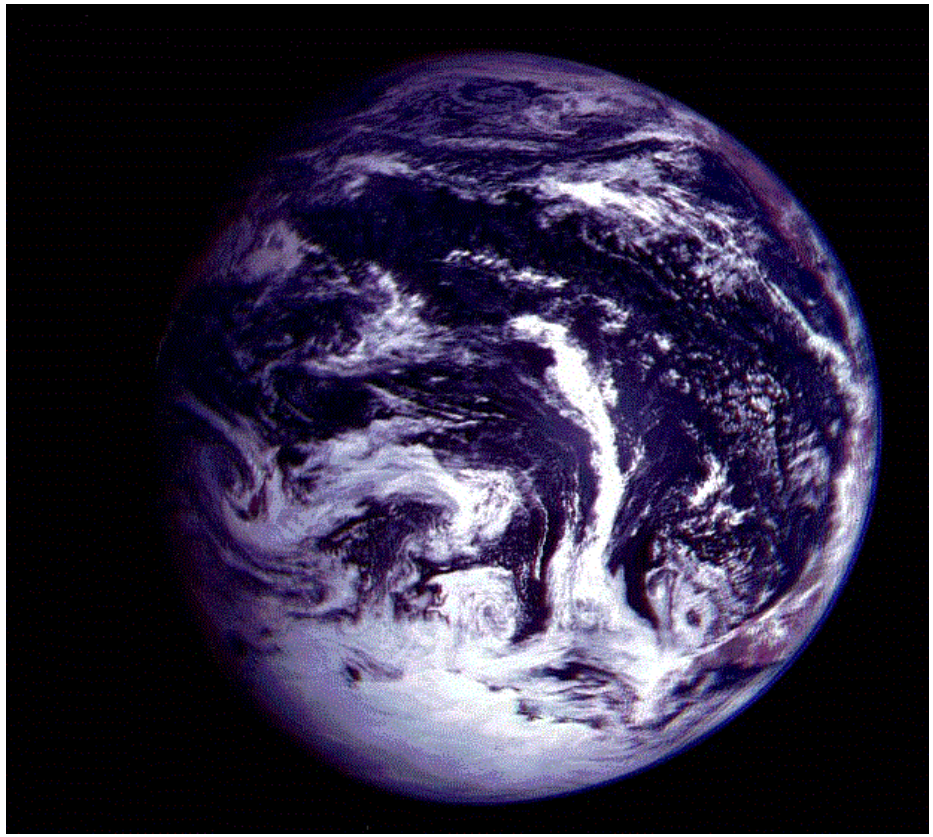
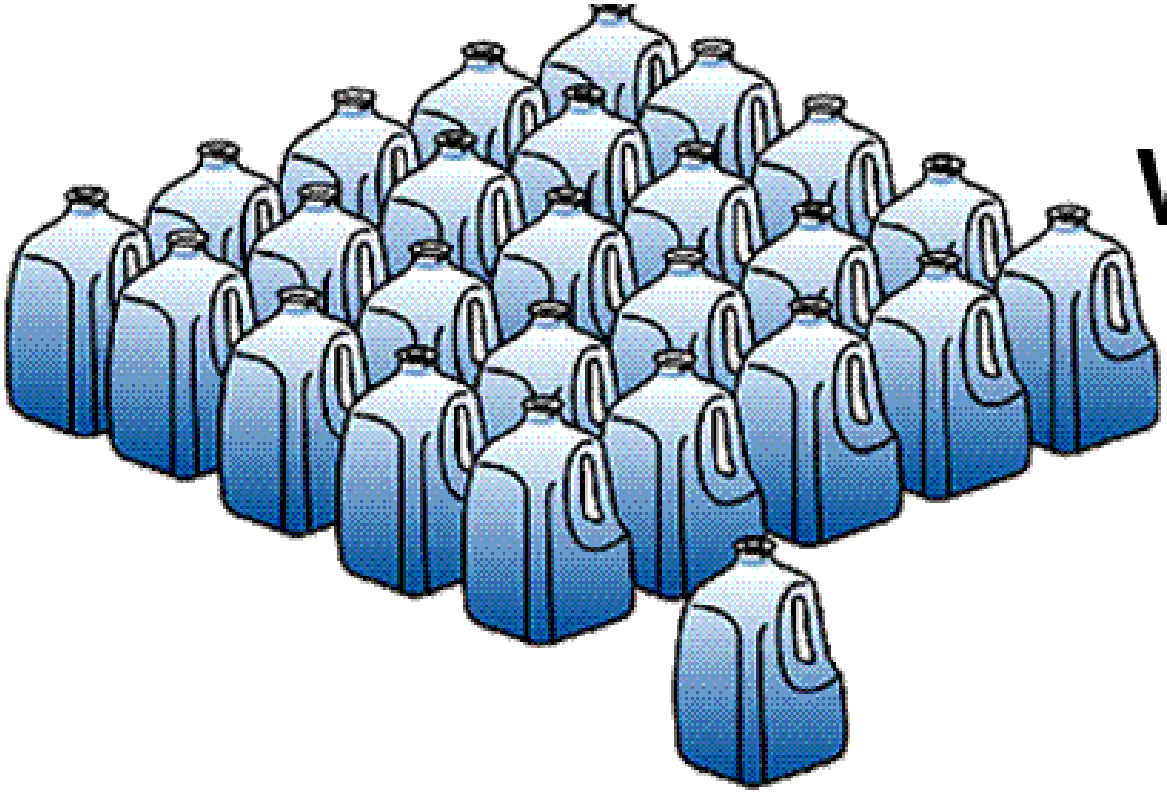
Mammalian Cell Cytotoxicity and Genotoxicity of New Drinking Water Disinfection By-Products

U.S. EPA Region 5 ORD STAR Seminar

Dr. Michael J. Plewa
Professor of Genetics
University of Illinois at Urbana-Champaign

Water is the best of all things.
Pindar (438 BC), Olympian Odes





World's Water Supply

100 liters
(26 gallons)

Total water
100%

3 liters (0.8 gallon)
Fresh water
3%

0.003 liter (1/2 teaspoon)

Readily available
fresh water 0.003%

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. However, there is an unintended consequence of disinfection, the generation of chemical disinfection by-products (DBPs).

Drinking Water Disinfection By-Products (DBPs)

- DBPs are compounds formed during drinking water disinfection as a result of the reaction between naturally occurring organic materials, synthetic organic contaminants and disinfectants.
- Between 51% and 92% of DBP products are unknown in the halogenated organic fraction (TOX) depending on the disinfection process.

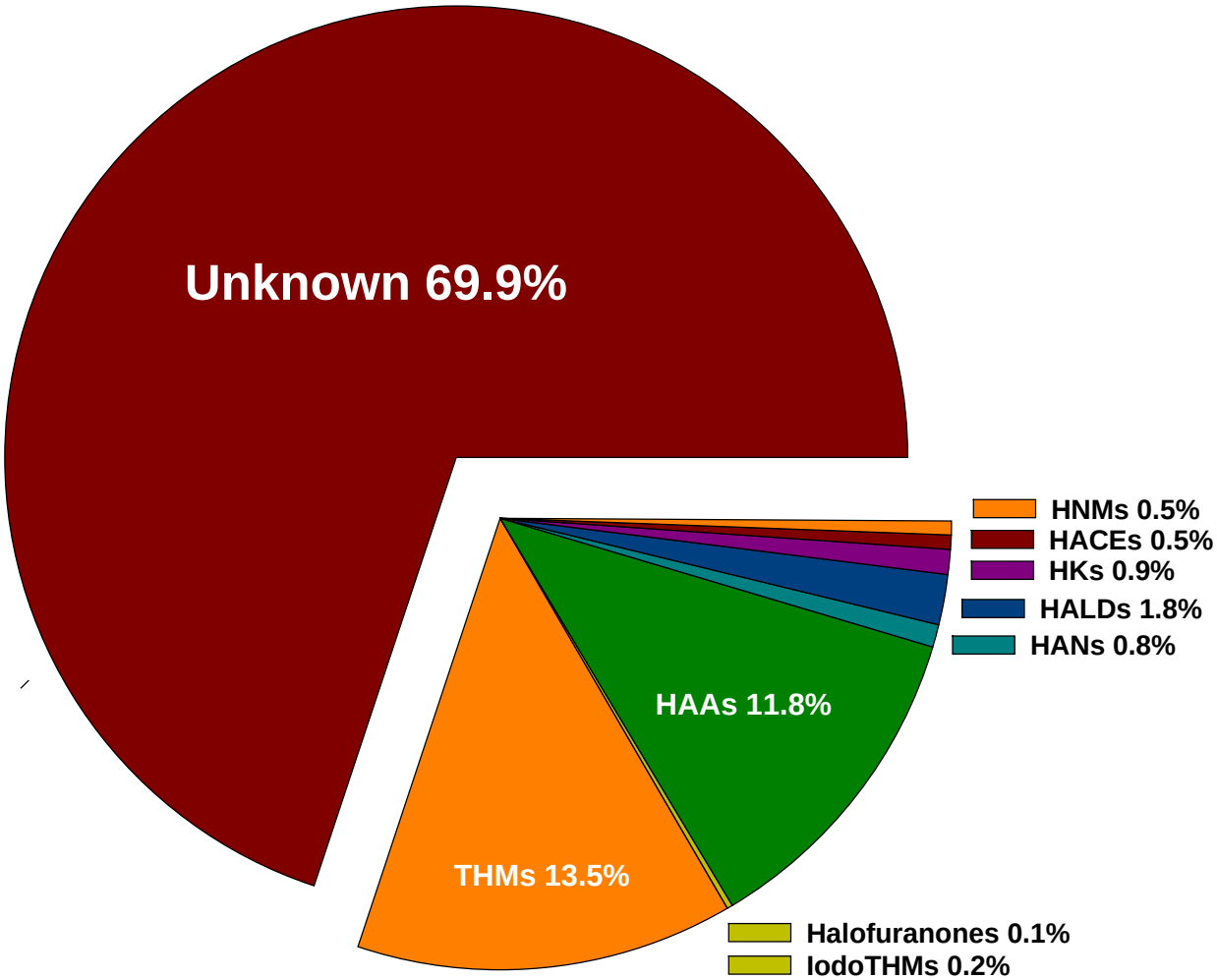
Regulation of DBPs

- The health risks due to DBPs are not fully known, however, a substantial number of these agents were demonstrated to be toxic in many biological assays. In 1979 the EPA began the formal regulation of DBPs.
- The regulation was extended in 1998 with the publication of the Stage 1 Disinfectants/Disinfection Byproducts Rule.
- Stage 2 of the Rule is in the process of finalization.
- Although over 600 DBPs have been isolated and identified, this represents only a fraction of the halogenated organic material that can be isolated after the disinfection of raw waters.

No DBP Toxicity Database

- In 1999 the U.S. EPA, the National Institute of Environmental Health Sciences and the U.S. Army called for a comprehensive biological and mechanistic DBP database.
- EPA employed a computer model-based structure-activity relationship (SAR) analysis of hundreds of DBPs. The SAR analysis was used to rank the carcinogenic potential of DBPs and identify a group of priority DBPs for further chemical and biological analysis.
- The EPA also conducted a Nationwide DBP Occurrence Study. A list of priority DBPs was drawn up of approximately 50 agents that were not included in the Information Collection Rule and were estimated to be the most toxic. A second group of approximately 20 DBPs of similar chemical structure to the priority compounds was also defined. Also from the surveyed water treatment plants, 28 new DBPs were structurally identified.
- There is virtually no toxicity data for most of these priority and new DBPs.

DBP Ignorance



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 Dr. S. Krasner.

In a Recent Issue of the Journal *Epidemiology*

- A panel of international experts stated, “These findings strengthen the hypothesis that the risk of bladder cancer is increased with long-term exposure to disinfection byproducts at levels currently observed in many industrialized countries.”
- *Epidemiology* 2004, 15:357-367

Objectives of This Study

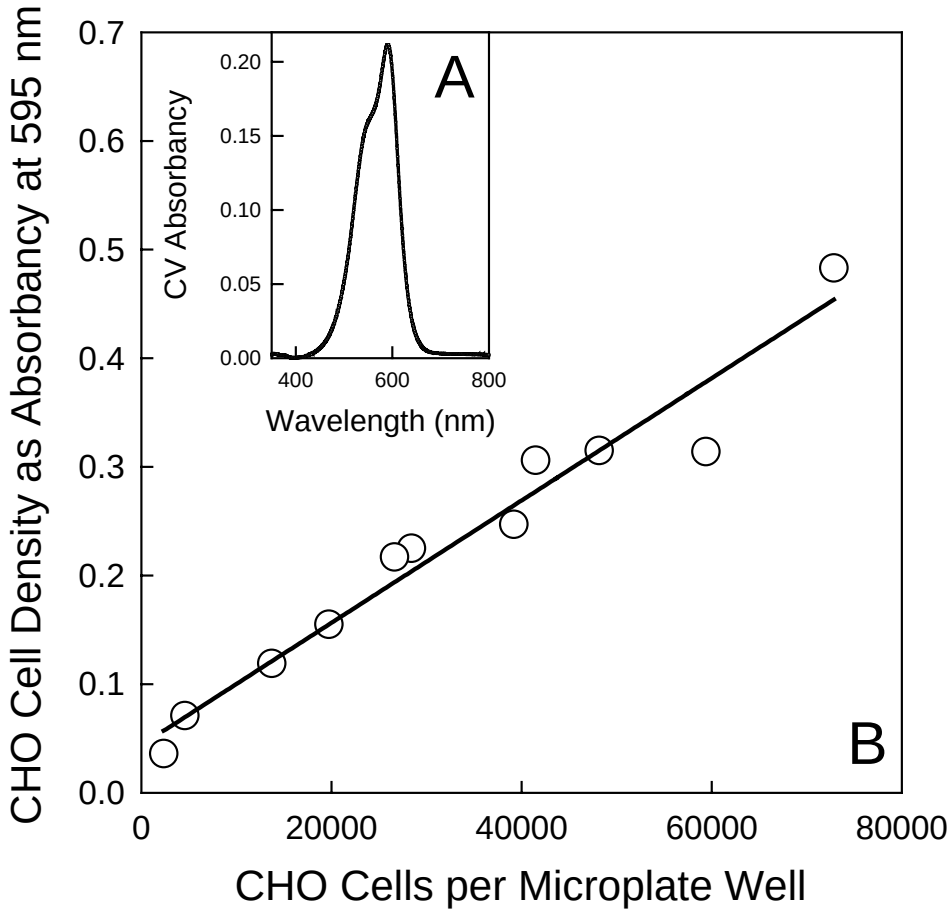
- Analyze the cytotoxicity of the individual DBPs with Chinese hamster ovary (CHO) cells.
- Determine the cytotoxic rank order of the DBPs.
- Analyze the genotoxicity of the individual DBPs with CHO cells.
- Determine the genotoxic rank order of the DBPs.
- Employ these data in a U.S. Environmental Protection Agency risk assessment program.

Mammalian Cell Chronic Cytotoxicity Assay

Chronic mammalian cell cytotoxicity is an important measure of the toxic impact of a test agent in which cells are continuously exposed throughout several cell divisions. Standard plating methods to measure toxicity are laborious, time consuming and require large amounts of sample. To address these problems we developed a rapid, semi-automated microplate-based, chronic cytotoxicity assay that measured the impact of a specific water DBP on cell survivorship.

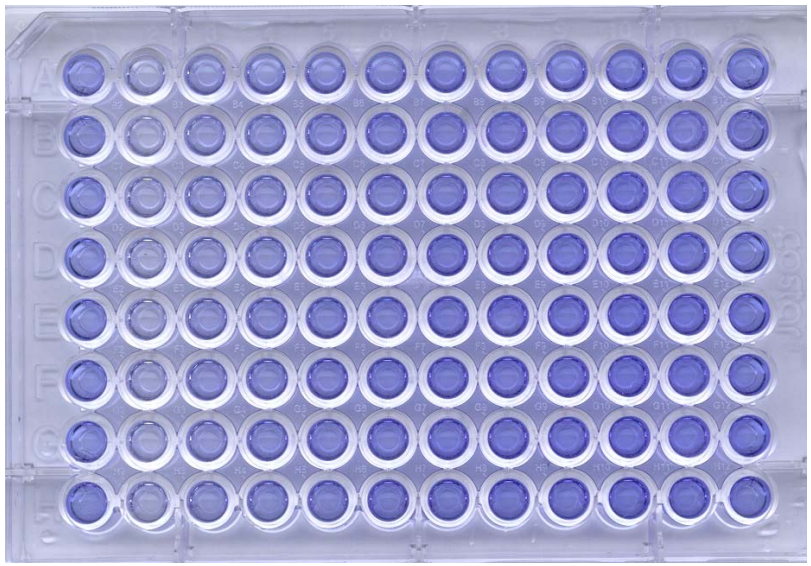
Mammalian Cell Chronic Cytotoxicity Assay

- CHO cells were exposed to a known concentration of a DBP in a microplate well for 72 h in a CO_2 incubator at 37°C.
- After incubation the cells were fixed, stained with crystal violet, washed and 50 µl DMSO was added to each well and analyzed with a microplate reader at 595 nm.
- The data were transferred onto an Excel spreadsheet and analyzed.
- The absorbancy and the cell density were significantly and highly correlated (r = 0.98, P < 0.001).

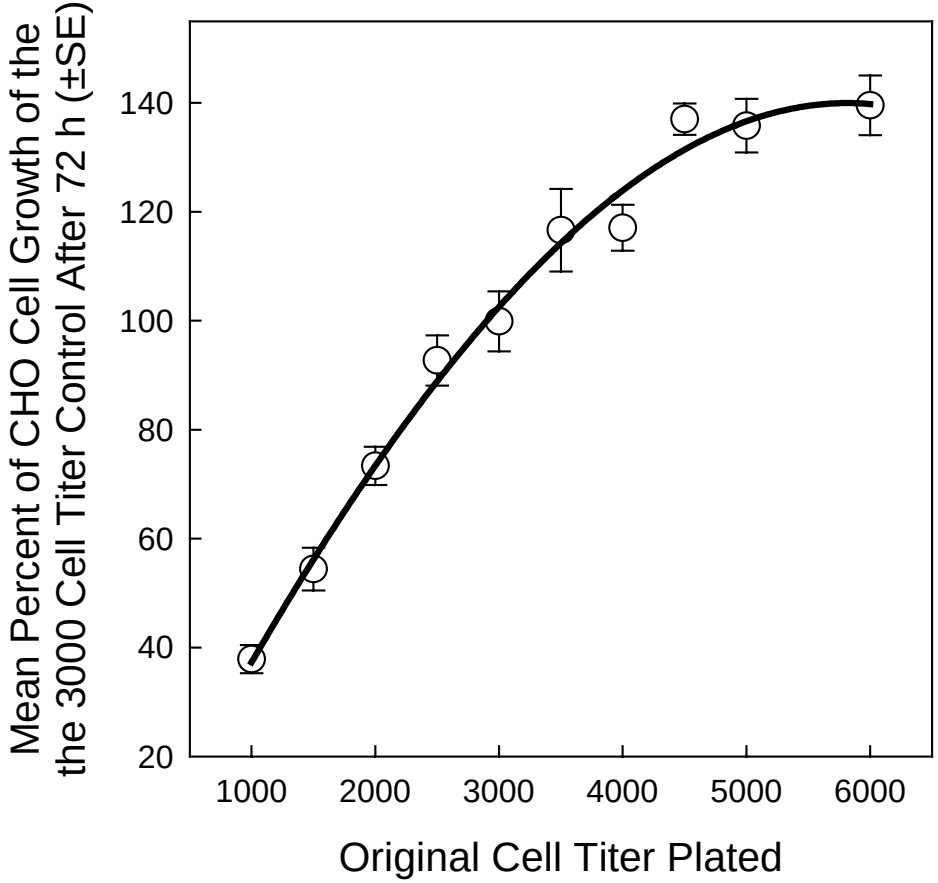


(A) Absorption spectrum of crystal violet in the range from 340 to 800 nm. (B) A comparison of the number of cells per microplate well determined with a Coulter counter and the absorbancy of identical wells after crystal violet staining.

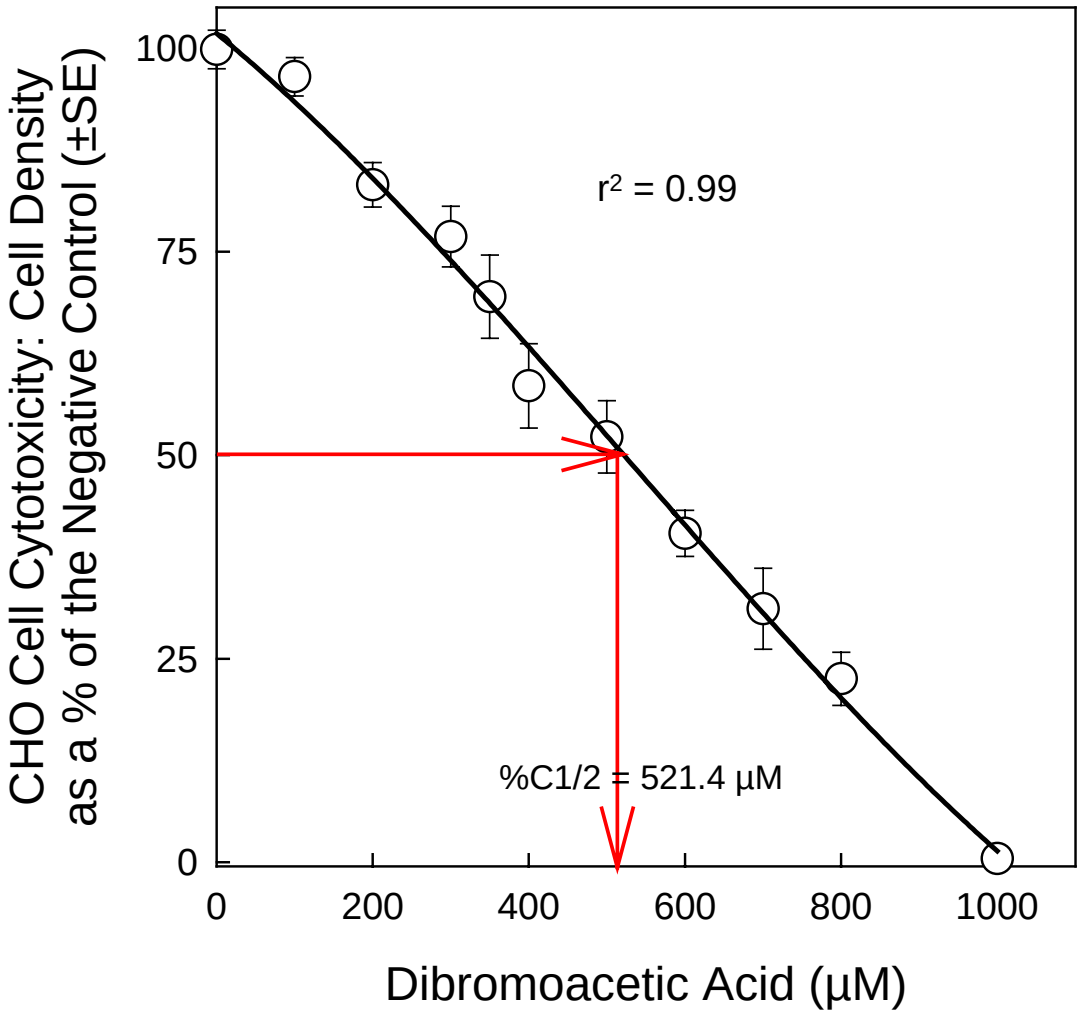
Determination of Optimal CHO Cell Plating Density



3000 CHO cells plated will grow to near confluency after 72h.



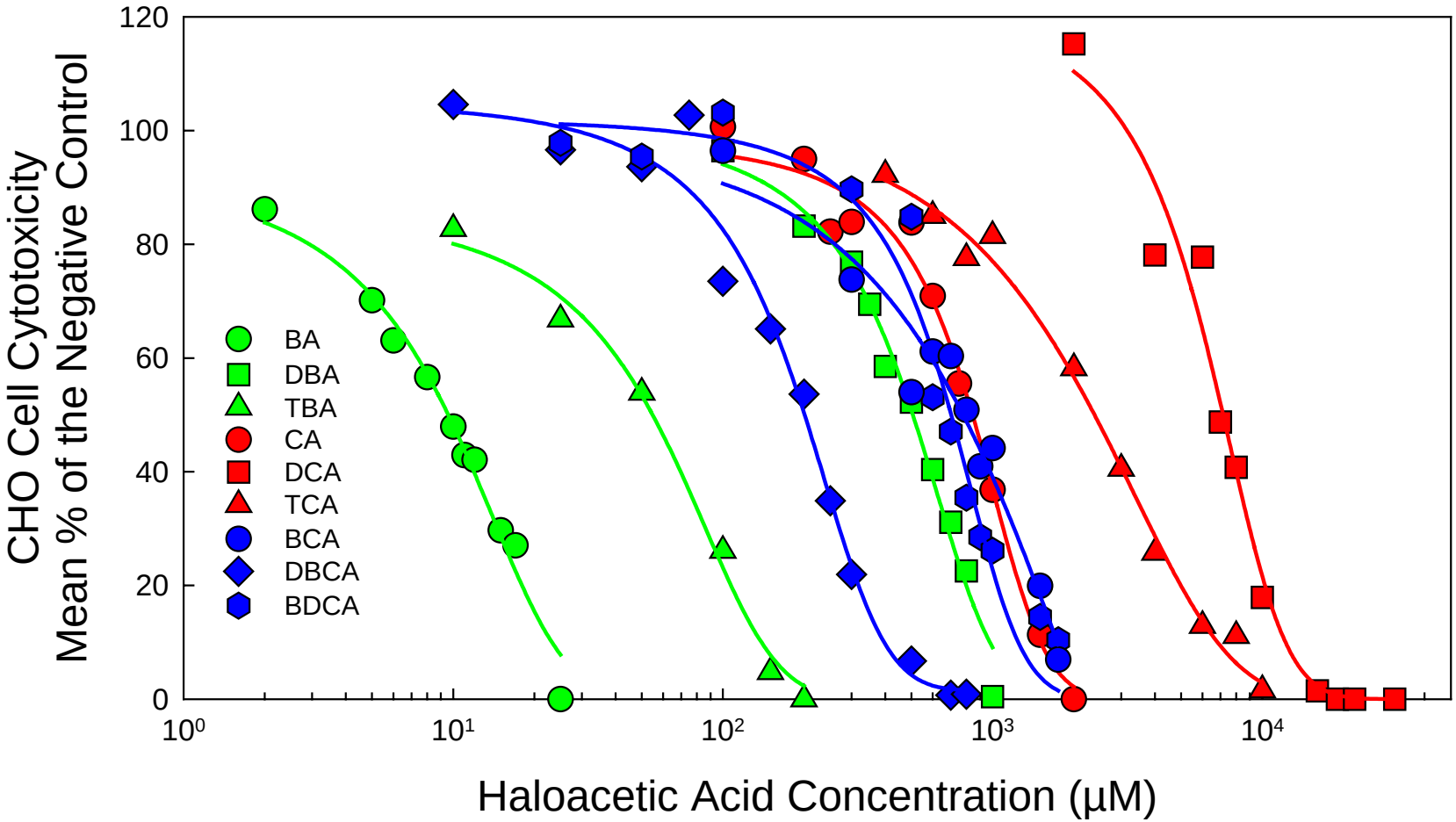
CHO Cell Cytotoxicity of Dibromoacetic Acid: %C½ Value



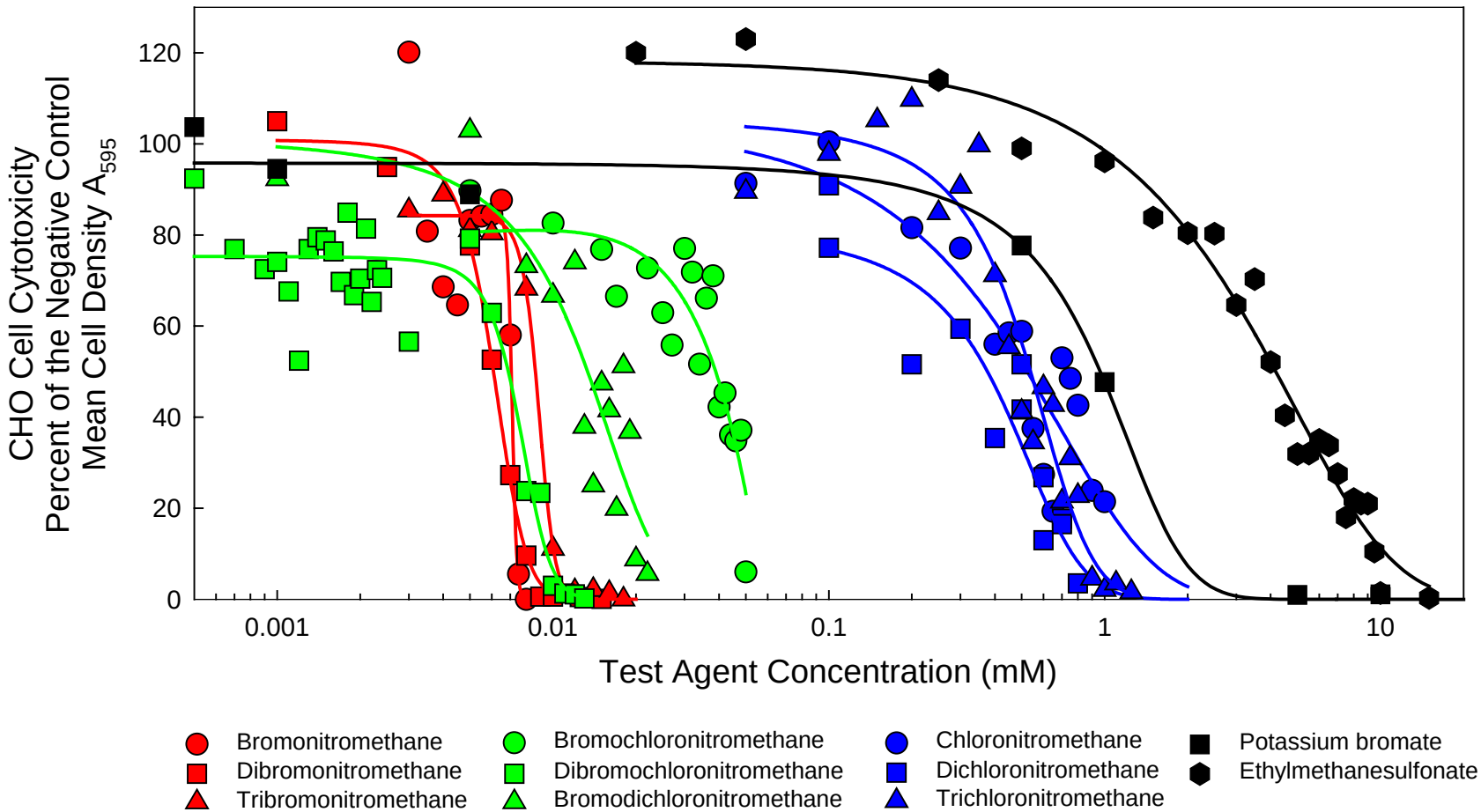
The %C½ value is the concentration of each test agent that reduced the CHO cell density by 50% as compared to the negative control.

The %C½ value is analogous to the LC_{50} measurement.

Haloacetic Acids: Comparison of CHO Cell Cytotoxicity (EPA Additivity Project)



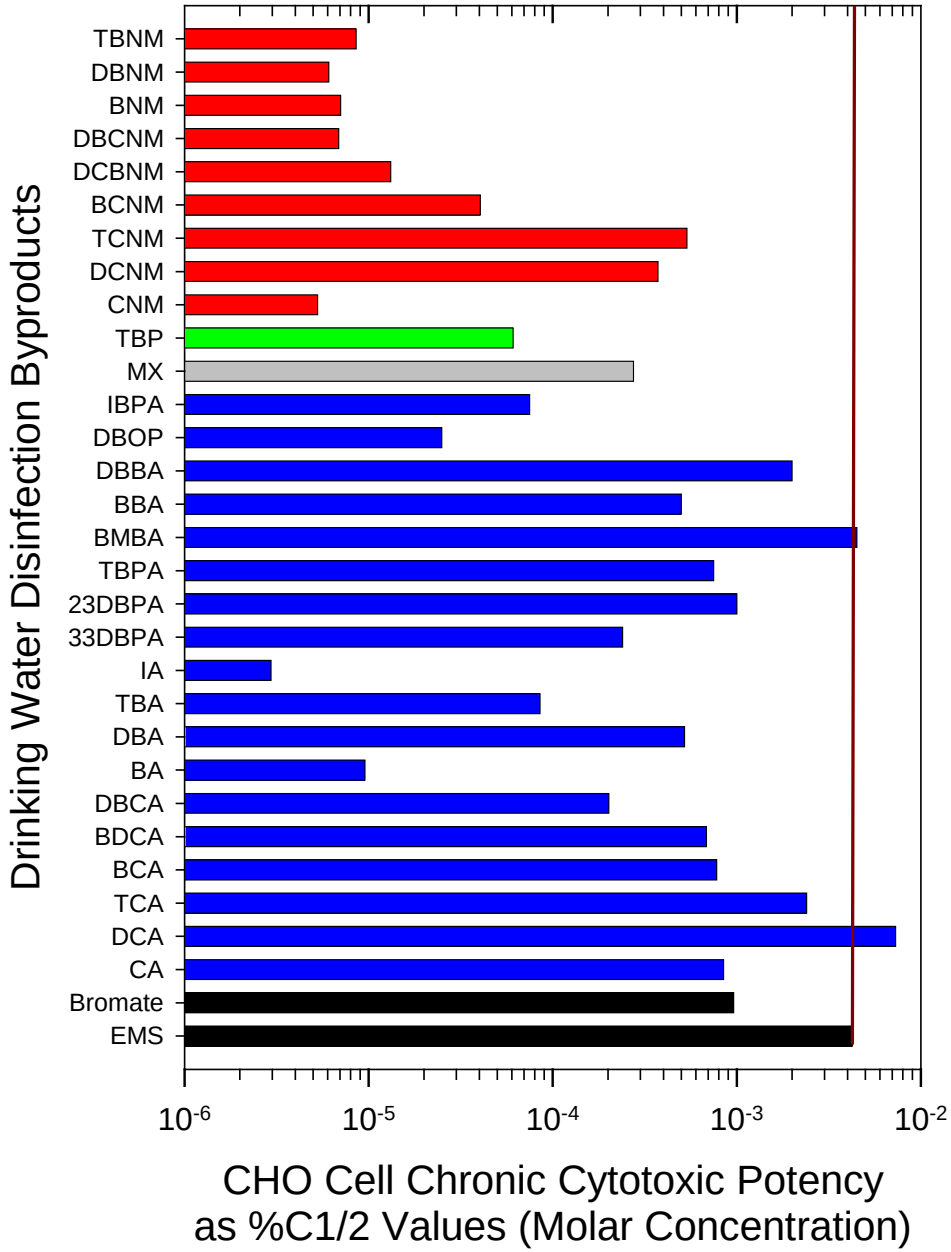
CHO Cell Cytotoxicity of Halonitromethanes



Comparison of DBP Chronic Cytotoxicity to CHO Cells

The halonitromethanes were more cytotoxic to mammalian cells than the haloacetic acids.

The brominated HNMs and HAAs were more cytotoxic than their chlorinated analogs.



DBP-Induced Mammalian Cell Cytotoxicity: Summary

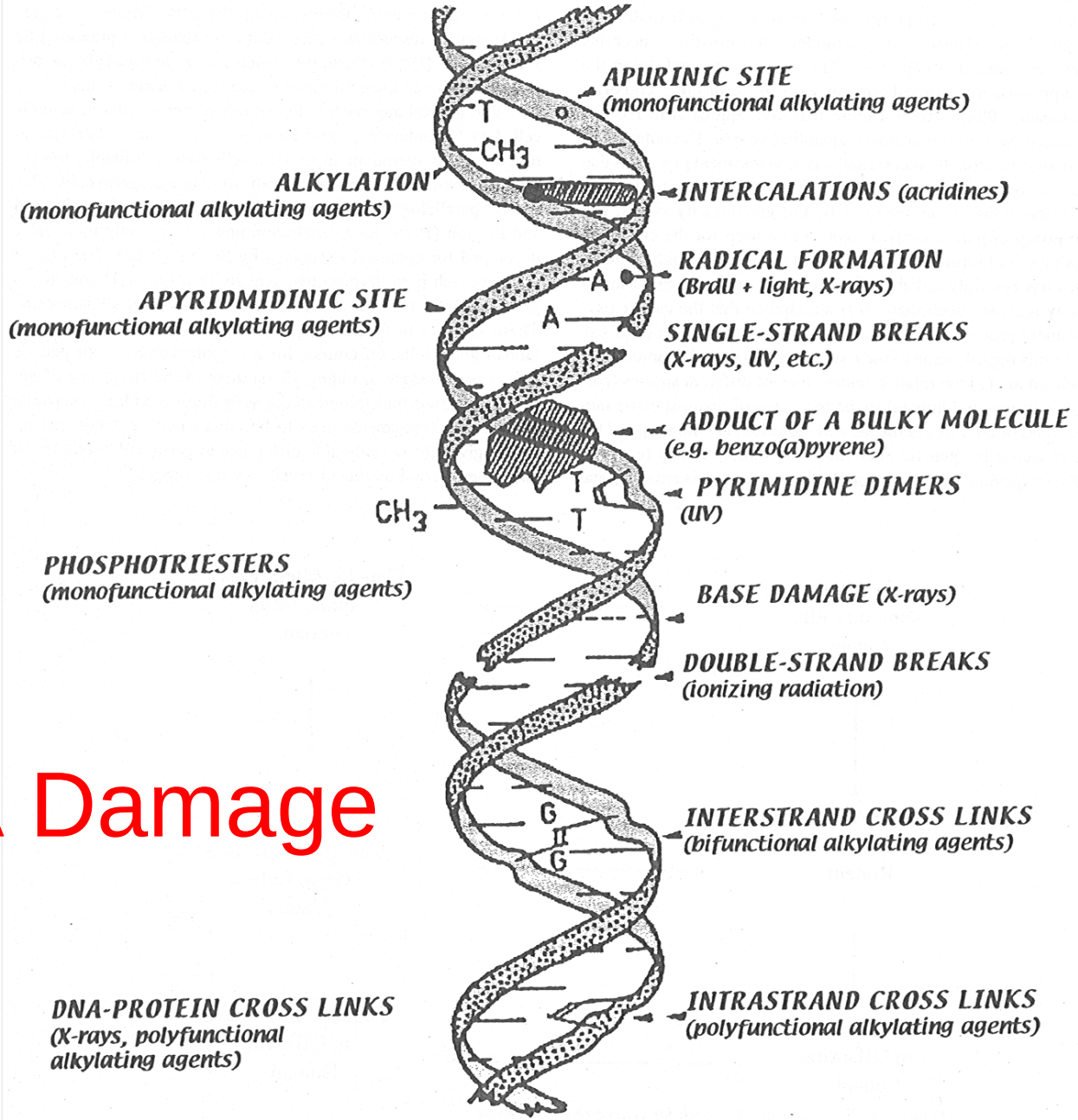
- Our microplate-based method allows for the analysis of a large number of concentrations per test agent and a large number of replicates per concentration.
- Chronic mammalian cell cytotoxicity is an important toxicological measurement and is being used for risk assessment by the US EPA.
- The cytotoxic potency (%C½ value) permits a quantitative comparison and rank ordering of the DBPs.
- The HNMs were more cytotoxic than HAAs.
- Brominated HNMs and HAAs were more cytotoxic than their chlorinated analogs.

Genomic DNA Damage Induced by Drinking Water Disinfection By-Products

Single Cell Gel Electrophoresis

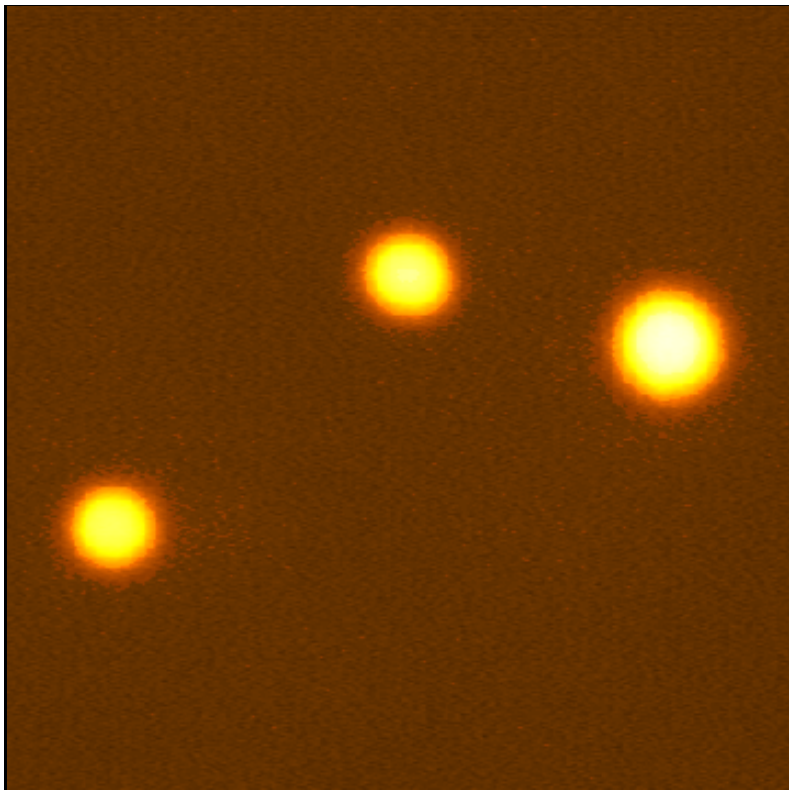
The target is the genome, not just a gene.

DNA Damage

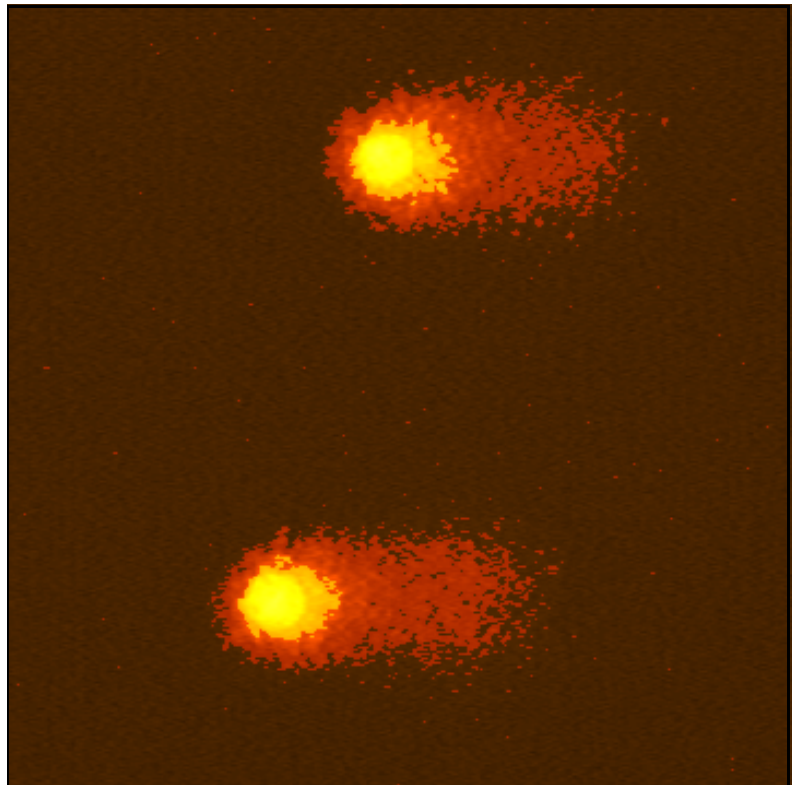

APURINIC SITE
(monofunctional alkylating agents)
T
CH3
ALKYLATION
(monofunctional alkylating agents)
INTERCALATIONS (acridines)
RADICAL FORMATION
(BrdU + light, X-rays)
A
APYRIDMIDINIC SITE
(monofunctional alkylating agents)
A
SINGLE-STRAND BREAKS
(X-rays, UV, etc.)
ADDUCT OF A BULKY MOLECULE
(e.g. benzo(a)pyrene)
T
PYRIMIDINE DIMERS
(UV)
CH3
T
PHOSPHOTRIESTERS
(monofunctional alkylating agents)
BASE DAMAGE (X-rays)
DOUBLE-STRAND BREAKS
(ionizing radiation)
G
INTERSTRAND CROSS LINKS
(bifunctional alkylating agents)
G
DNA-PROTEIN CROSS LINKS
(X-rays, polyfunctional
alkylating agents)
INTRASTRAND CROSS LINKS
(polyfunctional alkylating agents)

SCGE Analysis of 2AAAF

(in isolated nuclei from control and treated CHO cells)

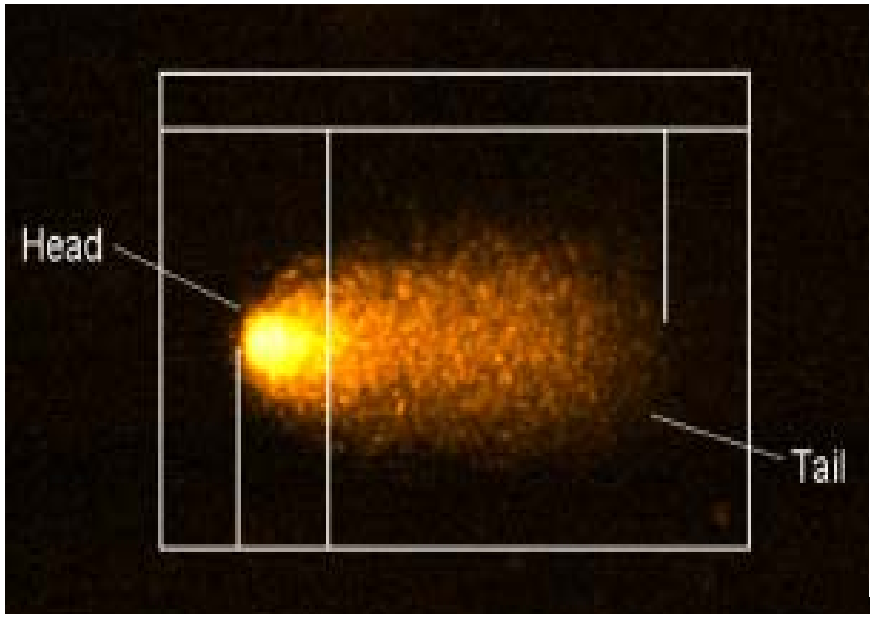


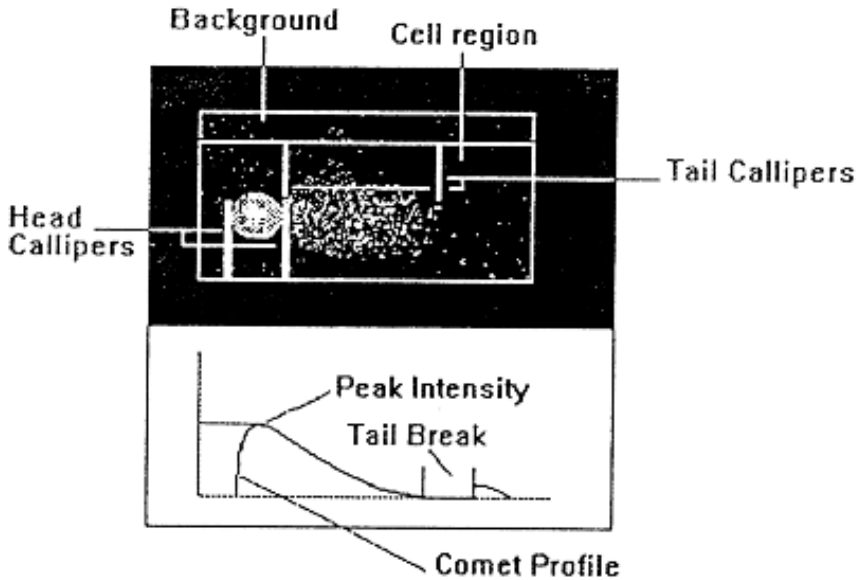
Negative Control



800 nM 2AAAF

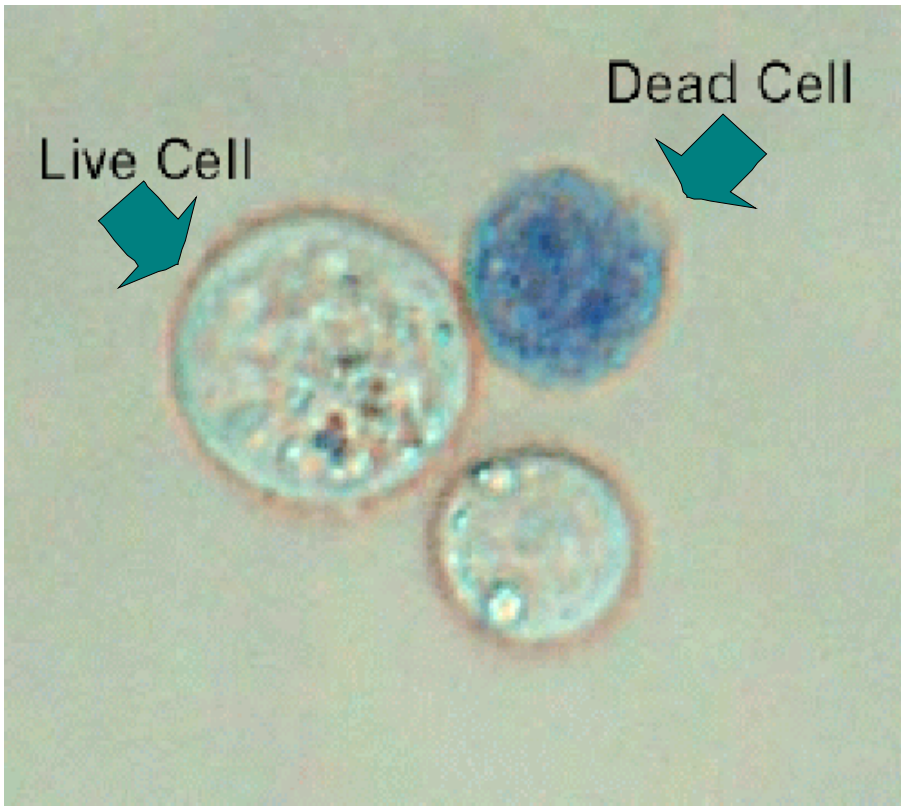
Computer Analysis of SCGE Images





- 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.
- **The tail moment** is the integrated value of DNA density multiplied by the migration distance.

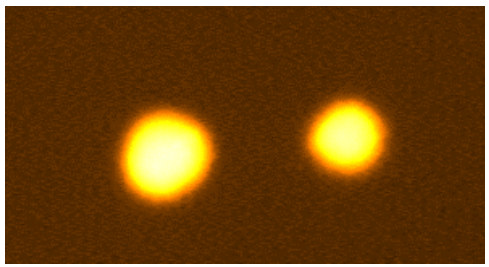
Acute Cytotoxicity



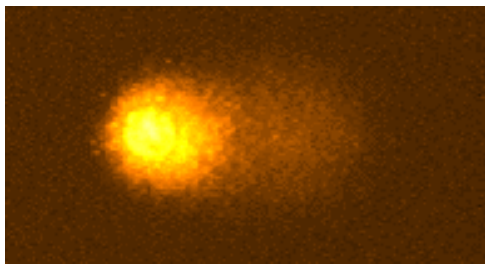
- From each treated cell suspension a 10 µl aliquot was stained with 10 µl of 0.05% trypan blue vital dye in PBS.
- The percent survival for each treatment group was determined by counting the dead cells (blue) and the live cells (clear).

Genomic DNA Damage Induced by Dibromonitro-methane

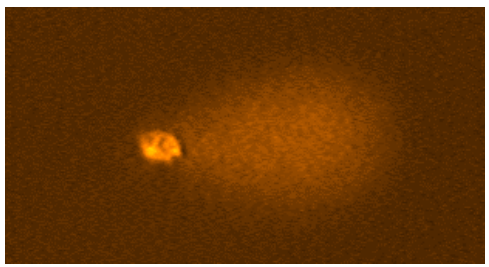
Control

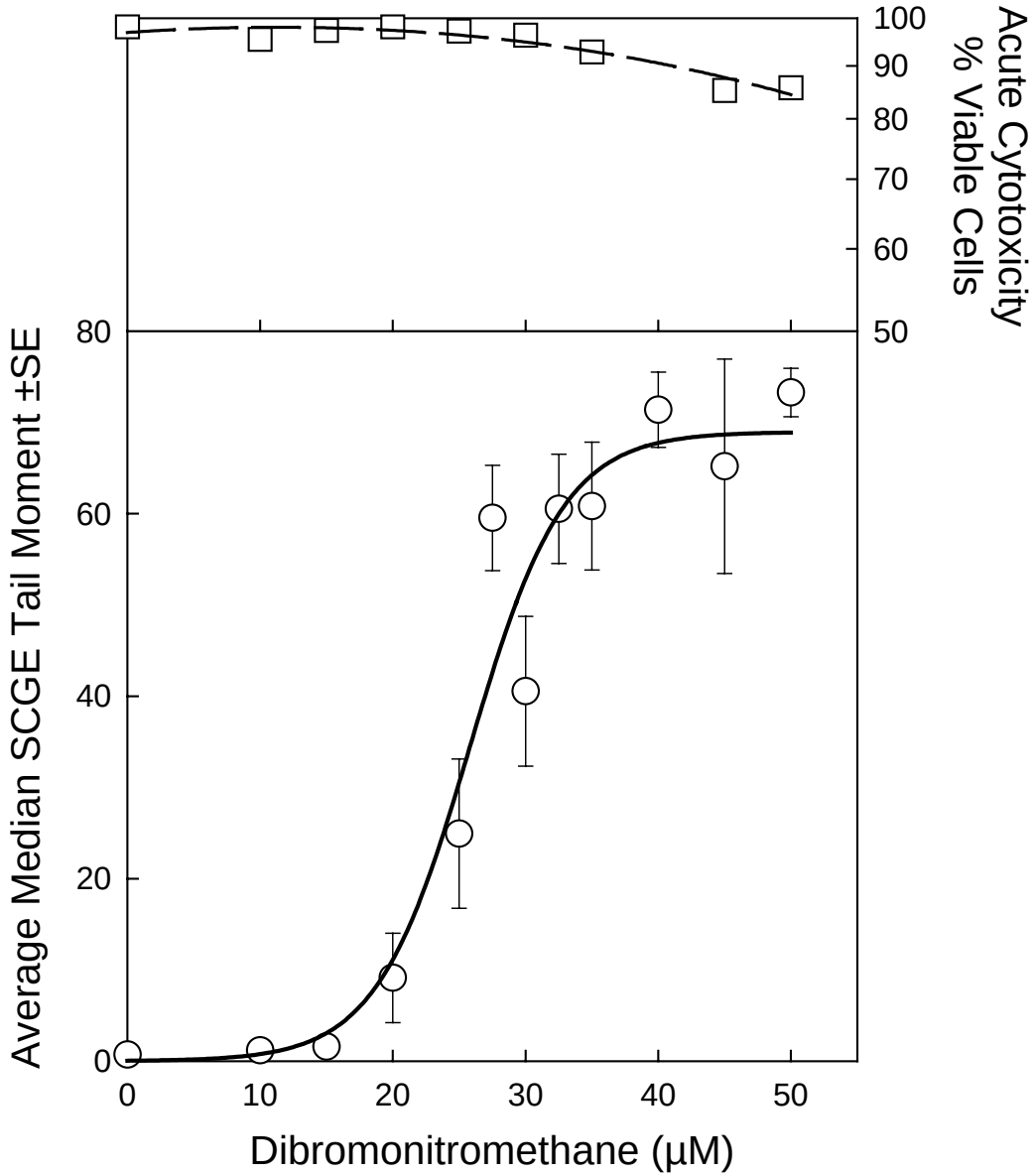


30 µM DBNM

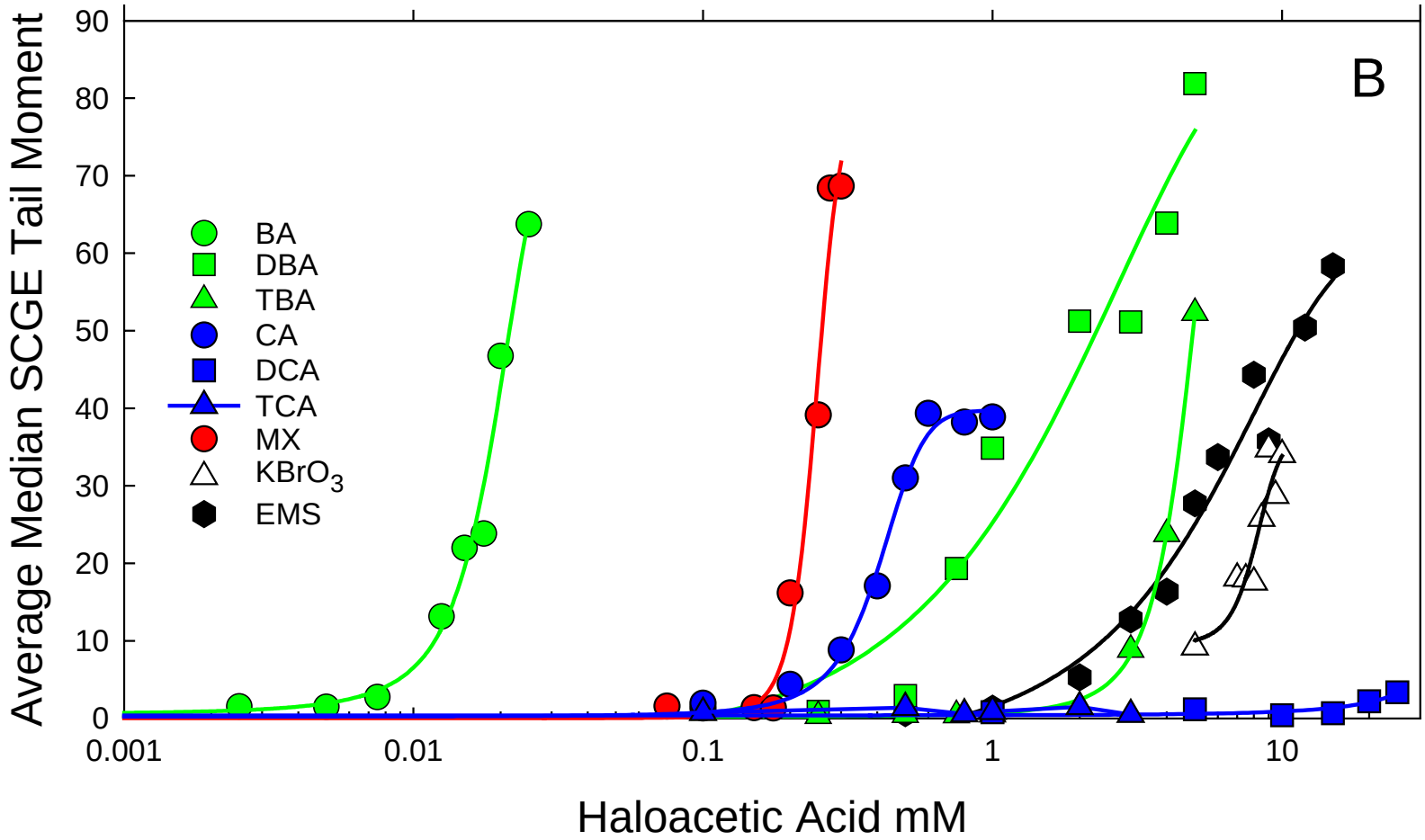


40 µM DBNM

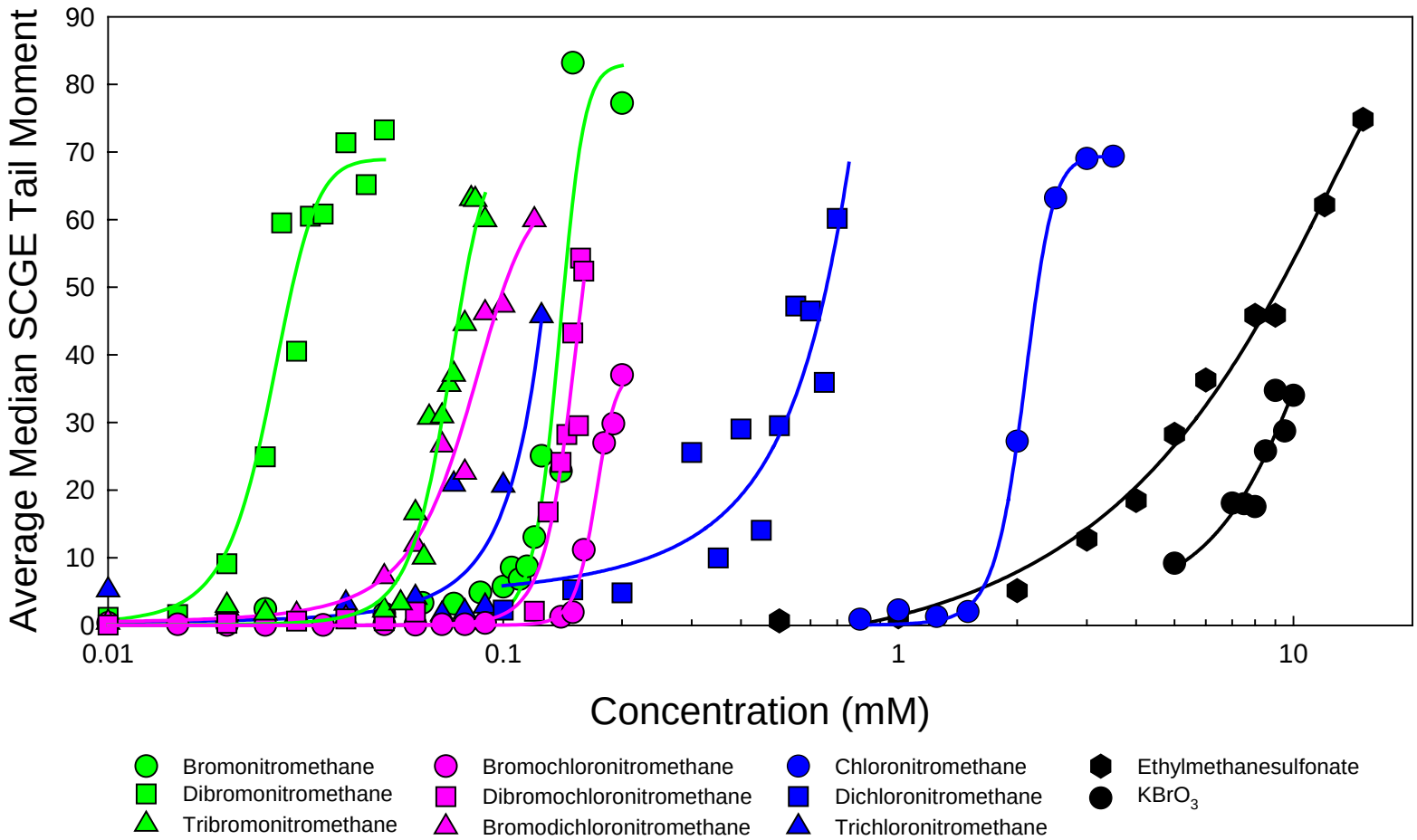




SCGE Analysis of Haloacetic Acids in CHO Cells

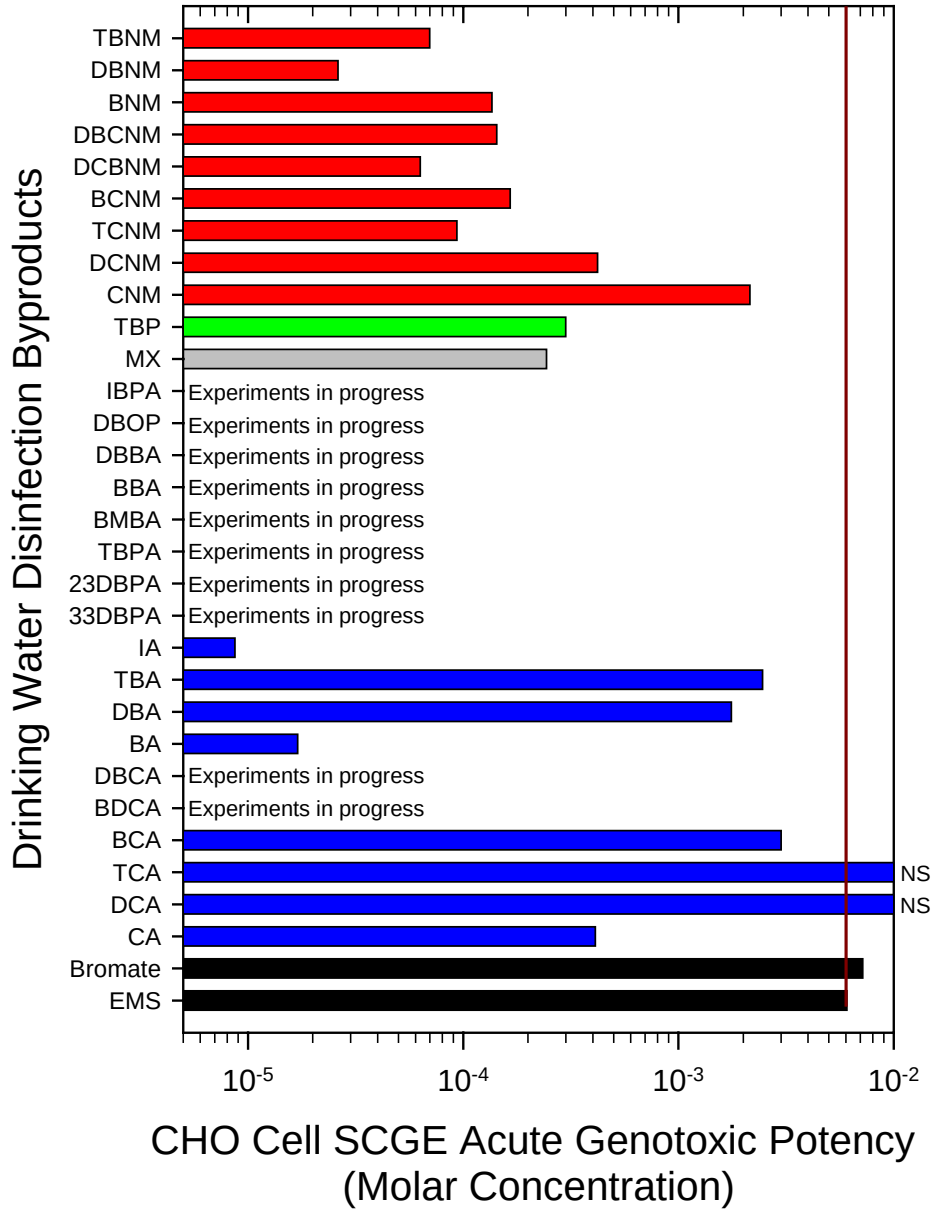


SCGE Analysis of Halonitromethanes in CHO Cells

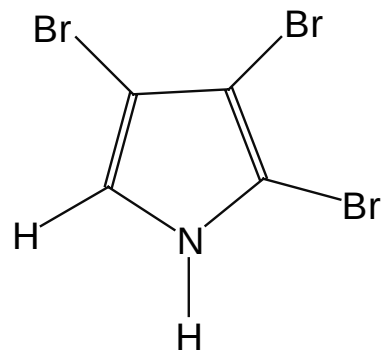


DBP CHO Cell Genotoxicity

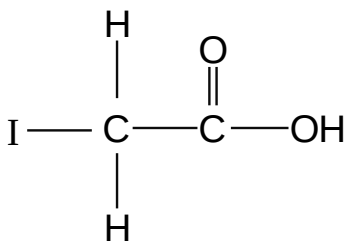
In general, the halonitromethanes were more genotoxic to CHO cells than the haloacetic acids.
The brominated HNMs and HAAs were more genotoxic than their chlorinated analogs.



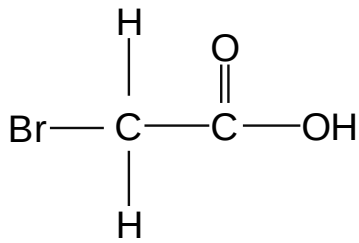
Newly Identified Disinfection By-Products (2002-2004)



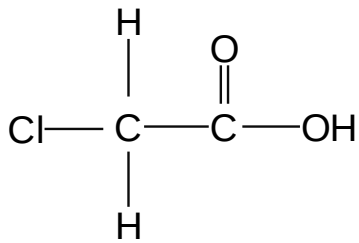
2,3,5-Tribromopyrrole



Iodoacetic acid

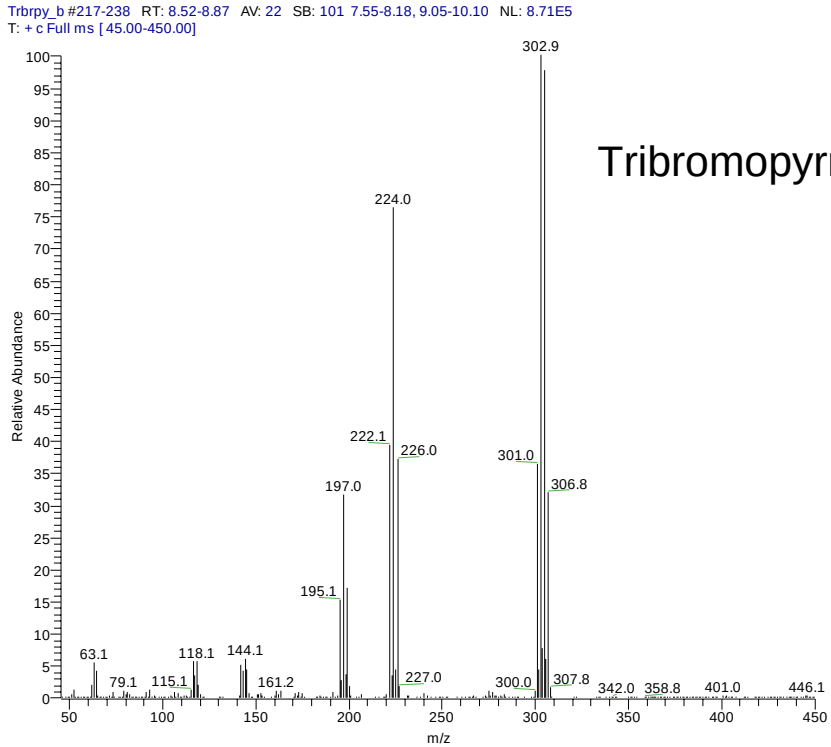


Bromoacetic acid

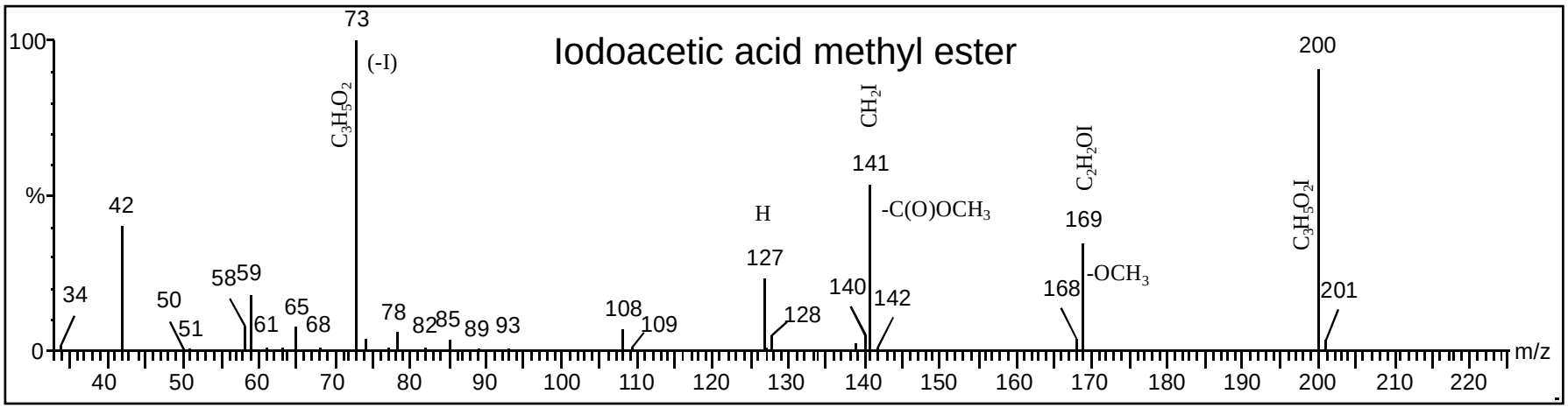


Chloroacetic acid

Mass Spectra of 2,3,5-Tribromopyrrole and Iodoacetic Acid

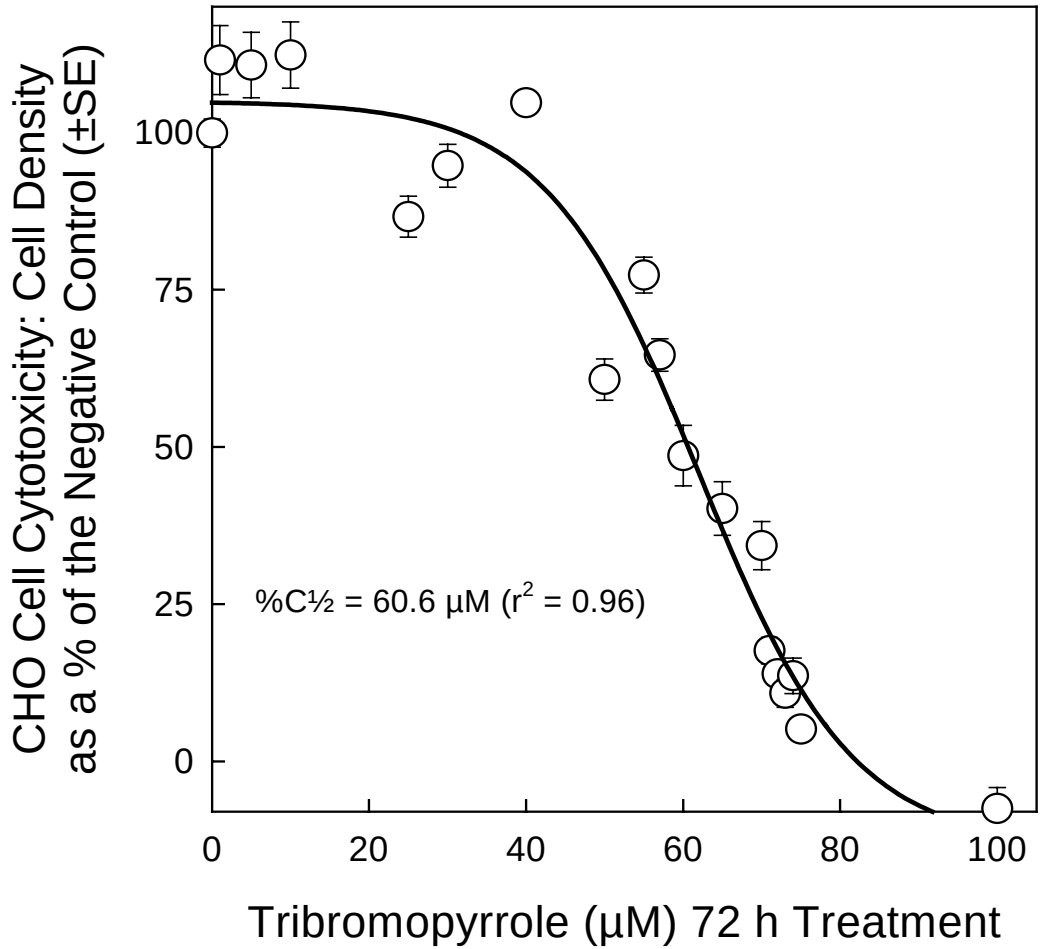


Tribromopyrrole



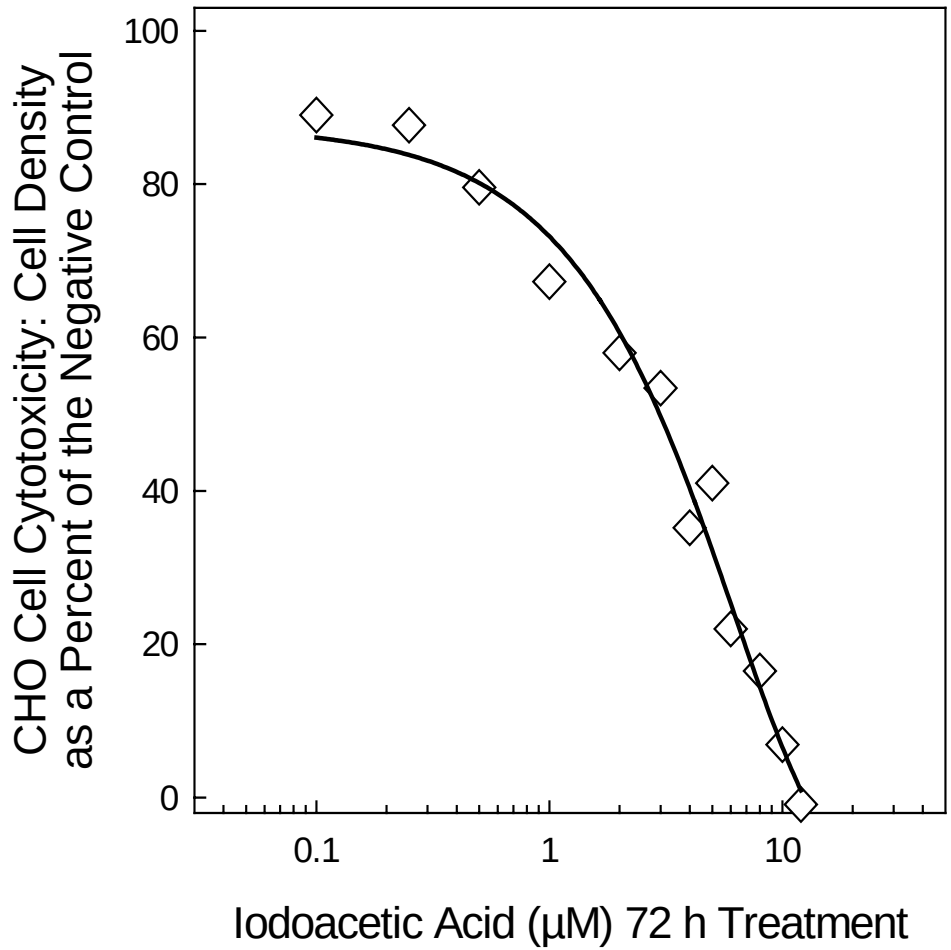
CHO Cell Chronic Cytotoxicity of Tribromopyrrole

- In the chronic CHO cell cytotoxicity assay the level of cell killing by tribromopyrrole during the 72 h period was similar to that of BCNM and TBA.
- The %C½ value was 60.6 µM.



CHO Cell Chronic Cytotoxicity of Iodoacetic Acid

- Iodoacetic acid was the most potent cytotoxic DBP among the 30 analyzed in our laboratory.
- The %C½ value for iodoacetic acid was 2.9 µM.



Rank Order of DBP CHO Cell Cytotoxicity

The halonitromethanes were more cytotoxic to mammalian cells than the haloacetic acids.

The brominated HNMs and HAAs were more cytotoxic than their chlorinated analogs.

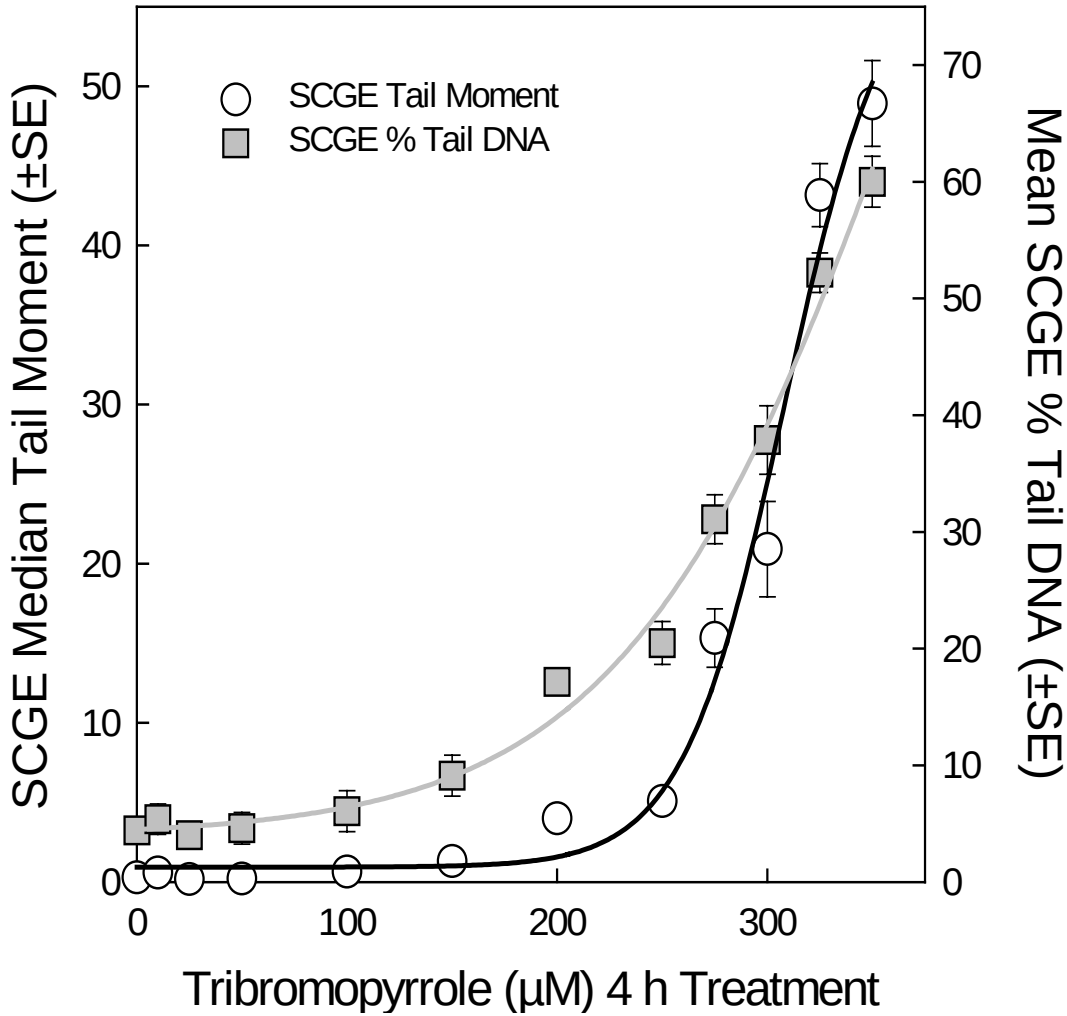
DBP	%C1/2 mM
DBNM	0.006
BNM	0.007
DBCNM	0.007
TBNM	0.008
BA	0.010
BDCNM	0.013
BCNM	0.041
TBA	0.085
MX	0.275
DCNM	0.373
DBA	0.521
CNM	0.529
TCNM	0.536
CA	0.848
PB	0.963
TCA	2.400
EMS	4.250
DCA	7.304

IA = 0.003

TBP =0.06

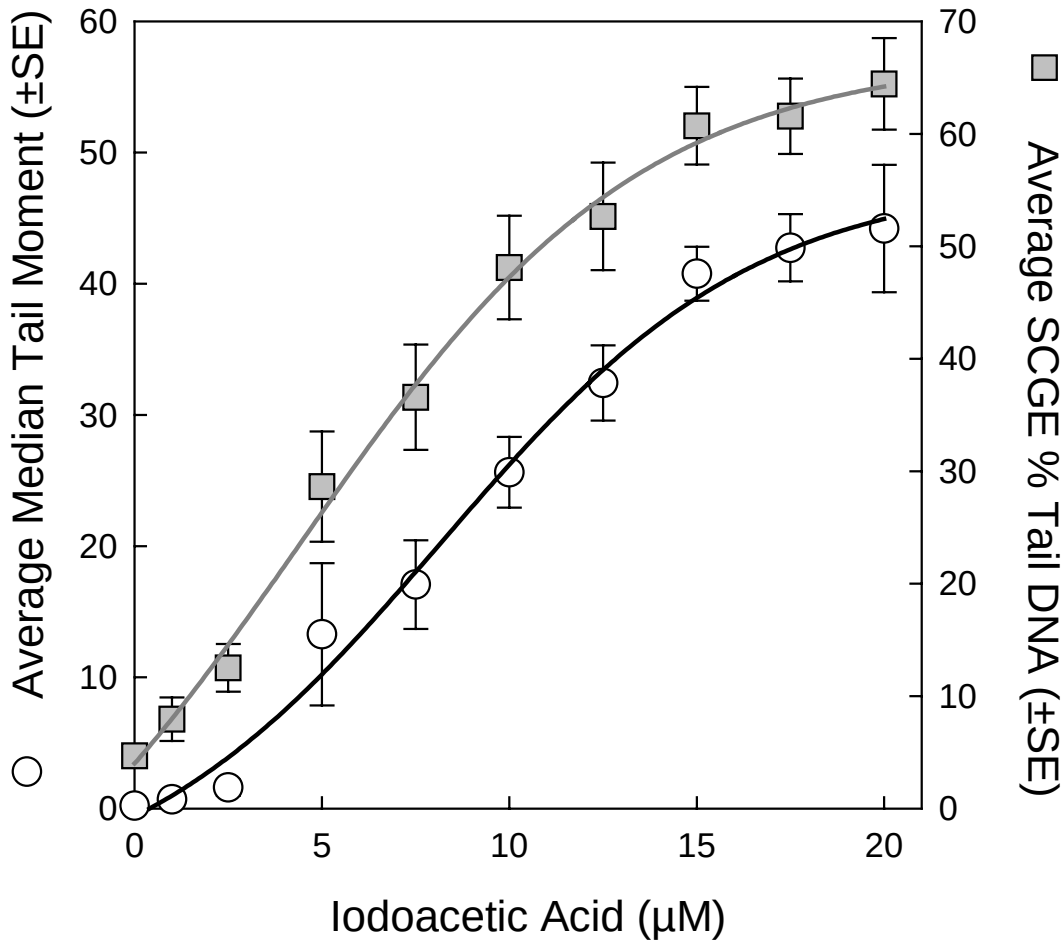
Genotoxicity of Tribromopyrrole

- Tribromopyrrole is a strong genotoxic agent in CHO cells.
- The TM SCGE genotoxic potency of TBP is 301.5 µM.



Genotoxicity of Iodoacetic Acid

- Iodoacetic acid is the most potent genotoxic DBP (of 22) that we analyzed in CHO cells.
- The TM SCGE genotoxic potency of iodoacetic acid is 7.9 µM.



Rank Order of DBP CHO Cell Genotoxicity

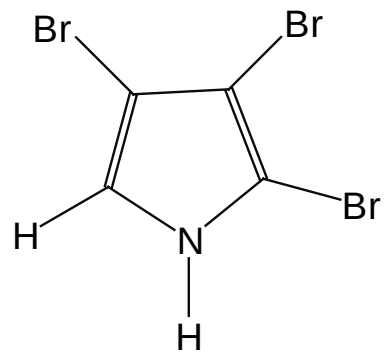
In general, the halonitromethanes were more genotoxic to CHO cells than the haloacetic acids.
The brominated HNMs and HAAs were more genotoxic than their chlorinated analogs.

DBP	CHO Genotoxic Potency (mM)
BA	0.017
DBNM	0.026
TBNM	0.070
TCNM	0.093
BNM	0.133
DBCNM	0.143
BCNM	0.166
MX	0.244
CA	0.411
BDCNM	0.413
DCNM	0.420
DBA	1.756
TBA	2.456
CNM	3.007
EMS	6.057
$KBrO_3$	7.195
DCA	NS
TCA	NS

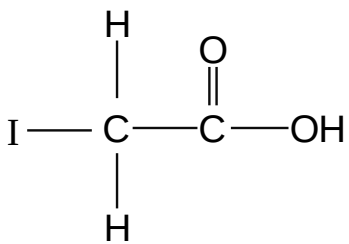
IA = 0.008

TBP = 0.301

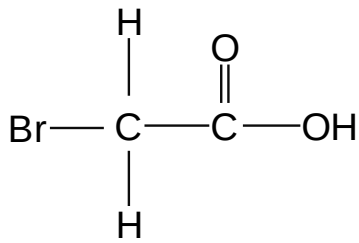
Newly Identified Disinfection By-Products (2002-2004)



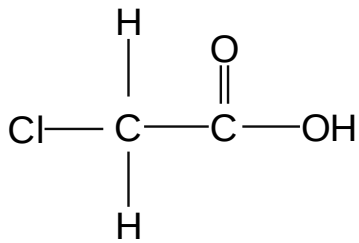
2,3,5-Tribromopyrrole



Iodoacetic acid



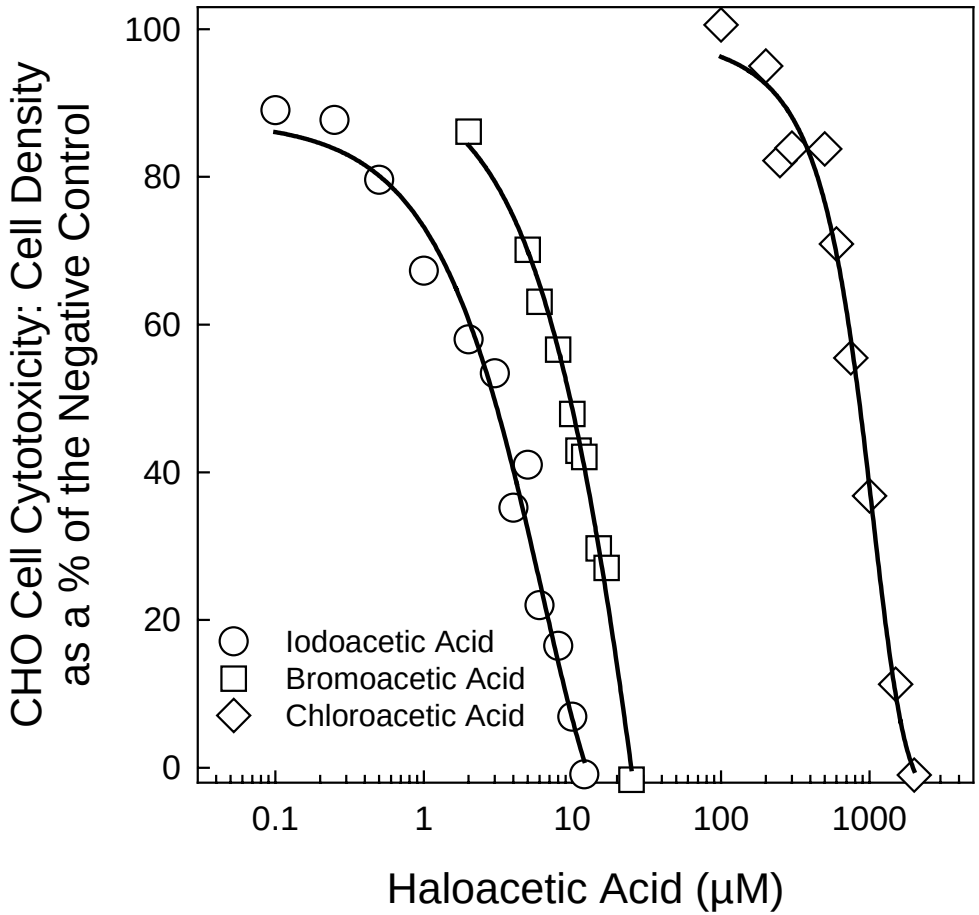
Bromoacetic acid



Chloroacetic acid

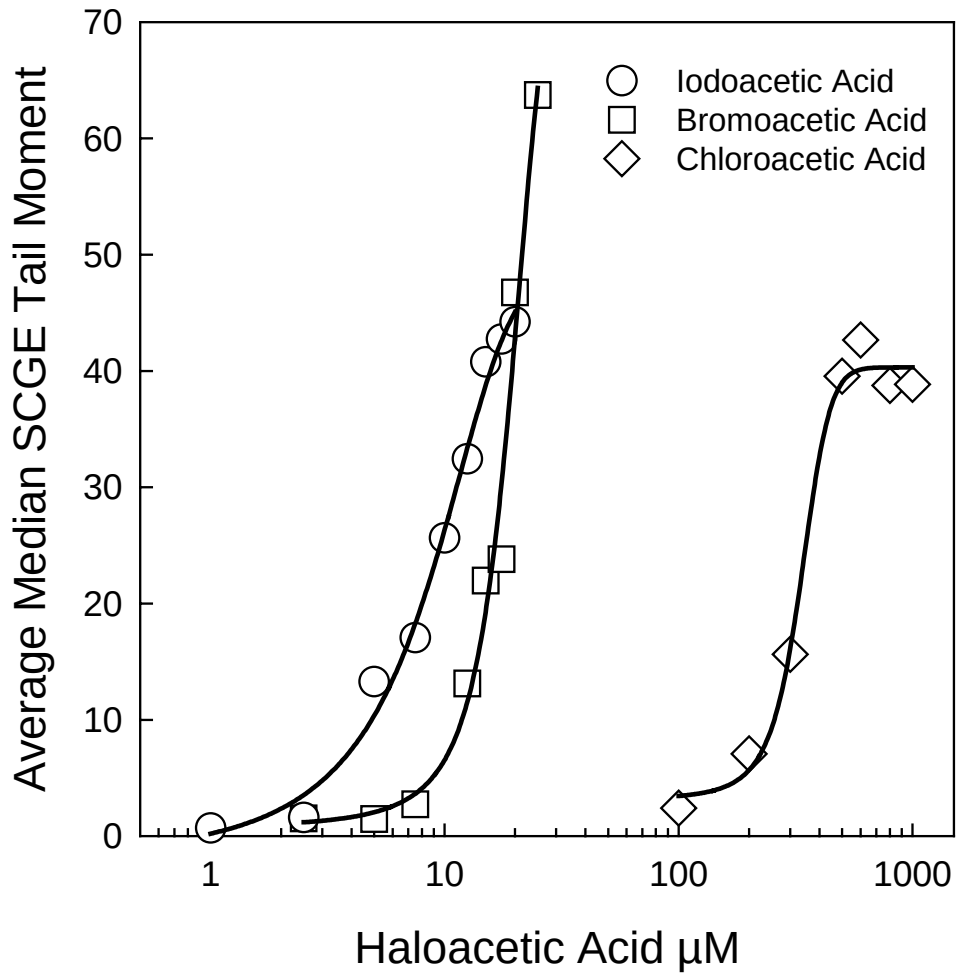
Comparative Cytotoxicity of the Monohalogenated Acetic Acids

- All of the mono-halogenated acetic acids were chronic toxins to CHO cells.
- The %C½ values for iodo-, bromo-, and chloro- acetic acid were 2.9, 10.0, and 848 µM, respectively.



Comparative Genotoxicity of the Monohalogenated Acetic Acids

- All of the mono-halogenated acetic acids were acute genotoxins to CHO cells.
- The SCGE genotoxic potencies for iodo-, bromo- and chloro-acetic acid were 7.9, 17, and 411 µM, respectively.



DBP-Induced Mammalian Cell Genotoxicity: Summary

- The mammalian cell microplate SCGE method allows for the quantitative genotoxic analysis of small amounts of test agent.
- In general the HNMs are more genotoxic than HAAs.
- The genotoxic potency for both the HNMs and HAAs is highest for the brominated analogs, followed by the bromo-chloro analogs, and then the chlorinated analogs.
- New DBPs, such as tribromopyrrole and iodoacetic acid, can be rapidly analyzed for their cytotoxic and genotoxic activity in mammalian cells.

Mechanisms of Haloacetic Acid Genotoxicity

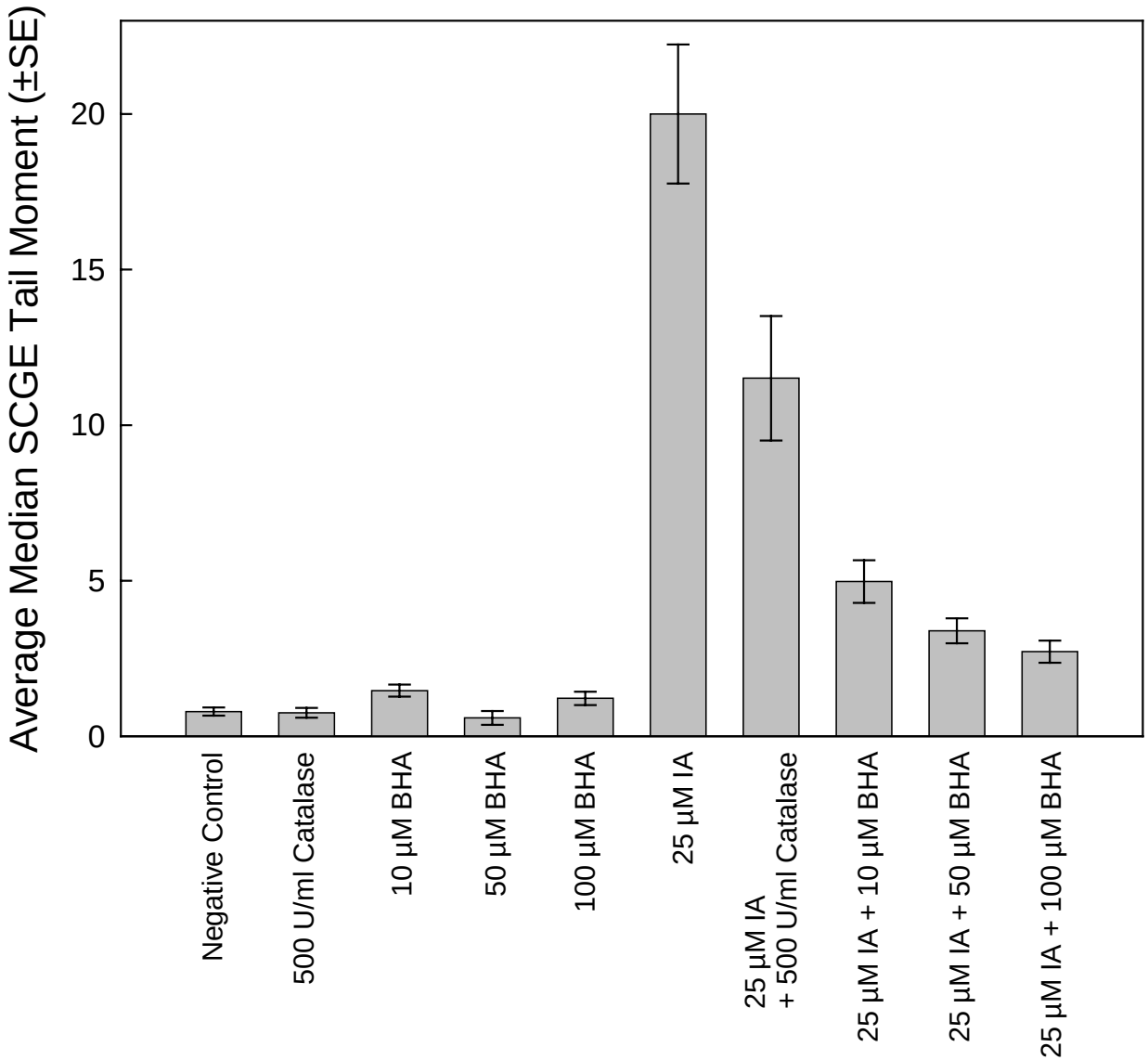
- No DNA adducts have been identified.
- Haloacetic acids (HAAs) induce mutation in *Salmonella* and mammalian cells.
- HAAs are good inducers of DNA strand breaks in mammalian cells.
- In rodent cancer studies HAAs caused peroxisome proliferation and may induce oxidative stress.

Effect of Catalase or BHA on Modulating Iodoacetic Acid Genotoxicity

- Catalase is an enzyme that specifically degrades H_2O_2.
- Butylated hydroxyanisole is a potent radical scavenger.
- We treated CHO cells with iodoacetic acid and catalase or BHA and determined SCGE DNA damage.

Inhibition of Iodoacetic Acid DNA Damage by Catalase or BHA

Experiments 072503EC, 073003EC,080503EC, 080803EC, 081003EC, 081203EC



Take-Home Message

- Chemical disinfection of water reduced the incidence of waterborne diseases.
- Disinfection unfortunately generates a large number of halogenated DBPs.
- Epidemiological studies have linked consumption of water containing DBPs with enhanced risks of spontaneous abortion, birth defects and cancer.

Take-Home Message

- The vast majority of DBPs that comprise TOX have not been chemically or biologically characterized.
- The U.S. EPA has a high priority to identify DBPs and determine their toxicity.
- We demonstrated that CHO cell microplate cytotoxicity and SCGE assays were sensitive and accurate when working with small amounts of DBPs.

Take-Home Message

- The halonitromethanes (HNM) were both more cytotoxic and genotoxic as compared to the haloacetic acids.
- The brominated and iodinated HAAs and HNMs were more cytotoxic and genotoxic than their chlorinated analogs.
- HAAs induce their genotoxic damage via an oxidative stress mechanism.
- The goal is to engineer drinking water disinfection technologies that generate the lowest levels of highly toxic DBPs. Our work is a step in this direction.

This research was funded in part by AWWARF grant 554,
U.S. EPA grants R825966, R83069501, and NSF grant INT97-26617

EPA Region 5
July 2004