EFFECTS OF pH AND TEMPERATURE ON THE GENOTOXICITY OF HALOGENATED DISINFECTION BY-PRODUCTS IN CHLORINATED WATER

by

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Effects of pH and temperature on the genotoxicity of halogenated disinfection by-products in chlorinated water.

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Abstract

Disinfection by-products (DBPs) are important environmental chemicals and the objective of this study was to assess the effects of pH, temperature and bromide concentration on the genotoxicity of DBPs in chlorinated water. Cells were exposed to humic acid samples and genotoxocity was assessed by chromosomal aberration assay using Chinese hamster lung (CHL) cells *in vitro*. A strong positive correlation between bromide concentration and the number of chromosomal aberrations formed was observed. Higher temperature values resulted in more chromosomal aberrations (14.6%) and a greater percentage of aberrant cells (24.6%) at pH 9 and, at higher bromide concentrations, more aberrations were formed at 25°C than 5°C for all pH values. There is some evidence that the number of aberrant cells is higher at 5°C at pH 7 than pH 5 or 9, however there does not appear to be any appreciable change in genotoxicity over the pH range tested.

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1. Introduction

Drinking water is an important carrier of infectious disease and clean drinking water is essential to human health. The importance of clean potable water was recognized early in human history and the earliest evidence of water treatment originates in the sedimentation apparatuses and wick siphons used by the early Egyptians in the 15-13th centuries B.C. (Baker and Taras 1981). Following the extensive aqueduct public water supply systems built by the Romans in the 3rd century B.C., there was little progress in water treatment and its connection to public health until the 17th and 18th centuries A.D. The invention of the microscope by Anton van Leeuwenhoek and the development of filters, first by Lu Antonio Porzio and later by Joseph Amy, played important roles in the development of water treatment, but it was the crucial realization in the 19th century of the connection between van Leeuwenhoek's "animacules", water and health that lead the way to modern water treatment (Baker and Taras 1981). In the early 20th century, cities in North America and Europe began disinfecting drinking water and using more complex and effective filtration systems and the basic water treatment system of coagulation, sedimentation, filtration and disinfection was established.

Water that is not disinfected contains bacteria, viruses and other microbes, often in sufficient quantities to cause disease or even death from short-term exposure to pathogens. Today, widespread treatment of drinking water, especially disinfection, in developed countries is regarded a major public health advancement and remains a victory over many waterborne diseases, including typhoid fever, dysentery and cholera (Bull and Kopfler 1991). Untreated or inadequately treated drinking water remains the greatest threat to public health and in many parts of the world safe drinking water is almost nonexistent, due to poverty, a lack of a treatment and delivery infrastructure and poor understanding of water contamination. The World Health Organization and United Nations Children's Fund (2004) estimate that about 1 billion people do

not have access to safe drinking water and about 2.6 billion have no adequate sanitation facilities. In lesser developed countries, where nearly half the population drinks contaminated water, waterborne diseases such as cholera and chronic dysentery kill millions of people; each year, water-related diseases cause an estimated 3.4 million deaths, mostly in children. The main killers are diarrhea and malaria (WHO and UN CF 2004).

It is difficult to overstate the importance of water disinfection or the role that it has played in protecting public health through the control of waterborne diseases. Despite the enormous contribution of disinfection to public health and the control of pathogens, there is evidence that disinfectants can also pose health risks. Over the past 30 years, a growing body of literature has identified the presence of numerous disinfection by-products (DBPs) (Richardson et al. 1999; Weinberg et al. 2002; Plewa et al. 2004; reviewed in Symons 2001a and 2001b), and many potential health hazards related to DBPs have been reported in the literature (reviewed in Richardson 1998; Bull 2000; Nieuwenhuijsen et al. 2000). Disinfection by-products are groups of compounds resulting from the chemical reaction of disinfectants with the organic matter in natural waters. This natural organic matter, which is generally measured by total organic carbon (TOC), serves as the organic DBP precursor and, for a number of DBPs, bromide ions serve as an inorganic DBP precursor. As a result, levels of DBPs are generally highest in treated waters from sources with high organic matter levels, such as rivers and lakes, and are lower when the source is groundwater (Weinberg et al. 2002). A number of other factors influence the types and amounts of DBPs formed at a location and DBP levels can vary greatly depending on water quality and treatment conditions. These factors include the type and amount of disinfectant, the application point in the treatment process, the type and concentration of organic matter in the water, pH, temperature, contact time with the disinfectant, and bromide levels (reviewed in Xie 2004).

Chlorine is the most commonly used chemical disinfectant in drinking water treatment. However, because of concern over the health effects associated with the by-products of chlorine disinfection, the use of alternative disinfectants, such as chloramines, ozone and chlorine dioxide, for primary and/or secondary disinfection is increasing (Bull 2000). It is important to note that each of these alternative disinfectants has also been shown to form its own set of DBPs. A nationwide DBP occurrence study conducted by the U.S. Environmental Protection Agency (US EPA) found that while alternative disinfectants reduce the levels of some regulated DBPs relative to chlorination, alternative disinfectants produce higher levels of some unregulated, potentially toxicologically significant DBPs (Weinberg et al. 2002). Each individual chemical disinfectant can form a mixture of DBPs and combinations of disinfectants, for example in primary and secondary disinfection, can form even more complex mixtures. As with exposure to any compound, human exposure to DBPs is a function of DBP concentration and exposure (exposure intensity and exposure time); these parameters are subject to both temporal and spatial change. More than 500 disinfection by-products have been identified in the literature thus far (Weinberg et al. 2002) and new drinking water DBPs are being identified and characterized as techniques improve and sampling efforts increase (Richardson et al. 1999; Plewa et al. 2004). However, little or no information is available for the majority of these compounds and there is great uncertainty about the identity and levels of DBPs that humans are exposed to through their drinking water. In addition, only a very limited number of DBPs have been studied for adverse health effects (Weinberg et al. 2002).

The two most prevalent groups of DBPs, trihalomethanes (THMs) and haloacetic acids (HAAs), are of particular concern to public health, because a number of studies have pointed to the possible association between these compounds and carcinogenesis and reproductive and other adverse health effects. Some epidemiological studies have linked cancers of the stomach, pancreas, kidney, bladder and rectum (Morris et al. 1992; Richardson 1998), as well as adverse reproductive outcomes, such as miscarriage and birth defects, to exposure to DBPs

(Nieuwenhuijsen et al. 2000). In a review of the genotoxic activity of organic chemicals in drinking water, Meier (1988) stated that the overwhelming majority of genotoxic agents in drinking water were generated during the chlorination stage of water treatment. Considering the assembled information on the genotoxicity and carcinogenic potential of chlorination by-products, it has been suggested that there should be a more judicious use of chlorine in the disinfection of drinking water (Minear and Amy 1996) and many nations have enacted either maximum contaminant level standards or guidelines for these two groups of compounds. The speciation and concentration of DBPs varies greatly from place to place, depending on raw water quality parameters, treatment processes, and characteristics of the distribution system. A survey of three Ontario municipalities reported total THM (TTHM) levels between 20-65 µg/L and levels of dichloroacetic acid and trichloroacetic acid between 3-20 µg/ L (Health Canada 1996a).

The over-arching question is how to obtain the cleanest and safest drinking water, while minimizing the risk from both microbial pathogens and disinfection by-products simultaneously. While the effects and mechanisms of many microbial pathogens are known, there is little information on the toxicological effects or mechanisms of most DBPs. Since there is so much information missing about disinfection by-products, the only way to confidently balance the risks between microbial pathogens and DBPs is through further research into DBPs and the water quality parameters that affect their formation. Given that DBP levels are largely determined by source water characteristics and that disinfectant consumption varies dramatically depending on those characteristics, additional information about DBPs and their effects could help water treatment facilities select the most appropriate disinfectant or adjust water quality parameters, such as pH, temperature or organic matter levels, where possible prior to disinfection. In either case, additional research in this area will help to put the threat of DBPs to human and animal health into perspective.

Although some epidemiological studies have demonstrated an association between DBPs and adverse health effects, it is unclear which DBPs pose the greatest risk. In the presence of bromide, brominated DBPs are preferentially formed during chlorine disinfection (Cowman and Singer 1996; Richardson et al. 2003). There is a general opinion that brominated DBP products are more genotoxic than their chlorinated analogues and the toxicity of these brominated DBPs is attracting increased attention (Kargalioglu et al. 2000; Plewa et al. 2002). DBP speciation is strongly affected by pH and although it is generally believed that the degree of mutagenicity gradually decreases as pH increases, recent work has shown that this is not always the case (Luk et al. 2006, in press). More work is needed in this area. Similarly, there is limited information on the effects of temperature on DBP formation kinetics and the genotoxicity of the resultant DBP mixture. The effects of these water quality parameters on DBP formation and speciation is especially relevant because a number of these factors can be controlled to some extent during the water treatment process.

Fundamental toxicological information can be obtained through the analysis of chromosome damage and cytogenetic endpoints are most frequently used in hazard identification and risk assessment (Obe et al. 2002; Sutiakova et al. 2004). The strong association between these end points and known mechanisms of oncogene activation or loss of tumour suppressor gene function places great importance on genotoxicity testing to evaluate the mutagenic and carcinogenic potential of consumer and industrial products, pharmaceutical and agricultural agents, and environmental samples. The *in vitro* chromosome aberration assay has been used for several decades to assess chromosomal damage and has become an integral part of genetic toxicology testing (Putman et al. 2001). Although the chromosome aberration assay is considerably more time and labour intensive and requires a far greater degree of training than other cytogenetic assays, such as the bacterial bioassay, it provides greater control of experimental variables, such as precise dose and consistent exposure time (Kargalioglu et al. 2000). Genetic assays based on mammalian cells are also more relevant to

the assessment of human risks than bacterial assays (Kirkland 1998). The chromosome aberration assay is one of the most sensitive and relevant means of identifying mutagens and carcinogens and the relevance of testing a chemical for the ability to induce chromosomal aberrations is now well established (Ishidate, Jr. 1988).

The objective of this study is to provide a direct assessment of the effects of pH and temperature on the genotoxicity of halogenated disinfection by-products in chlorinated water. Given the large number of brominated DBPs and the increased risk that they pose to human health, these two parameters were examined in the context of a broad range of bromide concentrations (Cowman and Singer 1996; Plewa et al. 2002; Richardson et al. 2003). In this study, the genotoxocity or the potential of chromosomal damage associated with DBPs in drinking water was evaluated using chromosome aberrations as cytogenetic endpoints. A secondary aim is to contribute to the body of knowledge on the risks associated with two important water quality parameters, pH and temperature, which in some situations may be controlled during drinking water treatment. Furthermore, the use of chromosomal aberration assays for genotoxic assessment of DBPs is still in its infancy and this study will provide comparison and verification of existing data. Additional work on chromosomal aberrations induced by chlorinated humic acid samples will help to clarify whether the use of the chromosome aberration test is a viable tool for the analysis of DBP risks. The determination of any significant genotoxic effects of chlorinated drinking water will raise concerns about the potential risk to exposed human and animal populations.

2. Factors Affecting Disinfection By-Product Formation

The speciation and concentration of disinfection by-products in water are affected by a number of water quality parameters and operating conditions, including natural organic matter, chlorine residual, chlorination time, inorganic bromide concentration, pH and temperature. To better understand DBPs in drinking water, it is important to understand DBP formation and speciation; this chapter will review the formation mechanisms of the three most prevalent groups of DBPs, trihalomethanes, haloacetic acids and trihaloacetaldehydes, and examine the effects of water quality conditions on DBP formation.

2.1 Trihalomethanes

Trihalomethanes (THMs) are small chlorinated and/or brominated organic compounds similar in structure to methane, which are formed when naturally occurring organic matter, including humic and fulvic substances, reacts with a disinfectant. Figure 2.1 illustrates the molecular structures of the four chlorinated/brominated trihalomethanes.

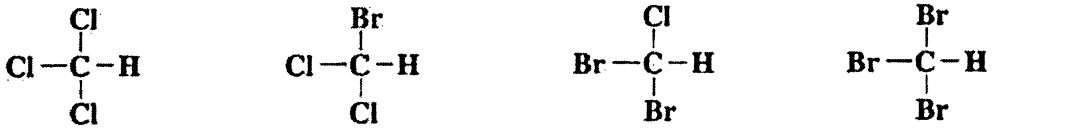


Figure 2.1: THM molecular structures (adapted from Bull and Kopfler 1991).

There is very limited information on the chemical structures of humic and fulvic substances and thus the mechanisms of DBP formation in chlorinated water are not well understood (Xie 2004). Organic substances with simple molecules are generally used to

illustrate the mechanisms of THM formation. For example, the formation of THMs can be illustrated by the reaction between propanone (acetone) and chlorine, as shown below:

(2.1) $CH_3COCH_3 + 3\ HOCl \rightarrow CH_3COCCl_3 + 3\ H_2O$

(2.2) $CH_3COCCl_3 + H_2O \rightarrow CH_3COOH + CHCl_3$

In chlorinated water, propanone is readily oxidized into trichloropropanone (Equation 2.1), which undergoes a hydrolysis reaction to form trihalomethanes (trichloromethane shown), especially at high pH (Equation 2.2) (Xie 2004). THMs can also be formed by the hydrolysis of many other trihalogenated DBPs and intermediate products, such as trihaloacetic acids, trihaloacetonitriles, trihaloacetaldeydes. In other words, some DBPs are intermediate products of chlorination reaction and others are end products; these intermediate products can be further oxidized by chlorine into end products (Reckhow and Singer 1990). If bromide ions are present, brominated intermediate products can form, which then result in the formation of brominated THMs. Bromine reacts readily with other elements and is primarily found in soluble inorganic bromide salts in aquatic and terrestrial environments. Bromide does not react with NOM directly, but inorganic bromide can be oxidized by chlorine to produce hypobromous acid or hypobromite; in this form, bromide can react with NOM to from brominated DBPs. Many monohalogenated and dihalogenated DBPs are intermediate products and, depending on a number of factors but mainly on low pH, they will remain as intermediates. Trihalomethanes are typically the end products of chlorination (Xie 2004).

Trihalomethanes were the first disinfection by-products identified in drinking water (Rook 1974). Although there is uncertainty regarding the health effects of THMs, these compounds are of special concern as they form the majority of disinfection by-products (Bull and Kopfler 1991). While animal studies have shown very high levels of THMs to be mutagenic and carcinogenic, the outcomes of epidemiological studies have been mixed. Some studies of

human populations have indicated higher incidences of bladder and colon cancer in areas where drinking water is high in THMs, while others have found associations between adverse reproductive outcome (e.g. miscarriage, birth defects, low birth weight) and high THM levels (Richardson 1998; Nieuwenhuijsen et al. 2000). Conversely, other studies have found no increased risk of cancer or adverse effects on pregnancy (Nieuwenhuijsen et al. 2000).

According to the United States Environmental Protection Agency and the Canadian Federal-Provincial-Territorial Subcommittee on Drinking Water (CDW), the trihalomethanes of significance in disinfected water are trichloromethane, dichlorobromomethane, chlorodibromomethane and tribromomethane. Under the auspices of the *Safe Drinking Water Act*, the current limit for THMs in the United States is 80 μg/L (US EPA 1998). In Canada, the CDW and Health Canada have established *Guidelines for Canadian Drinking Water Quality* (2006), which include a drinking water guideline of 100 μg/L for THMs. The Canadian guideline was established as interim until such time as the risks from other DBPs are established and is currently under review as mounting epidemiological and toxicological data becomes available. Trichloromethane and dichlorobromomethane are the first and second most dominant species of trihalomethanes (Health Canada 1996b).

2.2 Haloacetic Acids

Haloacetic acids (HAAs) are also organic compounds containing chlorine and/or bromine that are formed from organic matter during disinfection. The formation of HAAs can also be demonstrated by the reaction between propanone (acetone), as the model organic precursor, and chlorine:

(2.3) $CH_3COCH_3 + 3\ HOCl \rightarrow CH_3COCCl_3 + 3\ H_2O$

(2.4) $CH_3COCCl_3 + 2\ HOCl \rightarrow CHCl_2COCCl_3 + 2H_2O$

(2.5) $CHCl_2COCCl_3 + H_2O \rightarrow CHCl_2COOH + CHCl_3$

Propanone is oxidized into trichloropropanone (Equation 2.3), which can be further oxidized into tetra-, penta- (shown) or hexchloropropanone (Equation 2.4), especially at low pH. These chloropropanones can then undergo hydrolysis reactions to form mono-, di- (shown) and trichloroacetic acids (Equation 2.5) (Xie 2004). Trichloroacetic acid can also be formed by the reaction between generalized organic matter, CH_3-CO-R, where R is an oxidizable group, and chlorine, as shown in Equations 2.6 and 2.7 (Reckhow and Singer 1990):

(2.6) $CH_3COR + 3\ HOCl \rightarrow CCl_3COR + 3H_2O$

(2.7) $CCl_3COR + H_2O \rightarrow CCl_3COOH + HR$

If bromide ions are present, brominated intermediate byproducts can be formed, which then result in the formation of brominated HAAs (Xie 2004). Figure 2.2 shows the molecular structures of the two monohaloacetic acids, the three dihaloacetic acids and the four trihaloacetic acids.

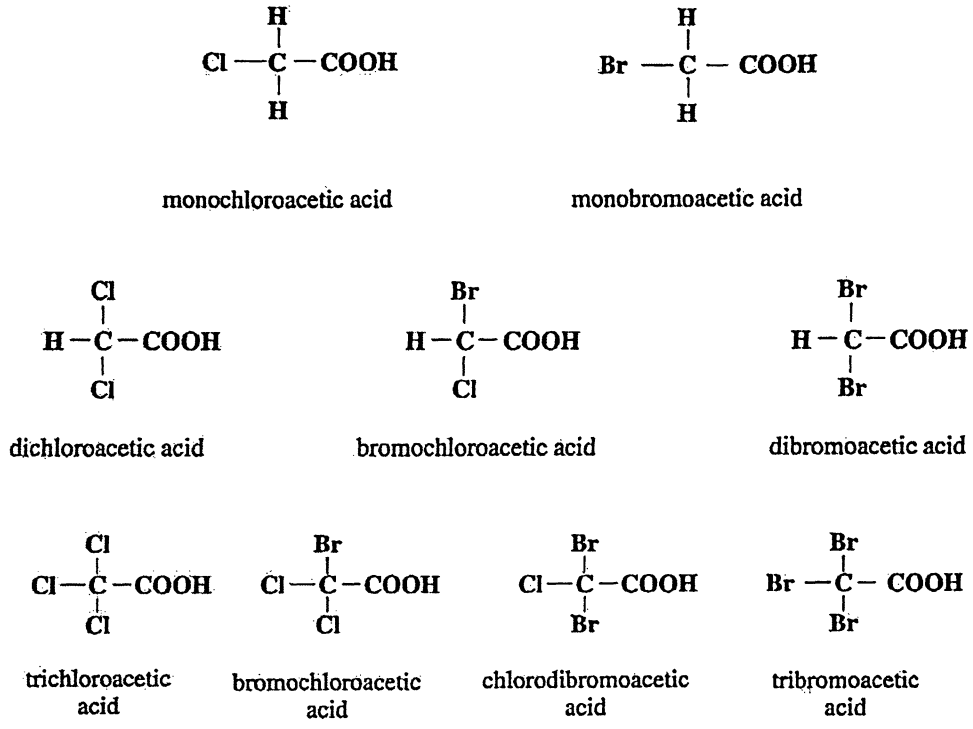


Figure 2.2: HAA molecular structures (adapted from Bull and Kopfler 1991)

Much less is known about HAAs than THMs, however animal studies have found that high levels of some haloacetic acids have carcinogenic potential and can result in adverse reproductive and developmental effects (U.S. EPA 1998; Nieuwenhuijsen et al. 2000). Most evidence suggests that the induction of mutations by haloacetic acids may depend on modifying processes of cell division and cell death rather than their weak mutagenic activites. Although dibromoacetic acid has been found to have an effect on male reproduction (i.e. marked atrophy of seminiferous tubules), data on the carcinogenicity of brominated HAAs are too preliminary to be useful in risk characterization (Cowman and Singer 1996; Nieuwenhuijsen et al. 2000).

According to the U.S. EPA (1998), the HAAs of concern in chlorinated water are chloroacetic acid, dichloroacetic acid and trichloracetic acid, as well as two brominated forms,

bromoacetic acid and dibromoacetic acid. Current *Safe Drinking Water Act* regulations limit the total level of HAAs to 60 μg/L in the United States. Although Health Canada, in its role as Secretariat to the CDW, is evaluating the potential health hazards of HAAs, there are currently no guidelines for HAAs in Canada. Dichloroacetic acid and trichloroacetic acid are the first and second most dominant species of haloacetic acids (Health Canada 1996b).

2.3 Trihaloacetaldehydes

Although typical concentrations are significantly lower than those of the THMs or the HAAs, trihaloacetaldehydes - trichloroacetaldehyde (chloral) and its brominated analogues - form the third largest group of DBPs in chlorinated water (Xie 2004). Figure 2.3 illustrates the molecular structures of trichloroacetaldehyde and the three brominated trihaloacetaldehydes, bromodichloroacetaldehyde, chlorodibromoacetaldehyde and tribromoacetaldehyde (bromal).

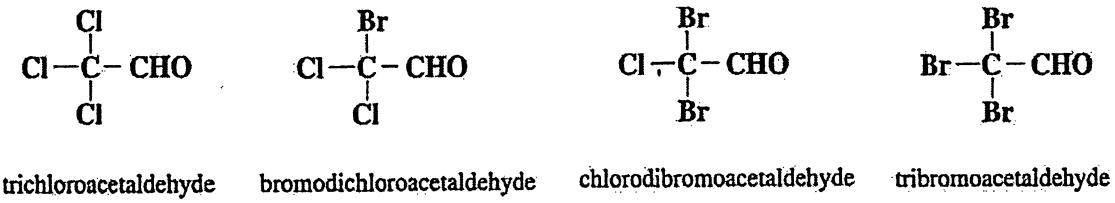


Figure 2.3: Trihaloacetaldehyde molecular structures (adapted from Bull and Kopfler 1991).

Unlike THMs and HAAs, which are formed when naturally occurring organic matter in the water reacts with a disinfectant, trihaloacetaldehydes are formed by a reaction between acetaldehyde and chlorine (aldehyde + chlorine + bromide → trihaloacetaldehydes). Monohaloacetaldehydes and dihaloacetaldehydes are also formed in chlorinated water,

however they are readily oxidized into trihaloacetaldehydes and so their occurrence is relatively limited. The three brominated trihaloacetalaldehydes are not commonly reported in drinking water monitoring, due to a lack of commercial standards and the fact that these compounds are not very stable (Xie 2004).

Table 2.1 lists the dominant groups of DBPs and the names, chemical formulas and common acronyms of their component compounds.

Table 2.1: Organic chlorination disinfection by-products (adapted from data in Bull and Kopfler 1991, US EPA 1998 and Xie 2004).

Disinfection By-Product Groups	Components	Formula	Acronyms
Trihalomethanes (THMs)	Trichloromethane (Chloroform)	$CHCl_3$	TCM, CF
	Bromodichloromethane	$CHBrCl_2$	BDCM
	Chlorodibromomethane	$CHBr_2Cl$	CDBM
	Tribromomethane (Bromoform)	$CHBr_3$	TBM, BF
Haloacetic Acids (HAAs)			
Monohaloacetic Acids	Monochloroacetic acid	$CH_2ClCOOH$	ClAA, MCAA
	Monobromoacetic acid	$CH_2BrCOOH$	BrAA, MBAA
Dihaloacetic Acids	Dichloroacetic acid	$CHCl_2COOH$	Cl_2AA, DCAA
	Bromochloroacetic acid	CHBrClCOOH	BrClAA, BCAA
	Dibromoacetic acid	$CHBr_2COOH$	Br_2AA, DBAA
Trihaloacetic acids	Trichloroacetic acid	CCl_3COO	Cl_3AA, TCAA
	Bromodichloroacetic acid	$CBrCl_2COOH$	$BrCl_2AA$, BDCAA
	Chlorodibromoacetic acid	$CBr_2ClCOOH$	$ClBr_2AA$, CDBAA
	Tribromoacetic acid	CBr_3COOH	Br_3AA, TBAA
Trihaloacetaldehydes	Trichloroacetaldehyde (Choral)	CCl_3CHO	Cl_3Ald, CH
	Bromodichloroacetaldehyde	$CBrCl_2CHO$	$BrCl_2Ald$
	Chlorodibromoacetaldehyde	CBr_2ClCHO	Br_2ClAld
	Tribromoacetaldehyde (Bromal)	CBr_3CHO	Br_3ClAld
Haloacetonitriles			
Dihaloacetonitriles	Dichloroacetonitrile	$CHCl_2CN$	Cl_2AN, DCAN
	Bromochloroactonitrile	CHBrClCN	BrClAN, BCAN
	Dibromoacetonitrile	$CHBr_2CN$	Br_2AN, DBAN

Table 2.1: Organic chlorination disinfection by-products (cont.)

Disinfection By-Product Groups	Components	Formula	Acronyms
Trihaloacetonitriles	Trichloroacetonitrile	CCl_3CN	Cl_3AN, TCAN
	Bromodichloroacetonitrile	$CBrCl_2CN$	$BrCl_2AN$, BDCAN
	Chlorodibromoacetonitrile	CBr_2ClCN	Br_2ClAN, CDBAN
	Tribromoacetonitrile	CBr_3CN	Br_3AN, TBAN
Halopropanones/ Haloacetones			
Dihalopropanones	Dichloropropanone (Dichloroacetone)	$CHCl_2COCH_3$	Cl_2PN
	Bromochloropropanone (Bromochloroacetone)	$CHBrClCOCH_3$	BrClPN
	Dibromopropanone (Dibromoacetone)	$CHBr_2COCH_3$	Br_2PN
Trihalopropanones	Trichloropropanone (Trichloroacetone)	CCl_3COCH_3	Cl_3PN
	Bromodichloropropanone (Bromodichloroacetone)	$CBrCl_2COCH_3$	$BrCl_2PN$
	Chlorodibromopropanone (Chlorodibromoacetone)	$CBr_2ClCOCH_3$	Br_2ClPN
	Tribromopropanone (Tribromoacetone)	CBr_3COCH_3	Br_3PN
Trihalonitromethanes	Trichloronitromethane (Chloropicrin)	CCl_3NO_2	Cl_3NM, CP
	Bromodichloronitromethane	$CBrCl_2NO_2$	$BrCl_2NM$
	Chlorodibromonitromethane	CBr_2ClNO_2	Br_2ClNM
	Tribromonitromethane	CBr_3NO_2	Br_3NM
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone		MX
	Brominated analogues		

2.4 Natural Organic Matter

Disinfection by-products are formed from the reaction between natural organic matter (NOM), which serves as the organic precursor, and chlorine or other disinfectants. It is not surprising, therefore, that increasing NOM levels in chlorinated water increases the formation of DBPs (Reckhow et al. 1990; Barrett et al. 2000). Two mechanisms underlie this positive correlation. First, increasing NOM levels will increase the level of DBP precursors, which in turn increases DBP formation. Secondly, increasing NOM levels will increase the chlorine demand of the water, which in turn requires a high chlorine dosage to maintain a proper chlorine residual in distribution systems. NOM consumes chlorine, making it unavailable for disinfection of pathogenic microorganisms or for oxidation of reduced metals, such as ferrous iron. The resultant high chlorine dosage leads to a further increase in the formation of DBPs (Minear and Amy 1996; Harrington et al. 1996). Figure 2.4 illustrates the positive relationship between NOM levels (expressed as concentration of phenolic carbon – a hydrophobic NOM fraction) and chlorine consumption. Figures 2.5, 2.6 and 2.7 demonstrate how this increase in chlorine demand corresponds to an increase in the formation of three DBPs, trichloromethane (chloroform), dichloroacetic acid and trichloroacetic acid (Harrington et al. 1996).

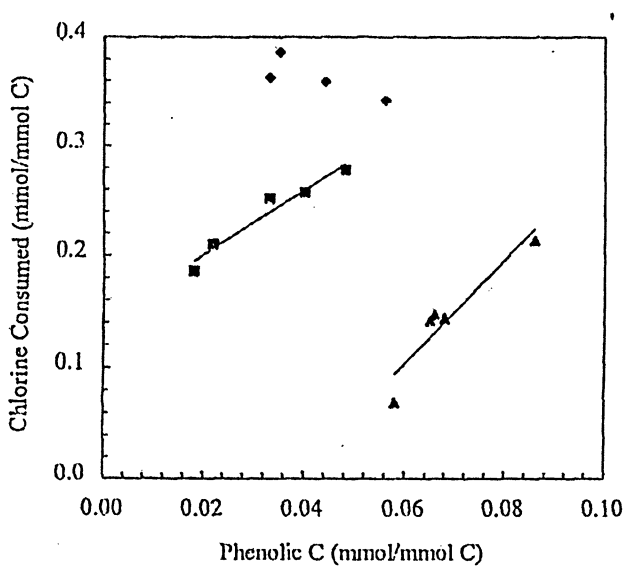


Figure 2.4: Relationship between chlorine consumption and initial concentration of phenolic carbon (Harrington et al. 1996)

■ Fulvic acids
♦ Humic acids
▲ Hydrophobic NOM

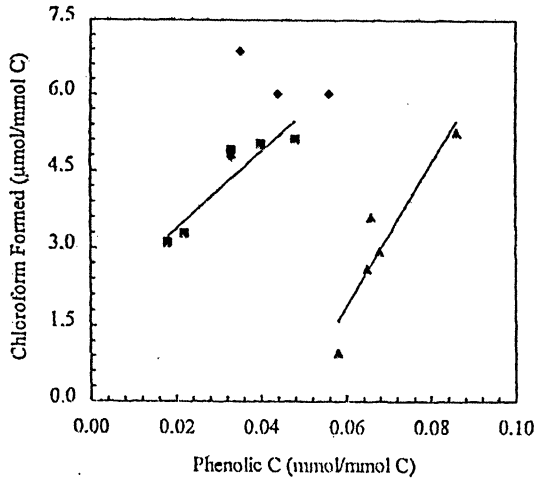


Figure 2.5

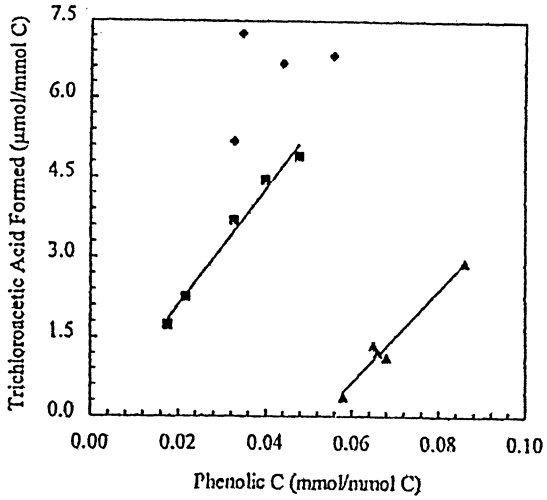


Figure 2.7

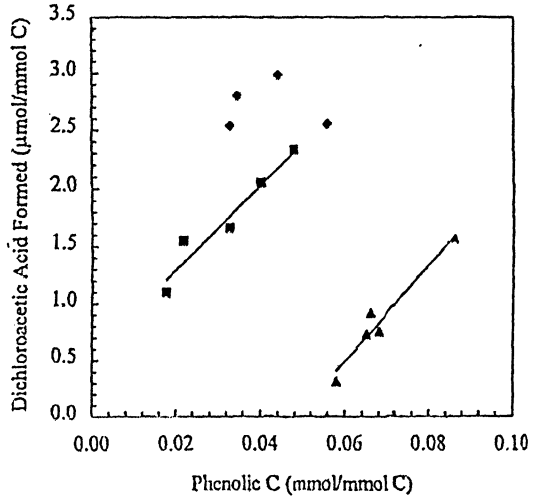


Figure 2.6

Figure 2.5: Relationship between chloroform formation and initial concentration of phenolic carbon (Harrington et al. 1996)

Figure 2.5: Relationship between dichloroacetic acid formation and initial concentration of phenolic carbon (Harrington et al. 1996)

Figure 2.7: Relationship between trichloroacetic acid formation and initial concentration of phenolic carbon (Harrington et al. 1996)

■ Fulvic acids
♦ Humic acids
▲ Hydrophobic NOM

NOM levels are generally measured and reported as total organic carbon (TOC) or dissolved organic carbon (DOC), however NOM can be separated into several fractions, including humic acids, fulvic acids, hydrophobic acids, hydrophobic neutrals, transphilic acids, transphilic neutrals, hydrophilic acids and hydrophilic neutrals (Minear and Amy 1996). The structural nature of the NOM species present in solution is an important factor in determining the influence of NOM on the chlorination process and DBP formation. Aquatic humic materials are thought to have moderate aromatic character (approximately 25% of the total carbon) with large numbers of carboxyl groups and phenolic groups, some alcohol OH groups, methoxyl groups, ketones and aldehydes. This is significant because activated aromatic structures, such as phenolics, are known to be especially reactive with chlorine, producing large amounts of chlorinated by-products (Reckhow et al. 1990). Aqueous chlorine species are electrophiles that tend to react with electron rich sites on organic structures, and activated aromatic rings, aliphatic β-dicarbonyls and amino nitrogen are all examples of electron-rich organic structures that react strongly with chlorine (Harrington et al. 1996). Reckhow et al. (1990) have shown that for aquatic humic substances, reactivity with chlorine tends to increase with the activated aromatic carbon content of the humic substance. That study also found that humic acids generally produce higher concentrations of DBPs than corresponding fulvic acids.

There is limited information on the effects of NOM on DBP speciation. In general, where water contains bromide, low levels of NOM result in higher percentages of brominated DBPs than high levels of NOM. This is because a higher NOM level requires a higher chlorine dose, which in results in a larger ratio between bromide and chlorine (Minear and Amy 1996). In typical drinking water treatment, a high level of NOM results in high levels of DBPs, including THMs and HAAs. Table 2.2 shows the organic content and diversity of drinking water sources.

Table 2.2: NOM characteristics of natural waters

Location	Source Type	TOC (mg/L)	Reference
Kanagawa, Japan	river	0.49	Urano et al. 1983
Charlesbourg, QB, Canada	river	2.47	Rodriguez and Sérodes 2001
Teays Aquifer, IL, USA	aquifer	2.9	Zhang et al. 2005
Manhattan, KS, USA	groundwater	2.9	Zhang et al. 2005
Jefferson Parish, LA, USA	river	3.0	Plewa et al. 2004
Lake Matthews, CA, USA	impoundment	3.1	Harrington et al. 1996
Lévis, QB, Canada	river	3.26	Rodriguez and Sérodes 2001
Lake Decatur, IL, USA	lake	3.4	Zhang et al. 2005
Sainte-Foy, QB, Canada	river	3.42	Rodriguez and Sérodes 2001
Andover, MA, USA	natural lake	3.5	Harrington et al. 1996
Buckingham, Canada	river	4.1	Health Canada 1996a
Istanbul, Turkey	lake	5.12	Toroz and Uyak 2005
Haworth, NJ, USA	impoundment	5.4	Harrington et al. 1996
Minot, ND, USA	groundwater	5.6	Zhang et al. 2005
Ottawa, Canada	river	5.9	Health Canada 1996a
Hull, Canada	river	5.9	Health Canada 1996a
Bangkok, Thailand	reclaimed from industrial wastewater	6.13	Musikavong et al. 2005
Sioux Falls River, SD, USA	river	10.5	Zhang et al. 2005
St. Paul Lake Vadnais, MN, USA	lake	12.8	Zhang et al. 2005
West Palm Beach, FL, USA	limestone aquifer	14.4	Harrington et al. 1996
Myrtle Beach, SC, USA	swamp canal	17.4	Harrington et al. 1996
Suwannee River, FL, USA	river	108	Kargalioglu et al. 2000
Commercially synthesized humic acid		1030	Itoh et al. 2001

2.5 Chlorine Dose and Chlorination Time

Chlorine serves as an inorganic DBP precursor and is the other major reactant in the formation of chlorinated DBPs. The amount of chlorine used in the water treatment process (chlorine dose) and the age of the treated water (chlorination time) are thus key factors in DBP formation and speciation (Bull 2000). Some DBPs are end products of chlorination reaction and others are intermediate products; these intermediate products can be further oxidized by chlorine into end products, as shown in the following equations (Xie 2004).

(*3.1*) NOM + HOCl → Intermediate products

(*3.2*) Intermediate products + HOCl → End products

Many monohalogenated and dihalogenated DBPs, except for monohaloacetic acids and dihaloacetic acids, are intermediate products (Williams et al. 1994). Depending on the amount of chlorine residual, which is required for pathogen control, and the age of the water/distance along the distribution system from the treatment plant, further chlorination of these intermediate products can result in the formation of dihalogenated and trihalogenated DBPs. Hence, in general, increasing chlorine dose increases the formation of THMs, HAAs and other chlorination disinfection end products in typical finished water (Xie 2004). Figure 2.8 shows the ranges and average concentrations of THMs in three utilities around Québec City, Canada (Rodriguez and Sérodes 2001) and illustrates the correlation between chlorine dose and THM concentrations. THM levels are consistently highest in Charlesbourg, the facility with the highest chlorine dose (2.81 mg/L). Similarly, at points further along the distribution system, THM concentrations appear to be higher in Sainte-Foy (chlorine dose: 1.93 mg/L) than in Levis (chlorine dose: 1.25 mg/L).

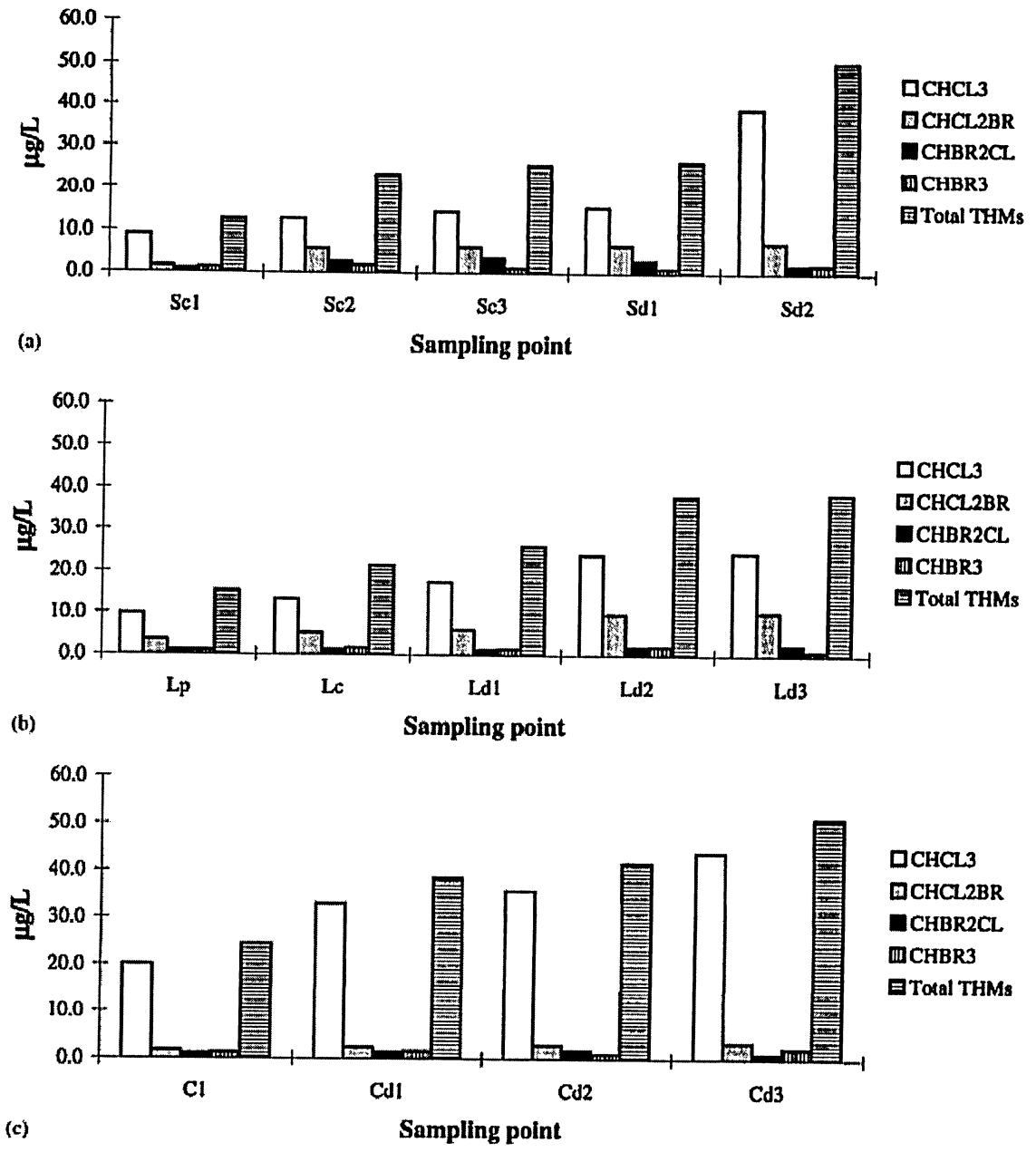


Figure 2.8: Variations in THM concentrations across three distribution systems: (a) Sainte-Foy [chlorine dose: 1.93 mg/L], (b) Levis [chlorine dose: 1.25 mg/L], (c) Charlesbourg [chlorine dose: 2.81 mg/L]. The sampling points (left to right) refer to increasing distance along the distribution system from the treatment facility (Rodriguez and Sérodes 2001).

Since many DBPs are formed by a series of reactions, increasing the reaction time will increase the formation of end-product DBPs. Conversely, increasing the reaction time may decrease the formation of intermediate-product DBPs, especially at high chlorine doses. DBPs such as trihalopropanones, trihaloacetaldehydes and trihalonitromethanes readily undergo hydrolysis reactions and increasing the reaction time, especially after DBP precursors have been exhausted, will favour hydrolysis reactions and reduce the concentrations of these DBPs (Xie 2004). Since THMs are the most prevalent DBPs and are typical hydrolysis end-products, their formation generally increases with increased reaction time. As shown in Table 2.3, a survey (Health Canada 1996a) of the spatial variation of DBPs in a distribution system following chlorine-chlorine treatment found that THM concentrations increased significantly along the distribution system, with a maximum level of trichloromethane at the final (D3) sample location at levels almost three times greater than those observed at the treatment plant. This is of significance since the majority of consumers receive their water at relatively distant points along the distribution system, where THM levels and associated risks are highest. It should be noted that the water taken by this treatment plant has very low levels of bromide (<0.002 mg/L) and accordingly the levels of brominated THMs observed were very low. Similar trends have been reported in other surveys (Whitaker et al. 2003; Rodriguez et al. 2004; Toroz and Uyak 2005).

Table 2.3: Yearly mean THM values (µg/ L) and chlorine reaction time: Hull, QB, Canada (chlorine-chlorine treatment). D1, D2 and D3 refer to distances along the distribution system away from the treatment plant. Adapted from Health Canada (1996a).

DBP	Treatment Plant	D1 (4 km)	D2 (11 km)	D3 (17 km)
Trichloromethane (Chloroform)	21.3	33.3	43.5	55.9
Bromodichloromethane	3.1	3.9	4.6	5.1
Chlorodibromomethane	0.3	0.3	0.3	0.3
Tribromomethane (Bromoform)	<0.1	<0.1	<0.1	<0.1
Total Trihalomethanes	**24.8**	**37.5**	**48.4**	**61.4**

Mature water, as determined relative to the time of disinfection at the treatment plant, has consistently higher levels of THMs compared to points on the distribution system closer to the treatment plant, however a more complex picture emerges with regard to HAAs. HAA levels are initially fairly constant, however they tend to decrease at distant points along the distribution system (around 18 km) (Health Canada 1996a). Other researchers have reported similar findings and have attributed this effect to bacterial degradation, however whether the degradation mechanism is biological or chemical has not been resolved conclusively (Williams et al. 1994; Williams et al. 1995).

2.6 pH

Many DBPs, including trihalopropanes, trihaloacetonitriles, trihaloacetaldehydes, trihalonitromethanes and trihaloacetic acids, undergo hydrolysis reactions to form trihalomethanes (THMs). Since a higher pH generally increases the rates of these hydrolysis reactions, a high pH usually results in higher levels of THMs, but lower levels of haloacetic acids (HAAs) and other halogenated DBPs (Xie 2004). Conversely, a low pH favours the formation of HAAs, trihaloacetaldehydes, trihalopropanones and other halogenated DBPs. The effects of pH on the formation of total trihalomethanes (TTHM) and two haloacetic acids, trichloroacetic acid (TCAA) and dichloroacetic acid (DCAA), are shown in Figure 2.9 (Xie 2004)

Urano et al. (1983) modeled the relationship between THM concentration and pH at fixed times and found that THM formation was approximately proportional to the logarithmic concentration of the hydroxide ion. The relations are expressed approximately by the straight lines converging at a point of pH 2.8 in the practical pH range of 5 to 9 (Figure 2.10).

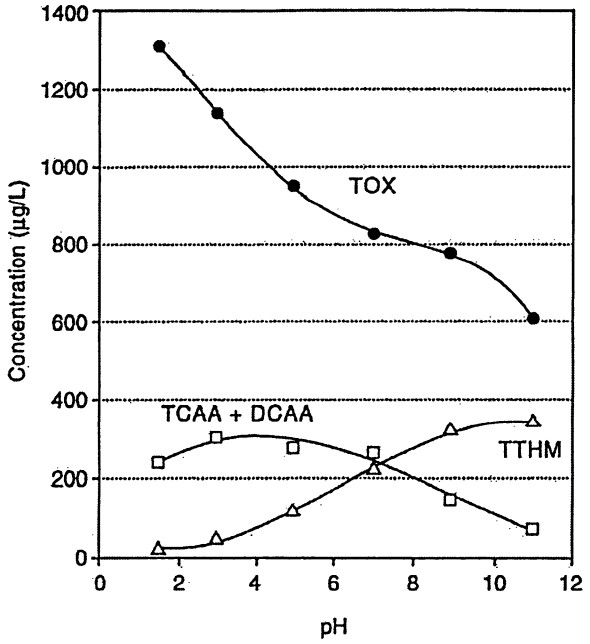


Figure 2.9: Formation of chlorination by-products as a function of pH (Xie 2004).

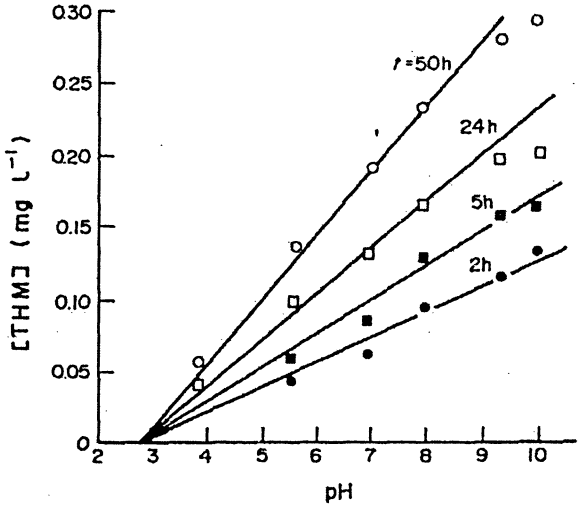


Figure 2.10: Relationship between THM concentration and pH. T=20°C, TOC=4.2 mg/L, Cl_2=50 mg/L (Urano et al. 1983).

The effects of pH on THM and HAA formation can be further illustrates with one of their precursors, propanone. At a low pH, hydrolysis reactions are slowed and trichloropropanone can be further oxidized into pentachloropropanone, which results in the formation of dichloroacetic acid. However, at a high pH, the hydrolysis of trichloropropanone significantly reduces the formation of pentachloropropanone and dichloroacetic acid (Xie 2004). pH also influences electron distribution within NOM structures and the distribution of aqueous chlorine species and is an important factor in the interaction between NOM and aqueous chlorine species. The kinetic competition between hydrolysis, oxidation and halogenation reactions also establishes pH as an important factor in determining the distribution of DBPs formed (Harrington et al. 1996).

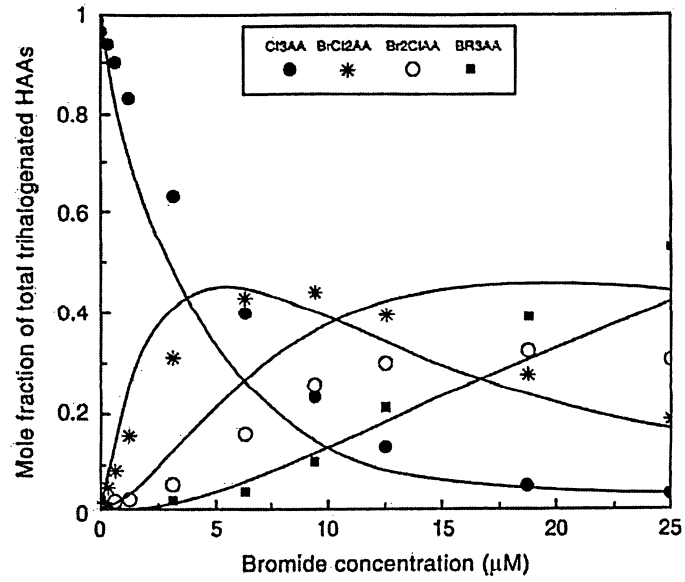
Meier et al. (1983) examined the effects of varying chlorination pH on mutagen formation and found that pH is a critical factor in mutagen formation, with a pattern of decreasing mutagenic activity with increasing pH. Observed TOX (total organic halide) levels were 445 mg/L at pH 2.75, 298 mg/L at pH 6.4 and 217 mg/L at pH 7.6 and the authors concluded that maximum mutagen formation occurs at pH below 7. This supports an earlier finding that raising the pH to the neutral range reduces mutagenicity somewhat, but that a very high pH (10-12) is required to greatly reduce mutagenicity (Meier et al. 1983). The observed pH dependency of mutagen formation is also consistent with the observation of Oliver (1978) who found that non-volatile chlorinated organic carbon formation following chlorination of humic acid decreased with increasing pH. Although it is generally believed that the degree of mutagenicity decreases as pH increases, recent work has shown that this is not always the case. In brominated humic acid samples, the genotoxic effects of TOBr and TOCl may counter each other, resulting in little appreciable difference in genotoxicity across the pH range (Luk et al. 2006, in press).

In general, the pH of water undergoing disinfection treatment fluctuates between approximately 6.3 and 9 (Health Canada 1996a; Whitaker et al. 2003; Toroz and Uyak 2005).

However, depending on the source water, pH levels can range from pH 5 to pH 9.5 (Rodiguez et al. 2004; Thompson 2005, pers. comm.).

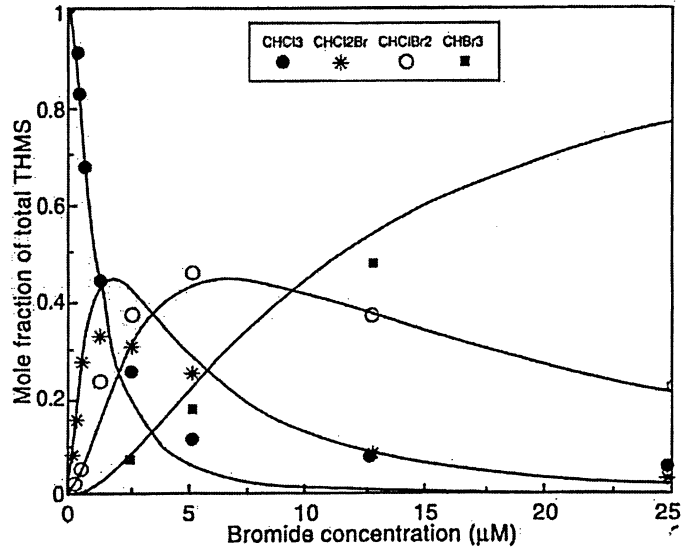
2.7 Bromine

Bromine is a halogen that readily reacts with other elements and dissolves easily in water. In its naturally occurring form, bromine is primarily found as soluble inorganic bromide salts in aquatic and terrestrial environments, although some organic bromine substances are produced by a number of marine organisms. Bromide does not react with NOM directly, but inorganic bromide can be oxidized by chlorine to produce hypobromous acid or hypobromite (Xie 2004). In this form, bromide acts as an additional DBP precursor and can react with NOM to form brominated DBPs. In general, bromide is much more reactive to NOM than chlorine and when water containing bromide is chlorinated, both brominated and chlorinated DBPs will be formed. When bromide is present, the formation of chlorinated species will be reduced, since the bromide will occupy the site for chlorine substitution (Cowman and Singer 1996). Richardson et al. (2003) confirmed through the use of gas chromatography/ mass spectrometry that there is a significant shift in speciation to brominated DBPs, dominated by tribromomethane and dibromoacetic acid, when there is an increase in the concentration of the original bromides. Cowman and Singer (1996) investigated the effect of bromide ion of the distribution of haloacetic acid species and found that bromochloro-, bromodichloro-, and dibromochloro-acetic acids were readily formed in waters containing as little as 0.1 mg/L bromide and were major components of the total HAA concentration at bromide concentrations found in raw drinking water (Figure 2.11). A similar trend was observed for THM speciation in brominated water (Figure 2.12) and the authors concluded that under these experimental conditions, bromine was approximately 10 times more reactive than chlorine in substitution reactions.



• trichloroacetic acid (Cl_3AA); * bromodichloroacetic acid ($BrCl_2AA$);
o dibromochloroacetic acid ($BrCl_2AA$); ■ tribromoacetic acid (Br_3AA)

Figure 2.11: Effects of bromide on HAA formation (Cowman and Singer 1996)



• trichloromethane ($CHCl_3$); * bromodichloromethane (CHCl2Br);
o chlorodibromomethane ($CHClBr_2$); ■ tribromomethane ($CHBr_3$)

Figure 2.12: Effects of bromide on THM formation (Cowman and Singer 1996)

Both TOC levels and bromide levels affect DBP formation, but it is the chlorine-to-bromide ratio and the bromide-to-TOC ratio that affect DBP speciation. In addition, temperature and reaction time can affect speciation of brominated DBPs when TOC concentrations are limited (see *2.8 Temperature* below). Cowman and Singer (1996) showed that an increase in bromide levels results in an increase in the formation of brominated HAAs and a reduction in the formation of chlorinated HAAs. However, brominated trihaloacetic acids, especially tribromoacetic acid, tend to be unstable and readily undergo hydrolysis reaction to form brominated THMs. Accordingly, the overall total molar concentration of trihaloacetic acids decreases as bromide levels increase and the total molar concentration of THMs, common hydrolysis by-products of many brominated DBPs, tends to be increase with higher bromide levels (Cowman and Singer 1996). Since the mass of bromine (atomic weight 79.9) is much greater than that of chlorine (atomic weight 35.5), the µg/L concentration of tribromomethane is more than double that of trichloromethane (Xie 2004). Therefore, under similar chlorination conditions, higher bromide levels could significantly increase the total concentration of the four regulated THMs. Figure 2.13 shows the impact of TOC and bromide ion levels on total THM (TTHM) formation in the Sacramento River, CA, USA (Krasner et al. 1996). It is noteworthy that as bromide concentrations increase, the range of TOC values that would enable compliance with the 80 µg/L TTHM maximum contaminant level established by the US EPA (1998) decreases. This has implications for precursor removal technologies, such as enhanced coagulation or granular activated carbon (GAC), which remove TOC, but not bromide, and result in a shift to more brominated DBPs. In particular, when organic precursor levels are limiting, HOBr will react with active sites more readily, leaving fewer active sites for chlorine substitution (Krasner et al.1996).

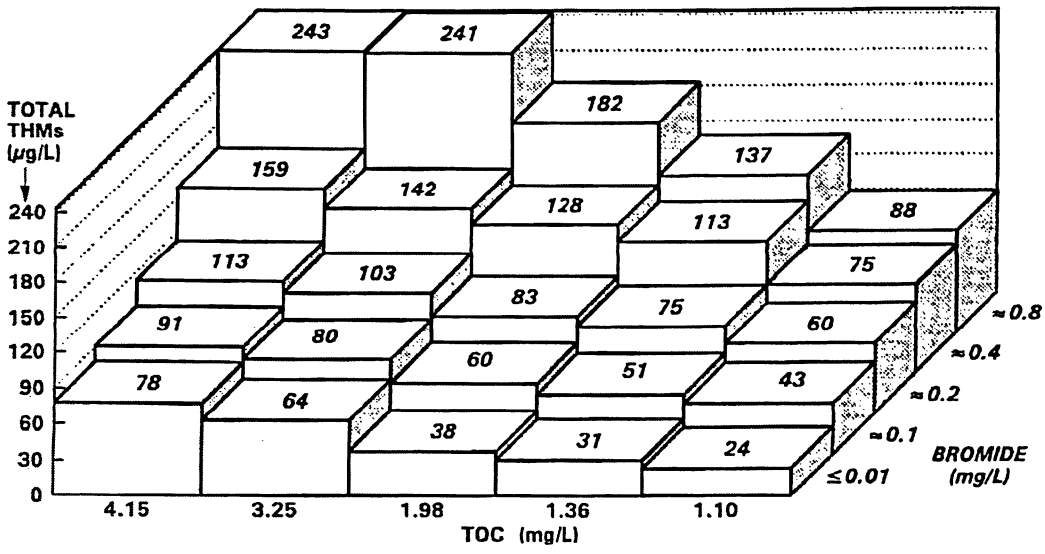


Figure 2.13: Impact of TOC and bromide ion on total THM formation (T = 25°C, pH = 8.2, incubation time = 3 hrs). (Krasner et al. 1996).

There is a general opinion that brominated DBPs are more cytotoxic and genotoxic than chlorinated DBPs and the toxicity of these brominated DBP products in now attracting increasing attention. Plewa et al. (2002) used Chinese hamster ovary (CHO) cells in microplate assays to assess the cytotoxicity and genotoxicity of a number of disinfection by-products. The rank order in decreasing chronic cytotoxicity that they observed was: bromoacetic acid (BA) >> 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) > dibromoacetic acid (DBA) > chloroacetic acid (CA) > $KBrO_3$ > tribromoacetic acid (TBA) > dichloroacetic acid (DCA) > trichloroacetic acid (TCA). The CHO cell cytotoxicity-response curves for the DBPs under investigation are illustrated in Figure 2.14 (Plewa et al. 2002). Alkaline single-cell gel electrophoresis (SCGE, comet assay) was used to assess the induction of DNA strand breaks by these compounds and the observed rank order in decreasing genotoxicity was: BA >> MX > CA > DBA > TBA > $KbrO_3$ > DCA > TCA. The authors concluded that the brominated haloacetic acids are more cytotoxic and genotoxic than their chlorinated analogues.

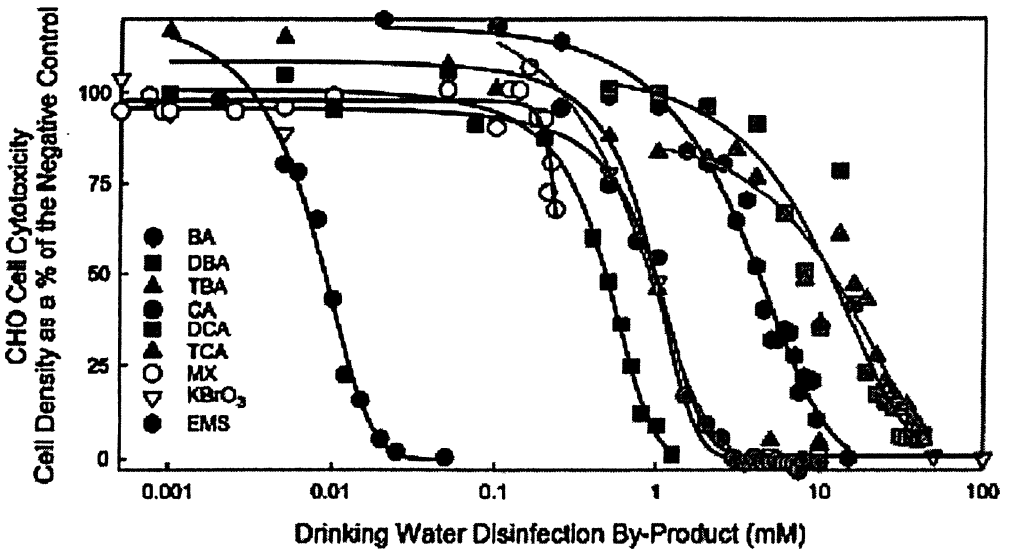


Figure 2.14: Log-linear plot of CHO cell cytotoxicity by DBPs (Plewa et al. 2002)

Similar trends were observed for halonitromethanes, a group of drinking water disinfection by-products that recently received high priority for health effects research by the US Environmental Protection Agency (EPA). Using CHO cells in microplate assays, Plewa et al. (2004) found that the brominated nitromethanes were more cytotoxic and genotoxic than their chlorinated analogues. Although the greater genotoxic potential of brominated DBPs has been substantially described, details about the nature of their contribution to health hazards and the effects of environmental factors, such as pH or temperature, remain largely unknown.

The U.S. EPA nationwide DBP occurrence study identified the presence of numerous brominated DBPs, many of which were previously unknown. A number of brominated acids (i.e. brominated propanoic, propenoic, butanoic, butenoic, oxopentanoic, heptanoic, nonanoic and butenedioic acids) and a brominated ketone (1-bromo-1,3,3-trichloropropanone), which were identified for the first time, were found to be wide-spread (Weinberg et al. 2002). Table 2.4 summarizes the range of bromide concentrations found in natural waters around in the world.

Table 2.4: Summary of previous surveys of bromide levels present in natural waters (adapted from Magazinovic et al. 2004)

Water source	Location	Bromide (μg/L)	Reference
Resevoirs	United States	6-83	Amy et al. 1993
	France, UK, Spain	30-70	Legube 1996
Other surface waters	United States	3-426	Amy et al. 1993
	United States	24-170	Pyen et al. 1980
	UK	100-120	Bourgine et al. 1993
	Germany	6-280	Haag and Hoigne 1982
	Germany	9-760	Rook et al. 1978
	France, UK, Spain	30-70	Legube 1996
	France	55-202	Lefebvre et al. 1995
	France	24-57	Welte and Montiel 1995
	Sweden	4-76	Lundstrom and Olin 1986
	Poland	400-700	Olsinska 1994
	Israel	2000	Rebhun et al. 1988
Groundwaters	United States	2-429	Amy et al. 1993
	Finland	30-100	Hiisvirta and Sauri 1995
	England	26-2226	Houghton 1946
	France, UK, Spain	40-140	Legube 1996
	France	190-647	Lefebvre et al. 1995
Shallow water wells & water wells near plugged oil/gas wells	United States	<0.5 mg/L-9.4 mg/L	Hudak and Wachal 2001
Sea	Atlantic	65000	Stumm and Morgan 1981

2.8 Temperature

Only recently have researchers started to look at the effects of temperature on DBP formation and a review of the literature reveals limited information on the relationship between temperature and DBP speciation and genotoxicity. Krasner et al. (1996) conducted a series of experiments that examined the impact of temperature and time on THM and HAA formation. Figure 2.15 shows the impact of temperature and time on THM formation when TOC (organic precursor) concentrations are more than an order of magnitude greater than bromide levels, the scenario typically observed in surface water treatment plants (Amy et al. 1993). Trichloromethane ($CHCl_3$) continues to form over time and approximately twice as much is formed at 25°C than at 10°C. On the other hand, tribromomethane ($CHBr_3$) forms quickly at 10°C and neither temperature nor time have any significant impact on $CHBr_3$ concentration. The formation of the two mixed bromo/chloro THM species, bromodichloromethane ($CHCl_2Br$) and chlorodibromomethane ($CHClBr_2$), increase with increasing time and temperature, however these increases were not as dramatic as those observed for trichloromethane. The initial formation of trichloromethane at 25°C was less than that of either bromodichloromethane or chlorordibromomethane, yet after 24 hours of chlorine contact time, the concentration of trichloromethane was the greatest. These findings suggest that there is a difference in the kinetics of halogenation between HOBr and HOCl. Halogenation by HOBr appears to be quicker and less impacted by other variable when TOC is in excess (Amy et al. 1993).

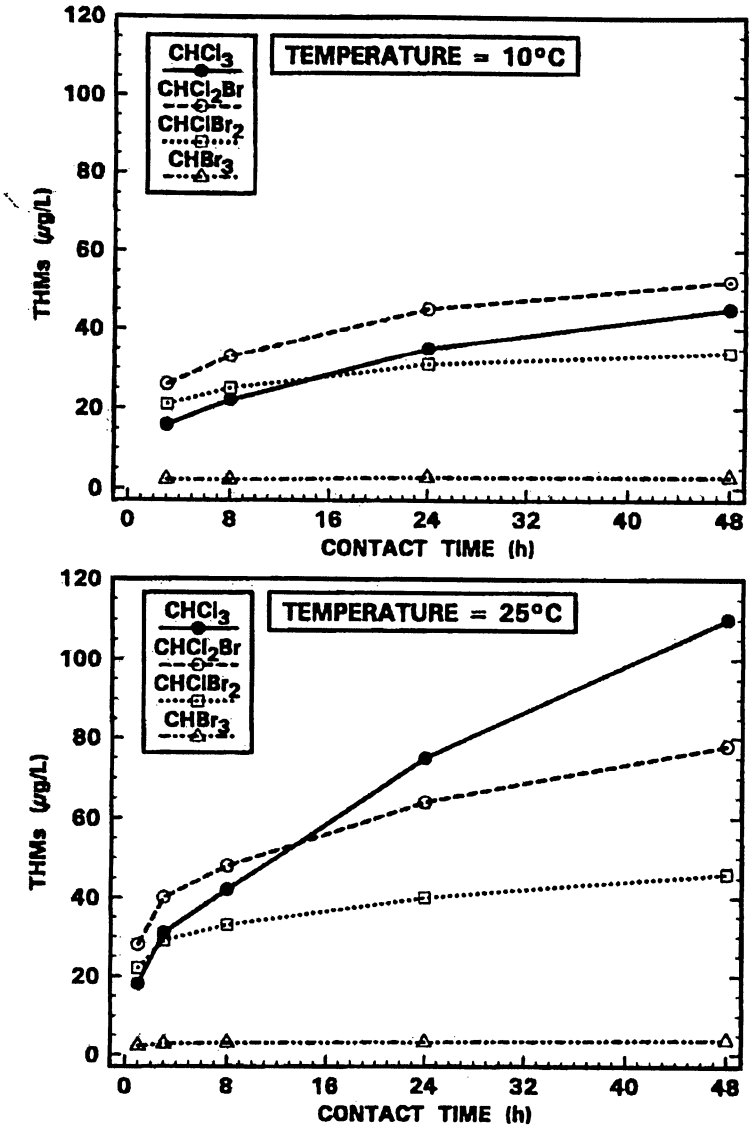


Figure 2.15: Impact of temperature and chlorine contact time on THM formation (low bromide). TOC: 3.25 mg/L; Bromide: 0.2 mg/L; pH: 8.2 (Krasner et al. 1996).

In coastal surface waters or in groundwater that is heavily impacted by salt-water incursion, TOC and bromide concentrations are more equivalent and tribromomethane formation appears to be strongly influenced by reaction time and temperature (Figure 2.16). Unlike Figure 2.15, where TOC was not a limiting factor for HOBr halogenation, Figure 2.16 shows that when less

TOC is available for halogenation, more HOBr becomes available compared to HOCl (Krasner et al. 1996).

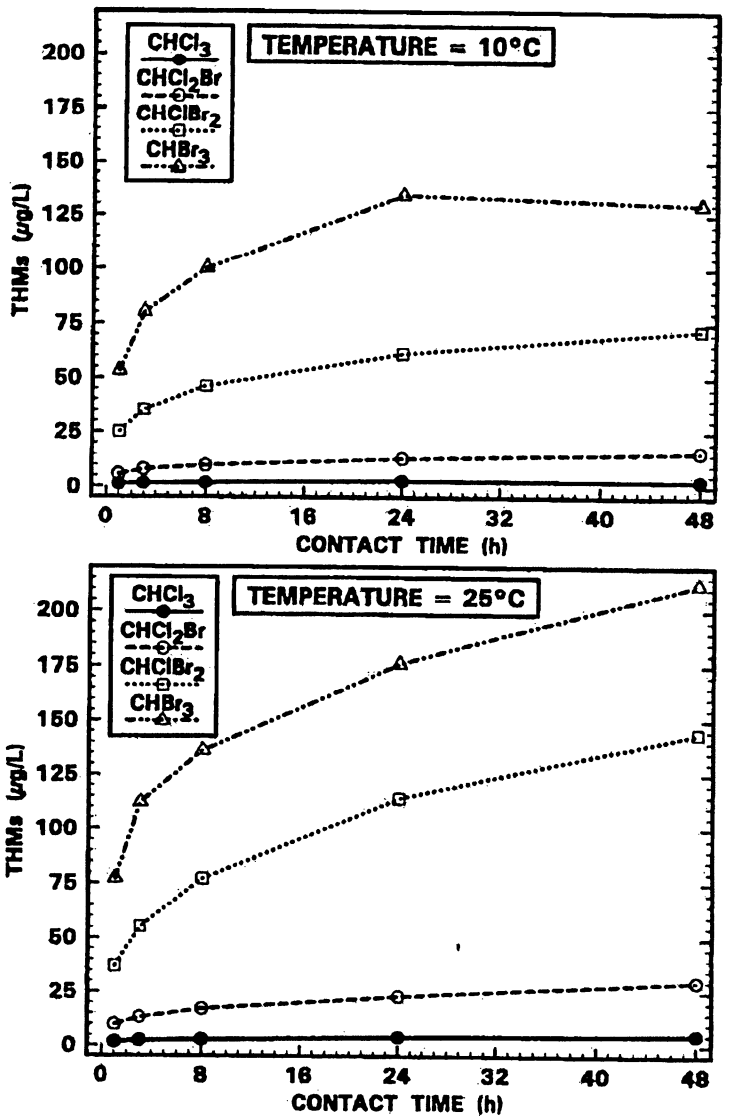


Figure 2.16: Impact of temperature and chlorine contact time on THM formation (high bromide). TOC: 2.0 mg/L; Bromide: 0.8 mg/L; pH: 8.2 (Krasner et al. 1996).

Observed effects of temperature on haloacetic acid formation were similar to those observed for the THMs. As with the THMs, when TOC levels are much greater than bromide levels, chlorinated HAAs are more impacted by temperature and time than brominated HAAs

(Krasner et al. 1996). Where TOC and bromide concentrations are more equivalent, brominated HAAs are more strongly influenced by temperature and time and there is a notable increase in the concentration of dibromoacetic acid. Krasner et al. (1996) also observed a positive correlation between temperature and chlorine contact time and chlorine demand. In samples where the TOC concentration (3.25 mg/L) was significantly greater than the bromide concentration (0.2 mg/L), the chlorine demand at 10°C varied between 1.95 and 3.52 mg/L for the 3 to 48 hour incubation period. At 25°C, the chlorine demand for these samples during the 3 to 48 hour incubation period ranged from 2.65 to 5.15 mg/L. Thus, increases in temperature or chlorine contact time result in a higher chlorine demand and, in turn, a lower Br^- to Cl^+ ratio.

A number of surveys have reported higher DBP concentrations in summer months than in winter months (Health Canada 1996a; Rodriguez et al. 2004; Toroz and Uyak 2005). Rodriguez et al. (2004) found that the highest average THM levels in the distribution system occurred during summer and fall (Figure 2.17). The authors noted that during the summer period, average water temperatures, chlorine doses and TOC levels were at their highest levels and it seems likely that much of the observed seasonal difference in THM levels can be attributed to higher levels of organic matter in surface waters during the summer months, and the resultant high chlorine demand. Health Canada (1996a) reported that in warmer months, several of the surveyed treatment facilities introduced a prechlorination step (increased the level of chlorine) to control algal growth. Figure 2.17 also shows that levels of brominated THMs were lower in the spring than in the summer and fall, which the authors ascribe to a possible decrease of bromide concentration in the raw water during the spring caused by an increase in the river flow rate after spring thaw.

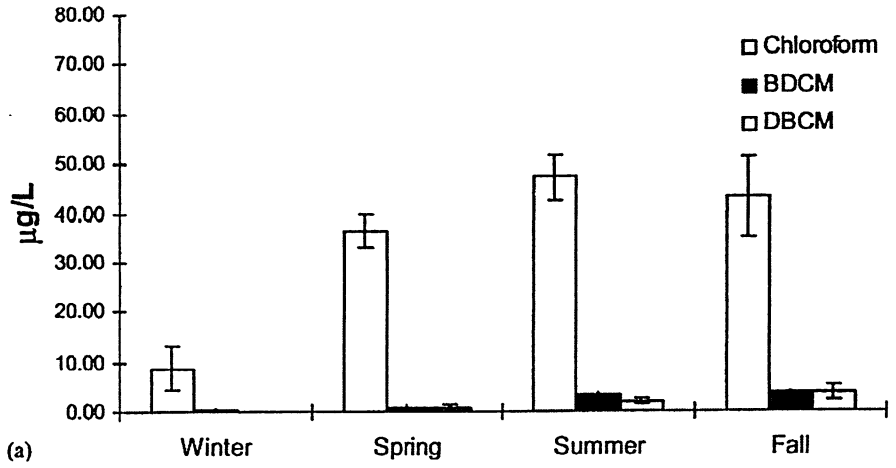


Figure 2.17: Variations in THM concentrations according to season [trichloromethane (chloroform), bromodichloromethane (BDCM) and dibromochloromethane (DBCM); error bars represent 95% confidence intervals] (Rodriguez et al. 2004).

Natural water undergoing disinfection generally fluctuates within a temperature range of 2°C to 25°C (Health Canada 1996a; Guasp and Wei 2003; Toroz and Uyak 2005). This range is more pronounced in treatment facilities that draw water from smaller bodies of water (Thompson 2005, pers. comm.). It should be noted that, for the most part, the temperature values reported in these surveys were recorded as the water entered the treatment plant or at the start of the treatment process. Although it is not surprising, given the enormous variability within a distribution system and the technical difficulties associated with such an undertaking, there is virtually no information on temperature fluctuations along the distribution system, which is where most DBPs are formed. In paved urban areas, the temperature of the water in the distribution system is reasonably consistent with the temperature of the soil surrounding the distribution systems and to some extent temperatures along the distribution system can be approximated from the temperatures of the surrounding soil.

Table 2.5 summarizes the effects of pH, temperature and bromide concentration, the water quality parameters under investigation in this study, on the formation, speciation and mutagenicity/genotoxicity of disinfection by-products.

Table 2.5: Effects of pH, temperature and bromide concentration on DBP formation, speciation and genoxicity.

Factor	Effect	Reference
PH	♦ ↑pH = ↑THMs (approximately proportional) ♦ ↓pH = ↑ HAAs, trihaloacetaldehydes, trihalopropanones + other halogenated DBPs	Urano et al. 1983; Xie 2004
	♦ ↑pH = ↓ mutagenic activity	Meier et al. 1983; Itoh and Maksuoka 1996
	♦ little difference in genotoxicity/mutagenicity across the pH range. **effects of pH on mutagenicity inconclusive**	Luk et al. 2006, in press
	♦ range of water undergoing disinfection treatment fluctuates between pH 5 and 9.	Rodriguez et al. 2004
Bromide	♦ ↑bromide = ↑ brominated DBPs = ↓ chlorinated DBPs	Cowman and Singer 1996; Richardson et al. 2003
	♦ brominated DBPs are more cytotoxic and genotoxic than their chlorinated analogues.	Plewa et al. 2002; Plewa et al. 2004
	♦ typical bromide range: 3-700 µg/L surface waters and groundwater; 65 000 µg/L seawater.	Magazinovic et al. 2004
Temperature (T)	♦ typically ↑T = ↑NOM = ↑chlorine demand = ↑chlorine dose = ↑DBP levels	Health Canada 1996a; Rodriguez et al. 2004; Toroz and Uyak 2005.
	♦ *low bromide*: ↑T = ↑ chlorinated THMs and HAAs = little effect on tribromomethane & brominated HAAs	Krasner et al. 1996
	♦ halogenation by HOBr appears to be less impacted by T and time than HOCl halogenation when TOC is in excess.	
	♦ *high bromide:* ↑T = ↑ all THMs and HAAs	Krasner et al. 1996
	♦ when less TOC is available for halogenation, more HOBr becomes available compared to HOCl; brominated DBPs are more strongly influenced by T.	
	♦ no information available on the effects of temperature on DBP genotoxicity.	
	♦ in temperate climates generally fluctuated between 2°C and 25°C.	Health Canada 1996a; Guasp and Wei 2003; Toroz and Uyak 2005

3. *In Vitro* Chromosome Aberration Assay

The past century has been witness to enormous advances in the study of genetics and leaps in the understanding of relationships between environmental contaminants and the effects of those contaminants on DNA and chromosomes. At the same time, a growing human population and increased industrialization and consumption have resulted in an escalating number of potentially genotoxic materials being released into the environment in ever-growing quantities. In order to determine human exposure limits and to confidently test new chemicals prior to their release in the market, it is necessary to understand the mechanisms of genotoxic chemicals and to develop the tools to properly assess these chemicals and their effects. Genetic toxicity focuses on the processes of mutagenesis, which include the induction of DNA damage, gene mutation and chromosome aberration; these genotoxic effects are considered important precursors to the development of adverse health effects such as cancer. As important primary screening tools for the assessment of genetic hazard, genetic toxicity test systems are categorised by the end points that they measure, such as gene mutation, chromosome damage or DNA damage (Putman et al. 2001). The strong association between these end points and known mechanisms of oncogene activation or loss of tumour suppressor gene function places great importance on genotoxicity testing to evaluate the mutagenic and carcinogenic potential of consumer and industrial products, pharmaceutical and agricultural agents, and environmental samples.

The *in vitro* cytogenetics assay has been used for several decades to assess chromosomal derangements caused by chemicals and radiation and has become an integral part of genetic toxicology testing (Putman et al. 2001). Chromosomal aberrations are the microscopically visible part of a wide spectrum of DNA changes generated by different repair mechanisms of DNA double strand breaks (DSB) (Obe et al. 2002). DNA molecules are

extremely long (human chromosome 1, for example, contains a DNA molecule 7.5 cm in length (Lodish et al. 2000)) and it is because of these enormous dimensions that DNA molecules in chromosomes are permanent targets of chemical or physical damage of diverse origin. Agents that damage chromosomal DNA can induce chromosomal aberrations (Miller and Therman 2001) and experimental work has shown that DNA double strand breaks (DSB) are the principal lesion in the process of chromosome aberration formation (Obe et al. 1992; Bryant 1998). DSB arise spontaneously at significant frequencies through a variety of cellular processes, and can be directly induced by ionizing radiation, certain antibiotics or DNA endonucleases (Obe et al. 1992; Pfeiffer et al. 2000). Spontaneously induced DSB can occur through accumulated single strand breaks caused by DNA replication and DNA excision repair, transposition, VDJ-recombination, antibody class switching, mitotic recombination or oxidative damage. The majority of chemical mutagens are not able to induce DSB directly, but may lead to other lesions in chromosomal DNA, which during repair or DNA synthesis may give rise to DSB and eventually to chromosomal aberrations (reviewed in Obe et al. 2002). Therefore, chromosome aberrations are not special phenomena resulting from specific cellular activity, but are large-scale alterations, which form the microscopically visible part of a wide spectrum of products generated by different DSB repair mechanisms.

The purpose of the chromosome aberration assay is to identify agents that cause chromosome aberrations in cultured mammalian cells, as well as to screen populations for chromosome anomalies arising as a result of environmental agents. More specifically, the aim of the *in vitro* cytogenetics assay is to evaluate the clastogenic or chromosome breakage potential of a test contaminant and its metabolites based upon their ability to induce chromosome aberrations in a culture (Putman et al. 2001). The use of cell cultures as a test system has been demonstrated to be an effective method of detection of chemical clastogens and the induction of chromosome breakage *in vitro* is an indication that the test article is

potentially genotoxic (Preston et al. 1981). The chromosomal aberration assay is vital because there is strong evidence to suggest that chromosome mutations and related events cause alterations in the oncogenes and tumour suppressor genes of somatic cells and are involved in cancer induction in humans and animals (Tlsty et al. 1995). Carcinogenesis is a multi-stage process and recessive mutations induced by chemical or physical agents can come to express their mutant phenotype if there is loss of heterozygosity. Malignant progression can also arise through translocation of genetic material, rearrangement, or chromosome loss leading to the elimination of tumour suppressor genes (Kirkland 1998). Epidemiological studies have shown that people with elevated frequencies of chromosome aberrations in their peripheral blood lymphocytes have a significantly elevated risk of developing cancer (Hagmar et al. 1998; Bonassi et al. 2000). Many types of cancers are associated with specific types of chromosome aberrations, which are etiologic for the cancer in question (Mitelman et al. 1997). Thus, chromosome damage is relevant for carcinogenesis and testing agents for their ability to induce chromosome aberrations has a firm place in screening strategies for mutagenic and carcinogenic agents (Kirkland 1998; Ishidate et al. 1998).

In vitro metaphase tests for chromosomal aberrations have undergone considerable changes over the past 30 years and different approaches developed in different parts of the world. Much of the progress over the past few decades has been in the development of standard techniques; test sample parameters, such as defining upper exposure limits for testing and length of treatment, and harvest times have been especially well discussed in the literature (reviewed in Kirkland 1998). Many of the early protocol differences were due to the fact that early guidelines (e.g. OECD 1983) were quite brief in their recommendations and thus were open to a variety of possible interpretations and study designs, all of which complied with the basic recommendations. The differences between protocols used in the UK, US and Japan have since been harmonized (Kirkland 1998). The *in vitro* cytogenetics assay is part of the

Organization for Economic Cooperation and Development's (OECD) guidelines as one of the three levels of *in vitro* tests for genotoxicity, as well as a part of the guidelines established by the International Conference on Harmonization (ICH) (OECD 1997, ICH 1997). In the United States, the *in vitro* chromosome aberration test is also used as part of the mutagenicity testing battery for Section 4 chemicals classified under the *Toxic Substances Control Act* (*TSCA*), as well as the three-test battery for agricultural chemicals overseen by the Office of Pesticide Programs (OPP). In Japan, the chromosome aberration assay is required for new chemicals as part of the guidelines for screening new chemical substances published by the Ministry of Health and Welfare, the Ministry of International Trade and Industry and the Agency of Environment (Putman et al. 2001). In Canada, the Guidelines for the Notification and Testing of New Substances in the *Canadian Environmental Protection Act* (1999) stipulate mutagenicity test procedures for the approval of new substances that are consistent with the guidelines put forth by the OECD (1997) and include *in vitro* chromosome aberration assays.

3.1 Chromosomal Aberrations

Two types of chromosomal abnormalities can be detected using the *in vitro* chromosome aberration assay: structural chromosome aberrations and numerical chromosome aberrations. Structural aberrations include deviations such as breaks and rearrangements and can result in a discontinuity in the chromosomal DNA. These discontinuities may be a) repaired, thereby restoring the original structure; b) rejoined inappropriately, forming rearrangements such as intra- or inter-changes; c) left unrejoined, causing a break or a deletion in the chromosome (Putman et al. 2001). Structural aberrations may be of two types, chromosome or chromatid. Chromosome-type aberrations are expressed as a breakage or a breakage and reunion of both chromatids at an identical site. The majority of chemical mutagen induced aberrations

are chromatid type, in which structural chromosome damage is expressed as breakage of single chromatids or breakage and reunion between chromatids (OECD 1997), as shown in Figure 3.1.

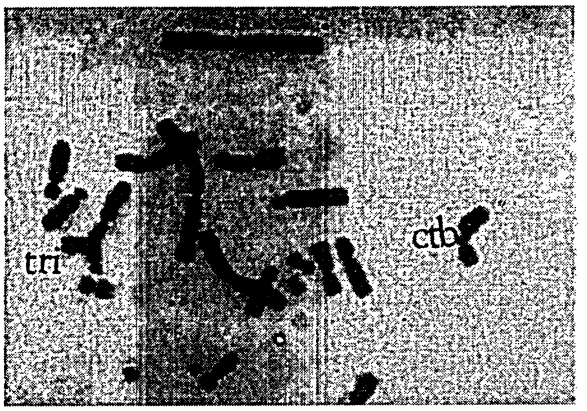


Figure 3.1: Chromatid-type Structural Aberrations. Chinese hamster ovary (CHO) chromosomes: chromatid break (ctb) and triradial chromatid rearrangement (tri) (Putman et al. 2001)

For the most part, structural aberrations are lethal to the cell or to the daughter cells in the first few cell cycles after their appearance. However, these structural deviations may also serve as an indicator of the occurrence of transmittable aberrations, such as balanced translocations, duplications, inversions or small deletions, and may play a role in tumor initiation and progression in somatic cells (Tlsty et al. 1995).

On the other hand, numerical aberrations are variations in the number of chromosomes in the nucleus from the normal number characteristic of the cell. Two examples of numerical aberrations are aneuploidy and polyploidy or endoreduplications (ICH 1997). Aneuploidy is a deviation in the chromsome number involving one or a few chromosomes, while polyploidy or endoreduplications is a variation in the complement of chromosomes involving the whole set of

chromosomes. Studies have generally found that numerical chromosome aberrations are not due to the direct interaction of an environmental agent with the chromosomal DNA (Putman et al. 2001). Although numerical aberrations do not seem to play a key role in the initiation of tumours and the physiological and genotoxic impacts of polyploidy and endoreduplication are less than clear, they may be indicative of the evolution of karyotypic instability within a population of tumour cells (de Mitchell et al. 1995). The *in vitro* cytogenetics assay was not designed to measure numerical aberrations and is not routinely used for that purpose.

3.2 Cell Selection

The chromosome aberration test system can be run using either established cell lines or primary cell cultures. The cell lines routinely used in this assay are Chinese hamster ovary (CHO) cells and Chinese hamster lung (CHL) cells grown in mono-layer cultures. There are a number of advantages to using established cell lines from frozen stocks. Cell lines are genetically more homogeneous than primary cell cultures and thus tend to show less inter-experimental variability within the cell types (Putman et al. 2001). Secondly, the established Chinese hamster cell lines are advantageous for use in the *in vitro* cytogenetics assay because they are easily cultured in standard media, have a small number of large chromosomes each with a more or less distinctive morphology, and have a relatively short cell cycle (Ishidate et al. 1988). On the other hand, primary cell cultures, such as human peripheral blood lymphocytes (HPBL), show some variability among donors in their response and sensitivity to test articles compared to established cell lines (Kirkland and Garner 1987; Kirkland 1992), but the relevance of these systems to human exposure and risk assessment cannot be overlooked.

During cell culturing, treatment and preparation of samples for mounting, there is extensive chromosome rearrangement and it is an inherent property of most established cell

lines that the chromosome number varies around a modal value (Kirkland 1992). As such, it is necessary to establish criteria for defining analyzable cells; current criteria are that cells with the modal chromosome number ± 2 chromosomes are acceptable for microscopic analysis (Putman et al. 2001). For CHL cells, which have a modal chromosome number of 25, all cells with chromosome numbers ranging from 23 to 27 are considered as acceptable samples for the reading. Figure 3.2 shows an untreated, normal Chinese hamster cell (CHL) cell with 25 chromosomes.

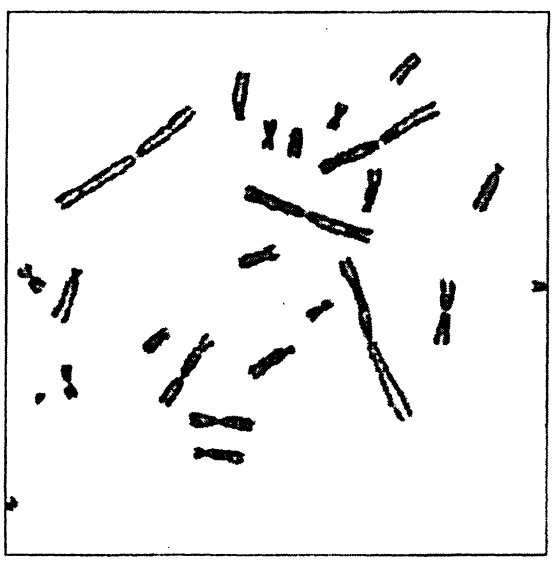


Figure 3.2: Normal Chinese hamster lung cell (Ishidate, Jr. 1988)

Prior to the start of the assay, frozen stocks should be established and cells checked for contamination by parasitic, pathogenic microorganisms, such as mycoplasma (ICH 1997). Since the objective of the chromosome aberration assay is to determine whether test chemicals induce aberrations in a culture, environmental factors, such as pH, cytotoxicity and osmolality, must be carefully monitored and adjusted to physiological levels if necessary, as extremes of these factors can cause chromosome aberrations (Putman et al. 2001). Failure to maintain

conditions at physiological levels could lead to positive results, which do not reflect intrinsic mutagenicity. As there are natural background levels of mutation, a negative control test is also required to assess the relative increases in the measured end-point, in this case structural aberrations. It is necessary to determine how many aberrations occur in the control (untreated or solvent control) sample, and subtract that base number from all of the test samples.

3.3 Experimental Approach

In general, chromosome aberration assays are conducted in two stages: the preliminary toxicity assay and the chromosome aberration assay (Putman et al. 2001). The preliminary toxicity assay serves as a dose range-finding assay for the definitive portion of the study. There are a number of protocols in place for determining which exposure concentrations should be tested in order to ensure that concentrations cover the range from the maximum (>50% reduction in cell growth or cloning efficiency or confluency) to little or no toxicity (OECD 1997, Putman et al. 2001). For some agents, which have been well-described in other studies, it is possible to forgo this phase and to refer to the dose-range described in previous work. In cases where the contaminant in question is wide-spread in the environment, either from long-time human use or disposal practices, it may be possible to use a dose range that reflects the concentrations of the chemical found in natural systems. In the chromosome aberration assay, the clastogenic potential of the contaminant is evaluated microscopically. After cells are exposed to the test substance for predetermined intervals, they are treated with a metaphase-arresting substance, harvested, stained and examined microscopically under a minimum magnification of 1000x for the presence of structural aberrations.

Although the *in vitro* chromosome aberration assay is an important tool for screening potential mutagens and carcinogens, there is not a perfect correlation between this test and

carcinogenicity. For example, a test chemical for which there is a negative result, indicating that the test chemical does not induce aberrations, may still be a carcinogen. There is growing evidence of carcinogens that are not detected by the *in vitro* cytogenetics test because they appear to act through mechanisms other than direct DNA damage (Tlsty et al. 1995, OECD 1997). Secondly, the *in vitro* chromosome aberration test is usually the most time consuming part of the standard battery of genotoxicity testing; it requires skill and considerable training for investigators to be fully competent to carry out this assay (Kirkland 1998). Despite these potential limitations, the chromosome aberration assay provides two distinct advantages. First, the test is very useful for comparisons of chemicals in the same class and provides a direct measure of their toxicity. Secondly, the test is not financially prohibitive and is feasible even for smaller laboratories. The test does not require the use of laboratory animals or the space to house and care for them, and there are fewer requirements for additional staff (animal care technicians, etc.) and equipment. The feasibility of the *in vitro* chromosome aberration assay provides an excellent opportunity to quickly and efficiently test a large number of potential genotoxins, under a wide range of conditions. This in turn allows for the prioritization of further research through laboratory or clinical trials.

4. Experimental Design

4.1 Health Effects and Toxicity

Water disinfection by-products have been recognized as an important class of hazardous environmental chemicals and the drinking water industry is required to minimize DBP formation, while ensuring adequate disinfection and pathogen control (U.S. E.P.A. 1998). Not only is it necessary to balance disinfection efficiency and DBP control, but also there are often additional issues concerning the risks and trade-offs between by-products of chlorination and those of alternative disinfectants. Concerns about the potential health effects of chlorination by-products have prompted investigations of the possible association between exposure to DBPs and incidences of cancers, and more recently, with adverse reproductive outcomes.

Individual epidemiological studies into the associations between DBPs and cancer have considered a wide range of populations and regions and, although suggestive, have been inconclusive. Morris et al. (1992) conducted a meta-analysis of pertinent case-control and cohort studies into the associations between DBPs and cancer and found a significant positive association between consumption of chlorinated by-products in drinking water and bladder and rectal cancer in humans. The meta-analysis of all cancer sites yielded a relative risk estimate for exposure to DBPs of 1.15; pooled relative risk estimates for organ-specific neoplasms were 1.21 for bladder cancer and 1.38 for rectal cancer. In a subsequent review of epidemiological evidence for the association between DBPs and cancer, Cantor (1994) noted that there is a general convergence of findings in that cancers of the bladder, colon and rectum have been associated with disinfection by-products far more often than have cancers of other sites. Cantor (1994) observed that although the quantitative estimates of cancer risk owing to DBPs are

highly uncertain, the growing body of toxicological and epidemiological data suggests that risk is likely to be elevated, especially for cancers of the rectum and bladder.

Nieuwenhuijsen et al. (2000) evaluated the toxicological and epidemiological data involving chlorination DBPs and adverse reproductive effects and concluded that although many of the findings are not definite, the evidence for associations between DBPs and adverse reproductive outcomes is growing. Although relatively few toxicological and epidemiological studies have been carried out examining the effects of DBPs on reproductive health outcomes, a number of studies have pointed toward as association between DBPs and low birth weight, spontaneous abortions, still births and birth defects – in particular central nervous system and major cardiac system defects, oral cleft, and respiratory and neural tube defects. The authors noted that the main limitation of most studies so far has been the relatively crude methodology, in particular for assessment of exposure, and dose selection in toxicology studies.

Early investigations of the health effects of DBPs relied primarily on epidemiological studies and analysis of cohort records, however it is now well established by both *in vivo* and *in vitro* bioassays that concentrated extracts of disinfected drinking water are toxic. Although research into the effects of exposure to DBPs is mounting, a comparative database on the quantitative cytotoxicity and genotoxicity of these compounds, established through a series of bioassays, is lacking (Plewa et al. 2002). The majority of DBPs present in drinking water have yet to be chemically defined. As a result, most experimental work on DBPs and toxicity has focused on assessing the genotoxocity of individual, or more commonly, groups of DBPs. In 1994, Koivusalo et al. took a new approach to assessing exposure by using estimates of mutagenic potency and genotoxic activity of drinking water based on *S. typhimurium* TA100 bacterial assays. The authors determined past exposure to drinking water carcinogens based on drinking water mutagenicity estimated from historic records of water parameters and found that there was a significant exposure-dependent response with the consumption of mutagenic

water and the development of kidney and bladder cancer. This opened the way for other studies that used the *S. typhimurium* mutagenicity assay to quantitatively compare the cytotoxicity of DBPs (DeMarini et al. 1994; Kargalioglu et al. 2000; Kargalioglu et al. 2002). However, the limitations of the bacterial bioassay soon became evident. Kargalioglu et al. (2000) noted that while the *S. typhimurium* assay is an excellent rapid qualitative mutagenicity assay, it cannot quantitatively determine the cytotoxicity of test agents and the authors questioned its relevance to the assessment of human health risks. In a comparison of DBP cytotoxicity, a rank order of decreasing cytotoxicity of the DBPs under investigation was established: MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone) >> bromoacetic acid (BA) >> tribromomethane > dibromoacetic acid (DBA) >> tribromoacetic acid (TBA) > trichloromethane >> dimethylsulfoxide (Kargalioglu et al. 2000).

Plewa et al. (2002) developed and calibrated assays for cytotoxicity and genotoxicity in Chinese hamster ovary (CHO) cell and used the cytotoxicity and mutagenicity data of selected DBPs in *Salmonella typhimurium* reported by Kargalioglu et al. (2000; 2002) for comparison. The mammalian cell assay used was a semi-automated micro-plate assay, where the concentration of the DBPs that repressed 50% of CHO cell growth with a 72-hour exposure was used as the measure of cytotoxicity. The rank order in decreasing chronic cytotoxicity measured in CHO cells was BA >> MX > DBA > chloroacetic acid > $KbrO_3$ > TBA > dichloroacetic acid > trichloroacetic acid. These results suggest that *S. tymphimurium* cannot quantitatively predict the relative cytotoxic or genotoxic risk of DBPs in mammalian cell systems. The micro-plate CHO bioassay method has been used in a number of other studies, including a 2000 study (Plewa et al. 2000) that found that halonitromethanes are more cytotoxic than haloacetic acids and a 2004 study (Plewa et al. 2004) that found that the brominated nitromethanes are more cytotoxic and genotoxic than their chlorinated analogues. Plewa et al. (2000) also compared the cytotoxicity of DBPs in *Salmonella tymphimurium* and CHO cells, and although the cytotoxic rank order for both cell systems was identical, with the monobrominated

species the most toxic and the tribrominated species the least toxic, the mammalian cells were approximately two orders of magnitude more sensitive than the bacteria cells. In all three studies, genotoxicity analyses of the DBPs were conducted using the single cell gel electrophoresis (SCGE) assay, which detects genomic DBA damage at the level of the individual nucleus. Overall, there was very good correlation between the cytotoxicity and genotoxicity assays. Very recent toxicological work has focused solely on mammalian cell assays to provide information relevant to human risk assessment (Plewa et al. 2004; Sutiakova et al. 2004).

These past toxicological studies have been useful in identifying the potential health effects of various individual by-products, but little is known about the effects of mixtures in low concentrations, such as those found in water treated for human consumption. A comparative database on the quantitative cytotoxicity and genotoxicity of these agents among a series of bioassays is lacking and the majority of DBPs present in drinking water have yet to be chemically defined. Information is markedly limited on the identification of brominated compounds and their health effects, which is noteworthy, since, in the presence of bromide, higher concentrations of brominated compounds are formed than chlorinated compound.

4.2 Chromosome Aberration Assay and DBP Toxicity

In his toxicological analysis of safe drinking water, Van Leeuwen (2000) noted that it is important to understand the potential genetic risks of chlorinated drinking water. The use of cell cultures as a test system has been demonstrated to be an effective method of detection of chemical clastogens and the induction of chromosome breakage *in vitro* is an indication that the test article is potentially genotoxic (Preston et al. 1981). There is strong evidence to suggest

that chromosome mutations and related events cause alterations in the oncogenes and tumour suppressor genes of somatic cells and are involved in cancer induction in humans and animals (Tlsty et al. 1995) and epidemiological studies have shown that people with elevated frequencies of chromosome aberrations in their peripheral blood lymphocytes have a significantly elevated risk of developing cancer (Hagmar et al. 1998; Bonassi et al. 2000). Thus, chromosome damage is relevant for carcinogenesis and testing agents for their ability to induce chromosome aberrations has a firm place in screening strategies for mutagenic and carcinogenic agents (Kirkland 1998; Ishidate et al. 1998).

Chromosome aberration assays have been used for almost 30 years in genotoxicity testing to evaluate the mutagenic and carcinogenic potential of consumer and industrial products, pharmaceutical and agricultural agents, and environmental samples (Ishidate, Jr. 1988; Kirkland 1998). However, it was only recently that attention has turned to using the chromosome aberration assay to assess the genotoxicity of disinfection by-products. Itoh and Matsuoka (1996) examined the relationships between chromosome aberrations induced by water treated with disinfectants and DBP nitro and carbonyl groups in Chinese hamster lung cells (CHL) *in vitro*. Their results indicate that the presence of carbonyl groups in treated water is associated with a large increase in chromosomal aberrations, while the contribution of the nitro group is small. The authors noted that the carbonyl group may be useful as a coarse indicator to compare activity inducing aberrations of water treated with different disinfectants. In a subsequent study, Itoh et al. (2001) looked at indices of potential carcinogenicity and examined variations in the toxicity of chlorinated water subsequent to chlorination by employing a chromosomal aberration test in Chinese hamster lung cells to measure initiating activity and a transformation test using mouse fibroblast cells to measure promoting activity. They found that activity inducing chromosomal aberrations of chlorinated humic acid gradually decrease with time after chlorination, while activity inducing transformations measured by the two-stage assay

gradually increase. This study suggested that further research is necessary to determine the relative carcinogenicity of tap water as a function of distance from the water purification plant.

Similarly, Nobukawa and Sanukida (2000) evaluated the genotoxic characteristics of chlorinated and brominated DBPs by Ames tests using *S. typhimurium* strains TA100 and TA98 and by chromosome aberration tests using cultured Chinese hamster lung (CHL) cells *in vitro*. They observed a significant increase in chromosomal aberrations in cells exposed to chlorinated water, compared to those exposed to unchlorinated water and a consistent increase in mutagenic potential as bromide levels increased in the sample. In a related study, Lu et al. (2002) examined the mutagenicity of chlorinated drinking water in rats *in vivo* and in human HepG2 cells *in vitro* and concluded from results obtained from several biological systems and endpoints that chlorinated drinking water may be a potential genetic hazard for humans. The chromosome aberration assay is one of the most sensitive and relevant means of identifying mutagens and carcinogens (Sutiakova et al. 2004).

4.3 Experimental Parameters and Study Objectives

Numerous studies have found potential health effects associated with DBPs, including carcinogenicity, adverse reproductive and developmental effects, and immunotoxic and neurotoxic effects (reviewed in Barrett et al. 2000; Nieuwenhuijsen et al. 2000). Much work remains to be done to determine the potential risks of the numerous compounds that have been identified in drinking water, as well as to determine research priorities on newly identified by-products. Although chromosome analysis is not as specific as molecular studies, which monitor effects on single genes, it does monitor the entire genome and increasing frequencies of chromosome aberrations in cells exposed to DBPs suggest a potential genetic hazard with implications for human health. Most studies to date have looked at either the effects of water

quality parameters, such as pH or chlorination time, on the formation of individual disinfection by-products or the health effects associated with individual DBPs. There is currently relatively little information about the impact of seasonal water quality changes or variability in operational water treatment strategies on the simultaneous occurrence of THMs, HAAs and a host of other disinfection by-products in a distribution system.

Previous studies have looked at the effects of some water quality parameters on the genotoxicty of DBPs, as assessed by the chromosome aberration assay; prominent and representative of these efforts is the work of Sadahiko Itoh and his colleagues in Japan. Among other factors, such as levels of natural organic matter, chlorine dose, chlorination time and bromide levels, pH and temperature have been identified as important factors in the formation and genotoxicity of DBPs. Meier et al. (1983) examined the effect of pH on the stability of mutagenic activity of chlorinated water and found that mutagenic activity of chlorinated humic acids decreases with increasing pH. Although Itoh and Matsuoka (1996) report results consistent with these findings, recent work by Luk et al. (2006, in press) has shown that this is not always the case. Additional study is needed of this important water quality parameter. Due to great variability in methodological conditions among laboratories and intrinsic individual variability, Forni (1992) recommended that the results of chromosome aberrations be compared within the same laboratory under the same methodological conditions. Thus, the effects of pH, in the range encountered during the water treatment process (pH 5-9), will be re-examined in the hopes of clarifying the impact of this important parameter. The effects of temperature variation on the genotoxicity of DBPs *in vitro* have not been investigated thus far and so aberration formation by chlorinated humic acid samples will be examined at 5°C and 25°C; these values parallel the extremes of the temperature range observed in temperate climates. Given the large number of brominated DBPs and the increased risk that they pose to human health, there is a real need for further study into the genotoxicty and risk posed by brominated

DBPs. Thus, pH and temperature will be looked at in the context of bromide concentrations ranging from 50-250 mg/L. The experimental parameters are outlined in Table 4.1

Table 4.1 Experimental Parameters

Temp. (°C)	pH	{Br^-} mg/L	{Cl_2} mg/L
5	5	50	1500
		100	1500
		150	1500
		200	1500
		250	1500
	7	50	1500
		100	1500
		150	1500
		200	1500
		250	1500
	9	50	1500
		100	1500
		150	1500
		200	1500
		250	1500
25	5	50	1500
		100	1500
		150	1500
		200	1500
		250	1500
	7	50	1500
		100	1500
		150	1500
		200	1500
		250	1500
	9	50	1500
		100	1500
		150	1500
		200	1500
		250	1500

The objective of this study is to provide a direct assessment of the effects of pH and temperature on the genotoxicity of halogenated disinfection by-products in chlorinated water. In this study, the genotoxocity or the potential of chromosomal damage associated with DBPs in

drinking water was evaluated using chromosome aberrations as cytogenetic endpoints. A secondary aim is to contribute to the body of knowledge on the risks associated with two important water quality parameters, pH and temperature, which in some situations may be controlled during drinking water treatment. Furthermore, the use of chromosomal aberration assays for genotoxic assessment of DBPs is still in its infancy and this study will provide comparison and verification of existing data. The determination of any significant genotoxic effects of chlorinated drinking water will raise concerns about the potential risk to exposed human and animal populations. It is important to note that because the identities of the mutagens produced by chlorination are not known, exposure levels cannot be defined and the *in vitro* assay, although useful for identifying potential hazards and for comparisons between compounds, cannot be used to quantify the magnitude of risk. However, additional work on chromosomal aberrations induced by chlorinated humic acid samples will help to clarify whether the use of the chromosome aberration test is a viable tool for the analysis of DBP risks.

5. Study Methods

The objective of this study was to evaluate the effects of pH, temperature and bromide ion concentration on the genotoxicity of disinfection by-products in chlorinated water, using structural chromosomal aberrations as the measured endpoint. Following exposure to experimental humic acid samples, cells were arrested in the first post-treatment metaphase of the cell cycle, fixed onto slides and examined for sample-induced aberrations.

5.1 Cell Culture

Chinese hamster (*Cricetulus griseus*) lung (CHL) cells were obtained from American Type Culture Collection (ATCC), Manassas, VA, U.S.A., and the cell line was initiated according to supplier's instructions. CHL cells are a fibroblast cell line from the lungs of newborn female Chinese hamsters. This cell line was derived from the CHL line established by Utakogi in 1970 and has 25 chromosomes (the chromosome number of the Chinese hamster is 2n=22). The cell line was maintained in MEM/EBSS (Medium Essential Medium (1X), w/ 2.00 mM/L Glutamine, w/ Earle's Balanced Salts), supplemented with 10% sterile filtered Fetal Bovine Serum (FBS) and 1% Gentamicin antibiotic (40 mg/mL, Sabex®), in a 5% CO_2 incubator at 37°C. The cell doubling time is approximately 15-16 hours and the cells proliferate in monolayers (Ishidate, Jr. 1988). Cells were sub-cultured (see *Appendix A – Cell Splitting*) every 3-6 days, depending on experimental work; the time between subcultures can be adjusted by increasing or reducing the volume of cells added after each split.

The cell line was maintained at all times in large (75 cm^2) stock flasks, which were supplemented with 1% Gentamicin. At the start of each trial, cells were sub-cultured into small cell culture flasks (25 cm^2), each of which served as one experimental treatment. For the experimental sub-culture, 0.5 mL of cell suspension and 5.5 mL of MEM supplemented with 10% FBS were added to each small flask. In order to avoid any potential interference with the mutagenicity of the humic acid sample, the MEM added to the experimental flasks was not supplemented with 1% Gentamicin antibiotic. The addition of 0.5 mL of cell suspension to each experimental flask was established in preliminary trials. In established cell lines, the monolayers must not reach confluency at any time during the incubation of seeded cultures prior to test article treatment or during the assay. The target cells in the assay are mitotically active and as the cells approach confluency, the growth rate slows down, thereby diminishing the number of target cells (Putman et al. 2001). In addition, as the monolayers become confluent and the growth rate slows, cell lines have a tendency to become karyotypically unstable and the background levels of chromosome rearrangements may increase (OECD 1997; Putman et al. 2001).

5.2 Sample Preparation and Addition

Natural organic matter in surface waters is primarily composed of humic and fulvic acids and it is these organic acids that react with chlorine and other disinfectants to form DBPs. In order to maintain experimental control and assure consistency, humic acid samples made from commercially produced humic acid powder (Acros Organics®, 50-60%) were used as the model substrate and organic DBP precursor in this study. Stock humic acid solutions were prepared by dissolving 3 g of commercially produced humic acid (Acros Organics® 50-60% as humic acid) in 1L of 0.1 M NaOH solution. This corresponds to a TOC of 1030 mg C/L, which has been

established in the literature as necessary for the detection of activity inducing chromosome aberrations during the experimental exposure time (Itoh et al. 2001). The humic acid solutions were first adjusted to pH 7 with H_2SO_4 (from approximately pH 12.2) and then adjusted to pH 5, 7 and 9 using phosphate-buffering salts, Na_2HPO_4 and KH_2PO_4 (Perrin and Dempsey 1974; Harris 2003). The humic acid stock solutions were kept at 4°C in amber glass bottles.

Humic acid test samples were created on the first day of each experimental trial by adding bromide ions and chlorine to the humic acid solution (see *Appendix B – Sample Preparation*). Bromide ions were introduced to the sample with potassium bromide (KBr) solution (14.9 mg/L $\Rightarrow$ $\{Br^-\}$ =10,000 mg/L) in 50 mg/L intervals from 50 mg/L to 250 mg/L. Hypochlorous acid (HOCl) solution (5% (0.67 M) $\Rightarrow$ $\{Cl_2\}$ = 47,503 mg/L, Fisher Scientific) was used to simulate the chlorine disinfectant used during the water treatment process and 1500 mg Cl_2/L was added to each sample; this chlorination level has been established for this procedure in previous work to simulate exposure over many years (Itoh et al. 2001). Since HOCl has a tendency to deteriorate, the standard ABTS™ (2,2' – Azino-di-(3-ethylbenzothiazoline-6-sulfonic acid, Calbiochem®) colour determination method was used in a UV-spectrophotometer (Genesys 10 UV, Thermo Electron Corp.) to measure the available chlorine in the stock solution at a wavelength of 405 nm (Childs and Bardsley 1975; Groome 1980). In an effort to replicate as closely as possible the conditions found in the water treatment process and to avoid the possibility of photosynthetic activity, sample bottles were covered with aluminum foil and were shaken continuously for approximately 24 hours at either 5°C or 25°C.

Following the 24-hour incubation, test samples were syringe filtered ((Acrodisc® 25 mm filter w/ 0.2 µm membrane) to remove all fine particles and bacteria and 1 mL of test sample was added to each experimental flask (see *Appendix C – Sample Addition*). Since the total volume of the growth medium prior to sample addition is 6 mL, 1 mL corresponds to 10 mM of test sample. A task group, assembled under the auspices of the International Commission for

Protection Against Environmental Mutagens and Carcinogens (Scott et al. 1991), examined the issue of test sample concentration and set a practical testing limit of 10 mM, based on the highest *in vitro* concentration needed to detect *in vivo* clastogens. The work of this committee marked the first time that a thorough scientific assessment of published cytogenetic data was used to determine an appropriate top dose (Kirkland 1998). This volume ensures that sample-induced cell cycle delay is kept within 50% (1.5 cell cycles – see *Chromosome Preparation* below) and that the culture medium concentration is maintained at 150 mg/L ± 10%.

A long-standing concern with chromosome aberration assays is whether test sample conditions, such as low pH, could result in increased frequencies of chromosome aberrations without any direct effect on the DNA. These sample conditions could, for example, interfere with DNA synthesis and repair or interact with various proteins and might produce results of questionable biological relevance (Shelby and Sofuni 1991; Kirkland 1998). Accordingly, following the sample incubation period at the specified pH and temperature, during which time the disinfection by-products form, samples were incubated at 37°C for 25 min. Immediately prior to sample addition, all samples were neutralized to pH 7 by the addition of either 1 M NaOH or 1 M HCl. Temperature and pH were adjusted to physiological values to prevent environmental cell shock and/or cell death.

5.3 Chromosome Preparation

A critical requirement of the *in vitro* chromosome aberration assay is that structural aberration assessment of chromosomes be made in the first post-treatment metaphase of the cell cycle. If damaged cells are capable of cycling and allowed to progress through more than one cell cycle, damaged chromosomes or fragments may be lost or converted from one type of damage into another, which can be misleading (Galloway et al. 1985; Kirkland 1998). For

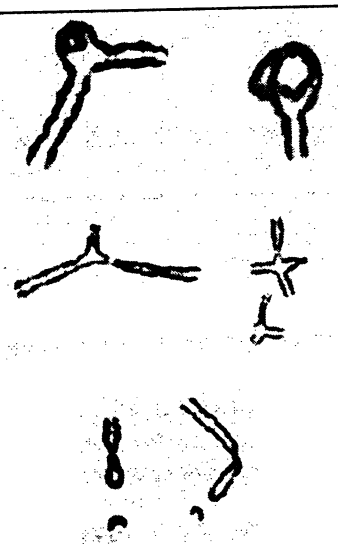
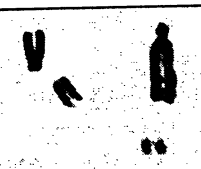
example, structural damage that appears as chromatid-type in the first post-treatment metaphase can emerge as chromosome-type damage in the second post-treatment metaphase (Galloway et al. 1985). In addition, some cell cycle delay often occurs following treatment with the test sample. Following standard protocol established in the literature (Ishidate, Jr. 1988; Galloway et al. 1997; Kirkland 1998; Putman et al. 2001) and endorsed by the OECD (1997), the ICH (1997), cells were harvested for microscopic analysis at a single time point 1.5 times the normal cell cycle from the initiation treatment. This harvest time balances the requirement for evaluating first-division metaphase chromosomes with the possibility of sample induced cell cycle delay. CHL cells have a cell cycle of approximately 15-16 hours; thus, cells were harvested 24 hours (16 x 1.5) after sample addition.

In order to obtain a sufficient number of metaphase chromosomes, the cells were treated with 0.2 µg/ mL Colcemid® solution (Calbiochem®), a spindle apparatus disrupting agent, two hours prior to cell harvest (see *Appendix D – Chromosome Preparation*). Although the underlying mechanism of the Colcemid® effect remains unclear, the Colcemid® solution stops cell division in metaphase at the time of cell harvest by depolymerising microtubules and inhibiting mitotic spindle formation (Lodish et al. 2000, Putman et al. 2001). The cells were harvested by Trypsinization (0.25% (1X) solution, w/out Calcium and Magnesium, w/ EDTA) and then treated with 0.075 M potassium chloride, a hypotonic buffer, in order to swell the cells. The KCl treatment ensures that chromosomes are well separated when plated onto the microscope slides. Following a 25-minute incubation at 37°C, the hypotonic solution was replaced with a fixative of 3:1 v/v methanol (>99.8%, Fisher Scientific) and glacial acetic acid (≥99.7%, Fisher Scientific). The suspended cells were then plated onto slides and allowed to air dry (see *Appendix E – Slide Preparation*). After air drying, the slides were stained with 2% Giemsa (7.415 g/L, Harleco®) prepared in pH 6.8 phosphate buffer for 20 minutes, rinsed with water, and allowed to dry. Once the slides were dry, a cover slip was placed over an area of high cell concentration and adhered with Permount®, a mounting medium (Fisher Scientific).

5.4 Analysis of Chromosomal Aberrations

Fifty well-spread metaphase cells (modal chromosome number ± 2) were analyzed per treatment under the microscope with a 100x oil-immersion lens (1000x magnification). Observed structural aberrations were enumerated and classified as chromatid gaps, chromatid breaks, chromatid exchanges, chromosome gaps, chromosome breaks or chromosome exchanges. Table 5.1 illustrates the different types of structural chromosome aberrations; this classification scheme was adapted from Ishidate, Jr. (1988) and is compatible with ICH (1997) and OECD (1997) guidelines. As shown in Table 5.1, chromatid exchanges include interchromosomal exchanges, such as triradials and quadriradials (symmetrical and asymmetrical interchanges), and intrachromosomal exchanges, such as rings. Chromosome exchanges include dicentrics and rings.

Table 5.1: Types of Structural Chromosome Aberrations (adapted from Ishidate, Jr. 1988)

Structural Chromosome Aberrations	Examples
chromatid gap (ctg)	
chromatid break (ctb)	
chromatid exchange (cte)	
chromosome gap (csg)	
chromosome break (csb)	
chromosome exchange (cse)	

During chromosomal aberration analysis, a cell that had any of the above-mentioned aberrations was enumerated as an aberrant cell. There has been some dispute in the literature about whether gaps should be included in the enumeration of aberrant cells (Ishidate, Jr. 1988; Putman et al. 2001; reviewed in Galloway et al. 1997), however an international multi-laboratory comparison study found that much of the dispute was a matter of semantics. Japanese laboratories usually include gaps when classifying aberrant cells and in the counts of total aberrations, while laboratories in the United States and the United Kingdom do not. Galloway et al. (1997) found that many of the "gaps" noted in U.S. and U.K. laboratories were small staining discontinuities less than the width of a chromatid or with visible material extending across the gap; these "gaps", which were excluded from analysis in U.S. and U.K. laboratories, were not recorded in Japanese laboratories. The study thus found that the Japanese definition of a gap met the description of a "break" in other laboratories and concluded that the inclusion of "Japanese gaps" in the analysis of aberrant cells was consistent with the U.S./U.K. method of analysis. Studies on chromosome aberrations in CHL cells, especially with regards to disinfection by-products, have been performed almost exclusively in Japan, while most studies from laboratories in the U.S. and the U.K have used human lymphocytes, and to a lesser extent CHO cells (reviewed in Kirkland 1998; Plewa et al. 2000; Itoh et al. 2001). Established cell lines provide a number of advantages over primary cell cultures; CHL and CHO cells are easily cultured in standard media, have a small number of large chromosomes each with a more or less distinct morphology, have relatively short cell cycles, and are more genetically homogeneous and thus show less inter-experimental variability than primary cell cultures. Cell selection is discussed in greater detail in Chapter 3 (*3.2 Cell Selection*).

Accordingly, this study included both chromatid and chromosome gaps in the determination of aberrant cells. A gap was defined as a clear discontinuity with no visible connecting material, generally equal to or more than the width of the chromatid.

5.5 Controls

To determine that the chromosome aberration test system is functioning properly, appropriate controls were included in the test assays. Solvent controls, in which cell cultures were treated with distilled water only, were performed at 5°C and 25°C to assess relative increases in structural chromosome aberrations, the measured end point. There can be substantial background/inborn levels of chromosome aberrations (Obe et al. 2002), thus it is important to ensure that the chromosome aberrations observed were induced by the test samples under investigation. The amount of solvent added to the control flasks was the same as the amount of test sample added to the experimental flasks (1 mL) and incubation times were consistent between control and experimental flasks (24 hours). It is well established in the literature that the *in vitro* cytogenetics assay is capable of detecting clastogenic activity (Ishidate, Jr. 1988; Itoh et al. 2001; Putman et al. 2001; Obe et al. 2002), therefore a positive control was not performed.

Previous work has shown that the mutagens produced during the chlorination of surface waters are direct-acting, and that if rat liver S9 fraction is included during the treatment period, activity is reduced (Wilcox and Williamson 1986). Therefore, for this experiment, treatments were carried out in the absence of S9.

6. Observations and Results

Tables 6.1 and 6.2 summarise the DBP-induced chromosome aberrations observed at 5°C and 25°C respectively. Observed structural aberrations were classified as chromatid gaps (ctg), chromatid breaks (ctb), chromatid exchanges (cte), chromosome gaps (csg), chromosome breaks (csb) or chromosome exchanges (cse) and tallied. A negative control was performed with DDW for each temperature value and is shown at the bottom of each table. The tables show the total number of aberrations, as well as an adjusted value; this value is the total number of aberrations minus the number of aberrations observed in the control. The adjusted value represents the number of aberrations induced by the test sample and accounts for background levels of chromosome aberrations. While some studies report the total number of aberrations, others report the total number of aberrant cells. Any given cell can have aberrations on more than one chromosome and, in this study, up to three aberrations were observed on one cell. In the tally of aberrant cells, a cell with aberrations is only counted once, regardless of the number of chromosomal aberrations. The total number of aberrant cells, the adjusted value, and the percent aberrant cells are shown in the tables.

Two slides were created for each sample investigated. In order to confirm that the aberration values observed are reproducible, 50 metaphase cells were examined from the second slide for five samples or one complete pH/temperature test run. The chromosome aberrations observed during the initial reading and the confirmation reading for pH 5/ 5°C are shown in Table 6.3. Figures 6.1 and 6.2 compare the initial and confirmation readings for the total number of aberrations and the total number of aberrant cells respectively. Within the error margins commonly accepted for biological cell assays, there is good consistency between the initial and confirmation readings, indicating that the initial readings are reliable and that the results are reproducible.

Table 6.1: Disinfection by-product induced chromosome aberrations (5°C)

Temp (°C)	pH	{Br^-} mg/L	{Cl_2} mg/L	Aberrations						Total No. Aberrat-ions	Adjusted Value (Aberrat-ions)	No. Aberrat-ions /100 cells	Total No. Aberrant Cells/ 50	Adjusted Value (Aberrant Cells)	% Aberrant Cells
				ctg	ctb	cte	csg	csb	cse						
5	5	50	1500	2	3	1	1	0	0	7	6	12	6	5	10
		100	1500	4	2	0	1	0	0	7	6	12	7	6	12
		150	1500	8	2	4	1	1	0	16	15	30	11	10	20
		200	1500	5	4	2	1	0	0	12	11	22	11	10	20
		250	1500	5	4	2	1	0	0	12	11	22	11	10	20
	7	50	1500	6	3	0	1	0	0	10	9	18	9	8	16
		100	1500	6	3	2	0	0	1	12	11	22	10	9	18
		150	1500	8	5	0	1	1	0	15	14	28	14	13	26
		200	1500	8	3	2	0	1	0	14	13	26	14	13	26
		250	1500	11	4	0	0	0	0	15	14	28	15	14	28
	9	50	1500	2	4	1	1	0	0	8	7	14	7	6	12
		100	1500	5	3	2	1	0	0	11	10	20	9	8	16
		150	1500	4	4	1	0	1	0	10	9	18	10	9	18
		200	1500	7	2	2	1	1	0	13	12	24	12	11	22
		250	1500	7	3	4	0	1	1	16	15	30	13	12	24
	Control			1	0	0	0	0	0	1			1 (2%)		

Table 6.2: Disinfection by-product induced chromosome aberrations (25°C)

Temp (°C)	pH	{Br^-} mg/L	{Cl_2} mg/L	Aberrations						Total No. Aberrat-ions	Adjusted Value (Aberrat-ions)	No. Aberrat-ions /100 cells	Total No. Aberrant Cells/ 50	Adjusted Value (Aberrant Cells)	% Aberrant Cells
				ctg	ctb	cte	csg	csb	cse						
25	5	50	1500	3	3	1	0	0	0	7	6	12	7	6	12
		100	1500	5	3	1	0	1	0	10	9	18	9	8	16
		150	1500	5	4	0	2	0	0	11	10	20	10	9	18
		200	1500	6	4	2	0	1	0	13	12	24	13	12	24
		250	1500	6	4	1	1	1	0	13	12	24	12	11	22
	7	50	1500	4	3	2	1	0	0	10	9	18	8	7	14
		100	1500	2	0	6	2	0	1	11	10	20	10	9	18
		150	1500	5	6	0	2	0	0	13	12	24	13	12	24
		200	1500	9	2	0	5	1	0	17	16	32	15	14	28
		250	1500	10	6	1	1	0	0	18	17	34	17	16	32
	9	50	1500	3	3	0	1	1	0	8	7	14	8	7	14
		100	1500	7	4	1	1	1	0	14	13	26	14	13	26
		150	1500	6	5	2	2	0	0	15	14	28	15	14	28
		200	1500	8	4	0	1	2	0	15	14	28	15	14	28
		250	1500	7	6	0	3	0	0	16	15	30	15	14	28
	Control			1	0	0	0	0	0	1			1 (2%)		

Table 6.3: Confirmation of chromosomal aberration results (pH 5/ 5°C)

Run	{Br^-} mg/L	{Cl_2} mg/L	Aberrations						Total No. Aberrat-ions	Total No. Aberrant Cells/ 50
			ctg	ctb	cte	csg	csb	cse		
Initial	50	1500	3	3	1	0	0	0	7	6
	100	1500	5	3	1	0	1	0	7	7
	150	1500	5	4	0	2	0	0	16	11
	200	1500	6	4	2	0	1	0	12	11
	250	1500	6	4	1	1	1	0	12	11
Confirmation	50	1500	3	4	0	0	0	0	7	6
	100	1500	4	3	1	0	0	0	8	7
	150	1500	5	4	2	2	0	0	13	10
	200	1500	7	6	0	1	1	0	15	12
	250	1500	6	6	1	0	1	0	14	13

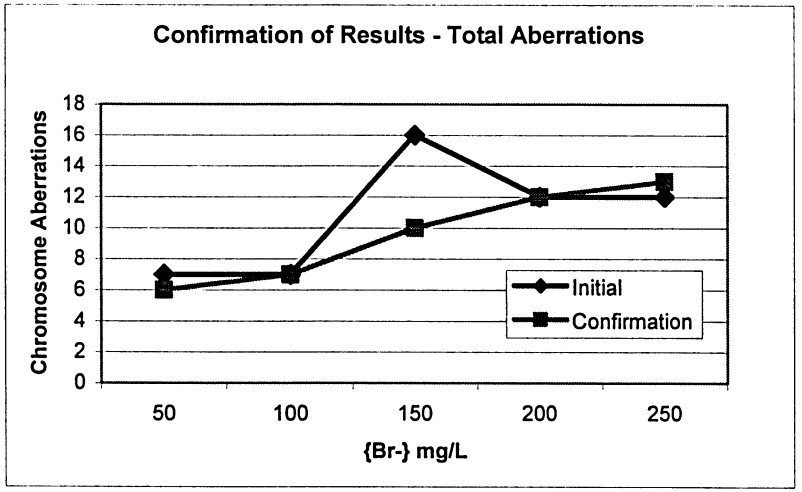


Figure 6.1: Confirmation of results – Total Number of Aberrations (pH 5/ 5°C)

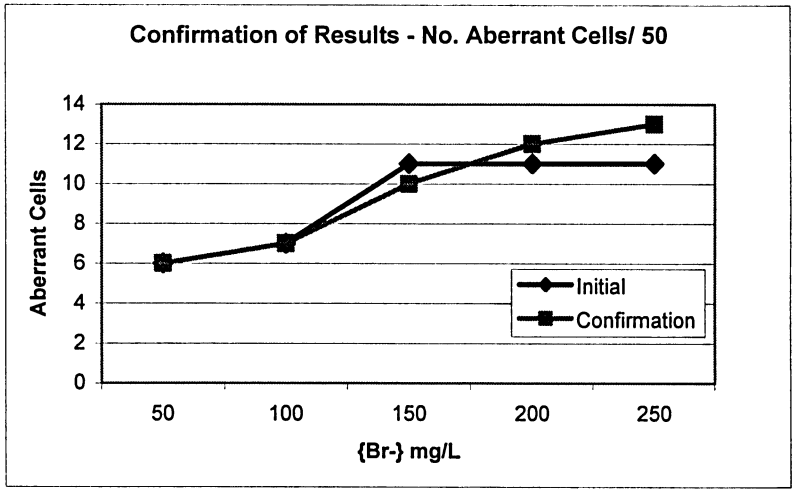


Figure 6.2: Confirmation of results – Total Number of Aberrant Cells (pH 5/ 5°C)

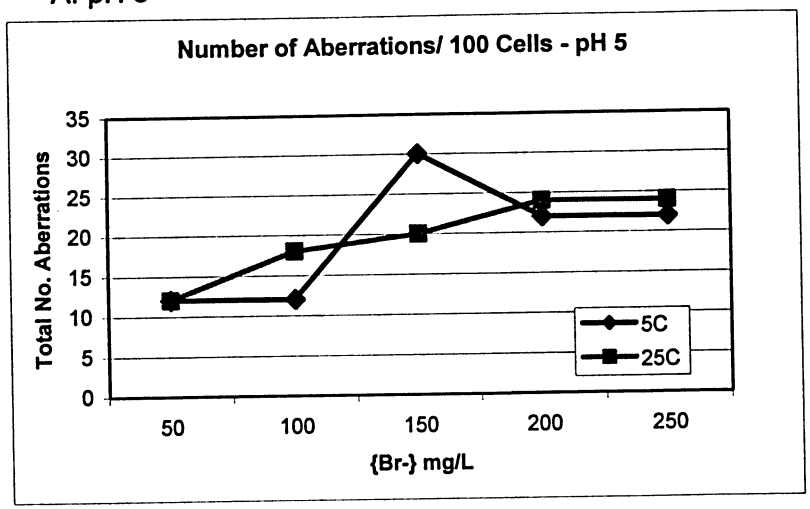
Effects of Temperature on Chromosomal Aberration Formation

Figure 6.3 shows the effects of temperature on DBP-induced chromosomal aberrations, as measured by the total number of aberrations/ 100 cells for each of the pH values under investigation. Meanwhile, Figure 6.4 illustrates the percent aberrant cells as a measure of the effect of temperature on chromosomal aberration formation. It appears that there are more aberrations formed and a higher percentage of aberrant cells at 25°C than 5°C for pH 9 (Figure 6.3 (C) and Figure 6.4 (C)). This difference is particularly noticeable when comparing the number of aberrant cells between the two samples. However, there does not appear to be an considerable difference between the number of aberrations or the percent aberrant cells formed at 5°C and 25°C for pH 5 or pH 7 (Figure 6.3 (A) and (B) and Figure 6.4 (A) and (B)). It is interesting to note that for all three pH values there appears to be greater aberrations induction potential at the higher end of the bromide concentration range (200 mg/L and 250 mg/L) at 25°C than at 5°C. Although in some cases this difference is relatively small (2-4 aberrations/ aberrant cells), it is consistent for both the total number of aberrations and the number of aberrant cells.

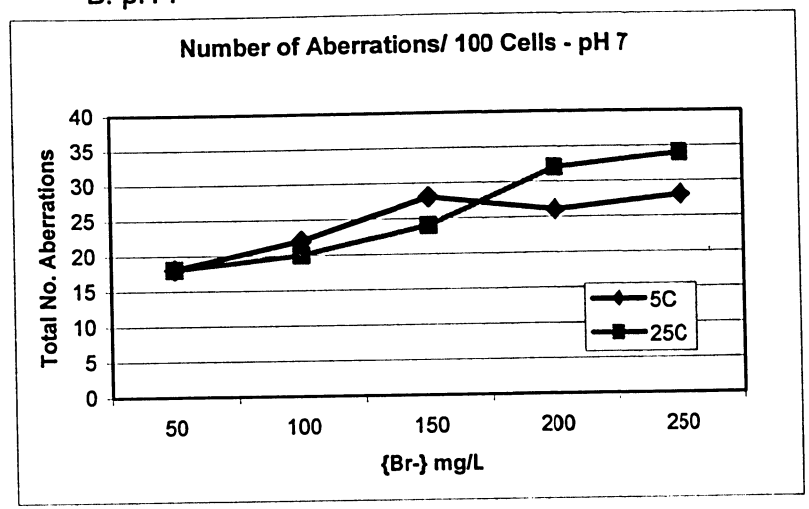
Effects of pH on Chromosomal Aberration Formation

The effects of pH on the formation of aberrations are illustrated in Figure 6.5 for total number of aberrations and Figure 6.6 for number of aberrant cells. For both the total number of aberrations and the number of aberrant cells, samples at pH 5 generally displayed the lowest levels of aberrations. This trend is more evident at 25°C where the there appears to be both less aberrations (Figure 6.5 (B) and a smaller percentage of aberrant cells (Figure 6.6 (B)) at pH 5. It is noteworthy that based on the confirmation reading shown in Figure 6.1, the high value for total number of aberrations at pH 5/ 5°C at 150 mg/L (Figure 6.5 (A)) seems to be an outlier of the general trend. At 5°C, the greatest numbers of aberrations are overwhelmingly formed at pH 7; this trend is most clearly visible with regard to the number of aberrant cells.

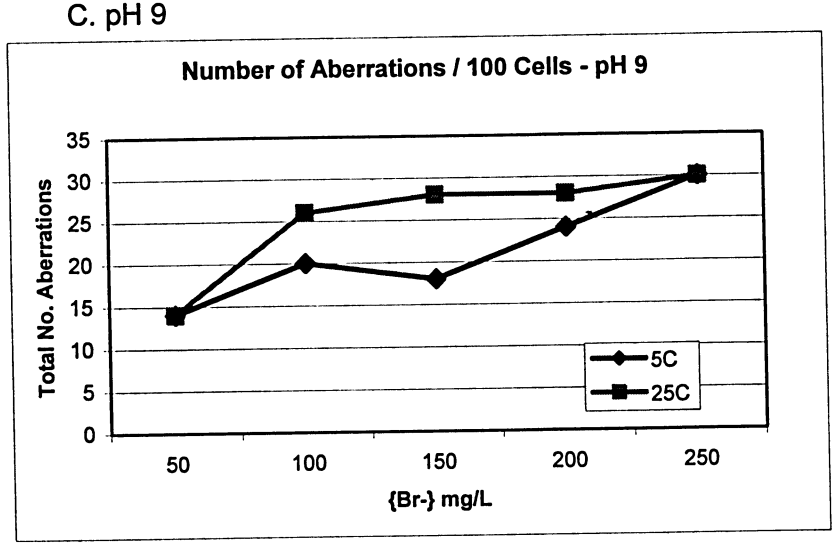
A. pH 5



B. pH 7



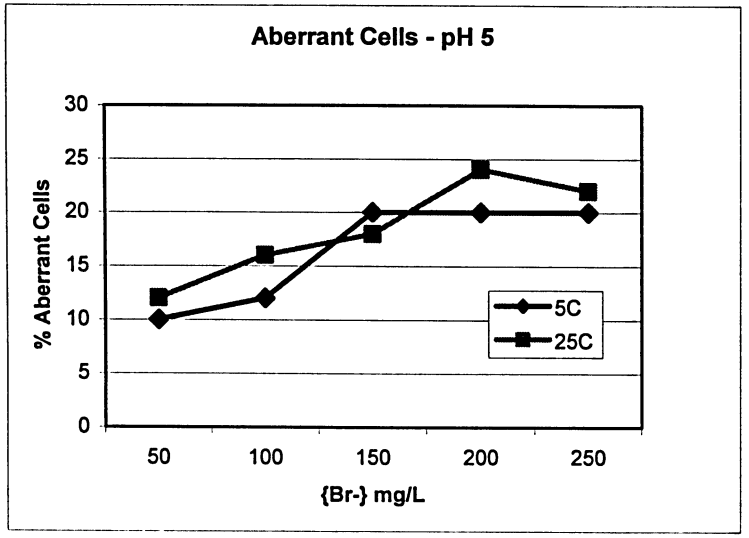
C. pH 9



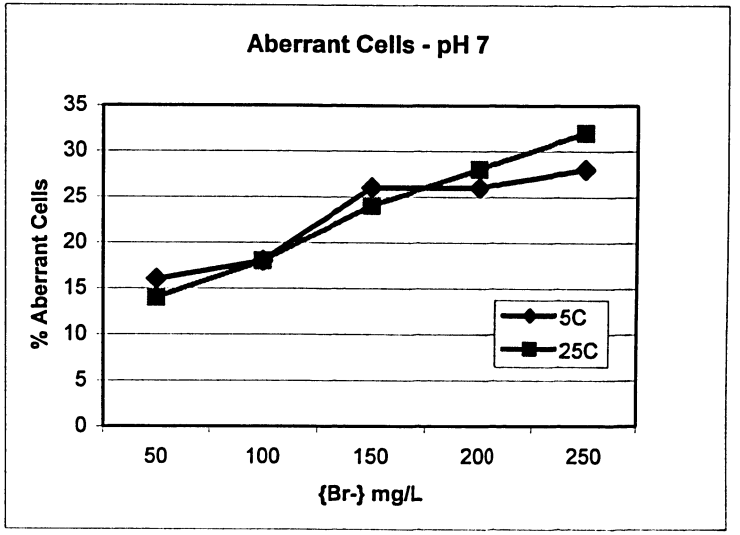
		Regression Equation	Goodness of Fit (R^2)
5°C	pH 5	$y = -1.8571x^2 + 14.143x - 2.4$	0.5879
	pH 7	$y = -0.8571x^2 + 7.5429x + 11.2$	0.9027
	pH 9	$y = 0.5714x^2 + 0.1714x + 14.4$	0.9017
25°C	pH 5	$y = -0.7143x^2 + 7.2857x + 5.6$	0.9793
	pH 7	$y = 0.2857x^2 + 2.6857x + 14.4$	0.9017
	pH 9	$y = -1.5714x^2 + 12.829x + 4$	0.9112

Figure 6.3: Effects of temperature on chromosomal aberrations (**Total Aberrations/ 100 Cells**)

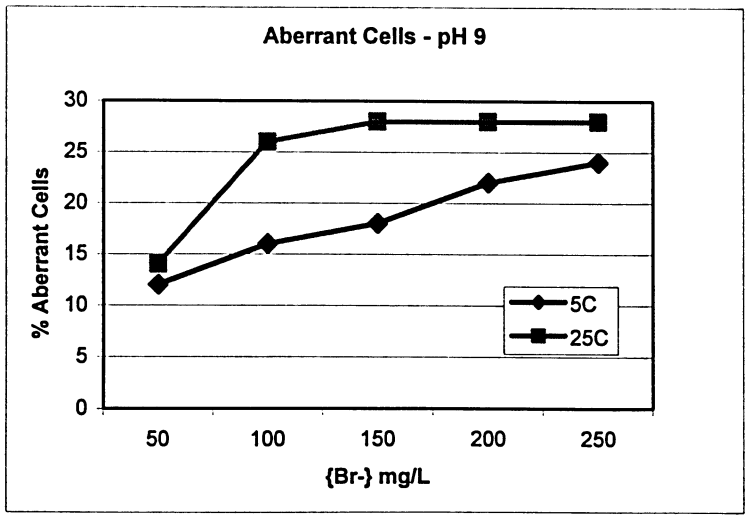
A. pH 5



B. pH 7



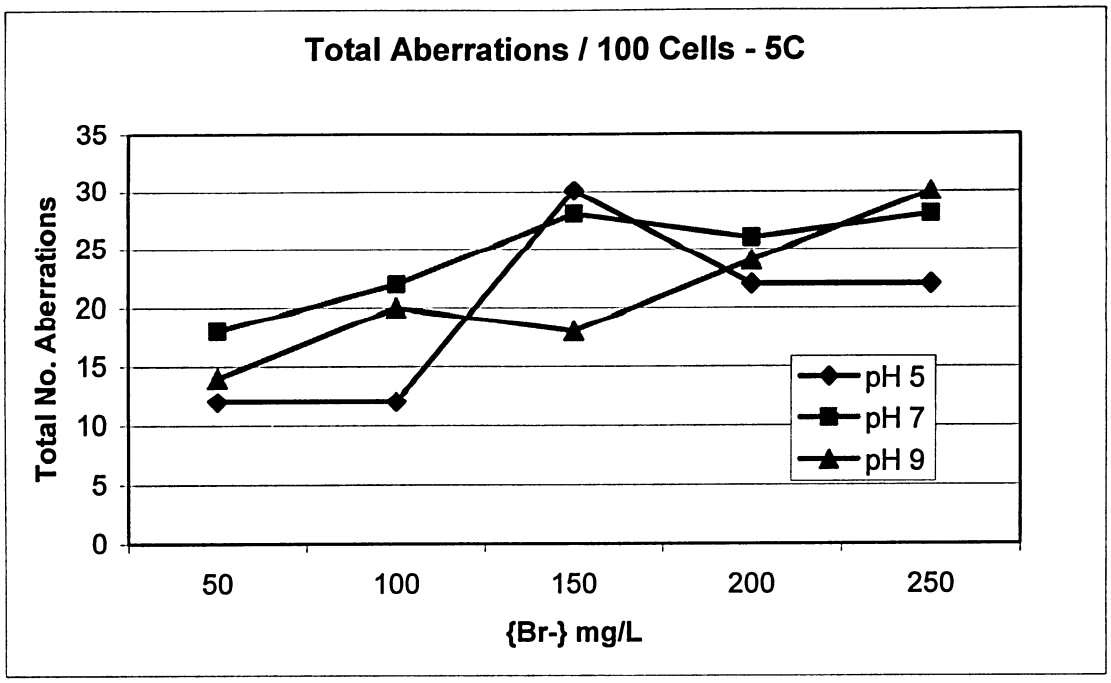
C. pH 9



		Regression Equation	Goodness of Fit (R^2)
5°C	pH 5	$y = -0.8571x^2 + 7.9429x + 2$	0.894
	pH 7	$y = -0.5714x^2 + 6.6286x + 9.2$	0.9159
	pH 9	$y = -0.1429x^2 + 3.8571x + 8.4$	0.99
25°C	pH 5	$y = -0.5714x^2 + 6.2286x + 6$	0.9098
	pH 7	$y = -0.1429x^2 + 5.4571x + 8.4$	0.9957
	pH 9	$y = -1.8571x^2 + 14.143x + 2.8$	0.9293

Figure 6.4: Effects of temperature on chromosomal aberrations (**% Aberrant Cells**)

A. 5°C



B. 25°C

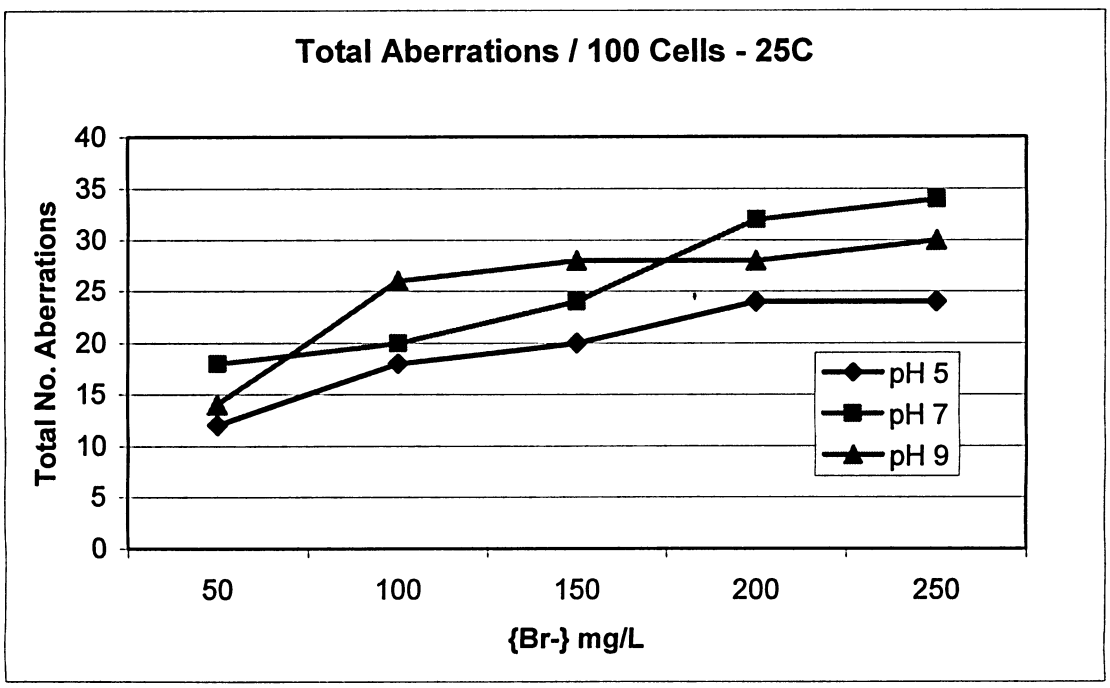
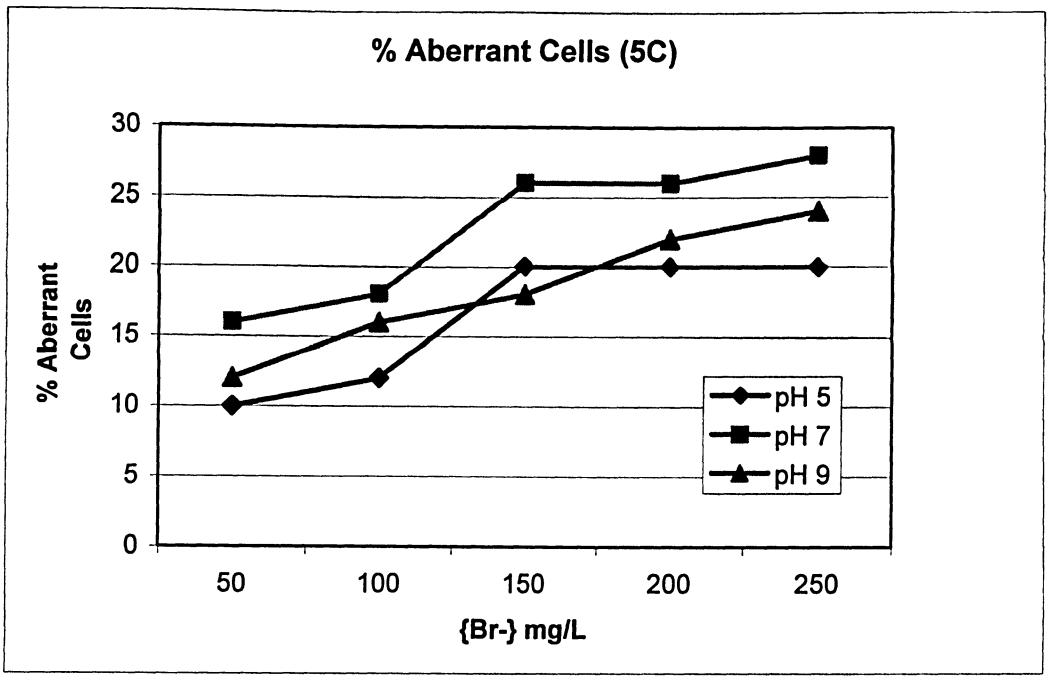


Figure 6.5: Effects of pH on chromosomal aberrations **(Total Aberrations / 100 Cells)**

A. 5°C



B. 25°C

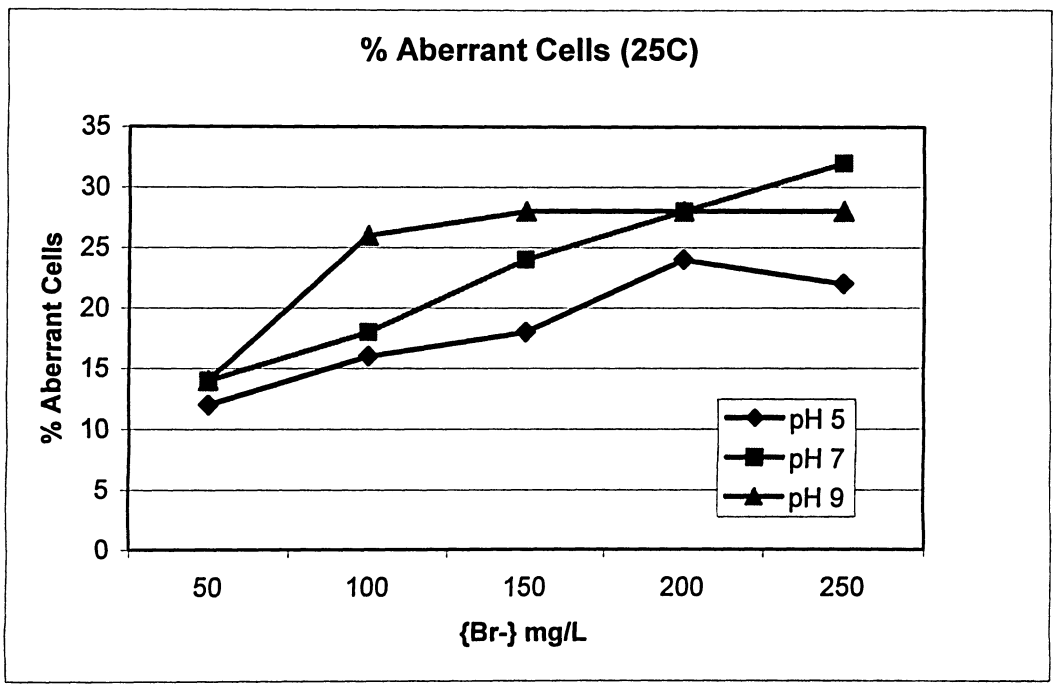


Figure 6.6: Effects of pH on chromosomal aberrations (**% Aberrant Cells**)

Figures 6.4 and 6.6 also demonstrate a trend with regard to bromide concentration. For pH 5 there is no difference between the aberrations formation at 200 mg/L and 250 mg/L and in terms of the number of aberrant cells both pH 5 and pH 9 plateau at the higher end of the bromide concentration range. In fact, pH 7 is the only parameter that shows a consistent increase in the number of aberrations and the number of aberrant cells with increasing bromide concentration. However, in terms of total number of aberrations at pH 9, there is an increase in aberrations formed with increasing bromide concentration.

Effects of Bromide Concentration on Chromosomal Aberration Formation

Although there are some fluctuations, a very strong general trend emerges with regard to bromide concentration and chromosomal aberrations. Examination of both the effects of temperature (Figures 6.3 and 6.5) and pH (Figures 6.4 and 6.6) reveals that there is a strong positive correlation between bromide concentration and both the total number of aberrant cells and the number of aberrations. Figures 6.3 and 6.4 list the regression equations, in the form of second-order polynomial quadratic equations, and the goodness of fit of these equations, as measured by the sum of the squares of residuals for the six data sets. The r^2 values, which average 0.9015 and range from 0.5879 to 0.9957, describe a reasonably good correlation between the data and the equations.

The images below are some examples of the CHL cells observed and analyzed in this study; Figure 6.7 shows cells at 400x magnification, while Figure 6.8 depicts cells at 1000x magnification. Individual chromosomes are visible at 400x magnification, while distinct aberrations on specific chromosomes can be observed at 1000x magnification.

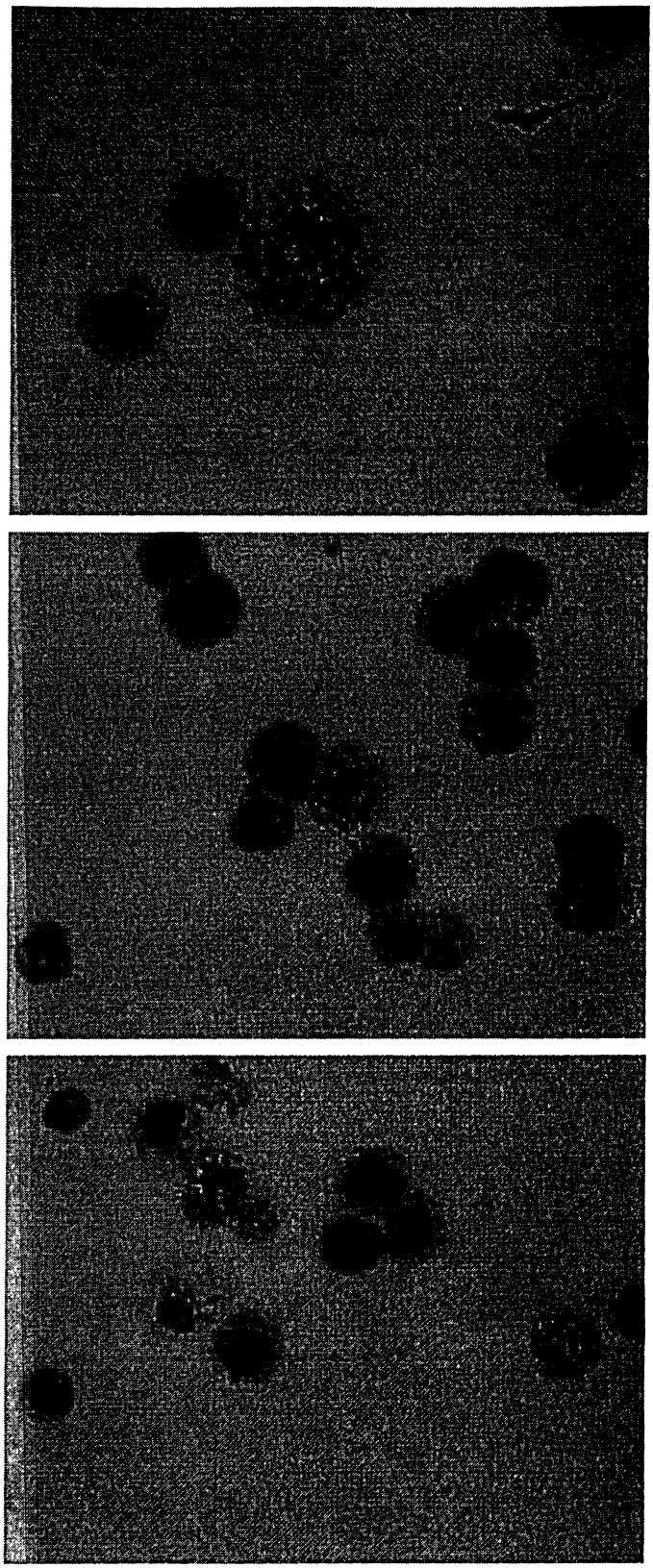


Figure 6.7: Chinese hamster lung cells (400x magnification)

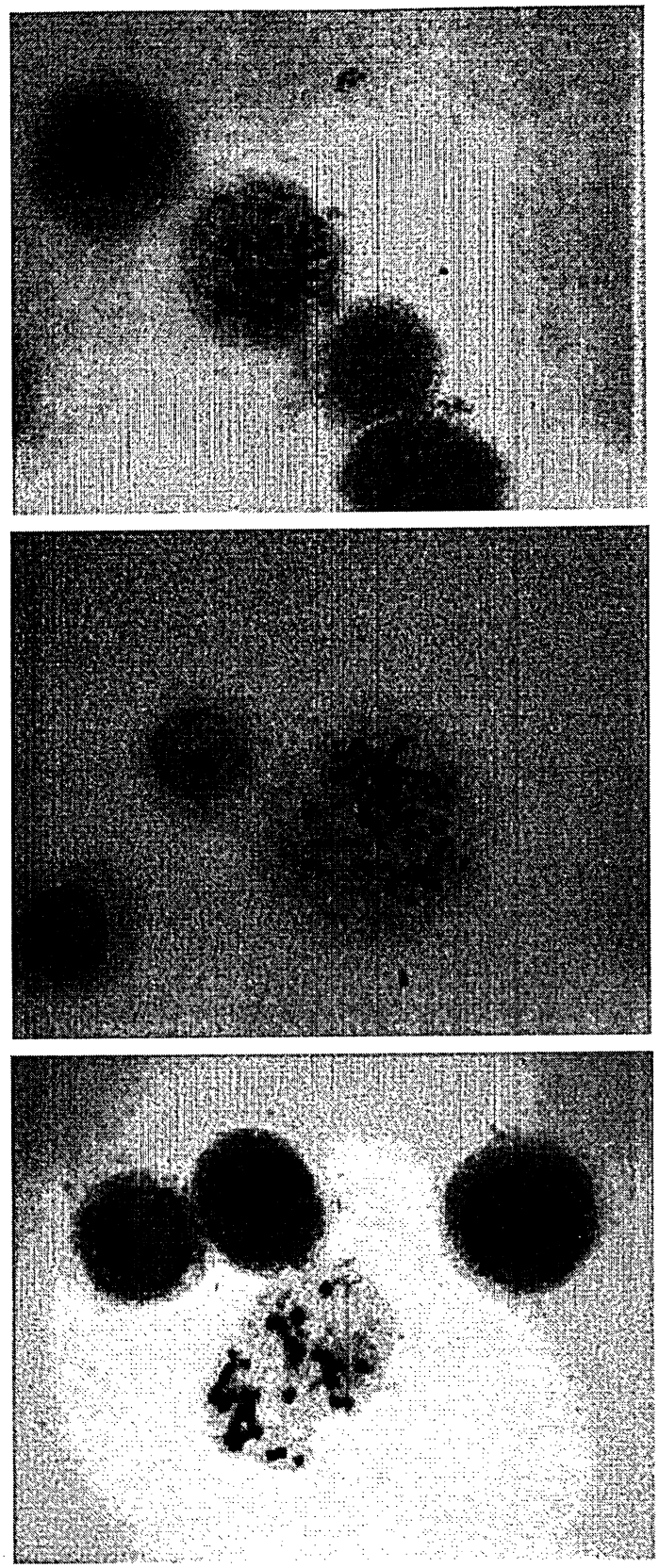


Figure 6.8: Chinese hamster lung cells (1000x magnification)

7. Discussion and Conclusions

Chlorination disinfection by-products are an important class of hazardous environmental chemicals and cytotoxicity in mammalian cells is a sensitive biological indicator for both specific environmental compounds and complex mixtures. Exposure to mutagenic chemicals can cause damage to cellular macromolecules and chromosomal aberrations are a direct indicator of genetic damage *in vitro* and *in vivo* (Obe et al. 2002). Carcinogenesis is a multi-stage process and recessive mutations induced by chemical or physical agents can come to express their mutant phenotype if there is loss of heterozygosity. Maligant progression can also arise through translocation of genetic material, resulting in a change of expression of proto-oncogenes, and deletions, rearrangements or chromosome loss leading to elimination of tumour suppressor genes (Kirkland 1998). Good examples of this are retinoblastoma and colorectal carcinoma (Morris et al. 1992). Thus, chromosome damage is relevant for carcinogenesis and the importance of testing for the ability of a chemical to induce chromosomal aberrations is now well established.

Epidemiological studies indicate a relationship between cancer incidence and high levels of chlorinated by-products in drinking water and attempts have been made to equate this carcinogenesis to specific components of drinking water, such as trihalomethanes or haloacetic acids. Assessment of the health effects of these compounds in drinking water is a formidable task and attempts to extend such associations into cause-and-effect relationships are made all the more difficult by our ignorance of the effects of the majority of other organic compounds in drinking water. In typical water, volatile organic compounds, such as THMs, represent only about 10% of the total organic material by weight and of the remaining 90%, it is estimated that between 80 and 90% of compounds are yet to be identified (Bull 2000). Compound identification among the non-volatiles is difficult and time-consuming and individual toxicological assessment of even a large minority of the total compounds in drinking water is a practical

impossibility (Richardson et al. 1999; Van Leeuwen 2000). Furthermore, such complex mixtures raise the prospect of additive or antagonistic effects or of enhancement of carcinogenesis and mutagenesis as promoters/co-carcinogens and co-mutagens (Richardson et al. 1999; Bull 2000). There is thus merit in attempting to provide a direct assessment of the genotoxic effects of DBP mixtures under conditions encountered during water treatment, as a preliminary step.

7.1 Effects of Temperature, pH and Bromide Concentration

The findings of this study demonstrate that chlorinated and brominated humic acid samples are capable of inducing *in vitro* chromosome damage in mammalian cells. The finding that chromosomal aberrations increased with higher bromide concentration is consistent with earlier work with bacterial assays and mammalian cell micro-plates that showed that brominated DBPs are more cytotoxic and genotoxic than their chlorinated analogues. Plewa et al. (2002) assessed the cytotoxicity and genotoxicity of a number of DBPs and found the following rank order in decreasing chronic cytotoxicity: bromoacetic acid (BA) >> 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) > dibromoacetic acid (DBA) > chloroacetic acid (CA) > $KBrO_3$ > tribromoacetic acid (TBA) > dichloroacetic acid (DCA) > trichloroacetic acid (TCA). SCGE was used to assess the induction of DNA strand breaks by these compounds and the observed rank order in decreasing genotoxicity was: BA >> MX > CA > DBA > TBA > $KbrO_3$ > DCA > TCA. The authors concluded that the brominated haloacetic acids are more cytotoxic and genotoxic than their chlorinated analogues. In a subsequent study, similar trends were observed for halonitromethanes and Plewa et al. (2004) concluded that the brominated nitromethanes are more cytotoxic and genotoxic than their chlorinated analogues. Using *S. typhimurium* TA100 assays, Nobukawa and Sanukida (2000) reported an increase in mutagenic potential when

bromide ions were substituted for chloride ions in humic acid samples. Mutagenicity of chlorinated water containing bromide ions was approximately twice that of chlorinated water without bromide. Although less is known about the brominated DBPs (e.g. HAAs – Cowman and Singer 1994), this study confirms that they have the greatest genotoxic potential. This also further strengthens the justification for use of chromosomal aberration assays as a tool for genotoxicity screening.

There is evidence that higher temperature values result in both more chromosomal aberrations and a greater percentage of aberrant cells at pH 9. For pH 9, the number of aberrant cells is on average 24.6% greater at 25°C than 5°C (range: 14-38%), while the average total number of aberrations is 14.6% greater at 25°C than at 5°C (range: 0-36%). In addition, at the higher end of the bromide concentration range (200 and 250 mg/L), more aberrations were formed at 25°C than 5°C for all three pH values. For example, at 200 mg/L bromide, 17% more aberrant cells and 8% more total aberrations were observed at 25°C than 5°C at pH 5; 7% more aberrant cells and 19% more total aberrations at pH 7; 21% more aberrant cells and 14% more total aberrations at pH 9.

Rodriguez et al. (2004) reported that the highest average total THM levels in the distribution system in Quebec City, Canada occur during the summer and fall. The authors note that during the summer period, average water temperatures, chlorination doses (pre- and post-), and raw water TOC are at their highest. This and a number of other surveys have reported higher concentrations of THMs and HAAs in summer months, but this has mainly been attributed to higher levels of organic matter and the resultant higher chlorine dose (Health Canada 1996a; Whitaker et al. 2003; Toroz and Uyak 2005). In the present study, there are no differences in the chlorine dose or the organic content between treatments at 5°C and 25°C and yet there are differences in the formation of chromosomal aberrations. Seasonal variations in temperature can affect reaction rates and higher temperatures generally correspond to increased rates of reaction. At higher water temperatures, rates of chlorine decay are higher

and higher chlorine doses are added at the pre- and post-chlorination stages to ensure acceptable levels of chlorine residual in the distribution system (Toroz and Uyak 2005). In this study, that could mean that the chlorine was used up more quickly or that rates of chlorine decay are higher, ultimately resulting in a greater number of brominated species at the end of the 24-hour formation period. The higher number of aberrations observed at 200 and 250 mg/L supports this idea, since there is greater opportunity for the formation of brominated species, which are more genotoxic.

Although there is evidence that the number of aberrant cells is higher at 5°C at pH 7 than pH 5 or 9 (Figure 6.6), overall there do not appear to be any appreciable changes in genotoxicity or strong trends over the pH range tested. The number of aberrant cells was 23-38% higher at pH 7 compared to pH 5 (average 29.2%), while the number of aberrant cells was 11-31% higher at pH 7 than pH 9 (average 19.2%). Taking into account the outlier at 150 mg/L {Br^-} at pH 5/5°C, a similar trend was observed for the total number of aberrations (Figure 6.5), however the differences are less pronounced. Whereas Meier et al. (1983) and Itoh and Matsuoka (1996) reported that mutagenic activity of chlorinated humic acid samples decreased with increasing pH, recent work by Luk et al. (2006, in press) found no appreciable difference in genotoxicity between samples at pH 5, 7 or 9. Luk et al. (2006, in press) noted that when sample pH is low, conditions favoured the formation of TOCl over TOBr, while at high pH this trend was reversed. Since, in general, genotoxicity is much more sensitive to TOBr than TOCl, these factors counter each other and overall genotoxicity remains largely unaffected. The complex mixtures found in drinking water are variable, thus it is important to improve understanding of DBP formation in each geographic region or under each set of experimental conditions. Although the effects of pH on the formation of certain DBPs has been described (e.g. more THMs are formed at a higher pH), it is important to note that that there is a multitude of DBPs present in both source water and humic acid samples in the laboratory. For example,

Rodriguez et al. (2004) report that trichloroacetic acid (TCAA) formation in chlorinated waters in higher at lower pH, whereas dichloroacetic acid (DCAA) formation does not appear to be affected by pH. Analysis of complex mixtures, such as water or humic acid samples, presents comparison problems even in the use of relatively straightforward cytogenetics assays. Where there is inconsistency, the observed variability between assays of mutagenesis is probably due to the cells' interaction with mutagenic and toxic components in the mixtures. The resulting apparent mutagenic induction capacity for a sample is likely to underestimate the activity actually present (Bull 2000). Comparisons with previous studies are further complicated by the age of the cell line. In this study, the cell line was newly initiated and was thus more resistant to environmental stresses than those cell lines that have been in use for many months or even years. Accordingly, this cell line displayed a faster growth rate and lower numbers of aberrations, both in control and experimental samples, than reported elsewhere in the literature (e.g. Itoh et al. 2001; Sutiakova et al. 2004). Although it may be possible to compare trends between studies, direct comparisons are often irrelevant since cell lines in different laboratories are at different stages of advancement.

Wilcox and Williamson (1986) report that the presence of serum in the treatment medium markedly reduced the clastogenic activity of samples in mammalian cell assays. Since in the present study treatment was carried out in medium supplemented with 10% FBS, the clastogenic activity of the humic acid samples may have been suppressed. This may have important implications for the transport and distribution of the mutagens in the body once they are ingested and Wilcox and Williamson (1986) suggest that this may indicate that mutagenic species readily bind to proteins.

7.2 Disinfection By-Product Control

While chlorine came under early scrutiny for the formation of disinfection by-products (Rook 1974), alternative chemical disinfectants such as ozone (O_3), chlorine dioxide (ClO_2) and chloramines (e.g. NH_2Cl – monochloramine) have each since been shown to form their own set of DBPs. Since DBPs are formed by all of these alternative chemical disinfectants, adoption of alternative disinfectants for DBP control often only means a trade-off from one group of DBPs for another. A recent study by the U.S. EPA surveyed drinking waters across the U.S, and found that while the use of alternative disinfectants lowered the levels of the regulated THMs and HAAs compared to chlorine, many other DBPs were formed at higher levels with these alternative disinfectants (Weinberg et al. 2002). A number of these DBPs have since been placed on the EPA's list of DBPs considered high priority for further study. For example, in the presence of NOM, ozonation produces several groups of organic by-products, including aldehydes, ketoacids and carboxylic acids (Weinberg 2002). In the presence of both NOM and bromide, organo-bromine compounds, such as tribromomethane, bromoacetic acids and bromoacetonitriles, can form (Cowman and Singer 1996; Richardson et al. 1999; Richardson et al. 2003) and studies have found these compounds to pose greater risk than their chlorinated analogues (Kargalioglu et al. 2000; Plewa et al. 2002). The major by-products associated with chlorine dioxide include chlorate (ClO_3^-) and chlorite (ClO_2^-) and the U.S. E.P.A. has recommended that the combined residuals of ClO_2, ClO_2^- and ClO_3^- be less than 1 mg/L. Although chloramination significantly reduces THM formation, cyanogen chloride and total organic halides (TOX) represent the important DBPs associated with chloramines (Minear and Amy 1996).

In addition to the water quality parameters discussed in Chapter 2, water treatment processes also significantly affect DBP formation, speciation and removal. These processes, which include pre-oxidation, coagulation, carbon adsorption, bio-filtration and membrane

filtration, can impact DBP levels directly or indirectly, by altering water quality (US EPA 2001). The most effective DBP control strategy is organic precursor removal and all of these processes reduce DBP levels by either removing NOM from the water or by altering its reactivity to chlorine (Xie 2004). NOM consists of a mixture of humic substances (humic and fulvic acids) and non-humic, hydrophilic material and it is the humic substances that are most reactive with disinfectants. NOM removal is strongly influenced by the size, structure and functionality of the organic mixture (Reckhow et al. 1990). Processes such as coagulation, adsorption and membranes remove NOM intact, while treatments with oxidants can cause NOM reaction sites to become inactive to further chlorination and thus affect subsequent chlorination. By removing NOM, these treatment processes significantly reduce the chlorine demand of the water and thus the chlorine dose and DBP formation potential associated with disinfection (US EPA 2001).

The Stage 1 Disinfectants and Disinfection Byproduct Rule issued by the U.S. EPA (1998) identifies two best available technologies (BATs) for DBP control: enhanced coagulation and granulated activated carbon (GAC) adsorption. The enhanced coagulation process is defined as an optimized coagulation process for removing DBP precursors (NOM) and generally involves the addition of high doses of alum, ferric chloride, ferric sulphate or ferrous sulphate as coagulants. Enhanced coagulation is normally practiced at a lower pH, which generally enhances water disinfection. At a lower pH, hypochlorous acid, a stronger chlorine species, becomes dominant, which tends to lowers the chlorine dose requirement and can improve the stability of the chlorine residual in the distribution system (US EPA 1999). On the other hand, the lower pH conditions associated with enhanced coagulation can significantly compromise the removal of manganese, as soluble manganese does not oxidize completely at low pH conditions. Enhanced coagulation also increases sludge production and may alter sludge characteristics, which could significantly increase the cost and difficulty of sludge handling and disposal. Furthermore, by lowering pH, enhanced coagulation could potentially result in

corrosion problems in the distribution system; the pH may need to be adjusted with Na_2CO_3 or NaOH to proper levels, which would increase the cost of the finished water (Xie 2004).

Carbon adsorption is another DBP control process and under the US EPA's Stage 1 Disinfectants and Disinfection Byproduct Rule (1998), granular activated carbon (GAC) with an empty bed contact time of 10 minutes (GAC10) is identified as one of the two BATs for DBP control. GAC is effective in removing NOM and can be used for DBP removal as well. Frequent GAC regeneration is required for most contactors and Xie (2004) reported that in bench scale studies in his laboratory, GAC generally reached saturation for HAAs in a few days. Membrane technologies, especially nano-filtration, are also very effective in DBP precursor removal, however, due to the high capital and operation costs, as well as the huge quantities of wastewater produced, membrane technologies are not identified as best-available technologies for DBP control in surface waters. Membrane technologies have traditionally been used for groundwater treatment and are only cost-effective in surface-water treatment for very small systems (population size 25-100) (US EPA 2001).

In recent years, many water treatment plants, especially those that serve large communities, have eliminated pre-chlorination and moved back the chlorination point to intermediate or post-chlorination (Bull 2000). While delaying the chlorination point does shorten chlorination time and can be a very effective way to control DBP levels in finished water, eliminating prechlorination can also affect iron and manganese removal and may cause algal and bio-film growth inside treatment units. Many treatment plants have attempted to mitigate some of these impacts on treatment processes by replacing pre-chlorination with pre-oxidation with alternative disinfectants or oxidants. Potassium permanganate, chlorine dioxide and ozone are all commonly used in pre-oxidation treatment (Bull 2000).

Despite their effectiveness in removing organic precursors, it is important to note that there are trade-offs associated with these treatment processes. For example, the use of ozone in pre-oxidation has been shown to significantly reduce the formation of THMs, trichloroacetic

acids and total organic halides (TOX). However, pre-ozonation causes the formation of acetone and acetaldehyde, which in turn significantly increases the formation potential of trichloroacetone and chloral hydrate (trichloroacetaldehyde hydrate) (Bull 2000). A more pressing concern lies in the increased formation of brominated DBPs following organic precursor removal. When raw water is chlorinated, naturally occurring bromide is oxidized into highly reactive hypobromous acid and hypobromite. Prior to NOM removal, a large quantity of NOM is available to react with both free chlorine and highly reactive hypobromous acid and hypobromite. At low bromide levels, the majority of DBPs formed are chlorinated and a smaller amount of brominated DBPs are formed. However, treatment processes, such as coagulation or adsorption, remove NOM, but not bromide in the water, resulting in a reduced NOM levels and relatively higher levels of bromide. Consequently, although total DBP levels are reduced following treatment, a greater proportion of those DBP formed will be brominated (Krasner et al. 1996). As discussed in Chapter 2 and demonstrated in the results of this study, there is a compelling body of work implicating brominated DBPs as far more dangerous mutagens and carcinogens than their chlorinated analogues.

The effectiveness and associated trade-offs of these treatment processes on DBP control depend on many factors, such as raw water quality, especially NOM characteristics and bromide concentration, the specifics of the treatment process, the type of water distribution system and the size of the community being served by the treatment plant. Given these many variables, it seems only logical to recommend that disinfection by-product control must be assessed at a local or regional scale. This calls for regulatory agencies and the scientific community to develop models and frameworks that enable water treatment facilities to assess the most effective and economically feasible methods of controlling DBP formation without compromising disinfection efficiency.

7.3 Regulations and Risk Assessment

Under the auspices of the *Safe Drinking Water Act*, the current limit for THMs in the United States is 80 μg/L, while total levels of HAAs are limited to 60 μg/L (US EPA 1998). The *Guidelines for Canadian Drinking Water Quality* (2006) include a THM guideline of 100 μg/L, however there are currently no HAA guidelines in Canada. Although the maximum contaminant levels (MCL) set by the US EPA and the guidelines established by Health Canada are very conservative, it is important to recognize that concern over increased cancer risk or higher incidences of adverse reproductive outcomes is from long-term exposure to disinfection by-products. Given that the vast majority of North Americans consume water containing DBPs and assuming a minimum 70-year exposure to DBPs, even a small increase in risk could have important implications across the population. A second important consideration is the fact that both regulatory agencies have only set MCLs/guidelines for total THMs and HAAs; individual THM and HAA levels aren't regulated. This is of significance because different compounds have different properties and levels of 80 μg/L or 100 μg/L can have very different implications for risk depending on the compound or mixture of compounds. The results of recent toxicological studies suggest that further considerations should be paid to the occurrence of brominated compounds, especially bromodichloromethane (Krasner et al. 1996). The occurrence of brominated compounds is especially important in coastal areas, where raw water can be affected by saline incursion, resulting in much higher levels of brominated species. It is also significant that while the maximum contaminant levels set by the U.S. EPA are enforceable standards, the Canadian guidelines set by the CDW are non-enforceable public health goals.

The humic acid samples tested for the ability to induce chromosomal aberrations in this study were very concentrated and questions naturally arise about the relevance of these

findings to human exposure. In its technical requirements for genotoxicity testing, the ICH (1997) noted that various conditions might produce positive *in vitro* results that may not be of biological relevance, suggesting that there are concentration thresholds below which genotoxic activity will not occur. Certainly there are many more chemicals that produce positive results *in vitro* than *in vivo* (Ishidate et al. 1988; Obe et al. 2002), which raised concerns that many *in vitro* positives are not relevant in terms of human exposure and that the exposure conditions *in vitro* are too extreme. These concentration thresholds may result from the reaction of the test chemical with non-DNA targets, such as interference with DNA replication or repair, interaction with peripheral proteins or cellular energy depletion, which from the point of view of human risk are likely to be of less importance than direct genotoxins (Scott et al. 1991). There may also be thresholds for effective detoxification of DNA-reactive compounds or their metabolites at lower concentrations that cannot be achieved at higher concentrations (Kirkland 1998). However, recommendations from the 1993 Melbourne International Workshop, a satellite to the International Conference on Environmental Mutagens, suggest that that it may be necessary to use highly concentrated samples in order to detect some important genotoxins (Galloway et al. 1994). Thus, in order to better understand whether the results of *in vitro* assays are relevant or not, there is a great need for mechanistic studies that can establish whether the aberrations induced *in vitro* are due to a process with or without a threshold.

Concentrated extracts prepared from chlorinated surface-derived waters or commercially prepared humic acid, have now been shown to possess genotoxic activity in a wide range of *in vitro* assays using bacterial, rodent and human cells. A number of DBPs are a major health concern because of their carcinogenic properties or associations with adverse health and reproductive effects. On the basis of these results, one must conclude that the chlorination of waters results in the generation of compounds that represent a qualitative carcinogenic risk to humans. *In vitro* assays, although useful for identifying potential hazards, cannot be used to

quantify the magnitude of the risk (Bull et al. 1993). Risk analysis relies either on risk extrapolation from animal experiments or on epidemiological studies and ideally requires information on exposure levels. Both approaches, however, are relatively insensitive and are not particularly reliable for evaluating low-dose exposures that are likely to represent a low risk of disease (Bull et al. 1993). Furthermore, because we do not know the identity of the mutagens produced by chlorination, we cannot define the level of exposure. It should be recognized that, for some time, it will be difficult or impossible to estimate accurately the risk associated with consumption of chlorinated drinking water (Van Leeuwen 2000). The World Health Association (WHO) has set guidelines for a number of these compounds, including bromate, bromodichloromethane and 2,4,6-trichlorophenol, based on an excess cancer risk of one in a population of 100 000. These guidelines were established under the assumption that the use of mathematical low-dose risk extrapolation in general provides an overestimation of the actual risk (WHO 1993). In its recommendations, WHO emphasizes that when a choice must be made between meeting guidelines for disinfectants and disinfection by-products on one hand and microbiological guidelines on the other, microbiological quality considerations must take precedence. In fact, the recommendations state that "efficient disinfection must never by compromised" (WHO 1993). Research in this area offers the prospect of a much clearer assessment of health hazards in drinking water. Disinfection of all surface waters used for human consumption is crucial and the health risks from pathogenic microorganisms far exceed those potential risks associated with the chemical disinfection by-products produced during water treatment. The challenge is, therefore, to minimize the potential risks from DBPs without compromising disinfection efficiency.

Appendices

1. Appendix A: Methods – Cell Splitting

2. Appendix B: Methods – Sample Preparation

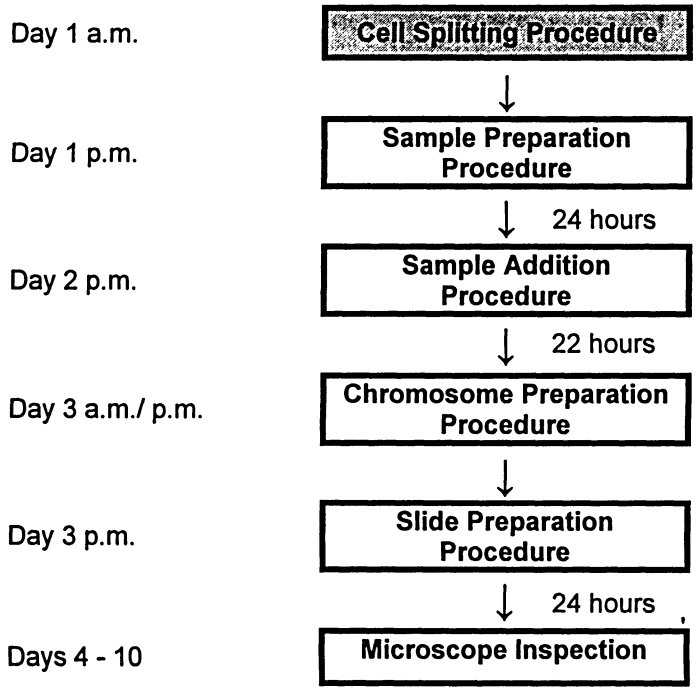
3. Appendix C: Methods – Sample Addition

4. Appendix D: Methods – Chromosome Preparation

5. Appendix E: Methods – Slide Preparation

Appendix A: Methods – Cell Splitting

In fresh media, Chinese hamster lung (CHL) cells double approximately every 15-16 hours. It is important to split the cells either before they become completely confluent or shortly thereafter, otherwise the cells will become crowded, detach and die. During the *Cell Splitting Procedure*, cells are sub-cultured into small cell culture flasks; each small flask serves as one experimental treatment.



Cell Splitting Procedure

30 Minutes Before Procedure:

1. Check the large "stock" cell culture flask under the microscope to ensure that there are a lot of cells and that they are ready for sub-culture (approximately 80-90% confluent).

2. Collect the following items from the fridge and place in a 37°C incubator or water bath:

    - Trypsin [0.25% (1X) solution, w/out Calcium and Magnesium, w/ EDTA]; small aliquots of Trypsin can be kept in the fridge, otherwise will need to thaw from frozen beforehand)
    - Phosphate Buffer [Phosphate buffered saline (1X) 0.0067 M (PO_4), w/out Calcium or Magnesium]
    - MEM/ EBSS [Medium Essential Medium (1X), w/ 2.00 mM/L Glutamine, w/ Earle's Balanced Salts] supplemented with 10% sterile filtered Fetal Bovine Serum and 1% Gentamicin antibiotic (40 mg/mL, Sabex®)
    - MEM/ EBSS [Medium Essential Medium (1X), w/ 2.00 mM/L Glutamine, w/ Earle's Balanced Salts] supplemented with 10% sterile filtered Fetal Bovine Serum

3. Place the following items in the Clean Bench and turn on the UV light:

    - 5 small cell culture flasks (25 cm^2)
    - 2 large cell culture flasks (75 cm^2)
    - 10-mL pipettes + pipettor
    - 2-mL pipettes + pipettor
    - 1000-µL micro-pipette tips + 1000-µL micro-pipettor
    - 2 10-mL centrifuge tubes
    - test-tube rack

Procedure:

1. Using a 10-mL pipette, withdraw all medium from the old cell culture flask and dispose in a waste beaker.

2. Gently rinse the cells with 2-mL of Phosphate Buffer and drain off completely with the pipette; repeat once, changing pipettes each time.

3. Add 2 mL of Trypsin solution to the flask, shake gently, and drain off very quickly with the pipette.

4. With a new pipette, add 1.2 mL of Trypsin to the culture flask and shake gently. Wait for a few minutes, periodically shaking the flask, until the solution becomes cloudy and the Trypsin separates the cells from the bottom of the flask.

5. Add 8.8 mL of MEM (no antibiotic) to the culture flask and shake the flask gently. Separate cells by aspirating the solution for approximately 30 rounds with a pipette.

6. Transfer 5 mL of the cell suspension into each of the centrifuge tubes and centrifuge the cells at 3000 rpm for 2 minutes. Disinfect the centrifuge tubes and hands and return to the Clean Bench.

7. Open the centrifuge tubes and discard the supernatant with a 2-mL pipette.

8. Add 6 mL of MEM (no antibiotic) into each of the centrifuge tubes.

9. Aspirate the contents of the centrifuge tubes approximately 35 rounds with a new 2-mL pipette.

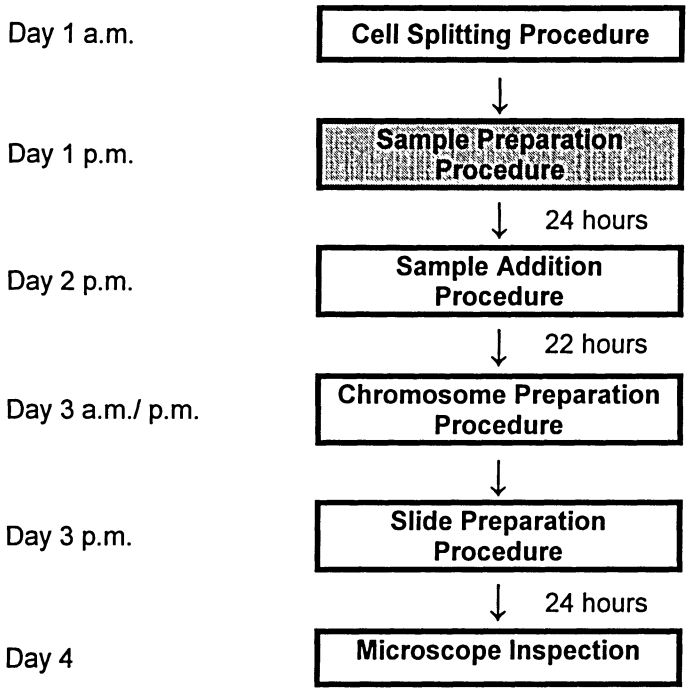
10. Dispense 5.5 mL of MEM (no antibiotic) into each of 5 small culture flasks. Add 0.5 mL of the cell suspension from the centrifuge tubes to each small culture flask.

11. Dispense 23 mL of MEM (with antibiotic) into each of 2 large culture flasks. These large culture flasks will be the source of cells for the next cycle of cell sub-culturing. Depending on the date of the next cell split, add 0.2 mL (6-7 days) to 0.5 mL (3-4 days) of cell suspension from the centrifuge tubes to each large cell culture flask.

12. Disinfect, dry and label all flasks and return to the 5% CO_2/ 37°C incubator.

Appendix B: Methods – Sample Preparation

Disinfection by-products (DBPs) are formed from the chemical reaction of chlorine with the organic matter in natural waters. In this study, humic acid is used as the organic DBP precursor and HOCl simulates the chlorine disinfectant used during the water treatment process. Bromide ions are commonly found in surface waters and brominated DBP species are of special concern; in this study, KBr provides Br^- ions, which serve as an additional inorganic precursor. These three elements of DBP formation come together during *Sample Preparation.*



Sample Preparation Procedure

1. Collect:
    - 1000-µL micropipette tips + 1000-µL micropipettor
    - 100-µL micropipette tips + 100 µL micropipettor
    - humic acid solution (at appropriate test pH)
    - HOCl solution (5% (0.67 M) $\Rightarrow$ $\{Cl_2\}$ = 47,503 mg/L)
    - KBr solution (14.9 mg/L $\Rightarrow$ $\{Br^-\}$ =10,000 mg/L)
    - aluminum foil
    - 5 sterile sample bottles

2. Measure the concentration of the HOCl with a spectrophotometer following standard ABTS procedure. The measured concentration may be lower than the original recorded concentration as HOCl tends to lose strength over time. However, the decrease should be maintained to within a 10% loss.

3. Create labels which include the following information:
   - pH + temperature
   - concentration of HOBr
   - concentration of HOCl
   - date

4. Wrap sample bottles in aluminum foil and affix labels.

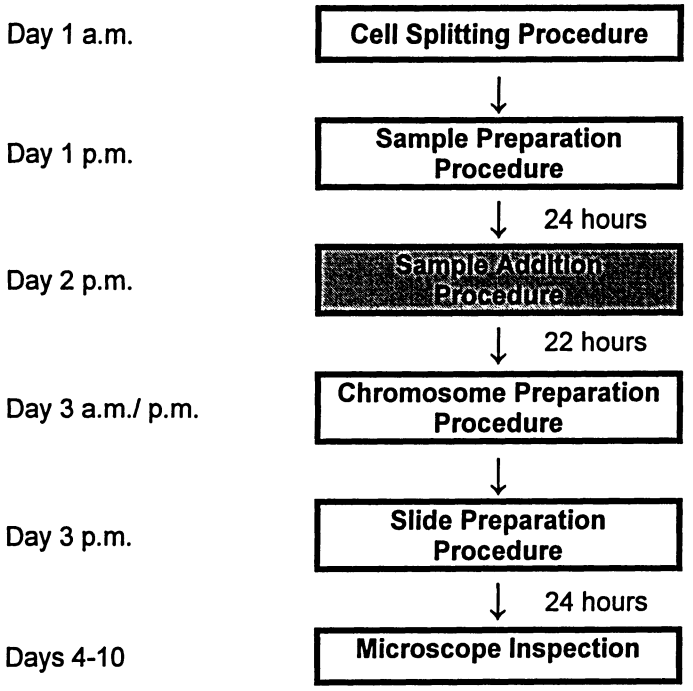
5. Make samples:

Sample Bottle	1	2	3	4	5
[Br^-] mg/L	50	100	150	200	250
[Cl_2] mg/L	1500	1500	1500	1500	1500
Volume KBr (14.9 mg/L)	20 µL	40 µL	60 µL	80 µL	100 µL
Volume HOCl (5% / 0.67 M)	126.3 µL	126.3 µL	126.3 µL	126.3 µL	126.3 µL
Volume of Humic Acid	3.854 mL	3.834 mL	3.814 mL	3.794 mL	3.774 mL
Total Volume	4 mL	4 mL	4 mL	4 mL	4 mL

6. Incubate samples at the appropriate temperature for approximately 24 hours. Samples must be shaken continuously during the incubation period.

Appendix C: Methods – Sample Addition

Following *Sample Preparation*, the samples are incubated for approximately 24 hours at the selected temperature. The samples must be shaken continuously throughout the incubation period, as it is during this period that the disinfection by-products (DBPs) form. During *Sample Addition*, the humic acid sample is syringe-filtered, to remove any bacteria from the sample, and added to the cell culture.



Sample Addition Procedure

30 Minutes Before Procedure:

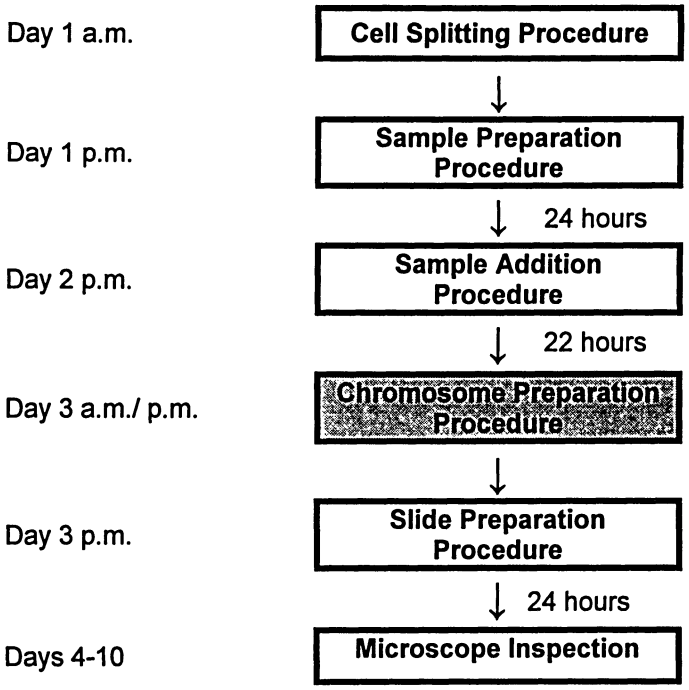
1. Place the following items in the Clean Bench and turn on the UV light:
   - 1000-µL micro-pipette tips + 1000-µL micropipettor
   - 5 syringes + 5 syringe filters (Acrodisc® 25 mm w/ 0.2 µm membrane)
   - 5 steam-sterilized sample bottles

Procedure:

1. Disinfect hands and arms with ethanol.
2. Take the small culture flasks out of the incubator, disinfect and wipe dry.
3. Label flasks and place them in the Clean Bench.
4. Outside of the Clean Bench, disinfect one sample bottle containing humic acid sample, wipe dry, and shake very well for complete mixing.
5. Disinfect hands and arms and bring the sample bottle to the Clean Bench.
6. Insert a syringe into a syringe filter and remove the syringe top.
7. Place the syringe and syringe filter on top of an empty sample bottle.
8. Pour 2-3 mL of sample into the syringe and press the sample into the empty sample bottle with the syringe top.
9. Transfer 1 mL of the filtered sample into the appropriate culture flask.
10. Cap the culture flask and shake contents gently.
11. Repeat procedure for all sample bottles.
12. Disinfect the culture flasks, wipe dry and return to the incubator.
13. Incubate cells for 22 hrs.

Appendix D: Chromosome Preparation

The objective of the *Chromosome Preparation* procedure is to arrest cells in the first post-treatment metaphase of the cell cycle, in order to assess sample induced structural damage to chromosomes, and to prepare the cells for plating onto slides. Cells are first treated with Colcemid® solution, which traps cells in metaphase, followed by potassium chloride treatment to expand cells, and finally the water in the cells is replaced with a fixative.



Chromosome Preparation Procedure

Two Hours Before Procedure:

1. Take the Colcemid® solution (200 mg/L, Calbiochem®) from the fridge. Disinfect hands and take the 5 small culture flasks from the incubator.
2. Pipette 1 mL of the Colcemid® solution into a 50-mL volumetric flask and fill to the top with DDW. Cover and shake well to mix contents.

3. Pipette 0.35 mL of the diluted Colcemid® solution into each of the culture flasks for a final concentration of 0.2 mg/L.
4. Disinfect culture flasks, wipe them dry and return to incubator for 2 hrs.
5. Drain off the rest of the diluted Colcemid® solution.
6. Take the Trypsin (0.25% (1X) solution, w/out Calcium and Magnesium, w/ EDTA) from the freezer and leave it on the bench to thaw.
7. Place 10 microscope slides in a tray and submerge in ethanol (for *Slide Preparation*).

30 Minutes Before Procedure:

1. Place the Trypsin, Phosphate Buffer (Phosphate Buffered Saline (1X) 0.0067 M (PO_4) w/out Calcium or Magnesium) and KCl (75 mM) in a 37°C incubator or water bath.
2. Collect items and place on bench:
   - pipette rack
   - 2-mL pipettes + pipettor
   - 10-mL pipettes + pipettor
   - test-tube rack
   - 5 centrifuge tubes
   - waste beaker with bleach
3. Label pipette rack and centrifuge tubes with sample information.
4. Set up 5 labelled 2-mL dedicated pipettes on the pipette rack, one for each sample.

Procedure:

Phosphate Buffer Wash:

1. Disinfect hands and retrieve the culture flasks from the incubator.
2. Drain off all liquid from the culture flasks with the designated pipettes.
3. Wash the cells in each cell once with 2 mL of Phosphate Buffer.
4. Cap the Phosphate Buffer and set it aside.

Trypsin Addition:

1. Rinse the cells **very quickly** with 2 mL of Trypsin (drain the Trypsin, as it takes too long to pipette out).
2. Dispense 1 mL of Trypsin into each culture flask and wait 5-6 minutes, periodically shaking flasks gently, until the cells separate from the bottoms of the flasks.
3. With the dedicated pipettes, aspirate the contents of each flask for approximately 20 rounds to separate the cells.
4. Using the same dedicated pipettes, transfer the entire contents of each flask into a labelled centrifuge tube. Throughout the procedure make sure that each sample is only handled with the appropriate designated pipette.

KCl Addition:

1. Very slowly add 7 mL of KCl solution to each centrifuge tube (**slowly drip** solution down the side of the tube).
2. Aspirate the contents of the centrifuge tubes very gently for about 20 rounds with the dedicated pipette).
3. Cap the centrifuge tubes and centrifuge at 3000 rpm for 3 minutes.
4. With the designated pipettes, drain off all but the bottom 0.5 mL of contents.
5. Because they have come into contact with the Trypsin, change all of the 2-mL designated pipettes for new ones.
6. Very slowly dispense 8 mL of KCl into each centrifuge tube.
7. Gently aspirate the contents of the centrifuge tubes 30+ rounds with the dedicated pipettes.
8. Cap the centrifuge tubes and place them on a test-tube rack.
9. Place the test-tube rack and sample in an incubator and incubate the samples for 25 minutes at 37°C.

During the 25 Minute Incubation:

1. Prepare the fixing reagent of 3:1 v/v methanol and glacial acetic acid; approximately 30 mL of fixing reagent is required per sample. The fixing reagent should be covered with Parafilm® when not in use.
2. Remove the microscope slides from the ethanol and place on tray lined with tissue paper to dry.

Addition of Fixing Reagent:

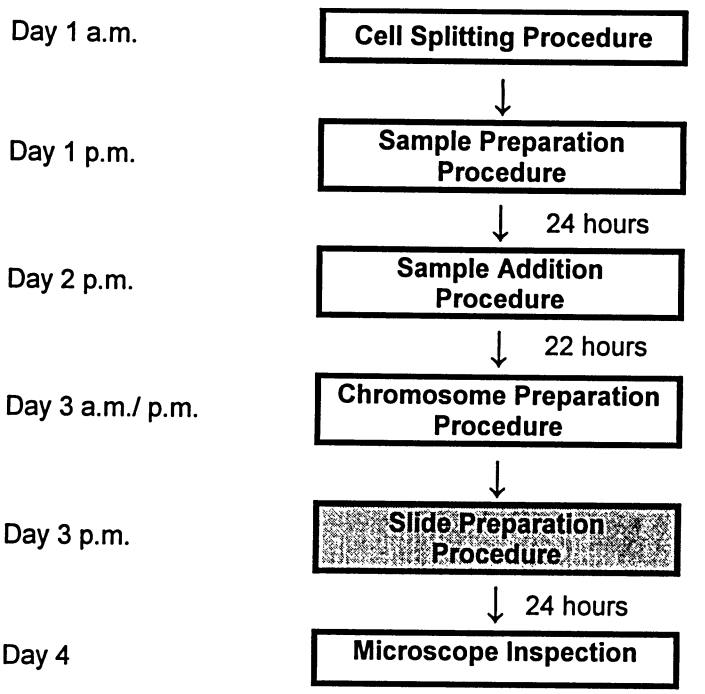
1. Take samples from the incubator and very gently aspirate each centrifuge tube 5-6 times with the designated pipette to resuspend cells.
2. Slowly add 2 mL of fixing reagent to the top of each sample.
3. Very gently aspirate the fixing reagent, which is lighter than the sample and will float on the top, into the bottom sample for 20-30 rounds.
4. Cap the centrifuge tubes and centrifuge at 3000 rpm for 3 minutes.

Final Cell Fixing:

1. Remove samples from the centrifuge and drain off all but the bottom 0.5 mL of contents with the designated pipettes.
2. Slowly add 8 mL of fixing reagent to each centrifuge tube.
3. Replace the 2-mL designated pipettes and very gently aspirate the fixing reagent into the bottom cells for 20-30 rounds.
4. Cap centrifuge tubes and centrifuge at 3000 rpm for 3 minutes.
5. Repeat the process THREE times, changing designated pipettes in each round. It is important to shake the fixing reagent prior to each addition.
6. In the last round, drain off all but the bottom 0.3-0.5 mL of contents using the designated pipettes.

Appendix E: Methods - Slide Preparation

During *Slide Preparation*, which immediately follows *Chromosome Preparation*, cells are plated onto slides, stained with Giemsa dye and mounted. Once the mounting medium has completely dried, the slides are ready for microscope inspection.



Methods – Slide Preparation

Slide Plating

1. During *Chromosome Preparation* the microscope slides were immersed in ethanol for approximately 3 hours and then allowed to air dry. Ensure that the slides are dry and line them up on tissue paper.
2. Label each slide in pencil with the following information:

    - pH value
    - Br^- concentration
    - Cl_2 concentration
    - Temperature
    - Date of Preparation

3. With a micro-pipette, very gently aspirate the cell sample in each centrifuge tube 2-3 times.

4. Place 4-6 drops of the cell sample onto each slide. It is advisable to plate each sample onto two slides (there should be just enough cell sample for 2 slides); this will act both as a safeguard and can be used to independently confirm chromosome aberration analysis.

5. Place the slide on the metal tray and allow the sample to dry completely (approximately 30 min.).

Cell Staining

1. Line up the glass slides on tissue paper on a flat surface.

2. Prepare 2% Giemsa dye by mixing 1 mL of Giemsa solution (7.415 g/L, Harleco®) and 49 mL of pH 6.8 Phosphate Buffer (Phosphate Buffered Saline (1X) 0. 0067 M (PO_4) w/out Calcium or Magnesium).

3. With a 1000-L micro-pipette, drop-wise apply a thick layer of dye onto each slide for adequate staining

4. Staining time is 20 minutes.

5. When finished staining, drain off the dye on the slides and rinse each slide very gently with 1 mL of DDW to remove excess stain.

6. Allow the slide to dry completely (approximately 1 hr.).

7. As Giemsa solution is highly flammable, the remaining Giemsa solution should be drained very slowly in the sink, followed by a lot of water for dilution.

Final Mounting

1. Inspect each slide closely to locate areas of high cell concentration

2. With a glass rod, place 1-2 drops of mounting medium (Permount®, Fisher Scientific) on top of 2 spots on each slide

3. Place a cover slip over each spot and allow the mounting medium to dry overnight prior to microscope inspection.

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