GENOTOXICITY INVESTIGATION OF ORGANIC

N-CHLORAMINES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Organic N-chloramines have long been recognised as disinfection by-products (DBPs) found in both chlorinated and chloraminated water, but have gained little attention from water authorities in the past. However, in recent years studies have shown that organic N-chloramines are molecules involved in inflammation and several chronic diseases including cancers. A recent study (Bull *et al.*, 2006) has suggested that organic N-chloramines can be potential health risks but due to a lack of available toxicological information toxicity studies of compounds in this group have been recommended as a priority in DBPs research.

The aim of this study was to investigate genotoxicity of individual organic N-chloramines utilising a mammalian cell-based genotoxicity assay to help determine which compound(s) should be subject to further *in vivo* studies. The flow cytometry-based micronucleus (FCMN) assay was optimised and validated for use as a rapid screening for genotoxicity of organic N-chloramine candidates. A number of assay validations were conducted on two mammalian cell lines (WIL2-NS and L5178Y) using model genotoxicants with various modes of action. Comparative studies on these two cell lines showed that WIL2-NS cells were suitable for the FCMN assay and therefore selected for use in all studies described in this thesis.

For the genotoxicity investigation of organic N-chloramines, 16 compounds were synthesised by chlorination of amine precursors. At least 3 concentrations (in μM range) were subjected to screening for genotoxicity using the validated FCMN assay and confirmed by microscopic counting of micronuclei. This study found that of the 16 compounds, 4 were genotoxic to WIL2-NS cells by both FCMN and microscopy based MN

assay. Oxidative stress was hypothesised as a possible genotoxic mechanism of these compounds and also was investigated in this study. Following exposure to the 4 genotoxic organic N-chloramines, it was found that although there was a small reduction of cellular glutathione the change in lipid peroxidation was not observed. This suggested that oxidative stress is unlikely to be a mechanism involved in genotoxicity of these organic N-chloramines.

The final part of this research demonstrated an application of using the optimised FCMN assay to identify genotoxic DBP precursors in Australian water. We collaborated with Curtin University, Western Australia on this aspect. Highly coloured surface water was collected, concentrated, and fractionated based on molecular weight (MW) of the organic contents by researchers at Curtin University. Eight MW fractions (pre- and post chlorination) were tested for genotoxicity using the FCMN assay. No genotoxicity was observed in all pre-chlorinated MW fractions while significant genotoxicity was seen in chlorinated products of several fractions of medium to high MW. This result indicated that these fractions contain materials that are precursors to genotoxic DBPs and may lead to future studies such as characterisation of the genotoxic DBP precursors for their removal prior to the disinfection process.

DECLARATION

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PUBLICATIONS IN SUPPORT THIS THESIS

Laingam, S., Froscio, S.M. and Humpage, A.R. (2008). Flow-cytometric Analysis of *in vitro* Micronucleus Formation: Comparative Studies with WIL2-NS Human Lymphoblastoid and L5178Y Mouse Lymphoma Cell Lines. *Mutatation Research*, 656 (1-2): 19-26.

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ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ATCC	American type culture collection
ADWG	Australian drinking water guidelines
AWQC	Australian water quality centre
BA	Bromoacetic acid
BaP	Benzo[a]pyrene
BrO_3^-	Bromate
$CHCl_3$	Chloroform
CHO	Chinese hamster ovary
CI	Confident interval
Cl_2	Chlorine
ClO_2	Chlorine dioxide
ClO_2^-	Chlorite
DBP(s)	Disinfection by-product(s)
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
DPD	N,N-diethyl-p-phenyl diamine
dsDNA	Double stranded deoxyribonucleic acid
DTNB	5, 5'′- dithiolbis-2-nitrobenzoic acid
EC30	Effective concentration at 30% of the untreated control
EC50	Effective concentration at 50% of the untreated control
ECL	Enterochromaffin-like
EDTA	Ethylenediaminetetraacetic acid

EPA	Environmental protection agency
ETOPO	Etoposide
FACS	Fluorescence-activated cell sorting
FAS	Ferrous ammonium sulphate
FBS	Foetal bovine serum
FCMN	Flow cytometry based micronucleus
$FeSO_4$	Ferrous sulphate
FSC	Forward scatter
GSH	Glutathione (Reduced form)
GSSG	Glutathione (Oxidised form)
H_2O_2	Hydrogenperoxide
HAAs	Haloacetic acids
HANs	Haloacetonitriles
HBSS	Hank balanced salt solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
HPSEC	High performance size exclusion chromatography
IARC	International agency for research on cancer
LOAELs	Lowest observed adverse effect levels
MDA	Malondialdehyde
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MN	Micronucleus
MW	Molecular weight

MX	3-chloro-4(dichloromethyl)-5-hydroxy-2(5H) furanone
N	Nucleus
NCl_3	Trichloramine
NCP	N-chloropiperidine
NDMA	N-nitrosodimethylamine
NH_2Cl	Monochloramine
NH_3	Ammonia
$NHCl_2$	Dichloramine
NOM	Natural organic matter
O_3	Ozone
OCl^-	Hypochlorite ion
OECD	Organization for economic co-operation and development
OR	Odds ratio
PBS	Phosphate buffered saline
PI	Propidium iodide
QSTR	Quantitative structure toxicity relationship
RO	Reverse osmosis
RPMI	Roswell park memorial institute medium
r	Spearman˚s coefficient
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Side scatter
SUCRO	Sucrose
TB	Trypan blue
TBARS	Thiobarbituric acid reactive substances

TDN	Total dissolved nitrogen
THMs	Trihalomethanes
US	The United States
VINB	Vinblastin
WHO	World health organization

1. GENERAL INTRODUCTION

For nearly a century, chlorine has been successfully used for the control of water-borne infectious diseases. Although consumption of chlorinated water has significantly reduced death and illness from water borne pathogens (Sadiq and Rodriguez, 2004), concern has been raised over the health risks associated with disinfection by-products (DBPs) that form during the chlorination processes . The DBP chloroform ($CHCl_3$) was first detected in chlorinated drinking water in the mid 1970s (Komulainen, 2004). Since then, several areas of DBP research have been conducted including DBP identification, toxicity and epidemiology studies, in order to determine the risks associated with DBP"s in drinking water.

Epidemiology studies have shown that there is a significant relationship between consumption of disinfected water and chronic adverse health effects, in particular an increased rate of bladder cancer among exposed populations. Toxicological investigations have focused on common DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs). Studies conducted *in vitro* and *in vivo* have shown that some of these types of DBPs are mutagenic and genotoxic; however, to date the data cannot provide a strong mechanistic link between such DBPs and formation of bladder cancer. Aside from THMs and HAAs it has been suggested that several hundred or perhaps thousands of other DBPs could be present in disinfected water.

In a recent study (Bull *et al.*, 2006) used quantitative structure toxicity relationship analysis (QSTR) to predict possible carcinogens and identified several groups of DBPs prioritised for further studies. These included haloquinones, halocyclopentenoic acids, nitrosamines, nitrosamides, halonitriles, halomines and organic N-haloamines. Of those

groups listed by Bull *et al*. (2006), although members of the organic N-haloamines (i.e. organic N-chloramines) were determined to be of concern, it was noted that currently there are insufficient toxicological data to make an informed judgment of their potential health risk when formed in drinking water.

This thesis describes *in vitro* toxicity and genotoxicity studies of organic N-chloramines. The thesis comprises 8 Chapters. This Chapter presents general introduction of relevant DBP research and literature reviews, while Chapter 2 describes background information on organic N-chloramines followed by the project rationale and specific aims. Chapter 3 explains the *in vitro* approach and assay validations for the study of cytotoxicity and genotoxicity of organic N-chloramines. The production of organic N-chloramines, their genotoxicity investigation and possible genotoxic mechanism are described in Chapters 4, 5 and 6 respectively. Chapter 7 shows an application of using the optimised cell based assay to help determine potential health risk of DBPs formed in Australian water and the overall research summary and final discussion is addressed in Chapter 8.

1.1.   Water Disinfection

The main purpose of disinfection is to reduce health risks from waterborne pathogenic organisms that cause human diseases. As a result, outbreaks of disease or death from waterborne pathogens of bacterial origin (e.g. cholera and typhoid) have declined dramatically since its introduction in the early 1900"s (Leclerc *et al.*, 2002).

The chemicals used for disinfection normally are substances that possess strong oxidative activity, which not only inactivate or remove the waterborne pathogens, but also provide other benefits when used in water treatment such as removing taste and odour causing-compounds and preventing biological regrowth in the water distribution system (Sadiq and Rodriguez, 2004).

A number of chemicals or processes can be used to disinfect water. Most drinking water produced from surface water supplies is currently disinfected with chlorine, or, to a much lesser extent, chloramine (a product of chlorine after reaction with ammonia), while chlorine dioxide, ozone and ultraviolet radiation have also been used to some extent (Tibbetts, 1995).

1.1.1.   Chlorine

Chlorine has been extensively used as a disinfectant in water industries because it is by far the cheapest means to disinfect water particularly in a large scale production. Chlorine has strong oxidising potential, which can kill a wide range of harmful water borne pathogens. When chlorine is added to water, a rapid hydrolysis takes place forming hypochlorous acid (HOCl) as shown in the equation 1 below. Depending on pH value, HOCl can partly degrade to hypochlorite ion (OCl$^-$) as shown in equation 2. Both HOCl and OCl$^-$ have very distinctive behaviour; HOCl is more reactive and is a stronger disinfectant than hypochlorite.

$$Cl2 + H_2O \rightarrow HOCl + H^+ + Cl^- \qquad \text{……. (1)}$$

$$HOCl \rightarrow H^+ + OCl^- \qquad \text{……. (2)}$$

Since chlorine is a strong oxidant that reacts not only with organisms but also other substances present in the water, the amount of chlorine required for an effective disinfection is dependent on quality of the water source. If the water contains high levels of substances such as organic materials, a higher amount of chlorine will be required. The term "break point chlorination" is the name given to the process where chlorine is added to water until the organic materials are totally oxidised. The excess chlorine added above this point will be present as free available chlorine. In general, after chlorination the chlorine residual in finished water is also maintained at low levels throughout the distribution system in order to protect against microbial recontamination.

1.1.2.   Chloramine

Chloramination is an alternative disinfection method using chloramine as a disinfectant. Chloramines are produced in the reaction between chlorine (Cl_2) and ammonia (NH_3). When ammonia is added to chlorine solution, 3 possible amine species i.e. monochloramine (NH_2Cl), dichloramine ($NHCl_2$) and trichloramine (NCl_3) can be formed depending on the pH of the reaction. Of these 3 amine species, monochloramine is the most desirable product used for water disinfection purposes. Monochloramine is the major product when the reaction takes place in a mild alkaline solution (eg pH 8.4). Reactions of chloramine in solution are shown in the equations below.

$$NH_3 + HOCl \rightarrow NH_2Cl + H_2O \qquad \text{……. (3)}$$

$$NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O \qquad \text{……. (4)}$$

$$NHCl_2 + HOCl \rightarrow NCl_3 + H_2O \qquad \text{……. (5)}$$
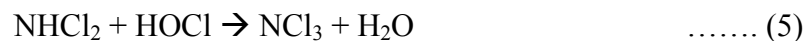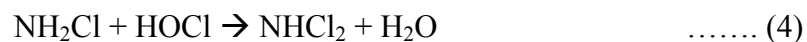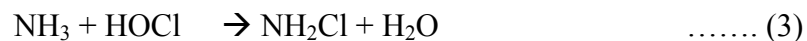
Although chloramines are weaker disinfectants than chlorine, they are more stable, hence providing benefits in extending disinfection efficiency throughout the water utility"s distribution system. It is recognised that the longer half life of chloramines also helps provide better protection against bacterial regrowth in systems with large storage tanks or dead-end water main and that chloramination reduced production rates of trihalomethanes (THMs) and haloacetic acids (HAAs), which are two common classes of DBPs regulated by health authorities. Another benefit of using chloramines for water disinfection is that drinking water produced from the plants using chloramine as a disinfectant has a rather pleasant taste and odour compared with those that use chlorine. For these reasons, chloramination has become a common method used in water industries, particularly in the

USA (Yoon and Jensen, 1996). Approximately 20% of US drinking water utilities have replaced chlorination with chloramination since 2002 (Sadiq and Rodriguez, 2004). In Australia, chloramination is mainly used for disinfection in long pipelines, where the longer half life is a advantage. However, a problem is that although chloramination can reduce formation rates of THMs and HAAs, it can increase the production of other N-containing DBPs, including N-chloramines.Chlorine dioxide

In recent years, interest has increased in the use of chlorine dioxide (ClO_2) as an alternative or in addition to chlorine, for water disinfection. Chlorine dioxide is a very effective bactericide and more effective than chlorine for the disinfection of viral contaminated water. It is a neutral chlorine compound, small and volatile molecule with a strong oxidative potency. The solubility of chlorine dioxide in water is very high (10 times more soluble in water than chlorine) especially at low temperatures (EPA, 1999). It does not hydrolyse when added into water, but remains as a dissolved gas in solution. For potable water application, chlorine dioxide is generated on site from reaction of sodium chlorite ($NaClO_2$) solution with gaseous chlorine (Cl_2), hypochlorous acid (HOCl) or hydrochloric acid (HCl) as shown in the equations below (EPA, 1999).

$$2NaClO_2 + Cl_2(g) \rightarrow 2ClO_2(g) + 2NaCl \qquad \text{……. (6)}$$

$$2NaClO_2 + HOCl \rightarrow 2ClO_2(g) + NaCl + NaOH \qquad \text{……. (7)}$$

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2(g) + 5NaCl + 2H_2O \qquad \text{……. (8)}$$
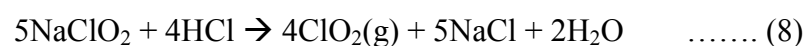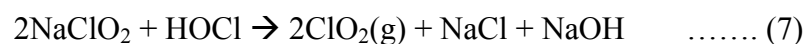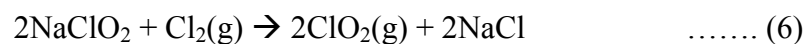
Advantages of using chlorine dioxide as a disinfectant include its efficacy in deactivating the chlorine-resistant pathogens, Giardia and Cryptosporidium (Korich *et al.*, 1990; Winiecka-Krusnell and Linder, 1998), the ability to remove and prevent biofilm formation, in addition to a reduction in unpleasant odour caused by phenolic compounds (Lalezary *et al.*, 1986). Also, compared with chlorine, chlorine dioxide is more effective for the removal of iron and manganese especially when these are found in complex compounds (Aieta and Berg, 1986).

Some disadvantages of using chlorine dioxide for water disinfection include its low stability because it can be easily decomposed when in contact with sunlight. Although chlorine dioxide is effective in deactivating Giardia, Cryptosporidium and some viruses, it is less effective for deactivation of rotaviruses and E. coli (Betancourt and Rose, 2004; De Luca *et al.*, 2008; Pereira *et al.*, 2008). In addition, water disinfection using chlorine dioxide is very expensive (i.e. 5 to 10 times higher cost) than chlorine (White, 1999).

1.1.3.   Ozone

Ozone (O_3) is the most powerful oxidant amongst all available disinfectants used in water treatment, and has been used for water disinfection purposes for a long period of time (von Gunten, 2003). O_3 is produced by an ozone generator by a conversion of O_2 using high voltage power (6,000-20,000V). O_3 is a very unstable molecule that can be reverted to O_2 shortly after its formation. Therefore it must be generated on-site and cannot be shipped to the water treatment plant.

Although ozone is a very effective disinfectant and has an excellent property in controlling odour and taste making ozonated water more palatable compared with other disinfected waters, ozonation is not a very popular method used in water utilities. It is by far the most expensive disinfection method and its application is not practical for a large scale drinking water production. Furthermore, due to its short life, ozone has a very short contact time to water (around 5 minutes), which may not be enough to provide an effective disinfection. In addition, not only does ozone not produce a disinfection residual, but it also can convert non-biodegradable organic matter to biodegradable compounds, hence providing additional nutrient in the distribution systems. Therefore, bacterial re-growth can be readily found in the system using only ozonation for water disinfection process (Alonso *et al.*, 2004)

1.2. Disinfection By-products (DBPs)

Disinfection by-products are formed by the non-specific reaction of disinfectant with natural organic matter (NOM) or bromide in water to produce various organic and inorganic compounds. Types of DBPs formed in disinfected waters are varied, dependent on many factors such as the composition of NOM, choices of disinfectant and their dosing rate, the presence of ions (i.e. bromide or iodide), temperature and pH of the water. To date, approximately 600 DBPs have been identified in disinfected drinking waters (Richardson, 2003). These DBPs have been categorised into many classes based on their molecular structures, formation and chemical properties. Some currently known DBPs and their occurrence in disinfected waters (mostly from chlorination) are described here and also summarised in Table 1-1 (page 13).

1.2.1.   Trihalomethanes

Trihalomethanes (THMs) were the first group of DBPs identified in chlorinated drinking water and have been well studied. Trichloromethanes are formed from reactions between chlorine and organic matter (NOM). When bromine is present in the water, tribromomethanes are also likely to form. THMs consist of 4 chemicals; chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), dibromochloromethane ($CHBr_2Cl$) and bromoform or tribromomethane ($CHBr_3$). Total THMs is a collective term representing the four members and traditionally have been used as surrogate markers for monitoring of DBP formation in water.

The US Environmental Protection Agency (EPA, 2001) has published the "Stage 1 Disinfectants/Disinfection Byproducts Rules" to regulate total THMs at a maximum allowable annual average level of 80 µg/L, while the WHO (2008) has a separate standard for individual members of the THMs i.e. 60 µg/L for bromodichloromethane, 100 µg/L for bromoform, 200 µg/L for chloroform. In Australia, the guideline level of THMs is set at 250 µg/L (NHMRC/ARMCANZ, 2004).

1.2.2.   Haloacetic acids

Haloacetic acids (HAAs) are another group of DBPs, mostly produced in chlorinated water. HAAs comprise 9 members (HAA9), but only 5 compounds (called HAA5) are regulated by health authorities, which include 3 chlorinated acetic acids (mono, di and trichloroacetic acids), and 2 brominated acetic acids (mono and dibromoacetic acids). HAAs are non-volatile compounds and can occasionally be found in water at higher

concentrations than THMs, depending on the pH value of the water. For example, when the pH is low, HAAs are preferably formed and when the pH is high THMs are the predominant products. As with the THMs, composition of NOM in the water source determines the amount of HAAs (Chang *et al.*, 2009; Chellam and Krasner, 2001; Serodes *et al.*, 2003; Uyak *et al.*, 2008).

HAA levels are also monitored in disinfected drinking water. The US EPA has established maximum contaminant levels of HAAs (or HAA5) of 60 µg/l (EPA, 1998), while WHO has set individual guideline value of 50 µg/L for dichloroacetic acid and 200 µg/L for trichloroacetic acid (WHO, 2004). In Australia, the guideline for monochloroacetic acid is 150 µg/L and for trichloroacetic is 100 µg/L (NHMRC/ARMCANZ, 2004).

1.2.3. Bromate

Bromate (BrO_3^-) is formed mainly in ozonated water when the source waters contain high levels of natural bromide. Formation of bromate can also be found in water disinfected by chlorine dioxide, particularly when the disinfection with chlorine dioxide is conducted in the presence of sunlight (Gordon *et al.*, 1990). Bromate can be found as a contaminant from the use of hypochlorite solution instead of gaseous chlorine (Bolyard *et al.*, 1992). In ozonated water, bromate can be detected at concentrations up to 25.1 µg/L, while in chlorine dioxide treated water significantly lower concentrations are usually seen. In the United States, the regulated concentration of bromate is set at 10 µg/L (WHO, 2008). In Australia, the guideline is set at 20 µg/L (NHMRC/ARMCANZ, 2004).

1.2.4.   Chlorite

Chlorite (ClO_2^-) is a common DBP produced in water treated with chlorine dioxide. Chlorite is a degradation product of chlorine dioxide when in contact with naturally occurring organic and inorganic matter. Levels of chlorite can be varied between 30% and 70% of the chlorine dioxide dose, depending on several factors such as oxidant demand, temperature, competitive side reactions with other chemicals or processes and generator efficiency. In the United States, chlorite is currently regulated at 1.0 mg/L (Richardson *et al.*, 2007). In Australia, the guideline value is set at 3.0 mg/L (NHMRC/ARMCANZ, 2004).

1.2.5.   Haloacetonitrile

Haloacetonitriles (HANs) is a group of DBPs consisting of dichloroacetonitrile, bromoacetronitrile, bromochloroacetonitrile, dibromoacetonitrile and tribromoacetonitrile. HANs are usually formed immediately during water disinfection, but decomposed quickly during hydrolysis reaction or reactions with residual disinfectants. The levels of HANs present in water are normally lower than THMs and HAAs. The guideline set by WHO (2008) for dichloroacetonitrile is 90 μg/L and for trichloroacetonitrile is 1 μg/L.

1.2.6. Halofuranones

The main DBP in this class is MX (3-chloro-4(dichloromethyl)-5-hydroxy-2(5H) furanone. Other analogues of MX such as its geometric isomer (EMX), as well as brominated analogues (BMXs) have also been identified in disinfected water. MX was first identified as a chlorination by-product in pulp mill effluent (Holmbom *et al.*, 1984) and was later found in chlorinated drinking water. It has gained interest in recent decades because it is the most potent mutagenic DBP in bacterial *in vitro* genotoxicity assays compared with other known DBP present in chlorinated water (Kronberg *et al.*, 1988).

Levels of MX found in disinfected water have been reported in ng/L concentration range. As high as 80 ng/L of MX was reported in Massachusetts, USA (Wright *et al.*, 2002), while a nationwide study of MX in the United State reported > 100 ng/L (as high as 850 ng/L) was found in many US water utilities (Weinberg *et al.*, 2002). However, due to a lack of toxicological information, there is no guideline for MX at present.

Table 1-1. Summary of DPBs and their occurrence in disinfected water

Regulated DBPs	High µg/L	Low µg/L	ng/L	Other
THMs				
Chloroform	√			
Bromodichlormethane	√			
Chlorodibromomethane	√			
Bromoform	√			
HAAs				
Chloroacetic acid		√		
Bromoacetic acid		√		
Dichloroacetic acid	√			
Dibromoacetic acid	√			
Trichloracetic acid	√			
Oxyhalides				
Bromate		√		
Chlorite	√			

Unregulated DBPs	High µg/L	Low µg/L	ng/L	Other
Halomethanes				
Chloronitromethane			√	
Bromonitromethane			√	
Dichloronitromethane			√	
Dibromonitromethane		√		
Bromochloronitromethane			√	
Trichloronitromethane		√		
Bromodichloronitromethane		√		
Dibromochloronitromethane		√		
Tribromonitromethane		√		
Iodo-acids				
Iodoacetic acid		√		
Bromoiodoacetic acid		√		
(*Z*)-3-Bromo-3-iodopropenic acid			√	
(*E*)-3-Bromo-3-iodopropenic acid			√	
2-Iodo-3-methlybutenedioic acid		√		
Other halo-acids				
Bromochloroacetic acid		√		
Bromodichloroacetic acid		√		
Dibromochloroacetic acid		√		
Tribromoacetic acid		√		
Iodo-THMs and other unregulated THMs				
Dichloroiodomethane		√		
Bromochloroiodomethane		√		
Dibromoiodomethane		√		
Chlorodiiodomethane		√		
Bromodiiodomethane		√		
Iodoform		√		
Dichloromethane		√		
Bromochloromethane				ND
Dibromomethane				ND

Data modified from (Richardson *et al.*, 2007).
ND = non-detect. NA = no occurrence data available.

Table 1-1. (continued)

Unregulated DBPs	High µg/L	Low µg/L	ng/L	Other
MX compounds				
MX			√	
EMX			√	
ZMX			√	
BMX-1			√	
BMX-2			√	
BMX-3			√	
BEMX-1			√	
BEMX-2			√	
BEMX-3			√	
Haloamides				
Chloroacetamide		√		
Bromoacetamide		√		
Iodoacetamide				NA
Dichloroacetamide		√		
Bromochloroacetamide		√		
Dibromoacetamide		√		
Bromoiodoacetamide		√		
Trichloroacetamide		√		
Bromodichloroacetamide		√		
Dibromochloroacetamide		√		
Tribromoacetamide		√		
Diiodoacetamide				NA
Chloroiodoacetamide				NA
Haloacetonitrile				
Chloroacetonitrile		√		
Bromoacetonitrile		√		
Iodoacetonitrile				NA
Dichloroacetonitrile		√		
Bromochloroacetonitrile		√		
Dibromoacetonitrile		√		
Trichloroacetonitrile		√		
Bromodichloroacetonitrile		√		
Dibromochloroacetonitrile		√		
Tribromoacetonitrile		√		
Halopyrroles				
2,3,5-Tribromopyrrole			√	
Nitrosamines				
N-nitrosodimethylamine			√	
N-nitrosopyrrolidine			√	
N-nitrosomorpholine			√	
N-nitrosopiperidine			√	
N-nitrosodiphenylamine			√	
Aldehydes				
Formaldehyde		√		
Acetaldehyde		√		
Chloroacetaldehyde		√		
Dichloroacetaldehyde		√		
Bromochloroacetaldehyde		√		
Trichloroacetaldehyde (chloral hydrate)		√		
Tribromoacetaldehyde		√		

1.2.7.   Emerging DBPs

Apart from the regulated DBPs, it has been speculated that a large portion of DBPs have not been identified (Figure 1.1). The ongoing concern that the types of cancer found in animal studies following exposure to the known DBPs is not correlated with the findings from human epidemiological studies (Bull *et al.*, 2006; Richardson, 2003; Richardson *et al.*, 2007), suggested that the new DBPs that have not yet been identified may link to the effects seen in humans.
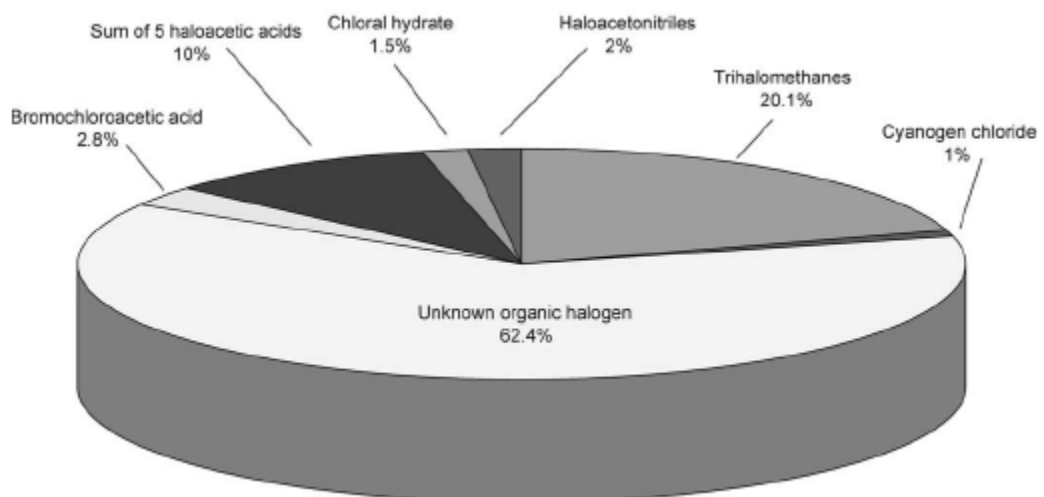
Figure 1.1. Halogenated DBPs as proportionate to total organic halogen.

Relative amounts of halogenated DBPs as proportionated to total organic halogen (TOX) in chlorinated drinking water. The diagram shows representative data of a treatment plant in the United States (Richardson, 2003).

The change in disinfection practices to avoid formation of THMs does not necessarily mean the disinfected water is safe for consumption. Instead, the more harmful DBPs may be produced (Muellner *et al.*, 2007). For example, although replacing chlorination with chloramination has caused a significant reduction of THMs in the finished water, chloramination has introduced the formation of nitrogen-containing DBPs (Nissinen *et al.*, 2002). (i.e. NDMA). The presence of NDMA (N-nitrosodimethylamine) in drinking water has caused a greater health concern as NDMA is known to be a potent carcinogen (Choi and Valentine, 2002; Najm and Trussell, 2001).

1.3. Health Risks Associated With DBPs

The detection of chloroform in disinfected drinking water in the 1970's (Bellar *et al.*, 1974; Rook, 1974) prompted epidemiology based-studies to determine whether there was an association between exposure to disinfected drinking water and adverse health effects. Most epidemiological studies in early years have focused on outcomes of long term exposure to disinfected drinking water and incidences of various kinds of cancer, particularly bladder cancer. Moreover, in recent years, many studies have suggested that exposure to disinfected water may be associated with adverse reproductive effects and developmental toxicity and child birth defects.

1.3.1. DBPs and Bladder Cancer

Whilst studies have shown that DBPs may have been associated with an increased incidence of various types of cancer such as rectal (Koivusalo *et al.*, 1997), colon (Doyle *et al.*, 1997), kidney (Koivusalo *et al.*, 1998) as well as certain types of leukaemia (Infante-Rivard *et al.*, 2002), the majority of previous epidemiological studies have found a strong

link between an increased incidence of bladder cancer and long term exposure to disinfected drinking water (Cantor *et al.*, 1987; King and Marrett, 1996; Koivusalo *et al.*, 1998; McGeehin *et al.*, 1993; Villanueva *et al.*, 2004; Zierler *et al.*, 1988).

A meta-analysis conducted from a pool of epidemiological studies (6,084 bladder cancer cases and 10,816 controls from 6 case-control studies, and 24 bladder cancer cases from 2 cohort studies) of populations in Europe and North America (Villanueva *et al.*, 2003) has shown that there is an association between long term consumption of chlorinated drinking water and bladder cancer, particularly in men [Odds ratio (OR) = 1.4; 95% Confident Interval (CI) = 1.1 – 1.9]. Although the observed relative risk was found to be relatively low, it was suggested that the population distributable risk could be very important as the vast majority of the population of industrialised countries are potentially exposed to chlorinated by-products for a long period of time.

A recent study (Villanueva *et al.*, 2004) has strengthened the hypothesis that the risk of bladder cancer is increased with long-term exposure to disinfection by-products. This study was conducted from analyses of pooled data collated from 6 case-control studies. Of the 6 studies, 2 were from the United States (Cantor *et al.*, 1998; Lynch *et al.*, 1989) and one each from Canada (King and Marrett, 1996), France (Cordier *et al.*, 1993), Italy (Porru *et al.*, 1996) and Finland (Koivusalo *et al.*, 1998). All 6 studies provided data on THM exposure (THMs was used as a marker of DBPs) and individual water consumption. A pool of 2806 cases and 5254 controls was analysed. There was a significant increase in bladder cancer in men exposed to an average of more than 1 μg/L THMs compared with those who had lower or no exposure [OR = 1.24; 95% CI = 1.09 – 1.41]. The risk was reported to be higher in those exposed to higher than 50 μg/L THMs [OR = 1.44; 95% CI = 1.20 – 1.73). In women, it was found that THMs exposure was not

associated with bladder cancer risk [OR = 0.95; CI = 0.76 – 1.20). This suggested a significant increase of bladder cancer risk in men associated with long-term exposure to DBPs at levels currently observed in many industrialised countries.

1.3.2. DBPs and Adverse Reproductive Outcomes

Aside from bladder cancer risk, in recent years there have been a number of reports on the association between DBP exposure and adverse reproductive outcomes. These include low birth weight, preterm delivery, spontaneous abortions, stillbirth (Bove *et al.*, 2002; Magnus *et al.*, 1999; Nieuwenhuijsen *et al.*, 2000) and birth defects, mainly central nervous system, respiratory, major cardiac, oral cleft (Dodds and King, 2001; Nieuwenhuijsen *et al.*, 2000), neural tube defects (Bove *et al.*, 2002; Nieuwenhuijsen *et al.*, 2000) and small for gestational age (Bove *et al.*, 2002).

Although many studies have suggested that exposure to DBPs is a factor for adverse reproductive outcomes, some inconsistent findings have been reported. A study by (Graves *et al.*, 2001) showed that there was no association with DBP exposure for several outcomes including low birth weight, preterm delivery, some specific congenital anomalies, and neonatal death. Inconsistent or very weak results for all congenital birth defects, all central nervous system anomalies, neural tube defects, spontaneous abortion and stillbirth were also reported (Nieuwenhuijsen *et al.*, 2000; Tardiff *et al.*, 2006). A pooled analysis of 5 epidemiological studies conducted during 1966 – 2001 also showed that the effects on respiratory system, major cardiac and oral cleft defects are heterogenous and inconclusive (Hwang and Jaakkola, 2003; Magnus *et al.*, 1999).

Various epidemiological studies pointed towards an association between THMs and low birth weight (Bove *et al.*, 2002; Dodds and King, 2001; Nieuwenhuijsen *et al.*, 2008; Wright *et al.*, 2004) but the evidence is inconclusive. There also is no evidence for an association between THMs and preterm delivery. Only moderate evidence for association with neural tube defects and spontaneous abortion was reported (Bove *et al.*, 2002). It is also suggested that another main limitation of most studies has been the relatively crude methodology, for the assessment of exposure in particular (Nieuwenhuijsen *et al.*, 2000), hence the overall evidence showing an association between THMs exposure and birth defects remain inconclusive (Dodds and King, 2001; Nieuwenhuijsen *et al.*, 2000)

At this point of time, although there have been some inconsistencies of the result findings from the literature, many studies have shown that the risks of DBPs associated with adverse reproductive/developmental toxicity effects may exist. However, with the available information to date, it is very difficult to confirm or deny the results observed from epidemiological studies. Firstly, many of the epidemiological links connecting DBPs to adverse health effects are tenuous as they are very difficult to prove (Smith, 2007). Secondly, DBPs in water do not appear as isolated compounds and normally manifest themselves as complex mixtures. Therefore, using only THMs as a marker for DBP loading may be acceptable to help estimate levels of exposure, but it does not necessary mean that the outcomes observed would reflect the final outcomes seen in the epidemiology. To better understand the effects of DBPs and health effects more toxicological studies of individual DBPs or mixtures of DBPs as well as studies on determination of the presence of individual DBPs in a water supply, the concentrations of each and their temporal and spatial variance to help predict possible additive, synergistic or antagonistic effects of the mixture would be required.

1.4. Toxicological Studies of DBPs

Along with extensive epidemiological studies, a large number of toxicological tools (i.e. mutagenicity, genotoxicity and carcinogenicity assays) have been used to help determine the carcinogenic risks of DBPs. Mutagenicity assays measure a change in DNA sequence (arising from gene mutation) while genotoxicity assays are used to determine damage that occurs at either the DNA or chromosome level.

Over the past 30 years, DBP toxicity research has focussed intensively on THMs. Chloroform is the main THM product and a great deal has been reported on its toxicity and carcinogenicity. A review of many *in vitro* and *in vivo* mutagenicity and genotoxicity studies demonstrates that chloroform is not mutagenic nor genotoxic, but it is carcinogenic in rodents causing tumours at various sites such as liver, kidney and intestine (IARC, 1999) It is also reported that chloroform and the other regulated THMs do not induce bladder cancer in animal studies. This indicates that THMs themselves may not be associated with the bladder cancer risks described in the epidemiological studies.

Another group of DBPs that have been extensively studied is HAAs. All 5 members of HAAs have been found to be mutagenic in bacteria and genotoxic in mammalian cells (IARC, 2004). The cytotoxicity and genotoxicity potencies of the brominated forms are higher than the chlorinated forms (Giller *et al.*, 1997; Plewa *et al.*, 2002). Bromoacetic acid has not been tested for carcinogenicity, while chloroacetic acid gave negative results in a carcinogenicity test in rodents. On the other hand, the remaining 3 members of the HAAs (dibromoacetic, dichloroacetic and trichloroacetic acids) have been shown to be

carcinogenic, producing liver tumours in mice, and leukemias and abdominal cavity mesotheliomas in rats (DeAngelo *et al.*, 1997).

Other than THMs and HAAs, a large number of mutagenicity and genotoxicity studies, and some carcinogenicity studies, of known/unregulated DBPs have been conducted. More details and a summary of these toxicity studies can be viewed in an article published by Richardson *et al.* (2007).

Although a wide range of data on DBPs exist, the current toxicological information does not support the epidemiological findings. For example, most of DBPs tested for carcinogenicity in animal models cause primarily liver cancer rather than bladder cancer (IARC, 1995; IARC, 1999; IARC, 2004), while the epidemiological studies have found an association between exposure to disinfected water and bladder cancer risks (Villanueva *et al.*, 2003). This lack of correlation between sites of tumours in animal cancer studies and human epidemiological studies using drinking is not well understood. In addition, the fact that bladder cancer risk in human has been associated with THM levels (King and Marrett, 1996; Villanueva *et al.*, 2007), while THMs themselves are not bladder carcinogens (IARC, 1999) suggests that the causative DBPs are yet to be identified.

1.5. Early Prioritisation Studies of DBP Research

Aside from the regulated DBPs, there is very little or no data on occurrence and or toxicity of other DBPs. Literature has suggested that over 600 compounds may form during the water disinfection processes (Richardson *et al.*, 1999) depending on the NOM content of the source water and the method of disinfection used. Given that it is both impractical

and uneconomical to conduct studies on all DBPs reported in the literature, prioritisation on which DBPs are to be tested is necessary.

The early prioritisation by US EPA aimed to create a list of DBPs for further research. Known DBPs were selected for prioritisation based on predictions of adverse health effects (cancer) by multidisciplinary expert panels, including toxicologists, structure-activity specialists and chemists (Woo *et al.*, 2002). The expert panel considered three main factors 1) the occurrence of the DBPs in drinking water, 2) the strength of the available toxicity data and 3) similarity of the DBPs to other carcinogenic compounds. Additionally, where toxicity data for a chemical was unavailable, computer based QSTR (qualitative structure-toxicity relationships) was used to assist the process based on the chemical"s molecular attributes. The study suggested approximately 50 DBPs of high priority, including brominated, chlorinated and iodinated species of halomethanes, brominated and chlorinated forms of haloacetonitriles, haloketones, haloacids, and halonitromethanes as well as analogs of MX for further occurrence studies.

Following the prioritisation of DBPs for further research, a US Nationwide DBP Occurrence study was conducted (Weinberg *et al.*, 2002). This study was of particular importance as it confirmed that the DBPs identified were commonly present in US drinking water. Another significant outcome taking place at this period of time was that a number of emerging DBPs were discovered (i.e. iodo-acids were identified for the first time (Plewa *et al.*, 2004). This has also led to a significant input in research on improving analytical technology and assay development.

The Nationwide DBP occurrence survey also showed that changes in disinfection practices to minimise the levels of regulated THMs resulted in the formation of various other types of DBPs. For example, iodo-THMs were found to be at highest concentration in a plant using chloramination. Dihaloaldehydes were highest at a plant using chloramines and ozone, while MX and BMXs were found to be at highest concentrations in plants that treated waters high in NOM and bromide using chlorine dioxide followed by chlorine (Krasner *et al.*, 2002; Weinberg *et al.*, 2002).

A great concern has also been raised in relation to chloraminated DBPs, particularly when NDMA was identified in chloraminated water (Tomkins and Griest, 1996). NDMA, a member of the N-nitrosamine group of nitrogenous carcinogens, has previously been most commonly found in food and cigarette smoke (Jakszyn *et al.*, 2006; Tricker *et al.*, 1991). NDMA is only one of many nitrogenous compounds, so its presence in disinfected water has indicated that other nitrogenous DBPs may also be present in water too. These new emerging DBPs may be responsible for adverse health effects, therefore should be taken into consideration for the subsequent prioritisation.

1.6.   Recent Prioritisation Study of DBP Research

In 2006, Bull *et al*. (2006) reported outcomes of a prioritisation study for DBP research using toxicological and chemical models. Nitrogenous DBPs (i.e. nitrosamines, organic N-chloramines) were included in this prioritisation study.

QSTR/ TOPKAT® was the main approach used for the analyses. TOPKAT® is a QSTR model that has proven useful and has been used in the examination of pharmaceutical and toxicological data. TOPKAT® was selected in the study because 1) it has a set of QSTR models that predict chronic lowest observed adverse effect levels (LOAELs), 2) it has databases for predicting carcinogenicity, and 3) it includes a developmental toxicity potential model and an Ames‟ mutagenicity model.

Two approaches were used in this prioritisation study. The first approach called "Feed-back process" involved analyses of the carcinogenic chemicals listed in the Carcinogen Potency Database (CPDB[1]) including those that have been shown to cause bladder cancer in one or more species including humans. The results of these analyses showed that the DBPs that might be formed from NOM that are most closely related to known bladder carcinogens were aromatic amines, heterocyclic amines, nitrosamines and quinones. Of these, amines were found to be very common among bladder carcinogens. The second approach is called "Feed-forward process", which involved two fundamental steps: 1) identification of significant molecular sub-structures of NOM and 2) prediction of

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[1] The CPDB is an online database summarising most of the cancer bioassays that have been conducted on chemicals in experimental animals. The website (http://potency.berkeley.edu/cpdb.html) is maintained by Gold and coworkers at the University of California, Berkeley.

probable DBPs from those structures through a combination of laboratory model compound studies and mechanistic organic chemistry.

The outcome of this study has suggested several DBPs of concern. These include 1) Haloquinones, 2) Halocyclopentenoic acids, 3) Organic N-chloramines, 4) Nitrosamines and nitrosamides and 5) Halonitriles and haloamides. It was noted that although organic N-chloramines were predicted as potential candidates for bladder carcinogens, there are insufficient toxicological data related to these compounds to inform judgments for individual members of the class. To be able to investigate further and to strengthen the prediction using QSTR/TOPKAT® model, more basic experimental toxicity and genotoxicity studies of organic N-chloramines are required. More details of the organic N-chloramines and their related literature reviews are discussed in the next Chapter.

2.   ORGANIC N-CHLORAMINES AND RESEARCH AIMS

Organic N-chloramines have long been recognised as potential health hazards in medicals and pharmaceuticals. It is also known that organic N-chloramines can form in water by the reaction of chlorine with primary or secondary amines - including α- amino acids and polypeptides, which are abundant in natural surface waters and municipal wastewater (Bull *et al.*, 2006). These compounds are both reactive, and have sufficient stability to be absorbed and delivered to target organs. The question has been raised as to whether these compounds could be causative of the chronic health effects associated with long term exposure to disinfected water as observed in epidemiological studies. This Chapter reviews literature on organic N-chloramines and related studies including several summary points suggested by a recent Quantitative Structure-Toxicity Relationship (QSTR) report by Bull *et al.* (2006). The project rationale, hypothesis and specific aims of this research are then detailed.

2.1.   Organic Nitrogen in Natural Water

The main precursors of organic N-chloramines are organic nitrogenous substances, which are essential elements found ubiquitously in natural aquatic systems. Dissolved organic nitrogen (DON) is the main component of the total dissolved nitrogen (TDN) in natural water, representing only a small fraction (0.5 – 10%) of the mass of NOM, while the major component of NOM is organic carbon (30 – 50%). Given organic carbon as the main composition of NOM, a large number of studies have focussed on various aspects of DOC, while relatively little is known about its nitrogenous fraction (Westerhoff and Mash, 2002).

Levels of organic nitrogen in water supplies can be substantially varied by location (Caraco and Cole, 2002) or season (Westerhoff and Mash, 2002). For example, in South-eastern US rivers, DON concentrations ranged between 0.42 – 1.08 mg N/L (Alberts and Takacs, 1999). In Florida, USA, up to 2.5 mg N/L of DON was determined in water collected from Lake Apopka (Tuschall and Brezonik, 1980). A study of 20 forested watersheds in Sweden and Finland demonstrated DON concentration range from 0.2 to 1.2 mg N/L (Arheimer *et al.*, 1996). At present, there is no published data on the prevalence of individual amino acids in natural source water. Only available information was obtained from a prediction by D. Reckhow (personal communication, unpublished data) as shown in Table 2-1.

Table 2-1. Levels of free amino acids in natural water source

Amino Acid	Abundance*
Glycine	11.70%
Alanine	11.70%
Valine	5.20%
Isoleucine	4.40%
Leucine	5.60%
Serine	10.80%
Threonine	7.00%
Methionine	1.00%
Aspartic Acid	10.20%
Glutamic Acid	10.00%
Lysine	1.60%
Ornithine	2.00%
Arginine	7.40%
Histidine	2.50%
Asparagine	0.40%
Glitamine	0.50%
Tryptophan	2.50%
Phenylalanine	3.40%
Tyrosine	2.20%

* Based on calculation of amino acid nitrogen/DOC ranges from Thomas (1997) and Bronk (2002). Data adopted from D. Reckhow (personal communication).

In Australia, very few studies of water quality, particularly in terms of providing information on organic nitrogen have been published in international journals (Harris, 2001). Only a summary report reviewing the export of total nitrogen and total phosphorous from an Australian catchment (Murray-Darling Basin) was published by Young *et al.* (1996). However, due to relatively low annual rainfall and a significant amount of land used for agricultural/farming purposes, it is strongly believed that the organic nitrogen content in Australian aquatic systems would be very high. Furthermore, since organic nitrogen can be released from algal cells (Fogg, 1971; Nagao and Miyazaki, 2002), frequent algal blooms in Australian waters may play a significant role in raising the levels of DON in natural water bodies.

2.2. Water Disinfection and Formation of Organic N-Chloramines

During water disinfections, organic N-chloramines are formed in the presence of chlorine or chloramines when primary or secondary amines are present in the source water (Mitch and Sedlak, 2002). In chlorinated water, the formation of organic N-chloramines in aqueous medium takes place rapidly by the transfer of chlorine from hypochlorite (HOCl) to the nitrogen of the amine (Donnermair and Blatchley, 2003). Two predominant products formed are monochloramines ($RNHCl$) and dichloramines ($RNCl_2$) as shown in reactions (1) and (2). In chloraminated water, organic N-chloramines are formed by a reaction between monochloramine (NH_2Cl) and organic amine (RNH_2) as shown in reaction (3). The formation of organic N-chloramines in disinfected water has been reported to be temperature and pH dependent (Deinzer *et al.*, 1978).

$$RNH_2 + HOCl \quad \rightarrow RNHCl + H_2O \qquad \text{.......} (1)$$

$$RNHCl + HOCl \quad \rightarrow RNCl_2 + H2O \qquad \text{.......} (2)$$

$$RNH_2 + NH_2Cl \quad \rightarrow RNHCl + NH_3 \qquad \text{.......} (3)$$
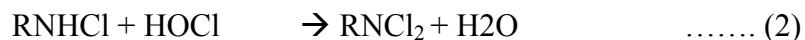
Previously, organic N-chloramines have been classified as "nuisance" compounds in water treatment industries because they exert a chlorine demand, reducing the disinfection activity of chlorine or chloramines. However, in recent years organic N-chloramines have gained attentions from health authorities as potential key molecules for chronic health effects as shown in biomedical literature (Barua *et al.*, 2001; Bernofsky, 1991; Davies *et al.*, 1993; Englert and Shacter, 2002; Kawai *et al.*, 2004; Marcinkiewicz, 1997; Midwinter *et al.*, 2004; Schuller-Levis and Park, 2004). While they have received attention in research community, there is a lack of toxicological studies of compounds in this group. Some reviews of published toxicological studies are addressed below.

2.3.   Toxicological Significance of Organic N-chloramines

Organic N-chloramines were first suggested as potential carcinogens in the 1980s (Scully and Bempong, 1982). Since that time, there have been very limited studies on chemistry and the occurrence of these substances in drinking water. Only a few of organic N-chloramines have been assessed toxicologically. One problem with the toxicological assessment of these compounds is that organic N-chloramines are thermally labile: many cannot be generated and isolated as a pure compound and as a result, very few compounds have been synthesised and tested for toxicity.

One compound, N-chloropiperidine (NCP) has been successfully synthesised and purified and thus often used as a model compound for organic N-chloramines. In bacteria, NCP has been shown to be a mutagen in Salmonella typhimurium strain TA 100 (Bempong and Scully, 1980). In Chinese hamster ovary (CHO) cells, NCP showed strong cytostatic and cytotoxic effects, suggesting that NCP may interfere with normal chemical cytogenetic programming of the cells leading to anomalous chromosome separation and nuclear distribution. In addition, it was shown that NCP could induce structural chromosome aberrations (breaks, exchanges, fragments, ring chromosomes and centromeric errors). The responses measured were found to be concentration-dependent (Scully and Bempong, 1982).

Apart from the NCP, most toxicological investigations of organic N-chloramines have used freshly produced compounds without purification for the studies. For example, Süssmuth (1982) conducted studies using a number of bacterial assays and found that chlorinated products of methionine, tyrosine, phenylalanine, cysteine and glycine were mutagenic in bacteria, while the parent compounds (no-chlorination) were non-mutagenic. Similarly, Nakamura *et al*. (1993) reported that chlorinated products of arginine, glycine, histidine, hydroxyproline, lysine, methionine, phenylalanine, proline, serine and threonine were also shown to have mutagenic effects in bacterial assays. The mutagenicity of these organic N-chloramines was observed without using metabolic activation system, which indicated that the organic N-chloramines are direct acting mutagens.

There has been very limited information in terms of *in vivo* toxicological studies of organic N-chloramines and there are no data that demonstrate carcinogenic effects of organic N-chloramines in experimental animals (Bull *et al.*, 2006). Aside from exposure to organic N-chloramines via disinfected drinking water, studies have shown that the

formation of organic N-chloramines can occur *in vivo*. Scully *et al.* (1985) reported that organic N-chloramines (eg. N-chloroglycine and N-choropiperidine) could be formed in stomach of rat following successive administration of the parent amines and chlorine. It was also reported that organic N-chloramines could also be formed when mixing gastric fluid with inorganic chloramines (Scully *et al.*, 1990). The presence of organic N-chloramines in stomach demonstrates the stability of some of these compounds at an extremely low pH. Since a large amount of fluid and nutrients can be absorbed in stomach, the presence of organic N-chloramines in stomach also indicates a significant delivery of these compounds at the site of entry.

Although there is very little information on *in vivo* toxicity of externally formed organic N-chloramines, the *in vivo* formation of organic N-chloramines by phagocytes during inflammatory processes has been discussed extensively in the biomedical literature and has been postulated to be potential contributors to the development of chronic diseases and cancers (Barua *et al.*, 2001; Bernofsky, 1991; Davies *et al.*, 1993; Englert and Shacter, 2002; Grisham *et al.*, 1984; Kawai *et al.*, 2004; Midwinter *et al.*, 2006; Midwinter *et al.*, 2004; Schuller-Levis and Park, 2004; Vissers *et al.*, 2001; Weitzman and Gordon, 1990). These studies have indicated toxicological significance of organic N-chloramines if present in disinfected drinking water and consumed by humans.

To date, while the toxicity mechanism of organic N-chloramines is not well understood, literature has suggested that due to the oxidative capacity like HOCl organic N-chloramines could play a critical role as intermediates for HOCl medicated cellular damage (Pattison and Davies, 2006). In terms of genotoxicity, it has been suggested that once enter the cells, organic N-chloramines (which are more stable than HOCl) can travel to the nucleus and may directly attack DNA molecules (Bull *et al.*, 2006). The direct

interaction between N-chloramines and DNA can progress to the evolution of nitrogen centred free radicals, which results in oxidation of DNA and/or dimers between DNA bases (Hawkins and Davies, 2002). Examples of the formation of organic N-chloramines (in drinking water) and their predicted toxicologically active compounds are illustrated in Figure 2.1.

Aside from experimental studies, QSTR/TOPKAT[®] has been used to assess toxicological potentials of organic N-chloramines (Bull *et al.*, 2006). Thirteen organic N-chloramines were selected from reactions of amino acids and pharmaceutical with chlorine and subjected to the TOPKAT[®] analysis. The study reported that although TOPKAT[®] estimated probabilities of carcinogenic effectsfor many N-chloramines, the program indicated a lack of structural fragment in the training sets of the carcinogenic activity or developmental toxicity and there are insufficient toxicological data related to compounds in this group to form judgements for individual members of organic N-chloramines. The study therefore recommended that further toxicity investigations on both individual compounds and their mixtures may help understand toxicology of organic N-chloramines.

NOTE:
This figure is included on page 33 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 2.1. Formation of organic N-chloramines in water and *in vivo* significance.

Figures were adapted from Bull et al. (2006). It is predicted that the first intermediate will

be produced in drinking water with subsequent reactions occurring *in vivo*. Example A

shows products of reaction of amino acid (primary amine) with HOCl. Example B shows

products of reaction of acetamide (secondary amine) with HOCl. Example C shows

products of reaction of aminopyrine with HOCl.

2.4. Organic N-Chloramines and Bladder Cancer Risk

To date, there is no data for identifying specific organic N-chloramines as likely bladder carcinogens. Due to a complete lack of toxicological data, the QSTR TOPKAT® model (Bull *et al.*, 2006) could not predict the carcinogenic risks of organic N-chloramines. However, the study has drawn several crucial points indicating that compounds in this class may play critical roles in adverse health effects. In terms of bladder cancer risks, it was suggested that organic N-chloramines may play a critical role due to a number of reasons. Firstly, organic N-chloramines are often rather stable molecules (more stable than either HOCl or monochloramine). The stability of some organic N-chloramines is sufficient to be absorbed and distributed to target tissue *in vivo*, allowing time for them to consequently produce toxicity effects. Secondly, the bladder has local metabolic capability, and hence could further facilitate toxicity of the absorbed organic N-chloramines (Bull *et al.*, 2006). To assist further investigation by QSTR analyses, more experimental toxicity investigations of individual compounds in this group are required.

2.5. Significance of Organic N-Chloramines: Australian Perspectives

In Australia, there have been no epidemiological studies that have examined the relationship between disinfection of drinking water and human cancer risk. However, a report on cancer prevalence in Australia in 2000 has shown that bladder cancer incidence was one of the most common cancers amongst Australians, representing 2,886 cases of 85,231 total cancer cases (McAvoy *et al.*, 2005). This report also shows that the bladder cancer incidence was found to be higher in men than women (2138 vs. 747 cases). Other dietary factors are also thought to contribute to bladder cancer incidence in developed

countries, but it is noted that the high bladder cancer incidence in Australian men is similar to that in other developed countries where chlorinated drinking water has been used for a long time. This indicates that Australia may face the same problems as the other countries in terms of DBPs and associated health risks.

Due to relatively low annual rainfall, it is believed that Australian water resources contain high levels of NOM and therefore types and concentrations of DBPs in Australian drinking water can be extremely varied. It has been noted that the problems regarding DBPs and health risks exist, but very few studies in this research area have been conducted in Australia. Most water quality centres in Australia only monitor THMs as a surrogate marker of DBPs. Therefore the information on the nature, prevalence and typical concentrations of DBPs in Australia is very limited. Furthermore, remarkably high THMs levels in Australian drinking water (Simpson and Hayes, 1998) has confirmed that the problems regarding DBPs in this country exist. In part, high levels of THMs indicate that other more toxic DBPs may be present in Australian drinking water too.

Frequent algal blooms as well as the increased number of drinking water sources that are impacted by municipal wastewater indicated that Australian water source are organic nitrogen rich, resulting in a possibly high levels of organic N-chloramines as a consequence of water disinfection. While organic N-chloramines have been shown to have significant implications on human health risks in the literature, the complete lack of information on toxicology, occurrence and fate of organic N-chloramines in drinking water supplies indicated that these areas of studies need to be of priority.

2.6.   Project Rationale

Organic N-chloramines have been predicted to be a potential health hazard (Bull *et al.*, 2006). Formation of organic N-chloramines occurs during water disinfection and can be present as DBPs in the finished drinking water. Although it is believed that organic N-chloramines could be a potential health threat, to date little information on the toxicology of organic N-chloramines is available and so any potential risk cannot be quantified.

The primary aim of the research described in this thesis is to investigate the genotoxicity of a number of organic N-chloramines. The work was motivated by a previous study, "Use of Toxicological and Chemical Models to Prioritized DBP Research" (Bull *et al.*, 2006). This report suggested a potential role for organic N-chloramines in the incidence of cancer associated with consumption of chlorinated drinking water, but also a lack of toxicological data with which to assess this hypothesis.

This project aimed to test two hypotheses:

1. That certain organic N-chloramines (in the µM concentration range) are genotoxic to mammalian cells and,

2. That oxidative stress is a mechanism involved in the genetic damage caused by the organic N-chloramines.

The specific aims of the work described in this thesis were:

1. To establish a rapid genotoxicity screening assay, the flow cytometry-based micronucleus assay (Chapter 3).

2. To synthesise organic N-chloramines by chlorination of pure amines and amino acids (Chapter 4).

3. To assess the genotoxicity of the synthesised organic N-chloramines by the validated genotoxicity assay (Chapter 5).

4. To investigate roles of organic N-chloramines in the induction of oxidative stress (Chapter 6).

5. To investigate cytotoxicity and genotoxicity of DBPs formed when molecular weight fractions of raw water are chlorinated (Chapter 7).

It was expected that this project would provide additional information on the toxicity and genotoxicity of organic N-chloramines. This basic information may be used as support data for the QSTR/TOPKAT® training sets for future risk assessment for this class of DBPs. It was also expected that the application of the developed assay for genotoxicity assessment of DBPs produced in Australian water would help narrow the scope of future studies by focussing research on the components that might be potential causes of adverse health effects.

3. FLOW CYTOMETRY-BASED MICRONUCLEUS ASSAY

3.1. Introduction

In vitro genotoxicity studies are usually used as the initial step to identify possible carcinogens prior to undertaking more complex *in vivo* carcinogenicity studies. Most genotoxicity assays focus on the investigation of damage or abnormalities at DNA or chromosome levels. A range of assays can be used including chromosome aberration assay (2003; Evseeva *et al.*, 2005; Shahabuddin *et al.*, 2005), sister chromatic exchange (Ipek *et al.*, 2003; Kocaman and Topaktas, 2008; Snyder and Green, 2001), micronucleus assay (Fenech, 2000), detection of DNA adducts (Austin *et al.*, 1996; Zhou *et al.*, 2007) and the comet assay (Buschini *et al.*, 2004; Landi *et al.*, 2003; Maffei *et al.*, 2005). Other methods such as gene mutation assays and fluorescence *in situ* hybridisation have also been used in genotoxicity assessment (Albertini *et al.*, 2000). To assess genotoxicity of a chemical, it is essential to select the appropriate assay(s) to be able to detect expected events, and further where the assay is to be used as a screening assay, the practicality of running large number of samples should also be considered.

For genotoxicity assessment of disinfection by-products, comet assay and micronucleus (MN) assay have been commonly used (Buschini *et al.*, 2004; Landi *et al.*, 2003; Liviac *et al.*, 2009; Maffei *et al.*, 2005; Pellacani *et al.*, 2005; Plewa *et al.*, 2002). A pilot study evaluating the comet and micronucleus assays was firstly conducted at the Australian Water Quality Centre (AWQC), with both assays being established for use. A number of factors resulted in the micronucleus assay being chosen for use in this project.

In order to assess genotoxicity of organic N-chloramines of unknown genotoxic mechanisms, it is necessary to use an assay that has both good sensitivity and more importantly that can detect genotoxicity from a wide range of mechanisms. While the comet assay measures clastogenic effects (i.e. DNA/chromosome breaks) (Klaude *et al.*, 1996), the micronucleus assay can detect both clastogenic and aneugenic effects (i.e. chromosome loss) of the test chemicals (Fenech, 2000). In addition, a comparative study on genotoxicity assessment of a large number of genotoxicants with unknown modes of actions suggested that the micronucleus assay is by far more sensitive than the comet assay (Hartmann *et al.*, 2001). Laboratory work also showed that the micronucleus assay was more practical and less complicated experimentally to use than the comet assay.

In the conventional micronucleus (MN) assay, the frequency of MN is determined using microscopy by counting cells with and without MN. Several hundred or thousand cells (if the occurrence of MN is rare) are required for the evaluation of micronucleus frequencies. As a result, analysis of MN by microscopy demands significant amount of time and is labour intensive, which is not practical for use as a screening assay for a large number of samples. In addition, since the process is conducted manually, the process is subjective and can introduce discrepancies in MN yields i.e. discrepancies of results between different laboratories and among different operators (Fenech *et al.*, 2003a).

To minimise the variations and to increase the screening capability, an automated approach for MN scoring using flow cytometry has been introduced (Nüsse and Kramer, 1984). Although the FCMN assay has potential use as a rapid genotoxicity screening assay, some disadvantages have been discussed (Nüsse and Marx, 1997). The main problem of the FCMN assay involves the interference from apoptotic cells and apoptotic bodies, which can interfere with the scoring of micronuclei during the measurement.

In the conventional micronucleus assay, it is suggested that performing the experiment using cells with impaired p53 could reduce false positives resulting from apoptosis (Meintieres *et al.*, 2001). Therefore it was hypothesised that the same principle may be applicable to the flow cytometry-based micronucleus assay too. However, at this point of time, although many cell types have been assessed for use in the FCMN assay (Avlasevich *et al.*, 2006; Bryce *et al.*, 2007; Sanchez *et al.*, 2000; Slavotinek *et al.*, 1995), comparative information on their sensitivity to genotoxicants in terms of cytotoxicity, genotoxicity and apoptosis is not available.

This chapter describes the set up, optimisation and validation of the FCMN assay. Two cell lines, WIL2NS and L5178Y were evaluated for use. Following exposure to several model genotoxicants with various modes of action, genotoxic and cytotoxic responses of the two cell lines were compared. A non-genotoxicant was used as negative control and included in the validation studies. Comparison of the apoptotic response between the two cell lines is also reported in this chapter. The information on genotoxicity, cytotoxicity and the ability to undergo apoptosis between these two cell lines is then used as a basis for recommending a suitable cell line for the FCMN assay for genotoxicity screening of organic N-chloramines.

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Mitomycin C (CAS No. 50-07-07), methyl methanesulfonate (CAS No. 66-27-3), vinblastine sulfate (CAS No. 143-67-9), etoposide (CAS No. 33419-42-0), benzo[a]pyrene (CAS No. 50-32-8) and sucrose (CAS No. 57-50-1) were purchased from Sigma-Aldrich (MO, USA). Details of chemicals with their modes of action are provided in Table 3-1. Sodium citrate (CAS No. 6132-04-3), citric acid (CAS No. 77-92-9), RNase A (Cat. No. R6513) and NaCl (CAS No. 7647-14-5) were also purchased from Sigma-Aldrich. Propidium iodide (PI) was obtained from Invitrogen (California, USA).

Table 3-1. Genotoxic and non-genoxic test chemicals used in assay validation

Chemical	Abbreviation	CAS No.	Genotoxicity outcomes
Mitomycin C	MMC	50-07-7	DNA cross links (Thomas *et al.*, 2003)
Methyl-methanesulfonate	MMS	66-27-3	dsDNA breaks (Beetstra *et al.*, 2005)
Vinblastine	VINB	143-67-9	Chromosome lag (Thomas *et al.*, 2003)
Etoposide	ETOPO	33419-42-0	dsDNA breaks (Fenech *et al.*, 2003b) and chromosome lag (Kallio and Lahdetie, 1996)
Benzo[a]pyrene**	BaP	50-32-8	dsDNA breaks (Loquet and Wiebel, 1982)
Sucrose	SUCRO	57-50-1	Non-genotoxic (Avlasevich *et al.*, 2006)

dsDNA = double stranded DNA, ** metabolic activation required

3.2.2.   Cell Culture

3.2.2.1.   General Maintenance

WIL2-NS cell line

The WIL2-NS (human lymphoblastoid) cell line was a gift from Dr B. Sanderson (Flinders University, South Australia). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich; MO, USA) supplemented with 10% foetal bovine serum (FBS), 10 mM HEPES, 1.5 g/L sodium bicarbonate, 0.06 mg/ml Penicillin G and 0.1 mg/ml Streptomycin.

L5178Y cell line

The mouse lymphoma cell line, L5178Y tk+/- (clone 3.7.2C), was obtained from the American Type Culture Collection (ATCC) (Manassas VA, USA). The cells were grown in Dulbecco‟s Modified Eagle‟s medium (Sigma-Aldrich; MO, USA) supplemented with 10% foetal bovine serum, 10 mM HEPES, 0.1% Pluronic, 0.06 mg/ml Penicillin G and 0.1 mg/ml Streptomycin.

Routine maintenance

Both cell lines were cultured at 37ºC in a humidified atmosphere with 5% CO_2. To maintain exponential growth phase, cells were passaged every 2-3 days maintaining a cell density of $1.0 \times 10^5 – 1.0 \times 10^6$ cells/ml. Each lot of cells was used within a period of 6 months or within the passage number no greater than 85. A new lot of cells were resuscitated from cryo-preserved stocks when required.

3.2.2.2. Measurement of Cell Growth

Since formation of MN takes place during cell division, in order to determine appropriate incubation time for the MN assay it is necessary to know rate of cell growth. To determine the rate of cell growth, cell counts were performed at time 0 and 24 hr using trypan blue (TB) exclusion assay. Briefly, 200 µl of cell suspension was added into a 1.5 ml eppendorf tube containing 400 µl TB buffer (0.1 % w/v TB in PBS[2] solution). Trypan blue (CAS 72-57-1), purchased from Sigma-Aldrich (MO, USA), is a dye commonly used to distinguish between viable and non-viable cells. Viable cells exclude the dye, while non-viable cells absorb the dye and appear blue in cytoplasm of the cells (Strober, 2001). Numbers of cells (both viable and non-viable) were counted microscopically using a hemocytometer (Improved Neubauer) as shown in Figure 3.1. Total cell number was calculated and reported as number of cells per millilitre, and viability was expressed as percentage of viable cells over the total cell count.

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[2] Phosphate Buffered Saline
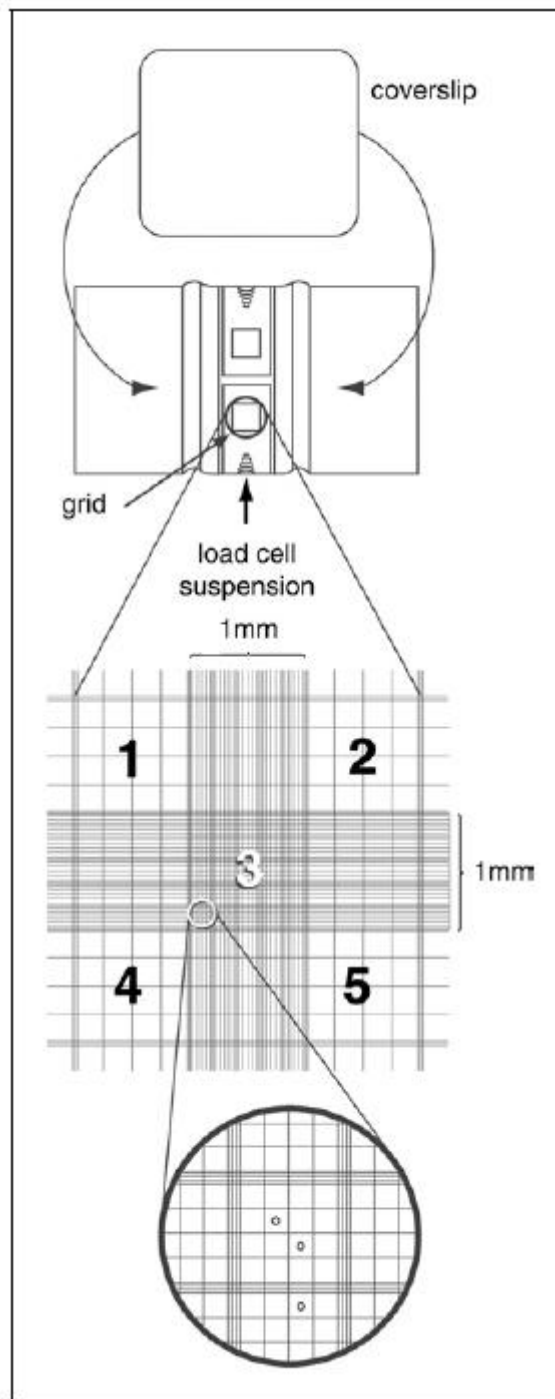
Figure 3.1. Hemocytometer (Improved Neubauer) used for cell count.

The coverslip is applied to slide and cell suspension is added to the counting chamber using a micropipette. Each counting chamber has a 3 x 3 mm grid (enlarged). Cells are counted from the four corner squares (1, 2, 4 and 5) and the central square (3) (Phelan, 2006).

3.2.3.   Flow Cytometry Settings for Measurement of Micronuclei

3.2.3.1.   Flow Cytometry Settings

To set up parameters for flow cytometric measurement of micronuclei, a suspension of micronuclei/nuclei was firstly prepared (described in section 3.2.4.2 below). The FACSCalibur™ flow cytometer (Becton Dickensn, San Jose, CA, USA) equipped with a 15 mW air-cooled argon ion laser emitting at a fixed wavelength of 488 nm was used in this study. Forward scatter (FSC) signals were collected by the 90° collection lens and focussed into a series of optical filters and the light spectrally split by a collection of dichroic mirrors and filters. Side scatter (SSC) signals were collected by the side scatter photomultiplier tube. The orange fluorescence signals from PI-stained DNA were collected by the FL2 photomultiplier tube (585 ± 42 nm). The optical layout of the the FACSCalibur™ flow cytometer is shown in Figure 3.2.

All parameters were presented on logarithmic scales. Detector voltages and threshold settings were adjusted for the stained sample (suspension of N and MN). Sheath fluid consisted of ISOTON® II Diluent (Beckman Coulter, USA) with three possible flow rates of low (12 ± 3 µl min^{-1}), medium (35 ± 5 µl min^{-1}) and high (60 ± 7 µl min^{-1}). Acquisition of data for suspensions of N and MN was performed using a low flow setting. Data were recorded and analysed using the FACStation Data Management system comprising a Power Macintosh® G3 computer with CellQuest™ software (Becton Dickenson, San Jose, CA, USA).

NOTE:
This figure is included on page 47 of the print copy of
the thesis held in the University of Adelaide Library.

Figure 3.2. Optical layout of the FACSCalibur™ flow cytometer.

The Red Diode Laser and FL4 PMT are optional extras and not present on the

FACSCalibur™ used at the AWQC and in this thesis. Picture adopted from Becton

Dickinson FACSCalibur™ System User˶s guide (1996), p56.

3.2.3.2. Gating Criteria for Measurement of Micronuclei

To determine gating regions for the measurement of micronuclei, bivariate dot plots using logarithmic scales were created (i.e. FSC vs. FL2 or SSC vs. FL2). Events were triggered on FL2 fluorescence; the fluorescence threshold was fixed at channel 5 and the G1 phase peak was set to channel 1000. Nuclei and micronuclei were discriminated from non-specific debris using the electronic gates shown in Figure 3.5. The nuclei region was set to channels 1000-2000 and the micronuclei region was defined as channels 10-100 (1-10% of DNA of G1 phase nuclei) (Nüsse *et al.*, 1994; Nüsse and Kramer, 1984). The frequency of MN was expressed as a percentage of the ratio between total micronuclei (Nmn) and total nuclei (Nn) [% MN = 100 (Nmn/Nn) based on the acquisition of 10,000 events.

3.2.3.3. Confirmation of Micronucleus by Flow Cytometry Sorting

To confirm that the events in regions R3 and R4 described in Figure 3.5D contain micronuclei and nuclei, particle sorting by flow cytometry followed by morphological investigation by microscopy was performed. Briefly, WIL2-NS cells were exposed to 15 µg/ml MMS for 24 hr and a nuclear suspension prepared (as described in section 3.2.4.2). Using the FACSCalibur™ and Cell Quest® software, electronic gating regions were defined as shown in Figure 3.6 and, where appropriate, events in each region were collected in a clean collection tube. The sorted particles were concentrated by passing the suspension through a 0.8 µM Poretics® polycarbonate membrane (Osmonic Inc.) using a vacuum apparatus (QIAGEN, Australia). The membrane was then placed on a glass slide. Twenty-five microlitres of 10 µg/ml PI in PBS solution was added on the membrane, sealed with a cover glass for 10 min at room temperature in the dark before analyses using fluorescence microscopy. Morphologies of the particles were observed and photographs taken using a fluorescence microscope (Olympus BX60) equipped with a digital camera (Olympus DP50) using 200X magnification.

3.2.4.   Micronucleus Assay

3.2.4.1.   Chemical Treatment

Two treatment protocols, with and without the metabolic activation by S9 were used in the validation studies as described below and summarised in Figure 3.3.

Protocol for direct acting genotoxicants (without S9)

This protocol was used for evaluation studies of 4 genotoxicants (MMC, MMS, VINB and ETOPO) and a non-genotoxicant (SUCRO). Twenty-four hours prior to experiment, cells (exponential growth phase, see section 3.2.2.1) were passaged into a new tissue culture flask (CELLSTAR®, Greiner bio-one). On the day of experiment, the cells were counted (TB exclusion assay, see section 3.2.2.2), and a suspension of 2.5×10^5 cells/ml was prepared in complete medium. The cells were seeded at 2 ml per well in a 24-well tissue culture plate (flat bottom for suspension cells, SARSTEDT, Sarstedt Inc.). The cells were incubated at 37ºC, 5% CO_2 for a 2 hr recovery. Following this recovery period, cells were treated (in triplicate) with the test chemicals by diluting a stock solution 100-fold in the well. Continuous exposure to the test chemical continued at 37ºC, 5% CO_2 for 24 hrs. Untreated cells (no treatment control) were included as required.

Protocol for metabolic activation by S9

BaP is an indirect genotoxicant, requiring metabolism by cytochrome P450 1A1 (Georgiadis *et al.*, 2005) to produce the active compound benzo[a]pyrene-7, 8-diol (Kim *et al.*, 1998). For experiments using benzo[a]pyrene (BaP), a short term treatment was used because preliminary experiments indicated that 24 hr exposure to S9 produced an

unacceptable level of cytotoxicity. S9 post mitochondrial fractions (MOLTOX™, USA) were prepared in culture medium supplemented with NADP and glucose-6-phosphate (both from Sigma-Aldrich; MO, USA) as per the company instruction. Briefly, 1 ml cell suspensions (5.0×10^5 cells/ml, prepared from a fresh 24 hr culture as above) were treated with BaP in the presence of 1 ml of S9 mix for 3 hrs. After treatment, cells were washed twice with serum-free culture medium, resuspended in 2 ml of growth medium in a new 24-well tissue culture plate and incubated at 37ºC, 5% CO_2 for 21 hrs.
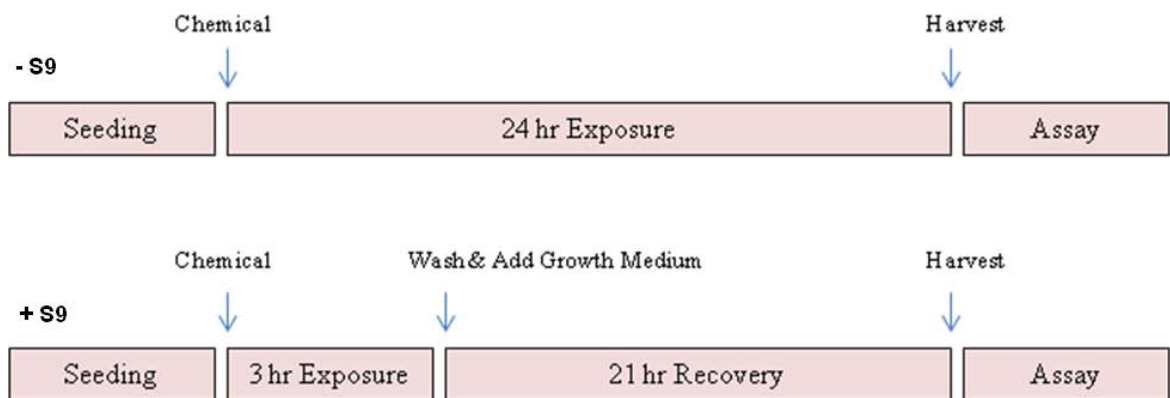
Figure 3.3. Summary of protocols used for *in vitro* genotoxicity assessment.

A 24 hr exposure experiment without metabolic activation (Top panel) was used for all test chemicals except BaP. A short-term exposure experiment with S9 metabolic activation (Bottom panel) was used for BaP treatment. Seeding (equilibration), exposure and recovery stages are conducted in cell culture environment (37ºC, 5% CO_2 in humidified atmosphere). Cells were harvested and assayed at room temperature with minimal exposure to UV light.

3.2.4.2.   Analysis of MN by Flow Cytometry

For analysis of MN by flow cytometry, the two-step treatment described by Nüsse and Kramer (1984) with minor modifications was used to prepare a suspension of nuclei (N) and micronuclei (MN). The two-step method allows a separation of MN from cells without disruption of mitotic and other nuclei. Briefly, 600 μl of cell suspension was transferred into a 1.5 ml eppendorf tube, centrifuged at 100 g (Eppendorf Centrifuge 5804) for 5 min and the supernatant removed.

The cell pellet was then resuspended in 600 μl of FCM-solution I (584 mg/L NaCl, 1000 mg/L sodium citrate, 10 mg/L RNAse A, 0.3 ml/L Nonidet P-40 and 10 mg/L PI), gently mixed by vortexing and incubated at room temperature for 60 min. The detergent (NP40) containing solution disrupted the cell membrane and released MN into the solution. Then 600 μl of FCM-solution II (85.6 g/L sucrose, 15 mg/L citric acid and 10 mg/L PI) was added directly to the tube, gently mixed and the suspension of N and MN transferred into a FCM tube. This step was to completely remove the cytoplasm adhering to nuclei while the osmotic pressure caused by the sucrose and citric acid helps prevent further disruption of mitotic nuclei.

Following incubation at room temperature for 30 min, the N/MN suspension was gently mixed and subjected to flow cytometric measurement using the FACSCalibur™ flow cytometer (Becton Dickensn, San Jose, CA, USA). Measurement and analysis of micronuclei was performed as described in section 3.2.3.2.

3.2.4.3. Analysis of MN by Microscopy

Using the same treatment samples, microscopic measurement of MN was performed in parallel with the scoring of MN by flow cytometry. Five hundred microlitres of cell suspension (the same cell samples used for the FCMN assay) was collected in 1.5 ml eppendorf tubes, centrifuged at 100 g for 5 min and the supernatant removed. The cell pellet was then resuspended in 20 µl FBS. A sample (5 µl) was thinly smeared on glass slides, using the same technique as described by Houwen (2002). The slide was air dried, fixed in absolute methanol and stained with Diff-Quik (Surgical and Medical Supplies, Australia). Stained slides were stored at room temperature in the dark until analysed.

Scoring of MN was performed using 1000X magnification (Olympus BX40). For each culture, 1000 cells were analysed (i.e. 1000 cells/culture x triplicate cultures = 3000 cells/chemical concentration) and total number of nuclei (Nn) and micronuclei (Nmn) was recorded. To be counted as a micronucleus the stained DNA had to be round in shape, of approximately 1 - 10% of the size of the main nucleus and exhibit similar staining characteristics as the main nuclei (Fenech, 2000). The frequency of micronuclei was calculated in the same manner as in the flow cytometry [i.e. %MN = 100 x (Nmn/Nn)].

3.2.5.   MTS-Cell Viability Assay

The MTS assay (The CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was used to determine cell viability. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium   salt]   is   reduced   by metabolically active cells into a coloured formazan product that is soluble in culture medium, which can be easily detected by measuring absorbance at 490 nm.

Following the chemical treatment, 100 µl of cell suspension was transferred into a well of a 96-well tissue culture plate (CELLSTAR®, greiner bio-one). Ten microlitres of the CellTiter 96® AQueous One Solution was added directly to the cell suspension. Cells were mixed, incubated at 37ºC, 5% CO^2 for 3 hrs and then absorbance was measured at 490 nm (Wallac VICTOR3 1420 Multilabel Counter, PerkinElmer™). Viability was expressed as a percentage of the absorbance of the test sample over the no-treatment control.

3.2.6.   Apoptosis Measurement

3.2.6.1.   Apoptosis Induction

To compare levels of apoptosis responses between WIL2-NS and L5178Y cells, two apoptosis inducing procedures, heat-shock treatment and exposure to etoposide, were utilised. Apoptosis induction by heat-shock was carried out by incubating the cells at 45ºC for 30 min. The cells were then returned to 37ºC for 1, 2, 3 or 4 hr. This procedure was also

described by VanderWaal et al. (1997). For etoposide treatment, cells were exposed to 0, 0.5, 1.0 or 1.5 μg/ml etoposide for 24 hr. Following the treatments, levels of caspase-3 in apoptosis-induced cells were determined using the flow cytometric NucView Assay.

3.2.6.2.   Flow Cytometric NucView Assay

Briefly, following induction of apoptosis, 200 μl of cell suspension was transferred into a flow cytometry tube. Two microlitres of NucView^{TM} 488 caspase-3 substrate (Biotium Inc., USA) was added directly to the cells and incubated at room temperature in the dark for 15 min. The NucViewTM 488 caspase-3 substrate is cleaved by caspase-3 in the cytoplasm of apoptotic cells to release a fluorescent DNA dye that is able to enter the nucleus and stain the DNA. Ten thousand cells were analysed using flow cytometry (from histogram plots of events registered in FL1 channel; Figure 3.9). Levels of apoptosis were expressed as percentages of the NucView positive cells over the total cell counts.

3.2.7.   Statistical Analysis

All statistical analyses and graphing were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). Unless otherwise stated, data plots represent mean ± SEM of 3 independent experiments. Kruskal-Wallis followed by Dunns post test was used to determine significant differences of treatments from negative controls. Non-parametric Spearman‟s coefficient (r) was used to assess the degree of correspondence of the micronucleus frequency results measured by flow cytometry and by microscopy. Statistical significance was accepted at $p < 0.05$.

3.3.   Results

3.3.1.   Doubling Times of WIL2-NS and L5178Y Cell Lines

The doubling time of WIL2-NS and L5178Y cell lines was determined. As shown in Figure 3.4, over 24 hr culture, cell population of WIL2-NS increased from the average of 2.7 x 10^5 cells /ml to 13.0 x 10^5 cells/ml, while L5178Y population increased from 2.8 x 10^5 cells /ml to 14.1 x 10^5 cells /ml. Both cell lines produce at least 2 population doublings over 24 hr, giving the estimated doubling time of 10-12 hours. Hence, both cell lines are suitable for a 24 hr MN assay protocol.
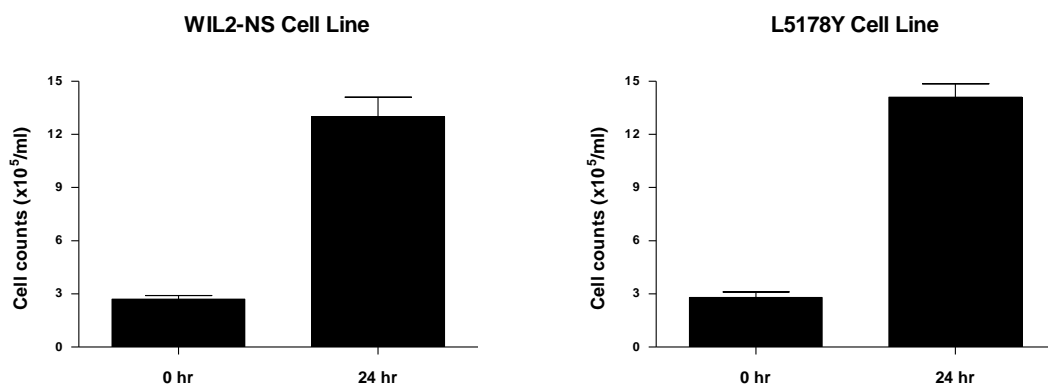
Figure 3.4. Growth rate of WIL2-NS and L5178Y cell lines.

Cell counts were determined using TB exclusion assay. Data are mean ± SEM of 4 individual experiments.

3.3.2.   Optimisation of the FCMN Assay

To establish the FCMN assay, a number of parameters required optimisation. This included the flow cytometer parameter settings and the confirmation of the electronic gating by flow cytometry sorting.

3.3.2.1.   Flow Cytometry Parameter Settings

The flow cytometer parameter settings were optimised in order to discriminate PI-stained nuclei and micronuclei from irregular shaped debris of unknown origin. A sequential gating procedure was used as shown Figure 3.5. The scatter plot (log SSC-H against log FSC-H) of PI stained nuclei and micronuclei showed that the majority of events fell within the R1 region (Figure 3.5A). The DNA distribution profile of R1, as shown in Figure 3.5B (or histogram plot of events against log FL2-H) was used for the adjustment of DNA signal and for the flow cytometry threshold setting. G1 phase nuclei were fixed at channel 1000, while the threshold was set at channel 5. Channel 2000 represents a peak of G2 phase nuclei having twice the DNA content of the G1 peak. Figure 3.5C represents the second gate (R2) of a dot plot (log SSC-H against log FL2-H). Particles outside this gate were determined as debris (see sorting below). All events in this R2 region were selected and an additional scatter plot of log FSC-H vs. FL2-H was created (Figure 3.5D). The N and MN gates were determined from this final scatter plot.
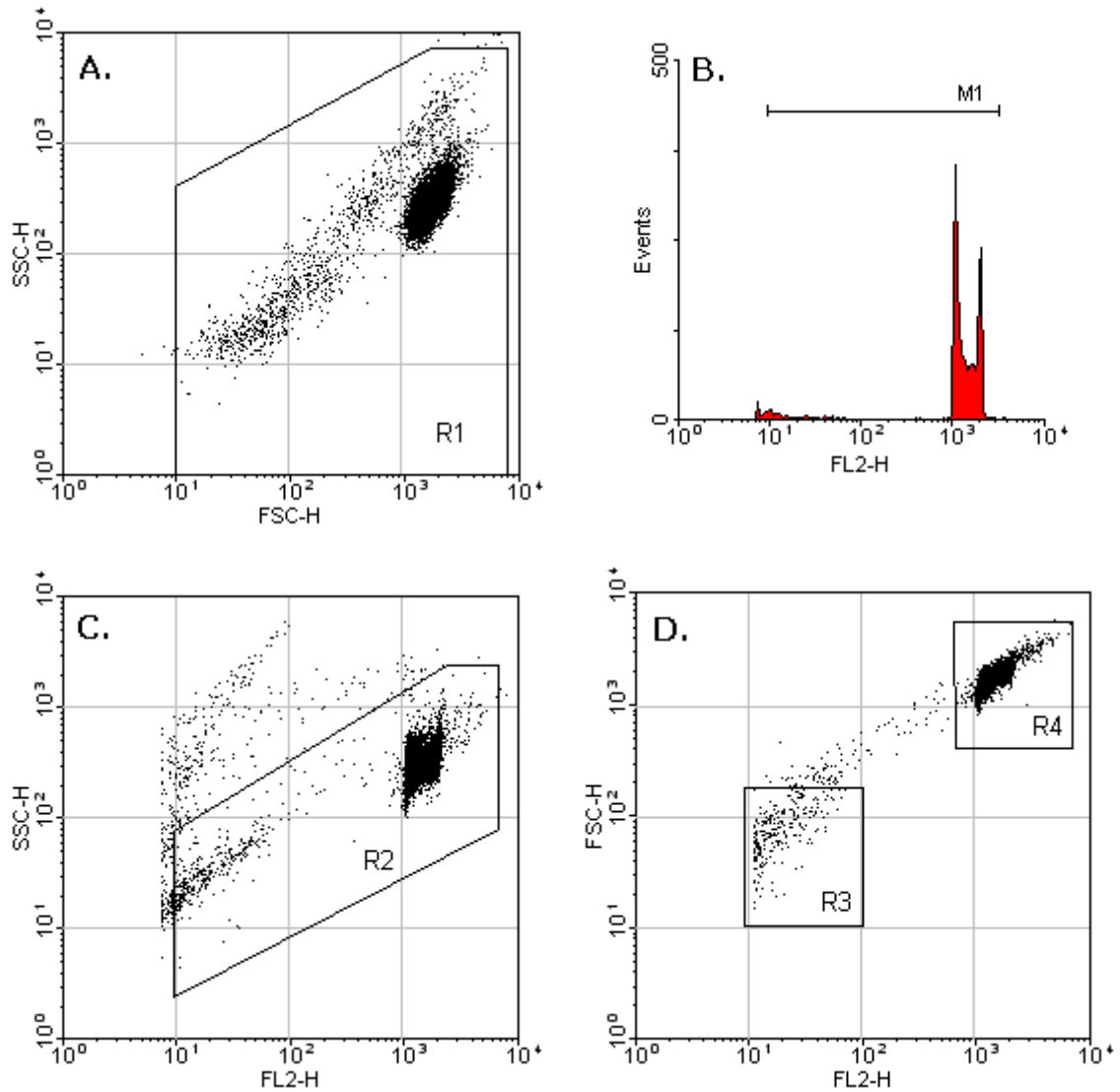
Figure 3.5 Gating criteria for flow cytometric measurement of micronuclei.

To be scored as micronuclei the events must fall within R1 region of the light scatter plots (A), FL2 range (M1 region; B), the SSC vs. FL2-H (R2 region; C) and FSC vs. FL2-H (D). R3 is the region that micronuclei are to be found (1-10% of G1 phase nuclei PI-fluorescence, FL2H) while R4 is the region of main nuclei (D).

3.3.2.2. Flow Cytometric Sorting of Nuclei/Micronuclei

To confirm that the regions detailed in section 3.3.2.1 above contained N and MN as expected, flow cytometry regions were sorted, collected, and the content assessed by fluorescence microscopy. As shown in Figure 3.6, particles collected from region R1 were observed to be round in shape, and each particle had similar size and fluorescence staining characteristics. Particles in R2 region were also round with similar fluorescence intensity to the particles in R1 but of smaller size. This confirms that particles in R1 and R2 are nuclei and micronuclei respectively. It was noted that particles sorted from R3 region were a mixture of small and irregular shaped characteristics, classified as debris or DNA artefacts.
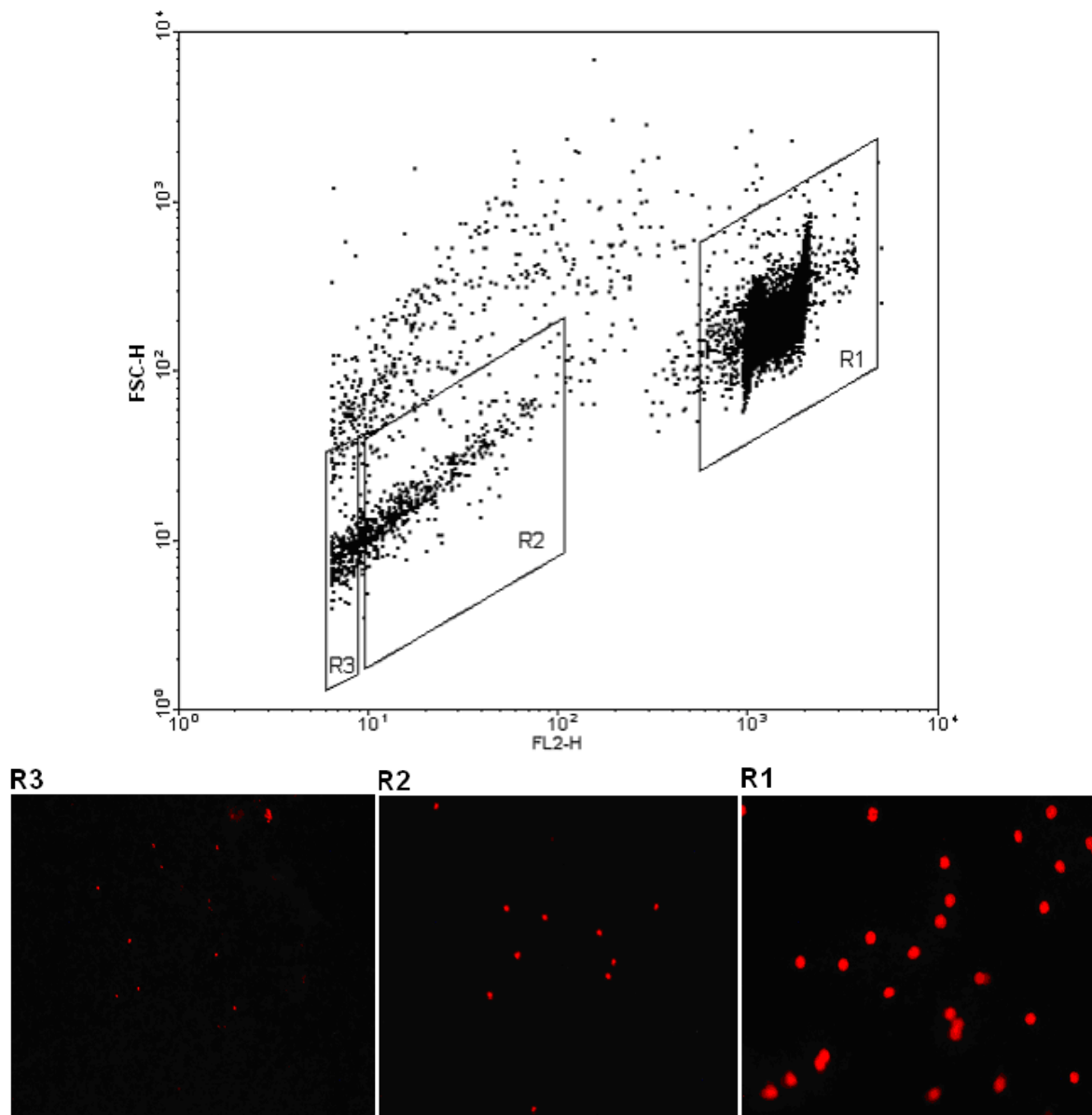
Figure 3.6. Flow cytometry sorting of PI-stained nuclei (N) and micronuclei (MN).

Top picture shows a dot plot (FSC-H vs. FL2-H) of the suspension of N/MN from WIL2-NS cells treated with 15 µg/ml MMS. Several sort regions are indicated (R1, R2 and R3). Pictures in the bottom panel show the morphologies of the sorted particles, corresponding to the defined sort region. Photos were taken under a fluorescence microscopy (200X magnification).

3.3.3.   FCMN Assay Validation

The FCMN assay was validated using a number of genotoxicants with various modes of action and a non-genotoxicant (Table 3-1). Cytotoxicity and MN formation in WIL2-NS and L5178Y cell lines was assessed following exposure to MMC, MMS, VINB, ETOPO, BaP and SUCRO as shown in Figure 3.7 and Figure 3.8, respectively, and is described in detail below. Spontaneous MN formation in no-treatment control cells is also discussed. For each compound tested, MN formation was also assessed by the microscopy based MN assay in parallel.

3.3.3.1.   Spontaneous MN Formation (no-treatment control)

The average MN frequency in untreated WIL2-NS cells was $1.9 \pm 0.32\%$ by FCM and $1.13 \pm 0.35\%$ (mean $\pm$ SD, n = 21) by microscopy. For L5178Y cells, the background MN frequency was $1.4 \pm 0.41\%$ by FCM and $0.92 \pm 0.28\%$ (mean $\pm$ SD, n = 21) by microscopy. Overall, results showed that background MN levels were slightly higher in WIL2-NS cells than in L5178Y cells and MN measured by flow cytometry provided higher values than microscopy. Measurement of MN over 21 cell passages indicated that spontaneous MN formation was stable in both cell lines.

3.3.3.2. Treatment with Mitomycin- C (MMC)

In WIL2-NS cells, MMC demonstrated concentration-dependent cytotoxicity and genotoxicity (Figure 3.7A). There was a statistically significant increase in micronuclei as assessed by flow cytometry (p = 0.0110, Kruskal-Wallis Test) with good agreement with microscopic measurement (r = 0.9804, Spearman's correlation). Viability was only reduced to $74 \pm 1.3\%$ (mean $\pm$ SEM, n = 3) at concentrations resulting in the increased formation of MN.

MMC treatment of L5178Y cells also showed a concentration-dependent response for both cytotoxicity and genotoxicity (Figure 3.8A). While viability remained >70%, an increase in micronucleus formation measured by flow cytometry was statistically significant (p = 0.0357, Kruskal-Wallis Test). The results were consistent with those obtained from microscopy (r = 0.9893, Spearman's correlation).

3.3.3.3. Treatment with Methyl Methanesulfonate (MMS)

Cytotoxicity screening of MMS on both cell lines showed that each cell line responded to MMS differently. L5178Y cells were less susceptible to the chemical than WIL2-NS cells. EC30 (the concentration that cause 30% reduction of cell viability) of L5178Y and WIL2-NS cell lines was 80 µg/ml and 20 µg/ml, respectively. Therefore, different concentration ranges of MMS were tested for micronucleus induction.

In WIL2-NS cells, treatment with MMS up to 20 µg/ml gave concentration-dependent responses in both cytotoxicity and micronucleus formation (Figure 3.7B). Flow cytometric measurement of MN demonstrated a significant increase of MN frequency ($p <$ 0.0111, Kruskal-Wallis Test) and the results were well correlated with the microscopy ($r =$ 0.9529, Spearman's correlation). Viability at these concentrations was $\geq 75 \pm 4.6\%$.

In L5178Y cells (Figure 3.8B), the cytotoxicity and genotoxicity pattern differed in comparison to WIL2-NS cells. An increase in viability of the cells was observed after the cells were treated with low concentrations of MMS (<60 µg/ml), but at the highest concentration (80 µg/ml) cytotoxicity was clearly observed (viability reduced to $72 \pm 4.8\%$). At the non-cytotoxic concentrations, flow cytometric measurement of MN showed a statistically significant increase ($p = 0.0285$, Kruskal-Wallis Test). At the cytotoxic concentrations, levels of MN were similar to or reduced compared to the lower concentrations. Thus, compared to the untreated control ($2.0 \pm 0.32\%$), no significant increase in MN formation was observed at 60 µg/ml ($5.4 \pm 0.9\%$), but the result was significant at 80 µg/ml ($8.0 \pm 2.7\%$). A similar pattern was also observed when MN were measured by microscopy.

3.3.3.4.   Treatment with Vinblastine (VINB)

WIL2-NS cells were slightly more sensitive to VINB (EC30 = 1.5 ng/ml) than the L5178Y cells (EC30 = 3.0 ng/ml). In WIL2-NS cells, treatment with VINB showed concentration-dependent cytotoxicity and genotoxicity (Figure 3.7C). MN formation as measured by flow cytometry was found to be statistically significant ($p = 0.0224$, Kruskal-Wallis Test). Viability was reduced from $90 \pm 3.5\%$ to $73 \pm 3.3\%$ at concentrations that

produced MN. There was good positive correlation between MN results obtained from the two measurement methods ($r = 0.9916$, Spearman‟s correlation).

Results showing the concentration-dependent cytotoxicity and genotoxicity in L5178Y cells are given in Figure 3.8C. In the range of concentrations tested (the highest caused $31 \pm 2.6\%$ reduction in viability), FCMN results showed statistically significant increases of MN ($p < 0.0216$, Kruskal-Wallis Test) with positive correlation with the results from microscopic measurement ($r = 0.9963$, Spearman‟s correlation).

3.3.3.5. Treatment with Etoposide (ETOPO)

Our preliminary cytotoxicity screening indicated that WIL2-NS cells were also more susceptible to ETOPO than L5178Y cells with EC30‟s of 0.45 µg/ml (WIL2-NS) and 2.0 µg/ml (L5178Y), respectively. Hence, different concentration ranges were tested on each cell line.

In WIL2-NS cells (Figure 3.7D), a significant increase in MN was observed in the FCMN assay following treatment with ETOPO ($p < 0.0211$, Kruskal-Wallis Test). The highest test concentration reduced viability to $66 \pm 4.1\%$. A positive correlation between results from FCMN and microscopy was observed ($r = 0.9034$, Spearman‟s correlation). However, while at the lowest concentration of ETOPO (0.15 µg/ml, producing $88 \pm 1.9\%$ viability) both measurement methods gave similar results, at the higher two concentrations (0.30 µg/ml and 0.45 µg/ml, producing viabilities $= 77 \pm 4.6\%$ and $66 \pm 4.1\%$, respectively) the FCMN method produced substantially higher MN counts than did microscopy. It was noted during microscopic examination that ETOPO treatment gave rise

to unusual morphologies i.e. enlarged cells with multi-nuclei or micronuclei, which made the analyses of MN more difficult.

In L5178Y cells (Figure 3.8D), concentration-dependent cytotoxicity and genotoxicity was observed following treatment with ETOPO, although higher concentrations ($\geq$ 1.0 µg/ml) were required when compared to the experiments on WIL2-NS cells. The increase of MN formation measured by FCM was also found to be statistically significant ($p < 0.0090$, Kruskal-Wallis Test) with a positive correlation with the microscopic result ($r = 0.9000$, Spearman''s correlation).

3.3.3.6. Treatment with Benzo[a]pyrene (BaP)

Treatment of WIL2NS or L5178Y with BaP (up to 24 µg/ml) for 24 hr did not show any cytotoxic or genotoxic effects in either of the cell lines (data not shown) indicating that the cells do not have the metabolic enzymes required to produce the active toxin. Hence, S9 was used in all experiments with BaP in this study.

In the presence of S9, treatment of WIL2-NS cells with BaP up to 24 µg/ml did not show cytotoxicity, but gave a concentration-dependent genotoxic response (Figure 3.7E). The FCMN results were found to be statistically significant ($p < 0.0018$, Kruskal-Wallis Test) with a positive correlation with microscopy ($r = 0.9684$, Spearman''s correlation).

In L5178Y cells (Figure 3.8E), treatment with BaP up to 24 µg/ml resulted in a reduction of viability to 69 ± 2.7%. Following the treatment, a statistically significant increase of MN induction was observed by the FCMN assay ($p = 0.0399$, Kruskal-Wallis

Test). The results from flow cytometry were in agreement with microscopy (r = 0.9747, Spearman‟s correlation).

3.3.3.7.   Treatment with Sucrose (SUCRO)

Sucrose, a non-genotoxicant, was used as a negative control. In both cell lines, treatment with SUCRO up to 5 mg/ml, the OECD[3] recommended highest test concentration for unknown compounds (OECD, 2004), did not show signs of cytotoxic or genotoxic effects in the FCMN assay (data not shown). To evaluate further, we treated both cell lines with SUCRO at higher concentrations to initiate cytotoxicity through osmotic stress mechanisms.

It was found that following treatment of SUCRO up to 32 mg/ml, WIL2-NS cells (Figure 3.7F) demonstrated a concentration-dependent cytotoxicity response (viability reduced to 73 ± 2.9 %) with no significant increase in MN when measured by both flow cytometry (p = 0.2758, Kruskal-Wallis Test) and microscopy (p = 0.4824, Kruskal-Wallis Test).

In L5178Y cells (Figure 3.8F), a reduction of viability (75 ± 3.6 %) was seen at 8 mg/ml with no further decrease at higher concentrations. It was also found that treatment with SUCRO up to 32 mg/ml did not show significant increases in MN when measured by both flow cytometry (p = 0.0908, Kruskal-Wallis Test) and microscopy (p = 0.1051, Kruskal-Wallis Test).

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[3] The Organization for Economic Co-operation and Development guideline for the testing of chemicals: *in vitro* micronucleus assay.
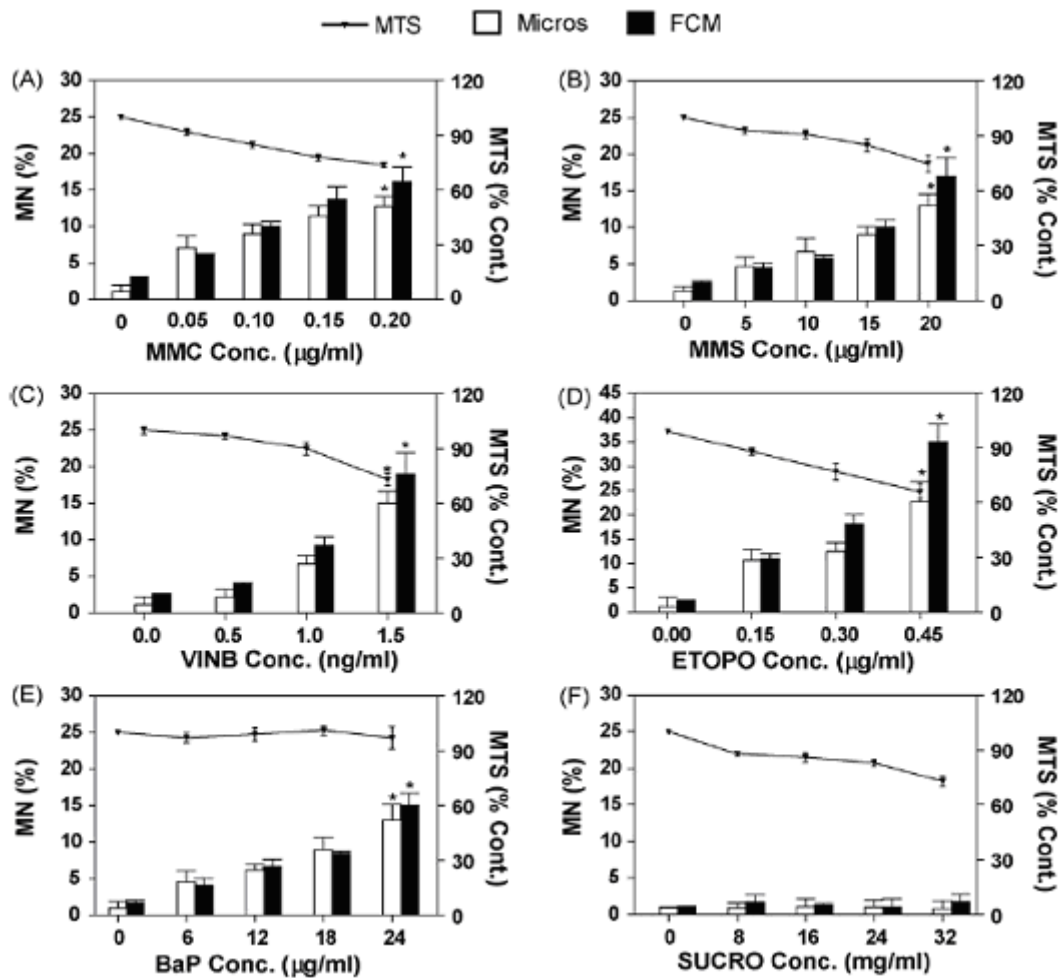
Figure 3.7. Cytotoxicity and micronuclei formation responses of WIL2-NS

following treatment with mitomycin-C (A), methyl methanesulfonate (B), vinblastine (C),

etoposide (D), benzo[a]pyrene (E) and sucrose (F). Right y-axis shows percent cell

viability, relative to control, as measured by the MTS assay (▼). Left y-axis is

micronucleus frequency, as a percentage of whole nuclei, measured by flow cytometry (■)

and microscopy (□). Each data point represents mean ± SEM of three independent

experiments. Picture taken from Laingam *et al.* (2008)
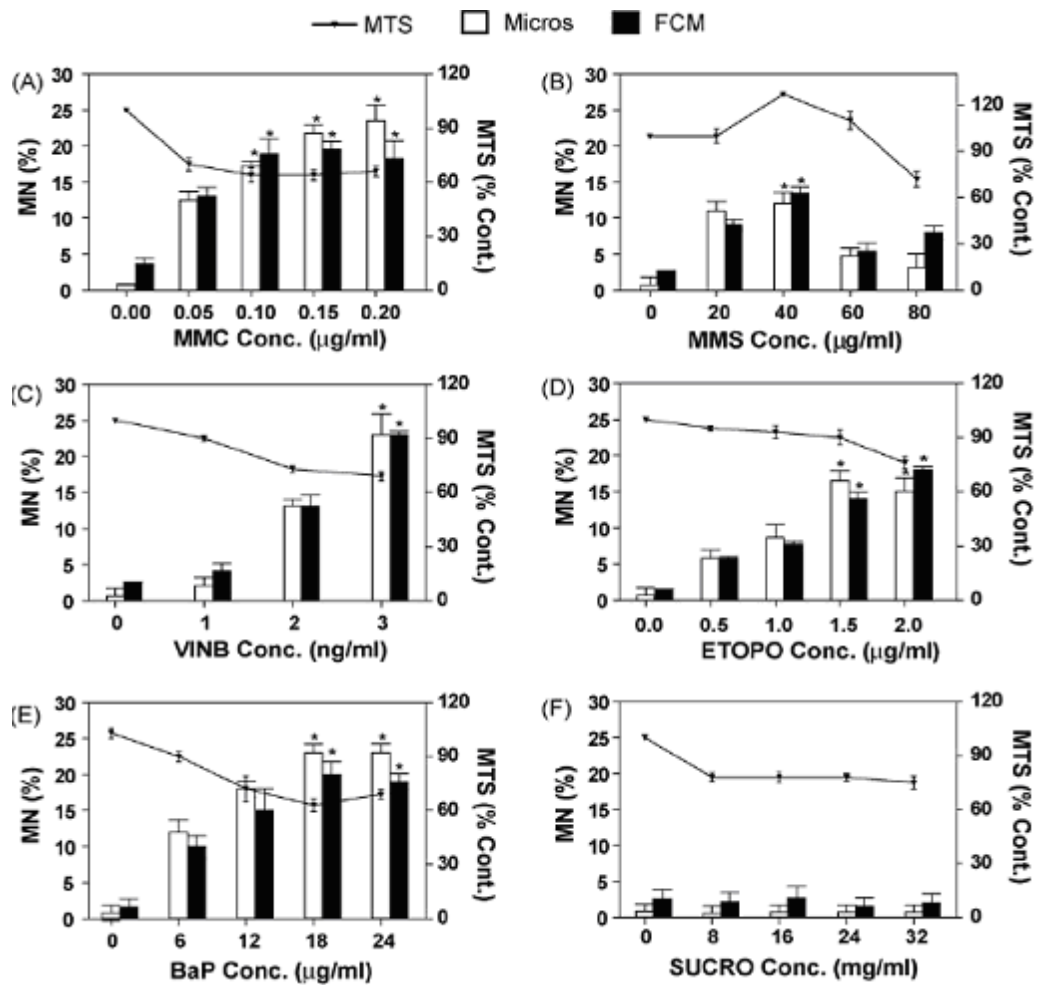
Figure 3.8. Cytotoxicity and micronuclei formation responses of L5178Y

following treatment with mitomycin-C (A), methyl methanesulfonate (B), vinblastine (C),

etoposide (D), benzo[a]pyrene (E) and sucrose (F). Right y-axis shows percent cell

viability, relative to control, as measured by the MTS assay (▼). Left y-axis is

micronucleus frequency, as a percentage of whole nuclei, measured by flow cytometry (■)

and microscopy (□). Each data point represents mean ± SEM of three independent

experiments. Picture taken from Laingam *et al.* (2008).

3.3.4.   Comparison of Apoptosis Response

To compare levels of apoptosis responses between WIL2-NS and L5178Y cells, we induced apoptosis using two procedures, heat-shock treatment and exposure to etoposide. Apoptosis was monitored by determination of caspase-3 activity in cells using the Flow Cytometric NucView™ assay. Figure 3.9 demonstrates the flow cytometry profile of apoptotic L5178Y cells at 0, 2 and 4 hr following heat-shock treatment. Cells were considered apoptotic if they fell in the M2 region shown in Figure 3.9 (B, D and F).

Comparison of the apoptotic responses of the two cell lines is shown in Figure 3.10. For heat-shock treatment (Figure 3.10A), it was found that numbers of apoptotic L5178Y cells increased with time to 77.4 ± 11.3% (mean ± SD, n = 2) of the population being apoptotic 4 hr after heat-shock. The induction of apoptotic cells was not seen in WIL2-NS cell within 3 hours after heat-shock; and only a small increase 14.6 ± 3.3% (mean ± SD, n = 2) was observed at 4 hr.

When apoptosis was induced by treatment with etoposide (Figure 3.10B), it was found that at all concentrations tested (up to 1.5 μg/ml) a greater amount of caspase-3 positive cells were seen in L5178Y cells than WIL2-NS cells.
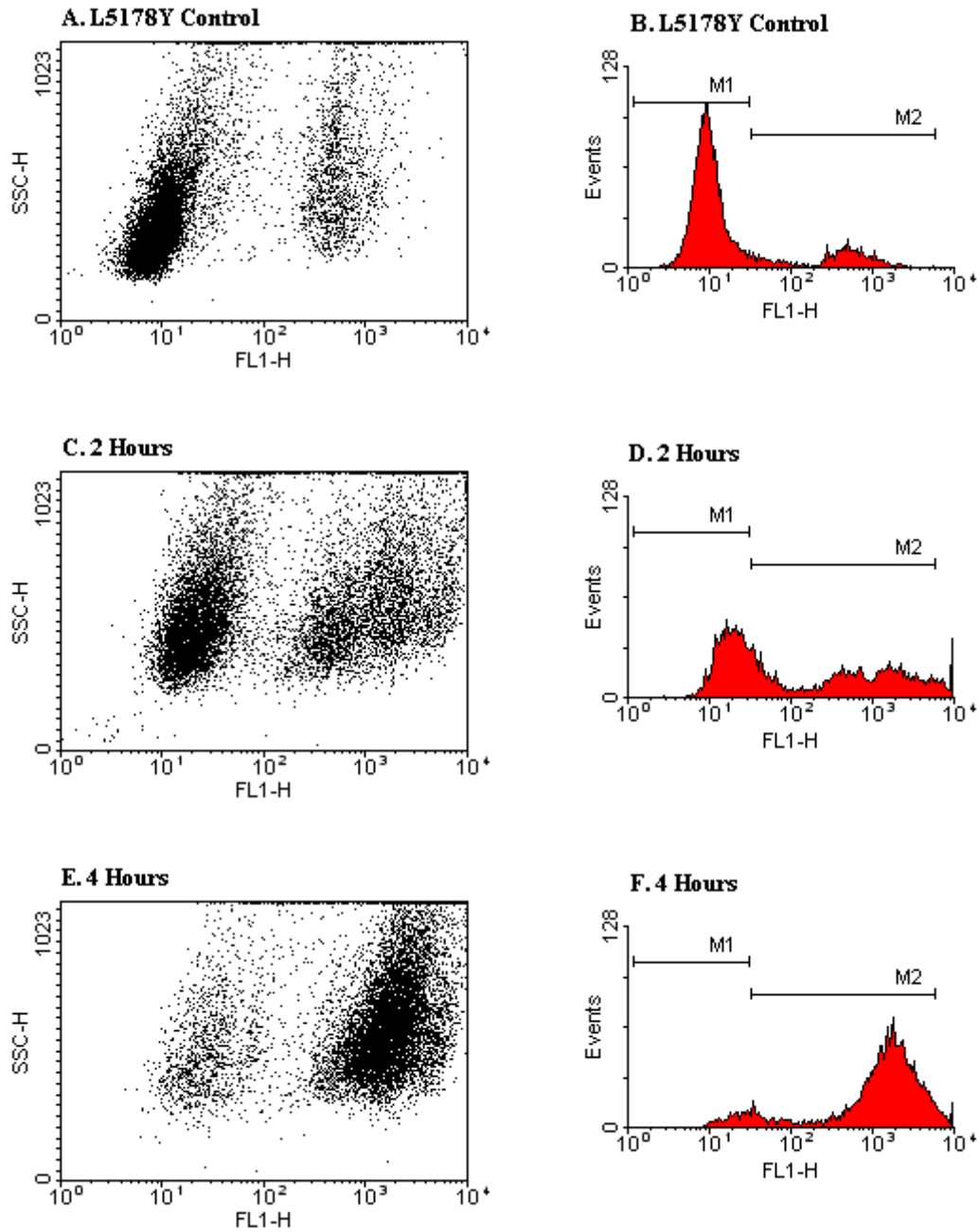
Figure 3.9. Apoptosis measurement by the flow cytometric NucView Assay.

Pictures show representative data of L5178Y control (A, B), cells after 2 hr post heat-shock (C, D) and cells after 4 hr post heatshock (E, F). M1 is the caspase-3 negative region and M2 is the caspase-3 positive region. Apoptosis is expressed as a percentage of M2/(M1+M2).
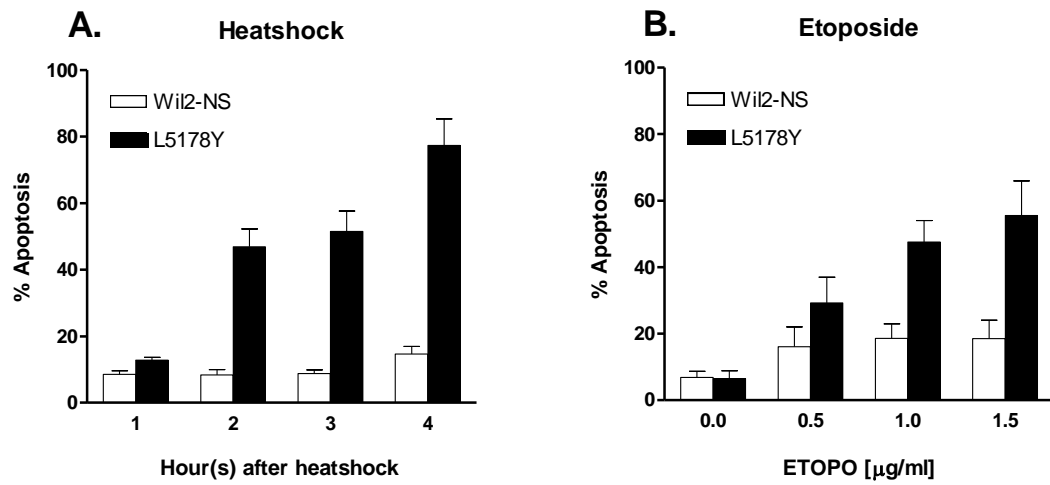
Figure 3.10. Comparisons of apoptosis responses between WIL2-NS and L5178Y.

Apoptosis levels were measured by theflow cytometric NucView assay following heatshock treatment (A), exposure to etoposide, ETOPO (B). Histograms show mean ± SD of data taken from 2 independent experiments.

3.4. Discussion

For use in an *in vitro* micronucleus assay, it is required that cells must complete at least 2 - 2.5 cell cycles (cell doublings) before commencing the measurement of micronuclei (OECD, 2004). Therefore, understanding the population doubling time of the cells was the first step in conducting in this study. Under the optimal cell culture conditions, both WIL2-NS and L5178Y cell lines were shown to have estimated doubling times of 10 – 12 hours, hence they completed at least 2 cell cycles within the 24 hour treatment protocol used in this study.

An advantage of the FACSCalibur™ system is the sorting facility, which allows isolation of certain particles for further studies. This application was used to confirm that the particles in the defined MN gate are micronuclei and those in the defined N gate are nuclei. The results observed in this study showed that the gating criteria described in section 3.2.3.2 and Figure 3.5 were consistent with the information obtained from previous studies (Nüsse *et al.*, 1994; Roman *et al.*, 1998). It is noted that some nuclear blebs were observed in both nuclei and micronuclei, which could have resulted from ruptures of nuclear envelops during the sorting and preparation process.

Preliminary studies in this project had shown that when highly cytotoxic concentrations were used in the FCMN assay, substantial interference from cellular debris or DNA artefacts made the interpretation of the FCMN results in MN regions very difficult. It was also observed that (1) when the concentrations below EC30 were tested the FCMN gave positive results in a concentration-dependent manner and that (2) the FCMN results were similar to the results from microscopy. Hence, in this study EC30 was used as

the top concentration for the FCMN assay. The same principle was also applied in a study of the Comet assay described by Henderson et al. (1998).

Both cell lines demonstrated concentration-dependent cytotoxic responses to all genotoxicants with the exception of the treatment of WIL2-NS cells with BaP, where no significant cytotoxicity was observed. Since a significant increase in genotoxicity was seen in WIL2-NS at 12-24 µg/ml and the solubility of BaP was very limited in culture medium, concentrations above 24 µg/ml were not included in our study.

MN induction in L5178Y and WIL2-NS cell lines was first compared using the FCMN assay. It was found that both cell lines were responsive to the genotoxicants tested. Significant increases in MN formation demonstrated a concentration-dependent response from genotoxic damage caused by a range of genotoxic mechanisms i.e. breakage of DNA strands (MMC, MMS, ETOPO and BaP) or inhibition of spindle fibre function (VINB) that affects chromosome segregation. The only exception was following MMS exposure where in the L5178Y cell line, an increase in MN formation at low concentrations (20 – 40 µg/ml), but instead a decrease in MN at high concentrations (60 – 80 µg/ml) was observed. The reduction of MN frequencies at high MMS concentrations (60 – 80 µg/ml) could be due to the fact that these concentrations were highly cytotoxic to the cells but was not shown by MTS assay. In addition, since MTS assay measures metabolic function of the cells, an increase in MTS cell viability following treatment with low MMS concentrations (i.e. 20 – 40 µg/ml) could possibly be a consequence of compensation for the disruption of cellular homeostasis caused by this chemical (Calabrese, 2001a; Calabrese, 2001b; Calabrese and Baldwin, 2001).

To determine the specificity of the FCMN assay, MN frequencies obtained from flow cytometry were compared with microscopy counting. As it was expected that scoring of MN by flow cytometry would give a higher result compared with the microscopy due to methodological bias (a cell containing 3 MN is normally scored as 1 positive unit by microscopy, but is scored as 3 positive units by flow cytometry) (Nüsse and Kramer, 1984), in this study MN frequencies by microscopy were calculated and expressed as percentages of total MN over total nuclei counts. Using this approach, if a disagreement of results was found, it would have indicated false positive results from the FCMN assay, which could be from necrotic/apoptotic nuclei. Overall, at the range of concentrations tested, the MN results measured by FCMN and microscopy were demonstrated to be similar.

The only obvious discrepancy was seen in WIL2-NS cells following the treatment with high concentration of ETOPO (0.45 µg/ml). It is worth noting that after treatment with ETOPO, WIL2-NS showed unusual morphology i.e. enlarged cells with multiple micronuclei and nuclei, which made microscopic scoring of MN very difficult and therefore it was possible that the results from microscopy were underestimated. However, the purpose of this study was to establish a micronucleus-based genotoxicity screening method to be used to look for unknown genotoxins in complex mixtures or genotoxicity in novel candidate compounds. In this context, the method does not need to produce results that quantitatively correspond with the responses observed using other counting methods, but rather positives and negatives should be detected at much the same concentrations of test compound that they are in more established methods.

For the investigation of apoptosis responses between the two cell lines, we found that WIL2-NS cells did not show as high levels of apoptosis as the L5178Y cells. The results demonstrating low apoptosis response on WIL2-NS cells lines in this study are consistent with those reported in the literature (Amundson *et al.*, 1993; Umegaki and Fenech, 2000; Xia *et al.*, 1994; Xia *et al.*, 1995). This advantage should be considered for selection of the cell line for the FCMN assay.

In conclusion, this Chapter described an establishment of the FCMN assay. For assay validations, two cell lines (WIL2-NS and L5178Y) were investigated using genotoxicants with various genotoxic mechanisms. Following exposure to the test chemicals, concentration-dependent responses were observed. The studies have shown that both cell lines can be used in the FCMN assay. However, when apoptosis responses of the cells were compared, it was found that WIL2-NS cells did not undergo apoptosis to the same extent as L5178Y cells. Since apoptotic nuclei are a major interference in the FCMN assay, we concluded that, of the two cell lines investigated, WIL2-NS is a more suitable cell line to use in the FCMN assay for rapid genotoxicity screening of unknowns.

Conclusion remark: The study described in this Chapter has been published in

Laingam S, Froscio SM and Humpage AR. Flow-cytometric analysis of *in vitro* micronucleus formation: Comparative studies with WIL2-NS human lymphoblastoid and L5178Y mouse lymphoma cell lines. Mutat. Res. 2008: 656 (1-2): 19-26.

4.   PRODUCTION OF ORGANIC N-CHLORAMINES

4.1.   Introduction

The organic N-chloramines chosen for toxicity and genotoxicity studies were based on the recommendation of Bull *et al.* (2006) and subsequent discussions (Bull 2007, personal communication). The compounds chosen, in most cases, are predicted to have high probability of being produced in disinfected drinking water based on the occurrence of precursors. The initial list includes 21 compounds (Appendix 2, A2-1), most of which are products from chlorination of amino acids and amines. However, since these compounds are not commercially available they need to be produced freshly prior to use and at the time of study 5 of the 21 candidates were not feasibly synthesised in our laboratory. Therefore, only 16 organic N-chloramine candidates were used as model compounds for genotoxicity investigation of organic N-chloramine in this study. The list of these compounds and chemical structures of their precursors are shown in Table 4-1 and Figure 4.1, respectively.

Table 4-1. List of the organic N-chloramine candidates and their precursors

No.	Organic N-chloramines	Amine Precursors	CAS No.
1	N- chloroleucine	L-Leucine	61-90-5
2	N- chloroisoleucine	L-Isoleucine	73-32-5
3	N- chloroglycine	L-Glycine	56-40-6
4	N- chlorophenylalanine	L-Phenylalanine	63-91-2
5	N- chloroserine	L-Serine	56-45-1
6	N- chlorohistidine	L-Histidine	71-00-1
7	Taurine monochloramine	L-Taurine	107-35-7
8	N, N'- dichlorolysine	L-Lysine	56-87-1
9	N-chloro-α-acetyllysine	N_α-Acetyl-L-lysine	1946-82-3
10	N- chloro-ε-acetyllysine	N_ε-Acetyl-L-lysine	692-04-6
11	N- chloroarginine	L-Arginine	74-79-3
12	N- chlorohistamine	Histamine	51-45-6
13	N- chloroethanolamine	Ethanolamine*	141-43-5
14	N- chloroalanylphenylalanine	Alanylphenylalanine	3061-90-3
15	N- chloroglutamate	L-Glutamic acid	56-86-0
16	N, N'- dichlorocystine	L-Cystine	56-89-3

All reagents are of analytical grade.

Ethanolamine (*) is in liquid form, density = 1.012 g/ml at 25°C.

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┌─────────────────────────────────────────────────┐
│                     NOTE:                         │
│   This figure is included on page 78 of the print │
│   copy of the thesis held in the University of    │
│          Adelaide Library.                        │
└─────────────────────────────────────────────────┘
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Figure 4.1. Molecular structure of amino acids and amine precursors

used for production of organic N-chloramines. Pictures adopted from Sigma-Aldrich

product catalogue (http://www.sigmaaldrich.com/catalog/ProductDetail, accessed 3/03/09).

The production of the compounds of interest can be achieved by a simple reaction between chlorine and the precursors. In aqueous medium, chlorination of amino acids or amines yields the N-Cl-compounds by direct transfer of Cl from the O of the HOCl to the unprotonated N of the amino acid or amine as shown below (Armesto *et al.*, 1993; Donnermair and Blatchley, 2003). Under a controlled condition (i.e. pH, temperature and concentration of chlorine and precursors), the product formed can be directly used for subsequent assays (Nakamura *et al.*, 1993).

$$\begin{array}{c} R' \\ \diagdown \\ R'' \diagup \end{array} N\!-\!H \ + \ HOCl \ \longrightarrow \ \begin{array}{c} R' \\ \diagdown \\ R'' \diagup \end{array} N\!-\!Cl \ + \ H_2O$$
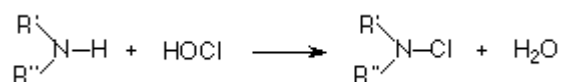
Amino acids (L-isomer) are used in this study for production of the N-chloramines as they are the major form present in natural water supplies (Abe *et al.*, 1993). Previous studies have reported mutagenicity of chlorination products of α-amino acids (Nakamura *et al.*, 1993; Süssmuth, 1982), while toxicity information of chlorinated products of amino acid containing 2 amino groups is not well understood. Nightingale (2000) reported that when the α-amino group of lysine is blocked, Nε dichloro lysine is formed during chlorination. It has been suggested that the Nε-dichloro lysine could decompose to free radicals that form protein-DNA cross links (Kulcharyk and Heinecke, 2001), indicating that this compound could be potentially genotoxic. Hence it was also included in this study.

Ethanolamine and histamine were included in the list for several reasons. Ethanolamine is abundant in decaying materials (Thomas, 1997), which could be present at high concentration in organic rich water. On the other hand, a high concentration of histamine is secreted (by mast cells) at inflammatory sites and is likely to be chlorinated by HOCl produced from oxidation of chloride in activated neutrophils at sites of inflammation

(Bull *et al.*, 2006; Peskin and Winterbourn, 2003; Thomas, 1979). Both chlorinated products of ethanolamine and histamine demonstrated a potent mutagenic activity in bacteria (Thomas *et al.*, 1987), but their genotoxicity in mammalian cells is not known.

To provide significant information for risk characterisation of the organic N-chloramines, the test concentration range of the organic N-chloramines should be comparable to those that might be present in actual disinfected waters. Since there have been no reports on the individual organic N-chloramine in the literature (Westerhoff and Mash, 2002), the chosen test concentration range has to be estimated from a predicted level of its precursor. Previous studies have suggested that micromolar concentrations of amino acids could be found in natural waters (Nweke and Scully Jr, 1989; Ram and Morris, 1980) and that they are not necessarily removed during water treatment process but instead they could also be released from microorganisms during water treatment process i.e. filtration (Nagata and Kirchman, 1990), resulting in them being present at the disinfection step (Rice and Gomez-Taylor, 1986). Therefore, it is predicted that organic N-chloramines produced could be found in micromolar concentration range too.

This chapter describes the production of organic N-chloramines by chlorination of amino acids and several amines. The reaction time and concentrations of amino acid; and amine precursors (in micromolar concentration range) were optimised. The optimal condition was then selected for further production of organic N-chloramines for further toxicity and genotoxicity investigations, which are detailed in the following Chapters.

4.2.   Materials and Methods

4.2.1.   Chemicals and Reagents

Chlorine Solution

Chlorine stock solution was prepared by bubbling chlorine gas in 1 L Milli-Q water to reach an approximate concentration of 3,000 mg/L and left to stabilise at 4ºC for 24 hr before use. Prior to each experiment, the chlorine stock solution was diluted 1/1000 in Milli-Q water and the chlorine concentration measured by the DPD/FAS titration method as described in section 4.2.3.

Preparation of Amino Acid/Amine Solution

All L-amino acids and amines (precursors of organic N-chloramines) were purchased from Sigma-Aldrich (MO, USA). The details are shown in Table 4-1. The stock solutions (100 mM), except ethanolamine, were prepared in sterile Milli-Q water and stored at 4°C for approximately 1 week. Histamine (100 mM) was stored at - 20°C. Ethanolamine was used as the concentrated liquid form supplied by Sigma-Aldrich (density = 1.012 g/ml). All solutions were equilibrated to room temperature prior to experiment.

4.2.2.   Production of Organic N-chloramines

Determination of Chlorine Contact Time

To select the optimal chlorine contact time, an experiment was set up by preparation of 100 µM of amino acid (or 828 µM for ethanolamine) solution in Hanks Balanced Salt Solution (HBSS) in a 1L Schott bottle and spiked with 4 mg/L chlorine solution. Samples (100 ml) were taken for chlorine residual measurement (using the method described in 4.2.3) at 1, 3, 5, 10 and 30 min.

Determination of Precursor Concentration

Organic N-chloramines were produced by chlorination of the amino acids and amine precursors. Briefly, 100 ml of amino acid/amine precursors (0 − 100 µM for all amino acids and histamine, 0 − 1657 µM for ethanolamine) was prepared in HBSS (pH 7.4) in a clean flask and spiked with 4 mg/L chlorine. Chlorination took place at room temperature for 3 min in the dark. Following the reaction time, chlorine residual was measured by the DPD/FAS method (section 4.2.3) and formation of mono-chloramine and di-chloramine was determined using the modified DPD/FAS protocol described in section 4.2.4.

4.2.3.   Measurement of Chlorine Residual

Chlorine residuals were determined using the standard DPD/FAS method for examination of chlorine in water and waste water (Eaton *et al.*, 1995). Briefly, 5 ml of buffer solution (6% w/v Na_2HPO_4, 9.5% w/v KH_2PO_4, 0.4% EDTA, pH 6.5) was added to 5 ml of N,N-diethyl-p-phenyl diamine (DPD) solution (0.55% w/v DPD, 0.1% w/v EDTA, 0.5% v/v H_2SO_4). The sample (100 ml) was then added to the solution and mixed, upon which a pink colour indicates the presence of free chlorine. The solution was then titrated to a colourless end-point with ferrous ammonium sulphate (FAS) solution (0.22% w/v FAS, 0.05% v/v H_2SO_4).

4.2.4.   Measurement of Chloramines

Organic N-chloramines were determined using a modified version of the DPD/FAS method (Eaton *et al.*, 1995). Briefly, 5 ml of buffer solution was added to 5 ml of DPD solution. The sample (100 ml) was then added to the solution and mixed. If the solution turned pink, free chlorine was present and the solution was titrated to the colourless end-point as described in the section above. If the solution remained colourless no free chlorine was present, upon which three drops of a 1.5% (w/v) potassium iodide solution was added. Potassium iodide acted to release chlorine bound as mono-chloramine turning the solution pink. The solution was then titrated back to the colourless end-point with FAS solution and the mono-chloramine residual concentration determined. The presence of di-chloramine was then determined by the addition of approximately 1 g solid potassium iodide following the mono-chloramine titration. This released chlorine bound as di-chloramine and turned

the solution pink if this chemical species was present. The solution was then titrated with

FAS solution to determine di-chloramine residual concentration.

4.3. Results

4.3.1. Optimal Chlorine Contact Time

Optimisation studies showed that reaction of chlorine with amines took place rapidly within the first few minutes of exposure (Figure 4.2). No chlorine residual was detected at 3 min post chlorination. Therefore, 3 min chlorine contact time was used in all further experiments.
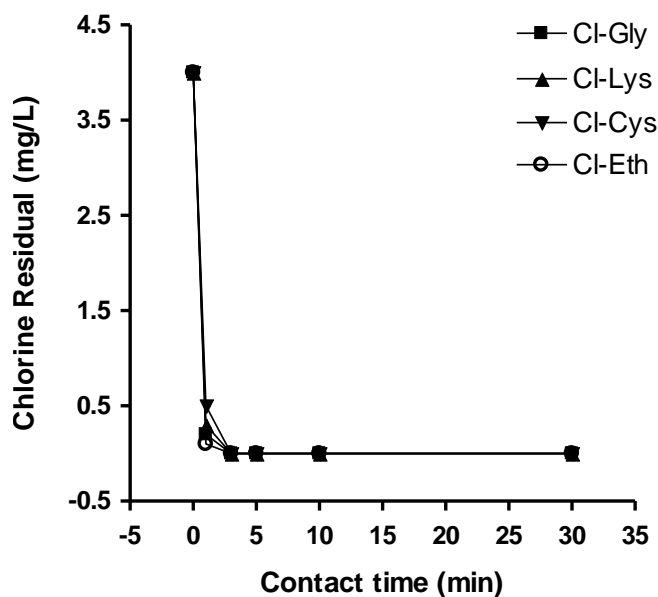
Figure 4.2. Amines and rate of chlorination.

Picture shows representative data of chlorinated glycine (Cl-Gly), lysine (Cl-Lys), cystine (Cl-Cys) and ethanolamine (Cl-Eth). Values on X-axis are time (min) of the reaction. Values on Y-axis are chlorine residual measured by the DPD/FAS method.

4.3.2. Optimal Precursor Concentration

Studies were carried out to determine the optimal concentration of amines required to produce mono-chloramine as the main product with minimal or no di-chloramine formation. Each of the amines was chlorinated (using 4 mg/L chlorine) over a 20 – 100 µM (or 165 – 1657 µM for ethanolamine) concentration range.

Figure 4.3 shows chlorination products of leucine, isoleucine, phenylalanine, glycine, serine, arginine, histidine and Nε- acetyl lysine. As seen, chlorine concentration decreased as amine concentration increased, and mono-chloramine was the only product formed. It was also found that the highest level of mono-chloramine was formed in the reaction containing 60 µM amines. There was no further increase of the mono-chloramine formation in the reaction containing > 60 µM amines.

Figure 4.4 shows chlorination products of glutamic acid, alanylphenylalanine, cystine, ethanolamine, histamine, taurine, lysine and Nα-acetyl lysine. While mono-chloramines are also the main product, di-chloramines at lower concentrations were also formed. It is noted that, except for the chlorination of ethanolamine, maximal mono-chloramine production was observed in the reaction of chlorine with the amine concentration at and above 60 µM. For the chlorination of ethanolamine, maximum mono-chloramine was seen at and above 828 µM.
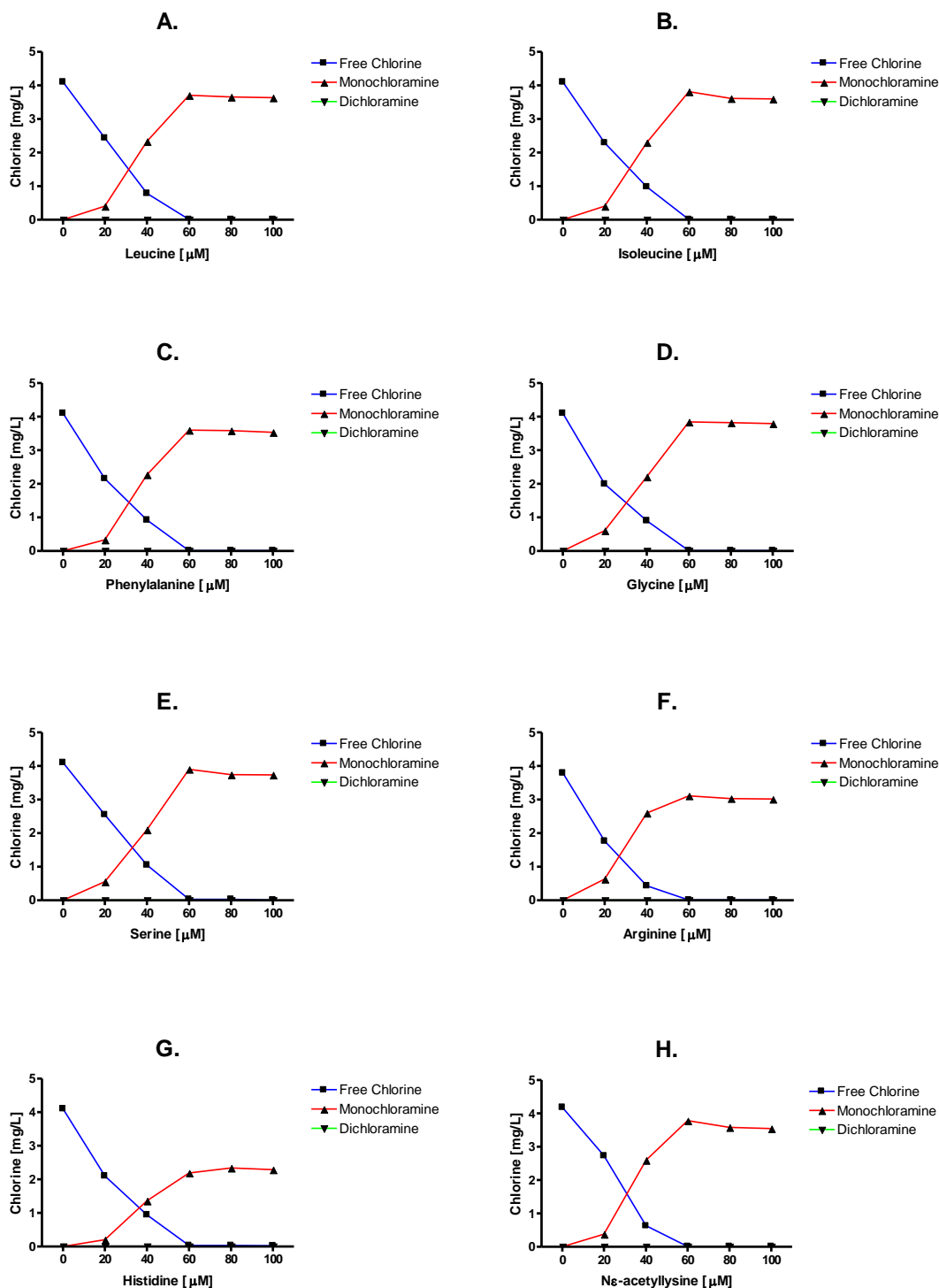
Figure 4.3. Production of organic N-chloramines (I)

Chlorination of leucine (A), isoleucine (B), phenylalanine (C), glycine (D), serine (E), arginine (F), histidine (G) and Nε-acetyl lysine (H) at pH 7.4. Amino acids prepared in HBSS, chlorinated with 4 mg/L chlorine, with 3 min contact time. Free chlorine, mono- and di-chloramines measured by DPD/FAS method.
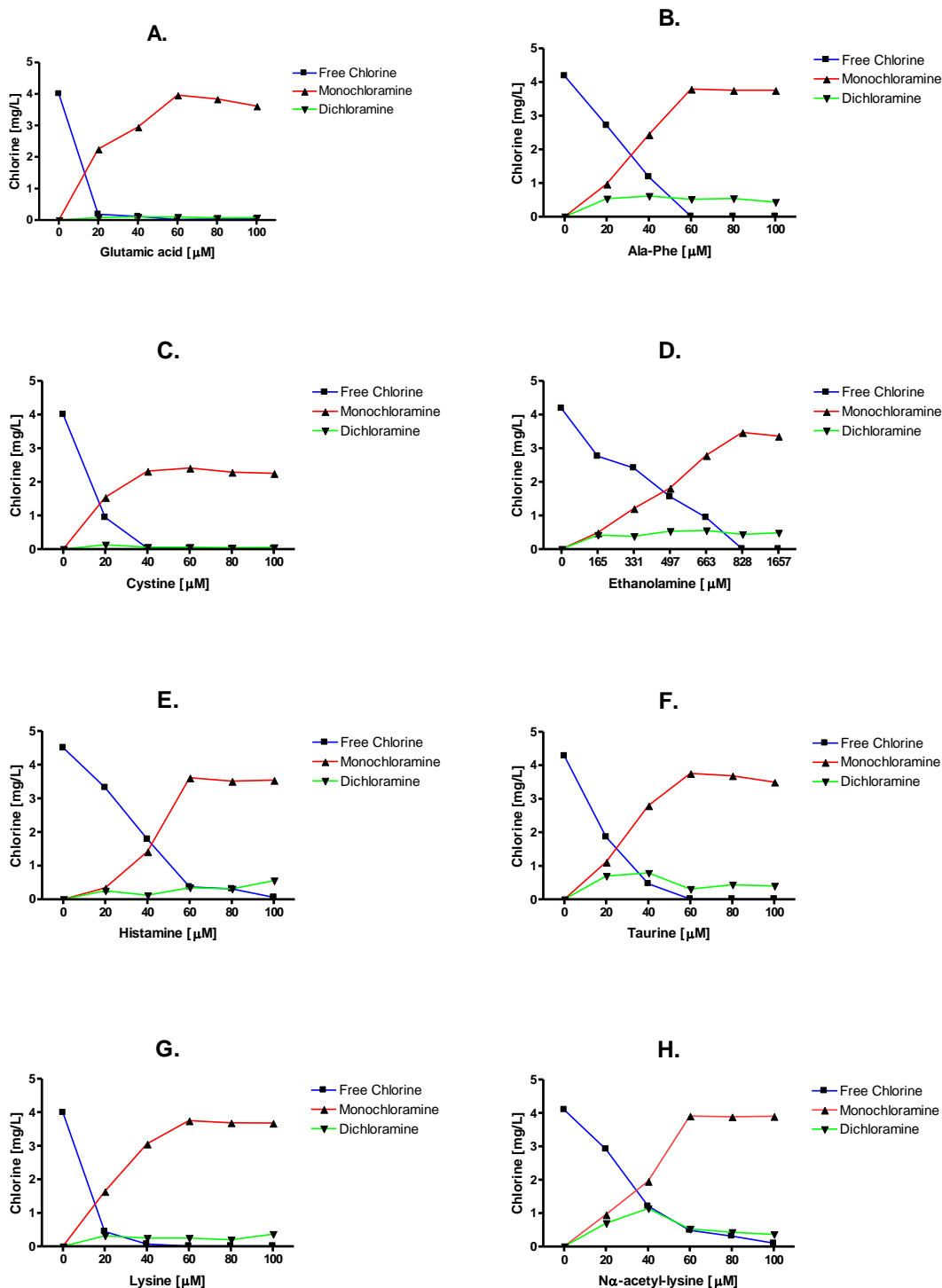
Figure 4.4. Production of organic N-chloramines (II)

Chlorination of glutamic acid (A), alanylphenylalanine (B), cystine (C), ethanolamine (D), histamine (E), taurine (F), lysine (G) and Nα-acetyl-lysine (H) at pH 7.4. Amino acids prepared in HBSS, chlorinated with 4 mg/L chlorine, with 3 min contact time. Free chlorine, mono- and di-chloramines measured by DPD/FAS method.

4.4.   Discussion

The formation of products from the reaction of chlorine with amino acids is known to be temperature, concentration and pH dependent (Deinzer *et al.*, 1978). All experiments were conducted under temperature- and pH-controlled conditions. The reaction conditions such as chlorine contact time and optimal concentration of the amine precursors were optimised. Reaction of chlorine with all amino acids and amines was shown to occur very rapidly, similar to results described in the literature (Fukayama *et al.*, 1986; Margerum *et al.*, 1979; Morris *et al.*, 1992). In addition, this study showed that at neutral pH, the amino acids and amines have similar reactivity with chlorine. This observation was also consistent with the results described in a previous study (Na and Olson, 2007).

For chlorination of amines, when the ratio of Cl:N is kept low, it is suggested that virtually all free chlorine is converted to mono-chloramine [i.e. 1:1 (N:Cl) molar ratio] (Abia *et al.*, 1998; Armesto *et al.*, 1994; Armesto *et al.*, 1993). Similarly, this study showed that mono-chloramines were predominant amongst all chlorinated products although low levels of di-chloramines were observed in some reactions (i.e. the chlorination of glutamic acid, alanylphenylalanine, cystine, ethanolamine, histamine, taurine, lysine and Nα-acetyl lysine). Since the aim was to select the optimal conditions to produce the organic N-chloramines for toxicity and genotoxicity testing in mammalian cell systems without further purification, the reaction containing di-chloramines may need to be taken into account when interpreting the observed outcomes.

In conclusion, this chapter described production of organic N-chloramines from chlorination of amino acids and amine precursors at neutral conditions. Chlorine contact time, concentrations of chlorine and the precursors were determined. This study has demonstrated optimal conditions for the production of organic N-chloramines. These conditions were chosen for further used to produce the compounds for cytotoxicity and genotoxicity testing, which will be addressed in the following Chapters.

5. TOXICITY AND GENOTOXICITY OF ORGANIC N-CHLORAMINES

5.1. Introduction

As described in previous Chapters, organic N-chloramines are predicted to be potential health risks (Bull *et al.*, 2006), but experimental toxicity investigations have been very limited. Although several compounds have been reported to be mutagenic in bacteria (Nakamura *et al.*, 1993; Süssmuth, 1982; Thomas *et al.*, 1987), information on their genotoxicity particularly on mammalian cells is not available. Furthermore, to investigate toxicity and genotoxicity of organic N-chloramines, selection of an appropriate concentration range is also important. Although the occurrence and fate of organic N-chloramines in disinfected waters is not available, based on the presence of precursors in natural waters (Ram and Morris, 1980; Thurman, 1985) it is predicted that organic N-chloramines in micromolar – millimolar concentration range could also be present in disinfected water (Bull *et al.*, 2006).

Because N-chloramines are reactive molecules, whose chlorine atom can transfer to other compounds, particularly in the culture medium (Antelo *et al.*, 1997; Ferriol *et al.*, 1991; Peskin *et al.*, 2004; Snyder and Margerum, 1982), a standard exposure protocol is not applicable for use in the toxicity and genotoxicity of the compounds. Midwinter *et al*. (2006) reported that the non-cell permeable taurine chloramine in HBSS had no effects on cellular toxicity, but in complete medium the effects were clearly observed, which suggested that cytotoxic effects were a consequence of chlorine exchange with other amines in the medium to form more permeable chloramines. Therefore, to minimise this

confounding factor, exposure to N-chloramines should be conducted in an amine-free medium.

This Chapter describes an assessment of the cytotoxicity and genotoxicity of organic N-chloramines using freshly produced organic N-chloramines (μM concentration range). The chloramine candidates were generated using the reaction conditions described in Chapter 4 (i.e. chlorination of 60 μM of all amines, except 828 μM for ethanolamine, with 4 mg/L chlorine at pH 7.4 for 3 min at room temperature in the dark). The cytotoxicity and genotoxicity screening protocol described in Chapter 3 was used, with some modification of the protocol to allow for short term exposure of the N-chloramines, and using an appropriate media as defined above. This modified treatment protocol was optimised and validated prior to use for the chloramine candidates and is also described in this Chapter.

5.2.   Materials and Methods

5.2.1.   Chemicals

Chlorine Solution

Chlorine stock solution was prepared (as described in Chapter 4) by bubbling chlorine gas in 1 L Milli-Q water to reach an approximate concentration of 3,000 mg/L and left refrigerated to stabilise at 4ºC for 24 hr before use. Prior to each experiment, the chlorine stock solution was diluted 1/1000 in Milli-Q water and the chlorine concentration measured by the DPD/FAS method as described in Chapter 4.

Organic N-chloramines

Organic N-chloramines were freshly produced using conditions described in Chapter 4. Briefly, samples (60 µM amines or 828 µM ethanolamine) were prepared in sterile Hanks Balanced Salt Solution, HBSS (Sigma-Aldrich, MO, USA) and chlorinated with 4 mg/L chlorine. Residual chlorine was measured using the DPD/FAS and mono-chloramines using the modified DPD/FAS methods (described in Chapter 4). All preparation steps were performed using aseptic technique.

5.2.2.   Cell Culture and Seeding Preparation

WIL2-NS cells were cultured and maintained as described in Chapter 3. One day prior to the experiment, cells were freshly passaged into a new flask and the culture continued for 24 hr. On the day of experiment, a cell count was performed using the TB exclusion assay (described in Chapter 3). Unless otherwise stated, the cells were washed once in HBSS and a 5.0 x 10^5 cells/ml suspension prepared in HBSS. One millilitre of the cell suspension was placed into each well of a 24-well tissue culture plate and cell recovery continued at 37°C, 5% CO_2 for 1 hr.

5.2.3.   Cell Treatment – Optimisation for Short Term Exposure

5.2.3.1.   Selection of Treatment Medium

Since chlorine transfer can occur between the N-chloramines to other amino acids in the culture medium (Antelo *et al.*, 1997; Ferriol *et al.*, 1991; Peskin *et al.*, 2004; Snyder and Margerum, 1982), the assay protocol described in Chapter 3 is not applicable for use in this study. Pilot studies were carried out to determine which treatment medium would be suitable for organic N-chloramine studies. Two treatment media (serum-free RPMI and HBSS) were investigated.

It was hypothesised that chlorination of treatment medium would reduce free available chlorine, hence decreasing cytotoxicity. To select an appropriate medium for use in N-chloramines studies, WIL2-NS cells (5.0 x 10^5 cells/ml) were prepared in 1) HBSS

and 2) RPMI, of which, 100 μl was placed into each well of a 96-well tissue culture plate. The cell recovery continued at 37°C, 5% CO^2 for 1 hr. One hundred microlitres of chlorine solution (serially diluted in the appropriate medium, either HBSS or RPMI) was added into each well. The cells were gently mixed and exposure continued at 37°C, 5% CO_2 for 3 hr. Following the 3 hr exposure, cytotoxicity was determined using the MTS assay as described in Chapter 3.

5.2.3.2.   Comparison between Short Term Exposure vs. Continuous Exposure

Experiments were also conducted to ensure that the short-term exposure protocol in HBSS medium would give genotoxicity results that are comparable to those obtained from the continuous exposure protocol as described in Chapter 3. To investigate this, MMS was used as a model genotoxicant. Two experimental protocols (short term and continuous) were run in parallel as described below.

Short-term Exposure

One millilitre of cell suspension (5.0×10^5 cells/ml prepared in HBSS) was placed in each well of a 24-well tissue culture plate and the cell recovery continued at 37°C, 5% CO_2 for 1 hr. Following the cell recovery, 1 ml of MMS (2X working stock i.e. 10 μg/ml, 20 μg/ml, 30 μg/ml and 40 μg/ml prepared in HBSS) was added into each well. The cells were mixed and incubation continued at 37°C, 5% CO_2 for 3 hr. Cells were washed once in HBSS, resuspended in 2 ml complete medium and placed back in a new 24-well tissue culture plate. Cell culture continued at 37°C, 5% CO_2 for 21 hr. The cells were harvested and genotoxicity determined using the FCMN assay as described in Chapter 3.

<u>*Continuous Exposure*</u>

One millilitre of cell suspension (5.0 x 10^5 cells/ml prepared in complete medium) was placed in each well of a 24-well tissue culture plate and the cell recovery continued at 37°C, 5% CO_2 for 1 hr. Following the cell recovery, 1 ml of MMS (2X working stock i.e. 10 µg/ml, 20 µg/ml, 30 µg/ml and 40 µg/ml also prepared in complete medium) was added into each well. The cells were mixed and incubation continued at 37°C, 5% CO_2 for 24 hr. The cells were harvested and genotoxicity determined using the FCMN assay as described in Chapter 3.

5.2.4. Cell Treatment for Genoxicity Studies of N-chloramines

Since organic N-chloramines are direct acting mutagens (Nakamura *et al.*, 1993; Scully and Bempong, 1982; Süssmuth, 1982), a short-term exposure protocol without S9 metabolic activation system was used for all experiments with organic N-chloramines in this current study. Cell treatment was performed in a 24-well tissue culture plate. A summary of the experimental set-up is shown in Figure 5.1. Briefly, 0.25, 0.50 and 1 ml of freshly produced organic N-chloramines was added directly into each appropriate well containing 1 ml of cell suspension. HBSS was then added into the first and second wells to give a final volume of 2 ml per well, thus giving the final organic N-chloramine dilutions of 1/8, 1/4 and 1/2, which were approximately equivalent to concentrations of 7.5 µM, 15 µM and 30 µM for all N-chloramines except N-chloroethanolamine, which were 103.5 µM, 207 µM and 414 µM, respectively. The cells were mixed and plates were incubated at 37°C, 5% CO_2 for 3 hr. Following the 3 hr treatment, cells from each well were transferred into a 1.5 ml eppendorf tube and washed once with HBSS. The cells were then

resuspended in 2 ml complete medium and placed back in a well of a new 24-well tissue culture plate. The cells were grown for 21 hr, 37°C, 5% CO_2 prior to harvest for cytotoxicity and genotoxicity measurements.

# 1 (1/8)	# 1 (1/4)	# 1 (1/2)		Pos. control	Pos. control
1 ml Cell 0.25 ml sample 0.75 ml HBSS	1 ml Cell 0.50 ml sample 0.50 ml HBSS	1 ml Cell 1 ml sample		1 ml Cell 1 ml sample	1 ml Cell 1 ml sample
# 2 (1/8)	# 2 (1/4)	# 2 (1/2)			
1 ml Cell 0.25 ml sample 0.75 ml HBSS	1 ml Cell 0.50 ml sample 0.50 ml HBSS	1 ml Cell 1 ml sample			
# 3 (1/8)	# 3 (1/4)	# 3 (1/2)		Neg. control	Neg. control
1 ml Cell 0.25 ml sample 0.75 ml HBSS	1 ml Cell 0.50 ml sample 0.50 ml HBSS	1 ml Cell 1 ml sample		1 ml Cell 1 ml sample	1 ml Cell 1 ml sample
# 4 (1/8)	# 4 (1/4)	# 4 (1/2)			
1 ml Cell 0.25 ml sample 0.75 ml HBSS	1 ml Cell 0.50 ml sample 0.50 ml HBSS	1 ml Cell 1 ml sample			

Figure 5.1. Experimental plan of chemical treatment in a 24-well tissue culture plate. At least 4 test samples (3 dilutions: 1/8, 1/4 and 1/2) were tested in each run. Duplicates of positive control (20 μM MMS) and negative control (no-treatment control) were also included in each experiment.

5.2.5.   Measurement of Cytotoxicity by MTS assay

The MTS assay was used to determine cytotoxicity of the organic N-chloramines as described in Chapter 3. Samples were assayed in triplicate. Cytotoxicity was expressed as a percentage reduction of MTS cell viability compared with the no treatment control.

5.2.6.   Measurement of MN Formation by Flow Cytometry

Six hundred microlitres of the cells from each well was transferred into a 1.5 ml eppendorf tube. The cells were centrifuged at 100 g for 5 min and supernatant removed. FCM solution 1 (600 µl) was added into the tube, gently mixed and placed in the dark at room temperature for 1 hr. Following this time, 600 µl of FCM solution 2 was added directly into the tube, the incubation continued in the dark at room temperature for 30 min prior to the measurement of MN by flow cytometry as described in Chapter 3.

5.2.7.   Measurement of MN Formation by Microscopy

The remaining cells in each well of the 24-well plate (approximately 800 µl) were used for preparation of slides for MN measurement by microscopy. The cells were collected in a 1.5 ml eppendorf tube, centrifuged at 100 g for 5 min and supernatant removed. The cell pellet was mixed in 5 µl FBS and thinly smeared onto 2 glass slides. The slides were air-dried, fixed in absolute methanol and stained with Dip-Quik. The stained slides were stored at room temperature in the dark until analysis. Microscopic counting of MN was conducted under a light microscope (1000X magnification). Approximately 1000

cells were counted per slide. MN formation was expressed as a percentage of MN containing cells over the total cell counts.

5.2.8. Statistical Analysis

All statistical analyses and graphing were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). Unless otherwise stated, data plots represent mean ± SEM of 3 independent experiments. Nonparametric one-way ANOVA (Kruskal-Wallis Test) followed by Dunns post test was used to determine significant differences of treatments from negative controls. Non-parametric Spearman's coefficient (r) was used to assess the degree of correspondence of the micronucleus frequency results measured by flow cytometry and by microscopy. Student t-test was used to compare a significant different between two experimental groups. Statistical significance was accepted at $p < 0.05$.

5.3. Results

5.3.1. Short-term Exposure to N-chloramines

5.3.1.1. Selection of Treatment Medium

Figure 5.2 shows cytotoxic effect of chlorine when experiments were conducted in RPMI (amine-rich medium) or HBSS (amine-free medium). The EC50 was used to compare toxicity of chlorine in each medium. It was found that EC50 of chlorine in RPMI was 38.5 mg/L compared with 7.9 mg/L in HBSS. This suggested that a large amount of chlorine has been used for chlorination of various amine-compounds (amino acids) in RPMI, which could potentially cause misinterpretation when investigating genotoxicity of the candidate N-chloramines. In addition, it is noted that in HBSS medium, toxicity of chlorine was not observed at or below 0.4 mg/L chlorine.

To further determine whether HBSS had an effect on cells, the viability of WIL2-NS cells following 3 hr suspension in HBSS was compared with the cells in complete medium, using the TB exclusion assay. There was no difference in percentage viability of WIL2-NS cells in HBSS for 3 hr compared with the cells in complete medium (93.9 ± 2.4% and 95.4 ± 1.3%, respectively), suggesting that HBSS can be used for 3 hr exposure protocol for genotoxicity studies of organic N-chloramines.
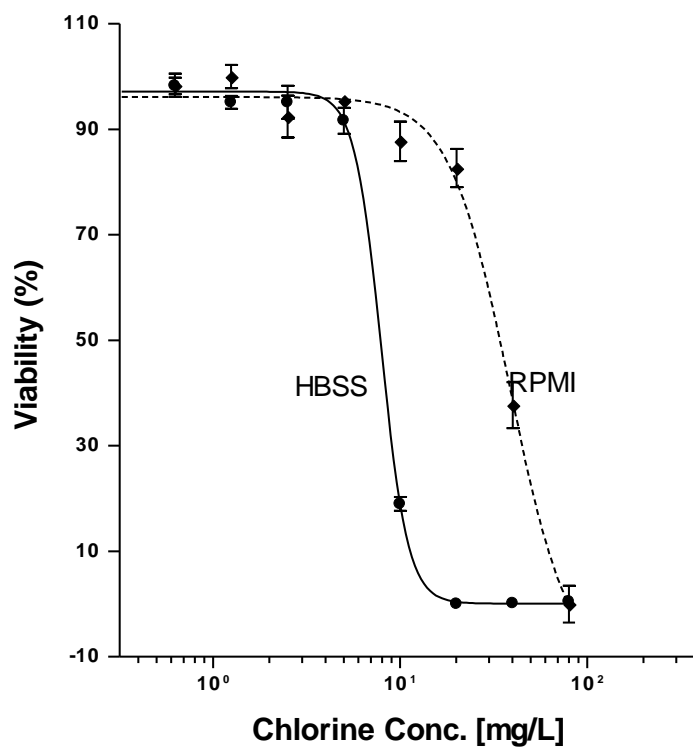
Figure 5.2. Comparison of cytotoxicity of chlorine in serum-free treatment media (HBSS vs. RPMI). RPMI contains various nutrients eg. amino acids (organic N-compounds) while HBSS contains only basic salts without N-compound. Cytotoxicity was measured using MTS assay. EC50 of chlorine in HBSS is 7.9 mg/L and in RPMI is 38.5 mg/L. Picture shows data of mean ± SEM, n =3.

5.3.1.2. Comparison of Continuous vs. Short-term Exposure Treatment

Evaluation of the short term exposure was performed by comparison of toxicity and genotoxicity responses of WIL2-NS cells following exposure to a genotoxicant (MMS). The results are shown in (Figure 5.3). Both treatment protocols gave a similar pattern in terms of reduction of viability ($r = 1.000$, p = 0.0165, Spearman correlation). Genotoxicity results obtained from the short-term treatment were also similar to those from the 24 hr continuous treatment ($r = 1.000$, $p = 0.0167$, Spearman correlation).
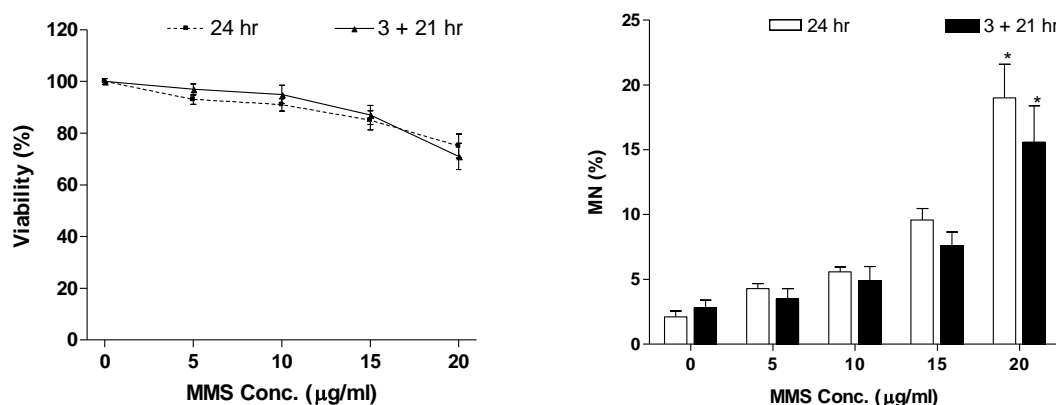
Figure 5.3. Comparative results between 24 hr and 3 hr exposure protocols.

Data are mean ± SEM of 3 experiments. Cytotoxicity (left) is determined using MTS assay and expressed as a percentage reduction of viability compared with the no-treatment control. Genotoxicity (right) is measured by the FCMN assay. Asterisk (*) represent a statistically significant genotoxicity from the untreated control at $p < 0.05$ (Kruskal-Wallis with Dunns post test).

5.3.2. Cytotoxicity of Organic N-chloramines

Cytotoxicity results of organic N-chloramines are summarised in Figure 5.4 and Table 5-1. Figure 5.4 shows concentration-dependent cytotoxic responses of MMS (positive control) and 5 organic N-chloramines. Statistically significant (One-way ANOVA, Kruskal-Wallis test) cytotoxic responses were observed in N-chloroglycine ($p = 0.0169$), N-chloroethanolamine ($p = 0.0249$), N-chlorohistamine ($p = 0.0188$) and N-chloro lysine ($p = 0.0116$). Although some cytotoxicity was observed at 15 µM and 30 µM in N-chloro- α-acetyllysine (Figure 5.4 G.), it was not shown to be statistically significant ($p = 0.0956$).

As shown in Table 5-1, cytotoxicity was not observed in N-chloroleucine ($p = 0.6389$), N-chloroisoleucine ($p = 0.8847$), N-chlorophenylalanine ($p = 0.9541$), N-chloroserine ($p = 0.6282$), N-chloroarginine ($p = 0.5511$), N-chlorohistidine ($p = 0.9368$), N-chloro-ε-acetyllysine ($p = 0.9207$), N-chloroglutamic acid ($p = 0.7329$), N-chloroalanylphenylalanine ($p = 0.9894$), N-chlorocystine ($p = 0.9896$) and N-chlorotaurine ($p = 0.9941$).

Further experiments using unchlorinated samples were conducted to confirm that the cytotoxicity observed in N-chloroglycine, N-chloroethanolamine, N-chlorohistamine and N-chlorolysine were a consequence of the chloramines, not the parent amines. Following exposure, no cytotoxicity was observed in WIL2-NS cells treated with 30 µM of glycine, histamine, lysine and 414 µM ethanolamine (Table 5-2).
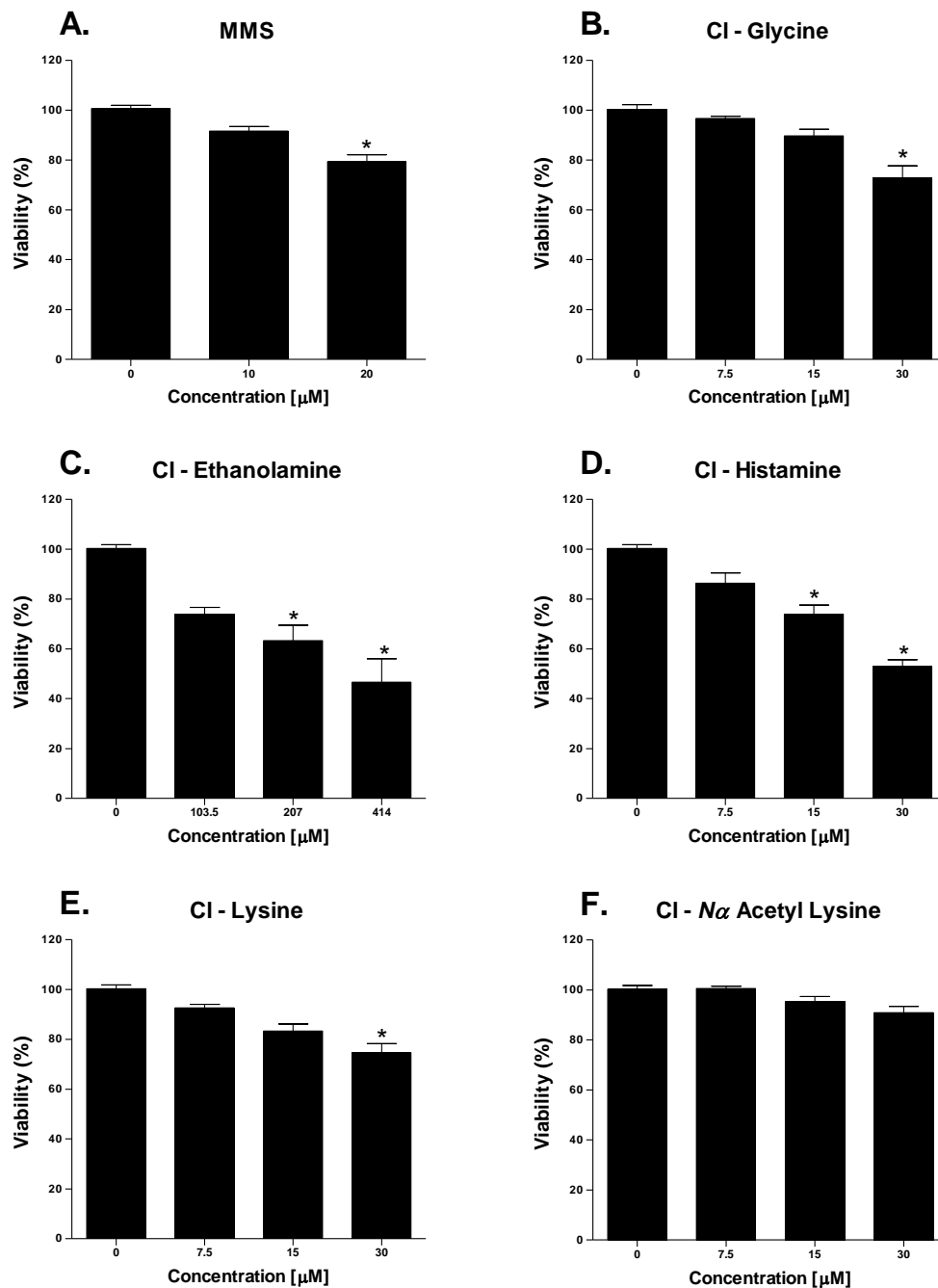
Figure 5.4. Cytotoxicity of organic N-chloramines.

MMS is a positive control. Data are mean ± SEM of 3 independent experiments. Kruskal-Wallis followed by Dunn's post test was used to determine statistically significant different of the test from the untreated control, $p < 0.05$ (*).

Table 5-1. Cytotoxicity of organic N-chloramines

Sample	Viability (%)	*p* value
Control (untreated)	100 ± 0.5	
Cl-Leucine	101 ± 1.2	0.6389
Cl-Isoleucine	99 ± 1.6	0.8847
Cl-Phenylalanine	101 ± 1.5	0.9541
Cl-Serine	99 ± 2.0	0.6282
Cl-Arginine	99 ± 2.3	0.5511
Cl-Histidine	100 ± 1.5	0.9368
Cl-Nε Acetyl Lysine	100 ± 1.8	0.9207
Cl-Nα Acetyl Lysine	96 ± 2.5	0.0956
Cl-Glutamic acid	100 ± 1.5	0.7329
Cl-Alanylphenylalanine	100 ± 2.6	0.9894
Cl-Cystine	101 ± 2.1	0.9896
Cl-Taurine	100 ± 1.7	0.9941

Data are percentages (Mean ± SEM, n =3) of viability of the maximum test concentration (30 µM). p values were obtained from statistical analyses using nonparametric One-way ANOVA (Kruskal-Wallis test).

Table 5-2. Determination of cytotoxicity of the pre-chlorinated amines

Sample	% Viability (mean ± SEM)	Significance (*t*-test)
Control (untreated)	100 ± 0.5	-
30 µM Glycine	98.9 ± 0.8	ns (*p* = 0.9306)
414 µM Ethanolamine	98.7 ± 0.9	ns (*p* = 0.0940)
30 µM Histamine	99.1 ± 0.9	ns (*p* = 0.2046)
30 µM Lysine	99.3 ± 0.4	ns (*p* = 0.1312)

5.3.3.   Genotoxicity of Organic N-chloramines

Measurement of MN by flow cytometry (FCMN) showed a concentration-dependent increase in MN formation with MMS (positive control) and 5 organic N-chloramines as shown in Figure 5.5. Statistically significant (one-way ANOVA: Kruskal-Wallis test) genotoxic responses were observed following treatment with MMS (p = 0.0273), N-chloroglycine (p = 0.0241), N-chloroethanolamine (p = 0.0273), N-chlorohistamine (p = 0.0379) and N-chlorolysine (p = 0.0241). Although a slight increase in MN was observed in the treatment with N-chloro-α-acetyllysine (Figure 5.5 F), this was not statistically significant (p = 0.2361).

Measurement of MN by microscopy confirmed the results from the FCMN that a concentration-dependent increase in MN was observed in MMS and the organic N-chloramines (Figure 5.5 A-E), except N-chloro-α-acetyllysine (Figure 5.5 F). Statistical analyses demonstrated a significant increase in MN in the treatment with MMS (p = 0.0270), N-chloroglycine (p = 0.0273), N-chloroethanolamine (p = 0.0265), N-chlorohistamine (p = 0.0211) and N-chlorolysine (p = 0.0249).

Of the 16 compounds tested, 12 did not show a statistically significant increase in MN formation. The results of MN frequencies measured by flow cytometry and microscopy of the highest tested concentration including the statistical p values are summarised in Table 5-3.
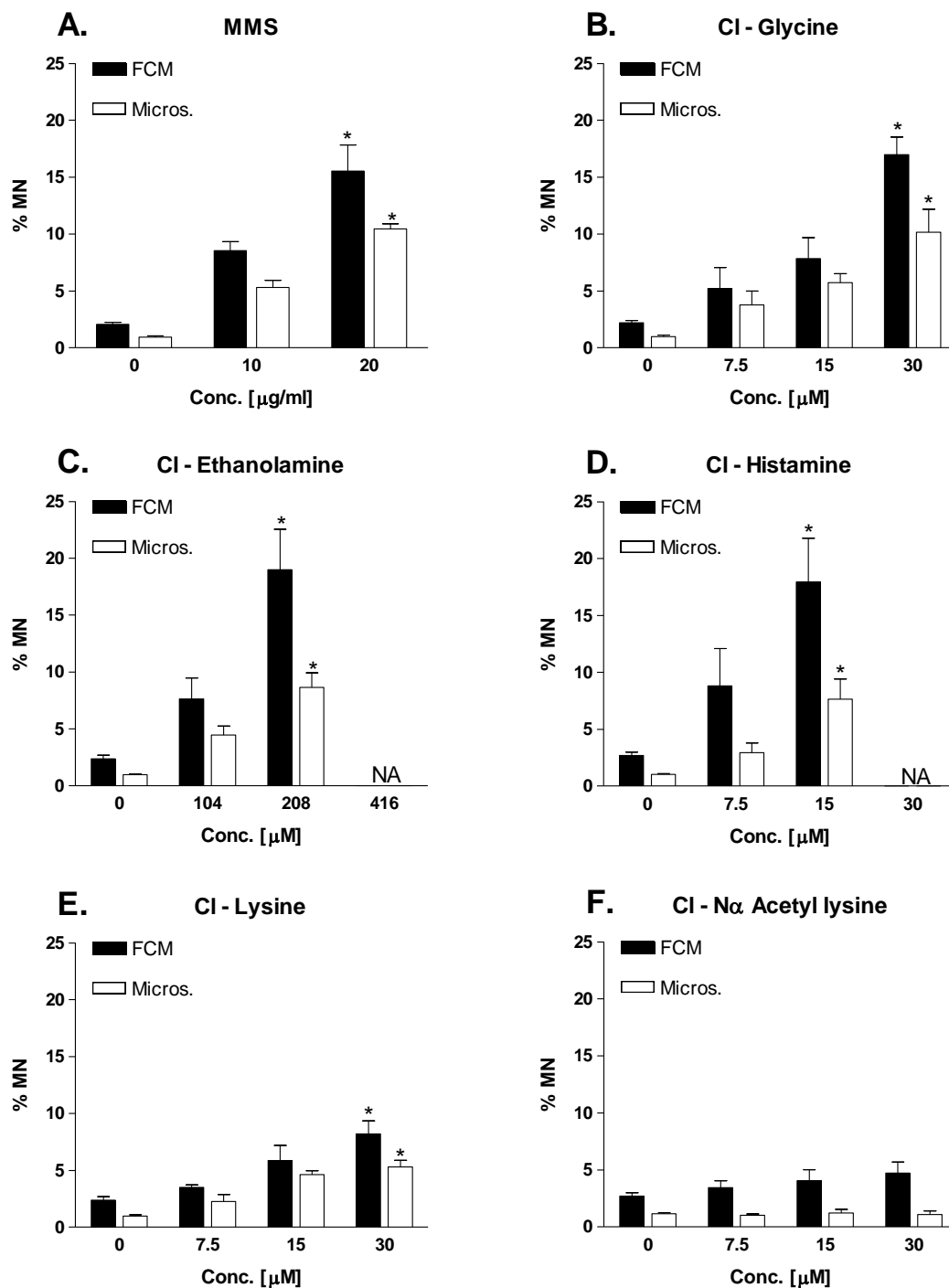
Figure 5.5. Genotoxicity of organic N-chloramines

by FCMN and microscopy based MN assay. MMS is a positive control. Data present are mean ± SEM of 3 independent experiments. Asterisk (*) indicates statistical significance (*p* < 0.05) of the "test" compared with the "no-treatment control". NA = not analysed (highly cytotoxic concentration).

Table 5-3. Genotoxicity of organic N-chloramines

Sample	% MN (FCM)	*p* value	% MN (Micros.)	*p* value
Control (untreated)	2.1 ± 0.31		0.9 ± 0.25	
Cl-Leucine	3.0 ± 0.61	0.5748	2.1 ± 0.95	0.2950
Cl-Isoleucine	2.7 ± 0.68	0.7038	1.3 ± 0.46	0.5361
Cl-Phenylalanine	3.4 ± 0.59	0.1524	2.3 ± 0.77	0.5677
Cl-Serine	2.9 ± 0.64	0.4465	1.6 ± 0.91	0.5361
Cl-Arginine	3.2 ± 0.40	0.3311	1.5 ± 0.67	0.5677
Cl-Histidine	3.6 ± 0.65	0.0878	2.0 ± 0.71	0.4545
Cl-Nε Acetyl Lysine	3.1 ± 0.58	0.1324	1.5 ± 0.69	0.6306
Cl-Nα Acetyl Lysine	4.7 ± 1.60	0.2361	1.3 ± 0.23	0.5677
Cl-Glutamic acid	3.2 ± 0.72	0.6612	1.0 ± 0.43	0.6737
Cl-Alanylphenylalanine	3.5 ± 0.56	0.1845	1.4 ± 0.49	0.2950
Cl-Cystine	3.5 ± 0.73	0.0924	1.0 ± 0.23	0.8140
Cl-Taurine	3.5 ± 1.10	0.2081	1.6 ± 0.69	0.3838

Data are % MN (mean ± SEM, n = 3) by flow cytometry, determined at the maximum test concentration (30 µM.). *p* values were obtained from statistical analyses using nonparametric one-way ANOVA (Kruskal-Wallis test).

To confirm that the genotoxicity observed in N--chloroglycine, N-chloroethanolamine, N-chlorohistamine and *N*-chlorolysine were a consequence of the chloramines, not the parent amines, the FCMN assay was conducted using un-chlorinated samples. Following exposure, no genotoxicity was observed in WIL2-NS cells treated with 30 µM of glycine, histamine, lysine and 414 µM ethanolamine. The results showed that none of these were genotoxic to the WIL2-Ns cells (Table 5-4).

Table 5-4. Determination of genotoxicity of the pre-chlorinated amines

Unchlorinated Sample	% FCMN (mean ± SEM)	Significance (*t*-test)
Control (untreated)	2.1 ± 0.31	-
30 µM Glycine	2.7 ± 0.68	ns ($p = 0.1963$)
414 µM Ethanolamine	2.9 ± 0.51	ns ($p = 0.1763$)
30 µM Histamine	2.5 ± 0.38	ns ($p = 0.1890$)
30 µM Lysine	2.6 ± 0.41	ns ($p = 0.1713$)

ns = not statistically significant when compared with the untreated control

5.4.   Discussion

A modified version of a treatment protocol in buffer solution has been previously described in a mutagenicity study of N-chloramines (Maron and Ames, 1983). It was hypothesised that this assay principle could be applied for the mammalian genotoxicity assay too. Hanks balanced salt solution (HBSS) has been used in mammalian cell based assays to maintain the pH and osmotic balance as well as to provide the cells with water and essential inorganic ions. Our study showed that HBSS is an appropriate medium for use in exposure treatment of organic N-chloramines. WIL2-NS cells could be maintained in HBSS for 3 hr without affecting the cell viability. As N-chloramines are small reactive molecules that could easily enter the cells, a 3 hr exposure time is considered suitable for the treatment protocol. Validation using MMS confirmed that the short-term exposure protocol was comparable to continuous exposure for 24 hr.

Cytotoxicity and genotoxicity screening of the 16 organic N-chloramine candidates showed that 4 compounds (chlorinated products of glycine, lysine, ethanolamine and histamine) were both cytotoxic and genotoxic in WIL2-NS cells and the responses were concentration-dependent. The rank order of both cytotoxicity and genotoxicity effects (based on the estimated concentration tested and the observed outcomes) were N-chlorohistamine > N-chloroglycine > N-chlorolysine > N-chloroethanolamine. The results reported here are consistent with previous studies using bacterial mutagenic assays (Nakamura *et al.*, 1993; Süssmuth, 1982; Thomas *et al.*, 1987).

When results are compared between chlorinated lysine, chlorinated-α-acetylate- and ε-acetylate-lysines, it was seen that significant effects occur only with chlorinated lysine. Low effects (but not statistically significant) occurred following chlorinated α-acetylated lysine and no effects in chlorinated ε-acetylated lysine. A significantly greater effect observed in the treatment of chlorinated lysine (N,Nᵋ-dichlorolysine) compared with its acetylated derivatives that prevent chloramines forming at one of the other of the amino group was interesting (see further discussion in Chapter 8). It was possible that the greater effects observed may indicate toxicological significance of number of chlorine atom in a molecule of N-chloramine once it enter the cells.

While chlorinated products of arginine, histidine, phenylalanine, and serine have previously been reported to be mutagens in bacteria (Nakamura *et al.*, 1993), our study showed that they are not genotoxic in WIL2-NS cells. Aside from the difference in test systems between studies, it is also noted that Nakamura *et al.* (1993) used much higher concentrations of test compounds, millimolar range compared to the micromolar concentrations used in this study.

Although previous optimisation work (Chapter 4) indicates that at the time of exposure mono-chloramines were the major chlorination products present, the contribution of other toxic products particularly after the chloramines enter cells cannot be excluded. Of the 4 genotoxic organic N-chloramines, 3 (chlorinated products of ethanolamine, histamine and lysine) contained low levels of di-chloramines (Chapter 4). These di-chloramines may play a part in the observed outcomes: a similar observation was also described by Thomas *et al.* (1987). They could also proceed through secondary reaction, producing other products such as aldehydes (Zgliczynski *et al.*, 1971). However, since all organic N-chloramines were freshly produced and immediately used in cell treatment, it was expected

that the secondary reactions may not occur prior to entering the cells. Question requiringanswer are whether these molecules once they enter cells directly attack DNA, transfer chlorine to other intracellular amines, protein or lipid whose products then eventually induce DNA damage: some of these have been investigated and described in the next Chapter.

In conclusion, this study reports on cytotoxicity and genotoxicity of the candidate organic N-chloramines using mammalian cell based system. The cytotoxicity and genotoxicity assay protocol was firstly modified for suitability to use in organic N-chloramine studies. Using the modified assay protocol, a series of organic N-chloramines of amino acids and simple primary amines were tested for cytotoxicity and genotoxicity. Of 16 organic N-chloramines tested, it was found that 4 compounds (chlorinated glycine, lysine, ethanolamine and histamine) were both cytotoxic and genotoxic to WIL2-NS cells. These compounds may be potential health risks and may require further toxicological studies. Their possible modes of action on mammalian cells have been studied and will be described in the next Chapter.

6. ORGANIC N-CHLORAMINES AND THE ROLE OF OXIDATIVE STRESS IN GENOTOXICITY

6.1. Introduction

Following the screening of organic N-chloramine candidates through flow cytometry MN assay (Chapter 5), 4 compounds were identified to have genotoxic effects in WIL2-NS cells. These compounds included N-chloroglycine, N, N$^\alpha$- dichlorolysine, N-chlorohistamine and N-chloroethanolamine. In this chapter, experiments are carried out to further investigate the mechanisms that may be involved in genotoxicity of these N-chloramines. Due to oxidative potential of chloramine molecules, it is hypothesised that following the cell entry oxidation between the compounds with cellular antioxidants may occur and induction of oxidative stress may be involved in the observed genotoxicity (as described in Chapter 5).

Oxidative stress is a term used to denote the situation caused by an imbalance between levels of oxidants (i.e. reactive oxygen and nitrogen species) and antioxidants in favour of the former, which can potentially lead to biological damage or injury (Sies, 1997). Oxidative stress can occur either in the cells as a result of cellular metabolism (Burcham, 1998) or from exposure to exogenous sources such as carcinogenic substances (Garza *et al.*, 2008), redox-cycling drugs (Simunek *et al.*, 2009) and ionizing radiation (Lenarczyk *et al.*, 2009). The damage caused by oxidative stress is believed to contribute to a number of pathological implications such as aging (Minelli *et al.*, 2009), carcinogenesis (Hayashi, 2008; Hebels *et al.*, 2009; Liu *et al.*, 2009), atherosclerosis (Higashi *et al.*, 2009) and neurodegeneration (Mytilineou *et al.*, 2002; Varadarajan *et al.*, 2000).

Under oxidative stress conditions, excessive reactive oxygen or nitrogen species overwhelm the ability of cellular antioxidant defence system and attack cellular macromolecules such as lipid, protein and DNA. DNA damage caused by these free radicals can occur by either 1) a direct attack to DNA molecules leading to strand breaks or 2) indirect effects i.e. via lipid peroxidation when a lipid peroxide product such as malondialdehyde (MDA) enters the nucleus forming MDA – DNA adducts (Munnia *et al.*, 2004), which can consequently result in DNA strand breaks (Plastaras *et al.*, 2000). Oxidative stress is suggested to be a mechanism involved in genotoxicity of a number of chemicals and metals, for example, organic mixtures of airborne particles (Gabelova *et al.*, 2007), polychlorinated biphenyls (Glauert *et al.*, 2008), cigarette smoke (Spencer *et al.*, 1995; Thorne *et al.*, 2009), iron (Abalea *et al.*, 1998), chromium (Shi and Dalal, 1989), cadmium (Koizumi and Li, 1992), arsenic (Scott *et al.*, 1993) and nickel (Athar *et al.*, 1987).

Investigation of cellular oxidative stress can be achieved using several methods including measurements of 1) low molecular weight antioxidants such as glutathione (Chiou and Tzeng, 2000) and ascorbic acid (Dhariwal *et al.*, 1990), 2) proteins and enzymes such as superoxide dismutase (Hassoun and Ray, 2003; Marklund and Marklund, 1974) and 3) peroxidation products such as MDA (Draper *et al.*, 1993). Measurement of the cellular antioxidant glutathione (GSH), which is one of the most abundant soluble antioxidant molecules found in cells has been commonly used as a marker for oxidative stress (Mytilineou *et al.*, 2002). GSH protects cells from injuries caused by oxidative stress. It has been shown that depletion of GSH can cause damage to mitochondria (Griffith and Meister, 1979; Jain *et al.*, 1991), which can consequently lead to cell death (Mytilineou *et al.*, 2002). Another commonly used method for determination of oxidative stress is measurement of MDA, a product of the reaction between free radicals and cellular lipid

(Ahmad *et al.*, 2008; Greilberger *et al.*, 2008; Mateos *et al.*, 2004; Papalambros *et al.*, 2007; Wijnhoven *et al.*, 2006). Both depletion of cellular GSH and an increase in MDA formation were used as indicators for determination of cellular oxidative stress in this current study.

Oxidative stress has been suggested as a possible genotoxic mechanism of several known DBPs. For example, a significant concentration-dependent GSH reduction was reported in mammalian cells following exposure to MX (Zeni *et al.*, 2004), dichloroacetate, trichloroacetate (Austin *et al.*, 1996; Hassoun and Ray, 2003) and hypochlorite solution (Hammerschmidt *et al.*, 2002). A significant increase in levels of MDA has also been reported in mouse liver cells following exposure to dichloroacetate and trichloroacetate (Austin *et al.*, 1996) and in the HepG2 cell line after treatment with a mixture of DBPs produced from different sequential water treatments (Shi *et al.*, 2009).

For organic N-chloramines in relation to disinfection by-products, while the formation and their potential toxicological effects have been predicted by experts (Bull *et al.*, 2006; Davies *et al.*, 1993; Hawkins and Davies, 1999) (as shown in Figure 6.1), little is known about their genotoxicity and the mechanisms that may be involved. It is possible that upon cell entry, both N-centred and chlorine radicals (Figure 6.1) may be involved in induction of cellular oxidative stress, which consequently leads to genotoxic damage.

In this Chapter, investigations are described into whether the four genotoxic N-chloramines (N-chloroglycine, N,N''-dichlorolysine, N-chlorohistamine and N-chloroethanolamine) induce cellular oxidative stress. Known genotoxic concentrations (obtained from Chapter 5) were tested. Two methods were used to determine oxidative stress: measurement of cellular GSH using the enzymatic recycling method based on the

principle described by Tietze (1969), and MDA levels using the quantitative TBARS Assay (Armstrong *et al.*, 1998).

NOTE:
This figure is included on page 115 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6.1. Formation of organic N-chloramine and predicted radical formation.

Picture modified from Bull et al. (2006). It is predicted that the first intermediate compound is produced in drinking water with subsequent reactions occurring *in vivo*. Both nitrogen-centred and chlorine radicals may play a role in cellular oxidative stress, which may lead to a cascade of cellular toxicity and genotoxicity.

6.2.   Materials and Methods

6.2.1.   Chemicals and Materials

The organic N-chloramines, N-chloroglycine, N,N''-dichlorolysine, N-chlorohistamine and N-chloroethanolamine, were prepared as described in Chapter 5. Ferrous sulphate was obtained from Ajax (Auburn, NSW Australia).

6.2.2.   Cell Culture and Seeding Preparation

WIL2-NS cells were cultured and maintained as described in Chapter 3. A day prior to the experiment, cells were freshly passaged in several 75 cm^2 tissue culture flasks and the culture continued for 24 hr. On the day of experiment, a cell count was performed using the TB exclusion assay (described in Chapter 3). The cells were pooled, washed in HBSS (Sigma-Aldrich, MO USA) and a 4.0 x 10^6 cell/ml suspension was prepared in HBSS. Cell recovery continued in the cell culture incubator (37°C, 5% CO_2) for 1 hr prior to exposure experiment.

6.2.3.   Glutathione Assay

Glutathione (GSH) levels were determined using the Trevigen''s HT Glutathione Assay Kit (TREVEGEN®, Gaithersburg, MD USA). The assay principle was based on the literature (Baker *et al.*, 1990; Tietze, 1969), utilising the optimised enzymatic recycling method as detailed in Figure 6.2. The glutathione assay involved several steps: 1) chemical

exposure and 2) sample preparation followed by the measurement of glutathione levels using spectrophotometry. The assay procedure is detailed below.

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|                      NOTE:                        |
|   This figure is included on page 117 of the      |
|   print copy of the thesis held in the            |
|   University of Adelaide Library.                 |
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Figure 6.2. Reaction scheme for the Glutathione assay

Picture adopted from HT Glutathione Assay Kit, Trevigen® Instruction manual. The measurement of GSH is based on the optimised enzymatic recycling method. Glutathione Reductase converts oxidised glutathione (GSSG) to reduced glutathione (GSH). The sulfhydril group of GSH reacts with 5, 5′- dithiolbis-2-nitrobenzoic acid (DTNB, or Ellman′s reagent) (Ellman, 1959) to produce a yellow coloured 5-thio-2-nitrobenzoic acid (TNB), which absorbs at 405 nm, and mixed disulfide (GSTNB), which is reduced by Glutathione Reductase to recycle the Glutathione and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of glutathione in the sample.

6.2.3.1.   Chemical Exposure

The chemical exposure procedure was performed in a 24-well tissue culture plate. Briefly, 1 ml of the 4.0 x 10^6 cells/ml cell suspension (described in section 6.2.2) was placed in each well of the tissue culture plate, followed by 1 ml of sample. The cells were mixed and exposure continued in the cell culture incubator (37°C, 5% CO_2) for 3 hr. Three dilutions (1/8, 1/4 and 1/2) of the freshly prepared stock of each sample were tested. Untreated control was included as required. Ferrous sulphate ($FeSO_4$) was used as positive control. All exposure experiments were conducted 3 times (3 independent experiments).

6.2.3.2.   Sample Preparation and Measurement of Total Glutathione

Following the 3 hr exposure, cells were collected in a conical tube and centrifuged at 300 g for 5 min. The cell pellet was resuspended in 500 µl cold 5% metaphosphoric acid (Sigma-Aldrich, MO USA), mixed thoroughly by repeated pipetting and placed on ice for 5 min. The cells were sonicated on ice for 5 x 20 sec at 50 mW using a BRASON Digital Sonifier® sonicator (ASIS Scientific Pty Ltd, Australia). The lysate was kept on ice for 5 min prior to transferring into a 1.5 ml eppendorf tube and centrifuged at 14000 g, 4°C for 5 min. The supernatant was then placed into a new 1.5 ml eppendorf tube and stored on ice for GSH measurement.

For the measurement of GSH, samples were diluted 10 fold in 1X assay buffer (freshly prepared from the 25X concentrate provided in the assay kit). The diluted sample (50 μl) or standards (0, 12.5, 25, 50 and 100 pmole, prepared by diluting the 4 μM GSSG standard in 1X assay buffer) was placed in each well of a microtitre plate, followed by 150 μl freshly prepared reaction mix (kit provided) containing glutathione reductase. Samples were assayed in duplicates, standards in triplicates. Absorbance (405nm) of the reaction was immediately recorded using the microplate reader (Wallac VICTOR[3] 1420 Multilabel Counter, PerkinElmer™). Repeat measurements were recorded at 2 min intervals over 10 min period.

In order to quantify levels of glutathione, a standard curve was generated. The average of the triplicate absorbance readings for each standard (or average of duplicate absorbance readings for each experimental sample) was plotted against incubation time. The slope of the regression was determined from the linear portion of each curve. The background slopes were subtracted from the slope values of standards and samples. The glutathione standard curve was generated from plots of the net slopes versus pmoles of glutathione standards. Quantification of glutathione in the experimental sample was obtained by comparing the net slope values of samples with the standards. Results were expressed as pmole GSH/well.

6.2.4.   Malondialdehyde Assay

The lipid peroxidation product (MDA) was measured using the OxiSelect™ TBARS Assay kit for MDA quantification, obtained from CELL BIOLABS, Inc. (San Diego, CA USA). The procedure was carried out following the manufacturer's instructions. The investigation of MDA levels was based on the reaction of MDA with thiobarbituric acid (TBA), producing the MDA-TBA adduct, which could be detected using fluorometry (Armstrong *et al.*, 1998). The MDA assay involved 1) chemical exposure and 2) sample preparation followed by the measurement of MDA as detailed below.

6.2.4.1.   Chemical Exposure

Three millilitres of the cell suspension prepared as described in section 6.2.2 was transferred into a small (25 cm^2) tissue culture flask, followed by 3 ml of the organic N-chloramine. The cells were mixed and incubated at 37°C, 5% CO_2 for 3 hr. For each sample, 3 dilutions (1/8, 1/4 and 1/2) of the freshly prepared stock were tested. Untreated control was included as required. Ferrous sulphate ($FeSO_4$) was used as a positive control. The experiments were conducted 3 times (3 independent experiments).

6.2.4.2.   Sample Preparation and Measurement of MDA

Following the 3 hr exposure, cells were collected in a conical tube, centrifuged at 200 g for 5 min and supernatant removed. The cell pellet was resuspended in 100 µl HBSS and transferred into a 1.5 ml eppendorf tube. The cells were lysed by vigorously mixing in

100 µl SDS lysis solution and incubated at room temperature for 5 min. TBA reagent (250 µl) was then added to each sample. The mixture was incubated at 95°C for 1 hr, followed by cooling in an ice bath for 5 min. The cell lysate was then centrifuged at 3000 rpm for 15 min (Eppendorf Centrifuge 5804). The supernatant (150 µl) was transferred into each well of a microtitre plate (in duplicate). Fluorescence of the MDA-TBA product (530 nm excitation and 580 nm emission) was measured using the microplate reader (Wallac VICTOR^3 1420 Multilabel Counter, PerkinElmer™).

In order to quantify MDA levels, the MDA standard was simultaneously prepared by serially diluting the MDA standard (Kit provided) in purified water, of which 150 µl was used for the measurement of MDA as described above. The standards were assayed in triplicate. The MDA standard curve was generated from plotting of values of fluorescence unit against the MDA concentration (Figure 6.6). The slope was determined using linear regression analysis and used for the calculation of MDA concentration in the test samples.

6.2.5.   Statistical Analysis

GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA) was used for all statistical tests and graphing. Unless otherwise stated, data plots represent mean ± SEM of 3 independent experiments. Kruskal-Wallis followed by Dunns post test was used to determine significant differences of treatments from untreated control. Statistical significance was accepted at $p < 0.05$.

6.3. Results

6.3.1. Glutathione Depletion

6.3.1.1. Glutathione Standard Curve

The glutathione standard curve was generated as described in materials and methods (section 6.2.4). Linearity of the standard curve is shown in Figure 6.3. Linear regression was analysed and slope as well as Y-intercept values were obtained. From the standard curve, the linear equation used for further calculation of total glutathione levels in the test sample is also determined ($Y = 0.0014X + 0.0011$).
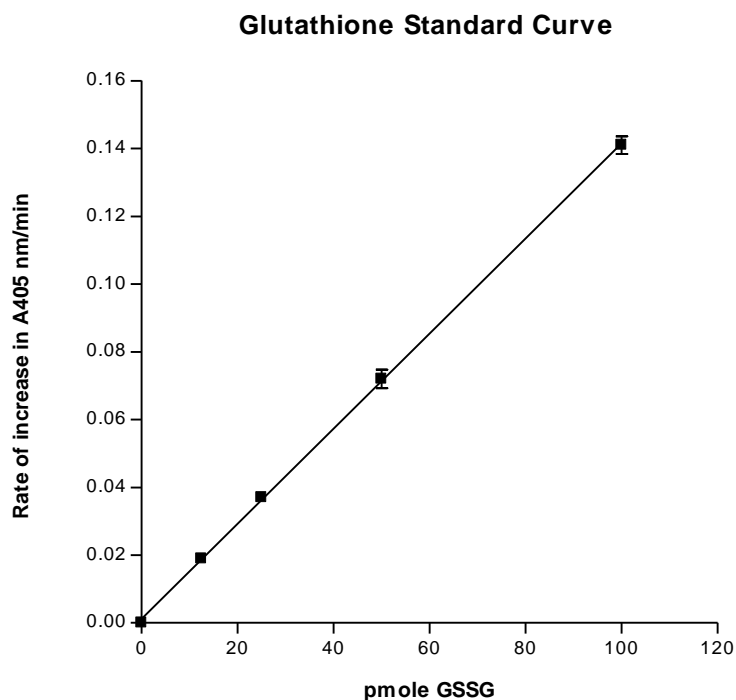
Figure 6.3. Representative total glutathione standard curve.

X-axis shows concentrations of standard in pmole/well. Y-axis shows values of rate of increase in the absorbance (405 nm) observed at 2 min intervals over 10 min period. Data are mean ± SD, n = 3. Linear regression, slope = 0.0014, Y intercept = 0.0011, r^2 = 0.9998.

6.3.1.2.   Reduction of Cellular Glutathione Levels Following $FeSO_4$ Treatment

The glutathione assay was set up using the optimised enzymatic recycling method as detailed in Figure 6.2. $FeSO_4$ was used as a positive control to induce free radicals and results are shown in Figure 6.4. Following treatment with $FeSO_4$ (10 µM − 100 µM), a significant concentration dependent reduction of cellular GSH was observed ($p = 0.0047$, Kruskal-Wallis Test). The concentration of $FeSO_4$ that demonstrated a significant reduction in GSH levels compared with the untreated control was 100 µM ($p < 0.05$, Dunns post test).
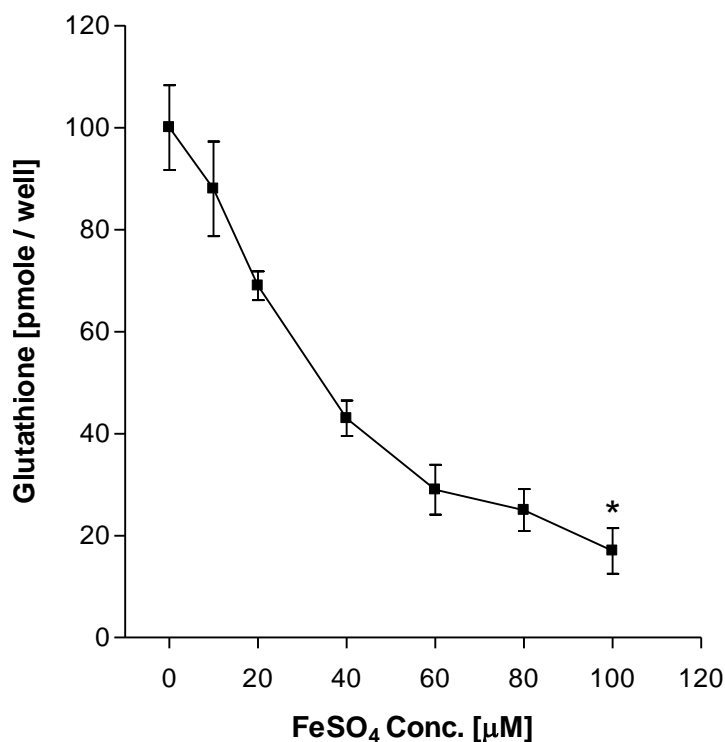
Figure 6.4. Effect of ferrous sulphate ($FeSO_4$) on intracellular glutathione levels. Data are expressed as percentages of untreated control (mean ± SEM of 3 independent experiments). Significant differences from control are indicated as * ($p < 0.05$).

6.3.1.3.   Effect of Organic N-chloramines on Cellular Glutathione Levels

Figure 6.5 shows reduction of cellular glutathione following treatment with organic N-chloramines. Data were expressed as percentages of the untreated control (raw data values of GSH are shown in Appendix 2, A2-2). Significant concentration-dependent glutathione depletion (analysed by Kruskal-Wallis Test) was observed in the treatment with N-chloroglycine (p = 0.0213), N,N''-dichlorolysine (p = 0.0153), N-chlorohistamine (p = 0.0439) and N-chloroethanolamine (p < 0.0185). Following treatment with 30 µM of N-chloroglycine, glutathione levels were reduced to 85 ± 3.9% (mean ± SEM, n = 3) of the control level (Figure 6.5A). Similar concentrations of N,N''-dichlorolysine (Figure 6.5B) and N-chlorohistamine (Figure 6.5C) reduced GSH to a similar extent. For cells treated with N-chloroethanolamine (Figure 6.5D), a significant reduction of glutathione was seen at or above 414 µM. For example 207 µM and 414 µM reduced GSH to 94 ± 1.0% and 87 ± 1.8% of the untreated control, respectively.
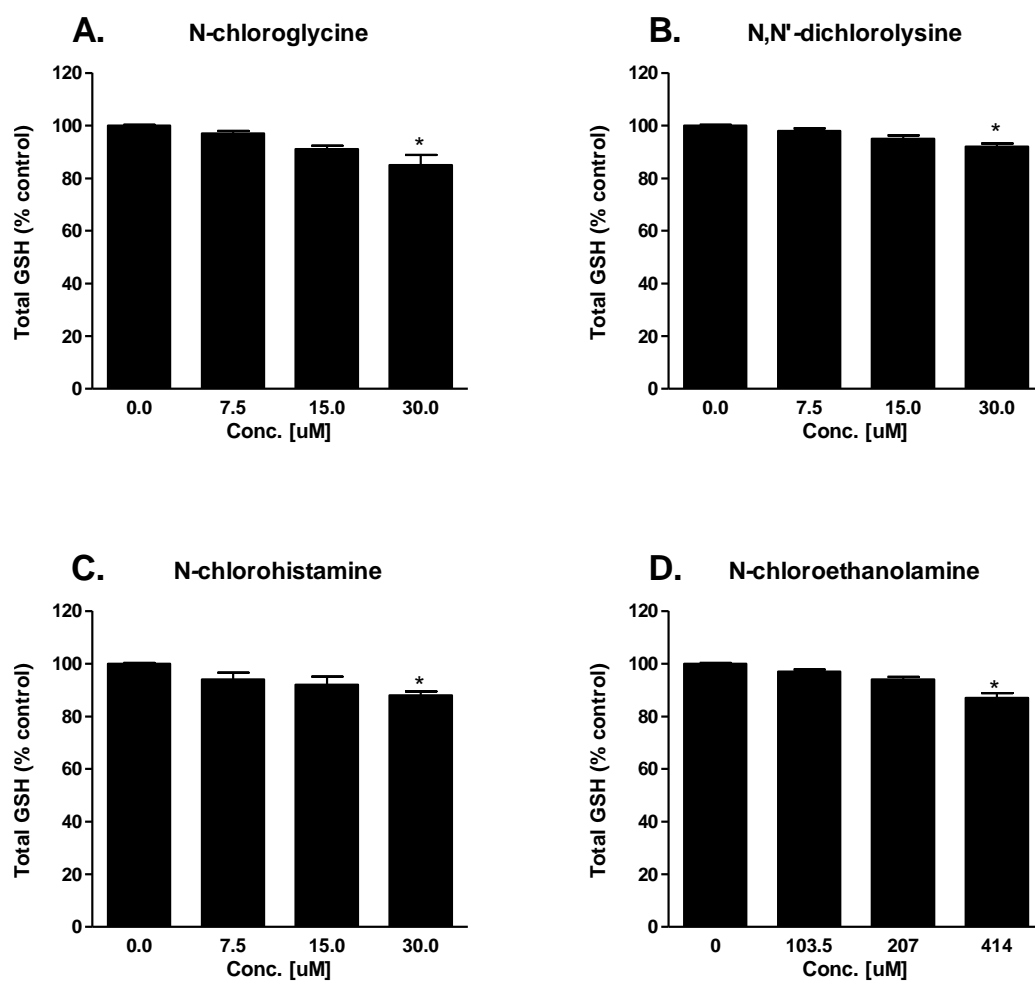
Figure 6.5. Levels of intracellular glutathione after treatment with organic N-chloramines. Glutathione was expressed as percentages of the untreated control. Data represent values of mean ± SEM of 3 independent experiments. Significant differences from control (Kruskal-Wallis Test, followed by Dunns post test) are indicated as * ($p < 0.05$).

6.3.2.   Analysis of Lipid Peroxidation Products

6.3.2.1.   MDA Standard Curve

The MDA standard curve was generated from fluorometric measurements of a dilution series of the MDA standard as described in materials and methods section 6.2.3. Linearity of the standard curve was observed in a $0 - 31.25$ µM range (Figure 6.6). Linear regression was analysed, and slope and Y-intercept values were obtained. From the standard curve, the linear equation used for further calculation of MDA levels in the test sample is also determined ($Y = 250.2X + 152.4$).
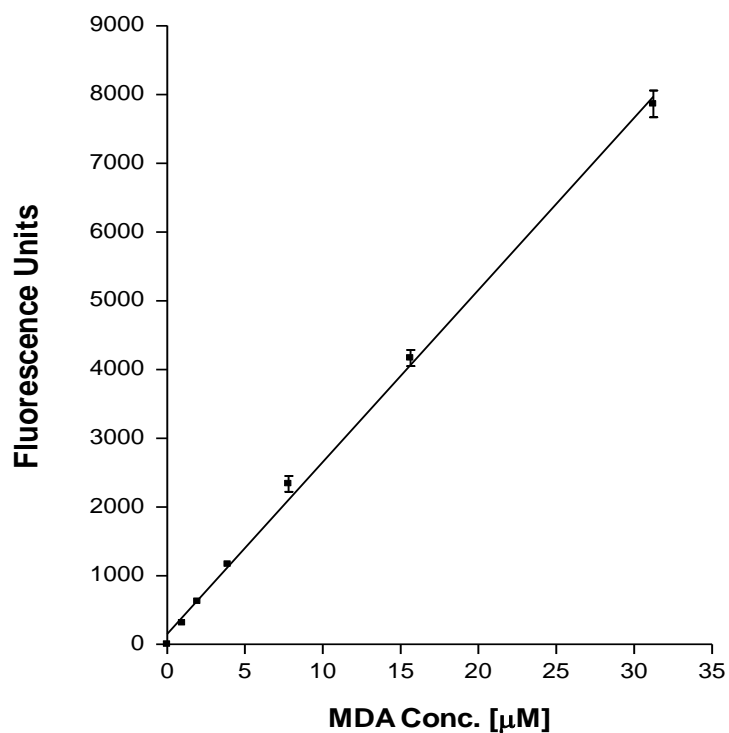
Figure 6.6. Representative MDA standard curve.

Fluorescence was measured using the microplate reader (excitation = 530 nm, emission = 580 nm). Data are mean ± SD, n = 3. Linear regression, slope = 250.2, Y intercept = 152.4, $r^2 = 0.9978$.

6.3.2.2.   Effect of FeSO₄ on MDA Production

Ferrous sulphate (FeSO₄) was also used as a positive control in the MDA assay. As shown in Figure 6.7, while the background MDA value of the untreated control was 6.7 ± 1.3 µM (mean ± SEM, n = 3), a significant concentration dependent increase in MDA was observed in the treatments with 10 µM – 80 µM FeSO₄ ($p = 0.0106$, Kruskal-Wallis Test). The minimum concentration of FeSO₄ that demonstrated a significantly increased MDA formation compared with the untreated control was 80 µM ($p < 0.05$, Dunns post test).
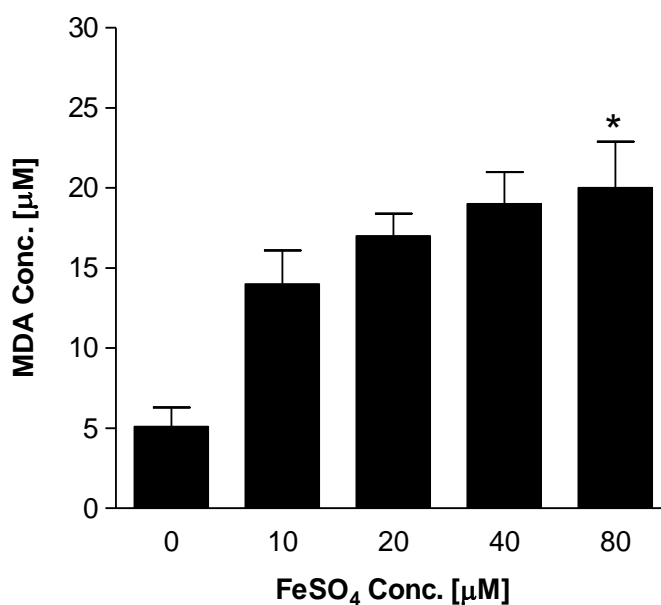
Figure 6.7. MDA induction in WIL2-NS cells following treatment with FeSO₄.

Data are mean ± SEM of 3 independent experiments (n = 3). Asterisk (*) shows significant difference ($p < 0.05$, Dunns post test) compared with the untreated control.

6.3.2.3.   Effects of Organic N-chloramines on Lipid Peroxidation

Following exposure to organic N-chloramines, cellular levels of MDA were measured. The results are shown in Figure 6.8. The background MDA levels in control cells (untreated) was 6.7 ± 1.6 µM (mean $\pm$ SEM, n =3). Within the range of concentrations tested, no significant increase in cellular MDA levels (analysed by Kruskal-Wallis Test) was observed following treatment with N-chloroglycine ($p = 0.8629$), N,N"-dichlorolysine ($p = 0.6446$), N-chlorohistamine ($p = 0.8617$) or N-chloroethanolamine ($p = 0.6676$).
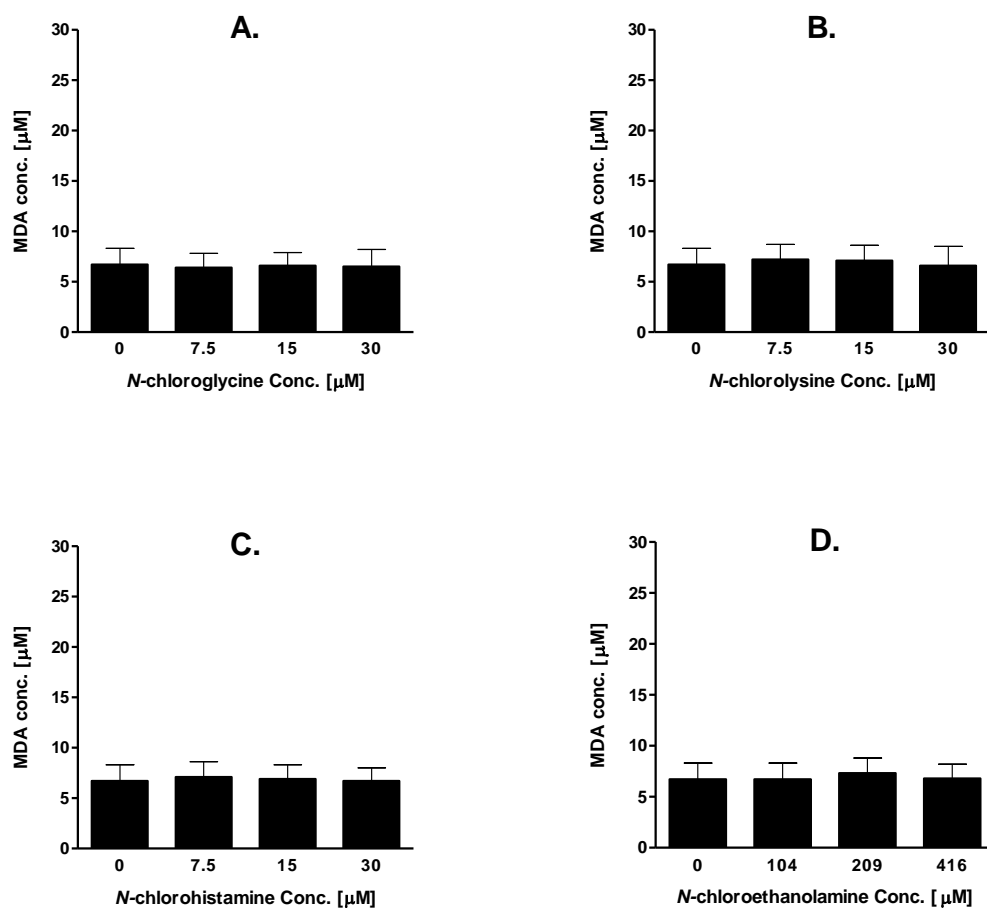
Figure 6.8. MDA levels in WIL2-NS cells following N-chloamine exposure

to N-chloroglycine (A), N,N''-dichlorolysine (B), N-chlorohistamine (C) and N-chloroethanolamine (D). Data are mean ± SEM of 3 independent experiments (n = 3).

6.4. Discussion

The oxidative capacity of chloramines has led to a hypothesis that cellular oxidation and oxidative stress may be involved in a mechanism of toxicity and genotoxicity of the four genotoxic organic N-chloramines described in previous Chapters. To investigate oxidative stress conditions, the GSH and the MDA assays were established using $FeSO_4$ as the positive control. In WIL2-NS cells, treatment with $FeSO_4$ demonstrated concentration-dependent responses in both reduction of GSH and increased formation of MDA. Similar responses were reported in Jurkat T cells following exposure to $FeSO_4$ as described by Erba et al. (2003).

A small but significant reduction of cellular GSH (i.e. up to 15% of the untreated control) which was observed in cells following treatment with the four organic N-chloramines suggested that these compounds reacted with cellular antioxidant. The reduction of GSH may be a consequence of an oxidation reaction of the thiol group in GSH molecules with the N-chloramines, producing glutathione sulphonamide (Carr *et al.*, 2001; Pullar *et al.*, 2001; Winterbourn and Brennan, 1997), and hence the GSH was less available to react with DTNB or Ellman‛s reagent (Ellman, 1959) in the GSH assay. A similar effect of other organic N-chloramines (i.e. chlorination products of taurine, glycine, Nα-acetyllysine, lysine, serine, aspartic acid and phenylalanine) on reduction of GSH levels *in vitro* has been reported in the literature (Peskin and Winterbourn, 2001; Raftery, 2007; Robaszkiewicz *et al.*, 2008).

This study has shown that while levels of cellular GSH decreased somewhat, changes in MDA levels were not observed in cells following treatment with the organic N-chloramines. It was noted that even at maximal N-chloramine concentrations used in this study, only 8% − 15% reduction of cellular GSH compared with the untreated control ($100 \pm 0.43\%$) was seen (i.e. $85 \pm 3.9\%$, $92 \pm 1.2\%$, $88 \pm 1.4\%$ and $87 \pm 1.2\%$ for the N-chloroglycine, N,N''-dichlorolysine, N-chlorohistamine and N-chloroethanolamine, respectively).

When the effects of cells treated with $FeSO_4$ were compared with the untreated control in both MDA and GSH assays, it was found that an increase in MDA formation was not observed until the cellular GSH was reduced to $87.8 \pm 2.1\%$ of the control (Figure 6.4 and Figure 6.7, 10 µM $FeSO_4$). This indicates that free radicals produced by the treatment of cells with these organic N-chloramines were scavenged prior to lipid peroxidation. Although studies have suggested that MDA can cause DNA damage via formation of MDA-DNA adducts (Burcham, 1998; Wang and Liehr, 1995), in this study, MDA levels remained unchanged in cells treated with the organic N-chloramines, suggesting that the genotoxicity observed was not the result of MDA-DNA adduct.

In conclusion, this study investigated oxidative stress as a possible genotoxic mechanism of the four organic N-chloramines identified as genotoxins in Chapter 5. The results show a slight reduction of cellular GSH following treatment with the organic N-chloramines but without formation of MDA, hence induction of cellular oxidative stress may not be a mechanism involved in genotoxicity of these compounds. To better understand the genotoxic mechanism of these organic N-chloramines, further studies are required. It is possible that N-centred radicals produced by the organic N-chloramines (Hawkins and Davies, 2002) could react with or add directly to DNA molecules, which

may result in base modification (Aruoma *et al.*, 1989) or form bulky DNA adducts (Randerath *et al.*, 1991). It is also possible that the N-chloramine could react with protein molecules (Davies *et al.*, 1993), especially those involved in DNA repair systems (Pero *et al.*, 1996), to cause the observed genotoxic events. In addition, since the MN observed in Chapter 5 could be either from clastogenic or aneugenic events (Fenech, 2000), apart from investigation of effects on DNA damage as discussed above, the loss of whole chromosomes due to exposure to the test compound could be possible. This also requires further investigation.

7. APPLICATION OF CELL BASED ASSAY FOR IDENTIFICATION OF PRECURSORS OF TOXIC DBPs IN AUSTRALIAN WATER

7.1. Introduction

While the previous Chapters have specifically investigated the cytotoxicity and genotoxicity of organic N-chloramines, the assays optimised for this work can also be applied to other areas of DBP research. One area is the identification of potential precursors of toxic DBPs in source water, which should aid in their removal prior to the disinfection process.

With relatively low annual rainfall in Australia, very high levels of NOM are present in natural waters. NOM is a complex mixture of organic compounds and acts as a precursor to DBPs. Hence high levels of DBPs have been reported in disinfected waters in Australia (Simpson and Hayes, 1998). Removal of NOM in the source water prior to the disinfection process has shown to be an effective approach in minimising DBP formation. To date, several methods such as coagulation (Szlachta and Adamski, 2008), adsorption (Bolto *et al.*, 2002), advanced oxidation processes (Lim *et al.*, 2007), filtration (de la Rubia *et al.*, 2008; Rojas *et al.*, 2008) or a combination of these methods (Zularisam *et al.*, 2009) have been used for removal of NOM in water supplies. Although these methods are effective and widely used in water industries, total removal of NOM has not been accomplished to date. As total removal of NOM is not possible, one alternative approach to this problem would be to try to target NOM that are known to be precursors for DBPs for their selective removal. This would first require characterisation of the precursor NOM.

Molecular weight (MW) characterisation of NOM components has been discussed in the literature using a technique called high-performance size exclusion chromatography (HPSEC) (Amy *et al.*, 1992; Becher *et al.*, 1985; Logan and Wagenseller, 2000; Nissinen *et al.*, 2001; Yoshioka *et al.*, 2007). The separation of water components by HPSEC is based on hydrodynamic properties related to molecular size. Samples are injected into a column containing porous gel materials, where small molecules (low MW) can gain greater access to the internal pores than large molecules (high MW) and hence the largest molecules are eluted first in the column, while the smallest molecules are eluted last (Potschka, 1993). Using this technique, NOM can be separated into various fractions.

In this Chapter MW fractions from a water sample from Western Australia with high NOM were analysed for potential cytotoxicity and genotoxicity responses. The samples were analysed both pre- and post-chlorination, to determine which fraction(s) contain precursors for toxic and/or genotoxic DBPs. This work was carried out in collaboration with researchers at Curtin University, Western Australia, who performed the HPSEC separations.

7.2.   Materials and Methods

7.2.1.   Water Sample and Preparation of MW Fractions

This preparative work was conducted by researchers at Curtin University, Western Australia. Information on water characteristics and the processes used for preparation of the MW fractions was provided as detailed below.

A water sample (1000 L) was collected from a Western Australian highly coloured surface water (dissolved organic carbon, DOC, 26.2 mg/L) in September 2006. The sample was filtered through a 0.45 μm membrane and then subjected to reverse osmosis (RO) to concentrate the organic matter in the sample. The final volume of RO concentrate was 16 L and the DOC concentration in the concentrate was approximately 1200 mg/L. The recovery of DOC after RO treatment was 92%.

A portion of this RO concentrate was then subjected to preparative HPSEC, following the method described by Peuravuori & Pihlaja (2004). A BioSep-SEC-S 3000 (300 x 21.2 mm i.d., Phenomenex) column was preceded by a BioSep SEC-S 3000 guard column (75 x 21.2 mm i.d., Phenomenex). The eluent comprised a 20 mM phosphate buffer with a flow rate of 4 ml/min and an injection volume of 1 ml. The high pressure liquid chromatography (HPLC) system was an Agilent 1100 equipped with a dual-loop autosampler, a diode array detector collecting data at 254 nm, and an automated fraction collector capable of collecting an infinite volume of individual fractions to enable the system to run without interruption for an extended period of time.

Eight individual MW fractions were collected as shown in Figure 7.1. Elution volumes were used as selection criteria for each MW fraction, and the automated fraction collector was able to collect the same MW fraction eluting at this elution volume, combining the fraction with identical MW fractions collected in previous runs. In total, 979 injections were conducted over a 3 month period. The total recovery of organic carbon from the preparative SEC step was 89%.

The large volumes of each separated MW fraction, also containing the concentrated HPSEC phosphate mobile phase, were each concentrated to approximately 1 L by reduced pressure distillation and desalted by ultrafiltration using a 1000 Da nominal MW cut-off membrane. Each fraction was then diluted to 1 L with purified water, prior to use. Measurement of dissolved organic carbon was also conducted at Curtin University. Data (provided by Brad Allpike) are shown in Table 7-1. Aliquots of 20 ml of each fraction were placed in glass containers and shipped to the Australian Water Quality Centre (AWQC) onice. Upon the arrival, 10 ml of all samples were filtered (0.45 μM) and stored at 4ºC until further processed.

7.2.2. Chlorination of MW Fractions

7.2.2.1. Modified Colorimetric DPD Method

The colorimetric DPD method described in the Standard Methods for the Examination of Water and Waste Water, published by the American Public Health Association (1998) was modified to allow measurement of small sample volumes. Briefly, 15 μl PBS and 15 μl DPD solution was added into each well of a microtitre plate, followed by 300 μl of freshly prepared chlorine standard (0 – 4 mg/L) or sample. The plate was

immediately placed in the microplate reader (Wallac VICTOR[3] 1420 Multilabel Counter, PerkinElmer™), during which time the plate was shaken (medium speed) for 10 second (at room temperature in the dark) and absorbance was measured at 490 nm. The chlorine standard curve was generated from scatter plots between absorbance and chlorine concentrations. Linear regression was used to calculate levels of chlorine residual in the samples. Standards were assayed in triplicates. Samples were assayed in duplicate.

7.2.2.2.   Determination of Chlorine Dose for Chlorination of MW Fractions

In order to find the appropriate chlorine dose for chlorination of MW fractions, several concentrations were tested. The predicted chlorine concentrations (shown in Table 7-1) were calculated based on a suggestion by Assoc. Prof. Anna Heitz, Curtin University, Western Australia (personal communication). All chlorination experiments were conducted in 1.5 ml eppendorf tubes. Briefly, 1350 µl of each MW fraction was mixed with 150 µl 10X HBSS (Appendix 1, A1-1) in a 1.5 ml eppendorf tube and spiked with various concentrations of chlorine solution as shown in Table 7-1. The reaction continued at room temperature in the dark (close lid) for 3 days. During which time, samples were taken at 1 hr, 24 hr, 48 hr and 72 hr for measurements of chlorine residual using the modified colorimetric DPD method as described in section 7.2.2.1. The conditions that provided no chlorine residual after 24 hr chlorination were selected for use to produce DBPs for subsequent cell based assays.

7.2.2.3. Chlorination of MW Fractions for Cytoxicity and Genotoxicity Assays

To produce chlorinated DBPs for cytotoxicity and genotoxicity assays, reaction conditions obtained from 7.2.2.2 (also shown in highlighted figures in Table 7-1) were used. Briefly, 1350 µl of each MW fraction (1 - 8) was placed in a 1.5 ml eppendorf tube containing 150 µl 10X HBSS. Chlorine solution was then added into each tube to the final concentrations of 36.6, 134.8, 167.4, 103.1, 83.6, 45.6, 25.5 and 137.1 mg/L for fraction 1 – 8, respectively. The solution were mixed by gently vortexing (10 sec) and reaction continued at room temperature in the dark for 24 hr. Prior to cell treatment, chlorine residual were measured using the modified colorimetric DPD method (as described in 7.2.2.1).

7.2.3. Cytotoxicity and Genotoxicity of Chlorinated MW Fractions

7.2.3.1. Cell Culture and Seeding Preparation

WIL2-NS cells were cultured and maintained as described in Chapter 3. A day prior to the experiment, cells were freshly passaged in a new flask and the culture continued for 24 hr. On the day of experiment, cell count was performed using the TB exclusion assay (described in Chapter 3). The cells were washed once in HBSS and 5.0×10^5 cell suspension prepared in HBSS. One millilitre of the cell suspension was placed into each well of a 24-well tissue culture plate and cell recovery continued at 37°C, 5% CO_2 for 1 hr.

7.2.3.2.   Cell Treatment

A short-term exposure protocol as described in Chapter 5 was used in this current study. Briefly, 125 µl, 250 µl and 500 µl of sample (either pre-chlorinated or post-chlorinated as described in 7.2.2.3) followed by 875 µl, 750 µl and 500 µl of HBSS was added into 1st, 2nd and 3rd well of a 24-well tissue culture plate containing the seeded cells (refer to section 7.2.3.1), giving the final dilution of 1/16 1/8 and 1/4, respectively. A similar experimental set up is shown in Chapter 5.

Bromoacetic acid (BA) (CAS No. 79-08-3, Sigma-Aldrich MO, USA) was used as a positive control. Working stocks (2X) were prepared in HBSS (12.5 µM, 25 µM and 50 µM) and 1 ml of each was added directly into 1st, 2nd and 3rd well of the seeded plate to give final concentrations of 6.25 µM, 12.5 µM and 25 µM, respectively. Untreated controls were included as required. The cells were mixed and plates were incubated at 37°C, 5% CO_2 for 3 hr. Following the 3 hr treatment, cells from each well were transferred into a 1.5 ml eppendorf tube and washed once with HBSS. The cells were then resuspended in 2 ml complete medium and placed back in a well of a new 24-well tissue culture plate. The cells were grown for 21 hr, 37°C, 5% CO_2 prior to harvest for cytotoxicity and genotoxicity measurements.

7.2.3.3.   Cytotoxicity Measurement

The MTS assay (as described in Chapter 3) was used to determine cytotoxicity of the samples.   Samples were assayed in triplicate. Cytotoxicity was expressed as a percentage reduction of MTS cell viability compared with the no treatment control.

7.2.3.4.   Measurement of MN Formation by Flow Cytometry

Six hundred microlitres of the cells from each well was transferred into a 1.5 ml eppendorf tube. The cells were centrifuged at 100 g for 5 min and supernatant removed. FCM solution 1 (600 µl) was added into the tube, gently mixed and placed in the dark at room temperature for 1 hr. Following this time, 600 µl of FCM solution 2 was added directly into the tube, the incubation continued in the dark at room temperature for 30 min prior to the measurement of MN by flow cytometry as described in Chapter 3.

7.2.3.5.   Measurement of MN Formation by Microscopy

The remaining cells in each well of the 24-well plate (approximately 800 µl) were used for preparation of slides for MN measurement by microscopy. The cells were collected in a 1.5 ml eppendorf tube, centrifuged at 100 g for 5 min and supernatant removed. The cell pellet was mixed in 5 µl FBS and thinly smeared onto 2 glass slides. The slides were air-dried, fixed in absolute methanol and stained with Dip-Quik. The stained slides were stored at room temperature in the dark until analysis. Microscopic counting of MN was conducted under a light microscope (1000X magnification). Approximately 1000

cells were counted per slide. MN formation was expressed as a percentage of MN containing cells over the total cell counts.

7.2.4. Data Analysis

All data analyses and graphing were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA). Unless otherwise stated, data plots represent mean ± SD of 2 independent experiments.

7.3.   Results

7.3.1.   Preparation of MW Fractions

Figure 7.1 shows the chromatogram of the size-exclusion chromatography (SEC) to isolate individual fractions of a surface water sample based on their molecular weight component. SEC uses porous particles to separate molecules of different sizes. Molecules that are smaller than the pore size can enter the pores and therefore have a longer path and longer transit time than larger molecules that cannot enter the particles. Hence, the highest MW molecules were isolated in fraction 1 while the lowest MW molecules were isolated in fraction 8.

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NOTE:
This figure is included on page 144 of the print copy of
the thesis held in the University of Adelaide Library.
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Figure 7.1. SEC-UV chromatogram of highly coloured surface water after reverse osmosis concentration, showing fractions collected in subsequent preparative SEC. kDA = kilodalton. Picture provided by Assoc. Prof. Cynthia Joll, Curtin University, Western Australia.

7.3.2.   Chlorination of MW Fractions

7.3.2.1.   Modified Colorimetric Microplate DPD Assay

Chlorine standard curve was generated from plots of absorbance (490 nm) versus chlorine concentrations as shown in Figure 7.2.   The data were analysed by linear regression to obtain slope and Y-intercept values. From the standard curve, the linear equation used for further calculation of residual chlorine in the test sample is also determined (Y = 0.1410X + 0.0060).
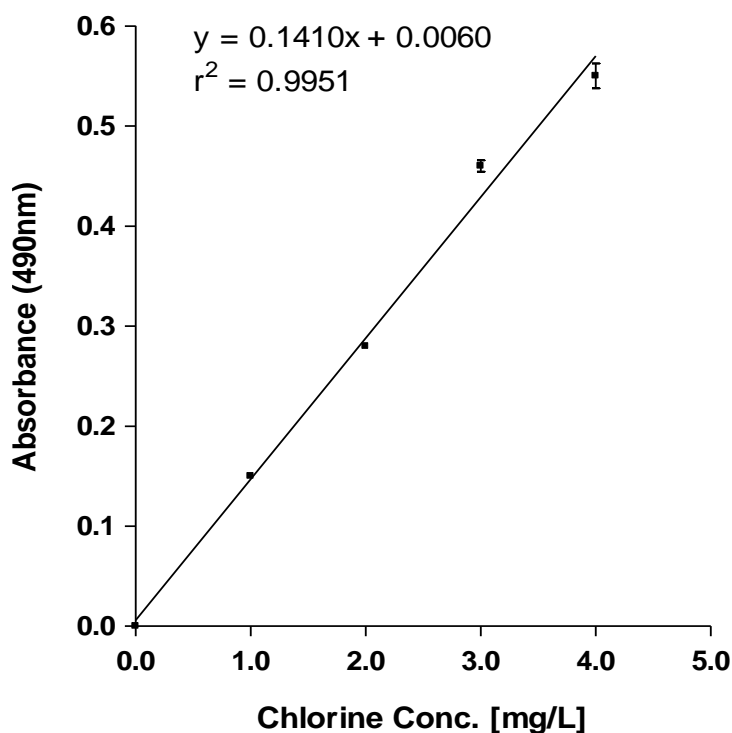
Figure 7.2. Representative chlorine standard curve by the modified colorimetric DPD method (microplate). Linear regression was used to determine slope (0.1410) and Y-intercept (0.0060). Data are values of mean ± SEM, n = 3.

7.3.2.2.   Determination of Chlorine Dose for Chlorination of MW Fractions

Six chlorine dosages were used to chlorinate the MW fractions. The dosages were selected based on individual DOC concentration of each fraction as shown in Table 7-1. Following the chlorination, chlorine residuals were measured at 1 hr, 24 hr, 48 hr and 72 hr using the colorimetric DPD method. It was found that, for all reactions, no chlorine residuals were detected at 72 hr (data not shown). The highlighted figures in Table 7-1 indicates the amount of chlorine consumed at 24 hr after chlorination. These chlorine doses were used for chlorination of MW fractions for cytotoxicity and genotoxicity assays.

Table 7-1. MW Fractions, DOC and predicted chlorine doses

MW Fraction	DOC* (mg/L)	Dose 1 (mg/L)	Dose 2 (mg/L)	Dose 3 (mg/L)	Dose 4 (mg/L)	Dose 5 (mg/L)	Dose 6 (mg/L)
Fraction 1	23.2	30.5	33.3	**36.6**	40.7	45.8	52.3
Fraction 2	84.5	112.3	122.6	**134.8**	149.8	168.5	192.6
Fraction 3	122.0	139.5	152.2	**167.4**	186.0	209.2	239.1
Fraction 4	66.7	79.6	86.8	95.5	**106.1**	119.3	136.4
Fraction 5	54.6	69.7	76.0	**83.6**	92.9	104.6	119.5
Fraction 6	32.7	34.2	37.3	41.1	**45.6**	51.4	58.7
Fraction 7	17.9	23.4	**25.5**	28.1	31.2	35.1	40.1
Fraction 8	60.4	80.0	87.3	96.0	106.7	120.0	**137.1**

DOC* values provided by Brad Allpike (Curtin University, WA). Predicted chlorine doses were calculated based on recommendation of Assoc. Prof. Anna Heitz (Curtin University, WA). Figures highlighted are chlorine doses that produced no chlorine residual after 24 hr contact time.

7.3.3. Cytotoxicity and Genotoxicity of Pre-chlorinated MW Fractions

Cytotoxicity screening of pre-chlorinated MW fractions was carried out. While the positive control (BA) demonstrated a concentration-dependent cytotoxic response i.e. viability reduced to $52.5 \pm 7.7\%$ (mean $\pm$ SD, n = 2) of the untreated control (100.1 $\pm$ 0.14%), the pre-chlorinated MW fractions No. 1 – 7 were not seen to be cytotoxic to WIL2-NS cells. Cell viabilities at the maximum concentration (dilution 1/4) of these MW fractions ranged from $97.5 \pm 1.1\%$ to $102.8 \pm 1.9\%$ (mean $\pm$ SD, n = 2), which were not different from the untreated control (100.1 $\pm$ 0.14%). It was noted that viability of the pre-chlorinated fraction 8 (at all dilutions) were greater than the control i.e. $117.3 \pm 12.1\%$, $122.4 \pm 13.7\%$ and $122.6 \pm 7.6\%$ (mean $\pm$ SD, n = 2) for dilution 1/16, 1/8 and 1/4, respectively (Graph results are shown in Appendix 2, A2-3).

Genotoxicity of each pre-chlorinated MW fraction was also investigated (data are also presented in Appendix 2, A2-4). It was found that while BA demonstrated an increase in MN formation measured by flow cytometry ($9.7 \pm 1.4\%$, mean $\pm$ SD, n = 2) compared with the control ($2.4 \pm 0.3\%$, mean $\pm$ SD, n = 2), there was no change in levels of MN in cells treated with pre-chlorinated MW fractions 1 – 7 i.e. the MN values of these samples ranged from $2.5 \pm 0.6\%$ to $3.1 \pm 0.2\%$. MN results obtained by microscopy were found to be consistent with the FCMN method. For the pre-chlorinated fraction 8, FCMN scatter plot of the pre-chlorinated fraction 8 demonstrated a distinct population that was different from the control (picture is shown in Appendix 2, A2-5), which was identified as bacteria by microscopy (gram positive bacilli). Bacterial contamination was also found in the original sample. This may explain the increase in cell viability reported previously. Due to

bacterial contamination, genotoxicity of pre-chlorinated fraction 8 was not analysed and fraction 8 was not included in subsequent experiments.

7.3.4. Cytotoxicity and Genotoxicity of Chlorinated MW Fractions

7.3.4.1. Cytotoxicity of Chlorinated MW Fractions

Cytotoxicity of chlorinated MW fractions is shown in Figure 7.3. Chlorinated fractions 2, 3 and 4 (Figure 7.3C-E) were observed to be cytotoxic with concentration dependent responses shown. No cytoxic response was seen following treatment with chlorinated fractions 1, 5, 6 or 7 (Figure 7.3B, F, G and H, respectively). At the highest concentration tested (dilution 1/4), the rank of cytotoxicity (expressed as percentages of control viability, mean ± SD, n = 2) was chlorinated fraction 4 (54.9 ± 13.6%), chlorinated fraction 3 (67.8 ± 4.1% and chlorinated fraction 5 (84.9 ± 7.7%), respectively.
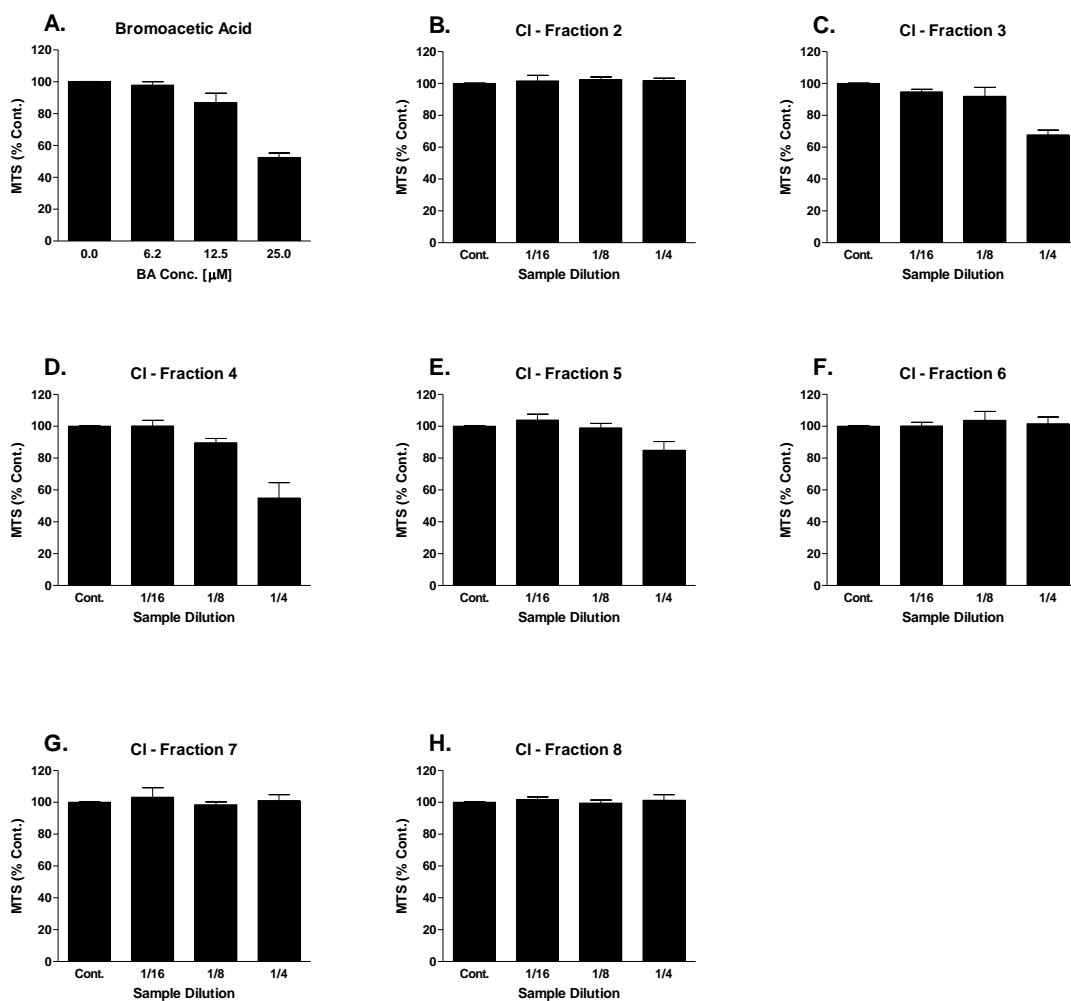
Figure 7.3. Cytotoxicity of chlorinated MW fractions.

Cytotoxicity is presented as a percentage reduction of MTS viability compared with the untreated control (Cont.). Values are mean ± SD of 2 independent experiments.

7.3.4.2.   Genotoxicity of Chlorinated MW Fractions

Figure 7.4 shows the genotoxicity results of chlorinated MW fractions measured by the FCMN and microscopy-based MN assays. A concentration-dependent genotoxic response was observed in cells treated with the positive control (BA) from both methods (Figure 7.4A). While a concentration-dependent increase in MN was clearly observed in chlorinated fractions 2, 3 and 4 (Figure 7.4C, D and E, respectively), only a slight increase in genotoxic response was seen in chlorinated fraction 5 (Figure 7.4F). Generally, the MN results obtained from both FCM and microscopy were consistent, except at the highest test concentration (dilution 1/4) of chlorinated fraction 2 and 3 (Figure 7.4C and D), where the FCMN results were greater than the microscopy results (i.e. 58.7 ± 5.7% and 56.8 ± 7.1 % by FCMN compared with   9.1 ± 1.8% and 7.9 ± 1.9% by microscopy, respectively). Genotoxicity was not observed in chlorinated fractions 1, 6 and 7 (Figure 7.4B, G and H, respectively).
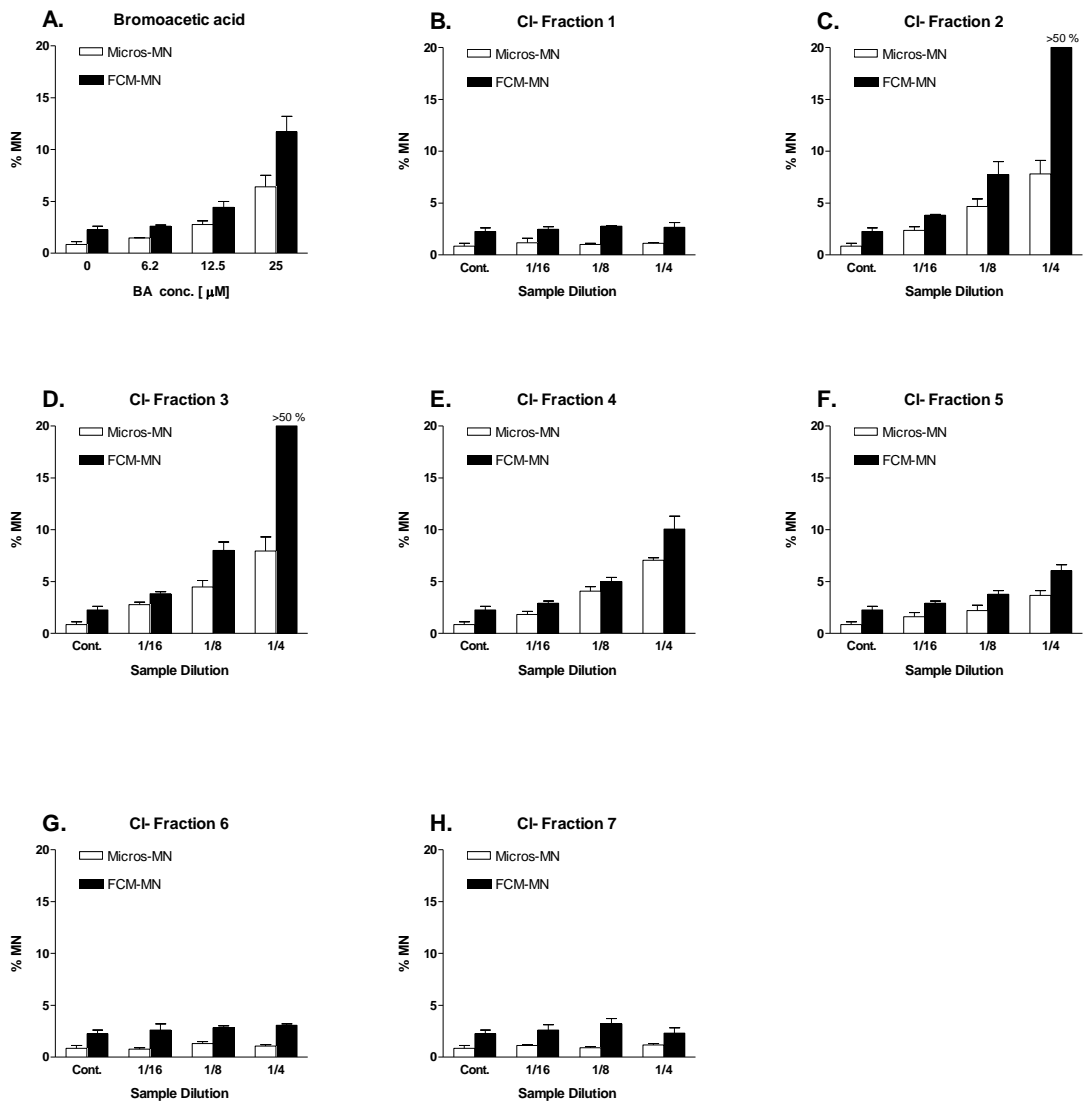
Figure 7.4. Genotoxicity of chlorinated MW fractions.

MN frequencies were measured using flow cytometry (black) and confirmed by microscopy (white) methods. Dilution 1/4 of chlorinated fraction 3 (C) and fraction 4 (D) were highly cytotoxic (results shown as > 50% MN by FCM). Data are presented as mean ± SD of 2 independent experiments.

7.4. Discussion

This Chapter reports a preliminary study using an application of HPSEC followed by cytotoxicity and genotoxicity cell based assays to help identify potential precursors of hazardous DBPs. This work was conducted in collaboration with researchers at Curtin University, Western Australia.

HPSEC is a technique used in NOM characterisation studies for evaluating various water treatment processes (Chow *et al.*, 1999; Gjessing *et al.*, 1998). In water treatment, HPSEC has been used in the characterisation of NOM both pre- and post treatment process to help optimise the treatment process (Vuorio *et al.*, 1998). HPSEC was used to prepare MW fractions from a Western Australian water sample as described in the first part of this Chapter. Curtin Water Quality Research Centre (Western Australia) has facilities for preparative SEC system. The method has been automated by use of a large volume autosampler and a state-of-the-art fraction collector, which allows isolation of sufficient NOM in each MW fraction to conduct detailed characterisation studies. A total of 8 MW fractions were isolated from the water sample and sent to the AWQC for further toxicity studies. Unfortunately, fraction 8 was later found to be bacterial contaminated and hence it was excluded from the cytotoxicity and genotoxicity studies.

Several studies have shown that chlorination of large MW NOM molecules could result in the formation of many low MW DBPs (i.e. THMs, phenol, ketones) (Christman *et al.*, 1980; Johnson *et al.*, 1982), which indicated the existence of chlorine-containing species or intermediate molecules during the process (Glaze and Peyton, 1978). Therefore, in order to determine toxicity and genotoxicity of chlorinated products including these intermediate molecules (compounds with intermediate half life, including some organic N-chloramines), the chlorination of MW fraction was performed using 24 hr chlorine contact time. It was expected that the effects observed would represent the outcomes of both stable DBPs and those with shorter half life. Comparative toxicity studies of the chlorinated MW products using 48 and 72 hr chlorine contact time should be included in future study to help understand the toxicity of these intermediate molecules.

Several chlorinated MW fractions (especially medium to high MW) demonstrated cytotoxicity and genotoxicity in mammalian cells. No effects were seen pre-chlorination in all MW fractions. The results of genotoxicity of chlorinated products of intermediate MW fractions present in this Chapter was consistent with the mutagenicity study of products obtained from chlorination of intermediate MW fractions isolated from a Norwegian water sample (Becher *et al.*, 1985). Although there are some variations such as contents in sources of water (i.e. DOC of the Australian water is 26.2 mg/L and the Norwegian water is 17.5 mg/L), method and the amount of chlorine doses used, a similarity in terms of mutagenicity and genotoxicity of these chlorinated intermediate MW fractions is interesting and may be worthwhile for further investigation.

Although the data presented in this study showed that high cytotoxicity and genotoxicity was observed, in respective order, in chlorinated F3, F2, F4 and F5, their toxicity potencies cannot be compared. At time of the study, apart from DOC information, other chemical compositions of each MW fraction was not available, and hence the maximal possible concentrations of chlorinated MW fractions were tested on the cytotoxicity and gentoxicity assays. For example, each MW fraction was chlorinated as "undiluted" sample and the chlorine dosages determined based on their DOC contents (i.e. 17.9 mg/L − 122 mg/L concentration range). The highest MW fraction (i.e. F3) showed highest toxicity, but this does not mean it is the most potent toxicant compared with the others. To determine cytotoxic and genotoxic potencies between these chlorinated samples, a defined concentration of the test sample i.e. in molar range is required, which is not applicable in these unknown samples.

At present, the compounds responsible for the genotoxicity of these chlorinated MW fractions are not known. Due to their health hazard potential, further studies are recommended in order to identify the components in these fractions to be able to help predict which precursors should be monitored or removed prior to disinfection process. The overall study should give a good indication for water authorities on how water treatment processes can be optimised for maximum removal of NOM, particularly the fractions that contain significant precursors of emerging DBPs. The application described in this study could be applied to use in other water sources including drinking water and/or recycled water.

Another outcome from this study was modification of the colorimetric DPD method to a microplate format in order to allow measurement of chlorine residual using a small sample volume. The standard colorimetric DPD method 4500-Cl-G (APHA, AWWA and WEF, 1998) described in Chapter 4 was adapted to a microplate set up to allow analysis of small sample volume. The modified colorimetric DPD method was used to screen chlorine residual of chlorinated MW fractions using 300 µl sample volume. In this microplate format, up to 96 samples (including chlorine standards) could be measured in a short time frame (5 – 10 min).

In conclusion, this Chapter has demonstrated an alternative approach for prioritisation studies in terms of NOM removal by using an application of HPSEC and the validated cell based assay to help identify potential toxic DBP precursors. This study has shown that medium to high MW fractions in NOM contain toxic DBP precursors, which may lead to further studies on their chemical identification or on development of effective removal technology targeting these specific fractions.

8.   SUMMARY AND GENERAL DISCUSSION

The aims of the research presented in this thesis were 1) to establish and validate the FCMN assay for rapid genotoxicity screening of DBPs, 2) to modify the FCMN assay for use in genotoxicity screening of organic N-chloramines and 3) to assess the genotoxicity of organic N-chloramines at the concentrations that may be present in disinfected drinking water. This research addressed the significance of some organic N-chloramines as potential health risks with genotoxic potencies identified at micromolar concentrations. Oxidative stress was hypothesised as a possible genotoxic mechanism of N-chloramines and was also investigated. Furthermore, the optimised FCMN assay was used to screen genotoxicity of DBPs produced from chlorination of molecular weight (MW) fractions of an Australian water sample. In this final Chapter the results presented in this thesis will be summarised and discussed in more general terms in relation to genotoxicity of DBPs, organic N-chloramines and possible future research that may benefit Australian water industries.

8.1.   Establishment of the FCMN Assay

The first aim of this research was to establish a procedure that was suitable for screening the genotoxicity of a large number of samples. From the literature review, we found that the micronucleus assay was very suitable for use in this study due to its broad ability in detection of both clastogenic and aneugenic effects of a test chemical (Fenech, 2000). However, while the traditional MN assay is recommended as a genotoxicity testing tool (Fenech, 1997; Fenech, 2006; OECD, 2004), counting MN microscopically is impractical for use as a genotoxicity screening method. Therefore, the FCMN that has been

previously described in several studies (Avlasevich *et al.*, 2006; Bryce *et al.*, 2007; Nüsse and Marx, 1997; Roman *et al.*, 1998; Sanchez *et al.*, 2000; Schreiber *et al.*, 1992) was adopted and validated for use to screen genotoxicity of organic N-chloramines.

One disadvantage of the FCMN assay is that if apoptotic nuclei are present in a sample they can be registered as MN during the measurement, causing a false positive interpretation (Avlasevich *et al.*, 2006; Nüsse and Marx, 1997). To minimise the interference from apoptotic nuclei, it was hypothesised that performing experiments in cell lines with an impaired apoptosis response may be a good alternative. Two cell lines (WIL2-NS and L5178Y) with mutated p53, a gene that plays a crucial role in the induction of apoptosis (Lowe *et al.*, 1993), were studied in comparison.

While both cell lines have mutated p53, our results showed that WIL2-NS cells had reduced apoptosis response (Chapter 3, section 3.3.4). The impaired ability to undergo apoptosis in WIL2-NS cells presented in this thesis is consistent with the previous studies (Xia *et al.*, 1994; Xia *et al.*, 1995). On the othe hand, although having mutated p53, a markedly higher apoptosis response was seen in L5178Y cells. It is also noted that the function of p53 protein was not investigated in this study, and therefore it could be possible that mutation of p53 in L5178Y has less effect on function of P53 protein than that of WIL2-NS cells. In addition, although the mechanism of apoptosis is mediated through a p53-dependent pathway (Shen and White, 2001), apoptosis could also be induced via a p53-independent pathway (Narine *et al.*, 2009; Wang *et al.*, 2009). Further studies are required in order to understand these mechanisms, but it is beyond the scope of our study.

It may be argued that genotoxicity assessment of a chemical should be conducted in cells with normal p53 function (Kirkland *et al.*, 2007); however, in view of a rapid

screening test, the practicality and low background of the assay are of primary consideration and assays based on cells with mutated p53 have been widely used in the literature for genotoxicity assessment of chemicals. WIL2-NS cells have been widely used in the traditional *in vitro* micronucleus assay (Beetstra *et al.*, 2005; Furuno-Fukushi *et al.*, 1996; Humpage *et al.*, 2000; Thomas *et al.*, 2003; Umegaki and Fenech, 2000; Wang *et al.*, 2007), but there is no report on this cell line for the FCMN application. Our study has shown that with limited ability to undergo apoptosis, WIL2-NS cell line is a suitable for the FCMN assay as a rapid genotoxicity screening of chemicals, which can be used as a frontline screening tool in a test battery for genotoxicity.

Another important issue that was considered when validating the FCMN assay for screening genotoxicity of N-chloramines was the use of organic N-free exposure medium. While initial validation of the FCMN assay with model genotoxicants was carried out in complete cell culture medium, it was realised that chlorine from N-chloro group of organic N-chloramines can transfer to other organic nitrogenous compounds in the exposure medium (i.e. amnio acids and protein), which could potentially confound the results by i.e. producing genotoxic events other than from the test chemical. This is critical when testing individual organic N-chloramines for genotoxic activity. This phenomenon has been shown previously, for example, by Peskin et al (2004) as discussed in Chapter 5. Therefore, the exposure protocol using N-free exposure medium (HBSS) was further optimised and we found this exposure protocol work well with WIL2-NS cells as shown in Chapter 5 (section 5.3.1). Similar considerations were described in a genotoxicity study described by (Maron and Ames, 1983).

8.2. Genotoxicity of Organic N-chloramines

A major focus of this work was to address potential toxicity and genotoxicity of organic N-chloramines that may be present in disinfected drinking water. Initially this study aimed to investigate cytotoxicity and genotoxicity of 21 organic N-chloramine candidates (as listed in Appendix 2, A2-1). These model compounds were selected based on their formation rates, stability, indications of predicted mutagenicity and the presence of set of diverse set of secondary functional groups that will affect formation and/or stability of the chloramines (Bull *et al.*, 2006). However, only 16 could be produced in our laboratory and therefore used in this research. Five compounds (N-chloro-2-methylbutylaldimine, N-chloroglycyladimine, N-chlorophenylacetaldimine, N-2(N' chloroimino)-propanoyl phenylalanine and N-chloro-α-acetylchloroarginine) requires more complex reaction conditions and purification, which was not feasible at time of this study, and hence they were not included in this thesis.

In order to assess the genotoxicity risk of organic N-chloramines in relation to consumption of disinfected drinking water, selection of the test concentration was considered critical. The concentrations tested should, in part, reflect the real life situation of these compounds being present in drinking water. Unfortunately, to date there is no available information on the occurrence of organic N-chloramines in disinfected water. The concentrations of organic N-chloramines (in micromolar range) used in this study were selected based on the concentration of their amine precursors that could be present in natural water source (Nweke and Scully Jr, 1989; Ram and Morris, 1980; Thomas, 1997). A survey study on occurrence of these compounds will help understand the risks they may pose to humans and therefore should be included in future studies.

Our study has shown that of 16 organic N-chloramines, 4 compounds (N-chloroglycine, N,N''-dichlorolysine, N-chlorohistamine and chloroethanolamine) are both cytotoxic and genotoxic in the mammalian cells (WIL2-NS). Similarly, genotoxicity of these four organic N-chloramines have been reported in pervious studies using bacterial models (Nakamura *et al.*, 1993; Thomas *et al.*, 1987).

At present, the health risks associated with these genotoxic N-chloramines in relation to consumption of drinking water is not known. However, histamine is a biomolecule produced (*in vivo)* by mast cells and secreted from the cells to surrounding tissues at inflammatory cites (Nilsson *et al.*, 1999), and thus N-chlorohistamine could be formed at sites of inflammation when chlorine or chlorine donating molecules (i.e. chlorinated DBPs) are present (Pattison and Davies, 2006). Further studies should be conducted in order to investigate toxicity effects of these inflammatory cells in the presence of chlorine or chlorinated disinfection by-products.

Apart from mast cells, enterochromaffin-like (ECL) cells are another main storage site of histamine. These cells are found at the mucosal layer of stomach and digestive tract (Hakanson *et al.*, 1986; Reite, 1969; Waldum *et al.*, 1991). Since some organic N-chloramines can be formed and survive in the extremely low pH condition of gastric content (Scully *et al.*, 1985), it is possible that the N-chlorohistamine could also be formed in the stomach following ingestion of disinfected drinking water (containing HOCl, chlorinated DBPs). In order assess health risks of this compound, *in vivo* formation of N-chlorohistamine following ingestion of chlorine or chlorinated DBPs may be worth investigating.

A molecule of lysine comprises 2 amino groups (attached to the α-carbon and the ε-carbon). In peptides, lysines are often found buried with only the ε- NH_2 exposed to the environment and this moeity is suggested to be highly susceptible to attack by chlorine during water disinfection (Handelman *et al.*, 1998). To clarify this point, apart from the normal lysine molecule, 2 analogues of lysine in which either α-NH_2 or ε-NH_2 were blocked by an acetyl group (see chemical structure in Chapter 4) were included in this study. Our study showed that there was a clear preference of reactivity on the different amino side chains with chlorine, which is consistent with a study reported by Handelman *et al.* (1998) and Nightingale *et al.* (2000).

It was also found that chlorination of α-acetylated lysine (chlorine attack at ε-NH_2) demonstrated a small increase in cytotoxic and genotoxic effects (Chapter 5). This indicated that ε-NH_2 of lysine is likely to form a toxic produce when in contact with chlorine. A significantly greater effect observed in the treatment of chlorinated lysine (N,N$^\varepsilon$-dichlorolysine) compared with its acetylated derivatives was rather interesting. It was possible that the greater effects observed may indicate a toxicological significance of the number of chlorine atoms in a molecule of an N-chloramine once it enters the cells. Because lysine in natural water can be present as a complex molecule, including in peptides (in which ε-NH_2 is the hot spot for chlorine attack) it is suggested that toxicity/genotoxicity of chlorinated lysine-containing peptides should be included in future studies.

A possible genotoxic mechanism of the 4 genotoxic N-chloramines (measurements of oxidative stress) was conducted as detailed in Chapter 6. As N-chloramines have oxidative capacity that, once they enter cells, can initiate secondary reactions with cellular antioxidants such as thiols (Carr *et al.*, 2001; Peskin and Winterbourn, 2001; Peskin and

Winterbourn, 2003; Prutz, 1998; Thomas *et al.*, 1986), it was hypothesised that a mechanism involved in genotoxicity of these compounds may be mediated through oxidative stress.

Depletion of cellular glutathione and lipid peroxidation were used as indicators to determine the level of oxidative stress in WIL2-NS cells following exposure to the cytotoxic / genotoxic N-chloramines. This study has shown that a small reduction of GSH occurred at all treatment concentrations with the 4 genotoxic N-chloramines while lipid peroxidation was not observed. The slight reduction of GSH without lipid peroxidation indicated that although the N-chloramines could react with the cellular GSH, the oxidative potency did not overwhelm the cellular antioxidant, and hence genotoxicity of these compounds was unlikely to be mediated through oxidative stress.

To date, the genotoxic mechanism of the N-chloramines is still not well understood. It has been suggested that following the entry of these compounds to the cell interior, some chemical modifications such as oxidation of thiols in protein molecules occurred, which may have a subsequent effect on modification of bases in DNA (Thomas *et al.*, 1987). A small reduction of intracellular glutathione present in this study has confirmed that oxidation of cellular thiols took place following the cell entry (Carr *et al.*, 2001; Peskin and Winterbourn, 2001; Peskin and Winterbourn, 2003; Prutz, 1998; Thomas *et al.*, 1986). Although our study demonstrated that cellular oxidative stress was not evident, the oxidation of the cellular antioxidant suggested that reactions with other cellular macromolecules could have occurred, but was not measured in this study.

Hawkins and Davies (2002) has shown that direct reaction of N-chloramines with plasmid DNA could damage the DNA backbone, which lead to both single- and double-

strand breaks via N-chloramine-mediated reactions as well as radicals from the nucleobases. The study also suggested that the initial formation of nitrogen-centred radicals on nucleobases could lead to backbone strand cleavage via hydrogen atom abstraction from the sugar-phosphate backbone of the DNA strand. Hence, it was possible that upon cell entry although oxidation of cellular glutathione took place, the majority of organic N-chloramine may rapidly enter the nucleus and react with nucleobases, resulting in strand breaks which consequently lead to MN formation as observed. To confirm this hypothesis, further investigations on organic N-chloramines and oxidative DNA damage, mediated through N-centred radical formation using techniques such as Electron Paramagnetic Resonance Spectroscopy (Hawkins and Davies, 1999; Hawkins and Davies, 2002) are required.

Within the concentration range tested (micromolar), 12 organic N-chloramines did not show cytotoxic nor genotoxic effects in WIL2-NS cells (Chapter 5; Table 5-1 and Table 5-3). While compounds such as chlorinated products of arginine, histidine, phenylalanine and serine were reported to have mutagenic effects in the bacterial Ames Test (Nakamura *et al.*, 1993), they did not show genotoxic effects in WIL2-NS cells in this study. This discrepancy may be due to two possibilities. Firstly, the concentration of compounds used in our study (micromolar) is much lower that that used in previous study (millimolar) (Nakamura *et al.*, 1993). Secondly, it is also possible that the different outcomes from our studies compared with the study conducted by Nakamura *et al.* (1993) may have been from the different assay sensitivity between micronucleuss assay and Ames test.

8.3.   Significance of This Study to Water Research in Australia

An application of the mammalian cell-based assay validated in this research was used to help identify which MW fraction(s) should be focussed for future studies in relation to identification of individual compound(s) that have potential in forming toxic DBPs, for future removal of these precursors prior to chlorination or monitoring of the toxic DBPs after the disinfection process. The methods described in Chapter 7 can be applied to use in water collected from different part of Australia, including South Australia.

Another objective of this research was to understand the relationship between amine precursors found in each of the MW fractions produced by the Curtin University team and the genotoxic organic N-chloramines identified following chlorination of the MW water fractions (Chapter 7). Unfortunately, at the time of this study, chemical analysis of the amine content of these MW fractions had not been done. It is hypothesised that the genotoxic MW fractions (reported in Chapter 7) may contain organic N-chloramines produced from anime precursors in the concentrates. Further studies are required to help identify whether organic N-chloramines play a role in the genotoxicity of these MW fractions isolated from Australian water.

8.4.   Conclusion

This thesis described optimisation and validation of the mammalian cell based FCMN assay, and its use for assessment of the genotoxicity of 16 organic N-chloramine candidates. This study reported that within the lower micromolar concentration range, 4 organic N-chloramines were genotoxic to mammalian cells (WIL2-NS). A slight reduction of cellular GSH without lipid peroxidation observed in the cells treated with these four N-chloramines has indicated that oxidative stress was unlikely to be a mechanism involved in their genotoxicity. These findings therefore suggest that formation of N-centred radicals that react preferentially with DNA bases may be a better hypothesis to investigate further. To understand what mechanisms are involved, further studies including direct reaction to DNA molecules to form DNA adducts are required. A main outcome of this research is the successful establishment of a mammalian cell based FCMN assay for use as a rapid genotoxicity screening test of water samples. The established assay could also be used to screen potential genotoxicants in various types of water samples, which will provide a great benefit for water industries in Australia.

8.5.   Future Research Direction

Previously, concerns in relation to potential health effects of organic N-chloramines have been raised by investigation of their roles in production of a variety of chronic diseases including some human cancers following their *in vivo* formation as a consequence of inflammatory processes (Daumer *et al.*, 2000; Halliwell *et al.*, 1987; Pero *et al.*, 1995). However, to date, there have not been any reports on their toxicology if consumed in drinking water. Our studies have shown that some organic N-chloramines can cause genotoxic damage in human cells even when exposure occurred at relatively low concentrations (micromolar), indicating the risk that these compounds may pose to humans. It is strongly recommended that *in vivo* studies should be undertaken in order to characterise the toxicological risks of these compounds. To this end, Dr Bull recently included unpublished results from this study on N-chloramines in a submission to the US National Toxicology Program recommending subchronic, chronic and carcinogenicity studies be carried out on N, N-dichlorolysine and N-chlorohistamine (R. J. Bull, personal communication).

Since the formation of organic N-chloramines could take place in both chlorinated and chloraminated drinking waters, further studies including their occurrence, fate of compounds in distribution systems, potential exposure and health risk assessments associated with these compounds should be of priority. A key question is whether these molecules are 1) those that occur in disinfected water or 2) those that might be formed after ingestion of water containing residual amounts of free chlorine, chloramines or other chlorinated compounds so that chlorine transfer can occur when in contact with biological amines. This area of study also needs to be investigated further.

An application of the cell based assay for described in this thesis (Chapter 7) may be applied to use for determination of toxicity of pre- and post-chlorination of MW fractions isolated from various water sources. In addition, this research has shown that several medium to high MW fractions have been shown to be precursors to toxic DBPs. At this point of time, identification of individual DBPs in these complex mixtures is not available. It could be possible that several N-chloramines may be present amongst these chlorinated products. Further toxicity identification of the DBPs in these fractions may indicate if organic *N*-chloramines were involved in the cytotoxicity and genotoxicity observed.

APPENDICES

Appendix 1: Media, Buffers and Solutions

A1-1. Cell Culture and Treatmen Media

RPMI Basic

RPMI 1640 (Sigma-Aldrich # R6504)	10.4 g
NaHCO$_3$ (Sigma-Aldrich # S5761)	2.0 g
HEPES (Sigma-Aldrich # H4034)	3.8 g
Sterile Milli-Q water	950 ml

Dissolve all ingredients in 1 L Schott bottle, adjust to pH 7.4 ± 0.05,

make to 1 L, filter (0.2 µM) and store at 4°C.

WIL2-NS Complete Medium

RPMI Basic	200 ml
Filtered (0.2 µM) FBS	20 ml
Penicillin G (Sigma-Aldrich # P7794)	0.06 mg/ml
Streptomycin (Sigma-Aldrich # S9137)	0.1 mg/ml

L5178Y Complete Medium

DMEM (Sigma-Aldrich # D6429)	200 ml
Filtered (0.2 µM) 100X HEPES[*]	1 ml
10% Pluronic acid (GIBCO)	2 ml
Filtered (0.2 µM) FBS	20 ml

[*]100X HEPES

HEPES (Sigma-Aldrich # H4034)	2.38 g
DMEM (Sigma-Aldrich # D6429)	10 ml

Dissolve the ingredients in a 50 ml conical tube, filter (0.2µM) and store at - 20°C.

10X PBS

NaCl (UNIVAR # A465)	80 g
KCl (Sigma-Aldrich # P5405)	2 g
KH_2PO_4 (BDH)	2 g
Na_2HPO_4 (Sigma-Aldrich # S5136)	11.5 g
Sterile Milli-Q water	1 L

Dissolve all ingredients in 1 L Schott bottle, adjust the pH to 7.4 ± 0.05,

Autoclave at 121°C for 15 min and store at room temperature up to 3 months.

1X PBS

10X PBS	100 ml
Sterile Milli-Q water	900 ml

Store at room temperature (200 ml per bottle).

10X HBSS

KCl (Sigmal-Aldrich # P5405)	4 g
KH_2PO_4 (BDH)	0.6 g
$NaHCO_3$ (UNIVAR # D3247)	3.5 g
NaCl (UNIVAR # A465)	80 g
Na_2HPO_4 (Sigma-Aldrich # S5136)	0.4788 g
D-glucose (Sigma-Aldrich # G5146)	10 g
Sterile Milli-Q water	1 L

Dissolve all ingredients in 1 L Schott bottle, adjust the pH to 7.4 ± 0.05,

filter (0.2 µM) and store at 4°C.

HBSS

10X HBSS	100 ml
Sterile Milli-Q water	900 ml

Store at 4°C (200 ml per bottle).

A1-2. Flow Cytometry Solutions

FCM Solution 1

NaCl (UNIVAR # A465)	0.584 g
Na-citrate (Sigma-Aldrich # S4641)	1 g
Nonidet P40 (Fluka # 74385)	300 μl
Sterile Milli-Q water	1 L

Dissolve all ingredients in 1 L Schott bottle and store at room temperature for up to 3 months.

FCM Solution 2

Citric acid (Sigma-Aldrich # C0759)	7.5 mg
Sucrose (Sigma-Aldrich # S0389)	42.8 g
Sterile Milli-Q water	500 ml

Dissolve all ingredients in 1 L Schott bottle and store at 4°C for up to 3 months.

A1-3. Chlorination Reagents

DPD (N,N-diethyl-p-phenyl diamine)

$NH_2\text{-}C_6H_4\text{-}N(C_2H_5)_2 . H_2SO_4$	1.1 g
EDTA (disodium)	0.2 g
H_2SO_4 (conc.)	2 ml
Milli-Q water	1 L

Dissolve all ingredients in 1 L bottle, store at room temperature (protected from light)

FAS (Ferrous ammonium sulphate)

$Fe(NH_4)_2(SO_4)_2 . 6H_2O$	1.106 g
H_2SO_4 (conc.)	0.25 ml
Milli-Q water	1 L

Dissolve all ingredients in 1 L bottle, store at 4°C (protected from light) and equilibrate at room temperature prior to use.

Buffer Solution pH 6.5

Na_2HPO_4	24 g
KH_2PO_4	46 g
Disodium EDTA	0.8 g
$HgCl_2$	0.02 g
Milli-Q water	1 L

Dissolve all ingredients in a 1 L bottle, store at room temperature (protected from light).

KI Solution

KI	0.5 g
Milli-Q water	100 ml

Prepare the solution in a dark bottle and store at room temperature.

A1-4. Glutathione Assay Reagens

1X Assay Buffer

25X Assay Buffer (TREVIGEN[®])	2 ml
Sterile Milli-Q water	48 ml

Reaction Mix

Reaction Mix (TREVIGEN[®])	1 bottle
Sterile Milli-Q water	8 ml

Reconstitute the reaction mix by gently mixing the content over a 15 min period. Immediately before use in the assay, vortex the vial of Glutathione Reductase (TREVIGEN[®]) and add 10 µl to the bottle of reaction mix.

5% Metaphosphoric Acid

Metaphosphoric Acid (Aldrich #23,927-5)	5 g
Sterile Milli-Q water	100 ml

Dissolve metaphosphoric in Milli-Q water and store at 4°C.

2 M 4-Vinylpyridine

      4-Vinylpyridine (Aldrich # V3204)   108 µl

      Ethanol   392 µl

      Prepare the reagent in a fume hood and use immediately.

A1-5. MDA Assay Reagens

1X TBA Acid Dilutent

      2X TBA acid diluents (OxiSelect™)   25 ml

      Sterile Milli-Q water   25 ml

TBA Reagents

      Thiobarbituric acid (OxiSelect™)   130 mg

      1X TBA acid diluents   25 ml

      Prepar the TBA reagent just before use by mixing vigorously until the powder has dissolved. Adjust the pH of the solution to pH 3.5 with sodium hydroxide solution.

Appendix 2: Additional Results

(Not Presented in Thesis Chapters)

A2-1. Organic N-chloramine candidates (RJ Bull, personal communication)

Twenty-one organic N-chloramines were suggested for further genotoxicity investigation.

Only 16 were used in this study. The results are presented in Chapter 4 and Chapter 5.

NO.	N-chloramine candidates	N-chloramines tested in this study
1	N- chloroleucine	N- chloroleucine
2	N- chloroisoleucine	N- chloroisoleucine
3	N- chloroglycine	N- chloroglycine
4	N- chlorophenylalanine	N- chlorophenylalanine
5	N- chloroserine	N- chloroserine
6	N chlorohistidine	N- chlorohistidine
7	Taurine monochloramine	Taurine monochloramine
8	N, N′- dichlorolysine	N, N′- dichlorolysine
9	N- chloro-α-acetyllysine	N- chloro-α-acetyllysine
10	N- chloro-ε-acetyllysine	N- Chloro-ε-acetyllysine
11	N- chloroarginine	N- chloroarginine
12	N- chlorohistamine	N- chlorohistamine
13	N- chloroethanolamine	N- chloroethanolamine
14	N- chloroalanylphenylalanine	N- chloroalanylphenylalanine
15	N-chloroglutamate	N- c hloroglutamate
16	N, N′- dichlorocystine	N, N′- dichlorocystine
17	N- chloro-2-methylbutylaldimine	NA
18	N- chloroglycyladimine	NA
19	N- chlorophenylacetaldimine	NA
20	N- 2(N' chloroimino)-propanoyl phenylalanine	NA
21	N- chloro-α-acetylchloroarginine	NA

A2-2. Intracellular GSH levels of WIL2-NS cells following treatment with N-chloramines (Raw data taken from Chapter 6).

Treatment	Total GSH (pmole/well)
Control (untreated)	286 ± 21
20 µM FeSO4	193 ± 7.9
40 µM FeSO4	70 ± 12.0
N-chloroglycine	
7.5 µM	277 ± 23
15 µM	261 ± 22
30 µM	243 ± 24
N-chlorolysine	
7.5 µM	280 ± 21
15 µM	273 ± 21
30 µM	262 ± 17
N-chlorohistamine	
7.5 µM	271 ± 25
15 µM	264 ± 25
30 µM	253 ± 20
N-chloroethanolamine	
104 µM	277 ± 20
209 µM	271 ± 22
418 µM	250 ± 21

Data represent values of mean ± SEM, n = 3.

A2-3. Cytotoxicity of pre-chlorinated MW fractions

(Additional results from Chapter 7)
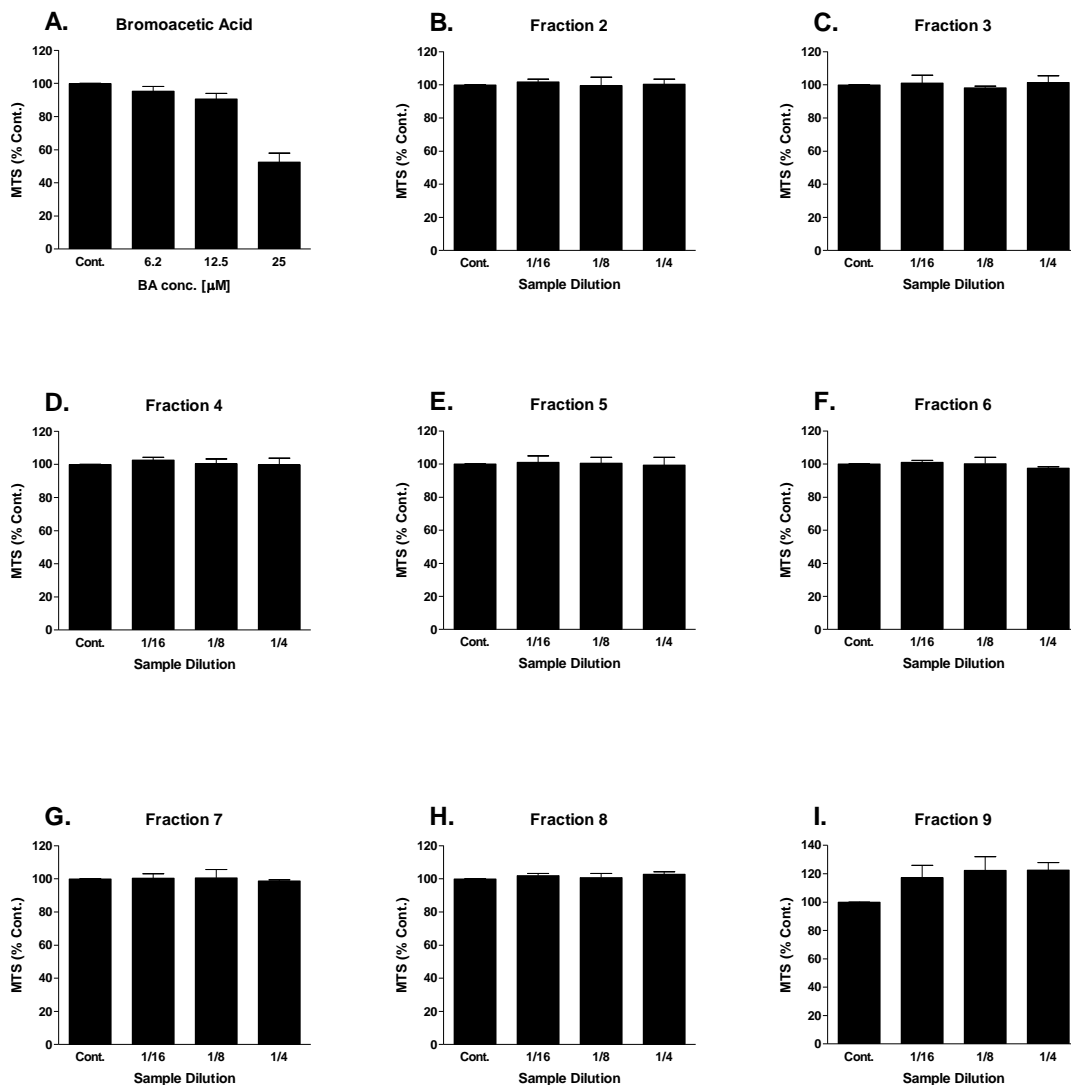
Cytotoxicity is presented as a percentage reduction of MTS viability over the untreated control (Cont.). Data are mean ± SD of 2 independent experiments.

A2-4. Genotoxicity of Pre-chlorinated MW fractions

(Additional results from Chapter 7)

Sample	% MN (FCM)	% MN (Microscopy)
Control (untreated)	2.4 ± 0.3	1.0 ± 0.2
BA 6.2 μM	2.9 ± 0.3	1.0 ± 0.2
BA 12.5 μM	3.8 ± 0.7	1.7 ± 0.2
BA 25 μM	9.7 ± 1.4	6.2 ± 0.4
Fraction 1	2.8 ± 1.0	1.0 ± 0.2
Fraction 2	2.5 ± 0.6	1.0 ± 0.2
Fraction 3	3.0 ± 0.9	0.8 ± 0.3
Fraction 4	3.1 ± 0.2	0.9 ± 0.2
Fraction 5	2.8 ± 0.3	0.9 ± 0.3
Fraction 6	2.5 ± 0.3	0.8 ± 0.3
Fraction 7	2.7 ± 0.4	0.9 ± 0.4
Fraction 8	77.0 ± 3.9*	1.1 ± 0.3**

BA = Bromoacetic acid. Data are mean ± SD of 2 independent experiments.

Fraction 8 showed high levels of debris in FCMN (*) and bacterial contamination in microscopy (**).

A2-5. FCMN Scatter Plots of Pre-chlorinated MW Fraction 8

(additional results from Chapter 7)
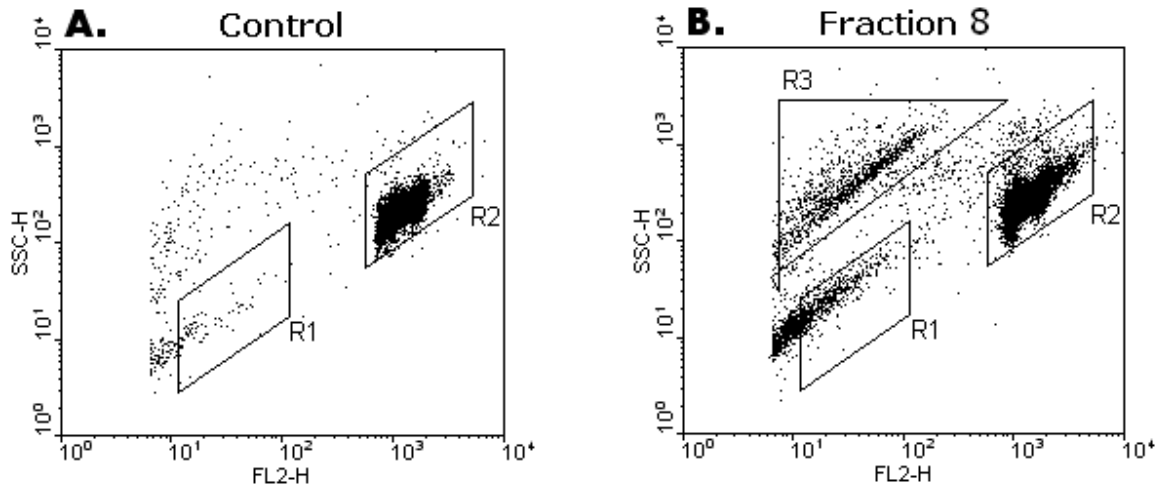
Flow cytometry profile (scatter plots: SSC vs. FL2-H) of control (A) vs. pre-chlorinated fraction 8 (B). R1 and R2 are MN and N regions. R3 contains population of unknown origin. Investigation of this pre-chlorinated sample indicated bacterial contamination.

BIBLIOGRAPHY

Abalea V., Cillard J., Dubos M.P., Anger J.P., Cillard P. and Morel I. (1998) Iron-Induced Oxidative DNA Damage and Its Repair in Primary Rat Hepatocyte Culture. *Carcinogenesis* 19(6):1053-1059.

Abe I., Nakamura K., Toyonaga T., Kobara Y. and Wasa T. (1993) Determination of D,L-Amino Acids in Natural-Water by Gas-Chromatography with Enantiomer Labeling. *Analytical Sciences* 9(6):775-778.

Abia L., Armesto X.L., Canle M., Garcia M.V. and Santaballa J.A. (1998) Oxidation of Aliphatic Amines by Aqueous Chlorine. *Tetrahedron* 54(3-4):521-530.

Ahmad R., Tripathi A.K., Tripathi P., Singh S., Singh R. and Singh R.K. (2008) Malondialdehyde and Protein Carbonyl as Biomarkers for Oxidative Stress and Disease Progression in Patients with Chronic Myeloid Leukemia. *In Vivo* 22(4):525-528.

Aieta E.M. and Berg J.D. (1986) A Review of Chlorine Dioxide in Drinking Water Treatment. *J. Am. Water Works Assoc.* 78(6):62-72.

Albertini R.J., Anderson D., Douglas G.R., Hagmar L., Hemminki K., Merlo F., Natarajan A.T., Norppa H., Shuker D.E., Tice R., Waters M.D. and Aitio A. (2000) Ipcs Guidelines for the Monitoring of Genotoxic Effects of Carcinogens in Humans. International Programme on Chemical Safety. *Mutat Res* 463(2):111-172.

Alberts J.J. and Takacs M. (1999) Importance of Humic Substances for Carbon and Nitrogen Transport into Southeastern United States Estuaries. *Organic Geochemistry* 30(6):385-395.

Alonso E., Santos A. and Riesco P. (2004) Micro-Organism Re-Growth in Wastewater Disinfected by Uv Radiation and Ozone: A Micro-Biological Study. *Environ Technol* 25(4):433-441.

Amundson S.A., Xia F., Wolfson K. and Liber H.L. (1993) Different Cytotoxic and Mutagenic Responses Induced by X-Rays in Two Human Lymphoblastoid Cell Lines Derived from a Single Donor. *Mutat Res* 286(2):233-241.

Amy G.L., Sierka R.A., Bedessem J., Price D. and Tan L. (1992) Molecular-Size Distributions of Dissolved Organic-Matter. *J. Am. Water Work Assoc.* 84(6):67-75.

Antelo J.M., Arce F., Crugeiras J. and Parajo M. (1997) Kinetic Study of the Chlorine Transfer from *N*-Chlorosuccinimide to Amino Compounds. *J. Physic. Org. Chem.* 10:631-636.

APHA, AWWA and WEF (1998) Chlorine (Residual), 4500-Cl G. Dpd Colorimetric Method, in *Standard Methods for the Examination of Water and Wastewater* (Clesceri L.S., Greenberg A.E. and Eaton A.D. eds), American Public Health Association, Washington DC.

Arheimer B., Andersson L. and Lepisto A. (1996) Variation of Nitrogen Concentration in Forest Streams Influences of Flow, Seasonality and Catchment Characteristics. *Journal of Hydrology* 179(1-4):281-304.

Armesto X.L., Canle M., Garcia M.V., Losada M. and Santaballa J.A. (1994) N Reactivity Vs O Reactivity in Aqueous Chlorination. *Int. J. Chem. Kinet.* 26(11):1135-1141.

Armesto X.L., Canle M. and Santaballa J.A. (1993) Alpha-Amino-Acids Chlorination in Aqueous-Media. *Tetrahedron* 49(1):275-284.

Armstrong D., Hiramitsu T. and Ueda T. (1998) In Vitro Screening for Antioxidant Activity, in *Methods in Molecular Biology, Free Radical and Antioxidant Protocols* (Armstrong D. ed) pp 315-324, Humana Press.

Aruoma O.I., Halliwell B., Gajewski E. and Dizdaroglu M. (1989) Damage to the Bases in DNA Induced by Hydrogen Peroxide and Ferric Ion Chelates. *J Biol Chem* 264(34):20509-20512.

Athar M., Hasan S.K. and Srivastava R.C. (1987) Evidence for the Involvement of Hydroxyl Radicals in Nickel Mediated Enhancement of Lipid Peroxidation: Implications for Nickel Carcinogenesis. *Biochem Biophys Res Commun* 147(3):1276-1281.

Austin E.W., Parrish J.M., Kinder D.H. and Bull R.J. (1996) Lipid Peroxidation and Formation of 8-Hydroxydeoxyguanosine from Acute Doses of Halogenated Acetic Acids. *Fundam Appl Toxicol* 31(1):77-82.

Avlasevich S.L., Bryce S.M., Cairns S.E. and Dertinger S.D. (2006) In Vitro Micronucleus Scoring by Flow Cytometry: Differential Staining of Micronuclei Versus Apoptotic and Necrotic Chromatin Enhances Assay Reliability. *Environ Mol Mutagen* 47(1):56-66.

Baker M.A., Cerniglia G.J. and Zaman A. (1990) Microtiter Plate Assay for the Measurement of Glutathione and Glutathione Disulfide in Large Numbers of Biological Samples. *Anal Biochem* 190(2):360-365.

Barua M., Liu Y. and Quinn M.R. (2001) Taurine Chloramine Inhibits Inducible Nitric Oxide Synthase and Tnf-Alpha Gene Expression in Activated Alveolar Macrophages: Decreased Nf-Kappab Activation and Ikappab Kinase Activity. *J Immunol* 167(4):2275-2281.

Becher G., Carlberg G.E., Gjessing E.T., Hongslo J.K. and Monarca S. (1985) High-Performance Size Exclusion Chromatography of Chlorinated Natural Humic Water and Mutagenicity Studies Using the Microscale Fluctuation Assay. *Environmental Science & Technology* 19(5):422-426.

Beetstra S., Thomas P., Salisbury C., Turner J. and Fenech M. (2005) Folic Acid Deficiency Increases Chromosomal Instability, Chromosome 21 Aneuploidy and Sensitivity to Radiation-Induced Micronuclei. *Mutat Res* 578(1-2):317-326.

Bellar T.A., Lichtenbert J.J. and Kroner R.C. (1974) The Occurrence of Organohalides in Chlorinated Drinking Waters. *J. Am. Water Works Assoc.* 66:703-706.

Bempong M.A. and Scully F.E., Jr. (1980) Mutagenic Activity of N-Chloropiperidine. *J Environ Pathol Toxicol* 4(2-3):345-354.

Bernofsky C. (1991) Nucleotide Chloramines and Neutrophil-Mediated Cytotoxicity. *Faseb J* 5(3):295-300.

Betancourt W.Q. and Rose J.B. (2004) Drinking Water Treatment Processes for Removal of Cryptosporidium and Giardia. *Vet Parasitol* 126(1-2):219-234.

Blaszczyk A., Osiecka R. and Skolimowski J. (2003) Induction of Chromosome Aberrations in Cultured Human Lymphocytes Treated with Ethoxyquin. *Mutat Res* 542(1-2):117-128.

Bolto B., Dixon D., Eldridge R. and King S. (2002) Removal of Thm Precursors by Coagulation or Ion Exchange. *Water Res* 36(20):5066-5073.

Bolyard M., Fair P.S. and Hautman D.P. (1992) Occurrence of Chlorate in Hypochlorite Solutions Used for Drinking Water Disinfection. *Environ. Sci. Technol.* 26:1663-1665.

Bove F., Shim Y. and Zeitz P. (2002) Drinking Water Contaminants and Adverse Pregnancy Outcomes: A Review. *Environ Health Perspect* 110 Suppl 1:61-74.

Bronk D.A. (2002) Dynamics of Don, in *Biogeochemistry of Marine Dissolved Organic Matter* (Hansell D.A. and Carlson C.A. eds), Academic Press, London.

Bryce S.M., Bemis J.C., Avlasevich S.L. and Dertinger S.D. (2007) In Vitro Micronucleus Assay Scored by Flow Cytometry Provides a Comprehensive Evaluation of Cytogenetic Damage and Cytotoxicity. *Mutat Res* 660(1-2): 78-91.

Bull R.J., Reckhow D.A., Rotello V., Bull O.M. and Kim J. (2006) Use of Toxicological and Chemical Models to Prioritize Dbp Research: Report for Awwa Research Foundation Project 2867. *Awwa Research Foundation*.

Burcham P.C. (1998) Genotoxic Lipid Peroxidation Products: Their DNA Damaging Properties and Role in Formation of Endogenous DNA Adducts. *Mutagenesis* 13(3):287-305.

Buschini A., Carboni P., Frigerio S., Furlini M., Marabini L., Monarca S., Poli P., Radice S. and Rossi C. (2004) Genotoxicity and Cytotoxicity Assessment in Lake Drinking Water Produced in a Treatment Plant. *Mutagenesis* 19(5):341-347.

Calabrese E.J. (2001a) Apoptosis: Biphasic Dose Responses. *Crit Rev Toxicol* 31(4-5):607-613.

Calabrese E.J. (2001b) Overcompensation Stimulation: A Mechanism for Hormetic Effects. *Crit Rev Toxicol* 31(4-5):425-470.

Calabrese E.J. and Baldwin L.A. (2001) Hormesis: A Generalizable and Unifying Hypothesis. *Crit Rev Toxicol* 31(4-5):353-424.

Cantor K.P., Hoover R., Hartge P., Mason T.J., Silverman D.T., Altman R., Austin D.F., Child M.A., Key C.R., Marrett L.D. and et al. (1987) Bladder Cancer, Drinking Water Source, and Tap Water Consumption: A Case-Control Study. *J Natl Cancer Inst* 79(6):1269-1279.

Cantor K.P., Lynch C.F., Hildesheim M.E., Dosemeci M., Lubin J., Alavanja M. and Craun G. (1998) Drinking Water Source and Chlorination Byproducts. I. Risk of Bladder Cancer. *Epidemiology* 9(1):21-28.

Caraco N.F. and Cole J.J. (2002) The Importance of Organic Nitrogen Production in Aquatic Systems: A Landscape Perspective, in *Aquatic Ecosystems: Interactive of Dissolved Organic Matter* (Findlay S. and Sinsabaugh R.L. eds), N. Y. Academic Press, New york.

Carr A.C., Hawkins C.L., Thomas S.R., Stocker R. and Frei B. (2001) Relative Reactivities of N-Chloramines and Hypochlorous Acid with Human Plasma Constituents. *Free Radical Biology and Medicine* 30(5):526-536.

Chang H.H., Tung H.H., Chao C.C. and Wang G.S. (2009) Occurrence of Haloacetic Acids (Haas) and Trihalomethanes (Thms) in Drinking Water of Taiwan. *Environ Monit Assess*.

Chellam S. and Krasner S.W. (2001) Disinfection Byproduct Relationships and Speciation in Chlorinated Nanofiltered Waters. *Environ Sci Technol* 35(19):3988-3999.

Chiou T.J. and Tzeng W.F. (2000) The Roles of Glutathione and Antioxidant Enzymes in Menadione-Induced Oxidative Stress. *Toxicology* 154(1-3):75-84.

Choi J. and Valentine R.L. (2002) Formation of N-Nitrosodimethylamine (Ndma) from Reaction of Monochloramine: A New Disinfection by-Product. *Water Res* 36(4):817-824.

Chow C.W.K., van Leeuwen J.A., Drikas M., Fabris R., Spark K.M. and Page D.W. (1999) The Impact of the Character of Natural Organic Matter in Conventional Treatment with Alum. *Water Science and Technology* 40(9):97-104.

Christman R.F., Johnson J.D., Pfaender F.K., Norwood D.L., Webb M.R., Hass J.R. and Bobenrieth M.J. (1980) Chapter 7, in *Water Chlorination: Environmental Impact and Health Effects* (Jolley R.L., Brungs W.A. and Cummings R.B. eds), Ann Arbor Sciences Publishers, Ann Arbor, MI.

Cordier S., Clavel J., Limasset J.C., Boccon-Gibod L., Le Moual N., Mandereau L. and Hemon D. (1993) Occupational Risks of Bladder Cancer in France: A Multicentre Case-Control Study. *Int J Epidemiol* 22(3):403-411.

Daumer K.M., Khan A.U. and Steinbeck M.J. (2000) Chlorination of Pyridinium Compounds. Possible Role of Hypochlorite, N-Chloramines, and Chlorine in the Oxidation of Pyridinoline Cross-Links of Articular Cartilage Collagen Type Ii During Acute Inflammation. *J Biol Chem* 275(44):34681-34692.

Davies J.M., Horwitz D.A. and Davies K.J. (1993) Potential Roles of Hypochlorous Acid and N-Chloroamines in Collagen Breakdown by Phagocytic Cells in Synovitis. *Free Radic Biol Med* 15(6):637-643.

De la Rubia A., Rodriguez M., Leon V.M. and Prats D. (2008) Removal of Natural Organic Matter and Thm Formation Potential by Ultra- and Nanofiltration of Surface Water. *Water Research* 42(3):714-722.

De Luca G., Sacchetti R., Zanetti F. and Leoni E. (2008) Comparative Study on the Efficiency of Peracetic Acid and Chlorine Dioxide at Low Doses in the Disinfection of Urban Wastewaters. *Ann Agric Environ Med* 15(2):217-224.

DeAngelo A.B., Daniel F.B., Most B.M. and Olson G.R. (1997) Failure of Monochloroacetic Acid and Trichloroacetic Acid Administered in the Drinking Water to Produce Liver Cancer in Male F344/N Rats. *Journal of Toxicology and Environmental Health* 52(5):425-445.

Deinzer M., Schaumburg F. and Klein E. (1978) Environmental Health Sciences Center Task Force Review on Halogenated Organics in Drinking Water. *Environ. Health Perspect.* 24:209-239.

Dhariwal K.R., Washko P.W. and Levine M. (1990) Determination of Dehydroascorbic Acid Using High-Performance Liquid Chromatography with Coulometric Electrochemical Detection. *Anal Biochem* 189(1):18-23.

Dickinson B. (1996) Facscalibur System User's Guide, p 56, Becton Dickinson Instrument System, San Jose, CA.

Dodds L. and King W.D. (2001) Relation between Trihalomethane Compounds and Birth Defects. *Occup Environ Med* 58(7):443-446.

Donnermair M.M. and Blatchley E.R., 3rd (2003) Disinfection Efficacy of Organic Chloramines. *Water Res* 37(7):1557-1570.

Doyle T.J., Zheng W., Cerhan J.R., Hong C.P., Sellers T.A., Kushi L.H. and Folsom A.R. (1997) The Association of Drinking Water Source and Chlorination by-Products with Cancer Incidence among Postmenopausal Women in Iowa: A Prospective Cohort Study. *Am J Public Health* 87(7):1168-1176.

Draper H.H., Squires E.J., Mahmoodi H., Wu J., Agarwal S. and Hadley M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malondialdehyde in Biological Materials. *Free Radic Biol Med* 15(4):353-363.

Eaton A.D., Clesceri L.S. and Greenberg A.E. (1995) *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington D.C.

Ellman G.L. (1959) Tissue Sulfhydryl Groups. *Arch Biochem Biophys* 82(1):70-77.

Englert R.P. and Shacter E. (2002) Distinct Modes of Cell Death Induced by Different Reactive Oxygen Species: Amino Acyl Chloramines Mediate Hypochlorous Acid-Induced Apoptosis. *J Biol Chem* 277(23):20518-20526.

EPA (1998) *Disinfectants and Disinfection Byproducts; Final Rule*. US Environmental Protection Agency.

EPA (1999) Chlorine Dioxide, in *Epa Guidance Manual. Alternative Disinfectants and Oxidants* pp 1-41.

EPA (2001) National Primary Drinking Water Standards, United States Environmental Protection Agency. Epa 816-F-01-007.

Erba D., Riso P., Criscuoli F. and Testolin G. (2003) Malondialdehyde Production in Jurkat T Cells Subjected to Oxidative Stress. *Nutrition* 19(6):545-548.

Evseeva T.I., Geras'kin S.A., Shuktomova, II and Taskaev A.I. (2005) Genotoxicity and Cytotoxicity Assay of Water Sampled from the Underground Nuclear Explosion Site in the North of the Perm Region (Russia). *J Environ Radioact* 80(1):59-74.

Fenech M. (1997) The Advantages and Disadvantages of the Cytokinesis-Block Micronucleus Method. *Mutat Res* 392(1-2):11-18.

Fenech M. (2000) The in Vitro Micronucleus Technique. *Mutat Res* 455(1-2):81-95.

Fenech M. (2006) Cytokinesis-Block Micronucleus Assay Evolves into A "Cytome" Assay of Chromosomal Instability, Mitotic Dysfunction and Cell Death. *Mutat Res* 600(1-2):58-66.

Fenech M., Bonassi S., Turner J., Lando C., Ceppi M., Chang W.P., Holland N., Kirsch-Volders M., Zeiger E., Bigatti M.P., Bolognesi C., Cao J., De Luca G., Di Giorgio M., Ferguson L.R., Fucic A., Lima O.G., Hadjidekova V.V., Hrelia P., Jaworska A., Joksic G., Krishnaja A.P., Lee T.K., Martelli A., McKay M.J., Migliore L., Mirkova E., Muller W.U., Odagiri Y., Orsiere T., Scarfi M.R., Silva M.J., Sofuni T., Surralles J., Trenta G., Vorobtsova I., Vral A. and Zijno A. (2003a) Intra- and Inter-Laboratory Variation in the Scoring of Micronuclei and Nucleoplasmic Bridges in Binucleated Human Lymphocytes. Results of an International Slide-Scoring Exercise by the Humn Project. *Mutat Res* 534(1-2):45-64.

Fenech M., Chang W.P., Kirsch-Volders M., Holland N., Bonassi S. and Zeiger E. (2003b) Humn Project: Detailed Description of the Scoring Criteria for the Cytokinesis-Block

Micronucleus Assay Using Isolated Human Lymphocyte Cultures. *Mutat Res* 534(1-2):65-75.

Ferriol M., Gazet J. and Adad M. (1991) Kinetics and Mechanisms of Chlorine Transfer from Chloramine to Amines in Aqueous-Medium. *Int. J. Chem. Kinet.* 23(4):315-329.

Fogg G.E. (1971) Extracellular Products of Algae in Freshwater. *Ergeb. Limno.* 5:1-25.

Fukayama M.Y., Tan H., Wheeler W.B. and Wei C.I. (1986) Reactions of Aqueous Chlorine and Chlorine Dioxide with Model Food Compounds. *Environ Health Perspect* 69:267-274.

Furuno-Fukushi I., Tatsumi K., Takahagi M. and Tachibana A. (1996) Quantitative and Qualitative Effect of Gamma-Ray Dose-Rate on Mutagenesis in Human Lymphoblastoid Cells. *Int J Radiat Biol* 70(2):209-217.

Gabelova A., Valovicova Z., Labaj J., Bacova G., Binkova B. and Farmer P.B. (2007) Assessment of Oxidative DNA Damage Formation by Organic Complex Mixtures from Airborne Particles Pm(10). *Mutat Res* 620(1-2):135-144.

Garza K.M., Soto K.F. and Murr L.E. (2008) Cytotoxicity and Reactive Oxygen Species Generation from Aggregated Carbon and Carbonaceous Nanoparticulate Materials. *Int J Nanomedicine* 3(1):83-94.

Georgiadis P., Topinka J., Vlachodimitropoulos D., Stoikidou M., Gioka M., Stephanou G., Autrup H., Demopoulos N.A., Katsouyanni K., Sram R. and Kyrtopoulos S.A. (2005)

Interactions between Cyp1a1 Polymorphisms and Exposure to Environmental Tobacco Smoke in the Modulation of Lymphocyte Bulky DNA Adducts and Chromosomal Aberrations. *Carcinogenesis* 26(1):93-101.

Giller S., Le Curieux F., Erb F. and Marzin D. (1997) Comparative Genotoxicity of Halogenated Acetic Acids Found in Drinking Water. *Mutagenesis* 12(5):321-328.

Gjessing E.T., Alberts J.J., Bruchet A., Egeberg P.K., Lydersen E., McGown L.B., Mobed J.J., Munster U., Pempkowiak J., Perdue M., Ratnawerra H., Rybacki D., Takacs M. and Abbt-Braun G. (1998) Multi-Method Characterisation of Natural Organic Matter Isolated from Water: Characterisation of Reverse Osmosis Isolates from Water of Two Semi-Identical Dystrophic Lakes Basins in Norway. *Water Research* 32(10):3108-3124.

Glauert H.P., Tharappel J.C., Lu Z., Stemm D., Banerjee S., Chan L.S., Lee E.Y., Lehmler H.J., Robertson L.W. and Spear B.T. (2008) Role of Oxidative Stress in the Promoting Activities of Pcbs. *Environ Toxicol Pharmacol* 25(2):247-250.

Glaze W.H. and Peyton G.R. (1978) Chapter 1, in *Water Chlorination: Environmental Impact and Health Effects* (Jolley R.L., Gorchev H. and Hamilton D.H.J. eds), Ann Argor Science Publishers, Ann Arbor, MI.

Gordon G., Slootmaekers B., Tachiyashiki S. and Wood D.W. (1990) Minimizing Chlorite Ion and Chlorate Ion in Water Treated with Chlorine Dioxide. *J. Am. Water Works Assoc.* 82:160-165.

Graves C.G., Matanoski G.M. and Tardiff R.G. (2001) Weight of Evidence for an Association between Adverse Reproductive and Developmental Effects and Exposure to Disinfection by-Products: A Critical Review. *Regul Toxicol Pharmacol* 34(2):103-124.

Greilberger J., Koidl C., Greilberger M., Lamprecht M., Schroecksnadel K., Leblhuber F., Fuchs D. and Oettl K. (2008) Malondialdehyde, Carbonyl Proteins and Albumin-Disulphide as Useful Oxidative Markers in Mild Cognitive Impairment and Alzheimer's Disease. *Free Radic Res* 42(7):633-638.

Griffith O.W. and Meister A. (1979) Potent and Specific Inhibition of Glutathione Synthesis by Buthionine Sulfoximine (S-N-Butyl Homocysteine Sulfoximine). *J Biol Chem* 254(16):7558-7560.

Grisham M.B., Jefferson M.M., Melton D.F. and Thomas E.L. (1984) Chlorination of Endogenous Amines by Isolated Neutrophils. Ammonia-Dependent Bactericidal, Cytotoxic, and Cytolytic Activities of the Chloramines. *J Biol Chem* 259(16):10404-10413.

Hakanson R., Bottcher G., Ekblad E., Panula P., Simonsson M., Dohlsten M., Hallberg T. and Sundler F. (1986) Histamine in Endocrine Cells in the Stomach. A Survey of Several Species Using a Panel of Histamine Antibodies. *Histochemistry* 86(1):5-17.

Halliwell B., Wasil M. and Grootveld M. (1987) Biologically Significant Scavenging of the Myeloperoxidase-Derived Oxidant Hypochlorous Acid by Ascorbic Acid. Implications for Antioxidant Protection in the Inflamed Rheumatoid Joint. *FEBS Lett* 213(1):15-17.

Hammerschmidt S., Buchler N. and Wahn H. (2002) Tissue Lipid Peroxidation and Reduced Glutathione Depletion in Hypochlorite-Induced Lung Injury. *Chest* 121(2):573-581.

Handelman G.J., Nightingale Z.D., Dolnikowski G.G. and Blumberg J.B. (1998) Formation of Carbonyls During Attack on Insulin by Submolar Amounts of Hypochlorite. *Anal Biochem* 258(2):339-348.

Harris G.P. (2001) Biogeochemistry of Nitrogen and Phophorus in Australian Catchments, Rivers and Estauries: Effects of Land Use and Flow Regulation and Comparisons with Global Patterns. *Mar. Freswater Res.* 52:139-149.

Hartmann A., Elhajouji A., Kiskinis E., Poetter F., Martus H., Fjallman A., Frieauff W. and Suter W. (2001) Use of the Alkaline Comet Assay for Industrial Genotoxicity Screening: Comparative Investigation with the Micronucleus Test. *Food Chem Toxicol* 39(8):843-858.

Hassoun E.A. and Ray S. (2003) The Induction of Oxidative Stress and Cellular Death by the Drinking Water Disinfection by-Products, Dichloroacetate and Trichloroacetate in J774.A1 Cells. *Comp Biochem Physiol C Toxicol Pharmacol* 135(2):119-128.

Hawkins C.L. and Davies M.J. (1999) Hypochlorite-Induced Oxidation of Proteins in Plasma: Formation of Chloramines and Nitrogen-Centred Radicals and Their Role in Protein Fragmentation. *Biochem J* 340 (Pt 2):539-548.

Hawkins C.L. and Davies M.J. (2002) Hypochlorite-Induced Damage to DNA, Rna, and Polynucleotides: Formation of Chloramines and Nitrogen-Centered Radicals. *Chem Res Toxicol* 15(1):83-92.

Hayashi M. (2008) Roles of Oxidative Stress in Xeroderma Pigmentosum. *Adv Exp Med Biol* 637:120-127.

Hebels D.G., Jennen D.G., Kleinjans J.C. and de Kok T.M. (2009) Molecular Signatures of N-Nitroso Compounds in Caco-2 Cells: Implications for Colon Carcinogenesis. *Toxicol Sci*.

Henderson L., Wolfreys A., Fedyk J., Bourner C. and Windebank S. (1998) The Ability of the Comet Assay to Discriminate between Genotoxins and Cytotoxins. *Mutagenesis* 13(1):89-94.

Higashi Y., Noma K., Yoshizumi M. and Kihara Y. (2009) Endothelial Function and Oxidative Stress in Cardiovascular Diseases. *Circ J* 73(3):411-418.

Holmbom B., Voss R.H., Mortimer R.D. and Wong A. (1984) Fractionation, Isolation and Characterization of Ames-Mutagenic Compounds in Kraft Chlorination Effluents. *Environ. Sci. Technol.* 18(5):333-337.

Houwen B. (2002) Blood Film Preparation and Staining Procedures. *Clin Lab Med* 22(1):1-14, v.

Humpage A.R., Fenech M., Thomas P. and Falconer I.R. (2000) Micronucleus Induction and Chromosome Loss in Transformed Human White Cells Indicate Clastogenic and Aneugenic Action of the Cyanobacterial Toxin, Cylindrospermopsin. *Mutat Res* 472(1-2):155-161.

Hwang B.F. and Jaakkola J.J. (2003) Water Chlorination and Birth Defects: A Systematic Review and Meta-Analysis. *Arch Environ Health* 58(2):83-91.

IARC (1995) *Monographs on the Evaluation of Carcinogenic Risks to Humans. Dry Cleaning, Some Chlorinated Solvents and Other Industrial Chemicals*. International Agency for Research on Cancer, Lyon, France.

IARC (1999) *Monographs on the Evaluation of Carcinogenic Risks to Human. Some Chemicals That Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances*. International Agency for Research on Cancer, Lyon, France.

IARC (2004) *Monograph on the Evaluation of Carcinogenic Risks to Humans. Som Drinking-Water Disinfectants and Contaminants, Including Arsenic*. International Agency for Research on Cancer, Lyon, France.

Infante-Rivard C., Amre D. and Sinnett D. (2002) Gstt1 and Cyp2e1 Polymorphisms and Trihalomethanes in Drinking Water: Effect on Childhood Leukemia. *Environ Health Perspect* 110(6):591-593.

Ipek E., Tuylu B.A. and Zeytinoglu H. (2003) Effects of Carvacrol on Sister Chromatid Exchanges in Human Lymphocyte Cultures. *Cytotechnology* 43(1-3):145-148.

Jain A., Martensson J., Stole E., Auld P.A. and Meister A. (1991) Glutathione Deficiency Leads to Mitochondrial Damage in Brain. *Proc Natl Acad Sci U S A* 88(5):1913-1917.

Jakszyn P., Agudo A., Berenguer A., Ibanez R., Amiano P., Pera G., Ardanaz E., Barricarte A., Chirlaque M.D., Dorronsoro M., Larranaga N., Martinez C., Navarro C., Quiros J.R., Sanchez M.J., Tormo M.J. and Gonzalez C.A. (2006) Intake and Food Sources of Nitrites and N-Nitrosodimethylamine in Spain. *Public Health Nutr* 9(6):785-791.

Johnson J.D., Christman R.F., Norwood D.L. and Millington D.S. (1982) Reaction Products of Aquatic Humic Substances with Chlorine. *Environ Health Perspect* 46:63-71.

Kallio M. and Lahdetie J. (1996) Fragmentation of Centromeric DNA and Prevention of Homologous Chromosome Separation in Male Mouse Meiosis in Vivo by the Topoisomerase Ii Inhibitor Etoposide. *Mutagenesis* 11(5):435-443.

Kawai Y., Morinaga H., Kondo H., Miyoshi N., Nakamura Y., Uchida K. and Osawa T. (2004) Endogenous Formation of Novel Halogenated 2'-Deoxycytidine. Hypohalous Acid-Mediated DNA Modification at the Site of Inflammation. *J Biol Chem* 279(49):51241-51249.

Kim J.H., Stansbury K.H., Walker N.J., Trush M.A., Strickland P.T. and Sutter T.R. (1998) Metabolism of Benzo[a]Pyrene and Benzo[a]Pyrene-7,8-Diol by Human Cytochrome P450 1b1. *Carcinogenesis* 19(10):1847-1853.

King W.D. and Marrett L.D. (1996) Case-Control Study of Bladder Cancer and Chlorination by-Products in Treated Water (Ontario, Canada). *Cancer Causes Control* 7(6):596-604.

Kirkland D., Pfuhler S., Tweats D., Aardema M., Corvi R., Darroudi F., Elhajouji A., Glatt H., Hastwell P., Hayashi M., Kasper P., Kirchner S., Lynch A., Marzin D., Maurici D., Meunier J.R., Muller L., Nohynek G., Parry J., Parry E., Thybaud V., Tice R., van Benthem J., Vanparys P. and White P. (2007) How to Reduce False Positive Results When Undertaking in Vitro Genotoxicity Testing and Thus Avoid Unnecessary Follow-up Animal Tests: Report of an Ecvam Workshop. *Mutat Res* 628(1):31-55.

Klaude M., Eriksson S., Nygren J. and Ahnstrom G. (1996) The Comet Assay: Mechanisms and Technical Considerations. *Mutat Res* 363(2):89-96.

Kocaman A.Y. and Topaktas M. (2008) The in Vitro Genotoxic Effects of a Commercial Formulation of Alpha-Cypermethrin in Human Peripheral Blood Lymphocytes. *Environ Mol Mutagen* 50(1):27-36.

Koivusalo M., Hakulinen T., Vartiainen T., Pukkala E., Jaakkola J.J. and Tuomisto J. (1998) Drinking Water Mutagenicity and Urinary Tract Cancers: A Population-Based Case-Control Study in Finland. *Am J Epidemiol* 148(7):704-712.

Koivusalo M., Pukkala E., Vartiainen T., Jaakkola J.J. and Hakulinen T. (1997) Drinking Water Chlorination and Cancer-a Historical Cohort Study in Finland. *Cancer Causes Control* 8(2):192-200.

Koizumi T. and Li Z.G. (1992) Role of Oxidative Stress in Single-Dose, Cadmium-Induced Testicular Cancer. *J Toxicol Environ Health* 37(1):25-36.

Komulainen H. (2004) Experimental Cancer Studies of Chlorinated by-Products. *Toxicology* 198(1-3):239-248.

Korich D.G., Mead J.R., Madore M.S., Sinclair N.A. and Sterling C.R. (1990) Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on Cryptosporidium Parvum Oocyst Viability. *Appl Environ Microbiol* 56(5):1423-1428.

Krasner S., Chinn R., Pastor S., Sclimenti M., Weinberg H., Onstad G. and Richardson S. (2002) The Occurrence of Disinfection by-Products of Health Concern in Drinking Water. *Epidemiology* 13(4):160.

Kronberg L., Holmbom B., Reunanen M. and Tikkanen L. (1988) Identification and Quantification of the Ames Mutagenic Compound 3-Chloro-4-(Dichloromethyl)-5-Hydroxy-2(5h)-Furanone and of Its Geometric Isomer (E)-2-Chloro-3-(Dichloromethyl)-4-Oxobutenoic Acid in Chlorine-Treated Humic Water and Drinking-Water Extracts. *Environmental Science & Technology* 22(9):1097-1103.

Kulcharyk P.A. and Heinecke J.W. (2001) Hypochlorous Acid Produced by the Myeloperoxidase System of Human Phagocytes Induces Covalent Cross-Links between DNA and Protein. *Biochemistry* 40(12):3648-3656.

Laingam S., Froscio S.M. and Humpage A.R. (2008) Flow-Cytometric Analysis of in Vitro Micronucleus Formation: Comparative Studies with Wil2-Ns Human Lymphoblastoid and L5178y Mouse Lymphoma Cell Lines. *Mutat Res* 656(1-2):19-26.

Lalezary S., Pirbazari M. and McGuire M. (1986) Oxidation of Five Earthy-Musty Taste and Odor Compounds. *J. Am. Water Works Assoc.* 78(3):62-69.

Landi S., Naccarati A., Ross M.K., Hanley N.M., Dailey L., Devlin R.B., Vasquez M., Pegram R.A. and DeMarini D.M. (2003) Induction of DNA Strand Breaks by Trihalomethanes in Primary Human Lung Epithelial Cells. *Mutat Res* 538(1-2):41-50.

Leclerc H., Schwartzbrod L. and Dei-Cas E. (2002) Microbial Agents Associated with Waterborne Diseases. *Crit Rev Microbiol* 28(4):371-409.

Lenarczyk M., Cohen E.P., Fish B.L., Irving A.A., Sharma M., Driscoll C.D. and Moulder J.E. (2009) Chronic Oxidative Stress as a Mechanism for Radiation Nephropathy. *Radiat Res* 171(2):164-172.

Lim S.M., Chiang K., Amal R., Fabris R., Chow C. and Drikas M. (2007) A Study on the Removal of Humic Acid Using Advanced Oxidation Processes. *Sep. Sci. Technol.* 42(7):1391-1404.

Liu J., Qu W. and Kadiiska M.B. (2009) Role of Oxidative Stress in Cadmium Toxicity and Carcinogenesis. *Toxicol Appl Pharmacol* 238(3):209-214.

Liviac D., Creus A. and Marcos R. (2009) Genotoxicity Analysis of Two Halonitromethanes, a Novel Group of Disinfection by-Products (Dbps), in Human Cells Treated in Vitro. *Environ Res*.

Logan B.E. and Wagenseller G.A. (2000) Molecular Size Distributions of Dissolved Organic Matter in Wastewater Transformed by Treatment in a Full-Scale Trickling Filter. *Water Environment Research* 72(3):277-281.

Loquet C. and Wiebel F.J. (1982) Geno- and Cytotoxicity of Nitrosamines, Aflatoxin B1, and Benzo[a]-Pyrene in Continuous Cultures of Rat Hepatoma Cells. *Carcinogenesis* 3(10):1213-1218.

Lowe S.W., Schmitt E.M., Smith S.W., Osborne B.A. and Jacks T. (1993) P53 Is Required for Radiation-Induced Apoptosis in Mouse Thymocytes. *Nature* 362(6423):847-849.

Lynch C.F., Woolson R.F., O'Gorman T. and Cantor K.P. (1989) Chlorinated Drinking Water and Bladder Cancer: Effect of Misclassification on Risk Estimates. *Arch Environ Health* 44(4):252-259.

Maffei F., Buschini A., Rossi C., Poli P., Forti G.C. and Hrelia P. (2005) Use of the Comet Test and Micronucleus Assay on Human White Blood Cells for in Vitro Assessment of Genotoxicity Induced by Different Drinking Water Disinfection Protocols. *Environ Mol Mutagen* 46(2):116-125.

Magnus P., Jaakkola J.J., Skrondal A., Alexander J., Becher G., Krogh T. and Dybing E. (1999) Water Chlorination and Birth Defects. *Epidemiology* 10(5):513-517.

Marcinkiewicz J. (1997) Neutrophil Chloramines: Missing Links between Innate and Acquired Immunity. *Immunol Today* 18(12):577-580.

Margerum D.W., Gray E.T. and Huffman R.P. (1979) Chlorination and Formation of N-Chloro Compounds in Water Treatment, in *Organometals and Organometalloids, Occurrence and Fate in the Environment* (Brinckman F.E. and Belloma J.M. eds) pp 278-291, American Chemical Society, Washington.

Marklund S. and Marklund G. (1974) Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *Eur J Biochem* 47(3):469-474.

Maron D.M. and Ames B.N. (1983) Revised Methods for the Salmonella Mutagenicity Test. *Mutat Res* 113(3-4):173-215.

Mateos R., Goya L. and Bravo L. (2004) Determination of Malondialdehyde by Liquid Chromatography as the 2,4-Dinitrophenylhydrazone Derivative: A Marker for Oxidative Stress in Cell Cultures of Human Hepatoma Hepg2. *J Chromatogr B Analyt Technol Biomed Life Sci* 805(1):33-39.

McAvoy B., Elwood M. and Staples M. (2005) Cancer in Australia--an Update for Gps. *Aust Fam Physician* 34(1-2):41-45.

McGeehin M.A., Reif J.S., Becher J.C. and Mangione E.J. (1993) Case-Control Study of Bladder Cancer and Water Disinfection Methods in Colorado. *Am J Epidemiol* 138(7):492-501.

Meintieres S., Biola A., Pallardy M. and Marzin D. (2001) Apoptosis Can Be a Confusing Factor in in Vitro Clastogenic Assays. *Mutagenesis* 16(3):243-250.

Midwinter R.G., Cheah F.C., Moskovitz J., Vissers M.C. and Winterbourn C.C. (2006) Ikappab Is a Sensitive Target for Oxidation by Cell-Permeable Chloramines: Inhibition of Nf-Kappab Activity by Glycine Chloramine through Methionine Oxidation. *Biochem J* 396(1):71-78.

Midwinter R.G., Peskin A.V., Vissers M.C. and Winterbourn C.C. (2004) Extracellular Oxidation by Taurine Chloramine Activates Erk Via the Epidermal Growth Factor Receptor. *J Biol Chem* 279(31):32205-32211.

Minelli A., Bellezza I., Conte C. and Culig Z. (2009) Oxidative Stress-Related Aging: A Role for Prostate Cancer? *Biochim Biophys Acta* 1795(2):83-91.

Mitch W.A. and Sedlak D.L. (2002) Formation of N-Nitrosodimethylamine (Ndma) from Dimethylamine During Chlorination. *Environ Sci Technol* 36(4):588-595.

Morris R.D., Audet A.M., Angelillo I.F., Chalmers T.C. and Mosteller F. (1992) Chlorination, Chlorination by-Products, and Cancer: A Meta-Analysis. *Am J Public Health* 82(7):955-963.

Muellner M.G., Wagner E.D., McCalla K., Richardson S.D., Woo Y.T. and Plewa M.J. (2007) Haloacetonitriles Vs. Regulated Haloacetic Acids: Are Nitrogen-Containing Dbps More Toxic? *Environ Sci Technol* 41(2):645-651.

Munnia A., Amasio M.E. and Peluso M. (2004) Exocyclic Malondialdehyde and Aromatic DNA Adducts in Larynx Tissues. *Free Radic Biol Med* 37(6):850-858.

Mytilineou C., Kramer B.C. and Yabut J.A. (2002) Glutathione Depletion and Oxidative Stress. *Parkinsonism Relat Disord* 8(6):385-387.

Na C. and Olson T.M. (2007) Relative Reactivity of Amino Acids with Chlorine in Mixtures. *Environ Sci Technol* 41(9):3220-3225.

Nagao F. and Miyazaki T. (2002) Release of Dissolved Organic Nitrogen from Scenedesmus Quadricauda (Chlorophyta) and Microcystis Novacekii (Cyanobacteria). *Aquat. Microb. Ecol.* 27(3):275-284.

Nagata T. and Kirchman D.L. (1990) Filtration-Induced Release of Dissolved Free Amino-Acids - Application to Cultures of Marine Protozoa. *Marine Ecology-Progress Series* 68(1-2):1-5.

Najm I. and Trussell R.R. (2001) Ndma Formation in Water and Wastewater. *Journal AWWA* 93(2):92-99.

Nakamura S.-I., Ugawa M. and Hirotaka Obana (1993) Genotoxicity of Chlorine-Treated Amino Acids. *Environmental Toxicology & Water Quality* 8(2):163-171.

Narine K.A., Keuling A.M., Gombos R., Tron V.A., Andrew S.E. and Young L.C. (2009) Defining the DNA Mismatch Repair-Dependent Apoptotic Pathway in Primary Cells:

Evidence for P53-Independence and Involvement of Centrosomal Caspase 2. *DNA Repair (Amst).*

NHMRC/ARMCANZ (2004) Australian Drinking Water Guidelines National Health and Medical Research Council, Agricultural and Resource Management Council of Australia and New Zealand.

Nieuwenhuijsen M.J., Toledano M.B., Bennett J., Best N., Hambly P., de Hoogh C., Wellesley D., Boyd P.A., Abramsky L., Dattani N., Fawell J., Briggs D., Jarup L. and Elliott P. (2008) Chlorination Disinfection by-Products and Risk of Congenital Anomalies in England and Wales. *Environ Health Perspect* 116(2):216-222.

Nieuwenhuijsen M.J., Toledano M.B., Eaton N.E., Fawell J. and Elliott P. (2000) Chlorination Disinfection Byproducts in Water and Their Association with Adverse Reproductive Outcomes: A Review. *Occup Environ Med* 57(2):73-85.

Nightingale Z.D., Lancha A.H., Jr., Handelman S.K., Dolnikowski G.G., Busse S.C., Dratz E.A., Blumberg J.B. and Handelman G.J. (2000) Relative Reactivity of Lysine and Other Peptide-Bound Amino Acids to Oxidation by Hypochlorite. *Free Radic Biol Med* 29(5):425-433.

Nilsson G., Costa J.J. and Metcalfe D.D. (1999) Mast Cells and Basophils, in *Inflammation: Basic Principles and Clinical Correlates* (Gallin J.I. and Snyderman R. eds) pp 97 - 117 Williams & Wilkins, Philadelphia.

Nissinen T.K., Miettinen I.T., Martikainen P.J. and Vartiainen T. (2001) Molecular Size Distribution of Natural Organic Matter in Raw and Drinking Waters. *Chemosphere* 45(6-7):865-873.

Nissinen T.K., Miettinen I.T., Martikainen P.J. and Vartiainen T. (2002) Disinfection by-Products in Finnish Drinking Waters. *Chemosphere* 48(1):9-20.

Nüsse M., Beisker W., Kramer J., Miller B., Schreiber G., Viaggi S., Weller E. and Wessels J. (1994) Measurement of Micronuclei by Flow Cytometry. *Method Cell Biol* 42:149-158.

Nüsse M. and Kramer J. (1984) Flow Cytometric Analysis of Micronuclei Found in Cells after Irradiation. *Cytometry* 5(1):20-25.

Nüsse M. and Marx K. (1997) Flow Cytometric Analysis of Micronuclei in Cell Cultures and Human Lymphocytes: Advantages and Disadvantages. *Mutat Res* 392(1-2):109-115.

Nweke A. and Scully Jr F.E. (1989) Stable N-Chloroaldimines and Other Products of the Chlorination of Isoleucine in Model Solutions and in a Wastewater. *Environ. Sci. Technol.* 23:989-994.

OECD (2004) Guideline for the Testing of Chemicals: Draft Proposal for a New Guideline 487: *In Vitro* Micronucleus Test.

Papalambros E., Sigala F., Georgopoulos S., Paraskevas K.I., Andreadou I., Menenakos X., Sigalas P., Papalambros A.L., Vourliotakis G., Giannopoulos A., Bakoyiannis C. and

Bastounis E. (2007) Malondialdehyde as an Indicator of Oxidative Stress During Abdominal Aortic Aneurysm Repair. *Angiology* 58(4):477-482.

Pattison D.I. and Davies M.J. (2006) Evidence for Rapid Inter- and Intramolecular Chlorine Transfer Reactions of Histamine and Carnosine Chloramines: Implications for the Prevention of Hypochlorous-Acid-Mediated Damage. *Biochemistry* 45(26):8152-8162.

Pellacani C., Branchi E., Buschini A., Furlini M., Poli P. and Rossi C. (2005) [Cytotoxicity and Genotoxicity of Drinking Water of Two Networks Supplied by Surface Water]. *Epidemiol Prev* 29(5-6 Suppl):34-36.

Pereira J.T., Costa A.O., de Oliveira Silva M.B., Schuchard W., Osaki S.C., de Castro E.A., Paulino R.C. and Soccol V.T. (2008) Comparing the Efficacy of Chlorine, Chlorine Dioxide, and Ozone in the Inactivation of Cryptosporidium Parvum in Water from Parana State, Southern Brazil. *Appl Biochem Biotechnol* 151(2-3):464-473.

Pero R.W., Olsson A., Sheng Y., Hua J., Moller C., Kjellen E., Killander D. and Marmor M. (1995) Progress in Identifying Clinical Relevance of Inhibition, Stimulation and Measurements of Poly Adp-Ribosylation. *Biochimie* 77(5):385-393.

Pero R.W., Sheng Y., Olsson A., Bryngelsson C. and Lund-Pero M. (1996) Hypochlorous Acid/N-Chloramines Are Naturally Produced DNA Repair Inhibitors. *Carcinogenesis* 17(1):13-18.

Peskin A.V., Midwinter R.G., Harwood D.T. and Winterbourn C.C. (2004) Chlorine Transfer between Glycine, Taurine, and Histamine: Reaction Rates and Impact on Cellular Reactivity. *Free Radic Biol Med* 37(10):1622-1630.

Peskin A.V. and Winterbourn C.C. (2001) Kinetics of the Reactions of Hypochlorous Acid and Amino Acid Chloramines with Thiols, Methionine, and Ascorbate. *Free Radic Biol Med* 30(5):572-579.

Peskin A.V. and Winterbourn C.C. (2003) Histamine Chloramine Reactivity with Thiol Compounds, Ascorbate, and Methionine and with Intracellular Glutathione. *Free Radic Biol Med* 35(10):1252-1260.

Peuravuori J. and Pihlaja K. (2004) Preliminary Study of Lake Dissolved Organic Matter in Light of Nanoscale Supramolecular Assembly. *Environ Sci Technol* 38(22):5958-5967.

Phelan M.C. (2006) Techniques for Mammalian Cell Tissue Culture. *Curr Protoc Protein Sci* Appendix 3:Appendix 3C.

Plastaras J.P., Riggins J.N., Otteneder M. and Marnett L.J. (2000) Reactivity and Mutagenicity of Endogenous DNA Oxopropenylating Agents: Base Propenals, Malondialdehyde, and N(Epsilon)-Oxopropenyllysine. *Chem Res Toxicol* 13(12):1235-1242.

Plewa M.J., Kargalioglu Y., Vankerk D., Minear R.A. and Wagner E.D. (2002) Mammalian Cell Cytotoxicity and Genotoxicity Analysis of Drinking Water Disinfection by-Products. *Environ Mol Mutagen* 40(2):134-142.

Plewa M.J., Wagner E.D., Richardson S.D., Thruston A.D., Jr., Woo Y.T. and McKague A.B. (2004) Chemical and Biological Characterization of Newly Discovered Iodoacid Drinking Water Disinfection Byproducts. *Environ Sci Technol* 38(18):4713-4722.

Porru S., Aulenti V., Donato F., Boffetta P., Fazioli R., Cosciani Cunico S. and Alessio L. (1996) Bladder Cancer and Occupation: A Case-Control Study in Northern Italy. *Occup Environ Med* 53(1):6-10.

Potschka M. (1993) Mechanism of Size-Exclusion Chromatography .1. Role of Convection and Obstructed Diffusion in Size-Exclusion Chromatography. *Journal of Chromatography* 648(1):41-69.

Prutz W.A. (1998) Interactions of Hypochlorous Acid with Pyrimidine Nucleotides, and Secondary Reactions of Chlorinated Pyrimidines with Gsh, Nadh, and Other Substrates. *Arch Biochem Biophys* 349(1):183-191.

Pullar J.M., Vissers M.C. and Winterbourn C.C. (2001) Glutathione Oxidation by Hypochlorous Acid in Endothelial Cells Produces Glutathione Sulfonamide as a Major Product but Not Glutathione Disulfide. *J Biol Chem* 276(25):22120-22125.

Raftery M.J. (2007) Detection and Characterization of N-Alpha-Chloramines by Electrospray Tandem Mass Spectrometry. *Anal Biochem* 366(2):218-227.

Ram N.M. and Morris J.C. (1980) Environmental Significance of Nitrogenous Organic Compounds in Aquatic Sources. *Environ. Intl.* 4:397-405.

Randerath K., Yang P.F., Danna T.F., Reddy R., Watson W.P. and Randerath E. (1991) Bulky Adducts Detected by 32p-Postlabeling in DNA Modified by Oxidative Damage in Vitro. Comparison with Rat Lung I-Compounds. *Mutat Res* 250(1-2):135-144.

Reite O.B. (1969) Phylogenetical Persistence of the Non-Mast Cell Histamine Stores of the Digestive Tract: A Comparison with Mast Cell Histamine. *Experientia* 25(3):276-277.

Rice R.G. and Gomez-Taylor M. (1986) Occurrence of by-Products of Strong Oxidants Reacting with Drinking Water Contaminants--Scope of the Problem. *Environ Health Perspect* 69:31-44.

Richardson S.D. (2003) Disinfection by-Products and Other Emerging Contaminants in Drinking Water. *Trac-Trends in Analytical Chemistry* 22(10):666-684.

Richardson S.D., Plewa M.J., Wagner E.D., Schoeny R. and Demarini D.M. (2007) Occurrence, Genotoxicity, and Carcinogenicity of Emerging Disinfection by-Products in Drinking Water: A Review and Roadmap for Research. *Mutat Res*.

Richardson S.D., Thruston Jr A.D., Caughran T.V., Chen P.H., Collette T.W. and Floyd T.L. (1999) Identification of New Ozone Disinfection Byproducts in Drinking Water. *Environ Sci Technol* 33:3368-3377.

Robaszkiewicz A., Bartosz G. and Soszynski M. (2008) Effect of N-Chloroamino Acids on the Erythrocyte. *Free Radic Res* 42(1):30-39.

Rojas J.C., Moreno B., Garralon G., Plaza F., Perez J. and Gomez M.A. (2008) Potabilization of Low Nom Reservoir Water by Ultrafiltration Spiral Wound Membranes. *Journal of Hazardous Materials* 158(2-3):593-598.

Roman D., Locher F., Suter W., Cordier A. and Bobadilla M. (1998) Evaluation of a New Procedure for the Flow Cytometric Analysis of in Vitro, Chemically Induced Micronuclei in V79 Cells. *Environ Mol Mutagen* 32(4):387-396.

Rook J.J. (1974) Formation of Haloforms During Chlorination of Natural Waters. *Water Treat. Exam.* 23:234-243.

Sadiq R. and Rodriguez M.J. (2004) Disinfection by-Products (Dbps) in Drinking Water and Predictive Models for Their Occurrence: A Review. *Sci Total Environ* 321(1-3):21-46.

Sanchez P., Llorente M.T. and Castano A. (2000) Flow Cytometric Detection of Micronuclei and Cell Cycle Alterations in Fish-Derived Cells after Exposure to Three Model Genotoxic Agents: Mitomycin C, Vincristine Sulfate and Benzo(a)Pyrene. *Mutat Res* 465(1-2):113-122.

Schreiber G.A., Beisker W., Bauchinger M. and Nusse M. (1992) Multiparametric Flow Cytometric Analysis of Radiation-Induced Micronuclei in Mammalian Cell Cultures. *Cytometry* 13(1):90-102.

Schuller-Levis G.B. and Park E. (2004) Taurine and Its Chloramine: Modulators of Immunity. *Neurochem Res* 29(1):117-126.

Scott N., Hatlelid K.M., MacKenzie N.E. and Carter D.E. (1993) Reactions of Arsenic(Iii) and Arsenic(V) Species with Glutathione. *Chem Res Toxicol* 6(1):102-106.

Scully F.E., Jr. and Bempong M.A. (1982) Organic N-Chloramines: Chemistry and Toxicology. *Environ Health Perspect* 46:111-116.

Scully F.E., Jr., Mazina K.E., Ringhand H.P., Chess E.K., Campbell J.A. and Johnson J.D. (1990) Identification of Organic N-Chloramines in Vitro in Stomach Fluid from the Rat after Chlorination. *Chem Res Toxicol* 3(4):301-306.

Scully F.E., Jr., Mazina K.E., Sonenshine D.E. and Daniel F.B. (1985) Reactions of Hypochlorite and Organic N-Chloramines in Stomach Fluid, in *Water Chlorination: Chemistry, Environmental Impact and Health Effects* (Jolley R.L., Bull R.J., Davis W.P. and Katz S. eds) pp 175-184, Lewis Publishers, Chelsea, Michigan.

Serodes J.B., Rodriguez M.J., Li H. and Bouchard C. (2003) Occurrence of Thms and Haas in Experimental Chlorinated Waters of the Quebec City Area (Canada). *Chemosphere* 51(4):253-263.

Shahabuddin M.S., Gopal M. and Vijayalaxmi K.K. (2005) The Genotoxicity of DNA Intercalating Drug 8-Methoxy Pyrimido [4',5':4,5]Thieno(2,3-B)Quinoline-4(3h)-One. *Drug Chem Toxicol* 28(3):345-357.

Shen Y. and White E. (2001) P53-Dependent Apoptosis Pathways. *Adv Cancer Res* 82:55-84.

Shi X.L. and Dalal N.S. (1989) Chromium (V) and Hydroxyl Radical Formation During the Glutathione Reductase-Catalyzed Reduction of Chromium (Vi). *Biochem Biophys Res Commun* 163(1):627-634.

Shi Y., Cao X.W., Tang F., Du H.R., Wang Y.Z., Qiu X.Q., Yu H.P. and Lu B. (2009) In Vitro Toxicity of Surface Water Disinfected by Different Sequential Treatments. *Water Res* 43(1):218-228.

Sies H. (1997) Oxidative Stress: Oxidants and Antioxidants. *Exp Physiol* 82(2):291-295.

Simpson K.L. and Hayes K.P. (1998) Drinking Water Disinfection by-Products: An Australian Perspective. *Wat. Res.* 32(5):1522-1528.

Simunek T., Sterba M., Popelova O., Adamcova M., Hrdina R. and Gersl V. (2009) Anthracycline-Induced Cardiotoxicity: Overview of Studies Examining the Roles of Oxidative Stress and Free Cellular Iron. *Pharmacol Rep* 61(1):154-171.

Slavotinek A., Miller E., Taylor G.M., Nusse M. and van Heyningen V. (1995) Micronucleus Frequencies in Lymphoblastoid Cell Lines Measured with the Cytokinesis-Block Technique and Flow Cytometry. *Mutagenesis* 10(5):439-445.

Smith M. (2007) Health Effects of Disinfection by-Products in Australian Drinking Waters. *Int. J. Water* 3(4):347-355.

Snyder M.P. and Margerum D.W. (1982) Kinetics of Chlorine Transfer from Chloramine to Amines, Amino Acids, and Peptides. *Inorg. Chem.* 21(7):2545-2550.

Snyder R.D. and Green J.W. (2001) A Review of the Genotoxicity of Marketed Pharmaceuticals. *Mutat Res* 488(2):151-169.

Spencer J.P., Jenner A., Chimel K., Aruoma O.I., Cross C.E., Wu R. and Halliwell B. (1995) DNA Damage in Human Respiratory Tract Epithelial Cells: Damage by Gas Phase Cigarette Smoke Apparently Involves Attack by Reactive Nitrogen Species in Addition to Oxygen Radicals. *FEBS Lett* 375(3):179-182.

Strober W. (2001) Trypan Blue Exclusion Test of Cell Viability. *Curr Protoc Immunol* Appendix 3:Appendix 3B.

Süssmuth R. (1982) Genetic Effects of Amino Acids after Chlorination. *Mutat Res* 105(1-2):23-28.

Szlachta M. and Adamski W. (2008) Assessing the Efficiency of Natural Organic Matter (Nom) Removal from Water by Coagulation. *Ochr. Sr.* 30(3):9-13.

Tardiff R.G., Carson M.L. and Ginevan M.E. (2006) Updated Weight of Evidence for an Association between Adverse Reproductive and Developmental Effects and Exposure to Disinfection by-Products. *Regul Toxicol Pharmacol* 45(2):185-205.

Thomas E.L. (1979) Myeloperoxidase-Hydrogen Peroxide-Chloride Antimicrobial System: Effect of Exogenous Amines on Antibacterial Action against Escherichia Coli. *Infect Immun* 25(1):110-116.

Thomas E.L., Grisham M.B. and Jefferson M.M. (1986) Preparation and Characterization of Chloramines. *Methods Enzymol* 132:569-585.

Thomas E.L., Jefferson M.M., Bennett J.J. and Learn D.B. (1987) Mutagenic Activity of Chloramines. *Mutat Res* 188(1):35-43.

Thomas J.D. (1997) The Role of Dissolved Organic Matter, Particularly Free Amino Acids and Humic Substances, in Freshwater Ecosystems. *Freshwater Biology* 38(1):1-36.

Thomas P., Umegaki K. and Fenech M. (2003) Nucleoplasmic Bridges Are a Sensitive Measure of Chromosome Rearrangement in the Cytokinesis-Block Micronucleus Assay. *Mutagenesis* 18(2):187-194.

Thorne D., Wilson J., Kumaravel T.S., Massey E.D. and McEwan M. (2009) Measurement of Oxidative DNA Damage Induced by Mainstream Cigarette Smoke in Cultured Nci-H292 Human Pulmonary Carcinoma Cells. *Mutat Res* 673(1):3-8.

Thurman E.M. (1985) *Organic Geochemistry of Natural Waters*. Martinus Nijhoff/Dr. W. Junk Publishers.

Tibbetts J. (1995) What's in the Water: The Disinfection Dilemma. *Environ. Health. Perspect.* 103(1):30-34.

Tietze F. (1969) Enzymic Method for Quantitative Determination of Nanogram Amounts of Total and Oxidized Glutathione: Applications to Mammalian Blood and Other Tissues. *Anal Biochem* 27(3):502-522.

Tomkins B.A. and Griest W.H. (1996) Determinations of N-Nitrosodimethylamine at Part-Per-Trillion Concentrations in Contaminated Groundwaters and Drinking Waters Featuring Carbon-Based Membrane Extraction Disks. *Anal Chem* 68(15):2533-2540.

Tricker A.R., Ditrich C. and Preussmann R. (1991) N-Nitroso Compounds in Cigarette Tobacco and Their Occurrence in Mainstream Tobacco Smoke. *Carcinogenesis* 12(2):257-261.

Tuschall J.R. and Brezonik P.L. (1980) Characterization of Organic Nitrogen in Natural-Waters - Its Molecular-Size, Protein-Content, and Interactions with Heavy-Metals. *Limnology and Oceanography* 25(3):495-504.

Umegaki K. and Fenech M. (2000) Cytokinesis-Block Micronucleus Assay in Wil2-Ns Cells: A Sensitive System to Detect Chromosomal Damage Induced by Reactive Oxygen Species and Activated Human Neutrophils. *Mutagenesis* 15(3):261-269.

Uyak V., Ozdemir K. and Toroz I. (2008) Seasonal Variations of Disinfection by-Product Precursors Profile and Their Removal through Surface Water Treatment Plants. *Sci Total Environ* 390(2-3):417-424.

VanderWaal R., Malyapa R.S., Higashikubo R. and Roti Roti J.L. (1997) A Comparison of the Modes and Kinetics of Heat-Induced Cell Killing in Hela and L5178y Cells. *Radiat Res* 148(5):455-462.

Varadarajan S., Yatin S., Aksenova M. and Butterfield D.A. (2000) Review: Alzheimer's Amyloid Beta-Peptide-Associated Free Radical Oxidative Stress and Neurotoxicity. *J Struct Biol* 130(2-3):184-208.

Villanueva C.M., Cantor K.P., Cordier S., Jaakkola J.J., King W.D., Lynch C.F., Porru S. and Kogevinas M. (2004) Disinfection Byproducts and Bladder Cancer: A Pooled Analysis. *Epidemiology* 15(3):357-367.

Villanueva C.M., Cantor K.P., Grimalt J.O., Malats N., Silverman D., Tardon A., Garcia-Closas R., Serra C., Carrato A., Castano-Vinyals G., Marcos R., Rothman N., Real F.X., Dosemeci M. and Kogevinas M. (2007) Bladder Cancer and Exposure to Water Disinfection by-Products through Ingestion, Bathing, Showering, and Swimming in Pools. *Am J Epidemiol* 165(2):148-156.

Villanueva C.M., Fernandez F., Malats N., Grimalt J.O. and Kogevinas M. (2003) Meta-Analysis of Studies on Individual Consumption of Chlorinated Drinking Water and Bladder Cancer. *J Epidemiol Community Health* 57(3):166-173.

Vissers M.C., Lee W.G. and Hampton M.B. (2001) Regulation of Apoptosis by Vitamin C. Specific Protection of the Apoptotic Machinery against Exposure to Chlorinated Oxidants. *J Biol Chem* 276(50):46835-46840.

Von Gunten U. (2003) Ozonation of Drinking Water: Part I. Oxidation Kinetics and Product Formation. *Water Res* 37(7):1443-1467.

Vuorio E., Vahala R., Rintala J. and Laukkanen R. (1998) The Evaluation of Drinking Water Treatment Performed with Hpsec. *Environment International* 24(5-6):617-623.

Waldum H.L., Brenna E., Sandvik A.K. and Petersen H. (1991) Trophic Effect of Histamine on the Stomach. *Scand J Gastroenterol Suppl* 180:137-142.

Wang J., Wang X.F., Zhang L.G., Xie S.Y., Li Z.L., Li Y.J., Li H.H. and Jiao F. (2009) Involvement of the Mitochondrial Pathway in P53-Independent Apoptosis Induced by P28gank Knockdown in Hep3b Cells. *Cytogenet Genome Res* 125(2):87-97.

Wang J.J., Sanderson B.J. and Wang H. (2007) Cyto- and Genotoxicity of Ultrafine Tio(2) Particles in Cultured Human Lymphoblastoid Cells. *Mutat Res* 628(2):99-106.

Wang M.Y. and Liehr J.G. (1995) Lipid Hydroperoxide-Induced Endogenous DNA Adducts in Hamsters: Possible Mechanism of Lipid Hydroperoxide-Mediated Carcinogenesis. *Arch Biochem Biophys* 316(1):38-46.

Weinberg H.S., Krasner S.W., Richardson S.D. and Thruston Jr A.D. (2002) The Occurrence of Disinfection by-Products (Dbps) of Health Concern in Drinking Water: Results of a Nationwide Dbp Occurrence Study.

Weitzman S.A. and Gordon L.I. (1990) Inflammation and Cancer: Role of Phagocyte-Generated Oxidants in Carcinogenesis. *Blood* 76(4):655-663.

Westerhoff P. and Mash H. (2002) Dissolved Organic Nitrogen in Drinking Water Supplies: A Review. *J Water SRT - Aqua* 51:415-448.

White G.C. (1999) Chlorine Dioxide, in *Handbook of Chlorination and Alternative Disinfectants* pp 1153-1202, Wiley & Son, New York.

WHO (2004) *Guideline for Drinking Water Quality*.

Wijnhoven H.J., Heunks L.M., Geraedts M.C., Hafmans T., Vina J.R. and Dekhuijzen P.N. (2006) Oxidative and Nitrosative Stress in the Diaphragm of Patients with Copd. *Int J Chron Obstruct Pulmon Dis* 1(2):173-179.

Winiecka-Krusnell J. and Linder E. (1998) Cysticidal Effect of Chlorine Dioxide on Giardia Intestinalis Cysts. *Acta Trop* 70(3):369-372.

Winterbourn C.C. and Brennan S.O. (1997) Characterization of the Oxidation Products of the Reaction between Reduced Glutathione and Hypochlorous Acid. *Biochem J* 326 (Pt 1):87-92.

Woo Y.T., Lai D., McLain J.L., Manibusan M.K. and Dellarco V. (2002) Use of Mechanism-Based Structure-Activity Relationships Analysis in Carcinogenic Potential Ranking for Drinking Water Disinfection by-Products. *Environ Health Perspect* 110 Suppl 1:75-87.

Wright J.M., Schwartz J. and Dockery D.W. (2004) The Effect of Disinfection by-Products and Mutagenic Activity on Birth Weight and Gestational Duration. *Environ Health Perspect* 112(8):920-925.

Wright J.M., Schwartz J., Vartiainen T., Maki-Paakkanen J., Altshul L., Harrington J.J. and Dockery D.W. (2002) 3-Chloro-4-(Dichloromethyl)-5-Hydroxy-2(5h)-Furanone (Mx)

and Mutagenic Activity in Massachusetts Drinking Water. *Environmental Health Perspectives* 110(2):157-164.

Xia F., Amundson S.A., Nickoloff J.A. and Liber H.L. (1994) Different Capacities for Recombination in Closely Related Human Lymphoblastoid Cell Lines with Different Mutational Responses to X-Irradiation. *Mol Cell Biol* 14(9):5850-5857.

Xia F., Wang X., Wang Y.H., Tsang N.M., Yandell D.W., Kelsey K.T. and Liber H.L. (1995) Altered P53 Status Correlates with Differences in Sensitivity to Radiation-Induced Mutation and Apoptosis in Two Closely Related Human Lymphoblast Lines. *Cancer Res* 55(1):12-15.

Yoon J. and Jensen J.N. (1996) Modeling the Distribution of Chloramines During Drinking Water Chloramination, in *Disinfection by-Products in Water Treatment. The Chemistry of Their Formation and Control* (Minear R.A. and Amy G.L. eds) pp 351-361, Lewis Publishers, New York.

Yoshioka T., Mostofa K.M.G., Konohira E., Tanoue E., Hayakawa K., Takahashi M., Ueda S., Katsuyama M., Khodzher T., Bashenkhaeva N., Korovvakova I., Sorokovikova L. and Dorbunova L. (2007) Distribution and Characteristics of Molecular Size Fractions of Freshwater Dissolved Organic Matter in Watershed Environments: Its Implication to Degradation. *Limnology* 8(1):29-44.

Young W.J., Marston F.M. and Davis J.R. (1996) Nutrient Exports and Land Use in Australian Catchments. *Journal of Environmental Management* 47(2):165-183.

Zeni O., Salvemini F., Di Pietro R., Buonincontri D., Komulainen H., Romano M. and Scarfi M.R. (2004) Induction of Oxidative Stress in Murine Cell Lines by 3-Chloro-4-(Dichloromethyl)-5-Hydroxy-2(5h)-Furanone (Mx). *Toxicol Lett* 147(1):79-85.

Zgliczynski J.M., Stelmaszynska T., Domanski J. and Ostrowski W. (1971) Chloramines as Intermediates of Oxidation Reaction of Amino Acids by Myeloperoxidase. *Biochim Biophys Acta* 235(3):419-424.

Zhou G.D., Moorthy B., Bi J., Donnelly K.C. and Randerath K. (2007) DNA Adducts from Alkoxyallylbenzene Herb and Spice Constituents in Cultured Human (Hepg2) Cells. *Environ Mol Mutagen* 48(9):715-721.

Zierler S., Feingold L., Danley R.A. and Craun G. (1988) Bladder Cancer in Massachusetts Related to Chlorinated and Chloraminated Drinking Water: A Case-Control Study. *Arch Environ Health* 43(2):195-200.

Zularisam A.W., Ismail A.F., Salim M.R., Sakinah M. and Matsuura T. (2009) Application of Coagulation-Ultrafiltration Hybrid Process for Drinking Water Treatment: Optimization of Operating Conditions Using Experimental Design. *Sep. Purif. Technol.* 65(2):193-210.