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Analytical and Toxicological Characterization of Novel Nitrogen Containing Disinfection Byproducts

by

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ABSTRACT

Disinfection byproducts (DBPs) are an unwanted consequence of water disinfection. Consumption of chlorinated drinking water has been associated with an increased risk of bladder cancer; however, the DBP or DBPs responsible has not been identified. The N-nitrosamines are a class of DBPs that are known rodent carcinogens. They are more potent than the currently regulated DBPs and may be capable of causing health effects at low ng/L concentrations. Due to these possible health concerns, the study of nitrosamines in drinking water is warranted.

Gas chromatography mass spectrometry (GC-MS) is the main technique used for nitrosamine analysis, but cannot directly detect thermally unstable or non-volatile nitrosamines. A liquid chromatography tandem MS (LC-MS/MS) method was developed that is capable of detecting GC-detectable nitrosamines, such as N-nitrosodimethylamine (NDMA), and GC-non-detectable nitrosamines. Using this method, N-nitrosodiphenylamine (NDPhA), a thermally unstable nitrosamine, was detected as a DBP from an authentic drinking water sample. A survey of 38 North American drinking water systems using the LC-MS/MS method found that NDMA was the most commonly detected nitrosamine (28 of 38 systems) followed by NDPhA (6 of 38 systems).

A real-time cell electronic sensing (RT-CES) technique was developed and demonstrated as a useful tool for DBP toxicity testing. NDPhA was more cytotoxic than NDMA in four cell lines. Further mechanistic analysis determined that NDPhA induces cell cycle arrest, which is different than other nitrosamines such as NDMA.

Studies on nitrosamine formation showed the important role of source water. Disinfectant type alone was not sufficient to determine which nitrosamines are formed. Additional studies determined that diphenylamine (DPhA) can form NDPhA, particularly in the presence of monochloramine. This reaction also formed phenazine and N-chlorophenazine. Further investigation determined that phenazine containing natural products produced by bacteria are also precursors for the phenazine containing DBPs.

These results suggest that the currently monitored nitrosamines are not widespread enough to be solely responsible for the observed increase in bladder cancer risk. However, there are several locations where elevated nitrosamine concentrations are a concern. Until the DBP cancer culprit is identified, reduction of overall DBP formation through source water management and careful monitoring of the disinfection process is the best practice to manage cancer risks posed by DBPs.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg/L	Microgram per litre
AOP	Advanced oxidative processes
APCI	Atmospheric pressure chemical ionization
CAS#	Chemical Abstracts Service number
CCL-3	Candidate Contaminant List (3rd version)
CE	Collision energy
CI	Cell Index
Cl-UDMH	Chlorinated unsymmetrical dimethylhydrazine
CXP	Cell exit potential
DBP	Disinfection byproduct
DMA	Dimethylamine
DP	Declustering potential
DPD	N,N-diethyl-p-phenylenediamine
DPhA	Diphenylamine
DPhA-d6	[6-H^2] Diphenylamine
EMS	Enhanced mass spectrometry
ESI	Electrospray ionization
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HAA	Haloacetic acid
IC_{50}	*In vitro* cytotoxicity at 50%
IV or ISV	Ionspray voltage

K_{OW}	Octanol/Water coefficient
LC	Liquid chromatography
LD_{50}	Lethal dose at 50%
LLE	Liquid-liquid extraction
LPUV	Low pressure ultraviolet
MAC	Maximum allowable concentration
MDL	Method detection limit
MeOH	Methanol
mg/L	Milligram per litre
MPUV	Medium pressure ultraviolet
MRL	Method reporting limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCI	National Cancer Institute
ND	Not detected
NDBA	N-nitrosodibutylamine
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
NDMA-d6	[6-H^2] N-nitrosodimethylamine
NDPA	N-nitrosodipropylamine
NDPA-d6	[14-H^2] N-nitrosodi-n-propylamine
NDPhA	N-nitrosodiphenylamine

NDPhA-d6	[6-H^2] N-nitrosodiphenylamine
ng/L	Nanogram per litre
NH_2Cl	Monochloramine
NIST	National Institute of Standards and Technology
NMEA	N-nitrosomethylethylamine
NMor	N-nitrosomorpholine
NOM	Natural organic matter
NPip	N-nitrosopiperidine
NPyr	N-nitrosopyrrolidine
NRU	Neutral red uptake
O_3	Ozone
OCl^-	Chlorine
PBS	Phosphate buffered saline
PTFE	Polytetrafluoroethylene
QSTR	Quantitative structure toxicity relationship
RRF	Relative response factor
RT-CES	Real time cell-electronic sensing
SD	Standard deviation
SDWA	Safe Drinking Water Act
SIM	Selective ion monitoring
SPE	Solid phase extraction
SPME	Solid phase microextraction
SUVA	Specific ultraviolet absorbance

THM	Trihalomethane
TIC	Total ion chromatogram
TN	Total nitrogen
TOC	Total organic carbon
TOF	Time-of-flight
TON	Total organic nitrogen
TOX	Total organic halogen
U.S. EPA	United States Environmental Protection Agency
UCMR-2	Unregulated Contaminant Monitoring Rule-2
UDMH	Unsymmetrical dimethylhydrazine
UV	Ultraviolet
WTP	Water treatment plant
XIC	Extracted ion chromatogram

1 INTRODUCTION

1.1 Importance of safe drinking water

Provision of clean drinking water is still an important issue around the world. In 2010, an estimated 780 million people did not have access to clean drinking water [1] and 2.2 million people die every year from waterborne diarrheal diseases [2]. Waterborne disease outbreaks also occur in developed nations [3]. In Canada, it is difficult to forget the disaster in Walkerton, Ontario in 2000, when 7 people died and over 2500 became ill after pathogenic *Escherichia coli* O157:H7 made its way into their tap water [4]. These statistics are troubling considering that it has been known for over 100 years that disinfection of drinking water with chlorine is effective in reducing the spread of waterborne disease. Introduction of chemical disinfection has played a significant role in reducing the incidence of diseases such as cholera and typhoid. Given the acute risk posed by microbiological agents, disinfection of drinking water is essential to maintain public health.

1.2 Disinfection Byproducts (DBPs)

1.2.1 Discovery of DBPs

Water disinfection can also lead to the formation of disinfection byproducts (DBPs), chemicals that form from the reaction of the disinfectant with natural organic matter (NOM) present in raw water (Figure 1.1).

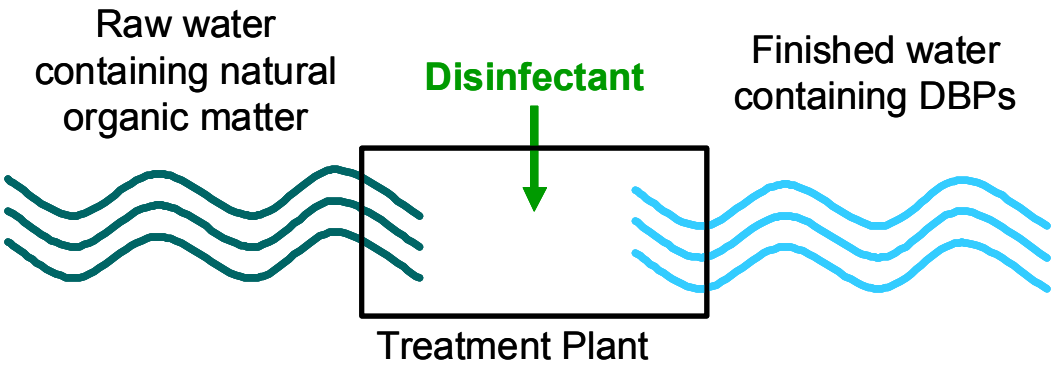

Figure 1.1: Schematic of disinfection byproduct formation during water treatment.

Chloroform, bromodichloromethane, dibromochloromethane, and bromoform [all trihalomethanes (THMs)] were the first DBPs identified independently by both Rook [5] and Bellar [6]. This discovery was aided by advances in analytical chemistry, specifically sample extraction techniques and instrument sensitivities [7]. In 1975, an occurrence study of THMs performed as part of the U.S. Safe Water Drinking Act (SWDA) found the presence of THMs in drinking water to be widespread [8]. In 1976, the National Cancer Institute (NCI) published the results of a two year rat cancer bioassay showing chloroform to be a carcinogen [9]. This led to the removal of chloroform from many consumer products. Epidemiological investigations into links between THM exposure and cancer risk in humans started appearing. In 1978, Canada set a guideline of 350 µg/L for total THMs (chloroform, bromoform, dibromochloromethane, bromodichloromethane), becoming the first country to set a THM guideline. In 1979 the U.S. followed suit, setting a total THM guideline of

100 µg/L as a running annual average [10]. The research and regulatory interest in DBPs in drinking water has been sustained until the present.

1.2.2 Identification of new DBPs

Since 1974, over 600 DBPs have been identified [11]. THMs make up the largest constituent of DBPs in chlorinated water, followed by haloacetic acids (HAAs). Many other classes of DBPs have been identified including other chlorinated DBPs and, more recently, nitrogen containing DBPs (N-DBPs). Despite such a large number of DBPs having been identified, these identified DBPs only take up a small portion of the overall composition of DBPs in drinking water. Analysis of drinking water has suggested that up to 60% of total organic carbon (TOC) and total organic halide (TOX) are still unidentified [12]. This suggests that there are a great number of DBPs forming during water disinfection that remain unknown.

1.2.3 Human health concerns surrounding DBPs

The human health risks posed by DBPs are uncertain. The effect of long-term, low-level exposure to these chemicals in drinking water is unknown. A number of epidemiology studies have been performed investigating associations between consumption of chlorinated drinking water and certain adverse health effects, including bladder cancer, colon cancer, spontaneous abortion and other adverse reproductive outcomes. These studies and the weight of evidence for each of these health outcomes has been reviewed by S.E. Hrudey [13, 14]. The accumulated evidence suggests that the strongest and most consistent association is between consumption of chlorinated drinking water and bladder cancer;

however, there is still uncertainty regarding the strength of this relationship. Several meta-analyses have been performed to try and increase the power of the already published studies, which have generally determined risk estimates above 1 which supports the association between consumption of chlorinated drinking water and bladder cancer. No DBP epidemiology study has found an odds ratio or rate ratio above 2.

Epidemiology studies involving DBPs have many challenges. Because DBPs are suspected to be toxic to humans, they cannot be directly given to human study participants like in a clinical trial. This then forces the use of less powerful epidemiology study designs such as cohort or retrospective studies, in which determining the epsoure of study participants to DBPs is much more difficult. In addition, many epidemiology studies also use THM or HAA data to calculate DBP exposure because it is the main DBP monitoring data available. However, THMs and HAAs are not themselves responsible for the increased bladder cancer risk, and there is no evidence that they are good surrogates for other DBPs present in tap water. These, and other challenges [14], may be affecting the ability of epidemiology studies to find a stronger association between DBPs and human health outcomes.

1.3 Searching for the DBP cancer culprit

1.3.1 THMs and HAAs

Identification of the DBP or DBPs that are responsible for the increase in bladder cancer risk observed in epidemiology studies has been a top research goal for nearly 40 years. Originally, it was believed that the THMs were the likely

culprit as they are typically the largest proportion of DBPs in chlorinated drinking water and because the 1976 NCI chloroform carcinogenesis bioassay [9] (Section 1.2.1) suggested that chloroform was a carcinogen. In 1986, studies showed that continuous dosing of chloroform in water to rats did not produce the carcinogenic effect observed in the NCI study [15]. The NCI study used corn oil as a delivery vehicle instead of water and later studies determined that the combination of chloroform dosed in corn oil causes cell death. The subsequent cell proliferation is what resulted in the tumorogenesis observed in the NCI study [16, 17]. It is now recognized that chloroform alone is not a DNA mutagen [18].

It has become clear that the two largest constituents of DBPs in chlorinated drinking water, THMs and HAAs, are not sufficiently toxic to be responsible for the health effects observed in epidemiology studies. Despite having known this for 10 years, THMs and HAAs remain the most commonly regulated DBPs and many research papers are published every year studying THM and HAA occurrence and formation and many water treatment plant operators still believe that THMs and HAAs will give you cancer. This misconception is what led the water treatment plant operators in Walkerton to stop dosing chlorine properly in an effort to reduce the concentrations of these DBPs [4].

1.3.2 Searching for a more plausible DBP bladder cancer culprit

If THMs and HAAs are not responsible for the observed increase in bladder cancer risk, then another DBP may be responsible. However, with over 600 DBPs already identified and many more identified every year, there are many

possibilities from which to choose from. Hampering this search is the lack of any toxicity information for many of these DBPs, making research prioritization extremely difficult.

Several strategies have been proposed to prioritize future DBP research. The first involves identifying as many DBPs in drinking water as possible, in an attempt to reduce the amount of unknown total organic halogen (TOX) and total organic carbon (TOC) in drinking water samples. Some of this comes from identification of new unknown peaks observed in samples already being tested for DBPs using chlorine and bromine isotopic patterns to narrow down the search. Some researchers advocate selecting putative DBPs that are likely to form based on the structure of NOM and other precursors [19]. With the development of more sensitive analytical instrumentation, there is no doubt that more and more DBPs will be detected at lower and lower concentrations. However, this begs the question, whether identification of all DBPs is an important or necessary goal.

Toxicological modeling using quantitative structure toxicity relationships (QSTR) has also been used to prioritize DBP research. Bull et al. (2006) used QSTR to predict DBP classes with sufficient potency to cause health effects at the low concentrations at which DBPs are typically detected [20]. Six groups were identified as having sufficient potency to cause health effects at the concentrations found in drinking water: halobenzoquinones, halocyclopentenoic acids, organic N-haloamines, halonitriles, haloamides, nitrosamides, and the N-nitrosamines.

1.4 N-nitrosamines as DBPs

1.4.1 Identification of N-nitrosamines as DBPs

In 1989, N-nitrosodimethylamine (NDMA) was detected in treated drinking water in Ohsweken, Ontario [21]. Subsequent experiments indicated it was a result of water disinfection making NDMA the first nitrosamine identified as a DBP. In 1998, NDMA was detected in treated drinking water in California [22]. Initially this was determined to be a result of contamination by rocket fuel, which contains an NDMA precursor. However, this sparked a state-wide survey for NDMA and several other locations were determined to have NDMA, but only as a result of water disinfection. Since then, nitrosamines have been detected in several other locations [23, 24], including Alberta [25-27], Japan [28], China [29], and the UK [30]. To date, a total of seven nitrosamines have been identified as DBPs: NDMA, N-nitrosopyrrolidine (NPyr), N-nitrosomorpholine (NMor), N-nitrosopiperidine (NPip), N-nitrosodiphenylamine (NDPhA), N-nitrosodibutylamine (NDBA), and N-nitrosodiethylamine (NDEA) (See Table 2.1 for structures).

1.4.2 Toxicity of N-nitrosamines

The toxicity of nitrosamines has been well studied as they have been detected historically in a wide range of foods (smoked meats, cheeses), beverages (beer) and consumer products (cigarettes, cosmetics) [31]. The nitrosamines are known rodent carcinogens and suspected human carcinogens [31]. As a class they are recognized to cause cancer in every major tissue in laboratory animals [32]. Nitrosamines also display tissue selectivity, listed in Table 1.1.

Nitrosamines are not direct acting carcinogens. Inside the body they are bioactivated by P450 enzymes via alpha hydroxylation formung a hydroxyl radical that methylates macromolecules such as DNA [33]. Failure to repair these methylated DNA bases may lead to carcinogenesis.

Table 1.1: Lifetime cancer risk estimates and target organs of the 7 identified nitrosamine DBPs

Nitrosamine	Abbrev.	10^{-6} upperbound lifetime cancer risk from drinking water consumption [34]	Target Organ [Ref]
N-nitrosodimethylamine	NDMA	0.7 ng/L	Liver [35]
N-nitrosopyrrolidine	NPyr	20 ng/L	Liver [36]
N-nitrosopiperidine	NPip	NA	Liver, esophagus, Jaw [36]
N-nitrosodiphenylamine	NDPhA	7000 ng/L	Bladder [37]
N-nitrosomorpholine	NMor	NA	Liver [38]
N-nitrosodibutylamine	NDBA	6 ng/L	Bladder, esophagus [39]
N-nitrosodiethylamine	NDEA	0.2 ng/L	Liver [35]

NA=Not available

The potency of nitrosamines is reflected in their upperbound one-in-a-million lifetime cancer risk estimates from consumption of nitrosamines in drinking water, which are typically in the low ng/L range (Table 1.1). This means

that they may be able to cause health effects even if they are present in drinking water at low ng/L concentrations. And more importantly for DBP research, it means that nitrosamines are much more potent compared to THMs and HAAs.

1.4.3 Regulatory response to N-nitrosamines

The regulatory response to nitrosamines in drinking water has varied depending on the jurisdiction. In North America, the Province of Ontario set a Maximum Allowable Concentration (MAC) for NDMA of 9 ng/L [40] and continues to routinely monitor NDMA in provincial drinking water. The Sate of California set Notification Levels for NDMA, N-nitrosodiethylamine (NDEA) and N-nitrosodipropylamine (NDPA) of 10 ng/L in response to the discovery of NDMA in some of its drinking water supplies [41]. The State of Massachusetts Office of Research and Standards has set a guideline of 10 ng/L for NDMA in drinking water [42]. Federally, the U.S. Environmental Protection Agency (U.S. EPA) included six nitrosamines [NDMA, NDEA, NDPA, NPyr, N-nitrosomethylethylamine (NMEA), and N-nitrosodibutylamine (NDBA)] on the Unregulated Contaminants Monitoring Rule-2 (UCMR-2) which required monitoring of these six nitrosamines in U.S. drinking water systems between 2007 and 2010 [43]. Five nitrosamines (NDMA, NDEA, NDPA, NPyr and NDPhA) were also added to the 3rd version of the Candidate Contaminant List which proposes water contaminants for possible future regulation [44]. On March 22, 2010, the U.S. EPA Administrator announced that nitrosamines were among a set of drinking water contaminants being considered for regulation as a group. In 2010, Health Canada proposed a MAC for NDMA in drinking water of 40 ng/L

[45]. The Australian National Health and Medical Research Council is currently considering an NDMA guideline of 100 ng/L in drinking water [46]. The World Health Organization has also set a guideline value for NDMA of 100 ng/L [47].

1.5 Rationale and scope of thesis

Removal or inactivation of microbial elements in drinking water is essential to protect the public from disease. Although disinfection practices may produce unwanted DBPs, the long-term effects of DBP exposure are much lower than the acute risk posed by waterborne pathogens. However, this is not to say that health risks posed by DBPs cannot be managed using proper risk management principles.

In the almost 40 years since the discovery of THMs as DBPs, it has become clear that neither the THMs nor HAAs are responsible for the adverse health effects cited by epidemiology studies. In the search for more plausible culprits, several promising DBP classes have been identified but it takes years to generate the requisite toxicological, occurrence and formation data necessary to characterize each class.

The discovery of the N-nitrosamines provided a unique opportunity in DBP research, as significant toxicological research had already been performed on this class. From this wealth of data, we know that nitrosamines are rodent carcinogens and probable human carcinogens, and are more potent than the currently regulated DBPs (THMs and HAAs). At the start of my Ph.D. studies, general interest in nitrosamines as DBPs was growing steadily. Some formation studies had been published, but occurrence data was lacking which limited

understanding how widespread nitrosamines might be in tap water. In addition, analytical methodology for nitrosamine detection was firmly based on gas chromatography (GC), which restricted research to the eight GC-detectable nitrosamines. While GC is a workhorse for DBP and other environmental contaminant analysis, the emergence of liquid chromatography (LC) and sensitive tandem mass spectrometry (MS/MS) techniques was opening the door for detection of a wider range of DBPs at lower concentrations. This is particularly important considering most studies only looked at NDMA and did not consider the formation of other nitrosamines that might also be important.

My research objectives for this thesis were to:

1. Develop a sensitive solid phase extraction – liquid chromatography–tandem mass spectrometry (SPE-LC-MS/MS) method to analyze for both GC-detectable and GC-non-detectable nitrosamines in treated drinking water (Chapter 2);
2. Use the newly developed SPE-LC-MS/MS method to determine the occurrence of both GC-detectable and GC-non-detectable nitrosamines in 38 North American drinking water distribution systems (Chapter 3);
3. Investigate the use of a real-time, *in vitro*, high throughput, label-free testing platform (real-time cell electronic sensing) to rapidly assess cytotoxicity of newly identified nitrosamine DBPs (Chapter 4);
4. Investigate nitrosamine formation by treating the same source water with different disinfectants (Chapter 5);

5. Investigate formation of new nitrosamine DBPs through chloramination of suspected precursors (Chapter 5); and
6. Investigate novel precursors for newly identified nitrogen containing DBPs (Chapter 6)

Finally, I will present the conclusions and implications of my research and suggest future research objectives regarding DBP research (Chapter 7).

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2 DEVELOPMENT OF AN SPE-LC-MS/MS METHOD TO DETECT N-NITROSAMINES IN DRINKING WATER*

2.1 Introduction

Several techniques have been developed for nitrosamine analysis in various matrices including beer, smoked meats, cheeses, milk, tobacco, rubber products and cosmetics [1, 2]. Analysis for nitrosamines present as disinfection byproducts (DBPs) in drinking water has focused on nine nitrosamines present in the U.S. EPA's Method 521 standard mix [3]: N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosomethylethylamine (NMEA), N-nitrosodipropylamine (NDPA), N-nitrosomorpholine (NMor), N-nitrosopiperidine (NPip), N-nitrosopyrrolidine (NPyr), N-nitrosodibutylamine (NDBA), and N-nitrosodiphenylamine (NDPhA) (Table 2.1). Analysis of these nitrosamines as DBPs in drinking water presents several analytical challenges in that they are polar and water soluble (see Table 2.1 for water solubilities), low in molecular weight (<200 Da) and are present at low ng/L concentrations, demanding highly sensitive analytical techniques to detect them. Historically, analysis of nitrosamines in drinking water has been performed using gas chromatography–mass spectrometry (GC-MS) methods combined with liquid-liquid extraction

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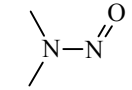
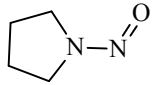
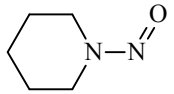
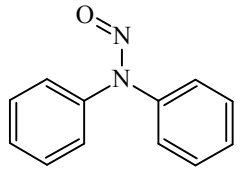
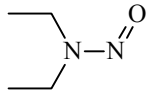
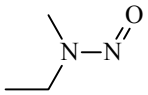
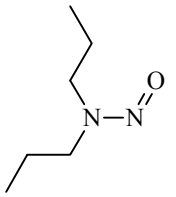
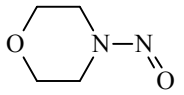
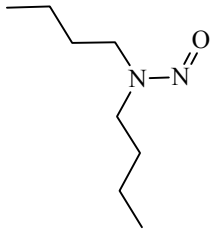
(LLE), solid phase extraction (SPE) or solid phase microextraction (SPME) for sample preconcentration.

Gas chromatography (GC) based techniques are commonly used for nitrosamine analysis both in water research labs and for regulatory analysis. Nitrosamine analysis methods using GC coupled with mass spectrometry (MS) [3-10], nitrogen-phosphorous detectors [7, 8] and flame ionization detectors [11] have been developed. The popularity of GC-MS is mainly due to the high sensitivity and selectivity offered by the selective ion monitoring (SIM) capability of the MS detector and the ruggedness needed to routinely detect nitrosamines below the guideline concentrations [11]. It still remains the methodology of choice for many routine analysis programs including the Ontario Ministry of the Environment's Drinking Water Surveillance Program [4] and the U.S. EPA's UCMR-2 program [12]. GC-MS analysis has shown itself to be an excellent method for detection of semi-volatile and thermally stable nitrosamines in drinking water. This focus has been narrowed even more to the eight semi-volatile nitrosamines present in the standard mix used for the U.S. EPA's nitrosamine analysis method (Method 521).

Table 2.1: Structures and solubilities of the nine nitrosamines included in this study

Nitrosamine	Abbrev.	Molecular formula	CAS #	Structure	Log octanol/water coefficient (Log Kow) [ref]	Water solubility (g/100 mL) [13]
N-nitrosodimethylamine	NDMA	$C_2H_6N_2O$	62-75-9		-0.57 [14]	∞
N-nitrosopyrrolidine	NPyr	$C_4H_8N_2O$	930-55-2		-0.19 [14]	∞
N-nitrosopiperidine	NPip	$C_5H_{10}N_2O$	100-75-4		0.63 [14]	7.7
N-nitrosodiphenylamine	NDPhA	$C_{12}H_{10}N_2O$	86-30-6		3.13 [15]	0.003
N-nitrosodiethylamine	NDEA	$C_4H_{10}N_2O$	55-18-5		0.48 [14]	10.6

N-nitrosomethylethylamine	NMEA	$C_3H_8N_2O$	10595-95-6		0.08 [16]	0.12
N-nitrosodipropylamine	NDPA	$C_6H_{14}N_2O$	621-64-7		1.36 [14]	0.98
N-nitrosomorpholine	NMor	$C_4H_6N_2O$	59-89-2		-0.44 [14]	∞
N-nitrosodibutylamine	NDBA	$C_8H_{18}N_2O$	924-16-3		1.92 [14]	0.12

However, use of GC precludes the direct analysis of thermally unstable or non-volatile nitrosamines. With the majority of total organic nitrogen (TON) from disinfected drinking water currently unidentified [17], it is likely that some nitrosamines may be undetectable by GC because they are thermally unstable or non-volatile. While GC is an excellent technique for semi-volatile nitrosamines such as NDMA, new methodologies are required to obtain a more global understanding of nitrosamine composition in drinking water. A good example of this is N-nitrosodiphenylamine (NDPhA) which is included in the U.S. EPA Method 521 standard mix but is not included in the GC-MS method because it is thermally unstable and decomposes in the GC injector.

Liquid chromatography–mass spectrometry (LC-MS) is a technique gaining prominence in environmental analysis [18]. LC-MS can determine a wider range of analytes compared to GC-MS due to the liquid phase separation at room temperature and electrospray ionization (ESI) for MS detection. This is of particular interest, as it is suspected that non-volatile, thermally unstable, and/or higher molecular weight nitrosamines are formed during water disinfection that are not directly detectable by GC. In order for LC-based methods to be considered comparable with GC methods, they must be robust and sensitive to detect nitrosamines at low ng/L concentrations. This must be accomplished despite LC having a lower resolving capability than GC, a possible increase in matrix effects due to ESI, and the higher cost especially when combined with MS/MS instruments. However, LC-MS methods may be the key to providing a more complete picture of the speciation and concentration of nitrosamines in drinking

water, which is important considering the toxicological potency of nitrosamines (Chapter 1).

In order to characterize nitrosamine DBPs in drinking water, we developed a method combining SPE preconcentration and micro-column LC separation with tandem mass spectrometry (MS/MS) using multiple reaction monitoring (MRM). To ensure the method was comprehensive for both GC-detectable and undetectable nitrosamines, we included the nine nitrosamines of the U.S. EPA Method 521 mix which includes both GC-detectable (NDMA, NDEA, NMEA, NDPA, NMor, NPyr, NPip, NDBA) and one thermally unstable nitrosamine (NDPhA) which is not directly detectable by GC. As no previous LC-MS/MS based methods had been developed for analysis of nitrosamine DBPs in drinking water, it was important to include nitrosamines like NDMA, NMor and NPip to show that the method could detect already identified nitrosamine DBPs at relevant concentrations. NDPhA was selected as the example non-volatile nitrosamine for method development it was already part of the standard mix.

2.2 Materials and Methods

2.2.1 General reagents

Methanol (AnalR grade) and dichloromethane (Omni-Solv grade) were purchased from VWR International (Mississauga, ON, Canada). Ammonium acetate (ACS reagent grade) and L-ascorbic acid (analytical grade) were supplied by Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were of analytical grade and obtained from Fisher Scientific (Nepean, ON, Canada) unless otherwise indicated.

2.2.2 Nine nitrosamine method

2.2.2.1 Method specific reagents

A standard solution (10 µg/mL each) containing the nine nitrosamines, NDMA, NMEA, NPyr, NPip, NMor, NDEA, NDPA, NDBA, and NDPhA, was purchased from Supelco (Oakville, ON, Canada). The structures, cancer risk and solubilities of the nine nitrosamines are shown in Table 2.1. Isotope-labeled standards ([6-H^2] N-nitrosodimethylamine, NDMA-d6, and [14-H^2] N-nitrosodi-n-propylamine, NDPA-d14) (98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). The SPE packing materials, Ambersorb 572 (Rohm & Hass, Philadelphia, PA, U.S.A.) and LiChrolut EN (Merck, Darmstadt, Germany), were obtained from Supelco and VWR International, respectively.

2.2.2.2 Standard solutions

A stock solution (1 µg/mL) containing the nine N-nitrosamines was prepared in methanol and stored at 4 ^{o}C. Working solutions (5–200 µg/L) were prepared with 1:1 methanol/water. Each working solution contained the surrogate standard NDMA-d6 (50 µg/L) and the internal standard NDPA-d14 (50 µg/L). All working solutions were freshly prepared prior to LC-MS/MS analysis.

The purity and stability of NDMA-d6 were determined by repeated analyses of NDMA-free water spiked with 40 ng/L of NDMA-d6. No NDMA (non-labeled) was detected using the SPE-LC-MS/MS (MRM) method described below. This confirmed that the NDMA determined was found only in the samples.

2.2.2.3 Extraction of Water Samples

Nitrosamines were extracted from water samples using a previously published method [5]. Briefly, the glass 6 mL SPE cartridges, fitted with a glass 'c' frit were packed with 350 mg of LiChrolut EN (bottom layer), 500 mg of Ambersorb 572 (middle), and glass wool (top). Ten packed SPE cartridges were loaded onto the top of a Visiprep Manifold (Supelco) and rinsed with 15 mL each of hexane and dichloromethane, and the residual organic solvents were removed under vacuum. The SPE cartridges were then conditioned with 15 mL each of methanol and water.

The pH of each 500 mL water sample was adjusted to pH 8 by the addition of 1 g of sodium bicarbonate. NDMA-d6 (100 µL of 200 µg/L) was then spiked into the sample (final concentration of 40 ng/L). The sample was passed through the SPE cartridge at a flow rate of 3–5 mL/min. The analytes absorbed on the SPE cartridge were eluted using 15 mL of dichloromethane. The organic eluent was collected and concentrated down to 200 µL under a high purity nitrogen stream in a 40 ^{o}C water bath. After concentration, the internal standard NDPA-d14 (100 µL of 200 µg/L) was added to the extract (final concentration of 40 ng/L) prior to LC-MS/MS analysis. The extracts were stored at 4 ^{o}C and analyzed using LC-MS/MS within a week. Nitrosamine-free water blanks were extracted in each set to ensure all reagents were nitrosamine-free. Samples of 10 or 40 ng/L spiked water were also included in each extraction set to ensure accuracy. The surrogate standard, NDMA-d6, was used to determine recovery; the internal standard, NDPA-d14, was used for quantification.

The effect of dichloromethane and methanol on analyte elution from the SPE cartridges was also examined when a water (Optima grade) sample spiked with 40 ng/L of NDMA-d6 was analyzed. NDMA-d6 recovery was 75% when dichloromethane was used as the solvent, whereas recovery of NDMA-d6 was only 40% with methanol. Therefore, dichloromethane was used to elute the analytes from the SPE cartridge.

2.2.2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Analysis was performed using an Agilent 1100 capillary liquid chromatograph (Agilent Technologies, Palo Alto, CA, U.S.A.) coupled directly to an API 4000 QTrap mass spectrometer (ABSciex, Concord, ON, Canada) with an ionspray ionization source. Analyst software for API 4000 QTrap was used for data acquisition and analysis. A Luna C_8 (2) capillary column (150 × 0.32 mm i.d., 5 μm) (Phenomenex, Torrance, CA, U.S.A.) was used for separation. The mobile phase was composed of solvent A [10 mM ammonium acetate and 0.01% acetic acid in water (Optima grade)] and solvent B (100% methanol). The solvent gradient program consisted of 60% of solvent B for 1 min, increasing solvent B from 60% to 90% over 5 min, and returning back to 60% of solvent B over 0.1 min, followed by a 13-min re-equilibration prior to the next sample injection. The flow rate was 6 μL/min. The sample injection volume was 1.2 μL.

Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were examined for ionization of the target nitrosamines. ESI produced characteristic ions of the parent compounds and product ions for all nitrosamines, whereas APCI could not generate the parent ion for the thermally

unstable nitrosamine NDPhA. The detection of both the parent and product ions is important for specific determination of nitrosamines at trace concentrations in water. Therefore, ESI was used to interface the LC with the mass spectrometer.

Positive ESI combined with the multiple-reaction monitoring (MRM) mode was used. The optimization of MS conditions was performed by infusing a mixture of N-nitrosamines (1 µg/mL each in 10 mM ammonium acetate in 90% MeOH: 10% water) using a syringe pump. The optimal ionspray parameters were: curtain gas (N_2) at 10, ion-source gas 1 at 13, and ionspray voltage at 4500 V. The declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were optimized for individual analytes (Table 2.2).

Table 2.2: Compound dependent parameters for the nine-nitrosamine method

Nitrosamines	MRM transition	Declustering potential (DP)	Collision Energy (CE)	Cell exit potential (CXP)
NDMA	75/43	56	25	6
NDMA-d6	81/46	56	25	6
NMEA	89/61	51	17	8
NPyr	101/55	51	25	8
NDEA	103/75	46	17	12
NPip	115/69	56	23	10
NMor	117/87	51	17	14
NDPA	131/89	46	15	14
NDPA-d14	145/97	46	15	14
NDBA	159/103	51	23	8
NDPhA	199/169	51	17	10

Standard solutions of 5 to 200 µg/L with NDPA-d14 (internal standard, 50 µg/L) and NDMA-d6 (surrogate standard, 50 µg/L) were analyzed. The relative response factors (RRFs) for the nine target nitrosamines and NDMA-d6 were

calculated based on the ratio of the relative peak area of the individual analytes to that of NDPA-d14. The reproducibility of RRFs was also determined. Routine quality control measures included the injection of a blank solution consisting of mobile phase to check for carry over after each sample, and a set of standard solutions of 5–200 µg/L to calculate the RRFs of each compound before and after a set of authentic drinking water samples.

2.2.2.5 Collection and analysis of authentic water samples

Water samples were collected from four locations within one distribution system, in which surface water was treated by a combination of chloramination and UV. Samples were collected in 1 L amber bottles that had been previously solvent washed with dichloromethane and baked at 180 °C overnight. The caps were lined with PTFE. Prior to sample collection, taps were allowed to run for at least 5 min to flush the pipes. Then the bottles and caps were rinsed four times with tap water after which the sample was collected with zero headspace. Twenty mg/L L-ascorbic acid was added to quench the disinfectant residual. Bottles were stored on ice and transferred to 4 °C once they reached the laboratory. Trip blanks were prepared and analyzed along with the drinking water samples.

2.3 Results and Discussion

2.3.1 Development of the nine nitrosamine method

Analysis of nitrosamines in drinking water requires high sensitivity and specificity in order to detect these DBPs at the low ng/L concentrations at which they are typically found in drinking water. To achieve this, a SPE preconcentration step was combined with LC separation, and specific detection by

MS/MS using MRM mode. To obtain the parent–product ion pairs for MRM detection, we first characterized the fragmentation behavior of the nine nitrosamines under MS/MS conditions (Figure 2.1). One major product ion was observed for each nitrosamine, which was used to generate an MRM transition for each nitrosamine.

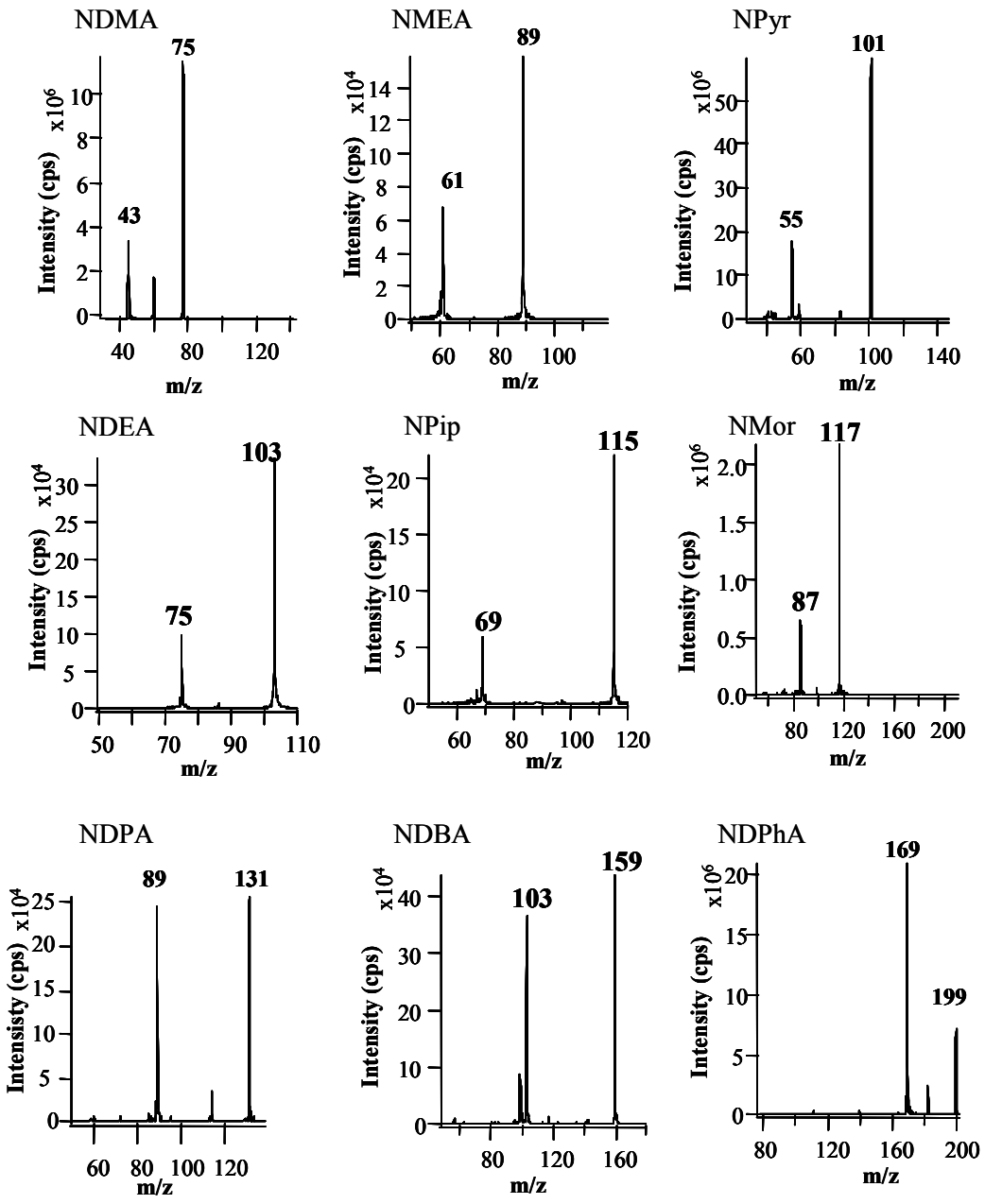


Figure 2.1: MS/MS spectra of the nine nitrosamines showing their parent ion and characteristic product ion.

The specificity of MRM detection for each nitrosamine was further improved using LC separation, which allowed separation of interference molecules that could have the same MRM transition as the analyte of interest.

Figure 2.2 shows the MRM chromatograms of NDPA (131/89) and NDPhA (199/169), when the extracts of a spiked pure water sample and an authentic drinking water sample were analyzed. In the spiked pure water sample, NDPA was detected at a retention time of 6.87 min. Analysis of the same MRM transition of the authentic water sample showed a peak at a retention time of 5.56 min. Spiking the standard into the sample extract confirmed that the peak at 5.56 min was not NDPA.

Similarly, the NDPhA standard (MRM transition 199/169) spiked in pure water was detected at retention time 7.94 min. However, MRM monitoring of transition 199/169 detected two peaks at 5.83 and 7.96 min in the authentic water sample extract. The peak at 7.96 min was identified as NDPhA based on the ion pair monitoring and retention time data. The other peak at 5.83 min was clearly separated from NDPhA, confirming that it is not NDPhA. These results demonstrate the importance and usefulness of the combination of LC separation with MS detection. Although the LC provides only partial separation of these nitrosamines, the retention times provide useful complementary information to MRM monitoring for identification of the analytes in the samples.

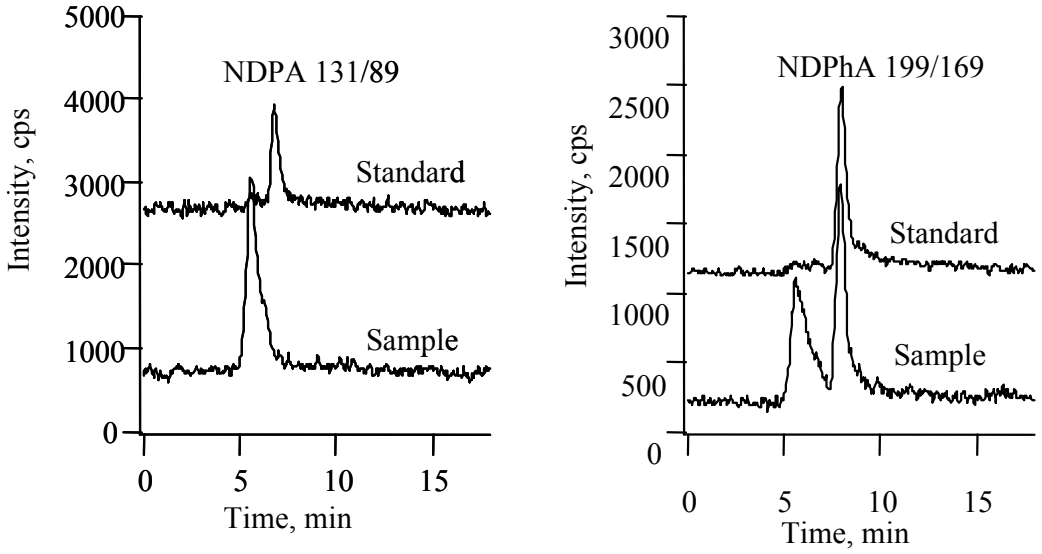


Figure 2.2: MRM chromatograms of a standard spiked Optima water extract and a drinking water sample showing the separation of NDPA and NDPhA from background interference. The standard concentration in the spiked Optima water sample was 5 ng/L of each nitrosamine.

The LC-MS/MS (MRM) method was further combined with SPE preconcentration to determine trace levels of nitrosamines in drinking water. To test the developed method, 40 ng/L of both GC-detectable (NDMA, NMEA, NDEA, NMor, NPyr, NPip, NDPA, and NDBA) and GC-undetectable (NDPhA) nitrosamines were spiked into water samples and then analyzed using the SPE-LC-MS/MS method (Figure 2.3). All nine nitrosamines, plus the surrogate (NDMA-d6) and internal standard (NDPA-d14) were clearly visualized in the MRM chromatograms. Identification and quantification was achieved using the MRM transitions of each nitrosamine. The SPE method was successfully integrated with the LC-MS/MS detection, enabling ultra–sensitive determination of these nitrosamines.

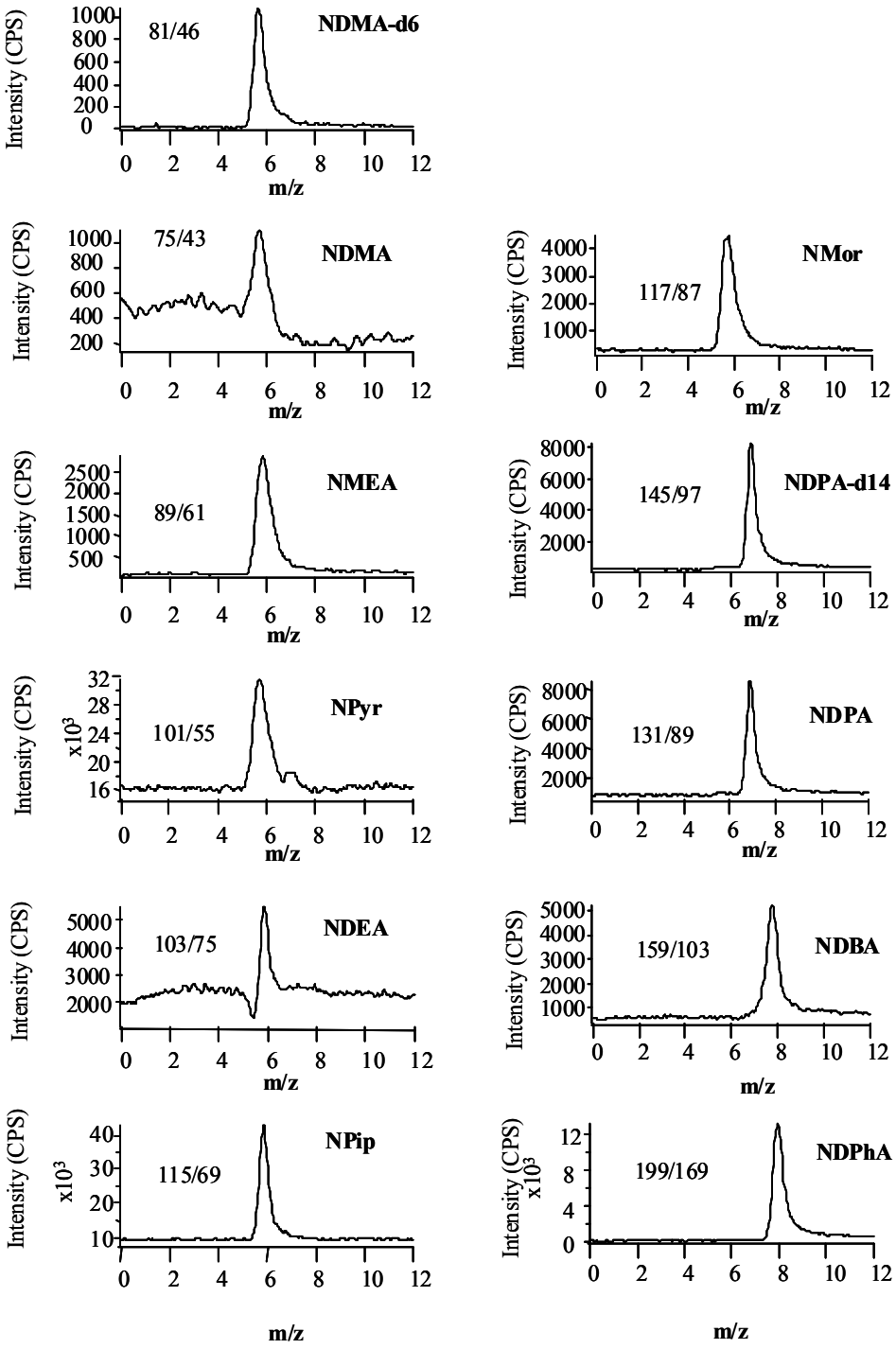


Figure 2.3: Chromatograms of the nine nitrosamines obtained from an Optima water sample spiked with the nine standards and two internal standards under the optimized conditions.

2.3.2 Identification of new nitrosamine DBPs in drinking water

The optimized SPE-LC-MS/MS was used to identify nitrosamines in drinking water samples. Both source water and treated water samples from a water treatment plant were analyzed using the SPE-LC-MS/MS (MRM) method. Four of the nine nitrosamines, NDMA, NPyr, NPip, and NDPhA, were detected in the treated water samples but not in the source water samples. The other five nitrosamines were not detected in either the source water or the treated water samples. Figure 2.4 shows the chromatograms obtained by monitoring the MRM transitions of NDMA (75/43), NPyr (101/55), NPip (115/69), and NDPhA (199/169) from the analysis of a treated water sample.

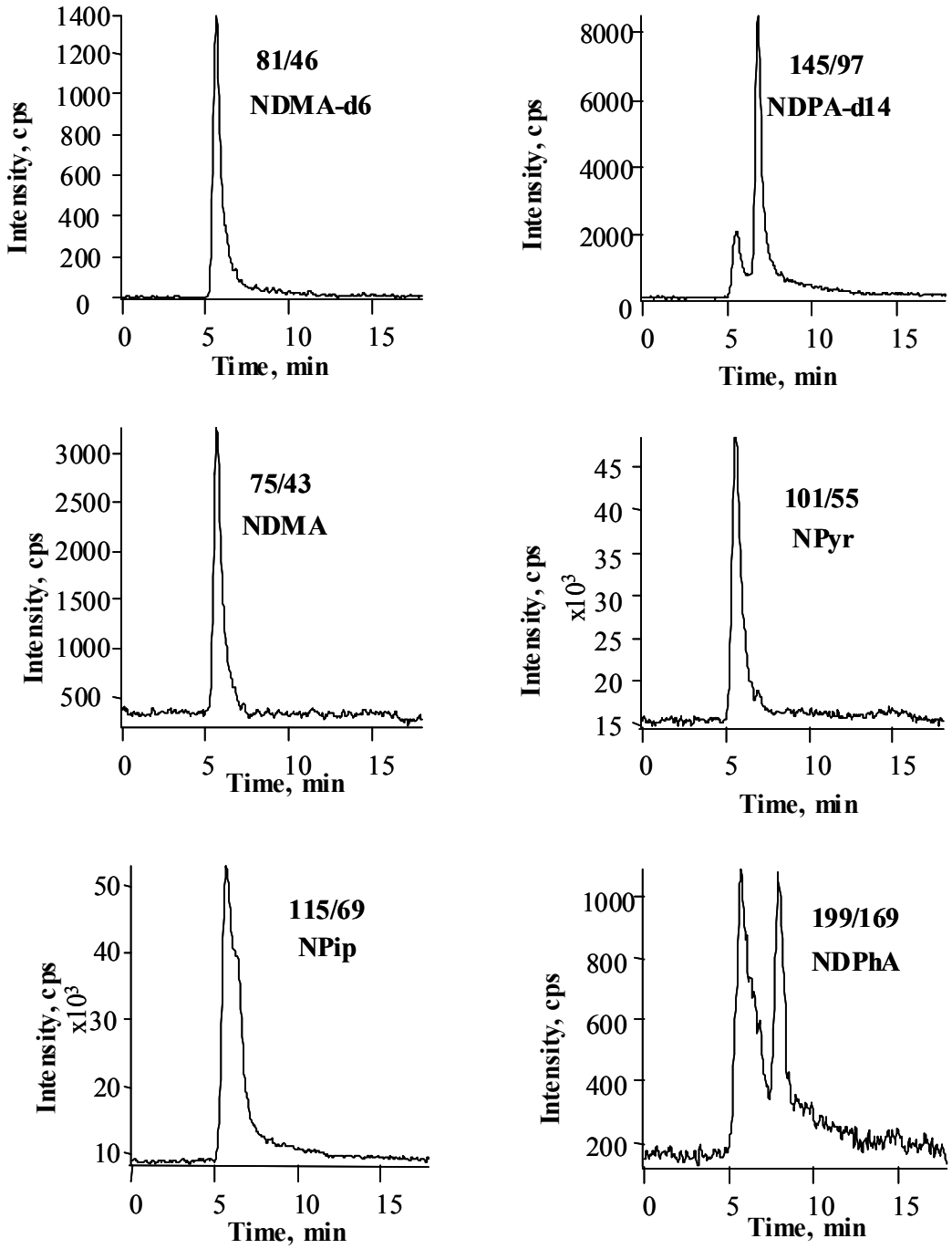


Figure 2.4: Detection of NDMA, NPip, NPyr and NDPhA in a treated drinking water sample collected from a drinking water distribution system.

Based on the retention times and the mass-to-charge (m/z) ratios of the ions, these peaks were identified as NDMA (75/43), NPyr (101/55), NPip

(115/69), and NDPhA (199/169). The identification of these peaks was initially confirmed by analysis of spiked samples showing the same retention time match of the standards with the target analytes and by product ion mass spectral analysis. NDMA, NPyr, and NPip were also confirmed using the established GC-MS method [5]. GC-MS is not able to analyze NDPhA directly due to thermal decomposition of this nitrosamine in the GC injector. The presence of NDPhA as a consequence of the water disinfection process was confirmed by analyzing pure water blanks, source water, and treated water samples, and comparing them with the standard (Figure 2.5). NDPhA was detected only in treated water collected from the distribution system but not in the blanks and the source water. This confirms NDPhA as a product of disinfection. Similar results were also obtained for NDMA, NPyr, and NPip. The results suggest that NDPhA, NDMA, NPyr, and NPip resulted from disinfection processes.

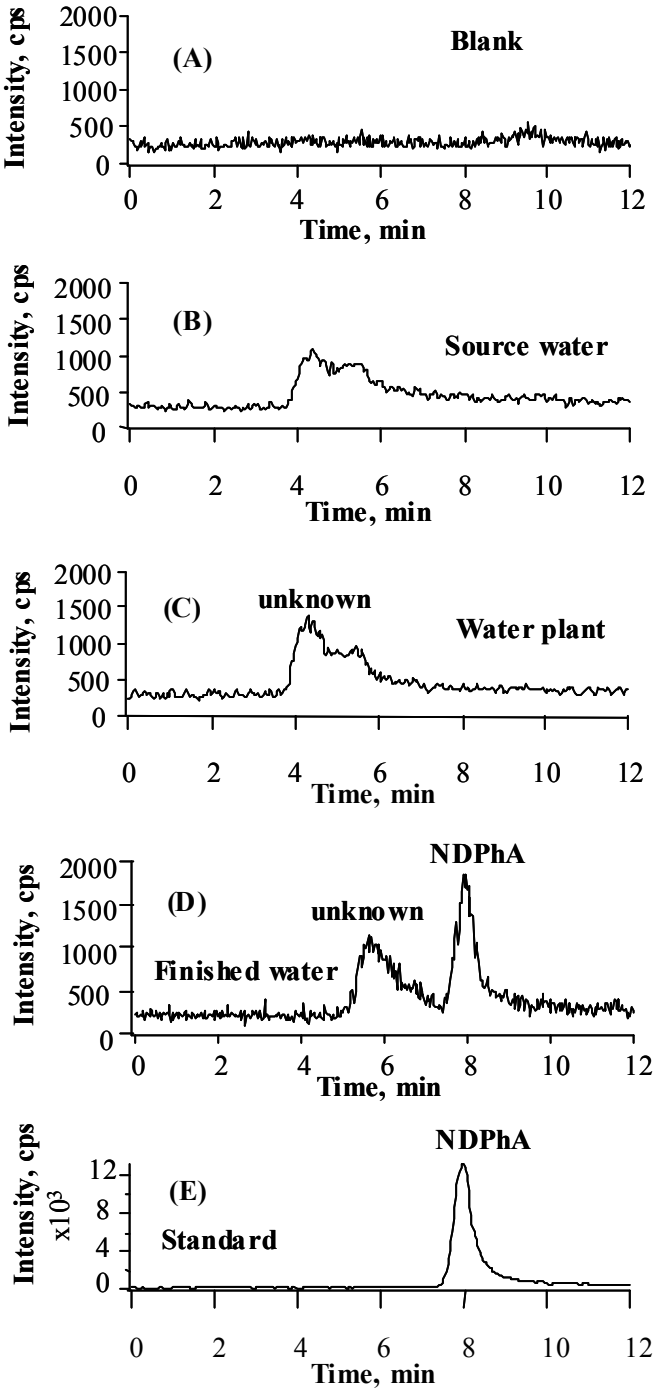


Figure 2.5: Determination of NDPhA in (A) blank, (B) source water, (C) treated water sample collected from the water treatment plant, (D) treated water sample collected from a location in the distribution system, and (E) NDPhA standard.

2.3.3 Quantification of nitrosamines in water

The key analytical parameters for quantification, including recoveries, method detection limits (MDLs), and relative response factors (RRFs) for the nine nitrosamines, are summarized in Table 2.4. The recoveries of the nine nitrosamines were obtained from triplicate analyses, when spiked samples containing 10 and 40 ng/L (each) of the nine standards in Optima water were extracted by SPE and analyzed by LC-MS/MS. The average recoveries ranged from 64.6% for NMor to 111% for NDPhA at 40 ng/L and from 41% for NPyr to 96% for NDBA at 10 ng/L. The recovery of NDMA-d6 at spiked concentration as low as 10 ng/L was 63% with a standard deviation of 6%. The MDLs of the nine compounds by the SPE-LC-MS/MS method were 0.2–3.1 ng/L (except 10.6 ng/L for NDEA), obtained from duplicate extractions of samples containing 10 ng/L and triplicate LC-MS/MS analyses of each extracts. These results are comparable with those obtained by GC-MS [5]. The excellent method detection limits make the ultra-trace analysis of nitrosamines possible.

Table 2.3: Relative response factors (RRF), recovery and method detection limit (MDL) of the nine N-nitrosamines and the surrogate standard NDMA-d6

Nitrosamine	MRM transition	RRF[a] (mean ± SD)	Recovery (%)[b] (mean ± SD)		MDL[c] (ng/L)
			40 ng/L	10 ng/L	
NDMA-d6	81/46	0.23 ± 0.02	77 ± 5	63 ± 6	N.A.
NDMA	75/43	0.17 ± 0.01	78 ± 6	42 ± 10	3.1
NMEA	89/61	0.67 ± 0.04	75 ± 2	77 ± 8	2.4
NPyr	101/55	2.49 ± 0.28	82 ± 7	41 ± 7	2.1
NDEA	103/75	0.39 ± 0.01	93 ± 1	<MDL	10.6[d]
NPip	115/69	3.50 ± 0.11	105 ± 9	81 ± 3	0.9
NMor	117/87	1.10 ± 0.10	65 ± 7	69 ± 7	0.2
NDPA	131/89	1.61 ± 0.12	82 ± 6	65 ± 6	0.2
NDBA	159/103	0.88 ± 0.07	76 ± 9	96 ± 6	3.1
NDPhA	199/169	4.75 ± 0.28	111 ± 3	56 ± 5	0.1

N.A. = Not Applicable
<MDL = Below the method detection limit
[a] The RRF is the average of six values obtained from different concentrations ranging from 5 to 200 μg/L.
[b] Recovery values were the mean of the triplicate analyses of spiked water samples containing either 40 or 10 ng/L.
[c] MDL was obtained from duplicate SPE extractions of water samples containing 10 ng/L nitrosamines and triplicate LC-MS/MS analysis of each SPE extract.
[d] MDL for NDEA was estimated from the analysis of a water sample spiked with 40 ng/L NDEA.

2.3.4 Distribution of four nitrosamine DBPs in a drinking water distribution system

Having detected four nitrosamines in the treated water samples, we further investigated the distribution of these nitrosamines in this distribution system. Water samples were collected from the water treatment plant and three other locations within the same distribution system (Distribution 1 to 3). The locations are numbered in order of increasing distance from the water plant. Being the furthest away from the water plant, Distribution 3 represents the longest residence

time in this distribution system. Table 2.5 shows the concentrations of NDMA, NPyr, NPip, and NDPhA in water samples collected at the four locations.

Table 2.4: Concentrations of NDMA, NPyr, NPip and NDPhA in a water distribution system.

Locations	Concentration (ng/L) (mean ± SD)			
	NDMA	**NPyr**	**NPip**	**NDPhA**
Water treatment plant	<MDL	18.0 ± 1.1	33.1 ± 0.3	<MDL
Distribution 1	51.7 ± 4.7	47.2 ± 1.5	59.8 ± 1.1	0.65 ± 0.05
Distribution 2	65.0 ± 2.7	43.9 ± 3.2	59.8 ± 5.6	1.86 ± 0.13
Distribution 3	108.2 ± 11.7	70.5 ± 5.1	117.8 ± 6.2	0.85 ± 0.01

Note: Distribution 1–3 are increasing distance from the water treatment plant.
<MDL = Below the method detection limit

In general, their concentrations increase with the distance from the water plant. This is probably because these nitrosamines are produced both during the initial water treatment at the plant and within the distribution system. Water treatment systems are required to maintain a disinfectant residual within the distribution system. Because of the presence of the residual disinfectant and natural organic matter in the water, DBPs may continue to form in the distribution system. Therefore, the concentrations of the observed nitrosamines increase initially with the distance from the water plant. This increase reaches a maximum because both formation and decomposition of DBPs take place. As is observed in Table 2.4, the concentration of NDPhA initially increases from the Water Treatment Plant to Distribution 1 and 2 and then decreases when it reaches Distribution 3.

2.4 Conclusions

The developed MRM-LC-MS/MS method provides high sensitivity, specificity, and the capability of detecting both GC-detectable and non-detectable nitrosamines. Using this method, four nitrosamine DBPs were detected (NDMA, NPyr, NPip, and NDPhA). NPip and NDPhA have not been previously reported as DBPs. Our laboratory had previously detected NDMA in this water system, and we observed NMor and NPyr in the treated water samples at levels close to the limit of detection by the GC-MS method [5]. We have clearly demonstrated that LC-MS/MS is comparable with GC-MS techniques for GC-detectable nitrosamines like NDMA. In addition, the detection of NDPhA shows that a wider range of nitrosamines can also be detected using LC-MS/MS, demonstrating that it is a powerful tool for the analysis of nitrosamines in drinking water.

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3 OCCURRENCE OF NINE N-NITROSAMINES IN THIRTY-EIGHT DRINKING WATER SYSTEMS IN CANADA AND THE U.S.A.*

3.1 Introduction

For a DBP to be a candidate for the increased bladder cancer risk observed in epidemiology studies, its occurrence must be widespread enough to expose large numbers of people. In most parts of the world, consumers receive their water from a relatively local source that is treated at a local water treatment plant. Every source water is slightly different in terms of water quality and precursor composition. Similarly, every water treatment plant uses different combinations of treatment processes to produce tap water. This results in every tap water having slightly different composition and concentration of DBPs. This was clearly demonstrated when treated water from two water treatment plants (WTPs) using the same source water was tested [1] for the presence of N-nitrosodimethylamine (NDMA). NDMA concentrations detected in the distribution system from one plant averaged around 100 ng/L over a period of two years, but were always below 5 ng/L in the treated water from the second plant. This shows the importance of monitoring of individual water systems and the need for occurrence studies to determine the population exposure to certain DBPs.

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* Parts of this chapter have been published

1. Boyd, J.M., Zhao, Y.Y., Wagner, M, Qin, F., Levallois, P., Legay, C., Charrois, J.W.A., Richardson, S.D., Hrudey, S.E., Li, X.F. Liquid chromatography-tandem mass spectrometry determination of N-nitrosodiphenylamine and N-nitrosodimethylamine in thirty-eight drinking water systems. Proceedings of the International Water Association's Biannual Water Congress, Montreal, Quebec Sept 2010.
2. Reprinted from *Trends in Analytical Chemistry*, 30/9, J.M. Boyd, S.E. Hrudey, X.-F. Li, S.D. Richardson, Solid-phase extraction and high-performance liquid chromatography mass spectrometry analysis of nitrosamines in treated drinking water and wastewater, 1410-1421, Copyright (2011), with permission from Elsevier

Although NDMA was first identified as a DBP in Ontario in 1989 [2], it was not until it was identified again in California in 1998 [3] that interest in nitrosamines as DBPs became widespread. However, outside of Ontario (which instituted an NDMA monitoring program in 1988), it was not known how common NDMA was in drinking water, or if any other nitrosamines formed as DBPs. In 2001, a North American survey of NDMA in drinking water found that a majority of systems tested had NDMA concentrations below 2 ng/L [4]. However, certain systems had elevated NDMA concentrations, particularly in the distribution systems. In 2004, Charrois *et al*. detected NDMA in a drinking water system in Alberta, Canada, at 180 ng/L, which at that time was the highest NDMA concentration ever detected in drinking water not contaminated with rocket fuel [5]. In addition, two new nitrosamines (NPyr and NMor) were also detected in the same drinking water system at low ng/L concentrations. A follow-up survey of Alberta drinking water in 2006 [6] found that NDMA was the most commonly detected nitrosamine; however, NMor was also detected, signaling the formation of nitrosamines other than NDMA. Ontario and California both have drinking water surveillance programs for NDMA based on provincial and state regulations, but these programs provide data for limited geographical areas. Overall, most nitrosamine occurrence studies have measured only NDMA concentrations. Only some looked at the other GC-detectable nitrosamines included in the U.S. EPA Method 521. It is uncertain whether these studies adequately describe the exposure of consumers to nitrosamines in tap water.

There are several reasons why determining the occurrence of nitrosamines in drinking water is potentially important. Firstly, nitrosamines are much more potent carcinogens than currently regulated DBPs such as THMs and HAAs. Therefore they may be capable of causing health effects at very low concentrations, possibly at the concentrations at which they are typically found in drinking water. For example, the U.S. EPA has set its 1 in 1,000,000 upperbound lifetime cancer risk estimate at 0.7 ng/L of NDMA in drinking water [7] (Table 1.1). Therefore, even low ng/L concentrations may be important. Secondly, it is suspected that nitrosamine concentrations, particularly in U.S. drinking water, may be increasing due to 20–30% of U.S. water treatment plants switching from chlorine to chloramine for disinfection. This switch has come as WTPs try to comply with the U.S. EPA's Stage 2 Disinfectants and DBP Rule which sets maximum contaminant levels for four trihalomethanes (THM4) and five haloacetic acids (HAA5) [8]. Changing disinfection from chlorine to chloramine has been shown to be effective in reducing the concentrations of THMs and HAAs which are regulated under the Stage 2 rule. However, chloramination has been associated with producing higher concentrations of certain groups of DBPs, such as nitrosamines [9] and iodo-DBPs [10], that are more toxic than the currently regulated DBPs. Therefore the reduction of regulated DBPs may result in higher concentrations of more toxic DBPs being produced, which provides an additional challenge for risk assessment.

Based on the Charrois *et al.* study [6], it is possible that NDMA will comprise a large part of nitrosamine exposure from drinking water. However, it is

uncertain how many other nitrosamines also form during water disinfection. A few have been identified, but the focus has remained squarely on the nine nitrosamines in U.S. EPA Method 521, although justification for the selection of these nine nitrosamines is missing and uncertain. With the development of new high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) methods, including the one described in Chapter 2, other thermally unstable nitrosamines can now be detected [11]. Using these methods, N-nitrosodiphenylamine (NDPhA), a thermally unstable nitrosamine, has been detected in a drinking water distribution system in Alberta; however, no information is available on the extent of NDPhA occurrence in drinking water. Other nitrosamines, including ones that have not yet been identified, may also be toxicologically relevant when considering total nitrosamine exposure from drinking water, but very little is known about the occurrence of nitrosamines other than NDMA.

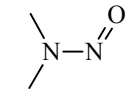
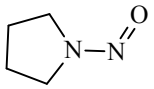
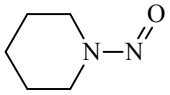
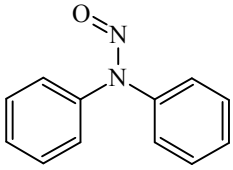
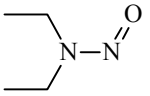
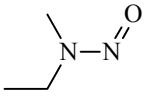
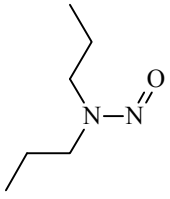
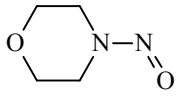
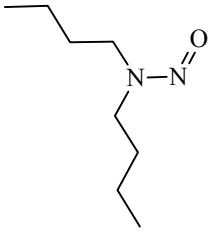
In the present study we investigated the concentrations of nine *N*-nitrosamines in 38 drinking water systems in Canada and the U.S.A. In particular, we determined NDPhA concentrations in drinking water systems in order to better understand human exposure and potential health concerns. In addition, we examined whether there were any differences in nitrosamine formation due to different treatment processes based on the water treatment plants studied.

3.2 Materials and Methods

3.2.1 Reagents

A standard solution containing nine nitrosamines (NDMA, NDEA, NMEA, NDPA, NMor, NPip, NPyr, NDBA and NDPhA) (Table 3.1) was obtained from Supelco (Oakville, ON, Canada). Isotopically labeled standards ([6-H^2] N-nitrosodimethylamine, NDMA-d6, and [14-H^2] N-nitrosodi-n-propylamine, NDPA-d14 (98%) were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). The solid phase extraction (SPE) packing materials, Ambersorb 572 (Rohm & Haas, Philadelphia, PA, U.S.A.) and LiChrolut EN (Merck, Darmstadt, Germany), were obtained from Supelco and VWR International (Mississauga, ON, Canada), respectively. Methanol (AnalR grade) and dichloromethane (Omni-Solv grade) were purchased from VWR International. Ammonium acetate (ACS reagent grade) was supplied by Sigma Aldrich (Oakville, ON, Canada). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Nepean, ON, Canada) unless otherwise indicated.

Table 3.1: Structures of the nine nitrosamines investigated in this study

Nitrosamine	Abbrev.	Molecular formula	Structure
N-nitrosodimethylamine	NDMA	$C_2H_6N_2O$	
N-nitrosopyrrolidine	NPyr	$C_4H_8N_2O$	
N-nitrosopiperidine	NPip	$C_5H_{10}N_2O$	
N-nitrosodiphenylamine	NDPhA	$C_{12}H_{10}N_2O$	
N-nitrosodiethylamine	NDEA	$C_4H_{10}N_2O$	
N-nitrosomethylethylamine	NMEA	$C_3H_8N_2O$	
N-nitrosodipropylamine	NDPA	$C_6H_{14}N_2O$	
N-nitrosomorpholine	NMor	$C_4H_6N_2O$	
N-nitrosodibutylamine	NDBA	$C_8H_{18}N_2O$	

3.2.2 Sample collection

Water samples (237 total) were collected from 38 drinking water systems in Canada and the U.S.A. between May 2006 and August 2007. Water systems were primarily chosen for sampling based on their use of chloramine as a disinfectant, as chloramination potentially increases nitrosamine formation and is commonly used. Systems using chlorination and combinations of chlorine and ozone were included for comparison. Also included in the study were systems that have previously had high nitrosamine concentrations to allow for temporal evaluation, as well as some systems that had not been previously tested for nitrosamines. In addition, systems were only included if willing to participate in the study. Some systems were simultaneously sampled for an iodo-DBP occurrence study [12].

In addition to the treated drinking water samples, several controls were also analyzed, including 32 pure water (Optima Grade) samples, 32 spiking controls, and 25 filter and trip blanks. Sample types collected included raw water, treated water at the WTP and at one or two points within the distribution system. Each system was sampled once within the study period. Specifically, samples of the raw water (source water), treated water at the WTP and from one or two locations within the distribution system were collected from 12 systems (1, 13, 25, 27, 30, 31, 32, 34, 35, 36, 37, and 38). Samples of raw water and treated water at the WTP were obtained from 16 systems (2, 3, 4, 6, 8, 9, 10, 12, 14, 15, 16, 17, 18, 22, 24, and 28). Samples of raw water and treated water at one distribution point were obtained from 6 locations (7, 11, 19, 20, 21, and 23). Samples from one distribution point were obtained from 4 systems (5, 26, 29, and 33).

The raw water samples collected from 34 out of 38 systems were used to assess raw water quality parameters including pH, turbidity, total organic carbon (TOC), UV absorbance at 254 nm (UV A254), specific UV absorbance (SUVA), and total nitrogen (TN) according to standard methods [13]. Table 3.2 summarizes the raw water quality results, water source, and the population served for each system.

Table 3.2: Water source, population served and raw water quality parameters of the 38 water systems studied

System Number	Population served	Water Source	TOC (mg/L)	TN (µg/L)	UV A254 (cm^{-1})	SUVA (L/mg-m)	pH
Chloramine disinfection							
1	5000–50,000	Surface	17.4	1490	NA	NA	NA
2	5000–50,000	Surface	5.3	NA	0.188	3.5	8.5
3	5000–50,000	Surface	3.3	NA	0.097	2.9	8.1
4	>50,000	Surface	5.6	NA	0.234	4.1	7.7
5	5000–50,000	Surface	NA	NA	NA	NA	7.1
6	>50,000	Surface	5.1	NA	0.174	3.4	8.1
7	>50,000	Surface	5.1	NA	0.123	2.4	7.9
8	>50,000	Ground	NA	NA	0.621	NA	7.5
9	>50,000	Surface	5.2	NA	0.123	2.3	7.5
10	>50,000	Surface	3.3	NA	0.100	3.0	8.0
11	>50,000	Surface	16.1	NA	0.625	3.9	7.4
12	>50,000	Surface	5.1	NA	0.177	3.5	7.0
13	>50,000	Surface	3.4	NA	0.110	3.2	7.7
14	>50,000	Surface	3.9	NA	0.085	2.2	8.1
15	5000–50,000	Surface	4.2	NA	0.203	4.9	8.4
16	>50,000	Surface	2.9	NA	0.119	4.1	8.0
17	5000–50,000	Ground	5.0	NA	0.174	3.5	8.2
18	>50,000	Ground	6.1	NA	0.252	4.2	7.4
19	>50,000	Surface	3.2	NA	0.114	3.6	7.7
20	>50,000	Surface	0.7	NA	0.011	1.6	7.9
21	>50,000	Surface	4.2	NA	0.203	4.9	8.4

22	>50,000	Surface	3.2	NA	0.111	3.4	7.8
23	>50,000	Ground	1.4	NA	0.062	4.6	7.0
24	>50,000	Surface	2.6	NA	0.074	2.9	7.7
Chlorine disinfection							
25	5000–50,000	Surface	5.6	366	0.566	NA	8.2
26	5000–50,000	Ground	NA	NA	NA	NA	NA
27	5000–50,000	Surface	4.0	326	0.514	NA	8.2
28	>50,000	Surface	3.5	NA	0.044	1.3	8.3
29	<5000	Ground	NA	NA	NA	NA	NA
30	>50,000	Surface	3.6	133	0.610	NA	7.0
31	>50,000	Surface	8.2	331	NA	NA	NA
32	5000–50,000	Surface	4.9	169	0.858	NA	7.4
33	<5000	Ground	NA	NA	NA	NA	NA
Chlorine then ozone disinfection							
34	5000–50,000	Surface	7.4	719	1.255	NA	7.9
35	5000–50,000	Surface	4.1	312	0.533	NA	8.3
Ozone then chlorine disinfection							
36	>50,000	Surface	5.1	464	1.066	NA	7.3
37	>50,000	Surface	5.1	176	1.08	NA	7.0
38	>50,000	Surface	4.0	300	0.521	NA	8.1

NA = data not available.

Water samples for nitrosamine analysis were collected in 1-L amber glass bottles with PTFE lined caps. Prior to sample collection all bottles were washed, then rinsed with dichloromethane and baked at 180 ºC overnight. Bottles were rinsed with tap water before being filled completely with zero headspace. Most samples had 0.1 g ascorbic acid added to quench the disinfectant residual. Samples were placed on ice until they reached the laboratory after which they were stored at 4 ºC until extraction. Some samples did not have ascorbic acid added after collection as they were collected as part of a larger study [12]. These samples were shipped overnight on ice to the laboratory and extracted the following day. Samples that had ascorbic acid added to them were also extracted as soon as possible after their arrival. Sample analysis using LC-MS/MS occurred within one month of extraction for most samples. A few were analyzed within two months due to a large number of samples collected at the same time and to instrument maintenance.

3.2.3 Nitrosamine extraction

Raw water samples were filtered through pre-baked glass microfibre filters (G/F, Whatman, particle retention >0.7 μm) to remove particulates that may clog the SPE materials. Extraction [5] and LC analysis [11] methods have been previously described in detail. Briefly, glass SPE cartridges were hand-packed with a layer of Lichrolut EN (350 mg) underneath a layer of Ambersorb 572 (500 mg) and capped by a glass wool plug. Five hundred mL of each water sample was spiked with the first internal standard (100 μL of 40 μg/mL NDMA-d6) and then was passed through an SPE cartridge. The first internal standard was

used to assess recovery of NDMA and as quality control for each set of extractions. Once the water had completely passed through, the cartridge was air dried and the nitrosamines eluted using 15 mL of dichloromethane. The eluant was concentrated under a stream of high purity nitrogen gas in a 40 ºC water bath using a Turbovap II evaporation system (Caliper Life Sciences, Hopkinton, MA, U.S.A.) to approximately 200 µL (avoiding dryness), after which the second internal standard was spiked in (100 µL of 40 µg/mL NDPA-d14). The second internal standard was used to correct for instrument response. The extract was stored in a glass insert (Agilent Technologies, Palo Alto, CA, U.S.A.) inside a 2 mL amber vial with a PTFE lined cap (Agilent Technologies) at 4 ºC until analysis. Pure water blanks (Omni-Solv grade water) and nitrosamine spiked samples were extracted along with authentic water samples for each set of extractions.

3.2.4 Liquid chromatography–tandem mass spectrometry analysis

An Agilent 1100 capillary liquid chromatograph (Agilent Technologies) coupled with an API 4000 QTrap mass spectrometer (ABSciex, Concord, ON, Canada) was used to analyze nitrosamines using the conditions that were developed in Chapter 2 [11]. The method detection limits (MDLs) for the nine nitrosamines were determined using a previously published method [14]. The MDLs were calculated to be: NDMA = 2.4 ng/L (standard deviation (SD) = 0.9), NDPhA = 0.11 ng/L (SD = 0.2 ng/L) and NMor = 0.07 ng/L (SD = 0.03). A separate NDMA MDL was calculated for Systems 1, 5, 26, 29, 31, and 33 as the MDL calculated for this set of samples was different from the rest of the survey

(NDMA = 5.2 ± 2.1 ng/L). NDMA-d6 was used to assess NDMA recovery for quality control of each set of extractions. The average NDMA-d6 recovery was 80 ± 6% (n=9). NDPhA recovery was assessed by the analysis of a series of spiked samples ranging in concentration from 2.5–250 μg/L. The average NDPhA extraction efficiency was determined to be 61 ± 3 % (n=9).

3.3 Results and Discussion

The raw water and treated water at different points within the distribution systems were collected wherever allowed. The concentrations of nitrosamines in the raw water were compared to those at the WTP and in the distribution systems to assess the concentration variations in the distribution systems and to confirm that the nitrosamines detected were due to disinfection. Raw water samples were collected from 34 systems and their quality parameters, including pH, turbidity, total organic carbon (TOC), UV absorbance at 254 nm (UV A254), specific UV absorbance (SUVA), and total nitrogen (TN) were obtained. Table 3.2 summarizes general information on the types of source water (ground water and surface water), the population served, and the raw water quality parameters.

Investigation of samples from the 38 drinking water systems showed the presence of NMor and NDPhA in addition to NDMA. As shown in Table 3.3, NMor was detected at the WTP of system 20 with a concentration of 2.4 ng/L and NDPhA was quantified in Systems 1, 11, 23, 33 and 35 with concentrations ranging from 0.19 to 1.8 ng/L. The TOC of raw water from systems 1, 11, and 35 were 17.6, 16.1, and 4.1 mg/L respectively, which are generally higher than those of the other raw water samples, suggesting that higher TOC may partly contribute

to NDPhA formation. This is the first study demonstrating the occurrence of NDPhA in drinking water because NDPhA is thermally unstable and cannot be measured with existing GC-MS methods.

Table 3.3: N-nitrosodiphenylamine (NDPhA) and N-nitrosomorpholine (NMor) concentrations detected in 6 of 38 drinking water systems

Water System Number	Sampling Location	NMor (ng/L ± SD[a])	NDPhA (ng/L ± SD[a])
Chloramine disinfection			
1	Distribution 1	<0.07	0.26 ± 0.09[d]
	Distribution 2	<0.07	1.8 ± 0.5[d]
11	WTP	<0.07[b]	0.30[b]
20	WTP	2.2 ± 0.35[c]	<0.11[c]
23	WTP	<0.07[c]	0.60 ± 0.35[c]
Chlorine disinfection			
33	Distribution	<0.07	0.70 ± 0.05
Chlorine then ozone disinfection			
35	Distribution	<0.07	0.19 ± 0.06

Samples were extracted in triplicate unless otherwise indicated.
[a]SD=standard deviation. Standard deviation indicates triplicate extraction of one sample.
[b]Samples extracted once.
[c]Samples extracted in duplicate.
[d]Two locations were sampled from the distribution system of system 1
WTP = Water treatment plant

Table 3.4 summarizes NDMA concentrations detected in raw source water, finished drinking water samples collected at the WTPs, and finished drinking water samples from the distribution systems. NDMA was detected in several finished drinking water samples collected at the WTPs at concentrations ranging from non-detectable to 29.0 ng/L, and in the distribution systems at concentrations ranging from non-detectable to 130 ng/L (Table 3.4). Nine out of 38 systems (24%) had NDMA concentrations above the California Notification

Level of 10 ng/L [15]. The highest NDMA concentrations detected in this study were in the treated water samples from theWTP (29 ng/L) and distribution system (87.4 and 130 ng/L) taken from System 1. While these concentrations are up to 13 times the California Notification Level, they do not exceed California's Response Level for NDMA of 300 ng/L (30 times the notification level) [16]. The Response Level is the concentration at which the California Department of Public Health recommends taking the water source out of service. These results are comparable to two previous studies that found most water systems had NDMA below the 10 ng/L level, but there are a few water systems with elevated NDMA [4, 6]. NDMA was found in treated ground water (System 26), suggesting the presence of NDMA precursors in ground water. NDMA was detected in a few raw water samples (2, 9, 25, 27, 32 and 38), at concentrations of 2.9, 4.7, 9.4, 4.2, 4.2, and 4.7 ng/L, respectively.

Table 3.4: N-nitrosodimethylamine (NDMA) concentrations detected in samples collected from 38 drinking water systems using chloramination, chlorination or combinations of chlorine and ozone for disinfection.

Location	NDMA ± SD (ng/L)[a,b,c]		
	Source	Water Treatment Plant (WTP)	Distribution
Chloramine disinfection			
1	<5.4[d]	29 ± 5	87 ± 24[e] 130 ± 17[e]
2	2.9[b]	27.5 ± 2.1[c]	NA
3	<2.4[b]	21.5 ± 0.7[c]	NA
4	<2.4[b]	21.0 ± 0[c]	NA
5	NA	NA	19 ± 10
6	<2.4[b]	14.0 ± 1.4[c]	NA
7	<2.4[b]	NA	9.6 ± 0.6[c]
8	<2.4[b]	9.5 ± 2.1[c]	NA
9	4.7[b]	8.8 ± 1.8[c]	NA
10	<2.4[b]	8.0 ± 1.6[c]	NA
11	<2.4[b]	NA	8.2 ± 0.1[c]
12	<2.4[b]	7.5 ± 1.4[c]	NA
13	<2.4[b]	7.3 ± 0.8[c]	<2.4[c]
14	<2.4[b]	7.1 ± 01.2[c]	NA
15	NA	5.8 ± 1.2[c]	NA
16	<2.4[b]	5.5 ± 0.3[c]	NA
17	<2.4[b]	5.4 ± 1.4[c]	NA
18	<2.4[b]	3.9 ± 0.8[c]	NA
19	<2.4[b]	NA	4.9 ± 1.7[c]
20	<2.4[b]	NA	4.8 ± 2.3[c]
21	<2.4[b]	NA	<2.4[c]
22	<2.4[b]	<2.4[c]	NA
23	<2.4[b]	NA	<2.4[c]
24	<2.4[b]	<2.4[c]	NA
Chlorine disinfection			
25	9.4 ± 8.2	17 ± 2	20 ± 4[e] 13 ± 2[e]
26	NA	NA	17 ± 1
27	4.2 ± 2.8	4.7 ± 2.1[c]	6.3 ± 5.8[e] 5.2[b,e]
28	<2.4[b]	5.5 ± 0.2[c]	NA
29	NA	NA	<5.4[d]
30	4.2 ± 3.7	<2.4	<2.4[e] <2.4[e]

31	<5.4[c,e]	<5.4[e]	<5.4[d]
32	<2.4	3.2 ± 4.5	<2.4[e] <2.4[b,e]
33	NA	NA	<5.4[d]
Chlorine then Ozone disinfection			
34	<2.4	<2.4	<2.4[e] <2.4[e]
35	<2.4	<2.4	<2.4[e] <2.4[e]
Ozone then Chlorine disinfection			
36	<2.4	<2.4[d]	12.1 ± 4.2[c,e] <2.4[b,e]
37	<2.4	3.5 ± 3.3	6.3 ± 1.1[e] <2.4[e]
38	4.7 ± 4.1	2.5 ± 4.3	<2.4[b,e] <2.4; 4.0[c,e]

[a]Standard deviation indicates triplicate extraction of one sample. Samples are in triplicate extraction unless otherwise indicated.
[b]Indicates single extraction
[c]Indicates duplicate extraction
[d]Samples were collected from two locations within the distribution system. The sampling location closest to the WTP is listed first.
[e]The method detection limit for systems 1, 5, 26, 29, and 31–33 was 5.4 versus 2.4 for the other systems.
NA= samples from these locations were not available.

Concentration variations within distribution systems were further examined in systems in which samples were collected from both the WTP and the distribution system (12 of 38 systems). In systems where two locations within the distribution system were sampled, the samples are ordered so that the location closest to the WTP is listed first (Table 3.4). The relative distance represents relative residence time in the distribution system. NDMA concentrations increased with the increase of the relative distance from the WTP in System 1 (Table 3.4), which is consistent with our previous observations of this system [5,

17]. Decreases in NDMA concentrations in the distribution system were observed in Systems 13 and 32. The NDMA concentrations detected in the distribution system samples of Systems 25 and 37 were different depending on the sampling location. These findings indicate that NDMA concentrations can change dramatically in the distribution system, indicating that nitrosamine concentrations at the WTPs are inadequate to estimate the exposure of consumers to NDMA in tap water. Therefore, it is necessary to sample different points within the distribution systems for exposure assessment.

Figure 3.1 summarizes NDMA concentrations in the WTP and distribution system obtained from three separate studies of System 1 conducted from 2003 to 2007 [1, 5, 6, 11]. These measurements consistently show 5 to 6 times higher NDMA in the distribution system than at the WTP, confirming the need to sample both WTP and distribution water for NDMA exposure assessment. The highest concentration of NDMA in a distribution system ever reported was from a water sample collected from this system in July 2003 (180 ng/L) [5]. Since then, NDMA concentrations have been reduced after modification of several water treatment processes.

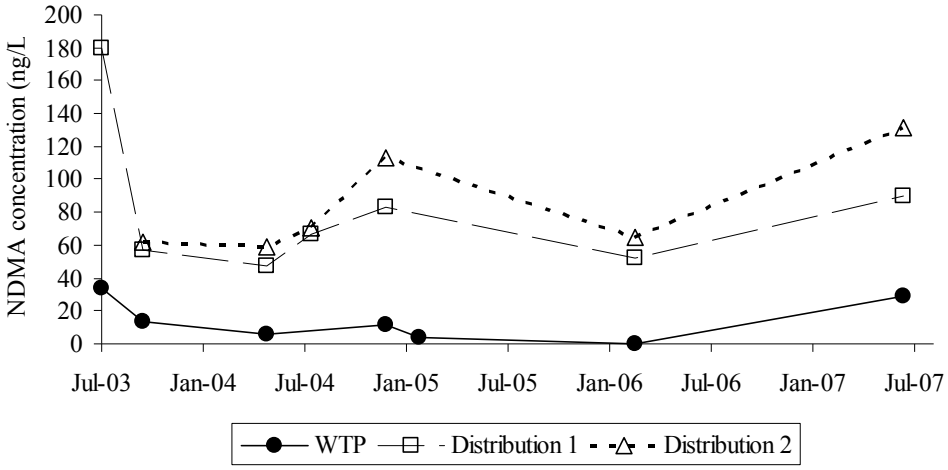


Figure 3.1: NDMA concentrations detected in water samples collected from System 1 between 2003 and 2007. Distribution 2 is farther away than Distribution 1 from the water treatment plant.

The type of disinfectant used has been shown to be an important determinant for nitrosamine formation. Chloramines typically produce more nitrosamines than other treatments [9], but this varies depending on the quality of the source water [17]. In the present study of 38 systems, 24 WTPs used chloramination, nine used chlorination, and five used a combination of chlorine and ozone for disinfection. The highest NDMA concentrations were detected in chloramine disinfected drinking water (Systems 1, 2, 3 and 4); however, we recognize that our sample population was selected to emphasize chloraminating systems.

Nitrosamine concentrations from systems using chloramine disinfection were further examined by taking into account the free chlorine contact time prior to the addition of ammonia. In general, increasing the free chlorine contact time resulted in decreased levels of NDMA formed (Figure 3.2). This finding supports

previous bench-scale experiments [1, 18]. This is important because decreasing free chlorine contact time is one strategy currently used for decreasing the formation of regulated DBPs; however, pursuing this strategy may increase NDMA formation. However, our results agree with previous studies [18] that demonstrate that increasing free chlorine contact time to >1 min or using preformed monochloramine can significantly reduce NDMA without complicated modifications to the treatment equipment.

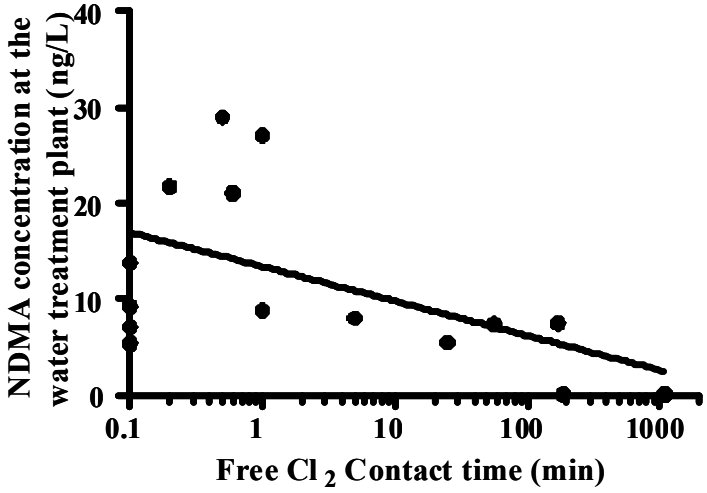


Figure 3.2: Effect of free chlorine contact time on NDMA concentrations produced by chloramine disinfection. NDMA concentrations are from treated water samples collected at the water treatment plant.The line indicates a trend.

In the time since this study was performed, the U.S. EPA has finished collecting nitrosamine occurrence data from across the U.S.A. as part of the Unregulated Contaminant Monitoring Rule-2 (UCMR-2) [19]. Six *N*-nitrosamines (NDMA, NDEA, NDPA, NPyr, NDBA, and NMEA) were included as part of the

UCMR-2. As a result, all U.S. water treatment plants serving a population over 100,000 people and a selection of 320 plants serving 10,001 to 100,000 people are required to monitor for these six nitrosamines during a 12-month period between January 2008 and December 2010 [20]. The finalized data set was not yet available at the time of writing (March 2012); however, an interim data set had been published online. The results support what had been observed in our study and in those by Charrois *et* al [6]. A summary of the interim results appeared in Boyd *et al.* [21]. NDMA was the most commonly detected nitrosamine out of the six tested, being detected at least once in 324/1198 systems. Only 10.2% of the samples analyzed had concentrations above the method reporting limit (MRL) of 2 ng/L (1841 detections above MRL out of 18040 analyses). NDEA, NDBA, NMEA and NPyr were also detected at least once in the study, although at much lower rates (0.02–0.26% of samples with nitrosamine concentrations above the MRL). NDPA was not detected above the MRL in any samples tested. Unfortunately, NDPhA was not included on the UCMR-2 testing list, so it is unknown whethe NDPhA occurrence across the U.S. reflects what was determined in our study.

In summary, this study provides some new findings that are useful for future design of population studies, regulatory considerations, and disinfection practices in utilities in order to meet current DBP regulations. NDPhA, a thermally unstable nitrosamine, is formed in chloraminated drinking water, noticeably in cases where the TOC of the raw water is high (4–18 mg/L). There is some evidence linking NDPhA to higher incidence of bladder cancer in rats [22]

indicating that its presence in drinking water deserves attention. NDMA is the most frequently detected nitrosamine, but only 24% of the systems studied have NDMA above the California Notification Level (10 ng/L). This study demonstrates that nitrosamine concentrations tend to be elevated in systems using chloramination with shorter free Cl_2 contact times or that use chloramination when disinfecting water with high TOC. These results suggest that disinfection processes can be optimized to reduce nitrosamine concentrations. Nitrosamine concentrations change between the WTP and distribution system, indicating that consumer exposure assessments of nitrosamines in drinking water should include samples from multiple locations within the drinking water system. This information will be useful for regulators and water treatment plant operators currently involved in the UCMR-2 or those considering a switch from chlorine to chloramine disinfection to reduce regulated DBP concentrations.

3.4 References

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4 RT-CES METHOD DEVELOPMENT AND APPLICATION TO TOXICITY TESTING FOR EMERGING NITROSAMINE DBPs*

4.1 Introduction

As described in Chapter 1, over 600 disinfection byproducts (DBPs) have been identified [1] and more are reported in the literature every year. For most of these newly identified DBPs, little or no toxicity information is available. Even for DBPs that have been identified for several years, key toxicological information may be lacking, which greatly impedes research and regulatory prioritization.

This lack of toxicity data is not limited to DBPs or even just environmental chemicals. Of the thousands of chemicals under the jurisdiction of the U.S. Environmental Protection Agency (U.S. EPA), only some have undergone toxicity testing [2]. For decades, rodent toxicity studies have been an integral component for toxicity testing and risk assessment determinations. However, animal testing is time consuming, laborious and expensive and it is not a feasible way to test the hundreds of DBPs that have not yet been tested. More and more emphasis is being placed on *in silico* (computer based) and *in vitro* testing to quickly and inexpensively screen new DBPs and other chemicals for research prioritization. In 2007, the U.S. National Research Council published "Toxicity Testing in the 21st Century: A Vision and a Strategy" [3], which

*Parts of this chapter have been published

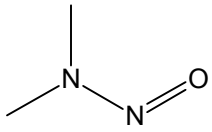
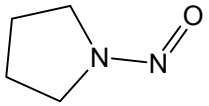
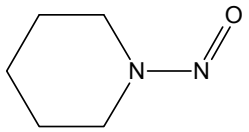
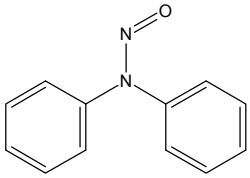
outlines the movement towards *in vitro* based toxicity testing for chemicals with the eventual aim of reducing or eliminating animal testing, reducing the time and cost to screen chemicals and increasing our knowledge of how chemicals exert their toxicity by analysis of multiple toxicity endpoints. The goals outlined in this report have been embraced by several regulatory agencies including the U.S. EPA, the National Institutes of Health, and the National Toxicology Program [4] and have lead to the establishment of *in vitro* screening programs such as ToxCast [5].

In recent years a number of techniques utilizing cellular impedance biosensors have been developed to provide real-time, label-free toxicity testing [6-12]. One of the most recently developed biosensor technologies is Real-Time – Cell Electronic Sensing (RT-CES) which has been demonstrated to provide sensitive monitoring of cellular responses in a real-time continuous manner [10, 11, 13]. This technique utilizes a series of microwells, the bottoms of which are 80% covered with microelectrodes to provide an advance in sensitivity in cell sensing compared to previous techniques. RT-CES measures cell viability by monitoring cell proliferation and morphology [10, 11, 13] using a dimensionless unit called the cell index (CI) which is based on the impedance changes caused by cells interacting with the microelectrodes. The CI values can then be used to generate *in vitro* cytotoxicity values (IC_{50}) which can be used similarly to *in vivo* LD_{50} values for toxicological ranking of chemicals. The results obtained using RT-CES have been shown to be comparable to more traditional cytotoxicity assays such as the MTT, neutral red uptake, lactose dehydrogenase and acid

phosphatase tests [10, 11, 14] and have been used to screen a number of chemicals including drugs and environmental metal contaminants [10, 11, 13]. RT-CES was included as one of nine *in vitro* assays used in phase 1 (2007–2009) of the ToxCast program [15].

In vitro toxicity testing of emerging DBPs has been primarily performed with microplate assays using both bacterial [16, 17] and mammalian cells [17-19]. No DBP toxicity data has been generated using cell electronic sensing based methods. However, there are definite advantages to being able to use these types of systems. To test the applicability of this system for DBP toxicity testing, we developed a method to assess the cytotoxicity of the four nitrosamines detected in treated drinking water [20] in Chapter 2: N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPyr), N-nitrosopiperidine (NPip) and N-nitrosodiphenylamine (NDPhA) (Table 4.1). The toxicity of nitrosamines has been studied extensively after these chemicals were detected in a number of foods and consumer products in the 1970s [21]. NDMA, NPyr and NDPhA are classified as probable human carcinogens [22] and NPip is considered a possible human carcinogen [21]. The available toxicity data concerning NDMA, NPyr and NPip will help to validate the results of the RT-CES method. As less information is available about NDPhA toxicity, the RT-CES results will help improve our understanding of the toxicity of this particular nitrosamine.

Table 4.1: Chemical structures and purity of the four nitrosamines used in this study

Compound	Molecular formula	CAS #	Standard Purity	Structure
NDMA	$C_2H_6N_2O$	62-75-9	99 %	
NPyr	$C_4H_8N_2O$	930-55-2	99 %	
NPip	$C_5H_{10}N_2O$	100-75-4	99.8 %	
NDPhA	$C_{12}H_{10}N_2O$	86-30-6	99.9 %	

The purpose of this study is to develop an RT-CES method for profiling the toxicity of chemicals with similar structures and properties. The continuous sensing and quantitative measurements of the RT-CES system combined with use of multiple cell lines will produce a panel of cytotoxicity profiles that will allow differentiation among similar chemicals such as nitrosamines.

4.2 Experimental

4.2.1 Materials and cell culture conditions.

Standards of NDMA, NDPhA, NPip, NPyr were purchased from Sigma Aldrich (Oakville, ON, Canada) (Table 4.1). Methanol and water (HPLC grade) were obtained from Fisher Scientific (Nepean, ON, Canada).

A549 (Human type II pneumocyte derived adenocarcinoma) cells were cultured in RPMI 1640. T24 (human bladder carcinoma) cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.) and were cultured in McCoy's 5A modified medium (ATCC). CHO-K1 (Chinese hamster ovary) cells were obtained from Dr. Tom Hobman (Department of Cell Biology, University of Alberta) and cultured in (1:1) Dulbecco's Modified Eagle medium (DMEM):Ham's F12 containing L-glutamine and HEPES buffer (Gibco Co., Invitrogen, Burlington, ON, Canada). HepG2 (human liver carcinoma) cells were cultured in DMEM containing high glucose, L-glutamine and sodium pyruvate. All cells were cultured in their respective medium supplemented with 10% fetal bovine serum (Sigma Aldrich) and 1% penicillin-streptomycin (Invitrogen) at 37°C with 5.5% CO_2.

4.2.2 RT-CES cytotoxicity testing

Cytotoxicity testing was performed using the real-time cell electronic sensing (RT-CES) 16X system (ACEA Biosciences Inc., San Diego, CA, U.S.A.). This system utilizes a 16X plate station, which can simultaneously monitor up to six 16-well E-plates. The 16X plate station is kept inside a CO_2 incubator at the conditions described above. The cytotoxic effect of the four nitrosamines was evaluated using four cell lines (A549, T24, CHO-K1, HepG2). Cell calibration on the RT-CES system was performed to determine the optimal cell seeding number for each cell line (Figure 4.1). The initial number of cells seeded into the microwells for each cell line was selected based on the cells reaching a CI of 1 at approximately 24 h and remaining within the log growth phase (linear dynamic

range) at 48 h (or at 24 h after addition of nitrosamines) when the IC_{50} was determined (Figure 4.1). The number of cells seeded for each cell line was: CHO-K1, 5000; A549, 5000; T24, 4000; HepG2, 15000. HepG2 cells do not form a perfect monolayer on the bottom of the microwells, which has been observed previously [23]. They grow slightly upwards, forming a small bump in the monolayer in the middle of the microwell, which resulted in lower CI values recorded for this cell line. The other three cell lines formed a monolayer on the bottom of the microwells.

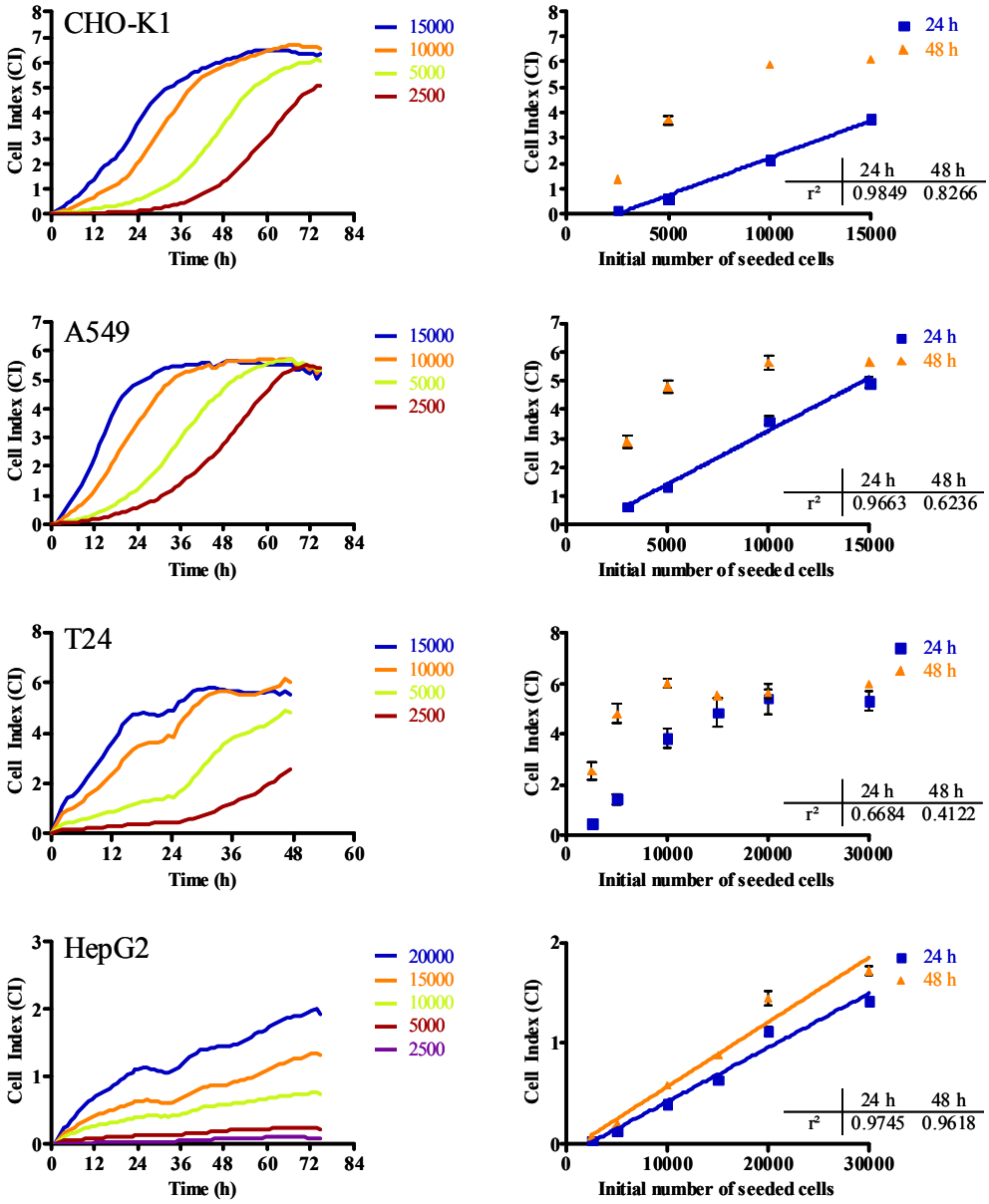


Figure 4.1: RT-CES curves and linear relationship between cell index (CI) and initial number of seeded cells. The linear relationships between RT-CES CI and the number of seeding cells are found during the log phase.

Nitrosamines were added to the cultures 24 h after cell seeding. NDMA, NPip, and NPyr were dissolved in the appropriate medium for each cell line

before being added to the wells. Because NDPhA does not completely dissolve in culture medium, it was dissolved in methanol first, and then diluted with the culture medium before being introduced to the cultures in the microwells. NDPhA was dissolved in methanol at a proportion of 0.5% methanol per 2.5 mM of NDPhA. Methanol controls were performed along with the NDPhA experiments to ensure that any observed cytotoxic effects were due to NDPhA and not methanol.

CI was measured automatically by the RT-CES system once per hour until the end of the experiment. A range of nitrosamine concentrations (0.2 to 300 mM) was separately tested, with at least five different concentrations tested per nitrosamine on each cell line. Each concentration was repeated at minimum in triplicate.

4.2.3 Alternative determination of IC_{50}

To validate IC_{50} obtained from the RT-CES method, the IC_{50} of NDMA was also tested using a microplate cytotoxicity assay that has been previously described [18]. The only modification made to this method was the addition of NDMA after the cells were allowed to grow for 24 h to maintain consistency between the microplate cytotoxicity assay and the RT-CES assay. The IC_{50} was determined after 24 h of NDMA exposure.

4.2.4 Cell enumeration, cell death, and cell cycle analysis.

CHO-K1 cells were grown in tissue culture dishes until they reached 60% confluence, at which point they were treated with the following nitrosamine concentrations: NDMA, 35 mM; NPyr, 30 mM; NPip, 10 mM; and NDPhA, 1.25

mM. These concentrations were approximately the IC_{50} concentrations obtained from the RT-CES experiments. The cells grew in the presence of nitrosamines for 24 h before harvesting. For enumeration of viable and dead cells, the harvested cells were diluted with phosphate-buffered saline (PBS) (Gibco Co.), re-suspended, and mixed. A 20 μL aliquot of the cell suspension was mixed with 50 μL of trypan blue (Gibco Co.) and 30 μL of PBS and incubated for 5 min. The number of dead cells and total cells was counted using a hemocytometer. Flow cytometric analysis of the harvested cells was performed as described previously [24]. Cell cycle analysis was performed using a Becton and Dickinson FACScanTM (Mountain View, CA, U.S.A.). QuestTM software (Becton and Dickinson) was used for data analysis.

4.2.5 Statistics

In vitro cytotoxicity at 50% (IC_{50}) was defined as the concentration that was required to reduce the CI measurement by 50% compared to the control after 24 h of nitrosamine exposure. IC_{50} values and 95% confidence intervals were calculated using Prism 4 (Graph Pad Software Inc., San Diego, CA, U.S.A.). Two-sided Mann Whitney tests were performed to compare between treatments and the control for cell cycle and cell death analysis. The confidence level was set at 95%.

4.3 Results and Discussion

4.3.1 Method design

The RT-CES system has been previously described in detail [10, 11, 13]. Briefly, the RT-CES 16X system used in this study utilizes electronic microchips

with 16 microwells on each E-plate. The bottom of each microwell has an approximate area of 20 mm^2 and approximately 80% of this area is covered by microelectrodes. The microelectrodes measure changes in impedance between the electrode and the solution. Cells attaching to the microelectrodes cause a change in electrical impedance compared to when no cells are bound to the microelectrode. Impedance is measured at three frequencies:10 kHz, 25 kHz and 50 kHz. The resistance (R) is then calculated from these measures of impedance (Z) using Equation 4.1:

$$Z = R + jX \qquad \textbf{Equation 4.1}$$

Where X indicates reactance and j is the square root of -1. The determined values of resistance are then converted into cell index (CI) via Equation 4.2:

$$CI = \max_{i=1,\ldots,N}\left[\frac{R_{cell}(f_i)}{R_b(f_i)} - 1\right] \qquad \textbf{Equation 4.2}$$

R_b indicates the frequency-dependent resistance of the microelectrode/solution interface with no cells attached, and R_{cell} indicates the frequency-dependent resistance of the microelectrode when cells are attached. Therefore, lower CI values indicate fewer cells are bound to the microelectrodes. Increases in CI can be attributed to increasing cell numbers (more cells attached to the microelectrodes); increased cell adhesion to microelectrodes; or cell

spreading (increased cell/electrode contact area). In this study, the RT-CES system was used as a sensing technique for measuring cytotoxicity based on CI changes resulting from changes in cell number, morphology, and/or cell detachment from the microelectrodes. By measuring these chemical-induced changes in the cell population, this technique allows for high throughput cytotoxicity profiling of environmental contaminants including those with similar chemical and physical properties.

Cells are the living component of the cell-electronic sensors which provide dynamic information on chemical toxicity. A range of cell lines was used to develop this chemical toxicity profiling technique for a better understanding of the cytotoxicity of individual chemicals, because different cell lines have different susceptibilities to each chemical. This can be accomplished by taking advantage of the parallel screening abilities of the RT-CES system to monitor multiple concentrations and cell lines at one time. To demonstrate the proof of principle, four cell lines, namely CHO-K1, A549, T24, and HepG2, were used in this study because they have been used previously in cytotoxicity testing assays [9, 17-19, 25-28] and all are easily available. In addition, CHO-K1 (Chinese hamster ovary) cells are non-cancerous and have been previously utilized in a number of cytotoxicity studies of other DBPs [17-19], which may be useful for comparisons between data sets in the future.

4.3.2 Continuous monitoring of cellular response to NDMA exposure

NDMA was initially used to demonstrate the monitoring method because it is well characterized toxicologically and is the most commonly detected

nitrosamine drinking water DBP. Figure 4.2 shows characteristic RT-CES traces of the four cell lines exposed to various concentrations of NDMA. The RT-CES system automatically recorded the CI from each microwell once every hour during the experiment. Controls without NDMA treatment (blue traces) indicate normal cell growth in the electronic microwells, providing real-time control of cell status (growth and attachment to the microelectrodes). CI provides a quantitative measurement of the numbers of cells on the microelectrodes as shown in Figure 4.1. The four cell lines were simultaneously tested with five different concentrations of NDMA, although in Figure 4.2 only four are shown. Figure 4.2 shows typical RT-CES curves (CI vs. exposure time) of the four cell lines (CHO-K1, A549, T24, and HepG2) responding to NDMA treatment. These curves clearly demonstrate cell-dependent toxic responses. NDMA treatment decreased the measured CI in all four cell lines. With sufficient concentrations of NDMA, CI can be reduced to zero; however, the range of concentrations that cause CI to decrease is cell-line dependent. CI at zero means no viable cells are attached to the microelectrodes, indicating the highest cytotoxicity. T24 cells showed the most severe toxic response to NDMA treatment compared to the other three cell lines. In this study, the lowest tested NDMA concentration that reduced CI to 0 was 13 mM for T24, 40 mM for A549, 50 mM for CHO-K1, and 200 mM for HepG2. Figure 4.2 also shows that the lowest concentrations that produce a change in CI compared to the control in CHO-K1 and A549 cells are 40 mM and 30 mM, respectively. After a period of time however, the cells were able to continue growing with a slope similar to that of the control cells during their log

phase at approximately 50 h into the experiment (or 26 h after addition of NDMA). A possible explanation for this observation is that the administered NDMA dose did not kill all the cells in the well or cause permanent damage to prevent them from growing. Thus the surviving cells could continue growing after they adjust to the conditions or the NDMA concentration in the well decreases sufficiently through metabolism or breakdown. The insensitivity of HepG2 cells to NDMA may be partially explained due to the tendency of these cells to grow in clumps and in a multilayer rather than a monolayer [23]. In the present study, we observed that these cells can grow upwards in the microwells unlike the other cell lines which form a monolayer. The formation of a multilayer by HepG2 would result in a slight underestimation of CI as not all cells in the microwell would be directly attached to the electrodes. This in turn, would result in a slight overestimation of the toxicant dose necessary to cause cytotoxic effects.

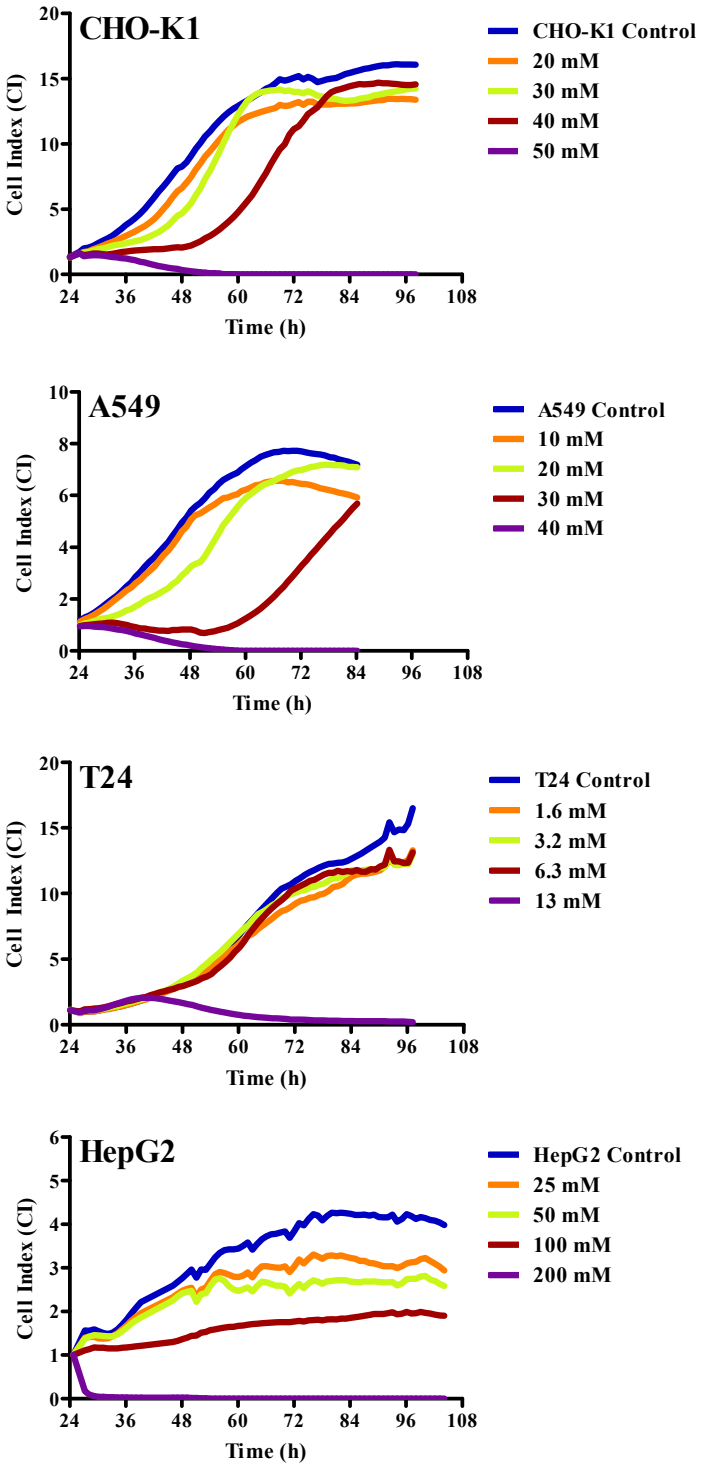
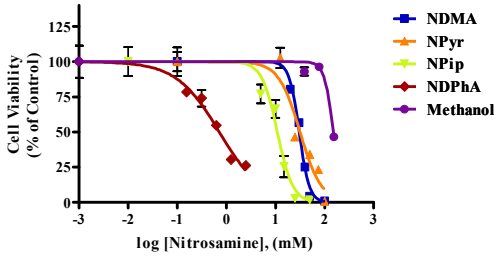


Figure 4.2: Typical RT-CES traces showing the effect of NDMA on the four cell lines. Cells were allowed to grow for 24 h prior to the introduction of the nitrosamine to the culture. CI was recorded every hour. Each trace at each concentration was an average of 3 replicates.

4.3.3 Profiling cytotoxicity of different nitrosamines

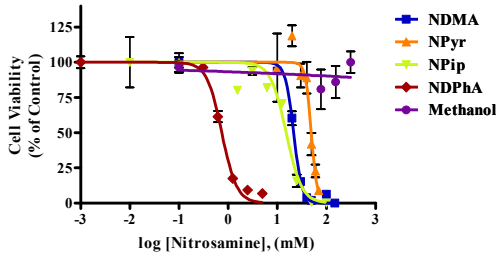
To demonstrate the capability of this method to provide continuous, rapid, and simultaneous monitoring of different chemicals, the four nitrosamines (NDMA, NPyr, NPip, and NDPhA) were simultaneously tested in the four cell lines. RT-CES response curves similar to those in Figure 4.2 were obtained for the remaining three nitrosamines in all four cell lines and show the continuous and real-time change of cell status during exposure to these compounds (data not shown). The RT-CES curves were converted to the viability curves shown in Figure 4.3. The viability curves are cell viablity vs. log concentration of the nitrosamine. The viability was determined as the CI of the treated cells relative to CI of the control at a given time. These viability curves are comparable to the dose response curves of the four nitrosamines in the four cell lines and different cytotoxicity profiles are clearly shown. The IC_{50} values of the nitrosamines in the four cell lines were also determined and are listed in Figure 4.3. These results clearly show that NDPhA has the highest toxicity in each cell line, followed by NPip. NDMA and NPyr had very similar IC_{50} values in CHO-K1 and T24 cells. NDMA was much less potent in HepG2 cells compared to NPyr, and the opposite was found in A549 cells. The IC_{50} data also demonstrate the cell-specific toxicity of the four nitrosamines and that T24 cells are the most sensitive to the four nitrosamines in all the tests. The order of sensitivity of the four cell lines is T24>A549>CHO>HepG2.

CHO-K1



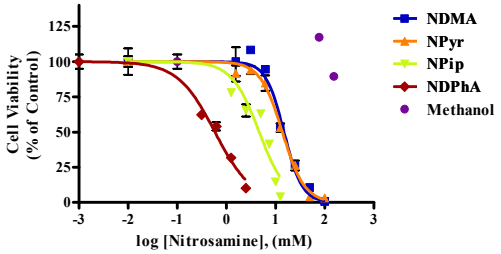
	NDMA	NPyr	NPip	NDPhA	Methanol
IC_{50} (mM)	31	32	11	0.72	152
95% CI	29 to 32	25 to 40	9 to 13	0.56 to 0.92	129 to 178

A549



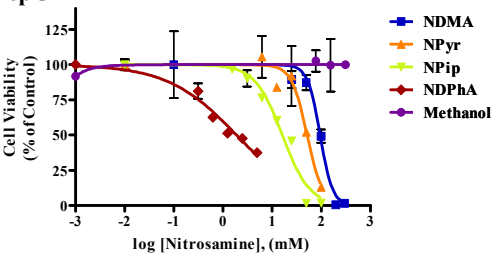
	NDMA	NPyr	NPip	NDPhA	Methanol
IC_{50} (mM)	22	50	15	0.75	NA
95% CI	21 to 22	45 to 55	13 to 19	0.69 to 0.82	NA

T24



	NDMA	NPyr	NPip	NDPhA	Methanol
IC_{50} (mM)	15	14	4.7	0.59	NA
95% CI	13 to 17	12 to 15	3.9 to 5.6	0.48 to 0.72	NA

HepG2



	NDMA	NPyr	NPip	NDPhA	Methanol
IC_{50} (mM)	95	52	16	1.9	NA
95% CI	77 to 118	38 to 70	13 to 20	1.5 to 2.4	NA

Figure 4.3: IC_{50} values obtained after the cells were incubated with the four nitrosamines for 24 h. Cells were grown for 24 h prior to treatment with nitrosamines. Each data point is the average of 3 replicates. Methanol was used as solvent for NDPhA.

Curves for methanol toxicity are also included in Figure 4.3 because methanol was used to dissolve NDPhA prior to introduction to the cell cultures. The methanol concentrations tested were the same concentrations used to dissolve the NDPhA doses used in this study. Methanol at the concentrations used had no significant impact on the cells, indicating that toxic effects observed in the NDPhA-treated cells was mainly due to NDPhA and not a solvent effect.

Previous studies have focused mainly on nitrosamine mutagenicity rather than overall cytotoxicity. Therefore there are few IC_{50} values for these four nitrosamines in the literature to compare to our values. One study looked at the inhibition of RNA synthesis in HepG2 cells by NDMA, without the addition of an S9 fraction and found an IC_{50} of 110 ± 24 mM [25], which is similar to our IC_{50} value of 95 mM (95% confidence interval = 77 to 118 mM) (Figure 4.3). However the RT-CES system has previously been shown to have similar sensitivity to more commonly used assays such as the MTT, neutral red uptake (NRU), lactose dehydrogenase [29] and acid phosphatase tests [30]. In particular, IC_{50} values generated by the RT-CES systems were shown to be most similar to the NRU [10], which is the cell cytotoxicity assay recommended by the National Institues of Health and National Institutes of Environmental Health Sciences [31] thus supporting the RT-CES measurement of IC_{50} as a valid cytotoxicity assay.

To validate the IC_{50} values obtained using the RT-CES system, we treated CHO-K1 cells with the IC_{50} concentration and looked for a 50% reduction in the number of cells compared to the control. Figure 4.4 shows that the treated groups had approximately 50% fewer cells than the concurrent controls. We also

determined an IC_{50} of 49 mM for NDMA in CHO-K1 cells using the microplate cell cytotoxicity method that was previously developed for screening DBP cytotoxicity [18]. This value obtained from the microplate cytotoxicity assay is comparable to the value of 31 mM obtained from the RT-CES test (Figure 4.3).

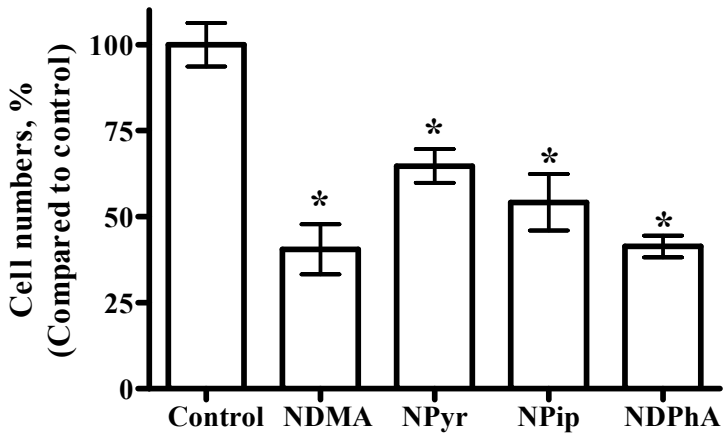


Figure 4.4: Relative numbers of CHO-K1 cells after 24 h of incubation with the nitrosamine compared to the control without nitrosamine. After 24 h of growth, the cells were treated with approximately the IC_{50} concentration of each nitrosamine as determined by the RT-CES experiments (the concentrations: NDMA, 35 mM; NPyr, 30 mM; NPip, 10 mM; NDPhA, 1.25 mM). After 24 h exposure the cells were harvested and counted using a hemocytometer. Bars represent the average of 4 replicates and error bars indicate the standard error. Asterisks indicate the mean of the nitrosamine treated cells is statistically lower compared to the mean of the control cells based on a two-sided Mann Whitney test with 95% confidence.

4.3.4 Effects of nitrosamines on cell viability and cell cycle

The higher cytotoxicity of NDPhA over NDMA (a potent carcinogen) is observed for the first time in this study. Previous studies of NDPhA focused on genotoxicity and mutagenesis and NDPhA was found to be non-mutagenic [32]. To explore the reasons behind the different nitrosamine cytotoxicities (or IC_{50}

values), particularly the high cytotoxicity of NDPhA, we examined the induction of cell death and cell cycle arrest by the four nitrosamines. Trypan blue staining results revealed that NPip and NDMA treatment resulted in a statistically significant increase in cell death compared to the control (Figure 4.5a) when the cells were treated separately with IC_{50} concentrations of NPip and NDMA. This suggests that these two nitrosamines cause a drop in CI by initiating cell death. This is consistent with previous research showing that NDMA and NPip induce apoptotic cell death in mammalian cells [33, 34]. NDPhA and NPyr treatment did not result in a statistically significant increase in cell death compared to the control, indicating that these two nitrosamines exert their toxicity through another mechanism other than cell death.

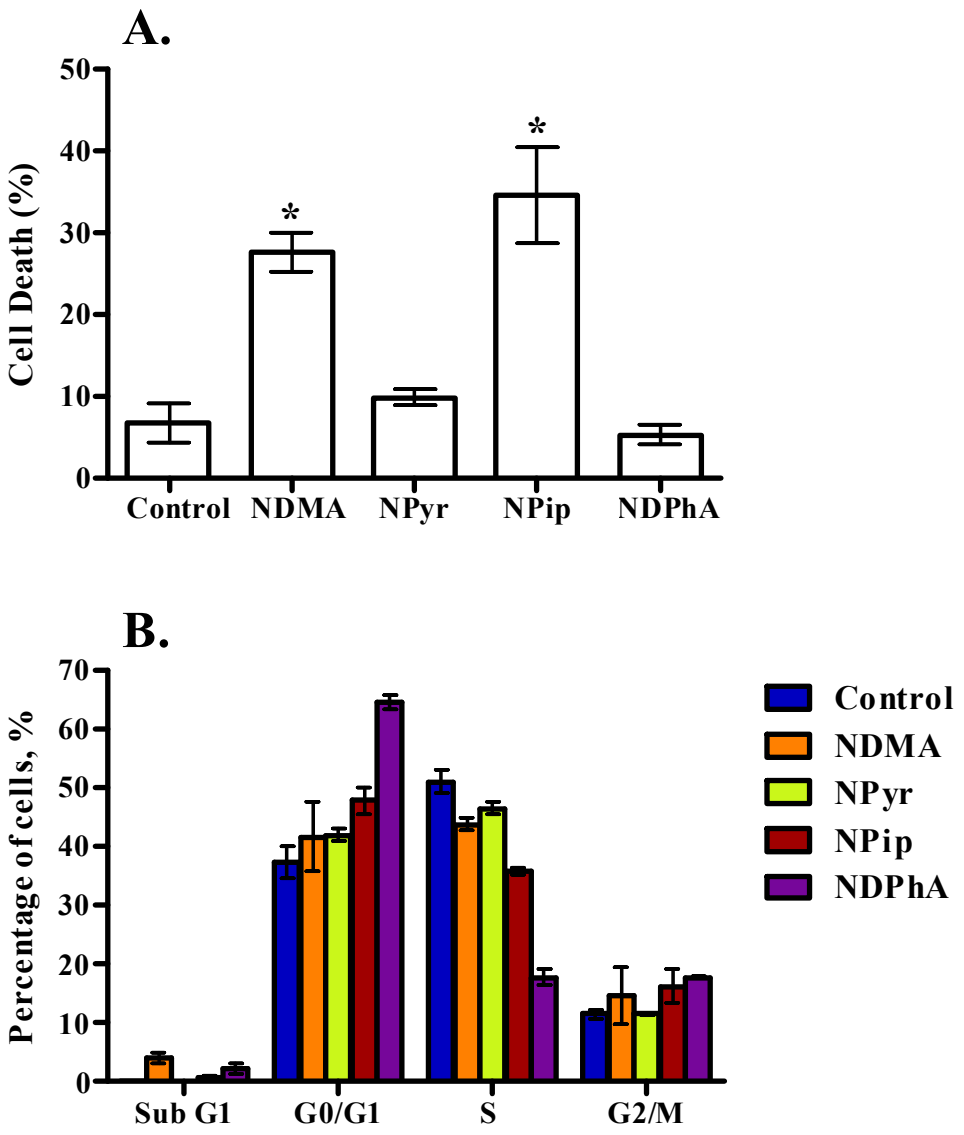


Figure 4.5: Effect of nitrosamine exposure on cell death and the cell cycle. (A). Trypan blue staining analysis of the percentage of cell death induced by the nitrosamine exposure related to the control. Bars represent the average of 4 replicates. Error bars indicate the standard error. (B). Flow cytometry analysis of cell cycle. Bars represent the average of 2 replicates. Error bars indicate the standard error. Asterisks indicate the mean of the nitrosamine treated cells is statistically higher compared to the mean of the control determined using a one-sided Mann Whitney test with 95% confidence.

Flow cytometry analysis was performed to evaluate the effects of the nitrosamines on the cell cycle. The results (Figure 4.5b) revealed that NDPhA treatment caused a higher percentage of cells to remain in G0/G1 and decreased the percentage of cells in S phase compared to the control (Figure 4.5b). This implies that NDPhA is likely inducing G0/G1 arrest which halts cell proliferation. Inhibition of cell proliferation by NDPhA would result in a lower CI value compared to the control because fewer cells would be available to attach to the microelectrodes. This is consistent with what was observed using the RT-CES system for the NDPhA treated cells. The effect of NDPhA on cell cycles has never been reported. NPyr induced decreases in CI were not adequately explained by either the cell death or cell cycle assays, suggesting that other mechanisms may be responsible. Further study is needed to elucidate the cellular effects of this nitrosamine.

The various profiles of nitrosamine-dependent and cell line dependent cytotoxicity that are detected by RT-CES may be related to different factors. For example, specific nitrosamines may require activation by specific P450 isozymes in order to cause toxic effects, and different types of cells may have different levels of P450 expression (Table 4.2) [32-40]. NDMA, NPyr and NPip are metabolized by P450 enzymes to form hydroxyl radical intermediates via α-hydroxylation. These intermediates are capable of methylating macromolecules such as DNA [35, 36] and the resulting DNA adducts may result in carcinogenesis, especially in tissues where particular P450 enzymes are highly expressed. P450 2E1 is the major isozyme for NDMA bioactivation [37]. P450

2E1 and 2A6 are also important for NPyr bioactivation [38]. NPip is metabolized by P450 2E1, 2A6 and 1A1 [39]. The expression of P450 enzymes important for metabolism of NDMA, NPyr, and NPip in HepG2 and A549 cell lines is available in the literature [41-44]. CHO-K1 cells do not express P450 enzymes. No data on the P450 expression of T24 cells is available. In contrast, NDPhA (Table 4.1) does not undergo P450 mediated α-hydroxylation [32] and thus has a different mechanism of action than NDMA, NPip and NPyr. There is much less toxicological information regarding NDPhA compared to the other three nitrosamines. It has been reported that NDPhA is a poor mutagen [32, 40], but NDPhA has also been shown to cause DNA damage via a mechanism that may rely on P450 activation [45]. This study and others demonstrate that various mechanisms may be associated with cytotoxicity of different nitrosamines although they all have the same reactive group in the molecules. The RT-CES method can provide cytotoxicity profiles that can be useful for directing other studies into the mechanisms of action and toxicity.

Table 4.2: P450 expression in A549 and HepG2 cell lines

Cell Line	Cell Type	P450 Isotype	Average mRNA levels (mean ± SD) [ref[	Derived tissue/cell line P450 expression ratio [ref]	Enzyme activity[a] (mean ± SD) [ref]
A549	Type II pneumocyte derived adenocarcinoma	1A1	0.40 ± 0.16[b] [41]	16 [41]	NA
		2A6	0.09 ± 0.03 [b] [41]	1.4 [41]	NA
		2E1	<0.01[b] [41]	>3500 [41]	NA
HepG2	Human liver carcinoma	1A1	2.93 ± 0.93[c] [43]	13.71 [43]	0.17 ± 0.09 [42]
		2A6	<0.01[d] [44]	>407[e]	0.05 ± 0.04 [44]
		2E1	0.38 ± 0.11[c] [43]	1464 [43]	ND [42]

NA = Data not available
ND = Not detectable
[a] 1A1: EROD (ethoxyresorufin *O*-deethylase) (pmol/(min mg protein))
2A6: coumarin 7-hydroxylase (pmol/(min mg protein))
2E1: Hydroxylation of *p*-nitrophenol
[b]Expressed relative to β-actin mRNA content as CYP mRNA/ β-actin mRNA) x 10^4
[c]Calculated on the basis that 40 pg of total RNA corresponds to one cell
[d]Expressed as (mRNA CYP molecules/ β-actin mRNA) x 10^3
[e]Calculated from data from reference [44]

In conclusion, this study has demonstrated an RT-CES method for cytotoxicity profiling of nitrosamines. The discovery of the unique toxicity of NDPhA demonstrates that an array of cellular response profiles on a panel of cell lines can provide more information about the toxicity of a chemical. The RT-CES technique is able to sensitively assess the cytotoxicity of compounds on a variety of cell lines concurrently in a rapid, label-free, automated and continuous manner. This *in vitro* assay may be useful for future toxicity profiling and assessments of chemicals in order to prioritize chemicals for regulation and to reduce animal use.

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5 INVESTIGATION OF FORMATION OF N-NITROSAMINES DURING DRINKING WATER TREATMENT*

5.1 Introduction

Understanding the formation of DBPs is necessary in order to reduce or prevent the formation of these chemicals during water treatment. However, DBP formation is a complicated process that is influenced by many variables including the pH, temperature, turbidity, natural organic matter (NOM) content and concentration in source water as well as the type and concentration of the disinfectants used and disinfectant contact time. For most DBPs, formation is not well understood except for the understanding that manipulation of one water treatment variable to reduce the formation of a particular group of DBPs will result in the promotion of formation of another group of DBPs. Thus, understanding DBP formation is central to a comprehensive risk management strategy to reduce health risks by all DBPs.

5.1.1 N-nitrosamine formation during water treatment

Following the identification of N-nitrosamines as DBPs, interest in how they formed from water disinfection was very high due to the potential health risks associated with consumption of these chemicals. N-nitrosamines are known to form from amines and nitrite at low pH via nitrosation [1]. While this pathway

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is relevant for formation of nitrosamines in the stomach, it is likely not significant for water treatment where the majority of processes occur between pH 5 and 8. Several studies have investigated the effect of different disinfection treatments on nitrosamine formation. In general, these studies have focused only on NDMA formation, and have focused on the corresponding secondary amine dimethylamine (DMA) as the main NDMA precursor.

5.1.1.1 Nitrosamine formation from chlorine and chloramine

Of the disinfectants studied, chloramination alone or chlorination of water containing ammonia usually produce higher concentrations of NDMA, a finding that has been replicated in most occurrence studies (Chapter 3). Several studies have investigated NDMA formation from DMA and chloramines. Initial versions of the pathway suggested the reaction of DMA and monochloramine to form unsymmetrical dimethylhydrazine (UDMH), which then reacted with another molecule of monochloramine to form NDMA [2, 3]. However, there were several issues with this model. It did not match with kinetic models for UDMH production; addition of monochloramine to UDMH did not produce the expected yields of NDMA; and it did not explain the presence of oxygen in NDMA [4]. Two studies by Schreiber and Mitch revised this pathway (Figure 5.1) showing that dichloramine rather than monochloramine was the important disinfectant species that reacted with DMA, forming chlorinated unsymmetrical dimethylhydrazine (Cl-UDMH) as an intermediate [5, 6]. Cl-UDMH, in the presence of dissolved oxygen, is then oxidized to form NDMA.

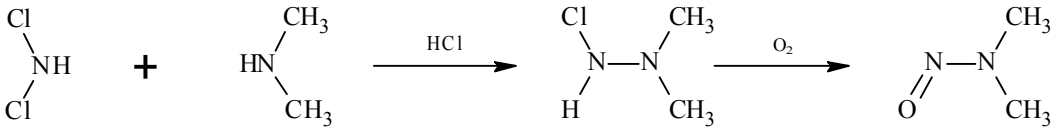


Figure 5.1: Proposed mechanism for the formation of NDMA from DMA and dichloramine. From [5, 6].

Another pathway for NDMA formation from chlorination of nitrate containing water has been reported [7]. The yields from this reaction are significantly lower than formation of NDMA from chloramination of DMA (Figure 5.1), suggesting it is not as important for nitrosamine formation.

5.1.1.2 Nitrosamine formation from alternative disinfectants

The effect of alternative disinfectants on nitrosamine formation has also been investigated, although not to the same degree as chlorine or chloramines. Chlorine dioxide (ClO_2) and ozone (O_3) are able to produce nitrosamines, but usually at lower concentrations than chloramination [8, 9]. Schmidt and Brauch showed that, in the case of O_3, this is most likely due to a smaller range of precursors that will react with O_3 to form nitrosamines [10]. However, the same study showed certain precursors, specifically N,N-dimethylsulfamide, can be converted to NDMA during ozonation resulting in the detection of NDMA concentrations up to 390 ng/L in selected water treatment plants.

In contrast, ultraviolet (UV) and advanced oxidative processes (AOP) have both been investigated as possible strategies to decrease or eliminate nitrosamine concentrations in water [11-14]. UV is known to break the N-N bond in nitrosamines, and thus has been employed to degrade these chemicals in water.

AOP oxidizes precursors, in theory making them unable to act as nitrosamine precursors.

5.1.1.3 Research questions regarding nitrosamine formation

Although many studies have investigated nitrosamine formation during water treatment, several questions remain. Firstly, most formation studies have focused on NDMA. Thus very little is known on the formation of other nitrosamine DBPs. It is generally assumed that they form from similar precursors (i.e. secondary amines) via similar pathways; however, this has not been demonstrated.

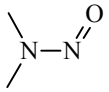
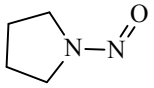
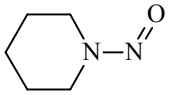
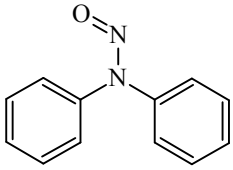
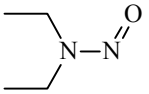
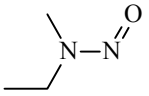
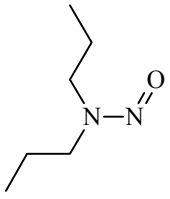
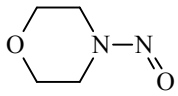
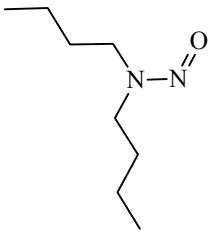
Secondly, formation studies have focused mainly on one NDMA precursor: DMA. This is, of course, necessary when deriving a reaction pathway for formation (Figure 5.1). However, to understand DBP formation, the complexity of the starting source water must be addressed. DMA is available in source waters, but not at large concentrations. Studies have indicated that free DMA makes up only a small part of total NDMA precursors [15, 16]. This raises the question whether the mechanistic studies truly reflect NDMA formation in drinking water. In some cases, a DMA moiety is incorporated into a larger structure which can be released into the water to react with the disinfectant [17, 18]. Another complicating factor is that the mix of precursors available in source water changes between different source waters. This can be further affected if the source water is impacted by anthropogenic sources. Wastewater effluents in particular have been shown to greatly increase nitrosamine concentrations in some locations. Therefore, while DMA is an ideal precursor for modeling purposes, it

likely does not represent the vast number of reactions occurring during water disinfection that produce NDMA.

Thirdly, studies investigating nitrosamine formation tend to focus on one precursor or one disinfectant type. Therefore, we know that certain precursors will form nitrosamines from chloramination, but we do not know what the effect of other disinfectants on the same precursors might be. A good example of this is the study investigating NDMA formation from ozonation of N,N-dimethylsulfamide mentioned earlier [10].

To address some of these issues, we set out to investigate the changes in nitrosamine formation when the same source water is disinfected with 11 different disinfectant combinations in parallel. This will allow the evaluation of the effect of different disinfectants on nitrosamine formation while maintaining the same precursor composition. In addition, it will allow the evaluation of the formation of eight nitrosamines other than NDMA (Table 5.1) by using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed in Chapter 2. We are particularly interested in the formation of the newly identified nitrosamine DBP, N-nitrosodiphenylamine (NDPhA).

Table 5.1: Structures of the nine nitrosamines included in this study

Nitrosamine	Abbrev.	Molecular formula	Structure
N-nitrosodimethylamine	NDMA	$C_2H_6N_2O$	
N-nitrosopyrrolidine	NPyr	$C_4H_8N_2O$	
N-nitrosopiperidine	NPip	$C_5H_{10}N_2O$	
N-nitrosodiphenylamine	NDPhA	$C_{12}H_{10}N_2O$	
N-nitrosodiethylamine	NDEA	$C_4H_{10}N_2O$	
N-nitrosomethylethylamine	NMEA	$C_3H_8N_2O$	
N-nitrosodipropylamine	NDPA	$C_6H_{14}N_2O$	
N-nitrosomorpholine	NMor	$C_4H_6N_2O$	
N-nitrosodibutylamine	NDBA	$C_8H_{18}N_2O$	

5.2 Materials and Methods

5.2.1 Formation of nine nitrosamines during disinfection of seven different source waters

5.2.1.1 Chemicals and materials

A standard solution containing 10 μg/mL each of N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosopyrrolidine (NPyr), N-nitrosopiperidine (NPip), N-nitrosomorpholine (NMor), N-nitrosodiethylamine (NDEA), N-nitrosodi-n-propylamine (NDPA), N-nitrosodi-n-butylamine (NDBA) and N-nitrosodiphenylamine (NDPhA) was purchased from Supelco (Oakville, ON, Canada). Isotopically labeled standards, [6-H^2] N-nitrosodimethylamine (NDMA-d6, 98%) and [14-H^2] N-nitrosodi-n-propylamine (NDPA-d14, 98%), were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Methanol (AnalR grade) and dichloromethane (Omni-Solv grade) were purchased from VWR International (Mississauga, ON, Canada). Ammonium acetate (ACS Reagent grade) was obtained from Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were of analytical grade and obtained through Fisher Scientific (Nepean, ON, Canada) unless otherwise indicated. Optima grade water (Fisher Scientific) was used for all water blanks and spiking samples. The SPE packing materials, Ambersorb 572 (Rohm & Hass;, Philadelphia, PA, U.S.A.) and LiChrolut EN (Merck, Darmstadt, Germany), were obtained from Supelco and VWR International, respectively.

5.2.1.2 Source water collection and characterization

Source water was collected from seven locations in Canada and the United States between April and September 2006. Total organic carbon (TOC),

ultrafdviolet absorbance at 254 nm (UV A254), pH, turbidity, and color were measured (Table 5.2) for all source waters. TOC, turbidity and color analysis were performed according to standard methods [19]. Water samples were kept at 4 °C before and after disinfection treatment.

Table 5.2: Source water quality from seven different locations.

Location	Source water	TOC[a] (mg/L)	UV A254[b] (cm^{-1})	pH	Turbidity (NTU[c])	Color (TCU[d])
1	River	2.0	0.039	8.3	1.8	14
2	Lake	5.7	0.329	7.3	6.1	118
3	River	5.7	0.275	8.3	25	210
4	River	5.9	0.127	8.0	0.75	18
5	River	7.3	0.223	7.7	0.84	35
6	Lake	15.9	0.464	8.8	30	230
7	River	23.9	0.933	7.4	4.68	235

[a]Total organic carbon
[b]UV absorbance at 254 nm
[c]Nephelometric turbidity units
[d]True color unit

5.2.1.3 Disinfection treatments

Eleven disinfection treatments were used in this study: chlorine (OCl^-), chloramine (NH_2Cl), chlorine dioxide (ClO_2), ozone (O_3), ozone followed by chlorine (O_3/OCl^-), low pressure ultraviolet (LPUV), LPUV followed by chlorine (LPUV/OCl^-), medium pressure ultraviolet (MPUV), MPUV followed by chlorine (MPUV/OCl^-), advanced oxidative processes (AOP), which is a combination of hydrogen peroxide (H_2O_2) and MPUV, and AOP followed by chlorine (AOP/OCl^-) (Figure 5.2).

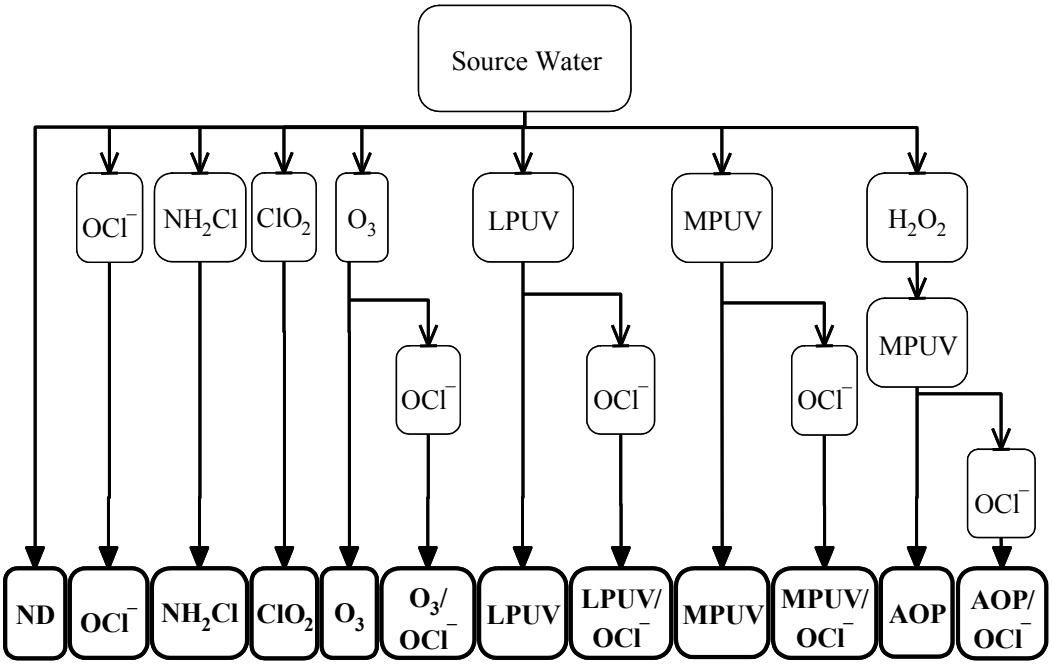


Figure 5.2: Schematic of disinfection treatment processes used in this study. ND: Not disinfected, OCl^-: Chlorination, NH_2Cl: chloramination, ClO_2: Chlorine dioxide, O_3: ozone, O_3/OCl^-: ozone followed by chlorine, LPUV: Low pressure ultraviolet; LPUV/OCl^-: LPUV followed by chlorine, MPUV: medium pressure ultraviolet, MPUV/OCl^-: MPUV followed by chlorine, AOP: Advanced oxidative processes, H_2O_2: hydrogen peroxide, AOP/OCl^-: AOP followed by chlorine.

Only clean and disinfectant demand-free glassware was used for these experiments. The disinfection protocol was designed to represent adverse scenarios in drinking water treatment with no coagulation pretreatment, high disinfectant doses and long contact times.

5.2.1.3.1 Disinfectant dosing

Demand for chemical disinfectants varies greatly depending on source water quality, which in turn affects the actual dose of disinfectant applied to water. Therefore, OCl^-, NH_2Cl and ClO_2 doses were based on maintaining a predetermined disinfectant residual concentration of 0.5 mg/L for OCl^-, 2.0 mg/L

for NH_2Cl and 0.5 mg/L for ClO_2 after 24 h contact time. The actual doses of these disinfectants applied to the source water are listed in Table 5.3.

Table 5.3: OCl^-, NH_2Cl and ClO_2 doses applied to each of the seven source waters

Location (source water)	Disinfectant Dose (mg/L)		
	Cl_2	NH_2Cl	ClO_2
1	2	3.5	2.5
2	12	7.5	8.5
3	9.5	3	8
4	6	4.5	7
5	8	3	6.5
6	25	6	11
7	33	10	20

Ozone, LPUV, MPUV and AOP do not leave disinfectant residuals and so were dosed the same for each of the source waters. The doses used were: O_3: 10 mg•min/L, LPUV: 100 mJ/cm^2, MPUV: 1000 mJ.(cm^2)$^{-1}$, AOP: MPUV, 1000 mJ.(cm^2)$^{-1}$ and H_2O_2,10 mg/L. For O_3/OCl^-, LPUV/OCl^-, MPUV/OCl^- and AOP/OCl^- treatments, the same OCl^- dose was used as for OCl^- only (Table 5.3). Raw water from location 3 was treated with OCl^-, NH_2Cl and ClO_2 only.

5.2.1.4 Disinfection protocols

5.2.1.4.1 Chlorine (OCl^-), chloramine (NH_2Cl) and chlorine dioxide (ClO_2)

For chlorination, a 40 ng/L stock solution of sodium hypochlorite (NaOCl) was prepared. Disinfection was performed in clean, chlorine demand-free glassware (Fisher Scientific). Immediately following OCl^- addition to the water sample, the free and combined chlorine residuals were measured using the N,N-diethyl-p-phenylenediamine (DPD) colorimetric standard method [19]. The

sample was then allowed to sit for 24 h. The chlorine residuals were measured again and then quenched with 75 mg/L sodium thiosulfate.

For chloramination, NH_2Cl stock solution (1000 mg/L) was prepared by mixing solutions of ammonium chloride (NH_4Cl) and NaOCl in a carbonate buffer (pH 9.4). Residual chloramine concentrations were determined by subtracting the combined chlorine residual from the free chlorine residual as measured using standard methods [19].

ClO_2 was generated by pumping a 25% NaOCl solution into an 18 N sulfuric acid (H_2SO_4) solution. The resulting gas was collected and pumped through a 15% NaOCl solution and then collected into ice-cold Milli-Q water to produce a ClO_2 solution. The ClO_2 residual was measured using U.S. EPA Method 327.0 (lissamine green B) [20]. NH_2Cl and ClO_2 residuals were quenched using 75 mg/L sodium thiosulfate.

5.2.1.4.2 Ozone (O_3) and ozone followed by chlorine (O_3/OCl^-)

Ozone gas, produced by an ozone generator, was bubbled directly through sample water in a semi-batch reactor. Ozone residual concentrations were monitored in samples taken from the semi-batch reactor until the dose of approximately 10 mg*min/L. Ozone residuals were measured using the indigo colorimetric standard method [19]. The contact time was the length of time required for the desired dose (10 mg*min/L) to be achieved. The molar absorptivity was 20,000 M^{-1} cm^{-1}. Residual ozone was displaced by bubbling oxygen through the sample water until the residual was confirmed to be below 0.05 mg/L. The O_3/OCl^- samples were further treated with OCl^-, using the same

dose as for OCl^- only samples (Table 5.3). OCl^- was allowed to react for 24 h, and then the residual was measured [19]. The residual was quenched with sodium thiosulfate.

5.2.1.4.3 Ultraviolet (UV) treatments

These experiments, utilizing low pressure UV (LPUV), LPUV followed by chlorine (LPUV/OCl^-), medium pressure UV (MPUV), and MPUV followed by chlorine (MPUV/OCl^-), were conducted using a collimated beam apparatus (Calgon Carbon Corporation; Pittsburgh, PA, U.S.A.). Aliquots of 250 mL of source water were treated using the appropriate dose (LPUV, 100 $mJ.(cm^2)^{-1}$; MPUV, 1000 $mJ.(cm^2)^{-1}$). UV contact time was determined by the source water absorbance spectrum and system dosing factors. LPUV/OCl^- and MPUV/OCl^- samples were further treated with OCl^-, using the dose applied to the OCl^- only samples (Table 5.3). OCl^- was allowed to react for 24 h. Then the residual was measured [19] and quenched with sodium thiosulfate.

5.2.1.4.4 Advanced oxidative processes (AOP)

AOP disinfection consisted of a combination of MPUV with hydrogen peroxide (H_2O_2) pretreatment. Ten mg/L H_2O_2 was added to each sample and the initial concentration measured using the I_3^- method [21]. The samples were irradiated using the MPUV procedure described above. The final H_2O_2 concentration was measured and 0.2 mg/L bovine catalase was added to quench residual H_2O_2 [22]. AOP/OCl^- samples were further treated with OCl^-, using the dose applied to the OCl^- only samples (Table 5.3). OCl^- was allowed to react for 24 h, then the residual was measured [19] and quenched with sodium thiosulfate.

5.2.1.5 Sample extraction, analysis and quantification

Prior to SPE extraction, the disinfected water samples were filtered through pre-baked (400 °C for 4 h) glass microfiber filters (GF/F; Whatman; particle retention >0.7 μm, 142 mm) to remove large particles and to avoid clogging of the SPE material. The SPE extraction of the filtered samples and LC-MS/MS analysis of the extracts was described in Chapter 2. Briefly, 500 mL of a water sample, spiked with 40 ng/L of NDMA-d6, was passed through a hand-packed SPE cartridge packed with 350 mg Lichrolut EN (bottom layer), 500 mg of Ambersorb 572 (middle), and glass wool (top). The SPE materials were air-dried and the nitrosamines eluted using 15 mL of dichloromethane. The eluent was further concentrated to approx. 200 μL under high purity nitrogen gas in a 40°C water bath after which the internal standard NDPA-d14 (40 ng/L) was spiked in. The extract was stored at 4°C until analysis.

Samples were analyzed using an Agilent 1100 capillary liquid chromatograph (Agilent Technologies, Palo Alto, CA, U.S.A.) coupled with an API 4000 QTrap mass spectrometer (ABSciex, Concord, ON, Canada). Positive electrospray ionization combined with the multiple-reaction monitoring (MRM) mode was used to analyze the samples. A C8 capillary column (150 x 0.32 mm i.d., 3 μm) (Phenomenex, Torrance, CA, U.S.A.) was used for separation. The sample injection volume was 1.2 μL. The optimal ionspray parameters were: curtain gas (N_2) at 10, ion-source gas 1 at 13, and ionspray voltage at 4500 V. The declustering potential (DP), collision energy (CE), and cell exit potential (CXP) were the same as in Chapter 2

Quantification was performed using NDPhA-d14 as the internal standard. Standard solutions of 5–200 µg/L of the nine target nitrosamines plus NDPA-d14 and NDMA-d6 were analyzed to determine the relative response factors (RRFs) for each nitrosamine. RRFs for the nine target nitrosamines and NDMA-d6 were calculated based on the ratio of peak area of individual nitrosamines to that of NDPA-d14.

The method detection limits (MDLs) and limits of quantification (LOQs) are shown in Table 5.4. The MDL and LOQ were calculated for each nitrosamine based on the analysis of seven independently prepared 10 ng/L laboratory fortified blanks. The MDL is defined as 3 times the standard deviation calculated from the seven replicate 10 ng/L laboratory fortified blanks, while the LOQ is defined as 10 times the standard deviation

Table 5.4: MRM ion pairs, method detection limits and limit of quantification for the nine nitrosamine method

Nitrosamine	MRM ion pair	MDL[a] (ng/L)	LOQ[b] (ng/L)
NDMA	75/43	2.4	8.1
NMEA	89/61	0.4	1.2
NPyr	101/55	1.5	4.9
NDEA	103/75	2.6	8.6
NPip	115/69	0.6	2.1
NMor	117/87	0.7	2.3
NDPA	131/89	0.3	1.0
NDBA	159/103	0.7	2.2
NDPhA	199/169	0.06	0.22
NDPA-d14	145/97	N/A[c]	N/A
NDMA-d6	81/46		

[a]Method detection limit
[b]Limit of quantification
[c]Not applicable

5.2.2 Investigation of NDPhA formation

Standard solutions of DPhA and NDPhA were obtained from Supelco (Oakville, ON, Canada). Isotopically labeled standards, NDPhA-d6 (98%) and DPhA-d6 (98%), were obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.) and C/D/N Isotopes (Pointe-Claire, PQ, Canada), respectively. Formic acid (50% solution), ammonium chloride, sodium hypochlorite solution (available chlorine 10–15 %), L-ascorbic acid, potassium dihydrogen phosphate and sodium hydroxide were ACS reagent grade or higher and purchased from Sigma-Aldrich (Oakville, ON, Canada). All reaction solutions were prepared using deionized water produced from a Millipore (Billerica, MA, U.S.A.) water purifying system.

5.2.2.1 SPE method

Oasis HLB cartridges (Waters, Mississauga, ON, Canada) were used to extract NDPhA and DPhA from water samples. Each packed SPE cartridge was initially rinsed with 10 mL each of dichloromethane, methanol and water. One g of sodium bicarbonate was added to 500 mL of the water sample (~pH 8). DPhA-d6 (100 µL of 50 µg/L) was then spiked into the sample and used as the surrogate standard to assess sample loss during extraction. The 500 mL water sample was then passed through the SPE cartridge at a flow rate of 4–6 mL/min using a vacuum system. The analytes absorbed on the SPE cartridge were eluted using 10 mL of dichloromethane. The organic eluent was collected and concentrated down to 200 µL under a high purity nitrogen stream in a 40 °C water bath. After concentration, NDPhA-d6 (100 µL of 50 µg/L) internal standard was added to the

extract prior to the LC-MS/MS analysis and was used for quantification. The recoveries for NDPhA and DPhA are shown in Table 5.5.

Table 5.5: MS parameters, limits of detection (LODs) and solid phase extraction recoveries for the NDPhA/DPhA specific method

	MRM transitions	DP	CE	CXP	Recovery ± SD (%)	LOD (µg/L)
NDPhA	199/169	56	17	10	111 ± 5	0.10
	199/66	56	49	10		
DPhA	170/93	66	35	6	94 ± 3	0.05
	170/65	76	47	10		

5.2.2.2 LC-MS/MS analysis

An Agilent 1100 HPLC (Palo Alto, CA, U.S.A.) was coupled directly to a QTrap 4000 mass spectrometer (AB Sciex, Concord, ON, Canada) with an ionspray ionization source. A Phenomenex Luna C8(2) column (100 x 2.0 mm i.d., 3 µm; Torrance, CA, U.S.A.) was used for separation. The mobile phase was composed of solvent A (0.1% formic acid in water) and solvent B (100% methanol) with a flow rate of 0.15 mL/min. The solvent gradient program consisted of increasing solvent B from 60% to 90% over 15 min, and returning back to 60% of solvent B over 0.1 min, followed by a 10 min re-equilibration prior to the next sample injection. The sample injection volume was 10 µL. Positive electrospray ionization combined with MRM mode was used for the analysis of DPhA and NDPhA. The selected MRM transitions, optimized MS conditions and instrument detection limits for NDPhA and DPhA are shown in Table 5.5.

5.2.2.3 Preparation of chloramine solutions

Stock solutions of chloramines were freshly prepared daily as previously described [2, 3, 6]. Briefly, ammonium chloride was dissolved in deionized water and adjusted to pH > 9.0 with sodium hydroxide. Then sodium hypochlorite was added slowly to a rapidly stirred solution until a Cl/N molar ratio of 0.7:1 was reached, at which monochloramine (NH_2Cl) is the predominant species in solution [23]. The pH was maintained above 9.0 to minimize the disproportionation of monochloramine (NH_2Cl) to dichloramine ($NHCl_2$). Concentrations of chloramines in stock solutions were standardized using the DPD ferrous titrimetric method [19].

5.2.2.4 NDPhA formation reactions

All glassware used in these experiments was rinsed with dichloromethane and baked at 200 °C for at least 12 h prior to use. Reactions were conducted at room temperature in 500 mL sealed amber bottles. Unless otherwise specified, all reaction solutions were buffered with the mixture of 10 mM potassium dihydrogen phosphate and sodium hydroxide (pH = 6.8). A predetermined amount of DPhA stock solution was added and fully dissolved into 500 mL of buffered Optima water. The reaction was initiated with the addition of chloramine solution. An aliquot of 500 mL of Optima water was also processed according to this procedure to serve as a control blank. During the reaction process, 0.5 mL of the reaction solutions was taken and mixed with internal standards at specified time intervals to directly measure the concentrations of DPhA and NDPhA by LC-MS/MS.

5.3 Results and Discussion

5.3.1 Nitrosamine formation from disinfection of seven source waters with 11 different disinfection treatments

This study investigated the formation of nitrosamines, with particular interest in NDPhA, when source water was disinfected without addition of any known precursors. Each of the seven representative source waters collected was treated with 11 different disinfection treatments in parallel, allowing for the comparison of nitrosamine formation during different disinfection processes in the same source water.

The seven locations were numbered in order from lowest to highest TOC, which ranged from 2.0–23.9 mg/L (Table 5.2). UV A254 readings ranged from 0.039 to 0.933 cm^{-1} (Table 5.2). Locations 3, 6 and 7 had increased values for UV A254, turbidity and color despite their TOC values.

NDMA, NMEA, NMor and NDPhA were detected above the MDL in some of the disinfected samples of the source waters from locations 2, 3, 4, 5, 6 and 7. No nitrosamines were detected in any of the treated samples of the source water from location 1. Location 1 also had the lowest concentrations of TOC and UV A254, which may partially explain why no nitrosamines were formed after disinfection of this source water. In keeping with this idea, location 7 had the highest values of TOC and UV A254 and the greatest number of nitrosamine species formed. However, this study did not find a significant relationship between NDMA formation and either TOC or UV A254.

Figures 5.3 and 5.4 show the formation of NDMA with different disinfection treatments. NDMA was the most frequently detected nitrosamine

with the highest concentrations. NDMA was also detected in untreated source waters from locations 2, 3, 4, 6 and 7, indicating that its presence is frequent but not entirely due to disinfection treatment. NDMA concentrations ranged from <MDL–53.5 ng/L in raw water and <MDL–118.1 ng/L in disinfected water.

NMor was detected in O_3 and O_3/ClO^- disinfected water from location 6. NDPhA was detected in ClO^- and NH_2Cl treated samples from location 3 and in O_3 and MPUV/ClO^- disinfected samples from location 7. NMEA was detected in ClO^- and MPUV/ClO^- disinfected samples from location 7. The highest concentrations of NMor, NMEA and NDPhA detected were 19.1, 0.55 and 0.23 ng/L, respectively. These results demonstrate that NDMA is the most frequently detected and has the highest concentration compared to other nitrosamines, suggesting that NDMA may serve as a surrogate for nitrosamine exposure assessment.

The effect of specific disinfection treatments on nitrosamine formation are discussed below.

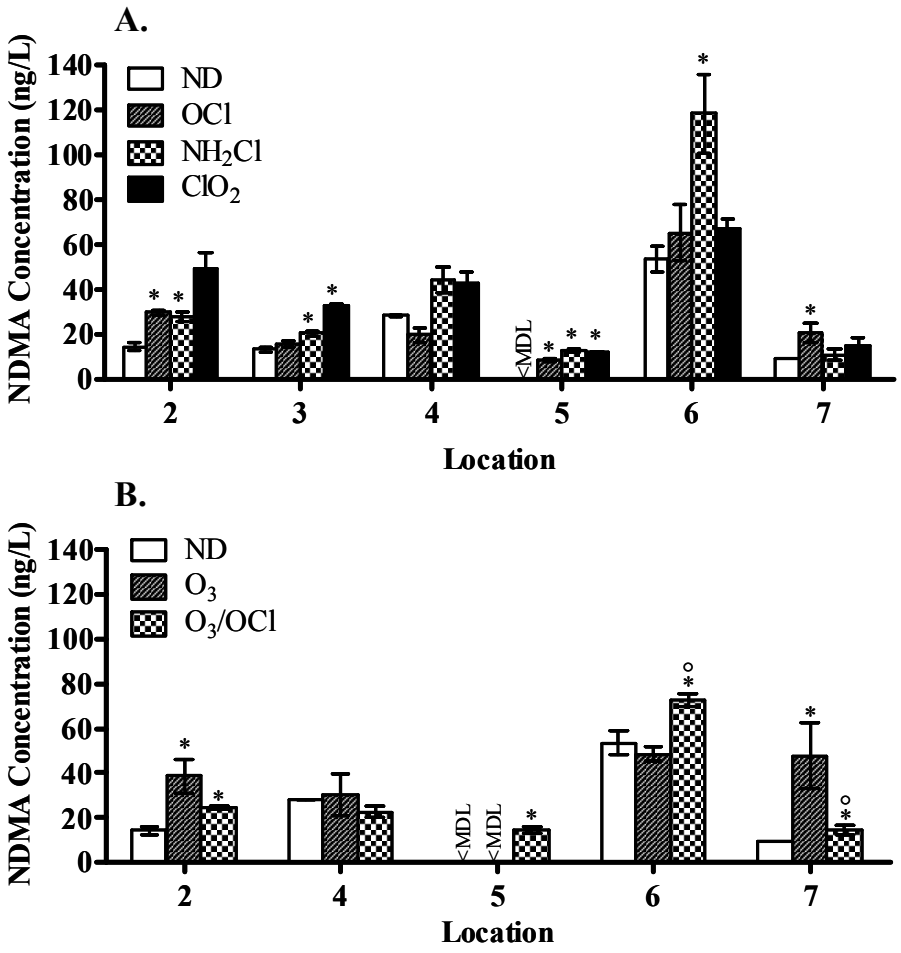


Figure 5.3: NDMA concentrations detected in 6 of 7 different source water samples treated with (A) OCl^-, NH_2Cl and ClO_2 and (B) with O_3 and O_3/OCl^-. ND = not disinfected. Error bars indicate standard error. <MDL indicates that NDMA concentrations for a particular treatment were not above the MDL. Statistical analysis was performed using a two-sided Mann-Whitney test at 95% confidence. * indicates statistical significance of a treatment compared to the control (ND). º indicates statistical significance of O_3/OCl^- disinfection compared to O_3. Statistical analysis was not performed on water from location 4 due to sample loss of the ND sample.

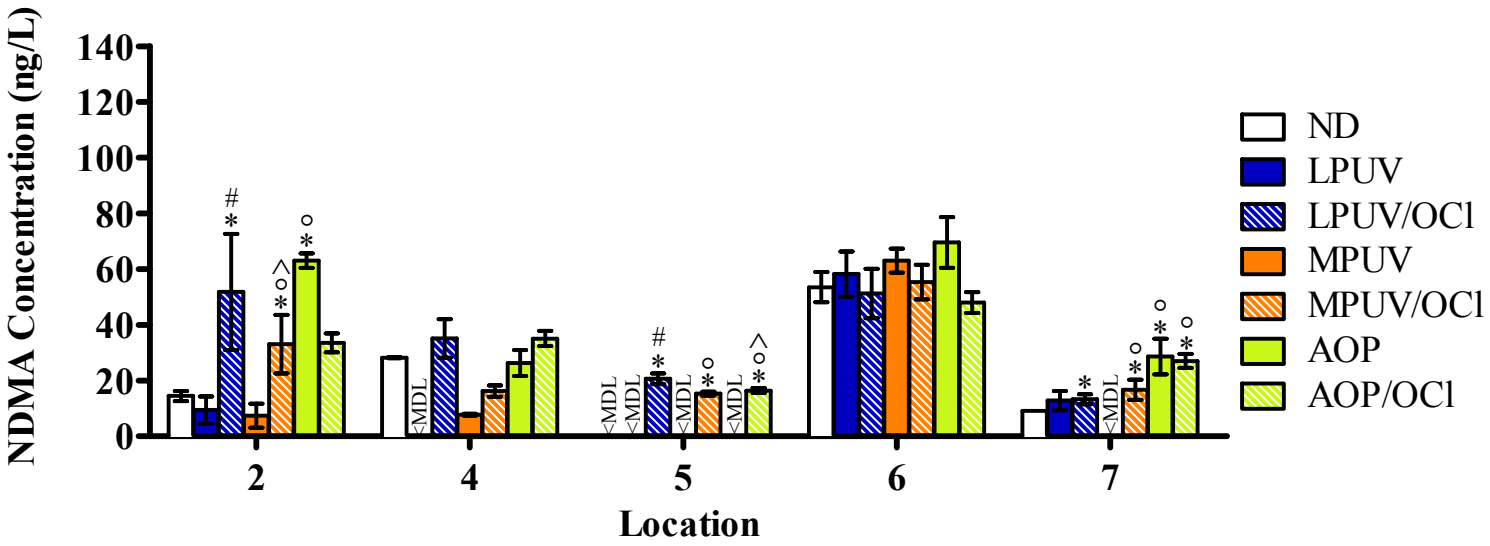


Figure 5.4: NDMA concentrations detected in 5 of 7 different source water samples treated with LPUV, LPUV/OCl⁻, MPUV, MPUV/OCl⁻, AOP and AOP/OCl⁻. ND = Not disinfected. Error bars indicate standard error. <MDL indicates that NDMA concentrations for a particular treatment were not above the MDL. Statistical analysis was performed using a two-sided two sample Mann-Whitney test at 95% confidence. * indicates statistical significance of a treatment compared to the control (ND); # indicates statistical significance of LPUV/OCl⁻ compared to LPUV; º indicates statistical significance of MPUV/OCl⁻, AOP or AOP/OCl⁻ compared to MPUV; ^ indicates statistical significance of AOP/OCl⁻ compared to AOP. Statistical analysis was not performed on water from location 4 due to some sample loss of the ND sample.

5.3.2 OCl^-, NH_2Cl and ClO_2

Figure 5.3A shows that chloraminated (NH_2Cl) source water from locations 2, 3, 4, 5 and 6 had higher concentrations of NDMA compared to the untreated source water (ND). Chlorination (OCl^-) also produced more NDMA compared to the untreated source water from locations 2, 5 and 7. Source waters from locations 2 and 5 produced similar amounts of NDMA after treatment with either OCl^- or NH_2Cl. The highest concentration of NDMA was produced with NH_2Cl disinfection of source water 6. In general, these results are consistent with previous reports that chloramination may produce more NDMA than chlorination [8].

It is interesting that ClO_2 treatment of source water from locations 3, 4, and 5 produced more NDMA compared to the untreated source waters, agreeing with the previous observation that ClO_2 treatment of reagent water containing DMA and ammonia formed NDMA [24]. Another study found that, in the absence of ammonia, ClO_2 pretreatment before chloramination reduced NDMA formation potential in reagent water containing certain NDMA precursors and in source water containing precursors of unknown identity [25]. However, this study also showed that the NDMA formation potential was not reduced when DMA and dimethylforamide (DMFA) were used as precursors, demonstrating that ClO_2 pretreatment may not remove all precursors with the same efficiency. Interestingly, ClO_2 pretreatment of water containing some tertiary amines, including trimethylamine (TMA), 3-(dimethylaminomethyl)indole (DMAI), and 4-dimethylaminoantipyrine (DMAP), resulted in an increase in DMA

concentration, suggesting that ClO_2 pretreatment can produce DMA, the NDMA precursor [25]. Our results are consistent with previous findings [24, 25] that ClO_2 can produce NDMA. Because the nature of precursors in source waters is usually not known, the effectiveness of ClO_2 to remove nitrosamines may require optimization according to source water quality.

NMEA was also detected in the OCl^- treated samples from source water 7, but was below the limit of quantification. OCl^- and NH_2Cl treated samples of source water 3 had NDPhA concentrations of <LOQ and 0.25 ± 0.05 ng/L, respectively.

5.3.3 O_3 and O_3/OCl^-

O_3 treatment of source waters 2 and 7 produced higher concentrations of NDMA compared to the untreated source waters (Figure 5.3B), agreeing with some recent results that NDMA is produced from the reaction of ozone with DMA, especially at higher pH [9]. O_3 treatment of source waters 5 and 6 did not produce NDMA, whereas the combination of O_3 treatment with OCl^- (O_3/OCl^-) produced significantly higher concentrations of NDMA compared to the untreated source waters 5 and 6 (Figure 5.3B). Furthermore, O_3/OCl^- treatment of source waters 5 and 6 produced higher concentrations of NDMA over O_3 treatment alone. These results suggest that O_3 oxidation of natural organic matter may release NDMA precursors in the source water, which may produce NDMA during OCl^- treatment. This is consistent with the reported observation that O_3 pretreatment can release DMA from NDMA precursors dimethylaminobenzene (DMAB), 3-(dimethylaminomethyl)indole (DMAI), and 4-

dimethylaminoantipyrine (DMAP) [25]. Source water from location 7 was interesting because O_3 disinfection produced higher amount of NDMA compared to O_3/ClO^- treatment. This may reflect the difference in precursors available in this source water and their reactivity with O_3 and OCl^-.

One previous study found that O_3 disinfection did not result in NDMA formation [8], but concluded that more work was required to confirm this result. Kinetic data has suggested that O_3 treatment should reduce NDMA formation by oxidizing any secondary amines present in source water [26]. This model relies on the assumption that O_3 treatment removes DMA by completely mineralizing the secondary amine, which is not always the case [9]. As such, recent experimental results have shown NDMA formation resulting from O_3 treatment of DMA containing water [9] and from combined O_3/chloramination treatment of water dosed with known tertiary amines [25]. Similarly to ClO_2 treatment, the efficiency of O_3 treatment for reducing/eliminating NDMA formation is influenced by the nature of the precursors in the source water. Our results and others [25] demonstrate that O_3 may produce precursors for NDMA under some conditions.

O_3 and O_3/OCl^- treated samples of source water 6 also produced NMor at concentrations (± SE) of 12.3 ± 0.2 and 19.1 ± 0.1 ng/L, respectively. This is consistent with previous results showing the formation of NDEA and NMEA resulting from O_3 treatment of reagent water containing the respective secondary amine [9]. These results demonstrate the importance of evaluating the formation of other nitrosamines during disinfection, because of the mixed organic compounds in source water.

5.3.4 Ultraviolet (UV)

Various types of UV radiation, including LPUV [14], MPUV [13] and pulsed UV techniques [11], have been shown to be a potentially useful technique for removal of nitrosamines from water. This is observed in our MPUV treatment of source waters 2, 4, and 7 (Figure 5.4). UV irradiation may also degrade natural organic matter and anthropogenic organic contaminants in the source water. DMA is one of the main UV degradation products of NDMA; however, DMA itself is not broken down by UV [13]. This suggests that UV degradation products of organic compounds containing DMA may serve as precursors for NDMA formation. Here, we investigated the formation of nitrosamines when source waters were treated with UV followed by OCl^-. Figure 5.4 shows that more NDMA was produced in the samples of source waters 2, 4, and 5 treated by LPUV/OCl^- than with LPUV alone. Similar results were obtained when source waters 2, 4, 5, and 7 were treated with MPUV/OCl^- compared to MPUV alone (Figure 5.4). This effect has also been observed from the treatment of pulsed UV irradiation followed by OCl^- for groundwater and NDMA spiked reagent water [11]. These results demonstrate that UV degradation products may serve as precursors that can form NDMA during subsequent chlorination steps. In addition, MPUV/OCl^- treatment of source water 7 also produced NMEA (<LOQ) and NDPhA (0.46 ± 0.08 ng/L). Source water 7 had the highest TOC values of the source waters studied (Table 5.2). These results suggest that the addition of chlorine to maintain a disinfectant residual following UV should be optimized to avoid nitrosamine formation, especially in cases of source water with high TOC.

5.3.5 Advanced oxidative processes (AOP)

AOP involves an oxidation (and sometimes also a reduction) step followed by UV irradiation. In the present study, AOP treatment consisted of H_2O_2 pretreatment followed by MPUV irradiation. The idea behind H_2O_2 pretreatment is to degrade nitrosamines and oxidize their precursors to reduce nitrosamine formation in subsequent disinfection steps. No NDMA was detected in the samples of source water 1 with or without AOP or AOP/ClO^- treatments, which could be explained by its low TOC. AOP treatment of the source waters 2 and 7 produced higher concentrations of NDMA compared to the non-treated source waters (Figure 5.4), whereas AOP did not significantly change NDMA concentration in source waters 4 and 6. Compared to MPUV alone, AOP treatment of the source waters 2 and 7 produced significantly higher amounts of NDMA over the untreated water. This implies that the H_2O_2 pretreatment may affect NDMA formation or breakdown during subsequent UV irradiation.

Similarly to the LPUV and MPUV combined with ClO^- treatments, the effect of chlorination following AOP treatment was also investigated. Prior to any treatment, no NDMA was detected in source water 5. AOP/OCl^- treatment of source water 5 clearly showed formation of NDMA (Figure 5.4). Increase of NDMA formation with AOP/ClO- was also observed in source water 4, but this was not observed in other samples. NDMA formation during AOP or AOP/ClO^- has not been studied in natural water. A previous AOP study of reagent water spiked with NDMA looked at combined oxidation and reduction steps of AOP (using 60Co irradiation prior to UV treatment), and the results indicated that this technology was likely to be most effective in water with low concentrations of

natural organic matter [12]. The different patterns of NDMA formation during AOP or AOP/ClO^- treatment of different source waters also indicate that natural organic matters and anthropogenic organic contaminants in the source water may affect NDMA removal by AOP or UV technologies. More research is needed for better understanding of AOP technologies for removal of nitrosamines.

The effect of source water quality on nitrosamine formation is clearly observed in the results described above. As in real water treatment plant situations, the identities and concentrations of organic precursors in source water are not known. TOC, UV A254, pH, turbidity, and color are common water quality parameters assessed by water treatment plants and were determined for all seven source waters in this study (Table 5.2). These parameters can affect nitrosamine formation [3, 5, 7, 9]; however, none of these parameters correlate with nitrosamine formation, likely because they do not adequately represent the chemical structures and properties of precursors. This suggests that determination of specific nitrosamine precursors may be more useful than relying on general water quality parameters.

5.3.6 Investigation of NDPhA formation

The previous results clearly show that NDPhA does not form as frequently as NDMA. However, it was the second most commonly detected nitrosamine in the above study and in the occurrence survey presented in Chapter 3. In addition, *in vitro* cytotoxicity testing showed that it is more cytotoxic than NDMA (Chapter 4). Therefore, we decided to focus on NDPhA formation separately from the other nitrosamines. Based on the structure of NDPhA (Table 5.1) and because

secondary amines have been previously shown to be nitrosamine precursors, we propose diphenylamine (DPhA) as a likely precursor of NDPhA from water chloramination.

5.3.6.1 NDPhA method development

In order to sensitively assess NDPhA and DPhA in water samples, we developed a new SPE-LC-MS/MS method specific for these analytes. The decision to develop a new method instead of using the nine nitrosamine method developed in Chapter 2 was due to a couple of factors. As method parameters had to be developed for DPhA, this provided an opportunity to improve NDPhA recoveries in the SPE step. NDPhA recoveries in the nine nitrosamine method were 56%, which was likely due to the SPE phases for the nine nitrosamine method being chosen to provide the highest NDMA recoveries. NDMA is typically the most difficult nitrosamine to extract from water due to its high hydrophilicity [27], but NDPhA is more hydrophobic meaning its retention on solid phase material will differ from that of NDMA.

The new method consisted of SPE using Oasis HLB cartridges, LC separation using a C8 column, followed by ESI-MS/MS detection using MRM. The HLB SPE cartridges provided DPhA recoveries of 94% and improved NDPhA recoveries to 111% (Table 5.5) which represented a substantial improvement over the nine nitrosamine method. MS conditions were optimized for NDPhA and DPhA (Table 5.5). NDPhA-d6 was chosen as the internal standard for sample quantification. The limits of detection were determined to be 0.05 and 0.10 µg/L for DPhA and NDPhA, respectively.

5.3.7 Identification of DPhA as a precursor of NDPhA

DPhA is likely to be a precursor for NDPhA based on their structures and on the results from other nitrosamine formation studies. DPhA is available in the environment as a precursor as it has been shown to be present in the environment as an environmental pollutant of surface and wastewater from its use as an insecticide, fruit preservative, and in the preparation of azo dyes, pharmaceuticals, rubber, and rocket fuel [28].

To confirm that environmental DPhA could act as an NDPhA precursor, we analyzed the raw water used by the drinking water treatment plant where we had detected NDPhA in the treated water in Chapter 2 [29]. DPhA was detected in the raw water at 1.3 ± 0.05 ng/L while no NDPhA was detected (Figure 5.5). When the raw water was treated with 1.0 mM of chloramines for 7 h, NDPhA was detected at a concentration of 0.37 ± 0.03 ng/L, while the DPhA concentration decreased to 0.37± 0.03 ng/L compared to the unchloraminated raw water.

The molar yield (Y_m) of NDPhA formation from DPhA in the raw water was calculated to be about 20% using Equation 5.1.

$$Y_m = C_{Nt}/(C_{D0} - C_{Dt}) \times 100\% \qquad \textbf{Equation 5.1}$$

where CD0 is the initial DPhA concentration (μM); CDt and CNt are the concentrations (μM) of DPhA and NDPhA in solution at a given reaction time (t), respectively. Previous studies that identified DMA as a precursor for NDMA showed a molar yield of <1% from DMA to NDMA [15, 30, 31]. Our results

suggest DPhA is converted more efficiently to NDPhA compared to the conversion of DMA to NDMA.

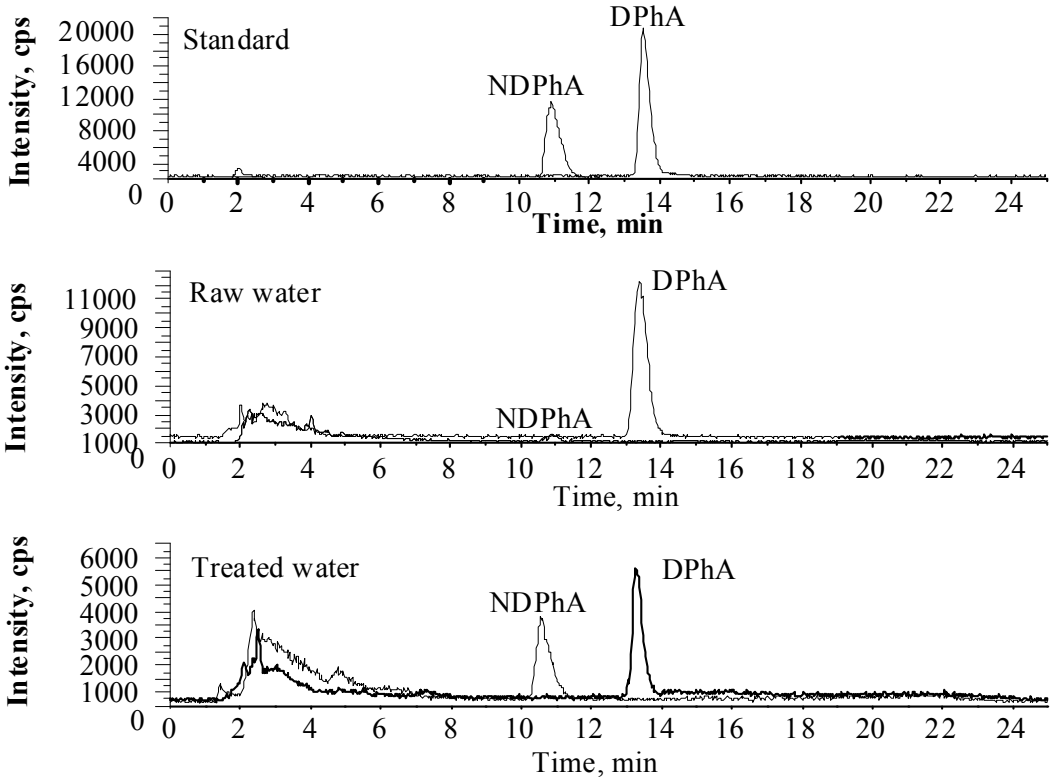


Figure 5.5: LC-MS/MS analysis of DPhA and NDPhA in standards, raw water, and chloraminated water.

5.3.8 NDPhA formation from DPhA during chloramination

To confirm that NDPhA forms from DPhA during chloramination, a series of experiments were performed in which DPhA was reacted with chloramines in laboratory grade water under highly controlled conditions. The first experiment involved reacting DPhA (1.0 μM) with chloramines (1.0 mM) to monitor the consumption of DPhA and formation of NDPhA over 14 h (Figure 5.6). Over the course of the experiment, DPhA levels gradually decreased coinciding with an

increase in NDPhA. After 10 h, 98% of the DPhA had been consumed producing a maximum NDPhA concentration of 0.15 μM.

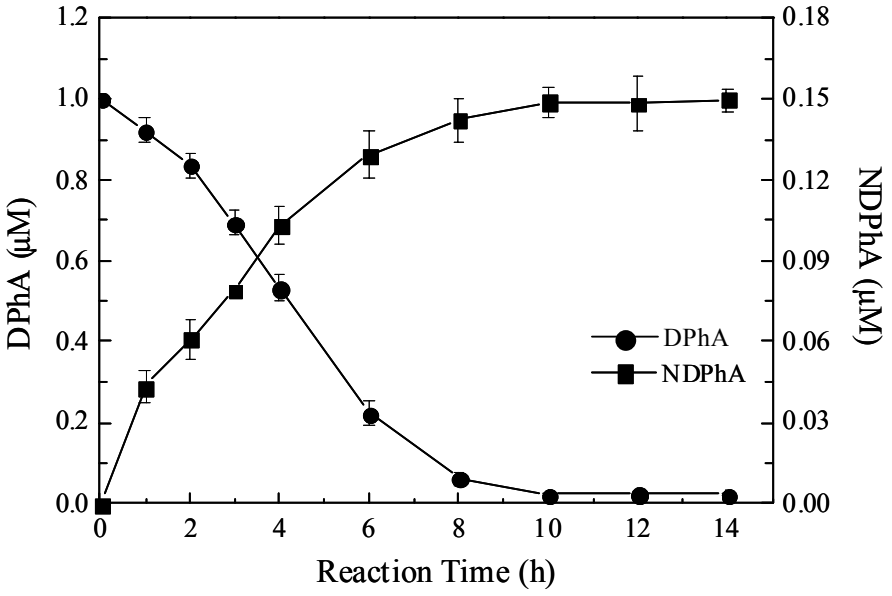


Figure 5.6: Time course of NDPhA formation from the reaction of 1.0 μM DPhA with 1.0 mM chloramines.

The molar yields from the data in Figure 5.6 ranged from 15–20% which is consistent with the molar yield calculation from the chloramination of raw water (Figure 5.5). Previous studies have determined that the molar yield of NDMA formed from DMA is less than 1% [15, 30, 31]. This is much lower than the molar yields we obtained from the formation of NDPhA from DPhA (15–20%). This difference may be attributed to pH effects and structure-dependent reactivity, because the pK_b values of DPhA and DMA are 13.8 and 3.3, respectively. DPhA (with aromatic groups) may be more reactive than DMA

(with methyl groups) with chloramines in water (around pH 7), resulting in higher yield of NDPhA from the secondary amine precursor.

5.3.9 Effect of initial DPhA and chloramine concentrations on NDPhA formation

The effect of DPhA concentration on NDPhA formation was studied by varying initial DPhA concentrations (from 0.02 to 1.0 μM) while maintaining a fixed concentration of chloramines (1.0 mM). As expected, a linear relationship between NDPhA formation and the initial DPhA concentration was observed (Figure 5.7) after 10 h of reaction time. The molar yields of NDPhA from DPhA range from 19% to 14% when the initial DPhA concentration increases from 0.02 to 1.0 μM.

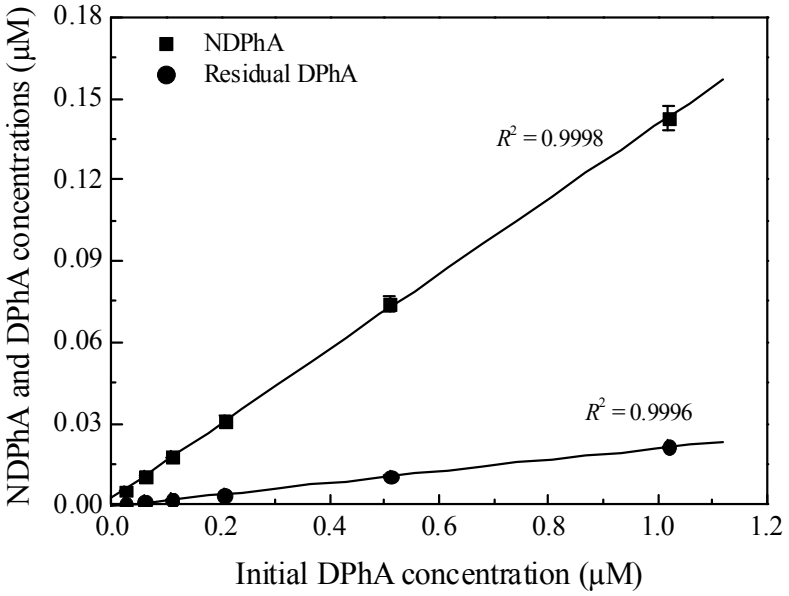


Figure 5.7: Effect of initial DPhA concentration on NDPhA formation and residual DPhA concentration.

The effect of initial chloramine concentration on NDPhA formation was also assessed. Varying chloramine concentrations (0.05 to 2.0 mM) were reacted with a fixed DPhA concentration (0.1 μM). Figure 5.8A shows that the rates of DPhA consumption increase with increasing initial chloramine concentration. Accordingly, the reaction time to reach equilibrium decreases from about 36 h to 4 h when the initial concentration of chloramines increases from 0.05 to 2.0 mM.

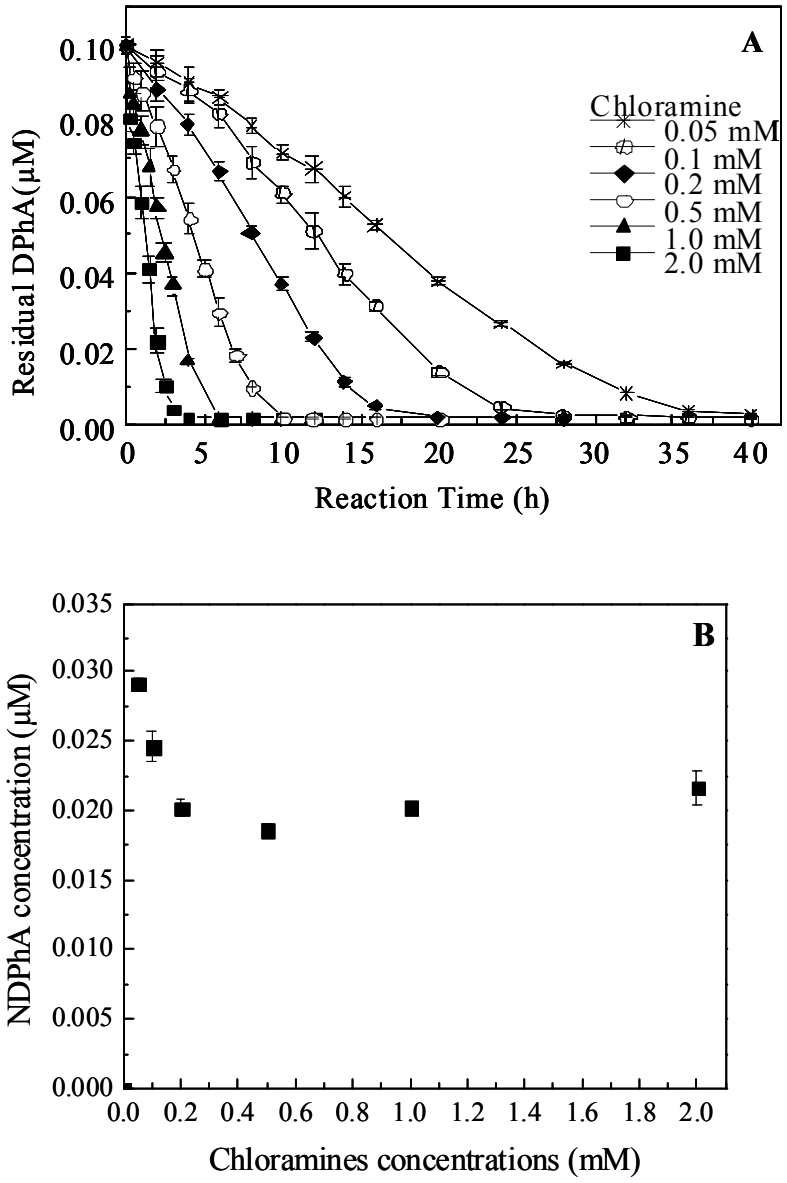


Figure 5.8: Effect of initial chloramine concentration on NDPhA formation from DPhA. (A) Residual DPhA concentration after reacting with different chloramine concentrations over time; (B) NDPhA concentrations produced at equilibrium from different initial chloramine concentrations.

The NDPhA concentrations formed during these reactions are shown in Figure 5.8B. The highest NDPhA concentrations were observed with initial

chloramine concentrations of 0.05 and 0.1 mM, corresponding to a 500- to 1000-fold molar excess of chloramines over the precursor DPhA. NDPhA concentrations did not increase significantly between initial chloramine concentrations of 0.1 to 2.0 mM. The molar yield of NDPhA remained between 15–19%. At pH 6.8, monochloramine is approximately 98% in the chloamines solution. Sufficient monochloramine in 0.1–2.0 mM solutions of chloramines to react with DPhA could explain why the yield of NDPhA remained stable.

5.3.10 Effect of chloramine speciation on NDPhA formation

In general, chloramines are obtained by mixing chlorine with ammonia. Monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3) are produced (Equations 5.2–5.5):

$Cl_2 + H_2O \rightarrow HOCl + HCl$ **Equation 5.2**

$HOCl + NH_3 \rightarrow NH_2Cl + H_2O$ **Equation 5.3**

$NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O$ **Equation 5.4**

$NHCl_2 + HOCl \rightarrow NCl_3 + H_2O$ **Equation 5.5**

The relative abundance of these species depends on the molar ratio of chlorine and ammonia (Cl:N) [23]. At Cl:N molar ratio < 1.5, both NH_2Cl and $NHCl_2$ coexist, while NH_2Cl is predominant at Cl:N < 1 and $NHCl_2$ is the major species at Cl:N from 1 to 1.5. At Cl:N molar ratio above 1.5, NCl_3 and free chlorine coexist. To study the effect of the Cl:N ratio on NDPhA formation from

DPhA, chloramine solutions were preformed prior to application to DPhA, as has been reported previously [5, 6].

Figure 5.9 shows NDPhA formation when a fixed concentration of DPhA (0.1 μM) reacts with chloramines (1.0 mM) prepared at varying Cl:N molar ratios after 12 h. NDPhA was barely detectable when DPhA was treated by chlorination (OCl^-) alone, without the addition of NH_3. NDPhA (>10 nM) was produced only by chloramination, albeit its concentration varied with different Cl:N ratios. The maximum concentration of NDPhA (20 nM, equal to a molar yield of 20%) was formed at the Cl:N molar ratio of 0.7:1, when NH_2Cl was predominant. This is different from the formation pathway that has been proposed for NDMA (Figure 5.1) where $NHCl_2$ is predicted to be most important [5, 6]. Therefore NDMA and NDPhA may have different mechanisms of formation.

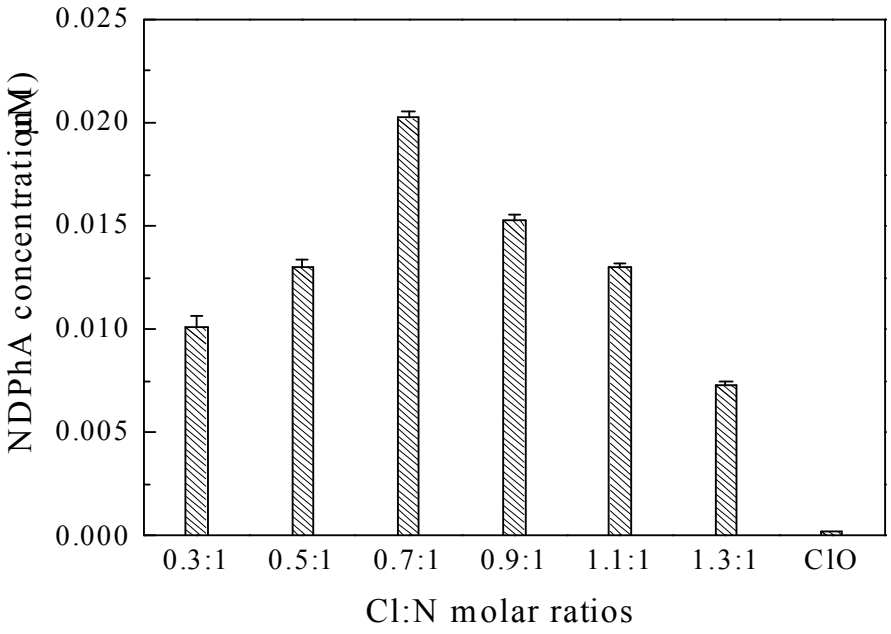


Figure 5.9: Effect of Cl:N ratio on NDPhA formation from DPhA (0.1μM) after 12 h.

5.3.11 Effect of pH on chloramination DBPs

The effect of solution pH on NDPhA formation was examined when DPhA (0.1 μM) was treated with 1.0 mM chloramines at different pH values. Figure 5.10 summarizes the concentrations of NDPhA and residual DPhA formed at equilibrium for pH 4.0–10.0. The amount of NDPhA formed increases dramatically with increasing pH; 64 times more NDPhA was produced at pH 10.0 than at pH 4.0. The molar yield of NDPhA from DPhA increased from about 1.4% to 90% over the same pH range.

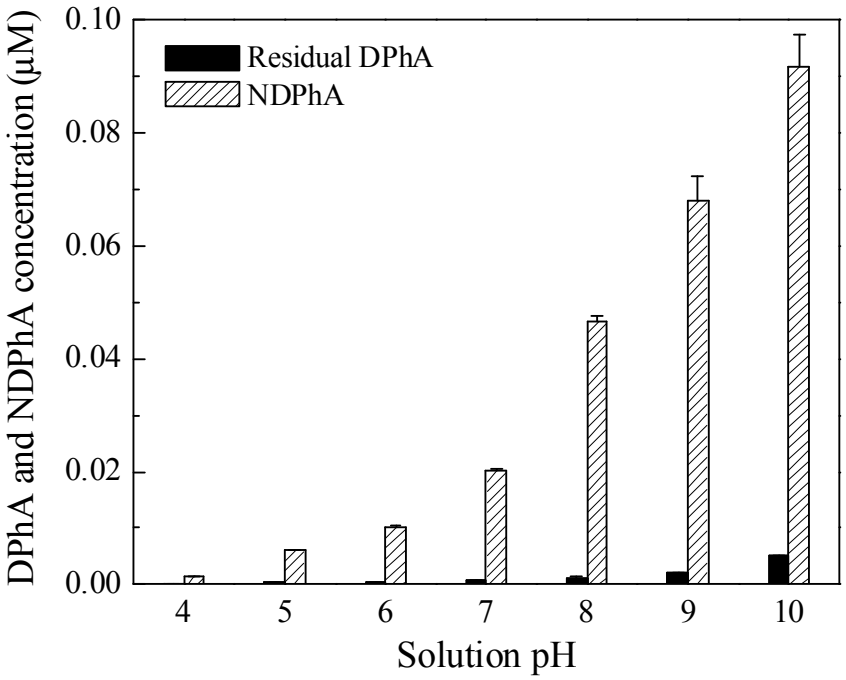


Figure 5.10: Effect of pH on NDPhA formation and residual DPhA concentration. DPhA (0.1 µM) was reacted with 1.0 mM chloramines at pH 4, 5, 6, 7, 8, 9, and 10 for 6 h.

The solution pH also affects chloramine speciation (Equations 5.2–5.5). Monochloramine is favoured at higher pH, while dichloramine is favoured at lower pH. Figures 5.9 and 5.10 show that the pH and concentration of monochloramine strongly impact NDPhA formation. At Cl:N 0.7:1, NDPhA formation can be reduced by 50% at pH 7 compared to pH 8. This could be due to monochloramine being more stable at higher pH which increases its chance to react with DPhA to produce more NDPhA.

Figure 5.10 also shows that the residual DPhA at equilibrium also increases when the pH is increased from 4.0 to 10.0. However, the removal of

DPhA was not equal to the corresponding formation of NDPhA at pH 4–8. DPhA and NDPhA in water at pH 4–10 were stable within the experimental period, thus the concentrations of DPhA and NDPhA in Figure 5.10 were not affected due to the stability of these compounds. The mass imbalance between DPhA removal and NDPhA formation suggests that other products may be formed during chloramination of DPhA in water.

5.3.12 Identification of new DBPs

To investigate other DBPs that may be produced from chloramination of DPhA, extracts of the reaction solutions (0.1 μM DPhA with 1.0 mM chloramines) at pH 6–8 were analyzed using full scan enhanced MS (EMS) (Figure 5.11) Two new peaks, A and B, were observed in the EMS total ion chromatogram in extracts from chloramination of DPhA at pH 7, in addition to NDPhA. These two peaks were not observed in blank samples (chloramination of pure water without DPhA). The product ion spectra of Peaks A and B are shown in Figure 5.11 (insert A and B). The molecular weight of 180 Da and the LC-MS/MS spectra suggest peak A is phenazine. GC-MS analysis of the same extract determined an exact match for phenazine with GC library databases. Further LC-MS/MS experiments using a phenazine standard confirmed that the retention time and mass spectrum of Peak A match those of the phenazine standard. These results support the identification of Peak A as phenazine.

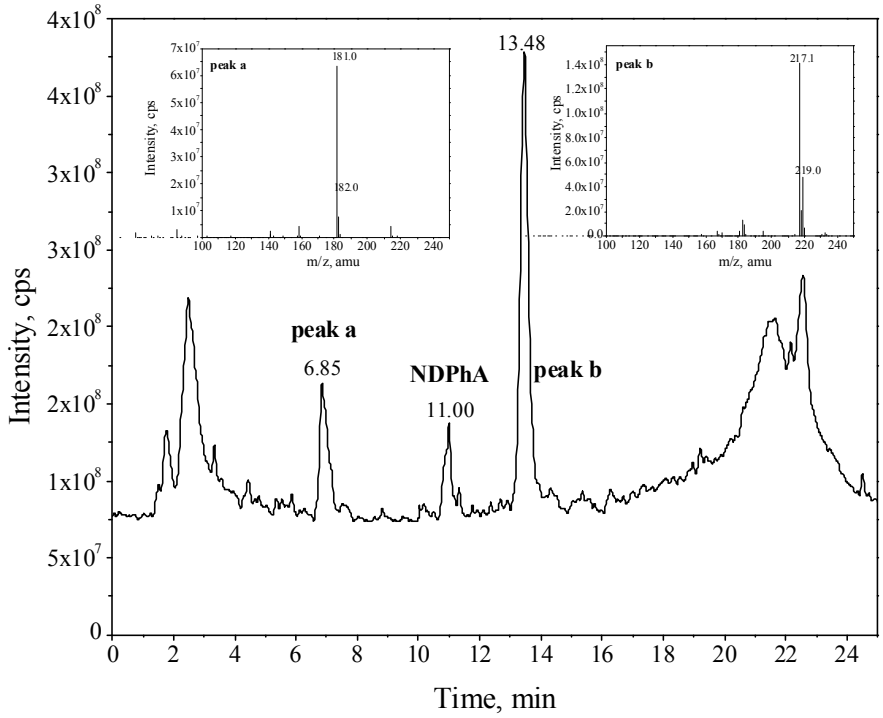


Figure 5.11: LC-MS total ion chromatogram of a chloraminated DPhA extract (pH 7). Inset A: EMS spectra of peak A. Inset B: EMS spectra of peak B. Sample prepared by reacting 0.1 µM DPhA with 1.0 mM chloramines at pH 7. After reaching equilibrium the reaction mixture was extracted and analyzed using LC-MS with enhanced scan mode.

The molecular ion of Peak B is m/z 216.8 ($[M+H]^+$) (Peak B, Figure 5.11), indicating the molecular weight is 216 Da and the molecular formula as $C_{12}N_2H_9Cl$. The major fragment ion at m/z 180.9 indicates a loss of 36 (HCl) from $[M+H]^+$ m/z 216.8 and this ion corresponds to the phenazine molecular ion $[M+H]^+$ (Peak A, Figure 5.11). This suggests that HCl is easily lost from peak B during MS/MS. We further examined whether the chlorine was situated on one of the nitrogens or on the phenazine ring itself. Analysis of Peak B using GC-MS did

not produce a spectrum match with 2-chlorophenazine in the NIST mass library, suggesting that Peak B is N-chlorophenazine. Further confirmation was not performed at this time because no standard is available and there is no GC-MS spectrum of N-chlorophenazine in the library. The peak at 2.3 min (Figure 5.11) was also detected in the blank, thus it was not further characterized.

Peaks A and B (phenazine and N-chlorophenazine) were then carefully examined using LC-MS/MS (MRM) mode. Figure 5.12 shows MRM chromatograms of the extracts of reaction mixtures of 1.0 mM chloramines with 0.1 μM DPhA at pH 6–9 after 5 h. At pH 6, Peak A and NDPhA are minor products and Peak B is predominant. At pH 7, NDPhA increases and Peak B decreases compared to pH 6. At pH 8, both NDPhA and Peak B are produced at similar concentration. At pH 9, NDPhA becomes predominant while Peak B is significantly reduced and Peak A is barely detected compared to pH 7. These results indicate pH-dependent DBP formation; therefore, different DBPs could be produced during chloramination depending on the pH of the water during treatment. Figure 5.12 also explains the mass imbalance observed in Figure 5.10.

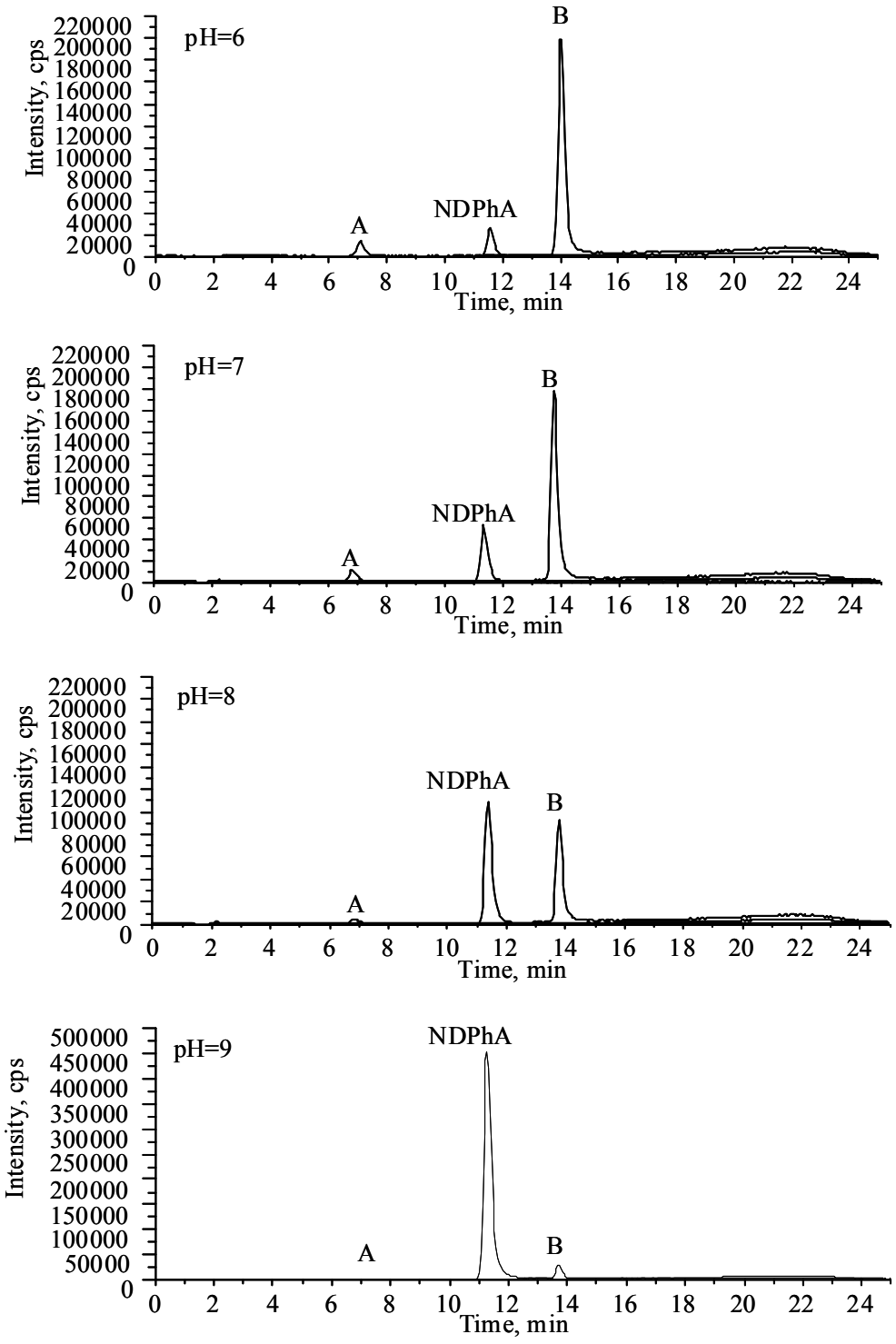


Figure 5.12: MRM chromatograms of Peaks A and B and NDPhA produced at pH 6–9. Sample prepared by reacting 0.1 µM DPhA with 1.0 mM chloramines at pH 6–9.

To confirm Peaks A and B as new DBPs, raw water used in Section 5.3.7 was treated with 1.0 mM chloramines for 12 h at pH 8.4 (without any buffer). Peak A and B are clearly detected in the treated water (Figure 5.13) but not in the source water, supporting phenazine and N-chlorophenazine as new N- and N-chloro DBPs, which have not been previously reported. The peak before Peak A was only detected in the treated source water and was not detected in reactions of DPhA with chloramines. This suggests that this peak likely results from other unknown precursor(s), not DPhA, warranting further investigation.

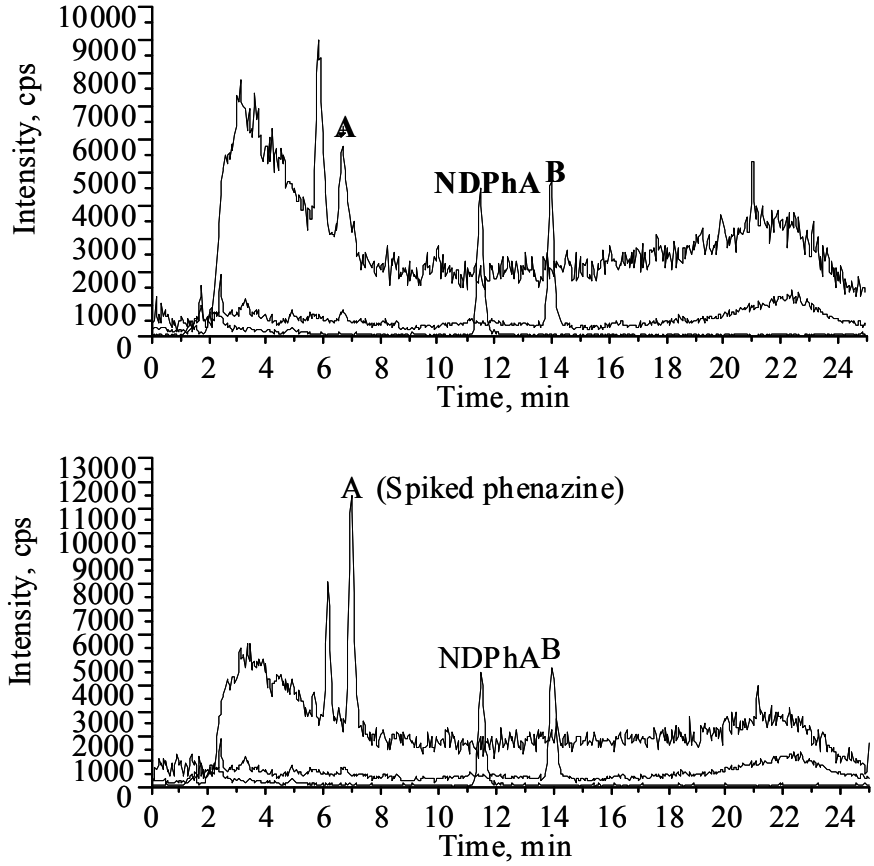


Figure 5.13: MRM chromatograms of NDPhA, Peak A (phenazine), and Peak B (N-chlorophenazine) in chloraminated raw water.

Based on this result, we propose a putative pathway for the production of NDPhA, phenazine and N-chlorophenazine from chloramination of DPhA (Figure 5.14). DPhA, through a number of possible intermediates, produces these three DBPs depending on the solution pH. At pH < 7, in addition to NDPhA, phenazine and its chlorinated derivative are produced. At pH>7, NDPhA formation significantly increases compared to acidic pH, while phenazine and N-chlorophenazine formation is much less. This pathway is consistent with the findings described above: 1) DPhA is a precursor; 2) NDPhA, phenazine, N-

chlorophenazine and other DBPs are formed after chloramination; and 3) water pH and chloramines are key factors in the formation of these DBPs.

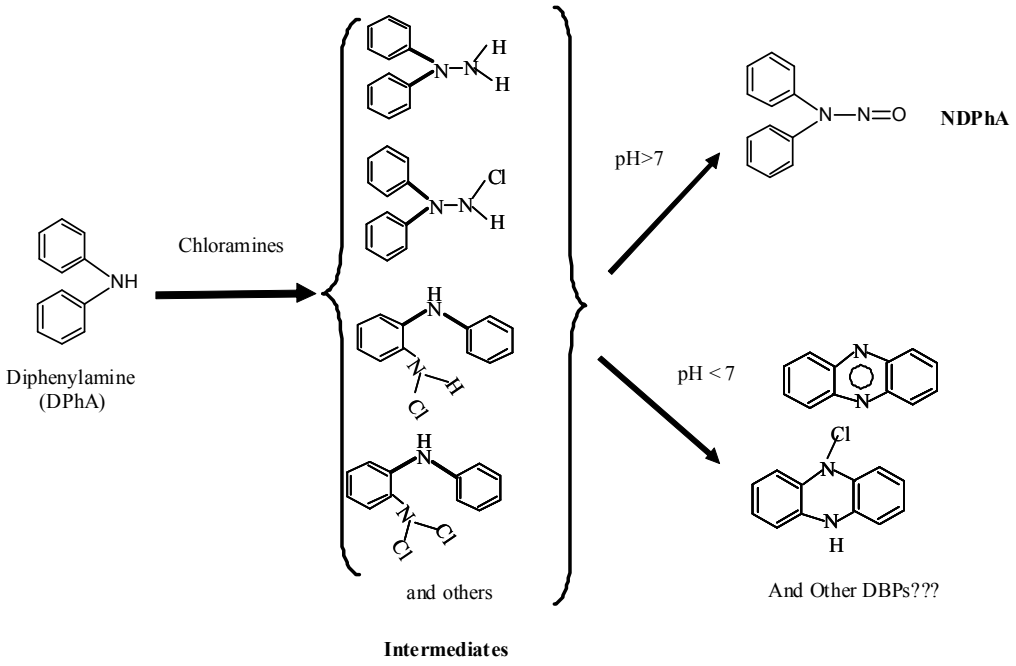


Figure 5.14: Putative pathways for the formation of NDPhA and other N-DBPs from chloramination of water containing DPhA

5.4 Conclusions

This study investigated several factors related to nitrosamine formation. The initial study aimed to systematically study the effect on nitrosamine formation from different disinfectants on the same source water. This study found that OCl^- and NH_2Cl can produce nitrosamines in the same source waters. However, NH_2Cl has the potential to produce much higher concentrations of nitrosamines than OCl^- alone, depending on the source water. Our results support previous observations that ClO_2 can also produce NDMA. UV removal of

nitrosamines is effective in source water with low turbidity and color. Subsequent addition of OCl^- following alternative treatments (UV or AOP or O_3) can increase NDMA concentration and lead to formation of other nitrosamines such as NMor and NDPhA. The formation of different nitrosamines in the source waters suggest that natural organic matter and/or anthropogenic contaminants may contain different subunits for formation of various nitrosamines; therefore, determination of the precursors of various nitrosamines in source water warrant further studies.

Secondly, we sought to better understand the formation of newly identified DBP, NDPhA, by performing chloramination experiments on its secondary amine, DPhA. This study demonstrated that DPhA can form NDPhA following chloramination at much higher molar yields than formation of NDMA from DMA. In addition, NDPhA formation is higher in conditions favouring monochloramines, which is different than NDMA. Finally, chloramination of DPhA can produce different intermediates depending on water pH, resulting in formation of different DBPs. Phenazine and N-chlorophenazine have never been reported as DBPs in drinking water and their occurrence toxicity and health effects are not known.

Taken together, one major theme can be found in these two studies. The quality of source water (and by extension, the type and concentration of precursors) is very important in determining DBP formation. General indicators of raw water quality are not sufficient to predict nitrosamine formation, even for NDMA. Therefore, ensuring the cleanliness of raw water prior to disinfection is one possible strategy to reduce DBP formation during water treatment. Changing

disinfection strategies has been shown to be ineffective in preventing DBP formation; it just changes the composition of DBPs formed. This was observed in both of our studies, in which different disinfection treatments could produce other nitrosamines such as NMor or NDPhA. Even in our study of NDPhA formation from DPhA, just changing the pH (not the disinfectant type) resulted in the promotion of two other DBPs, phenazine and N-chlorophenazine. Therefore, it is extremely important for water treatment plants to understand their source water and how it reacts with disinfectants and how that will change when water treatment processes are changed. Without this understanding, changes to the water treatment train in attempts to prevent formation of certain DBPs may result in the formation of other more toxic ones.

5.5 References

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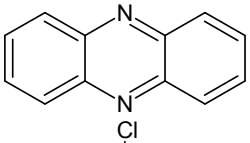
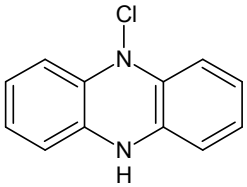
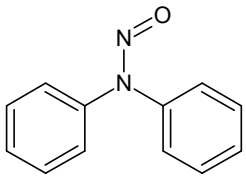
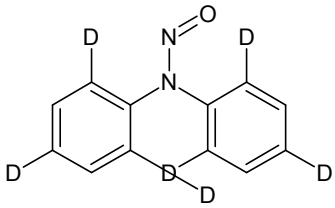
6 DISINFECTION BYPRODUCT FORMATION FROM BACTERIAL PRECURSORS

6.1 Introduction

In Chapter 5, we identified two new N-DBPs, phenazine and N-chlorophenazine (Table 6.1) [1]. Both of these DBPs form from the chloramination of diphenylamine (DPhA); based on their chemical structures, we hypothesize that phenazine containing chemicals should be ideal precursors for chloro-phenazine DBPs. Like DPhA, phenazine containing chemicals are readily available in the environment. Phenazines are produced commercially as dyes [2]. They are also secreted by certain bacterial species including *Pseudomonas* species, *Streptomyces* spp. and *Pantoea agglomerans* [3]. Pseudomonads are found both in the environment and in some distribution system biofilms [4]. This suggests that DBPs such as phenazine and N-chlorophenazine may use a phenazine precursor secreted by bacteria, thus indicating a bacterial source of DBP precursors.

Table 6.1: Structure, MRM transition and charged monoisotopic mass for phenazine, N-chlorophenazine, NDPhA and NDPhA-d6

Chemical	Structure	Formula	Charged monoisotopic mass (Da)
Phenazine		$C_{12}N_2H_8$	181.07602
N-chlorophenazine		$C_{12}N_2H_9Cl$	217.052702
N-nitrosodiphenylamine (NDPhA)		$C_{12}N_2H_{10}O$	199.08659
NDPhA-d6		$C_{12}H_4D_6N_2O$	205.12425

Microorganisms have been previously investigated as sources of DBP precursors. Several studies have looked at trihalomethane formation from various algae species [5]. Fang *et al.* produced a variety of DBPs including THMs, haloacetic acids (HAAs), chloral hydrate, trichloronitromethane, dichloroacetonitrile, 1,1-dichloropropanone, and haloacetonitriles from disinfection of the blue-green algae *Microcystis aeruginosa* [6]. Few studies have investigated DBP formation from bacteria, and none have looked at phenazine containing DBPs. Mitch and Sedlak examined the formation of N-nitrosodimethylamine (NDMA) from chloramination of bacteria and yeast (*Saccharomyces cerevisiae, Escherichia coli* and *Gordona amarae)* as precursors,

but did not detect any NDMA even after 10 days[7]. Pseudomonads, like the previously investigated microorganisms, are available in the environment to serve as phenazine containing DBP precursors. In addition, they can also form biofilms on the insides of distribution system pipes and so may serve as a source of DBP precursors within the distribution system.

This study was designed to examine whether phenazine containing DBPs can form from bacteria during chlorination and chloramination. To test this, we used *Pseudomonas fluorescens,* an opportunistic Pseudomonad, as a model as it is commonly found in soil and has also been isolated from biofilms in drinking water distribution systems.

6.2 Materials and Methods

6.2.1 Chemicals and standards

N-nitrosodiphenylamine (NDPhA) was obtained from Fluka Chemicals (Sigma Aldrich, Oakville, ON, Canada). Formic acid (LC-MS grade) and phenazine (98% purity) were obtained from Sigma Aldrich. NDPhA-d6 (98%) was obtained from Cambridge Isotope Laboratories (Cambridge, MA, U.S.A.). All other chemicals were of analytical grade and obtained from Fisher Scientific (Nepean, ON, Canada).

6.2.2 Growth and isolation of *P. fluorescens*

P. fluorescens was revived from cold storage onto LB agar plates. These primary streak plates were incubated for 48 h at room temperature. Then colonies from these plates were restreaked onto new LB agar plates and allowed to grow for 48 h at room temperature. Once individual colonies were observed, they were

transferred to 100 mL LB broth and grown for 48 h at room temperature being shaken at 170 rpm. After 48 h, media was split between 2–50 mL conical centrifuge tubes and spun down at 4000 rpm for 10 min. The supernatant was collected, transferred to new 50 mL tubes and spun down again. The cells were resuspended in 1X PBS and spun down at 4000 rpm for 10 min. This step was repeated two more times to ensure all the LB broth had been removed. Cells were finally resuspended in 1X PBS.

6.2.3 Preparation of disinfectant solutions

Stock solutions of chloramines (100 mM) were freshly prepared daily as previously described [1]. Solutions were prepared in 100 mL volumetric flasks. Ammonium chloride (1.0698 g) was dissolved in deionized water and adjusted to pH > 9.0 with 960 µL of 6.25 M sodium hydroxide. Sodium hypochlorite (10–15%) was then added dropwise to the solution while stirring. The final Cl:N molar ratio was 0.7:1.

Stock solutions of chlorine were freshly prepared daily by mixing sodium hypochlorite in deionized water.

6.2.4 Disinfection of bacteria

Resuspended bacteria were added to 500 mL amber bottles containing 1X PBS. Disinfectant was added and the solution was stirred for 5 min. Afterwards, the bottles were left to react at room temperature for 24 h (unless otherwise specified). After 24 h, ascorbic acid was added to each bottle to quench the disinfectant residual. Samples were stored at 4 °C until extraction.

6.2.5 Sample extraction

Solid phase extraction was performed as described in Chapter 5 [1]. Samples were filtered using Whatman GF/F glass filters (particle retention >0.7 μm, 142 mm) to remove bacterial particles that would block up the SPE cartridges. Samples were extracted using Waters Oasis HLB (Waters, Milford, MA, U.S.A.) cartridges mounted onto a Supelco Visiprep manifold. The cartridges were preconditioned using washes of dichloromethane, methanol and Optima grade water. The samples were then run through the cartridges at a flow rate of 2–3 mL/min. Once the sample had been run through, one more column volume of Optima grade water was used to wash the cartridges. Then two washes of 0.5 mL methanol and 3-5 mL washes of dichloromethane were used to elute the cartridges. The eluant was concentrated to 0.1–0.2 mL using high purity nitrogen. Fifty microlitres of 200 μg/mL of NDPhA-d6 were added to each sample, followed by 0.2 mL methanol and 0.15 mL deionized water, to obtain a final composition of 70% methanol. The extracts were stored at 4 °C until analysis.

6.2.6 Sample Analysis

Sample analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC separation was performed using an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, U.S.A) and a Phenomenex C8(2) Luna column with a C8 guardcolumn (Phenomenex, Torrance, CA, U.S.A.). LC mobile phases were A: water with 0.1% formic acid, B: methanol. The LC gradient consisted of 60% B for 3 min, ramped to 90% B

over 15 min, returning to 60% B over 0.1 min, and hold at 60% B for 8 min for a total run time of 25 min. The flow rate was 0.150 mL/min and the injection volume was 10 μL.

Two MS systems were used in this study. Quantitative analysis was performed using an AB Sciex 4000 QTrap (ABSciex, Concord, ON, Canada). The method consisted of an MRM survey scan followed by an information dependent acquisition-Enhanced product ion (IDA-EPI) scan. Optimized instrument dependent parameters are shown in Table 6.2. MRM transitions and compound dependent parameters for each analyte are shown in Table 6.3.

Table 6.2: Instrument dependent parameters for triple quadrupole MS and time-of-flight MS analysis in this study

Parameter	4000 QTrap	5600 Triple TOF
Ionspray voltage (V)	5500	5500
Curtain gas	30	30
Gas 1	60	50
Gas 2	50	50
Temperature	400	400

Table 6.3: Compound dependent parameters for the 4000 QTrap MRM method

Chemical	MRM transitions	DP	CE	CXP
N-chlorophenazine	217/181	51	33	10
	217/128	46	57	8
	219/181	51	33	10
	219/128	46	57	8
Phenazine	181/77	96	55	12
	181/154	91	45	8
N-nitrosodiphenylamine	199/169	56	17	10
	199/66	56	49	10
N-nitrosodiphenylamine-d6	205/175	46	27	10

High resolution analysis was performed using an ABSciex 5600 TripleTOF. The method consisted of a high resolution time-of-flight (TOF) scan (100–1000 Da) followed by an IDA-EPI (50–1000 Da) scan. The compound dependent parameters were as follows: declustering potential (DP) = 80, collision energy (CE) = 30, collision energy spread (CES) = 15. The instrument dependent parameters are shown in Table 6.2. The same LC method was used for both MS methods.

Some samples were run on a 5600 TripleTOF at ABSciex in Concord, Ontario. A Shimadzu UFLC XR system using a Phenomenex Kinetex C18 (10 X 2.1 mm X 2.5 μm) column was used for separation. LC mobile phases were A: water with 0.1% formic acid, B: methanol. LC gradient consisted of 40% B to 90% B in 10 min at 0.3 mL/min. Total run time was 10 min. Injection volume was 10 μL. MS conditions were: Positive ESI, DP = 80 V, CE = 35 V, CES = 15 V; TOF-MS mass range = 100–1000 Da; TOF-MS/MS Mass range = 50–1000 Da.

6.3 Results and Discussion

6.3.1 Formation of N-chlorophenazine from chloramination of *P. fluorescens*

In this study, we examined whether phenazine containing DBPs could form during disinfection of *P. fluorescens.* We first investigated formation during chloramination as phenazine and N-chlorophenazine formed from chloramination of DPhA. Figure 6.1 shows the MRM chromatograms (217/181) (4000 QTrap) for extracts of *P. fluorescens* alone (black trace) and *P. fluorescens* treated with chloramine (red trace). A peak was detected at 17 min in the *P. fluorescens* + chloramine trace, which corresponds with our N-chlorophenazine results from Chapter 5. Peaks were also observed at the same retention time in the three other MRM transitions for N-chlorophenazine (not shown) confirming the unknown peak matched the fragmentation and chlorine isotope pattern for N-chlorophenazine. This peak was a result of disinfection as no peaks were observed at the same retention time in the *P. fluorescens* alone trace. This information, taken together, supports the identification of the peak as N-chlorophenazine. Therefore, the hypothesis that N-chlorophenazine can form from bacterial precursors is confirmed.

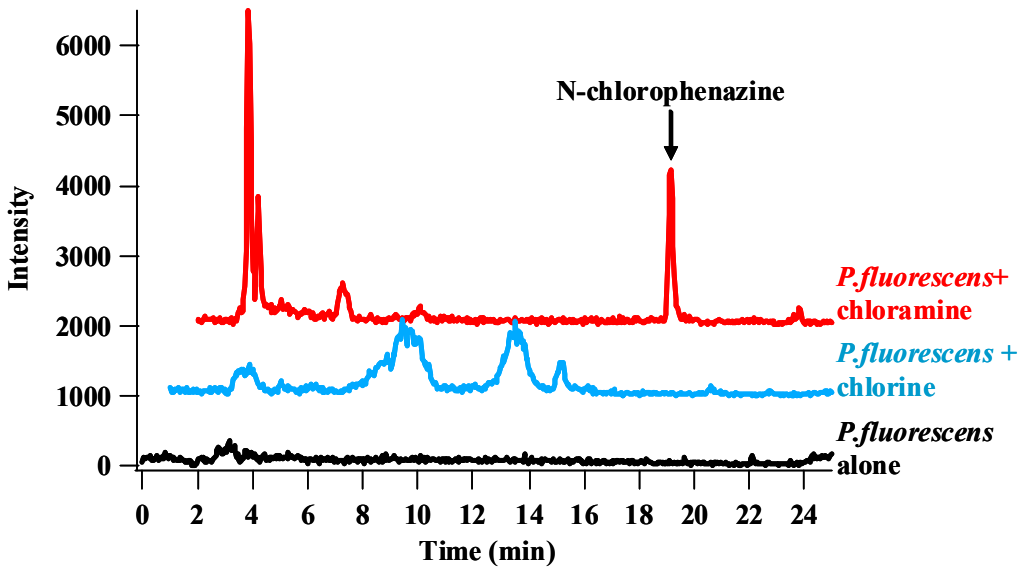


Figure 6.1: MRM (217/181) traces of chloraminated or chlorinated *Pseudomonas fluorescens*. Disinfectant dose was 3.4 mM. *P. fluorescens* dose: 1.43e+9 cells/mL. The N-chlorophenazine peak is denoted by an arrow.

To further verify the observed peak as N-chlorophenazine, we analyzed the chloraminated *P. fluorescens* samples using high resolution mass spectrometry (Qq-TOF) to obtain accurate mass measurements of the peak. The theoretical charged accurate mass of N-chlorophenazine is 217.05270 Da (Table 6.1). The experimentally measured mass of the peak was 217.0526. Analysis using PeakView software found only one compatible formula ($C_{12}H_{10}N_2Cl$, Accuracy -0.5 ppm) (Figure 6.2) which corresponds to the $[M+H]^+$ state of N-chlorophenazine. This result further supports the identification of N-chlorophenazine detected in the extract of the chloraminated *P. fluorescens* sample. This is the first evidence of N-DBP precursors originating from bacteria.

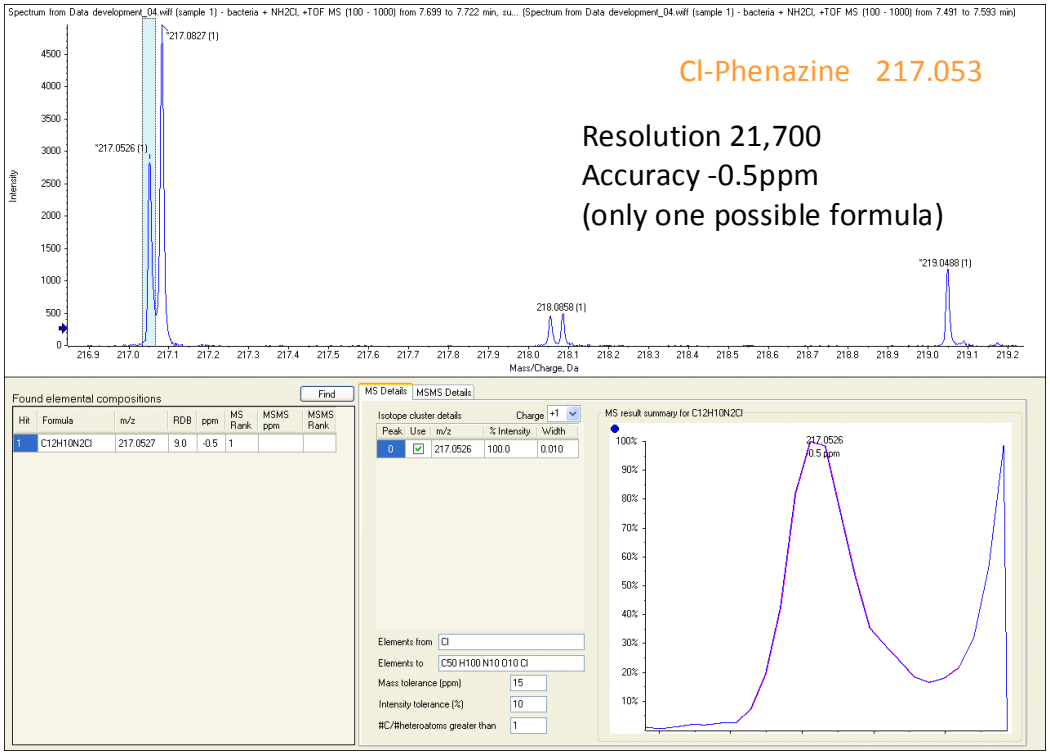


Figure 6.2: Accurate mass analysis of suspected N-chlorophenazine peak in a chloraminated *Pseudomonas fluorescens* sample.

6.3.2 Factors influencing N-chlorophenazine formation during chloramination of *P. fluorescens*

6.3.2.1 Effect of P. fluorescens concentration

Next, the effect of *P. fluorescens* cell numbers on the production of N-chlorophenazine was investigated. Different *P. fluorescens* concentrations (1.1e+5 cells/mL to 1.1e+9 cells/mL) were disinfected with the same chloramine concentration (100 mg/L) (Figure 6.3). As standards for N-chlorophenazine are not available, a standard curve could not be generated for quantification. Instead, the amount of N-chlorophenazine is expressed as a peak area ratio between N-chlorophenazine and the internal standard (NDPhA-d6) (left y-axis). Phenazine

standards are available, therefore phenazine concentration is shown on the right y-axis.

N-chlorophenazine was detected in samples containing 1.1e+7 cells/mL of *P. fluorescens* and above. N-chlorophenazine concentrations increased with increasing phenazine concentrations. When *P. fluorescens* was not chloraminated, phenazine was detected but N-chlorophenazine was not. This indicates that chloramination is necessary for the production of N-chlorophenazine (Figure 6.3).

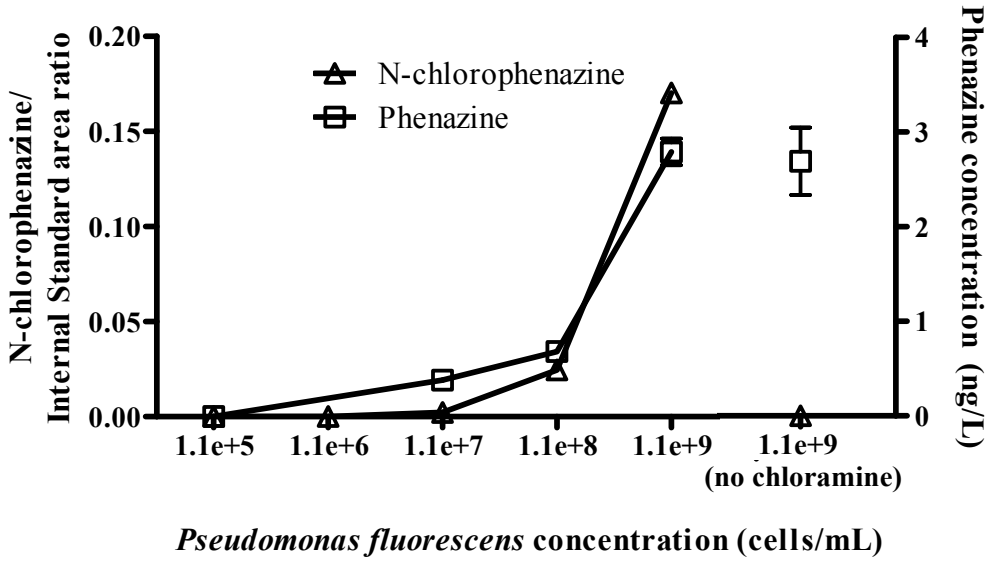


Figure 6.3: Effect of *Pseudomonas fluorescens* cell number on formation of N-chlorophenazine during chloramination. Chloramine dose was 100 mg/L.

6.3.2.2 Effect of chloramine concentration

The effect of chloramine dosing concentration on the formation of N-chlorophenazine from *P. fluorescens* was investigated. *P. fluorescens* (1.4e+7 cells/mL) samples were dosed with three different chloramine concentrations (51,

103 and 206 mg/L) (Figure 6.4). N-chlorophenazine was produced with all three chloramine concentrations; however, the highest intensity was observed with 51 mg/L, followed by 103 mg/L. This suggests that higher chloramine concentrations may form other byproducts more favorably than N-chlorophenazine or that N-chlorophenazine is not stable at higher chloramine concentrations. Phenazine concentrations remained stable through all four treatment groups.

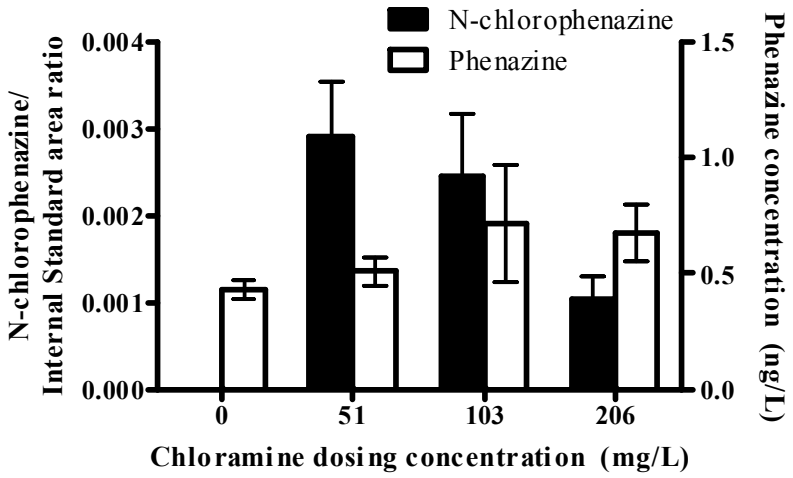


Figure 6.4: Effect of chloramine dosing concentration on the formation of N-chlorophenazine from *Pseudomonas fluorescens. P. fluorescens* dosing concentration was 1.4e+7 cells/mL

6.3.2.3 Effect of contact time length

Drinking water often sits for long periods of time in reservoirs and within the distribution system. This results in a longer period of time for disinfectants to react with precursors to form DBPs. For nitrosamines we have previously determined that concentrations increase with increasing distance from the water treatment plant (Chapter 2). To assess contact time length on formation of N-chlorophenazine, *P. fluorescens* was chloraminated at the same conditions as

shown in Figure 6.1 and then left for 90 days before analysis. Following sample extraction and analysis, no N-chlorophenazine was detected indicating that any N-chlorophenazine that formed likely decayed due to the long contact time.

6.3.3 DBP formation from chlorination of *Pseudomonas fluorescens*

Formation of N-chlorophenazine from chlorination of *P. fluorescens* was also assessed, as chlorine is very commonly used as a water disinfectant in North America. Figure 6.1 shows the MRM chromatograms (217/181) for *P. fluorescens* disinfected with chlorine (blue trace) and *P. fluorescens* alone (black trace). N-chlorophenazine was not observed in the chlorinated *P. fluorescens* samples; however, three new peaks were observed. All three peaks were also observed in the 219/181 MRM chromatogram at ratios of 3:1 indicating the presence of chlorine. This suggests that these unknown peaks may also be chlorophenazines with chlorine substituted in different locations around the phenazine ring.

To investigate this possibility, chlorine was reacted with phenazine alone (Figure 6.5) to try and reproduce the three unknown peaks observed in Figure 6.1. With only one precursor available for the chlorine to react, a greater quantity of monochlorinated phenazines would be formed than by using *P. fluorescens*.

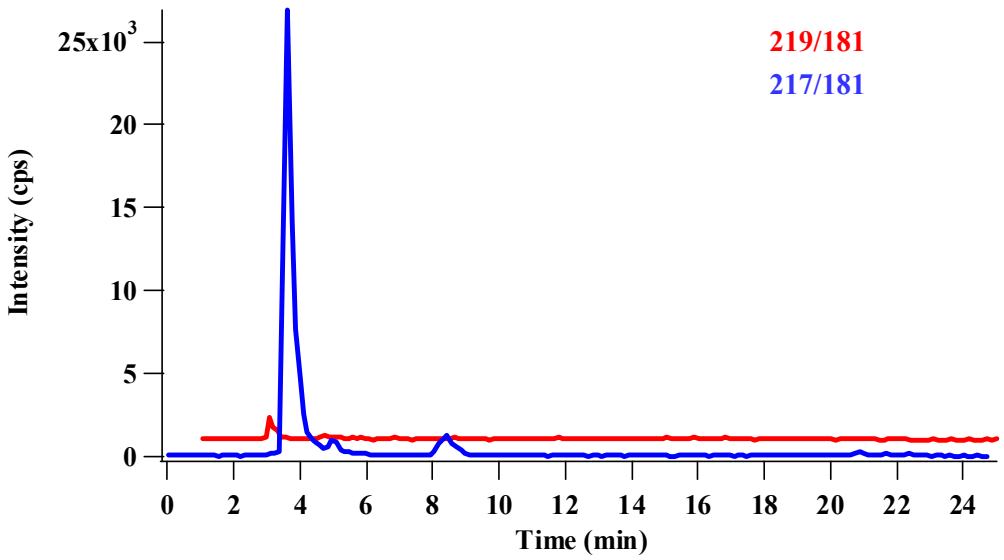


Figure 6.5: Chlorination of phenazine. MRM traces for N-chlorophenazine transitions 217/181 (blue) and 219/181 (red) are shown.

Chlorination of phenazine alone (Figure 6.5) did not produce the three unknown peaks observed following chlorination of *P. fluorescens* (Figure 6.1). However, previous research has shown that synthesis of monochlorophenazines is difficult due to the seeming low reactivity of the phenazine ring towards electrophilic substitution [8]. Chlorination of phenazine in particular has been reported to produce very low yield mixtures of singly and multiply chlorinated phenazines [8-10]. The results are not conclusive in determining if the unknown peaks are chlorophenazines. If they are chlorophenazines, then they may have been produced at concentrations too low for the method to detect.

Being unable to easily produce chlorinated phenazines via chlorination of phenazine, the identity of the unknown peaks was further investigated by analyzing chlorinated *P. fluorescens* samples using high resolution mass spectrometry. Figure 6.6 shows the extracted chromatogram of 217.0 Da +/- 1.0.

Five peaks (1–5) were observed instead of just the three observed in Figure 6.1, as all m/z 217.0 ions (+/- 1.0 Da) are shown, not just those producing m/z 181 or 128 product ions. Accurate mass spectra were obtained for peaks 1–5 (Figure 6.6B). Interestingly, four of the five peaks have an accurate mass near to 216.1. The extracted chromatograms of the determined accurate masses from peaks 1–5 are shown in Figure 6.7.

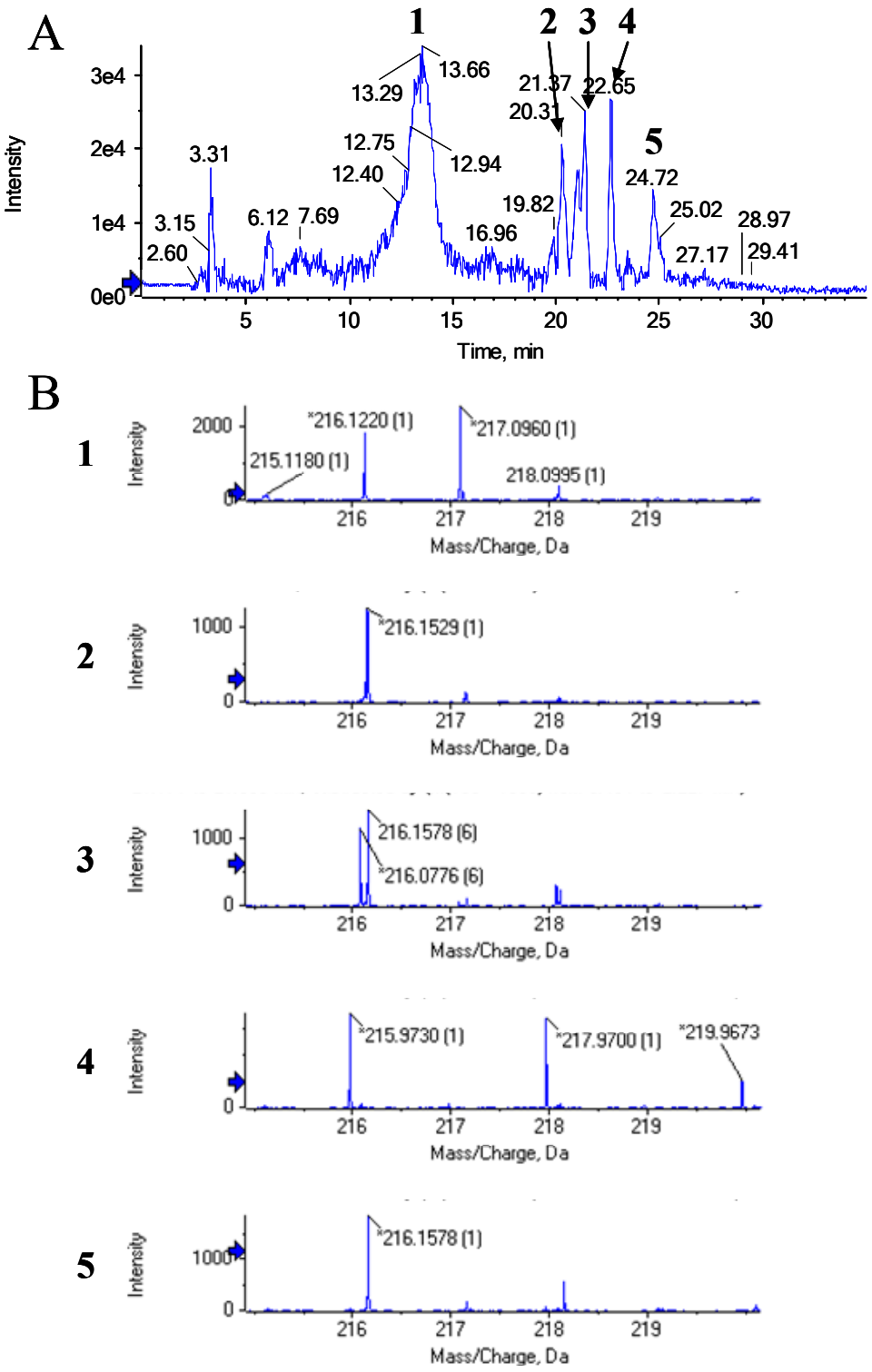


Figure 6.6: TripleTOF analysis of chlorinated *P. fluorescens* extracts. (A) XIC of 217.0 +/- 1.0 Da (B) MS spectra of peaks 1–5 focused on m/z 214.0–220.0.

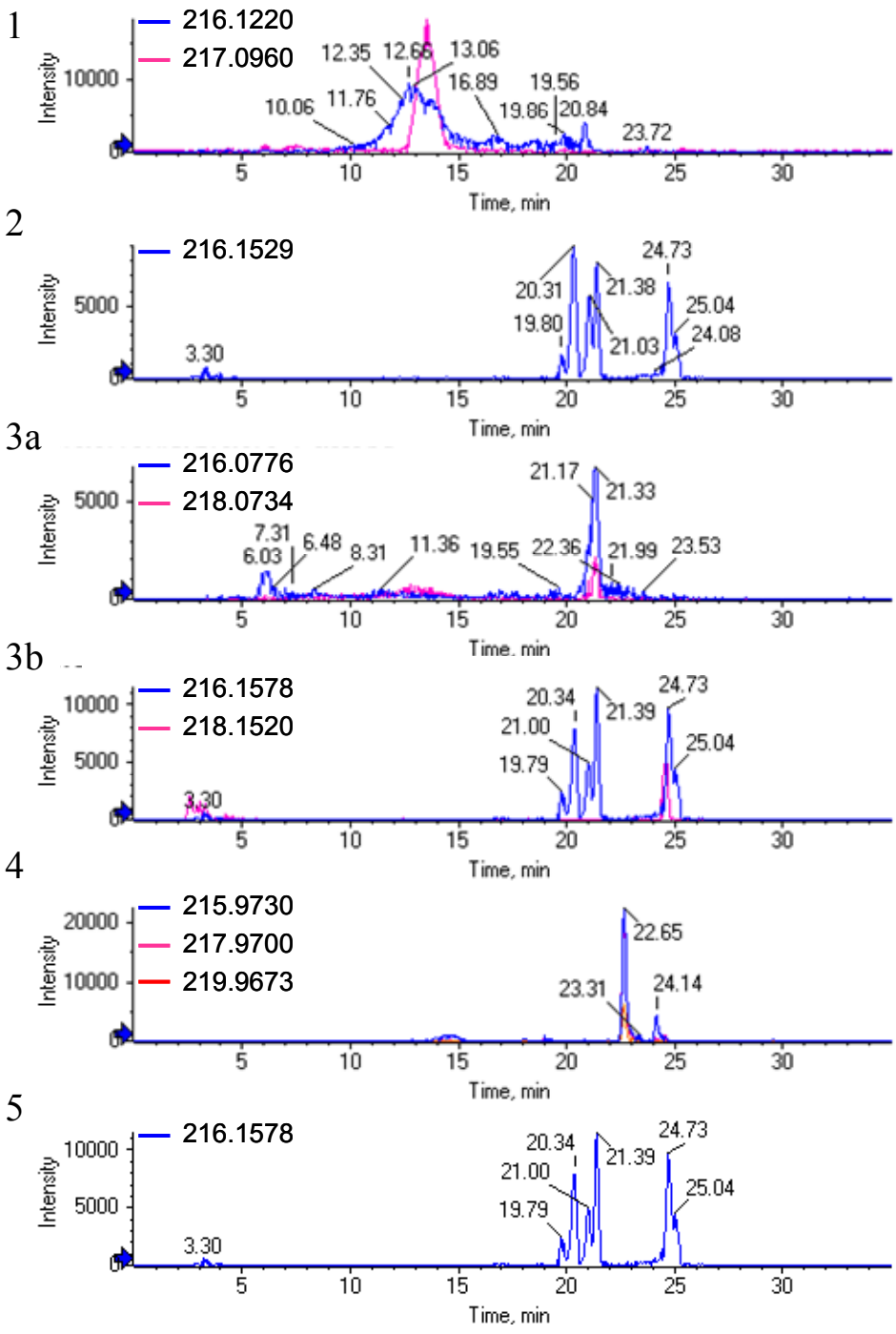


Figure 6.7: TOF-MS extraction of the accurate masses identified from the 5 peaks observed in chlorinated *Pseudomonas fluorescens* samples (Figure 6.6a). Extraction windows are +/- 0.005 Da.

Based on the extracted accurate mass results (Figure 6.7) it is clear that a number of compounds are present in the spectra, some co-eluting. It is uncertain if these peaks represent chlorinated phenazines, as the charged monoisotopic mass for 1- and 2-chlorophenazine (m/z 215.03705) was not observed. However, the unknown peaks were observed in the N-chlorophenazine MRM transition window on the 4000Qtrap which does suggest that they may be chlorophenazines.

Peak 1 (Figure 6.7, chromatogram 1) is a composite of m/z 216.1220 and 217.0960. Peak 2 consists of m/z 216.1529 alone. Extraction of m/z 216.1529 showed a number of peaks within the extraction window. No clear chlorine isotope patterns were observed even though it was one of the three unknown peaks observed on the 4000QTrap. A number of MS peaks were observed for Peak 3: m/z 216.0776, 218.0724, 216.1578, and 218.1520. Peaks for m/z 216.0776 and 218.0724 were observed at the same retention time at a 3:1 ratio suggesting the presence of chlorine (Figure 6.7, chromatogram 3a). However, the peaks for m/z 216.1578 and 218.1520 had slightly different retention times, suggesting they are from different analytes; m/z 216.1578 was also observed in Peak 5. Peak 4, one of the three unknown peaks from Figure 6.1, also exhibits a chlorine isotope pattern. Ions of m/z 215.9700, 217.9300 and 219.9656 are observed at intensity ratio of approximately 3:3:1 and elute at the same time (Figure 6.7, chromatogram 4). This suggests the possible presence of three chlorines on this molecule. The major constituent of Peak 5 was m/z 216.1578. Determination of the identity of these peaks is underway. The chlorine signatures observed in these results confirm my results from the 4000Qtrap.

6.4 Conclusions

This study is the first to demonstrate formation of phenazine containing DBPs from bacterial sources. While much attention is paid to natural organic matter in source water, microorganisms themselves have not been well studied as N-DBP precursors. This could potentially be important both in source water and within the distribution system. N-chlorophenazine was produced from chloramination of *P. fluorescens*. The amount formed depended on precursor and disinfectant concentrations. However, N-chlorophenazine was not formed during chlorination. Other peaks were observed that are currently being identified. These results are useful for determining the formation of phenazine containing DBPs during water disinfection.

The toxicity of phenazine containing DBPs and their potential effect on human health is unknown. Chlorinated phenazines have been investigated as herbicides and fungicides, with mono-halogenated phenazines shown to be more toxic than di-, tri- or tetra-halogenated phenazines [11]. The substitution position of the halogen atom on the phenazine ring is also important as 1-chlorophenazine was shown to be more toxic than 2-chlorophenazine [11]. No toxicity information is available on N-chlorophenazine. More research is needed to determine the possible human health effects of the presence of these DBPs in drinking water.

6.5 References

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7 CONCLUSIONS AND SYNTHESIS

7.1 Introduction

Disinfection of drinking water is effective at preventing the spread of waterborne disease. However, it also produces disinfection byproducts (DBPs). The health effects of DBPs are unclear. Many DBPs have been shown to be carcinogenic in chronic rat toxicity assays; however, the effect of long-term exposure to low doses of these chemicals on humans in unknown. Epidemiology data has only been able to consistently link consumption of chlorinated drinking water with an increased risk of bladder cancer, but the DBP or DBPs responsible for this effect remain unclear. What is clear is that regulated DBPs such as trihalomethanes (THMs) and haloacetic acids (HAAs), which typically also make up the highest proportion of identified DBPs, do not have sufficient potency for causing the increased bladder cancer risk.

At the start of my Ph.D., nitrosamines were considered a possible culprit for the increased bladder cancer risk. They were much more potent rodent carcinogens than THMs or HAAs and had been identified in drinking water in California and Alberta [1, 2]. My work aimed to fill some of the research gaps in nitrosamine knowledge including improved methodology to detect a wider range of nitrosamines in drinking water (Chapter 2), investigation of the occurrence of nine nitrosamines other than NDMA (Chapter 3), development of new methodologies to rapidly screen newly identified DBPs (Chapter 4), investigation of nitrosamine formation from different disinfectants on the same source water and from suspected precursors (Chapter 5), and investigation of microbial sources

of DBP precursors (Chapter 6). This chapter presents summaries of the major findings from my research (Chapters 2-6), conclusions based on my thesis work as a whole and suggestions for future research directions.

7.2 Advancements in Knowledge

7.2.1 Chapter 2: Development of an SPE-LC-MS/MS method to detect nine N-nitrosamines in drinking water

Prior to 2006, analysis of N-nitrosamines in drinking water was done almost exclusively using gas chromatography–mass spectrometry (GC-MS) [3]. While this technique is sensitive and robust for the eight thermally stable and volatile nitrosamines included in U.S. EPA Method 521 [4], it precludes the direct detection of thermally unstable or non-volatile nitrosamines. I hypothesized that thermally unstable or non-volatile nitrosamines may form during water treatment but were not being detected by the current GC-MS methodologies. To test this hypothesis, I developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to detect eight GC-detectable and one GC non-detectable nitrosamine (NDPhA). Using electrospray ionization, I was also able to analyze for N-nitrosodiphenylamine, a thermally unstable nitrosamine that breaks down in the GC injector. Combined with a solid phase extraction (SPE) preconcentration step, this method is capable of detecting nitrosamines at low ng/L concentrations, which is comparable with GC-MS methods. Using this method, four nitrosamines were detected in treated drinking water from an Alberta water treatment plant (WTP): NDMA, N-nitrosopyrrolidine (NPyr), N-nitrosopiperidine (NPip), and NDPhA. NPip and NDPhA had not been reported as DBPs previously. The detection of NDPhA demonstrated that GC non-detectable nitrosamines also form

during water treatment and that LC-MS/MS based techniques can be used to sensitively detect ng/L concentrations of DBPs in drinking water samples.

7.2.2 Chapter 3: Occurrence of nine N-nitrosamines in thirty-eight drinking water systems in Canada and the U.S.A.

Although N-nitrosamines are potent enough to cause health effects at low ng/L concentrations and had been detected in select water systems in North America in 2006, little was known about their occurrence in drinking water. Of the two occurrence studies published, one was five years old and only investigated NDMA [5]. The other looked at eight volatile nitrosamines, but focused only on drinking water in Alberta [6]. Both of these studies used GC-MS for analysis, thus no data was available for NDPhA. Using the SPE-LC-MS/MS method developed in Chapter 2, I analyzed treated drinking water from 38 water distribution systems in North America. NDMA was the most commonly detected nitrosamine, with the highest concentrations observed in chloramination systems. Although NDMA was detected above the method detection limit in 76% of samples, only 24% were above the California Notification Limit of 10 ng/L. Higher NDMA concentrations were detected in systems using a free chlorine contact time between 0 and 1 min, compared to systems using no free chlorine contact time or >1 min contact time. Interestingly, NDPhA was the second most commonly detected nitrosamine (16% of systems). NMor was also detected once. This was the first study to investigate the occurrence of both GC-detectable and GC non-detectable nitrosamines in drinking water across North America.

7.2.3 Chapter 4: RT-CES method development and application to toxicity testing for emerging nitrosamine DBPs

Toxicity data is lacking for the majority of the over 600 identified DBPs. High-throughput screening techniques are needed to prioritize these DBPs for future research. Real-time cell electronic sensing (RT-CES) monitors changes in cell numbers, size and adhesion using microelectrodes in real time. This technique is label free and can simultaneously monitor 16- or 96-well plates for several days. It has been used previously to test the toxicity of several classes of environmental contaminants [7-9] and has been included as part of the U.S. Environmental Protection Agency's (U.S. EPA) ToxCast program [10]. In this chapter, I developed an RT-CES method to screen the toxicity of the four nitrosamine DBPs detected in drinking water distribution systems in Chapter 2. Using this method, I screened the cytotoxicity of the four nitrosamines on a panel of four cell lines. The order of sensitivity of the four cell lines to the four nitrosamines was: T24>A549>CHO>HepG2. The differences in sensitivity are likely due to the differing P450 expression within the four cell lines. NDPhA was 10X more cytotoxic than NDMA in all four cell lines; an interesting result considering that NDMA is considered to be one of the most toxic nitrosamines. Further analysis showed that NDMA causes cytotoxicity by causing cell death whereas NDPhA causes cell cycle arrest at G0/G1. NDPhA cannot undergo alpha-hydroxylation like NDMA, which may account for the different mechanisms of toxicity observed for these two nitrosamines. This is the first report of the effect of NDPhA on the cell cycle.

7.2.4 Chapter 5: Investigation of the formation of N-nitrosamines during water treatment

A number of studies have investigated nitrosamine formation during water treatment. However, these studies typically focus only on NDMA and one or two disinfectants. Thus it is unknown how nitrosamine formation is affected when different disinfectants are applied to the same group of precursors. There is also almost no information on the formation of nitrosamines other than NDMA. I collected source waters from seven locations in North America, representing a range of water quality, and treated them with 11 different disinfectants alone. Using the method developed in Chapter 2, I monitored the reactions for the formation of nine different nitrosamines. Four nitrosamines were detected in the study: NDMA, NDPhA, NMor and NMEA. NDMA was by far the most commonly formed nitrosamine. In general, chloramination produced higher NDMA concentrations than chlorination but this was source water specific. In addition, treatment of precursors with UV or AOP can reduce NDMA concentrations, but application of chlorine following these treatments can re-form NDMA. This study demonstrated how important source water quality is in determining NDMA formation.

Only a few treatments produced NDPhA making it difficult to obtain any insights into its formation. Therefore I took a closer look at NDPhA formation. Based on itschemical structure, I predicted diphenylamine (DPhA) as the precursor for NDPhA formation. I first confirmed that DPhA existed in the source water, then demonstrated that DPhA is an NDPhA precursor and that monochloramine is more important for NDPhA formation than dichloramine. This

is different from NDMA formation which has been shown to form from dichloramine. Chloramination of diphenylamine also produced two other DBPs, which were identified as phenazine and N-chlorophenazine. Phenazine and N-chlorophenazine had not been reported as DBPs previously.

7.2.5 Chapter 6: Formation of N-DBPs from bacterial precursors

Following the identification of phenazine and N-chlorophenazine in Chapter 5, I further investigated the formation of these two DBPs. Phenazine containing compounds are likely to be precursors for these DBPs in addition to DPhA. Phenazines are available in the environment anthropogenically and are produced by some bacterial species. *Psuedomonas fluorescens* is one type of bacteria that produces phenazine-containing natural products. It is present in the environment and in distribution system biofilms possibly providing a source of precursors in the distribution system. Little is known about the formation of DBPs from microorganisms. Some studies have shown DBP formation from algae, but the effect of bacterial precursors on DBP formation is largely unknown. To investigate this, *P. fluorescens* was disinfected with chloramine which resulted in the production of N-chlorophenazine. This confirmed that bacteria can serve as DBP precursors. N-chlorophenazine was not observed when *P. fluorescens* was treated with chlorine. Instead, other peaks were observed which display a chlorine isotope pattern. This suggests the formation of other chlorinated phenazines, although their exact structures require further confirmation.

7.3 Conclusions

The N-nitrosamines are one class of DBPs with sufficient potency to cause health effects at low ng/L concentrations. My work has produced new knowledge and expanded on the existing studies in several ways. I identified a new nitrosamine DBP, NDPhA, which is not directly detectable using common GC-based screening techniques. I showed that NDPhA is 10 times more cytotoxic than NDMA in *in vitro* toxicity tests and exerts its toxicity in a manner distinct from other nitrosamines. My results show that NDPhA was the second most commonly detected nitrosamine in a study of North American drinking water, suggesting it should be taken into account when considering exposure to nitrosamines in drinking water.

My work on nitrosamine formation demonstrated that source water and the type of disinfectants are the key factors in determining nitrosamine formation. Every disinfection process creates its own suite of DBPs, and small changes in the disinfection process may reduce the concentrations of certain DBPs but will produce other DBPs which may be more or less toxic. For example, changes in chlorine contact time will affect NDMA formation; pH changes may reduce NDPhA formation but increase the concentration of phenazine containing DBPs. Changing disinfection procedures to reduce formation of a particular DBP is much easier than changing the source water. However, my results clearly demonstrate the need for individual water systems to thoroughly evaluate the effect of changes in the treatment train on DBP formation.

The precursor content of source water is also extremely important in determining DBP formation. My nitrosamine formation study showed that application of chloramines did not guarantee nitrosamine formation. However, no strong relationships were found between general source water parameters and nitrosamine formation potential. Our finding that DPhA may serve as a precursor for several different classes of DBPs (NDPhA and phenazines) further demonstrates the complicated nature of DBP formation. Identification of precursor sources, such as bacteria for N-chlorophenazine, may help to determine what types of DBPs might form during water disinfection. In addition, protection of source water contamination from agriculture, industry, and algal blooms to reduce the amount of available precursors will also help reduce DBPs in treated water.

Finally, my work has demonstrated the usefulness of utilizing new technologies for DBP research. I demonstrated that LC-MS/MS can detect a wider range of nitrosamines in drinking water at concentrations comparable to GC-MS. This will allow for better characterization of nitrosamines in drinking water which will aid exposure assessment. I also demonstrated the usefulness of high throughput cell sensing techniques in assessing the cytotoxicity of new DBPs.

7.4 Future Research

The public health concerns surrounding the presence of DBPs in drinking water are valid due to the large number of people who use tap water each day. However, the DBP or DBPs responsible for the increased risk of bladder cancer has not been identified. My results, along with other studies, indicate that

nitrosamines are not widespread enough in tap water to be solely responsible for the increased bladder cancer risk. For the select locations where nitrosamine concentrations are elevated, it will be important to monitor these locations and attempt to reduce their nitrosamine concentrations. Interest in nitrosamines remains high and many jurisdictions are in the process or have already implemented NDMA guidelines or regulations in drinking water.

The search for the DBP responsible for the observed increased bladder cancer risk will continue. Therefore a cautious outlook of reducing overall DBP formation will help until the ultimate culprit is determined. The role of distribution system biofilms in contributing to DBP formation should be investigated, as this could provide a significant source of DBP precursors. The need for rapid toxicity testing of new DBPs, including mechanistic data, is still necessary for most currently identified DBPs. Further investigation of the toxicity of DBP mixtures is also warranted as it is likely that the health effects attributed to DBPs may be part of a mixture rather than a single DBP or class of DBPs. In addition, epidemiology studies need to be generated on DBPs other than THMs and HAAs. Due to the issues with obtaining exposure data, this may mean a shift to more prospective cohort studies than case control studies.

Finally, it is important to better translate the knowledge generated within the research community to water treatment operators. If water treatment operators continue to believe that THMs in drinking water will give you cancer, then we risk increasing concentrations of more toxic DBPs in drinking water, or failure to

properly disinfect microbial threats as was seen in Walkerton. The provision of clean, microbially safe water must remain the ultimate goal of water treatment.

7.5 References

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