Elsevier Editorial System(tm) for Toxicology
Manuscript Draft

Manuscript Number: TOX-11-251R1

Title: Potential Carcinogenic Hazards of Non-regulated Disinfection By-products: Haloquinones, Halo-Cyclopentene and Cyclohexene Derivatives, N-Halamines, Halonitriles and Heterocyclic Amines

Article Type: Review Article

Keywords: Chlorination by-products, Structure-toxicity relationships, Haloquinones, N-Chloramines, Halonitriles, Halonitriles, Heterocyclic amines

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Abstract: Drinking water disinfectants react with natural organic material (NOM) present in source waters used for drinking water to produce a wide variety of by-products. Several hundred disinfection by-products (DBPs) have been identified, but none have been identified with sufficient carcinogenic potency to account for the cancer risks projected from epidemiological studies. In a search for DBPs that might fill this risk gap, the present study projected reactions of chlorine and chloramine that could occur with substructures present in NOM to produce novel by-products. A review of toxicological data on related compounds, supplemented by use of a quantitative structure toxicity relationship (QSTR) program TOPKAT® identified chemicals with a high probability of being chronically toxic and/or carcinogenic among 489 established and novel DBPs. Classes of DBPs that were specifically examined were haloquinones (HQs), related halo-cyclopentene and cyclohexene (HCP&H) derivatives, halonitriles (HNs), organic N-chloramines (NCls), haloacetamides (HAMs), and nitrosamines (NAs). A review of toxicological data available for quinones suggested that HQs and HCP&H derivatives appeared likely to be of health concern and were predicted to have chronic lowest observed adverse effect levels (LOAELs) in the low μg/kg day-1 range. Several HQs were predicted to be carcinogenic. Some have now been identified in drinking water. The broader class of HNs was explored by considering current toxicological data on haloacetonitriles and extending this to halopropionitriles. 2,2-Dichloropropionitrile has been identified in drinking water at low concentrations, as well as the more widely recognized haloacetonitriles. The occurrence of HAMs has been previously documented. The very limited toxicological data on HAMs suggests that this class would have toxicological potencies similar to the dihaloacetic acids. Organic N-halamines are also known to be produced in drinking water treatment and have biological properties of concern, but no member has ever been characterized toxicologically beyond bacterial or in vitro studies of genotoxicity. The documented formation of several nitrosamines from secondary amines from both natural and industrial sources prompted exploration of the formation of additional nitrosamines. N-Diphenylnitrosamine was identified in drinking waters. Of more interest, however, was the formation of phenazine (and subsequently N-chorophenazine) in a competing reaction. These are the first heterocyclic amines that have been identified as chlorination by-products. Consideration of the amounts detected of members of these by-product classes and their probable toxicological potency suggest a prioritization for obtaining more detailed toxicological data of HQs > HCP&H derivatives > NCls > HNs. Based upon a

ubiquitous occurrence and virtual lack of in vivo toxicological data, NCls are the most difficult group to assign a priority as potential carcinogenic risks. This analysis indicates that research on the general problem of DBPs requires a more systematic approach than has been pursued in the past. Utilization of predictive chemical tools to guide further research can help bring resolution to the DBP issue by identifying likely DBPs with high toxicological potency.

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4/27/11

Kendall B. Wallace, Ph.D.
Managing Editor
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Dear Dr. Wallace,

Thank you for the opportunity to make revisions to our manuscript TOX-11-251, "Potential Carinogenic Hazards of Non-regulated Disinfectant By-products: Haloquinones, Halo-Cyclopentene and Cyclohexene Derivatives, *N*-Halamines, and Heterocyclic Amines".

We have addressed the points raised by Reviewer 1 as follows:

We incorporated a discussion of the epidemiology studies identified toward the end of the discussion section. We call attention to the fact that it was necessary to write a review critical of the notion that the associations of THM exposure with polymorphisms in xenobiotic metabolizing enzymes establish that these compounds can really account for the magnitude of the bladder cancer risk identified. There is essentially a gap in risk assessments based upon animal studies and epidemiological studies of almost two orders of magnitude that should be accounted for when only considering THM exposure. In addition, there is no direct evidence that any of the THMs are bladder carcinogens. It is important to note that we do not question the validity of the association of bladder cancer risk with chlorinated drinking water. We just believe that the association with THMs is a trivial correlation that adds little specificity to the virtually identical correlation one gets with chlorinated water, itself. Our thesis is that both the magnitude of the risk and the specificity for the bladder would be much better accounted for by some other identified by-product(s) in the very complex mixture that is produced in chlorinated drinking water (and perhaps some that are yet to be identified). We agree that the Cantor et al. study is an important paper in the area of disinfectant by-product work. However, Dr. Cantor is careful not to claim that THMs are causal for the bladder cancer. I have discussed this with him on many occasions. He is well aware that other by-products are substrates for the drug metabolizing enzymes, especially the glutathione transferases, which are clearly of the most interest. Several of the chemical classes we have identified in this paper could have both the potency and specific toxicological properties that could produce bladder carcinogenesis and are or very likely to be metabolized by glutathione transferases.

Addressing the problem identified by the reviewers required significant additions to the text and a number of new references to point out why the actual rates of metabolism at

the low doses from drinking water would not be significantly different among the polymorphisms for GSTZ1 or CYP2E1 on kinetic grounds. In fact, we cite a paper that demonstrates that clearly in exposed humans for the THMs based on phenotypic expression of cytochrome P450 2e1. The GSTT1 is a slightly different case because is it null in some individuals and potentially takes on an all-or-none connotation. However, the K_m of the BDCM for this enzyme is so high that this enzyme will not effectively compete with cytochrome P450 2e1 for its metabolism at blood concentrations anticipated from THM exposure from drinking water.

In response to the reviewer's comments, we did include all the references cited by the reviewer except the Font-Ribera et al. 2010 reference which really did not deal with cancer risk with any specificity.

The Li et al., 2010 reference in the bibliography is incorrect. It should have been 2011 and has been corrected.

The Bull et al. 2006 reference has been available from the indicated date as a report of the Water Research Foundation (formerly Awwa Research Foundation). The reference has been rewritten in a way that makes it clear that it is available as a published report.

We appreciate the comments of Reviewer 1, as well. We have more diligently sought out and corrected grammatical and typographical errors.

We thank the reviewers for their helpful comments. We hope they have been adequately addressed.

Sincerely,

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***Response to Reviews**

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Potential Carcinogenic Hazards of Non-regulated Disinfection By-products: Haloquinones, Halo-Cyclopentene and Cyclohexene Derivatives, *N*-Halamines, Halonitriles, and Heterocyclic Amines

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Abstract

Drinking water disinfectants react with natural organic material (NOM) present in source waters used for drinking water to produce a wide variety of by-products. Several hundred disinfection by-products (DBPs) have been identified, but none have been identified with sufficient carcinogenic potency to account for the cancer risks projected from epidemiological studies. In a search for DBPs that might fill this risk gap, the present study projected reactions of chlorine and chloramine that could occur with substructures present in NOM to produce novel by-products. A review of toxicological data on related compounds, supplemented by use of a quantitative structure toxicity relationship (QSTR) program TOPKAT® identified chemicals with a high probability of being chronically toxic and/or carcinogenic among 489 established and novel DBPs. Classes of DBPs that were specifically examined were haloquinones (HQs), related halo-cyclopentene and cyclohexene (HCP&H) derivatives, halonitriles (HNs), organic *N*-chloramines (NCls), haloacetamides (HAMs), and nitrosamines (NAs). A review of toxicological data available for quinones suggested that HQs and HCP&H derivatives appeared likely to be of health concern and were predicted to have chronic lowest observed adverse effect levels (LOAELs) in the low μg/kg day^{-1} range. Several HQs were predicted to be carcinogenic. Some have now been identified in drinking water. The broader class of HNs was explored by considering current toxicological data on haloacetonitriles and extending this to halopropionitriles. 2,2-Dichloropropionitrile has been identified in drinking water at low concentrations, as well as the more widely recognized haloacetonitriles. The occurrence of HAMs has been previously documented. The very limited toxicological data on HAMs suggests that this class would have toxicological potencies similar to the dihaloacetic acids. Organic *N*-halamines are also known to be produced in drinking water treatment and have biological properties of concern, but no member has ever been characterized toxicologically beyond bacterial or *in vitro* studies of genotoxicity. The documented formation of several nitrosamines from secondary amines from both natural and industrial sources prompted exploration of the formation of additional nitrosamines. *N*-Diphenylnitrosamine was identified in drinking waters. Of more interest, however, was the formation of phenazine (and subsequently *N*-chorophenazine) in a competing reaction. These are the first heterocyclic amines that have been identified as chlorination by-products. Consideration of the amounts detected of members of

these by-product classes and their probable toxicological potency suggest a prioritization for obtaining more detailed toxicological data of HQs > HCP&H derivatives > NCls > HNs. Based upon a ubiquitous occurrence and virtual lack of *in vivo* toxicological data, NCls are the most difficult group to assign a priority as potential carcinogenic risks. This analysis indicates that research on the general problem of DBPs requires a more systematic approach than has been pursued in the past. Utilization of predictive chemical tools to guide further research can help bring resolution to the DBP issue by identifying likely DBPs with high toxicological potency.

Keywords

Chlorination by-products, Structure-toxicity relationships, Haloquinones, *N*-Chloramines, Halonitriles, Heterocyclic amines

1. Introduction

Chlorination of drinking water has been consistently linked with an increased risk of bladder cancer in different geographic areas around the world (IARC, 2004) and less consistent associations were found with cancers of other organs. The odds ratios obtained in epidemiological studies are small, but the numbers of cases that would be attributable to chlorination could be relatively large compared to other chemical exposures from the environment (Morris et al., 1992; Poole, 1997; Villanueva et al., 2002; 2004). The USEPA's background document for the Stage II Disinfection By-product Rule (USEPA, 2003a & b) implies a lifetime cancer risk from chlorinated drinking water of approximately one additional cancer per thousand population per lifetime (Bull and Reckhow, 2008).

Regulation of disinfectant by-products (DBPs) from the chlorination of drinking water has focused upon trihalomethanes (THMs) and haloacetic acids (HAAs). The members of these classes that have been studied in experimental animals are weak carcinogens (Bull et al. 2006). Therefore, they would appear as improbable causes of risks of the magnitude that have been suggested by epidemiological data. At the concentrations that are found in drinking water and assuming an equivalent potency of THMs and HAAs for humans as found in experimental animals, the potencies of these chemicals are at least two orders of magnitude too weak as carcinogens to significantly contribute to the risk associated with chlorination of drinking water (more fully documented in discussion section). This simple comparison raises the question of whether much more potent carcinogens are produced in the chlorination of drinking water than those routinely measured in response to regulation.

Water from surface sources contains organic and inorganic chemicals that are largely of natural origin. Natural organic matter (NOM) in water is a complex mixture comprised of small amounts of nutrients (e.g. simple amino acids and sugars) to relatively large conglomerates of biological products (Leenheer et al., 2000). Within this amorphous material, a substantial portion of the total organic carbon (TOC) is comprised of complex polymeric structures known as humic and fulvic acids. The structures of these organic acids include a large variety of moieties comprised of substituted phenols, furans, and heterocycles connected by aliphatic carbon chains. There are numerous carbonyl and hydroxyl substitutions. Substructures within fulvic and humic acids have been described by degradation studies, including the use of

pyrolysis (e.g. Martin et al., 1994) and functional groups detected using a variety of direct and indirect methods (e.g. Ritchie and Perdue, 2003). From these studies numerous substructures have been identified (Schulten and Schnitzer, 1998). Utilizing these substructures to predict the types the nature of reactions that will occur with chlorine or chloramine allows prediction of both halogenated and non-halogenated organic by-products to be tractable. A key question is whether by-products formed with alternate forms of disinfection (e.g. chloramine, chlorine dioxide, or ozone) differ significantly from those formed with free chlorine. Epidemiological data suggest that risk from bladder cancer is reduced when chloramine is introduced in place of free chlorine (Zierler et al., 1988; McGeehin et al., 1993). Similar findings have been reported when ozone was used in advance of treating water with chlorine (Chevrier et al. 2004). However, these case control studies of alternative disinfectants have focused exclusively on bladder cancer. Therefore, they do not indicate absence of carcinogenic risk at other sites (e.g. GI tract, kidney), which may be produced by products that differ from those derived from simple chlorination.

In the present study, chemicals that were probable products of reactions of the disinfectants with substructures within NOM, as well as selected members of established DBP classes have been evaluated for probable carcinogenic properties. The toxicology of compounds closely related to these DBPs was explored by an extensive literature search that included related compounds as well as the DBPs of concern. This was supplemented by a quantitative structure toxicity relationships (QSTR) analysis to provide a basis for judging whether these classes were likely to add significantly to hazards already assigned to DBPs that have been subject to regulation, the THMs and HAAs. Several compounds and classes were identified as candidates of sufficient potency to be plausible causes of cancer outcomes. In the meantime, several of these DBPs have been identified in drinking waters (Zou et al.. 2000; Krasner et al., 2006; Zhao et al., 2010). Therefore, the present paper is an assessment of whether these DBPs are likely to contribute to cancer risk.

2. Methods

2.1. Literature sources of information on formation of DBPs

Chemicals and chemical classes that are established by-products of disinfection were identified in the review of Richardson (1998) and subsequent original contributions by her laboratory and her associates (Richardson 1999a & b; Richardson et al. 2003). A large number of papers in the primary literature that documented formation of by-products by reaction with compounds that could be viewed as probable constituents of NOM were also reviewed. A more comprehensive review of DBPs and their health effects was recently published by (Richardson et al. (2007). The source of information on specific DBPs is identified as each class is discussed.

2.2. Prediction of By-Product Formation from Substructures of NOM.

Many DBPs are produced in disinfection that have not been chemically identified. This is evidenced by less than half of the total organic halogen being accounted for by established chlorination by-products. Therefore, a major part of this effort was the prediction of reaction products of chlorine or chloramine with natural organic matter (NOM). The approach taken was to predict intermediates and products of reactions between substructures of NOM. This allowed a broader evaluation of the possible DBPs that would arise from chloramination or chlorine disinfection. In the present paper, to conserve space, a description of the pathways postulated to be responsible for the formation of novel DBPs are provided only for those compounds that were subsequently found to be of toxicological interest.

Only a small portion of the chemicals studied are discussed in this paper. A detailed list of the chemicals subjected to study is provided in Bull et al. (2006). Some of the by-products discussed were identified in drinking water in a subsequent report (Li et al., 2011) and are more explicitly addressed here.

2.3. Literature Evaluations of Probable Toxicity of Predicted By-Products

Toxicological data that addressed the comparative toxicity of chemicals in the same or related chemical class were considered most important in determining the likely potency of putative DBPs as chronic toxicants or carcinogens. Data that addressed mechanisms of toxicity, structural and/or functional group contributions to toxicological potency, coupled with some descriptive toxicological studies within the class was considered the most dependable means of

identifying DBPs of potential interest. This was accomplished by reviewing individual scientific papers, authoritative reviews of the toxicology of particular classes, and selected databases. The most useful database was the Cancer Potency Database (CPDB)(Gold et al., 2010). Seven groups of DBPs appeared most interesting and were explored in some detail; haloquinones (HQs), halo-cyclopentene & cyclohexene derivatives (HCP&H), including halofuranones, *N*-chloramines (NCls), haloacetamides (HAMs), halonitriles (HNs), haloaldehydes (HAs), nitrosamines (NAs), and heterocyclic amines.

2.4. Quantitative Structure Toxicity Relationships (QSTR)

Many DBPs belong to chemical classes that have received little or no toxicological characterization. To supplement our analysis of the literature, we employed a QSTR program. TOPKAT® (Accelrys, 2001) was chosen among several commercial programs that were available that approach structure-activity relationships in somewhat different ways. TOPKAT® was selected primarily because of the diversity of endpoints that can be addressed with the series of models that are part of this software package. The main point of this effort was to identify compounds that would have effects at low doses (i.e. high toxicological potency). Therefore, the model in TOPKAT® that predicts chronic lowest observed adverse health levels (LOAELs) in rats provided a means of estimating the toxicological potency of each DBP.

Within the TOPKAT® model formulations, descriptors are utilized that relate to the rates of absorption of a chemical such as molecular bulk, shape, and symmetry. These are combined with descriptors that quantify the chemical properties of a compound. The chemical properties utilized by TOPKAT® relate to the electropological state (E-state) values developed by Kier and Hall (Accelrys, 2001) to quantify the electronic attributes of molecular structure governing molecular interactions that give rise to toxicological effects.

The predictions of individual models or sub-models within TOPKAT® are very dependent upon their individually constituted training sets. For example, there are separate models for mice and rats and for males and females within each of these species for predictions of carcinogenicity. In addition, there are different models based on the two large databases that are available, those of the National Toxicology Program (NTP) and the U.S. Food and Drug Administration (FDA). TOPKAT® makes independent predictions from the two databases.

Widely varying predictions are frequently observed among models based on these differing databases. Divergence in the predictions occurs largely because classes of chemicals are differentially represented in these databases. Differences also occur because related chemicals in the training sets were only studied in a single sex, in a single species, or in different mouse or rat strains.

TOPKAT® provides three ways of judging the confidence that can be placed in a prediction. The first is an indication of whether or not the prediction is within the optimum predictive space (OPS) of the model. The program distinguishes between predictions that are outside the OPS, but within permissible ranges, and those that exceed the permissible range. In this project, those predictions that exceed the permissible range were interpreted as "no prediction" and there was no attempt to compile lists of compounds that were predicted to be non-carcinogens. Secondly, within each model's database, the program provides a measure of the similarity of the query structure with the chemicals that were included in the equation on which the prediction is based. Measures of similarity ranged from 0.000 (identity) to 1.0 (no similarity). Finally, the program indicates when a molecular fragment in the query structure is not represented in the model's database. These qualifications on a prediction are identified in the assembled database and carried over to tables presented in this paper.

Explicitly, the criteria for identifying putative DBPs as high priority for research on the basis of their occurrence drinking water and characterization of their toxic properties were as follows:

1. They are from a chemical class that has recognized toxicological properties and a data base for estimating whether the derivative forms identified as DBPs might be of greater or lesser toxicological potency.
2. Chemicals with an actual or predicted chronic LOAEL < 1 mg/kg were considered to be of high interest, irrespective of whether or not they were predicted to be carcinogens.
3. Carcinogens or predicted carcinogens with doses that produce a 50% increase in cancer in a lifetime (TD_{50}s – available online, Gold et al. 2010) or actual or predicted chronic LOAELs of < 10 mg/kg were considered to be of high interest.
4. A probability of 0.7 was required for a positive prediction of carcinogenicity with any given QSTR model. Predictions that were outside the permissible limits of the OPS of the specific model used were discarded.

5. A positive prediction of carcinogenicity by TOPKAT® was accepted only if at least one chemical in the training set of the model used had a similarity index of < 0.2.
6. Predictions of carcinogenic activity were considered of greater interest the more consistent the prediction was among species (rats and mice) and sex. Both the NTP and FDA models for each species and sex were utilized. Positive prediction of carcinogenic activity in two species in addition to other criteria described above was required before a compound was identified as a high priority for study as a probable carcinogen. A positive prediction within either database satisfied these criteria. In effect, this treats the databases (or training sets) for the FDA and NTP models as complimentary rather than competing models. This is justified because disagreement among models based on the NTP and FDA databases was most commonly attributable to the absence of chemicals of the class in one or the other of the databases. To increase the stringency of the predictions, we required that a positive prediction meeting the criteria described above was observed in two species to classify a compound as a predicted carcinogen based solely on QSTR analysis.
7. Finally, a DBP predicted to be a carcinogen and to be positive with respect to Ames' mutagenicity was ranked higher in priority than a chemical not meeting the mutagenesis criteria, but having a similar LOAEL.

In the overall project, a total of 489 chemicals were evaluated. Only a fraction of these chemicals are discussed in this paper. Emphasis in this paper was on DBPs that were considered highly probable to form or have been detected in drinking water, but individually have little or no chemical-specific toxicological data available. The entire list of chemicals examined and associated databases are available in a report published by the Water Research Foundation (Bull et al., 2006).

3. Results

3.1 Testing the relationship between predicted chronic LOAEL and carcinogenic potency

The predicted chronic LOAEL was the primary measure of toxicological potency in the absence of empirical data. We were concerned that using the same cut-off for carcinogens as other chronic toxicities might inappropriately exclude important carcinogens because the chronic LOAEL might be a poor predictor of carcinogenic potency. Gaylor and Gold (1995) described a relationship between maximum tolerated dose (MTD) and carcinogenic potency. This hypothesis was tested by determining how close TD_{50}s of carcinogens in the CPDB were approximated by predicted chronic LOAELs. Figure 1A compares the TD_{50}s 111 randomly selected chemicals that are in the Carcinogen Potency Database (Gold et al., 2010) with chronic LOAELs for the same chemicals predicted with TOPKAT®. Seventeen of these compounds were found to be outside the optimum predictive space of the TOPKAT® model, so the correlation developed included 94 of these chemicals. While there was a significant correlation ($r = 0.42$), it is far from an ideal relationship. A virtually identical correlation was observed between the TD_{50} and predicted chronic LOAEL of the 46 halogenated chemicals in the group (data not shown). The predicted LOAEL was approximately equivalent to the TD_{50} at 30 mg/kg day^{-1}. However, there was a departure of one order of magnitude in the two values at 0.1 mg/kg day^{-1}, and a further divergence at lower doses. This implies that potent carcinogens are frequently active at doses which do not present with obvious evidence of chronic toxicity. Thirty-four of the compounds examined in Figure 1A were also in the TOPKAT® training set. The relationship between the chronic LOAEL with the TD_{50} for these compounds was better ($r = 0.72$) (Figure 1B). Nevertheless, the best fit line still deviates by about an order of magnitude at 0.1 mg/kg day^{-1}. The tendency for the TD_{50} to be significantly less than the LOAEL was compensated for by identifying the DBP as a high priority for research if it was identified as a carcinogen and had a predicted chronic LOAEL of < 10 mg/kg day^{-1}.

3.2. Haloquininones

3.2.1 Formation of Haloquinones from Substructures of NOM

Haloquinones (HQs) may arise from the reaction of chlorine with a variety of naturally-occurring organic compounds. The approach is described in more detail with this class to illustrate the approach. Formation of DBPs in other classes are described in less detail for the sake of brevity. However, the predicted pathways of formation are detailed in Bull et al. (2006).

One likely reaction pathway is by substitution of chlorine on the ring of the preformed quinone. Electrophilic aromatic substitution of chlorine for hydrogen may also occur in parallel with oxidation of the hydroquinone to a quinone. The degree to which the former occurs before the latter determines the extent of halogenation of the HQ product. When substitution is kinetically favored the product is a fully halogenated *p*-quinone or *o*-quinone structure from 1,4- and 1,2-dihydroxybenzene, respectively (Figure 2a and 2b) as supported by Sarkanen and Dence (1960). As chlorine atoms are added to the ring, the acidity of the ring OH groups will increase. This should help to keep the reaction rate high even at neutral pH where the anionic form may not be dominant. If this is the major pathway, there must be subsequent loss of HCl, largely giving oxygenated aliphatic products. This is based on the low TOX yield (i.e. < 1 M/M) reported for chlorinated solutions of 1,4- and 1,2-dihydroxybenzene (Reckhow & Singer, 1985).

The fully halogenated quinones could arise from other simple precursors as well. For example, catechol or hydroquinone derivatives with carboxylic acid groups directly bound to a ring carbon are prime candidates. These should slowly decarboxylate, with accompanying halogen substitution in the presence of chlorine, leaving the corresponding halogen substitution product (e.g., see Larson & Rockwell, 1979), which can then be transformed into the *p*-quinone and *o*-quinones. Again, the presence of chlorine on the aromatic ring prior to decarboxylation should elevate the equilibrium concentration of the reactive anionic form of the carboxylic acid. In addition, there are many larger substituted structures that can degrade to phenolic-carboxylic acid forms that can also enter this reaction pathway. Many naturally-occurring aromatic compounds and macromolecules (e.g., lignins, tannins) are potentially susceptible to production of HQ precursors upon treatment with chlorine.

Logical subsequent degradation pathways for tetrachloro-*o*-quinone are shown in Figure 3. The degradation roughly follows the scheme proposed by Zou et al. (2000). These authors were investigating a rather minor sub-pathway leading to the potent mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). The alternative terminal section of the pathway (shown with a dashed line) involves simple decarboxylation of the intermediate, which may be more likely under conditions of low chlorine residual. This produces 1,1,3,3-tetrachloropropanone which quickly gives rise to dichloroacetic acid and chloroform (details not shown) (Reckhow et al., 1985). While model compound data do not support either of these as the major pathway for HQ degradation, they may explain some of the observed TOX formation.

Heasley et al (2004) have demonstrated formation of HQs and related compounds on reaction of monochloramine with phenolic precursors. The outline of the reactions of 2,4,6-trichlorophenol with either NH_2Cl or $NHCl_2$ giving rise to a mixture of 2,6-dichloro-1,4-benzoquinone (DCBQ) with 55% yield and 2,6-dichloro-1,4-benzoquinone-4(*N*-chloro)imine with 35% yield is shown in Figure 4. Under the same conditions, reactions of the chloramines with 2,4,6-trichloro-*m*-cresol or 2,4,6-trichloro-3-methoxyphenol resulted in analogous products (i.e. with 3-methyl or 3-methoxy groups) with similar yields. Therefore, the use of chloramine in disinfection increases the variety of haloquinone structures that are likely to be generated in drinking water. The use of free chlorine vs. chloramine also has the potential of differentially affecting the stability of the HQ. At acid pH, excess chlorine will result in ring scission, which is unlikely to occur with chloramine. Analogous stabilization of cyanogen chloride has been demonstrated in chloraminated water (Na and Olson, 2004). On the other hand, at the alkaline pH that is usually sought with chloramine disinfection, chlorine will be lost from the ring. This suggests that lower degrees of halogen substitution are likely with chloramine.

Table 1 provides the frequency at which HQs and other DBPs were detected in 18 drinking waters (Zhao et al. 2010; Li et al. 2011). Four HQs were identified, DCBQ, 2-6-dibromo-1,4-benzoquinone (DBBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), and 2,3,6-trichloro-1,4-benzoquinone (TCBQ). DCBQ and DBBQ were detected in all systems and ranged from 2.2 to 295 and 0.5 to 37.9 ng/L, respectively. The remaining HQs were detected less frequently and at lower concentrations.

3.2.2. Toxicological Evaluation of Haloquinones (HQs)

Table 2 provides a list of quinone and quinone-like structures that were predicted to occur as DBPs. Only the ones identified in the previous section have been sought in drinking water. Predictions of all the members provides some perspective on how broad this class of DBPs could be and also provide some perspective on how important this group might be toxicologically.

There are few toxicological studies of haloquinones and essentially no chronic studies in the literature. On the other hand, non-halogenated polyphenols and quinones have been studied extensively because of evidence that they play an important role in the responses to a number of

well known carcinogens, including benzene and polyaromatic hydrocarbons (Bolton et al., 2000). As a result, there is a rich literature describing likely modes of action of quinones.

The metabolism and distribution of the metabolites of polyphenols and quinones deliver several toxic metabolites to certain cells (Figure 5). The first step in quinone metabolism is conjugation with glutathione which occurs primarily in the liver. The importance of this step is illustrated by the fact that the nephrotoxicity of 1,4-benzoquinone is increased with each glutathione substitution until 3 sites on the ring are occupied, adding the fourth glutathione-conjugation decreases nephrotoxicity (Lau et al., 1988). The conjugates are actively taken up in cells that express GSH transporters. Secondly, the glutathione conjugates are metabolized to cysteine conjugates that are also transported into cells (e.g. renal proximal tubular cells) that express gamma glutamyl transferase (γ-GT). Within cells, the polyphenol form of the compound is oxidized to the quinone form by the enzyme NAD(P)H:quinone oxidoreductase (NQO1). The quinone or the semiquinone radical can directly interact with macromolecules (Lin et al. 1999). The cysteinyl forms can also be converted to forms that alkylate macromolecules (Bolton et al., 2000), however, this can be prevented by *N*-acetylation of the cysteine residue, which reduces toxicity. Further, the parent polyphenol and quinone forms and their respective conjugates participate in redox cycling reactions that lead to the formation of reactive oxygen species (ROS). Mutation spectra in treated human cells or bacteria are consistent with DNA damage induced by hydroxyl radical, implicating ROS in the genotoxicity of quinones (Jeong et al., 1999). Halogen substitution appears to enhance the toxicity of these conjugates. Conjugation favors the oxidized quinone form (Monks et al. 1990) and the electron withdrawing effects of halogen substitutions will increase their activity as oxidants (Monks and Lau, 1997). The formation of the cysteine conjugate enhances the oxidation of the hydroquinone form, but is suppressed by *N*-acetylation of the cysteine residues (Monks et al., 1990).

An *in vitro* QSTR study of *p*-benzoquinones was conducted in rat hepatocytes and PC12 cells and it was found that the two chlorinated congeners, 2,3,5,7-tetrachloro-1,4-benzoquinone and 2,5-dichloro-1,4-benzoquinone, were substantially more active than the non-halogenated compounds (Siraki et al., 2004). The two HQs were especially potent in producing ROS, doubling fluorescence measures by two-fold with sub-μM concentrations. They were also more cytotoxic than non-chlorinated congeners, but these differences were as little as 2-fold whereas

they were 10-250X more potent in inducing ROS formation. These differences were predictable based upon the shifts in redox potential that occur with halogenation.

The details of the distribution of quinones and the corresponding hydroquinones can be complex *in vivo*. Monks et al. 1990 found that the chloroquinone conjugates were less potent as nephrotoxicants than the hydroquinone conjugates. The authors attributed this to non-specific loss of the quinone structure prior to accessing the interior of cells. Thus, the equilibrium between hydroquinone and quinone structures can have a significant impact on the systemic toxicity resulting from exposure.

Hydroquinones and the respective quinones are not generally detected as mutagens in *Salmonella* test systems but it is clear that they are capable of damaging DNA through the generation of hydroxyl radical (Monks and Lau, 1997). The HQs do induce aneuploidy in human cells (Imai et al., 2009). 1,4-Benzoquinone has also been shown to form adducts with DNA and to increase mutation frequency in a human kidney cell line (Gaskal et al., 2005). Recently, halogenated quinones were found to participate in a metal-independent degradation of hydroperoxides to produce a quinone ketoxy radical, which may be a candidate for adduct formation by these compounds (Zhu et al., 2009).

TOPKAT® indicated that some of the HQ structures predicted to form by chlorine or chloramine reaction with NOM were likely to be carcinogenic (Table 2). Low chronic LOAELs (< 1 mg/kg day^{-1}) were predicted for most of the haloquinones. Consistent with the literature on non-halogenated hydroquinones and quinones, TOPKAT® modeling predicted most of the halogenated congers would be negative in *Salmonella*-based mutagenicity tests.

Halogen substitution and the formation of the benzoquinone 4-(*N*-chloro)imine derivative with chloramine have substantial effects on the polarity of quinones. The data in Figure 6 were developed with the program Molinspiration (www.molinspiration.com) which predicts octanol/water (O/W) partition coefficients based upon chemical structure. First, formation of the quinones increases the O/W partition coefficient of the corresponding hydroquinones. Second, halogenation progressively increases the O/W partition coefficient, with bromination increasing it more effectively than chlorination. Third, as expected, substitution of an additional non-polar group to the ring (modeled with a methyl group) increases the coefficient. Finally, it can be seen that the substitution of the 4-(*N*-chloro)imine group greatly increases the O/W partition coefficient.

Based upon the review of the literature on related compounds, we conclude that the production of HQs as DBPs likely represents a cancer risk.

3.3. Halocyclopentanes, Halocyclohexenes and Halofuranones

3.3.1 Formation of Halocyclopentene and Halocyclohexene Derivatives and Halofuranones

It was predicted that reaction of chlorine with substructures within NOM would give rise to a large variety of chemicals including the cyclopentene and cyclohexene derivatives that were likely to be produced with the chlorination of drinking water. Most of the chemicals considered were arrived at through consideration of orcinol (3,5-dihydroxytoluene) as a probable structural fragment in NOM (Bull et al., 2006) and included ketone, dione and carboxylic acid derivatives. The halofuranones are the most broadly recognized related by-products in this group. The total concentrations of the halofuranones were found in a recent survey to be substantially greater than anticipated from prior studies (Krasner et al., 2006). The highest concentration of total halofuranones was 2.38 µg/L.

A variety of halocyclopentene and halocyclohexene derivatives were predicted to form. Examples of predicted products include 3,5-dichloro-1-hydroxy-4-ketocyclopent-2-enoic acid, 3,5-dichloro-hydroxy-4-ketocyclopent-2-enoic acid, 2,3,6- trichloro-4,5-diketophenylprop-2-enol, and 1,2,2,4-tetrachloro-3-hydroxycyclopent-4-enoic acid (Table 2). Gong et al. (2005) demonstrated formation of 2,2,4-trichloro-5-methoxycyclopent-4-ene-1,3-dione through reaction of chlorine with syringaldehyde. This cyclopentenedione was subsequently shown to occur in drinking water, although concentrations were not provided. The compound was shown to be a direct-acting mutagen (i.e. most active in the absence of S9 fraction of rat liver homogenates). Thus, this grouping of DBPs could be important contributors to carcinogenic risk from chlorinated drinking water.

3.3.2 Toxicological Evaluation of Halocyclopentene, Halocyclohexene, and Halofuranones

Because of its low concentrations, MX alone, is unlikely to contribute significantly to the cancer risk associated with chlorinated drinking water. However, the finding of higher concentrations of other halofuranones raises the possibility that the total of MX-related

compounds may make a significant contribution to risk. MX is fairly potent as a carcinogen with a TD_{50} of 0.583 mg/kg day^{-1} (Gold et al., 2010). Interest in this group should be increased by the predicted formation of cyclopentenoic acid derivatives that bear structural resemblance to the halofuranones and which are predicted to have some of the same toxicological characteristics.

There is a database of carcinogenesis assays on chemicals that involve small modifications of the basic furan ring (NTP, 1990; 1993; 1994; 1999) that may provide the basis of a QSTR study of the carcinogenic properties of this group of chemicals. Table 3 presents estimates of carcinogenic potency of furan and a number of modifications of furan, including MX. Of the compounds listed, furan is the most potent carcinogen. It is a multispecies, multiple target organ carcinogen. It produces cholangiosaracomas, hepatocellular carcinomas, mononuclear leukemia in male and female rats, hepatocellular carcinomas, and pheochromocytomas in male and female mice. However, furan is not mutagenic in *Salmonella* tester strains, although it does produce clastogenic effects in mammalian cells (NTP, 1993). With the exception of MX, modification of the furan structure substantially reduces the carcinogenic potency. The carcinogenic potencies of tetrahydrofuran and furfural are two to three orders of magnitude less than that of furan. Evidence that furfuryl alcohol is systemically carcinogenic has to be considered marginal, as the nose was the only target organ in an inhalation study. Thus, it may be that the structure-activity studies of mutagenic effects of MX and related compounds in *Salmonella* (LaLonde & Leo, 1994) are not reliable indicators of carcinogenic potency for furan derivatives. Therefore, DBPs that are related to furan should be studied more systematically as carcinogens.

A very low predicted LOAEL was associated with 3,5-dichloro-1-hydroxy-4-ketocyclopent-2-enoic acid by TOPKAT®, but the prediction was outside the predictive space of the model. It was not predicted to be a carcinogen. This compound was predicted to be formed from reaction of phenol with excess chlorine (Bull et al., 2006) and would be favored in chlorinated water while formation of 2,3,5,6-tetrachloro-1,4-benzoquinone would be favored with chloramine (Heasley et al., 2004). 2,2,4-Trichloro-5-methoxycyclopent-4-en-1,3-dione was identified by Gong et al. (2005) and had a sub-mg/kg predicted chronic LOAEL, but was not predicted to be a carcinogen. Although the prediction of the LOAEL for some of the halocyclopentenes were outside of the OPS of the model, the predictions for MX were also outside the OPS. MX has been shown experimentally to be a carcinogen (Komulainen et al. 1997).

3.4. N-Halamines

3.4.1 Formation of N-Halamines.

It has long been recognized that primary and secondary amines will react with aqueous chlorine (e.g., HOCl or OCl^-) and inorganic chloramine (e.g., NH_2Cl or $NHCl_2$) to form organic *N*-chloramines.

$$R-NH_2 \xrightarrow{HOCl} R-NHCl \xrightarrow{HOCl} R-NCl_2$$

$$R_2-NH \xrightarrow{HOCl} R_2-NCl$$

Equilibrium considerations are closely tied to the basicity of the amine. As the amine becomes a stronger base, it will tend to form more stable chloramines (Pitman et al., 1969). The primary amine groups of the common amino acids are quite basic and therefore readily donate their electrons to electrophilic reactants such as chlorine. Amidines are also quite basic (pKa ≥ 11.2), especially the guanidine derivatives (pKa ~ 13.5).

Subsequent reaction with chlorine leads to the formation of *N*-dihalo species. This reaction is much slower than the initial halogenation, due to the much lower basicity of the *N*-chloro compounds. Disproportionation of two monohalamine molecules can lead to the formation of a dihalamine, however, this is less likely in highly dilute drinking waters containing a free chlorine residual.

There is little question that *N*-halamines are formed in the disinfection of drinking water as the total chloramine concentration is routinely measured as combined chlorine. This measurement generally exceeds the amount of inorganic *N*-chloramine and is generally considered to reflect *N*-chloramines that are formed with various primary and secondary amines that are present in the water. However, no surveys of individual organic *N*-chloramines appear in the literature.

3.4.2. Toxicological Evaluation of N-Halamines.

A search of the literature revealed a very limited toxicological data, primarily in the form of mutagenicity assays of *N*-chloramines of α-amino acids (Thomas et al., 1987). In *Salmonella*

TA100, the dichloramines of histamine, ethanolamine, and putrescine were most potent, whereas histamine was most active among the monochloramine derivatives.

Laingam et al. (2011) studied the ability of the *N*-chloramines of several amino acids to induce micronuclei in mammalian cells. As suggested by the results of Nightingale et al. (2000) with the ε-amino group of lysine, ethanolamine and α-acetyllysine formed significant amounts of dichloramine at equivalent molar concentrations with chlorine, whereas only monochloramines were observed with glycine, ε-acetyllysine, and histidine. Thus, α-amino groups are much less likely to form dichloramines at physiological pH than those which are not adjacent to a carboxyl group. Increased induction of micronuclei was observed with most of the *N*-chloramines. *N*-Chlorohistamine was more potent than the others. An interesting finding was that the *N*-chloramines formed with lysine were substantially more potent than that of the two acetylated derivatives. This suggests that chloramination of both nitrogens might lead to bifunctional interactions with macromolecules, perhaps resulting from the formation of crosslinks (i.e. DNA to protein, protein to protein, or DNA with DNA).

Organic *N*-chloramines have received attention in the biomedical literature as 1) mediators of inflammatory and anti-inflammatory effects that result from activation of neutrophils (Bernofsky, 1991; Davies et al., 1993; Marcinkiewicz, 1997; Barua et al., 2001; Vissers et al., 2001; Englert & Shacter, 2001; Kawai et al. 2004; Midwinter et al., 2004; Schuller-Levi & Park, 2004) and 2) they have been invoked as reactive metabolites produced by neutrophils that could account for a variety of chronic side effects of certain drugs (Utrecht et al., 1991 & 1995; Miyamoto et al., 1997). These studies suggest that organic *N*-halamines are toxicologically active, but provide little information on which to base a risk assessment related to their occurrence in drinking water.

Illustrative examples of organic *N*-halamine formation and their decay to reactive intermediates are provided in Figure 7. In each case, the initial reaction is shown and further progression in the development of selected secondary intermediates or terminal products is depicted. In the first two examples, the initial reactions should be viewed as occurring in drinking water, the subsequent formation of radical species is suggested to result from metabolic processing *in vivo*. The first reaction depicted would be typical of amino acids and other primary amines. The figure illustrates the generation of nitrogen and chlorine radical products and the regeneration of free chlorine, each of which could contribute to toxicity. The reaction with

acetamide shows that the corresponding *N*-chloramides can be converted into carbon-centered radicals *in vivo*, as well. Finally, the formation of a less stable *N*-chloramine with a drug (aminopyrine) that contains tertiary amine group is depicted. In this case, a di-cation is formed that reacts with an additional molecule of aminopyrine to form a cation radical. In the latter case, it is probable that the low stability and high polarity of the initial chloramine should limit its systemic absorption. As a result, exogenous sources of this *N*-halamine are unlikely to represent an *in vivo* hazard.

The reason for concern about the toxicology of organic *N*-halamines is that they are 1) less polar than the parent compound (see Fig. 6) and 2) that some are sufficiently stable to be distributed systemically once absorbed (Hawkins & Davies, 1999). It is of importance to note that the *N*-chloro group can be transferred to other chemicals, or in the case of RNA and DNA, from initially formed, less stable sites on purine and pyrimidine rings, to the exocyclic nitrogens (Hawkins & Davies, 2002). As a consequence, less stable compounds formed in water may transfer the *N*-chloro group as the chemical is ingested. Of course, some organic *N*-chloramine formation can be expected upon ingestion of a free chlorine residual because of the rich sources of amines in the oral cavity and esophagus.

The importance of transfer of the *N*-chloro group among different amino acids was elegantly illustrated by Peskin et al. (2004), who assessed the relative abilities of the chloramines of histamine, glycine, and taurine to inhibit intracellular glycerylaldehyde-3-phosphate dehydrogenase activity (GAPDH) when added to incubation media individually or in combination. The *N*-chloramine of glycine inhibited GAPDH, but that of taurine did not. However, if taurine-*N*-chloramine was added in the presence of glycine, the enzyme was inhibited (Figure 8). This indicates that the *N*-chloro-group of the polar, non-penetrating *N*-chloramine had transferred the *N*-chloro group to the permeant *N*-chloramine, glycine-*N*-chloramine, in a biological milieu.

While the initial formation of *N*-chloramines is more rapid when the amino group is α to a carboxyl function, the formation of the initial *N*-chloro group on an amine without an α-carboxyl group facilitates addition of the second *N*-chloro group (Nightingale et al., 2000). As a consequence, the *N*-chloro groups on the epsilon amino groups of lysine in proteins appear to be responsible for the production of nitrogen-centered free radicals that play a role in inactivation of the proteins (Hawkins and Davies, 1999).

TOPKAT does not recognize the *N*-halamine or *N*-haloamide groups, reflecting the absence of systemic toxicological studies with compounds in these classes. As a consequence, QSTR projections for these two classes were not useful.

3.5. Halonitriles and Haloamides

3.5.1. Formation of Halonitriles and Haloamides

As described in the previous section, most amino acids undergo initial, rapid formation of mono and di-*N*-chloramines. Due to the presence of an adjacent carboxyl group, *N*-halo substituted α-amino acids undergo a Grob fragmentation to yield an aldehyde or ketone and a nitrile. Many amino acids are known to yield halogenated aldehydes and nitriles (e.g., chloral hydrate and dichloroacetonitrile) following reaction with chlorine (Figure 9). The degree to which this happens depends on the presence of an activating side chain (e.g. R3). After reaction, the amine nitrogen leaves a functional group (carbonyl or nitrile) that is electron withdrawing. When combined with a similarly electron withdrawing side chain, the α-carbon can become an active site for halogen substitution.

The haloacetonitriles have long been recognized as chlorination by-products (Trehy & Bieber, 1981). Attempts were made to identify halopropionitriles as DBPs (Li et al., 2011), but only in the case of 2,2-dichloropropionitrile was it possible to synthesize and purify a standard for the project. The concentrations of 2,2-dichloropropionitrile in a drinking water are compared with the haloacetonitriles in Table 1.

HAMs have been detected in drinking water (Krasner et al., 2006). Concentrations in the range of 2-3 µg/L were reported for mono- di- and tri- haloacetamides. The brominated trihaloacetamides have not been measured as no standards are currently available. Studies with amino acids as precursors suggest the formation of halopropionamides as well as haloacetamides (see Figure 9).

3.5.2. Toxicological Evaluation of the Halonitriles.

Chloroacetonitrile (CAN), bromoacetonitrile (BAN), dichloroacetonitrile (DCAN), bromochloroacetonitrile (BCAN), dibromoacetonitrile (DBAN), and trichloroacetonitrile (TCAN) have been studied toxicologically to varying degrees.

The haloacetonitriles are known to be mutagenic and to act as tumor initiators in the mouse skin (Bull et al., 1985). Several other studies have established the genotoxicity of haloacetonitriles in various systems, the SOS chromotest, Ames-fluctuation assay, newt micronucleus test (Le Curieux et al., 1995), DNA strand breaks in cultured human lymphoblastic cells (Lin et al., 1986), and aneuploidy in *Drosophila* (Osgood and Sterling, 1991).

Table 4 provides predicted chronic LOAELs, predictions of carcinogenicity, and Ames' system mutagenicity for HNs. Within the HN class, compounds with 3-carbon chains were prominent in terms of their potential toxicological potency as reflected in the predicted LOAELs. 2,2-Dichloropropionitrile was found at 8 ng/L in a drinking water sample (Li et al., 2011). Four haloacetonitriles were also identified (DCAN, BCAN, DBAN and TCAN) in the same drinking waters. The predicted LOAELs for the haloacetonitriles were outside the permissible limits of the OPS, but experimental data indicate that the estimates are about an order of magnitude lower than those predicted for members of the class that have been partially characterized toxicologically (Bull et al., 2006). The brominated analogs of these chemicals did not meet the stringent criteria used to identify those chemicals most likely to be carcinogenic. However, DBAN was recently shown to be carcinogenic in mice and rats (NTP, 2010). Neoplastic changes were noted in the oral cavity (rats) and the forestomach (mice) and glandular stomach (rats).

DBAN produced cancer in rats at a daily dose of 4 mg/kg, which results in a calculated unit risk value of approximately 0.2 µg/L (unit risk is defined as the concentration in drinking water that would increase lifetime risk of cancer by 1 additional cancer per million over a lifetime). Based on these data, the HNs may be approximately 3-10 X as potent as the THMs.

If it is assumed to be of equivalent potency to DBAN, the concentrations of 2,2-dichloropropionitrile found would be unlikely to increase cancer risk significantly even though they are mutagenic compounds.

3.5.3. Toxicological Evaluation of Haloamides

Acetamide has been classified as possibly carcinogenic in humans (IARC, 1999b) based upon the induction of liver tumors in rats and malignant lymphoma in mice (Fleischman et al., 1980) when given at 1.18 or 2.36% in the diet. Formamide administered by gavage at doses as low as 20 mg/kg day^{-1} induced clear evidence of malignant lymphoma in male mice, but no neoplastic changes were observed in rats (NTP, 2008). Acetamide, itself, is not mutagenic (Kennedy, 1986), nor does it damage DNA (Sakano et al., 2004), but its metabolite, acetohydroxamine, does produce adducts in the presence of metals. Therefore, there is evidence of toxicological effect with the amide class independent of halogen substitution.

The literature on the toxicology of haloamides is limited. A cancer bioassay has been conducted with chloropropamide by the National Cancer Institute (NCI, 1978). No evidence of carcinogenicity was observed in B6C3F1 mice to concentrations of 10,000 ppm of their diet or F344 rats to concentrations of 6000 ppm.

Analogs of dichloropropionamide are utilized as "safeners" that block the effects of thiocarbamate and chloroacetanilide herbicides (Walton and Casida, 1995). One of these, dichloramid (*N*,*N*-diallyl-2,2-dichloroacetamide), has a reasonably complete data base. NOAELs of 5 mg/kg day^{-1} from a 1 year study in dogs, 6.8 in a 2 year study in rats, and 7.0 in an 18 month study in mice have been established (U.S. EPA, 2005). The corresponding LOAELs were 20, 32.8, and 70.7 mg/kg day^{-1}, respectively. The NOAELs for reproductive and developmental studies were higher. The liver effects observed in rats are reminiscent of dichloroacetic acid, which is one of the metabolites of this compound.

Two haloacetamides have been found in chlorinated drinking water (Krasner et al., 2006) and are included in Table 4. The remaining compounds evaluated were propionamides that have yet to be sought in drinking water. Five of six propionamides met our criteria as probable carcinogens (the exception being the 3,3-dibrominated propionamide) and were predicted to be Ames' test mutagens. The predicted chronic LOAELs ranged from 0.5 to 3.2 mg/kg day^{-1}, approximately one order of magnitude below that of dichloramid.

3.6. Haloaldehydes

3.6.1. Formation of Haloaldehydes

Haloacetaldehydes have been known to arise in the chlorination of drinking water for some time. Several were identified and some measured by Krasner et al. (2006). Chloral hydrate (trichloroacetaldehyde) occurred frequently with median concentrations of 1, a 75th percentile concentration of 4, and a maximum concentration of 16 µg/L. Corresponding concentrations for dichloroacetaldehyde were 1, 4, and 14 µg/L, respectively. Bromochloroacetaldehyde was less frequently detected, with a maximum concentration of 1.3 µg/L and a mean among 12 systems of about 0.5 µg/L. The maximum concentration of tribromoacetaldehyde detected was 0.9 µg/L. Iodobutanal was found in two treatment plants and note was made of two tentatively identified haloaldehydes, dichloropropenal and 4-chloro-2-butenal.

3.6.2 Toxicological Evaluation of Haloaldehydes

Chloral hydrate is a weak carcinogen only in mice (IARC, 2004) and is of little toxicological interest as a carcinogen at the low concentrations detected in drinking water. Chloroacetaldehyde has been identified as a reactive intermediate of several well known carcinogens (e.g. vinyl chloride) and has been well established as a mutagen (IARC, 2004). The CPDB (Gold et al., 2010) indicates a TD_{50} for chloroacetaldehyde of 36.1 mg/kg day^{-1}, based upon the study of Daniel et al. (1992). The genotoxicity of other saturated (Loforth, 1978; Bignami et al., 1980) and unsaturated haloaldehydes (Rosen et al., 1980; Meier et al., 1985; Segall et al., 1985) has been studied extensively and have been found positive as mutagens. However, the *in vivo* toxicological studies of the haloaldehydes found in drinking water appear to be limited to chloroacetaldehyde and chloral hydrate.

Eighteen haloaldehydes were evaluated that were predicted to be carcinogens and to have chronic LOAELs of less than 10 mg/kg day^{-1}. Table 5 includes only those 4 haloaldehydes that met all of our criteria (valid predictions in 2 species with members in the training set with similarity indices of less than 0.2). It is notable that all of the compounds identified by the TOPKAT® analysis as being interesting as carcinogens were chlorinated, but brominated analogs that did not meet these criteria should also should be considered of importance. This reflects the much smaller variety of brominated compounds in the NTP and FDA databases. In general, brominated analogs can be considered at least as potent as the corresponding chlorinated analogs.

The 4-chlorobutenal and dichloropropenal tentatively identified by Krasner et al. (2006) met neither the chronic LOAEL or predicted carcinogenicity criteria.

3.7. Phenazine and Chlorophenazines

3.7.1. Formation of Phenazine and Chlorophenazines

In an investigation of the formation of *N*-nitrosodiphenylamine, Li and coworkers (2011) identified phenazine and an *N*-chloro derivative of phenazine in chloraminated water. Phenazine is formed in a competing reaction with *N*-nitrosodiphenylamine formation at more acid pH. These compounds are of interest because, to our knowledge, this is the first heterocyclic amine that has been demonstrated to be formed in chloraminated water.

3.7.2 Toxicological Evaluation of Phenazine and Chlorophenazines

There are no toxicological data on phenazine or chlorophenazines, *per se*, in the open scientific literature. Derivatives of phenazine are used as dyes (e.g. eurhodines, toluylene red, indulines, and saframines). It is also a moiety in natural products produced by several bacteria on a branch of the shikimic acid pathway (Price-Whelan et al., 2006). Carbon-bonded chlorophenazines have been evaluated as pesticides (Cross et al, 1968) with the monochloro substituted compounds being the most effective. A phenazine-water complex has been shown to abstract an electron from DNA bases in isolation (Choudhury and Basu, 2005). Some phenazine derivatives can be very active as redox agents (Davis and Thornalley, 1983). In the case of phenazine methosulfate, a radical cation is produced as it oxidizes reduced pyridine nucleotides within cells. Some N^6,N^{10}-dioxide phenazines are being evaluated as anti-tumor agents and are cytotoxic in the low µM range, *in vitro*. Simpler brominated 2-amino or 2-hydroxy analogs appear to be selectively cytotoxic under hypoxic as opposed to normoxic conditions (Lavaggi et al., 2008). These studies raise concerns in hazard assessment, but provide little information for risk assessment.

Phenazine and chlorophenazines were subject to QSTR analysis. The predicted chronic LOAEL for phenazine was 64.2 mg/kg (95% CI = 15.7- 262 mg/kg) (Table 6). This indicates that its probable chronic toxicity would occur at somewhat lower doses than chloroform, for

example. Phenazine was predicted to be a carcinogen, but not a mutagen in the Ames' test. Given the relatively high LOAEL, phenazine is unlikely to be a potent carcinogen.

N-Chlorophenazine was detected in drinking water (Li et al., 2011). There are no toxicological data available on this chemical and, as indicated earlier, the *N*-Cl group is not recognized by TOPKAT®. Nevertheless, QSTR consistently predicted *N*-chlorophenazine to be carcinogenic. This was more consistent than the predictions for phenazine, itself, in that 7 of the 8 models employed predicted a probability that it was carcinogenic >0.974. It was also predicted to be a mutagen. Predictions in TOPKAT® are based upon electropological characteristics of the molecule and therefore the prediction is made independently of the specific atoms in the molecule. Nevertheless, there were no *N*-chloramines in the training sets. Similarity indices were outside our criteria in 7 of the 8 models.

Substitution of chlorine on carbons of phenazine resulted in lower predicted chronic LOAELs, with LOAELs decreasing with the degree of chlorine substitution (Table 6). None were predicted to be carcinogenic or mutagenic (data on Ames mutagenicity not shown). None of the carbon-substituted phenazines were found in drinking waters (Li et al., 2011).

3.8. Formation of other classes of DBPs

There are many other documented and predicted DBPs that could be formed with chlorine or chloramine. In the interest of brevity, consideration has been limited to a set arrived at through our analysis that could have the potential of contributing to cancer risks of the magnitude suggested by epidemiological studies. The absence of other compounds from our analysis should not eliminate them from further consideration.

4. Discussion

The initial impetus for this project was to identify novel DBPs that could be formed by the reaction of chlorine or chloramine with substructures that are known to occur in NOM (Bull et al., 2006). We also considered several chemicals that have recently demonstrated to occur in drinking water for which there are reasons to be concerned about their hazards. All of these chemicals were assessed by review of effects of related compounds and/or through formal consideration of structure-activity relationships. A second group of chemicals is discussed

because they are drawn from a class which has not been studied in either humans or experimental animals (e.g. organic *N*-chloramines) despite the widespread recognition of their occurrence in chlorinated drinking water.

HQs were identified as a class of chemicals for which data on related compounds (hydroquinones and benzoquinones) imply high toxicological potency and probable carcinogenic properties. Halogen substitution on quinones could substantially increase their potency.. QSTR analysis of the HQs support the conclusion that they could present substantive hazards to health even at the low concentrations that seem to be present in drinking water. Now that data demonstrate the occurrence of four members of the class in drinking water (Zhao et al., 2010), it is probable that other members of this class will be found in drinking water based on the common occurrence of similar precursor structures in natural waters (Bull et al., 2006).

The toxicity of benzoquinones is modified significantly by halogenation. First, halogenation increases the O/W partition coefficient. Second, the introduction of halogens enhances metabolic activation of quinones. An early step in the metabolic activation of benzoquinones and polyphenols is conjugation with glutathione that is mediated by glutathione transferases. Toxicity of benzoquinones (most commonly assessed in the rat kidney) increases progressively with up to 3 glutathione substitutions. A fourth glutathione substitution decreases toxicity (Lau et al., 1988). These glutathione substitutions allow the compound to be transported into cells. Within cells, the glutamate and glycine residues are hydrolyzed from GSH leaving an adducted cysteine that readily forms an electrophilic sulfenamide cation that can react with macromolecules (Commandeur et al., 1995). Third, hydroquinones and their corresponding quinones can be involved in redox cycling to produce reactive oxygen species (ROS) that can contribute to adverse health outcomes, depending upon their redox potentials. A fourth mechanism involves the ability of the semiquinone and quinones to react with macromolecules (Lin et al., 1999). In the case of the latter two mechanisms, the addition of two or three chlorines to the glutathione-conjugated benzoquinone shifts the equilibrium towards the quinone form, which increases toxicity (Monks et al., 1990), but may result in non-specific degradation of the quinone before it reaches cellular targets. The transport processes that allow glutathione-conjugates to concentrate within cells is coupled with generation of reactive intermediates and the effect of halogen substitution on the redox properties of the quinone, all of which will dramatically influence toxicological properties of hydroquinones (Monks and Lau, 1998). Fifth,

the modification of the quinone structure by chloramine, which adds an *N*-chlorimine group, further increases the O/W partition coefficient. This combination of altered chemical and physical properties should focus attention on the HQ-4-(chloro)imine compounds that can be formed with chloramine (Heasley et al., 2004). Sixth, the toxic effects of the cysteine conjugates of the HQs is suppressed by *N*-acetylation and reactivated by *N*-deacetylation. Slow acetylators have long been known to be at greater risk for bladder cancer (Garcia-Closas et al., 2005).

Formation of the *N*-chloroimine derivatives of HQs could be sensitive to relatively small changes in source water characteristics and disinfection practice. Excess free chlorine would be expected to increase halogen substitution to the point of destabilizing the quinone ring structure. This will give rise to familiar terminal by-products of chlorination, such as the THMs and HAAs (Bull et al., 2006). Variation in treatment conditions is likely to complicate this relationship, as well. Chloramine has been shown to react with phenol or *m*-cresol to form several HQs, but it appears that the formation of members of the HQ-(chloro)imine class will be unique to chloramine (Heasley et al., 2004). A benzoquinone-imines are strong electrophiles and have been found to be the metabolite responsible for hepatotoxicity produced by acetoaminophen (Miner and Kissinger, 1979). This suggests that current regulatory strategies to control risk from chlorination by-products based upon the THMs and HAAs as surrogates might be misguided, since the use of chloramine instead of chlorine may result in a unique class of DBPs that could contribute to carcinogenic risk.

An attempt was made to address organic *N*-chloramines as a group of well recognized DBPs are formed, but for which chemical characterization has been limited. In the course of exploring nitrosamine formation, an *N*-chloramine of phenazine was discovered as a by-product formed by chloramine reactions with diphenylamine. Literature searches revealed general concerns about *in vivo* formation of *N*-chloramines and *N*-bromamines in the biomedical literature as products and potential mediators of damage in inflammatory processes. As these compounds are known to be formed in drinking water, characterizing the toxicological properties of 2 or 3 compounds with varying chemical properties (e.g. stability to hydrolysis, lipophilicity) within this group should be given high priority.

The recent availability of chronic data on DBAN (NTP, 2010) reinforce screening data that suggest that halonitriles probably represent carcinogenic hazards. Subchronic data also indicate that there are some mild thyroid effects for DBAN (Poon et al., 2003). Formation of longer

chain halonitriles have been demonstrated in laboratory studies of reactions of chlorine with isoleucine (Nweke & Scully, 1989). Therefore, the possibility that there is a larger halonitrile pool of DBPs needs to be explored. QSTR analyses of 3-carbon halonitriles indicated that some could be of interest as potential carcinogens if they occurred in a concentration range of 0.1-10 μg/L. Dihaloacetonitriles are present within this range. However, the modest concentrations of 2,2-dichloropropionitrile (Li et al., 2011) preliminarily suggest that longer chain HNs may not occur at sufficient concentrations to be of concern.

The QSTR evaluation was generally consistent with two prior efforts for prioritization of DBP research. Moudgal et al. (2000) also utilized TOPKAT®, but limited their analysis to simple identification of chemicals that were probable carcinogens or developmental toxicants. Woo et al. (2002) utilized expert judgment SAR analysis to identify those DBPs that are most likely to be carcinogenic and provided a relative estimate of potency based upon related chemicals. While the predictive approach used in the Woo et al. study was more analogous to that of the present study, its analysis was limited to DBPs that have been shown to occur in drinking water. They identified MX and some other halofuranone derivatives as a high priority class. A second tier of chemicals that included selected unsaturated and saturated halonitriles, and halopropanones (e.g. 1,3-dichloropropanone), and haloaldehydes (e.g. dichloroacetaldehyde) were identified as likely to be a moderately active multispecies/target carcinogen at low doses. Thus, the findings of our analysis were generally consistent with those of Woo et al. (2002).

The present study differed from the two prior efforts in that it did not limit the analysis to chemicals actually demonstrated in drinking water, but included chemicals whose formation was considered likely based on predictions of reactions with organic substructures within NOM. This effort resulted in the identification of several potential DBPs that could be of considerable interest as general toxicants and/or as carcinogens (Zhao et al. 2010; Li et al., 2011). Specifically, this work calls attention to the HQs and non-halofuranone members of the halocyclopentene class as compounds that are likely to have high toxicological potency and with properties suggesting they could help explain epidemiological associations of chlorinated drinking water with cancer. There have been no toxicological data confirming that the group can express those toxicological properties *in vivo*. Moreover, established or related classes of DBPs were identified that have a high probability of occurring in drinking water that have received very limited toxicological characterization (*N*-halamines, haloamides, halonitriles, and

haloaldehydes). These classes of DBPs should be considered as potential contributors to cumulative risks. Finally, the first heterocyclic amine DBP and a chlorinated derivative (chlorophenazine and *N*-chlorophenazine) to be identified in drinking water were also evaluated.

The initial impetus for this study was the perception that regulated DBPs (i.e. the THMs and HAAs) were too weak as carcinogens to account for the cancer risks that have been associated with chlorinated drinking water. This comparison was based on the potency of the individual regulated DBPs in animal studies relative to the population attributable risk (PAR) derived from meta-analyses of epidemiological studies (Bull et al., 2006). There are a number of epidemiological studies that address selected polymorphisms in enzymes that are known to metabolize the THMs and HAAs that might modify the gap between risks calculated for the THMs and HAAs from animal studies and the epidemiological studies of chlorinated water (often using individual or collective concentrations of THMs as the dose-measure). As illustrated from this project and other data in the literature, there is clearly an opportunity for hundreds of other by-products to co-occur with the regulated DBPs. Many of these should also correlate with cancer risk and selection of one set of DBPs without ruling out others can result in purely trivial attributions to that class. The key question is which of these associations are causal?

If only those by-products within the THM class that have been shown to both be carcinogenic and genotoxic (i.e. excludes chloroform and dibromochloromethane) the risk attributed to this class at the highest exposure level identified in a recent survey of U.S. and Canadian drinking waters (Krasner et al., 2006) is approximately 20×10^{-6} per lifetime based upon the upper bound unit risks calculated for animals studies by U.S. EPA (2003a). This risk is dominated by calculations based on the original BDCM bioassay using corn oil gavage (19×10^{-6}). The risk attributable to BDCM will undoubtedly decrease significantly once the newer drinking water study of BDCM (NTP, 2005) is taken into account. The 2005 bioassay was negative at a dose that should have produced a small, but significant increase in tumor incidence if a linear low dose extrapolation were applied to the results of the corn oil gavage study. Calculations of risk posed by the dihaloacetic acids [bromochloroacetic acid (BCA) and dibromoacetic acid (DBA)] that are both carcinogenic and genotoxic based upon recent bioassays (NTP, 2007; 2009) can add an additional upper bound risk of 56×10^{-6} per lifetime. While DCA have been found genotoxic in some studies, the concentrations necessary to produce

these effects are so high relative to the systemic concentrations that produce cancer in animals as to make them irrelevant to *in vivo* carcinogenesis (Moore and Harrington-Brock, 2000). This conclusion is bolstered by the fact that DCA has been shown to selectively stimulate the growth of already initiated cells (Stauber and Bull, 1997), which can completely account for the development of liver cancer (Miller et al., 2000) at much lower systemic concentrations. TCA is also excluded as a non-mutagen. It is notable that it produces liver cancer in mice, not rats. TCA induced liver cancer is associated with peroxisome proliferation, a mode of action that does not appear active in humans (Gonzalez and Shah, 2007). It is important to observe that median risk based upon most likely estimates of the linear low dose extrapolation and using median drinking water exposures would decrease these cancer risk estimates substantially.

In contrast, an analysis by the U.S. EPA (1998) suggested that the population attributable risk (PAR) to chlorinated drinking water ranged from 2 to 17% of bladder cancer cases in the U.S. This equates to a range of 700 and 6,000 additional cases of cancer per 10^6 per lifetime in men and somewhat lower risk in women (Bull et al., 2006). The comparison with the risks derived from animal data indicates that it is not reasonable to attribute a cancer risk of this magnitude to THMs or HAAs in drinking water without clear explanation of why humans should be so much more sensitive to these chemicals than animals.

A recent epidemiological study by Cantor et al. (2010) found interactions in THM association with bladder cancer with several polymorphisms in enzymes that are involved in the metabolism of THMs and HAAs. Evidence of a mutagenic metabolite of BDCM produced as an intermediate by a glutathione transferase isozyme, *GSTT1*, has been reported (Pegram et al. 1997). Cantor et al. (2010) found that individuals not expressing this enzyme were at lesser risk for bladder cancer that has been associated with the consumption of chlorinated drinking water. In addition, the CC variant of CYP2E1, an enzyme known to be a somewhat more efficient catalyst in the metabolism of THMs, was found at higher frequency in bladder cancer cases and displayed an interaction with THM levels, while those who carried a mutation that results in the substitution of a histidine for arginine at position 76 of the protein did not. This mutation is slightly less effective as a catalyst, but the main effect appears to be suppressed expression as a protein. The expression of less active variants of *GSTZ1* towards the dihaloacetic acids were observed among bladder cancer cases exposed to higher levels of THMs. Most interesting was an increase in odds ratios (OR) for bladder cancer to 5.9 in individuals with *GSTT1* present and

the *GSTZ1* DT/TT variant when exposure was stratified against total THM concentrations. These are important observations, but they do not force the conclusion that THMs are responsible for bladder cancer.

First, it has to be recognized that kinetics dictate that there will be very small differences in THM and or HAA metabolism among these enzyme variants at the low systemic concentrations that would result from the amounts that are obtained from water by any route of absorption. Cytochrome P450 2e1 (the *CYP2E1* protein product) has be highest affinity for THMs (K_m = 0.15 µM) and has a high maximum rate of catalysis (V_{max} = 28.3 nmol/h/mg protein) (Lipscomb et al., 2004). At an oral dose equivalent to 72 µg/kg, peak concentrations of BDCM in blood were 2.6 ng/L (equivalent to 0.016 nM) and 90.5 ng/L (0.76 nM) from a dermal exposure of an hour to a dose-equivalent concentration were observed in human volunteers (Leavens et al., 2004). These concentrations are roughly two orders of magnitude below the K_m and the relative V_{max} between the variants is so small that there would be no appreciable difference in their metabolism of the THMs at concentrations that occur in drinking water. It is notable that Backer et al. (2008) found no evidence that cytochrome P450 2e1 activity had any effect on THM levels 10 minutes following a shower with household drinking water.

The K_m of DCA for *GSTZ1* is 71.4 µM and the V_{max} is 1334 nmol/min. mg protein. (Tong et al. 1998b). This reflects the huge capacity for oxidation of the dihaloacetic acids in humans. Again, the systemic concentrations of the HAAs at exposures found in drinking water are several orders of magnitude below this K_m (Schultz and Shangraw, 2006). Therefore, it is difficult to see how expression of the different variants of *GSTZ1* could affect the disposition of these compounds at low doses as the relative specific activity of the four variants studied to date differ by only by a factor of five (Board et al. 2001).

GSTT1 is known to be active in producing a mutagenic metabolite with the brominated THMs. The apparent K_m for chloroform is sufficiently high and the V_{max} low, essentially rendering this pathway irrelevant even at the high carcinogenic doses (e.g. 90 mg/kg) in animals. On the other hand, it is effective with brominated THMs and the lesser risk attributed to individuals that do not express this enzyme is a more attractive explanation for differences in humans susceptibility than the polymorphisms of *CYP2E1* and *GSTZ1*. However, the low activity and high K_m of THMs for this enzyme mean that it will not compete well with cytochrome P450 2e1 in the metabolism of brominated THMs at low doses (Ross and Pegram,

2004). It will become important primarily when THM concentrations begin to exceed their K_m for cytochrome P450 2e1.

The differences in metabolism of THMs and HAAs at doses that are obtained from drinking water will not be appreciably different among individuals in the population based on the polymorphisms. This is because at systemic concentrations much below the K_m the amount of the enzyme that is present is not rate limiting to metabolism. Therefore, virtually the same amount of metabolism will occur irrespective of the polymorphism.

Second, it is important that many by-products of chlorination interact with glutathione. These include halonitriles (Lin and Guion, 1989), halopropanoic acids (Tong et al., 1998a), chloropicrin (Schneider et al. 1999), the halofuranones (Clark and Chipman, 1995; Febry et al., 2011), halopropanols (Hammond et al., 1999), halopropanones (Merrick et al., 1987) and as illustrated in this paper, the HQs. The isoforms of glutathione transferase involved in forming unstable or stable conjugates of some of these by-products have not been characterized.

Third, there have been suggestions that the relative importance of ingestion, inhalation, or dermal exposure to THMs has not been adequately considered. Villanueva et al. (2007) found individuals with dermal and inhalation exposure to THMs (largely through showering and bathing) had higher risks of bladder cancer than those who only ingested chlorinated drinking water. Indeed a high rate of absorption of BDCM through human skin was demonstrated by Leavens et al. (2007) that would support such a relationship. However, polymorphisms in the enzymes that metabolize the THMs still do not significantly close the gap in attribution of bladder cancer risk to THM exposure.

A follow-up to the Villanueva et al. study associated brominated THM concentrations in expelled breath with an increased frequency of micronucleated lymphocytes and increased levels of urinary mutagenicity (Salmonella strain YG1024) following a session of swimming (Kogevinas et al., 2010). This suggested the involvement of by-products with significant vapor pressure. The association with the *GSTT1* was not confirmed. In contrast, there was an apparent increase in micronuclei in exfoliated urothelial cells in individuals with the normal *CYP2E1* variant vs. the alleles that express the form with the arginine to histidine mutation in position 76. An increase in peripheral blood lymphocytes with micronuclei was observed in a group that expressed the *GSTZ1* GG variant vs. those with the AG-AA variant. The actual expression of these proteins in these individuals was not measured. Most important, however, is that neither

paper provides an explanation of how variation in these isoforms could be a sufficient basis of alterations of metabolism of THMs (or HAA) at doses likely to be derived from consuming and bathing with drinking water which have concentrations documented in their studies.

Richardson et al. (2010) reported analyses of water in chlorinated and brominated Spanish swimming pools from which subjects were drawn in the epidemiological study of Kogevinas et al. (2010). They identified a wider variety of by-products than are ordinarily found in chlorinated drinking water. It is notable that some of the DBPs other than the THMs that were identified have significant vapor pressures and could contribute to inhalation risks. Among these by-products were chemicals that ranged from relatively non-polar (e.g. haloketones) to non-polar (HQs) that could well be absorbed through the skin. From the present study, several of these compounds are expected to have a high toxicological potency. Some of the chemicals could well present a significant hazard by dermal or inhalation exposure.

For the above reasons, the thesis that regulated DBPs are of insufficient carcinogenic potency to account for the cancer risk associated with the chlorination of drinking water has not been refuted by recent epidemiological studies. It is suggested that some of the compounds identified for study in this manuscript might account for some of the discrepancy in potency. The discrepancy in target organ specificity is also of concern, but this difference may lie in species differences in sensitivity. On the other hand, the metabolism of several of the DBPs identified here (specifically the HQs and related compounds) have characteristics that parallel the schemes of the well-known bladder carcinogens in the aromatic amine class and should be investigated.

Acknowledgments

This work was supported in part by Water Research Foundation Contracts No. 2867 and 4089. In-kind contributions in the former case were by MoBull Consulting, the University of Massachusetts Amherst, Municipal Water District of Southern California and East Bay Municipal Water District. The second project was conducted by the University of Alberta (UA), with in-kind contributions from UA, MoBull Consulting, Australian Water Quality Centre, and Curtin University. In addition, we thank the following cooperating utilities: Charleston Water System, Charleston, SC, USA; Louisville Water Company, Louisville, KY, USA; Santa Clara Valley Water District, San Jose, CA, USA; Water Corporation of Western Australia, Perth, Australia; Alberta Research Council, Vegreville, Alberta, Canada; Institute for Biological Sciences, NRC, Ottawa, Ontario, Canada

Figure 1. Relationship between the rat TD_{50} and the predicted chronic LOAEL. Panel A: Relationship for 94 chemicals randomly selected from the Carcinogen Potency Database (CPDB)(Gold et al., 2010). Panel B: The relationship between actual NOAELs and chemicals found the CPDB. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 2a. Formation of tetrachloro-*p*-quinone from 1,4-dihydroxybenzene.

Figure 2b. Mechanism of halo-*o*-quinone formation from 1,2-dihydroxy-tetrachlorobenzene. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 3. Degradation of tetrachloro-*o*-quinone to haloacids, chloroform and CO_2. Two alternative pathways exist, the one with solid arrows leads to chloroform and CO_2, the alternate pathway leads to dichloroacetic acid and chloroform. The *p*-quinone is degraded by similar mechanisms. Details available in Bull et al. (2006)

Figure 4. Illustration of the formation of halogenated quinones by reaction of chlorine or chloramines with phenols and alternative formation of an quinone-4-(*N*-chloro)imine with chloramine. (Drawn from data in Heasley et al. 2004)

Figure 5. Mechanisms by which haloquinones may bind to macromolecules contributing to cytotoxicity or/or genotoxicity. A key to toxicity induced by quinones is that the glutathione and cysteine conjugates, produced largely in the liver, are transported via blood and bile to tissues where they may be concentrated in cells that express γ-glutamyl transferase (γ-GT) or glutathione transporters. Once in these tissues, they are further metabolized to thiol compounds that may be directly toxic or that are metabolized to reactive intermediates. This is illustrated in the γ-GT-expressing cells. Similar metabolic processing can occur in cells that concentrate glutathione (not shown). *N*-acetylation of the cysteine conjugate detoxifies the compound. This latter reaction may occur on the amino group of one or more cysteine residues.

Figure 6. Predicted changes in octanol/water partition coefficients with halogen substitution on hydroquinones, quinones, methyl-quinone, and quinone-4(*N*-chloro)imines. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 7. Formation of *N*-halamines and illustration of how they may represent partially toxicologically activated compounds. With primary and secondary amines (A and B), it is anticipated that the first intermediate will be produced in drinking water and the remaining reactions occurring in vivo as the result of metabolic activation. *N*-chloramine of tertiary amine is unstable and reactive intermediates will be produced in water. Example A – products of reaction of amino acids with HOCl (Davies et al., 1993). Example B – products of reaction of acetamide with HOCl (Hawkins and Davies, 1998). Example C – products of reaction of aminopyrine with HOCl (Utrecht et al., 1995).

Figure 8. Illustration of the transfer of *N*-chloro groups from a non-permeant amino acid (illustrated as *N*-chlorotaurine above) to a permeant amino acid to effect oxidation of intracellular thiols. The *N*-chloramino acid can also react with macromolecules to produce carbon- or nitrogen-centered radicals as illustrated in Figure 4.

Figure 9. General scheme for carbonyl and cyano formation from chlorination of amines and amino acids. Compound I is a *N*-chloramine, II a *N*-dichloramine, III, an imine, IV, a *N*-chloroamide, V is either an aldehyde (R_1 – H) or a ketone (R_1= -$[CH_2]_{0-X}CH_3$.)

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Potential Carcinogenic Hazards of Non-regulated Disinfection By-products: Haloquinones, Halo-Cyclopentene and Cyclohexene Derivatives, *N*-Halamines, Halonitriles, and Heterocyclic Amines

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Abstract

Drinking water disinfectants react with natural organic material (NOM) present in source waters used for drinking water to produce a wide variety of by-products. Several hundred disinfection by-products (DBPs) have been identified, but none have been identified with sufficient carcinogenic potency to account for the cancer risks projected from epidemiological studies. In a search for DBPs that might fill this risk gap, the present study projected reactions of chlorine and chloramine that could occur with substructures present in NOM to produce novel by-products. A review of toxicological data on related compounds, supplemented by use of a quantitative structure toxicity relationship (QSTR) program TOPKAT® identified chemicals with a high probability of being chronically toxic and/or carcinogenic among 489 established and novel DBPs. Classes of DBPs that were specifically examined were haloquinones (HQs), related halo-cyclopentene and cyclohexene (HCP&H) derivatives, halonitriles (HNs), organic *N*-chloramines (NCls), haloacetamides (HAMcNs), and nitrosamines (NAs). A review of toxicological data available for quinones suggested that HQs and HCP&H derivatives appeared likely to be of health concern and were predicted to have chronic lowest observed adverse effect levels (LOAELs) in the low µg/kg day^{-1} range. Several HQs were predicted to be carcinogenic. Someeveral have now been identified in drinking water. The broader class of HNs was explored by considering current toxicological data on haloacetonitriles and extending this to halopropionitriles. 2,2-Dichloropropionitrile has been identified in drinking water at low concentrations, as well as the more widely recognized haloacetonitriles. The occurrence of HAcNs HAMs haves been previously documented as occurring in drinking water. The very limited toxicological data on HAcNs HAMs suggests that this class would have toxicological potencies similar to the dihaloacetic acids. Organic *N*-halamines are also known to be produced in drinking water treatment and have biological properties of concern, but no member has ever been characterized toxicologically beyond bacterial or *in vitro* studies of genotoxicity. The documented formation of several nitrosamines from secondary amines from both natural and industrial sources prompted exploration of the formation of additional nitrosamines. *N*-Diphenylnitrosamine was identified in drinking waters. Of more interest, however, was the formation of phenazine (and subsequently *N*-chorophenazine) in a competing reaction. These are the first heterocyclic amines that have been identified as chlorination by-products.

Consideration of the amounts detected of ~~each of~~ members of these by-product classes and their probable toxicological potency suggest~~s~~ a prioritization for obtaining more detailed toxicological data of HQs > HCP&H derivatives > NCls > HNs. Based upon a ubiquitous occurrence and virtual lack of *in vivo* toxicological data, NCls are the most difficult group to assign a priority as potential carcinogenic risks. This analysis indicates that research on the general problem of DBPs requires a more systematic approach than has been pursued in the past. Utilization of predictive chemical tools to guide further research can help bring resolution to the DBP issue by identifying likely DBPs with high toxicological potency.

Keywords

Chlorination by-products, Structure-toxicity relationships, Haloquinones, *N*-Chloramines, Halonitriles, Heterocyclic amines

1. Introduction

Chlorination of drinking water has been consistently linked with an increased risk of bladder cancer in different geographic areas around the world (IARC, 2004) and less consistent associations were found with cancers of other organs. The odds ratios obtained in epidemiological studies are small, but the numbers of cases that would be attributable to chlorination could be relatively large compared to other chemical exposures from the environment (Morris et al., 1992; Poole, 1997; Villanueva et al., 2002; 2004). The USEPA's background document for the Stage II Disinfection By-product Rule (USEPA, 2003a & b) implies a lifetime cancer risk from chlorinated drinking water of approximately one additional cancer per thousand population per lifetime (Bull and Reckhow, 2008).

Regulation of disinfectant by-products (DBPs) from the chlorination of drinking water has focused upon trihalomethanes (THMs) and haloacetic acids (HAAs). The members of these classes that have been studied in experimental animals are weak carcinogens (Bull et al. 2006). Therefore, they would appear as improbable causes of risks of the magnitude that have been suggested by epidemiological data. At the concentrations that are found in drinking water and assuming an equivalent potency of THMs and HAAs for humans as found in experimental animals, the potencies of these chemicals are at least two orders of magnitude too weak as carcinogens to significantly contribute to the risk associated with chlorination of drinking water (~~based on linear extrapolation of data from animals~~more fully documented in discussion section). This simple comparison raises the question of whether much more potent carcinogens are produced in the chlorination of drinking water than those routinely measured in response to regulation.

Water from surface sources contains organic and inorganic chemicals that are largely of natural origin. Natural organic matter (NOM) in water is a complex mixture comprised of small amounts of nutrients (e.g. simple amino acids and sugars) to relatively large conglomerates of biological products (Leenheer et al., 2000). Within this amorphous material, a substantial portion of the total organic carbon (TOC) is comprised of complex polymeric structures known as humic and fulvic acids. The structures of these organic acids include a large variety of moieties comprised of substituted phenols, furans, and heterocycles connected by aliphatic carbon chains. There are numerous carbonyl and hydroxyl substitutions. Substructures within

fulvic and humic acids have been described by degradation studies, including the use of pyrolysis (e.g. Martin et al., 1994) and functional groups detected using a variety of direct and indirect methods (e.g. Ritchie and Perdue, 2003). From these studies numerous substructures have been identified (Schulten and Schnitzer, 1998). Utilizing these substructures to predict the types the nature of reactions that will occur with chlorine or chloramine allows prediction of both halogenated and non-halogenated organic by-products to be tractable. A key question is whether by-products formed with alternate forms of disinfection (e.g. chloramine, chlorine dioxide, or ozone) differ significantly from those formed with free chlorine. Epidemiological data suggest that risk from bladder cancer is reduced when chloramine is introduced in place of free chlorine (Zierler et al., 1988; McGeehin et al., 1993). Similar findings have been reported when ozone was used in advance of treating water with chlorine (Chevrier et al. 2004). However, these case control studies of alternative disinfectants have focused exclusively on bladder cancer. Therefore, they do not indicate absence of carcinogenic risk at other sites (e.g. GI tract, kidney), which may be produced by products that differ from those derived from simple chlorination.

In the present study, chemicals that were probable products of reactions of the disinfectants with substructures within NOM, as well as selected members of established DBP classes have been evaluated for probable carcinogenic properties. The toxicology of compounds closely related to these DBPs was explored by an extensive literature search that included related compounds as well as the DBPs of concern. This was supplemented by a quantitative structure toxicity relationships (QSTR) analysis to provide a basis for judging whether these classes were likely to add significantly to hazards already assigned to DBPs that have been subject to regulation, the THMs and HAAs. Several compounds and classes were identified as candidates of sufficient potency to be plausible causes of cancer outcomes. In the meantime, several of these DBPs have been identified in drinking waters (Zou et al.. 2000; Krasner et al., 2006; Zhao et al., 2010). Therefore, the present paper is an assessment of whether these DBPs are likely to contribute to cancer risk.

2. Methods

2.1. Literature sources of information on formation of DBPs

Chemicals and chemical classes that are established by-products of disinfection were identified in the review of Richardson (1998) and subsequent original contributions by her laboratory and her associates (Richardson 1999a & b; Richardson et al. 2003). A large number of papers in the primary literature that documented formation of by-products by reaction with compounds that could be viewed as probable constituents of NOM were also reviewed. A more comprehensive review of DBPs and their health effects was recently published by (Richardson et al. (2007). The source of information on specific DBPs ~~is literature~~ is identified as each ~~the particular~~ class ~~of DBPs~~ is discussed.

2.2. Prediction of By-Product Formation from Substructures of NOM.

Many DBPs are produced in disinfection that have not been chemically identified. This is evidenced by less than half of the total organic halogen being accounted for by established chlorination by-products. Therefore, a major part of this effort was the prediction of reaction products of chlorine or chloramine with natural organic matter (NOM). The approach taken was to predict intermediates and products of reactions between substructures of NOM. This allowed a broader evaluation of the possible DBPs that would arise from chloramination or chlorine disinfection. In the present paper, to conserve space, a description of the pathways postulated to be responsible for the formation of novel DBPs are provided only for those compounds that were subsequently found to be of toxicological interest.

Only a small portion of the chemicals studied are discussed in this paper. A detailed list of the chemicals subjected to study is provided ~~in the project report (~~in Bull et al.~~,~~ (2006). Some of the by-products discussed were identified in drinking water in a subsequent ~~project~~ report (Li et al., 2011) and are more explicitly addressed here.

2.3. Literature Evaluations of Probable Toxicity of Predicted By-Products

Toxicological data that addressed the comparative toxicity of chemicals in the same or related chemical class were considered most important in determining the likely potency of putative DBPs as chronic toxicants or carcinogens. Data that addressed mechanisms of toxicity, structural and/or functional group contributions to toxicological potency, coupled with some

descriptive toxicological studies within the class was considered the most dependable means of identifying DBPs of potential interest. This was accomplished by reviewing individual scientific papers, authoritative reviews of the toxicology of particular classes, and selected databases. The most useful database was the Cancer Potency Database (CPDB)(Gold et al., 2010). Seven groups of DBPs appeared most interesting and were explored in some detail; haloquinones (HQs), halo-cyclopentene & cyclohexene derivatives (HCP&H), including halofuranones, *N*-chloramines (NCls), haloacetamides (~~HAcNs~~HAMs), halonitriles (HNs), haloaldehydes (HAs), nitrosamines (NAs), and heterocyclic amines.

2.4. Quantitative Structure Toxicity Relationships (QSTR)

Many DBPs belong to chemical classes that have received little or no toxicological characterization. To supplement our analysis of the literature, we employed a QSTR program. TOPKAT® (Accelrys, 2001) was chosen among several commercial programs that were available that approach structure-activity relationships in somewhat different ways. TOPKAT® was selected primarily because of the diversity of endpoints that can be addressed with the series of models that are part of this software package. The main point of this effort was to identify compounds that would have effects at low doses (i.e. high toxicological potency). Therefore, the model in TOPKAT® that predicts chronic lowest observed adverse health levels (LOAELs) in rats provided a means of estimating the toxicological potency of each DBP.

Within the TOPKAT® model formulations, descriptors are utilized that relate to the rates of absorption of a chemical such as molecular bulk, shape, and symmetry. These are combined with descriptors that quantify the chemical properties of a compound. The chemical properties utilized by TOPKAT® relate to the electropological state (E-state) values developed by Kier and Hall (Accelrys, 2001) to quantify the electronic attributes of molecular structure governing molecular interactions that give rise to toxicological effects.

The predictions of individual models or sub-models within TOPKAT® are very dependent upon their individually constituted training sets. For example, there are separate models for mice and rats and for males and females within each of these species for predictions of carcinogenicity. In addition, there are different models based on the two large databases that are available, those of the National Toxicology Program (NTP) and the U.S. Food and Drug

Administration (FDA). TOPKAT® makes independent predictions from the two databases. Widely varying predictions are frequently observed among~~between~~ models based on these differing databases. Divergence in the predictions occurs largely because classes of chemicals are differentially represented in these databases. Differences also occur because related chemicals in the training sets were only studied in a single sex, in a single species, or in different mouse or rat strains.

TOPKAT® provides three ways of judging the confidence that can be placed in a prediction. The first is an indication of whether or not the prediction is within the optimum predictive space (OPS) of the model. The program distinguishes between predictions that are outside the OPS, but within permissible ranges, and those that exceed the permissible range. In this project, those predictions that exceed the permissible range were interpreted as "no prediction" and there was no attempt to compile lists of compounds that were predicted to be non-carcinogens. Secondly, within each model's database, the program provides a measure of the similarity of the query structure with the chemicals that were included in the equation on which the prediction is based. Measures of similarity ranged from 0.000 (identity) to 1.0 (no similarity). Finally, the program indicates when a molecular fragment in the query structure is not represented in the model's database. These qualifications on a prediction are identified in the assembled database and carried over to tables presented in this paper.

Explicitly, the criteria for identifying putative DBPs as high priority for research on the basis of their occurrence drinking water and characterization of their toxic properties were as follows:

1. They are from a chemical class that has recognized toxicological properties and a data base for estimating whether the derivative forms identified as DBPs might be of greater or lesser toxicological potency.
2. Chemicals with an actual or predicted chronic LOAEL < 1 mg/kg were considered to be of high interest, irrespective of whether or not they were predicted to be carcinogens.
3. Carcinogens or predicted carcinogens with doses that produce a 50% increase in cancer in a lifetime (TD_{50}s – available online, Gold et al. 2010) or actual or predicted chronic LOAELs of < 10 mg/kg were considered to be of high interest.

4. A probability of 0.7 was required for a positive prediction of carcinogenicity with any given QSTR model. Predictions that were outside the permissible limits of the OPS of the specific model used were discarded.
5. A positive prediction of carcinogenicity by TOPKAT® was accepted only if at least one chemical in the training set of the model used had a similarity index of < 0.2.
6. Predictions of carcinogenic activity were considered of greater interest the more consistent the prediction was among species (rats and mice) and sex. Both the NTP and FDA models for each species and sex were utilized. Positive prediction of carcinogenic activity in two species in addition to other criteria described above was required before a compound was identified as a high priority for study as a probable carcinogen. A positive prediction within either database satisfied these criteria. In effect, this treats the databases (or training sets) for the FDA and NTP models as complimentary rather than competing models. This is justified because disagreement among models based on the NTP and FDA databases was most commonly attributable to the absence of chemicals of the class in one or the other of the databases. To increase the stringency of the predictions, we required that a positive prediction meeting the criteria described above was observed in two species to classify a compound as a predicted carcinogen based solely on QSTR analysis.
7. Finally, a DBP predicted to be a carcinogen and to be positive with respect to Ames' mutagenicity was ranked higher in priority than a chemical not meeting the mutagenesis criteria, but having a similar LOAEL.

In the overall project, a total of 489 chemicals were evaluated. Only a fraction of these chemicals are discussed in this paper. Emphasis in this paper was on DBPs that were considered highly probable to form or have been detected in drinking water, but individually have little or no chemical-specific toxicological data available. The entire list of chemicals examined and associated databases are available in a report published by the Water Research Foundation ~~to the American Water Works Association Research Foundation~~ (Bull et al., 2006).

3. Results

3.1 Testing the relationship between predicted chronic LOAEL and carcinogenic potency

The predicted chronic LOAEL was the primary measure of toxicological potency in the absence of empirical data. We were concerned that using the same cut-off for carcinogens as other chronic toxicities might inappropriately exclude important carcinogens because the chronic LOAEL might be a poor predictor of carcinogenic potency. Gaylor and Gold (1995) described a relationship between maximum tolerated dose (MTD) and carcinogenic potency. This hypothesis was tested by determining how close TD_{50}s of carcinogens in the CPDB were approximated by predicted chronic LOAELs. Figure 1A compares the TD_{50}s 111 randomly selected chemicals that are in the Carcinogen Potency Database (Gold et al., 2010) with chronic LOAELs for the same chemicals predicted with TOPKAT®. Seventeen of these compounds were found to be outside the optimum predictive space of the TOPKAT® model, so the correlation developed included 94 of these chemicals. While there was a significant correlation ($r = 0.42$), it is far from an ideal relationship. A virtually identical correlation was observed between the TD_{50} and predicted chronic LOAEL of the 46 halogenated chemicals in the group (data not shown). The predicted LOAEL was approximately equivalent to the TD_{50} at 30 mg/kg day^{-1}. However, there was a departure of one order of magnitude in the two values at 0.1 mg/kg day^{-1}, and a further divergence at lower doses. This implies that potent carcinogens are frequently active at doses which do not present with obvious evidence of chronic toxicity. Thirty-four of the compounds examined in Figure 1A were also in the TOPKAT® training set. The relationship between the chronic LOAEL with the TD_{50} for these compounds was better ($r = 0.72$) (Figure 1B). Nevertheless, the best fit line still deviates by about an order of magnitude at 0.1 mg/kg day^{-1}. The tendency for the TD_{50} to be significantly less than the LOAEL was compensated for by identifying the DBP as a high priority for research if it was identified as a carcinogen and had a predicted chronic LOAEL of < 10 mg/kg day^{-1}.

3.2. Haloquininones

3.2.1 Formation of Haloquinones from Substructures of NOM

Haloquinones (HQs) may arise from the reaction of chlorine with a variety of naturally-occurring organic compounds. The approach is described in more detail with this class to

illustrate the approach. Formation of DBPs in other classes are described in less detail for the sake of brevity. However, the predicted pathways of formation are detailed in Bull et al. (2006).

One likely reaction pathway is by substitution of chlorine on the ring of the preformed quinone. Electrophilic aromatic substitution of chlorine for hydrogen may also occur in parallel with oxidation of the hydroquinone to a quinone. The degree to which the former occurs before the latter determines the extent of halogenation of the HQ product. When substitution is kinetically favored the product is a fully halogenated *p*-quinone or *o*-quinone structure from 1,4- and 1,2-dihydroxybenzene, respectively (Figure 2a and 2b) as supported by Sarkanen and Dence (1960). As chlorine atoms are added to the ring, the acidity of the ring OH groups will increase. This should help to keep the reaction rate high even at neutral pH where the anionic form may not be dominant. If this is the major pathway, there must be subsequent loss of HCl, largely giving oxygenated aliphatic products. This is based on the low TOX yield (i.e. < 1 M/M) reported for chlorinated solutions of 1,4- and 1,2-dihydroxybenzene (Reckhow & Singer, 1985).

The fully halogenated quinones could arise from other simple precursors as well. For example, catechol or hydroquinone derivatives with carboxylic acid groups directly bound to a ring carbon are prime candidates. These should slowly decarboxylate, with accompanying halogen substitution in the presence of chlorine, leaving the corresponding halogen substitution product (e.g., see Larson & Rockwell, 1979), which can then be transformed into the *p*-quinone and *o*-quinones. Again, the presence of chlorine on the aromatic ring prior to decarboxylation should elevate the equilibrium concentration of the reactive anionic form of the carboxylic acid. In addition, there are many larger substituted structures that can degrade to phenolic-carboxylic acid forms that can also enter this reaction pathway. Many naturally-occurring aromatic compounds and macromolecules (e.g., lignins, tannins) are potentially susceptible to production of HQ precursors upon treatment with chlorine.

Logical subsequent degradation pathways for tetrachloro-*o*-quinone are shown in Figure 3. The degradation roughly follows the scheme proposed by Zou et al. (2000). These authors were investigating a rather minor sub-pathway leading to the potent mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) ~~(3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone).~~ The alternative terminal section of the pathway (shown with a dashed line) involves simple decarboxylation of the intermediate, which may be more likely under conditions

of low chlorine residual. This produces 1,1,3,3- tetrachloropropanone which quickly gives rise to dichloroacetic acid and chloroform (details not shown) (Reckhow et al., 1985). While model compound data do not support either of these as the major pathway for HQ degradation, they may explain some of the observed TOX formation.

Heasley et al (2004) have demonstrated formation of HQs and related compounds on reaction of monochloramine with phenolic precursors. The outline of the reactions of 2,4,6-trichlorophenol with either NH_2Cl or $NHCl_2$ giving rise to a mixture of 2,6-dichloro-1,4-benzoquinone (DCBQ) with 55% yield and 2,6-dichloro-1,4-benzoquinone-4(*N*-chloro)imine with 35% yield is shown in Figure 4. Under the same conditions, reactions of the chloramines with 2,4,6-trichloro-*m*-cresol or 2,4,6-trichloro-3-methoxyphenol resulted in analogous products (i.e. with 3-methyl or 3-methoxy groups) with similar yields. Therefore, the use of chloramine in disinfection increases the variety of haloquinone structures that are likely to be generated in drinking water. The use of free chlorine vs. chloramine also has the potential of differentially affecting the stability of the HQ. At acid pH, excess chlorine will result in ring scission, which is unlikely to occur with chloramine. Analogous stabilization of cyanogen chloride has been demonstrated in chloraminated water (Na and Olson, 2004). On the other hand, at the~~in~~ alkaline pH that is usually sought with chloramine disinfection, chlorine will be lost from the ring. This suggests that lower degrees of halogen substitution are likely with chloramine.

Table 1 provides the frequency at which HQs and other DBPs were detected in 18 drinking waters (Zhao et al. 2010; Li et al. 201~~0~~). Four HQs were identified, DCBQ, 2-6-dibromo-1,4-benzoquinone (DBBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), and 2,3,6-trichloro-1,4-benzoquinone (TCBQ). DCBQ and DBBQ were detected in all systems and ranged from 2.2 to 295 and 0.5 to 37.9 ng/L, respectively. The remaining HQs were detected less frequently and at lower concentrations.

3.2.2. Toxicological Evaluation of Haloquinones (HQs)

Table 2 provides a list of quinone and quinone-like structures that were predicted to occur as DBPs. Only the ones identified in the previous section have been sought in drinking water.

Predictions of all the members provides some perspective on how broad this class of DBPs could be and also provide some perspective on how important this group might be toxicologically.

There are few toxicological studies of haloquinones and essentially no chronic studies in the literature. On the other hand, non-halogenated polyphenols and quinones have been studied extensively because of evidence that they play an important role in the responses to a number of well known carcinogens, including benzene and polyaromatic hydrocarbons (Bolton et al., 2000). As a result, there is a rich literature describing likely modes of action of quinones.

The metabolism and distribution of the metabolites of polyphenols and quinones deliver several toxic metabolites to certain cells (Figure 5). The first step in quinone metabolism is conjugation with glutathione which occurs primarily in the liver. The importance of this step is illustrated by the fact that the nephrotoxicity of 1,4-benzoquinone is increased with each glutathione substitution until 3 sites on the ring are occupied, adding the fourth glutathione-conjugation decreases nephrotoxicity (Lau et al., 1988). The conjugates are actively taken up in cells that express GSH transporters. Secondly, the glutathione conjugates are metabolized to cysteine conjugates that are also transported into cells (e.g. renal proximal tubular cells) that express gamma glutamyl transferase (γ-GT). Within cells, the polyphenol form of the compound is oxidized to the quinone form by the enzyme NAD(P)H:quinone oxidoreductase (NQO1). The quinone or the semiquinone radical can directly interact with macromolecules (Lin et al. 1999). The cysteinyl forms can also be converted ~~by~~ to forms that alkylate macromolecules (Bolton et al., 2000), however, this can be prevented by *N*-acetylation of the cysteine residue, which reduces toxicity. Further, the parent polyphenol and quinone forms and their respective conjugates participate in redox cycling reactions that lead to the formation of reactive oxygen species (ROS). Mutation spectra in treated human cells or bacteria are consistent with DNA damage induced by hydroxyl radical, implicating ROS in the genotoxicity of quinones (Jeong et al., 1999). Halogen substitution appears to enhance the toxicity of these conjugates. Conjugation favors the oxidized quinone form (Monks et al. 1990) and the electron withdrawing effects of halogen substitutions will increase their activity as oxidants (Monks and Lau, 1997). The formation of the cysteine conjugate enhances the oxidation of the hydroquinone form, but is suppressed by *N*-acetylation of the cysteine residues (Monks et al., 1990).

An *in vitro* QSTR study of *p*-benzoquinones was conducted in rat hepatocytes and PC12 cells and it was found that the two chlorinated congeners, 2,3,5,7-tetrachloro-1,4-benzoquinone and

2,5-dichloro-1,4-benzoquinone, were substantially more active than the non-halogenated compounds (Siraki et al., 2004). The two HQs were especially potent in producing ROS, doubling fluorescence measures by two-fold with sub-µM concentrations. They were also more cytotoxic than non-chlorinated congeners, but these differences were as little as 2-fold whereas they were 10-250X more potent in inducing ROS formation. These differences were predictable based upon the shifts in redox potential that occur with halogenation.

The details of the distribution of quinones and the corresponding hydroquinones can be complex *in vivo*. Monks et al. 1990 found that the chloroquinone conjugates were less potent as nephrotoxicants than the hydroquinone conjugates. The authors attributed this to non-specific loss of the quinone structure prior to accessing the interior of cells. Thus, the equilibrium between hydroquinone and quinone structures can have a significant impact on the systemic toxicity resulting from exposure.

Hydroquinones and the respective quinones are not generally detected as mutagens in *Salmonella* test systems but it is clear that they are capable of damaging DNA through the generation of hydroxyl radical (Monks and Lau, 1997). The HQs do induce aneuploidy in human cells (Imai et al., 2009). 1,4-Benzoquinone has also been shown to form adducts with DNA and to increase mutation frequency in a human kidney cell line (Gaskal et al., 2005). Recently, halogenated quinones were found to participate in a metal-independent degradation of hydroperoxides to produce a quinone ketoxy radical, which may be a candidate for adduct formation by these compounds (Zhu et al., 2009).

TOPKAT® indicated that some of the HQ structures predicted to form by chlorine or chloramine reaction with NOM were likely to be carcinogenic (Table 2). Low chronic LOAELs (< 1 mg/kg day^{-1}) were predicted for most of the haloquinones. Consistent with the literature on non-halogenated hydroquinones and quinones, TOPKAT® modeling predicted most of the halogenated congers would be negative in *Salmonella*-based mutagenicity tests.

Halogen substitution and the formation of the benzoquinone 4-(*N*-chloro)imine derivative with chloramine have substantial effects on the polarity of quinones. The data in Figure 6 were developed with the program Molinspiration (www.molinspiration.com) which predicts octanol/water (O/W) partition coefficients based upon chemical structure. First, formation of the quinones increases the O/W partition coefficient of the corresponding hydroquinones. Second, halogenation progressively increases the O/W partition coefficient, with bromination increasing

it more effectively than chlorination. Third, as expected, substitution of an additional non-polar group to the ring (modeled with a methyl group) increases the coefficient. Finally, it can be seen that the substitution of the 4-(*N*-chloro)imine group greatly increases the O/W partition coefficient.

Based upon the review of the literature on related compounds, we conclude that the production of HQs as DBPs likely represents a cancer risk.

3.3. Halocyclopentanes, Halocyclohexenes and Halofuranones

3.3.1 Formation of Halocyclopentene and Halocyclohexene Derivatives and Halofuranones

It was predicted that reaction of chlorine with substructures within NOM would give rise to a large variety of chemicals including the cyclopentene and cyclohexene derivatives that were likely to be produced with the chlorination of drinking water. Most of the chemicals considered were arrived at through consideration of orcinol (3,5-dihydroxytoluene) as a probable structural fragment in NOM (Bull et al., 2006) and included ketone, dione and carboxylic acid derivatives. The halofuranones are the most broadly recognized related by-products in this group. The total concentrations of the halofuranones were found in a recent survey to be substantially greater than anticipated from prior studies (Krasner et al., 2006). The highest concentration of total halofuranones was 2.38 µg/L.

A~~There are a~~ variety of halocyclopentene and halocyclohexene derivatives were predicted to form. Examples of predicted products include 3,5-dichloro-1-hydroxy-4-ketocyclopent-2-enoic acid, 3,5-dichloro-hydroxy-4-ketocyclopent-2-enoic acid, 2,3,6- trichloro-4,5-diketophenylprop-2-enol, and 1,2,2,4-tetrachloro-3-hydroxycyclopent-4-enoic acid (Table 2). Gong et al. (2005) demonstrated formation of 2,2,4-trichloro-5-methoxycyclopent-4-ene-1,3-dione through reaction of chlorine with syringaldehyde. This cyclopentenedione was subsequently shown to occur in drinking water, although concentrations were not provided. The compound was shown to be a direct-acting mutagen (i.e. most active in the absence of S9 fraction of rat liver homogenates). Thus, this grouping of DBPs could be important contributors to carcinogenic risk from chlorinated drinking water.

3.3.2 Toxicological Evaluation of Halocyclopentene, Halocyclohexene, and Halofuranones

Because of its low concentrations, ~~the furanone~~ MX alone, is unlikely to contribute significantly to the cancer risk associated with chlorinated drinking water. However, the finding of higher concentrations of other halofuranones raises the possibility that the total of MX-related compounds may make a significant contribution to risk. MX is fairly potent as a carcinogen with a TD_{50} of 0.583 mg/kg day^{-1} (Gold et al., 2010). Interest in this group should be increased by the predicted formation of cyclopentenoic acid derivatives that bear structural resemblance to the halofuranones and which are predicted to have some of the same toxicological characteristics.

There is a database of carcinogenesis assays on chemicals that involve small modifications of the basic furan ring (NTP, 1990; 1993; 1994; 1999) that may provide the basis of a QSTR study of the carcinogenic properties of this group of chemicals. Table 3 presents estimates of carcinogenic potency of furan and a number of modifications of furan, including MX. Of the compounds listed, furan is the most potent carcinogen. It is a multispecies, multiple target organ carcinogen. It produces cholangiosaracomas, hepatocellular carcinomas, mononuclear leukemia in male and female rats, hepatocellular carcinomas, and pheochromocytomas in male and female mice. However, furan is not mutagenic in *Salmonella* tester strains, although it does produce clastogenic effects in mammalian cells (NTP, 1993). With the exception of MX, modification of the furan structure substantially reduces the carcinogenic potency. The carcinogenic potencies of tetrahydrofuran and furfural are two to three orders of magnitude less than that of furan. Evidence that furfuryl alcohol is systemically carcinogenic has to be considered marginal, as the nose was the only target organ in an inhalation study. Thus, it may be that the structure-activity studies of mutagenic effects of MX and related compounds in *Salmonella* (LaLonde & Leo, 1994) are not reliable indicators of carcinogenic potency for furan derivatives. Therefore, DBPs that are related to furan should be studied more systematically as carcinogens.

A very low predicted LOAEL was associated with 3,5-dichloro-1-hydroxy-4-ketocyclopent-2-enoic acid by TOPKAT®, but the prediction was outside the predictive space of the model. It was not predicted to be a carcinogen. This compound was predicted to be formed from reaction of phenol with excess chlorine (Bull et al., 2006) and would be favored in chlorinated water while formation of 2,3,5,6-tetrachloro-1,4-benzoquinone would be favored with chloramine (Heasley et al., 2004). 2,2,4-Trichloro-5-methoxycyclopent-4-en-1,3-dione was identified by Gong et al. (2005) and had a sub-mg/kg predicted chronic LOAEL, but was not predicted to be a

carcinogen. Although the prediction of the LOAEL for some of the halocyclopentenes were outside of the OPS of the model, the predictions for MX were also outside the OPS. MX has been shown experimentally to be a carcinogen (Komulainen et al. 1997).

3.4. N-Halamines

3.4.1 Formation of N-Halamines.

It has long been recognized that primary and secondary amines will react with aqueous chlorine (e.g., HOCl or OCl^-) and inorganic chloramine (e.g., NH_2Cl or $NHCl_2$) to form organic *N*-chloramines.

$$R-NH_2 \xrightarrow{HOCl} R-NHCl \xrightarrow{HOCl} R-NCl_2$$

$$R_2-NH \xrightarrow{HOCl} R_2-NCl$$

Equilibrium considerations are closely tied to the basicity of the amine. As the amine becomes a stronger base, it will tend to form more stable chloramines (Pitman et al., 1969). The primary amine groups of the common amino acids are quite basic and therefore readily donate their electrons to electrophilic reactants such as chlorine. Amidines are also quite basic (pKa ≥ 11.2), especially the guanidine derivatives (pKa ~ 13.5).

Subsequent reaction with chlorine leads to the formation of *N*-dihalo species. This reaction is much slower than the initial halogenation, due to the much lower basicity of the *N*-chloro compounds. Disproportionation of two monohalamine molecules can lead to the formation of a dihalamine, however, this is less likely in highly dilute drinking waters containing a free chlorine residual.

There is little question that *N*-halamines are formed in the disinfection of drinking water as the total chloramine concentration is routinely measured as combined chlorine. This measurement generally exceeds the amount of inorganic *N*-chloramine and is generally considered to reflect *N*-chloramines that are formed with various primary and secondary amines that are present in the water. However, no surveys of individual organic *N*-chloramines appear in the literature.

3.4.2. Toxicological Evaluation of N-Halamines.

A search of the literature revealed a very limited toxicological data, primarily in the form of mutagenicity assays of *N*-chloramines of α-amino acids (Thomas et al., 1987). In *Salmonella* TA100, the dichloramines of histamine, ethanolamine, and putrescine were most potent, whereas histamine was most active among the monochloramine derivatives.

Laingam et al. (2011) studied the ability of the *N*-chloramines of several amino acids to induce micronuclei in mammalian cells. As suggested by the results of Nightingale et al. (2000) with the ε-amino group of lysine, ethanolamine and α-acetyllysine formed significant amounts of dichloramine at equivalent molar concentrations with chlorine, whereas only monochloramines were observed with glycine, ε-acetyllysine, and histidine. Thus, α-amino groups are much less likely to form dichloramines at physiological pH than those which are not adjacent to a carboxyl group. Increased induction of micronuclei was observed with most of the *N*-chloramines. *N*-Chlorohistamine was more potent than the others. An interesting finding was that the *N*-chloramines formed with lysine were substantially more potent than that of the two acetylated derivatives. This suggests that chloramination of both nitrogens might lead to bifunctional interactions with macromolecules, perhaps resulting from the formation of crosslinks (i.e. DNA to protein, protein to protein, or DNA with DNA).

Organic *N*-chloramines have received attention in the biomedical literature as 1) mediators of inflammatory and anti-inflammatory effects that result from activation of neutrophils (Bernofsky, 1991; Davies et al., 1993; Marcinkiewicz, 1997; Barua et al., 2001; Vissers et al., 2001; Englert & Shacter, 2001; Kawai et al. 2004; Midwinter et al., 2004; Schuller-Levi & Park, 2004) and 2) they have been invoked as reactive metabolites produced by neutrophils that could account for a variety of chronic side effects of certain drugs (Utrecht et al., 1991 & 1995; Miyamoto et al., 1997). These studies suggest that organic *N*-halamines are toxicologically active, but provide little information on which to base a risk assessment related to their occurrence in drinking water.

Illustrative examples of organic *N*-halamine formation and their decay to reactive intermediates are provided in Figure 7. In each case, the initial reaction is shown and further progression in the development of selected secondary intermediates or terminal products is depicted. In the first two examples, the initial reactions should be viewed as occurring in drinking water, the subsequent formation of radical species is~~are~~ suggested to result from

metabolic processing *in vivo*. The first reaction depicted would be typical of amino acids and other primary amines. The figure illustrates the generation of nitrogen and chlorine radical products and the regeneration of free chlorine, each of which could contribute to toxicity. The reaction with acetamide shows that the corresponding *N*-chloramides can be converted into carbon-centered radicals *in vivo*, as well. Finally, the formation of a less stable *N*-chloramine with a drug (aminopyrine) that contains tertiary amine group is depicted. In this case, a di-cation is formed that reacts with an additional molecule of aminopyrine to form a cation radical. In the latter case, it is probable that the low stability and high polarity of the initial chloramine should limit its systemic absorption. As a result, exogenous sources of this *N*-halamine are unlikely to represent an *in vivo* hazard.

The reason for concern about the toxicology of organic *N*-halamines is that they are 1) less polar than the parent compound (see Fig. 6) and 2) that some are sufficiently stable to be distributed systemically once absorbed (Hawkins & Davies, 1999). It is of importance to note that the *N*-chloro group can be transferred to other chemicals, or in the case of RNA and DNA, from initially formed, less stable sites on purine and pyrimidine rings, to the exocyclic nitrogens (Hawkins & Davies, 2002). As a consequence, less stable compounds formed in water may transfer the *N*-chloro group as the chemical is ingested. Of course, some organic *N*-chloramine formation can be expected upon ingestion of a free chlorine residual because of the rich sources of amines in the oral cavity and esophagus.

The importance of transfer of the *N*-chloro group among different amino acids was elegantly illustrated by Peskin et al. (2004), who assessed the relative abilities of the chloramines of histamine, glycine, and taurine to inhibit intracellular glycerylaldehyde-3-phosphate dehydrogenase activity (GAPDH) when added to incubation media individually or in combination. The *N*-chloramine of glycine inhibited GAPDH, but that of taurine did not. However, if taurine-*N*-chloramine was added in the presence of glycine, the enzyme was inhibited (Figure 8). This indicates that the *N*-chloro-group of the polar, non-penetrating *N*-chloramine had transferred the *N*-chloro group to the permeant *N*-chloramine, glycine-*N*-chloramine, in a biological milieu.

While the initial formation of *N*-chloramines is more rapid when the amino group is αalpha to a carboxyl function, the formation of the initial *N*-chloro group on an amine without an αalpha-carboxyl group facilitates addition of the second *N*-chloro group (Nightingale et al.,

2000). As a consequence, the *N*-chloro groups on the epsilon amino groups of lysine in proteins appear to be responsible for the production of nitrogen-centered free radicals that play a role in inactivation of the proteins (Hawkins and Davies, 1999).

TOPKAT does not recognize the *N*-halamine or *N*-haloamide groups, reflecting the absence of systemic toxicological studies with compounds in these classes. As a consequence, QSTR projections for these two classes were not useful.

3.5. Halonitriles and Haloamides

3.5.1. Formation of Halonitriles and Haloamides

As described in the previous section, most amino acids undergo initial, rapid formation of mono and di-*N*-chloramines. Due to the presence of an adjacent carboxyl group, *N*-halo substituted α-amino acids undergo a Grob fragmentation to yield an aldehyde or ketone and a nitrile. Many amino acids are known to yield halogenated aldehydes and nitriles (e.g., chloral hydrate and dichloroacetonitrile) following reaction with chlorine (Figure 9). The degree to which this happens depends on the presence of an activating side chain (e.g. R3). After reaction, the amine nitrogen leaves a functional group (carbonyl or nitrile) that is electron withdrawing. When combined with a similarly electron withdrawing side chain, the α-carbon can become an active site for halogen substitution.

The haloacetonitriles have long been recognized as chlorination by-products (Trehy & Bieber, 1981). Attempts were made to identify halopropionitriles as DBPs (Li et al., 2011), but only in the case of 2,2-dichloropropionitrile was it possible to synthesize and purify a standard for the project. The concentrations of 2,2-dichloropropionitrile in a drinking water are compared with the haloacetonitriles in Table 1.

~~HAcNs~~ HAMs have been detected in drinking water (Krasner et al., 2006). Concentrations in the range of 2-3 µg/L were reported for mono- di- and tri- haloacetamides. The brominated trihaloacetamides have not been measured as no standards are currently available. Studies with amino acids as precursors suggest the formation of halopropionamides as well as haloacetamides (see Figure 9).

3.5.2. Toxicological Evaluation of the Halonitriles.

Chloroacetonitrile (CAN), bromoacetonitrile (BAN), dichloroacetonitrile (DCAN), bromochloroacetonitrile (BCAN), dibromoacetonitrile (DBAN), and trichloroacetonitrile (TCAN) have been studied toxicologically to varying degrees.

The haloacetonitriles are known to be mutagenic and to act as tumor initiators in the mouse skin (Bull et al., 1985). Several other studies have established the genotoxicity of haloacetonitriles in various systems, the SOS chromotest, Ames-fluctuation assay, newt micronucleus test (Le Curieux et al., 1995), DNA strand breaks in cultured human lymphoblastic cells (Lin et al., 1986), and aneuploidy in *Drosophila* (Osgood and Sterling, 1991).

Table 4 provides predicted chronic LOAELs, predictions of carcinogenicity, and Ames' system mutagenicity for HNs. Within the HN class, compounds with 3-carbon chains were prominent in terms of their potential toxicological potency as reflected in the predicted LOAELs. 2,2-Dichloropropionitrile was found at 8 ng/L in a drinking water sample (Li et al., 2011). Four haloacetonitriles were also identified (DCAN, BCAN, DBAN and TCAN) in the same drinking waters. The predicted LOAELs for the haloacetonitriles were outside the permissible limits of the OPS, but experimental data indicate that the estimates are about an order of magnitude lower than those predicted for members of the class that have been partially characterized toxicologically (Bull et al., 2006). The brominated analogs of these chemicals did not meet the stringent criteria used to identify those chemicals most likely to be carcinogenic. However, DBAN was recently shown to be carcinogenic in mice and rats (NTP, 2010). Neoplastic changes were noted in the oral cavity (rats) and the forestomach (mice) and glandular stomach (rats).

DBAN produced cancer in rats at a daily dose of 4 mg/kg, which results in a calculated unit risk value of approximately 0.2 µg/L (unit risk is defined as the concentration in drinking water that would increase lifetime risk of cancer by 1 additional cancer per million over a lifetime). Based on these data, the HNs may be approximately 3-10 X as potent as the THMs.

I~~However, i~~f it is assumed to be of equivalent potency to DBAN, the concentrations of 2,2-dichloropropionitrile found would be unlikely to increase cancer risk significantly even though they are mutagenic compounds.

3.5.3. Toxicological Evaluation of Haloamides

Acetamide has been classified as possibly carcinogenic in humans (IARC, 1999b) based upon the induction of liver tumors in rats and malignant lymphoma in mice (Fleischman et al., 1980) when given at 1.18 or 2.36% in the diet. Formamide administered by gavage at doses as low as 20 mg/kg day^{-1} induced clear evidence of malignant lymphoma in male mice, but no neoplastic changes were observed in rats (NTP, 2008). Acetamide, itself, is not mutagenic (Kennedy, 1986), nor does it damage DNA (Sakano et al., 2004), but its metabolite, acetohydroxamine, does produce adducts in the presence of metals. Therefore, there is evidence of toxicological effect with the amide class independent of halogen substitution.

The literature on the toxicology of haloamides is limited. A cancer bioassay has been conducted with chloropropamide by the National Cancer Institute (NCI, 1978). No evidence of carcinogenicity was observed in B6C3F1 mice to concentrations of 10,000 ppm of their diet or F344 rats to concentrations of 6000 ppm.

Analogs of dichloropropionamide are utilized as "safeners" that block the effects of thiocarbamate and chloroacetanilide herbicides (Walton and Casida, 1995). One of these, dichloramid (*N,N*-diallyl-2,2-dichloroacetamide), has a reasonably complete data base. NOAELs of 5 mg/kg day^{-1} from a 1 year study in dogs, 6.8 in a 2 year study in rats, and 7.0 in an 18 month study in mice have been established (U.S. EPA, 2005). The corresponding LOAELs were 20, 32.8, and 70.7 mg/kg day^{-1}, respectively. The NOAELs for reproductive and developmental studies were higher. The liver effects observed in rats are reminiscent of dichloroacetic acid, which is one of the metabolites of this compound.

Two haloacetamides have been found in chlorinated drinking water (Krasner et al., 2006) and are included in Table 4. The remaining compounds evaluated were propionamides that have yet to be sought in drinking water. Five of six propionamides met our criteria as probable carcinogens (the exception being the 3,3-dibrominated propionamide) and were predicted to be Ames' test mutagens. The predicted chronic LOAELs ranged from 0.5 to 3.2 mg/kg day^{-1}, approximately one order of magnitude below that of dichloramid.

3.6. Haloaldehydes

3.6.1. Formation of Haloaldehydes

Haloacetaldehydes have been known to arise in the chlorination of drinking water for some time. Several were identified and some measured by Krasner et al. (2006). Chloral hydrate (trichloroacetaldehyde) occurred frequently with median concentrations of 1, a 75th percentile concentration of 4, and a maximum concentration of 16 µg/L. Corresponding concentrations for dichloroacetaldehyde were 1, 4, and 14 µg/L, respectively. Bromochloroacetaldehyde was less frequently detected, with a maximum concentration of 1.3 µg/L and a mean among 12 systems of about 0.5 µg/L. The maximum concentration of tribromoacetaldehyde detected was 0.9 µg/L. Iodobutanal was found in two treatment plants and note was made of two tentatively identified haloaldehydes, dichloropropenal and 4-chloro-2-butenal.

3.6.2 Toxicological Evaluation of Haloaldehydes

Chloral hydrate is a weak carcinogen only in mice (IARC, 2004) and is of little toxicological interest as a carcinogen at the low concentrations detected in drinking water.
Chloroacetaldehyde has been identified as a reactive intermediate of several well known carcinogens (e.g. vinyl chloride) and has been well established as a mutagen (IARC, 2004). The CPDB (Gold et al., 2010) indicates a TD_{50} for chloroacetaldehyde of 36.1 mg/kg day^{-1}, based upon the study of Daniel et al. (1992). The genotoxicity of other saturated (Loforth, 1978; Bignami et al., 1980) and unsaturated haloaldehydes (Rosen et al., 1980; Meier et al., 1985; Segall et al., 1985) has been studied extensively and have been found positive as mutagens. However, the *in vivo* toxicological studies of the haloaldehydes found in drinking water appear to be limited to chloroacetaldehyde and chloral hydrate.

Eighteen haloaldehydes were evaluated that were predicted to be carcinogens and to have chronic LOAELs of less than 10 mg/kg day^{-1}. Table 5 includes only those 4 haloaldehydes that met all of our criteria (valid predictions in 2 species with members in the training set with similarity indices of less than 0.2). It is notable that all of the compounds identified by the TOPKAT® analysis as being interesting as carcinogens were chlorinated, but brominated analogs that did not meet these criteria should also should be considered of importance. This reflects the much smaller variety of brominated compounds in the NTP and FDA databases. In general, brominated analogs can be considered at least as potent as the corresponding chlorinated analogs.

The 4-chlorobutenal and dichloropropenal tentatively identified by Krasner et al. (2006) met neither the chronic LOAEL or predicted carcinogenicity criteria.

3.7. Phenazine and Chlorophenazines

3.7.1. Formation of Phenazine and Chlorophenazines

In an investigation of the formation of *N*-nitrosodiphenylamine, Li and coworkers (2011) identified phenazine and an *N*-chloro derivative of phenazine in chloraminated water. Phenazine is formed in a competing reaction with *N*-nitrosodiphenylamine formation at more acid pH. These compounds are of interest because, to our knowledge, this is the first heterocyclic amine that has been demonstrated to be formed in chloraminated water.

3.7.2 Toxicological Evaluation of Phenazine and Chlorophenazines

There are no toxicological data on phenazine or chlorophenazines, *per se*, in the open scientific literature. Derivatives of phenazine are used as dyes (e.g. eurhodines, toluylene red, indulines, and saframines). It is also a moiety in natural products produced by several bacteria on a branch of the shikimic acid pathway (Price-Whelan et al., 2006). Carbon-bonded chlorophenazines have been evaluated as pesticides (Cross et al, 1968) with the monochloro substituted compounds being the most effective. A phenazine-water complex has been shown to abstract an electron from DNA bases in isolation (Choudhury and Basu, 2005). Some phenazine derivatives can be very active as redox agents (Davis and Thornalley, 1983). In the case of phenazine methosulfate, a radical cation is produced as it oxidizes reduced pyridine nucleotides within cells. Some N^6,N^{10}-dioxide phenazines are being evaluated as anti-tumor agents and are cytotoxic in the low µM range, *in vitro*. Simpler brominated 2-amino or 2-hydroxy analogs appear to be selectively cytotoxic under hypoxic as opposed to normoxic conditions (Lavaggi et al., 2008). These studies raise concerns in hazard assessment, but provide little information for risk assessment.

Phenazine and chlorophenazines were subject to QSTR analysis. The predicted chronic LOAEL for phenazine was 64.2 mg/kg (95% CI = 15.7- 262 mg/kg) (Table 6). This indicates that its probable chronic toxicity would occur at somewhat lower doses than chloroform, for

example. Phenazine was predicted to be a carcinogen, but not a mutagen in the Ames' test. Given the relatively high LOAEL, phenazine is unlikely to be a potent carcinogen.

N-Chlorophenazine was detected in drinking water (Li et al., 2011~~0~~). There are no toxicological data available on this chemical and, as indicated earlier, the *N*-Cl group is not recognized by TOPKAT®. Nevertheless, QSTR consistently predicted *N*-chlorophenazine to be carcinogenic. This was more consistent than the predictions for phenazine, itself, in that 7 of the 8 models employed predicted a probability ~~of~~ that it was carcinogenic >0.974. It was also predicted to be a mutagen. Predictions in TOPKAT® are based upon electropological characteristics of the molecule and therefore the prediction is made independently of the specific atoms in the molecule. Nevertheless, there were no *N*-chloramines in the training sets. Similarity indices were outside our criteria in 7 of the 8 models.

Substitution of chlorine on carbons of phenazine resulted in lower predicted chronic LOAELs, with LOAELs decreasing with the degree of chlorine substitution (Table 6). None were predicted to be carcinogenic or mutagenic (data on Ames mutagenicity not shown). None of the carbon-substituted phenazines were found in drinking waters (Li et al., 2011).

3.8. Formation of other classes of DBPs

There are many other documented and predicted DBPs that could be formed with chlorine or chloramine. In the interest of brevity, consideration has been limited to a set arrived at through our analysis that could have the potential of contributing to cancer risks of the magnitude suggested by epidemiological studies. The absence of other compounds from our analysis should not eliminate them from further consideration.

4. Discussion

The initial impetus for this project was to identify novel DBPs that could be formed by the reaction of chlorine or chloramine with substructures that are known to occur in NOM (Bull et al., 2006). We also considered several chemicals that have recently demonstrated to occur in drinking water for which there are reasons to be concerned about their hazards. All of these chemicals were assessed by review of effects of related compounds and/or through formal consideration of structure-activity relationships. A second group of chemicals is~~are~~ discussed

because they are drawn from a class which has not been studied in either humans or experimental animals (e.g. organic *N*-chloramines) despite the widespread recognition of their occurrence in chlorinated drinking water.

HQs were identified as a class of chemicals for which data on related compounds (hydroquinones and benzoquinones) imply high toxicological potency and probable carcinogenic properties. Halogen substitution on quinones could substantially increase their potency. ~~as toxicants and probable carcinogens~~. QSTR analysis of the HQs support the conclusion that they could present substantive hazards to health even at the low concentrations that seem to be present in drinking water. Now that data demonstrate the occurrence of four members of the class in drinking water (Zhao et al., 2010), it is probable that other members of this class will be found in drinking water based on the common occurrence of similar precursor structures in natural waters (Bull et al., 2006).

The toxicity of benzoquinones is modified significantly by halogenation. First, halogenation increases the O/W partition coefficient. Second, the introduction of halogens enhances metabolic activation of quinones. An early step in the metabolic activation of benzoquinones and polyphenols is conjugation with glutathione that is mediated by glutathione transferases. Toxicity of benzoquinones (most commonly assessed in the rat kidney) increases progressively with up to 3 glutathione substitutions. A fourth glutathione substitution decreases toxicity (Lau et al., 1988). These glutathione substitutions allow the compound to be transported into cells. Within cells, the glutamate and glycine residues are hydrolyzed from GSH leaving an adducted cysteine that readily forms an electrophilic sulfenamide cation that can react with macromolecules (Commandeur et al., 1995). Third, hydroquinones and their corresponding quinones can be involved in redox cycling to produce reactive oxygen species (ROS) that can contribute to adverse health outcomes, depending upon their redox potentials. A fourth mechanism involves the ability of the semiquinone and quinones to react with macromolecules (Lin et al., 1999). In the case of the latter two mechanisms, the addition of two or three chlorines to the glutathione-conjugated benzoquinone shifts the equilibrium towards the quinone form, which increases toxicity (Monks et al., 1990), but may result in non-specific degradation of the quinone before it reaches cellular targets. The transport processes that allow glutathione-conjugates to concentrate within cells is coupled with generation of reactive intermediates and

the effect of halogen substitution on the redox properties of the quinone, all of which will dramatically influence toxicological properties of hydroquinones (Monks and Lau, 1998). Fifth, the modification of the quinone structure by chloramine, which adds an *N*-chlorimine group, further increases the O/W partition coefficient. This combination of altered chemical and physical properties should focus attention on the HQ-4-(chloro)imine compounds that can be formed with chloramine (Heasley et al., 2004). Sixth, the toxic effects of the cysteine conjugates of the HQs is suppressed by *N*-acetylation and reactivated by *N*-deacetylation. Slow acetylators have long been known to be at greater risk for bladder cancer (Garcia-Closas et al., 2005).

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Formation of the *N*-chloroimine derivatives of HQs could be sensitive to relatively small changes in source water characteristics and disinfection practice. Excess free chlorine would be expected to increase halogen substitution to the point of destabilizing the quinone ring structure. This will give rise to familiar terminal by-products of chlorination, such as the THMs and HAAs (Bull et al., 2006). Variation in treatment conditions is likely to complicate this relationship, as well. Chloramine has been shown to react with phenol or *m*-cresol to form several HQs, but it appears that the formation of members of the HQ-(chloro)imine class will be unique to chloramine (Heasley et al., 2004). A benzoquinone-imines are~~is a~~ strong electrophiles and have been found to be ~~is~~ the metabolite ~~held~~ responsible for hepatotoxicity produced by acetoaminophen (Miner and Kissinger, 1979). This suggests that current regulatory strategies to control risk from chlorination by-products based upon the THMs and HAAs as surrogates might be misguided, since the use of chloramine instead of chlorine may result in a unique class of DBPs that could contribute to carcinogenic risk.

An attempt was made to address organic *N*-chloramines as a group of well recognized DBPs are formed, but for which chemical characterization has been limited. In the course of exploring nitrosamine formation, an *N*-chloramine of phenazine was discovered as a by-product formed by chloramine reactions with diphenylamine. Literature searches revealed general concerns about *in vivo* formation of *N*-chloramines and *N*-bromamines in the biomedical literature as products and potential mediators of damage in inflammatory processes. As these compounds are known to be formed in drinking water, characterizing the toxicological properties of 2 or 3 compounds with varying chemical properties (e.g. stability to hydrolysis, lipophilicity) within this group should be given high priority.

The recent availability of chronic data on DBAN (NTP, 2010) reinforce screening data that suggest that halonitriles probably represent carcinogenic hazards. SRecent subchronic data also indicate that there are some mild thyroid effects for DBAN (Poon et al., 2003). Formation of longer chain halonitriles have been demonstrated in laboratory studies of reactions of chlorine with isoleucine (Nweke & Scully, 1989). Therefore, the possibility that there is a larger halonitrile pool of DBPs needs to be explored. QSTR analyses of 3-carbon halonitriles indicated that some could be of interest as potential carcinogens if they occurred in a concentration range of 0.1-10 μg/L. Dihaloacetonitriles are present within this range. However, the modest concentrations of 2,2-dichloropropionitrile (Li et al., 2011) preliminarily suggest that longer chain HNs may not occur at sufficient concentrations to be of concern.

The QSTR evaluation was generally consistent with two prior efforts for prioritization of DBP research. Moudgal et al. (2000) also utilized TOPKAT®, but limited their analysis to simple identification of chemicals that were probable carcinogens or developmental toxicants. Woo et al. (2002) utilized expert judgment SAR analysis to identify those DBPs that are most likely to be carcinogenic and provided a relative estimate of potency based upon related chemicals. While the predictive approach used in the Woo et al. study was more analogous to that of the present study, its analysis was limited to DBPs that have been shown to occur in drinking water. They identified MX and some other halofuranone derivatives as a high priority class. A second tier of chemicals that included selected unsaturated and saturated halonitriles, and halopropanones (e.g. 1,3-dichloropropanone), and haloaldehydes (e.g. dichloroacetaldehyde) were fell into a intermediate category identified as likely to be a moderately active multispecies/target carcinogen at low doses. Thus, the findings of our analysis were generally consistent with those of Woo et al. (2002).

The present study differed from the two prior efforts in that it did not limit the analysis to chemicals actually demonstrated in drinking water, but included chemicals whose formation was considered likely based on predictions of reactions with organic substructures within NOM. This effort resulted in the identification of several potential DBPs that could be of considerable interest as general toxicants and/or as carcinogens (Li et al., 2010; Zhao et al. 2010; Li et al., 2011). Specifically, this work calls attention to the HQs and non-halofuranone members of the halocyclopentene class as compounds that are likely to have high toxicological potency and with properties suggesting they could help explain epidemiological associations of chlorinated

drinking water with cancer. THowever, there have been no toxicological data that confirmsconfirming that the group can express those toxicological properties *in vivo*. Moreover, established or related classes of DBPs were identified that have a high probability of occurring in drinking water that have received very limited toxicological characterization (*N*-halamines, haloamides, halonitriles, and haloaldehydes). These classes of DBPs should be considered as potential contributors to cumulative risks. Finally, the first heterocyclic amine DBP and a chlorinated derivative (chlorophenazine and *N*-chlorophenazine) to be identified in drinking water were also evaluated.

The initial impetus for this study was the perception that regulated DBPs (i.e. the THMs and HAAs) were too weak as carcinogens to account for the cancer risks that have been associated with chlorinated drinking water. This comparison was based on the potency of the individual regulated DBPs in animal studies relative to the population attributable risk (PAR) derived from meta-analyses of epidemiological studies (Bull et al., 2006). There are a number of epidemiological studies that address selected polymorphisms in enzymes that are known to metabolize the THMs and HAAs that might modify the gap between risks calculated for the THMs and HAAs from animal studies and the epidemiological studies of chlorinated water (often using individual or collective concentrations of THMs as the dose-measure). As illustrated from this project and other data in the literature, there is clearly an opportunity for hundreds of other by-products to co-occur with the regulated DBPs. Many of these should also correlate with cancer risk and selection of one set of DBPs without ruling out others can result in purely trivial attributions to that class. The key question is which of these associations are causal?

If only those by-products within the THM class that have been shown to both be carcinogenic and genotoxic (i.e. excludes chloroform and dibromochloromethane) the risk attributed to this class at the highest exposure level identified in a recent survey of U.S. and Canadian drinking waters (Krasner et al., 2006) is approximately 20 x 10^{-6} per lifetime based upon the upper bound unit risks calculated for animals studies by U.S. EPA (2003a). This risk is dominated by calculations based on the original BDCM bioassay using corn oil gavage (19 x 10^{-6}). The risk attributable to BDCM will undoubtedly decrease significantly once the newer drinking water study of BDCM (NTP, 2005) is taken into account. The 2005 bioassay was negative at a dose that should have produced a small, but significant increase in tumor incidence

if a linear low dose extrapolation were applied to the results of the corn oil gavage study. Calculations of risk posed by the dihaloacetic acids [bromochloroacetic acid (BCA) and dibromoacetic acid (DBA)] that are both carcinogenic and genotoxic based upon recent bioassays (NTP, 2007; 2009) can add an additional upper bound risk of 56 x 10^{-6} per lifetime. While DCA have been found genotoxic in some studies, the concentrations necessary to produce these effects are so high relative to the systemic concentrations that produce cancer in animals as to make them irrelevant to *in vivo* carcinogenesis (Moore and Harrington-Brock, 2000). This conclusion is bolstered by the fact that DCA has been shown to selectively stimulate the growth of already initiated cells (Stauber and Bull, 1997), which can completely account for the development of liver cancer (Miller et al., 2000) at much lower systemic concentrations. TCA is also excluded as a non-mutagen. It is notable that it produces liver cancer in mice, not rats. TCA induced liver cancer is associated with peroxisome proliferation, a mode of action that does not appear active in humans (Gonzalez and Shah, 2007). It is important to observe that median risk based upon most likely estimates of the linear low dose extrapolation and using median drinking water exposures would decrease these cancer risk estimates substantially.

In contrast, an analysis by the U.S. EPA (1998) suggested that the population attributable risk (PAR) to chlorinated drinking water ranged from 2 to 17% of bladder cancer cases in the U.S. This equates to a range of 700 and 6,000 additional cases of cancer per 10^6 per lifetime in men and somewhat lower risk in women (Bull et al., 2006). The comparison with the risks derived from animal data indicates that it is not reasonable to attribute a cancer risk of this magnitude to THMs or HAAs in drinking water without clear explanation of why humans should be so much more sensitive to these chemicals than animals.

A recent epidemiological study by Cantor et al. (2010) found interactions in THM association with bladder cancer with several polymorphisms in enzymes that are involved in the metabolism of THMs and HAAs. Evidence of a mutagenic metabolite of BDCM produced as an intermediate by a glutathione transferase isozyme, *GSTT1*, has been reported (Pegram et al. 1997). Cantor et al. (2010) found that individuals not expressing this enzyme were at lesser risk for bladder cancer that has been associated with the consumption of chlorinated drinking water. In addition, the CC variant of CYP2E1, an enzyme known to be a somewhat more efficient catalyst in the metabolism of THMs, was found at higher frequency in bladder cancer cases and displayed an interaction with THM levels, while those who carried a mutation that results in the

substitution of a histidine for arginine at position 76 of the protein did not. This mutation is slightly less effective as a catalyst, but the main effect appears to be suppressed expression as a protein. The expression of less active variants of *GSTZ1* towards the dihaloacetic acids were observed among bladder cancer cases exposed to higher levels of THMs. Most interesting was an increase in odds ratios (OR) for bladder cancer to 5.9 in individuals with *GSTT1* present and the *GSTZ1* DT/TT variant when exposure was stratified against total THM concentrations. These are important observations, but they do not force the conclusion that THMs are responsible for bladder cancer.

First, it has to be recognized that kinetics dictate that there will be very small differences in THM and or HAA metabolism among these enzyme variants at the low systemic concentrations that would result from the amounts that are obtained from water by any route of absorption. Cytochrome P450 2e1 (the *CYP2E1* protein product) has be highest affinity for THMs (K_m = 0.15 µM) and has a high maximum rate of catalysis (V_{max} = 28.3 nmol/h/mg protein) (Lipscomb et al., 2004). At an oral dose equivalent to 72 µg/kg, peak concentrations of BDCM in blood were 2.6 ng/L (equivalent to 0.016 nM) and 90.5 ng/L (0.76 nM) from a dermal exposure of an hour to a dose-equivalent concentration were observed in human volunteers (Leavens et al., 2004). These concentrations are roughly two orders of magnitude below the K_m and the relative V_{max} between the variants is so small that there would be no appreciable difference in their metabolism of the THMs at concentrations that occur in drinking water. It is notable that Backer et al. (2008) found no evidence that cytochrome P450 2e1 activity had any effect on THM levels 10 minutes following a shower with household drinking water.

The K_m of DCA for *GSTZ1* is 71.4 µM and the V_{max} is 1334 nmol/min. mg protein. (Tong et al. 1998b). This reflects the huge capacity for oxidation of the dihaloacetic acids in humans. Again, the systemic concentrations of the HAAs at exposures found in drinking water are several orders of magnitude below this K_m (Schultz and Shangraw, 2006). Therefore, it is difficult to see how expression of the different variants of *GSTZ1* could affect the disposition of these compounds at low doses as the relative specific activity of the four variants studied to date differ by only by a factor of five (Board et al. 2001).

GSTT1 is known to be active in producing a mutagenic metabolite with the brominated THMs. The apparent K_m for chloroform is sufficiently high and the V_{max} low, essentially rendering this pathway irrelevant even at the high carcinogenic doses (e.g. 90 mg/kg) in animals.

On the other hand, it is effective with brominated THMs and the lesser risk attributed to individuals that do not express this enzyme is a more attractive explanation for differences in humans susceptibility than the polymorphisms of *CYP2E1* and *GSTZ1*. However, the low activity and high K_m of THMs for this enzyme mean that it will not compete well with cytochrome P450 2e1 in the metabolism of brominated THMs at low doses (Ross and Pegram, 2004). It will become important primarily when THM concentrations begin to exceed their K_m for cytochrome P450 2e1.

The differences in metabolism of THMs and HAAs at doses that are obtained from drinking water will not be appreciably different among individuals in the population based on the polymorphisms. This is because at systemic concentrations much below the K_m the amount of the enzyme that is present is not rate limiting to metabolism. Therefore, virtually the same amount of metabolism will occur irrespective of the polymorphism.

Second, it is important that many by-products of chlorination interact with glutathione. These include halonitriles (Lin and Guion, 1989), halopropanoic acids (Tong et al., 1998a), chloropicrin (Schneider et al. 1999), the halofuranones (Clark and Chipman, 1995; Febry et al., 2011), halopropanols (Hammond et al., 1999), halopropanones (Merrick et al., 1987) and as illustrated in this paper, the HQs. The isoforms of glutathione transferase involved in forming unstable or stable conjugates of some of these by-products have not been characterized.

Third, there have been suggestions that the relative importance of ingestion, inhalation, or dermal exposure to THMs has not been adequately considered. Villanueva et al. (2007) found individuals with dermal and inhalation exposure to THMs (largely through showering and bathing) had higher risks of bladder cancer than those who only ingested chlorinated drinking water. Indeed a high rate of absorption of BDCM through human skin was demonstrated by Leavens et al. (2007) that would support such a relationship. However, polymorphisms in the enzymes that metabolize the THMs still do not significantly close the gap in attribution of bladder cancer risk to THM exposure.

A follow-up to the Villanueva et al. study associated brominated THM concentrations in expelled breath with an increased frequency of micronucleated lymphocytes and increased levels of urinary mutagenicity (Salmonella strain YG1024) following a session of swimming (Kogevinas et al., 2010). This suggested the involvement of by-products with significant vapor pressure. The association with the *GSTT1* was not confirmed. In contrast, there was an apparent

increase in micronuclei in exfoliated urothelial cells in individuals with the normal *CYP2E1* variant vs. the alleles that express the form with the arginine to histidine mutation in position 76. An increase in peripheral blood lymphocytes with micronuclei was observed in a group that expressed the *GSTZ1* GG variant vs. those with the AG-AA variant. The actual expression of these proteins in these individuals was not measured. Most important, however, is that neither paper provides an explanation of how variation in these isoforms could be a sufficient basis of alterations of metabolism of THMs (or HAA) at doses likely to be derived from consuming and bathing with drinking water which have concentrations documented in their studies.

Richardson et al. (2010) reported analyses of water in chlorinated and brominated Spanish swimming pools from which subjects were drawn in the epidemiological study of Kogevinas et al. (2010). They identified a wider variety of by-products than are ordinarily found in chlorinated drinking water. It is notable that some of the DBPs other than the THMs that were identified have significant vapor pressures and could contribute to inhalation risks. Among these by-products were chemicals that ranged from relatively non-polar (e.g. haloketones) to non-polar (HQs) that could well be absorbed through the skin. From the present study, several of these compounds are expected to have a high toxicological potency. Some of the chemicals could well present a significant hazard by dermal or inhalation exposure.

For the above reasons, the thesis that regulated DBPs are of insufficient carcinogenic potency to account for the cancer risk associated with the chlorination of drinking water has not been refuted by recent epidemiological studies. It is suggested that some of the compounds identified for study in this manuscript might account for some of the discrepancy in potency. The discrepancy in target organ specificity is also of concern, but this difference may lie in species differences in sensitivity. On the other hand, the metabolism of several of the DBPs identified here (specifically the HQs and related compounds) have characteristics that parallel the schemes of the well-known bladder carcinogens in the aromatic amine class and should be investigated.

Acknowledgments

This work was supported in part by Water Research Foundation Contracts No. 2867 and 4089. In-kind contributions in the former case were by MoBull Consulting, the University of Massachusetts Amherst, Municipal Water District of Southern California and East Bay Municipal Water District. The second project was conducted by the University of Alberta (UA), with in-kind contributions from UA, MoBull Consulting, Australian Water Quality Centre, and Curtin University. In addition, we thank the following cooperating utilities: Charleston Water System, Charleston, SC, USA; Louisville Water Company, Louisville, KY, USA; Santa Clara Valley Water District, San Jose, CA, USA; Water Corporation of Western Australia, Perth, Australia; Alberta Research Council, Vegreville, Alberta, Canada; Institute for Biological Sciences, NRC, Ottawa, Ontario, Canada

Figure 1. Relationship between the rat TD_{50} and the predicted chronic LOAEL. Panel A: Relationship for 94 chemicals randomly selected from the Carcinogen Potency Database (CPDB)(Gold et al., 2010). Panel B: The relationship between actual NOAELs and chemicals found the CPDB. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 2a. Formation of tetrachloro-*p*-quinone from 1,4-dihydroxybenzene.

Figure 2b. Mechanism of halo-*o*-quinone formation from 1,2-dihydroxy-tetrachlorobenzene. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 3. Degradation of tetrachloro-*o*-quinone to haloacids, chloroform and CO_2. Two alternative pathways exist, the one with solid arrows leads to chloroform and CO_2, the alternate pathway leads to dichloroacetic acid and chloroform. The *p*-quinone is degraded by similar mechanisms. Details available in Bull et al. (2006)

Figure 4. Illustration of the formation of halogenated quinones by reaction of chlorine or chloramines with phenols and alternative formation of an quinone-4-(*N*-chloro)imine with chloramine. (Drawn from data in Heasley et al. 2004)

Figure 5. Mechanisms by which haloquinones may bind to macromolecules contributing to cytotoxicity or/or genotoxicity. A key to toxicity induced by quinones is that the glutathione and cysteine conjugates, produced largely in the liver, are transported via blood and bile to tissues where they may be concentrated in cells that express γ-glutamyl transferase (γ-GT) or glutathione transporters. Once in these tissues, they are further metabolized to thiol compounds that may be directly toxic or that are metabolized to reactive intermediates. This is illustrated in the γ-GT-expressing cells. Similar metabolic processing can occur in cells that concentrate glutathione (not shown). *N*-acetylation of the cysteine conjugate detoxifies the compound. This latter reaction may occur on the amino group of one or more cysteine residues.

Figure 6. Predicted changes in octanol/water partition coefficients with halogen substitution on hydroquinones, quinones, methyl-quinone, and quinone-4(*N*-chloro)imines. Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with permission.

Figure 7. Formation of *N*-halamines and illustration of how they may represent partially toxicologically activated compounds. With primary and secondary amines (A and B), it is anticipated that the first intermediate will be produced in drinking water and the remaining reactions occurring in vivo as the result of metabolic activation. *N*-chloramine of tertiary amine is unstable and reactive intermediates will be produced in water. Example A – products of reaction of amino acids with HOCl (Davies et al., 1993). Example B – products of reaction of acetamide with HOCl (Hawkins and Davies, 1998). Example C – products of reaction of aminopyrine with HOCl (Utrecht et al., 1995).

Figure 8. Illustration of the transfer of *N*-chloro groups from a non-permeant amino acid (illustrated as *N*-chlorotaurine above) to a permeant amino acid to effect oxidation of intracellular thiols. The *N*-chloramino acid can also react with macromolecules to produce carbon- or nitrogen-centered radicals as illustrated in Figure 4.

Figure 9. General scheme for carbonyl and cyano formation from chlorination of amines and amino acids. Compound I is a *N*-chloramine, II a *N*-dichloramine, III, an imine, IV, a *N*-chloroamide, V is either an aldehyde (R_1 – H) or a ketone (R_1= -$[CH_2]_{0-X}CH_3$.)

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Table

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Table 1. Predicted DBPs that have been shown to occur in drinking water[a]

Predicted DBP	Frequency of detection	Range of concentrations, ng/L	Notes
Haloquinones (HQs)	18/18		Favored with chlorine
DCBQ	18/18	2.2–275	
DBBQ	13/18	0.5–37.9	(enhanced with O_3)
DCMBQ	7/18	0.9–6.5	
TCBQ	7/18	2.9–9.1	
Halonitriles (HN)	18/18		
DCAN	18/18	100–2950	Favored with chlorine
BCAN	12/18	<3–2100	
DBAN	8/18	<4–2520	
TCAN	6/18	<2–210	
2,2-Dichloropropionitrile	1/18	8	Formation potential detected with other waters in lab exp
N-Nitrosamines	14/16		Favored with chloramine
NDMA	14/16	<2.4–130	
N-Nitrosomorpholine	6/38	<0.07–2.2	
N-Nitrosodiphenylamine	6/38	<0.11–1.8	Favored pH > 8
Heterocyclic amines			
Phenazine	NS[b]	NQ[c]	Favored pH < 8
N-Chlorophenazine	NS	NQ	Major product

[a] Source: Li et al. (2011) ©2011 Water Research Foundation. Reprinted with permission.
[b] not included in the survey of 18 water supplies
[c] concentrations not quantified

Table 2. Predicted LOAELs, carcinogenic and mutagenic activity of quinone and quinone-like structures predicted to form by reaction of chlorine or chloramine with substructures of natural organic matter (NOM)

Chemical	LOAEL mg/kg day^{-1}	Predicted carcinogen	Predicted mutagen
2,6-Dichloro-1,4-benzoquinone	0.049†	no	no
2,6-Dibromo-1,4-benzoquinone	0.159	no	no
2,3,6-Trichloro-1,4-benzoquinone	0.033	yes	no
2,3,5,6-Tetrachloro-1,4-benzoquinone	0.005	no	no
2,6-Dichloro-3-methyl-1,4-benzoquinone	0.079	yes	no
3,4,5,6-Tetrachloro-*o*-quinone	0.0047†	no	no
2,3,6-Trichloro-1,4-benzoquinone-4-(*N*-chloro)imine	0.073‡	yes	equivocal
2,3-Dichloro-4-prop-(2)-enol-*o*-quinone	0.064	yes†	yes
2,3,6-Trichloro-4-prop-(2)-enol-*o*-quinone	0.0065†	no	no
2,3,5-Trichloro-4,5-diketobenzoic acid	0.073	no	no
1,2,3-Triketo-4,4,5,6-tetrachlorocyclohex-5-ene	0.050	no	no*
1,3,3,5,5-Pentachloro-4-keto-5-hydroxycyclohexene	0.21*	no	no
2,3.4-Trichlorocyclohexadien-5-one carboxylic acid	0.22	no	no
1,2,2,4-Tetrachloro-3-hydroxy-cyclopent-4-enoic acid	0.19†	no	no
3,5-Dichloro-1-hydroxy-4-ketocyclopent-enoic acid	0.00025†	no	yes†
2,2,4-Trichloro-5-methoxycyclopent-4-ene-1,3-dione	0.24	no	yes†

*Outside the optimal predictive space of the model, but within permissible limits
†Outside the optimal predictive space of the model and exceeds permissible limits
‡Query structure contains fragment/s not represented in the training set

Table 3. Comparisons of the carcinogenic properties and potency of furan derivatives §

Compound	**Rat TD_{50}***	**Mouse TD_{50}***	**Mutagenicity†**
Furan	0.396	2.72	neg
Tetrahydrofuran	407	1300	neg
Furfural	683	197	pos
Furfuryl alcohol‡	Some evidence/equiv	Some/no evidence	neg
MX	0.583	Not tested	pos
Hexachlorocyclopentadiene	No evidence	No evidence	neg‡

§ Source: Bull et al. (2006). ©2006 Awwa Research Foundation. Reprinted with persmission
* TD_{50}s drawn from Gold et al. (2010)
† Ames' test mutagenicity only.
‡ Increased sister chromatid exchange in CHO cells in vitro, other tests negative.

Table 4. Predicted LOAELs, carcinogenic and mutagenic activity of halonitriles or haloamides shown or predicted to form by reaction of chlorine or chloramine with substructures of NOM

Chemical	LOAEL mg/kg day^{-1}	Predicted carcinogen	Predicted mutagen
Dichloroacetonitrile	0.029†	yes	yes
Dibromoacetonitrile	0.095	no	yes
Trichloroacetonitrile	4.5†	yes	yes†
3-Bromopropionitrile	0.89†	yes	yes
3,3-Dichloropropionitrile	0.77†	yes	yes
2,3-Dibromopropionitrile	0.15	yes	yes
3,3,3-Trichloropropionitrile	3.9†	yes	no
Dichloroacetamide	3.2	yes	yes
Bromochloroacetamide	1.5	yes	yes
2,3-Dichloropropamide	0.5	yes	yes
2,3-Dibromopropamide	1.8	yes	yes
2-Bromo-3-chloropropamide	2.2	yes	yes

*Outside the optimal predictive space of the model, but within permissible limits
†Outside the optimal predictive space of the model and exceeds permissible limits

Table 5 Predicted LOAELs, carcinogenic and mutagenic activity of haloaldehydes predicted to form by reaction of chlorine or chloramine with substructures of natural organic matter (NOM)

Chemical	LOAEL mg/kg day^{-1}	Predicted carcinogen	Predicted mutagen
2,3,4-Trichlorobutanal	0.96	no	
2,3-Dichloropropenal	0.97	yes	yes
2-Chloropropenal	1.7	yes	yes
Dichloroacetaldehyde	5.8	yes	yes

*Outside the optimal predictive space of the model, but within permissible limits
†Outside the optimal predictive space of the model and exceeds permissible limits

Table 6. Predicted LOAELs and predicted carcinogenicity of phenazine, *N*-chlorophenazine, and *C*-chlorophenazines.

Compound	Predicted carcinogen?	Met Criteria	Simil. Index	LOAEL mg/kg day^{-1}	Met criteria	Simil. Index
Phenazine	Yes	Yes	0.089	64.2	Yes	0.013
N-Chlorophenazine	Yes‡	No	0.140	114	No‡	0.082
1-Chlorophenazine	No	None	2 < 0.2	31	Yes	0.074
2-Chlorophenazine	No	2-species	3 < 0.2	31.8	Yes	0.074
1,4-Dichlorophenazine	No	None	3 < 0.2	15	Yes	0.108
2,3-Dichlorophenazine	No	None	3 < 0.2	16.6	Yes	0.101
2,3,7,8-Tetrachlorophenazine	No	None	2 < 0.2	4.1	Yes	0.157

‡Query structure contains fragment/s not represented in the training set

Figure
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Figure 1 A

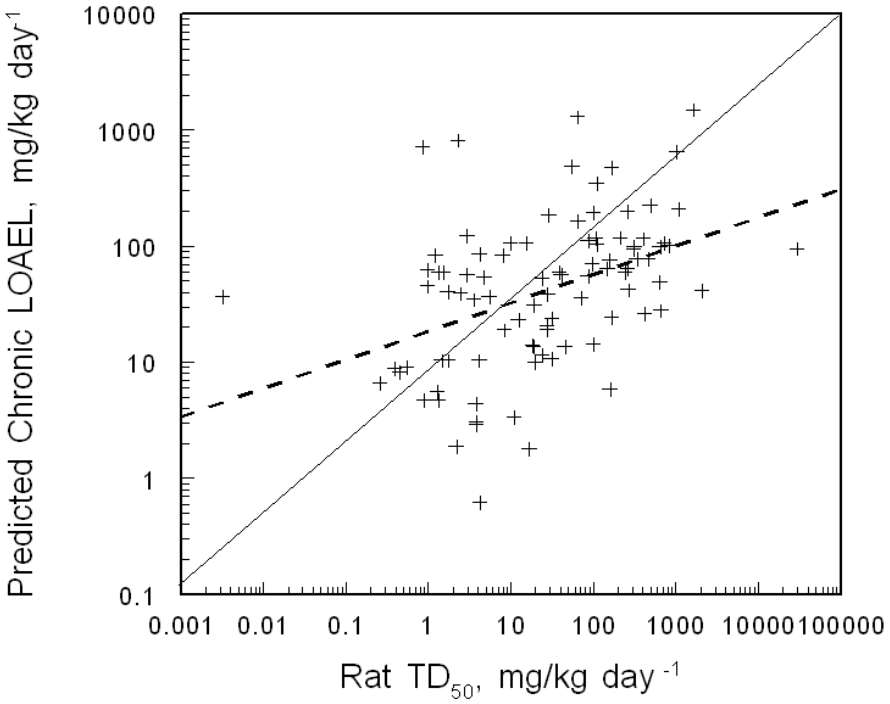

Figure 1 B

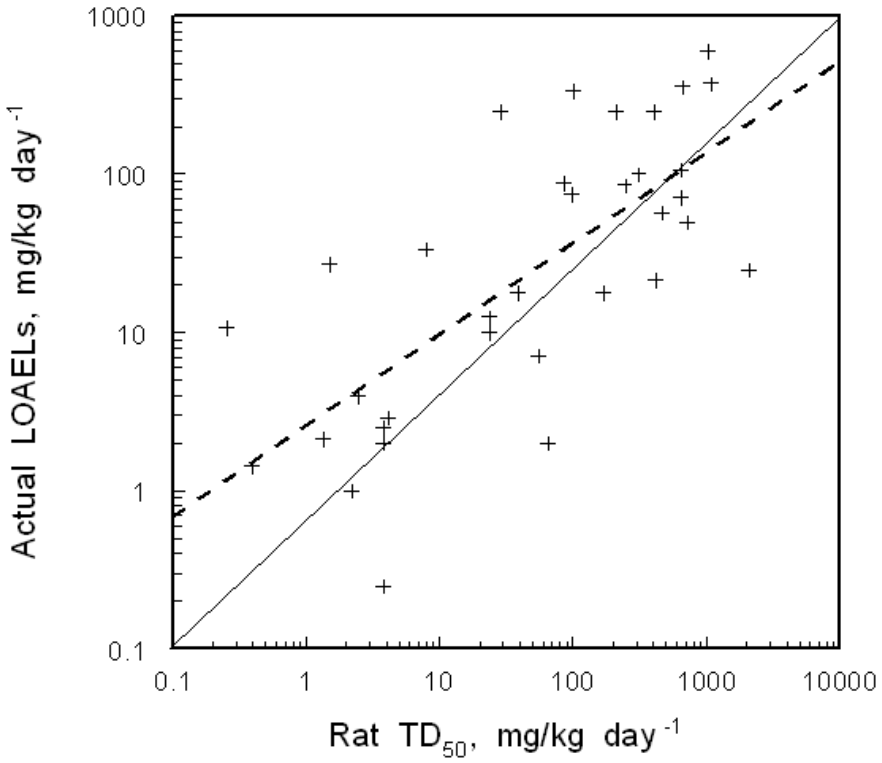

Figure 2 A

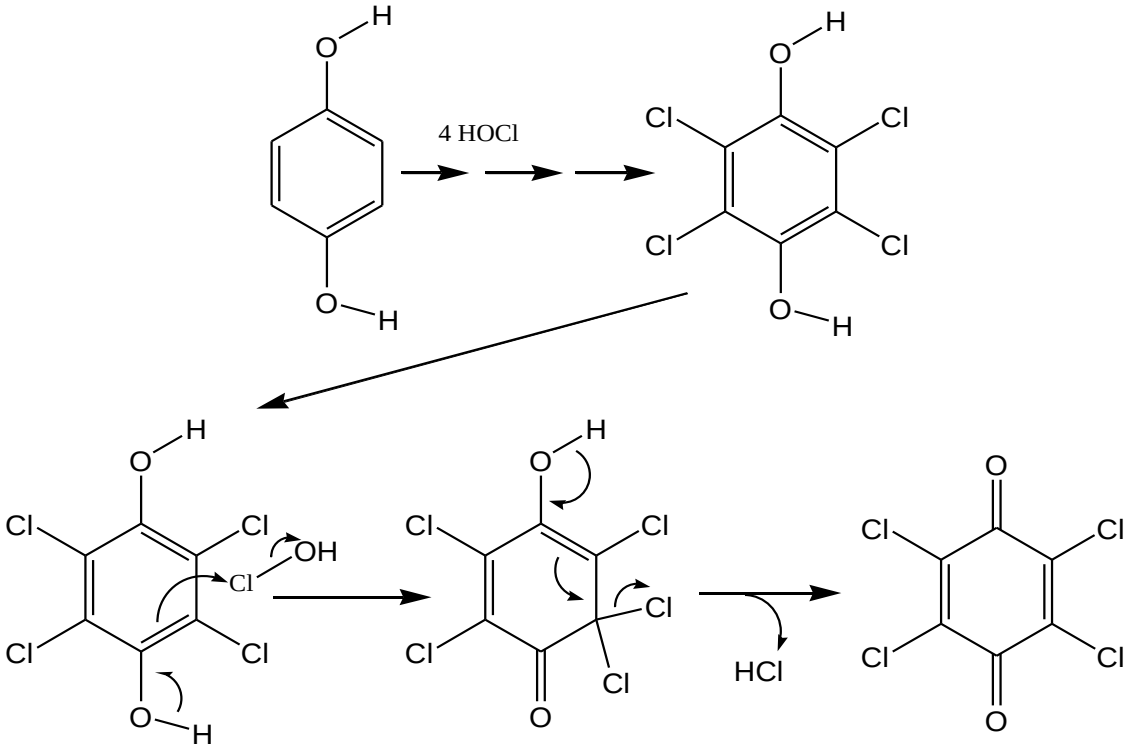

Figure 2 B

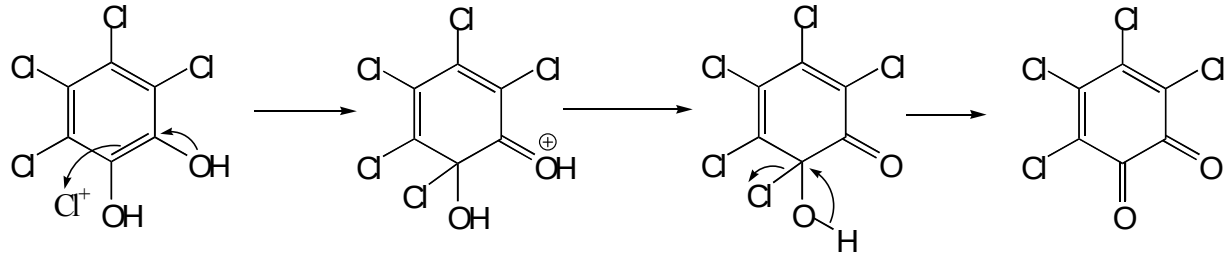

Figure 3

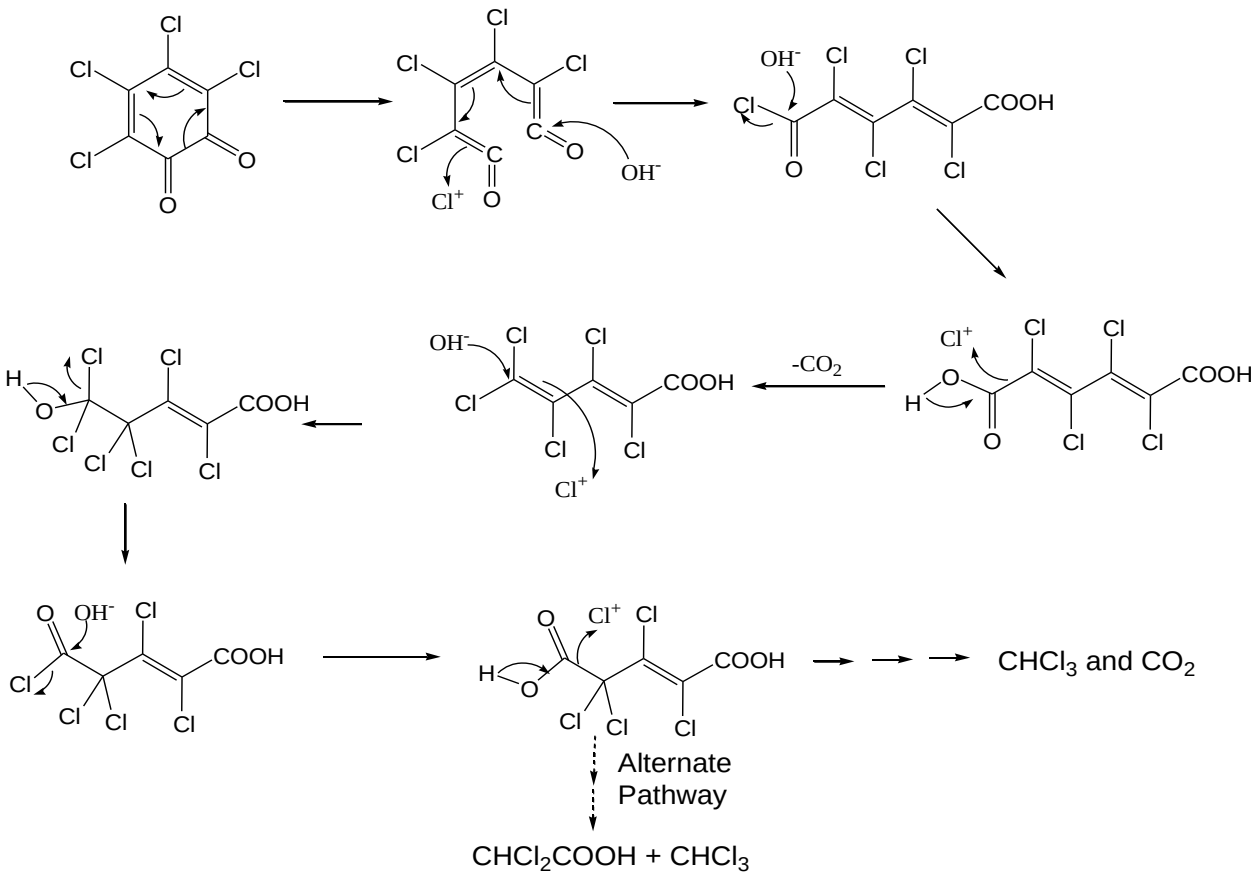
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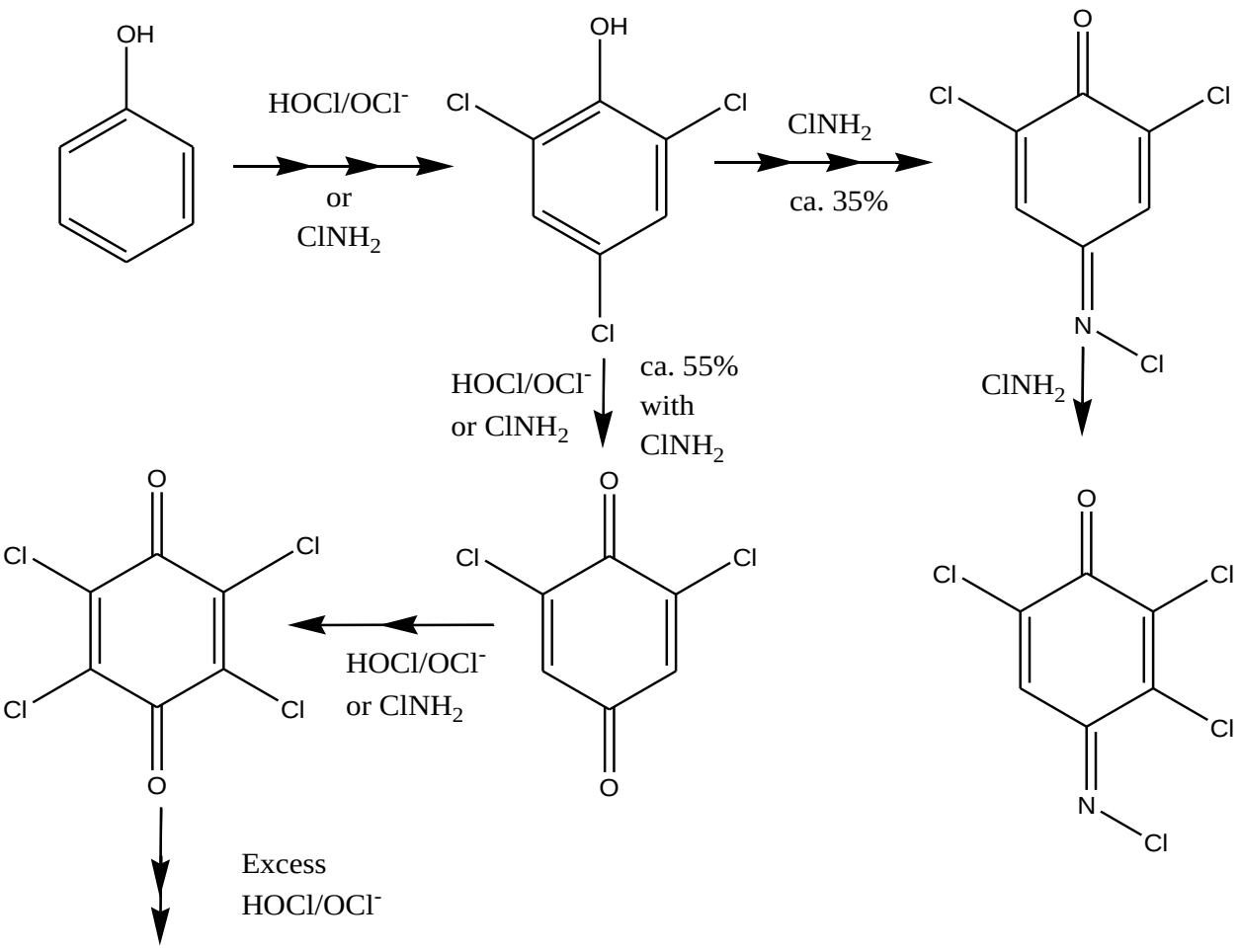
Figure 4



Haloacids, THMs, other aliphatic products

Figure 5

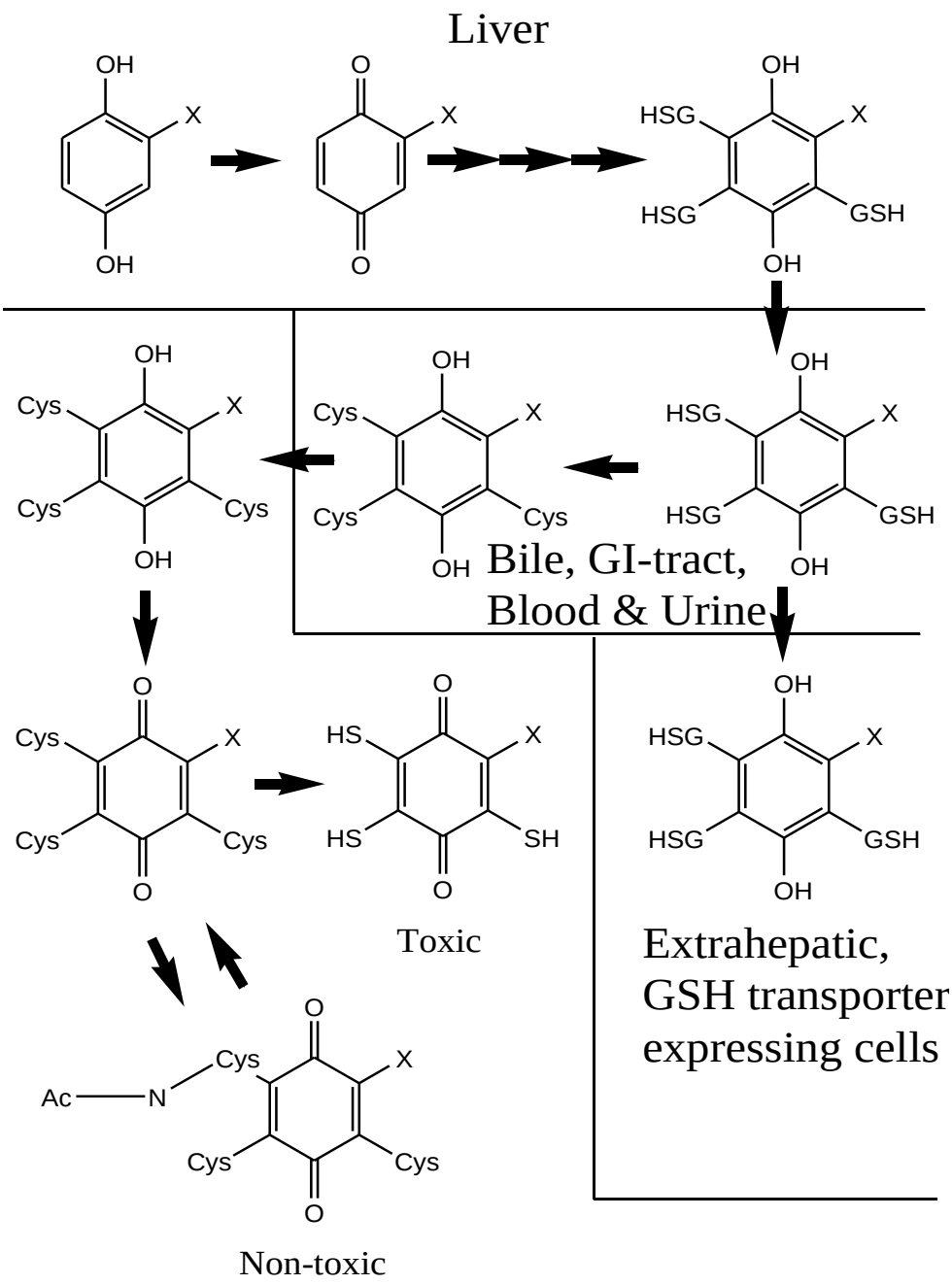

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Figure 6

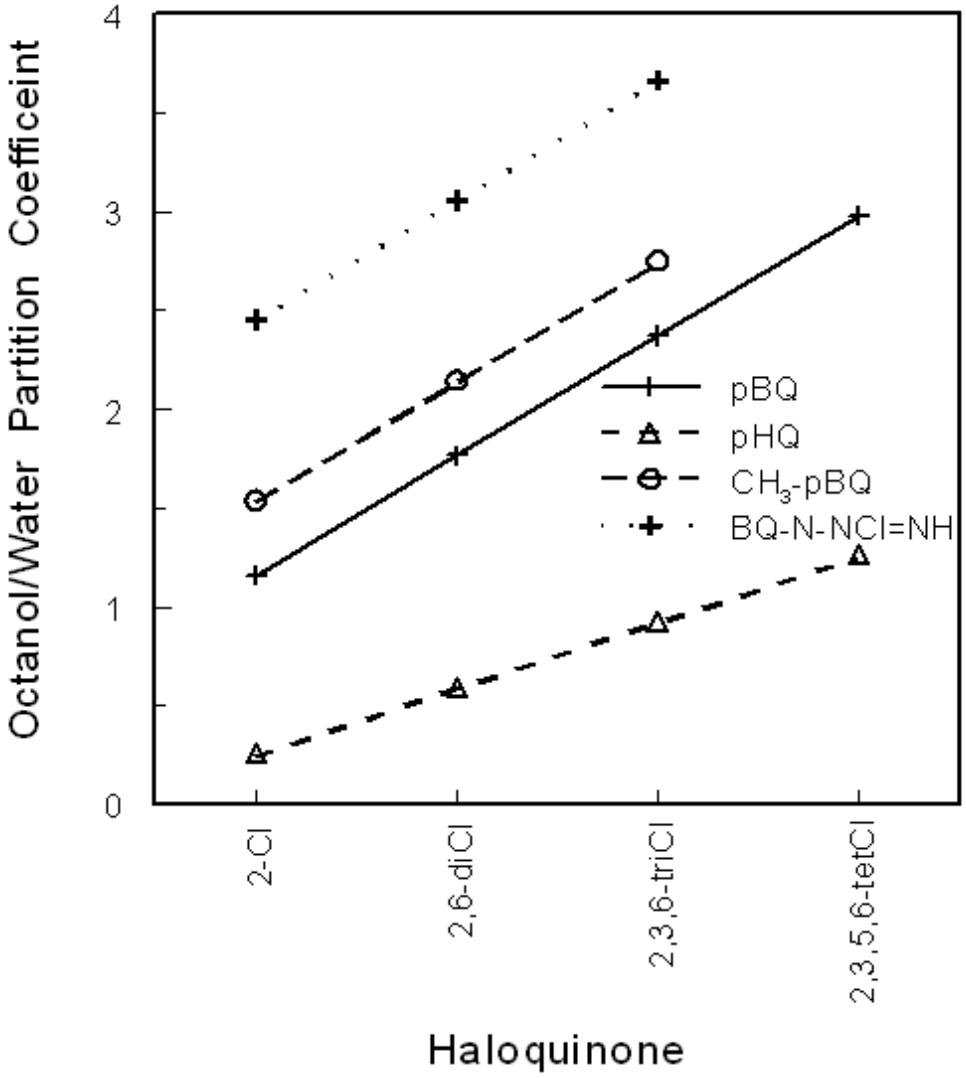
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Figure 7

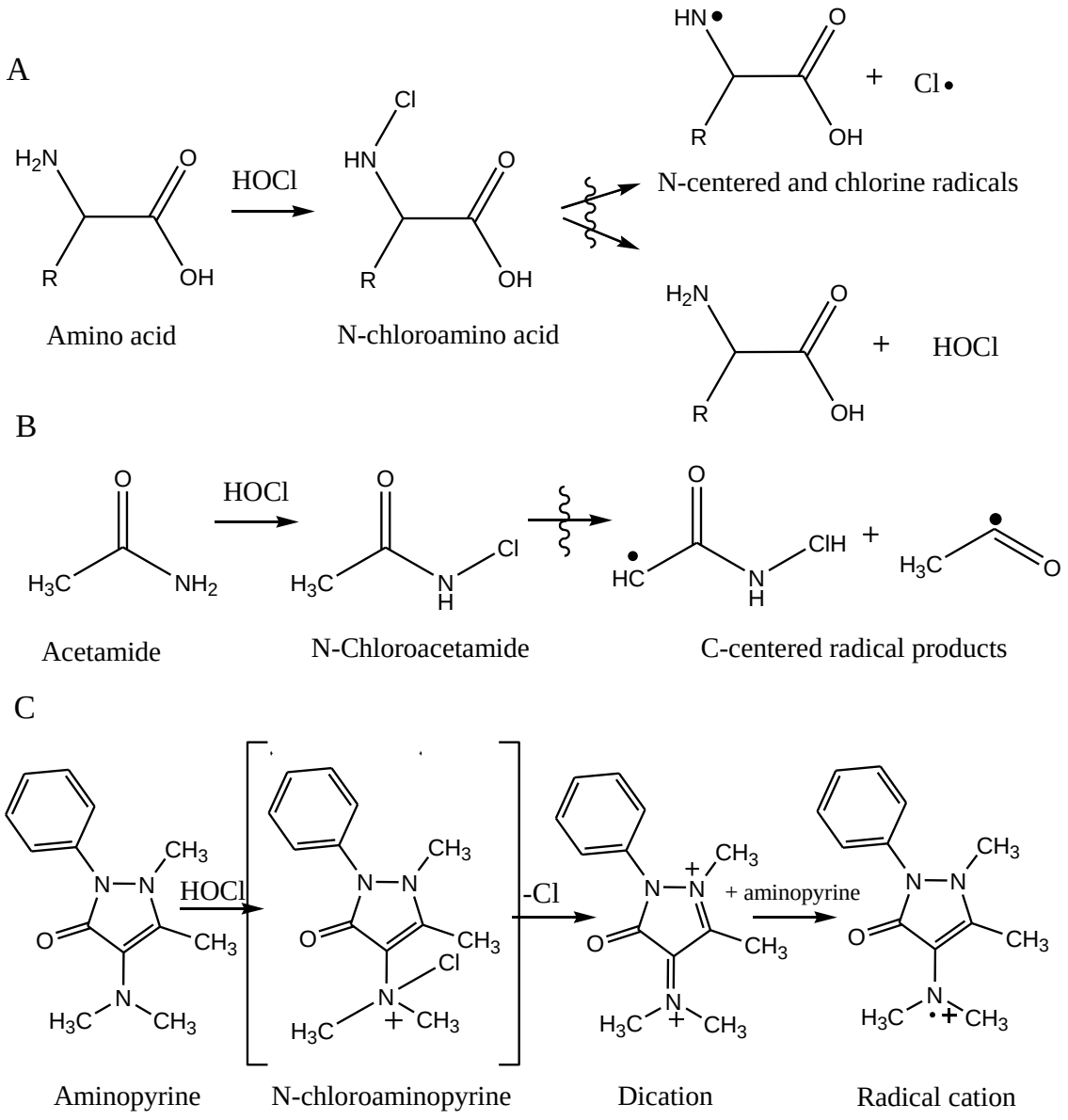
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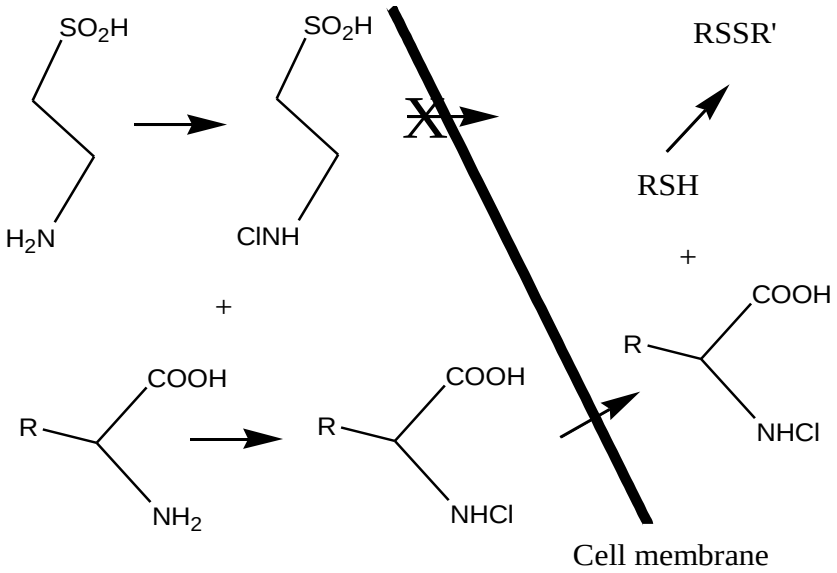
Figure 8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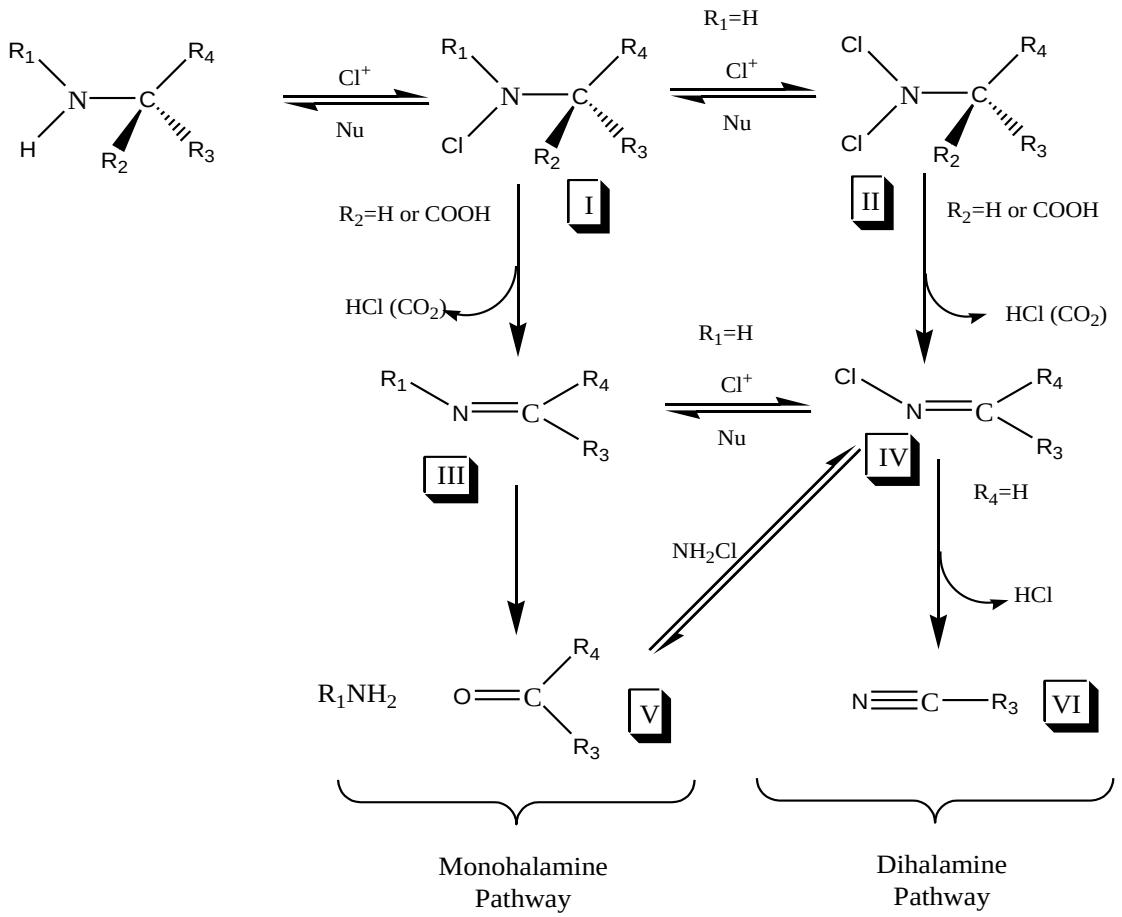
NHCl
Cell membrane

Figure 9


R1
R4
N
C
H
R2
R3
Cl+
Nu
Cl
I
R1=H
II
R2=H or COOH
HCl (CO2)
III
IV
R4=H
HCl
NH2Cl
R1NH2
O
V
VI
Monohalamine Pathway
Dihalamine Pathway

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ELSEVIER

Toxicology

Conflict of Interest Policy

Manuscript number (if applicable):

Article Title: Potential carcinogenic hazards of non-regulated disinfection by-products: Haloquinones, halo-cyclopentene and cyclohexene derivatives, N-halamines, and heterocyclic amines

Author name: Richard J. Bull

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Author name: XING-FANG LI

[illegible]ations

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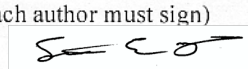

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