Development of a Research Strategy for Integrated Technology-Based Toxicological and Chemical Evaluation of Complex Mixtures of Drinking Water Disinfection Byproducts

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Chemical disinfection of water is a major public health triumph of the 20th century. Dramatic decreases in both morbidity and mortality of waterborne diseases are a direct result of water disinfection. With these important public health benefits comes low-level, chronic exposure to a very large number of disinfection byproducts (DBPs), chemicals formed through reaction of the chemical disinfectant with naturally occurring inorganic and organic material in the source water. This article provides an overview of joint research planning by scientists residing within the various organizations of the U.S. Environmental Protection Agency Office of Research and Development. The purpose is to address concerns related to potential health effects from exposure to DBPs that cannot be addressed directly from toxicological studies of individual DBPs or simple DBP mixtures. Two factors motivate the need for such an investigation of complex mixtures of DBPs: *a*) a significant amount of the material that makes up the total organic halide and total organic carbon portions of the DBPs has not been identified; and *b*) epidemiologic data, although not conclusive, are suggestive of potential developmental, reproductive, or carcinogenic health effects in humans exposed to DBPs. The plan is being developed and the experiments necessary to determine the feasibility of its implementation are being conducted by scientists from the National Health and Environmental Effects Research Laboratory, the National Risk Management Research Laboratory, the National Exposure Research Laboratory, and the National Center for Environmental Assessment. *Key words:* analytical chemistry, complex mixtures, disinfection byproducts, drinking water, reverse osmosis, toxicology. *Environ Health Perspect* 110(suppl 6):1013–1024 (2002).
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Disinfection of drinking water is rightly hailed as one of the major public health triumphs of the 20th century. As a result of chemical disinfection of water, dramatic reductions have been observed in both mortality and morbidity from waterborne diseases (Regli et al. 1993). Recent outbreaks of waterborne disease, both in the United States and in South America, serve as vivid reminders of the necessity of ensuring that public water supplies are treated properly to guard against infectious microbial contamination. Traditionally, drinking water disinfection has involved the use of chlorine at two separate points in the treatment process, for both pretreatment (disinfection at the beginning of the treatment process) and posttreatment (additional disinfection near the end of the treatment process to maintain a disinfectant residual in the distribution system), resulting in the production of a wide variety of disinfection byproducts (DBPs) (Richardson 1998). The concentrations and bromo/chloro speciation of the DBPs are influenced by source water characteristics such as pH, total organic carbon (TOC) concentration, bromide concentration, chlorine dose, and temperature (Fair 1995; Krasner 2001; Singer 1995).

Because of concern over the potential human health effects of the trihalomethanes (THMs) and the other DBPs formed when chlorination is used for both pre- and posttreatment, many treatment plants in the United States have switched to the use of only a single postchlorination treatment. There is also considerable interest in the use of ozone to disinfect drinking water because relative to chlorination, ozonation typically results in decreased concentrations of regulated DBPs such as THMs. An additional benefit of ozone is that it is a more effective biocide than chlorine, particularly for chlorine-resistant microbes such as *Cryptosporidium* oocysts. However, when ozone is used in the United States to disinfect water in public utility systems, a posttreatment is usually applied to ensure that microbial contamination is controlled during residence time in the distribution system (i.e., the time from treatment at the plant to the tap in the home). Typical posttreatments include chlorination and chloramination. Disinfection strategies that involve ozone can be characterized as resulting in a decrease in the total amount of halogenated DBPs formed; an alteration in the speciation of the DBPs formed (i.e., increased formation of brominated species when natural bromide levels are high); and formation of a few DBPs of toxicological concern that are not usually formed by chlorination processes alone.

Disinfected water contains more than 500 chemical DBPs (Richardson 1998); the routinely measured/monitored DBPs are listed in Table 1. However, it should be noted that many DBPs are still unidentified; for chlorination, the known DBPs might account for < 50% of the mass of total organic halide (TOX) (Stevens et al. 1989). At the dose levels tested in laboratory experiments, a number of these DBPs were either carcinogenic or caused target-organ toxicity, including reproductive/developmental toxicity. These laboratory dose levels are high compared with the low levels, ppb (μg/L) to ppt (ng/L), found in drinking water. The vast majority of DBPs have not been investigated toxicologically; in

Table 1. Chemical analyses for routinely monitored DBPs and water parameters.

Bromide
Bromate
Chlorate
Iodide
Iodate
Four THMs[a] (chloroform, bromodichloromethane, dibromochloromethane, bromoform)
Nine HAAs[a] (monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, dibromoacetic acid, tribromoacetic acid, bromochloroacetic acid, bromodichloroacetic acid, dibromochloroacetic acid)
Four HANs[a] (trichloroacetonitrile, dichloroacetonitrile, bromochloroacetonitrile, dibromoacetonitrile)
Chloropicrin
Chloral hydrate
Two halopropanones[a] (1,1-dichloropropanone, 1,1,1-trichloropropanone)
Four aldehydes (formaldehyde, acetaldehyde, glyoxal, methyl glyoxal)
DOC
TOX

[a]Within the chemical classes of THMs, HAAs, HANs, halopropanones, and aldehydes, chemical analyses will be conducted for each individual chemical listed. Using the THMs as an example, individual analyses will be conducted for chloroform, bromodichloromethane, dibromochloromethane and bromoform.

fact, fewer than 20 individual DBPs have been subjected to toxicity studies usable for risk assessment (U.S. EPA 2000a). Additionally, there are concerns over the toxic contribution of the unknown fraction. It should also be noted that little is known about the potential interactions among the DBPs, with this being particularly true when looking beyond the THMs and the haloacetic acids (HAAs).

In contrast to what might be extrapolated from laboratory animal studies on individual DBPs, some of the epidemiologic studies conducted to date suggest associations between human consumption of chlorinated drinking water and adverse health outcomes, including reproductive and developmental effects such as increased spontaneous abortions and low birth weight (Bove et al. 1995; Gallagher et al. 1998; Klotz and Pyrch 1999; Kramer et al. 1992; Waller et al. 1998), and bladder and rectal cancer (Cantor 1997; Cantor et al. 1998, Freedman et al. 1997; King and Marrett 1996; McGeehin et al. 1993). In the absence of a definitive epidemiologic data set, estimation of the human health risks posed by exposures to DBPs can be developed through toxicological evaluations of the complex mixture itself or of the components of the mixture. Given these options, toxicological evaluation of the complex mixture rather than assessment of each individual chemical separately is preferred because this option better characterizes the real human exposure. Importantly, assessment of the complex mixture accounts for any compounded effects

from exposure to the low levels of the multiple DBPs, characterized and unknown, found in drinking water.

As most animal DBP toxicity data are from single-chemical studies and the results of the human epidemiologic studies are from exposure to DBP mixtures, there is a need to conduct scientifically sound and defensible animal experiments with DBP mixtures. Such studies should

• focus on those end points identified as of concern from epidemiologic studies
• provide information that bridges the gap between single-chemical studies in experimental animals and human exposure to multiple DBPs by incorporation, where feasible, of dosimetry in the mixtures experiments
• improve our ability to estimate the risk(s) from complex mixtures of DBPs at low exposure levels and multiple routes of exposure
• provide useful information in areas of concern with regard to DBP mixtures, such as any change in health risk(s) associated with moving from chlorination disinfection strategies to alternative disinfection strategies.

The majority of the toxicological research currently ongoing with DBP mixtures is with simple, defined mixtures (for a review, see Simmons et al. 2001). Such work fills an important data gap, as it provides information about the nature of the interactions (additive, greater than additive, less than additive) among DBPs known to be toxicologically important. Further, it indicates the appropriateness, or lack thereof, of the default risk assessment assumptions of dose additivity for noncancer health effects and response additivity for cancer. However, there are simply too many possible combinations of DBPs for it to be feasible to evaluate more than selected subsets as simple, defined mixtures. Additionally, and very importantly, even if it were technically feasible and financially possible to examine all possible combinations of all known DBPs, this leaves the large unknown fraction unevaluated for potential health effects.

Working with drinking water concentrates allows evaluation of the mixture as a whole. Most previous concentrations have used methodologies that have resulted in the loss of the volatile fraction, so the concentrates or extracts tested consisted of the semivolatiles and nonvolatiles (Simmons et al. 2001). Historically, health effects research with concentrated drinking water samples, typically prepared by adsorption on polymer (XAD) resins, has emphasized detection of mutagenicity. There has been markedly little toxicological evaluation of complex mixtures of DBPs in experimental animals. One exception is a research project, conducted some years ago, to evaluate concentrates of disinfected

water collected from five U.S. cities. Despite evidence of *in vitro* mutagenicity (Loper et al. 1978), little evidence of carcinogenicity was exhibited in a mouse skin initiation/promotion assay by reverse osmosis (RO) concentrates of disinfected water that had undergone organic solvent extraction followed by removal of the organic solvent (Bull et al. 1982). Pregnant CD-1 mice were gavaged from gestation day 7 (GD7) to GD14 with concentrates from the same finished water sources that had been prepared in the same manner (Kavlock et al. 1979). A volatile organic mixture, based on the known chemical levels in drinking water, was restored to the water concentrates. The authors reported no adverse effects in the fetus attributable to either the drinking water concentrates or the volatile fraction. Based on these results, longer-term multigenerational dosing and evaluation of both female and male reproductive toxicity, as well as developmental toxicity, were incorporated into the present research design.

Although epidemiologic and toxicological studies suggest that human health effects from DBP exposure are of concern, these studies have not demonstrated conclusively a causal relationship between the typically low environmental DBP levels to which humans are exposed and human health risks. If it is assumed, however, that low-level DBP exposures cause human health effects, as suggested in some epidemiologic studies, several hypotheses can be posed to explain the discrepancies between the epidemiologic results and the expected lack of effects in epidemiologic studies based on currently available rodent single-chemical bioassays, including

• There is an effect from exposure to the complex mixture of DBPs that is at least additive (if not synergistic) in nature, so studies involving low levels of individual DBPs are inadequate to explain the health effects found in the positive epidemiologic data.
• Of the more than 500 DBPs identified, relatively few have undergone evaluation for reproductive and developmental toxicity in experimental animals, and the experimental animal and human data will be reconciled when sufficient numbers of individual DBPs have been tested.
• Animal studies differ from human exposures through differences in physiology, biochemistry, anatomy, and lifestyle factors (e.g., high-fat diets) that prevent animal studies from demonstrating the same outcomes observed in humans.
• Typically, laboratory studies expose animals by only a single route, usually oral, so effects due to combined oral-dermal-inhalation exposures, as in the case of human exposure to DBPs, are not observed.

- Effects observed in the subset of positive epidemiologic studies are the result of confounding factors (exposure to other environmental pollutants present in the source water or exposure to industrial pollutants in urban areas or pesticides in agricultural areas), so animal studies focused solely on DBPs will not corroborate epidemiologic findings.
- The positive epidemiologic findings are due to factors other than exposure to environmental chemicals.

Although the above hypotheses are not mutually exclusive, this project will test the first hypothesis, that adverse health effects exist and are attributable to exposure to the complex mixture of DBPs. We will evaluate the effects of the complex mixture of DBPs through a "mixture as a whole" approach, and using the chemical analysis of the mixture, attempt to understand whether additive, greater than additive, or less than additive interactions are occurring. Further, to the extent possible, we will examine possible confounding of the experimental results by other environmental factors in the drinking water. Results from recent and ongoing research activities with DBPs (Richardson et al. 2002) will provide useful information on the relative importance of the various routes of exposure and will provide toxicity information on previously unevaluated DBPs. In summary, a critical data gap in DBP mixtures research is toxicological evaluation in experimental animals of those end points of concern identified from epidemiology studies. These end points are reproductive/developmental effects and cancer. These experiments need to use, to the extent technically possible and feasible, real-world complex mixtures of DBPs. The overarching research program goal may be stated as follows: based on sound, defensible research on complex mixtures of DBPs, the level of comfort associated with ubiquitous human exposure to the byproducts of drinking water disinfection is either decreased or increased. Alternatively, this may be stated: estimation of the potential health risks associated with exposure to the mixtures of DBPs formed during disinfection of drinking water will be improved by the provision of sound, defensible experimental data on environmentally relevant mixtures of DBPs.

Research Plan

The research plan described in this article is designed to evaluate real-world complex mixtures of DBPs formed by chemical disinfection of bromide-containing water. The proposed research will *a*) develop health effects and chemical identity information for surface water disinfected by chlorination; *b*) develop health effects and chemical identity information for the same surface water disinfected by an alternative disinfection scheme (the alternative disinfection scheme used in preliminary experimentation was preozonation followed by postchlorination as the secondary chemical treatment); and *c*) compare the potential human health risks associated with consumption of water disinfected by different treatment scenarios.

This initial research plan proposes a succession of studies that are logical steps to follow but that may be difficult to implement because of a number of challenging technical issues. Although some of the procedures included in the plan have been performed often (e.g., water treatment operations, toxicological assays), aspects of this research are innovative and novel. Thus, this research effort has been divided into three phases; each preceding stage is expected to influence the structure, practicality, and implementation of the next stage. The first phase (pretrial run phase) involved experiments to

- determine whether bromide and iodide spiking of the source water selected for the trial run phase was needed and, if needed, the appropriate levels with which to spike the source water (see "Addition of Bromide and Iodide to the Source Water," below)
- develop methods for RO membrane concentration of water (see "Concentration by Reverse Osmosis," below), and
- determine the treatment parameters related to the palatability of the drinking water concentrates to rats.

The second phase (trial run phase), involves experiments to

- assess the logistics of concentration of large volumes of water
- compare XAD-resin extraction to RO membrane concentration

- measure water consumption rates in rodents to calculate accurately the volumes of concentrated water required for the full study (the third phase)
- perform initial *in vivo* and *in vitro* toxicological experiments
- study the chemical stability of the concentrated water, and
- perform initial chemical analysis of the DBPs.

In the third phase (the full study), we use the information gained and knowledge learned from the first two phases, as well as the initial peer review of a concept proposal for this research, to determine the feasibility of the proposed experimental plan for the full study and, assuming feasibility; revise the proposed research plan for the full study; and conduct the full study to gather extensive toxicological data on relevant health end points and more fully characterize the chemical composition of the DBPs.

Figure 1 provides the basic elements of the full study and their sequence, as outlined in the concept proposal. They are as follows:

- Select a surface source water and determine an appropriate level of bromide and iodide spiking if needed.
- By splitting the source water into two streams, disinfect and produce finished drinking water by two disinfection systems to allow comparison of both the DBPs and health consequences of alternative disinfection scenarios.
- Concentrate these two treated waters by procedures and methods developed and evaluated in the pretrial and trial runs and transport the concentrated waters to other laboratories.
- Perform extensive chemical analyses both to qualitatively assess which DBPs/chemicals are present and to quantitatively measure as many of these DBPs/chemicals as possible.
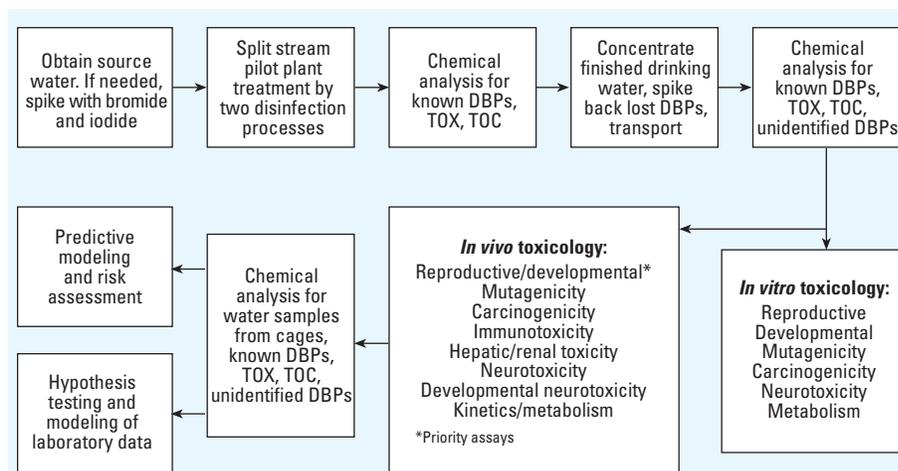

Figure 1. Flow diagram for the proposed full study. The basic elements of the full study and their sequence are illustrated.

- Perform *in vivo* toxicological assays and short-term *in vitro* experiments with the concentrated waters, with a targeted focus on reproductive and developmental end points, including other important end points and potential target organs, to the maximum extent possible.
- Perform statistical analyses and modeling to test for statistically significant effects and to generate estimates of the proportions of any observed toxicity that can be attributed to the known and unknown components of the mixtures.
- Use these data to estimate the risk posed by these complex mixtures and the relative differences, if any, between the two water treatments. These steps may be iterative over time. Aspects of the steps are described in more detail below. It is expected and anticipated that elements of the full study will be modified to reflect both new knowledge gained as a result of the preliminary experiments leading up to the full study and new advances in our knowledge of the potential human health consequences of exposure to DBPs.

Participants

A multidisciplinary team of investigators from a wide array of disciplines is needed for successful completion of this project. Necessary areas of expertise include water treatment engineering; DBP source and occurrence information; analytical chemistry; target organ toxicology; mixtures toxicology; statistical analysis and experimental design; and, risk assessment. In addition to the steering committee (the authors of this article), a number of individuals from the research laboratories of the Office of Research and Development, the National Health and Environmental Effects Research Laboratory (NHEERL), the National Risk Management Research Laboratory (NRMRL), the National Exposure Research Laboratory (NERL), and the National Center for Environmental Assessment (NCEA) participated in development of the research concept proposal. These individuals are listed on the first page of this article.

Water Source

The source water selected for the first and second phases is East Fork Lake (EFL, also known as Harsha Lake). It is a large reservoir on the Little Miami River at the East Fork State Park in Ohio. This surface water is representative of many in the country with higher than average TOC, typically 5–8 mg/L. The Water Industry Data Base of the American Water Works Association (AWWA) showed the 50th percentile for surface water TOC to be 4.0 mg/L (Federal Register 1994). Normal seasonal changes are expected for the reservoir,

which can alter somewhat the water quality over the course of a year (TOC, temperature, water stage, turbidity, etc.). Although uniform formation conditions (UFCs) (Summers et al. 1996) for chlorine and oocyst inactivation conditions for ozone are expected to be relatively constant, other conditions may vary (chlorine and ozone demand [and therefore doses] may change, coagulation doses vary with turbidity, bromide spiking may change as ambient bromide levels change, etc.). Therefore, temporal changes in finished water quality are expected, requiring monitoring over time to relate consumer equivalents to animal equivalents.

One source water cannot serve to represent all typical surface waters. More than one source water (ideally many more than one) should be used to gain a comprehensive picture of the potential health risks resulting from long-term low-level exposure to the highly complex, incompletely characterized mixtures of DBPs formed by chemical disinfection of water. It is our hope that the methods developed for use in the full study will prove useful, and that they will then be applied to other water sources to build a representative database.

Keeping in mind that there is no one ideal water source, EFL does offer several advantages. Among these are the fact that it is a reservoir and hence of more consistent water quality than a free-flowing water source such as a river. It is the water source for Clermont County's Bob McEwen Water Treatment Plant (5 million gallons per day (mgd) operational, 10 mgd design). This has two advantages. It means that the water being used in the initial stages of this project is being consumed by a large number of individuals, adding to the environmental realism of the effort. Additionally, the historical water-quality database of this water treatment plant can be used to better understand the degree of variability that might be expected over time. In addition to these factors, another consideration was location of a water source within reasonable trucking distance of the site of water treatment and concentration. The first and second phases of water treatment and concentration are taking place at the U.S. EPA facility in Cincinnati, Ohio. There are two surface waters with truck access within driving distance of the facility. One is EFL, the other is the Ohio River. EFL was selected because, compared with the Ohio River, it has higher TOC levels with resultant higher formation of DBPs, and as a reservoir, it has more consistent water quality and lower day-to-day variation in turbidity, TOC, bromide, etc. than the free-flowing Ohio River. Careful attention must be paid to the criteria used to select the source water for the full study.

These selection criteria will be provided in the publication of the full study data.

Addition of Bromide and Iodide to the Source Water

Based on the bromide concentration of the selected surface water(s), the intake water(s) will be spiked (if necessary) with sufficient levels of bromide and iodide to ensure representative formation of brominated and iodinated species. EFL is a low-bromide water (typically 10–15 µg/L); when it is used, higher bromide with a consistent concentration is obtained by spiking. Bromate, bromodichloromethane, dibromoacetic acid, and other brominated DBPs are of health concern (Richardson et al. 2002), and their concentrations depend in part on the bromide concentration of the water being treated. Chlorination of EFL water that contains ambient concentrations of bromide typically results in formation of mainly chlorinated byproducts such as chloroform, dichloroacetic acid and trichloroacetic acid, and chloral hydrate (Miltner RJ. Personal communication). Bromide spiking is based on three goals. The first goal is to achieve a bromide-spiking level that results in reasonable formation of the mixed chloro-bromo species of the THMs but does not result in too much of a shift from chloroform to bromoform. Representative concentrations of the four THMs in surface waters are given in the Stage 1 D/DBP Rule (Federal Register 1994). Thus, determination of the amount of bromide required to produce a representative mixture of the four THMs (chloroform, bromodichloromethane, dibromochloromethane, bromoform), and consequently of HAAs and haloacetonitriles (HANs), is a goal of the preliminary studies. The second goal is to have the total THM level close to the 80-µg/L maximum contaminant level (MCL) in the 1× water (i.e., disinfected water prior to concentration) produced by chlorination. Although the bromide concentration to be added to the selected surface water(s) is based on DBP formation under chlorination, the same bromide and iodide spike is used for the other disinfection treatments under consideration to allow proper comparison of alternative disinfection schemes. The third goal is to have the bromate level of the 1x water close to the Stage 1 MCL of 10 µg/L. The resulting bromide concentration will account for both the ambient and spiked bromide.

The rationale for spiking with iodide is that bromide and iodide have been found to co-occur in waters from different locations that have been tested for the presence of iodide (Khiari et al. 1999). In addition, there has been recent concern that iodo-THMs and other iodinated byproducts could be toxicologically important. An effort by the U.S. EPA

Office of Water to prioritize all known DBPs according to probable adverse health effects resulted in the selection of two of the iodo-THMs (bromochloroiodomethane and dichloroiodomethane) in a "top 50" list of such DBPs. As a result, these iodo-THMs and others were quantified in waters across the United States in a recently conducted nationwide DBP occurrence study (Gonzalez et al. 2001; Onstad et al. 2001; Weinberg et al. 2002). Thus, the rationale for concurrent spiking of iodide with bromide is 2-fold: having iodide present with bromide is approximating a real-world water situation, and having iodide present in realistic proportion to bromide might give rise to toxicologically important DBPs. The iodide concentration is typically 10–15% of the bromide concentration (Khiari et al. 1999); thus, an iodide concentration corresponding to 15% of the bromide spike has been selected for use in this study. If the selected source water for the full study contains sufficient bromide and iodide for representative formation of brominated and iodinated DBPs, spiking with bromide and iodide will not be necessary.

Water Disinfection Treatment Schemes

The choice of treatment was among the more difficult decisions faced in the planning process. Although we strongly preferred to have more than two treatment scenarios, production and evaluation of more than two treatment scenarios at one time is not currently technically feasible. If the project proves successful, we hope to use the methods developed here to evaluate other disinfection schemes. Given the limitation of being able to carry out only two treatments in this initial project, chlorination and ozonation/chlorination were chosen for the preliminary experiments; chlorine is still the most frequently used disinfectant in the United States, and ozonation/chlorination is the most prevalent scenario when ozone is used. Had an additional treatment been possible at this stage, chloramination, most likely in combination with ozonation, would have been selected, as many treatment plants have moved to this disinfection scenario. An ozonated water was included because many water utilities have moved to ozonation to meet DBP MCLs and to control *Giardia* cysts and *Cryptosporidium* oocysts. Surveys of water treatment plants conducted in 1996 by the American Water Works Association Research Foundation and in 1998 by the AWWA Water Quality Division Disinfection Systems Committee (AWWA 2000) indicate that chlorination/chlorination (i.e., pretreatment with chlorine/posttreatment with chlorine) is still the most prevalent disinfectant scenario and impacts the largest part of the U.S. population. In addition, ozonation/chlorination is more prevalent than ozonation/chloramination. Our plans for work beyond that outlined here are dependent on the successful completion of the present research. Once the methods are proven successful, they can be used to examine both a variety of source waters and a variety of disinfection scenarios.

For the chlorination scenario, a postchlorination scenario was selected for the preliminary experiments because many water utilities have moved to this scenario to meet DBP MCLs (Figure 2). Chlorination occurs under UFC conditions (a free chlorine concentration of 1 mg/L after 24 hr at pH 8) (Summers et al. 1996). For the ozonation/chlorination scenario (Figure 2), the level of ozone will be set to achieve 1 log inactivation of *Cryptosporidium* oocysts, based on previous inactivation studies (Owens et al. 2001). Demand studies will be conducted to determine the ozone dose required to provide CTs (mean dissolved ozone concentration in the contactor × the contactor's residence time based on tracer studies) that give 1 log inactivation of *Cryptosporidium* oocysts (Owens et al. 2001). An additional advantage to the use of chlorination as the posttreatment with ozonation is it allows a comparison to the postchlorination scenario that minimizes the number of variables that differ between the two treatment schemes. Similar to the chlorination-only scenario, the water is chlorinated at UFC (Summers et al. 1996). Based on previous pilot-scale ozonation/chlorination studies of EFL water without bromide spiking, under UFCs (Miltner et al. 1996), the ozonation/chlorination plant is expected to produce lower concentrations of DBPs than the chlorination plant, as ozone will oxidize some chlorine-reactive DBP precursor material.

A conventional filter was selected because at the time this project was initiated, the majority of U.S. water treatment plants that employed ozonation used conventional filtration (Rice R. Personal communication). Additionally, use of conventional filtration across the two treatment schemes being compared decreases the number of variables that differ between the two and increases the opportunity to attribute observed differences between the two methods of disinfection. Given the experimental complexity anticipated, it will be easier to examine and attribute risks if only a single aspect of the treatment changes. It should be noted that the Stage 1 D/DBP Rule recommends that utilities employing ozonation also employ downstream biological filtration to remove some of the ozone byproducts prior to distribution, as these DBPs are nutrients for possible bacterial regrowth in the distribution system.

Concentration by Reverse Osmosis

The concentration of DBPs for evaluation of mutagenicity is typically done by XAD-resin techniques. For XAD concentration, compounds are adsorbed onto the resin of choice and eluted with an organic solvent that is then removed by evaporation. The remaining organics can then be redissolved in an appropriate solvent for testing. The hydrophilic and volatile fractions of the natural organic matter (NOM) and DBPs are lost in the XAD procedure. The percentage of DBPs lost is a function of the nature of each DBP, the chosen resin, and the operating conditions. If standard protocols are used, the loss due to breakthrough/operating conditions is minimal. The popularity of XAD-resin concentration methods has been due, in part, to their ability to concentrate the organics easily without inorganic interferences (Wilcox et al. 1986). However, the amount of water
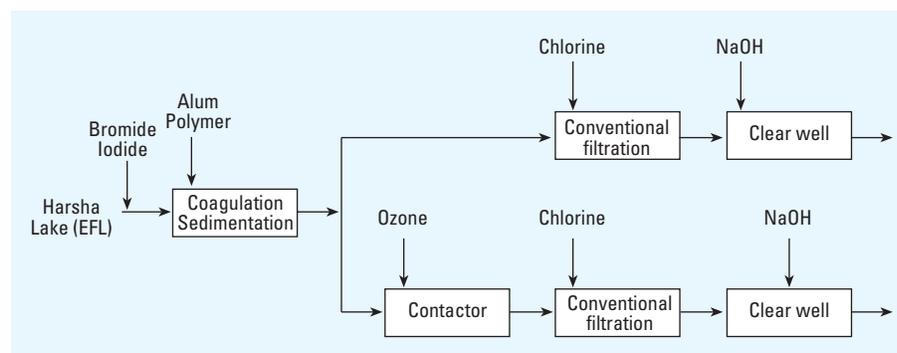
Figure 2. Schematic of the water treatment plant and the disinfection scenarios used in the preliminary experiments. Bromide and iodide were added prior to the water entering the coagulation/settling process. The same water source was used for both treatment scenarios; after coagulation/settling, the water stream was split, and part entered the postchlorination treatment scenario and part entered the ozonation/chlorination treatment scenario. Chlorine was added at the same point in the treatment process for both the postchlorination disinfection scheme and the ozonation/chlorination disinfection scheme.

(~6000 gallons per treatment scenario) that needs to be concentrated for the proposed *in vivo* toxicology study outlined here (reproductive, developmental, and cancer end points) far exceeds the amount of water that can be reasonably concentrated using XAD resins. In addition, it is difficult to redissolve the organics into the water matrix required for the *in vivo* study. XAD resins can be used to concentrate DBPs in drinking waters for mutagenicity testing because a water matrix is not required.

RO membranes have been used previously to concentrate NOM (Gjessing et al. 1998; Odegaard and Koottatep 1982; Serkiz and Perdue 1990; Sun et al. 1995). Larger quantities of water can be concentrated more quickly by RO membranes than by XAD techniques. Membrane procedures will also keep the concentrated organics in a water matrix, unlike the XAD technique where the compounds are dissolved in organic solvents. For membrane concentration, NOM and DBP recovery is a function of the nature of the NOM or DBP, membrane type, pH, temperature, and final concentration. A molecule has a greater chance of passing the membrane material if it is small and uncharged. Volatile DBPs are typically neutral, low-molecular-sized molecules; therefore, they are expected to be lost to various degrees during the membrane-concentration procedure. Given the toxicological importance of volatile DBPs (e.g., the THMs) based on the decreased incidence of adverse reproductive outcomes when chlorinated water is heated or allowed to sit before consumption (Waller et al. 1998), the concentrate will need to be spiked, to the extent possible, with the lost volatile DBPs. The feasibility of returning to the concentrates those DBPs lost during the concentration procedures is being evaluated in the preliminary experiments. In addition to DBP losses during concentration, degradation and volatilization losses within the holding basins for specific DBPs will have to be tracked. This includes both organic and inorganic DBPs and also applies to shipping and animal cage–holding times. The development of appropriate methodology for production of water concentrates for *in vivo* animal toxicological assessment is perhaps the most difficult challenge for implementation of the full study. The preliminary studies are centered around evaluation of RO techniques and assessment of the parameters for its use. As outlined in the concept proposal (Simmons JE. Personal communication), RO concentration would take place after disinfection.

As mentioned above and confirmed in preliminary experiments, concentration of disinfected water by RO results in DBP losses. Although known DBPs can be measured before and after RO concentration and spiked back if they are lost during the concentration process, this adds to the technical difficulty of the process. Additionally, only known and measured chemicals can be restored, and approximately 50% of TOX comprises unknown chemicals. Realistically, relatively few compounds can be spiked back. Candidates include THMs and other DBPs such as HAAs, HANs, and bromate, i.e., those for which fast analytical turnaround time may be expected and compounds that can be purchased in sufficient quantities. Spike-back is expected to be difficult; however, the importance of returning such toxicologically significant compounds as bromodichloromethane and chloroform justifies the effort of appropriate spike-back.

Degree of Concentration by Reverse Osmosis

A 100-fold (100×) concentration factor was selected as the initial target RO concentration. The rationale for this degree of concentration is as follows:

• Animals are not expected to drink severely concentrated water because of taste aversion. Decreased water consumption by treated animals adds a confounding factor (Simmons et al. 1994) that may be difficult to properly account for in the data. To protect against confounding of experimental results by decreased water consumption, palatability studies are included in the preliminary experiments.

• Mixtures studies should be conducted, to the extent feasible, in the low-dose region of health effects dose–response curves.

• Water consumption demands are such that concentration greater than 100× does not appear feasible at this time. In addition, concentration to much greater than 100× is likely to require XAD-resin methods, with an expected increased loss of volatiles. XAD-resin extraction procedures are generally not compatible with generation of sufficient extract for extended *in vivo* assessment.

• Concentration beyond 100× by RO would be expected to lead to unacceptable increases in various inorganic constituents, which may cause fouling by precipitation/scaling, result in elevated osmotic pressures, or confound the results of the subsequent health studies.

These considerations resulted in an initial target of 100× concentration. Results from the preliminary experiments will be examined to assess the feasibility of using more highly concentrated samples.

Analytical Chemistry Analyses on Water and Water Concentrates

Extensive analysis and quantitation of the DBPs is a major goal of this work. Analyses that will be run on the raw source water, the in-plant waters, and 1x finished waters during operation of the plants include measurements of: temperature, pH, turbidity, particle counts, ultraviolet absorbance at 254 nm, TOC, biodegradable dissolved organic carbon (DOC), hardness, alkalinity, coliform bacteria, and bacterial endospores. Additionally, both quantitative and qualitative chemical analyses will be run variously on the raw water, the in-plant waters, the 1× waters during operation of the treatment plants, the 1× finished waters, and the RO concentrates. The quantitative analyses for those DBPs that tend to be routinely monitored are listed in Table 1.

A number of DBPs not routinely quantified will also be measured in the 1× finished waters and in the RO concentrates. Gas chromatography–mass spectrometry (GC–MS) analyses will be performed to provide qualitative comprehensive chemical/DBP identification for all chemicals and DBPs detected. This comprehensive chemical/DBP identification effort will provide a more complete picture of the DBPs present in the drinking water. Similarly, GC–MS analyses conducted during the preliminary experiments will aid in evaluation of DBP stability during bulk concentrate storage and placement on animal cages. A comparison of chromatographic peaks can be used to assess whether nontargeted chemicals in the unknown fraction are being lost or concentrated, or if new chemicals are formed. Although not quantitative, it will give an approximate percentage loss during this storage/rat cage time and will complement the quantitative information obtained for the targeted DBPs. The analytic chemistry results from both the quantitative and qualitative analyses will be used to determine the effectiveness of the concentration procedures (what chemicals are lost and what chemicals are preserved and to what extent) and to evaluate chemical stability during concentrate storage and during animal-cage placement.

In addition to quantitation of the routinely measured DBPs and qualitative analysis by GC–MS, we have proposed the inclusion of quantitative analysis for more than 50 DBPs not routinely measured. These are the more than 50 priority unregulated DBPs (Table 2) recently quantified for a nationwide DBP occurrence study (Weinberg et al. 2002). The DBPs were selected based on structure-activity analysis according to probable adverse health effects. The health end point for the selection exercise was cancer. Also included are two chemicals, methyl bromide and methyl-*t*-butyl ether, that are not recognized as DBPs but are of concern in drinking water. A few more chemicals were added so ozone and chlorine dioxide DBPs could be better represented. Addition of these quantitative analyses will allow a more complete characterization of the drinking water.

Analytical Chemistry Issues for Background Chemical Contamination

The background chemical contamination of the tested source water is an important issue, as these chemicals will be concentrated along with the DBPs. The EFL reservoir has a relatively low background chemical load but is expected to contain pesticides (mainly herbicides), as the surrounding watershed is largely agricultural. Existing data on background pesticide/herbicide concentrations are being sought. Through the U.S. Geological Survey (USGS) water quality monitoring program, pesticides have been/are being monitored at EFL. Other background chemicals of concern include fuel hydrocarbons such as benzene, toluene, and xylenes, as the water intake area is close to a public boat ramp. Previous measurements of these hydrocarbon chemicals by NRMRL, U.S. EPA, have indicated they are rarely present (Miltner RJ. Personal communication).

In Vivo Toxicological Assessments Planned for the Full Study

The Sprague-Dawley rat is the proposed experimental subject. Although the Fischer-344 (F-344) rat is more sensitive to chemically mediated pregnancy loss (Bielmeier et al. 2001; Narotsky et al. 2001), the choice of Sprague-Dawley rats was based on several factors: the high priority of the reproductive and developmental end points; the reproductive toxicology guidelines specify that strains with low fecundity (such as the F-344) shall not be used; the database developed by NHEERL on the male reproductive effects of DBPs has been developed in the Sprague-Dawley rat; and the Sprague-Dawley rat is expected to produce sufficient pups to conduct the proposed full study as outlined below. The route of exposure is oral; the water concentrates will be provided to the rats as their sole source of drinking water.

Based on the results of the more recent epidemiology studies (Bove et al. 1995; Gallagher et al. 1998; Klotz and Pyrch 1999; Kramer et al. 1992; Waller et al. 1998) and the feasibility of conducting *in vivo* exposures, first priority was assigned to reproductive and developmental end points, followed by cancer end points. Cancer end points, although important, were assigned a lower priority, given the limited feasibility of obtaining enough water concentrate to conduct an *in vivo* 2-year cancer bioassay. Additional end points are "piggy-backed" onto the reproductive and developmental end points where feasible. The piggy-backed experiments are an important component of the overall study design, as they greatly increase the spectrum of target organs to be examined in the full study. They include target organs known to

Table 2. Chemical analyses for nonroutinely monitored DBPs.

3,3-Dichloropropenoic acid (3,3-dichloroacrylic acid)
Bromoacetonitrile [590-17-0][a]
Chloroacetonitrile [107-14-2]
Tribromoacetonitrile [75519-19-6]
Bromodichloroacetonitrile [60523-73-1]
Dibromochloroacetonitrile [144772-39-4]
Chloropropanone (chloroacetone) [78-95-5]
1,3-Dichloropropanone (1,3-dichloroacetone) [534-07-6]
1,1- Dibromopropanone (1,1-dibromoacetone)
1,1,3-Trichloropropanone (1,1,3-trichloroacetone) [921-03-9]
1-Bromo-1,1-dichloropropanone (1-bromo-1,1-dichloroacetone)
1,1,1,3-Tetrachloropropanone (1,1,1,3-tetrachloroacetone) [16995-35-0]
1,1,3,3-Tetrachloropropanone (1,1,3,3-tetrachloroacetone) [632-21-3]
1,1,3,3-Tetrabromopropanone (1,1,3,3-tetrabromoacetone)
1,1,1,3,3-Pentachloropropanone (pentachloroacetone)
Hexachloropropanone (hexachloroacetone) [116-16-5]
Dimethylglyoxal (2,3-butanedione)
Chloroacetaldehyde [107-20-0]
Dichloroacetaldehyde [70-02-7]
Bromochloroacetaldehyde
Tribromoacetaldehyde [115-17-3]
3-Chloro-4-(bromochloromethyl)-5-hydroxy-2(5H)-furanone (BMX-1)
3-Chloro-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone (BMX-2)
3-Bromo-4-(dibromomethyl)-5-hydroxy-2(5H)-furanone (BMX-3)
(E)-2-Chloro-3-(bromochloromethyl)-4-oxobutenoic acid (BEMX-1)
(E)-2-Chloro-3-(dibromomethyl)-4-oxobutenoic acid (BEMX-2)
(E)-2-Bromo-3-(dibromomethyl)-4-oxobutenoic acid (BEMX-3)
3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)
3-Chloro-4-(dichloromethyl)-2-(5H)-furanone (red-MX)
(E)-2-Chloro-3-(dichloromethyl)-butendioic acid (ox-MX)
(E)-2-Chloro-3-(dichloromethyl)-4-oxobutenoic acid (EMX)
2,3-Dichloro-4-oxobutenoic acid (mucochloric acid) [87-56-9]
Chloromethane (methyl chloride) [74-87-3]
Bromomethane (methyl bromide) [74-83-9][b]
Dibromomethane [74-95-3]
Bromochloromethane [74-97-5]
Bromochloroiodomethane
Dichloroiodomethane
Dibromoiodomethane
Chlorodiiodomethane
Bromodiiodomethane
Iodoform [75-47-8]
Chlorotribromomethane
Carbon tetrachloride [56-23-5]
1,1,1,2-Tetrabromo-2-chloroethane
Bromochloromethyl acetate
Chloroacetamide [79-07-2]
Bromoacetamide [683-57-8]
Dichloroacetamide [683-72-7]
Dibromoacetamide
Trichloroacetamide [594-65-0]
Bromonitromethane [563-70-2]
Chloronitromethane
Dibromonitromethane
Bromochloronitromethane
Dichloronitromethane
Bromodichloronitromethane
Dibromochloronitromethane
Tribromonitromethane (bromopicrin)
2-Hexenal [505-57-7]; [6728-26-3]
5-Keto-1-hexanal
Methylethyl ketone (2-butanone) [78-93-3]
Cyanoformaldehyde
6-Hydroxy-2-hexanone
Methyl-*tert*-butyl ether [1634-04-4][b]
Benzyl chloride

[a]Chemical Abstracts Service (CAS) numbers listed when available. [b]Not a DBP but an important water contaminant.

be affected by exposure to DBPs (liver, kidney) and those where database limitations preclude judgment regarding their potential sensitivity to either single DBPs or DBP mixtures (immunotoxicity, neurotoxicity, developmental neurotoxicity). Given the labor-intensive nature of production of the water concentrates and the costs, both direct and indirect, associated with production of sufficient concentrate quantities to conduct the proposed *in vivo* studies, it is important to gain as much toxicological information as possible from a single study.

The full experiment is projected to consist of a minimum of four treatment groups: *a*) a control group that receives distilled deionized water (analyzed to ensure there are no remaining volatiles); *b*) a chlorination group that receives the chlorinated water concentrate; *c*) an ozonation group that receives the ozonated/chlorinated water concentrate; and *d*) a positive control group testing a chemical (either dibromoacetic acid or dichloroacetic acid) known to be positive at the given dose level for several priority end points. If feasible, a concentrated raw-water control group will be included that receives concentrated source water (i.e., source water concentrated but not chemically disinfected) to control for the effects of the background contaminants present in the source water. The feasibility of the concentrated-source water control depends on the availability of a procedure for establishing the microbial purity of the concentrated source water that does not also remove the background contaminants of interest. The number of rats per treatment group (see discussion below) will be influenced by both the available volumes of water concentrates and statistical power considerations.

The full study was designed based on the assumption that the preliminary experiments would indicate that the needed quantities of water could be prepared and that the rats will consume 100× water without toxicologically significant self-imposed water restriction. If decreased water consumption is noted in the preliminary experiments, an additional restricted-water control group may be needed. The usefulness of a concurrent restricted-water control group in understanding the health effects associated with a complex mixture of chemicals administered in the drinking water has been demonstrated (Simmons et al. 1994). In a study of this nature, the appropriate control group must be considered carefully. Although the untreated, unconcentrated surface water (1×, raw water) might appear to be a reasonable control, issues of microbial and viral contamination might prevent its use, and this control would not control for background contamination as well as the concentrated-source water control mentioned above. Another possibility is to

use the disinfected, unconcentrated water (1×, disinfected by chlorination, and 1×, disinfected by ozonation/chlorination) as controls. In actuality, these represent very low dose treatment groups rather than controls. The size of the full study as presently planned makes it difficult to include two additional treatment groups without blocking the experimental design.

The full study can be logically broken into three parts:
• Phase I: assays to be conducted between GD0 and postnatal 21 (PND21).
• Phase II: assays to be conducted by carrying the F1 offspring from PND21 to PND90, with evaluation of the reproductive capabilities, female and male, of the F1 generation.

• Phase III: assays to be conducted by carrying the F1 offspring from PND21 to PND360.

The proposed full study, as presented in the research concept proposal, is outlined in a flow diagram (Figure 3), with each phase detailed in a table (Table 3) that describes the relationship of water exposure, GD, PND, and toxicology assay. Table 3 also identifies which tests and assays are conducted on the same rat (dam or pup).

In addition to the *in vivo* assays proposed in Table 3 and Figure 3, complementary *in vitro* assays are also proposed. Included among these are reproductive/developmental assays using embryo cultures or cultures of isolated seminiferous tubules. Research by Hunter et al. (1996) has shown
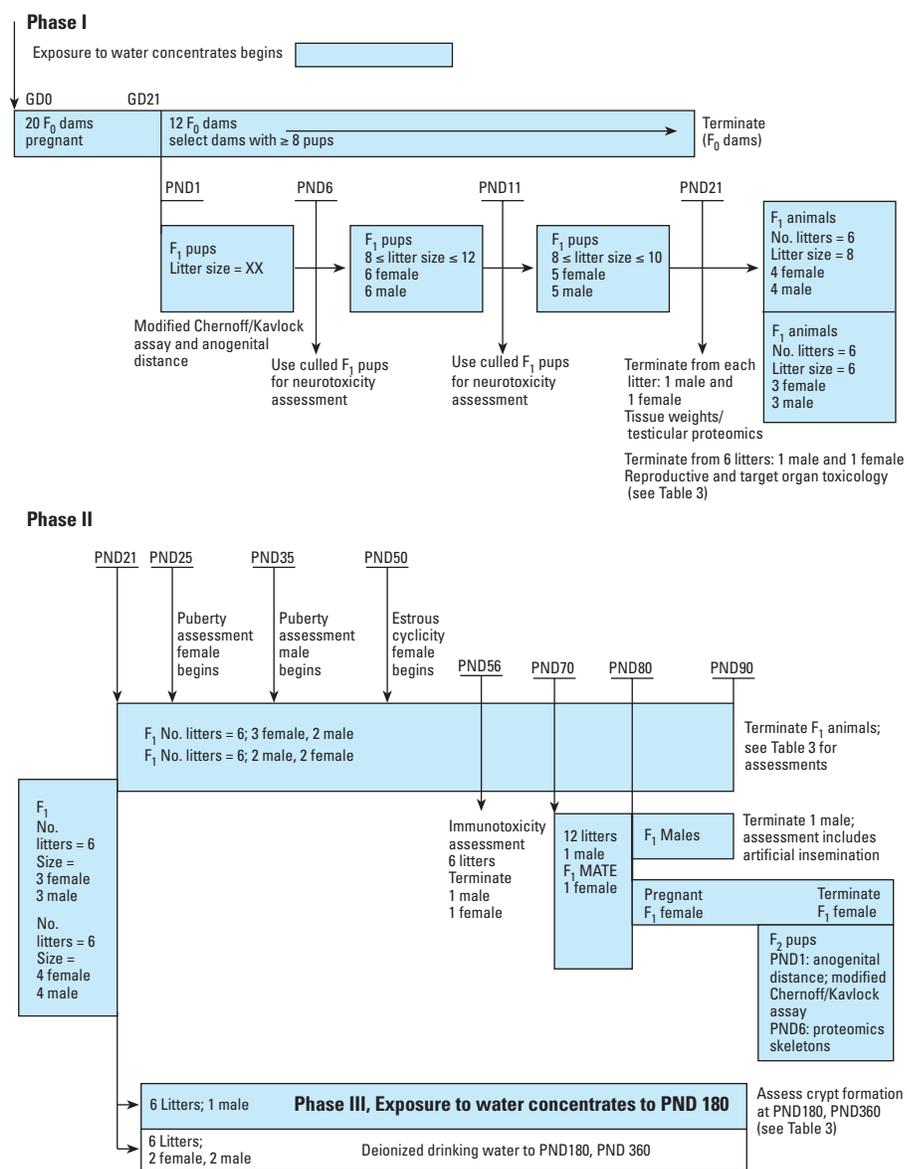

Figure 3. Flow diagram for the toxicology assays. The relationship between exposure to the water concentrate, GD, PND, and toxicology assay is illustrated. The movement of experimental animals between Phases I, II, and III of the toxicology assays is shown.

Table 3. The relationship between water exposure, GD, PND, and toxicology assay in the proposed toxicological evaluations.[a,]

GD or PND	Toxicological evaluation or experimental activity
Phase I. Toxicological evaluation: exposure during pregnancy and lactation	
	Forty adult female Sprague-Dawley rats (60 days of age) arrive in facility—the F_0 rats. These rats are monitored daily for estrus cycling. Regularly cycling females are mated overnight with proven-fertile chemically naïve rats. Twenty sperm-positive females are selected and begin receiving water concentrate on GD0.
GD0	Exposure to water concentrate begins.
GD1–GD21	Pregnant rats receive water concentrate throughout gestation; water and feed consumption are monitored; maternal weight gain, gestation length, and pregnancy rate are measured.
PND1–PND21	Water and feed consumption of dams are monitored; dams are weighed periodically.
PND1	Twelve F_0 dams with more than 8 pups are selected (ideally, selection will achieve 12 litters with at least 12 pups/litter); pups are weighed, anogenital distance is measured; external defects are evaluated (noninvasive). Dams and litters not selected are terminated, with numeration of implantation sites in dams.
PND2	Ontogeny of the righting reflex (noninvasive)
PND3	Ontogeny of the righting reflex (noninvasive)
PND4	Ontogeny of the righting reflex (noninvasive)
PND6	Pups are weighed; external defects are evaluated; anogenital distance is measured (noninvasive).
PND6	Any of the 12 selected litters with more than 12 pups are culled to 12 (6 males and 6 females). Culled pups (1 male and 1 female from each litter if available) are killed for assessment of alterations in regional markers of differentiation and apoptosis.
PND11	Any of the 12 litters with more than 10 pups are culled to 10 (5 males and 5 females). Culled pups (1 male and 1 female from each litter if available) are killed for qualitative and quantitative neuropathology.
PND14	Male pups are assessed for retained nipples.
PND17	Functional observational battery and motor activity assessment are conducted. Those pups that will be terminated on PND21 are not assessed (noninvasive).
PND21	F_0 dams are killed; implantation sites in dams are counted. In all 12 F_1 litters, anogenital distance is measured. From all 12 F_1 litters, one male and one female pup are terminated. From 6 litters, one additional male and one additional female pup are terminated. In both dams and pups, body and all major organ weights are measured; reproductive organ weights and histopathology in dams and pups are assessed; endocrine function is evaluated by measurement of TSH and T_4 and by histopathological evaluation of the thyroid and the adrenal glands; testicular proteomics in male pups are evaluated. In both dams as well as male and female pups, blood and liver samples are taken for hepatotoxicity and nephrotoxicity evaluation; tissue samples are preserved for both indicator DBP analysis and for *in vitro* metabolism for assays; in pups, brains are assayed for alterations in regional markers of differentiation and apoptosis.
End of Phase I	

Distribution of the remaining F_1 pups is shown on the toxicology flow diagram (Figure 3). At the end of Phase I there are 6 litters with 6 pups (3 male and 3 female) and 6 litters with 8 pups (4 male and 4 female). From the 6 litters with 8 pups each, 2 male and 2 female pups from each litter are placed on deionized drinking water; these rats are assayed for colonic crypts at PND180 and PND360. From the 6 litters that have 6 pups each (3 male and 3 female), 1 male from each litter is placed in Phase III toxicological evaluation. These rats are exposed to the water concentrate until PND180, when they are terminated and assayed for colonic crypts. All other pups are placed in Phase II toxicological evaluation.

Phase II. Toxicological evaluation; reproductive maturation of the F_1 generation	
PND21	The remaining pups at PND21 are divided into those going to Phase II toxicological evaluation and those going to Phase III toxicological evaluation (see Phase III description, below). A total of 30 females and 24 males enter Phase II (see Figure 3, Phase II).
PND21 to termination	All F_1 animals receive water concentrate until termination. Water and feed consumption and body weight are monitored until termination.
PND25	Daily assessment for vaginal opening in F_1 females begins.
PND35	Daily assessment for preputial separation in F_1 males begins.
PND50–PND70	Daily monitoring of F_1 females is conducted for estrus cycling.
PND63–PND85	One male and 1 female from each of 6 litters are evaluated for immunotoxicity. Rats are immunized with T-cell–dependent antigen sheep erythrocytes on PND63 and PND80. On PND68, rats are monitored for primary (IgM) antibody titers and on PND86 for recall (IgG) antibody titers.
PND70–PND80	Daily mating (2–3 hr/day in light cycle between cycling F_1 females and F_1 males from separate litters); time to pregnancy and pregnancy rate are measured.
PND90	F_1 males and nonpregnant F_1 females are terminated. All major organs, including reproductive organs, are weighed. Reproductive organ histopathology is evaluated. Further assessments include the following: in females: enumeration of implantation sites; in males: enumeration of testicular and epididymal sperm numbers; testicular testosterone measurement, epididymal sperm motility and morphology, and ability to produce pregnancy through artificial insemination. Endocrine function is evaluated by measurement of TSH and T_4 and by histopathological evaluation of the thyroid and the adrenal glands; testes are frozen for proteomics analysis; serum hormones (LH, testosterone, FSH, prolactin, inhibin) are measured; males and females are evaluated for aberrant colonic crypts. Blood and liver samples are taken for hepatotoxicity and nephrotoxicity; tissue samples are taken for metabolism assays; tissue samples are taken for indicator DBP analysis; brains are assayed for alterations in regional markers of differentiation and apoptosis; quantitative and quantitative neuropathology is assessed; immunotoxicity assessment includes proliferative potential of T and B lymphocytes and natural killer cell activity; blood is analyzed for serum levels of preformed immunoglobulins M, G, and A.
GD0–GD21, F_1 dams	Pregnant rats receive water concentrate throughout gestation; water and feed consumption are monitored; maternal weight gain, gestation length, and pregnancy rate are measured.
PND1–PND6, F_2 pups	Water and feed consumption of dams are monitored; dams are weighed periodically.
PND1, F_2 pups	Pups are weighed, anogenital distance is measured; external defects are evaluated (noninvasive).
PND2, F_2 pups	Ontogeny of the righting reflex (noninvasive)
PND3, F_2 pups	Ontogeny of the righting reflex (noninvasive)
PND4, F_2 pups	Ontogeny of the righting reflex (noninvasive)
PND6, F_2 pups	F_1 dams and F_2 pups are killed. Assessments include the following: pups: weighed, external defects evaluated, anogenital distance measured, skeletal evaluation conducted; dams: weighed; implantation sites enumerated; reproductive organs evaluated; evaluation for aberrant colonic crypts conducted. Blood and liver samples are taken for hepatotoxicity and nephrotoxicity; tissue samples are taken for metabolism assays and indicator DBP analysis; brains are assayed for alterations in regional markers of differentiation and apoptosis; qualitative and quantitative neuropathology is assessed.
End of Phase II	

(continued)

Table 3. *(Continued)*

GD or PND	Toxicological evaluation or experimental activity
Phase III. Development of aberrant colonic crypts	
PND21	A total of 30 rats enter Phase III. Only 6 male rats (from separate litters) will continue to receive water concentrate. These 6 rats will receive water concentrate until termination. The other 24 rats (12 males and 12 females) will receive deionized water, beginning at PND21. These rats will receive deionized water until termination (see Figure 3, Phase III).
Various times between PND21 and PND180	Functional observational battery and motor activity assessment are conducted. Periodic testing is conducted between PND21 and PND180, with the timing and frequency of these assessments based on the results of the PND17 testing in Phase I (noninvasive).
PND158–PND180	The 6 male rats that continue to receive water concentrate are evaluated for immunotoxicity. Rats are immunized with T-cell–dependent antigen sheep erythrocytes and monitored for primary (IgM) antibody titers and for recall (IgG) antibody titers.
PND180	The 6 rats that continued to receive water concentrate are terminated. Six males and 6 females that received deionized water beginning on PND21 are terminated. Both sets of rats are evaluated for presence of aberrant crypts; major organs are evaluated for tumors; endocrine function is evaluated by TSH and T_4 and by histopathological evaluation of the thyroid and the adrenal glands. Blood and liver samples are taken for hepatotoxicity and nephrotoxicity; tissue samples are taken for metabolism assays; tissue samples are taken for indicator DBP analysis (water concentrate rats only); brains are assayed for alterations in regional markers of differentiation and apoptosis; qualitative and quantitative neuropathology is assessed. Immunotoxicity assessment includes proliferative potential of T and B lymphocytes and natural killer cell activity; blood is analyzed for serum levels of preformed immunoglobulins M, G, and A.
PND360	The remaining 6 males and 6 females that received deionized water beginning on PND21 are terminated. Rats are evaluated for presence of aberrant colonic crypts; major organs are evaluated for tumors; endocrine function is evaluated by measurement of TSH and T_4 and by histopathological evaluation of the thyroid and the adrenal glands. Blood and liver samples are taken to assess hepatotoxicity and nephrotoxicity; tissue samples are taken for metabolism assays; brains are assayed for alterations in regional markers of differentiation and apoptosis; qualitative and quantitative neuropathology is assessed. Immunotoxicity assessment includes proliferative potential of T and B lymphocytes and natural killer cell activity; blood is analyzed for serum levels of preformed immunoglobulins M, G, and A.
End of Phase III	

Abbreviations: FSH, follicle-stimulating hormone; LS, luteinizing hormone; T_4, tetraiodothyronine; TSH, thyroid-stimulating hormone. [a]This table illustrates one exposure group. The full experiment, as planned, has four exposure groups (distilled deionized water control group, chlorination concentrate, ozonation/chlorination concentrate, positive control).

HAAs are toxic to the developing embryo. Isolated seminiferous cultures were selected to evaluate effects on spermatogenesis. Mutagenicity assays include both the standard *Salmonella* assay with and without metabolic activation, and the *Salmonella* assay with glutathione *S*-transferase (GST)–transfected bacteria. Inclusion of *in vitro* mutagenicity assays with the standard *Salmonella* strains is based on the literature database demonstrating that water samples concentrated by extraction methods that result in the loss of volatile organic matter are mutagenic (Simmons et al. 2001). Inclusion of assays that employ a *Salmonella* strain transfected with rat theta-class GST T1-1 is based on the demonstrated mutagenicity of DBPs in this assay system. Brominated THMs are potent mutagens in this assay but not the standard *Salmonella* assay, indicating the important role of metabolic activation through conjugation with glutathione in brominated THM-mediated genotoxicity (Pegram et al. 1997). *In vitro* assays for carcinogenicity and neurotoxicity have also been proposed. Additionally, blood and tissue samples will be prepared and stored (quick-frozen in liquid nitrogen and stored at –80°C) for possible future analyses. This is important, given the degree of difficulty and effort involved in the preparation of the concentrated water samples for subchronic *in vivo* toxicological assessment.

Meaning of Positive Result(s) and Possible Future Directions

A positive result for any end point demonstrates toxicity that can be attributed to the chemicals present in the water concentrate. Although this project is meant to assess health effects due to DBPs, it must be remembered that any background chemical contamination of the source water must also be considered, as the concentration procedures used to concentrate the DBPs will also concentrate background contaminants of the source water.

With positive results from both water treatment scenarios, or positive results from one water treatment scenario and negative results from the other treatment scenario, the comparative toxic potency of the two water treatment scenarios can be evaluated. This will provide useful information with regard to shifts or alterations in toxic outcome that might be expected with shifts in water treatment scenario.

The analytical chemistry information will be used to develop predictive models. The results of the predictive models will be compared with the observed toxicological outcomes (positive or negative). The expected toxic potency of the mixtures under an assumption of additivity (both dose and response) will be calculated. These predicted toxicity estimates will make use of statistical procedures and risk assessment techniques such as the Gennings additivity model (Gennings et al. 1997).

Possible future directions resulting from a positive toxicology outcome include
• Use of the chemical characterizations to develop mechanistic hypotheses with respect to chemicals or subsets of chemicals expected to be responsible for the observed toxic effects. These hypotheses may be tested experimentally in follow-up studies.
• Dose–response assessments (tests at 50, 25, and 1×) to determine the shape of the dose–response curve as the total mixture decreases. However, the number of rats and the related water concentrate volumes required for dose–response assessment will prove technically challenging.
• A positive toxicological result from either disinfection scheme may lead to research to identify those factors that reduce the formation of toxic chemicals. Factors for consideration, besides the bromide and iodide levels, include pH, ozone dose, chlorine dose, and temperature, among others. A positive result in the mutagenicity assays conducted with *Salmonella*, particularly *Salmonella* transfected with GST-theta, may lead to bioassay-directed fractionation. This type of research can pinpoint the mutagenic fractions and aid in identification of those chemicals responsible for the mutagenic response.

Meaning of Negative Result(s) and Next Steps

If all the toxicological assays are negative, then no toxicity was observed that could be attributed to exposure to these complex mixtures of DBPs. The toxicology assays are weighted toward reproductive and developmental end points and include exposure during gestation of the F_1 generation to production of F_2 pups. The inclusion of the positive control chemical adds weight to the assumption that if reproductive or developmental effects were observable in animals, we would have detected them with

our study design. (This statement has the most validity under an assumption of common mode of action.) Statistical power calculations (see below) are being conducted in advance of the full study. Given that the full study has sufficient statistical power, a lack of concordance of animal results and the epidemiologic studies might suggest a research focus on the chemicals suspected of being background contaminants in the waters examined by epidemiologic investigation. Alternatively, hypotheses that could be developed include the following: that the epidemiologic end points do not have a chemical basis; that the experimental animal used is less sensitive than humans to the adverse health effects associated with exposure to complex mixtures of DBPs; or that higher dose levels are required to elicit adverse health effects when exposure is limited to the oral route. The other health end points (e.g., immunotoxicology) are less fully examined in the proposed experimental design, so it is possible further testing would reveal a positive effect. Cancer is a particular concern, as technical limitations prohibit the conduct of a traditional 2-year bioassay. With regard to the issue of susceptible subpopulations, this research addresses the susceptibility of the developing embryo and the young animal. Questions regarding potential enhancement of susceptibility due to nutrition, disease, or advanced age are not considered here.

The preliminary results will be used to calculate a sample size for the full study that allows for a reasonable Type II error rate (beta), i.e., the probability of not detecting an effect when one is truly present. The sample size currently suggested, 20 sperm-positive females culled to 12 dams with litter size greater than 8 on PND1, is based on standard guidelines for reproductive/developmental studies. Note that a small Type I error rate (alpha) is also required; Type I error is the probability of a false positive result, i.e., detecting an effect when one is really not there. As it is desirable for alpha to be low, it is usually set at $p < 0.05$. Generally, alpha is set at a nominal level, and beta is calculated for the statistical test(s) of interest, using estimates of sample size and variance of the toxic end point to be measured. Both alpha and beta can be reduced with larger sample sizes and by selecting end points that can be tested using a one-tailed statistical test. Because the concentration methods discussed herein will necessarily limit the amount of water available to dose the animals and thus limit the number of animals in the study, sample sizes will necessarily be small. Power calculations then become important as a method for choosing among the possible toxic end points for the full study. For example, if sample size is limited by the amount of water to $n = 10$, and alpha is required to be < 0.05, then beta is affected by the choice of toxic end point,

including the sensitivity of that end point and our ability to correctly estimate the expected value and variance of that end point. Thus, the projected power of the statistical test to detect an effect will be critical to the design of the full study and will rely on data from the trial run and from previous toxicity studies.

Potential Risk Assessment Uses of the Data

The data developed through the conduct of this project are important for risk assessment in three ways: *a*) as toxicity data for estimating DBP risks from exposure to the complex mixture that can be used to compare health risks across treatment trains and to contrast with risk estimates using epidemiologic data; *b*) as complex mixture data that can be used in conjunction with existing data on single DBPs and defined mixtures to evaluate the toxicity of the unidentified fraction of the DBPs produced; and *c*) as exposure and toxicity data that can be used to aid in the development and refinement of emerging mixture risk assessment methodologies (e.g., for evaluation of the accuracy of component-based approaches to estimating DBP risks; as exposure data on a variable complex mixture over time).

Estimation of complex mixtures health risks. The problem of characterizing health risks from exposure to DBPs across different drinking water treatment trains is being investigated. The most recent effort uses a component-based approach, response addition, to estimate these risks (U.S. EPA 2000a). Response addition assumes each component of the mixture acts toxicologically in a functionally independent way for a given effect; thus, at low exposure levels, the risks of the individual chemicals can be added together to estimate the risk of the adverse effect for the whole mixture (U.S. EPA 2000b). To include potential toxicity from exposure to the unidentified fraction in the risk assessment, the chemicals most likely to be present in the fraction were identified. A quantitative structure–activity relationship (QSAR) model (Moudgal et al. 2000) was then used to estimate the toxicity of those chemicals. This information was used to estimate risk from exposure to the unidentified fraction, which was added to the estimated risk from the known chemicals to produce a whole-mixture risk estimate. This approach, although a reasonable and defensible method, may not accurately estimate the real health risks. The data from the proposed full study will be useful in testing the response addition assumption and QSAR approach, along with other assumptions and methods, to find the best technique for DBP mixtures health risk estimation. An additional goal is to compare health risk estimates based on animal toxicology data with those from the epidemiologic

literature. Hypotheses can then be tested regarding why differences exist between estimates from the two data sources (e.g., contributions to toxicity by non-DBP contaminants in the water or from inhalation and dermal exposures to DBPs), leading to further research efforts.

Toxicity of the unidentified fraction. Another potential use of these data is to develop a method to attribute a certain proportion of the toxicity to known chemicals, and thus attribute the rest of the toxicity to the unidentified portions of the DBPs produced. The risk from exposure to the known chemicals may be estimated in several ways, including the response-addition method shown above. Other methods, such as dose-addition approaches (e.g., relative potency factors) or multiple-chemical dose–response models, may also be employed. An example of the latter, Gennings et al. (1997), uses data on the four THMs to demonstrate a modeling approach that detects departures from additivity for the mixture. The same model may be useful in predicting the toxicity from exposure to the known chemicals, including the THMs, which could then be contrasted with the toxicity observed from exposure to the whole mixture. The model is based on one definition of additivity given by Berenbaum (1985), that is, in a combination of c chemicals, let d_i represent the concentration/dose of the ith component alone that yields a fixed response and let D_{xi} represent the concentration/dose of the ith component in combination with the c agents that yields the same response. According to this definition, if the substances combine under dose addition (with no interaction effects), then

$$d_1/D_{x1} + d_2/D_{x2} + d_i/D_{xi} = 1.$$

The experimental data necessary and sufficient to support the estimation of this additivity surface are single-chemical dose–response data. The additivity surface (estimated using the single-chemical data) can be used to predict the response under additivity at the mixture point of interest. For defined mixtures (i.e., all components of the mixture are known) with monotonically increasing dose–response curves, the response is considered synergistic when the observed response is greater than the predicted response under additivity; the response is considered antagonistic when the observed response is less than the predicted response under additivity. When the mixture contains unidentified chemicals and an assumption of dose addition for the known chemicals in the mixture is deemed reasonable, the observed experimental response from exposure to the complex mixture could be compared with the response predicted by this model, and the difference used

to estimate the toxicity of the unidentified fraction. The development of such a statistical estimation process that contends with unidentified fractions of complex mixtures may have application beyond the study of DBPs.

Present Status

Substantial progress has been made to date. The research concept proposal was completed and has undergone external peer review. In addition to a number of smaller preliminary experiments, a large trial-run experiment has been completed that involved the laboratories of 15 scientists (L. Claxton, NHEERL; A. DeAngelo, NHEERL; S. Hunter, NHEERL; S. Krasner, MWDSC; G. Klinefelter, NHEERL; R. Miltner, NRMRL; M. Narotsky, NHEERL; R. Pegram, NHEERL; G. Rice, NCEA; S. Richardson, NERL; K. Schenck, NRMRL; J. E. Simmons, NHEERL; T. Speth, NRMRL; L. Teuschler, NCEA; H. Weinberg, UNC-Chapel Hill) in five locations around the United States (Cincinnati, Ohio; Athens, Georgia; La Verne, California; Chapel Hill, North Carolina; and Research Triangle Park, North Carolina). We are currently in the final stages of analyzing the data resulting from this trial run experiment. Results from the trial run led us to design and conduct a follow-up experiment to assess the potential for artifact formation during the water concentration procedures used in the trial run. Results from the artifact formation experiment will aid our interpretation of the trial run results.

In conclusion, we have designed a research strategy for integrated technology-based toxicological and chemical evaluation of the highly complex mixtures of chemicals formed during chemical disinfection of water. Experiments necessary to the ability to conduct the full study, as proposed here, have been conducted and their results are being used to inform and guide the next steps in the process.

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