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Method to assess component contribution to toxicity of complex mixtures: Assessment of puberty acquisition in rats exposed to disinfection byproducts

Shahid Parvez¹, Glenn E. Rice^{2,*}, Linda K. Teuschler³, Jane Ellen Simmons⁴, Thomas F. Speth⁵, Susan D. Richardson⁶, Richard J. Miltner⁵, E. Sidney Hunter III⁴, Jonathan G. Pressman⁵, Lillian F. Strader⁴, Gary R. Klinefelter⁴, Jerome M. Goldman⁴, Michael G. Narotsky⁴

1. Indiana University Richard M. Fairbanks School of Public Health, Department of Environmental Health Sciences, IUPUI Campus, Indianapolis, IN 46202, USA

2. National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268, USA

3. Linda Teuschler and Associates, St. Petersburg, FL 33707, USA

4. National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA

5. National Risk Management Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268, USA

6. Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, USA

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ABSTRACT

A method based on regression modeling was developed to discern the contribution of component chemicals to the toxicity of highly complex, environmentally realistic mixtures of disinfection byproducts (DBPs). Chemical disinfection of drinking water forms DBP mixtures. Because of concerns about possible reproductive and developmental toxicity, a whole mixture (WM) of DBPs produced by chlorination of a water concentrate was administered as drinking water to Sprague–Dawley (S–D) rats in a multigenerational study. Age of puberty acquisition, i.e., preputial separation (PPS) and vaginal opening (VO), was examined in male and female offspring, respectively. When compared to controls, a slight, but statistically significant delay in puberty acquisition was observed in females but not in males. WM-induced differences in the age at puberty acquisition were compared to those reported in S–D rats administered either a defined mixture (DM) of nine regulated DBPs or individual DBPs. Regression models were developed using individual animal data on age at PPS or VO from the DM study. Puberty acquisition data reported in the WM and individual DBP studies were then compared with the DM models. The delay in puberty acquisition observed in the WM-treated female rats could not be distinguished from delays predicted by the DM regression model, suggesting that the nine regulated DBPs in the DM might account for much of the delay observed in the WM. This method is applicable to mixtures of other types of chemicals and other endpoints.

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* Corresponding author. E-mail: rice.glenn@epa.gov (Glenn E. Rice).

Introduction

Although chemical disinfection effectively produces potable drinking waters, such processes generally form low concentrations (\leq mg/L) of numerous disinfection byproducts (DBPs) (Richardson et al., 2007, 2008). These DBP mixtures include hundreds of known compounds, as well as compounds that have not yet been identified chemically and comprise the unidentified fraction. Two chemical classes of DBPs, the trihalo-methanes (THMs) and haloacetic acids (HAAs), together typically account for approximately 40% of the total mass of organic halogen (TOX) formed in disinfected drinking waters (Richardson et al., 2008; Krasner et al., 2006). The unidentified fraction, which can comprise more than 50% of the TOX measured in some treated drinking waters (Pressman et al., 2010; Weinberg et al., 2002; Krasner et al., 2006), likely includes highly polar and high molecular weight compounds (Richardson et al., 2002).

A number of epidemiologic studies have evaluated associations between exposure to chemically disinfected drinking water and adverse reproductive and developmental effects. Some report significant associations between DBP exposure and still birth (King et al., 2000; Dodds et al., 2004), low birth weight (Danileviciute et al., 2012; Gallagher et al., 1998; Wright et al., 2003), premature birth (Bove, 1996; Hinckley et al., 2005), and pregnancy loss (Waller et al., 1998, 2001; Savitz et al., 1995, 2006). Other studies report negative results (e.g., Nieuwenhuijsen et al., 2009; Hrudey, 2009). Using animal and *in vitro* bioassays, toxicologists have studied the reproductive and developmental effects of a small number of individual DBPs (Colman et al., 2011; US EPA, 2000a; Hunter and Tugman, 1995; Hunter et al., 1996); there are even fewer *in vivo* developmental toxicity studies of complex DBP mixtures (Kavlock et al., 1979; Simmons et al., 2002; Teuschler and Simmons, 2003; Narotsky et al., 2008, 2013).

To address potential health concerns that cannot be answered by toxicological research on individual DBPs or defined mixtures, the U.S. EPA's Four Lab Study investigated the toxicity of environmentally realistic complex DBP mixtures. An important objective of this study was a thorough assessment of rodent reproductive and developmental endpoints, integrating these bioassay results with extensive quantitative and qualitative analyses of the chemicals present in the complex DBP mixture(s) (Simmons et al., 2002, 2004, 2008). To meet this objective, a multi-generational reproductive and developmental bioassay was conducted with a chlorinated concentrate in Sprague–Dawley (S–D) rats. The water concentrate was water produced using a procedure that concentrated a natural source water by reverse osmosis procedures; aliquots of this water concentrate were chlorinated as needed for the bioassay (Pressman et al., 2010). In the bioassay, this whole, complex DBP mixture (whole mixture, WM) was administered to the treatment group as the sole source of drinking water. When compared with controls, the WM-treated female offspring (F1 generation) experienced a slight, but significant delay in puberty acquisition, which was measured as their age at vaginal opening (VO) ($p < 0.05$); the WM-treated F1 males did not exhibit a significant delay in puberty acquisition, which was measured as their age at preputial separation (PPS) (Narotsky et al., 2013).

In a companion multi-generational bioassay, a defined mixture (DM) that contained the four regulated THMs and the

five regulated HAAs was administered to S–D rats as the sole source of drinking water. Significant, dose-dependent delays in puberty acquisition were observed in both F1 males and females (Table 1) (Narotsky et al., 2015). This DM contained chloroform (CHCl_3), bromodichloromethane (BDCM), dibromochloromethane (DBCM), bromoform (CHBr_3), chloroacetic acid (CAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromoacetic acid (BAA), and dibromoacetic acid (DBAA). U.S. EPA regulations limit the sum of the four THMs to 80 $\mu\text{g/L}$ and the sum of the five HAAs to 60 $\mu\text{g/L}$ in U.S. drinking waters (US EPA, 2006). Some bioassays of individual DBPs (i.e., DBAA, bromochloroacetic acid [BCAA], and BDCM), administered as single chemicals in drinking water of S–D rats, also report significant delays in puberty acquisition (Table 1) (Klinefelter et al., 2004; Sloan et al., 2005; Christian et al., 2002).

Here, we present a method developed to assess the contribution of constituent chemicals and subset mixtures to the toxicity of highly complex environmental mixtures. We use assessment of puberty acquisition in rats exposed to DBP mixtures to illustrate methodology that allows comparison of the health effects from constituent chemicals and subset mixtures contained within more complex mixtures to the health effects associated with highly complex environmental mixtures. We specifically compare differences in the age of puberty acquisition in rats and evaluate whether any of the tested individual DBPs or the DM can account for the observed difference in the age at puberty acquisition associated with the WM. The method relies on component-based and whole-mixture approaches for determining if some of the delay in puberty acquisition is potentially due to the unknown fraction in the WM (Rice et al., 2008) and assumes that the relative proportions of the chemicals common to both the whole mixture and the defined mixture are similar.

1. Materials and methods

Fig. 1 depicts our method for comparing differences (relative to concurrent controls) in the age at puberty acquisition associated with the DM to those differences associated with the WM and with individual DBPs. After completing the WM and DM bioassays, we undertook a literature search targeting studies that administered DBPs via the drinking water and reported age at puberty acquisition. Because differences among species and strains/stocks could influence the age of puberty acquisition, we targeted studies reporting the same endpoints (i.e., age at VO or PPS) in the same test species/stock (S–D rats).

1.1. DBP bioassays reporting the age at puberty acquisition in S–D rats

Table 1 details the five studies, three studies of individual DBPs and two studies of DBP mixtures, meeting the search criteria. Four of the five studies (the exception being Klinefelter et al., 2004) met or exceeded the 20 litters/treatment recommended by toxicity testing guidelines (US EPA, 1998; OECD, 2012) and all met or exceeded the recommended number of offspring/sex/litter for VO or PPS evaluation.

Table 1 – Summary of pubertal developmental studies.

Experimental details	DBAA	BCAA	BDCM	DM	WM
Reference	Klinefelter et al. (2004)	Sloan et al. (2005)	Christian et al. (2002)	Narotsky et al. (2015)	Narotsky et al. (2013)
Concentration/dose group (mg/L)	0, 4, 40, 400	0, 30, 300, 600	0, 50, 150, 450	0, 70, 140, 280	0, 36
Concentration as TOX (mmol/L) ^a	0, 0.04, 0.40, 4	0, 0.35, 3.50, 7	0, 0.92, 2.75, 8.24	0, 1.30, 2.60, 5.20	0, 1.03
Number of litters per group (P0)	12, 12, 12, 12	25, 25, 25, 25	30, 30, 30, 30	24, 25, 24, 24	39, 57
Day of weaning	22	21	23	26	21
Exposure period (P0)	GD 15 to F1 adulthood	10 weeks pre mating to F1 adulthood	10 weeks pre mating to F1 adulthood	GD 0 to F1 adulthood	GD 2 to F1 adulthood
Male rat					
Number of rats evaluated per litter ^b	2	1	1	2–4	2–4
Mean ± SD day of PPS	44.4 ± 2, 45.6 ± 3, 46.3 ± 2, 48.1 ± 3 *	41.8 ± 2, 42.0 ± 2, 41.8 ± 2, 43.1 ± 2 *	47 ± 3, 47.2 ± 3, 48.9 ± 2, 49.7 ± 3	45.5 ± 1.7, 46.8 ± 1.6, 48.4 ± 3 *, 51.2 ± 2.6 *	46.3 ± 2, 46.8 ± 2
Mean ± SD body weight (g) at puberty (g)	237 ± 21, 254 ± 29, 249 ± 21, 223 ± 30	222 ± 20, 220 ± 15, 209 ± 19, 192 ± 23	Data not available	244 ± 14, 249 ± 17, 251 ± 27, 208 ± 21	270 ± 23, 269 ± 20
Mean ± SD body weight (g) at weaning	53 ± 6, 54 ± 5, 49 ± 5, 45 ± 10	Data not available	46.7 ± 6, 50.1 ± 8, 42.1 ± 8, 40.9 ± 8	81 ± 6, 80 ± 8, 76 ± 5, 60 ± 8	59 ± 5, 59 ± 6
Female rat					
Number of rats evaluated per litter ^a	1	1	1	3	2
Mean ± SD day of VO	33.3 ± 2, 32.9 ± 1, 32.7 ± 2, 36.2 ± 2 *	30.8 ± 2, 31.9 ± 2, 31.5 ± 2, 32.2 ± 2 *	33.8 ± 2, 33.3 ± 2, 34.6 ± 2, 35.8 ± 2	34 ± 2, 34.9 ± 2, 35.5 ± 2 *, 39.9 ± 3 *	34.1 ± 2, 34.9 ± 2 *
Mean ± SD body weight (g) at puberty	117 ± 16, 122 ± 13, 109 ± 11, 126 ± 24	108 ± 16, 113 ± 14, 110 ± 13, 105 ± 15	Data not available	120 ± 10, 122 ± 10, 124 ± 11, 116 ± 12	128 ± 14, 133 ± 16
Mean ± SD body weight (g) at weaning	48 ± 6, 52 ± 7, 46 ± 6, 45 ± 9	Data not available	45.5 ± 5, 46.5 ± 8, 40.8 ± 7, 37.6 ± 7	76 ± 6, 75 ± 7, 72 ± 6, 57 ± 7	56 ± 6, 56 ± 5

DBAA: dibromoacetic acid; BCAA: bromochloroacetic acid; BDCM: bromodichloromethane; DM: defined mixture; WM: concentrated whole mixture; GD: gestation day; SD: standard deviation; TOX: total organic halogen; PPS: preputial separation; VO: vaginal opening.

^a Sample calculation for molar TOX concentration (mmol/L): Example: Bromodichloromethane (BDCM) Given dose of BDCM is 50 mg/l; molecular weight of BDCM is 163.9; molecular formula of BDCM is CHBrCl₂; number of chlorine and bromine atoms in BDCM are 2 and 1, respectively.

Step 1 Calculate the molar concentration of BDCM (mmol/L)

= Given dose (mg/L) of BDCM/Molecular Weight of BDCM = 50 mg/L/163.9 = 0.305 mmol/L

Step 2 Calculate the molar concentration of individual halogens

Molar concentration of Chlorine = (Molar concentration of BDCM × Number of Chlorine atoms) = 0.305 mmol/L × 2 = 0.601 mmol/L

Molar concentration of Bromine = (Molar concentration of BDCM × Number of Bromine atoms) = 0.305 mmol/L × 1 = 0.305 mmol/L

Step 3 Calculate the Total Organic Halogen (TOX) concentration

=Sum of molar concentrations of individual halogens=0.601 mmol/L+0.305 mmol/L=0.92 mmol/L

If we use the individual molar concentration of each halogen (from Step 2) and multiply them with their atomic weights, they will represent their individual concentrations on weight basis. For example, chlorine concentration on weight basis will be equal to its molar concentration × 35.5 (i.e., 0.601 mmol/L × 35.5 = 21.66 mg/L).

^b A few rats were removed from the analysis (died or did not report data), hence actual number of rats evaluated per litter may have varied during the analysis.

* Author reported significant delay from concurrent control ($p \leq 0.05$).

1.1.1. Single DBP studies

Klinefelter et al. (2004) examined the age at PPS and VO in rats treated with DBAA from Gestation Day (GD) 15 through adulthood of the F1 generation. Body weights were recorded on day of weaning (Postnatal Day [PND] 22) and day of puberty acquisition. In the F1 generation, the authors reported significant delays in PPS and VO in the high-dose group compared to controls ($p < 0.05$).

Sloan et al. (2005) examined the age at PPS and VO in rats treated with BCAA from 10 weeks before mating until the F1 generation acquired puberty. Rat body weights were measured on the day of puberty acquisition, but not at weaning. In both F1 males and females, the authors reported delays in puberty acquisition in the high-dose group compared to controls ($p < 0.05$).

Christian et al. (2002) examined the age at PPS and VO in rats treated with BDCM. BDCM treatment was initiated 10 weeks prior

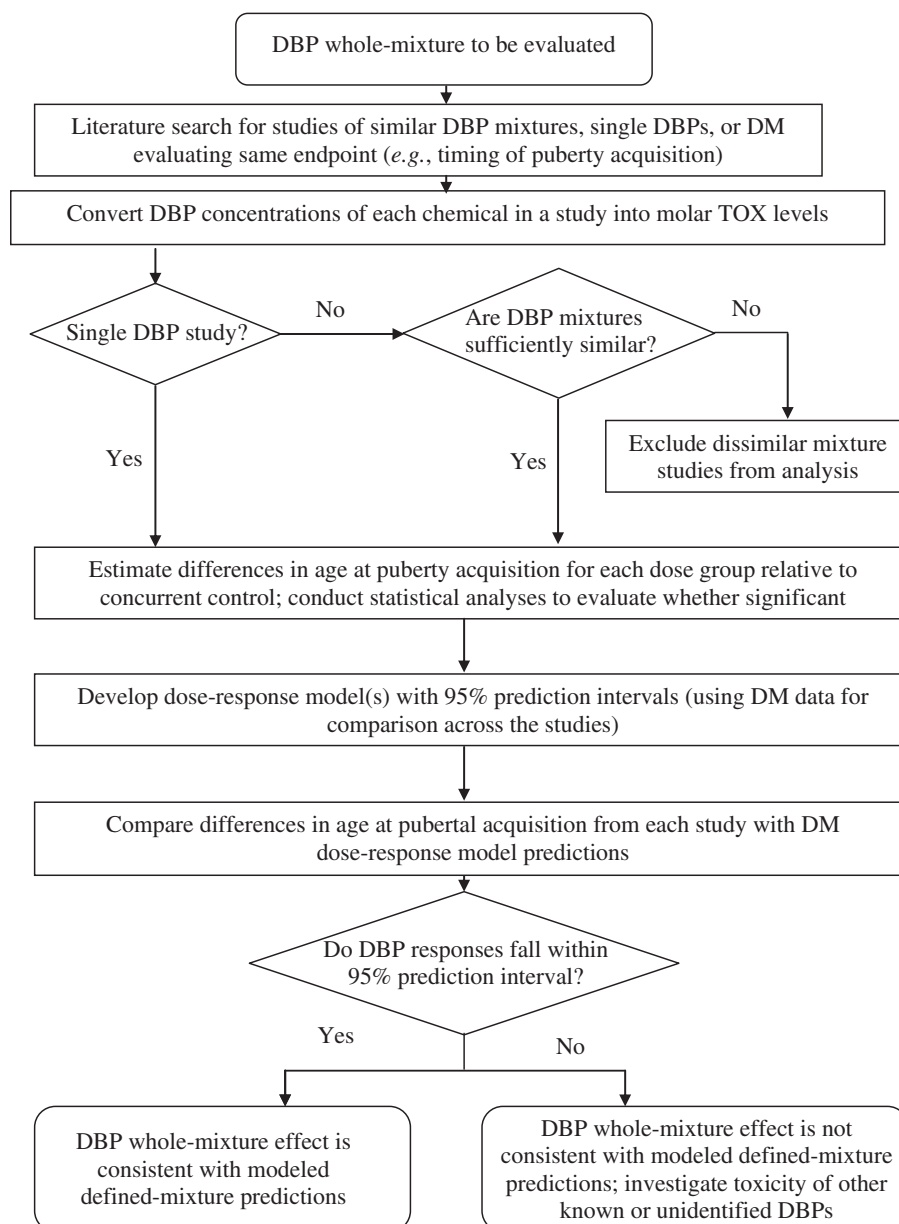


Fig. 1 – Flow diagram for comparing results of single DBP, defined-mixture, and whole-mixture bioassays. DBP: disinfection byproduct; DM: defined mixtures; TOX: total organic halogen.

to mating and continued until the F1 generation acquired puberty. Body weights were reported for the day after weaning but not at puberty. In the F1 generation, the authors reported no significant differences in age at PPS or VO in any dose group ($p > 0.05$).

We note that other published reports on the effects of DBPs either on additional reproductive/developmental endpoints in S-D rats or in other *in vivo* experimental models (examples include the reported effects of bromochloroacetic acid, dichloroacetic acid and dibromoacetic on sperm in S-D rats (Klinefelter et al., 2002; Linder et al., 1994a, 1994b, 1997a, 1997b) are not reported here, as we focused on puberty attainment in the S-D rat.

1.1.2. DBP mixture studies

Narotsky et al. (2015) administered the DM, comprised of nine regulated DBPs, as drinking water to pregnant rats from GD 0

through weaning of their offspring; treatment of the F1 generation via drinking water continued to the end of the study. Body weights both on the day of weaning (PND 26) and the day of puberty acquisition were reported. Significant delays in PPS and VO were observed in the medium and high-dose groups compared to controls ($p < 0.05$).

Narotsky et al. (2013) administered the WM as drinking water to pregnant rats from GD 2 until their litters were weaned; F1 rat exposures continued through adulthood. Body weights both on the day of weaning (PND 21) and the day of puberty acquisition were reported. In F1 females, the age at VO was slightly but significantly delayed in the treatment group ($p < 0.05$); however, among males, no significant difference in age at PPS was reported. The results of chemical analyses of the WM are reported by Pressman et al. (2010).

1.2. Calculation of TOX concentration, a common dose metric across studies

Use of a common metric is required for comparisons across the studies. Because DBP dose levels among these five studies were not directly comparable, we converted all reported DBP concentrations in each study to molar TOX levels. Thus, water concentrations were expressed on a uniform molar TOX scale. To calculate molar TOX levels, we estimated the contribution of each halogen atom (*e.g.*, chlorine, bromine, and iodine) in one mole of each DBP. We used a three-step approach to estimate molar TOX concentrations (Table 1, Footnote ‘a’). In Step 1, the molar concentration of each DBP was estimated by dividing the mass concentration by its molecular weight. In Step 2, the molar concentration of each halogen present in each DBP was calculated by multiplying the total number of halogen atoms by the molar concentrations of each DBP. Finally, in Step 3, the molar halogen concentrations were summed to estimate the TOX concentration (mmol/L).

1.3. Sufficient similarity analysis of mixtures

We also used molar TOX estimates to examine whether the chemical compositions of the WM and DM were “sufficiently similar” and thus it would be appropriate to compare the associated health outcomes (US EPA, 2000b; Rice et al., 2009). For the nine DBPs common to both the DM and the WM, we summed the TOX contribution of each chemical to create the 9-DBP TOX for that mixture. Then, for both mixtures, we estimated the percent TOX contribution of each of the nine DBPs to the respective 9-DBP TOX for that mixture. We evaluated similarity between WM and DM by comparing these percent TOX contributions of nine DBPs.

1.4. Estimation of differences in age at puberty acquisition

The mean age at VO or PPS in the control groups differed across studies. Therefore, the difference from concurrent controls was used as a standardized metric to enable comparisons of responses in the WM and individual DBP studies with the DM data. For statistical modeling using individual animal data, the difference in age of puberty acquisition for each rat was calculated by subtracting the concurrent control group’s mean age of puberty acquisition from the individual rat’s age at puberty acquisition.

1.5. Statistical analyses

1.5.1. Test for significant delay

The age at puberty acquisition for concurrent treatment groups was compared against concurrent controls. Analyses of covariance (ANCOVA) were performed on age at VO and PPS, adjusting each using either weaning body weight or pubertal body weight as covariates at 95% confidence intervals ($p < 0.05$) (SAS 9.1, Proc Mixed). Analyses of variance (ANOVA), unadjusted for body weight, were also performed ($p < 0.05$). Upon finding significance in ANOVA or ANCOVA, *t*-tests (two-tailed) for significance ($p < 0.05$) was also performed to compare treated groups versus concurrent controls using unadjusted and body weight-adjusted data on age at puberty acquisition.

1.5.2. Body-weight adjustments

Age at puberty acquisition across concurrent treatment groups was examined using ANCOVA, with body weight as a covariate. Different study authors have used body weights measured at different times as the covariate. Some use the body weight reported at weaning (Christian et al., 2002; Goldman et al., 2000); others use the body weight reported at puberty acquisition (Blystone et al., 2007; Klinefelter et al., 2004; Tyl et al., 2004). Because of the varied approaches among researchers, ANCOVA models using each of these body weight measures were developed. ANOVA models, without body-weight adjustments, were also developed.

1.5.3. Defined mixture regression modeling

To compare age at puberty acquisition in the DM study with results from the WM and individual DBP studies, dose-response models for the DM study result were developed based on individual animal data using regression analysis (SAS 9.1, Proc Reg). Regression models were developed by regressing dose on differences in age at puberty acquisition between treated and control male and female rats; separate regression models were developed for males and females, one using age of puberty acquisition data adjusted for body weight measured at day of weaning, a second using age of puberty acquisition data adjusted for body weight measured on the day of puberty acquisition, and a third using unadjusted data (a total of six models, three for each sex). The data were fit using polynomial functions of n^{th} degrees, each including an intercept term. Model selection was based on consideration of goodness of model fit, judged by r^2 estimates, and parsimony. Using the modeling results, we calculated 95% prediction intervals for responses that corresponded to the differences in age at puberty acquisition reported in the WM study, and the three individual DBP studies.

2. Results

2.1. Sufficient similarity analysis

Table 2 summarizes the individual organic halogen and TOX concentrations for the nine regulated DBPs and BCAA in the five studies utilized. Results of the simple sufficient similarity analysis of the nine DBPs common to the DM and WM suggest that the percent halogen distributions of the nine DBPs in these two mixtures follow similar patterns (Table 2). The percentage of the TOX associated with CHBr_3 and BAA is $\leq 1\%$ for both mixtures. The concentrations of CAA and BAA were below their minimum reporting levels (MRLs) in the WM (in the WM, the MRL values used in TOX analysis are presented). The main difference between the DM and WM occurs with the TOX associated with CAA and TCAA. The percentage of TOX associated with CAA in the DM was approximately six times higher than in the WM. The percentage of TOX associated with TCAA was approximately three times higher in the WM than in the DM. Except for these differences, the two mixtures appear to be sufficiently similar based on the chemical composition of these nine DBPs; thus, comparing differences in age at puberty acquisition associated with these mixtures was judged to be reasonable.

Table 2 – Illustration of individual organic halogens and TOX at medium dose (Group-III) of each single DBP and mixture studies.

Study	Chemical(s)	DBP Conc. (mg/L)	DBP Conc. (mmol/L)	TOCl (mmol/L)	TOBr (mmol/L)	TOX (mmol/L)	Percent TOX fraction (%)
DBAA	Dibromoacetic acid	40	0.18	0	0.4	0.4	NA
BCAA	Bromochloroacetic acid	300	1.73	0.17	0.17	3.5	NA
BDCM	Bromodichloromethane	150	0.92	1.83	0.92	2.75	NA
DM	Chloroform	44.77	0.375	1.124	0.000	1.124	43
	Bromodichloromethane	25.96	0.158	0.317	0.158	0.475	18
	Dibromochloromethane	8.59	0.041	0.041	0.082	0.124	5
	Bromoform	0.68	0.003	0.000	0.008	0.008	<1
WM ^a	Chloroacetic acid	14.07	0.149	0.149	0.000	0.149	6
	Dichloroacetic acid	27.03	0.210	0.419	0.000	0.419	16
	Trichloroacetic acid	13.71	0.084	0.252	0.000	0.252	10
	Bromoacetic acid	1.64	0.012	0.000	0.012	0.012	<1
	Dibromoacetic acid	3.54	0.016	0.000	0.033	0.033	1
	Chloroform	7.12	0.060	0.179	0.000	0.179	40
	Bromodichloromethane	3.28	0.020	0.040	0.020	0.060	13
	Dibromochloromethane	0.79	0.004	0.004	0.008	0.011	3
	Bromoform	0.06	0.000	0.000	0.001	0.001	<1
	Chloroacetic acid	0.05 ^b	0.001	0.001	0.000	0.001	<1
	Dichloroacetic acid	4.46	0.035	0.069	0.000	0.069	15
	Trichloroacetic acid	6.90	0.042	0.127	0.000	0.127	28
	Bromoacetic acid	0.03 ^b	0.000	0.000	0.000	0.000	<1
	Dibromoacetic acid	0.19	0.001	0.000	0.002	0.002	<1

TOX: total organic halogen; DBP: disinfection byproduct; TOCl: total organic chloride; TOBr: total organic bromide; DBAA: dibromoacetic acid; NA: not applicable; BCAA: bromochloroacetic acid; BDCM: bromodichloromethane; DM: defined mixture; WM: whole mixture; MRL: minimum reported level.

^a The organic halogen concentrations are shown only for nine of the regulated DBPs (9-DBP TOX).

^b Chloroacetic acid and bromoacetic acid concentrations were below MRL in the WM, and for illustration, their reported MRL values were used. The WM chemistry data are taken from Pressman et al. (2010). The chemical compositions of the remaining DBPs in the WM were either not known or not included (other DBPs concentrations were not relevant for this paper). The percent TOX fraction (%) for each DBP was calculated using total TOX of only nine DBPs. The mean DBP concentration (mg/L) of the WM may differ slightly from Pressman et al. (2010) median concentration because we have incorporated other identified DBPs from Pressman et al. (2010) in the remaining TOX fraction.

2.2. Observed and body weight-adjusted age at puberty acquisition

Fig. 2 shows the mean observed and body weight-adjusted differences from concurrent control in age at puberty acquisition in the five studies. In the lower dose groups of the three individual DBP studies, age at puberty acquisition was comparable to controls, whereas in the higher dose groups, significant delays in puberty acquisition were consistently observed. In the DM study, delays in puberty acquisition increased with dose; in the medium and high-dose groups, these delays were significant in both sexes. In the WM study, significant delays in puberty acquisition were observed for females (mean \pm standard error 0.78 ± 0.3 days), but the delay in males (0.39 ± 0.3 days) did not reach significance. In almost all cases, use of pubertal body weight as a covariate increased the estimated mean age at puberty acquisition at the highest dose level. When body weight at weaning was used as a covariate, the estimated mean age at puberty acquisition decreased for both males and females, especially in the high dose groups. The results of significance tests shown in Fig. 2 were consistent with the results reported in the individual DBP studies using either weaning body weight or pubertal body weight as a covariate; however, some results reported here are inconsistent with those in the individual DBP studies, when the covariates were not included.

2.3. Defined mixture regression models

Eqs. (1) to (4) show the regression models developed for males and females in the DM bioassay by regressing the differences from concurrent control in age at puberty acquisition (shown in Fig. 2) on dose with body weight as a covariate, along with the model-fit statistics. The regression models were adjusted using a covariate of either body weight measured at puberty (Eqs. (1) and (2)), or body weight measured at weaning (Eqs. (3) and (4)) in male and female rats, respectively. Based on the r^2 statistic, the statistical fits of the regression models using dose as an independent variable improved substantially when body-weight adjustments were employed (comparing Eqs. (1), (2), (3), and (4) to Eq. (5) and (6)).

$$\text{Difference in PPS}_{\text{adjusted}} = -16 + 0.15 \times \text{Dose} + 0.26 \times \text{Dose}^2 + 0.07 \times \text{Pubertal Body weight} \quad (r^2 = 0.57) \quad (1)$$

$$\text{Difference in VO}_{\text{adjusted}} = -4.74 - 0.16 \times \text{Dose} + 0.25 \times \text{Dose}^2 + 0.10 \times \text{Pubertal Body weight} \quad (r^2 = 0.61) \quad (2)$$

$$\text{Difference in PPS}_{\text{adjusted}} = -11 + 0.81 \times \text{Dose} + 0.06 \times \text{Dose}^2 - 0.13 \times \text{Weaning Body weight} \quad (r^2 = 0.40) \quad (3)$$

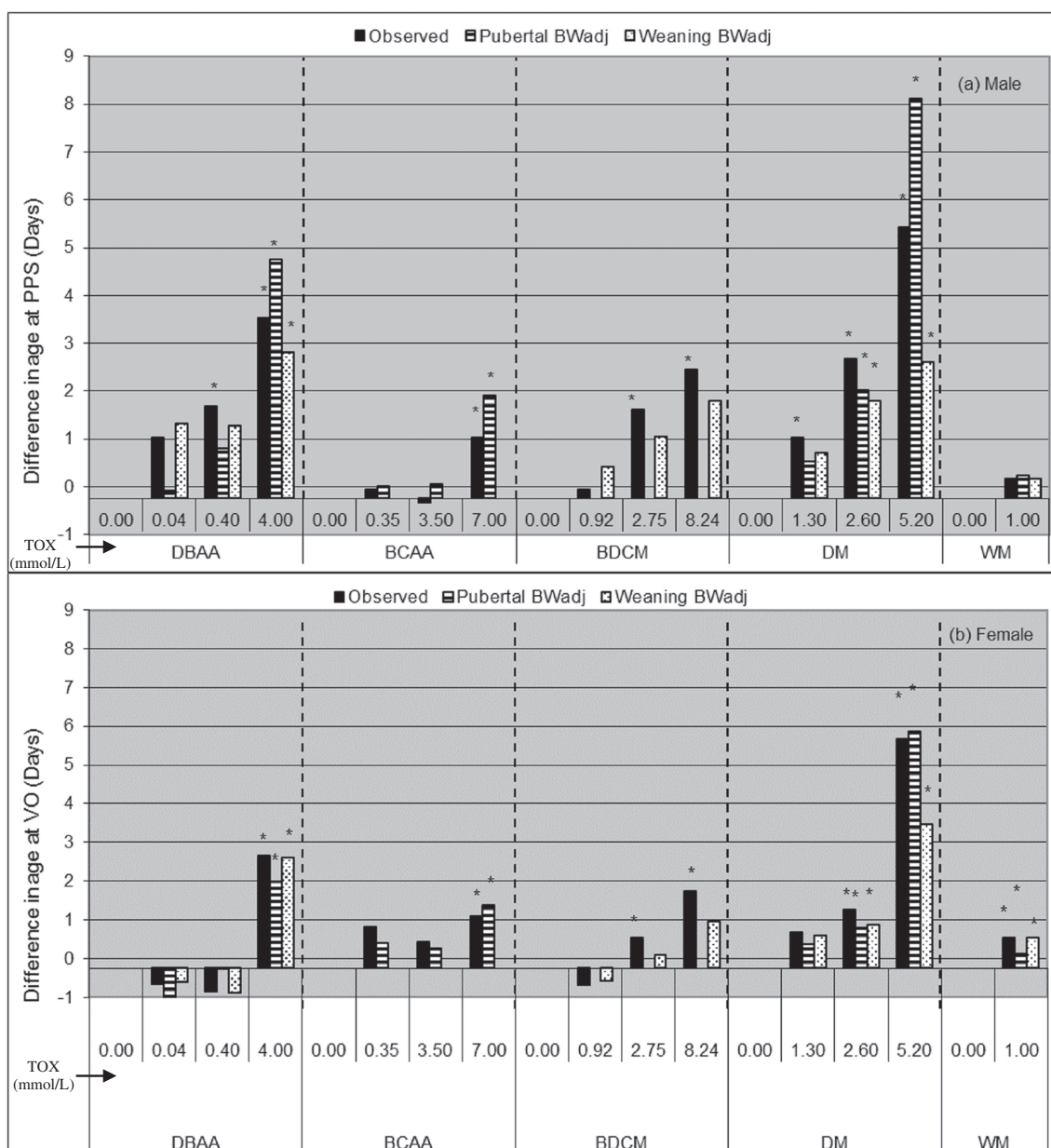


Fig. 2 – The unadjusted and body weight-adjusted mean differences in age at puberty acquisition between treated and control groups across the male (a) and female (b) studies. Note: The x-axis represents the concentrations as TOX (mmol/L) for each dose level. Asterisk “*” indicates significant difference from concurrent control. In the BDCM study, body weight at puberty acquisition was not reported. In the BCAA study, body weight at weaning was not reported. BCAA was present in whole mixture but absent in defined mixture. The analysis for significant difference was done using weaning and pubertal body weights as covariate, and the outcomes are consistent with the reported individual study results; however, a test for significant difference with unadjusted (observed) day of puberty acquisition showed additional significance in DM male rats at 1.3 mmol/L, in DBAA male rats at 0.04 mmol/L, in BDCM male rats at 2.75 and 8.24 mmol/L, respectively, and in BDCM female rats at 8.24 mmol/L. BWadj: adjusted body weight PPS: preputial separation; VO: vaginal opening; TOX: total organic halogen; DBAA: dibromoacetic acid; BCAA: bromochloroacetic acid; BDCM: bromodichloromethane; DM: defined mixture; WM: whole mixture.

$$\text{Difference in VO}_{\text{adjusted}} = -11 - 0.31 \times \text{Dose} + 0.06 \times \text{Dose}^2 - 0.14 \times \text{Weaning Body weight} \quad (r^2 = 0.52) \quad (4)$$

$$\text{Difference in PPS}_{\text{unadjusted}} = -0.16 + 1.0 \times \text{Dose} \quad (r^2 = 0.33) \quad (5)$$

$$\text{Difference in VO}_{\text{unadjusted}} = -0.16 + 1.0 \times \text{Dose} \quad (r^2 = 0.42) \quad (6)$$

2.4. Comparing observed and body weight-adjusted age at puberty acquisition between the DM and other studies

The differences in age at puberty acquisition reported in the WM and individual DBP bioassays were compared to the 95% prediction intervals from the DM models. The regression models adjusted with weaning body weight as a covariate

were not selected for comparison because the model fits achieved in the models with pubertal body weight as a covariate were better. Fig. 3a and b depict the pubertal body weight-adjusted difference in age at puberty acquisition at different TOX concentrations in male and female rats, respectively. The deviation of age at puberty acquisition from the DM regression model line shows the difference in puberty acquisition predicted by the DM model at the corresponding TOX concentration. Because the differences in puberty acquisition for the WM fall within the 95% prediction intervals, they are considered to be consistent with the DM model results. The single chemical responses that fell outside the lower 95% prediction interval, particularly BCAA, differed from DM model; however, BCAA is not a DM component and was judged to not be of toxicological concern because of small differences in age at puberty acquisition compared to the DM.

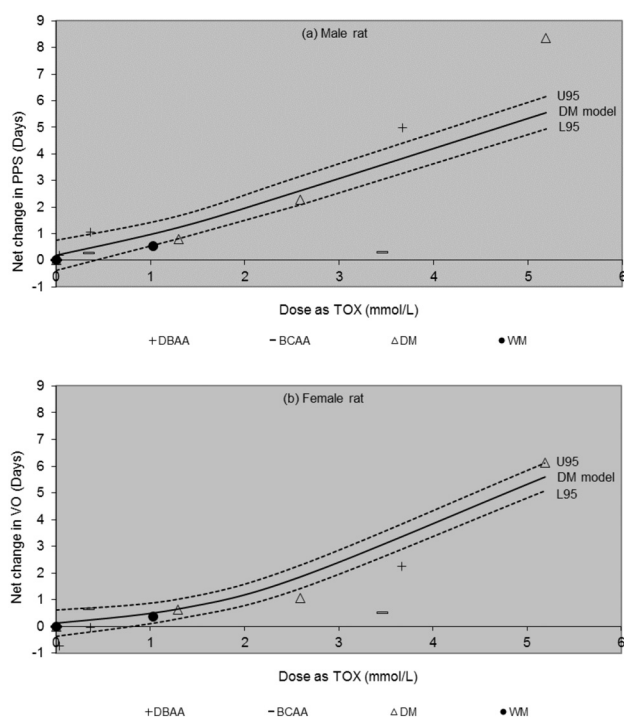


Fig. 3 – Comparison of pubertal body weight-adjusted differences in age at PPS and VO across studies with pubertal body weight-adjusted defined mixture regression models.
Note: The WM associated differences in puberty acquisition were compared with the DM regression model at 1.0 mmol/L TOX concentration. In female rats, the mean pubertal body weight-adjusted difference was 0.36 day, just slightly lower than the DM regression model line. The BDCM study did not report pubertal body weight-adjusted data and are not present in the figure. PPS: preputial separation; VO: vaginal opening; WM: whole mixture; DM: defined mixture; TOX: total organic halogen; BDCM: bromodichloromethane; DBAA: dibromoacetic acid; BCAA: bromochloroacetic acid.

3. Discussion

Here, we present a method to evaluate the contribution of component chemicals to the toxicity of a mixture. The method is illustrated with evaluation of delays in puberty acquisition in rats posed by known and unidentified DBPs present in the WM. The delays in puberty acquisition observed in the WM-exposed rats could not be distinguished statistically from those estimated by the DM model; i.e., these delays appeared consistent with those attributed to the nine DBPs in the DM. Significant differences in puberty acquisition also were reported in bioassays of the following individual DBPs: DBAA, BCAA, and BDCM (Klinefelter et al., 2004; Sloan et al., 2005; Christian et al., 2002); DBAA and BDCM also were components of the DM. A significant delay in PPS was observed in DM-treated males (Narotsky et al., 2015); however, in the WM bioassay, the age at PPS in treated males did not differ significantly from controls, even though the WM had a larger sample size than the DM.

Exposures of regulated DBPs have been associated with human reproductive and developmental outcomes (e.g., Waller et al., 1998; Windam et al., 2003; Hinckley et al., 2005; Wright et al., 2003; Hoffman et al., 2008). It is not known whether these outcomes are associated with identified DBPs or other factors such as co-occurring DBPs. Our analysis suggests that, in the rat, the slight delay in VO observed in the WM bioassay may be associated mainly with nine of the DBPs regulated currently in the United States, and that these nine DBPs may influence the adverse effect of DBP mixtures.

The timing of puberty acquisition in rats is influenced by a number of factors, including hormones secreted from the hypothalamus, adrenal glands and thyroid glands, body size, genetics, and environmental factors, including toxicant exposures (Ashby et al., 1997; Goldman et al., 2000). Given the many factors influencing the age of puberty acquisition, understanding the biological implications of a modest puberty delay observed in the WM-treated female rats is not straightforward. In the WM study, the treated females' serum estradiol and progesterone levels and the treated males' testosterone levels were comparable to controls (Narotsky et al., 2013). Decreased body weight is also associated with delayed puberty; however, that study reported no differences in body weights of treated and control animals. Because the WM study reported no differences in key hormonal levels and body weights between treated and control rats, we anticipated no significant reproductive effects in rats as a consequence of the slight delay in puberty in treated females. Considering the lack of effect on other endpoints associated with sexual maturation, it appears inappropriate to extrapolate such a modest developmental effect from rats to humans.

Body weight may significantly impact age at puberty acquisition, as heavier rats are believed to acquire puberty earlier than lighter rats, although this relationship is not always observed (Goldman et al., 2000). Our analysis suggests that pubertal body weight-adjusted models fit the data better than the weaning body weight-adjusted models.

The TOX estimation approach used in this manuscript facilitated the comparisons among studies of individual DBPs and DBP mixtures. We also employed this approach in the sufficient similarity analysis between DM and WM. Our analysis of sufficient similarity relied on *ad hoc* comparisons

of the contributions to TOX of each mixture's components. These remained constant across the various dose groups. Although the TOX fraction of two compounds (CAA and TCAA) differed between the DM and WM, we judged these mixtures to be sufficiently similar based on the remaining seven chemicals in the DM.

Several other researchers have developed approaches that identify the components influencing the toxicity of complex environmental mixtures. In an analysis of wood-preserving waste mixtures that contained polycyclic aromatic hydrocarbons (PAH) and pentachlorophenol, Cizmas et al. (2004) analyzed the mixtures chemically using gas chromatography/mass spectrometry (GC/MS) and evaluated the mixtures toxicologically using *Salmonella*/microsome and *Escherichia coli* prophage induction assays. Eide et al. (2002) used pattern recognition and multivariate regression modeling to test PAH mixtures derived from gasoline. These mixtures were analyzed chemically through GC/MS and chemical fingerprinting, and toxicologically evaluated using the Ames *Salmonella* assay. These chemical and toxicity data served as inputs to a principal component analysis to evaluate similarities among the mixtures, and regression analyses were used to predict the mixtures' mutagenicity based on chemical composition. Rice et al. (2008) proposed a component-based method to estimate the risk associated with exposures to known DBP fractions in a mixture. Using animal bioassay data, they compared the predicted risk associated with mixture components to the effects observed *in vivo* following exposures to whole DBP mixtures. Each of these approaches integrates the results of a chemical analysis with toxicity data in an attempt to identify components that influence the toxicity of a mixture. Whereas Cizmas et al. (2004) and Eide et al. (2002) rely on *in vitro* toxicology data, both Rice et al. (2009) and the present method rely on test animal bioassay data.

When compared to *in vivo* data, *in vitro* data require fewer resources; consequently, more samples of the mixture, fractions of the mixture, variations of the defined mixtures and individual mixture components can be tested at different concentrations using *in vitro* toxicity measures. Also, such studies typically can test more replicates (*i.e.*, increased *n*) than *in vivo* studies. The conduct of additional toxicity tests (*e.g.*, using *in vitro* or *in silico* studies) on the whole mixture, additional fractions, additional defined mixtures, and individual chemical components could be used as inputs to the method proposed here, likely increasing the confidence in the results of such an application. Further, increases in sample sizes would increase the reliability of the sample statistics. *In vitro* studies would be particularly useful to develop testable hypotheses for *in vivo* studies. The relevance to humans would be increased by validated *in vitro* to *in vivo* extrapolation methods. As these methods are generally still in the developmental stage, typically risk assessors consider animal bioassays (*in vivo* data) to be more relevant than *in vitro* data for predicting human health effects. Thus, differences among these approaches are due, in part, to an explicit trade-off between the number of samples that can be tested, given resource constraints, and relevancy of the samples to human health.

Although a modest but significant delay in puberty acquisition was observed in WM-treated female rats, we cannot discern a difference between the nine DBPs in the DM and the

WM. However, the contribution of the unidentified DBPs could be important for other health effects in different DBP mixtures, and the present method could be used to elucidate such differences in toxicity between a defined mixture and a mixture of concern. We suggest that the regression-modeling approach discussed in this manuscript may be applied to analyze the toxicity of other complex environmental mixtures. For the vast majority of complex environmental mixtures, relevant data will be gathered from existing literature, as the cost and time necessary for *de novo* data collection will be prohibitive. Thus, it is highly likely that the sample sizes (in this case number of litters per group) will vary among the data sets used in the analysis. The impact of varying sample sizes between different data sets on model uncertainty is an area that will benefit from future research. However, such an approach as the present one will provide useful information to risk assessors, managers, and regulators. It allows risk management, risk remediation, and risk prevention efforts to focus on those components of the mixture responsible for the majority of mixture toxicity, focusing limited resources.

4. Conclusions

We developed a regression modeling-based method to evaluate the contribution of component chemicals to the toxicity of complex mixtures and used the method to examine the onset of puberty in S–D rats prenatally exposed to DBPs. The delays in puberty acquisition observed in the WM-exposed rats could not be distinguished statistically from those estimated by the DM regression model, suggesting that the nine regulated DBPs in the DM might account for much of the delay observed in the WM-exposed rats. The illustrated regression modeling technique presents a promising new way to compare the health effects among complex environmental mixtures and components of such mixtures. Although further adjustments to this approach may be needed due to differences among specific mixtures (*e.g.*, chemical composition, nature of dose–response data, and differences in modes of exposure) or differences in available toxicity tests, it provides a conceptual approach to evaluate contributions of components and mixture fractions to whole mixture toxicity. This approach could provide useful information to health risk assessors and risk managers to reduce health risks associated with exposures to some environmental mixtures.

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