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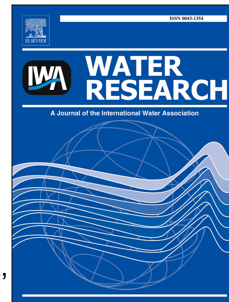

Bioanalytical and Chemical Evaluation of Disinfection By-Products in Swimming Pool Water

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PII: S0043-1354(14)00285-1

DOI: 10.1016/j.watres.2014.04.002

Reference: WR 10604

To appear in: *Water Research*

Received Date: 5 December 2013

Revised Date: 26 March 2014

Accepted Date: 2 April 2014

Please cite this article as: Yeh, R.Y.L., Farré, M.J., Stalter, D., Tang, J.Y.M., Molendijk, J., Escher, B.I., Bioanalytical and Chemical Evaluation of Disinfection By-Products in Swimming Pool Water, *Water Research* (2014), doi: 10.1016/j.watres.2014.04.002.

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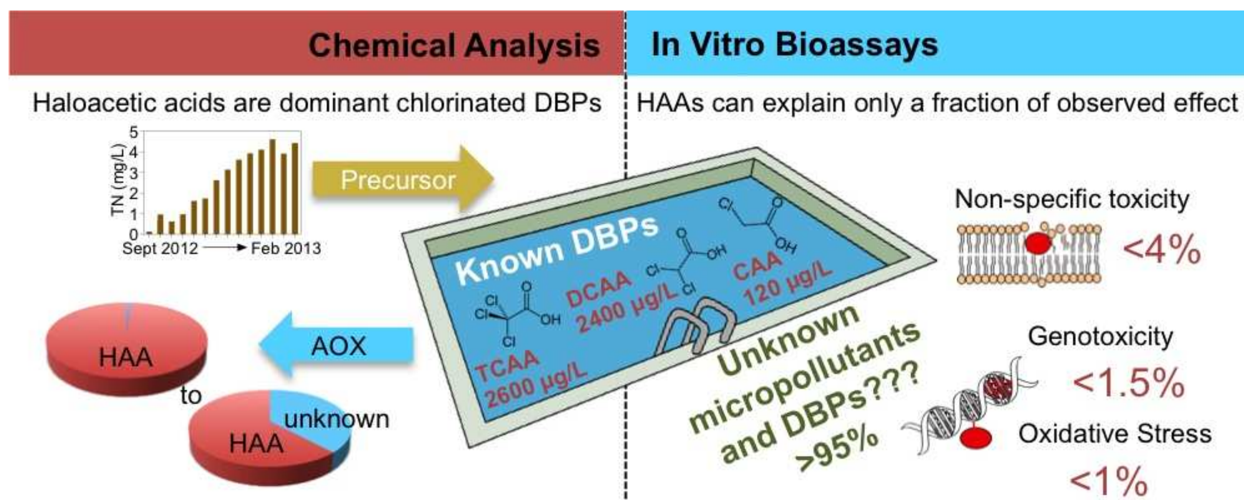

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1 # Bioanalytical and Chemical Evaluation of Disinfection
2 # By-Products in Swimming Pool Water

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24 **Keywords**

25 *In vitro* bioassay, cytotoxicity, genotoxicity, oxidative stress, disinfection by-products,
26 haloacetic acids

27

28 **Abstract**

29 Pool water disinfection is vital to prevent microbial pathogens. However, potentially
30 hazardous disinfection by-products (DBP) are formed from the reaction between
31 disinfectants and organic/inorganic precursors. The aim of this study was to evaluate the
32 presence of DBPs in various swimming pool types in Brisbane, Australia, including
33 outdoor, indoor and baby pools, and the dynamics after a complete water renewal.
34 Chemical analysis of 36 regulated and commonly found DBPs and total adsorbable
35 organic halogens as well as *in vitro* bioassays targeting cytotoxicity, oxidative stress and
36 genotoxicity were used to evaluate swimming pool water quality. Dichloroacetic acid and
37 trichloroacetic acid dominated in the pool water samples with higher levels (up to 2600
38 µg/L) than the health guideline values set by the Australian Drinking Water Guidelines
39 (100 µg/L). Chlorinated DBPs occurred at higher concentrations compared to tap water,
40 while brominated DBPs decreased gradually with increasing pool water age. Biological
41 effects were expressed as chloroacetic acid equivalent concentrations and compared to
42 predicted effects from chemical analysis and biological characterisation of haloacetic
43 acids. The quantified haloacetic acids explained 35 to 118 % of the absorbable organic
44 halogens but less than 4 % of the observed non-specific toxicity (cytotoxicity), and less
45 than 1 % of the observed oxidative stress response and genotoxicity. While the DBP
46 concentrations in Australian pools found in this study are not likely to cause any adverse
47 health effect, they are higher than in other countries and could be reduced by better
48 hygiene of pool users, such as thorough showering prior to entering the pool and avoiding
49 urination during swimming.

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51 # 1. INTRODUCTION

52 Chemical disinfectants minimise microbial pathogen growth in swimming pools and thus
53 prevent potential adverse health effects in human. However, chemical disinfectants can
54 interact with natural organic matter and organic micropollutants brought in by swimmers
55 to form potentially hazardous compounds known as disinfection by-products (DBPs)
56 (Richardson et al. 2010). More than 600 DBPs have been identified in drinking water
57 (Richardson et al. 2007) and many DBPs were also found in swimming pool water
58 (Chowdhury et al. 2014). Identified DBPs (Plewa et al. 2008) as well as swimming pool
59 water samples (Glauner et al. 2005, Liviac et al. 2010, Plewa et al. 2011) were shown to
60 be carcinogenic or mutagenic.

61 Epidemiological studies on chronic exposure to DBPs in drinking water suggested
62 an association between bladder cancer and exposure to chlorinated drinking water
63 (Villanueva et al. 2007, Cantor et al. 2010). Increased trihalomethane (THM)
64 concentrations and some positive biomarkers of genotoxicity were observed in swimmers
65 after a regular training session (Kogevinas et al. 2010).

66 The considerable research that has been done on DBPs in chlorinated drinking
67 water has led to the definition of health-based guideline values (GV) for several DBPs in
68 drinking water by the World Health Organisation (WHO 2011), the Australian National
69 Health and Medical Research Council (NHMRC 2011), the United States Environmental
70 Protection Agency (U.S. EPA 2011), and the European Union
71 (European Parliament and European Council 2009). There are few GVs for DBPs in
72 swimming pool water apart from THMs, e.g., the German Norm (DIN 19643-1 2011),
73 but the WHO recommended reading across drinking water GVs while considering
74 differences in exposure route and amount of ingested water (WHO 2006).

75 Although there are currently guidelines for managing risks in recreational water in
76 Australia they do not include DBPs in swimming pools (NHMRC 2008). The Australian
77 Drinking Water Guidelines (ADWG) have only set GV in drinking water for 10 out of 23
78 of the recognised DBPs due to the limited knowledge on occurrence and toxicity
79 (Appendix A, Table S1 (NHMRC 2011)).

80 Drinking water commonly serves as source water in pools, and it contains organic
81 and inorganic (e.g., bromide and iodide) precursors for DBP formation as well as

82 previously formed DBPs during disinfection at water treatment plants. Pool users further
83 introduce anthropogenic organic micropollutants (e.g., cosmetic products such as
84 sunscreen, deodorant and lotions) and natural organic matter from bodily excretions such
85 as saliva, urine and sweat. Other factors such as filling water quality, pool type (i.e.,
86 outdoors versus indoors), intensity of usage, temperature, pH, disinfectant used,
87 disinfection process and contact time can all contribute to the overall complicated
88 chemistry of swimming pool water (WHO 2006).

89 Although more than 600 DBPs have been identified, there are still many unknown
90 DBPs. Hua et al. (2007) showed that about 45 % of the halogenated DBPs (measured as
91 adsorbable organic halogens (AOX)) attributed to known DBPs during chlorination,
92 indicating that 55 % were still unknown. This highlights the need to have a
93 multidisciplinary approach to investigate the complex mixture effect of swimming pool
94 water.

95 The goal of this study was to identify and quantify relevant DBPs by chemical
96 analysis of various swimming pool waters across Brisbane, Australia. Precursors were
97 measured in the form of total nitrogen (TN), total organic nitrogen (TON) and total
98 organic carbon (TOC). TN is a measure of both organic and inorganic nitrogen while
99 TON measures organic compounds including proteins, amino acids, and urea. TOC is a
100 measure of the organic carbon in water and is mainly composed of humic substances that
101 contribute to the formation of DBPs. As representative DBPs, 27 volatile DBPs and 8
102 haloacetic acids (HAA) were quantified.

103 Cell-based *in vitro* bioassays are useful in water quality assessment and
104 complement chemical analysis of DBPs as they can capture the effects of heterogeneous
105 mixtures of known and unknown compounds and give information on specific endpoints
106 relevant for human or environmental health (Escher and Leusch 2012). The reactive
107 properties of known DBPs lead to a focus of biological assessment on genotoxicity and
108 carcinogenicity in previous studies (Richardson et al. 2007, Plewa et al. 2012).

109 Liviac et al. (2010) evaluated the genotoxicity of pool water extracts and found
110 highest effects when the water was treated with bromochlorodimethylhydantoin, followed
111 by chlorination and a combination of free chlorine and UV produced the samples with
112 lowest genotoxicity, albeit still higher than tap water. Plewa et al. (2011) investigated the

113 cytotoxicity of pool water extracts and found higher toxic effects towards mammalian
114 cells than for chlorinated tap water. We demonstrated the applicability of bioanalytical
115 tools for the investigation of DBP formation and the toxicity of DBPs formed in a full-
116 scale drinking water treatment plant (Neale et al. 2012) and in lab-based experiments on
117 the formation potential of DBPs from different organic matter precursor fractions (Farré
118 et al. 2013).

119 Not only genotoxicity but also the non-specific cytotoxicity and the oxidative
120 stress response were found to be good indicators of the formation of DBPs (Neale et al.
121 2012, Farré et al. 2013). We assessed non-specific toxicity with the bioluminescence
122 inhibition assay with *Vibrio fischeri* (Tang et al. 2013b) and the induction of oxidative
123 stress response using the AREc32 assay (Wang et al. 2006). With respect to genotoxicity,
124 the bacterial assay umuC was applied to address the SOS response, an early indicator of
125 DNA damage (Oda et al. 1985), and the CellSensor™ p53RE-*bla* HCT-116 assay
126 (Knight et al. 2009) to measure the p53 activation in mammalian cells, which is an
127 adaptive stress response to DNA damage that triggers repair, cell cycle arrest and
128 apoptosis (Bieging and Attardi 2012).

129 By complementing chemical analysis (precursor analysis, DBP analysis and
130 halogen-specific AOX analysis) with bioanalytical tools, it was the goal of this study to
131 give a comprehensive assessment of DBP formation and a profile of potential adverse
132 effects in swimming pool water.

133

134 # 2. MATERIALS AND METHODS

135 ## 2.1. Chemicals

136 All 27 volatile DBPs and 8 HAAs measured for this study, their commercial sources and
137 other chemicals used are described in Appendix A, Section S1.

138 ## 2.2. Pool water sample

139 Samples were taken from municipal swimming pools in Brisbane, Australia with
140 permission granted by the Brisbane City Council. The identity of the pools had to remain
141 confidential; they are coded Pool 1 to Pool 7 and a description of the sites is given in

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142 Appendix A, Table S2. All pools source the water from local drinking water, which was
143 surface water treated by coagulation, chlorination and polished with chloramination
144 (Neale et al. 2012) and used chlorination, mainly by addition of sodium hypochlorite, two
145 pools used salt-chlorination where chlorine was formed in-situ from NaCl by electrolysis.
146 According to the Queensland Health Swimming and Spa Pool Water Quality and
147 Operational Guidelines (Queensland Government 2004), Brisbane pools operate with
148 coated mesh filters or sand filters and the filter types are reported in Appendix A, Table
149 S2. None of the pools use activated carbon treatment, ozone treatment and flocculation.
150 The operators are required to keep the pH between 7.2 and 7.8, total alkalinity between
151 80 and 200 mg/L. All of the pools use electronic dosing system for adjusting pH and
152 chlorine levels in the pools The minimum free chlorine recommended above 26 ºC is 2
153 mg/L for indoor pools and 3 mg/L for outdoor pools (Queensland Government 2004) and
154 most pools met these requirements (Appendix A, Table S2). The first campaign involved
155 6 months sampling for bioanalytical and precursor analysis of Pool 1 taken fortnightly in
156 spring and summer from 28 September 2012 to 7 February 2013 between 7:30 am to
157 8:30 am. The pool had been emptied over the winter and renovated and was filled with
158 tap water in the beginning of September 2012. 17 Samples were taken including two tap
159 water samples and one MilliQ water control; coded O1 to O17 (Appendix A, Table S3).
160 All samples were characterised for precursors, AOX and with bioassays. Volatile DBPs,
161 N-nitrosodimethylamine (NDMA) and HAAs were quantified in sample O14.

162 A second sampling was undertaken in Pool 1 over one daily cycle in autumn: 9
163 samples were taken on 4 April 2013 from 6 am to 6 pm approximately every 1.5 hours
164 and one on-site tap water control (samples D1 to D10). Analysis of AOX, volatile DBPs
165 and HAAs and bioassays were performed with these samples.

166 The third sampling campaign was undertaken in autumn/winter from 1 May 2013
167 to 18 July 2013 and involved 6 additional swimming pools (unheated and heated outdoor,
168 indoor and baby pools) sampled between 8 to 9 am. A total of 26 samples including
169 controls and on-site tap water were taken; coded S1 to S26. AOX and HAAs were
170 quantified in these samples and all bioassays were performed.

171 Specific details on pool water sampling in preparation of both chemical analysis
172 and bioanalytical assessment is given in Appendix A, Sections S1 and S2.

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173 **2.3. Chemical analysis**

174 **2.3.1. DBP precursors**

175 Non-purgeable organic carbon (NPOC, in the following abbreviated as total organic
176 carbon, TOC) and total nitrogen (TN) were measured using a Shimadzu TOC-L total
177 organic carbon analyser with a TNM-L total nitrogen analyser and ASI-L auto-sampler.
178 Ammonia, nitrite and total mono-nitrogen oxides (NOx) were measured on a Lachat flow
179 injection analyser. Total organic nitrogen (TON) was calculated as the difference
180 between TN and the sum of inorganic nitrogen species (ammonia, nitrite and nitrate).
181 Bromide (Br^-) was quantified using ion chromatography.

182 **2.3.2. Adsorbable Organic Halides (AOX)**

183 AOX analysis was performed as described by Farré et al. (2013). A summary of the
184 experimental method is given in Appendix A, Section S1. The concentration obtained
185 from the ion chromatograph and the concentration factors of the initial samples in
186 absorber solution were used to calculate the concentration of adsorbable organic chloride
187 (AOCl; µg/L Cl^-), adsorbable organic bromide (AOBr; µg/L Br^-), and adsorbable
188 organic iodide (AOI; µg/L I^-) in the samples.

189 For mass balance calculations the mass concentrations of halogens were
190 converted into molar concentrations (AOX_{total} in µM) using the molecular weights of Cl
191 (35.45 g/mol), Br (79.90 g/mol) and I (126.90 g/mol). The quantified DBPs were also
192 converted to molar concentrations of halogens (AOX_{known} in µM) by accounting for the
193 stoichiometry of each formed DBP (e.g., 1 µM of $CHCl_3$ translates to 3 µM AOCl) and
194 summing up the molar concentrations of AOCl. The unexplained AOX ($AOX_{unknown}$) then
195 equals the difference of AOX_{total} minus AOX_{known}.

196 **2.3.3. DBP analysis**

197 Volatile DBPs were extracted by liquid–liquid extraction using methyl-*t*-butylether
198 (MtBE). 27 Volatile DBPs (Appendix A Section S1) were quantified using an Agilent
199 7890A gas chromatograph with an electron capture detector (GC-ECD; Santa Clara, CA)
200 that has double injection for identification and confirmation of DBPs in two different
201 chromatographic columns according to the methods described in (Krasner et al. 2006,

202 Farré et al. 2013). NDMA was measured according to Farre et al. (2011). HAAs were
203 measured with GC-ECD using the U.S. EPA Method 552.3 (U.S. EPA 2003) after liquid–
204 liquid extraction using MtBE followed by derivatization. Details on the methods are
205 given in Appendix A, Section S1.

206 2.4. Bioassays

207 2.4.1. Sample preparation for bioassays

208 Initially a routine solid phase extraction (SPE) procedure that has been used previously
209 for disinfected water samples (Neale et al. 2012) was applied for the sample series O.
210 This method only allows enrichment of non-volatile DPBs but loses any volatile DBPs,
211 which cannot be tested in conventional microtitre plate bioassays due to loss of
212 compound during incubation and cross-contamination of neighbouring wells (Neale et al.
213 2012). Samples were extracted with SPE at pH 3 using 1 g Oasis HLB (Waters Australia)
214 as described in Appendix A, Section S2.

215 However, initial analysis of pool water sample series O showed high abundance
216 of haloacetic acids, which are known not to enrich well at pH 3. Thus, several solid
217 phases were compared at pH 1 and the recovery of AOX and biological effects were
218 evaluated to select the best suitable SPE phase. This evaluation is described in
219 Appendix A, Section S2 and the method chosen for the D and S-series of samples was
220 HLB at pH 1.

221 2.4.2. Non-specific toxicity: Bioluminescence inhibition (Microtox) assay

222 The Microtox assay or bioluminescence inhibition test with the bacteria *Vibrio fischeri*
223 provides an indication of non-specific toxicity. The assay was conducted in accordance
224 with the standard operating procedure of the International Organization for
225 Standardization (ISO11348-3 1998) modified for 96-well microplates as described by
226 Tang et al. (2013b).

227 2.4.3. Oxidative stress response: AREc32 assay

228 The AREc32 reporter cell line is based on the MCF7 breast cancer cell line with a
229 luciferase gene attached (Wang et al. 2006). The AREc32 assay was performed according
230 to Escher et al. (2012).

231 **2.4.4. Genotoxicity: umuC assay**

232 Developed by Oda et al. (1985), the umuC assay is widely used for water quality
233 assessment for detection of genotoxicity and assessing induction of DNA repair
234 mechanisms with the *Salmonella typhimurium* bacteria TA1535/pSK1002 genetically
235 modified to produce β-galactosidase. The umuC assay was performed according to
236 Macova et al. (2011).

237 **2.4.5. CellSensor p53RE-*bla* HCT-116 assay for genotoxicity**

238 The HCT-116 epithelial human colon carcinoma cells were stably transfected with a β-
239 lactamase reporter gene controlled by p53 to detect agonists and antagonists of the p53
240 pathway and are commercially available from Invitrogen, Carlsbad, USA. The CellSensor
241 p53RE-*bla* HCT-116 cells were cultivated and the bioassay was performed according to
242 the protocol provided by Invitrogen (Invitrogen 2010) with an extended exposure period
243 of the cells of 48 h. In parallel, cytotoxicity was assessed with resazurin. More details on
244 the assay method are given in Appendix A, Section S3.

245 **2.4.6. Data evaluation of bioassay results**

246 Effect concentrations (EC) in units of relative enrichment factor (REF) were derived from
247 full log-logistic (cytotoxicity) or linear (oxidative stress and genotoxicity) concentration-
248 effect curves with a minimum of eight different concentrations as serial or linear
249 dilutions. Each reported EC value is the average of at least two independent experiments
250 performed on different days. The EC_{50} corresponds to the REF of sample at which 50 %
251 of cytotoxicity was observed, and $EC_{IR1.5}$ to the REF of sample that caused an induction
252 ratio of 1.5. The REF is equivalent to the "concentration factor" that Plewa et al. (2011)
253 used in previous studies to characterize the toxicity of pool water.

254 The results of the bioassays on the samples tested in the present study were
255 compared with a one-way ANOVA with Tukey's Multiple Comparison Test using
256 GraphPad's Prism 6.0 (http://www.graphpad.com/).

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257 **2.4.7. Bioanalytical Equivalent Concentration for comparison of chemical analysis**
258 **and bioanalysis**

259 Bioanalytical equivalent concentrations (BEQ) can be used to compare the
260 predicted biological effect, which is calculated from the concentration C_i quantified by
261 chemical analysis and relative effect potency REP_i of all known chemicals i, with the
262 measured biological effect (Escher and Leusch 2012). CAA was selected as the reference
263 compound due to its higher potency in most bioassays than DCAA and TCAA, despite its
264 occurrence in swimming pool waters was lower than DCAA and TCAA. All biological
265 effects were expressed as CAA equivalent concentration $CAA\text{-}EQ_{chem}$ (equation 1) or
266 $CAA\text{-}EQ_{bio}$ (equation 2), which is the ratio of the effect concentration of CAA divided by
267 the effect concentration of the sample.

268 $$CAA-EQ_{chem}=\sum_i C_i\cdot REP_i \quad (1)$$

269 $$CAA-EQ_{bio}=\frac{EC(CAA)}{EC(sample)} \quad (2)$$

270

271 # 3. RESULTS AND DISCUSSION

272 ## 3.1. DBP Precursors

273 ### 3.1.1. Nitrogen containing precursors

274 Precursors can react with chemical disinfectants to form DBPs thus it is important to
275 monitor the precursors and not only the DBPs formed. The trend of DBP precursors was
276 observed as time-series study at Pool 1 over a period of 6 months from September 2012
277 to February 2013 starting with a complete refill of the pool water with fresh tap water
278 (Appendix A, Table S7).

279 An increasing accumulation of TN over the sampling period is notable in Figure
280 1. Tap water contained approximately 1 mg/L TN because the chlorinated drinking water,
281 which was used to initially fill the pool in September 2012, is chloraminated as last
282 polishing step (Neale et al. 2012). While the tap water and the pool had the same
283 concentration of TN after it was freshly filled with tap water, the TN concentrations

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284 increased more than four fold over the sampling period (Figure 1). Initially up to 25 % of
285 TN was TON, while after a couple of months this ratio decreased to 5 to 10 %
286 (Appendix A, Figure S2).

287 The observed trend indicated (a) a constant input of both organic and inorganic
288 nitrogen into the pool water and (b) that at least a fraction of TN remained trapped and
289 accumulated over time from the day it was completely replenished with on-site tap water.
290 Judd and Bullock (2003) found that nitrogen accumulated in pools over time as nitrate,
291 which could be a possible explanation for the observed overall TN increase. In addition,
292 it has been suggested that the decomposition of chloramine in the presence of organic
293 matter can decrease the proportion of nitrogen gas (N_2) and increase the proportion of
294 NH_3 and NO_3^- (Judd and Bullock 2003).

295 Probably the most significant nitrogen source is urea in urine (De Laat et al.
296 2011). It has also been highlighted that many cosmetic products brought into the
297 swimming pool by swimmers contain nitrogen. They can all interact with free chlorine
298 and produce compounds such as trichloramine and nitrogen-containing DBPs such as
299 nitrosamines. However, trichloramine is known to be volatile and potentially an
300 inhalative irritant (Florentin et al. 2011). In the present study it is likely that the formation
301 of trichloramine is actually a removal pathway for DBPs due to its volatile property
302 (Schmalz et al. 2011).

303 **3.1.2. Inorganic precursors**

304 Inorganic bromide and iodide can lead to the formation of toxicologically more relevant
305 DBPs (Plewa et al. 2008). In previous work, we have demonstrated that in the source
306 water used to fill the investigated swimming pools iodide was below the limit of
307 detection because of the chlorination conditions in the drinking water plant that
308 completely oxidised the iodide to iodate, which is ineffective for formation of DBPs
309 (Neale et al. 2012) and therefore we concentrated in the present study on bromide.

310 Bromide was detected in the tap water samples (0.018 mg/L) in similar
311 concentrations as in previous work (Neale et al. 2012), but not in the pool water (Figure
312 1A). However, the bromide peak (at a retention time of 8.1 min) in the chromatogram
313 was close to the nitrate peak (at 8.4 min), which is more concentrated in the pool water
314 than in the tap water and might have hampered a clear detection. On the other hand no

315 additional source of bromide and iodide apart from the tap water is expected during
316 operation of the pools.

317 **3.1.3. Organic matter as precursor**

318 The level of TOC was similar to that of the tap water and remained constant throughout
319 the sampling period (3.1 – 3.9 mg/L, Figure 1 and Appendix A, Table S7). The measured
320 values are at the lower end of the range of TOC concentrations measured in 23 indoor
321 public pools in the United States, which ranged from 3 to 23.6 mg/L with median of 7.1
322 mg/L (Kanan and Karanfil 2011). A study in French indoor pools found TOC ranging
323 from 1.8 to 7.3 mg/L (Bessonneau et al. 2011).

324 Judd and Bullock (2003) studied the fate of chlorine and organic materials in
325 swimming pools by mimicked human body fluid and found TOC attained steady-state
326 levels between 6.5 – 28 mg/L after >200 h, which suggested that mineralisation occurred.
327 While a constant input of urea and other body fluids was evidenced by TN measurements
328 in the present study, the concentrations of TOC measured were fairly uniform across time
329 and pools and are thus expected to have reached steady state despite the continuous input.

330 **3.2. Adsorbable organic halogens (AOX)**

331 AOX is the collective term for the organic compounds containing halogens. AOX form
332 during the water chlorination but also occur naturally. A summary of all measured AOX
333 values is given in Appendix A, Table S8. The concentration of AOCl, AOBr and AOI of
334 Pool 1 over a period of 6 months starting with the filling of the pool with tap water is
335 depicted in Figure 1B. AOCl was the most dominant form of total AOX across all water
336 samples and reached up to 15 µM.

337 AOBr concentrations gradually decreased over time in pool water samples
338 whereas the on-site tap water consistently exhibited the highest concentration (0.7 µM,
339 Figure 1B). It appeared that AOBr in on-site tap water was gradually degraded and/or
340 volatilised over time. The brominated HAAs (BDCAA, DBCAA and TBAA) tend to
341 decompose to form the corresponding THMs (BDCM, DBCM, and TBM) via
342 decarboxylation but this process is slow at ambient temperature (Cammann and Hubner
343 1993, Zhang and Minear 2002b). This indicates that the final decomposition products of
344 AOBr might be predominantly THMs, which quickly escape the pool water via

345 volatilisation. As no additional Br^- as AOBr precursor seems to enter the pool, this would
346 explain the gradual decrease in AOBr concentration over time.

347 AOI was present in some of the swimming pool water samples but in very low
348 concentrations close to the limit of detection (0.004 µM).

349 Comparison of various different swimming pools (Appendix A, Figure S3)
350 demonstrated similar or even higher concentrations of AOCl in swimming pool samples
351 and lower concentrations in corresponding on-site tap water, while AOBr was
352 consistently lower in pool than in the corresponding on-site tap waters. The range of
353 AOCl of a factor of ten between highest and lowest concentration in different pools
354 (Appendix A, Figure S3) confirms that different properties of the pools can affect the
355 concentration of AOX, such as source water quality, pH, temperature, contact time,
356 disinfectant dose and type, age of the pool and frequency of use, which can all affect the
357 formation of DBPs (WHO 2006).

358 **3.3. Occurrence of DBPs in pool water**

359 Only 12 out of 27 volatile DBPs measured in samples D2 to D10 of Pool 1 (taken over
360 one daily cycle in autumn) were above the detection limit of 0.1 µg/L (Figure 2A).
361 Highest concentrations were observed for trichloromethane (chloroform, TCM, 75 ±
362 7.1 µg/L) and chloral hydrate (CH, 21 ± 1.8 µg/L). Of the trihalomethanes TCM,
363 bromodichloromethane (BDCM) and dibromochloromethane (DBCM) were above the
364 limit of detection. In addition low levels of trichloronitromethane (TCNM) were found.
365 The TCM and CH levels were in a similar range but slightly higher than in a previous
366 study of 85 Korean pools (Lee et al. 2010). Further studies reporting THM levels
367 typically much higher than in the filling water are compiled in the review by Chowdhury
368 et al. (2014).

369 Of the haloketones very little 1,1-dichloropropanone (1,1-DCP) was detected but
370 concentrations of 1,1,1-trichloropropanone (1,1,1-TCP) were close to 10 µg/L.

371 Dichloroacetonitrile (DCAN) was more than a factor of 10 higher than the
372 trichloroacetonitrile (TCAN) and bromochloroacetonitrile (BCAN), which are known to
373 hydrolyse fast. Kim et al. (2002) have previously reported that DCAN formation was
374 presumably increased due to degradation of urea and proteins from humans. The DCAN

375 concentrations were of similar level than measured in previous work (Hansen et al. 2012)
376 but the TCAN concentrations were much lower. As the formation of haloacetonitriles is
377 strongly pH-dependent (Hansen et al. 2012) but the actual pH was not known in the
378 various pools, we cannot differentiate if TCAN and BCAN were less formed or were
379 formed but hydrolyzed rapidly.

380 Of the analysed haloacetamides, only dibromoacetamide (DBAM) and
381 trichloroacetamide (TCAM) were above the limit of detection. None of the volatile DBPs
382 exceeded the GV of the ADWG but CH was detected close to its GV (Appendix A, Table
383 S1). NDMA was below its detection limit of 5 ng/L and was not included in further
384 analysis.

385 The HAAs detected in all pools were dominated by DCAA and TCAA with
386 concentrations as high as 2400 µg/L and 2600 µg/L respectively (Figure 2B). The
387 ADWG GV is 100 µg/L each for DCAA and TCAA (Appendix A, Table S1). As HAAs
388 were the DBPs exceeding the GV, further chemical analysis focused on the group of
389 HAAs and HAAs were also characterised in all bioassays.

390 A comparison of the HAA concentrations found in the different pools reveals that
391 they vary by a factor of ten (Appendix A, Figure S4). In general at the same site, the
392 indoor pools appeared to have higher levels than the outdoor pools and baby pools had
393 higher levels than other pools. The differences for CAA, DCAA and TCAA were larger
394 between pool sites than between pool types and were not statistically significant (one-
395 way ANOVA with Tukey's Multiple Comparison Test between pool types, $p > 0.1$).

396 DCAA and TCAA accounted for almost 95 % of the sum of the eight HAA
397 concentrations (HAA8) detected in the swimming pools in this study, which is similar to
398 previous work by Simard et al. (2013). The measured concentrations of DCAA and
399 TCAA in both outdoor and indoor pools in this study were higher than previous studies
400 (WHO 2006, Lee et al. 2010, Cardador and Gallego 2011, Simard et al. 2013). The most
401 recent study by Simard et al. (2013) in Canada consisted of 54 outdoor and indoor
402 swimming pools. The highest measured total HAA concentrations (HAA9 = HAA8 +
403 TBAA) were 1195 µg/L in indoor pools and 2224 µg/L in outdoor pools (Simard et al.
404 2013) whereas in the present study the highest HAA8 were 3931 µg/L in indoor pools
405 and 2772 µg/L in outdoor pools. Cardador and Gallego (2011) reported an average

406 DCAA and TCAA concentration at 148 µg/L (outdoor) and 83 µg/L (indoor) in
407 comparison to 584 µg/L (outdoor) and 982 µg/L (indoor) in the present study.

408 3.4. Fraction of AOX explained by HAA

409 Between 35 – 118 % of AOX_{total} obtained from pool waters could be explained by sum of
410 HAA halides (AOX_{known}) with DCAA and TCAA explaining the predominant fraction of
411 AOX_{known} (Table 1 and Appendix A, Figure S5). Over 100 % could be explained in some
412 water samples, which might have been resulted from not capturing everything in the
413 AOX_{total} analysis of pool water samples, e.g., if breakthrough due to high concentrations
414 occurred. The variability of the ratio of AOX_{known}/AOX_{total} could not be linked to whether
415 the pool is outdoor or indoor.

416 Overall it is exceptional to be able to explain such a high fraction of AOX_{total} by
417 known DBPs and by only one group of DBPs. In chlorinated and chloraminated drinking
418 water typically less than 50 % of AOX_{total} is AOX_{known} (Hua and Reckhow 2007, Brown
419 et al. 2011).

420 The high concentrations of DCAA and TCAA and the fact that they explain a large
421 fraction of AOX indicate that these compounds might be the final decomposition
422 products of certain intermediate DBPs for DCAA and TCAA. Previous studies found that
423 TBAA undergoes decomposition to form tribromomethane in aqueous solution while
424 TCAA does not decompose to the analogue TCM (Heller-Grossman et al. 1993, Zhang
425 and Minear 2002a).

426 3.5. Bioanalytical assessment

427 The summary of bioassay responses in all samples tested is given in Figure 3. The EC_{50}
428 and $EC_{IR1.5}$ values were plotted on an inverse logarithmic scale to place samples with
429 higher effects on top of each graph and lower effect on the bottom. A high EC means that
430 a sample has to be highly enriched to cause an effect, thus the potency is low, while a low
431 EC means that the effect can be observed at low enrichment. If the EC is 1 the effect
432 threshold occurs in the native sample. With a few exceptions, all of the samples had to be
433 enriched to cause an effect (Figure 3).

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434 Previous studies on chlorinated and chloraminated drinking water, surface water and
435 stormwater (Macova et al. 2011, Escher et al. 2012, Tang et al. 2013a, Escher et al. 2014)
436 are shown in Figure 3 for benchmarking the pool water quality against various natural
437 water sources and drinking water. From an initial visual inspection it can be seen that all
438 pool water samples were active at a relative enrichment factor (REF) of 1 (equivalent to
439 the native sample) to 100 (equivalent to 100 fold enriched sample). All effects of pool
440 water samples were higher than of ultrapure water (control). In addition to the data
441 reported in Figure 3, a day course was also sampled in pool 1. There were consistent
442 results and no temporal trends over the course of one day, therefore the detailed data of
443 these experiments are only reported in Appendix A, Section S4.

444 The highest responses observed were non-specific toxicity and oxidative stress, which
445 is consistent with previous studies on chlorinated and chloraminated drinking water
446 indicating that Microtox and AREc32 are more sensitive bioassays than umuC (Neale et
447 al. 2012, Farré et al. 2013). This does not necessarily mean that the endpoints of
448 cytotoxicity and oxidative stress response are more relevant than genotoxicity because
449 sensitivity is a function of both the relevance of the target endpoint and the performance
450 of the cellular assay.

451 **3.5.1. Non-specific toxicity**

452 All swimming pool water samples showed higher non-specific toxicity than the ultrapure
453 water control (one-way ANOVA with Tukey's Multiple Comparison Test, $p < 0.0001$,
454 Figure 3A). There was no significant difference between on-site tap water and any of the
455 pool water samples ($p = 0.91$ for tap water vs. outdoor pools, $p = 0.55$ for tap water vs.
456 indoor pools and $p = 0.95$ for tap water vs. baby pools). There was no difference between
457 the different pool water types ($p = 0.09$ for outdoor vs. indoor pools, $p = 0.63$ for outdoor
458 vs. baby pools and $p = 0.98$ for indoor vs. baby pools).

459 On-site tap water and chlorinated drinking water appears in the same order of
460 magnitude, with no significant difference (Figure 3A, $p = 0.61$; t-test, the chlorinated
461 drinking water was not added in the ANOVA analysis as these data are from various
462 previous studies). This is expected as on-site tap water was sourced from the same
463 drinking water treatment plant where the chlorinated and chloraminated drinking water
464 samples were taken. Levels of effect in outdoor pool water were similar to tap water on-

465 site and chloraminated drinking water despite other DBPs known to be dominant in
466 drinking water (Richardson et al. 2007).

467 The small variability between the same pool types but different sites is
468 presumably caused by a large number of factors, including differences in location and age
469 of construction of the pools, water age, shading by trees and buildings, treatment type,
470 type and numbers of users etc. However, given that all pools were in the same city with
471 associated similar temperatures and solar irradiation, it is not astonishing that the
472 differences of effects between sites were overall small.

473 The ultrapure water had similar low effect as surface water, which can be
474 explained by lack of chlorination for both samples and very low levels of other organic
475 micropollutants. A previous study by Neale et al. (2012) observed increasing non-specific
476 and reactive toxicity with the addition of chlorine at a drinking water treatment plant and
477 suggested formation of DBPs (reaction of chlorine with organic/inorganic compounds)
478 contributed to the observable toxicity.

479 **3.5.2. Oxidative stress response**

480 The AREc32 cell line was demonstrated to be sensitive to chemicals inducing oxidative
481 stress (Natsch 2010) and has been previously applied for water quality monitoring
482 (Escher et al. 2013). All water samples, including the pool water samples, drinking water
483 and surface water, induced the oxidative stress response within the same order of
484 magnitude but effects were higher than in ultrapure water (Figure 3B).

485 All swimming pool water samples showed higher oxidative stress response than
486 the ultrapure water control but the difference had a lower statistical significance level
487 than for the Microtox assay (one-way ANOVA with Tukey's Multiple Comparison Test,
488 $p < 0.02$), A general trend showed highest responses in baby pools, followed by indoor
489 pools, then outdoor pools, similarly to that of non-specific toxicity and genotoxicity.
490 However, the one-way ANOVA with Tukey's Multiple Comparison Test revealed that
491 there was no significant difference between on-site tap water and any of the pool water
492 samples ($p = 0.91$ for tap water vs. outdoor pools, $p > 0.99$ for tap water vs. indoor pools
493 and $p > 0.99$ for tap water vs. baby pools) and between the different pool water types ($p =$
494 0.88 for outdoor vs. indoor pools, $p = 0.89$ for outdoor vs. baby pools and $p > 0.99$ for
495 indoor vs. baby pools).

496 An increased oxidative stress response was also observed in extracts of drinking
497 water dosed to ARE-HepG2 cells although at much higher enrichments than in the
498 present study (Wang et al. 2013).

499 **3.5.3. Genotoxicity**

500 Genotoxicity without (-S9) and with (+S9) metabolic activation was measured using the
501 umuC assay. Genotoxicity with metabolic activation (+S9) was evaluated only with Pool
502 1 time-series study but showed responses below the detection limit, suggesting the
503 majority of DBPs formed are direct genotoxicants which can be detoxified by
504 metabolism, which is also consistent with previous work (Neale et al. 2012, Farré et al.
505 2013). Therefore in the remaining samples only genotoxicity without metabolic activation
506 (-S9) was assessed (Figure 3C).

507 The ultrapure water control had significantly lower genotoxic effects than the
508 swimming pool water samples with the exception of outdoor pool water (one-way
509 ANOVA with Tukey's Multiple Comparison Test, ultrapure water vs. tap water on-site, p
510 = 0.0014, ultrapure water vs. outdoor pools, p = 0.12), ultrapure water vs. indoor pools, p
511 = 0.0017, ultrapure water vs. baby pools, p = 0.0033). Indoor pools had higher genotoxic
512 responses than outdoor pools (p = 0.044) and even the tap water on-site was less
513 genotoxic than the outdoor pool water (p = 0.033). This observation is also consistent
514 with previous findings by Liviac et al. (2010) who explained the lower observed
515 genotoxicity in outdoor pools by photodegradation and possibly higher volatilisation.

516 The genotoxic response of on-site tap water and chlorinated drinking water were also
517 similar (t-test, p = 0.218). However, genotoxicity was commonly observed at an REF >
518 10 and needed an order of magnitude higher enrichment to elicit effects than for
519 cytotoxicity. Chlorinated drinking water spanned across two orders of magnitude,
520 possibly dominated by brominated/ iodinated species that have been shown to be more
521 genotoxic and cytotoxic compared to their chlorinated counterparts (Pals et al. 2011,
522 Plewa et al. 2011). Overall, the genotoxicity results had similar trend to non-specific
523 toxicity response and DBPs were directly reactive without the need of metabolic
524 activation to cause DNA damage.

525 Many of the halogenated DBPs and N-DBPs are direct genotoxicants with exceptions
526 such as nitrosamines that require metabolic activation to elicit genotoxic effect (Wagner

527 et al. 2012). NDMA was not detected and thus it is likely that nitrosamines are not
528 contributing to the mixture effects.

529 Only 12 samples were assessed with the p53 assay but most showed high cytotoxicity
530 (shown in red in Figure 3D), which masked the induction of p53 and therefore $EC_{IR1.5}$
531 values could only be obtained for five samples. As we have used the p53 assay for the
532 first time in the present study we have no data for other water samples to benchmark
533 against. For those samples that were valid, the induction of p53 occurred at lower REFs
534 compared to the umuC assay.

535 The umuC is a bacterial bioassay that is indicative of the SOS response after DNA
536 damage, while the mammalian p53 pathway is broader activated in response to DNA
537 damage but also non-genotoxic mechanisms such as hypoxia or mitotic spindle damage
538 (Stenius and Hogberg 1999, Simmons et al. 2009). p53 not only initiates a series of DNA
539 repair mechanisms, among them regulators of the cell cycles and genes for repair
540 enzymes but also regulates apoptosis and is thus referred to as the tumor suppressor.
541 Accordingly, p53 induction is regarded as excellent predictor of genotoxic carcinogens
542 (Duerksen-Hughes et al. 1999) but cannot be directly compared with bacterial
543 genotoxicity assays. It has previously been shown that bacterial mutagenicity of DBPs
544 does not correlate well with cytotoxicity and genotoxicity measured in mammalian cells
545 (Plewa et al. 2002, Plewa et al. 2004).

546 **3.6. Comparison of chemical analysis with bioanalytical tools**

547 By comparing chemical analysis and bioanalytical measurements, it is possible to yield
548 complementary information on how much of the effects measured in swimming pool
549 water samples can be explained by known chemicals, i.e., HAAs in this study (Escher
550 and Leusch 2012). As HAAs appear of high relevance for swimming pool DBPs due to
551 their high occurrence and are also formed by various different chemical disinfectants,
552 especially at highest levels with chlorination (Richardson et al. 2007), we also evaluated
553 the effects of 12 individual HAAs in the four *in vitro* bioassays. The results are
554 summarised in Appendix A, Section S4. The bioassay results of HAAs of this study
555 (Appendix A, Table S12 and Figure S6) were within a factor of 10 to the chronic

556 cytotoxicity and genotoxicity of HAAs in Chinese Hamster Ovary Cells (Plewa et al.
557 2010), as is shown in the Appendix A, Figure S7.
558 The relative effect potencies (REP) resulting from the EC values tabulated in the
559 Appendix A, Table S12, can be used to convert the concentrations of HAAs quantified
560 with chemical analysis into CAA-EQ ($CAA\text{-}EQ_{chem}$) using Eq. 1.
561 The $CAA\text{-}EQ_{bio}$ can be obtained directly from the bioassay results using Eq. 2. Table
562 2 summarises the CAA-EQ for Microtox, AREc32 and umuC. HAAs could explain up to
563 4 % of non-specific toxicity (Table 2 and Figure 4), which is higher compared to a
564 previous study by Tang et al. (2013b) where a maximum of 1 % of bioanalytical
565 equivalents could be explained by known chemicals after quantifying 269 organic
566 micropollutants (no DBPs) and detecting up to 56 chemicals in ten environmental
567 samples.
568 The contribution of HAAs to oxidative stress response varied by several orders of
569 magnitude (Table 2 and Figure 4). Oxidative stress is an important mode of action for
570 HAAs (Pals et al. 2013). HAAs could only explain $\leq$1 % of oxidative stress response,
571 which is similar to a recent study that quantified 269 individual chemicals in
572 environmental samples including drinking water (Escher et al. 2013).
573 HAAs only attributed to about 1 % of genotoxicity observed with the umuC assay
574 (Table 2 and Figure 4), indicating that there are unknown contributors to genotoxic
575 effects in swimming pool waters. Results for the p53 assay were less clear, as mainly
576 CAA dominated the effects while the higher concentrated DCAA had an $EC_{IR1.5}$ at the
577 same concentration as the EC_{10} for cytotoxicity and for TCAA no $EC_{IR1.5}$ could be
578 derived due to overwhelming cytotoxicity (Appendix A, Section S4).
579 Although DCAA and TCAA were the dominant DBPs in pool samples, previous
580 studies had demonstrated that DCAA in drinking water exhibited genotoxicity only at
581 high concentrations and was not considered to play a primary role in contributing to
582 overall effect (Richardson et al. 2007, Plewa et al. 2010). Genotoxicity is of minor
583 importance for pool water as evidenced by the lower effects observed than in chlorinated
584 and chloraminated drinking water. It has been suggested that the water with high level of
585 bromide results in high level of brominated DBPs and are generally more cytotoxic and
586 mutagenic, therefore more relevant than chlorinated DBPs (Richardson et al. 2007,

587 Zwiener et al. 2007). In addition, this could also be a possible explanation for the relative
588 high toxic effects seen in tap water as it has much higher AOBr than pool water samples
589 (Figure 1).

590 3.7. Temporal trends

591 In addition to the comparison of different pool types, we have also assessed the temporal
592 trends in pool 1 by sampling approximately fortnightly over six month and one daily
593 course. The bioassay results were consistent with the one-time sample and did not show
594 any trend. Therefore these results are only presented in Appendix A, Section S5.

595 4. CONCLUSIONS

596 Despite that more than 600 DBPs have been identified, the known DBPs constitute only
597 the tip of an iceberg. There are still many unidentified DBPs and potential toxicity of
598 many identified DBPs is still unknown. The high concentration of HAAs (especially
599 DCAA and TCAA) in the pool water as well as the good match between HAAs and AOX
600 suggested that HAAs could be used as indicator chemicals to define guideline values for
601 monitoring swimming pool water quality.

602 Although bioassays have the ability to capture mixture effect, the sample
603 preparation by SPE has its own limitation as it can only retain non-volatile and semi-
604 volatile compounds, and not able to capture volatile compounds. Our group is presently
605 working on developing methods to assess volatile chemicals with cell-based bioassays
606 (Stalter et al. 2013) and to extract volatiles from water samples without use of excessive
607 amounts of solvents (work in progress).

608 The levels of DBPs and effects found in the present study are not likely to cause
609 any adverse health impacts for casual swimmers although HAA levels exceeded guideline
610 values for drinking water up to 26 times but those GVs are based on drinking 2 L of
611 water for a lifetime. While swimming pool water is occasionally swallowed, it is not
612 expected that large quantities were ingested.

613 The levels of DBPs could be significantly decreased if the input of DBP precursors
614 were reduced by improving the hygiene of pool users, that is, by implementing measures
615 such as thorough showering prior to pool usage and avoiding urinating into pools

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616 altogether (Chowdhury et al. 2014). The steady increase of total nitrogen in the pool that
617 was freshly filled with tap water is an indication of these anthropogenic inputs.

618 All current risk/safety assessment and guidelines are based on single-chemical
619 testing. The present study demonstrated that the swimming pool is a complex
620 environment with mixtures of DBPs and we can also expect the presence of
621 anthropogenic micropollutants from consumer products (Chowdhury et al. 2014). Thus it
622 is important to understand the correlation of bioanalytical measures with chemical
623 analysis in order to gain the full picture of chemical hazard. Future integration of
624 bioanalytical tools into water quality assessment to complement current chemical analysis
625 could provide an improved assessment of the risks and thus facilitate better management
626 practices.

627 Acknowledgements

628 We thank Tim Flood from the Brisbane City Council for permission to sample pool
629 waters and the pool operators and Marcel Schlamowitz for assistance with sampling. We
630 thank Shane McCarty, Mriga Dutt, and Eva Glenn from Entox for assistance with the
631 bioassays, Marion Revalor from AWMC and Renu Patel from QHFSS for assisting with
632 chemical analysis. We thank Christian Zwiener and Christina Schmalz for helpful
633 discussions.

634 Appendix A. Supplementary Data

635 Supplementary data related to this article can be found at
636 http://dx.doi.org/10.1016/j.watres…...

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875 **Figure Captions**

876 Figure 1. Summary of (A) precursor analysis (TN, total nitrogen; TON, total organic
877 nitrogen; TOC, total organic carbon; Br^{-}, bromide) and (B) AOX in the time series
878 samples of Pool 1. On-site tap water (O1) was taken on the 28 September 2012.
879
880 Figure 2. (A) Occurrence of 12 out of 27 volatile DBPs measured in nine water samples
881 (D2 – D10) taken on 4 April 2013. (B) Occurrence of HAA in 17 swimming pool water
882 samples including Pool 1 outdoor (average of O2, O4, O6, O8, O11 and O14), Pool 2
883 outdoor (S24), Pool 2 indoor (S25), Pool 3 indoor (S13), Pool 5 covered outdoor (average
884 of S7 and S22), Pool 5 baby pool (average of S9 and S23), Pool 6 outdoor (S20), Pool 6
885 indoor (S21), Pool 7 outdoor (S17) and Pool 7 indoor (S18). The boxes extend from the
886 25th to 75th percentile with the median marked as line in the middle. The whiskers
887 extend from the 5th to 95th percentile. The ADWG guideline values (GV) are shown for
888 comparison.
889
890
891 Figure 3. Summary of the bioassay results ((A) Microtox, (B) AREc32, (C) umuC, (D)
892 p53) for all samples and comparison with results from previous studies (marked with *,
893 excluded from the ANOVA (Macova et al. 2011, Escher et al. 2012, Tang et al. 2013a,
894 Escher et al. 2014)). The boxes extend from the 25th to 75th percentile with the median
895 marked as line in the middle. The whiskers extend from the 5th to 95th percentile. The
896 LODs are marked with red dashed lines. For p53, the activation was in many cases
897 masked by the cytotoxicity, therefore the EC_{10} values for cytotoxicity are plotted in red
898 for comparison.
899
900 Figure 4. Comparison of $CAA\text{-}EQ_{bio}$ from bioassays and $CAA\text{-}EQ_{chem}$ from chemical
901 analytical data.
902

Tables

Table 1. AOX analysis (AOX_{total}) in comparison with AOX_{known} calculated from the detected DBPs (Table S9).

Sample ID	Site ID	Sample Type	AOX_{total} (µM)	AOX_{known} (µM)	Percentage that can be explained by HAAs		
					AOCl (%)	AOBr (%)	AOX (%)
S7	Pool 5	Covered outdoor pool	27.9	27.5	100	8.5	97
S9	Pool 5	Baby indoor pool	81.5	86.4	108	4.5	103
S13	Pool 3	Indoor pool	11.2	5.9	54	-	51
S17	Pool 7	Outdoor pool	43.3	46.4	108	13	106
S18	Pool 7	Indoor pool	65.7	65.2	100	14	98
S20	Pool 6	Outdoor pool	29.6	19.9	68	19	67
S21	Pool 6	Indoor pool	45.4	20.4	45	-	45
S22	Pool 5	Covered outdoor pool	31.6	28.9	92	35	91
S23	Pool 5	Baby indoor pool	84.7	35.8	44	2.5	41
S24	Pool 2	Outdoor pool	29.8	36.3	124	8.5	119
S25	Pool 2	Indoor pool	47.5	37.1	79	18	77

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Table 2. Comparison of $CAA\text{-}EQ_{chem}$ and $CAA\text{-}EQ_{bio}$ for Microtox,AREc32 and umuC.

Sample ID	$CAA\text{-}EQ_{chem}$ [Microtox] (µM)	$CAA\text{-}EQ_{bio}$ [Microtox] (µM)	Quantified chemicals explain % CAA-EQ [Microtox]	$CAA\text{-}EQ_{chem}$ [AREc32] (µM)	$CAA\text{-}EQ_{bio}$ [AREc32] (µM)	Quantified chemicals explain % CAA-EQ [AREc32]	$CAA\text{-}EQ_{chem}$ [umuC] (µM)	$CAA\text{-}EQ_{bio}$ [umuC] (µM)	Quantified chemicals explain % CAA-EQ [umuC]
S7	3.6	348	1.0 %	1.23	824	0.15 %	3.84	396	1.0 %
S9	11.0	1279	0.9 %	3.74	1144	0.33 %	12.04	1764	0.7 %
S13	1.2	332	0.4 %	0.52	971	0.05 %	0.79	177	0.4 %
S17	6.3	158	4.0 %	2.18	293	0.74 %	6.26	649	1.0 %
S18	9.7	533	1.8 %	3.52	3272	0.11 %	8.81	621	1.4 %
S20	2.4	134	1.8 %	0.94	563	0.17 %	3.27	270	1.2 %
S21	3.0	377	0.8 %	0.82	2317	0.04 %	2.02	568	0.4 %
S22	3.3	353	0.9 %	1.10	1480	0.07 %	4.36	742	0.6 %
S23	2.0	156	1.3 %	0.44	14422	0.003 %	6.12	973	0.6 %
S24	4.2	146	2.9 %	1.41	448	0.32 %	5.57	634	0.9 %
S25	3.6	307	1.2 %	0.90	23075	0.004 %	5.94	1012	0.6 %

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A

TN (mg/L) TON (mg/L) TOC (mg/L)

0 1 2 3 4 5

Br^- (mg/L)

0.00 0.01 0.02 0.03 0.04 0.05

O1 O2 O3 O4 O5 O6 O7 O8 O9 O10 O11 O12 O14 O13

tap 9/2012 → 2/2013 milliQ

B

AOCl (μM)

0 2 4 6 8 10 12 14 16

AOBr (μM)

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

O1 O2 O3 O4 O5 O6 O7 O8 O9 O10 O11 O12 O14 O13

tap 9/2012 → 2/2013 milliQ

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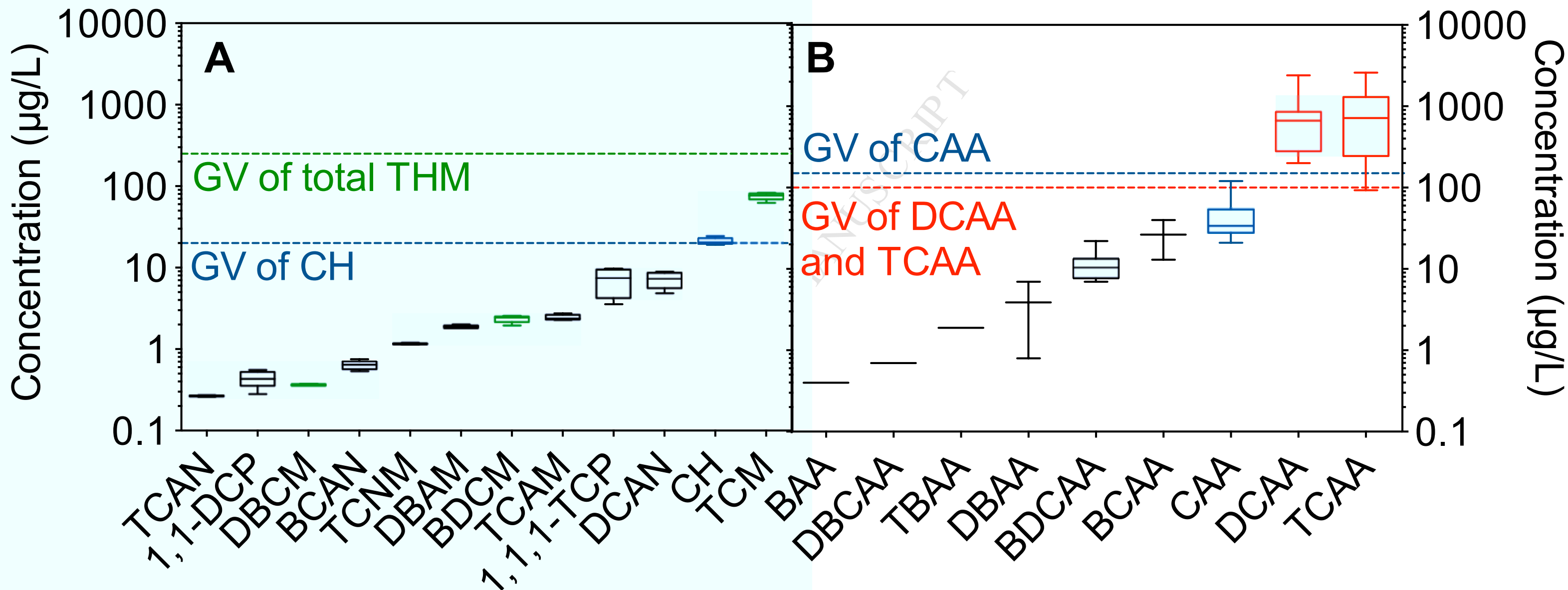

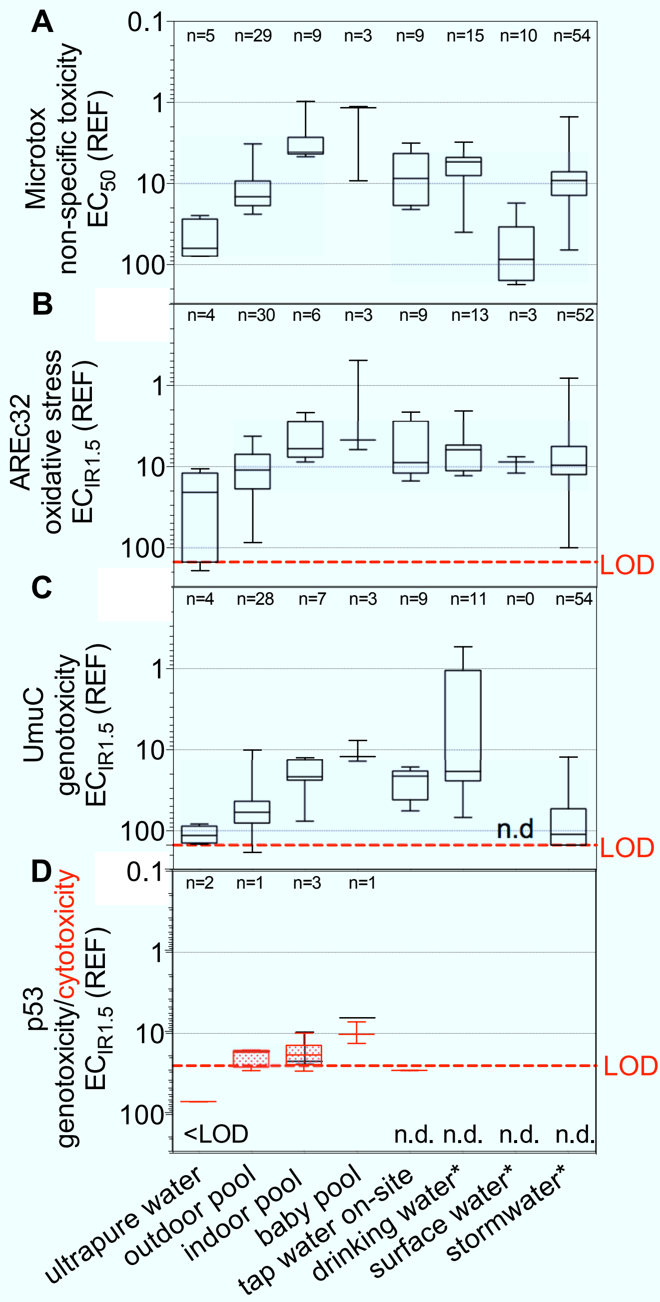
A
B
C
D
Microtox
non-specific toxicity
EC50 (REF)
AREc32
oxidative stress
ECIR1.5 (REF)
UmuC
genotoxicity
ECIR1.5 (REF)
p53
genotoxicity/cytotoxicity
ECIR1.5 (REF)
0.1
1
10
100
n=5 n=29 n=9 n=3 n=9 n=15 n=10 n=54
n=4 n=30 n=6 n=3 n=9 n=13 n=3 n=52
n=4 n=28 n=7 n=3 n=9 n=11 n=0 n=54
n=2 n=1 n=3 n=1
LOD
n.d
<LOD
n.d. n.d. n.d. n.d.
ultrapure water
outdoor pool
indoor pool
baby pool
tap water on-site
drinking water*
surface water*
stormwater*

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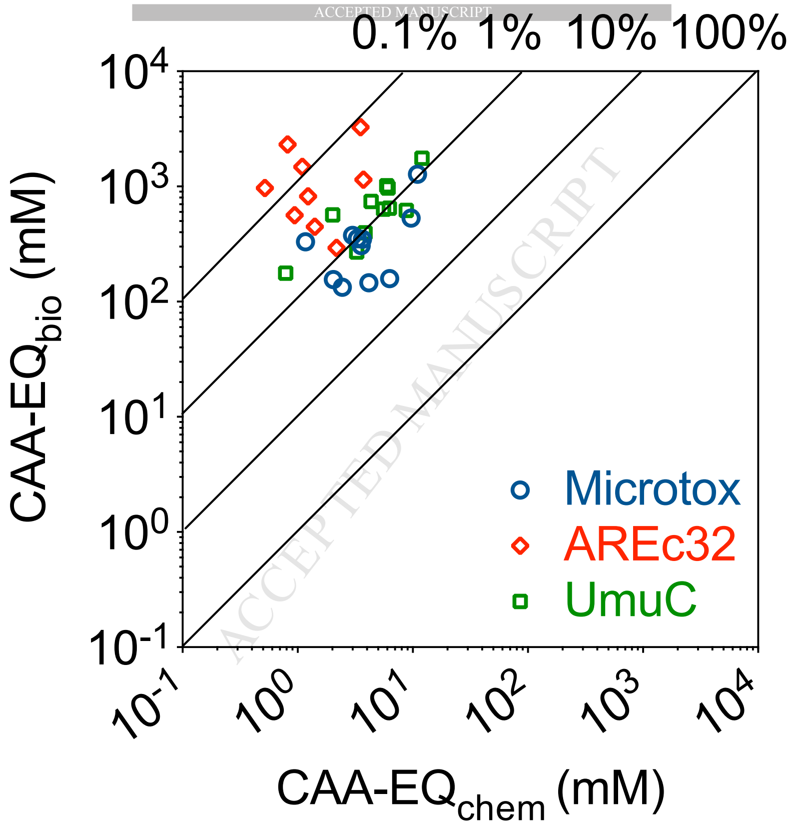

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Highlights

- Combination of DBP quantification and bioassays in swimming pool water
- Increasing load of total nitrogen related to swimmers' activity
- 35 to 118 % of total organic halogens were explained by haloacetic acids
- Haloacetic acids serve as good indicators for chemical pool water quality
- Nevertheless, haloacetic acids accounted for less than 4 % of biological effect

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Appendix A. Supplementary Data

Bioanalytical and Chemical Evaluation of Disinfection By-Products in Swimming Pool Water

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Table S1. Regulated DBP values of Australia, USA, Europe in comparison to WHO recommendations (Table adapted from Farré et al (2012)). HAA5 refers to the sum of the five HAA listed on the left.

DBPs	WHO (µg/L)	ADWG (µg/L)	U.S. EPA (µg/L)	EU (µg/L)
HAA: Haloacetic Acids				
DBAA: Dibromoacetic acid	-	-	60 as HAA5	-
BAA: Monobromoacetic acid	-	-		-
CAA: Monochloroacetic acid	20	150		-
TCAA: Trichloroacetic acid	-	100		-
DCAA: Dichloroacetic acid	50	100		-
THM: Trihalomethanes				
BDCM: Bromodichloromethane	60	250 as total THM	80 as total THM	100 as total THM
TBM: Tribromomethane (bromoform)	100			
DBCM: Dibromochloromethane	100			
TCM: Trichloromethane (chloroform)	300			
Other Regulated DBPs				
Bromate	10	20	10	10
Chlorite	700	800	1000	-
DCAN: Dichloroacetonitrile	20	-	-	-
DBAN: Dibromoacetonitrile	70	-	-	-
NDMA: N-Nitrosodimethylamine	0.1	0.1	-	-
CH: Chloral hydrate	10	20	-	-
Chlorate	700	-	-	-
Cyanogen chloride (Cyanide)	70	80	-	-

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Section S1. Additional information on materials and methods

Chemicals

DBP class	DBP	Abbreviation
Trihalomethanes (THM)	Bromodichloromethane	BDCM
	Dibromochloromethane	DBCM
	Tribromomethane (bromoform)	TBM
	Trichloromethane (chloroform)	TCM
Iodo-trihalomethanes (I-THM)	Bromochloroiodomethane	BCIM
	Bromodiiodomethane	BDIM
	Chlorodiiodomethane	CDIM
	Dibromoiodomethane	DBIM
	Dichloroiodomethane	DCIM
	Triiodomethane (iodoform)	TIM
Halonitromethanes (HNM)	Trichloronitromethane	TCNM
	Tribromonitromethane	TBNM
Haloketones (HK)	1,1-dichloropropanone	1,1-DCP
	1,1,1-trichloropropanone	1,1,1-TCP
Haloacetonitriles (HAN)	Bromochloroacetonitrile	BCAN
	Dibromoacetonitrile	DBAN
	Dichloroacetonitrile	DCAN
	Trichloroacetonitrile	TCAN
Haloacetamides (HACAm)	Bromochloroacetamide	BCAM
	Bromodichloroacetamide	BDCAM
	Bromoiodoacetamide	BIAM
	Chloroiodoacetamide	CIAM
	Dibromoacetamide	DBAM
	Dichloroacetamide	DCAM
	Dibromochloroacetamide	DBCAM
	Diiodoacetamide	DIAM
	Tribromoacetamide	TBAM
	Trichloroacetamide	TCAM
Chloral hydrate (CH)	Chloral hydrate	CH
Haloacetic acids (HAA)	Bromoacetic acid	BAA
	Chloroacetic acid	CAA
	Bromodichloroacetic acid	BDCAA
	Dibromoacetic acid	DBAA
	Bromochloroacetic acid	BCAA
	Dichloroacetic acid	DCAA
	Trichloroacetic acid	TCAA
	Dibromochloroacetic acid	DBCAA

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DBP class	DBP	Abbreviation
	Tribromoacetic acid	TBAA
	Iodoacetic acid*	IAA
	Bromoiodoacetic acid*	BIAA
	Chloroiodoacetic acid*	CIAA

*only applied to bioanalytical assessment.

EPA 501/601 THMs Calibration Mix (TCM, DBCM, BDCM and TBM at 100 µg/mL in methanol), EPA 551B Halogenated Volatiles Mix (BCAN, DBAN, DCAN, 1,1-DCP, 1,1,1-TCP, TCAN and TCNM at 2000 µg/mL in acetone); TIM, DCAM and TCAM and CH were obtained from Sigma-Aldrich (Castle Hill, Australia). TBNM and remaining HACAm standards including BCAM, BDCAM, DBAM, CIAM, DBCAM, BIAM, DIAM, TBAM and I-THMs, including DCIM, BCIM, DBIM, CDIM and BDIM were purchased at the highest level of purity from Orchid Cellmark (New Westminster, BC, Canada). HAAs including BAA, CAA, BDAA, TBAA, DBAA, BCAA, DCAA, TCAA and DBCAA were purchased from Sigma-Aldrich, Australia. IAA (Sigma-Aldrich, Australia), BIAA and CIAA (Orchid Cellmark) were also purchased for bioanalytical assessment, but were not included in chemical analysis.

L-ascorbic acid, ACS reagent ≥99% (Sigma–Aldrich, St Louis, MO) was used to quench the measured residual disinfectants at twice the required stoichiometric concentration during the analysis of THMs, I-THMs, HNM, HK, HAN, HACAms and CH. For this analysis 1,2-dibromopropane (97% Sigma-Aldrich) was also used as internal standard. Analytical grade 99.9% methyl tertiary butyl ether (MtBE) was purchased from Sigma-Aldrich (Castle Hill, Australia) and used as extraction solvent. Sodium sulphate anhydrous (10–60 mesh) was purchased from Mallinckrodt chemicals (Phillipsburg, USA). Ammonium chloride (NH_4Cl; Sigma-Aldrich, Castle Hill, Australia, ACS reagent 99.9% purity) was used as quenching agent for HAA analysis. Sodium thiosulphate ($N_2S_2O_3$; Sigma-Aldrich, Castle Hill, Australia, ACS reagent 99%) was used to quench the residual disinfectant prior to extraction for bioanalytical assessment as well as AOX analysis. Commercial N,N diethyl-1,4 phenylenediamine sulphate (DPD) test kits (Hach, Notting Hill, Australia) were used for the analysis of free and total chlorine.

Pool water sample

For precursor analysis, total organic carbon (TOC) and dissolved organic nitrogen (DON), samples were collected in 40 mL standard volatile organic analysis (VOA) vials. Samples for adsorbable organic halide (AOX) analysis were taken in 125 mL MilliQ-washed HDPE plastic bottles and frozen until analysis (−20°C). For analysis of volatile DBPs samples were collected in acid-washed 60 mL VOA vials headspace-free, quenched with ascorbic acid solution (2.48 mg/L per 1 mg/L free chlorine), transported at 4°C and analysed within 24 h.

Samples O14 and D2 to D10 were collected headspace-free in acid washed 40 mL glass vials quenched with ascorbic acid, transported on ice and sent to Advanced Water

Management Centre (AWMC) laboratory at The University of Queensland (UQ) for analysis of volatile DBP analysis within 24 h.

For HAA analysis, water was sampled in solvent-washed 200 mL amber glass bottles containing 0.2 g NH_4Cl (99.9%) as quenching agent to remove disinfectant residual and stop DBP formation (U.S. EPA 2003, Hong et al. 2008). HAA samples were analysed within 2 weeks after sampling (stored at 4°C) except sample S8, S10, S14 (within 7 weeks, stored at −20°C). Selected pool water samples were also collected in solvent-washed 200 mL amber glass bottles containing 0.2 g NH_4Cl (99.9%) as quenching agent and sent to Queensland Health Forensic Scientific Services (QHFSS) laboratory for HAA analysis.

For bioanalytical assessment, 2 to 4 L of pool water was sampled in 1 L solvent-washed amber glass bottles and quenched with sodium thiosulphate ($Na_2S_2O_3$) after free chlorine reading using Pocket Colorimeter™ II, Chlorine (Free and Total; Hach, USA) to determine the amount of $Na_2S_2O_3$ to use.

Quantification of bromide

Bromide was quantified using a Dionex ICS-2100 Ion Chromatograph (Thermo Fisher Scientific, Australia). 1000 µL of the sample was injected and detected by a conductivity detector. A Dionex IonPac AS11-HC column (with IonPac AG11-HC guard column) was used with eluent generated using a Dionex Eluent Generator Cartridge III with 30 – 75 mM KOH and a flow rate of 1 mL min^{-1}.

Quantification of Adsorbable Organic Halides (AOX)

The samples for AOX analysis were quenched using sodium sulfite (≥98% purity; Sigma-Aldrich, Castle Hill Australia) acidified to pH 2 using HNO_3 (99.9% purity; Sigma-Aldrich, Castle Hill, Australia) and then enriched on two consecutive activated carbon cartridges (40 mg activated carbon per glass column with 2 mm inner diameter; CPI International, California, USA) using the Mitsubishi TX-3AA Adsorption Module. The cartridges were then washed with 10 mL of 5 g/L nitrate (NO_3^-) as potassium nitrate (KNO_3; ≥99% purity; Sigma-Aldrich, Castle Hill, Australia) and 2 mL MilliQ water to remove inorganic halides.

Activated carbon was transferred for combustion inside a Mitsubishi AQF-2100 Automated Quick Furnace unit in the presence of oxygen for 260 seconds at 1000°C. The gases from the pyrolysis process containing the hydrogen halides and halogens were collected in a Mitsubishi GA-210 absorption unit and absorbed in 10 mL of the absorption solution (ultrapure water with 0.003% hydrogen peroxide) for reducing halogen gases to halide ions. The absorption solution also contained phosphate (1 mg/L) as internal standard to take into account volume variations of absorption solution injected into the IC by the absorption module. Subsequently, 1000 µL of the absorption solution was injected into a Dionex ICS-2100 Ion Chromatograph (Thermo Fisher Scientific, Australia) to quantify the concentrations of chloride (Cl^-), bromide (Br^-) and iodide (I^-) by a conductivity detector. A Dionex IonPac AS11-HC column (with IonPac AG11-HC guard column) was used with eluent generated using a Dionex Eluent Generator Cartridge III with 30–75 mM KOH and a flow rate of 1 mL min^{-1}.

Quantification of volatile DBPs

For the analysis of volatile DBPs with gas chromatography-electron capture detection (GC-ECD) the sample was liquid–liquid extracted within 24 h of sampling. 30 mL aliquots were extracted in duplicates by first adjusting the pH to 3.5 using 0.2 M sulphuric acid. Subsequently, the sample was extracted by adding 3 mL methyl-*t*-butyether (MtBE) containing 200 µg/L of 1,2-dibromopropane as an internal standard and 10 g of pre-baked sodium sulfate (at 500°C). Samples were vortexed for 1 min. After settling for 5 min, 1.5 mL of the MtBE layer was transferred to two GC vials for double injection.

Analysis was performed on an Agilent 7890A GC-ECD at 300ºC. 2 µL sample volume were injected by parallel double-pulsed splitless injection at 140ºC. Chromatographic separations on the GC-ECD were performed using an Agilent DB-5 column for quantification (30 m x 0.25 mm i.d., 1.0 µm film thickness), and an Agilent DB-1 column for confirmation (30 m x 0.25 mm i.d., 1.0 µm film thickness). The oven temperature program was used as follows: 35°C for 25 min, ramped to 100°C at 2°C/min and held for 2 min, then ramped to 200ºC at 5°C/min, and the final ramp reaches 280°C at 50°C/min leading to a total run time of 81.1 minutes.

Precision was measured using relative percent difference (RPD) of the duplicate analyses of each sample. RPD was less than 10% for all duplicates. The coefficient of variation of all the internal standard responses for the complete set of samples was less than 15%. A calibration curve was prepared before extraction ranging from 0.1 to 500 µg/L. Recovery ranged between 80-120%. The detection limit was 0.1 µg/L for all DBPs except the HACAms, which was 0.5 µg/L.

Quantification of HAAs

The U.S. EPA Method 552.3 (U.S. EPA 2003) includes nine HAAs (bromoacetic acid (BAA), chloroacetic acid (CAA), bromodichloroacetic acid (BDCAA), tribromoacetic acid (TBAA), dibromoacetic acid (DBAA), bromochloroacetic acid (BCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA) and dibromochloroacetic acid (DBCAA)). TBAA was not included in the analysis of samples older than two weeks because it is the least stable HAA ester and degrades to TBM (U.S. EPA 2003). Samples were adjusted to pH 0.5 and extracted by liquid–liquid extraction using MtBE followed by derivatization (methylation) via acidic methanol. Analysis of the DBPs was carried out with gas chromatography using parallel double-pulsed splitless injection at 140ºC followed by electron capture detection (an Agilent 7890A GC-ECD) at 290ºC. Chromatographic separations on the GC-ECD were performed using a DB-5 column (30 m x 0.25 mm i.d., 1.0 µm film thickness, Agilent), while a DB-1 column (30 m x 0.25 mm i.d., 1.0 µm film thickness, Agilent) was used for confirmation. Reported are averages between both results. The oven temperature program was used as follows: 35°C for 25 min, ramp to 145°C at 5°C/min and hold for 2 min and then ramp to 260ºC at 20°C/min and hold for 10 min.

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Table S2. Information of pool properties obtained from pool managers on-site. The swimming pools are not completely emptied and refilled on a regular basis but undergo system circulation as well as constant replenishment with tap water. The amount of tap water for replenishment is unknown in the pools investigated but common practise is to replenish whenever there is loss due to evaporation.

ID	Pool Type	Size	Disinfection Method	Target pH (measured frequency per day)	Filtration System	UV Treatment	Chlorine Stabiliser	Temp (^0C)	Free chlorine residual (mg/L as Cl_2) Mean ± SD (n)	Last complete water exchange	Regular replenishment	Visitors per day
1	Outdoor	50 m	Chlorination, no addition of cyanuric acid	7.5 (4)	Sand	No	No	Ambient	2.0±1.2 (22)	Sep 2012	100 - 130 kL per week	500 - 1000
1	Baby Outdoor	Small wading	Chlorination	7.5 (4)	Sand	No	No	Ambient	4.8 (1)		Combined with above	
2	Outdoor	50 m	Chlorination	7.5 – 7.6 (4)	Diatomaceous Earth	No	Yes	28°C	0.8±0.8 (2)			500 - 800
2	Indoor	20 m	Chlorination	7.5 – 7.6 (4)	Sand	No	Yes	32°C	3.1±1.2 (2)			100 - 500
3	Indoor	25 m	Sodium Hypochlorite	7.3 – 7.5 (4)	Diatomaceous Earth	Yes	No	32°C	3.8±0.1 (2)	2009		
4	Outdoor	50 m	Sodium Hypochlorite	7.5 – 7.8 (4)	Perlite	No	No	27°C	0.08 (1)	Feb 2012		300 - 1000
5	Covered Outdoor	25 m	Sodium Hypochlorite	7.2 – 7.8 (5)	Sand	No	No	Ambient	5.0±4.1 (2)	2013		
5	Baby	12 m	Salt-	7.2 – 7.8	Diatomaceou	No	No	32°C	1.0±0.2	2013		

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	Indoor		Chlorination [a]	(5)	s Earth				(2)			
6	Outdoor	25 m	Salt-Chlorination [a]	7.2 – 7.6 (5)	Diatomaceous Earth	No	No	27°C	2.6 (1)	2008		100 - 200
6	Indoor	25 m	Chlorination	7.2 – 7.6 (5)	Diatomaceous Earth	No	No	32°C	0.9 (1)	2008		300 - 500
7	Outdoor	25 m	Salt-Chlorination [a]	7.2 – 7.8 (5)	Diatomaceous Earth	No	Yes	27.5°C	5.3 (1)	2009		600 - 1400
7	Indoor	14 m	Chlorination	7.2 – 7.8 (5)	Diatomaceous Earth	Yes	No	33°C	3.4 (1)	2009	Combined with above	

[a]Salt-chlorination refers to a process where chlorine is formed in situ in water supplemented with NaCl by electrolysis.

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Table S3. Sample codes and information on the sampling date and time.

Sample ID	Site ID	Sample Type	Sample Date
First sampling campaign – temporal trends in one pool over 6 months			
O1	Pool 1	Onsite tap water	28 Sep 2012
O2	Pool 1	Outdoor pool	28 Sep 2012
O3	Pool 1	Outdoor pool	11 Oct 2012
O4	Pool 1	Outdoor pool	25 Oct 2012
O5	Pool 1	Outdoor pool	01 Nov 2012
O6	Pool 1	Outdoor pool	22 Nov 2012
O7	Pool 1	Outdoor pool	05 Dec 2012
O8	Pool 1	Outdoor pool	20 Dec 2012
O9	Pool 1	Outdoor pool	07 Jan 2013
O10	Pool 1	Outdoor pool	14 Jan 2013
O11	Pool 1	Outdoor pool	24 Jan 2013
O12	Pool 1	Outdoor pool	31 Jan 2013
O13	N/A	Ultrapure water	31 Jan 2013
O14	Pool 1	Outdoor pool	07 Feb 2013
O15	Pool 1	Baby outdoor pool	07 Feb 2013
O16	Pool 1	Outdoor pool (at outlet of pool)	07 Feb 2013
O17	Pool 1	Onsite tap water	07 Feb 2013
Second sampling campaign – day course on an autumn day			
D1	Pool 1	Onsite tap	04 Apr 2013, 7:30 am
D2	Pool 1	Outdoor pool	04 Apr 2013, 6:15 am
D3	Pool 1	Outdoor pool	04 Apr 2013, 7:55 am
D4	Pool 1	Outdoor pool	04 Apr 2013, 9:15 am
D5	Pool 1	Outdoor pool	04 Apr 2013, 10:45 am
D6	Pool 1	Outdoor pool	04 Apr 2013, 12:15 pm
D7	Pool 1	Outdoor pool	04 Apr 2013, 1:45 pm
D8	Pool 1	Outdoor pool	04 Apr 2013, 3:15 pm
D9	Pool 1	Outdoor pool	04 Apr 2013, 4:45 pm
D10	Pool 1	Outdoor pool	04 Apr 2013, 6:00 pm
Third sampling campaign – samples from various swimming pools taken from 01 May 2013 to 18 July 2013 between 8 am to 9 am			
S1	Pool 4	Onsite tap water	01 May 2013
S2	Pool 4	Outdoor pool	01 May 2013
S3	Pool 2	Onsite tap water	01 May 2013
S4	Pool 2	Outdoor pool	01 May 2013
S5	Pool 2	Indoor pool	01 May 2013
S6	Pool 5	Onsite tap water	06 Jun 2013

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S7	Pool 5	Covered outdoor pool	06 Jun 2013
S8	Pool 5	Covered outdoor pool (repeat)	06 Jun 2013
S9	Pool 5	Baby indoor pool	06 Jun 2013
S10	Pool 5	Baby indoor pool (repeat)	06 Jun 2013
S11	Blank	Ultrapure water	06 Jun 2013
S12	Pool 3	Onsite tap water	13 Jun 2013
S13	Pool 3	Indoor pool	13 Jun 2013
S14	Pool 3	Indoor pool (repeat)	13 Jun 2013
S15	Blank	Ultrapure water	13 Jun 2013
S16	Pool 7	Onsite tap water	16 Jul 2013
S17	Pool 7	Outdoor pool	16 Jul 2013
S18	Pool 7	Indoor pool	16 Jul 2013
S19	Pool 6	Onsite tap water	16 Jul 2013
S20	Pool 6	Outdoor pool	16 Jul 2013
S21	Pool 6	Indoor pool	16 Jul 2013
S22	Pool 5	Covered outdoor pool	18 Jul 2013
S23	Pool 5	Baby indoor pool	18 Jul 2013
S24	Pool 2	Outdoor pool	18 Jul 2013
S25	Pool 2	Indoor pool	18 Jul 2013
S26	Blank	Ultrapure water	18 Jul 2013

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Section S2. Additional information on the sample preparation

Sample preparation for bioassays

Samples were enriched with solid phase extraction in preparation for the bioassays. After quenching the chlorine at the pools, the samples were acidified in the laboratory to pH 3 (sample series O) or 1 (sample series D and S) by using concentrated H_2SO_4 (95-98%, ACS grade; Sigma-Aldrich, Castel Hill, Australia). 1 g Oasis HLB in 20 mL cartridges (Oasis HLB: Hydrophilic-lipophilic-balanced reversed-phase sorbent for acids, bases and neutrals, Waters, Milford, MA, USA) were used for solid phase extraction (SPE) of 2 L water samples. The cartridges were conditioned with 10 mL MtBE, followed by 20 mL methanol and 10 mL pH 1 MilliQ water before running the water samples through (ca. 10 mL per minute). DBP compounds were retained in the stationary phase Oasis HLB. The cartridges were then dried with nitrogen gas then eluted from the cartridge with 20 mL methanol and 10 mL MtBE under gravity. The eluates were evaporated to low volume under nitrogen gas and the solvent was exchanged into methanol to yield an enrichment factor of the SPE of 8000 to 10000 in the final extract.

Relative enrichment factor

For each sample, the enrichment factor of the SPE was calculated using equation S1, which represents the ratio of the volume of the water sample to the volume of the SPE extract.

$$\text{enrichment factor}_{SPE} = \frac{V_{sample}}{V_{extract}} \qquad \text{(S1)}$$

For testing samples in the different bioassays, a portion of the enriched sample extracts was added to the microtiter plate and serially diluted by test medium to obtain a concentration-effect curve. Equation S2 was used to calculate the dilution factor in each well of the microtiter plate.

$$\text{dilution factor}_{bioassay} = \frac{\text{volume extract added to bioassay}}{V_{bioassay}} \qquad \text{(S2)}$$

The final relative enrichment factor REF was calculated with equation S3, as the product of the enrichment factor and the dilution factor in the bioassay. The REF represents the enrichment or dilution of the original sample in each bioassay.

$$REF = \text{enrichment factor}_{SPE} \cdot \text{dilution factor}_{bioassay} \qquad \text{(S3)}$$

Recovery of AOX and biological effects after different SPE methods

Prior to evaluating the recovery of AOX after SPE, the recovery of the AOX analyser was tested. The typically used method of washing with KNO_3 incurred substantial loss of recovery, presumably due to the dominance of HAA, which could be improved by substituting KNO_3 with HNO_3 (Table S4). All SPE recovery experiments were performed with the KNO_3 washing method but the absolute recovery of the AOX analyzer cancels out because samples were measured before and after SPE.

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Table S4. Recovery of AOX analysis.

	CAA	DCAA	TCAA	BAA	IAA
No wash	110 %	112 %	102 %	105 %	100 %
Wash with KNO_3	36 %	61 %	104 %	39 %	99 %
Wash with HNO_3	98 %	116 %	107 %	87 %	106 %

Various solid phases were evaluated for their efficacy of extraction by evaluating AOX recovery and compatibility with bioassays. The tested phases included HLB at pH 1 and 3 (1 g sorbent per 20 mL cartridge, 60 µm particle size; Waters, Australia), XAD2/8 (3.125 mL of each sorbent per 20 mL cartridge; sorbents prepared as described by Plewa et al. (2012), a mixed cartridge containing 2 g coconut charcoal (CC, Supelclean; Sigma Aldrich, Australia) and 1 g HLB (Sigma-Aldrich, Australia), Isolute ENV+ (highly cross linked polystyrene based polymer, 1 g per 20 mL cartridge, John Morris, Australia), Lichrolut EN (40 - 120 µm, 0.5 g per 6 ml cartridge, Merck Millipore, Australia), WAX (0.5 g per 6 mL cartridge, 60 µm particle size, Oasis WAX: Mixed-mode weak anion exchange sorbent for strong acids, Waters) and MAX (0.5 g per 6 mL cartridge, 60 µm particle size, Oasis MAX: Mixed-mode anion exchange sorbent for acids, Waters, Australia). Conditioning, enrichment and elution with HLB pH 3, CC, Lichrolut, ENV+ were performed as described for HLB pH 1 above. The sample to sorbent and sorbent to solvent ratio was kept constant for conditioning, enrichment and elution (i.e., 0.5 g of sorbent per litre of sample). XAD2/8 were conditioned and eluted as described by Plewa et al. (2012). Briefly, the XAD resins where first consecutively washed with 0.1 N NaOH, ultrapure water and methanol. Next, three consecutive 24-h Soxhlet extractions were applied: 1st with methanol, 2nd with ethyl acetate and 3rd with methanol. After purification, resins were stored in methanol until the methanol was replaced by ultrapure water prior to use. 20 mL polypropylene SPE tubes (Sigma Aldrich, Australia) were packed with 3.125 mL XAD-2 resin followed by 3.125 mL XAD-8 resin (Sigma-Aldrich, Australia) separated by polyethylene frits (20 µm porosity; Sigma-Aldrich, Australia). These sorbent volumes were chosen to meet the sample water to resin ratio applied in Plewa et al. (2012). The cartridges were conditioned with three resin volumes of ultrapure water, two resin volumes of 0.1 N HCl, one resin volume of 0.1 N NaOH and one resin volume of ultrapure water (pH 1). After extraction of 2 L sample water (pH 1), the cartridges were washed with 40 mL of ethyl acetate (flow rate ≤ 2 mL/min), which was collected in a separatory funnel to allow discarding the aqueous bottom layer and next dried with sodium sulphate (Sigma-Aldrich, Australia). The extract was stored at −80°C until the next day when it was blown down to dryness under a gentle stream of nitrogen and the extract redissolved in methanol. SPE using WAX was applied according to Taniyasu et al. (2005) and modified after Bernad et al. (2011). WAX and MAX cartridges were conditioned with 10 mL methanol, 10 mL 0.1 % NH_4OH in methanol (WAX) or 0.35 % HCOOH in methanol (MAX) and 10 mL of ultrapure water. For elution the same organic solvents were used as for conditioning.

Recovery of adsorbable organic chloride (AOCl) and adsorbable organic bromide (AOBr) in a sample of pool 1 was increased from 10% to 51 % and 4 % to 122 % by decreasing the pH of the SPE with HLB from 3 to 1 (Table S5, Figure S1). This observation can be rationalised by the acidity constants for HAAs, which range from 0.6 to 3.0 (Table S6). It can be assumed that only the neutral form of the HAA sorbs well to the SPE material and therefore decreasing the pH increases the fraction of neutral species of HAAs and thus their retention on the solid phase.

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Table S5. Recovery of AOX in pool water samples (pool 1) extracted with various solid phase extraction methods.

Solid phase	pH	AOCl (nM)	AOBr (nM)	AOI (nM)	AOCl % recovery	AOBr % recovery
HLB	3	1206±27	1.0±0.1	3.9	10	4
HLB	1	6044±2341	32.3±0.1	<4	51	122
XAD2/8	1	4000±508	14.1±0.2	<4	34	53
CC-HLB	1	8496±2386	12.1±0.6	<4	72	46
ENV+	1	14430±131	35.9±8.6	<4	122	136
Lichrolut	1	14739±473	13.0±0.1	<4	125	49
WAX	1	14307±2424	6.4±5.7	4.3	242	61
MAX	1	11703±2667	<1	<4	198	

Further SPE experiments were therefore performed only at pH 1. The XAD2/8 resin, which is favoured in many studies (Park et al. 2000, Claxton et al. 2008), did not perform well with only 34 % and 54 % recovery of AOCl and AOBr, respectively. The two-bed cartridge of CC and HLB had better recoveries but also very high blank toxicity in the Microtox assay (Figure S1), presumably due to leaching of residues on the charcoal that are not necessarily AOX but toxic organics.

WAX and MAX had over 200 % recovery, which must be an experimental artefact that could not be explained given that the ion exchanger should not contain any halogen functional groups. Of the three polymer-based neutral phases (Oasis HLB, Isolute ENV+ and Lichrolut) recovery was excellent for AOCl with ENV+ and Lichrolut and for AOBr with HLB and ENV+, while the others only gave around 50% recovery. However, one must also account for the background toxicity in the final decision for a solid phase. All MilliQ blanks had an EC_{50} around or over 100, which is considered acceptable. The ratio between the EC_{50} of the blank and that of the pool water gives an indication on how much toxic chemicals can be captured in the pool water matrix and what is the window of detection. This ratio was 9 for Oasis HLB, 4 for Isolute ENV+ and 13 for Lichrolut. Thus differences between the three polymers appear not to be large and not systematic. We opted for HLB pH 1 as the first batch of pool water samples had already been extracted with HLB pH 3 but Isolute ENV+ and Lichrolut appear equally suitable.

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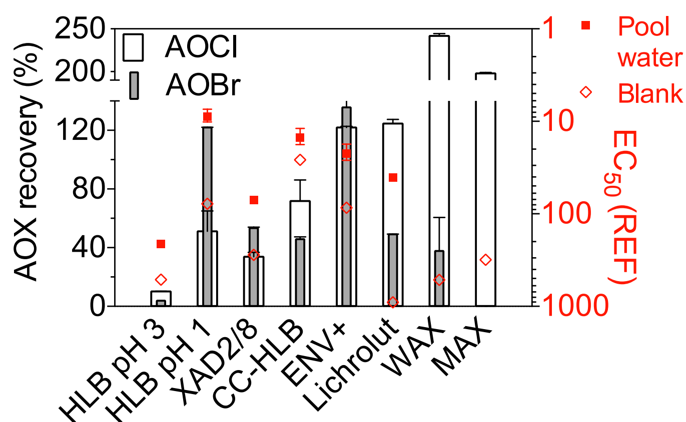

Figure S1. Percentage of AOX recovery with various SPE methods and EC_{50} values for pool water and blank (MilliQ water) in the Microtox assay. The native pool water sample contained 420 μg/L Cl^- as AOCl, 2.1 μg/L Br^- as AOBr and 0.8 μg/L I^- as AOI.

Table S6. Physicochemical properties of the haloacetic acids.

Haloacetic acids		Octanol-water partition coefficient $logK_{ow}$	Acidity constant pK_a [c]
BAA	Bromoacetic acid	0.41[a]	
BCAA	Bromochloroacetic acid	0.61[b]	1.4
CAA	Chloroacetic acid	0.22 [a]	2.97
DBAA	Dibromoacetic acid	0.7	1.39
DCAA	Dichloroacetic Acid	0.92 [a]	1.41
TCAA	Trichloroacetic acid	1.33 [a]	0.66
IAA	Iodoacetic acid	0.85 [a]	2.95

[a]$logK_{ow}$ values taken from PhysProp (http://www.epa.gov/oppt/exposure/pubs/episuitedl.htm, also accessible via http://www.syrres.com); [b]$logK_{ow}$ values taken from KOWWIN™ v. 1.67 (U.S.EPA 2008), [c]acidity constants estimated with SPARC (Hilal et al. 2005).

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Section S3. Additional information on bioassays

CellSensor p53RE-*bla* HCT-116 assay for genotoxicity

A disadvantage of bacteria-based methods to assess genotoxicity (e.g., Ames assay or umuC assay) is that extrapolation of data from bacteria to mammalian cells is difficult but necessary to interpret the data with regard to human health relevance. Therefore, we additionally used the p53 induction as marker for genotoxicity in a mammalian cell line. The p53 adaptive stress response pathway is involved in cellular processes such as DNA repair, apoptosis and cell cycle arrest and can serve as indicator for exposure to genotoxic compounds (Duerksen-Hughes et al. 1999).

The commercially available CellSensor™ p53RE-bla HCT-116 cell-based assay developed by Invitrogen was used in this project to measure the p53 activation. The HCT-116 epithelial human colon carcinoma cells are stably transfected with a β-lactamase reporter gene controlled by p53 making it possible to detect agonists and antagonists of the p53 pathway. This assay applies the LiveBLAzer FRET-B/G CCF4-AM (Fluorescence Resonance Energy Transfer) substrate, which is fluorescent at different wavelengths (460 and 530 nm), for quantification of the β-lactamase. The esterified and lipophilic CCF4-AM dye is able to pass the cell membrane and enter the cell, after cleavage by cellular esterases, however, the substrate is retained in the cell. This green fluorescent charged CCF4 form undergoes hydrolysis of its lactam ring catalyzed by β-lactamase leading to the blue fluorescent product (Hallis et al. 2007). Advantages of this assay include the lack of background β-lactamase activity in mammalian cells and the use of a ratio dye (Hallis et al. 2007).

Experiments were performed by simultaneously measuring the p53 activity as well as the cellular mitochondrial activity by means of the resazurin conversion using 50 μM final resazurin concentration. Mitochondrial activity is a reliable indicator of cell viability required for interpretation of the p53 data. Chemical reduction of resazurin to resorufin occurs in cells with active mitochondrial metabolism.

Cells were plated in black-coated clear-bottom 384-well Falcon BD plates and were allowed to adhere to the wells for >6 hours. Dosing was performed by adding 8 μL of assay medium containing 2.5% solvent and test compounds to the cells (32 μL assay medium) leading to a final solvent concentration of 0.5%. Plates were sealed with Breathe-Easy sealing membrane (Sigma-Aldrich, Australia) and incubated for 48 h at 37°C and 5% CO_2 after dosing. Afterwards, 8 μL of the LiveBLAzer mix and resazurin solution was added and incubated for 2.5 h at room temperature before measurements.

Fluorescent measurements were performed with the FLUOstar Omega plate reader (BMG Labtech) and MARS version 2.10 software. The fluorescent excitation resulting from resazurin exposure for 2.5 h was measured at 590 nm after emission of 544 nm. CCF4 was excited at 405 nm and emission was measured at 460 (blue channel) and 530 nm (green channel). These wavelengths were chosen based on recommendations by Invitrogen (2010). The fluorescence data were corrected by the average of 8 cell free blank wells (assay medium without cells). The blue/green emission ratio indicated the p53 induction and the

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induction ratio was calculated by dividing the blue/green emission ratio of a sample by the average emission ratio of the solvent control.

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Section S4. Additional results

Table S7. Sample details and DBP precursor analysis (TN, TOC, TON, Br^-) of first campaign (Pool 1 time-series study over a period of 6 months) including on-site tap water and MilliQ water collected for this study.

Sample ID	TN (mg/L)	TON (mg/L)	TOC (mg/L)	Br^- (µg/L)
O1	0.64	0.16	3.53	270
O2	0.61	0.09	3.14	<4.5
O3	0.96	0.22	3.22	<4.5
O4	1.61	0.43	3.41	<4.5
O5	1.73	0.28	3.55	<4.5
O6	2.62	0.65	3.23	<4.5
O7	3.13	0.55	3.11	<4.5
O8	3.61	0.24	3.22	<4.5
O9	3.92	0.31	3.52	<4.5
O10	4.10	0.27	3.60	<4.5
O11	4.61	0.34	3.74	<4.5
O12	3.90	0.27	3.32	<4.5
O13	0.12	0	0.06	<4.5
O14	4.42	0.63	3.87	<4.5
O15	4.37	0.56	3.87	<4.5
O16	4.40	0.55	3.87	<4.5
O17	0.95	0.15	3.55	<4.5

Table S8. Summary of AOX analysis.

Sample ID	AOCl (µM)	AOBr (µM)	AOI (µM)	AOX (µM)
O1	2.71	0.74	0.026	3.47
O2	7.87	0.40	<0.004	8.27
O3	7.18	0.25	<0.004	7.43
O4	9.08	0.15	<0.004	9.24
O5	8.00	0.12	<0.004	8.12
O6	9.18	0.07	<0.004	9.25
O7	5.43	0.03	<0.004	5.46
O8	15.13	0.10	0.005	15.24
O9	5.86	0.02	<0.004	5.88
O10	9.86	0.05	<0.004	9.91
O11	11.35	0.04	<0.004	11.39
O12	12.12	0.05	0.005	12.17

O13	1.64	<0.001	<0.004	1.64
O14	10.13	0.04	<0.004	10.17
O15	8.92	0.02	<0.004	8.95
O16	12.89	0.05	0.004	12.95
O17	3.04	0.66	0.016	3.71
D1	1.36	0.21	<0.004	1.58
D2	5.84	0.013	<0.004	5.86
D3	5.59	0.013	<0.004	5.60
D4	2.33	0.010	<0.004	2.34
D5	4.33	0.009	<0.004	4.35
D6	3.13	0.006	<0.004	3.14
D7	2.60	0.007	<0.004	2.61
D8	3.27	0.006	<0.004	3.28
D9	4.86	0.013	<0.004	4.87
D10	3.27	0.008	<0.004	3.28
S6	1.9	0.23	0.02	2.2
S7/S8	27.5	0.20	0.00	27.7
S9/S10	79.7	0.86	0.00	80.6
S11	-0.2	0.00	<0.004	-0.2
S12	7.0	1.03	0.01	8.1
S13/S14	10.8	0.14	0.02	11.0
S15	0.2	<0.001	<0.004	0.2
S16	6.4	0.89	0.01	7.3
S17	43.0	0.15	0.01	43.1
S18	65.3	0.19	0.02	65.5
S19	4.3	0.54	0.04	4.9
S20	29.3	0.10	0.02	29.4
S21	45.1	0.10	0.02	45.3
S22	31.5	0.06	0.01	31.5
S23	82.0	1.34	0.01	83.3
S24	29.1	0.34	<0.004	29.4
S25	46.9	0.30	0.04	47.2

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Table S9. Summary of volatile DBP analysis results for Pool 1 outdoor (sample D2 to D10). Values are reported in unit μg/L. The limit of detection was 0.1 μg/L.

Sample ID	D2	D3	D4	D5	D6	D7	D8	D9	D10
Volatile DBPs detected (μg/L)									
TCM	64.5	68.3	71.0	73.0	76.9	82.0	82.9	82.7	83.7
BDCM	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.5	2.5
DBCM	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3
TBM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DCIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
BCIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DBIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
CDIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
BDIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TIM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TCAN	<0.1	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.3
DCAN	5.4	4.9	5.7	6.3	7.3	8.6	8.7	8.9	8.4
BCAN	0.5	0.5	0.6	0.6	0.7	0.7	0.8	0.7	0.6
DBAN	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.3	<0.1	<0.1
CH	21.5	19.2	19.4	20.3	22.7	23.8	23.0	20.0	19.5
TCNM	1.2	1.3	1.2	1.2	1.2	1.2	1.2	1.2	1.2
TBNM	1.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
1,1- DCP	0.3	0.3	0.4	0.4	0.5	0.5	0.5	0.4	0.4
1,1,1-TCP	3.6	3.9	4.6	5.8	7.4	8.5	9.3	9.6	9.2
DCAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
BCAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TCAM	2.4	2.4	2.5	2.5	2.6	2.9	2.8	3.1	3.1
DBAM	1.8	1.8	1.8	1.9	1.9	2.0	<0.1	2.0	<0.1
CIAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

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Sample ID	D2	D3	D4	D5	D6	D7	D8	D9	D10
BDCAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
BIAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DBCAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
TBAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
DIAM	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

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Table S10. Chemical analysis of 8 HAA, results obtained from QHFSS for 11 representative swimming pool water samples. Concentrations are reported in unit µg/L.

DBP	ADWG GV[a]	S7	S9	S13	S17	S18	S20	S21	S22	S23	S24	S25
BAA	N/A	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BCAA	N/A	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
BDCAA	N/A	7	16	<0.5	8	11	8	<0.5	9	14	12	22
CAA	150	34	110	26	64	120	40	<0.5	32	<0.5	44	<0.5
DBAA	N/A	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
DBCAA	N/A	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
DCAA	100	810	2400	230	1400	2100	480	770	710	400	880	830
TCAA	100	790	2600	110	1300	1700	650	460	950	1600	1200	1300

[a](NHMRC 2011). N/A refers to no current guideline values available.

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Table S11. Summary of bioassay results for all water samples (n.a. = not analyzed). The EC values are presented as average and standard deviations of a minimum of two independent experiments performed on two different days.

Sample ID	EF_{SPE}	Extraction pH	Microtox EC_{50} ± SD	AREc32 $EC_{IR1.5}$ ± SD	umuC $EC_{IR1.5}$ ± SD	p53 $EC_{IR1.5}$ ± SD
O1	8000	pH 3	16.3 ±6.2	13.3±1.8	18.2±1.3	n.a.
O2	7999	pH 3	22.2±3.9	21.9±2.1	46.2±0.6	n.a.
O3	7998	pH 3	11.2±4.4	43.7±6.9	47.1±1.3	n.a.
O4	8000	pH 3	23.1±3.9	35.9±4.2	59.2±9.8	n.a.
O5	7999	pH 3	21.1±0.2	23.9±3.9	62.0±11.9	n.a.
O6	7999	pH 3	27.5±6.9	86.3±27	78.1±34.1	n.a.
O7	7999	pH 3	26.0±3.0	6.7±0.4	59.1±1.4	n.a.
O8	8000	pH 3	25.5±3.6	5.4±0.3	87.8±3.3	n.a.
O9	7999	pH 3	25.5±3.7	5.6±0.5	81.1±4.4	n.a.
O10	8000	pH 3	19.5±2.0	10.9±1.4	51.9±3.8	n.a.
O11	7999	pH 3	21.6±1.2	16.3±1.5	71.2±22.3	n.a.
O12	7999	pH 3	28.4±2.7	15.4±1.4	101.9±58.6	n.a.
O13	7998	pH 3	90.1±4.9	10.6±1.0	121.4±16.6	n.a.
O14	7998	pH 3	33.8±0.9	5.9±1.6	67.0±1.1	n.a.
O15	8000	pH 3	19.5±2.0	3.5±0.3	48.3±4.8	n.a.
O16	7999	pH 3	21.9±0.8	4.2±0.2	42.1±18.7	n.a.
O17	7999	pH 3	28.4±2.7	8.8±0.7	18.4±3.5	n.a.
D1	10020	pH 1	20.3±7.4	9.8±3.7	44.2±14.8	n.a.
D2	10007	pH 1	14.7±0.4	16.8±9.3	30.1±1.1	n.a.
D3	9976	pH 1	9.1±1.1	9.6±4.2	50.8±34.5	n.a.
D4	9977	pH 1	19.4±1.3	10.5±3.5	124.6±15.2	n.a.
D5	9990	pH 1	8.6±0.5	6.0±2.2	264.9±258.5	n.a.
D6	9988	pH 1	9.4±0.5	8.1±3.1	83.6±12.5	n.a.
D7	10013	pH 1	9.5±0.9	7.9±1.4	59.2±8.5	n.a.
D8	10005	pH 1	10.4±1.1	11.9±1.1	46.6±7.2	n.a.
D9	9997	pH 1	14.4±0.02	10.9±3.5	60.1±42.5	n.a.
D10	9996	pH 1	16.3±0.2	20.5±6.7	194.6±49.6	n.a.
S1	10233	pH 1	3.1±0.1	6.2±0.3	56.6±4.4	n.a.
S2	9968	pH 1	5.8±0.3	6.9±0.8	35.8±2.5	n.a.
S3	9881	pH 1	3.9±0.8	14.9±1.9	37.1±5.4	n.a.
S4	9936	pH 1	3.2±0.5	7.2±0.9	10.1±0.7	n.a.
S5	10018	pH 1	0.9±0.1	7.2±2.2	12.5±1.0	n.a.
S6	10332	pH 1	11.5±0.8	10.9±2.9	29.5±1.9	n.a.
S7	10442	pH 1	4.1±0.6	8.5±2.9	34.0±2.3	n.a.

Sample ID	EF_{SPE}	Extraction pH	Microtox EC_{50} ± SD	AREc32 $EC_{IR1.5}$ ± SD	umuC $EC_{IR1.5}$ ± SD	p53 $EC_{IR1.5}$ ± SD
S8	10428	pH 1	3.6±0.3	8.6±1.3	34.1±4.3	16.5±5.2
S9	10420	pH 1	1.1±0.1	6.1±1.5	7.6±0.8	6.4±13.4
S10	10414	pH 1	1.2±0.1	4.7±1.1	12.2±0.9	n.a
S11	10293	pH 1	29.9±3.2	25.5±5.2	145.8±35.1	>25
S12	10368	pH 1	4.5±0.6	2.1±0.1	18.3±1.3	n.a.
S13	10381	pH 1	2.6±2.3	7.2±0.3	76.0±23.3	22.1±3.9
S14	10222	pH 1	2.8±2.4	8.7±1.0	21.7±2.4	n.a.
S15	10262	pH 1	27.1±0.6	191.5±286.7	126.3±42.6	>25.
S16	10338	pH 1	5.3±0.3	2.3±0.2	16.4±1.9	n.a.
S17	10406	pH 1	9.1±0.5	24.0±3.4	20.8±3.9	>25
S18	10299	pH 1	2.7±0.08	2.2±0.2	21.7±2.8	>25
S19	10189	pH 1	8.7±0.9	3.2±0.2	21.2±1.7	n.a.
S20	10416	pH 1	10.7±0.01	12.5±2.1	49.9±11.3	>25
S21	10359	pH 1	3.8±0.01	3.0±0.2	23.8±3.4	>25
S22	10215	pH 1	4.1±3.2	4.7±0.4	18.2±1.5	>25
S23	10174	pH 1	9.2±11.2	0.48±0.05	13.9±1.9	>25
S24	10348	pH 1	9.9*	15.7±1.3	21.3±2.1	>25
S25	10377	pH 1	4.7±4.0	0.3±0.02	13.3±1.4	9.6±9.0
S26	10068	pH 1	5.3±5.1	16.3±1.7	82.3±27.4	n.a.

*only one replicate.

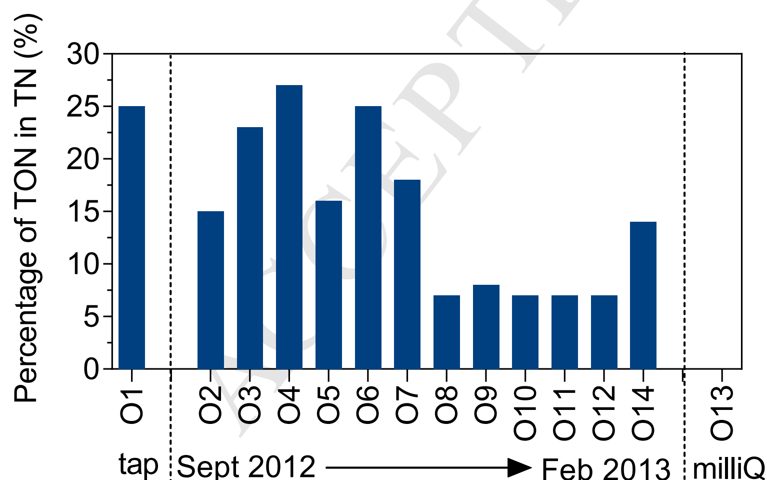

Figure S2. Percentage of TON in TN over the course of 6 months (samples O1 to O14). On-site tap water was taken on the 28 Sept 2012, same as first pool water sample.

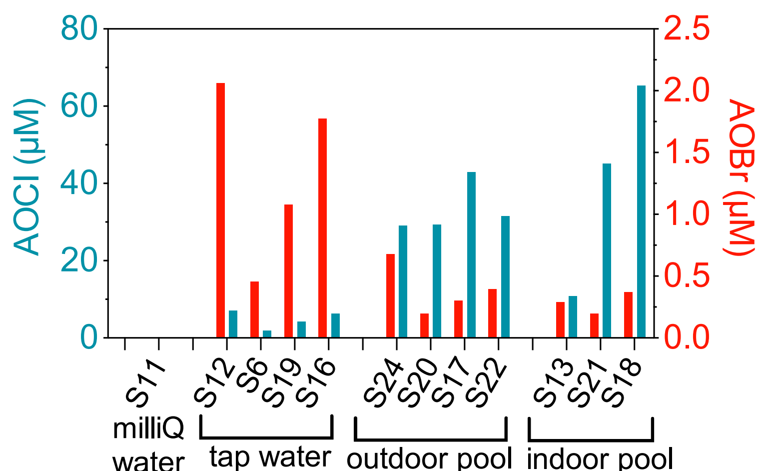

Figure S3. AOX results for the ultrapure (milliQ) water control, on-site tap water (sample S12, S6, S19 and S16), outdoor pool water (sample S24, S20, S17 and S22), indoor pool water (sample S13, S21 and S18).

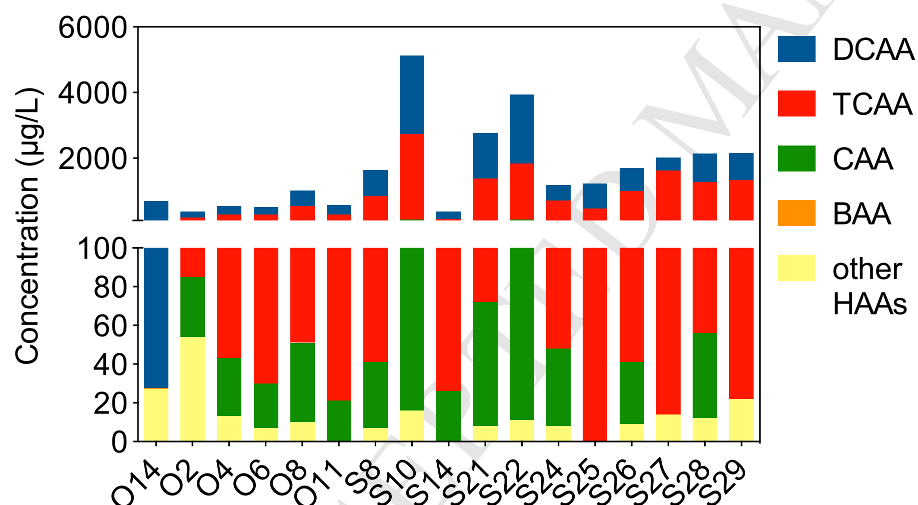

Figure S4. Composition of HAAs in 17 swimming pool water samples from 10 different swimming pools (outdoor, indoor and baby). Pool 1 outdoor (O2, O4, O6, O8, O11 and O14), Pool 2 outdoor (S24), Pool 2 indoor (S25), Pool 3 indoor (S13), Pool 5 covered outdoor (average of S7 and S22), Pool 5 baby pool (average of S9 and S23), Pool 6 outdoor (S20), Pool 6 indoor (S21), Pool 7 outdoor (S17) and Pool 7 indoor (S18).

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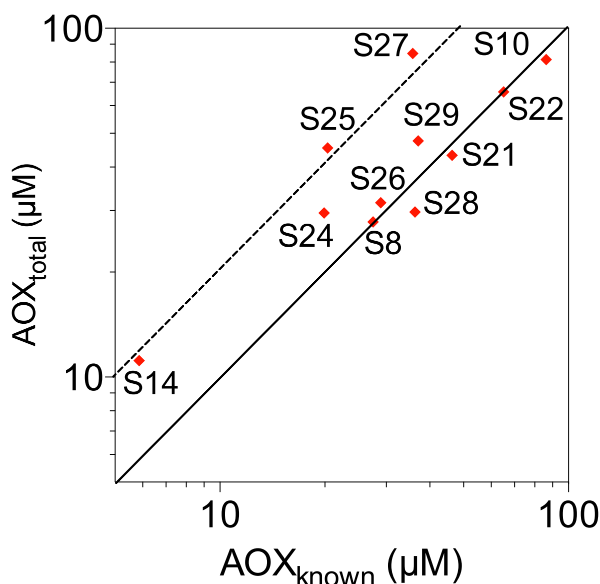

Figure S5. A comparison of AOX_{total} from AOX analysis with AOX_{known} calculated from identified DBPs of 11 representative pool water samples (S8, S10, S14, S21, S22, S24, S25, S26, S27, S28 and S29). The drawn line is the 1:1 line, the dashed line represents 50 % AOX_{total} of total is AOX_{known}.

Section S4. Toxicity of HAA reference compounds.

Table S12 summarises non-specific toxicity and reactive endpoints results of the 12 individual HAAs. Iodinated DBPs were most potent followed by mixture of chlorinated/brominated species with the chlorinated HAAs the least toxic. While non-specific toxicity (EC_{50}) spanned a range of two orders of magnitudes, reactive endpoints ($EC_{IR1.5}$) varied much more, over four orders of magnitude.

The oxidative stress response of CAA, BAA and IAA was recently characterised with the ARE-GeneBLAzer assay (Pals et al. 2013). CAA was 800 times less potent with AREc32 than with ARE-GeneBLAzer, and BAA and IAA were 40 times less potent, indicating that HAAs require metabolic activation to exert oxidative stress because the ARE-GeneBLAzer is based on the metabolically active HepG2 cell line, while AREc32 is derived from the metabolically inactive MCF cell line.

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Table S12. EC values and REP in relation to CAA of all tested HAA.

Abbre-viation	Microtox		AREc32		umuC			p53	
	EC_{50} ± SD (mM)	REP	$EC_{IR1.5}$ ± SD (mM)	REP	$EC_{IR1.5}$ ± SD (mM)	REP	EC_{10} cyto-toxicity (mM)	$EC_{IR1.5}$ ± SD (mM)	REP
BAA	0.10 ± 0.01	14.9	0.06 ± 0.007	109	0.5 ± 0.11	27.6		Not tested	
IAA	0.04 ± 0.01	32.7	0.04 ± 0.003	166	0.06 ± 0.01	230.7		Not tested	
CAA	*1.44 ± 0.4*	*1.0*	*7.0 ± 0.6*	*1.00*	*13.5 ± 5.3*	*1.0*	*0.5*	*0.086 ± 0.015*	*1.0*
BDCAA	2.15 ± 0.3	0.7	39.7 ± 28.7	0.18	1.2 ± 0.07	11.5	4.5	3.8 ± 3.7	0.02
TBAA	0.68*	2.3	3.7 ± 0.5	1.90	0.3 ± 0.006	53.7		Not tested	
DBAA	0.68 ± 0.06	2.1	0.5 ± 0.04	15.0	4.2 ± 1.4	3.2	1.4	0.44 ± 0.30	0.19
BCAA	2.70 ± 0.5	0.5	0.8 ± 0.1	8.36	1.1 ± 0.2	11.8	1.0	>10	
DCAA	2.99 ± 0.02	0.5	51.2 ± 5.8	0.14	140 ± 24	0.1	6.5	6.4 ± 2.5	0.01
BIAA	0.29 ± 0.01	4.9	0.08 ± 0.01	88.4	0.05 ± 0.01	265		Not tested	
CIAA	0.03 ± 0.00	43.9	0.18 ± 0.01	39.3	0.27 ± 0.07	50.7		Not tested	
TCAA	27.8 ± 0.2	0.1	>50	-	26.2 ± 5.8	0.5	7.2	>10	
DBCAA	3.16*	0.5	3.30 ± 0.2	2.13	1.1 ± 0.1	11.8		Not tested	

*only one replicate.

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There were no obvious trends observed between non-specific toxicity and the reactive endpoints (Table S12, Figure S6). Of particular interest are those HAAs where the $EC_{IR1.5}$ of the reactive endpoint occurred at lower concentrations than the EC_{10} of cytotoxicity. BIAA and BCAA were highly specific for both oxidative stress response and genotoxicity measured with the umuC. TBAA and BDCAA were highly specific for genotoxicity with the umuC but did not respond strongly in oxidative stress, while DBAA was specific to oxidative stress and did not induce genotoxicity. In contrast, only few $EC_{IR1.5}$ values could be derived for the p53 assay because cytotoxicity masked the induction (Figure S6, Table S12).

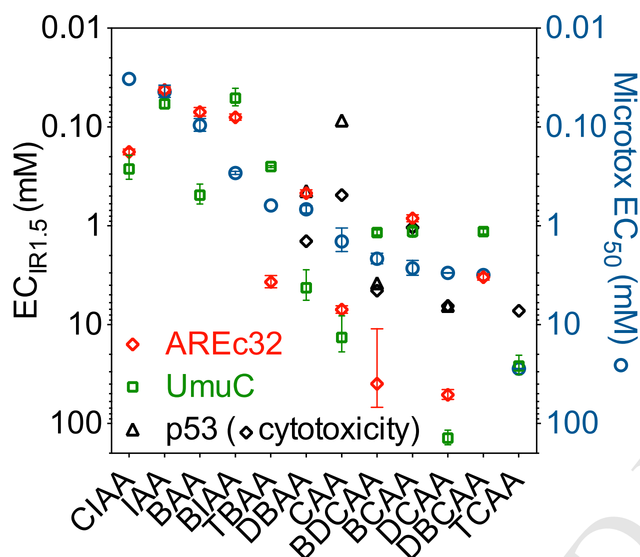

Figure S6. Summary of bioassay results of HAAs. For p53, also the EC_{10} for cytotoxicity is shown for comparison.

The chronic cytotoxicity and genotoxicity of HAAs were previously assessed in Chinese Hamster Ovary Cells by Plewa et al. (2010). As Figure S7 shows, the effect concentrations are in the same order of magnitude in the two studies.

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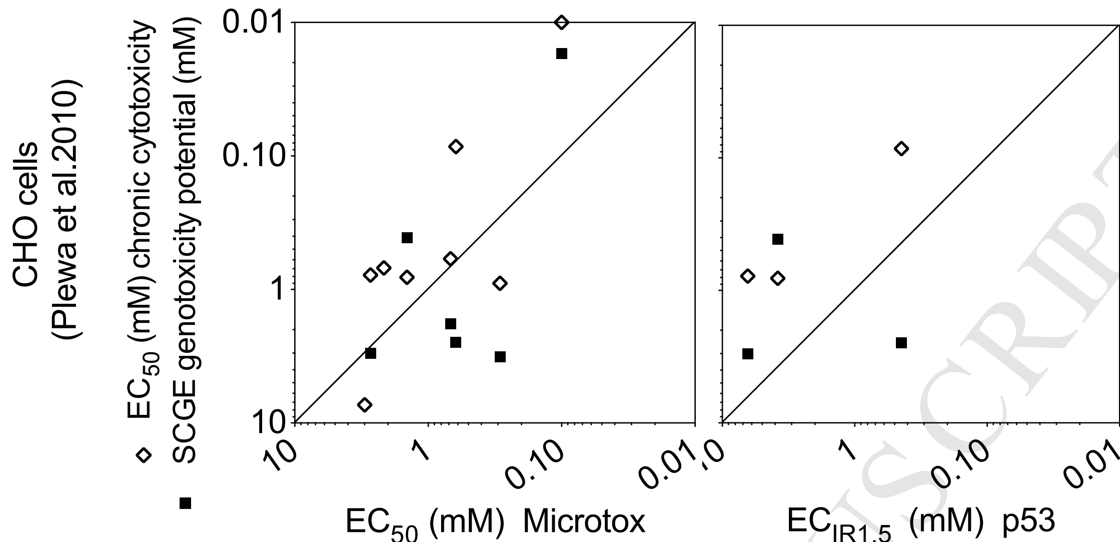

Figure S7. Comparison of the bioassay results of HAAs with the chronic cytotoxicity and genotoxicity of HAAs in Chinese Hamster Ovary Cells (Plewa et al. 2010).

Dad et al. (2013) suggested HAA is dependent on the carbon-halogen bond length as well as the bond dissociation energy where increasing bond length means lower dissociation energy required to react with target molecule. Greater bond length was linked to greater cytotoxicity and genotoxicity which was found follow the pattern of IAA > BAA >> CAA (Dad et al. 2013). Interestingly, this is the same general trend we see in our bioassay results for the cytotoxicity assay Microtox. It is relevant to note that the detected HAAs are those of lower effect potency. The REPs in relation to CAA were calculated (Table S12) for a quantitative assessment of the contribution of detected HAAs to the overall effects.

This topic is discussed in the main MS and Figure 4 shows the data associated with Table 2. Due to the high cytotoxicity it was not possible to derive meaningful $CAA\text{-}EQ_{bio}$ and $CAA\text{-}EQ_{chem}$ for the p53 assay (Table S13).

Table S13. Comparison of $CAA\text{-}EQ_{chem}$ and $CAA\text{-}EQ_{bio}$ for p53. Please note the limitations of this analysis due to cytotoxicity disturbing the induction in both HAAs and water samples.

Sample ID	$CAA\text{-}EQ_{chem}$ [p53] (µM)	$CAA\text{-}EQ_{bio}$ [p53] (µM)	Quantified chemicals explain % CAA-EQ [p53]
S7	0.44	5.23	8.5%
S9	1.42	13.40	11%
S13	0.30	3.91	7.7%

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S17	0.82	<3.4	-
S18	1.49	<3.4	-
S20	0.47	<3.4	-
S21	0.08	<3.4	-
S22	0.41	<3.4	-
S23	0.04	<3.4	-
S24	0.56	<3.4	-
S25	0.09	8.96	1.0%

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Section S5. Temporal trends

A part of the study was undertaken to observe temporal trends of an outdoor pool (Pool 1) in the course of 6 months and in the course of within a day. The results of bioassays in comparison to TN, TOC and TON from 6 months study are compared in Figure S8.

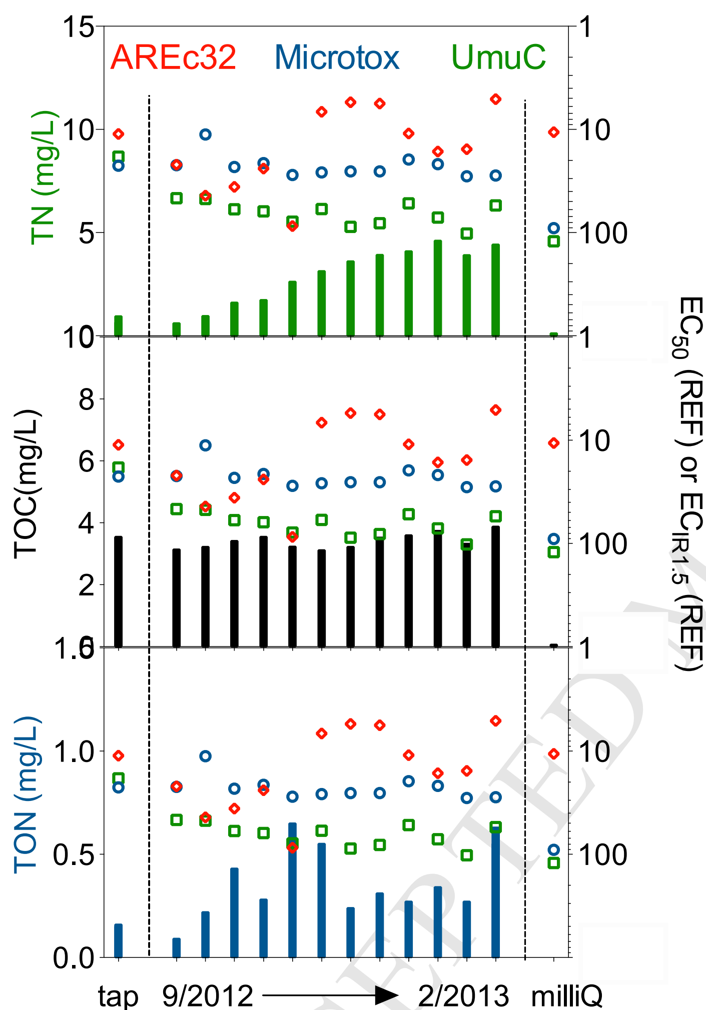

Figure S8. Concentrations of total nitrogen (TN), total organic carbon (TOC) and total organic nitrogen (TON) in comparison to bioassays (nonspecific and reactive toxicity expressed as EC_{50} or $EC_{IR1.5}$ in units of REF) of swimming pool water samples (pool 1) collected from September 2012 to February 2013.

There was no change of bioassay responses over time for cytotoxicity and genotoxicity but the oxidative stress response increased over time. The relative order of potency changed with the oxidative stress response becoming the dominant effect starting December (when the summer started). It should also be noted that the MilliQ blank could have possible contamination as it showed high effects, even higher than some of the pool water samples. Thus, we cannot conclude an overall trend nor whether effects were representative. While TN

increased with time, there was no such associated increase in TOC and TON, nor effects (Figure S8).

Within a day samples were taken on the 4 April 2013 in Pool 1, the results of AOX and bioassay did not have a trend (Figure S9). AOCl varied across the day but no trend could be observed. The bioassay results demonstrated again that AREc32 and Microtox were more sensitive than umuC but there was no diurnal variability. No p53 was tested with these samples.

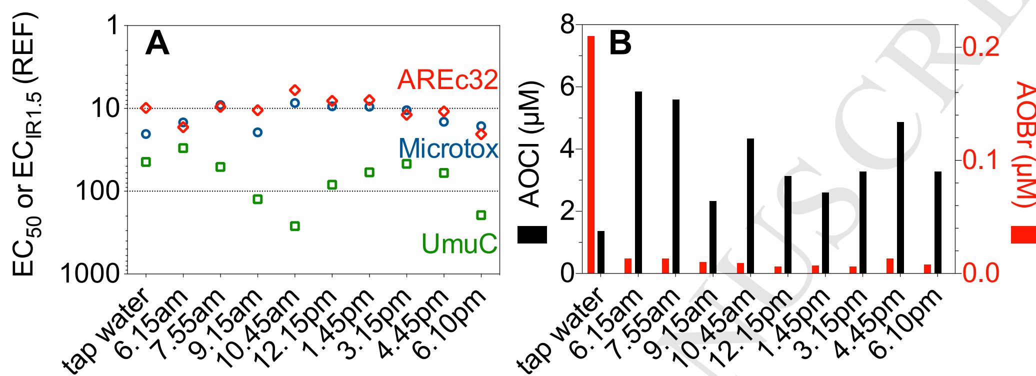

Figure S9. (A) Bioassay results (non-specific and reactive toxicity expressed as EC_{50} or $EC_{IR1.5}$ in units of REF) and (B) concentration of AOCl and AOBr of swimming pool water samples (pool 1), samples D1 to D10 collected on 04 April 2013 over the course of one day.

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