Developmental effects and genotoxicity of ten water disinfection by-products in zebrafish

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ABSTRACT

Disinfection by-products (DBPs) are contaminants produced during drinking water disinfection. Several DBPs have been implicated in a variety of toxic effects, mainly carcinogenic and genotoxic effects. Moreover, DBPs exposure has also been associated to an increased risk of developmental effects. In this study, the developmental toxicity and genotoxicity of 10 DBPs (4 trihalomethanes (THMs), 5 haloacetic acids (HAAs) and sodium bromate) in the zebrafish embryo model was evaluated. Embryos exposed during 72 hours were observed for different endpoints such as growth, hatching success, malformations and lethality. THMs exposure resulted in adverse developmental effects and a significant reduced tail length. Two HAAs, tribromoacetic acid and dichloroacetic acid along with sodium bromate were found to cause a significant increase in malformation rate. Chloroform, chlorodibromomethane and sodium bromate produced a weak induction of DNA damage to whole embryos. However,

developmental effects occurred at a range of concentrations (20-100µg/mL) several orders of magnitude above the levels that can be attained in the foetal blood in humans exposed to chlorinated water. In conclusion, the teratogenic and genotoxic activity observed by some DBPs in zebrafish reinforce the view that there is a weak capacity of disinfection products to cause developmental effects at environmentally relevant concentrations.

Keywords: Comet assay, developmental toxicity, haloacetic acids, trihalomethanes, water disinfection by-products, zebrafish.

INTRODUCTION

Disinfection by-products (DBPs) are contaminants formed as a consequence of chemical disinfection of public drinking waters. The disinfectants, such as chlorine, can react with natural organic matter in surface waters leading to the formation of a complex mixture of DBPs. So far, more than 600 DBPs have been identified and reported in the literature; nevertheless, they represent less than half of all possible environmental DBPs. The most prevalent DBPs include the four trihalomethanes (THMs), chloroform, bromoform, chlorodibromomethane (CDBM) and bromodichloromethane (BDCM), the group of haloacetic acids (HAAs) and bromate anion. DBPs in drinking water are generally present at sub-µg/L or low- to mid-µg/L levels (Richardson *et al.* 2007) and some countries have regulated the levels of some DBPs in drinking water (Table 1, U.S.EPA 2006). Moreover, World Health Organization (WHO) guidelines exist as well as European Union DBP standards (Table 1, WHO 2004 and EU Directive 98/83/EC 1998).

Several DBPs have been confirmed as mutagenic, genotoxic and/or carcinogenic in different test systems (Richardson *et al.* 2007). There is an increasing concern about the association of DBPs exposure to adverse developmental effects. A number of individual DBPs have been found to cause developmental toxicity in mammalian assays at high doses (Ruddick *et al.* 1983; Narotsky *et al.* 1996; Christian *et al.* 2001a). Some DBPs induce specific congenital malformations of the cardiovascular and neurological system, but, in general, foetal body weight reduction is often reported as the major effect (Epstein *et al.* 1992; Hunter III *et al.* 1996). Recent studies had evaluated the developmental effects of complex mixtures and appeared to exert no adverse developmental effects (Narotsky et al. 2012). The epidemiologic studies found inconsistent results or very weak associations for congenital anomalies/birth defects, central nervous system anomalies, neural tube defects and spontaneous abortion. However, these studies suggested a positive association with some measure of growth retardation (Reviewed in Graves *et al.* 2001; Tardiff *et al.* 2006; Colman *et al.* 2011).

Chemical treatment of public water supplies is designed to kill pathogens that may exist in the drinking water, so the risk-benefit balance of water disinfection is considered positive. However some factors as the huge magnitude of the population affected, the distorted perception by the population of chemical risks, the availability of several alternative water treatment methodologies, the uncertainties

in the hazard characterization of DBPs and the intrinsic limitations of the epidemiological studies, warrant the requirement of a better knowledge about DBPs toxicity and mechanisms of action (Colman *et al.* 2011). In this study, we have explored the capabilities of the zebrafish embryo model in order to identify and characterize the potential embryotoxic and genotoxic effects of some DBPs.

Zebrafish (*Danio rerio*) is a prominent model vertebrate in developmental genetics, toxicology and ecotoxicology (Postlethwait *et al.* 2000; Hill *et al.* 2005; Scholz *et al.* 2008). Size, easy husbandry, high fecundity and fast development represent the main benefits of using zebrafish over other vertebrate species. In addition, zebrafish embryos are transparent and develop outside the mother. Hence, morphological structures and internal organs can be easily visualized (Zhang *et al.* 2003). Moreover, the mechanisms of embryogenesis are well conserved along the vertebrates and there are several studies that confirm the ability of zebrafish model to predict the teratogenic potential of chemicals in mammals (Brannen *et al.* 2010; Padilla *et al.* 2012; Selderslaghs *et al.* 2012). Hence, zebrafish is increasingly used for assessing developmental toxicity of chemicals.

Currently, “whole-mixture” approaches are being used to address concerns related to the potential adverse health effects of DBPs exposure. Scientists from the US EPA office of Research and Development proposed an experimental design for a multigenerational reproductive/developmental bioassay to optimize the probability of detecting adverse effects (Simmons *et al.* 2008). In the present study, the capability of the zebrafish embryo, as alternative model, was explored in order to investigate the developmental effects and genotoxicity of disinfection by-products. The selected DBPs -four trihalomethanes (chloroform, bromoform, chlorodibromomethane and bromodichloromethane), five haloacetic acids (dichloroacetic acid, trichloroacetic acid, dibromoacetic acid, tribromoacetic acid and bromochloroacetic acid) and sodium bromate – represented the most prevalent compounds and data were available for comparison with other test systems and models.

MATERIALS AND METHODS

Chemicals and test media

Dibromoacetic acid (DBA), trichloroacetic acid (TCA), chloroform, bromoform and sodium bromate were purchased from Sigma-Adrich (St.Louis, MO). Dichloroacetic acid (DCA) and tribromoacetic acid

(TBA) were delivered from Tokyo Chemical Industry (Tokyo, Japan). Bromochloroacetic acid (BCA), bromodichloromethane (BDCM) and chlorodibromomethane (CDBM) were purchased from Alfa Aesar (Karlsruhe, Germany). Buffered embryo medium (17.4 mM NaCl; 0.23 mM KCl; 0.12 mM $MgSO_4 \cdot 7 H_2O$; 0.18 mM $Ca(NO_3)_2$; 1.5 mM HEPES; pH 7.4) was used as the medium for all solutions during the experiments to keep the pH stable and constant between assays (Gustafson *et al.* 2012).

Embryo exposure

All stock solutions were prepared with buffered embryo medium except for THMs that were initially prepared in 100% dimethylsulfoxide (DMSO) and subsequently diluted in buffered embryo medium with a final DMSO concentration of 0.1% (v/v).

For all substances, a concentration range-finding experiment was conducted with a constant spacing factor 2. The range finding test allow us to select the final tested concentrations based on the presence of a 0 and 100% effect level (for both malformation and mortality).Each substance was tested in 5-7 concentrations with a negative control, test medium only or solvent control with 0.1% of DMSO. Exposure concentrations are anticipated to be stable during all the test duration.

Zebrafish embryos were collected by natural spawning and staged according to Kimmel *et al.* (1995). Fertilization success was checked and only batches of eggs with at least a fertilization rate of 80% were used. Exposures of embryos began at 4 hours post-fertilization (hpf) and were incubated at 27 ± 1ºC on a 14-h light and 10-h dark cycle for 72 hours. The exposure was semi-static and solutions were renewed every 24 hours.

Embryos were exposed to HAAs and sodium bromate in a 6-well culture plate (Greiner Bio-one, Germany). Ten embryos were randomly distributed into wells and filled with 5 ml of each solution. Each 6-well plate held five different concentrations of the test compound and the negative or solvent control. In order to prevent losses by volatilization, THMs were tested in 20 ml glass vials hermetically sealed. Ten embryos per vial and treatment were exposed with 10 ml of each test solution. For each substance, three independent exposure experiments were conducted using eggs from independent spawning events (n=3).

Evaluation of developmental effects

At 8, 28, 52 and 76 hpf, mortality of embryos was checked using a stereomicroscope (SMZ-168, Motic). According to Nagel (2002) four four endpoints were considered as indicators of mortality: coagulation of eggs, non-development of somites, non-detachment of the tail and no presence of heartbeat. Dead embryos were removed daily after assessment of mortality rate. The fraction of dead embryos at the end of the test was used to calculate LC_{50} values.

The teratogenic effects were evaluated and recorded as described in Teixidó et al. (2013) (Table 2). The fraction of abnormal embryos was determined for each concentration and control group in order to calculate EC_{50} value. The frequency of teratogenic effects in all tested groups of a test substance was also analysed. If the following criteria were fulfilled: (a) concentration-response relationship and (b) the endpoint is observed in ≥50% of all embryos showing malformations, the effect was considered to be a distinctive or identifying malformation (fingerprint endpoint) for this substance (Weigt *et al.* 2011). Embryos were observed using a stereo microscope, and images were obtained with a camera (Moticam 2000, Motic). Image processing was performed in Image J 1.41 (available at http://rsb.info.nih.gov/ij/) and Adobe Photoshop CS3 (Adobe Systems Inc., USA). Test concentrations are expressed in nominal concentrations.

Hatching success and tail length measurement

From 48 hpf the embryos are able to hatch. Hatching success was recorded at 76 hpf as the percentage of embryos that hatched respect surviving embryos. The malformations observed in the embryos could affect its movement and consequently reduce or delay the hatching. Therefore, motility was also assessed by touch evoked response as a complementary endpoint.

Embryos that have not yet hatched were dechorionated. All embryos were anesthetized with buffered tricaine methanesulfonate (0.5 mM, Sigma-Aldrich, St. Louis, MO) and photographed (Moticam 2000, Motic) positioned on their lateral side in order to measure the distance between the anus and the posterior end of the notochord, defined as tail length (Bachmann 2002). The minimum concentration to inhibit growth (MCIG) is defined as the minimum concentration to significantly produce a decrease in tail length.

Cell isolation and alkaline comet assay

After exposure for 72 h, seven surviving fish embryos for the treatments and control groups were processed for cell isolation and comet assay. Cell isolation was carried out mechanically according to the protocol by Kosmehl *et al.* (2006). The EC_{50} values from teratogenic effects were used as the test concentrations for genotoxicity testing.

The alkaline single cell gel electrophoresis (SCGE) or comet assay was performed as described by Singh *et al.* (1988) with some modifications. Fifty microliters of cell suspension were added to a tube containing 100 µL of 0.9% low melting point agarose (37 ºC). The suspension was added to an agarose precoated slide and gently covered with a cover slide to make a micro gel. The gel was allowed to solidify 10 min at room temperature and 6 min at −20 ºC. Immersion in lysis buffer (2.5 M NaCl, 100 mM disodium EDTA and 10 mM Tris, pH 10) containing Lauryl Sarcosine 1% (v/v), Triton 1% (v/v) and DMSO 10% (v/v) in the dark was performed for 1,5 h at 4 ºC. The slides were then placed in a horizontal gel electrophoresis unit, immersed in cold alkaline electrophoresis buffer (300 mM NaOH and 1 Mm Na_2EDTA, pH > 13.5), and left in solution for 20 min at 4 ºC. After electrophoresis in the same buffer at 25 V and 300 mA for 20 min, slides were neutralized by washing three times with 0.4 M Tris buffer at pH 7.5.

DNA was stained with 20 µl of DAPI solution (4′,6-diamidino-2-phenylindole) and immediately analysed using a Nikon E600 fluorescence microscope. DNA damage as % of DNA in the tail was measured using the Comet Assay IV software (Perceptive Instruments, Suffolk, UK). Zebrafish in vivo exposure to methyl methanesulfonate (MMS) during 72 h (from 4 hpf to 76 hpf) was used as a positive control for comet assay.

Data evaluation

Concentration-response curves for mortality and teratogenicity were plotted for compounds that showed a clear concentration-response relationship. It has been demonstrated that ignoring control mortality, even at lower than 10%, can lead to biased estimation of LC_{50} (lethal effects) and EC_{50} (teratogenic effects) (Hoekstra 1987). Therefore, data were corrected for control mortality with Abbott's formula: Pc = (P – Pi) / (100 – Pi) x 100 where Pc is the corrected percentage, P is the percentage mortality of the treated embryos and Pi is the percentage mortality of the control embryos

(Abbott 1987).

Concentration-response curves were calculated using probit analysis (SPSS 15.0). Confidence intervals were set at 95%. Based on LC_{50} and EC_{50} values, a teratogenic index (TI) was calculated as the ratio LC_{50}/EC_{50}. In case no TI could be calculated, the compound was considered to be a non-teratogen although it could still be embryotoxic. EC_{20} values were calculated for comparison between zebrafish embryo and human exposure data.

Statistical analysis was performed with SPSS 15.0. One-way analysis of variance (ANOVA) followed by post hoc multi-comparison with the Bonferroni's test was used to analyse homogeneous data of the continuous variables. Kruskal-Wallis test was used to analyse non-homogeneous data followed Dunnett's post hoc test. Significance was accepted when $p < 0.05$.

RESULTS AND DISCUSSION

Exposure of zebrafish embryos to trihalomethanes

Exposure to the THMs selected in this study resulted in adverse developmental effects in zebrafish. The cumulative concentration-response curves for lethality and teratogenesis are shown in figure 1A. The vehicle control (0.1% DMSO) was not toxic to embryos. The estimated LC_{50}, EC_{50}, EC_{20} and TI values at 76 hpf are represented in table 3. Malformations that occurred with the highest frequency included cardiac edema and tail developmental abnormalities (Table 4 and Figure 1B) and are in accordance with those reported by Brennan *et al.* (2005) for tadpoles. In our study, the EC_{20} values range between 0.11 mM (23 mg/L) for chlorodibromethane and 0.7 mM (84.7 mg/L) for chloroform. Using the EC_{20} to compare the chemicals a ranking of the compounds in order of decreasing potency can be established as: Chlorodibromomethane > Bromoform ≈ Bromodichloromethane > Chloroform. CDBM (LC_{50}= 0.48 mM, 100 mg/L and EC_{50}= 0.16 mM, 33.3 mg/L) resulted to be 3-fold times more potent than chloroform (LC_{50}= 2.4 mM, 286.5 mg/L and EC_{50}= 0.84 mM, 100.3 mg/L). This order of toxicity was also found by Mattice *et al.* (1981) after exposure of common carp embryos to THMs. All THMs inhibited the growth of the embryo (Table 3), effect that is often observed when there is a cardiovascular impairment (Billiard *et al.* 1999). Delayed hatching was also observed after THMs exposure and could be due to an inhibition of enzymes involved in hatching or a decreased mobility in the embryo (Von Westernhagen 1988). The developmental abnormalities and the concentration

dependent sedative effect observed after THMs exposure partially or completely inhibit movement which may be required for proper hatching (Figure 3).

The EC_{50} reported in this study for chloroform (EC_{50}=0.85 mM) and BDCM (EC_{50}= 0.26 mM) were similar to those reported for FETAX in Brennan *et al.* (2005) (0.92 mM and 0.4 mM respectively). Based on the TI values (from 2.5 to 3.6), all THMs were found to produce teratogenic effects in zebrafish. Studies in mammalians suggest evidence of a fetotoxic response for THMs but not a teratogenic effect (Thompson *et al.* 1974; Ruddick *et al.* 1983). Other mammalian studies have reported a decreased fetal growth, delayed ossification and craniofacial abnormalities in rats treated by chloroform inhalation (Schwetz *et al.* 1974; Murray *et al.* 1979). Rats exposed to chloroform via drinking water have only showed to have an impaired postnatal growth (Lim *et al.* 2004). Brown-Woodman *et al.* (1998) reported a no effect concentration for chloroform of about 1.05 mM in rat whole embryo culture (WEC), similar to the EC_{20} concentration reported in our study (EC_{20}= 0.7 mM).

Exposure of zebrafish embryos to five haloacetic acids

The only HAAs that induced some morphological effects were DCA and TBA at high concentrations (Figure 2B and Table 3). Embryos exposed to DCA exhibited moderate pericardial oedema whereas no lethality was observed up to 46.5 mM (Figure 2A and B, Table 4). The estimated LC_{50}, EC_{50}, EC_{20} and TI-values are represented in table 3. DCA were previously found to be developmental toxic in zebrafish by Hassoun *et al.* (2005). Craniofacial abnormalities (reduced mouth and jaw formation), skeletal muscle deformations and yolk sac and cardiac oedema were observed at 144 hpf after an exposure of DCA in a concentration range between 8 and 32 mM. However, (Weber *et al.* 2004) did not found any embryotoxic effect up to a concentration of DCA of about 124 mM in *Xenopus laevis*. DCA have been shown to produce cardiovascular defects when administered to pregnant dams at high dose levels (Epstein *et al.* 1992; Johnson *et al.* 1998).

In our study, TBA exposure caused curvatures of the spinal column, swelling of the pericardia and small eyes (Figure 2B, Table 4) with an EC_{20} of 4.4 mM (1,300 mg/L, Table 3). The current data are in agreement with results from Hunter III *et al.* (1996) that reported that brominated acetic acids are potentially more toxic than chlorinated species to developing embryo. Developmental toxicity studies with frog (Bantle *et al.* 1999) reported a teratogenic index of about 3.86 after TBA treatment, a higher

value compared with our results with zebrafish embryos (TI= 2.2). In vivo, TBA exposure was found to not produce any adverse developmental effect (NTP, National Toxicology Program 1998a). HAAs have been shown to produce neural tubes defects, prosencephalic and pharyngeal arch hypoplasia, heart and eye defects in rat and mice whole embryo culture (Hunter III *et al.* 1996; Andrews *et al.* 2004), but at a lower range concentration (0.05-2 mM) compared to zebrafish (10-30 mM).

BCA exposure did not produce any adverse developmental effects at the highest concentration tested (30 mM, Table 3). In BCA-exposed tadpoles, some gut abnormalities and decreased growth were observed only at higher concentrations (57.7 mM and 46 mM, respectively)(Brennan *et al.* 2005). BCA was found to not produce any adverse developmental effect in vivo in mammals (NTP, National Toxicology Program 1998b). In our study, TCA exposure did not caused developmental effects (Table 3). In vivo, administered TCA to pregnant dams at high dose levels has been shown to produce cardiovascular defects and low weight (Smith *et al.* 1989; Johnson *et al.* 1998). Zebrafish embryo exposure to DBA only produced a decrease in tail length (Table 3). DBA was found to not produce any adverse developmental effect in vivo in mammals (Christian et al. 2001b).

Exposure of zebrafish embryos to sodium bromate

Sodium bromate showed embryotoxic effects on zebrafish only at very high concentration (LC_{50}= 65.4 mM, Table 3), probably by an irrelevant unespecific effect. Small eyes and pericardial oedema were the predominant sodium bromate-induced malformations in zebrafish embryos (Figure 2B and Table 4). As shown in table 3, the EC_{20} value after exposure to sodium bromate was 51.7 mM, The 96-h LC_{50} (8.32 mM) reported for frog embryos after sodium bromate treatment resulted to be about 8 times lower than the LC_{50} reported for zebrafish embryos in this study (65.4 mM). Studies in mammals have been shown that sodium bromate did not cause any adverse developmental effect (Wolf & Kaizer 1996).

Genotoxicity of water disinfection by-products

DNA-damage caused by water disinfection by-products was investigated using the comet assay. Genotoxicity could represent a potential mechanism leading to developmental disorders and embryo mortality, and also it has been associated with carcinogenic effects provoked by DBPs. The four

THMs, the TBA and sodium bromate were selected for the comet assays as they exhibit embryo toxicity in zebrafish. The test concentration used was the EC_{50} values from teratogenic effects. Chloroform (0.85 mM) and CDBM (0.16 mM) produced a significant DNA damage compared to control solvent group (Figure 4B). If compared to the DNA damage produced by the positive control (Figure 4A), disinfection by-products displayed a weak positive response. Exposure to the positive control (methyl methanesulfonate –MMS-) produced a concentration-dependent increase in the mean percentage of DNA in the tail ($r=0.89$, $p<0.01$; Figure 4A) and ranged between 4 and 37 %. The highest tested concentration of MMS (25 mg/L) produced less than 10% of embryo mortality, however all embryos showed developmental effects (data not shown). Many in vitro techniques have been used to investigate the mutagenic and genotoxic properties of THMs and HAAs (Richardson *et al.* 2007). These studies have shown that THMs are weak inducers of DNA damage (Landi *et al.* 2003; Zhang *et al.* 2012) and that glutathione S-tranferase-theta (GSTT1-1) activity mediated transformation of brominated THMs to mutagenic intermediates (Pegram *et al.* 1997). Although, it has not been found yet the homolog GSTT1-1 gene for zebrafish, embryos possess a lower GST activity that could play a role not only in the weak mutagenicity observed but also in the low teratogenicity observed. HAAs caused DNA breaks in Chinese hamster ovary (CHO) cells (Plewa *et al.* 2010) and in human derived hepatoma cell line (Zhang *et al.* 2012). In our study, the strongest effect was found in sodium bromate treated embryos with a median damage of 8 % of DNA in the tail (Figure 4C). It has been reported that bromate induced DNA damage (SCGE assay) in mammalian cells through oxidative damage (Priestley *et al.* 2010).

Exposure to DBPs in humans has been quantitatively assessed by measuring the concentration of DBPs and its metabolites in blood, urine and exhaled breath after the oral intake of chlorinated water and after the dermal or inhalatory exposure during shower and bath. The reported blood levels in exposed humans are in the pg/mL range, with the highest levels of about 300 pg/ml for chloroform after a shower with a tap water surpassing the current standards (Nieuwenhuijsen *et al.* 2000; Nuckols *et al.* 2005). Transplacental crossing of some DBPs in blood at concentrations equal to or greater than in maternal blood has been demonstrated in rodents and humans (Dowty *et al.* 1976; Danielsson *et al.* 1986; Christian *et al.* 2001a). In this study, effect concentrations of a 20% were between 0.11-0.7mM (20-100µg/mL) after DBPs exposure in zebrafish, several orders of magnitude

above the levels that human embryos would be exposed through their mother blood. It should be noted that it has yet not been demonstrated that plasma concentrations in mammals relate to toxicity effect concentrations similar as exposure concentrations to fish embryo test.

One of the alleged weaknesses of the zebrafish embryo as a model for teratogenicity in mammals is the difference in metabolic activity towards exogenous substances. This is especially relevant in the case of xenobiotics whose toxicity is mediated by their metabolites. It is well known that some of the toxic effects of halogenated short-chain hydrocarbons are mediated by intermediate electrophilic metabolites. Zebrafish have a total of 94 CYP genes, distributed among 18 gene families, most of which are direct orthologs of human CYPs. Most of these CYPs are expressed in embryos during various time courses along the first 48 hours after fertilization. Indeed, some maternally-derived CYPs RNA transcripts are present in the unfertilized egg (Goldstone *et al.* 2010). Jones *et al.* (2010) have further demonstrated the expression of several xenobiotic metabolizing genes similar to human (CYP1A, CYP2B6, CYP3A5, UGT1A1) and their functional capacity metabolizing some model compounds during the early development. Therefore, the zebrafish embryo is endowed with a wide spectrum metabolic capacity, but the capability of metabolic transformation in comparison to mammalian models is still not fully understood. (Hill *et al.* 2012). To our knowledge, there are no data about the capacity of zebrafish embryo to metabolize THMs or HAAs. However, there is evidence of the capacity to bioactivate THMs by other fish species (Räbergh & Lipsky 1997; Vega-López *et al.* 2012).

Conclusions

Effect concentrations in zebrafish embryos support previous observation of a weak teratogenic and genotoxic potential of DBPs.The proximity of effect concentration of lethality and malformations suggest that probably the teratogenic effects are related to unspecific embryo toxicity. The effects are observed only at concentration levels well above those that can be attained in the foetal blood in humans exposed to chlorinated water, providing further evidence for only a weak teratogenic potential of DBP products.. However, more studies are needed to explore the involvement of metabolism in the potential DBP toxicity and to extent our knowledge about exposure to mixtures and the possible developmental effects and genotoxicity. Finally, our study indicates that zebrafish embryos are as

sensitive as other test systems and can be used as a potential screening and prioritization tool to assess large number of disinfection by-products.

Tables

Table 1. DBP regulations and guideline values (highest concentration allowed in drinking water).

Table 2. Lethal and teratogenic effects evaluated in zebrafish embryos at 76 hpf.

Table 3. The LC50, EC50 values are represented with their 95% confidence intervals (CI) and teratogenic index (TI) of all water disinfection by-products tested. Minimum concentration that inhibits growth (MCIG) and EC20 effect concentrations at 76 hpf are also represented for all substances. –, indicates that could not be calculated. Abbreviations used: CDBM (chlorodibromomethane),BDCM (bromodichloromethane), DCA (dichloroacetic acid), TCA (trichloroacetic acid), DBA (dibromoacetic acid), TBA (tribromoacetic acid) and BCA (bromochloroacetic acid).

Table 4. Frequency of endpoints observed at 76hpf in zebrafish embryos exposed to DBPs. Σt represents the sum of embryos in all test concentrations showing teratogenic effects for each specific endpoint. The percentatge of embryos showing each endpoint was calculated respect the total number of embryos with teratogenic effects found in all test concentrations. Bold values indicate that the endpoint followed a concentration-response relationship and was observed in ≥50% of all embryos showing malformations. Abbreviations used: CDBM (chlorodibromomethane), BDCM (bromodichloromethane), TBA (tribromoacetic acid), DCA (dichloroacetic acid).

Figures

Figure 1. (A) Concentration-response curves for mortality (o, full line) and teratogenesis (x, dotted line) for chloroform, bromoform, chlorodibromomethane and bromodichloromethane exposure from 4-76hpf. % Effect (Mean± S.D.) is shown versus the logarithm of concentration tested. (B) Representative images of fish embryos exposed to chloroform, bromoform, chlorodibromomethane and bromodichloromethane at 76hpf.

Figure 2. (A) Concentration-response curves for mortality (o, full line) and teratogenesis (x, dotted line) for tribromoacetic acid, dichloroacetic acid and sodium bromate exposure from 4-76hpf. % Effect

(Mean ± S.D.) is shown versus the logarithm of concentration tested. (B) Representative images of fish embryos exposed to dichloroacetic acid, tribromoacetic acid and sodium bromate at 76hpf.

Figure 3. Percentage of embryos (Mean ± S.E.M) displaying reduced motility after THMs exposure and hatching success of these embryos at 76 hpf. Motility of the embryos was checked after dechorionation if the embryo had not hatched at 76 hpf. * Significant different from solvent control group (0), $p<0.05$.

Figure 4. (A) Genotoxicity following exposure of whole embryos to increasing concentrations of methyl methanesulfonate (MMS,from 4 to 76 hpf). DNA damage expressed as % of DNA in tail in primary cells derived from 76-h-old zebrafish embryos (B) Genotoxicity following exposure to the EC_{50} of chloroform (0.85 mM), bromoform (0.2 mM), bromodichloromethane (BDCM, 0.26 mM) and chlorodibromomethane (0.16 mM). (C) Genotoxicity following exposure to the EC_{50} of tribromoacetic acid (TBA, 5.7 mM) and sodium bromate (49.2 mM). For each treatment and control groups, 100 cells were investigated, while 2 X 50 cells on two replicate slides were tested for treatments plots. *Significant different from control group (Dunnett's test with $p<0.05$).

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Table

Table 1. DBP regulations and guideline values (highest concentration allowed in drinking water).

U.S. EPA, Safe Drinking Water Act (SDWA)[a]

DBP	MCL (mg/L)
Total THMs	0.08
Haloacetic acids (HAA5)	0.06
Bromate	0.01

World Health Organization (WHO) guidelines[b]

DBP	Guideline value (mg/L)
Chloroform	0.2
Bromoform	0.1
Chlorodibromomethane	0.1
Bromodichloromethane	0.06
Dichloroacetic acid	0.05[c]
Trichloroacetic acid	0.2
Bromate	0.01[c]

European Union Standards

DBP	Standard value (mg/L)
Total THMs	0.1
Bromate	0.01[c]

[a] The total THMs represent the sum of the concentrations of four trihalomethanes: chloroform, bromoform, bromodichloromethane, and chlorodibromomethane. The haloacetic acids represent the sum of monochloro-, dichloro-, trichloro-, monobromo-, and dibromoacetic acid (U.S.EPA 2006).
MCL: maximum contaminant levels.
[b] WHO guidelines on THMs state that for authorities wishing to establish a total THM standard to account for additive toxicity the sum of the ratio of the concentration of each THM to its respective guideline value should not exceed unity.
[c] Provisional guideline value
[d] Where possible, without compromising disinfection, EU member states should strive for a lower value. This value must be met, at the latest, 10 calendar years after the issue of Directive (EU Directive 98/83/EC 1998); within 5 years of the Directive, a value of 0.025 mg/L must be met.

Table 2. Lethal and teratogenic effects evaluated in zebrafish embryos at 76 hpf.

Type	*Physiological/dysmorphogenic effect*	*Description*
Lethal	Coagulated egg	Denaturated fish egg. No clear structures of the embryo are observable anymore.
	Non-detachment of the tail from the yolk	From 16 hpf the tail begins to detach from the yolk and to extend.
	Non-development of somites	The somites are structures of the early trunk or tail segments that will eventually form the skeletal muscle, skin and cartilage.
	Lack of heartbeat	
Teratogenic	Malformation of the chorda	No tail, malformation of chorda or spinal cord.
	Malformation of the eyes	Abnormal pigmentation, small eyes or asymmetric eyes.
	Malformation of the ear	Formation of no, one or more than two otoliths per sacculus. Absence or abnormally shaped vesicles.
	Malformation of the head	Brain necrosis, hemorrhage or small head.
	Malformation of the heart	Pericardial oedema, big heart, hemorrhage or abnormal chambers.
	Malformation of the tail	Hemorrhage, tail necrosis, bent tail, bent or twisted tip tail.
	Abnormal pigmentation	

Table 3. The LC_{50}, EC_{50} values are represented with their 95% confidence intervals (CI) and teratogenic index (TI) of all water disinfection by-products tested. Minimum concentration that inhibits growth (MCIG) and EC_{20} effect concentrations at 76 hpf are also represented for all substances.

Substance	CAS-No	LC_{50} mM (95% CI)	EC_{50} mM (95% CI)	TI	EC_{20} mM (95% CI)	MCIG mM
Chloroform	67-66-3	2.1 (1.75 – 2.31)	0.85 (0.75 – 0.97)	2.5	0.7 (0.6 – 0.8)	1.26
Bromoform	75-25-2	0.52 (0.45 – 0.60)	0.20 (0.17 – 0.23)	2.6	0.15 (0.12 – 0.17)	0.1
BDCM	75-27-4	0.93 (0.80 – 1.06)	0.26 (0.20 – 0.32)	3.6	0.17 (0.10 – 0.22)	0.3
CDBM	124-48-1	0.45 (0.38 – 0.54)	0.16 (0.13 – 0.19)	2.8	0.11 (0.08 – 0.13)	0.06
DCA	79-43-6	> 46.5	28.9 (24.5 – 33.5)	-	22.1 (16.1 – 25.8)	46.5
TCA	76-03-9	> 42.8	> 42.8	-	> 42.8	> 42.8
DBA	631-64-1	> 20	> 20	-	> 20	10
TBA	75-96-7	12.7 (11.6 – 13.9)	5.7 (4.8 – 6.5)	2.2	4.4 (3.2 – 5.2)	2.6
BCA	5589-96-8	> 30	> 30	-	> 30	> 30
Sodium Bromate	7789-38-0	65.4 (57.6 – 73.0)	49.2 (42.0 – 56.6)	1.3	40.7 (25.3 – 46.0)	68.3

–, indicates that could not be calculated. Abbreviations used: CDBM (chlorodibromomethane),BDCM (bromodichloromethane), DCA (dichloroacetic acid), TCA (trichloroacetic acid), DBA (dibromoacetic acid), TBA (tribromoacetic acid) and BCA (bromochloroacetic acid).

Table 4. Frequency of endpoints observed at 76hpf in zebrafish embryos exposed to DBPs. Σt represents the sum of embryos in all test concentrations showing teratogenic effects for each specific endpoint. The percentage of embryos showing each endpoint was calculated respect the total number of embryos with teratogenic effects found in all test concentrations. Bold values indicate that the endpoint followed a concentration-response relationship and was observed in ≥50% of all embryos showing malformations.

	Chloroform		Bromoform		CDBM		BDCM		TBA		DCA		NaBr	
Malformation	Σ_t	(%)	Σ_t	(%)	Σ_t	(%)	Σ_t	(%)	Σ_t	(%)	Σ_t	(%)	Σ_t	(%)
Chorda	9	24.3	0	0.0	16	28.1	25	36.3	18	27.7	0	0.0	3	12.0
Ear	2	5.4	9	23.7	0	0.0	9	13.0	25	38.5	0	0.0	1	4.0
Head	4	10.8	14	36.8	19	33.3	9	13.0	19	29.2	0	0.0	1	4.0
Eyes	29	**78.4**	25	**65.8**	30	**52.6**	23	33.3	44	**67.7**	8	22.9	20	**80.0**
Heart	28	**75.7**	23	**60.5**	30	**52.6**	39	**56.5**	40	**61.5**	**31**	**88.6**	24	**96.0**
Tail	29	**78.4**	27	**71.0**	31	**54.4**	37	**53.6**	48	**73.8**	12	34.3	10	40.0

Abbreviations used: CDBM (chlorodibromomethane),BDCM (bromodichloromethane), TBA (tribromoacetic acid), DCA (dichloroacetic acid).

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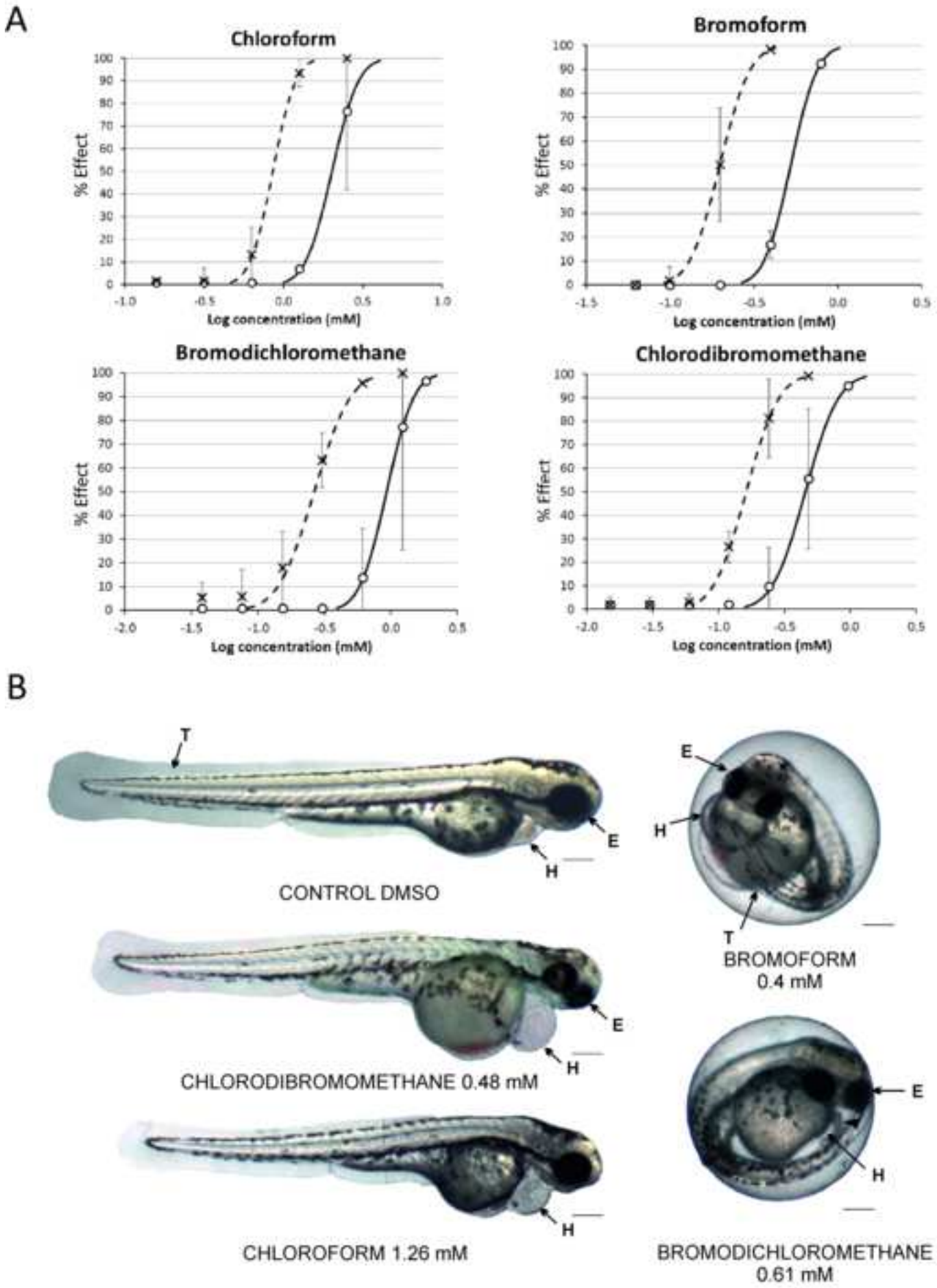

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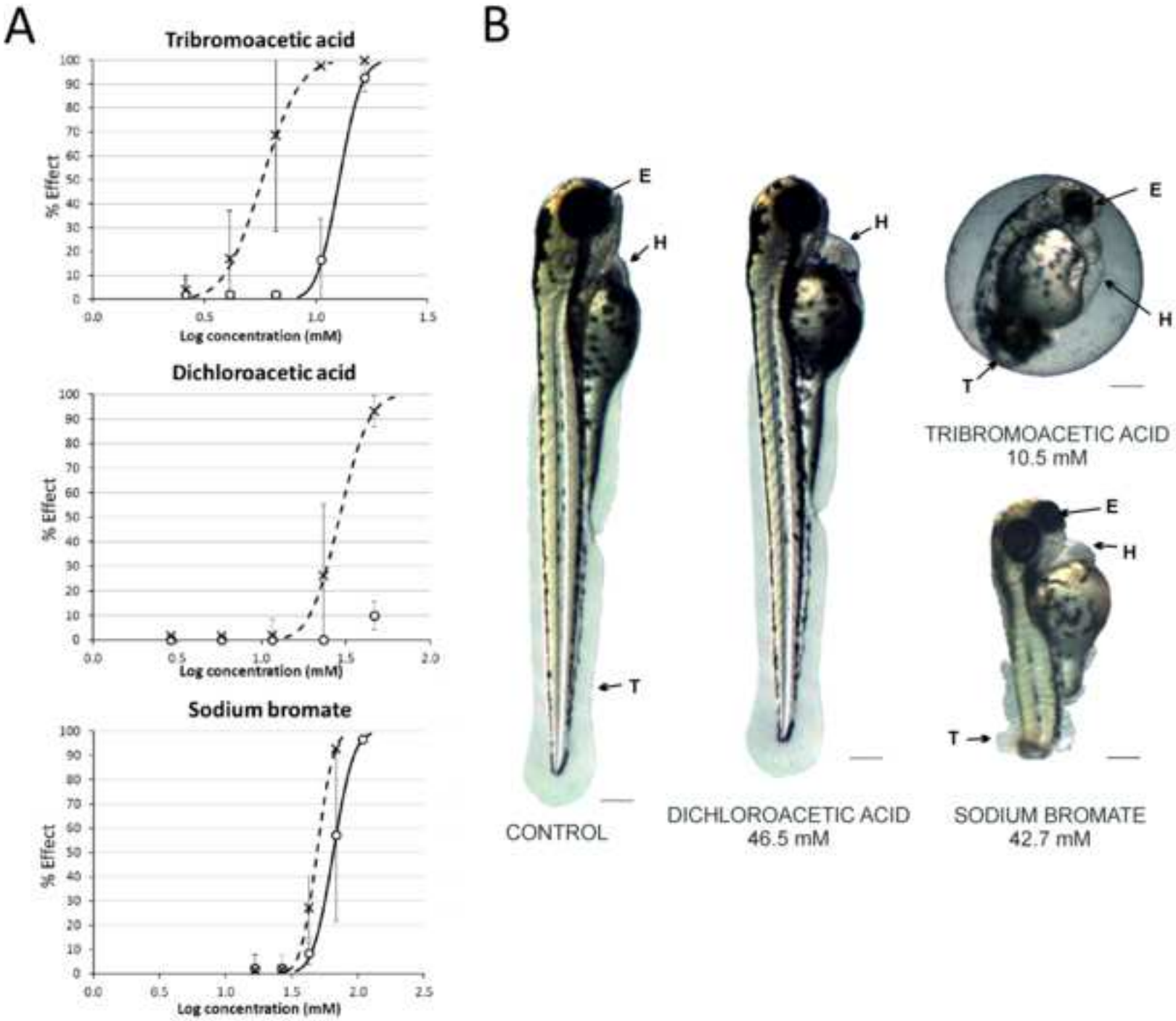

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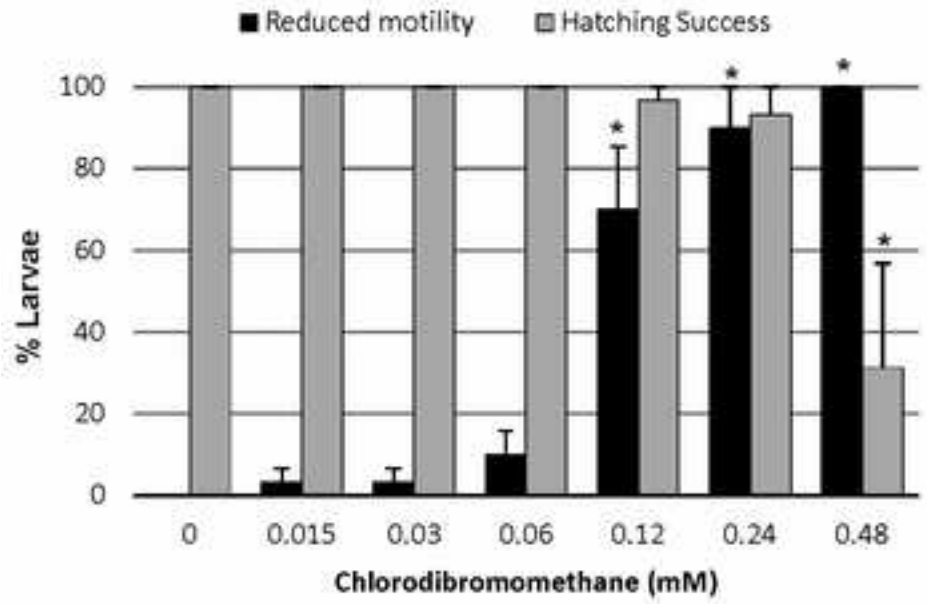

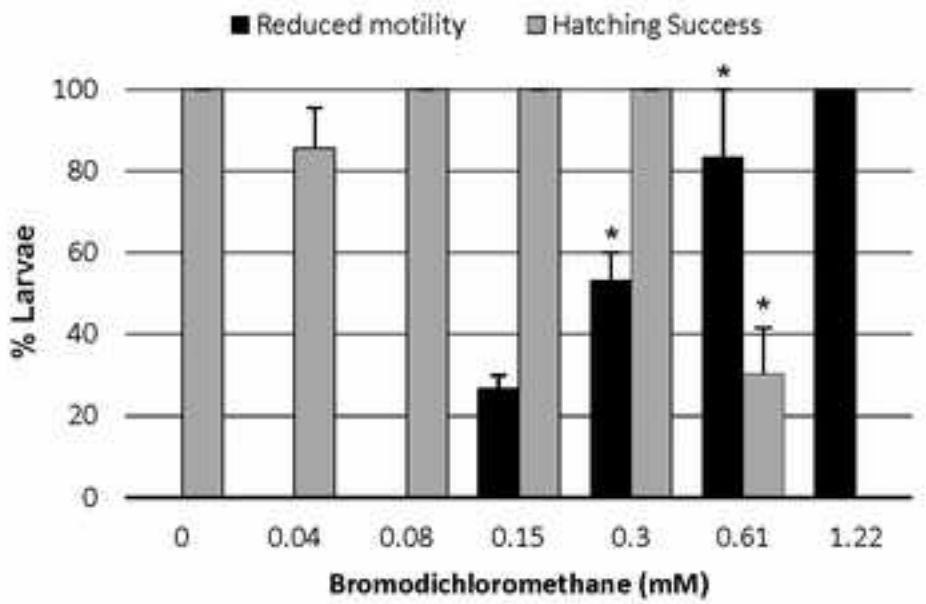

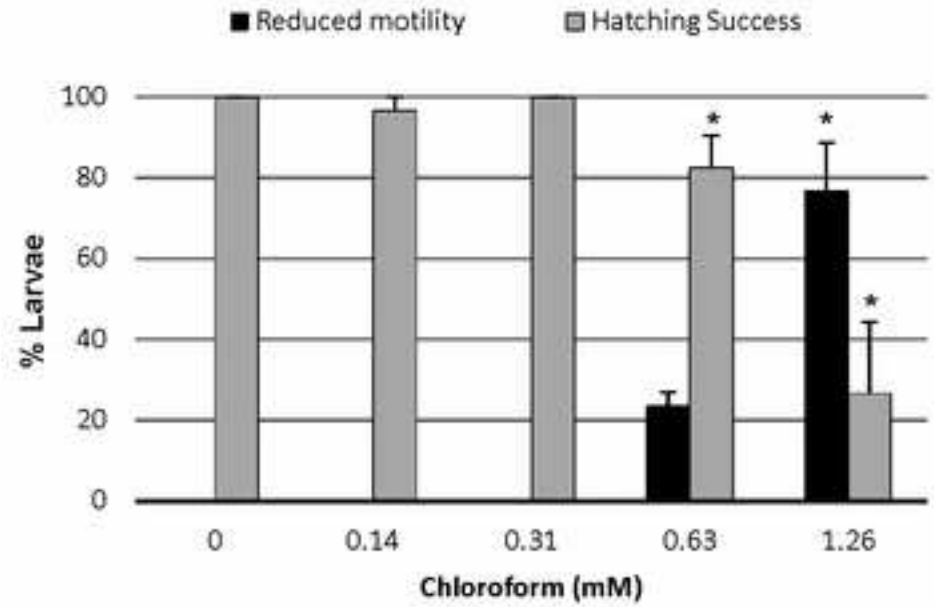

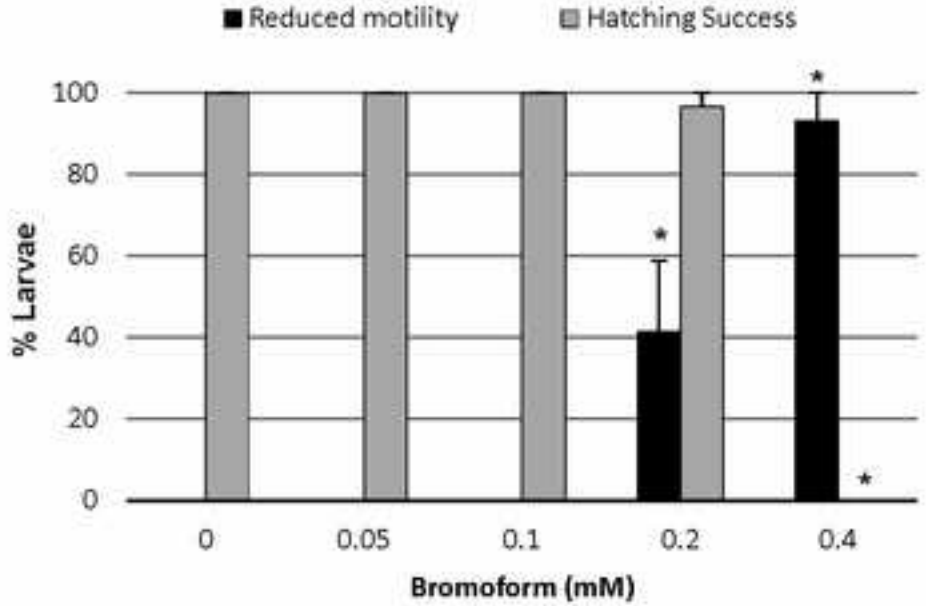

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