THE PENNSYLVANIA STATE UNIVERSITY
The Graduate School
College of Environmental Engineering

THE TERATOGENIC EFFECTS OF SIX DRINKING WATER DISINFECTION BYPRODUCTS ON ZEBRAFISH (DANIO RERIO) EMBRYOS

A Thesis in
Environmental Pollution Control

by
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ABSTRACT

Disinfection byproducts (DBPs) are produced as a result of treating water using disinfectants. Hundreds of DBPs have been identified with very little or no toxicological data available thus necessitating the use of additional methods for hazard estimation. In the framework of this study, five methodological points were broached: the variability of the selected endpoints during embryogenesis of control zebrafish embryos, the dose optimization and dose response of six DBPs, their relevance for the analysis of DBP toxicity, the most sensitive developmental stages of zebrafish embryos, and the potential for recovery after exposure to DBPs. The research was conducted using zebrafish (Danio rerio) embryos from 0 to 72 hours post fertilization (hpf). Observations of embryo development were made at different developmental stages, for which morphological, physiological, and behavioral endpoints were selected and quantified for untreated control and exposed embryos. The sensitivity, recovery, and endpoints of six model DBPs (iodoacetonitrile, bromoacetonitrile, iodoacetic acid, bromochloroacetic acid, dibromoacetic acid, and chloroacetic acid), with little known information on their toxic effects, were established. Lethal, sublethal (heart rate/edema, and spontaneous movements), and teratogenic effects were detected for all the studied compounds. The most sensitive developmental stage occurred between 0-5hpf, and recovery after exposure to DBPs was possible. The findings of this study will aid in a better understanding of the risks associated with these DBPs.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>BAN</td>
<td>Bromoacetonitrile</td>
</tr>
<tr>
<td>BCAA</td>
<td>Bromochloroacetic acid</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>CAA</td>
<td>Chloroacetic acid</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>Calcium carbonate</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Cl₂</td>
<td>Chlorine</td>
</tr>
<tr>
<td>DBAA</td>
<td>Dibromoacetic acid</td>
</tr>
<tr>
<td>DBP</td>
<td>Disinfection byproducts</td>
</tr>
<tr>
<td>DCAA</td>
<td>Dichloroacetic acid</td>
</tr>
<tr>
<td>DDI</td>
<td>Distilled and double de-ionized</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferases</td>
</tr>
<tr>
<td>HAA</td>
<td>Haloacetic acids</td>
</tr>
<tr>
<td>HAN</td>
<td>Haloacetonitriles</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>HT₅₀</td>
<td>Mean hatching rate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetic acid</td>
</tr>
<tr>
<td>IAN</td>
<td>Iodoacetonitrile</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lowest dose that kills 50%</td>
</tr>
<tr>
<td>MX</td>
<td>3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone</td>
</tr>
<tr>
<td>N-DBP</td>
<td>Nitrogenous water disinfection byproducts</td>
</tr>
<tr>
<td>NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Ammonia</td>
</tr>
<tr>
<td>NOEC</td>
<td>No-observable effect concentration</td>
</tr>
<tr>
<td>THM</td>
<td>Trihalomethanes</td>
</tr>
<tr>
<td>YSL</td>
<td>Yolk syncytial layer</td>
</tr>
</tbody>
</table>
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1.0 Introduction

1.1 Drinking Water Disinfection Byproducts

1.1.1 Background

Providing microbially safe drinking water is an important public health issue, and the use of chemical disinfection in the 20th century is rightly regarded as a major public health triumph. The main target of fresh water disinfection is to kill microbial and viral pathogens in order to eliminate waterborne diseases such as cholera and typhoid (Richardson et al., 2002). However, chemical disinfection has also produced an unintended health hazard, the potential for reproductive/developmental effects that may be linked to chemical disinfection byproducts (DBPs) produced during disinfection. Chemical disinfectants are effective for killing harmful microorganisms in drinking water, but at the same time, disinfectants are also powerful oxidants and react with both naturally occurring and synthetic organic matter along with bromide and iodide naturally present in most source waters (rivers, lakes, and many ground waters), forming DBPs. Chlorine, ozone, chlorine dioxide, and chloramines are the most common disinfectants in use today, and each produces its own suite of chemical DBPs (Richardson, 2005). While reducing the public health risk of acute infection by waterborne pathogens, the unintended generation of DBPs poses a chronic health risk. DBPs represent an important class of environmentally hazardous chemicals that carry long-term human health implications.

The two major classes of DBPs are trihalomethanes (THMs) and haloacetic acids (HAAs). Both are the most observed DBPs after the chlorination of wastewater and
drinking water. A third important class is the haloacetonitriles (HANs). At least four of these compounds are carcinogenic (chloroform, bromodichloromethane, bromoform, and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX)) (Richardson et al., 2000).

1.1.2 Haloacetic Acids

The HAAs represent the second largest group of DBPs generated by drinking water disinfection. The base forms of haloacetic acids are carboxylic acids in which halogen atoms can take the place of hydrogen atoms in acetic acid (CH$_3$COOH). There exist 9 common HAAs which can be arranged in 3 different groups of haloacetic acids. The number of halogen atoms which replace the hydrogen atoms at the alpha position define the 3 groups: monohaloacetic-, dihaloacetic-, and trihaloacetic-. Exposure to some HAAs have been shown to have toxic and mutagenic effects or lead to tumors. The chlorinated and brominated haloacetic acids have been evaluated for their mutagenicity and cytotoxicity in various animal studies. In general, brominated haloacetic acids are significantly more cytotoxic and genotoxic than their chlorinated analogues (Plewa et al., 2004). Therefore, the US Environmental Protection Agency (USEPA) and the World Health Organization (WHO) publish guidelines for some HAAs in drinking water (Agus et al., 2009).
1.1.3 Haloacetonitriles

Haloacetonitriles (HANs) are small nitrogenous water DBPs and most exist with two halogen substitutions (OECD, 1998). HANs are formed when organic material containing nitrogen is present during chlorination and chloramination. After chlorination, HANs are usually formed at lower concentrations than that of THMs and HAAs (Agus et al., 2009).

1.1.4 Chlorine

Chlorine is the most abundant and reactive of all halogens. Chlorination is the process of adding chlorine (Cl$_2$) into water:

$$\text{Cl}_2 + \text{H}_2\text{O} \rightarrow \text{HOCl} + \text{HCl}$$

Hypochlorites (mostly NaOCl) are often used in place of Cl$_2$ due to lower hazards during handling. The results of the reaction above are hypochlorous (HOCl) and hydrochloric acid (HCl). Hypochlorous acids react with many organic components and efficiently destroy many forms of bacteria and viruses. As a result of the chlorine reaction with organic matter, DBPs will be formed.

Chlorine has been the most used disinfectant for drinking water in the last 100 years. In the USA, it is still the primary disinfectant due to its strong oxidizing nature, long lasting residue, ease of application, and low cost. To date, more than 300 reported DBPs are formed from chlorination (Liang et al., 2003).
1.1.5 Chloramines

Chloramines are formed when ammonia is added to hypochlorous acid:

\[ \text{NH}_3 + \text{HOCl} \rightarrow \text{NH}_2\text{Cl} + \text{H}_2\text{O} \]

DBPs which are formed during chloramination often contain nitrogen. Another important fact is that in chloraminated water the concentration of formed THMs and HAAs are much lower than in chlorinated water. Chloramines are a weaker and more stable water disinfectant than chlorine. Chloramination of water forms the DBPs which are produced during the chlorination of water (Rieder, 2007) and nitrogenous DBPs, along with a new class of DBP, the iodoacids.

1.1.6 Iodoacids

Iodoacids are a new, toxicologically significant class of DBP that were identified as part of a U.S. Nationwide Occurrence Study (Weinberg et al., 2002).

1.1.6.1 Iodoacetic Acid

Iodoacetic acid, one of the five iodoacids identified for the first time in chloraminated drinking water, has recently been shown to be more genotoxic and cytotoxic to mammalian cells than all DBPs that have been studied (Hunter et al., 1996).
1.2 Defining Teratology

Teratology is the branch of embryology and pathology that deals with abnormal development and the production of congenital anomalies. Teratogen refers to any agent that causes a structural abnormality following fetal or embryologic exposure. Teratogens act with specificity in that they produce specific abnormalities at specific times during embryo development, as well as being species specific. To date, very few chemicals are proven teratogens. Usually, an increased prevalence of a particular birth defect leads to the discovery of a teratogenic agent. The number of suspected teratogens is on the rise due to an increase in the number of synthetic chemical compounds in use along with clinical recognition of subtle malformations as teratogenic effects (Shepard, 1979).

Teratogenic agents include: infectious (rubella, varicella) and physical (ionizing agents, hyperthermia) agents, maternal health factors (diabetes), environmental chemicals (organic mercury compounds, DBPs), and medications/drugs (prescription, recreational). Many agents can exhibit teratogenic effects under certain circumstances. Species differences, along with the dose and timing of exposure to a particular agent often determine the severity of the damage and the type of defect that occurs. The dose response is a threshold phenomenon: the greater the dose, the greater the effect. The time of exposure is another important concept, as certain stages of embryonic and fetal development are more vulnerable than others. In general, the stages of gestation can be divided into 3 developmental periods: early embryonic development, organogenesis, and late embryonic development. The period of organogenesis is the time when most structural teratogens induce their effect (Figure 1) (Tyl, 2000).
The etiology of the congenital malformations includes both genetic and environmental factors; but there is another large category labeled as unknown, (e.g., polygenic, multifactorial, spontaneous errors of development, and synergistic interactions of teratogens). To induce malformations, teratogens must cross the placenta or reach the developing embryo through some other route, which makes phenomenon of teratogenesis applicable to all organisms including those in which embryonic development occurs outside the female. Animal based studies of developmental toxicology provide the initial information on whether a drug or chemical constitutes a teratogenic risk substance. Typically, a range of doses administered via the most appropriate route is given to pregnant animals during the period of embryonic organogenesis and the outcomes are compared to untreated control animals (Ali, 2007).
1.3 Fish as Model Organisms

As the most numerous and phylogenetically diverse group of vertebrates, fish teach us important principles about fundamental processes in vertebrate evolution, development, and disease. Zebrafish (*Danio rerio*) have been used predominately in developmental biology and molecular genetics, but their value in toxicology is recognized (Hill et al., 2005). Fish have served as useful sentinels to detect environmental hazards, and as efficient, cost-effective model systems for mechanistic toxicology and risk assessment for many decades (Kane et al., 1996). To evaluate the toxicity of a chemical, it is essential to identify the endpoints of toxicity and their dose-response relationships, elucidate the mechanisms of toxicity, and determine the toxicodynamics of the chemical. In addition to detailed toxicological investigations of a single chemical, there also is a need for high-throughput, large-scale screening for toxicity of several hundred chemicals at a time (Hill et al., 2005).

1.3.1 Advantages of Zebrafish Models

More is probably known about “what is normal” in the zebrafish than any other fish species. This includes morphological, biochemical, and physiological information at all stages of early development and in juveniles and adults of both sexes. This makes using the zebrafish ideal for toxicology research where the objective is to identify adverse effects of chemical exposure (Hill et al., 2005). There are numerous advantages for the use of zebrafish as a toxicological model species (Spitsbergen et al., 2003). The main benefits of using zebrafish as a toxicological model over other vertebrate species are their size, husbandry, and early morphology. Unlike other fish species, zebrafish adults are
only approximately 1-1.5 inches long, which greatly reduces housing space and husbandry costs (Westerfield, 1995). The minute size of the larval and adult zebrafish minimizes costs through low quantities of dosing solutions and thereby creates limited volumes of waste for disposal (Hill et al., 2002). In addition, small embryos allow reasonable sample sizes to be tested together using a single cell-culture plate or series of Petri dishes to provide several experimental replicates at one time. This allows the creation of high-throughput screens for toxicity testing in which zebrafish grow and develop in small microformat screening plates. From the egg stage, zebrafish embryos can survive for several days in a single well of a 384-well plate through the absorption of yolk, and can be visually assessed for malformation (MacRae et al., 2003).

Aside from their size, this species is invaluable because of their high fecundity and transparent embryos. One pair of adult fish is capable of laying 200-300 eggs in one morning; if appropriately maintained, they can provide this yield every 5-7 days (Skidmore, 1965). The rapid maturation of zebrafish (sexual maturation occurs around 100 days) also allows easy experimentation for assessing chemicals for teratogenicity (Hill et al., 2005). Their optical transparency allows for easy development staging, identification of phenotypic traits during mutagenesis screening, and assessment of endpoints of toxicity during toxicity testing (Oxtoby et al., 1993). Whole-mount larval staining can be performed rather than first having to dissect the tissue or stain sections. The visualization of gene expression throughout the larvae is possible due to the optical clarity of the zebrafish tissues, which allows successful penetration for light microscopy (Andreasen et al., 2002b).
Zebrafish development has been well characterized (Kimmel, 1989; Kimmel et al., 1995). Because zebrafish eggs remain transparent from fertilization to when the tissues become optically dense and pigmentation is limited (at approximately 30-72 hours post fertilization (hpf)), unobstructed observations of the main morphological changes up to and beyond pharyngulation is possible. Therefore, using little magnification, adverse effects of chemical exposure on development of the brain, notochord, heart, and jaw, trunk segmentation, and measurements of size can be assessed quantitatively. Also, unlike rodents, embryological development can be continually followed in live individuals rather than in vitro maintenance of embryos and fetuses. In addition, zebrafish embryos that are malformed, missing organs, or displaying organ dysfunction, can usually survive substantially past the time in which those organs start to function in healthy individuals. For example, mutant strains of zebrafish such as silent heart, still heart, and slo mo (Chen et al., 1996), and toxicant-exposed embryos with heart abnormalities (Antkkiewicz et al., 2005) survive well beyond 24hpf when the heart normally begins to beat (Kimmel et al., 1995). This is in contrast to rodent embryos with malformed hearts that tend to die in utero (Hill et al., 2005).
1.4 Objective of Research

The aim of the present study was to determine the effects of six DBPs: chloro-, iodo-, dibromo-, and bromochloro- acetic acids as well as bromo- and iodo- acetonitrile on zebrafish Danio rerio embryos. The DBPs tested were selected for their prominence in drinking water and classification as teratogens on account of previous studies or due to the lack of experimentation performed on newly classified DBPs.

In this study, fertilized eggs of wild strain zebrafish that have reached the four-cell stage were subjected to various concentrations of the individual DBPs which were chosen based on a series of dose and time optimization tests. Embryos were examined under a laser capture microscope at various time points up to 72hpf. The findings of this study will aid in a better understanding of the risks associated with these DBPs.
2.0 Materials and Methods

2.1 Egg Production

Fertilized zebrafish eggs were obtained from adult zebrafish (*Danio rerio*) bred under standardized conditions. The genitors were purchased from Aquatica Tropicals, Plant City, FL at the juvenile stage and were adapted in the Levenson fish laboratory at the Penn State College of Medicine, Department of Pharmacology, Hershey, PA. They were used as genitors from 6 months old to 1.5 years old. Fish were kept in a dark room with a 12:12 hour day/night regimen in aquariums containing 100L of continuously renewed natural distilled and double de-ionized water (DDI). The dilution rate was adjusted in order to reach a total hardness of 100mg CaCO$_3$/L and a conductivity of 300µS/cm (S=Siemens), in a range of pH between 7.5 and 8.5. The water temperature was kept at 28.5°C. Adult fish were fed 2 times a day, 7 days a week, with brine shrimp (Brine Shrimp Direct, Ogden, UT) and salmon starter (Aquatic Habitats, Apopka, FL).

On the day before the experiment, phenotypic zebrafish females (18) and males (18) from the main aquarium were placed separately in specific spawning aquariums. A male and female zebrafish were each placed in one of 18 spawning aquariums equipped with 4mm-holed mesh bottoms to protect the eggs from being eaten. Spawning was induced in the morning once the light was turned on. The eggs were collected after 30-40 minutes from the bottom of each aquarium and were observed with a dissecting microscope to select embryos from genitors with less than 30% of unfertilized or division-blocked eggs. Next, the eggs were rinsed twice separately with DDI water and pooled. After egg collection, the adult zebrafish were returned to the main aquarium.
2.2 Test Solutions and Dose Optimization/Dose Response

Bromochloroacetic acid (BCAA) (97%), chloroacetic acid (CAA) (99%), dibromoacetic acid (DBAA) (97%), iodoacetic acid (IAA) (99%), bromoacetonitrile (BAN) (97%), and iodoacetonitrile (IAN) (98%) were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO.

The molarity of each DBP was converted to µM and designated as the high concentration. The high concentration test solutions were prepared by dissolving a known quantity of the DBP in 1L of DDI water and stirred vigorously. Then, 1µL of that test solution was mixed into another 1L of DDI water, for each DBP. For the lower concentrations, three 1:2 serial dilutions were performed for each DBP. The following concentrations were prepared (Table 1):

<table>
<thead>
<tr>
<th></th>
<th>BAN</th>
<th>IAN</th>
<th>BCAA</th>
<th>CAA</th>
<th>DBAA</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>14.4</td>
<td>13.8</td>
<td>11.4</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Medium-High</td>
<td>7.2</td>
<td>6.9</td>
<td>5.7</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
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<td>Medium</td>
<td>3.6</td>
<td>3.45</td>
<td>2.85</td>
<td>3.75</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>Low</td>
<td>1.8</td>
<td>1.73</td>
<td>1.43</td>
<td>1.88</td>
<td>1.88</td>
<td>1.88</td>
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</tbody>
</table>

Table 1. Range of concentrations (µM) prepared per DBP for dose optimization and dose response tests.
The initial dose-response relationship for each DBP was established by using the LD$_{50}$ (the standardized measure for expressing and comparing the toxicity of chemicals). A total of 180 zebrafish embryos were distributed into a 6-well plate, with 30 embryos and 10mL of the DBP per well. This was done for each DBP and concentration. The plates were incubated at 28.5°C for 72 hours after which the embryos were determined to be viable or nonviable. After the LD$_{50}$ for each DBP was identified, the medium concentration for each DBP was then designated as the new high concentration and three 1:2 serial dilutions were performed from this to create three lower concentrations. The following concentrations were prepared and used for the subsequent experiments (Table 2):

<table>
<thead>
<tr>
<th></th>
<th>BAN</th>
<th>IAN</th>
<th>BCAA</th>
<th>CAA</th>
<th>DBAA</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>3.6</td>
<td>3.45</td>
<td>2.85</td>
<td>3.75</td>
<td>3.75</td>
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</tr>
<tr>
<td>Medium-High</td>
<td>1.8</td>
<td>1.73</td>
<td>1.43</td>
<td>1.88</td>
<td>1.88</td>
<td>1.88</td>
</tr>
<tr>
<td>Medium</td>
<td>0.9</td>
<td>0.86</td>
<td>0.71</td>
<td>0.94</td>
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<td>Low</td>
<td>0.45</td>
<td>0.43</td>
<td>0.36</td>
<td>0.47</td>
<td>0.47</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Table 2. Range of concentrations (µM) per DBP tested after initial LD$_{50}$ identification.
2.3 Experimental Design

2.3.1 Development of Control Embryos

Thirty embryos were randomly distributed into each well of a 15.5 mL 6-well Falcon polystyrene tissue culture plate (Fisher Scientific, Pittsburgh, PA). Each well was filled with 10mL of DDI water. The water was totally renewed daily by transferring embryos into new 6-well plates. The plates were incubated at 28.5°C, with a photoperiod of 12h:12h light: dark.

The development of zebrafish embryos was observed until 72 hours post fertilization (hpf), after which the embryos were euthanized with Finquel (MS-222/tricaine) (Sigma-Aldrich, St Louis, MO) (200mg/L) solution. Normally, at this time all the control embryos are hatched and are free non-feeding larvae (Fraysse et al., 2006). The nonviable embryos (as defined later) were removed at each observation time.

2.3.2 Embryological Defects of DBPs

For each of the six DBPs, four concentrations (Table 2) were tested plus one control and two replicates for each condition. The complete test includes 25 conditions and 30 embryos per condition at the beginning of the exposure. The exposure procedure was the same as previously described for the experiment with control embryos. Following the egg collection, 30 embryos were randomly transferred into each well of an appropriated and clearly labeled plate. After 4, 8, 12, 18, 24, 48, and 72hpf of exposure, dead embryos were recorded and removed.
2.3.3 Sensitivity of Developmental Stages

Additional experiments were performed to better evaluate the developmental stage that is most sensitive to teratogens. The low concentration for each DBP: BAN 0.45µM, BCAA 0.36µM, IAN 0.43µM, CAA 0.47µM, DBAA 0.47µM, and IAA 0.47µM was used to determine the most sensitive time period. In the first experiment, 30 embryos were placed into each well of a 6-well polystyrene tissue culture plate along with DDI water. After 6hpf, all nonviable embryos were removed and the viable embryos were transferred to the low concentration for each DBP (Figure 2). The embryos were observed every 3 hours after being placed into the DBP until 72hpf. At each stage dead embryos were recorded and removed.

![Figure 2: Sensitivity test of zebrafish embryos using the low concentration for each DBP.](image)

30 embryos were placed in each well with 10mL of DDI from 0-6hpf. Then all viable embryos were transferred to 10mL of the low concentration per DBP from 6-72hpf. All viable embryos were recorded at 72hpf.
The second experiment monitoring sensitivity was performed as described above. This experiment allowed the embryos to acclimate in DDI water for the first 3 (Figure 3A), 12 (Figure 3B), or 24hpf (Figure 3C) after which all nonviable embryos were removed and all viable embryos were transferred to the low concentration for each DBP. The embryos were observed every 3 hours after being placed into the DBP until 72hpf. At each of these time points all dead embryos were recorded and removed.

Figure 3: Sensitivity test of zebrafish embryos using the low concentration for each DBP. (a) 30 embryos were placed into each well with 10mL of DDI from 0-3hpf, then all viable embryos were transferred to 10mL of the low concentration per DBP from 3-72hpf. (b) 30 embryos were placed into each well with 10mL of DDI from 0-12hpf, then all viable embryos were transferred to 10mL of the low concentration per DBP from 12-72hpf. (c) 30 embryos were placed into each well with 10mL of DDI from 0-24hpf, then all viable embryos were transferred to 10mL of the low concentration per DBP from 24-72hpf. All viable embryos were recorded at 72hpf.
2.3.4 Recovery

The high concentration for each DBP: BAN 3.6µM, BCAA 2.85µM, IAN 3.45µM, CAA 3.75µM, DBAA 3.75µM, and IAA 3.75µM, was used to determine the level of recovery. In the first experiment, 30 embryos were immediately placed in each well of a 6-well polystyrene tissue culture plate along with the high concentration for each DBP. After 12hpf, all nonviable embryos were removed and the viable embryos were placed in DDI water for the remaining 60hpf for a total of 72hpf (Figure 4).

![Diagram](image)

**Figure 4:** Recovery of zebrafish embryos was tested using the high concentration of each DBP. 30 embryos were placed into each well along with 10mL of the high concentration of each DBP for the first 12hpf. Then all viable embryos were placed into 10mL of DDI water for the remaining 60hpf for a total of 72hpf.
The second experiment monitoring recovery was performed as described above. This experiment allowed the embryos to acclimate in DDI water for the first 6hpf after which all nonviable embryos were removed and all viable embryos were placed into the high concentration of each DBP for 12hpf. After 18hpf the nonviable embryos were once again removed and the viable embryos were placed back into DDI water for the remaining 54hpf for a total of 72hpf monitored (Figure 5).

![Diagram](image.png)

**Figure 5:** Recovery of zebrafish embryos was tested using the high concentration of each DBP. 30 embryos were placed into each well along with 10mL of DDI water for the first 6hpf. Then all viable embryos were placed into 10mL of the high concentration of each DBP for 12hpf. Next all viable embryos were placed back into 10mL of DDI water for the remaining 54hpf for a total of 72hpf.

2.4 Endpoint Selection and Observation

The observations of embryos were made at distinct stages which represent important steps of zebrafish development (Table 3). They were classified into three toxicological categories: lethal, sublethal, and teratogenic (Fraysse et al., 2006), connected to seven observation times (4, 8, 12, 18, 24, 48, and 72hpf). The choice of the selected endpoints was based on the possibility of an easy measurement and handling. The distinction between normal and abnormal development was established using the zebrafish embryogenesis description published by Kimmel et al. (1995).
Table 3 represents all the selected endpoints. Two categories were used: quantitative and semiquantitative. For the latter, only their occurrence for each observation time was considered.

<table>
<thead>
<tr>
<th>Developmental period*</th>
<th>Epiboly</th>
<th>Segmentation</th>
<th>Pharyngula</th>
<th>Hatching</th>
<th>Early larval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (hpf)</td>
<td>4-10 hpf</td>
<td>10-24 hpf</td>
<td>24 hpf</td>
<td>48 hpf</td>
<td>72 hpf</td>
</tr>
<tr>
<td>Characterization</td>
<td>Epiboly in progression</td>
<td>Somite pairs form Tail development</td>
<td>Primary organ rudiments</td>
<td>Extended tail</td>
<td>Developed somites Head, eyes, and otolith formed</td>
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<td>Toxicological endpoints</td>
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<td></td>
<td></td>
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<td></td>
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<td>Lethal</td>
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</tr>
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<td>√</td>
<td>√</td>
<td>√</td>
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</tr>
<tr>
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<td></td>
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<td>Heartbeat</td>
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<td>Spontaneous movement</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td></td>
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</tr>
<tr>
<td>Development of the eyes</td>
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<td></td>
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</tr>
<tr>
<td>Development of the otolith</td>
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<tr>
<td>Blood tail circulation</td>
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<tr>
<td>Heart rate</td>
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<td>Edema (pericardial, yolk sac)</td>
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</tr>
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<td>Tail malformation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Length of tail</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Defined by Kimmel et al. (1995).

Table 3. Main development period of early development of zebrafish and corresponding toxicological endpoints considered
The observations were performed directly in the wells using a Zeiss PALM Robo Microscope. The rate of nonviable embryos was determined by counting at 4hpf, 8hpf, 12hpf, and 18hpf the unfertilized eggs and the eggs blocked at blastomere stage. For the following steps, embryos and pro-larvae were considered dead when no heartbeat was detected. Except at 72hpf, all the observations were performed on live embryos, rolled up in the chorion and tossed by spontaneous movements. No quantification of the morphological impairment can be easily objectively proposed. Therefore, for all morphological features checked in Table 1, only the most obvious abnormalities, compared with control, were recorded.

The 24hpf embryos present spontaneous lashing movements of the tail. Each movement was counted using a manual counter for 20sec. At 48hpf, the heart is formed by two chambers and presents a regular heart rate. This endpoint was calculated using a manual counter for 1min.

From 48hpf the embryos are able to hatch. The number of hatched pro-larvae was recorded until 72hpf. A pro-larva is considered hatched when the entire body (from tail to head) is out of the chorion. The hatching rate was calculated for each well as the percentage of hatched larvae from the 30 embryos per well. Then, the number of hatched embryos in each replicate was pooled to calculate the mean hatching time (HT$_{50}$).

At the end of the experiment, pro-larvae were photographed (Zeiss PALM Robo Microscope) positioned on the lateral side (Figure 6). Image analysis allowed the quantification of three different endpoints: the tail (number of somites) length, the frequency of tail curvature (lordosis – abnormal ventral curvature of the vertebral column, accompanied by abnormal calcification of the afflicted vertebrae, kyphosis –
abnormal outward curvature of the vertebral column), and the pericardial area (membranous sac surrounding the heart). The tail length was measured from the beginning of the first somite (anterior part) to the end of the most posterior one (Figure 6A). The tail curvature was estimated by drawing a straight line along the margin of the back axis (Figure 6B). This approach helps easily distinguish lordosis (drawn line upper the back margin) and kyphosis (drawn line down the back margin). The pericardial area was measured by circling the pericardial space using conventional image software (Adobe Illustrator CS4, Adobe Systems Inc.). All the picture treatments were performed using this software and the quantification (length and area) were made with SigmaScan Pro 5.0 (Systat Software Inc., San Jose, CA).

Figure 6. Zebrafish free larva at 72hpf (A) and picture analysis performed. Tail length (A); tail curvature (B1, normal morphology; B2, lordosis-arrow head); pericardial area (C). Scale bar: 0.2mm. Adapted from Fraysse, 2005.
2.5 Statistical Analysis

Statistical comparisons made between varying concentrations of one DBP were made by one-way ANOVA followed by Dunnett’s post hoc multiple comparisons tests (with concentrations compared to water control data), with $p<0.05$ considered statistically significant. Statistical analysis of similar concentrations of multiple DBPs were made by one-way ANOVA followed by student Neuman Keuls post hoc testing, with $p<0.05$ considered statistically significant. ANOVA statistical comparisons were made using SigmaStat 3.1 (Systat Software Inc, San Jose, CA).
3.0 Results

3.1 Dose Optimization/Dose Response

At the beginning of the experiment 750 embryos were used for each optimization experiment over a 72hpf period. The actual concentration ranges used for each DBP can be seen in Table 1. Each concentration range per DBP varied, therefore the high dose for each DBP were labeled “high” while the lower concentrations were labeled “medium-high”, “medium”, and “low”, respectively. The initial dose-response relationship for each DBP was established by using the LD$_{50}$ (the standardized measure for expressing and comparing the toxicity of chemicals). For each of the six DBPs, the LD$_{50}$ (the dose that kills half or 50% of the animals tested) fell within the low concentration range (Figure 7 and Figure 8). All the DBPs tested at each concentration in this experiment were statistically different from the control: BAN, CAA, DBAA (P=0.029); BCAA, IAN, IAA (P<0.001).
Figure 7. Initial dose response curve experiment. For each DBP, 50% or more of the embryos died at the low concentration. BAN=1.8μM, IAN=1.88 μM, BCAA=1.43 μM, CAA=1.88 μM, DBAA=1.88 μM, and IAA=1.88 μM.
In the second dose optimization experiment, the medium concentration for all six DBPs became the high concentration, and three lower concentrations per DBP were tested (Table 2). The LD$_{50}$ fell within the medium-high range for BCAA, IAN, CAA, and DBAA. BAN and IAA had 50% lethality in the medium range (Figure 9 and Figure 10). Therefore, the LD$_{50}$ for all six DBPs were: BCAA 1.43µM, BAN 0.9µM, IAN 1.73µM, IAA 0.94µM, CAA 1.88µM, DBAA 1.88µM. All the DBPs used at each concentration in this experiment were statistically different from the control: BAN (P=0.002), CAA (P=0.029), DBAA (P=0.029), BCAA (P=0.007), IAN (P=0.029), and IAA (P=0.029).
Figure 9. Final results for the dose response experiment. For DBPs BAN and IAA, 50% or more of the embryos died at the medium concentration, 0.9 µM and 0.47 respectively. For DBPs BCAA, IAN, CAA, and DBAA, 50% or more zebrafish embryos died at the medium-high concentration, 1.43 µM, 1.73 µM, 1.88 µM, and 1.88 µM respectively.
3.2 Development of Control Embryos

A total of 120 embryos were used for this experiment. The initial mortality, only due to involution of the blastomere stage, was 12%. No dead embryos were observed during the following steps. Considering the 72hpf period data, very few morphological abnormalities were observed. The development of the embryos was characterized with only 2% of free pro-larvae with a weak kyphosis and one organism was not pigmented (albino).
3.2.1 Spontaneous Movements

Between 0 and 5 movements per 20 seconds per embryo at 24hpf were recorded. The values are discrete and the distribution is not symmetric over the mean (Figure 11). The embryos moved 2 or 3 times per 20 seconds, 85% for all embryos. At 24hpf, the movements of the tail are considered spontaneous because they are induced by the development of the motoneurons without any control by the central nervous system (Kimmel et al., 1995).

![Spontaneous Movements of Control Embryos](image)

**Figure 11. The frequency of spontaneous movements for the control group.** The embryos moved 2 or 3 times per second, 85% for all embryos at 24hpf.
3.2.2 Heart Rate, Pericardial Area, and Tail Length

The three parameters: heart rate, pericardial area, and tail length, show a Gaussian distribution and are described by a Normal law. The heart rate is described by discrete values. The mean for the control group was 139 beats/min, with a coefficient of variation of 6.4%. The recorded values ranged from 136 to 142 beats/min. The pericardial area ranged between 0.016 and 0.037mm². The mean of the control was 0.026 mm². The tail length for the control group ranged between 2.37 and 2.89 mm. The mean was 2.67 mm and the coefficient of variation was 3%.

3.2.3 Hatching Rate and Frequency

For the control group, 100% of the embryos were hatched at 72hpf and there was a time lag between the first and the last hatch of 24 hours. The hatching process is not synchronous and a wide difference can appear between multi-well plates. For example, at 48hpf, the percentage of hatched larvae ranged from 20% to 80% per plate. The estimated HT₅₀ (IC₉₅%) is 65hpf (60-68hpf) with correlation factors (R²) of 0.98.

3.3 Embryological Defects of DBPs

Table 4 presents the results for the six DBPs. The presented values are NOEC (no-observable effect concentration), that is, the highest tested concentrations without effect on the considered endpoints.

Under the experimental conditions, regardless of the DBP, the early embryo mortality was due to non-development of the blocked embryos at the blastomere stage. These observations were recorded at 4hpf and at 8hpf. The frequency of undeveloped
embryos was independent of the DBP concentration. The mean proportion of viable embryos after 8hpf was 89% for BAN, CAA, DBAA and BCAA, 96% for IAN, and 86% for IAA. These values are in accordance with the various ISO guidelines requiring at least 70% of initial survival (Fraysse, 2004). Moreover, at 8hpf, no morphological abnormalities or development delays were detected whatever the DBP and concentration, suggesting the requirement for a minimal duration exposure to result in discernable changes.

<table>
<thead>
<tr>
<th>From 4 to 24hpf</th>
<th>BAN (µM)</th>
<th>BCAA (µM)</th>
<th>IAN (µM)</th>
<th>IAA (µM)</th>
<th>CAA (µM)</th>
<th>DBAA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulate eggs</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
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</tr>
<tr>
<td>Development delay</td>
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<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Heartbeat</td>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Edema (pericardial, yolk sac)</td>
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<td>0.93</td>
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<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>Spontaneous movements</td>
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<td>—</td>
<td>1.73</td>
<td>—</td>
<td>—</td>
<td>1.88</td>
</tr>
<tr>
<td>Development of the eyes</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Development of the otoliths</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<table>
<thead>
<tr>
<th>At 48hpf</th>
<th>BAN (µM)</th>
<th>BCAA (µM)</th>
<th>IAN (µM)</th>
<th>IAA (µM)</th>
<th>CAA (µM)</th>
<th>DBAA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood tail circulation</td>
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<td>0.43</td>
<td>1.88</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>0.43</td>
<td>0.46</td>
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<td>1.88</td>
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<table>
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<th>At 72hpf</th>
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<th>IAN (µM)</th>
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<th>DBAA (µM)</th>
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<tr>
<td>Dead pro-larva</td>
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<tr>
<td>Hatching rate</td>
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<tr>
<td>Tail malformations</td>
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<tr>
<td>Length of tail</td>
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Table 4. The effects of DBPs (BAN, BCAA, IAN, IAA, CAA, and DBAA) on zebrafish early life stages. The values are the highest tested concentration (µM) without effect on the considered endpoint. —, indicates no-effect at the highest tested concentration (NOEC).+
3.3.1 Effects of Iodoacetonitrile

A significant increase in the number of movements per 20 seconds was measured for the high concentration (3.45 µM) after 24hpf. Whereas no embryos of the control group moved 5 times or more during this time, 45% of the 3.45 µM exposed embryos did. Moreover, pericardial edemas were found for 73% of the embryos exposed to this concentration.

At 48hpf, the morphology of the embryos exposed to the high concentration (3.45 µM) was widely impaired. 52% of the embryos had a large pericardial edema, a weak pigmentation, tail curvatures (lordosis), and a lack of caudal blood circulation.

Moreover, at this concentration 15% were dead (no heart beat). IAN induces a heart rate reduction proportional to the exposure concentration. The lowest treatment statistically significant was 0.86 µM with 133 beats/min. The heart rate reached 125 beats/min for 3.45 µM (Figure 12). Finally, the edemas detected at 48hpf were still observable, but only for the embryos exposed to 3.45 µM. The pericardial area increase was 1.4 times compared to control.
At 72hpf only 6% of the embryos exposed to 3.45 µM of IAN hatched, and 12% died. But no effect on the hatching time was noted.

All the pro-larvae exposed to IAN had tail deformations for all ranges of the tested concentrations. At 72hpf the pro-larvae tails were weakly twisted, as with the other parameters, a positive correlation with the exposure concentration was observed. For the three first concentrations: 0.43 µM, 0.86 µM, and 1.73 µM, 11%, 25%, and 47% of pro-larvae showed mainly kyphosis; 100% of the embryos exposed to 3.45 µM presented lordosis (Figure 13). Moreover, at this concentration, shorter tails (reduction of 4.2%, P<0.01) and smaller pericardial areas (reduction of 16%, P<0.01) were measured (Figure 14).
Figure 13. Effect of IAN on frequency of tail deformations (lordosis, kyphosis, and total of impaired embryos).

Figure 14. Abnormal development of zebrafish embryo exposed to IAN. 100% of the embryos exposed to 3.45 µM of IAN at 72hpf present lordosis and shortening of the tail.
3.3.2 Effects of Iodoacetic Acid

The first observed effect of IAA on embryo development was pericardial edema. At 24hpf pericardial edema were noted for 47% of the embryos exposed to 3.75 μM.

The major development abnormalities appeared at 48hpf, with mainly pericardial and yolk sac edemas and a lack of caudal blood circulation. The frequency of impaired embryos increased with the exposure concentration: 23% of the controls and 0.46 μM exposed embryos presented weak deformations, and the proportion significantly increased up to 86% at concentrations of 1.88 μM and 3.75 μM. Embryos from both higher concentrations had the same edema frequency, but those exposed to 3.45 μM were much more deformed (Figure 15). IAA induces a heart rate reduction similar to that of IAN, where the reduction is proportional to the exposure concentration. The lowest treatment statistically significant was 1.88 μM, with 132 beats/min. The heart rate reached 121 beats/min for 3.75 μM (Figure 16).

At 72hpf, 83% of all the pro-larvae exposed to 3.75 μM of IAA had tail deformations, with mainly lordosis. Moreover at this concentration, shorter tails (reduction of 3.3%, P<0.01) and smaller pericardial areas (reduction of 14%, P<0.01) were measured.
Figure 15: Abnormal development of zebrafish embryo exposed to IAA. 47% of embryos exposed to 3.75 µM of IAA had severe pericardial edema.

IAA Heart Rate

![Bar chart showing heart rate (Beats/min) for different concentrations of IAA (µM).]

Figure 16. Effect of IAA on heart rate at 48hpf (mean ± SD).
3.3.3 Effects of Bromoacetonitrile

A significant increase in the number of movements per 20 seconds was measured for the high concentration (3.6 µM) at 24hpf. Whereas no embryos of the control group moved 5 times or more during this time, 37% of the 3.6 µM exposed embryos did. Pericardial edemas were measured for 59% of the embryos exposed to this concentration.

At 48hpf, the morphology of the embryos exposed to the high concentration (3.6 µM) was widely impaired. 46% of the embryos had a large pericardial edema and a weak pigmentation. Moreover, at 3.6 µM, 9% were dead (no heart beat). BAN induced a change in heart rate proportional to the exposure concentration. The lowest treatment statistically significant was 1.8 µM, with 128 beats/min. The heart rate reached 115 beats/min for 3.6 µM (Figure 17).

![BAN Heart Rate](image)

**Figure 17.** Effect of BAN on heart rate at 48hpf (mean ± SD).
At 72hpf only 10% of the embryos exposed to 3.6 µM of BAN hatched, and 7% died. But no effect on the hatching time was registered. All pro-larvae exposed to BAN had tail deformation for all ranges of the tested concentrations. For the three first concentrations: 0.45 µM, 0.9 µM, and 1.8 µM, 9%, 28%, and 42% of pro-larvae showed mainly kyphosis. 100% of the organisms exposed to 3.6 µM presented lordosis (Figure 18). Moreover, at this concentration shorter tails (reduction of 2.9%, P<0.01) and smaller pericardial areas (reduction of 12%, P<0.01) were measured. The edemas detected at 48hpf were still observable but only for the embryos exposed to 3.6 µM. The pericardial area increase was 1.9 times compared to control (Figure 19).

![Frequency of BAN Tail Deformations](image)

**Figure 18.** Effect of BAN on frequency of tail deformations (lordosis, kyphosis, and total of impaired embryos).
3.3.4 Effects of Bromochloroacetic Acid

The first observed effect of BCAA on embryo development was at 72hpf. Minimal pericardial edemas were recorded with their frequency increasing with the exposure concentrations. A significant effect was measured for 2.85 µM, and the percentage of impaired embryos was 40%. Morphological aspects and intensity of the deformations were similar to those observed after an exposure of 0.43 µM of IAN. The pro-larvae presented a shorter tail at the concentration of 2.85 µM with a length reduction of 4.5%.
3.3.5 Effects of Dibromoacetic Acid

One of the initial observed effects of DBAA on embryonic development was a decrease in the number of spontaneous movements at 24hpf. In relation to the increase of the concentration, the proportion of embryos waved by one movement or more per 20 second changed from 96% for the control to 15% (3.75 µM). Except for embryos exposed to 0.94 µM, all the treated groups presented statistical differences from the control.

At 48hpf, DBAA induced a heart rate increase proportional to the exposure concentration. The lowest treatment statistically significant was 1.88 µM, with 157 beats/min. The increase reached 163 beats/min for 3.75 µM (Figure 20).

![DBAA Heart Rate](image)

**Figure 20. Effect of DBAA on heart rate at 48hpf (mean ± SD).**

At 72hpf, the pro-larvae presented a shorter tail at the concentration of 3.75 µM with a length reduction of 5.8%. The most important morphological effect of DBAA at 72hpf was detected in the pericardial area. At the low concentration (0.46 µM) this area
was smaller than the control, corresponding to only 73% of the value of the control pericardia (P<0.01). Thereafter, it increased gradually at 0.94 µM with 81% (P<0.05), until having a comparable surface with the control at the high concentration 3.75 µM (94%, P=0.48).

3.3.6 Effects of Chloroacetic Acid

The first observed effect of CAA on embryo development was at 72hpf. Weak pericardial edema were recorded with their frequency increasing with the exposure concentrations. A significant effect was measured at 3.75 µM, and the percentage of impaired embryos was 37%. Morphological aspects and intensity of the deformations were similar to those observed after an exposure of 0.43 µM of IAN. The pro-larvae presented a shorter tail at the concentration of 3.75 µM with a length reduction of 3.9%.

3.4 Sensitivity

The low concentration for each DBP: BAN 0.45µM, BCAA 0.36µM, IAN 0.43µM, CAA 0.47µM, DBAA 0.47µM, and IAA 0.47µM was used to determine the most sensitive time period after an acclimation period of 3, 6, 12, and 24hpf in DDI water (Figure 3A, Figure 2, Figure 3B, and Figure 3C, respectively). A significant effect was measured at the low concentration for each DBP, after a 3hpf acclimation period in DDI water (P<0.01). The death rate of the zebrafish embryos ranged from 13.33% to 86.67% after 72hpf. There were no significant mortality rates for the 6, 12 and 24hpf DDI acclimation periods for each DBP (Figure 21).
3.5 Recovery

The high concentration for each DBP: BAN 3.6µM, BCAA 2.85µM, IAN 3.45µM, CAA 3.75µM, DBAA 3.75µM, and IAA 3.75µM, was used to determine the level of recovery. In the first recovery experiment the embryos were exposed to each DBP for 12hpf, after which all viable embryos were placed in DDI water for 60hpf for a total of 72hpf (Figure 4). After 72hpf, at the high concentration for each DBP, there was a significant effect measured between the mortality of zebrafish embryos that were exposed solely to the DBP as compared with the embryos that were exposed to the DBP for 12hpf before being placed into DDI water for the remaining 60hpf (P<0.01). For
each DBP there was at least a 15% increase in the mortality of the embryos that were subjected to just the DBP (Figure 22).

**Recovery: Experiment I**

![Graph showing mortality percentages for different concentrations of DBPs.]

**Figure 22. Recovery of zebrafish embryos after 12hpf in high concentration of each DBP.** The % mortalities were significantly decreased for those embryos that were exposed to the DBPs for 12hpf before being placed in DDI water for the remainder of the 72hpf, as opposed to those zebrafish embryos that were subjected to the DBPs for 72hpf.

In the second recovery experiment, the embryos were placed in DDI water for 6hpf after which all viable embryos were placed in each DBP for 12hpf. The remaining viable embryos were then placed back into DDI water for 54hpf for a total of 72hpf (Figure 5). After 72hpf at the high concentration for each DBP, there was a significant effect measured between the mortality of zebrafish embryos that were exposed solely to the DBPs as compared with the embryos that were first acclimated in DDI water for 6hpf before being placed in the high concentration for 12hpf and then back to DDI water for
the remainder of the 72hpf (p<0.01). For each DBP there was at least a 40% increase in the mortality of the embryos that were subjected to just the DBP (Figure 23).

**Figure 23.** Recovery of zebrafish embryos after a 6hr acclimation period in DDI, followed by 12hpf in the high concentration per DBP, and then back to recovery in DDI water for the remainder of the 72hpf. There is a significant difference in the % mortalities for those zebrafish embryos that were solely exposed to the DBP.
4.0 Discussion

In the framework of this study, five methodological points were broached: (1) the variability of the selected endpoints during embryogenesis of control zebrafish embryos, (2) the dose optimization and dose response of the six DBPs, (3) their relevance for the analysis of DBP toxicity, (4) the most sensitive developmental stages, and (5) the potential for recovery after an exposure to DBPs.

4.1 The Selected Endpoint – Case of Control Zebrafish Embryos

The frequency of abnormalities, not including mortalities, detected among control embryos, including the control groups used during DBP toxicity experiments, was very low, less then 1%. It has already been reported that appropriate rearing conditions (temperature, water quality) and healthy genitors are suitable for a very low embryo mortality and low morphological heterogeneity. However, good rearing conditions are not sufficient to avoid any early mortality. In the various standards and guidelines, the validity threshold for the control is 30% of undeveloped embryos at 24hpf (Fraysse et al., 2004). In this study, the early mortality rates were between 5% and 25%.

4.1.1 Spontaneous Movements

The modifications of the spontaneous movements and the heart rate of zebrafish during early development have been widely documented from a genetic, physiological, and morphological point of view.

These spontaneous movements may be due to the development of functional neuromuscular junctions or spontaneous depolarization of the muscle cell membrane.
The development of the locomotor network of zebrafish has been extensively reviewed and it is reported that at the 14-somite stage, at 20hpf, the first movements can be recorded, corresponding to the beginning of the somite innervations. There is a peak of activity at the 20-somite stage (≈24hpf) with 7 movements per 20 sec at a temperature of 28.5°C (Drapeau et al., 2002). For this developmental stage, in this experiment at 28.5°C, 85% of the embryos moved 2 or 3 times per 20 sec. Then the embryo activity decreased linearly at a rate of 0.5 movements per 20 sec during the following 6-7 hours. These movements may be due to the presence of functional neurons adjoining the somite, covered by action potential, but without any biochemical control mechanisms developed yet. The first reflex movement is recorded at the 26-somite stage (≈27hpf). After the prim-15 stage (≈35hpf), the motoneuronal system is installed and embryos present very few non-controlled movements (Myers et al., 1997).

4.1.2 Heart Rate

The first beats of the heart are arrhythmic and uncontrolled (Kimmel et al., 1995). At 20hpf, the heart tube is formed, not fully connected to the developing circulatory system but able to contract itself (Fraysse et al., 2004). The two cardiac chambers (ventricle, atrium) begin to be differenced at 30hpf, and at 36hpf the heartbeat is pronounced and regular (Barrionuevo and Burggren., 1999). The heart rate is directly linked to the ambient temperature. The values measured during this study, at 28.5°C, are 139±3 beats per minute. Barrionuevo and Burggren (1999) have measured this parameter at 25, 28, and 31°C, and recorded respectively 17.5, 20.8, and 25.8 beats/10 sec (105, 145, 155bpm, respectively). Also for 28°C, Kimmel et al., (1995) reported a value of 30
beats/10 sec (180bpm). Hassoun, Kariya, and Williams (2005) show 91.9bpm at 32hpf and 121.8bpm at 55hpf.

4.1.3 Hatching

Hatching is considered to be a key point in the life cycle of fish but not for an ontogenic point of view (Kimmel et al., 1995). For the zebrafish, it does not correspond to a defined embryonic stage. It is a sporadic event and spreads over a period of 24 hours or more, as shown in the results of this experiment. Whether or not an embryo has hatched, its development progresses and generally organisms that have hatched early are not more developed than ones remaining in the chorion (Kimmel et al., 1995). Despite this variability, the HT\textsubscript{50} calculated for the control groups was 65±3hpf.

4.2 Embryotoxicity of DBPs

In the present study of representative DBPs, note of the paper by Reider (2007) shows that there are literally hundreds of DBPs. The model system used in the present study is one of the few using the early zebrafish embryo (for example, see the study of a single DBA (DCA) by Hassoun et al., 2005). The use of intact amphibian embryo has also been utilized by Weber et al., (2004). The use of the isolated rodent fetus is closer to the potential application to the human condition, but the model system cannot be readily used for large sampling of DBP effects.
4.2.1 Effects of Iodoacetonitrile

Iodoacetonitrile (IAN) is a type of haloacetonitrile (HAN), which are toxic, nitrogenous water disinfection byproducts (N-DBPs) (Reider, 2007) and are observed with chlorine, chloramine, or chlorine dioxide disinfection which induce acute genomic DNA damage (Muellner et al., 2007).

IANs toxic effects are not or little known for all organisms. Muellner et al. (2007) analyzed seven HANs for their acute genotoxicity and chronic cytotoxicity in animal experiments with Chinese hamster ovary (CHO) cell line assays. IAN showed no mutagenic effect but at low concentrations IAN was shown to be the second most cytotoxic HAN and the most genotoxic HAN of the seven analyzed. Ahmed et al. (1989) studied the mechanism of HAN toxicity by inhibition of rat hepatic glutathione S-transferases (GST) in vitro. IAN was shown to inhibit GST activity in an irreversible manner.

In the present study, a reduction of pericardial area from the low concentration was observed, and then this parameter increased as the DBP concentration increased. The IAN heart rate could be explained by the effect on the pericardial area at 72hpf. Hu et al. (2000) showed that the ventricular-to-body weight ratio increases when heart rate decreases. Moreover, the zebrafish mutant strain island beat (isl), which presents a defect in L-type calcium channels involved in the myocardial contraction, has a ventricular size diminution (Yelon et al., 2002). A consequence of the heart size reduction is a smaller pericardial volume (Kimmel et al., 1995). On the other hand, the blood flow decrease leads to an increase of the pericardial area (Billiard et al., 1999). These results could reflect the succession and the combination of both these phenomena.
The first one acts mainly at the low concentration and the next is replaced by the second one when the concentration increases. At the high concentration (3.45 µM), the cardiovascular system seems to be widely impaired, particularly with no blood circulation in the tail at 48hpf, leading to unhatched embryos and lethal impairments.

Another effect of this DBP is trunk deformities (lordosis and kyphosis). Nyuyen et al. (1997) have hypothesized that the twisted axis was the result of uncontrolled contractions of the axis musculature and/or a deformation of the vertebral elements themselves. The first expression of the **AChE** in the primary motoneurons of the zebrafish embryo takes place at the 9-somite stage (≈16hpf) and is propagated slowly in the anterior-posterior way until the beginning of the pharyngula period (≈25-27hpf) (Hanneman et al., 1989). A mutation of the AChE gene (**ache**) has been identified for zebrafish, which suppresses acetylcholine (ACh) hydrolysis. The **ache** mutant strain presents normal spontaneous movement frequency at 24hpf. This result can explain the lack of effect of IAN on embryos at this developmental stage, except at the high concentration (3.45 µM) which induced a small effect. The first signs of impaired motility appear at 27hpf, with spasmodic movements. Moreover, at 48hpf, the differentiation of the axial musculature is delayed, muscular cells become necrotized, the muscle fibers are disorganized, and the motoneuron dendritic extensions are notably reduced (Behra et al., 2002). Thus, Ach accumulation, induced by AChE inhibitors or lack of AChE (**ache** mutant), seems to induce a very intense body axis defect in relation to two main causes, a muscular tissue disorganization and a neuromuscular system defect. Such an effect (shorter and twisted tail) at the low concentration (0.43 µM) has been observed in this experiment suggesting involvement of the neuromuscular effect of IAN.
4.2.2 Effects of Iodoacetic Acid

Iodoacetic acid (IAA) is a DBP formed from source water with a high bromide/iodide concentration that was disinfected with chloramines. IAA has recently been shown to be more cytotoxic and genotoxic to mammalian cells than all DBPs that have been studied, including the regulated HAAs and bromate (Plewa et al., 2004). IAA is more than 250 times more cytotoxic than chloracetic acid and it is a factor of 2X more genotoxic than bromoacetic acid, which is the most genotoxic of the regulated HAAs. Low levels of IAA caused these effects, which was similar to concentrations of IAA that caused developmental effects (neural tube closures) in mouse embryos (Hunter et al., 1996). IAA's toxic effects are relatively unknown for all organisms.

The pericardial area increase induced at the high concentration (3.75 µM) could be linked to cardiovascular impairment. Also, it is not surprising to observe a heart rate reduction at 48hpf for the high IAA concentrations. The associated decrease of blood flow could be at the origin of the pericardial edema (Billiard et al., 1999) as well as weak myocardial contractions (Couillard, 2002).

4.2.3 Effects of Bromoacetonitrile

Bromoacetonitrile (BAN) is a type of haloacetonitrile (HAN), which are toxic, small nitrogenous water disinfection byproducts (N-DBPs) (Reider et al., 2007) which induce acute genomic DNA damage (Muellner et al., 2007) and are observed with chlorine, chloramine, or chlorine dioxide disinfection. According to a short term reproductive and developmental toxicity experiment of bromoacetonitrile (BAN) administered in drinking water to Sprague-Dawley rats there was a significant aversion to
BAN in the water and suggests that BAN may be a possible mild renal toxicant and a potential reproductive toxicant (NTP, 1997). Muellner et al. (2007) analyzed seven HANs due to their acute genotoxicity and chronic cytotoxicity in animal experiments with Chinese hamster ovary (CHO) cell line assays. BAN was shown to be cytotoxic at low concentrations. BANs toxic effects on fish are relatively unknown.

In this experiment the results for BAN were comparable to that of IAN, which is consistent with the class of DBPs that these are from, the HANs. The toxicity of BAN was less potent then that of IAN which is also reflected in the findings from Muellner et al. (2007).

4.2.4 Effects of Bromochloroacetic Acid

Bromochloroacetic acid (BCA) is formed from the disinfection of drinking water with chlorine or chloramine and bromine. Reproductive and developmental toxicity experiments on Sprague-Dawley rats administered drinking water containing BCAA showed statistically significant decreases in the live number of fetuses per litter. A number of other reproductive outcomes were reported to be adversely affected but were not significantly different from the controls (NTP, 1998). Other developmental studies on Sprague-Dawley rats and CD-1 mice administered BCAA resulted in dymorphogenesis and primarily rotational and heart defects and to a lesser extent prosencephalic, visceral arch, and eye defects (Andrews et al., 2004 and Hunter III et al., 2006). A study testing BCAA on frog embryogenesis (Xenopus) showed that only at high concentrations development was affected (Weber et al., 2004). BCAA’s toxic effects on fish are little or unknown.
In this study the morphological aspects and intensity of the deformations were similar to that of IAN at 0.43 µM (the low concentration). This demonstrates that the HANs are more toxic at low concentrations then HAAs, but that they have similar teratogenic effects. It is also consistent with other studies demonstrating that BCAA is a mutagen.

4.2.5 Effects of Dibromoacetic Acid

Dibromoacetic acid (DBAA) is formed by the reaction of chlorine oxidizing compounds with natural organic matter in water containing bromide (Melnick et al., 2007). Carcinogenic as well as reproductive and developmental effects have been reported following exposure to HAAs. DBAA is teratogenic when administered to rats throughout major organogenesis (Hunter III et al., 1996). Studies testing DBAA on frog embryogenesis (Xenopus) did not produce any significant mortality or malformation at any of the concentrations tested (Weber et al., 2004). DBA’s toxic effects on fish are relatively unknown.

In this study, the effects of DBAA on zebrafish embryos are mainly opposite of what occurred with the other five DBPs. At 24hpf the number of spontaneous movements decreased and at 48hpf the heart rate increased proportional to the exposure concentration. An increase in the heart rate of zebrafish embryos has been seen by Hassoun et al. (2005) in the study of the developmental toxicity of DCAA but at rather high doses in the zebrafish model system. Exposure of embryos to 8-32mM of DCAA resulted in significant increases in the heart rate and blood flow of the 55 and 80hpf embryos.
4.2.6 Effects of Chloroacetic Acid

Chloroacetic acid (CAA) is present in drinking water disinfected with chlorine. It is ranked as one of the most hazardous compounds (worst 10%) to ecosystems and human health. Three of eight basic tests to identify chemical hazards have not been conducted on this DBP, or are not publicly available according to US EPA’s 1998 hazard data availability study. This DBP also lacks at least some of the data required for a safety assessment. Of the data available, CAA has been found to be a suspected human health hazard; cardiovascular or blood toxicant, kidney toxicant, neurotoxicant, respiratory toxicant, and skin or sense organ toxicant (EPA, 1988).

Reproductive and developmental toxicity experiments on pregnant Sprague-Dawley rats given CAA in drinking water show a significant decrease in body weight gain, but no adverse reproductive, developmental, or teratogenic effects were reported (Johnson et al., 1998). Exposure to CAA in CD-1 mice showed affected embryonic development and embryo lethality as well as arch and heart defects. A predominant feature of CAA-induced effects was a constriction of the outflow tract of the heart leading to an enlargement of the proximal portion of the heart up to the region of the bulboventricular fold (Hunter III et al., 1996). CAA exhibited teratogenic potential in non-mammalian developmental toxicity screening assays with Hydra attenuate (Fu et al., 1990).

In this study, the morphological aspects and intensity of the deformations were similar to that of IAN at 0.43 µM (the low concentration). This demonstrates that the HANs are more toxic at low concentrations than HAAs, but that they have similar teratogenic effects. It is also consistent with other studies demonstrating that CAA is a mutagen.
4.3 Dose Optimization/Dose Response

In this study, spectrums of concentrations were used to determine the optimal concentration for each DBP. In this case, the optimal concentration is the lethal concentration (LD), an indication of the lethality of each of the DBPs. The ‘lethal dose’ represents a concentration at which a given percentage of subjects will die. The most commonly used lethality indicator is the LD$_{50}$. An LD$_{50}$ value is the amount of a solid or liquid material necessary to kill 50% of test animals (e.g., zebrafish embryos) in one dose. For each DBP tested in the first dose optimization study, the LD$_{50}$ fell in the low concentration range. This demonstrated that the DBPs were 100% more lethal than they were originally expected to be.

The science of toxicology is based on the principle that there is a relationship between a toxic reaction (the response) and the amount of poison received (the dose). An important assumption in this relationship is that there is almost always a dose below which no response occurs or can be measured. A second assumption is that once a maximum response is reached any further increases in the dose will not result in any increased effect.

In this study, the low concentration for each chemical was still potent enough to cause either mutations or death for 20% of the embryos. There was no concentration of any DBP where there was no response occurring to be measured. The medium-high and high concentrations of each DBP could be associated with the maximum response. At least 50% of the embryos died and 30% of the viable embryos had mutations.
4.4 Sensitivity

Toxicants affect organisms by impairing lifecycle traits that are most sensitive to these toxicants, a concept also adopted by national and international legislative authorities for deriving safe standards for contaminants in soil and water. During lifecycle tests with a variety of toxicants, certain developmental stages have consistently been more sensitive than others. The possibility of focusing research efforts on these more sensitive stages promises success in searching for quicker and less costly ways of predicting chronic toxicity of chemicals to fish. Several investigators proposed that chronic toxicity to fish might be predicted by use of shorter tests with early developmental stages. In studies with selected toxicants, these early stages were shown to be among the most sensitive in the lifecycle (Pickering et al., 1970, Pickering et al., 1972, McKim et al., 1975, Eaton et al., 1978, and Sauter et al., 1978). The greater sensitivity of the early life stages compared to the later stages provides toxicologists with an accurate, efficient tool for predicting chronic effects of environmental pollutants in a short period of time.

The present experiment shows that the most sensitive period for zebrafish embryos is in the first 0-5hpf of life. This period of life is the blastula stage, specifically the 1K cell stage at 3hpf. One can usually, but not always, see a yolk syncytial layer (YSL), irregular in form and containing a total of about 20 nuclei within a single ring around the blastodisc margin. There are fewer than 1024 blastomeres after division 10 because the first-tier (marginal) EVL cells from the previous stage joined together in the YSL. Moreover, because of the manner of YSL formation, the cells making up the first EVL tier at this stage are descendants of those that were in the second tier a stage earlier.
(Kimmel et al., 1995). At this stage, the toxic mortality effects of all six DBPs at the low concentration ranged from 13.33% to 86.67% after 72hpf. After 6hpf, there were no mortalities except those embryos exposed to IAN, but the % mortalities of those embryos exposed to IAN decreases to 21%, 14%, and 8% for 6hpf, 12hpf, and 24hpf respectively.

This is consistent with the life cycle of the zebrafish. At 6hpf of life, the zebrafish embryo is in the gastrula stage of life, specifically the shield stage. During this stage the embryonic shield and the germ ring have formed. Both the epiblast and hypoblast are locally thickened at the shield (Kimmel et al., 1995), perhaps making the embryos at this stage less permeable, and therefore, less sensitive to DBPs. This is reflected in the sensitivity experiment where embryos 6hpf and beyond, subjected to DBPs, are more likely to remain viable.

4.5 Recovery

A recovery experiment or the reversibility of toxicant-induced injury after prolonged exposure is important for understanding cumulative toxicology, reorganization of physiological parameters, regeneration of normal biological status in an organism, and repair mechanisms. The inclusion of such studies in a project helps identify the progression, regression, or amelioration of toxicant-induced injury after termination of exposure (Barile, 2007).

In this experiment the embryos that were exposed for 12hpf to DBPs before being placed into DDI water had a greater survival rate than the embryos placed solely in the DBPs for the full 72hpf. This demonstrates that recovery is possible for the embryos after the 12hpf stage of life. This could be due to the rules of dose-response. The longer
the embryo is subjected to the chemical, the more likely that the toxicant will have a lethal effect.

In the second recovery experiment the embryos that were acclimated in DDI water before being placed into the DBPs and then back into DDI water had a higher survival rate than the embryos placed solely in the DBPs and the embryos that were placed in the DBPs for 12hpf before being placed in DDI water. This is consistent with the sensitivity experiment which demonstrated that between 0 and 5hpf the embryos were the most sensitive to DBPs. Therefore, acclimating the zebrafish embryos in DDI water through the most sensitive part of the life cycle before placing them in the DBPs allows for more viable embryos. After 6hpf of life, the embryo is more protected from outside threats combined with the least amount of time in the DBP solutions these embryos would have a greater survival rate.
5.0 Conclusion

Use of chemical oxidants such as chlorine and chloramines as drinking water disinfectants is a well-established and successful treatment practice to combat waterborne disease. As a result of these practices, the incidence of waterborne disease has been greatly reduced. While the addition of chemical oxidants to drinking water yields a measurable benefit in public health protection, it may also result in the introduction of other potential risks. The chemical oxidants react with naturally occurring organic and inorganic substances to form byproducts. These byproducts have been shown to produce adverse health effects in laboratory animal studies. Since their discovery, DBPs have become one of the major driving forces in drinking water regulations, research and water utility operations throughout the world. In 1979, the U.S. Environmental Protection Agency (EPA) regulated the most prevalent group of byproducts, trihalomethanes (THMs), as surrogates for the range of byproducts that could be formed. This initial regulation triggered substantial research related to the occurrence of disinfection byproducts and their potential health effects. The list of DBPs that can occur in treated drinking waters has grown from a few THMs to a long list of halogenated and non-halogenated organic and inorganic compounds. The EPA is now in the process of revising and enhancing its regulation of disinfection byproducts to include compounds other than trihalomethanes. The European Union directive on drinking water is also under revision, as is the Canadian Drinking Water Safety Act. These regulatory processes require a careful balance of reducing exposure to chemical byproducts of disinfection while maintaining control of waterborne pathogens. This list is expected to continue to
grow as the analytical techniques are improved, as more information on their toxicity is developed and as more occurrence studies are conducted. (Fawell et al., 1996).

In the present study all six DBPs tested resulted in some form of mutation in the zebrafish embryos. HANs, especially IAN appear to be the most toxic of all the chemicals tested. The iodoacids are found to be the most potent genotoxic DBP in the zebrafish embryos. This is consistent with findings that the iodoacids are the most potent DBPs to mammalian cells (Plewa et al., 2004). The rank order of the six DBPs tested in this study in order of increasing potency is: CAA<BCAA<DBAA<IAA<BAN<IAN. This is consistent with the reactivity trends most often observed in chemistry: Cl<Br<I. The most sensitive developmental stages of the zebrafish embryo occur between 0-5hpf and there is a potential for recovery after exposure to DBPs.

The zebrafish is considered an excellent model for toxicological assessment and is a valid approach to determining the teratogenic effects of DBPs due to the high-throughput assays which allow a broader range of DBPs to be tested in an effective manner (Sabaliauskas et al., 2006). The zebrafish embryo was originally used to study the genetics of development due to its transparency, quick embryonic development, easy production of high numbers of embryos and similarity with human development. In fact, the zebrafish embryo constitutes a complete, developing vertebrate organism, and it allows testing predictability for toxicity not only in the context of cellular function, but also at the level of organ and organism toxicity. Moreover, the anatomic and genomic similarity with humans, 80% genetic homology, foresees test predictability for human toxicity (Carroll et al., 2003). All of these characteristics make the zebrafish especially suitable for the assessment of toxicity and highly prone to yield additional information to
the cell-based assays. Humans and fishes share many developmental pathways, organ systems, and physiological mechanisms, making conclusions relevant to human biology.
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